Genetic Approaches to Virulence Factor Expression in *Campylobacter jejuni*

Taraq M. Arain

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Department of Microbiology,
University of Glasgow

This work is dedicated to all those who have stood by me and helped get me this far.

I can think of no better way to demonstrate my gratitude.

"In art, nothing worth doing can be done without genius, in science even a very moderate capacity can contribute something to a supreme achievement"

.....*Bertrand Russell*, 1917

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LIST OF ABBREVIATIONS USED

List of Abbreviations Used

A	Adenosine
Am	Ampicillin
ATP	Adenosine triphosphate
BA	Oxoid blood agar base + 7% defibrinated sheep blood
BHI	Brain heart infusion
bp	base pair(s)
BRL	Bethesda Research Laboratories
BSA	Bovine serum albumin
C	Cytidine
cAMP	Cyclic adenosine 3', 5'-monophosphate
CD_{50}	50% cell death
cfu	colony forming units
CHO	Chinese hamster ovary
CjT	*Campylobacter jejuni* enterotoxin
cm	Centimeter(s)
cm^2	Square centimeters
Co	Cobalt
cos	Cohesive end site
CT	Cholera toxin
CTAB	Hexadecyltrimethyl ammonium bromide
Cys-HCl	Cysteine hydrochloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dTTP	deoxythymidine triphosphate
ed.	Editor(s)
ELISA	Enzyme-linked immunosorbent assay
eltA	Gene encoding the A subunit of LT
eltB	Gene encoding the B subunit of LT
EtBr	Ethidium bromide

FEMS	Federation of European Microbiological Societies
G+C	Guanine plus cytosine ratio
G	Guanosine
Gal	Galactose
GalNAc	*N* -acetylgalactosamine
Glc	Glucose
GM_1	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide
h	hour(s)
H_2S	Hydrogen sulphide
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG	Immunoglobulin class G
Inc	Plasmid incompatibility group
IU	International unit(s)
kb	1000 base pairs
kDa	Kilodalton(s)
Km	Kanamycin
λ	Bacteriophage lambda
LT	*E.coli* heat-labile toxin
Ltd.	Limited company
M	Mole(s)/molar
Mg	Magnesium
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimole(s)/millimolar
mob	Mobilisation site
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide	
NA	Nutrient agar
NCTC	National Collection of Type Cultures (U.K.)
NeuAc	*N* -acetlyneuraminic acid
ng	Nanogram(s)
nm	nanometer(s)
°C	Degrees Celsius
OD	Optical density
ODU	Optical density units

Oligo	Oligodeoxyribonucleotide
ori	Origin of DNA replication
PBS	Phosphate buffered saline
pfu	Plaque forming unit(s)
phage	Bacteriophage
phoA	Phosphatase gene
PVP	Polyvinylpyrrolidone
^{R}	Antibiotic resistance
RNA	Ribonucleic acid
RNase	Ribonuclease
RPLA	Reversed passive latex agglutination
rpm	Revolutions per minute
^{S}	Antibiotic sensitivity
SDS	Sodium dodecyl sulphate
SHMT	Serine hydroxymethyltransferase
SIgA	Secretory immunoglobulin class A
SLT-I	Shiga-like toxin type I (Verotoxin type 1)
SLT-II	Shiga-like toxin type II (Verotoxin type 2)
T	Thymidine
TBE	Tris-borate buffer
Tc	Tetracycline
TE	Tris-EDTA buffer
Tn	Transposon
toxA	Gene encoding A subunit of CT
toxB	Gene encoding B subunit of CT
tra	Transfer genes
U	Uridine
U.K.	United Kingdom
U.S.A.	United States of America
UV	Ultra-violet
$^{v}/_{v}$	Volume : volume ratio
VT1	Verotoxin type 1 (Shiga-like toxin type I)
VT2	Verotoxin type 2 (Shiga-like toxin type II)
$^{w}/_{v}$	Weight : volume ratio
Zn	Zinc
µg	Microgram(s)

μM	Micromole(s)/micromolar
μm	micrometer(s)
Δ	Deletion
%	Per cent
^{32}P	Radioactive isotope of phosphorus
::	Novel joint

SUMMARY

Summary

A modification of a blood-containing agar medium used for cultivation of *Campylobacter jejuni* is described which promoted or enhanced the haemolytic activity of this organism. Of several types of blood tested, sheep erythrocytes were most sensitive to the actions of the *C.jejuni* haemolytic factor. A procedure is also reported for the assay of haemolysin released after ultrasonic disintegration of *C.jejuni* cells. Of 40 strains representative of the *C.jejuni /coli* group, all exhibited the capacity for haemolysin production. Tentative evidence for a relationship between this haemolysin and the ß-lysin of *Staphylococcus aureus* was not confirmed at the genetic level. A link between the ability to lyse erythrocytes and a microbial requirement for iron was not established.

The ability of *C.jejuni* cell extracts to induce the death of cultured mammalian cells was also assessed by a standard trypan blue assay and by relation to a technique detecting the activity of dehydrogenase enzymes in active mitochondria. Reported also is an evaluation of a reversed phase latex agglutination technique for the detection and assay of the *C.jejuni* enterotoxin.

Two statistically complete libraries of random *C.jejuni* chromosomal DNA sequences were generated in *Escherichia coli*, one based in a plasmid shuttle vector and the other in a cosmid vector. Attempts to isolate the gene encoding the *Campylobacter* haemolysin from both of these genetic libraries proved unsuccessful. Several clones were identified, however, which hybridized with a mixed oligonucleotide probe complementary to a highly conserved region of the mature polypeptide of *Vibrio cholerae* enterotoxin and *E.coli* heat-labile toxin.

Attempts were made at the isolation of transposon-generated mutants of *C.jejuni*. Transfer of Tn*5*, Tn*10* and Tn*phoA* from several delivery vectors to the *Campylobacter* chromosome

using protocols employing conjugation or electroporation were, however, unsuccessful. Electroporation experiments did, nevertheless, provide indications for a natural transformation system which allowed entry of DNA into the *C.jejuni* cell without the requirement for an artificially induced state of competence.

1. INTRODUCTION

1.1. General description of the genus

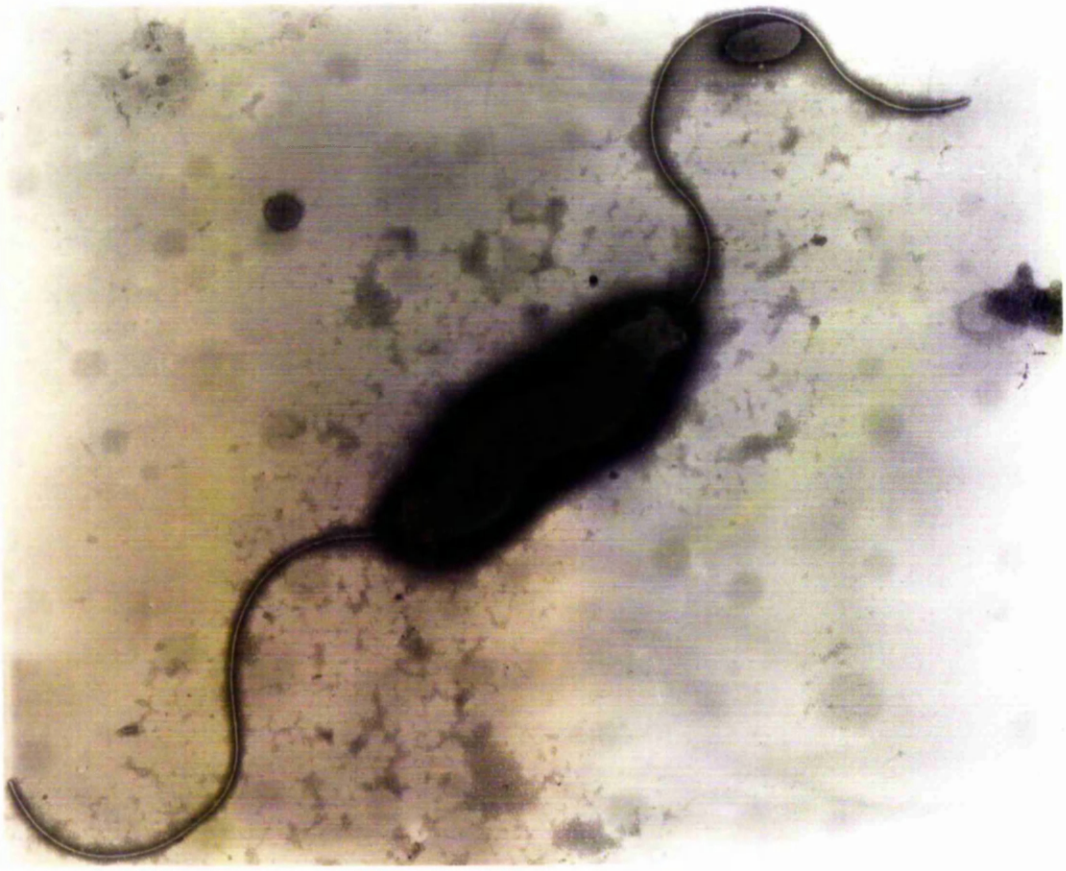
Smibert (1984) described members of the genus *Campylobacter* as slender Gram-negative bacteria, 0.2 μm to 0.5 μm wide and 0.5 μm to 5 μm long, occurring as spiral, curved or 'S'-shaped cells when observed microscopically. His description also noted that cells may be monotrichous or amphitrichous, exhibiting a characteristic darting and corkscrew-like motility. This typical morphology of a *C.jejuni* cell is well demonstrated in Figure 1. Microaerophily was noted as a prominent feature of the majority of the thirteen species included in the genus by Penner (1988), a notable exception being the aerotolerant species, *Campylobacter cryaerophila* (Neill *et al.*, 1985).

A respiratory type of metabolism is utilised, with energy being obtained from amino acids or intermediates of the tricarboxylic acid cycle. Oxidative or fermentative breakdown of carbohydrates has not been reported (Smibert, 1984). Members of the genus are known to exhibit a wide variation in their tolerance of growth temperatures, a characteristic usefully employed for taxonomic purposes. For example, *C.cryaerophila* has the ability to grow at a temperature as low as 15°C, while a group referred to as the "thermophilic campylobacters" grow well at 42°C (Penner, 1988).

A common feature of all *Campylobacter* species is their ability to produce the oxidase enzyme, whilst all except *C.cryaerophila* can reduce nitrates (Penner, 1988). Being inert with regard to any attack on carbohydrates greatly limits the application of useful biochemical tests to differentiate between species. Most valuable of those tests available are the detection of the catalase enzyme, production of hydrogen sulphide and the hydrolysis of indoxyl acetate as described by Mills and Gherna (1987). The guanine-plus-cytosine (G + C) content of genomic DNA is generally regarded as being amongst the lowest known for prokaryotes, ranging from 28 to 39 moles per cent (Smibert, 1984).

Figure 1: Normal Morphological features of *C.jejuni*
(Negatively stained preparation at a magnification of 30000X)

This electron micrograph was very generously provided by Dr. A. Curry
of the Public Health Laboratory, Withington Hospital, Manchester.



1.1.1. The thermophilic campylobacters

According to the classification put forward by Penner in 1988, the thermophilic group of campylobacters had four representatives, namely *C.jejuni*, *C.coli*, *C.laridis* and *C.upsaliensis*. Of these, *C.upsaliensis* was the latest addition, being described by Sandstedt *et al.* (1983) as an isolate from diarrhoeic dogs but as yet unconfirmed as a human pathogen. The other three members were closely related in terms of G + C content and biochemical reactions, all reducing nitrates and producing catalase. *C.laridis*, predominantly isolated from seagulls, has been implicated in cases of human diarrhoea (Simor and Wilcox, 1987) and bacteriaemia (Tauxe *et al.*, 1985). It can be distinguished from *C.jejuni* and *C.coli* by its inability to hydrolyse indoxyl acetate and through its resistance to nalidixic acid. However, resistant strains of *C.jejuni* and *C.coli* have been described both by Walder *et al.* (1983) and Lior (1984), probably arising through exposure of patients to the antibiotic norfloxacin (Altwegg *et al.*, 1987).

Intense speculation persisted for many years concerning the distinction between *C.jejuni* and *C.coli*. Skirrow (1977), during his pioneering studies to confirm the association of *Campylobacter* spp. with human enteritis, referred simply to the *C.jejuni-C.coli* group, since shortcomings had been noted in the rationale of both Smibert's listing of a single species as *C.fetus* subspecies *jejuni* (1974) and Veron and Chatelain's consideration of two separate species, *C.jejuni* and *C.coli* (1973). A breakthrough in this matter was achieved only after the finding of Harvey (1980) that *C.jejuni* had the capability to hydrolyse hippurate to produce glycine and benzoic acid, whilst *C.coli* had no effect on this substrate.

1.2. Historical perspective

Originally implicated as the causal organisms of abortion in sheep (Mcfadyean and Stockman, 1913), microaerophilic vibrios, later named *Vibrio fetus* by Smith and Taylor (1919), had also been

associated with abortion in cattle (Smith, 1918). The first speculative link with any form of diarrhoeal illness came with Smith and Orcutt's isolation of these "vibrios", as they described them, from the livers and spleens of calves suffering with diarrhoea (1927). Their investigations, together with those of Jones *et al.* (1931), established that the organism causing bovine enteritis resembled *V.fetus* in microscopic appearance but not in its serological reactions. These enteritis strains were therefore categorised separately as *V.jejuni.* Similar organisms were found by Doyle (1944) to be involved in swine dysentery, and were given the name, *V.coli.*

In 1946 came the first association of microaerophilic vibrios with human illness after Levy's report of an outbreak of gastroenteritis affecting 350 persons. He described the isolation of these vibrios from blood cultures and showed that similar organisms could also be detected in faecal smears. It was not until 1957, however, that Elizabeth King discovered the ability of *V.fetus* isolated from patients with septicaemia to grow optimally at 37°C whilst bloodstream isolates, where diarrhoea was a prominent feature, grew best at 42°C. Later, in 1962, she recognised this latter group of "related vibrios" to be identical to the *V.jejuni* and *V.coli* previously described in animals. Although she promoted the view that these organisms might be present not only in the bloodstream, but also in the gut, attempts at their isolation from stool samples were unsuccessful, primarily due to an uncontrollable overgrowth by other faecal bacteria.

Following the work of Sebald and Véron (1963), the microaerophilic vibrios were assigned to a new genus called *Campylobacter.* The first complete taxonomic account of this genus appeared a decade later (Veron and Chatelain, 1973) and set a precedent for the current nomenclature of the major enteritis-associated strains as *C.jejuni* and *C.coli.*

During the same year, the Belgian research group of Butzler *et al.* (1973) pioneered a technique to isolate *Campylobacter* spp.

from human faecal samples. Their approach was based on the ability of the slender *Campylobacter* cells to pass through a filter with pore diameters of 0.45 μm, which would, however, retain the majority of other members of the faecal flora present in the sample. Using this method, they isolated campylobacters from the faeces of 5.1% of children suffering from diarrhoea. Four years later, Skirrow (1977) described a culture medium made selective for *C.jejuni /C.coli* by addition of vancomycin, polymixin B and trimethoprim which was incubated microaerophilically at 43°C. From a group of 803 patients with diarrhoea, 57 (7.1%) were found to be excreting campylobacters in their faeces. Numbers of *Campylobacter* isolations have been rising steadily since then. Much of the early increase was undoubtedly due to the increased awareness of a newly described disease and the availability of methods to define its microbiological diagnosis, although Skirrow (1987 a) argued that these factors were unlikely to have affected figures in more recent years.

1.3. Modern perspective

Acute diarrhoeal disease continues to be a major cause of morbidity and mortality, accounting for the deaths of nearly five million children under the age of five per year in Africa, Asia and Latin America (Snyder and Merson, 1982). These authors calculated that, for the same age group and geographical boundaries, the total number of diarrhoeal episodes per annum exceeded seven hundred million. The pattern of disease in the more developed parts of the world, however, is very different, better living conditions and nutrition contributing to a much decreased level of both morbidity and mortality (Guerrant *et al.*, 1990).

Campylobacter enteritis is currently recognised as the most commonly reported cause of acute diarrhoea in the United Kingdom (Galbraith, 1988). Although not considered a notifiable disease, data on laboratory confirmed infections is collected both on a local and national basis for purposes of public health. About 300 laboratories in England and Wales submit information to the

Communicable Desease Surveillance Center (CDSC) based in London. The CDSC reported nearly 29000 cases of laboratory-confirmed *Campylobacter* infections in 1988 (Richmond, 1990), compared with about 24000 isolations of *Salmonella* spp. in the same year (Annual Report of the Chief Medical Officer of the Department of Health, 1989). This trend was also followed by reports from Scotland where numbers of isolates in 1988 were 2906 and 2585 for *Campylobacter* spp. and *Salmonella* spp. respectively (Communicable Diseases Surveillance, 21st January 1989). The annual trends in the numbers of laboratory reports citing *Campylobacter* spp. and *Salmonella* spp. in England and Wales are indicated in Fig. 2.

Reports from other countries, including New Zealand (Watson*et al.*, 1979), Sweden (Svedhem and Kaijser, 1980), the Gambia (Billingham, 1981), Bangladesh (Glass *et al.*, 1983) and the United States (Riley and Finch, 1985) suggest that the high prevalence of infection with *C.jejuni* is a feature of both industrialised countries and of the third world.

Although mortality is rare, except in elderly or debilitated patients (Symonds, 1983), the major importance of *Campylobacter* enteritis lies in its social and economic implications. In 1982, Skirrow estimated that with the prevailing incidence of laboratory verified infections, 20000 working days were lost per year, a seemingly conservative estimate based on an average incapacity of three days per episode. The annual isolation rate in 1986 was double that of 1982, so it may be assumed that the figure for lost working days might approach, or even exceed, 40000 days per annum. In a study of 53 laboratory confirmed cases, Sockett and Pearson (1988) reported that 49 patients within the group made a total of 107 consultations with a general practitioner. As a result, 40 were prescribed medication and five were admitted to hospital for a total of 41 days. Direct costs amounted to over seven thousand pounds sterling, two thirds attributible to health care services and one third to environmental health departments. This

Figure 2: Laboratory Reports of Infections Caused by *Campylobacter* spp.
and *Salmonella* spp. England and Wales. 1978 - 1988

(Data from the Annual Report of the Chief Medical Officer of the Department of Health, 1989)

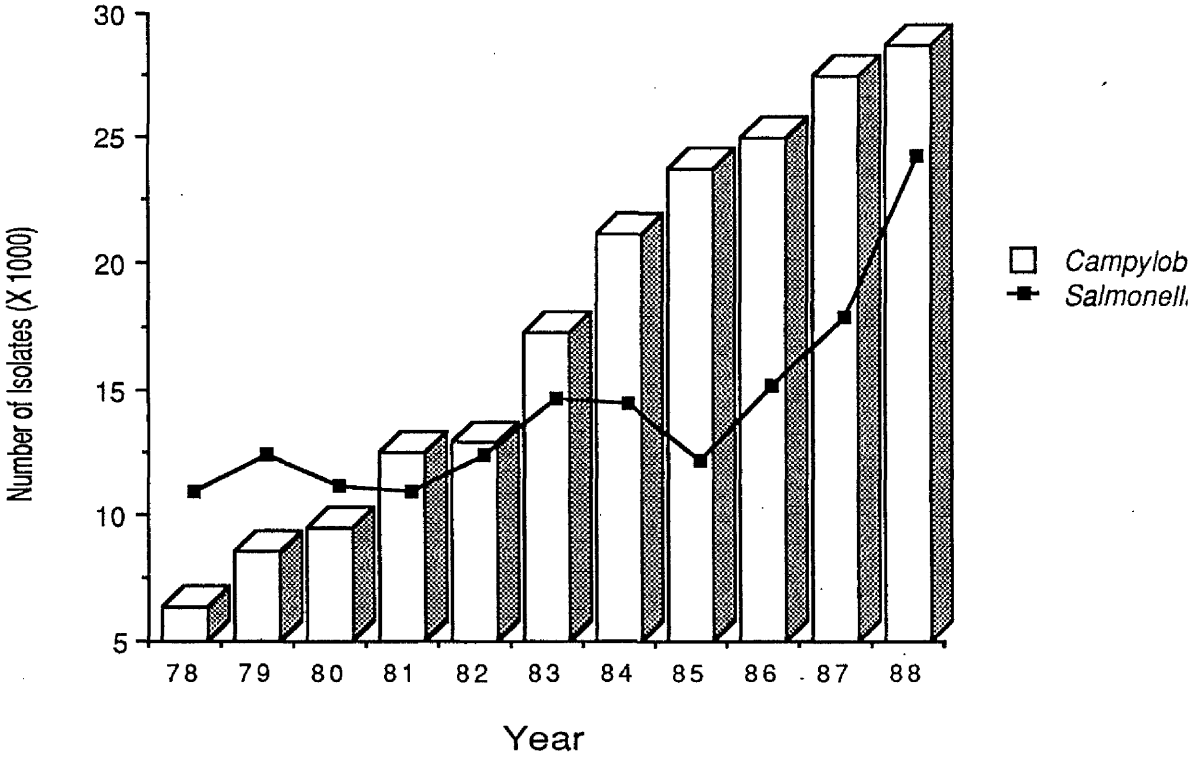

30
25
20
15
10
5
Number of Isolates (X 1000)
78
79
80
81
82
83
84
85
86
87
88
Year
Campylob
Salmonell

figure did not take into account any indirect costs associated with lost earnings and productivity. Extrapolation of this figure to account for the 29000 episodes of *Campylobacter* enteritis reported in 1988 exemplifies the very real economic burden posed by this diarrhoeal illness.

1.4. *Campylobacter jejuni*

On primary isolation, *C.jejuni* can occur in two colonial forms as first reported by Smibert (1965). One type is convex, round and shiny with an entire edge, while the other is flatter with an irregular boundary. Moist agar plates have been demonstrated by Karmali *et al.* (1981 a) to encourage a swarming type of growth. This finding was supported by the work of Buck and Kelly (1981) who noted that the moisture content of solidified media affected both colony size and swarming.

Smibert (1984) reported the average wavelength of the *C.jejuni* spiral morphology to be 1.12 μm and the average amplitude as 0.48 μm. It should be noted, however, that on exposure to unfavourable conditions, for example prolonged maintenance of cultures in aerobic conditions, and in older cultures, cells may form spherical or coccoid bodies which are considered to be degenerate and non-viable forms (Moran and Upton, 1985; Karmali *et al.*, 1981 a).

C.jejuni has an optimal growth temperature of 42°C, although it can also grow at 37°C. Growth, however, is impeded at 25°C. The majority of strains demonstrate phosphatase activity, whilst about 40% hydrolyse casein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Further descriptive details can be found in the review by Penner (1988).

1.4.1. Biotypes of *C.jejuni*

Skirrow and Benjamin (1980) distinguished between two biotypes of *C.jejuni* according to a test for hydrogen sulphide (H_2S) production, an approach extended by Lior (1984) through use of a

more rapid test for H_2S production and a sensitive DNA hydrolysis procedure. The biotypes thus distinguished have since been shown to differ according to the presence of the enzyme γ-glutamyl amino-peptidase in biotype II strains, with no detectable activity in strains of biotype I (Elhariff and Megraud, 1986). In addition, the work of Karmali *et al.* (1984) may show promise in defining further biotypes of *C.jejuni* by testing for the deamination of D-asparagine using an ammonia electrode assembly.

1.4.2. Serotypes of *C.jejuni*

A number of serotyping schemes have been developed to provide an investigative tool for purposes of epidemiology. Penner and Hennessy (1980) employed a passive haemagglutination technique for detection of antigenic specificities. The extracted thermostable antigens used were later proven by various researchers to be lipopolysaccharide somatic O-antigens (Naess and Hofstad, 1984; Mills *et al.*, 1985; Preston and Penner, 1987). In their protocol, Penner *et al.* (1983), used 42 antisera raised against the majority of North American isolates, so that many strains from other countries remained "untypeable". Illingwoth and Fricker (1987) developed a less laborious procedure incorporating a slide coagglutination test using antigen-sensitized erythrocytes and antibody-coated cells of *Staphylococcus aureus*. This method demonstrated a high level of sensitivity.

Several other serotyping strategies have been proposed. That of Kahlich *et al.* (1985) used antisera prepared against surface antigens of live cells. Hébert *et al.* (1983), on the other hand, employed the technique of direct immunofluorescence, a procedure also later used by Hodge *et al.* (1986). As determined by Smibert (1984), over 85% of human isolates were typeable using a simple slide agglutination technique described by Lior *et al.* (1982) and Rogol *et al.* (1983) which employed absorbed antisera directed against thermostable antigens.

1.5. *Campylobacter* enteritis

Gastrointestinal disease caused by *C.jejuni* can conveniently be separated into four stages: incubation period, onset, diarrhoeal stage and recovery.

1.5.1. Incubation period

From clinical data available to them, Butzler and Skirrow (1979) estimated an incubation period of three to five days, which has since been confirmed by investigations of outbreaks or human volunteer studies, as detailed below. Nevertheless, infection is not an inevitable outcome of exposure to *C.jejuni* whilst not all infected individuals develop any symptoms. In a waterborne outbreak in Vermont, about 20% of the population at risk actually became infected (Vogt *et al.*, 1982), whilst the report of Skirrow *et al.* (1981) concerning an improperly prepared restaurant meal indicated an attack rate of only 15%. A study by Porter and Reid (1980) of an outbreak resulting from the distribution of unpasteurised milk showed that 50% of those exposed to the milk were excreting *C.jejuni* in the faeces, although 40% of these individuals had no direct symptoms. One fifth of infections in another milkborne outbreak at a student hall of residence were also found to be symptomless (Jones *et al.*, 1981).

Human volunteer studies have shown incubation periods of three days (Steele and McDermott, 1978) and four days (Robinson, 1981). Results of the more extensive volunteer experiments of Black *et al.* (1988) indicated that the rate of infection increased from 50% to 100% when the size of the inoculum was raised from 800 colony forming units (cfu) to $1X10^8$ cfu. However, the size of inoculum had no effect on the severity of illness or incubation period (2-3 days).

1.5.2. Onset

A characteristic feature of the onset of illness is the abruptness with which it occurs. The majority of patients develop abdominal pain and diarrhoea, although in about one third of cases,

influenza-like symptoms including malaise, headache, shivering, dizziness and generalised muscle cramps can persist for up to 24 hours (Symonds, 1983). In a few cases, high fever of around 40°C has been associated with delerium in adults or febrile convulsion in children (Havalad *et al.*, 1980). Although nausea is a common feature, vomitting is rare.

1.5.3. Diarrhoeal stage

Stools typically become fluid, bile-stained, mucoid and watery in the more severe cases. From the second day of diarhoea onwards, they also appear blood-stained, especially in children. During the peak of this stage, ten or more bowel motions per day have been reported (Blaser *et al.*, 1979; Svedham and Kaijser, 1980). Diarrhoea can continue for eight days, although in otherwise healthy young adults it may persist for up to two weeks (Symonds, 1983).

The other notable feature at this time is severe abdominal pain which patients often find more discomforting than the diarrhoea itself (Skirrow, 1977). Jewkes *et al.* (1981) observed that this pain could last for up to three days and could be severe enough to warrant surgical examination for suspected appendicitis. Such cases have also been reported by Skirrow (1977) and Kendall and Tanner (1982). In most instances, inflammation of the ileum and jejunum but not the appendix was discovered, although Symonds (1983) noted that such inflammation could occasionally lead to genuine appendicitis.

Other complications which may occur include infection of the colon with histological findings very similar to those of salmonellosis or shigellosis (Lambert *et al.*, 1979), pancreatitis (Gallagher *et al.*, 1981), cholecystitis (Darling *et al.*, 1979; Mertens and De Smet, 1979), haemolytic uraemic syndrome (Delans *et al.*, 1984; Chamovitz *et al.*, 1983) and proctitis in practising homosexual men (Quinn *et al.*, 1980). Reactive arthritis is now also recognised as a complication of campylobacter enteritis, as described by Berden *et al.* (1979). Arthritis usually appears within

seven to fourteen days following the onset of diarrhoea, with the knee joint being the most common target, although other joints like the wrist and ankle may also be affected.

Neonatal infections, usually presenting as passage of blood from the rectum with little or no diarrhoea, are usually acquired from a mother who has a history of diarrhoea near or at the time of delivery (Mawer and Smith, 1979). A case of meningitis caused by *C.jejuni /coli* has been described in a neonate by Thomas *et al.* (1980).

1.5.4. Recovery stage

On subsidence of diarrhoea, patients often complain of weight loss and continued abdominal pain. Milder relapses of illness were recorded in a quarter of cases studied by Blaser *et al.* (1979), although Schofield and Mandel (1983) reported that all 200 patients in their investigations were free of symptoms within 12 days of onset.

1.6. Reservoirs and sources of transmission

1.6.1. Animals

Skirrow's assertion (1982, 1987 b) that campylobacter enteritis is a zoonosis has now been widely accepted. Both wild and domestic animals form the major reservoir of infection. *C.jejuni* has been isolated from a wide range of zoo animals including felines, ungulates, pigeons and other birds in a study conducted by Leuchtefeld *et al.* (1980 a). A high rate of incidence has been noted in wild birds, with an isolation rate of 35% from waterfowl in North America (Leuchtefeld *et al.*, 1980 b) and 50% of pigeons in Great Britain (Fenlon, 1981). Wild birds may indeed constitute the main natural reservoir for this organism (Skirrow, 1982).

In developed countries, intestinal infection with *C.jejuni* is usually associated only with illness and symptomless excretion is uncommon. In the developing countries, however, prolonged carriage

in the human intestine may play an important role in the transmission of infection. Blaser *et al.* (1983 a) found that 1.5% to 17.7% of children in Africa and Asia not suffering from diarrhoea were excreting *C.jejuni* in their faeces, whilst up to 32% of symptomless infants in Bangladesh were discovered to be similarly colonised by Glass *et al.* (1983). Rajan and Mathan (1982) have also documented the occurance of *C.jejuni* in healthy populations of Southern India. Spread of infection from person to person by the faecal-oral route is not commonplace in developed countries, probable due to the sensitivity of the organism to oxygen (Symonds, 1983). Only on rare occasions have there been any reports of direct transmission from an infected mother to her child or from children with profuse diarrhoea (Blaser and Reller, 1981). No evidence exists to implicate the spread of infection via food handlers who are excreting *C.jejuni* following a bout of illness and there is consequently no justification in detaining them from their work so long as normal practices of hygiene are followed.

The spread of campylobacters from animals to man may occur through occupational contact or via domestic pets. Blaser *et al.* (1980 a) documented the case of a young man who developed enteritis soon after beginning work at a cattle feedlot. Enteritis has also been reported in persons who have been in close contact with infected puppies (Blaser *et al.*, 1978) and cats (Skirrow *et al.*, 1980). House flies (Rosef and Kapperud, 1983; Wright, 1983), cockroaches (Umunnabuike and Irokanulo, 1986) and wild birds (Hudson *et al.*, 1990) have been shown to be possible sources for the transmission of *C.jejuni.*

1.6.2. Foodstuffs

The majority of infections, however, occur through ingestion of contaminated foodstuffs. *C.jejuni* is known to occur in the intestinal flora of most commercially raised poultry, spreading to the carcasses during slaughter (Blaser *et al.*, 1983 a). Raw or undercooked poultry has been implicated in several outbreaks, including one in England after a catered banquet (Skirrow *et al.*,

1981). In their study, Simmons and Gibbs (1979) found that large numbers of organisms were recovered from broiler chicken carcasses after slaughter and could survive subsequent processing, refrigeration and thawing.

Over 50 outbreaks have been reported due to raw or improperly pasteurised milk (Galbraith, 1988), of which 13 were previously reviewed by Robinson and Jones (1981). The volunteer study conducted by Robinson (1981) demonstrated that as few as 500 viable organisms in milk could lead to illness, while Blaser *et al.* (1980 b) reported that *C.jejuni* could survive in milk at 4°C for up to three weeks. It is not clear whether the organisms enter the milk through contamination with bovine faecal material or via a cow suffering from campylobacter mastitis, a pathological condition first reported by Hutchinson *et al.* (1985).

In general, red meat is found to be less frequently contaminated. Whilst campylobacters are regularly isolated from abattoir carcasses (Stern, 1981), the level of contamination has reduced considerably by the time the meat reaches its retail outlets.

1.6.3. Water supplies

An increasing risk to public health is the isolation of *C.jejuni* from sources of potable water. Contaminated supplies have resulted in large outbreaks, as in Bennington, Vermont, where nearly 3000 residents became infected (Vogt *et al.*, 1982). Immediately prior to the outbreak, heavy rains may have led to contamination by surface water of the 80 year old municipal supply system. Another outbreak in central Sweden involved about 2000 persons and was probably caused by backflow of river water through a water main (Mentzing, 1981). *C.jejuni* has been isolated from both fresh and salt water, invariably being present as a result of faecal contamination (Knill *et al.*, 1978). Blaser *et al.* (1980 b) have reported the survival of *C.jejuni* in unchlorinated water for several weeks when maintained at 4°C.

1.7. Proposed pathogenic mechanisms

Klipstein *et al.* (1985) considered it appropriate to distinguish the overt symptoms of campylobacter enteritis as either watery-type diarrhoea or invasive, bloody-type diarrhoea. They suggested that three potential pathogenic properties may be of importance: enterotoxin production, cytotoxin production and invasiveness. Konkel and Jones (1989) also speculated that these three factors may be of importance in the disease process. Previously, McKendrick *et al.* (1982) had proposed that the various clinical features prevalent in campylobacter enteritis may indeed be the results of toxin-mediated effects.

1.7.1. Enterotoxin production

Symptoms akin to a watery-type diarrhoea may be mediated through the elaboration of an enterotoxin. Ruiz-Palacios *et al.* (1983) demonstrated that some strains of *C.jejuni* produced a factor with properties very similar to those of cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT). This finding was confirmed by Fernandez *et al.* (1983) who demonstrated a net secretory flux of sodium ions from the plasma to the lumen of adult rat jejunal segments when perfused with *C.jejuni* culture supernatants. As a consequence, many of the techniques used for the assay of *C.jejuni* enterotoxin (CjT) have been adapted from those employed for CT, including those based on elongation of Chinese hamster ovary (CHO) cells and rounding of Y-1 mouse adrenal cells (Klipstein and Engert, 1984; McCardell *et al.*, 1984). Ruiz-Palacios *et al.* (1983) found that CjT led to the accumulation of fluid in the rat intestinal lumen, and determined that this occurred through mechanisms analagous to those known to occur with CT and LT, namely the stimulation of adenylate cyclase in the intestinal mucosa leading to disruption of normal ion transport in the enterocytes. However, CjT was found to differ from CT and LT through its inability to induce fluid secretion in the ligated ileal loops of rabbits. From their collected data, Ruiz-Palacios and

colleagues proposed that similarities existed between CjT and the A subunit of CT, whilst no such similarities could be demonstrated with the B subunit of CT responsible for receptor binding.

This view has, however, been challenged by the development of procedures to detect CjT using enzyme-linked immunosorbent assays employing GM1 ganglioside as solid phase and antibodies against either CT or LT (Klipstein and Engert, 1984; Klipstein *et al.*, 1985). An assay of this type performed by Klipstein and Engert (1985) demonstrated that in common with CT and LT, the *C.jejuni* enterotoxin does possess a B subunit allowing binding to the GM1 ganglioside, thereby facilitating internalisation of the adenylate cyclase activator.

In their study, Ruiz-Palacios and co-workers (1983) reported that CHO cell elongation and fluid accumulation in ligated rat ileal loops could be inhibited by preincubation of enterotoxin samples with antisera to LT or CT. McCardell *et al.* (1984) also demonstrated a reaction of partial identity between cholera and campylobacter enterotoxins by slide gel immunodiffusion tests. Nevertheless, there is evidence to suggest that dissimilarities may exist between CjT and CT or LT which are not apparent from the results of immunological studies alone. Under conditions of optimal and reduced stringency, Olsvik *et al.* (1984) were unable to demonstrate the hybridisation of gene probes specific for subunits A and B of both CT and LT to chromosomal DNA extracted from strains of *C.jejuni* known to produce enterotoxin. From this finding, it was concluded that the gene coding for enterotoxin production in *C.jejuni* differs in a substantive manner from that coding for either CT or LT.

Daikoku *et al.* (1989) observed that freshly isolated strains of *C.jejuni* produced high concentrations of enterotoxin when examined by ELISA whilst strains which had undergone storage for periods exceeding one year only exhibited weak enterotoxin activities. The same group has since succeeded in the partial purification and characterisation of this enterotoxin (Daikoku *et al.*,

1990). Crude toxin was purified 8.7-fold by sequential steps of culture supernatant ultrafiltration, precipitation with 80% ammonium sulphate and gel filtration through a Sephadex G-100 column. The resultant purified toxin preparation yielded three bands at 68, 54 and 43 kilodaltons (kDa) on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and was able to enhance the adenylate cyclase activity of HeLa cell membranes. After passage through an affinity gel column coupled with anti-CT goat immunoglobulin (IgG) and elution of the bound component, only one band of 68 kDa resulted after SDS-PAGE, suggesting an immunological relationship of this polypeptide to cholera toxin. When this procedure was repeated using an affinity gel column coupled with gangliosides, two bands of 68 kDa and 54 kDa resulted on SDS-PAGE, from which it was concluded that both of these polypeptides might be of importance in the process of binding the toxin to the ganglioside receptors on the surface of the cell. These findings reinforce those of Klipstein and Engert (1985) who reported that the holotoxin, when treated with guanidine, constituted three protein peaks after filtration through Sephadex G-75, the largest of which cross-reacted with LT.

The frequency with which enterotoxigenic strains are encountered varies considerably in reports from around the world. Goossens *et al.* (1985 a) determined that nearly all strains tested in their Belgian study were positive for enterotoxin production, while the work of Ruiz-Palacios *et al.* (1983) indicated that in Mexico, 70% of strains elaborated enterotoxin. However, in Sweden, only 32% of strains were enterotoxigenic (Lindblom *et al.*, 1989). Investigations undertaken by Perez-Perez *et al.* (1989) were unable to demonstrate enterotoxigenic activities in any of the 22 isolates tested using both CHO cell and ELISA methodologies. The discrepancies reported by different laboratories regarding the presence or absence of enterotoxin may be a result of several disparate but linked factors. Some *Campylobacter* isolates simply may not express any enterotoxin-like properties or else such activities may be produced in levels too low to be assayed using

procedures currently available (Perez-Perez *et al.* , 1989). Alternatively, variations in technical performance of the assays themselves in individual laboratories may play a significant role (Lindblom *et al.*, 1989).

1.7.2. Cytotoxin production

The current understanding of the cytotoxins of *C.jejuni* can only be described as "confused" since a number of toxic factors with diverse specificities have been reported. This is not entirely unexpected as widely differing culture conditions have been employed, using various strains and a variety of assay systems. A concerted effort is required to clarify the situation.

Prescott *et al.* (1981) proposed that production of a cytolytic exotoxin may have accounted for damage to the intestinal epithelium observed in gnotobiotic beagles. However, it was not until 1983 that the first report appeared of the *in vitro* cytotoxic effects of cell free filtrates of *C.jejuni* (Yeen *et al.*, 1983). They found that of four human *Campylobacter* isolates initially tested, three led to progressive and reproducible cell rounding, loss of adherance and cell death in HeLa, MRC-5 and HEp-2 human cell lines. Cytopathic effects were not detected in monkey (Vero, MK2 and PMK) or mouse (L929) cell cultures. Seven further isolates were subsequently tested in human cell lines only, of which five resulted in cell death. The toxic factor was found to be heat stable and trypsin sensitive, suggesting that it was protein in nature.

In the following year, Pennie *et al.* (1984) demonstrated a similar effect on HeLa cells when using filtrates of polymixin B treated campylobacters. Of ten filtrates, five resulted in death of 50% of the cells (CD_{50}) at titers of 1:4. Concentration of the campylobacters ten-fold prior to polymixin B treatment led to a ten-fold increase in cytotoxin titer. Antiserum to shiga toxin did not neutralise the cytopathic effect.

CHO cells were also determined to be susceptible to a

cytotoxic factor derived from the culture supernatants of 33 out of 39 strains of *C.jejuni* and all eight *C.coli* strains when grown in a biphasic system (Goossens *et al.*, 1985 b). Again, cell death was not prevented by preincubation of the supernatants with antiserum to purified shiga toxin. As a point of interest, the six strains which exhibited no cytotoxic activity were all isolated from patients with predominantly watery-type diarrhoea, though no data was presented on the clinical symptoms associated with the cytotoxin-positive isolates.

McCardell *et al.* (1986) reported the production of two distinct factors cytotoxic for CHO cells. After purification by fast protein liquid chromatography, "cytotoxin 1" was found to have a molecular weight of 70000 daltons and was insensitive to heat and trypsin, whilst being inactivated by normal rabbit serum. This factor had haemagglutinating activity but showed no haemolytic properties. In contrast, "cytotoxin 2" proved to be heat labile, sensitive to trypsin and was not inactivated by normal rabbit serum. Although antiserum to the cytotoxin of *Shigella dysenteriae* had no effect, sera raised against the *V.cholerae* non-01 cytolysin could neutralise the cytolethal effects of cytotoxin 2.

When 60 strains of *C.jejuni/C.coli* were tested, all produced cytotoxin 1 and 27 produced cytotoxin 2. The authors speculated that the former may be involved in attachment of the organisms to mammalian cells, much as observed with other haemagglutinating factors, while the latter might provide a mechanism for obtaining iron. In addition, they noted that a previously non-haemolytic strain, when injected into a rabbit ileal loop and re-isolated from the blood three hours later, did produce haemolysin. This might suggest that production of haemolysin may be an inducible virulence factor.

In a later study, Guerrant *et al.* (1987) examined *C.jejuni* isolated from twelve patients with inflammatory diarrhoea and found that a significant cytopathic effect occurred in HeLa cells with the supernatants of five of these strains after polymixin B

treatment. However, analysis of the data indicates that whereas CD_{50} titers of 1:4, and in one case 1:8, were reported, these are not as convincing as they seem since controls containing polymixin B alone resulted in titers of 1:2.

Cytotoxicity was generally equivalent or greater in CHO cells, though Vero and WI-38 cell lines were unaffected. Antisera prepared against the toxins of a number of microorganisms, including shiga-like toxins I and II, and the cytotoxins of *Clostridium difficile, Aeromonas* spp. and *V.cholerae* non-01 strains could not neutralise the cytopathic effect. Heating to 60°C reduced cytotoxicity, with 70% of toxic activity being destroyed after treatment with trypsin, an effect specifically blocked by trypsin inhibitor. Cytotoxin preparations had no enterotoxic properties when tested in the rabbit ligated ileal loop model.

In the same year, Pang *et al.* (1987) searched for cytotoxic factors in culture filtrates of freshly isolated strains of *C.jejuni* using HeLa, HEp-2 and MRC-5 cell lines. Of the strains tested, 50% were found to be positive to a titer of 1:8. However, the effect could not be reproduced in cell lines from other animals. In addition, a radioactive chromium release assay was performed whereby HeLa cells were labelled with ^{51}Cr, which binds to intracellular organelles, washed to remove unbound label, and then incubated with dilutions of toxin sample. Radioactivity was then measured in the supernatant and from osmotically lysed cells. The observed cytopathic effects correlated well with a significant release of ^{51}Cr into the supernatant, suggesting that the cytotoxin affects membrane integrity leading to cell death.

Cytotoxin preparations again had no enterotoxic properties when tested in ligated rabbit ileal loops. The authors also noted that the capacity to produce cytotoxin diminished with repeated subculture; in one case, all cytotoxic activity was lost after only four subcultures of the isolate.

In contradiction to these results, Johnson and Lior (1984)

found that the majority of strains which they examined produced a factor cytotoxic in low titer to Vero cells. Again, activity was lost after heating. Klipstein *et al.* (1985) demonstrated cytotoxicity in both Vero and HeLa cells in strains isolated from patients in whom invasive, bloody-type diarrhoeal symptoms were evident.

In the latest report on the subject of cytotoxins, Johnson and Lior (1988) described a new cytotoxic factor in CHO, Vero, HeLa and HEp-2 cell lines. After screening 718 strains of *Campylobacter* spp., 41% gave rise to cellular elongation followed by cell death with 96 hours of incubation. This factor, termed "cytolethal distending toxin" (CLDT) by the authors, was distinct from *C.jejuni* enterotoxin since limited fluid accumulation was observed in ligated rabbit ileal loops, leading instead to mucosal inflammation and haemorrhagic reactions. No neutralisation was demonstrated with antisera to CT, *C.difficile* cytotoxin or the *Campylobacter* cytotoxin which they had previously described in 1986. No media supplements, elaborate growth procedures or protracted techniques for extraction were required for demonstration of CLDT.

1.7.3. Invasiveness

The initial interaction between a pathogenic microoganism and its host involves attachment to the eukaryotic cell surface. Although the mechanisms of bacterial adherence are diverse, most in their simplest form involve an interaction between a receptor molecule and an adhesin. Some microorganisms remain and successfully multiply at the cell surface, whereas others penetrate deeper into the underlying tissues. Organisms which establish infection by following the orogastric route encounter various adverse enviroments, being exposed at times to extremes of pH, high bile salt concentrations, varied nutrient availabilities and a range of digestive enzymes (Finlay and Falkow, 1989). Membranes of the gastrointestinal tract are also protected by the constant movement of lumen contents, presence of mucus and activity of a resident commensal microbial population (Smith, 1977).

The presence of blood or leukocytes in the faeces of patients infected with *C.jejuni* presents strong evidence for the invasive capacity of this pathogen (Black *et al.*, 1988; Duffy *et al.*, 1980). Such clinical evidence has been corroborated by *in vivo* demonstration of direct tissue invasion in chicken embryos (Field *et al.*, 1986), adult chickens (Ruiz-Palacios *et al.*, 1981) and hamsters (Humphrey *et al.*, 1985). Manninen *et al.* (1982) found that all 15 of the *Campylobacter* strains which they studied associated with cultured HeLa cells whereas Newell and Pearson (1983) established that both of their strains associated with INT 407 cultured cells. However, later investigations determined that fewer than 50% of human faecal isolates could associate with HeLa cells (Fauchere *ét al.*, 1986). Using transmission electron microscopy, it was demonstrated that campylobacters associated with eukaryotic cells were present internally within endocytic vacuoles (Fauchere *et al.*, 1986).

Konkel and Jones (1989) showed that *Campylobacter* strains could also adhere to and invade cultured HEp-2 cells. Uptake of bacteria was inhibited by cytochalasin B, suggesting that the process of internalisation requires an active participation of the host cell in a manner likened to phagocytosis. This fungal metabolite disrupts subplasmalemma microfilaments which are involved in translocation of the plasma membrane during phagocytosis (Axline and Reaven, 1974), and has been shown to inhibit uptake of other gastrointestinal pathogens, including *Shigella flexneri* (Hale *et al.*, 1979) and *Salmonella typhimurium* (Kihlstrom, 1980). Konkel and Jones (1989) attempted to identify the nature of antigens responsible for adherence and invasion. They found that neither trypsin nor proteinase K treatment affected either capability. They further reported that *Campylobacter* lysates subjected to mild oxidation with sodium metaperiodate, known to cleave carbohydrate hydroxyl groups without affecting polypeptide chain structure (Bobbit, 1956), significantly affected the lysate's ability to inhibit adherence and invasion. From this, it

was concluded that the antigen responsible for internatlisation was glycoprotein or carbohydrate in nature.

DeMelo *et al.* (1989) verified the inhibitory effects of cytochalasin B on the energy dependant process of phagocytosis and added that similar inhibition resulted by treatment of cells with iodoacetate and dinitrophenol, which respectively block glycolysis and the Krebs cycle. Their studies also established that, in common with *Salmonella* spp. (Popiel and Turnbull, 1985) and *Yersinia pseuodtuberculosis* (Small *et al.*, 1987), campylobacters were maintained in the cell cytoplasm within endocytic vacuoles, although subsequent fusion with phagolysosomes led to a decrease in numbers of enveloped bacteria. Bacteria could neither survive nor multiply within cultured HEp-2 cells since loss of viability occurred after only nine hours of incubation. In contrast, Kiehlbauch *et al.* (1985) discovered that phagocytosis of *C.jejuni* strains by human monocytes and murine macrophages may actually have resulted in promoting intracellular survival of the bacteria, as determined by vital staining with acridine orange, for up to six or seven days.

The intestinal epithelium is covered by a layer of mucus consisting primarily of a mixture of proteins and high molecular weight glycoproteins referred to collectively as mucins (Clamp, 1977). Investigators have demonstrated the chemotactic attraction of *C.jejuni* to mucus (Hugdahl and Doyle, 1985) and have suggested that colonisation of enterocytes may be a result of favourable adaptation to the mucus of intestinal crypts (Lee *et al.*, 1986). DeMelo and Pechere (1988) found that pretreatment of HEp-2 cell monolayers with bovine submaxillary mucin prior to challenge with *C.jejuni* increased both association and internalisation of bacteria.

Nonetheless, intestinal mucus has also been shown to trap pathogens like *Trichinella* (Bells *et al.*, 1984) and *Salmonella* spp. (Magnusson and Sjernstrom, 1982) prior to their expulsion. Secretory immunoglobulin A (sIgA) forms an antibody monolayer at the interface between the mucus and the intestinal lumen. It is

considered to exert a protective effect by binding to the bacterial surface and causing aggregation (Gibbons, 1982). McSweegan *et al.* (1987) employed an *in vitro* adhesion assay using INT 407 cells overlaid with a crude preparation of rabbit intestinal mucus. They found that binding of bacteria to components of the mucus significantly reduced adherence to the underlying cells. When the monolayers were overlaid with mucus obtained from rabbits previously colonised with the same *C.jejuni* strain, and therefore containing specific sIgA, adherence was completely inhibited. Thus, a combination of mucus and sIgA formed an effective barrier to intestinal cell attachment.

