Molecular Genetics Approaches to the Investigation of *Campylobacter jejuni*

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by

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For Mum and Dad

ABSTRACT

Campylobacter jejuni is one of the leading bacterial agents of gastrointestinal illness throughout the world. The clinical importance of C.jejuni has led to a number of investigations into the pathogenesis of the organism. Much controversy remains, however, about the pathogenesis of *C.jejuni* enteritis and the precise mechanism of infection has yet to be fully elucidated. A molecular genetic approach has yielded a great deal of information concerning the pathogenesis of other bacteria, but, until recently, such an approach has not been very successful with *Campylobacter* spp.

In order to identify *C.jejuni* genes encoding potential virulence determinants, it is necessary to develop workable strategies and systems for cloning and investigating *Campylobacter* DNA. This thesis describes the utilisation of two different methods for the isolation of *C.jejuni* genes. In the first method, a Lambda ZAP II library of *C.jejuni* strain H132 DNA was screened with convalescent antiserum to isolate novel genes for antigenic proteins which are likely to be synthesized *in vivo*. The second method used PCR with degenerate oligonucleotide primers (PCRDOP) to isolate a fragment of a known conserved gene.

Several Lambda ZAP II clones of *C.jejuni* DNA which synthesized products recognised by convalescent antiserum were successfully isolated and rescued into the pBluescript phagemid. *In vitro* transcription/translation and immunoprecipitation analysis confirmed that the clones could synthesize products which reacted with convalescent antiserum. The transposon, Tn*phoA*, was used to mutagenise the ORFs and identify any which could encode exported *C.jejuni* peptides. It was revealed that at least three of the positive clones could produce *C.jejuni* proteins which were exported from *E.coli*. The ORFs were partially sequenced to determine any similarities with known exported products which may be involved in virulence. One of the ORFs could encode a protein with N-terminal similarity to *Campylobacter* flagellins and, upstream from this gene, an ORF encoding an ATP-binding transport protein was found.

PCRDOP was used successfully to isolate a fragment of the *C.jejuni recA* gene. The fragment was sequenced and amino acid substitutions were found which may cause the RecA protein to have constitutive protease activity for the LexA repressor. The entire *C.jejuni* H132 *recA* gene was isolated by PCR and found also to lack an upstream LexA suppressor binding-site. The potential significance of these observations is discussed.

It is hoped that the development of molecular genetics techniques for the investigation of *C.jejuni* physiology and pathogenesis will lead to a greater understanding of the mechanisms by which *Campylobacter* cause disease.

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CONTENTS

Contents 1

Abbreviations 4

Chapter One: General Introduction

1.1 Perspective 5

1.2 The organism 6

1.3 Clinical features of *C.jejuni* infection 7

1.4 Epidemiology of *C.jejuni* 7

1.5 Pathogenesis of *C.jejuni* 8

1.5.1 Colonisation 9

1.5.2 Invasion 12

1.5.3 Cytotoxins 16

1.5.4 LPS 20

1.5.5 Enterotoxin 21

1.6 Genetics 23

1.7 Aims and objectives 30

Chapter Two: A Lambda ZAP II expression vector-based library as a method of cloning *C.jejuni* antigen genes

2.1 Introduction 31

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions 40

2.2.2 Chromosomal DNA extraction 40

2.2.3 Digestion of *C.jejuni* H132 DNA 41

2.2.4 Preparation of Lambda ZAP II vector arms 41

2.2.5 Partial fill-in of staggered ends and ligation 42

2.2.6 Amplification of the library 43

2.2.7 Absorbing the antiserum 43

2.2.8 Immunological screening 44

2.2.9 Preparation of DNA probes 44

2.2.10 Radioactive labelling of probes 44

2.2.11 Treatment of filters 45

2.2.12 Non-radioactive probes 45

2.2.13 *In vivo* excision of the pBluescript SK- phagemid 46

2.3 Results

2.3.1 Preparation of partially digested and size-selected genomic H132 DNA 47

2.3.2 Preparation of Lambda ZAP II vector arms 48
2.3.3 Ligation of vector and insert 48
2.3.4 Amplification of the library 49
2.3.5 Immunological screening 50
2.3.6 DNA screening 51
2.3.7 *In vivo* excision of the pBluescript SK- phagemid 52
2.4 Discussion 60

Chapter Three: Analysis of positive clones
3.1 Introduction 63
3.2 Materials and methods
3.2.1 Phagemid miniprep procedure 64
3.2.2 DNA maxiprep procedure using Qiagen 64
3.2.3 DNA maxiprep using caesium chloride density gradient 64
3.2.4 Standard DNA manipulations 65
3.2.5 Southern blotting 66
3.2.6 SDS-PAGE 67
3.2.7 Western blotting 68
3.2.8 *In vitro* transcription/translation 68
3.2.9 Immunoprecipitation 69
3.2.10 Double-stranded DNA sequencing 69
3.3 Results
3.3.1 Initial confirmation and relative comparison of clones 71
3.3.2 Southern blots to verify absence of *flaA* from positive clones 72
3.3.3 Mapping 73
3.3.4 SDS-PAGE and Western blotting 74
3.3.5 *In vitro* transcription/translation and immunoprecipitation 75
3.3.6 Sequencing 77
3.4 Discussion 94

Chapter Four: Tn*phoA* mutagenesis to detect *C.jejuni* genes encoding proteins which can be exported from *E.coli*
4.1 Introduction 97
4.2 Materials and methods
4.2.1 Bacterial strains and vectors 100
4.2.2 Electroporation 100
4.2.3 Isolation of Tn*phoA* insertions into multicopy plasmids 100
4.2.4 Mapping using restriction enzyme analysis and PCR 101
4.3 Results
4.3.1 Tn*phoA* mutagenesis of pBluescript SK- clones 103

4.3.2 Mapping the Tn*phoA* mutants 104
4.3.3 *In vitro* transcription/translation analysis 105
4.3.4 Sequencing 108
4.4 Discussion
4.4.1 Summary of Tn*phoA* analysis of pMK11 133
4.4.2 Summary of Tn*phoA* analysis of pMK14 135
4.4.3 Summary of Tn*phoA* analysis of pMK3 136
4.4.4 Future work 137

Chapter Five: PCR with degenerate oligonucleotide primers as a method for cloning conserved *C.jejuni* genes: isolation of the *C.jejuni* strain H132 *recA* gene

5.1 Introduction 138
5.2 Materials and methods
5.2.1 Bacterial strains, vectors and growth conditions 141
5.2.2 PCRDOP method 141
5.2.3 DNA manipulation and analysis 142
5.2.4 Colony PCR screening for recombinants 142
5.2.5 Electroporation of *C.jejuni* 143
5.3 Results
5.3.1 Primer design 144
5.3.2 PCRDOP 144
5.3.3 Cloning of *C.jejuni recA* fragment 144
5.3.4 Sequence analysis 145
5.3.5 Mutagenesis of *C.jejuni recA* gene 145
5.3.6 Isolation of entire *C.jejuni* H132 *recA* gene 147
5.4 Discussion
5.4.1 Primer design 156
5.4.2 Sequence analysis 156
5.4.3 Mutagenesis 157
5.4.4 Future work 158

Chapter Six: General Discussion

6.1 Screening a Lambda ZAP II library of *C.jejuni* genomic DNA with convalescent antiserum 161
6.2 PCRDOP to isolate a fragment of the *C.jejuni* H132 *recA* gene 163

References 166

ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair
BFA	blood-free agar
BFSA	blood-free selective agar
BSA	bovine serum albumin
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxynucleotidyl triphosphate
ddNTP	dideoxynucleotidyl triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetraacetate
GCG	genetics computer group
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
LA	Luria-Bertani agar
LB	Luria-Bertani broth
mRNA	messenger RNA
MHB	Mueller-Hinton broth
min	minute
PCR	polymerase chain reaction
PCRDOP	PCR using degenerate oligonucleotide primers
RNA	ribonucleic acid
tRNA	transfer RNA
sdH_2O	sterile distilled water
sec	second
UV	ultraviolet
VAIN	variable atmosphere incubator
X-gal	5-bromo-4-chloro-indolyl-β-D--galactopyranoside
XP	5-bromo-3-chloro-indolylphosphate

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Perspective. At the beginning of this century, a spiral organism was associated with infectious abortion in cattle and was named *Vibrio fetus* [1]. In 1931, a similar organism was described as the causative agent of winter dysentery in cattle and was named *Vibrio jejuni* [2]. These organisms were considered pathogens of animals only until *Vibrio fetus* was cultured from human blood in 1947 [3]. In 1957 Elizabeth King studied the human strains and found that a distinct group, with an unusually high optimum growth temperature, were associated with human diarrhoeal illness [4]. These organisms were provisionally called "related vibrios", but were later considered to be represented by the two species *Campylobacter jejuni* and *Campylobacter coli*, the term campylobacter being derived from the Greek for curved rod [5].

The importance of *Campylobacter* species, as the leading bacterial agents of gastrointestinal illness throughout the world, was not suspected until the 1970's when culture medium selective for campylobacters was developed [6]. Incubation of faecal suspensions on the selective medium (which contained vancomycin, polymyxin B and trimethoprim) at 43°C in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% hydrogen yielded colonies of *Campylobacter* species from 57 (7.1%) of 803 patients with diarrhoea [6]. This suggested that *Campylobacter* species were possibly the commonest identifiable cause of infectious diarrhoea in Britain. Since this observation, many epidemiological studies have confirmed the importance of campylobacters as one of the major bacterial enteric pathogens worldwide [reviewed in refs. 7, 8 and 9].

In the 1980's, *Campylobacter jejuni* was isolated from 4 to 9% of patients with diarrhoea in the United States [10] and the annual incidence in the populations of Britain and the U.S. as a whole has been estimated at approximately 1% [9, 11]. The incidence of *Campylobacter* enteritis is more prevalent than that caused by *Salmonella* spp., *Shigella* spp. or *Yersinia enterocolitica* [12].

In developed regions, where naturally acquired immunity is uncommon, *C.jejuni* infections occur more frequently in young adults and often cause an inflammatory disease [7]. This involves bloody diarrhoea,

characteristic of an invasive organism, abdominal pain and fever [13]. In developing countries affected people primarily have episodes of loose and watery stools and infants under 12 months are most susceptible to the disease [14], although breast feeding imparts partial immunity [15]. One explanation for this different clinical presentation is that a high rate of exposure to *Campylobacter* species early in life ellicits the development of a rapid and effective immune response against the organism. This results in the prevention of the inflammatory diarrhoea seen in developed countries [16].

Several species of *Campylobacter* have now been described, *C.jejuni* being responsible for the majority of cases (80-90%) of *Campylobacter* enteritis in developed countries. *Campylobacter coli* accounts for 5-10% of such cases, but is more common (up to 40%) in developing countries [14]. Other species which have been associated with diarrhoea in humans include *C.upsaliensis*, *C.lari* and *C.hyointestinalis* [17]. *C.fetus* is a well known veterinary pathogen, causing reproductive tract infection and abortion in sheep and cattle, but rarely produces human diarrhoeal illness [17]. However, *C.fetus* can occasionally cause bacteremia and other extraintestinal infections in immunocompromised patients [18]. In this thesis, *C.jejuni* is the species referred to most often, but the term *Campylobacter* species will be used when the discussion is thought to apply to all of the species.

The clinical importance of *C.jejuni* has led to a number of investigations into the pathogenic mechanisms of the organism. Much controversy remains, however, about the pathogenesis of *C.jejuni* enteritis and the precise mechanism of infection has yet to be fully elucidated.

1.2 The organism. *C.jejuni* are Gram-negative bacteria that grow best at 42°C in microaerobic conditions of 3-15% O_2 and 3-5% CO_2 [19]. The organism obtains energy from amino acids or tricarboxylic acid cycle intermediates, not carbohydrates, and can be grown on Mueller-Hinton agar without serum or blood added [20]. The cells are curved or spiral rods that vary in size from 0.2 to 0.5μm in width and 0.5 to 8μm in length. Cells in old cultures, and those exposed to atmospheric oxygen, tend to form spherical bodies which some investigators have found to be viable but non-culturable [21]. It has been speculated that transition to these coccoid forms enables the survival of the organism in adverse environmental conditions [22]. Active campylobacters are characterised by a rapidly darting motility, with a corkscrew-like motion, which occurs by means of a single polar flagellum at one or both ends of the cell [23].

1.3 Clinical features of *C.jejuni* infection. The most common clinical features of *C.jejuni* enteritis in non-immune subjects include fever, diarrhoea and abdominal pain. The incubation period varies between 1 and 7 days and volunteers have become ill following ingestion of as few as 500 organisms, while others resist doses of 100,000 organisms [24]. Usually there is headache, malaise, fever and aching back and limbs 12 to 48 hours before the onset of intestinal symptoms. Abdominal pain is very characteristic of the disease and often becomes cramping just before the onset of diarrhoea.

The diarrhoea can be profuse, watery and frequent. After 2 or 3 days, patients from developed regions may experience a dysentry which mimicks inflammatory bowel disease, with mucus, blood and faecal leukocytes in the stools. Infection can cause a range of intestinal pathology, from small changes of the mucosa to colitis, involving loss of mucus, epithelial ulceration, crypt abscesses in the epithelial glands, haemorrhagic lesions and inflammation and oedema of the full thickness of the intestinal wall [25]. *C.jejuni* enteritis is usually a self-limiting disease which does not last for longer than a week. However, in about 20% of patients, a recurrence of symptoms may be noted, particularly of the abdominal pain [26].

Extraintestinal complications of the disease occur rarely, but have included: erythema nodosum [27]; reactive arthritis [28]; cholecystitis [29]; appendicitis, including recovery of *C.jejuni* from the appendix [30]; meningitis [31]; haemolytic-uremic syndrome [32]; bacteraemia [33] and Guillain-Barré syndrome (GBS) [34]. GBS is the commonest cause of acute neuromuscular paralysis in developed regions and up to two-thirds of cases are preceded by symptoms of an infectious illness [35]. Of these preceding infectious illnesses, *C.jejuni* enteritis is the most common.

Treatment with specific antibiotics, notably erythromycin, may help to eradicate *C.jejuni* from the faeces of infected patients [36], however, early administration is recommended for the antibiotics to have a significant effect. It is debatable whether treatment should be given for uncomplicated *C.jejuni* enteritis, since an increase in the frequency of antibiotic resistance in *Campylobacter* species has been reported [37].

1.4 Epidemiology of *C.jejuni*. The major reservoirs of *C.jejuni* are animals, both domestic and wild, in which they live as commensals. In general, the most important source of infection with *C.jejuni* is ingestion of contaminated food, such as under-cooked poultry and red meat. Fresh food prepared with the same utensils as uncooked meat and poultry products can also be a source of infection [8]. The pattern of infection is mainly sporadic, with occasional outbreaks in closely associated populations due to consumption

of the same contaminated product [38, 9] . The consumption of unpasteurised or improperly pasteurised milk has been widely reported to cause outbreaks of *Campylobacter* enteritis [39, 9]. Some outbreaks have been associated with untreated or contaminated water supplies, for example at a boys school in Britain the organism was thought to have originated from avian faecal contamination of a water-holding tank [40]. A large outbreak, involving 2000 to 3000 people, occurred in Vermont in 1978, due to consumption of contaminated water [41]. Poultry meat is the most common source of *C.jejuni* infection in developed countries [8] and the rise of incidence of *Campylobacter* enteritis in industrialised regions has been associated with the recent rise in chicken consumption [42].

In temperate climates there is a higher incidence of the disease in summer, which probably reflects the fact that *C.jejuni* does not actually grow below 25°C, although it can survive for 2 to 5 weeks in bovine milk or water kept at 4°C [43]. It has also been speculated that this higher incidence could be due to the seasonal popularity of barbeques [8]. Campylobacters are inactivated after a few seconds at 63°C and would not be expected to survive thorough meat cooking procedures [8].

In developing countries, infections due to *C.jejuni* and closely related organisms, such as *C.coli*, are endemic, with higher reported infection rates in young children than in the rest of the population [14]. Several groups have shown that immunity to infection occurs after about the age of 6 months, but that this immunity does not prevent asymptomatic carriage of the organism [44]. Subjects in these areas experience a milder disease with a watery, non-inflammatory diarrhoea, whereas travellers tend to experience the symptoms described domestically [14].

1.5 Pathogenesis of *C.jejuni*. The mechanisms by which *C.jejuni* causes disease are not well understood. On entry into the host, *C.jejuni* must multiply and colonise, resisting host defence mechanisms. Bacterial colonisation of mucosal surfaces depends on the bacteria being able to maintain close proximity to the mucosa (association) and to attach (adhesion) to avoid being swept away. The main site of colonisation by campylobacters is the small intestine, but the colon is also colonised and most medical investigations utilise colonoscopy [43].

In colonising the intestine, *C.jejuni* can cause pathological changes resulting in the clinical symptoms observed in patients, notably diarrhoea. The specialised features that the infecting organism uses in these events are known as "virulence determinants" and those used by *Campylobacter* species are generally not yet well characterised.

1.5.1 Colonisation. Campylobacters are susceptible to the bactericidal activity of gastric acid. However, ingestion in association with food, especially vehicles with rapid wash-through such as water, or with high buffering capacity such as milk, aids the passage of the organism through the gastric barrier [45]. *C.jejuni* then colonises the distal ileum and colon.

Association with the intestinal mucosa is a prerequisite for colonisation and establishment of disease. The presence of one or more flagella is required for the penetration and colonisation of this mucus layer which covers the epithelium of the small intestine. *C.jejuni* are unusually motile within intestinal mucus and nonmotile, aflagellate variants are unable to colonise the gastrointestinal tracts of experimentally infected mice or human volunteers [46; 24]. When Ferrero and Lee compared the motilities of *C.jejuni* and conventional rod-shaped bacteria in a viscous environment, they observed that *C.jejuni* continued to be motile in viscous, mucus-like solutions which immobilised the other bacteria [47]. Recently it has been observed that the velocity of *C.jejuni* increases in high-viscosity growth medium and that this increased velocity is associated with longer periods of straight swimming followed by pauses, instead of the punctuating tumbles observed in non-viscous tissue culture medium [48].

C.jejuni have been shown to colonise the intestinal mucosa of adult mice by association with the mucus blanket, the mucus-filled crypts of Lieberkuhn and crypts of the large bowel [49]. The same group postulated that the organism does not require adherence to exert its final pathogenic potential, since it can achieve close enough association with the epithelial cells by colonising the mucus alone. Fauchere *et al.* also found that less than half of the *C.jejuni* and *C.coli* strains isolated from human faeces could adhere to HeLa cells (derived from human cervical carcinoma) *in vitro* [50]. McSweegan and Walker demonstrated that some strains colonised the mucus substrate in large numbers but attached in relatively low numbers to an intestinal cell-line (INT 407); while, for other strains, binding to the INT 407 cells was relatively high [51]. However, adhesion to the cell surface seems necessary for subsequent invasion to occur and potential adhesins have been and are being sought.

It has been suggested that the flagella and lipopolysaccharide (LPS) extracted from *C.jejuni* may be important adherence factors [52]. However, ultrastructural observation by transmission electron microscopy of HEp-2 cells (derived from human laryngeal epidermoid carcinoma) infected with *C.jejuni* revealed no close apposition between flagella and the plasma membrane [53]. It was also demonstrated that preincubation of

Campylobacter outer membrane preparations with monoclonal antibodies (mAbs) directed against LPS or flagella does not inhibit their binding to INT 407 cell membranes. However, binding to INT 407 cell membranes was detectable using LPS-specific mAbs, indicating that LPS is associated with, but is not itself, a binding structure [54].

Studies using non-flagellated mutants of *C.jejuni* indicated that either motility or the presence of the major flagellin subunit, FlaA, are required for invasion, but not adherence, to intestinal epithelial cells [55, 56]. Therefore, it seemed that flagella do not play a major role in adhesion to epithelial cells. However, recent experiments with *C.jejuni* non-motile mutants which have intact flagella suggest that the FlaA protein on an immobilised filament is capable of mediating adherence to INT 407 cells without invasion occurring [57]. Whereas mutants in the *flaA* gene, which lacked the FlaA protein and had severely truncated flagella (made solely from FlaB) were both non-adherent and non-invasive. Fully motile mutants were also found which were defective in both adherence and invasion, indicating that further adhesins are responsible for mediating a motility-dependent invasion process [57].

Moser and Schroder [58] found that flagella were resistant to proteinase K activity but that pretreatment of *C.jejuni* outer membrane preparations with proteinase K almost completely abolished binding to INT 407 cells. They also found that oxidation of LPS with sodium meta-periodate did not significantly reduce binding. Experimental results therefore indicate that, although the flagella may mediate some adherence to the intestinal epithelium, structures other than LPS and flagella are primarily involved in the close adhesion required for invasion.

Moser *et al.* [54] found that the purified 42kDa major outer membrane protein (MOMP), which had been previously described as a porin [59, 60], could be involved in the binding of *C.jejuni* to INT 407 cell membranes. The MOMP elicits a strong immune response in human *C.jejuni* infections [61, 62] and the MOMP from *C.jejuni* strain 85H has been shown to exhibit antigenic similarity with the *E.coli* OmpC porin [63]. Proteinase K treatment altered the apparent molecular mass of the MOMP from 42kDa to 24kDa, possibly abolishing its putative adhesive function [58], but without a monoclonal antibody against MOMP available for blocking experiments, or a defined mutant in the gene for MOMP, its role in adhesion cannot be confirmed.

deMelo and Péchère investigated the binding of isolated cell-surface antigens from *C.jejuni* to HEp-2 monolayers [64]. Investigation of glycine acid extracts revealed proteins of 28, 32, 36 and 42kDa which bound HEp-2 cells (only a 36kDa protein from a less invasive strain was found to bind).

They found that the surface proteins with molecular masses of 28 and 32kDa bound to human HEp-2 and HeLa cell monolayers, but not to animal cell cultures and that binding correlated with the ability of the invasive *C.jejuni* strain to penetrate. Further investigations have confirmed that a protein of approximately 28kDa from enteropathogenic *C.jejuni* strains adheres specifically to HeLa cells and is among the most immunogenic bacterial constituents for infected patients [65, 66]. This potential adhesin has been called PEB1 and the gene encoding for this protein has been cloned and sequenced [67]. The deduced amino acid sequence exhibits significant homology to amino acid transport systems in Gram-negative *Enterobacteriaceae*, but PEB1 appears to contain a second signal peptidase processing site which distinguishes it from these amino acid-binding proteins and may make it accessible to the bacterial cell surface where it could also perform a cell binding function.

The pilus is a virulence determinant which is found in numerous Gram-negative bacteria, and often mediates adhesion of the bacterium to its target host cells [68]. Recently, it has been demonstrated that *C.jejuni*, *C.coli* and *C.fetus* are capable of producing a pilus-like appendage in response to incubation in the presence of bile salts [69]. A *C.jejuni* gene, termed *pspA*, was identified which is involved in the production or regulation of pilus production. Mutation of this gene resulted in non-piliated mutants which were not impaired in their ability to adhere to or invade INT 407 cells. The mutants also retained the ability to colonise ferrets, but, nevertheless, had a significantly reduced virulence in the ferret model. The data published to date therefore indicates that the *C.jejuni* pilus is not a primary adhesin for host intestinal cells.

The observations that *Campylobacter* colonies grown on media containing bile salts became tightly aggregated and a uniform suspension could not be made of them, and also that the pili frequently formed bundles, could indicate that the primary function of *Campylobacter* pili is the formation of microcolonies. Enteropathogenic *E.coli* cells utilise bundle-forming pili to form microcolonies which participate in localised adhesion to the host cells [70]. Formation of such microcolonies appears to be necessary for further stages of the infection to take place, possibly because a minimum number of bacteria must cooperate in order to bring about a particular event such as invasion.

Grant *et al.* [56] suggested that binding of campylobacters to host cells occurs in stages, similar to those proposed for *S.typhimurium* [71]. The bacteria are brought into contact with host intestinal cells through motility and reversible binding of the bacteria to the cells occurs. This reversible binding can then facilitate binding in an irreversible manner,

for which the bacteria utilise specific adhesins. Only irreversibly bound campylobacters may then be internalised. The observations of Yao *et al.* [57] indicate that invasion is also dependent on the motility of the organism in an unknown manner. It is clear that adherence of *C.jejuni* is a complex, multifactorial process, involving some adhesins that do not lead to invasion.

1.5.2 Invasion. The tissue damage and inflammation, which occur during *C.jejuni* infection, may occur as a result of invasion of the epithelial cells by *Campylobacter* cells and/or by the action of toxins produced by the organism. Clinical data suggests that intestinal epithelial invasion can occur in *Campylobacter* enteritis, with bloody diarrhoea and leukocytes and inflammatory cells in the stools. Intracellular *C.jejuni* have been observed in colonic epithelial cells of patients with *Campylobacter* colitis [72] and direct tissue invasion is also supported by *in vivo* models, in which *C.jejuni* was orally administered to chickens [73], hamsters [74], mice [75] and monkeys [76]. On the basis of these observations, studies have been carried out in mammalian cell-lines. Penetration of HeLa cells [65] and HEp-2 cells [77] has been demonstrated and pretreatment of HEp-2 cells with mucin enhanced internalisation of fresh isolates of *C.jejuni* [78]. Similarly, *C.jejuni* has also been found to invade INT 407 (human embryonic intestine) cells [79] and a colon carcinoma cell-line (Caco-2) [80, 81]. Both these cell types have enterocyte-like differentiation patterns, with brush-border microvilli being exhibited in a polarised fashion. Caco-2 cells also form tight junctions and express apical surface enzymes characteristic of intestinal enterocytes, thus they represent a unique model for differentiated enterocytes in the intestine.

More recently, attempts have been made to find the invasion ligands on the surface of *C.jejuni*. Konkel and Cieplak [79] have found that *C.jejuni* synthesizes at least 14 new proteins following cocultivation with intestinal epithelial cells *in vitro* and also that chloramphenicol, a selective inhibitor of bacterial protein synthesis, significantly reduced the invasion, but not the binding, of *C.jejuni* to INT 407 cells. Furthermore, antiserum against cocultivated bacteria specifically inhibited the internalisation, but not the binding, of *C.jejuni* in a dose-dependent fashion, whereas antiserum against *C.jejuni* cultured in medium alone reduced invasion by only 50% at the highest concentration tested [82]. *C.jejuni* was also found to invade both HEp-2 cells and INT 407 cells in significantly higher numbers after it was passed through swine intestinal cells [83]. These findings strongly suggest that one or more of the proteins which have their synthesis enhanced during cultivation with

intestinal epithelial cells, also has a functional role in invasion. These *de novo* synthesized proteins have yet to be identified, however there is some evidence that a glycoprotein or carbohydrate may be involved in invasion. Konkel and Joens [77] found that the number of intracellular *C.jejuni* decreased when *Campylobacter* whole-cell lysates were adsorbed onto HEp-2 cell monolayers, although adherence of the bacteria to the epithelial cells was not affected. Following oxidation of the lysates with sodium *meta*-periodate, their inhibitory capacity was significantly reduced, implying that the *Campylobacter* invasive ligand may have a carbohydrate moiety.

Only the *C.jejuni* flagellum has been shown consistently to be involved in invasion (although it is not a primary adhesin) [55, 56]. Experiments with mutants in the *flaA* gene demonstrated that non-motile, non-flagellated *C.jejuni* mutants are unable to invade tissue culture cells. More recently, *C.jejuni* non-motile mutants have been constructed which have intact but paralysed flagella [57]. These mutants in the *pflA* gene (paralysed flagella) were found to adhere to INT 407 cells with up to 46% of the efficiency of wild type *C.jejuni*, but they could not invade. It therefore seems that motility, rather than the FlaA protein, is necessary for invasion of *C.jejuni* into INT 407 cells.

Some invasive pathogenic bacteria, such as *Salmonella*, have been shown to express products that bind to host cell receptors and facilitate subsequent internalisation in a process which has been described as parasite-directed endocytosis [84, 85], while others, such as *Shigella*, are thought to cross the mucosa through naturally phagocytic M cells [86].

Investigations into the mechanism(s) of internalisation of *C.jejuni* into tissue culture cells have yet to reveal the details of the process involved. Conflicting results have been obtained by different groups using different tissue culture cell types. deMelo, Gabbiani and Péchère [53] found that cytochalasin B, which inhibits endocytosis by causing actin depolymerisation, significantly decreased the number of *C.jejuni* internalised by HEp-2 cells and they observed a network of microfilaments under the host cell plasma membrane at the sites of *C.jejuni* binding. However, Konkel *et al.* [87] did not find dense concentrations of actin in association with the sites of *C.jejuni* attachment to HEp-2 cells but the same group did find that cytochalasin D, a specific inhibitor of microfilament formation, reduced the internalisation of *C.jejuni* by INT 407 cells [88]. They observed accumulation of actin in association with attached *C.jejuni* in these intestinal epithelial cells, but they noted that the cytochalasin D treatment did not prevent this actin accumulation.

Konkel *et al.* also found that dansylcadaverine, which inhibits receptor recycling, had a marked inhibitory effect on invasion of INT 407 cells [88]. Oelschlaeger *et al.* [89] found that microtubule depolymerisation reduced invasion of *C.jejuni* into INT 407 cells by approximately 80% and that inhibitors of coated-pit formation also inhibited endocytosis of *C.jejuni* strain 81-176 in INT 407 cells. However, Russell and Blake [90] found no inhibition of invasion of *C.jejuni* into Caco-2 cells by cytochalasin D and that microtubule inhibitors vincristine and vinblastine did not reduce the invasiveness of *C.jejuni* 81-176 in Caco-2 cells and they found no decrease in the numbers of intracellular *C.jejuni* following treatment with monodansylcadaverine, which inhibits transglutamase activity needed for coated-pit formation.

Russell and Blake also appeared to find that chloramphenicol did not inhibit invasion of *C.jejuni* into monolayers of Caco-2 cells, implying that protein synthesis was not required for internalisation. These results appear to contradict all the previous work by other groups, but may be due to differences in experimental conditions under which the assays were conducted, methods of quantifying adherent and internalised *C.jejuni* and the strains used. The results could also reflect fundamental differences in the mechanisms of bacterial invasion of the different cell-lines, i.e. HEp-2, INT 407 and Caco-2 cells.

Such conflicting reports mean that the details of the mechanism(s) involved in the invasion of host cells by *C.jejuni* are still not certain. Nevertheless, recent observations have provided the first insights into the host cell signalling events required for uptake of *C.jejuni* into intestinal epithelial cells. Recent work has shown that pretreatment of Caco-2 cell monolayers with filipin III significantly reduces the ability of *C.jejuni* to enter, but not adhere to, these cells [91]. Filipin III disrupts the small (50-100nm) plasma membrane invaginations known as caveolae which are found in a variety of mammalian cells and are implicated in certain types of clathrin-independent endocytosis and signal transduction. Furthermore, pretreatment of the Caco-2 cells with staurosporine, a broad spectrum inhibitor of eukaryotic kinases, and genistein, a more specific inhibitor of tyrosine kinases, also caused a reduction in levels of *C.jejuni* invasion [91]. These inhibitors had little effect on *C.jejuni* binding to the Caco-2 cells or on Caco-2 cell invasion by *Salmonella*, in agreement with published findings [92]. Wooldridge *et al.* [91] also observed that the fungal toxin wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, and cholera toxin, which is an antagonist of the $G\alpha_s$ subfamily of heterotrimeric G-proteins, reduced uptake, but not binding, of *C.jejuni* by Caco-2 cells. Pre-incubation of the Caco-2 cell monolayer with pertussis

toxin, which uncouples the $G\alpha_1$ and $G\alpha_0$ subfamilies of heterotrimeric G-proteins, did not inhibit uptake of the bacteria and *Salmonella* invasion was not inhibited by any of these toxins. From this data, a possible mode of *C.jejuni*-induced endocytosis has been proposed in which an unidentified cell surface receptor(s) present within caveolae is stimulated by contact with motile *C.jejuni* and activates tyrosine protein kinases and/or heterotrimeric G-proteins (of the $G\alpha_s$ subfamily), which are known to localise to caveolae [93, 94]. Both of these molecules are known to stimulate phosphatidylinositol 3-kinase activity, which can in turn lead to activation of the small GTP-binding protein Rac [95]. Since activated Rac mediates membrane ruffling, which has been documented to enable uptake of enteroinvasive bacteria [96], it is speculated that *C.jejuni* may also invade by this process.

C.jejuni can survive for extended periods within INT 407 epithelial cells [88] and they have also been shown to survive in mononuclear phagocytes. Kiehlbauch *et al.* [97] found that *C.jejuni* cells were readily internalised by human peripheral blood monocytes, but were not rapidly killed following ingestion. The more rapid death of *C.jejuni* in the absence of phagocytic cells appeared to indicate that phagocytosis by macrophages may enable *C.jejuni* to avoid some host defence mechanisms, since campylobacters have been shown to be sensitive to the bactericidal activity of serum [98].

Campylobacter spp. have also been observed to translocate across polarised Caco-2 cell layers [56, 80, 81]. The organism is capable of either transcytosis via a cytoplasmic pathway following initial invasion of the host cell, or translocation between cells [56, 81]. Resistance to killing by macrophages would be useful to campylobacters once they had crossed the intestinal epithelium. However, only one *C.jejuni* strain was used in the phagocytosis experiments and studies *in vivo* have revealed that macrophages can have a significant role in the defence against *C.jejuni* infection. Mice depleted of macrophages prior to inoculation with *C.jejuni* isolated from a case of human diarrhoea had significantly increased mortality [99]. Furthermore, four human isolates of *C.jejuni* were phagocytosed by murine peritoneal macrophages at rates which corresponded with the rates of clearance from the bloodstreams of adult Balb/c mice and 11 day old chicken embryos [100]. Therefore, macrophages may provide an important defence against bloodstream infection by some strains of *C.jejuni*.

C.jejuni has also been shown to be efficiently killed by polymorphonuclear leukocytes (PMNLs) in the presence of IgM and complement, but is phagocytosed and killed less efficiently in the absence of specific

immunoglobulin [101]. It can be proposed that the pathogenic mechanism of *C.jejuni* enteritis involves invasion and translocation across the intestinal mucosa to the sub-epithelial tissues where, in the absence of a specific immune response, further replication and the elaboration of toxic substances and subsequent inflammation occurs.

The tissue damage and inflammation caused by the mechanisms proposed above may, perhaps, result in diarrhoea through disruption of the structural integrity of the intestinal eptithelium so that net absorption can no longer occur. Another suggestion is that the immune system of the host may elaborate the inflammatory response and cause net secretion to occur into the intestine. The invading *C.jejuni* could activate the complement cascade, which includes production of anaphylotoxins (C3a and C5a). These, in turn, cause the release of leukotrienes (including histamine) from mast cells, which increase vascular permeability and cause smooth muscle contraction. White blood cells infiltrating the tissue from the newly permeable blood vessels could cause further damage and inflammation.

Everest *et al.* [102] have found that prostaglandin E2 is present in the fluid secreted from the intestinal epithelium of rabbit ileal loops on infection with inflammatory *C.jejuni* strains. They also showed that these loop fluids could cause increased cAMP production in Caco-2 cells which was inhibited by antiserum against prostaglandin E2 but not by anti-cholera toxin antiserum. Cholera toxin is known to increase intracellular cAMP levels and this increase of the second messenger is thought to be one of the causes of the net fluid secretion seen in severe diarrhoea.

Kaur *et al.* [103] have demonstrated that, during *C.jejuni* infection of ligated rat ileal loops, Ca^{2+} and protein kinase C could also be used as second messengers in the stimulation of intestinal fluid secretion. Thus, it can be suggested that intestinal inflammation following invasion and transcytosis by *C.jejuni* could also lead to active intestinal fluid secretion.

1.5.3 Cytotoxins. The damage to intestinal epithelial cells observed in cases of inflammatory diarrhoea may also be partly due to the action of *Campylobacter* cytotoxin(s) and several studies suggest that *C.jejuni* can elaborate such toxins. Perhaps not unsurprisingly, different research groups using different *C.jejuni* strains and mammalian cell lines have found different cytotoxic activities. Several groups have reported a 68-70kDa cytotoxin which does not affect monkey kidney (Vero) cells, but which is toxic to HeLa, human diploid lung (MRC-5) and HEp2 cells [104, 105], and/or CHO (Chinese hamster ovary) and HeLa cells [106, 107, 108, 109]. Another

cytotoxin has been described which is active against Vero cells and this toxin also seems to have activity against HeLa cells [106, 110, 111, 112]. Other cytotoxins in the literature include: cytolethal distending toxin (CLDT) [113, 114, 115], Shiga-like toxin [116] and haemolysins [117].

68kDa cytotoxin. In 1983, Wong *et al.* [104] found a *C.jejuni* cytotoxic activity which affected HeLa, HEp-2 and MRC tissue culture cells, but not Vero or other animal cell lines, was heat-labile and trypsin-sensitive and was predominantly associated with culture supernatants. The same group later found that the toxin could produce diarrhoea in rabbits using RITARD (removable intestinal tie adult rabbit diarrhoea) and was more than 30kDa in size [105]. Notably, they discovered that expression of the cytotoxin was lost on subculturing of the *C.jejuni* strains. Johnson and Lior [106] also detected a cytotoxin specific for CHO and HeLa cells which was inactive against Vero cells.

Guerrant *et al.* [107], investigated 12 clinical isolates of *C.jejuni* and found the polymyxin B extracts of five to be cytotoxic to HeLa cells, and six to have cytotoxic effects on CHO cells. The toxin was heat-labile, trypsin-sensitive, in the 50-70kDa weight range and not neutralisable with anti-Shiga-like toxin, anti-*C.difficile* toxin or anti-cholera toxin antisera. Two distinct factors which were cytotoxic for CHO cells have also been reported [118], one of which was heat-stable and trypsin-resistant with a molecular weight of 70kDa and a pI of 9.0 and caused haemagglutination of rabbit reticulocytes. The observed heat-stability and trypsin-resistance of this toxin was in contrast to other reports. The second factor, which was observed to be heat-labile and trypsin-sensitive, was haemolytic for rabbit red blood cells.

Mahajan and Rodgers [119] purified a 68kDa *C.jejuni* cytotoxin which was heat-labile, trypsin-sensitive and had activity against CHO and INT 407 cells. They demonstrated that the toxin adhered to chicken fibroblast and INT 407 cells, probably via a glycoprotein receptor. Other groups have also reported a heat-labile, trypsin-sensitive cytotoxin which was active against CHO and HeLa cells [108, 109], but they did not test the effect of the toxin against Vero cells. Daikoku *et al.* [108] also found that expression of the cytotoxin was lost on subculturing or long-term storage of the *C.jejuni* strains.

In summary, a *C.jejuni* cytotoxin of approximately 68kDa has been found to have activity against HeLa, CHO, Hep-2 and INT 407 cells, but not Vero cells. The toxin is heat-labile, trypsin-sensitive and is not neutralised by anti-Shiga toxin, anti-cholera toxin or anti-*C.difficile* toxin antisera.

Cytotoxin which affects Vero cells. There are fewer reports on the cytotoxin which can affect Vero cells. Johnson and Lior [110, 106] reported that Vero cells were more sensitive than HeLa cells to a *C.jejuni* toxin, which could not be neutralised with anti-*Clostridium* toxin or anti-Shiga-like toxin antisera. Klipstein *et al.* [111] found that HeLa cells were more sensitive to the toxin than Vero cells, but attributed this to a difference in the number of cells tested. However, there has been another observation of a stronger cytotoxic effect on HeLa cells than Vero cells using bacterial sonicates after polymyxin B treatment of *C.jejuni* strains [112].

Florin and Antillon [120] observed that Vero cells were more susceptible than HeLa cells, as Johnson and Lior found originally, and that MRC-5 fibroblasts were even more sensitive. In preliminary experiments, they found a toxic fraction from an FPLC column which gave a major band at approximately 100kDa on SDS-polyacrylamide gel electrophoresis, but no further results have been reported.

Cytolethal distending toxin. In 1988, Johnson and Lior [113] demonstrated the production of a cytotoxin, which they named cytolethal distending toxin (CLDT), from 237 out of 583 *C.jejuni* strains tested. CLDT was distinct from previous cytotoxins since it was lethal to CHO, Vero, HeLa and HEp-2 cells but did not affect Y-1 cells, specific assay conditions were necessary for its effect to be seen and its effect on tissue culture cells could take 120 hours to develop. More recently, a receptor-based ELISA has been developed for detection of CLDT [114] which gives a result in a few hours. Ligand blotting identified the CLDT receptor on HeLa cells to be a doublet of 59kDa and 45kDa and the receptor on CHO cells to be a single peptide of 59kDa [114].

Shiga-like cytotoxin. Moore *et al.* [116] reported that low levels of a Shiga-like cytotoxin (SLT) were produced by some *Campylobacter* isolates and was neutralised by monoclonal antibody to the B subunit of *E.coli* Shiga-like toxin-1 and rabbit anti-Shiga toxin. However, SLT was genetically distinct from the SLT-1 toxin produced at high levels by certain *E.coli* and, as non-pathogenic *E.coli* K12 has also been reported to produce low levels of SLT-1, the significance of a low level of SLT-like acivity in *C.jejuni* is questionable.

Haemolysin. Hossain, Stewart-Tull and Freer [117] have observed haemolysin production by Group C (produce cholera-like watery diarrhoea) and Group D (produce dysentery-like, bloody, mucoid diarrhoea) *C.jejuni* strains. Both

the Group C and Group D haemolysins were trypsin-sensitive and were most active against rabbit erythrocytes and least active against chicken erythrocytes. The Group D haemolysin was heat-stable, while the Group C haemolysin was heat-labile at 100°C, and both strain types synthesized haemolysin during the stationary phase of growth. The haemolysin-producing strains were found to be more virulent in experiments with chicken embryos, suggesting some involvement of the *C.jejuni* haemolysin in releasing iron to the pathogen. This is the only report of secreted haemolysin activity from *C.jejuni*, although there have been several reports of contact-haemolysis [121, 122, 123, 124].

It has been suggested that contact haemolysis by *Campylobacter* strains is a two-step process involving successive alpha- and beta-haemolysis [122]. Green zones around *C.jejuni* colonies on rabbit blood agar plates appeared within 48 hours at 37°C and this activity was designated alpha-haemolysis. After 6 days at 37°C or 3 days at 42°C, beta-haemolysis occurred, producing clear zones on blood agar plates around colonies of the same *C.jejuni* strains. Tay *et al.* [123] found that larger zones of beta-haemolysis occurred on plates solidified with agarose (instead of agar) and incubated aerobically at 42°C. They also developed a microtitre plate assay system for measuring levels of contact haemolysis.

Recently, an expression library of *C.jejuni* genomic DNA in *E.coli* has been screened for haemolytic activity on blood agar plates [125]. Three positive clones were obtained which all contained the same open reading frame, but the derived amino acid sequence had no similarity with haemolysins. In fact, the gene responsible for conferring the haemolytic phenotype on the *E.coli* host cells encoded a protein with significant homology to periplasmic siderophore-binding proteins and it was concluded that the haemolysis observed was an indirect effect of overexpression of this protein.

Incidence of cyotoxic acivity. Reports on the incidence of cytotoxic activity have attempted to correlate symptoms of inflammatory diarrhoea with production of cytotoxin. In two American studies, 42% [107] and 32% [126] of *C.jejuni* isolates, from patients with inflammatory diarrhoea, produced cytotoxin. A larger study of 316 clinical isolates yielded 72% cytotoxin-producing strains, but the type of diarrhoea was not specified [106]. In contrast, Florin and Antillon found that, out of 18 Costa Rican children with inflammatory diarrhoea, only two had *C.jejuni* strains which produced cytotoxin [120]. In other studies concerning cytotoxin production by *C.jejuni* isolates from adults, in which the type of diarrhoea was not specified, the frequencies have varied from 18% to 87% [104, 105, 108, 112,

126]. The different frequencies reported make it difficult to draw any definite conclusions about the correlation between cytotoxin production and type of disease.

Production of cytotoxins which may bind to receptors on host cell membranes is, therefore, a potential virulence trait that may be involved in the inflammatory diarrhoea caused by several *C.jejuni* isolates, but the function of cytotoxin(s) at a detailed level is still unclear. A promising step has been made towards elucidating the function of CLDT with the recent cloning of the *C.jejuni cdt* genes, which were shown to encode CLDT and were found by hybridisation to be present in most *C.jejuni* strains tested [115]. However, no genes have yet been identified that could encode other cytotoxins.

1.5.4 LPS. Lipopolysaccharide (LPS) is an endotoxin which forms part of the cell wall of Gram-negative bacteria. The toxic component of LPS is the lipid portion, called lipid A, which is embedded in the outer membrane and exerts its effects when the bacteria lyse. The toxicity of lipid A is caused primarily by its ability to activate the host complement cascade and cytokine system. The overproduction of these compounds can cause fever and increase vascular permeability and, in extreme cases, involving bacteria infecting and releasing LPS into the bloodstream, septic shock can occur through collapse of the circulatory system [128]. It is possible that *C.jejuni* may induce local inflammation in intestinal epithelial tissue through the effects of LPS activation of the host complement cascade and cytokine system.

As discussed previously (section 1.5.2), activated components of the complement cascade can activate mast cells to release prostaglandins and leukotrienes. Everest *et al.* [102] observed that inoculation of rabbit ileal loops with inflammatory strains of *C.jejuni* caused increases in the levels of prostaglandin E_2 and leukotriene B_4 in the loop fluids which also correlated with infiltration of neutrophils. Leukotriene B_4 is known to be chemotactic for PMNLs, which include neutrophils, and the infiltration of neutrophils is a characteristic of the acute inflammatory response [129]. PMNLs are themselves potent sources of prostaglandins, leukotrienes and other inflammatory mediators which cause characteristic local blood vessel dilation and swelling from influx of cells and proteins.

Large numbers of complement complexes attaching to PMNLs may cause the release of phagolysosomal contents and hence damage to host tissues. There has been a report of *C.jejuni* strains evoking low level release of toxic oxygen metabolites from activated neutrophils [130]. Prostaglandin E2 also decreases active sodium and chloride absorption and increases fluid

secretion in both the small intestine and the colon by activation of adenylate cyclase and, hence, increased levels of cyclic AMP [131].

The LPS molecule also forms the basis of the thermo-stable antigen serotyping system of *C.jejuni* [132, 133] and interesting features of *C.jejuni* LPS include N-acetylneuraminic acid (NeuAc) residues which occur in a mode of attachment which is commonly encountered in mammalian glycolipids and glycoproteins [134]. Such NeuAc residues are known to confer low or no immunogenicity on the capsular polysaccharide of certain *E.coli* strains because of structural mimicry of mammalian host components [135]. This low immunogenicity may, therefore, preclude the LPS of some *C.jejuni* strains from playing a major role in activating the host immune response to effect the symptoms of inflammatory diarrhoea.

C.jejuni LPS can consist predominantly of a low molecular weight fraction which is similar to the lipooligosaccharides found in *Neisseria* and *Haemophilus* species, but some strains also possess high molecular weight LPSs equivalent to the *Salmonella* smooth-type LPSs [134]. Such a strain, possessing both the *Salmonella* smooth-type high molecular weight LPS and the lipooligosaccharide-type low molecular weight LPS, constitutes a complexity which is unique among Gram-negative bacteria.

1.5.5 Enterotoxin. Enterotoxins, similar to cholera toxin, have been looked for in *C.jejuni* as a possible cause of the watery diarrhoea observed on infection with non-inflammatory strains. *C.jejuni* enterotoxin (CJT) is reported to be a large protein made up of a 15kDa A subunit and a 60kDa B subunit [136]. It appears to be very similar to *E.coli* heat-labile enterotoxin (LT) and cholera toxin (CT), since it has also been reported to be heat-labile, increase intracellular cyclic AMP, and cause increased fluid secretion in rat ileal loops [137]. The B subunits of CJT, LT and CT are all immunologically related and all bind GM1-ganglioside [138].

The first evidence for the production of CJT was the observation that the supernatant of a prototype virulent strain induced elongation and increased intracellular cyclic AMP levels in Chinese hamster ovary (CHO) cells and intraluminal fluid secretion in rat ileal loops [137]. Subsequently, an enzyme-linked immunosorbent assay (ELISA), based on binding to anti-cholera toxin antibody, was used to screen cell-free supernatants from *C.jejuni* strains [136]. Most strains tested were positive and also caused rounding in a Y-1 mouse adrenal cell assay.

Other groups have reported cholera-like enterotoxin production by *C.jejuni* through the development of a double sandwich ELISA using GM1-ganglioside and/or hyperimmune serum to *E.coli* LT [138] and the development of new media [139]. McCardell, Madden and Stanfield [140] reported that

the production of CJT is enhanced when *C.jejuni* is grown in medium supplemented with iron in the form of ferric chloride.

Although several groups have demonstrated the presence of an enterotoxin in clinical isolates of *C.jejuni*, the proportion of toxin-producing strains varies. In 1983, Ruiz-Palacios *et al.* [137] reported that they found cholera-like enterotoxin in 24 out of 32 strains from patients with diarrhoea and 1 out of 6 carriers. McCardell, Madden and Lee [136] isolated the enterotoxin from 7 out of 8 clinical isolates and Johnson and Lior [110] found over 85% of *C.jejuni* and *C.coli* strains had cytotonic activity (cholera-like toxin). While Mathan *et al.* [141] found cholera-like enterotoxin in 7 out of 22 strains tested and Wadstrom *et al.* [142] could not find a cytotonic toxin in strains isolated from Swedish tourists coming from various developing countries. These varied results might reflect differences in media used and procedures of isolation applied. There is also evidence that *Campylobacter* spp. lose the ability to produce toxin after several passages or long storage times [120].

Attempts to correlate production of enterotoxin with watery diarrhoea, which is thought to occur due to the increased intracellular cyclic AMP, were successful in one US study [111]. Enterotoxin-producing strains were also isolated more frequently from children with watery diarrhoea than from cases of inflammatory diarrhoea in Costa Rica [120] and more frequently from symptomatic patients than from asymptomatic carriers in Mexico [143], but there was no significant difference in the number of enterotoxin-producing strains in symptomatic and asymptomatic Indian children [128].

An oligonucleotide complementary to the conserved coding region for a GM1-ganglioside binding site on LT and CT was found to hybridise, under low stringency, to a 2.6kb *Sau*3A fragment from several *C.jejuni* strains, whether they were enterotoxigenic or not [144]. Since the CT-LT/Btrp88 oligonucleotide, but not the whole *eltB* gene (which encodes LT), hybridised with *C.jejuni* DNA, it was suggested that the similarities between CJT and LT/B or CT/B were confined to relatively small regions, probably encoding protein moieties which interact with common host targets. However, other groups have not found an enterotoxin produced by *C.jejuni*, find no anti-CJT antibody response and cannot demonstrate any DNA sequence similarity with CT or LT [145, 146].

The conflicting results may, in part, be explained by the discovery that the production of enterotoxin was lost after storage of the *Campylobacter* strains for 1 year at -70°C [120]. As well as the finding that increasing the concentration of iron in the media enabled the production of enterotoxin in previously negative strains [140] and that

non-producing strains were converted to enterotoxin-producers after passage through the gut of a rat [148].

More recently, a detailed comparison of the immunological properties and ganglioside recognitions by *C.jejuni* enterotoxin and cholera toxin [149] found some specific differences between the two. Antibody against CJT recognised the 68, 54 and 43kDa polypeptides of CJT and the 11kDa subunit of CT, whereas antibody against CT recognised the 68 and 54kDa, not the 43kDa, polypeptides of CJT and the 11kDa subunit of CT. The immunological reactions were also weaker between heterogenous combinations of toxins and antiserum than those between homogenous combinations. Furthermore, although both CJT and CT bound preferentially to GM1 ganglioside than to other gangliosides, CJT did not bind to GD1b whereas CT preferred GD1b.

The isolation of the gene for CJT and, hence, the construction of isogenic mutants, could provide convincing evidence that it has a role in pathogenicity, but this has not yet been achieved. Some researchers in the *Campylobacter* field doubt that *C.jejuni* produces an enterotoxin at all [146].

Conclusions. In conclusion, the lack of detectable toxin production in many clinical isolates, and the low titres of toxins produced by *C.jejuni*, suggest that *C.jejuni* enteritis is not solely a toxin-mediated disease. However, the details of the mechanism(s) by which *C.jejuni* produces the observed clinical symptoms are not yet known. The data appears to indicate that different strains of the organism may use different combinations of mechanisms, and that the mechanism used may also depend on the host immune response.

1.6 The genetics of *C.jejuni*.

The chromosome of *C.jejuni* has an average moles percent G + C content of 30.1 to 33.0 (*E.coli* has a GC content of 50%) and the use of pulsed-field gel electrophoresis, to accurately size large restriction fragments of the *C.jejuni* genome, revealed an average genome size of 1718kb (the *E.coli* genome is approximately 4600kb) [149].

Plasmids of varying sizes are present in *C.jejuni* and, out of 628 isolates examined by Tenover *et al.* [150], 31% were noted to contain plasmid DNA. They have not been shown to specify virulence determinants, as some plasmids do in other bacteria, but are often associated with resistance to antibiotics, notably tetracycline resistance [150, 151]. Some of these antibiotic resistance genes have been cloned from *C.jejuni* and expressed in *E.coli*. The tetracycline resistance gene from *C.jejuni* was found to be significantly homologous to the DNA encoding class M

tetracycline resistance from *Streptococcus* sp. and both were expressed in *E.coli* [152]. In addition, the sequence of the kanamycin resistance gene *aphA*-3 from *C.coli* was found to be identical to the corresponding gene in *Streptococcus* sp. [153]. These data suggest that *Campylobacter* sp. may have acquired certain tetracycline and kanamycin resistance genes from Gram-positive bacteria (or *vice versa*).

The recognised importance of the flagellum in the virulence of *C.jejuni* has concentrated the efforts of some groups to its investigation and led to the first reported isolation of a *Campylobacter* structural gene in 1989 [154]. Part of the *C.jejuni* flagellin gene was cloned from an expression library of *C.jejuni* DNA constructed in Lambda gt11. Immunoscreening of approximately 150,000 recombinant plaques, with flagellin-specific antiserum, resulted in one positive clone being obtained, containing phage DNA with an insert of only 850bp [154]. This 850bp contained 410bp of the flagellin gene, which encoded approximately 15kDa of the flagellin protein.

