CONSTRUCTION OF A PLASMID SHUTTLE VECTOR FOR *CAMPYLOBACTER HYOINTESTINALIS*

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"Of the events of war, I have not ventured to speak from any chance information, nor according to any notion of my own. I have described nothing but what I saw myself, or learned from others of whom I made the most careful and particular enquiry."

Thucydides. Peleponnesian War

"I've jazzed mine up a little."

Scott Waterman. Ph.D. Thesis. 1992.

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Chapter 2: Materials and Methods

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published by any other person, accept where due reference is made in the text. I consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan, if accepted for the award of the degree.

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ABSTRACT

Campylobacter hyointestinalis has been associated with proliferative enteritis in swine (Gebhart *et al.*, 1983, 1985; Lambert *et al.*, 1984) and cattle (Diker *et al.*, 1990), however, no genetic study of *C. hyointestinalis* has been previously reported and little is known about its surface structures. This study has been concerned with the initial characterization of the composition of the *C. hyointestinalis* outer membrane, and the construction of plasmid shuttle vectors for the future analysis and expression of *C. hyointestinalis* genes.

Examination of the outer membrane proteins, lipopolysaccharides (LPSs), and flagellins of ten strains of *C. hyointestinalis* revealed that *C. hyointestinalis* possessed features in common with other *Campylobacter* species, in particular its close relative *C. fetus*. The LPS of *C. hyointestinalis* shows considerable heterogeneity between strains, with some exhibiting some higher M_r LPS similar to that observed in *C. fetus*. All strains express two major outer membrane proteins. Three strains differed from the norm in displaying some variation in the apparent molecular weights (M_rs) of the proteins. The flagellin of *C. hyointestinalis* was determined to be of a similar size (62 kDa) to the flagellins of *C. jejuni* and *C. coli*; antisera raised against *C. hyointestinalis* detected flagellin of *C. jejuni* and *C. coli* by immunoblot. A single 2.5 kb cryptic plasmid was isolated from four of the ten strains screened.

Attempts to mobilize the *C. jejuni/C. coli* shuttle vector pILL550, and a number of broad-host-range vectors, into *C. hyointestinalis*, were unsuccessful. It is thought that this might be because the replicons of these vectors were inoperative in a *C. hyointestinalis* host. A series of *C. hyointestinalis* candidate shuttle vectors based upon the 2.5 kb cryptic plasmid (to provide the appropriate genetic material for replication in *C. hyointestinalis*) were constructed. Despite repeated attempts, none of

these *C. hyointestinalis* candidate shuttle vector constructs could be efficiently mobilized into *C. hyointestinalis*. This suggested that *C. hyointestinalis* might possess a barrier to the introduction of foreign DNA *via* conjugation; the most likely being a restriction/modification system.

The entire 2.5 kb cryptic plasmid has been sequenced to determine the location of its replicon in order to eliminate the possibility that the lack of success in constructing a shuttle vector was due to the absence of a complete replication region. A candidate shuttle vector containing the replicon in an intact form could function, and two further shuttle vectors were constructed. After treatment with a chemical mutagen, *C. hyointestinalis* transconjugants were obtained with these plasmids. One of these isolates was also shown to be transformable by *E. coli*-modified plasmid DNA at a high efficiency, and was therefore assumed to be restrictionless mutant. Plasmid shuttle vector DNA extracted from this mutant could not, however, be transformed into the wild-type *C. hyointestinalis* type strain, implying that the DNA modification system of the restriction mutant had also been mutated.

A putative origin of transfer (*oriT*) was identified on the cryptic plasmid. This *oriT* contained a putative "nick region" which was demonstrated to have been mistaken, by *E. coli* K-12, for the "nick region" of the *oriT* of the broad-host-range plasmid RP4, on rare occasions. Mating experiments between various *Campylobacter* species demonstrated that a *C. hyointestinalis* shuttle vector could be mobilized (most probably from the putative *oriT*) from *C. coli* to *C. fetus* and to a *C. hyointestinalis* restriction mutant. The *C. hyointestinalis* shuttle vectors were efficiently mobilized into both subspecies of *C. fetus*, which implied that *C. fetus* did not possess a restriction system against foreign DNA introduced *via* conjugation. An analysis of the restriction barriers that exist between different *Campylobacter* species was finally undertaken.

In summary, a number of *C. hyointestinalis*-specific shuttle vectors were constructed which could be mobilized into a *C. hyointestinalis* strain only when it had been mutated for its restriction system. *C. hyointestinalis* appears to differ from other

characterized *Campylobacter* species studied in its possession of a restriction system which prevents the introduction of foreign DNA *via* conjugation.

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LIST OF ABBREVIATIONS

A	: adenine
aa	: amino acid
Ap	: ampicillin
ATP	: adenosine 5′-triphosphate
BHK	: baby hamster kidney
bp	: base pair(s)
BSA	: bovine serum albumin
C	: cytosine
CLOs	: *Campylobacter*-like organisms
Cp	: cephalothin
cpm	: counts per minute
Da	: dalton(s)
DNA	: deoxyribonucleic acid
DNase	: deoxyribonuclease
dNTP	: deoxyribonucleoside triphosphate
ddNTP	: dideoxyribonucleoside triphosphate
DTT	: dithiothreitol
EDTA	: ethylene-diamine-tetra-acetic acid
EtBr	: ethidium bromide
G	: guanine
HeLa	: human cervical carcinoma cell(s)
HEp	: human epithelial cell(s)
IHF	: integration host factor
IMVS	: Institute of Medical and Veterinary Science

INT	: human intestinal epithelial cells
IPTG	: isopropyl-β-D-thiogalactopyranoside
kb	: kilobase pairs
kDa	: kilodalton(s)
Km	: kanamycin
LB	: Luria broth
LPS	: lipopolysaccharide
LT	: heat labile toxin
M.	: methylase
Mdal	: megadalton(s)
MDBK	: bovine kidney cell(s)
MOMP	: major outer membrane protein
MNNG	: N-methyl-N′-nitro-N-nitrosoguanidine
M_r	: relative molecular mass
mRNA	: messenger ribonucleic acid
N	: any nucleoside
NA	: nutrient agar
Nal	: nalidixic acid
NB	: nutrient broth
NCTC	: National Collection of Type Cultures
nos.	: numbers
nt	: nucleotide
OD	: optical density
oligo(s)	: oligodeoxynucleotide(s)
ORF	: open reading frame
PAGE	: polyacrylamide gel electrophoresis
PEG	: polyethylene glycol-6000
PK	: pig kidney cell(s)
Pmb	: polymyxin B

R.	: restriction endonuclease
R	: resistant
RBS	: ribosomal binding site
rRNA	: ribosomal ribonucleic acid
RF	: replicative form
RITARD	: removable intestinal tie adult rabbit diarrhoea procedure
RNA	: ribonucleic acid
RNase	: ribonuclease
rpm	: revolutions per minute
RT	: room temperature
S	: sensitive
SD	: Shine-Dalgarno
SDS	: sodium dodecyl sulphate
Sm	: streptomycin
T	: thymine
Tc	: tetracycline
TEMED	: N,N,N′,N′-tetramethyl-ethylene-diamine
Tmp	: trimethoprim
Tn	: transposon
Tris	: Tris (hydroxymethyl) aminomethane
tRNA	: transfer ribonucleic acid
u	: unit(s)
U	: uracil
UV	: ultraviolet
Vm	: vancomycin
v/v	: volume per volume
w/v	: weight per volume
X-gal	: 5-Bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside

CHAPTER 1

INTRODUCTION

1.1 The Genus *Campylobacter*

Members of the genus *Campylobacter* are small Gram-negative, microaerophilic, spirally curved rods that have a single unsheathed polar flagellum at one or both ends of the cell, and that exhibit a characteristic rapid and darting corkscrew type of motility (Smibert, 1978). When they were first discovered, the organisms were classified in the genus *Vibrio* on the basis of their comma-shaped morphology and rapid motility (McFayden and Stockman, 1913; Smith and Taylor, 1919; King, 1957). In 1963 Sebald and Véron discovered that these organisms differed greatly from the type species of the genus *Vibrio, Vibrio cholerae*, in both phenotype and genotype. These differences were sufficient for the reclassification of these organisms into a new genus, *Campylobacter*, within the family *Spirillaceae* (Sebald and Véron, 1963; Véron and Chatelain, 1973).

The genus *Campylobacter* currently includes eleven species and subspecies (Vandamme *et al.*, 1991). These campylobacters are 0.5 to 8 μm long and 0.2 to 0.5 μm in diameter (Sebald and Véron, 1963; Karmali and Skirrow, 1984). They are non-sporeforming, non-fermentative, oxidase-positive, usually sensitive to oxygen, and use amino acids and tricarboxylic acid cycle intermediates as their principal energy sources (Smibert, 1974, 1984; Karmali and Skirrow, 1984). The guanine-plus-cytosine (G+C) content of the genus ranges from 30 to 46 mol%, and is among the lowest known for bacteria (Sebald and Véron, 1963; Smibert, 1974, 1978, 1984; Neill *et al.*, 1979; Owen and Leaper, 1981; Vandamme *et al.*, 1991).

1.1.1 *Campylobacter* taxonomy

The classification of the genus *Campylobacter* has always been somewhat difficult. Because these organisms do not catabolize carbohydrates and are inert with regard to most of the traditional biochemical tests used for the identification of bacteria, only a relatively small number of tests are available for their identification and classification (Roop *et al.*, 1984, 1985). A convenient practice in the past has been to divide the species into two groups based on catalase production. The application of catalase testing for the classification of campylobacters was first described by veterinarians (Bryner and Frank, 1955) who found that the test permitted the differentiation of *Campylobacter fetus*, the bovine pathogen of interest, from commensals now classified as *Campylobacter sputorum* biovar *bubulus*.

Campylobacter species can also be separated into two further groups, the thermophilic and the non-thermophilic, according to the range of temperatures at which they will grow (Morris and Patton, 1985). The thermophilic species grow at 42°C but not at 25°C and the converse applies to the non-thermophilic species. In addition, there are other biochemical tests like H_2S production on lead acid strips or in iron-containing media, growth tolerance in varying concentrations of NaCl, glycine or bile, nitrate or nitrite reduction, sodium hippurate hydrolysis, indoxyl acetate hydrolysis, and sensitivity to nalidixic acid or cephalothin, which are commonly used to differentiate between species and subspecies. These differences are summarized in Table 1.1.

Recent developments which have facilitated the rapid generation of partial 16S ribosomal ribonucleic acid (rRNA) sequences have allowed the phylogenetic relationships among bacteria to be determined (Lane *et al.*, 1985). It was concluded that *Campylobacter pylori* was not related at the genus level to *Campylobacter jejuni*, *Campylobacter coli*, *C. fetus* subsp. *fetus*, *Campylobacter laridis*, or *C. sputorum* biovar *sputorum*, and that these latter species represented the true genus *Campylobacter* (Romaniuk *et al.*, 1987). *C. pylori* was more closely related to *Wollinella succinogenes* than to the other species and has now been reclassified as *Helicobacter pylori* (Goodwin *et al.*, 1989).

TABLE 1.1 Differentiation between species of the family *Campylobacter*

Taxon	Catalase	Nitrate reduction	Nitrite reduction	H_2 requirement[a]	Urease	H_2S production (TSI)[b]	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Growth at: 15°C	25°C	42°C	Growth in: 3.5% NaCl	1% glycine	0.1% TMAO (anaerobic)[c]	Susceptibility to:[d] Nalidixic acid	Cephalothin	G+C content (mol%)
C. fetus subsp. *fetus*	+*	+	-	-	-	-	-	-	-	+	-	-	+	-	R	S	33-35
C. fetus subsp. *venerealis*	+	+	-	-	-	-	-	-	-	+	-	-	-	-	R	S	33-34
C. hyointestinalis	+	+	-	V	-	+	-	-	-	+	+	-	+	+	R	S	33-36
C. sputorum biovar sputorum	-	+	+	-	-	+	-	-	-	-	+	-	+	V	S	S	30-31
C. sputorum biovar bubulus	-	+	+	-	-	+	-	-	-	-	+	+	+	+	R	S	29-30
C. sputorum biovar fecalis	+	+	+	-	-	+	-	-	-	-	+	-	+	+	R	S	30-32
C. jejuni subsp. *jejuni*	+	+	-	-	-	-	+	+	-	-	+	-	+	-	S	R	30-33
C. jejuni subsp. *doylei*	V	-	-	-	-	-	V	+	-	-	-	-	+	-	S	S	30-31
C. coli	+	+	-	-	-	-	-	+	-	-	+	-	+	-	S	R	30-33
C. lari	+	+	-	-	V	-	-	-	-	-	+	-	+	+	R	R	30-32
"C. upsaliensis"	W	+	-	-	-	-	-	+	-	-	+	-	V	-	S	S	32-36
C. mucosalis	-	+	+	+	-	+	-	-	-	-	+	-	+	-	R	S	36-38
C. concisus	-	+	+	+	-	+	-	-	-	-	+	-	+	-	R	R	37-41
C. curvus	-	+	+	+	-	+	-	+	-	-	+	-	+	ND	S	ND	45-46
C. rectus	-	+	+	+	-	+	-	+	-	-	W	-	+	ND	S	ND	45-46
A. cryaerophilus	+	+	-	-	-	-	-	+	+	+	-	-	-	-	V	R	28-29
A. nitrofigilis	+	+	-	-	+	ND	-	-	+	+	-	+	-	ND	S	S	28-29

[a]: For microaerophilic growth

[b]:TSI: triple sugar iron agar

[c]:TMAO: trimethylamine-*N*-oxide hydrochloride

[d]:Susceptibility was determined using 30µg discs

*:+: positive reaction; -: negative reaction; W: weak reaction; V: variable reaction; ND: not determined; S: susceptible; R: resistant

The data in this Table were taken from Vandamme and De Ley (1991).

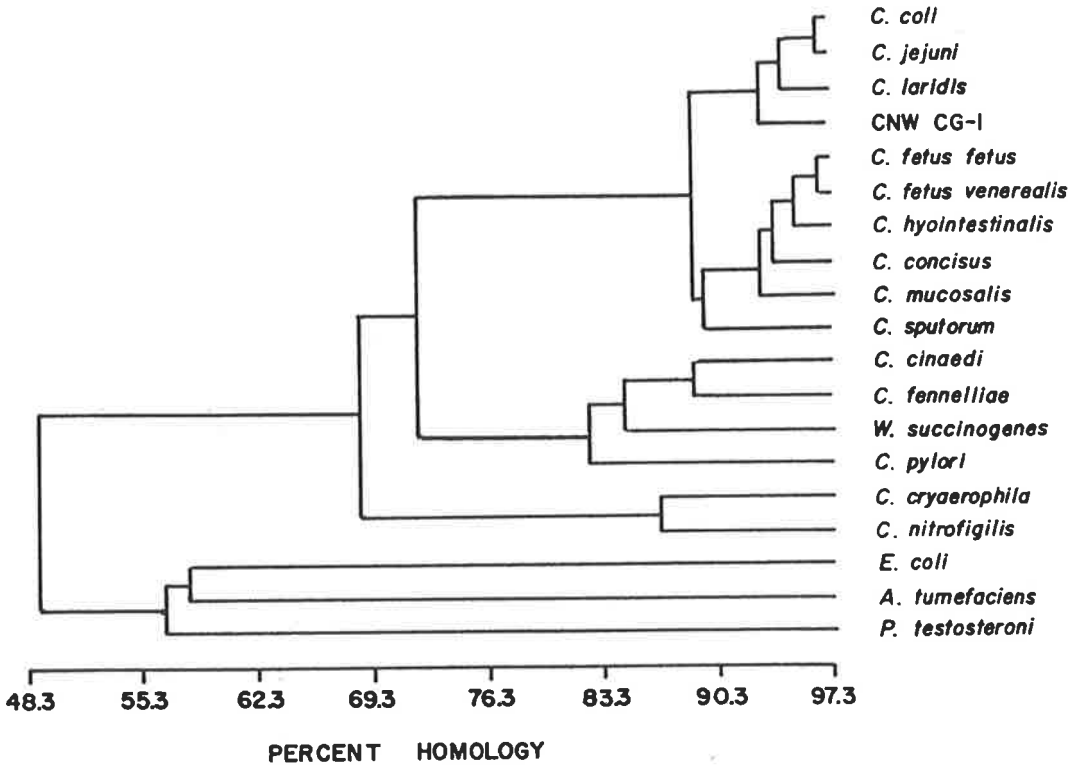
A study of the taxonomic structure of the genus *Campylobacter*, based on partial 16S rRNA sequence analysis, revealed that the species could be divided into three major homology groups (Thompson *et al.*, 1988). The species represented in each homology group are shown (Figure 1.1). The first homology group contained *C. fetus* (the type species of the genus *Campylobacter*), *Campylobacter hyointestinalis, C. sputorum, C. jejuni, C. coli, C. lari, "Campylobacter upsaliensis", Campylobacter concisus,* and *Campylobacter mucosalis*. The second rRNA homology group contained *C. pylori, Campylobacter fennelliae,* and *Campylobacter cinaedi. W. succinogenes* also belonged to this second rRNA homology group. The third rRNA homology group consisted of *Campylobacter nitrofigilis* and *Campylobacter cryaerophila.*

In another phylogenetic study of the genus *Campylobacter*, a close relationship was found between *Wolinella curva, Wolinella recta, Bacteroides gracilis,* and *Bacteroides ureolyticus* on the one hand, and the so-called true campylobacters belonging to the rRNA group I of Thompson *et al.* (1988), on the other (Paster and Dewhirst, 1988).