Specialised antigen-sampling structures called M cells in the epithelium covering lymphoid follicles of the intestinal mucosa have been shown to attach to and internalise *V.cholerae* (Owen *et al.*, 1986). The epithelium of the Peyer's patches has an extensive population of M cells over the lyphoid follicles, while the opposing villus epithelium consists entirely of absorptive cells. Walker *et al.* (1988) showed by transmission electron microscopy that *C.jejuni* inoculated into ligated rabbit ileal loops selectively adhered to the luminal surface of M cells and were transported through them to the underlying follicles. They inferred that M cells may provide a means for the transportation of *Campylobacter* from the gut lumen into the general blood system and proposed that this may underline mechanisms of bacteriaemia known to occur in humans (Spelman *et al.*, 1986) and rabbits (Caldwell *et al.*, 1983).

Levine and colleagues (1983) considered that translocation of *C.jejuni* from the mucosal surface to the mesenteric lymph nodes was a major step in pathogenesis. Such translocation has been described in gnotobiotic mice (Fauchere *et al.*, 1985; Yrios and Balish, 1986; Youssef *et al.*, 1987).

Frequent reports of polymicrobial infections in campylobacter enteritis are encountered. In their Norwegian study, Lassen and Kapperud (1984) described several mixed infections. Out of 249 cases, 25 were infected jointly with *C.jejuni* and

Salmonella, 13 with *C.jejuni* and *Shigella* and two with all three enteropathogens. Similarly, in Finland, Pitkanen *et al.* (1983) determined that 11 out of 186 patients diagnosed with *Campylobacter* enteritis were also excreting *Salmonella.* Mixed infections have also been reported in Israel where 3.3% of *Campylobacter* infections occurred concomitantly with *Salmonella, Shigella* or *E.coli* (Melamed *et al.*, 1985).

These observations prompted the studies of Buckholm and Kapperud (1987) on interactions between *C.jejuni* and other coinfectants. They found that in pure culture, none of the six *Campylobacter* strains were capable of intracellular localisation in monolayers of the human epithelial cell lines, HEp-2 and A-549. However, when coinfected with other enteropathogenic bacteria, four strains attained an invasive potential. When coinfected with *S.typhimurium*, 30% of cells harboured both bacteria, 14% contained campylobacters only and 2% were invaded solely by salmonellas. A significantly lower degree of internalisation was induced by *Shigella sonnei*, *Shigella flexneri*, *Shigella boydi* and an enteroinvasive strain of *E.coli.* Interestingly, *E.coli* JH-13 was capable of inducing invasion of *C.jejuni* although it was itself completely noninvasive. No evidence was presented on whether the effects exerted by the coinfectants was upon the campylobacters themselves or on the host cells.

Most recently, DeMelo and Pechere (1990) identified *Campylobacter* surface exposed proteins which may play an important role in interactions with host cell membranes. They found that four polypeptides with molecular masses of 28, 32, 36 and 42 kDa, extracted from a strain known to be invasive, consistently interacted with HEp-2 cells. Although flagella had previously been implicated in mechanisms of *Campylobacter* adhesion (McSweegan and Walker, 1986; Moser and Hellmann, 1989), no evidence was found for association of flagellin to HEp-2 cells by DeMelo and Pechere (1990).

1.8. Current knowledge on genetics

1.8.1. Plasmids

Plasmids were first described in *Campylobacter* spp. by Austen and Trust (1980). Several surveys were subsequently undertaken to determine whether plasmid fingerprinting could be employed as a tool in epidemiological studies. Amongst the first of these was an investigation by Bradbury *et al.* (1983) which reported that 11 out of 40 *C.jejuni* isolates (27.5%) harboured plasmids ranging in molecular weights from two up to 62 megadaltons (MDa), with most strains carrying more than one plasmid species and no two strains exhibiting the same plasmid profile. A similar plasmid carriage rate was reported in strains isolated from a wide range of wild and domestic animals (Bradbury and Munroe, 1985). A somewhat more extensive survey of 688 *C.jejuni* and *C.coli* strains isolated over a period of two years in Seattle by Tenover *et al.* (1985) revealed a plasmid incidence rate of 33%, with sizes in the range of two kb up to 162 kb. In agreement with previous findings, most of these strains carried multiple plasmids. However, intensive and extensive efforts by all of these researchers failed to associate plasmid carriage with any virulence properties. Indeed, resistances to two antibiotics are the only plasmid specified functions known to date.

1.8.1.1. Tetracycline resistance

Reported incidences of tetracycline resistance in *C.jejuni* range from 38% in Israel (Michel *et al.*, 1983) and 12% in Canada (Karmali *et al.*, 1981 b) to no apparent resistance in Sweden (Walder and Forsgren, 1978). Taylor *et al.* (1980) were the first to domonstrate that the tetracycline resistance determinant could be transferred to other strains of *C,jejuni* and to other members of the genus. Such transfer was associated with the concomitant movement of a 45 kb plasmid to the new host. In a further study (Taylor *et al.*, 1981), the same group reported that plate or filter mating methods yielded an approximately 100 fold higher frequency of transfer than did broth mating methods, whereas attempts at

transfer of the plasmid to *E.coli* C and a restrictionless strain of *E.coli* K were unsuccessful.

Restriction enzyme fingerprints and G + C content of the plasmid have been determined, the latter indicating a very close correlation with the G + C content of *Campylobacter* chromosomal DNA (Taylor *et al.*, 1983). Restriction patterns of plasmids from three different tetracycline resistant isolates showed that much of the molecular structure was highly conserved, although two contained four kilobases of DNA additional to that of the prototype plasmid, pMAK175 (Taylor *et al.*, 1986). Taylor (1986) reported the cloning of the tetracycline resistance determinant in *E.coli* by ligation of a partial digest of plasmid pUA466 into vector pUC8 (Vieira and Messing, 1982). Homology was demonstrated between the *C.jejuni* resistance determinant and the class M tetracycline resistance gene from *Streptococcus* spp. This gene specifies resistance to tetracycline at the protein synthesis level rather than encoding an active efflux mechanism as occurs among the TET proteins of *E.coli* (Burdett, 1986).

Taylor *et al.* (1987) showed that the resistance determinant encoded a 68 kDa protein in *E.coli* maxicells and *in vitro* transcription-translation systems, with formation of altered proteins after insertion of *Tn* 1000 into the cloned gene. Later mapping and sequence determination revealed 76% homology with *tet*M (Manavathu *et al.*, 1988).

Sougakoff *et al.* (1987) sequenced the tetracycline resistance gene from *C.coli* plasmid pIP1433 (see next section) and also determined that it had 76% sequence identity with *tet*M. They referred to this *Campylobacter* gene as *tet*O. Since *C.jejuni* and *C.coli* resistance determinants were highly homologous, Ng *et al.* (1987) proposed that both should be designated as members of class O. In the same year, Zilhao *et al.* (1988) demonstrated homology to the *tet*O gene in six *Streptococcus* strains and two *Enterococcus* strains. In their concluding remarks, they proposed that *tet*O probably originated in Gram-positive bacteria. This argument was

based on the higher G + C content of *tet*O (40%) compared with that of *Campylobacter* genomic and plasmid DNA (31 to 33%) and complementarity of the *tet* O ribosome binding site to the 3'-OH terminus of the *Bacillus subtilis* 16S rRNA.

1.8.1.2. Kanamycin resistance

Enzymes modifying aminoglycoside antibiotics are classified according to the chemical reaction catalysed and the site of modification on the antibiotic molecule. 3'-aminoglycoside phosphotransferases, APH(3'), catalyse phosphorylation of the hydroxyl group at position 3' of aminohexose I of kanamycin and related molecules (Davies and Smith, 1978). Six such phosphotransferases have previously been described. Of these, *aphA*-I, *aphA*-II and *aphA*-IV are restricted to Gram-negative bacteria. Prior to the work reported below, the *aphA*-III gene had been described as being resident solely in Gram-positive organisms (Courvalin and Carlier, 1981).

Lambert *et al.* (1985) described a *C.coli* plasmid, pIP1433, which conferred resistance to both tetracycline and kanamycin. The gene encoding kanamycin resistance had extensive structural relationships with that coding for the enzyme conferring resistance to kanamycin in staphylococci and streptococci, 3'-aminoglycoside phosphotransferase type III, previously unknown in Gram-negative organisms. The nucleotide sequence of this gene was later reported and its expression in *E.coli* and *B.subtilis* studied (Trieu-Cuot *et al.*, 1985). The promoter sequence consisted of the hexanucleotides TTGACA and TATAAT separated by a region of 17 base pairs (bp). Transcription in all three bacterial hosts started at the same site located 406 bp upstream from the ATG initiator. It was proposed that kanamycin resistance in *Campylobacter* had occurred through the *in vivo* acquisition of this gene from a Gram-positive bacterium.

Papdopoulou and Courvalin (1988) later investigated the occurrance of *aphA*-III in 160 strains of *Campylobacter* spp.

isolated from humans and animals throughout the world. Six were found to be resistant to both kanamycin and tetracycline, of which four were *C.coli* and the other two *C.jejuni*. The *aphA*-III genes were located on transmissible plasmids ranging in sizes from 45 kb to 61 kb except in one case, where transfer of the smaller 31 kb resident plasmid did not lead to kanamycin resistance in the transconjugants. The authors speculated that the kanamycin resistance determinant might therefore have resided in the chromosome, but no further evidence was put forward for a possible transposition event.

Subsequent screening by Tenover and Elvrum (1988) of 225 isolates of *C.jejuni* and 54 isolates of *C.coli* revealed resistance to kanamycin in five *C.jejuni* and six *C.coli* strains, all of which contained plasmids of different sizes. A probe specific for *aphA*-III hybridised to DNA from only nine of these strains. The remaining two strains showed no homology to probes specific for genes encoding 3'-aminoglycoside phosphotransferase types I, II or III. A 10 kb fragment from one of these plasmids was ligated into vector pBR322 and transformed into *E.coli*, where APH(3') activity was adequately expressed. The nucleotide sequence of this novel kanamycin resistance gene, *aphA*-VII, was later determined. Its G + C content was found to be consistent with that of the *C.jejuni* chromosome, thereby indicating its origin within this genus (Tenover *et al.*, 1989). In addition, the promoter and ribosomal binding site of this gene were typical of the types found in Gram-negative species.

1.8.2. Chromosomal restriction endonuclease analysis

Differentiation of *C.jejuni* isolates on the basis of plasmid content may be applicable in only a few cases because of the limited number of strains harbouring plasmids. In similar circumstances, problems with classification of *Leptospira* and *Rhizobium* species have been resolved by analysis of chromosomal restriction endonuclease digest banding patterns in agarose gels (Mielenz *et al.*, 1980; Marshall *et al.*, 1981). Bradbury *et al.* (1984)

used this technique in conjunction with a conventional serotyping procedure to investigate an outbreak of campylobacter enteritis. Whilst serotyping was incapable of unequivocally determining a relationship between 29 bovine isolates, 20 human isolates and one strain from a milk source, restriction patterns of all bovine and human strains were indistinguishable, confirming an association of the cattle with outbreak of enteritis in man. However, the restriction profile of the milk isolate differed considerably from the other patterns, disqualifying it as a possible means for transmission of the bacteria.

Owen and Beck (1987) addressed the major drawback of this procedure, that of objective and reliable comparison of the complex pattern of bands obtained, by employing a laser scanning densitometer and microcomputer programs to analyse the patterns and estimate fragment sizes. With this modification of the basic method, the authors demonstrated a significant difference between the *C.jejuni* type strain, NCTC 11351, and a number of nitrate negative variants isolated from human gastric epithelium.

1.8.3. Use of probes specific for ribosomal RNA genes

Several reports have described the utility of methods based upon compositional analysis of ribosomal RNA (rRNA) or DNA encoding rRNA (rDNA) for identification of a range of organisms (Lane *et al.*, 1985; Giovannoni *et al.*, 1988; Gobel *et al.*, 1987). The nucleotide sequence of rRNA is highly conserved in several regions of 16S RNA with a lower degree of conservation in surrounding regions (Woese *et al.*, 1983; Gray, Sankoff and Cedergren, 1984). Moureau *et al.* (1989) employed an oligonucleotide probe complementary to the 3' end of *C.jejuni* 16S RNA to differentiate between five different *Campylobacter* reference species, an approach originally proposed by Grimont and Grimont (1986).

Hybridisation data also suggested the presence of at least three copies of 16S rDNA in the *Campylobacter* genome. While

Moureau *et al.*, had utilised an oligonucleotide probe 23 nucleotides in length (23-mer) specific for the 3' terminus, an earlier report by Romaniuk and Trust (1987) had described a 17-mer probe complementary to the 5' domain. Comparable results were obtained in both studies, with agreement on the presence of more than one copy of the 16S rDNA gene.

1.8.4. Phase and antigenic variation of *Campylobacter* flagella

Flagella have been implicated as important virulence factors for several enteropathogenic microorganisms. As an example, the polar flagellum of *V.cholerae* allows the initial means of colonization of the intestine by providing the motility necessary to negotiate the mucus barrier (Guentzal and Berry, 1975). Furthermore, the flagellum acts as an adhesin and thereby forms one of the initial points of contact of the bacterium with the target gastrointestinal cells (Attridge and Rowley, 1983). In addition, the flagella of *S.typhimurium* have been shown to enhance virulence in a manner independent of simply providing the means for motility and chemotaxis, functioning by either protecting the bacterial cell from macrophage killing mechanisms or in some other way which apparently promotes the ability of *S.typhimurium* to multipy within macrophages (Carsiotis *et al.*, 1984; Weinstein *et al.*, 1984).

1.8.4.1. Phase variation

Flagella have also been implicated as virulence factors in the case of *C.jejuni*. Newell *et al.* (1984) demonstrated that a spontaneous aflagellated (Fla^-) variant of strain 81168 was less efficient at colonising the gastrointestinal tract of infant mice compared with the flagellated (Fla^+) form. In the human volunteer study performed by Black *et al.* (1988), equal numbers of Fla^+ and Fla^- forms of strain A3249 were orally administered to one individual, but only flagellated types were isolated from subsequent faecal samples. Caldwell *et al.* (1983) used the RITARD procedure to challenge rabbits with Fla^+ and Fla^- variants of strain 81168 and found that motile campylobacters were isolated from stool cultures

from all animals infected with aflagellate bacteria, indicating that the Fla$^-$ phenotype was quite unstable. These findings were confirmed at the genetic level by the work of Nuijten *et al.* (1989). They demonstrated the absence of both flagellin and flagellin-specific messenger RNA in a non-motile *C.jejuni* variant using Western (or immuno-) and Northern (or RNA) blot analysis respectively. However, Southern blots of DNA from flagellate and aflagellate cells when probed with a DNA sequence specific for the carboxy-terminal portion of the flagellin gene were identical, indicating that the aflagellate non-motile form of *C.jejuni* carried an unexpressed flagellin gene. Flagellin expression in *C.jejuni* was thus shown to be regulated at the transcriptional level.

Such on/off switching, or phase variation, has been documented for a number of bacterial and parasitic pathogens (Borst and Greaves, 1987). These include the phase variation of flagellar antigen types of *Salmonella* (Zieg *et al.*, 1977), and the variation of pilin in *Neisseria gonorrhoeae* via a complex cassette system (Segal *et al.*, 1986; Swanson *et al.*, 1986). Phase variations have also been described for various virulence determinants in *Bordetella pertussis* (Weiss and Falkow, 1984) and strains of *E.coli* pathogenic for the urinary tract (Eisenstein, 1981).

1.8.4.2. Antigenic variation

Harris *et al.*, (1987) demonstrated that campylobacters were capable of producing flagella of different antigenic specificities. Later in the same year, this group (Logan *et al.*, 1987) isolated the flagellins from three strains of *C.jejuni* producing antigenically distinct flagella and from the *C.coli* VC167 strain described in their previous report which produced either phase one (P1) or phase two (P2) flagella. Tryptic and chymotryptic digests of these flagellins followed by SDS-PAGE showed that the *C.jejuni* flagellins were structurally similar and that the *C.coli* P1 and P2 flagellins also shared a high degree of structural relatedness. However, major differences existed in the electrophoretic profiles of *C.jejuni* and *C.coli* flagellins. Analysis of the amino terminal

sequence indicated a high level of conservation amongst the 20 N-terminal amino acids of all *Campylobacter* flagellins. Although the *Campylobacter* flagellin sequence was longer, there was also a limited relatedness to the flagellins of *Salmonella* and *Bacillus* species, suggesting evolution of the flagellin gene from a longer ancestral gene as a result of several deletion events.

Further studies identified a highly conserved sequence of chromosomal DNA from *C.coli* VC167 which was shared with members of the *Enterobacteriaceae* (Guerry *et al.*, 1988). The region of homology was confined to a 700 bp sequence. Phase two *C.coli* cells were then isolated by growth of phase one cells in anti-phase one serum. These cells were subsequently grown in anti-phase two serum and phase one cells reisolated. DNA extracted from the original and revertant phase one cells and the intermediate phase two cells of this series were probed with *S.typhimurium* DNA. This revealed the occurrance of a reversible DNA rearrangement, with the probe hybridising to a 9.4 kb band from phase one cells and an 8.4 kb band from cells of phase two. These results were confirmed by hybridisation profiles of chromosomal DNA blots probed with pGK109 DNA.

Logan *et al.* (1989) reported that the observed antigenic variation was demonstrable *in vivo* by infecting rabbits with *C.coli* cells producing either phase one or phase two cells. Campylobacters isolated from the faeces were then analysed by molecular weight determination and hybridization pattern with the pGK109 probe. Results indicated that rabbits infected with P2 cells shed cells of the same type throughout the experiment. Rabbits infected with P1 cells initially excreted P1 cells, but a transition to P2 forms was noted within two to three days, indicating that an underlying biological preference for phase two flagella was apparent.

Guerry *et al.* (1990) went on to demonstrate that the *C.coli* VC167 chromosome contains two tandemly-arranged full length flagellin genes, *flaA* and *flaB* with 91.5% mutual homology at the nucleotide level. The data presented indicated that these two genes

were transcribed under the control of separate promoters, but were expressed concomitantly within the same cell regardless of whether P1 or P2 flagella were being produced, with the *flaA* gene being expressed at much higher levels than the *flaB* gene in both phases. The *flaA* gene product was the major component of the flagellar filament and the *flaB* gene product was not assembled. Evidence was also presented which indicated that the shift from phase one to phase two cells involved the insertion of a partial flagellin-coding cassette into *flaA* at some point downstream of the amino terminal sequences.

Wassenaar *et al.* (1991) have shown that mutants of *C.jejuni* containing an inactivated *flaA* gene are immotile, whereas those with a defective *flaB* gene remain motile. Moreover, mutants lacking motile flagella were unable to adhere to, and penetrate into, INT-407 human intestinal cells *in vitro*.
phosphorylation reaction in contrast to the methylation processes more commonly encountered in prokaryotic systems (Ambler and Rees, 1959; Sastry *et al.*, 1985). The identity of the flagellin as either P1 or P2 could not be determined by reference to the molecular weight data because of aberrant migration characteristics in SDS-PAGE gels, presumeably due to these modifications. When compared with the amino acid sequences of flagellins from *B.subtilis* and *S.typhimurium,* conserved residues were noted at both the amino and carboxy terminals, with the internal region being highly variable. The N-terminus of the *Salmonella* flagellin is known to be involved in regulatory functions (Wei and Joys, 1985) whilst both the N- and C-termini have been shown to function in transport and assembly of the monomers into the flagellar filament (Homma *et al.*, 1987).

1.8.5. Cloning of chromosomally encoded genes

1.8.5.1. Cloning of proline biosynthesis genes

Lee *et al.* (1985) were the first to describe the cloning and expression of a *Campylobacter* chromosomal gene in *E.coli*. A

C.jejuni genomic library was prepared in vector pBR322 and transformed into *E.coli* HB101. Three clones were identified which could complement a nutritional defect for biosynthesis of proline.

Synthesis of proline in *E.coli* has been shown to occur essentially via three enzymatic steps, as shown in Fig. 3 (Vogel and Davies, 1952; Baich, 1971). The genes *proA* and *proB* form a single operon at minute six of the *E.coli* chromosome while *proC* maps further at minute nine (Bachman and Low, 1980). A similar genetic linkage was also described in *S.typhimurium* by Mahan and Csonka (1983).

Lee, Walker and Guerry (1985) reported that their clones were able to complement two strains deleted for both *proA* and *proB*, but no complementation was achieved for a *proC* mutant, suggesting a linkage relationship for these genes as recorded in *E.coli* and *S.typhimurium*. No conclusive evidence was presented to show whether expression in *E.coli* was occurring via a *Campylobacter* promoter.

1.8.5.2. Cloning of the *glyA* gene

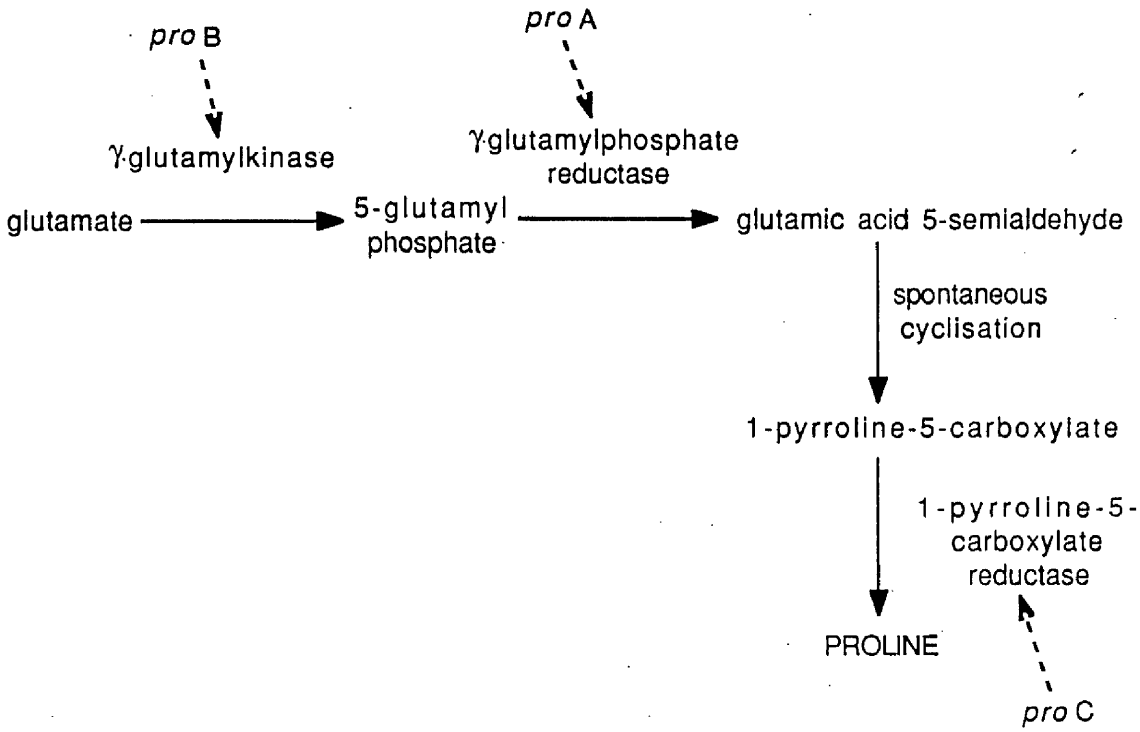
From a genomic library of *C.jejuni* DNA, several clones were identified which contained the complete *glyA* structural gene (Chan *et al.*, 1988). This gene encodes serine hydroxymethyltransferase (SHMT), an enzyme catalysing the reversible conversion of serine to glycine, with tetrahydrofolate acting as a C1 acceptor by its conversion of 5,10-methylenetetrahydrofolate (Blakley, 1969). The functional gene was confined to a 2.3 kb *Hin*dIII fragment, from which successful expression was obtained in a specialised vector from which active transcription could only proceed if the insert contained a functional promoter. Thus, the SHMT gene of *C.jejuni* was probably transcribed in *E.coli* from its own promoter.

1.8.5.3. Cloning of genes coding for rRNA

C.jejuni ribosomal RNA genes (rDNA) were cloned in *E.coli* HB101 by Rashtchian *et al.* (1987). The gene coding for 16S rRNA

Figure 3: Pathway of Proline Biosynthesis in Prokaryotes

(Adapted from the Reports of Baich, 1971 and Vogel & Davies, 1952)


pro B
pro A
γ-glutamylkinase
γ-glutamylphosphate reductase
glutamate
5-glutamyl phosphate
glutamic acid 5-semialdehyde
spontaneous cyclisation
1-pyrroline-5-carboxylate
1-pyrroline-5-carboxylate reductase
PROLINE
pro C

was located on a 1.7 kb *Hin*dIII fragment, while the 5S and 23S rRNA genes were on *Hin*dIII fragments of 1.65 and 5.5 kb respectively. The fragment containing the 16S rRNA gene was characterised further by restriction endonuclease mapping and sequencing. Examination of the deduced nucleotide sequence of the 3' end of *C.jejuni* 16S rRNA revealed a high degree of similarity to that of *E.coli* 16S rRNA, confirming the accepted view that this portion is highly conserved in many species (Woese *et al.*, 1983).

1.8.5.4. Cloning of the flagellin gene

Nuijten *et al.* (1989) prepared a *C.jejuni* genomic library in λgt11, a vector system developed by Young and Davis (1983) which expresses inserted DNA as a protein fused to ß-galactosidase and allows both efficient expression of the inserted DNA and screening of large numbers of clones. This library was screened by incubating nitrocellulose filter replicas of recombinant plaques in the presence of flagellin-specific antiserum. Immunoscreening of approximately 150000 recombinant plaques gave rise to only one positive clone which contained an 850 bp insert. Western blot analysis of the protein product recognised by the anti-flagellin serum identified a 130 KDa moiety representing a flagellin-β-galactosidase fusion protein. In addition to the findings already recorded, Southern blot analysis of *C.jejuni* chromosomal DNA revealed the presence of three *Hin*dIII fragments reacting positively with a probe constructed from the 850 bp insert. This indicated that the sequences present in the probe occurred more than once in the genome.

A markedly different strategy was employed by the group of Logan *et al.* (1989) to clone the flagellin gene from *C.coli*. Genomic DNA fragments generated by complete digestion with *Hin*dIII were initially fractionated on sucrose gradients. After agarose gel electrophoresis, each fraction was transferred to nitrocellulose and probed with an oligonucleotide specific for residue numbers four to nine of the *C.coli* flagellin. A fraction containing DNA fragments of

approximately 2.0 to 2.5 kb gave the strongest signal and was cloned in vector pBR322 and transformed into *E.coli* DH5. Of the 2000 resultant colonies, five clones were identified which hybridised to the flagellin-specific oligonucleotide. Evidence for posttranslational modifications of the *Campylobacter* flagellin and the presence of two flagellin genes has already been detailed elsewhere. Data indicated that the *flaA* gene represented the major component of the flagellar filament and that the *flaB* gene product could only be assembled in the presence of *flaA*, presumably because of a lack of sufficient gene product synthesised under the direction of the *flaB* promoter.

1.8.6. Development of shuttle vectors for genetic analysis

Recognising the need for a genetic exchange system which would permit the introduction of genes into *Campylobacter* cells, Labigne-Roussel *et al.* (1987) developed a shuttle cloning vector which could be mobilized from *E.coli* to *C.jejuni, C.coli* and *C.fetus* at a frequency of 10^{-4} transconjugants per donor by complementation *in trans* with the transfer functions of an IncP plasmid.

This vector was constructed by inserting the 1.427 kb *Cla*I-*Hin*dIII fragment of *Ccoli* plasmid pIP1433 containing the gene encoding 3'aminoglycoside phosphotransferase III for kanamycin resistance into pBR322. Into this recombinant plasmid, pILL502, was then inserted the entire length of a high copy number cryptic *C.coli* plasmid, pIP1445, to supply *Campylobacter* replication functions (forming pILL505). A 760 bp *Bam*HI fragment containing the *ori*T sequence was then added from pEYDG1 (originally derived from RK2 - see Yakobson and Guiney, 1984) to give the shuttle vector, pILL512. From this initial shuttle vector construct, various plasmids were subsequently generated with deletions of portions of the pIP1445 DNA region. From these, one was selected of minimum size which still retained the ability to replicate in *C.jejuni*. Addition of a polylinker sequence and removal of *Pst*I, *Bam*HI and *Sal*I endonuclease sites adjacent or internal to the *ori*T sequence

resulted in an 8.5 kb shuttle cloning vector with unique restriction sites for *Bam*HI, *Cla*I, *Eco*RI, *Pst*I, *Sal*I and *Sma*I. The construction and structure of this vector, designated pILL550, are presented in Fig. 4 and Fig. 5.

Figure 4: Construction of *E.coli-Campylobacter* Shuttle Vectors

(Adapted from the report of Labigne-Roussel *et al.*, 1987)

Restriction endonucleases used:
Bam : *Bam*HI
Cla : *Cla*I
Hin : *Hin*dIII

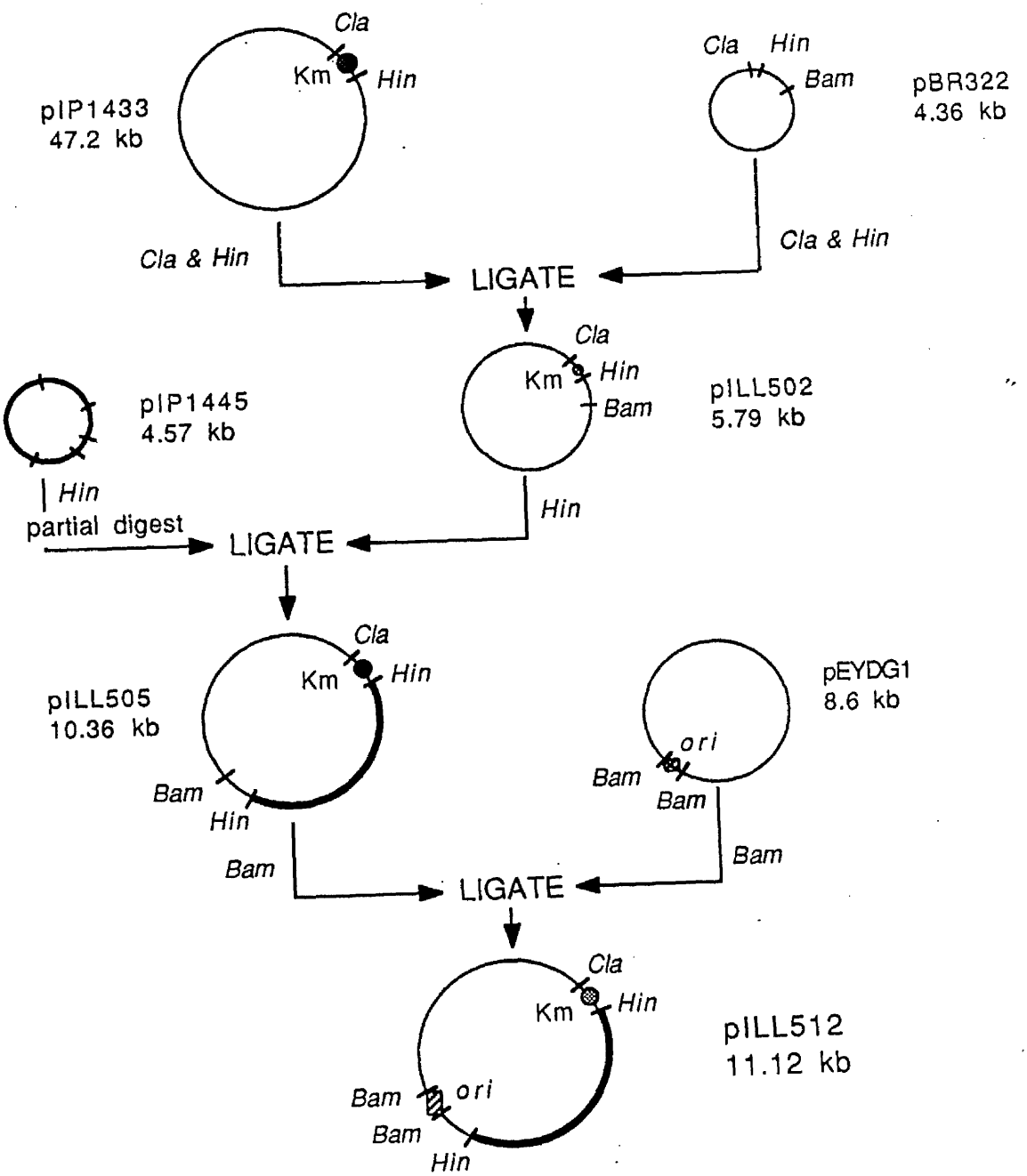
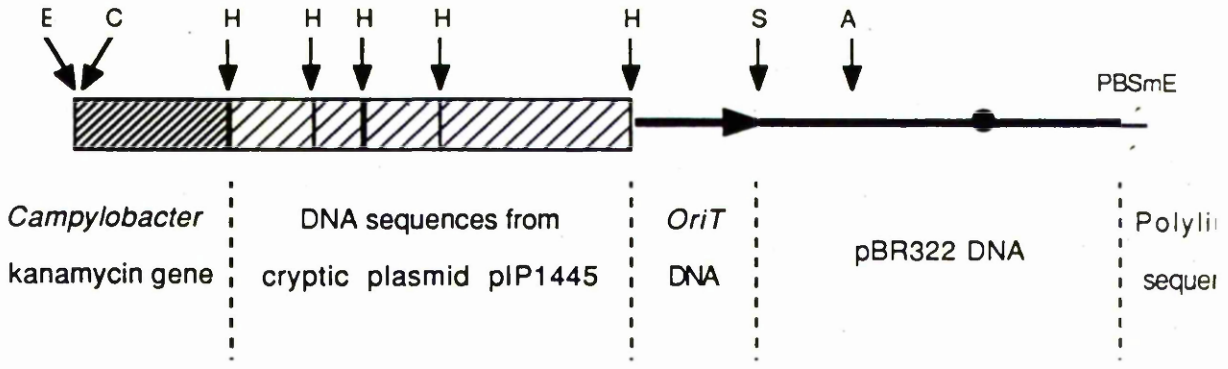

Cla
Km
Hin
pIP1433
47.2 kb
Cla Hin
Bam
pBR322
4.36 kb
Cla & Hin
Cla & Hin
LIGATE
Cla
Km
Hin
Bam
pILL502
5.79 kb
pIP1445
4.57 kb
Hin
partial digest
Hin
LIGATE
Cla
Km
Hin
pILL505
10.36 kb
Bam
Hin
Bam
pEYDG1
8.6 kb
ori
Bam
Bam
Bam
LIGATE
Cla
Km
Hin
pILL512
11.12 kb
Bam
ori
Bam
Hin

Figure 5: Restriction Endonuclease Map of Shuttle Cloning Vector pILL550

(Adapted from the report of Labigne-Roussel *et al.*, 1987)



Restriction endonuclease sites:

A: *Aval*	B: *Bam*HI	C: *Cla*I
E: *Eco*RI	H: *Hin*dIII	P: *Pst*I
S: *Sal*I	Sm: *Sma*I	

AIMS OF THE RESEARCH

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These studies were directed towards the identification of one or more factors expressed by *Campylobacter jejuni* which might contribute towards the virulence of the organism. Although several properties have been proposed as being of potential importance in this regard, a definitive role has not, as yet, been established for any of these in the disease process.

Molecular cloning techniques have not been applied to the investigation of *C.jejuni* virulence determinants. Such procedures would allow the study in a foreign host of a single determinant independently of other factors which might otherwise give rise to confusion. The expression of virulence properties in a host previously devoid of such pathogenic potential would clarify the role of individual factors produced by *Campylobacter.*

Four major objectives were to be realised. Firstly, putative virulence properties had to be identified and methods for their detection assessed with regard to accuracy, reproducibility and ease of operation. Secondly, a statistically complete library of *C.jejuni* chromosomal DNA sequences had to be generated in *E.coli.* A major obstacle was the maintenance of the integrity of library segments in this foreign host. Thirdly, either the gene product associated with the virulence determinant, or the gene itself, had to be identified amongst the clones obtained. Finally, the technique of transposon mutagenesis was to be employed in order to inactivate one specific virulence property and note the effects on the overall phenotype.

2. MATERIALS AND METHODS

2.1. *E.coli* K12 strains

E.coli K12 strains employed during the course of these investigations are listed in Table 1.

2.2. Isolation of *Campylobacter* spp. from faecal samples

Faecal samples were made available by the Microbiology Departments of Law Hospital (Carluke, Scotland) and Ruchill Hospital in Glasgow and by the Pathology Department of the Western General Infirmary, Leeds. The specimens were plated onto Oxoid blood agar base supplemented with 10% ($v/_v$) defibrinated sheep blood (Becton Dickinson) and made selective by addition of vancomycin (10 μg/ml), polymixin B (2.5 international units/ml), trimethoprim (5 μg/ml), cephalothin (15 μg/ml) and amphotericin B (2 μg/ml) as suggested by Blaser *et al.* (1979). These antibiotics were supplied as a dry powder mixture yielding the above concentrations when reconstituted (Blaser-Wang *Campylobacter* selective supplement; Oxoid). Plates were incubated microaerophilically at 42°C for three days. Isolates were identified as members of the *C.jejuni-coli* group by Gram-stained morphology and positive catalase and oxidase reactions, performed according to the guidelines of Ullmann (1979). Strains of *Campylobacter* spp. used in these studies are listed in Table 2.

2.2.1. Growth of campylobacters on solid media

Routine growth of *Campylobacter* strains was performed on blood agar (BA) base (Oxoid) supplemented with 7% ($v/_v$) defibrinated sheep blood (Becton Dickinson). Plates were adequately dried prior to use to prevent swarming. Incubation was performed in anaerobic jars at 42°C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N).

2.2.2. Growth of campylobacters in liquid media

A starter culture was prepared by emulsifying several

Table 1: *E.coli* K12 Strains Used in this Study

***E.coli* Strain**	**Genotype**	**Reference**
C600	F⁻*thi*-1 *thr*-1 *leu* B6 *lac* Y1 λ⁻ *ton* A21 *sup* E44	Appleyard (1954)
DH1	F⁻*rec* A1 *end* A1 *gyr* A96 *thi*-1 λ⁻ *hsd* R17($r_K^-m_K^+$) *sup* E44 *rel* A1	Hanahan (1983)
DH5	F⁻*end* A1 *rec* A1 *hsd* R17 *sup* E44 ($r_K^-m_K^+$) *thi*-1 λ⁻ *gyr* A96 *rel* A1	Hanahan (1985)
DH5αMCR™	F⁻*mcr* A *mcr* B *mrr* φ80d *lac* ZΔM15Δ(*lac* ZYA-*arg* F) U169 *end* A1 *rec* A1 *hsd* R ($r_K^-m_K^+$) *sup* E44 *thi*-1 *gyr* A96 *rel* A1 λ⁻	Bethesda Research Laboratories
HB101	F⁻*hsd* S20($r_B^-m_B^+$) *rec* A13 λ⁻ *leu* B6 *ara*-14 *pro* A2 *lac* Y1 *gal* K2 *rps* L20 *xyl*-5 *mtl*-1 *sup* E44	Boyer & Roulland-Dussoix (1969)
JM83	F⁻*ara* Δ(*lac-pro* AB) *rps* L φ80d *lac* ZΔM15	Vieira & Messing (1982)
LE392	F⁻*hsd* R514($r_K^-m_K^+$) *sup* E44 *sup* F58 *lac* IY1 or Δ(*lac* IZY)6 *gal* K2 *gal* T22 *met* B1 *trp* R55 λ⁻	Murray *et al.* (1977)

Table 2: *Campylobacter* Strains Used in this Study

Isolate Number	Source of Strain or Faecal Sample	Strain and Characteristics
NCTC 11168		*C.jejuni* biotype 1
NCTC 11352	Dr. D.S Tompkins,	*C.laridis* type strain
NCTC 11353	Microbiology Department,	*C.coli*
NCTC 11366	University of Leeds	*C.coli* type strain
NCTC 11392		*C.jejuni* biotype 2
NCTC 11457		*C.laridis*
346/84		*C.jejuni* (Tc^r)
350/84	Dr. M.B. Skirrow,	*C.jejuni* (Tc^r)
408/84	Microbiology Department,	*C.jejuni* (Tc^r)
409/84	Worcester Royal Infirmary,	*C.coli* (Tc^r)
410/84	Worcester	*C.coli* (Tc^r)
435/84		*C.jejuni* (Tc^r)
503/84		*C.jejuni* (Tc^r)
1430	Mr. R. Bellhouse,	*C.jejuni* biotype 2
1866	Veterinary Investigation Center, Lincoln	*C.jejuni*
2677		*C.jejuni /coli*
4293		*C.jejuni /coli* (Tc^r)
6696		*C.jejuni /coli*
41161	Pathology Department,	*C.jejuni /coli* (Tc^r)
49775	Western General Infirmary,	*C.jejuni /coli*
51517	Leeds	*C.jejuni /coli*
52156		*C.jejuni /coli*
54713		*C.jejuni /coli*
55278		*C.jejuni /coli*
57449		*C.jejuni /coli*

(Table continued overleaf.....)

Table 2 (continued)

Isolate Number	Source of Strain or Faecal Sample	Strain and Characteristics
01110	Microbiology Departments of Law Hospital, Carluke and Ruchill Hospital, Glasgow	*C.jejuni*
01987		*C.jejuni*
03584		*C.jejuni*
03587		*C.jejuni /coli*
03611		*C.jejuni*
03987		*C.jejuni /coli*
04676		*C.jejuni /coli*
05030		*C.jejuni /coli*
05803		*C.jejuni*
05982		*C.jejuni*
12444		*C.jejuni /coli*
45340		*C.jejuni*
45457		*C.jejuni*
48609		*C.jejuni*
51513		*C.jejuni /coli*
51680		*C.jejuni*

loopfuls of *Campylobacter* growth in 1 ml of brain heart infusion (BHI; Difco) broth and using this as inoculum for 100 ml of the same medium. This was incubated in a microaerophilic atmosphere in an anaerobic jar secured on an orbital shaker running at 100 revolutions per minute (rpm) for 48 h. Seeding of subsequent cultures was performed with 1 ml of the starter culture per 100 ml of fresh broth. Several passages of the strain in fresh broth were often required to attain a high density of growth in liquid medium. Prior to each passage, the inoculum was checked for purity by Gram-stained appearance.

2.2.3. Long term storage of *Campylobacter* strains

Several loopfuls of an overnight culture of the *Campylobacter* strain were removed from a BA plate and emulsified in 1.5 ml of BHI broth containing 40% ($v/_v$) glycerol. This was placed immediately at -70°C. Cultures were grown from frozen stocks by thawing rapidly at 37°C and spreading 150 µl of the bacterial suspension on a pre-warmed BA plate. Incubation was performed as above.

2.2.4. Hippurate hydrolysis test

When appropriate, strains were confirmed as being *C.jejuni* or *C.coli* using a rapid hippurate hydrolysis test. This method was first reported for presumptive identification of group B streptococci (Hwang and Ederer, 1975) and its use for differentiation of campylobacters was proposed by Harvey (1980).

A loopful of an 18 to 24 h plate culture was emulsified in 400 µl of 1% sodium hippurate (Sigma) and incubated at 37°C for 2 h, the tube being shaken at regular intervals. An overlay solution comprising 3.5% ($w/_v$) ninhydrin (Sigma) dissolved in a 1:1 mixture of acetone and butanol was then carefully applied and incubation continued for a further ten min. A positive reaction, indicating the breakdown of hippurate to glycine by a hippuricase enzyme, was demonstrated by the appearance of a purple interface between the

two layers. A pale blue or colourless interface was regarded as a negative reaction.

2.2.5. Evaluation of extracellular DNase production by *C.jejuni* 11168

Two detection methods were utilised to assess whether *C.jejuni* 11168 could secrete enzymes capable of degrading extraneous plasmid DNA, a problem recognised by the manufacturers of the Gene Pulser equipment (Bio-Rad Laboratories) to be of particular relevance due to the high cell concentrations employed in electroporation procedures. The protocols used were essentially as described by Hänninen (1989). DNase agar (Difco) containing 0.01% ($^{w}/_{v}$) toluidine blue O was adjusted to pH 7.3 with 50 mM Tris buffer and poured in petri dishes. DNase production was tested by heavily inoculating a small area with bacterial growth from an overnight BA culture. Plates were incubated miroaerophilically at 42°C for 48 h.

For the agar diffusion method, a turbid suspension of cells was prepared in 0.5 ml of 0.1M Tris-HCl buffer (pH 7.3) containing 1.6 X 10^3 IU/ml polymixin B (Sigma). This was incubated at 4°C for 15 min. Wells of approximately 0.5 cm diameter cut in DNase toluidine blue O plates were then filled with this suspension and plates incubated aerobically at 37°C for 24 h.

In both cases, after incubation, a pink zone with a definite edge resulting was considered a positive reaction, with *Staphylococcus aureus* (Oxford strain; obtained from the Department of Microbiology strain collection) being used as a positive control. For the agar diffusion method, a well filled with Tris-HCl buffer (pH 7.3) containing polymixin B (1.6 X 10^3 IU/ml) acted as a negative control.

2.3. Demonstration of haemolysin directly on agar

C.jejuni normally demonstrates no potential for haemolytic activity on BA plates. However, it was found that addition of

cysteine hydrochloride (Cys-HCl) at a final concentration of 6.5 mM to Oxoid blood agar base promoted the formation of haemolytic zones in the presence of 7% sheep blood after 48 h microaerophilic incubation at 42°C. This effect was also observed at 37°C after 72 h incubation.

To determine the spectrum of sensitivity of erythrocytes from various species to the *C.jejuni* haemolysin, BA plates, either with or without added Cys-HCl, was supplemented with defibrinated blood from adult chicken, donkey, guinea pig, rabbit (all from Serotec, Oxford) and horse (Becton Dickinson). In addition, goat erythrocytes in alsevers solution (Serotec), heparinised ox and citrated mouse blood were also tested. The latter two were obtained respectively from the Veterinary School, Glasgow and the Microbiology Department animal house facility.

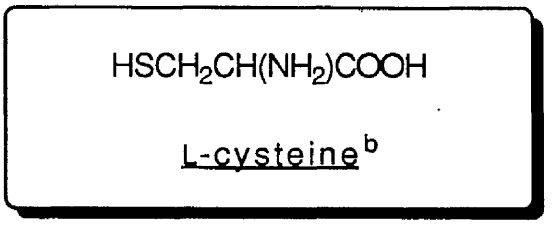
Compounds structurally related to L-cysteine were also investigated for the ability to promote haemolysis in BA base containing 7% sheep cells. Filter sterilised solutions of L-cysteine methyl ester hydrochloride, L-cysteine ethyl ester hydrochloride, 5-methyl-L-cysteine and L-S,S´ methylenebiscysteic acid (all from Sigma) were incorporated into plates at concentrations of 0.05, 0.1 and 0.2 mg/ml. Plates were inoculated for single colonies and incubated as above. L-cystine dihydrochloride was added directly to the medium at 0.1 mg/ml prior to autoclaving; higher concentrations were found to precipitate out of the medium (solubility equivalent to 0.112 mg/ml at 25°C; Budavari *et al.*, 1989).

2.3.1. Role of iron regulation and release of haemolysin

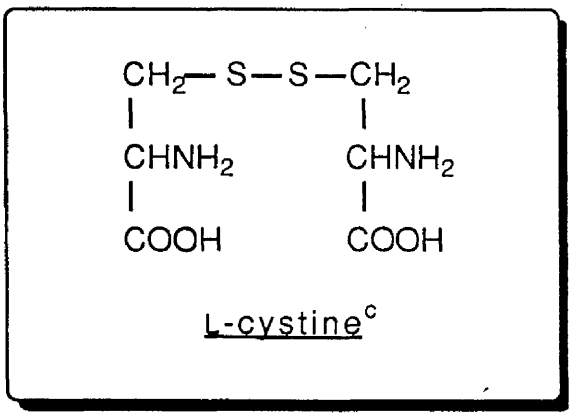
Three metal chelating agents were independently tested for their ability to promote haemolysis. Disodium EDTA (pH 8.0) was added to 20 ml of molten blood agar base supplemented with 7% defibrinated sheep blood to give doubling concentrations of EDTA ranging from 0.05 mM to 50 mM. Plates were poured, set and dried according to normal practice.

Figure 6: Chemical Structures of L-Cysteine, L-Cystine and Related Compounds [a]

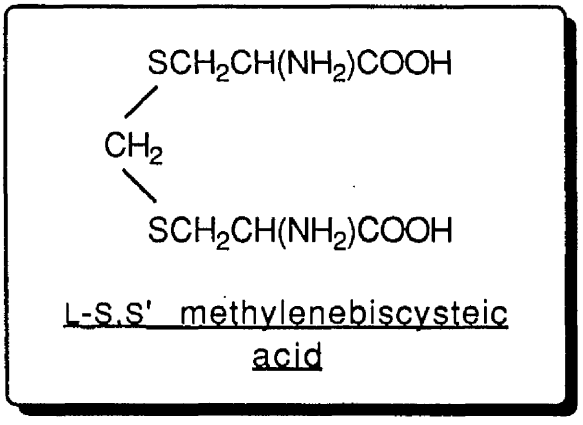
From: Budavari *et al.* (1989). The MERCK Index, Eleventh Edition.