In the same year a different approach was used successfully to clone flagellin genes of *C.coli* [155]. N-terminal amino acid sequences of cleaved flagellin polypeptides were used to synthesize oligonucleotides, which were subsequently labelled and used to probe a pBR322 library of *C.coli* inserts in the size range 2.0 to 2.5kb. Comparison of the DNA sequence of *C.coli* flagellin with the amino acid sequence revealed that post-translational modification occurred at numerous serine residues. This was thought to contribute to the inability of *E.coli* to express immunologically detectable levels of recombinant *C.coli* flagellin.

Nuijten *et al.* [156] used the 850bp fragment to probe Southern blots and genomic libraries of *C.jejuni* strain 81116 DNA and, hence, cloned the entire flagellin genes *flaA* and *flaB*. These two genes were 95% identical, however only *flaA* was expressed in motile *C.jejuni* 81116 bacteria. In *C.coli* strain VC167 the *flaA* and *flaB* genes were 91.9% identical [157] and both gene products were required for a fully active flagellar filament. Mutants defective in the *flaA* gene were partially motile and produced a severely truncated filament, while *flaB* mutants were only slightly less motile than wild type *C.coli* cells and produced a flagellum indistinguishable in length from the wild type. The *flaA* gene was found to be transcribed from a σ^{28} promoter, similar to flagellin genes in the family *Enterobacteriaceae* and in *Bacillus* spp., while the *flaB* gene was transcribed from a σ^{54} promoter.

Cloning and sequence analysis of the flagellin gene *fla1* of *C.jejuni* strain TGH9011 [158] revealed that there was sequence heterogeneity within the internal regions of the flagellin gene and its protein, while the amino

and carboxyl ends were highly conserved between *C.jejuni* strains TGH9011 and 81116 and *C.coli*. *C.jejuni* strain 81116 FlaA protein exhibited 86% identity with *C.coli* flagellin and 75% identity with *C.jejuni* TGH9011 Fla1 protein.

Further analysis of the distribution and polymorphism of flagellin genes from isolates of *C.jejuni* and *C.coli* belonging to 13 different Lior serogroups identified 10 unique polymorphic groups [159]. Within most of the serogroups examined, isolates contained *flaA* genes with conserved primary structures, but between the different serogroups, the *flaA* genes showed considerable polymorphism in their central regions. All serogroups examined contained a second *fla* gene corresponding to *flaB*.

Further analysis of the σ^{54} promoter of the *C.coli* gene coding for the minor FlaB flagellin has revealed that it is subject to environmental regulation [160]. Expression from the σ^{54} promoter was found to increase during growth at 42°C, and in the presence of 10mM $MgSO_4$ on defined medium, this resulted in increased synthesis of FlaB protein accompanied by an increase in cell motility. Investigations of the *C.jejuni flaB* gene have indicated that the *flaB* messenger in *C.jejuni* 81116 is also produced from a σ^{54} promoter, similar to that found in *C.coli* [161], and that flagellin gene expression seems to be regulated in such a way that one gene is transcribed at a time. Promoters which are σ^{54}-dependent usually have upstream activator sequences which can be affected by *trans*-acting factors [162]. These findings suggest that campylobacters may be able to regulate their motility by varying the synthesis of alternate flagellins in response to the environmental niche of the cells.

The precise roles of other putative virulence determinants of *C.jejuni*, involved in colonisation, adhesion, invasion, cytotoxin and enterotoxin production, have not yet been elucidated. It has not been possible to construct isogenic mutants, each modified in a single different virulence determinant, which would enable the importance of every step in the pathogenic process to be determined.

Various difficulties have been encountered in attempts to clone, maintain and express *Campylobacter* genes in *E.coli* host strains. Attempts to subclone portions of the *C.coli* flagellin gene into *E.coli* expression vectors did not yield immunologically detectable gene products [155]. This may have been due to the inability of *E.coli* to post-translationally modify the flagellin molecules in the same manner as *C.coli*. However, it was also noted that the plasmid constructs were unstable in many of the subcloning experiments, often producing deletions [155]. Consequently, many of the *C.jejuni* chromosomal genes which have been cloned and expressed in *E.coli* are so-called "housekeeping" genes, which appear to be expressed from their

own promoters in *E.coli* host cells and have been isolated by functional complementation of *E.coli* mutants. Such genes include: the proline biosynthesis genes (*proA* and *proB*) [163, 164], *glyA* (serine biosynthesis) [165], *argH* (biosynthesis of arginine) [166] and *leuB* (leucine biosynthesis) [167]. The *C.jejuni fur* (iron-dependent gene regulation) [168] and catalase (*katA*) genes [169] were also isolated by complementation and the *C.jejuni* arylsulphatase gene (*astA*) [170] and the *hipO* gene (which encodes the hippuricase enzyme that distinguishes *C.jejuni* from other *Campylobacter* species) [171], were isolated from libraries of *C.jejuni* chromosomal DNA by assaying for the activity of the enzymes encoded by these genes.

The complete sequence of the *C.jejuni glyA* gene has been published [172] and sequencing upstream of the *glyA* gene revealed the *C.jejuni* lysyl-tRNA synthetase (*lysS*) gene, which was also found to be expressed from its own promoter in an *E.coli* minicell system [173]. However, it was discovered in earlier experiments that the beta-lactamase gene, contained on pBR322, is not expressed in *Campylobacter* cells [174]. This, and other similar findings, suggested that there were some transcriptional/translational barriers to the expression of *Campylobacter* sp. genes in *E.coli*. However, some genes have been successfully isolated by using *C.jejuni* expression libraries in Lambda gt11, Lambda ZAP and pBluescript [67, 154, 175, 176, 177, 178].

In 1995, the *peb4A* gene was found by screening a Lambda gt11 library of chromosomal DNA from *C.jejuni* strain 81-176 in *E.coli* Y1090 cells with antibody raised against purified PEB4 [175]. In the same year, the *tig* gene for *C.jejuni* trigger factor (involved in cell division) was isolated from a Lambda ZAP library using antiserum raised against glycine-extractable *C.jejuni* proteins [176]. Very recently, antiserum against whole *C.jejuni* cells was used to screen a pBluescript SK+ library and successfully isolate a gene encoding an 18kDa peptidoglycan-associated lipoprotein (PAL) [177] and antiserum against a glycine-extracted 29kDa protein, used to screen a pBluescript SK+ library, detected the gene for HisJ (histidine periplasmic binding protein) [178]. As described earlier, an 850bp insert was transcribed and translated into part of a flagellin protein, which was subsequently recognised by flagellin-specific antiserum [154]. It was suggested that larger clones were not identified because they may have contained regulatory sequences toxic for *E.coli*, however, this does not explain why larger parts of the structural gene were not found.

There is some evidence that *Campylobacter* genomic DNA can be unstable in *E.coli*-derived cloning vectors. Attempts to subclone the proline

biosynthesis genes from a 14.5kb insert in pBR322 into pBR328 were unsuccessful, although eventually a deletion derivative was cloned by digestion with *Eco*RV and self-ligation [163]. Louie and Chan [164] discovered that plasmid preparations from *E.coli proA* mutant cells which had been transformed with plasmids carrying the *C.jejuni proA* gene, yielded smaller plasmid DNAs, of several different sizes, than those with which the *E.coli* strain had originally been transformed. Similarly, the sizes of recombinant cosmids (containing *C.jejuni* genomic DNA) which became stabilised in *E.coli leuB* mutant cells during complementation experiments, ranged from 12.9 to 15.4kb, compared to the originally packaged 45 to 50kb molecules [167]. Recently, attempts to clone an 8.3kb *Pvu*II fragment which included *cdt* gene sequences were unsuccessful, although several other clones were isolated which contained *cdtB* and *cdtC* genes or *cdtA* plus some 5′ sequence [115]. The observation that spontaneous deletions can occur in clones containing *C.jejuni* chromosomal DNA, possibly associated with particular destabilising *Campylobacter* sequences, highlights the difficulties inherent in cloning and maintaining *C.jejuni* DNA sequences in *E.coli* under conditions in which no positive selection is possible (problems encountered in cloning *Campylobacter* genes are discussed further in the introduction of Chapter 2).

Investigations into the precise role of each putative virulence determinant of *Campylobacter* species were also hindered by the absence of a natural genetic exchange mechanism, which would allow the introduction of recombinant genes into *Campylobacter* cells. No transfer of plasmids between *E.coli* and *Campylobacter* species by conjugation or transformation was found prior to 1987 [150, 152]. This suggested that, either DNA transfer itself was being blocked, or replication and maintenance of *Campylobacter* plasmids was blocked in unrelated Gram-negative organisms and *E.coli* plasmids could not be maintained in *Campylobacter* sp. In contrast, plasmids were known to be transferred by conjugation within the *Campylobacter* genus itself.

However, in 1987 Labigne-Roussel, Harel and Tompkins [174] constructed a shuttle cloning vector by cloning replication functions of a *C.coli* cryptic plasmid and the origin of transfer of the broad-host-range plasmid RK2 (IncP) into pBR322 sequences, thus ensuring replication of the plasmid in both *Campylobacter* species and *E.coli*. The vector can be mobilised from *E.coli* to *Campylobacter* cells by conjugation, when complemented *in trans* by an IncP plasmid, showing that it was the maintenance of *E.coli* vectors in *Campylobacter* sp. which could not occur previously.

The shuttle vector was subsequently used for mutagenesis, in which a *C.coli* kanamycin resistance gene was first inserted into *Campylobacter* DNA fragments encoding 16S rRNA, cloned in *E.coli*. The disrupted sequences were then returned to *C.jejuni* via conjugation and the gene encoding kanamycin resistance was rescued by chromosomal integration, resulting in the gene replacement of one of the 16S rRNA sequences of *C.jejuni* and the loss of the vector [179]. This same shuttle mutagenesis system was used in mutational analysis of the flagellin gene, *flaA*, of *C.coli* [180]. The number of transconjugants obtained using the shuttle vector system was 10^{-4} per *E.coli* donor cell present at the beginning of the mating.

Attempts to mutagenise the *Campylobacter* genome using the shuttle vector carrying the Gram-negative (Tn5) or Gram-positive (Tn917) transposons were unsuccessful. Recently, manipulations of transposon sequences have been carried out in an attempt to obtain transposon mutagenesis in *C.jejuni*. The Tn*phoA* kanamycin resistance gene was replaced with the kanamycin resistance gene from *C.coli* and the transposase gene (*tnp*) was placed under the control of the *C.coli*-derived chloramphenicol acetyl transferase (*cat*) gene promoter [181]. However, no transposition was observed and it was speculated that the transposon sequences and/or transposase may not be able to interact with host factors necessary for the transposition event to occur, due to significant protein differences or the complete absence of a required host factor in *Campylobacter* spp. It has been suggested that there may be a kanamycin transposon which can insert into the chromosome of *C.fetus* but this has not yet been fully investigated [182].

Although the absence of a transposon mutagenesis system which functions in *Campylobacter* may have hindered the discovery of unique pathogenic determinant genes, other techniques have been developed which enable investigation of conserved or related genes. PCR with degenerate oligonuceotide primers, designed against regions of proteins which are conserved between different bacteria, has been utilised successfully to clone several *C.jejuni* genes. These include: *sodB* (encodes iron superoxide dismutase) [183], *gyrA* (encodes DNA gyrase A subunit) [184], *flhA* (involved in the secretion and/or assembly of flagellin) [185], *recA* [186], the *cdt* genes for cytolethal distending toxin [115], *htrA* [182] and genes for two-component regulators [J.M.Ketley and J.Henderson, personal communication].

Since broad host range plasmids are not maintained in *Campylobacter* cells, they have become useful as suicide vectors. *C.jejuni* mutants in the genes *sodB*, *flhA* and *recA* have all been isolated by inserting the *Campylobacter* kanamycin resistance gene into the *C.jejuni* gene carried by pBluescript or pUC18, then transforming these constructs into *Campylobacter*

cells. Selection on media containing kanamycin was found to yield mutant *C.jejuni* in which double cross-over events had occurred to leave a single copy of the gene of interest, mutated by the insertion of the kanamycin resistance gene.

Transformation of *C.jejuni* can be carried out by conjugation [174], electroporation [187] or natural transformation [188]. In comparing natural transformation and electroporation, Wassenaar and co-workers [189] found that only two out of nine *C.jejuni* strains were naturally competent and that natural transformation could only occur with *C.jejuni* chromosomal DNA and subsequent recombination into the chromosome. They agreed with Wang and Taylor [188] that, similar to natural transformation in *Neisseria gonorrhoea* and *Haemophilus influenzae*, specific receptors on the surface of the *C.jejuni* cell recognise specific uptake sequences in *C.jejuni* DNA and that this accounted for the inability to introduce the shuttle vector pILL550 into *C.jejuni* by natural transformation. *C.jejuni* strain 81116 was highly naturally competent, with 1μg of *C.jejuni* DNA being accepted with an efficiency of 2×10^5, and *C.jejuni* strain 719 accepted *C.jejuni* 81116 DNA with an efficiency of 3.4×10^3. Three out of nine strains were transformable by electroporation and the shuttle vector pILL550 could be transformed into *C.jejuni* by this method. However, *C.jejuni* strain 480 was the only strain to accept pILL550 DNA isolated from both *E.coli* and *C.jejuni* strains. Strain 480 also gave the highest transformation efficiencies and these were up to 205 transformants per μg DNA (per 10^8cfu) for pILL550 from *C.jejuni* and 800 transformants per μg DNA (per 10^8cfu) for pILL550 from *E.coli*.

In conclusion, natural transformation is the most efficient method for introducing *C.jejuni* chromosomal DNA into competent strains such as 81116. However, electroporation would be the preferred method for strain 480, which has the additional advantage that it is transformable with shuttle vector DNA of *E.coli* or *C.jejuni* origin. But, for many strains of *C.jejuni*, and for most experiments requiring transformation from an *E.coli* origin, conjugation would be the preferred technique.

1.7 Aims and objectives. The work presented in this thesis consists of two projects in which different techniques were utilised for cloning and investigating *C.jejuni* genes. The first project was devised in 1990, when investigations into the genetics of *Campylobacter* were still at a very early stage and the few *C.jejuni* genes which had been cloned were: *proA/proB* [163], rRNA genes [190], *glyA* [165] and *fla* [154, 156]. We were interested in isolating genes for *C.jejuni* virulence determinants, but it was realised that virulence determinant genes may only be expressed *in*

vivo. Thus it was decided that convalescent antiserum, from a patient who had contracted campylobacter enteritis, should be used to screen an expression library of *C.jejuni* genomic DNA, derived from the strain which had infected the patient. Although the antiserum was likely to detect proteins expressed *in vivo*, clearly these may not be involved in virulence. However, they would still be of some interest because so little was known about the biochemistry of *C.jejuni* at the time.

The second project was devised in 1991 and developed from the successes which had recently been achieved in the utilisation of the polymerase chain reaction (PCR) to amplify segments of DNA from low concentrations. It was realised that conserved sequences of known proteins could be used to design degenerate oligonucleotide PCR primers (DOPs) which could then be used to amplify the corresponding gene fragment from *Campylobacter* spp. Researchers in this laboratory had achieved some success with the PCRDOP technique in amplifying segments of *C.jejuni* two-component regulator genes and the *htrA* gene for a stress-response protein. It was decided to attempt to clone the *C.jejuni recA* gene using this PCRDOP method because *recA* is ubiquitous within many bacteria and contains several highly conserved regions. Furthermore, there was some evidence that RecA may be involved in virulence of *Vibrio cholerae*, by helping to increase the number of copies of the cholera toxin virulence cassette [191].

The ultimate aim in developing these diverse techniques in this laboratory was to identify potential virulence determinants of *C.jejuni* and to investigate their roles in the pathogenic process of diarrhoeal disease by constructing *Campylobacter* mutants, each with a single defined mutation in a virulence gene. Such mutants could be compared with the wild-type organism in *in vitro* assays and, ultimately, in *in vivo* models, leading to a better understanding of the factors involved in the pathogenicity of *C.jejuni*.

CHAPTER TWO

A LAMBDA ZAP II EXPRESSION VECTOR-BASED LIBRARY AS A METHOD FOR CLONING *CAMPYLOBACTER JEJUNI* ANTIGEN GENES

2.1 INTRODUCTION

A genomic library is a collection of recombinant clones which contain all of the DNA present in an individual organism. Genomic libraries are prepared by purifying total cell DNA then cleaving this DNA into fragments which can be cloned into a suitable vector. In an ideal library the entire genome of the organism to be studied will be represented as a set of overlapping independently cloned fragments. Such fragments can be created either by partial digestion of the genomic DNA with an enzyme which has a frequently occurring recognition sequence or by mechanical shearing.

Mechanical shearing maximises the randomness of the DNA fragments since it does not rely on the distribution of recognition sites, but the short single-stranded termini created by the shearing process have to be digested with exonuclease (such as *Bal*31) to produce blunt ends for cloning. Ligation of blunt-ended DNA fragments is not very efficient because ligase has to wait for chance associations to bring the ends together. However, it is possible for linkers containing a suitable restriction site to be ligated onto the ends of larger blunt-ended DNA molecules, this particular reaction can be performed efficiently because linker oligonucleotides can be made in large amounts and added to the ligation mixture in high concentration. The resulting hybrid molecules can then be treated with restriction endonuclease to produce fragments which can be incorporated into vector molecules cut with the same restriction endonuclease, or with another endonuclease which produces complementary ends. Due to these complications, partial digestion with a frequently cutting enzyme is more often the method chosen. Such an enzyme is *Sau*3A, which cuts frequently because it recognises a sequence of just four bases, 5′-GATC-3′.

After the genomic DNA has been cleaved to give random fragments of various sizes in the general range required, it is often necessary to further isolate fragments of more specific size. This is because small fragments ligate more efficiently than larger molecules and clones with small inserts transform with a higher efficiency. If there is no size

selection, then in a random cloning exercise the recombinants will have a preponderence of small inserts. DNA fragments of the desired size can be obtained by centrifugation on a sucrose gradient or by preparative gel electrophoresis.

The next step is to choose the vector for the library. Various factors which might influence the choice of vector to be used for the library include: the source of the DNA (i.e. restriction barriers), type of selection/screening mechanism to be employed, size of the fragment, and the proposed use of the cloned fragment. Generally, fragments of greater than 10kb are not conveniently cloned into plasmid vectors. As insert size increases, the transformation frequency of chimeric plasmids is lower and the probability that the cloned DNA will undergo rearrangements in the host is higher. If, for example, the purpose of the experiment is to clone 10-40kb contiguous regions of genomic DNA, then a lambda or cosmid vector would be a more suitable choice. To generate a library of bacterial DNA fragments considerably larger than 20kb, cosmids are the preferred option.

Cosmids are hybrids between a phage DNA molecule and a bacterial plasmid, and are designed around the fact that the enzymes that package the lambda DNA molecule into the phage protein coat need only the *cos* sites in order to function. Thus, the cosmid itself can be quite small - just 5kb is sufficient for the *cos* sites, selectable marker and plasmid replication origin. Lorist 6, for example, is a 5.2kb moderate copy number (approximately 10 copies per cell) cosmid vector, which contains a kanamycin resistance gene, T7 and SP6 promoters close to the cloning site and transcription terminators to minimise possible effects of cloned sequences on plasmid replication or stability [192]. The *in vitro* packaging reaction will work with any molecule that carries *cos* sites separated by 37-52kb of DNA. Cosmids therefore have a relatively large capacity for inserted DNA before the packaging cut-off point of about 52kb is reached. Cloned prokaryote DNA of this length would be useful in examining the structure of chromosomes and reconciling physical linkage of genes with genetic linkage data. However, cosmids are known to accumulate deletions which may result from selection of a shorter size for faster replication and/or metabolic imbalances caused by the increased dosage of a particular gene on the cosmid that affects the growth of host cells.

If the final objective is to examine a single gene or control region, then smaller fragments would be more appropriate, and plasmids may prove more useful as vectors. The major advantage of plasmids is their ease of handling, with rapid methods for small- and large-scale production of relatively pure preparations. However, the high copy number of many plasmids can lead to the instability of some clones, for example those

carrying genes for proteins which adversely affect the host cell when present in large quantities.

Bacteriophage lambda has been used extensively as a library vector, not only due to its ability to maintain larger inserts, but also because the efficiency of lambda phage packaging and subsequent infection of *E.coli* exceeds the efficiency of plasmid transformation 100-fold. This facilitates the construction of primary libraries containing greater than 1 $x10^6$ independent clones. Lambda phage plaques can also be screened when plated at high plaque density, producing little background with DNA or antibody probes. Screening libraries of lambda recombinants by plaque hybridisation usually gives cleaner results than screening libraries of bacteria containing cosmid or plasmid recombinants by colony hybridisation. Furthermore, it may be desired to retain and store an amplified genomic library. With phage, the initial recombinant DNA population is packaged and plated out. It can be screened at this stage, or the plates can be washed to give an amplified library of recombinant phage. The amplified library can then be stored almost indefinitely. With bacterial colonies containing cosmids it is also possible to store an amplified library, but bacterial populations cannot be stored for as long as phage populations without some loss of viability. However, the large size of lambda phage vectors complicates the restriction mapping and sequence analysis of genes after cloning. Therefore, DNA inserts are usually subcloned from the lambda phage vector into plasmid vectors after initial isolation.

Cloned DNA fragments can be identified by several methods and, to some degree, the cloning approach will also be determined by the method of screening employed. Identification of the appropriate DNA fragments may be based upon hybridisation to previously cloned homologous genes, generation of synthetic probes from a known amino acid sequence, functional activity of a cloned gene, or immunological screening to detect the protein coded by the gene. Hybridisation probing with a labelled DNA probe is usually the preferred method for identification of a particular recombinant from a library. The technique is simple to perform and can be fine-tuned to the degree of homology expected between the probe and its target sequence by altering the stringency of washes. Many recombinants can be screened per experiment, allowing large genomic libraries to be screened in a short time. Nevertheless, the requirement for a probe that is at least partly complementary to the desired gene sometimes makes it impossible to use hybridisation in clone identification. This was particularly true in the case of *Campylobacter* virulence genes, since many of the virulence mechanisms had yet to be elucidated. The one major virulence factor to have been isolated was the flagellum and hybridisation had been used to

find the flagellin genes of *C.coli*. N-terminal amino acid sequences of cleaved flagellin peptides were used to synthesize oligonucleotides, which were subsequently labelled and used successfully to probe a pBR322 library of *C.coli* inserts in the size range 2.0 to 2.5kb [155]. The A-T rich nature of *Campylobacter* DNA and the frequency of rare codons also hindered the application of heterologous probing. Limited success had been achieved with an oligonucleotide designed to be complementary to the conserved coding region for a GM1-ganglioside binding site on *E.coli* heat-labile enterotoxin (LT) and cholera toxin (CT). The CT-LT/Btrp88 oligonucleotide, but not the whole *eltB* gene (which codes for LT), was found to hybridise to a 2.6kb *Sau3A* fragment from several *C.jejuni* strains [144]. However, it has not been shown that this *Sau*3A fragment contained any gene of interest.

An alternative to hybridisation probing for *Campylobacter* genes was immunological screening. The distinction is that, whereas with hybridisation probing the cloned DNA fragment is itself identified directly, an immunological method will detect the protein coded by the gene. Immunological techniques therefore assume that the cloned gene is being expressed, and that the protein is not normally present in the vector strain. This technique had been used with some success to isolate a portion of the flagellin gene from an expression library of *C.jejuni* DNA constructed in lambda gt11 [154]. In my project, the intention was to use convalescent antiserum, from a patient who had contracted inflammatory diarrhoea, to screen a library of genomic DNA made from the same *C.jejuni* strain which caused the patient's disease. It was hoped that the antiserum would identify immunogenic proteins that may be involved in an inflammatory infection. The advantage of using convalescent antiserum is that it may detect genes for virulence factors which would normally only be expressed *in vivo*. These could include outer membrane or cytoplasmic proteins which play an important role in pathogenic processes such as adhesion to and invasion of host cells, resistance to the host immune system, and acquisition of growth limiting nutrients *in vivo*.

A further factor to consider in the design and utilisation of a genomic library is the potential number of clones that will need to be screened. Clarke and Carbon [193] derived a statistical formula to calculate the number of clones required to give a specific probability of finding the clone of interest in a library. For a probability, P, of having the fragment of DNA carrying the gene of interest in a library, the number of clones, N, to be screened can be calculated as:

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where f is the average size of the cloned fragments divided by the total genome size (in base pairs).

Campylobacter jejuni has a small genome of only about 1700 kb, therefore, assuming an average size of 4 kb for the cloned fragments, one can determine that a library consisting of just 1955 independent clones should have a 99% probability of every DNA sequence being represented. It is important to emphasize, however, that these calculations are estimates of library size. Factors such as distribution of restriction sites and the effect of gene expression on the host can raise or lower the number of clones carrying the gene, and therefore the necessary library size. Moreover, various difficulties have been reported in attempts to clone, maintain and express *Campylobacter* genes in *E.coli* host strains.

At the time this project was devised (1990) very few genes had been cloned from *C.jejuni*. In 1985, the proline biosynthesis genes *proA* and *proB* were cloned from *C.jejuni* by selection for complementation in a *proAproB* mutant of *E.coli* [163], and the *glyA* gene for serine hydroxymethyltransferase was similarly isolated by complementation in 1988 [165]. Attempts to subclone the proline genes from a 14.5 kb insert in pBR322 into pBR328 using *Eco*RV, *Eco*RI and *Cla*I restriction enzymes were unsuccessful. However, a deletion derivative was isolated by digestion with *Eco*RV and self-ligation. The same group were unable to identify clones capable of complementing a leucine auxotrophic marker in *E.coli* and it was suggested that not all *C.jejuni* genes could be expressed in *E.coli*. During the development of shuttle cloning vectors, in 1987, Labigne-Roussel and co-workers [174] found that *Campylobacter* cells harbouring the chimeric plasmid pILL512 did not express the beta-lactamase gene from pBR322. This gene can be expressed in diverse Gram-negative species, including all members of the *Enterobacteriaceae*, *Haemophilus* species and *Neisseria* species, but is not expressed in Gram-positive bacilli (*Bacillus subtilis*) nor in *Bacteroides fragilis*. It was concluded that fundamental differences in gene expression may exist between *Enterobacteriaceae* and *Campylobacter* species.

The perceived problem with expressing *Campylobacter* genes in *E.coli* was further highlighted in attempts to subclone portions of the *C.coli* flagellin gene into *E.coli* expression vectors. No gene products were immunologically detectable in these experiments. This may have been due to the inability of *E.coli* to post-translationally modify the flagellin molecules in the same manner as *C.coli*. However, it was also noted that the plasmid constructs were unstable in many of the subcloning experiments, often producing deletions [155]. As described earlier, Nuijten *et al.* [154] reported problems in cloning *C.jejuni* flagellin genes from an

expression library of *C.jejuni* DNA constructed in lambda gt11. Further reports of difficulties in cloning and maintaining *C.jejuni* sequences in *E.coli* hosts in 1989 came from Calva and co-workers who were attempting to clone the putative enterotoxin gene [144].

Thus, it seemed that the success of the use of complementation to clone genes from amino acid pathways such as *proA*, *proB* and *glyA* (which were apparantly transcribed from their own promoters), was probably due to the strong selective pressure on *E.coli* to maintain the *Campylobacter* genes in the absence of that particular amino acid. It was also thought likely that promoters of most housekeeping genes of *C.jejuni* would function in *E.coli* cells [172], but there was still doubt as to whether other genes may have unusual promoter sequences that are recognised much less efficiently in *E.coli*.

Various theories were put forward to explain the difficulties of cloning *Campylobacter* genes. The observation that shuttle vector DNA isolated from *E.coli* was resistant to *MboI* digestion, while that isolated from *Campylobacter* recipients was completely digested, suggested that DNA was not methylated at the adenosine residue of the 5'-GATC-3' recognition sequence in *C.jejuni*. Reciprocally, shuttle plasmids isolated from *C.jejuni* were resistant to *EcoRI* digestion [174]. These differences in methylation of DNA between *E.coli* and *Campylobacter* may result in instability, since base-specific DNA methylation can alter DNA-protein interactions in a number of ways and has several important functions, including effects on gene expression. Methylation of the adenosine residue in the sequence 5'-GATC-3' is important in mismatch repair. Repair enzymes recognise the newly synthesized DNA strand behind the replication fork because this is unmethylated, while fully methylated DNA is not a substrate for repair. Calva has reported some increased stability of *C.jejuni* DNA recombinants by cloning them in a *mutL* derivative of *E.coli* (deficient in methyl-directed DNA repair) [194].

The A-T rich nature of *Campylobacter* DNA was also considered to be involved in the instability of *Campylobacter* clones in *E.coli*. A-T rich sequences could be recognised as strong promoters in *E.coli* and regions containing 70-80% A-T residues that are also rich in static bends may serve as upstream activators of promoters [195]. Such strong promoters are only stable in vectors which are protected from excessive transcription by efficient terminator signals. Such a vector is pJDC9, which was developed for cloning genes from *Streptococcus pneumoniae*, which also has a low G-C content and was found not to generate stable DNA inserts greater than 2kb in common *E.coli* vectors [196].

When it was found that some genes from *Campylobacter* specify mRNA with a high percentage of suboptimal or rare codons, it was thought that this might limit the synthesis of *Campylobacter* peptides in *E.coli* [180]. However, the subsequent successful expression of polypeptides from *C.jejuni* flagellin genes cloned in pEX expression vectors (fusion proteins produced by pEX recombinants precipitate inside the cell and are therefore protected against proteolysis) and of the *cat* gene, suggested that a high percentage of rare codons does not necessarily limit expression. It is possible that *E.coli* may not be able to post-translationally modify proteins in the same way as *Campylobacter*, since certain accessory factors which could be required for the processing of some *Campylobacter* gene products might be absent.

To maximise the probability of cloning and expressing *C.jejuni* virulence factor genes in *E.coli*, it was decided to use a lambda expression vector for making the library presented here. The advantages of lambda were detailed earlier and include the high packaging efficiency of lambda and the ease of transfection of appropriate *E.coli* hosts. This facilitates the production of libraries covering the entire genome, improving the likelihood of isolating rare clones. Moreover, lysis of the host cells to form plaques, instead of relying on the formation of colonies, could facilitate the cloning of *C.jejuni* genes which may be toxic to *E.coli*. Immunoscreening of plaques also gives less background than with colonies.

The vector chosen was Lambda ZAP II (Figure 2.1, p.53) [197]. Lambda ZAP II has 21 restriction sites in the polylinker contained within the N-terminal portion of the *lacZ* gene and can accept up to 10kb of foreign DNA at six unique cloning sites: *Sac*I, *Xba*I, *Spe*I, *Eco*RI, *Not*I and *Xho*I. Because the multiple cloning site is in the N-terminal region of the *lacZ* gene, insertion of a DNA fragment into the cloning site prevents functional β-galactosidase from being produced. The *E.coli* host strain, XL1-Blue, harbours the F′ episome which has a partial *lacZ* gene (with the M15 deletion mutation) able to complement the amino terminus encoding portion of the *lacZ* gene on the Lambda ZAP II vector to generate a functional β-galactosidase protein when no insert is present. Thus, recombinant phage can be recognised by the production of white plaques when they are plated out with a *lac*- host in the presence of isopropyl-β-D-thio-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), whereas nonrecombinants produce blue plaques due to the action of the β-galactosidase enzyme on X-gal.

DNA sequences cloned into the polylinker can be expressed as fusion proteins under the control of the *lac* promoter and, hence, be detected using antibodies. This made the Lambda ZAP II system particularly

attractive for cloning *C.jejuni* genes for subsequent screening with convalescent antiserum, since it is possible that some *Campylobacter* promoters are not recognised in *E.coli*. The F' also carries the *lac* repressor gene (*lacIq*) which blocks transcription from the *lacZ* promoter in the absence of the inducer IPTG, thus controlling expression of fusion proteins which may be toxic to the *E.coli* host.

Lambda ZAP II was also designed to eliminate the need to subclone DNA inserts from the lambda phage into a plasmid by restriction digestion and ligation [197]. It can be automatically excised and recircularised *in vivo* to generate the pBluescript SK- phagemid vector containing the cloned insert DNA. This technique eliminates the time involved in subcloning and allows the rapid production of plasmid DNA for use in restriction analysis, mapping, sequencing and expression. It was realised that this system could be especially useful for cloning *C.jejuni* genes, since *C.jejuni* DNA tends to lack suitable restriction enzyme sites and can be unstable in subcloning experiments.

Lambda ZAP II incorporates the initiation and termination signals for f1 bacteriophage plus strand replication on either side of the potential pBluescript SK- DNA, which also includes the ampicillin resistance gene, the ColE1 origin of replication, the multiple cloning site and partial *lacZ* gene (see Figure 2.1). The F' episome in *E.coli* strain XL1-Blue contains genes for the expression of bacterial F' pili which are required for filamentous phage infection of *E.coli* host cells. The *E.coli* XL1-Blue host is superinfected with the Lambda ZAP II clone to be rescued and a filamentous helper phage (R408 is preferred [198]). The gene II protein from the helper phage nicks the Lambda ZAP II DNA at the initiator signal. This nick is then used as an initiation site for DNA synthesis. Replication proceeds from the initiator signal toward the terminator signal within the Lambda ZAP II vector. At the terminator site, the displaced strand is nicked and circularised by gene II protein. The circularised single-stranded pBluescript SK- DNA is packaged and secreted from the *E.coli* in preference to the helper phage. R408 helper phage has a deletion in the hairpin structure required for packaging of single stranded DNA, thus it provides the proteins required for packaging single stranded plasmid DNA, but is not itself packaged efficiently [198]. Circularisation of the single-stranded pBluescript SK- DNA automatically recreates a functional f1 origin, so that single stranded DNA can subsequently be rescued from the phagemid for rapid DNA sequence analysis.

This chapter describes the construction of a Lambda ZAP II expression vector-based library of *C.jejuni* H132 DNA, which could then be utilised as a resource in this laboratory. The initial library construction was

carried out as part of a three month project during a taught MSc. course. I screened the library with convalescent antiserum and obtained plaques which appeared to contain proteins that were bound by the antiserum. However, on re-screening, most of these positives (which were designated MK1-MK9) did not effectively bind convalescent antiserum and were, therefore, not investigated further. The objective of the work presented here was to improve the immunological screening of the Lambda ZAP II library and obtain new positive plaques which could be confirmed by re-screening. The pBluescript SK- plasmids, containing *C.jejuni* H132 DNA inserts, could then be rescued from the Lambda ZAP II clones in these positive plaques.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and growth conditions. *Campylobacter jejuni* H132 was provided to us by the World Health Organisation reference laboratory in Belgium. It was originally isolated from stools of a 10 month old child with bloody diarrhoea and colitis (confirmed by colonoscopy). Colonies were grown at 37°C in an atmosphere containing 5% O_2, 10% CO_2 and 85% N_2, provided by a variable atmosphere incubator (VAIN) (Don Whitley Scientific Ltd.), on Mueller-Hinton agar (MHA) (Oxoid). *E.coli* XL1-Blue [*endA1*, *hsdR17* (rk-, mk+), *supF44*, *thi-1*, lambda-, *recA1*, *gyrA96*, *relA1*, *lac-*, [F', *proAB*, *lacIqZ*ΔM15, Tn10(*tet*^{R})]] from Stratagene was used as the host for cloning experiments. Stock colonies were grown at 37°C on Luria-Bertani agar (LA) plates [10g/L NaCl, 10g/L tryptone, 5g/L yeast extract, 20g/L agar; pH7.0] with 15μg/ml tetracycline. Liquid cultures (for Lambda ZAP II phage infection) were started from these colonies and grown at 30°C, with shaking at 200rpm, to OD_{600} 0.5 in LB broth [as for LA plates, but without agar] supplemented with 0.2%(w/v) maltose and 10mM $MgSO_4$.

2.2.2 Chromosomal DNA extraction. Confluent overnight cultures of H132 were harvested from 15cm MHA plates, washed with TES [10mM Tris-HCl pH7.5, 5mM NaCl, 0.5mM EDTA] and suspended in lysis buffer [10mM Tris-HCl pH7.8, 5mM EDTA, 0.5% (w/v) SDS]. Proteinase K was added to a final concentration of 50μg/ml and the lysis mix was incubated for 1 hour at 37°C with gentle shaking. If lysis was incomplete, the concentration of SDS was increased to 1% (w/v) and the mixture incubated for a further 30 min. at 37°C. Immediately after lysis, the mixture was extracted with an equal volume of TES-saturated phenol, followed by two chloroform [chloroform:isoamylalcohol 24:1] extractions. Total DNA was precipitated by adding one tenth volume 3M sodium acetate (pH5.4) and 2 to 3 volumes ethanol to the final supernatant. The precipitate was poured through a sieve tube (a hot needle was used to make a few holes in the bottom of a 30ml universal tube) which retained the high molecular weight DNA. The retained DNA was rinsed with ethanol and resuspended in 9.5ml sterile distilled water (sdH_2O), to which 0.5ml 20x TES and 0.1ml RNAse (10mg/ml) were then added. After the mixture had been incubated at 60°C for at least an hour, it was again extracted once with phenol, followed by repeated chloroform extraction until the interface was clear. One tenth volume 3M sodium acetate (pH5.4) was added to the final supernatant, and approximately 0.6 volume of cold isopropanol was added while spooling the DNA with a glass rod. The spooled DNA was

rinsed with ethanol at room temperature, allowed to air dry, resuspended in TES and stored at 4°C.

2.2.3 Digestion of *C.jejuni* H132 DNA. To determine the most suitable restriction enzyme to use for partial digestion of the H132 DNA, test digestions were performed with several different enzymes: *Eco*RI, *Sal*I, *Xba*I, *Xho*I, *Bam*HI, *Sac*I, and *Spe*I (Gibco-BRL Life Technologies Ltd.). 1μg of H132 genomic DNA was incubated with 10 units of enzyme, in the buffer recommended by the manufacturer, at 37°C for 3 hours and the digestion products were analysed on a 0.7% (w/v) agarose gel. *Sau*3A was the enzyme chosen for the partial digest.

Conditions which gave the greatest proportion of *Sau*3A fragments in the 2.5-9kb range were determined by incubating 1μg of H132 DNA with serial dilutions of *Sau*3A at 37°C for $1^1/_2$ hours. Larger scale preparations of partially digested H132 DNA were then carried out. The reactions were stopped by the addition of stop mix [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 40% (w/v) sucrose, 100mM EDTA] and were run on a 0.7% (w/v) agarose gel containing 0.5μg/ml ethidium bromide. DNA fragments in the size range 2.5-9kb were isolated by electrophoresis onto a dialysis membrane. The membrane was washed with 100μl sterile distilled water to elute the DNA. The DNA was precipitated with one tenth volume of 3M sodium acetate (pH5.4) and 3 volumes of cold ethanol. After centrifugation at 12,000xg for 20min., the DNA was washed with 70%(v/v) ethanol and dried under vacuum. The pellet was dissolved in a minimum volume of TE [10mM Tris-HCl, 1mM EDTA; pH7.6] and stored at -20°C.

2.2.4 Preparation of Lambda ZAP II vector arms. Lambda ZAP II vector was supplied by Stratagene. The *cos* ends were ligated prior to digestion, by overnight incubation at 4°C of 10μg (10μl) of Lambda ZAP II arms with 8 units (8μl) of T4 DNA ligase in 50mM Tris-HCl (pH7.5), 7mM $MgCl_2$, 1mM dithiothreitol and 1mM ATP (40μl total volume). The ligase activity was inactivated by heating the sample at 68°C for 15 min.

A pilot *Xho*I digestion of the Lambda ZAP II DNA was carried out to determine the minimum time which gave complete digestion of the vector. 2.5μg (10μl) were incubated with 12.5 Units of *Xho*I in a total volume of 25μl. After $1^3/_4$ hours the first 0.5μg (5μl) sample was removed and further samples were taken at 15 min. intervals. The digests were stopped by the addition of 0.5μl of 10xSTE [100mM Tris-HCl, 1M NaCl, 10mM EDTA; pH8.0] to each 5μl sample. 0.4μg (4μl) from each timepoint was packaged using Stratagene's Gigapack II Plus. The kit contains two extracts prepared from

bacteria infected with two different lysogenic bacteriophage lambda. The two lysogens are each mutated in different genes required for assembly of bacteriophage particles. When the lysates of the two cell cultures are mixed, complementary components of the packaging reaction are provided and the Lambda ZAP II is packaged into mature phage particles. 1µl and 2µl aliquots of packaged *Xho*I-digested Lambda ZAP II were mixed with 200µl XL1-Blue cells (OD_{600} 0.5, see section 2.2.1) and added to 3ml NZY top agar at 48°C. This mixture was plated out onto 9cm NZY agar plates. NZY broth contains 5g/l NaCl, 2g/l $MgSO_4.7H_2O$, 5g/l yeast extract and 10g/l casein hydrolysate adjusted to pH7.5 with 10M NaOH. For NZY plates 15g of agar were added per litre of NZY broth, prior to sterilisation by autoclaving. Top agar consisted of autoclaved NZY broth containing 0.7% (w/v) agarose.

The digestion time which gave less than 0.1% of the plating efficiency of uncut Lambda ZAP II was used to digest 6µg of *cos*-ligated Lambda ZAP II to completion. Digested Lambda ZAP II was phenol/chloroform extracted once and chloroform extracted twice. DNA was precipitated with $^1/_{10}$th volume 3M sodium acetate (pH5.4) and 3 volumes ethanol. After centrifugation at 12,000xg in a microcentrifuge for 20 min., the pellet was washed with 70% (v/v) ethanol, resuspended in 5µl TE and stored at -20°C.

2.2.5 Partial fill-in of staggered ends and ligation. Since the H132 genomic DNA was digested with *Sau*3A (^GATC) and the Lambda ZAP II vector was digested with *Xho*I (C^TCGAG), it was necessary to partially fill-in the staggered ends for the inserts to be successfully ligated with the vector and to prevent vector religation. 5µg (5µl) of Lambda ZAP II vector DNA were filled in using 5µl 1mM dCTP, 5µl 1mM dTTP, 1.8µl 10x ligase buffer [500mM Tris-HCl, 70mM $MgCl_2$, 10mM dithiothreitol, 10mM ATP; pH7.5] and 0.5 unit (1µl) Klenow enzyme. 2.5µg (5µl) of H132 insert DNA were filled in using 5µl 1mM dATP, 5µl 1mM dGTP, 1.8µl 10x ligase buffer, and 0.5 unit (1µl) Klenow. Both reactions were carried out for 30 min. at room temperature. The reactions were stopped by placing the tubes at 65°C for 30 min. Each DNA sample was phenol/chloroform extracted once and choroform extracted twice. The DNA was precipitated with $^1/_{10}$th volume 3M sodium acetate (pH5.4) and 3 volumes of ethanol. The pellet was washed with 70% (v/v) ethanol, resuspended in 5µl TE and stored at -20°C.

Cut and partially filled-in vector (1µg) and insert (1µg) DNA was ligated using 3 Units of T4 DNA ligase, in ligase buffer, for 16 hours at 4°C. Half of this ligation reaction was packaged using Gigapack II Plus packaging extract (Stratagene). 1µl of a 10^{-1} dilution of the final

packaged reaction was plated with 200µl OD_{600} 0.5 XL1-Blue cells (section 2.2.1). The phage and bacteria were preincubated together at 37°C for 15 min., added to 3ml NZY top agar with 15µl 0.5mM IPTG and 50µl 0.612M X-gal and plated onto 9cm NZY agar plates.

2.2.6 Amplification of the library. The resulting library was amplified by inoculating 200µl aliquots of XL1-Blue cells (OD_{600} 0.5) with 1µl of packaged library, plating out in 3ml NZY top agar onto 9cm NZY agar plates and incubating at 37°C for 8 hours. The bacteriophage were recovered by washing the plates overnight at 4°C with SM buffer [100mM NaCl, 8mM $MgSO_4$, 50mM Tris-HCl, 0.01% (w/v) gelatin; pH7.5]. The suspension was recovered from each plate, chloroform added to 5% (v/v), then left at room temperature for 15 min. After centrifugation for 5 min. at 3500xg, the supernatants were recovered, pooled and chloroform added to 0.3% (v/v). This amplified library was stored in 1ml aliquots at 4°C.

2.2.7 Absorbing the antiserum. The convalescent antiserum was absorbed against *E.coli* XL1-Blue sonicate prior to its use in screening. A 5ml overnight culture of *E.coli* XL1-Blue was centrifuged for 5 min. at 3500xg and the pellet was resuspended in 0.5ml sdH_2O. This cell suspension was sonicated at 0°C for 60 sec. at full power to prepare a cell lysate which was diluted to 5ml with sdH_2O. A nitrocellulose filter (Schleicher and Schuell) was floated in the cell lysate, first with one side facing downwards and then the other, for 15 min. each side. The *E.coli* lysate-coated nitrocellulose filter was transferred to 10ml PonceauS [0.2% (w/v) PonceauS, 3% (w/v) trichloroacetic acid, 3% (w/v) sulphosalicylic acid] and incubated for a further 10 min. to fix proteins to the filter. Washes were carried out in three changes of 20ml TBS-Tween [0.15M NaCl, 0.01M Tris-HCl, 0.2% (v/v) Tween-20; pH7.4]. The filter was then incubated for 2 hours at room temperature with 10ml TBS-Tween containing 3% (w/v) bovine serum albumin (BSA) and 1% (w/v) casein. Three washes were carried out in 20ml TBS-Tween and the filters were stored in TBS [0.15M NaCl, 0.01M Tris-HCl; pH7.4] at 4°C.

The antiserum to be absorbed was diluted to 10x its working concentration in TNT [0.15M NaCl, 0.01M Tris-HCl, 0.05% (v/v) Tween-20; pH7.4] plus 3% (w/v) BSA and 1% (w/v) casein and incubated with the lysate-coated filter for 1 hour at room temperature. The absorbed antiserum was stored at 4°C with sodium azide added to a final concentration of 10mM.

2.2.8 Immunological screening. An adaptation of the method described by Sambrook *et al.* [199] was used to screen the library. 200µl of XL1-Blue cells (OD_{600} 0.5) were infected with 1µl of packaged library DNA, plated out in top agar and incubated at 42°C for 4 hours. Nitrocellulose filters, previously soaked in 10mM IPTG, were laid onto the plates and incubation continued for 5 hours at 37°C. The filters were rinsed with TNT (described above), transferred to 20ml fresh TNT and washed with gentle agitation for 30 min. to remove cell debris and agar. Washed filters were blocked for 30 min. (MSc.), or overnight (PhD.), at 4°C in 20ml TNT, 3% (w/v) BSA and 1% (w/v) casein. They were incubated for overnight (MSc.), or 6 hours (PhD.), at room temperature with convalescent antiserum diluted 1:25 in blocking buffer or with mouse anti-β-galactosidase antiserum (Sigma) diluted 1:1000 in blocking buffer. The filters were washed three times (10 min. per wash) in 20ml TNT then incubated for two hours at room temperature, in the dark, with peroxidase-coupled goat anti-human IgG or goat anti-mouse IgG antibody (both from Sigma) diluted 1:2000 in blocking buffer. The washes were repeated as before, but with a final brief rinse in TBS. Developing solution was 30mg 4-chloronaphthol dissolved in 10ml methanol, plus 30ml TBS and 40µl H_2O_2. Each filter was incubated in the dark for up to 20 min. with 10ml of developing reagent.

2.2.9 Preparation of DNA probes. DNA was digested with restriction endonucleases to isolate recombinant DNA from the plasmid vector. Fragments were separated by electrophoresis through a 1% (w/v) low melting point agarose gel (Flowgen) and recombinant DNA fragments were excised from the gel. Sterile distilled water was added at a ratio of 1.5ml sdH_2O to 1g of gel. The tubes containing this DNA were placed in a boiling waterbath for 7 min., then equilibrated to 37°C for at least 10 min. before storing at -20°C. Prior to radioactive labelling, the DNA fragments were reboiled for 3 min. and kept at 37°C for a further 10 min.

2.2.10 Radioactive labelling of probes. The fragments were labelled with [^{32}P]dCTP by the random primer method of Feinberg and Vogelstein [200]. The reagents were added in the following order: 5µl sdH_2O, 16.6µl DNA (10-25ng), 3µl OLB, 1.2µl BSA (10mg/ml), 3µl [^{32}P]dCTP (10µCi/µl), 1.2µl Klenow fragment of DNA polymerase I (1 unit/µl). The reaction was allowed to proceed at room temperature for 4 hours. OLB consisted of solutions A, B and C in a ratio of 2:5:3. Solution A was 1.21M Tris-HCl (pH8.0), 0.121M $MgCl_2$, 1.8% (v/v) β-mercaptoethanol, 0.483mM dGTP, 0.483mM dATP and 0.483mM dTTP. Solution B was 2M HEPES, titrated to pH6.6 with 10M NaOH. Solution C was the

hexadeoxyribonucleotides (Pharmacia), evenly suspended in 3mM Tris-HCl, 0.2mM EDTA (pH7.0) at 90 A_{260}units/ml.

2.2.11 Treatment of filters. 200µl XL1-Blue cells (OD_{600} 0.5) were preincubated with 0.5µl packaged library for 15 min. at 37°C, plated out in top agar and incubated overnight at 37°C. This gave a plaque density of approximately 1000 per plate. The plates were chilled at 4°C for 30 min. before being overlayed with Hybond-N (nylon membrane) filters (Amersham) for 60 seconds. The filters were rinsed in denaturing solution [0.5M NaOH, 1.5M NaCl] at room temperature for 5 min. and neutralised in 0.5M Tris (pH7.4), 1.5M NaCl for 5 min. They were rinsed in 2x SSC [20x SSC is 3M NaCl, 0.3M Na citrate; pH7.0] and allowed to dry on paper towels. DNA was fixed to the filters by exposing them to UV light on a Fotodyne transilluminator for 30 sec. Filters were blocked for 1 hour at 65°C in 6x SSC, 0.25% (w/v) Marvel (Cadbury's skimmed milk powder). Radiolabelled probes were boiled for 5 min., added to the filters in fresh 6x SSC, 0.25% (w/v) Marvel and incubation continued at 65°C overnight. After this hybridisation period, three 10 min. washes in 3x SSC, 0.1% (w/v) SDS were carried out at 65°C, followed by four 10 min. stringent washes in 1.5x SSC, 0.1% (w/v) SDS at 65°C. Filters were air dried and wrapped in Saranwrap for autoradiography with Fuji RX medical X-ray film at -80°C for two days.

2.2.12 Non-radioactive probes. The Boehringer Mannheim Nonradioactive DNA Labelling and Detection Kit was used according to the manufacturer's instructions. This method involved labelling the probe DNA using random primers (as in 2.2.10) but digoxigenin-11-dUTP was the labelling molecule, instead of [^{32}P]dCTP. 0.3-0.5µg (15µl) DNA was mixed gently with 2µl hexanucleotides (50 A_{260}units/ml), 2µl dNTPs [1mM dATP, 1mM dCTP, 1mM dGTP, 0.65mM dTTP, 0.35mM digoxigenin-11-dUTP; pH6.5] and 1µl Klenow enzyme (2 units/µl), in that order, and incubated at 37°C for 6 hours. Before use, the digoxigenin-labelled probes were denatured by boiling for 5 min.

Library plaques to be probed were lifted onto Hybond-N filters, denatured, neutralised, rinsed in 2 x SSC and fixed by UV, as they were for the radioactive method (2.2.11), but they were blocked in non-radioactive hybridising solution [5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 1% (w/v) manufacturer's blocking reagent] for 2 hours at 65°C. Filters were then incubated with the denatured digoxigenin-labelled probes in fresh hybridising solution at 65°C overnight and washes carried out as for the radioactive method (section 2.2.11).

Digoxigenin-labelled probe remaining bound to target plaque DNA after stringent washes was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase. The filters were rinsed briefly in buffer 1 [0.1M Tris-HCl, 0.15M NaCl; pH7.5] and incubated for 30 min. with 100ml buffer 2 [0.5% (w/v) blocking reagent in buffer 1] at room temperature. Each filter was again rinsed with buffer 1 and incubated for 30 min. with 20ml antibody-conjugate (diluted 1:5000 in buffer 1). They were then soaked in buffer 3 [0.1M Tris-HCl, 0.1M NaCl, 0.05M $MgCl_2$; pH9.5] for 2 min. and positive plaques were detected in a chromogenic reaction with 45µl 4-nitrobluetetrazoliumchloride (NBT) and 35µl 5-bromo-4-chloro-3-indolyl-phosphate freshly added to 10ml buffer 3. A localised blue colour reaction was produced in the presence of the bound alkaline phosphatase.

2.2.13 *In vivo* excision of the pBluescript SK- phagemid. Selected phage plaques were cored from the agar plate and vortexed in 500µl SM buffer (described in 2.2.6) plus 4% (v/v) chloroform to release lambda phage particles from the agar plug. This Lambda ZAP II recombinant phage stock was stored at 4°C. To excise the pBluescript SK- phagemid, 200µl of XL1-Blue cells (OD_{600} 1.0) were combined with 200µl of the cored Lambda ZAP II phage stock or 3µl amplified Lambda ZAP II stock (>1 x 10^5 phage particles) and 1µl (>1 x 10^6 pfu/µl) R408 helper phage (Stratagene). After incubating at 37°C for 15 min., 5ml of 2 x YT media [10g/l NaCl, 10g/l yeast extract, 16g/l Bacto-tryptone; pH7.5] were added and incubation continued for 5 hours at 37°C with shaking. The tube was heated at 70°C for 20 min. to inactivate the parent lambda phage and kill the host bacteria. The tube was centrifuged for 5 min. at 3500xg and the supernatant contained the pBluescript SK- phagemid packaged as a single stranded filamentous phage particle. 10µl of this phagemid suspension was added to 200µl XL1-Blue host cells (OD_{600} 1.0) and incubated at 37°C for 15 min. Infected cells (100µl) were plated on LA plates containing 100µg/ml ampicillin (Calbiochem), hereafter referred to as LA-AMP plates. Following overnight incubation at 37°C, colonies containing the pBluescript SK- double-stranded phagemid with cloned DNA inserts were restreaked onto fresh LA-AMP plates.

