CAMPYLOBACTER JEJUNI VIRULENCE MECHANISMS: CHARACTERISATION AND REGULATION

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Doctor of Philosophy
at the University of Leicester

by

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To my husband, Martin,

and to my family

and to my family

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Campylobacter jejuni virulence mechanisms: characterisation and regulation

A. M. Brás

ABSTRACT

Campylobacter jejuni is a major cause of gastrointestinal illness throughout the world. Despite its importance as a human pathogen, the current understanding of *C. jejuni* virulence mechanisms or the means by which the bacterium regulates the expression of virulence remains limited. In this thesis, two different areas were investigated and are presented separately. In Part I, studies focused on the characterisation of *C. jejuni* translocation across an epithelial-like barrier. It was previously demonstrated that many strains of *C. jejuni* are able to translocate across polarised Caco-2 cell monolayers. A small number of these strains are not detectable within cells but are nevertheless able to translocate across the monolayer, presumably using a paracellular route. The possibility of paracellular translocation (without invasion) was investigated by looking at changes in the permeability across differentiated Caco-2 cell monolayers. The data obtained suggest that *C. jejuni* does not significantly alter the permeability of the monolayer, causing no permanent damage to the host cells, at least in the short term. In the long term, however, *C. jejuni* infection leads to a drop in the monolayer resistance, suggesting host cell damage. Although paracellular translocation seems unlikely to occur in the early stages of infection, it may occur later as consequence of host cell damage caused during invasion.

In Part II, studies focused on the role played by the environment on the regulation of *C. jejuni* virulence. Two-component regulatory systems are involved in the regulation of numerous cell functions in response to environmental stresses, including virulence. Recently, the polymerase chain reaction with degenerate oligonucleotide primers was used to isolate regulatory genes responsive to environmental stimuli. The fragment isolated was identified as homologous to the members of the family of response regulators. The DNA fragment was subsequently used to probe a genomic library. The complete gene, *regX1*, was isolated, sequenced and mutated. In order to determine the role of *regX1* in *C. jejuni*, the phenotype of two *regX1* mutants was investigated. The results suggest that RegX1 may respond to changes in temperature and that RegX1 may be involved in the control of genes related to growth, host cell interaction and *in vivo* colonisation. Furthermore, a putative histidine protein kinase partner to RegX1 was identified downstream *regX1*.

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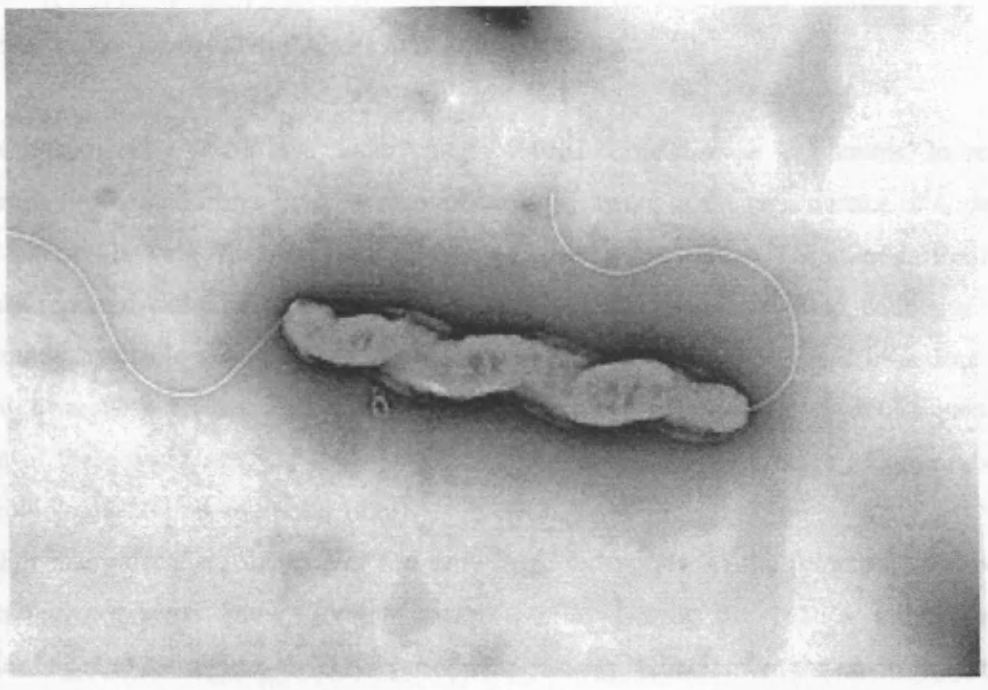
ABBREVIATIONS

A_{595nm}	Absorbance at a wave length of 595nm
A	Adenine
A (mA)	Ampère (milliAmpère)
a.a	Amino acid residue
AMP	Adenosine monophosphate
Ap	Ampicillin
Ap^R	Resistant to ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
BPB	Bromophenol blue
Bq	Becquerel
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
cAMP	Cyclic AMP
cfu	Colony forming units
cfu/gc.c	cfu per gram of caecal content
Cm	Chloramphenicol
Cm^R	Resistant to chloramphenicol
CO_2	Carbon dioxide
CsCl	Cesium chloride
1-D	One-dimensional protein electrophoresis
2-D	Two-dimensional protein electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide trophosphate
dsDNA	Double stranded DNA
EPB	Electroporation buffer
EtBr	Ethidium bromide
F	Faradays
FBS	Foetal bovine serum
g	Angular velocity as multiples of the acceleration due to gravity (g =9.8 $m.s^{-2}$)
g	Grams
G	Guanine

h	Hour(s)
H_2O	Water
HK	Histidine protein kinase
IgA	Immunoglobulin A
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)
MH	Mueller Hinton agar or broth
min	Minute(s)
Mr	Molecular weight
mRNA	Messenger ribonucleic acid
n	Nano (10^{-9})
NA	Nutrient agar
NB	Nutrient broth
Ω	Ohms
$O.D_{600nm}$	Optical density at a wave length of 600nm
OM	Outer membrane
OMP	Outer membrane protein
Orf	Open reading frame
p	Pico (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PCRDOP	Polymerase chain reaction with degenerate oligonucleotide primers
pfu	Plaque forming units
PolyB	Polymyxin B
ΔR	Variation of electrical resistance
RILT	Rabbit (or rat) ileal loop test
RITARD	Removable intestinal tie adult rabbit diarrhoea
RNA	Ribonucleic acid
RR	Response regulator
s	Second(s)
SD	Standard deviation
sIgA	Secreted immunoglobulin A
ssDNA	Single stranded DNA
T	Thymine
Tc	Tetracycline
TER	Transmonolayer electrical resistance
TJ	Tight junction
Tm	Trimethoprim
UV	Ultraviolet
V	Volts
$^{v}/_{v}$	Volume per volume
Vm	Vancomycin
$^{w}/_{v}$	Weight per volume



"The role of the infinitely small in nature is infinitely large"

Louis Pasteur

GENERAL INTRODUCTION

1. *Campylobacter jejuni* AND CAMPYLOBACTERIOSIS

Campylobacter jejuni is a major cause of food-borne disease in humans. In recent years, public health authorities have become increasingly aware of the significance of *C. jejuni* induced enteritis or campylobacteriosis[1]. Based on present knowledge, *C. jejuni* is the most commonly reported cause of sporadic bacterial enterocolitis in industrialised countries. The data currently available does not indicate a rise in the incidence of *C. jejuni* infection. The increased awareness results instead from the improvement in laboratory techniques for detection of these organisms and from better surveillance (ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993).

For many decades, *Salmonella spp* have been recognised as the principal food-borne pathogen and are better known by the general public. During the 1980's, public health authorities began to learn more about the prevalence of campylobacters[2] in the environment, the illness they cause and more appropriate laboratory techniques for culturing and identifying these organisms. *Campylobacter spp* have since emerged as important human pathogens. Among the members of the genus, *C. jejuni* is by far the species most frequently isolated with human infection. Because *C. jejuni* is not usually associated with large outbreaks and campylobacteriosis is rarely fatal, *C. jejuni* is not generally a source of interest for the mass media and hence, the level of public awareness remains limited (Norcross *et al.*, 1992; ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993). With improved surveillance and an increase in reporting cases of campylobacteriosis to health authorities, more coverage about campylobacters can be expected in the scientific and general media. This will certainly have a significant impact in informing the lay public about *C. jejuni* and campylobacteriosis.

The genus *Campylobacter* includes other important pathogenic species (Table 1). *C. coli* and *C. fetus* are probably the species of most economical importance, after *C. jejuni*. *C.*

[1] The term "campylobacteriosis" refers to disease induced by members of the genus *Campylobacter*. In this thesis, however, the term will be used to refer to *C. jejuni*-induced infection only.

[2] The term "campylobacters" usually refers to the species *C. jejuni* and *C. coli*. In this thesis, however, the term will be used to refer to *C. jejuni* only.

coli is closely related to *C. jejuni* and induces the same type of infection in humans. *C. fetus* typically causes bactereamia in elderly men with chronic underlying illness, but it is not a significant human pathogen (Mishu *et al.*, 1992). Instead, *C. fetus* is a major cause of septic abortions in domestic animals and it is associated with infection of the reproductive tract in cattle (Mishu *et al.*, 1992).

Table 1. *Campylobacter* species of clinical importance (*in* Butzler *et al.*, 1992).

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

It is now well established that *C. jejuni* constitutes a major public health problem (Tauxe, 1992; ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993). In the last decade, considerable interest focused on *C. jejuni* and on *C. jejuni*-induced enteritis. Major progress in characterising the clinical disease and the epidemiology of infections has occurred but the pathogenic mechanisms remain poorly understood. Moreover, the genetic characterisation of *C. jejuni* pathogenesis is still at an early stage and only now is the regulation of virulence beginning to be unravelled. In this thesis, I investigated questions regarding both the characterisation and the regulation of *C. jejuni* virulence. The two different areas were studied separately and therefore, the thesis is organised in two parts to reflect the two major objectives of this study. The first main objective was to investigate one pathway by which campylobacters may penetrate the sub-epithelial tissues of the intestine. In Part I, I describe data that characterises the process of *C. jejuni* translocation across polarised cell monolayers. The second main objective was to investigate the regulatory systems campylobacters utilise to adapt to changing environments. In Part II, I describe the identification and characterisation of a two-component regulatory system in *C. jejuni*. Each part contains a specific introduction to the presented work and my findings are discussed in detail. Although each objective addresses a different aspect of the pathogenesis of *C. jejuni*, they are closely related. Penetration of the

intestinal tissue is an important component of *C. jejuni* infection and in order to penetrate successfully, the bacterium must adapt to the differing conditions found in the lumen and tissue. Therefore, the General Introduction contains a detailed description of what is known about *C. jejuni* pathogenesis and the General Conclusions brings the work of Parts I and II into context of this description of the pathogenesis of infection.

2. THE MICROORGANISM

2.1 *C. jejuni* biology

Campylobacters are small Gram-negative bacteria, 0.2-0.5μm wide and 0.5-8μm long (Ketley, 1997). The cells are characteristically shaped as spiral curved rods (Blaser, 1989; Griffiths & Park, 1990; Ketley, 1995, 1997). Under unfavourable conditions or upon ageing, the cells become coccoid in shape. These forms are associated with a transition from a viable and culturable (corresponding to the rod-shaped cells) to a viable but non-culturable form (coccoid-shaped cells). In the same culture, not all bacteria are round in shape and a mix of rod and coccoid-shaped cells occurs. Not all coccoid forms are viable non-culturable (VNC) organisms and in fact, most are not viable (Rollins & Colwell, 1986; Jones *et al.*, 1991; Ketley, 1995, 1997). Besides shape, motility is another characteristic feature of these organisms. They possess a single unsheathed flagellum at one or both ends of the cell. They are actively motile with a typically rapid darting and spinning type of motion (Blaser, 1989; Griffiths & Park, 1990; Ketley, 1995, 1997).

C. jejuni is unable to metabolise carbohydrates and the bacterium obtains energy from amino acids and intermediates of the tricarboxylic acid cycle (Penner, 1988). These organisms are sensitive to acid conditions and do not survive at a pH below five (ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993). *C. jejuni* is non-sporulating although it has been suggested that the VNC forms correspond to a dormant-like stage, enabling the bacteria to survive in adverse conditions (Ketley, 1995, 1997).

CO_2 enriched atmospheres are generally favoured by members of the genus. *C. jejuni*, like most species in the genus, is microaerophilic requiring an oxygen concentration between 3-15% and a carbon dioxide concentration between 3-5%. Some species (*C. fetus*) require anaerobic conditions for optimal growth (Penner, 1988; Griffiths & Park, 1990; Ketley, 1995, 1997).

C. jejuni varies in tolerance of growth temperature from those considered optimum. The bacterium grows best at 42°C, grows well at 37°C and does not grow at temperatures below 30°C. Other species tolerate temperatures ranging from 15°C (for instance, *C. fetus*) to 42°C (for instance *C. jejuni* and *C. coli*). Members of the genus, for example *C. jejuni* and *C. coli*, that grow best at 42°C are designated thermophilic campylobacters (Penner, 1988; Griffiths & Park, 1990; Ketley, 1995, 1997).

2.2 Genetics of *C. jejuni*

C. jejuni has a small genome of AT rich DNA. The chromosome is approximately 1.7Mbp, as determined by pulse field gel electrophoresis, with a G+C content ranging from 30 to 33 mol% (Nuijten *et al.*, 1990; Taylor, D.E., 1992b). Such a small genome is consistent with some of *C. jejuni*'s phenotypic properties, for example, the requirement for complex growth media and the inability to metabolise carbohydrates or to degrade complex substances (Griffiths & Park, 1990; Taylor, D.E., 1992b). Plasmids have been detected in several different strains of *C. jejuni* (Taylor, D.E., 1992a) and their DNA contains a similar A+T content to chromosomal DNA. Many strains contain plasmids, although the copy number as well as the plasmid size are variable (Bradbury *et al.*, 1983; Tenover *et al.*, 1985). Conjugative plasmids containing genes encoding resistance to chloramphenicol, kanamycin or tetracycline are found in some strains. These plasmids usually range in size from 45 to 50kb and are transferrable within the genus. Restriction maps show that except for the presence of the genes encoding the normal plasmid functions, only the resistance determinants are located on the plasmids (Taylor, D.E., 1992a).

Compared to other enteropathogens, the number of DNA sequences from *C. jejuni* available in the GenBank remains comparatively low. *C. jejuni* genes are often difficult to clone and subsequently analyse. Examples of the problems encountered by different groups of researchers include instability of cloned genes, failure to express the gene of interest and the lack of a system for generalised mutagenesis (Taylor, D.E., 1992b; Tompkins, 1992). Nevertheless, alternative strategies have been devised and a few genetic tools are now available.

One of the initial problems experienced consists in creating genomic libraries (Taylor, D.E., 1992b). *C. jejuni* genes are difficult to clone in *E. coli* as clones containing large inserts often collapse. Several possible reasons have been suggested to explain such problems. Due to the high A+T content of *C. jejuni* DNA, cloned fragments may resemble *E. coli* promoter

sequences, leading to the instability and collapse of large clones. Long patches of AT repeats found along the chromosome may promote recombinational events and deletion of sequences when the clones are introduced in a foreign background. In addition, different patterns of methylation between *C. jejuni* and *E. coli* may also result in the instability of clones (Labigne-Roussel *et al.*, 1987; Taylor, D.E., 1992b). Nevertheless, these difficulties can be avoided using genomic libraries with small inserts or bacteriophage vectors (Ketley, 1995).

Another difficulty has been to express *C. jejuni* genes in *E. coli*. Difficulties in gene expression may be caused by the *C. jejuni* promoter structure not being recognised by *E. coli* transcriptional machinery or/and lack of accessory gene products (Taylor, D.E., 1992b). Problems with gene expression may also be due to differences in codon usage between *C. jejuni* and *E. coli* (Wang & Taylor, 1990a; Taylor, D.E., 1992b). However, some genes from amino acid biosynthetic pathways were isolated by complementation of an *E. coli* mutant by a cloned gene of *C. jejuni* (Tompkins, 1992). Such housekeeping genes are highly conserved across species boundaries and encode similar functions in many different bacterial species. Clearly for more specialised genes, for example flagellins or porins, such situation would not apply (Taylor, D.E., 1992a). Nevertheless, the success of these strategies was attributed to the use of the strong selective pressure on *E. coli* to grow in the absence of a particular amino acid. Another problem with the expression of some gene products, for example, flagellin, corresponds to the host's inability to carry out the necessary post-translational modifications (Taylor, D.E., 1992b).

In addition to the instability of clones and to the difficulties in expressing and processing *C. jejuni* genes, other obstacles have hindered the identification of virulence determinant genes of *C. jejuni*. A major difficulty has been the lack of a method to randomly mutagenise the chromosome (Taylor, D.E., 1992b). Transposons of either Gram-negative or Gram-positive origin have not been found to transpose in a *C. jejuni* background, although Tn*phoA* was found to successfully mutagenise cloned *C. jejuni* DNA in *E. coli* (Ketley, 1995). Although there are reports of several bacteriophages specific of *C. jejuni*, both lytic and lysogenic, no method of generalised transduction has been identified. Nevertheless, alternative methods have been developed. Yao and colleagues (Yao *et al.*, 1994) successfully adapted a method of insertional mutagenesis previously applied to *Haemophilus influenzae*. This method consists of restricting *C. jejuni* chromosomal DNA with an endonuclease followed by the self-ligation of the various fragments, creating loops. The loops are subsequently digested with a second endonuclease and ligated to a kanamycin-resistance cassette. This DNA is transformed into *C. jejuni*, enabling homologous recombination to occur between identical sequences present in the loops and in the chromosome. Recombinants are screened for the expression of kanamycin resistance and tested in various assays to analyse the loss of certain features, for

example, motility. This method is not as effective as transposon mutagenesis as it depends on the use of restriction enzymes as well as on the position of the restriction sites. Alternatively, other authors have used the polymerase chain reaction (PCR) with degenerate primers designed to anneal to conserved regions within families of proteins (Wren *et al.*, 1992; Miller, S. *et al.*, 1993). This method only allows the isolation of genes encoding known proteins with highly conserved domains and thus, unlike transposon mutagenesis, is not useful for identifying novel gene loci.

Although the use of classical molecular genetic approaches to study *C. jejuni* is difficult, some tools are available. Plasmid vectors with replicons from *E. coli* cannot replicate and are not maintained in *C. jejuni*. Instead, they are useful as suicide vectors and are used widely in strategies for the insertional mutagenesis of chromosomal genes (Taylor, D.E., 1992b). Alternatively, shuttle vectors that contain replicons from both *E. coli* and *Campylobacter* origin have been constructed and proven to work (Labigne-Roussel *et al.*, 1987; Wang & Taylor, 1990a, b; Yao *et al.*, 1993). The shuttle vectors developed by these authors carry an origin of transfer from RK2 plasmids and can be mobilised from *E. coli* into *C. jejuni*. Genes encoding resistance to kanamycin (Labigne-Roussel *et al.*, 1987) and chloramphenicol (Wang & Taylor, 1990a) isolated from *C. coli* or tetracycline isolated from *C. jejuni* (Taylor, D.E., 1986) are included in these vectors to provide selective markers. All three resistance genes are expressed in both *E. coli* and *C. jejuni* (Taylor, D.E., 1992b).

The transfer of DNA into *C. jejuni* cells is a necessary step in the assessement of gene function. Broad host range plasmids from the IncP incompatibility group were found to conjugate into *C. jejuni*. Electrotransformation is also possible, although the transformation yield is usually low (Miller, J.F. *et al.*, 1988) and success appears to depend on the strain. Wang & Taylor demonstrated that *C. jejuni* can naturally take up DNA without any previous treatment (Wang & Taylor, 1990b). Natural transformation is common among Gram-positive bacteria but only a few Gram-negative are naturally competent, for example *Haemophilus spp* and *Neisseria gonorrhoeae*. Gram-negative bacteria take up DNA by binding to a small (11bp for *Haemophilus spp* and 10bp for *N. gonorrhoeae*) specific DNA sequence. There is preliminary evidence that the mechanism by which DNA uptake occurs is similar between *C. jejuni* and the other Gram-negative bacteria but the recognised sequence is still unknown (Ketley, 1995). However, transformability of *C. jejuni* by either electroporation or natural transformation is strain-dependent (Wassenaar *et al.*, 1993a).

3. *C. jejuni* INFECTION: THE DISEASE

3.1 Historical background

Campylobacter enteritis was first reported at the end of the last century. In 1880, Theodor Escherich described spiral organisms present in the faeces of children that could not be cultured on solid media. These organisms were probably *Campylobacter spp.* As Escherich's findings were published in German journals, campylobacters remained unrecognised as human pathogens for several decades (Goossens & Butzler, 1992). By 1909, *Campylobacter spp* were considered pathogens of veterinary importance only, causing foetal and reproductive tract infection and abortion in sheep and cattle (Blaser *et al.*, 1983; Cover & Blaser, 1989). These organisms were designated *Vibrio fetus* and correspond to the modern *C. fetus*. Only in the late 1940's, *Campylobacter spp* (*V. fetus*) were isolated from blood cultures of debilitated patients. Campylobacters were related to human infections but considered as rare and only perhaps opportunistic human pathogens (Walker *et al.*, 1986; Blaser, 1989). By the late 1950's, Elizabeth King noted that blood cultures from patients with diarrhoea contained two distinct groups of organisms that she could distinguish on the basis of thermophilic characteristics (Walker *et al.*, 1986; Blaser, 1989). The organisms that grew better at 42°C than at 37°C are now thought to be *C. jejuni*. King speculated that these latter organisms could be the cause of diarrhoeal illness. She also considered that they could occur more commonly than their infrequent isolations from blood cultures would indicate (Blaser, 1989). However, because campylobacters are fastidious and slow growing, they could not be isolated from faeces. It was not until in the 1970's that the development of selective media enabled widespread isolation of these organisms from stools and consequently they were recognised as a cause of intestinal illness (Blaser, 1989). *Vibrio fetus* and related organisms were grouped in a new genus named *Campylobacter* as they show fundamental differences (for instance G+C content of DNA, oxygen requirements for growth and cell morphology) from the other members of the genus *Vibrio* (Penner, 1988). Over the past decade, the importance of "King's vibrios" was confirmed and *C. jejuni* is now recognised as a major bacterial pathogen worldwide (Blaser, 1989; Goossens & Butzler, 1992; Ketley, 1997).

3.2 Epidemiology of *C. jejuni* infection

The epidemiology of *C. jejuni* infections varies markedly between different regions of the globe. In industrialised countries, the pattern of the incidence of campylobacteriosis is sporadic as opposed to epidemic. However, common source outbreaks have been described. Sporadic cases are most likely related with consumption of meat, mainly poultry, whereas outbreaks are usually milk or water-borne (Skirrow & Blaser, 1992; Taylor, D.N., 1992). Nevertheless, the origin of infection is rarely identified; this is particularly true for the numerous cases occurring sporadically (Ketley, 1995; Ketley, 1997);

Campylobacteriosis tends to show seasonality in western countries. Cases occur all year round but the incidence is higher during the Summer months which coincides with an increase in outdoor activities. Barbecues, for instance, are often related with family outbreaks because of improper cooking and a higher probability of cross-contamination between cooked and uncooked meat, either by using the same utensils or just through handling. Another event is hiking and consumption of untreated spring water contaminated by the excreta of wild or farm animals (Skirrow & Blaser, 1992; Ketley, 1995).

In industrialised countries, campylobacteriosis affects people of all ages but the isolation rate of *C. jejuni* peaks for children less than one year old and for young adults. For unknown reasons, the incidence of infection in adults less than 45 years old is higher in men than in women (Blaser, 1989; Ketley, 1995).

In developing countries, the pattern of campylobacteriosis incidence does not show seasonality, which may relate to local climatic conditions. Epidemics have not been reported but this might be explained by poor surveillance (Taylor, D.N., 1992). Campylobacteriosis is frequently asymptomatic in developing countries. Although the incidence is several orders of magnitude higher than in industrialised countries (Taylor, D.N., 1992), the rate of asymptomatic infections are 3 to 4 times the rate of symptomatic cases. Based upon studies carried out with children from Bangladesh and India, it was recognised that *C. jejuni* could be isolated from healthy children as often as from children who had diarrhoea (Blaser, 1989). The frequent asymptomatic carriage is attributed to a natural immunity developed by exposure to *C. jejuni* early in life. In developing countries, the majority of infections occurs in the first two years of life (Blaser *et al*, 1983). Studies have shown that people continuously exposed to *C. jejuni* or persistent consumers of raw milk develop immunity to the bacterium and are less likely to develop severe campylobacteriosis (Black *et al*, 1988; Blaser *et al*, 1983; Blaser, 1989). Thus, acquired immunity might be the reason for the high rate of asymptomatic infections in developing areas.

The high rate of asymptomatic infection has raised the question of whether *C. jejuni* strains isolated in developing countries should be considered pathogenic (Blaser *et al.*, 1983; Black *et al.*, 1988; Taylor, D.N., 1992). Some authors have suggested that differences between *C. jejuni* strains found in developed or developing areas account for the differences in epidemiology and clinical disease between these regions. However, travellers visiting developing areas often contract *C. jejuni*-induced enteritis and develop an illness with the same symptoms experienced as *C. jejuni* enteritis acquired in developed countries. Furthermore, no biochemical differences have been found between the isolates from these two areas. The same serotypes have been isolated in both developed and developing countries. This seems to indicate that the isolates from developing areas are indeed pathogenic. The differences in epidemiology and clinical disease will probably be due to differences in host characteristics. Immunity to *C. jejuni* seems to be the identifiable host characteristic distinguishing populations in the two areas (Blaser, 1989).

3.3 Transmission of *C. jejuni* infection

C. jejuni is a food-borne pathogen (ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993). Transmission to humans occurs mainly by ingestion of contaminated meat products, milk or water (Fig.1). Experimental infection in human volunteers showed that the ingestion of as few as 500 organisms are sufficient to produce illness. Other volunteers only developed symptoms after ingesting at least 10^6 organisms in a glass of milk (Black *et al*, 1988; Walker *et al*, 1986).

C. jejuni exists in the intestinal tract of most domestic and many wild animal species (Blaser *et al*, 1983; Blaser, 1989). Animals affected, including animals used in food production are generally asymptomatic carriers and are thought to constitute the main source of infection. The surface of carcasses frequently becomes contaminated by intestinal contents during processing in slaughter houses. Subsequently, improper handling and/or cooking of the contaminated meat results in the transmission of the organism to the consumer. Although cattle and lamb are important reservoirs to these bacteria, poultry constitutes a major vector of *C. jejuni*. Studies show that 40 to 85% of poultry are *C. jejuni* positive and that 50% of the cases of *C. jejuni* infection are due to consumption of poultry (Doyle & Jones, 1992). The consumption of unpasteurised milk contaminated by excreta can also result in infection. In

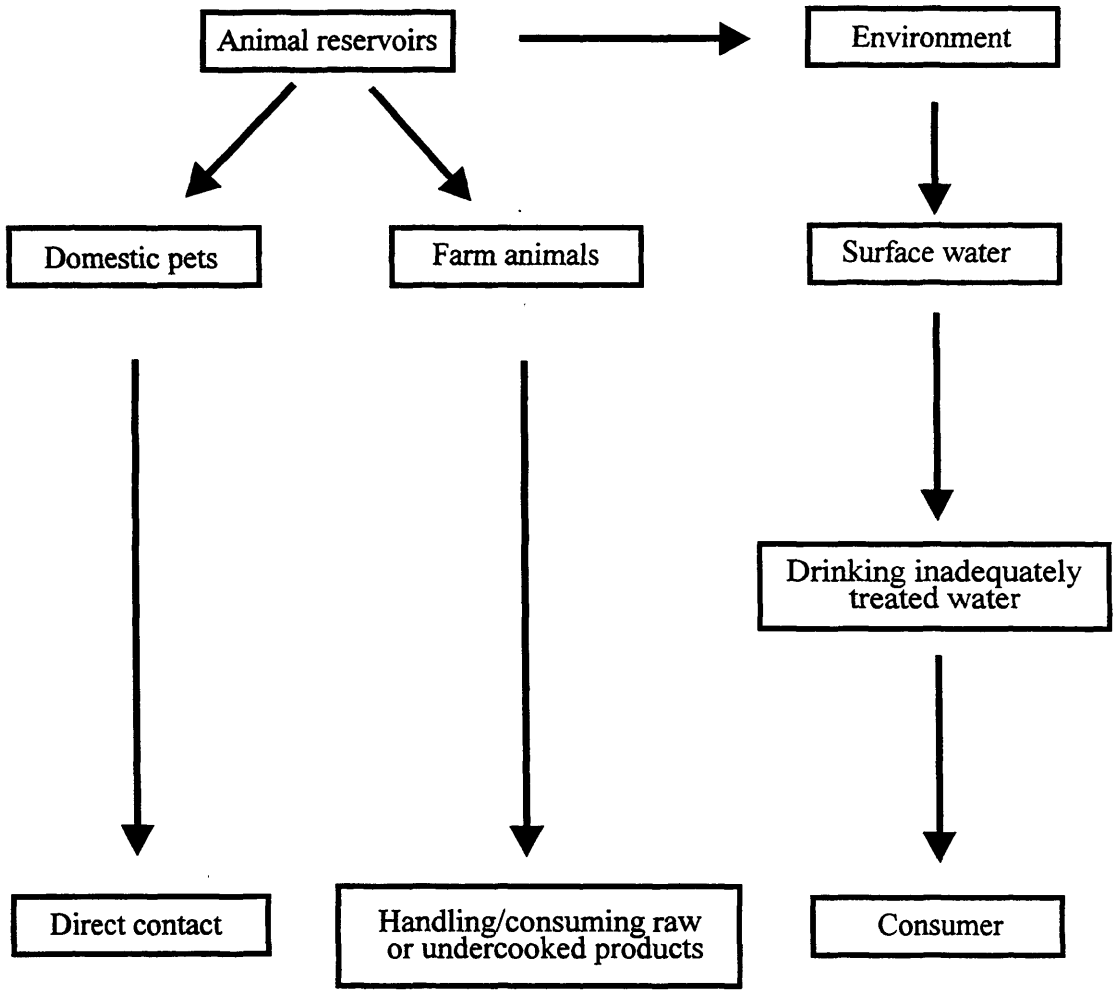


Fig.1. Transmission of *C. jejuni* to humans (adapted from Butzler *et al.*, 1992).

addition, untreated water contaminated by excreta from wild or farm animals constitutes another means of infection.

Transmission can also occur through direct contact with infected animals. Pets such as puppies, kittens, turtles, birds and rodents are vehicles of infection but they are not major vectors of *C. jejuni* (Blaser *et al*, 1983; Blaser, 1989). Campylobacteriosis is essentially a food-borne disease as the vast majority of infections are transmitted to humans through the ingestion of contaminated foods (Skirrow & Blaser, 1992). Person-to-person transmission is uncommon but it has been reported among young children or among carers of patients with *C. jejuni*-induced enteritis (Blaser *et al*, 1983; Blaser, 1989; Butzler *et al*, 1992).

Transmission of *C. jejuni* to humans shows geographic variation. In industrialised countries, transmission occurs mainly by the ingestion of contaminated food, particularly contaminated poultry. The same is observed in the less developed countries. However, in the poorest regions, transmission occurs mainly by contact with domestic animals usually kept in the household (Taylor, D.N., 1992).

3.4 Clinical features of *C. jejuni* infection

The clinical features of *C. jejuni* infection also show geographical variation. In industrialised countries, the most common presentation of *C. jejuni* infection is acute enterocolitis. The incubation period ranges from one to seven days. The predominant symptoms are high fever, abdominal pain and profuse diarrhoea (Table 2). Vomiting is rare. The infection can produce watery diarrhoea or slimy stools containing fresh blood and pus or mucus. In these latter cases, a microscopic analysis of stool samples shows an inflammatory exudate with leukocytes; it is also possible to see numerous campylobacters which are easily recognisable by their characteristic morphology and motility (Butzler *et al.*, 1992). The sites of tissue injury include the jejunum, ileum and colon (Black *et al.*, 1988; Blaser, 1989). Sigmoidoscopy usually reveals mucosal abnormalities ranging from oedema and hyperemia, either with or without petechial hemorrhage, to mucosal friability. *C. jejuni* can produce prolonged and severe illness lasting several weeks, but most patients recover after one week (Blaser, 1989). Most infections are self-limiting although patients can suffer a relapse, with symptoms usually not so severe as with the first attack. Extraintestinal infections have been described, including cases of meningitis, cholecystis and urinary tract infection (Butzler *et al*, 1992; Walker *et al*, 1986); bacteraemia occurs in the early stages of the disease, probably more often than reported

(Butzler *et al*, 1992). Complications are uncommon but there has been reports associating *C. jejuni* enteritis with Reiter's syndrome, reactive arthritis and Guillain-Barré syndrome (Walker *et al.*, 1986; Butzler *et al.*, 1992; Skirrow & Blaser, 1992).

Table 2. Symptoms of *Campylobacter* enteritis (*in* Butzler *et al*, 1992).

Disease phase	Symptoms	Duration
Prodromal	Malaise, headache, dizziness, anorexia, myalgia, arthralgia, fever	Few hours to few days
Diarrhoeic	Abdominal cramps, profuse diarrhoea, watery or slimy stools containing inflammatory exsudate, leukocytes and fresh blood	2 to 10 days
Recovery	Abdominal pain may persist	3 days to 3 weeks
Complications	Cholecystitis, urinary tract infection, bacteraemia, meningitis, arthritis, Reiter's syndrome, Guillain-Barré syndrome	

In contrast, in developing countries, infection is most commonly asymptomatic or associated with a milder illness of much shorter duration than in industrialized countries (Blaser, 1989; Skirrow & Blaser, 1992; Taylor, D.N., 1992). The primary symptomatology is watery diarrhoea without fever. No blood or leukocytes occur in the stools.

3.5 Diagnosis of campylobacteriosis

The symptoms and signs of *C. jejuni* enteritis are not so distinctive that a physician can readily differentiate them from infections caused by other enteric pathogens (Blaser, 1989; Butzler *et al*, 1992). In cases where *C. jejuni* produces a mild infection, symptoms might last for no longer than 24h and can resemble a viral gastroenteritis. In more severe *C. jejuni* infections, the symptoms are not distinguishable clinically from those induced during *Salmonella*, *Shigella* or *Yersinia* enteritis (Blaser, 1989). Severe illness can often mimic and be wrongly treated as a case of appendicitis or ulcerative colitis (Blaser, 1989). Despite these

problems, *C. jejuni* enteritis can be rapidly diagnosed by identifying the organisms in fresh faeces, either by Gram-staining or dark-field microscopy (Blaser, 1989; Butzler *et al*, 1992). Campylobacters can be easily distinguished from other organisms due to the characteristic morphology and motility. Nevertheless, these rapid means are not sufficient. The isolation of the organism from stools by selective filtration or by culturing on selective media is considered the basis for diagnosis (Blaser, 1989; Kaijser & Megraud, 1992). The first method allows isolation of all *Campylobacter* species as it is based on size selection but is of low sensitivity. The accuracy of the results depends on the concentration of bacteria in the stool sample ($>10^4$) (Kaijser & Megraud, 1992). On the other hand, culturing the organism on selective media shows higher sensitivity but only allows isolation of some *Campylobacter* species, including *C. jejuni* and *C. coli*. Considering that these species are the most common causative agents of campylobacteriosis, the second method has been considered sufficient for routine diagnosis (Kaijser & Megraud, 1992). Serology is not generally useful because of the long delay in waiting for convalescent serum (Blaser, 1989). However, in culture-negative cases of *C. jejuni* infection, for example extraintestinal complications such as reactive arthritis, serologic diagnosis is recommended (Butzler *et al*, 1992).

3.6 Treatment of campylobacteriosis

C. jejuni infections are usually self-limiting. In mild cases, *C. jejuni* is excreted for only a few days after the onset of symptoms and most patients recover in less than a week. Treatment consists mainly of replacing the lost liquid, protecting the patient from dehydration. However, in severe illness, *C. jejuni* excretion can last from two to seven weeks and chemotherapy is justifiable. In these cases, the use of erythromycin has been advised (Blaser, 1989; Butzler *et al.*, 1992). *C. jejuni* strains resistant to this antibiotic have been rarely reported (Blaser *et al.*, 1983; Butzler *et al.*, 1992; Tenover *et al.*, 1992).

3.7 Prevention of *C. jejuni* infection

Prevention of *C. jejuni* infection depends primarily on the interruption of transmission to humans. Given its growth requirements (section 2.1), *C. jejuni* is unlikely to survive well

outside a host. *C. jejuni* is also unlikely to grow or survive well in foods (Doyle & Jones, 1992; ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993). Bacteria die rapidly at ambient temperatures and do not survive storage at 25°C. They are sensitive to atmospheric levels of oxygen and to dehydration. However, these organisms may survive for weeks on the surface of meats stored at 4°C. *C. jejuni* is sensitive to freezing but some cells remain viable on the surface of poultry kept at -20°C. The bacterium may also remain viable for hours on moist hands or surfaces (Griffiths & Park, 1990; Doyle & Jones, 1992; Norcross *et al.*, 1992; ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993). Nevertheless, *C. jejuni* is sensitive to heat, to acidic conditions and is rapidly killed by chlorine-based desinfectants (Griffiths & Park, 1990; Norcross *et al.*, 1992; ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993).

Meats can be contaminated by a large number of organisms and there are reports showing the isolation of 10^8 bacteria per chicken on display in supermarkets (Doyle & Jones, 1992; ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993). Therefore, control measures should focus on the prevention of contamination and cross-contamination of food or utensils used to deal with food. The best methods will be to promote adherence to sensible public health precautions such as consumption of treated water and pasteurised milk, hygienic handling of all raw and properly cooked meats (especially poultry). In the long term, prevention will be based on the reduction of the reservoirs and the control of infection at all stages of food processing (Norcross *et al.*, 1992; ACMSF (Advisory Committee on the Microbiological Safety of Food), 1993).

4. THE PATHOGENESIS OF *C. jejuni*

C. jejuni is a food-borne pathogen. The organism enters the host by ingestion of contaminated food or drink, reaches the intestinal tract and colonises the intestinal mucus. Experimental evidence supports bacterial adherence to mucosal surfaces, indicates invasion of epithelial cells and/or the production of toxin(s). In either case, the bacterium damages the epithelial cell function and perturbs the normal absorptive capacity of the intestine to induce diarrhoea. Moreover, *C. jejuni* may translocate across the epithelium and penetrate the lamina propria and mesenteric lymph nodes, inducing extraintestinal complications. It is not clear whether the same strain can express several of these virulence attributes or not. However,

several of these mechanisms may be used individually or in concert to produce infection, depending on the host status and characteristics of the infecting strain.

4.1 Colonisation of the intestinal mucosa

C. jejuni enters the host through the faecal-oral route. In association with solid or liquid food, the organism travels through the stomach where it may or may not survive the effects of the hydrochloric acid and proteolytic enzymes. Once past the gastric barrier, *C. jejuni* reaches the intestine where it colonises the distal ileum and the colon. Since the surface of the intestinal tract is covered with a thick layer of mucus, association with intestinal mucus is thought to be the first step for colonisation, followed by attachment to the epithelial cells.

4.1.1 Association with intestinal mucus

In the intestine, the mucous blanket lining of the gastrointestinal tract is likely to be the initial contact point between the host and the bacterium. Association with mucus is an initial step for the colonisation of the intestine, involving adhesion to the surface and penetration into the mucus gel. Lee and co-workers showed that two human isolates of *C. jejuni* colonised the mucus on the outer surface and deep within the intestinal crypts in a mouse caecal model (Lee *et al.*, 1986). Previously, another group also showed *C. jejuni* present on, in and below the mucous gel in the lower ileum of infected neonatal mice (Field *et al.*, 1981).

Motility is likely to be an important colonisation factor. Mucus forms a highly viscous gel, mainly composed of a heterogeneous mixture of proteins, polysaccharides and large molecular weight glycoproteins or mucins, resulting in a structured network. Bacteria can be trapped in this environment which contains secretory immunoglobulins (sIgA) (McSweegan *et al.*, 1987) and other substances that either kill bacteria (*e.g.*, lysozyme or lactoperoxidase) or inhibit their growth (*e.g.*, iron chelators like lactoferrin) (Salyers & Whitt, 1994). Motility is essential for the bacterium to traverse this gel and to reach the mucosal surface where the organism damages epithelial cell function. Morooka *et al.* compared nonspecific nonmotile mutants to wild type cells in their ability to colonise the intestinal tract of neonatal mice (Morooka *et al.*, 1985). They showed that a nonmotile mutant which had the complete flagellar

structure did not colonise the intestine of mice. Other studies also showed that nonspecific nonmotile mutants were either unable to colonise or showed poor colonisation of the intestinal tract of experimentally challenged hamsters (*cit. in* Nachamkin & Yang, 1992) and suckling mice (Newell *et al.*, 1985). Furthermore, genetically defined nonmotile mutants did not colonise or colonised poorly the intestinal tract of experimentally challenged chickens (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993b). Thus, motility is an important physical property for the colonisation of the intestinal tract. However, this implies that the bacterium must be able to move within the highly viscous mucus gel. In fact, *in vitro* studies showed that *C. jejuni* retains its motility in viscous solutions usually capable of immobilising flagellated bacteria, including *Vibrio cholerae*, *Salmonella enteritidis* or *E. coli.* At low viscosities, *C. jejuni* seems to move like other flagellated bacteria with the flagella propelling them. At high viscosities, when the flagella can no longer overcome the viscous drag on the cell, the spiral form becomes more accentuated and motility becomes less random. The bacterium moves farther in longer path lengths and faster, tracking along the mucus strands. Changes in flagellar conformation and/or rotation occur, which combined with the spiral shape may enable the bacteria to move more effectively in intestinal mucus (Ferrero & Lee, 1988). This point of view is supported by other work. Lee and colleagues (Lee *et al.*, 1986) examined under phase-contrast microscopy scrapings of mucosa from caecal tissue of antibiotic-treated mice infected with human isolates of *C. jejuni.* They observed that *C. jejuni* was highly motile in the intestinal tissue but exhibited different types of motility. When no tissue was present in the preparations for microscopy, the direction of motility was random. If the bacterium appeared associated with the mucus, the organism moved extremely rapidly across the field of view and in parallel streams in the mucus gel. It is possible that the fact that *C. jejuni* is spiral-shaped, microaerophilic and possesses motility in viscous matrixes constitute important ecological advantages in the intestine of the host (Lee *et al.*, 1986; Ferrero & Lee, 1988).

Chemotaxis is also an important colonisation factor. Bacterial movement is generally directed by chemotactic responses (Takata *et al.*, 1992) and the intestinal mucus has been reported to be chemotactic for *C. jejuni*, in addition to serine and fucose, two major constituents of mucins (*cit. in* McSweegan & Walker, 1986). If more chemoattractant is present in the mucus layer than in the lumen, a gradient of concentration of chemoattractant may drive *C. jejuni* into penetrating the mucus gel and thus moving towards the epithelial cells (Takata *et al.*, 1992). This possibility was supported by de Melo and Pechère who showed that *C. jejuni* internalisation by HEp-2 cells was enhanced in the presence of mucin (de Melo & Pechère, 1988). Consequently, these authors proposed that mucin promoted infectivity and was essential for intestinal colonisation. Furthermore, nonchemotactic, fully motile *C. jejuni* mutants were unable to colonise the neonatal mouse intestinal tract (Takata *et al.*, 1992).

In summary, colonisation may require chemotactic-driven motility (Takata *et al.*, 1992). The persistence of *C. jejuni* in the intestinal mucus probably results from the interplay between motility and chemotaxis. Pathology may require subsequent interaction with epithelial cells. Whether the bacterium actually reaches the epithelial cells might depend on the interactions with the mucus gel.

4.1.2 Adhesion to the mucosal surfaces

Adhesion reflects the ability of the pathogen to interact with the host surfaces. It might be the first stage of an invasive process or/and a way for the bacterium to get close enough to the cells to facilitate toxin action. In the absence of adhesion, the bacterium would have difficulty in remaining in contact with the mucus and would be washed away by the mucus secretions and peristaltic motion (Williams *et al.*, 1988). It is possible that *C. jejuni* ability to colonise intestinal mucus could prevent this, but certainly, it would be less likely that invasion would occur (see section 4.2) or/and that bacterial toxins would reach their target (see section 4.4.5).

The adhesion of *C. jejuni* to epithelial-like cells *in vitro* is well documented (Wooldridge & Ketley, 1997). Adhesion seems necessary for colonisation of the intestine and subsequent pathogenesis. However, Lee *et al.* (1986) postulated that adhesion of *C. jejuni* to tissue was unlikely to be an important factor. They suggested that colonisation of the intestine occurs by association with intestinal mucus. In this environment, the bacteria may achieve close enough proximity to the mucosa to exert their final pathogenic potential. Nevertheless, *C. jejuni* invasiveness is well established and adhesion seems a prerequisite for invasion *in vitro*.

The ability of *C. jejuni* to adhere *in vitro* to a variety of epithelial-like cell lines has been demonstrated by several different groups of researchers (Wooldridge & Ketley, 1997). In comparative studies, bacteria were found to adhere with equal efficiency to cultured cells of human and non-human origin whereas some strains were found to be unable to adhere *in vitro* (Fauchère *et al.*, 1986; Konkel & Joens, 1989).

The pattern of adherence to HeLa and INT 407 cells was described as localised adhesion (Fauchère *et al.*, 1986; Konkel *et al.*, 1992a). Electron microscopic observations showed bacteria in close apposition to the host cell. Following apical association, the host microvilli appeared damaged or absent (de Melo *et al.*, 1989; Russell *et al.*, 1993). Fauchère and co-workers also observed bacteria dispersed over the surface of HeLa cells. It is not clear

whether this distribution corresponded to a different pattern of adherence or to a pre-adherence stage (Fauchère *et al.*, 1986).

C. jejuni adhesion does not require bacterial *de novo* protein synthesis because treatment of bacteria with a selective inhibitor of bacterial protein synthesis (chloramphenicol) does not affect binding to INT 407 cells. Moreover, non-viable campylobacters are still able to attach to host cells. This suggests that bacteria express the adherence factors constitutively (Konkel & Cieplak, 1992).

Adhesion is described as a dynamic process. The interaction requires participation of the pathogen as well as the host. This process is usually mediated by bacterial adhesins that interact with the host receptors. The nature of *C. jejuni* adhesins and the corresponding host receptors remains unclear. Since the bacterial outer membrane is the interface between host and bacterium, flagella, LPS and OMP have been extensively examined for the role of putative adhesins (section 4.4). Some authors attempted to completely block adhesion in order to determine the nature of the bacterial factors involved. Prior to the adhesion assay, the host cells were incubated with purified flagella or LPS (McSweegan & Walker, 1986; Wassenaar *et al.*, 1991). In every case, a reduction in the ability of *C. jejuni* to bind cultured cells was noted but binding was never totally abolished. Therefore, adhesion of *C. jejuni* to cultured cells may be mediated by multiple adhesins.

Flagella may have two roles in the adherence process (section 4.4.3). Both roles are very difficult to assess separately. Flagella-mediated motility is necessary for the bacterium to reach the mucosal surface. *C. jejuni* aflagellate mutants are unable to adhere to epithelial cells *in vitro* (McSweegan & Walker, 1986; Yao *et al.*, 1994). Aflagellate mutants are also unable to colonise the intestinal tract of a chicken model (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993b). Additionally, the flagellum may have a secondary role in adhesion. Some authors have suggested that one of the flagellum subunits, FlaA, may have adhesive properties but the evidence in support of this hypothesis remains ambiguous (Grant *et al.*, 1993; Yao *et al.*, 1994). The importance of the flagellum as a putative virulence determinant is discussed in detail in section 4.4.3.

In addition to the flagellum, two outer membrane proteins, PEB1 and PEB4, as well as LPS have been implicated as potential adhesins. However, the results that suggested their involvement in adhesion have been subsequently thought to result from experimental artefacts (sections 4.4.1 and 4.4.2).

4.2 Invasion

Firm adherence to the host cell surface facilitates the establishment of *C. jejuni* in the new niche. However, the success of the pathogen within the host depends on its capacity to multiply in sufficient numbers to ensure survival and transmission into another host. The intracellular environment, rich in nutrients and free of competitors, provides a good site for proliferation (Williams *et al.*, 1988; Finlay & Falkow, 1989).

The ability of *C. jejuni* to invade epithelial cells is well documented. Invasion has been observed *in vivo* with experimentally challenged infant *Macaca mulatta* (Russell *et al.*, 1993), three-day-old chicks (Ruiz-Palacios *et al.*, 1981) and newly hatched chicks (Welkos, 1984). In addition, van Spreeuwel *et al.* detected intracellular *C. jejuni* in colonic epithelial cells in biopsy specimens from patients with colitis (Spreeuwel *et al.*, 1985). *In vitro*, invasiveness has also been demonstrated with a variety of cultured cell lines. Comparative studies showed that internalisation occurs more efficiently when the cell line used is of human origin (Konkel *et al.*, 1992a). Some researchers have attempted to enhance the relevance of *in vitro* invasion assays by using primary swine intestinal cells (Babakhani & Joens, 1993) or the Caco-2 cell line which shows spontaneous enterocyte-like differentiation (Everest *et al.*, 1992; Russell & Blake, 1994; Szymanski *et al.*, 1995).

Although enteroinvasiveness is an important feature of campylobacteriosis, not all strains are invasive. In addition, the relative ability of invasive isolates to enter epithelial cells is variable and strain-dependent (Konkel & Joens, 1989; Everest *et al.*, 1992; Konkel *et al.*, 1992a; Oelschlaeger *et al.*, 1993). Extensive *in vitro* passaging of clinical isolates often leads to a decreased invasiveness, recoverable at times by *in vivo* passaging (Welkos, 1984; Konkel *et al.*, 1990; Cawthraw *et al.*, 1996). Fresh clinical isolates tend to be more invasive but the phenotype of the isolate does not always correlate to the clinical picture induced by those isolates. Everest *et al.* found that a significant number of isolates from patients with non-inflammatory diarrhoea were able to invade Caco-2 cells (Everest *et al.*, 1992). However, they also found that all isolates from patients with colitis invaded Caco-2 cells. In a similar study by other researchers, no significant difference was found between the propensity of isolates from either inflammatory or non-inflammatory diarrhoea to invade HEp-2 cells (*cit. in* Wooldridge & Ketley, 1997).

4.2.1 Invasion mechanism

The cellular processes and requirements underlying *C. jejuni* invasion have been investigated extensively through the use of electron microscopy and biochemical inhibitors of eukaryotic processes and structures. Current evidence shows that the uptake of *C. jejuni* by epithelial cells occurs via a phagocytosis-like mechanism. Electron microscopic observations show the engulfment of bacteria by membrane invaginations and formation of an endocytic vacuole approximately 1h post-infection. Furthermore, cellular pseudopods were observed enveloping attached bacteria (Fauchère *et al.*, 1986; de Melo *et al.*, 1989; Konkel *et al.*, 1992a, b; Russell *et al.*, 1993). An active participation of the host cell is also supported by the inhibition of bacterial internalisation following the treatment of HEp-2 host cells with inhibitors of energy-producing pathways (iodoacetate which blocks glycolysis; dinitrophenol which blocks the Krebs cycle) (de Melo *et al.*, 1989). Host *de novo* protein synthesis is not essential for *C. jejuni* uptake because treatment of INT 407 host cells with a selective eukaryotic protein synthesis inhibitor (cycloheximide) had no effect on the internalisation of bacteria (Konkel & Cieplak, 1992; Oelschlaeger *et al.*, 1993).

C. jejuni internalisation appears associated with the deposition of dense layers of actin-like filaments, as visualised by transmission electron microscopy. The deposition of the fibrillar material occurs beneath the invaginated plasma membrane, at the sites of bacterial close attachment and entry, (de Melo *et al.*, 1989; Konkel *et al.*, 1992b; Russell *et al.*, 1993). Immunofluorescence microscopy with fluorescein-conjugated phalloidin confirmed the assembly of actin filaments in association with attached bacteria (Konkel *et al.*, 1992b). In addition to electron microscopy techniques, biochemical inhibitors of actin polymerisation (cytochalasin B and D) were used to further investigate the involvement of microfilaments with the bacterial uptake. In this regard, conflicting reports were published. In an early study, de Melo and colleagues described the inhibition of *C. jejuni* internalisation by HEp-2 cells in the presence of cytochalasin B (de Melo *et al.*, 1989). These effects were subsequently corroborated by another group, with a different inhibitor, cytochalasin D, and a different cell line, INT 407, (Konkel *et al.*, 1992b). However, Oelschlaeger *et al.* (1993) concluded that the involvement of microfilaments in *C. jejuni* endocytosis is strain- and possibly cell line-dependent. These authors found that cytochalasin D did not affect the internalisation of one strain (*C. jejuni* 81-176) by INT 407 cells but caused a 50% reduction in the internalisation of another strain. In another study, Russell and co-workers reported that endocytosis of *C. jejuni* 81-176 by Caco-2 cells was unaffected by either cytochalasin B or D and therefore, was microfilament independent (Russell & Blake, 1994). The ambiguity of the results may be attributed partly to the use of different assay conditions (different concentrations and

incubation times) but, more likely, to differences among cell lines (differences in the nature and availability of receptors). Despite the contradictory results obtained with chemical inhibitors, electron microscopic observations appear conclusive about the cytoskeletal rearrangements occurring during *C. jejuni* uptake.

To further evaluate the involvement of host cytoskeketal components in the internalisation mechanism, the participation of microtubules was also investigated. According to Oelschlaeger *et al.*, the invasive ability of *C. jejuni* was reduced by microtubule depolymerisation (following the treatment of the host cells with colchicine, vincristine or vinblastine). Thus, *C. jejuni* entry into INT 407 cells may occur via either a microfilament- (see above) or microtubule-dependent pathway, possibly as consequence of the different surface receptors in a particular cell line (Oelschlaeger *et al.*, 1993). Konkel *et al.* also reported a variable and inconsistent effect of colchicine on the invasion of INT 407 cells by *C. jejuni*, which may reflect the participation of a microtubule-dependent pathway in some, as yet unknown, way (Konkel *et al.*, 1992b). Other researchers have not found any evidence of microtubule involvement (Russell & Blake, 1994).

4.2.2 Bacterial factors

In order to invade, *C. jejuni* must interact with a specific receptor(s) on the host cell surface, through specific factors. The inability of epithelial cells to phagocytose implicates some sort of bacterial modulation of host processes, facilitating subsequent uptake (Finlay & Falkow, 1989). As yet, neither the bacterial factors nor the host receptor involved in these interactions have been identified.

In competitive invasion assays, *C. jejuni* lysates adsorbed to HEp-2 cells specifically inhibited *C. jejuni* invasion (Konkel & Joens, 1989; Konkel *et al.*, 1990). Pretreatment of these lysates with proteinase K had no effect on the inhibition of invasion. However, periodate oxidation of the lysates prior to adsorption significantly affected the lysate blocking effect (Konkel & Joens, 1989). These findings indicated that the factors involved in invasion appear to depend upon an intact carbohydrate moiety. Konkel and colleagues reported that invasion of HEp-2 cells was competitively inhibited by a monoclonal antibody against *C. jejuni* (Konkel *et al.*, 1990). Proteinase K or sodium periodate treatment of the lysates changed the mobility of the antigens, reflecting the partial degradation of the molecules. These findings suggested that the invasive ligand is a glycoprotein (Konkel & Joens, 1989; Konkel *et al.*, 1990). Immunogold electron microscopy showed that the antibody bound to epitopes on the bacterial surface and

flagella of invasive strains but only to the flagella of non-invasive strains. The authors proposed that the bacterial surface and the flagella share a common epitope. Furthermore, there is a difference in antigens exposed on the surface of invasive and non-invasive strains. The antigens specific to invasive strains are likely to take some part in the process of internalisation (Klipstein *et al.*, 1985; Konkel *et al.*, 1990).

Other groups have attempted to determine the nature of the invasive ligand by investigating the *C. jejuni* protein synthetic response to cultured cells. Konkel and colleagues reported that the expression of several proteins was induced or preferentially enhanced in response to cocultivation with INT 407, Caco-2 or HEp-2 cells (Konkel & Cieplak, 1992; Konkel *et al.*, 1993). Some of those novel proteins also appeared in response to cultivation in INT 407-conditioned medium (Konkel & Cieplak, 1992). These findings raised two possibilities. First, the protein profile was, at least partly, altered in response to some unknown attributes of the conditioned medium. Such attributes could be the presence of epithelial cell-derived products as well as physicochemical properties of the medium. Second, the proteins synthesised only in the presence of the host cells could play a role in the internalisation process. The second possibility was supported by the fact that chloramphenicol-treated *C. jejuni* bound to INT 407 cells but failed to invade (Wassenaar *et al.*, 1991; Konkel & Cieplak, 1992). The need for metabolically active bacteria was also demonstrated by the inability of non-viable organisms (heat-killed or treated with a respiratory inhibitor, sodium azide) to enter INT 407 cells (Konkel & Cieplak, 1992). Additionally, rabbit antiserum prepared against *C. jejuni* cultured with the epithelial cells specifically inhibited the internalisation of *C. jejuni* in a dose-dependent fashion (Konkel *et al.*, 1993). The serum blocking effect further supported the potential involvement of the newly synthesised proteins in the internalisation process.

In another study, Panigrahi and colleagues examined changes in the bacterial protein profile in response to cultivation in rabbit ileal loops (Panigrahi *et al.*, 1992). They found that *C. jejuni* expressed a few novel proteins in the ileal loops which were not expressed *in vitro*. These novel proteins were reported to have similar molecular weights to some of those newly expressed by *C. jejuni* in presence of cultured cells (Konkel & Cieplak, 1992; Konkel *et al.*, 1993). It is, therefore, possible that a correlation may exist between the *in vitro* observations and the situation in the rabbit ileal loops. The novel proteins may indeed be expressed in response to contact with enterocytes (Panigrahi *et al.*, 1992; Konkel *et al.*, 1993). Panigrahi and colleagues also described how two of the newly synthesised proteins in the rabbit ileal loops, detected at 48h post-infection, appeared to elicit an immune response in humans as they reacted with convalescent sera from patients with *C. jejuni* infection. Nevertheless, the identity of the novel proteins expressed in presence of cultured cells or in the rabbit ileal loops has yet to be determined.

C. jejuni invasion of epithelial cells is dependent on flagella-mediated motility. *In vitro* studies show that non-motile flagellar mutants show a greatly reduced ability to penetrate INT 407 and Caco-2 cells (Wassenaar *et al.*, 1991; Grant, C.C.R. *et al.*, 1993; Russell & Blake, 1994; Yao *et al.*, 1994). When motility dependent effects are eliminated by centrifugation of the bacteria onto the target cells, the level of penetration usually rises but to levels lower than those seen with the wild type. *In vivo*, the flagella were observed spirally wrapped around the bacteria during entry into colonic epithelial cells from *Macaca mulatta* (Russell *et al.*, 1993). Subsequently, based on the *in vivo* observations associated with the *in vitro* inability of aflagellate mutants to invade, these authors postulated a functional involvement for the flagella in the invasion process. According to Russell and Blake, only one flagellum would wrap around the organism during entry, while the second polar flagellum would propel the bacterium into the cells in a drill-like rotation (Russell & Blake, 1994). Such hypothesis is supported by the fact that immobilised flagella facilitate adherence but abolish invasion, implicating a requirement for flagella rotation during entry (section 4.4.3; McSweegan & Walker, 1986; Yao *et al.*, 1994).

4.2.3 Host receptor

The use of antagonists of clathrin-coated pit formation (monodansylcadaverine and g-strophantin) has a marked inhibitory effect on the uptake of *C. jejuni* by INT 407 cells (Konkel *et al.*, 1992b; Oelschlaeger *et al.*, 1993). These results suggest a role for clathrin-coated pits in the invasion pathway. The inhibition of endosome acidification which potentially affects bacterial uptake by preventing receptor recycling to the cell surface has little or no effect on the internalisation of *C. jejuni* (Konkel *et al.*, 1992b; Oelschlaeger *et al.*, 1993). However, these results contrast a report that entry of *C. jejuni* into Caco-2 cells is independent of clathrin-coated pits (Russell & Blake, 1994). This work is consistent with a more recent study investigating host cell signal transduction involved in the endocytosis of *C. jejuni* (Wooldridge *et al.*, 1996). Here, treatment of Caco-2 cells with an agent (filipin III) which disrupts caveolae (a non-clathrin-coated plasma membrane invagination) inhibits the uptake of *C. jejuni*, implicating the involvement of clathrin-independent endocytosis. *C. jejuni* uptake by Caco-2 cells is also impaired by treatment of the host cells with inhibitors of protein phosphorylation (staurosporine), tyrosine protein kinases (genistein), phosphatidylinositol 3-kinase or PI 3-kinase (wortmannin) and a subfamily of heterotrimeric G-proteins (cholera toxin) (Wooldridge *et al.*, 1996). None of these compounds affects bacterial attachment to Caco-2 cells. The effects

observed are specific to *C. jejuni* as any of the compounds tested interfered in the uptake of *Salmonella typhimurium* by Caco-2 cells. Based on their findings, Wooldridge *et al.* (1996) postulated that *C. jejuni* host receptor localises to caveolae. Subsequent interactions between the bacterial ligand and the host cell receptor will trigger a sequence of signalling events which will be transduced across the host cell membrane. Such events have not yet been identified but may involve G-proteins and tyrosine phosphorylation, the outcome being actin rearrangements and subsequent endocytosis.

4.2.4 Intracellular residence and survival

Once inside the eucaryotic cell, *C. jejuni* remains inside the endosome. In the long term, bacteria are visualised free in the cytoplasm when the host cell begins to deteriorate (72h post-infection of INT 407 cells) (Fauchère *et al.*, 1986; de Melo *et al.*, 1989; Konkel *et al.*, 1992b; Russell *et al.*, 1993). The host cell may contain from 2 to 5 bacteria (Fauchère *et al.*, 1986), although as many as 30 bacteria may be observed per cell at 96h post-inoculation (Konkel *et al.*, 1992b). Endosomes are frequently located close to the host cell nucleus or in the Golgi area (Fauchère *et al.*, 1986; Konkel *et al.*, 1992b). According to de Melo and colleagues, (1989), three hours after infection, there was mobilisation of lysosomes in the phagosome area. After a longer period, they started to observe what looked like phagosome-lysosome fusions. Inside the fused phagosome-lysosome, bacteria lost their viable S-shape and became coccoid, myelinic structures were present and there was intense acid phosphatase activity. These events were also observed by other groups who, in addition, reported the presence of viable bacteria within other phagosomes (Fauchère *et al.*, 1986; Konkel *et al.*, 1992b). *C. jejuni* was shown to survive inside INT 407 cells for at least 96h. At this time, the degree of host cell degeneration was very high and it was no longer possible to continue evaluating *C. jejuni* viability within INT 407 cells (Konkel *et al.*, 1992b).

Recently, a mutant in the superoxide dismutase gene (*sodB*) was isolated. A *sodB*$^-$ mutant showed reduced ability to survive inside INT 407 cells when compared to the parental strain (Pesci *et al.*, 1994). Since the catalase gene (*katA*) has also been identified in *C. jejuni* (Grant, K.A. & Park, 1995), it is possible that *C. jejuni* possesses a defence system against oxidative stress which enables the bacterium to survive intracellularly. Intracellular survival may, therefore, be influenced by reactive oxygen species. In contrast to *Salmonella typhimurium*, the defence system of *C. jejuni* against oxidative stress would not require the

product of *htrA* (high temperature requirement) because a *htrA*⁻ mutant is insensitive to oxidative stress and invades Caco-2 cells as well as the parental strain (Henderson, 1996).

During infection, host cells are reported to undergo pronounced cytopathic effects, detectable at 72h post-infection (Konkel *et al.*, 1992b). Cells become vacuolated and rounded and viability declines. Russell and colleagues also described cell damage in colonic epithelial cells from *Macaca mulatta*, characterised by cytoplasmic swelling, loss of microvilli, evidence of premature apoptosis and exfoliation into the lumen (Russell *et al.*, 1993).

Early studies found no evidence of *C. jejuni* replication within the host cell (de Melo *et al.*, 1989). However, a more recent study reported the presence of dividing bacterial forms within infected INT 407 cells at 96h post-inoculation (Konkel *et al.*, 1992b). Intracellular replication is also implied by the appearance of multiple bacteria within the vacuoles. *C. jejuni* not only possesses the ability to survive inside epithelial cells, even for long periods (up to 96h, according to (Konkel *et al.*, 1992b)), but also appears to retain the potential to replicate.

4.3 Epithelial translocation

Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract to extraintestinal sites, such as the submucosa, blood and the reticuloendothelial system (Berg, 1992).

C. jejuni is able to translocate across polarised epithelial-like Caco-2 cell monolayer (Everest *et al.*, 1992; Konkel *et al.*, 1992c). *In vivo*, *C. jejuni* has been observed in the submucosa and in the lamina propria in experimentally challenged chicks (Welkos, 1984) and infant macaque monkeys (Russell *et al.*, 1993). These observations, in conjunction with clinical features of campylobacteriosis (tissue damage and inflammation) and occasional *C. jejuni*-induced extraintestinal infections, indicate that translocation is likely to be of relevance for *C. jejuni* pathogenesis. However, *C. jejuni* invasion has been reported to cause pronounced cytopathic effects to the host cells. Invasion followed by cell lysis may be another way for *C. jejuni* to gain access to tissues underlying the epithelium.

C. jejuni translocation across Caco-2 cell monolayers requires *de novo* bacterial protein synthesis. Chloramphenicol-treated bacteria show a reduced ability to translocate. However, chloramphenicol treatment fails to completely abrogate translocation, suggesting that some of the factors required may be expressed constitutively. Furthermore, non-viable organisms (gentamicin-treated) also fail to cross the monolayer, corroborating the need for metabolically

active bacteria (Konkel *et al.*, 1992c). The ability of *C. jejuni* to cross a Caco-2 cell monolayer also depends on a functional flagellum. Grant and colleagues demonstrated that *flaA*$^-$ mutants (*flaA*$^-$*flaB*$^-$ and *flaA*$^-$*flaB*$^+$) were unable to traverse the cell monolayer (Grant, C.C.R. *et al.*, 1993). Therefore, either motility, or the flagellin FlaA, or both, are essential for *C. jejuni* translocation.

The route followed by *C. jejuni* during translocation is not well defined. A single report suggested that *C. jejuni* may translocate via the M cells (Walker *et al.*, 1988). Given that *C. jejuni* is an invasive organism, translocation probably also occurs through the cytoplasm of the epithelial cells. Furthermore, transmission electron microscopy showed *C. jejuni* present in intercellular spaces of Caco-2 (Konkel *et al.*, 1992c) and INT 407 (Oelschlaeger *et al.*, 1993) cell monolayers and cells from intestinal epithelium of experimentally challenged chicks (Welkos, 1984) and infant *Macaca mulatta* (Russell *et al.*, 1993). These observations suggest that *C. jejuni* may also translocate via a paracellular route.

4.4 *C. jejuni* putative virulence determinants

The virulence determinants of *C. jejuni* have not been well determined. Research has focused on bacterial surface exposed components as more likely to be responsible for association and invasion of the host cells. Experimental evidence provides support for a few potential candidates, including outer membrane proteins (OMP), lipopolysaccharides (LPS), flagellins and aggregative fimbriae or pili. However, their role in pathogenesis has not been clearly demonstrated. Toxins are also important bacterial virulence determinants and *C. jejuni* may produce several.

4.4.1 Outer membrane proteins

In preliminary studies, two immunogenic OMP, PEB1 (or CBF1) and PEB4 (or CBF2), with apparent molecular masses of 28 and 31 kDa, respectively, were considered potential adhesins (Pei *et al.*, 1991; Kervella *et al.*, 1993). The two proteins were antigenically cross-reactive with convalescent-phase serum from *C. jejuni* infected patients (Dubreuil *et al.*, 1990; Pei *et al.*, 1991). PEB1 was shown to bind to HeLa cells (Fauchère *et al.*, 1992). Moreover,

preincubation of HeLa cells with PEB1 blocked the adhesion of *C. jejuni* (Kervella *et al.*, 1993). Subsequently, immunogold labelling showed that only PEB1 was surface exposed (Pei *et al.*, 1991). Cell fractionation studies indicated that PEB4 was associated with the periplasmic surface of the inner membrane. In view of the cellular location of the two proteins, Dubreuil and colleagues suggested that PEB4 may form a complex with PEB1 and co-adhere to the host cell membrane (Dubreuil *et al.*, 1990) but this hypothesis has not been proven.

In order to genetically characterise the two "adhesin-like" proteins, the respective genes were isolated and sequenced. DNA sequence analysis revealed surprisingly that the HeLa cell binding proteins were homologous to components of bacterial transport systems. PEB1 (*pebIA*) shared homology with the binding component of bacterial amino acid transport systems (Pei & Blaser, 1993). The gene encoding PEB4 was similar to a gene encoding a part of a protein export system (*cit. in* Ketley, 1997). Furthermore, PEB1 was not required in the colonisation of the chicken model of infection (*cit. in* Ketley, 1997). Based on the genetic and colonisation results, it has been suggested that the proteins binding to cultured cells is an artefact and consequently, PEB1 and PEB4 are unlikely to be adhesins.

4.4.2 Lipopolysaccharides

LPS are major heat-stable antigens of *C. jejuni*. Most *C. jejuni* serotype reference strains (i.e. serostrains) are found to only contain the low-Mr LPS fraction (consisting of the lipid A and the core oligosaccharide) (Preston & Penner, 1987). However, *C. jejuni* low-Mr LPS are apparently linked to a short single oligosaccharide unit (equivalent to a single O-antigen repeating unit), more closely resembling the lipooligosaccharides or LOS occurring in *Neisseria spp* and *Haemophilus influenzae* than the rough type LPS found in enterobacteria (Mills *et al.*, 1992). In addition, a third of *C. jejuni* serostrains contain smooth-like type LPS (consisting of the O side chain or O-antigen in addition to the low-Mr LPS fraction) (Preston & Penner, 1987).

The role of LPS in *C. jejuni* virulence is not clear. A single report has indicated that LPS may act as an adhesin (McSweegan & Walker, 1986). McSweegan and Walker observed that purified LPS specifically bound to INT 407 cells whereas oxidised LPS (which lost the O antigen due to treatment with sodium *meta*-periodate) did not. Moreover, treatment of INT 407 cells with purified LPS blocked bacterial adhesion. According to McSweegan and Walker, these results indicated that LPS display adhesive properties which are dependent upon the carbohydrate fraction. However, other researchers (Konkel & Joens, 1989) consider that the

LPS concentrations used in the blocking assays were too high and could be toxic to the host cells. Indeed, McSweegan and Walker did not check on the viability of the host cells at the end of the assay. Consequently, any interactions with the bacteria could have been inhibited and the blocking effect of LPS may have been an artefact (Konkel & Joens, 1989). Furthermore, Moser and Hellmann reported that only approximately 10% of the total binding of different *C. jejuni* strains to the murine small intestinal epithelial membranes was found to be mediated by LPS (Moser & Hellmann, 1989).

4.4.3 Flagella

4.4.3.1 Characteristics. *C. jejuni* flagellum is formed by two flagellin proteins, FlaA and FlaB. The genetics of flagellar production reveals that the two flagellin genes, *flaA* and *flaB*, are adjacent on the chromosome in a head-to-tail configuration. The genes are of equal size (~1.7kb) and share 95% sequence identity, most heterogeneity occurring at the 5' and 3' regions and in a small central region of the genes. The *flaA* and *flaB* genes are transcribed independently and transcription of the two genes is regulated by different types of promoter: *flaA* by a σ^{28} (which in *E. coli* transcribes genes involved in chemotaxis, motility and flagella); *flaB* by a σ^{54} (which is usually activated in response to nitrogen starvation). Transcription of σ^{54} is subjected to environmental regulation in response to conditions such as temperature, pH and inorganic salt and divalent cation concentrations (Taylor, 1992; Ketley, 1995, 1997).

FlaA and FlaB have estimated molecular masses of ~59kDa but the observed molecular mass on protein gels is approximately 62kDa due to post-translational modification of the flagellins by glycosylation (Doig *et al.*, 1996a). Under the conditions tested, FlaA is expressed at much higher levels than FlaB but both proteins are incorporated into the whole flagellar filament. Characterisation of the phenotype of mutants in either one or both flagellin genes shows that a *flaA*$^-$*flaB*$^+$ mutant produces a short, truncated flagellum and is non-motile; a *flaA*$^+$*flaB*$^-$ mutant produces a full-length flagellum, morphologically similar to the wild type flagellum but with slightly reduced motility; a *flaA*$^-$*flaB*$^-$ mutant does not produce a flagellum (Taylor, 1992; Ketley, 1995, 1997).

Flagellar expression is subject to antigenic variation thought to arise from post-translational modification by glycosylation (Doig *et al.*, 1996a). Flagellar expression is also subjected to phase variation with cells undergoing bidirectional transition between flagellated and aflagellated phenotypes (Taylor, 1992).

4.4.3.2 Putative role in pathogenesis. The flagellum is the most intensively investigated virulence determinant of *C. jejuni*. Its relevance in pathogenesis is well accepted but the precise role has yet to be determined. Flagella are certainly important as mediators of motility. Flagellar characteristics combined with the spiral shape of the bacterial cell confer a distinctive darting motility. This type of motility is believed to be essential for the bacterium to penetrate into the intestinal mucus and to come into contact with the mucosa (section 4.1.1). Aflagellate and flagellate non-motile mutants were shown to be unable to colonise or only able to colonise poorly the intestinal tracts of experimentally challenged hamsters (*cit. in* Nachamkin & Yang, 1992), suckling mice (Morooka *et al.*, 1985; Newell *et al.*, 1985) and chickens (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993).

In addition to the role of motility mediator, the flagellum may play other, secondary, roles in *C. jejuni* pathogenesis; the difficulty of assessing these roles separately from motility confuses interpretations. Some authors proposed that the flagellum may contain an adhesin (Newell *et al.*, 1985; McSweegan & Walker, 1986) or that one of the flagellins, FlaA, may be involved in the internalisation process *in vitro* (Grant, C.C.R. *et al.*, 1993; Yao *et al.*, 1994). Given the differing conclusions of the reports, it is difficult to decide on the exact involvement of the flagellum in *C. jejuni* pathogenesis.

Szymanski and colleagues studied the role of motility in *C. jejuni* infectivity in a viscous environment similar to the mucus lining of the intestinal tract (Szymanski *et al.*, 1995). They found that the attachment of *C. jejuni* to Caco-2 cells increased in the presence of viscous solutions. Because *C. jejuni* moves faster and does not tumble so often in a viscous environment (Lee *et al.*, 1986; Ferrero & Lee, 1988), the increased attachment rate may result from an increase in the frequency of impacts between the bacterium and the host cell. On contact, the flagellum may interact with the host cell surface, facilitating an initial and transient adhesion. Electron microscopy showed flagellar tips interacting with INT 407 cells prior to internalisation (Konkel *et al.*, 1992a). The results from other studies also suggest the involvement of the flagellum in adhesion. McSweegan and Walker found that removal of *C. jejuni* flagella by shearing reduced bacterial adherence to INT 407 cells, whereas treatment of bacteria with potassium cyanide to immobilise flagella increased adhesion (McSweegan & Walker, 1986). Newell and colleagues demonstrated that a flagellate, but non-motile, variant showed an increase in adherence ability to INT 407 cells relative to the parent strain or aflagellate variants (Newell *et al.*, 1985).