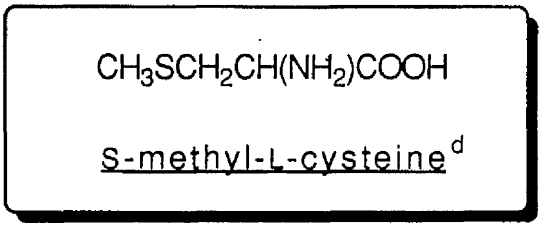
L-cysteine[b]



L-cystine[c]



L-S,S' methylenebiscysteic acid



S-methyl-L-cysteine[d]

[a] L-cysteine ethyl ester is not shown

[b] hydrochloride salt was used in these studies

[c] dihydrochloride salt was used in these studies

[d] the hydrochloride salt of this compound is referred to above as L-cysteine methyl ester HCL

Plates containing doubling concentrations of 8-hydroxyquinoline (dissolved in ethanol; Sigma) ranging from 0.05 mM to 12.5 mM and 2,2'-dipyridyl (Sigma) ranging from 1.17 μM to 300 μM were similarly prepared. All plates were inoculated in duplicate with *C.jejuni* 11168 and incubated microaerophilically at 42°C for up to five days.

2.3.2. Demonstration of haemolysin from cell extracts

C.jejuni 11168 was inoculated into 50 ml of BHI broth or BHI broth containing 6.5 mM Cys-HCl and incubated with shaking in a microaerophilic atmosphere at 42°C for 12, 24, 36, 48 or 72 h. Cells were harvested by centrifugation at 6×10^3 X g for 10 min at 4°C in a Sorvall SS-21 rotor, gently resuspended in 5 ml of ice cold PBS and then disrupted by sonication in an MSE 100 ultrasonic disintegrator. Two cycles of sonication were applied, each lasting five min, with a two min interval between cycles. Sonicates were centrifuged at 1×10^4 X g for 20 min in a Sorvall SS-21 rotor running at 4°C. The cell extract was passed through a 0.45 μm membrane filter (Acrodisc; Gelman Sciences) and 45 μl added to 4 mm diameter wells cut in agar media.

2.3.3. Assay of haemolysin by tube or microdilution methods

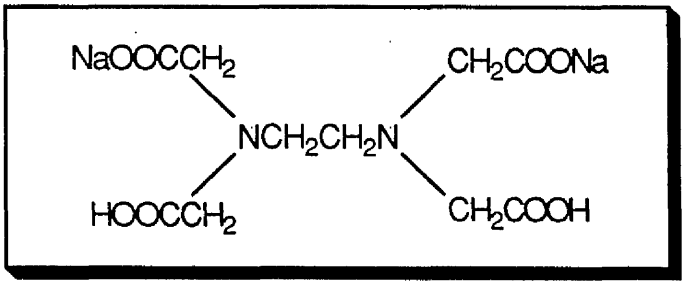
Cell extracts of 12, 24, 36, 48 or 72 h cultures of *C.jejuni* 11168 were prepared as above. After harvesting cells from the culture medium, supernatants were decanted and passed through 0.45 μm filters (Acrodisc; Gelman Sciences). Assay of haemolysis was attempted by a number of tube and microtitration procedures, as detailed in the Results section.

2.4. Evaluation of latex agglutination in testing for enterotoxin production

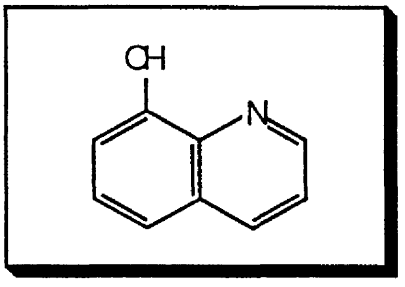
The utility of a reversed passive latex agglutination procedure designed for the detection of *Vibrio cholerae* enterotoxin (CT) and *E.coli* heat labile enterotoxin (LT) was assessed for the

Figure 7: Chemical Structures of Disodium EDTA, 8-Hydroxyquinoline and 2,2'-Dipyridyl

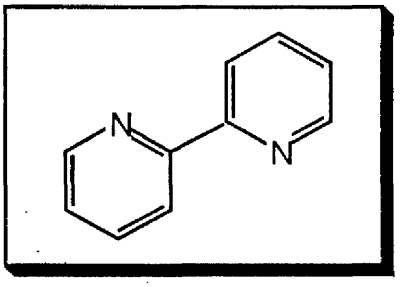
From: Budavari *et al.* (1989). The MERCK Index, Eleventh Edition.



Disodium EDTA



8-Hydroxyquinoline



2,2'-Dipyridyl

determination of enterotoxin production by *Campylobacter* spp. Cultures of *C.jejuni* 11168 grown in BHI broth for 24, 48 and 72 hours were harvested by centrifugation at 1 X 10^4 rpm for 10 min in a Sorvall SS-34 rotor running at 4°C. Supernatants were passed through 0.45 µm pore size membrane filters (Acrodisc; Gelman Sciences). The cell pellets were resuspended to give a density of 0.75 optical density units (ODU; 650 nm) in saline containing 1 X 10^4 IU/ml polymixin B (Sigma). Following incubation at 37°C for 30 min, a 1 ml volume of the treated cell pellets was centrifuged at 4 X 10^3 rpm for 20 min in an Heraeus microfuge and the supernatants filtered as above. In addition, three fresh clinical isolates were also tested in a similar manner. All three had been sub-cultured on laboratory media only once after primary isolation from faecal material to ensure purity of the cultures and had then been stored at -70°C in BHI broth containing 40% ($^v/_v$) glycerol. After removal from storage, the strains were grown on BA plates to again ensure purity and were then inoculated into BHI broth.

All reagents, including control *V.cholerae* enterotoxin and latex particles sensitized with purified rabbit IgG against CT, were supplied by Oxoid Diagnostic Reagents (VET-RPLA kit). Duplicate two-fold dilutions of test supernatants and control enterotoxin were performed in 25 µl volumes in round-bottomed microtitre plates (Sterilin) using PBS containing 0.5% ($^w/_v$) bovine serum albumin (BSA) as diluent. To one set of dilutions was added 25 µl of sensitized latex, whilst to the other was added an equal volume of control latex coated with non-immune rabbit globulins. Each well was examined for agglutination of the latex particles after incubation at room temperature for 24 h.

2.5. Tissue culture cytotoxicity assays

2.5.1. Continuous culture of cell lines

HeLa and Vero cells (Flow Laboratories) were propagated as monolayers within plastic tissue culture flasks in Eagle's minimal essential medium (E-MEM) supplemented with 2 mM glutamine and

10% ($v/_v$) foetal calf serum (both purchased from Flow Laboratories). Penicillin (100 IU/ml) and streptomycin (100 IU/ml) were added to prevent microbial contamination. A sterile stream of 5% CO_2, 95% air (British Oxygen Company) was passed over the surface of the medium until a colour change from pink to yellow was observed, indicating that a hydrogen ion concentration suitable for cellular proliferation had been attained.

To maintain the cell lines in continuous culture, monolayers were washed with sterile PBS after discarding the overlying medium and 5 to 10 ml of trypsinizing solution (0.05% $w/_v$ trypsin and 0.02% $w/_v$ EDTA; Flow Laboratories) added to cover the base of the flask. Cells were detached by gently tapping the flask after 5 min at room temperature and cell aggregates broken up by vigorous pipetting of the suspension, aliquots of which were immediately introduced into fresh medium. For general maintenance of the cell lines, monolayers were stripped and re-seeded every five to six days.

2.5.2. Cytotoxicity assay procedure

2.5.2.1. Preparation of eukaryotic cells

Cell monolayers were detached as described above, resuspended in 20 ml of fresh medium in a sterile plastic centrifuge tube (Sterilin) and harvested by centrifugation at room temperature in an MSE Minor benchtop centrifuge at 1.5×10^3 rpm for 5 min. Cells were resuspended in 1 ml of fresh medium and an aliquot diluted in 0.1% ($w/_v$) trypan blue solution, a stain rendering dead cells blue. A small volume was applied to a haemocytometer well and the number of unstained cells per ml of undiluted suspension calculated. The stock suspension was then diluted in fresh medium to yield a final viable cell density of 1×10^5 cells/ml.

2.5.2.2. Preparation of *C.jejuni* cell extracts grown in BHI broth

In addition to *C.jejuni* 11168, three fresh clinical isolates were also tested for cytotoxicity. All three had been sub-cultured on laboratory media only once after primary isolation from faecal material to ensure purity of the cultures and had then been stored at -70°C in BHI broth containing 40% ($^{v}/_{v}$) glycerol. Subsequent to removal from storage, the strains were grown on BA plates to again ensure purity and were then inoculated into liquid medium.

All *Campylobacter* strains were grown in BHI broth for 24 or 48 h. Cells were harvested by centrifugation for 15 min at 4°C in a Sorvall SS-21 rotor at 1 X 10^4 rpm. The supernatant was removed, sterilised by filtration (0.22 µm; Acrodisc) and retained at 4°C for subsequent testing. The cells were resuspended in PBS to an OD of 0.4 ODU (600 nm). Polymixin B was added at a final concentration of 1.5 mg/ml. After 1 h at 37°C, the cells were pelleted by centrifugation for 20 min at 1.2 X 10^4 rpm in an Heraeus microfuge at 4°C. The supernatant was aspirated and sterilised by passage through a 0.22 µm pore size filter (Acrodisc; Gelman Sciences).

2.5.2.3. Preparation of *C.jejuni* cell extracts grown on BA

Campylobacter strains were grown as confluent lawns on BA plates or BA plates supplemented with 6.5 mM Cys-HCl for 24 to 48 h. Growth was washed off with 2 ml of sterile PBS and resuspended to an OD of 0.4 ODU (600 nm). Polymixin B treatment and centrifugation were performed as described above. As a negative control, the surface of an uninoculated BA plate and BA/Cys-HCl plate were flooded with 2 ml of sterile PBS. This sample was then processed exactly as above.

2.5.2.4. Preparation of *E.coli* cell extracts

Verotoxin 2 producing *E.coli* strain E32511 (kindly provided by Dr. G.A. Willshaw, Central Public Health Laboratory, London) was grown aerobically at 37°C in BHI broth. Cells were harvested and treated with polymixin B as outlined above. This extract acted as a

cytotoxin positive control when tested against the Vero cell line. In addition, *E.coli* genetic strains DH1 and DH5 were also tested for cytotoxicity.

2.5.2.5. Assay procedures

Suspensions of HeLa or Vero cells in E-MEM were introduced into wells of sterile 96-well flat-bottom microtiter plates (Flow Laboratories). Volumes of 100 µl were used containing either 2×10^3 or 1×10^4 cells. The final column of wells in each plate received 200 µl of E-MEM only. Plates were maintained at 37°C in an atmosphere of 5% CO_2, 95% air for 4 h to allow the cells to adhere. Serial twofold dilutions of *C.jejuni* polymixin B-treated filtrates made in E-MEM were then added in quadruplicate to the monolayers in 100 µl volumes and the plates returned to the CO_2/air atmosphere at 37°C for 24 hours. Polymixin B-treated extracts of verotoxin producing *E.coli* strains served as positive controls for assays using Vero cells. Polymixin B-treated BHI broth and PBS containing polymixin B acted as negative controls for both cell lines.

Two assay protocols were followed to assess the extent of cytotoxicity. In the first, 20µl of 0.1% ($^w/_v$) trypan blue solution were added to each well and dead cells differentiated from living cells by viewing under an inverted microscope (Olympus Optical Company).

The second method involved the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma), the tetrazolium ring of which is cleaved by dehydrogenase enzymes in the mitochondria of living cells to produce a dark blue formazan product (Mosmann, 1983). Following incubation, 20 µl of 5 mg/ml MTT (prepared in PBS and filtered through a 0.22 µm Acrodisc filter) was added to each well and incubation continued at 37°C for a further 4 h. The overlying medium was then removed by rapid inversion of the plate and the formazan product solubilised by addition of 100 µl of 0.04 N HCl in dimethylsulphoxide. The plates

were examined spectrophotometrically at 620 nm using a Titertek Multiscan MC photometer employing mode1 and filter 7 of the apparatus. Cytotoxicity was calculated as follows:

$$\% \text{ Cell death} = 1 - \left(\frac{\text{OD of test wells}}{\text{OD of negative control wells}} \right) \times 100$$

All wells contained similar concentrations of cultured mammalian cells. Cells in the control wells had not been exposed to the cytotoxin being tested.

2.6. DNA Manipulation procedures

2.6.1. Small-scale isolation of plasmid DNA

The method used was based on that communicated by Ish-Horowicz and reported by Maniatis *et al.* (1982). It is essentially a modification of the alkaline lysis procedure of Birnboim and Doly (1979).

A single bacterial colony was inoculated into 5 ml of nutrient broth (Oxoid) containing the appropriate antibiotic and incubated overnight at 37°C on a rotary platform. A culture volume of 1.5 ml was transferred to a microfuge tube and cells harvested by centrifugation at $1.3 \times 10^4 \times g$ in an Heraues Biofuge A microfuge. The supernatant was removed to leave the pellet as dry as possible and the pellet resuspended by vortexing in 100 µl of an ice-cold solution containing 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl (pH 8.0) to which lysozyme (Sigma) was added at a concentration of 4 mg/ml immediately prior to use. After 5 min at room temperature, 200 µl of an ice cold solution of 0.2N NaOH and1% SDS was added and the tube inverted slowly several times. After a period of five min on ice, 50 µl of potassium acetate solution (described in Appendix 2) was added and the contents of the tube mixed by vortexing. The tube was left on ice for a further 5 min and then centrifuged as above for 5 min. The supernatant was transferred to a fresh microfuge tube containing 400 µl of Tris-saturated phenol (see Appendix 2). After vortexing for 10 seconds,

the tube was again centrifuged as described above and the aqueous phase transferred to a fresh tube containing 400 µl of a 24:1 mixture of chloroform and isoamyl alcohol. After thorough mixing, the phases were separated by centrifugation at $1.3 \times 10^4 \times g$ for 2 min. The supernatant was removed to a fresh tube and mixed with 800 µl of ice-cold ethanol. The tube was maintained at -70°C for 30 min, after which it was centrifuged at $1.3 \times 10^4 \times g$ for 20 min. The supernatant was removed and the pellet washed gently with 150 µl of 70% ethanol. After brief centrifugation, the ethanol was discarded and the pellet dried by vacuum desiccation. The pellet was finally dissolved in 50 µl of TE buffer. When required, any remaining RNA was removed by digestion at 37°C for 1 h with one unit of T_1 RNase (BRL).

2.6.2. Large-scale isolation of plasmid DNA

This procedure was again based upon that described by Birnboim and Doly (1979) and, although many steps are similar to those described above, the protocol will be recorded in full for clarity.

A single bacterial colony was used to inoculate 5 ml of nutrient broth (NB; Oxoid) containing the appropriate antibiotic and incubated on a rotary shaker at 37°C for 5 to 6 h. This was used as the inoculum for one litre of antibiotic-supplemented NB which was incubated overnight with shaking at 37°C. Cells were harvested by centrifugation at 9×10^3 rpm for 10 min in a Sorvall GS-3 rotor at 4°C. The supernatant was poured off to leave the pellet as dry as possible. This was resuspended by vortexing in 20 ml of a solution containing 50 mM glucose, 10 mM EDTA (pH 8.0) and 25 mM Tris-HCl (pH 8.0) to which lysozyme (Sigma) had been added at a concentration of 4 mg/ml. After 5 min at room temperature, 40 ml of an ice-cold solution of 0.2 N NaOH, 1% ($^w/_v$) SDS was added and the contents of the tube mixed gently. Following a period of 10 min at 0°C, 30 ml of ice-cold potassium acetate solution (described in Appendix 2) was added and, after thorough mixing, incubation on ice continued for a further 15 min. Following centrifugation as noted

above, the supernatant was carefully filtered through a layer of tissue paper to remove any coarse particles. After mixing with 0.6 volumes of isopropanol, the tube was again centrifuged as above. The pellet was gently washed with 5 ml of 70% ethanol and then dissolved in 10 ml of TE buffer. This was transferred to a tube containing 5 ml of Tris-saturated phenol and 5 ml of chloroform/isoamyl alcohol mixture, mixed and centrifuged in a Sorvall SS-34 rotor for 10 min at 5000 rpm at room temperature. The aqueous layer was removed to a fresh tube, mixed with 10 ml of 5 M ammonium acetate and incubated on ice for 15 min. After centrifugation at 10000 rpm for 10 min at 4°C, the RNA-containing pellet was discarded and the supernatant mixed with 10 ml of isopropanol. Following a period of 10 min on ice, this was again centrifuged as detailed above and the pellet washed with 70% ethanol and dried by vacuum desiccation. The pellet was finally resuspended in 1 ml of TE buffer containing 10 units of T_1 RNase (BRL) and incubated at 37°C for 1 h.

2.6.3. Large-scale isolation of chromosomal DNA

The protocol used was a modification of that described by Ausubel *et al.* (1987).

Cells of *C.jejuni* 11168, grown microaerophilically at 42°C over 36 h, were harvested by centrifugation for 15 min at 10000 rpm in a Sorvall GS-A rotor at 4°C. After discarding the supernatant, the cells were resuspended in 9.5 ml of TE buffer to which was added 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K (Sigma). The suspension was thoroughly mixed and incubated at 37°C for 1 h to allow cell lysis. After this period, 1.8 ml of 5 M NaCl was added and the suspension again mixed. Immediately, 1.5 ml of hexadecyltrimethyl ammonium bromide (CTAB / NaCl solution; see Appendix 2) was mixed into the suspension and the tube incubated at 65°C for 20 min. In the presence of NaCl concentrations exceeding 0.5 M, CTAB complexes with residual proteins and polysaccharides but not nucleic acids. An equal volume of Tris-saturated phenol was then added and the

phases slowly mixed to emulsion. These were separated by centrifugation at 5000 rpm in a Sorvall SS-34 rotor at room temperature and the upper aqueous phase removed to a fresh tube with a wide-bored pipette. This was followed by separation of the aqueous phase with an equal volume of chloroform/isoamyl alcohol. The upper phase was carefully mixed with 0.6 volumes of isopropanol. With slow rotation of the tube, a DNA precipitate was obtained which was removed by centrifugation for 10 min at 10000 rpm in a Sorvall SS-34 rotor at room temperature. After discarding the supernatant, the pellet was washed with 5 ml of 70% ethanol and this wash solution removed by brief centrifugation. The pellet was dried by vacuum desiccation and finally allowed to redissolve overnight at 4°C in 2 ml of TE buffer.

2.6.4. Small-scale isolation of chromosomal DNA

For small-scale isolation of chromosomal DNA, *Campylobacter* cells were propagated as lawns on sheep blood agar plates incubated for 24 h at 42°C. Bacterial growth was harvested in 1.5 ml of BHI broth. Thereafter, the protocol reported by Ausubel *et al.* (1987), essentially a scaled-down version of that described above, was followed. The final DNA pellet was redissolved in 100 µl of TE buffer.

2.6.5. Transformation of *E.coli* strains

2.6.5.1. Preparation of competent cells

This protocol was adapted from that described by Mandel and Higa (1970). Fresh competent cells were prepared as required.

A single *E.coli* colony was used to inoculate 10 ml of SOC medium (see Appendix 1) and incubated overnight on a rotary platform at 37°C. A 200 µl aliquot of this culture was then used to inoculate 50 ml of SOC medium which was incubated under similar conditions until the absorbance at 650 nm reached 0.2 ODU (for rec^+ strains) or 0.5 ODU (for rec^- strains), whereupon the culture

was immediately chilled on ice for 10 min. Cells were harvested by centrifugation for 10 min at 4000 rpm in a Sorvall SS-34 rotor at 4°C. The cell pellet was resuspended in 20 ml of ice-cold 80 mM $CaCl_2$, incubated on ice for 25 min and thereafter harvested as indicated above. The final cell pellet was gently resuspended in 500 µl of 80 mM $CaCl_2$.

2.6.5.2. Transformation procedure

Dagart and Ehrlich (1979) reported that prolonged incubation of competent *E.coli* cells in $CaCl_2$ improved their transformation efficiency. With the strain most frequently used in this study, JM83, no significant increase in transformation efficiency was noted when cells stored for 1 h or 24 h were transformed with monomers of pUC19 (Boehringer Mannheim). Thus, in all cases, competent cells were stored on ice in 80 mM $CaCl_2$ for 1 h only prior to transformation.

To 20 µl of competent cells in a chilled sterile microfuge tube was added 1 µl of plasmid DNA. After incubation on ice for 30 min (absorption stage), the suspension was heat-shocked at 42°C for 45 seconds and returned to ice for a further 2 min. To allow an expression stage, 80 µl of SOC medium was introduced and the tube incubated with gentle shaking at 37°C for 1 h. Cells were then gently spread on the surface of a nutrient agar plate supplemented with the appropriate antibiotic. To ensure that competent cells were incapable of growing on these selective plates to provide a negative control, the above procedure was repeated by substituting 1 µl of TE buffer for plasmid DNA. In addition, the capability of the cells to take up extraneous DNA was demonstrated by transforming a 20 µl aliqout of cells with 1 µl of control plasmid DNA encoding resistance to the antibiotic in use. All plates were incubated overnight at 37°C.

2.6.6. Determination of nucleic acid concentrations

Nucleic acid concentrations of genomic DNA preparations

were ascertained by electrophoresis in an agarose gel and reference to doubling dilutions of a standard preparation of bacteriophage lambda DNA (BRL; Bethesda Research Laboratories) run in the same gel.

Nucleic acid concentration of restriction endonuclease digested plasmid DNA was determined in a similar manner. However, in this case, doubling dilutions of a standard λ DNA digest were run in the same gel and reference was made to the concentration of the band(s) in this standard digest most closely corresponding to the size of the plasmid DNA fragment(s).

2.6.7. Agarose gel electrophoresis

Electrophoresis of DNA was performed in an horizontal gel apparatus supplied by LKB-Pharmacia. Gels were cast either in 400 cm^2 or 84 cm^2 moulds using 250 ml and 55 ml of molten agarose (type II-A, medium electroendosmosis; Sigma) respectively. An agarose concentration of 0.7% was used for studies involving plasmid and endonuclease digested genomic DNA whilst 0.35% agarose was used for native chromosomal DNA. In all cases, the agarose was made up in 0.5X Tris-borate buffer (89 mM Tris-HCl, 89 mM boric acid, 1.25 mM EDTA, pH8.2; TBE).

DNA mixed with loading buffer (60% sucrose, 0.1% bromophenol blue) was introduced into slots in the gel and run towards the anode at 80 to 100 volts until the bromophenol blue tracking dye had travelled four-fifths the length of the gel. DNA molecular size markers were run in one or more slots of each gel. In specific instances, primarily when employing 0.35% agarose gels loaded with genomic DNA, a voltage of 30 volts was applied overnight.

After electrophoresis, the gel was immersed in a solution of ethidium bromide (0.5 μg/ml) for 15 min, excess stain removed by immersion in distilled H_2O for 10 min and DNA visualised by shortwave (254 nm) ultraviolet (UV) transillumination. Monochrome

photography was performed with a Polaroid MP4 camera assembly using type 667 film exposed through a Kodak Wrattan number 16 red filter. Colour photography was performed with a 35 mm camera using Kodak Ektachrome 100 transparency film from which positive prints were subsequently produced.

2.6.8. Restriction endonuclease digestion of plasmid DNA

All restriction endonucleases were supplied by Bethesda Research Laboratories (BRL). Buffers of ionic composition and strength appropriate for each enzyme were supplied by the manufacturers at ten times the required concentration. A list of enzymes used, cleavage sites and buffers is presented in Table 3.

Typically, 1.5 µl of a small-scale preparation of plasmid DNA was digested with 2 to 10 units of restriction endonuclease in single strength buffer made up to a final volume of 20 µl with sterile deionised water (d.H_2O). Digestion was allowed to proceed for a period of at least 2 h, or alternatively overnight, at 37°C.

When the concentration of plasmid DNA was known, digestion reactions were performed with a ten-fold excess of restriction endonuclease, on the assumption that one unit of enzyme was capable of cleaving all available sites in 1 µg of DNA within 1 h. When required, restriction endonucleases in digestion mixtures were inactivated by sequential extractions with Tris-buffered phenol and chloroform/isoamyl alcohol.

2.6.9. Estimation of DNA molecular size

Lambda DNA (BRL), which has a molecular size of 48.502 kb, was cleaved with *Hin*dIII to yield fragments of the following computer assessed sizes (kb; information supplied by Sigma and based on the findings of Murray and Murray, 1975): 23.13, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564 and 0.125.

The final concentration of λ DNA in the reaction mixture was 55.2 nanograms per microlitre (ng/µl). This digest was preheated

Table 3: Restriction Endonucleases Used in this Study [a]

Enzyme	Recognition Sequence [b]	Buffer
*Bam*HI	5' G*GATCC 3'	REact™3
*Bgl*II	5' A*GATCT 3'	REact™3
*Eco*RI	5' G*AATTC 3'	REact™3
*Eco*RV	5' GAT*ATC 3'	REact™2
*Hin*dIII	5' A*AGCTT 3'	REact™2
*Pst*I	5' CTGCA*G 3'	REact™2
*Sal*I	5' G*TCGAC 3'	REact™10
*Sau*3A	5' *GATC 3'	REact™4

[a] all restriction endonucleases and buffers were obtained from Bethesda Research Laboratories

[b] * indicates the point of cleavage within a recognition sequence

to 65°C for 5 min before use, cooled to 4°C, and 275 to 330 ng loaded into each gel slot. Following electrophoresis, staining and visualisation of the DNA with UV transillumination, a graphical relationship was established between molecular size and mobility by plotting the $\log_{10}$ conversion of DNA fragment length versus the distance migrated from the origin.

To assess the sizes of *Sau*3A fragments of genomic DNA, 250 ng of preheated high molecular weight markers were loaded into each slot. These markers were supplied by BRL and had been prepared by digesting λ DNA with various restriction enzymes to yield fragments of the following sizes (kb) : 48.5, 38.4, 33.5, 29.9, 24.8, 22.6, 19.4, 17.1, 15.0, 12.2, 10.1, 8.6, 8.3.

2.6.10. Radioactively-labelled DNA size markers

To 550 ng of λ *Hin*dIII digest was added 5 µl of REact™ 2 buffer (BRL) and 10 µl of Å buffer, the latter consisting of deoxycytidine triphosphate (dCTP), deoxygaunosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), each at a final concentration of 100 µM, in 1.25 M Tris-HCl (pH 8.0) and 0.125 M $MgCl_2$. The final volume of the mixture was made up to 48 µl with $d.H_2O$. After addition of 1 µl of α-^{32}P-deoxyadenosine triphosphate (dATP; 10 µCi/µl; Amersham) and 1 µl of the Klenow fragment of *E.coli* DNA polymerase I (BRL), the reaction mixture was kept at room temperature for 20 min. Following incubation, 10 µl of loading dye was added and 12.5 to 15 µl loaded into each gel slot, equivalent to 115 to 138 ng of DNA.

2.6.11. Dialysis of DNA samples

Prior to ligation, all DNA samples were dialysed overnight at 4°C in a BRL microdialysis unit. Briefly, this consists of an upper and lower perspex assembly separated by a replaceable dialysis membrane with a molecular weight cutoff of 14×10^3 to 16×10^3 (BRL). The upper assembly has six separate compartments allowing up to six different DNA samples to be dialysed simultaneously. The

DNA to be dialysed was pipetted onto the upper exposed surface of the dialysis membrane in one or more of these compartments. TE buffer was drawn continuously through the lower assembly from a reservoir at the rate of 6.6 ml/hour using an ISCO Tris™ pump.

2.6.12. Concentration of DNA with 2-butanol

As an alternative to precipitation of DNA with ethanol or isopropanol and redissolution in a smaller volume of buffer, the concentration of dilute DNA solutions was also increased on occasions by extraction with 2-butanol; water molecules, but not solutes and DNA, preferentially partition into the organic butanol phase (Stafford and Bieber, 1975). An equal volume of butanol was added to the DNA solution, mixed well, and the phases separated by centrifugation at $1.6 \times 10^3 \times g$ for 2 min in a bench centrifuge. The upper organic phase was discarded and the extraction procedure repeated until the desired reduction in volume was achieved. To remove any traces of butanol, the sample was mixed with water-saturated ether, the phases allowed to separate without centrifugation, and the upper ether phase discarded. Traces of ether were evaporated off by incubation at 65°C for 10 min. All samples concentrated in this manner were dialysed extensively before any further manipulation.

2.6.13. Purification of large-scale plasmid preparations

Large-scale purification of plasmid DNA was performed according to the method of Gomez-Marquez *et al.* (1987).

Briefly, 600 µl of DNA in TE buffer was mixed with 200 µl of 5X equilibration buffer (25 mM EDTA, 5 M NaCl, 250 mM Tris-HCl, pH 8.0) and 200 µl of a loading solution consisting of 20 mM EDTA, 40% (w/v) sucrose and 0.25 % (w/v) bromophenol blue. This mixture was applied to the top of a 25 cm bed height column of Sephacryl S-1000 Superfine gel matrix (Pharmacia) and eluted using an ISCO Tris™ pump at a rate of 9.5 ml/h in single strength equilibration buffer containing 1 mM sodium azide to prevent microbial

contamination. The eluate was collected in 750 µl volumes in microfuge tubes. A 10 µl aliquot of each fraction was then mixed with loading dye and subjected to electrophoresis through 0.7% agarose. Fractions containing chromosomal DNA, undigested RNA and open forms of the plasmid were discarded, whilst those containing closed circular plasmid molecules were pooled together. The volume of the pooled eluate was decreased as far as possible by butanol extraction followed by sequential steps of ethanol precipitation and washing of the resulting pellet with 70% ethanol. The plasmid DNA was finally redissolved in a minimum volume of TE buffer and dialysed overnight.

2.6.14. Dephosphorylation of vector DNA

In order to minimise vector recircularisation during ligation steps in cloning procedures, calf intestinal phosphatase (CIP; Boehringer Mannheim) was used to remove 5′ terminal phosphates from endonuclease-generated ends of both plasmid and cosmid vectors. This increased the frequency of ligation to insert termini and thereby enhanced the population of insert-bearing clones.

Vector DNA was cleaved with the appropriate restriction endonuclease and the enzyme inactivated by sequential extractions with Tris-saturated phenol and chloroform/isoamyl alcohol. DNA was recovered by ethanol precipitation with 0.25 M sodium acetate (pH 5.2) and resuspended in d.H_2O. Dephosphorylation was performed at 37°C for 1 h in the presence of 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, 50 mM Tris-HCl (pH9.0) and a two-fold excess of CIP. The amount of CIP to be added was calculated on the basis that one mole of linear DNA contains two moles of 5' ends and one picomole (pmol) of 5′ ends can be dephosphorylated by 0.01 units of CIP (Maniatis *et al.*, 1982). Inactivation of the enzyme was achieved by addition of EDTA at a final concentration of 15 mM and incubation at 68°C for 1 h. Following phenol and chloroform/isoamyl alcohol extractions, the DNA was ethanol precipitated in the presence of 2.5 M ammonium acetate and finally resuspended in an appropriate volume of TE buffer.

2.6.15. Test ligations of vector DNA

To ensure that dephosphorylation had occurred to a satisfactory extent and that the dephosphorylated vector molecule was capable of ligating to insert fragments, test ligations were performed using bacteriophage λ DNA cleaved with a restriction endonuclease producing compatible cohesive termini. Reactions were set up in microfuge tubes as follows :

i) 50 ng of dephosphorylated vector DNA
ii) 50 ng of dephosphorylated vector DNA + 1 U T_4 DNA ligase
iii) 50 ng of restriction endonuclease digested λ DNA
iv) 50 ng of restriction endonuclease digested λ DNA + 1 U T_4 DNA ligase
v) 50 ng of dephosphorylated vector DNA + 50 ng of restriction endonuclease digested λ DNA + 1 U T_4 DNA ligase

After addition of 1 µl of 10X ligation buffer (see below) to every tube, the volume of each reaction was made up to 10 µl with d.H_2O. All tubes were incubated at 14°C for 16 h. Samples of each reaction were then subjected to electrophoresis through 0.7% agarose. On occasions, in addition to using λ DNA fragments, test ligations were also performed between dephosphorylated vector and DNA from an unrelated plasmid.

The outcome of a typical test ligation experiment is presented in the Results section.

2.6.16. Ligation of DNA

Vector and insert DNA molecules were ligated under the influence of one unit of T_4 DNA ligase (Boehringer Mannheim) in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM dithiothreitol (DTT; Sigma) and 50 µg/ml bovine serum albumin (BSA; Pentax fraction V;

Sigma). Ligations were performed in 10 µl final volumes. Immediately prior to incubation at 14°C for 14 to 16 h, 1 µl of 10 mM ATP (Sigma) was added to the reaction.

The relative concentrations of insert and vector DNA were controlled so as to produce the greatest number of hybrid molecules, according to the findings of Dugaiczyk *et al.* (1975). These authors demonstrated that two factors were of importance when predicting whether the products of a ligation reaction would be circular or linear. The first of these, designated "i", the total concentration of all complementary DNA termini in the solution, is determined as:

$$i = 2 N_o M \times 10^{-3} \quad \text{(ends/ml)}$$

where N_o is Avogadro's number (6.023×10^{23}) and M is the molar concentration of the DNA molecules.

The second factor, designated "j", is the effective concentration of the two ends of the same molecule, and is found thus :

$$j = \left(\frac{3}{2\pi lb}\right)^{\frac{3}{2}}$$

where l is the length of the DNA molecule and b is the minimal length of DNA which can form a circle.

For phage λ, Dugaiczyk *et al.* determined the value of j to be 3.6×10^{11} ends/ml. Thus, the above equation can be rewritten to calculate the value for j for a DNA molecule of any molecular weight :

$$j = j_\lambda \left(\frac{MW_\lambda}{MW}\right)^{\frac{3}{2}}$$

The molecular weight of a molecule can be determined from the relationship :

1 kb of double stranded DNA = 6.5×10^5 daltons.

Dugaiczyk *et al.* demonstrated that when cloning into phage λ vectors, the formation of linear oligomers required for efficient packaging was favoured when $i/_i$ values for both insert and vector DNA molecules were less than one. These conditions were adopted for cloning experiments involving the use of cosmid vectors.

With plasmid vectors, however, the hybrid molecules must circularise in order to transform competent *E.coli* cells at high efficiency. Dugaiczyk *et al.* determined that in this case, reactions in which the $i/_i$ value was below 0.8 yielded predominantly linear molecules which subsequently underwent circularisation. Thus, the concentrations of plasmid vector and insert DNA were determined which would satisfy these parameters.

2.6.17. Isolation of DNA from agarose gels

DNA fragments were isolated from agarose gels by one of the two methods detailed below.

2.6.17.1. Electroelution of DNA into dialysis bags

This procedure was used to isolate fragments generated by either complete or partial restriction endonuclease digestion of DNA and was as described by Maniatis *et al.* (1982). Briefly, the digested DNA was subjected to electrophoresis under standard conditions as outlined earlier, the gel stained in EtBr for 3 min only and the DNA visualised under UV transillumination for as short a period of time as possible. These latter precautions were designed to minimise any potential damage to the DNA. The gel segment containing the DNA of interest was excised and transferred to a small length of dialysis tube sealed at one end. The dialysis tubing had been boiled in a solution containing 2% sodium bicarbonate and 1 mM EDTA, rinsed extensively with distilled H_2O and then autoclaved in distilled H_2O. It was thoroughly rinsed prior to use. The gel slice was covered with a minimum volume of half strength

TBE buffer so that it was in constant contact with the buffer. The open end of the tubing was then sealed and the dialysis bag placed under half strength TBE buffer in an horizontal gel electrophoresis tank. A current of 100 volts was applied for 3 h, allowing the DNA to pass out of the gel slice. The polarity of the current was briefly reversed to remove any DNA adhering to the inner walls of the bag and the buffer surrounding the gel slice removed to a microfuge tube. The inner walls of the bag were then washed with a small volume of half strength electrophoresis buffer. The pooled DNA solution was then sequentially extracted with an equal volume of Tris-saturated phenol and chloroform/isoamyl alcohol. The DNA was recovered by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.2) and finally resuspended in a minimum volume of TE buffer.

2.6.17.2. Recovery of DNA from low melting point agarose

The method used was essentially as described by Maniatis *et al.* (1982) and was employed for isolation of DNA fragments generated by complete digestion with restriction endonucleases. The digest was subjected to electrophoresis through 0.7% low melting point agarose (Sigma) running at 4°C in half strength TBE buffer. In all other respects, electrophoresis was performed as described previously. The gel was stained in EtBr and DNA visualised by UV transillumination, observing the precautionary measures detailed above. The gel segment containing the DNA of interest was then excised and transferred to a microfuge tube. Five volumes of a solution containing 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA were added and the gel slice melted by heating at 68°C for 10 min. This solution was then extracted twice with an equal volume of Tris-saturated phenol which had been warmed to 37°C, followed by an extraction with chloroform/isoamyl alcohol. The DNA was finally recovered by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.2), dissolved in a minimum volume of TE buffer and dialysed overnight at 4°C.

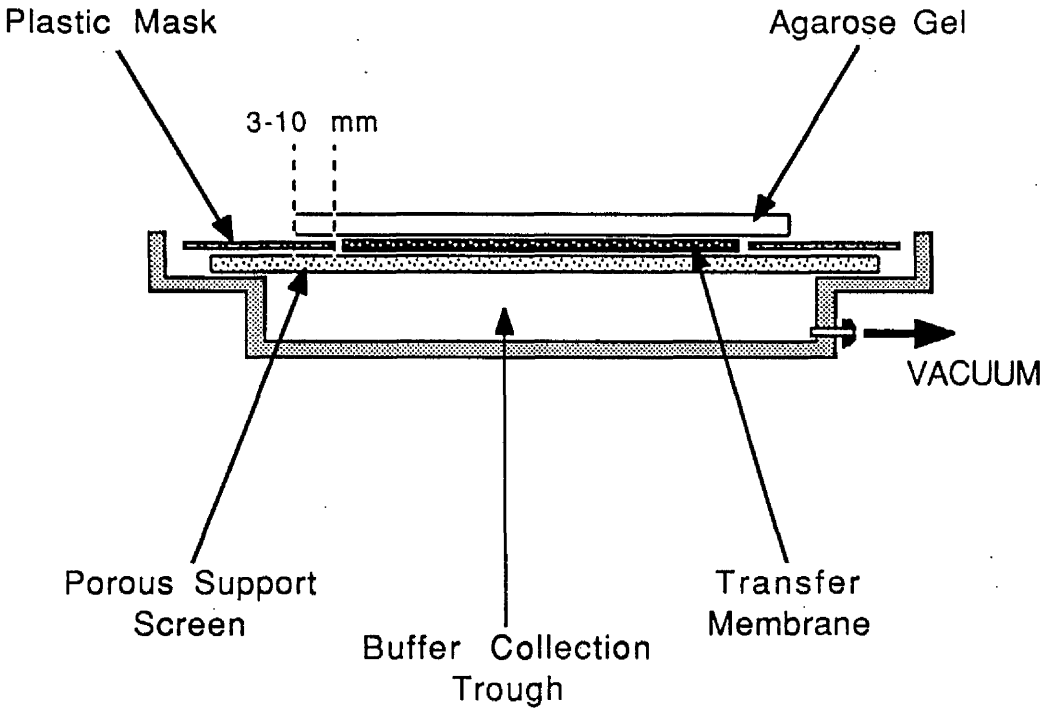
2.6.18. Southern blotting

Transfer of DNA from agarose gels to Hybond-N™ nylon membranes (Amersham) was achieved either by vacuum blotting or capillary transfer techniques.

2.6.18.1. Vacuum transfer

The DNA fragments to be transferred were subjected to electrophoresis in 0.7% agarose according to the standard protocol. Radioactively labelled λ *Hin*dIII fragments were run in the same gel as size markers. The VacuGene vacuum blotting apparatus (LKB Pharmacia) was assembled as directed by the manufacturer's instructions. The base of the VacuGene unit contained a porous screen supporting a waterproof mask in which a "window" had been cut. The Hybond-N™ membrane, cut to the correct size, was positioned within this window and the gel placed carefully on top. The upper frame of the unit was then secured and a vacuum of 40 cm of H_2O applied to immobilise the gel. The general set-up of the VacuGene unit is illustrated in Fig. 8.

A volume of 0.25 M HCl was poured onto the gel to cover it completly. After 4 min, this solution was removed and the gel submerged under denaturation solution, consisting of 1.5 M NaCl and 0.5 M NaOH, for 3 min. This was then replaced by neutralising solution containing 2 M NaCl and 1 M Tris-HCl (pH 5.0). After a further 3 min, the neutralising solution was removed and 20X SSC (see Appendix 2) poured into the unit so that the gel was submerged to twice its depth. Transfer was allowed to occur for 50 min, following which the transfer solution and gel were removed from the unit. Once the vacuum pump had been switched off, the nylon membrane was transferred to a sheet of 3MM paper (Whatman™) and allowed to dry for one hour. The dried membrane was then wrapped in Saran Wrap (Dow Chemical Company) and the face of the membrane carrying the DNA was exposed to shortwave UV radiation for 5 min.


Plastic Mask
Agarose Gel
3-10 mm
VACUUM
Porous Support Screen
Buffer Collection Trough
Transfer Membrane

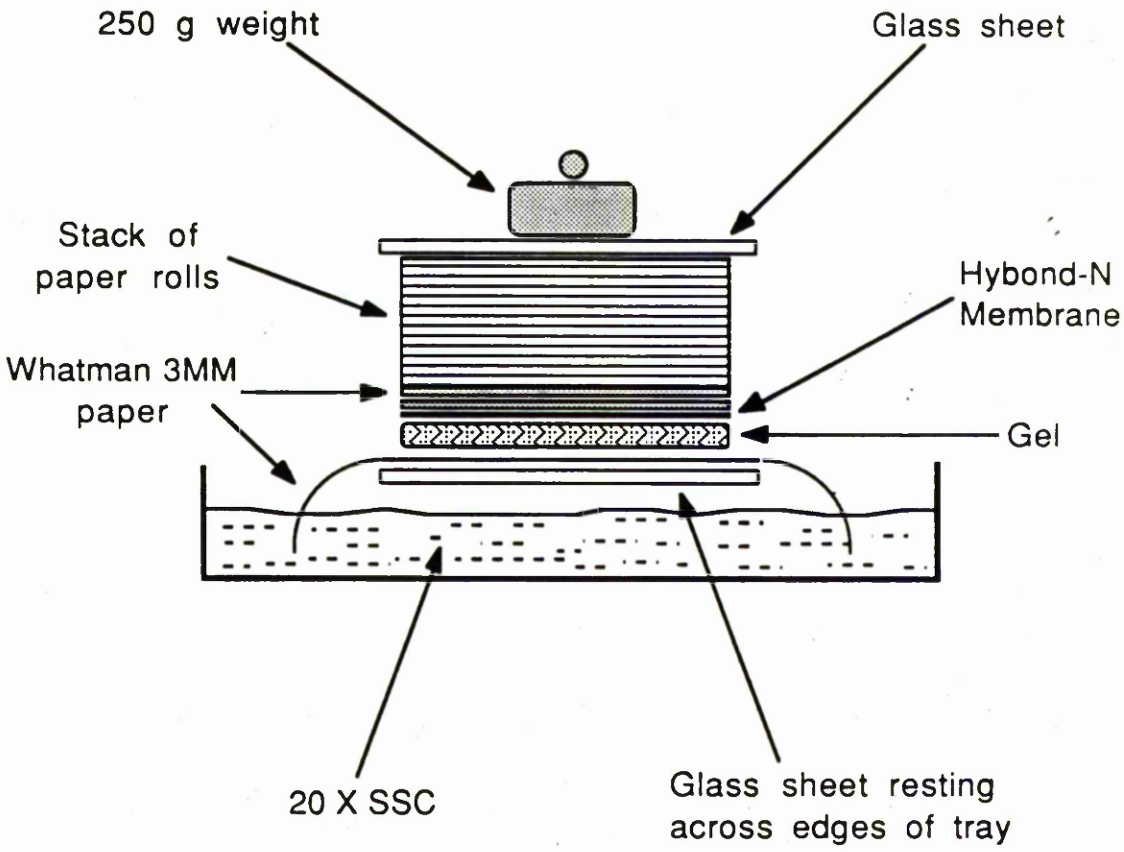
2.6.18.2. Capillary transfer

Capillary transfer of DNA to Hybond-N™ membranes was achieved as described by Sambrook *et al.* (1989). Following electrophoresis, the gel was soaked for 10 min in 0.2 M HCl, rinsed in distilled H_2O and then agitated gently in 1.5 M NaCl, 0.5 M NaOH for 45 min. It was then soaked for a further 45 min in a solution containing 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl, again with gentle agitation. A "transfer pyramid" was then set up as illustrated in Fig. 9 and capillary transfer of DNA to a piece of Hybond-N™ membrane, prewetted in 20X SSC for 5 min, was allowed to occur for 16-18 h. The paper towels, 3MM papers and gel were removed, and the nylon membrane soaked in 6X SSC for 5 min. The membrane was then dried at room temperature for 1 h, wrapped in Saran Wrap and the DNA fixed to the filter by UV irradiation.

2.6.19. Labelling of DNA restriction endonuclease fragments

The random priming procedure developed by Feinberg and Vogelstein (1983; 1984) was used to label DNA fragments for use as probes. Plasmid DNA was cleaved with the appropriate restriction endonuclease and the fragments separated by electrophoresis through 0.7% low melting point agarose running in half strength TBE buffer. After EtBr staining and visualisation of the DNA by UV transillumination, the band of interest was excised and transferred to a preweighed sterile microfuge tube. Sterile d.H_2O was added at a ratio of 3 ml per gram of agarose and the DNA denatured at 100°C for 7 min. The microfuge tube was then placed at 37°C for 15 min.

To a fresh microfuge tube was added 10 µl of oligo-labelling buffer (OLB; Feinberg and Vogelstein, 1984; see Appendix 2) containing random sequence synthetic hexanucleotides and all deoxynucleoside triphosphates except ATP. In the presence of radiolabelled ATP, the hexanucleotide primers which hybridised to the denatured DNA template would be extended to generate double


250 g weight
Glass sheet
Stack of paper rolls
Hybond-N Membrane
Whatman 3MM paper
Gel
20 X SSC
Glass sheet resting across edges of tray

stranded DNA uniformly labelled on both strands. To this was added 2 µl of 10 mg/ml BSA, 10-30 µl of denatured DNA in agarose (dependent upon the concentration of DNA in the gel slice) and d.H_2O to make the reaction volume up to 46 µl. Finally, 2 µl of ^{32}P-dATP (10 µCi/µl; Amersham) and 2 µl of the Klenow fragment of *E.Coli* DNA polymerase I (1 U/µl) were added and the reaction incubated overnight at room temperature. The labelled probe solution was heated to 100°C for five min prior to use.

2.6.20. Conditions for hybridisation

Hybridisation was performed under conditions of either high or low stringency as detailed below.

2.6.20.1. High stringency

The Hybond-N™ membrane was placed in a heat-sealable polyethylene bag. Prehybridisation solution was added at a ratio of 50 µl per cm^2 of filter, the solution containing 6.6X SSC, 4X Denhardts reagent (1X = 0.2% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), 50% formamide (deionised as described by Maniatis *et al.*, 1982) and 0.4% SDS. Prior to sealing the bag, 0.1 mg of heat-denatured salmon sperm DNA, prepared according to Maniatis *et al.* (1982), was added per cm^2 of membrane. The filter was incubated overnight at 42°C with constant, gentle agitation. The prehybridisation solution was then replaced by an equal volume of hybridisation solution prepared as above, but with the salmon sperm DNA being substituted by denatured labelled probe. The bag was resealed and reincubated overnight with gentle agitation at 42°C.

The filter was then removed from the bag and washed twice at room temperature for 30 min in a solution containing 2X SSC and 0.1% SDS, followed by two washes at 68°C for 1 h in 1X SSC, 0.1% SDS. The membrane was then blotted dry, sealed in a polyethylene bag and exposed to a sheet of Kodak X-OMAT film at -70°C with an intensifying screen for various periods of time until an acceptable

autoradiographic image had been produced. X-OMAT film was developed for 5 min in 17.7% ($v/_v$) Kodak LX24, development stopped by immersion in a bath of 3% ($v/_v$) acetic acid and the image finally fixed in 20% ($v/_v$) Kodak Unifix for a further 5 min.

2.6.20.2. Low stringency

The procedure was exactly as described for high stringency conditions except that the prehybridisation and hybridisation solutions contained only 20% deionised formamide. In addition, post-hybridisation washes were performed twice at room temperature for 30 min in a solution of 2X SSC and 0.1% SDS, followed by two washes at 50°C for 1 h in fresh 2X SSC, 0.1% SDS.

2.6.21. Use of a *S.aureus* beta-lysin probe

E.coli C600 harbouring plasmid pDC007 was obtained from Dr. T.H. Birkbeck, Department of Microbiology, University of Glasgow. This plasmid consists of a 2.2 kb DNA fragment specifying the ß-lysin determinant (Hlb) from *S.aureus* strain CN6708 cloned into the *Hin*dIII site of pBR322 (Coleman *et al.*, 1986). The ability of this clone to cause haemolysis of sheep erythrocytes was ensured by sub-culturing onto a BA plate. After 48 h incubation at 37°C, the plate was placed immediately at 4°C to induce the "hot-cold" haemolysis phenomenon. Haemolysis became apparent after 24 h at this temperature and intensified considerably after maintenance of the plate at 4°C for a further two days. The 2.2 kb *Hin*dIII fragment of pDC007 was used as a genetic probe for *S.aureus* ß-lysin.