2.3 RESULTS

2.3.1 Preparation of partially digested and size-selected genomic H132 DNA. *C.jejuni* genomic DNA from the inflammatory strain H132 was partially digested to create a series of overlapping fragments for insertion into the Lambda ZAP II vector. To eliminate fragments of less than 2.5kb, the partial digest products were size-selected from agarose gels.

Genomic DNA from *C.jejuni* strain H132 was isolated as described in 2.2.2. The absorbance at 260nm of the *C.jejuni* H132 DNA showed that its concentration was **0.5µg/µl**, with a total yield from four 15cm plates of **0.5mg**.

In order to verify that restriction enzymes could digest the *C.jejuni* DNA, and determine the restriction enzyme most suitable for generating overlapping genomic fragments, test digestions were carried out with several of the restriction enzymes which also had recognition sites in the polylinker of Lambda ZAP II, as detailed in 2.2.3. Figure 2.2 shows that *Eco*RI (G^AATTC), *Sal*I (G^TCGAG), *Xba*I (T^CTAGA) and *Xho*I (C^TCGAG) did not cut the *C.jejuni* H132 DNA, while *Bam*HI (G^GATCC), *Sac*I (GAGCT^C) and *Spe*I (A^CTAGT) gave several discrete bands over 2kb in size and only *Sau*3A (^GATC) gave a smear of low molecular weight digestion products. It was expected that the enzyme which recognised a sequence consisting of only four bases (*Sau*3A) would cut much more often than all the other enzymes which had six-base recognition sequences, since the probability of four bases occurring in a certain order is much higher than that of six bases. Four of the enzymes tested did not cut *C.jejuni* H132 DNA at all, even though two of them recognised sites containing a high proportion of A/T residues (*C.jejuni* DNA has 68% A/T content). However, it has been previously observed that *Eco*RI does not digest *C.jejuni* DNA [174] and it is to be expected that some other restriction enzymes may also be unable to cut *C.jejuni* DNA in its native state of methylation.

Having chosen *Sau3A*, it was necessary to determine the preferred conditions for partial digestion of the *C.jejuni* H132 DNA. Test digests were performed with serial dilutions of *Sau*3A incubated with 1µg of H132 DNA at 37°C for $1^1/_2$ hours. Digestion products were analysed on a 0.7% (w/v) agarose gel (Figure 2.3) and, from these results , it was decided to use 0.06 units of *Sau*3A per µg of H132 DNA to give the greatest proportion of fragments in the size range 2.5-9kb.

Several *Sau*3A digests of 10µg aliquots of H132 DNA were carried out and fragments between 2.5 and 9kb isolated as described in 2.2.3. The final yield from 100µg of H132 DNA was **20µg** of 2.5-9.0kb H132 DNA *Sau*3A

fragments, at a concentration of **0.5µg/µl**. Figure 2.4 shows 1µg of this size-selected DNA separated on a 0.7% (w/v) agarose gel.

2.3.2 Preparation of Lambda ZAP II vector arms. The ends of lambda DNA arms are marked by complementary 12 nucleotide single-stranded sequences called *cos* sites. When lambda infects a cell, its chromosome circularises by hybridisation of these single-stranded extensions then, in the lytic response, the chromosome replicates to form concatemers which are packaged into capsid particles. Cleavage occurs at tne left *cos* site to generate a free end that is inserted first into the capsid. The insertion of DNA continues until the right *cos* site is encountered, when it is cleaved to regenerate the other single-stranded end. The *cos* sites are therefore essential for packaging Lambda ZAP II DNA. Since the *Xho*I-digested Lambda ZAP II had to be filled-in prior to ligation with the *Sau*3A-digested *C.jejuni* DNA, it was necessary to ligate the *cos* ends prior to digestion. Otherwise these single-stranded sequences may also have been filled-in to some extent.

The *cos* ends of 10µg (10µl) of Lambda ZAP II DNA were ligated by overnight incubation with 8 units of T4 DNA ligase in a total reaction volume of 40µl (method 2.2.4). As expected, the mixture became very viscous due to the formation of Lambda ZAP II concatemers.

A pilot digestion of 2.5µg (10µl) of the *cos*-ligated Lambda ZAP II was carried out, which covered a range of lengths of time of digestion with *Xho*I (method 2.2.4). This test digest was necessary because underdigestion would result in increased background of intact Lambda ZAP II forming blue plaques, with no *C.jejuni* inserts. However, leaving the Lambda ZAP II DNA for too long with the restriction enzyme could result in some non-specific endonuclease activity, damaging the lambda arms.

0.4µg (4µl) of vector was packaged at each timepoint, 0.4µg of undigested Lambda ZAP II was also packaged as a control. Dilutions of packaged vector were used to infect *E.coli* XL1-Blue. The digestion time which gave less than 0.1% of the plating efficiency of uncut Lambda ZAP II was found to be $2^1/_2$ hours at 37°C. 6µg of cos-ligated Lambda ZAP were digested under these conditions. Following purification, the yield of *Xho*I-digested Lambda ZAP II was **5µg** at a concentration of **1µg/µl**.

2.3.3 Ligation of vector and insert. The staggered ends produced by *Sau*3A digestion of the H132 DNA and *Xho*I digestion of the Lambda ZAP II vector were partially filled-in prior to ligation, as described in 2.2.5. This was necessary to create compatible pairs of nucleotides at the staggered

ends, but also prevented self-ligation of the vector or multiple insertion of H132 DNA fragments. The following diagram shows the inserted nucleotides in bold:

*Xho*I-digested Lambda ZAP II	*Sau*3A-digested *C.jejuni* H132 DNA
TCGACNN--//--NNG**TC**	GATCNN--//--NN**GA**
CTGNN--//--NNCAGCT	**AG**NN--//--NNCTAG

A test ligation, of 1μg of filled-in Lambda ZAP II vector arms with 0.5μg of filled-in H132 genomic DNA, yielded 1.46 x 10^6 recombinant plaque forming units (pfu) and 2.9 x 10^5 nonrecombinant pfu per μg of arms. This was a satisfactory yield, but was only five-fold above the number of blue nonrecombinant background plaques. Therefore, the ligation procedure was repeated using 1μg of insert DNA with 1μg of vector. Half of the ligation reaction was packaged and dilutions of this packaged material were plated out. 1μl, out of the 500μl packaged library DNA, contained typically 1935 recombinants and 190 nonrecombinants. This was a yield of **1.9 x 10^6 recombinant pfu/μg arms**, approximately 10-fold above nonrecombinant background. The total number of recombinant pfu in 500μl of packaged library should therefore be 9.5 x 10^5.

Assuming that the average size of H132 *Sau*3A fragment inserted into Lambda ZAP II was 4kb, then the library contained a total of 4 x 9.5 x 10^5 = **3.8 x 10^6 kb** of H132 genomic DNA. This is approximately **2 x 10^3** times the size of the *C.jejuni* genome.

2.3.4 Amplification of the library. After amplification, as described in 2.2.6, the library had a titre of 2 x 10^5 recombinants/μl and 1.9 x 10^4 nonrecombinants/μl (10-fold more recombinants than background). Therefore, the amplification procedure resulted in a very high titre stock of the library, which could be plated out and screened many times. However, there was a possibility of distortion of the amplified library occurring, since not all recombinants in the library population would be propagated equally well and plaques which formed more slowly would be underrepresented in the amplified library. Such slow growing plaques may contain Lambda ZAP II clones with large *C.jejuni* DNA inserts, but there was also concern that some *C.jejuni* sequences may be toxic or unstable in the *E.coli* host (as discussed in the introduction, section 2.1). Consequently, the screening procedures presented here were all carried out on the original, unamplified library.

2.3.5 Immunological screening. Convalescent antiserum from the patient who had contracted the *C.jejuni* inflammatory strain, H132, was used to screen the Lambda ZAP II library of *C.jejuni* H132 DNA. It was anticipated that such antiserum would be able to detect *C.jejuni* antigens which may usually only be expressed during infection of the human host. The *C.jejuni* H132 DNA was cloned into the *Xho*I site of Lambda ZAP II, thus *Campylobacter* genes inserted in frame with the *lacZ* translation start could be expressed when the inducer IPTG was present and subsequently be detected by the antibodies.

1µl of packaged library material was used for each plate to be screened and was plated out as described in 2.2.7. The plates were incubated at 42°C for 4 hours then overlayed with IPTG-impregnated nitrocellulose filters and incubated for a further 5 hours at 37°C. The filters were blocked for 30 min. then incubated with convalescent antiserum overnight, as detailed in 2.2.7. In order to verify that the IPTG induction of the *lacZ* promoter was occuring, the library was also screened with anti-β-galactosidase monoclonal antibody.

The β-galactosidase production by the plaques was clearly detected using this method (Figure 2.5). Convalescent antiserum gave a higher background than the monoclonal antibody, despite being preabsorbed against *E.coli* cell sonicate (Figure 2.6). However, positives that were visible above background (two are shown in Figure 2.6) were cored and stored at 4°C (method 2.2.13). Control filters incubated with peroxidase-coupled secondary antibody alone were completely white, confirming that the background was due to nonspecific binding of the convalescent antiserum (data not shown). Nine putative positives were isolated in total by this method, which was carried out during my 3-month MSc. project, and were designated MK1-MK9.

During this PhD., background binding of the α-H132 convalescent antiserum was successfully reduced by: (i) increasing the incubation time of the filters with blocking buffer from 30 mins. to overnight, (ii) reducing incubation with primary antiserum from overnight to 6 hours at room temperature and (iii) repeating the pre-absorption of antiserum with *E.coli* sonicate-coated filters (data not shown). The potential positive clones from this second screening method were designated MK10 - MK14. The consistent typical yield of library plaques which produced proteins bound by convalescent antiserum was 1 per 1000 screened.

The putative positives were plated out with *E.coli* XL1-Blue and screened again with convalescent antiserum, using the same screening method as was used for the whole library. This is standard procedure to reveal

any false positives which might have been picked. Re-testing of the putative positive cored plaques confirmed that MK3, MK11, MK12, MK13, and MK14 were all positive, while the MK6 prep contained half positives and half negatives (due to inaccurate coring of the original positive). A well isolated MK6 positive plaque was cored and used in the subsequent amplification and rescue procedures. The other clones did not bind convalescent antiserum as well on rescreening as they had appeared to initially and on some filters high background binding of the convalescent antiserum obscured the results. Therefore, these clones were not investigated further.

2.3.6 DNA screening. The usefulness of the Lambda ZAP II library of *C.jejuni* H132 DNA was also demonstrated in DNA hybridisation screening with *Campylobacter* sequences obtained by other means. *C.jejuni* genomic DNA had been used for amplification of conserved gene fragments using PCR with degenerate oligonucleotide primers (PCRDOP) (see Chapter Five). This technique had been used successfully to isolate fragments of several *C.jejuni* genes, including a 250bp fragment of a gene encoding a two component response regulator, *regX1*, and a 473bp fragment of the *Campylobacter* equivalent of *htrA* from *Salmonella typhimurium*. A 320bp DNA fragment, which was similar to the *E.coli* response regulator gene *creB*, was also provided to us by St.Bartholomew's Hospital. The *creB* gene fragment was from an unknown organism with AT-rich DNA, but was not from *C.jejuni* and, therefore, I used it as a negative control.

0.5µg of each of the *regX1*, *htrA* and *creB* clones was digested with 1 unit of *Hin*dIII and 1 unit of *Pst*I at 37°C for 2 hours. 0.5µg of the flagellin (*flaA*) clone pTNS#A (kindly provided by Trudy Wassenaar, Utrecht) was digested with 2 units of *Hin*dIII to isolate the pBluescript SK- vector as a positive control. The digest products from the recombinant clones were electrophoresed on a 1% (w/v) low-melting point agarose gel. A fragment of *Vibrio cholerae* strain CVD103 *recA* gene was used to screen the library as another negative control. The bands corresponding to the *C.jejuni* DNA fragments, the *creB* fragment, the *V.cholerae recA* fragment and the pBluescript positive control from pTNS#A, were subsequently cut from the gel and melted in sdH_2O (method 2.2.9).

The radioactive labelling reactions were carried out as detailed in method 2.2.9. The reagents were mixed and incubated at room temperature for 4 hours. Library filters to be probed were prepared as detailed in method 2.2.11 and incubated overnight at 65°C with the labelled DNA. After the final 10 min. stringent wash in 1.5x SSC, 0.1% (w/v) SDS at 65°C (method 2.2.11), the filters were air-dried, wrapped in a single layer of

Saranwrap and placed against X-ray film in front of an intensifying screen in a film cassette. The film was developed after 2 days at -80°C and the results are presented in Figure 2.7. The pBluescript DNA hybridised to all the library plaques as a good positive control (Figure 2.7a). The *htrA* probe hybridised to three plaques which were subsequently cored from the plate (Figure 2.7b), but no plaque binding by the *creB*, *V.cholerae recA* or *regX1* probes was detected (data not shown).

Non-radioactive labelling reactions were also carried out with all the gene fragments. Using this method (details in method 2.2.12), two further *htrA*-binding plaques were detected (data not shown). Re-probing of the library with *regX1* was also successful (Ana Bras-Goldberg, personal communication). The PCRDOP fragment of the *V.cholerae* CVD103 *recA* gene, and the AT-rich *creB* fragment, failed to hybridise with any library plaques and were good negative controls, demonstrating that there was no background spotting of the filters (not shown).

2.3.7 *In vivo* excision of the pBluescript SK- phagemid. Lambda ZAP II was designed to allow *in vivo* excision and recircularisation of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. This is possible because Lambda ZAP II incorporates the pBluescript SK- vector, flanked by the signals for initiation and termination of DNA synthesis from the f1 bacteriophage origin of replication. When *E.coli* XL1-Blue is coinfected with Lambda ZAP II and R408 f1 bacteriophage, helper proteins from the f1 phage enable single-stranded pBluescript SK- to be synthesized, packaged and secreted from the host (see Introduction 2.1). The single-stranded pBluescript SK-, incorporating the cloned insert DNA, is used to infect fresh *E.coli* host cells and spread on LA plates containing ampicillin to select for colonies containing the now double-stranded phagemid (Methods section 2.2.13).

200µl OD_{600} 1.0 XL1-Blue cells were infected with 1µl R408 helper phage and 200µl of phage stock for each of the clones MK3, MK6, MK11, MK12, MK13 and MK14. After the rescue process detailed in method 2.2.13, colonies containing the pBluescript SK- double-stranded phagemid were obtained for clones MK3, MK6 and MK11. These pBluescript SK- clones, which contained *C.jejuni* H132 insert DNA, were designated pMK3, pMK6 and pMK11 (the "p" prefix denotes plasmid/phagemid clones). Since excision efficiency is directly related to the Lambda ZAP II phage titer [197], the other clones were amplified to produce high titer phage stock and 3µl of amplified phage stock were used successfully to rescue pMK12, pMK13 and pMK14.

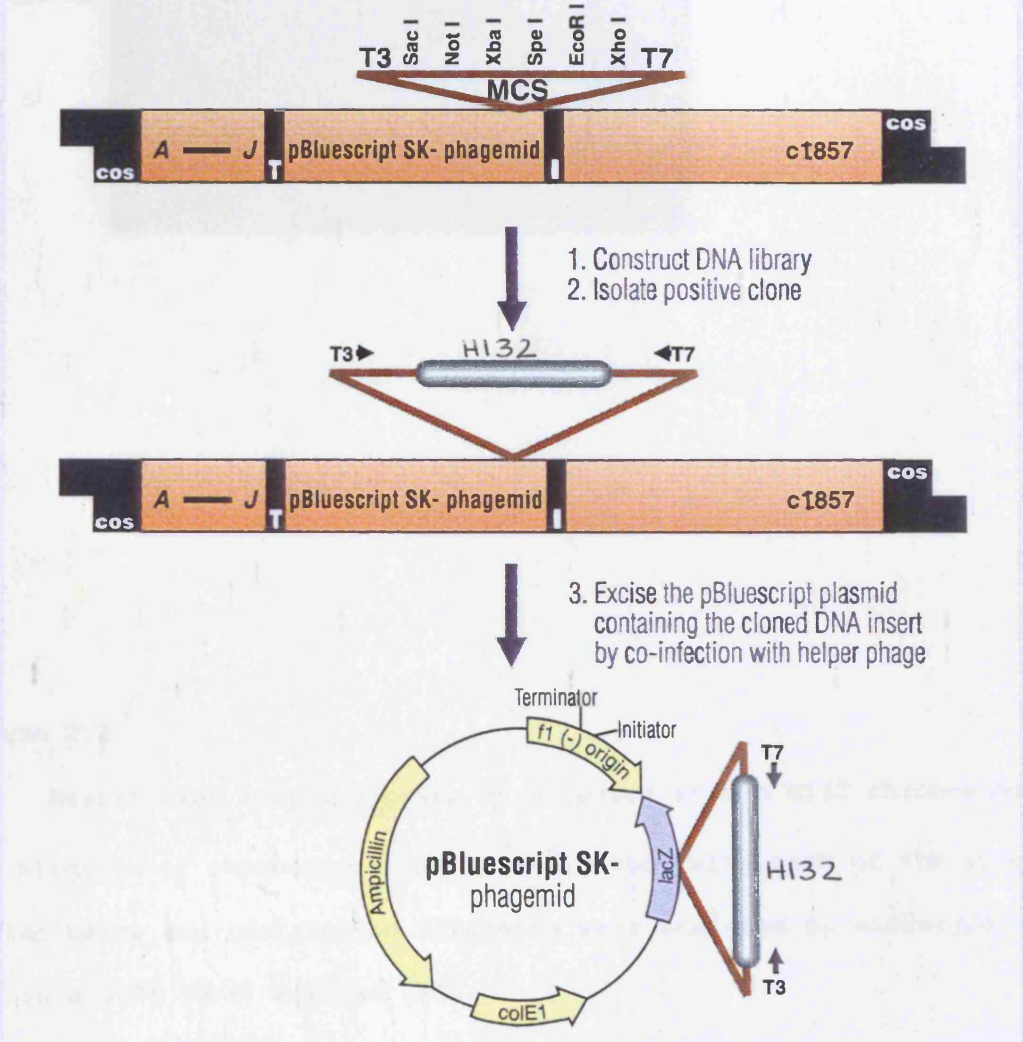
Figure 2.1

Diagram of Lambda ZAP II, showing pBluescript SK- vector excision, adapted from 1997/1998 Stratagene catalogue.

A) Lambda ZAP II containing the pBluescript SK- phagemid, which has a multiple cloning site for construction of the DNA library. "T" refers to the f1 terminator of replication and "I" refers to the f1 initiator, which allow the *in vivo* excision of pBluescript SK-. T3 and T7 primer binding sites, which flank the multiple cloning site, are shown.

B) Lambda ZAP II containing *C.jejuni* H132 DNA (solid red box) cloned into the *Xho*I site of pBluescript SK-.

C) Excised pBluescript SK- phagemid with the *C.jejuni* H132 DNA still cloned in the *Xho*I site. Also shown are the ampicillin resistance gene, the colE1 origin of replication, the f1 origin of replication and the partial *lacZ* gene, into which the *C.jejuni* H132 DNA is inserted.


T3
Sac I
Not I
Xba I
Spe I
EcoR I
Xho I
T7
MCS
cos
A — J
T
pBluescript SK- phagemid
I
c1857
cos
1. Construct DNA library
2. Isolate positive clone
T3
H132
T7
cos
A — J
T
pBluescript SK- phagemid
I
c1857
cos
3. Excise the pBluescript plasmid containing the cloned DNA insert by co-infection with helper phage
Terminator
Initiator
f1 (-) origin
T7
Ampicillin
pBluescript SK-
phagemid
lacZ
H132
T3
colE1

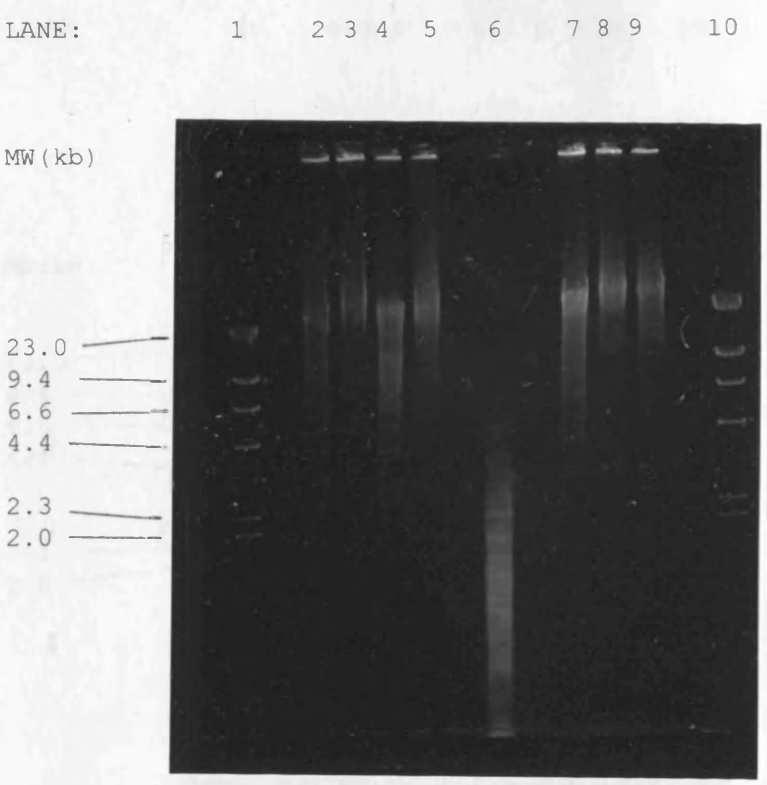


Figure 2.2

Restriction enzyme digests of *C.jejuni* strain H132 chromosomal DNA. 1µg aliquots of chromosomal DNA were digested with each of the enzymes listed below and restriction fragments were analysed by electrophoresis at 80V on a 0.7% (w/v) agarose gel.

Lanes 1 and 10: λ *Hin*dIII molecular weight markers (sizes, in kb, shown on the left-hand side); Lanes 2, 3, 4, 5, 6, 7, 8 and 9 contain 1µg H132 DNA digested with *Bam*HI, *Eco*RI, *Sac*I, *Sal*I, *Sau*3A, *Spe*I, *Xba*I and *Xho*I, respectively.

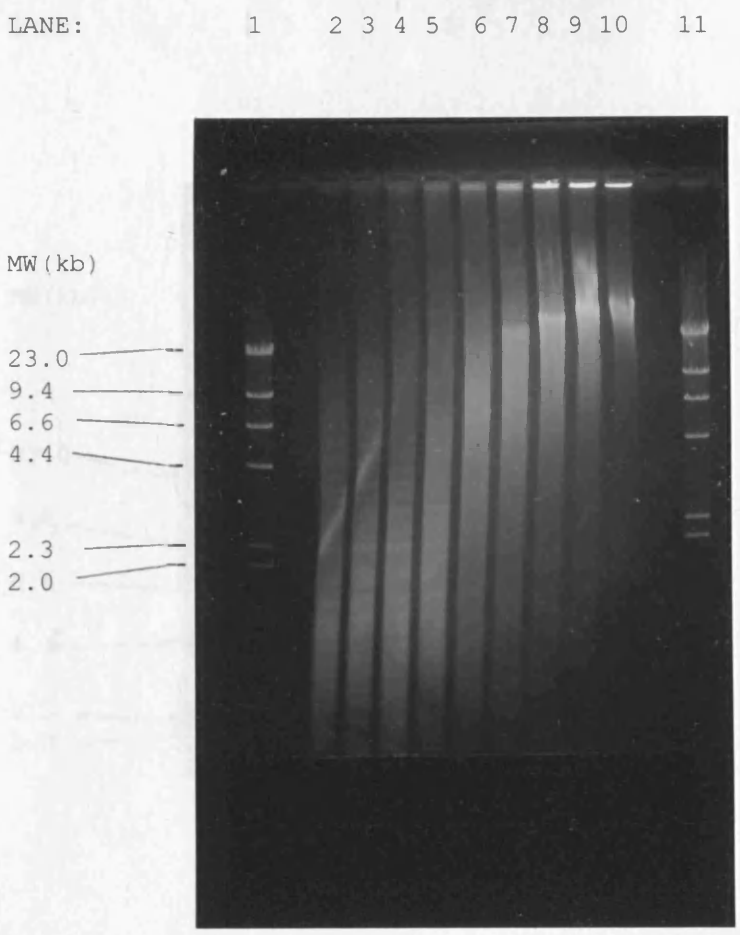


Figure 2.3

Restriction digests to determine the optimum conditions for partial digestion of *C.jejuni* DNA. *C.jejuni* strain H132 chromosomal DNA was digested with serial dilutions of *Sau*3A and analysed by electrophoresis at 70V on a 0.7% (w/v) agarose gel.

Lanes 1 and 11: λ *Hin*dIII molecular weight markers (sizes, in kb, shown on the left-hand side); Lanes 2, 3, 4, 5, 6, 7, 8 and 9 contain 1μg of H132 DNA digested with 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016 and 0.008 units of *Sau*3A, respectively; Lane 10: 1μg uncut H132 DNA.

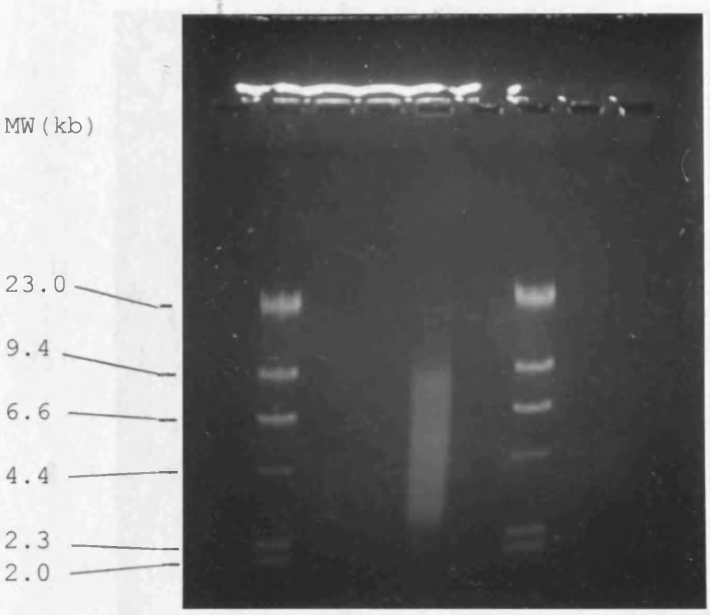


Figure 2.4

Result of size-fractionation of digested *C.jejuni* DNA. *Sau*3A fragments of *C.jejuni* strain H132 DNA, between 3 and 10kb in size, were isolated and analysed by electrophoresis at 80V on a 0.7% (w/v) agarose gel.

Lanes 1 and 3: λ *Hin*dIII molecular weight markers (sizes, in kb, shown on the left-hand side); Lane 2: 2μl (1μg) of purified and size-selected H132 *Sau*3A fragments.

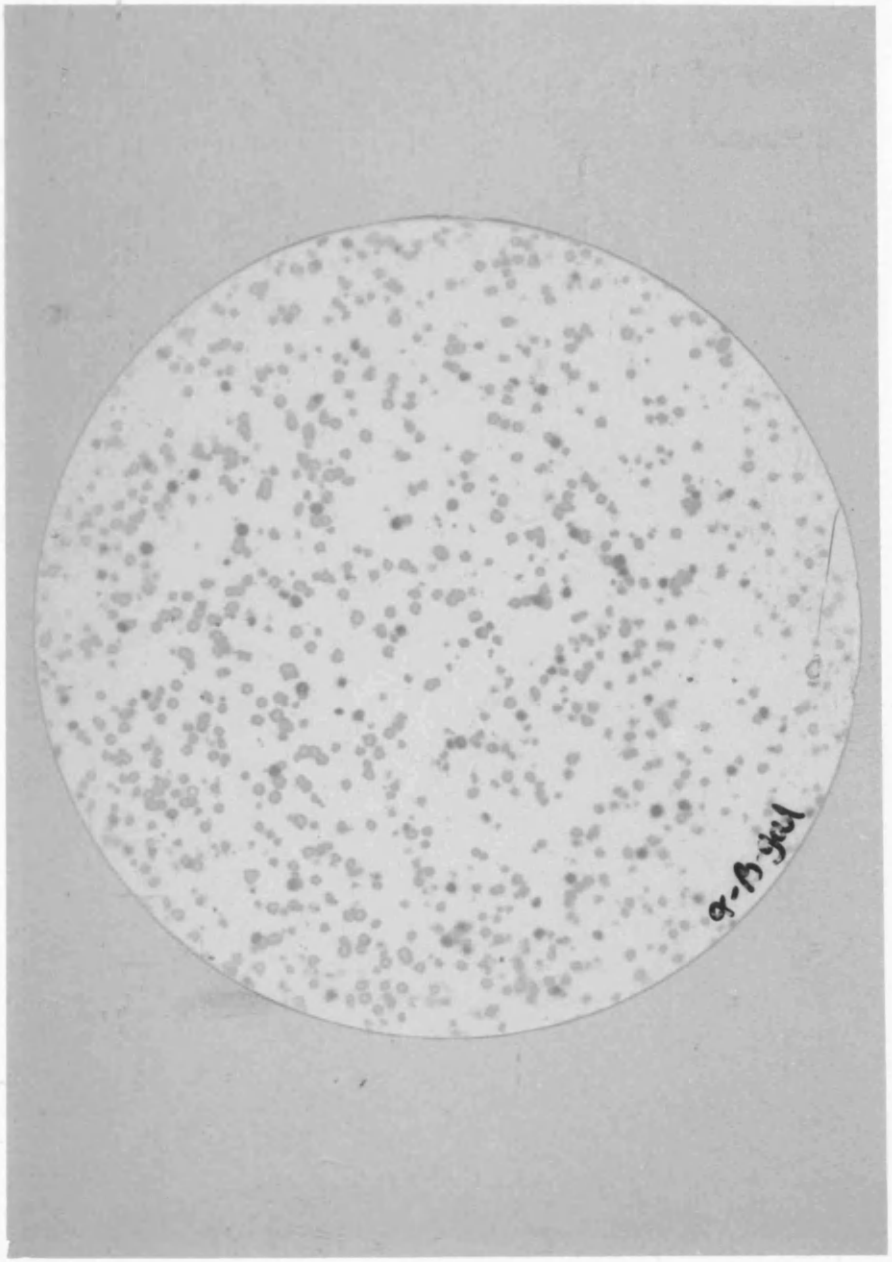


Figure 2.5

Figure to show *lacZ* expression from Lambda ZAP II library clones, [adapted from M. Kiernan, MSc. project report, 1991]. Plaques obtained from 1µl of the *C.jejuni* H132 library were lifted onto Hybond N membrane and screened with anti-β-galactosidase monoclonal antiserum.

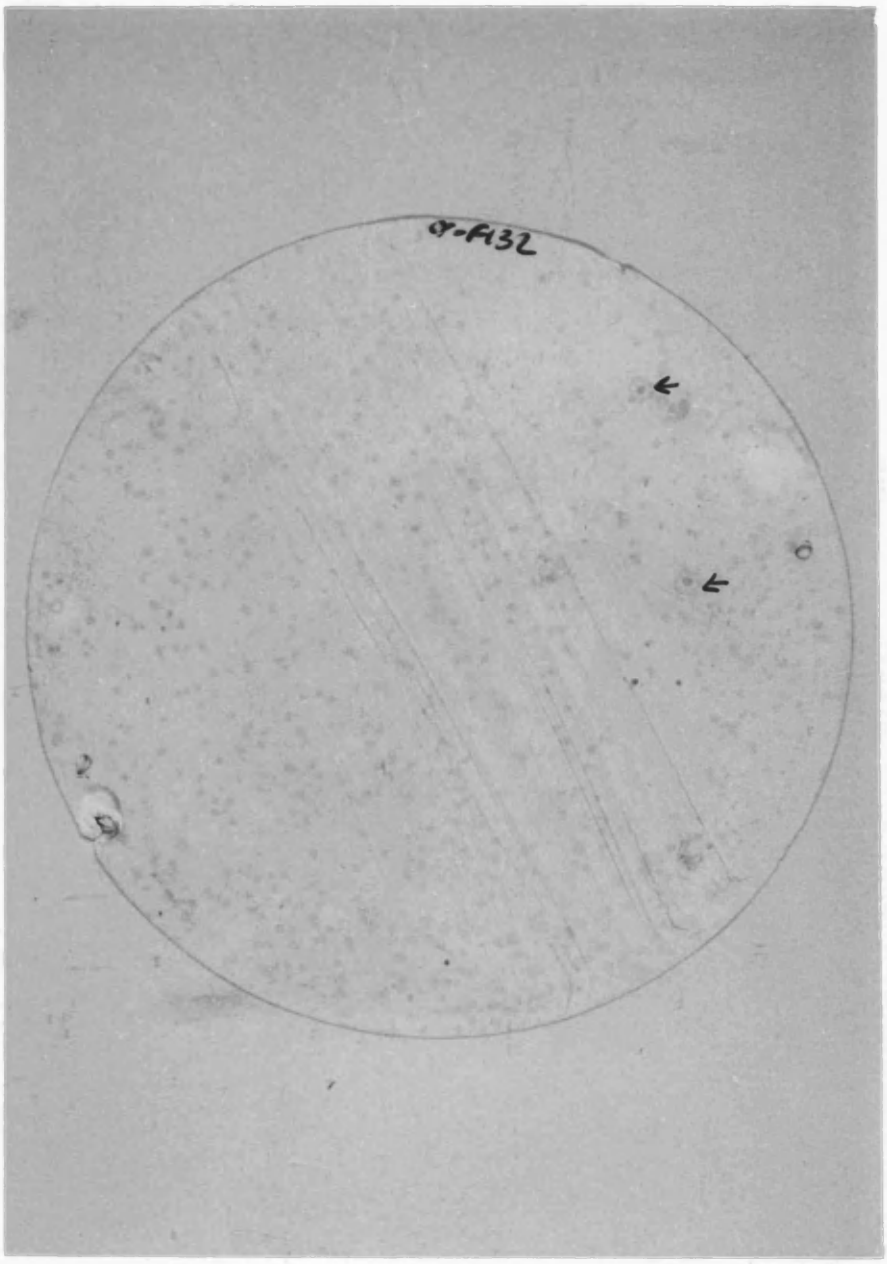
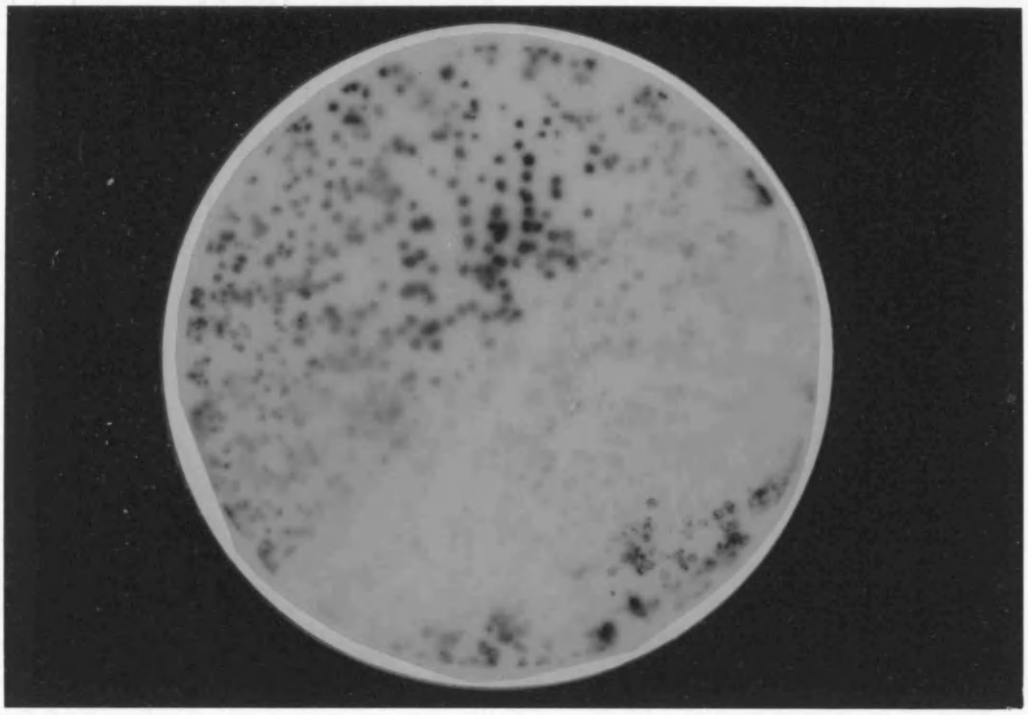


Figure 2.6

Screening Lambda ZAP II library with convalescent antiserum. This figure shows plaques obtained from 1µl of the *C.jejuni* H132 library, which have been lifted onto Hybond N membrane and screened with anti-*C.jejuni* strain H132 convalescent antiserum [adapted from M Kiernan, MSc. project report, 1991]. Two putative positive plaques are indicated with arrows.

A)



B)

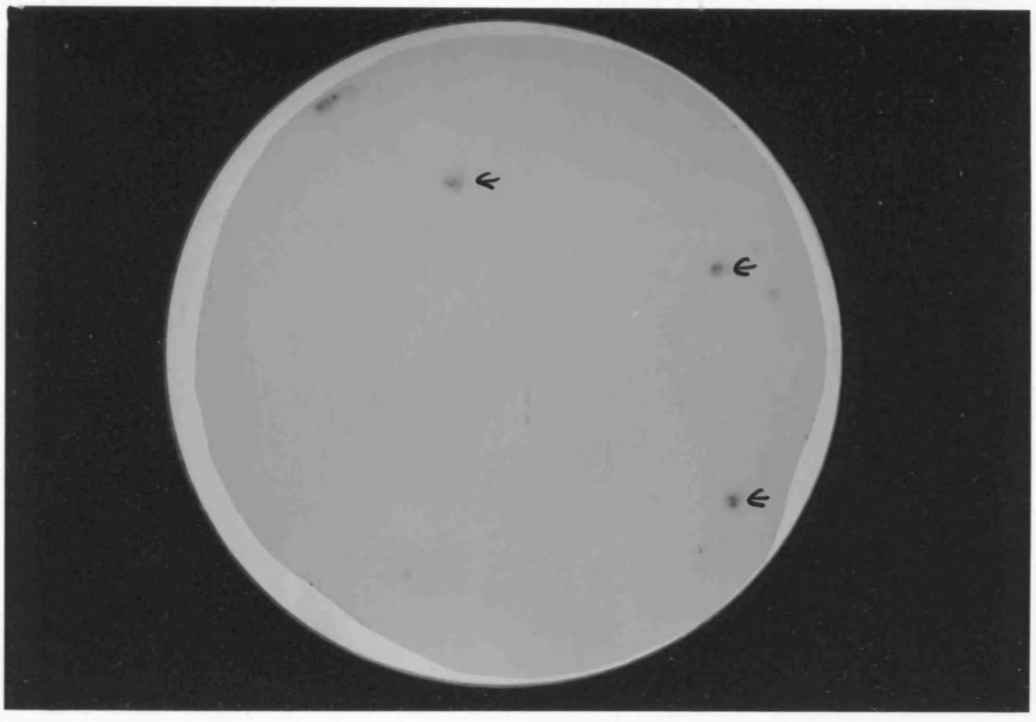


Figure 2.7

Library screening with radioactive labelled probes. Library plaques were transferred to Hybond N membrane and subsequently probed with radiolabelled pBluescript (positive control) or a fragment of *C.jejuni htrA*. Figure 2.7a shows library plaques probed with pBluescript, while Figure 2.7b shows library plaques probed with *htrA* (arrows indicate positives).

2.4 DISCUSSION

As discussed earlier, various difficulties have been described in attempts to clone, maintain and express *Campylobacter* genes in *E.coli* host strains. At the time this project was devised, there were few genetic tools available which had been used successfully with *Campylobacter spp.* The most innovative and useful system which had been developed was a series of shuttle vectors which contained both *E.coli* and *Campylobacter* origins of replication plus a *Campylobacter* antibiotic resistance gene. However, attempts to mutagenise the *Campylobacter* genome using shuttle vectors carrying transposons Tn5 or Tn917 were unsuccessful [179].

After this project had begun, further examples were reported of the difficulties inherent in cloning and maintaining *C.jejuni* DNA sequences in *E.coli* under conditions in which no positive selection was possible. In 1993, Louie and Chan [164] reported that plasmids containing a 2.3kb *Hin*dIII fragment which contained the putative *proA* gene were capable of complementing a *pro*A mutant of *E.coli*. However, plasmid DNA isolated from those transformants revealed size heterogeneity and all of the plasmid DNAs were smaller than the corresponding plasmid vectors transformed. Moreover, the frequency of Amp^+ transformants on plates with proline was 10-fold higher than the transformation frequency on ampicillin plates without proline. This discrepancy observed between the complementation and transformation frequencies suggested the possibility that sequences located close to *pro*A were involved in the destabilisation of the plasmid DNA.

Similarly, in 1992, Labigne and co-workers [167] reported the instability in *E.coli* of cosmid recombinant clones of *C.jejuni* DNA carrying leucine biosynthesis genes. The sizes of the recombinant hybrid pasmids capable of complementing the auxotrophic defect in leucine biosynthesis of the *E.coli* strain HB101 were much smaller (12-15kb) than expected. Initially 40-50kb fragments were packaged into phage lambda particles and introduced in *E.coli* cells, following which they rapidly underwent major rearrangements. The successful cloning of the *Campylobacter* leucine biosynthesis gene in *E.coli* can be attributed to the fact that the recombinant clones were replicated under selective conditions, ensuring the maintenance and the rescue of genes required for the growth of defective *E.coli* cells.

In this study, an expression vector-based library of *C.jejuni* DNA has been developed which can be screened using antisera and DNA probes. Lambda ZAP II was the chosen vector, because it can be automatically excised and recircularised *in vivo* to generate the pBluescript SK- phagemid vector containing the cloned insert DNA. This technique could reduce the problems

associated with subcloning *Campylobacter* DNA. Also, the multiple cloning site of Lambda ZAP is in the N-terminal region of the *lacZ* gene, thus convalescent antiserum can be used to screen plaques for β-galactosidase fusion proteins produced under the control of the *lac* promoter. This approach is, therefore, not dependent on the expression from *Campylobacter* promoters in the *E.coli* host.

As discussed in the introduction (section 2.1), according to the Clarke and Carbon equation:

$$N = \frac{\ln (1 - P)}{\ln (1 - f)}$$

(where N = no. of independent recombinants, P = probability of including any sequence, and f = $^{\text{size of single cloned fragment}}/_{\text{size of genome}}$), a library in which the average size of recombinant fragment was approximately 4kb would need to consist of at least 1955 independent clones (totalling 7820kb) to have a 99% probability of containing the *C.jejuni* sequence of interest. The library constructed in this project consisted of 3.8×10^6 kb (9.5×10^5 clones) and should therefore contain overlapping clones covering the entire *C.jejuni* strain H132 genome. In practice, however, this may not be the case, since *Sau*3A sites may not be randomly distributed throughout the *C.jejuni* chromosome, some *C.jejuni* DNA sequences may be toxic for *E.coli* and the *C.jejuni* DNA fragments of below 2.5kb were eliminated prior to ligation into Lambda ZAP II. However, a library of 3.8×10^6 kb, from an organism with a chromosome of only approximately 1720kb, should cover the majority of the genome.

Screening the library with convalescent antiserum is likely to detect *Campylobacter* proteins which are expressed *in vivo*, some of which may be virulence determinants. To date, such screening of the library has yielded 13 putative positive clones, approximately one positive for every thousand plaques screened. Re-testing of these positive plaques was slightly confused in some cases by high backgrounds on the test filters. Overnight blocking was found to reduce backgrounds considerably, however further improvements could be made to the convalescent antiserum used to screen the library. These may include pre-absorbing the antiserum more thoroughly with *E.coli* XL1-Blue sonicate, perhaps involving three or more successive incubation periods with fresh sonicate-coated filters. Different dilutions of antisera could be tested to find the optimum concentration for the screening process and/or the purified IgG fraction of the antiserum could be used instead of whole antiserum (the second antibody is anti-human IgG).

The library has also been screened successfully with labelled *C.jejuni* DNA probes. The entire *C.jejuni regX1* gene, for a member of the family of two-component sensor/regulators, has been isolated and rescued

from the library (Ana Bras-Goldberg, personal communication). A *C.jejuni regX1*$^-$ mutant is currently being tested in *in vitro* virulence assays (Ana Bras-Goldberg, personal communication).

The four putative *htr*A positives were re-tested using the non-radioactive method. One of them gave a strong positive response and attempts were made to rescue this clone into the pBluescript SK- phagemid. Rescue attempts with both cored plaque prep and amplified phage stock failed and further attempts to rescue the phagemid at 30°C also failed. Previous work with *S.typhimurium htrA* suggested that *htrA* could be stably maintained only at 30°C in low copy number vectors (S.Chatfield, personal communication). Therefore, although a clone containing part of an *htr*A gene homologue was isolated from the Lambda ZAP II library, it could not be rescued into the pBluescript SK- phagemid because it was toxic to the *E.coli* host in high copy number. In fact, it was later discovered that the promoter and upstream region of the *C.jejuni htrA* gene could not even be cloned at 30°C in the low copy number vector pLG39 (John Henderson, personal communication). This demonstrates a limitation of the Lambda ZAP II library, that *C.jejuni* sequences which are lethal to the *E.coli* host are unlikely to be isolated in their entirity from this library. However, the Lambda ZAP expression vector-based library of *C.jejuni* DNA has generally proved itself to be a useful resource, having been screened successfully with both antisera and DNA probes.

CHAPTER THREE

ANALYSIS OF POSITIVE CLONES

3.1 INTRODUCTION

Chapter two described the successful screening of the Lambda ZAP II library with convalescent antiserum. Several positive clones were isolated and rescued into the pBluescript SK- vector. The next step was to establish that these clones contained *C.jejuni* DNA inserts. It was then necessary to determine whether some of the clones contained inserts that overlapped and to find the approximate extent of the shared sequence. It was also necessary to determine whether they had protein products in common which reacted with convalescent antiserum.

One of the most immunodominant antigens in patients with *Campylobacter* enteritis is the 62kDa flagellin protein and antiflagellin antibodies are thought to contribute to the protective immune response of the host [62, 201, 202]. Therefore, it was possible that the positive clones identified from the Lambda ZAP II library by their reaction with convalescent antiserum could contain all or part of the flagellin genes *flaA* and *flaB*. Since several other research groups were investigating these genes, we were interested in obtaining genes for virulence determinants other than flagellin. Thus, it was also necessary to verify that the *C.jejuni* inserts did not contain the *fla* genes.

This chapter describes the experiments that were carried out in order to address these questions.

3.2 MATERIALS AND METHODS

3.2.1 Phagemid miniprep procedure. A single colony from the rescue procedure was grown to high density at 37°C in 3ml of LB (method 2.2.1) containing 100μg/ml ampicillin (LB-AMP). The alkaline lysis method of Birnboim and Doly [203] was used. Briefly, the cells were harvested by centrifugation at 12,000xg for 2 min., resuspended in 200μl lysis buffer [50mM glucose, 10mM EDTA, 4mg/ml lysozyme (Sigma), 25mM Tris-HCl; pH8.0] and left for 5 min. at room temperature. 400μl of freshly made 0.2M NaOH, 1% (w/v) SDS were added, mixed by inversion and incubated for 5 min. on ice. 300μl of 7.5M ammonium acetate (pH 7.8) were added, mixed gently for a few seconds and kept on ice for 10 min. After centrifugation for 10 min. at 12,000xg, the supernatant was removed and 0.6 volumes of isopropanol added to it. The DNA was left to precipitate for 10 min. at room temperature and harvested at 12,000xg for 10 min. The pellet was washed with 70% (v/v) ethanol, dried and resuspended in 20μl TE [10mM Tris-HCl, 1mM EDTA; pH8.0].

3.2.2 DNA maxiprep procedure using Qiagen. The Qiagen plasmid midi prep kit was used according to the manufacturer's instructions. Briefly, 100ml LB-AMP was inoculated with the bacterial clone of interest and incubated overnight at 37°C, with shaking at 200rpm. The cells were harvested by centrifugation at 3,900xg for 15min. at 4°C and resuspended in 4ml buffer P1 [50mM Tris-HCl, 10mM EDTA, 100μg/ml RNase A; pH8.0]. Cell lysis was carried out by adding 4ml P2 [0.2M NaOH, 1% w/v SDS] and incubating on ice for 5min. 4ml of P3 [3M potassium acetate, pH5.5] was added, mixed by inversion and incubated on ice for a further 10 min. to precipitate cell debris, denatured proteins, chromosomal DNA and SDS. After centrifugation at 3,900xg for 15min. at 4°C, the supernatant was loaded onto a Qiagen-tip 100 column, which had been equilibrated with buffer QBT [0.75M NaCl, 50mM 3-(N-morpholino) propane sulphonic acid (MOPS), 15% (v/v) ethanol, 0.15% (v/v) Triton X-100; pH7.0]. The Qiagen column was washed twice with 10ml buffer QC [1M NaCl, 50mM MOPS, 15% (v/v) ethanol; pH7.0] and the DNA was finally eluted with 5ml of QF [1.25M NaCl, 50mM Tris-HCl, 15% (v/v) ethanol; pH8.5]. DNA was precipitated with 0.7 volumes isopropanol and harvested by centrifugation at 12,000xg for 15min. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in 40μl sdH_2O.

3.2.3 DNA maxiprep using caesium chloride density gradient. 100ml of *E.coli* cells containing the plasmid of interest were harvested as above (3.2.2) and resuspended in 5ml lysis buffer (see 3.2.1). 10ml of 0.2M NaOH, 1% (w/v)

SDS were added and mixed by inverting the tube several times. After 5 min. on ice, 7.5ml of 7.5M ammonium acetate (pH7.8) were added, mixed gently and left on ice for a further 20 min. The precipitate was removed by centrifugation at 3,900xg for 20 min. at 4°C. 0.7 volumes isopropanol was added to the supernatant and incubated for 30 min. at room temperature. The precipitate was isolated by centrifugation at 15,000xg for 10 min. and resuspended in 4ml sdH_2O. 5ml of 4.4M LiCl were added to precipitate some of the RNA in the sample and this was removed by centrifugation at 8,000xg for 10 min. To the 9ml of supernatant were added 6ml of isopropanol and the precipitated DNA was isolated by centrifugation at 15,000xg for 20 min. The DNA pellet was dissolved in 1.6ml sdH_2O and added to 1.76g CsCl (Sigma) and 30mg sarkosyl (Sigma). 60µl of 10mg/ml ethidium bromide were added and mixed gently by pipetting until the caesium chloride had dissolved. A 2ml syringe was used to load the sample into a centrifuge tube (Beckman Quick-Seal) and the top was heat-sealed. Centrifugation was carried out at 356,160xg at 20°C for 16 hours. The plasmid band was harvested using a syringe and needle carefully pushed through the side of the centrifuge tube. An equal volume of CsCl-saturated isobutanol was mixed with the isolated DNA and the bottom layer was retained. The plasmid DNA could then be precipitated by adding water and isopropanol in the following ratio:- H_2O:isopropanol:DNA at 0.54:0.5:0.4, respectively. After centrifugation at 12,000xg for 20 min., the DNA pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in 20µl sdH_2O.

3.2.4 Standard DNA manipulations.

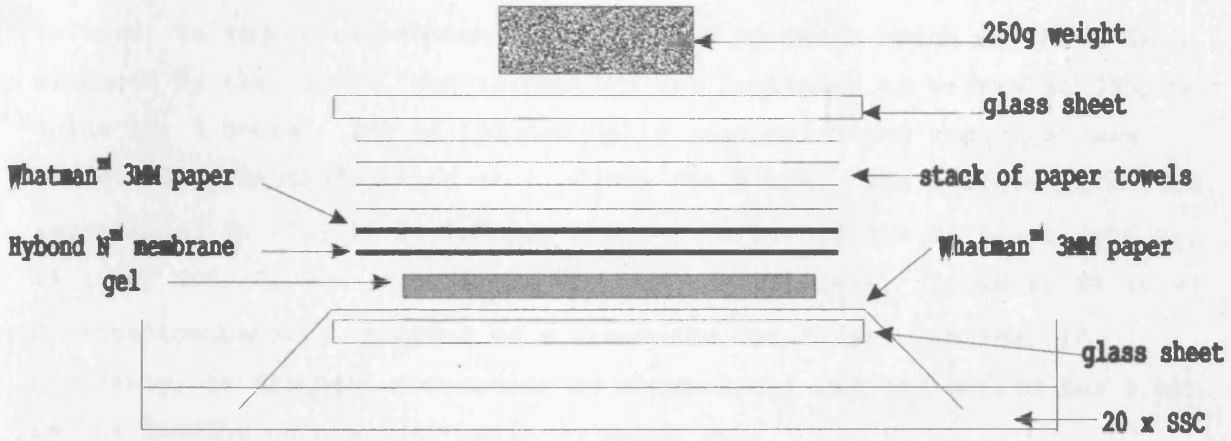
Restriction enzyme digestion. Restriction enzyme digests were carried out using enzymes and buffers from Gibco-BRL Life Technologies Ltd., according to the manufacturer's instructions. After digestion, each DNA sample was extracted once with TES-saturated phenol then extracted twice with chloroform:isoamylalcohol at 24:1. The DNA was precipitated with one tenth volume of 3M sodium acetate (pH5.4) and 3 volumes of cold ethanol, washed with 70% (v/v) ethanol and dried under vacuum. The pellet was dissolved in a minimum volume of sdH_2O and stored at -20°C.