Initial host cell adhesion promoted by the flagellum, the first step of a two-step adhesive process, is weak and reversible (McSweegan & Walker, 1986). The bacterium can become free and thus, is released into the intestinal lumen. Alternatively, once the initial binding is accomplished, the bacterium remains immobilised long enough to establish contacts

at multiple points and to attach irreversibly. Other adhesin(s) may be involved in the second step of this two-step adhesive process. Moser and Hellmann reported that only up to 30% of the total binding of several *C. jejuni* strains to murine small intestinal membranes (brush borders) was flagella dependent (Moser & Hellmann, 1989). Treatment of INT 407 cells with purified exogenous flagella did not block *C. jejuni* adherence nor invasion of the host cells (McSweegan & Walker, 1986; Wassenaar *et al.*, 1991). Moreover, attempts to totally abrogate adhesion *in vitro* failed (section 1.2.2; McSweegan & Walker, 1986; Wassenaar *et al.*, 1991). Although flagella show an ability to bind to cultured cells, it is likely that *C. jejuni* possesses other adhesin(s).

Following the irreversible binding of bacteria, internalisation is the next step. Only irreversibly bound bacteria are internalised. It is also possible that a flagellar constituent is important in the transition between reversible and irreversible attachment to the host cell surface. *flaA*$^-$ mutants are unable to invade or invade poorly Caco-2 and INT 407 cells (Wassenaar *et al.*, 1991; Russell & Blake, 1994; Yao *et al.*, 1994). Conversely, the isogenic *flaA*$^+$*flaB*$^-$ mutants invade INT 407 cells as efficiently as the wild type (Wassenaar *et al.*, 1991). Russell and Blake proposed that one flagellum wraps around the bacterial cell during entry, while the other propels the bacterium into the host cell (Russell & Blake, 1994). Therefore, this may explain why mutants with paralysed flagella which are unable to rotate (*flaA*$^+$*flaB*$^+$*pflA*$^-$) adhere, but do not invade INT 407 cells (Yao *et al.*, 1994). Alternatively, FlaA *per se* may have a more direct involvement and be required for the internalisation process (Wassenaar *et al.*, 1991; Grant, C.C.R. *et al.*, 1993; Russell & Blake, 1994; Yao *et al.*, 1994).

Based on the data described in this section, the role(s) of the flagellum in *C. jejuni* virulence is not yet clearly defined. It is possible, however, to conclude that the flagellum plays several key roles in pathogenesis, including promoting the transport through mucus towards the surface of epithelial cells and increasing the efficiency of attachment and invasion of the host cell (Szymanski *et al.*, 1995).

4.4.4 Aggregative fimbriae

C. jejuni produces peritrichous pilus-like appendages which cause the bacteria to aggregate. The production of these structures is induced when the bacteria are grown in the presence of bile salts, in particular deoxycholate and chenodeoxycholic acid. A gene encoding a prepilin peptidase (*pspA*) was isolated and mutated. The inactivation of *pspA* results in loss of pilus synthesis. The ability of *pspA*$^-$ mutant to adhere to and invade INT 407 cells is not

affected when compared to the parental strain. In addition, the non-piliated mutant is able to colonise the intestinal tract of a ferret animal model of infection, but does cause significantly milder disease symptoms. This suggests that the aggregative pili may have a yet unkown role in *C. jejuni* virulence (Doig *et al.*, 1996b).

4.4.5 Toxin production

Extensive research has addressed toxin production by *C. jejuni*. The studies include tests with animal models and the responses of *in vitro* cultured cell lines as well as immunassay techniques and the use of genetic probes specific for genes encoding toxic factors. *C. jejuni* toxins have been compared structurally and immunologically to other toxins known from other enteropathogens in an attempt to determine their role in pathogenesis. A comparison between *C. jejuni* enteritis and infections caused by other enteropathogens provides a possible mechanistic basis for the clinical signs of campylobacteriosis. Researchers found that the induction of watery diarrhoea may be attributed to the activity of an enterotoxin in a situation akin to that caused by *Vibrio cholerae* (Wassenaar, 1997). In addition, they found that an inflammatory diarrhoea is suggestive of cytotoxic activity and, thus, has similarity to *Shigella spp* or *Clostridium difficile* infections (Wassenaar, 1997).

At present, the literature is confusing about whether *C. jejuni* is toxigenic or not. This is partly because of the wide variety of strains, bioassays and culturing conditions employed. In a recent review on toxin production (Wassenaar, 1997), Wassenaar reported that *C. jejuni* may produce different types of exotoxins, including an enterotoxin and at least five different cytotoxins. Nevertheless, it is not yet clear how many toxins are really produced, what their mode of action is and what relevance they have in disease.

4.4.5.1 *C. jejuni* enterotoxin. According to several reports, *C. jejuni* produces an enterotoxin. The frequency of production by strains obtained as clinical isolates as well as from poultry varies widely between studies. Researchers apply different methods to different strains maintained and cultured in different ways. All these varying parameters may influence enterotoxin production and detection.

The enterotoxin, termed CJT, is produced by certain strains when grown under the appropriate conditions of added amino acids (Ruiz-Palacios *et al.*, 1983) or added iron

(McCardell *et al.*, 1986). CJT is a heat-labile periplasmic protein (Ruiz-Palacios *et al.*, 1983; Calva *et al.*, 1989) and several groups have attempted to purify the toxin by affinity chromatography using anti-CT antibodies or ganglioside GM1 (because CJT apparently binds GM1; see below). The purified toxin migrates on non-denaturing protein gel as a single band of approximately 70kDa. Under denaturing conditions, two bands of 90 and 50kDa or three bands of 68, 54 and 43kDa have been obtained (*cit. in* Wassenaar, 1997).

CJT is immunologically similar to cholera toxin (CT; Ruiz-Palacios *et al.*, 1983) and to *E. coli* heat-labile enterotoxin (LT; Klipstein & Engert, 1984) but is genetically distinct from both enterotoxins (*cit. in* Wassenaar, 1997). CJT is suspected to bind the same plasma membrane receptors that CT and LT do, i.e, GM1 ganglioside.

C. jejuni enterotoxin was reported to cause elongation of CHO cells and rounding of Y-1 cells. In addition, the toxin raised intracellular cyclic AMP (cAMP) levels in CHO cells (Ruiz-Palacios *et al.*, 1983; Klipstein & Engert, 1984; Johnson & Lior, 1988). Maximum enterotoxigenic activity was detected when cultures were at early stationary phase of growth (Daikoku *et al.*, 1989). When applied *in vivo*, *C. jejuni* enterotoxin caused fluid and electrolyte accumulation in the ileal loop of rat (Ruiz-Palacios *et al.*, 1983; Klipstein & Engert, 1984; Klipstein *et al.*, 1985) and rabbit (*cit. in* Wassenaar, 1997). This is thought to be caused by an increase in intracellular cAMP levels, a mechanism similar to the one utilised by both cholera and LT toxins (*cit. in* Wassenaar, 1997). However, other authors have suggested that the increase in intracellular cAMP levels and fluid secretion may be caused by host-derived mediators of secretion following intestinal inflammation and host cell invasion (Everest *et al.*, 1993).

The clinical significance of the enterotoxin was evaluated by attempting to correlate CJT production and watery diarrhoea. A correlation was established in a number of cases (*cit. in* Wassenaar, 1997). Studies carried out in Costa Rica, Mexico and USA showed that enterotoxigenic strains were more frequently isolated from watery diarrhoea cases than from inflammatory diarrhoea or asymptomatic cases. However, this convenient correlation was not always true and not proven in studies carried out in India, Algeria or Brussels (*cit. in* Wassenaar, 1997). A different approach to establish the relevance of CJT was attempted by determining the seroconversion of patients to anti-CJT positive following *C. jejuni* infection. The results obtained were ambiguous and the seroconversion of patients was not always demonstrated (*cit. in* Wassenaar, 1997).

4.4.5.2 *C. jejuni* cytotoxins. Several groups have demonstrated that *C. jejuni* possesses cytotoxic activity. However, some confusion concerning both the number and specificity of the cytotoxic factors remains. A direct comparison of the published data is difficult as a wide variety of strains, bioassay systems and cultural conditions are employed in these studies. In a recent review on toxin production by *Campylobacter* (Wassenaar, 1997), Wassenaar considered that *C. jejuni* may produce five different cytotoxins according to two criteria: cell specificity and molecular weight. These cytotoxins include a 70kDa cytotoxin inactive on Vero cells; a cytotoxin active on Vero and HeLa cells; a cytotoxin neutralised by anti-shiga toxin; a cytolethal distending cytotoxin; a cytotoxin with hemolytic activity.

Based on the work from several authors, *C. jejuni* elaborates a cytotoxin with an estimated molecular mass of approximately 70kDa and whose most consistently analysed characteristic is a lack of activity on Vero cells. In contrast, the cytotoxin is toxic to a number of mammalian cell lines: CHO (the cell line most frequently tested), HeLa, HEp-2, MRC and INT 407 (*cit. in* Wassenaar, 1997). Only one group tested toxicity on an *in vivo* assay. They showed that the toxin can produce diarrhoea in rabbits using the RITARD model (*cit. in* Wassenaar, 1997).

The 70kDa cytotoxin is trypsin-sensitive and heat-labile although McCardell and co-workers (1986) described an otherwise identical toxin but trypsin-resistant and heat-stabile; the discrepancy may arise from differences in experimental conditions. This cytotoxin is immunologically and biologically distinct from shiga toxin and *C. difficile* cytotoxins (Everest *et al*, 1992; Guerrant *et al.*, 1987; Moore *et al*, 1988). Solubilisation experiments indicate that the cytotoxin is produced during early exponential phase and released during stationary phase (*cit. in* Wassenaar, 1997). The toxin appears to be membrane-associated as cytotoxic activity was detected in the LPS fraction of bacterial lysates (*cit. in* Wassenaar, 1997). The expression of the 70kDa cytotoxin was described as unstable and it is lost upon subculturing or storage of the strains (*cit. in* Wassenaar, 1997).

A second cytotoxin was described by few authors and referred to as the Vero/HeLa cytotoxin. This toxin is distinct from the 70kDa toxin mainly for its toxicity to the Vero cell line. HeLa cells were also found sensitive to this toxin although the authors disagree on which cell line is more susceptible (*cit. in.* Wassenaar, 1997). This cytotoxin can not be neutralised with anti-*Clostridium* or anti-shiga antitoxins.

In addition to the cytotoxins described above, there is one report that describes the production of a shiga-like cytotoxin by *C. jejuni*. Moore and colleagues (1988) described a cell associated cytotoxin immunologically resembling shiga toxin. These authors also reported that some of the strains they tested produced other cytotoxins distinct from shiga-like toxin. A correlation to the type of diarrhoea experienced clinically was not demonstrated.

Another cytotoxin, distinct from all the others previously described, was first reported by Johnson & Lior (1988). This toxin is known as cytolethal distending toxin (CLDT) and was isolated from patients with inflammatory diarrhoea. Recently, Pickett and co-workers reported the isolation and characterisation of the genes encoding CLDT (Pickett *et al.*, 1996). CLDT is toxic to a number of mammalian cell lines including CHO, HeLa, HEp-2, and Vero, causing cell distention and ultimate cytolethality (Johnson & Lior, 1988). The toxin induces the accumulation of cAMP in CHO cells 24h post-treatment. Nevertheless, CLDT is reported to be distinct from CJT as it causes cell death 96h post-treatment. Moreover, in contrast to CJT, this cytotoxin has no effect on Y-1 mouse adrenal tumour cells (section 4.4.5.1). Recently, a receptor for CLDT was detected on CHO and HeLa cell membranes (*cit. in* Wassenaar, 1997). CLDT was tested on a number of *in vivo* assays, comprising the infant mouse assay, the rabbit and rat RILT assays and the permeability factor assay in rabbit skin. The only responsive *in vivo* assay was the rat RILT test with CLDT producing hemorrhagic fluid and local inflammation (Johnson & Lior, 1988). CLDT cytotoxic activity was only neutralised with homologous rabbit antitoxin and not by anti-CT, anti-Vero, anti-*C. difficile* cytotoxins and anti *C. jejuni* Vero/HeLa cytotoxin (Johnson & Lior, 1988).

Finally, there is preliminary evidence indicating that *C. jejuni* produces one or more cytotoxins with haemolytic activity. A group reported contact-haemolysis on blood agar plates. This cytotoxin was toxic to CHO cells. These findings were later confirmed by two other groups (*cit. in* Wassenaar, 1997). Secreted haemolytic activity was also observed. One report detected both heat-labile and heat-stable haemolytic activity in culture filtrates from a number of *C. jejuni* strains. The strains were isolated from patients with dysenteric and watery diarrhoea. Rabbit erythrocytes were most sensitive to the culture filtrates in contrast to chicken erythrocytes, which were least sensitive. Further analysis revealed that the two haemolysins were trypsin-sensitive and showed different isoelectric points (pI 6.95 and 4.9) (*cit. in* Wassenaar, 1997).

4.4.5.3 Summary. *C. jejuni* may produce an enterotoxin that is heat-labile, raises the intracellular cAMP levels and is immunologically related to CT and LT. *C. jejuni* may also produce a number of different cytotoxins active on different mammalian cell lines. Strains that produce both an enterotoxin and a cytotoxin, have also been described (Klipstein *et al.*, 1985; Daikoku *et al.*, 1989). Nevertheless, the subject remains controversial and a correlation between *C. jejuni* phenotypes and clinical features has not always been established (Ruiz-Palacios *et al.*, 1983; Klipstein & Engert, 1984; Klipstein *et al.*, 1985; Guerrant *et al.*, 1987;

Johnson & Lior, 1988; Moore *et al.*, 1988; Daikoku *et al.*, 1989; Wassenaar, 1997). It is possible, however, that the repertoire of *C. jejuni* toxins may include one enterotoxin and at least five different cytotoxins (Table 3).

Table 3. Putative toxins produced by *C. jejuni* (adapted from Wassenaar, 1997)

Toxins	~ MW (kDa)	Heat sensitivity	Toxin neutralisation	Sensitive cell line	Responsive animal model
Enterotoxin CJT	70kDa	heat-labile	Anti-CT and anti-LT antitoxin	CHO; Y-1	rat and rabbit RILT
70kDa cytotoxin	70kDa	not clear (see text)		HeLa; HEp-2; MRC; INT 407	RITARD
Vero/HeLa cytotoxin		heat-labile		HeLa; Vero	
Shiga-like cytotoxin			Anti-shiga antitoxin	HeLa	
CLDT cytotoxin				CHO; HeLa; HEp-2; Vero	
Haemolytic cytotoxin				CHO	

4.5 Host defences

C. jejuni is susceptible to acid pH and therefore, the gastric hydrochloric acid provides a bactericidal barrier. Volunteers ingesting the organisms with sodium bicarbonate experience a high rate of illness (Black *et al.*, 1988). Volunteers receiving antimotility agents showed that peristalsis is also an important clearance mechanism. Secretion of mucus and immunoglobulin A (sIgA) were demonstrated to prevent *C. jejuni* invasion of INT 407 cells *in vitro* (McSweegan *et al.*, 1987). Another prompt and consistent nonspecific host defence is reducing the availability of iron. *C. jejuni* apparently does not produce siderophores but produces a transport system that may be involved in the import of scavenged siderophores (enterochelin). *C. jejuni* uses ferritin as the iron-storage protein (*cit. in* Ketley, 1997).

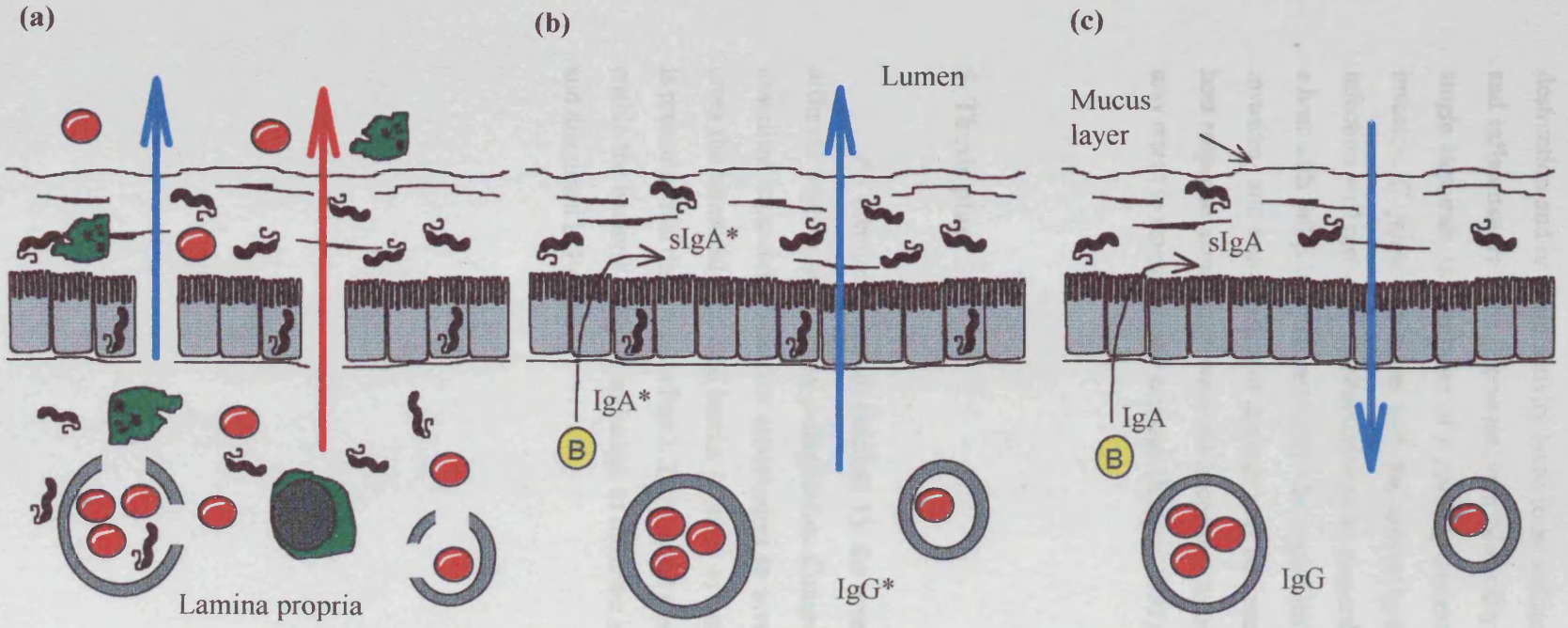
In addition to the nonspecific host defence mechanisms, *C. jejuni* triggers host specific defence responses. The flagella, a 44kDa porin and a 31kDa outer membrane protein elicit a constant and specific antibody response (Logan & Trust, 1983; Wu *et al.*, 1991; Newell & Nachamkin, 1992). These constituents are recognised consistently by antisera from convalescent patients.

Phagocytosis of *C. jejuni* during infection is important to decide the outcome of infection. The inflammatory response often associated with campylobacteriosis is accompanied by infiltration of polymorphonuclear leucocytes (PMNLs) in the intestinal epithelium. Antibody- and complement-opsonised *C. jejuni* are readily phagocytosed by PMNLs, trigger an oxidative response and are killed (Bär *et al.*, 1991; Wooldridge & Ketley, 1997). Mononuclear phagocytes are also often present in the lamina propria but their relevance on *C. jejuni* clearance is not well understood. There are reports of *C. jejuni* internalisation by macrophages in the intestinal tract of experimentally challenged chicks (Ruiz-Palacios *et al.*, 1981). *In vitro*, bacteria are readily phagocytosed by mice macrophages and human peripheral blood monocytes but appear to remain viable intracellularly for up to 6-7 days. However, *C. jejuni* looses the viable S-shape and becomes coccoid within 4 to 8h. Neither specific immunoglobulins nor complement seem to be required for efficient phagocytosis of *C. jejuni* by macrophages (Kiehlbauch *et al.*, 1985; Wooldridge & Ketley, 1997). In the blood stream, the bacterium becomes antibody- and/or complement- opsonised and is phagocytosed. Most strains are serum sensitive (Blaser *et al.*, 1985) but *C. jejuni* can induce bacteraemia, suggesting that bacteria may survive in the blood stream within white blood cells (Wooldridge & Ketley, 1997). The fact that *C. jejuni* undergoes phase and antigenic variation which avoids the activation of the complement cascade (Williams *et al.*, 1988) will certainly assist the bacterium to survive in the blood stream.

4.6 A model which correlates the clinical disease and the virulence mechanisms

The pathophysiological consequences of *C. jejuni* infection can range from watery diarrhoea to a severe dysentery-like illness. The different outcomes of infection may result from different mechanisms of virulence but their actual contribution to the clinical features of disease is not well established. The possibility of strains expressing specific phenotypes, for example, enteroinvasive, enterotoxigenic, etc is not acceptable as a correlation between the isolate phenotype and the clinical symptoms is often not established (*cit. in* Ketley, 1997). In contrast, it has been suggested that different outcomes of infection may be modulated by the host status in conjunction with the strain features (Fig.2). In a host who has not been previously exposed to *C. jejuni*, bacteria become established in the intestinal tract by colonising the mucus layer and attaching to the mucosa. They release toxin(s), invade the epithelial cells and traverse the epithelium by translocation. Consequently, *C. jejuni* causes tissue damage, inducing an

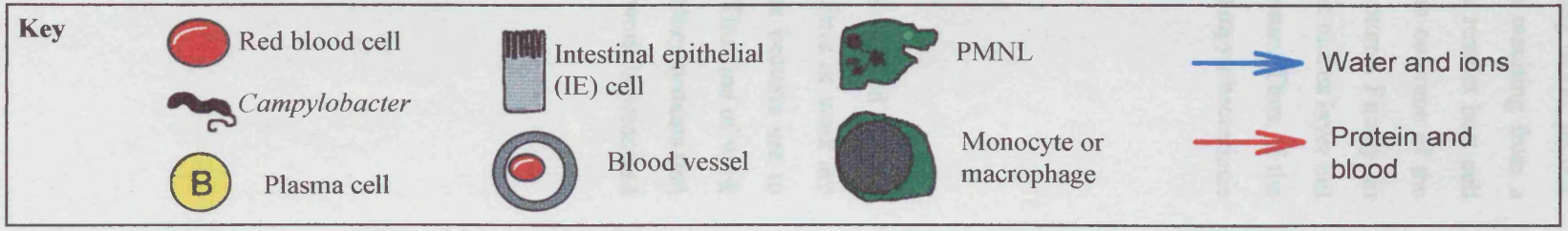
Fig.2. Hypothetical model to explain the different clinical outcomes of infection by *C. jejuni*. (a): no immunity; (b): partial immunity; (c): full immunity. See text for details (*in* Wooldridge & Ketley, 1997).



- No protective antibody response
- Colonisation of mucus layer
- IE cell invasion and translocation
- Infection of lamina propria
- Inflammatory response (IR) and tissue damage
- IR-mediated secretion and serosal fluid exudation
- Inflammatory diarrhoea

- Partially-protective (*) antibody response
- Colonisation of mucus layer
- Invasion of host IE cells
- Little/no penetration into lamina propria
- Pertubation of intestinal fluid absorption
- Watery diarrhoea

- Fully protective antibody response
- No infection or colonisation of mucus only
- Little/no invasion of host IE cells
- Host maintains net absorption of fluid
- No diarrhoea



inflammatory response with the recruitment of phagocytes to the site of infection. Cell destruction and/or toxin activity leads to an inflammatory diarrhoea, containing blood, protein and inflammatory cells. However, in a host with partial immunity, perhaps resulting from a single exposure, the presence of a partially protective immune response may restrict host cell invasion. *C. jejuni* associates with the mucus layer and produces toxins. The outcome of the infection will probably be characterised by non-inflammatory diarrhoeal symptoms. Finally, in a host with full protective immunity, the organisms may still associate with the mucus layer but invasion- and toxin-mediated damage will be restricted by the immune response. Thus, if the host response actually prevents the bacterium to associate with the mucus, campylobacteriosis may result in asymptomatic carriage (Ketley, 1997).

5. Thesis plan

As mentioned before (section 1), the work developed in this thesis focused on two different aspects of *C. jejuni* pathogenesis. Consequently, the two different lines of work are described separately. The first aspect aims to investigate the mechanisms the bacteria use to cross the intestinal epithelial barrier in order to reach the underlying tissues. This line of work is presented and discussed in Part I. The second aspect aims to study the regulatory systems that enable the bacteria to sense and adapt to different environments. This line of work is presented and discussed in Part II.

PART I

Translocation of *C. jejuni*: characterisation of the pathway.

CHAPTER 1
Introduction

1.1 Translocation across an epithelial barrier

Bacterial translocation has already been defined in a previous chapter (General Introduction; section 4.3) as the passage of viable bacteria from the gastrointestinal tract, across the epithelial cell barrier to underlying tissues. Indigenous bacteria are continuously translocating in low numbers from the gastrointestinal tract to the mesenteric lymph-node-complex, even in immunocompetent hosts. Usually, they are promptly killed *en route* or *in situ* in lymphoid organs by the host immune defences and cause no damage to the mucosa (Wells *et al.*, 1988; Berg, 1992). Translocation is also a mechanism used by pathogenic bacteria to pass through epithelial barriers and to disseminate throughout the host. Many pathogens including species of *Yersinia*, *Listeria*, *Salmonella*, *Shigella*, *Neisseria*, *Haemophilus influenzae* and *Brucella* use this method to enter deeper tissues in the host. The translocation of pathogens usually results in damage to the host epithelium (Wells *et al.*, 1988; Finlay & Falkow, 1989).

Bacteria may translocate through the cytoplasm of the epithelial cell via a transcellular route (Fig.2). Transcellular translocation is named transcytosis. Alternatively, the bacteria may pass between the enterocytes via a paracellular route (Mostov & Simister, 1985). Bacteria attempting this latter route will encounter a physical obstacle placed by the junctional complex. An increase in the permeability across the junctional complex or the wider disruption of the intestinal mucosa will facilitate the paracellular crossing.

1.2 Morphological features of the normal intestinal tract

1.2.1 Anatomy of the normal intestinal tract

In view of the possibility of bacteria penetrating morphologically intact intestinal mucosa, a brief review of the anatomy of the intestinal tract is relevant at this point (based on Wells *et al.*, 1988). To date, most evidence indicates that bacterial translocation occurs in the

area of the small intestine. In order to traverse the small intestine, the bacterium must penetrate the mucus gel (see General Introduction, section 4.1.1) which covers the intestinal surface. Beneath the mucus layer, there is the mucosa or mucous membrane (Fig.1a). The mucosa is characterised by the presence of intestinal villi and crypts. In addition, the mucosa includes the lamina propria and the muscularis mucosae.

Intestinal villi are finger-shaped projections of the mucosa (Fig.1a). Villi are covered by a columnar epithelium which consists of absorptive columnar epithelial cells (enterocytes) interspersed with goblet cells (mucus-secreting), intraepithelial leukocytes and APUD cells (secrete hormones and possibly other substances). The epithelial cells are covered apically by minute finger-like projections called microvilli (Figs 1b and 2) and are directly involved in the absorption of nutrients from the lumen to the capillaries in the lamina propria. The entire epithelium rests on a basement membrane (Figs 1b and 2). M cells are also part of the mucosa. These cells phagocytose bacteria and other antigens within the intestinal lumen and present them to the underlying lymphoid tissue in the lamina propria. They are responsible for inducing the production of sIgA (see below). The lamina propria lies beneath the basement membrane and forms the core of the villus.

At the bases of villi, the mucosa contains crypts of Lieberkühn (Fig.1a). Crypts are tubular structures lined by an epithelium consisting mainly of undifferentiated cells which differentiate to originate the cells in the columnar epithelium; the newly differentiated cells migrate up to the villus surface to the cell extrusion zone where replacement is required. In addition, the crypt epithelium contains absorptive cells, goblet cells, endocrine cells and Paneth's cells (produce lysozyme and peptides known to be toxic to most bacteria called cryptdins) (Fig.1a).

The mucosa is protected by different mechanisms which are briefly summarised below (based on Wells *et al.*, 1988). An important defence mechanism corresponds to the protective role carried out by the mucus layer. The mucus is produced by the goblet cells and acts both as a lubricant that prevents matter from damaging the cells and as a barrier to bacterial adhesion (see General Introduction, section 4.1.1). The mucus also contains substances that either kill bacteria (*e.g.*, lysozyme or lactoperoxidase) or inhibit their growth (*e.g.*, iron chelators like lactoferrin). Moreover, the mucosa has a specialised immune system called gastrointestinal-associated lymphoid tissue (GALT). One of the roles of GALT is the production of sIgA (secreted IgA). In addition to the mucosa-associated antibody response, there is a mucosa-associated cell-mediated response which includes macrophages, mucosa-specific mast cells and other types of lymphocytes like the intraepithelial lymphocyte (Salyers & Whitt, 1994).

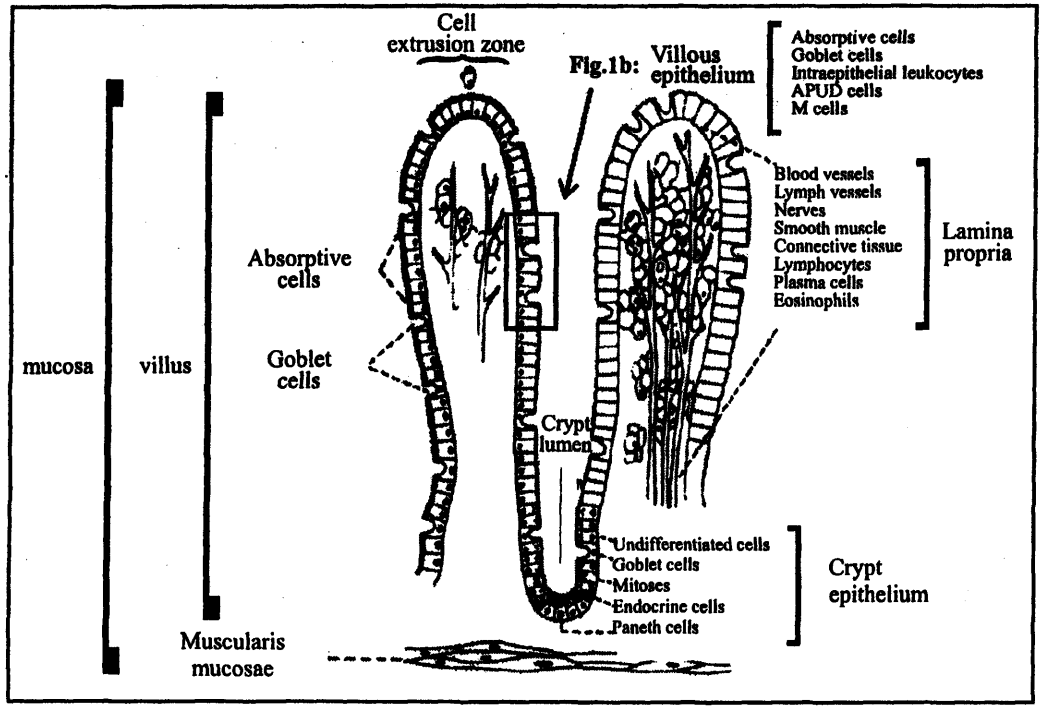


Fig.1a

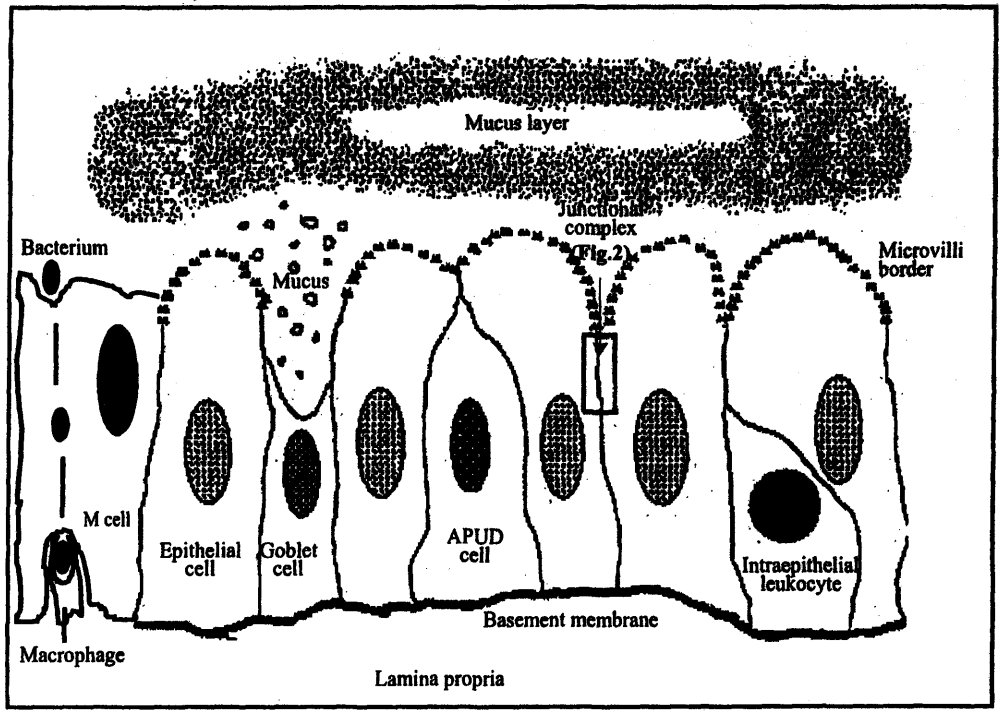


Fig.1b

Fig.1. Diagrammatic representation of the epithelium in the small intestine (adapted from Madara & Trier, 1987; Wells *et al.*, 1988). Fig.1a summarises the characteristic features of the mucosa, including villi and lamina propria, crypts of Lieberkühn and the muscularis mucosae. The main constituents of the lamina propria, the villous epithelium and the crypt epithelium are also shown (see text). Fig.1b corresponds to the rectangle in Fig.1a and is a diagrammatic representation of the various constituents from the villous epithelium (see text).

1.2.2 The junctional complex

The intercellular space between epithelial cells is bridged by a set of adhesive structures forming the junctional complex. These structures are the tight junction (TJ), the adherens junction, the desmosomal junction and the gap junction. Morphologically, the adhesive complex appears as a series of punctate fusions between the outer leaflets of adjacent epithelial cell membranes (Fig.2).

The TJ is the most apical structure in the complex. It forms a ring around the epithelial cells and regulates the paracellular permeability of the epithelium. Freeze-fractured plasma membranes from epithelial cells show that the TJ comprises an anastamosing web of strands (Madara, 1988). Within the TJ ring, the lateral membranes of adjacent cells closely approximate one another. An integral membrane protein, occludin, is thought to contribute to the formation of membrane contact between two adjacent cells and is probably responsible for the occluding barrier. Occludin interacts with a complex of proteins (ZO-1, ZO-2) located on the cytoplasmic sides of the TJ which are thought to bind several cytoskeleton-associated proteins, for example, cingulin, 7H6 antigen and actin (Marmorstein *et al.*, 1992; Gumbiner, 1996).

The TJ has two interrelated functions in the epithelium. The "fence" function where the strands prevent diffusion and subsequent intermixing of lipids and proteins between the outer leaflets of the apical and basolateral plasma membrane. Therefore, the "fence" function preserves the epithelium's polarity. The "gate" function where the interaction between the strands minimises the intercellular space, increasing transepithelial resistance to the movement of ions and molecules, regulating their passage through the paracellular way. Therefore, the "gate" function oversees the epithelium's paracellular permeability (Madara, 1988; Balda *et al.*, 1993; Mandel *et al.*, 1993; Gumbiner, 1996). Rather than an absolute barrier, the TJ forms a dynamic one. Several signal transduction mechanisms (*e.g.*, calcium, protein kinase C, tyrosine kinase, cyclic AMP) may lead to the alteration of TJ's structure to adjust its permeability (Sears & Kaper, 1996).

1.3 Mechanisms of translocation: examples

Several enteropathogenic bacteria are known to move across epithelial barriers. It is a way commonly used to access the underlying tissues and may permit further local or systemic

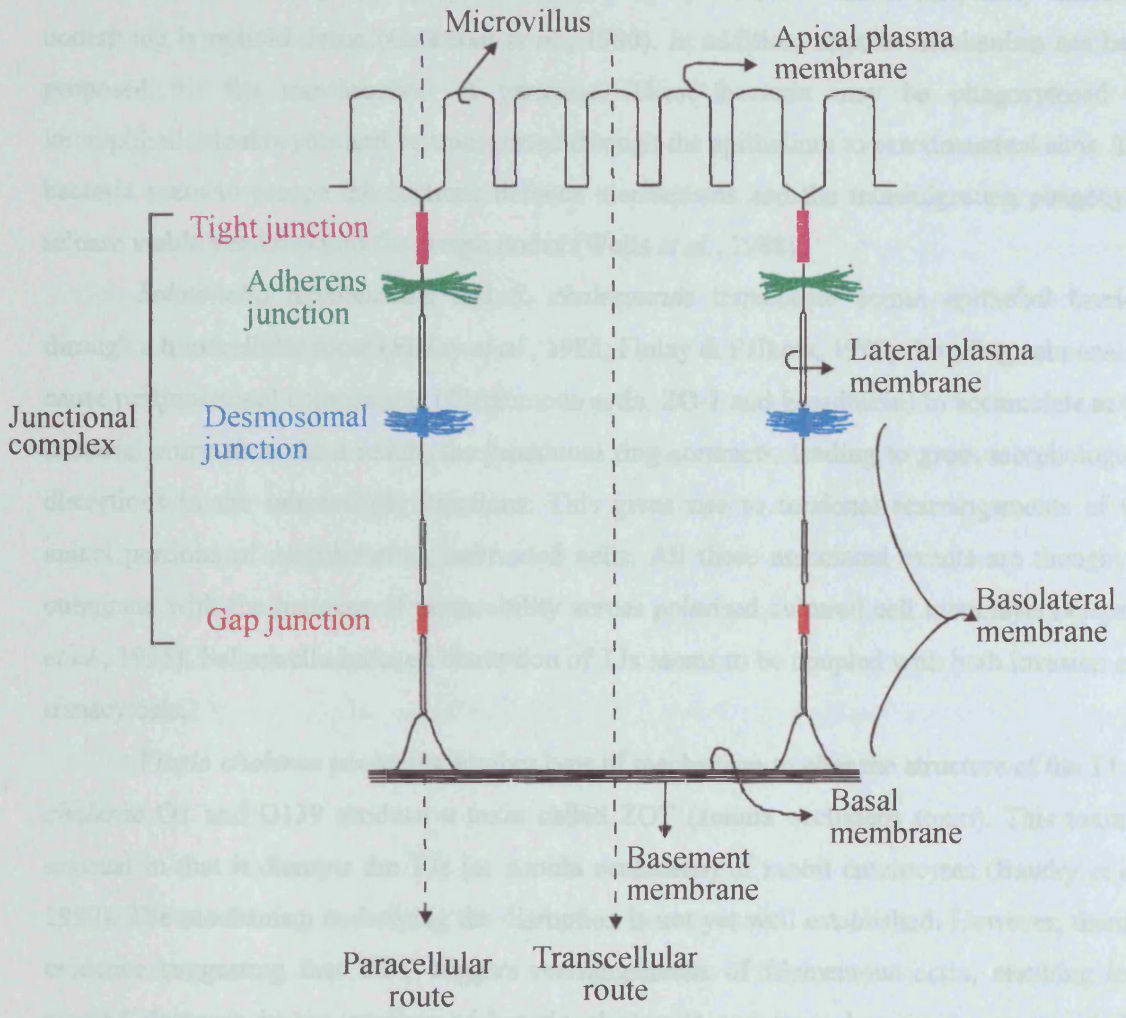


Fig.2. Schematic representation of transporting epithelial cells connected by the junctional complex. Microvilli project into the intestinal lumen and the apical membrane is responsible for the uptake of ions and nutrients from this compartment. The lateral membrane is the site of cell-cell contact and communication whereas the basal membrane is attached to the basement membrane. The basolateral membrane is involved in the generation of ion gradients across the apical membrane that facilitate vectorial uptake and transport of ions and solutes. Transporting epithelial cells are structurally and functionally polarised. The junctional complex, particularly the tight junction, is responsible for maintaining polarisation. The gap junction mediates cell-cell communication.

multiplication. In these cases, translocation is an important virulence determinant (Wells *et al.*, 1988; Finlay & Falkow, 1989). Different types of mechanisms are used by enteropathogens to traverse the host intestinal epithelium. *Yersinia enterocolitica* appears to transit across the intestinal mucosa through the cytoplasm of the phagocytic M cells and subsequently enters the underlying lymphoid tissue (Grützkau *et al.*, 1990). In addition, another mechanism has been proposed for the translocation of yersinias. These bacteria may be phagocytosed by interepithelial leukocytes and be transported through the epithelium to extraintestinal sites. The bacteria seem to escape the immune defence mechanisms and the transmigrating phagocytes release viable yersinias into the lymph nodes (Wells *et al.*, 1988).

Salmonella typhimurium and *S. choleraesuis* translocate across epithelial barriers through a transcellular route (Finlay *et al.*, 1988; Finlay & Falkow, 1990). Invading salmonellas cause perijunctional components (filamentous actin, ZO-1 and E-cadherin) to accumulate at the bacterial entry sites. As a result, the junctional ring contracts, leading to gross morphological distortions in the intercellular junctions. This gives rise to torsional rearrangements of the apical portions of neighbouring, uninvaded cells. All these associated events are thought to culminate with the increase of permeability across polarised cultured cell monolayers (Jepson *et al.*, 1995). Salmonella-induced disruption of TJs seems to be coupled with both invasion and transcytosis.

Vibrio cholerae possesses another type of mechanism to alter the structure of the TJ. *V. cholerae* O1 and O139 produce a toxin called ZOT (zonula occludens toxin). This toxin is unusual in that it disrupts the TJs (or zonula occludens) of rabbit enterocytes (Baudry *et al.*, 1992). The mechanism underlying the disruption is not yet well established. However, there is evidence suggesting that ZOT triggers rearrangements of filamentous actin, resulting in a marked decrease in the number of junctional strands and in a drop in the transepithelial resistance (reviewed in Sears & Kaper, 1996).

1.4 *C. jejuni* translocation

The ability of *C. jejuni* to translocate through the epithelial barrier is supported by *in vivo* observations. *C. jejuni* was shown in the submucosa and lamina propria in the intestinal tract of experimentally challenged chicks (Welkos, 1984) and infant macaque monkeys (Russell *et al.*, 1993) (General Introduction; section 4.3). Furthermore, *C. jejuni* occasionally induces extraintestinal infections, for example, bacteraemia and, thus, *C. jejuni* may reach new

potentially colonisable sites within the host by translocating across the intestinal epithelium. Based on these reports, translocation may be a significant virulence mechanism. Despite its potential importance, the present understanding of the processes underlying *C. jejuni* translocation is limited.

C. jejuni is an enteroinvasive organism known to cause pronounced cytopathic effects to the host cells (Konkel *et al.*, 1992a; Russell *et al.*, 1993). Host cell lysis may account for the presence of bacteria in the submucosa. Nevertheless, the bacterium may actively transcytose through the cytoplasm of epithelial cells. Unfortunately, the two possibilities (release by lysed host cells or active transcytosis) cannot be distinguished by *in vivo* observations. According to Walker and colleagues, transcytosis across the cytoplasm of M cells is a possible route through which *C. jejuni* may enter the submucosa (Walker *et al.*, 1988). These authors reported that *C. jejuni* selectively associated with M cells in the ligated ileal loops of rabbits and transcytosed across the M cell cytoplasm. The bacterial factors and host receptors involved in this uptake by M cells have not been identified. In contrast, *C. jejuni* was not observed near the M cells of experimentally infected *Macaca mulatta* (Russell *et al.*, 1993). The reason for this may be because *C. jejuni* interacts differently with the two species or because electron microscopy is not an effective way of looking at a lot of tissue. Certainly, it is not surprising if *C. jejuni* is phagocytosed by M cells as this is their function (section 1.2.1). Nevertheless, *C. jejuni* may translocate via M cells as this has been postulated to be a route for microbial translocation (Wells *et al.*, 1988).

In vitro, the ability of *C. jejuni* to translocate through an epithelial-like barrier is well established (Everest *et al.*, 1992; Konkel *et al.*, 1992b; Oelschlaeger *et al.*, 1993). Transmission electron microscopy revealed *C. jejuni* present in intercellular spaces of Caco-2 monolayers (Konkel *et al.*, 1992b) and INT 407 monolayers (Oelschlaeger *et al.*, 1993). These observations were consistent with reports that *C. jejuni* was frequently observed in direct contact with intercellular junctions from intestinal epithelial cells of experimentally challenged chicks (Welkos, 1984) and infant *Macaca mulatta* (Russell *et al.*, 1993). According to these reports, *C. jejuni* may show an affinity for the intercellular junctions. Furthermore, Everest and colleagues reported a group of *C. jejuni* isolates from patients with non-inflammatory diarrhoea which translocated through polarised Caco-2 cell line (Everest *et al.*, 1992). This group of isolates were unusual in that they were not isolated from Caco-2 cell cytoplasm at any time during the course of the invasion assay. Consequently, it was proposed that *C. jejuni* may traverse the Caco-2 cell monolayer through the paracellular route. It is not understood however, how the bacterium would negotiate its way through the junctional complex.

1.5 Aims of the project

Preliminary work was undertaken in this laboratory to characterise *C. jejuni* interactions with cultured cells. In previous observations, Paul Everest analysed a number of *C. jejuni* fresh clinical isolates. He defined three types of strains based on their interactions with a polarised epithelial-like Caco-2 cell monolayer. He observed that some strains were invasive and therefore, could be isolated from the Caco-2 cell cytoplasm. These strains were never isolated from the medium underneath the monolayer (Inv^+ $Trans^-$). Some strains were able to invade and to translocate through Caco-2 cell monolayer and therefore, the most probable route followed during translocation would be across the cell cytoplasm (Inv^+ $Trans^+$). Alternatively, they could have been released by lysed host cells as the consequence of cytopathic effects produced during bacterial invasion. In this case, host cell lysis had to occur within 4-6h post-infection. Some strains were non-invasive, although they were isolated from the medium underneath the monolayer, meaning that they translocated (Inv^- $Trans^+$; Everest *et al.*, 1992).

The aim of my project was to extend the studies initiated by P. Everest to further characterise the strain types, with particular focus on the third group (Inv^- $Trans^+$). The behaviour of the Inv^- $Trans^+$ group of strains differed from that of the majority of strains that are found in the cytoplasm prior to translocation. This observation suggested that these strains may be able to take a paracellular route, thus moving across the tight junctions. This hypothesis was supported by *in vivo* observations of *C. jejuni* close to intercellular junctions in the intestinal tract of chicks (Welkos, 1984) and infant *Macaca mulatta* (Russell *et al.*, 1993). Therefore, my studies focused on investigating whether *C. jejuni* penetrated the tight junction barrier. The possibility of Inv^- $Trans^+$ strains demonstrating that *C. jejuni* may follow a paracellular route during translocation was of great interest. This could explain how non-invasive strains still penetrate the epithelium and cause inflammatory diarrhoea.

To study whether *C. jejuni* penetrated the TJ barrier, it was necessary to use a polarised cell monolayer with functional TJs such as Caco-2 cell line. Caco-2 cells display a structural and functional spontaneous enterocyte-like differentiation and when grown on a surface, the cells form a polarised monolayer with TJs (section 2.1); (Pinto *et al.*, 1983; Grasset *et al.*, 1984).

Initially, it was important to re-confirm the work of P. Everest and thus, all three types of strains (Inv^+ $Trans^-$; Inv^+ $Trans^+$; Inv^- $Trans^+$) were analysed comparatively in their ability to translocate across Caco-2 cell monolayers. Subsequently, it was necessary to look at the effects of *C. jejuni* on Caco-2 cell monolayers during invasion and translocation to obtain evidence of a mechanism by which the bacteria may follow a paracellular route. This was achieved by

analysing the integrity of the monolayer's tight junctions during bacterial translocation by two different methods.

The first approach consisted of conducting electrical resistance measurements across Caco-2 cell monolayer. Due to the enterocyte-like features of the Caco-2 cultured cells, they are able to transport ions from the apical to the basolateral surface, leading to a difference of potential across the monolayer. A transmonolayer electrical resistance develops, mainly because of the presence of tight junctions (Grasset *et al.*, 1984). The level of resistance depends predominantly on the presence of effective TJs, which varies with the stage of cell differentiation and with the integrity of the monolayer. If the cells are damaged or the tight junctions disrupted, the electrical resistance will decrease. Thus, transmonolayer electrical resistance is a very sensitive way to evaluate tight junction integrity. I analysed comparatively transmonolayer resistance variation during Caco-2 cell infection by the three types of strains (see above). This should enable the detection of any changes occurring in cell or TJ permeability during invasion and/or translocation.

The second approach examined the translocation of a selective extracellular radiolabelled marker through Caco-2 cell monolayer infected with the different types of *C. jejuni* strains. This data can be correlated with the observations gathered with the resistance measurements to determine whether *C. jejuni*-Caco-2 cell interactions would result in a transient or permanent disruption of the monolayer.

CHAPTER 2

Materials and Methods

2.1. Caco-2 cell line

Caco-2 (reference number 86010202 in the European Collection of Animal Cell Cultures) is a continuous cell line derived from a human colon adenocarcinoma isolated from a 72 year old caucasian male (*cit. in* Pinto *et al.*, 1983). Caco-2 cells spontaneously differentiate like enterocytes, expressing several markers characteristic of small intestinal villus cells. They develop brush-border microvilli, acquire tight junctions and transport ions and amino acids from the apical to the basolateral membrane and across the cytoplasm. When Caco-2 cells grow on a surface, they form a polarised monolayer characterised by the presence of structures shaped like domes. Due to the presence of tight junctions, cellular transport only occurs from the cellular apical to the basolateral side and thus, water and ions accumulate under the monolayer causing domes to form (Pinto *et al.*, 1983; Grasset *et al.*, 1984, 1985).

2.2. Bacterial Strains

Campylobacter jejuni: all *C. jejuni* strains used in this report were isolated from stool samples during routine bacteriological screening at the Department of Microbiology, St. Pieters University Hospital, Brussels and characterised by Everest *et al.*, (1992). The main features of these strains are summarised on Table 1.

***Escherichia coli* DH5α**: F^-, ϕ80d*lacZ*ΔM15, *recA1, endA1, gyrA96, thi-1, hsdR17*(r_K^- m_K^+), *supE44, relA1, deoR,* Δ(*lacIZYA-argF*) U169 (Hanahan, 1983).

Table 1. Characteristics of *C. jejuni* clinical isolates (adapted from Everest *et al.*, 1992). All *C. jejuni* strains were isolated from stool samples during routine bacteriological screening at St. Pieters University Hospital in Brussels. Four strains were isolated from children with colitis whereas five strains were isolated from children with non-inflammatory diarrhoea. All strains were tested for the production of toxins by studying the effects of cell-free supernatants (enterotoxin) and cell sonicates (cytotoxin) on CHO cells. All strains were also tested for their ability to associate with, invade and translocate across Caco-2 cells.

		Clinical symptoms		Toxin production		Caco-2 cell		
Strain reference	***C. jejuni* serotype**	**colitis**	**non-inflammatory diarrhoea**	**enterotoxin**	**cytotoxin**	**association (cpm/monolayer)**	**invasion (cfu/ml)**	**translocation (cpm/ml)**
B415	I		+	+	-	215	0	+
E116	II	+		+	+	234	$1.3x10^6$	+
H104	I		+	+	-	143	0	+
K105	I		+	+	+	705	0	+
L115	I	+		+	+	232	$8.3x10^5$	-
N82	I		+	+	-	134	$1.0x10^5$	+
O69	II		+	+	+	186	0	+
O81	II	+		+	+	420	$1.2x10^3$	-
P71	I	+		-	+	227	$2.3x10^4$	+

Salmonella choleraesuis BRD394 is a mouse and pig virulent strain and was provided by P. Everest (Department of Biochemistry, Imperial College, London).

2.3. Media

Dulbecco's modified eagle medium (DMEM) (Gibco BRL) was supplied ready made, containing 4.5g/l of glucose. DMEM was supplemented with 10% ($^{v}/_{v}$) foetal bovine serum (FBS; Gibco BRL) and it was kept at 4°C.

Mueller Hinton (MH) Broth was prepared by dissolving 21g of Mueller Hinton broth powder (Oxoid) in 1 l of distilled water and was autoclaved at 121°C for 15min.

Mueller Hinton Agar was prepared as MH broth but with 15g/l BBL grade A bacteriological agar added.

Campylobacter Blood-Free Selective Agar was prepared by dissolving 22.75g of campylobacter blood-free selective agar base (Oxoid) in 0.5 l of distilled water and was autoclaved at 121°C for 15min.

2.4. Maintenance of the Caco-2 cell line

All manipulations involving tissue culture were carried out in a laminar vertical flow cabinet (Medical Air Technology Ltd). All glass and plastic materials used were sterile; all solutions were autoclaved at 121°C for 15min unless otherwise stated.

Caco-2 cells were grown routinely in 260ml volume plastic flasks (Nunc) containing DMEM supplemented with 10% ($^{v}/_{v}$) of FBS. The cultures were incubated at 37°C in 5% CO_2 with 95% humidity. Prior to subculture, semi-confluent stock cultures were thoroughly washed in prewarmed PBS containing 0.001M EDTA. The cells in the monolayer were dissociated by adding 2.5ml of 0.25% trypsin solution (Gibco BRL) containing 0.001M EDTA to the culture. The flask was incubated at 37°C and occasionally shaken until cells became separated.

Prewarmed DMEM with 10% ($^{v}/_{v}$) FBS was added to inactivate the trypsin and new stock cultures were started by transferring approximately 10^5 trypsinised cells into 260ml volume flask containing 30ml of DMEM with 10% ($^{v}/_{v}$) FBS. Approximately 3 to 4 days post-inoculation when the cells reached semi-confluence, the cells were either subcultured or left to further differentiate. In the latter case, the cells were allowed to continue growing and by the 6th or 7th day of culture the cells reached confluence. During cell growth, the medium was replaced every 3-4 days. Domes would form at the regions of more active growth. Their number increased with time and they would become evenly distributed throughout the cell monolayer. When cells started to form domes indicating that they were differentiating, DMEM supplemented with 20% ($^{v}/_{v}$) FBS was used and replaced every 2 days.

2.5. Freezing Caco-2 cells

Caco-2 cells were grown in standard conditions. Semi-confluent monolayers were thoroughly washed in prewarmed PBS and trypsinised as described above (section 2.4). The cells were centrifuged for 3-4min at room temperature, at 320 *g* in a Heraeus Megafuge 1R. After removing the supernatant carefully, the cells were resuspended in 1ml of FBS containing 10% ($^{v}/_{v}$) of DMSO (dimethylsulphoxide). The cell suspension was transferred into 1.8ml cryotubes (Nunc) and transferred successively to -20°C, then to-70°C and finally stored in liquid nitrogen up to one year. Frozen stocks were thawed at room temperature and mixed with 30ml DMEM containing 10% ($^{v}/_{v}$) of FBS to start fresh cultures.

2.6. Bacterial culture

All *C. jejuni* strains were grown in either DMEM supplemented with 10% ($^{v}/_{v}$) FBS, MH medium or campylobacter blood-free selective agar. Bacterial cultures were incubated at 37°C in a microaerophilic atmosphere of 6% hydrogen, 5% carbon dioxide, 5% oxygen and 84% nitrogen in a variable atmosphere incubator (VAIN, Don Whitley Scientific). Cultures in MH broth were shaken at approximately 200 rpm on a Gyrotory Shaker-Model G2 (New Brunswick Scientific Co, Inc.).

E. coli and *S. choleraesuis* strains were grown on either LB or NB (Part II; section 2.3) and incubated at 37°C. Liquid cultures were shaken at approximately 240 rpm on a G10 Gyrotory Shaker (New Brunswick Scientific Co, Inc.).

2.7. Translocation assay

2.7.1 Preparing the bacterial inoculum

C. jejuni strains were grown overnight in 5ml of DMEM supplemented with 10% ($^{v}/_{v}$) of FBS at 37°C in a microaerophilic atmosphere with constant shaking. The $O.D_{600nm}$ of the cultures was determined using a Pharmacia Ultraspec III spectrophotometer. Bacterial cells were harvested by centrifuging at 3200 *g* for 10min at room temperature and were resuspended in DMEM at 0.3-0.5 O.D units/ml.

E. coli and *S. choleraesuis* were grown overnight in 5ml of DMEM supplemented with 10% ($^{v}/_{v}$) of FBS at 37°C with constant shaking. The $O.D_{600nm}$ of the cultures was determined using a Pharmacia Ultraspec III spectrophotometer. Bacterial cells were harvested by centrifuging at 3200 *g* for 10min at room temperature and were resuspended in DMEM at 0.1 O.D units/ml.

Prior to inoculating the cell monolayer, the viable count of the cultures was determined by withdrawing 0.1ml from each bacterial suspension and plating serial dilutions on either MH or campylobacter blood-free selective agar. The plates were incubated overnight at 37°C, as described previously (section 2.6).

2.7.2 Preparing Caco-2 cell monolayers

Caco-2 cells were grown (section 2.4) on 12mm diameter semipermeable filters of 0.45μm or 3.0μm pore size, in transwell units (Costar; Fig.3). After reaching confluence, the stage of cell differentiation was monitored by measuring regularly the electrical resistance across the Caco-2 cell monolayer (Fig.3). The transmonolayer resistance was assessed using a Millicell electrical resistance system (Millicell-ERS; Millipore) and the resistance values were calculated using the equation:

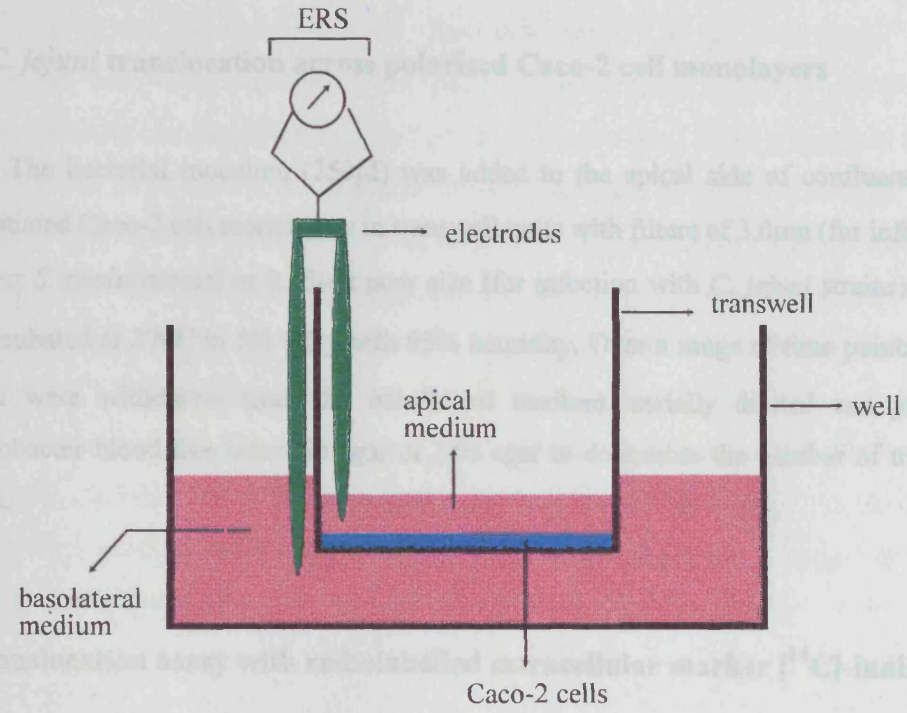


Fig.3. Schematic representation of a transwell unit and the use of Millicell-ERS meter to measure the electrical resistance across an epithelial-like monolayer. The transwell unit contains a semipermeable filter and is inserted inside a larger well unit. Both units contain medium. Caco-2 cells are seeded on the filter in the transwell unit. The transmonolayer electrical resistance is measured by simultaneously immersing the electrodes from the Millicell-ERS meter in the apical and basolateral media.

[measured monolayer resistance - measured blank resistance (filter without cells)] x area of the filter ($0.6cm^2$ for 12mm diameter filters) = transmonolayer resistance (Ω/cm^2)

The blank electrical resistance values were usually approximately $60\Omega/cm^2$. The cell monolayers were considered fully differentiated when showing electrical resistances of at least $250\Omega/cm^2$ (on 0.45μm filters) or $200\Omega/cm^2$ (on 3.0μm filters); typically, this occurred 10 to 14 days after the membrane was seeded.

2.7.3 *C. jejuni* translocation across polarised Caco-2 cell monolayers

The bacterial inoculum (250μl) was added to the apical side of confluent and fully differentiated Caco-2 cell monolayers in transwell units with filters of 3.0μm (for infection with *E. coli* or *S. choleraesuis*) or 0.45μm pore size (for infection with *C. jejuni* strains). The cells were incubated at 37°C in 5% CO_2 with 95% humidity. Over a range of time points, 100μl of medium were withdrawn from the basolateral medium, serially diluted and plated onto campylobacter blood-free selective agar or MH agar to determine the number of translocated bacteria.

2.8 Translocation assay with radiolabelled extracellular marker [^{14}C]-inulin

The bacteria and the Caco-2 cells were prepared as described in sections 2.7.1 and 2.7.2. Prior to Caco-2 cell infection, $2.5x10^{-3}$ MBq [^{14}C]-inulin were added to each bacterial suspension. The translocation assay was performed as described in section 2.7.3. The basolateral medium was withdrawn and processed for liquid scintillation (section 2.9).

2.9 Liquid scintillation spectrometry

The suspension recovered from underneath each Caco-2 cell monolayer grown in transwell units (section 2.8) was thoroughly mixed with Optiphase (LKB) scintillant in a 1:10 ratio (supernatant:Optiphase). The radioactivity associated with the suspension was determined in a Packard Tri-Star liquid scintillation spectrometer.

CHAPTER 3

Translocation of *C. jejuni* Across the Epithelial-like Caco-2 Cell Line

3.1 Introduction

The strains characterised by P. Everest showed a range of different phenotypes regarding the translocation across Caco-2 cell monolayers (section 1.5; Everest *et al.*, 1992). Previous observations suggested that one group of strains (Inv^+ $Trans^-$) appeared to follow a paracellular route and thus, may move through tight junctions. In order to determine if *C. jejuni* could take a paracellular route by altering tight junction permeability, two different methods were used. Both methods enable the assessment of the integrity of the Caco-2 cell monolayer and tight junctions during infection. The first method consisted of monitoring the variation of the electrical resistance across the monolayer which correlates with the variation of the monolayer's permeability. The second method looked for the translocation of a radiolabelled extracellular marker, [^{14}C]-inulin (Jepson *et al.*, 1995). When added apically to the cells, [^{14}C]-inulin is only found on the basolateral side if the cells are physically damaged or if the tight junctions are disrupted.

3.2 Variation of Caco-2 transmonolayer electrical resistance during translocation of *C. jejuni* strains

The effects of *C. jejuni* on the permeability of Caco-2 cell monolayers were assessed by studying the variation of Caco-2 transmonolayer electrical resistance during the course of infection with *C. jejuni*. As mentioned before (section 1.5), Grasset *et al.* (1984) showed that Caco-2 cells possess a measurable transepithelial electrical resistance. The level of resistance depends predominantly on the presence of effective tight junctions, i.e., on tight junction permeability.

The variation of transmonolayer electrical resistance was followed while translocation assays were carried out. Translocation assays were performed on confluent, differentiated Caco-2 cells, cultured on semipermeable filters in transwell units. Differentiation was evaluated

by regularly measuring the electrical resistance across the cells (section 2.7.2). The resistance values gradually increased until reaching a plateau, indicating the presence of tight junctions. This was usually achieved between the 10^{th} and the 14^{th} day of culture. However, invasion assays using the Caco-2 cell line proved that the highest rate of invasion was obtained after 16 to 17 days of culture. Thus, Caco-2 cell monolayers were only used in translocation assays after, at least, 16 days of culture and 2 to 6 days post-differentiation.

The magnitude of the resistance values varied with several factors including the batches of cells, medium or FBS. They also varied with the pore size of the filter used (Finlay & Falkow, 1990). Caco-2 monolayers at 16 to 17 days old were used when transmonolayer electrical resistances had reached at least $200\Omega/cm^2$ or $250\Omega/cm^2$ when cultured on 3.0μm or 0.45μm pore size filters, respectively. The electrical resistance of the blank (a transwell unit containing medium and no cells) was approximately $60\Omega/cm^2$.

C. jejuni strains from the different types described by Everest *et al.* (1992) were tested in the translocation assay (section 2.7). *E. coli* DH5α, an avirulent strain which does not penetrate the monolayer, was used as a control to check the integrity of the monolayer. *S. choleraesuis* which has been shown to translocate across polarised monolayers of Caco-2 cells (Finlay & Falkow, 1990) and MDCK (Finlay *et al.*, 1988), was used as a positive control of translocation. Campylobacters were resuspended to the same $O.D_{600nm}$ (0.3-0.5 O.D units/ml) and added to the apical side of each polarised monolayer. However, the viable counts performed at time point zero (section 2.7.1) showed that the actual number of viable bacteria added per well varied between bacterial cultures. Therefore, the monolayers were infected with different size inocula. Translocated bacteria were assayed by viable count of the bacteria present in the basolateral medium after apical infection. At several time points, aliquots of basolateral medium were plated on campylobacter blood-free selective medium and incubated overnight at 37°C in a variable atmosphere (section 2.6). In parallel, the permeability of the monolayer was assessed by conducting transmonolayer electrical resistance measurements (Fig.3, section 2.7.2). The variation of electrical resistance across uninfected or *E. coli* DH5α infected monolayers was used as negative control. The variation of electrical resistance across *S. choleraesuis* infected monolayers was used as positive control.

During the translocation assays, the measurements of electrical resistance were found to generate some problems. To obtain accurate measurements, it was necessary i) that the electrodes would be totally immersed in the medium but without disrupting the monolayer and ii) that the electrodes would be kept absolutely steady during the measurement. Ideally, the electrodes should be adapted to a stand like, for example, the electrodes of a pH meter. However, the shape of the transwells and the small volume of medium used made this

impossible. During the course of the experiment, it was extremely important to avoid mixing the apical and basolateral media and therefore, prior to each measurement, the electrode's position had to be re-adjusted. These technical difficulties may have contributed to the variability of data between experiments.

3.2.1 Translocation of *C. jejuni* strains across polarised Caco-2 monolayers

The ability of different *C. jejuni* clinical isolates (Table 1, section 2.2) to translocate across Caco-2 polarised monolayers was analysed (section 2.7). The results presented in Fig.4 and 5 correspond to two independent translocation experiments, A (Fig.4) and B (Fig.5). Translocation rates varied between assays and some strains did not always translocate. In contrast, the results obtained with *S. choleraesuis* were very consistent. Approximately 10^4 cfu/ml of *S. choleraesuis* were isolated from the basolateral medium 2h after apical infection with 10^8 cfu/ml; the translocated numbers increased to 10^5 cfu/ml by the end of the assay. The numbers of translocated *C. jejuni* varied between 10^2 to 10^3 cfu/ml following apical infection with 10^7-10^9 cfu/ml. In some assays, 10^4 cfu/ml of translocated E116 and L115 were isolated from the basolateral medium. The inoculum size does not seem to influence much the translocation yield. In experiment A, inocula of 10^6 cfu/ml of B415 or 10^7 cfu/ml of H104 resulted in similar or higher translocation rates, respectively, than an inoculum of 10^9 cfu/ml of N82 (Fig.4). In experiment B, the largest inoculum added to Caco-2 cells yielded the lowest translocation rate (Fig.5).

3.2.2. Analysing the Caco-2 monolayer transepithelial resistance in the presence of bacterial cells

The integrity of infected Caco-2 monolayers was evaluated by measuring the transmonolayer electrical resistance during translocation. The results shown in Figs 6-14 and 15-21 are representative of the variability obtained between assays and correspond to two independent translocation experiments, A (Fig.6-14) and B (Fig.15-21). The electrical resistance across monolayers infected with 10^8 cfu/ml of *S. choleraesuis* declined continuously, throughout the assay (8h), leading to a variation in the resistance values (ΔR) of approximately

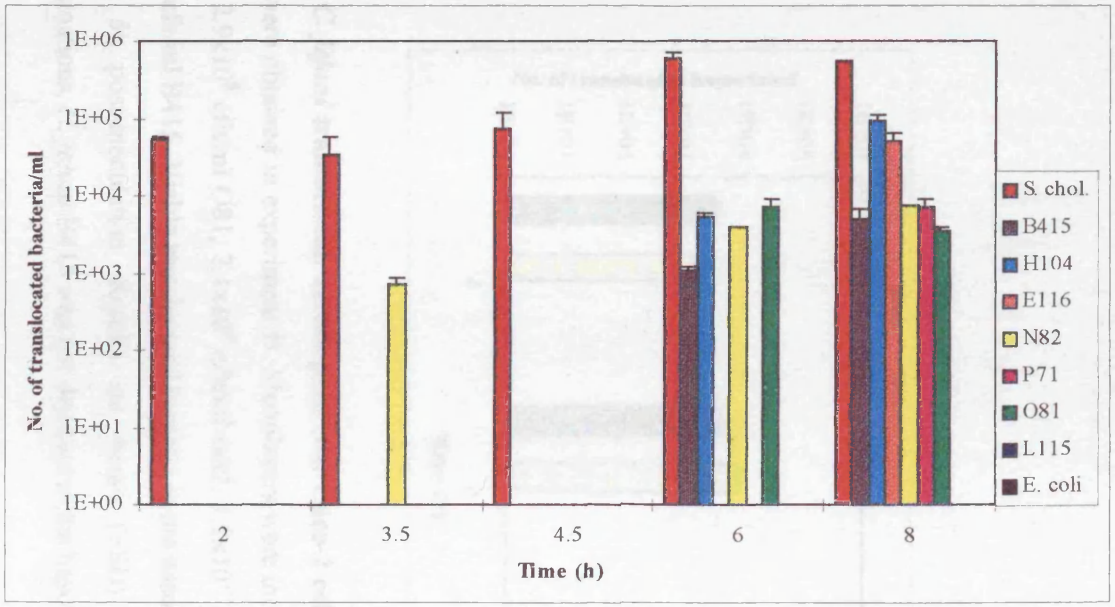


Fig.4. *C. jejuni* translocation across polarised Caco-2 cell monolayers. The results shown on Fig.4 were obtained in experiment A. Monolayers were inoculated apically with $1.6x10^8$ cfu/ml *E. coli* DH5α or $2.3x10^8$ cfu/ml *S. choleraesuis* and $6.4x10^6$ cfu/ml B415, $1.7x10^7$ cfu/ml H104, $4.4x10^9$ cfu/ml N82, $8x10^7$ cfu/ml L115. It was not possible to determine the inoculum size of E116, P71 and O81 because the plates were not countable. Viable translocated bacteria in the basolateral medium were quantitated at 2, 3.5, 4.5, 6 and 8h post-inoculation. Results are means (±SD) of triplicate determinations. *E. coli* DH5α and *C. jejuni* L115 were not detected in the basolateral medium at any time.

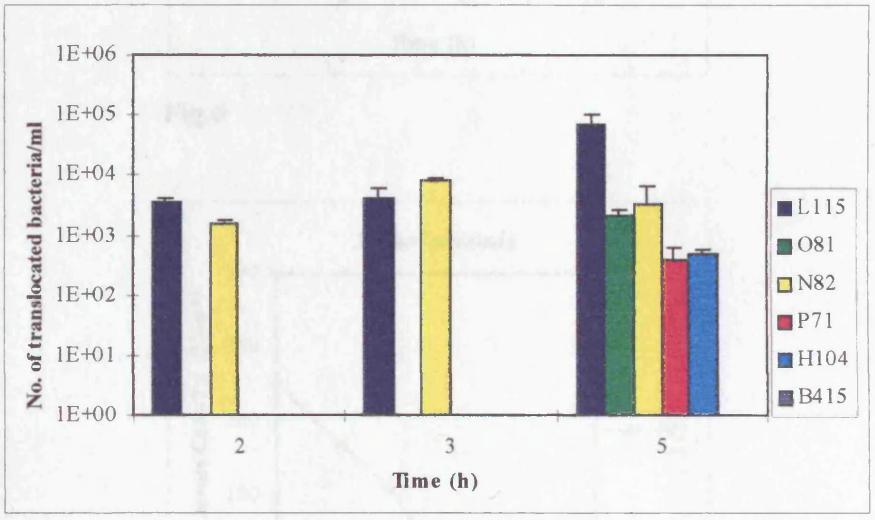


Fig.5. *C. jejuni* tranlocation across polarised Caco-2 cell monolayers. The results shown on Fig.5 were obtained in experiment B. Monolayers were inoculated apically with $4.1x10^9$ cfu/ml L115, $2.9x10^8$ cfu/ml O81, $7.1x10^9$ cfu/ml N82, $1.2x10^{10}$ cfu/ml P71, $3x10^8$ cfu/ml H104 and $6x10^7$ cfu/ml B415. Viable translocated bacteria in the basolateral medium were quantitated at 2, 3 and 5h post-inoculation. Results are means (±SD) of triplicate (duplicates for O81) determinations. *C. jejuni* B415 was not detected in the basolateral medium at any time.

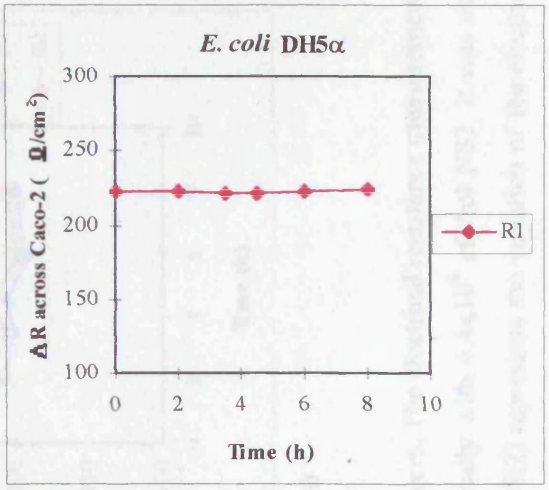


Fig.6

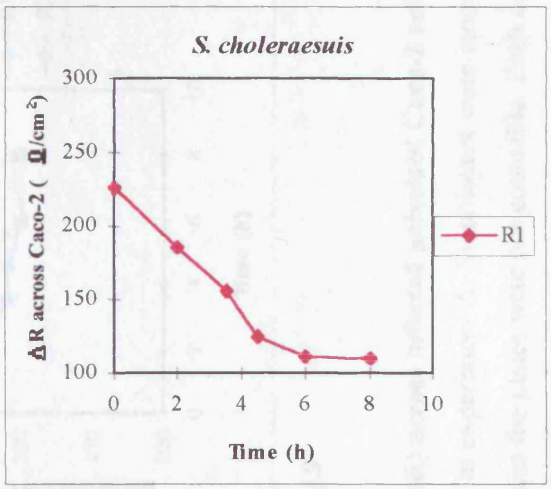


Fig.7

Figs 6-7. Variation of the electrical resistance (ΔR) across infected polarised Caco-2 cell monolayers. The electrical resistance measurements were taken during the translocation assay represented in Fig.4, in experiment A. Monolayers were inoculated apically with $1.6x10^8$ cfu/ml *E. coli* DH5α or $2.3x10^8$ cfu/ml *S. choleraesuis*. Each curve (R1) represents the variation of the electrical resistance across the polarised Caco-2 cell monolayer in one transwell unit.