2.6.22. Labelling of oligonucleotide probe

A mixed oligonucleotide probe designed to be complementary to the highly conserved codons 84 to 91 of the mature polypeptide of the CT and LT/B subunits (Calva *et al.*, 1989) was synthesized and purified by staff at the Public Health Laboratory Service Centre for Applied Microbial Research, Porton Down. The nucleotide

sequence of this probe is reproduced in Table 4.

Radiolabelling was performed by phosphorylation catalysed by bacteriophage T_4 polynucleotide kinase (PNK). The reaction mixture consisted of 3 µl of oligonucleotides, 5 µl of γ-^{32}P-ATP (10 µCi/µl; Amersham), 1 µl of PNK (10 U/µl; Boehringer) and 1 µl of 10X PNK buffer (0.1 M $MgCl_2$, 0.05 M DTT, 0.7 M Tris-HCl, pH 7.6). This was incubated at 37°C for 45 min, followed by 10 min at 65°C to terminate the reaction.

2.6.23. Hybridisation conditions with an oligonucleotide probe

Fragments of *C.jejuni* chromosomal DNA generated by restriction endonuclease digestion with *Hin*dIII, *Bgl*II, *Eco*RI and *Pst*I were transferred to Hybond-N™ membranes as described previously. Included as a positive control was plasmid pJYL2299 which contains a 5.1 kb *Pst*I fragment encoding the *E.coli* heat-labile holotoxin gene cloned into pBR322 (Yamamoto and Yokota, 1980). The filter was sealed in a polyethylene bag containing prehybridisation solution (6X SSC, 1X Denhardts reagent, 0.5% SDS, 0.05% sodium pyrophosphate and 200 µg/ml preboiled salmon sperm DNA) at a ratio of 50 µl per cm^2 of membrane. This was then incubated overnight at 37°C with gentle agitation.

Prehybridisation solution was replaced by an equal volume of hybridisation solution made up as above, but with labelled probe substituting for salmon sperm DNA. Hybridisation was allowed to proceed overnight at room temperature with gentle agitation. The filter was then washed twice at room temperature in several volumes of 6X SSC, 0.05% sodium pyrophosphate. This was followed by two 15 minute stringent washes at either 25°C, 37°C or 42°C, as detailed in the Results section. Autoradiography was performed as previously described.

2.7. Construction of cosmid-based genomic library

The various stages involved in the production of a library of

Table 4: Nucleotide Sequence of the CT-LT Mixed Oligonucleotide Probe

(From Calva *et al.*, 1989)

Position		84	85	86	87	88	89	90	91
Amino acids		Lys	Leu	Cys	Val	Trp	Asn	Asn	Lys
CT/B	5'	AAG	TTA	TGT	GTA	TGG	AAT	AAT	AAA
LT/B	5'	AAA	TTA	TGT	GTA	TGG	AAT	AAT	AAA
Oligonucleotide	3'	TTC/T	AAT	ACA	CAT	ACC	TTA/G	TTA/G	TTT

The oligonucleotide probe was designed to be complementary to the codons 84-91 of the CT/B and LT/B subunits

C.jejuni chromosomal DNA fragments in a cosmid vector are summarized in Fig. 10. Each of these steps is also described in detail below.

2.7.1. Preparation of vector

Cosmid vector, pHC79 (Hohn and Collins, 1980), was cleaved with a two-fold excess of *Bam*HI restriction endonuclease and dephosphorylated as previously described. The final DNA concentration was determined to be 3.2 mg/ml. Test ligations were performed as described in section 2.6.15.

2.7.2. Preparation of *Campylobacter* chromosomal DNA fragments

Hohn and Murray (1977) determined that for DNA to be packaged *in vitro*, its length must be between 78% and 107% of the length of the wild type λ genome, that is, between 38.5 kb and 52 kb. Thus, fragments for ligation to pHC79 were required to be between 32 kb and 45.6 kb.

C.jejuni 11168 chromosomal DNA was extracted, purified, dialysed and quantitated as detailed previously. Conditions for partial digestion of this high molecular weight DNA were established by a modification of the procedure of Maniatis *et al.* (1982). A reaction mixture was prepared containing 10 µg of DNA and 15 µl of REact™ 4 buffer in a total volume of 150 µl. Nine microfuge tubes were placed on ice. To tube 1 was added 30 µl of diluted DNA solution, whilst tubes 2 to 8 received 15 µl. One unit of *Sau*3A (BRL) was added to tube 1, the contents mixed and 15 µl transferred to tube 2. Two-fold dilution of the enzyme was thereafter continued up to tube 8, from which 15 µl was discarded. The concentration of *Sau*3A in the tubes thus ranged from 0.5 U/µg DNA to 1.95×10^{-3} U/µg DNA. Tube 9 acted as a negative control with no enzyme. All tubes were incubated at 37°C for 1 h, then returned to ice. EDTA was added to a final concentration of 20 mM

and the contents of each tube mixed with 3.5 µl of gel loading dye. A 10 µl aliquot of each sample was then subjected to electrophoresis through 0.35% agarose at 30 V over a period of 16 h. Fragments of *Hin*dIII digested λ DNA served as molecular size markers. The gel was stained with EtBr and DNA visualised by UV transillumination. The enzyme concentration producing maximum intensity of fluorescence in the 27 to 41 kb region of the gel was identified as 7.5×10^{-3} U/µg DNA.

A second series of enzyme dilutions was then set up in four tubes, labelled *a*, *b*, *c* and *d*. A reaction mixture was prepared containing 10 µg of uncut chromosomal DNA and 3.75 µl of REact™ 4 in a total volume of 37.5 µl. To tube *a* was added 15 µl of this mixture, whilst tubes *b*, *c* and *d* received 7.5 µl. The contents of tube *a* were mixed with 0.03 U of *Sau*3A and 7.5 µl transferred to tube *b*, mixed, and the same volume again transferred to tube *c*, from which 7.5 µl was then discarded. Again, the final tube in the series received no enzyme. The enzyme concentration in each tube was therefore 7.8×10^{-3} U/µg DNA, 3.91×10^{-3} U/µg DNA and 1.95×10^{-3} U/µg DNA. Incubation, termination of reaction and electrophoresis were performed as described earlier. The enzyme concentration yielding greatest fluorescence in the 32 kb to 45 kb region of the gel was 7.8×10^{-3} U/µg DNA.

Partially digested DNA was then prepared on a larger scale employing the conditions determined above, keeping time, temperature, enzyme concentration and DNA concentration constant. The reaction was scaled up by a factor of 15, so that 150 µg of genomic DNA was mixed with 56.25 µl of REact™ 4 in a final volume of 562.5 µl, to which was added 1.17 U of *Sau*3A. Upon termination of reaction, an aliquot was subjected to electrophoresis through a 0.35% agarose gel to ensure that fragments of the correct size distribution had been produced.

2.7.3. Isolation and purification of large DNA restriction fragments

Two methods were evaluated for efficiency at separation of DNA fragments, specifically to isolate fragments in the range of 27 kb to 41 kb.

2.7.3.1. Electroelution into dialysis bags

Into a 1 mm X 20 mm slot in a 0.4% agarose gel was loaded 1 µg of high molecular weight DNA markers (BRL). After electrophoresis at 25 V for 18 h, the region of the gel containing the 29.9 kb, 33.5 kb, 38.4 kb and 48.5 kb bands was excised and the DNA extracted exactly as detailed in section 2.6.17.1. Successful recovery was assessed by electrophoresis of a portion of the extracted DNA under conditions similar to those descried above.

2.7.3.2. Vertical preparative gel electrophoresis

The BRL 1100 PG preparative gel electrophoresis apparatus was set up precisely as instructed by the manufacturers. Prior to use, the elution assembly and electrophoresis buffer were purged of dissolved gases under vacuum. Preliminary experiments were conducted with a 1 cm high agarose gel running at 50 V in single strength TBE buffer. This was loaded with 1 µg of BRL high molecular weight DNA markers by gently layering the sample under the buffer at the gel interface. Eluant flow rate was maintained at 5 ml/h with 250 µl samples being collected over a period of 5 h. In an attempt to optimise separation of the DNA fragments, subsequent experiments were performed using agarose concentrations of 0.4%, 0.6% and 0.8% incorporated into gels ranging in height from 1 cm to 2.5 cm. In addition, the eluant flow rate was varied between 5 and 8 ml/hour with a voltage of 30 to 100 V/cm.

2.7.4. Ligation, packaging and transfection

To 1 µl of a diluted preparation of dephosphorylated cosmid (equivalent to 2 µg of DNA) was added 7 µl of *Sau*3A-generated

chromosomal fragments (0.224 µg DNA). The mixture was incubated at 42°C for 1 h, as suggested by Perbal (1984), to allow efficient formation of *cos* sites. After cooling to 9°C, 1 µl of 10X ligation buffer and 1 µl of 10 mM ATP was added. From this ligation mixture was removed a 1 µl aliquot for subsequent electrophoresis and replaced by a similar volume of T_4 DNA ligase (1 U/µl). The reaction thus contained a 52X molar excess of vector as compared with chromosomal fragments, with

$j_{vector} = 7.20 \times 10^{12}$ ends/ml
$i_{vector} = 5.76 \times 10^{13}$ ends/ml
$j_{fragments} = 5.11 \times 10^{11}$ ends/ml
$i_{fragments} = 1.11 \times 10^{12}$ ends/ml

The $j/_i$ ratios for vector and fragments were thus 0.125 and 0.46 respectively.

Incubation was performed at 9°C for 16 h, following which a further 1 µl aliquot was removed and analysed by electrophoresis through 0.4% agarose together with the aliquot set aside earlier.

Ligated DNA was packaged into bacteriophage λ particles using commercial *in vitro* kits supplied by Amersham or Stratagene (Gigapack II Plus). Protocols supplied by the manufacturers were followed precisely, using the remaining 9 µl of ligation mixture for each reaction. After packaging, the mix was diluted to 500 µl with SM buffer (see Appendix 2), mixed gently with 20 µl of chloroform and stored at 4°C until required.

The packaging efficiency of each *in vitro* kit was ascertained by introduction of wild type *c1857 Sam7* λ DNA to a reaction mixture, following guidelines furnished by the manufacturers. Efficiency was calculated according to the following relationship:

Packaging efficiency =

$$\frac{\text{no. of plaques X dlution factor X total packaging volume}}{\text{no. of µg packaged X no. of µl plated}}$$

Transfection was achieved by mixing a 10 µl aliquot of the reaction with 100 µl of SM and 200 µl of an *E.coli* LE392 (see Table 1 for genotype) culture grown in terrific broth (TB; Tartof and Hobbs, 1987; see Appendix 1) in the presence of 10 mM $MgCl_2$ and 0.2% ($^{w}/_{v}$) maltose. Preliminary experiments were performed with cultures grown under these conditions for 7, 10 and 16 h. In addition, a number of rec- *E.coli* strains were evaluated for use as bacterial hosts, including DH1, DH5 and DH5αMCR™ (BRL). The genotypes of these strains are presented in Table 1.

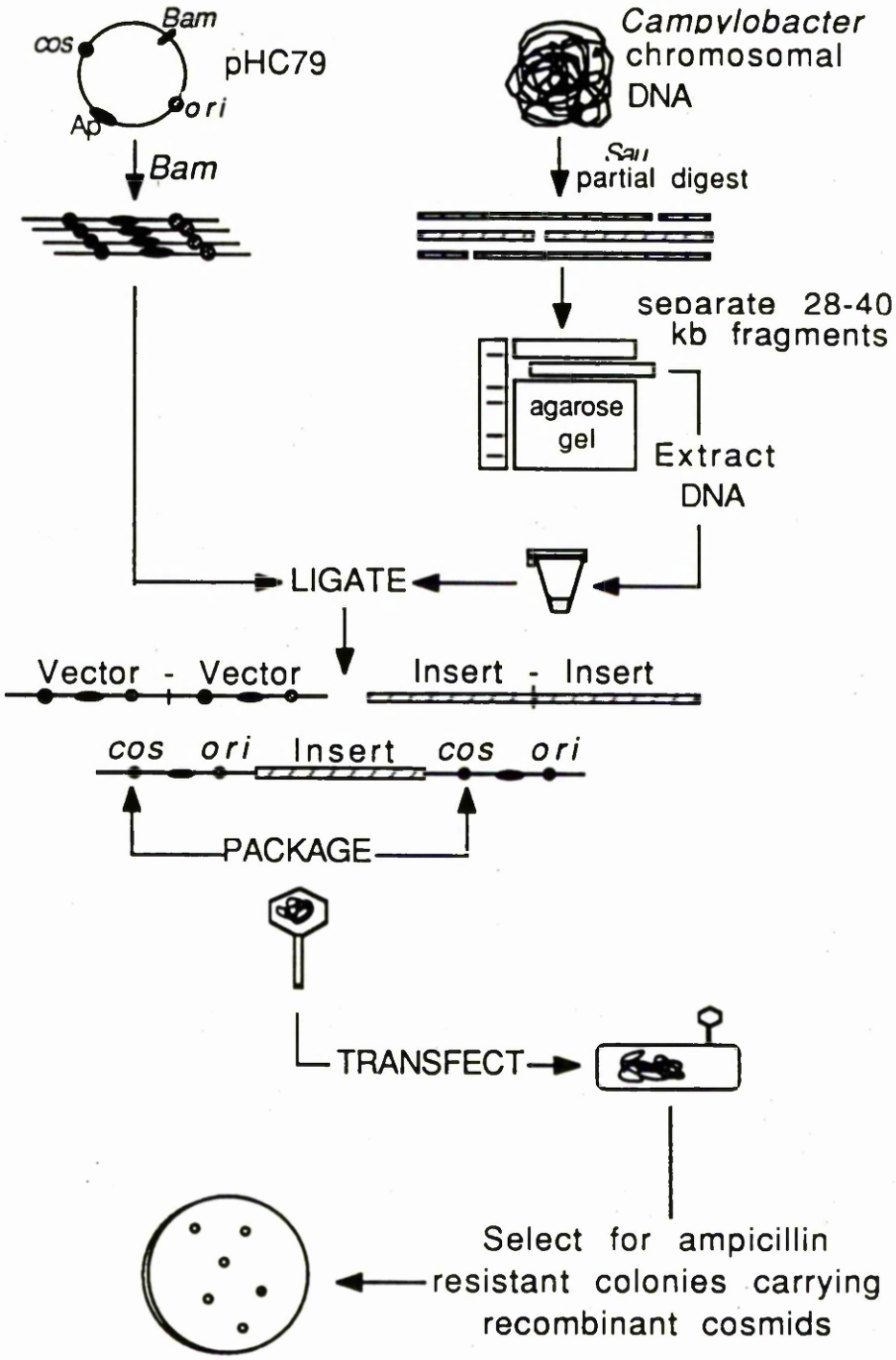
The bacteriophage particles were allowed to adsorb by incubating the infected culture at 37°C for 20 min, following which 1 ml of TB was added and incubation continued for a further 60 min to allow a period for expression of the ampicillin resistance gene. The infected culture was then spread onto the surfaces of two TB agar plates containing 65 µg/ml ampicillin which were incubated for 24 h at 37°C. A number of the resulting colonies were grown overnight in 5 ml of NB containing a similar level of antibiotic. Cosmid DNA was isolated, digested with restriction endonucleases and analysed by electrophoresis using procedures previously described. Each clone was stored at -20°C in NB containing 40% glycerol ($^{v}/_{v}$) as soon as practicable.

2.7.5. Screening for *C.jejuni* haemolysin gene

Library clones were propagated in 96-well flat bottom microtitre plates for 24 h in 300 µl volumes of NB supplemented with 65 µg/ml ampicillin. Each was then transferred, with the aid of a 48 position multipoint inoculator, to the surface of a SBA + Cys-HCl plate containing ampicillin at the above concentration. The *E.coli* host strain used for preparation of the gene library, grown in similar conditions but in the absence of antibiotics, was sub-

Figure 10: Schematic Representation of Steps Involved in Cloning of *Campylobacter* Chromosomal DNA in Cosmid pHC79

Restriction endonucleases used:
Bam : *Bam*HI
Sau : *Sau*3A


Bam
cos
pHC79
ori
Ap
Bam
Campylobacter
chromosomal
DNA
Sau
partial digest
separate 28-40
kb fragments
agarose
gel
Extract
DNA
LIGATE
Vector - Vector
Insert - Insert
cos ori Insert cos ori
PACKAGE
TRANSFECT
Select for ampicillin
resistant colonies carrying
recombinant cosmids

cultured in an analagous manner to act as a negative control. Plates were incubated at 37°C for 5 days and were examined every 24 h.

The ability of the medium to support haemolysis was checked by inoculating plates from the same batch as that used for screening purposes with *C.jejuni* 11168 and incubating at 42°C for 72 h in a microaerophilic atmosphere.

2.7.6. Screening for *C.jejuni* enterotoxin gene

Clones were transferred to duplicate Hybond-N™ membranes using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories). Briefly, a sheet of nylon membrane was clamped between the 96-well gasket and 96-well sample template and 125 µl volumes of overnight cultures grown in microtitre plates drawn onto the filter by vacuum. Positive controls on each membrane included pJYL2299 DNA and cultures of *E.coli* DH5αMCR™ harbouring pJYL2299, whilst negative controls were provided by cultures of the same *E.coli* strain devoid of any extrachromosomal DNA elements.

Immobilised bacterial cells were lysed essentially as described by Woods (1984). With the cell-bearing face uppermost, the membranes were placed firstly upon sheets of Whatman™ 3MM paper wetted with a solution of 0.5 M NaOH, 1.5 M NaCl for 15 min, then onto dry 3MM paper, and finally onto 3MM paper wetted with 1 M Tris-HCl (pH7.0), 1.5 M NaCl for 3 min. The filters were then suberged briefly in 3X SSC and left to dry at room temperature for 1 h. Nucleic acids were fixed to the nylon support by exposure to UV illumination for 5 min. The membranes were then washed in several volumes of 3X SSC, 0.1% SDS at 65°C for 18 h, changing the wash solution every 6 h. They were then prehybridised and hybridised with the CT-LT mixed oligonucleotide probe under conditions detailed previously.

2.8. Preparation of plasmid-based gene library

Several steps were involved in the production of a plasmid-

based *C.jejuni* gene library, as detailed below and summarized schematically in Fig. 11.

2.8.1. Preparation of vector

Shuttle vector pILL550 DNA was isolated from one litre of culture and purified by Sephacryl S-1000 column chromatography. It was cleaved with *Bam*HI restriction endonuclease and dephosphorylated according to procedures already detailed.

2.8.2. Preparation of *Campylobacter* chromosomal DNA fragments

Production of DNA fragments in the range of 8 kb to 12 kb was essentially as described by Maniatis *et al.* (1982). A reaction mixture was prepared containing 5 µg of chromosomal DNA and 15 µl of REact™ 4 buffer (BRL) in a final volume of 150 µl made up with d.H_2O. To one tube was added 30 µl of this mixture, whilst 15 µl was distributed to each of eight other tubes. One unit of *Sau*3A (BRL) was added to tube 1, the contents mixed and 15 µl transferred to tube 2. Dilution of the enzyme was performed in this manner up to tube 8, from which the final 15 µl was discarded. The concentration of *Sau*3A thus ranged from 2 U/µg DNA to 0.016 U/µg DNA. Tube 9 acted as a negative control to which no enzyme was added. All tubes were incubated at 37°C for 1 h, following which they were put on ice and EDTA added to each at a final concentration of 20 mM. Loading dye was added and an aliquot from each tube subjected to electrophoresis through 0.7% agarose, with *Hin*dIII-generated λ DNA fragments acting as molecular size markers.

Upon staining with EtBr and UV transillumination, the enzyme concentration yielding greatest fluorescence in the 8 to 12 kb region of the gel was determined to be 0.06 U/µg DNA. Using this concentration of enzyme, the above reaction was scaled up by a factor of ten, keeping the time of incubation, temperature and DNA concentration constant. Following incubation, a 6 µl aliquot was

analysed by electrophoresis through 0.7% agarose to ensure that fragments of the correct size distribution had been obtained. The reaction mixture was then extracted sequentially with Tris-saturated phenol and chloroform/isoamyl alcohol and the final volume reduced to about 100 µl with several gentle extractions with 2-butanol. This was dialysed overnight at 4°C against TE buffer.

A 10-40% sucrose gradient was then prepared in a polyallomer tube using an MSE gradient maker. The 10% and 40 % sucrose solutions were made up in 1 M NaCl, 5 mM EDTA and 20 mM Tris-HCl (pH 8.0). The dialysed DNA was loaded onto the surface of this gradient and the tube centrifuged at 26500 rpm in a Sorvall AH-627 swinging bucket rotor for 18 h at 20°C. The gradient was then fractionated by carefully pushing a glass capillary into the bottom of the centrifuge tube and slowly pumping out the gradient, heavier fractions first. These were collected in 750 µl volumes and 12 µl aliquots analysed by electrophoresis through 0.7% agarose.

Those fractions containing DNA of the correct size distribution were pooled, the volume of solution doubled by addition of TE buffer and the DNA precipitated overnight at -20°C by addition of two volumes of ethanol. The solution was split equally amongst several microfuge tubes and DNA recovered by centrifugation at 12500 rpm, the final pellet being redissolved in TE buffer.

2.8.3. Ligation of vector and chromosomal DNA fragments

To 1 µl of dephosphorylated vector (equivalent to 0.5 µg of DNA) was added 6 µl of chromosomal fragments (0.2 µg DNA). After cooling to 14°C, 1 µl volumes of 10X ligation buffer, 10 mM ATP and T_4 DNA ligase (1 U/µl)were added. The reaction thus contained a 3X molar excess of vector as compared with chromosomal fragments, with

$$j_{\text{vector}} = 4.74 \times 10^{12} \text{ ends/ml}$$
$$i_{\text{vector}} = 1.09 \times 10^{13} \text{ ends/ml}$$

$j_{fragments}$ = 3.71×10^{12} ends/ml

$i_{fragments}$ = 3.71×10^{12} ends/ml

The $j/_i$ ratios for vector and fragments were thus 0.43 and 1.0 respectively.

Incubation was performed at 14°C for 16 h, after which 1 µl aliquots were transformed into competent cells of *E.coli* DH1. Several colonies appearing on NA supplemented with 50 µg/ml kanamycin were inoculated into 10 ml of nutrient broth containing a similar level of antibiotic. Plasmids isloated from these cultures by the small-scale procedure were digested with the enzymes, *Pst*I and *Eco*RI. Clones were stored at -20°C in NB containing 40% glycerol ($v/_v$).

2.8.4. Screening for the *C.jejuni* haemolysin gene

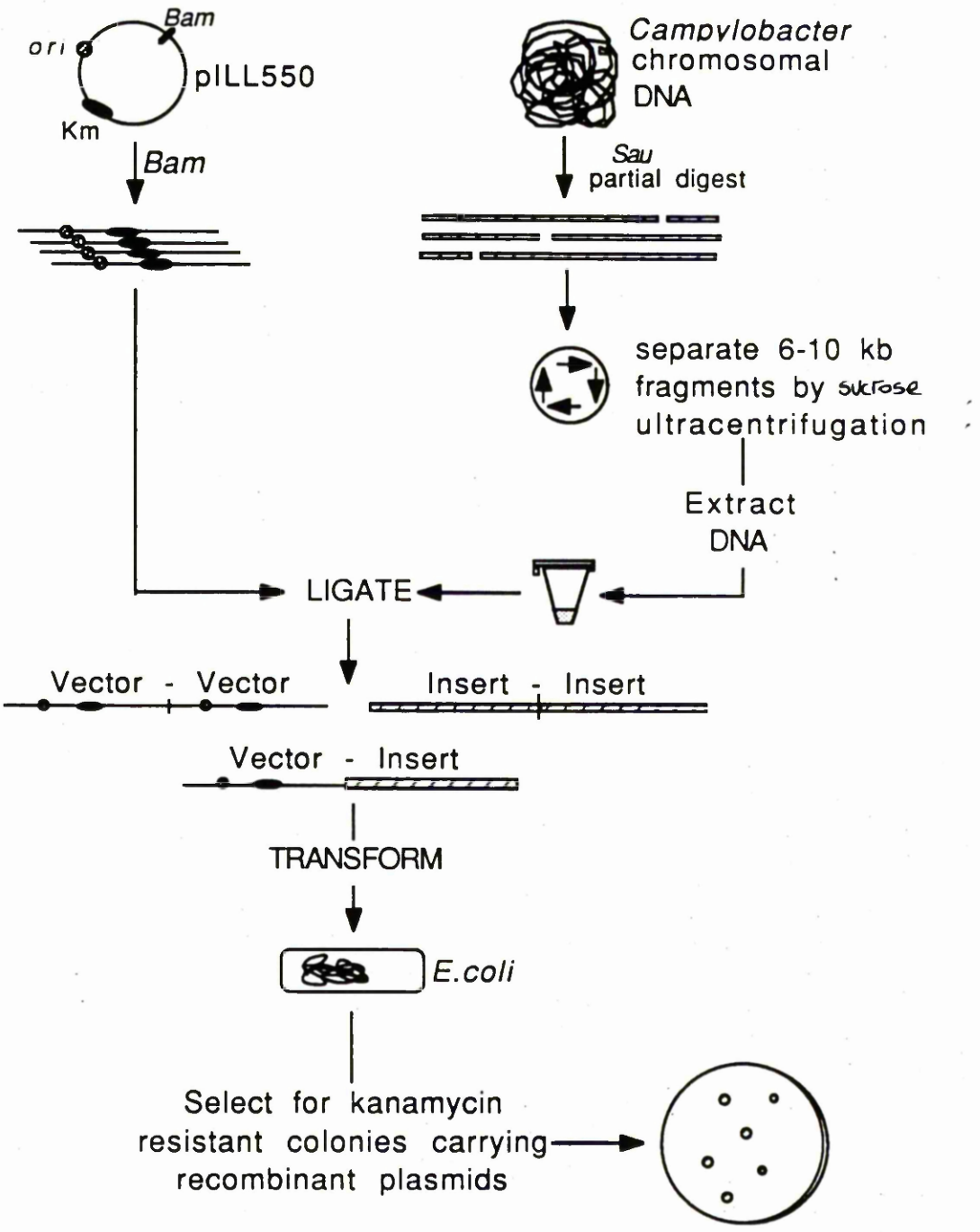
All clones were grown for 24 hours in 300 µl volumes of NB supplemented with 50 µg/ml kanamycin in 96-well microtitre plates. Each was then transferred to the surface of a BA/Cys-HCl plate containing kanamycin at the above concentration using the 48 position multipoint inoculator. *E.coli* DH1, grown as above but in the absence of antibiotics, was sub-cultured in a similar manner to act as a negative control. Plates were incubated at 37°C for five days and were examined every 24 h.

To ensure that the medium was able to support haemolysis, *C.jejuni* 11168 was inoculated onto several plates from the same batch as that used for screening the clones. Incubation was performed at 42°C for 72 h in a microaerophilic atmosphere.

In an attempt to release haemolysin from within the *E.coli* cells, clones were transferred from the BA/Cys-HCl plates used above to 85 mm diameter circles of Hybond-N™ membrane. Filters were suspended in saturated chloroform vapour for 1 h and subsequently incubated at 37°C for 15 min to remove residual chloroform. They were then placed onto fresh BA/Cys-HCl plates

Figure 11: Schematic Representation of Steps Involved in Cloning of *Campylobacter* Chromosomal DNA in pILL550

Restriction endonucleases used:
Bam : *Bam*HI
Sau : *Sau*3A


Bam
ori
pILL550
Km
Bam
Campylobacter
chromosomal
DNA
Sau
partial digest
separate 6-10 kb
fragments by sucrose
ultracentrifugation
Extract
DNA
LIGATE
Vector - Vector
Insert - Insert
Vector - Insert
TRANSFORM
E.coli
Select for kanamycin
resistant colonies carrying
recombinant plasmids

with the face supporting bacterial growth in contact with the agar. Plates were incubated in this fashion at 37°C for five days.

2.9. Transposon mutagenesis (conjugation on solid media)

The conditions reported below provided consistent and reproducible transfer of shuttle vector pILL550 from *E.coli* to *C.jejuni*.

C.jejuni was seeded as a confluent lawn on BA plates and incubated microaerophilically overnight at 42°C. Bacterial growth was removed from the lawn with a 48 position multipoint inoculator and transferred to fresh BA plates. These were again incubated overnight as above.

E.coli transposon donor strains (and the mobilisation helper strain, if required) were inoculated into 10 ml of NB containing the appropriate antibiotic(s) and incubated overnight with shaking at 37°C. Care was exercised with pNK81 in using 50 μg/ml ampicillin and only a low concentration of tetracycline (5 μg/ml). The *tet* gene of Tn*10* has a negative gene dosage effect and this, allied with the multicopy nature of pNK81, leads to expression of only a low overall level of tetracycline resistance (D.C. Coleman, personal communication). A 200 μl aliquot of this culture was used as inoculum for 100 ml of BHI broth which was subsequently incubated under microaerophilic conditions for 5 h on an orbital shaker at 37°C. The culture was then transferred onto the spots of *Campylobacter* growth using the multipoint inoculator and the plates incubated microaerophilically at 42°C for 16 to 18 h. Spots of growth were then removed to BA plates supplemented with cefoperazone (32 μg/ml; Oxoid) and bacteriophage T_1 (100 μl of stock suspension spread over the surface of the plate; supplied by J.G. Coote). These conditions selected against the survival of the *E.coli* donor strain. The plates were incubated overnight as described above. Spots of growth were then transferred to BA plates containing 32 μg/ml cefoperazone and either kanamycin (25 μg/ml) or tetracycline (10 μg/ml) to select for inheritance of Tn*5*

or Tn*10* respectively. Plates were incubated under microaerophilic conditions at 42°C and were checked on a daily basis for up to five days.

Cefoperazone and bacteriophage T_1 selection were found to be ineffective in arresting growth of some *E.coli* strains harbouring transposon delivery vectors. *E.coli* HB101, however, proved to be totally susceptible to this antibacterial combination. Vectors being carried by strains not inhibited by cefoperazone/bacteriophage T_1 were thus isolated and transformed into *E.coli* HB101 using procedures as described earlier. Table 5 lists the transposon delivery vectors used in this study and indicates, where appropriate, those which needed to be transferred from their original hosts to *E.coli* HB101. In Table 6 are presented relevant details of the transposons and delivery vectors.

2.10. Electroporation

Campylobacter cells were prepared for electroporation according to the guidelines reported by Miller *et al.* (1988). *C.jejuni* 11168 was grown in BHI broth for 24 h at 42°C under microaerophilic conditions on an orbital shaker. The optical density of the culture was determined and BA plates inoculated with a culture volume equivalent to 1×10^9 colony forming units (cfu) per plate. Plates were incubated microaerophilically at 42°C for seven hours. Cells were harvested in electroporation buffer (EPB; 272 mM sucrose, 15% $^{v}/_{v}$ glycerol made up in deionised water) and pelleted at 6000 X g for 10 min in a Sorvall SS-34 rotor at 4°C. Cells were then gently resuspended in an equal volume of EPB, pelleted once again as above, and finally resuspended in ice-cold EPB to an optical density of 0.7 ODU (650 nm), equivalent to approximately 5×10^9 cfu/ml.

Plasmid DNA was isolated by the large-scale protocol detailed in section 2.6.2 and dialysed extensively against TE buffer. Transposon delivery vectors pNK81, pSUP2021, pUW964 and pRK404::Tn*5* were isolated from *E.coli* strains listed in Table 5

Table 5: Transposon Delivery Vectors and their *E.coli* Host Strains [a] Used in this Study

E.coli Host Strain Used	Plasmid or Transposon Vector	Original *E.coli* Host Strain
HB101	pRK2013	HB101
HB101	pLG221	C600
HB101	pUW964	HB101
WA803	pGS9	WA803
HB101	pSUP2021	S17-1
HB101	pNK81	C600
SM10	pRT291	SM10
(See Results Section)	pRT733	SM10λpir
HB101	pRK404::Tn*5*	C600
HB101	pFC1	DH1

[a] Genotypes of *E.coli* strains are presented in Table 1.

Table 6: Transposons and Delivery Vectors Used in this Study

DELIVERY VECTOR	SIZE (kb)	TRANSPOSON TYPE	RELEVANT CHARACTERISTICS	ANTIBIOTIC RESISTANCE	REFERENCE
pRK2013	48.0	-	Mobilisation helper plasmid. ColE1replicon; P-group *tra* genes	Kn	Figurski & Helinski (1979)
pLG221	99.0	Tn*5*	Derivative of collb*drd-1*; conjugation functions expressed constitutively; IncIa	Kn	Boulnois (1981)
pUW964	73.0	Tn*5* ; Tn*7*	Tn*5* & Tn*7* inserted on pRK2013	Kn; Sm; Tm; Sp	Weiss *et al.* (1983)
pGS9	30.5	Tn*5*	p15A replicon; N-group *tra* genes	Kn; Cm	Selveraj & Iyer (1983)
pSUP2021	13.2	Tn*5*	Based on pBR325; mob+	Kn; Cm; Ap	Simon *et al.* (1983)
pNK81	16.0	Tn*10*	Tn*10* inserted in *his*G on pBR333 (Sutcliffe, 1978)	Ap; Tc	Foster *et al.* (1981)
pRT291	27.7	Tn*phoA* *phoA* gene replaces most of Tn*5* IS50L	Tn*phoA* inserted on pRK290; IncP; tra-; mob+; fusions used to detect membrane or exported proteins	Kn; Tc	Taylor *et al.* (1989)
pRT733	ND	Tn*phoA*	Tn*phoA* inserted on pJM703.1 (Miller & Mekalanos, 1988); contains *oriR6K* (functions only in presence of *pir* gene of λ-*pir* prophage); tra-; mob+	Kn; Ap	Taylor *et al.* (1989)
pRK404:: Tn*5*	ND	Tn*5* (Cm^r)	Tn*5* inserted on pRK404 (Ditta *et al.*, 1985); mob+	Tc; Cm	J.G. Coote, Glasgow [a]

(Continued overleaf.....)

Table 6 (continued)

DELIVERY VECTOR	SIZE (kb)	TRANSPOSON TYPE	RELEVANT CHARACTERISTICS	ANTIBIOTIC RESISTANCE	REFERENCE
pFC1	8.9	Tn*5* (Cm^r)	Tn*5* derivative (Sasakawa & Yoshikawa, 1987) inserted into Tc^r gene of pBR322	Cm; Ap	F.C. Craig, Glasgow [a]

[a] Department of Microbiology, University of Glasgow

Abbreviations used: Ap, ampicillin; Cm, chloramphenicol; Kn, kanamycin; Sm, streptomycin; Tc, tetracycline; Tm, trimethoprim; mob, mobilisation; tra, transfer; Inc, incompatability group.

Antibiotics were used at the following final concentrations:

Ap, 50 μg/ml; Cm, 10 μg/ml; Kn, 50 g/ml; Sm, 25 μg/ml; Tc, 10 μg/ml.

The preparation and use of these antibiotics is described in more detail in the Appendix section.

whilst pILL550 was isolated both from *E.coli* DH1 and *C.jejuni* 11168 (See Section 1.8.6. and Fig 5).

To a 185 µl aliquot of cells in a chilled microfuge tube was added 1 µg of DNA in a volume of 15 µl of TE buffer. The mixture of cells and DNA was then transferred to an ice-cold electroporation cuvette (0.2 cm electrode gap; Bio-Rad Laboratories). High voltage pulses were applied to the sample by a Bio-Rad Gene Pulser apparatus using the 25 microfarad (µF) capacitor at a field strength of 12.5 kilovolts per cm (kV/cm). Settings of a pulse controller (Bio-Rad Laboratories) connected to the Gene Pulser were varied between 100 Ω and 400 Ω. Control cuvettes were included to which either no pulse was applied or 15 µl of TE buffer replaced the DNA solution. Following delivery of the pulse, the cuvette was maintained on ice for 10 min, after which the sample was removed to a BA plate and incubated microaerophilically at 42°C for 4 h. This allowed a period for plasmid maintenance and expression of antibiotic resistance. Cells were harvested and diluted 1 in 5 and 1 in 50 in BHI broth and gently spread on BA plates containing kanamycin (25 µg/ml) or tetracycline (7.5 µg/ml) to select for Tn*5* and Tn*10* respectively. Plates were incubated microaerophilically at 42°C for three days.

As an additional control to ensure that the transposon delivery vectors were capable of transformation by this method, *E.coli* DH1 cells, prepared according to directions furnished by Bio-Rad Laboratories, were subjected to electroporation as above using the 200 Ω resistor. Outgrowth was performd in 1 ml of SOC medium incubated at 37°C for 1 h. Dilutions were then spread onto appropriate selective NA plates.

2.11. Construction of a shuttle cosmid vector

A schematic representation of the various stages involved in the production of a shuttle cosmid vector is shown in Fig. 12. These steps are described in detail below.

Plasmid pILL550 was cleaved with *Eco*RI and 5' phosphate ends removed by calf intestinal phosphatase treatment. Test ligations were performed to ensure that dephosphorylation had occurred to a satisfactory extent whilst the vector still maintained the ability to ligate to phosphorylated fragments of λ DNA cut with the same restriction enzyme.

The cosmid pBTI-1 (Rose and Broach, 1990; 11.1 kb; Ap^r & Tc^r), obtained from BRL, was transformed into *E.coli* JM83. Cosmid DNA was isolated from 100 ml of nutrient broth containing 50 µg/ml ampicillin inoculated with this culture and purified by Sephacryl S-1000 column chromatography. The portion of DNA containing the *cos* site was excised as a 2.6 kb *Eco*RI fragment from a 0.7% agarose gel and nucleic acids extracted by electroelution into a dialysis bag. After concentration with 2-butanol, the DNA was dialysed extensively against TE buffer.

2.11.1. Ligation of vector and insert fragments

To 0.2 µg of the pILL550 *Eco*RI digest were added 360 ng of the preparation containing the *cos* site fragment. To this mixture was added 1 µl of 10X ligation buffer, 1 µl of 10 mM ATP and one unit of T_4 DNA ligase. The final reaction volume was then made up to 10 µl with $d.H_2O$. The reaction thus contained a 5.9X molar excess of vector as compared with chromosomal fragments, with,

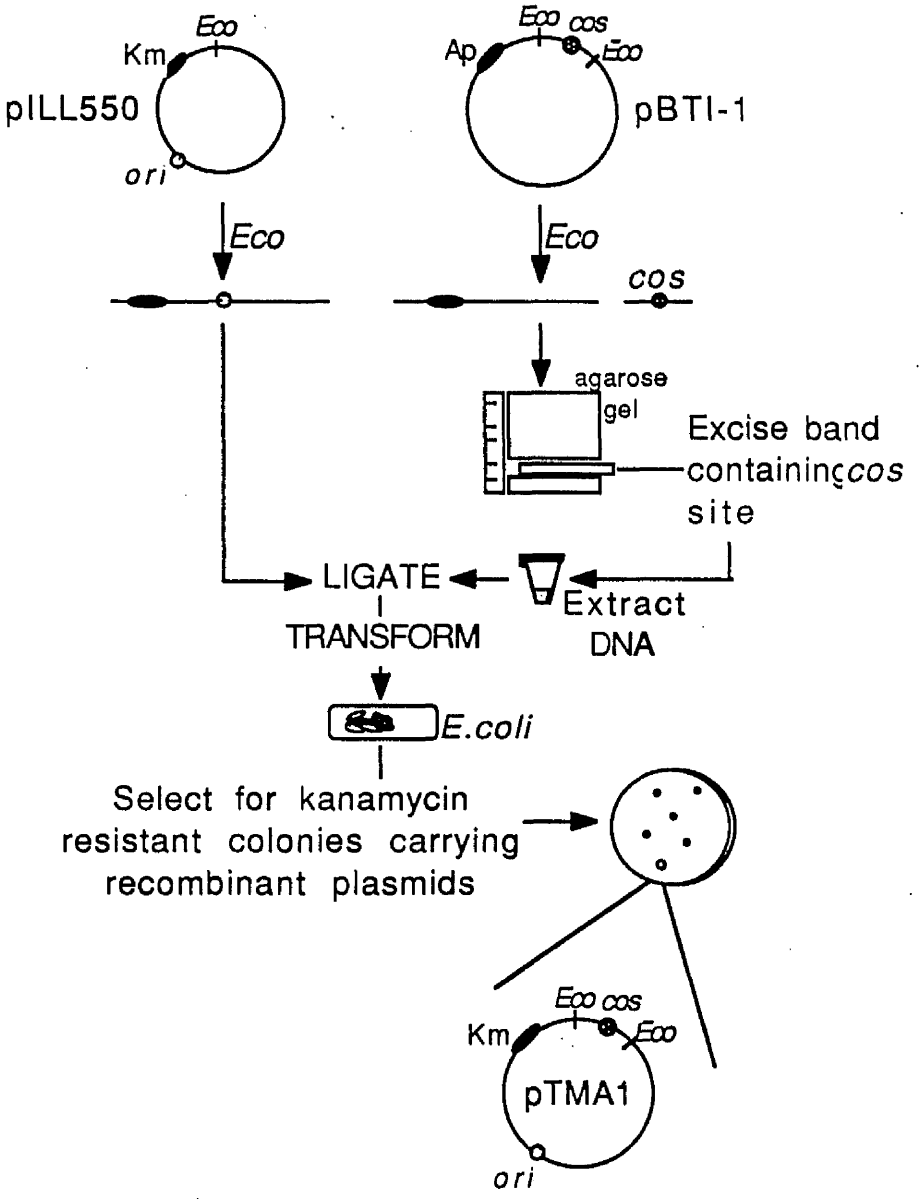
$$j_{vector} = 4.74 \times 10^{12} \text{ ends/ml}$$
$$i_{vector} = 4.36 \times 10^{12} \text{ ends/ml}$$
$$j_{insert} = 2.8 \times 10^{13} \text{ ends/ml}$$
$$i_{insert} = 2.57 \times 10^{13} \text{ ends/ml}$$

The $j/_i$ ratios for both vector and fragments were thus 1.09.

Incubation was performed at 14°C for 16 h. Aliquots of 1 µl of the ligation reaction were used to transform competent cells of *E.coli* JM83. Colonies appearing on NA plates supplemented with 50 µg/ml of kanamycin were grown in 10 ml volumes of nutrient broth

containing a similar level of antibiotic. Plasmid DNA was isolated by the small-scale protocol and digested with *Eco*RI.

Figure 12: Schematic Representation of the Construction of Shuttle Cosmid pTMA1


Eco
Km
pILL550
ori
Eco
cos
Ap
Eco
pBTI-1
Eco
Eco
cos
agarose
gel
Excise band
containing cos
site
LIGATE
TRANSFORM
Extract
DNA
E.coli
Select for kanamycin
resistant colonies carrying
recombinant plasmids
Eco
cos
Km
Eco
pTMA1
ori

3. RESULTS

3.1. *C.jejuni* haemolysin

3.1.1. Demonstration of haemolysin directly on agar medium

To determine the concentration of Cys-HCl resulting in greatest promotion of haemolytic activity, continuous gradient plates were prepared containing Cys-HCl concentrations ranging from zero at one edge of the plate to 16 mM at the opposing edge. *C.jejuni* strains 11168 and 11392 were inoculated as a single streak from the low to the high concentration of amino acid. Plates were inspected after incubation for three days at 42°C in a microaerophilic atmosphere. The approximate concentration of Cys-HCl which promoted haemolysis to the greatest extent was identified. This figure was confirmed by incorporating Cys-HCl at various concentrations at and around this value into individual plates of BA and noting haemolysis around single colonies. The optimal concentration of Cys-HCl was determined in this manner as 6.5 mM. Fig. 13 shows that *C.jejuni* 11168 exhibits no potential for haemolysis on normal BA medium (top half of the divided petri dish), but when the same medium was supplemented with 6.5 mM Cys-HCl, zones of haemolysis were clearly visible around areas of bacterial growth (bottom half of the petri dish). An enlarged view of colonies of *C.jejuni* growing on BA/Cys-HCl is presented as Fig. 14.

All strains of *C.jejuni*, *C.coli* and *C.jejuni /coli* listed in Table 2 were found to be haemolytic when grown on BA supplemented with 7% defibrinated sheep blood and 6.5 mM Cys-HCl.

the basal medium contributed any beneficial or detrimental effects upon haemolysis. The agar bases used were BHI agar, tryptone soy agar, Brucella agar, diagnostic sensitivity test agar (all Oxoid) and Columbia agar (Difco). All were prepared according to manufacturers' instructions and supplemented with 7% defibrinated sheep blood. Tests were performed both on media with and without added Cys-HCl.

Figure 13: Divided Plate Demonstrating Lysis of Sheep Erythrocytes Around *C.jejuni* Colonies Growing on BA Supplemented with Cys-HCl But Not in the Absence of Cys-HCl

The top half of the plate contained blood agar base (Oxoid) supplemented with 7% whole sheep blood. The bottom half of the plate contained medium identical to that in the top half, except supplemented additionally with 6.5 mM Cys-HCl. Both halves were inoculated with *C.jejuni* 11168 to give single colonies and incubated microaerophilically at 37°C for three days.

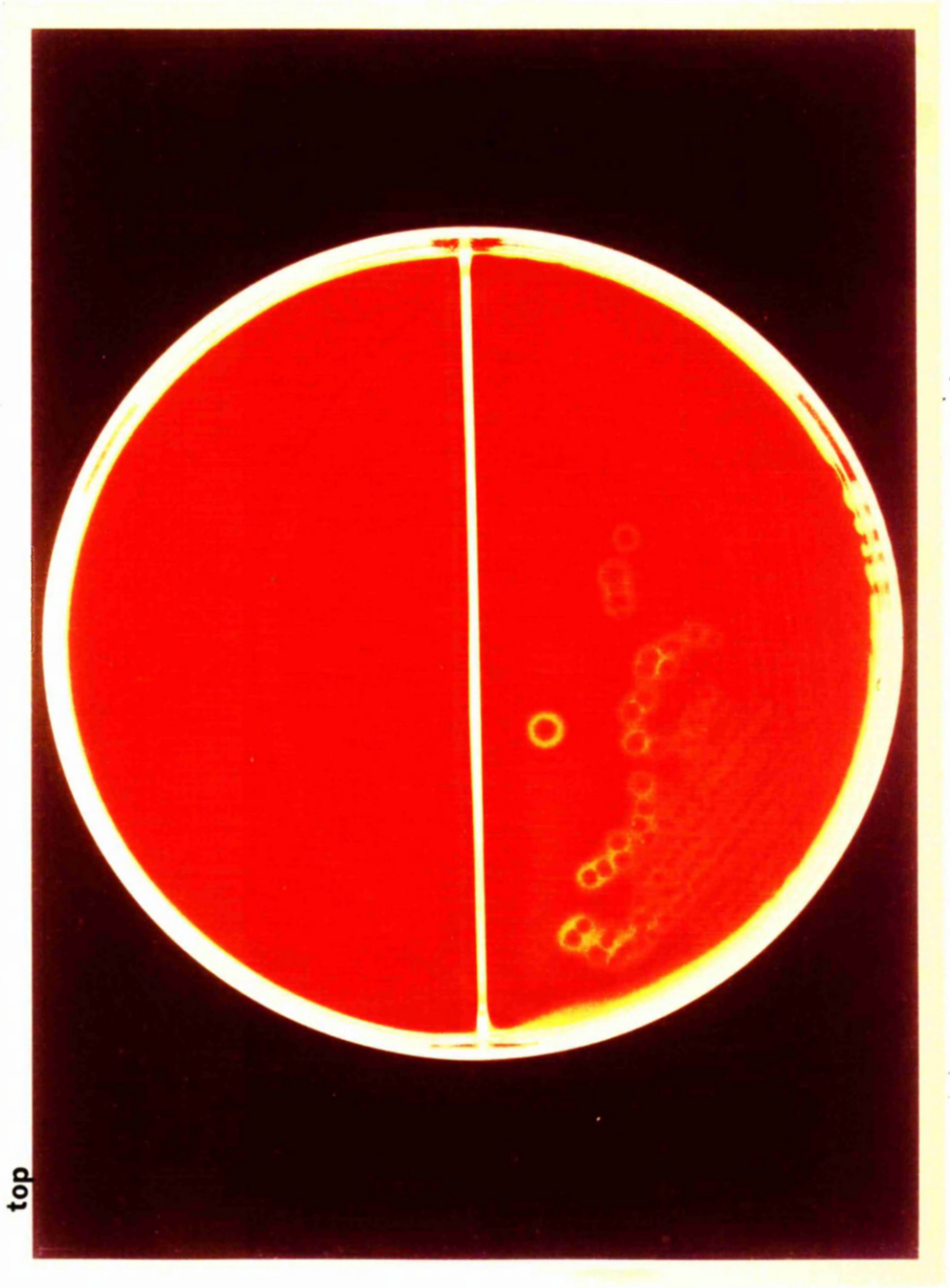
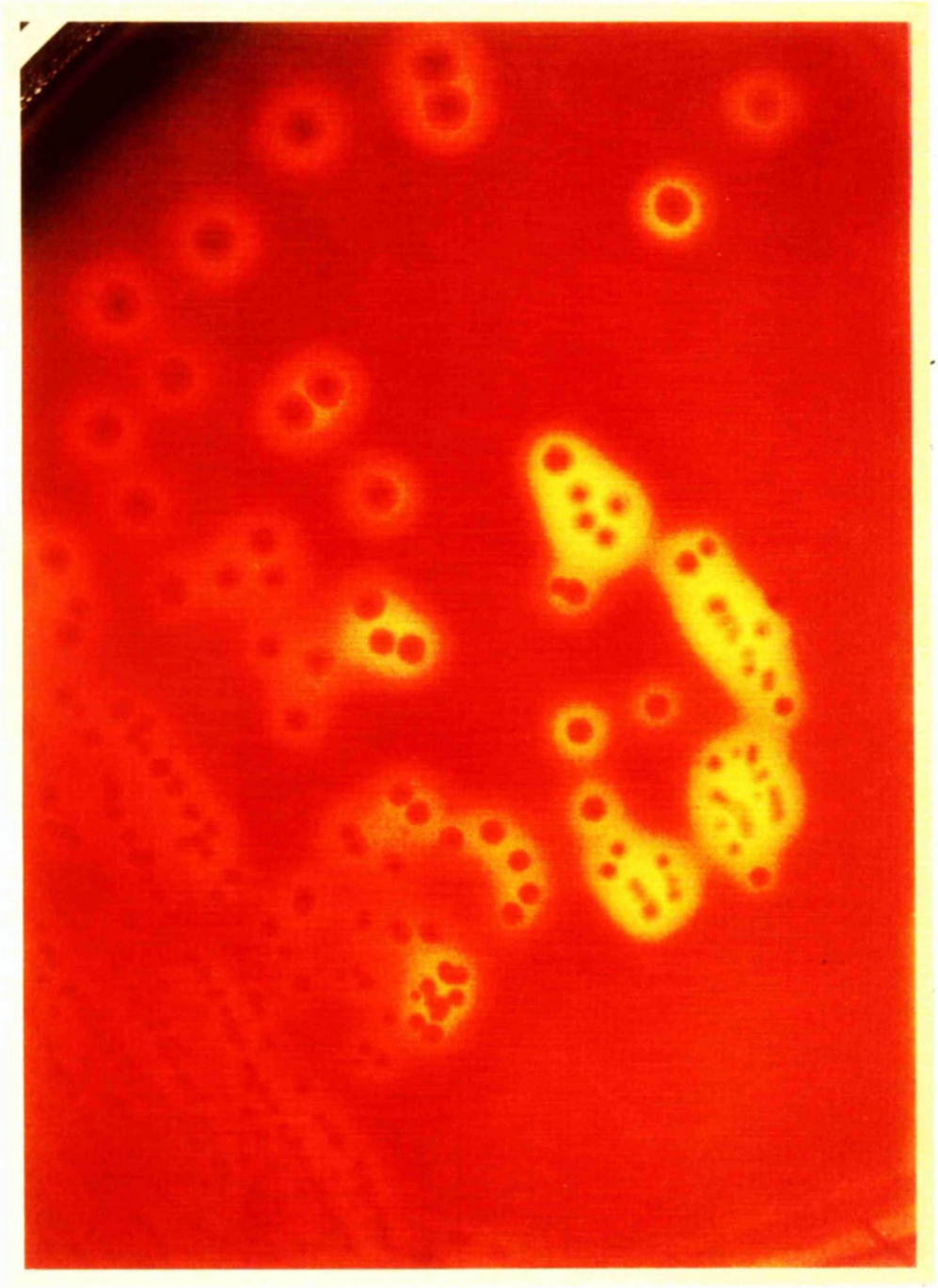

top

Figure 14: Enlarged View of *C.jejuni* Colonies Growing on BA Medium Supplemented with 6.5 mM Cys-HCl

The plate contained blood agar base (Oxoid) supplemented with 7% whole sheep blood and 6.5 mM Cys-HCl. *C.jejuni* 11168 was inoculated to give single colonies and the plate incubated at 37°C for three days.