The most extensive study of the taxonomic structure of the genus *Campylobacter* included more than 70 strains of *Campylobacter* species and related taxa (Vandamme *et al.*, 1991). In order to study the genotypic coherence of these organisms and their phylogenetic relationships with other bacteria, the DNA-rRNA hybridization technique of De Ley and De Smedt (1975) and the immunotyping technique of Falsen (1983) were used. It was proposed that the amended genus *Campylobacter* should be limited to *C. fetus, C. hyointestinalis, C. concisus, C. mucosalis, C. sputorum, C. jejuni, C. coli, C. lari,* and "*Campylobacter upsaliensis*". *W. curva* and *W. recta* were transferred to the genus as *Campylobacter curvus* comb. nov., and *Campylobacter rectus* comb. nov. respectively. It was concluded that *B. gracilis* and *B. ureolyticus* were generically misnamed, and that they were closely related to the genus *Campylobacter. C. nitrofigilis, C. cryaerophila,* and an unnamed *Campylobacter* species strain constituted a new genus, for which the name *Arcobacter* was proposed.

FIGURE 1.1 Phylogenetic relationships of *Campylobacter* species, *W. succinogenes*, and representatives of the major branches of the purple phototrophic bacteria, based on analysis of partial base sequences of 16S rRNA.

The horizontal scale is the percent homology calculated by the Numerical Taxonomy System (NT-SYS) analysis program (Rohlf *et al.*, 1979). The Figure is taken from Thompson *et al.* (1988).


C. coli
C. jejuni
C. laridis
CNW CG-I
C. fetus fetus
C. fetus venerealis
C. hyointestinalis
C. concisus
C. mucosalis
C. sputorum
C. cinaedi
C. fennelliae
W. succinogenes
C. pylori
C. cryaerophila
C. nitrofigilis
E. coli
A. tumefaciens
P. testosteroni
48.3
55.3
62.3
69.3
76.3
83.3
90.3
97.3
PERCENT HOMOLOGY

1.1.2 The *Campylobacter* species and their associated diseases

The genus *Campylobacter* contains several important species that are either proven pathogens or that are currently considered to be associated with diseases in humans and animals. The type species of the genus, *C. fetus*, is divided into two subspecies; *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*. The two subspecies are indistinguishable on the basis of DNA-DNA reassociation and partial 16S rRNA sequence analysis, and the division into two subspecies is based on the ability to grow in 1% glycine and differences in pathogenesis (Belland and Trust, 1982; Roop *et al.*, 1984; Smibert, 1984; Romaniuk *et al.*, 1987).

C. fetus subsp. *venerealis* causes infertility and abortions in cattle (Smibert, 1978), is transmitted via coitus, and releases an endotoxin which causes a chronic inflammation of the female genital tract and subsequent embryonic death (Osborne, 1965; Samuelson and Winter, 1966). Colonization of the mucosal epithelium is limited to the reproductive tract. *C. fetus* subsp. *venerealis* is thought to be a mutant of *C. fetus* subsp. *fetus* which has adapted to a specific ecological niche in the bovine reproductive tract (Véron and Chatelain, 1973).

C. fetus subsp. *fetus* is orally transmitted, causes sporadic abortions in cattle and sheep, and septicaemia in humans (Smibert, 1984). The infection in cattle is believed to arise from bacteria in the intestine acquired through ingestion of food or water contaminated with bacteria from faeces, aborted foetuses, or vaginal discharges of the aborting animal. Bacteraemia develops following ingestion, and the organisms, which have a particularly high affinity for placental tissue, invade the uterus and multiply in the immunologically immature foetus. A subacute placentitis develops and the foetus is usually aborted during the last six weeks of gestation (Jensen *et al.*, 1961).

C. jejuni is part of the normal intestinal flora of cattle, sheep, dogs, cats, poultry and other animals (Smibert, 1984). It is recognized as the causative agent of one of the most common forms of acute gastroenteritis in humans (Skirrow, 1977, 1982). The clinical disease caused by *C. jejuni* varies in severity with symptoms ranging from a mild infection mimicking viral enteritis, to a serious illness accompanied by high fever,

abdominal cramps, nausea, vomiting and persistent bloody diarrhoea (Blaser *et al.*, 1983b). It has been reported that about 80% of cases of acute *C. jejuni* enteritis require hospitalization for one to ten days but no fatalities have been reported (Tee *et al.*, 1986). The disease is transmitted to man by direct faecal ingestion, contaminated water supplies and the food chain (Blaser *et al.*, 1983b). Major outbreaks have been recorded following contamination of water supplies, milk, chicken and seafood (Skirrow, 1982; Blaser *et al.*, 1983b). Infected cats and dogs have been suggested as sources of infection for man (Blaser *et al.*, 1978; Svedhem and Norkrans, 1980). Recently, Steele and Owen (1988) isolated a group of atypical, nitrate-negative, *C. jejuni* strains from patients in Central and South Australia and named them *C. jejuni* subsp. *doylei*. The name *C. jejuni* subsp. *jejuni* was preserved for the genuine *C. jejuni* strains.

C. coli is part of the normal flora of pigs and poultry and causes diarrhoea in humans. *C. coli* is physiologically similar to *C. jejuni* and most diagnostic laboratories do not differentiate between them. Studies in Canada and England indicate that *C. coli* accounts for 3 to 5% of human cases of *Campylobacter* infections (Karmali and Skirrow, 1984).

C. lari occurs in the intestines of seagulls, humans, dogs, and horses and occasionally causes bacteraemia and diarrhoea in humans (Karmali and Fleming, 1979; Nachamkin *et al.*, 1984; Tauxe *et al.*, 1985; Simor and Wilcox, 1987).

C. hyointestinalis is frequently isolated from the intestines of pigs with lesions of proliferative enteritis (Lomax *et al.*, 1982; Gebhart *et al.*, 1983, 1985). Recent isolations from patients with proctitis and diarrhoea clearly implicate the species as an infrequent pathogen of humans (Fennel *et al.*, 1986; Edmonds *et al.*, 1987; Minet *et al.*, 1988).

C. mucosalis is more frequently isolated from the intestines of pigs with non-haemorrhagic proliferative enteritis (Lawson and Rowland, 1974; Rowland and Lawson, 1975; Lawson *et al.*, 1976; Lomax and Glock, 1982; Gebhart *et al.*, 1983) than from those with haemorrhagic proliferative enteritis (Love *et al.*, 1977; Lawson *et al.*, 1979; Yates *et al.*, 1979; Gebhart *et al.*, 1983). It has also been isolated from the oral cavity of healthy pigs (Lawson *et al.*, 1975). Attempts to produce typical lesions in conventional or

specific pathogen-free-pigs with pure cultures of *C. mucosalis* have generally been unsuccessful (Lawson and Rowland, 1984).

C. sputorum, which is divided into three biovars (*sputorum, bubulus*, and *fecalis*), occurs as part of the normal flora of the oral cavity of humans, bovine genitalia, and sheep faeces, respectively (Roop *et al.*, 1985).

C. concisus has been isolated from the gingival crevices of humans with periodontal disease, but its pathogenicity is unknown (Tanner *et al.*, 1981).

"*Campylobacter upsaliensis*" is the name suggested for a group of catalase-negative or weakly catalase-positive strains of *Campylobacter* that have been isolated from healthy and diarrhoeic dogs and cats (Sandstedt *et al.*, 1983).

C. curva and *C. rectus* (transferred to the genus *Campylobacter* from the genus *Wolinella* (Vandamme *et al.*, 1991)) have been isolated from humans with both oral and non-oral infections (Paster and Dewhirst, 1988).

1.1.3 *C. hyointestinalis*

In 1983, strains isolated from the intestines of pigs suffering from proliferative enteritis were found to belong to the genus *Campylobacter* (Gebhart *et al.*, 1983). The majority of the isolates belonged to a proposed new species called *C. hyointestinalis*, because of its porcine intestinal origin (Gebhart *et al.*, 1985). It was shown that *C. hyointestinalis* and *C. mucosalis* were the most common isolates, whereas *C. jejuni* and *C. coli* were only occasionally cultured, from the intestines of diseased pigs (Gebhart *et al.*, 1983). An indirect fluorescent antibody assay was developed to identify *Campylobacter* species in lesions of porcine proliferative enteritis (Chang *et al.*, 1984). Ileal frozen sections from 29 pigs with histologic lesions of proliferative enteritis showed specific fluorescent staining with rabbit antisera raised against *C. hyointestinalis* or *C. mucosalis* bacteria (*C. hyointestinalis* in all 29 sections and *C. mucosalis* in 24). *C. hyointestinalis* bacteria were seen in large numbers and were broadly distributed in intestinal luminal exudate, mucosal necrotic tissues, surface epithelium, lamina propria, and proliferative cryptal epithelium. Numerous *C. hyointestinalis* were always present in

the apical cytoplasm of proliferative cryptal epithelium. Fluorescent *C. mucosalis* bacteria were seen less frequently and were distributed focally in the mucosa. Ileal sections from 13 pigs without proliferative enteritis had no fluorescent staining for *C. hyointestinalis* and *C. mucosalis* indicating that these organisms were not present. On the basis of phenotypic and DNA relatedness it was confirmed that isolates from the intestines of hamsters with proliferative enteritis (Gebhart *et al.*, 1985) and the faeces of cattle (Ursing *et al.*, 1984; Diker *et al.*, 1990), were *C. hyointestinalis*.

Before 1986, no isolates of *C. hyointestinalis* were reported in humans. However, the recent isolations from patients with proctitis and diarrhoea suggest that *C. hyointestinalis* may be an opportunistic enteropathogen of humans (Fennell *et al.*, 1986; Edmonds *et al.*, 1987; Minet *et al.*, 1988).

By DNA hybridization, *C. hyointestinalis* showed a closer relationship to *C. fetus* than to any other catalase-positive *Campylobacter* species (Roop *et al.*, 1984). Like *C. fetus*, it was resistant to nalidixic acid and susceptible to cephalothin. Of the two *C. fetus* subspecies, however, it was more similar to *C. fetus* subsp. *fetus* in that it tolerated 1% glycine and some strains could grow at both 25°C and 42°C (Gebhart *et al.*, 1985).

1.1.4 Proliferative enteritis

Proliferative enteritis is a transmissible disease of weaned pigs which occurs in many swine-producing countries around the world including the United States (Dodd, 1968; Glock, 1981; Lomax *et al.*, 1982), Britain (Rowland and Rowntree, 1972; Rowland and Lawson, 1974), Canada (Nielsen, 1955; Yates *et al.*, 1979), Australia (Love *et al.*, 1977), New Zealand (O'Hara, 1972), India, Taiwan (Chu and Hong, 1973), Japan (Kubo *et al.*, 1984), Finland (Rhako and Saloniemi, 1972), Sweden (Martinsson *et al.*, 1974; Jonsson and Martinsson, 1976), and Denmark (Esmbo, 1951).

The disease was characterized by segmental mucosal hyperplasia, haemorrhage, necrosis, and other inflammatory changes of the small and large intestines (Dodd, 1968; Rowland and Rowntree, 1972). Mucosal hyperplasia was associated with proliferation of

the cryptal epithelial cells within which many slender, curved intracytoplasmic bacteria were consistently present (Rowland and Lawson, 1974; Jonsson and Martinsson, 1976; Kurtz *et al.*, 1980). The disease is clinically manifested in swine herds by a diarrhoeic condition which may be either haemorrhagic or relatively non-haemorrhagic. The haemorrhagic condition is characterized by acute dysentery and sudden death, whereas the non-haemorrhagic diarrhoeic condition is characterized by acute or chronic intermittent diarrhoea with a progressive wasting syndrome. In some countries like Australia and Taiwan, infected swine herds manifest primarily the haemorrhagic condition (Chu and Hong, 1973; Love *et al.*, 1977). This contrasts with Denmark and Sweden where the non-haemorrhagic diarrhoeic condition predominates (Esmbo, 1951; Martinsson *et al.*, 1974; Jonsson and Martinsson, 1976). In the United States and Great Britain, both conditions have been reported in swine herds (Rowland and Rowntree, 1972; Kurtz *et al.*, 1980; Gebhart *et al.*, 1983).

The haemorrhagic condition occurs primarily among young and adult swine in breeding stocks (Rowland and Rowntree, 1972; Love *et al.*, 1977; Lomax and Glock, 1982) and may spread to other feeder pigs and finishing pigs in the herd (Rowland and Rowntree, 1972). The disease is more frequently seen in closed swine herds, in specific-pathogen-free herds, and in pigs raised in confinement conditions (Rowland and Rowntree, 1972; Chu and Hong, 1973; Love *et al.*, 1977; Rowland and Lawson, 1981; Lomax *et al.*, 1982; Taylor, 1983).

In acute outbreaks bleeding usually occurs during the initial episode of the disease. This affects a high proportion of breeder pigs with the rapid onset of severe haemorrhagic dysentery which can result in death from 8 hours to several days after the onset of dysentery (Rowland and Rowntree, 1972; Love *et al.*, 1977). The outbreak may be associated with the introduction of carrier animals onto the premises (Lomax *et al.*, 1982). Complete recovery can occur in a few weeks (Rowland and Lawson, 1981; Taylor, 1983) but some pigs may be retarded from further growth and become 'poor doers' (Rowland and Rowntree, 1972). Depending upon the severity of the outbreaks, the morbidity in the herd may range widely from 12 to 56% (Rowland and Rowntree, 1972;

Love *et al.*, 1977), whereas the mortality ranged from 13 to 20% in one episode (Rowland and Rowntree, 1972), to 45 to 50% in another episode (Love *et al.*, 1977).

After the initial outbreak the disease may remain endemic in the herd and become a sporadic disease which affects only the replacement stock of young gilts and boars newly introduced into the units (Love *et al.*, 1977; Love and Love, 1977). An endemic situation has been reported in central boar and gilt testing stations in Britain (Jackson, 1980). Among thousands of pigs, the occurence of the disease was sporadic, and the mortality over five years averaged only 0.53%. Nevertheless, the disease accounted for more than 70% of all the deaths in the station.

The non-haemorrhagic condition primarily affects postweaning and young feeder pigs (Biester and Schwarte, 1931; Martinsson *et al.*, 1974; Jonsson and Martinsson, 1976; Lomax *et al.*, 1982). The pigs suffer from a diarrhoea which is often intermittent, tends to be chronic, and lasts at least several days to three weeks (Lomax *et al.*, 1982). The affected pigs gradually lose weight, show dehydration and wasting, and consequently are stunted and emaciated (Biester and Schwarte, 1931; Jonsson and Martinsson, 1976; Lomax *et al.*, 1982; Randolph and McCoy, 1982). The mortality is usually low (Glock, 1981) and most pigs recover within six weeks after the onset of clinical signs (Rowland and Lawson, 1974). However, the recovery of the lost weight is costly to the producer, and there may be an extension of at least six weeks of growing time before a marketable weight is attained (Horrox, 1977). A survey of the occurence of the disease in swine in Iowa herds showed that about 80% of epizootics in that state were caused by the non-haemorrhagic form of the disease (Lomax *et al.*, 1982). The condition occurs epizootically or sporadically (Biester and Schwarte, 1931; Dodd, 1968; Rowland and Rowntree, 1972; Lomax *et al.*, 1982) and is possibly associated with the introduction of carrier animals into a susceptible herd or with the introduction of susceptible replacement animals into an endemic herd.

The consistent presence in all of these conditions of intracellular *Campylobacter*-like organisms (CLOs) suggested that they were the aetiological agent (Rowland and Lawson, 1975, 1986; Lawson *et al.*, 1985; McOrist *et al.*, 1987, 1989). The identity and

origin of the intracellular CLOs and their relationship to the pathogenesis of the disease have not been resolved. Bacteriologic culture of the lesions frequently yields *C. hyointestinalis* and *C. mucosalis*, and neither of these organisms is numerous in normal porcine intestines (Lawson and Rowland, 1974; Gebhart *et al.*, 1983; Ohya *et al.*, 1985). *C. jejuni* and *C. coli* have also been isolated from the lesions; however, these bacteria are considered part of the normal flora (Rosef *et al.*, 1983; Rowland and Lawson, 1986). So far, however, attempts to reproduce proliferative enteritis in conventional or gnotobiotic piglets with any of these cultured *Campylobacter* species or combinations of the cultures did not cause any significant disease (Kashiwazaki *et al.*, 1971; Boosinger *et al.*, 1985; McCartney *et al.*, 1987).

Proliferative enteritis has been reproduced only by orally dosing pigs with homogenates of naturally affected mucosa (Roberts *et al.*, 1977; Mapother *et al.*, 1987). No particular *Campylobacter* species was clearly associated with the disease in those experiments. Therefore, experimental and cultural results have not identified the intracellular organism as one of the *Campylobacter* species that can be grown from porcine intestines.

Results obtained by using immunohistology to identify the intracellular organism have been controversial (Chang *et al.*, 1984; Lawson *et al.*, 1985). Antigens prepared against whole cells of *C. hyointestinalis* or *C. mucosalis* did not react with intracellular organisms in indirect immunofluorescence assays (Lawson *et al.*, 1985). In the same study, antisera prepared against intracellular CLOs purified from lesions by selective filtration did not react with intracellular bacteria in sections of lesions from other pigs. Also, a monoclonal antibody prepared against the intracellular CLO reacted with intracellular bacteria in sections of lesions from pigs with proliferative enteritis but not with purified *C. hyointestinalis*, *C. mucosalis* or *C. coli* (McOrist *et al.*, 1987). A comparison of whole cell and outer membrane preparations of each of the *Campylobacter* species, and of the intracellular bacteria purified directly from the lesions, established that specific antibodies to the intracellular organisms did not react with antigens of cultured *Campylobacter* species (McOrist *et al.*, 1989).

A recent study using DNA probes specific to the intracellular CLO found that several of the CLO-specific probes hybridized with porcine mucosa obtained from pigs with proliferative enteritis but not with non-diseased mucosa or with any of the commonly isolated porcine *Campylobacter* species (Gebhart *et al.*, 1991). These data suggest that the intracellular CLO associated with proliferative enteritis may be a novel bacterium not yet identified or cultured.