DNA ligation. Ligations of DNA were carried out overnight at 15°C with T4 DNA ligase (Gibco-BRL) in a total volume of 40µl, using buffer supplied by the manufacturer [50mM Tris-HCl (pH7.6), 10mM $MgCl_2$, 1mM dithiothreitol, 5%(w/v) PEG 8000 and 1mM ATP].

Agarose gel electrophoresis. The products of these manipulations were separated by electrophoresis in gels consisting of 0.8-1.0% (w/v) agarose dissolved

in TAE buffer [40mM Tris-acetate, 1mM EDTA; pH7.6] containing 0.5μg/ml ethidium bromide, visualised by ultraviolet radiation on a Fotodyne transilluminator and photographed.

3.2.5 Southern blotting. DNA fragments generated by complete restriction enzyme digestion of H132 and *E.coli* DH5α chromosomal DNA, or of recombinant plasmids, were electrophoresed on a 0.8% (w/v) agarose gel, and transferred to Hybond-N membrane by the method of Dalgleish [204]. Gels were photographed then depurinated in 0.25M HCl for 7 min. at room temperature. After rinsing briefly in distilled H_2O (dH_2O), the gels were agitated gently in denaturing solution [0.5M NaOH, 1.5M NaCl] for 25 min., rinsed again in dH_2O then neutralised for 25 min. in 3M NaCl, 0.5M Tris-HCl (pH7.4). The blotting apparatus was set up according to the diagram below:



A sheet of filter paper (Whatman 3MM) was placed over a glass plate so that the ends hung down into the tray. 20xSSC [3M NaCl, 0.3M sodium citrate; pH7.0] was poured over the filter paper, ensuring that no air bubbles were trapped between the paper and the glass. The neutralised gel was placed onto the paper, again avoiding air bubbles, and was surrounded with plastic film. A piece of Hybond-N membrane (Amersham) was wetted with 3xSSC and placed over the gel, followed by a similarly wetted piece of filter paper (Whatman 3MM). Any air bubbles were rolled out using a glass rod. A stack of paper towels (Kimdri, Kimberly-Clark) was placed on top, followed by a glass plate and a weight, to keep the towels in intimate contact with the gel. The capillary transfer was allowed to proceed for $2^1/_2$ to 3 hours, replacing wet towels with dry every 15 min. After blotting, the nylon membrane was rinsed briefly in 3xSSC and dried. DNA was fixed to the membrane by exposure to UV on a Fotodyne transilluminator for 40 seconds.

Membranes to be probed with radioactive DNA probes were prehybridised for 1 hour at 65°C in pre-wash containing 1.5xSSPE [0.27M NaCl, 1.5mM NaH_2PO_4, 1.5mM EDTA; pH7.7], 0.5% (w/v) Marvel milk powder (Cadbury's), 1% (w/v) SDS and 6% (w/v) polyethylene glycol (PEG) 8000. Probes were prepared as detailed in the previous chapter (section 2.2.10). Boiled probe was added to the membrane in fresh pre-wash and further treatments were carried out as detailed in section 2.2.11.

Membranes to be probed with non-radioactive probes were treated according to the manufacturer's instructions (see section 2.2.12).

3.2.6 SDS - PAGE. SDS polyacrylamide gel electrophoresis was carried out essentially according to the method of Laemmli [205]. 5ml of LB-AMP were inoculated with 50µl of overnight culture of each of the clones to be tested. Incubation was continued at 37°C with shaking at 200rpm, until the cultures were at OD_{600} 0.4. 50µl of 0.1M IPTG were then added to each culture, to induce expression of any fusion proteins which may have been produced by the clones, and incubation was continued as before at 37°C for a further 3 hours. 1ml of induced cells (and uninduced controls) was harvested by centrifugation at 12,000xg for 3 min. The pellets were each resuspended in 20µl of 2x SDS-gel loading buffer [0.25M Tris-HCl (pH6.8), 2% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol]. Samples were sonicated for 30 sec. on ice, if necessary, to fragment contaminating chromosomal DNA and boiled for 5 min. before loading onto an SDS-polyacrylamide gel.

SDS-PAGE was carried out using the Mini-Protean II vertical gel system of Bio-Rad. 13% acrylamide main gel was made by mixing 2.7ml buffer A [0.75M Tris-HCl (pH8.8), 0.2% (w/v) SDS], 220µl dH_2O, 2.34ml Protogel [30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (National Diagnostic)], 190µl 10mg/ml ammonium persulphate (APS) and 15µl N,N,N',N'-tetramethylethylene-diamine (TEMED, Sigma). This mixture was poured between the glass plates to within 2cm of the top and overlayed with 200µl of isobutanol. The 5% acrylamide stacking gel solution was made while the main gel was polymerising and consisted of 1ml buffer B [0.25M Tris-HCl (pH6.8), 0.2% (w/v) SDS], 650µl dH_2O, 350µl Protogel, 50µl APS (10mg/ml) and 4µl TEMED. The isobutanol was poured off the polymerised main gel and the gel surface rinsed with dH_2O before the stacking gel was poured on top. The sample well comb was inserted and the gel allowed to polymerise for about an hour. The comb was removed and the wells rinsed with dH_2O before the gel assembly was clamped into the tank apparatus. The tank was filled with running buffer [25mM Tris-HCl, 0.25M glycine, 0.1% (w/v) SDS; pH8.3] and samples

loaded into each well. Electrophoresis was carried out at a constant 15mA until the marker dye front reached the end of the stacking gel, then the current was increased to 25mA. When the marker dye had reached the bottom of the main gel, the apparatus was disassembled and the gel was ready for electroblotting.

3.2.7 Western blotting. A dry piece of nitrocellulose (BA85, Schleicher and Schuell) was layed on top of the gel and used to lift it onto a piece of Whatman 3MM filter paper which had been soaked with transfer buffer [39mM glycine, 48mM Tris-HCl (pH8.3), 0.037% (w/v) SDS, 20% (v/v) methanol] and layed on top of a piece of foam. 3ml of transfer buffer were poured over the nitrocellulose and any bubbles rolled out with a glass rod. A second piece of wetted 3MM paper was layed on top of the nitrocellulose, again avoiding bubbles, and another piece of foam was layed on top of this. The whole sandwich was placed into the blotting apparatus, with the nitrocellulose towards the positive electrode. The tank was filled with transfer buffer and electro-transfer was carried out at 400mA at 4°C for $1^1/_2$ hours.

The lane containing molecular weight markers was cut off the nitrocellulose filter and stained with Ponceau S solution [0.2% (w/v) Ponceau S (Sigma), 3% (w/v) trichloracetic acid]. The rest of the filter was incubated overnight at 4°C in blocking buffer [0.15M NaCl, 10mM Tris-HCl (pH7.4), 0.05% (v/v) Tween 20 (Sigma), 3% (w/v) bovine serum albumin (Sigma), 1% (w/v) casein (Sigma)]. The filter was covered with convalescent antiserum diluted 1:50 in blocking buffer and incubated for 6 hours at room temperature with gentle agitation. After three 10 minute washes in TBS containing 0.05% (v/v) Tween 20, the secondary antiserum [peroxidase-conjugated goat anti-human IgG (Sigma)] was added, diluted 1:2000 in blocking buffer, and incubation continued at room temperature in the dark for 2 hours. The filter was washed three times, as before, and finally rinsed in TBS. Protein bands bound by convalescent antiserum could then be visualised by staining in 15mg 4-chloro-1-naphthol dissolved in 5ml methanol to which 15ml TBS and 20µl H_2O_2 had been added immediately before use.

3.2.8 *In vitro* transcription/translation. The Promega *E.coli* S30 extract system for circular DNA templates was used to perform coupled transcription/translation from RNA- and chromosomal-free DNA, prepared using caesium chloride gradients (3.2.3). Promega's S30 extract is prepared by a modification of the method described by Zubay [206]. 0.4pmol of plasmid DNA was made up to 12µl with sdH_2O and mixed with 12µl S30

extract, 16µl amino acid mixture minus methionine and 0.4µl ^{35}S-methionine (Amersham). This mixture was incubated at 37°C for 2 hours, then 20µl was removed and added to 80µl acetone on ice. After 15 min., the precipitated proteins were harvested by centrifugation at 12,000xg for 5 min. The pellet was dried, resuspended in 20µl 2x SDS-gel loading buffer (see 3.2.6) and stored at -20°C. 2-5µl of this sample was electrophoresed by SDS-PAGE (see 3.2.6) and the gel was dried under vacuum. To visualise the radioactive proteins produced from the DNA clones, dried gels were exposed to Fuji X-ray film at room temperature for 2 days.

3.2.9 Immunoprecipitation. 20µl of the *in vitro* transcription/translation sample was added to 25µl of convalescent antiserum and 0.5ml RIPA buffer [0.15M NaCl, 5mM EDTA, 50mM Tris-HCl (pH7.4), 0.1% (w/v) SDS, 0.2% (w/v) sodium deoxycholate, 0.2% (v/v) Triton X-100, 0.1% (w/v) BSA] and mixed gently by pipetting. After 1 hour incubation on ice, with occasional mixing by inversion, 2mg protein A Sepharose beads (Sigma) in 20µl RIPA were added and incubation continued at 4°C on a rocking platform for 2 hours. The beads were harvested by centrifugation at 3,900xg for 30 sec., washed three times with 0.5ml RIPA and finally resuspended in 20µl 2x SDS-gel loading buffer and stored at -20°C. 10µl of this sample was separated by SDS-PAGE, as above (3.2.8).

3.2.10 Double stranded DNA sequencing. DNA was sequenced by the chain termination method of Sanger *et al.* [207]. The sequencing reactions were carried out according to the Sequenase Version 2.0 protocol (United States Biochemical Corporation) using T7 DNA polymerase to incorporate [α^{35}S]dATP into synthesized DNA strands. Alkaline denaturation was used in the initial stages of the project then, from 1992, the heat denaturation method was used as described by A.S. Anderson *et al.* [208]. Briefly, 7µl (3µg) DNA were mixed with 1µl (0.5pmol) primer and incubated at 95°C for 5 min. After centrifuging the sample for a few seconds to reincorporate any condensation, 2µl of 5x Sequenase buffer [0.2M Tris-HCl, 0.1M $MgCl_2$, 0.25M NaCl; pH7.5] were added and mixed. Primer was then annealed to the DNA template by incubating at 37°C for 15 min. To the annealed template and primer were added 1µl 0.1M dithiothreitol (DTT), 2µl diluted labelling mix [7.5mM dGTP, 7.5mM dCTP, 7.5mM dTTP, diluted 5-fold with sdH_2O], 0.5µl [^{35}S]dATP [10µCi/µl and 10µM dATP at 1000Ci/mmol (Amersham)] and 2µl diluted T7 polymerase (diluted 1:8 in cold enzyme dilution buffer). The reagents were mixed thoroughly and incubated for 4-5 min. at room temperature.

Meanwhile, 2.5µl aliquots of each termination mix were pre-heated to 37°C. Each termination mix consisted of 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 50mM NaCl and 8µM of the relevant dideoxynucleotide (i.e. ddGTP, ddATP, ddCTP or ddTTP). 3.5µl aliquots of the labelling reactions were removed and transferred to each of the four termination mixes. Incubation was continued at 37°C for 4-5 min. then 4µl of stop solution [95% (v/v) formamide (Sigma), 20mM EDTA, 0.05% (w/v) bromophenol blue (Sigma), 0.05% (w/v) xylene cyanol (Sigma)] were added to each of the termination reactions. The reactions were stored on ice or at -20°C until used.

The radiolabelled DNA fragments were separated by vertical gel electrophoresis using the Gibco-BRL Model S2 Sequencing Gel Electrophoresis System with wedge spacers or the Bio-Rad Sequi-Gen nucleic acid sequencing cell with flat spacers. The gel consisted of 0.5g/ml urea, 1xTBE [90mM Tris, 90mM boric acid, 2.5mM EDTA] and 150µl/ml Accugel [40% (w/v) 19:1 acrylamide:bisacrylamide (National Diagnostics)].

The urea was dissolved in a minimum of sdH_2O on a heated stirrer and 36ml 5xTBE plus 27ml Accugel were added. The gel mix was made up to 180ml and 70ml was removed to be used for plugging the base of the Bio-Rad apparatus. 175µl of 25% (w/v) APS plus 175µl of TEMED were added to polymerise the plug and, when this was set, 198µl of 25% APS plus 33µl of TEMED were added to the main gel mix. This was poured carefully between the gel plates and allowed to polymerise. The gel was pre-run at a constant power of 120W in 1xTBE running buffer until up to temperature (50°C). The sample wells were washed with running buffer and sequencing samples were heated at 95°C for 3 mins before 4µl of each was loaded on to the gel.

After electrophoresis, the sequencing gel was fixed for 30 min. in 10% (v/v) acetic acid, 12% (v/v) methanol and transferred to Whatman 3MM filter paper. The gel was covered with plastic film and dried under vacuum at 80°C for 5 hours before autoradiography with Fuji RX medical X-ray film at room temperature overnight.

Nucleotide sequences were analysed using the University of Wisconsin Genetics Computer Group software (GCG) on the Leicester University Irix system. The FASTA program, which uses the Pearson and Lipman algorithm, was used for searching the databases for similar sequences.

3.3 RESULTS

3.3.1 Initial confirmation and relative comparison of clones. One of the first priorities, after the isolation of positive clones, was to confirm that the inserts were definitely *C.jejuni* DNA, not contaminating DNA from another organism. To show that this was the case, Southern blots were carried out using the inserts as probes for *Cla*I-digested chromosomal DNA. Several enzymes were tested for their ability to digest *C.jejuni* DNA and *Cla*I was chosen because it gave a wide range of fragment sizes. *Cla*I-digested *C.jejuni* chromosomal DNA was run on an agarose gel in lanes next to a negative control of *Cla*I-digested *E.coli* DNA and blotted onto Hybond N membrane, as detailed in method 3.2.5. The pattern of *Cla*I-digested bands revealed by agarose gel electrophoresis is shown in Figure 3.1.

The membrane-bound *C.jejuni* and control *E.coli* DNA was probed separately with inserts derived from each positive clone. Figure 3.2 shows that the insert probes of those clones tested against *E.coli* DNA hybridised only with *C.jejuni* chromosomal bands and four of the clones (pMK11, pMK12, pMK13 and pMK6) hybridised with the same 7.5kb fragment. pMK14 insert DNA also hybridised very weakly with the 7.5kb fragment, but more strongly with three fragments of 2.2, 3 and 4kb (suggesting that the insert contained at least two, probably three, *Cla*I sites). The pMK3 insert hybridised with a unique fragment of 3.5kb. These results confirmed that at least pMK11, pMK12 and pMK13 did not contain *E.coli* DNA inserts, and suggested that four of the positive clones could include DNA sequences from the same *Cla*I fragment of the *C.jejuni* genome.

Further Southern blots were carried out to verify that clones pMK11, pMK12, pMK13, pMK14 and pMK6 contained overlapping inserts. *Apa*I and *Hinc*II restriction enzymes were used to cut the *C.jejuni* inserts out of the pBluescript SK- vector and the fragments were separated by electrophoresis and blotted onto Hybond N membrane (Figure 3.3). Membranes were probed separately with the radiolabelled inserts of pMK3, pMK12, pMK13, and pMK14 and the results are displayed in Figure 3.4. The radiolabelled insert of pMK12 hybridised with the inserts of pMK6, pMK11 and pMK13; pMK13 hybridised with pMK6, pMK11, pMK12 and pMK14; pMK14 hybridised only with pMK13; while pMK3 did not hybridise with any of the other clones. These Southern blot results confirmed that the inserts of pMK6, pMK11, pMK12 and pMK13 contained overlapping fragments of the *C.jejuni* genome and suggested that one end of the pMK13 insert could also overlap with pMK14. The insert of pMK3 was unique. The table below summarises these results.

CLONE	PROBE pMK12	pMK13	pMK14	pMK3
pMK6	+	+	-	-
pMK11	+	+	-	-
pMK12	+	+	-	-
pMK13	+	+	+	-
pMK14	-	+	+	-
pMK3	-	-	-	+

Table to show which *C.jejuni* DNA inserts hybridised with eachother. "+" indicates that the probe bound the insert of that clone, while "-" indicates no such binding occurred.

3.3.2 Southern blots to verify absence of *flaA* from positive clones. Flagellin is the major component of *C.jejuni* flagella, which are essential for colonisation of the intestinal mucosa [46]. The *C.jejuni* flagellin protein has consistently been shown to be immunogenic during human infections with the organism and several groups have focussed their genetic studies on the *flaA* and *flaB* genes [209]. The kind provision by Trudy Wassenaar of pTNS#A (Figure 3.5) made it possible to probe the positive clones with a labelled fragment of *flaA* to determine whether any of them contained a flagellin gene homologue. pTNS#A is a pBluescript construct which contains the first 700bp of the *flaA* gene interrupted by the *C.jejuni* kanamycin resistance gene (Figure 3.5). Digestion of pTNS#A with *Hin*dIII and *Eco*RV yielded four fragments which were separated by electrophoresis. Probe 1 consisted of pBluescript vector alone, probe 2 consisted of the first 163bp of *flaA* plus 2kb of *C.jejuni* DNA upstream of *flaA* and probe 3 contained the kanamycin resistance gene and the remaining 537bp of the *flaA* fragment (these two fragments resulted in a doublet on agarose gel electrophoresis). *Hin*dIII digests of the *C.jejuni* clones were probed with the pTNS#A fragments to give a detailed picture of which parts of the inserts, if any, were bound by the *fla*A fragment. These digests are shown in Figure 3.6 and the Southern blots are shown in Figure 3.7.

As expected, probe 1 (pBluescript vector DNA) hybridised strongly with all the vector bands on the Southern blot of the *Hin*dIII-digested positives, and with the pBluescript control. The sizes of these bands varied slightly according to the distance to the first *Hin*dIII site in each insert: 3.2kb for pMK11, pMK13 and pMK14; 3.8kb for pMK6 and pMK12; and 3.6kb for pMK3. Probe 1 also hybridised with a pBluescript-containing partial digest product of 6.6kb in the lanes for pMK13 and pMK14 and, less

strongly, with a 1kb *Hin*dIII fragment of pMK6. Since pMK6 and pMK12 were later found to be identical by sequencing, it was suspected that this 1kb fragment was contaminating R408 helper phage (it was too small to be a partial digest product containing the 2.9kb pBluescript SK- vector).

The Southern blots incubated with probes 2 and 3 of pTNS#A also showed hybridisation only with the *Hin*dIII fragments containing the pBluescript SK- vector DNA. The signal strength was weaker than that with probe 1, especially for the Southern incubated with probe 3, and it was most likely that the hybridisation seen with these two Southerns was due to contamination of probes 2 and 3 with pBluescript. No other DNA fragments from the positive clones hybridised with the probes. Moreover, when the reverse experiment was performed, using the cloned *C.jejuni* DNA inserts as probes for *Hin*dIII and *Eco*RV digested pTNS#A, only the pBluescript band was bound by the inserts (data not shown). Therefore, the insert fragments had no homology with the 2.7kb of *C.jejuni* DNA in pTNS#A, which included the first 700bp of *fla*A and 2kb of upstream sequence.

3.3.3 Mapping. Restriction enzyme digests were carried out as detailed in methods section 3.2.4. Figure 3.8 shows the pBluescript SK- vector with the restriction sites of interest. Note that the *C.jejuni Sau*3A fragments were cloned into the *Xho*I site. Figure 3.10 summarises the digest results.

*Hin*dIII was found to be a useful enzyme to use as it had several cleavage sites within each genomic fragment and, hence, could distinguish the minor differences between the overlapping fragments (Figure 3.9). *Hin*dIII was expected to cut the *C.jejuni* DNA frequently because of the AT rich nature of such DNA (the recognition site for this enzyme is A^AGCTT). Clones pMK6, pMK11, pMK12 and pMK13 had very similar *Hin*dIII digestion patterns. They all had two particular *HindIII* fragments in common with apparent molecular weights of 1.2kb and 0.7kb. The only differences between pMK6, pMK12 and pMK13 were that pMK6 had an extra 1kb *Hin*dIII fragment and pMK13 had extra bands of 0.7kb and 0.4kb, when compared to pMK12. Other enzymes with sites in the inserts included *Eco*RV (GAT^ATC), *Xba*I (T^CTAGA) and *Cla*I (AT^CGAT). All of these enzymes have recognition sites with a greater proportion of A and T residues.

The enzyme digests confirmed that pMK6, pMK11, pMK12, and pMK13 had large overlapping segments (Figure 3.10). pMK11 contained approximately 1.5kb more sequence upstream of pMK6, pMK12 and pMK13, but less downstream sequence, the insert ending just after the 1.2kb *Hin*dIII fragment. pMK12 and pMK6 both had the same fusion point with the reverse primer end of pBluescript SK- (just before the 0.7kb *Hin*dIII fragment), while pMK13 possessed approximately 400bp more sequence upstream of pMK12 and pMK6 and

extended approximately 700bp further downstream. The identity of the sequence overlap between the clones pMK14 and pMK13 could not be deduced by comparison of the restriction enzyme maps, but, since pMK13 has more downstream *C.jejuni* DNA than pMK11, pMK12 and pMK6, it is possible for this sequence to overlap that of pMK14 without including sequence shared with the other clones.

3.3.4 SDS-PAGE and Western blotting. As the clones had been picked from the library by their protein products binding convalescent antiserum, it was hoped that Western blotting (using convalescent antiserum to probe *C.jejuni* proteins run on SDS-PAGE and immobilised on nitrocellulose) could be used to reveal the sizes of these proteins. It was anticipated that the overlapping clones might have an open reading frame (ORF) in common and, hence, all produce an immunogenic protein of the same size. SDS-PAGE and Western blot analysis were carried out as detailed in methods 3.2.6 and 3.2.7.

Only two Western blots, out of the several which were carried out, yielded protein bands which bound convalescent antiserum. In the first Western blot experiment, convalescent antiserum recognised a 60kDa protein from pMK6 and a 50kDa protein from pMK11, but did not bind any proteins from pMK3, pMK13 or pMK14 (Figure 3.11). In the second experiment, convalescent antiserum reacted with a 45kDa protein from pMK13 and a 50kDa protein from both pMK6 and pMK12 (data not shown). It was encouraging that the convalescent antiserum bound the same protein bands from pMK6 and pMK12, which appeared to be separate isolates of the same clone. It was also interesting that the antiserum bound a lower molecular weight protein from pMK11, whose insert overlaps with the first 2kb of the inserts in pMK6 and pMK12 (see Figure 3.10). It was possible that pMK11 could have produced a truncated version of the same protein as that synthesized by the other two clones. However, pMK13 would be expected to produce the same protein as pMK6 and pMK12, since it incorporates all the same sequence, but instead the antiserum appeared to recognise a protein of lower molecular weight. It was also inconsistent that the antiserum bound a 60kDa protein from pMK6 in the first experiment, but then a 50kDa protein in the second. In order to clarify the results, further Western blots were carried out following the same protocol, but no reaction with convalescent antiserum was achieved in these experiments. Such inconsistent and unrepeatable results could not be relied upon and so another method of analysing the proteins produced by the positive clones was sought.

3.3.5 *In vitro* transcription/translation and immunoprecipitation. As an alternative to Western blotting, it was decided to use the approach of *in vitro* transcription/translation. This technique involves the synthesis of peptides directly from the DNA of the clone of interest, using *E.coli* strain S30 cell extract and ^{35}S-methionine to label the proteins produced [206]. Immunoprecipitation would then allow the peptides which bound convalescent antiserum to be visualised. Methods 3.2.8 and 3.2.9 give the details of these techniques.

Figure 3.12 shows the electrophoretic separation of products synthesized by *in vitro* transcription/translation of the positive clones. Controls were included of pBluescript alone and a pBluescript subclone (designated pMK1.2) containing the 1.2kb *Hin*dIII fragment common to pMK11, pMK12, pMK6, and pMK13. As expected, all the samples contained the 30kDa β-lactamase band (from the pBluescript SK- ampicillin resistance gene). Other background protein bands, identified by examination of the pBluescript control lanes (Figure 3.12 and, more clearly, Figure 3.13, lane 8), had molecular weights of 42kDa, 35kDa, 28kDa, 23kDa and 13kDa. Several unique proteins were synthesized by the positive clones. The two most abundant unique proteins produced by pMK11 had molecular weights of 65kDa and 40kDa, while pMK12, pMK6 and pMK13 all produced a protein doublet around 74kDa, a product of 25kDa and one of 13.5kDa. It was possible that the 65kDa pMK11 protein was a truncated version of the 74kDa protein synthesized by the other clones, since the ORF could have been positioned so that it continued further into these clones but was cut short in pMK11 at a *Sau*3A site just after the 1.2kb fragment (see Figure 3.10). The 40kDa pMK11 product could have been synthesized from an ORF in the 1.4kb of the pMK11 insert which does not overlap with pMK12, pMK6 or pMK13. The patterns of protein synthesis of pMK6 and pMK12 were identical, additional confirmation that these were separate isolates of the same clone. The abundant protein of 13.5kDa produced by pMK6, pMK12 and pMK13 was also synthesized by pMK14, which suggested that it was unlikely to have been a product from the *C.jejuni* DNA, since pMK14 has no homology with pMK6 or pMK12. A relatively abundant 46kDa product was synthesized by both pMK13 and pMK14, while pMK11, pMK12 and pMK6 all generated a product of 45kDa (less abundant in pMK11). Products unique to pMK14 were a 31kDa and a 29kDa protein. pMK3, which had demonstrated no homology with any of the other positives during Southern blot experiments, yielded one novel product of 14kDa.

In all the immunoprecipitation experiments, convalescent antiserum bound to both the 65kDa protein from pMK11 and the 74kDa protein from pMK12, pMK6 and pMK13 (see Figures 3.13, 3.14 and 3.15). This further

supported the theory that there could be an ORF common to all these clones. Although conflicting results in Figure 3.13 appeared to indicate that a 65kDa product synthesized by pMK12 and pMK6 was also recognised by convalescent antiserum, in several previous experiments only the 74kDa, a 46kDa and sometimes the 30kDa β-lactamase products from these clones had been bound by the antiserum (Figure 3.15 and data not shown). A 69kDa protein was also produced by pMK14 and showed a weaker reaction with convalescent antiserum (Figure 3.14), although, in previous experiments only a doublet at 80kDa had been bound by the same antiserum (Figure 3.15 and data not shown). The antiserum recognised as a doublet the 45kDa and 46kDa proteins synthesized by pMK11, pMK12, pMK6, pMK13 and pMK14 but, since pMK14 was shown not to have homology with pMK11, pMK12 or pMK6 and the 1kb overlap between pMK13 and pMK14 was too small to produce a 46kDa protein, these could have been background proteins from pBluescript SK- or contaminating *E.coli* XL1-Blue genomic DNA. Alternatively, the 45/46kDa material may have been caused by premature termination of the larger translation products, to form truncated polypeptides, or by the use of internal translational starts. pMK3 did not appear to produce any proteins which were recognised by convalescent antiserum, suggesting that it may have been a false positive. Alternatively, pMK3 could contain an ORF with a *C.jejuni* promoter which was not recognised by the *E.coli* S30 extract.

In summary, the convalescent antiserum generally showed patterns of immunoreactivity with the products from the positive clones which supported results gained from Southern blotting and mapping. pMK6 and pMK12 had identical protein profiles and synthesized one unique protein of 74kDa which consistently reacted with convalescent antiserum. pMK11 produced two distinct proteins. One had a molecular weight of 40kDa and did not react with convalescent antiserum. This product could have been generated from an ORF positioned outside of the region which overlapped with the other clones. The other product did react with convalescent antiserum and was 65kDa in size. This could have been a truncated version of the immunogenic 74kDa protein synthesized by pMK6 and pMK12. pMK13 had a similar protein profile to pMK6 and pMK12, with the same product at 74kDa which was detected by convalescent antiserum, as would be expected for a clone which overlaps the others. pMK14 synthesized two unique proteins of 29kDa and 31kDa, which were not detected by convalesent antiserum, and a product of 69kDa which the antiserum did react with. A less abundant 80kDa protein from pMK14 had also previously reacted with convalescent antiserum. pMK13 and pMK14 also had an immunogenic band in common at 46kDa, but this was too large a protein to have been produced by the 1kb overlap between the two clones alone. The unique clone, pMK3, produced a 14kDa band which was not

synthesized by any of the other clones , but no products from the *in vitro* transcription/translation of pMK3 were immunoreactive with convalescent antiserum.

3.3.6 Sequencing. Initial sequence analysis was performed using the M13 reverse primer, to generate sequence reading through the *lacZ-C.jejuni* DNA fusion joint of each clone, and the M13 forward primer, which could reveal the sequence from pBluescript SK- T7 promoter through to the *C.jejuni* DNA (see Figure 3.8). Using these primers to obtain sequence at both ends of the insert would enable us to prove whether pMK6 was identical to pMK12. Figure 3.16 displays some of the initial sequence found.

The only clone which could have produced a *lacZ* fusion protein was pMK14, the others all had stop codons shortly after the fusion joint. This suggested that immunogenic proteins were transcribed from internal *C.jejuni* promoters or at least that *C.jejuni* translation signals were working in *E.coli* XL1-Blue. The inserts of pMK6 and pMK12 were confirmed to have identical sequences at each end and, therefore, both these clones contained the same *Sau*3A fragment from the *C.jejuni* genome. A *Hin*dIII site was found 31 bases into the pMK12 insert which could be the first site of the 0.7kb *Hin*dIII fragment which was common to pMK11, pMK12 and pMK13.

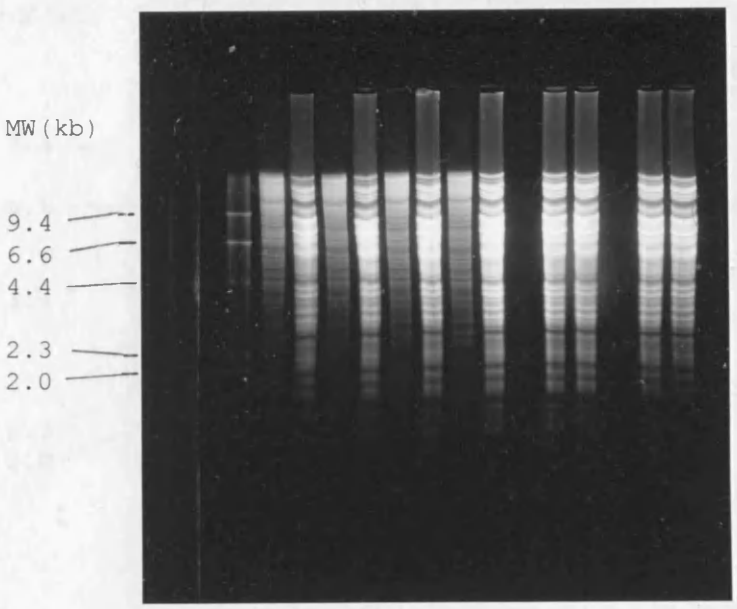


Figure 3.1

Southern blot to show that pBluescript clones contained *C.jejuni* DNA. This figure shows the digested genomic DNA which was probed with inserts of clones pMK11, pMK12, pMK13, pMK14, pMK6 and pMK3 (Figure 3.2). *C.jejuni* strain H132 and *E.coli* strain DH5α (negative control) chromosomal DNAs were digested with *Cla*I, electrophoresed at 80V on a 0.8% (w/v) agarose gel and subsequently transferred to Hybond N membrane.

Lane 1: λ *Hin*dIII molecular weight markers (sizes, in kb, shown on left-hand side); Lanes 2, 4, 6 and 8: 1μg *Cla*I-digested *E.coli* DH5α chromosomal DNA; Lanes 3, 5, 7, 9, 11, 12, 14 and 15: 1μg *Cla*I-digested *C.jejuni* H132 chromosomal DNA; Lanes 10 and 13: no DNA.

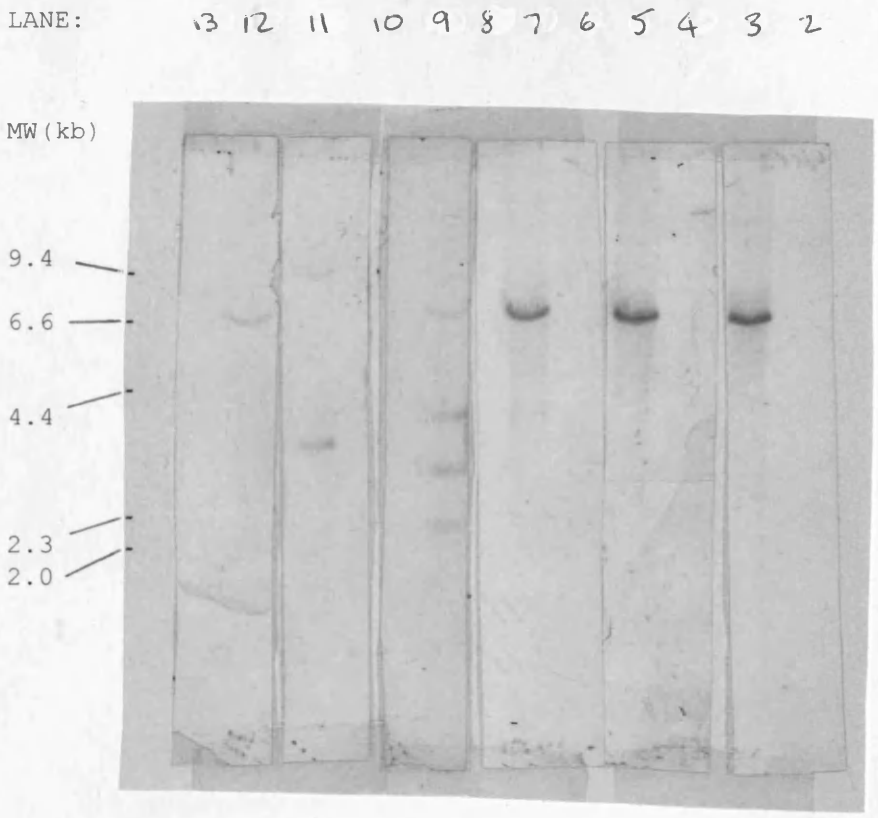


Figure 3.2

Southern blot to show that *C.jejuni* DNA had been cloned. Digoxigenin-labelled inserts of pBluescript clones were used as probes for *Cla*I-digested *C.jejuni* H132 and *E.coli* DH5α chromosomal DNAs (Figure 3.1).

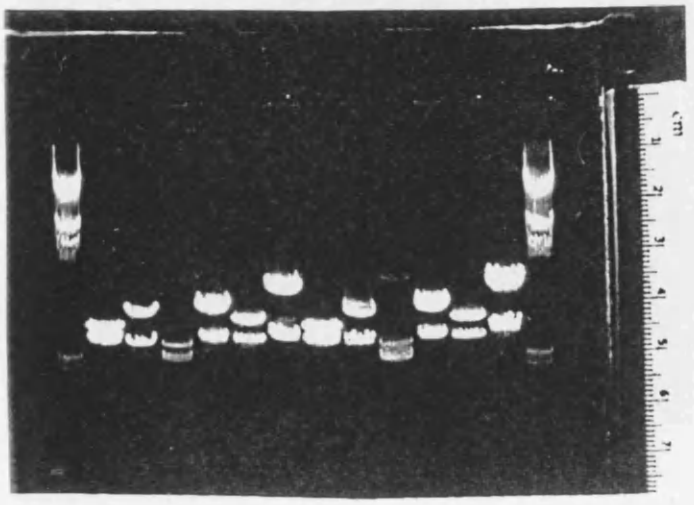
Lanes 13 and 12: insert of pMK6 used as probe; Lane 11: insert of pMK3 used as probe; Lanes 10 and 9: insert of pMK14 used as probe; Lanes 8, 7 and 6: insert of pMK13 used as probe; Lanes 5 and 4: insert of pMK12 used as probe; Lanes 3 and 2: insert of pMK11 used as probe.

Figure 3.3

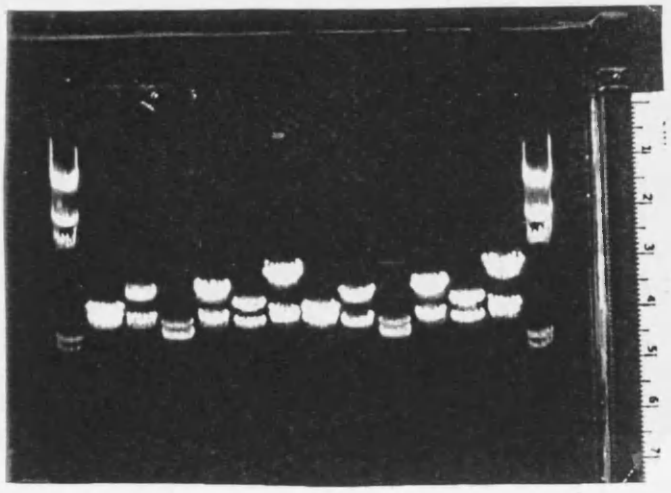
Southern blots to determine whether pBluescript clones contained overlapping inserts. This figure shows a thermal print of restriction digests of pMK11, pMK12, pMK13, pMK14, pMK6 and pMK3, which were subsequently probed with the *C.jejuni* DNA inserts of the clones (Figure 3.4). *Apa*I and *Hinc*II digests of pBluescript clones were electrophoresed at 100V on 1% (w/v) agarose gels and subsequently transferred to Hybond N membrane.

Lanes 1, 14, 15 and 28: λ *Hin*dIII molecular weight markers (sizes, in kb, shown on the left-hand side); Lanes 2-27 all contain 1.5μg of *Apa*I and *Hinc*II digested DNA from the following clones: Lanes: 2, 8, 16 and 22: pMK11; Lanes 3, 9, 17 and 23: pMK12; Lanes 4, 10, 18 and 24: pMK14; Lanes 5, 11, 19 and 25: pMK6; Lanes 6, 12, 20 and 26: pMK3; Lanes 7, 13, 21 and 27: pMK13.

LANE: 1 2 3 4 5 6 7 8 9 101112 1314



LANE: 151617 181920 212223 2425262728



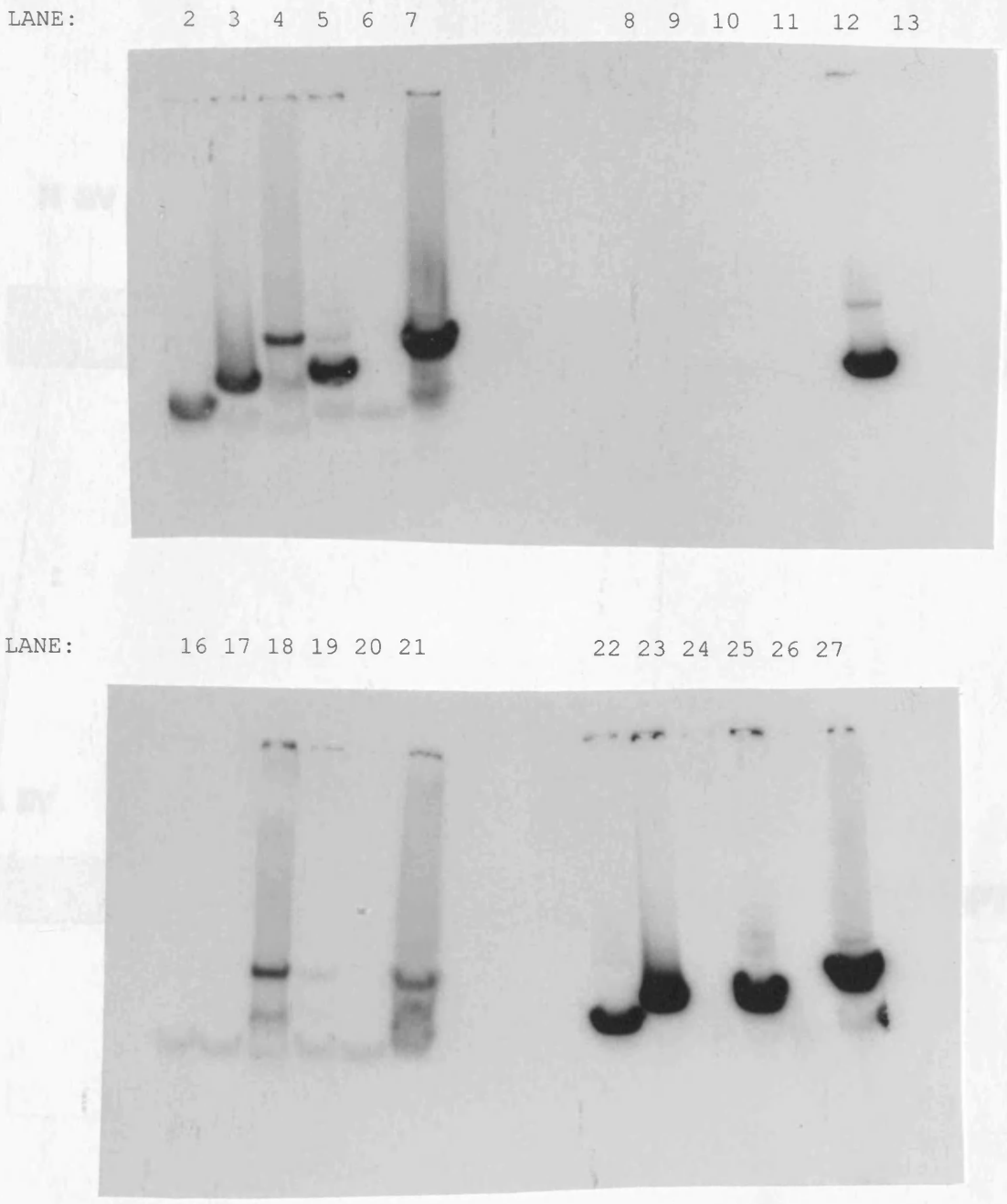


Figure 3.4

Figure to show the cross-hybridisation between the positive clone inserts. A Southern blot was performed, using radiolabelled inserts of pMK3, pMK12, pMK13 and pMK14 as probes for *Apa*I and *Hinc*II digests of all six positives (Figure 3.3).

Lanes 2-7: probed with insert of pMK13; Lanes 8-13: probed with insert of pMK3; Lanes 16-21: probed with insert of pMK14; Lanes 22-27: probed with insert of pMK12.

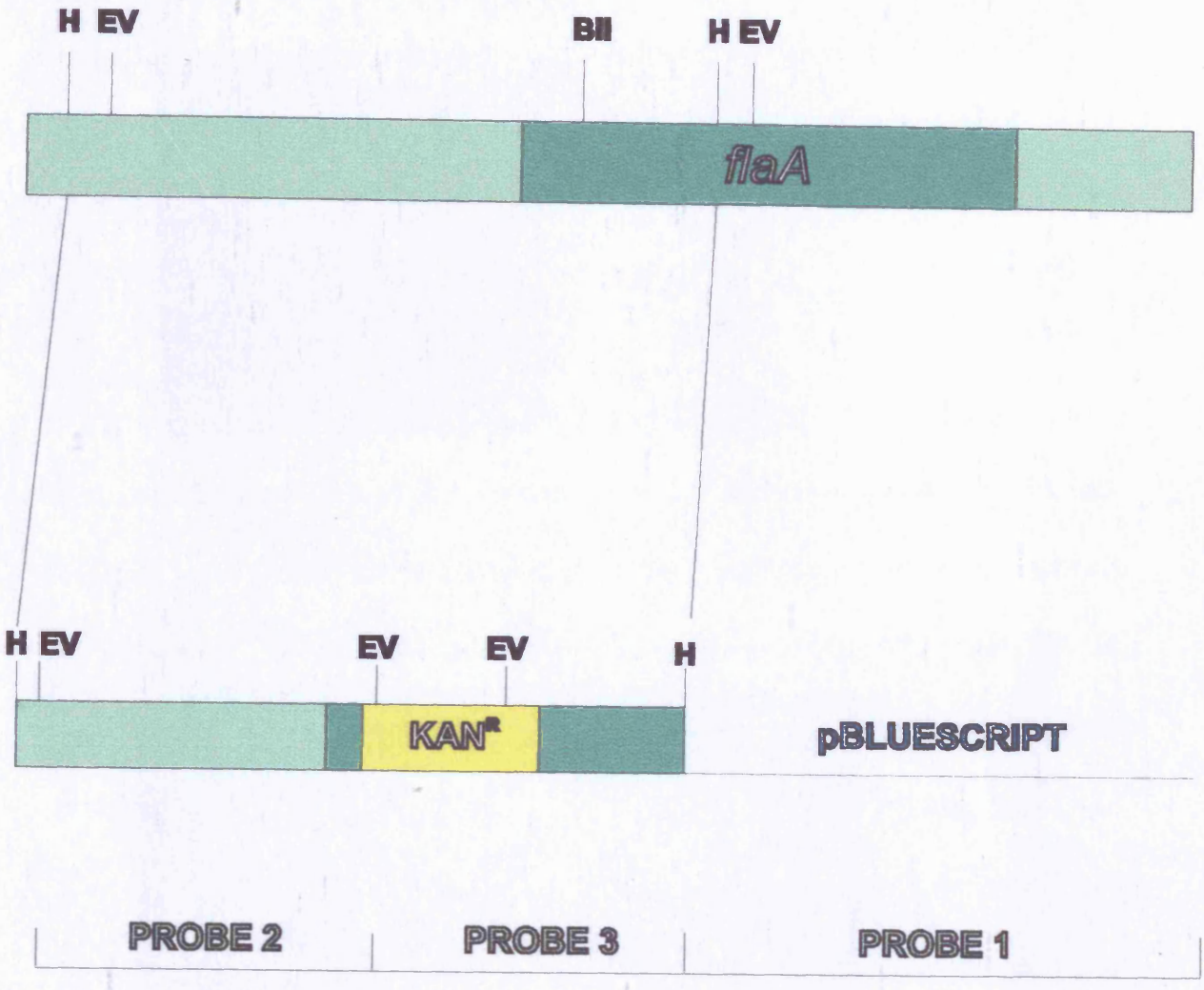


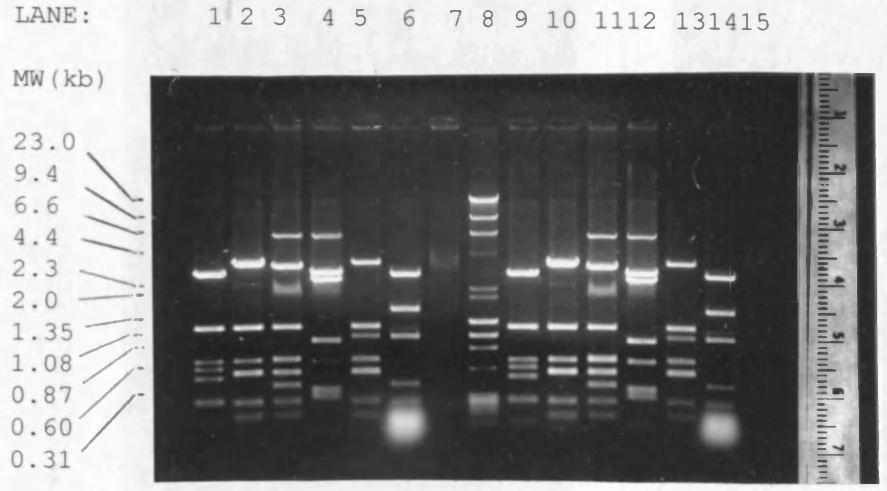
Figure 3.5

Diagram to show derivation of pTNS#A, with relevant restriction sites, adapted from T.M. Wassenaar *et al* [55]. The *C.jejuni flaA* gene is shown as a dark green box, flanking *C.jejuni* DNA is shown as light green boxes, the kanamycin resistance gene is shown as a yellow box and pBluescript vector as a simple line. The fragments from which each probe was derived are indicated. "H" denotes *Hin*dIII site; "EV" denotes *Eco*RV site; "BII" denotes *Bgl*II site.

Figure 3.6

Southern blots to show that clones pMK11, pMK12, pMK13, pMK14, pMK6 and pMK3 did not contain the *C.jejuni flaA* gene. This figure shows positive clone DNA, digested with *Hin*dIII and electrophoresed at 80V on a 0.8% (w/v) agarose gel. The separated fragments were subsequently transferred to Hybond N and probed with fragments of pTNS#A (Figure 3.7).

Lanes 8 and 23: λ *Hin*dIII and ϕX174 *Hae*III molecular weight markers (sizes, in kb, shown on the left-hand side); Lanes 1, 9 and 16: 1μg pMK11 digested with *Hin*dIII; Lanes 2, 10 and 17: 1μg pMK12 digested with *Hin*dIII; Lanes 3, 11 and 18: 1μg pMK13 digested with *Hin*dIII; Lanes 4, 12 and 19: 1μg pMK14 digested with *Hin*dIII; Lanes 5, 13 and 20: 1μg pMK6 digested with *Hin*dIII; Lanes 6, 14 and 21: 1μg pMK3 digested with *Hin*dIII; Lanes 7, 15 and 22: 1μg pBluescript digested with *Hin*dIII.


LANE: 1 2 3 4 5 6 7 8 9 10 1112 131415
MW(kb)
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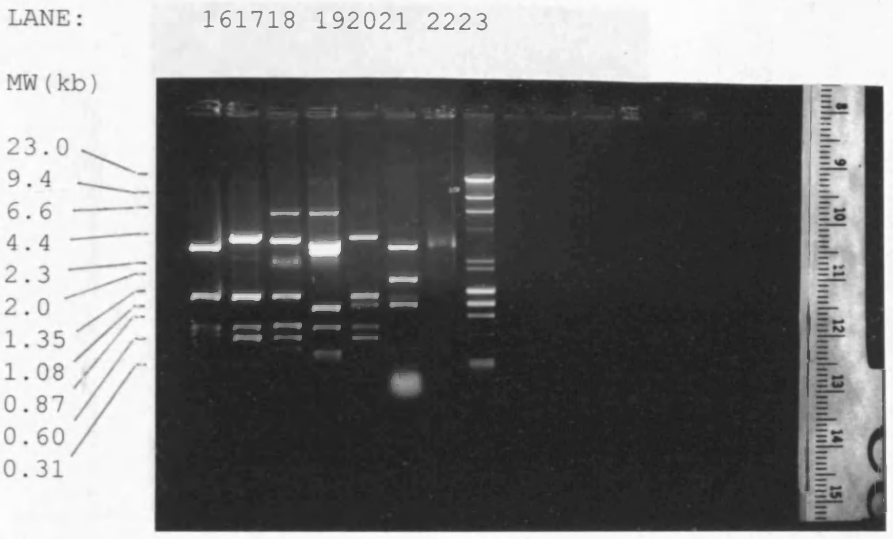
9.4
6.6
4.4
2.3
2.0
1.35
1.08
0.87
0.60
0.31


LANE: 161718 192021 2223
MW(kb)
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9.4
6.6
4.4
2.3
2.0
1.35
1.08
0.87
0.60
0.31

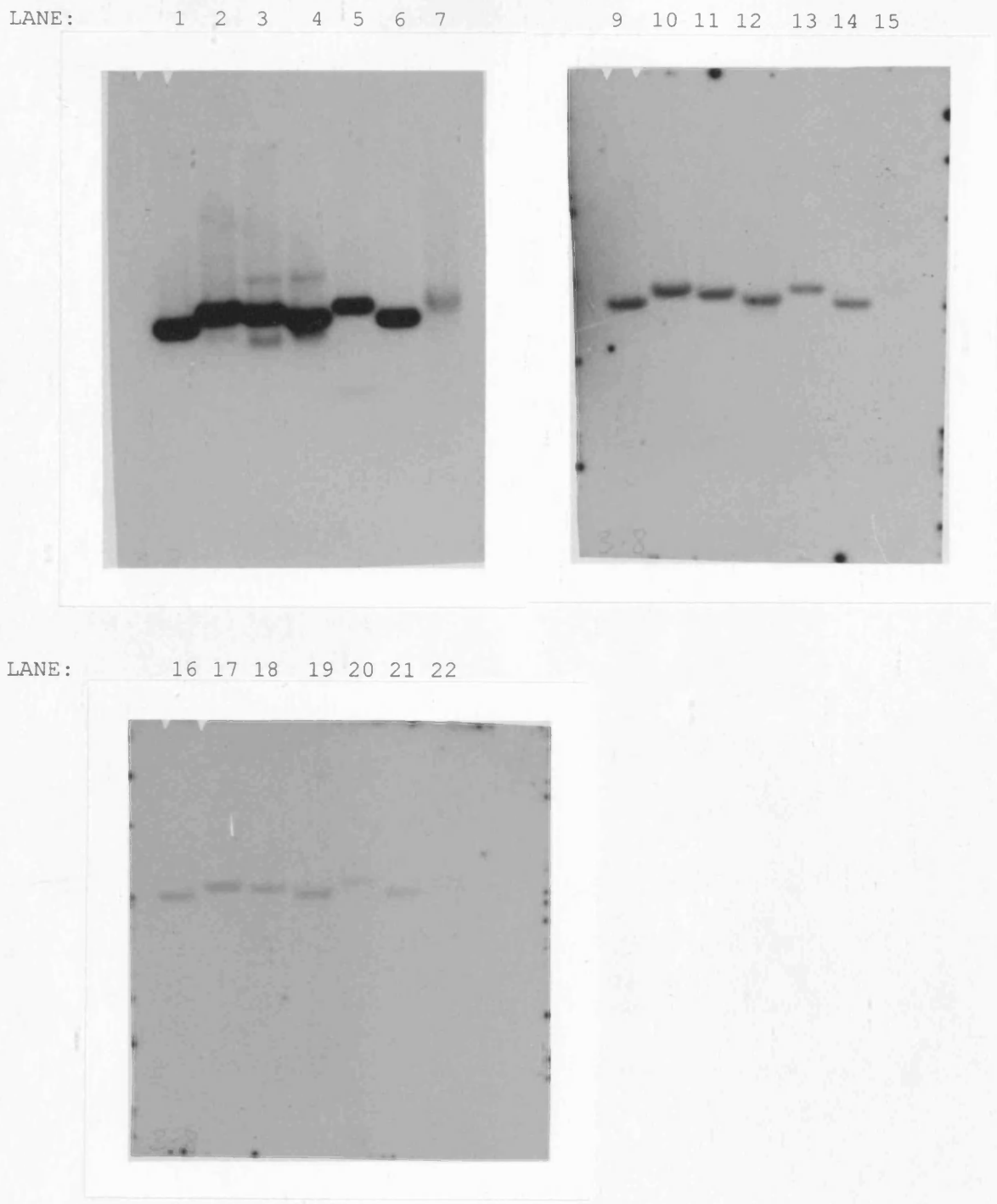


Figure 3.7

Southern blots using fragments of pTNS#A as probes for *Hin*dII digested positive clones (Figure 3.6).