With these different agar basal media, when supplemented only with 7% sheep blood, no haemolysis was observed around *Campylobacter* colonies after up to 3 days incubation at 42°C, as had been found when using blood agar base. However, on addition of 6.5 mM Cys-HCl, lysis of sheep erythrocytes was supported to an equal extent by all commercial agar bases tested. The one exception was *Brucella* agar base, where the zones of haemolysis were greatly reduced and only barely discernible around the edges of single colonies.

Haemolytic activity was limited to erythrocytes from ruminant species, with sheep erythrocytes exibiting the greatest zones of haemolysis after 48 h incubation, whilst ox and goat erythrocytes showed narrower zones. In all cases, haemolysis was only evident in media supplemented with Cys-HCl. No zones of haemolysis were demonstrated, either in the presence or absence of Cys-HCl, when erythrocytes from the following sources were tested: human, rabbit, horse, donkey, mouse, guinea-pig and chicken. These results are summarized in Table 7.

Haemolysis was not promoted when Cys-HCl was substituted, at concentrations of 0.05, 0.1 and 0.2 mg/ml, by its methyl or ethyl esters, nor by 5-methyl-L-cysteine or L-S,S´ methylenebiscysteic acid. Similarly, no haemolysis was noted when L-cystine dihydrochloride, at a concentration of 0.1 mg/ml, replaced Cys-HCl in BA plates.

3.1.2. Role of iron regulation and release of haemolysin

The iron binding properties of cysteine have previously been documented (Dawson *et al.*, 1986). It was postulated that the presence of cysteine may have reduced the amount of available iron in the medium, thereby leading to the induction of haemolysin production in order to release free iron from red blood cells. This hypothesis was tested by incorporating compounds known to chelate iron at various concentrations into BA medium. These experiments

Table 7: Spectrum of Erythrocyte Senitivity to Haemolysin of *C.jejuni* NCTC 11168

Erythrocyte Species	Hemolysis	
	Without Cysteine	With Cysteine
Sheep	No	Yes
Ox	No	Yes
Goat	No	Yes
Human	No	No
Rabbit	No	No
Horse	No	No
Donkey	No	No
Mouse	No	No
Guinea pig	No	No
Chicken	No	No

Table 8: Effect of Disodium EDTA on Growth of *C.jejuni* 11168 and Haemolysis of Sheep Erythrocytes in Blood Agar Base

EDTA Concentration (mM)	Growth	Haemolysis
50	-	N/A
25	-	N/A
12.5	-	N/A
6.25	-	N/A
3.13	-	N/A
1.56	+	very slight
0.78	+	very slight
0.39	+	very slight
0.2	+	very slight
0.1	+	very slight
0.05	+	very slight

Abbreviations used: N/A, not applicable

Table 9: Effect of 8-Hydroxyquinoline on Growth of *C.jejuni* 11168 and Haemolysis of Sheep Erythrocytes in Blood Agar Base

8-hydroxyquinoline Concentration (mM)	Growth	Haemolysis
12.5	-	N/A
6.25	-	N/A
3.13	-	N/A
1.56	-	N/A
0.78	-	N/A
0.39	+	-
0.2	+	-
0.1	+	-
0.05	+	-

Abbreviations used: N/A, not applicable

Table 10: Effect of 2,2' dipyridyl on Growth of *C.jejuni* 11168 and Haemolysis of Sheep Erythrocytes in Blood Agar Base

2,2' dipyridyl Concentration (μM)	Growth	Haemolysis
300	-	N/A
150	-	N/A
75	+	-
37.5	+	-
18.75	+	-
9.38	+	-
4.69	+	-
2.34	+	-
1.17	+	-

Abbreviations used: N/A, not applicable

were conducted in the absence of Cys-HCl.

The maximal concentrations of the three chelating agents used, disodium EDTA, 8-hydroxyquinoline and 2,2' dipyridyl, which allowed growth of *C.jejuni* 11168 were 1.56 mM, 0.39 mM and 75 µM respectively. Over the range of concentrations of 8-hydroxyquinoline and 2,2' dipyridyl at which growth was supported, no promotion of haemolytic activity was demonstrated. In the case of disodium EDTA, very slight haemolysis was observed around single colonies at all concentrations which allowed *Campylobacter* growth. The extent of this haemolysis did not intensify after a further four days incubation at 42°C, or after the plates were kept at 4°C for 10 days. The results of these investigations are presented in Tables 8, 9 and 10.

3.1.3. Demonstration of haemolysin from cell extracts

Extracts of 12, 24, 36, 48 or 72 h *C.jejuni* cultures obtained after sonication were tested by introduction into wells prepared both in blood agar base supplemented with 7% defibrinated sheep blood and in the medium described by Richardson *et al.* (1986) for demonstration of haemolysin production by *V.cholerae.* This latter medium consisted of 1% purified agar (Oxoid) supplemented with 50 mM Tris (pH 7.6), 0.15 M NaCl and 7% defibrinated sheep blood. The extracts were also tested in these two media when 3% sheep erythrocytes washed four times in PBS at room temperature was used instead of whole sheep blood. The effects of the addition of Cys-HCl to these media when supplemented with either whole blood or washed erythrocytes was also determined. *C.jejuni* cell extracts were thus tested in a total of eight media. These are described in detail below:

a) 1% purified agar (Oxoid) supplemented with 50 mM Tris (pH 7.6), 0.15 M NaCl, 7% defibrinated sheep blood (Richardson *et al.*, 1986)
b) as a) but whole sheep blood replaced by 3% sheep erythrocytes washed in PBS
c) as a) but containing in addition 6.5 mM Cys-HCl
d) as b) but containing in addition 6.5 mM Cys-HCl
e) blood agar base supplemented with 7% defibrinated sheep blood
f) as e) but whole sheep blood replaced by 3% sheep erythrocytes washed in PBS
g) as e) but containing in addition 6.5 mM Cys-HCl
h) as f) but containing in addition 6.5 mM Cys-HCl

Incubation conditions were as described in Materials and Methods section 2.3.2.

Extracts demonstrated no haemolytic activity when introduced into wells cut in blood agar plates containing either whole sheep blood or washed sheep erythrocytes, irrespective of whether the cultures had been grown in the presence of Cys-HCl or whether Cys-HCl was present in the plates themselves. In contrast, when the same extracts were added to wells in the medium described by Richardson *et al.* (1986), small zones of haemolysis were observed. This is depicted in Fig. 15, where zones of clearing are clearly discernible around wells cut in this latter medium supplemented with 7% defibrinated sheep blood (as described under 'a' in the scheme presented above) which were filled with 24 h, 36 h and 72 h culture sonicates of *C.jejuni* 11168.

After repetition of the experiment several times, it was noted that roughly equivalent zone diameters were obtained from 12, 24 and 36 h cultures, whilst smaller zones resulted when 48 and 72 h cultures were used. No advantage, as reflected by an increase in the extent of haemolysis, was obtained by using sheep erythrocytes which had been washed and resuspended in PBS instead

Figure 15: Demonstration of Haemolysis by *C.jejuni* Culture Extracts

The medium used was that of Richardson *et al.* (1986) supplemented with 7% sheep blood. This is described in the Materials and Methods section.

a → c: Extracts from sonicated *C.jejuni* cells

a: 12 h BHI broth culture b: 36 h BHI broth culture
c: 72 h BHI broth culture

d → f: *C.jejuni* culture supernatants

d: 12 h BHI broth culture e: 36 h BHI broth culture
f: 72 h BHI broth culture

g & h: negative controls

g: BHI broth h: BHI broth + 6.5 mM Cys-HCl

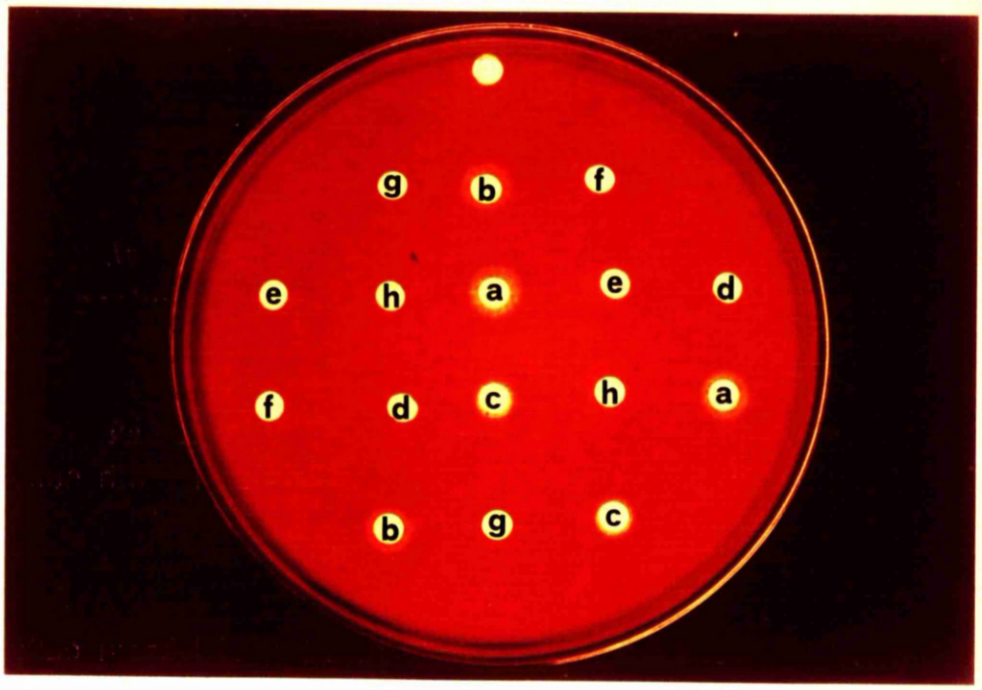

g
b
f
e
h
a
e
d
f
d
c
h
a
b
g
c

Table 11: Extent of Haemolysis of Whole Sheep Blood and Washed Sheep Erythrocytes by Extracts of *C.jejuni* 11168 Sonicates [a]

Culture Conditions	Mean Diameter of Haemolytic Zone (mm) [b]			
	Whole Blood [c]	Washed Erythrocytes [d]	Whole Blood + Cys-HCl [e]	Washed Erythrocytes + Cys-HCl [e]
12 h BHI broth	6.0	6.75	6.75	6.5
36 h BHI broth	6.0	6.5	7.25	6.25
12 h BHI broth + Cys-HCl	6.5	6.25	7.0	6.5
36 h BHI broth + Cys-HCl	6.25	6.25	6.75	6.0

[a] Medium used was as described by Richardson *et al.* (1986)

[b] Mean of four determinations

[c] 7% sheep blood

[d] 3% washed sheep erythrocytes in PBS

[e] 6.5 mM Cys-HCl added to medium

of whole sheep blood. In addition, the introduction of Cys-HCl, either to the *Campylobacter* growth medium or to the agar base used to suspend the red blood cells, did not lead to any appreciable increase in zone sizes. Zone diameters were measured for 12 and 36 h culture extracts introduced into wells cut in media supplemented with 7% whole sheep blood or 3% washed sheep erythrocytes, either in the presence or absence of Cys-HCl (corresponding to a, b, c and d in the above scheme). This data is presented in Table 11.

3.1.4. Assay of haemolysin by tube or microdilution methods

Dilution buffers of different compositions as used by various researchers were tested. These included PBS containing 0.1% ($^{w}/_{v}$) bovine serum albumin (Sigma; Geoffroy *et al.*, 1987), Tris-buffered saline (150 mM NaCl buffered with 20 mM Tris-HCl, pH 7.0; Smyth *et al.*, 1975) and the toxin diluent described by Bernheimer (1988) which consisted of 0.15 M NaCl in 10 mM Tris-HCl (pH 7.2) containing 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.2% gelatin. Titrations were performed in microtiter plates with doubling dilutions of the test sample, each well containing a final volume of 50 µl. An equal volume of 1.5% ($^{v}/_{v}$) sheep erythrocytes washed in the appropriate buffer was then added. The macrodilution glass tube protocol proposed by Bernheimer (1988) was also employed, using 1 ml volumes each of diluted test sample and washed erythrocyte suspension.

On no occasion was a haemolytic reaction observed when *C.jejuni* cell extracts were tested in aqueous buffers of various compositions using either macrodilution or microdilution techniques. This was true of both culture supernatants and extracts of cells obtained by sonication or polymixin B treatment. Supplementation of the growth medium or the aqueous dilution buffers with Cys-HCl similarly had no beneficial effects. All tests were subjected to extended incubation at 37°C for several days, although this, in addition, did not lead to the demonstration of a haemolytic reaction.

3.1.5. Effects of metal ions on haemolysis

Metal salts were individually added at concentrations of 0.5X, 1X and 2X those described by various researchers (detailed below) to BA plates both in the presence and absence of 6.5 mM Cys-HCl. Concentrations of salts tested (mM) were:

$CaCl_2$	10	20	40	(König *et al.*, 1987)
$CuSO_4$	0.3	0.6	1.2	(Avigad and Bernheimer, 1976)
$FeCl_2$	2.5	5	10	(Avigad and Bernheimer, 1976)
$ZnCl_2$	0.13	0.25	0.5	(Bashford *et al.*, 1986)
$MgCl_2$	5	10	20	(Bernheimer *et al.*, 1974)

Inoculation and incubation of plates was as described in Materials and Methods section 2.3.1.

The addition of these various metal salts at different concentrations to BA plates did not promote haemolytic activity of *C.jejuni* upon sheep erythrocytes. These salts also had no beneficial or detrimental effects at the concentrations tested on the extent of haemolysis observed around colonies of *C.jejuni* growing on BA plates supplemented with Cys-HCl.

3.1.6. Actions of miscellaneous compounds on haemolysis

Various compounds were tested both with and without Cys-HCl to determine their effects upon haemolysis of *C.jejuni* on BA plates. Mercaptobenzothiazole (MERCAP; Sigma), a sulphydryl agent exhibiting properties of a chelating agent (Palmer and Roberts, 1967) was tested at concentrations of 10 μM, 5 μM and 1 μM. The reducing agents glutathione (reduced form) and dithiothreitol were tested at concentrations of 3 mM, 6 mM and 10 mM. Finally, the sulphur containing amino acid, methionine, was used at 1 mM, 5 mM and 10 mM.

These compounds were found to have no effects in enhancing or inhibiting the haemolysis exhibited by *C.jejuni* on BA/Cys-HCl

plates. They did not themselves lead to promotion of such a haemolytic reaction in the absence of Cys-HCl.

3.1.7. Relationship of haemolysin to *S.aureus* beta-toxin

A reduction in the extent of sheep erythrocyte lysis was observed on BA/Cys-HCl plates around the growth of a streak of *C.jejuni* where the zone of haemolysis was in proximity to wells filled with anti-staphylococcal ß-lysin serum. Only slight reduction was observed under similar conditions with normal rabbit serum. A photograph of the plate demonstrating these results is presented as Fig. 16. A similar result was obtained when this experiment was repeated.

Using experimental conditions similar to those described above, no interactions were noted between the haemolysis demonstrated by *C.jejuni* on a BA/Cys-HCl plate and purified preparations of the ß-and δ-lysins of *S.aureus*.

3.1.8. Probing of the *C.jejuni* genome with the Hlb$^+$ probe

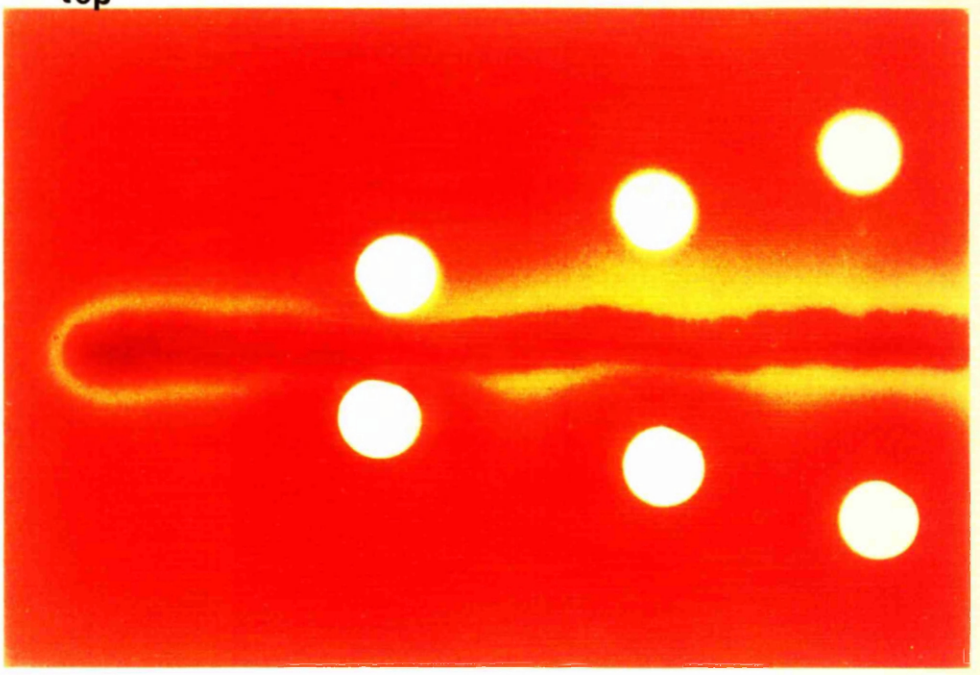
Chromosomal DNA isolated from *C.jejuni* 11168 was digested with restriction endonuclease *Bgl*II (BRL) and run into a 0.7% agarose gel along with a preparation of *Hin*dIII-digested plasmid pDC007. Radioactively labelled fragments of λ DNA served as molecular size markers. A photograph of this gel appears as Fig. 17. Transfer of nucleic acids onto Hybond-N™ membrane was achieved using the VacuGene apparatus. The filter was probed with the 2.2 kb Hlb$^+$ *Hin*dIII fragment of pDC007 which had been end-labelled under the influence of the Klenow fragment of *E.coli* DNA polymerase as detailed in Materials and Methods section 2.6.19. Both low and high stringency hybridization and washing conditions were applied; details of these procedures have been presented elsewhere. The filter was exposed to X-ray film at -70°C for varying periods of time, up to a maximum of 20 days.

Under both low and high stringency conditions, strong hybridization occurred between the Hlb$^+$ 2.2 kb *Hin*dIII probe and the

Figure 16: Interactions Between the Zone of Haemolysis Around Growth of *C.jejuni* 11168 and Preparations of Anti-Staphylococcal ß-Lysin and Normal Rabbit Serum

BA medium supplemented with 6.5 mM Cys-HCl was used.
The set of wells in the upper portion of the photograph were filled with normal rabbit serum.
The set of wells in the lower portion of the photograph were filled with anti-staphylococcal ß-lysin.
Between the two sets of wells was growth of *C.jejuni* 11168.

top



same fragment in a *Hin*dIII digest of the plasmid from which it was excised, pDC007. Weaker signals were also observed at low stringency with the 4.4 kb band, which represented pBR322 DNA, and with remnants of undigested plasmid at 6.6 kb. Under both sets of conditions, no hybridization was observed with *Bgl*II-digested *Campylobacter* chromosomal DNA, even after 20 days exposure of the Hybond-N™ membrane to X-ray film. Photographs of an autoradiograph produced at low stringency and the gel from which the nucleic acids were transferred are presented as Fig. 17 and Fig. 18.

3.2. Evaluation of extracellular DNase production by *C.jejuni* 11168

Both of the methods used to detect the presence of extracellular DNase produced unequivocal results. On DNase agar plates containing 0.01% ($^{w}/_{v}$) toluidine blue O, a pink zone was clearly discernible around the region of growth of the positive control, *S. aureus* Oxford strain. Similarly, large pink halos resulted around wells filled with polymixin B-treated extracts of this *S.aureus* strain, but no reaction was visible around wells filled with Tris-HCl buffer containing polymixin B. No such zones were evident when either protocol was employed for testing *C.jejuni* 11168. This strain of *C.jejuni* thus did not elaborate an extracellular DNase enzyme. Fig. 19 shows both DNase detection procedures with the appropriate positive and negative controls.

3.3. Evaluation of latex agglutination in testing for enterotoxin production

Enterotoxin was not detected in culture supernatants or polymixin B-treated cell extracts of *C.jejuni* NCTC 11168 or recently isolated clinical strains of *C.jejuni* using the Oxoid VET-RPLA kit, irrespective of the age of the cultures when tested. The addition of Cys-HCl to the growth medium similarly had no beneficial effects. Control *V.cholerae* enterotoxin gave rise to positive agglutination in all wells up to and including that containing a

Figure 17: *C.jejuni* 11168 Chromosomal DNA Cleaved with *Bgl*II for Hybridization with the Hlb$^+$ Probe

Lane No.	
1	^{32}P-labelled λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)
2	*Hin*dIII digest of plasmid pDC007
3→6	*C.jejuni* 11168 chromosomal DNA cut with *Bgl*II
7	^{32}P-labelled λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)

(Concentration of agarose used was 0.7%)

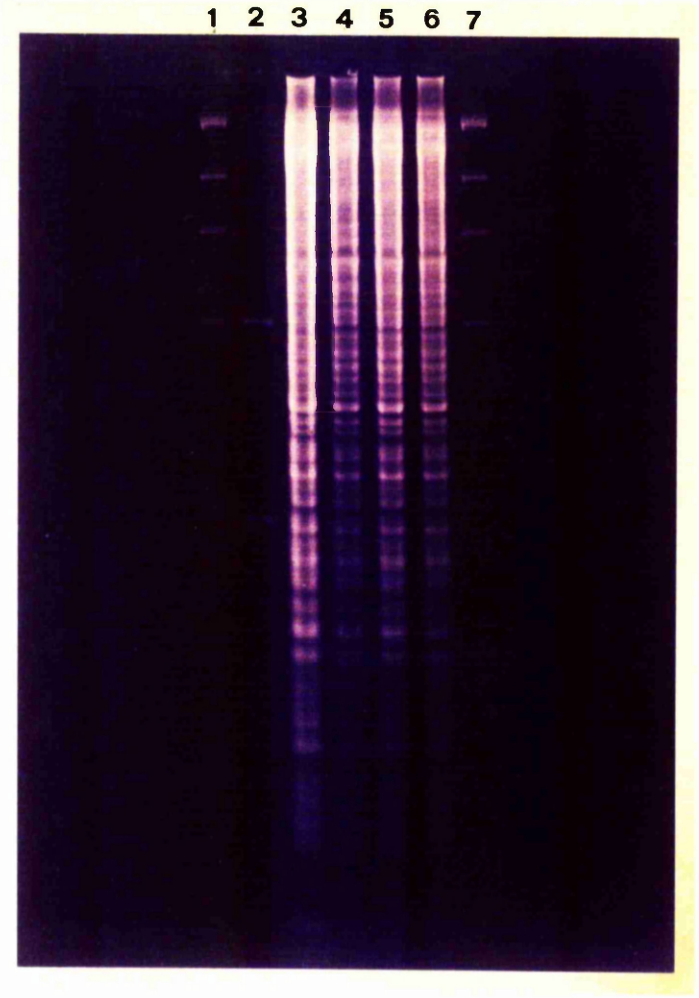

1 2 3 4 5 6 7

Figure 18: Hybridization of *Campylobacter* Chromosomal DNA with Hlb$^+$ Probe - Low Stringeny Conditions

(autoradiography performed at -70°C for 4 days)

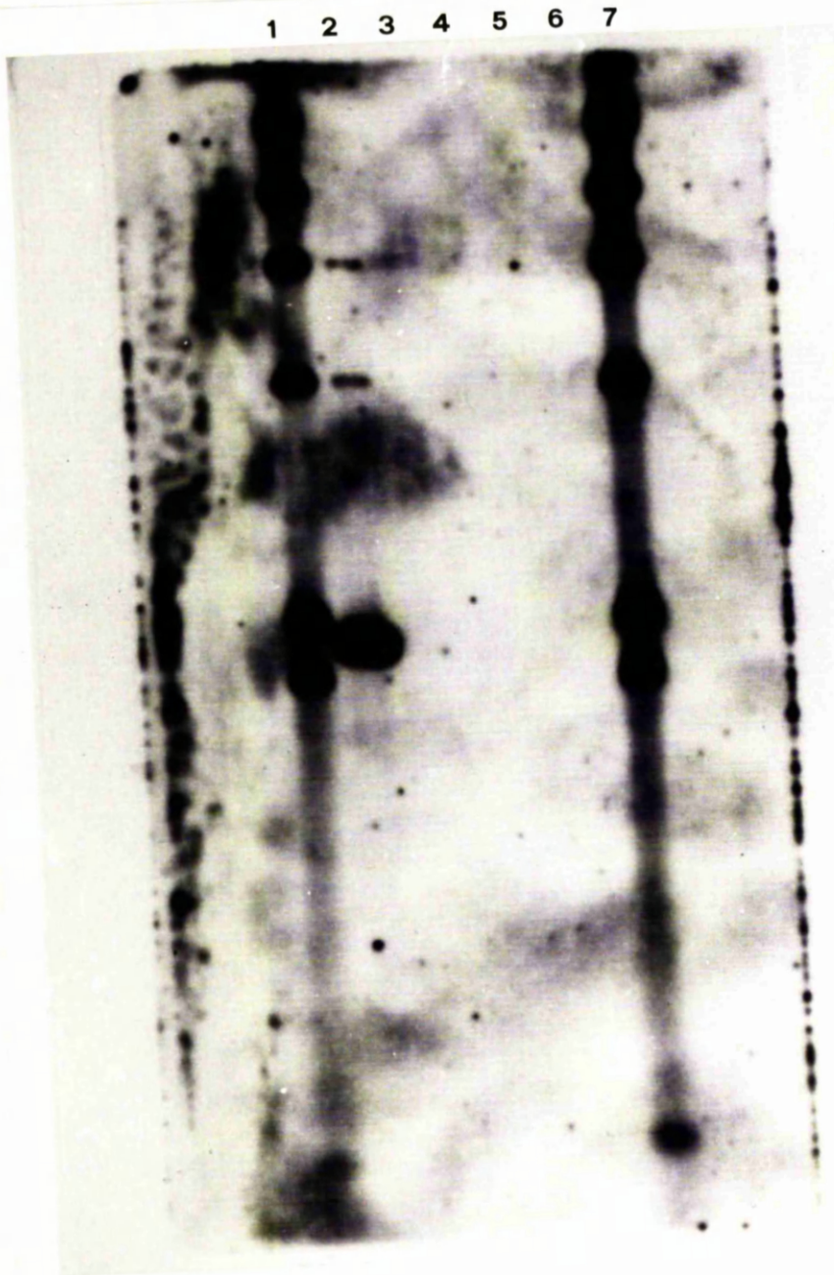

1 2 3 4 5 6 7

Figure 19: Evaluation of Extracellular DNase Production by *S.aureus* and *C.jejuni* on DNase Agar Plates Containing Toluidine Blue O

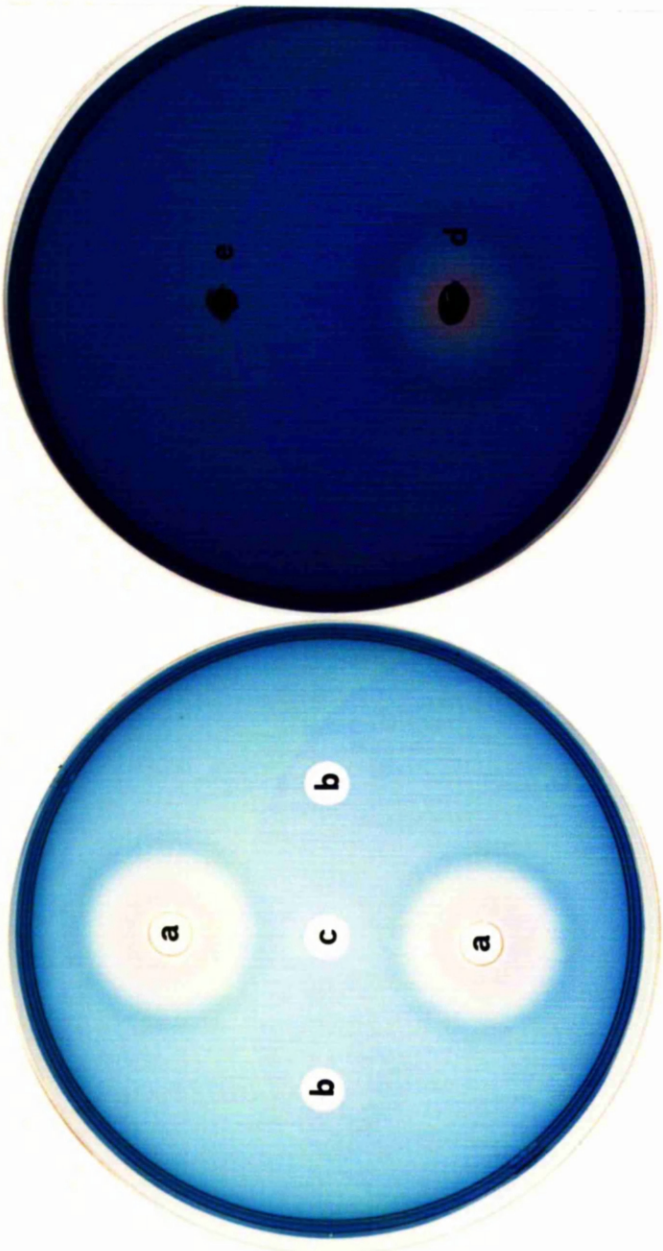
a: *S.aureus* Oxford strain polymixin B-treated extract

b: *C.jejuni* 11168 polymixin B-treated extract

c: Tris buffer + polymixin B

d: *S.aureus* Oxford strain

e: *C.jejuni* 11168


a
d
b
a
c
a
b

$^1/_{128}$ dilution of the toxin. No agglutination was noted in any wells containing control latex coated with non-immune rabbit globulins.

3.4. Determination of cytotoxicity of *C.jejuni* and *E.coli* strains

It became apparent from early experiments that campylobacters could not be harvested from BA plates or BA/Cys-HCl plates for cytotoxicity testing since significant cytotoxicity was demonstrated by 'negative' controls consisting solely of PBS which had been flooded onto the surfaces of sterile plates (≥ 50% cell death resulted from neat and $^1/_2$ dilutions of these samples). However, on no occasion was significant cytotoxicity (≥ 50% cell death) exhibited by BHI broth or PBS containing polymixin B at a concentration of 1.5 mg/ml so that all subsequent procedures thus used *Campylobacter* strains which had been grown in BHI broth.

In preliminary experiments using the HeLa cell line, the cytotoxic capacity of *C.jejuni* polymixin B-treated extracts was determined both by the trypan blue and MTT protocols. Comparable results were obtained with both of these methods. However, the trypan blue procedure was more laborious since each well of the microtiter plate had to be viewed individually under an inverted microscope and was also assumed to be more prone to errors of interpretation. Trials conducted with Vero cells and polymixin B-treated extracts of verotoxin 2 producing *E.coli* strain E32511 determined that the MTT assay procedure functioned adequately in determining the extent of cell death, as demonstrated in Table 12 and Fig. 20. Later experiments were therefore conducted using the MTT protocol only.

Polymixin B-treated extracts of *C.jejuni* 11168 consistently demonstrated > 50% cell death at neat and $^1/_2$ dilutions when tested in both HeLa and Vero cell lines. Similar results were obtained with 24 and 48 h cultures. The addition of Cys-HCl to the bacterial growth medium had no effect on the cytotoxicity titers. No appreciable activity was seen in culture supernatants. Results for cytotoxicity using cultures of HeLa cells

and Vero cells appear in Table 13 and Table 14. This data is also presented graphically as Fig. 21 and Fig. 22.

Three fresh faecal isolates of *C.jejuni* were also evaluated for cytotoxicity. All three had been sub-cultured on laboratory media a minimum number of times before inoculation into BHI broth. As can be seen from Table 15 and Fig. 23, titers of 2 to 4 were obtained for these strains. Extracts of *E.coli* genetic strains DH1 and DH5 were found to demonstrate significant cytotoxicity in undiluted samples (cytotoxicity of 48.1 ± 3.6% and 45.7 ± 4.6% respectively). These results are also represented in Table 15 and Fig. 23.

3.5. Transposon mutagenesis

As was stated in Materials and Methods section 2.9., not all *E.coli* strains were susceptible to the antibacterial effects of cefoperazone in combination with bacteriophage T_1. Most delivery vectors harboured in such strains, nevertheless, were easily transferred into the fully susceptible strain, HB101, by transformation. However, this strategy could not be employed with pRT733 (which carried Tn*phoA*) since this vector had an origin of replication derived from R6K, ensuring its replication only in *E.coli* strains carrying the λ-*pir* prophage. Whilst the original strain harbouring this plasmid, *E.coli* SM10λpir, which was resistant to cefoperazone and bacteriophage T_1, fulfilled this requirement, no other strain was available which could do so. Thus, further experimentation using pRT733 was abandoned.

In addition, *E.coli* strain S17-1 was also unaffected by the selective conditions employed. This strain, developed by Simon *et al.* (1983), contained the transfer genes of the broad host range IncP plasmid RP4 integrated into the chromosome, allowing high frequency mobilization of plasmids, like pSUP2021, which contained the P-type *mob* site. The pSUP2021 delivery vector was thus transformed into HB101 and mobilization attempted by tri-parental mating using the *mob* functions of pRK2013.

Table 12: Cytotoxicity of Verotoxin Producing *E.coli* E32511 and *C.jejuni* 11168 for Vero Cells Using MTT as Indicator of Cell Viability

Bacterial Culture	Dilution of Cell Extract	Percentage Cell Death [a]	Standard Error of Mean
E.coli E32511	Neat	100	2.4
	1/2	100	2.7
	1/4	84.9	2.7
	1/8	73.4	2.9
	1/16	58.9	3.8
	1/32	53.7	3.5
	1/64	51.2	6.1
	1/128	37.9	5.8
C.jejuni 11168	Neat	93.8	3.6
	1/2	87.4	4.9
	1/4	37.8	6.4
	1/8	18.9	4.1
	1/16	7.1	5.9
	1/32	0	0
	1/64	0	0
	1/128	0	0

[a] Mean of four determinations

Figure 20: Cytotoxicity Assays of Verotoxin Producing *E.coli* and *C.jejuni* Using Vero cells and MTT[a]

[a] Mean of four determinations

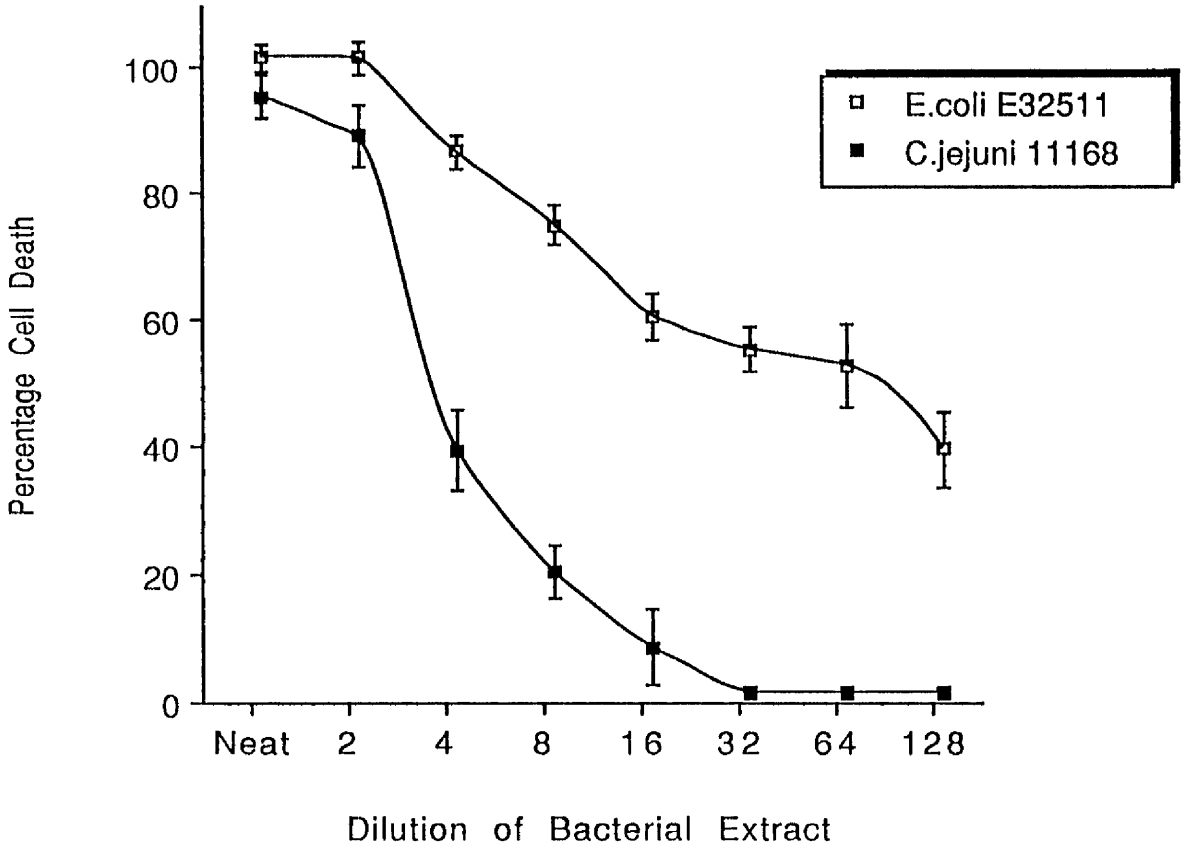

Percentage Cell Death
100
80
60
40
20
0
Neat
2
4
8
16
32
64
128
Dilution of Bacterial Extract
E.coli E32511
C.jejuni 11168

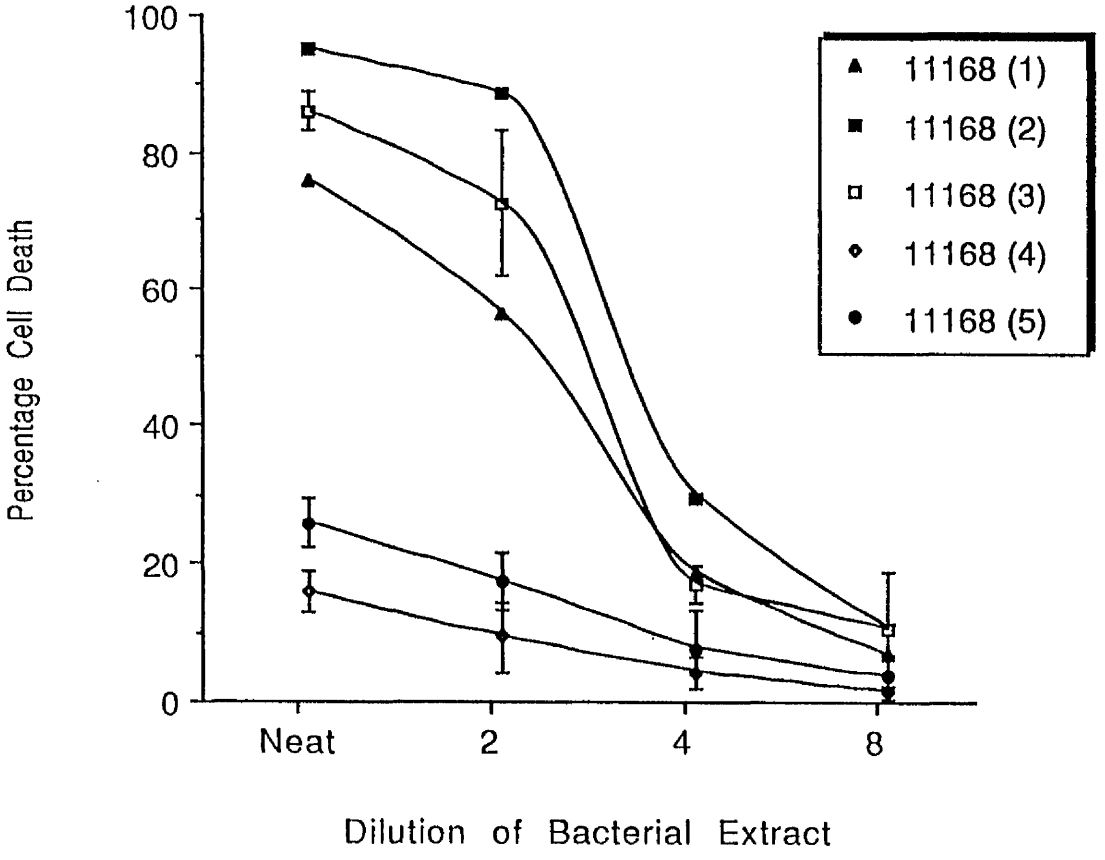
Table 13: Cytotoxicity for HeLa Cells of *C.jejuni* 11168 Culture Supernatants and Polymixin B-Treated Cell Extracts

Bacterial Culture	Dilution of Cell Extract	Percentage Cell Death [a]	Standard Error of Mean
Poly B Treated Cell Extract 24 Hours/BHIB	Neat	74.2	2.0
	1/2	54.5	4.6
	1/4	17.1	8.4
	1/8	5.2	6.5
Poly B Treated Cell Extract 48 Hours/BHIB	Neat	93.6	2.9
	1/2	87.3	2.7
	1/4	28.2	3.2
	1/8	9.1	7.2
Poly B Treated Cell Extract 48 Hours/BHIB + Cys-HCl	Neat	84.6	2.8
	1/2	71.0	10.8
	1/4	15.5	2.7
	1/8	9.1	8.2
Supernatant 48 Hours/BHIB	Neat	14.4	3.2
	1/2	7.9	5.1
	1/4	2.8	2.3
	1/8	0	0
Supernatant 48 Hours/BHIB + Cys-HCl	Neat	24.3	3.6
	1/2	15.8	4.2
	1/4	6.2	5.7
	1/8	2.1	3.1

[a] Mean of four determinations

Figure 21: Cytotoxicity for HeLa Cells of *C.jejuni* 11168 Culture Supernatants and Polymixin B-Treated Cell Extracts[a]

[a] Mean of four determinations



Some standard error bars have been omitted to aid clarity

11168 (1) : polymixin B treated cell extract - 24 hours in BHIB
11168 (2) : polymixin B treated cell extract - 48 hours in BHIB
11168 (3) : polymixin B treated cell extract - 48 hours in BHIB + 6.5 mM Cys-HCl
11168 (4) : supernatant - 48 hours in BHIB
11168 (5) : supernatant - 48 hours in BHIB + 6.5 mM Cys-HCl

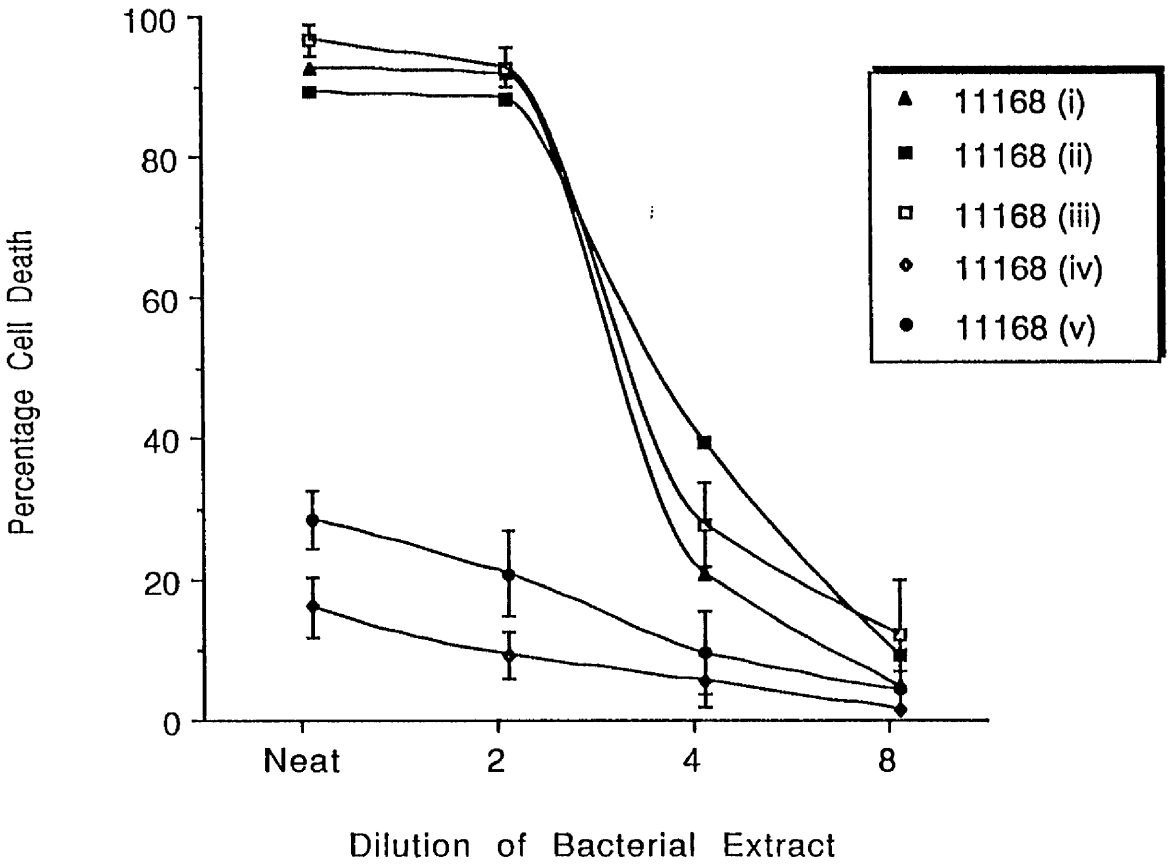
Table 14: Cytotoxicity for Vero Cells of *C.jejuni* 11168 Culture Supernatants and Polymixin B-Treated Cell Extracts

Bacterial Culture	Dilution of Cell Extract	Percentage Cell Death [a]	Standard Error of Mean
Poly B Treated Cell Extract 24 Hours/BHIB	Neat	91.2	2.6
	1/2	90.4	3.7
	1/4	19.3	7.8
	1/8	3.5	3.4
Poly B Treated Cell Extract 48 Hours/BHIB	Neat	87.7	2.8
	1/2	86.8	2.7
	1/4	38.0	3.7
	1/8	7.9	7.4
Poly B Treated Cell Extract 48 Hours/BHIB + Cys-HCl	Neat	95.2	2.1
	1/2	91.2	2.9
	1/4	26.5	5.9
	1/8	10.8	7.8
Supernatant 48 Hours/BHIB	Neat	14.8	4.3
	1/2	7.8	3.5
	1/4	4.1	3.7
	1/8	0	0
Supernatant 48 Hours/BHIB + Cys-HCl	Neat	27.2	4.2
	1/2	19.4	6.1
	1/4	8.1	5.9
	1/8	2.8	2.8

[a] Mean of four determinations

Figure 22: Cytotoxicity for Vero Cells of *C.jejuni* 11168 Culture Supernatants and Polymixin B-Treated Cell Extracts[a]

[a] Mean of four determinations



Some standard error bars have been omitted to aid clarity

11168 (i) : polymixin B treated cell extract - 24 hours in BHIB
11168 (ii) : polymixin B treated cell extract - 48 hours in BHIB
11168 (iii) : polymixin B treated cell extract - 48 hours in BHIB + 6.5 mM Cys-HCl
11168 (iv) : supernatant - 48 hours in BHIB
11168 (v) : supernatant - 48 hours in BHIB + 6.5 mM Cys-HCl

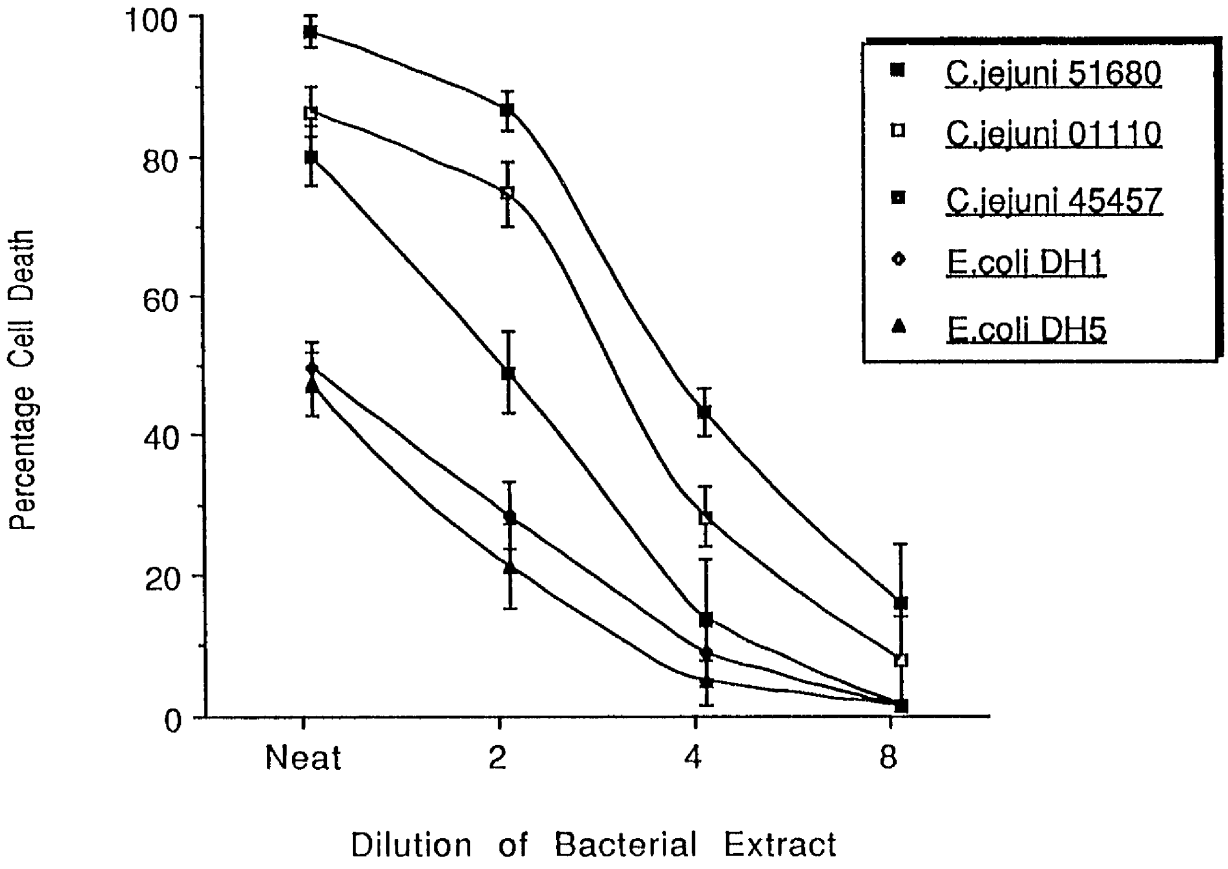
Table 15: Cytotoxicity of Fresh Faecal Isolates of *C.jejuni* and of *E.coli* Genetic Strains DH1 and DH5 for Vero Cells Using MTT as Indicator of Cell Viability

Bacterial Culture	Dilution of Cell Extract	Percentage Cell Death [a]	Standard Error of Mean
C.jejuni 51680	Neat	96.3	2.3
	1/2	85.1	2.8
	1/4	41.7	3.4
	1/8	14.6	8.6
C.jejuni 01110	Neat	84.9	3.6
	1/2	73.1	4.7
	1/4	26.9	4.3
	1/8	6.5	6.2
C.jejuni 45457	Neat	78.6	4.3
	1/2	47.3	5.7
	1/4	12.2	8.6
	1/8	0	0
E.coli DH1	Neat	48.1	3.6
	1/2	27.1	4.7
	1/4	7.4	3.9
	1/8	0	0
E.coli DH5	Neat	45.7	4.6
	1/2	19.8	6.1
	1/4	3.3	3.2
	1/8	0	0

[a] Mean of four determinations

Figure 23: Cytotoxicity of Fresh Faecal Isolates of *C.jejuni* and *E.coli* Genetic Strains DH1 and DH5 for Vero Cells Using MTT as Indicator of Cell Viability [a]

[a] Mean of four determinations


100
80
60
40
20
0
Percentage Cell Death
Neat
2
4
8
Dilution of Bacterial Extract
C.jejuni 51680
C.jejuni 01110
C.jejuni 45457
E.coli DH1
E.coli DH5

Conjugative transfer of the pILL550 shuttle vector from *E.coli* to *C.jejuni* was consistently achieved using the method described in section 2.9. of Materials and Methods, as evidenced by the recovery of colonies on plates containing kanamycin at every position of the multipoint inoculator after mating. These were confirmed as *C.jejuni* by positive reactions to the oxidase and hippurate hydrolysis tests and by Gram stain morphology. Additionally, a representative selection of these colonies was shown to harbour a plasmid of approximately 8.5 kb in size, whilst the original *C.jejuni* 11168 culture was devoid of any extrachromosomal DNA.