1.1.5 Pathogenesis of *Campylobacter* infections

The mechanisms of pathogenicity of *Campylobacter* species have yet to be fully elucidated. However, several possible mechanisms of infection have been suggested. *C. jejuni* has been shown to be enteroinvasive in humans (Mandall *et al.*, 1984) and in laboratory animals (Manninen *et al.*, 1982; Yrios and Balish, 1986; Boosinger and Powe, 1988). Approximately 50% of *C. jejuni* strains tested have also been shown to adhere to and penetrate HeLa cells but no correlation was detected between the strains associating with HeLa cells and those producing febrile, bloody diarrhoea (Fauchère, 1986). A study of the *Campylobacter* species isolated from the intestines of pigs with proliferative enteritis found that *C. hyointestinalis* attached to but did not invade HEp-2 cells, and that *C. mucosalis* did not (Konkel and Joens, 1989). An earlier study found that *C. mucosalis* attached to and persisted intracellularly in PK(15), MDBK, BHK, HeLa, and INT-407 cells, whereas *C. hyointestinalis* did not (Rajasekhar *et al.*, 1988). In addition to adhesins, factors such as chemotaxis and motility have also been suggested to be important in *Campylobacter* virulence (Morooka *et al.*, 1985; McSweegan and Walker, 1986; Hugdahl *et al.*, 1988).

It is possible that the pathogenicity of *C. jejuni* is related to the production of toxins as some *C. jejuni* strains can produce a cytotoxin (Johnson and Lior, 1984) and others have been shown to secrete a potent cholera-like enterotoxin (Ruiz-Palaicos *et al.*, 1983; Goossens *et al.*, 1985; McCardell *et al.*, 1986). Recently, *C. jejuni* enterotoxin has been partially purified (Daikoku *et al.*, 1990) and the DNA sequence encoding it has been shown to be related to that of the cholera toxin of *V. cholerae* and the LT enterotoxins of

Escherichia coli (Calva *et al.*, 1989). However, this enterotoxin did not seem to enhance the virulence of strains producing it, as the proportion of enterotoxigenic strains isolated from patients with watery diarrhoea was not significantly different to that from patients with no watery diarrhoea (Belbouri and Mégraud, 1988).

Although none of the factors described above appears alone to be directly responsible for the pathogenicity of *C. jejuni* it may be that a combination of spiral shape and darting motility assist the ability to adhere to intestinal mucosa, and that chemotaxis and toxin production are also both necessary to induce infection and illness.

1.1.6 Membrane proteins of *C. jejuni* and their antigenic properties

The membrane proteins of *C. jejuni* have been studied in some detail (Logan and Trust, 1982; Newell *et al.*, 1984). *C. jejuni* has been shown to possess a unique protein profile following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with the most striking feature being a single major outer membrane protein (MOMP) which varied among strains from 43 to 46 kDa in size (M_r). There were also at least a dozen minor but readily distinguishable proteins.

The 43 to 46 kDa MOMP and two other polypeptides of 37 and 76 kDa have been shown to be exposed on the cell surface by labelling of the membrane proteins with a radioiodination procedure which utilized immobilized lactoperoxidase and glucose oxidase (Logan and Trust, 1982). Other surface-exposed proteins of 27 and 64 kDa have also been identified (Newell *et al.*, 1984). The MOMP has been well characterized and identified as a pore-forming protein, or porin (Huyer *et al.*, 1986) which is thought to be the monomeric form of a native trimer involved in the formation of small hydrophobic channels in the outer membrane. The *C. jejuni* porin has been shown to be heat-modifiable, the modified form having a M_r of 43 kDa with that of the non-modified form being 31 kDa. This purified porin protein was incorporated into a black lipid bilayer and formed small permeable channels which permitted the passage of solutes with a molecular weight of less than 360 (Page *et al.*, 1989). This is considerably smaller than the ~600 molecular weight limit of *E. coli* porins (Benz *et al.*, 1985). In general, such

membrane channels are believed to affect bacterial antibiotic susceptibility, as the relatively small channels formed by the *C. jejuni* porin may hinder the permeability of the large molecules (Page *et al.*, 1989).

There are a number of unique *C. jejuni* membrane proteins that are recognized as being immunogenic. These include antigens of M_r 31, 36, and 48 kDa, whereas some of the other *C. jejuni* antigens were shared by other *Campylobacter* species. *C. jejuni* and *C. coli* were found to have the largest number of antigens in common (namely proteins of 20, 40, and 50 kDa and the 62 kDa flagellin). *C. lari* had four and *C. fetus* two antigenic determinants in common with *C. jejuni* (Logan and Trust, 1983; Wenman *et al.*, 1985). Sera from patients convalescing from infection with *C. jejuni* recognized proteins of 66 (flagellin), 46, and 12 kDa with a 43 kDa porin also being weakly immunogenic (Nachamkin and Hart, 1985).

Four surface proteins, from an invasive strain of *C. jejuni*, with apparent M_r of 28, 32, 36, and 42 kDa, have been identified as binding to HEp-2 cell monolayers (De Melo and Pechère, 1990). In contrast, only the 36 kDa protein from a less-invasive strain bound to HEp-2 cells. The 32 kDa protein associated only with the HEp-2 and HeLa cell lines, and this correlated directly with the ability of the invasive strain to invade these cell lines. It has been suggested that binding of these surface-exposed proteins may play a role in *C. jejuni* host-cell interactions and ultimate invasion (De Melo and Pechère, 1990).

Isolates of *C. jejuni* grown under conditions of iron deprivation showed conspicuous changes in their outer membrane protein composition (Field *et al.*, 1986). Three proteins with apparent M_r of 74, 76, and 82 kDa were consistently present in the outer membrane of cells grown in low-iron medium. Although the functions of these iron-regulated outer membrane proteins remain to be determined, it is not unlikely that one or more of them serve as receptors for the siderophores used by *Campylobacter* strains.

1.1.7 The S-layer of *C. fetus*

C. fetus has been shown to produce an S-layer which takes the form of crystalline lattices that cover the entire cell surface in hexagonal, tetragonal and oblique patterns (Dubreuil *et al.*, 1988; Fujimoto *et al.*, 1989). The S-layer has been demonstrated to be antiphagocytic and required in the pathogenesis of *C. fetus* infections (McCoy *et al.*, 1975); it may have a role in invasion and survival within the host (Blaser *et al.*, 1987; Fujimoto *et al.*, 1989; Pei and Blaser, 1990). Cells possessing the S-layer were refractory to ingestion by macrophages, except in the presence of specific antisera; in the absence of the S-layer maximum phagocytosis occurred without a requirement for opsonins (McCoy *et al.*, 1975). The presence of the S-layer was associated with increased virulence of *C. fetus* in experimentally infected mice (Pei and Blaser, 1990). The *C. fetus* S-layer was composed of high M_r protein subunits for which sizes of 98, 100, 127, and 149 kDa were demonstrated by SDS-PAGE (Pei *et al.*, 1988; Dubreuil *et al.*, 1988).

It has been recently observed that during *in vitro* passage, strains of *C. fetus* could lose their S-layer (Fujimoto, 1989) and variants could be obtained which produced S proteins with M_r values different from those of their parent (Dubreuil *et al.*, 1990; Wang *et al.*, 1990; Fujimoto *et al.*, 1991). The M_r changes of the S proteins seen in the variant strains were associated with both morphological and antigenic changes in the S-layer (Dubreuil *et al.*, 1990; Fujimoto *et al.*, 1991). These observations support the hypothesis that the pattern and antigenicity of the *C. fetus* S-layer is determined by the particular type of S protein. Furthermore, the presence of two different S-layer patterns on a single bacterial cell indicates that multiple S proteins can be produced and expressed on a single cell (Fujimoto *et al.*, 1991).

1.1.8 Lipopolysaccharides (LPS) of *Campylobacter* species

Analyses of the migration patterns of *C. jejuni* LPSs determined by SDS-PAGE and silver staining indicated that they were of low M_r and devoid of O side chains (Logan and Trust, 1984; Mills *et al.*, 1985; Perez-Perez and Blaser, 1985; Blaser *et al.*, 1986b). This type of LPS profile is found in pathogens such as *Neisseria, Haemophilus*, and

Bordetella species (Diena *et al.*, 1978; Inzana, 1983; Peppler, 1984). In contrast to the situation with these bacteria, in which the presence of low M_r LPS is associated with a low degree of serotypic variation, the LPS of *Campylobacter* species appears to be antigenically diverse (associated with a large degree of serotypic variation) (Penner and Hennessy, 1980; Penner *et al.*, 1983). *C. jejuni* LPS antigens display unique serological specificity. The reactions of LPSs with homologous and heterologous antisera indicated that they were strain-specific antigens but in some cases cross-reactions were observed (Preston and Penner, 1987). The lipid A of low M_r LPS shares some antigenic determinants with the core region of the LPS of other enteric bacteria such as *Salmonella* species and *V. cholerae* (Perez-Perez *et al.*, 1986). Immunoblotting with homologous antisera has shown that some strains of *C. jejuni* have a series of high M_r LPS components characteristic of core-attached O-side chains of various lengths (Preston and Penner, 1987). Strains of *C. fetus* have also been shown to contain LPS with a minimal core region and several higher M_r complexes (Logan and Trust, 1984; Perez-Perez and Blaser, 1985).

1.2. Flagella

1.2.1 The flagella of *C. coli*

The best characterized surface antigen of *C. jejuni* and *C. coli* is the flagellum. *C. jejuni* flagella have been shown to consist of identical protein subunits of M_r of approximately 62 kDa (62 to 66 kDa depending upon the strain) (Logan and Trust, 1986). The flagellin protein carried unique strain-specific epitopes as well as determinants which were shared not only between *C. jejuni* strains but also with *C. coli* and *C. fetus* (Logan and Trust, 1986; Nachamkin and Hart, 1986).

It has been demonstrated that some *C. jejuni* strains could interconvert between flagellated and non-flagellated states (Caldwell *et al.*, 1985). It was further found that *C. jejuni* and *C. coli* strains showed reversible antigenic variation of their flagellin subunits (associated with a M_r change from 61.5 to 59.5 kDa); both subunit forms were

structurally different (Harris *et al.*, 1987; Logan *et al.*, 1987). Such antigenic variation is known to occur in *Salmonella* strains where the phase transition involves reversible inversion of a 995 bp DNA sequence upstream of one of the flagellin structural genes (Simon *et al.*, 1980).

The basis for switching of *C. coli* flagellar antigens has been associated with a reversible DNA rearrangement of a 700 bp DNA segment, identified by hybridization with *Salmonella typhimurium* LT2 genomic DNA (Guerry *et al.*, 1988). This was used as a probe following the observation that when total DNA from *S. typhimurium* LT2, *E. coli*, and *Shigella flexneri* was nick-translated and used to probe *C. coli* or *C. jejuni* DNA, the same discrete bands were observed, indicating that these bands most likely represented a set of sequences common to the different organisms. However, such a rearrangement has not been detected in *C. jejuni*. The gene encoding the flagellin protein of *C. coli* has been cloned and sequenced and was shown to contain an open reading frame encoding a protein with a M_r of 58,945 (Logan *et al.*, 1989). Hybridization data suggested the presence of a second flagellin copy located adjacent to the first on the *C. coli* chromosome. DNA probes containing a complete flagellin gene, and various internal regions of the *C. coli* flagellin gene, were hybridized to DNA from 30 strains of *C. coli* or *C. jejuni* representing 20 serogroups. The results indicated a high overall degree of homology among all the strains examined with the most variable regions occuring within the middle of the genes (Thornton *et al.*, 1990).

It was found that two flagellin genes, with 91.9% sequence homology, located in tandem and separated by a short intervening sequence, were present in *C. coli*. The two genes, called *flaA* and *flaB*, were transcribed by separate σ^{28} and σ^{54} promoters respectively (Guerry *et al.*, 1990). Mutational analysis and primer extension experiments indicated that the two genes were expressed concomitantly in the same cell, regardless of the antigenic phase of expressed flagella. Gene replacement mutagenesis techniques were used to generate *flaA*$^+$ *flaB* and *flaA flaB*$^+$ mutants. A flagellar filament composed exclusively of the *flaA* gene product was indistinguishable in length from that of the wild-type and the organism showed a slight reduction in motility (Guerry *et al.*, 1991). The

flagellar filament composed exclusively of the *flaB* gene product was severely truncated in length and motility was adversely affected to a marked extent. Thus, while expression of both flagellins together was not necessary for motility, both products were required for a fully functional flagellar filament. Although the wild-type flagellar filament is a heteropolymer of the *flaA* and *flaB* gene products, immunogold electron microscopy suggested that the *flaB* epitopes were poorly surface-exposed along the length of the wild-type filament.

1.2.2 The flagella of *C. jejuni*

It has been suggested that the reversible expression of *C. jejuni* flagella may be regulated at the transcriptional level (Nuijten *et al.*, 1989). A 410 bp sequence of the flagellin structural gene was cloned as part of a fusion with the *E. coli* K-12 β-galactosidase gene and when this sequence was used to probe total RNA of flagellated and non-flagellated variants of the same *C. jejuni* strain, it detected an RNA transcript of the flagellin gene only in the flagellated variant. Hybridization of the partial sequence of the flagellin gene to the total DNA of *C. jejuni* indicated that there might be more than a single gene encoding the flagellin protein in the *C. jejuni* genome (Nuijten *et al.*, 1989). Further study confirmed that there were indeed two copies of the flagellin gene, which were called *flaA* and *flaB*, as in *C. coli* (Nuijten *et al.*, 1990b). Each gene consisted of 1731 bp, the genes occurred as tandem repeats, and they were 95% identical. Only mRNA that was transcribed from *flaA* was detected in flagellated cells. σ^{28}-specific promoter sequences were found upstream of the transcription initiation site. The analysis of the flagellin protein sequence showed that the amino-terminal and the carboxyl-terminal regions were very similar to those of other bacterial flagellins. The conserved regions could form α-helices with a non-polar backbone of residues at one side of the surface of both helices. It was suggested that because these domains were conserved they might be involved in either or both of polymerization or transport of flagellins, and that they were important for maturation and stability of the flagellum.

Similarly, Fischer and Nachamkin (1991) showed that two common regions, called C1 and C2, comprising 170 amino acids of the amino-terminus and 100 amino acids of the carboxyl terminus of the *C. jejuni* flagellin, respectively, exhibited 94% and 96% homology to the corresponding regions of the *C. coli* flagellin. A variable region, called V1, comprising the middle of the *C. jejuni* protein, showed 61% homology with the equivalent region of *C. coli*. Several areas within the V1 region corresponded to predicted surface-exposed regions and may represent areas in which surface epitopes are located.

The two flagellin genes of *C. jejuni* have been inactivated by homologous recombination of mutant forms (Wassenaar *et al.*, 1991). Mutants in which *flaB* but not *flaA* was inactivated remained motile. In contrast a defective *flaA* gene led to non-motile bacteria. Invasion studies showed that mutants without motile flagella had lost their potential to adhere to, and penetrate into, human intestinal cells *in vitro*. Invasive properties could be partially restored by centrifugation of the mutants onto the tissue culture cells, indicating that motility was important for invasion. Low level penetration was, however, possible without flagella. This was in agreement with earlier data indicating that factors, namely LPS (McSweegan and Walker, 1986; Konkel and Joens, 1989) and proteins in the range of 26 to 32 kDa (De Melo and Pechere, 1990), other than the flagella are involved in invasion.

1.3. Molecular cloning of *C. jejuni* genes in *E. coli*

Molecular cloning of *C. jejuni* genes in *E. coli* K-12 has met with limited success probably due to the poor expression of *C. jejuni* genes in *E. coli* K-12 (Chan *et al.*, 1988; Nuijten *et al.*, 1989). A 4.4 kb DNA fragment from *C. jejuni* capable of complementing the proline biosynthetic genes of *E. coli* K-12 has been cloned in pBR322 (Lee *et al.*, 1985). Cloning of the *C. jejuni* genome using pBR322 in *E. coli* K-12 has also been employed for the isolation of a genomic DNA sequence expressing serine hydroxylmethyltransferase and lysyl-tRNA synthetase (Chan *et al.*, 1988; Chan and Bingham, 1992). These systems, however, relied not only on expression of *C. jejuni*

genes in *E. coli* K-12, but also on the ability of these genes to functionally complement equivalent *E. coli* K-12 genes. Molecular cloning has also been used to isolate and characterize the 16S rRNA genes of *C. jejuni* (Rashtchian *et al.*, 1987). The plasmid-encoded kanamycin phosphotransferase and chloramphenicol-resistance determinants from *C. coli*, and the tetracycline-resistance determinant from *C. jejuni*, have been cloned and expressed in *E. coli* K-12 (Trieu-Cuot *et al.*, 1985; Taylor *et al.*, 1987; Wang and Taylor, 1990a).

1.3.1 Genetic analysis of *C. jejuni*

To facilitate the genetic analysis of *C. jejuni*, molecular techniques have been used to develop a gene transfer system. A plasmid shuttle vector, pILL550 (Figure 1.2), has been constructed which can be mobilized from *E. coli* K-12 to *C. jejuni*, *C. coli*, and *C. fetus* (Labigne-Roussel *et al.*, 1987). The chimaeric plasmid was constructed using the *E. coli* K-12 plasmid pBR322 into which was cloned a fragment of the *C. coli* plasmid pIP1445 encoding kanamycin-resistance, which was expressed in both *Campylobacter* species and in *E. coli* K-12. To ensure replication in *Campylobacter*, a small multicopy plasmid from *C. coli* was included. By incorporating the origin of transfer (*oriT*) from the broad-host-range IncP plasmid RK2 the vector was able to be mobilized into a number of *Campylobacter* species. The newly constructed shuttle vector therefore had the ability to replicate and was selectable, in both *E. coli* K-12 and *C. jejuni*. For conjugative transfer of this vector from *E. coli* K-12 to *Campylobacter* species, however, an IncP plasmid was required *in trans*. This shuttle vector has been used to isolate mutants in the *C. jejuni* genome by replacement of wild-type DNA by homologous, but mutated, DNA (Labigne-Roussel *et al.*, 1988). A gene encoding *C. jejuni* 16S rRNA was cloned into a modified form of this vector which lacked the *Campylobacter* replicon, inactivated by transposon mutagenesis, and the construct then used as a suicide vector to replace the wild type *C. jejuni* gene with the gene mutated *in vitro*. It should be possible to improve this method of gene replacement mutagenesis or allelic exchange based on two recent observations. Firstly, *C. jejuni* and *C. coli* may be transformed with plasmid

FIGURE 1.2 Restriction endonuclease-generated map of pILL550.