Lanes 1-7: blot probed with pTNS#A probe 1 and exposed to autoradiographic film for 3 hours at -80°C; Lanes 9-15: blot probed with probe 2 of pTNS#A and exposed to autoradiographic film overnight at -80°C; Lanes 16-22: blot probed with probe 3 of pTNS#A and incubated overnight with autoradiographic film at -80°C.

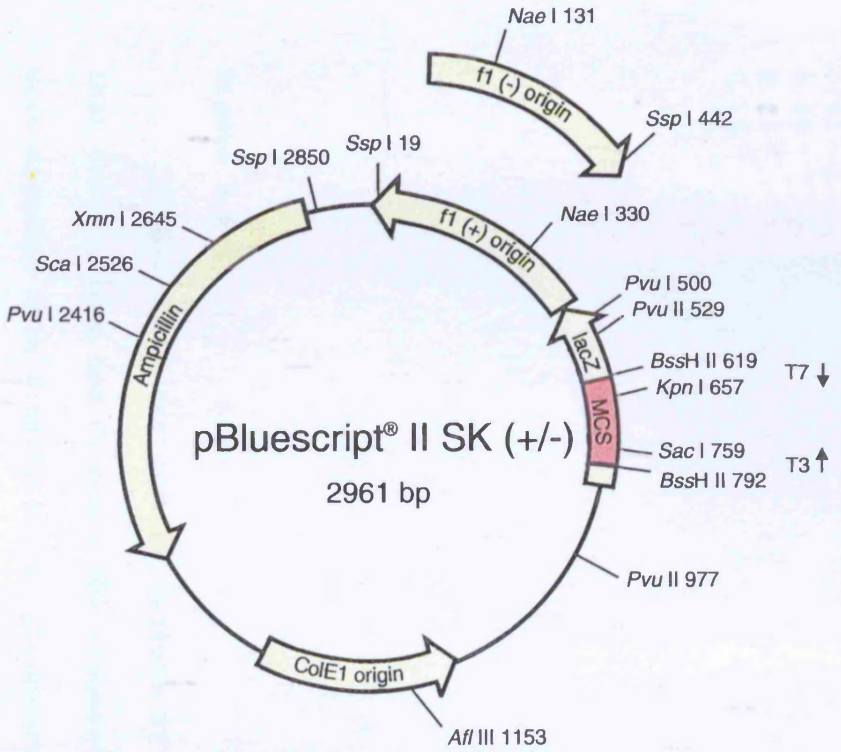
Figure 3.8

Adapted from 1997/1998 Stratagene catalogue.

A) Circular map of the pBluescript SK- phagemid. 6-462bp: f1(-) filamentous phage origin of replication, allowing recovery of the antisense strand of the *lacZ* gene (note that the f1 origin is in the "-" orientation in pBluescript SK-); 657-759bp: MCS (multiple cloning site) flanked by T3 and T7 promoters; 490-938bp: partial *lacZ* gene which provides α-complementation for blue-white colour selection of recombinant phagemids and inducible *lac* promoter for fusion protein expression; 1032-1912bp: ColE1 plasmid origin of replication; 1975-2832bp: *blaM* ampicillin-resistance gene.

B) Sequence of the MCS, showing restriction enzyme sites and the sequences of M13 primers. The T3 and T7 promoters are underlined. The *C.jejuni* strain H132 insert DNA was cloned into the *Xho*I site of the MCS.

A)



B)

M13 reverse primer
5′-GGAAACAGCTATGACCATG-3′

MET T3 promoter +1 *Sac*I *Xba*I *Bam*HI *Pst*I
5′-GGAAACAGCT**ATG**ACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
3′-CCTTTGTCGATACTGGTACTAATGCGGTTCGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCCGGCGAGATCTCGATCACCTAGGGGGCCCGACGTCC

816 β-galactosidase →
*Eco*RI *Eco*RV *Hin*dIII *Cla*I *Hinc*II *Xho*I *Apa*I *Kpn*I
5′-AATTCGATATTCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAA -3′
3′-TTAAGCTATAAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAATGTT -5′

657 +1 T7 promoter 3′-TGACCGGCAGCAAAATG-5′
M13 forward primer

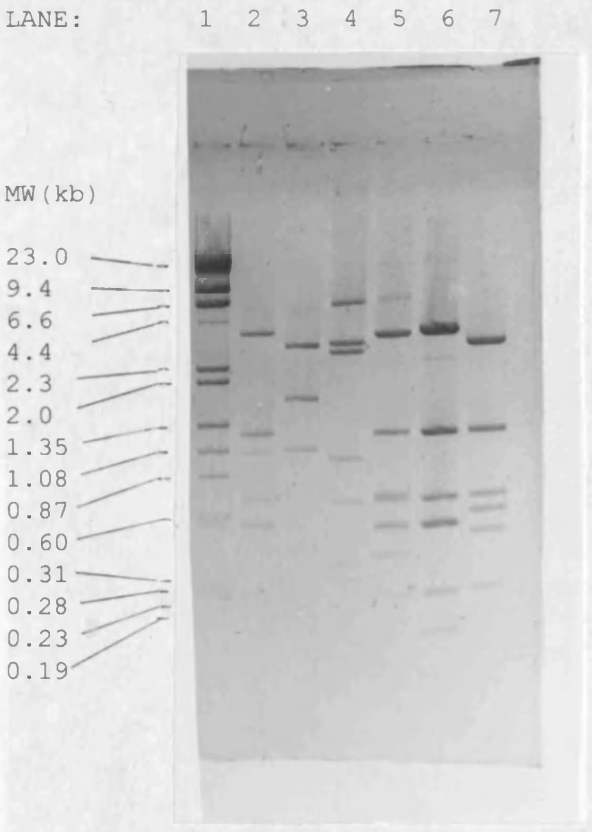


Figure 3.9

"Fingerprint" restriction digests of the pBluescript clones, showing that some of them had *C.jejuni* DNA fragments in common. Positive clones were digested with *Hin*dIII and electrophoresed on a 0.8% (w/v) agarose gel at 80V.

Lane 1: λ *Hin*dIII and ϕX174 *Hae*III molecular weight markers (sizes, in kb, shown on the left-hand side); Lane 2: 1μg *Hin*dIII digested pMK6; Lane 3: 1μg *Hin*dIII digested pMK3; Lane 4: 1μg *Hin*dIII digested pMK14; Lane 5: 1μg *Hin*dIII digested pMK13; Lane 6: 1μg *Hin*dIII digested pMK12; Lane 7: 1μg *Hin*dIII digested pMK11.

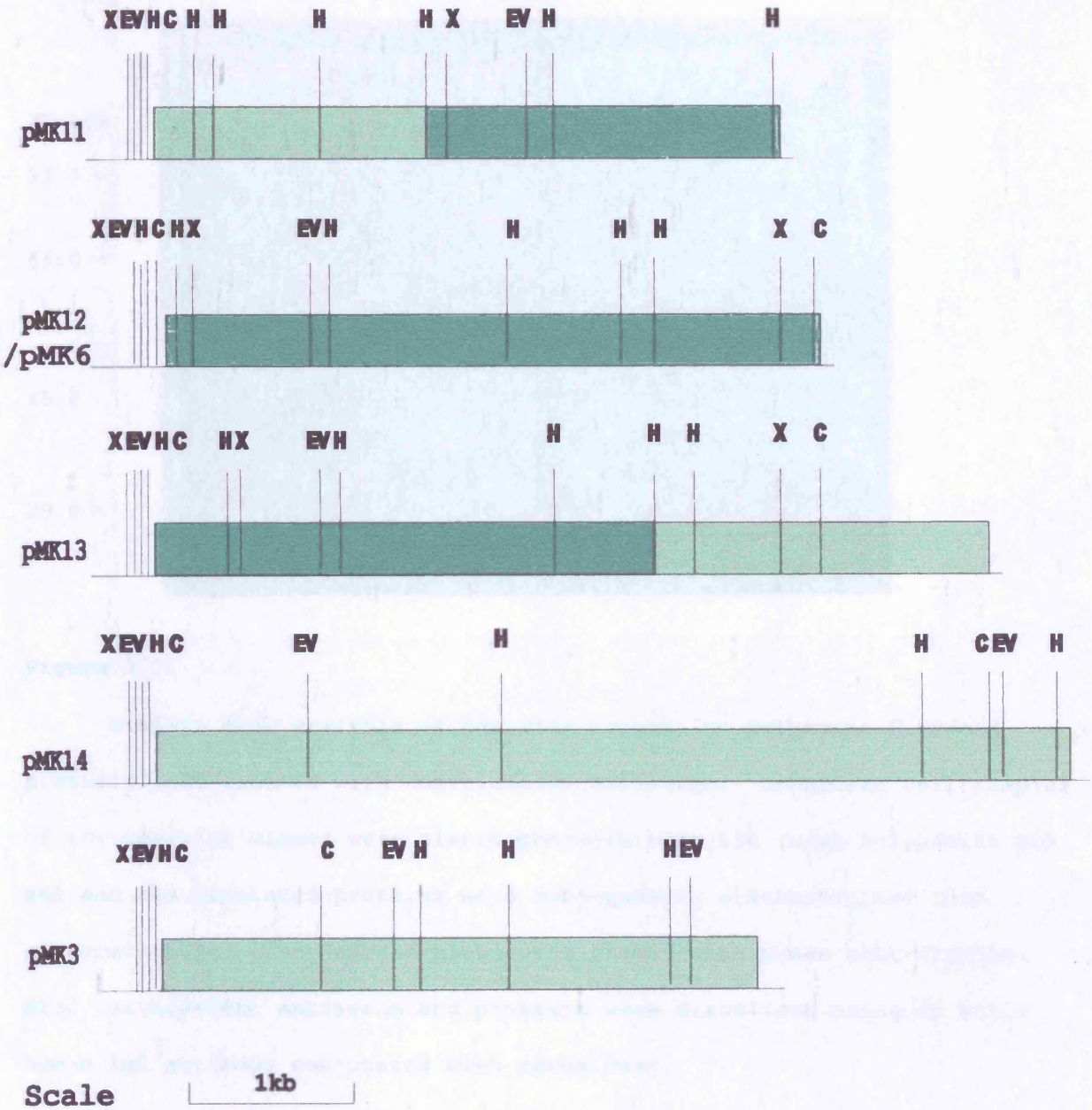


Figure 3.10

Non-exhaustive restriction maps of pMK11, pMK12/pMK6, pMK13, pMK14 and pMK3. "X" indicates *Xba*I restriction site, "EV" indicates *Eco*RV restriction site, "H" indicates *Hind*III restriction site and "C" indicates *Cla*I restriction site. The green boxes indicate *C.jejuni* insert DNA, while pBluescript SK- is shown as a simple horizontal line. The dark green area shows the putative extent of regions of overlapping sequence between clones pMK11, pMK12 and pMK13.

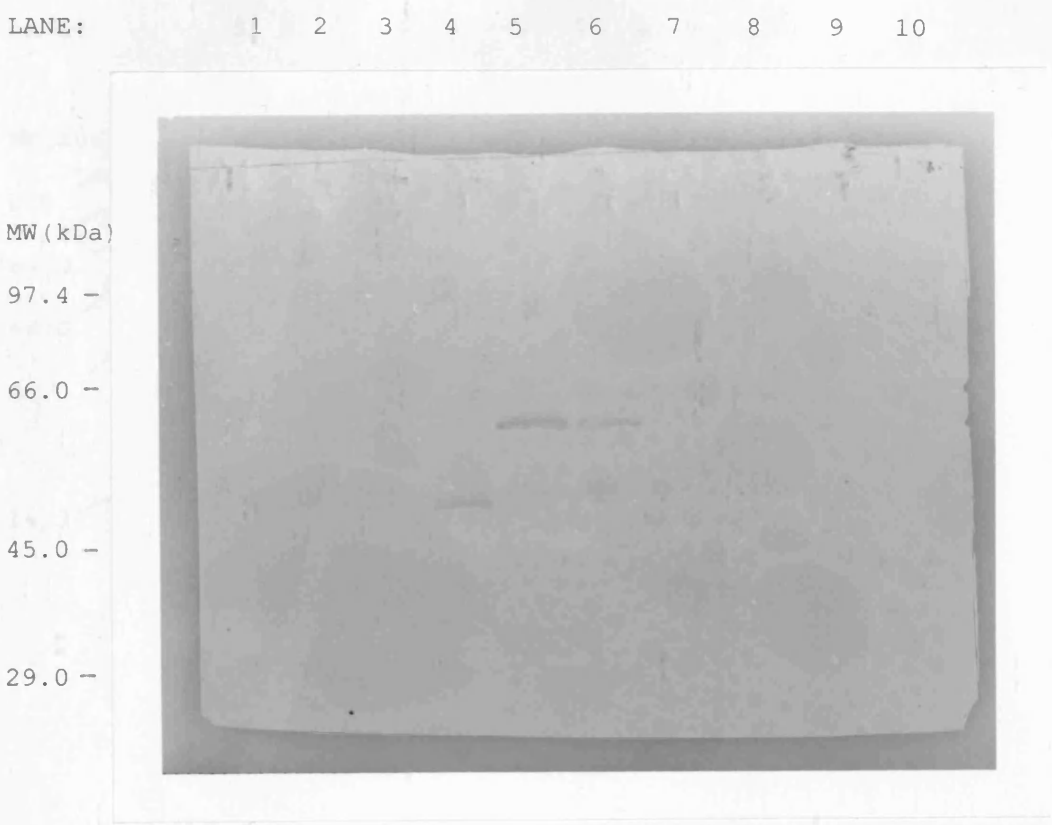


Figure 3.11

Western blot analysis of positive clones, to determine *C.jejuni* products that reacted with convalescent antiserum. Denatured cell samples of the positive clones were electrophoresed on a 13% (w/v) acrylamide SDS gel and the separated proteins were subsequently electroblotted onto nitrocellulose. The Western blots were probed with human anti-*C.jejuni* H132 convalescent antiserum and proteins were visualised using an anti-human IgG antibody conjugated with peroxidase.

Lane 1: protein molecular weight markers (sizes, in kDa, shown on the left-hand side); Lane 2: cell proteins from pMK14 in *E.coli* XL1-Blue; Lane 3: cell proteins from pMK13 in *E.coli* XL1-Blue; Lane 4: cell proteins from pMK11 in *E.coli* XL1-Blue; Lanes 5 and 6: cell proteins from pMK6 in *E.coli* XL1-Blue; Lanes 7 and 8: cell proteins from pMK3 in *E.coli* XL1-Blue; Lane 9: cell proteins from pMK2 (a putative positive which was not investigated further); Lane 10: cell proteins from *E.coli* strain XL1-Blue alone.

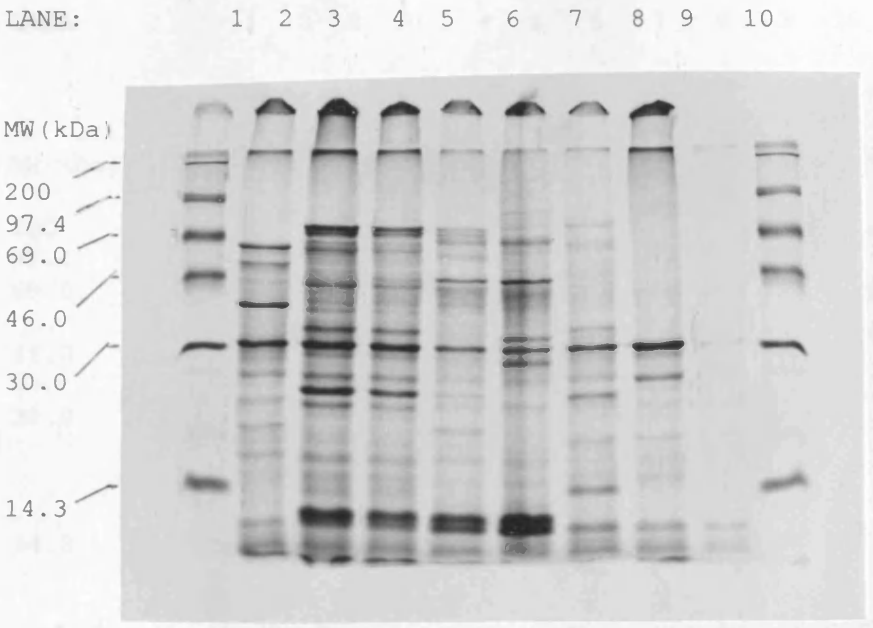


Figure 3.12

Figure to show proteins synthesized by positive clones. An *in vitro* transcription/ translation reaction was carried out with positive clone DNA and the products electrophoresed on a 13% (w/v) acrylamide SDS-PAGE gel. The gel was dried and exposed to autoradiographic film overnight at room temperature.

Lanes 1 and 10: protein molecular weight markers (sizes, in kDa, shown on the left-hand side); Lane 2: proteins synthesized from 0.05pmol (0.206µg) pMK11; Lane 3: proteins synthesized from 0.05pmol (0.222µg) pMK12; Lane 4: proteins synthesized from 0.05pmol (0.225µg) pMK6; Lane 5: proteins synthesized from 0.05pmol (0.164µg) pMK13; Lane 6: proteins synthesized from 0.05pmol (0.178µg) pMK14; Lane 7: proteins synthesized from 0.05pmol (0.214µg) pMK3; Lane 8: proteins synthesized from 0.05pmol (0.137µg) pBluescript subclone containing the 1.2kb *Hin*dIII fragment common to four of the positives (pMK1.2); Lane 9: 0.05pmol (0.1µg) pBluescript DNA control. Arrows indicate non-background products discussed in the text.

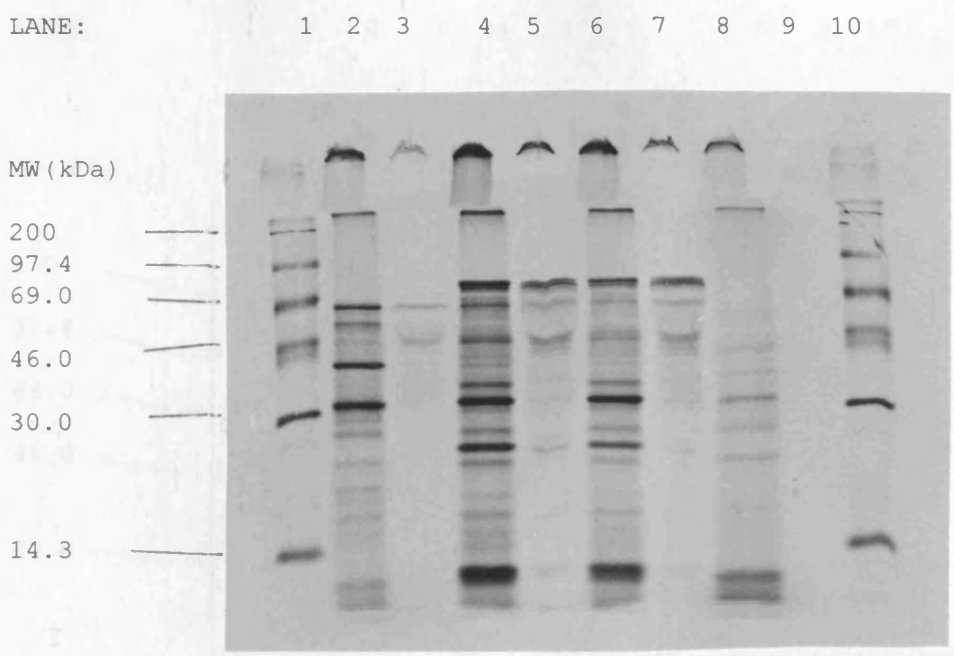


Figure 3.13

Figure to show proteins synthesized from clones pMK11, pMK12 and pMK6 and immunoprecipitated by convalescent antiserum. *In vitro* transcription/translation products and those *in vitro* transcription/translation products which were immunoprecipitated by convalescent antiserum were separated by SDS-PAGE [13% (w/v) acrylamide]. The gel was dried and exposed to autoradiographic film overnight at room temperature.

Lanes 1 and 10: protein molecular weight markers (sizes in kDa, shown on the left-hand side); Lane 2: proteins synthesized from 0.05pmol pMK11; Lane 3: immunoprecipitated proteins from 0.1pmol pMK11; Lane 4: proteins synthesized from 0.05pmol pMK12; Lane 5: immunoprecipitated proteins from 0.1pmol pMK12; Lane 6: proteins synthesized from 0.05pmol pMK6; Lane 7: immunoprecipitated proteins from 0.1pmol pMK6; Lane 8: proteins synthesized from 0.05pmol pBluescript; Lane 9: immunoprecipitated proteins from 0.1pmol pBluescript.

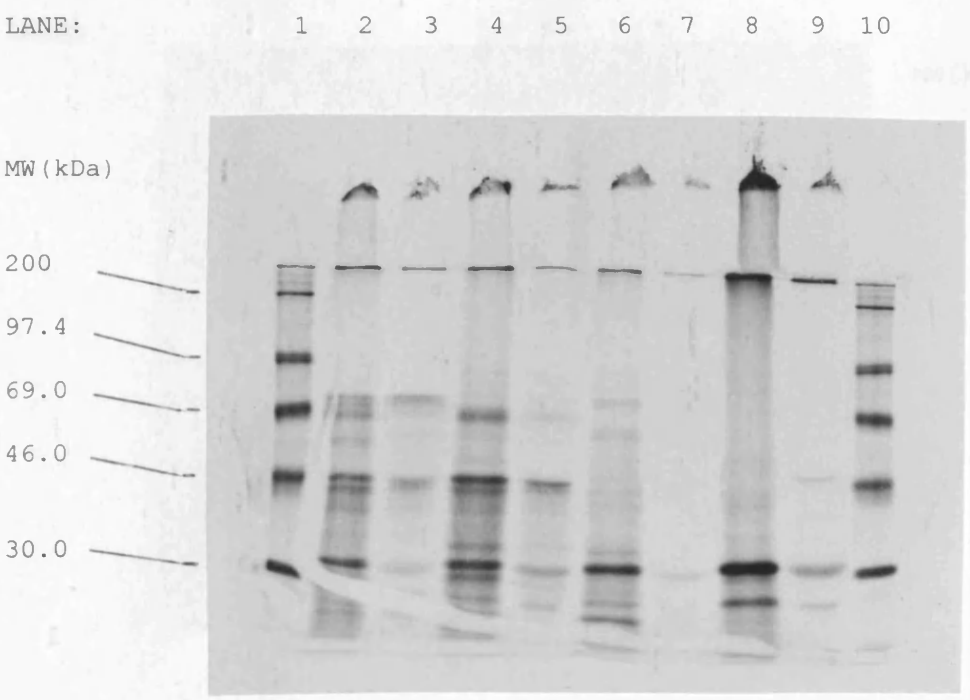


Figure 3.14

Figure to show proteins synthesized from clones pMK13, pMK14 and pMK3 and immunoprecipitated by convalescent antiserum. *In vitro* transcription/translation products and those *in vitro* transcription/translation products which were immunoprecipitated by convalescent antiserum were separated by SDS-PAGE [13% (w/v) acrylamide]. The gel was dried and exposed to autoradiographic film overnight at room temperature.

Lanes 1 and 10: protein molecular weight markers (sizes, in kDa, shown on the left-hand side); Lane 2: proteins synthesized from 0.05pmol pMK13; Lane 3: immunoprecipitated proteins from 0.1pmol pMK13; Lane 4: proteins synthesized from 0.05pmol pMK14; Lane 5: immunoprecipitated proteins from 0.1pmol pMK14; Lane 6: proteins synthesized from 0.05pmol pMK3; Lane 7: immunoprecipitated proteins from 0.1pmol pMK3; Lane 8: proteins synthesized from 0.05pmol pMK1.2; Lane 9: immunoprecipitated proteins from 0.1pmol pMK1.2.

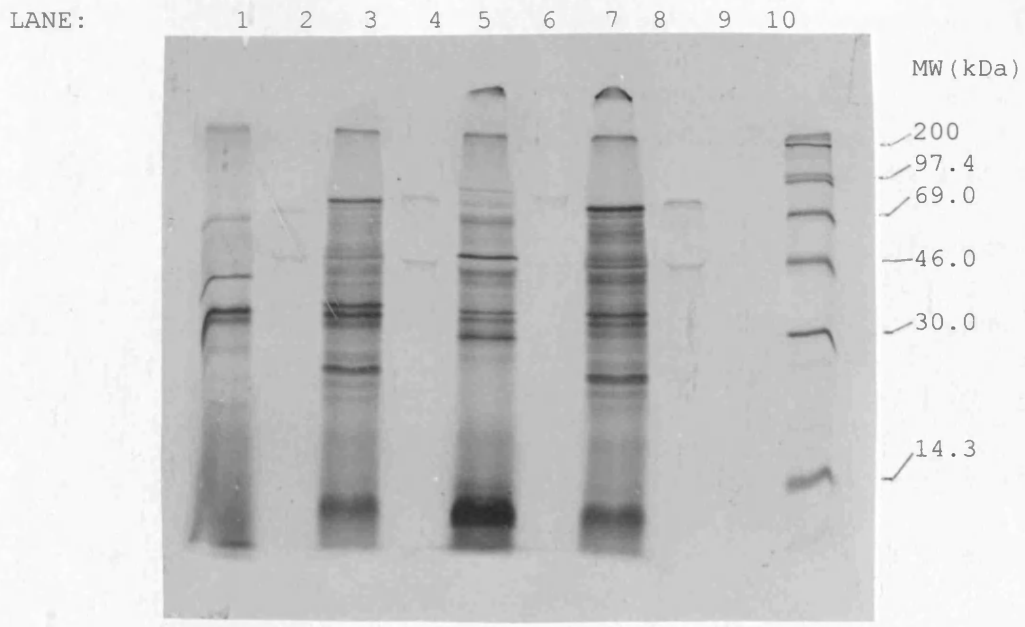


Figure 3.15

Figure to show proteins synthesized from clones pMK11, pMK12, pMK6 and pMK14 and immunoprecipitated by convalescent antiserum. Proteins produced by *in vitro* transcription/translation and those *in vitro* transcription/translation products which were immunoprecipitated by convalescent antiserum, were separated by SDS-PAGE [13% (w/v) acrylamide gel]. The gel was dried and exposed to autoradiographic film overnight at room temperature.

Lane 1: proteins synthesized from pMK11; Lane 2: immunoprecipitated proteins from pMK11; Lane 3: proteins synthesized from pMK12; Lane 4: immunoprecipitated proteins from pMK12; Lane 5: proteins synthesized from pMK14; Lane 6: immunoprecipitated proteins from pMK14; Lane 7: proteins synthesized from pMK6; Lane 8: immunoprecipitated proteins from pMK6; Lane 9: immunoprecipitated proteins from pUC19; Lane 10: protein molecular weight markers (sizes, in kDa, shown on the right-hand side).

Figure 3.16

Initial sequence analysis to determine whether *lacZ* fusion proteins could be synthesized by the positive clones and whether some of the clones contained identical 5′ or 3′ *C.jejuni* DNA sequence. This figure shows the first 38bp of *C.jejuni* DNA sequence, from the points of fusion with pBluescript SK-, in clones pMK11, pMK12, pMK6, pMK13 and pMK14.

"RP" denotes sequence obtained with the M13 reverse primer; "FP" denotes sequence obtained with the M13 forward primer; "*" denotes a stop codon in frame with the *lacZ* gene; "^" indicates point of fusion of *C.jejuni* H132 DNA with the pBluescript SK- vector; "//" indicates undetermined sequence between the 5′ and 3′ termini. Vector sequences are underlined.

pMK11

RP - CG A^TC CTA GTA ATA AAG ATT ATC TTC AAA AAA TAG ATA CGC // ATC CTG CAA CTG GAA ATA GTG GGG TTG TTG TTA CAG AG^T CG - 3′

3′ - GC T^AG GAT CAT TAT TTC TAA TAG AAG TTT TTT ATC TAT GCG // TAG GAC GTT GAC CTT TAT CAC CCC AAC AAC AAT GTC TC^A GC - **FP**

pMK12

RP - CG A^TC CAA ATG TAT ATC AAG AAA TAG GTA TTA ACA AGC TTT // AAT ATA AAT GAA ATA GTT ATA GAA GAA AAA AAT ACA GA^T CG - 3′

3′ - GC T^AG GTT TAC ATA TAG TTC TTT ATC CAT AAT TGT TCG AAA // TTA TAT TTA CTT TAT CAA TAT CTT CTT TTT TTA TGT CT^A GC - **FP**

pMK6

RP - CG A^TC CAA ATG TAT ATC AAG AAA TAG GTA TTA ACA AGC TTT // AAT ATA AAT GAA ATA GTT ATA GAA GAA AAA AAT ACA GA^T CG - 3′

3′ - GC T^AG GTT TAC ATA TAG TTC TTT ATC CAT AAT TGT TCG AAA // TTA TAT TTA CTT TAT CAA TAT CTT CTT TTT TTA TGT CT^A GC - **FP**

pMK13

RP - CG A^TC TTG ATA TAG CTA CGA TTA ATA TAT TAG AAG AAT ACT // TTA GGT AAA GAT ATA GTA GGC AAT GCT TTT GTA ACC GA^T CG - 3′

3′ - GC T^AG AAC TAT ATC GAT GCT AAT TAT ATA ATC TTC TTA TGA // AAT CCA TTT CTA TAT CAT CCG TTA CGA AAA CAT TGG CT^A GC - **FP**

pMK14

RP - CG A^TC CCA TGT GCA AAA CGA CTT TTT AAG CGT TCG GTT ATA // ATT TTT AGT ATT AGA GTC ATC ATC ACT TGG CTT AGA GA^T CG - 3′

3′ - GC T^AG GGT ACA CGT TTT GCT GAA AAA TTC GCA AGC CAA TAT // TAA AAA TCA TAA TCT CAG TAG TAG TGA ACC GAA TCT CT^A GC - **FP**

3.4 DISCUSSION

The previous chapter described the screening of a Lambda ZAP II expression library of *C.jejuni* DNA with human convalescent antiserum and the successful isolation of several positive clones. In this chapter, Southern blotting revealed that, out of six clones picked, four of them have overlapping inserts (pMK11, pMK12, pMK6 and pMK13) and one, pMK13, may also overlap with the end of the insert of a fifth positive clone, pMK14. Further Southern blots, using radiolabelled fragments of pMK14 as probes for restriction enzyme digests of pMK13, would verify the link between these two clones. It seems likely that the four overlapping clones could have an ORF in common, encoding the same protein which reacted with convalescent antiserum.

An important immunodominant antigen in *Campylobacter* species is the flagellin protein, which has a molecular weight of 60kDa, and antibodies against this protein are known to provide part of the convalecent host's protective immmune response against further infection with *Campylobacter* [62]. It was possible that the single locus common to the overlapping positive clones could be the *flaA* or *flaB* gene for flagellin (these genes show 95% sequence identity with each other), although it has been reported that *E.coli* is unable to express detectable levels of the protein [180]. In order to ascertain whether the flagellin gene was present in the positives, a radiolabelled fragment of the *flaA* gene was used as a probe for *Hin*dIII digests of the positive clones in a Southern blot experiment. The 2.7kb of *C.jejuni* DNA, which incorporated the first 700bp of *flaA* and 2kb of upstream sequence, was found not to hybridise with the *C.jejuni* DNA inserts. Therefore, none of the positive clones contained this first section of *flaA*, which included the promoter of the gene. Initial sequence analysis, using the M13 reverse primer, also revealed that the overlapping clones pMK11, pMK12, pMK6 and pMK13 could not have synthesized *lacZ* fusion proteins. Therefore, it was unlikely that any of the positives contained the rest of the *flaA* gene, since any immunogenic protein would have to be synthesized from its own *C.jejuni* promoter.

Restriction enzyme mapping enabled the detail of the overlapping sequences to be elucidated. Enzymes with recognition sites rich in A and T residues were found to cut most often in the *C.jejuni* DNA (as expected for DNA with only 30% to 33% G and C residues). *Hin*dIII digestion allowed a "fingerprint" of each clone to be compiled and adjacent *Hin*dIII fragments of 0.7kb and 1.2kb were found to be common to pMK11, pMK12, pMK6 and pMK13 (Figure 3.10). If the antiserum picked out these clones because they

produced the same immunogenic protein, then the ORF for that protein could be within these fragments.

In an effort to find out more about the immunogenic protein(s) synthesized by the positive clones, Western blotting was carried out, using the convalescent antiserum to try to detect immunoreactive proteins in whole cell lysates. No reproducible results were obtained with this method, however, the alternative method of *in vitro* transcription/translation and immunoprecipitation of the *in vitro* products using convalescent antiserum was successful. These results showed that the convalescent antiserum bound a 74kDa protein which was transcribed and translated from pMK12, pMK6 and pMK13 DNA. The antiserum also bound a 65kDa protein from pMK11 DNA and it was possible that this could have been a truncated version of the 74kDa protein synthesized by the other clones, since pMK11 contains the same sequences as pMK12 and pMK6, but the pMK11 insert ends at a *Sau*3A site approximately 50bp after the 1.2kb *Hin*dIII fragment (see Figure 3.10). A 69kDa protein produced by pMK14 DNA was also immunoprecipitated, but the unique 14kDa protein produced by pMK3 did not react with convalescent antiserum. pMK11 also produced a nonimmunogenic protein of 40kDa. If the 65kDa protein was a truncated version of the 74kDa product from pMK12, then the gene encoding the 40kDa protein could be situated upstream of the ORF for the 65kDa product, in the 1.4kb region which is not shared by the other clones.

Initial sequence analysis confirmed that pMK12 and pMK6 were separate isolates of the same clone and from now on both will be referred to as pMK12. Sequencing also revealed that most of the clones could not produce a *lacZ* fusion protein. However, pMK14 could have produced a fusion product and initial sequencing revealed the first 75 amino acids which may have been transcribed from the insert DNA, under the control of the *lacZ* promoter. No significant homologies were found when the database was screened with this peptide, further sequencing is necessary to obtain the rest of the gene. An *in vitro* transcription/translation experiment in which anti-β-galactosidase antiserum is used to detect the *lacZ* portion of fusion proteins would determine whether the pMK14 product which reacts with convalescent antiserum is the one being transcribed from the *lacZ* promoter. Since the Lambda ZAP II clones which were detected by convalescent antiserum were not synthesizing *lacZ* fusion proteins, they could contain genes for immunogenic proteins which are transcribed from their own *C.jejuni* promoters in *E.coli* strain XL1-Blue. Alternatively, internal *C.jejuni* translational starts could be functioning in *E.coli*, so that, although a *C.jejuni* promoter downstream of the *lacZ* gene would not produce

its own mRNA, a single long in-frame mRNA product synthesized from the *lacZ* promoter would still result in the translation of the *C.jejuni* protein.

Various difficulties had previously been reported with cloning and expressing *C.jejuni* genes in *E.coli* (see Introduction), but the results presented here support the theory that some *C.jejuni* promoters may be functional in *E.coli* cells [172]. The reported lack of stability of *Campylobacter* clones in *E.coli* has also not been observed with the positives isolated from the Lambda ZAP II library. The unique method of directly subcloning inserts from Lambda ZAP into pBluescript SK- and use of the XL1-Blue host strain, which is endonuclease and restriction deficient (but still retains the methylation system) and a *recA* mutant, seems to have been successful in facilitating the cloning of *Campylobacter* genes.

Further sequencing is necessary in order to find and analyse the ORFs in the positive clones. The gene common to pMK11, pMK12 and pMK13 could encode an immunogenic virulence determinant. The ORFs encoding the 40kDa (from pMK11), the 74kDa (from pMK11, pMK12 and pMK13) and the 69kDa protein (from pMK14) may even form a group of genes involved in virulence which are only expressed when environmental signals indicate to the *Campylobacter* cell that it is in a suitable host organism. The next chapter describes efforts made to obtain further sequence by transposon mutagenesis. The transposon used, Tn*phoA*, can also identify genes for secreted and transmembrane proteins.

CHAPTER FOUR

TN*PHOA* MUTAGENESIS TO DETECT *C.JEJUNI* GENES ENCODING PROTEINS WHICH CAN BE EXPORTED FROM *E.COLI*

4.1 INTRODUCTION

The last two chapters described the isolation of six *C.jejuni* DNA clones from a Lambda ZAP II library by screening with convalescent antiserum and the subsequent initial analysis of these clones. Four of the clones contained the same 2kb portion of *C.jejuni* DNA and one of them, pMK11, had 1kb more upstream sequence while another, pMK13, had 1kb more downstream sequence which may, in turn, overlap with one end of a further positive clone, pMK14. The overlapping clones produced a protein of 74kDa which was precipitated by convalescent antiserum, except for pMK11 which produced an immunogenic protein of 65kDa. It was thought likely that this 65kDa product was a truncated version of the 74kDa protein, since pMK11 contained more upstream sequence but less downstream sequence than the other clones. It was probable that the four overlapping clones had all been picked from the library because they all encoded the same antigenic determinant recognised by the convalescent antiserum. Convalescent antiserum will recognise proteins which are synthesized by *C.jejuni in vivo* and, hence, could be involved in virulence.

The pathogenic process of *Campylobacter* infection includes survival from gastric acid in the stomach, chemotaxis and motility into the mucus lining the intestinal wall and subsequent adhesion to and colonisation of the mucosa. These events could be facilitated by extracellular enzymes such as proteases and mucinases as well as surface structures such as flagella, pili, adhesins, invasins and outer membrane proteins. Ultimately, elaboration of secreted toxins, such as cytolethal distending toxin and other cytotoxins and possibly heat-labile enterotoxin, may occur. Thus, many different secreted protein factors could be utilised by *C.jejuni* during pathogenesis. As mentioned previously, the one major structural virulence factor to have been identified and genetically analysed in *C.jejuni* is the flagellum and the flagellin protein is immunodominant after infection. We know that the positive *C.jejuni* clones from the Lambda ZAP II library do not contain *flaA* or *flaB*, but it is hoped that they may contain a gene for some other virulence determinant. A transposon probe

for protein export signals, Tn*phoA*, can be used to help identify bacterial virulence determinants, since many (but not all) are exported from the bacterial cell.

Tn*phoA*, a derivative of Tn5, was constructed by Manoil and Beckwith [210] to study protein export in bacteria. Previously, Hoffman and Wright [211] had constructed a set of plasmids that could be manipulated *in vitro* to fuse the gene for the *E.coli* periplasmic protein alkaline phosphatase to different cloned genes. One of these plasmids was the source of the alkaline phosphatase gene used in the construction of *TnphoA* (represented schematically in Figure 4.1, p.113). The promoterless gene for *E.coli* alkaline phosphatase was inserted near the left end of IS50L in Tn5. The inserted *phoA* gene also lacks the region coding for the signal peptide which allows secretion of the alkaline phosphatase. When Tn*phoA* transposes into a gene, if it is in the correct reading frame, a hybrid protein can be produced which has alkaline phosphatase fused to the amino terminal sequence of the protein encoded by the disrupted gene. This hybrid protein exhibits no alkaline phosphatase activity, unless the target gene encodes a secreted or membrane-spanning protein, thus substituting for the missing signal sequence of the enzyme. Since alkaline phosphatase activity can be detected using media containing the indicator dye XP (5-bromo-3-chloro-indolyl phosphate), which produces a blue product in the presence of the active enzyme, Tn*phoA* mutagenesis is a relatively simple method of identifying genes encoding exported proteins. Tn*phoA* insertions can also be used to identify the position and orientation of cloned genes for exported proteins.

Broad-host-range delivery plasmids have been used successfully to introduce Tn*phoA* into other Gram-negative species, in addition to *E.coli*, and transposition of Tn*phoA* has allowed the identification of new genes encoding transmembrane and periplasmic proteins [for review see 212]. *Vibrio cholerae* genes involved in virulence have been identified using Tn*phoA* mutagenesis directly into the chromosome [213]. Insertion mutants with active *phoA* gene fusions were selected as colonies which appeared blue on media containing XP and, therefore, contained insertions in genes that encoded secreted proteins. Since many virulence determinants need to interact with host factors, most of them will be exported and Tn*phoA* can thus provide a strong enrichment for insertion mutations in virulence genes.

In the case of *Campylobacter*, however, not even the broad-host-range Tn5 class of transposons will transpose and this prevents the random Tn*phoA* mutagenesis of chromosomal genes *in vivo*. The alternative approach is to use Tn*phoA* to obtain fusions of *phoA* to potential virulence genes that have

been cloned onto plasmid vectors and transformed into *E.coli* host cells. Such an investigation was carried out on positive clones which were previously isolated from the Lambda ZAP II library of *C.jejuni* DNA and rescued into the pBluescript SK- phagemid. The kanamycin resistance gene on Tn*phoA* enabled us to avoid isolating insertions into the chromosome of the host cell, since high concentrations of kanamycin in the selective agar could be used to enrich for cells carrying transposon insertions into the multicopy phagemids. Temperature sensitive F' was used as a suicide vector to deliver the transposon to pMK3, pMK11 and pMK14. Incubation at 42°C resulted in loss of the F'ts on transfer of Tn*phoA* to the recombinant plasmid containing *C.jejuni* DNA.

The objective of this experiment is to use Tn*phoA* to identify and characterise any ORFs for exported proteins which may be present in the positive library clones. The first aim is to create Tn*phoA* mutants of pMK3, pMK11 and pMK14 which have exported alkaline phosphatase activity and determine the position and orientation of Tn*phoA* in the mutants. The protein products of the mutants can then be analysed to determine whether products which reacted with convalescent antiserum have been lost. Ultimately sequence analysis of the positive clones will be performed, using Tn*phoA* primers to define the ORFs that produced the exported proteins.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and vectors. Bacterial strain *E.coli* CC118 [Δ*phoA 20, araD139* Δ*(ara,leu)7697, ΔlacX74, galE, galK, thi, rpsE, rpoB, argE*$_{am}$, *recA1*] has no endogenous alkaline phosphatase activity, due to a deletion in the *phoA* gene. *E.coli* CC118(F'ts::Tn*phoA*) contains the temperature sensitive F' plasmid carrying the Tn*phoA* transposon and is, therefore, kanamycin resistant, but not at high kanamycin concentrations since F' is a single copy episome. The phagemid, pBluescript SK-, was the cloning vector into which the *C.jejuni* inserts were subcloned *in vivo* from Lambda ZAP II and *E.coli* XL1-Blue [*endA1, hsdR17* (rk-, mk+), *supE44, thi-1*, lambda-, *recA1, gyrA96, relA1, lac-*, [F', *proAB, lacIqZ*ΔM15, Tn10(*tet*^{R})]] from Stratagene was used as the host for cloning the library positives (as described in method 2.2.1).

4.2.2 Electroporation. Transformations were routinely carried out by electroporation. 1ml of overnight culture was used to inoculate 100ml of fresh LB broth (section 2.2.1) containing appropriate antibiotics [50μg/ml kanamycin for CC118(F'ts::Tn*phoA*) or 15μg/ml tetracycline for XL1-Blue]. This culture was incubated at 37°C with shaking at 200rpm, until it was at OD_{600} 0.5, when the cells were harvested by centrifugation at 3,900xg for 15min. at 4°C. The two pellets were each resuspended in 10ml of cold sdH_2O on ice and the centrifugation repeated. Each pellet was then resuspended in 1ml of cold sdH_2O on ice and centrifuged at 12,000xg for 30sec. at approximately 6°C. This washing and centrifugation step was repeated 3 times to remove as much salt as possible from the cell suspension. After the final centrifugation, the cells were resuspended in 300μl cold sdH_2O and placed on ice.

The electroporation apparatus (Bio-Rad Gene Pulser and Pulse Controller) was set at 250V and 1000 Ohms. 50μl of washed cell suspension was electroporated with 0.1-0.2μg of plasmid DNA (washed with 70% v/v ethanol to remove residual salt and resuspended in sdH_2O) and 1ml of SOC medium [20g/L tryptone, 5g/L yeast extract, 0.5g/L NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose; pH7.0] was added immediately afterwards. The cells were allowed to recover at 37°C for 1 hour with gentle shaking and dilutions were then plated onto selective media.

4.2.3 Isolation of Tn*phoA* insertions into multicopy plasmids. *E.coli* strain CC118(F'ts::Tn*phoA*) was transformed with the pBluescript SK- clones of *C.jejuni* DNA by electroporation, as described above. 200μl and 20μl

aliquots of recovered cells were plated onto LA plates (section 2.2.1) containing 100μg/ml ampicillin, and 50μg/ml kanamycin to select for CC118(F'ts::Tn*phoA*) cells containing pBluescript SK- clones, and incubated at 37°C overnight. Single colonies were then used to inoculate 5ml LB-AMP (method 3.2.1) and incubated at 37°C with shaking at 200rpm overnight. In order to isolate CC118 cells carrying the multicopy pBluescript SK- clones with Tn*phoA* inserted, 1ml, 100μl and 10μl aliquots of this overnight culture were plated onto selective indicator plates of LA containing 100μg/ml ampicillin, 300μg/ml kanamycin and 40μg/ml XP (LA-A6KXP plates). These plates contained a high concentration of kanamycin to enrich for cells carrying insertions of the transposon into the multicopy plasmid and were incubated overnight at 42°C to encourage loss of the single copy F'ts plasmid. The indicator, XP, allows the detection of alkaline phosphatase activity by turning blue under the action of this enzyme. The colour change indicates that active alkaline phosphatase is being produced under the control of a promoter for a gene encoding an exported protein present on the pBluescript SK- clone.

3.5ml LB broth was used to wash the colonies from each plate by rocking at 4°C for 30min. Alternatively, colonies were restreaked onto fresh selective plates and isolated single colonies were then used to inoculate 4ml LB broth containing 100μg/ml ampicillin and 50μg/ml kanamycin and incubated overnight at 37°C with shaking at 200rpm. DNA was isolated from these cells by the miniprep method in section 3.2.1 and was finally resuspended in 20μl sdH_2O. 1μl of each of these DNA samples was used to transform 50μl *E.coli* XL1-Blue or *E.coli* CC118 cells by electroporation (section 4.2.2). After recovery, these transformed cells were plated onto LA-A6KXP plates and incubated at 37°C overnight. Blue colonies and a few white controls were restreaked onto fresh LA-A6KXP plates. This extra re-transformation step ensures the loss of untransformed CC118(F'ts::Tn*phoA*) and insertions of the transposon into the chromosome of CC118 and, furthermore, helps ensure that no host cells contain a mixture of the original pBluescript SK- clone together with Tn*phoA*-mutated clones.

4.2.4 Mapping using restriction enzyme analysis and PCR. Restriction enzyme analysis was performed on DNA prepared from the Tn*phoA* mutants using enzymes and buffers supplied by Gibco-BRL Life Technologies Ltd., as detailed previously in section 3.2.4.

Further confirmation of the position of the inserted transposon was gained by PCR using primers designed against each end of Tn*phoA* and those hybridising with pBluescript SK- DNA. The amplification reactions were

carried out as follows: 10µl aliquots of 10mM Tris-HCl (pH 8.3), 50mM KCl, 10mM $MgCl_2$, 0.1mg/ml bovine serum albumin (BSA), 1mM each dNTP, 20pmol each primer, 0.05 Unit Taq DNA polymerase and 10ng template DNA were overlaid with 40µl mineral oil and transferred to a Techne PHC-3 programmable heating block. The reactions were subjected to 35 cycles which consisted of denaturing at 94°C for 1 min., primer annealing at 60°C for 1 min. and extension at 72°C for 3 min. A final cycle of 60°C for 2 min. and 72°C for 10 min. was carried out to complete the extension of any unfinished PCR products. The reactions were analysed by electrophoresis on a 1% (w/v) agarose gel.

4.3 RESULTS

4.3.1 Tn*phoA* mutagenesis of pBluescript SK- clones. Separate experiments were carried out in which each of the pBluescript SK- positive clones pMK11, pMK14 and pMK3 were transformed into *E.coli* strain CC118 (F'ts::Tn*phoA*). This transformation introduced the pBluescript SK- clones into the presence of the temperature sensitive F' plasmid which carries *TnphoA*. Transformed cells (see method 4.2.2) were isolated by plating onto LA plates containing both 100μg/ml ampicillin, to select for the pBluescript SK- clones, and 50μg/ml kanamycin, to maintain the presence of F'ts::Tn*phoA*. Colonies obtained after overnight growth at 37°C were then used to inoculate LB-AMP and grown overnight at 37°C. Glycerol stocks of the culture could be made at this stage and stored long term at -80°C.

In order to isolate transpositions of Tn*phoA* into the pBluescript SK- clones, dilutions of this culture were plated onto media containing 100μg/ml ampicillin and six times the standard concentration of kanamycin (as detailed in methods section 4.2.3) to select for CC118 cells in which Tn*phoA* had transposed into the multicopy pBluscript clone. The plates were incubated overnight at 42°C, a non permissive temperature for replication of the F'ts plasmid. These plates also contained XP, a chromogenic substrate which is turned blue in the presence of exported alkaline phosphatase. Each of the pBluescript SK- clones yielded several blue and many white colonies on these selective plates. However, at this stage each CC118 cell could contain a mixture of both pBluescript SK- clone and Tn*phoA* mutated clone, this was confirmed by preparing plasmids from a few of the blue colonies on these selective plates and analysing them by agarose gel electrophoresis. Each DNA preparation contained both the pBluescript SK- clone and a higher molecular weight species which was not present in the control lane of pBluescript SK- clone alone. It was also possible that, at this stage, some colonies were blue due to insertion of Tn*phoA* into the periplasmic β-lactamase protein for ampicillin resistance, transcribed from the *blaM* gene on pBluescript SK-. In order to produce a homogenous plasmid population of Tn*phoA*-mutated pBluescript SK- clones, with no Tn*phoA* insertions in *blaM*, it was necessary to make DNA from isolated colonies on selective plates and retransform this DNA into fresh *E.coli* CC118 cells. Dilutions of the transformation mixture were plated onto freshly made LA-A6KXP selective plates (as detailed in method 4.2.3) and incubated overnight at 37°C. Resulting blue and white colonies were restreaked onto fresh LA-A6KXP plates and plasmids were prepared from those which displayed stable colour inheritance.

Further analysis was carried out on two blue and two white Tn*phoA* mutants of pMK11, one blue and two white Tn*phoA* mutants of pMK14 and one blue pMK3 Tn*phoA* mutant. The blue Tn*phoA* mutants were numbered 1 and 2, e.g. pMK11.1 and pMK11.2, while the white Tn*phoA* mutants were mumbered 3 and 4, e.g. pMK11.3 and pMK11.4. Six white Tn*phoA* mutants of each of the subclones pMK1.2 and pMK0.7 were also isolated. These pBluescript subclones contained the 1.2kb and 0.7kb *Hin*dIII fragments which were common to the positive clones pMK11, pMK12 and pMK13 (as mentioned in the previous chapter). No stable blue Tn*phoA* mutants were obtained for either of the subclones, suggesting that neither of them contain an entire gene for a protein which could be exported from *E.coli*. However, it was possible that the translational start for the protein could lie just outside the subcloned DNA but still be present in all four of the positive library clones. Furthermore, it was not certain that *C.jejuni* export signals could function efficiently in *E.coli*, therefore, it was still possible that the positive clones all had an ORF for an immunogenic protein in common.

4.3.2 Mapping the Tn*phoA* mutants. Restriction enzymes were used to digest the DNA from each Tn*phoA* mutant and comparison with restriction enzyme digests of the original clones revealed the general position of Tn*phoA* within the mutant plasmids. The enzymes used for this preliminary mapping included *Eco*RV, which does not have a site in Tn*phoA*, and *Bam*HI, which cuts Tn*phoA* once, 4.97kb from the *phoA* end (see Figure 4.1). Restriction enzyme digests, however, could give only limited information on the position of Tn*phcA* in the mutants, especially since the *C.jejuni* DNA inserts of the plasmids did not have sites for many different restriction enzymes. In order to determine the exact position and orientation of Tn*phoA* within the mutant clones, PCR was the preferred technique.

The 15 nucleotide primer, PA1, with homology to the 5′ terminus of *phoA* had the sequence: 5′-CTGAGCAGCCCGGTT-3′. Figure 4.1 shows the binding site position of this primer, 77bp into the alkaline phosphatase coding region of Tn*phoA*. The 16 nucleotide primer, PB1, was designed against the other end of the transposon (79bp from the 3′ terminus of Tn*phoA*) and had the sequence: 5′-GTTAGGAGGTCACATG-3′ (see Figure 4.1). Figure 4.2 shows the sequences and positions of primers with binding sites on the pBluescript SK- vector. Primers SK and RP read towards the 5′ terminus of the *C.jejuni* insert, which was adjacent to the partial *lacZ* gene on pBluescript SK-, while the T7 and FP primers read towards the 3′ end of the insert. These primers were used in PCR reactions with PA1 or PB1 (see method 4.2.4) in order to determine the distance of Tn*phoA* in the *C.jejuni* insert from the flanking pBluescript SK- vector DNA.