All efforts at transposon transfer from *E.coli*-resident delivery vectors pLG221 (Tn*5*), pGS9 (Tn*5*), pSUP2021 (Tn*5*), pNK81 (Tn*10*), pRT291 (Tn*phoA*), pRK404::Tn*5* and pFC1 (Tn*5*) to the *C.jejuni* chromosome were unsuccessful. Experiments with each of these vectors were attempted on at least three occasions.

Similarly, the first two experiments employing the Tn*5* carrying vector pUW964 proved to be unpromising. However, on the third occasion, when the mating mixture was transferrred to plates containing kanamycin, cefoperazone and bacteriophage T_1 (to select for campylobacters which had gained the transposon but to select against the *E.coli* strain), slight bacterial growth was evident at the position of contact of three of the 48 points of the multiple inoculator after microaerophilic incubation at 42°C for 4 days. When sub-cultured onto BA plates containing various concentrations of kanamycin and incubated microaerophilically for three days, no growth was observed at a level of 50 µg/ml, only slight growth was again evident at 25 µg/ml whilst good growth resulted only on plates supplemented with 10 µg/ml of the antibiotic. Colonies were confirmed as *Campylobacter* spp. by the tests detailed above. To determine whether transfer of Tn*5* had indeed occurred into the *Campylobacter* chromosomal DNA, or whether these colonies represented some form of spontaneous mutation or other aberrant phenomenon, total DNA was extracted using the small-scale

chromosomal DNA isolation procedure detailed in section 2.6.4. of Materials and Methods and run into a 0.7% agarose gel. This was then blotted onto Hybond™ nitrocellulose filter and subsequently probed under conditions of high and low stringency with a probe derived from the largest *Hin*dIII fragment resulting after cleavage of pUW964, representing internal sequences of Tn*5*. On the same filter had been included *Hin*dIII and *Sal*I digested pUW964 DNA as well as the large *Hin*dIII fragment itself and *Hin*dIII cleaved DNA isolated from *C.jejuni* 11168. As expected, strong hybridization of the probe was observed to pUW964 DNA fragments containing Tn*5* sequences. However, even after prolonged exposure of up to 20 days at -70°C, no hybridization was noted to DNA from *C.jejuni* 11168 or the three *Campylobacter* clones under investigation.

3.6. Electroporation

Electroporation performed with both 400 ng and 4 µg of pILL550 DNA isolated from *E.coli* DH1 yielded no kanamycin resistant *Campylobacter* colonies. Additionally, no transformants were obtained when attempts were made at electroporation of DNA from various transposon delivery vectors at similar concentrations into *C.jejuni*. Nevertheless, vector DNA molecules were shown to be capable of electroporation-mediated transformation into *E.coli* DH1 cells, as indicated in Table 16.

Electroporation of *C.jejuni* 11168 with shuttle plasmid pILL550 DNA isolated from the same species resulted in a maximum of 1.18×10^4 kanamycin resistant colonies per µg of DNA. No transformants were obtained when sterile TE buffer was used instead of the DNA preparation. An unexpected finding was the isolation of 50 resistant colonies per µg of DNA (mean of two observations) when no pulse had been applied. These were confirmed as *Campylobacter* using the tests detailed above. The results of these experiments are presented in Table 17, whilst Fig. 24 shows the relationship between the number of transformants obtained per µg of DNA, the applied pulse and the time constant.

Table 16: Electro-transformation of *E.coli* DH1 with Transposon Delivery Vector DNA

Vector DNA	DNA Added (ng)	Pulse [a] (Ω)	Number of Transformants per µg DNA
pNK81	400	200	1.3×10^4
pSUP2021	400	200	2.7×10^5
pUW964	400	200	8.9×10^4
pRK404::Tn5	400	200	4.1×10^4

[a] Instrument settings: 25 microfarad capacitor at a field strength of 12.5 kilovolts per cm.

Table 17: Electro-transformation of *C.jejuni* 11168 with pILL550 DNA isolated from the Same Species

DNA Added (ng)	Pulse (Ω) [a]	Time Constant (msec)	Number of Transformants per µg DNA [b]
400	-	-	5.0×10^1
400	100	2.4	1.2×10^4
400	200	4.6	8.0×10^3
400	400	8.7	7.5×10^3

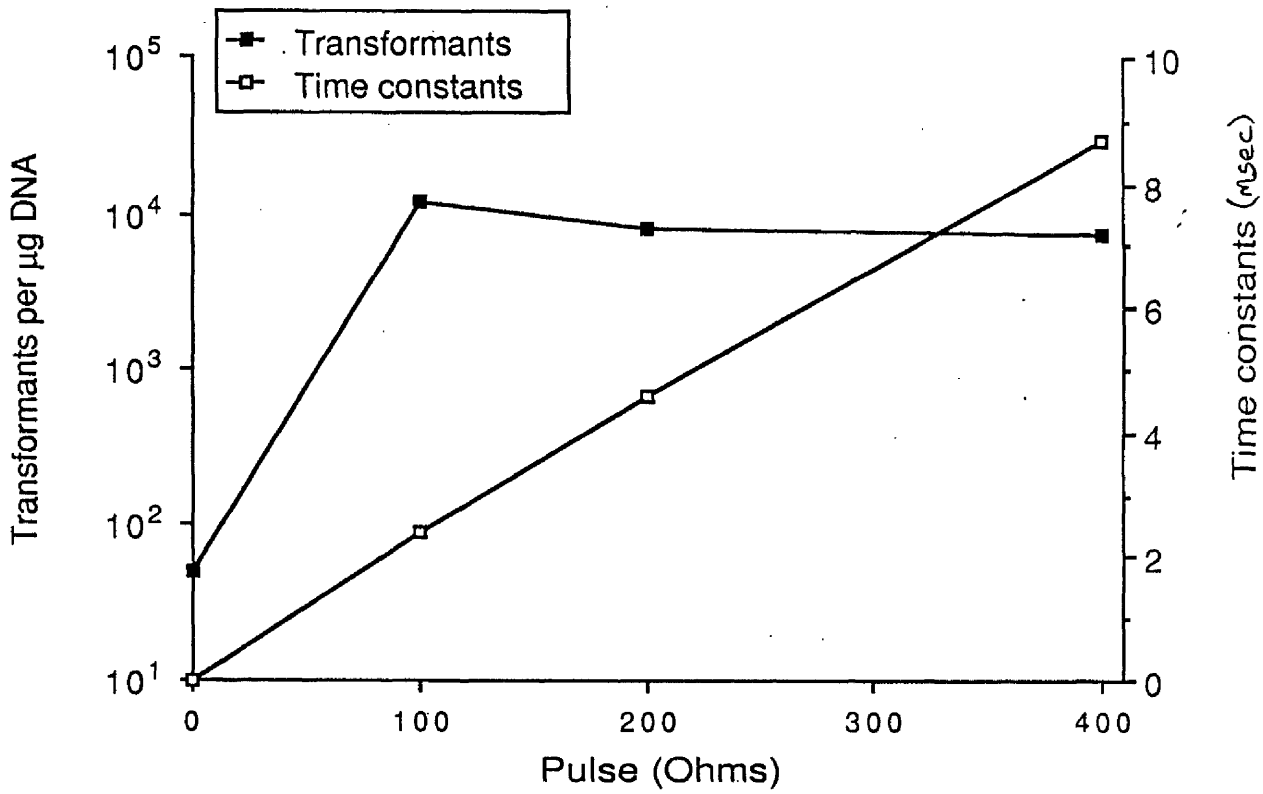
[a] Instrument settings: 25 microfarad capacitor at a field strength of 12.5 kilovolts per cm

[b] Mean of two determinations from two separate experiments

Figure 24: Electro-transforation of *C.jejuni* 11168 with pILL550 DNA isolated from the Same Species [a,b]

[a] Instrument settings:
25 microfarad capacitor at a field strength of 12.5 kilovolts per cm

[b] Means of two determinations from two separate experiments


Transformants
Time constants
Transformants per µg DNA
Time constants (msec.)
Pulse (Ohms)
10^5
10^4
10^3
10^2
10^1
10
8
6
4
2
0
0
100
200
300
400

3.7. Hybridization of *C.jejuni* chromosomal DNA with an oligonucleotide probe

Cleavage of plasmid pJYL2299 with *Pst*I produced two fragments, 5.1kb and 4.4 kb (Fig. 25), the larger of which hybridized strongly with the CT/LT oligonucleotide probe (Fig. 26). DNA sequences within this fragment encode the *E.coli* LT toxin. Samples of *C.jejuni* chromosomal DNA digested with four different restriction endonucleases were also included on this gel.

Initial experiments were performed with a post-hybridization stringent wash at 25°C. At this temperature, several hybridization signals were discernible in each of the lanes containing *Campylobacter* DNA and a high degree of non-specific hybridization was also evident (Fig. 26). Increase of the wash temperature to 37°C demonstrated a marked beneficial effect in that non-specific signals were greatly reduced, making interpretation of hybridization patterns much easier (Fig. 27). Under these conditions, two bands were discernible when DNA had been cut with *Hin*dIII, *Bgl*II and *Eco*RV, although no obvious signal was visible with *Pst*I. A further increase in temperature to 42°C increased specificity further, with only one band now being perceptible in each lane which had previously proved positive, although the strength of the hybridization signal became considerably weaker (Fig. 28).

3.8. Construction of cosmid-based genomic library

3.8.1. Test ligations of dephosphorylated cosmid pHC79 DNA

A preparation of cosmid pHC79 DNA, dephosphorylated with calf intestinal phosphatase and digested with *Bam*HI, was ligated under conditions as described in Materials and Methods section 2.6.15. On this occasion, however, ligation was assessed to both λ and pILL550 DNA cleaved with the same restriction enzyme. The pattern of DNA obtained after electrophoresis of aliquots of these reactions is shown in Fig. 29.

Figure 25: *C.jejuni* 11168 Chromosomal DNA Cleaved with Four Different Restriction Enzymes for Hybridization with the Oligonucleotide Probe

Lane No.	
1	^{32}P-labelled λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)
2	pJYL2299 digested with *Pst*I
3	*C.jejuni* 11168 chromosomal DNA cut with *Hin*dIII
4	*C.jejuni* 11168 chromosomal DNA cut with *Bgl*II
5	*C.jejuni* 11168 chromosomal DNA cut with *Eco*RV
6	*C.jejuni* 11168 chromosomal DNA cut with *Pst*I
7	^{32}P-labelled λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)

(Concentration of agarose used was 0.7%)

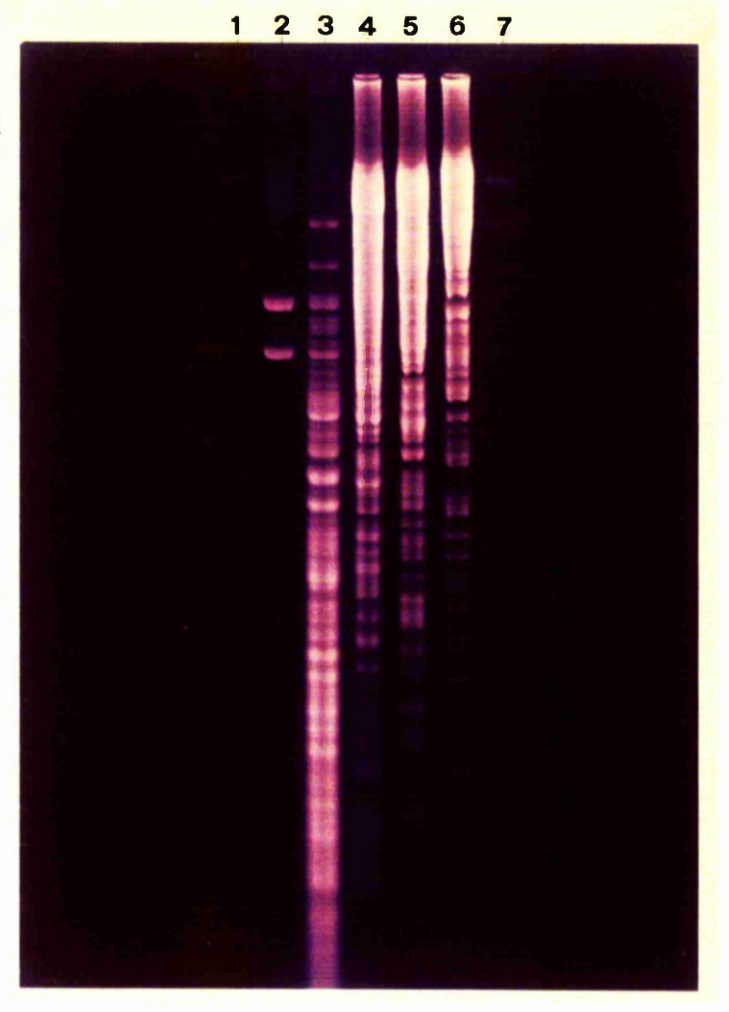

1 2 3 4 5 6 7

Figure 26: Hybridization of *Campylobacter* Chromosomal DNA with Oligonucleotide Probe - 25°C Stringent Wash

(DNA transferred from gel shown in figure 25; autoradiography performed at -70°C for 4 days)

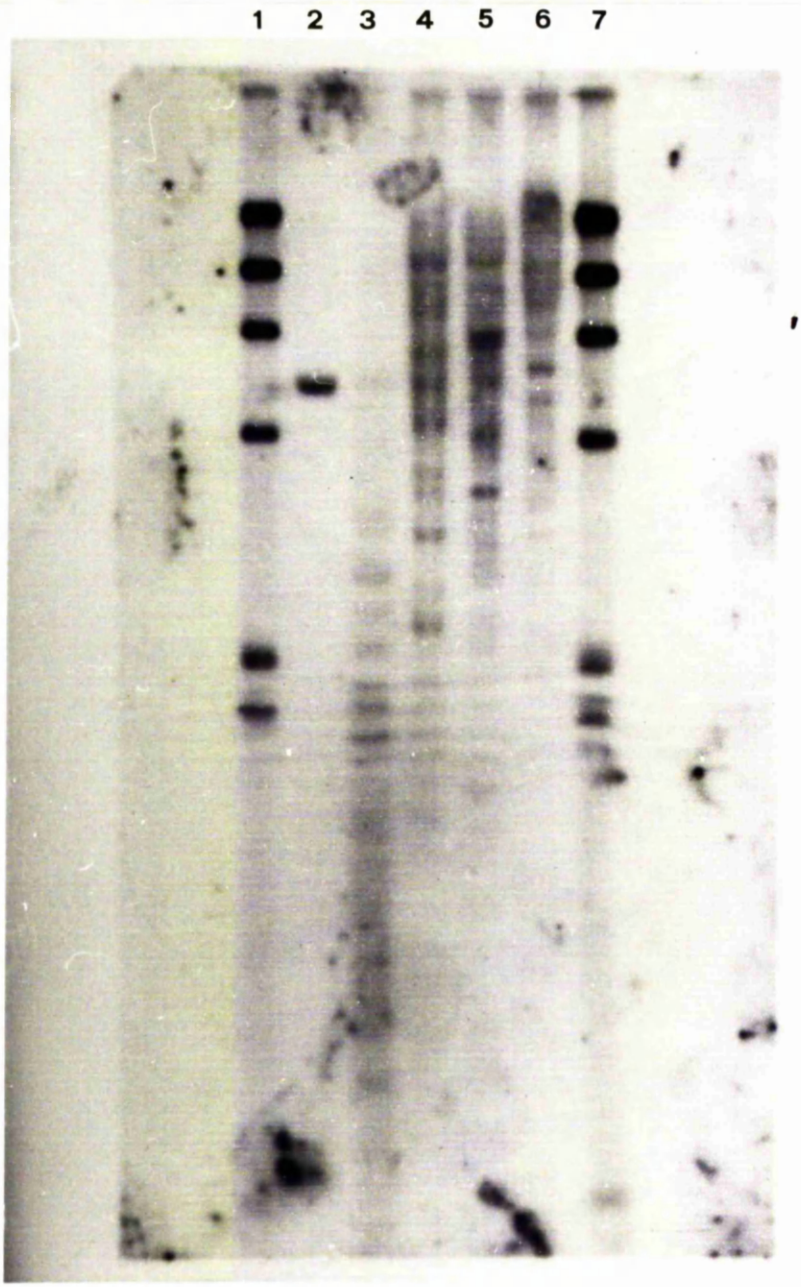

1 2 3 4 5 6 7

Figure 27: Hybridization of *Campylobacter* Chromosomal DNA with Oligonucleotide Probe - 37°C Stringent Wash

(autoradiography performed at -70°C for 4 days)

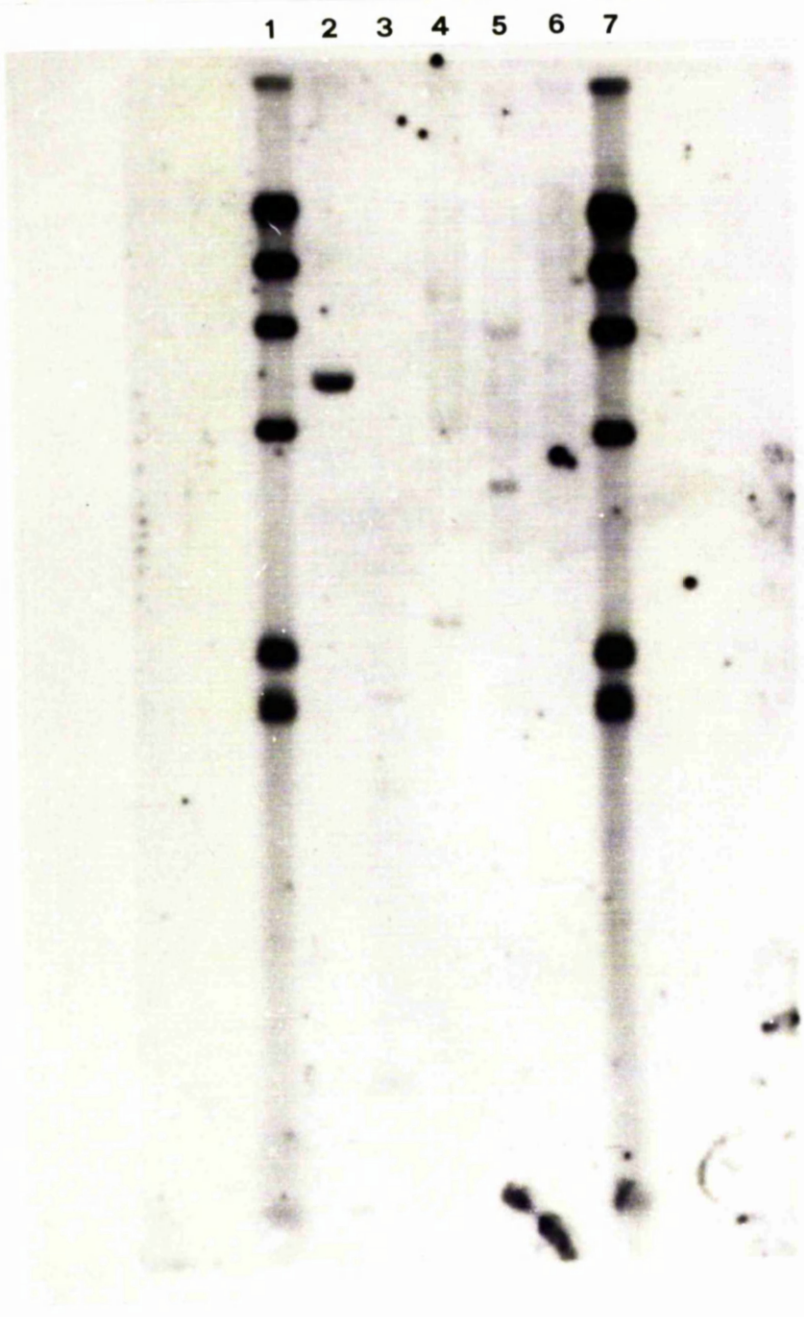
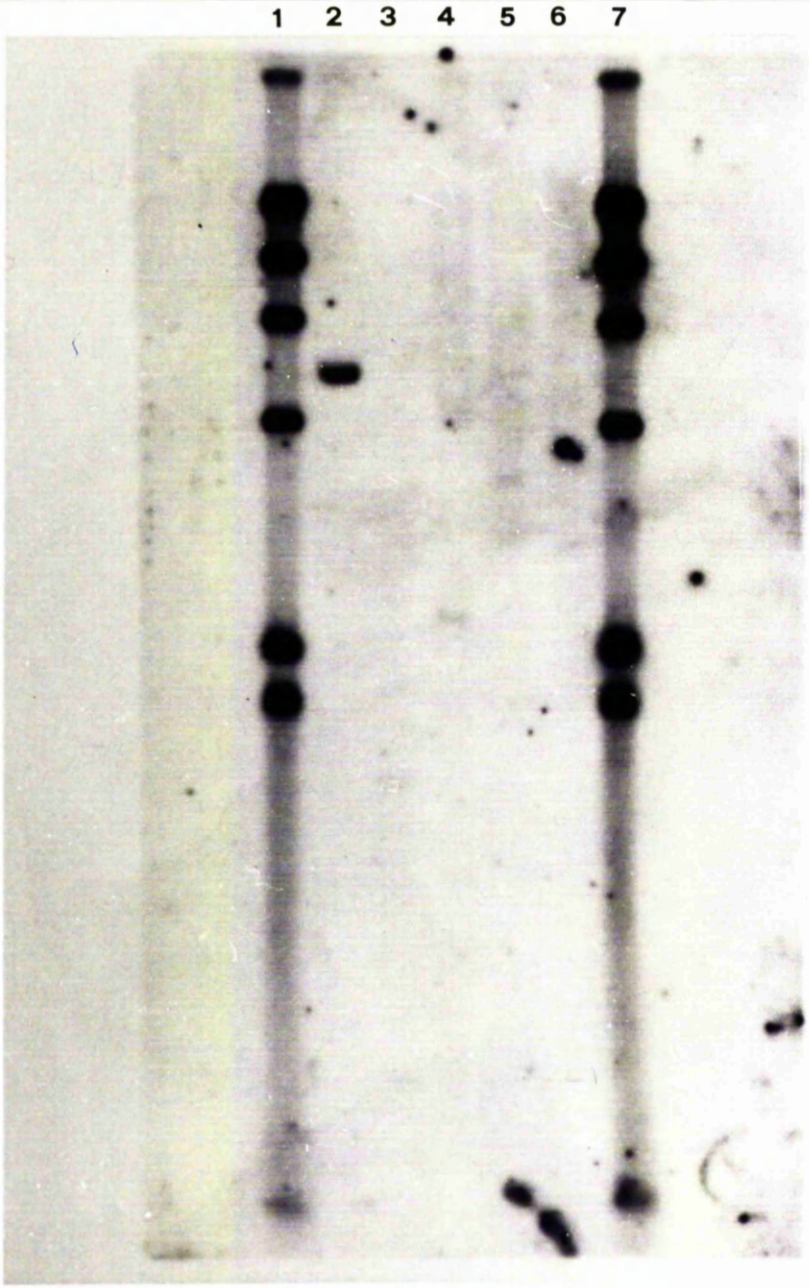

1 2 3 4 5 6 7

Figure 28: Hybridization of *Campylobacter* Chromosomal DNA with Oligonucleotide Probe - 42°C Stringent Wash

(autoradiography performed at -70°C for 4 days)


1 2 3 4 5 6 7

When incubated with T_4 DNA ligase, both λ and pILL550 DNA exhibited higher molecular weight complexes (compare lane 4 with lane 5 and lane 6 with lane 7 of Fig. 29). Complete dephosphorylation of the pHC79 vector was indicated by the total absence of such high molecular weight molecules in the presence of ligase since dephosphorylated 5' ends do not act as substrates for this enzyme, so preventing vector recircularisation (lanes 1 and 2 are identical). Ability of the 3' hydroxyl ends of the vector to ligate to phosphorylated 5' ends of passenger DNA was indicated by the presence in such reactions of recombinant molecules not visualised when either of the component DNAs were incubated separately with ligase. This can be seen by the greater intensity of fluorescence of high molecular weight bands in lane 8 compared with lane 4 (for ligation of pHC79 with λ fragments) and the appearance of extra bands in lane 9 as compared to lane 7 (indicating ligation of vector to pILL550).

3.8.2. Isolation and purification of large DNA restriction fragments

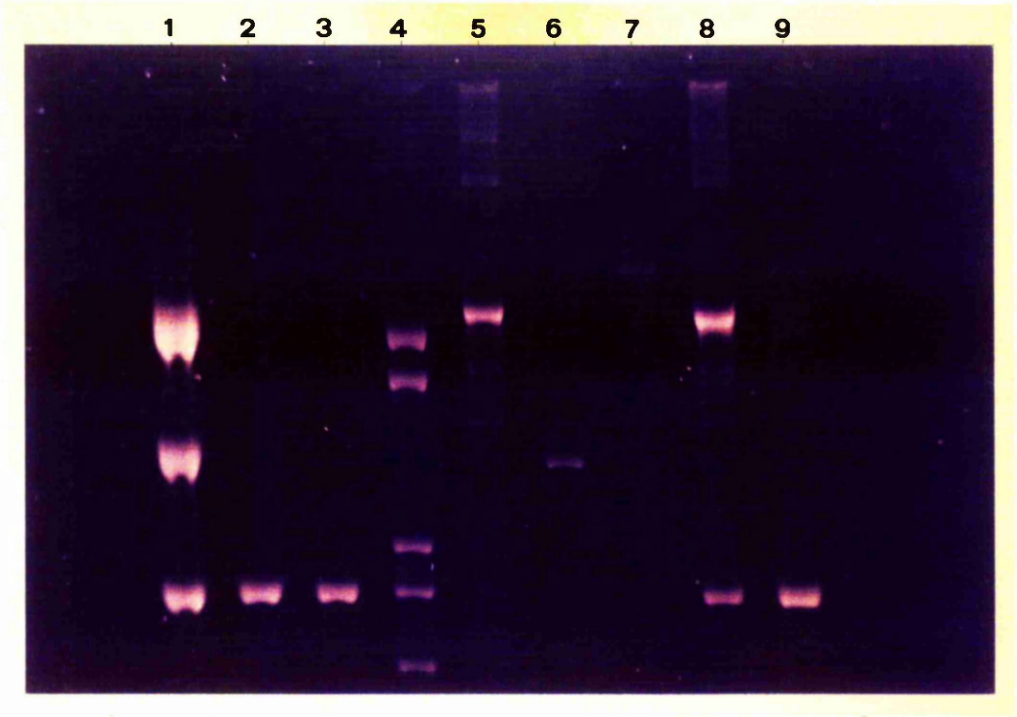
Of the two methods which had been evaluated for efficiency at separation of large DNA fragments, vertical preparative gel electrophoresis proved to be inferior with regard to separation of DNA of the required size range. The BRL apparatus did not allow satisfactory separation of test fragments, in the form of BRL high molecular weight markers, when used under standard conditions specified by the manufacturers. In addition, no benefit was derived when the length of the vertical gel and its agarose concentration were varied or when eluant flow rates and voltages applied during the electrophoresis run were altered.

Conversely, separation of DNA fragments in a 0.4% agarose gel, excision of the region containing the required large DNA fragments and extraction of DNA from the agarose by electroelution into dialysis bags permitted efficient recovery of high molecular weight molecules. Due to its technical ease and good yield of DNA,

Figure 29: Test Ligations of Dephosphorylated Cosmid Vector pHC79

(concentration of agarose used was 0.7%)

Lane No.	Vector and/or Passenger DNA	T_4 DNA Ligase
1	λ *Hin*dIII digest 23.13, 9.416 and 6.557 kb bands	No
2	50 ng *Bam*HI-digested/dephosphorylated pHC79	No
3	50 ng *Bam*HI-digested/dephosphorylated pHC79	Yes
4	50 ng *Bam*HI-digested λ DNA	No
5	50 ng *Bam*HI-digested λ DNA	Yes
6	30 ng *Bam*HI-digested pILL550 DNA	No
7	30 ng *Bam*HI-digested pILL550 DNA	Yes
8	50 ng *Bam*HI-digested/dephosphorylated pHC79 + 50 ng *Bam*HI-digested λ DNA	Yes
9	50 ng *Bam*HI-digested/dephosphorylated pHC79 + 30 ng *Bam*HI-digested pILL550 DNA	Yes


1
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7
8
9

this method was adopted for any subsequent work involving the isolation of large DNA fragments. *Campylobacter* chromosomal DNA fragments in the 32 kb to 45 kb size range produced in the presence of 7.8×10^{-3} units of *Sau*3A per μg of DNA were separated using this procedure.

3.8.3. Packaging efficiency of commercial packaging extracts

An experiment assessing the efficiency of the Stratagene Gigapack® II Plus packaging extract resulted in an average of 104 plaques (mean value from three plates) at a dilution of 10^{-5}. A total of 0.5 μg of wild type *c1857 Sam7* λ DNA had been introduced to the reaction, the total volume of which was 500 μl. Applying the equation presented in Materials and Methods section 2.7.4., the packaging efficiency of the extract was thus determined to be 4.16×10^8 plaque forming units per μg of DNA (pfu/μg). This compared favourably with the 1×10^9 pfu/μg efficiency claimed by the manufacturers.

Efficiency of the Amersham *in vitro* packaging kit was similarly determined to be 3.36×10^8 pfu/μg, with an average of 84 plaques (mean value from three plates) at the 10^{-5} dilution.

3.8.4. Ligation, packaging and transfection

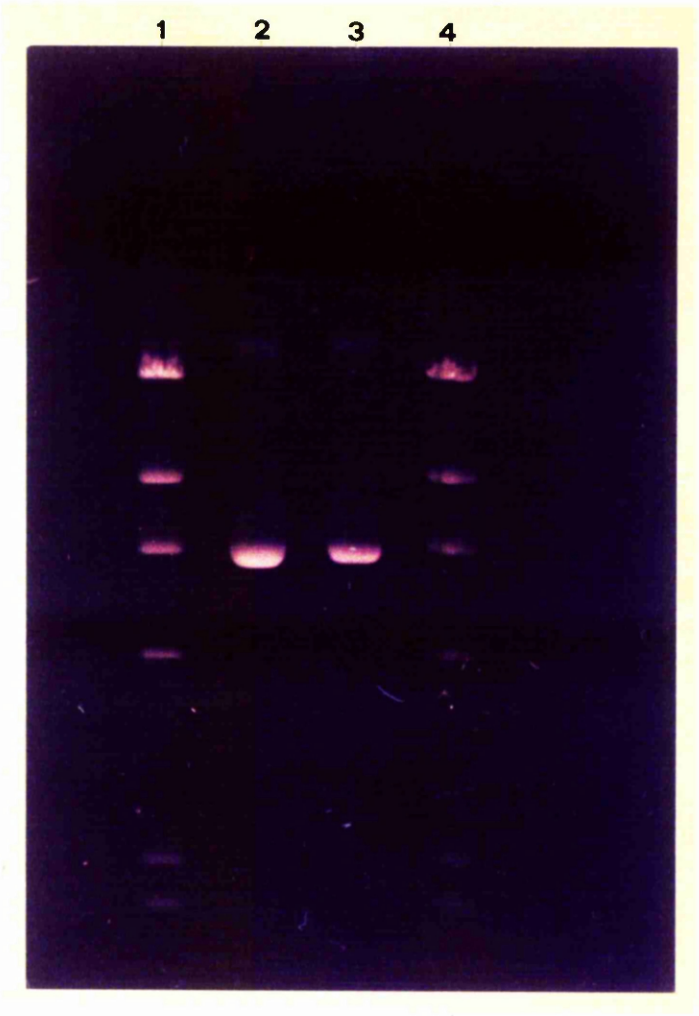
The effects of overnight incubation of dephosphorylated *Bam*HI-digested pHC79 DNA and *Sau*3A-generated *Campylobacter* chromosomal DNA fragments in the presence of T_4 DNA ligase are shown in Fig. 30. A difference is discernible between the reaction mixture before and after addition of the ligase enzyme, with an apparent reduction in the amount of pHC79 and an increase in the size of the high molecular weight species in the latter case.

In preliminary experiments, ligation reactions were packaged using the Amersham *in vitro* kit, employing *E.coli* DH1 as the transfection host organism. No colonies appeared with the negative control where an equal volume of SM buffer replaced the ligation mixture. Under these conditions, and also when the host

Figure 30: *In vitro* Ligation of Cosmid pHC79 and *Campylobacter* Chromosomal DNA Fragments

(concentration of agarose used was 0.4%)

Lane No.	
1	λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)
2	2 μg *Bam*HI/dephosphorylated pHC79 + 0.224 μg *Sau*3A chromosomal DNA fragments
3	Above reaction after overnight incubation in the presence of one unit of T_4 DNA ligase
4	λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)


1
2
3
4

organism was substituted by *E.coli* DH5, very few colonies resulted after plating of the packaged reaction on media containing 65 µg/ml ampicillin (roughly 50 per reaction, or about 0.1 clones per µl of the mix). Small-scale preparations of DNA from these colonies revealed the presence of cosmid/chromosomal fragment ligation products of roughly 48 to 50 kb. However, these clones tended to lose the chromosomal insert after only two or three sub-cultures on antibiotic containing media. One such clone is shown in Fig. 31, where *Pst*I cleaved cosmid DNA isolated before and after three consecutive daily transfers on NA plates containing ampicillin was run in adjacent lanes of a 0.7% agarose gel.

When packaging was repeated using the Stratagene Gigapack® II Plus kit and transfected into an overnight culture of *E.coli* DH5αMCR™ cells, the number of clones per µl of packaged mixture increased to 0.44 (mean of two observations). A similar efficiency was achieved with a 10 h culture of the same strain, resulting in 0.51 clones/µl of reaction (mean of two findings). However, when host cells were used which had been incubated for only 7 h at 37°C, a significant increase was noted in the number of ampicillin resistant colonies obtained (0.85 clones/µl; mean of three experiments). These findings are summarized in Table 18 and presented graphically as Fig. 32.

All subsequent transfection experiments were thus conducted using 7 h cultures of *E.coli* DH5αMCR™. Two large-scale transfection experiments using 100 µl of packaged mixture gave rise to 68 antibiotic resistant colonies each (or 0.68 clones/µl).

A total of 715 clones were obtained after several transfections had been performed. The isolation of extrachromosomal DNA by the small-scale procedure described in section 2.6.1. of Materials and Methods and cleavage with *Pst*I revealed each clone to harbour different recombinant molecules, as reflected by the diverse restriction digeston patterns observed (Fig. 33). The molecular sizes of *Pst*I-generated bands from 10 randomly

Figure 31: Loss of Insert DNA from a Cosmid Clone Prepared Using the Amersham *in vitro* Packaging Kit

(Host organism: *E.coli* DH1; agarose concentration: 0.7%)

Lane No.	
1	λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)
2	DNA isolated from clone prior to sub-culturing
3	DNA isolated from the same clone after three sussessive daily transfers on NA + 65 µg/ml ampicillin

DNA in lanes 2 and 3 was cut with *Pst*I.

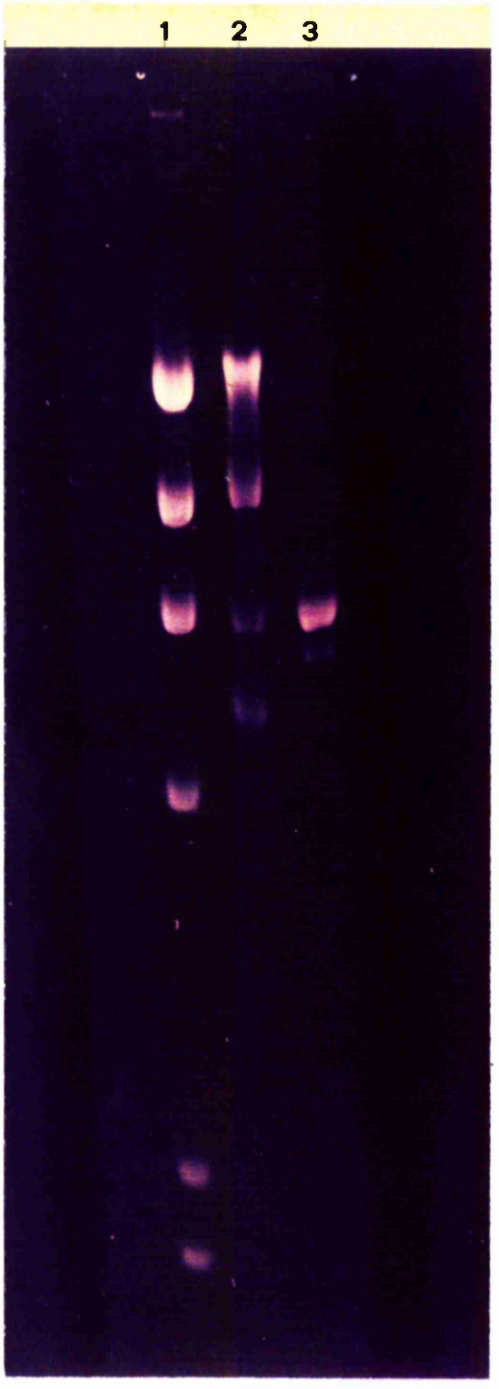

1
2
3

Table 18: Transfection of 16, 10 and 7 Hour Cultures of *E.coli* DH5αMCR™ With Packaged Recombinant Cosmid/Chromosomal Fragment DNA [a]

Age of Host Culture (Hours)	Volume of Packaging Mix Plated (μl)	Number of Clones Obtained	Mean Number of Clones Obtained	Mean Number of Clones per μl
16	50	19	22	0.44
	50	25		(± 0.06)
10	50	28	25.5	0.51
	50	23		(± 0.05)
7	50	48	42.7	0.85
	50	41		(± 0.08)
	50	39		

[a] Packaged using Stratagene Gigapack® II Plus kit

Figure 32: Transfection of 16, 10 and 7 Hour Cultures of *E.coli* DH5αMCR™ With Packaged Recombinant Cosmid/Chromosomal Fragment DNA[a]

[a] Packaged using Stratagene Gigapack® II Plus kit

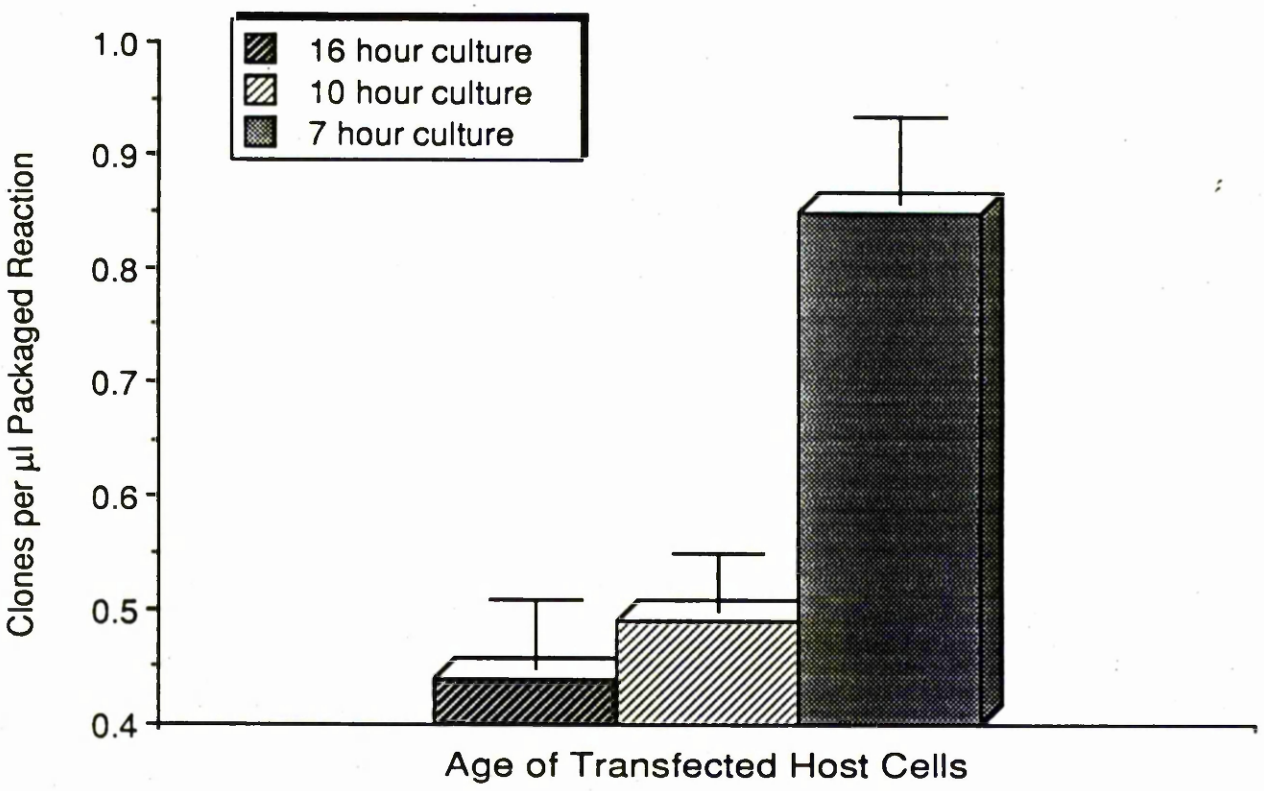

16 hour culture
10 hour culture
7 hour culture
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Clones per µl Packaged Reaction
Age of Transfected Host Cells

Figure 33: Digestion of Recombinant Cosmid DNA from Seven Independent Clones with *Pst*I

Lane No.	
1 & 9	λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)
2 → 8	Cosmid DNA extracted from randomly chosen clones cleaved with *Pst*I

(Concentration of agarose used was 0.7%)

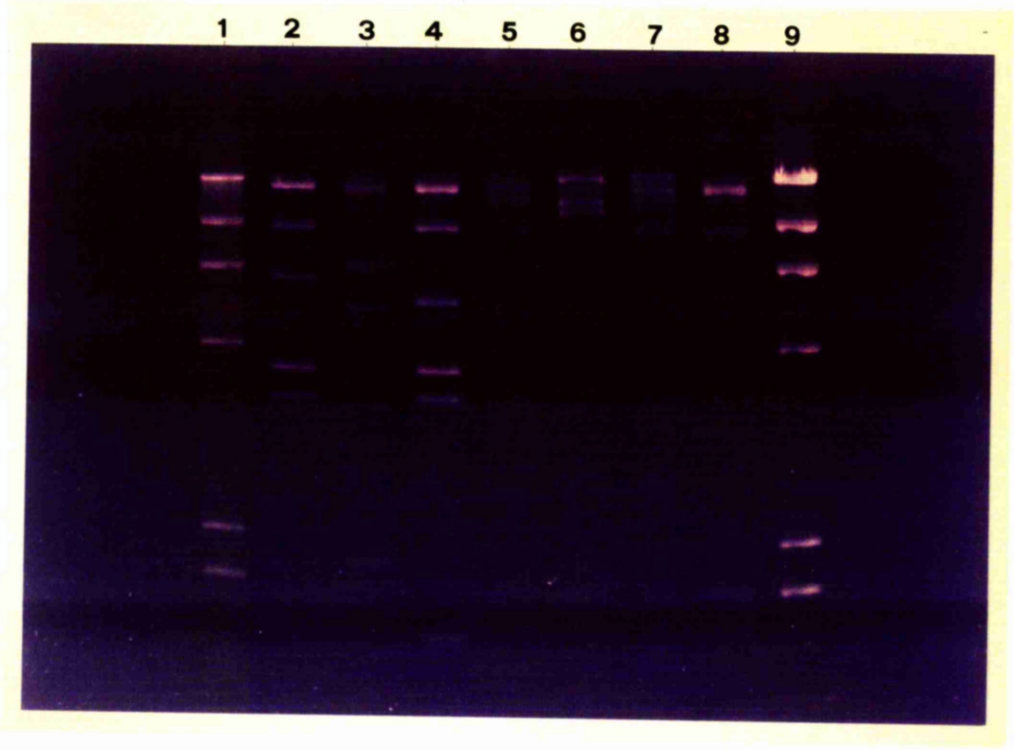

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Table 19: Determination of Total Insert Sizes of Recombinant Cosmids

Clone Designation	Sizes of PstI Fragments (kb)	Total Size of Recombinant (kb)	Total Insert Size (kb) [a]
a	14.45 9.77 6.73 5.25 4.07 3.55	43.82	37.39
b	13.18 8.28 7.21 5.56 4.70	38.93	32.50
c	11.22 9.77 9.12 7.16 5.56 4.07	46.90	40.47
d	14.45 9.55 6.61 6.31	36.92	30.49
e	17.38 15.85 4.27	37.50	31.07
f	16.5 13.9 10.4 2.1	42.9	36.47
g	16.5 8.75 5.9 4.30 4.1	39.55	33.12
h	20.35 17.75 8.45	46.55	40.12
i	25.13 23.0	48.13	41.70
j	19.20 8.45 9.15	36.80	30.37
Mean Values		41.8 kb	35.37 kb

[a] figures obtained by subtraction of size of pHC79 vector (6.43 kb)

chosen clones were determined by procedures as detailed in Materials and Methods section 2.6.9. This data is summarized in Table 19. The mean size of insert fragments harboured by the clones was calculated to be 35.37 kb.