The *Campylobacter aphA-3* gene is indicated by a dotted box. DNA sequences from the *C. coli* cryptic plasmid pIP1445 are shown as white boxes and the sizes (bp) of their *Hin*dIII fragments are given. The *oriT* DNA sequence from RK2 is indicated by a thin arrowed line. DNA sequences from pBR322 are indicated by a black line. Redrawn from Labigne-Roussel *et al.* (1987).

pILL550
EcoRI, ClaI
HindIII
HindIII
HindIII
HindIII
HindIII
SalI
AvaI
PstI, BamHI, SmaI, EcoRI
700
340
600
1600
1kb

DNA by electroporation at a frequency as high as 10^6 transformants per μg (Miller *et al.*, 1988). Secondly, most *C. coli* and some *C. jejuni* strains are naturally competent during the exponential phase of growth and can be transformed by naked DNA without the requirement for any special treatment (Wang and Taylor, 1990b). Thus, these systems open further the way for genetic analysis of *Campylobacter* species.

1.4 Plasmids

1.4.1 Plasmids in *Campylobacter*

Plasmids have been shown to specify virulence determinants in a variety of bacteria (Elwell and Shipley, 1980). Although many workers have described the physical isolation of plasmids from *C. jejuni* and *C. coli* (Austen and Trust, 1980; Ambroiso and Lastovica, 1983; Bradbury *et al.*, 1983; Tenover *et al.*, 1985), only antibiotic resistance and, in certain special cases, enterotoxin production have been shown to be plasmid-encoded in these strains. Both Taylor *et al.* (1980, 1981), and Tenover *et al.* (1983), independently described conjugative plasmids of approximately 57 kb encoding tetracycline resistance. In a subsequent study, Tenover *et al.* (1985) screened 688 isolates of *C. jejuni* and *C. coli* for the presence of plasmid DNA, and found that 32% harboured plasmid DNA, ranging in size from 2.0 to 162 kb. Only tetracycline resistance was found to correlate with the presence of plasmids.

1.4.2 Narrow-host-range plasmid replication

The autonomous replication of plasmids takes place in a controlled manner such that a plasmid, in a particular host under a given set of growth conditions, is maintained with a defined average number of copies per cell. The means for correcting deviations from their characteristic copy numbers is determined by the plasmids themselves. All narrow-host-range plasmids studied so far control their own replication by specifying a negative feedback loop (Nordström, 1985; Novick, 1987). The genes and sites required for autonomous replication and its control constitute the basic replicons of plasmids.

They generally consist of an origin of replication, "*cop*" and "*inc*" genes involved in the control of the initiation of replication, and (in most cases) "*rep*" genes encoding proteins required for replication and its control. The basic plasmid replicons are usually about 2 to 3 kb in length.

To date, few of the known plasmids have been studied in any detail, but a pattern has emerged in which most of the basic replicons fall into one of two types with respect to the manner in which they are controlled.

1.4.3 ColE1-like replication, and counter-transcript RNA control

One type of plasmid replication strategy uses a small RNA molecule as the main inhibitor in the control of the initiation of replication (De Wilde *et al.*, 1978; Muesing *et al.*, 1981; Rosen *et al.*, 1981; Stougaard *et al.*, 1981; Tomizawa *et al.*, 1981). The target of this RNA is an overlapping RNA transcribed from the opposite strand which is required as a primer, or as a messenger for a Rep protein, for the initiation of replication (Lacatena and Cesareni, 1981; Stougaard *et al.*, 1981; Tomizawa and Itoh, 1981). The resulting inhibitory effect is due to the formation of an RNA-RNA duplex between the counter-transcript RNA and the complementary sequence of the target RNA. This RNA-RNA duplex is initiated by base pairing between complementary unpaired loops that were formed in both RNAs by secondary folding (Figure 1.3) (Light and Molin, 1982; Brady *et al.*, 1983; Lacatena and Cesareni, 1983).

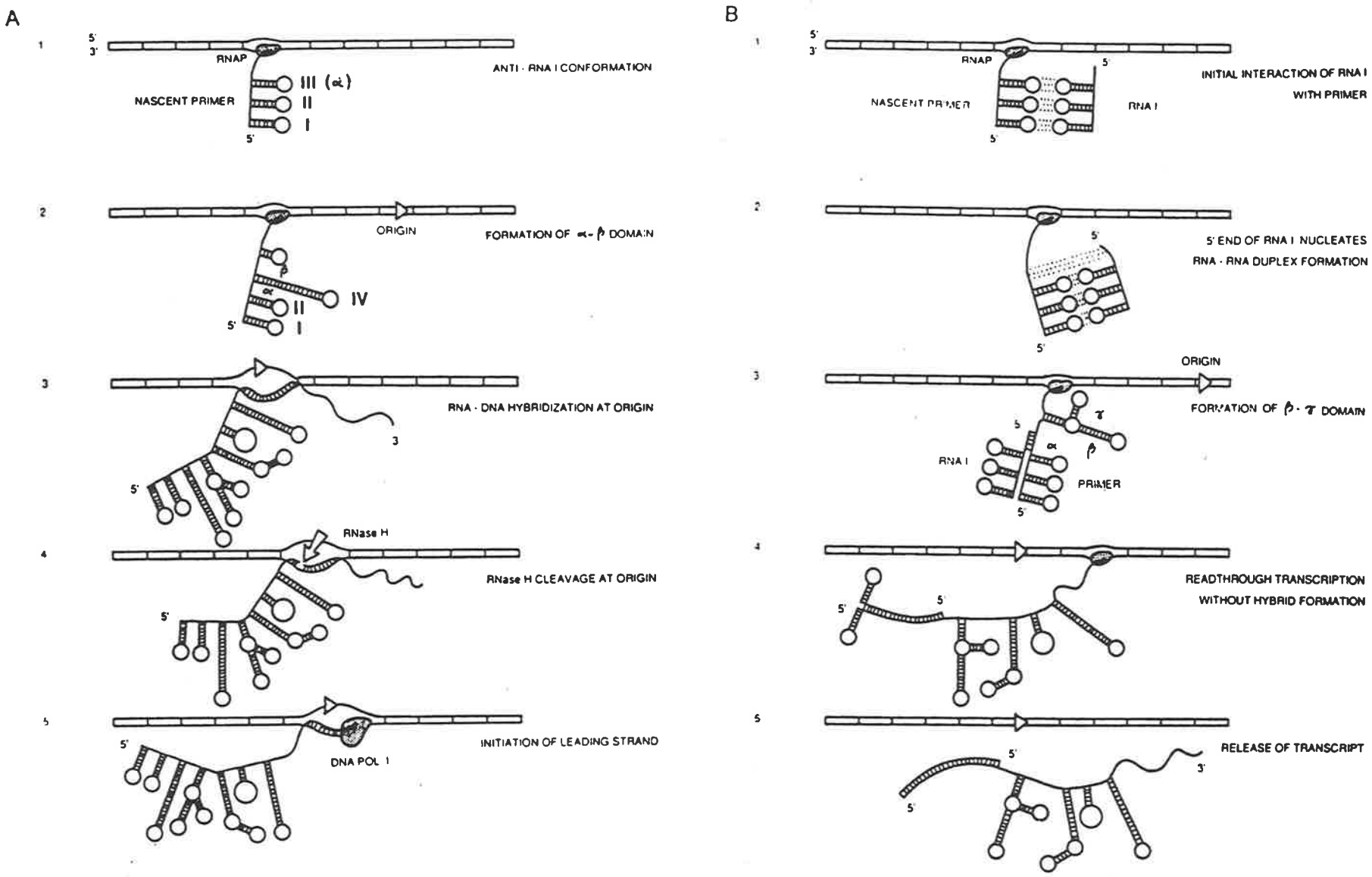
This type of control, which was called the inhibitor-target mechanism (Novick, 1987), is found in basic replicons of some small plasmids, such as ColE1 and related plasmids (Polisky *et al.*, 1985), which included p15A, pMB1, RSF1010, CloDF13 (Selzer *et al.*, 1983) and NTP16 (Lambert *et al.*, 1987), in the staphylococcal plasmid pT181 and its relatives (Kumar and Novick, 1985; Novick *et al.*, 1985), and in basic replicons of large conjugative plasmids belonging to the IncF incompatibility groups (Nordström *et al.*, 1984; Rownd *et al.*, 1985; Bergquist *et al.*, 1986; Saadi *et al.*, 1987). Several multicopy cloning vectors such as the well-known plasmids pACYC184 and pBR322 are derived from p15A and pMB1 (Chang and Cohen, 1978; Balbas *et al.*, 1986).

FIGURE 1.3 Major secondary structural features of RNAs involved in ColE1 replication control.

(Panel A) Primer formation and DNA strand initiation. (1) Initial elongation of the primer precursor: the transcript assumes a conformation consisting of three stem-loop domains. The 3′-proximal domain is called α. (2) Further elongation results in structural alteration due to interaction of the α and β domains to form stem-loop IV, which eliminates stem-loop III. Stem-loop IV formation precludes interaction of the β domain with other downstream primer domains. (3) The primer precursor forms a persistent hybrid in the origin region. (4) RNAase H cleaves the hybridized RNA at specific loci. (5) DNA polymerase (POL I) adds dNTPs to the processed primer terminus. RNAP, RNA polymerase.

(Panel B) Inhibition of primer precursor processing by RNA I. (1) RNA I interacts with nascent primer in a reversible reaction between complementary nucleotides located in loop regions. (2) Full-length pairing between RNA I and primer is nucleated from the 5′ terminus of RNA I. (3) Pairing precludes interaction of the α and β domains; the β domain is free to interact with downstream domain, γ. This interaction alters other, but not all, downstream primer domains. (4) Because of altered RNA conformation, hybrid formation does not occur. (5) Primer transcript is released from the template.

The Figure is taken from Polisky (1988).


A
1
5'
3'
RNAP
III (α)
II
I
NASCENT PRIMER
5'
ANTI - RNA I CONFORMATION
2
ORIGIN
FORMATION OF α - β DOMAIN
β
α
II
IV
I
5'
3
RNA - DNA HYBRIDIZATION AT ORIGIN
3
5'
4
RNase H
RNase H CLEAVAGE AT ORIGIN
5'
5
5'
DNA POL I
INITIATION OF LEADING STRAND
B
1
5'
3'
RNAP
5'
NASCENT PRIMER
RNA I
5'
INITIAL INTERACTION OF RNA I
WITH PRIMER
2
5'
5'
5' END OF RNA I NUCLEATES
RNA - RNA DUPLEX FORMATION
3
ORIGIN
FORMATION OF β - τ DOMAIN
5'
τ
α
β
RNA I
PRIMER
5'
4
5'
5'
READTHROUGH TRANSCRIPTION
WITHOUT HYBRID FORMATION
5
5'
5'
3'
RELEASE OF TRANSCRIPT

In the ColE1-like plasmids, the counter-transcipt RNA acts by preventing the processing of the preprimer RNA by ribonuclease H (RNaseH) (Itoh and Tomizawa, 1980). In the case of the large conjugative plasmids and pT181, the counter-transcript RNA binds to the mRNA leader sequences and prevents the translation of a Rep protein which is required and rate-limiting for replication (Stougaard *et al.*, 1981; Kumar and Novick, 1985). In all three systems, the counter-transcript RNA is able to act *in trans* and is responsible for the expression of an incompatibility phenotype.

The replication of ColE1 is initiated in a 600 bp region, the origin (*ori* or *oriV*), and progresses unidirectionally in the θ-shaped manner of Cairns-type replication (Inselberg, 1974; Backman *et al.*, 1978; Oka *et al.*, 1979; Tomizawa and Masukata, 1987). For the whole process, ColE1 requires only proteins encoded by the host bacterium, *E. coli* K-12 (Tomizawa *et al.*, 1974; Donoghue and Sharp, 1978). For initiation at the origin, a DNA-dependent RNA polymerase, RNase H, and DNA polymerase I (PolI), as well as DNA gyrase and topoisomerase I, are necessary (Itoh and Tomizawa, 1978, 1980; Hillenbrand and Staudenbauer, 1982; Minden and Marians, 1985). Gyrase participates in the opening of the DNA double strand and may provide the topological driving force for movement of the replication fork (Orr and Staudenbauer, 1981; Minden and Marians, 1985). By the modulation of the degree of plasmid superhelicity, topoisomerase I may favour the recognition of the primer promoter by RNA polymerase to make the primer transcript (Minden and Marians, 1985). By the combined action of RNA polymerase and RNase H, a processed primer transcript is formed which is used by Pol I for the synthesis of the leading strand over a length of about 400 nucleotides (Sakabiara and Tomizawa, 1974; Itoh and Tomizawa, 1980). DNA polymerase III (Pol III), single-stranded binding proteins, DnaB helicase, DnaB-complexed DnaC protein, DnaG primase, and the preprimosome organiser i, n, n′, n′′ are essential for extension of the leading strand and also for the discontinuous synthesis of the lagging strand (Staudenbauer *et al.*, 1979; Fouser and Bird, 1983; Minden and Marians, 1985, 1986; Masai *et al.*, 1986).

In ColE1, transcription of preprimer RNA begins about 500 bases upstream of the replication origin (Tomizawa *et al.*, 1974). To initiate DNA replication, the preprimer RNA must hybridize to its DNA template within *oriV*. This RNA-DNA hybrid serves as a substrate for RNase H, which cleaves the preprimer RNA to form the primer onto which deoxynucleotides are added (Itoh and Tomizawa, 1980).

ColE1 is negatively regulated by RNA I, a small untranslated RNA molecule encoded within the DNA region that is used to transcribe the RNA primer, but in the opposite direction to the primer (Hashimoto-Gotoh and Inselbury, 1979; Oka *et al.*, 1979; Shepard *et al.*, 1979; Itoh and Tomizawa, 1980). Because RNA I is complementary to the preprimer RNA, the two can hybridize. When the preprimer RNA hybridizes to RNA I, it cannot hybridize to the DNA and therefore cannot be used to form a primer (Itoh and Tomizawa, 1980; Tomizawa and Itoh, 1981; Tomizawa *et al.*, 1981).

In addition to the counter-transcript RNA, plasmids ColE1 and R1 code for proteins which modulate the control of replication. These proteins repress transcription of the precursor for primer RNA and therefore regulate the copy number of the plasmid. For ColE1, the gene for this protein has been designated *rop* or *rom* (Cesareni *et al.*, 1982; Som and Tomizawa, 1983); for the IncFII plasmids, like R1, it is called *copB* (Molin *et al.*, 1981) or *repA2* (Liu *et al.*, 1983; Dong *et al.*, 1985).

1.4.4 Iteron mediated replication

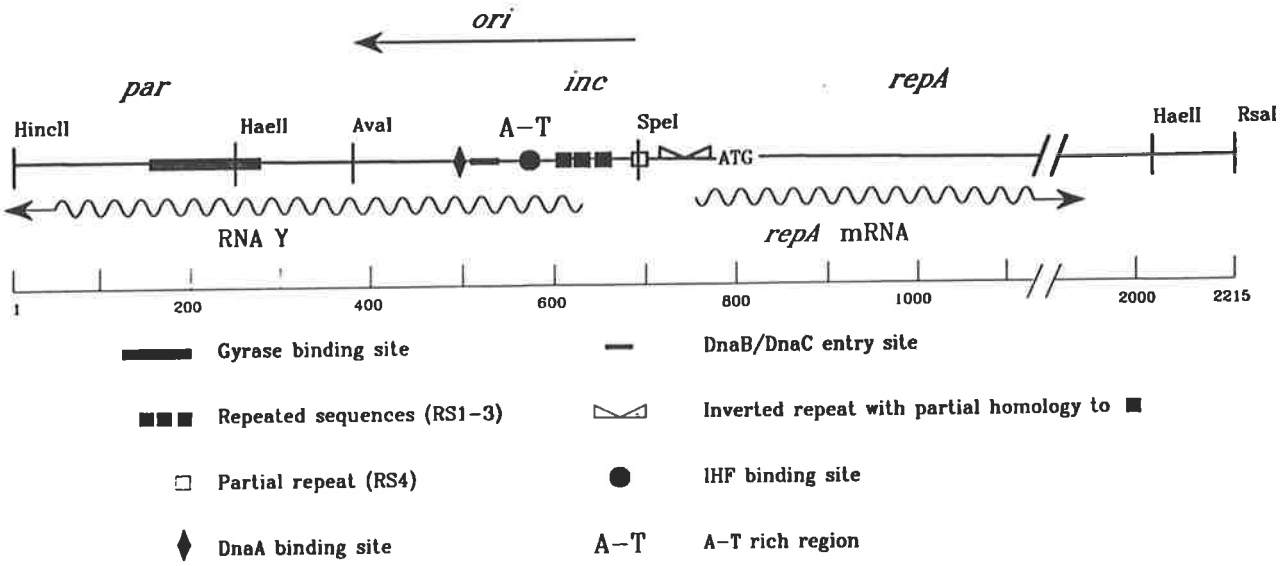
The second group of basic replicons uses a series of direct DNA repeats, called iterons, each about 20 bp long, as the main incompatibility determinant. These replicons show similarities with regard to the location of the repeats near the origin of replication and near a *rep* gene, to the size of the Rep protein (between 29 and 40 kDa), to the binding sites for the DnaA protein, and to A+T-rich sequences (Scott, 1984; Linder *et al.*, 1985; Filutowicz *et al.*, 1987). This type of replicon structure is found in the plasmids F (RepFIA replicon) (Kline, 1985), P1 (Chattoraj *et al.*, 1984, 1985), R6K (Filutowicz *et al.*, 1985), RK2 (Stalker *et al.*, 1982), pSC101 (Linder *et al.*, 1985), and $R_{ts}1$ (Itoh *et al.*, 1987).