PCR amplification of the two blue Tn*phoA* mutants of pMK11, pMK11.1 and pMK11.2, revealed that the distance between PA1 and the vector primer FP was approximately 0.4kb for pMK11.1 (Figure 4.3a, lane 2), while pMK11.2 had a distance of approximately 3.25kb between PB1 and the FP primer (Figure 4.3a, lane 3). Therefore, each of these blue Tn*phoA* mutants had the transposon inserted at opposite ends of the pMK11 insert and in opposite orientations. Two Tn*phoA* mutants, pMK11.3 and pMK11.4, which yielded white colonies on indicator media, were found to have the transposon inserted in the opposite orientation to the blue mutants. The distance between PA1 and FP was approximately 3.1kb for pMK11.3 (Figure 4.3a, lanes 4 and 5) and the distance between PB1 and FP was approximately 0.6kb for pMK11.4 (Figure 4.3a, lane 6). This result implies that there may be two ORFs in pMK11 which encode for proteins with export signals which function in *E.coli*. These genes appear to be situated at opposite ends of the *C.jejuni* DNA in pMK11 and in opposite orientations, both of them reading from the vector ends of the *C.jejuni* fragment towards the centre of the insert. Figure 4.4 shows the position and orientation of Tn*phoA* in each of the mutants.

Figure 4.3 also includes the PCR analysis of Tn*phoA* mutants of pMK14. The blue Tn*phoA* mutant of pMK14, pMK14.1, yielded a 0.25kb band in PCR amplification with primers PB1 and FP (Figure 4.3b, lane 2). This gives a distance of approximately 100bp from the 3′ end of Tn*phoA* to the pBluescript SK- vector. In pMK14.1 the transposon was, therefore, inserted with the alkaline phosphatase gene reading towards the FP end of pBluescript SK-. White Tn*phoA* mutant, pMK14.3, had the transposon inserted so that the distance between PA1 and FP was 0.25kb (Figure 4.3b, lanes 3 and 4), while the other white clone, pMK14.4, had Tn*phoA* inserted in the same orientation, approximately 100bp further into the *C.jejuni* insert DNA (Figure 4.3b, lanes 5 and 6). Thus, the mutants which had the transposon inserted so that the alkaline phosphatase was reading away from the FP end of pBluescript SK- were white, while the one which was reading from the 5′ end of the *C.jejuni* insert towards the FP end of pBluescript SK- was blue. Figure 4.5 summarises these results in a schematic diagram of the transposon insertions into pMK14.

PCR and restriction enzyme mapping of the blue Tn*phoA* mutant of pMK3, pMK3.1, revealed that the transposon had inserted in the position and orientation shown in Figure 4.6. The distance between the PA1 and RP primers was approximately 1.3kb, as revealed by PCR (Figure 4.3b, lane 7).

4.3.3 *In vitro* transcription/translation analysis. The technique of *in vitro* transcription/translation had previously been used successfully to show the

proteins which could be synthesized from the positive clones using an *E.coli* transcription/translation system. Immunoprecipitation with convalescent antiserum had also demonstrated that some of these proteins could be immunogenic to a human host (see results section 3.3.5). In order to determine whether the insertion of Tn*phoA* had disrupted any of the genes for these proteins, *in vitro* transcription/translation experiments were carried out with DNA isolated from some of the Tn*phoA* mutants. Immunoprecipitations with convalescent antiserum and with monoclonal anti-alkaline phosphatase antiserum were used to find out whether the proteins which bound convalescent antisera were also those which, when mutated with Tn*phoA*, could enable the export of alkaline phosphatase hybrid proteins. The methods used were described in sections 3.2.8 and 3.2.9 of the previous chapter.

***In vitro* transcription/translation of pMK11.1 and pMK11.2.** Figures 4.7 and 4.8 show the results of two experiments in which the Tn*phoA* mutants of pMK11 were used as templates in the *in vitro* transcription/translation reaction and immunoprecipitations of the products were performed. The pattern of protein synthesis showed that the blue Tn*phoA* mutant, pMK11.1, had lost the immunogenic 65kDa protein, suggesting that the transposon disrupted the ORF for this product. The immunogenic 40kDa product remained intact, as expected for pMK11.1, confirming previous conclusions that pMK11 contained two ORFs.

The anti-alkaline phosphatase monoclonal antibody appeared to bind a 47kDa product from pMK11.1, and it did not bind the same proteins as convalescent antiserum. Manoil and Beckwith [210] documented the appearance of a protein the size of mature alkaline phosphatase (47kDa) during investigations of *lacZ-phoA* fusions and concluded that this protein may have been a degradation product of the full-length hybrid that had lost β-lactamase sequences. One of the immunoprecipitation experiments with anti-alkaline phosphatase antiserum showed a doublet at 47/48kDa for pMK11.1, not just a single band, (Figure 4.8, lane 9) suggesting that both a hybrid protein of 48kDa and a degradation product of 47kDa could have been present in the *in vitro* transcription/translation sample. It can be concluded that pMK11.1 has Tn*phoA* inserted in frame near the beginning of the gene encoding the 65kDa immunogenic protein and it is possible that the resulting hybrid protein is approximately 48kDa in size.

pMK11.2 had Tn*phoA* inserted approximately 150bp into the opposite end of the *C.jejuni* DNA insert, but the SDS-PAGE did not show that pMK11.2 had lost any of the proteins which were produced by pMK11 (Figure 4.7, lane 6, and Figure 4.8, lane 12). However, an extra product of 60kDa was

synthesized by pMK11.2 which was not also produced by pMK11 or pMK11.1. The 60kDa band could have been a hybrid alkaline phosphatase protein, although the immunoprecipitation with anti-alkaline phosphatase antibody did not support this theory. The 60kDa protein was also not immunoprecipitated by convalescent antiserum. Since Tn*phoA* was inserted only approximately 150bp from the pBluescript SK- vector sequences in this case, a fusion protein of 60kDa would have to be transcribed from the *lacZ* promoter.

Bands which the two transposon mutants, pMK11.1 and pMK11.2, had in common included a 29kDa protein, that was most probably the product of the kanamycin resistance gene on *TnphoA*, and a band of approximately 45kDa, which bound the convalescent antiserum. It is not known at this stage whether the 45kDa protein bound by the convalescent antiserum in the *in vitro* transcription/translation samples was a product of the *C.jejuni* insert DNA, or a background product as detected previously (see Figure 3.13 and results section 3.3.5).

***In vitro* transcription/translation of pMK14.1 and pMK14.4.** Figure 4.9 shows the *in vitro* transcription/translation and immunoprecipitation results for the Tn*phoA* mutants of pMK14, pMK14.1 (blue) and pMK14.4 (white). The patterns of protein synthesis for both pMK14.1 and pMK14.4 differed from that of pMK14 without the transposon and they both demonstrated the loss of the 69kDa protein, which had been previously shown to have some reaction with convalescent antiserum (Figure 3.13 and results section 3.3.5). In pMK14.1, an extra band appeared at 75kDa, which also bound both convalescent and anti-alkaline phosphatase antiserum. The transposon in this mutant was positioned just 80bp from the end of the insert DNA and so did not completely disrupt the *C.jejuni* ORF into which it inserted. However, the 75kDa band had a lower molecular weight than would be expected for a complete hybrid with the 69kDa protein, since alkaline phosphatase alone is 47kDa in size. Therefore, if the 75kDa product was an alkaline phosphatase fusion protein, then the *C.jejuni* portion would be approximately 28kDa in size. pMK14.1 also had the 47kDa alkaline phosphatase band present among the *in vitro* transcription/translation reaction products, and both pMK14.1 and pMK14.4 had the kanamycin resistance protein from Tn*phoA* at 29kDa.

The white Tn*phoA* mutant, pMK14.4, lost the 69kDa protein and gained one of 26kDa. This result suggests that the *C.jejuni* sequence upstream of the transposon encoded 26kDa of protein and, therefore, corroborates the evidence obtained with pMK14.1, which has Tn*phoA* inserted approximately

100bp from the point of insertion for pMK14.4 and apparently synthesized a fusion protein containing approximately 28kDa of *C.jejuni* product.

***In vitro* transcription/translation of pMK3.1.** Figures 4.10 and 4.11 (courtesy of Dr. Anne Wood) show the results from *in vitro* transcription/translation experiments with a blue Tn*phoA* mutant of pMK3. Comparison of the patterns of protein synthesis from pMK3 and the blue Tn*phoA* mutant, pMK3.1, (Figure 4.10) reveal that pMK3.1 lost a 23kDa product but gained a band at approximately 60kDa. This suggested that the transposon inserted into a *C.jejuni* ORF for a protein of approximately 23kDa, resulting in a 60kDa fusion product. It had been previously thought that the 23kDa product was a background band (see Figure 3.12 and results section 3.3.5), but the Tn*phoA* mutagenesis showed that this was not the case for pMK3. *In vitro* transcription/translation products from pMK3.1 also included the 29kDa kanamycin resistance protein from Tn*phoA* and the 47kDa band corresponding to the alkaline phosphatase moiety of the fusion protein.

The 60kDa alkaline phosphatase fusion protein and the 47kDa breakdown product were immunoprecipitated by anti-alkaline phosphatase antiserum, but there was no reaction of any original or mutated products with convalescent antiserum (see Figure 4.11). This confirms the previous evidence which suggested that pMK3 did not synthesize a protein that could bind convalescent antiserum under these reaction conditions (results section 3.3.5).

4.3.4 Sequencing. The Tn*phoA* transposon mutagenesis showed successfully that the positive clones, which had originally been isolated from a Lambda ZAP II library due to their apparent recognition by convalescent antiserum, could synthesize exported/transmembrane proteins in *E.coli*. *In vitro* transcription/translation and immunoprecipitation determined the sizes of proteins which had been disrupted by the transposon and the position and orientation of Tn*phoA* within the mutants was found using PCR. The ultimate analysis which had to be performed was sequencing of the positive clones and Tn*phoA* mutants. DNA sequencing using primer PA1 would enable the ORFs into which Tn*phoA* had inserted to be found and the amino acid sequences in the proteins encoded by those ORFs could also then be deduced. Comparison with known DNA and amino acid sequences in the databases may reveal what type of proteins and their genes had been isolated from *C.jejuni* using these methods.

The primer PA1 was used to determine the sequences from the alkaline phosphatase encoding end of the transposon into the positive clone DNA and primer PB1 was designed against the other end of the transposon, to enable

the sequence of *C.jejuni* insert DNA to be continued further and to verify the position of Tn*phoA*. Vector primers FP, T7, RP and SK were also used to confirm sequences which were close to these ends of the *C.jejuni* DNA inserts. Primers were then designed using sequence information already obtained to enable the extension of sequence data further into the *C.jejuni* DNA inserts and also to confirm the sequencing already performed by giving the complementary DNA sequences of the opposite strands.

Sequence analysis of pMK11. DNA sequence of one of the blue Tn*phoA* mutants of pMK11, pMK11.2, revealed that the transposon had inserted in frame into a putative gene for an ATP-binding transport protein. Approximately 727bp of DNA sequence from the fusion point with the RP end of pBluescript SK- have been obtained to date, but the complementary strand has not been confirmed for the furthest 200bp from the vector. Approximately 242 amino acids have been deduced from the sequence of the putative open reading frame, ORF11.2, and the protein was designated PEP11.2. Figure 4.12 shows the sequence data and deduced amino acid sequence. On searching the database for similar protein sequences, PEP11.2 was found to have 33% identity (and 52% similarity) with the *Agrobacterium tumefaciens* multi-drug resistance ChvD protein, 30% identity with the *Staphylococcus epidermidis* erythromycin resistance protein, 29% identity with the *Streptomyces fradiae* tylosin resistance protein and similarity with bacterial oligopeptide transport proteins. As can be seen in Figure 4.13, showing the alignment of ChvD with the ORF11.2 deduced amino acid sequence (PEP11.2), the similarity is lost after amino acid 203 in PEP11.2 (ChvD consists of 286 amino acid residues). It is possible that a sequence error caused this apparent loss of similarity, since the last 200bp of sequence have not yet been confirmed. Notable areas of conservation between PEP11.2 and ChvD include regions 50-LSGGE-54 and 70-ILLLDEPT-77 (numbering corresponds to the amino acid positions in PEP11.2). These regions are conserved throughout the family of ATP-binding transport proteins and constitute part of a potential ATP-binding domain [214, 215].

The sequence which apparently gives active, exported, alkaline phosphatase is not in frame with the partial *lacZ* gene on pBluescript SK-. There is a deduced methionine residue encoded by ATG bases, 43bp into the *C.jejuni* insert DNA, but no apparent Pribnow box or Shine-Dalgarno sequence before the fusion point with the vector. The peptide also lacks a signal sequence, or any hydrophobic regions long enough to cross the bacterial membrane. Therefore, it is not clear how the active alkaline phosphatase was produced from pMK11.2 to give blue colonies on selective media containing XP.

Similarly, sequence data for the other blue transposon mutant of pMK11, pMK11.1, revealed that there could be 35 *C.jejuni* amino acids upstream of the alkaline phosphatase gene, but these do not form part of an ORF and contain no possible methionine or valine start codon. This result implies that the Tn*phoA* mutant cannot synthesize active alkaline phosphatase, but the clone has been replated onto selective media containing the XP substrate (LA-A6KXP) and it has been verified that blue colonies are produced. However, the transposon is inserted in an ORF reading in the opposite direction, towards the pBluescript SK- vector. Figure 4.14 shows the sequence obtained to date of this ORF, ORF11.1a, and a further 773bp of upstream sequence. In total, 2414bp of *C.jejuni* DNA sequence have been obtained, from the *Sau*3A site at which the *C.jejuni* DNA insert of clone pMK13 is known to start (see Figure 3.10 and Figure 4.17), to the fusion point of the 3′ end of the pMK11 insert with the pBluescript SK- vector. Screening the database with the 2414bp of *C.jejuni* DNA sequence revealed that bases 1822 to 1895 are 91% identical with the *E.coli* gene *glyW*, which encodes tRNA-gly. Figure 4.15 shows the alignment of the *C.jejuni* region which encodes tRNA-gly with *E.coli glyW*.

ORF11.1a, into which Tn*phoA* is inserted in pMK11.1, encodes 282 amino acids from *C.jejuni* DNA, plus a possible further 10 amino acids from the pBluescript SK- vector, and begins with a methionine residue. Comparison of the possible peptide product of this ORF, PEP11.1a, with amino acid sequences in the database does not reveal significant similarity with any known proteins. *In vitro* transcription/translation analysis demonstrated that, in pMK11.1, Tn*phoA* had disrupted the gene for a 65kDa product, therefore, further DNA sequence was sought upstream of the ORF for PEP11.1a. Another ORF was discovered, reading in the same direction as that encoding PEP11.1a, and is shown as ORF11.1b in Figure 4.14. 184 amino acids (PEP11.1b) were deduced from the new sequence, beginning with a methionine residue encoded by the ATG codon at 1533bp from the fusion point with the FP end of pBluescript SK- (refer to the sequence data in Figure 4.14). There is a ribosome binding site sequence, AGGA, in an optimal position 9bp upstream of the ATG codon and a typical σ^{54} promoter, $TTGGN_{10}GC$, commences at 1604bp. Searching the database with the deduced amino acid sequence revealed that PEP11.1b had 28% identity (and 46% similarity) with *Campylobacter coli* flagellins A and B and 27% identity with the *C.jejuni* flagellins. Figure 4.16 shows the alignment of PEP11.1b with *C.coli* strain VC167-T2 flagellin. According to the motifs program on the GCG molecular biology package at Leicester University, there is no consensus signal sequence in the first 50 amino acids of the peptide, although there is a chain of 28 uncharged amino acid residues from Leu^{7} to Leu^{34}. Therefore,

similarly to PEP11.2, it is not clear how the alkaline phosphatase hybrid protein is being exported from the *E.coli* host.

Further sequence upstream of ORF11.1a had been sought because Tn*phoA* had caused the loss of a 65kDa product in pMK11.1. Another open reading frame (ORF11.1b) was found, but, even if ORF11.1b continued through ORF11.1a to the pBluescript SK- vector (FP end), it could not synthesize a protein of more than 60kDa. Therefore, the identity of the 65kDa product is still unclear. Figure 4.17 summarises the results obtained from sequence analysis of pMK11 and its Tn*phoA* mutants, in a diagram to show the positions of Tn*phoA* insertion and possible ORFs.

Sequence analysis of pMK14. Sequencing the blue Tn*phoA* mutant of pMK14, pMK14.1, revealed that the transposon had inserted into a potential ORF, with the alkaline phosphatase gene in frame with the *C.jejuni* coding sequence. Figure 4.18 shows 898bp of pMK14 insert DNA sequence and the deduced amino acid sequence of PEP14.1, which could be encoded by the revealed ORF. There is an ATG start codon from base 751 to 749 and a well-positioned putative ribosome binding site, AGGA, begins 10bp upstream. Similarly to PEP11.1b, there are five consecutive A residues between the ATG and AGGA sequences. Searching the database with the deduced sequence of 314 amino acids revealed 36.7% identity of amino acid residues 13 to 250 with residues 34 to 274 of *Saccharomyces cerevisiae* Met5 protein [o-acetylhomoserine(thiol)-lyase] and 33% identity of residues 34 to 234 with residues 12 to 252 of the 386 amino acid *E.coli* protein MetB, cystathionine γ-synthase [o-succinylhomoserine(thiol)-lyase]. The alignment of PEP14.1 with *E.coli* MetB is shown in Figure 4.19. The MetB protein catalyses the second step in methionine biosynthesis and is encoded by the *metB* gene. In *E.coli* the MetB protein is cytoplasmic, which would not generate active alkaline phosphatase in an in-frame fusion with *TnphoA*. However, according to the GCG motifs program, the amino acid sequence GIPVASGMAAC, which incorporates residues 59 to 69 in PEP14.1, is a possible prokaryotic membrane lipoprotein lipid attachment site. This sequence can be recognised by a specific lipoprotein signal peptidase, which then cleaves the protein in front of the cysteine (C) residue, to which a glyceride fatty acid lipid becomes attached. Such a post-translational modification so far into this protein seems unlikely, however, since the signal peptide for such a modification would usually be found before the thirtyfifth amino acid residue of a protein. Nevertheless, there is a significant region of hydrophobicity (according to the Chou-Fasman prediction) from amino acid Leu^{37} to Asn^{87}, which may form one or more membrane spanning domains.

Sequence analysis of pMK3. Figure 4.20 shows the DNA sequence analysis of the blue Tn*phoA* mutant of pMK3 (courtesy of Dr. Wood), which revealed that the transposon had inserted in frame into an ORF of approximately 680bp, which could encode a protein of 23kDa, as confirmed by *in vitro* transcription/translation. The deduced amino acid sequence was not found to have significant identity with any peptide sequences in the database, but, according to the motifs program on the Leicester University GCG software, the sequence 4-NAKFFGKKSRGKS-16 is an ATP-binding site motif. Without knowing the function of the rest of the protein, the significance of this motif is unclear.

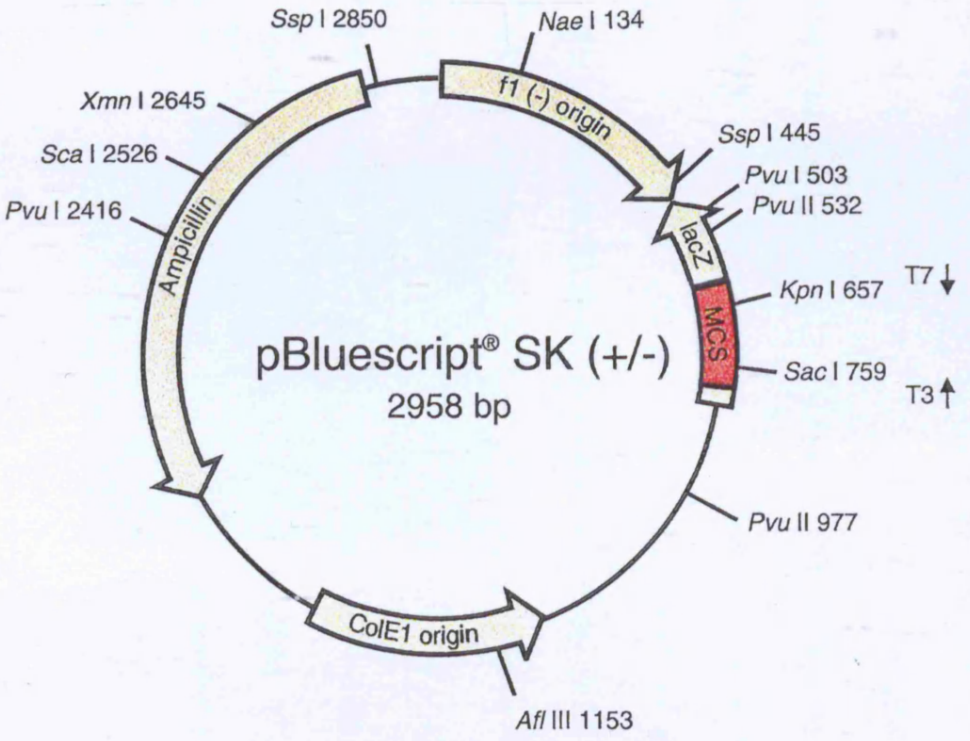
Figure 4.1

Schematic representation of the Tn*phoA* transposon, showing restriction enzyme sites and primer binding sites of PA1 and PB1, which were used in PCR mapping and sequence analysis. The truncated alkaline phosphatase gene, which lacks a promoter and N-terminal protein export sequences, is shown in blue and the kanamycin resistance gene, which allows selection for cells carrying the transposon, is shown in yellow. $IS50_L$ and $IS50_R$ are shown as black boxes, representing the left-hand and right-hand insertion sequences which contain the repeat sequences that allow transposition to occur. The size of Tn*phoA*, in kb, is shown across the top of the figure.

0
1
2
3
4
5
6
7
7.73
PB1
phoA
IS 50L
Kan'
IS 50R
PA1
ClaI-0.61kb
PvuII-0.62kb
EcoRI-0.77kb
ClaI-0.87kb
ClaI-1.01kb
EcoRI-1.1kb
XhoI-2.4kb
PstI-2.6kb
HindIII-3.1kb
PvuII-3.34kb
BglII-3.43kb
PstI-3.65kb
PvuII-3.7kb
PvuII-4.46kb
PstI-4.57kb
XhoI-4.75kb
BamHI-4.97kb
BglII-6.21kb
PvuII-6.31kb
HindIII-6.5kb
PstI-7.05kb
XhoI-7.24kb
HincII-7.55kb

Figure 4.2

Diagram of the pBluescript SK- phagemid vector (adapted from the 1997/1998 Stratagene catalogue), showing the sequence of the multiple cloning site and binding sites of the primers used in PCR mapping and sequence analysis. Note that the f1 origin is in the "-" orientation in pBluescript SK- and the *C.jejuni* H132 DNA was cloned into the *Xho*I site. The M13 forward primer is referred to as the M13 -20 primer in this figure.



Reverse primer
5' GGAAACAGCTATGACCATG 3'

T3 primer
5' AATTAACCCTCACTAAAGGG 3'

SK primer
5' CGCTCTAGAACTAGTGGATC 3'

BstX I Eag I Spe I Sma I EcoR I
MET T3 promoter +1 → Sac I Sac II Not I Xba I BamH I Pst I

5' GGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
3' CCTTTGTCGATACTGGTACTAATGCGGTTCGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAA

816
β-Galactosidase →

759

Bsp106 I Cla I Hinc II Acc I Eco0109 I Dra II
EcoR V Hind III Sal I Xho I Apa I Kpn I

AATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAA 3' (+)
GCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAATGTT 5' (-)

657
← +1 T7 promoter

3' CTATGGCAGCTGGAGCT 5'
KS Primer

3' CGGGATATCACTCAGCATAATG 5'
T7 Primer

3'TGACCGGCAGCAAAATG 5'
M13 -20 Primer

Figure 4.3

PCR mapping to find position and orientation of Tn*phoA* in pBluescript SK- clones pMK11.1, pMK11.2, pMK11.3, pMK11.4, pMK14.1, pMK14.3, pMK14.4 and pMK3.1. PCR samples were electrophoresed at 90V on 1% (w/v) agarose gels.

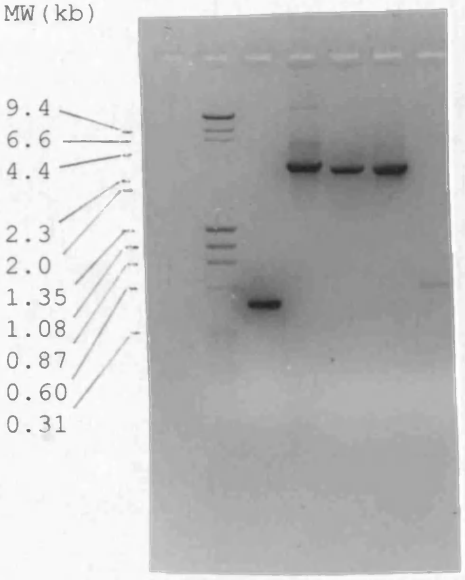
A) Lane 1: λ *Hin*dIII and ϕX174 *Hae*III molecular weight markers (sizes, in kb, shown on left-hand side); Lane 2: pMK11.1 amplified with PA1 and FP; Lane 3: pMK11.2 amplified with PB1 and FP; Lanes 4 and 5: pMK11.3 amplified with PA1 and FP; Lane 6: pMK11.4 amplified with PB1 and FP.

B) Lanes 1 and 9: λ *Hin*dIII and ϕX174 *Hae*III molecular weight markers (sizes, in kb, shown on left-hand side); Lane 2: pMK14.1 amplified with PB1 and FP; Lanes 3 and 4: pMK14.3 amplified with PA1 and FP; Lanes 5 and 6: pMK14.4 amplified with PA1 and FP; Lane 7: pMK3.1 amplified with PA1 and FP; Lane 8: pBluescript amplified with PA1 and FP (negative control).

A)

LANE: 1 2 3 4 5 6

MW(kb)



B)

LANE: 1 2 3 4 5 6 7 8 9

MW(kb)

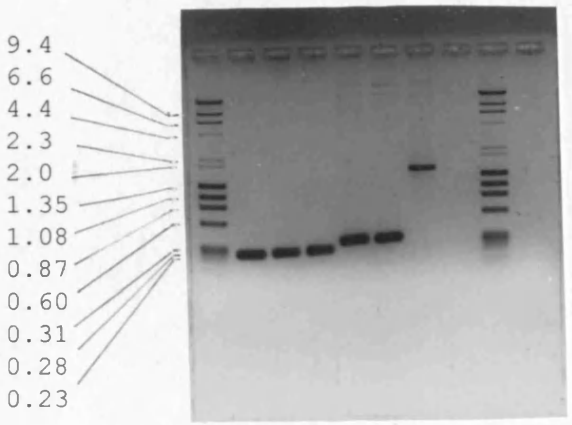


Figure 4.4

Diagram to show position and orientation of Tn*phoA* in each of the transposon mutants of pMK11. The distance between the forward primer (FP) and Tn*phoA* is shown in red, while the distance to the reverse primer (RP) is shown in black. The genes carried by pBluescript and their direction of transcription are indicated as arrows and the partial *lacZ* gene flanking the *C.jejuni* insert DNA is shown in dark blue. The partial alkaline phosphatase gene carried by Tn*phoA* is indicated as a light blue box for blue Tn*phoA* mutants and a white box for white Tn*phoA* mutants.

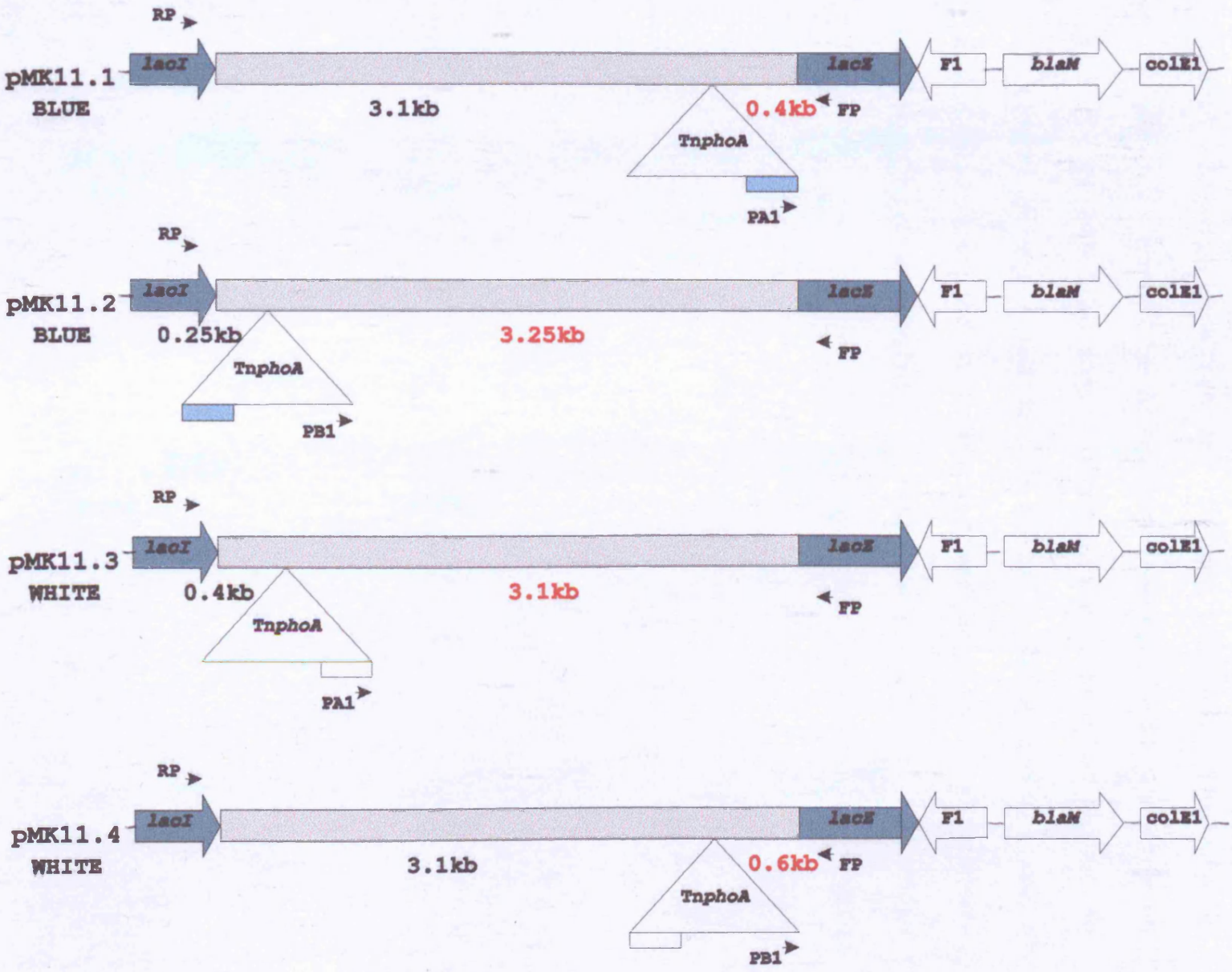

RP
pMK11.1
BLUE
lacI
3.1kb
lacZ
0.4kb
FP
TnphoA
PA1
F1
blaM
colE1
RP
pMK11.2
BLUE
lacI
0.25kb
TnphoA
PB1
3.25kb
lacZ
FP
F1
blaM
colE1
RP
pMK11.3
WHITE
lacI
0.4kb
TnphoA
PA1
3.1kb
lacZ
FP
F1
blaM
colE1
RP
pMK11.4
WHITE
lacI
3.1kb
TnphoA
PB1
lacZ
0.6kb
FP
F1
blaM
colE1

Figure 4.5

Diagram to show position and orientation of Tn*phoA* in the transposon mutants of pMK14. The distance from the forward primer (FP) to the primer indicated on Tn*phoA* is shown in red, while the distance from the reverse primer (RP) is shown in black. The partial alkaline phosphatase gene on Tn*phoA* is shown as a light blue box for the blue Tn*phoA* mutant and a white box for the white mutants. The pBluescript genes are also represented (as in Figure 4.4).

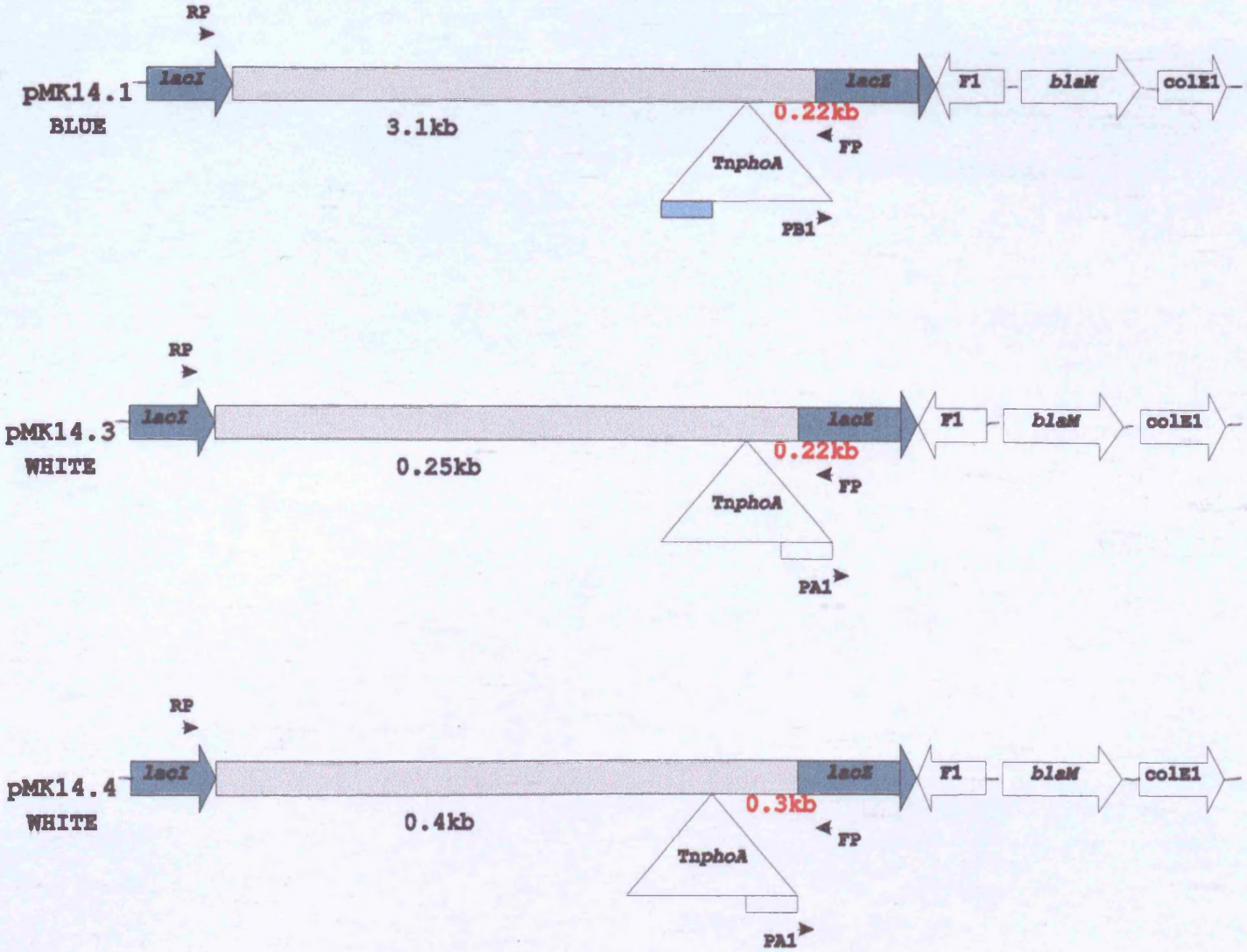

RP
pMK14.1
BLUE
lacI
lacZ
F1
blaM
colE1
3.1kb
0.22kb
FP
TnphoA
PB1
RP
pMK14.3
WHITE
lacI
lacZ
F1
blaM
colE1
0.25kb
0.22kb
FP
TnphoA
PA1
RP
pMK14.4
WHITE
lacI
lacZ
F1
blaM
colE1
0.4kb
0.3kb
FP
TnphoA
PA1

Figure 4.6

Diagram to show the position and orientation of Tn*phoA* in the blue tranposon mutant of pMK3, pMK3.1. The distance from Tn*phoA* primer PA1 to vector primer RP is shown in red, while the distance to the forward primer (FP) is shown in black. As in Figures 4.4 and 4.5, the partial alkaline phosphatase gene is represented as a light blue box.

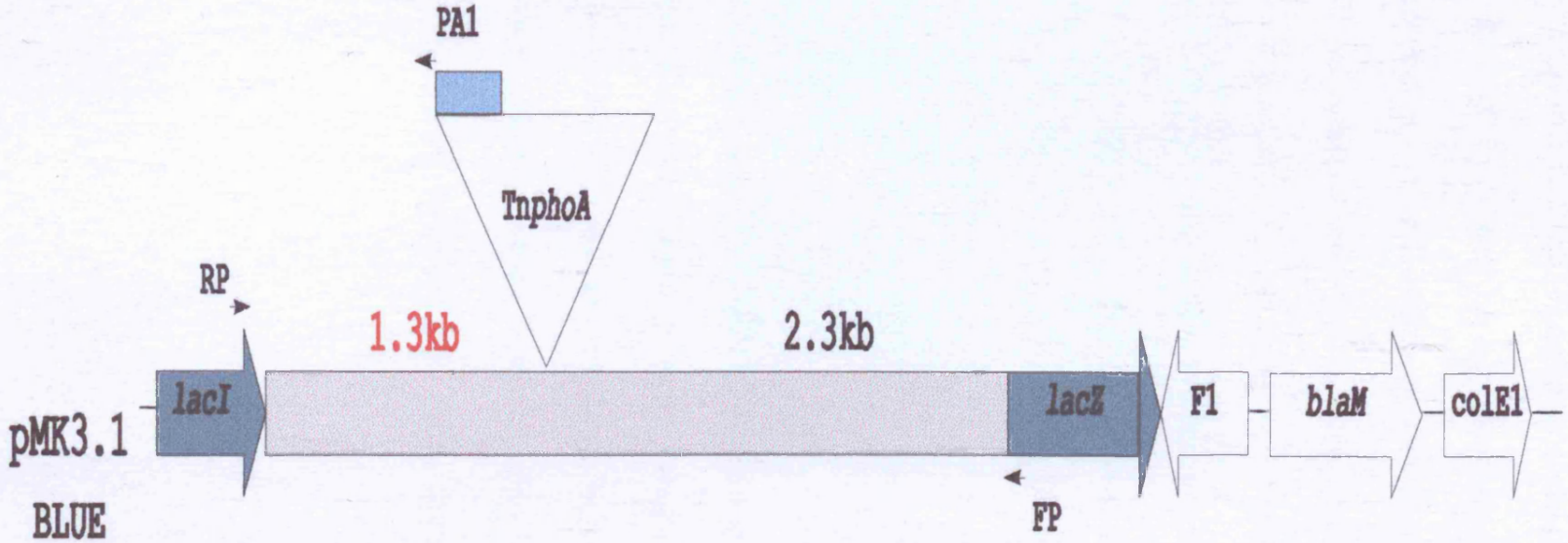

PA1
TnphoA
RP
1.3kb
2.3kb
lacI
lacZ
F1
blaM
colE1
pMK3.1
BLUE
FP

Figure 4.7

In vitro transcription/translation and immunoprecipitation analysis of Tn*phoA* mutants of pMK11. Reaction products were separated by SDS-PAGE on a 13% (w/v) acrylamide gel, which was then dried and exposed to autoradiographic film overnight.

Lane 1: protein molecular weight markers (sizes, in kDa, shown on left-hand side); Lane 2: proteins synthesized from 0.05pmol pMK11; Lane 3: proteins synthesized from 0.05pmol pMK11.1; Lane 4: proteins synthesized from 0.1pmol pMK11.1 and immunoprecipitated with convalescent antiserum; Lane 5: proteins synthesized from 0.1pmol pMK11.1 and immunoprecipitated with α-alkaline phosphatase antiserum; Lane 6: proteins synthesized from 0.05pmol pMK11.2; Lane 7: proteins synthesized from 0.1pmol pMK11.2 and immunoprecipitated with convalescent antiserum; Lane 8: proteins synthesized from 0.1pmol pMK11.2 and immunoprecipitated with α-alkaline phosphatase antiserum.

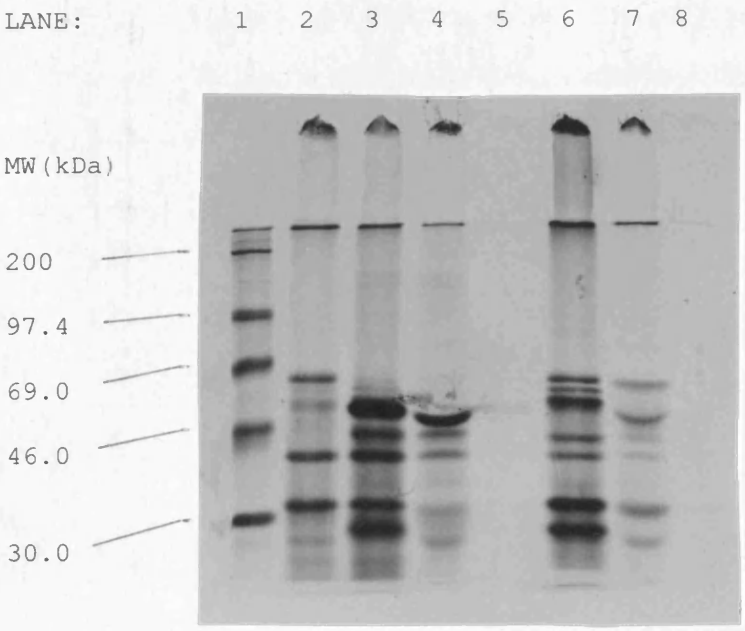

LANE: 1 2 3 4 5 6 7 8
MW(kDa)
200
97.4
69.0
46.0
30.0

Figure 4.8

In vitro transcription/ translation and immunoprecipitation analysis of Tn*phoA* mutants of pMK11. Reaction products were separated by SDS-PAGE on a 13% (w/v) acrylamide gel, which was dried and incubated overnight with autoradiographic film.

Lanes 1, 2 and 15: protein molecular weight markers (sizes, in kDa, shown on left-hand side); Lane 3: proteins synthesized from 0.05pmol pBluescript; Lane 4: α-alkaline phosphatase immunoprecipitated proteins from 0.1pmol pBluescript (negative control); Lane 5: convalescent antiserum immunoprecipitated proteins from 0.1pmol pBluescript (negative control); Lane 6: proteins synthesized from 0.05pmol pMK11; Lane 7: α-alkaline phosphatase immunoprecipitated proteins from 0.1pmol pMK11; Lane 8: convalescent antiserum immunoprecipitated proteins from 0.1pmol pMK11; Lane 9: proteins synthesized from 0.05pmol pMK11.1; Lane 10: α-alkaline phosphatase immunoprecipitated proteins from 0.1pmol pMK11.1; Lane 11: convalescent antiserum immunoprecipitated proteins from 0.1pmol pMK11.1; Lane 12: proteins synthesized from 0.05pmol pMK11.2; Lane 13: α-alkaline phosphatase immunoprecipitated proteins from 0.1pmol pMK11.2; Lane 14: convalescent antiserum immunoprecipitated proteins from 0.1pmol pMK11.2.

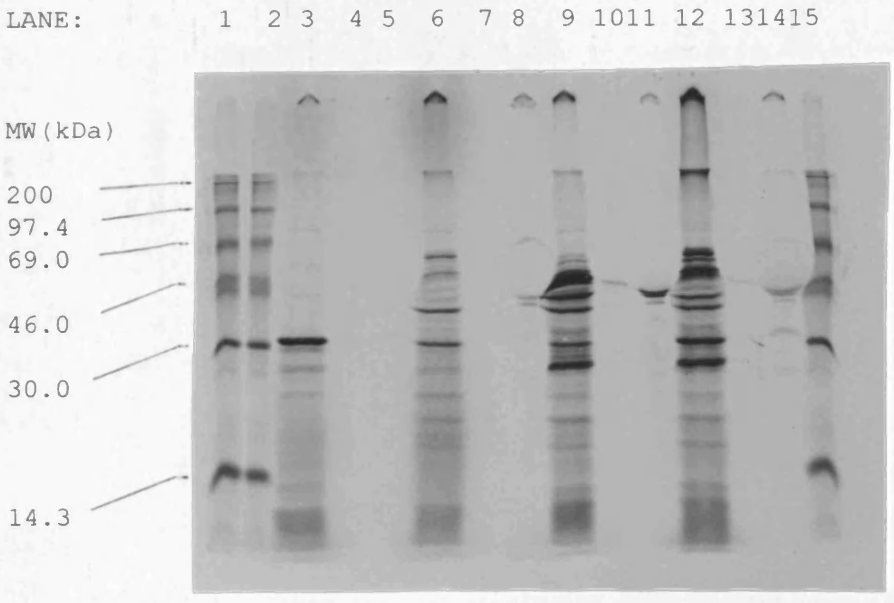
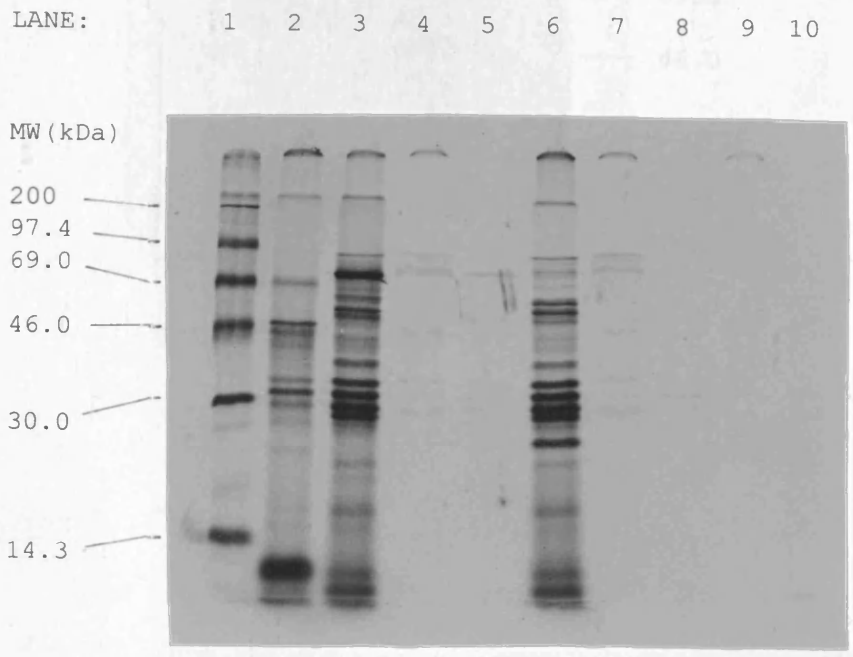

LANE: 1 2 3 4 5 6 7 8 9 1011 12 131415
MW(kDa)
200
97.4
69.0
46.0
30.0
14.3

Figure 4.9

In vitro transcription/ translation analysis of the Tn*phoA* mutants of pMK14. Reaction products were separated by SDS-PAGE on a 13% (w/v) acrylamide gel, which was dried and incubated overnight with autoradiographic film.

Lane 1: protein molecular weight markers (sizes, in kDa, shown on left-hand side); Lane 2: proteins synthesized from 0.05pmol pMK14; Lane 3: proteins synthesized from 0.05pmol pMK14.1; Lane 4: proteins from 0.1pmol pMK14.1 immunoprecipitated by convalescent antiserum; Lane 5: proteins from 0.1pmol pMK14.1 immunoprecipitated by α-alkaline phosphatase antiserum; Lane 6: proteins synthesized from 0.05pmol pMK14.4; Lane 7: proteins from 0.1pmol pMK14.4 immunoprecipitated by convalescent antiserum; Lane 8: proteins from 0.1pmol pMK14.4 immunoprecipitated by α-alkaline phosphatase antiserum; Lane 9: proteins from 0.1pmol Tn*phoA* mutant of pMK1.2 immunoprecipitated by convalescent antiserum (negative control); Lane 10: proteins from 0.1pmol Tn*phoA* mutant of pMK1.2 immunoprecipitated by α-alkaline phosphatase antiserum (negative control).


LANE: 1 2 3 4 5 6 7 8 9 10
MW(kDa)
200
97.4
69.0
46.0
30.0
14.3

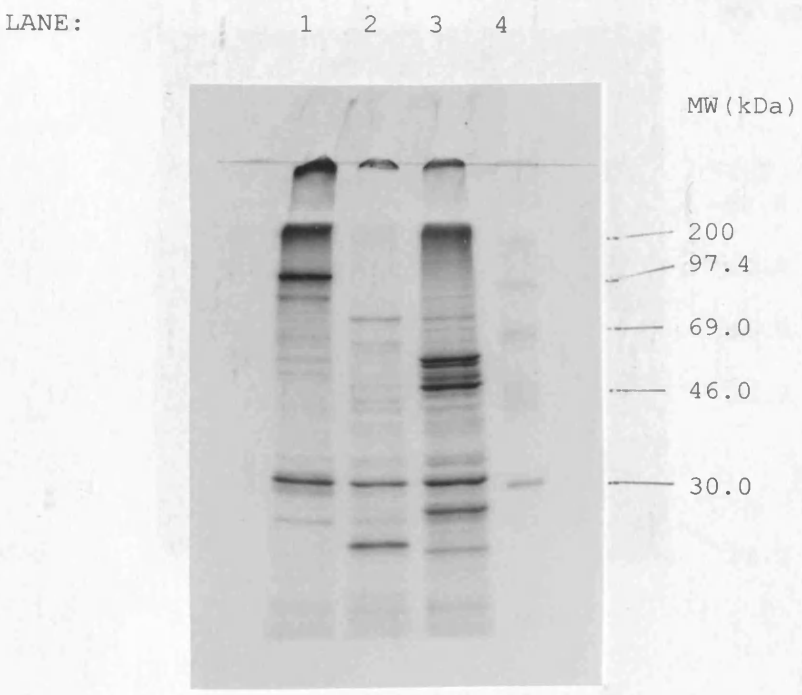


Figure 4.10

In vitro transcription/ translation analysis of the blue Tn*phoA* mutant of pMK3, pMK3.1. Reaction products were separated by SDS-PAGE on a 12% (w/v) acrylamide gel, which was dried and exposed to autoradiographic film overnight.

Lane 1: proteins synthesized from pGEM (plasmid similar to pBluescript); Lane 2: proteins synthesized from pMK3; Lane 3: proteins synthesized from pMK3.1; Lane 4: protein molecular weight markers (sizes, in kDa, shown on right-hand side).

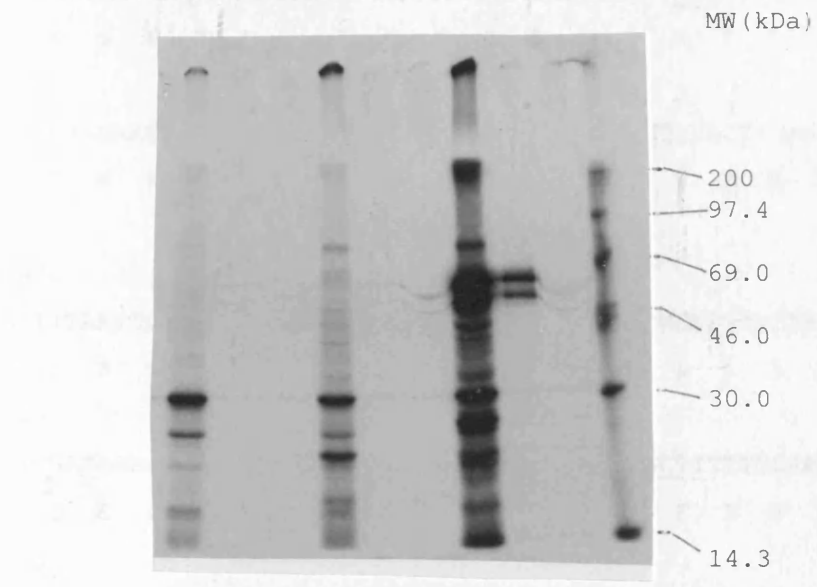


Figure 4.11

In vitro transcription/ translation and immunoprecipitation analysis of the Tn*phoA* mutants of pMK3. Reaction products were separated by SDS-PAGE on a 12% (w/v) acrylamide gel which was exposed to autoradiographic film for 4 days.

Lane 1: proteins synthesized from pBluescript; Lane 2: α-alkaline phosphatase immunoprecipitated pBluescript proteins (negative control); Lane 3: convalescent antiserum immunoprecipitated pBluescript proteins (negative control); Lane 4: proteins synthesized from pMK3; Lane 5: α-alkaline phosphatase immunoprecipitated pMK3 proteins; Lane 6: convalescent antiserum immunoprecipitated pMK3 proteins; Lane 7: proteins synthesized from pMK3.1; Lane 8: α-alkaline phosphatase immunoprecipitated pMK3.1 proteins; Lane 9: convalescent antiserum immunoprecipitated pMK3.1 proteins; Lane 10: protein molecular weight markers (sizes, in kDa, shown on right-hand side).