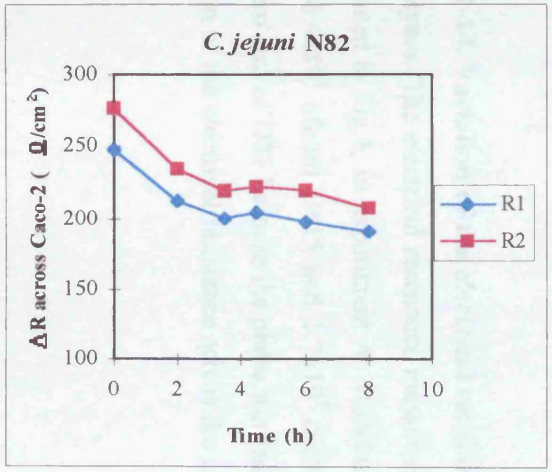


Fig.8

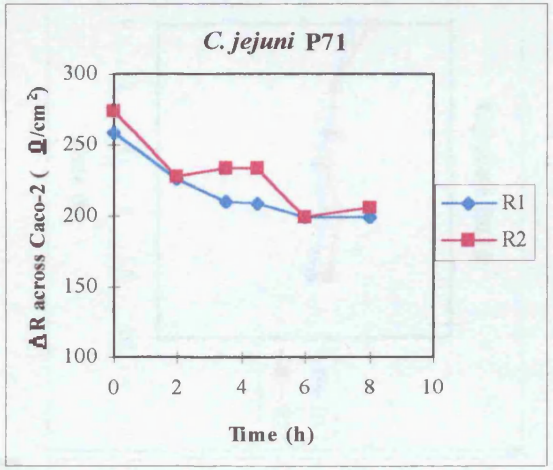


Fig.9

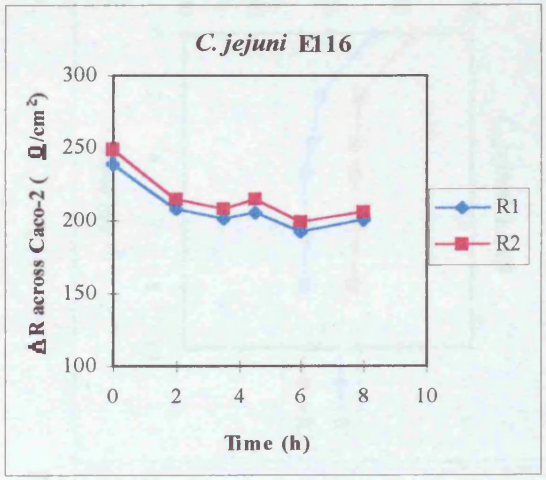


Fig.10

Figs 8-10. Variation of the electrical resistance (ΔR) across infected polarised Caco-2 cell monolayers. The electrical resistance measurements were taken during the translocation assay represented in Fig.4, in experiment A. Monolayers were inoculated apically with 4.4×10^9 cfu/ml N82. It was not possible to determine the inoculum size of P71 and E116 because the plates were not countable. Each curve (R1, R2) represents the variation of the electrical resistance across the polarised Caco-2 cell monolayer in one transwell unit.

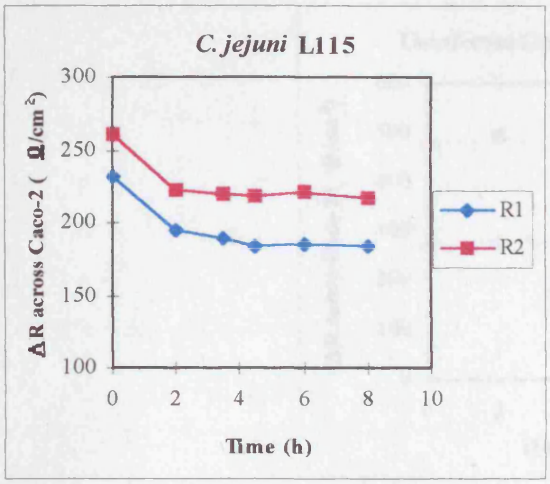


Fig.11

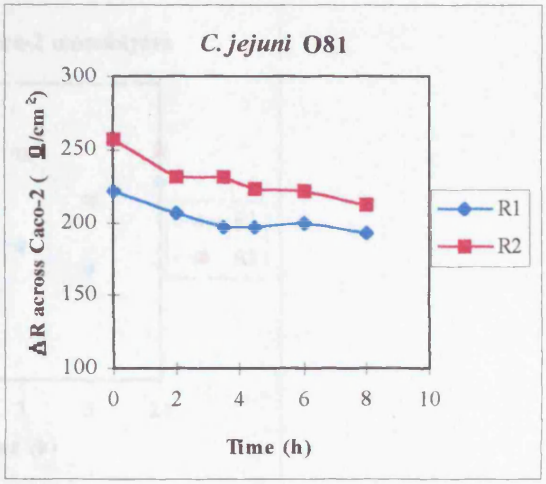


Fig.12

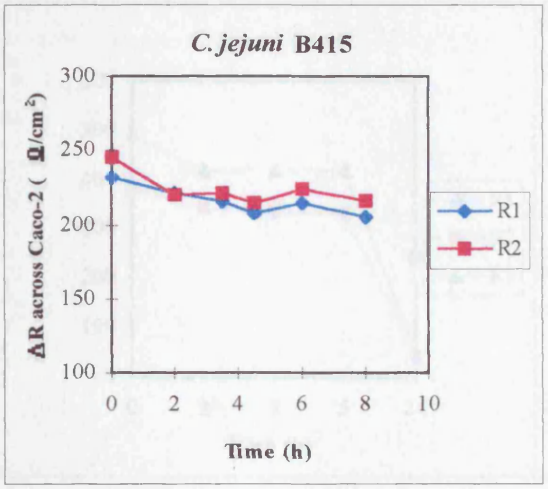


Fig.13

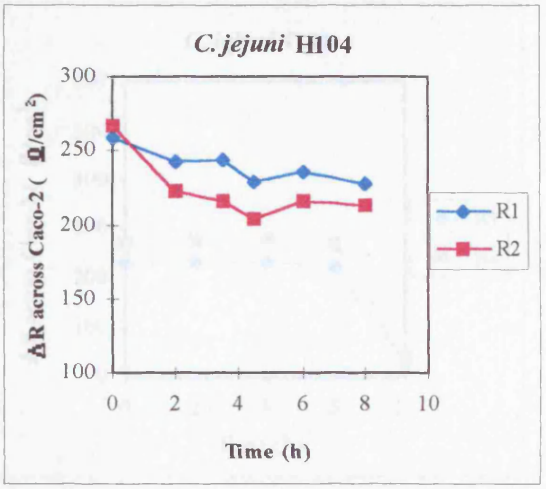


Fig.14

Figs 11-14. Variation of the electrical resistance (ΔR) across infected polarised Caco-2 cell monolayers. The electrical resistance measurements were taken during the translocation assay represented in Fig.4, in experiment A. Monolayers were inoculated apically with $8x10^7$ cfu/ml L115, $6.4x10^6$ cfu/ml B415 and $1.7x10^7$ cfu/ml H104. It was not possible to determine the inoculum size of O81 because the plates were not countable. Each curve (R1, R2) represents the variation of the electrical resistance across the polarised Caco-2 cell monolayer in one transwell unit.

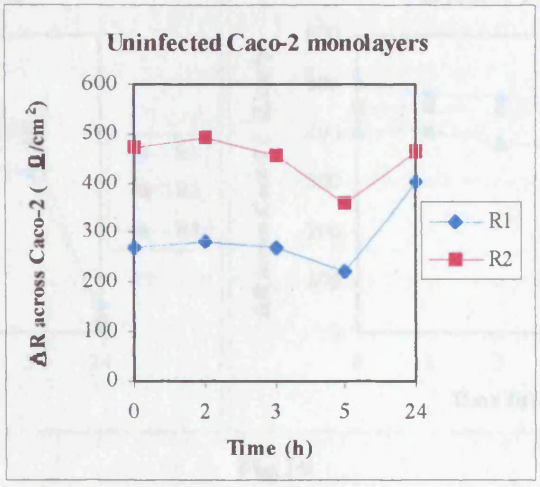


Fig.15

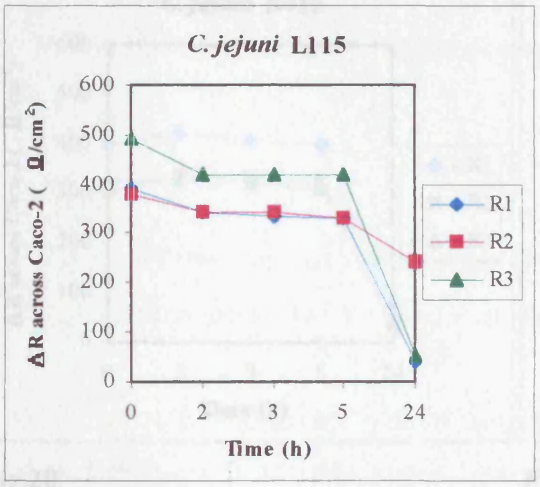


Fig.16

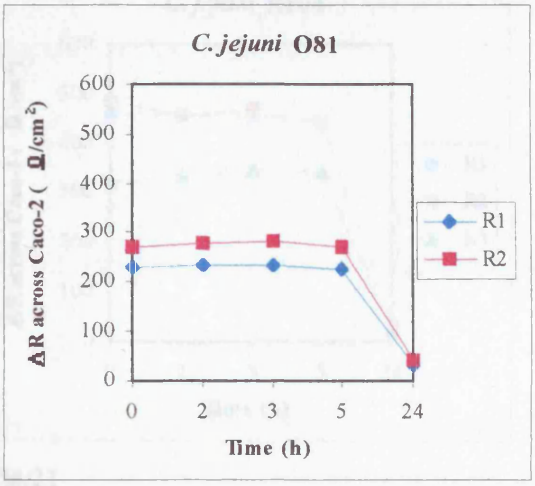


Fig.17

Figs 15-17. Variation of the electrical resistance (ΔR) across infected polarised Caco-2 cell monolayers. The electrical resistance measurements were taken during the translocation assay represented in Fig.5, in experiment B. Monolayers were inoculated apically with $4.1x10^9$ cfu/ml L115 and $2.9x\ 10^8$ cfu/ml O81. Uninfected monolayers were used as a control of the integrity of the monolayers. Each curve (R1, R2, R3) represents the variation of the electrical resistance across the polarised Caco-2 cell monolayer in one transwell unit.

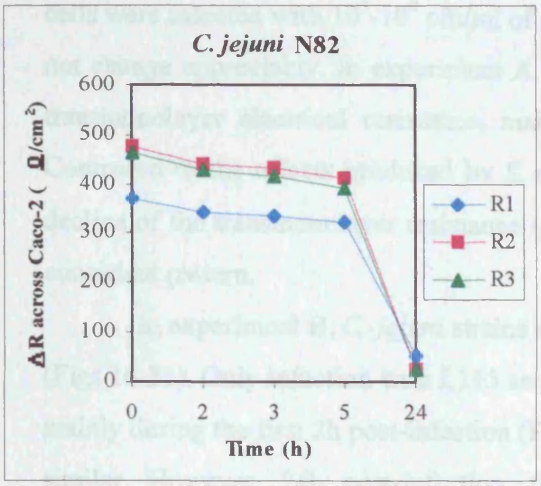


Fig.18

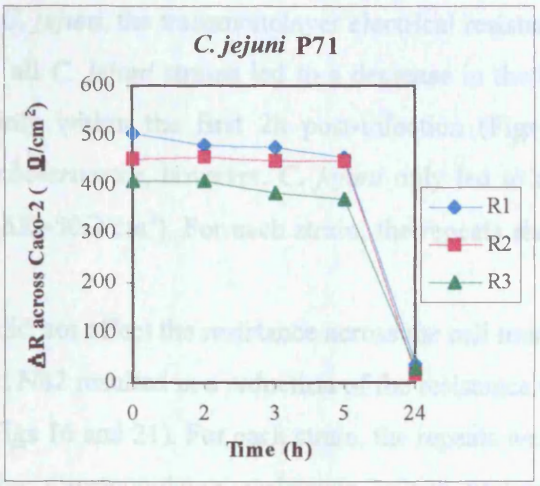


Fig.19

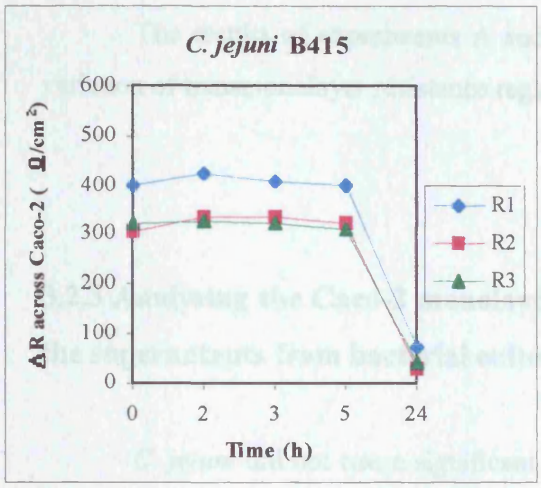


Fig.20

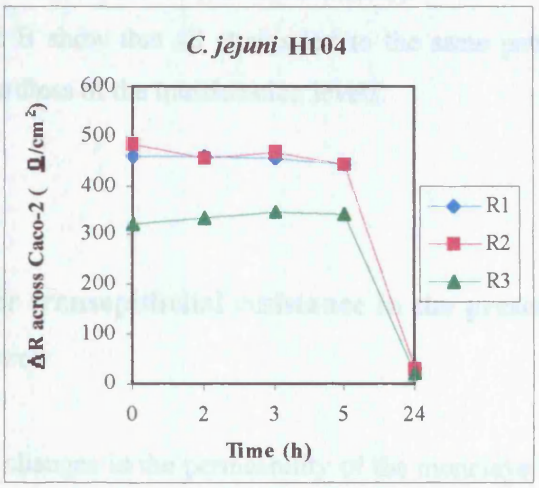


Fig.21

Figs 18-21. Variation of the electrical resistance (ΔR) across infected polarised Caco-2 cell monolayers. The electrical resistance measurements were taken during the translocation assay represented in Fig.5, in experiment B. Monolayers were inoculated apically with $7.1x10^9$ cfu/ml N82, $1.2x10^{10}$ cfu/ml P71, $6x10^7$ cfu/ml B415 and $3x10^8$ cfu/ml H104. Each curve (R1, R2, R3) represents the variation of the electrical resistance across the polarised Caco-2 cell monolayer in one transwell unit.

125Ω/cm^2 (Fig.7). In contrast, the addition of 10^8 cfu/ml of *E. coli* DH5α to the apical side of the monolayers had no effect on the transmonolayer electrical resistance (Fig.6). When Caco-2 cells were infected with 10^7-10^9 cfu/ml of *C. jejuni*, the transmonolayer electrical resistance did not change appreciably. In experiment A, all *C. jejuni* strains led to a decrease in the Caco-2 transmonolayer electrical resistance, mainly within the first 2h post-infection (Figs 8-14). Compared to the effects produced by *S. choleraesuis*, however, *C. jejuni* only led to a small decline of the transmonolayer resistance (ΔR~50Ω/cm^2). For each strain, the repeats showed a consistent pattern.

In experiment B, *C. jejuni* strains did not affect the resistance across the cell monolayer (Figs 16-21). Only infection with L115 and N82 resulted in a reduction of the resistance values, mainly during the first 2h post-infection (Figs 16 and 21). For each strain, the repeats were very similar. However, 24h post-infection, the transmonolayer resistance values underwent a massive drop, suggesting the monolayer's degeneration. These effects could be induced by a bacterial secreted toxin or by the bacterial cells themselves (see section 3.2.3).

The results of experiments A and B show that all strains led to the same pattern of variation of transmonolayer resistance regardless of the translocation levels.

3.2.3 Analysing the Caco-2 monolayer transepithelial resistance in the presence of the supernatants from bacterial cultures

C. jejuni did not cause significant changes in the permeability of the monolayer in the short term (5 to 8h). In the long term, however, polarised monolayers infected with *C. jejuni* for 24h showed electrical resistance values ≤ 37.5Ω/cm^2 (Figs 22-27). In order to determine whether the products resulting from bacterial metabolism caused the degeneration of the Caco-2 monolayers at 24h post-infection, the effect of bacteria-free supernatants on the transepithelial resistance of polarised Caco-2 monolayers was investigated. The supernatants tested were obtained from the cultures of N82, P71, L115 and B415 (section 2.2) used in the assay corresponding to Figs 8, 9, 11 and 13 (section 3.2.2). The supernatants were obtained from overnight cultures incubated at 37°C in DMEM with 10% FBS, in a variable atmosphere (section 2.7.1). Half volume of each sample was autoclaved at 121°C for 15min to determine whether temperature could abolish any effect produced by the supernatants. The samples (autoclaved and non-autoclaved) were added to polarised Caco-2 cell monolayers grown on 0.45μm pore size filters, in duplicate transwell units. The transmonolayer electrical resistance

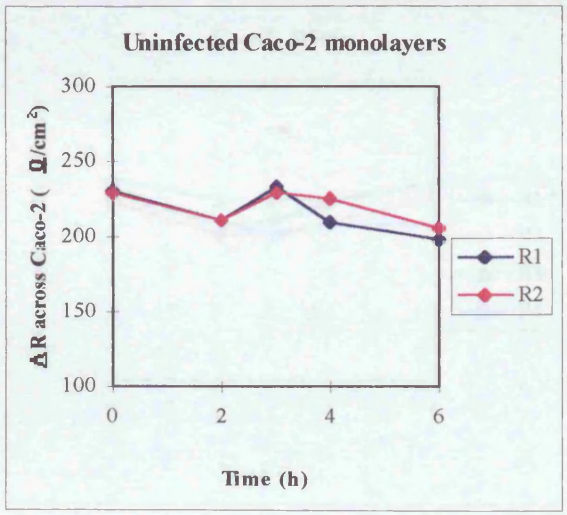


Fig.22

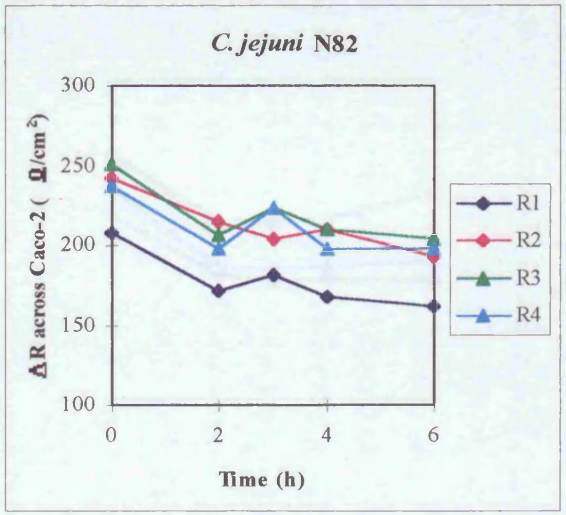


Fig.23

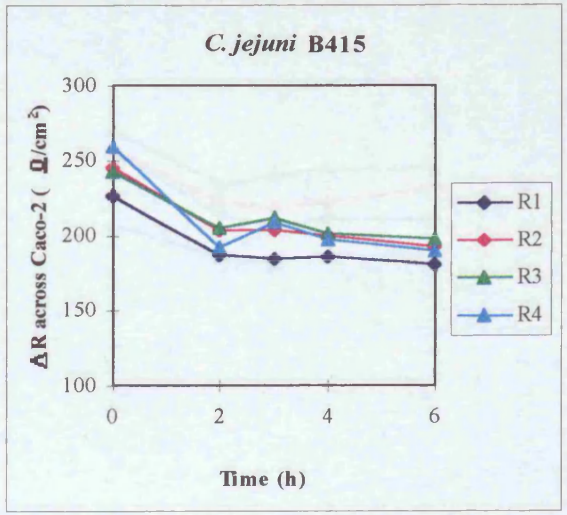


Fig.24

Figs 22-24. Variation of the electrical resistance (ΔR) across polarised Caco-2 cell monolayers following the replacement of the apical medium with bacteria-free supernatant. The supernatants were isolated from the bacterial cultures of *C. jejuni* N82 and B415 used in the assay represented in Figs 4, 8 and 13; the apical medium of uninfected monolayers was replaced with fresh DMEM+10%FBS. Each curve represents the variation of the electrical resistance across the polarised Caco-2 cell monolayer in one transwell unit. R1 and R2 correspond to duplicate mesurements in the presence of non-autoclaved supernatants; R3 and R4 correspond to duplicate measurements in the presence of autoclaved supernatants.

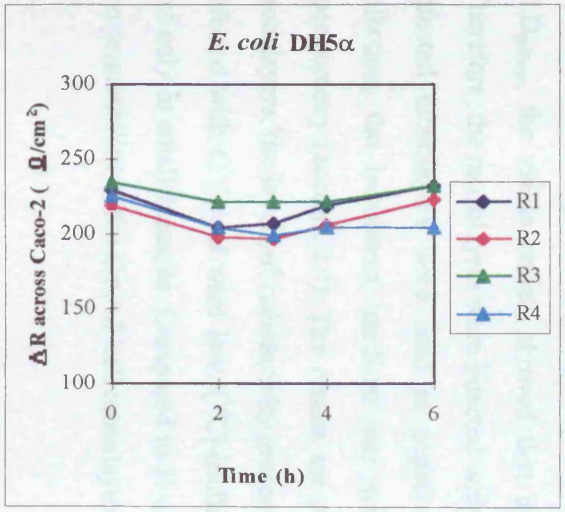


Fig.25

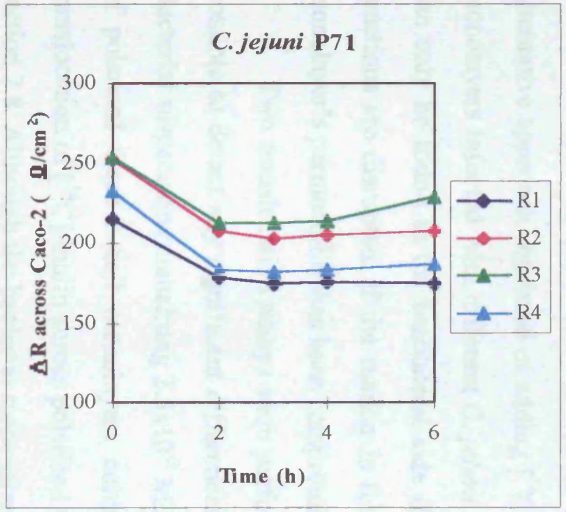


Fig.26

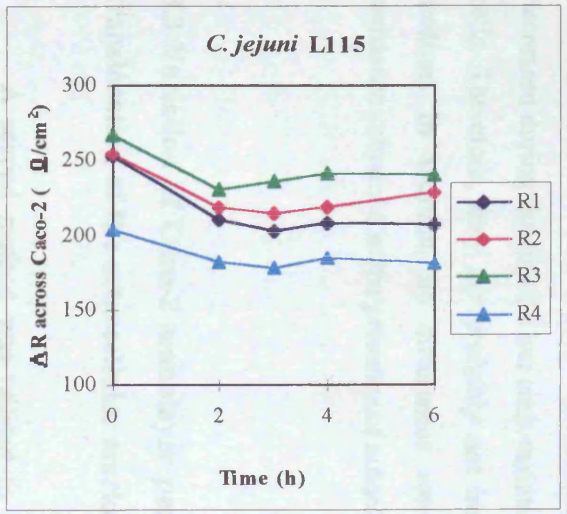


Fig.27

Figs 25-27. Variation of the electrical resistance (ΔR) across polarised Caco-2 cell monolayers following the replacement of the apical medium with bacteria-free supernatant. The supernatants were isolated from the bacterial cultures of *E. coli* DH5α and *C. jejuni* P71 and L115 used in the assay represented in Figs 4, 9 and 11. Each curve represents the variation of the electrical resistance across the polarised Caco-2 cell monolayer in one transwell unit. R1 and R2 correspond to duplicate mesurements in the presence of non-autoclaved supernatants; R3 and R4 correspond to duplicate measurements in the presence of autoclaved supernatants.

was regularly measured during the course of the experiment, as described previously (section 2.7.2). As shown in Figs 22-27, the results obtained did not vary from those obtained following the infection of the monolayers with bacterial cells (Figs 8-14). The transmonolayer resistance decreased during the first 2h but such variations were also obtained in the presence of bacterial cells. Therefore, they are probably not important and may be caused by elements in the medium. In addition, no differences were noted between the transmonolayer electrical resistances obtained in the presence of autoclaved and non-autoclaved supernatants.

3.3 Variation of Caco-2 monolayer permeability during translocation of *C. jejuni*: translocation of the extracellular marker[^{14}C]-inulin

A second method was utilised to assess changes in monolayer permeability. This alternative approach consisted of adding [^{14}C]-inulin to the apical side of polarised Caco-2 cell monolayers infected with different *C. jejuni* strains. [^{14}C]-inulin cannot enter Caco-2 cells and can only be found in the basolateral side if the cells are physically damaged or if the tight junctions are disrupted. If the marker is found underneath the monolayer, it means that the monolayer's permeability has been compromised.

Two translocation assays were performed simultaneously to determine whether it was possible to detect any significant differences between the different types of *C. jejuni* strains. Bacterial suspensions containing $2.5x10^{-3}$ MBq [^{14}C]-inulin were added apically to two batches of polarised Caco-2 cell monolayers cultured for 16 and 18 days. The assay to check translocation of [^{14}C]-inulin across polarised Caco-2 monolayers was performed as described in section 2.8. Although the bacterial suspensions added to each transwell unit contained the same $O.D_{600nm}$, the viable counts showed that the bacterial viability in the suspensions varied. Therefore, the monolayers were infected with 10^5 to 10^8 cfu/ml. Uninfected and *S. choleraesuis* infected monolayers were used as negative and positive controls, respectively. 7h post-infection, the basolateral medium was withdrawn and processed for liquid scintillation spectrometry (section 2.9). The results are shown in Figs 28-29. Compared to the uninfected monolayers, the levels of radioactivity present in the basolateral medium of Caco-2 monolayers infected with *C. jejuni* were low. [^{14}C]-inulin had traversed the Caco-2 polarised monolayers but only in small amounts. Compared to *S. choleraesuis*, infection with *C. jejuni* did not alter the permeability of the Caco-2 cell monolayer.

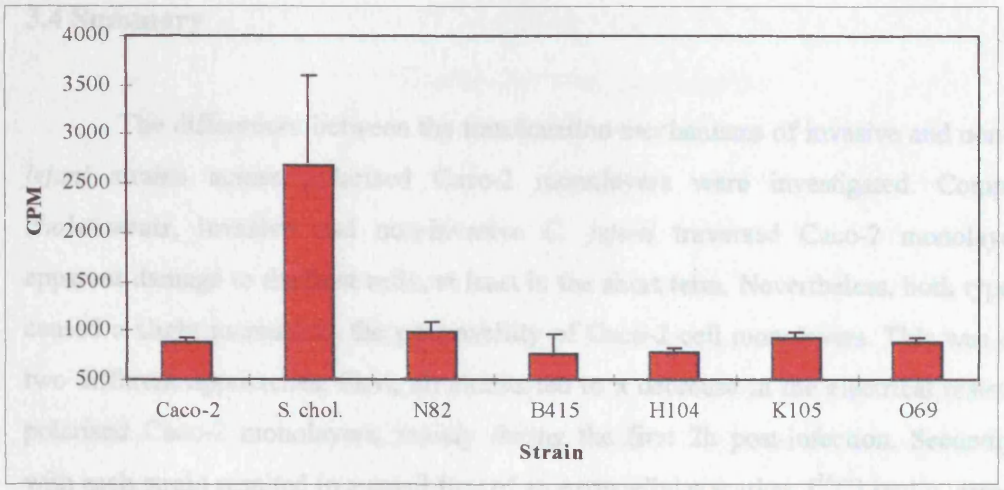


Fig.28. Translocation of [^{14}C]-inulin across infected polarised Caco-2 cell monolayers. The monolayers were cultured for 16 days. Prior to addition to the apical side of Caco-2 cell monolayers, [^{14}C]-inulin was mixed with bacterial suspensions with $2x10^8$ cfu/ml *S. choleraesuis*, $2.6x10^8$ cfu/ml N82, $4x10^7$ cfu/ml B415, $7x10^5$ cfu/ml H104, $2.1x10^8$ cfu/ml K105 or $8x10^5$ cfu/ml O69. The presence of [^{14}C]-inulin in the basolateral medium was evaluated by liquid scintillation spectrometry 7h post-inoculation. Results are means (±SD) of triplicate (duplicate for *S. choleraesuis* and uninfected) determinations.

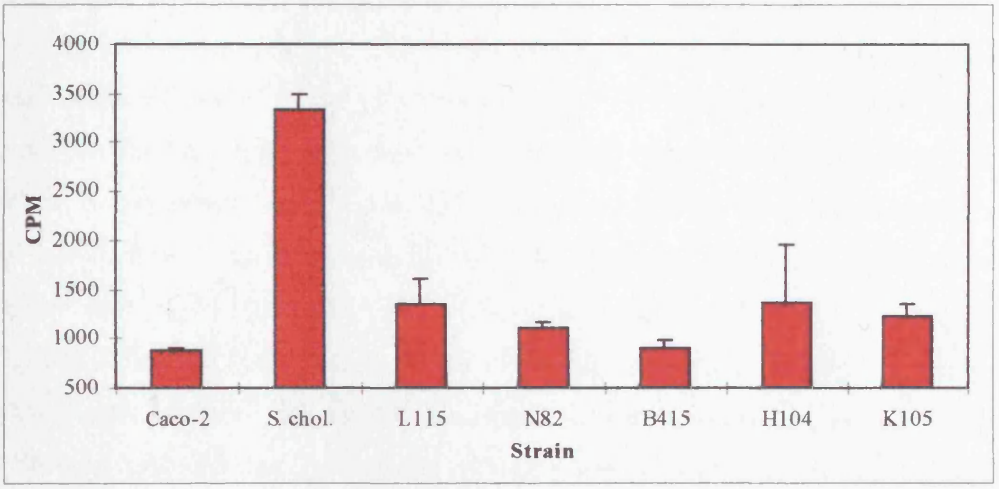


Fig.29. Translocation of [^{14}C]-inulin across infected polarised Caco-2 cell monolayers. The monolayers were cultured for 18 days. Prior to addition to the apical side of Caco-2 cell monolayers, [^{14}C]-inulin was mixed with bacterial suspensions with $2x10^8$ cfu/ml *S. choleraesuis*, $9x10^6$ cfu/ml L115, $2.6x10^8$ cfu/ml N82, $4x10^7$ cfu/ml B415, $7x10^5$ cfu/ml H104 or $2.1x10^8$ cfu/ml K105. The presence of [^{14}C]-inulin in the basolateral medium was evaluated by liquid scintillation spectrometry 7h post-inoculation. Results are means (±SD) of triplicate (duplicate for K105, *S. choleraesuis* and uninfected) determinations.

3.4 Summary

The differences between the translocation mechanisms of invasive and non-invasive *C. jejuni* strains across polarised Caco-2 monolayers were investigated. Compared to *S. choleraesuis*, invasive and non-invasive *C. jejuni* traversed Caco-2 monolayers without apparent damage to the host cells, at least in the short term. Nevertheless, both types of strains caused a slight increase in the permeability of Caco-2 cell monolayers. This was observed by two different approaches. First, all strains led to a decrease in the electrical resistance across polarised Caco-2 monolayers, mainly during the first 2h post-infection. Secondly, infection with each strain resulted in a small flux of an extracellular marker, [^{14}C]-inulin, across polarised Caco-2 cell monolayers. Invasive and non-invasive *C. jejuni* seemed to interact with Caco-2 cells in a similar manner.

CHAPTER 4

Discussion and Conclusions

The main objective of Part I was to investigate the possibility of a paracellular route for the translocation of *C. jejuni* across polarised Caco-2 cell monolayers. Previous observations (Everest *et al.*, 1992) suggested that *C. jejuni* could at least follow two possible routes during translocation: i) an intracellular route with the bacteria moving across the host cell cytoplasm or ii) a paracellular route with the bacteria moving between the host cells and through TJs. Inv^+ strains could probably take either the transcellular (i) or the paracellular (ii) pathway whereas Inv^- $Trans^+$ strains would probably be limited to the paracellular route (ii). Given that epithelial cells are connected together by TJs (and by the other adhesive structures within the junctional complex), translocation through the intercellular spaces was expected to disrupt the complex and TJs. Consequently, the cell monolayer would loose its electrical resistance properties and the paracellular permeability would rise. This was shown to occur with other enteropathogens, for example *Salmonella spp.* (Finlay *et al.*, 1988; Finlay & Falkow, 1990). However, *C. jejuni* Inv^+ and Inv^- strains traversed the Caco-2 cell monolayer without causing apparent damage to the monolayer. Compared to the effects produced by *S. choleraesuis*, *C. jejuni* caused little change to the transmonolayer electrical resistance (TER) within 5 to 8h post-infection. A small decline in the resistance values was noted in experiment A (Figs 8-14). This variation seemed specific to *C. jejuni* given that the addition of *E. coli* DH5α to the cell monolayers did not cause any drop in the resistance values (Fig.6). In experiment B, only L115 (Inv^+ $Trans^-$, Fig.16) and N82 (Inv^+ $Trans^+$, Fig.18) led to a reduction in the resistance values, mainly during the first 2h post-infection. Despite the variability observed between experiments A and B, the data obtained indicated that *C. jejuni* did not cause notable changes in the permeability of the monolayer, at least in the short term (5 to 8h). Since this project (Part I) has been initiated, another group reported the use of electrical resistance measurements across Caco-2 cell monolayer infected with Inv^+ $Trans^+$ strains (Konkel *et al.*, 1992b). The results they reported are in agreement with my findings.

When comparing experiments A and B, no correlation was found between the variation of TER and translocation. In fact, L115 caused a consistent decrease in the TER, whether translocation occurred or not. All other strains tested translocated in both experiments, although the effects produced on the TER varied. No differences were detected between the effects

produced by the Inv^+ and Inv^- strains on the TER. The various types of strains led to similar outcomes.

The translocation yields were not found to correlate to the proposed phenotype. All the *C. jejuni* strains tested translocated across Caco-2 cell monolayer, including the $Trans^-$ group (Figs 4 and 5). Therefore, the results obtained in experiments A and B (Figs 4 and 5) did not support the $Trans^-$ phenotype proposed by Everest *et al.* (1992). In fact, these phenotypes may be cell line-dependent as a strain characterised by P. Everest as an Inv^- $Trans^+$, (K105), was reported to invade HEp-2 cells in another study (de Melo & Pechère, 1988). Moreover, translocation rates varied in a non-consistent way between assays. In experiment B but not A, O81 ($Trans^-$) translocated at higher levels than P71 or H104 ($Trans^+$). Some strains did not always translocate (*e.g.* L115 and B415). In the case of L115 (Inv^+ $Trans^-$), there was a possible correlation between the inoculum size and the fact that the bacteria were isolated from the medium underneath the cell monolayer. The addition of 10^9 cfu/ml to the host cells resulted in bacterial translocation whereas the addition of 10^7 cfu/ml did not. It was possible that a larger inoculum of an invasive strain could lead to host cell lysis and subsequently, the released intracellular bacteria could pass through the filter in the transwell unit. Nevertheless, another Inv^+ $Trans^-$ strain (*e.g.* O81) was isolated from the basolateral medium in both assays, A and B, and when a smaller inoculum, 10^6 cfu/ml, was added to the host cells. Furthermore, Caco-2 cell lysis would lead to disruption of TJs between Caco-2 cells. Consequently, the electrical resistance across the monolayer would decrease considerably. This was not observed during infection with L115 (Figs 8 and 16, section 3.2.2). In the case of B415 (Inv^- $Trans^+$) there was no correlation between translocation and inoculum size.

Compared to *S. choleraesuis*, the translocation of *C. jejuni* strains occurred at a lower rate and at a later time. The numbers of translocated *S. choleraesuis* ranged from 10^4 cfu/ml at 2h post-infection to 10^5 cfu/ml at the end of the assay (8h). In contrast, the numbers of translocated *C. jejuni* ranged in average from 10^2 to 10^3 cfu/ml. Only two of the tested strains, E116 (Inv^+ $Trans^+$) and L115 (Inv^+ $Trans^-$), translocated at a rate of 10^4 cfu/ml. Although N82 translocated at 2h post-infection, most *C. jejuni* strains were isolated at least 5h post-infection. These findings were in contrast with a report that 10^5 cfu/ml of translocated *C. jejuni* were detected 3h after inoculation (Konkel *et al.*, 1992b). These authors also claimed to detect 100-500 organisms in the medium under the monolayer 15min after inoculation (Konkel *et al.*, 1992b). Nevertheless, it has been documented that different strains of *C. jejuni* invade and translocate at different rates (Everest *et al.*, 1992; Konkel *et al.*, 1992b).

When the effects of *C. jejuni* translocation on Caco-2 monolayer were analysed in the long term (24h post-infection), infected monolayers showed electrical resistance values ≤

37.5Ω/cm^2 (Figs 16-21). Host cell degeneration was described *in vivo*, in the intestinal tract of experimentally challenged *Macaca mulatta* at 32h post-infection (Russell *et al.*, 1993). Host cell degeneration is also consistent with the tissue damage and inflammation often found in cases of campylobacteriosis. Konkel *et al.* (Konkel *et al.*, 1992a) also reported that *C. jejuni* infection produced cytopathic effects on INT 407 cells at 48h post-infection. The results obtained in this study (section 3.2.2) showed that the disruption of the Caco-2 cell monolayers occurred earlier during the course of the assay. At 24h post-inoculation, the TER values indicated a massive disruption in the cell monolayer. These effects were observed in the presence of both invasive and non-invasive strains and thus, could relate to translocation. Alternatively, they could result from host cell lysis caused by bacterial invasion. Such an alternative would contest the existence of the Inv$^-$ phenotype proposed by Everest *et al.* (1992) for strains B415 and H104. The disruption of the cell monolayer could also arise from the accumulation of bacterial toxin(s) (Table 1; section 2.2). However, bacterial supernatants from overnight cultures did not affect the monolayer's permeability. This suggested two possibilities: the putative toxin(s) would only be produced under conditions met during infection or the disruption resulted from the direct interaction with bacterial cells rather than from the effects of bacterial secreted products. However, it was important to have analysed the variation of TER in the presence of bacterial supernatants for 24h to directly compare to the cytopathic effects produced by bacterial cells.

Despite the small variation in TER, it is important to note that within the first 8h, *C. jejuni* traversed the cell monolayer without apparently altering its permeability. Konkel and colleagues observed that bacteria pass through and between Caco-2 cells (Konkel *et al.*, 1992b). This implied that these organisms penetrate through TJs. According to Takeuchi, TJs between guinea pig epithelial cells were capable of resealing after bacterial penetration (Takeuchi, 1967). It could be that during the early stages of infection, *C. jejuni* would lead to a small leakage in the monolayer rather than to a massive disruption. However, the translocation of [^{14}C]-inulin occurring in the presence of *C. jejuni* was considerably smaller when compared to *S. choleraesuis* (Figs 28 and 29, section 3.3). In fact, only small amounts from [^{14}C]-inulin translocated through the cell monolayers in the presence of *C. jejuni* when compared to uninfected monolayers. The fact that Caco-2 cells were inoculated with different numbers of bacteria did not appear very important. Infection with 10^7 cfu/ml of B415 or 10^8 cfu/ml of N82 resulted in a lower flux of marker than infection with 10^5 cfu/ml of H104. No differences were detected between the effects produced by Inv$^+$ and Inv$^-$ types of *C. jejuni* strains.

The data obtained in this project (Part I) does not provide a basis for paracellular translocation as a main translocation pathway. It is more likely that *C. jejuni* penetrates the cell

monolayer via a transcellular route, given that the paracellular permeability remains unaffected at 8h post-infection. This is consistent with the data obtained with the resistance measurements and with the translocation of [^{14}C]-inulin. It will be important, though, to support the current data by using transmission electron microscopy to examine the translocation of the three types of strains across Caco-2 cell monolayers.

On the basis of the results obtained in chapter 3, an hypothetical scenario could be proposed which considers that *C. jejuni* may follow, at least, two routes during translocation. Several authors described the deposition of actin filaments at the sites of *C. jejuni* close apposition to the host cell membrane (de Melo *et al.*, 1989; Konkel *et al.*, 1992a; Russell *et al.*, 1993); (General Introduction, section 4.2.1). Initial host cell cytoskeletal rearrangements could account for the small variation in the TER values observed in the short term infection. During these early stages, *C. jejuni* may transcytose in small numbers without causing massive disruption of the Caco-2 cell monolayer. Alternatively, *C. jejuni*-induced actin reorganisation could lead to more flexible TJs, enabling the bacteria to take a paracellular route. As described by Takeuchi for other bacteria, TJs could reseal upon *C. jejuni* passage (Takeuchi, 1967). In the long term, the consequences of bacterial invasion on the host cell would be more drastic and the drop in TER would indicate a greater and more permanent disruption. In this case, transcytosis rates would increase and paracellular translocation could arise from the opportunity created by invasion and transcytosis. This hypothesis could be tested by simultaneously labelling of *C. jejuni* and junctional constituents (*e.g.* F-actin, ZO-1 and E-cadherin) with fluorescent dyes. This approach would enable bacterial movement to be followed in conjunction with host cellular rearrangements. These studies could provide insight into the mechanisms underlying transcytosis and paracellular translocation. The isolation of mutants deficient in the ability to translocate would allow to address epithelial translocation at the molecular level.

In addition to continue investigating translocation, it will be important to further characterise the three groups of strains defined by P. Everest. The construction of a subtraction library between the different strains could allow identification of possible genetic differences and correlation with the phenotypes observed. Additionally, the comparison between two-dimensional protein profiles isolated from bacteria recovered from infected Caco-2 cells could potentially enable the identification of differences between strains.

Conclusions

The objectives of my project included the further characterisation of translocation of strains previously observed to translocate across Caco-2 cells and to investigate the possibility of a paracellular route for the translocation of *C. jejuni*. The results obtained regarding the ability of the different strains to translocate across Caco-2 cell monolayer do not support the

Trans$^-$ phenotype proposed by P. Everest. Instead, it appears that all strains are capable of translocating, although the efficiency may vary. In addition, the data obtained in chapter 3 does not support the possibility of paracellular translocation. The results show that translocation occurs but without causing host cell lysis or the disruption of tight junctions. Caco-2 cell transmonolayer resistance measurements consistently show that paracellular permeability remains unaffected at 8h post-infection. This is in agreement with the [^{14}C]-inulin translocation measurements which also show that *C. jejuni* does not alter the monolayer's integrity at 7h post-infection. Indeed, my conclusions have been confirmed separately by another report (Konkel *et al.*, 1992b). Based on these observations, paracellular translocation may not be the main pathway taken by *C. jejuni*. Instead, the bacterium is more likely to invade and transcytose. Following entry into the host cell, the initial cytoskeletal rearrangements induced by the bacterium may account for the small variation of TER detected in the short term. These rearrangements are well documented both *in vitro* and *in vivo* (de Melo *et al.*, 1989; Konkel *et al.*, 1992a; Russell *et al.*, 1993) and have been reported to occur at least 32h post infection (Russell *et al.*, 1993). Therefore, this may be why Caco-2 host cell viability was not greatly affected within 8h post-infection. In the long term, however, host cell degeneration caused by bacterial invasion is probably the cause for the massive drop of TER observed. Clearly, these explanations need confirming but future work will certainly clarify this issue.

PART II

Isolation and characterisation of a response regulatory protein in *C. jejuni.*

CHAPTER 1
Introduction

1.1 Environmental control of pathogenicity: the stimulus-response pathway

Bacterial pathogens are highly specialised microrganisms. They have evolved a set of distinct strategies that enable them to gain entry to a host, find a privileged niche and multiply. Since not all sites within the host are suitable, the pathogen may become exposed to a series of other microenvironments. Each transition may involve severe fluctuations in physical and chemical parameters like pH, nutrition or oxygen availability (*e.g.*, enteropathogens in the gastrointestinal tract). The pathogen's success depends on its ability to promptly respond to the adaptive demands imposed by host-associated microenvironments (DiRita & Mekalanos, 1989; Miller, J.F. *et al.*, 1989; Bliska *et al.*, 1993; Dorman, 1995). Furthermore, it is essential to survive the pressures of the external environment between infections (Parkinson & Kofoid, 1992; Parkinson, 1993). Therefore, to survive in multicomponent, dynamic environments, the pathogen constantly senses and adapts to its surroundings (DiRita & Mekalanos, 1989; Dorman, 1991, 1995; Dorman & Bhriain, 1993). Each environmental parameter not only informs the bacterium of its current situation but the organism has the ability to turn the sensed changes into cues to elicit the appropriate cellular adjustments. The process of bacterial infection consists of adaptations by the pathogen to different microenvironments within the host. From an ecological point of view, bacterial pathogenicity is set up as a stimulus-response pathway. In a hostile environment, one possible action is to move to more favourable sites. However, adaptive responses frequently involve changes in gene expression.

Bacteria have sophisticated mechanisms that couple environmental signals with the regulation of gene expression. These mechanisms enable them to keep their gene expression profile well timed in respect to the environmental adaptive demands. This is not only true for pathogens but also for their non-pathogenic counterparts. Regulatory mechanisms that assist the pathogen during infection operate in a similar manner to those which aid the non-pathogen in the free-living state (Dorman, 1991, 1995; Dorman & Bhriain, 1993).

Certain common themes are used repeatedly by both pathogenic and non-pathogenic bacteria in signal transduction. One of these themes concerns the family of histidine protein kinases-response regulators (HK/RR; Stock *et al.*, 1989). This system operates by transmitting

signals concerning the state of the environment to the bacterial response machinery. Signal transduction occurs through a phosphotransfer mechanism.

Part II of this thesis focuses on the regulation of gene expression through HK/RR regulatory systems and how these systems are part of hierarchical regulatory networks within the bacterial cell. Against this background, what is known about environmental gene regulation in *C. jejuni* will be described.

1.2 Two-component regulatory system

The "two-component" designation arises from the fact that two types of proteins play central roles in the signal transduction events associated with this system. However, this designation should not be taken too literally. "Two-component" regulatory systems are rarely this simple and often include additional components.

The two proteins at the centre of each two-component signalling pathway are a histidine protein kinase (HK) and a response regulatory protein (RR) (Gross *et al.*, 1989; Stock *et al.*, 1989, 1990). They belong to two functionally important families of proteins within the bacterial cell, which are characterised by the presence of specific domains of conserved amino acid sequence. The HK is usually a transmembrane protein whereas the RR is invariably a cytoplasmic protein (Gross *et al.*, 1989; Stock *et al.*, 1989; Parkinson, 1993). Each component contains two functional domains connected via a linker of variable sequence and length (Fig.1). Different nomenclatures have been attributed to the various functional domains. According to the nomenclature used by Parkinson and Kofoid (1992), the HK contains i) a periplasmic domain located at the protein aminoterminus (N-terminus) responsible for monitoring a specific environmental parameter; this domain is referred to as the input domain; and ii) a cytoplasmic domain located at the protein carboxyterminus (C-terminus) responsible for transmitting the signal to the cognate RR; this domain is referred to as the transmitter domain. As to the RR, it contains i) the N-terminal domain that receives the signal from the HK's transmitter domain and thus, is referred to as the receiver and ii) a C-terminal domain responsible for triggering the adequate adaptive response, termed the output domain (Parkinson & Kofoid, 1992).

As mentioned above, the HK and the RR families are characterised by the presence of specific domains of conserved amino acid sequence. These domains correspond to the HK's transmitter and to the RR's receiver domains (Parkinson & Kofoid, 1992; Stock *et al.*, 1989). In contrast, the HK's input and RR's output domains are variable and specific for each protein.

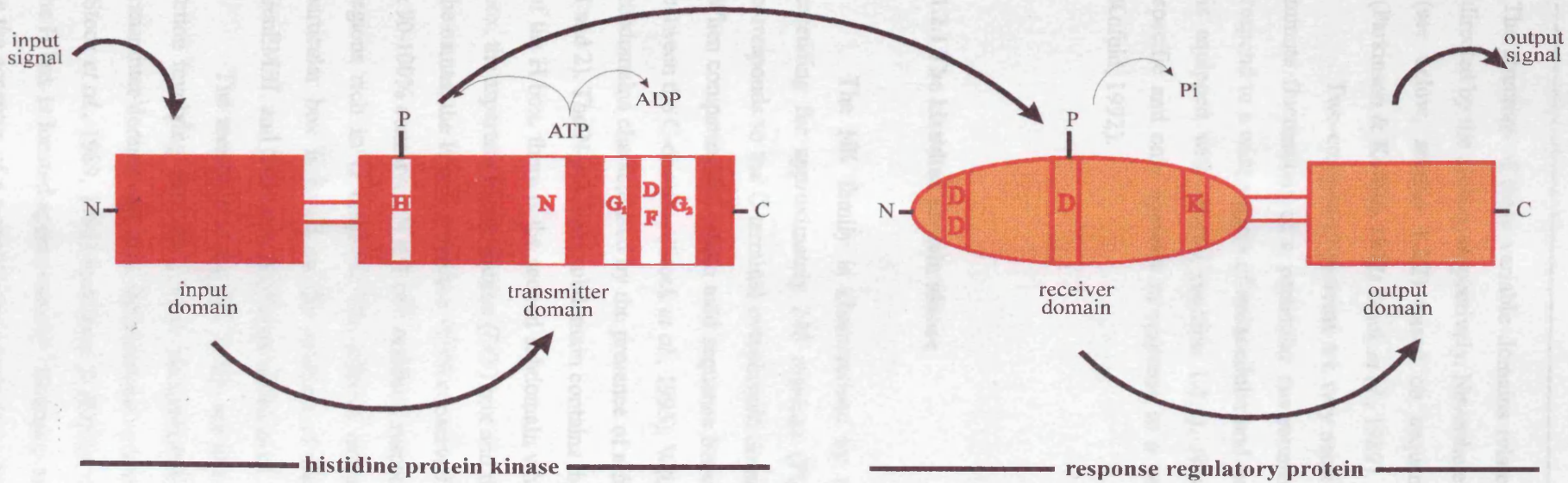


Fig.1. Two-component signalling pathway. Specific environmental signals are perceived by the input domain of a HK. Subsequently, the signal is communicated to the transmitter domain and the HK autophosphorylates at the conserved histidine residue (H). The phosphorylated transmitter domain transfers the information to the cognate RR's receiver by transferring the phosphoryl group to the conserved aspartate residue, D-57. The phosphorylated RR is activated and the RR's output domain triggers the adequate cellular response. The conserved motifs in both the transmitter (N, D/F and G1+G2 boxes) and the receiver (D-12, D-13 and K-109) are directly involved in the mechanism of communication (section 1.2.4; adapted from Parkinson & Kofoid, 1992).

The structure of these variable domains relates to the signal monitored and to the response directed by the system, respectively. Nevertheless, RR proteins can be grouped into subfamilies (see below, section 1.2.2) based on sequence similarities between the output domains (Parkinson & Kofoid, 1992; Stock *et al.*, 1989).

Two-component systems are very sensitive regulatory mechanisms, capable of sensing minute fluctuations of a particular environmental parameter. They enable the bacterium to respond to a wide variety of intracellular and extracellular signals and thus, each microrganism is equipped with several (section 1.2.5). However, each two-component system is highly specific and only operates in response to a certain signal (Gross *et al.*, 1989; Parkinson & Kofoid, 1992).

1.2.1 The histidine protein kinase

The HK family is characterised by a region of conserved amino acid sequence extending for approximately 240 residues (Fig.2) (Stock *et al.*, 1989, 1995). This region corresponds to the C-terminal cytoplasmic domain of the protein, i.e., the transmitter domain. When comparing the amino acid sequence between HKs, there is approximately 25% identity between the C-domains (Stock *et al.*, 1995). Within the conserved C-terminal domain, there are subdomains characterised by the presence of a few highly conserved amino acid residues (Figs 1 and 2). The N-proximal subdomain contains the so-called histidine box (H box). Downstream of the H box, there is the second subdomain which contains several boxes: the asparagine (N) box, the aspartate/phenylalanine (D/F) box and the glycine (G1 and G2) boxes. As indicated by the names, the H box contains a 100% conserved histidine residue, the N and D/F boxes contain a 90-100% conserved N and D/F residues, respectively and the G1 and G2 boxes correspond to regions rich in G residues. The relative occurrence of each amino acid residue within a particular box is based on the analysis of the amino acid sequence from 68 HK's from GenEMBL and SwissProt databases (Stock *et al.*, 1989, 1995).

The members of the HK family are also defined by sharing a common mechanism of action involving transient protein phosphorylation. The kinase activity is performed by the transmitter domain and thus, the structural conservation correlates to a functional conservation (Stock *et al.*, 1989, 1995; Parkinson & Kofoid, 1992). The conserved histidine residue within the H box is located approximately 110 amino acid residues into C-terminus and is thought to be the acceptor of a γ-phosphoryl group from ATP. This is based on studies using CheA (HK

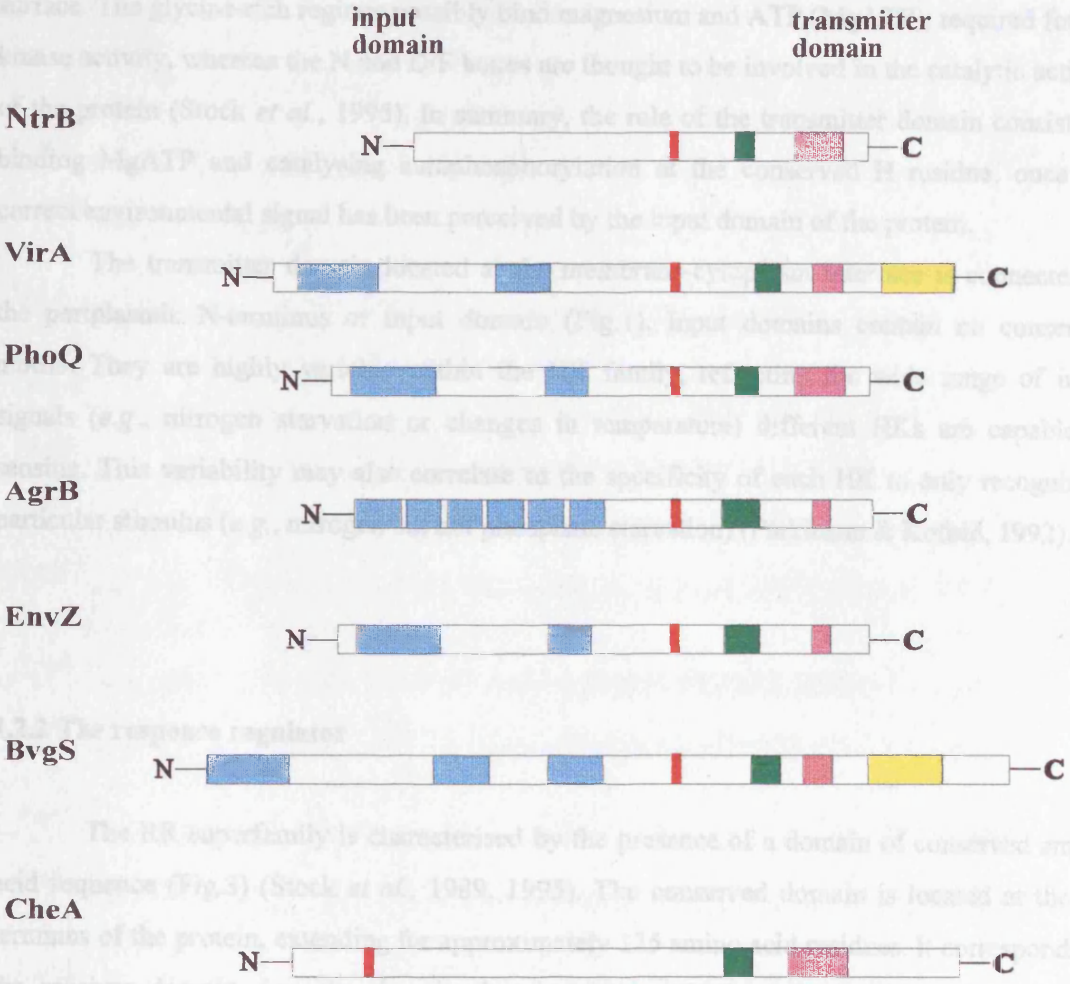


Fig.2. Domain organisation of HKs. Hydrophobic putative membrane-spanning sequences are indicated by blue boxes. Regions containing the H, N and D/F + G1+G2 boxes are shown on the figure, as red, green and magenta, respectively. The yellow boxes correspond to domains homologous to a receiver domain (see section 1.2.5.3). Transmitter domains between different HK share approximately 25% homology. There is, however, considerable variability in the length and amino acid sequence of the linkers between the conserved motifs. Input domains are highly variable in length and amino acid sequence, although they are commonly located in the membrane. Some HKs are cytoplasmic and are not the detector of the system (NtrB, CheA; see sections 1.2.5.2 and 1.2.5.3).

for the chemotaxis system) and NtrB (HK for the nitrogen assimilation system). The N, D/F and G1+G2 motifs are thought to be arranged in a tertiary structure to form a nucleotide binding surface. The glycine-rich regions possibly bind magnesium and ATP (MgATP), required for the kinase activity, whereas the N and D/F boxes are thought to be involved in the catalytic activity of the protein (Stock *et al.*, 1995). In summary, the role of the transmitter domain consists of binding MgATP and catalysing autophosphorylation at the conserved H residue, once the correct environmental signal has been perceived by the input domain of the protein.

The transmitter domain located at the membrane-cytoplasm interface is connected to the periplasmic N-terminus or input domain (Fig.1). Input domains contain no conserved motifs. They are highly variable within the HK family, reflecting the wide range of input signals (*e.g.*, nitrogen starvation or changes in temperature) different HKs are capable of sensing. This variability may also correlate to the specificity of each HK to only recognise a particular stimulus (*e.g.*, nitrogen but not phosphate starvation) (Parkinson & Kofoid, 1992).

1.2.2 The response regulator

The RR superfamily is characterised by the presence of a domain of conserved amino acid sequence (Fig.3) (Stock *et al.*, 1989, 1995). The conserved domain is located at the N-terminus of the protein, extending for approximately 125 amino acid residues. It corresponds to the receiver domain, i.e., the domain that interacts with the partner HK, and receives a phosphoryl group from the HK phosphohistidine (Fig.1). Therefore, the structural conservation among receiver domains indicates that the mechanism of communication between RR and partner HK has been conserved throughout evolution of the system (Stock *et al.*, 1989, 1995; Parkinson & Kofoid, 1992). Sequence alignments between any two RR conserved domains show approximately 20 to 30% identical amino acid residues (Stock *et al.*, 1989). Within the RR conserved domain, there are some amino acid residues that are particularly highly conserved (Fig.1) (Stock *et al.*, 1989). Based on the amino acid sequence of CheY (a RR in the chemotaxis system), these amino acids are aspartate-12 (Asp-12 or D-12), aspartate-13 (Asp-13 or D-13), aspartate-57 (Asp-57 or D-57) and lysine-109 (Lys-109 or K-109). The conserved aspartate residues (D) form an acid pocket with the Asp-57 located inside the pocket. The Asp-57 is the phosphoaccepting residue. The other conserved residues appear to contribute to binding magnesium which is essential for the phosphotransfer reaction (Parkinson & Kofoid, 1992; Stock *et al.*, 1995). In addition, there are other sites of highly conserved hydrophobic

Fig.3. Domain organisation of RRs. The N-terminal receiver domains are represented by yellow boxes. These regions are very conserved among RRs and contain the highly conserved amino acids D-12, D-13, D-57 and K-109. The C-terminal output domains are more variable but RRs responsive to similar signals share a considerable degree of sequence similarity. On the basis of this similarity, the RRs are organised into subfamilies. Some RRs contain two receiver domains in tandem (FrzZ).

residues that are thought to be involved in protein dimerisation or intramolecular contact (section 1.2.4.3) (Stock *et al.*, 1989; Volz, 1995).

The receiver domain is connected to the effector or output domain which shows no conserved domains (Fig.1) (Stock *et al.*, 1989, 1995). The output domain is specific for a RR and its structure correlates to the task for which the RR is responsible (Stock *et al.*, 1989, 1995; Parkinson & Kofoid, 1992). Typically, the output domain has DNA-binding activities, but there is considerable variation in structure and function among the RR proteins. Most RR proteins can be further classified into subfamilies by homology between the C-domains (Fig.3). Different authors group the RR in different ways. One type of classification (Gross *et al.*, 1989; Stock *et al.*, 1989; Parkinson & Kofoid, 1992) considers that a first group of RR is defined by the lack of an output domain. The receiver domain may occur alone like with CheY and SpoOf or in tandem like with FrzZ. A second group comprises DNA-binding proteins which bind to specific target sequences upstream of the genes they regulate. This group can itself be further subdivided into subgroups by sequence homology between output domains. Frequently, these structural homologies imply similarities in the way these RR interact with the genes under their control. This is the case of the PhoB/PhoP subfamily or the BvgA subfamily. A third group includes RR with unique output domains which do not resemble the output domain from any other known RR and includes CheB whose output domain functions as a methylesterase (Gross *et al.*, 1989; Stock *et al.*, 1989; Parkinson & Kofoid, 1992).

1.2.3 Phosphatase activity

The HK, which senses a specific environmental signal, transfers this signal to the cognate RR via the transfer of a phosphoryl group. Once phosphorylated, the RR becomes activated and triggers the appropriate cellular response. As long as the RR remains phosphorylated, the cellular response persists even if no longer necessary (Bourret *et al.*, 1991). The dephosphorylation of the RR is necessary to avoid continued stimulation and resets the system by restoring the phosphorylated RR (RR-P) to the unphosphorylated state, destroying outdated sensory data (Bourret *et al.*, 1991). The mechanism is not well understood but phosphatase activity requires MgATP, presumably as a cofactor to stabilise conformational rearrangements (Parkinson, 1993).

There are several routes to control the rate of dephosphorylation of RR-P and different components in the two-component system may exhibit phosphatase activity (Stock *et al.*, 1989, 1995; Bourret *et al.*, 1991; Parkinson & Kofoid, 1992; Perego & Hoch, 1996). In most cases,

the HK enhances greatly the dephosphorylation of RR-P. One molecule appears to sense both the presence and absence of the signal, playing the dual function of kinase and phosphatase. The two opposing activities supposedly result from different conformational states of the molecule (see section 1.2.4) (Stock *et al.*, 1989, 1995; Bourret *et al.*, 1991; Parkinson & Kofoid, 1992; Perego & Hoch, 1996).

RR have also been shown to be able to autodephosphorylate (Stock *et al.*, 1989, 1995; Bourret *et al.*, 1991; Parkinson & Kofoid, 1992; Perego & Hoch, 1996). This ability is thought to influence to some extent the stability of the phosphorylated RR. The RR-P stability varies greatly between two-component systems and probably correlates to the timing requirements for the implementation of a particular response (Bourret *et al.*, 1991). For example, CheY-P and CheB-P (chemotaxis) are the most unstable of the known RR, with half-lives measured in terms of milliseconds. PhoB-P (phosphate metabolism) or NtrC-P (nitrogen assimilation) show half-lives measured in terms of minutes. The stability of the RR-P appears to depend on whether the system is "designed" to operate in rapid time scale or not. In contrast, OmpR-P (osmoregulation) shows a half-life of approximately 1 hour. Although the response to changes in osmolarity is very fast in terms of switching the porin synthesis pattern, the actual change in membrane porin composition takes one cell generation. In long term responses like sporulation, the implementation of the response requires a long time scale to avoid premature decisions (Bourret *et al.*, 1991).

The system may also contain auxiliary phosphatases that accelerate the dephosphorylation process (Stock *et al.*, 1989, 1995; Bourret *et al.*, 1991; Perego & Hoch, 1996). The aspartate phosphatases are highly specific and are essential additional components of the two-component system. They play critical functions by adjusting the cellular level of RR versus RR-P and thus, enable two-component systems to control activities precisely (Perego & Hoch, 1996).

The existence of aspartate phosphatases as separate proteins must relate to the complexity of the signals (Perego & Hoch, 1996). The two-component system regulating sporulation of *Bacillus subtilis* is possibly among the most intricate systems and depends upon the activity of multiple kinases and regulators. In addition, this system is connected intimately with the two-component system regulating bacterial competence and the secretion of degradative enzymes (Stock *et al.*, 1989; Perego & Hoch, 1996). Therefore, the complexity of signals that govern whether the bacterium initiates sporulation or develops competence cannot be accomodated by simply regulating the input kinase. Recently, at least two aspartate phosphatases sharing amino acid sequence homology have been demonstrated to specifically dephosphorylate one RR (SpoOF) in the sporulation system (Perego & Hoch, 1996). These authors have found another five aspartate phosphatases showing approximately 40 to 50%

homology at the amino acid sequence level in *B. subtilis*. Perego and Hoch (1996) have not determined the substrate for these phosphatases but postulated that there may be a family of phosphatases as there are families of HKs and RRs.

As mentioned above, the mechanism underlying the dephosphorylation reaction remains poorly understood. Nevertheless, the reaction appears to be catalysed by the RR. The apparent role of the partner kinase or aspartate phosphatase is to bind the RR-P and introduce conformational instability into the site of phosphorylation so that the RR-P looses the phosphoryl group (Parkinson & Kofoid, 1992; Perego & Hoch, 1996). Considering this type of mechanism, the process of binding to the DNA might result equally in sufficient allosteric activity to stimulate the dephosphorylation of the RR. This could be a way to ensure that the RR-P would only induce a single round of transcription. In addition, the allosteric activity exerted by the DNA could explain the differences in the lifetime among RR-P (Perego & Hoch, 1996).

Phosphatase activity has not been detected in every system. This is the case of VirA/VirG, a two-component system that is involved in the regulation of virulence in *Agrobacterium tumefaciens* (Parkinson & Kofoid, 1992; Dorman, 1994c). This may simply mean that the investigators have not yet been able to identify a constituent in the VirA/VirG system with phosphatase activity. Alternatively, it reflects a need to maintain the transcriptional regulation for a long period.

1.2.4 HK-RR signalling properties

Given the structural description of the elements in a two-component system in the above sections, the mechanisms by which these elements interact will now be considered. HK/RR systems are very important functionally in the bacterial cell. They operate by transmitting signals concerning the state of the environment to the bacterial response machinery through a phosphotransfer mechanism. HK and associated RR communicate via two distinctive domains, the HK's transmitter and the RR's receiver (sections 1.2.1 and 1.2.2). Transmitter and receiver domains are structurally and functionally conserved among the HK and RR families, i.e., they are modular. Therefore, the two domains have been termed communication modules (Parkinson & Kofoid, 1992).

The mechanism of communication between transmitter-receiver modules depends upon phosphorylation and dephosphorylation reactions (Stock *et al.*, 1989, 1995; Parkinson & Kofoid, 1992; Parkinson, 1993). The two domains are usually located at the centre of the

signalling pathway in terms of the information flux. The activity of the transmitter is determined by the information flux from the input domain. The activity of the receiver depends upon the equilibrium between the kinase and phosphatase functions and determines the activity of the output domain. In summary, the two communication domains have in common the ability to modulate autophosphorylation and to mediate a flux of information that is regulated by their phosphorylation state (Parkinson & Kofoid, 1992).

1.2.4.1 Input-transmitter domain interactions. The autophosphorylation of the transmitter occurs upon propagation of sensory information from the input domain. The sensing mechanism is not yet established for many of the known two-component systems but is believed to result in conformational changes in the sensor protein (Parkinson & Kofoid, 1992; Parkinson, 1993). The way these conformational changes are induced depends on the system but the whole mechanism of propagation is still poorly understood. In the case of osmoregulation, the sensor HK (EnvZ) is thought to detect variations in environmental osmolarity by sensing changes in the fluidity or curvature of the cytoplasmic membrane (Parkinson, 1993). Alternatively, the sensors from the chemotaxis system (non-HK; see section 1.2.5.3) detect attractants or repellents in the environment by directly binding to these compounds (Parkinson, 1993).

A model has been proposed by Parkinson and Kofoid (1992) to explain the stimulation of autokinase activity by analogy with the sensors of the chemotaxis system. Currently, very little is known about second or tertiary structures of HK but there has been much research into the sensing mechanism of the non-HK chemotaxis sensors (MCPs). Parkinson and Kofoid (1992) postulate that the two subdomains within the transmitter domain (section 1.2.1), the H box (which they call T_L motif) and the N, D/F and Gs boxes (which they call T_R motif) interact to create an active or inactive autokinase. Prior to stimulation, the input domain remains at a relaxed conformation whereas T_L and T_R fold and bind in a tense conformation. The kinase remains inactive. Following stimulation, the input domain acquires a tense conformation leading to the relaxation of the transmitter which becomes exposed to ATP. The ATP probably functions as an allosteric effector, destabilising the T_L-T_R association and keeping the transmitter available to associate with the cognate RR (section 1.2.4.2). Based on the Parkinson and Kofoid (1992) hypothesis, transmitters in a relaxed conformation would exhibit kinase activity (autokinase ON mode) whereas transmitters in a tense conformation would exhibit phosphatase activity (autokinase OFF mode). The environmental stimulus would modulate phosphatase/kinase activity by shifting the OFF/ON equilibrium.

The phosphorylation of the histidine residue in the HK may not be an intramolecular reaction (Parkinson, 1993; Stock *et al.*, 1995). It has been proposed that kinases function as homodimers, the kinase of one monomer catalysing the phosphorylation of the histidine residue in the other monomer. Therefore, HK autophosphorylation apparently involves several intermediate reactions including kinase dimerisation, nucleotide binding and histidine phosphorylation (Stock *et al.*, 1995). The biological significance of a HK as a monomer has not been established and will probably vary with the system. *In vitro* studies show that intramolecular autophosphorylation does not occur at a significant rate (Stock *et al.*, 1995). *In vitro* studies also indicate that at least the kinases for chemotaxis (CheA) and nitrogen assimilation (NtrB) occur in the cell as dimers. However, nothing is actually known about the quaternary structure of HKs (Stock *et al.*, 1995).

1.2.4.2 Transmitter-receiver domain interactions. Once the HK has autophosphorylated, the phosphoryl group is transferred from the transmitter phosphohistidine to the aspartate in the cognate receiver. The molecular basis of these interactions has not yet been clearly established (Parkinson & Kofoid, 1992; Stock *et al.*, 1995).

The phosphotransfer reaction is catalysed by the RR (Parkinson & Kofoid, 1992; Stock *et al.*, 1995). The transmitter-receiver interaction probably involves reversible, specific association between the two communication modules (Fig.4) (Parkinson & Kofoid, 1992). Indeed, specificity is a very important aspect in these interactions since the phosphotransfer determines the overall precision of the response. Receivers can accept phosphoryl groups from certain small-molecule phosphodonors (*e.g.*, phosphoramidate, acetyl phosphate and carbamoyl phosphate) (Parkinson & Kofoid, 1992; Parkinson, 1993; Stock *et al.*, 1995) but not directly from ATP (Parkinson & Kofoid, 1992; Parkinson, 1993; Stock *et al.*, 1995; Appleby *et al.*, 1996). However, receivers have the ability to distinguish between the phosphodonors and they show much higher affinity for the partner HK than for the small-molecule phosphodonors (Stock *et al.*, 1995). It has been proposed that receivers recognise the local structure at the transmitter phosphohistidine site. Although the structural determinants have not yet been identified, the region of variable amino acid sequence around the H box and preceeding the N box are thought to participate in the recognition event (Parkinson & Kofoid, 1992; Stock *et al.*, 1995). Neither of these hypotheses has yet been tested or proven. Nevertheless, a transmitter-receiver docking mechanism (Fig.4) is thought most likely to occur, involving stereospecific

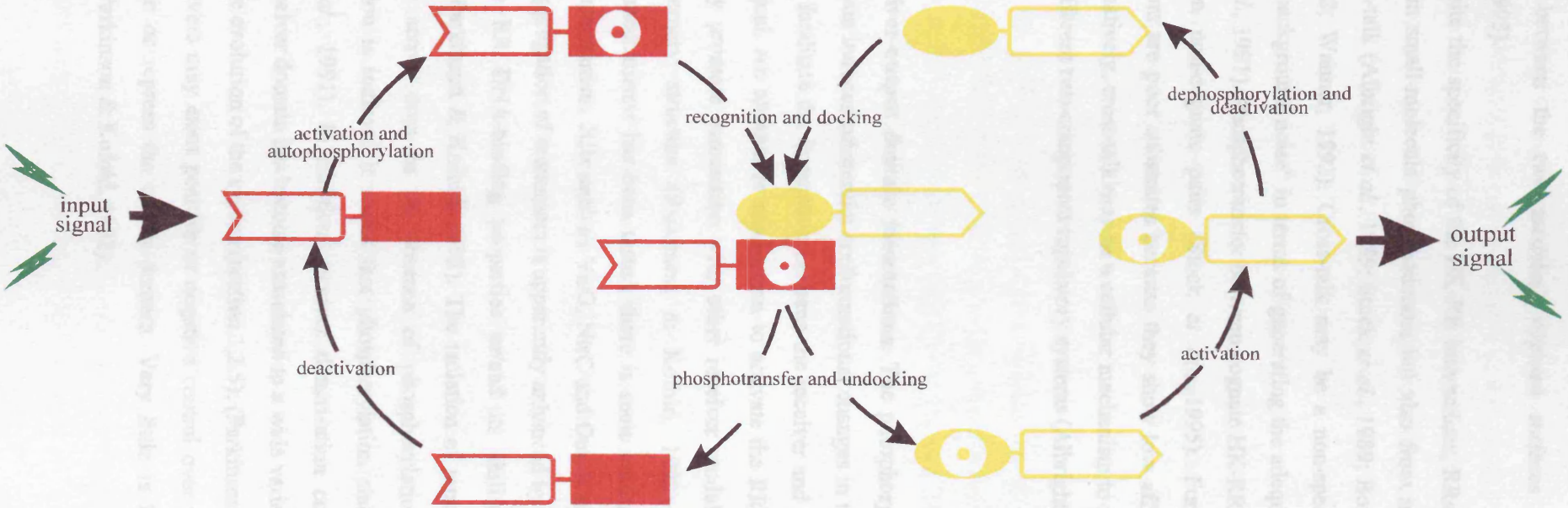


Fig.4. Signalling between communication modules. The flux of information between HKs and RRs involves docking between communication modules and subsequent release. Each step is potentially reversible but information flow only occurs in the directions indicated. Phosphotransfer depends on reversible equilibria between the phosphorylation states of the transmitter and the receiver (adapted from Parkinson & Kofoid, 1992).

interactions between the two modules' exposed surfaces (Parkinson & Kofoid, 1992; Parkinson, 1993).

Despite the specificity of the HK-RR interactions, RRs can accept phosphoryl groups not only from small-molecule phosphodonors but also from non-partner HKs. This event is termed cross-talk (Albright *et al.*, 1989; Stock *et al.*, 1989; Bourret *et al.*, 1991; Parkinson & Kofoid, 1992; Wanner, 1992). Cross-talk may be a non-specific event, leading to a non-significant "background noise" in terms of generating the adequate cellular adaptive responses (Bourret *et al.*, 1991). Phosphotransfer between cognate HK-RR pairs is at least two-fold faster than between non-cognate pairs (Stock *et al.*, 1995). Furthermore, the small-molecule phosphodonors are poor substrates because they show low affinity for the RR (Stock *et al.*, 1995). Alternatively, cross-talk may be a cellular mechanism to integrate environmental stimuli by linking different two-component regulatory systems (Albright *et al.*, 1989).