The most thoroughly-studied plasmid from this group is pSC101. This plasmid which was originally isolated from *Salmonella panama*, was used as a cloning vector in the first recombinant DNA experiment (Cohen *et al.*, 1973; Cohen and Chang, 1977). It has a relatively low copy number of six to seven copies per chromosome and is extremely stable, confering resistance to tetracycline on its host.

Replication of the 9,263 bp pSC101 (Bernardi and Bernardi, 1984) is unidirectional, and starts at a unique origin (Cabello *et al.*, 1976; Yamaguchi and Yamaguchi, 1984b) (Figure 1.4). In contrast to the ColE1-type plasmids, pSC101, as well as other plasmids with a *rep* gene, do not require Pol I for replication initiation (Cabello *et al.*, 1976; Lane, 1981; Scott, 1984). The basic replicon, which has a maximum size of 1.3 kb, consists of a 250 bp *cis*-acting segment which constitutes the origin and a sequence of about 1 kb encoding the 37.5 kDa initiation protein RepA (Churchward *et al.*, 1983; Linder *et al.*, 1983; Vocke and Bastia, 1983a; Armstrong *et al.*, 1984; Yamaguchi and Yamaguchi, 1984a, 1984b). The chromosomal replication origin (*oriC*) of *E. coli* K-12 and other members of the family *Enterobacteriaceae* show limited but significant homologies to the *cis*-acting fragment of pSC101 (Armstrong *et al.*, 1984; Yamaguchi and Yamaguchi, 1984a). The homologous region consists of a 13 bp repeat, described by Bramhill and Kornberg (1988) as the entry sites for the DnaB/DnaC complex to initiate the opening of the duplex, together with the binding site for the DnaA protein (Fuller *et al.*, 1984). The 13-mer repeat is part of an 82 bp 84% A+T-rich region containing a binding site for the integration host factor (IHF) (Gamas *et al.*, 1986), that forms a hinge between regions of poly(dA) and poly(dT) (Koo *et al.*, 1986; Tan and Harvey, 1987) which may melt rapidly (Wada and Suyama, 1986). Stenzel *et al.* (1987) showed that IHF bent pSC101 DNA at this site and that the integrity of the site is essential for replication. Three 18 bp iterons which act as binding sites for the plasmid-encoded RepA protein (Vocke and Bastia, 1983a, 1983b) are located immediately adjacent to the A+T-rich region (Vocke and Bastia, 1983a; Armstrong *et al.*, 1984; Yamaguchi and Yamaguchi, 1984a). Additional binding sites for the RepA protein are

FIGURE 1.4 Genetic organization of the pSC101 *Hinc*II-*Rsa*I fragment containing the essential origin of replication.

The promoter of RNA Y overlaps the repeated sequences RS2 and RS3 and that for *repA* overlaps the inverted repeat upstream of the gene. The 3′ end of the *repA* transcript has not been determined. A termination of RNA Y occurs at the location shown in the Figure, and another one is found further downstream. The Figure is taken from Manen and Caro (1991).


ori
par
inc
repA
HincII
HaeII
AvaI
A–T
SpeI
ATG
HaeII
RsaI
RNA Y
repA mRNA
1
200
400
600
800
1000
2000
2215
Gyrase binding site
DnaB/DnaC entry site
Repeated sequences (RS1–3)
Inverted repeat with partial homology to ■
Partial repeat (RS4)
IHF binding site
DnaA binding site
A–T
A–T rich region

found outside the origin in front of the *repA* gene (Linder *et al.*, 1985; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985).

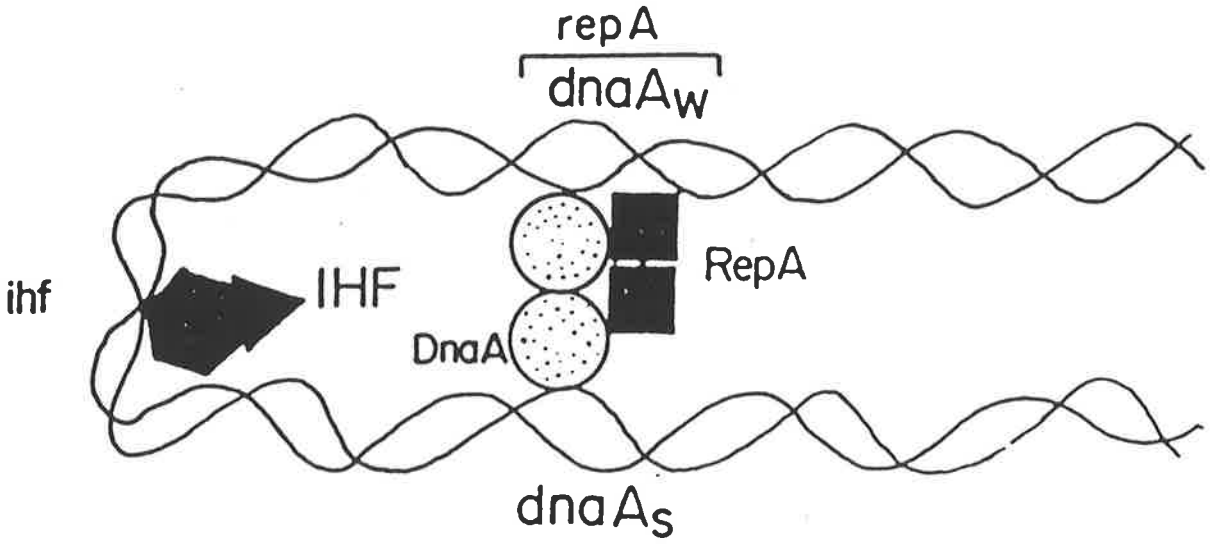
The RepA protein functions positively in replication by binding to the three direct repeats in the origin (Vocke and Bastia, 1983b; Yamaguchi and Masamune, 1985). This binding may be the beginning of a replisome formation as is seen at the *E. coli* K-12 chromosomal *oriC* (Funnel *et al.*, 1987; Sekimizu *et al.*, 1987; Bramhill and Kornberg, 1988). To form a correct protein-protein and protein-DNA complex, RepA may interact with host proteins like DnaA (Fuller *et al.*, 1984). RepA could also direct such essential host proteins to the start site of replication.

For replication initiation, the host DnaA protein is essential not only for the *E. coli* K-12 chromosome but also for pSC101 (Felton and Wright, 1979; Frey *et al.*, 1979; Hasunuma and Sekiguchi, 1979; Fuller *et al.*, 1984; Bramhill and Kornberg, 1988). Specific binding of DnaA to its recognition site has been demonstrated for the chromosomal origin and for the origin of pSC101 (Fuller *et al.*, 1984). With respect to *oriC*, the DnaA protein seems to be involved with the correct RNA-primer formation by RNA polymerase and/or DnaG primase (Messer, 1987; Rokeach *et al.*, 1987; Seufert and Messer, 1987).

Stenzel *et al.* (1991) have discovered that DNA bending of pSC101 induced by IHF *in vitro* promotes the interaction of the DnaA protein with two physically separated binding sites called $dnaA_S$ and $dnaA_W$. The cooperative interaction at a distance, most probably causes looping out of the *ihf* site. They also showed that the binding of RepA protein to its cognate sites promoted enhanced binding of the DnaA protein to the physically distant $dnaA_S$ site, probably also by DNA looping. The addition of RepA to a binding reaction containing IHF and DnaA further enhances the binding of DnaA protein to the $dnaA_S$ site. Thus, the three DNA-binding proteins interact with the origin, generating a higher order structure *in vitro*. On the basis of the known requirement for all three proteins for replication initiation, a model has been proposed for the structure of a preinitiation complex at the replication origin (Figure 1.5).

FIGURE 1.5 DNA looping model for the structure of the preinitiation complex at the replication origin of pSC101.

A simplified model showing the relative locations of the *dnaA*$_S$, *dnaA*$_W$, and *repA* sites at the origin and the observed interaction by DNA looping between the *dnaA*$_S$ and *dnaA*$_W$/*repA* sites in the presence of IHF, RepA, and DnaA, is given. The model shows looping out of the *ihf* site, implying that IHF-induced DNA bending promotes protein-protein interaction without direct contact between IHF and DnaA. The Figure is taken from Stenzel *et al.* (1991).


repA
dnaAw
ihf
IHF
DnaA
RepA
dnaAs

The RepA protein negatively regulates the initiation of pSC101 replication by its intracellular concentration (Linder *et al.*, 1985; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985). The promoter region of the *repA* gene is overlapped by a palindromic arrangement of three repeats (Yamaguchi and Yamaguchi, 1984a; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985), which are similar to the sequences of the iterons within the origin (Vocke and Bastia, 1983a; Armstrong *et al.*, 1984; Yamaguchi and Masamune, 1985). By binding to the inverted repeats, RepA competes with RNA polymerase for the *repA* promoter sequence and inhibits the transcription of *repA* by autoregulation (Linder *et al.*, 1985; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985). Since the binding of RepA to the promoter region has priority over binding to the origin (Vocke and Bastia, 1983b), the concentration of RepA can be maintained below a critical level when the correct plasmid copy number is reached (Vocke and Bastia, 1985). The iterons may also control replication in a different way by involving steric hindrance resulting from folding of the replicon around the *ori* region (Nordström, 1990). The steric hindrance model of replication is supported by the demonstration that replication of plasmid RK2 is inhibited *in vitro* as well as *in vivo* by the addition of iteron-containing DNA; this inhibition is not relieved by even drastic increases in the concentration of the Rep protein TrfA (Kittell and Helinski in press; cited by Nordström 1990).

Apart from regulation of the copy number, the RepA protein determines the incompatibility between pSC101 and related plasmids by binding to the three iterons of the origin (Churchward *et al.*, 1983; Linder *et al.*, 1983; Yamaguchi and Yamaguchi, 1984a). Such an incompatibility mechanism can be explained by a passive adsorption of replication proteins according to the titration model of Tsutsui *et al.* (1983).

1.5 Processing of plasmid DNA during bacterial conjugation

Bacterial conjugation is a highly specific process whereby DNA is transferred from donor to recipient bacteria by a mechanism involving cell-to-cell contact. This process is usually encoded by conjugative plasmids, which have been isolated from a diverse range of Gram-negative bacteria and include members of more than 20

incompatibility groups (Bukhari *et al.*, 1977; Datta, 1979; Bradley, 1980). It is a particularly important mechanism of genetic exchange, since transfer can occur not only between members of the same species but also between unrelated organisms. Plasmid-encoded conjugative pili are necessary for establishing cell-to-cell contact so that DNA can be transferred, either by retraction of the pili to bring the cells into wall-to-wall contact (Manning and Achtman, 1979; Willetts, 1980), or by fusion of the cell envelopes locally so that a transmembrane "pore" is formed. The general model for initiation of conjugative DNA synthesis proposes that cleavage at the *nic* site within the plasmid transfer origin (*oriT*) allows the creation of a single strand by subsequent strand displacement through the rolling-circle-type of replication. A prerequisite for the initial nicking reaction is the formation of a specialized nucleoprotein structure at *oriT*, the relaxosome.

The *oriT* site was first recognized on the F plasmid by deletion mapping experiments and was located at one end of the transfer region (Willetts, 1972; Guyer and Clark, 1976; Guyer *et al.*, 1977). Similar experiments indicated that IncP (RK2 and RP4) and IncN (R46 and N3) plasmids also possess an *oriT* region located in analogous positions with respect to their conjugation genes (Guiney and Helinski, 1979; Brown and Willetts, 1981; Al-Doori *et al.*, 1982).

The RK2/RP4 *oriT* region has been identified and subcloned as a 250 bp sequence (Guiney *et al.*, 1988; Pansegrau *et al.*, 1988). The site of the single strand nick, believed to be the initiation site for DNA transfer, is located 8 bp from an imperfect 19 bp inverted repeat (Pansegrau *et al.*, 1990b). The plasmid-encoded TraJ protein, which is essential for transfer, binds to the proximal arm of the inverted repeat (Ziegelin *et al.*, 1989). During the initiation of conjugative transfer of DNA, the proteins TraI, TraJ, and TraH interact with and assemble the relaxosome at *oriT* (Pansegrau *et al.*, 1990a). The data indicate that TraI recognizes specific bases in the region between the inverted repeat and the *nic* site, since mutations in these bases abolished nicking but do not affect TraJ binding (Waters *et al.*, 1991). TraJ binding may alter the DNA conformation, allowing TraI to interact with the *nic* region (Waters *et al.*, 1991). Site-specific nicking results in

the covalent attachment of the TraI protein to the 5′-terminal nucleotide of the nicked strand (Pansegrau *et al.*, 1990b). TraH stabilizes the TraJ·TraI·*oriT* relaxosome structure via protein-protein interactions (Pansegrau *et al.*, 1990a).

After nicking of the strand destined for transfer at *oriT*, the two DNA strands of a plasmid must be unwound to allow transmission of a single strand to the recipient cell. Analysis of Hfr or F-prime donor strains shows that a single strand of DNA is transferred with a leading 5′ terminus (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968; Ihler and Rupp, 1969). By selectively labelling DNA in either the donor or the recipient cell, it was demonstrated that DNA is synthesised on the transferred F strand in the recipient (Ohki and Tomizawa, 1968; Vapnek and Rupp, 1970) and that the strand retained in the donor serves as a template for DNA synthesis to replace that transferred (Vapnek and Rupp, 1970, 1971). Although the rolling-circle model for DNA transfer (Gilbert and Dressler, 1968) predicts transfer of a linear single-stranded DNA of greater than unit length, it has been concluded that plasmids are transferred as discrete strands of unit length (Willetts and Wilkins, 1984). If so, a mechanism must exist for the precise religation of the 5′ and 3′ termini at *oriT*. It has been proposed that upon nicking at *oriT*, the 5′ terminus would be covalently linked to a membrane protein, and after completion of DNA transfer, this protein would recognize the 3′ terminus and ligate it to the 5′ terminus (Willetts and Wilkins, 1984).

1.6 Aims of this thesis

There has been very little work performed to study *Campylobacter* species at the molecular level. Most of this work has generally concentrated on the human pathogens *C. jejuni* and *C. coli*, and to a lesser extent *C. fetus*. Attempts to clone and express genes from *Campylobacter* species in *E. coli* K-12 have met with limited success. To facilitate the analysis of gene expression of *Campylobacter* species, a plasmid shuttle vector, pILL550, has been constructed which can be mobilized from *E. coli* K-12 into a number of campylobacters. Studies have also been performed to characterize the surface structures which comprise the outer membranes of *C. jejuni*, *C. coli*, and *C. fetus*.

No genetic study of *C. hyointestinalis* has yet been undertaken. It has not been demonstrated whether pILL550 is a suitable cloning vector for *C. hyointestinalis*. A detailed examination of the surface structures of *C. hyointestinalis* has not been performed.

The objectives of this study are to characterize the components of the outer membrane of *C. hyointestinalis* strains and to construct a *C. hyointestinalis* plasmid shuttle vector which can be mobilized efficiently into *C. hyointestinalis* and be utilized for the cloning and expression of *C. hyointestinalis* genes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth media

The following nutrient media were used for bacterial cultivation. Nutrient broth (NB) (Difco), prepared at double strength (16 g/l) with added sodium chloride (NaCl) (5 g/l) or Luria broth (LB), were the general growth media for *E. coli* K-12 strains. Luria broth (LB) is composed of bacto-tryptone (10 g/l) (Difco), bacto-yeast (5 g/l) (Difco) and NaCl (5 g/l). 2 x YT medium was prepared as described by Miller (1972). Minimal A medium (M13 minimal media) was also prepared as described by Miller (1972) and supplemented prior to use with $MgSO_4$, glucose and thiamine-HCl to concentrations of 0.2 mg/ml, 2 mg/ml and 50 µg/ml, respectively.

Nutrient agar (NA) is composed of Lab-Lemco powder (Oxoid) (10 g/l), peptone (Oxoid) (10 g/l), NaCl (5 g/l) and Agar (Media Makers) (15 g/l). Soft agar contains equal volumes of NB and NA. H agar consisted of bacto-tryptone (16 g/l) (Difco), NaCl (8 g/l) and bacto-agar (12 g/l) (Difco). H top agar was like H agar but contained 8 g/l bacto-agar.

Antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap) 25 µg/ml; kanamycin (Km) 50 µg/ml; tetracycline (Tc) 10 µg/ml for *E. coli* K-12 and *S. typhimurium* strains. Nalidixic acid (Na) 50 µg/ml; cephalothin (Cp) 15 µg/ml; vancomycin (Vm) 10 µg/ml; polymyxin B (Pmb) 5 µg/ml; trimethoprim (Tm) 5 µg/ml; streptomycin (Sm) 100 µg/ml; kanamycin (Km) 50 µg/ml were used for *Campylobacter* species.

Incubations were at 37°C unless otherwise specified. Normally, liquid cultures were grown in 20 ml McCartney bottles.

Campylobacter strains were grown on NA with 5% defibrinated sheep blood, or on Muller-Hinton-II agar (BBL), and incubated in a microaerophilic environment (10% CO_2, 85% N_2, 5% O_2) in gas jars using a Gas Generating Kit sachet (Oxoid) with an O_2-absorbing catalyst for 48 h at 37°C.