That a particular sequence of interest is represented in a gene library of random chromosomal fragments is dependent upon both the total genome size and the average size of the cloned fragments. In studies with the *E.coli* genome, such a likelihood was determined by Clarke and Carbon (1976) by application of elementary statistical procedures. These authors defined the following relationship:

$$N = \frac{\ln (1-P)}{\ln \left[1 - \left(\frac{I}{G} \right) \right]}$$

Where N is the number of independent clones
P is the probability of a particular sequence being present
I is the average size of cloned fragments
G is the size of the entire genome

The molecular size of the *C.jejuni* genome has recently been determined by pulsed field gel electrophoresis as being 1.721×10^6 bp (Chang and Taylor, 1990). By substituting the available figures into the above equation, the probability of a given sequence being present in the cosmid-based gene library prepared was determined as:

$$715 = \frac{\ln (1 - P)}{\ln \left[1 - \left(\frac{35.37 \times 10^3}{1.721 \times 10^6} \right) \right]}$$

$\therefore$ P = 0.999

Assuming that completely random cleavage had been achieved with *Sau*3A treatment, the likelihood of a given sequence being represented in the gene library was thus greater than 99.9%.

The clones were found to be fairly stable. *Pst*I-generated fragment patterns of cosmid DNA isolated from six independent clones after consecutive sub-culture for five days on NA supplemented with 65 µg/ml ampicillin were identical to the patterns demonstrated by DNA isolated before sub-culture (results not shown).

3.8.5. Screening for the haemolysin gene

All 715 clones were sub-cultured onto BA/Cys-HCl plates, along with the *E.coli* host strain, DH5αMCR™. Haemolysis was not observed on any occasion, even after prolonged incubation of five days. Haemolysis was clearly evident after 72 h incubation around colonies of *C.jejuni* 11168 which had been sub-cultured onto BA/Cys-HCl plates of the same batch as that used to screen the clones.

3.8.6. Screening for the enterotoxin gene

DNA recovered from 48 of the 715 clones hybridized with the CT/LT oligonucleotide probe on both of duplicate filters. These clones are identified in Table 20, together with strengths of duplicate hybridization signals exhibited. Any clones which revealed an apparent positive signal on one filter only were disregarded from further studies. Both *E.coli* DH5αMCR™ harbouring plasmid pJYL2299 and an extracted preparation of pJYL2299 DNA produced strong signals, whilst no hybridization was detectable between the probe and plasmid-free DH5αMCR™ cells. Fig. 34 depicts one of these autoradiographs, showing positive signals to pJYL2299 DNA and to released DNA from one clone, but no

hybridization to the *E.coli* cells alone.

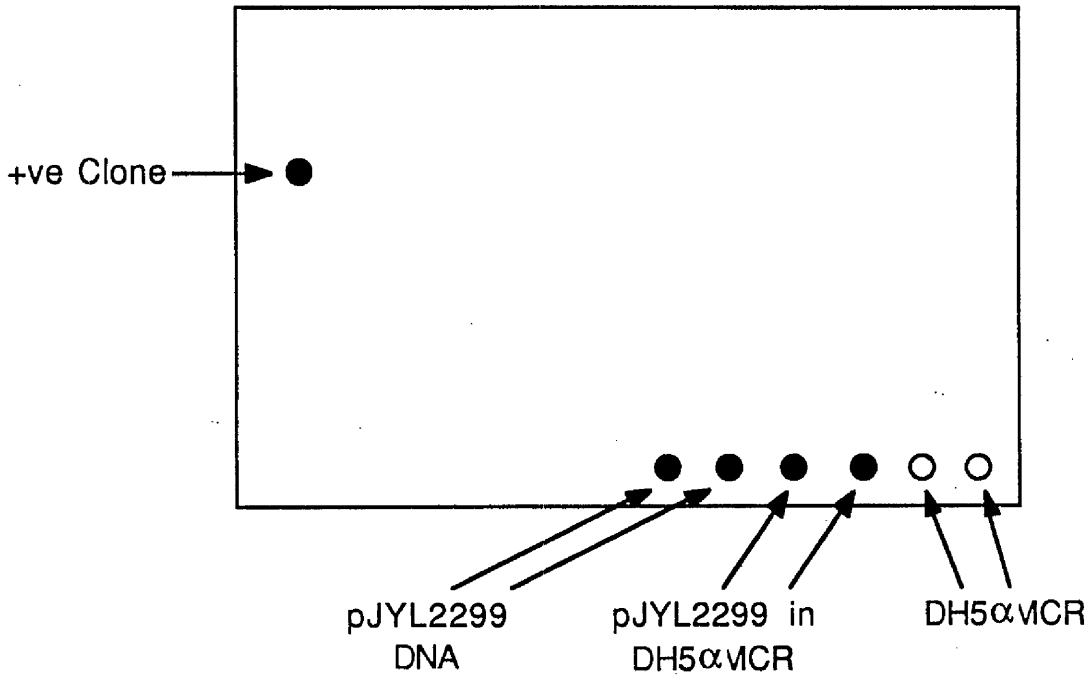
Plasmid DNA isolated by the small-scale protocol from each of these 48 clones and digested with *Pst*I was subjected to electrophoresis through 0.7% agarose. Radioactively labelled *Hin*dIII generated fragments of λ DNA acted as molecular size markers. Also included on the same gel were *Pst*I cleaved plasmid pJYL2299 and *Bam*HI digested cosmid pHC79. DNA from the gel was transferred to Hybond-N™ nitrocellulose membrane by capillary transfer and hybridized with the oligonucleotide probe using procedures detailed previously. Subsequent exposure of the membrane to X-ray film for ten days revealed the presence of one faint band in each of the lanes occupied by clones 89, 118, 330, 362 and 474. These bands appeared to be of differing molecular sizes. A schematic representation of the results is presented in Fig. 35 along with a photograph of the actual blot in Fig. 36.

3.9. Plasmid-based gene library

A total of 1420 clones were obtained by ligation of *Sau*3 A fragments of *Campylobacter* chromosomal DNA with *Bam*H I digested shuttle vector pILL550 after transformation into *E.coli* DH1. Plasmid DNA isolated from five randomly chosen clones and digested with the restriction enzymes, *Pst*I and *Eco*RI, revealed insert DNA fragments of varying sizes. Fragments of 4.8 kb and 3.4 kb represented vector DNA and were common to all samples (Fig. 37). The molecular size of each restriction fragment was determined as described in section 2.6.9. of Materials and Methods. This data is detailed in Table 21. The mean size of total insert DNA was calculated in this manner to be 8.64 kb. Clones did not lose insert fragments after five daily passages on antibiotic containing media.

The probability of a particular sequence of interest being represented in this library was calculated by reference to the relationship given above:

Figure 34: Hybridization of DNA Released from Clones of Cosmid-Based Library with the CT/LT Oligonucleotide Probe



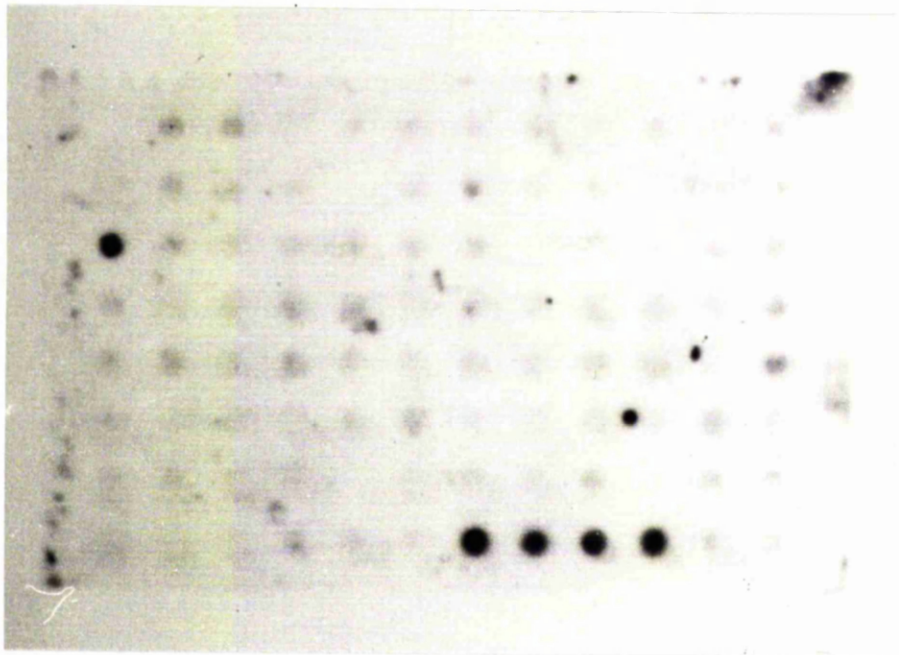


Figure 35: Schematic Representation of Hybridization of Extracted Cosmid DNA from Presumptive Positive Clones with CT/LT Oligonucleotide Probe

(Photograph is presented as Figure 36)

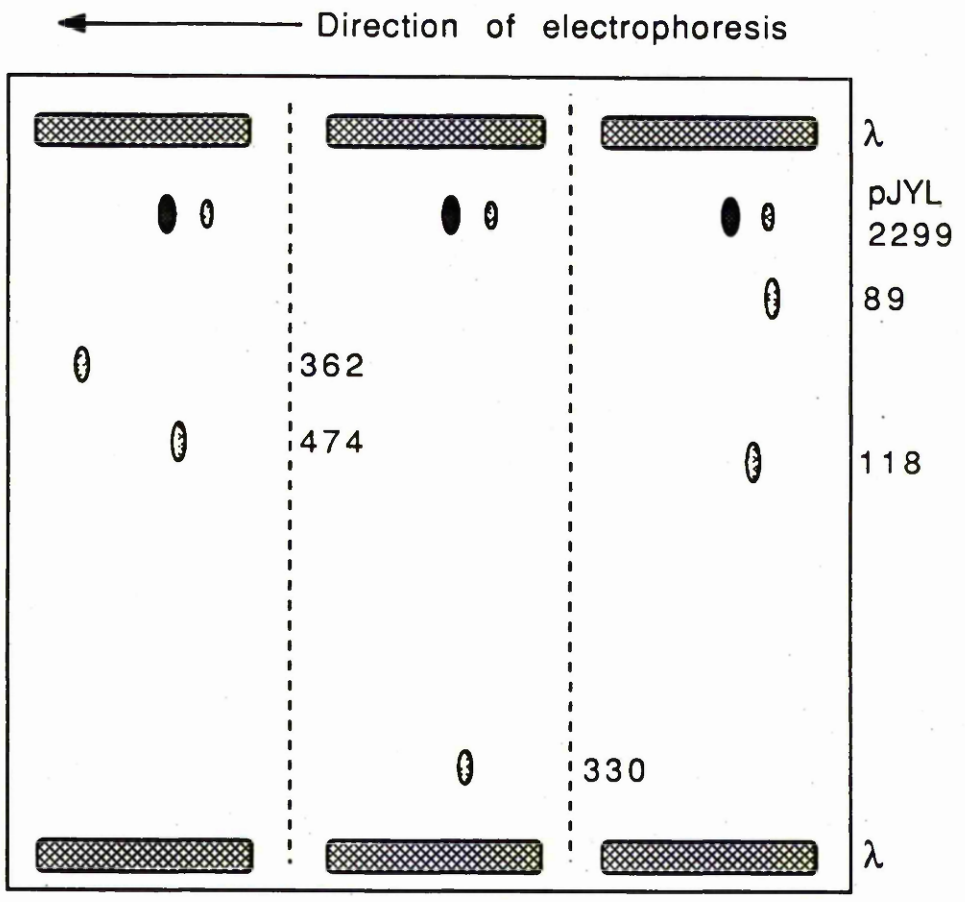

Direction of electrophoresis
λ
pJYL
2299
89
362
474
118
330
λ

Figure 36: Hybridization of Extracted Cosmid DNA from Presumptive Positive Clones with CT/LT Oligonucleotide Probe

Arrows point to proposed regions of hybridization.

(Schematic representation is presented as Figure 35)

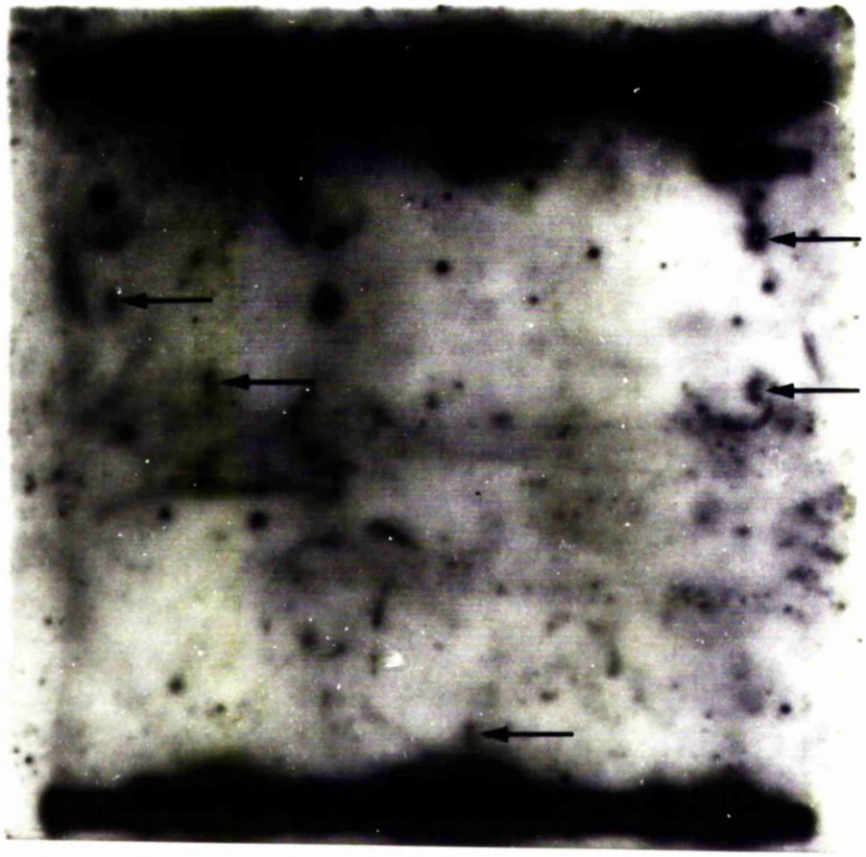


Table 20: Clones from Cosmid-Based Library Demonstrating Positive Hybridisation with CT/LT Oligonucleotide Probe on Duplicate Filters

Clone Number	Hybridisation Results for Duplicate Samples [a]	Positive on Re-blotting
69	± . ±	
89	± . ±	Yes
93	± . +	
97	± . ++	
118	± . ++	Yes
125	++ . ±	
145	++ . ±	
160	+ . +	
181	± . +	
183	++ . ±	
193	++ . ±	
198	± . ++	
199	+ . ±	
201	+ . ++	
209	± . ++	
210	+ . ±	
214	± . ++	
224	± . +	
225	+ . ±	
240	++ . ±	
247	+ . +	
254	± . ++	
259	± . ++	
265	++ . ±	
273	± . +	
275	+ . +	

(Table continued overleaf.....)

Table 20 (continued)

Clone Number	Hybridisation Results for Duplicate Samples [a]	Positive on re-blotting
286	+ . ±	
291	± . ++	
298	+ . +	
324	± . ++	
330	± . +	Yes
337	+ . ±	
340	± . +	
342	++ . ±	
350	+ . ++	
362	± . ++	Yes
379	± . ±	
474	± . ±	Yes
483	+ . ±	
498	± . +	
503	± . ±	
505	± . +	
507	+ . +	
515	± . ±	
529	± . ±	
585	± . ±	
612	+ . ±	
654	± . ±	

[a] Strengths of hybridisation signals were graded according to the following scheme:

± : weak positive signal

+ : good positive signal

++ : strong positive signal

$$1420 = \frac{\ln (1 - P)}{\ln \left[1 - \left(\frac{8.64 \times 10^3}{1.721 \times 10^6} \right) \right]}$$

$$\therefore P = \underline{0.999}$$

The completeness of the library was thus established, assuming that cleavage of the chromosomal DNA had occurred in a totally random fashion.

3.9.1. Screening of the plasmid-based library for haemolysin gene

All clones were transferred to BA plates containing 6.5 mM Cys-HCl. Whilst haemolysis was noted on plates inoculated with *C.jejuni* 11168 after 72 h incubation at 42°C, no such effect was observed around growth of any of the clones, even after 7 days at 37°C.

Transfer of fresh sub-cultures of all clones onto Hybond-N™ membranes, lysis with chloroform and re-testing for haemolysis again proved unsuccessful. There was, however, no means to assess whether lysis of the *E.coli* cells had occurred to a satisfactory degree by this procedure.

3.10. Construction of a shuttle cosmid

Of 48 kanamycin resistant colonies resulting after transformation of *E.coli* JM83 with the pIII550/*cos* fragment ligation products, two were found to harbour recombinants of about 11 kb, the others containing only recircularised vector molecules. Extrachromosomal DNA isolated from the two positive clones was digested separately with *Bam*HI and *Eco*RI and run in a 0.7% agarose gel, together with pILL550 cleaved with the same endonucleases. Fig. 38 depicts a photograph of this gel. When cleaved with both enzymes, pILL550 appeared as a single 8.5 kb fragment; the

Figure 37: Digestion of Recombinant Plasmid DNA from Five Independent Clones of pILL550-Based Library with *Pst*I and *Eco*RI

Lane No.	
1	λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)
2 → 6	Plasmid DNA extracted from randomly chosen clones cleaved with *Pst*I and *Eco*RI

(Concentration of agarose used was 0.7%)


1
2
3
4
5
6

Table 21: Determination of Total Insert Sizes of Recombinant Plasmids from pILL550-Based Library

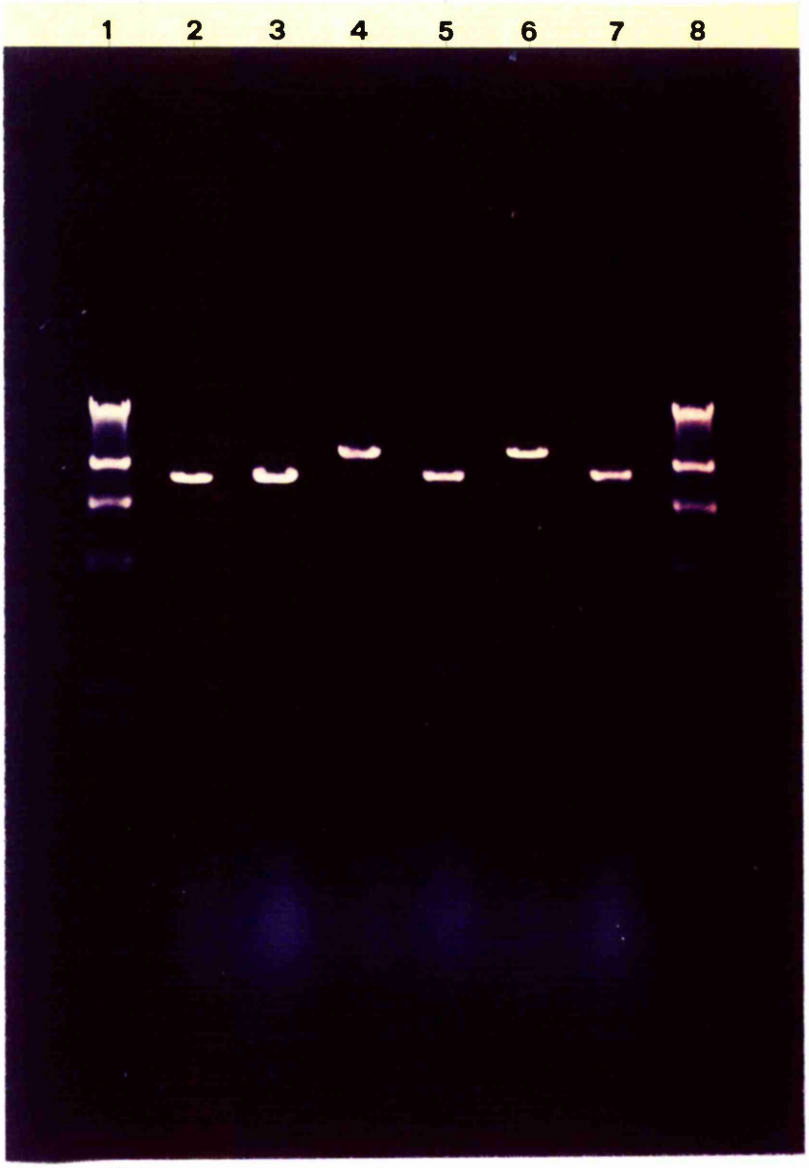
Clone Designation	Sizes of Fragments (kb)	Total Size of Recombinant (kb)	Total Insert Size (kb) [a]
(i)	4.8 3.4 3.2 2.9 1.9 1.6	17.8	9.3
(ii)	4.8 3.4 2.9 2.1 2.0 1.5	16.7	8.2
(iii)	4.8 4.2 3.4 1.74 1.66	15.80	7.3
(iv)	6.4 4.8 3.4 2.7 2.4	19.7	11.2
(v)	4.8 4.2 3.4 3.3	15.7	7.2
Mean Values		17.14 kb	8.64 kb

[a] figures obtained by subtraction of size of pILL550 vector (8.5 kb)

recombinants demonstrated two fragments when cut with *Eco* RI, of 8.5 kb and 2.6 kb, with a total molecular size of 11.1 kb (as seen in samples digested with *Bam*HI). Thus, the *cos* containing fragment derived from pBTI-1 had successfully been inserted into the *Eco* RI site of pILL550. This recombinant was designated as shuttle cosmid pTMA1.

Figure 38: Digestion of pILL550/*cos* Fragment Recombinants with *Eco*RI and *Bam*HI

Lane No.	
1	λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)
2	pILL550 digested with *Bam*HI
3	pILL550 digested with *Eco*RI
4	Recombinant No. 1 cut with *Bam*HI
5	Recombinant No. 1 cut with *Eco*RI (bands are visible at 8.5 kb & 2.6 kb)
6	Recombinant No. 2 cut with *Bam*HI
7	Recombinant No. 2 cut with *Eco*RI (bands are visible at 8.5 kb & 2.6 kb)
8	λ *Hin*dIII digest (23.13, 9.416, 6.557, 4.361, 2.322 & 2.027 kb fragments)


1
2
3
4
5
6
7
8

4. DISCUSSION

4.1. *Campylobacter* haemolysin

In Bergey's Manual of Determinative Bacteriology (1974), Smibert described colonies of *C.jejuni* growing on blood agar as being nonhaemolytic. This was the generally accepted view and no evidence emerged to challenge it until 1985, when Fricker *et al.* presented their findings, at the Third International Workshop on *Campylobacter* Infections, of a thermostable non-lipopolysaccharide substance released by *C.jejuni* strains which demonstrated a hot-cold lysis phenomenon on horse erythrocytes when tested by a tube method. The following year, the same group of investigators (Park *et al.*, 1986) reported that attempts to clearly show haemolysis of sheep or cattle erythrocytes on agar media had been unsuccessful, even when synergistic attack on red blood cells, as described by Christie *et al.* (1944; otherwise referred to as the CAMP reaction), had been attempted. During the same year, McCardell *et al.* (1986), reported that they had distinguished two different factors cytotoxic for CHO cells, one of which lysed rabbit erythrocytes. This latter toxin was determined to be heat labile, sensitive to the action of trypsin and was neutralised by antibodies raised against the cytolysin of the non-01 serogroup of *V.cholerae*. No further reports emerged from this group concerning either of the factors which they had described.

During the period when the experimental work reported here was being performed, therefore, no detailed evidence for a *Campylobacter* haemolysin had been presented as a peer-reviewed article. Recently, however, Arimi *et al.* (1990) formally reported the findings of their investigations of the haemolytic activity of *Campylobacter* spp. on blood agar plates, stating that clearing on such medium inoculated with *C.jejuni* had frequently been observed in their laboratory. They reported that incubation at 42°C for four days allowed them to visualise appreciable erythrocyte lysis around *Campylobacter* growth. Indeed, a similar observation that weak and very indisinct haemolysis could sometimes be distinguished around colonies of *Campylobacter* spp. on sheep erythrocyte-supplemented

agar media had inspired these present studies. However, this author was unable to prove the reproducibility of the phenomenon under these conditions.

The addition of L-cysteine hydrochloride to blood-containing agar media to enhance or induce haemolysin production by *Campylobacter* spp. has not previously been reported. A group of closely related cytolysins, the so-called oxygen-labile haemolysins, are known to be activated by compounds containing the thiol group. Bernheimer and Avigad (1970) determined that, in the case of streptolysin O, several compounds were capable of providing this particular group, including reduced glutathione, sodium thioglycolate, dithiothreitol, 2-mercaptoethanol and cysteine. Other such factors are elaborated by *Streptococcus pneumoniae* (pneumolysin), *Clostridium tetani* (tetanolysin) *Clostridium botulinum* types C and D (botulinolysin), *Bacillus cereus* (cereolysin) and *Listeria monocytogenes* (listeriolysin), as reviewed by Bernheimer (1976). In all of these cases, addition of the appropriate reducing agent to a wholly or partially purified preparation of the toxin led to activation. Thus, although the use of cysteine has been well documented for the activation of cytolytic proteins, no reports of its addition to blood containing agar media for this purpose were encountered by this author.

It would be very attractive to presume that the haemolysin produced by *C.jejuni* was of the same group as these oxygen-sensitive toxins, although the only indication for this would be the involvement of cysteine in the apparent activation, or enhancement of activity, of this factor. Other reducing agents, including glutathione and dithiothreitol, were not able to reproduce the effect. In addition, this particular collection of toxins is produced only by Gram-positive bacteria, and even then by genera which are closely related at the taxonomic level (Bernheimer, 1976).

Addition of Cys-HCl at a concentration of 6.5 mM to agar-containing medium supplemented with 7% sheep blood had a striking effect and this is well depicted in Figures 13 and 14. After three

days incubation at 42°C, very well defined zones of haemolysis could be seen in the presence of Cys-HCl, though no haemolysis was visible on the same basal medium and blood combination in its absence. This was of variance with the report of Arimi *et al.* (1990) who were apparently able to detect clearing on normal blood agar plates after three days. Their medium consisted of Heart Infusion (Difco) to which had been added 1.2% agar. They found Oxoid blood agar base to be unsatisfactory for their investigations, speculating that that this base might contain inhibitory substances or lack essential ingredients. In the study presented here, however, this particular basal medium was used routinely and found to consistently support haemolysis after Cys-HCl supplementation. In fact, of several commercially available agar bases used, only one, Difco Brucella agar, proved to be unsatisfactory for this purpose. Upon the others, including BHI agar, tryptone soy agar, Brucella agar, diagnostic sensitivity test agar (all Oxoid) and Columbia agar (Difco), lysis of red blood cells was only noted once Cys-HCl had been added. Reproducible detection of clearing was not observed on any occasion in its absence.

Of the various compounds tested, L-cysteine hydrochloride was unique in possessing the ability to promote haemolysis. Other reducing agents, like dithiothreitol, glutathione and 2-mercaptobenzothiazole did not reproduce the effect. The same was true of cystine, the oxidised form of cysteine, and methionine, another sulphur-containing amino acid. Structurally related compounds, like the ethyl and methyl esters of cysteine (including the hydrochloride salt of the latter ester) were also inert in this respect, as was L-S,S' methylenebiscysteic acid.

Sheep erythrocytes were found to be sensitive to the *Campylobacter* lysin both in this study and by Arimi *et al.* (1990). There was also agreement on the fact that washing of erythrocytes to eliminate any inhibitory substances was unnecessary. Nevertheless, whilst the group of Arimi *et al.* described horse erythrocytes to be equally sensitive, the study presented here

determined red cells of equine origin to be completely unaffected by the haemolysin. The spectrum of activity upon a range of erythrocytes from various birds and mammals revealed an interesting pattern. All were insensitive to the effects of the *Campylobacter* lysin except sheep, ox and goat cells. Notably, included in the list of resistant species was blood from the rabbit, which had been reported as the indicator cells by McCardell *et al.* (1986) when they had described factors cytotoxic for CHO cells, as discussed above.

Arimi *et al.* (1990) could distinguish lysis of cells at 42°C only, with no effects at 37°C, except with one strain of *C.jejuni* out of 50 tested. Under conditions used in this present study, however, lysis was demonstrable under both conditions, although increased incubation was necessary at the lower temperature.

The finding that only erythrocytes from ruminant species appeared to be sensitive to lysis was interesting in conjunction with the observation that, on occasions, the extent and intensity of haemolysis increased upon retention of BA/Cys-HCl plates at room temperature or 4°C for several hours after incubation at 42°C. Indeed, in some cases, the only manifestation of a haemolytic tendency was a zone of green discoloration around *Campylobacter* growth; progression to full clearing could be achieved by either continuing incubation at 42°C or by transferring the plate to 4°C for 24 h.

It was suggested that these facts pointed to a similarity with the β-lysin elaborated by *S.aureus*. Various investigators have noted the specificity of action of this toxin for certain erythrocyte species. Sheep and ox erythrocytes were found to be universally sensitive to lysis whilst much reduced activity, or no activity at all, was demonstrable upon cells from other species such as rabbit, guinea pig and man (Wiseman and Caird, 1967; Wiseman, 1970; Bernheimer, 1974). Bernheimer *et al.* (1974) reported that goat erythrocytes were lysed to an equal extent as those from the ox, although Wiseman (1970) and Wiseman and Caird (1967) reported

goat cells to be less sensitive. The membranes of ox, sheep and goat red blood cells all contain high levels of sphingomyelin, accounting for roughly 50% of the total phospholipids present (Bernheimer, 1974; Bernheimer *et al.*, 1974) whilst having very little lecithin (Van Deenen and De Gier, 1964). In fact, the staphylococcal β-lysin is often also referred to as a sphingomyelinase or sphingomyelin choline phosphohydrolase. The β-lysin exhibits a phenomenon referred to as "hot-cold" lysis, whereby haemolysis is absent or incomplete at 37°C but proceeds rapidly when the reaction is cooled to 4°C (Wiseman, 1970; Easmon and Goodfellow, 1990). Wadström *et al.* (1974) considered that the hot-cold lysis phenomenon of erythrocytes from ruminant species was a direct consequence of the high sphingomyelin content, in agreement with Wiseman's assertion (1970) that contraction of the membrane at a lowered temperature increased the circumference of perforations, thereby allowing haemoglobin to leak out. It was later suggested that stabilization of the red cell membrane by divalent cations was temperature dependent and this may have been responsible for the observed phenomenon (Smyth *et al.*, 1975).

Wiseman (1970) reported that optimal activity upon sheep cells occurred in the presence of 1 mM to 10 mM Mg^{2+} and Co^{2+}, with Zn^{2+} ions being inhibitory at the same concentrations. No such relationship was found with the *Campylobacter* haemolysin, although it must be realised that all of the work reported with *S.aureus* β-toxin was performed with purified or partially purified preparations; the complex mixture of ingredients present in BA plates would not allow an accurate assessment of metal ion requirements of a lysin released directly from the bacterial cell.

Antiserum prepared against the β-lysin reduced the zone of haemolysis around growth of *C.jejuni* to a greater extent than that demonstrated by normal rabbit serum. However, this was not substantiated at the genetic level since hybridization was not achieved, even under conditions of reduced stringency, between *Campylobacter* chromosomal DNA and a probe specific for the

staphylococcal β-lysin gene.

Haemolysins have been implicated as virulence factors in several Gram-positive and Gram-negative bacterial species, for instance, *E.coli* (Hughes *et al.*, 1983; Waalwuk *et al.*, 1983; Cavalieri *et al.*, 1984), *Serratia* (König *et al.*, 1987; Poole and Braun, 1988), *S.pneumoniae* (Walker *et al.*, 1987), *Vibrio parahaemolyticus* (Honda *et al.*, 1976), *Aeromonas hydrophila* (Thelestam and Ljungh, 1981; Stelman *et al.*, 1986) and *Shigella* (Clerc *et al.*, 1987; Kato *et al.*, 1989).

All strains of *C.jejuni*, *C.coli* and *C.jejuni /coli* available in this study were found to elaborate a diffusible haemolysin when tested on BA/Cys-HCl plates. This does not, however, detract from the possibility that the *C.jejuni* lysin may be a potential virulence factor, since all strains had been isolated from cases of human or animal disease. A parallel situation exists with the intracellular pathogen, *Listeria monocytogenes*, all virulent strains of which were reported to produce an extracellular haemolysin (Geoffroy, *et al.*, 1987; Kuhn *et al.*, 1988), whilst nonhaemolytic transposon-generated mutants were avirulent in a mouse infection model (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987). It would be worthwhile to determine whether haemolysin production is a feature of other *Campylobacter* species known to cause diarrhoeal disease, like *C.laridis* (Tauxe et al., 1985; Simor and Wilcox, 1987; Borczyk *et al.*, 1987), *C.fetus* subspecies *futus* (Harvey and Greenwood, 1983), *C.concisus* (Vandamme *et al.*, 1989), *C.cryaerophila* (Tee *et al.*, 1988), *C.cinaedi* and *C.fennelliae* (Totten *et al.*, 1985). Also of interest would be to ascertain whether *Campylobacter* species not associated with human or animal disease are able to lyse red blood cells, such as the various biovars of *C.sputorum* (Roop *et al.*, 1986), the saprophytic free-living species described by Laanbroek *et al.* (1977) and *C.nitrofigilis,* found in association with plant roots in salt marshes (McLung and Patriquin, 1980; McLung *et al.*, 1983).

The work of Lebek and Gruening (1985) with *Shigella* spp.

and investigations with *E.coli* by Clerc *et al.* (1987) suggested that a relationship might exist between the haemolysins formed by these organisms and stimulation of bacterial growth, probably through an increase in the level of available iron in the environment due to erythrocyte lysis, as has been indicated by other authors (Linggood and Ingram, 1982; Waalwijk *et al.*, 1983). L-cysteine may have been having the effect of reducing available iron levels in the growth medium, thereby activating or inducing the production of a haemolysin from *Campylobacter* cells so as to release iron from erythrocytes. However, the effect of Cys-HCl addition to BA plates could not be reproduced by several agents known to have metal-binding properties. Although the compounds were all capable of retarding bacterial growth, this could not definitely be attributed to chelation of iron only, since these agents also have the capacity to bind other metal ions, one or several of which may have been required for *Campylobacter* cell multiplication. The addition of exogenous iron to BA plates did not induce a haemolytic response. More importantly, however, addition of iron to BA/Cys-HCl plates did not abolish or even appreciably reduce the extent of haemolysis observed as compared to a control plate not supplemented with iron. Thus, it seems unlikely that haemolysin production and/or release is regulated by levels of iron in the environment external to the bacterial cell.

Assay of the haemolysin proved to be extremely difficult. Exhaustive efforts to demonstrate lysis of sheep blood or washed sheep erythrocytes by *C.jejuni* culture supernatants and cell extracts using conventional tube or microdilution methods were completely unsuccessful. Towards the end of these studies, however, a procedure was developed which allowed a demonstration of haemolytic activity from cell extracts. This protocol used extracts of sonicated cells in an agar medium originally described for the *V.cholerae* haemolysin (Richardson *et al.*, 1986). The levels of the *Campylobacter* lysin detected in this manner were low. Certainly, the magnitude of the haemolytic reaction produced by *C.jejuni* growing on BA/Cys-HCl plates was in no way comparable

to that observed on non-supplemented BA medium inoculated, for example, with strains of *Streptococcus pyogenes* or *S.aureus*. Nevertheless, low levels of production do not necessarily preclude this factor from a possible contribution towards virulence. A similar case exists with the haemolysin produced by *Gardnerella vaginalis,* which Kretzschmar *et al.* (1991) recently promoted as having an important role in the pathogenicity of this organism.

The role of L-cysteine in promoting the haemolytic activity of *C.jejuni* thus still remains to be elucidated. Cysteine levels up to 44 μM (0.54 mg/100 ml of venous plasma) have been reported in the blood plasma of normal adults (Brigham *et al.*, 1960) so this amino acid is available in the bloodstream. The utility of a capacity to lyse erythrocytes may not be immediately evident for an organism associated primarily with diarrhoeal disease. However, *C.jejuni* has also been reported in connection with several extraintestinal infections, including meningitis (Thomas *et al.*, 1980), urinary tract infection (Davis and Penfold, 1979) and cholecystitis (Darling *et al.*, 1979). In addition, transient bacteriaemia is a noteworthy finding with *Campylobacter* enteritis (Blaser *et al.*, 1986; Guerrant *et al.*, 1978; Kasten *et al.*, 1991).

4.2. *Campylobacter* cytotoxin

An investigaton into cytotoxin production by *C.jejuni* was undertaken primarily to establish whether this might prove promising as a phenotype for which to screen a genomic library. Several reports of *C.jejuni* cytotoxic factors have appeared since 1983, many of which were often contradictory in terms of target cell specificities and the effects of antibodies raised against toxins formed by other bacterial genera. A more detailed account of these various reports has already been presented in the Introduction section and will not be reproduced here.

One further report emerged whilst these studies were underway of a *Campylobacter* toxin whose activity could be neutralised by rabbit anti-Shiga toxin and by monoclonal antibody to

the B subunit of Shiga-like toxin I of *E.coli* (Moore *et al.*, 1988). *E.coli* produces two phage-encoded but antigenically distinct cytotoxins, referred to as Shiga-like toxin type I (synonyms: SLT-I; Verotoxin type 1; VT1) and Shiga-like toxin type II (synonyms: SLT-II; Verotoxin type 2; VT2), with antiserum against purified Shiga toxin derived from *Shigella dysenteriae* 1 completely inhibiting the cell-damaging properties of SLT-I but not SLT-II, as described by Scotland *et al.* (1985), Strockbine *et al.* (1986) and Karmali *et al.* (1986).

Low levels of cytotoxicity were found in 21 out of 36 *C.jejuni* and *C.coli* strains examined by Moore *et al.* (1988), of which 52% were capable of neuralization with the monoclonal or rabbit antibodies. The authors presented several observations suggesting that the *Campylobacter* Shiga-like toxin may not play a role in the disease process, including the fact that some strains producing this factor had been isolated from patients without overt symptoms of infection. Other strains clearly associated with an inflammatory-type diarrhoea did not produce any cytolethal factor. Also of concern to them was the low overall level of toxin production.

All efforts to detect homology under low stringency conditions between total DNA from a *C.jejuni* strain producing Shiga-like toxin and a probe encompassing the structural genes for SLT-I were unsuccessful (Moore *et al.*, 1988). This was in agreement with the report of Seriwatana *et al.* (1988) who similarly were unable to demonstrate hybridization of SLT-I and SLT-II with DNA from thirty *C.jejuni* isolates from Thailand.

The findings of Moore's group were at complete variance with those of other researchers who had previously attempted neutralisation studies with antibodies against Shiga or Shiga-like toxin, including Goossens *et al.* (1985), Guerrant *et al.* (1987) and Johnson and Lior (1988). In all these cases, pre-treatment of either the target cells or the *Campylobacter* toxin preparation with antiserum could not abolish the cytolethal effects observed. This

illustrates further the complexity and contradictory nature of the current literature concerning the cytotoxins formed by *C.jejuni*.

As was alluded to above, the studies presented here were not designed to determine the proportion of cytotoxin positive strains available, nor to characterise a *Campylobacter* cytotoxic factor. Of interest was whether such a toxin could reproducibly be detected by a method adaptable for the purpose of screening a library of random genomic sequences. The protocol employing trypan blue for detection of cell death, as used by Guerrant *et al.* (1987), proved to be very laborious since each well of the test had to be viewed individually under an inverted microscope. This was considered impractical and unsatisfactory considering that a plasmid-based gene library would consist of roughly 1500 clones (at a 99.9% probability of a particular sequence being represented). A method was sought, therefore, which would allow the processing of a number of samples simultaneously and which could be performed in 96-well microtiter plates so that a degree of automation could be achieved by reading the results in a multiwell spectrophotometer. These advantages were clearly fulfilled with the protocol described by Mosmann (1983). This was a colorimetric assay using a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to assess viability. Tetrazolium salts are particularly useful for this purpose since the tetrazolium ring is cleaved only by active mitochondrial dehydrogenase enzymes present in living cells (Slater *et al.*, 1963). MTT, a pale yellow substrate, was cleaved by all metabolically active cells but not by dead cells or erythrocytes to yield a dark blue formazan product (Mosmann, 1983).

The results indicated that the MTT procedure, when used with appropriate modifications, was a useful technique in determining cytotoxicity. Problems were encountered initially when the protocol was performed exactly as described by Mosmann due to the fact that the crystals of formazan end product were difficult to solubilise in isopropyl alcohol. This problem was

ascribed to the precipitation of serum proteins from the medium after addition of the organic solvent and was overcome by Denizot and Lang (1986) through use of serum-free media and pure isopropanol. The two cell lines used in these present studies, however, grew optimally in the presence of 10% foetal calf serum. Although it was possible to reduce the concentration of exogenous serum added to the growth medium, this would have had to be performed in a stepwise fashion, lowering the serum content very gradually, and would necessarily have taken some considerable time to achieve. An alternative was sought, and was provided by the modifications reported by Carmichael *et al.* (1987), who used the MTT technique to assess chemosensitivity in established lung cancer and Chinese hamster cell lines. These researchers used DMSO as a solubilizing agent for the formazan crystals, and their protocol was found to be reliable when used with HeLa and Vero cells.

Once the suitability of the modifications proposed by Carmichael's group was established, the assay system was evaluated using a known cytotoxin-positive organism, *E.coli* E32511, a strain producing Verotoxin 2, tested against the Vero cell line. Reprodicible results were obtained, both with replicate assays performed on the same day and when the assay was repeated on another occasion. The final readings were performed with a Titertek Multiscan MC photometer, results of the scan of a whole 96-well plate being obtained in a fraction of the time previously required with the trypan blue dye exclusion technique.

C.jejuni NCTC 11168 and three other *C.jejuni* strains which had recently been isolated from cases of human gastroenteritis were then tested. These latter organisms had been sub-cultured on laboratory media a minimum number of times before being stored at -70°C. The rationale for such caution derived from the finding by Pang *et al.* (1987) that the capacity for cytotoxin production diminished considerably with repeated sub-culture, with all activity being lost after the eighth passage of the strain on artificial medium. All isolates were tested within four weeks of

storage. This was prudent with regard to the later report by Daikoku *et al.* (1989) that much reduced levels of cytotoxic activity were detectable after prolonged storage of strains.

With the NCTC strain, titers between 2 and 4 were consistently obtained. Of the fresh isolates, *C.jejuni* 51680 exhibited the highest titer of 4; the other two strains, *C.jejuni* 45457 and *C.jejuni* 01110, were active at culture extract dilutions of $^1/_2$ and between $^1/_2$ and $^1/_4$ respectively. All had been tested with the Vero cell line. No significant difference in cytotoxic response had been noted between the Vero and HeLa lines when incubated with extract from the 11168 strain. Vero cells were used in most experiments merely because a positive control, in the form of *E.coli* E32511, was available for this cell line. Addition of Cys-HCl to the *Campylobacter* growth medium did not enhance cytotoxin production in the same manner as had been observed for the haemolysin. This was true of both polymixin B extracts and sonicates.

Low level of cytotoxin formation by *C.jejuni* was a common feature of most articles on the subject. For instance, Yeen *et al.* (1983) reported cytopathic effects at a filtrate dilution of $^1/_8$ in human but not animal cell lines from eight out of eleven strains examined. Similarly, Pennie *et al.* (1984) could only demonstrate cell death at a titer of four in 50% of campylobacters tested. Using HeLa cells, Guerrant *et al.* (1987) noted reciprocal titers of roughly two with eleven isolates (after allowance had been made for the cell-damaging activity of a control containing polymixin B alone), although one strain, C31, did demonstrate activity at a titer of eight. In CHO cells, six out of nine of the same cultures were inactive in relation to the negative control, whilst two had titers of about two. *C.jejuni* C31 produced a cytolethal effect at a dilution of $^1/_{32}$.

Further improvements to the MTT protocol might conceivably have increased sensitivity of the assay and thereby led to higher observed cytotoxin titers. Carmichael *et al.* (1987) considered that

optimal conditions should be determined for each cell line employed in terms of the number of eukaryotic cells used per well and the duration of the incubation period with the toxin preparation. The authors contended that control cells without exposure to toxin should be in the exponential phase of growth at the moment when the assay was processed. In the studies presented here, however, a fixed number of cells was used with a set period of incubation. By serially increasing cell numbers in adjacent columns of a 96-well microtiter plate, for example, a response curve relating the number of cells per assay well to the optical density of formazan product could be prepared. This would be useful in ascertaining the optimal cell concentration for each line.

The monolayer of cells in each well was left in contact with the *Campylobacter* toxin preparation for 24 h. This might not have been adequate time to allow cell death and subsequent loss of dehydrogenase activity. A *C.jejuni* extract known to exhibit cytolethal effects could be incubated with an optimal number of cells, determined as suggested above, for varying periods of time before processing the assay. A maximal period of three to four days might be possible before replenishment of cell nutrients would become necessary.

Moore *et al.* (1988) doubted the importance in the disease process of the Shiga-like cytotoxin described, as related above. This view has been shared by various other authors, including Cover and Blaser (1989), who proposed that *Campylobacter* enteritis could not be a solely toxin-mediated complaint since this factor was not demonstrable in a large percentage of clinical isolates and because an anti-toxin antibody response was absent in all patients suffering from inflammatory diarrhoea. Walker *et al.* (1986) were also unable to recognise a definitive role for toxin production as a virulence characteristic. Similar conclusions must be reached after reviewing the results of cytotoxicity testing performed in this study. Athough only a very small number of strains had been examined, all were associated with human disease. Low cytotoxin

titers could not be ascribed to continuous passage upon laboratory media or prolonged storage of strains, since all possible precautions had been taken to prevent this. Naturally, the possibility cannot be discounted that current conditions used for bacterial growth, extraction of cytolethal factors and assay for cellular damage may be inadequate for the *in vitro* determination of *C.jejuni* toxin activity.

4.3. Physical detection of enterotoxin production

As with the cytotoxin, enterotoxin production was considered a putative virulence factor suitable for screening a gene library. Again, however, this was dependent upon the availability of a method for assessing enterotoxic activity which could be adapted for testing a large number of clones.

Daikoku *et al.* (1990) stressed that the enterotoxin elaborated by *C.jejuni* (CjT) related strongly to the pathogenesis of this organism. Others have been more cautious, noting the production of this factor without being able to recognise an unequivocal role in the disease process (Newell, 1984; Walker *et al.*, 1986; Lindblom *et al.*, 1989). No correlation was discovered, for example, between the levels of enterotoxin formed by strains isolated from both symptomatic and asymptomatic children living in southern India (Mathan *et al.*, 1984).

Ruíz-Palacios *et al.* (1983) determined that the *Campylobacter* enterotoxin was similar in physiological function to cholera toxin (CT) and *E.coli* heat-labile toxin (LT). All three factors share several common properties. These include susceptibility to inactivation by elevated temperatures (Johnson and Lior, 1984; Walker *et al.*, 1986), induction of electrolyte and fluid accumulation in ligated ileal loops of the rat (Klipstein and Engert, 1984; Klipstein *et al.*, 1986) and rabbit (McCardell *et al.*, 1984) and finally a correlation of such events with elevation of intracellular cAMP levels associated with increased adenylate cyclase activity (Daikoku *et al.*, 1990).