1.2.4.3 Receiver-output domain interactions. The phosphorylation of the aspartate in the receiver domain induces substantial conformational changes in the RR (Parkinson, 1993). This is thought to facilitate the interaction between the receiver and the output domains, leading to an output signal. An alternative mechanism to activate the RR proposes that conformational variation may promote association with other receiver modules leading to changes in the protein quaternary structure (Parkinson & Kofoid, 1992). Although neither of these hypothetical mechanisms has been tested, there is some evidence in support of the receiver dimerisation mechanism. RRs such as VirG, NtrC and OmpR have been shown to function as multimers. Aggregation of monomers is apparently enhanced by phosphorylation which in turn enhances the RR DNA-binding properties or/and its ability to interact with the RNA polymerase (Parkinson & Kofoid, 1992). The isolation of mutants in the RR genes that are constitutively active even in the absence of phosphorylation suggests that the role of phosphorylation is indirect. It seems that phosphorylation stabilises a conformational state (Bourret *et al.*, 1991). In addition, receiver dimerisation could explain how the highly conserved receiver domain has become associated to a wide variety of different output domains throughout the evolution of the system (section 1.2.5); (Parkinson & Kofoid, 1992).

Receivers may exert positive or negative control over the output function, i.e., they either activate or repress the output domain. Very little is known about this controlling mechanism (Parkinson & Kofoid, 1992).

1.2.4.4 Properties of the signal transduction. Because the communication modules are central to the transduction of signal, the transmitter-receiver interactions are most likely to be the critical step in the signalling pathway. These include the rate of phosphorylation of the transmitter (section 1.2.4.1), the rate and specificity of the phosphotransfer reaction (section 1.2.4.2) and the equilibrium between phosphorylation and dephosphorylation of the receiver (Parkinson, 1993; Stock *et al.*, 1995).

The intensity of a response is determined by the level of the stimulus, i.e., the response is smoothly graded (Parkinson, 1993). Signal transduction depends upon simple switches: HKs have kinase or phosphatase activity; RRs have phosphorylated or dephosphorylated states. Each transition occurs through reversible covalent inter- and/or intramolecular modifications, leading to qualitative changes of signal, i.e., stimulation versus inhibition, induction versus repression. The regulatory system can deal with both the presence and the absence of environmental signal. In fact, it is the environmental signal that modulates the ratio between opposing activities. These types of transition are thought to provide a faster response than would a shift between repressor and inducer activities allocated to different proteins (Parkinson, 1993).

1.2.5 The design of HK-RR circuits

An ever-growing number of two-component regulatory systems has been identified in a range of both Gram-positive and Gram-negative bacteria (Parkinson & Kofoid, 1992). These systems mediate adaptive responses to a wide variety of environmental signals (Table 1). The basic functional elements have been maintained in all two-component systems. They are a signal detector, a HK that autophosphorylates, a regulator with phosphorylation sensitive activity, a target for the regulatory functions and a mechanism to interrupt the response by dephosphorylating the regulator. All these functional modules have been arranged to suit a list of cellular functions. As different HK/RR systems are better understood, the concept of a "typical" two-component system should probably be dismissed. It is my opinion that there is not a "typical" two-component system but that each system has unique features additional to the basic functional modules. As yet, no two systems have been found to be organised in the same manner (Bourret *et al.*, 1991; Appleby *et al.*, 1996). Two-component systems follow a modular design but each circuit has diverged to serve a particular sensory task. Nevertheless, many authors consider the simpler systems (*e.g.*, osmoregulatory system) as "paradigms" in order to explain the basic elements of a two-component system.

1.2.5.1 The signal. Sensing the signal is the starting point of all two-component regulatory processes or of any signal transduction mechanism that couples the status of the environment (intra or extracellular) with gene expression. The detector may sense chemical signals by directly binding a ligand (*e.g.*, chemotaxis) or may sense a physical parameter by detecting changes in fluidity or curvature of the cytoplasmic membrane (*e.g.*, osmolarity) (Parkinson, 1993). In some systems, the sensing mechanism is well understood but in some systems, particularly those which detect physical conditions, the mechanism remains speculative. Nevertheless, the detection mechanism appears to always induce conformational changes in the detector, leading to the stimulation of the signal transduction pathway. The signal is transduced by reversible protein phosphorylation. Systems like the one controlling chemotaxis utilise two types of "language" to transduce the signal: reversible protein methylation followed by reversible protein phosphorylation (Parkinson, 1993).

1.2.5.2 The detector. Every two-component system possesses a detector. The detector is commonly a transmembrane protein but in systems that detect intracellular signals, the detector may be a cytoplasmic protein. This is the case of the regulatory mechanism for nitrogen assimilation whose signal is the intracellular ratio between nitrogen and carbon and the detector is an UT/UR enzyme (uridylyltransferase/uridylyl-removing) (Bourret *et al.*, 1991).

1.2.5.3 The HK. The HK is an essential element to transduce the signal. Most systems depend upon the activity of a single kinase but in cases such as sporulation with highly complex signal transduction pathways, the system includes multiple kinases. There are also cases where kinases such as BvgS (kinase from BvgS/BvgA system that regulates virulence in *Bordetella spp.*) have the transmitter region fused to a receiver domain which is subsequently fused to a C-terminal domain (Gross *et al.*, 1989; Stock *et al.*, 1990). In these cases, upon autophosphorylation at the transmitter's conserved histidine, the kinase successively transfers the phosphoryl group to an aspartate residue at the kinase's receiver, to another histidine at the kinase's C-terminal domain and finally, to the RR conserved aspartate residue (Appleby *et al.*, 1996).

The HK is frequently both the detector and a transmembrane protein but there are exceptions. The regulatory mechanism for nitrogen assimilation utilises a UT/UR enzyme

whereas the regulatory mechanism for chemotaxis utilises transmembrane chemoaccepting proteins (MCPs) for the signal detection. In the latter, one kinase integrates signals sensed by multiple detectors and in both systems, the kinases (NtrB and CheA, respectively) are located in the cytoplasm. Another common feature among the HKs is their autophosphorylation activity. However, some HKs are assisted by other proteins during the phosphorylation process. An example consists of CheA whose autophosphorylation is enhanced by CheW (Parkinson, 1993).

1.2.5.4 The regulator. The regulator is invariably a cytoplasmic protein. In most systems, the RR is a transcriptional activator but RRs like PhoP or BvgA (section 1.2.5.5) may be both a transcriptional activator and repressor. Alternatively, CheY and CheB (chemotaxis) act post-translationally upon other proteins rather than acting at the transcriptional level. Regardless of the target, the activation of the regulator apparently depends on an induced conformational state stabilised by phosphorylation. Usually, there is only one RR per system but there are a few systems that involve two RR, for example chemotaxis (CheY and CheB) and sporulation (SpoOF and SpoOA).

1.2.5.5 The target: involvement of two-component systems in the regulation of pathogenicity. As mentioned at the beginning of section 1.2.5, two-component systems are involved in the transduction of a wide variety of signals and thus, they control a large number of cellular functions (Table 1). One of these functions concerns pathogenicity (Dziejman & Mekalanos, 1995).

Temperature is one of the environmental signals that modulate the activity of the two-component system BvgS/BvgA (Table 1). This two-component system has been shown to regulate the expression of a set of virulence determinants of *Bordetella pertussis* (*bvg*: *Bordetella* virulence gene) via a multi-step phosphorelay mechanism (Appleby *et al.*, 1996). BvgS is a transmembrane protein and contains an input domain, a transmitter domain fused to a receiver domain (homologous to the N-terminal region of a RR; section 1.2.2) which in turn is fused to a terminal C-region. Once activated, BvgS autophosphorylates at the transmitter conserved histidine residue. Subsequently, the phosphoryl group is successively transferred to an aspartate residue at the protein's receiver domain, to another histidine residue at the

Table 1. Two-component signalling systems (adapted from Parkinson & Kofoid, 1992).

Adaptive response	Two-component system		Organism[b]
	HK[a]	RR[a]	
Chemotaxis	CheA	CheY, CheB	Bsu, Eco, Pae, Sty, etc
Carbon utilisation	DctB	DctD	Rle, Rme
	PgtB	PgtA	Sty
	UhpB	UhpA	Eco, Sty
Nitrogen utilisation	NtrB	NtrC	Atu, Eco, Kpn, etc
Phosphate utilisation	PhoR	PhoB	Bsu, Eco, Kpn, Pae, Sdy, etc
	CreC	CreB	Eco
Nitrogen fixation	FixL	FixJ, Orf138	Rme, etc
Photosynthesis		RegA	RcA
Redox changes	HydH	HydG	Eco, Sty
	NarQ, NarX	NarL	Eco
	ArcB	ArcA	Eco
Stress from antibiotics	RteA	RteB	Bth
Stress from heavy metals	CutS	CutR	Sli
Stress from osmolarity	EnvZ	OmpR	Eco, Sty
Stress from starvation	AgrB	AgrA	Sau
	DegS	DegU	Bsu
Stress from turgor pressure	KdpD	KdpE	Eco
Antibiotic synthesis	VanS	VanR	Efa
Capsule synthesis	AlgR2	AlgR1, AlgB	Pae
	RcsC	RcsB	Eco
Fruiting-body formation	FrzE	FrzZ	Mxa
Gene transfer	ComP	ComA	Bsu
Sporulation	KinA, KinB, KinC	SpoOA, SpoOF	Bsu
Virulence	PhoQ	PhoP	Sty
	Xcc2	Xcc1	Xca
	BvgS	BvgA	Bpe, etc
	LemA		Psy
	VirA	VirG	Atu, etc

[a]: In some systems, the HK or the RR has not yet been identified.

[b]: Organism abbreviations: Atu, *Agrobacterium tumefaciens*; Bpe, *Bordetella pertussis*; Bsu, *Bacillus subtilis*; Bth, *Bacteroides thetaiotaomicron*; Eco, *Escherichia coli*; Efa, *Enterococcus faecium*; Kpn, *Klebsiella pneumoniae*; Mxa, *Myxococcus xanthus*; Pae, *Pseudomonas aeruginosa*; Psy, *Pseudomonas syringae* pv *syringae*; Rca, *Rhodopseudomonas capsulata*; Rme, *Rhizobium meliloti*; Sau, *Staphylococcus aureus*; Sdy, *Shigella dysenteriae*; Sli, *Streptomyces lividans*; Sty, *Salmonella typhimurium*; Xca, *Xanthomonas campestris* pv *campestris*.

protein's C-terminal region and finally to the RR conserved aspartate in BvgA. Transcription of *bvgA* is initiated from at least three promoters: a BvgA-independent (P_2) and two BvgA-dependent (P_1 and P_3) promoters. Temperature stimulation is thought to occur by inducing transcription of *bvgA* off P_1, the strongest promoter, and by shutting down transcription off the P_2 promoter. This event is regulated by the activated form of BvgA. As the intracellular levels of BvgA increase, the various BvgA-dependent virulence determinants are activated in a temporally regulated manner. There is some evidence suggesting that BvgA positive regulation is indirect, i.e., BvgA appears to act upon subordinate regulator(s) in a cascade of regulatory events (section 1.3) (Dorman, 1994c; Uhl & Miller, 1995).

The plasmid-borne (unlike most of the known two-component systems) VirA/VirG two-component system is involved in the regulation of virulence (*vir*) genes in *Agrobacterium tumefaciens* (Heath *et al.*, 1995). *vir* genes are present in a region of the Ti (tumor inducing) plasmid and code for proteins required for the sensing of plant wound metabolites as well as the transfer and integration of a region of the Ti plasmid into the plant cell chromosome. *vir* genes are not transferred into the plant cell but are essential for the crown-gall tumour formation. VirA is a transmembrane protein with an input domain and a transmitter domain which is fused to a receiver domain (homologous to the N-terminal region of the RR VirG). VirA autophosphorylates in response to plant signal molecules: phenolic compounds and monosaccharides. Moreover, VirA also responds to phosphate starvation and to acid pH. Following autophosphorylation at the transmitter conserved histidine residue, the phosphoryl group is successively transferred to an aspartate residue at the protein's receiver domain, to another histidine residue at the protein's C-terminal region and finally to the RR, VirG, conserved aspartate. Two promoters affect the transcription of *virG*: P1, that is induced by the phenolic compounds in a VirA/VirG-dependent manner or by phosphate starvation in a VirA/VirG-independent manner; P2 that is possibly induced by low pH. The plant wound metabolites are sensed by bacteria via VirA/VirG and the environment is recognised as a favourable one for plant cell colonisation by bacteria. Consequently, VirA/VirG activate the *vir* genes (Heath *et al.*, 1995).

The regulation of virulence gene expression in *S. typhimurium* is thought to be modulated by many different environmental signals (*e.g.*, low pH, osmolarity, starvation, etc) via a large number of two-component systems (Groisman & Heffron, 1995). Low pH is thought to be one of the signals sensed by PhoQ/PhoP which positively regulates the transcription of genes essential for intracellular survival within acidified macrophages (*pag*: PhoP activated genes) and represses the transcription of genes essential for invasion (*prg*: PhoP repressed genes). Osmolarity also controls the expression of virulence through EnvZ/OmpR and anaerobiosis has also been implicated through the two-component system ArcB/ArcA. In fact, it

has been postulated that perhaps as many as 50 two-component systems may be involved in the regulation of the virulence cascade of events in *S. typhimurium* (Groisman & Heffron, 1995).

These are only a few examples of pathogens relying upon two-component regulatory systems to control the expression of virulence in response to environmental signals but there are many more (Dziejman & Mekalanos, 1995).

It is important to note that two-component systems do not function in isolation within the cell. Virulence gene expression is under multifactorial environmental control. Pathogens often use regulatory cascades to express virulence determinants in different stages of the infection process rather than expressing them constitutively. Therefore, two-component systems are often coordinately integrated with other regulatory mechanisms (section 1.3) and are subjected to cross-regulation (DiRita & Mekalanos, 1989; Mekalanos, 1992; Wanner, 1992; Gross, 1993; Dorman, 1994a).

1.3 Coordinate control of transcriptional responses: regulatory networks

1.3.1 Bacterial transcriptional regulators

Bacteria are highly adaptable organisms and this may be the reason why they are successful colonisers of a large variety of ecological niches (Dorman, 1995). Bacteria constantly gather information from their surroundings and adjust their physiology and behaviour accordingly. These adaptations frequently imply changes in the transcriptional profile of the genome. Therefore, gene expression is largely determined by the signals sent in by the environment. In order to efficiently accomplish the necessary cellular adjustments, bacteria have developed precise and fine-tuned regulatory mechanisms that respond to specific environmental signals. One of these mechanisms is the two-component regulatory systems (section 1.2) but the bacterial cell possesses other regulatory systems that elicit changes in gene expression. A few examples of such systems are described below.

The AraC-like family of transcriptional regulators include proteins that share conserved amino acid sequence (at the C-terminus) with the regulator of the arabinose biosynthesis operon. AraC-like proteins have a N-terminal carbohydrate-binding domain (the sensor domain) and a C-terminal DNA-binding domain (the effector domain). The members of the AraC-like family have been subgrouped in two classes according to their roles: one class concerns the regulation of genes involved in carbohydrate metabolism (*e.g.*, cellobiose or rhamnose) and one

class contains regulators of virulence genes (*e.g.*, genes involved in the defence against superoxide stress) (Dorman, 1994a).

The LysR-like family of transcriptional regulators include proteins that share conserved amino acid sequence (located at the N-terminus) with the regulator of the lysine biosynthesis operon. The LysR-like proteins contain a N-terminal DNA-binding domain. They have been implicated in the regulation of genes concerned with amino acid biosynthesis (*e.g.*, lysine, methionine or tryptophan), antibiotic resistance, initiation of chromosome replication, oxidative stress response, iron-dependent virulence gene expression in *V. cholerae* or plasmid-encoded virulence genes in *Salmonella* (Dorman, 1994a).

The Lrp (leucine responsive regulatory protein) transcriptional regulator associates with leucine and the Lrp-leucine complex controls the expression of a regulon responsive to leucine. Lrp-leucine acts as either an activator or a repressor of genes involved in biosynthesis of some amino acid residues (isoleucine, leucine, valine or serine) and the synthesis of porins (OmpF and OmpC). (Dorman, 1994a).

Fur (ferric uptake regulation) transcriptional regulator represses genes of the iron assimilation pathways in presence of ferrous iron. This protein also regulates the expression of genes not directly involved in the uptake of iron, including bacterial virulence determinants (*e.g.*, expression of exotoxin A in *Pseudomonas aeruginosa* or expression of shiga-like toxin type I in *E. coli*) (Mekalanos, 1992; Gross, 1993). Fur contains a N-terminal DNA binding motif that binds to specific DNA sequences (the Fur box) in the promoters of the genes under its control (Dorman, 1994a).

The above examples refer to only a few of the bacterial regulatory systems that control the expression of specific operons or regulons. However, each of these operons or regulons is not isolated in the bacterial cell but are interconnected such that the bacterial cell exists as an integrated unit (Fig.5). Regulatory proteins follow a hierarchical organisation and are ranked in terms of their pleiotropy. The effects of specific regulators, i.e., lower ranked, can be influenced by other higher ranked regulators. These are the so-called global regulators because they influence the expression of a large number of unrelated promoters (Dorman, 1995). Crp (cyclic AMP receptor protein) is an example of a global regulator. Crp is usually an activator and only binds to specific DNA sequences on the bacterial chromosome and in the presence of a cofactor, cyclic AMP (cAMP). The DNA-binding motif is located at the C-terminus of the protein. Crp may antagonise other regulators, for example, the repressor of the lactose operon, or may activate the expression of the regulator for a certain operon, for example, the regulator of the maltose regulon. Global regulators often connect distinct operons or regulons (Dorman, 1994b). However, the bacterial cell possesses an even higher rank of regulators. These have been termed universal regulators because of their apparently unlimited capacity to influence

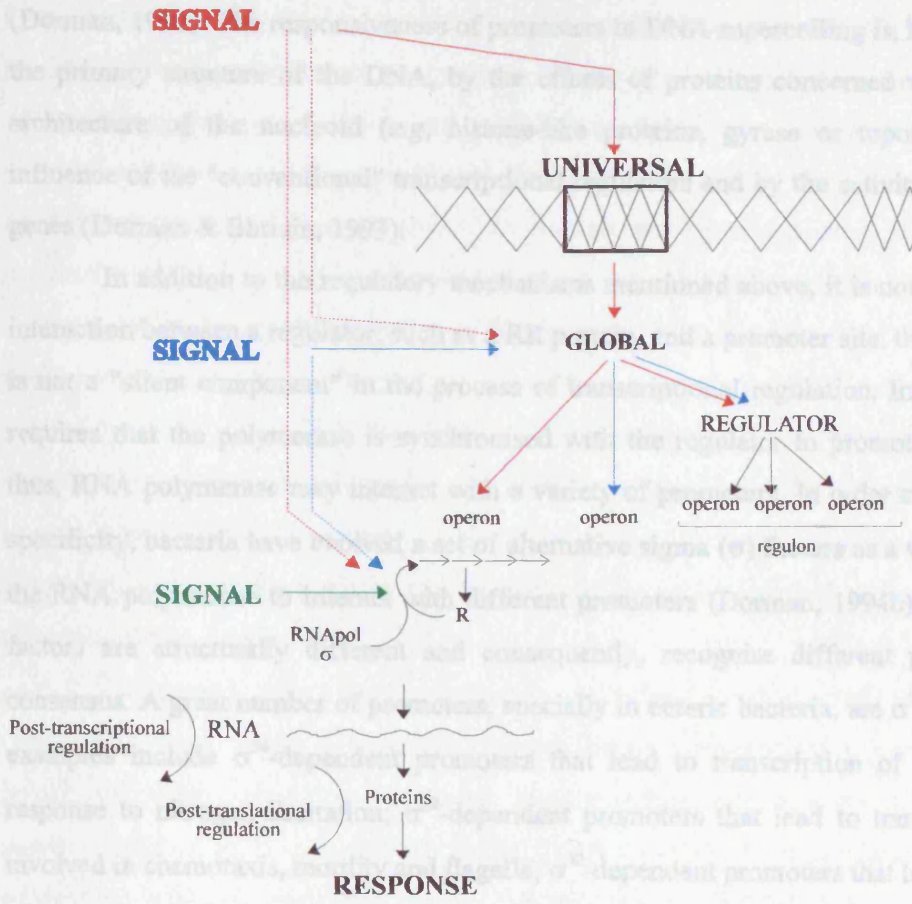


Fig.5. Schematic representation of networking in the regulation of gene expression. Transcriptional regulation of gene expression is organised as a cascade of events. Each level depends upon one or more regulatory proteins which may or may not be directly affected by the same signal. The different regulators follow a hierarchical organisation according to their pleiotropy. They are specific regulators if they only influence a specific set of genes. However, these genes may be organised as an operon or a regulon and thus, there are different ranks of specific regulators. Higher in the hierarchy, there are global regulators that control unrelated sets of genes through their specific regulators. As the scale ascends, the complexity of the interconnections increases and global regulators are subordinate to even higher ranked ones: the universal regulators (*e.g.* DNA topology). At all steps, transcription is coupled with the RNA polymerase/σ factor.

gene expression: universal regulators do not depend on the recognition of specific DNA sequences but on DNA architecture (Dorman, 1995). DNA topology is considered an universal regulator and is apparently influenced by environmental signals like temperature and osmolarity (Dorman, 1991). The responsiveness of promoters to DNA supercoiling is, however, limited by the primary structure of the DNA, by the effects of proteins concerned with organising the architecture of the nucleoid (*e.g*, histone-like proteins, gyrase or topoisomerase), by the influence of the "conventional" transcriptional regulators and by the activities of neighbouring genes (Dorman & Bhriain, 1993).

In addition to the regulatory mechanisms mentioned above, it is noteworthy that in the interaction between a regulator, such as a RR protein, and a promoter site, the RNA polymerase is not a "silent component" in the process of transcriptional regulation. Instead, transcription requires that the polymerase is synchronised with the regulator to promote transcription and thus, RNA polymerase may interact with a variety of promoters. In order to achieve promoter specificity, bacteria have evolved a set of alternative sigma (σ) factors as a way to "reprogram" the RNA polymerase to interact with different promoters (Dorman, 1994b). The alternative σ factors are structurally different and consequently, recognise different promoter sequence consensus. A great number of promoters, specially in enteric bacteria, are σ^{70}-dependent. Other examples include σ^{54}-dependent promoters that lead to transcription of genes involved in response to nitrogen limitation; σ^{28}-dependent promoters that lead to transcription of genes involved in chemotaxis, motility and flagella; σ^{32}-dependent promoters that lead to transcription of genes involved in response to heat shock; σ^{38}-dependent promoters that lead to transcription of genes during stationary phase (Dorman, 1994b). The use of σ factors brings yet another level of complexity into the hierarchy of regulators (Fig.5).

It is not within the scope of this chapter or of Part II to extensively cover the vast subject of bacterial gene regulation. Instead, it is only envisaged to focus on environmentally directed transcriptional regulation of gene expression and particularly regulation dependent on the two-component systems. Therefore, all regulatory systems mentioned in section 1.3.1 (and throughout chapter 1) refer to the regulation of gene expression at the transcriptional level. In fact, given the wide variety of mechanisms bacteria utilise for transcriptional regulation of gene expression, it seems that this process is the target of choice to control gene expression (Dorman, 1994b). Nevertheless, it is important to emphasise that regulation may occur at other levels, for example, post-transcriptionally (*e.g.*, by antisense RNA) or post-translationally (*e.g.*, by phosphorylation).

1.3.2 Bacterial regulatory networks

Changes in gene expression usually result from the activation or repression of transcription carried out by regulatory proteins. Because bacteria inhabit multicomponent environments, the adaptations may require the products of many genes. These genes may be organised as operons or may belong to progressively more complicated arrangements like regulons and stimulons (Dorman, 1994a). Cellular responses may also imply that the expression of different genes will be up- and/or down-regulated simultaneously. No single regulatory mechanism is able to achieve all this. Instead, single regulatory circuits are coordinately integrated in global regulatory networks (Gottesman, 1984; Dorman, 1994a). Each circuit has a highly specific regulator. Transcription is tightly controlled and gene expression occurs at precise moments. The different circuits are subsequently linked through more pleiotropic regulators to generate a network of coordinated events. The different regulatory circuits are multilayered and as the scale ascends, the complexity of the interconnections increases. It is for the global and universal regulators to supervise the gene networking (section 1.3.1; Fig.5) (Dorman, 1994a, 1995).

The osmotic stress response stimulon provides a good example for gene networking (Dorman, 1994a). Under hyperosmotic stress, bacteria accumulate a variety of compatible solutes and thus, genes concerned with osmoprotectant synthesis are induced. One of the operons induced in *E. coli* is the *kdpABC* (high-affinity ATP-dependent uptake system for potassium) which is regulated by the HK/RR system, KdpD/KdpE. In addition, another immediate response to changes in osmolarity is the variation in the ratio of porins in the membrane. These adjustments are regulated by a distinct HK/RR system, EnvZ/OmpR. KdpE and OmpR are examples of specific regulators. However, the EnvZ/OmpR system is not only part of this stimulon but can also respond to other signals such as pH, temperature or oxygen availability. The regulation of expression of the porins is also controlled by other regulators, including Lrp transcriptional regulation and cAMP/Crp global regulation (section 1.3.1). Moreover, one of the operons concerned with osmoprotection is the *proU* operon (*proVWX*; uptake of glycine-betaine). The activation of this operon is directly influenced by changes in DNA topology that is considered an universal regulator. The expression of the *proU* operon (*S. typhimurium*) as well as of porins (*E. coli* and *S. typhimurium*) are also induced by anaerobiosis, a different stimulon. Therefore, the signal "high osmolarity" elicits overlapping between regulons (*e.g.*, Lrp, cAMP/Crp) and stimulons (*e.g.*, osmotic stress, anaerobiosis). There are other stimulons like heat-shock response, cold-shock response or oxidative stress which depend upon the integration of different regulatory mechanisms. The bacterial cell exists as an integrated unit and successful survival is determined by the sensitive equilibrium between

forces attempting to activate transcriptional units and those which repress them. The interplay of all the different operons, regulons and stimulons produce dynamic and highly adaptable multifactorial phenotypes (Dorman, 1994a, 1995).

1.4 Environmental regulation of *C. jejuni* pathogenicity

As discussed in the General Introduction (section 4), the mechanisms by which *C. jejuni* causes infection remain unclear. Despite its significance as a human pathogen, there are no well characterised virulence determinants. Consequently, very little is known about the regulation of virulence gene expression in *C. jejuni*. Nevertheless, as with other bacterial species, environmental parameters are likely to be important (Dorman, 1994c).

C. jejuni needs to sense, interpret and adapt to different conditions encountered during its association with the host as well as during the free-living state. Furthermore, bacteria will need to recognise the right time and the right site to switch on the expression of their virulence determinants. By analogy with other bacterial species, they will probably rely upon specific cues to identify a host-associated environment and therefore, it is most likely that environmental conditions are involved in the control of *C. jejuni* pathogenicity.

The current knowledge on environmental regulation of *C. jejuni* pathogenicity is very limited although there is some evidence in its support. As mentioned before (General Introduction; section 4.4.3.1), the relevance of the flagellum in *C. jejuni* pathogenesis is well established. Transcription of one of *C. jejuni* flagellin genes, *flaB*, is regulated by σ^{54} and has been shown to be subjected to environmental regulation in response to conditions such as temperature, pH and inorganic salt and divalent cation concentrations (Taylor, 1992; Ketley, 1995, 1997). As to *flaA*, its transcription is regulated by σ^{28}. FlaA biosynthesis or secretion is apparently regulated by FlbA (flagella biogenesis), a protein from the LcrD/FlbF family of regulators (Miller, S. *et al.*, 1993). Although LcrD-like proteins have been shown to be important in the virulence of *Yersinia pestis*, *Shigella flexneri* and *S. typhimurium*, FlbA behaves more like the FlbF protein which is involved in the expression, secretion or assembly of flagella in *Caulobacter crescentus* (*cit. in* Miller *et al.*, 1993).

C. jejuni aggregative pili are also under environmental control and are expressed in response to compounds found in host-associated environments such as bile salts (Doig *et al.*, 1996). Since aggregative pili are considered putative virulence determinants (General Introduction; section 4.4.4), the presence of bile salts may be the signal sensed by bacteria to produce them.

Iron may also be important as an environmental signal. A Fur-like transcriptional regulator (section 1.3.1) was identified in *C. jejuni* (Wooldridge *et al.*, 1994). It has been proposed that Fur-like regulator controls the transcription of *sodB* and *katA* genes (General Introduction; section 4.2.4). Given that these genes may be implicated in the ability of *C. jejuni* to survive intracellularly, it is possible that iron and the Fur-like protein play an important role in environmental control of *C. jejuni* pathogenicity.

In view of these findings and by analogy with other bacterial pathogens, it is highly probable that *C. jejuni* virulence gene expression is under environmental regulation. However, it is necessary to investigate this further and to identify regulatory mechanisms responsible for directing *C. jejuni* adaptive responses.

1.5 Aims of the project

The primary aim of my project was to determine whether *C. jejuni* utilised regulatory systems from the two-component family. In recent years, it has become apparent from the study of control of bacterial virulence gene expression that there are several themes commonly used by pathogens. One of these themes is the fact that bacteria frequently use similar environmental cues to identify a particular host compartment (Mekalanos, 1992; Dorman, 1994c; Dziejman & Mekalanos, 1995). Another common theme appears to be the use of the same signal transduction systems to elicit adaptive responses to the environmental pressures (Miller, J.F. *et al.*, 1989; Mekalanos, 1992; Dorman, 1994c).

C. jejuni presumably encounters a variety of microenvironments, both when free-living or inside the host. In order to survive and multiply, campylobacters need the mechanisms to interpret and respond to external signals. It has become clear that two-component regulatory systems have a role in providing pathogens with a way of adapting and responding to the conditions found inside the host. Hence, it is highly probable that *C. jejuni* also utilises two-component regulatory systems. In order to isolate a putative RR-like gene in *C. jejuni*, a genomic library was screened using a DNA probe previously isolated and found to encode a peptide with sequence similarity to a RR receiver domain. A RR-like gene was isolated from the library and was sequenced. Subsequently, two mutants were isolated and their phenotypes were investigated to determine the role played by this RR-like protein in *C. jejuni*.

It is clear from section 1.4 that much remains to be uncovered on the mechanisms by which gene expression in *C. jejuni* is regulated. Like other bacteria, *C. jejuni* will certainly utilise complex networks of regulators to control all cellular events. The work developed in this

thesis represents an attempt to start identifying the components of such networks. However, to understand how they function, both specifically and in context of overall regulatory networks, is the ultimate objective to be achieved.

CHAPTER 2
MATERIALS & METHODS

2.1 Bacterial strains

2.1.1 *Campylobacter jejuni* strains

81116 (NCTC 11828), is a strain obtained as an isolate from a patient with diarrhoea (Palmer *et al.*, 1983);

AB1 (this study) derived from 81116 following the insertion of a *C.coli* kanamycin resistance gene (Trieu-Cuot *et al.*, 1985) in the *regX1* gene;

AB2 (this study) derived from 81116 following the insertion of a *C.coli* kanamycin resistance gene (Trieu-Cuot *et al*, 1985) in the *regX1* gene;

2T is a 81116-derived strain, following the insertion of a *C.coli* kanamycin resistance gene in a *htrA*-like gene (Henderson, 1996);

O81, is a strain isolated from stools during routine bacteriological screening by staff at the Department of Microbiology, St. Pieters University Hospital, Brussels (see section 2.2, Part I);

P71, is a strain isolated from stools during routine bacteriological screening by staff at the Department of Microbiology, St. Pieters University Hospital, Brussels (see section 2.2, Part I).

2.1.2 *Escherichia coli* K-12 strain derivatives

XL-1 Blue F'[*proAB, lacI*^{q}ZΔM15, Tn10], *recA1, endA1, gyrA96, thi-1, hsdR17*(r_k^-, m_k^+), *relA1,* Δ (*lac-pro*), *supE44* (Bullock *et al.*, 1987);

DH5α F$^-$, ϕ80d*lacZ*ΔM15, *recA1, endA1, gyrA96, thi-1, hsdR17*(r_k^-, m_k^+), *supE44, relA1, deoR,* Δ (*lacIZYA-argF*) U169 (Hanahan, 1983);

SM10 F^-, *thi-1*, *thr-1*, *leuB6*, *tonA21*, *lacYI*, *supE44*, *recA*::RP4-2-Tc::Mu, Km^r (Simon *et al*, 1983).

2.2 Vectors and oligonucleotide primers

2.2.1 Plasmids and bacteriophages

pUC18 and 19 plasmids: high copy number cloning vectors containing a multiple cloning site polylinker within the region encoding the α-peptide of the *lacZ* (β-galactosidase) gene. When transformed into host strains containing the ΔM15 mutation of the *lacZ* gene and in the presence of the *lacZ* operon inducer IPTG (isopropyl thio-β-D-galactoside), the vectors will complement the deletion in the host strain to produce a functional β-galactosidase protein. This allows blue/white colour selection of pUC18 or 19-derived clones on X-gal (5-bromo-4-chloro-3-indolyl phosphate) / IPTG containing plates. The insertion of foreign DNA into the multiple cloning site interrupts the α-peptide and prevents complementation, resulting in the formation of white colonies, whereas an intact polylinker leads to α-complementation and the production of blue colonies. These plasmids also carry the gene coding for β-lactamase, conferring resistance to ampicillin as another means for selection of derived clones (Yanisch-Perron *et al.*, 1985).

pBluescript II SK +/- phagemid: high copy-number cloning vector derived from pUC19, carrying a larger multiple cloning site polylinker. The polylinker is flanked by T3 and T7 bacteriophage promoters. pBluescript also contains the f1 bacteriophage origin of replication, thus enabling the production of single stranded DNA in the presence of f1 as a helper phage. This phagemid carries the gene coding for β-lactamase, conferring resistance to ampicillin as means for selection of derived clones (Short *et al.*, 1988).

pUOA15 plasmid: pUC13-derived shuttle vector (Wang & Taylor, 1990), containing a replication origin of *C.coli* plasmid pIP1445 and an origin of transfer of the RK2 plasmid. In addition, pUOA15 carries the genes coding for ampicillin resistance (only expressed in *E. coli*) and tetracycline resistance (expressed in both *E. coli* and *C. jejuni*; (Taylor, 1986)) and the α-peptide of the *lacZ* (β-galactosidase) gene.

pTNS#A plasmid: pBluescript derivative (see below), 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).

λ ZAP II bacteriophage: cloning vector derived from λ ZAP vector, both developed and manufactured by Stratagene. λ ZAP II lacks the Sam 100 mutation present in λ ZAP and therefore is able to invade non-*sup*F host strains (Short *et al.*, 1988).

R408 phage: f1-derived filamentous phage (Stratagene).

2.2.2 Oligonucleotide primers

The oligonucleotide primers listed in Table 2 specifically anneal to: DNA sequences flanking the multiple cloning sites from pBluescript and pUC19 vectors, DNA sequences in *regX1* gene (R1, R2, R3 and R4) or to the *C. coli* kanamycin resistance gene (R and F):

Table 2. DNA sequence from oligonucleotide primers used in PCR and sequencing reactions.

Oligonucleotide Primer	DNA Sequence (5'→3')
VECTOR	**SPECIFIC**
P1	TCC CAG TCA CGA CGT
P2	ATG TTG TGT GGA ATT GTG
P2/*Sst*I	CAC GAG CTC ATG TTG TGT GGA ATT GTG
P1L	GGG TTT TCC CAG TCA CGA CGT TGT
P2L	TAT GTT GTG TGG AAT TGT GAG CGG
M13 reverse (M13R)	GGA AAC AGC TAT GAC CAT G
M13 forward (M13F)	GTA AAA CGA CGG CCA GT
regX1	**SPECIFIC**
R1	GAA GAT CTA AAT CAG ACA ATC ATA GG
R2	GAA GAT CTT TAC CTG GAA TTG ATG
R3	GTG GTT GAG GAT GAT CCT GA
R4	TAA TAT AGT CGT CAC CAC CG
KANAMYCIN	**RESISTANCE GENE SPECIFIC**
R	CCA TCG ATT TTT AGA CAT CTA AAT CTA GGT AC
F	CCA TCG ATA CCC AGC GAA CCA TTT GAG G

2.3 Media

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

Luria-Bertani (LB) broth (Roth, 1970) was prepared by adding 10g of bacto-tryptone (Difco), 5g of bacto-yeast extract (Difco) and 10g of NaCl to 0.95 l of deionised water. The pH was adjusted to 7.0 with 5N NaOH. The volume was adjusted to 1 l and the medium was autoclaved at 121 °C for 15min.

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

Nutrient broth (NB) was prepared by dissolving 13g of nutrient broth 'E' powder (Lab M) in 1 l of deionised water and was autoclaved at 121°C for 15min.

Nutrient agar (NA) was prepared as NB but with 15g/l of grade A bacteriological agar (BBL) added.

Mueller Hinton (MH) broth was prepared by dissolving 21g of Mueller Hinton broth powder (Oxoid) in 1 l of deionised water and was autoclaved at 121°C for 15min.

Mueller Hinton agar was prepared as MH broth but with 15g/l of grade A bacteriological agar (BBL) added.

Campylobacter blood-free selective agar was prepared by dissolving 22.75g of campylobacter blood-free selective agar base (Oxoid) in 0.5 l of deionised water and was autoclaved at 121°C for 15min.

SOB medium was prepared by dissolving 20g/l of bactotryptone (Difco), 5g/l of yeast extract (Oxoid), and by adding 0.01M NaCl, 0.005M KCl, 0.01M $MgCl_2$ and 0.01M $MgSO_4$. The final volume was adjusted with deionised water. The medium was autoclaved at 121°C for 15min 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 (at ~60°C).

NZY top agar was prepared by dissolving 5g/l of yeast extract (Oxoid), 10g/l of casein enzymatic hydrolysate (N-Z-Amine A; Sigma), and by adding 0.085M NaCl, 0.008M $MgSO_4$ and 7g/l of agar (BBL). The pH of the medium was adjusted to pH 7.5 with 5N NaOH and the volume was adjusted to 1 l with deionised water. The medium was autoclaved at 121°C for 15min.

2xYT broth was prepared by dissolving 10g/l of yeast extract (Oxoid), 16g/l of bactotryptone (Difco) and by adding 0.17M NaCl. The volume was adjusted to 1 l with deionised water and the medium was autoclaved at 121°C for 15min.

2.4 Antibiotic supplements

All antibiotics were purchased from Sigma. Prior to use, all antibiotic stock solutions prepared in distilled water were filter sterilised.

Table 3. Antibiotic supplements.

Antibiotic	Working concentration
Ampicillin (Ap)	0.2mg/ml
Chloramphenicol (Cm)	0.02mg/ml
Kanamycin (Km)	0.05mg/ml
Tetracycline (Tc)	0.015mg/ml
Polymyxin B (PolyB)	2.5IU/ml
Trimethoprim (Tm)	0.005mg/ml
Vancomycin (Vm)	0.01mg/ml

2.5 Culture conditions of bacteria

All *C. jejuni* strains were grown on MH medium without antibiotics or supplemented with Vm, PolyB and Tm (section 2.4) or campylobacter blood-free selective agar. Bacterial cultures were incubated at 37 or 42°C, in a microaerophilic atmosphere of 6% hydrogen, 5% carbon dioxide, 5% oxygen and 84% nitrogen in a variable atmosphere incubator (VAIN, manufactured by Don Whitley

Scientific). Cultures in MH broth were shaken at approximately 200 rpm on a Gyrotory Shaker-Model G2 (New Brunswick Scientific Co, Inc., Edison, NJ, USA).

All *E. coli* strains were grown in LB, NB, LA or NA with the appropriate supplements and incubated at 37°C. Liquid cultures were shaken at approximately 240 rpm on a G10 Gyrotory Shaker (New Brunswick Scientific Co, Inc., Edison, NJ, USA).

2.6 Miscellaneous buffers and solutions

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

10x Phosphate buffered saline (PBS): 1.37M NaCl, 0.27M KCl, 0.015M KH_2PO_4 and 0.08M Na_2HPO_4. After adjusting the pH of the buffer to pH7.4 with 2N HCl, the final volume was adjusted by the addition of distilled water. The buffer was autoclaved at 121°C for 15min.

SM buffer: 0.01M NaCl, 0.008M of $MgSO_4.7H_2O$, 0.05M Tris-HCl pH7.5 and 0.1g/l of gelatin (Difco). The final volume was adjusted by the addition of distilled water. The buffer was autoclaved at 121°C for 15min.

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 volume was adjusted to the final amount with distilled water. The buffer was autoclaved at 121°C for 15min.

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

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 volume of the buffer was adjusted to the final amount with distilled water and autoclaved at 121°C for 15min.

CsCl saturated butan-2-ol was prepared by saturating approximately 50ml of distilled water with CsCl. Approximately 150ml of butan-2-ol were added to the CsCl saturated water and shaken.

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. This solution was not autoclaved.

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 by the addition of distilled water. The solution was autoclaved at 121°C for 15min.

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 liquified 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.

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 tubes were boiled for 15min 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 15min.

10% ($^{w}/_{v}$) SDS was prepared by adding sodium lauryl sulphate to distilled water and heated until it dissolved. This 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 in the dark to prevent damage by light, at -20°C.

2.7 Methods for the manipulation of DNA

All procedures involving the manipulation of DNA utilised solutions and materials that had been previously autoclaved at 121°C for 15min unless otherwise stated.

2.7.1 Preparation of chromosomal DNA

2.7.1.1 Growth conditions. *Escherichia coli* cells were grown in 100ml nutrient broth supplemented with the appropriate antibiotic and vigorously shaken overnight at 37°C. The bacterial culture was transferred into a sterile 50ml Falcon tube and the cells were harvested by centrifugation for 10min at 4°C, at 3200 *g* in a Heraeus Megafuge 1R.

Campylobacter jejuni cells were grown on campylobacter blood-free selective agar overnight at 37°C, in microaerophilic conditions. The bacterial cells were harvested from four plates showing fully confluent growth, resuspended in 25ml of PBS buffer, transferred into a sterile 50ml Falcon tube and centrifuged for 10min at 4°C, at 3200 *g* in a Heraeus Megafuge 1R.

2.7.1.2 DNA extraction. The cells were resuspended in 9.5ml of TE buffer. 0.5ml of 10% SDS and 0.05ml of 10mg/ml proteinase K were added and the suspension was incubated for 1h at 37°C. After clearing, 1.8ml of 5M NaCl was added and the suspension was thoroughly mixed. This was followed by the addition of 1.5ml of CTAB/NaCl solution and incubation for 20min at 65°C. An equal volume of chloroform/isoamyl alcohol was added, the contents gently mixed by inverting the tube successively and centrifuged for 15min at room temperature at 3200 *g* in a Heraeus Megafuge 1R. The upper layer was removed with a wide bore pipette, transferred into another sterile 50ml Falcon tube and mixed thoroughly with an equal volume of phenol/chloroform solution. The suspension was centrifuged for 15min at room temperature and the upper layer recovered. The phenol/chloroform extraction was repeated twice. Finally, the chromosomal DNA was precipitated following the addition of 0.6 volumes of propan-2-ol. The precipitate was transferred using a sealed Pasteur pipette into 1ml of 70% ($^{v}/_{v}$) ethanol. After pelleting the chromosomal DNA at room temperature, the supernatant was discarded, the DNA air-dried and resuspended in distilled water. The chromosomal DNA preparation was stored at 4°C.

2.7.2 Large scale preparation of plasmid DNA

2.7.2.1 Large scale preparation of plasmid DNA by ion-exchange chromatography. Bacterial strains containing the plasmid of interest were grown overnight with vigorous shaking at 37°C, in 100ml broth supplemented with the appropriate antibiotic. Bacteria were harvested by centrifugation for 10min, at 4°C, 16923 *g* in a Sorvall centrifuge, using a GSA rotor. The cells were resuspended in 4ml of buffer P1 (100μg/ml RNAse A, 0.05M Tris/HCl, 0.1M EDTA, pH8.0) and left on ice for 5min. 4ml of buffer P2 (0.2M NaOH, 1% ($^{w}/_{v}$) SDS) were added and mixed thoroughly but gently. The cells were then lysed at room temperature for 5-10min. When the suspension cleared, 4ml of ice-cold buffer P3 (2.55M potassium acetate, pH4.8) were added and the cell lysate was placed on ice for 5min. The tube was centrifuged for 30min at 4°C, 31070 *g* in a Sorvall centrifuge, using an SS34 rotor. The supernatant was transferred into a clean tube and centrifuged again for 10min to obtain a non-turbid particle-free lysate. The supernatant containing the plasmid DNA was removed promptly and further purified by anion-exchange chromotography on a column of silicagel (Qiagen). Qiagen column purification was carried out at room temperature and the column was allowed to empty by gravity flow. A Qiagen-tip 100 was firstly equilibrated with 3ml of buffer QBT (0.75M NaCl, 0.05M MOPS, 15% ($^{v}/_{v}$) ethanol, pH7.0, 0.15% ($^{v}/_{v}$) Triton X-100) and the supernatant containing the plasmid DNA was applied. The column was then washed with 10ml of buffer QC (1.0M NaCl, 0.05M MOPS, 15% ($^{v}/_{v}$) ethanol, pH7.0), followed by DNA elution with 5ml of buffer QF (1.25M NaCl, 0.05M MOPS, 15% ($^{v}/_{v}$) ethanol, pH8.2). In order to maximise the yield of plasmid DNA, the chromatography column was reused. The DNA was precipitated with 0.7 volumes of propan-2-ol. The tube was left at room temperature for 20min and centrifuged for 30min at 4°C, 31070 *g* in a Sorvall centrifuge, using an SS34 rotor. The pellet was washed in 1ml of 70% ($^{v}/_{v}$) ethanol and centrifuged for 10min at 4°C. The DNA pellet was dried in a vacuum dessicator and resupended in distilled water.

2.7.2.2 Large scale preparation of plasmid DNA by CsCl continuous gradients. The bacterial strain containing the plasmid of interest was grown in 100ml broth supplemented with the appropriate antibiotic and incubated overnight at 37°C with vigorous shaking. The bacterial culture was centrifuged for 10min at 4°C, 16923 *g* in a Sorvall centrifuge, using a GSA rotor. The bacterial pellet was resuspended in 3ml of solution I (100μg/ml RNAse A, 0.05M Tris/HCl, 0.1M EDTA, pH8.0) and placed on ice. 6ml of solution II (0.2M NaOH, 1% ($^{w}/_{v}$) SDS) were added to the suspension and the cells were incubated at room temperature to lyse. The cell lysate was gently but thoroughly mixed with 4.5ml of ice-cold solution III (3M sodium acetate, pH5.2) and placed on ice. The tube was

centrifuged for 30min at 4°C, 31070 *g* in a Sorvall centrifuge, using an SS34 rotor. The supernatant was transferred into a clean tube and centrifuged again for 10min to obtain a non-turbid particle-free lysate. The supernatant was removed promptly and the DNA precipitated with 0.7 volumes of propan-2-ol and incubation on ice for 30min. The precipitated DNA was pelleted by 30min centrifugation at 4°C, 31070 *g* in a Sorvall centrifuge, using a SS34 rotor. The DNA precipitate was gently washed in 3ml of 70% ($^{v}/_{v}$) ethanol and centrifuged for a further 15min. The supernatant was discarded and the DNA pellet was dried in a vacuum dessicator.

The dried DNA pellet was resuspended in 0.775ml of distilled water. An equal volume of 3% ($^{w}/_{v}$) N-lauryl sarcosine was added, followed by 1.76g of CsCl. Finally, 0.06ml of 10mg/ml EtBr were added to the solution. A Beckman 11x32mm Quickseal™ tube (ref. 344625) was loaded, balanced to a balance tube within 5mg, sealed and placed in the TLA100.2 rotor of a Beckman TL-100 ultracentrifuge. The tubes were centrifuged overnight at 20°C, 250000 *g*.

After removing the top of the Quickseal™ tube with a heated scalpel blade, the plasmid DNA band was carefully recovered from the CsCl gradient through the side of the tube by aspirating through a 27G 20mm syringe needle attached to a 1ml syringe. The solution containing the plasmid DNA was placed in a 1.5ml microcentrifuge tube and the EtBr was removed by adding one volume of CsCl-saturated butan-2-ol, mixing well and discarding the top layer which contained the EtBr. This was repeated 4-5 times until all the EtBr was removed and the plasmid DNA sample became colourless.

The plasmid DNA was precipitated at room temperature by the addition of distilled water and propan-2-ol to a final ratio of 0.54 of distilled water to 0.5 of propan-2-ol to 0.4 of DNA solution. The tube was centrifuged for 30min at room temperature at 13000 *g*. The pellet was gently washed in 1ml of 70% ($^{v}/_{v}$) ethanol and spun for a further 10min at 13000 *g*. The ethanol was removed, the DNA pellet dried in a vacuum dessicator and resuspended in distilled water.

2.7.3 Mini scale preparation of plasmid DNA

The small scale isolation of DNA is based on the method of Birnboim & Doly (Birnboim & Doly, 1979). A 1.5ml aliquot of overnight bacterial culture grown in broth supplemented with the appropriate antibiotic and incubated at 37°C with vigorous shaking was centrifuged for 5min at 4°C, 13000 *g* in a micro-centrifuge. The bacterial pellet was resuspended in 0.1ml of solution I (100µg/ml RNAse A, 0.05M Tris/HCl, 0.1M EDTA, pH8.0) and placed on ice. 0.2ml of solution II (0.2M NaOH, 1% ($^{w}/_{v}$) SDS) were added to the suspension which was lysed at room temperature. The cell lysate

was gently but thoroughly mixed with 0.15ml of ice-cold solution III (3M Sodium Acetate, pH5.2) and placed on ice. The tube was centrifuged for 15min at 4°C, 13000 *g* in a micro-centrifuge, the supernatant transferred into a clean 1.5ml microcentrifuge tube and one volume of phenol/chloroform solution was added. After vortexing vigorously, the suspension was centrifuged for 3-4min at room temperature to allow the phases to separate. The upper aqueous phase was carefully removed and transferred into a clean 1.5ml tube. 0.1ml of distilled water were added to the phenol/chloroform layer and the extraction was repeated. The second aqueous layer was removed and mixed with the first. The DNA was precipitated by the addition of 2.5 volumes of 100% ethanol, followed by the incubation at -70°C for 30min. The tube was centrifuged for 30min at 4°C, the supernatant discarded and the pellet gently shaken in 1ml of 70% ($^{v}/_{v}$) ethanol. Following a further 15min centrifugation, the supernatant was removed, the DNA pellet was dried in a vacuum dessicator and resuspended in distilled water.

2.7.4 Ethanol precipitation of DNA

In order to increase the DNA concentration in solution, to remove an excess of salt or to purify it, the DNA was precipitated by adding 1/10 volume of 3M sodium acetate pH5.2 and 2.5 volumes of 100% ethanol (kept at -20°C) to the sample. Whenever the sample consisted of a small DNA fragment, 1µl of 10mg/ml tRNA (type A from *E. coli*, Sigma) was also added to the tube as a co-precipitant. Following a 30min incubation at -70°C, the sample was centrifuged at 13000 *g* in a micro-centrifuge. The supernatant was discarded and the pellet was gently washed in 1ml of 70% ($^{v}/_{v}$) ethanol (kept at -20°C). After a further 15min centrifugation, the supernatant was removed, the DNA pellet was dried in a vacuum dessicator and resuspended in distilled water.

2.7.5 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 using the formula that 1.0 A_{260} unit equals 50µg/ml of double stranded DNA or 40µg/ml of single stranded DNA. The ratio of A_{260}/A_{280} was also determined in order to assess the purity of the DNA; a ratio of 1.8 was considered to indicate purity.

2.7.6 Electrophoresis of DNA

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 in 1xTAE buffer (section 2.6) to a final concentration of 0.8%-1.2% ($^{w}/_{v}$) depending on the size range to be separated. EtBr was added to the molten agarose to a final concentration of 0.5μg/ml. The gel was cast on a 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 1xTAE buffer. Prior to loading, 2μl of 5xTAE sample buffer (5xTAE buffer (section 2.6), 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 (0.25μg) were used and consisted of commercially prepared λ DNA restricted with *Hin*dIII (Gibco-BRL; 23130bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027 bp, 564bp and 125bp) and ϕX174 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, fitted with a cassette to accept 12.5x10cm Tmax film (Kodak).

2.7.7 Restriction endonuclease digestion of DNA

The restriction endonucleases were provided by GIBCO-BRL at a concentration of 8 units/μl and the corresponding restriction buffers were provided at a 10x normal concentration. For most of restriction enzyme reactions, 0.2-0.5 units of restriction enzyme were added to 0.2-0.5μg of DNA sample in a 0.01ml reaction containing 1x concentration of the respective enzyme buffer. The digestion conditions were as suggested by the manufacturer for each endonuclease and usually consisted of a 1-2h 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 times prolonged. The restriction reaction was terminated by adding 2μl of 5xTAE sample buffer and the digest was loaded onto an agarose gel for analysis. In instances where the DNA was to be used in a cloning experiment, the reaction was terminated by inactivating the restriction endonuclease. Inactivation conditions

consisted of heating the sample at 65°C for 10min or, when the endonuclease was heat-resistant, by extraction with phenol/chloroform. In the latter case, distilled water up to 0.1ml was added to the reaction and then mixed with 1 volume of phenol/chloroform. After vortexing and centrifuging the sample for 3min at room temperature, 13000 *g* in a micro-centrifuge, the upper layer was transferred into a clean 1.5ml tube and the DNA ethanol precipitated (section 2.7.4).

2.7.8 Purification of DNA fragments from agarose gels

2.7.8.1 Qiagen Qiaex™ DNA gel extraction kit. The Qiaex™ DNA gel extraction kit method relies upon the natural affinity of DNA for silica and glass in the presence of high salt concentrations. All buffers were supplied by the manufacturers.

The DNA fragments were initially separated on an agarose gel (section 2.7.6). Small blocks of agarose containing the target fragment of interest were excised, transferred into 1.5ml tubes and weighed. For each 0.1g of agarose, 0.3ml of QX1 (3M NaI, 4M $NaClO_3$, 0.005M Tris-HCl, pH7.5, 0.1% ($^{w}/_{v}$) Na_2SO_3) buffer were added to the block of agarose and mixed with 0.01ml of Qiaex beads. The tube was vigorously vortexed and incubated at 55°C for 10min; during incubation the sample was re-vortexed to maintain bead suspension. The tube was centrifuged for 1min at room temperature, at 13000 *g* in a micro-centrifuge. The supernatant was discarded and the beads-DNA pellet vigorously washed twice in 0.5ml of QX2 (8M $NaClO_3$) buffer. After discarding the supernatant, the pellet was vigorously washed twice in 0.5ml of QX3 (0.1M NaCl, 0.001M EDTA, 0.01M Tris-HCl, pH7.5, 70% ($^{v}/_{v}$) ethanol). The supernatant was carefully removed and the beads-DNA were air-dried briefly. The DNA bound to the Qiaex beads was eluted by vigorously vortexing in 20µl distilled water and incubating at 55°C for 5min. The tube was centrifuged briefly and the supernatant, containing the DNA, was transferred into a clean 1.5ml tube. In order to maximise the recovery yield, the beads were resuspended in a further 20µl of distilled water and the elution procedure was repeated.

2.7.8.2 Polyallomer wool filtration. The DNA fragments were initially separated on an agarose gel (section 2.7.6). Small blocks of agarose containing the fragment of interest were excised and transferred into 0.5ml tube, pierced in the base with a syringe needle, containing a few strands of polyallomer wool (Interpret™; obtained from a local tropical fish shop). The 0.5ml tube was placed inside a 1.5ml tube whose cap had been removed and centrifuged at room temperature for 5min,

13000 *g*. The liquid collected in the 1.5ml tube was transferred to a fresh tube, and the tubes containing the polyallomer wool and agarose centrifuged twice 5min. The liquid fractions collected after the three centrifugations were mixed together. Following a phenol/cloroform extraction, the DNA was ethanol precipitated, vacuum dried and resuspended in distilled water (section 2.7.4).

2.7.9 Dephosphorylation of linearised plasmid DNA

When plasmid DNA to be used as vector in a cloning experiment had compatible ends the 5' phosphate group was removed with calf intestinal alkaline phosphatase (CIP; Pharmacia) to prevent self-ligation. The re-linearised vector DNA was resuspended in 17.5µl of distilled water followed by the addition of 2µl of 10xCIP buffer (0.010M $ZnCl_2$, 0.010M $MgCl_2$, 0.1M Tris-HCl; pH8.3) and 0.5µl (0.5 unit) of 1000 units/ml CIP. The tube was incubated for 1h at 37°C and the reaction was interrupted by adding 0.5µl of 0.5M EDTA and 80µl of distilled water. The CIP was inactivated by phenol /chloroform extraction and the DNA was ethanol precipitated (section 2.7.4).

2.7.10 Ligation

In a ligation reaction, insert and vector DNA were mixed to an equimolar ratio in the smallest possible volume. The reactions were carried out in the presence of T4 ligase (Pharmacia), at the concentration of 5 units/µl, as was the respective 5x buffer. In a 10µl final volume reaction, 6µl of DNA species were mixed with 1µl of 10mg/ml ATP, 2µl of 5x ligation buffer and 1µl of T4 ligase. The ligation mixture was incubated either at room temperature for 2-3h or at 16°C overnight. The DNA was ethanol precipitated in the presence of tRNA (section 2.7.4).

2.7.11 Transformation of plasmid DNA: electrotransformation of plasmid DNA into *E. coli* strains

2.7.11.1. Cell preparation. To prepare electrocompetent cells, an overnight culture of *E. coli* cells was diluted 1:100 in 50ml of prewarmed nutrient broth and incubated at 37°C with vigorous shaking.

The culture was grown to 0.5-0.6 $O.D_{600nm}$. The flask was transferred promptly onto ice, swirling continuously to rapidly stop growth. The culture was then transferred into an ice-cold 50ml Falcon tube and centrifuged for 10min at 4°C, 3200 *g* in a Heraeus Megafuge 1R. The bacterial cells were resuspended in 10ml of ice-cold distilled water followed by another centrifugation. The pellet was resuspended in 1ml of ice-cold distilled water, transferred into a 1.5ml tube and centrifuged for 1min at 4°C, at 13000 *g* in a micro-centrifuge. After discarding the supernatant, the pellet was washed another 3-4 times with ice-cold distilled water. The bacterial cells were finally resuspended in 0.4ml of ice-cold distilled water and stored on ice until ready to use.

2.7.11.2 Electroporation. Prior to electroporation, the ligated DNA was ethanol precipitated (section 2.7.4) to remove salts and resuspended in 10µl of distilled water. In a 1.5ml tube, 40µl of electrocompetent cells were mixed with 5µl 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 25µF and with a resistance load of 1000Ω. 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 a 1ml of SOB or SOC medium (section 2.3) in a glass test tube and incubated with shaking for at least 1h at 37°C. The culture was transferred into a 1.5ml tube and centrifuged briefly at room temperature, at 13000 *g*. The cell pellet was resuspended in 0.1ml of the recovery medium (SOB or SOC). Serial dilutions were prepared and plated out on LA or NA containing the appropriate antibiotic selection for transformants.

2.7.12 Transformation of plasmid DNA: electrotransformation of plasmid DNA into *C. jejuni* strains

2.7.12.1 Cell preparation. *C. jejuni* 81116 cells from a frozen stock kept at -80°C were plated onto MH agar or campylobacter blood-free selective medium and incubated overnight at 42°C or 37°C, respectively, in a microaerophilic atmosphere (section 2.5). 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, resuspended in 10ml of ice-cold electroporation buffer

(EPB, 0.272M sucrose and 15% ($^{v}/_{v}$) glycerol). The cell suspension was transferred into a sterile 50ml Falcon tube and centrifuged for 30min at 4°C, 3200 *g* in a Heraeus Megafuge 1R. The supernatant was discarded and the cells washed twice in 10ml of ice-cold EPB. The bacterial cells were finally resuspended in 0.3ml of ice-cold EPB and used immediately.

2.7.12.2 Electroporation. Prior to electroporation, a 10µl aliquot of cells was withdrawn and used to determine the initial viability of cells. Serial dilutions were plated out onto MH agar or campylobacter blood-free selective medium and incubated overnight at 42°C or 37°C, in a microaerophilic atmosphere (section 2.5). In a 1.5ml tube, 50µl of electrocompetent cells were mixed with 5µl DNA and stored on ice for 10min. 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 content was resuspended in a 0.1ml of SOB or SOC medium (section 2.3) and plated out onto MH agar or campylobacter blood-free selective medium and incubated overnight at 42°C or 37°C, in a microaerophilic atmosphere (section 2.5). In addition, the percentage of cell killing due to electroporation was assessed by electroporating cells with no DNA and plating out serial dilutions onto MH agar or campylobacter blood-free selective medium; the plates were incubated overnight at 42°C or 37°C, in a microaerophilic atmosphere (section 2.5).

After an overnight recovery, the cells were washed off the plates with SOB or SOC medium (section 2.3) and centrifuged for 5min at room temperature, 3200 *g* in a Heraeus Megafuge 1R. The bacterial pellet was resuspended in the same medium (SOB or SOC) and plated onto MH agar or campylobacter blood free selective medium containing the appropriate antibiotic selection for transformants. The plates were incubated at 42°C or 37°C, in a microaerophilic atmosphere (section 2.5) for up to a week.

2.7.13 Natural transformation of *C. jejuni* strains

C. jejuni 81116 cells from a frozen stock kept at -80°C were plated onto campylobacter blood-free selective medium and incubated overnight at 37°C in a microaerophilic atmosphere

(section 2.5). Having passed the strain 2-3 times onto fresh plates, a culture was prepared in MH broth and incubated overnight at 37°C in a microaerophilic atmosphere. 0.2ml of the overnight culture were mixed with 5µl of DNA and transferred into a sterilin tube containing 1.5ml of campylobacter blood-free selective agar. The bacterial suspension in the biphasic medium was incubated overnight at 37°C in a microaerophilic atmosphere (section 2.5) with no shaking. The bacterial cells were plated onto campylobacter blood free agar containing the appropriate antibiotic selection for transformants.

2.7.14 Conjugation of plasmid DNA from *E. coli* strains into *C. jejuni* strains

2.7.14.1 Cell preparation. *E. coli* donor cells were prepared from a MH culture incubated at 37°C overnight shaking. The culture was diluted 1:50 in 10ml of MH broth and incubated at 37°C to 0.5-1.0 $O.D_{600nm}$. *C. jejuni* recipient cells were prepared from a 5ml culture in MH broth, incubated at 42°C overnight in a microaerophilic atmosphere (section 2.5) shaking. The bacterial culture was centrifuged for 10min at room temperature, 3200 *g* in a Heraeus Megafuge 1R. Bacterial cells were resuspended in 5ml of fresh MH medium and incubated for 1-2h. After incubation, the culture was centrifuged for 10min at room temperature, 3200 *g* in a Heraeus Megafuge 1R and the supernatant was discarded.

2.7.14.2 Cell mating. The bacterial pellet of the recipient strain was resuspended in 0.5ml of donor cells culture grown to the $O.D_{600nm}$ of 0.5-1.0 units. The cell suspension was transferred into a 1.5ml tube and centrifuged for 5min at room temperature, at 13000 *g* in a micro-centrifuge. The bacterial pellet was then resuspended in 0.1ml of MH broth and carefully placed on a sterile cellulose nitrate filter, 25mm diameter and 0.45µm pore size (Whatman) previously laid on a plate containing MH agar. The plates were incubated at 42°C in a microaerophilic atmosphere (section 2.5). After an overnight incubation, the filters were gently washed with 1ml of prewarmed MH broth, centrifuged for 5min at room temperature, at 13000 *g* in a micro-centifuge and plated onto MH agar containing the appropriate antibiotic selection for conjugants. Polymyxin B and trimethoprim were also added to the selection medium to eliminate the donor cells. The plates were incubated at 42°C in a microaerophilic atmosphere (section 2.5).

2.7.15 Polymerase chain reaction (PCR)

2.7.15.1 PCR to amplify DNA from plasmids. In a 0.5ml tube, the following was mixed: 1µl of 10xPCR buffer (0.1M Tris-HCl, 0.5M KCl, 0.1M $MgCl_2$, 1mg/ml BSA (DNAse free, Pharmacia), pH8.3), 1µl of 10xdNTP mix (0.01M dATP, 0.01M dCTP, 0.01M dGTP, 0.01M dTTP (ultrapure, Pharmacia)), 10pmoles of each primer and distilled water to a final volume of 9µl. 0.5 units of Taq polymerase were added, followed by 0.5µl (0.001µg) of plasmid DNA template. The whole mixture was overlayed by 15µl of mineral oil (Sigma), vortexed, centrifuged briefly and placed in a Hybaid OmniGene thermal cycler. Each PCR was programmed according to the characteristics of the DNA template and primers. In a typical reaction using vector-specific primers and 1kb of DNA template to amplify, the reactions were programmed to cycle through three different stages: stage one comprising one cycle at a denaturing temperature of 96°C for 2min, an annealing temperature of 60°C for 2min, an extension temperature of 72°C for 2min; stage two comprising thirty cycles at a denaturing temperature of 94°C for 1min, an annealing temperature of 60°C for 1min, an extension temperature of 72°C for 1min; stage three comprising one cycle at an annealing temperature of 60°C for 1min, an extension temperature of 72°C for 2min. Every reaction was carried out with a negative and, whenever possible, a positive control. When the aim of the PCR was to confirm the size of a clone insert or to confirm that the cloning experiment had worked, the 10µl reaction was electrophoresed on an agarose gel, following the addition of 2µl of 5xTAE sample buffer. When the PCR product was to be used in a cloning experiment, the PCR was carried out on a larger scale. 1/10 volume of the reaction was mixed with 5xTAE sample buffer and electrophoresed on an agarose gel to check whether the reaction worked. The remaining PCR product was purified as described below (section 2.7.16).

2.7.15.2 PCR to amplify DNA from bacterial colonies. Colony PCR was used to quickly check whether a cloning experiment worked, having the advantage of allowing the screen of a large number of transformants without requiring a lot of time and work. This method is based on that described by (Güsson & Clackson, 1989).

In a colony PCR, the template was prepared by resuspending a bacterial colony in 0.1ml of distilled water. The cell suspension was boiled for 5min to lyse the bacterial cells and centrifuged for 5min at room temperature, 13000 *g* in a micro-centrifuge. The supernatant was then transferred to a clean 1.5ml tube. The PCR was carried out as described above (2.7.15.1), using 2µl of colony lysate

as the template. The primers used were specific for the vector, annealing to sequences flanking the multiple cloning site (section 2.2.2). The PCR products were analysed by agarose gel electrophoresis.

2.7.16 Purification of PCR products

The purification of PCR products was carried out by ion-change chromatography, using a kit provided by Qiagen. Following the PCR reaction, the mineral oil was removed and 1volume of buffer QP (0.4M NaCl, 0.05M MOPS, pH7.0) was added to the sample. A Qiagen-spin 20 column was placed in a 1.5ml tube and the column matrix equilibrated by adding 0.8ml of buffer QP, with excess buffer being removed by a brief centrifugation at 1000 *g* in a micro-centrifuge. The sample was applied to the column which was centrifuged for 3min at 1000 *g*. The column was then washed twice with 0.8ml of propan-2-ol, by centrifugation at 1000 *g* for 3min and then washed twice again with 0.8ml of buffer QB (0.75M NaCl, 0.05M MOPS, 15% ($^{v}/_{v}$) ethanol, pH7.0). The bound DNA was eluted with 0.8ml of buffer QF (1.25M NaCl, 0.05M MOPS, 15% ($^{v}/_{v}$) ethanol, pH8.2) which was centrifuged through the column. The DNA in the eluate was precipitated with the addition of 0.8 volumes of propan-2-ol and centrifuged for 30min at 13000 *g*. The supernatant was discarded, the DNA pellet washed in 1ml of 70% ($^{v}/_{v}$) ethanol, dried in a vacuum dessicator and finally resuspended in distilled water.

2.7.17 Construction of a library of nested deletions

A library of nested deletions on double-stranded DNA was generated using the double stranded Nested Deletion Kit from Pharmacia. This protocol is based on the method described by Henikoff (1984). All solutions, enzymes and their respective buffers were supplied in the kit.

The DNA clone was double digested with two restriction endonucleases, creating a 5' and a 3'overhangs. In this digestion 5µg of DNA was mixed with 5 units of each enzyme in the presence of 2µl of the appropriate restriction buffer at 10x concentration, in a final volume of 20µl. The reaction was incubated overnight at 37°C. The digestions were performed simultaneously when both endonucleases were active in the same buffer. Otherwise, the DNA was incubated with the enzyme which created a 5' overhang first. Following inactivation of the enzyme (section 2.7.7) and ethanol precipitation of the DNA (section 2.7.4), the DNA was digested with the second restriction enzyme to

create a 3' overhang on the end. In order to check whether the DNA restriction was complete, 1µl of the digestion was loaded on a 0.8% agarose gel. Once the digestion was complete, the restriction endonucleases were inactivated by phenol/chloroform extraction, the DNA was ethanol precipitated and resuspended in 19µl of distilled water and 1µl of 0.2M $MgCl_2$.

In the next step, the nested deletions were generated. The S1 nuclease/buffer mix was prepared (for a total of 10 samples: 19.8µl of distilled water, 9.9µl of S1 buffer, 0.3µl of S1 nuclease), and 3µl was aliquoted into 1.5ml tubes before being placed on ice. A 2x Exonuclease III (ExoIII)/buffer mix was also prepared, initially excluding the nuclease (for a total of 10 samples: 1.7µl of distilled water, 5µl of 0.3M NaCl, 3.3µl of ExoIII buffer), thoroughly mixed with 10µl of the double digested DNA and placed in a water bath at 30°C. At this stage, 2µl were transferred and thoroughly mixed into the 3µl of S1 nuclease/buffer mix which was prealiquoted into tubes. The tube was labelled as corresponding to the time point zero (t=0) and stored on ice. 0.5µl of ExoIII were then added to main reaction tube and mixed. At pre-determined time points, 2µl of the ExoIII nuclease/buffer mix were transferred and thoroughly mixed into the 3µl of S1 nuclease/buffer mix and stored on ice. After withdrawing all the time points predicted to create a useful library, the tubes kept on ice were incubated for 30min at room temperature for the S1 nuclease to digest the single stranded overhangs generated by ExoIII. The S1 nuclease activity was terminated by adding 1µl of S1 stop solution and incubated for 10min 65°C. In order to determine whether the deletion reactions had worked and which aliquots to choose, 3µl from each time point were mixed with 0.6µl of 5xTAE sample buffer and ran on a 1% agarose gel. Single digested vector (used in the clone) and clone were included on the gel as controls. The 3µl of deletion reactions left in the chosen aliquots were recircularised by adding 17µl of ligation mix (87.2µl of distilled water, 16µl of ligation buffer, 32µl of 25% PEG, 0.4µl of T4 ligase) and incubated overnight at 16°C. The ligations were ethanol precipitated in the presence of tRNA (section 2.7.4), resuspended in 10µl of distilled water. 5µl of each ligation were subsequently used to electroporate into *E. coli* DH5α cells (section 2.7.11).

2.7.18 DNA sequencing

The DNA was prepared for sequencing using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (ABI, Applied Biosystems) and all reagents were supplied with the kit. In a 0.5ml tube, 1µg of dsDNA was mixed with 9.5µl of reaction premix containing 4µl of 5xTACS buffer (terminator ammonium cycle sequencing buffer: 0.4M Tris-HCl, 0.01M $MgCl_2$, 0.1M $(NH_4)_2SO_4$, pH9.0), 1µl of the dNTP mix (750µM dITP, 150µM dATP, 150µM dCTP, 150µM dTTP), 1µl of each

dyedeoxy™ terminator and 0.5µl of AmpliTaq DNA polymerase (4 units). 3.2pmoles of primer in distilled water were added to give a final volume of 20µl. The reaction was overlayed with 20µl of mineral oil and the tube was vortexed, briefly centrifuged to collect the reaction and placed in a Hybaid OmniGene thermal cycler programmed to undergo the following conditions: 25 cycles of denaturing temperature of 96°C for 30 seconds, annealing temperature of 50°C for 15 seconds and extension temperature of 60°C for 4min. After the temperature cycling finished, the reaction was transferred into a clean 1.5ml tube containing 0.08ml of distilled water. The dyedeoxy terminators were removed using 0.1ml of 68:18:14 phenol/water/chloroform solution at room temperature (previously shaken to ensure homogeneity). The tube was vigorously vortexed and briefly centrifuged. The lower organic phase containing the coloured terminators was discarded and the aqueous layer was reextracted with 0.1ml of the phenol/water/chloroform. After vortexing and centrifugation, the upper layer was transferred into a clean 1.5ml tube. The extension products were ethanol precipitated (section 2.7.4), the pellet dried and stored at -20°C. The DNA pellet from the sequencing reaction was sent to the Protein and Nucleic Acid Chemistry Laboratory at the University of Leicester for automated electrophoretic separation and sequence determination. The sequence data was received via the University MacFS Network and analysed using the Wisconsin Package, (Version 8, September 1994) via the Genetics Computer Group.

2.7.19 λZAP II genomic library

2.7.19.1 Plating out λZAP II library. A 5ml LB broth containing 2mg/ml of maltose and 0.01M of $MgSO_4$ was inoculated with a single colony of *E. coli* XL1-Blue isolated from a fresh LB plate containing tetracycline. After a 30°C overnight incubation with vigorous shaking, the culture was diluted in prewarmed 0.01M of $MgSO_4$ to an $O.D_{600nm}$ of 0.5 units. 0.2ml of bacterial cells were transferred to sterile test tubes and mixed with 1µl (~10^6 pfu/ml) of λ phage suspension in SM buffer. The tubes were incubated at 37°C for 20min. Prior to plating out, 4ml of molten (at 50°C) NZY top agar were mixed with the bacterial/phage suspensions and quickly overlayed onto prewarmed (37°C) LB agar plates (plate size: 8.2cm). The plates were incubated overnight at 37°C.

2.7.19.2 λZAP II phage stock preparation. The λZAP II library was plated onto LA plates to a density of approximately 1x10^3 plaques per plate. Isolated plaques were removed using sterile wide

bore Gilson P1000 tips to extract plugs of agar-containing the plaques. The plugs were transferred into 1.5ml tubes and the phages were resuspended in 1ml of SM buffer containing 0.02ml of chloroform. The phage suspensions were incubated at room temperature for 1-2h, followed by vigorous vortexing to completely release the phage into the SM buffer. Bacterial debris was pelleted by 5min centrifugation at room temperature, 13000 *g* in a micro-centrifuge. The supernatant corresponding to the phage stock was stored at 4°C for more than a month with no loss of viability.

To titer the phage stock, serial dilutions of the stock were prepared in SM buffer and used to transfect XL1-Blue host cells as described above (section 2.7.19.1). The number of viable phage in the starting stock was calculated by determining the number of plaques appearing per plate.

2.7.19.3 *In vivo* excision of phagemid from λZAP II. A 5ml LB broth containing 2mg/ml of maltose and 0.01M of $MgSO_4$ was inoculated with a single colony of *E. coli* XL1-Blue isolated from a fresh LA plate containing tetracycline. After a 30°C overnight incubation with vigorous shaking, the culture was diluted in prewarmed 0.01M of $MgSO_4$ to an $O.D_{600nm}$ of 1.0 unit. 0.2ml of bacterial cells were transferred into a 50ml Falcon tube containing 0.2ml of the λ recombinant phage stock (approximately $5x10^5$ pfu/ml) and 1µl of R408 helper phage (approximately $1x10^9$ pfu/ml). The tube was incubated at 37°C for 15min. 5ml of 2x YT medium were added to the mixture and incubated 3h at 37°C with constant shaking. The tube was heated at 70°C for 20min to kill the bacteria and it was centrifuged for 5min at 4°C, 3200 *g* in a Heraeus Megafuge 1R. The supernatant was transferred into a sterile 1.5ml tube to be stored at 4°C as a stock containing pBluescript packaged as filamentous phage.