2.2 Chemicals and reagents

Chemicals were Analar grade. Phenol, polyethylene glycol-6000 (PEG), sodium dodecyl sulphate (SDS) and sucrose were from BDH Chemicals. Tris was Trisma base from Boehringer Mannheim. Caesium chloride (Cabot) was technical grade. Ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA) was Analar analytical grade from Ajax Chemicals. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was from Aldrich. Sodium lauryl sarcosinate (Sarkosyl) was from Ciba-Geigy Corp.

Antibiotics were purchased from Sigma (ampicillin, cephalothin, kanamycin sulphate, nalidixic acid, polymyxin B, trimethoprim, vancomycin), and Calbiochem (tetracycline). All other anti-microbial agents (antibiotics) were purchased from Sigma Chemical Co., BDH Chemicals Ltd., Glaxo or Calbiochem.

The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulphate (Bio-Rad), ultra pure N, N′-methylene bis-acrylamide and urea (BRL).

The four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and their corresponding dideoxy-ribonucleotide triphosphate homologues (ddATP, ddCTP, ddGTP and ddTTP), were obtained from Boehringer-Mannheim. Adenosine-5′-triphosphate, sodium salt (ATP), herring sperm DNA and dithiothreitol (DTT) were obtained from Sigma. X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were purchased from Boehringer-Mannheim.

M13 sequencing primer and [^{32}P]-dCTP, at a specific activity of 1,700 Ci/mMole were obtained from BRESATEC (Adelaide). The -35 sequencing primer was obtained from New England Biolabs. [^{35}S]-Methionine (1,270 Ci/mmole), and [^{35}S]-dATP (>1000 Ci/mmole) were purchased from Amersham. Sequenase™ was purchased from United States Biochemical Corp.

2.3 Enzymes

Deoxyribonuclease I (DNase I) and lysozyme were obtained from Sigma. Pronase and Proteinase K were from Boehringer-Mannheim.

Restriction endonucleases *Aat*II, *Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Kpn*I, *Hin*dIII, *Not*I, *Pst*I, *Pvu*I, *Pvu*II, SphI, *Xba*I, and *Xho*I, were purchased from either Boehringer-Mannheim, New England Biolabs, Pharmacia or Amersham.

DNA modifying enzymes were purchased from the following suppliers: New England Biolabs (T4 DNA ligase), Pharmacia (Reverse transcriptase), Amersham (T4 DNA polymerase) and Boehringer-Mannheim (DNA polymerase I, Klenow fragment of DNA polymerase I, Polynucleotide kinase, and molecular biology grade calf intestinal alkaline phosphatase).

2.4 Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides (oligos) were synthesized using reagents purchased from Applied Biosystems or Ajax Chemicals (acetonitrile). Synthesis was performed on an Applied Biosystems 381A DNA synthesizer. Oligos were routinely of a purity such that no further purification was required.

2.5 Maintenance of bacterial strains

For long term storage, all strains were maintained as lyophilized cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to a 10 ml bottle of NB and incubated with shaking overnight at the

appropriate temperature. The other half was streaked onto two nutrient agar plates and incubated overnight at the appropriate growth temperature. Antibiotics were added to the media when appropriate. If the colony morphology was uniform, single colonies were selected and picked from these plates for subsequent storage or use. Short-term storage of strains in routine use was as a suspension of freshly grown bacteria in glycerol (32% v/v) and peptone (0.6% w/v) at -70°C. Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension onto a nutrient agar, or blood agar plate (with or without antibiotic as appropriate) followed by incubation overnight just prior to use.

Bacterial strains were prepared for long-term storage by suspension of several colonies in a small volume of sterile skimmed milk. Approximately 0.2 ml aliquots of this thick bacterial suspension were dispensed into sterile 0.25 x 4 in freeze-drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilized in a freeze-drier. After the vacuum was released, the cotton wool plugs were pushed well down the ampoule and a constriction was made just above the level of the plug. The ampoules were evacuated to a partial pressure of 30 microns and then sealed at the constriction without releasing the vacuum. Finally, the ampoules were labelled and stored at 4°C.

2.6 Bacterial strains and plasmids

The *Campylobacter* strains used are listed in Table 2.1. Table 2.2 describes the *Escherichia coli* K-12, *Salmonella typhimurium* LT2, and *Enterococcus faecalis* strains used in this study. The plasmid cloning vectors which were used in this study are listed in Table 2.3.

C. hyointestinalis isolates 45104, 48138, 48869, 49226, and 49905, were obtained from the Institute for Medical and Veterinary Science, Adelaide, Australia. All Australian isolates had been obtained from 1-2 year old Aboriginal children presenting at the Alice Springs Hospital, between December 1984 and January 1986, with diarrhoea. *C. jejuni* was also cultured from the stool of the individual from which isolate 49905 was

TABLE 2.1 Strains of *Campylobacter* used in this work

Strain	Source
C hyointestinalis NCTC 11608	NCTC
C hyointestinalis NCTC 11562	NCTC
C hyointestinalis NCTC 11563	NCTC
C hyointestinalis NCTC 11609	NCTC
C hyointestinalis NCTC 11610	NCTC
C hyointestinalis 45104	IMVS, Adelaide
C hyointestinalis 48138	IMVS, Adelaide
C hyointestinalis 48869	IMVS, Adelaide
C hyointestinalis 49226	IMVS, Adelaide
C hyointestinalis 49905	IMVS, Adelaide
C. coli NCTC 11366	NCTC
C. jejuni NCTC 11168	NCTC
C. fetus subsp. *fetus* NCTC 10842	NCTC
C. fetus subsp. *venerealis* NCTC 10342	NCTC

NCTC: National Collection of Type Cultures, Colindale, London, England.

IMVS: Institute of Medical and Veterinary Science, Adelaide, Australia.

TABLE 2.2 Non-*Campylobacter* strains used in this work

Strain	Genotype/phenotype	Source
Escherichia coli K-12 strains		
DH1	F$^-$, *gyrA-96, recA-1, relA-1, endA-1, thi-1, hsdR-17, supE-44,* λ^-	B. Bachmann
DS410	F$^-$, *minA, minB, rpsL*	D. Sherratt
JM101	F′ [*traD-36, proA, lacI^q, lacZ,* ΔM15], *supE, thi-1,* Δ[*lac-proA,B*]	A. Sivaprasad
S17-1	RP4-2-Tc::Mu-Km::Tn*7*/ *pro, hsdR*	U. Priefer
CB617	R64-11/*gal, thi, thr, endA, hsdR4, hsdM*$^+$	M.Bagdasarian
Non-*E. coli* K-12 strains		
Salmonella typhimurium C5		J. Hackett
S. typhimurium LT2 SL2981	*zzc-65*::Tn*10* (pSLT::Tn*10*)	B.A.D. Stocker
Enterococcus faecalis CG110	[chromosomal Tn*916*]	D. Clewell

TABLE 2.3 Plasmids and bacteriophage cloning vehicles used in this work

Plasmid/bacteriophage	Antibiotic-resistance marker	Reference
pHC79	Ap, Tc	Hohn and Collins (1980)
pPM2101	Tc	Sharma *et al.* (1989)
pILL550	Km	Labigne-Roussel *et al.* (1987)
RP4	Ap, Km, Tc	Datta *et al.* (1971)
pKT230	Km, Sm	Bagdasarian *et al.*(1981)
pKT231	Km, Sm	Bagdasarian *et al.*(1981)
pcos2EMBL	Km, Tc	Poustka *et al.* (1984)
pGEM5zf(+)	Ap	Promega Notes 10 (1987)
M13mp18	Ap	Messing and Vieira (1982)

obtained, while *E. coli* expressing heat-labile or heat-stable toxins were cultured from the stools of the children yielding isolates 45104 or 48869, respectively. The stool from which isolate 48318 was obtained was not examined for the presence of aerobic bacterial enteropathogens. The stools from which isolates 49226 and 49905 were obtained were negative for aerobic bacterial enteropathogens.

2.7 Transformation procedure

Transformation was performed essentially according to the method described by Brown *et al.* (1979). *E. coli* K-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaken culture (in NB) was diluted 1:20 into LB and incubated with shaking until the culture reached an OD of 0.6 (4×10^8 cells/ml). The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in a half-volume of cold 100 mM $MgCl_2$, centrifuged again, and resuspended in a tenth-volume of cold 100 mM $CaCl_2$. The suspension was allowed to stand for 60 min on ice before addition of DNA. Competent cells (0.2 ml) were then mixed with DNA [volume made to 0.1 ml with 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)] and left on ice for a further 30 min. The cell/DNA mixture was heated at 42°C for 2 min and then 3 ml NB was added followed by incubation with shaking at 37°C for 1-2 h. The culture was then plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were included as a control.

2.8 Electroporation procedure

Electroporation of *Campylobacter* species was performed as described by Miller *et al.* (1988). *Campylobacter* cells were grown for 16 h and harvested in ice-cold electroporation buffer (272 mM sucrose, 15% (w/v) glycerol (Ajax), 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4; pH 7.4) washed, and gently resuspended in the same buffer at a concentration of 5×10^9 cells/ml. Some 100 µl of cells were mixed with 1 µl (0.5 µg) of plasmid DNA and dispensed into a 0.2 cm electroporation cuvette (Bio-Rad). Cuvettes were pulsed using a Gene Pulser apparatus (Bio-Rad) with 2500 V from a 25 µF capacitor

and with a Pulse Controller set at a resistance of 200 Ω. Cuvettes were then placed on ice for 5 min. The cell suspensions were taken from the cuvettes and spread onto blood agar plates containing selective antibiotics and incubated under microaerophilic conditions for 48 h at 37°C.

2.9 Natural transformation procedure

Campylobacter transformation was performed by a modification of either the agar surface or biphasic method described by Wang and Taylor (1990b):

Method 1: For transformation on the surface of blood agar, fresh recipient cells (16 h growth on blood agar) were harvested in normal saline and resuspended at a concentration of about 10^8 cells/ml and mixed with plasmid DNA (10 μg in 10 μl) and spread onto blood agar. The plates were incubated for 8 h at 37°C in microaerophilic conditions. The cells were then harvested in normal saline (2 ml) and aliquots were plated onto selection plates directly or the culture was concentrated by centrifugation and plated.

Method 2: For transformation in a biphasic system, cell suspensions (5 x 10^8 cells/ml of Mueller-Hinton Broth) were transferred (0.2 ml per tube) to Eppendorf tubes containing 1.0 ml Mueller-Hinton agar. Plasmid DNA samples were added and the tubes incubated for 8 h at 37°C in microaerophilic conditions. The aqueous phase harbouring the cells was harvested and transformants were selected on blood agar plates containing appropriate antibiotics as described previously.

2.10 Bacterial conjugation

Overnight broth cultures of *E. coli* K-12 donor strains were grown in NB and diluted 1:20 and grown to early exponential phase with slow agitation. *Campylobacter* recipient strains were grown for 16 h on blood agar plates and harvested in saline (1ml).

Donor and recipient bacteria were mixed at a ratio of 1:10 and the cells pelleted by centrifugation (5000 rpm, 5 min, bench centrifuge). The pellet was gently resuspended in 200 µl of broth and spread onto a cellulose acetate membrane filter (0.45 µm, type HA, Millipore Corp.) on a blood agar plate. This plate was incubated for 8 hr at 37°C in microaerophilic conditions where the conjugation involved *Campylobacter* strains. The cells were then resuspended in 10 ml saline and samples plated onto selective agar and incubated overnight at 37°C in microaerophilic conditions when selecting for *Campylobacter* transconjugants.

2.11 Plasmid DNA extraction procedures

Plasmid DNA was isolated by one of the three following procedures:

Method 1: Triton X-100 cleared lysates were prepared from 10 ml overnight cultures by a modification of the procedure of Kahn *et al.* (1979). Cells were resuspended in 0.4 ml 25% (w/v) sucrose in 50 mM Tris-HCl, pH 8.0. Lysozyme (50 µl, 10 mg/ml freshly prepared in H_2O) and 50 µl of 0.25M EDTA, pH 8.0 were added to cells in Eppendorf tubes and left to stand on ice for 15 min. 0.5 ml TET buffer (50 mM Tris-HCl, 66 mM EDTA, pH 8.0, 0.4% Triton X-100) was added followed by a brief mixing by inversion of the tubes. The chromosomal DNA was then pelleted by centrifugation (20 min, 4°C, Eppendorf). The supernatant was extracted twice with TE saturated phenol (pH 7.5) and twice with diethyl-ether. Plasmid DNA was precipitated by the addition of an equal weight of propan-2-ol after the mixture was allowed to stand at -70°C for 30 min. The precipitate was collected (10 min, Eppendorf), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and resuspended in 50 µl 1 x TE buffer.

Method 2: Large scale plasmid purification was performed by the three step alkali lysis method (Garger *et al.*, 1983). Cells from a one litre culture were

harvested (6,000 rpm, 15 min, 4°C, GS-3, Sorvall) and resuspended in 24 ml solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). Freshly prepared lysozyme (4 ml of 20 mg/ml in solution 1) was mixed with the cell suspension and incubated at room temperature (RT) for 10 min. Addition of 55 ml of solution 2 (0.2 M NaOH, 1% (w/v) SDS), followed by a 5 min incubation on ice resulted in total lysis of the cells. After the addition of 28 ml solution 3 (5M potassium acetate, pH 4.8) and incubation on ice for 15 min, protein, chromosomal DNA and high molecular weight RNA were removed by centrifugation (8,000 rpm, 20 min, 4°C, GSA, Sorvall). The supernatant was then extracted with an equal volume of a TE-saturated-phenol, chloroform, isoamyl alcohol mixture (25:24:1). Plasmid DNA from the aqueous phase was precipitated with 0.6 volume of 100% (v/v) propan-2-ol at RT for 10 min and collected by centrifugation (10,000 rpm at 4°C, 35 min, GSA, Sorvall). After washing in 70% (v/v) ethanol, the pellet was dried *in vacuo* and resuspended in 4.8 ml TE. Plasmid DNA was purified from contaminating protein and RNA by centrifugation on a two step CsCl ethidium bromide gradient according to Garger *et al.* (1983). The DNA band was removed by side puncture of the tube with a 19 gauge needle attached to a 1 ml syringe. The ethidium bromide was extracted using isoamyl alcohol. CsCl was then removed by dialysis overnight against three changes of 2 litres 1 x TE at 4°C. DNA was stored at 4°C.

Method 3: Small scale plasmid purification was performed by the three-step alkali lysis method using a modification of the method of Garger *et al.* (1983). Overnight bacterial cultures (1.5 ml) or cells grown on blood agar plates (1 plate; harvested in 1 ml saline) were transferred to a microfuge tube, harvested by centrifugation (45 sec, Eppendorf), and resuspended in 0.24 ml solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM

EDTA). The addition of 0.55 ml solution 2 (0.2 M NaOH, 1% (w/v) SDS) followed by a 5 min incubation on ice resulted in cell lysis. After the addition of 0.28 ml solution 3 (5M potassium acetate, pH 4.8) and a 5 min incubation on ice, protein, chromosomal DNA and high molecular weight RNA were collected by centrifugation (90 sec, Eppendorf). The supernatant was transferred to a fresh tube and extracted once with TE-equilibrated phenol and once with diethyl ether. Plasmid DNA was precipitated by the addition of 2 volumes of 100% ethanol and a 2 min incubation at RT. The DNA was collected by centrifugation (15 min, Eppendorf), washed with 70% (v/v) ethanol and dried *in vacuo*. The pellet was resuspended in 50 µl 1 x TE.

2.12 Analysis and manipulation of DNA

2.12.1 DNA quantitation

The concentration of DNA in solutions was determined by measurement of absorption at 260 nm and assuming an A_{260} of 1.0 is equal to 50 µg DNA/ml (Miller, 1972).

2.12.2 Restriction endonuclease digestion of DNA

Cleavage reactions with the restriction enzyme *Acc*I, *Kpn*I, and *Xba*I were performed using Low-salt buffer (10 x: 100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol); digestions with *Bgl*II, *Cla*I, *Hin*dIII, and *Pvu*II used Medium-salt buffer (10 x: 0.5 M NaCl, 100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol); digestions with *Bam*HI, *Pst*I, *Eco*RI, *Eco*RV, *Not*I, *Pvu*I, *Sph*I, and *Xho*I used High-salt buffer (10 x: 1 M NaCl, 0.5 M Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol). Restriction digests with other enzymes were effected with these buffers also, but with the addition of either NaCl, KCl or Tris-HCl as described by the manufacturers. 0.1-0.5 µg of DNA or purified restriction fragments were incubated with

2 units of each restriction enzyme in a final volume of 20 µl, at 37°C, for 1-2 hr. The reactions were terminated by heating at 65°C for 10 min. Prior to loading onto a gel, a one tenth volume of tracking dye (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 0.1 mg/ml RNase A) was added.

2.12.3 Calculation of restriction fragment size

The sizes of restriction enzyme fragments were calculated by comparing their relative mobilities with those of *Eco*RI-digested *Bacillus subtilis* bacteriophage SPP1 DNA fragments. The sizes of the SPP1 *Eco*RI standard fragments were calculated by BRESATEC. The sizes (kilobases, kb) used were: 8.51; 7.35; 6.11; 4.84; 3.59; 2.81; 1.95; 1.86; 1.51; 1.39; 1.16; 0.98; 0.72; 0.48; 0.36.

2.12.4 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at RT on horizontal, 0.6%, 0.8% or 1% (w/v) agarose gels (Seakem HGT), 13 cm long, 13 cm wide and 0.7 cm thick. Gels were run at 100 V for 4-5 hr in either 1 x TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA, final pH 8.8), or 1 x TAE buffer (40 mM acetate, 40 mM Tris and 2 mM EDTA). After electrophoresis the gels were stained in distilled water containing 2 µg/ml ethidium bromide. DNA bands were visualized by trans-illumination with UV light and photographed using either Polaroid 667 positive film or 665 negative film.