1 73

GATCCTAGTAATAAAGATTATCTTCAAAAAATAGATACGCTT**ATG**ACTTTGATTGATAGTAAAGATGCTTGG

D P S N K D Y L Q K I D T L M T L I D S K D A W

74 146

AGTATAGAATCTAAAATAATACGCGTTTTAAAAGAATTTAGTTTACTTGAGTATGAAAATCGCATTATATCT

S I E S K I I R V L K E F S L L E T E N R I I S

TnphoA

147 219

ACTTTAAGTGGTGGAGAAATACGCCGCGTTGGGCTTTGCATACTTTTACTAAAAAATCCTGATATTTTATTG

T L S G G E I R R V G L C I L L L K N P D I L L

220 292

CTTGATGAACCAACTAATCATTTAGATGTATATATGACAAGCTTTTTGGAAGAATTGCTAAAAAATTCAAAG

L D E P T N H L D V Y M T S F L E E L L K N S K

293 365

ATGTGTGTGATTTTTATTTCCCATGATAGATATTTTATTGACGCTATAGCACACAAGTGTGTAGAAGTAGAG

M C V I F I S H D R Y F I D A I A H K C V E V E

366 438

CAGGGTAAGCTTAGTATTTTTAAAGGCGGATATGCAAACTATTTAGAGAAAAAAACACAAATTTTGCAAAGC

Q G K L S I F K G G Y A N Y L E K K T Q I L Q S

439 511

CTTGCTAAGAGCCATGAAACTCTTTTAAAGCAATTAAAAAGCGAAGAAGAATGGCTTAGAAGAGGGGTTAAA

L A K S H E T L L K Q L K S E E E W L R R G V K

512 584

GCAAGACTTAAAAGAAATGAAGGACGAAAAGAACGCATTTTTAAAATGCGTGAAGAAGCGAAAAAAAATCCT

A R L K R N E G R K E R I F K M R E E A K K N P

585 657

GGGGCAATCAAACGCTTAAAACTTGAAATTTCAAGAGCGGCTTTAAATTTTAATGGAGAAAAAACGATCATC

G A I K R L K L E I S R A A L N F N G E K T I I

658 730

GTAAAAAAATGCTTTTTGAGCTTAAAAATTTTAAGCAAAAATATAAACAATAAAAATTTTTTTAAAGATTTT

V K K C F L S L K I L S K N I N N K N F F K D F

738

TCAAAAAT-3′

S K

Figure 4.12

Sequence of the 5′ end of the *C.jejuni* DNA insert of pMK11. The coding strand sequence is shown, with the deduced amino acid sequence from ORF11.2. The putative methionine start is shown underlined and in bold type. *Hin*dII restriction enzyme sites are underlined. Also shown is the point of insertion of Tn*phoA* in mutant pMK11.2. The numbers show the distance, in bp, from the 5′ end of the insert.

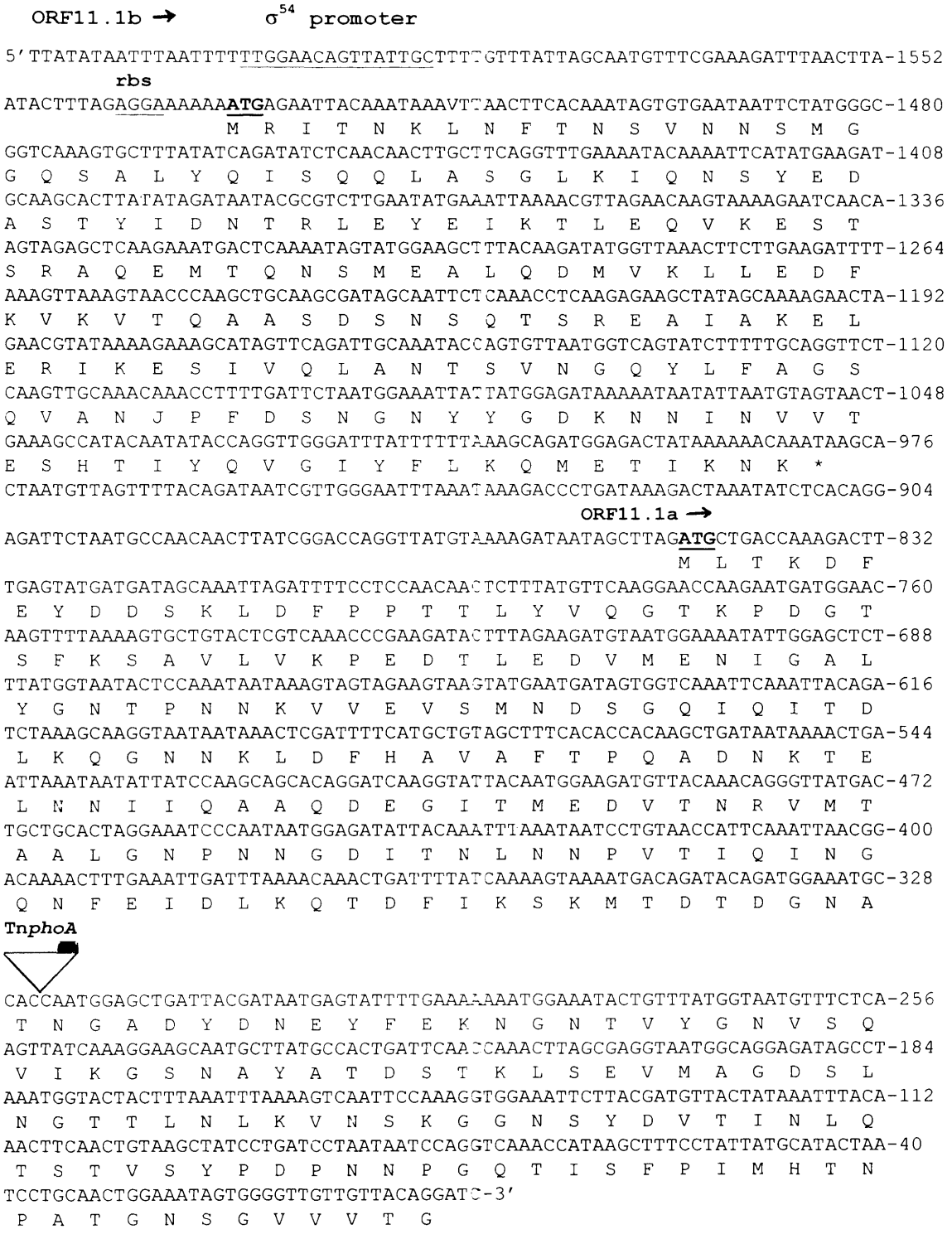
```
            1                                                          59
PEP11.2     DPSNKDYLQKIDTLMTLIDSKDAWSIESKIIRVLKEFSLLEYENRIISTLSGGEIRRVG
                        | ::|||:: |::|::: :::::::    ::: :::|||||| |||:
ChvD VDRYNELMMNYSDETADEGAKLQDMIDSQNLWDLENQVEMAMDALRCPP-GDSAVTGLSGGERRRVA
     1                                                                 67
            60                                                         118
PEP11.2     LCILLLKNPDILLLDEPTNHLDVYMTSFLEELLKNSKMCVIFISHDRYFIDAIAHKCVE
            || |||::||:||||||||||:  :::|||: |::    |::|:|||||:|:::  |:|
ChvD        LCKLLLSQPDLLLLDEPTNHLDAETIAWLEKHLRDYPGAVMMITHDRYFLDNVTGWILE
            68                                                         126
            119                                                        176
PEP11.2     VEQGKLSIFKGGYANYLEKKTQILQSLAKSHETLLKQLKSEEEWLRRGVKARLKRNEGR
            :::|:   ::|:|::||: |:: :|  |:::::  |::::|:|||: :: ||| :::::|
ChvD        LDRGRGIPYEGNYSAYLQAKAKRMQQEAREDASRQKAISREQEWIASSPKARQTKSKAR
            127                                                        184
            177                                                    232
PEP11.2     KERIFKMREEAKKNPGAIKRLKLEISRAALNFNGEKTIIVKKCFLSLKILSKNINN
              |  :: |:|:::: :  :: : ::
ChvD        IRRYDELVEAAENRRPGDAQIVIPVAERLGRVVIEAENLTKSYGDRVLIENLTFKLPPG
            185                                                        242
```

Figure 4.13

Amino acid sequence alignment of PEP11.2 with the *A.tumefaciens* ChvD protein. Fusion of the first 59 amino acids of PEP11.2 with the partial alkaline phosphatase from *TnphoA*, in the mutant pMK11.2, allowed the export of the alkaline phosphatase enzyme moiety. ":" indicates similar amino acid, "|" indicates identical amino acid. Amino acids corresponding to highly conserved ATP-binding regions are underlined.

Figure 4.14

Sequence of the 3′ end of the *C.jejuni* DNA insert of pMK11. The coding strand sequence is shown, with the deduced amino acid sequences from ORF11.1a and ORF11.1b. The putative methionine starts are underlined and in bold type, while the σ^{54} promoter and possible ribosome binding site (rbs) of ORF11.1b are underlined. "*" indicates a stop codon. Also shown is the site of Tn*phoA* insertion in pMK11.1. The numbers show the distance, in bp, from the 3′ end of the insert.


ORF11.1b →
σ54 promoter
5′TTATATAATTTAATTTTTTGGAACAGTTATTGCTTTTGTTTATTAGCAATGTTTCGAAAGATTTAACTTA-1552
rbs
ATACTTTAGAGGAAAAAAATGAGAATTACAAATAAAVTTAACTTCACAAATAGTGTGAATAATTCTATGGGC-1480
M R I T N K L N F T N S V N N S M G
GGTCAAAGTGCTTTATATCAGATATCTCAACAACTTGCTTCAGGTTTGAAAATACAAAATTCATATGAAGAT-1408
G Q S A L Y Q I S Q Q L A S G L K I Q N S Y E D
GCAAGCACTTATATAGATAATACGCGTCTTGAATATGAAATTAAAACGTTAGAACAAGTAAAAGAATCAACA-1336
A S T Y I D N T R L E Y E I K T L E Q V K E S T
AGTAGAGCTCAAGAAATGACTCAAAATAGTATGGAAGCTTTACAAGATATGGTTAAACTTCTTGAAGATTTT-1264
S R A Q E M T Q N S M E A L Q D M V K L L E D F
AAAGTTAAAGTAACCCAAGCTGCAAGCGATAGCAATTCTCAAACCTCAAGAGAAGCTATAGCAAAAGAACTA-1192
K V K V T Q A A S D S N S Q T S R E A I A K E L
GAACGTATAAAAGAAAGCATAGTTCAGATTGCAAATACCAGTGTTAATGGTCAGTATCTTTTTGCAGGTTCT-1120
E R I K E S I V Q L A N T S V N G Q Y L F A G S
CAAGTTGCAAACAAACCTTTTGATTCTAATGGAAATTATTATGGAGATAAAAATAATATTAATGTAGTAACT-1048
Q V A N J P F D S N G N Y Y G D K N N I N V V T
GAAAGCCATACAATATACCAGGTTGGGATTTATTTTTTAAAGCAGATGGAGACTATAAAAAACAAATAAGCA-976
E S H T I Y Q V G I Y F L K Q M E T I K N K *
CTAATGTTAGTTTTACAGATAATCGTTGGGAATTTAAATAAAGACCCTGATAAAGACTAAATATCTCACAGG-904
ORF11.1a →
AGATTCTAATGCCAACAACTTATCGGACCAGGTTATGTAAAAGATAATAGCTTAGATGCTGACCAAAGACTT-832
M L T K D F
TGAGTATGATGATAGCAAATTAGATTTTCCTCCAACAACTCTTTATGTTCAAGGAACCAAGAATGATGGAAC-760
E Y D D S K L D F P P T T L Y V Q G T K P D G T
AAGTTTTAAAAGTGCTGTACTCGTCAAACCCGAAGATACTTTAGAAGATGTAATGGAAAATATTGGAGCTCT-688
S F K S A V L V K P E D T L E D V M E N I G A L
TTATGGTAATACTCCAAATAATAAAGTAGTAGAAGTAAGTATGAATGATAGTGGTCAAATTCAAATTACAGA-616
Y G N T P N N K V V E V S M N D S G Q I Q I T D
TCTAAAGCAAGGTAATAATAAACTCGATTTTCATGCTGTAGCTTTCACACCACAAGCTGATAATAAAACTGA-544
L K Q G N N K L D F H A V A F T P Q A D N K T E
ATTAAATAATATTATCCAAGCAGCACAGGATCAAGGTATTACAATGGAAGATGTTACAAACAGGGTTATGAC-472
L N N I I Q A A Q D E G I T M E D V T N R V M T
TGCTGCACTAGGAAATCCCAATAATGGAGATATTACAAATTTAAATAATCCTGTAACCATTCAAATTAACGG-400
A A L G N P N N G D I T N L N N P V T I Q I N G
ACAAAACTTTGAAATTGATTTAAAACAAACTGATTTTATCAAAAGTAAAATGACAGATACAGATGGAAATGC-328
Q N F E I D L K Q T D F I K S K M T D T D G N A
TnphoA
CACCAATGGAGCTGATTACGATAATGAGTATTTTGAAAAAAATGGAAATACTGTTTATGGTAATGTTTCTCA-256
T N G A D Y D N E Y F E K N G N T V Y G N V S Q
AGTTATCAAAGGAAGCAATGCTTATGCCACTGATTCAACCAAACTTAGCGAGGTAATGGCAGGAGATAGCCT-184
V I K G S N A Y A T D S T K L S E V M A G D S L
AAATGGTACTACTTTAAATTTAAAAGTCAATTCCAAAGGTGGAAATTCTTACGATGTTACTATAAATTTACA-112
N G T T L N L K V N S K G G N S Y D V T I N L Q
AACTTCAACTGTAAGCTATCCTGATCCTAATAATCCAGGTCAAACCATAAGCTTTCCTATTATGCATACTAA-40
T S T V S Y P D P N N P G Q T I S F P I M H T N
TCCTGCAACTGGAAATAGTGGGGTTGTTGTTACAGGATC-3′
P A T G N S G V V V T G

```
                  1812
C.jejuni tRNA-gly 5'-CTTATTTTTTGCGGGAATAGCTCAGG-GGTAGAGCACA
                              ||||||||||||||  ||||||||||
E.coli glyW                   GCGGGAATAGCTCAGTTGGTAGATCACG

C.jejuni tRNA-gly     ACCTTGCCAAGGTTGGGGTCGCGGGTTCGAATCTCGTT
                      ||||||||||||| |||||||| |||||| |||||||
E.coli glyW           ACCTTGCCAAGGTCGGGGTCGCGAGTTCGAGTCTCGTT
                                              1912
C.jejuni tRNA-gly     TCCCGCTCCATAGAATAAAAAATATA-3'
                      |||||||||
E.coli glyW           TCCCGCTCC
```

Figure 4.15

Alignment of the *C.jejuni* gene for tRNA-gly with *E.coli glyW*. "|" indicates *E.coli* nucleotide identical to one present in the *C.jejuni* gene and "-" indicates position in *C.jejuni* sequence where no nucleotide exists to correspond with the one in the *E.coli* sequence. The numbered *C.jejuni* nucleotides show the distance, in bp, from the 3' end of the insert.

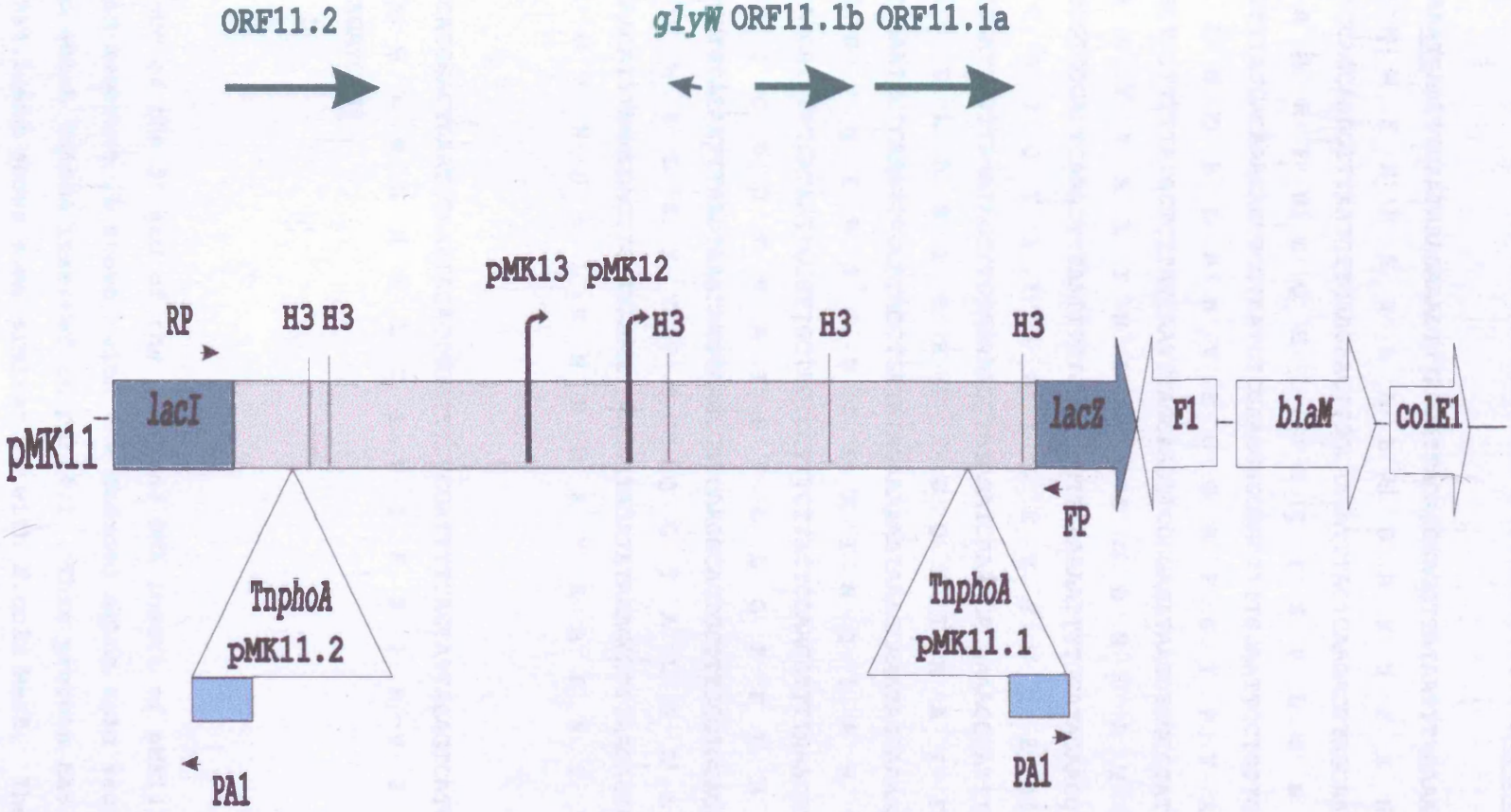
```
                1                                                   52
PEP11.1b        MRITNKLNFTNSVNNSMGGQSALYQISQQLASGLKIQNSYEDASTYIDNTRL
                 ||:::::  |:  ||  : :|| |   :|:|||:|::: :|||:   :::|
C.coli FlaB     MGFRINTNVAALNAKANSDLNSRALDQSLSRLSSGLRINSAADDASGMAIADSL
               1                                                    55
                53                                                  103
PEP11.1b         EYEIKTLEQVKESTSRAQEMTQNSMEALQDMVKLLEDFKVKVTQAASDSNS
                   : :|||:|: :::: | :: |:: :|::: :|:|:::|:|:|:|||| |  |
C.coli FlaB      RSQANTLGQAISNGNDALGILQTADKAMDEQLKILDTIKTKATQAAQDGQS
                56                                                  107
               104                                                  153
PEP11.1b         QTSREAIAKELERIKESIVQLAN-TSVNGQYLFAGSQVANKPFDSNGNYYG
                   :|  :  :: |: |:: ::|| || ||  |: |:   |  |:  :
C.coli FlaB      LKTRTMLQADINRLMEELDNIANTTSFNGKQLLSGG-FTNQEFQIGSSSNQ
               108                                                  155
               154                                      188
PEP11.1b         DKNNINVVTGAGTESHTIYQVGIYFLKQME---TIKNK
                        |  :  : | :: |   :       ||||
C.coli FlaB      TIKASIGATQSSKIGVTRFETGSQSFSSGTVGLTIKN
               156                                      192
```

Figure 4.16

Alignment of deduced amino acid sequence of PEP11.1b with *C.coli* flagellin protein sequence. ":" denotes similar amino acid in *C.coli* Fla as in PEP11.1b, while "|" denotes an identical amino acid.

Figure 4.17

Diagram to show the positions of Tn*phoA* insertions into pMK11 and the possible ORFs in the *C.jejuni* DNA insert. The position and orientation of Tn*phoA* in each of the blue mutants, pMK11.1 and pMK11.2, are indicated. The partial alkaline phosphatase gene is shown as a light blue box and the position of the PA1 primer is indicated. The *Sau*3A sites at which overlapping clones pMK12 and pMK13 start, are indicated by curved arrows. "H3" denotes a *Hin*dIII restriction site. The possible ORFs are shown as green arrows. ORF11.2 encodes a peptide with similarity to ATP-binding transport proteins, *glyW* encodes tRNA-gly, ORF11.1b encodes a peptide with similarity to *Campylobacter* flagellins and ORF11.1a encodes a peptide with no similarity to sequences in the database.


ORF11.2
glyW ORF11.1b ORF11.1a
pMK13 pMK12
RP
H3 H3
H3
H3
H3
pMK11
lacI
lacZ
F1
blaM
colE1
FP
TnphoA
pMK11.2
TnphoA
pMK11.1
PA1
PA1

898

5'ATGCTTTCTTCTTTCTGTATCGTGCTTGGCACGCTTAATCTACTCGTATATACTCATATTCTATAA-832

ATTTACATTTTCAAATAGCCTAGTTTAAAGCCGATAAAAAGCTTTTTAACATTTTTTTATTGATACAA-764

rbs

TA**AGGA**TAAAAA**ATG**AATTTCAATAAAGAAACTTTAGCATTACACGGAGCTTATAATTTTGAAAATT-697

PEP14.1→ M N F N K E T L A L H G A Y N F E N L

TGGATCAAGCTGCAGCAAGGTTTAATCTTCAAGAACTTGGCAATATTTACTCAAGACTTAGCAATCCT-629

D Q A A A R F N L Q E L G N I Y S R L S N P

ACAAGCGATGTTTTAGGACAAAGACTTGCTAATGTCGAAGGAGGGGCTTTTGGAATTCCTGTTGCTAG-561

T S D V L G Q R L A N V E G G A F G I P V A S

CGGTATGGCAGCTTGTTTTTATGCTCTTATCAATTTAGCAAGTTCGGGAGATAATGTCGCGTATTCGA-493

G M A A C F Y A L I N L A S S G D N V A Y S N

ACAAAATTTATGGTGGGACTCAAACTTTAATTTCTCACACACTTGAAAAATTTTGGATAGAAGCTAGG-425

K I Y G G T Q T L I S H T L E K F W I E A R

GAATTTGATATCGATGATTTAGATAGCTTGGAAAAAGTTATAGATCAAAACACAAAAGCGATTTTTTT-357

E F D I D D L D S L E K V I D Q N T K A I F F

CGAAAGTCTTTCAAATCCTCAAATTGCCATAGCTGATATAGAAAAAATAAACCAAATAGCAAAAAAAC-289

E S L S N P Q I A I A D I E K I N Q I A K K H

ATAAAATCGTTAGCATTTGTGATAATACCGTTGCTACTCCTTTCTTACTCCAACCTTTTAAACATGGC-221

K I V S I C D N T V A T P F L L Q P F K H G

GTGGATGTAATCGTGCATAGTTTAAGTAAATATGTAAGCGGTCAAGGCACTGCTTTGGGTGGAGCACT-153

V D V I V H S L S K Y V S G Q G T A L G G A L

TATAGAAAGAAAAGATTTAAACGACTTGCTTAAAAATAACGATAGATATAAAGCTTTTAACACTCCTG-85

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

TnphoA

ATCCAAGTTATCATGGACTGAATTTAAATACACTTGATTTGCCGATTTTTAGTATTAGAGTCATCATC-17

P S Y H G L N L N T L D L P I F S I R V I I

ACTTGGCTTATAGATC-3'

T W L R D

Figure 4.18

Sequence of the 3' end of the *C.jejuni* DNA insert of pMK14. The coding strand sequence is shown, with the deduced amino acid sequence from the ORF into which Tn*phoA* inserted in pMK14.1. This protein has been labelled PEP14.1 and shows some similarity with *E.coli* MetB. The putative methionine start is underlined and in bold type, while a possible ribosome binding site (rbs) is in bold type only. A putative -35 sequence is underlined. Also shown is the site of insertion of Tn*phoA* in pMK14.1.

```
           1                                   34
PEP14.1     MNFNKETLALHGAYNRENLDQAAARFNLQELGNIYSRLSNPTSDVLGQRLAN-52
              |                                :: ||| :|||:||: : ||:
E.coli MetB  GLNDDEQYGCVVPPIHLSSTYNFYGFNEPRAHDYSRRGNPTRDVVQRALAE-62
          12

PEP14.1      VEGGAFGIPVASGMAACFYALINLASSGDNVAYSNKIYGGTQTLISHTLEK-103
             :|||| :: :::||:|   : : : ::|| :: ::::|||:  |::   ::
E.coli MetB  LEGGAGAVLTNTGMSAIHLVTTVFLKPGDLLVAPHDCYGGSYRLFDSLAKR-113

PEP14.1      FWIEAREFDIDDLDSLEKVIDQNTKAIFFESLSNPQIAIADIEKINQIAKK-154
                :   | :| ::|  ::::::| || || ||| : ::||:|| ::|::
E.coli MetB  GCYRVLFVDQGDEQALRAALAEKPKLVLVESPSNPLLRVVDIAKICHLARE-164

PEP14.1      HKIVSICDNTVATPFLLQPFKHGVDVIVHSLSKYVSGQGTALGGALIERKD-205
               ||: |||  :| | :|:  |:|:::|| :||::|::::::|::|::
E.coli MetB  VGAVSVVDNTFLSPALQNPLALGADLVLHSCTKYLNGHSDVVAGVVIAKDP-215

                                    234            250
PEP14.1      LNDLLKNNDRYKAFNTPDPSYHGLNLNTLDLPIFSIRVIITWLRD-vector
                           |       | |  |
E.coli MetB  DVVTELAWWANNIGVTGGAFDSYLLLRGLRTLVPRMELAQRNAQA-260
```

Figure 4.19

Alignment of deduced amino acid sequence of PEP14.1 with *E.coli* MetB protein sequence. ":" indicates similar amino acids in each protein; "|" indicates the same amino acid.

Figure 4.20

Sequence of the ORF of pMK3, into which Tn*phoA* inserted to give an exported alkaline phosphatase fusion protein, with derived amino acid sequence. A putative σ^{54} promoter is shown in bold type (the σ^{54} consensus sequence is $TTGGN_{10}GC$ and the promoter of this ORF is **A**$TGGN_{10}GC$), while the methionine start is in bold type and underlined. A putative TATA box is also underlined. "*" indicates the stop codon at the end of the ORF and the numbers refer to the number of base pairs from the 5′ fusion point of the *C.jejuni* insert DNA with pBluescriptSK-.

701

5′-ACACCATCATTCATGTAAATGGAACTTTAAAAAAGATGCTTTTTTCTAGC-750

ATAGATGAAAATATACGCATCATTCGCTCTGAAAAAGTAATCGCTAGAAA-800

TTTAGTTCAAGAAGCGCTAGAATGCCGCATTCACAAAAATACCTAAGGAA-850

GAATTTTGACAAAAAATGAAGAAAAAGCCTTACGCGTAAAATACCTAAGA-900

σ^{54} *promoter*

AATCTTGAAAAATTTTTCA**ATGGCGCTATATCTGC**ACTTTAAAAAAGAAG-950

ATTTTGATAAAACTAAATTTGAAGAAAGA**ATG**TTAAAAAATGCTAAATTT-1000

M L K N A K F

TTTGGAAAAAAATCCCGCGGTAAATCTTTAACTCAACCTATGCAAAAAAT-1050

F G K K S R G K S L T Q P M Q K I

CTTGAATTTTTTGGTCAATGCTTGTTTAGATTTTTCCAAAGAAAAAAGCG-1100

L N F L V N A C L D F S K E K S E

AACTTTTAAATCTTTGCCAATGCTTTAGATTAAGCAAAAAAAAACAAGGT-1150

L L N L C Q C F R L S K K K Q G

GGAGAAAAAAGAACACACCAAAATTATTTAAAGGATTATGAAATGATTAA-1200

G E K R T H Q N Y L K D Y E M I N

TGTATTTTTTGATATGAATGGAACTTTAATCGATAGCGCAAATGCCATTT-1250

V F F D M N G T L I D S A N A I S

CTTGTGCAGTCAATGAAATAAGACAGGATTTAAACCTAGCCCCACTTTCA-1300

C A V N E I R Q D L N L A P L S

AGAGAAATCATTATGCAAACCATCAATACTCCTAATATTGACTGGGCAAA-1350

R E I I M Q N I N T P N I D W A K

AGNACTTTATAATATAGAAAATTTCCATCATTCNAGTTTTAAAGAAGGCT-1400

X L Y N I E N F H H S S F K E G Y

ACGAAAAATATTTTGTCAAACACTATGAACAAAGCGTGGTGCTTTTTGAA-1450

E K Y F V K H Y E Q S V V L F E

GGGGTGAAAGAACTGTTAGAATTTTTAAAAAGCAAAAATTGTTTTTTAGC-1500

G V K E L L E F L K S K N C F L A

TATTGCTACTAACGCCCCGCAAAGCTCACTTTCACACATACTTAAAAAAC-1550

I A T N A P Q S S L S H I L K K H

ACGATATCATTCCTTATTTTGATAAAATTTTAGGCGTAAGTNCTAGGCAT-1600

D I I P Y F D K I L G V S X R H

AGAACCCAAACCTCATCCTATGAAGCTAGAGCTTTTAAAAAGCGAGGCTC-1650

R T Q T S S Y D A R A F K K R G S

CATATAAAACAAGCGT-1666

I *

4.4 DISCUSSION

Tn*phoA* mutagenesis of the *C.jejuni* pBluescript SK- clones, which were originally isolated from a Lambda ZAP II library by screening with convalescent antiserum, successfully resulted in the identification of ORFs for proteins that could be exported from *E.coli*. This was the first report of Tn*phoA* mutagenesis of *C.jejuni* sequences. Tn*phoA* mutants of pMK11, pMK14 and pMK3 all yielded blue colonies on selective media containing the substrate XP. Only exported alkaline phosphatase would be capable of this modification of XP, therefore these mutants should contain Tn*phoA* inserted in frame, downstream of sequences encoding a signal peptide which is active in *E.coli* or in an ORF that encodes for a protein which is exported by other means.

PCR was found to be the most useful method of determining the position and orientation of the transposon within the *C.jejuni* DNA inserts. Such mapping revealed that one of the blue Tn*phoA* mutants, pMK11.1, had the transposon inserted in the 1.2kb *Hin*dIII fragment shared by positive clones pMK11, pMK12 and pMK13. *In vitro* transcription/translation analysis of pMK11.1 showed that this mutant no longer synthesized a 65kDa protein, which had previously demonstrated immunoprecipitation with convalescent antiserum. This evidence from Tn*phoA* mutagenesis suggests that the gene for an exported immunogenic protein is common to the positive clones pMK11, pMK12 and pMK13, thus explaining the isolation of these overlapping clones by convalescent antiserum. *In vitro* transcription/translation and immunoprecipitation had previously shown that only a 74kDa product from the *C.jejuni* insert of pMK12 hybridised with convalescent antiserum. Therefore, it seems likely that the full-length immunogenic protein has a molecular weight of 74kDa and pMK11 contains a partial gene which encodes a truncated product of 65kDa.

4.4.1 Summary of Tn*phoA* analysis of pMK11. Sequencing of the blue Tn*phoA* mutant, pMK11.1, revealed that Tn*phoA* had inserted into a putative ORF, although apparently not with the alkaline phosphatase gene in frame. The sequence obtained to date suggests that Tn*phoA* inserted in the opposite orientation to the direction of transcription from the *C.jejuni* ORF, which was designated ORF11.1a. The deduced sequence of 282 amino acids encoded by ORF11.1a did not have significant homology with any known protein sequence in the database. This sequence was also too short to have a molecular weight of 65kDa, but it is likely that sequencing error caused the apparent stop codons upstream, since complementary strand sequence has not yet been obtained. However, the sequence of both strands has been

obtained from 990bp (142bp upstream of ORF11.1a) to 2414bp and another ORF, designated ORF11.1b, was found to be reading in the same direction as ORF11.1a. Both strands of the sequence between the two ORFs has not yet been elucidated, but it is possible that they may form a single ORF, which begins at the ATG start codon of ORF11.1b. The deduced sequence of 188 amino acids from ORF11.1b had 28% identity with the N-terminal region of *C.coli* FlaA and FlaB and 27% with *C.jejuni* flagellin. Therefore, it may be significant that ORF11.1b has a typical σ^{54} promoter, which is the same type of promoter as that possessed by the *C.coli flaB* gene [160]. The highly specialised σ^{54} promoters are usually found upstream of genes involved in the fixation and assimilation of nitrogen, amino acid transport components, degradative enzymes and the pilin genes of *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. Only *Caulobacter crescentus* also has flagellin genes controlled by σ^{54} promoters. The homology does not appear strong enough for the positive clone peptide to be flagellin itself, since there is known to be a high degree of homology between the flagellins of different strains of *C.jejuni* and *C.coli*, for example the first 188 amino acids of *C.jejuni* strain 81116 FlaB contains only six residues different from those of *C.coli* strain VC167 FlaB [158]. Moreover, the deduced amino acid sequence from ORF11.1a, which is thought to continue from ORF11.1b, has no homology with flagellin. Also, Southern blotting experiments previously showed that the first 700bp of *flaA* plus 2kb of upstream sequence show no hybridisation with the positive clones. If my speculations are correct, then ORF11.1b continues through ORF11.1a and encodes an entire protein of 74kDa in pMK12 and pMK13. This product has a higher molecular weight than the 62kDa flagellin.

In order to clarify the results obtained to date, sequence analysis could be continued downstream of ORF11.1a, using pMK12 and pMK13 as templates, which would reveal whether the reading frame continues further and could encode a 74kDa product. Further Tn*phoA* mutants could be sought in which the 65kDa/74kDa products are lost from clones pMK11, pMK12 and pMK13. Mapping and sequencing of mutants in the ORFs encoding the 65kDa and 74kDa products should determine unequivocally whether these ORFs are the same and are possessed by the positive clones pMK11, pMK12 and pMK13. The DNA and amino acid sequences found can be used to search the databases for homology with known sequences. The function of the discovered protein(s) could also be analysed by mutagenesis of the *C.jejuni* gene *in vivo*. A suicide vector can be used to introduce the Tn*phoA* mutated ORF into *C.jejuni*, where homologous recombination between the mutant gene and the functional chromosomal gene can occur. Cells containing the mutant gene can be selected by their resistance to kanamycin and changes in the

phenotype of the organism investigated. The information obtained to date encourages the impression that a virulence determinant may have been cloned, since Tn*phoA* has detected an exported product, an ORF has been found with some similarity to *flaB* and convalescent antiserum binds to proteins synthesized *in vitro* from the positives. However, the mutation analysis is necessary before any firm conclusions can be made.

The second blue Tn*phoA* mutant of pMK11, pMK11.2, had the transposon inserted in frame in a putative ORF and the deduced amino acid sequence, PEP11.2, had 33% identity with *Agrobacterium tumefaciens* ChvD protein and similarity with other ATP-binding transport proteins. These proteins usually have a membrane-bound portion and, as such, could synthesize a hybrid protein in which alkaline phosphatase was exposed on the cell surface and therefore able to dephosphorylate the XP substrate, although the GCG motifs program did not detect a putative signal sequence. The sequence data suggests that a fusion protein could not be synthesized from the *lacZ* promoter, but the *in vitro* transcription/translation analysis shows that a 60kDa product is synthesized by pMK11.2, which is not produced by pMK11 or pMK11.1. If this product was the active alkaline phosphatase hybrid protein, it would have to start in the vector, because the *C.jejuni* sequence to the transposon is not long enough to encode it. However, the 60kDa product was not immunoprecipitated by anti-alkaline phosphatase antibody, therefore, it is possible that this is not the alkaline phosphatase hybrid protein. There are 45 deduced amino acid residues between the putative methionine start of PEP11.2 and the Tn*phoA* insertion site. This would give a possible alkaline phosphatase hybrid protein size of approximately 52kDa and there is such a band in the *in vitro* transcription/translation reaction (see Figure 4.8), but no firm conclusions can be drawn from this because of the absence of immunoprecipitation by the anti-alkaline phosphatase monoclonal antiserum.

It could be significant that a gene for an ATP-binding transport protein may have been found upstream of a gene with some similarity to *fla*, but the function of this protein still has to be confirmed. Also, the presence of the gene for tRNA-gly, situated between ORF11.1b and ORF11.2 and in the opposite orientation to the other ORFs (see Figure 4.17), may indicate that these genes are not connected functionally. Mutation of the chromosomal gene for PEP11.2, by the method described previously for PEP11.1, may provide some evidence of this product's utilisation by *C.jejuni* cells. Since no stop codons are encountered in the reading frame of ORF11.2 from the fusion point with the vector to the putative methionine start codon, it is also possible that pMK11 may not contain the entire

gene. The Lambda ZAP II library could be screened for clones containing the upstream sequence, so that the entire ORF can be verified.

4.4.2 Summary of Tn*phoA* analysis of pMK14. The blue transposon mutant of pMK14, pMK14.1, had the transposon inserted in frame into an ORF which could encode a protein with 30% identity to the *E.coli* methionine biosynthesis enzyme, cystathionine γ-synthase. There is a putative ribosome binding site 10bp upstream of the ATG methionine codon which begins at 754bp into the *C.jejuni* insert, reading towards the fusion point with the vector at the 5' end of the insert. However, in the *in vitro* transcription/translation experiments, pMK14.1 was shown to be no longer able to synthesize the immunogenic 69kDa protein, but if the ORF begins at base 754, then it could not encode a product of more than approximately 35kDa. Furthermore, the calculated molecular weight of the deduced peptide upstream of the transposon insertion point is approximately 26kDa, which is the correct size for synthesizing a fusion protein of approximately 75kDa. A protein band of 75kDa was observed in *in vitro* transcription/translation experiments with pMK14.1. It is possible that a larger hybrid product, consisting of alkaline phosphatase fused with a 69kDa protein, was synthesized initially but quickly degraded. Some degradation of hybrid proteins occurred in all the *in vitro* transcription/translation experiments, as evidenced by the presence of the 47kDa mature alkaline phosphatase band. Instability of some alkaline phosphatase hybrid proteins has also been previously documented [210]. However, Tn*phoA* could affect synthesis of the 69kDa product for another reason, such as the polar effects of the transposon insertion. This means that, since Tn*phoA* carries transcriptional terminators, if it inserts into the first gene in an operon, it will eliminate expression not only of that gene but also of downstream genes. It can be concluded that insertion of Tn*phoA* into pMK14 yields a 75kDa hybrid protein, which consists of the 47kDa alkaline phosphatase fused with 28kDa of a *C.jejuni* product, but also eliminates synthesis of a 69kDa protein due to polar effects. The hybrid 75kDa protein is recognised by both convalescent antiserum and the anti-alkaline phosphatase antiserum and appears to have significant similarity with *E.coli* MetB over its central region (see Figure 4.19). *E.coli* MetB consists of 386 amino acids and has a molecular weight of 41.5kDa. Since the *C.jejuni* ORF on pMK14 reads into the vector, the whole gene has not been cloned and the final size of the *C.jejuni* protein *in vivo* is therefore not known.

4.4.3 Summary of Tn*phoA* analysis of pMK3. A blue Tn*phoA* mutant of pMK3 was found to have the transposon inserted in frame into an ORF encoding a 23kDa protein, as determined by sequencing and *in vitro* transcription/translation. This protein did not hybridise with convalescent antiserum, thus corroborating previous data which suggested that pMK3 did not synthesize *in vitro* any proteins which could bind the antiserum. No significant similarity was found between the protein and sequences in the database, but it does have a putative ATP-binding site, the significance of which is not known.

4.4.4 Future work. The combined techniques of screening a Lambda expression library of *C.jejuni* DNA with convalescent antiserum, *in vitro* transcription/translation with immunoprecipitation and Tn*phoA* mutagenesis have enabled the isolation of at least three new *C.jejuni* genes. The complete identification of the ORFs has not yet been confirmed, but one of them could encode an ATP-binding transport protein and another could encode a product which has some similarity with flagellin at its N-terminal end. The next stage of investigation would be to create *C.jejuni* mutants in these genes (as described for ORF11.1) and ascertain any functions which such mutants will have lost in comparison to wild type cells. Our particular interest would be in virulence functions such as motility, adherence and invasion, as discussed further in Chapter 6.

CHAPTER FIVE

PCR WITH DEGENERATE OLIGONUCLEOTIDE PRIMERS AS A METHOD FOR CLONING CONSERVED *C.JEJUNI* GENES: ISOLATION OF THE *C.JEJUNI* STRAIN H132 *RECA* GENE

5.1 INTRODUCTION

The RecA protein of *E.coli* is known to be essential for homologous recombination, DNA-damage repair and the initiation of the SOS response. The RecA protein promotes a variety of ATP-dependent interactions of homologous DNA molecules. These include: the annealing of complementary single-stranded DNA (ssDNA), the formation of D-loops between linear ssDNA and circular double-stranded DNA (dsDNA) and the conversion of linear dsDNA and circular ssDNA to linear ssDNA and circular dsDNA (Figure 5.1, p148). RecA also promotes the recombination of homologous duplex molecules through formation of a single-stranded crossed connection, known as a "Holliday" junction, between the two linear dsDNA molecules, at least one of which must have a ssDNA tail [216, 217] (Figure 5.1). The major biochemical function of RecA protein is that it binds to ssDNA, in association with ATP, to form a nucleoprotein filament. This is also the first step in the process of recombination, when the filament is called the presynaptic complex. Upon recognising homologous duplex DNA, the presynpatic complex promotes both pairing and DNA strand exchange in the 5′ to 3′ direction relative to the displaced ssDNA, exchanging strands between homologous DNA molecules. Thus, RecA has a central role in recombination and the single-strand exchange involved in recombination-repair. Recombination-repair is the retrieval of a copy of a damaged DNA molecule from an undamaged source and the subsequent recombination to replace the damaged section of DNA.

In addition to its DNA strand-exchange functions, RecA also has activity as a protease under certain conditions. When DNA damage occurs, due to UV irradiation for example, the RecA protein serves as a co-protease to cleave the LexA repressor of the SOS regulon, thus activating the SOS response. The response takes the form of increased capacity to repair damaged DNA, achieved by inducing synthesis of the excision-repair system (including the products of genes *uvrA* and *uvrB*) and the Rec recombination-repair pathways (including RecA itself). The SOS response also includes the inhibition of cell division. Excision-repair removes mispaired or

damaged bases from DNA and synthesizes a replacement stretch of normal DNA. The *uvrA* and *uvrB* genes code for the components of a repair endonuclease that makes an incision either side of the damaged site. An excision exonuclease can then remove the damaged stretch of DNA and DNA polymerase I synthesizes a new DNA segment. The *lexA* gene itself is also repressed by its LexA product, so that, when induction of the protease activity of RecA ceases, it loses its ability to cleave LexA which rapidly accumulates in the uncleaved form and again suppresses expression of the SOS genes. RecA activated by DNA damage also has a protease activity on the UmuD protein, causing it to become activated. Mutants in the *umuD* gene are not susceptible to UV-induced mutagenesis and UmuD is, therefore, thought to be involved in the error-prone SOS DNA repair system, which allows DNA chain growth across damaged segments at the cost of fidelity of replication.

At the time this project was devised, the *recA* genes from a variety of Gram-negative bacteria had been isolated, mostly by complementation of *E.coli recA* mutants [218]. These included the *recA* genes of *Neisseria gonorrhoeae* [219], *Vibrio cholerae* [220, 221], *Shigella flexneri*, *Proteus vulgaris*, *Erwinia carotovora* [222] and *Pseudomonas aeruginosa* [223]. Analogues of *recA* had also been cloned from a few Gram-positive bacteria, including *Bacillus subtilis* [224] and *Mycobacterium tuberculosis* [225]. Sequence analysis of the *recA* gene, and the deduced amino acid sequence of RecA, has revealed a high degree of amino acid conservation throughout the RecA protein.

It has previously been shown that *C.jejuni* is capable of incorporating DNA into its chromosome by homologous recombination, supporting the theory that this organism has a *recA*-like gene [55, 179]. The natural transformation ability of *C.jejuni* depends upon the recombination of homologous sequences on the transformed DNA into the chromosome [188, 189]. In *Neisseria gonorrhoeae*, antigenic variation of the pilin protein can occur by the uptake of non-expressed pilin DNA sequences from lysed cells by natural transformation and subsequent recombination of these sequences into expressed pilin genes [226]. RecA is essential for such genetic rearrangements which enable the pathogen to avoid host defence mechanisms and, therefore, RecA can have an indirect role in virulence. Alm, Guerry and Trust have demonstrated that *Campylobacter flaA*$^-$ *flaB*$^+$ mutants, which have greatly reduced motility, become highly motile upon passage due to both intergenomic and intragenomic recombination [227]. They found that, when selective pressure for the mutational kanamycin cassette was maintained, natural transformation combined with intergenomic recombination allowed restoration of motility, while simple intragenomic recombination occurred between the homologous *fla*

genes when no kanamycin selection was present. Thus, it has been shown that *C.jejuni* can utilise natural transformation and homologous recombination to rescue a phenotype which is essential for virulence.

RecA has also been shown to have other roles in virulence. *V.cholerae recA* mutants were unable to amplify the genetic element carrying the *ctxA* and *ctxB* genes for cholera toxin, thus decreasing the pathogenicity of the organism [191]. More recently, *Salmonella typhimurium recA* mutants were found to be avirulent, due to an increased susceptibility to oxidative killing by macrophages [228]. Thus, RecA can play an important role in both the amplification of a virulence cassette and in the resistance of host defence mechanisms (and other stresses encountered in the environment) due to its functions in homologous recombination and DNA repair.

The construction of *Campylobacter recA* mutants would enable the significance of recombination events in the virulence of *C.jejuni* to be investigated. However, the *C.jejuni recA* gene had not been cloned by 1991 and it was therefore decided to attempt to isolate the *recA* gene. The strategy employed here involves amplification of a gene fragment using PCR with degenerate oligonucleotide primers (PCRDOP) [229]. This method had been used successfully to amplify fragments of two-component regulator genes and *htrA* from *C.jejuni* (J.Henderson and J.M.Ketley, personal communication) and it was wished to extend the utilisation of the technique. The *recA* gene was a suitable candidate for PCRDOP because of the highly conserved nature of the RecA protein. PCRDOP involves the design of degenerate oligonucleotide primers from regions of conserved amino acid sequence in a protein, from which primers can be designed with as little degeneracy as possible. This chapter describes the design of the primers and the results obtained from the PCRDOP experiment with *C.jejuni* DNA.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains, vectors and growth conditions. The *C.jejuni* inflammatory strain H132 was used as the source of chromosomal DNA for PCR experiments (prepared as described previously in method 2.2.2). The plasmid vector pUC19 was used for subcloning PCR products and *E.coli* strain JM105 [*endA1, hsdR4, thi, rpsL, sbcB15,* Δ(*lac-pro*), (F', *traD36, proAB, lacIqZ*ΔM15)] was the host for such plasmid constructs. *E.coli* strains were grown in LB at 37°C. Ampicillin (100μg/ml) was used for the selection of plasmid-containing strains, and IPTG and X-Gal were used to detect recombinant clones.

C.jejuni which were to be transformed by electroporation were grown for 18 hours on *Campylobacter* blood free selective agar [BFSA (Oxoid, Basingstoke)], in an atmosphere provided by a VAIN (as described in method 2.2.1).

5.2.2 PCRDOP method. The amino acid sequences of RecA proteins from twelve different organisms were aligned to find the regions of highest identity. The coding strand primer, RECA1, was based on the RecA protein consensus sequence 93-Phe-Ile-Asp-Ala-Glu-His-Ala-99 (FIDAEHA), in which the numbering corresponds to the *E.coli* RecA protein sequence, and its sequence was:

5'CGGGATCCTT(T,C)AT(C,A,T)GA(T,C)GCNGA(A,G)CA(C,T)GC-3'.

The recognition sequence of restriction enzyme *Bam*HI was incorporated at the 5' end of the primer for cloning purposes and is underlined. The two final 5' residues (CG) are a clamp which increases the efficiency of restriction enzyme digestion.

The complementary strand primer, RECA2, was based on the protein consensus sequence 196-Ile-Gln-Asn-Ile-Phe-Ile-191 (IQNIFI) and its sequence was:

5'-CTCCTGCAGAT(C,T)TG(G,A)TT(G,T,A)AT(G,A)AA(G,T,A)-3'.

The *Pst*I restriction enzyme site, incorporated for cloning the PCRDOP fragment, is underlined and, again there is a 5' clamp (CTC).

PCR reactions were prepared according to standard procedures (see method 4.2.4) and were subjected to 1 cycle of 94°C for 2 min., 45°C for 1 min., and 72°C for 2 min.; then 40 cycles of 94°C for 1 min., 50°C for 1 min., and 72°C for 1 min.; and, finally, 1 cycle of 55°C for 1 min. and 72°C for 7 min. (no ramping was necessary).

5.2.3 DNA manipulation and analysis.

Enzyme digests. Restriction enzymes were purchased from Gibco-BRL and were used in the manner specified by the suppliers (methods section 3.2.4). Restriction enzyme digested PCRDOP fragments were purified using the Promega Magic Minipreps™ DNA purification system, while digested pUC19 was phenol/chloroform extracted and ethanol precipitated as described previously (section 3.2.4).

Isolation of DNA fragments. Isolation of PCR and restriction enzyme digest products was carried out by electrophoresis in low melting point agarose gel and subsequent extraction of DNA fragments using Qiaex (Qiagen) according to the manufacturer's instructions.

Dephosphorylation. To approximately 4µg of plasmid DNA (16µl) were added 5µl 10x One-Phor-All buffer (Pharmacia), 4µl (4 units) calf intestinal alkaline phosphatase (Pharmacia) and 25µl sdH_2O. After incubating at 37°C for 1 hour, the reaction mixture was phenol/chloroform extracted and the dephosphorylated DNA was isolated by ethanol precipitation, as described previously.

Ligation. Ligations were carried out overnight at 15°C, as described in method 3.2.4.

Sequencing. Sequencing of the double-stranded DNA templates was performed by the dideoxynucleotide chain termination method of Sanger *et al.* [207], with T7 polymerase enzyme as described previously (method 3.2.10). The nucleotide sequence of the *recA* gene fragment was determined on both strands from the M13 forward and reverse primers.

5.2.4 Colony PCR screening for recombinants. In order to find clones containing the 350bp PCRDOP fragment, white colonies were picked and resuspended in 0.4ml sdH_2O. A small amount of this suspension was dotted onto a fresh LA-AMP plate and the rest was boiled for 5 min. The boiled cells were centrifuged at 12,000xg for 2 min. and 5µl of the supernatant were used in the PCR. The PCR reactions were performed according to standard procedures (see method 4.2.4) with vector primers P1 (5'-TCCCAGTCACGACGT-3') and P2 (5'-ATGTTGTGTGGAATTGTG-3') which flank the pUC19 multiple cloning site. The reaction products were electrophoresed on a 1%(w/v) agarose gel

containing 0.5μg/ml ethidium bromide and visualised on a UV transilluminator (method 3.2.4).

5.2.5 Electroporation of *C.jejuni*. The mutated *recA* pUC19 clone, pCJRK1, was introduced into *C.jejuni* by electroporation. *C.jejuni* strain 81116 was grown for 18 hours at 37°C on BFSA plates in an atmosphere provided by a VAIN and strain H132 was grown in the same conditions, except without the addition of selective supplement to the agar base (BFA plates). The cells were resuspended in 10ml of cold sterile wash buffer [272mM sucrose, 15%(v/v) glycerol] and harvested by centrifugation for 30min. at 3500xg and 4°C. They were resuspended on ice in a fresh 10ml of wash buffer then centrifuged as before. The pellet was resuspended in 1ml of wash buffer, centrifuged at 12,000xg for 5min., then finally resuspended in 1ml of wash buffer and kept on ice for imminent use.

50μl of washed *C.jejuni* cells were added to 5μl of sdH_2O (negative control), 5μl of pCJRK1 and 5μl of pTNS#A (positive control; kanamycin cassette inserted into fragment of *flaA* descibed previously in results section 3.3.2) in separate sterile microcentrifuge tubes on ice. A 1μl aliquot of the negative control sample was removed for a viable count at this stage. These cells were diluted to 10^{-4}, 10^{-5} and 10^{-6} and plated onto BFSA plates for strain 81116 and BFA plates for strain H132. Each mixture was elecroporated at 2.5KV, 200 Ohms and 25μFD and the time constant was noted. 100μl of SOC buffer were added immediately and the cells were allowed to recover overnight at 37°C in the VAIN on BFA plates. The recovered cells were harvested into 1ml of SOC buffer and 1μl of each sample was removed, diluted and plated onto BFA plates, as before, for a second viable count. The rest of the cells were plated onto BFA plates containing 50μg/ml kanamycin and incubated at 37°C for two days in a VAIN.

5.3 RESULTS

5.3.1 Primer design. The RecA protein sequences from a wide cross-section of different bacteria were aligned and regions of high similarity were highlighted. The sequences aligned were from *Mycobacterium tuberculosis*, *E.coli*, *Pseudomonas aeruginosa*, *Bordatella pertussis*, *N.gonorrhoeae*, *Thiobacillus ferrooxidans*, *Proteus mirabilis*, *Serratia marcescens*, *Aquaspirillum magnetotacticum*, *Anabaena variabilis*, *Methylobacillus flagellatum* and *Synechococcus* sp. Regions of similarity were sought which were separated by up to 200 amino acid residues and had a low number of possible codons, so that primers could be designed with as little degeneracy as possible, and some similarity between the DNA sequences at the chosen primer sites was also sought. A high amount of sequence similarity was particularly desirable for the 3′ end of each primer, since perfect homology at this end would allow efficient elongation. The RECA1 and RECA2 primer sequences (and the amino acids from which they were derived) are detailed in method 5.2.2.

5.3.2 PCRDOP. PCRDOP amplification using the RECA1 and RECA2 primers yielded a product of the desired size from all the bacterial genomic extracts tested, which included: *E.coli* strain DH5α, *Vibrio cholerae* strain CVD103, *C.jejuni* strains H132, D217, L1/1 and V161 (Figure 5.2), and also strains of *Listeria monocytogenes* and *Yersinia enterolitica* (data not shown). Due to the degenerate nature of the RECA primers, several PCRDOP products were obtained with each genomic DNA sample (Figure 5.2). Nevertheless, the expected 350bp product can be clearly identified. The amplified fragment from *C.jejuni* strain H132 was subsequently cloned into pUC19 and analysed.