XL1-Blue host cells were grown in 5ml of LB broth containing 2mg/ml of maltose and 0.01M of $MgSO_4$ and incubated overnight at 30°C with shaking. To plate the rescued phagemid, 0.2ml of bacterial culture diluted to an $O.D_{600nm}$ of 1.0 were mixed with 10µl of phagemid stock diluted in SM to 10^0, 10^{-1} and 10^{-2}. The mixtures were incubated at 37°C for 15min. After the incubation, the bacterial/phage suspension was plated onto LB plates containing ampicillin and incubated overnight at 37°C. The ampicillin resistant bacterial colonies appearing on the plates containing the double stranded pBluescript with the library insert DNA and were streaked onto fresh LA/ampicillin plates. pBluescript plasmid DNA was prepared as described before (section 2.7.2).

2.7.20 DNA hybridisation

DNA hybridisation was performed using the Nonradioactive DNA Labelling and Detection Kit supplied by Biochemica Boehringer Mannheim. This method is based on the detection of digoxigenin-11-dUTP (dig-dUTP) labelled DNA. The protocols for labelling and detecting DNA were supplied with the kit.

2.7.20.1 Labelling the probe with digoxigenin-11-dUTP. Prior to labelling, 1μg of linearised DNA was denatured by boiling for 5min and then chilled quickly on ice/NaCl for 3min. The denatured DNA was thoroughly mixed with 2μl of 10x hexanucleotide mixture (supplied with the kit), 2μl of 10x dNTP labelling mixture (1mM dATP, 1mM dCTP, 1mM dGTP, 0.65mM dTTP, 0.35mM dig-dUTP, pH6.5; supplied with the kit) and distilled water to 19μl final volume. 1μl (2units) of Klenow enzyme was added to this mixture. The tube was briefly centrifuged and incubated overnight at 37°C. The labelling reaction was stopped with the addition of 2μl of 0.2M EDTA, pH8.0. The newly synthesized DNA probe was precipitated in the presence of 2.5μl of 4M LiCl, 1μl of 10mg/ml tRNA, 75μl of prechilled 100% ethanol and incubated for 30min at -70°C. The tube was centrifuged for 30min at 4°C, at 13000 *g* in a micro-centrifuge. The supernatant was discarded and the pellet gently washed in 40μl of 70% (v/v) ethanol. Following a further 15min centrifugation, the DNA was dried in a vacuum dessicator and resuspended in 50μl of TE buffer. After determining the DNA concentration (section 2.7.5), the labelled probe was stored at -20°C where it remained viable for more than a year.

2.7.20.2 DNA transfer to nylon membranes

(a) recombinant phage plaques. The λ phage library (Kiernan, 1997; see section 3.2), was plated onto LA plates to a plaque density of approximately $1x10^3$ plaques per plate and incubated overnight (section 2.7.19). Following the overnight incubation, the plates were chilled at 4°C for 30–45min to harden the agar and the plaques were transferred onto a nylon filter (Hybond N, Amersham International PLC) by placing the filter onto the agar plate for 1min. Using a hot needle, the filter and the agar were pierced two and three times on diametrically opposite sides, respectively, to orientate the filter. After adsorption, the filters were carefully lifted, soaked plaque side up for 5min on 3MM chromatography paper (Whatman) saturated with denaturing solution (0.5M NaOH, 1.5M NaCl) and promptly put onto 3MM chromatography paper saturated with neutralisation solution (1.5M NaCl,

1M Tris-HCl, pH8.0) for another 5min. The filters were then transferred on paper towels and air-dried. The phage DNA was cross-linked onto the filters by wrapping in Saran Wrap™ and exposing them for 30 seconds to ultraviolet light at an intensity of 70mJ/cm^2 (Amersham UV crosslinker).

(b) transferring target DNA for southern blotting. The target bacterial DNA was digested and electrophoresed on an agarose gel as described above (sections 2.7.6 and 2.7.7). The gel was photographed with a ruler along one of the sides. Unused areas of the gel were trimmed away with a scalpel. The DNA was denatured by two 15min incubations in denaturing solution (0.5M NaOH, 1.5M NaCl) at room temperature with constant shaking. The gel was neutralised by immersing twice for 15min in neutralising solution (1.5M NaCl, 1M Tris-HCl, pH8.0) at room temperature with constant shaking. The DNA was ready to transfer and the apparatus was construted as follows: a glass plate, larger than the gel, was wrapped with 3MM chromatography paper (Whatman) and placed across a tray filled with 20x SSC (3M NaCl, 0.3M Na-citrate, pH7.0). The 3MM chromatography paper was left with the ends immersed in the 20x SSC and it was allowed to slowly become saturated. All air bubbles were removed using a glass pipette. The gel was inverted (i.e., wells side down) on top of the 3MM paper-wrapped glass plate. A piece of nylon membrane (Hybond N, Amersham International PLC) was cut to be about 2-3mm larger than the agarose gel, pre-soaked in distilled water, followed by 20x SSC before being placed on the gel; all air bubbles trapped between the gel and the membrane were removed. Two pieces of 3MM chromatography paper cut to the same size as the nylon membrane were pre-soaked in 20x SSC and placed onto the membrane, again removing all air bubbles. A stack of 6-7 sheets of Quickdraw™ (Sigma) absorbent paper was placed on top of the 3MM chromatography paper and a glass plate was put on the stack with a 500ml weight placed on the top to apply pressure. After 3h, the Quickdraw™ and 3MM paper were removed and the positions of the gel slots were marked on the membrane with a ball-point pen. The nylon membrane was placed on paper towels and air-dried. The DNA was cross-linked onto the membrane by wrapping it in Saran Wrap™ and exposing for 30 seconds to ultraviolet light at an intensity of 70mJ/cm^2 (Amersham UV crosslinker).

2.7.20.3 DNA hybridisation. After immobilising the target DNA, the nylon filters were immersed in prehybridisation solution (5x SSC, 0.1% N-laurylsarcosine sodium salt, 1% ($^w/_v$) blocking reagent which was supplied with the kit) for 2h at 68°C in a Hybaid dual hybridisation oven with constant

rotation. The filters were then ready to hybridise. The labelled probe (section 2.7.20.1) was denatured by boiling for 3min and added to the hybridisation solution (same composition of prehybridisation solution) at the concentration of 0.05µg/ml (section 2.7.20.2 a) or 0.026µg/ml (section 2.7.20.2 b). The filters were immediately immersed in the hybridisation solution and placed overnight in the oven at 68°C. The following day, the filter was washed twice 5min in wash solution 1 (2x SSC, 0.1% ($^{w}/_{v}$) SDS) at room temperature, followed by two 15min washes in wash solution 2 (0.1x SSC, 0.1% ($^{w}/_{v}$) SDS) at 68°C.

2.7.20.4 Detecting probe DNA-target DNA hybrids. The filters were briefly washed in Buffer 1 (0.1M Tris-HCl, 0.15M NaCl, pH7.5) and blocked for 30min in freshly prepared Buffer 2 (0.5% ($^{w}/_{v}$) blocking reagent in buffer 1). After another quick wash in Buffer 1, the filters were incubated with an anti-digoxigenin-alkaline phosphatase conjugate diluted 1:5000 in Buffer 1 for 30min. The unbound antibody-enzyme conjugate was removed by two 15min washes in Buffer 1, followed by a brief wash in Buffer 3 (0.1M Tris-HCl, 0.1M NaCl, 0.05M $MgCl_2$, pH9.5) to equilibrate the membrane. The bound probe was detected by incubating the membrane in 10ml of colour solution (45µl of 75mg/ml nitroblue tetrazolium salt (NBT, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene]-ditetrazolium-chloride) solution and 35µl of 50mg/ml of X-Phosphate (5-bromo-4-chloro-3-indolyl phosphate) solution in 10ml of Buffer 3) in the dark. When development was complete, the colour reaction was stopped by rinsing the membrane with Buffer 4 (0.1M Tris-HCl, 0.001M EDTA, pH8.0). The membrane was quickly dried by transferring it onto paper towels (avoiding exposure to light that causes the membrane to become brown) and stored wrapped in foil paper until ready to photograph.

2.8 Methods for the manipulation of proteins

2.8.1 One-dimensional polyacrylamide gel electrophoresis

2.8.1.1 Preparation of total protein extracts from *C. jejuni*. Bacterial strains were grown overnight in 5ml of MH broth, in a microaerophilic atmosphere at 37 or 42°C, with constant shaking. The cultures were chilled on ice and their $O.D_{600}$ determined using a Pharmacia Ultraspec III

spectrophotometer. Bacterial cells were harvested by 10min centrifugation at 4°C, 3200 *g* in a Heraeus Megafuge 1R and resuspended in Tris-$MgCl_2$ buffer (resuspending buffer: 0.1M Tris-HCl, 0.01M $MgCl_2$, pH7.8) to 1 O.D unit/ml in a final volume of 0.05ml. After transferring into a 1.5ml tube, 0.05ml of 2x 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) were added to the suspension and the tube was incubated at room temperature for 10-15min to allow cell lysis. The suspension was vigorously vortexed to help disrupt cell membranes and chromosomal DNA. The suspension was boiled for 5min, the tube vortexed and briefly centrifuged at 13000 *g* in a micro-centrifuge. The total protein extract was stored at -20°C.

2.8.1.2 Preparation of membrane protein extracts from *C. jejuni*. Bacterial strains were grown overnight in 5ml of MH broth, in a microaerophilic atmosphere at 37°C, with constant shaking. The cultures were chilled on ice and their $O.D_{600}$ determined using a Pharmacia Ultraspec III spectrophotometer. Bacterial cells were harvested by a 10min centrifugation at 4°C, 3200 *g* in a Heraeus Megafuge 1R and resuspended in Tris-$MgCl_2$ buffer (0.1M Tris-HCl, 0.01M $MgCl_2$, pH7.8) to 3 O.D units/ml in a final volume of 0.3ml. The suspension was subdivided into 1.5ml tubes, 0.1ml per each tube. The cells were disrupted by sonicating twice for 15sec with a 15sec interval, using a Soniprep sonicator (MSE) with a 0.3cm diameter probe. Non-disrupted organisms were removed by centrifugation at 13000 *g* in a micro-centrifuge at 4°C for 15min, and the supernatants were transferred to a thickwall polycarbonate TLS-100 tube (ref.: 343778). The tubes were placed in a TLS55 swing-out rotor of a Beckman TL-100 ultracentrifuge and centrifuged for 15min at 4°C, 259182 *g*. The pellet was washed in 0.1ml of Tris-$MgCl_2$ buffer and centrifuged for a further 15min at 4°C at 259182 *g*. The membrane protein pellet was resuspended finally in 0.05ml of 0.05M sodium phosphate pH7.2 and transferred into a 1.5ml tube containing 0.05ml of 2x SDS-PAGE sample buffer. The tubes were left at room temperature for 10-15min to allow the proteins to solubilise and stored at -20°C.

2.8.1.3 One-dimensional electrophoresis of proteins. Total and 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 7.2x10.2cm or a large Protean II apparatus producing "maxi-gels" with

dimensions of 16x16cm. All gels were of 0.75mm thickness. The stacking gels were of 5% ($^{v}/_{v}$) final acrylamide concentration and the separating gels of 11% ($^{v}/_{v}$) final acrylamide concentration (Table 4), using 30%:0.8% ($^{w}/_{v}$) acrylamide mix (Protogel, ready mixed form supplied by National Diagnostics, in the ratio of 30:0.8 acrylamide : bis-acrylamide). Electrophoresis was carried out in the presence of SDS-PAGE running buffer: 0.025M tris, 0.192M glycine, 0.19% ($^{w}/_{v}$) SDS, pH8.3-8.6. "Mini-gels" were routinely electrophoresed at a constant current of 15mA. "Maxi-gels" were routinely electrophoresed for 5h at a constant current of 25mA or overnight at a constant voltage of 40V.

Table 4. Recipes for the preparation of SDS-polyacrylamide gels.

	"Mini-gel"		"Maxi-gel"	
	5% Stacking gel	**11% Separating gel**	**5% Stacking gel**	**11% Separating gel**
2x Buffer A (0.75M Tris-HCl, 0.2% ($^{w}/_{v}$) SDS, pH8.8)		2.7ml		13.5ml
2x 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)	0.1ml	0.19ml	0.5ml	0.95ml
TEMED (N,N,N',N',-tetramethylethylenediamine; Sigma)	0.008ml	0.015ml	0.04ml	0.075ml

2.8.1.4 Coomassie brilliant blue PAGE gel staining. Prior to electrophoresis, non-radiolabelled protein samples and protein molecular weight standards (MW-SDS-200 kit, Sigma; 205kDa (Myosin, rabbit muscle), 116kDa (β-galactosidase, *E. coli)*, 97kDa (Phosphorylase-b, rabbit muscle), 66kDa (Bovine plasma albumin), 45kDa (Ovalbumin), 29kDa (Carbonic anhydrase, bovine erythrocytes)) were boiled for 3min and loaded on the gel. 10μl of the stainable molecular weight standards were loaded onto "mini-gels" (or 15μl 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; the solution

was reused) 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 onto 3MM chromatography paper (Whatman), using a BioRad gel drier programmed to dry at 80°C.

2.8.2 Protein analysis on two-dimensional gel electrophoresis

2.8.2.1 Preparation of total protein extracts from *C. jejuni*. Bacterial strains were grown overnight in 5ml of MH broth, in a microaerophilic atmosphere at 37°C, with constant shaking. The cultures were chilled on ice and their $O.D_{600nm}$ determined using a Pharmacia Ultraspec III spectrophotometer. A volume equivalent to 1.5 $O.D_{600nm}$ units was withdrawn from the overnight cultures, bacterial cells were centrifuged for 10min at 4°C, 3200 *g* in a Heraeus Megafuge 1R and resuspended in 0.1ml of sonication buffer (0.01M Tris-HCl, 0.001M EDTA, 0.001M PMSF (phenylmethansulfonyl fluoride), pH8.0). Bacterial cells were disrupted by sonication as described above (section 2.8.1.2). Non-disrupted organisms were removed by 5min centrifugation at 4°C, 13000 *g* in a micro-centrifuge and the supernatants were transferred to a 1.5ml tube and stored at -20°C. Prior to electrophoresis, an aliquot with the appropriate amount of protein (see below, section 2.8.2.2) was mixed in at least a 1:4 ratio with sample solution (containing 13.5g of urea, 0.5ml of 2-mercaptoethanol, 0.5ml of pharmalyte 3-10 (Pharmacia), 0.13ml of triton X-100, 0.2g of bromophenol blue (BPB; Sigma) and distilled water to make the final volume of 25ml) to make a final volume of 20µl. The sample could be stored at -20°C or applied on the first dimensional gel (see below, section 2.8.2.3).

2.8.2.2 Quantitation of proteins using Bradford's colorimetric method. The amount of protein in the supernatants was quantitated using the Bradford method (Ausubel *et al.*, 1992). A BSA (bovine serum albumin) 1mg/ml stock solution was prepared in sonication buffer (section 2.8.2.1) and diluted to specific final concentrations. 20µl from each BSA solution of known concentration or 2µl 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 concentration of protein in the *C. jejuni* samples.

2.8.2.3 Two-dimensional electrophoresis of proteins. The two-dimensional (2-D) electrophoresis, based on the horizontal system for 2-D electrophoresis originally devised by Görg (Görg, 1993), was carried out using the Immobiline DryStrip kit for 2-D electrophoresis with Immobiline DryStrip and ExcelGel™ SDS, manufactured by Pharmacia. The protocol followed was as instructed by the manufacturer. All the solutions utilised were freshly prepared.

Total cellular proteins (section 2.8.2.1) were separated on the first dimensional electrophoresis by isoelectric focusing (IEF), using precast 11cm long Immobiline DryStrip polyacrylamide gels (stored at -20°C) containing a pH gradient ranging from 3-10. Before electrophoresis, an appropriate number of Immobiline DryStrip polyacrylamide gels were rehydrated overnight in rehydration solution (containing 12g of urea, 0.13ml of triton X-100, 0.13ml of pharmalyte 3-10, 0.05g of dithiothreitol (DTT), 0.0005g of Orange G and distilled water to make the final volume of 25ml) in a reswelling cassette. After rehydration, the Immobiline DryStrips were transferred to the electrophoresis apparatus (Multiphor II) and one sample per strip was applied at the anodic end. First dimensional electrophoresis was carried out for a total of 16h and programmed to undergo four phases consisting of: phase 1 at 300V, 1mA, 5W, 1Vh; phase 2 at 300V, 1mA, 5W, 1350Vh, phase 3 at 2000V, 1mA, 5W, 5750Vh and phase 4 at 2000V, 1mA, 5W, 13000Vh. Prior to starting the second dimensional electrophoresis, the strips were equilibrated in a two step process with gentle rocking: 10min in equilibration solution 1 (0.1M Tris-HCl pH6.8, 18g of urea, 15ml of glycerol, 1%SDS, 0.125g of DTT and distilled water to 50ml final volume) and 10min in equilibration solution 2 (0.1M Tris-HCl pH6.8, 18g of urea, 15ml of glycerol, 1% ($^{w}/_{v}$) SDS, 2.25g of iodoacetamide(Sigma), a few grains of BPB and distilled water to 50ml final volume). The strips were placed on filter paper moistened with distilled water and drained by transferring them onto dry filter paper. At this stage the whole apparatus was reassembled for the second run, following manufacturers' instructions. The samples were then separated on the second dimensional electrophoresis by molecular weight, using precast 24.5x11cm ExcelGel SDS polyacrylamide gels (stored at 4°C) containing a gradient ranging from 8-18% and precast ExcelGel SDS anodic and cathodic buffer strips (stored at 4°C). 20µl of the Coomassie brilliant blue stainable molecular weight standards (section 2.8.1.4; prepared by diluting 1:10 in sample solution) were loaded onto an extra sample application piece placed at the end of each strip. The second dimensional electrophoresis was carried out at a constant temperature of 15°C and was programmed to undergo three phases consisting

of: phase 1 at 600V, 20mA, 30W, for 30min; phase 2 at 600V, 50mA, 30W, for 5min, phase 3 at 600V, 50mA, 30W, for 2h. When the SDS electrophoresis finished, the ExcelGel was processed for the detection of proteins.

2.8.2.4 Detection of proteins by silver staining. The detection method used for visualising 2-D electrophoresed proteins was based on the method described by Heukeshoven & Dernick (Heukeshoven & Dernick, 1986). All incubations were at room temperature with gentle rocking.

When the second dimensional electrophoresis completed, the SDS gel was removed and immersed in fixing solution (40% ($^{v}/_{v}$) ethanol and 10% ($^{v}/_{v}$) glacial acetic acid) for 30min, transferred into incubation solution (30% ($^{v}/_{v}$) ethanol, 68g/l sodium acetate, 0.52% ($^{v}/_{v}$) of 25% ($^{w}/_{v}$) glutaraldehyde, and 2g/l sodium thiosulfato) for 30min and washed for 5min three times in distilled water. The gel was then soaked in silver staining solution (1g/l $AgNO_3$ (ICN), 0.02% ($^{v}/_{v}$) formaldehyde) for 40min. The gel was then incubated with the developing solution (25g/l Na_2CO_3, 10% ($^{v}/_{v}$) formaldehyde) for 15min. The reaction was stopped by rinsing the gel twice for 5-10min with stop solution (14.6g/l EDTA) and the gel was rinsed three times for 5min with distilled water. For long term storage, after immersing the gel in preserving solution (10% ($^{v}/_{v}$) glycerol) for 20min, it was wrapped with a cellophane preserving sheet soaked in preserving solution and left at room temperature to dry.

2.9 Analysis of lipopolysaccharides (LPS)

2.9.1 Preparation of LPS

Bacterial strains were grown overnight in 5ml of MH broth at 37°C with constant shaking. The cultures were chilled on ice and their $O.D_{600}$ determined using a Pharmacia Ultraspec III spectrophotometer. Bacterial cells were harvested by centrifuging 500µl of culture (0.3$O.D_{600}$ units for *C. jejuni* cultures and 0.5 $O.D_{600}$ units for *E. coli* cultures) for 10min at 4°C, 3200 *g* in a Heraeus Megafuge 1R. The cells were resuspended in 100µl of 1x SDS-PAGE sample buffer (section 2.8.1.1) and incubated at room temperature for 10min to allow cell lysis and the solubilisation of proteins. Following a vigorous vortex to help cell membrane and DNA disruption, the suspension was boiled for 10min and left to return to room temperature. The proteins in the suspension were digested by the

addition of 10µl of 2.5mg/ml proteinase K during 2h incubation at 60°C. The samples were stored at -20°C.

2.9.2 Electrophoresis of LPS

A 10-15µl aliquot of LPS extract were loaded onto a SDS-polyacrylamide "mini-gel" (5% ($^{v}/_{v}$) stacking and 11% ($^{v}/_{v}$) separating gels, section 2.8.1.3), together with 10µl of the Coomassie brilliant blue stainable protein molecular weight standards (section 2.8.1.4). LPS samples were not boiled prior to electrophoresis but the molecular weight standards were boiled for 3min. The electrophoresis was carried out at constant current of 15mA for about 2h. Electrophoresis was stopped when the dye front entered the bottom tank buffer.

2.9.3 Silver staining of LPS

Following electrophoresis, the SDS-PAGE was removed and the LPS visualised by silver staining (Hitchcock & Brown, 1983). All incubations were at room temperature with gentle rocking.

The gel was fixed for 2h in fixing solution (40% ($^{v}/_{v}$) ethanol and 5% ($^{v}/_{v}$) glacial acetic acid), transferred into oxidative solution (40% ($^{v}/_{v}$) ethanol, 5% ($^{v}/_{v}$) glacial acetic acid and 0.7% ($^{v}/_{v}$) HIO_4) for 5min and washed for 10min three times in distilled water. The gel was then soaked in silver staining solution (28ml of 0.1M NaOH (freshly made), 2ml of concentrated NH_4OH, 5ml of 20% ($^{w}/_{v}$) $AgNO_3$ (ICN), freshly made and slowly added whilst stirring) and distilled water to 150ml final volume) for 10min. After another three 5min washes in distilled water, the gel was incubated with the colour solution (10mg citric acid and 100µl formaldehyde in 200ml of distilled water) and kept in the dark until the bands developed. The reaction was stopped by rinsing the gel several times with distilled water. The gel was then photographed. For long term storage, it could be transferred to 3MM chromatography paper (Whatman) and dried on a gel drier at room temperature.

2.10 Methods for utilising tissue culture

All manipulations involving tissue culture were carried out in a laminar vertical flow cabinet (manufactured by Medical Air Technology Ltd). All glass and plastic materials used were sterile; all solutions utilised were previously autoclaved at 121°C for 15min unless otherwise stated.

2.10.1 Cell line

Caco-2 (derived from a colon carcinoma; *cit. in* Pinto *et al.*, 1983) was the cell line used as an *in vitro* model to test *C. jejuni*-host interactions. Caco-2 cells display structural and functional enterocyte-like differentiation, forming polarised monolayers when grown on a surface. The description of Caco-2 cell line and information regarding the methodology to grow and maintain the cell line is given in Part I, chapter 2.

2.10.2 Association assay

2.10.2.1 Preparing the bacteria. *C. jejuni* strains were incubated overnight in 5ml of DMEM (Dulbecco's modified Eagle medium; Part I, chapter 2) supplemented with 10% ($^{v}/_{v}$) of FBS (foetal bovine serum; Part I, chapter 2) at 37°C in a microaerophilic atmosphere with constant shaking. The $O.D_{600nm}$ of the cultures was determined using a Pharmacia Ultraspec III spectrophotometer. Bacterial cells were harvested by a 10min centrifugation at room temperature, 3200 *g* in Heraeus Megafuge 1R and resuspended in DMEM to 0.3-0.5 O.D units/ml.

E. coli DH5α was also incubated overnight in 5ml of DMEM supplemented with 10% ($^{v}/_{v}$) of FBS at 37°C with constant shaking. The $O.D_{600nm}$ of the cultures was determined using a Pharmacia Ultraspec III spectrophotometer. Bacterial cells were harvested by 10min centrifugation at room temperature, 3200 *g* in Heraeus Megafuge 1R and resuspended in DMEM to 0.1 O.D units/ml.

In order to determine the initial viability of the cultures, 0.1ml of each suspension was withdrawn, serial dilutions were plated out on an adequate medium and the plates incubated at 37°C (section 2.5).

2.10.2.2 Preparing Caco-2 cell monolayers. Caco-2 cells were grown in 12 well (flat bottom) plates (Corning Cell Wells) containing DMEM supplemented with 10% ($^{v}/_{v}$) of FBS at 37°C, in 5% CO_2

with 95% humidified atmosphere. The cultures were used when fully differentiated, about 12-14 days post-confluence, when domes appeared evenly distributed throughout the cell monolayer.

2.10.2.3 *C. jejuni* association to Caco-2 cell monolayers. Prior to the experiment, the medium above the Caco-2 cells was removed and replaced with 250µl of bacterial suspension. The monolayers were incubated at 37°C in 5% CO_2 with 95% humidified atmosphere. After 30min, Caco-2 cell monolayers were thoroughly washed four times with prewarmed PBS to remove non-associated bacteria. Caco-2 cells were lysed by adding 250µl of 0.5% ($^{w}/_{v}$) sodium deoxycholate (freshly prepared in PBS). The mix of Caco-2 cells and bacteria was briefly vortexed to disrupt Caco-2 cell DNA and serial dilutions were plated out on either MH or campylobacter blood-free selective agar to determine the number of adherent and internalised bacteria.

2.10.3 Invasion assay

Bacterial strains and Caco-2 cell monolayers were prepared as described above (sections 2.10.2.1 and 2.10.2.2). Prior to the experiment, the medium above the Caco-2 cells was removed and replaced with 250µl of bacterial suspension. The monolayers were incubated at 37°C in 5% CO_2 with 95% humidified atmosphere. After 3h, Caco-2 cell monolayers were thoroughly washed four times with prewarmed PBS to remove non-associated bacteria. Non-internalised bacteria were killed by incubating the cell monolayers with 250µl of 250µg/ml of gentamicin (freshly prepared in DMEM) at 37°C in 5% CO_2 with 95% humidified atmosphere. After 3h, Caco-2 cell monolayers were thoroughly washed four times with prewarmed PBS and cells were lysed by adding 250µl of 0.5% ($^{w}/_{v}$) sodium deoxycholate (freshly prepared in PBS). The mix of Caco-2 cells and bacteria was briefly vortexed to disrupt Caco-2 cell DNA and serial dilutions were plated out on either MH or campylobacter blood-free selective agar to determine the number of internalised bacteria.

CHAPTER 3
Isolating a Response Regulator Gene

3.1 Introduction

To determine whether *C. jejuni* utilised two-component signal transduction systems, a genomic library was screened for the presence of response regulatory genes, using a DNA probe previously isolated in a collaboration with B. Wren (Wren *et al.*, 1992). Degenerate oligonucleotide primers were designed based on the sequence of the conserved domains from *E. coli* PhoB, the regulator of the phosphate regulon and *Salmonella typhimurium* PhoP, a regulator of virulence genes (Stock *et al.*, 1989). The degenerate oligonucleotide primers were used in a polymerase chain reaction (PCRDOP) to amplify gene fragments from the chromosome of *C. jejuni* strain F132. PCRDOP products of about 300bp were purified from an agarose gel and cloned into the plasmid vector pUC19, using the *Hin*dIII and *Pst*I restriction sites tailored during PCR. A 320bp fragment in pJMK39 (Fig.6) was sequenced and the predicted amino acid sequence showed homology to the RR superfamily (Wren *et al.*, 1992; Fig.7). The PCRDOP fragment (referred to as *regX1*) was used to probe the *C. jejuni* genomic library and a complete open reading frame was isolated. In order to identify other components in this regulatory system, the flanking sequences of this open reading frame were investigated. Characterisation of the *regX1* locus may facilitate to determine the role of *regX1* by providing more sequence data for sequence comparisons as well as to use in the construction of a defined mutant.

3.2 Isolating *regX1* gene from the chromosome

In order to isolate the whole *regX1* gene from the *C. jejuni* chromosome, *regX1* PCRDOP fragment was used as a non-radioactive probe to screen a genomic library. The library was constructed by M. Kiernan (Kiernan, 1997) using *C. jejuni* strain F132 chromosomal DNA, partially digested with *Sau*3A endonuclease and λZAP II vector DNA, digested with *Xho*I endonuclease. Chromosomal DNA fragments between 3-10kb were cloned into the polylinker of pBluescript within the λZAP II vector.

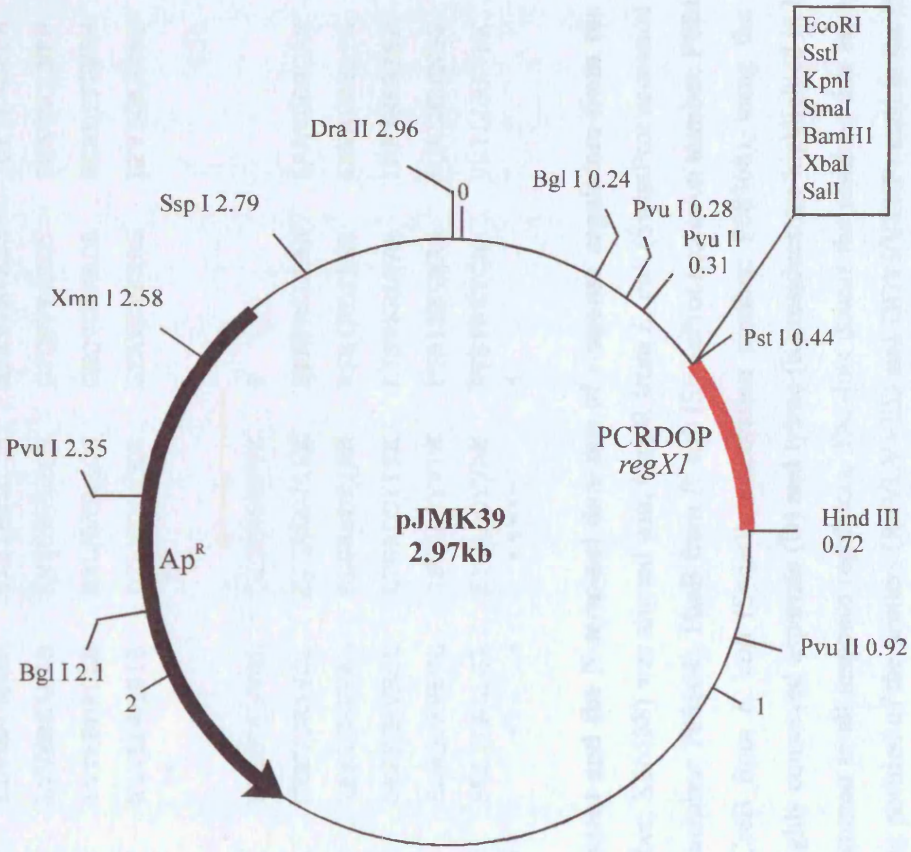


Fig.6 Map of the plasmid pJMK39. pJMK39 resulted from the cloning of *regX1* PCRDOP fragment into the *Hin*dIII/*Pst*I sites in pUC19. Restriction enzymes positions are in kb.

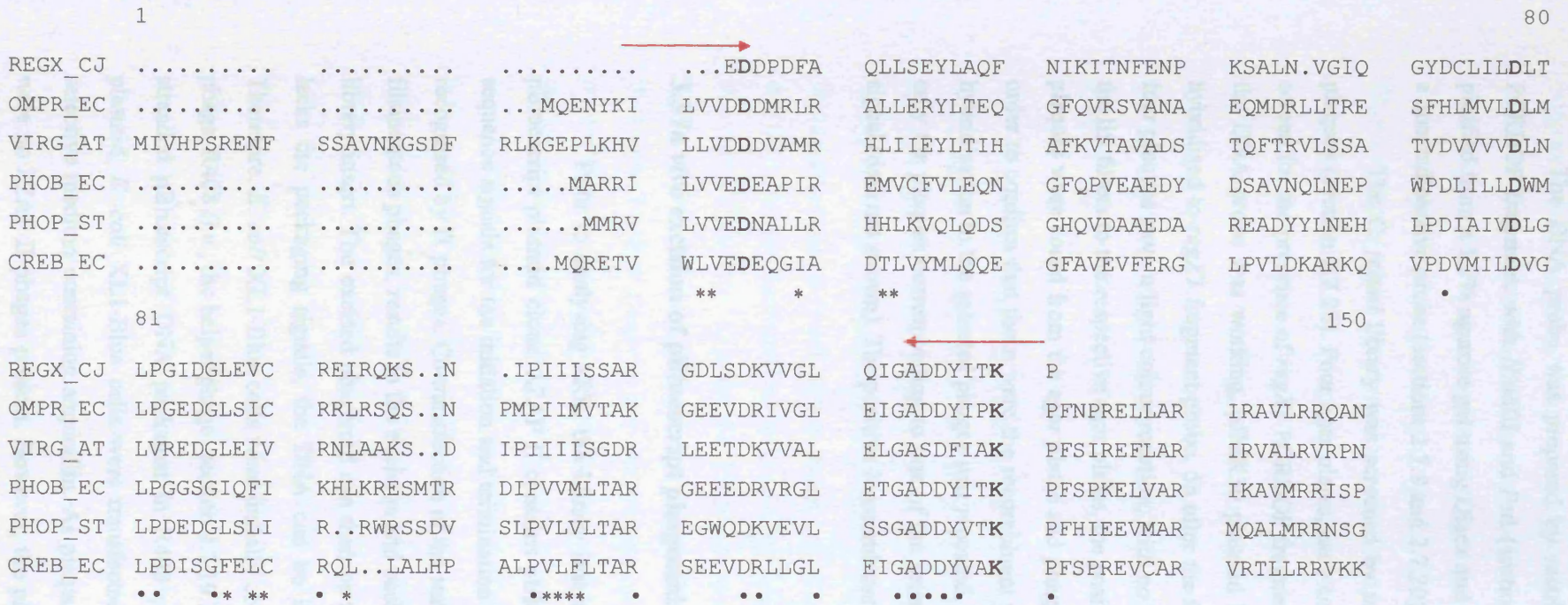


Fig.7. Similarity between *C. jejuni regX1* PCRDOP fragment and the N-terminal domains of response regulators from the PhoP subfamily. *regX1* predicted amino acid sequence (EMBL accession number: X66588) was aligned with OmpR from *E. coli* (SwissProt accession number: P03025), VirG from *Agrobacterium tumefaciens* (SwissProt accession number: P06664), PhoB from *E. coli* (SwissProt accession number: P08402), PhoP from *S. typhimurium* (SwissProt accession number: P14146) and CreB from *E. coli* (SwissProt accession number: P08368), using the program PILEUP (UWGCG Package) in the Genetics Computer Group. The highly conserved aspartate (D) and lysine (K) residues are highlighted in bold. The asterisks correspond to the residues forming the hydrophobic cores common to all response regulators. The dots denote the residues that are identical in at least five members of the group. The arrows represent the annealing position of the primers (RI/VLVVED and DDYI/VTKP) used in the PCRDOP to isolate *regX1* fragment.

The DNA probe was prepared by restricting pJMK39 (Fig.6), which contains the PCRDOP fragment, with *Hin*dIII and *Pst*I (section 2.7.7). The 320bp PCRDOP fragment was purified from a 1.2% agarose gel using Qiaex and labelled with digoxigenin-11-dUTP to use as a non-radioactive probe (sections 2.7.8 and 2.7.20).

The *C. jejuni* library was screened by *in situ* hybridisation to λ recombinant phage plaques (section 2.7.20). Four agar plates, each containing around $1x10^3$ plaques, were used to screen for the presence of *regX1* PCRDOP fragment homologous sequences. To check whether the DNA probe was working, pJMK39 plasmid DNA was cross-linked to a nylon filter and hybridised to *regX1* fragment probe. 5h after the filters were incubated in developing solution, four plaques gave a light colour reaction, with no background (data not shown). By comparing the lift filters to the respective agar plates, the positive recombinant phages were identified. The plaques were cored from the agar plates and phage stocks were prepared (section 2.7.19.2). In order to confirm that these were the recombinant phages carrying *regX1* sequences, the *in situ* hybridisation to the selected phages was repeated. Following the second hybridisation reaction, only the plaques corresponding to one of the selected phages reacted, giving a strong positive signal (data not shown). The positive λ recombinant, λRB, was selected for further analysis.

3.3 *In vivo* excision of pBluescript phagemid from λZAP II vector

Prior to analysing λRB, the library insert was rescued from the λ recombinant as a pBluescript plasmid clone. λZAP II contains pBluescript plasmid DNA inserted between the sequence signals for the initiation and termination of DNA synthesis and the packaging signals recognised by f1 phages. Co-transfection of the same host cell by λZAP II and f1, or f1-derived filamentous phages, results in the excision and packaging of pBluescript plasmid containing the library insert. The excised phagemid can then be transfected into another host and, because it lacks the packaging signals, the DNA can be isolated as double stranded plasmid DNA. Therefore, *E. coli* XL1-Blue cells were initially co-transfected with the λRB and an f1-derived phage, R408 (i.e., the helper phage; section 2.7.19.3). The isolated phage stock contained single stranded pBluescript DNA packaged in R408 phage. In order to obtain double stranded plasmid, *E. coli* XL1-Blue cells were transfected with this phage stock and plated out on selective medium containing ampicillin. As plaques did not form, it was concluded that there were no λZAP II phages present. However, the plates contained two different size ampicillin resistant colonies, one group of colonies growing much more slowly than the other. This

suggested that the phage stock probably contained a mix of both recombinant pBluescript phagemid and helper phage that had co-transfected the host cells. Thus, only the larger colonies that presumably contained recombinant pBluescript alone were selected. Plasmid DNA was isolated by ion-exchange chromatography (section 2.7.2.1) and analysed on a 0.8% agarose gel. This confirmed that only recombinant pBluescript plasmid DNA had been isolated (data not shown) and the plasmid clone was named pλRB.

3.4 Confirming the presence of *regX1* locus on pλRB and on the chromosome of *C. jejuni*

Before proceeding further, the presence of *regX1* gene within pλRB clone was confirmed by PCR and southern blotting experiments. Simultaneously, *C. jejuni* strain 81116 chromosome was also screened for the presence of *regX1* gene. Although the library was constructed using chromosomal DNA from *C. jejuni* F132, this strain has not been genetically characterised and it is not known whether F132 is transformable. In contrast, *C. jejuni* 81116 has been characterised and has been used in other studies to make mutants (*e.g.*, Wassenaar *et al.*, 1991). Therefore, *C. jejuni* 81116 was chosen to continue the studies on the *regX1* gene. PCR and hybridisation experiments using the strain 81116 chromosomal DNA aimed to determine whether there are any polymorphisms between the two strains, F132 and 81116, concerning this locus. Such event has been shown to occur for other loci, for example, within the lipopolysaccharide biosynthesis loci (A. Wood, personal communication).

Since the DNA sequence of *regX1* PCRDOP fragment had been determined previously (Fig.7, section 3.1), a PCR reaction (section 2.7.15.1) was used to confirm the presence of *regX1* fragment homologous sequence within pλRB clone and *C. jejuni* 81116 genome. Two primers, R3 and R4, were designed specifically to anneal to the *regX1* fragment (see section 2.2.2 and Appendix 4) and used to amplify it under the following conditions: a first set of reactions comprising one cycle at a denaturing temperature of 96°C for 1min, an annealing temperature of 60°C for 1min and an extension temperature of 72°C for 0.5min; a subsequent set of reactions comprising thirty cycles at a denaturing temperature of 94°C for 1min, an annealing temperature of 60°C for 1min and an extension temperature of 72°C for 0.5min; a last set of reactions comprising one cycle at an annealing temperature of 60°C for 1min and an extension temperature of 72°C for 2min. A positive control was included consisting of pJMK39 plasmid. The PCR products were analysed by electrophoresis on a 1.2% agarose gel and by

comparing to the positive control, a 300bp band corresponding to *regX1* fragment was present in both the pλRB clone and 81116 genome, although the positive band was faint in 81116 sample (Fig.8).

regX1 DNA probe (section 3.2) was also hybridised to pλRB clone and to *C. jejuni* 81116 chromosomal DNA digests. pλRB DNA was cleaved with *Pvu*II (see section 3.5), generating a 4.2kb DNA band containing the insert (and flanking sequences) and a 2.7kb DNA band corresponding to vector alone. 81116 chromosomal DNA was restricted, in single digestions, with *Bcl*I, *Bgl*II and *Cla*I. All samples were separated on a 0.8% agarose gel, along with pUC19 and pBluescript DNA linearised with *Pst*I as negative controls and pJMK39 DNA cleaved with *Hin*dIII and *Pst*I as the positive control. The DNA-DNA hybridisation was performed using a non-radioactive DNA labelling and detection system, with a digoxigenin-11-dUTP labelled DNA probe (section 2.7.20.1). The blot revealed that the probe hybridised to the 4.2kb pλRB band (Fig.9) as expected. The probe also hybridised to all genomic digests: a 2.9kb *Bcl*I band, a 4.8kb *Bgl*II band and a 2.6kb *Cla*I band (Fig.10). The hybridisation results indicated that *regX1* gene is present in both the pλRB clone and *C. jejuni* 81116 chromosome, thus corroborating the results previously obtained with PCR.

3.5 Determining the restriction map of the pλRB clone

pλRB plasmid was characterised by restriction analysis. Single and double restriction enzyme digests of ion-exchange chromatography purified plasmid were performed as described before (section 2.7.7), enabling the generation of a partial restriction map of the clone (Fig.11).

3.6 Locating the PCRDOP fragment sequence within the pλRB clone

The analysis of the restriction map showed that the isolated library clone contained around 3.8kb of *C. jejuni* DNA. In order to locate the *regX1* gene within the insert, pλRB was double digested with *Xba*I and *Hin*dIII and with *Pst*I and *Pvu*II (section 2.7.7). The digests were separated on a 0.8% agarose gel and probed with digoxigenin-11-dUTP labelled *regX1* PCRDOP fragment (section 2.7.20.1). The 1.5kb *Xba*I/*Hin*dIII and 3.2kb

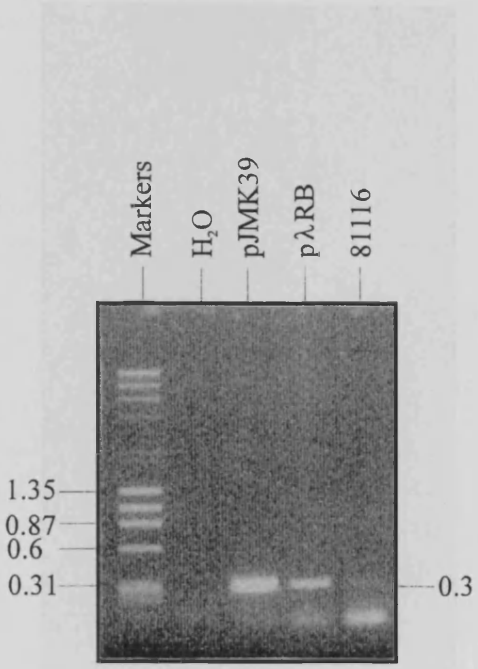


Fig.8. PCR to confirm the presence of *regX1* within the library clone pλRB and *C. jejuni* 81116 chromosome. pλRB and *C. jejuni* 81116 DNA were used as templates in two PCR reactions with R3 and R4 primers. A negative control using no template (H_2O) and a positive control using pJMK39 as template were prepared in parallel. The PCR products were separated on 1.2% agarose gel, showing a 0.3kb band in the lanes corresponding to the positive control (pJMK39), pλRB and the chromosome of 81116. The band amplified from 81116 chromosome was fainter than the band amplified from the other templates. The molecular markers (kb) are represented on the left of the figure.

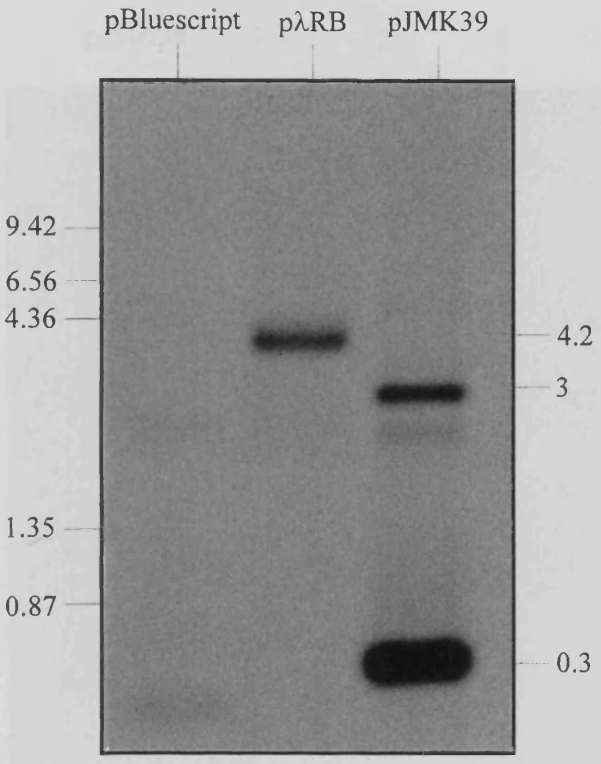


Fig.9. Southern blot to confirm the presence of *regX1* within the library clone pλRB. pλRB DNA was restricted with *Pvu*II and hybridised to digoxigenin-11-dUTP labelled PCRDOP *regX1* fragment. The probe was also hybridised to *Pst*I linearised pBluescript and to *Hin*dIII/*Pst*I double cleaved pJMK39 as negative and positive controls, respectively. The blot shows that the probe hybridised to two bands in the positive control: the 0.3kb band which corresponds to the PCRDOP fragment and to single cut vector (~3kb). The probe also hybridised to the 4.2kb band of pλRB which contains the clone's insert and, thus, confirms the presence of *regX1* within the clone. The sizes of the molecular markers (kb) are represented on the left of the figure.

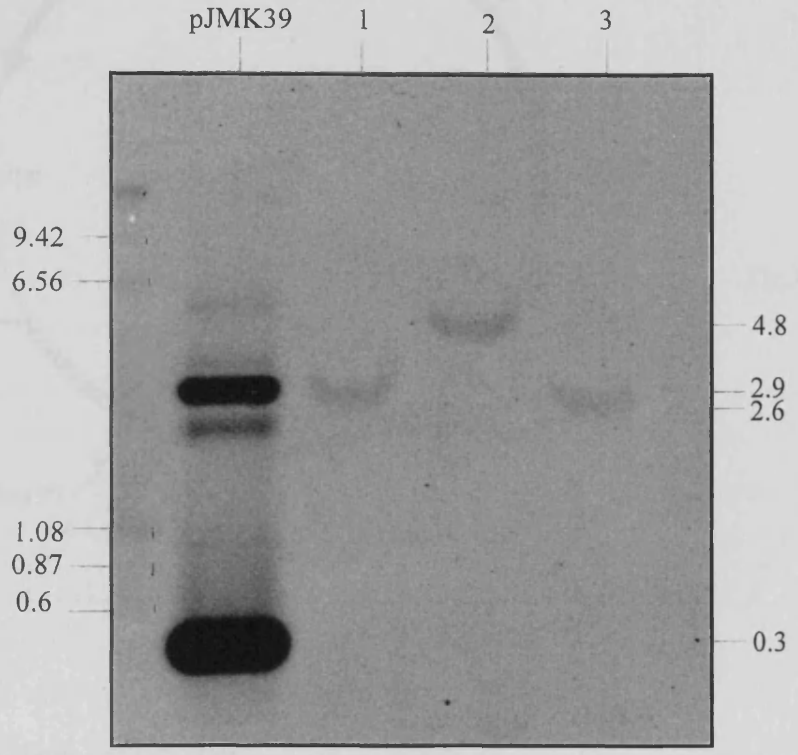


Fig.10. Southern blot to confirm the presence of *regX1* within the chromosome of *C. jejuni* 81116. Chromosomal DNA of 81116 was restricted, in single digestions, with *Bcl*I (1 on the figure) *Bgl*II (2 on the figure) and *Cla*I (3 on the figure). The chromosomal digests as well as *Hin*dIII/*Pst*I double cleaved pJMK39 (positive control) were hybridised to digoxigenin-11-dUTP labelled PCRDOP *regX1* fragment. The blot shows that the probe hybridised to two bands in the positive control: the 0.3kb band which corresponds to the PCRDOP fragment and to single cut vector (~3kb, not labelled on the figure). The probe also hybridised to a 2.9kb *Bcl*I band, a 4.8kb *Bgl*II band and to a 2.6kb *Cla*I band, thus, confirms the presence of *regX1* within 81116 chromosome. The sizes of the molecular markers (kb) are represented on the left of the figure.

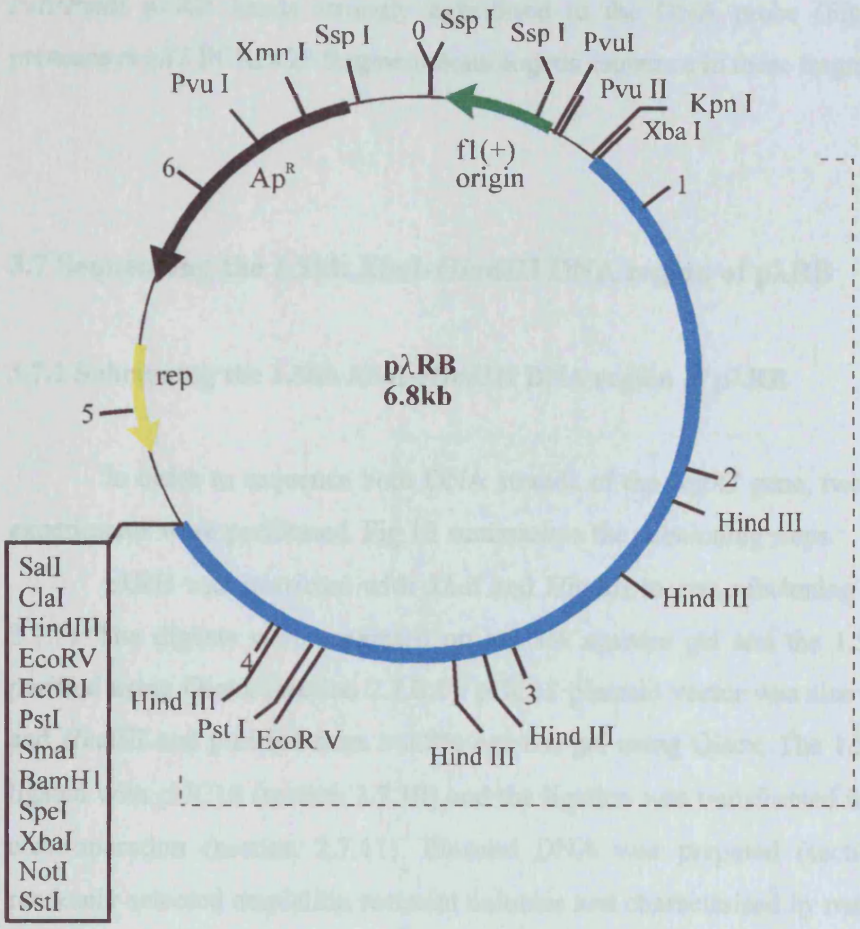


Fig.11b

Fig.11a. Map of the *C. jejuni* library clone, pλRB. The restriction enzymes positions are in kb.

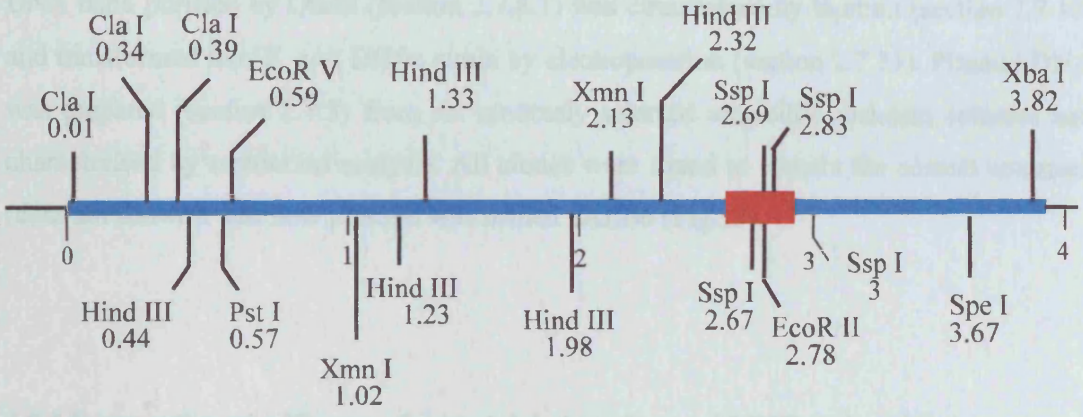


Fig.11b. Partial restriction map from pλRB's insert. The red box corresponds to the PCRDOP fragment. Sizes (kb) do not relate to those in Fig.11a and zero corresponds to the start of the genomic insert upstream *regX1* (see Fig.20).

PstI/PvuII pλRB bands strongly hybridised to the DNA probe (Fig.12), indicating the presence *regX1* PCRDOP fragment homologous sequence in these fragments.

3.7 Sequencing the 1.5kb *Xba*I-*Hin*dIII DNA region of pλRB

3.7.1 Subcloning the 1.5kb *Xba*I-*Hin*dIII DNA region of pλRB

In order to sequence both DNA strands of the *regX1* gene, two parallel subcloning experiments were performed. Fig.13 summarises the subcloning steps.

pλRB was restricted with *Xba*I and *Hin*dIII in one subcloning experiment (section 2.7.7). The digests were separated on a 0.8% agarose gel and the 1.5kb DNA band was purified using Qiaex (section 2.7.8.1). pUC18 plasmid vector was also restricted with *Xba*I and *Hin*dIII and purified from a 0.8% agarose gel using Qiaex. The 1.5kb DNA insert was ligated with pUC18 (section 2.7.10) and the ligation was transformed into *E. coli* DH5α by electroporation (section 2.7.11). Plasmid DNA was prepared (section 2.7.3) from six randomly selected ampicillin resistant colonies and characterised by restriction analysis. All clones were found to contain the correct construct (data not shown). The new plasmid was named pALB3R (Fig.13).

In a parallel subcloning experiment, pλRB was digested with the restriction enzyme *Hin*dIII (section 2.7.7). The digests were separated on a 0.8% agarose gel and the 4.5kb DNA band purified by Qiaex (section 2.7.8.1) was circularised by ligation (section 2.7.10) and transformed into *E. coli* DH5α strain by electroporation (section 2.7.11). Plasmid DNA was prepared (section 2.7.3) from six randomly selected ampicillin resistant colonies and characterised by restriction analysis. All clones were found to contain the correct construct (data not shown). The new plasmid was named pALB6 (Fig.13).

3.7.2 Preparation of a library of nested deletions from pALB3R and pALB6 clones

Plasmid DNA from pALB3R and pALB6 was prepared by ion-exchange chromatography (Qiagen; section 2.7.2.1). A library of nested unidirectional deletions of double-stranded DNA from pALB3R and pALB6 was generated (section 2.7.17), according

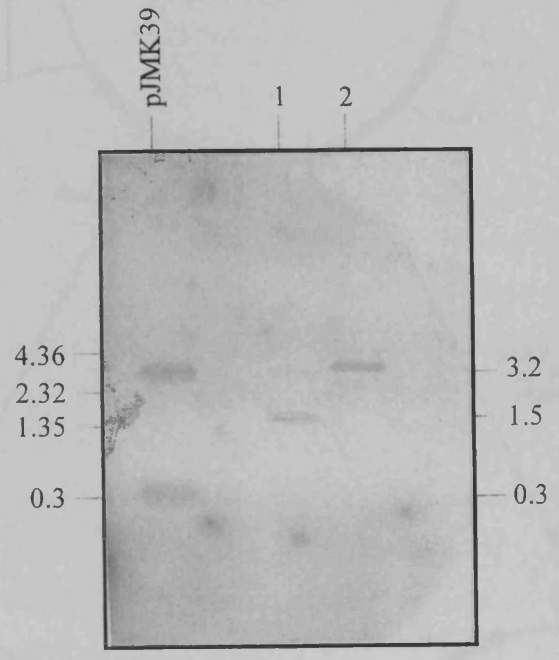


Fig.12. Southern blot to locate *regX1* within pλRB's insert. pλRB was double digested with *Xba*I/*Hin*dIII (1 on the figure) and *Pst*I/*Pvu*II (2 on the figure) and the digests were hybridised to digoxigenin-11-dUTP labelled PCRDOP *regX1* fragment. The blot shows that the probe hybridised to two bands in the positive control: the 0.3kb band which corresponds to the PCRDOP fragment and to single cut vector (~3kb, not labelled on the figure). The probe also hybridised to the 1.5kb *Xba*I/*Hin*dIII and the 3.2kb *Pst*I/*Pvu*II bands. The sizes of the molecular markers (kb) are represented on the left of the figure.

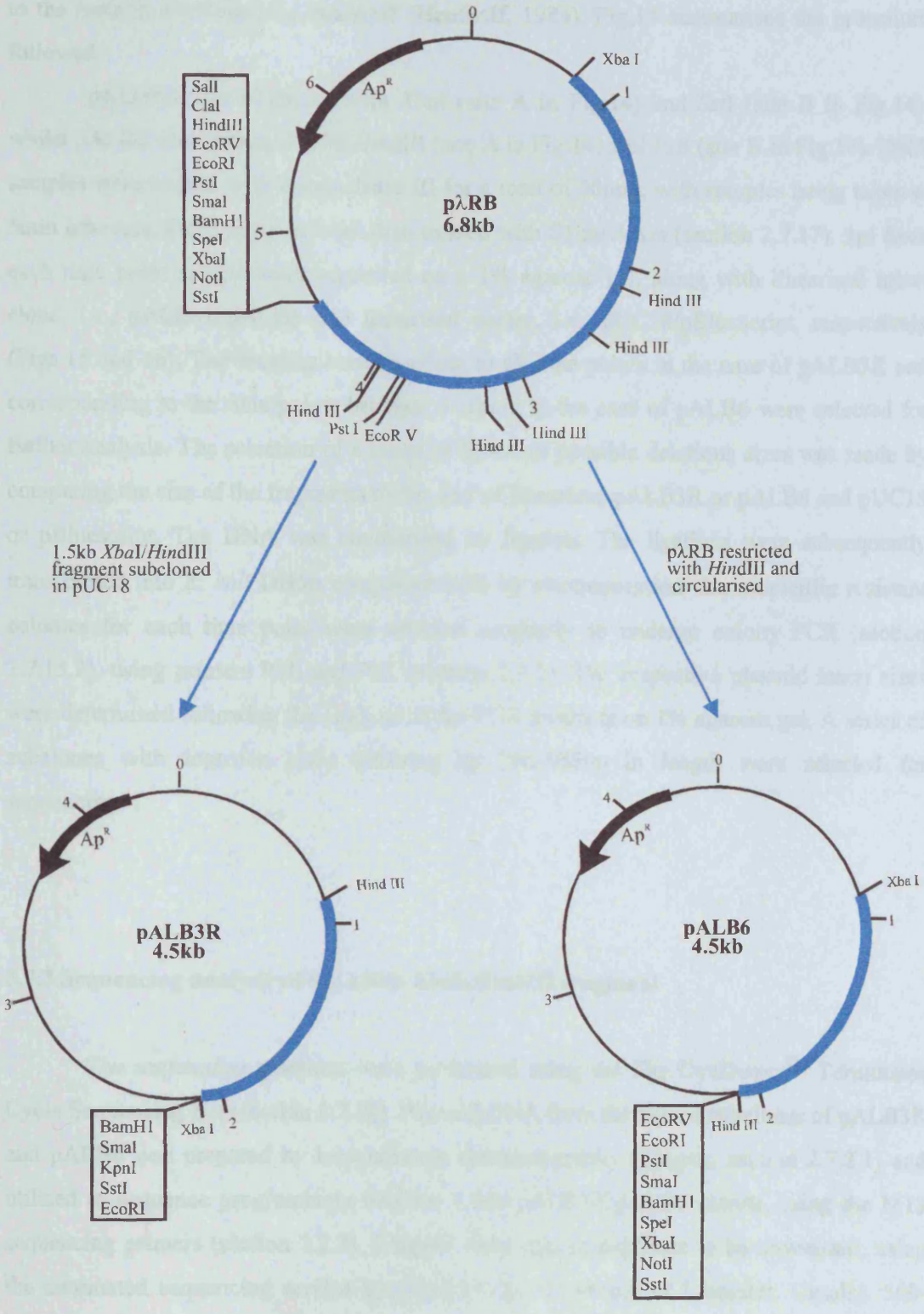


Fig.13. Construction of pALB3R and pALB6 plasmids. The two pλRB-derived plasmids were constructed in order to sequence both strands of the 1.5kb *Xba*I/*Hin*dIII fragment containing PCRDOP *regX1* fragment (see text).

to the method developed by Henikoff (Henikoff, 1984). Fig.14 summarises the procedure followed.

pALB3R was restricted with *Xba*I (site A in Fig.14) and *Sst*I (site B in Fig.14), whilst pALB6 was restricted with *Hin*dIII (site A in Fig.14) and *Pst*I (site B in Fig.14). DNA samples were treated with Exonuclease III for a total of 30min, with samples being taken at 5min intervals. DNA samples were then treated with S1 nuclease (section 2.7.17). 3µl from each time point sample were separated on a 1% agarose gel, along with linearised intact clone, i.e., pALB3R/pALB6 and linearised vector, i.e., pUC18/pBluescript, respectively (Figs 15 and 16). The samples corresponding to all time points in the case of pALB3R and corresponding to the time points between 5-20min in the case of pALB6 were selected for further analysis. The selection of a range of different possible deletions sizes was made by comparing the size of the fragments to the size of linearised pALB3R or pALB6 and pUC18 or pBluescript. The DNA was circularised by ligation. The ligations were subsequently transformed into *E. coli* DH5α competent cells by electroporation. Six ampicillin resistant colonies for each time point were selected randomly to undergo colony PCR (section 2.7.15.2), using primers P1L and P2L (section 2.2.2). The respective plasmid insert sizes were determined following the analysis of the PCR products on 1% agarose gel. A series of subclones with insertion sizes differing by 200-300bp in length were selected for sequencing.

3.7.3 Sequencing analysis of the 1.5kb *Xba*I-*Hin*dIII fragment

The sequencing reactions were performed using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (section 2.7.18). Plasmid DNA from the nested subclones of pALB3R and pALB6 was prepared by ion-exchange chromatography (Qiagen; section 2.7.2.1) and utilised to sequence progressively into the 1.5kb pALB3R/pALB6 inserts, using the M13 sequencing primers (section 2.2.2). Samples were sent in duplicate to be sequenced, using the automated sequencing service provided by the University of Leicester. Usually, 500-600bp of reliable DNA sequence were obtained. The sequence data from pALB3R/pALB6 was analysed using the Wisconsin Package, (Version 8, September 1994) via the Genetics Computer Group. The results are presented in Fig.17.

The 1.5kb DNA fragment was sequenced and the information obtained indicated the presence of one complete open reading frame (Orf1) and the N-terminal region of another

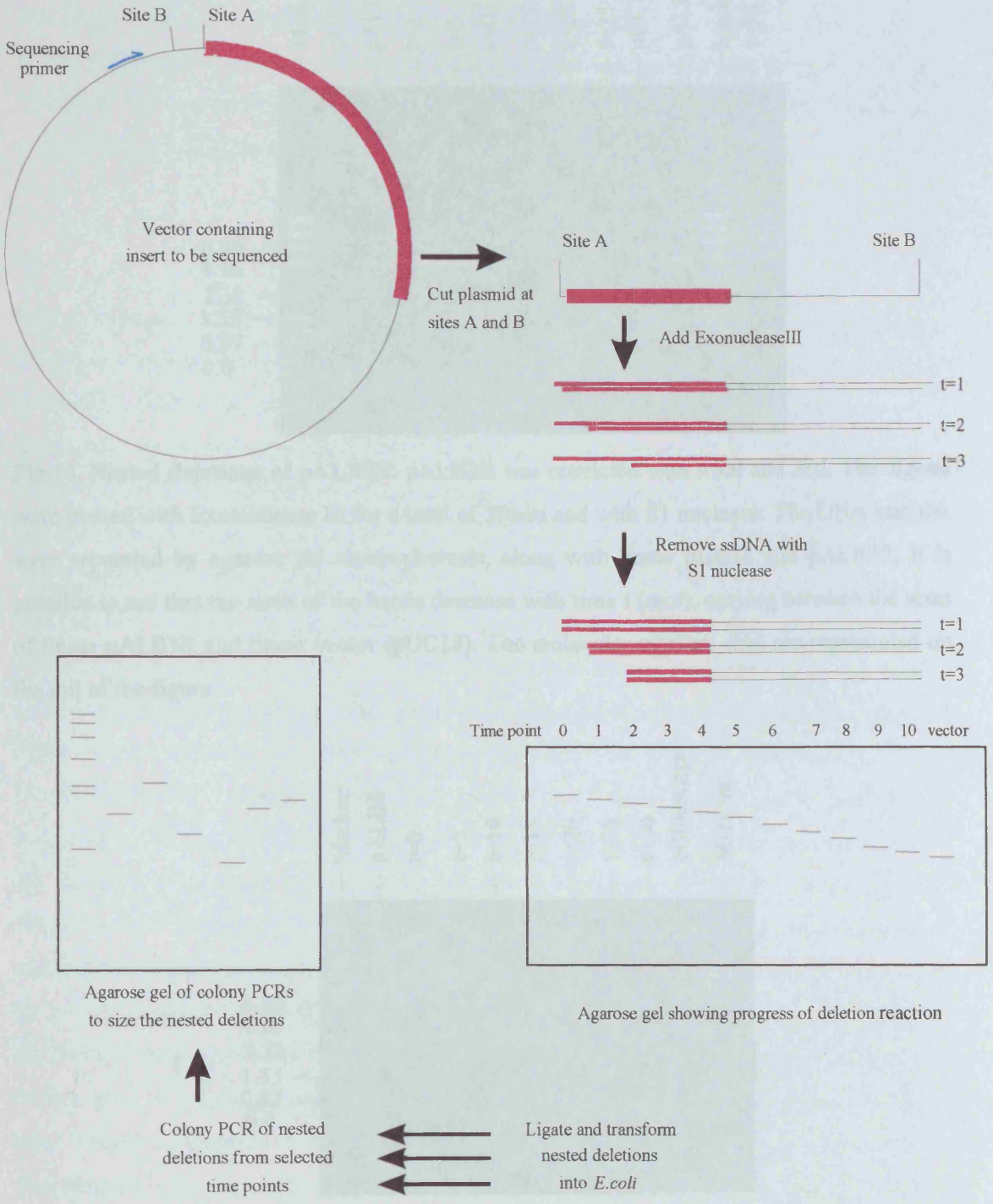


Fig.14. Schematic illustration of the steps involved in the preparation of a library of nested deletions (Henikoff, 1984).

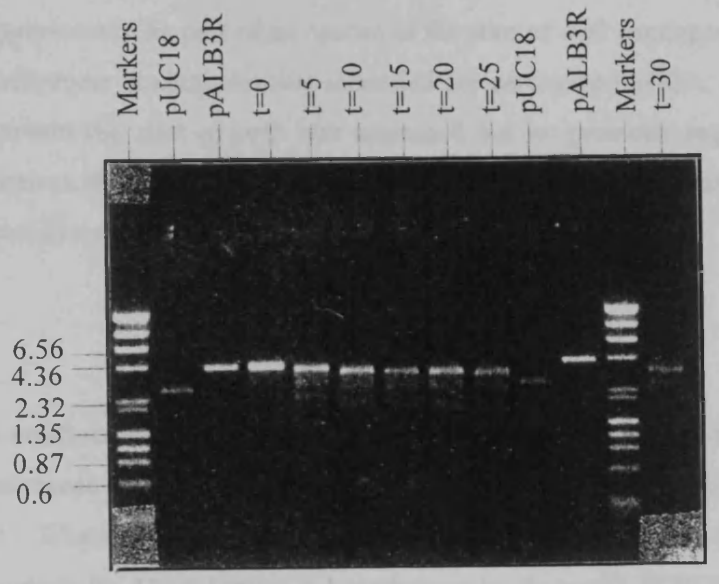


Fig.15. Nested deletions of pALB3R. pALB3R was restricted with *Xba*I and *Sst*I. The digests were treated with Exonuclease III for a total of 30min and with S1 nuclease. The DNA samples were separated by agarose gel electrophoresis, along with linear pUC18 and pALB3R. It is possible to see that the sizes of the bands decrease with time t (min), varying between the sizes of linear pALB3R and linear vector (pUC18). The molecular markers (kb) are represented on the left of the figure.

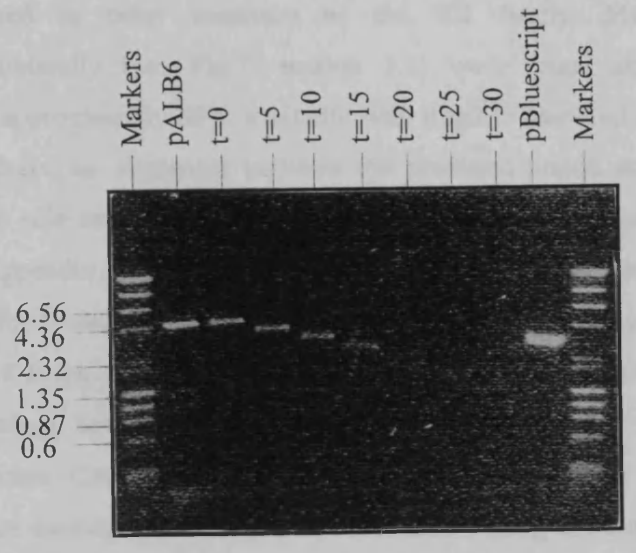


Fig.16. Nested deletions of pALB6. pALB6 was restricted with *Hin*dIII and *Pst*I. The digests were treated with Exonuclease III for a total of 30min and with S1 nuclease. The DNA samples were separated by agarose gel electrophoresis, along with linear pBluescript and pALB6. It is possible to see that the sizes of the bands decrease with time t (min), varying between the sizes of linear pALB6 and linear vector (pBluescript). The molecular markers (kb) are represented on the left of the figure.

(Orf2). The two genes could be part of an operon as the start of *orf2* overlapped the end of *orf1*. A potential ribosome binding site was identified for each gene (AGGA; Fig.17). The 130bp region upstream the start of *orf1* was examined but no promoter sequences were identified. Nevertheless, the region contained a series of AT repeats which could correspond to putative promoter-like structures.

3.7.3.1 Sequence analysis of *orf1*. *orf1* is 671bp long. The encoded protein deduced from the nucleic acid sequence contained 223 amino acids and had a predicted molecular weight of 24.5kDa (Orf1). When compared to the sequence of the pJMK39 insert (Wren *et al.*, 1992), *orf1* did include the DNA sequence homologous to the *regX1* PCRDOP fragment. Therefore, *orf1* was identified as corresponding to the whole *regX1* gene. Comparative sequence analysis using FASTA (Pearson & Lipman, 1988) and BLASTP (Altschul *et al.*, 1990) revealed extensive homology between the predicted amino acid sequence of RegX1 and proteins from the RR family available in GenEMBL and SwissProt databases. The similarities were greatest in the N-terminus but considerable homology could be seen throughout the entire sequence. RegX1 contained approximately 33 to 38% identical amino acids when compared to other members of the RR family. Members of the OmpR/PhoB/PhoP subfamily (see Fig.7, section 3.1) were found among the most homologous, sharing approximately 60% similarity with RegX1. Based on the results from the comparative analysis, an alignment between the predicted amino acid sequence of RegX1 and the amino acid sequence from the six most similar RR proteins was compiled and is presented in Appendix 1. Further comparative sequence analysis was carried out on the protein to search for evidence of the role of RegX1. As discussed before (section 1.2.2), the N-terminus of a RR is the most conserved domain within the protein. However, there is a certain level of homology between the C-terminus domain of regulators that control the same adaptive responses. Consequently, a putative role for RegX1 was investigated by searching the sequence databases (GenEMBL and SwissProt) using the C-terminus domain alone (Appendix 1). However, the search failed to provide any evidence in support of a possible role for RegX1 and it only permitted the conclusion that RegX1 belonged to the OmpR/PhoB/PhoP subfamily of RR (section 3.1; Wren *et al.*, 1992).

In addition to the comparative sequence alignments, the predicted RegX1 protein was examined for the presence of any distinctive features using the MOTIFS computer program. However, the program was unable to identify any motif signature.