For preparative gels Sea Plaque (Seakem) low-gelling-temperature agarose at a concentration of 0.6% (w/v) was used for separation of restriction fragments, which were recovered by the following methods:

Method 1: DNA bands were excised and the agarose melted at 65°C. Five volumes of 20 mM Tris-HCl, 1 mM EDTA, pH 8.0 buffer were added and the agarose extracted with phenol:water (1:1) and then phenol:chloroform (1:1). Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one tenth volume of

3 M sodium acetate, pH 5.0. DNA was collected by centrifugation (15 min, Eppendorf), washed once with 70% (v/v) ethanol and dried *in vacuo* before being resuspended in 1 x TE buffer.

Method 2: After electrophoresis the required DNA bands were excised and then placed inside dialysis tubing. This was then positioned in an electrophoretic tank filled with 1/2 x TAE buffer. A current was applied causing the DNA to move out of the gel and into the buffer contained in the dialysis tubing. The DNA was then precipitated with two volumes of ethanol and one tenth volume of 3 M sodium acetate, pH 5.0.

Method 3: After electrophoresis the required DNA bands were excised and placed in an Eppendorf tube containing siliconized glass wool covering a hole pierced in the bottom of the tube. This was inserted into another Eppendorf tube and centrifuged for 15 min at half the maximum speed. The DNA contained in the resulting solution was collected by precipitation as previously described.

2.12.5 Dephosphorylation of DNA using alkaline phosphatase

0.1-0.5 µg of digested plasmid DNA was incubated with 1 unit of alkaline phosphatase, for 30 min at 37°C. The reaction was terminated by the addition of EDTA, pH 8.0 to a final concentration of 3 mM followed by heating at 65°C for 10 min. The reaction mix was then extracted twice with hot (56°C) TE-saturated phenol and twice with diethyl ether. DNA was precipitated overnight at -20°C with two volumes of ethanol and one tenth volume of 3 M NaAc pH 8.0. The precipitate was collected by centrifugation (15 min, Eppendorf), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and dissolved in 1 x TE buffer.

2.12.6 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were filled in using the Klenow fragment of *E. coli* DNA polymerase I. Typically, 1 µg of digested DNA, 2 µl of 10 x nick-translation buffer (Maniatis *et al.*, 1982), 1 µl of each dNTP (2 mM) and 1 unit Klenow fragment were mixed and incubated for 30 min. The reaction was stopped by heating at 65°C for 10 min, followed by removal of unincorporated dNTPs and enzyme by centrifugation through a Sepharose CL-6B column.

Sepharose CL-6B columns were prepared by placing glass wool into an Eppendorf tube which had a hole punched in the bottom. 1 ml of Sepharose CL-6B (equilibrated with 1 x TE buffer) was added, and the tube within another carrier tube was centrifuged at 2000 rpm for 2 min (Hermle bench centrifuge). This column was washed 2 x with H_2O before use.

2.12.7 End-filling with T4 DNA polymerase

Plasmid DNA was cleaved and cohesive ends converted to blunt ends with T4 DNA polymerase in a final volume of 25 µl containing 2 µg DNA, 2 units T4 DNA polymerase, 1 µl of each dNTP (2mM) and 1 µl of 10 x T4 DNA polymerase buffer (Maniatis *et al.*, 1982). After a 5 min incubation at 37°C, the reaction was stopped by heating at 65°C for 10 min. Salt, unincorporated nucleotides and enzyme were removed by passage through a Sepharose CL-6B column, as described above.

2.12.8 *In vitro* cloning

DNA to be subcloned (3 µg) was cleaved in either single or double restriction enzyme digests. This was combined with 1 µg of similarly cleaved vector DNA, then ligated by 2 units of T4 DNA ligase in a volume of 50 µl in a final buffer composition of 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.6 mM ATP (ligation buffer) for 16 h at 4°C. The ligated DNA was then used directly for transformation of *E. coli* K-12 strains. Transformants were screened for insertional

inactivation of the appropriate drug resistance (Ap or Tc), wherever possible, prior to plasmid DNA isolation.

2.12.9 Nick translation

Nick translation reactions with DNA polymerase I were modified from Maniatis *et al.* (1982) and carried out as follows: 25 µCi α-[^{32}P]-dCTP (1.700 Ci/mmole in ethanol) was dried *in vacuo* in an Eppendorf tube, resuspended with 80 µl water, 10 µl of 10 x nick translation buffer (500 mM Tris-HCl, pH 7.2, 100 mM $MgCl_2$, 1 mM DTT, 500 µg/ml BSA) 1 µl each of 2 mM dATP, dGTP, dTTP. DNA (1 µg) and DNase (10 mg/ml; 1 µl) was added and incubated at 37°C for 10 min. DNA polymerase I (5 units) was added to the mix, and the mixture allowed to incubate at 16°C for 2 h. [^{32}P]-labelled DNA was separated from unincorporated label by centrifugation through a mini-column of Sepharose CL-6B.

2.12.10 Southern transfer and hybridization

Bidirectional transfers of DNA from agarose gels to nitrocellulose paper (Schleicher and Schuell) were performed as described by Southern (1975) as modified by Maniatis *et al.* (1982).

Prior to hybridization with radio-labelled probe, filters were incubated for 4 h at 44°C in a pre-hybridization solution containing 50% (v/v) formamide, 50 mM sodium phosphate buffer, pH 6.4, 5 x SSC (0.34 M NaCl, 75 mM sodium citrate, pH 7.0), 5 x Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% fraction V BSA) and 83 µg/ml single stranded Herring sperm DNA (Sigma) (Maniatis *et al.*, 1982). Pre-hybridization fluid was discarded and replaced with fresh hybridization buffer (as for pre-hybridization solution, with the exclusion of Herring sperm DNA). Denatured probe (approximately 10^6 cpm/µg) was added and hybridization allowed to occur for 16-24 h at 44°C.

Filters were washed twice with shaking at 37°C for 30 min in 2 x SSC, containing 0.1% (w/v) SDS. This was followed by two further washes in 0.1 x SSC plus 0.1% (w/v)

SDS at 65°C. After drying in air (15 min, RT), the filters were covered in plastic wrap and placed on film for autoradiography at -70°C with intensifying screens.

2.12.11 Kinasing single-stranded DNA and hybridization

Single stranded DNA (primers) were kinased using γ-[^{32}P]-dATP. The reaction mix consisted of 100 mM DTT, 1 µl 10 x kinase buffer (10 x: 500 mM Tris pH 7.4 and 100 mM $MgCl_2$), 3 units of polynucleotide kinase, 10 µl of γ-[^{32}P] and 60 µg of primer. This reaction mix was made to 10 µl in water and incubated at 37°C for 30 min. After incubation the labelled oligonucleotide was ethanol precipitated, dried *in vacuo* and resuspended in water. Before use, the oligonucleotide was heated to 65°C for 10 min.

2.13 Analysis and manipulation of RNA

2.13.1 RNA preparation

RNA was prepared by a method modified from that described by Aiba *et al.* (1981). Bacteria harbouring the plasmid of interest were subcultured 1:10 and grown to OD_{650} = 1. For *Campylobacter* strains harbouring plasmids, cells were grown for 16 h at 37°C in microaerophilic conditions. Five mls of culture, or 1 plate of *Campylobacter* cells, were centrifuged and the pellet was resuspended in 0.5 ml of solution A (0.02 mM NaAc pH 5.5, 0.5% SDS, 1 mM EDTA). This was extracted 3 to 4 times with hot (65°C) phenol (equilibrated with a solution containing: 0.02 mM NaAc, 0.02 mM KCl, 0.01 mM $MgCl_2$ at a pH of 5.2). Nucleic acids were then precipitated with two volumes of ethanol and one-tenth volume of 3 M NaAc. To remove contaminating DNA, the precipitate obtained was resuspended in water and incubated at 37°C for 10-15 min with DNase buffer (10 x: 200 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$) and 1 µl of DNase enzyme (10 U/µl). The solution was re-extracted with phenol, the RNA precipitated, dried *in vacuo* and resuspended in water.

2.13.2 Quantitation of RNA

The concentration of RNA in solutions was determined by measurement of absorption at 260 nm and assuming an A_{260} of 1.0 is equal to 40 µg RNA/ml (Miller, 1972).

2.13.3 Primer extension analysis

A synthetic 18mer oligonucleotide primer was radioactively labelled with γ-[^{32}P]-ATP at the 5′ end by T4 polynucleotide kinase. The kinasing reaction consisted of 60 ng of primer, 20 µCi γ-[^{32}P]-ATP, kinase buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 5 mM DTT) and 2 U T4 polynucleotide kinase in a volume of 10 µl and was incubated at 37°C for 30 min. The reaction mixture was made up to 80 µl and precipitated with 2 volumes of ethanol, one-tenth volume of 3M NaAc pH 5.5 and 20 mg glycogen for 16 h at -20°C. Labelled primer was centrifuged for 15 min, washed in 70% ethanol, dried *in vacuo*, and resuspended in water.

The kinased primer (5 ng) and RNA (60 µg) were precipitated together at -20°C with 3 volumes of ethanol and one-twentieth volume of 4 M NaCl. After centrifugation, pellets were washed in 70% ethanol and dried *in vacuo*. Primer was hybridized to RNA by resuspending pellets in 10 µl hybridization mix (10 mM Tris pH 8.3, 200 mM KCl), heating at 80°C for 3 min and incubation at 42°C for 60-90 min.

Extension of annealed primer was achieved by the addition of 24 µl extension mix (10 mM Tris pH 8.3, 14 mM $MgCl_2$, 14 mM DTT, 700 µM each dNTPs) and 10 U AMV reverse transcriptase, followed by a 60 min incubation at 42°C.

Reactions were treated with DNase-free RNaseA, phenol/chloroform extracted and precipitated for 16 h with 3 volumes ethanol and one-tenth volume 3 M NaAc pH 5.5 at -20°C. The pellets were recovered, washed in 70% ethanol, and dried before resuspension in 5 µl H_2O and 5 µl formamide loading buffer. Samples were boiled at 100°C for 3 min prior to loading on a 6% (w/v) polyacrylamide/urea sequencing gel. After electrophoresis, labelled bands were visualized by autoradiography (Section 2.14.3).

2.14 Protein analysis

2.14.1 Minicell procedures

Minicells were purified and the plasmid-encoded proteins labelled with [^{35}S]-methionine as described by Kennedy *et al.* (1977) and modified by Achtman *et al.* (1979). This involved separation of minicells from whole cells (500 ml overnight culture in LB) by centrifugation through two successive sucrose gradients, pre-incubating in minimal medium to degrade long lived mRNAs corresponding to chromosomally encoded genes, then pulse-labelling with [^{35}S]-methionine in the presence of methionine assay medium (Difco). Minicells were subsequently solubilized by heating at 100°C in 100 µl of 1 x sample buffer (Lugtenberg *et al.*, 1975) and analysed by SDS-PAGE.

2.14.2 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on either 11-20% (w/v) polyacrylamide gradients (for proteins) or uniform 20% (w/v) polyacrylamide gels (for lipopolysaccharides) using a modification of the procedure of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Samples were heated at 100°C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 5 h (11-20% (w/v) gradient gels) or 10 mA constant current for 16 h (20% (w/v) PAGE gels). Proteins were stained with gentle agitation of gels for 16 h at RT in 0.06% (w/v) Coomassie Brilliant Blue G250 (dissolved in 5% (v/v) perchloric acid). Destaining was accomplished with several changes of 5% (v/v) acetic acid, with gentle agitation of for 24 h.

Size markers (Pharmacia) were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa).

2.14.3 Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 h on a Bio-Rad gel drier. [^{35}S]-methionine and [^{35}S]d-ATP autoradiography was performed at RT for 1-7 days without intensifying screens using Kodak XR-100 film. For autoradiography with [^{32}P]-phosphate-labelled DNA, the gels were exposed to film for 6-72 h at -70°C, using intensifying screens.

2.14.4 Whole cell preparation

One plate of *Campylobacter* cells grown for 16 h was harvested in saline (5 ml) and 1 ml (10^9 cells) was placed in a microfuge tube and the cells were collected by centrifugation (30 sec, Eppendorf). The cell pellet was resuspended in 200 µl of 1 x sample buffer (Lugtenberg *et al.*, 1975) and heated at 100°C for 3 min prior to analysis by SDS-PAGE gel-electrophoresis. The remainder of the sample was stored at -20°C for future use.

2.14.5 Small scale cell envelope isolation

Whole membrane material was isolated from plates of mid-exponential phase cultures of *Campylobacter* species by the method of Manning *et al.* (1982). The cells were harvested in saline (2 ml) and were centrifuged for 10 min in a bench centrifuge and washed with 10 ml 30 mM Tris-HCl pH 8.1. The pellet was resuspended in 0.2 ml 20% (w/v) sucrose, 30 mM Tris-HCl pH 8.1, and the suspension transferred to SM24 tubes (Sorvall) on ice. After the addition of 20 µl lysozyme (1 mg/ml in 0.1 M EDTA pH 7.3) incubation was continued on ice for 30 min. The cells were then placed in an ethanol-dry ice bath for 30 min. After thawing, 3 ml of 3 mM EDTA pH 7.3 was added and the cells sonicated in a Branson sonicator (four 15 sec pulses on 50% cycle). Unlysed cells were pelleted and removed by centrifugation (5000 rpm, 5 min, 4°C, Sorvall). Membrane material was pelleted by centrifugation (20000 rpm, 60 min, 4°C, Sorvall) and resuspended in 100 µl 1 x sample buffer (Lugtenberg *et al.*, 1975). Samples were stored at -20°C. 10 µl amounts were loaded onto SDS-PAGE gels.

2.14.6 Isolation of cell envelope outer membrane

Cell envelope outer membrane samples were prepared by differential solubilization of the inner membrane using the method described by Filip *et al.* (1973). Whole membrane material extracted by the method of Manning *et al.* (1982) was resuspended in 3 ml of distilled water and made to 20 ml with 1% (w/v) sodium lauryl sarcosinate in 7 mM EDTA. The suspension was incubated for 20 min at 37°C. The suspension was then centrifuged (38,000 rpm, 2 h, Ti60, Beckman ultracentrifuge) and the pellet was resuspended in 0.01 M Tris-HCl pH 7.4 and centrifuged (38,000 rpm, 2 h, Ti60, Beckman ultracentrifuge). The resulting pellet (Sarkosyl-insoluble) was resuspended in 1.0 ml of distilled water and stored at 4°C or -20°C.

2.14.7 Preparation of whole cell lysates (LPS preparation)

Whole cell lysates were prepared by the method of Hitchcock and Brown (1983). Cells were grown for 16 h on blood agar plates in microaerophilic conditions or in NB. 1.5 ml of the cells grown in NB, or 5 x 10^9 cells of the plate-grown cells harvested in saline (1 ml), were pelleted in an Eppendorf centrifuge for 5 min. The pellets were solubilized in 50 µl of lysing buffer containing 2% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 1 M Tris-HCl pH 6.8, and 0.1% (w/v) bromophenol blue. Lysates were heated at 100°C for 10 min. 2.5 µg of Proteinase K solubilized in 10 µl of lysing buffer was added to each sample and incubated at 60°C for 2-4 h. Samples were stored at -20°C and treated at 100°C for 3 min prior to loading onto SDS-PAGE gels.

2.14.8 Isolation of flagella

Flagella were purified as described by Logan and Trust (1983). Cells (10^{11}) were harvested into 20 mM Tris pH 7.3, centrifuged for 10 min in a bench centrifuge, and resuspended in 20 ml distilled water. The suspension was homogenized twice for 30 sec in an Ultra-Turrax homogenizer (Janke & Kunkel KG) and unbroken cells were removed by centrifugation (7,000 rpm, 10 min, SS34, Sorvall). The pellet was washed in distilled

water and the suspension centrifuged as before. The supernatants were pooled and centrifuged (38,000 rpm, 1 h, Ti60, Beckman ultracentrifuge). The flagella pellet was resuspended in distilled water (1 ml) adjusted to pH 2.0 and held at 0°C for 15 min to ensure complete flagellum disassociation. Material insoluble at pH 2.0 was removed by centrifugation (38,000 rpm, 1 h, Ti60, Beckman ultracentrifuge); the supernatant was adjusted to pH 7.0 with NaOH and held at 0°C for 30 min to allow reaggregation of the flagella.

2.14.9 LPS silver staining

Silver staining of LPS in polyacrylamide gels was performed using the method of Tsai and Frasch (1982). The following procedure was used: i) fixation overnight in 40% (v/v) ethanol, 10% (v/v) acetic acid; ii) oxidation for 5 min with 0.7% (w/v) periodic acid in 40% (v/v) ethanol, 10% (v/v) acetic acid; iii) washing with water (4 x, 30 min each); iv) staining for 10 min, in a solution containing 28 ml 0.1 N NaOH, 2 ml 28% (w/v) NH_4OH and 5 ml 20% (w/v) $AgNO_3$ in a total volume of 150 ml; v) developing in a solution of 50 mg citric acid and 0.5 ml formaldehyde per litre of water. The citric acid was dissolved in water and heated to 37°C and formaldehyde added just before use. Deionized water which had been passed through a series of Millipore filters (Milli-Q) and had a conductivity of not less than 18 MΩ/cm was used to rinse all glassware and in preparation of solutions.

2.14.10 Re-electrophoresis of LPS

LPS was re-electrophoresed as decribed by Logan and Trust (1984). After an initial SDS-PAGE separation with duplicate gels, one gel was fixed and rapidly silver-stained for LPS. The duplicate unfixed gel was aligned with the stained gel, and areas containing the bands of interest were excised from the unfixed gel and placed in the wells of a fresh gel. After the addition of 20 µl of lysing buffer (2% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 1 M Tris-HCl pH 7.6, 0.1% (w/v) bromophenol blue; pH 6.8), the samples were subjected to electrophoresis, and the gel was stained.