At the time when the studies reported here were initiated, three methods were available for detection of the *Campylobacter* enterotoxin, all of which had been adapted from procedures originally used for CT and LT. Undoubtedly the most laborious involved the isolation of segments of the ileum of a rat or rabbit with ligatures, each segment subsequently being injected separately with the toxin preparation and appropriate controls. Twenty four hours after closing the abdomen, during which time neither food or water was allowed, the animals were sacrificed and the volume of fluid aspirated from each segment carefully measured. Ligated ileal loop techniques of this type, as applied to a number of gastrointestinal pathogens, have been reviewed in detail by Bergdoll (1988). This procedure would, of course, be completely impossible when the testing of greater than one thousand clones was to be performed.

Another method involved the detection of toxin-mediated morphological changes in cultured eukaryotic cells. Many investigators have used monolayers of the CHO cell line, in which cytototonic toxins induced elongation of the cells (Klipstein and Engert, 1984a; McCardell *et al.*, 1984; Lindblom *et al.*, 1989; Fendri *et al.*, 1991). A positive effect upon Y-1 mouse adrenal cells was demonstrated as rounding of the cells, though Johnson and Lior (1984) found this test to be clearly inferior to that employing CHO cells. Similar drawbacks as have been discussed above for determination of cytotoxicity by trypan blue exclusion were envisaged also for the use of tissue culture procedures in the detection of a cytotoxic effect from a large population of *E.coli* clones.

The functional similarities between CjT and both CT and LT have been substantiated also by immunological relatedness. A number of authors, including McCardell *et al.* (1984), Klipstein and Engert (1984a; 1985) and Ruíz-Palacios *et al.* (1985) described the inhibition of physiological effects on CHO cells by pre-incubation of the CjT preparation with anti-CT or anti-LT antiserum. The ELISA

protocol described by Klipstein *et al.* (1986), based upon such observations, could conceivably have been considered as an assay system suitable for screening an extensive gene bank. Two potential shortcomings were recognised. Firstly, some of the necessary reagents, like the solid phase ganglioside GM_1 and alkaline phosphatase-conjugated goat IgG antiserum, were expensive, and would have become prohibitively so when testing several hundred clones. More importantly, however, a source was not available for substanial quantities of the second antibody required, rabbit anti-LT antiserum.

At the time when these studies were conducted, a kit manufactured by the Japanese company, Denka Seiken Ltd., and marketed by Oxoid Ltd., became available commercially (VET-RPLA kit). This had been designed for the assay of CT and LT in culture supernatants and cell extracts using uniform latex particles sensitized with purified antibodies against CT. The close immunological relationship shared by CjT, CT and LT meant that this kit should therefore also have been able to detect CjT. Such a test was considered ideal for library screening purposes, being extremely fast and simple to perform and having the added advantage that reagents were readily available and stable when stored as directed. In addition, the kit was received pre-packaged with a positive control in the form of dried *V.cholerae* enterotoxin which merely required reconstitution before use. This not only provided verification of the reactivity of the sensitized latex particles, but also provided a visual reference for patterns of agglutination which classified the results from negative to strongly positive, as illustrated in the kit protocol. A negative control was also included, consisting of a latex suspension coated with non-immune rabbit globulins.

However, although positive and negative controls behaved exactly as expected, enterotoxin was not detected in culture supernatants, or in polymixin B-treated and sonicated extracts of the *C.jejuni* NCTC 11168 strain. In addition, no positive reaction

was achieved with three fresh clinical isolates. Incubation for 24, 48 and 72 h, with or without supplementation of the medium with Cys-HCl, gave similar results. This was considered inconsistent with previous findings of functional and immunological similarities between CjT and CT. Several reasons may have accounted for these negative results. Firstly, Denka Seiken Ltd. state the sensitivity of the VET-RPLA test to be 1-2 ng/ml, so that if levels of CjT in the samples were below this level, they would not have been detectable. Unfavourable growth conditions might have led to little or no production of enterotoxin, although these conditions were no different to those employed by investigators who had previously reported detection of CjT by other methods (as reported above). Nevertheless, Wadstrom *et al.* (1983) noted that *C. jejuni /coli* isolates from Sweden apparently did not form an enterotoxin, while a more recent report by Perez-Perez *et al.* (1989) also found that enterotoxin was absent from all 22 American isolates studied. Variations in reported incidences of CjT production by different laboratories may be ascribed to low levels of expression *in vitro* or to the diverse assay and growth conditions employed.

Although the VET-RPLA kit proved unsuitable as a method for the detection of *Campylobacter* enterotoxin in these studies, further evaluation of its potential should still be considered. For instance, production of CjT could be assessed in various liquid growth media as well as from cells harvested from BA and BA/Cys-HCl plates. In addition, a larger number of isolates should also be tested, in conjunction with an established procedure for toxin detection, like the CHO cell elongation method. Whilst the studies presented here were in progress, Scotland *et al.* (1989) reported an evaluation of the VET-RPLA test kit for detection of the heat-labile toxin of *E.coli.* These authors showed that the kit correctly identified 50 strains of *E.coli* known to produce LT-I, whilst no reaction was demonstrated with a further 50 isolates known to be toxin-negative.

4.4. Cloning of *Campylobacter* genes

The development of *in vitro* gene cloning methodologies during the early 1970s, by the isolation and linkage of individual segments of DNA to a replicon indigenous to a particular host cell, enabled the widspread application of molecular biology techniques to the analysis of genetic material derived from either prokaryotic or eukaryotic organisms (Cohen *et al.*, 1973; Cohen, 1975). Examples of the use of such procedures for the cloning of virulence factors of various Gram-positive and Gram-negative bacterial species, including *E.coli, Neisseria, Haemophilus, Pseudomonas, Streptococcus, Staphylococcus, Bacillus and Corynebacterium,* were reviewed in detail by Macrina (1984). The genetic manipulation of single or multiple virulence genes has been invaluable in understanding the complex and often multifactorial nature of microbial pathogenesis (Finlay and Falkow, 1989).

The application of molecular cloning technology to *Campylobacter* spp. was hampered initially by the absence of a system which allowed either inter- or intragenic exchange of chromosomal genetic material. For many years, the analysis of *Campylobacter* at the genetic level was limited to studies with plasmids which could be transmitted only to other members of the same genus (Austen and Trust, 1980; Taylor *et al.*, 1981; Tenover *et al.*, 1983). Lee *et al.* (1985) were the first researchers to demonstrate the expression of *C.jejuni* genomic DNA in a foreign host, but even since then, very few reports have emerged describing the cloning of other chromosomal determinants. A detailed account of these reports was presented in the Introduction section. As yet, the cloning of a putative *C.jejuni* virulence property has not been reported.

The description of a shuttle vector capable of mobilization from *E.coli* to *C.jejuni* when complemented in *trans* by an IncP plasmid permitted, for the first time, the bi-directional transfer of genetic information between these two organisms (Labigne-Roussel *et al.*, 1987). Although Lee *et al.* (1985) had achieved complementation of the proline requirements of auxotrophic *E.coli*

cells with *C.jejuni* DNA cloned into the pBR322 vector, this approach did not allow the isolation of the gene responsible for leucine biosynthesis. The reasons proposed by the authors for this lack of success are discussed in greater detail below.

For the investigations presented here, therefore, the approach favoured was that of producing a gene library using a shuttle vector so that, if expression of the gene of interest was not achieved in *E.coli*, then the library could be transferred to a suitable *Campylobacter* host deficient in that particular trait. Of specific interest were *C.jejuni* putative virulence genes since no progress had been reported in cloning these factors. Whilst work proceeded on the generation of a gene library using plasmid pILL550, a shuttle cosmid was prepared which could also be transferred between *C.jejuni* and *E.coli.* This vector, designated pTMA1, consisted of pILL550 into which was inserted a *cos* site derived from pBTI-1 (Rose and Broach, 1990) and was capable of accepting insert fragments ranging in size from approximately 26.5 kb to 40.5 kb. This shuttle cosmid was not, however, used for cloning purposes due to restrictions in the time available for completion of the studies presented here. Further improvements to this vector were considered after Ishiura *et al.* (1989 a) demonstrated that the *in vivo* packaging of cosmids with two *cos* sites was 7-20 times higher than that of cosmids which contained only one *cos* site. A shuttle cosmid containing two *cos* sequences would therefore be packaged with greater efficiency, and could be prepared, for example, by introduction of the 1.5 kb *Eco*RI fragment of pPR691 (Jiang *et al.*, 1987) into the corresponding restriction endonuclease site of pILL550.

Various properties have been proposed as being of potential importance in the pathogenesis of *Campylobacter* diarrhoea. These were reviewed at depth in the Introduction section. As discussed above, cytotoxin and enterotoxin production were originally considered as worthwhile targets for such studies since they had been fairly well characterised and methods were available for their

detection. These methods were not, however, always applicable to the screening of an extensive library exceeding 1500 separate sequences.

The first major obstacle in the cloning of *C.jejuni* virulence properties was therefore in identifying suitable methods for their production. This aspect has already been covered in detail above. During the period when such investigations were being performed, the phenomenon of cysteine enhanced haemolysis was discovered. Haemolysins had been described as potential virulence factors in other Gram-negative bacteria causing diarrhoea, including *E.coli* (Cavalieri *et al.*, 1984), *Shigella* spp. (Clerc *et al.*, 1987; Kato *et al.*, 1989) and *Vibrio parahaemolyticus* (Honda *et al.*, 1976). The prospect of cloning the haemolysin was attractive not only because this putative virulence determinant had not previously been described, but also due to the anticipated ease of screening of any gene library constructed. This would have involved merely plating all clones obtained onto BA/Cys-HCl plates and observing haemolysis. It was therefore decided to proceed with library construction employing the pILL550 shuttle vector for isolation of the haemolysin gene whilst continuing investigations into suitable methods for toxin detection to enable the eventual screening of the library for other virulence properties.

Production of the library proved to be fairly straightforward, using well established methods for vector and chromosomal insert preparation, ligation and tranformation of competent *E.coli* DH1 cells. The 1420 clones obtained were statistically proven to constitute a complete library of the *C.jejuni* genome, each recombinant DNA molecule carrying an insert fragment of roughly 8.64 kb. However, when the clones were sub-cultured onto BA/Cys-HCl medium, none were found to be haemolytic, even after allowing extended incubation in the hope that this might lead to release of haemolysin from lysed, dying colonies. Transfer of each clone to nitrocellulose membrane and treatment with chloroform vapour to chemically induce lysis of the

bacterial cells also proved unsuccssful. Although a very crude technique, this procedure was the only means available to release intracellular material from such a large number of clones. The alternative would have been to pool several clones together, break open the collected cells by sonication or use of a French press, and test each pooled extract individually for the presence of haemolysin, a necessarily laborious and lengthy procedure.

Several factors may have contributed to the inability to isolate a haemolytic clone. Most obvious of these was that cleavage of *Campylobacter* chromosomal DNA with *Sau*3A did not produce a completely random set of fragments due to a non-random distribution of sites in the DNA, so that the library was not, in effect, complete. Under-representation of individual clones has been noted and can render a particular sequence "unclonable", even when the size of the library was calculated to be statistically significant (Little, 1987).

Alternatively, some form of functional barrier may have existed within the *E.coli* cell preventing the expression of all *Campylobacter* sequences, as suggested by Lee *et al.* (1985) to explain why proline but not leucine biosynthesis genes were identified from their library. Kreft *et al.* (1983) experienced comparable problems when trying to clone the haemolysin determinant of *Bacillus cereus* in *E.coli.* This factor, cereolysin, was readily secreted by its native producer, but only weak activity was detected around growth of haemolysin positive *E.coli* HB101 clones. The authors suggested that decreased expression of the cereolysin gene in the foreign host, or else impaired processing, transport and secretion of the product, may have accounted for the low level of activity observed.

Transcription of genes in prokaryotes is catalysed by DNA dependent RNA polymerase, which binds to DNA at the promotor site, with transcription commencing upstream of the structural genes at a specific initiation point. The Shine-Dalgarno sequence at the 5' end of the RNA transcript then binds to RNA in the ribosome

and translation can subsequently proceed. These processes, deduced from studies with *E.coli*, are described in more detail by Slater (1985). However, based on the inability to express certain *C.jejuni* genes in *E.coli*, a number of researchers have commented that a fundamental difference in gene expression must exist between the *Enterobacteriaceae* and *Campylobacter* species. In addition to the report by Lee *et al.* (1985), Labigne-Roussel *et al.* (1987) were unable to obtain expression in *C.jejuni* of the beta-lactamase gene derived from plasmid pBR322 which was known to be fully expressed in several Gram-negative species, including *Haemophilus*, *Neisseria* and members of the *Enterobacteriaceae* family. Guerry *et al.* (1990) similarly could not demonstrate expression of *Campylobacter* flagellin in *E.coli*, and postulated that post-translational modification of the gene product might not have occurred, or else differences in codon utilization had contributed to the lack of expression.

Randall and Hardy (1984) described various steps involved in protein export from bacteria. Generally, recognition of a signal sequence by a signal recognition particle in the cytosol, blocking further protein synthesis, is followed by interaction with a membrane-bound docking protein. Further elongation then proceeds at the membrane. After translocation, the leader sequence is proteolytically removed and the soluble protein finally released. As described by Hirst and Welch (1988), proteins traversing the Gram-negative envelope must cross two membranes, the inner containing phospholipids and many biosynthetic and transport proteins, whilst the outer consists of a bilayer of phospholipids, proteins and lipopolysaccharides. Separating these two membranes is an aqueous periplasmic compartment filled with transport binding proteins and enzymes. Secreted proteins may either pass through both membranes via the periplasmic space, as in the case of newly synthesized toxin subunits in *V.cholerae* (Hirst and Holmgren, 1987) or may, as reported by Lory *et al.* (1983), leave the cell via special zones where inner and outer membranes are in contact.

In fact, Hirst and Welch (1988) have emphasized that multiple mechanisms have evolved, each specifying the secretion of an individual protein or family of proteins. The complexity of extracellular secretion thus illustrates that, even if successful expression of the *C.jejuni* haemolysin gene had been achieved in *E.coli,* its release might have been impeded by various factors, for example the manner of folding to allow insertion into the membrane, or transloction across the envelope.

The original rationale of producing a gene library using the pILL550 shuttle vector had been that, if expression of the gene of interest was not achieved in *E.coli*, then the individual genetic sequences could be transferred back to a suitable *Campylobacter* host deficient in that particular trait. However, problems were encountered in this case since all *C.jejuni /coli* strains tested had been haemolytic. In addition, as will be discussed in more detail below, all attempts to generate a non-haemolytic variant by transposon mutagenesis had met with failure.

Thus, no success was achieved at isolating a haemolysin positive clone from the pILL550-based library. At this point, testing of strains for cytotoxin production had proven negative, whilst detection of enterotoxin with the VET-RPLA procedure had also been unsuccessful. It would have been possible at this stage to evaluate the utility of other methods for detection of CjT, like CHO cell elongation, although the size of the library involved made screening using such procedures prohibitive.

An alternative strategy was therefore sought which might circumvent these problems. The major drawback with the plasmid-based library appeared to be the large number of clones involved. Of the available cloning vehicles, cosmids had the highest capacity for insert DNA. These vectors were developed by Collins and Hohn (1978) to overcome the inefficient transformation of competent bacterial cells with large recombinant DNA molecules. Cosmids are simply plasmids containing the lambda *cos* sequence,

which is recognised by λ-specific packaging proteins and head precursors. The vector used in these studies, pHC79, consisted of pBR322 into which the *cos* site had been inserted (Hohn and Collins, 1980). Recombinant molecules containing foreign DNA inserts could be packaged into capsids if the total size was between 78% and 107% of the length of wild type λ (Hohn and Murray, 1977) and if a *cos* site was available at either end of the molecule. The vector pHC79 (6.43 kb) could thus accommodate insert fragments of approximately 31.5 kb to 45.5 kb.

Commercially available *in vitro* packaging kits are prepared from two induced lysogens whose prophages have different genetic mutations for assembly of mature phage particles. Mutations preventing cell lysis are carried by both prophages. Upon induction, a specific mutation in one lysogen allows accumulation of phage pre-heads, whilst in the other lysogen, proteins required for DNA insertion accumulate. All required components for the packaging reaction are thus present when these two extracts are mixed together. Once transduction into *E.coli* has been initiated, the packaging endonuclease converts the *cos* sequences into cohesive ends which ligate to form a circular molecule within the bacterial cell, thereafter being capable of replication as a plasmid (Hohn and Hinnen, 1980).

Initial attempts at cosmid library construction using a combination of the Amersham *in vitro* packaging kit and *E.coli* strains DH1 or DH5 as the host cells resulted in unsatisfactorily low numbers of transfectant colonies. Although these were shown to contain extrachromosomal DNA elements of roughly 50 kb, the recombinant molecules proved to be very unstable, the overall size becoming reduced by serial passage on medium containing ampicillin. The gene encoding beta-lactamase was thus retained, whilst other portions of the molecule not seeming to confer any advantage in the prevailing environmental conditions were shed. Reanney *et al.* (1983) similarly noted reduction of plasmid size upon continuous culture of the host *E.coli* strain, finding that the

generally slower growth rates of cells containing plasmid DNA gave an advantage to plasmid-free populations. It may thus be concluded that the replication of a large plasmid confers more of a burden upon the metabolic functions of the host cell than a small one, so that, in the presence of a specific selective pressure, like the presence of a particular antibiotic, it would only be necessary to maintain those genes conferring an advantage, whilst "accessory" DNA would be shed.

This situation was not, however, acceptable for library construction, since insert DNA fragments would easily be lost. Two factors seemed to be of importance with regard to the previous findings of other researchers. Firstly, cosmid-based clones were noted to be generally unstable by Little (1987), often spontaneously rearranging or becoming smaller in overall size. Similarly, Vicente *et al.* (1985) found that their haemolysin positive recombinants consisting of pHC79 and *L.monocytogenes* chromosomal sequences were also unstable. Other researchers working both with eukaryotic (Chia *et al.*, 1982; Steinmetz *et al.*, 1982) and prokaryotic sequences (Yelton *et al.*, 1985) encountered high frequency deletions in recombinant cosmid DNA using standard *recA* *E.coli* hosts.

Secondly, as reported by Calva *et al.* (1989), some *C.jejuni* genomic DNA sequnces simply could not be maintained in *E.coli*. These investigators rapidly lost titer of a library constructed in coliphage λ1059, whereas a similar library of *S.typhi* chromosomal segments remained stable. The authors suggested that *C.jejuni* recombinant clones might prove to be less susceptible to rearrangement or deletion in methylcytosine-specific and methyladenine-specific restriction minus systems as described by Raleigh *et al.* (1988). Indications of differences in modification of DNA between *Campylobacter* and *Escherichia* species were provided by the work of Labigne-Roussel *et al.* (1987), who found that shuttle plasmid pILL512 (a description of this vector is provided in Introduction section 1.8.6.) isolated from *E.coli* was

resistant to digestion with the restriction enzyme, *Mbo* I, while the same DNA extracted from *C.jejuni* was easily cleaved, suggesting that methylation at the adenosine residue of the GATC recognition sequence did not occur in *Campylobacter.* However, pILL512 DNA isolated from *C.jejuni,* but not *E.coli,* was resistant to digestion with *Eco* RI, indicating that modification of one or more sites of the GAATTC sequence might occur in *C.jejuni.* In addition, Miller *et al.* (1988) reported that *C.jejuni* strain C31 was incapable of electroporation-mediated transformation with pILL512 DNA isolated from *E.coli* HB101, but could be efficiently transformed with DNA of the same plasmid when extracted from *C.jejuni* cells. This again suggested the presence of a *Campylobacter* system capable of restricting heterologous sequences but which allowed modification of endogenous DNA.

It was thus considered that the use of an *E.coli* host strain containing appropriate mutations or deletions in order to prevent restriction of methylated DNA might prove beneficial in maintaining the stability of cosmid clones and producing a more representative gene library. The strain chosen was BRL's DH5αMCR™. This lacked *hsdR* activity, a restriction endonuclease capable of restricting DNA not protected by adenine methylation at specific sequences, and *hsdM* activity, mutation of which blocks sequence specific methylation (Bickle, 1982). In addition, the strain was *mcrA*$^-$, *mcrB*$^-$ and *mrr*$^-$ by introduction of an *mcrA* mutation and deletion of the *mcrB* gene, possibly as well as the *mrr* gene (Jessee and Bloom, 1988). These genes are known to be involved in the restriction of methylated DNA (Blumenthal, 1985; Heitman and Model, 1987).

Kretz and Short *et al.* (1989) proposed that the production of a fully representative library of methylated DNA using a bacteriophage or cosmid vector would be difficult unless restriction systems were removed from both the *E.coli* host strain and the packaging extract. They contended that DNA foreign to the *E.coli* lysogen used to prepare the extracts would be susceptible to

the restriction activities of that organism. This was in agreement with the earlier finding by Rosenberg (1985) that restriction activity in extracts prepared from certain strains of *E.coli* contributed to the failure to isolate all clones of interest from cosmid-based libraries. Thus, the Stratagene Gigapack™ packaging system, which lacks *hsd, mrr, mcrA* and *mcrB* functions, was favoured for use with the DH5αMCR™ strain.

This combination of host strain and packaging extract resulted in a total of 715 clones carrying average *Campylobacter* DNA insert fragments of 35.37 kb, giving a library of statistically complete size. Clones were also much more stable compared to those prepared with the Amersham *in vitro* packaging kit using *E.coli* DH1 or DH5 as the plating strains. It was concluded that modification systems played an important role in causing instability of large recombinant molecules, like cosmid clones, in some genotypically defined *E.coli* strains, at least when the insert DNA fragments contained *C.jejuni* genomic sequences. As a general recommendation, therefore, any investigations involving the cloning of *Campylobacter* genes should be limited to using *E.coli* hosts deficient in *hsd, mcrA, mcrB* and *mrr* activities and, if phage or cosmid vectors are to be employed, packaging should be performed with extracts prepared in comparable restriction free *E.coli* strains.

An interesting observation was that the efficiency of transfection of DH5αMCR™ was dependent upon the age of the *E.coli* culture when transfection was initiated. The protocol included with the Amersham *in vitro* packaging kit recommended the use of a fresh overnight culture, as suggested by Maniatis *et al.* (1982). It was found, however, that the efficiency of transfection increased by a factor of two if a fresh 7 h culture was used instead of one grown overnight. The reasons for this were not clear but it might be speculated that the number of phage receptor sites available per viable bacterial cell were probably greater in the 7 h culture than after overnight incubation, the latter probably containing a

significant proportion of non-viable organisms.

Screening of the pHC79 based library for the *Campylobacter* haemolysin gene, using methods developed earlier, proved unsuccessful. Speculations regarding the failure to identify haemolytic clones from the library generated using pILL550 as vector applied equally in this case.

Synthetic oligonucleotides have previously been employed for the detection of heat-labile and heat-stable enterotoxins (Hill *et al.*, 1985; Echeverria *et al.*, 1987) and Shiga-like toxin (Karch and Meyer, 1989) produced by *E.coli* isolates associated with intestinal disease. As was related above, a considerable degree of similarity, both functional and immunological, exists between the heat-labile enterotoxins produced by *C.jejuni*, *E.coli*, and *V.cholerae.* Calva *et al.* (1989) designed a mixed oligonucleotide probe complementary to codons 84 to 91 of *toxB*, the gene coding for the B subunit of CT. This region was proposed as being of importance in binding to ganglioside GM_1 and was highly conserved between *toxB* and *eltB,* the corresponding gene encoding the B subunit of LT (Lockman and Kaper, 1983; Leong *et al.*, 1985). Calva *et al.* (1989) demonstrated hybridization of the oligonucleotide probe with chromosomal DNA from thirteen enterotoxigenic and non-enterotoxigenic *C.jejuni* strains isolated from Mexican children. Comparing their results with those obtained by Olsvik *et al.* (1984), who were unable to demonstrate hybridization of polynucleotide probes specific for *toxA, toxB, eltA* or *eltB* to *C.jejuni* genomic DNA, Calva *et al.* surmised that similarities between CjT and the B subunits of CT and LT may be confined to relatively small regions.

The findings of Calva *et al.* (1989) thus enabled the screening of a library of *C.jejuni* chromosomal DNA sequences for the gene encoding CjT using an oligonuceotide probe. Such a probe was synthesized according to specifications reported by these authors and its ability to hybridize with total genomic DNA extracted from *C.jejuni* NCTC 11168 was assessed. The initial problem that of determining conditions which would produce an

optimal hybridization signal, so that these conditions could subseqently be applied to library screening. In his review on the use of synthetic oligonucleotides as probes, Binnie (1990) noted that several factors were important in this regard. Firstly, the temperature at which hybridization was allowed to proceed needed to be high enough to reduce non-specific binding to non-target DNA whilst still permitting specific formation of hybrid molecules. In addition, the monovalent ion concentration needed to be under strict control, generally being high during hybridization, but being decreased for subsequent washing steps. The reduced ionic concentration of the buffers employed for these washing steps, together with regulation of the temperature at which these final stages occurred, served to remove probe which had bound non-specifically to non-complementary sequences. As can be seen from Figures 26, 27 and 28, increasing the temperature at which post-hybridization washing was performed greatly reduced the extent of non-specific attachment of the CT/LT mixed oligonucleotide probe.

When optimal hybridization and washing conditions had been determined, screening of the cosmid-based clones was initiated. An extra step was incorporated into the procedure, as suggested by Woods (1984), once Hybond-N™ membranes supporting DNA from lysed bacteria had been exposed to UV radiation. This involved extensive pre-washing of the filters at high temperature in order to reduce binding of the probe to any remaining bacterial debris present. Nevertheless, the possibility existed that false negative signals would still occur despite all the precautions adopted. To circumvent this, duplicate filters were prepared and only clones exhibiting signals on both filters were considered positive. It should be noted, however, that significant variation was observed in the intensities of signals obtained for individual clones on each of the two filters, as indicated in Table 20. The identification of positive clones would have been greatly aided if a high degree of background hybridization could have been avoided (see Figure 36). This might have been achived, for instance, by removing

unincorporated [$\gamma-^{32}$P]ATP from the labelled oligonucleotide molecules by chromatography through Whatman DE-52 cellulose matrix, as recommended by Wallace and Miyada (1987).

The initial number of putative positive clones was found to be unexpectedly high. Extrachromosomal DNA was isolated from each of these clones and re-probed, revealing faint hybridization of the oligonucleotide to bands of different molecular sizes from clone numbers 89, 118, 330, 362 and 474. These clones were therefore proposed as harbouring recombinant cosmid molecules carrying genetic sequences specific for CjT. The validity of the positive signals should, nevertheless, be confirmed by probing DNA from these five clones once again with the CT/LT mixed oligonucleotide. In addition, they could also be screened with a secondary oligonucleotide mixture corresponding to some other conserved region of *toxB*. Clones proving positive with two independent and non-overlapping sets of probes should have a greater likelihood of containing the gene of interest (Binnie, 1990).

To further localize the gene encoding CjT, the restriction endonuclease generated DNA fragment from one or more of the positive clones which hybridized with the oligonucleotide probe could be isolated and ligated into an appropriate plasmid vector. Alternatively, extrachromosomal DNA from positive clones could be partially digested with a suitable restriction enzyme and sub-cloned into a plasmid vector, the resulting recombinant molecules then being transformed into competent *E.coli* cells. Re-screening with the oligonucleotide probe would thus allow the identification of a smaller portion of the *Campylobacter* genomic DNA fragment which could be associated with enterotoxin production.

Of interest would be to ascertain whether any of the clones found to be positive by oligonuleotide screening actually expressed the *C.jejuni* enterotoxin gene in a foreign host. This could be achieved by determining whether culture supernatants or cell

extracts led to elongation of CHO cells or demonstrated a positive response with the ELISA technique. Definitive evidence for expression of the *Campylobacter* enterotoxin gene in *E.coli* could present several new lines of investigation. For instance, if initial yields of enterotoxin proved to be low, then they might be increased by insertion of the cloned gene into a high expression plasmid vector, as described, for example, by Balbas and Bolivar (1990) and Rangwala *et al.* (1991). Additionally, determination of the positions of specific restriction endonuclease sites within the coding region would enable an internal DNA fragment to be identified which could be employed as a polynucleotide probe to ascertain whether this gene was indeed present in both enterotoxigenic and non-enterotoxigenic strains, as reported by Calva and fellow researchers (1989).

Labigne-Roussel *et al.* (1988) described a shuttle mutagenesis technique for the creation of precise insertional mutations in the *Campylobacter* genome using cloned DNA sequences. A suicide vector was used, designated pILL560, which possessed the same transfer capabilities of the shuttle plasmid, pILL550, but lacked specific *Campylobacter* replication functions. Into this vector was sub-cloned the *C.jejuni* gene encoding 16S rRNA, which was subsequently disrupted by insertion of a portion of DNA containing a kanamycin resistance gene. Transfer of this recombinant molecule into cells of *C.jejuni* resulted in genomic integration of the resistance determinant by a double crossover recombination event and consequent loss of the chromosomal gene. This approach could very usefully be employed to obtain mutants of *C.jejuni* deficient in the capacity to produce enterotoxin. The contribution of this toxic factor towards the pathogenicity of *Campylobacter* spp. could then be assessed by comparison of the potential of toxin positive and negative isogenic mutants to cause disease in a suitable animal model.

4.5. Transposon mutagenesis

Transposable genetic elements have proven to be very useful tools in the study of bacterial genetics (Kleckner *et al.*, 1977). In general, transposons can be described as discrete segments of DNA which ensure their own maintenance by insertion into another genetic element which is autonomously maintained (Starlinger, 1980). The translocation event does not require extensive homology between the transposon itself and the target DNA (Brown and Evans, 1991). As discussed by Bruijn and Lupski (1984), the insertion of a transposon into a gene is normally a stable event and leads to inactivation of that gene, presenting a new physically identifiable genetic marker at the site of insertion.

In the studies presented here, the transposons Tn*5* and Tn*10* were employed, both of which can have a few to several hundred target sites in any given gene (Berg *et al.*, 1989). Transposon Tn*5* has an overall molecular size of 5.7 kb and contains two flanking inverted repeats, each of approximately 1.5 kb (IS*50*), which encode genes responsible for transposition and regulation (Berg and Berg, 1983). Mazodier *et al.* (1985) demonstrated that the central portion contained three antibiotic resistance genes. Of these, aminoglycoside resistance is frequently used for selection purposes in prokaryotes, with kanamycin generally being favoured over neomycin since a single chromosomal copy of Tn*5* can confer resistance to concentrations exceeding 50 µg/ml of kanamycin (Berg *et al.*, 1989). The other two genes confer resistance to streptomycin, which is only expressed in certain *E.coli* mutants and in some non-enteric bacteria (Mazodier *et al.*, 1986), and bleomycin (Genilloud *et al.*, 1984; Mazodier*et al.*, 1985).

Pugsley (1986) described Tn*10* as approximately 9 kb in molecular size, comprising a central region coding for tetracycline resistance flanked by virtually identical copies of the 1.33 kb IS*10* insertion sequence. One of these copies, IS*10*R, codes for the transposase protein which, in conjunction with host factors, is responsible for IS*10* and Tn*10* transposition. The movement of Tn*10* from one site to another is non-replicative, so that

transposition events may not lead to increased copy number of IS*10* -containing elements (Foster, 1981).

The most direct approach to transposon-mediated mutagenesis involves the use of transposon donor vehicles carrying replicons which cannot be maintained in the target cell. During the period when the studies presented here were begun, no reports existed which cited the use of this mutagenesis technique for *Campylobacter* spp. An evaluation of the utility of various transposon delivery vectors carrying both Tn*5* and Tn*10*, specifically aimed at the creation of haemolysin negative mutants of *C.jejuni*, was thus initiated. The construction of such mutants would have allowed an understanding of the contribution towards virulence of haemolysin production by testing haemolytic and non-haemolytic variants in an appropriate animal model. A similar approach had proven useful in experimental studies of the role of *L.monocytogenes* haemolysin in virulence (Gaillard *et al.*, 1986). Of equal importance was the value of such a mutant as a host for a shuttle vector-based gene library of *Campylobacter* sequences from which expression of the haemolysin gene had not been achieved in *E.coli.* This procedure would, of course, also have been of use in the study of other putative *Campylobacter* virulence determinants.

Several of the chosen vectors had been employed in mutagenesis studies in a variety of other bacterial species. For instance, pUW964-mediated mutagenesis has been reported in *Bordetella pertussis* (Weiss *et al.*, 1983) and *Bradyrhizobium arachis* (Wilson *et al.*, 1987). The vectors pGS9, pUW964, pSUP2021 and pLG221 have all been used for mutagenesis studies involving several species of the genus *Pseudomonas* (Cuppels, 1986; Sokol, 1987; Viebrock and Zumft, 1987; Boulnois *et al.*, 1985) whilst pGS9 and pSUP2021 have been used also in *Azospirillum* spp. (Singh and Klingmuller, 1986; Vanstockem *et al.*, 1987). The 7.7 kb Tn*phoA*, when inserted in the proper orientation into a gene for a cell envelope protein, fuses alkaline phosphatase to the amino terminus of the protein product of that gene (Manoil and Beckwith,

1985). This transposon has been used to identify surface protein mutants of *Salmonella* (Finlay *et al.*, 1988) and osmotically regulated cell envelope proteins in *E.coli* (Gutierrez *et al.*, 1987). The pRT733 and pRT291 vectors were developed for the study of such proteins in *V.cholerae* (Miller & Mekalanos, 1988; Taylor*et al.*, 1989). The use of pRT733 to generate random fusions of alkaline phosphatase to extracytoplasmic proteins produced by *Erwinia amylovora* has recently been reported (Coleman *et al.*, 1991), providing evidence that sequences encoding signal peptides are present in this phytopathogenic bacterium.

All attempts at transfer of Tn*5*, Tn*phoA* and Tn*10* from a number of different delivery vectors to the *C.jejuni* chromosome proved unsuccessful. On the one occasion when colonies of *C.jejuni* were obtained which might have contained Tn*5* (derived from pUW964), it was demonstrated by hybridisation experiments that chromosomal DNA from these colonies did not in fact contain sequences related to the transposon. These colonies probably appeared as a result of spontaneous mutation allowing resistance to low levels of kanamycin.

Care had been taken to determine experimental conditions which would allow efficient transfer of the pILL550 shuttle vector between *E.coli* and *C.jejuni*. These conditions could then be applied to the transfer of delivery vectors between these two genera. If the efficiency of the transfer event was therefore discounted as a contributing factor, then failure to obtain confirmed transposon-generated mutants might have been due to degradation of transposon DNA sequences upon entry into the *Campylobacter* cell. Both Labigne-Roussel *et al.* (1987) and Miller *et al.* (1988) presented experimental data which suggested the presence of a *Campylobacter* restriction/modification system, as has been discussed above.

Tn*5* is known to encode both a transposase, which promotes the movement of the transposon, and a transposition inhibitor (Biek and Roth, 1980). These are transcribed respectively from

promoters referred to as m1 and m2. McCommas and Syvanen (1988) demonstrated that adenosine methylation of the m1 promoter by the *E.coli dam* system reduced transcription from this promoter, thereby causing a relative increase in the levels of inhibitor, leading eventually to a situation where transposition was almost totally blocked. In *C.jejuni*, transcription from Tn*5* promoters may not proceed at all, so that transposition might not result through lack of the transposase protein. Alternatively, this protein might not function in the *Campylobacter* host. Another possibility is that, as with *E.coli dam+* strains, a *Campylobacter* modification system may retard transcription from the m1 promoter, allowing sufficient inhibitor to accumulate so as to prevent transposition from occurring. Similar arguments may be extended to explain why transposition of Tn*phoA* and Tn*10* was not observed in *C.jejuni*.

It was also conceivable that expression of the transposon antibiotic resistance genes was not achieved in *Campylobacter*. This problem could be circumvented by the construction, for example, of transposon variants in which the original resistance determinant was replaced by a *Campylobacter* gene encoding resistance to kanamycin. The pILL550 vector would provide a convenient source for this particular gene. The *C.jejuni* gene encoding resistance to tetracycline, which has been cloned and sequenced (Taylor *et al.*, 1987; Manavathu *et al.*, 1988), could also be used as a marker for selection.

Whilst these studies were in progress, Labigne-Roussel *et al.* (1988) reported similar findings, in that all efforts aimed at the creation of transposon-mediated *Campylobacter* mutants were unsuccessful. Their approach had been to attempt the transfer of Tn*5* or Tn*917* from a suicide shuttle vector. This prompted the development of the shuttle mutagenesis procedure described in detail above. The technique, whilst originally applied to the mutagenesis of the cloned *Campylobacter* 16S rRNA gene, could be extended to transposition-mediated mutagenesis of randomly cloned

Campylobacter chromosomal DNA fragments using *E.coli* cells as hosts (Labigne-Roussel *et al.*, 1988). This promises to be a powerful method to achieve *in vivo* insertional inactivation of *Campylobacter* genes and is suggested for future investigations designed to obtain mutants deficient in single putative virulence determinants.

4.6. Electroporation

Electroporation was considered as an alternative approach for the transfer of transposon delivery vectors into *C.jejuni*. The procedure involves the reversible permeabilization of cell membranes by the application of a high voltage potential across the membrane (Knight and Scrutton, 1986). Shigekawa and Dower (1988) described the utility of this technique for the introduction of macromolecules into both eukaryotic and prokaryotic cells. It has also been applied to the genetic transformation of several bacterial genera, including *Escherichia* (Calvin and Hanawalt, 1988; Fiedler and Wirth, 1988), *Enterococcus* (Cruz-Rodz and Gilmore, 1990; Friesenegger *et al.*, 1991), *Lactobacillus* (Chassey and Flickinger, 1987), *Streptococcus* (Powell *et al.*, 1988) and the filamentous cyanobacterium, *Anabaena* (Thiel and Poo, 1989). In addition, Wirth *et al.* (1989) reported the electroporation-mediated transformation of 15 different Gram-negative species of various bacterial families, including the *Enterobacteriaceae*, *Pseudomonadaceae* and *Rhizobiaceae*.

Electroporation was used by Miller *et al.* (1988) to introduce pILL512 (see Introduction section 1.8.6. for a description of this plasmid) into cells of *C.jejuni*. Conditions for the electroporation protocol used in the studies presented here were similar to those described by these authors (with the exception that a Bio-Rad Pulse Controller was connected in order to allow the use of small sample volumes and narrow-gap electrodes), as were guidelines concerning cell growth and harvesting. Due to the possibility of restriction barriers, Bio-Rad, the manufacturers of the Gene Pulser apparatus,

recommended that initial experiments be performed with DNA isolated from the same species as that being transformed. Failing this, the use of high concentrations of DNA was suggested in order to circumvent this problem. However, in the investigations described here, this latter strategy proved unsuccessful for transformation of *C.jejuni* cells with plasmid pILL550 isolated from *E.coli* DH1, even when up to 4 μg of DNA was used. Bio-Rad noted in addition that the high cell densities required would exacerbate any problems associated with degradation of vector DNA by secreted bacterial nucleases. *In vitro* tests to detect extracellular DNAse determined that the *C.jejuni* strain used, NCTC 11168, did not produce this enzyme. In contrast, a transformation efficiency of 1.18×10^4 transformants per μg DNA was obtained with the use of only 400 ng of the same plasmid extracted from *C.jejuni.*

Miller *et al.* (1988) had reported similar observations and argued that a *Campylobacter* restriction and modification system was probably responsible for the degradation of DNA derived from *E.coli.* This, together with other factors discussed above, might have contributed to the lack of success in using electroporation for delivering transposons to *Campylobacter* cells. Nevertheless, the usefulness of this approach in another bacterial species has since been demonstrated by Lai *et al.* (1990), who were able to introduce suicide plasmid pSUP2021 bearing Tn*5*, a vector also employed in the studies presented here, into *Brucella abortus.*

Negative controls were, of course, included on each occasion that an electroporation experiment was performed. One such control involved the use of sterile TE buffer instead of an equal volume of DNA preparation, which did not lead, on any occasion, to the isolation of bacterial colonies on selective plates. In a second control, pILL55O DNA (isolated from *C.jejuni*) was added to cells of *C.jejuni* 11168 exactly as for other electroporation experiments, except that a pulse was not discharged from the electroporation apparatus. This was included to demonstrate that DNA could enter

into the bacterium only after cell membrane permeabilization through the application of a high voltage potential. However, on both occasions when this control was used, small numbers of colonies, confirmed as *C.jejuni*, were found on medium containing kanamycin, at a mean frequency of 50 colonies per µg of DNA. This indicated that *Campylobacter* cells were capable of transformation with DNA derived from the same genus without the need for artificial induction of a state of competence. Such "natural" transformation systems have been described for several bacterial species, as reviewed by Stewart and Carlson (1986). In *Haemophilus* spp. (Danner *et al.*, 1980) and *Neisseria gonorrhoeae* (Goodman and Scocca, 1988), uptake is dependent on the presence of specific sequences on the incoming DNA of 11 bp and 10 bp respectively. In a study published after the investigations presented here had been completed, Wang and Taylor (1990) demonstrated that most *C.coli* and some *C.jejuni* strains were also naturally competent for the uptake of DNA derived from closely related species. In addition, these authors found that plasmid DNA isolated from *Campylobacter* spp. and *E.coli* were transformed with roughly equivalent efficiencies, suggesting that incoming DNA was protected from the action of *Campylobacter* restriction endonucleases reported by Labigne-Roussel *et al.* (1987) and Miller *et al.* (1988). Such protection of transforming DNA molecules has been observed in other natural transformation systems (Smith *et al.*, 1981).

Wassenaar *et al.* (1991), unlike Miller *et al.* (1988), were able to introduce DNA isolated from *E.coli* into *C.jejuni* by electroporation, and suggested that this discrepancy may be due to strain-dependent modification of *E.coli* DNA by campylobacter. This may also explain why the results presented in this thesis conflict with those of Wang and Taylor (1990), in that transformation of *C.jejuni* 11168 was not achieved with plasmid DNA isolated from *E.coli*.

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APPENDICES

Appendix 1 - Liquid media

SOC Medium
(Sambrook *et al.*, 1989)

To 950 ml of d.H_2O were added:

bacto-tryptone (Difco)	20 g
bacto-yeast extract (Difco)	5 g
NaCl	0.5 g

Once the solutes had dissolved, 10 ml of 0.25 M KCl was added and the pH of the solution adjusted to 7.0. The final volume was then made up to one litre and the medium sterilised by autoclaving.

Before use, 5 ml of a sterile solution of 2M $MgCL_2$ (autoclaved) and 20 ml of sterile 1 M glucose (filtered through a 0.22 µm Acrodisc filter) were added to the cooled medium.

Terrific Broth
(Tartof and Hobbs, 1987)

To 900 ml of d.H_2O were added:

bacto-tryptone (Difco)	12 g
bacto-yeast extract	24 g
glycerol	4 ml

The medium was sterilised by autoclaving once the solutes had completely dissolved. Before use, 100 ml of an autoclaved solution containing 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 was added.

Appendix 2 - Miscellaneous Reagents and Solutions

Å Buffer

The composition of this buffer was as follows:

Tris-HCl (pH 8.0)	1.25 M
$MgCl_2$	0.125 M
dCTP	100 µM
dGTP	100 µM
dTTP	100 µM

Calf Intestinal Phosphatase buffer

(Maniatis *et al.*, 1982)

The composition of the buffer was as follows:

$MgCl_2$	1 mM
$ZnCl_2$	0.1 mM
Spermidine	1 mM
Tris-HCl (pH 9.0)	50 mM

CTAB/NaCl Solution

(Ausubel *et al.*, 1987)

In 80 ml of $d.H_2O$ was dissolved 4.1 g of NaCl. The solution was heated to 65°C and 10 g of hexadecyltrimethyl ammonium bromide (CTAB; purchased from Sigma) slowly added to it with constant mixing. Once dissolved, the final volume was adjusted to 100 ml.

Denhardt's Reagent (50X)

(Sambrook *et al.*, 1989)

To 80 ml of $d.H_2O$ were added:

Ficoll (type 400)	1 g
Polyvinylpyrrolidone (PVP)	1 g
Bovine serum albumin (BSA; Fraction V)	1 g

The solution was made up to 100 ml with d.H_2O, filtered, and stored at -20°C. BSA and PVP were obtained from Sigma; Ficoll was obtained from Pharmacia.

Gel Loading Buffer
(Sambrook *et al.*, 1989)

Gel loading buffer at a strength of 6X consisted of the following components made up in d.H_2O:

bromophenol blue	0.25%
xylene cyanol FF	0.25%
sucrose	40% ($^{w}/_{v}$)

Oligo-Labelling Buffer (OLB)
(Feinberg and Vogelstein, 1984)

Four solutions were made separately:

Solution O: Tris-HCl (pH 8.0) 1.25 M
$MgCl_2$ 1 mM

Solution A: To 1 ml of Solution O were added
18 µl 2-mercaptoethanol
5 µl dCTP
5 µl dGTP
5 µl dTTP
(all triphosphates were at a concentration of 100 mM in 0.2 mM EDTA, 3 mM Tris-HCl, pH 7.0).

Solution B: 2 M Hepes buffer adjusted to pH 6.6 with 4 M NaOH

Solution C: Random hexadeoxyribonucleotides suspended in TE buffer at 90 ODU/ml.

To make OLB buffer, solutions A, B and C were mixed in the

ratio, 10:25:15. The buffer was stored at -20°C.

Phosphate buffered saline (PBS)
(Sambrook *et al.*, 1989)

To 900 ml of d.H_2O were added:

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

After the solutes had dissolved, the buffer was adjusted to pH 7.4 with HCl, made up to one litre, and sterilised by autoclaving.

Polynucleotide Kinase Buffer (PNK)

This buffer had the following composition:

$MgCl_2$	0.1 M
Dithiothreitol	0.05 M
Tris-HCl (pH 7.6)	0.7 M

Potassium Acetate Solution
(Sambrook *et al.*, 1989)

This solution consisted of the following:

5 M potassium acetate	120 ml
Glacial acetic acid	23 ml
H_2O	57 ml

SM Buffer
(Sambrook *et al.*, 1989)

To 90 ml of d.H_2O were added:

NaCl	0.58 g
$MgSO_4.7H_2O$	0.20 g
1 M Tris-HCl (pH 7.5)	5.0 ml

2% sterile gelatin solution	0.5 ml

The buffer was made up to 100 ml with d.H_2O and then sterilised by autoclaving.

20X SSC
(Sambrook *et al.*, 1989)

To 800 ml of d.H_2O were added the following:

NaCl	175.3 g
Sodium citrate	88.2 g

After the solutes had dissolved completely, the buffer was adjusted to pH 7.0 with 10 N NaOH, made up to one litre and sterilised by autoclaving.

TE Buffer
(Sambrook *et al.*, 1989)

The composition of the buffer was as follows:

Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Tris-borate Buffer (TBE)
(Sambrook *et al.*, 1989)

To 800 ml of distilled d.H_2O were added:

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml

After the solutes had dissolved, the pH was adjusted to pH 8.2 if necessary, and the solution made up to one litre. This represented a 5X concentrated stock solution. For agarose gel electrophoresis, TBE was routinely used at a working concentration of 0.5X.

Tris-Saturated Phenol
(Sambrook *et al.*, 1989)

To 100 ml of water saturated liquified phenol (Rathburn Chemical Company) in a separating funnel was added 8-hyhroxyquinoline to a final concentration of 0.1%. An equal volume of 0.5 M Tris-HCl (pH 8.0) was added and the phases emulsified by repeated inversion. When separated, the upper phase was discarded and the lower phase was mixed with another 100 ml of 0.5 M Tris-HCl (pH 8.0). The phenolic phase was then extracted twice with 0.1 M Tris-HCl (pH 8.0) and the pH ensured as being >7.8 (as determined by use of pH paper). The final equilibrated phenol was stored under a solution of 0.1 M Tris-HCl (pH 8.0) containing 0.2% β-mercaptoethanol at 4°C in a light-proof bottle.

Appendix 3- Antibiotic Solutions

Antibiotic [a]	Stock Solution [b]	Usual Working Concentration
Ampicillin	25 mg/ml in d.H_2O	50 µg/ml
Chloramphenicol	30 mg/ml in ethanol	10 µg/ml
Kanamycin	25 mg/ml in d.H_2O	50 µg/ml
Streptomycin	20 mg/ml in d.H_2O	25 µg/ml
Tetracycline	10 mg/ml in ethanol	10 µg/ml

[a] all antibiotics were purchased from Sigma.

[b] Stock solutions of antibiotics dissolved in d.H_2O were sterilised by filtration through a 0.22 µm Acrodisc filter. Antibiotics dissolved in ethanol were not sterilised. All stock solutions were stored in light-proof bottles at -20°C.

Appendix 4 - Composition of BRL REact™ Buffers Used in These Studies

REact™ Buffer	Composition at Working Concentration (1X)
REact™2	50 mM Tris-HCl (pH 8.0) 10 mM $MgCL_2$ 50 mM NaCl
REact™3	50 mM Tris-HCl (pH 8.0) 10 mM $MgCL_2$ 100 mM NaCl
REact™4	20 mM Tris-HCl (pH 7.4) 5 mM $MgCL_2$ 50 mM KCl
REact™10	100 mM Tris-HCl (pH 7.6) 10 mM $MgCL_2$ 150 mM NaCl

REact™ buffers appropriate for each restriction endonuclease were supplied by BRL at 10X working concentration.