Fig.17. DNA sequence from *regX1* (*orf1*) and *orf2*. The amino acid sequence is shown on the line below the DNA sequence. Putative ribosome binding sites (RBS) are highlighted. The DNA sequence upstream *regX1* is presented in Appendix 3 (see section 3.8).

```
  5' TAAAAAATTTAAAATTTTACTAGAATTTTAATTTTAAAAATTCAAAAATATAATTTTAGC
2471 ---------+---------+---------+---------+---------+---------+ 2530
  3' ATTTTTTAAATTTTAAAATGATCTTAAAATTAAAATTTTTAAGTTTTTATATTAAAATCG

     AAAAATAAAATCAATAATTTATCAAATTTTAAATAAAATTATGTAAAGATTTTAAAGACT
2531 ---------+---------+---------+---------+---------+---------+ 2590
     TTTTTATTTTAGTTATTAAATAGTTTAAAATTTATTTTAATACATTTCTAAAATTTCTGA

                     RBS
     ACATAAAAATTTACAAGGACACTAGAATGATTAATGTGTTGATGATAGAAGATGATCCTG
2591 ---------+---------+---------+---------+---------+---------+ 2650
     TGTATTTTTAAATGTTCCTGTGATCTTACTAATTACACAACTACTATCTTCTACTAGGAC
                               M  I  N  V  L  M  I  E  D  D  P  D
                        RegX1-->

     ACTTTGCACAATTATTATCTGAATATTTAGCGCAATTTAATATTAAAATCACAAATTTTG
2651 ---------+---------+---------+---------+---------+---------+ 2710
     TGAAACGTGTTAATAATAGACTTATAAATCGCGTTAAATTATAATTTTAGTGTTTAAAAC
       F  A  Q  L  L  S  E  Y  L  A  Q  F  N  I  K  I  T  N  F  E

     AAAATCCTAAAAGTGCTTTAAATGTGGGTATTCAAGGCTATGATTGTCTGATTTTAGATT
2711 ---------+---------+---------+---------+---------+---------+ 2770
     TTTTAGGATTTTCACGAAATTTACACCCATAAGTTCCGATACTAACAGACTAAAATCTAA
       N  P  K  S  A  L  N  V  G  I  Q  G  Y  D  C  L  I  L  D  L

     TAACTTTACCTGGAATTGATGGTCTTGAAGTTTGTAGAGAAATCCGACAAAAAAGCAATA
2771 ---------+---------+---------+---------+---------+---------+ 2830
     ATTGAAATGGTCCTTAACTACCAGAACTTCAAACATCTCTTTAGGCTGTTTTTTCGTTAT
       T  L  P  G  I  D  G  L  E  V  C  R  E  I  R  Q  K  S  N  I

     TTCCTATTATCATCTCATCGGCTAGAGGTGACTTAAGCGATAAAGTTGTGGGGCTTCAAA
2831 ---------+---------+---------+---------+---------+---------+ 2890
     AAGGATAATAGTAGAGTAGCCGATCTCCACTGAATTCGCTATTTCAACACCCCGAAGTTT
       P  I  I  I  S  S  A  R  G  D  L  S  D  K  V  V  G  L  Q  I

     TCGGTGCTGATGATTATTTACCAAAGCCTTACGATCCAAAAGAAATGTATGCAAGGATTA
2891 ---------+---------+---------+---------+---------+---------+ 2950
     AGCCACGACTACTAATAAATGGTTTCGGAATGCTAGGTTTTCTTTACATACGTTCCTAAT
       G  A  D  D  Y  L  P  K  P  Y  D  P  K  E  M  Y  A  R  I  M
```

Fig.17. Cont.

```
     TGAGTCTTATTCGTCGCACAAAAAGAGTTGAACACACTAATAATGAAAATATTAACTCTG
2951 ---------+---------+---------+---------+---------+---------+ 3010
     ACTCAGAATAAGCAGCGTGTTTTTCTCAACTTGTGTGATTATTACTTTTATAATTGAGAC
       S  L  I  R  R  T  K  R  V  E  H  T  N  N  E  N  I  N  S  A

     CTTTCAAAATTGATGAAAGAAGACATGAGATCACCTATGAAGATAAGGTTTTAACACTTA
3011 ---------+---------+---------+---------+---------+---------+ 3070
     GAAAGTTTTAACTACTTTCTTCTGTACTCTAGTGGATACTTCTATTCCAAAATTGTGAAT
       F  K  I  D  E  R  R  H  E  I  T  Y  E  D  K  V  L  T  L  T

     CTCCTGCTGAATTTGAAATTTTAGAATACCTTATCCAACAACACGGATATAGTGTTTCAA
3071 ---------+---------+---------+---------+---------+---------+ 3130
     GAGGACGACTTAAACTTTAAAATCTTATGGAATAGGTTGTTGTGCCTATATCACAAAGTT
       P  A  E  F  E  I  L  E  Y  L  I  Q  Q  H  G  Y  S  V  S  R

     GAGAACAGCTTGTAAGTCGCTGTAAAAATCTAAAAGATAAAGATTCTAAAAGCCTTGATG
3131 ---------+---------+---------+---------+---------+---------+ 3190
     CTCTTGTCGAACATTCAGCGACATTTTTAGATTTTCTATTTCTAAGATTTTCGGAACTAC
       E  Q  L  V  S  R  C  K  N  L  K  D  K  D  S  K  S  L  D  V

     TTATCATCGGTCGCTTAAGGGTAAAAATAGGAGATAGTTCAAAATCTCCAAAACATATTT
3191 ---------+---------+---------+---------+---------+---------+ 3250
     AATAGTAGCCAGCGAATTCCCATTTTTATCCTCTATCAAGTTTTAGAGGTTTTGTATAAA
       I  I  G  R  L  R  V  K  I  G  D  S  S  K  S  P  K  H  I  F

                                    RBS
     TTTCTGTTAGAGGTATAGGATATAAACTGATAGGATGACAAAAAATTATTCTATTCATAC
3251 ---------+---------+---------+---------+---------+---------+ 3310
     AAAGACAATCTCCATATCCTATATTTGACTATCCTACTGTTTTTTAATAAGATAAGTATG
       S  V  R  G  I  G  Y  K  L  I  G  *

                                          M  T  K  N  Y  S  I  H  T
                                 Orf2-->

     CAAACTGATTATTTTATTTGTTGTTACTTTTTTCTTGGTTTGTGTGCTTTTTATCGTTCT
3311 ---------+---------+---------+---------+---------+---------+ 3370
     GTTTGACTAATAAAATAAACAACAATGAAAAAAGAACCAAACACACGAAAAATAGCAAGA
       K  L  I  I  L  F  V  V  T  F  F  L  V  C  V  L  F  I  V  L

     TTTAAAAATAGAAGGGAATACCTATAATGTAGAAGAAAGTTTAAAACAAGAAAATCTTAT
3371 ---------+---------+---------+---------+---------+---------+ 3430
     AAATTTTTATCTTCCCTTATGGATATTACTTCTTCTTTCAAATTTTGTTCTTTTAGAATA
       L  K  I  E  G  N  T  Y  N  E  E  E  S  L  K  Q  E  N  L  I
```

Fig.17. Cont.

```
     AAAAAATTTACTCATCTCCTATGAAAACACTTCAGGTGTTGAAATAGGAGCTTATCTTGG
3431 ---------+---------+---------+---------+---------+---------+ 3490
     TTTTTTAAATGAGTAGAGGATACTTTTGTGAAGTCCACAACTTTATCCTCGAATAGAACC
      K  N  L  L  I  S  Y  E  N  T  S  G  V  E  I  G  A  Y  L  G

     AAACAGCGGTTTTAACGCTATACAAAATCCCAATCTTGTAAAAGCTATAAGAAATAATGG
3491 ---------+---------+---------+---------+---------+---------+ 3550
     TTTGTCGCCAAAATTGCGATATGTTTTAGGGTTAGAACATTTTCGATATTCTTTATTACC
      N  S  G  F  N  A  I  Q  N  P  N  L  V  K  A  I  R  N  N  G

     ACAATCACTTTTTAAAGCAGGCGGAGAGCTTTGTACTCTTTCTTCTTTAAAATATCATAG
3551 ---------+---------+---------+---------+---------+---------+ 3610
     TGTTAGTGAAAAATTTCGTCCGCCTCTCGAAACATGAGAAAGAAGAAATTTTATAGTATC
      Q  S  L  F  K  A  G  G  E  L  C  T  L  S  S  L  K  Y  H  S

     CAATCTTTATTTTGATGTTCAATGTAAAGATTTTGATGGCTTGTATGAAGAAAATACTAG
3611 ---------+---------+---------+---------+---------+---------+ 3670
     GTTAGAAATAAAACTACAAGTTACATTTCTAAAACTACCGAACATACTTCTTTTATGATC
      N  L  Y  F  D  V  Q  C  K  D  F  D  G  L  Y  E  E  N  T  S

     TGATAGGGTTTATAATCTTTTATTGATAGGATTTTTCTCTTTTTCTTTGTTGGTTGTTTT
3671 ---------+---------+---------+---------+---------+---------+ 3730
     ACTATCCCAAATATTAGAAAATAACTATCCTAAAAAGAGAAAAAGAAACAACCAACAAAA
      D  R  V  Y  N  L  L  L  I  G  F  F  S  F  S  L  L  V  V  F

     TATGTATTTTTCTGTATTAAAATCCTTAGAACCTTTAAAAAAATTAAGAAGACAAGTTGC
3731 ---------+---------+---------+---------+---------+---------+ 3790
     ATACATAAAAAGACATAATTTTAGGAATCTTGGAAATTTTTTTAATTCTTCTGTTCAACG
      M  Y  F  S  V  L  K  S  L  E  P  L  K  K  L  R  R  Q  V  A

     CGAAGTTGCAAATGGGGAACAACCTGATTTTCTAGACTATCAAGAAGATGAAGTAGGCAA
3791 ---------+---------+---------+---------+---------+---------+ 3850
     GCTTCAACGTTTACCCCTTGTTGGACTAAAAGATCTGATAGTTCTTCTACTTCATCCGTT
      E  V  A  N  G  E  Q  P  D  F  L  D  Y  Q  E  D  E  V  G  K

     GA
3851 ---------+-- 3852
     CT
```

3.7.3.2 Sequence analysis of *orf2*. The 1.5kb DNA fragment also contained the first 570bp of a second *orf*, encoding the N-terminal 190 amino acids of the protein Orf2 deduced from the nucleic acid sequence. An examination of the predicted amino acid sequence revealed the presence of two regions containing a large patch of hydrophobic amino acids (between amino acids 11 and 32; between amino acids 135 and 174), long enough to traverse the membrane (the minimum being 20 amino acids) and thus, forming possible transmembrane domains. The analysis of Orf2 using the computer program TMpred that specifically looked for transmembrane domains (Hofmann & Stoffel, 1993) indicated the presence of two hydrophobic peaks corresponding to the amino acid sequences of these two regions. The results are shown in Fig.18 and strongly suggest the presence of two transmembrane domains located in the stretches of hydrophobic residues previously identified. The program was tested by using RegX1 as a control. RegX1 was checked to determine whether the program would identify any possible transmembrane regions but the analysis showed clearly that the RR protein did not contain any (Fig.19). Therefore, RegX1 is probably located in the cytosol like all the RR proteins identified to date (section 1.2).

Following the structural analysis, the predicted amino acid sequence was used to search through GenEMBL and SwissProt databases using FASTA (Pearson & Lipman, 1988) and BLASTP (Altschul *et al.*, 1990). The search showed that Orf2 presented approximately 25% identity to two proteins: a sensor protein isolated from *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and a sensory transduction histidine kinase isolated from *Synechocystis sp* (unpublished) (Appendix 2). Thus, it is possible that Orf2 may be the partner HK for RegX1.

3.8 Sequencing the 2.3kb region 5' to *regX1*

In order to identify which genes laid upstream of *regX1* and whether they would be of importance for this study, the 2.3kb region 5' to *regX1* was sequenced on one strand.

3.8.1 Preparing a library of nested deletions from the 2.3kb region

pλRB DNA prepared by ion-exchange chromatography (Qiagen; section 2.7.2.1) was used to prepare a library of nested deletions of double-stranded DNA (section 2.7.17),

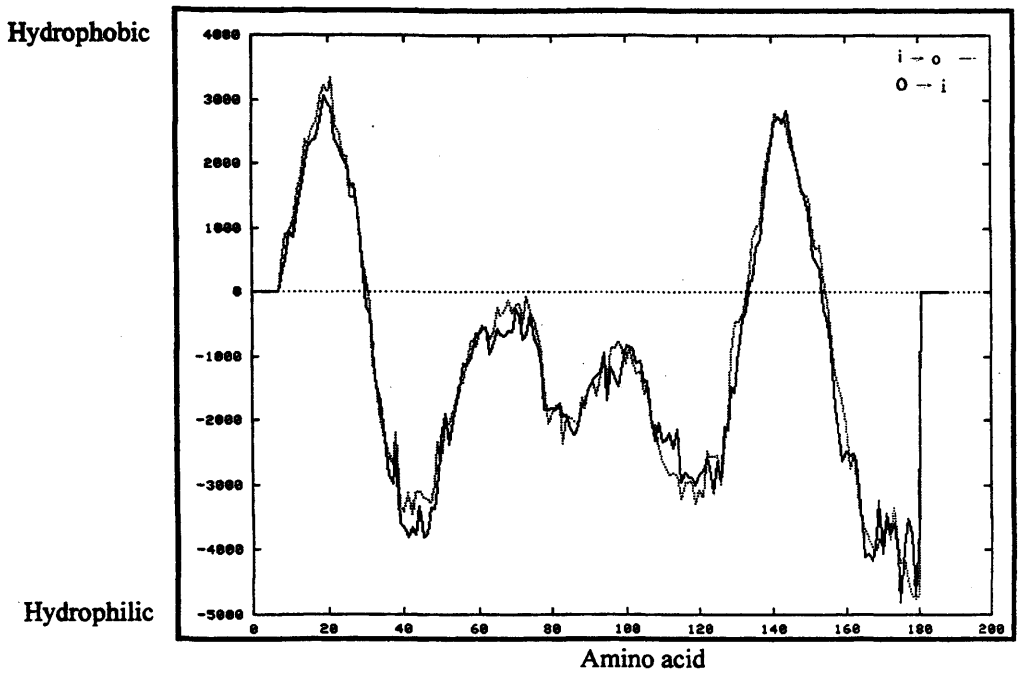


Fig.18. TMpred for the predicted Orf2. TMpred program makes a prediction of membrane-spanning regions and their orientation (inside to outside helices: i→o or outside to inside helices: o→i). Based on this prediction, Orf2 appears to contain two transmembrane helices, between amino acids 11-32 and 135-174. The preferred orientation is the N-terminus outside.

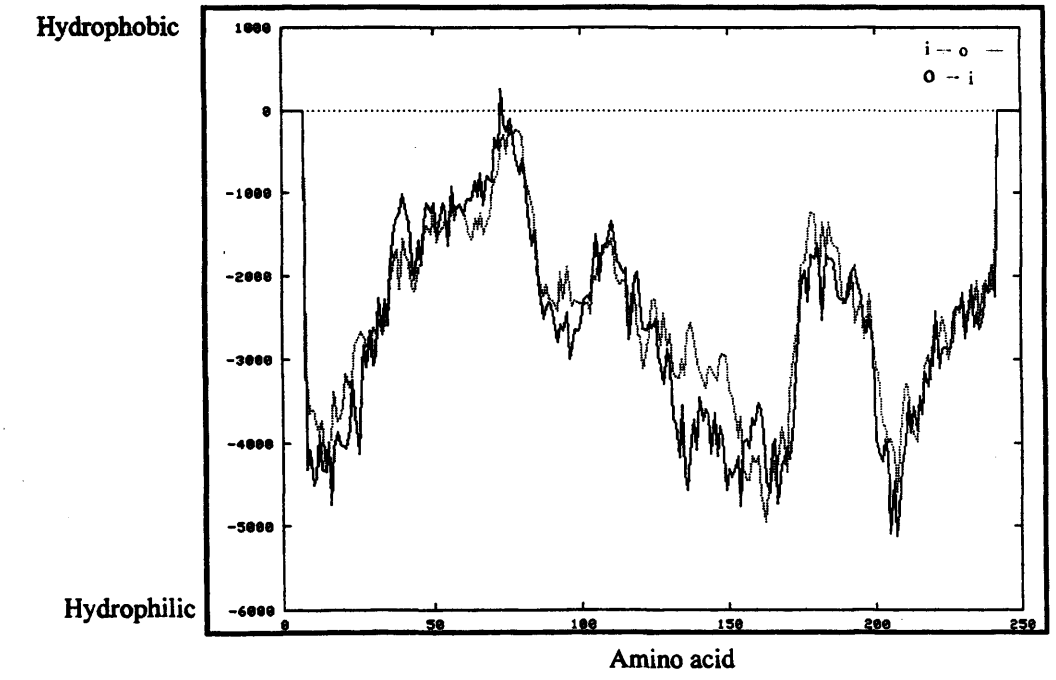


Fig.19. TMpred for the predicted RegX1. Based on the prediction by TMpred, RegX1 does not contain any transmembrane helices. Like all RRs known to date, RegX1 is a cytoplasmic protein.

according to the method developed by Henikoff (1984). The procedure was the same as described in section 3.7.2 (Fig.14) except for the following differences. pλRB was restricted with *Eco*RI (site A in Fig.14) and *Sst*I (site B in Fig.14). The DNA sample was treated with Exonuclease III for a total of 50min, with samples being taken at 5min intervals. Samples corresponding to time points between 5-40min were circularised and transformed into *E. coli* DH5α competent cells by electroporation. For each time point, plasmid DNA was prepared (section 2.7.3) from six ampicillin resistant colonies selected randomly and restricted with *Pvu*II. The digests were separated on a 0.8% agarose gel and the insert size determined for each subclone. A series of subclones with a difference of 200-300bp in length were selected and plasmid DNA prepared by ion-exchange chromatography (Qiagen; section 2.7.2.1).

3.8.2 Sequencing the 2.3kb region

The sequencing reactions were performed as described previously, using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (section 2.7.18). pλRB subclones were used to sequence progressively into the pλRB insert, using the M13 reverse sequencing primer (section 2.2.2). Samples were sent in duplicate to be sequenced using the automated sequencing services provided by the University of Leicester. Usually, 500-600bp of reliable DNA sequence were obtained. The sequence data was studied using the Wisconsin Package, (Version 8, September 1994) via the Genetics Computer Group and the results are presented in Appendix 3.

Two open reading frames, Orf3 and Orf4, were identified. Searches through the protein sequence databases GenEMBL and SwissProt, using FASTA (Pearson & Lipman, 1988) and BLASTP (Altschul *et al.*, 1990) revealed that Orf3 showed high homology to the heat-shock protein DnaJ from a variety of bacterial species and yeast, containing 45 to 50% identical amino acids and approximately 60% similarity. A sequence alignment between Orf3 predicted amino acid sequence and the sequence from the five most similar DnaJ proteins was compiled and is presented in Appendix 3. As to Orf4, no significant homology to any other protein available in the databases searched was found. None of the genes were considered to relate to the objectives of this project (Part II) and no further studies were carried out. A schematic representation of the orientation of all the open reading frames present in pλRB is shown in Fig.20.

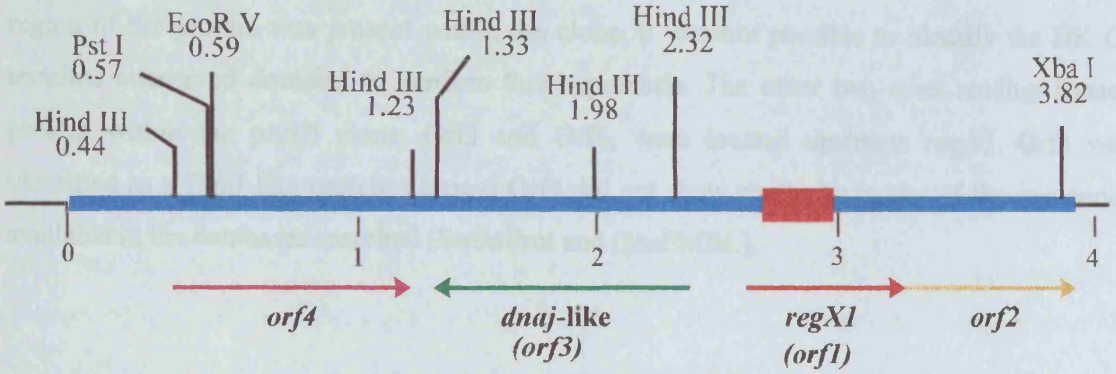


Fig.20. Diagram showing the organisation of the open reading frames identified in pλRB. The red box represents the PCRDOP fragment. The arrows show the Orfs found in the insert of pλRB. The restriction enzymes positions are in kb.

3.9 Summary

A *C. jejuni* genomic library was probed for the presence of RR proteins, using a gene fragment (*regX1*) with sequence homology to genes encoding RR proteins of the OmpR subfamily. Following the library screen, one positive clone carrying a 3.8kb insert was isolated and named pλRB. The entire DNA sequence of pλRB clone was determined, allowing the identification of four open reading frames. Orf1 and Orf2 were orientated in the same direction and the end of the first overlapped the beginning of the second. This suggested that the two genes could be part of the same operon. Sequence analysis established that *orf1* corresponded to the complete *regX1* gene. It was demonstrated that RegX1 belonged to the family of RR proteins but no similarities high enough to suggest a role were noted. Sequence analysis of Orf2 suggested that this peptide could correspond to the RegX1 partner HK. As only the N-terminal region of the protein was present within the clone, it was not possible to identify the HK C-terminal conserved domains to confirm this hypothesis. The other two open reading frames present within the pλRB clone, Orf3 and Orf4, were located upstream *regX1*. Orf3 was identified as a DnaJ-like protein whereas Orf4 did not show similarity to any of the sequences available in the databases searched (SwissProt and GenEMBL).

CHAPTER 4

Isolating a *regX1* Mutant

4.1 Introduction

In order to characterise the *regX1* gene, it was necessary to isolate a defined mutant to investigate the role of the gene in *C. jejuni*. It had been established previously that the predicted amino acid sequence of *regX1* showed the structural features of a response regulatory protein. However, further comparative sequence analysis had failed to provide significant clues to a specific role in the bacterial cell. Therefore, to elucidate the specific role of *regX1* in *C. jejuni*, a mutant was isolated by inactivating the *regX1* gene in the bacterial chromosome.

The strategy for the construction of a *regX1* mutant involved two stages. Firstly, a defined mutation was engineered by inverse PCR (IPCR). Specific primers were designed to construct the mutation at the region thought to correspond to the catalytic site thus, causing the permanent inactivation of the mutated RegX1 protein. Secondly, after constructing the mutation in *regX1* in *E. coli*, the construct containing the modified fragment was introduced into *C. jejuni* to allow homologous recombination to occur between the chromosomal and the mutated copies of the gene. Initially, the modification was introduced in the *regX1* PCRDOP fragment and the modified fragment was introduced into *C. jejuni*, using two different vectors: a conjugative vector and a suicide vector. Later, the whole *regX1* gene was isolated from the library clone pλRB and used as template to IPCR. The modified gene was subsequently transferred into *C. jejuni* by electroporation and the mutation was transferred into the chromosomal wild type copy of the gene by homologous recombination.

4.2 Inverse PCR mutagenesis of pJMK39: construction of pJMK44

In a first step to isolate a *regX1* mutant, the *regX1* PCRDOP fragment was modified in the region containing the catalytic site of a response regulator, the Asp-51 (D-51, corresponding to D-57 of CheY; see section 1.2.2). The modification was introduced by inverse PCR (Wren *et al.*, 1994), with the oligonucleotide primers R1 and R2 (section 2.2.2; Appendix 4) and using

pJMK39 as the DNA template (Appendix 4). R1 and R2 were designed so the 3' region annealed to different strands in the middle of the *regX1* fragment and extended in opposite directions, amplifying the flanking vector sequences. The 5' region of each primer overlapped and carried a mutation comprising the introduction of a unique *Bgl*II restriction site, two stop codons and a 5bp deletion (Fig.21).

Initially, to test the primers and optimise the PCR conditions, a 10µl pilot reaction was prepared (section 2.7.15.1). The PCR products were analysed on a 0.8% agarose gel and the results showed a single band of about 3kb, the size of pJMK39. The PCR was then prepared in a larger scale of 100µl of final volume, using 0.003µg of pJMK39 DNA and 100 pmoles of each primer R1 and R2; all the other reagents were scaled up. The reaction was programmed as described in section 2.7.15.1, using 60°C for 1min as annealing conditions and 72°C for 0.5min for the extension. After the PCR, 10µl of the total reaction volume were analysed on a 0.8% agarose gel to confirm whether the reaction had worked and the remaining PCR product was purified by ion-change chromatography (Qiagen; section 2.7.16). In order to circularise the IPCR-modified pJMK39, the linear product was restricted with *Bgl*II overnight to create cohesive ends (section 2.7.7). The restricted product was phenol/chloroform extracted and ethanol precipitated (section 2.7.4). The restricted DNA was circularised with DNA ligase (section 2.7.10) and following ethanol precipitation, was transformed into *E. coli* DH5α competent cells by electroporation (section 2.7.11). The transformants were selected on ampicillin containing plates. Plasmid DNA was isolated from six colonies (section 2.7.3) and restricted with *Bgl*II. The digests were separated on a 0.8% agarose gel. All six colonies possessed the correct size plasmid containing a unique *Bgl*II restriction site (data not shown). One of the clones was selected randomly and it was named pJMK44 (Appendix 4). In order to verify whether the IPCRM of pJMK39 occurred as initially planned, with the introduction of the two stop codons, a 5bp deletion and a unique *Bgl*II restriction site, pJMK44 plasmid DNA was prepared by ion-exchange chromatography and the insert DNA was sequenced, using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (section 2.7.18). The sequence data from pJMK44 was analysed using the Program Manual for the Wisconsin Package (Version 8, September 1994), via the Genetics Computer Group and it confirmed that the *regX1* fragment was modified as expected. The isolation of the pJMK44 was performed during a supervised third year project by Mr Andrew Wall.

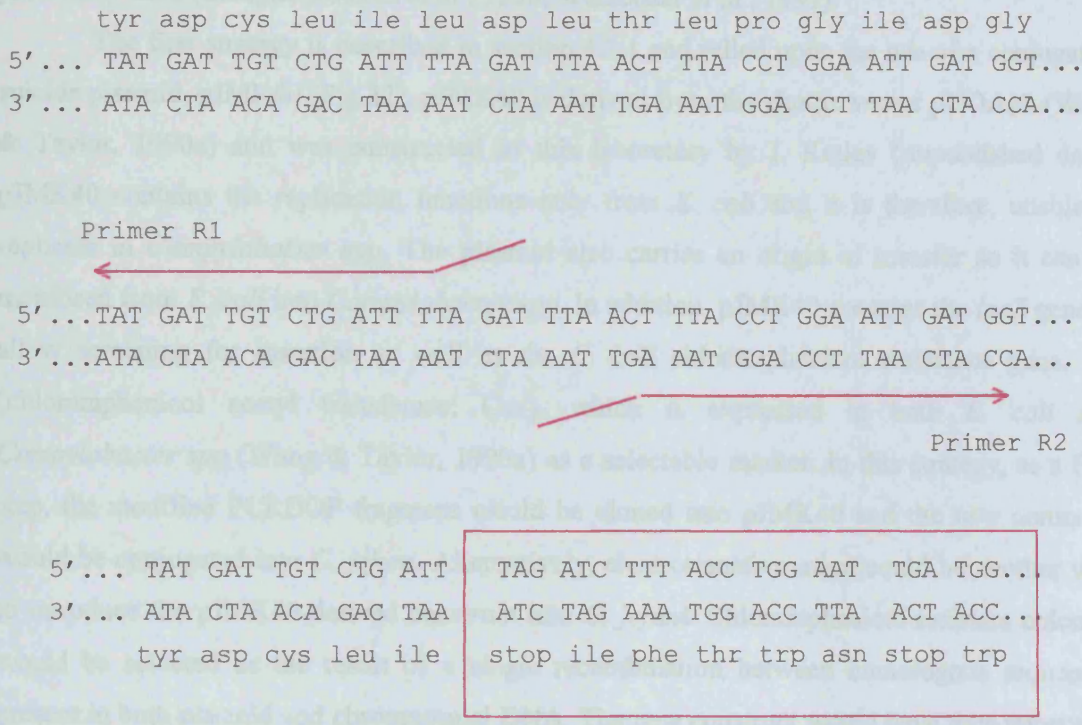


Fig.21. Diagram showing the DNA and amino acid sequence modifications introduced by IPCRM. *regX1* sequence equivalent to the PCRDOP fragment (in pJMK39 or pALB3) was used as template in IPCR, using R1 and R2 oligonucleotide primers. R1 and R2 were designed so the 3' region annealed to different strands in the middle of the *regX1* fragment and extended in opposite directions, amplifying the flanking vector sequences. The 5' region of each primer overlapped and carried the mutation that resulted in the introduction of a unique *Bgl*II restriction site, two stop codons and a 5bp deletion. The modified sequence is shown boxed on the figure.

4.3 Gene replacement mutagenesis of *C. jejuni*: ways of selecting a *regX1* mutant

The mutation introduced by IPCRM was not directly selectable as the *regX1* mutant phenotype was not known. Therefore, it was necessary to create a selective environment in which to isolate a *regX1* mutant. This was achieved using two different strategies, both previously used (Labigne-Roussel *et al.*, 1988; Wassenaar *et al.*, 1991).

The first strategy is described in section 4.3.1 and relied upon the use of a conjugative suicide plasmid, pJMK40 (Fig.22). pJMK40 is derived from the shuttle vector pUOA18 (Wang & Taylor, 1990a) and was constructed in this laboratory by J. Ketley (unpublished data). pJMK40 contains the replication functions only from *E. coli* and it is therefore, unable to replicate in *Campylobacter spp.* The plasmid also carries an origin of transfer so it can be mobilised from *E. coli* into *Campylobacter spp.* In addition, pJMK40 contains the *lacZ* gene to allow screening for insertion as well as the *C. coli* chloramphenicol resistance gene, *cat* (chloramphenicol acetyl transferase; Cm^r), which is expressed in both *E. coli* and *Campylobacter spp* (Wang & Taylor, 1990a) as a selectable marker. In this strategy, as a first step, the modified PCRDOP fragment would be cloned into pJMK40 and the new construct would be conjugated into *C. jejuni.* Alternatively, electrotransformation could be another way to introduce the pJMK40-derived construct into *C. jejuni.* Chloramphenicol resistant colonies would be selected as the result of a single recombination between homologous sequences present in both plasmid and chromosomal DNA. The new construct would have thus integrated in the bacterial chromosome. Given the duplication of sequences, this integration would probably create instability and a second recombination would be expected. The plasmid could cross out either carrying the original modified insert or the homologous wild type chromosomal sequence. Thus, all chloramphenicol resistant colonies would have to undergo a second screen for loss of resistance, the chances being that approximately 50% would be *regX1* mutants as a result of the mutation being in the centre. Southern blot on chromosomal DNA would establish whether an extra *Bgl*II site would be present in the *regX1* gene, establishing which of the transconjugants were *regX1* mutants.

The alternative strategy (section 4.3.2) used the pJMK44 as a suicide vector to transfer the mutation into *C. jejuni* chromosome. pJMK44 is a pUC19-derived construct and it cannot replicate in *Campylobacter spp.* Therefore, following the expected recombination between homologous sequences present in the plasmid and chromosomal DNA, the plasmid would be eventually lost, i.e., it would suicide. In order to provide this approach with a way to select for recombinants, the kanamycin resistance gene, *aphA-3* (aminoglycoside phosphotransferase; Km^r) would be cloned into pJMK44, using the unique *Bgl*II restiction site introduced by

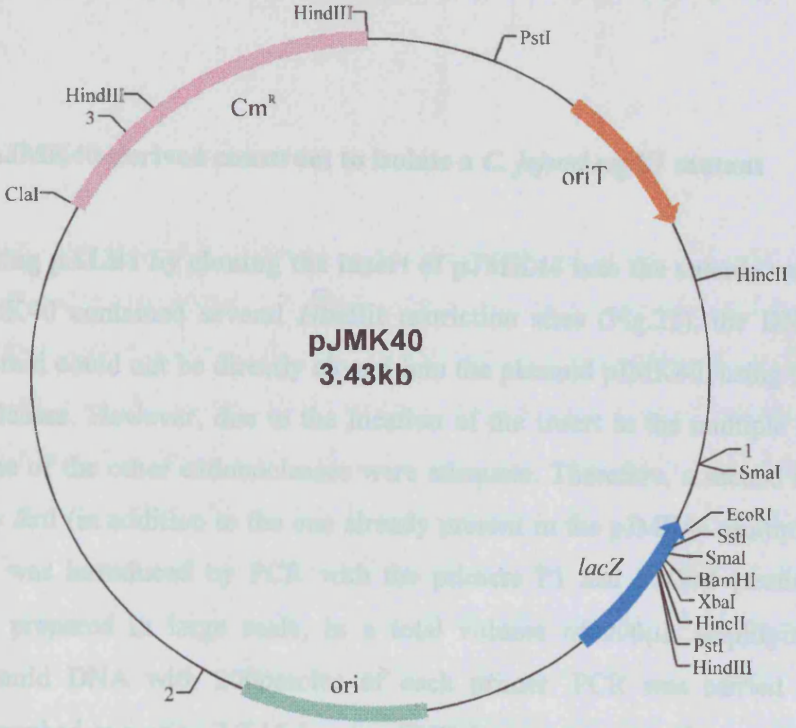


Fig.22. Map of the plasmid pJMK40. pJMK40 is a conjugative suicide plasmid derived from pUOA18 (Wang & Taylor, 1990a). OriT corresponds to the origin of transfer; ori corresponds to origin of replication.

IPCRM. *aphA-3* was first isolated from *C. coli* and is expressed in both *E. coli* and *Campylobacter spp* (Trieu-Cuot *et al.*, 1985). The new construct would be transferred into *C. jejuni* by electrotransformation. Kanamycin resistant colonies would presumably result from a double recombination event between the pJMK44 and the chromosomal *regX1* sequences. The putative mutants would be analysed by PCR with primers specifically annealing to the *aphA-3* gene; southern blotting would also confirm whether the transformants would be *regX1* mutants.

4.3.1 Using pJMK40-derived construct to isolate a *C. jejuni regX1* mutant

4.3.1.1 Isolating pALB1 by cloning the insert of pJMK44 into the suicide vector pJMK40. Because pJMK40 contained several *Hin*dIII restriction sites (Fig.22), the DNA insert from pJMK44 plasmid could not be directly cloned into the plasmid pJMK40, using the *Hin*dIII and *Pst*I endonucleases. However, due to the location of the insert in the multiple cloning site of pJMK44, none of the other endonucleases were adequate. Therefore, a second restriction site, recognised by *Sst*I (in addition to the one already present in the pJMK44 multiple cloning site; Appendix 4) was introduced by PCR with the primers P1 and P2/*Sst*I (section 2.2.2). The reaction was prepared in large scale, in a total volume of 200µl, amplifying 0.006µg of pJMK44 plasmid DNA with 200pmoles of each primer. PCR was carried out under the conditions described in section 2.7.15.1, using 60°C for 1min as annealing conditions and 72°C for 0.5min for the extension. Negative and positive controls were included, the former corresponding to a 10µl reaction with no template DNA and the latter corresponding to a 10µl reaction using 0.001µg of pJMK39 plasmid as template DNA. The PCR products were purified by ion-exchange chromatography (Qiagen; section 2.7.16) and resuspended in distilled water. An aliquot of the purified DNA was analysed in a 1.2% agarose gel, along with the non-purified controls. Like the positive control, only one DNA band of approximately 300bp corresponding to the insert of pJMK39/pJMK44 had been amplified (data not shown). The DNA fragment was digested overnight with *Sst*I (section 2.7.7). After a phenol/chloroform extraction to remove the enzyme, the DNA was ethanol precipitated. In parallel, the vector DNA, pJMK40, was restricted with *Sst*I. An aliquot of the digested plasmid was analysed on a 0.8% agarose gel to determine whether the restriction was complete. The enzyme was removed by phenol/chloroform extraction and the DNA was ethanol precipitated. The insert DNA was ligated to vector DNA as described before (section 2.7.10) and following ethanol precipitation, half of the ligation was transformed into competent *E. coli* XL1-Blue cells by electroporation

(section 2.7.11). Transformants were selected on X-gal/IPTG containing plates, supplemented with chloramphenicol. Six white chloramphenicol resistant colonies were randomly selected for further analysis. Plasmid DNA was prepared in small scale (section 2.7.3) from these transformants and tested by restriction digestion with *Sst*I. The digests were separated on a 1.2% agarose gel, generating two bands with the expected sizes of 4.5kb and 300bp, proving the introduction of an *Sst*I restriction site downstream the *Hin*dIII site and cloning of the 300bp pJMK44 insert into pJMK40 (data not shown). The new plasmid construct was named pALB1 (Appendix 4).

4.3.1.2 Conjugation of pALB1 from *E. coli* SM10 into *C. jejuni* P71 and *C. jejuni* O81. Prior to conjugating the new construct into *C. jejuni*, pALB1 was transformed into *E. coli* strain SM10 by electroporation (section 2.7.11). The transformants were selected on chloramphenicol containing plates. Four chloramphenicol resistant colonies were selected randomly and plasmid DNA was prepared in a small scale. The plasmids were cleaved with *Sst*I and the digests separated on a 1.2% agarose gel, along with *Sst*I restricted pALB1. In all cases, the restriction pattern obtained was the same as the one obtained with pALB1, confirming that the plasmid had been successfully transformed into *E. coli* SM10 (data not shown). In parallel, the shuttle vector pUOA15 (section 2.2.1; (Wang & Taylor, 1990b)) was utilised as a positive control for the conjugation. pUOA15 contains a tetracycline resistance gene that can be expressed in both species (and an ampicillin resistance gene that is only expressed in *E. coli*), *E. coli* and *Campylobacter* replication functions and an origin of transfer. Thus, prior to utilisation as a control, pUOA15 was electrotransformed into *E. coli* strain SM10 (section 2.7.11).

As for the recipient *C. jejuni* strain, there were only two strains that had been successfully used for conjugation in the laboratory, P71 and O81 (section 2.2, Part I; K. Wooldridge, personal communication). Both strains were used in parallel and pALB1 was conjugated from *E. coli* SM10 cells into *C. jejuni* P71 and O81 cells.

The donor and the recipient cells were prepared for conjugation (section 2.7.14.1). Conjugation was set up by resuspending the recipient cells, P71/O81, in 500µl of donor cells culture grown to an $O.D_{600nm}$ of 0.67 for SM10 (pALB1) and 0.5 for SM10 (pUOA15) (section 2.7.14.2). The *E. coli-C. jejuni* mixtures were pelleted, resuspended in 100µl of MH broth and placed onto sterile filters on agar plates without selection. The plates were incubated overnight at 42°C in a microaerophilic atmosphere. Following incubation, the filters were gently washed with 1ml of prewarmed MH broth, centrifuged and plated onto MH agar supplemented with

chloramphenicol (pALB1) or tetracycline (pUOA15). Polymyxin B and trimethoprim were also included to eliminate the *E. coli* SM10 donor cells. The plates were incubated at 42°C in a microaerophilic atmosphere. The results obtained showed that the pUOA15 was transferred into both recipient strains, with more efficient transfer into strain P71 (around 10^3 cfu/ml) than into strain O81 (around 10^2 cfu/ml). However, even after four days incubation, no chloramphenicol resistant conjugants were obtained. The conjugation of pALB1 into *C. jejuni* was not repeated.

4.3.1.3 Electrotransformation of pALB1 into *C. jejuni* 81116. Transformation of pALB1 plasmid into *C. jejuni* 81116 cells by electroporation was attempted several times. 81116 competent cells were always freshly prepared from MH agar plates incubated for 18h at 42°C in a microaerophilic atmosphere (section 2.7.12.1). 50µl of competent 81116 cells were electrotransformed with 4, 6.5 and 10µg of pALB1 plasmid DNA (section 2.7.12.2). 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 pUOA15, respectively. After electroporation, the cells were resuspended in 100µl of SOB medium and plated immediately onto MH agar plates. All plates were incubated overnight at 42°C in a microaerophilic atmosphere. The initial viability of the competent cells was determined as ranging from 10^8 to 10^{10}cfu/ml. The percentage of killing was calculated as approximately 10%. Following recovery, all plates usually contained confluent growth and which was carefully washed off with SOB or SOC medium. The bacterial suspensions were centrifuged, resuspended in the same medium and plated onto MH agar plates supplemented with the appropriate antibiotic selection. The plates were incubated at 42°C in a microaerophilic atmosphere. After five days, there were still no chloramphenicol resistant colonies on the plates, including the positive control. Therefore, this approach was not continued.

4.3.2 Using pJMK44-derived construct to isolate a *C. jejuni regX1* mutant

4.3.2.1 Isolating pALB2 by cloning *aphA-3* gene into pJMK44. The *aphA-3* (*C. coli*-derived kanamycin resistance gene; Trieu-Cuot *et al.*, 1985) gene was obtained from pJMK30. pJMK30 is a pUC19 derivative, containing the kanamycin resistance gene cloned as a cassette, with a

pUC19-derived multiple cloning site on each flank (Fig.23). pJMK30 DNA, isolated by ion-exchange chromatography (Qiagen; section 2.7.2.1), was used as template in a PCR (section 2.7.15.1) with vector primers P1 and P2 (section 2.2.2) to isolate the kanamycin cassette. The reaction was prepared in large scale, in a total volume of 200µl. 0.006µg of pJMK30 plasmid DNA were amplified with 200pmoles of each primer. PCR was performed by using 60°C for 1min as annealing conditions and 72°C for 0.5min for the extension. A negative control was included consisting of a 10µl reaction with no template DNA. The PCR products were purified by ion-exchange chromatography (Qiagen; section 2.7.16) and resuspended in distilled water. An aliquot of the purified DNA was analysed on a 0.8% agarose gel, showing one band of approximately 1.4kb, the size of the kanamycin cassette (data not shown). The DNA was restricted overnight with *Bam*H1 (section 2.7.7). After phenol/chloroform extraction of the enzyme, the DNA was ethanol precipitated.

pJMK44 DNA, isolated by ion-exchange chromatography (section 2.7.2.1), was restricted with *Bgl*II (section 2.7.7), which cleaves the plasmid in the insert (Appendix 4). An aliquot of the digested plasmid was analysed on a 0.8% agarose gel to determine whether the restriction was complete. The endonuclease was extracted by phenol/chloroform treatment and the DNA ethanol-precipitated.

The *Bam*H1-cleaved kanamycin cassette was ligated to *Bgl*II cleaved pJMK44 (section 2.7.10). The ligation was ethanol precipitated and half of it was transformed into competent *E. coli* XL1-Blue cells by electroporation (section 2.7.11). Transformants were selected on plates supplemented with ampicillin and kanamycin. Plasmid DNA was isolated in small scale (section 2.7.3) from twelve randomly selected ampicillin and kanamycin resistant colonies and characterised with a double restriction digestion with *Pst*I and *Sst*I (section 2.7.7). The digests were separated in a 1% agarose gel, generating two bands with the expected sizes of approximately 3 and 1.4kb (data not shown). The new plasmid construct was named pALB2 (Appendix 4).

4.3.2.2 Electrotransformation of pALB2 into *C. jejuni* 81116. As an alternative approach to mutate *C. jejuni regX1*, pALB2 plasmid was used as the delivery vector and transformed into *C. jejuni* 81116 cells by electroporation. 81116 competent cells were always freshly prepared using the protocol described in section 2.7.12.1. 50µl of competent 81116 cells were electrotransformed with pALB2 DNA (section 2.7.12.2). Different quantities of plasmid were electroporated. As electroporation negative and positive controls, 50µl of competent 81116

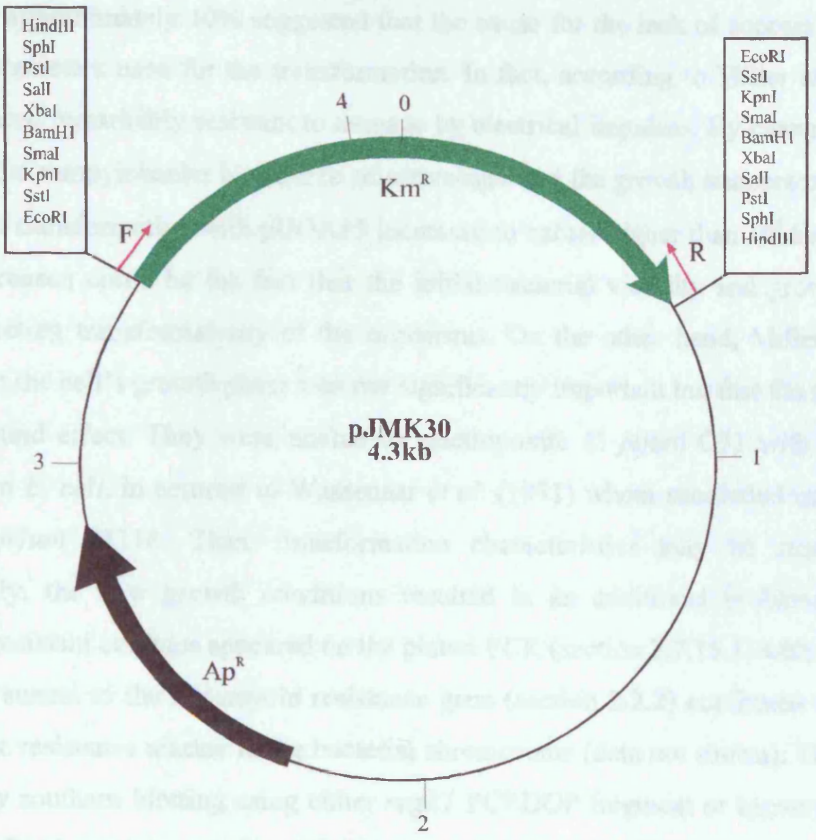


Fig.23. Map of the plasmid pJMK30. pJMK30 is a pUC19 derivative, containing *C. coli* kanamycin resistance gene cloned as a cassette. F and R are primers designed to specifically anneal to Km^R cassette.

cells were electroporated in the absence of DNA and in the presence of 1μg of pUOA15, respectively. Initially, electrocompetent 81116 cells were prepared from MH agar plates incubated for 18h at 42°C in a microaerophilic atmosphere. Under these conditions, previously found adequate for transformation of *C. jejuni* (Miller *et al.*, 1988), the positive control appeared not to transform. The fact that the percentage of killing during electroporation was found to be approximately 10% suggested that the cause for the lack of success was not likely to be the parameters used for the transformation. In fact, according to Miller *et al.* (1988), *C. jejuni* appeared remarkably resistant to damage by electrical impulses. By changing the growth medium to the campylobacter blood-free selective agar and the growth temperature to 37°C, the efficiency of transformation with pUOA15 increased to values higher than 10^5 transformants/μg DNA. The reason could be the fact that the initial bacterial viability and growth stage were altered, affecting transformability of the organisms. On the other hand, Miller *et al.* (1988) reported that the cell's growth phase was not significantly important but that the source of DNA had a profound effect. They were unable to electroporate *C. jejuni* C31 with plasmid DNA isolated from *E. coli*, in contrast to Wassenaar *et al.* (1991) whom succeeded using a different strain, *C. jejuni* 81116. Thus, transformation characteristics may be strain dependent. Unfortunately, the new growth conditions resulted in an additional problem: non-specific kanamycin resistant colonies appeared on the plates. PCR (section 2.7.15.1) with primers which specifically anneal to the kanamycin resistance gene (section 2.2.2) confirmed the absence of the antibiotic resistance marker in the bacterial chromosome (data not shown). This was further supported by southern blotting using either *regX1* PCRDOP fragment or kanamycin resistance cassette as DNA probes (section 2.7.20; data not shown). This problem was eventually eliminated by increasing the final concentration of the antibiotic on the selection plates from 30μg/ml (Wassenaar *et al.*, 1991) to 50μg/ml. Nevertheless, *regX1* mutants were never isolated, even following post-electroporation incubation periods for up to a week.

4.4 Mutagenesis of *C. jejuni*: a new mutagenesis vector

In order to increase the probability of a recombination event to occur between the homologous sequences present in both plasmid vector and chromosome, it was decided to use a new plasmid containing larger flanking sequences on both sides of the mutation. The new plasmid was constructed by subcloning a fragment from pλRB's insert into the plasmid vector pUC19 (Appendix 4). pλRB was restricted with *Xba*I and *Hin*dIII (section 2.7.7). The digests

were separated on a 0.8% agarose gel and the 1.5kb DNA band was purified using Qiaex (section 2.7.8.1). pUC19 was also restricted with the endonucleases *Xba*I and *Hin*dIII and purified from a 0.8% agarose gel using Qiaex (section 2.7.8.1). The 1.5kb DNA insert was ligated to pUC18 (section 2.7.10) and the ligation was transformed into *E. coli* XL1-Blue by electroporation (section 2.7.11). Plasmid DNA was prepared (section 2.7.3) from six randomly selected ampicillin resistant colonies and characterised by restriction analysis. All clones were found to contain the correct construct (data not shown). The new plasmid was named pALB3.

pALB3, like pJMK39, is a pUC19 derivative and it cannot replicate in *Campylobacter spp.* Therefore, pALB3 could be used as a mutagenesis suicide vector. In addition, it contains a 1.5kb *C. jejuni* DNA fragment, including the whole *regX1* gene and flanking sequences. Therefore, recombination between the copy of the gene present in the plasmid and in the chromosomal DNA was expected to be more likely.

Gene inactivation remained as the strategy to employ to make a *regX1* mutant. As before, the mutation would be constructed in two stages. Firstly, the same defined mutation (section 4.2) consisting of a unique *Bgl*II restriction site, two stop codons and a 5bp deletion, would be engineered by IPCR with the R1 and R2 oligonucleotide primers and pALB3 as the DNA template. The kanamycin resistance gene, *aphA-3*, isolated from pJMK30 plasmid, would be subcloned into the unique *Bgl*II restriction site and the new construct would be transformed into *C. jejuni* 81116 by electroporation.

4.4.1 IPCRM of pALB3: construction of pALB4

Initially, a defined mutation consisting of a unique *Bgl*II restriction site, two stop codons and a 5bp deletion, was engineered by IPCRM (Fig.21). A 10µl pilot PCR was prepared (section 2.7.15.1). The PCR products were analysed on a 0.8% agarose gel, showing that a unique band of about 4.5kb, the size of pALB3, had been amplified (data not shown). The PCR was then prepared in a larger scale of 100µl of final volume, using 0.003µg of pALB3 DNA and 100pmoles of each primer, R1 and R2; the rest of the reagents were scaled up. The reaction was programmed as described before (section 2.7.15.1), using 60°C for 1min as the annealing parameters and 72°C for 6min as extension parameters. After PCR, 10µl of the total reaction volume were analysed on a 0.8% agarose gel to confirm whether the reaction had worked and the remaining PCR product was purified by ion-change chromatography (Qiagen; section 2.7.16). In order to circularise the IPCRM-modified pALB3, the linear product was restricted

with *Bgl*II overnight to create the cohesive ends (section 2.7.7). The restricted DNA was phenol/chloroform extracted and ethanol precipitated. The restricted DNA was circularised with DNA ligase (2.7.10) and following ethanol precipitation, the ligation mixture was transformed into *E. coli* XL1-Blue competent cells by electroporation (2.7.11). The transformants were selected on ampicillin containing plates. Plasmid DNA isolated from six colonies was restricted with *Bgl*II and the digests separated on a 0.8% agarose gel. All six colonies contained the correct size plasmid cleaved only once by *Bgl*II (data not shown). One of the clones was selected randomly and named pALB4 (Appendix 4). In order to verify whether the IPCRM of pALB3 occurred as initially planned, with the introduction of the two stop codons, a 5bp deletion and a unique *Bgl*II restriction site, pALB4 plasmid DNA was prepared by ion-exchange chromatography and the insert DNA was sequenced, using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit. The sequence data from pALB4 was analysed using the Program Manual for the Wisconsin Package (Version 8, September 1994), via the Genetics Computer Group and it confirmed that the modification had ocurred as expected.

4.4.2 Isolating pALB5 by cloning *aphA-3* gene into pALB4

pALB4 DNA, isolated by ion-exchange chromatography, was restricted with *Bgl*II (section 2.7.7), which cleaves the plasmid within the insert (Appendix 4). An aliquot of the digested plasmid was analysed on a 0.8% agarose gel to determine whether the restriction had completed. The restricted DNA was extracted by phenol/chloroform treatment and the DNA ethanol precipitated.

The *Bam*H1 cleaved kanamycin cassette from pJMK30 (Fig.23) was ligated to *Bgl*II cleaved pALB4 (section 2.7.10). The ligation was ethanol precipitated and half of it was transformed into competent *E. coli* XL1-Blue cells by electroporation (section 2.7.11). Transformants were selected on plates supplemented with ampicillin and kanamycin. Ten ampicillin and kanamycin resistant colonies were selected randomly for further analysis by colony PCR. The template DNA was prepared as described in section 2.7.15.2 and amplified with the primers R and F (designed to specifically anneal to the kanamycin cassette, section 2.2.2), using 58°C for 1min as the annealing conditions and 72°C for 1min as the extension conditions. A negative control was introduced consisting of a 10µl reaction with no template DNA. As a positive control, pJMK30 was used in a 10µl reaction. The colony PCR products were analysed on a 0.8% agarose gel which showed that six of the selected ten clones contained

the kanamycin cassette (data not shown). One of the positive clones was picked randomly for further analysis. Plasmid DNA was prepared in small scale and further characterised by restriction digestion with *Xba*I and *Hin*dIII. The digests were separated on a 0.8% agarose gel, generating two bands with the expected sizes of approximately 4.5 and 1.4kb (data not shown). The new plasmid construct was named pALB5 (Appendix 4).

4.4.3 Transfer of pALB5 into *C. jejuni* 81116 by natural transformation

Wang & Taylor (Wang & Taylor, 1990b) demonstrated that most *Campylobacter spp* can be naturally transformed with chromosomal DNA, at an efficiency of 10^3-10^4 transformants/μg of DNA, while shuttle vector DNA transformed at a much lower rate. According to their studies, *Campylobacter spp*, like a few other Gram-negative bacteria, are likely to bind and uptake DNA containing specific recognition sequences (*cit. in* Wang & Taylor, 1990b). In view of their results, these specific recognition sequences could be present in the 2.9kb insert of pALB5 and thus, natural transformation was considered as a feasible method to introduce the mutated *regX1* gene into *C. jejuni*. pALB5 DNA was naturally transformed into *C. jejuni* 81116 as described before (section 2.7.13). Different DNA concentrations (1, 2.5 and 5μg) of pALB5 were added to 81116 cells. Two different transformation controls were used: approximately 1μg of pTNS#A plasmid DNA, (Wassenaar *et al.*, 1991; section 2.2.1), as well as 0.5μg of chromosomal DNA from *C. jejuni* 2T (a 81116-derived mutant containing a kanamycin resistance cassette inserted in *htr*A, a gene involved in bacterial heat-shock responses) isolated in this laboratory (Henderson, 1996; section 2.1.1). Following an overnight incubation in the presence of the DNA, the cells were plated onto campylobacter blood-free selective agar supplemented with the appropriate antibiotic selection. Natural transformation of 2T chromosomal DNA yielded 2.4×10^2 kanamycin resistant colonies, whilst no resistant colonies were obtained with pTNS#A DNA. These results indicated that the strain 81116 was naturally transformable but that *E. coli*-derived plasmid DNA was probably not taken up, at least under the conditions utilised. In fact, this was very much in agreement with Wang & Taylor's findings (Wang & Taylor, 1990b). According to these authors, *Campylobacter spp* uptake *E. coli*-derived plasmid DNA very inefficiently and this may have been the reason why pALB5 and pTNS#A did not lead to 81116-derived kanamycin resistant colonies.

4.4.4 Electrotransformation of pALB5 into *C. jejuni* 81116

Transformation of pALB5 into *C. jejuni* 81116 cells by electroporation was attempted during two parallel experiments (expts), A and B. 81116 competent cells were freshly prepared from four and six campylobacter blood-free selective agar plates showing confluent growth and incubated at 37°C for 18h or at 42°C for 8h, respectively. The cells were prepared using the protocol described in section 2.7.12.1. 50µl of competent 81116 cells were electrotransformed with different quantities of pALB5 plasmid DNA: 0.5, 1, 2.5, 5µg of DNA for attempt A and 2.5, 5 and 7.5µg of DNA for attempt B. As negative control, 50µl of competent 81116 cells were electroporated in the absence of DNA. As positive control, 50µl of competent 81116 cells were electroporated in the presence of 1µg (A) or 2µg (B) of pTNS#A plasmid.

pTNS#A (section 2.2.1) is a pBluescript derivative, containing a segment of the *flaA* gene (one of the flagellin genes) from *C. jejuni* interrupted by the *aphA-3* gene (kanamycin resistance gene) and isolated by Wassenaar *et al.* (1991). Because pTNS#A was constructed in a similar way to pALB5 and because it was used to mutate *flaA* in *C. jejuni* chromosome by double recombination (Wassenaar *et al.*, 1991), it appeared a better control to monitor the various steps for the mutagenesis of *regX1* than pUOA15.

During the electroporation, the time constants did not vary widely and ranged from 4.4 to 4.6ms in both electroporations. Following the electric impulse, the cells were resuspended in 100µl of SOB medium and plated onto campylobacter blood-free selective agar plates. All plates were incubated overnight at 42°C in a microaerophilic atmosphere. The values corresponding to cell viability before and after the impulse are presented in Table 5. Following recovery, all plates showed confluent bacterial growth which was washed off thoroughly with SOB medium. The bacterial suspensions were centrifuged, resuspended in the same medium and plated onto campylobacter blood-free selective agar plates, supplemented with 50µg/ml of kanamycin. The plates were incubated at 42°C in a microaerophilic atmosphere. The results obtained are shown in Table 5.

Table 5. Electrotransformation of pALB5 into *C. jejuni* 81116 cells, obtained in experiments A and B.

Expts	Initial cell viability (cfu/ml)	Cell viability after electric impulse (cfu/ml)	Total n° of pTNS#A transformants	Total n° of pALB5 kanamycin-resistant transformants per amount of plasmid added				
				0.5µg	1µg	2.5µg	5µg	7.5µg
A	$6.4x10^9$	$1.1x10^9$	2	0	0	0	51	–
B	$1.8x10^{11}$	$9.6x10^9$	371	_	_	0	2	8

Resistant colonies started growing after 2 days incubation for the positive control and 3 to 4 days incubation for the pALB5 transformants. All transformants had typical *Campylobacter* morphology as confirmed by Gram staining and dark field microscopy. All the putative *regX1* mutants were replated onto campylobacter blood-free selective agar supplemented with 50μg/ml of kanamycin and incubated overnight at 42°C in a microaerophilic atmosphere. Surprisingly, not all the clones grew back on the kanamycin containing plates; only 16 of the 51 colonies in A and only 4 of the 8 colonies obtained with electroporation of 7.5μg of pALB5 in B remained kanamycin resistant. It seemed that approximately 30% of the clones behaved as true kanamycin resistant clones and were saved for further analysis. For a preliminary selection, the clones were analysed by colony PCR, followed by southern blotting for a final confirmation (see section 4.4.5).

4.4.5 Analysis of the pALB5 transformants: searching for a *regX1* mutant

4.4.5.1 Analysis of pALB5 transformants by colony PCR. pALB5 transformants were tested for the presence of the kanamycin resistance cassette in the *regX1* gene by colony PCR (section 2.7.15.2), using two pairs of oligonucleotide primers. Primers R and F (section 2.2.2) were used to amplify the kanamycin resistance cassette from the chromosome and primers R3 and R4 (section 2.2.2) were used to check whether the chromosomal *regX1* gene had been disrupted. In the PCR with primers R and F, two reaction controls were included; a negative control with no DNA template and a positive control corresponding to 0.001μg of pALB5 plasmid as the DNA template. In the PCR with primers R3 and R4, an additional positive control was included which corresponded to 0.001μg of pJMK39 plasmid as the DNA template. The PCR was performed using 55°C for 2min as the annealing conditions and 72°C for 3min as extension conditions. The colony PCR products were separated on a 1% agarose gel. In experiment A, 15 out of the 16 clones tested by colony PCR with the primers R and F showed a band of the approximate size of the kanamycin resistance cassette (Fig.24). When the same clones were amplified with the primers R3 and R4, only one clone contained the kanamycin cassette in the *regX1* gene (clone 14, Fig.25). In experiment B, 3 of the 4 clones tested contained the kanamycin resistance cassette (Fig.24), only one of which contained the antibiotic marker inserted into the *regX1* gene (clone 3, Fig.25). The two positive clones were replated onto selective plates and fresh colonies were used in a second colony PCR with the same two pairs of oligonucleotide primers. The results obtained confirmed both clones (clone 14 from expt A

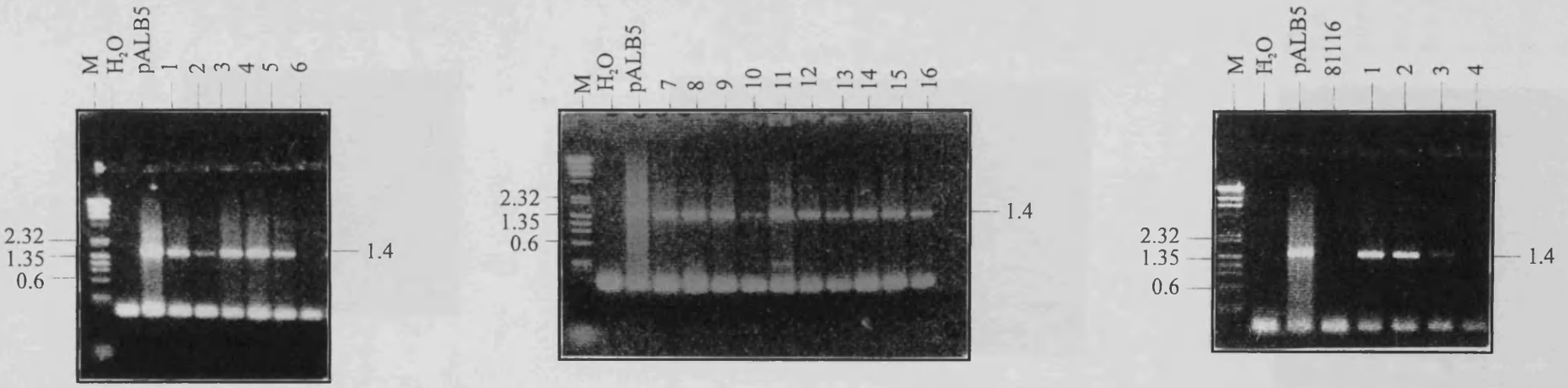


Fig.24. Colony PCR to identify putative *regX1* mutants. The Km^R colonies obtained by electroporation of pALB5 into *C. jejuni* 81116 underwent colony PCR with R/F primers which amplify the Km^R gene. A reaction with no template (H_2O) and a reaction with pALB5 as DNA template were the negative and positive controls, respectively. Standard molecular markers (M; kb) are on the left of the figure. PCR-amplified bands (kb) are on the right of the figure. The numbers on the top correspond to the clones isolated in each experiment (see text). A 1.4kb band was amplified in the positive control pALB5, in 15 of the 16 clones obtained in experiment A and in 3 of the 4 clones obtained in experiment B, indicating the presence of the Km^R cassette on the chromosome from these clones.

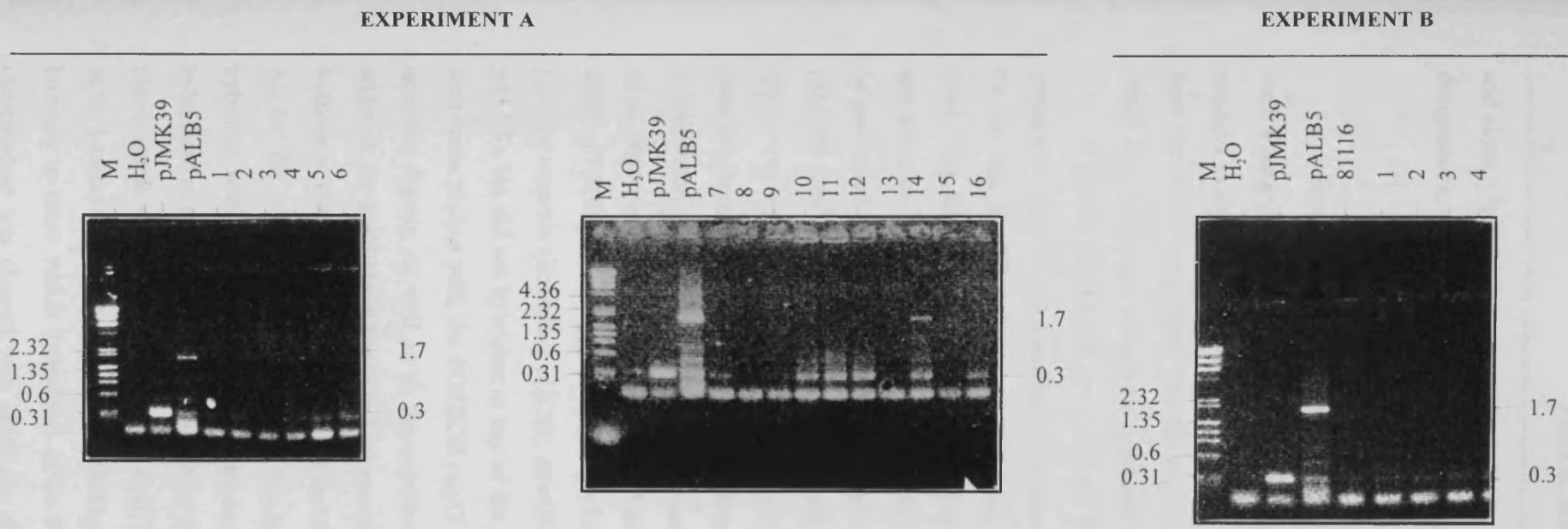


Fig.25. Colony PCR to identify putative *regX1* mutants. The Km^R colonies obtained by electroporation of pALB5 into *C. jejuni* 81116 underwent colony PCR with R3/R4 primers which amplify the *regX1* PCRDOP fragment. A reaction with no template (H_2O) was the negative control. Two reactions with pJMK39 and pALB5 as DNA templates were the positive controls. In experiment B, a third positive control corresponding to the chromosome of *C. jejuni* 81116 was included. Standard molecular markers (M; kb) are on the left of the figure. PCR-amplified bands (kb) are on the right of the figure. The numbers on the top correspond to the clones isolated in each experiment (see text). A 1.7kb band was amplified in the positive control pALB5, in 1 of the 15 clones containing the Km^R cassette obtained in experiment A and in 1 of the 3 clones containing Km^R cassette obtained in experiment B, indicating that the Km^R cassette only disrupted the *regX1* gene on those two clones: clone 14 in A and clone 3 in B. A 0.3kb band corresponding to intact *regX1* fragment was visualised in the positive controls pJMK39 and 81116 and in the clones which did not contain the Km^R cassette inserted in *regX1*. The 0.3kb band is also present in clone 14 but it probably corresponds to a non-specific band.

and clone 3 from expt B) as *regX1* mutants. The two potential *regX1* mutants were temporarily designated as #1 and #2 and were further analysed by southern blotting.

4.4.5.2 Analysis of the putative *regX1* mutants by southern blotting. Southern blotting was required to confirm that mutants #1 and #2 were true *regX1* mutants. The chromosomal DNA from the two clones, along with the appropriate controls, was probed with labelled PCRDOP *regX1* DNA fragment, kanamycin resistance cassette or linearised pUC19.

Chromosomal DNA from mutants #1 and #2, as well as from the parent strain, was prepared by the method described in section 2.7.1 and analysed by agarose gel electrophoresis. To perform each of the three hybridisation experiments, approximately 5μg of chromosomal DNA from all the strains were restricted separately with different endonucleases, *BclI*, *BglII* and *MspI*, overnight. Different DNA controls were included, according to the hybridisation to be performed; pUC19 linearised with *PstI* was used in the hybridisation to pUC19 DNA probe; pJMK39 restricted with *HindIII* and *PstI* was used in the hybridisation to pUC19 and PCRDOP *regX1* fragment DNA probes; pALB5 restricted with *PvuII* was used in all hybridisations. Samples were separated on a 0.8% agarose gel, along with the controls. The DNA-DNA hybridisation was performed using the nonradioactive DNA labelling and detection system, using digoxigenin-11-dUTP labelled DNA probes (section 2.7.20). The membrane probed with linear pUC19 DNA revealed that the probe hybridised to the ~2.7kb pUC19 band from all positive controls (linearised pUC19, *HindIII* and *PstI* restricted pJMK39 and *PvuII* restricted pALB5), but did not hybridise to any of the genomic digests (data not shown). In contrast, the membrane probed with the PCRDOP *regX1* fragment showed that the probe hybridised to all genomic digests, as well as to the expected DNA bands in the control digests (Fig.26). In addition, the highlighted bands in both recombinants underwent a size increase when compared to those in the parent strain, indicating that the *regX1* gene was disrupted in the recombinants. As for the membrane hybridised to the kanamycin resistance cassette, the probe did not hybridise with the parental strain chromosomal digests (Fig.27). In the recombinant clones, it hybridised to the same bands recognised by the PCRDOP *regX1* fragment DNA probe when the chromosome was cleaved with *BclI* or *BglII* because there is not a site for these endonucleases in the kanamycin cassette. The highlighted bands in both recombinants underwent a similar size increase to those which hybridised to the PCRDOP *regX1* fragment DNA probe. When the chromosome was cleaved with *MspI*, the situation became more complex. *MspI* cleaves the Km^R cassette at four sites. In addition, *MspI* appears to cleave 81116 chromosomal *regX1* once

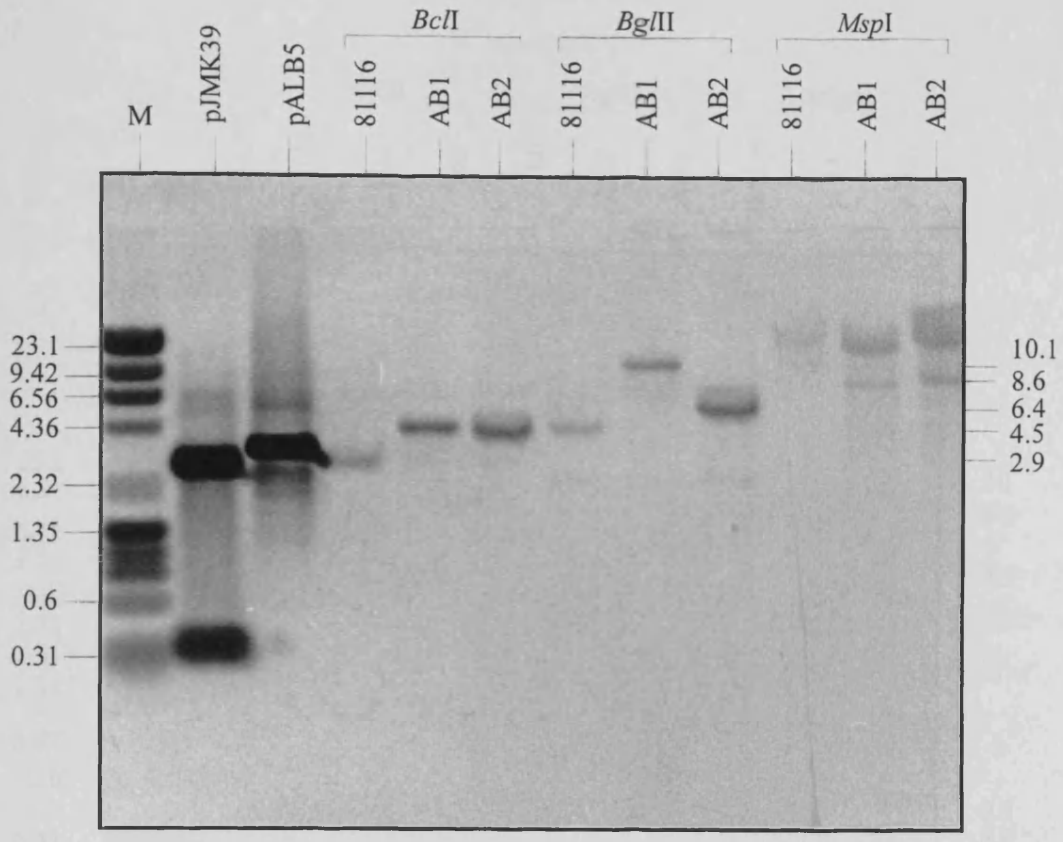


Fig.26. Southern blotting to confirm the disruption of *regX1* in AB1 and AB2. Chromosomal DNA of *C. jejuni* 81116, AB1 and AB2 was restricted with *Bcl*I, *Bgl*II and *Msp*I and the digests were hybridised to digoxigenin-11-dUTP labelled PCRDOP *regX1* fragment. *Hin*dIII/*Pst*I restricted pJMK39 and *Pvu*II restricted pALB5 were included as positive controls. Standard molecular markers (M; kb) are on the left of the figure. The bands (kb) recognised by the DNA probe are on the right of the figure. PCRDOP *regX1* fragment probe hybridised to both 0.3kb and ~3kb pJMK39 bands (not labelled on the figure) which correspond to PCRDOP *regX1* fragment and single cut plasmid, respectively. The probe also recognised the 2.9kb pALB5 insert (not labelled on the figure). In the *Bcl*I digests, the probe hybridised to a wild type ~2.9kb band and to a mutant ~4.5kb band. In the *Bgl*II digests, the probe hybridised to a wild type ~4.5kb band, to AB1 ~10.1kb band and to AB2 ~6.4kb band. In the *Msp*I digests, it was not possible to determine accurately the size of the bands the probe hybridised to but in general, the size changes occurring between 81116 and the mutants hybridisation profiles indicated the insertion of the Km^R cassette into the *regX1* gene.

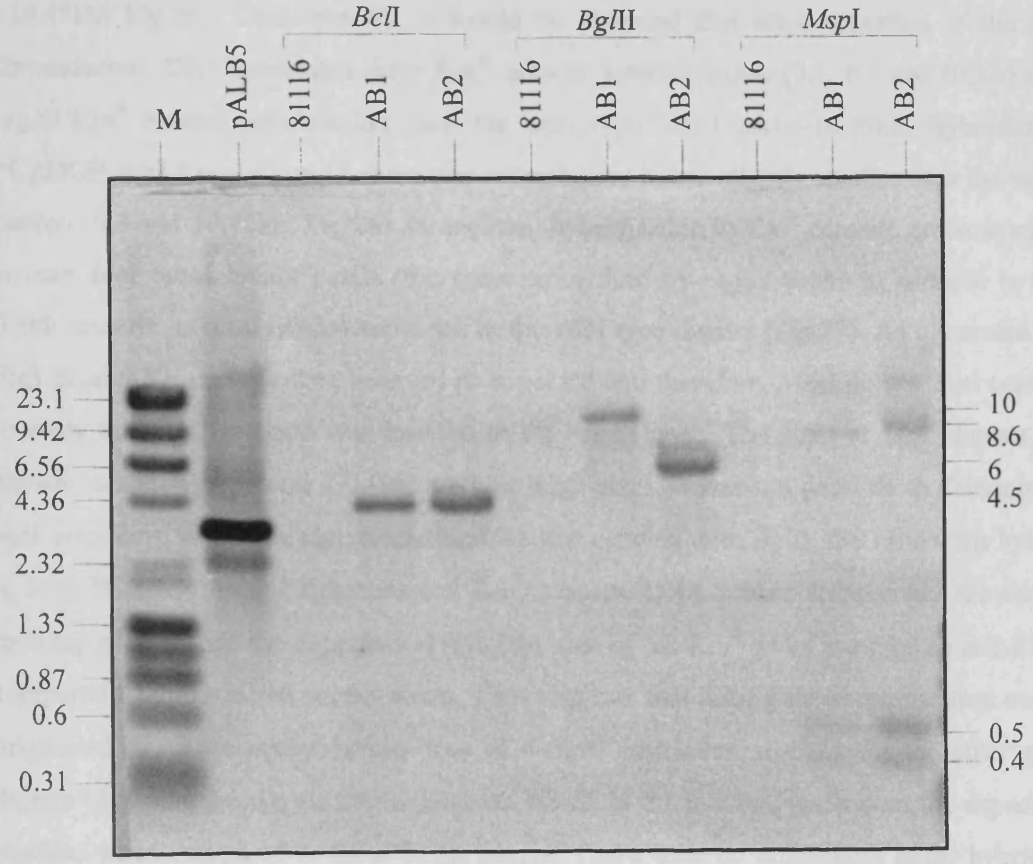


Fig.27. Southern blotting to confirm the disruption of *regX1* in AB1 and AB2. Chromosomal DNA of *C. jejuni* 81116, AB1 and AB2 was restricted with *Bcl*I, *Bgl*II and *Msp*I and the digests were hybridised to digoxigenin-11-dUTP labelled Km^R cassette. *Pvu*II restricted pALB5 was also hybridised to Km^R cassette probe and used as a positive control. Standard molecular markers (M; kb) are on the left of the figure. The bands (kb) recognised by the DNA probe are on the right of the figure. The probe hybridised to a~2.9kb pALB5 band (not labelled on the figure) which correspond to the plasmid's insert. The probe did not hybridise to any of the wild type digests as expected. In the *Bcl*I digests, the probe hybridised to a mutant ~4.5kb band. In the *Bgl*II digests, the probe hybridised to a AB1 ~10kb band and to AB2 ~6kb band. AB1 seems to have lost a *Bgl*II site during recombination. In the *Msp*I digests, the probe hybridised to mutant ~8.6, ~0.5 and ~0.4kb bands. In both mutants, *regX1* was disrupted by the insertion of the Km^R cassette.