5.3.3 Cloning of *C.jejuni recA* fragment. The purified 350bp PCRDOP fragment was digested with restriction enzymes *Bam*HI and *Pst*I and ligated into *Bam*HI and *Pst*I-digested pUC19 (method 5.2.3). The ligated pUC19 and PCRDOP fragment was transformed into *E.coli* strain JM105 by electroporation (method 4.2.2). Recombinant JM105 colonies, containing pUC19 with the inserted PCRDOP fragment, were white on selective plates containing IPTG and X-Gal, while nonrecombinants were blue. Fourteen white colonies were screened using colony PCR with primers P1 and P2 to find clones which had 350bp inserts into pUC19 (method 5.2.4). All of the white colonies screened had inserts of the expected size (data not shown).

In order to ensure that the recombinants contained *C.jejuni* DNA, Southern hybridisation was carried out at high stringecy, using the

digoxigenin-labelled 350bp inserts to probe restriction enzyme digests of *C.jejuni* chromosomal DNA. A single positive fragment in each chromosomal digest was bound by the *recA* probe and these were a 3.9kb *Ssp*I fragment, a 0.8kb *Hae*III fragment, a 12kb *Eco*RV fragment and a 0.4kb *Cla*I fragment (data not shown). This result confirmed that a *C.jejuni* DNA fragment had been isolated.

5.3.4 Sequence analysis. Since the 350bp PCRDOP product could possibly contain non-*recA* sequences, due to the degeneracy of the PCRDOP primers, it was necessary to confirm by sequencing that a *recA* gene fragment had been cloned. Sequence analysis of the *C.jejuni* insert DNA of four of the pUC19 clones with 350bp inserts, revealed that they all contained the same DNA fragment and that the true size of the fragment was 311bp. The *C.jejuni recA* fragment had significant homology with *recA* genes from other organisms (Figure 5.3) and showed 66.6% identity with the *recA* sequence of *B.subtilis* and 60.6% identity with that of *E.coli*. The deduced protein sequence also showed closer identity with Gram-positive *B.subtilis* RecA (75%) than with *E.coli* RecA (71.1%) (see Figure 5.4). The percentages of identity and similarity of the *C.jejuni* RecA protein fragment with the RecA proteins from five species of bacteria are detailed in Table 5.1.

The *C.jejuni* RecA protein retains the conserved amino acid residues which are essential for the function of the protein. For example, the glycine (G) residue at position 156 and the arginine (R) residue at 168 are required for homologous recombination and the prolines at 118 and 150 are thought to be important in dictating the protein structure by controlling folding events. The conserved region of hydrophobic amino acids from positions 139 to 143 consitutes the ATP hydrolysis B site and the proximal highly conserved region from 144 to 148 is thought to be involved in nucleotide binding [230]. The region from amino acid 156 to 164 also contains only conserved amino acids, in particular the two glycine residues at positions 156 and 159, and is a DNA-binding domain [230]. Significant amino acid changes in the *C.jejuni* RecA protein, compared with *E.coli* RecA, include the replacement of *E.coli* amino acids Glu^{159} (E), Ala^{180} (A) and Gln^{185} (Q) with Asp^{157} (D), Thr^{178} (T) and Lys^{183} (K) in the *C.jejuni* RecA fragment (see Figure 5.4). These substitutions are thought to result in the constitutive cleavage of LexA, even in the absence of DNA damage (discussed later).

5.3.5 Mutagenesis of *C.jejuni recA* gene. In order to determine the function of the *recA* gene in *C.jejuni*, an attempt was made to mutagenise the *recA* genes of *C.jejuni* strains 81116 and H132. A plasmid, pCJRK1, was constructed in

which a kanamycin resistance cassette was inserted into the unique *Bgl*II site in the 311bp *recA* fragment from the PCRDOP experiment. A pUC19 clone containing the *C.jejuni recA* fragment was restriction enzyme digested with *Bgl*II and subsequently dephosphorylated to prevent intramolecular ligation of the *recA* clone (method 5.2.3). 10µl (0.4µg) of *Bam*HI digested kanamycin cassette was then ligated with 4µl (0.2µg) of *Bgl*II digested and dephosphorylated *recA* clone. *E.coli* strain XL1-Blue was electroporated with 5µl of the ligation mixture, as described in method 4.2.2, and transformed cells were plated onto LA plates containing 100µg/ml ampicillin and 300µg/ml kanamycin (LA-A6K plates) to select for *recA* pUC19 clones with the kanamycin gene inserted (pCJRK1). Single colonies were used to inoculate LB containing 100µg/ml ampicillin and 50µg/ml kanamycin and incubated at 37°C overnight with shaking at 200rpm. These cultures were used to perform small-scale plasmid DNA preparations of pCJRK1 (as in method 3.2.1) and to prepare 1ml glycerol stocks for long term storage at -80°C.

The vector carrying the mutated *recA* gene, pUC19, has no *Campylobacter* origin of replication, therefore, it is unable to replicate and acts as a suicide vector when introduced into *C.jejuni*. Similarly, pTNS#A contains the vector pBluescript, which also cannot be maintained in *Campylobacter* cells. pTNS#A carries 0.7kb of *flaA* (and 2kb of upstream sequence) which has been interrupted by the kanamycin resistance gene and was used as a positive control in this experiment, since it had been previously shown to successfully disrupt the flagellin genes of *C.jejuni* 81116 when introduced directly by electroporation [55]. pCJRK1 and pTNS#A were transformed into *C.jejuni* 81116 and *C.jejuni* H132 by electroporation (method 5.2.5).

Viable counts revealed that 1µl of electroporation mixture which had been allowed to recover on non-selective plates overnight contained approximately 1×10^8 viable *C.jejuni* cells, giving a total of 1×10^{11} viable cells resuspended in 1ml of SOC buffer. Plating the rest of the recovered electroporated cells onto BFA plates containing 50µg/ml kanamycin yielded a total of **311** colonies of *C.jejuni* strain 81116 electroporated with pTNS#A and **216** colonies of *C.jejuni* strain H132 electroporated with pTNS#A. There were no colonies on kanamycin plates onto which *C.jejuni* strains 81116 and H132 had been plated which had been electroporated with sdH_2O (negative control) or pCJRK1. Therefore, the yield of putative *flaA* mutants was 3.11×10^{-9} for *C.jejuni* 81116 and 2.16×10^{-9} for *C.jejuni* H132, but no putative *recA* mutants were isolated for either strain.

5.3.6 Isolation of entire *C.jejuni* H132 *recA* gene. In order to enable the construction of a *C.jejuni* H132 *recA* mutant by homologous recombination, it was necessary to isolate more of the *C.jejuni recA* gene. The Lambda ZAP II library of *C.jejuni* H132 DNA was screened with the radiolabelled *recA* fragment, by the method described in sections 2.2.9, 2.2.11 and 2.3.6. Five positive plaques were obtained and the pBluescript clones were rescued from them. However, before the analysis of these clones was taken further, the sequence of the entire *C.jejuni* 81-176 *recA* gene was published [186]. It was then possible to isolate the entire *C.jejuni* H132 *recA* gene by designing PCR primers, CJREC1 and CJREC2, from the published *Campylobacter recA* sequence and amplifying the 1.2kb gene from strain H132 genomic DNA. The *C.jejuni* 81-176 *recA* gene sequence and the CJREC1 and CJREC2 primers are shown in Figure 5.5. *Bam*HI and *Pst*I restriction enzyme sites were added to the 5′ ends of the primers to facilitate the cloning of the *recA* gene into the pBluescript vector and recombinant clones were successfully isolated. The first 688bp of the H132 *recA* gene have been sequenced and show 90% identity with the corresponding region of the *C.jejuni* 81-176 *recA* gene. The nucleotide differences between the two *C.jejuni* genes are conserved with respect to the amino acids which they encode, so that the protein sequences are identical (see Figure 5.6).

Figure 5.7 shows the alignment of the upstream regions of *recA* genes from *E.coli*, *Shigella flexneri*, *Erwinia carotovora*, *Proteus vulgaris*, *N.gonorrhoeae* and *C.jejuni*. Similarly to *N.gonorrhoeae*, the sequence upstream from the *C.jejuni recA* ORF contains no consensus LexA binding region (5′-CTGTN$_9$CAG-3′). This suggests that the *C.jejuni recA* gene is not under the control of the LexA repressor (discussed later).

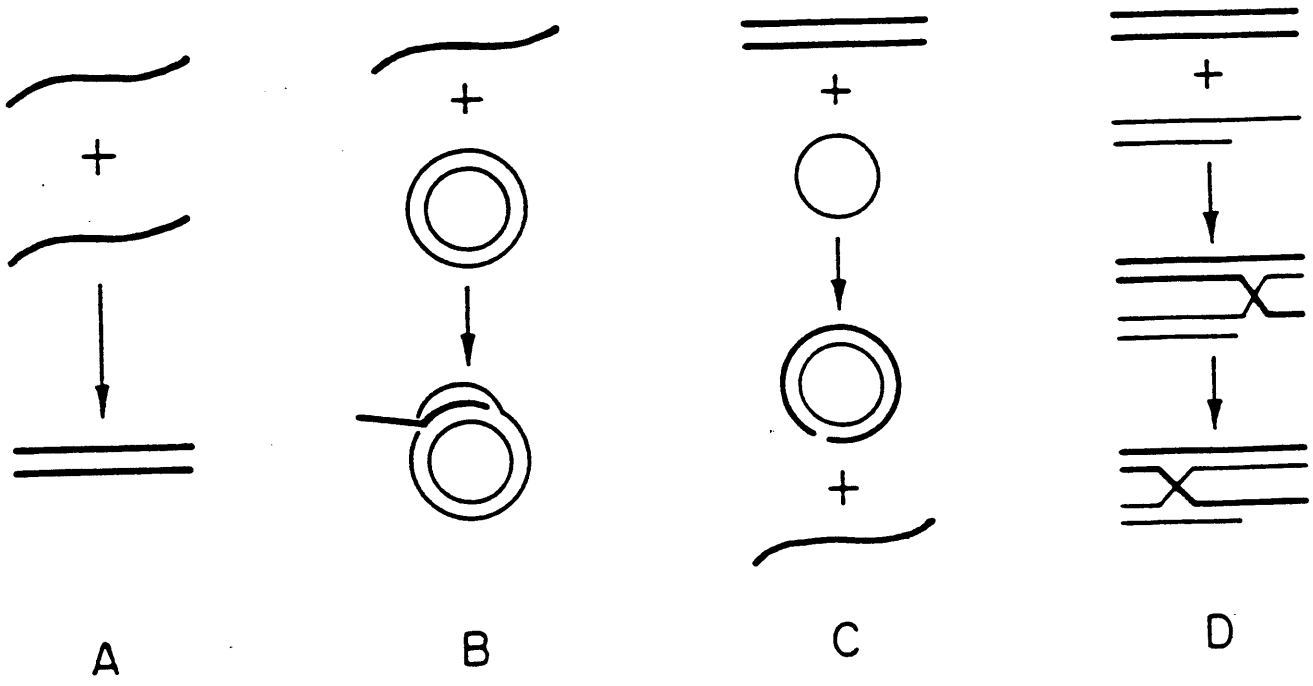


Figure 5.1

Schematic diagram to show interactions between DNA strands promoted by RecA. A) annealing of complementary ssDNA to form dsDNA. B) D-loop formation from linear ssDNA and circular dsDNA. C) conversion of linear dsDNA and circular ssDNA into nicked circular dsDNA and linear ssDNA. D) reaction of two linear dsDNA molecules, one of which has a ssDNA tail, to form the cross-stranded structure designated the Holliday junction. Continued reciprocal strand transfer results in movement of the Holliday junction, designated branch migration. Adapted from ref. 217.

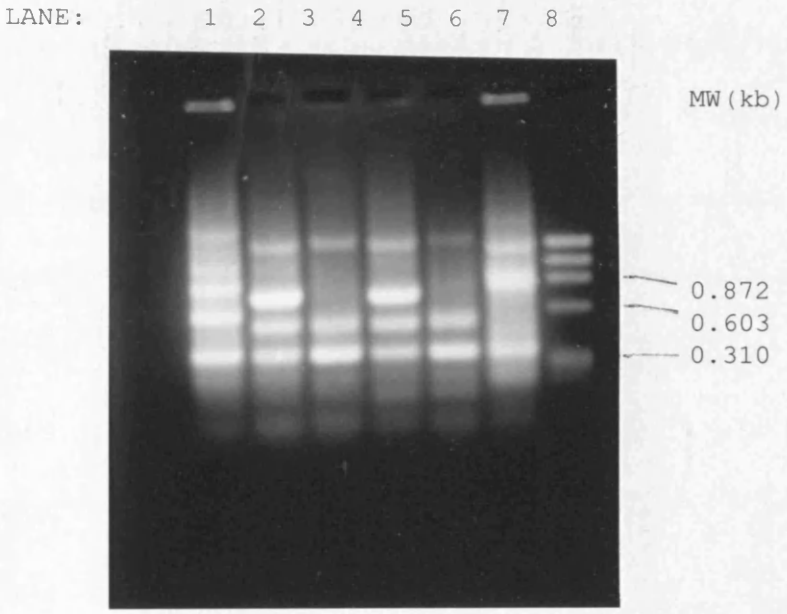


Figure 5.2

PCRDOP to isolate a fragment of the *C.jejuni recA* gene. Products of a PCRDOP reaction, using primers RECA1 and RECA2 against genomic DNA, were separated by electrophoresis on a 1.5% (w/v) agarose gel.

Lane 1: sdH_2O negative control; Lane 2: *E.coli* DH5α genomic DNA used as template; Lane 3: *C.jejuni* H132 genomic DNA; Lane 4: *C.jejuni* D217 genomic DNA; Lane 5: *C.jejuni* L1/1 genomic DNA; Lane 6: *C.jejuni* V161 genomic DNA; Lane 7: *V.cholerae* CVD103 genomic DNA; Lane 8: ϕX174 *Hae*III molecular weight markers (sizes, in kb, shown on right-hand side).

Figure 5.3

Alignment of bacterial *recA* gene fragment DNA sequences. The top line contains the *C.jejuni recA* fragment. Sequences which served as primer-binding sites are underlined, but note that the DNA sequence at these sites may not be the true *in vivo* sequence. Aligned with the *C.jejuni* DNA sequence are the corresponding *recA* sequences from *Bacillus subtilis* (Bs), *Erwinia carotovora* (Eca), *Vibrio anguillarium* (Va), *Neisseria gonorrhoeae* (Ng) and *Escherichia coli* (Eco). Dashes (-) indicate nucleotides that are identical to the corresponding nucleotide in *C.jejuni*. Dots (.) indicate that there is no nucleotide in that position.

```
 5'-  1
Cj    TTATAGATCGCTGAGCATGCGCTTGATGTAAAATATGCTA-40
Bs    -----C--T--G---------T-A---CCGGT---C--GC
Eca   --C--T--T--------C--TT-A--CCCT-TC------C
Va    -----C--T--A--A-----C-----CCC--TT-------
Ng    ---G-C--T--C--A--C--CT-C---CCCGTT--C--CC
Eco   -----C--T-----A--C-----G--CCC--TC--C--AC

Cj    AAAATTTGGGTGTAAATACAGATGATTTGTATGTTTCTCA-80
Bs    ----GC-C-----T--C-TC--A--GC-T-TAC-G-----
Eca   ----AC-A--C---G---TT---A-CC--CTCTG------
Va    ----AC----------C-TC-----GC---TG-----A--
Ng    GC--AC-C--C-----AGTC--A--GC-T--CC-G--C--
Eco   GT--AC----C--CG---TC--CA-CC--CTGTGC--C--

Cj    GCCTGATTTTGGAGAACAAGCCTTAGAAATTGTAGAAACT-120
Bs    ------CACA--C--G--G--GC-T-------CG---G-A
Eca   -------AC---T---------C--------TGT--TG-A
Va    A------ACG--C--------GC-T------TGT--TG-C
Ng    ---C---ACC--C-----G--T--G-----CTGC--C--G
Eco   ---G--CACC--C--G--G--AC-G-----CTGT--CG-C
                        BglII
Cj    ATAGCAAGAAGTGGCGCAGTAGATCTTATCGTAGTAGATA-160
Bs    T-G-TTC----C--G-----T--CA--G----T--C--CT
Eca   T--T-TC-TTC---T-----T---G-------T--T--CT
Va    T-G--CC-TTC---T--CA-T---G-------CA-T--CT
Ng    C-C-TCC-TTCG----GCA-----A-GG-A--C--C---T
Eco   C-G--GC-TTC----------CG-------C--T--CT

Cj    GCGTTGCAGCACTTACCCCAAAAGCAGAAATTGAAGGCGA-200
Bs    CT--A--C--T--CGTT--G-----G--------------
Eca   C---------TT-A--A--------------------T--
Va    CT--G--T-----G--A--------T-----------T--
Ng    C---A--C-----CGT---C-----T-----------T--
Eco   C---G--G-----G--G--G-----G-----C--------

Cj    TATGGGCGATCAACATGTAGGACTTCAAGCAAGACTTATG-240
Bs    C-----A---TCG-----C--TT-A------C-CT-A---
Eca   G--T--T---TC---C--T--TT-AGCT--TC-TA-----
Va    A---------AGC--CA-G--T--A-----CC-TA-GC-T
Ng    ------G--CAGC--C--C-----G--G--GC-C--G---
Eco   A--C-----CTCT--CA-G---C--GCG---C-TA-G---

Cj    TCTCAAGCTCTAAGAAAACTTACAGGTATAGTTCATAAAA-280
Bs    --------G--TC-T--G---T----GGCCA--A-C---T
Eca   AG-------A-GC-T--GR-AG-G----.-C--G-A---C
Va    --------GA-GC-T--GT-A--G----ACC-AA-AC-GT
Ng    -----G---T-GC-C-----G--C--ACACA-CA-A----
Eco   AGC--G--GA-GC-T--G--GG-G----ACC-GA-GC-GT

                                        311
Cj    T.GAATACCACAGTAATATTCATAAACCAAAT - 3'
Bs    C.---G--A-TC-CG--T-----T--------
Eca   -CC--C--TCTGC-T--T-----C--------
Va    C.C--CTG--TGTGT--C-----C-----G--
Ng    C.C-----GCTG--TG-G-----C--------
Eco   C.C--C--GCTGC-G--C-----C-----G--
```

```
     91       100       110       120       130
Cj   FIDAEHALDVKYAKNLGVNTDDLYVSQPDFGEQALEIVET
Bs   ---------PV--QK----IEE-LL----T-------A-A
Eca  ---------PI--QK---DI-N-LC----T-------CDA
Va   ---------PI---K----I-E-LV----T-------CDA
Ng   -V-----F-PV--RK---KVEE--L----T-------CD-
Eco  ---------PI--RK---DI-N-LC----T-------CDA

             140       150       160       170
Cj   IARSGAVDLIVVDSVAALTPKAEIEGDMGDQHVGLQARLM
Bs   LV------IV--------V-----------S---------
Eca  LS------V-----------------EI--S----A--M-
Va   L-----I-V--I--------------E---S-M-----ML
Ng   LV---GI-MV--------V-----------S---------
Eco  L-------V-----------------EI--S-M--A--M-

             180       190  194
Cj   SQALRKLTGIVHKMNTIVIFINQI
Bs   -------S-AIN-SK-IA------
Eca  ---M---A-NLKNS--LL------
Va   ---M-----NLKQS-CMC------
Ng   ---------HIK-T--L-V-----
Eco  ---M---A-NLKQS--LL------
```

Figure 5.4

Alignment of deduced amino acid sequence of the *C.jejuni* RecA fragment with RecA from other bacteria. Dashes (-) in the sequence refer to amino acids that are identical to the corresponding amino acid in *C.jejuni* RecA. Underlined amino acids are referred to in the text. Amino acids in bold are substitutions thought to result in constitutive protease activity. The numbers refer to the positions of amino acid residues in the *C.jejuni* RecA protein sequence. Organism abbreviations are as in Figure 5.3.

	% IDENTITY with *C.jejuni* RecA	% SIMILARITY with *C.jejuni* RecA
Bacillus subtilis	75.0	82.7
Neisseria gonorrhoeae	73.1	86.5
Vibrio anguillarum	73.0	84.6
Escherichia coli	71.1	83.6
Erwinia carotovora	71.1	81.7

Table 5.1

Table to show percentages of identity and similarity of the RecA protein sequences from five different bacteria with the *C.jejuni* H132 RecA protein fragment.

Figure 5.5

DNA and deduced amino acid sequence of the *C.jejuni* 81-176 *recA* gene (from ref. 186). The binding sites of the primers, CJREC1 and CJREC2, which were used to amplify the *C.jejuni* H132 *recA* gene are underlined. "*" denotes the stop codon at the end of the *recA* gene.

CJREC1

5′-GGAGAATGTGCAATAGTAATTTTTTTCATCTATGCTTCCTTGTAAATCTTTGCTTTAATT 60

CTAGCAAAATAAATCATTATTTAATTTTATTTTGTTAGAATTCTAGCTAAAAATTTAGAA 120

TTTTTAAGGAAAGTTATGGATGATAATAAAAGAAAATCTCTAGACGCTGCCCTAAAAAGT 180

CJRecA → M D D N K R K S L D A A L K S 15

TTAGATAAAACCTTTGGAAAAGGCACTATTTTAAGACTAGGGGATAAAGAAGTTGAGCAA 240

L D K T F G K G T I L R L G D K E V E Q 35

ATCGATAGCATAGGCACAGGTTCAGTTGGGCTTGATCTTGCTTTAGGTATAGGCGGTGTT 300

I D S I G T G S V G L D L A L G I G G V 55

CCAAAAGGAAGAATTATAGAAATTTATGGACCTGAAAGTTCAGGTAAAACCACTCTAACT 360

P K G R I I E I Y G P E S S G K T T L T 75

TTACATATTATCGCAGAATGCCAAAAAGCAGGTGGAGTTTGTGCTTTTATCGATGCAGAA 420

L H I I A E C Q K A G G V C A F I D A E 95

CATGCGCTTGATGTAAAATATGCTAAAAATTTGGGTGTAAATACAGATGATTTGTATGTT 480

H A L D V K Y A K N L G V N T D D L Y V 115

TCTCAGCCTGATTTTGGAGAACAAGCCTTAGAAATTGTAGAAACTATAGCAAGAAGTGGC 540

S Q P D F G E Q A L E I V E T I A R S G 135

GCAGTAGATCTTATCGTAGTAGATAGCGTTGCAGCACTTACTCCAAAAGCAGAAATTGAA 600

A V D L I V V D S V A A L T P K A E I E 155

GGCGATATGGGCGATCAACATGTAGGACTTCAAGCAAGACTTATGGCTCAAGCTCTAAGA 660

G D M G D Q H V G L Q A R L M S Q A L R 175

AAACTTACAGGTATAGTTCATAAAATGAATACCACAGTAATTTTTATCAACCAAATTCGT 720

K L T G I V H K M N T T V I F I N Q I R 195

ATGAAAATCGGTGCTATGGGTTATGGCACTCCTGAAACTACAACAGGTGGAAATGCATTA 780

M K I G A M G Y G T P E T T T G G N A L 215

AAAATCTATGCTTCTGTGCGTTTAGATGTTAGAAAAGTAGCAACCTTAAAACAAAACGAA 840

K I Y A S V R L D V R K V A T L K Q N E 235

GAACCTATAGGAAACCGCGTTAAAGTAAAAGTAGTTAAAAATAAAGTTGCTCCTCCATTC 900

E P I G N R V K V K V V K N K V A P P F 255

AGACAAGCTGAATTTGATGTGATGTTTGGAGAGGGTTTAAGCCGTGAAGGTGAATTGATC 960

R Q A E F D V M F G E G L S R E G E L I 275

GATTATGGTGTAAAACTTGATATCGTAGATAAAAGTGGTGCGTGGTTTTCTTATAAAGAT 1020

D Y G V K L D I V D K S G A W F S Y K D 295

AAAAAACTTGGACAAGGTAGAGAAAATTCAAAAGCTTTCTTAAAAGAAAACCCTGAAATT 1080

K K L G Q G R E N S K A F L K E N P E I 315

GCACATGAAATCACAAAAGCAATTCAAAATTCTATGGGAATAGAAGGTATGATCAGCGGT 1140

A D E I T K A I Q N S M G I E G M I S G 335

CJREC2

AGCGAAGATGACGAAGGAGAAGAATAATGTTAGTAATTGAAGATGTTAGAGCCTATGAAG 1200

S E D D E G E E * M L V I E D V R A Y E V

343

TTCTTGATAGTAGAGGAAATCCAACCGTAAAAGCCGAAGTTACGCTAAGCGATGGAAGTG 1260

L D S R G N P T V K A E V T L S D G S V

TAGGTGCG-3′ 1268

G A

Figure 5.6

Alignment of the first 715bp of DNA sequence of the *C.jejuni* H132 *recA* gene with the *C.jejuni* 81-176 *recA* gene. The deduced amino acid sequence, which is the same for both strains, is also shown. "-" denotes identical nucleotide in the 81-176 sequence to that in the corresponding position of the H132 sequence. Primers referred to in the text are underlined and the *BglII* site, which was used for insertion of the kanamycin resistance cassette, is shown in bold type.

```
        28      CJREC1
H132    ATCTATGCTTCCTTGTAAATCTTTGCTTTAATTCTAGCAAAATAAATCAT
81-176  -------------------------------------------------- 77

H132    TATTTAATTTTATTTGGTTATAATTCTAACTAAAAATTTAGAATTTTTAA
81-176  ---------------T----G-------G--------------------- 127

H132    GGAAAGTT ATG GAT GAT AAT AAA AGA AAA TCT CTA GAC G
81-176  -------- --- --- --- --- --- --- --- --- --- --- - 166
                 M   D   D   N   K   R   K   S   L   D     10
H132    CT GCC CTA AAA AGT TTA GAT AAA ACC TTT GGA AAA GGC
81-176  -- --- --- --- --- --- --- --- --- --- --- --- --- 203
        A   A   L   K   S   L   D   K   T   F   G   K   G  23
H132    ACT ATC TTA AGA CTA GGG GAT AAA GAA GTC GAG CAA AT
81-176  --- --T --- --- --- --- --- --- --- --T --- --- -- 241
         T   I   L   R   L   G   D   K   E   V   E   Q   I 36
H132    C GAT AGC ATA GGC ACA GGC TCA GTG GGA CTT GAT CTT
81-176  - --- --- --- --- --- --T --- --T --G --- --- ---  278
           D   S   I   G   T   G   S   V   G   L   D   L   48
H132    GCT TTA GGT ATA GGC GGT GTC CCA AAA GGA AGA ATT AT
81-176  --- --- --- --- --- --- --T --- --- --- --- --- -- 316
         A   L   G   I   G   G   V   P   K   G   R   I   I 61
H132    A GAA ATT TAT GGG CCT GAA AGT TCA GGT AAA ACC ACT
81-176  - --- --- --- --A --- --- --- --- --- --- --- --- ---  353
           E   I   Y   G   P   E   S   S   G   K   T   T   73
H132    CTA ACT CTA CAC ATT ATC GCA GAA TGC CAA AAA GCA GG
81-176  --- --- --- --T --- --- --- --- --- --- --- --- -- 391
         L   T   L   H   I   I   A   E   C   Q   K   A   G 86
                                 RECA1
H132    T GGG GTT TGT GCT TTT ATA GAC GCT GAG CAT GCG CTT
81-176  - --A --- --- --- --- --C --T --A --A --- --- ---  428
           G   V   C   A   F   I   D   A   E   H   A   L   98
H132    GAT GTA AAA TAT GCT AAA AAT TTG GGT GTA AAT ACA GA
81-176  --- --- --- --- --- --- --- --- --- --- --- --- -- 466
         D   V   K   Y   A   K   N   L   G   V   N   T   D 111
H132    T GAT TTG TAT GTT TCT CAG CCT GAT TTT GGA GAA CAA
81-176  - --- --- --- --- --- --- --- --- --- --- --- ---  503
           D   L   Y   V   S   Q   P   D   F   G   E   Q   123
H132    GCC TTA GAA ATT GTA GAA ACT ATA GCA AGA AGT GGC GC
81-176  --- --- --- --- --- --- --- --- --- --- --- --- -- 541
         A   L   E   I   V   E   T   I   A   R   S   G   A 136
            BglII
H132    A GTA GAT CTT ATC GTA GTA GAT AGC GTT GCA GCA CTT
81-176  - --- --- --- --- --- --- --- --- --- --- --- ---  578
           V   D   L   I   V   V   D   S   V   A   A   L   148
H132    ACC CCA AAA GCA GAA ATT GAA GGC GAT ATG GGC GAT CA
81-176  --T --- --- --- --- --- --- --- --- --- --- --- -- 616
         T   P   K   A   E   I   E   G   D   M   G   D   Q 161
H132    A CAT GTA GGA CTT CAA GCA AGA CTT ATG TCT CAA GCT
81-176  - --- --- --- --- --- --- --- --- --- --- --- ---  653
           H   V   G   L   Q   A   R   L   M   S   Q   A   173
H132    CTA AGA AAA CTT ACA GGT ATA GTT CAT AAA ATG AAT AC
81-176  --- --- --- --- --- --- --- --- --- --- --- --- -- 691
         L   R   K   L   T   G   I   V   H   K   M   N   T 186
                             RECA2
H132    C ACA GTA ATA TTC ATA AAC CAA AT
81-176  - --- --- --- --T --C --- --- -- 715
           T   V   I   F   I   N   Q   I 194
```

Figure 5.7

Figure to show the alignment of the upstream regions of *recA* genes from *E.coli* (Eco), *Shigella flexneri* (SF), *Erwinia carotovora* (Eca), *Proteus vulgaris* (PV), *N.gonorrhoeae* (NG) and *C.jejuni* (CJ). The ATG start codons and ribosome binding sites are shown in bold. The LexA repressor binding sites are underlined. Similarly to *N.gonorrhoeae*, the sequence upstream from the *C.jejuni recA* ORF contains no consensus LexA binding region (5'-CTGTN$_9$CAG-3'). This suggests that the *C.jejuni recA* gene is not under the control of the LexA repressor.

RBS

ECo GTGGCAACAA TTTCTACAAA ACACTTGATA CTGTATGAGC ATACAGTATA ATTGCTTCAA CAGAACATAT TGACTATCCG GTATTACCCG GCATCAC**AGG A**GTAAAA**ATG**

RBS

SF GTGGCAACAA TTTCTACAAA ACACTTGATA CTGTATGAGC ATACAGTATA ATTGTTTCAA CAGAACATAT TGACTATCCG GTATTACCCG GCATGAC**AGG A**GTAAAA**ATG**

RBS

ECa CCTTGATGCT GTATGCGCAT ACAGTATAAT GTCAGTAATT ATTCAGCAAT TCATAATTAA GCCGCCGCGT AGAGACAGTA GCTTTACGCC GCATGAC**AGG A**GCAAAA**ATG**

RBS

PV CTTGATACTG TATGATTATA CAGTATAATG AGTTTCAACA AGCAAAATCA TATACGTTTT AATGGTAGTG ACCCATTTTT ATGCTTCACT GCCCAG**AGGG A**GATAAC**ATG**

RBS

NG AGCCCCCGAA AGGCAAACAT GCGATAATGA CCGACCGATT CATATTCCGC GCCGCAGCCC GTGTTGCGCC GCAGCCCACA TACCGCATTT GTTCC**GGAG**T AACCCCC**ATG**

RBS

CJ TCTATGCTTC CTTGTAAATC TTTGCTTTAA TTCTAGCAAA ATAAATCATT ATTTAATTTT ATTTTGTTAG AATTCTAGCT AAAAATTTAG AATTTTTA**AG GA**AAGTT**ATG**

5.4 DISCUSSION

Using degenerate PCR primers, based on two conserved regions of the RecA protein, allowed the amplification of gene fragments from several different bacterial species and the successful isolation of a 311bp internal fragment of the *C.jejuni* strain H132 *recA* gene. The PCRDOP technique is clearly a very useful and less time consuming method of obtaining *recA* sequences. In fact, after this study had been devised, it was reported that PCRDOP had been used successfully to isolate *recA* fragments from Gram-positive bacteria [231, 232], organisms for which methods such as complementation and hybridisation had failed.

5.4.1 Primer design. The primers used were designed from areas of homology which were found on alignment of the RecA protein sequences from twelve different microorganisms. Dybvig *et al.* [231] chose the same N-terminal area of similarity (the amino acid sequence 91-FIDAEHA-97, in which the numbers refer to the positions of the amino acids in the *C.jejuni* RecA protein) and nearly the same C-terminal sequence (192-NIFIA-188, whereas I used 194-IQNIFI-189) for the design of the degenerate primers which they used to amplify *recA* fragments from Gram-positive bacteria. Duwat, Ehrlich and Gruss [232] used the sequences 95-EHALDP-100 and 211-GTTTEP-206 for designing the primers to amplify the *Lactococcus lactis recA* fragment. More recently, Guerry *et al.* used the same technique to isolate a 180bp fragment of the *C.jejuni* 81-176 *recA* gene, with primers designed from the conserved peptides 62-EIYGP-66 and 122-EGTDP-118. They used the *recA* fragment to probe *Hin*dIII digested *C.jejuni* DNA and thus clone the entire *C.jejuni recA* gene [186]. A 470bp fragment of the *Helicobacter pylori recA* gene has also been isolated using PCRDOP, with the primers designed from the conserved peptides 62-EIYGPE-67 and 219-AYKFLAN-213 [233].

5.4.2 Sequence analysis. Sequence analysis of the cloned *C.jejuni* H132 *recA* gene fragment revealed a higher degree of similarity with *B.subtilis recA* than with the *E.coli recA* gene. This may be due to the A/T rich nature of *C.jejuni* DNA, but other *Campylobacter* genes have also been found to have greater similarity with those of Gram-positive organisms. These include the kanamycin resistance gene found on a *C.coli* plasmid, which shows a high degree of identity with the *aphA*-3 gene, previously found only in Gram-positive cocci [234]. In addition, tetracycline resistance determinants from *C.coli* and *C.jejuni* plasmids demonstrate 75-76% identity with the *tetM* gene of *Streptococcus pneumoniae* [235]. The amino acid sequence also showed greater similarity with the *B.subtilis* RecA (75%) than with the

E.coli RecA protein (71.1%), which suggests that the DNA similarity is not solely due to its A/T rich nature. Most of the amino acid changes were conserved in terms of their function, as has been found for the RecA proteins from other bacteria [230]. However, the deduced amino acid sequence from the *C.jejuni* H132 *recA* fragment contained some substitutions which could indicate that the *Campylobacter* RecA has elevated levels of protease activity in the absence of DNA-damaging agents and, thus, is not under the control of the LexA repressor protein. These substitutions are *E.coli* amino acids Glu^{159}, Ala^{180} and Gln^{185} which have been replaced by Asp^{157}, Thr^{178} and Lys^{183} in the *C.jejuni* RecA fragment.

In *E.coli*, the protease activity of RecA results in cleavage of the LexA repressor of the *recA* gene when DNA damage occurs. Organisms which have the same, or similar, amino acid substitutions to those found in the *C.jejuni* protein include *Neisseria gonorrhoeae* [236], *Bacteroides fragilis* [237] and *Thiobacillus ferrooxidans* [238] and these organisms appear not to be subject to LexA control. It has been speculated that the control of the *recA* gene in *N.gonorrhoeae* may have evolved to give transformation and recombination systems which facilitate the evasion of host defence mechanisms [236]. In *N.gonorrhoeae*, pilin antigenic variation can occur, not only by intragenomic recombination, but also by the uptake of silent pilin DNA sequences from lysed cells which subsequently recombine with expressed pilin genes in living piliated cells.

Recently, the sequence of the entire *C.jejuni* strain 81-176 *recA* gene has been published [186]. The sequence is shown in Figure 5.5 and the positions of the two PCRDOP primers, RECA1 and RECA2, are underlined. The deduced amino acid sequence from this gene shows greatest similarity with the *N.gonorrhoeae* RecA (66% identity) and investigation of the region upstream of the *C.jejuni recA* ORF reveals that there is no consensus LexA binding site (5'-CTGTN_9CAG-3') present (see Figure 5.7). Therefore, it is possible that *C.jejuni*, which is also naturally transformable, has evolved to increase the ability to recombine with exogenous DNA without initiating the SOS response.

5.4.3 Mutagenesis. The final aim of this study was to determine the role of the *recA* gene in *C.jejuni* by characterising a defined *recA* mutant strain. An attempt was made to construct this *recA* mutant by combining the PCRDOP fragment with a selectable marker and insertionally inactivating the *recA* gene by homologous recombination. No mutants were achieved with this method, possibly because the 144bp and 168bp regions of *C.jejuni* DNA flanking the kanamycin cassette were not long enough for homologous recombination to occur. Experiments with other PCRDOP fragments in this

laboratory has revealed similar results. It was found that the 250bp *virG* fragment and a 750bp *cheY* fragment (with flanking regions of 400 and 350bp) were not capable of mutagenesis by homologous recombination in *C.jejuni* and longer gene segments had to be isolated to allow mutagenesis to be carried out. In contrast, a C.jejuni *htrA* mutant had been produced previously using a PCRDOP fragment of 473bp, with flanking sequences of approximately 230bp on each side of the kanamycin cassette. It was the successful production of this *htrA* mutant which encouraged the attempts to mutagenise other genes using small gene fragments.

The pTNS#A positive control construct had over 1kb of *C.jejuni* DNA on either side of the kanamycin resistance gene and kanamycin resistant *C.jejuni* colonies were obtained for this plasmid. The successful isolation of putative flagellin gene mutants of *C.jejuni* strain H132 indicates that this organism contains sequences which are homologous to the flagellin genes of other *Campylobacter* strains.

5.4.4 Future work. Now that the entire *C.jejuni* H132 *recA* gene has been cloned, future work will involve cloning the kanamycin resistance gene into the *Bgl*II site of the H132 *recA* gene and introducing this construct into *C.jejuni* by electroporation, as before, in the hope that a *recA* mutant will then be obtained by homologous recombination. As discussed in the introduction, RecA has an indirect role in the virulence of some pathogenic microorganisms. Guerry *et al.* showed that, as expected, natural transformation of *C.jejuni* is dependent on a functional RecA protein, since no transformation was detectable in *recA* mutant strains [186]. They also found that the *C.jejuni* 81-176 *recA* mutant colonised rabbits almost as well as the wild type organism and produced a protective immune response in the rabbits. However, their paper concentrated on the use of the *recA* mutant for a vaccine and, thus, did not explore the potential role of RecA in the pathogenesis of *C.jejuni*. It would be of interest to discover whether the *C.jejuni* H132 *recA* mutant had reduced virulence in human volunteer studies and in animal models (ferrets, for example), since it was originally isolated as an inflammatory strain (as mentioned in previous chapters).

RecA mutants of other pathogenic bacteria have been found to be avirulent. A *S.typhimurium recA* mutant was found to be avirulent due to an increased susceptibility to oxidative killing by macrophages [228] and *recA* mutation may have a similar effect in *C.jejuni*. *V.cholerae recA* mutants have reduced virulence due to their inability to amplify the virulence cassette containing the cholera toxin genes [191]. As yet, such a virulence cassette has not been identified in *C.jejuni* and no

overproduction of a toxin, or other virulence determinant, has been observed *in vivo* or *in vitro*.

As described earlier, in *N.gonorrhoeae*, antigenic variation of the pilin protein occurs via rearrangements of the pilin genes which must be dependent upon the RecA protein [226]. The phase variation of the *N.gonorrhoeae* pilus, i.e. switching between Pil^+ and Pil^-, can also occur via homologous recombination. Uneven homologous exchange between two pilin genes can lead to synthesis of a large form of the pilin protein which is not processed and assembled into pili. Recently, *Campylobacter* species were shown to produce pilus-like appendages when they were grown in the presence of bile salts, but the pilin subunit gene has not yet been isolated. Thus, it is not known whether *Campylobacter* spp. possess multiple copies of pilin genes which could be involved in the phase variation and/or antigenic variation of the pilus by recombination events. *Campylobacter* proteins which are known to undergo variation due to genetic rearrangements are the S-layer proteins (SLPs) of *C.fetus*. The SLPs form a crystalline layer of closely packed protein subunits on the surface of *C.fetus* and this surface layer has a critical role in resistance to the host immune response [239]. Reciprocal recombination events among the eight SLP gene cassettes, which encode 97-149kDa SLPs, results in the variation in size and antigenicity of the predominant SLP [240]. Dworkin and Blaser [241] recently showed that SLP expression utilises a single promoter which is situated between two oppositely oriented SLP gene cassettes. The gene cassette which is expressed can be varied by inversion of the 6.2kb segment of DNA containing the promoter, resulting in expression of the opposite SLP gene cassette. Dworkin and Blaser [241] found that variation could also occur by the recombination of another SLP gene cassette (there are eight in total) into at least one of the sites flanking the single promoter. They state that flanking homologies may be necessary for inversion of the 6.2kb DNA segment, consistent with the hypothesis that *C.fetus sapA* DNA rearrangement is RecA-dependent. Although *C.jejuni* does not possess the S-layer, this example of a RecA-dependent antigenic variation system shows that such a process can occur in *Campylobacter*.

A *C.jejuni* virulence determinant which has been shown to exhibit phase and antigenic variation, and which possesses two homologous genes for its constituent protein, is the flagellum. In *Salmonella typhi*, intragenic recombination can occur between a pair of repeats in the middle of the *fla* gene, leading to the appearance of a new flagellin serotype [242]. It has been demonstrated that *C.coli flaA* mutants can regain a functional flagellum by homologous recombination between the mutated *flaA* and normal

flaB genes [227]. Moreover, the process of natural transformation combined with intergenomic recombination were capable of rescuing the motile phenotype when selective pressure for the mutational kanamycin cassette was maintained [227]. The presence of a functional RecA protein must be crucial for such recombinational rescue of the fully motile phenotype, which is essential for the pathogenicity of *Campylobacter* spp. However, the antigenic variation of *Campylobacter* flagellin is known to be due to post-translational modifications, not homologous recombination events [243, 244]. Wassenaar *et al.* have also shown that expression of the two flagellin genes in *C.jejuni* 81116 is regulated at the transcriptional level, since the two flagellin genes are transcribed from different promoters [161]. They suggested that one flagellin gene (*flaA* or *flaB*) was transcribed at a time so that different types of flagella, composed of different proportions of FlaA and FlaB proteins, were synthesized according to environmental conditions. Investigations of the synthesis of FlaA and FlaB in the *recA* mutant would enable the differential transcription theory (as opposed to recombination events) to be confirmed.

The successful isolation of the *C.jejuni* H132 *recA* gene means the completion of the essential first step in the investigation of the function of *Campylobacter* RecA and the evaluation of the contribution of RecA to the virulence of *C.jejuni*.

CHAPTER SIX

GENERAL DISCUSSION

The major objective of research into *C.jejuni* is to discover ways of preventing *C.jejuni* from causing disease. A molecular genetic approach has yielded a great deal of information concerning the pathogenesis of other organisms, but, until recently, such an approach had not been very successful with *Campylobacter* spp. As a first step in the utilisation of molecular genetic techniques, it was necessary to develop workable strategies and systems for identifying *C.jejuni* genes encoding potential virulence determinants. The preceding chapters have described the use of two different methods for the isolation of *C.jejuni* genes. In the first method, an expression vector-based library of *C.jejuni* DNA was screened with convalescent antiserum in order to isolate genes for unknown antigenic proteins, whereas the second method used PCR with degenerate primers to isolate a fragment of a known gene. Both of these techniques were successful in finding *C.jejuni* genes of interest, which may provide some insight into the pathogenesis and biology of *C.jejuni*.

6.1 Screening a Lambda ZAP II library of *C.jejuni* genomic DNA with convalescent antiserum.

The advantages of the library screening technique are that the convalescent antiserum can detect products expressed by library clones which are synthesized by *C.jejuni in vivo* and that novel *C.jejuni* genes may be found using this method. The construction of the library and screening with convalescent antiserum were described in Chapter Two. Several Lambda ZAP II clones of *C.jejuni* H132 DNA, which synthesized products that reacted with convalescent antiserum, were successfully rescued into the pBluescript SK- phagemid vector. The initial analysis of the positive clones was described in Chapter Three and involved mapping the *C.jejuni* DNA inserts and Southern blot experiments to show that some of the positive clones contained overlapping *C.jejuni* sequences. It seemed likely that the clones with overlapping inserts would all contain the same ORF encoding a protein which was recognised by convalescent antiserum. *In vitro* transcription/translation and immunoprecipitation experiments confirmed that most of the clones synthesized products which reacted with convalescent antiserum. These experiments also showed that the overlapping clones pMK12 and pMK13 could synthesize the same immunogenic product, while pMK11 could synthesize a truncated version of the protein. This was one of

the first reports of the successful use of a cell-free *E.coli* expression system with *C.jejuni* DNA.

In order to find those ORFs synthesizing products bound by convalescent antiserum, the time consuming subcloning and analysis of the open reading frames of positive clones was the next logical step. However, subcloning was particularly difficult in this case, due to the lack of suitable restriction enzyme sites in the *C.jejuni* DNA inserts. Thus, transposon mutagenesis was used to identify the ORFs which encoded proteins that reacted with convalescent antiserum, by causing the loss of *in vitro* transcription/translation products which were immunoprecipitated by the antiserum. The transposon Tn*phoA* was used which also shows whether the products can be exported from *E.coli* cells. Many gene products involved in virulence are either present on the surface of the bacterial cell or excreted from the cell, where they could interact with the host. Therefore, Tn*phoA* mutagenesis may identify exported products which are involved in virulence of *C.jejuni*. However, not all virulence determinants are exported and not all exported products which react with convalescent antiserum are virulence determinants. Furthermore, proteins which are exported from *C.jejuni* are not necessarily also exported from *E.coli*, since *C.jejuni* signal sequences might not function in other organisms and/or accessory factors may be required for export which are not synthesized in *E.coli* laboratory strains. Thus Tn*phoA* mutagenesis has some limitations. Nevertheless, in this study, clones that reacted with convalescent antiserum have been isolated from a Lambda ZAP II library of *C.jejuni* DNA and blue Tn*phoA* mutants of positive clones were obtained. The isolation and characterisation of the Tn*phoA* mutants was described in Chapter Four and this was the first report of the successful mutagenesis of *C.jejuni* DNA using *TnphoA*.

It was revealed that at least clones pMK11, pMK14 and pMK3 could produce *C.jejuni* proteins which were exported from *E.coli*. pMK11 contained two ORFs for exported proteins, at opposite ends of the *C.jejuni* DNA insert but reading in the same direction. The truncated ORF at the 3′ end of the *C.jejuni* DNA insert of pMK11 could encode a protein with some similarity to *Campylobacter* Fla at its N-terminal end. According to *in vitro* transcription/translation experiments with pMK12 and pMK13, which contain the entire gene, this protein is likely to have a molecular weight of 74kDa. The other ORF, at the 5′ end of the insert, could encode a protein with similarity to ATP-binding transport proteins. The blue Tn*phoA* mutant of pMK14 had the transposon inserted in an ORF which encodes a protein with some similarity to methionine biosynthesis enzymes and the blue Tn*phoA* mutant of pMK3 contained an ORF for an unidentified exported protein with

an ATP-binding site. The use of *TnphoA*, in tandem with *in vitro* transcription/translation and immunoprecipitation, allowed the rapid identification of ORFs for exported proteins which reacted with convalescent antiserum and, hence, are likely to be synthesized *in vivo*.

The identification of a gene for an ATP-binding transport protein upstream of a gene for a protein with some similarity to a known virulence determinant of *C.jejuni*, may indicate that this locus is involved in the pathogenesis of the organism. It is possible that the transport protein enables the export of the 74kDa protein, which could then form a structure on the surface of the *C.jejuni* cell. In *Shigella flexneri*, the export of the Ipa polypeptides (invasion plasmid antigens which are essential for the invasion process) is dependent on the expression of genes upstream from the *ipa* locus [245]. Similarly, the ATP-binding transport protein responsible for secretion of *E.coli* haemolysin is encoded by genes (*hlyB* and *hlyD*) linked to the structural haemolysin gene (*hlyA*) and to a gene (*hlyC*), which encodes a protein involved in the activation of the toxin [246]. The full sequences of the *C.jejuni* ATP-binding transport protein gene and the gene for the 74kDa product have to be determined but, once this is completed, construction of defined mutants in these genes can be carried out and the mutants investigated to discover the biological functions of the proteins. It would be of interest to discover whether the mutants were still capable of colonisation, adhesion and invasion of host cells, in *in vitro* and *in vivo* models. It may be found that the 74kDa protein is not exported by the ATP-binding transport protein encoded by the upstream gene, but the transport protein could, nevertheless, be involved in the export of another product of interest. The presence of the *glyW* gene between the two ORFs, and transcribed in the opposite direction to them, may indicate that they are not connected.

6.2 PCRDOP to isolate a fragment of the *C.jejuni* H132 *recA* gene. The advantages of the PCRDOP technique are the simplicity and speed with which gene fragments can be cloned using this method. The disadvantages are that only gene fragments may be isolated using PCRDOP, so more time consuming methods may have to be used to find the rest of the gene, and that the technique is limited to finding genes which have already been cloned from several different bacterial species and found to be conserved between them. Therefore, it is not possible to isolate novel genes using this method. This thesis describes the successful isolation of a *C.jejuni* H132 *recA* gene fragment using PCRDOP. The labelled *recA* fragment was used to screen the Lambda ZAP II library of *C.jejuni* DNA and picked out several positive plaques. However, the subsequent publication of the *C.jejuni* 81-176 *recA*

sequence enabled the rapid cloning of the entire *C.jejuni* H132 *recA* gene using PCR with primers designed from the published sequence.

Some interesting similarities were noted between the *C.jejuni* RecA and the RecA protein from *N.gonorrhoeae*. They both have amino acid substitutions which may enable the RecA protein to act as a protease constitutively. The result of constitutive cleavage of the LexA repressor would be to prevent the repression of SOS response genes. Constitutive derepression of the SOS regulon may be useful to an organism which is exposed to high levels of stress during infection of the host, since the SOS response is important in the repair of DNA damage. The *C.jejuni* and *N.gonorrhoeae recA* genes also lack upstream binding sites for the LexA repressor. The absence of LexA repression of *recA* gene transcription would enable *C.jejuni* to synthesize relatively high levels of RecA. This may be necessary because of the constitutive protease activity of the protein, since it has been shown that the binding and cleavage of LexA is in competition with the recombination activity of RecA [247].

In order to determine the function(s) of the RecA protein, future work should involve the construction of defined mutants of the chromosomal *C.jejuni recA* gene. As discussed previously, Guerry *et al* [186] found that *C.jejuni recA* mutants were not prevented from colonising rabbits or stimulating an immune response which protected against subsequent infection with wild-type *C.jejuni*. However, the production of diarrhoeal disease by *C.jejuni* involves more than just colonisation. Virulence determinants involved in the pathology of the disease, such as adhesins, invasins, toxins, and stimulators of inflammation (see Introduction, section 1.5) may be encoded by genes which are subject to amplification, in a similar manner to the *ctx* virulence cassette of *V.cholerae* [191]. RecA could also play a role in the stress response necessary for the intracellular survival of *C.jejuni*. In addition, *recA* mutants may be avirulent in animal models and human volunteer studies due to increased susceptibility to reactive oxygen metabolites from the host immune system, similarly to *S.typhimurium* [228].

Investigation of *C.jejuni recA* mutants may also lead to a greater understanding of the way in which antigenic variation occurs in *Campylobacter*. The apparent derepression of the *C.jejuni recA* gene may indicate that natural transformation and recombination are important functions for the survival of the organism. As discussed previously (section 5.4.4), it has been shown that variation of the flagellin proteins can occur via recombination of *fla* gene sequences, although posttranslational modifications are thought to be the major mechanism of antigenic variation of Fla proteins. Antigenic variation of the LPS O antigens has also been demonstrated to occur *in vivo* [248]. When three

consecutive *C.jejuni* isolates from the same patient were sent for serotyping, it was found that there were marked differences in the specificities of the O antigens between the first and third isolates, suggesting the possibility of a host effect on the expression of LPS antigens. Investigation of the role of RecA in the antigenic variation of LPS may have to be postponed until the loci responsible for LPS biosynthesis have been characterised and it has been determined whether recombination could occur between LPS genes. A region of the chromosome containing genes which are likely to have a role in LPS biosynthesis has recently been isolated and, therefore, this characterisation could soon be achieved [A.Wood and J.M.Ketley, personal communication]. Future experiments with the *C.jejuni recA* mutants could involve the selection of particular O antigens with anti-O serotype antibodies, in order to attempt to drive a change of serotype. If the serotype could be changed in wild type *C.jejuni*, but not in the *recA* mutants, then it may be concluded that RecA does have an important role to play in the antigenic variation and, hence, the virulence of *C.jejuni*.

The molecular genetic approach to the investigation of the pathogenic mechanisms of *C.jejuni* has yielded some useful information, especially concerning the role of the flagellum. However, the molecular genetic methodologies are not as well-developed as in other common bacterial pathogens, probably due to the problems of instability and lack of expression of some *C.jejuni* genes in *E.coli* host cells. Therefore, the development of novel molecular genetic techniques is beneficial for the further investigation of *C.jejuni* pathogenesis. The methods of Tn*phoA* mutagenesis and PCRDOP, described in this study, are two such techniques. In the future, Tn*phoA* mutagenesis could be utilised during the investigation of genes identified by the proposed *Campylobacter* genome sequencing project.

Perhaps sequencing the genome will reveal how *C.jejuni* causes disease in humans. There is good evidence that motility and invasion play a role in pathogenesis, but the role of *Campylobacter* toxins, and the precise mechanisms of infection, have yet to be elucidated. It is hoped that advances in the understanding of the physiology and pathogenesis of *C.jejuni* will lead to strategies for preventing debilitating infection from occurring.

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