(in contrast to chromosomal F132 *regX1* as shown in Fig.11) as judged by the fact that PCRDOP *regX1* fragment DNA probe hybridised to two bands in 81116 digests (~10.1 and ~10.45kb; Fig.26). Consequently, it would be expected that *Msp*I digestion of the mutants chromosomal DNA generated three Km^R cassette internal bands (0.1, 0.4 and 0.5kb) and two *regX1*/Km^R bands, both smaller than the wild type ~10.1 and ~10.45kb. Hybridisation to PCRDOP *regX1* probe would show two recombinant bands slightly smaller than the wild type bands (10.1 and 10.45kb; Fig.26). In contrast, hybridisation to Km^R cassette probe would show at least four recombinant bands (the same recognised by *regX1* probe in addition to 0.4 and 0.5kb cassette internal bands) and none in the wild type digests (Fig.27). As illustrated in both Figs 26 and 27, these probes behaved as expected and therefore, *Msp*I digests also contributed to show that Km^R cassette was inserted in the *regX1* gene. The sizes of *Msp*I digests are not shown on the Figs 26 and 27. Due to their large sizes, it was not possible to determine them with accuracy. When the chromosome of #1 was cleaved with *Bgl*II, the bands that hybridised to both PCRDOP *regX1* fragment and Km^R cassette DNA probes surprisingly showed a size increase greater than the expected ~1.4kb (the size of the Km^R gene; see Figs 26 and 27) when compared to those in the parent strain. This suggests that during the recombination event that originated #1, there probably was loss of a *Bgl*II restriction site. As for the other genomic digests (*Bcl*I and *Msp*I), all the highlighted bands in the mutants underwent the expected size changes when compared to those in the parent. There were no differences in the hybridisation profiles to *Bcl*I and *Msp*I digests between #1 and #2. In summary, the two clones resulted from the allelic replacement of the wild type *regX1* gene by the insertionally mutated copy and were true *regX1* mutants. The two mutants #1 and #2 were named AB1 and AB2, respectively.

4.5 Summary

In order to charaterise the role of *regX1* in *C. jejuni*, different strategies were employed to isolate a *regX1* mutant. In a first step to construct the mutant, a defined mutation was engineered in the gene by IPCRM, in an *E. coli* background. The defined mutation was designed to disrupt RegX1 catalytic site, thus, permanently inactivating the protein. Subsequently, a kanamycin resistance cassette was subcloned in the middle of *regX1* and the whole fragment (*regX1* with the kanamycin resistance gene) was cloned in different vectors to be utilised as suicide mutagenesis vectors. Eventually, two mutants were obtained following the electroporation of pALB5 (a pUC19-derivative containing a ~2.9kb insert) into *C. jejuni* 81116

and homologous recombination between plasmid and chromosome homologous sequences. The two mutants were named AB1 and AB2.

CHAPTER 5

Characterising the Phenotype of the *regX1* Mutants

5.1 Introduction

In order to determine the role of *regX1* in the bacterial cell, a mutation was constructed by inactivating the chromosomal copy of the gene. Two mutants were isolated and named AB1 and AB2. The phenotype of the mutants was investigated to identify changes that could unravel the environmental factors responsible for activating *regX1* and *regX1*-dependent genes. Comparative sequence analysis indicated that a close *regX1* homologue had not yet been found in other bacteria. Thus, there was no evidence suggesting the type of signal *regX1* gene would respond to and no clues as to the possible phenotype of a *regX1* mutant. Consequently, the mutant strains were investigated for any phenotypic differences when compared to the parental strain 81116. AB1 and AB2 were analysed for changes in colony morphology, *in vitro* growth rates and protein profile. Both mutants were also tested in *in vitro* assays for possible changes in the ability to interact with an enterocyte-like cell line and AB1 was studied in an *in vivo* model to determine its ability to colonise the intestine of chicken.

5.2 Colony morphology of AB1 and AB2

AB1 and AB2 were analysed for changes in colony morphology. The *regX1* mutants and the parental strain were plated on either campylobacter blood-free selective agar or MH agar plates and incubated at 37 or 42°C in a microaerophilic atmosphere. In all of the conditions tested, the mutants grew more slowly and formed smaller colonies than the parental strain (Fig.28). Changes in motility and LPS (lipopolysaccharide) profile of the mutant strains were investigated as possible causes for this phenotype.

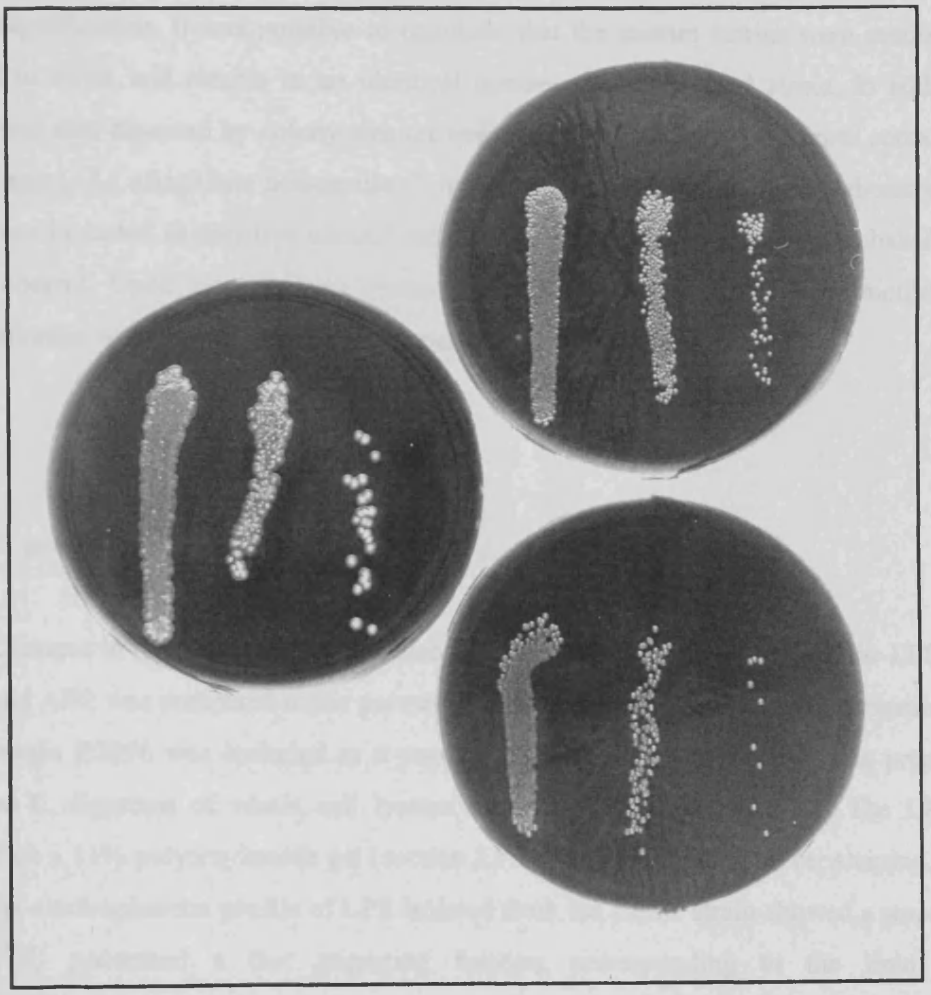


Fig.28. Colony morphology of *C. jejuni* 81116, AB1 and AB2. The strains 81116 (left), AB1 (top on the right) and AB2 (bottom on the right) were plated on campylobacter blood-free selective medium and incubated overnight at 37°C in a microaerophilic atmosphere.

5.2.1 Motility of AB1 and AB2

Since the size of colonies could be influenced by motility, *regX1* mutants were analysed by dark field microscopy for changes in motility. An aliquot from an overnight culture in MH broth incubated in a microaerophilic atmosphere was transferred onto a microscope slide and examined by phase contrast, using a Zeiss Axioskop microscope at a 1000X magnification. It was possible to conclude that the mutant strains were motile and appeared to swim and tumble in an identical manner to the parental strain. In addition, motility was also assessed by colony size on semi-solid MH medium (MH broth containing 7.5g/l of agar). An aflagellate non-motile *C. jejuni* mutant (constructed in the laboratory by J. Cox) was included as negative control and the wild type strain 81116 was included as a positive control. Once more, it was observed that both mutant strains were motile and formed colonies with the same shape as the parental strain (data not shown).

5.2.2 LPS profile of AB1 and AB2

Changes in LPS composition can also influence the colony size. Hence, the LPS profile of AB1 and AB2 was compared to the parental strain LPS profile. The enteropathogenic *E. coli* (EPEC) strain E2036 was included as a positive control. The LPS fraction was prepared by proteinase K digestion of whole cell lysates as described in section 2.9.1. The LPS were separated on a 11% polyacrylamide gel (section 2.9.2) and visualised by silver staining (section 2.9.3). The electrophoretic profile of LPS isolated from the EPEC strain showed a smooth-type LPS. EPEC possessed a fast migrating fraction corresponding to the lipid A and oligosaccharide core and a ladder-like pattern of slow migrating bands corresponding to LPS with increasing numbers of side O chain repeats. In contrast, the three *C. jejuni* strains appear to lack the high-Mr bands corresponding to the O polysaccharide chain. Only the low-Mr bands corresponding to the lipid A and oligosaccharide core were visualised. Nevertheless, it looked like there were no differences in the LPS profiles of the *regX1* mutants and the parental strain (Fig.29). All three strains appeared to have rough-type LPS although the colonies grown on MH and campylobacter blood-free agar had the shiny appearance of typical smooth-type LPS. Thus, it was possible that the O polysaccharide chain was not visualised by the silver staining but would only be detectable by immunoblotting as demonstrated by other groups (Preston & Penner, 1987). This was not analysed any further as Penner antiserum (Penner serotype 6) for immunoblotting was not available. Nevertheless, the primary aim had been fulfilled.

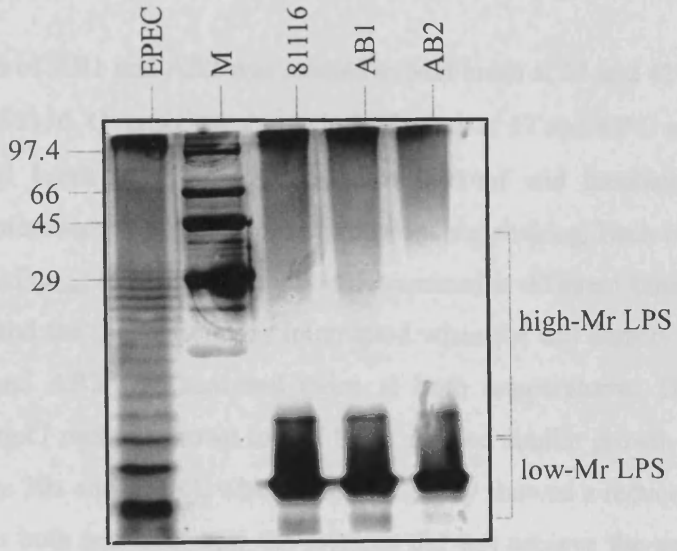


Fig.29. LPS profiles of *C. jejuni* 81116, AB1 and AB2. LPS were prepared from overnight cultures in MH broth incubated at 37°C by proteinase K digestion. The samples were separated on 11% polyacrylamide gel and silver stained. LPS from EPEC were used as positive control for the presence of high-Mr LPS. The silver stained profile of *C. jejuni* only contains the low-Mr LPS. The molecular markers (M; kDa) are on the left.

Subsequently, the LPS composition of AB1 strain was examined in more detail, in another laboratory, and no differences were found (Dr A. Moran at the Department of Microbiology, University College Galway, personal communication).

5.3 Characterisation of the growth rate of AB1 and AB2 in MH broth

The growth of AB1 and AB2 was studied in MH broth at 37 and 42°C and compared to the parental strain 81116. Overnight cultures in MH broth at 37 and 42°C were diluted in 20ml of prewarmed MH broth to a final $O.D_{600nm}$ of 0.01/ml and incubated at 37 or 42°C, respectively, in a microaerophilic atmosphere with constant shaking. Each culture was prepared in duplicate. The $O.D_{600nm}$ for each culture was determined at different times during the course of the experiment and the incubation was interrupted when the cell density started to decrease. Growth of AB1 and AB2 was analysed twice at both temperatures. The results obtained revealed that the *regX1* mutants grown in MH broth showed similar growth rates at 37°C to the parental strain (Figs 30a and 31a-c), whereas at 42°C, they showed a reduced growth rate (Figs 30b and 31a-c). At both temperatures, the mutants did not achieve the parental level of cell density and at 37°C, they appeared to enter stationary phase at an earlier time. To investigate whether the inoculum size influenced the growth pattern of the strains, 81116 and AB1 cultures were inoculated with different numbers of viable cells. Overnight cultures in MH broth at 37°C were diluted in 20ml of prewarmed MH broth and incubated at 37°C in a microaerophilic atmosphere with constant shaking. Cultures of 81116 were diluted to a final concentration of 10^6, 10^7 and 10^8 cfu/ml while cultures of AB1 were diluted to a final concentration of 10^5, 10^6 and 10^7 cfu/ml. At different times throughout incubation, an aliquot was withdrawn from each culture and serial dilutions were plated onto campylobacter blood-free selective agar. The plates were incubated at 37°C in a microaerophilic atmosphere. The results from the viable counts are presented in Figs 32 and 33. The data is inconclusive and further studies are required to understand how the inoculum size might affect the final $O.D_{600nm}$ achieved by AB1 at late logarithmic phase.

In view of the differences observed in the growth rates of the mutant and parental strains, variation of cell density was followed when the mutants were exposed to temperature shift during growth. Overnight cultures in MH broth grown at 37°C were diluted in 20ml of prewarmed MH broth to a final $O.D_{600nm}$ of 0.01/ml and incubated at 37°C, in a microaerophilic atmosphere with constant shaking. The cultures were prepared in duplicate. The $O.D_{600nm}$ for

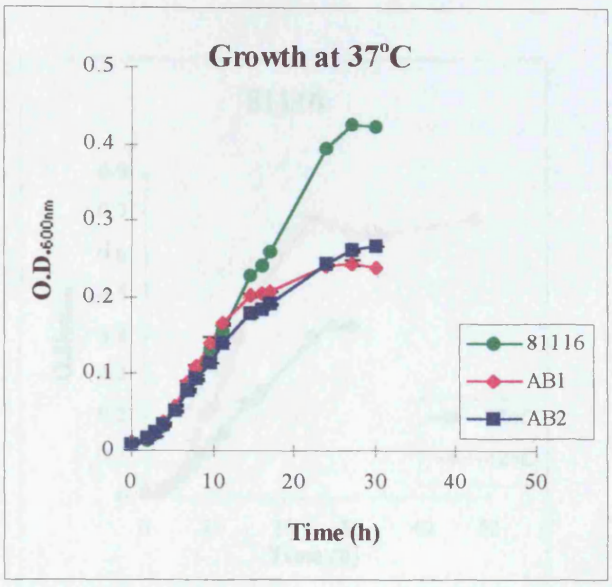


Fig.30a. Growth pattern of the parent and mutant strains incubated at 37°C. The growth profile of 81116, AB1 and AB2 was analysed when the strains were incubated in MH broth at 37°C. Each point represents the mean (±SD) of the $O.D_{600}$ of two cultures. The mutants entered stationary phase earlier than 81116 and never achieved the parental level of cell density.

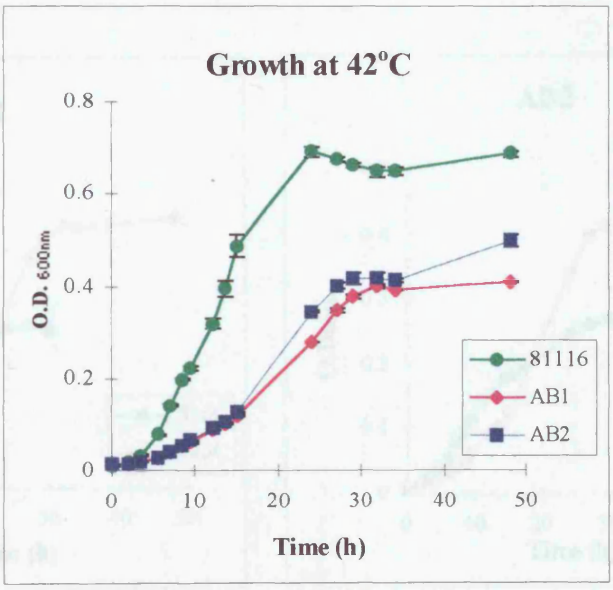


Fig.30b. Growth pattern of the parent and mutant strains incubated at 42°C. The growth profile of 81116, AB1 and AB2 was analysed when the strains were incubated in MH broth at 42°C. Each point represents the mean (±SD) of the $O.D_{600}$ of two cultures. The mutants showed a slower growth rate than 81116 and never achieved the parental level of cell density.

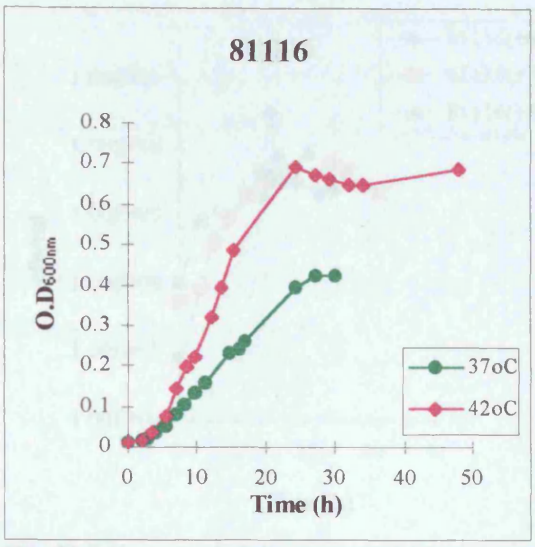


Fig.31a

Fig.31a. Growth pattern of 81116 incubated at different temperatures. The growth profile of 81116 was analysed when the strain was incubated in MH broth at 37 or 42°C. 81116 showed a higher growth rate at 42°C than at 37°C.

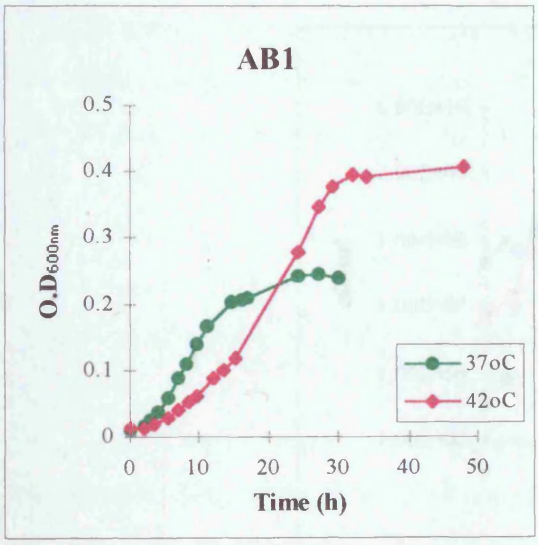


Fig.31b

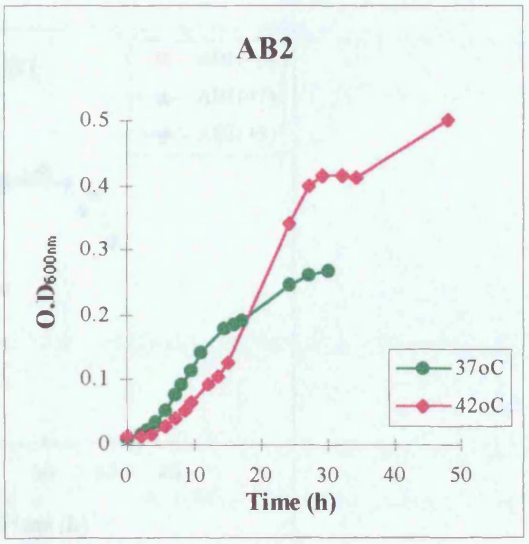


Fig.31c

Figs 31b-c. Growth pattern of the mutant strains incubated at different temperatures. The growth profile of AB1 and AB2 was analysed when the strains were incubated in MH broth at 37 or 42°C. The mutants achieved higher cell densities when incubated at 42°C.

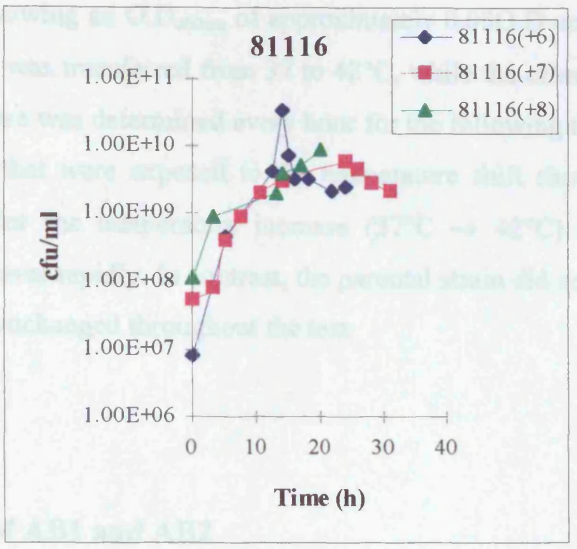


Fig.32. Influence of the inoculum size in the growth of 81116. The growth profiles of 81116 cultures inoculated with $7.8x10^6$ (+6), $5.3x10^7$ (+7) and $1.1x10^8$ (+8) cfu/ml were compared. The cultures were incubated at 37°C in MH broth. Growth was followed by determining the viable count at specific time points.

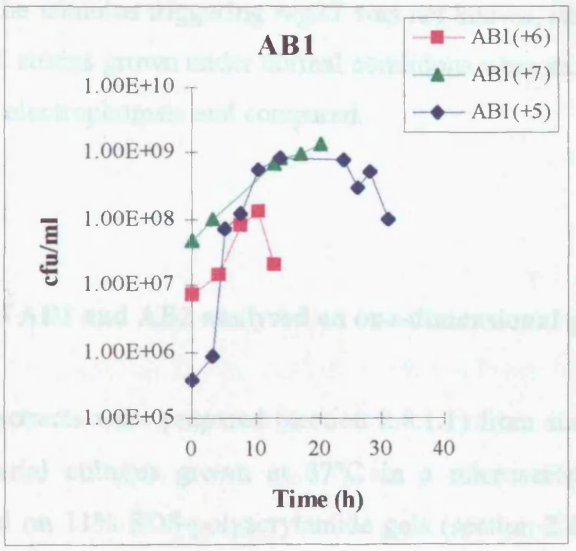


Fig.33. Influence of the inoculum size in the growth of AB1. The growth profiles of AB1 cultures inoculated with $3.8x10^5$ (+5), $7x10^6$ (+6) and $4.6x10^7$ (+7) cfu/ml were compared. The cultures were incubated at 37°C in MH broth. Growth was followed by determining the viable count at specific time points.

each culture was determined at different times. When the cultures reached exponential growth after 5h incubation, showing an $O.D_{600nm}$ of approximately 0.05O.D units, one of the duplicate cultures of each strain was transferred from 37 to 42°C, while the other remained at 37°C. The $O.D_{600nm}$ for each culture was determined every hour for the following three hours. The cultures of the mutant strains that were exposed to the temperature shift showed an obvious loss of viability one hour after the temperature increase (37°C → 42°C) (Fig.34). However, the cultures seemed to recover rapidly. In contrast, the parental strain did not loose viability and the growth rate remained unchanged throughout the test.

5.4 Protein profile of AB1 and AB2

In the previous chapter, it was established that RegX1 belonged to the response regulatory family of proteins and thus, it should play a regulatory function in the bacterial cell. Inactivation of *regX1* could probably affect the expression of the genes under its control. Therefore, AB1 and AB2 mutant strains were compared with the parental strain 81116 for changes in protein profiles. Differences would be more likely to be observed if the mutants were exposed to the appropriate environmental stimulus in the presence of a radiolabel (like [^{35}S]-methionine). As the stimulus triggering *regX1* was not known, stained protein profiles of the mutant and parental strains grown under normal conditions were analysed on one (1-D) and two (2-D)-dimensional electrophoresis and compared.

5.4.1 Protein profile of AB1 and AB2 analysed on one-dimensional gel electrophoresis

Total protein extracts were prepared (section 2.8.1.1) from stationary (overnight) and exponential (6h) bacterial cultures grown at 37°C in a microaerophilic atmosphere. The samples were separated on 11% SDS-polyacrylamide gels (section 2.8.1.3) and visualised by Coomassie Brilliant blue staining (section 2.8.1.4). Several differences were observed in the protein profile obtained from stationary cultures, with additional proteins being induced in the mutants, while others were repressed (Fig.35). No differences were detected between the different growth phases and all changes were the same whether the bacteria were in exponential (data not shown) or stationary growth phase (Table 6). The protein profiles from the mutants incubated at different temperatures were also compared. Total protein extracts were prepared

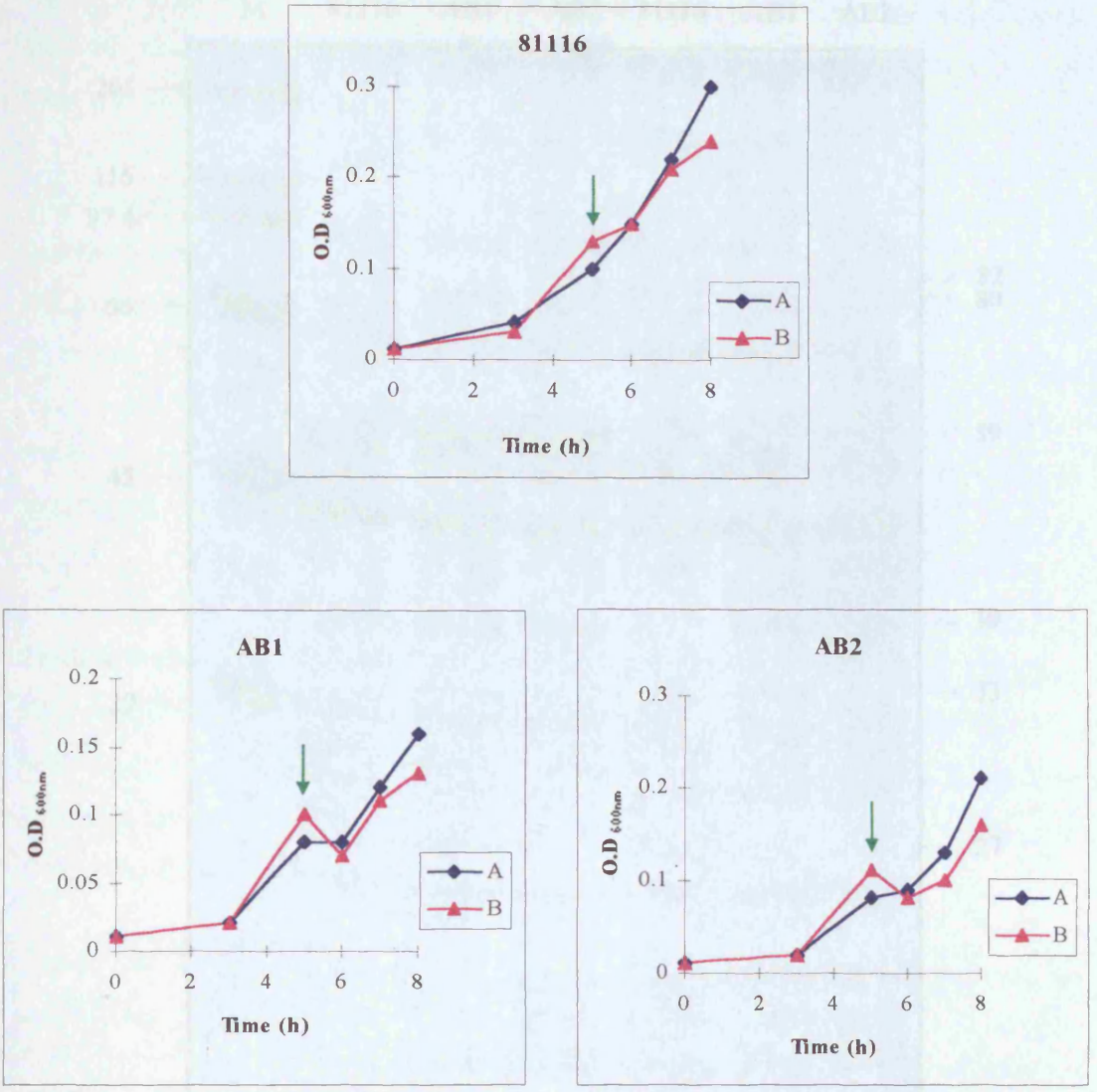


Fig.34. Growth profile of 81116, AB1 and AB2 following a temperature shift. The growth of the strains was analysed when they were exposed to a temperature shift from 37°C to 42°C. Curve A corresponds to the culture control and represents growth at 37°C; curve B corresponds to the culture undergoing temperature increase. The temperature was shifted from 37°C to 42°C 5h post-incubation (indicated by the green arrow).

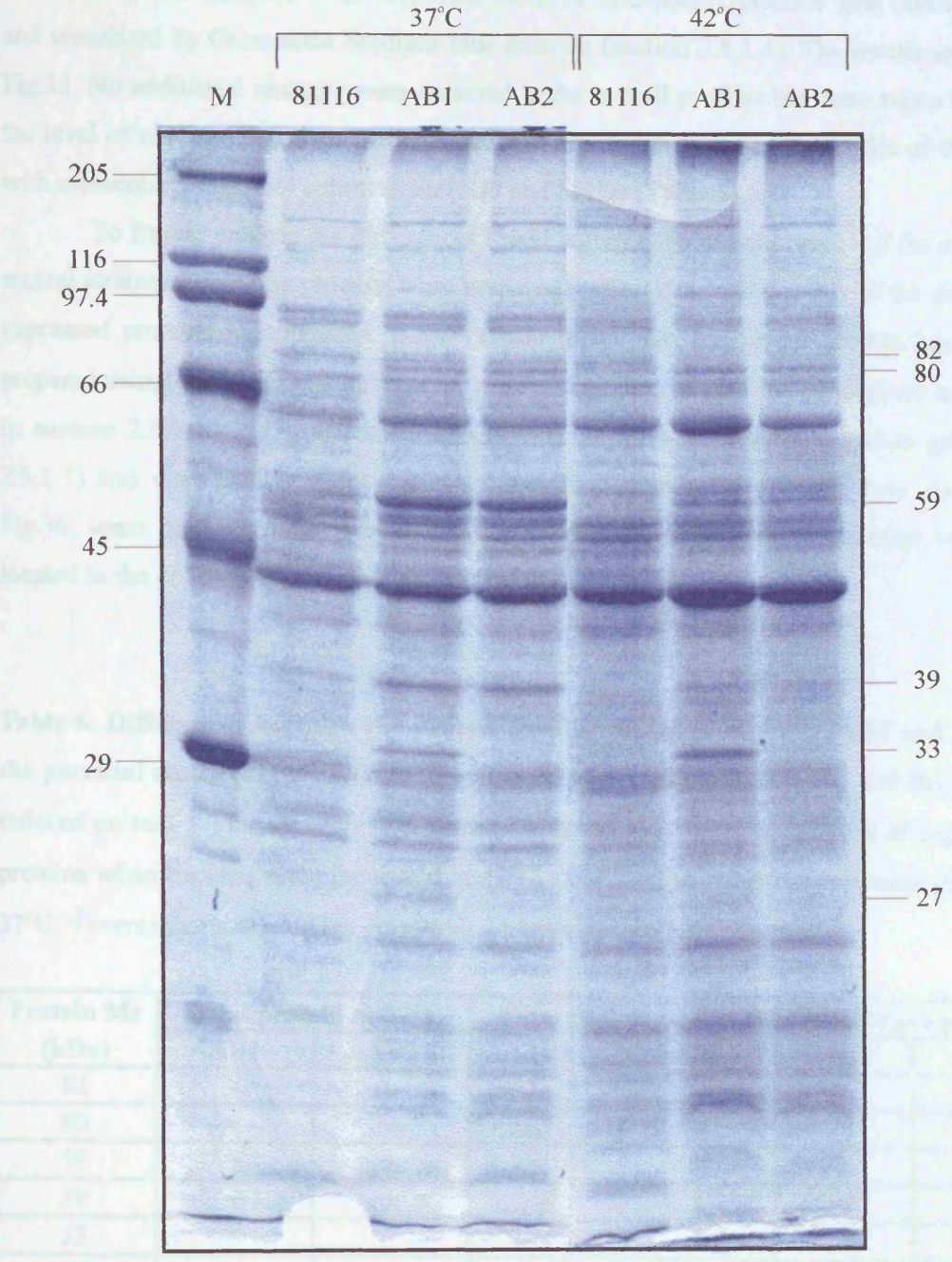


Fig.35. Total protein profile of *C. jejuni* 81116, AB1 and AB2. Total proteins were prepared from bacteria incubated overnight at 37°C or 42°C. The proteins were visualised by Coomassie blue staining. The proteins differentially expressed (kDa) are shown on the right of the figure. Standard molecular markers (M; kDa) are shown on the left of the figure.

(section 2.8.1.1) from overnight cultures grown at 37 and 42°C in a microaerophilic atmosphere. The samples were separated on 11% SDS-polyacrylamide gels (section 2.8.1.3) and visualised by Coomassie Brilliant blue staining (section 2.8.1.4). The results are shown in Fig.35. No additional changes were detected in the overall profiles but there was a decrease in the level of expression of two of the additional proteins detected in the profile of the mutants, with molecular weights of approximately 39 and 59kDa (Table 6).

To further analyse the differences found between the protein profile of the parental and mutant strains, membrane proteins were prepared to determine whether any of the differentially expressed proteins were located in the cellular membrane. Membrane protein fractions were prepared using overnight cultures grown at 37°C in a microaerophilic atmosphere as described in section 2.8.1.2. The samples were separated on 11% SDS-polyacrylamide gels (section 2.8.1.3) and visualised by Coomassie Brilliant blue staining (section 2.8.1.4). As shown in Fig.36, some of the proteins whose expression had been altered in the mutants were in fact located in the cellular membrane fraction (Table 6).

Table 6. Differences between the protein profiles of *regX1* mutants (AB1 and AB2) and the parental strain (81116) on one-dimensional electrophoresis (Figs 35 and 36). (+) means induced protein. (-) means repressed protein. ↓ means a decrease in the level of expression of proteins when bacteria were incubated at 42°C, compared to when bacteria were incubated at 37°C. √ corresponds to proteins present in the cellular membrane fraction.

Protein Mr	Total protein at 37°C		Total protein at 42°C		Membrane protein	
(kDa)	AB1	AB2	AB1	AB2	AB1	AB2
82	-	-	-	-		
80	+	+	+	+		
59	+	+	↓	↓		
39	+	+	↓	↓	√	√
33	+		+		√	
27	+		+			

5.4.2 Protein profile of AB1 analysed on two-dimensional gel electrophoresis

In view of all the differences observed in the one-dimensional protein profile, a two-dimensional analysis was undertaken. Therefore, total protein extracts were prepared (section 2.8.2.1) from stationary (overnight) 81116 and AB1 cultures incubated at 37°C in a

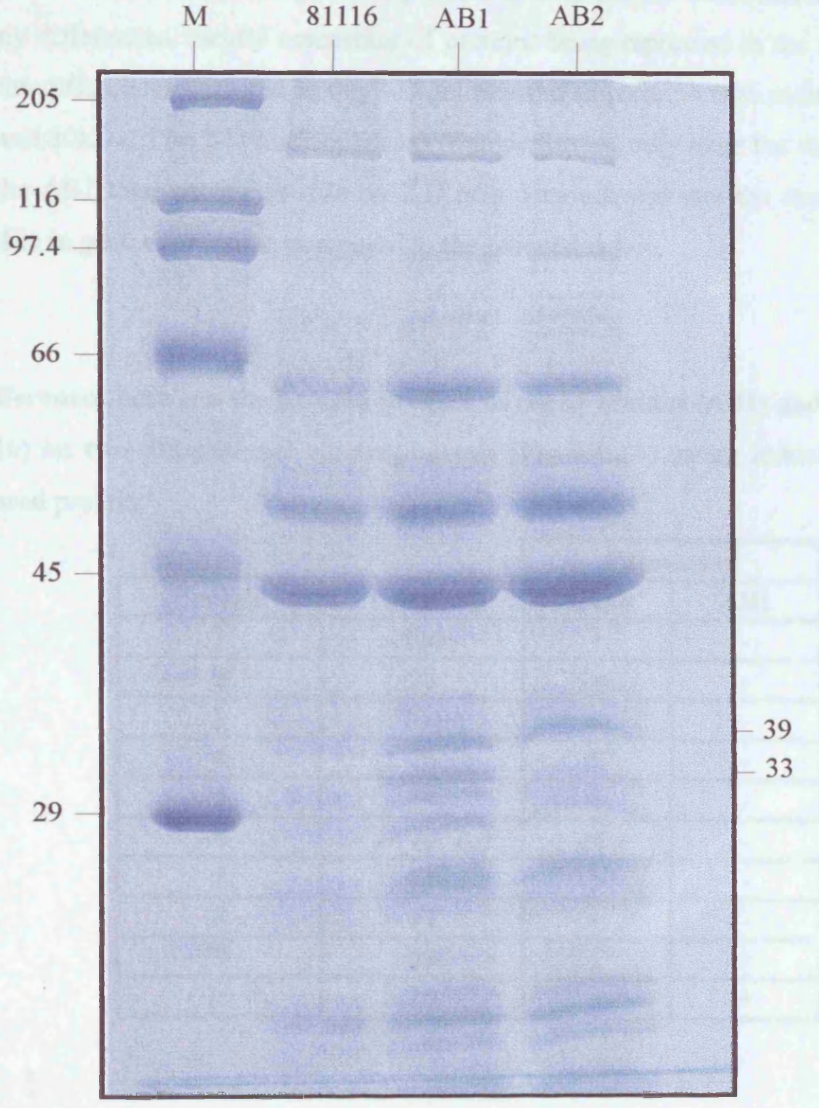


Fig.36. Membrane protein profile of *C. jejuni* 81116, AB1 and AB2. Membrane proteins were prepared from bacteria incubated overnight at 37°C. The proteins were visualised by Coomassie blue staining. The proteins differentially expressed (kDa) are shown on the right of the figure. Standard molecular markers (kDa) are shown on the left of the figure.

microaerophilic atmosphere. Approximately 5μg of total protein per sample were separated by 2-D electrophoresis as described previously (section 2.8.2.3). The proteins were visualised by silver staining (section 2.8.2.4; Fig.37). Although the samples were quantitated before the electrophoresis (section 2.8.2.2), there was more protein from 81116 sample on the gel and consequently, it was difficult to compare the profiles of both strains. Nevertheless, the 2-D gel revealed many differences, mostly consisting of proteins being repressed in the mutant (Table 7). Most of the differences detected in the 2-D gel referred to proteins with molecular weights between 55 and 80kDa. The 2-D electrophoresis was performed only once but this preliminary analysis of the AB1 total protein profile on 2-D gels demonstrated that the mutant showed a different profile in gene expression, compared to the parental strain.

Table 7. Differences between the protein profiles of *regX1* mutant (AB1) and the parental strain (81116) on two-dimensional electrophoresis (Fig.37). (+) means induced protein. (-) means repressed protein.

		Expression	
Protein	**Protein Mr**	**81116**	**AB1**
1	116	+	-
2	98	+	-
3	105	+	-
4	76	+	-
5	74	+	-
6	70	+	-
7	61	+	-
8	57	+	-
9	33	-	+
10	25	-	+

5.5 Interaction of AB1 and AB2 with cultured enterocyte-like cells

The ability of the mutant strains to interact with the host cells was investigated using an enterocyte-like cell line, Caco-2, as an *in vitro* model. It was demonstrated previously that *C. jejuni* 81116, like many other *C. jejuni* strains, adhered to and invaded tissue culture cells (Newell *et al.*, 1985). Hence, AB1 and AB2 were compared to the parental strain in their ability to bind and enter Caco-2 cells.

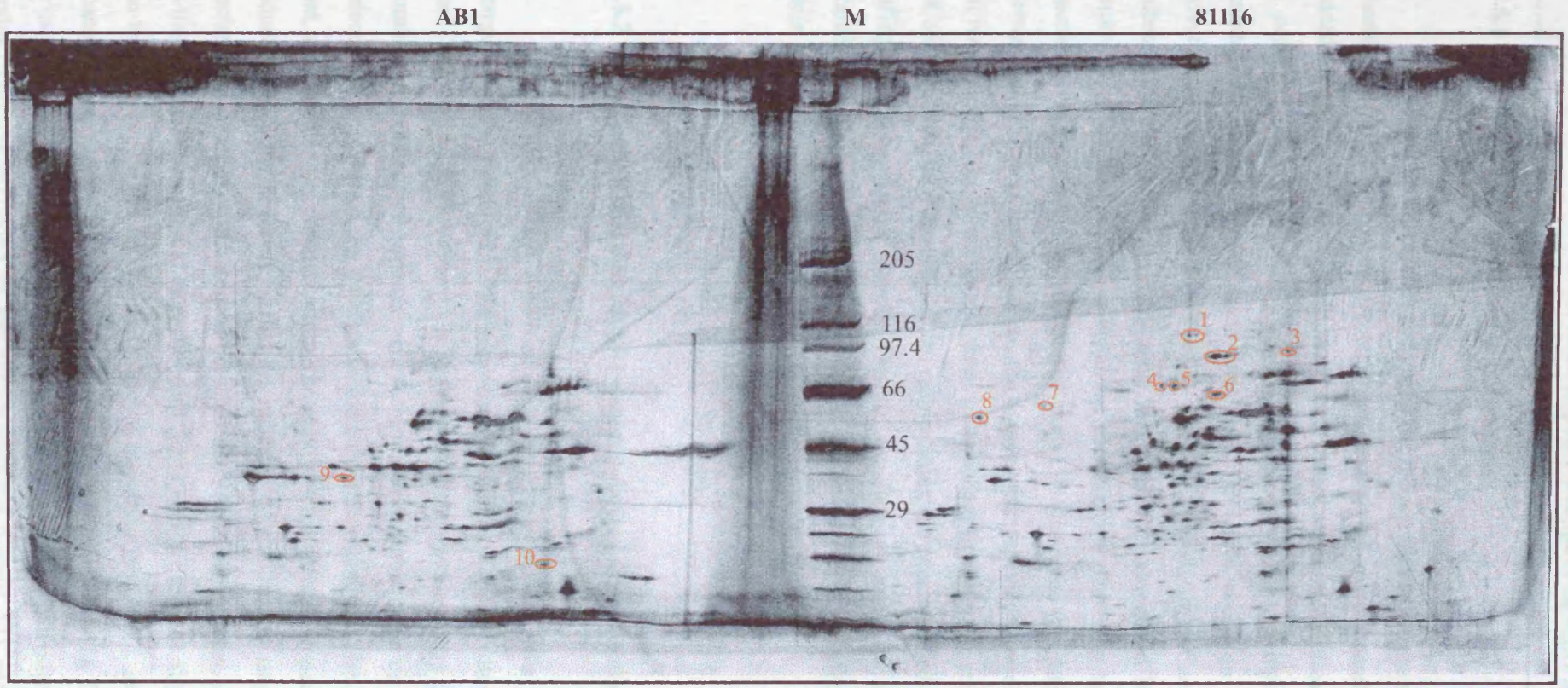


Fig.37. Protein profile of *C. jejuni* 81116 and AB1. Approximately 5µg of total protein of 81116 and AB1 were separated by 2-D electrophoresis. The proteins were visualised by silver staining. The proteins numbered in the profile of 81116 have been repressed in the mutant AB1 whereas the proteins numbered in the profile of AB1 have been derepressed in AB1. The proteins' numbers relate to those in Table 7. Standard molecular markers (M; kDa) are shown at the centre of the figure.

5.5.1 Caco-2 cell adherence and invasion assay

AB1 and AB2 strains were tested for their ability to adhere to and invade Caco-2 cells (sections 2.10.2 and 2.10.3). Bacterial suspensions of approximately 0.3 $O.D_{600nm}$ units were added apically to differentiated Caco-2 cell monolayers onto triplicate wells of 12-well plates. *E. coli* DH5α was included as a negative control and 81116 was included as a positive control. Internalisation was assayed by determining the viable count on blood-free campylobacter selective agar of gentamicin-protected bacteria isolated from Caco-2 lysates. The level of adhesion was assayed by determining the number of associated bacteria, i.e. adherent (not removed by the washes) and internalised bacteria, and deducting the numbers of internalised bacteria (obtained in the internalisation assay). It was noted that adhesion and invasion varied between experiments. Nevertheless, the assays were repeated several times and the data based on two independent adhesion and three independent invasion experiments is presented in Figs 38 and 39. Statistical analysis of the results using the *t*-test showed no statistically significant differences between the strains. Despite the lack of statistical significance, the mutants consistently showed approximately 2-fold enhanced ability to adhere to and invade Caco-2 cells.

5.5.2 Translocation of AB1 and AB2 across polarised Caco-2 cell monolayers

The ability of the *regX1* mutants to translocate across a polarised monolayer was compared to the parent 81116 in a translocation assay. The procedure followed is described in Part I (section 2.7). Viable bacteria in the basolateral medium were quantified every 1.5h. The numbers of translocated bacteria were determined by viable count on campylobacter blood-free selective agar plates of the bacteria in the basolateral medium. The results obtained indicated that, compared to the parental strain, the ability of the mutants to penetrate the monolayer was reduced (Fig.40). However, statistical analysis of the results using the *t*-test showed no statistically significant differences between the strains. In addition, the mutants were detected in the basolateral medium at an earlier time (2h) than the parent (3.5h). At the same time points, the transmonolayer electrical resistance was measured (see Part I, section 3.2). No significant changes were detected during the course of the experiment (Fig.41). There was a small increase in the transmonolayer resistance values following the first 2-3.5h post-infection with the mutant strains. This corresponded to the values with the highest SD and are most likely due to

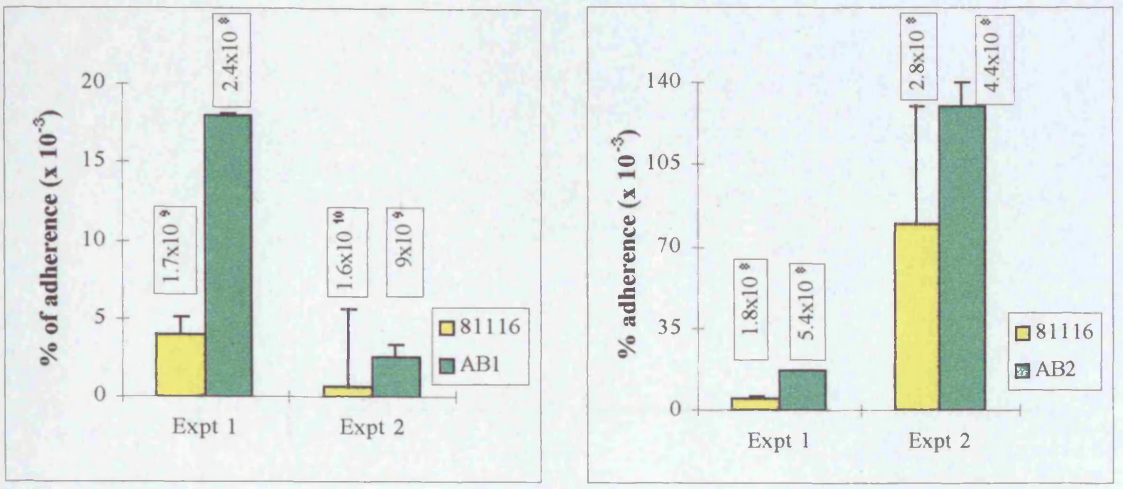


Fig.38. *C. jejuni* adherence to Caco-2 cell monolayers. The ability to adhere to Caco-2 cell monolayers was compared between 81116 and the mutants. The values are the mean (±SD) percentages of the number of adherent *C. jejuni* in the inocula derived from independent experiments. In each experiment, the means refer to triplicate determinations. Inocula added to the monolayers in each experiment are shown inside the boxes.

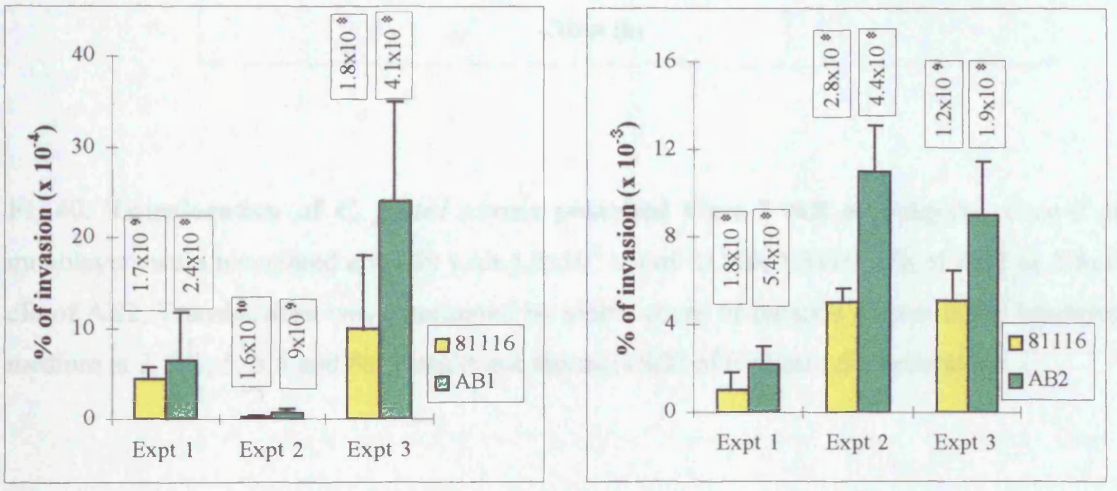


Fig.39. *C. jejuni* invasion of Caco-2 cell monolayers. The ability to invade Caco-2 cell monolayers was compared between 81116 and the mutants. The values are the mean (±SD) percentages of the number of internalised *C. jejuni* in the inocula derived from independent experiments. In each experiment, the means refer to triplicate determinations. Inocula added to the monolayers in each experiment are shown inside the boxes.

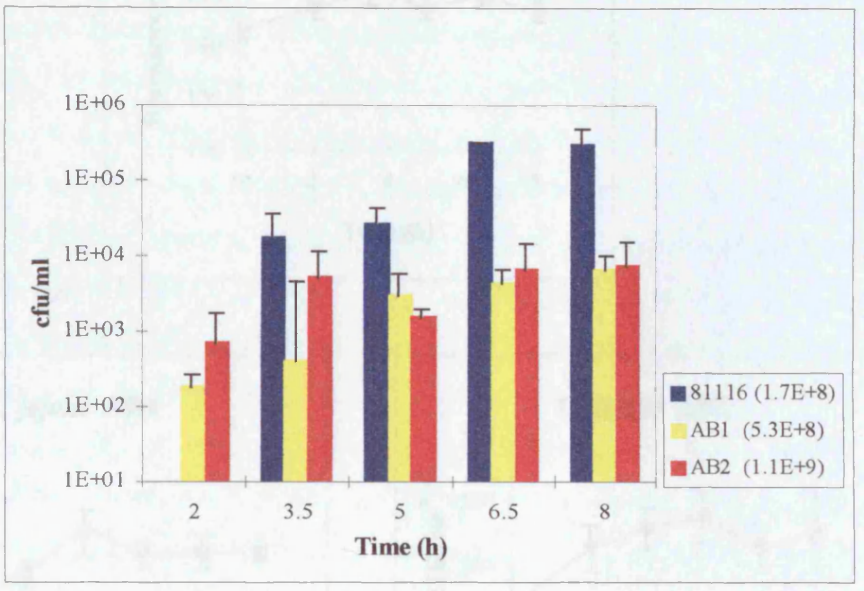


Fig.40. Translocation of *C. jejuni* across polarised Caco-2 cell monolayers. Caco-2 cell monolayers were inoculated apically with $4.2x10^7$ cfu of 81116, $1.3x10^8$ cfu of AB1 or $2.8x10^8$ cfu of AB2. Translocation was determined by viable count of bacteria present in the basolateral medium at 2, 3.5, 5, 6.5 and 8h. Results are means (±SD) of triplicate determinations.

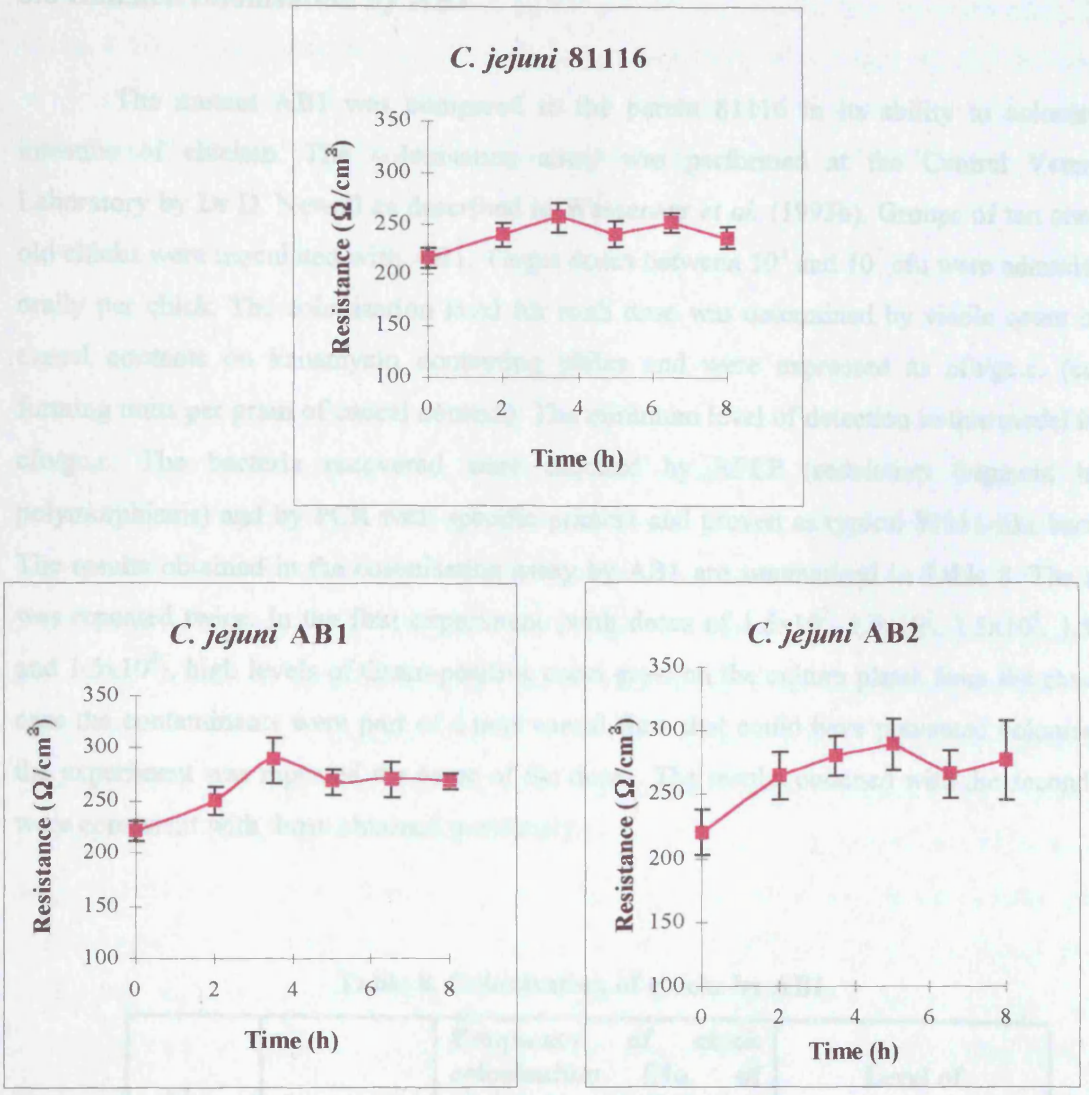


Fig.41. Variation of transmonolayer electrical resistance across infected Caco-2 monolayers. Caco-2 cell monolayers were infected with *C. jejuni* 81116 ($4.2x10^7$ cfu; positive control) and *C. jejuni regX1* mutants ($1.3x10^8$ cfu of AB1; $2.8x10^8$ cfu of AB2). Results are means (±SD) of triplicate determinations.

measurement errors. The data indicated that the infection of the cell monolayer with 81116, AB1 or AB2 did not significantly alter its integrity.

5.6 Chicken colonisation by AB1

The mutant AB1 was compared to the parent 81116 in its ability to colonise the intestine of chicken. The colonisation assay was performed at the Central Veterinary Laboratory by Dr D. Newell as described in Wassenaar *et al.* (1993b). Groups of ten one-day-old chicks were inoculated with AB1. Target doses between 10^2 and 10^7 cfu were administered orally per chick. The colonisation level for each dose was determined by viable count of the caecal contents on kanamycin containing plates and were expressed as cfu/gc.c. (colony forming units per gram of caecal content). The minimum level of detection in this model is 100 cfu/gc.c. The bacteria recovered were checked by RFLP (restriction fragment length polymorphisms) and by PCR with specific primers and proven as typical 81116-like bacteria. The results obtained in the colonisation assay by AB1 are summarised in Table 8. The assay was repeated twice. In the first experiment (with doses of $1.5x10^2$, $1.5x10^3$, $1.5x10^4$, $1.5x10^5$ and $1.5x10^6$), high levels of Gram-positive cocci grew on the culture plates from the caeca. In case the contaminants were part of a new caecal flora that could have prevented colonisation, the experiment was repeated for some of the doses. The results obtained with the second trial were consistent with those obtained previously.

Table 8. Colonisation of chicks by AB1.

Expts	Dose (cfu)	Frequency of chick colonisation (No. of birds from which *C. jejuni* was recovered/No of infected chicks)	Level of colonisation (cfu/gc.c.)
Expt 1	$1.5x10^2$	0/10	ND [b]
	$1.5x10^3$	3/10	10^2
	$1.5x10^4$	5/10	10^2-10^3
	$1.5x10^5$	3/9 [a]	10^2
	$1.5x10^6$	9/9 [a]	10^5
Expt 2	$8.5x10^3$	1/10	10^2
	$8.5x10^4$	3/10	10^2
Expt 3	$1.4x10^6$	7/10	10^4
	$1.4x10^7$	10/10	10^4

(a): one of the chicks in the group died.

(b): not detected, i.e., below the sensitivity of the test: between 0-99 cfu/gc.c. (see above).

The colonisation levels obtained for 81116 (Wassenaar *et al.*, 1993b) and AB1 are directly compared in Fig.42. The data shows that AB1 does not colonise the chicken intestine as well as the parent strain. The results published on the wild type 81116 (Wassenaar *et al.*, 1993b) established that a challenge dose of $5x10^3$ cfu resulted in 100% colonisation and a challenge dose of $5x10^5$ cfu allowed the maximum level of colonisation observed, with 10^8-10^9 cfu/gc.c. being recovered. In contrast, a challenge dose of 10^3 cfu of AB1 resulted in only 30% of colonisation and the maximum levels of colonisation observed for AB1 were of approximately 10^4-10^5 cfu/gc.c, obtained for an administered dose of 10^7-10^6 cfu.

5.7 Summary

The possible role played by *regX1* in the bacterial cell was investigated by characterising the phenotype of two *regX1* mutants, AB1 and AB2. The results obtained showed that the mutation of *regX1* affected *in vitro* microaerophilic growth. Although the mutant's growth rate at 37°C is similar to the parental strain, they showed a reduced growth rate at 42°C. At both temperatures, the mutants did not achieve the parental level of cell density and at 37°C they appeared to enter stationary phase at an earlier time. The mutation also interfered with gene expression, as would be expected for the mutation of a RR protein, resulting in a number of proteins being induced or repressed. The expression of some of these proteins was also found to be altered at 42°C. The mutants also interacted differently with an epithelial-like cell line. They adhered to and invaded Caco-2 cell monolayers more efficiently than the parent strain. In contrast, they translocated across Caco-2 cell monolayers less efficiently than the parent strain. In addition, the mutation resulted in AB1 having a reduced ability to colonise the intestine of chicken.

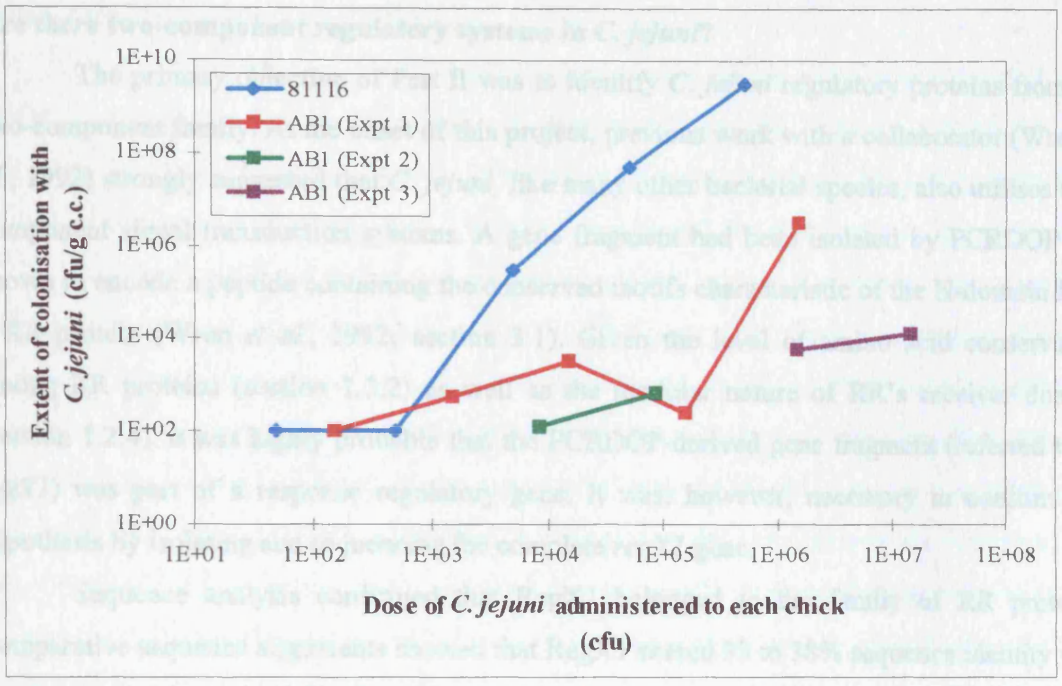


Fig.42. Colonisation of 1 day old chicks with *C. jejuni*. The ability of 81116 and AB1 to colonise the intestine of 1 day old chicks was compared. Colonisation was evaluated by both the level of caecal colonisation (i.e., number of viable bacteria recovered from the chicks caecal content) and by the frequency of chick colonisation (i.e., colonisation of all chicks tested; see Table 8). Each point in the graph represents the mean value of number of viable bacteria recovered from the caecum of ten chicks.

CHAPTER 6

Discussion and Conclusions

Are there two-component regulatory systems in *C. jejuni*?

The primary objective of Part II was to identify *C. jejuni* regulatory proteins from the two-component family. At the onset of this project, previous work with a collaborator (Wren *et al.*, 1992) strongly suggested that *C. jejuni*, like many other bacterial species, also utilises two-component signal transduction systems. A gene fragment had been isolated by PCRDOP and shown to encode a peptide containing the conserved motifs characteristic of the N-domain from a RR protein (Wren *et al.*, 1992; section 3.1). Given the level of amino acid conservation among RR proteins (section 1.2.2) as well as the modular nature of RR's receiver domain (section 1.2.4), it was highly probable that the PCRDOP-derived gene fragment (referred to as *regX1*) was part of a response regulatory gene. It was, however, necessary to confirm this hypothesis by isolating and sequencing the complete *regX1* gene.

Sequence analysis confirmed that RegX1 belonged to the family of RR proteins. Comparative sequence alignments showed that RegX1 shared 33 to 38% sequence identity with members of this protein family and particularly with members of the OmpR/PhoB/PhoP subfamily (section 3.7.3.1; Appendix 1; Stock *et al.*, 1989). Furthermore, the N-terminus from a second *orf* (*orf2*) was found adjacent to *regX1* on the same *C. jejuni* genomic clone (Fig.17, section 3.7.3). The *orf2* start codon overlaps the *regX1* stop codon, indicating that the two genes may be part of one operon. The analysis of the predicted amino acid sequence showed that Orf2 contained two transmembrane domains and thus, Orf2 is probably a transmembrane protein (Fig.18, section 3.7.3.2). Since HKs are most frequently transmembrane proteins and they are often adjacent on the chromosome to the associated RR, Orf2 might be the partner HK to RegX1. Moreover, comparative sequence alignments between the Orf2 predicted peptide and proteins available in GenEMBL and SwissProt databases revealed that Orf2 showed approximately 25% identity to two sensory proteins (Appendix 2). Structural features of the N-terminus region as well as the comparative sequence alignments indicated that Orf2 might be a HK. It will be, however, necessary to identify the HK conserved motifs in the C-terminus region of Orf2 to conclude that it belongs to the HK family of proteins. The Orf2 C-domain has not yet been isolated but whilst writing this thesis, an MSc student, M. Franklin, has been attempting to isolate the whole Orf2 from a *C. jejuni* genomic library for sequence analysis (M.

Franklin, unpublished data). Nevertheless, it is likely that Orf2/RegX1 are part of a two-component system and that *C. jejuni* utilises these regulatory mechanisms. RegX1 was the first RR to be described in *C. jejuni*. Other *C. jejuni* RR-like proteins have since been isolated (Henderson, 1996), corroborating the initial prediction of *C. jejuni* utilisation of two-component regulatory systems.

What is the role of RegX1 in *C. jejuni*?

The next question to be addressed was what is the nature of the role of RegX1 in *C. jejuni*? The basic two-component circuit is characterised by a signal, a sensor, a regulator and a target. So far, the RR protein had been identified and a putative partner HK had been isolated. It was necessary to determine the signal that activated RegX1 and the regulator's target.

RRs responsible for directing identical cellular responses share a high degree of similarity between C-domains (section 1.2.2). The hope was that comparative sequence alignments between the amino acid sequence from the C-domain of RegX1 and of other RR proteins available in GenEMBL and SwissProt databases would provide information regarding a possible role for RegX1. However, the sequence alignments failed to provide any information and only allowed the conclusion that RegX1 belonged to the OmpR/PhoB/PhoP subfamily of RRs (section 3.7.3.1; Appendix 1). In contrast, another RR protein recently isolated in this laboratory was identified as a CheY-like protein by comparative sequence alignments. The identity of the protein was later confirmed by mutation and analysis of the mutant's phenotype (J. Marchant, unpublished data). As for RegX1, determining its role in the bacterial cell required the mutation of the *regX1* locus by insertional inactivation (section 4.4). The phenotypic characterisation of *regX1* mutants could provide the answer to two major questions: what is the cellular target(s) of RegX1 and what is the environmental signal that activates RegX1-dependent pathway(s)?

Mutation of *regX1*

The construction of *regX1* mutants was performed in several stages (section 4.1) consisting of i) the disruption of the RR's Asp-51 by IPCRM in an *E. coli* background, ii) subcloning of a kanamycin cassette to interrupt the gene, iii) and replacing *C. jejuni* wild type chromosomal *regX1* by the disrupted copy of the gene through homologous recombination. The last stage was the limiting step in the isolation of the mutants (sections 4.3.1 and 4.3.2).

The first vector used to introduce the disrupted *regX1* gene in *C. jejuni* was a conjugative suicide vector, pALB1 (section 4.3.1). pALB1 is similar to another conjugative suicide vector, pILL560, used previously by Labigne-Roussel and colleagues to mutate *C. jejuni* 16S rRNA genes (Labigne-Roussel *et al.*, 1988). In contrast to pILL560, pALB1 failed to

deliver the mutation into the target locus. A possible reason to explain the lack of success of pALB1 versus pILL560 could be the use of different strains (O81 or P71 instead of C31, respectively). However, the fact that conjugation of the shuttle vector pUOA15 into strains O81 and P71 was possible (section 4.3.1.2) proved that plasmid DNA could be mobilised from *E. coli* SM10 into *C. jejuni* O81 and P71. A second possible reason arises from the strategy for the selection of transformants. In contrast to pALB1, the pILL560-derived construct carries the antibiotic marker (a kanamycin resistance gene) in the vector's insert and thus, the marker remains in the chromosome following either a single or a double recombination between the plasmid and the chromosome homologous sequences. The pILL560-based strategy allows selection of single and/or double recombinants whereas pALB1-based strategy only allows selection of single recombinants. According to several reports (Labigne-Roussel *et al.*, 1988; Wassenaar *et al.*, 1991; Wassenaar *et al.*, 1993a), *C. jejuni* merodiploids are apparently very unstable. In both cases, the authors did not obtain single recombinants, even though they employed different strategies to mutagenise different genes of *C. jejuni*. In recent years, this has also been the experience in this laboratory and 81116-derived single recombinants have never been isolated. In view of these reports, the means of selection might have been the reason why no pALB1-derived *regX1* mutants were detected and isolated, either by conjugation or electroporation. Furthermore, electroporation of *E. coli*-derived plasmids into *C. jejuni* 81116 occurs but at a low frequency (Wassenaar *et al.*, 1993a), which may have also prevented the isolation of pALB1-derived electrotransformants.

Despite the requirement for a two step selection and dependence on generating a merodiploid, the pALB1-based strategy was initially selected for two reasons. First, it did not involve the introduction of an antibiotic resistance gene in the bacterial chromosome which could potentially result in polar effects. Second, it would help to understand whether the mutation of *regX1* would be lethal for the bacterial cell. If so, the second cross over would fail to result in a mutant and the merodiploid would always revert to wild type. Alternatively, the pALB2-based strategy was technically simpler and involved one screening only. The introduction of a kanamycin resistance cassette into the chromosome could potentially result in polar effects but this type of approach had succeeded previously when used by Wassenaar *et al.* (1991) to isolate the flagellin mutants. Nevertheless, pALB2-based strategy was also not successful (section 4.3.2). By analysing the data obtained with the shuttle vector pUOA15 (section 4.3.2.2), it was possible to select the best conditions to electroporate *C. jejuni* 81116. Since the positive control (i.e., pUOA15) was electroporated and maintained in the strain, this suggested that the problem resided in the recombination event rather than in the transformation parameters or transformability of the strain. Wassenaar *et al.* (1991) utilised DNA fragments 0.7 and 1.6kb long to recombine into the chromosome of *C. jejuni* 81116. Moreover,

Richardson and Park did not detect homologous recombination into the chromosome of *C. coli* UA585 when the plasmid-borne homologous sequences (*katA*; Grant & Park, 1995) were less than 286bp long (Richardson & Park, 1997). It was possible that the efficiency in producing a *regX1* mutant was greatly influenced by the small size (320bp) of the DNA fragment expected to recombine into the bacterial chromosome. In fact, an increase in the size of the fragment from 320bp to 1.5kb led to the recombination event and to the isolation of two *regX1* mutants, AB1 and AB2 (section 4.4). In view of the data obtained with pALB5, the major obstacle to the production of a mutant was apparently the size of the DNA fragment available for homologous recombination into the chromosome.

During the selection that led to the isolation of AB1 and AB2, a total of 20 kanamycin resistant colonies were analysed (see section 4.4.4). Although colony PCR revealed that most of the clones (18 clones) contained the kanamycin resistance cassette (Fig.24, section 4.4.5.1), only two clones contained the cassette inserted into the *regX1* gene (AB1 and AB2; Fig.25, section 4.4.5.1). Where did pALB5 integrate in 81116 chromosome? The colony PCR with each pair of primers (R/F and R3/R4) was set in parallel (section 4.4.5.1). This excluded the possibility of these clones being merodiploids that underwent a second recombination event, during which the vector containing the mutated copy of the gene crossed out. Instead, these clones may result from a heterologous recombination. In this case, the colony PCR with primers R3 and R4 should have generated two bands: a band of ~0.3kb corresponding to the wild type copy of the chromosomal-equivalent *regX1* PCRDOP fragment and a band of ~1.7kb corresponding to the mutated copy of *regX1*. This was not the case and none of the clones possessed the 1.7kb band (Fig.25, section 4.4.5.1). The nature of these clones is not currently understood.

In order to confirm that AB1 and AB2 were true *regX1* mutants, the two clones were analysed by colony PCR (section 4.4.5.1) and southern blotting (section 4.4.5.2). The hybridisation reactions showed that the *Bgl*II restriction profile of AB1 chromosome changed and it appears that there was loss of one *Bgl*II site (Figs 26 and 27, section 4.4.5.2). In contrast, no abnormalities were detected in the restriction profiles with either *Bcl*I or *Msp*I. The highlighted bands in both chromosomal digests underwent the expected ~1.4kb size increase when compared to those in the parent chromosomal digests, corresponding to the insertion of the kanamycin cassette. This suggested that AB1 did not undergo massive chromosomal rearrangements. Nevertheless, the reason for the loss of the *Bgl*II site has not been determined but sequencing the *regX1* locus of the mutants would establish if there are differences in the way the insertional inactivation occurred in AB1 compared to AB2. As for AB2, the recombinant behaved as expected and the chromosomal bands that hybridised to both *regX1*

PCRDOP fragment and kanamycin resistance cassette DNA probes underwent the expected ~1.4kb size increase when compared to the parent hybridisation profiles.

What is the phenotype of *regX1* mutants?

The phenotype of the two *regX1* mutants was analysed and a number of differences were detected between the parental and mutant strains. The mutation in *regX1* interferes with gene expression, as would be expected from the mutation of a regulatory protein, resulting in a number of proteins being induced and repressed. This was observed by both one- and two-dimensional electrophoretic analysis of protein profiles (Figs 35 and 37, section 5.4). Therefore, RegX1 may be both a transcriptional activator and repressor. In addition, the level of induction of gene expression in the mutants was growth temperature-dependent. The differentially expressed ~59 and ~39kDa proteins were induced at a higher level when the mutants were grown at 37°C than at 42°C (Fig.35, section 5.4.1).

At least two of the induced proteins with Mr of approximately ~39 and ~33kDa were located in the cell membrane (Fig.36, section 5.4.1). Surprisingly, the 33kDa protein was absent in the profile of AB2. Although the mutants were constructed in the same way, their phenotypes are slightly different. The reason for this has not yet been determined but as mentioned above, comparative sequence analysis of the mutants *regX1* locus could establish the putative differences in the way the insertional inactivation occurred between AB1 and AB2. The level of induction of the ~39kDa membrane protein was also growth temperature-dependent and was higher when the mutants were grown at 37°C than at 42°C (Fig.35, section 5.4.1).

The data regarding the protein profiles clearly demonstrates how the mutation in *regX1* altered gene expression. The differentially expressed proteins are presumably under the control of RegX1. An approach to identify these proteins consists of deriving the N-terminal sequence, followed by the construction of DNA probes to isolate the respective encoding genes from a genomic library. In fact and whilst writing this thesis, another student, S. Chatterjee, has further examined the two-dimensional protein profile of AB1 to identify the genes encoding those proteins. Her results have confirmed that a number of changes occur in the gene expression profile of AB1 (S. Chatterjee, unpublished data). Eleven proteins with Mr ranging from 41 to 25kDa and pI between 5.5 and 7.5 are differentially expressed between AB1 and the parent. Some of these proteins (five proteins) are repressed in the mutant whereas other proteins (two proteins) are induced. Four proteins with Mr of approximately 41kDa and pI of 5.5-6.0, 6.0-6.5 and 6.5-7.0 and with Mr of approximately 6.5kDa and pI of 7.0-7.5 are repressed in 81116 at 42°C but are induced in AB1 at 42°C. At least two of these proteins (Mr~41kDa and pI:6.0-6.5 and 6.5-7.0) have been sequenced. N-terminal sequencing data revealed that the two bands

corresponded to the same protein. Comparative sequence alignments showed that the 41kDa protein has no significant homology to proteins already available in databases. Nevertheless, a degenerate oligonucleotide primer has been designed in an attempt to construct a DNA probe which will subsequently be used to isolate the complete gene. Furthermore, one of the proteins of approximately 25kDa which is absent in the mutant's profile has also been sequenced and identified as RegX1. This demonstrates that the mutation has definitely inactivated the gene and *regX1* is not expressed by AB1. In addition, it also shows that the cellular basal level of expression of the regulator is high, since its selection for N-terminal sequencing was based on the intensity of the band on the gel. Why would the bacterial cell produce a large amount of a RR protein? It may be because *C. jejuni* permanently requires RegX1, i.e., RegX1 plays a central role in the bacterial cell or it may be because RegX1 acts upon multiple targets. As yet, there is no evidence that offers an explanation for this and these explanations are speculative. When more of the differentially expressed proteins have been identified, it may be easier to understand the importance of RegX1 in the bacterial cell. It will also be important to identify the membrane-bound 39 and 33kDa proteins (Fig.36, section 5.4.1), as bacterial membrane components are likely to be involved during pathogen-host cell interactions.

Having established that the mutation in *regX1* clearly affected gene expression, another question emerged: what processes are these genes involved in?

Are RegX1-dependent genes involved in growth?

RegX1 may be involved in the regulation of genes involved in growth at higher temperatures. At 42°C, but not 37°C, the mutation clearly alters growth rate in *in vitro* microaerophilic conditions (Figs 30a and 30b, section 5.3). The changes in the growth rate may result from the inability of the mutants to respond to higher temperatures given that their growth rate declines when they are shifted from 37°C to 42°C (Fig.34, section 5.3). However, the mutants seem to recover rapidly and 2h after the temperature shift, their growth rate appears similar to the rate observed for the cultures kept at 37°C, i.e., similar to the parental growth rate. This suggests that it is sufficient that AB1 and AB2 are incubated at 37°C during early exponential growth phase to acquire the ability to respond to a higher temperature. Additionally, the mutants are incapable of achieving the parental level of cell density when cultured at both temperatures and enter stationary phase earlier when cultured at 37°C. The mutant strains do not arrest growth at a lower cell density than the parental strain due to a decrease in nutrients available in the growth medium because 81116 always grew to higher cell density (Fig.30a, section 5.3). The time at which cultures reached stationary phase did not

depend on the inoculum size because no major effects were noticed on the growth pattern of AB1 by varying the size of the inocula (Fig.33, section 5.3).

Are RegX1-dependent genes important in host cell interaction?

RegX1 may also be involved in the regulation of genes important in host cell interaction. When tested *in vitro*, no statistically significant differences were detected in the ability of the mutants to interact with the Caco-2 cell line. However, the mutants consistently adhered to and invaded Caco-2 cells approximately 2-fold more efficiently than the parent (Figs 38 and 39, section 5.5.1). As yet, there is no experimental evidence relating the changes in the membrane protein profile of the mutants and their interactions with Caco-2 cells. It is conceivable however, that these changes may be involved in the enhanced ability of AB1 and AB2 to adhere to Caco-2 cells. An increased adherence could subsequently lead to enhanced internalisation. However, it is not known if the increase in internalisation is a consequence of an increase in adherence. A possible approach to determine whether these changes in membrane constituents would lead to an increase in the mutants' adherence to and invasion rates of Caco-2 cells would be to incubate host cells with pre-labelled bacteria. Comparison of the radioactive protein profiles between the strains could determine whether the parent expressed any of the genes constitutively activated in the mutants.

The possible involvement of RegX1 in the regulation of genes important in host cell interaction is also supported by the translocation phenotype of the mutants. During translocation across Caco-2 cell monolayers, AB1 and AB2 were detected in the basolateral medium at an earlier time (2h post-inoculation) than the parent (3.5h post-inoculation; Fig.40, section 5.5.2). This may arise from the mutants adhering to Caco-2 cells 2-fold more efficiently than the wild type. Consequently, they may start translocation sooner after infection. However, despite the lack of statistical significance, AB1 and AB2 consistently translocated across Caco-2 cell monolayers in lower numbers than the parent. This could be explained if the level of reversible attachment (see General Introduction, section 4.4.3.2) by the mutants was higher than by the parent, even though they would bind the host cells more often.

Are RegX1-dependent genes important in *in vivo* colonisation?

RegX1 or RegX1-dependent genes seemed to be required for the colonisation of the intestine of chicken. Compared to the parent, AB1 is a poor coloniser (Table 8, section 5.6). The results published on the wild type 81116 (Wassenaar *et al.*, 1993b) established that a challenge dose of 10^3 cfu resulted in 100% colonisation (i.e., all chicks tested were colonised) and a challenge dose of $5x10^5$ cfu allowed the maximum level of colonisation observed, with 10^8-10^9 cfu/gc.c. being recovered. In contrast, a challenge dose of 10^3 cfu of AB1 resulted in

only 30% of colonisation and the maximum levels of colonisation observed for AB1 were of approximately 10^4-10^5 cfu/gc.c, obtained for an administered dose of 10^7-10^6 cfu (Fig.42, section 5.6). So, has the inactivation of RegX1 influenced the ability of *C. jejuni* to colonise the chicken intestine?

No differences in motility were detected between the mutant and the parent (section 5.2.1), although the methods used only allowed a qualitative evaluation. Colonisation ability has also been shown to depend on LPS profile in *S. california* and *S. typhimurium* (Craven *et al.*, 1993). In the case of AB1 and 81116, no differences in LPS profile were noted between the two strains (Fig.29, section 5.2.2). Nevertheless, immunoblotting would have been a more sensitive method to analyse *C. jejuni* LPS profile than silver staining (Preston & Penner, 1987). The growth characteristics of AB1 may also account for the colonisation results obtained. The physiological temperature of chickens is 42°C and, as discussed above, AB1 grows less efficiently at 42°C than the parent and never reaches the same level of cell density. Testing AB1 in a mammalian model whose physiological temperature is 37°C could indicate if the phenotype is related to temperature. If the mutant colonises the intestine of a mammal as efficiently as the wild type, temperature may be responsible for the results obtained with the chicken model. However, this would only be speculative as such a conclusion would require a more indepth understanding of the factors involved in the colonisation of the intestine of both models. This is not the case and so it would still not be possible to reject this hypothesis. Nevertheless, AB1 has recently been tested in a rabbit model by M. Franklin. The results show that although the difference between the ability of 81116 and AB1 to colonise the rabbit intestinal tract is not so marked as in the chicken model, the mutant does not perform as well as the parent. It is possible that the slower growth rate of AB1 at 42°C is an important factor in decreasing the success of the mutant in colonising the chicken gastrointestinal tract. The results obtained in the rabbit model may also result from the fact that AB1 is unable to achieve the parental level of cell density at 37°C. This hypothesis could be tested if the genes under the control of RegX1 were isolated and mutated. By characterising each mutant's phenotype, it could be possible to identify the genes responsible for AB1 growth features. Subsequently, it would be possible to investigate whether the mutant(s) in growth-related gene(s) and *regX1* mutant showed the same colonisation characteristics and therefore, determine whether colonisation and growth profiles were related. However, it is not currently known which genes RegX1 regulate and whether RegX1 acts upon one or more operons. A more immediate but not so useful approach to clarify this matter would be to make the *in vitro* growth assays as relevant as possible from the *in vivo* point of view. To what extent may one actually extrapolate *in vivo* bacterial growth features from *in vitro* ones? Because the intestinal tract may be the natural

habitat of *C. jejuni*, it would be useful to compare growth profiles between AB1 and 81116, using intestinal mucus from the chicken or rabbit as a growth medium and incubate the strains in microaerophilic conditions at 42°C or 37°C, respectively. Such assay would still be different from the chicken or rabbit intestinal environment but could be a better means to evaluate to what extent the *in vitro* AB1 growth features may influence its performance in the intestinal tract of the animal models.

Alternatively, the changes in gene expression between AB1 and 81116 may be responsible for the different phenotypes regarding the ability to colonise the intestine of chickens. Stern and colleagues reported that two human clinical isolates (A-74/O and B-73) did not colonise the intestine of chicken although A-74 became a coloniser (A-74/C) after being repeatedly passaged (Stern *et al.*, 1988). Subsequently, Meinersmann and colleagues compared the outer membrane antigenic profiles between the non-colonising A-74/O and the colonising A-74/C variants (Meinersmann *et al.*, 1990). These authors used serum obtained from rabbits immunised with OMP from either A-74/O or A-74/C in western blots to identify antigenic differences between the variants. In fact, they identified a 69kDa antigen found in the colonising but not in the non-colonising variant. Considering that 81116 and AB1 show different membrane protein profiles, one could speculate that the protein differences may be responsible for the differences in colonisation ability. A possible approach to establish whether this is true would involve the identification of differences between *in vitro* and *in vivo*-induced protein profiles. This could be accomplished by introducing semipermeable chambers containing either AB1 or 81116 into the intestine of chicks and carrying out a colonisation assay (Wassenaar *et al.*, 1993b). Comparison between the 2-D protein profile of the two strains could allow the identification of proteins differentially expressed *in vitro* and *in vivo* between mutant and parental strains.

In summary, several phenotypic differences were found between the parental and mutant strains. In view of all the information gathered in chapter 5, the two initial questions are reiterated: what is the cellular target(s) of RegX1 and what is the environmental signal that activates RegX1-dependent pathway(s)?

What is the cellular target of RegX1?

RegX1 may be involved in the regulation of genes involved in growth, host cell interaction, chicken colonisation. It is important that the genes belonging to *regX1*'s operon are identified. Their characterisation may allow the identification of both the input signal and the target of the system. It is not possible, however, to exclude the possibility that RegX1 may

control the expression of different operons. Considering the phenotypic features of the *regX1* mutants, this may be the case.

What is the input signal in Orf2/RegX1 system?

The nature of the input signal is not yet clear but according to Konkel and colleagues, the effect of growth temperature on deposition of surface proteins and adherence may indicate the presence of a regulatory system that is directly affected by temperature (Konkel *et al.*, 1992). The results presented in chapter 5 show how the mutation altered growth at 42°C as well as the ability of AB1 to colonise the intestine of chickens. In addition, the mutation affects gene expression in a temperature-dependent manner. Temperature certainly seems to be a potential candidate for an input signal. Assuming that RegX1 is autoregulated, the environmental signal activating RegX1 could be identified by constructing a fusion between the *regX1* promoter and promoterless *cat* (chloramphenicol acetyl transferase) gene. The construct would need to be cloned into a shuttle vector with a *Campylobacter*-derived origin of replication and introduced into the mutants. Subsequently, transcription of the *cat* gene through the *regX1* promoter could be examined by assaying chloramphenicol acetyl transferase activity in response to several growth parameters such as different temperatures, pH or osmotic stress. A similar approach was employed by Wooldridge *et al.* (1994) to study the regulation of the *C. jejuni fur* homologue.

Orf2/RegX1 circuit

Based on the results discussed in chapter 6, two models are proposed as a possible Orf2/RegX1 circuit (Figs 43a and 43b). In these models, temperature is proposed as the putative environmental stimulus underlying Orf2/RegX1 signal transduction events.

C. jejuni is found *in vivo* at 42°C when colonising the intestine of chicken and does not cause any significant pathology, in contrast to the colonisation of the human intestine at 37°C. Temperature is perhaps an important environmental cue for the bacterium to identify its surroundings. Therefore, the fact that growth at higher temperatures has been altered by the mutation is of interest. RegX1 may regulate, directly or indirectly, a set of genes important in temperature-related growth characteristics. A number of genes have been repressed in *regX1* mutants. If they were involved in temperature-related growth, their repression would affect the growth pattern of the mutants not only at 42°C but perhaps at 37°C, too. Furthermore, AB1 is unable to efficiently colonise the intestine of chicken. Currently, very little is understood about *C. jejuni* colonisation determinants but their expression is presumably induced by specific substances present in the chicken intestinal tract (Stern *et al.*, 1988). It may be that the mutation in *regX1* has led to the repression of bacterial factors necessary in the recognition process

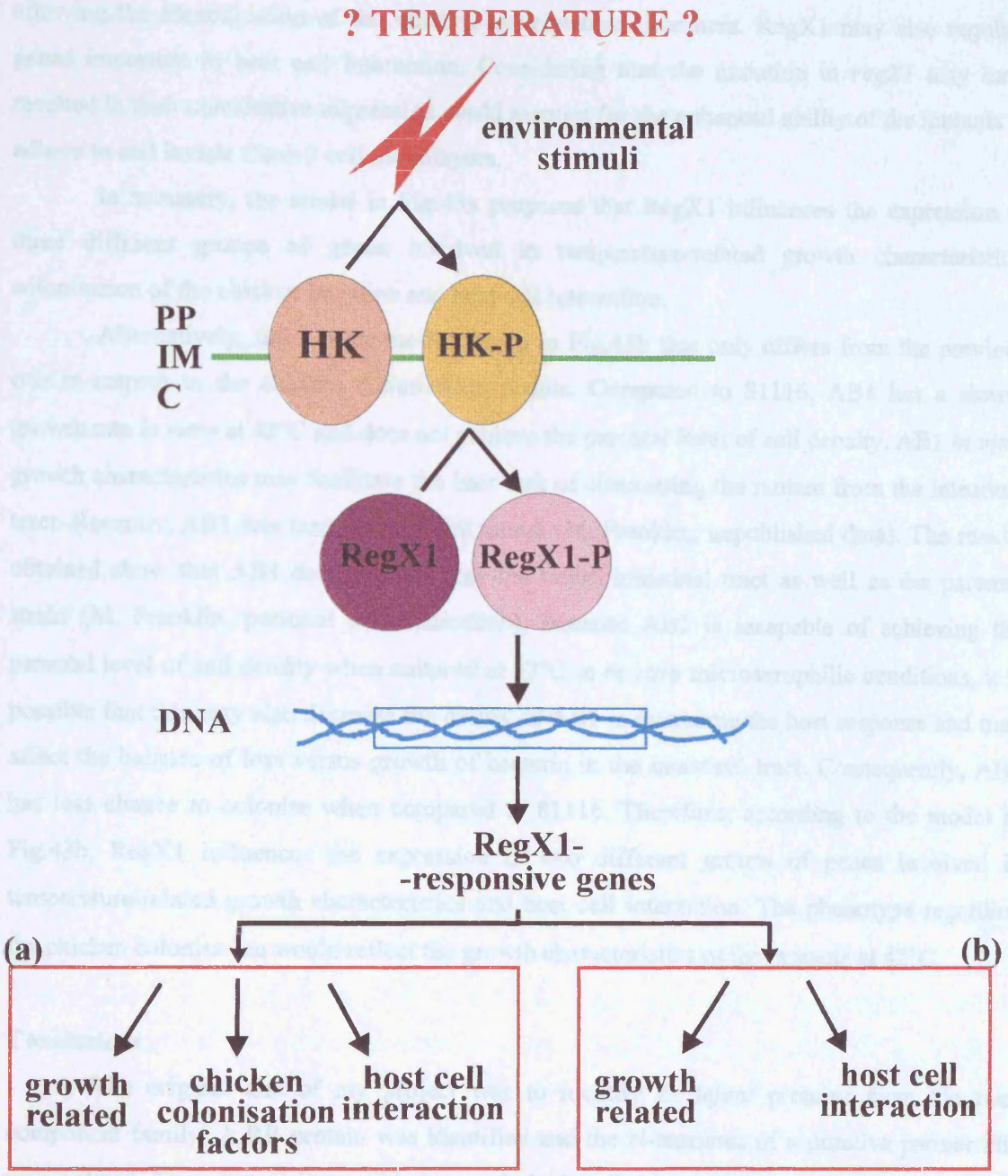


Fig.43. Signal pathway model for RegX1. The model proposes that temperature is the environmental signal responsible for activating the system. As a result, the activated RegX1 will act upon different sets of genes. The only difference between (a) and (b) is that the model in (b) suggests that the phenotype regarding chicken colonisation is due to growth-related set(s) of genes. The abbreviations in the figure are: PP, periplasm; IM, inner membrane; C, cytoplasm.

allowing the identification of the chicken-associated environment. RegX1 may also regulate genes important in host cell interaction. Considering that the mutation in *regX1* may have resulted in their constitutive expression could account for the enhanced ability of the mutants to adhere to and invade Caco-2 cell monolayers.

In summary, the model in Fig.43a proposes that RegX1 influences the expression of three different groups of genes involved in temperature-related growth characteristics, colonisation of the chicken intestine and host cell interaction.

Alternatively, there is the model shown in Fig.43b that only differs from the previous one in respect to the chicken colonisation results. Compared to 81116, AB1 has a slower growth rate *in vitro* at 42°C and does not achieve the parental level of cell density. AB1 *in vitro* growth characteristics may facilitate the host task of eliminating the mutant from the intestinal tract. Recently, AB1 was tested in a rabbit model (M. Franklin; unpublished data). The results obtained show that AB1 does not colonise the rabbit intestinal tract as well as the parental strain (M. Franklin, personal communication). Because AB1 is incapable of achieving the parental level of cell density when cultured at 37°C in *in vitro* microaerophilic conditions, it is possible that this may also decrease the ability of AB1 to overcome the host response and may affect the balance of loss versus growth of bacteria in the intestinal tract. Consequently, AB1 has less chance to colonise when compared to 81116. Therefore, according to the model in Fig.43b, RegX1 influences the expression of two different groups of genes involved in temperature-related growth characteristics and host cell interaction. The phenotype regarding the chicken colonisation would reflect the growth characteristics of the mutants at 42°C.

Conclusions

The original aim of my project was to identify *C. jejuni* proteins from the two-component family. A RR protein was identified and the N-terminus of a putative partner HK was isolated. Therefore, it is possible to conclude that *C. jejuni* utilises two-component signal transduction pathways. Since the work that led to Part II has been initiated, other *C. jejuni* RR proteins have been described, confirming the initial hypothesis underlying my project.

In addition to structural analysis, RegX1 and RegX1-dependent pathway(s) was examined by investigating the phenotype of two *regX1* mutants. A complex picture has emerged as to what the role of *regX1* may be. Clearly, some of the interpretations of the results need further investigation but with future work, it is possible that RegX1 will be implicated in the regulation of important pathway(s) in *C. jejuni*.

In view of *regX1* mutant reduced ability to colonise the intestine of chickens, RegX1 has been renamed RacR (reduced ability to colonise) and the genes in the *racR*-dependent operon(s) will be termed *rag* (RacR activated genes) or *rrg* (RacR repressed genes).

GENERAL CONCLUSIONS

At the outset of the work described in this thesis, the ultimate objective was to contribute to our understanding of *C. jejuni* pathogenesis. Two projects were designed and investigated in parallel. Each project focused on a potentially relevant aspect of *C. jejuni* pathogenesis: the characterisation and the regulation of *C. jejuni* virulence mechanisms. The specific objectives introduced, investigated and discussed in Part I consisted of characterising the possibility of *C. jejuni* penetrating the epithelium through a paracellular route and, in Part II, consisted of identifying *C. jejuni* regulatory proteins from the two-component family and determining whether these regulators were important in the control of the interactions between *C. jejuni* and the host. The two related pieces of work have provided experimental data that can be put into the context of *C. jejuni* pathogenesis and used to add further detail to the putative model (General Introduction, section 4.6) of how this organism causes disease.

C. jejuni is a food-borne pathogen. The organism enters the host through the oral-faecal route and can colonise not only humans but also a variety of animals. Inside a host, *C. jejuni* is exposed to different microenvironments before reaching its niche. By analogy with other enteropathogens, two-component regulatory systems presumably have a role with providing the bacterium with a way to adapt to the different conditions found inside the host. Chemotactic factors may guide the microrganism towards the right niche within the host via the two-component system regulating chemotactic responses (J. Marchant, unpublished data). In the intestine, it is important that the bacterium recognises the different host conditions. *C. jejuni* infects a variety of hosts but the pathological consequences of colonisation are host specific; this may result from different host responses to a set of bacterial factors or due to a host-dependent difference in bacterial response. In the latter situation, assuming that the various host-associated microenvironments are specific to that particular host, RRs like RegX1 (RacR) may enable the bacterium to distinguish between, for example, the intestine of humans and chickens. The recognition process is, perhaps, dependent on the ability to respond to changes of temperature. In the intestine of chickens, at 42°C, RegX1 may activate the expression of genes related with growth at higher temperatures and/or chicken colonisation determinants. In a human host, at 37°C, RegX1 may repress these genes and activate others involved in host cell interaction. In addition, other two-component regulatory systems may be necessary for the bacterial adaptation and survival, for example, the OmpR-dependent system to respond to

changes in osmolarity. Ultimately, the bacterium will need all its sensory and regulatory machinery to successfully colonise a host.

By analogy with the closely related *Helicobacter pylori*, two-component regulatory systems may be of great importance in the regulation of gene expression in *C. jejuni*. The complete genomic sequence of *H. pylori* was published recently (Tomb *et al.*, 1997), revealing that *H. pylori* utilises a reduced number of global regulatory proteins when compared to *E. coli*. *H. pylori* uses two-component systems and four HK and seven RR were found. These numbers are similar to those found in *H. influenzae* which has a similar size genome to *H. pylori* (and *C. jejuni*) but represent a third of those found in *E. coli* whose genome is also bigger. The entire genome of *C. jejuni* has not yet been sequenced. Therefore, it is only possible to speculate that a similar situation could be applied to *C. jejuni*, given its closeness to *H. pylori*. If this will be the case, RR proteins may be of great importance in transcriptional regulation of gene expression in *C. jejuni* in response to environmental pressures. To date, five RRs have been identified in *C. jejuni*: RacR, CheY, RegX3, a PhoP-like (C. Jones, personal communication) and a CpxR (considered by others as OmpR-like).

Once the organism is in an intestinal niche, for example, the intestine of a susceptible human, the bacterium will utilise the regulatory mechanisms required to control the expression of the necessary virulence determinants. *C. jejuni* colonises the intestinal mucus layer and attaches to the mucosa. The bacterium may release toxin(s) and invade the epithelial cells. As a consequence of cell damage due to cytotoxins or host cell invasion or, alternatively, due to direct translocation, campylobacters gain access to tissues underlying the intestinal epithelium. The data presented in this thesis supports the notion that translocation probably occurs mainly through the transcellular pathway. However, it is possible that paracellular translocation occurs at a later stage of the infection process and only arises from the opportunity created by the cytopathic effects produced by cytotoxins or during invasion and transcytosis. These different virulence characteristics culminate with *C. jejuni* disrupting the epithelial cell function and causing tissue damage and inflammation. Tissue damage and inflammation lead to inflammatory diarrhoea.

In summary, according to the objectives of Part II, a RR (RacR) was characterised and shown to be important in the cell physiology of *C. jejuni* and may play a role in adaptation to growth in the intestine. According to the objectives of Part I, it was shown that once *C. jejuni* is in the intestine, translocation through the paracellular route may be only occurring late, during the infection. *C. jejuni* seems more likey to take the transcellular pathway to exit the host cell and enter the underlying tissue. Questions remain as to the nature of the signal that activates RacR and the identity of the members of the regulon that RacR controls. Furthermore, once the

bacterium is adapted to growth in the intestine, it is necessary to determine the mechanisms by which *C. jejuni* enters and exits the host cell. Current and future studies address these questions which, when answered, will contribute towards a complete understanding of how *C. jejuni* colonises the intestine and causes disease.

APPENDICES

APPENDIX 1

Comparative Sequence Analysis of *regX1*

Based on the results from the comparative sequence analysis discussed in section 3.7.3.1, a multiple alignment between the predicted amino acid sequence of RegX1 and the amino acid sequences from the six most homologous RR was compiled. The alignment was obtained using the computer program PILEUP (Devereux *et al.*, 1984). Information regarding the name, organism, role if known and accession number for the proteins in the alignment were presented in Table 9. The highly conserved aspartate (D) and lysine (K) residues are highlighted in bold. The asterisks correspond to the residues forming the hydrophobic cores common to all response regulators.

```
          1                                                                  80
REGX1_CJ  .........  .........  ..MINVLMIE  DDPDFAQLLS  EYLAQFNIKI  TNFGNPKSAL  .NVGI.....  ....QGYDCL
OMPR_CJ   .........  .........  ..MTNILMIE  DDLELAEITA  EYLEKFDMKV  DIAHEPYIGL  SKLAL.....  ....KEYQLI
OMPR_EC   .........  ........MQ  ENYK.ILVVD  DDMRLRALLE  RYLTEQGFQV  RSVANAEQMD  RLL.......  ..TRESFHLM
OMPR_ST   .........  ........MQ  ENYK.ILVVD  DDMRLRALLE  RYLTEQGFQV  RSVANAEQMD  RLL.......  ..TRESFHLM
VIRG_AT   .........  MAGQDPRLRG  EPLKHVLVID  DDVAMRHLIV  EYLTIHAFKV  TAVADSKQFN  RVL.......  ..CSETVDVV
BAER_EC   .........  ...MTELPID  ENTPRILIVE  DEPKLGQLLI  DYLRAASYAP  TLISHGDQVL  PYVR......  ...QTPPDLI
VANR_EF   .........  .........  .MSDKILIVD  DEHEIADLVE  LYLKNENYTV  FKYYTAKEAL  ECID......  ...KSEIDLA
                                   ****       *  **    **                                      **
```

```
          81                                                                                         160
REGX1_CJ  ILDLTLPGID  GLEVCREIRQ  KS.NIPIIIS  SARGDLSDKV  VGLQIGADDY  LPKPYDPKEM  YARIMSLIRR  TKRVEHTNNE
OMPR_CJ   ILDLSLPGLD  GLEVCEEIRK  KY.DTPIIVS  SARHDITDKV  NALELGADDY  LPKPYNPKEL  QARIKSHLRR  ISNTKSAIAK
OMPR_EC   VLDLMLPGED  GLSICRRLRS  QSNPMPIIMV  TAKGEEVDRI  VGLEIGADDY  IPKPFNPREL  LARIRAVLRR  QANELPGAPS
OMPR_ST   VLDLMLPGED  GLSICRRLRS  QSNPMPIIMV  TAKGEEVDRI  VGLEIGADDY  IPKPFNPREL  LARIRPVLRR  QANELPGAPS
VIRG_AT   VVDLNLGRED  GLEIVRSLAT  KSDVPIIIIS  GARLEEADKV  IALELGATDF  IAKPFGTREF  LARIRVALRV  RP...SVART
BAER_EC   LLDLMLPGTD  GLTLCREIRR  FS.DIPIVMV  TAKIEEIDRL  LGLEIGADDY  ICKPYSPREV  VARVKTILRR  CKPQRELQQQ
VANR_EF   ILDIMLPGTS  GLTICQKIRD  KH.TYPIIML  TGKDTEVDKI  TGLTIGADDY  ITKPFRPLEL  IARVKAQLRR  YKKFSGVKEQ
          **           * **  *          ****                                   *     *  **

          161                                                                                        240
REGX1_CJ  NIN.......  .SAFKIDERR  HEITYEDK.V  LTLTPAEFEI  LEYLIQQHGY  SVSREQLVSR  CKNLKDK..D  S.KSLDVIIG
OMPR_CJ   SVK.......  ..DLVYDQYK  HIITMKGQ.E  LTLTNAEFDI  LSYLIKKEGG  VVSREELVYN  CSSISED..S  SNKSIDVIIR
OMPR_EC   QEE...AVIA  FGKFKLNLGT  REMFREDEP.  MPLTSGEFAV  LKALVSHPRE  PLSRDKLMNL  ARGREYSAME  ..RSIDVQIS
OMPR_ST   QEE...AVIA  FGKFKLNLGT  REMFREDEP.  MPLTSGEFAV  LKALVSHPRE  PLSRDKLMNL  ARGREYSAME  ..RSIDVQIS
VIRG_AT   KDR...RSFS  FADWTLNLRR  RRLISEEGSE  VKLTAGEFNL  LVAFLEKPRD  VLSREQLLIA  SRVREEEVYD  ..RSIDVLIL
BAER_EC   DA....E...  .SPLIIDEGR  FQASWRGK.M  LDLTPAEFRL  LKTLSHEPGK  VFSREQLLNH  LY.DDYRVVT  D.RTIDSHIK
VANR_EF   NE....NVIV  HSGLVINVNT  HECYLNEK.Q  LSLTPTEFSI  LRILCENKGN  VVSSELLFHE  IWGDEYFSKS  N.NTITVHIR

          241                                          250
REGX1_CJ  RLRVKIGD.S  SKSPKHIFSV  RGIGYKLIG.  .......... .
OMPR_CJ   EF........  ..........  ..........  .......... .
OMPR_EC   RLRRMVEE.D  PAHPRYIQTV  WGLGYVFVPD  GSKA...... .
OMPR_ST   RLRRMVEE.D  PAHPRYIQTV  WGLGYVFVPD  GSKA...... .
VIRG_AT   RLRRKLEG.D  PTTPQLIKTA  RGAGYFFDAD  VDVSYGGVMA A
BAER_EC   NLRRKLESLD  .AEQSFIRAV  YGVGYRWEAD  ACRIV..... .
VANR_EF   HLREKMNDTI.  DNPKYIKTV  WGVGYKIEK.  .......... .
```

In addition, another multiple alignmnent was compiled using the same computer program PILEUP (Devereux *et al.*, 1984) as before. This second alignment compared the amino acid sequence of the C-terminus regions from RegX1 and from the eight most homologous RR. Information regarding the name, organism, role if known and accession number for the proteins in the alignment were presented in Table 9.

```
          151                                                                 230
REGX1_CJ  TKRVEHTNNE  .NINSA....  .FKIDERRHE  ITYEDKVLTL  TPAEFEILEY  LIQQHGYSVS  REQLVSRCKN  LKD...KDSK
PHOP_EC   NSG.......  .LASQVISLP  PFQVDLSRRE  LSINDEVIKL  TAFEYTIMET  LIRNNGKVVS  KDSLMLQLYP  DAE..LRESH
PHOP_ST   NSG.......  .LASQVINIP  PFQVDLSRRE  LSVNEEVIKL  TAFEYTIMET  LIRNNGKVVS  KDSLMLQLYP  DAE..LRESH
BASR_ST   HNN.......  .QGESELTVG  NLTLNIGRHQ  AWRDGQELTL  TPKEYALLSR  LMLKAGSPVH  REILYNDIYN  WDN..EPSTN
OMPR_CJ   ISNT..KSAI  AKSVKDLVYD  ....QYKH.I  ITMKGQELTL  TNAEFDILSY  LIKKEGGVVS  REELVYNCSS  ISED..SSNK
BAER_EC   CKP..QRELQ  QQDAE....S  PLIIDEGRFQ  ASWRGKMLDL  TPAEFRLLKT  LSHEPGKVFS  REQLLNHLY.  .DDYRVVTDR
VANR_EF   YKK..FSGVK  EQNENVIVHS  GLVINVNTHE  CYLNEKQLSL  TPTEFSILRI  LCENKGNVVS  SELLFHEIWG  .DEYFSKSNN
OMPR_EC   QANEL.PGAP  SQEEAVIAFG  KFKLNLGTRE  MFREDEPMPL  TSGEFAVLKA  LVSHPREPLS  RDKLMNLARG  ..REYSAMER
OMPR_ST   QANEL.PGAP  SQEEAVIAFG  KFKLNLGTRE  MFREDEPMPL  TSGEFAVLKA  LVSHPREPLS  RDKLMNLARG  ..REYSAMER

          231                                                  279
REGX1_CJ  SLDVIIGRLR  VKIGD.SSKS  PKHIFSVRGI  GYKLIG....  ..........
PHOP_EC   TIDVLMGRLR  KKIQAQYPQE  V..ITTVRGQ  GYLFELR...  ..........
PHOP_ST   TIDVLMGRLR  KKIQAQYPHD  V..ITTVRGQ  GYLFELR...  ..........
BASR_ST   TLEVHIHNLR  DKV....GKS  R..IRTVRGF  GYMLVATEES  ..........
OMPR_CJ   SIDVIIREF.  ..........  ..........  ..........  ..........
BAER_EC   TIDSHIKNLR  RKLESLD.AE  QSFIRAVYGV  GYRWEADACR  IV........
VANR_EF   TITVHIRHLR  EKMNDTI.DN  PKYIKTVWGV  GYKIEK....  ..........
OMPR_EC   SIDVQISRLR  RMVEE.DPAH  PRYIQTVWGL  GYVFVPDGSK  A.........
OMPR_ST   SIDVQISRLR  RMVEE.DPAH  PRYIQTVWGL  GYVFVPDGSK  A.........
```

Table 9. Two-component response regulator proteins used in the amino acid sequence alignements between the predicted RegX1 and the nine most homologous proteins.

Protein	Organism	Function	Accession Number
BaeR	*E. coli*	Unknown	P30846
BasR	*S. typhimurium*	Virulence	P36556
OmpR	*E. coli*	Osmoregulation	P03025
OmpR	*S. typhimurium*	Osmoregulation	P08981
OmpR	*C. jejuni*	Homologous to OmpR from *Pseudomonas aeruginosa*	P27271
PhoP	*E. coli*	Regulation of acid phosphatase activity	P23836
PhoP	*S. typhimurium*	Virulence	P14146
RegX1	***C. jejuni***		**This study**
VanR	*Enterococcus faecium*	Resistance to vancomycin	Q06239
VirG	*A. tumefaciens*	Virulence	P07545

APPENDIX 2

Comparative Sequence Analysis of *orf2*

As discussed in section 3.7.3.2, Orf2 contained two putative transmembrane domains. The results obtained from database searches indicated that the similarities found between Orf2 and other proteins arose mainly from the amino acid sequence from these regions. The proteins Orf2 was compared to were arranged in two groups: one (A) comprising of GlcB, a protein involved in glucose specific phosphotransferase system (PTS; GLCB_SC; accession number X93360); the other (B) comprising a sensory transduction histidine kinase from *H. influenzae* (HK_HI; accession number P45336) and a sensory transduction histidine kinase from *Synechocystis sp* (HK_SYN). The comparison was produced by the computer program BESTFIT.

A

Orf2 x GLCB_SC

```
95                                                                    175
AGGELCTLSSLKYHSNLYFDVQCKDFDGLYEENTSDRVYNLLLIGF.FSFSLLVVFMYFSVLKSLEPLKKLRRQVAEVANGE
||..: .:  |. : .: |...  |:  ||:  ..||  .||:|.. :.:.|:  |::  ::|.|: ||.  |:  ||.. :
AGTSFLIMHLLHVQIGMTFSGGFIDYI.LYGLLSWDRSNALLVIPVGIAYALIYYFLFTFLIKKLN.LKTPGREDKEVESKD
1                                                                      49
```

B

HK_SYN x **Orf2** x HK_HI

```
  7                          36        311                                351
  SVRRSLLLKFVLSYFCLSSVVVASIFLVAN....//...LVLVGLVSTALLLIAVYLLSIKITKPILLITKAAIDISSGD
  |:: .|:: ||:.:| :: :.:. : : :|        |:|:|:.| .||:: :|:  :|  .|:  : :.. ::..|:.
1 SIHTKLIILFVVTFFLVCVLFIVLLKIEGN 30//135 LLLIGFFSFSLLVVFMYFSVLKSLEPLKKLRRQVAEVANGE 175
                                          ::| | |:.:|::.: : : |.| |:|:|..:|..  .|:
                                         QMWIWFASLPILIIVLGWLIHKELRPIKRLSQEVQTRKSGD
                                         161                                201
```

APPENDIX 3

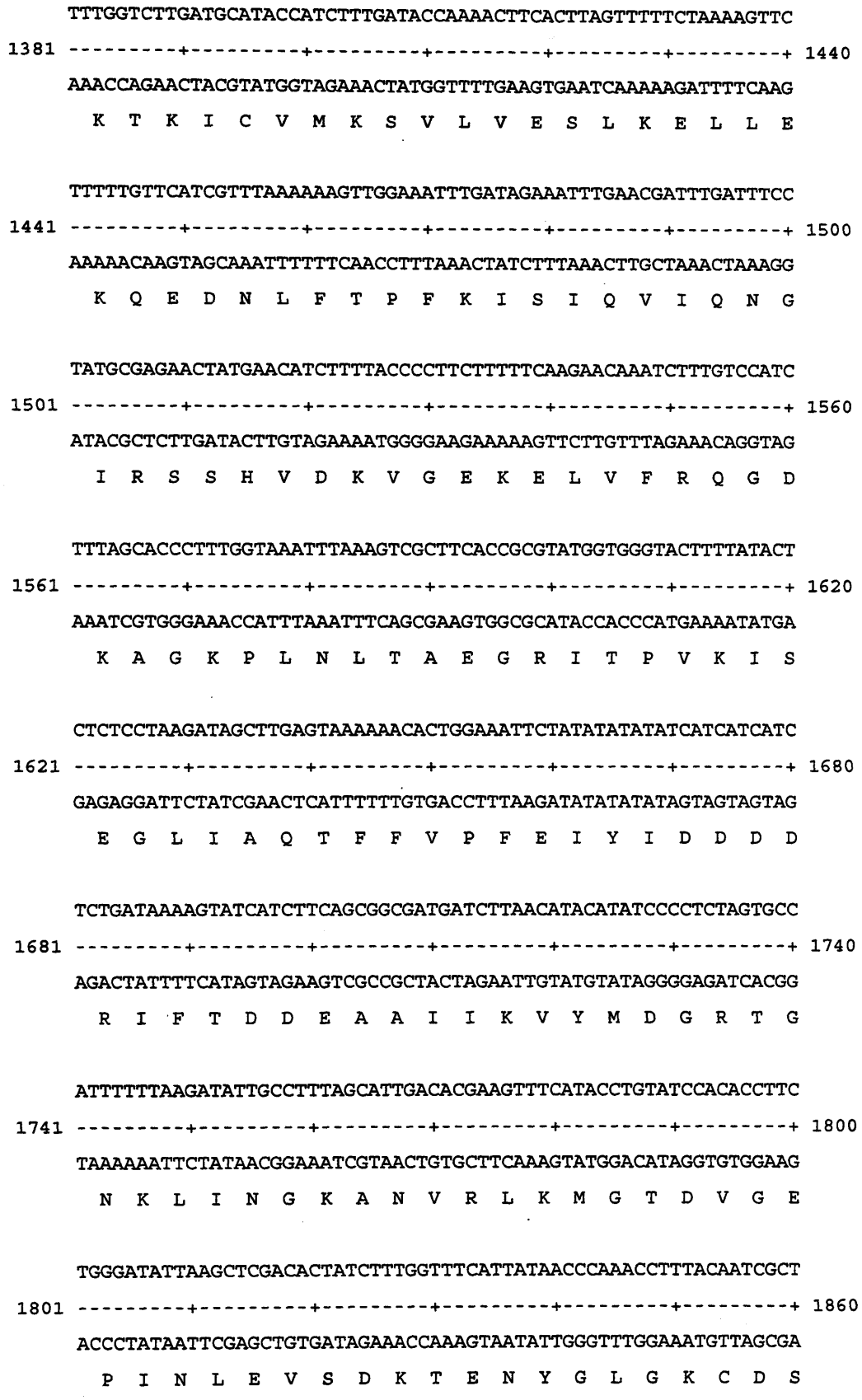
DNA Sequence of pλRB's 2.3kb Region 5' to *regX1*

As mentioned in section 3.8.2, the sequence data from pλRB's 2.3kb region 5' to *regX1* was analysed using the Wisconsin Package, (Version 8, September 1994) via the Genetics Computer Group. The results are presented below, comprising the predicted amino acid sequences and possible ribosome binding sites (RBS).

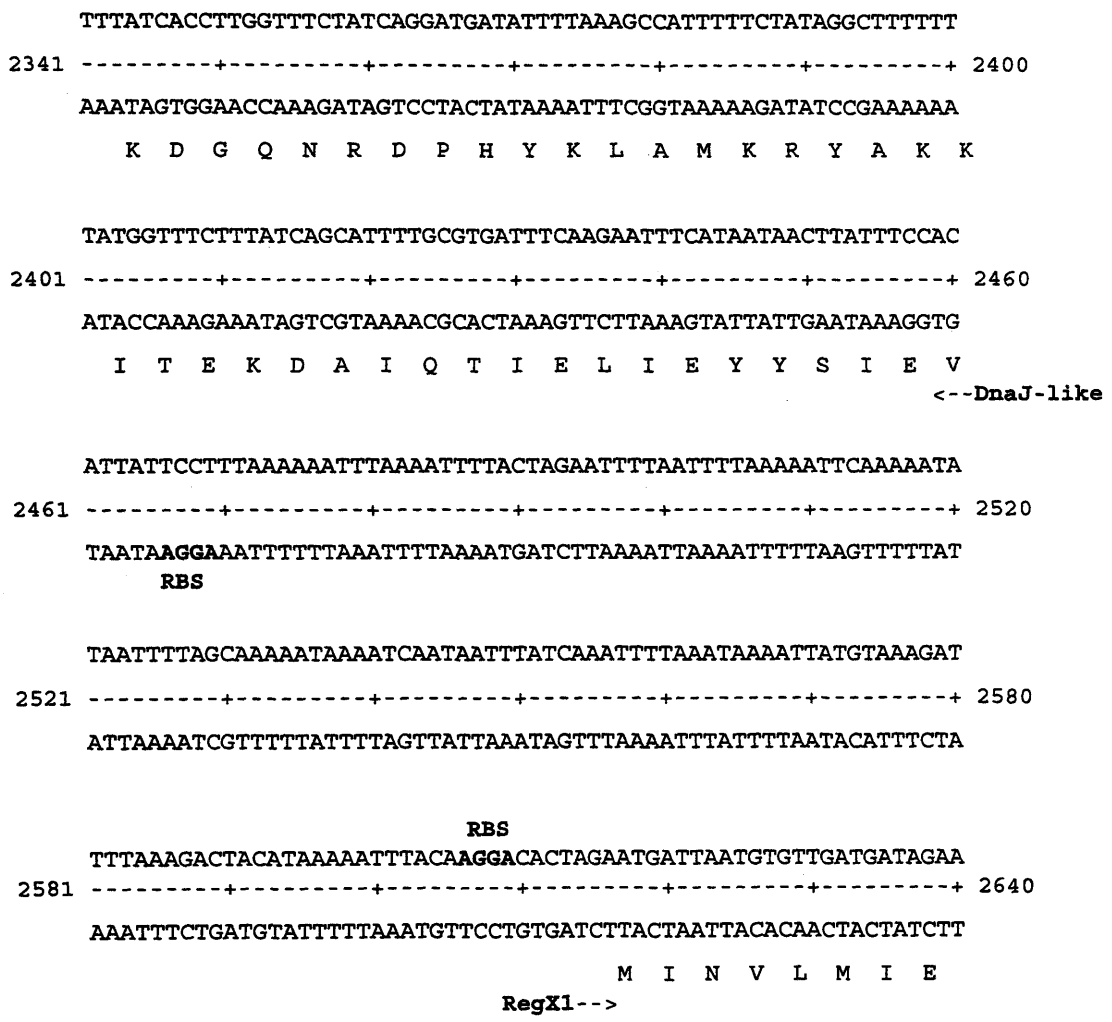
```
5' TCAAAGATATCGATGTATCTGGAGTATTAAGATACAGATATGAAAGTAGCAATCCATGGA
 1 ---------+---------+---------+---------+---------+---------+ 60
3' AGTTTCTATAGCTACATAGACCTCATAATTCTATGTCTATACTTTCATCGTTAGGTACCT
     K  D  I  D  V  S  G  V  L  R  Y  R  Y  E  S  S  N  P  W  S

   GCAATGCAAATTTTGGTTCTGGTATTTCTGGAAAACAAGATCATAAATACAGAGCACAAG
61 ---------+---------+---------+---------+---------+---------+ 120
   CGTTACGTTTAAAACCAAGACCATAAAGACCTTTTGTTCTAGTATTTATGTCTCGTGTTC
     N  A  N  F  G  S  G  I  S  G  K  Q  D  H  K  Y  R  A  Q  V

   TTAACTTCAGCGGTGCTATATCAGACAACTTTAAAGCTTTCGTTCAATTTGACTACAACT
121 ---------+---------+---------+---------+---------+---------+ 180
   AATTGAAGTCGCCACGATATAGTCTGTTGAAATTTCGAAAGCAAGTTAAACTGATGTTGA
     N  F  S  G  A  I  S  D  N  F  K  A  F  V  Q  F  D  Y  N  S

   CACAAGATGGTGGGTTATGGCACAGATAGCATCAGGTAACACAAGCGATACTTTTAACAA
181 ---------+---------+---------+---------+---------+---------+ 240
   GTGTTCTACCACCCAATACCGTGTCTATCGTAGTCCATTGTGTTCGCTATGAAAATTGTT
     Q  D  G  G  L  W  H  R  *

   GTTCCGTCAATTATATTTTAACTTATACAAATGAAGATGTTGGCTACAAGCGTAATCGCT
241 ---------+---------+---------+---------+---------+---------+ 300
   CAAGGCAGTTAATATAAAATTGAATATGTTTACTTCTACAACCGATGTTCGCATTAGCGA

   GGGTAAACAACAACTAAACACTATCTTGGACTGACAATGGTATCGATGGTTTAGGTTGGA
301 ---------+---------+---------+---------+---------+---------+ 360
   CCCATTTGTTGTTGATTTGTGATAGAACCTGACTGTTACCATAGCTACCAAATCCAACCT
```

```
    ACAGGTGTTAAAGTAGTAAACAACAGTATCGATGGTTTAACTCTAGCTGCTTTTGCAATG
361 ---------+---------+---------+---------+---------+---------+ 420
    TGTCCACAATTTCATCATTTGTTGTCATAGCTACCAAATTGAGATCGACGAAAACGTTAC
                                                            M
                                                       Orf4 -->

    GATAGTTTCAATGAAGCTTCAGATACTACTGTAACAATTACTCAAGATAATAATCAAAAA
421 ---------+---------+---------+---------+---------+---------+ 480
    CTATCAAAGTTACTTCGAAGTCTATGATGACATTGTTAATGAGTTCTATTATTAGTTTTT
    D  S  F  N  E  A  S  D  T  T  V  T  I  T  Q  D  N  N  Q  K

    ATTACAGGAGTTCAATTTAATCGTGGAAATCCTAAAGGTGATAGTGATGTAAGTGGTGCT
481 ---------+---------+---------+---------+---------+---------+ 540
    TAATGTCCTCAAGTTAAATTAGCACCTTTAGGATTTCCACTATCACTACATTCACCACGA
    I  T  G  V  Q  F  N  R  G  N  P  K  G  D  S  D  V  S  G  A

    TTAGATTGGAGCAAAAATATTTATGGTGCTGCAGCTATCGGCTCTTATGATATCGCTGGT
541 ---------+---------+---------+---------+---------+---------+ 600
    AATCTAACCTCGTTTTTATAAATACCACGACGTCGATAGCCGAGAATACTATAGCGACCA
    L  D  W  S  K  N  I  Y  G  A  A  A  I  G  S  Y  D  I  A  G

    GGACAATTCAACCCACAATTATGGTTAGCTTATATGAGTGATAATGCATTCTTATATGCT
601 ---------+---------+---------+---------+---------+---------+ 660
    CCTGTTAAGTTGGGTGTTAATACCAATCGAATATACTCACTATTACGTAAGAATATACGA
    G  Q  F  N  P  Q  L  W  L  A  Y  M  S  D  N  A  F  L  Y  A

    TTAGATGCAGCTTATAGCACAACTATCTTTGATGGAATTAACTGGACTATCGAAGGTGCT
661 ---------+---------+---------+---------+---------+---------+ 720
    AATCTACGTCGAATATCGTGTTGATAGAAACTACCTTAATTGACCTGATAGCTTCCACGA
    L  D  A  A  Y  S  T  T  I  F  D  G  I  N  W  T  I  E  G  A

    TACCTAGGAAATTCTGTAGATAATAAACTTAAAGATAGATTAGATGCAGCAAATGGTAAT
721 ---------+---------+---------+---------+---------+---------+ 780
    ATGGATCCTTTAAGACATCTATTATTTGAATTTCTATCTAATCTACGTCGTTTACCATTA
    Y  L  G  N  S  V  D  N  K  L  K  D  R  L  D  A  A  N  G  N

    TTCTTTGCTTTAAGAGGCACTGTTGAGGTAAATGGGTGGGGATGCAAGTCTTGGTGGGTT
781 ---------+---------+---------+---------+---------+---------+ 840
    AAGAAACGAAATTCTCCGTGACAACTCCATTTACCCACCCCTACGTTCAGAACCACCCAA
    F  F  A  L  R  G  T  V  E  V  N  G  W  G  C  K  S  W  W  V
```

```
    TATACTATGGGTAAAAAAGATAAAGTTACTTTAACCACAATTGAAGATCAAGGTAATCTT
841 ---------+---------+---------+---------+---------+---------+ 900
    ATATGATACCCATTTTTTCTATTTCAATGAAATTGGTGTTAACTTCTAGTTCCATTAGAA
    Y  T  M  G  K  K  D  K  V  T  L  T  T  I  E  D  Q  G  N  L

    GGCTCTTTGCTTGCAGGTGAAAAAATTTTCTATACTAATGGTTCTAATTTAAATGGCGAT
901 ---------+---------+---------+---------+---------+---------+ 960
    CCGAGAAACGAACGTCCACTTTTTTAAAAGATATGATTACCAAGATTAAATTTACCGCTA
    G  S  L  L  A  G  E  K  I  F  Y  T  N  G  S  N  L  N  G  D

    ATTGGTAGAAATATCTTTGGTTATGTAACTGCTGGATATACTTTTAATGAAACAGTTCGC
961 ---------+---------+---------+---------+---------+---------+ 1020
    TAACCATCTTTATAGAAACCAATACATTGACGACCTATATGAAAATTACTTTGTCAAGCG
    I  G  R  N  I  F  G  Y  V  T  A  G  Y  T  F  N  E  T  V  R

    GTTGGTGCTGACTTTGTATACGGTGGAACAAAAACAAATATAATTGGTCAAGGTGGTAAA
1021 ---------+---------+---------+---------+---------+---------+ 1080
    CAACCACGACTGAAACATATGCCACCTTGTTTTTGTTTATATTAACCAGTTCCACCATTT
    V  G  A  D  F  V  Y  G  G  T  K  T  N  I  I  G  Q  G  G  K

    AAACTTGAAGCTGTTGCAAGAGTAGATTACAAATACTCTCCAAAACTTAACTTCTCAGCA
1081 ---------+---------+---------+---------+---------+---------+ 1140
    TTTGAACTTCGACAACGTTCTCATCTAATGTTTATGAGAGGTTTTGAATTGAAGAGTCGT
    K  L  E  A  V  A  R  V  D  Y  K  Y  S  P  K  L  N  F  S  A

    TTCTACTCTTATGTAAATGTTGATACAGATCCTGAAAGCACTCATCATGATGCTGTAAGA
1141 ---------+---------+---------+---------+---------+---------+ 1200
    AAGATGAGAATACATTTACAACTATGTCTAGGACTTTCGTGAGTAGTACTACGACATTCT
    F  Y  S  Y  V  N  V  D  T  D  P  E  S  T  H  H  D  A  V  R

    CTTCAAGCTCTTTACAAATTCTAAGAAGCTTTTAAGTCTAACTTCAAGGCGGGGTTTTGG
1201 ---------+---------+---------+---------+---------+---------+ 1260
    GAAGTTCGAGAAATGTTTAAGATTCTTCGAAAATTCAGATTGAAGTTCCGCCCCAAAACC
    L  Q  A  L  Y  K  F  *

    TCCGCCTTTTTTTATGCCTGATTTTTAAAACTTAAATTTATATAAAAAGATTTTCCCAAG
1261 ---------+---------+---------+---------+---------+---------+ 1320
    AGGCGGAAAAAAATACGGACTAAAAATTTTGAATTTAAATATATTTTTCTAAAAGGGTTC

    CTAAAAAGCTTTAGGAAAATTAAGACTTAAACCAATTAGCGATTTTTTCAAAAAGTCCTT
1321 ---------+---------+---------+---------+---------+---------+ 1380
    GATTTTTCGAAATCCTTTTAATTCTGAATTTGGTTAATCGCTAAAAAAGTTTTTCAGGAA
      *  F  A  K  P  F  N  L  S  L  G  I  L  S  K  K  L  F  D  K
```


TTTGGTCTTGATGCATACCATCTTTGATACCAAAACTTCACTTAGTTTTTCTAAAAGTTC
1381 ---------+---------+---------+---------+---------+---------+ 1440
AAACCAGAACTACGTATGGTAGAAACTATGGTTTTGAAGTGAATCAAAAAGATTTTCAAG
K T K I C V M K S V L V E S L K E L L E
TTTTTGTTCATCGTTTAAAAAAGTTGGAAATTTGATAGAAATTTGAACGATTTGATTTCC
1441 ---------+---------+---------+---------+---------+---------+ 1500
AAAAACAAGTAGCAAATTTTTTCAACCTTTAAACTATCTTTAAACTTGCTAAACTAAAGG
K Q E D N L F T P F K I S I Q V I Q N G
TATGCGAGAACTATGAACATCTTTTACCCCTTCTTTTTCAAGAACAAATCTTTGTCCATC
1501 ---------+---------+---------+---------+---------+---------+ 1560
ATACGCTCTTGATACTTGTAGAAAATGGGGAAGAAAAAGTTCTTGTTTAGAAACAGGTAG
I R S S H V D K V G E K E L V F R Q G D
TTTAGCACCCTTTGGTAAATTTAAAGTCGCTTCACCGCGTATGGTGGGTACTTTTATACT
1561 ---------+---------+---------+---------+---------+---------+ 1620
AAATCGTGGGAAACCATTTAAATTTCAGCGAAGTGGCGCATACCACCCATGAAAATATGA
K A G K P L N L T A E G R I T P V K I S
CTCTCCTAAGATAGCTTGAGTAAAAAACACTGGAAATTCTATATATATATCATCATCATC
1621 ---------+---------+---------+---------+---------+---------+ 1680
GAGAGGATTCTATCGAACTCATTTTTTGTGACCTTTAAGATATATATATAGTAGTAGTAG
E G L I A Q T F F V P F E I Y I D D D D
TCTGATAAAAGTATCATCTTCAGCGGCGATGATCTTAACATACATATCCCCTCTAGTGCC
1681 ---------+---------+---------+---------+---------+---------+ 1740
AGACTATTTTCATAGTAGAAGTCGCCGCTACTAGAATTGTATGTATAGGGGAGATCACGG
R I F T D D E A A I I K V Y M D G R T G
ATTTTTTAAGATATTGCCTTTAGCATTGACACGAAGTTTCATACCTGTATCCACACCTTC
1741 ---------+---------+---------+---------+---------+---------+ 1800
TAAAAAATTCTATAACGGAAATCGTAACTGTGCTTCAAAGTATGGACATAGGTGTGGAAG
N K L I N G K A N V R L K M G T D V G E
TGGGATATTAAGCTCGACACTATCTTTGGTTTCATTATAACCCAAACCTTTACAATCGCT
1801 ---------+---------+---------+---------+---------+---------+ 1860
ACCCTATAATTCGAGCTGTGATAGAAACCAAAGTAATATTGGGTTTGGAAATGTTAGCGA
P I N L E V S D K T E N Y G L G K C D S

```
     ACATTTTTCACTAGCCTTTTCTCCAATTCCTTGACAATCAGGGCAAGTTTGGGCAAAAGT
1861 ---------+---------+---------+---------+---------+---------+ 1920
     TGTAAAAAGTGATCGGAAAAGAGGTTAAGGAACTGTTAGTCCCGTTCAAACCCGTTTTCA
       C  K  E  S  A  K  E  G  I  G  Q  C  D  P  C  T  Q  A  F  T

     GATAAAACCTTGAGAAACCCCAACCTGTCCTCTACCTTGACACTTTGGACAAGTTTGAAG
1921 ---------+---------+---------+---------+---------+---------+ 1980
     CTATTTTGGAACTCTTTGGGGTTGGACAGGAGATGGAACTGTGAAACCTGTTCAAACTTC
       I  F  G  Q  S  V  G  V  Q  G  R  G  Q  C  K  P  C  T  Q  L

     CTTTCCATCTTTAGCCCCTGTTCCATTACAAGTTTTACAAGAGCATTTATAAGTAAAGTC
1981 ---------+---------+---------+---------+---------+---------+ 2040
     GAAAGGTAGAAATCGGGGACAAGGTAATGTTCAAAATGTTCTCGTAAATATTCATTTCAG
       K  G  D  K  A  G  T  G  N  C  T  K  C  S  C  K  Y  T  F  D

     TATATTTTTCTTACAACCAAAAACCGCTTCTTTAAAACTTAATTTTAAATTAACGATAAA
2041 ---------+---------+---------+---------+---------+---------+ 2100
     ATATAAAAAGAATGTTGGTTTTTGGCGAAGAAATTTTGAATTAAAATTTAATTGCTATTT
       I  N  K  K  C  G  F  V  A  E  K  F  S  L  K  L  N  V  I  F

     ATCTGAAGGGATTTTCTCATCATTTGAACTTTTTCTACGACGCGATGAGCCAAAGCCTTC
2101 ---------+---------+---------+---------+---------+---------+ 2160
     TAGACTTCCCTAAAAGAGTAGTAAACTTGAAAAAGATGCTGCGCTACTCGGTTTCGGAAG
       D  S  P  I  K  E  D  N  S  S  K  R  R  R  S  S  G  F  G  E

     TCCAAAAAAACTAGAAAATATATCTCCTAAATCCTCAAAACCGGCAAAACCTGAGCTACT
2161 ---------+---------+---------+---------+---------+---------+ 2220
     AGGTTTTTTTGATCTTTTATATAGAGGATTTAGGAGTTTTGGCCGTTTTGGACTCGATGA
       G  F  F  S  S  F  I  D  G  L  D  E  F  G  A  F  G  S  S  S

     AGAACCAAATCCACCGCCTTTTAAAGCATCTTTTCCGTATCTATCATAAATAGCTCGCTT
2221 ---------+---------+---------+---------+---------+---------+ 2280
     TCTTGGTTTAGGTGGCGGAAAATTTCGTAGAAAAGGCATAGATAGTATTTATCGAGCGAA
       S  G  F  G  G  G  K  L  A  D  K  G  Y  R  D  Y  I  A  R  K

     TTCATCATTGGTTAAAACTTCATAGGCTTCATTAACAAGCTTGAATTTATCTTCTGCTTC
2281 ---------+---------+---------+---------+---------+---------+ 2340
     AAGTAGTAACCAATTTTGAAGTATCCGAAGTAATTGTTCGAACTTAAATAGAAGACGAAG
       E  D  N  T  L  V  E  Y  A  E  N  V  L  K  F  K  D  E  A  E
```


TTTATCACCTTGGTTTCTATCAGGATGATATTTTAAAGCCATTTTTCTATAGGCTTTTTT
2341 ---------+---------+---------+---------+---------+---------+ 2400
AAATAGTGGAACCAAAGATAGTCCTACTATAAAATTTCGGTAAAAAGATATCCGAAAAAA
K D G Q N R D P H Y K L A M K R Y A K K
TATGGTTTCTTTATCAGCATTTTGCGTGATTTCAAGAATTTCATAATAACTTATTTCCAC
2401 ---------+---------+---------+---------+---------+---------+ 2460
ATACCAAAGAAATAGTCGTAAAACGCACTAAAGTTCTTAAAGTATTATTGAATAAAGGTG
I T E K D A I Q T I E L I E Y Y S I E V
<--DnaJ-like
ATTATTCCTTTAAAAAATTTAAAATTTTACTAGAATTTTAATTTTAAAAATTCAAAAATA
2461 ---------+---------+---------+---------+---------+---------+ 2520
TAATAAGGAAATTTTTTAAATTTTAAAATGATCTTAAAATTAAAATTTTTAAGTTTTTAT
RBS
TAATTTTAGCAAAAATAAAATCAATAATTTATCAAATTTTAAATAAAATTATGTAAAGAT
2521 ---------+---------+---------+---------+---------+---------+ 2580
ATTAAAATCGTTTTTATTTTAGTTATTAAATAGTTTAAAATTTATTTTAATACATTTCTA
RBS
TTTAAAGACTACATAAAAATTTACAAGGACACTAGAATGATTAATGTGTTGATGATAGAA
2581 ---------+---------+---------+---------+---------+---------+ 2640
AAATTTCTGATGTATTTTTAAATGTTCCTGTGATCTTACTAATTACACAACTACTATCTT
M I N V L M I E
RegX1-->

As mentioned in section 3.8.2, Orf3 shares 45 to 50% identity and approximately 60% similarity with DnaJ proteins from a variety of bacterial species. Based on the results from the comparative sequence analysis, a multiple alignment between the predicted amino acid sequence of Orf3 and the amino acid sequences from the six most homologous DnaJ proteins was compiled. The alignment was obtained using the computer program PILEUP (Devereux *et al.*, 1984). The organism abbreviations are: CB, *Coxiella burnetii*; CJ, *C. jejuni*; EC, *E. coli*; ER, *Erysipelothrix rhusiopathiae*; HI, *H. influenzae*; LL, *Lactococcus lactis*; SA, *Staphylococcus aureus*.

```
          1                                                                  80
DNAJ_CJ   ..VEISYYEI  LEITQNADKE  TIKKAYRKMA  LKYHPDRNQG  DKEAEDKFKL  VNEAYEVLTN  DEKRAIYDRY  GKDALKGGGF
DNAJ_EC   .MAKQDYYEI  LGVSKTAEER  EIRKAYKRLA  MKYHPDRNQG  DKEAEAKFKE  IKEAYEVLTD  SQKRAAYDQY  GHAAFEQ..G
DNAJ_HI   .MAKKDYYEV  LGLQKGASED  EIKRAYKRLA  SKHHPDKNQG  SKEAEEKFKE  INEAYEVLGD  DQKRAAYDQY  GHAAFEQ..G
DNAJ_CB   .MAKRDYYEV  LGVNLNATEA  EVKKAFRRLA  MKYHPDRNPG  DKDAEVKFKE  AREAYEVLCD  SRKRASYDQF  GHAGVEQTFG
DNAJ_LL   .MNNTEYYER  LGVDKNASQD  EIKKAYRKMS  KKYHPDLNKE  E.GAEEKYKE  VQEAYETLSD  EQKRAAYDQY  GEAGANGGFG
DNAJ_SA   .MAKRDYYEV  LGISKDASKD  EIKKAYRKLS  KKYHPDINKE  E.GADEKFKE  ISEAYEVLSD  DNKRATIDQF  GHDGPQ.GFG
DNAJ_ER   MADKRDFYEI  LGVSKSATDA  EIKKAYRQLA  KKYHPDINKE  D.GAEAKFKE  VQEAYEVLSD  SQKRANYDQF  GHAAFDQGAG

          81                                                                 160
DNAJ_CJ   GSSSSGFAG.  .........F  EDLGDIFSSF  FGEGFGSSRR  RKSSNDEKIP  SDFIVNLKLS  FKEAVFGCKK  NIDFTYKCSC
DNAJ_EC   GMGGGGFG..  .GGADFS...  DIFGDVFGDI  F.G..GGRGR  QRAA....RG  ADLRYNMELT  LEEAVRGVTK  EIRIPTLEEC
DNAJ_HI   G.GAGGFGGG  FGGADFG...  DMFGDIFGDI  FGG..GGRGR  QRVV....RG  EDLRYDLEIS  LEEAVKGTTK  DIQINTLAHC
DNAJ_CB   GAGAGGFGFG  ....DLG...  DIFGDIFGDI  FGG..ARGGQ  AREQ....RG  ADLAYELVLS  LEEAVHGLSR  TIKVPTWINC
DNAJ_LL   GGGFGGASGF  SGFGGSSGGF  GGFEDIFSSF  FGG..GGA..  QVNPNAPRQG  DDLQYRINLK  FEEAIFGVEK  QVKYNREELC
DNAJ_SA   GQGFNG.SDF  GGFSGFGG..  GGFEDIFSSF  FGG..GS...  QRDPNAPQKG  DDLQYTMTLT  FEEAVFGTTK  EISIRKDVTC
DNAJ_ER   GFG.......  ...GGFSGGF  DDFGDIFSSF  FGG..GGGGQ  RRNPNGPMKG  QDRFMSMRID  FMEAVFGANK  SVTLNVDEEC
```

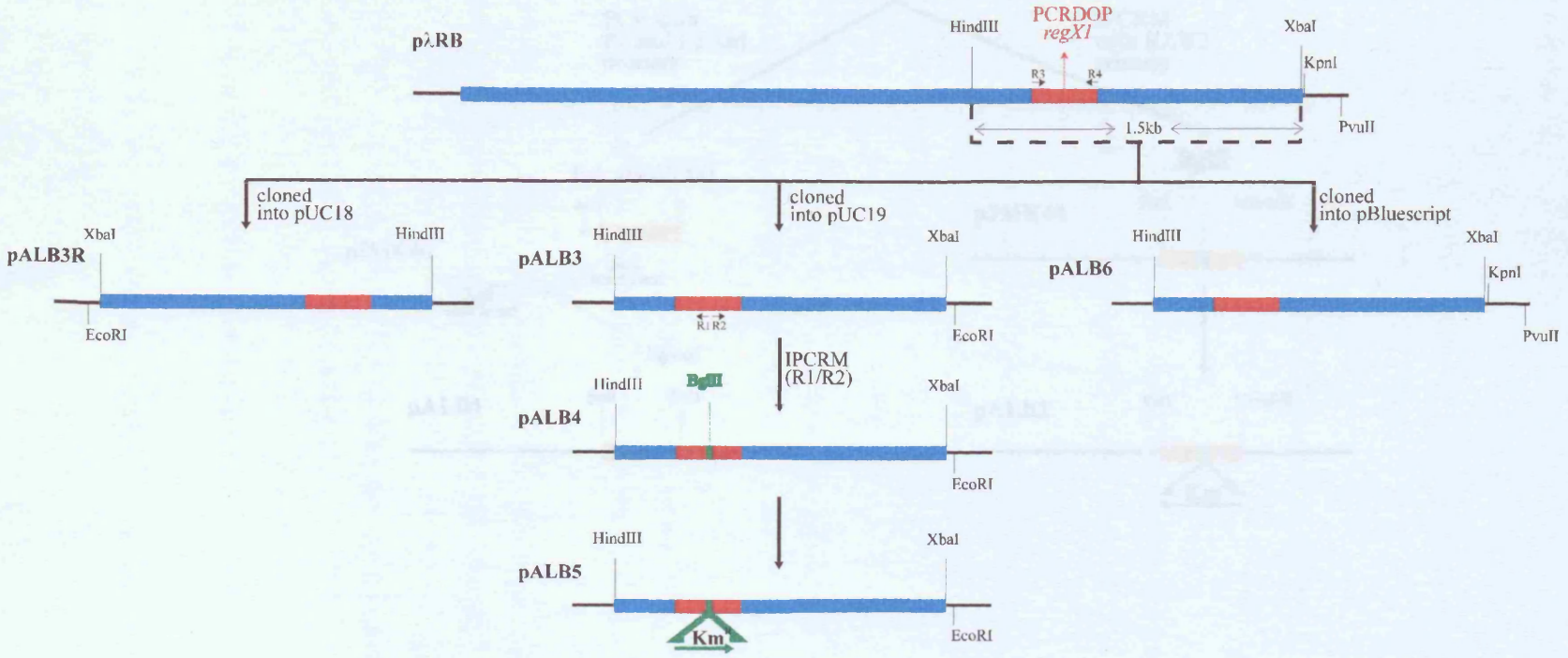
```
          161                                                                                                  240
DNAJ_CJ   KTCNGTGAKD  G.KLQTCPKC  QGRGQVGVSQ  ....GFITFA  QTCPDCQGIG  EKASEKCSDC  KGLGYNETKD  SVELNIPEGV
DNAJ_EC   DVCHGSGAKP  GTQPQTCPTC  HGSGQVQMRQ  ....GFFAVQ  QTCPHCQGRG  TLIKDPCNKC  HGHGRVERSK  TLSVKIPAGV
DNAJ_HI   DSCGGSGAEK  GSKVETCPHC  HGSGRIRRQQ  ....GFFVSE  SICPTCHGSG  KKIEKPCRNC  HGEGRVHKKE  NLSVKIPAGV
DNAJ_CB   KTCNGSGAK.  GSSPATCPRC  NGSGQMRMQH  ....GFLQVQ  QTCSVCRGRG  QVIKDPCTDC  HGQGRQQQTK  TLSVKIPPGI
DNAJ_LL   HTCGGSGAKR  GTHPETCHKC  GGRGQINVVR  DTPLGRMQTQ  VTCDVCNGTG  KEIKEKCETC  HGSGHEKVAH  TVKVTVPAGV
DNAJ_SA   ETCHGDGAKP  GTSKKTCSYC  NGAGHVAVEQ  NTILGRVRTE  QVCPKCNGSG  QEFEEACPTC  HGKGTENKTV  KLEVKVPEGV
DNAJ_ER   TSCHGSGAHS  KDDIKTCSRC  GGTGQTVTQQ  RTPFGVFQSQ  ATCPDCGGSG  KTITKRCGEC  HGKGFNTKRV  EVDIKIPAGI

          241                                                                                                  320
DNAJ_CJ   DTGMKLRVNA  KGNILKNGTR  .GDMYVKIIA  AEDDTFIRDD  DDIYIEFPVF  FTQAILGESI  KVPTIRGEAT  LNLPKGAKDG
DNAJ_EC   DTGDRIRLAG  EGEAGEHGAP  AGDLYVQVQV  KQHPIFEREG  NNLYCEVPIN  FAMAALGGEI  EVPTLDGRVK  LKVPGETQTG
DNAJ_HI   DTGNQLRLAG  KGAAGENGAP  AGDLYVVIHV  REHNIFERDG  SNLYCEVPIS  FATAALGGEI  EVPTLDGRVK  LKIPAETQTG
DNAJ_CB   DTGDRIRLAG  EGEAGLFGAP  PGDLYVQVRV  KPHPLFHREG  NDLHSEVPID  FTTAALGGEM  EIPTLDGSVR  LTIPPETQGG
DNAJ_LL   ETGQKMRLQG  QGDAGVNGGP  YGDLYVVFQV  EASDKFERDG  AEIYYKMPMD  FVQAALGDEI  EVPTVHGNVK  LKIPAGTQTG
DNAJ_SA   DNEQQIRLAG  EGSPGVNGGP  AGDLYVVFRV  KPSETFKRDG  DDIYYKLNVS  FPQAALGDEI  KIPTLNNEVM  LTIPAGTQTG
DNAJ_ER   VTGQQLRVSG  KGERGANGGP  NGDLFIEIVV  GTHKHFRREG  NDIHINIPLS  VIDATLGTEI  EVPTVHGDVK  LTIPAGTQPN

          321                                                                                                  400
DNAJ_CJ   QRFVLEKEGV  KDVHSSRIGN  QIVQISIKFP  TFLNDEQKEL  LEKLSEVLVS  KMVCIKTKKD  FLKKSLIGLS  LNFPKAF*..
DNAJ-EC   KLFRMRGKGV  KSVRGGAQGD  LLCRVVVETP  VGLNERQKQL  LQELQESFGG  PTGEHNSPR.  ..SKSFFDGV  KKFFDDLTR.
DNAJ-HI   KLFRMRGKGV  ASTRSGYAGD  LICRIVVETP  VNLTSEQKEL  LHKLEESLQG  KDLSKHAPK.  ..SSGFLDGV  KKFFDNLGKS
DNAJ_CB   KQFRLRGKGV  KALRSGAVGD  LICHIVVETP  VKLSPEQKDY  LKQFAELLK.  KDEKNHSPR.  ..TRNWFGTV  ..........
DNAJ_LL   ANFRLKGKGA  PKLRGSGNGD  QYVIINIVTP  KNLNQAQKEA  LQAFAKA...  SGVEVSGSG.  ..KKGFFDKF  K.........
DNAJ_SA   KQFRLKEKGI  KNVHGYGYGD  LYVDIKVVTP  TKLTDRQKEL  MKEFAQL...  NGEEINE.Q.  ..PSNFKDRA  KRFFKGE...
DNAJ_ER   TKFRLREKGV  QDLRSGRMGD  QYVEVKLEVP  TKLTDRQKEL  MKEFAQL...  NGEEINE.Q.  ..PSNFKDRA  KRFFKGE...
```

```
          401                                                       450
DNAJ_CJ   ..........  ..........  ..........  ..........  ..........
DNAJ_EC   ..........  ..........  ..........  ..........  ..........
DNAJ_HI   DK........  ..........  ..........  ..........  ..........
DNAJ_CB   ..........  ..........  ..........  ..........  ..........
DNAJ_LL   ..........  ..........  ..........  ..........  ..........
DNAJ_SA   ..........  ..........  ..........  ..........  ..........
DNAJ_ER   TKLSRQQREH  LEAL......  KETEVKG...  ...DSVFDRF  KKAFK.....
```

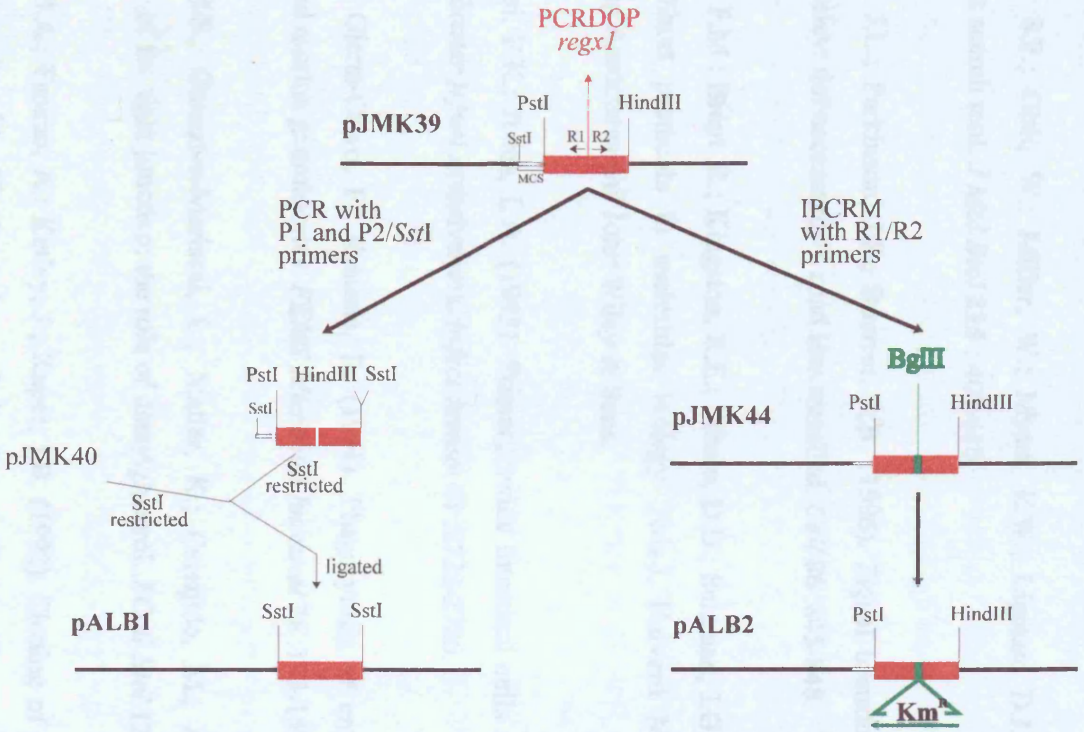
APPENDIX 4

Plasmid constructs

Appendix 4.1. pλRB-derived constructs



Appendix 4.2. pJMK39-derived constructs



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