2.14.11 Western transfer and protein blotting

The procedure used was a modification of that described by Towbin *et al.* (1979). Samples were subjected to SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell) at 200 mA for 2 h in a Trans-Blot Cell (Bio-Rad). The transfer buffer used was 25 mM Tris-HCl pH 8.3, 192 mM glycine and 5% (v/v) methanol. After transfer, the nitrocellulose sheet was incubated for 30 min in 5% (w/v) skim milk powder in TTBS (0.05% (v/v) Tween 20, 20 mM Tris-HCl, 0.9% (w/v) NaCl) to block non-specific protein binding sites. The antiserum was diluted 1/1000 in TTBS, 0.02% (w/v) skim milk powder and incubated with the filter with gentle agitation at RT for 2-16 h. The antibody was removed by washing the nitrocellulose sheet three times for 10 min in TTBS with shaking. Bound antibody was detected using an anti-antibody coupled with horseradish peroxidase and peroxidase substrate. This was accomplished by incubating the filter for 2-16 h (gentle agitation with goat anti-rabbit IgG coupled with horseradish peroxidase (KPL) at a dilution of 1/5000 in TTBS plus 0.2% (w/v) skim milk powder). The filter was then washed four times (5 min intervals) with TTBS, followed by two 5 min washes in TBS (20 mM Tris-HCl, 0.9% (w/v) NaCl). The antigen-antibody complexes were then visualised using peroxidase substrate (9.9 mg 4-chloro-1-naphthol dissolved in 3.3 ml -20°C methanol added to 16.5 ml TBS containing 15 µl hydrogen peroxide). This was added to the filter, and development proceeded for 10-15 min with shaking, as described by Hawkes *et al.* (1982).

2.14.12 Colony transfer and blotting with antiserum

A nitrocellulose disc (9 cm diameter) was placed onto agar plates containing the colonies to be screened. Once the colonies had adhered to the disc (3 min), the cell debris was removed from the nitrocellulose with a jet of saline (0.9% (w/v) NaCl). The nitrocellulose sheet was then treated exactly as described earlier for the protein blotting procedure (Section 2.14.11)

2.14.13 Western transfer and LPS blotting

LPS components separated by SDS-PAGE were transferred to nitrocellulose by the method described by Preston and Penner (1987). After SDS-PAGE, the gel was equilibrated in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol for 30 min and then applied to nitrocellulose sheets (Schleicher and Schuell). Blotting was performed for 18 h at 0.1 A. The nitrocellulose sheet was then treated with the primary and secondary antisera as described previously for protein blotting.

2.15 M13 cloning and sequencing procedures

2.15.1 Preparation of M13 replicative form (RF) DNA

Fresh 2 x YT broth (10 ml) was inoculated with 10 µl of an overnight culture of JM101 (in M13 minimal medium). A single plaque of M13mp18 picked from an H agar plate with a sterile toothpick was added to this bottle. The culture was grown at 37°C with vigorous shaking for 6 h. Bacterial cells were removed by centrifugation (5,000 rpm, 10 min, bench centrifuge) and the supernatant added to 1 litre NB containing 10 ml of a shaken overnight culture of JM101. Following incubation for 14 h at 37°C with shaking, replicative form (RF) DNA was prepared as described above for plasmid DNA purification.

2.15.2 Cloning with M13mp18

The M13 vector, M13mp18, (Messing and Vieira, 1982; Vieira and Messing, 1982) was used for selective cloning of restriction enzyme-generated DNA fragments. Stocks of M13 vector cleaved with various enzyme combinations and alkaline phosphatase-treated were stored at 4°C, after heat inactivation of enzymes. Plasmid DNA was cut with the appropriate enzyme combinations for subcloning into the M13 vector. The reaction mixtures consisted of the DNA to be cloned (100 ng) and the DNA vector (20 ng) in a final volume of 10 µl of ligation buffer. Ligation with T4 DNA ligase (2 U) was carried out overnight at 4°C.

2.15.3 Transfection of JM101

The *E. coli* strain JM101 was made competent for transformation/transfection as described in Section 2.7. Competent cells (0.2 ml) were added directly to the ligation mixes and incubated on ice for 30 min. This was followed by a 2 min heat shock at 42°C. Cells were then transferred to sterile test tubes to which was added a mixture of JM101 indicator cells (200 µl), 100 mM IPTG (40 µl) and 2% (w/v) X-gal in N, N′-dimethyl formamide (40 µl) and finally 4 ml H top agar. The mixture was poured as an overlay onto an H agar plate and incubated overnight at 37°C.

2.15.4 Screening M13 vectors for inserts

White plaques were picked from X-gal/IPTG plates with sterile toothpicks and added to 1 ml 2 x YT broth in microfuge tubes containing a 1:100 dilution of an overnight culture of JM101. These tubes were incubated for 5 hr at 37°C. The cells were pelleted by centrifugation (30 sec, Eppendorf). RF DNA, suitable for restriction analysis, was prepared by the miniprep method (Section 2.11, method 3). After restriction enzyme digestion, DNA was examined on 1% (w/v) agarose gels.

2.15.5 Purification of single-stranded template DNA

M13 RF DNA containing appropriate inserts were reintroduced into JM101 and single white plaques from this transfection picked with sterile toothpicks to inoculate 2 ml 2 x YT broth containing 20 µl of an overnight culture of JM101. After vigorous shaking at 37°C for 6 h, the culture was transferred to Eppendorf tubes and centrifuged for 10 min. The supernatant was transferred to clean tubes and recentrifuged for 5 min. Three methods of lysing phage and collecting single-stranded phage were employed. They are as follows:

Method 1: A 1 ml aliquot of the supernatant from each tube was withdrawn and mixed in a fresh tube with 0.27 ml 20% (w/v) polyethylene glycol (PEG), 2.5 M NaCl. These tubes were then incubated at RT for 15 min. The

phage were pelleted by centrifugation for 5 min in an Eppendorf centrifuge and the supernatant discarded. Following another short spin (10 sec), the remainder of the PEG/NaCl supernatant was removed with a drawn out Pasteur pipette. The pellets were resuspended in 0.2 ml TE buffer. Redistilled TE-saturated phenol (0.1 ml) was then added to the phage suspension and the tubes were briefly vortexed. After standing for 15 min at RT, the tubes were centrifuged for 2 min and 0.15 ml of the top phase transferred to clean tubes. To the aqueous phase 6 µl of 3 M NaAc pH 5.0 and 400 µl absolute ethanol was added. Single-stranded DNA was precipitated at -20°C overnight, followed by centrifugation for 15 min in an Eppendorf centrifuge. DNA pellets were washed once with 1 ml 70% (v/v) ethanol followed by centrifugation. After drying *in vacuo* the pellets were resuspended in 25 µl 1 x TE buffer and stored at -20°C until required.

Method 2: This method was the same as method 1 to the stage of phage precipitation; phage was resuspended in 300 µl of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). 300 µl of TE-saturated phenol was added and the mix was vortexed sporadically for 10 min. The tubes were centrifuged for 3 min, and then the aqueous phase was extracted with chloroform:isoamyl alcohol (24:1). The liberated single-stranded DNA was precipitated by the addition of a one-tenth volume of 5 M $NaClO_4$ and 1 volume of isopropanol. Single-stranded DNA was precipitated at -20°C overnight, followed by centrifugation for 15 min in an Eppendorf centrifuge. DNA pellets were washed once with 1 ml 70% (v/v) ethanol followed by centrifugation. After drying *in vacuo* the pellets were resuspended in 50 µl 1 x TE buffer and stored at -20°C until required.

Method 3: After pelleting of cells, 1 ml of the phage-containing supernatant was added to a microfuge tube containing 250 µl of a 20% (w/v) PEG/3.5 M ammonium acetate solution. This was vortexed and incubated on ice for 30 min. The phage were collected by centrifugation (15 min, Eppendorf) and all the supernatant carefully removed. The pellet was dissolved in 100 µl 1 x TE buffer. 50 µl redistilled phenol was added and the tube vortexed for 2 min followed by incubation at RT for 5 min. 50 µl chloroform was then added, the tube was vortexed for 2 min, spun in a microfuge for 5 min and the upper aqueous phase was transferred to a fresh tube. The phenol/chloroform phase was extracted with 100 µl 1 x TE buffer, spun as before and the aqueous phases combined. The combined aqueous phases were then extracted with an equal volume of phenol/chloroform (three more times). The aqueous phase was then extracted with chloroform and 250 µl transferred to a microfuge tube containing 125 µl 7.5 M ammonium acetate. After addition of 0.75 ml 95% (v/v) ethanol the tubes were stored overnight at -20°C. The DNA was collected by centrifugation (15 min, Eppendorf), and the pellet washed twice with 95% (v/v) ethanol. The pellet was dried *in vacuo* before resuspending the DNA in 20 µl distilled water.

2.15.6 Dideoxy sequencing protocol with Sequenase™

The dideoxy chain termination procedure of Sanger *et al.* (1977) was modified to encompass the use of Sequenase™ (modified T7 DNA polymerase) in place of Klenow enzyme (Tabor and Richardson, 1987). All reagents were stored at -20°C. Two types of labelling and termination mixes were used, namely the dGTP mixes and the dITP mixes. The contents of the dGTP mixes were as follows :

Labelling Mix (dGTP):	7.5 µM dGTP, dCTP and dTTP
ddG Termination Mix (dGTP):	80 µM dNTP, 8 µM ddGTP, 50 mM NaCl
ddA Termination Mix (dGTP):	80 µM dNTP, 8 µM ddATP, 50 mM NaCl
ddC Termination Mix (dGTP):	80 µM dNTP, 8 µM ddCTP, 50 mM NaCl
ddT Termination Mix (dGTP):	80 µM dNTP, 8 µM ddTTP, 50 mM NaCl

The dITP mixes were used to reduce gel artifacts due to secondary structures in DNA synthesized in the sequencing reaction (Barnes *et al.*, 1983; Gough and Murray, 1983). The dITP mixes were as follows :

Labelling Mix (dITP):	15 µM dITP, 7.5 µM dCTP, 7.05 µM dTTP
ddG Termination Mix (dITP):	160 µM dITP, 80 µM dATP, dCTP dTTP, 1.6 µM ddGTP, 50 mM NaCl
ddA Termination Mix (dITP):	160 µM dITP, 80 µM dATP, dCTP dTTP, 8 µM ddATP, 50 mM NaCl
ddC Termination Mix (dITP):	160 µM dITP, 80 µM dATP, dCTP dTTP, 8 µM ddCTP, 50 mM NaCl
ddT Termination Mix (dITP):	160 µM dITP, 80 µM dATP, dCTP dTTP, 8 µM ddTTP, 50 mM NaCl

Normally the labelling mix was diluted 1:5 with water to obtain the working concentration, however, to read long sequences in a single reaction, a dilution of 1:2 was used. The synthetic primer was annealed to the template by incubating 7 µl template (5-10 nmoles), 1 µl primer (500 nmoles) and 2 µl 5 x Sequenase buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The mixture was heated in a metal block

at 65°C for 3 minutes and then the block containing the tubes was allowed to cool to RT. To the annealed mixture, 2 µl of the appropriately diluted labelling mix, 1 µl DTT (0.1 M), 0.5 µl [α-^{35}S]-dATP (1000 Ci/mmol) and 2 µl of diluted Sequenase™ (1:8 dilution in 1 x TE buffer) were added, spun, mixed, respun and then incubated for 5 minutes at RT. 3.5 µl of this mix was then aliquoted into four microfuge tubes, prewarmed to 37°C, labelled A, C, G and T, each containing 2.5 µl of the corresponding termination mix, then spun briefly to start the termination reaction. After 5 minutes at 37°C, 4 µl Stop solution (95% (w/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol) was added to each of the reactions. Reaction mixes were heated to 100°C for 2 min and immediately 1.2 µl loaded onto the sequencing gel. For re-running, these samples were kept at -20°C for up to 2 weeks and heated to 100°C for 3 min prior to loading.

2.15.7 Double-stranded sequencing

Plasmid DNA (2-4 µg/ml) was diluted to a volume of 18 µl with water. The DNA was denatured by the addition of 2 µl of 2 M NaOH and incubated for 5 min at RT. To this mix, 8 µl of 5 M ammonium acetate (pH 7.5) was added, with 100 µl of 100% ethanol to precipitate the DNA. The supernatant was removed and the pellet washed in 70% ethanol. The pellet was dried *in vacuo* and dissolved in 7 µl of water. 2 µl of sequencing buffer (1 x Sequenase buffer) and 1 µl of primer (0.5 pmol) was added to the DNA and the mixture heated to 37°C for 20 min. The labelling and termination reactions were run exactly as described for M13 single-stranded template DNA.

2.15.8 DNA sequencing gels

Polyacrylamide gels for DNA sequencing were prepared using glass plates 33 x 39.4 cm and 33 x 42 cm. Spacers and combs were high density polystyrene (0.25 mm thick). The gel mix contained 70 ml acrylamide stock [5.7% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 8 M urea in 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA, pH 8.3)], plus 420 µl 25% ammonium persulphate and 110 µl

TEMED (N, N, N′, N′-tetramethyl-ethylene-diamine, Sigma). After thorough mixing the gel mix was poured into a clean gel sandwich and the comb inserted. Polymerization took place for 60 min, with the gel in a horizontal position. The gel was mounted onto the sequencing apparatus and a waterjacket was attached to the outside plate of the gel. This consisted of a plastic bag wedged between two 0.3 cm thick spacers and a third plate. The plastic bag was filled with 1 x TBE buffer and this was sufficient to evenly distribute heat throughout the gel. Gels were pre-electrophoresed at 700 V for 30 min. After the samples had been loaded the gel was electrophoresed at a constant voltage (700 V) for 15 min, and the voltage was then increased to 1200 V (33 mA). After 4 h the samples were reloaded into a second set of wells on the same gel. The gel was further electrophoresed, initially at 700 V, then 1200 V for 2.5 h by which time the bromophenol blue dye front from the second loading had reached the bottom of the gel. Plates were separated and tissue paper was used along the borders of the gel to hold it to the plate during the fixation procedure which involved slowly washing the gel using 2 litres of 10% (v/v) acetic acid, 20% (v/v) ethanol in a 60 ml syringe. The gel was then dried at 100°C for 20 min. Plastic wrap was used to cover the gel before placing on film for autoradiography. Autoradiography was performed at RT, without the use of intensifying screens, for 16-24 h.

2.15.9 Analysis of DNA sequences

Sequencing data was analysed using the following computer programs: V6.0 of the LKB DNA and protein analysis programs, DNASIS and PROSIS, the MailfastA program from EMBL, and the multiple alignment programs CLUSTAL (Higgins and Sharp, 1988) and MACAW (Schuler *et al.*, 1991). The protein bank screened was Swissprot (January, 1990).

2.16 Mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)

Two methods were employed for the mutagenesis of *Campylobacter* strains. They are as follows:

Method 1: Attempts to isolate restriction mutants of *C. hyointestinalis* were made using a modification of the method described by Morooka *et al.* (1985). *C. hyointestinalis* cells were grown for 16 h on blood agar, harvested in saline (5 ml) at a concentration of 5 x 10^8 cells/ml. Solutions of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Aldrich) (1 mg/ml in 0.1 M citrate buffer pH 5.5) were added to the suspensions at final concentrations of 5, 10, 25, 50, and 100 µg/ml. Mutagenesis was performed at 37°C for 1 h under microaerophilic conditions. The treated cells were harvested and washed with saline, and the surviving cells were cultured on blood agar plates. After incubation at 37°C for 48 h the cultures were harvested in saline (1 ml) and used as recipients in a mating mix with *E. coli* K-12 donor cells as described earlier in the procedure for bacterial conjugation (Section 2.10). *C. hyointestinalis* exconjugants were selected on blood agar plates containing the appropriate antibiotics.

Method 2: Restriction mutants of *C. hyointestinalis* were obtained by a modification of the method described by Eisenstark (1965). *C. hyointestinalis* cells were grown for 16 h on blood agar, harvested in saline (1 ml), and spread onto three blood agar plates. Two separate drops of MNNG (Aldrich) at 50 µg/ml were placed on the surface of each plate. The plates were then incubated under microaerophilic conditions for 48 h at 37°C. Zones of growth surrounding the areas of killing were harvested with a sterile loop and resuspended in 200 µl of a suspension of *E. coli* K-12 donor cells (5 x 10^8 cells). The mating mix was spread onto a nitrocellulose disc as described earlier in the procedure for bacterial conjugation (Section 2.10)

and exconjugants were selected on blood agar plates containing the appropriate antibiotics.

2.17 Heat inactivation of restriction systems

Heat attenuation of *Campylobacter* restriction systems was attempted using a modification of the method described by Engel (1987). Cells were grown for 16 h and harvested in saline (1 ml) and the suspensions incubated at 50°C for either 10, 20, or 30 min, respectively. After incubation the cells were pelleted by centrifugation in a bench centrifuge and resuspended in saline (100 µl) before use as recipients in bacterial conjugation experiments by the method described earlier (Section 2.10).

2.18 Preparation of rabbit antisera

Adult rabbits were obtained from the Central Animal House of the University of Adelaide for production of antisera to whole cells by a combined subcutaneous/intravenous immunization protocol. For the primary inoculation, cells were harvested in normal saline (10^7/ml), washed, and mixed with an equal volume of 4% (w/v) sodium alginate. 0.25 ml of the mixture was deposited subcutaneously and 0.15 ml of 4% (w/v) $CaCl_2$ was injected into the same site, without removing the needle, but using a new syringe. This was repeated at three other sites. At 3-4 days intervals thereafter, increasing doses of live cells (10^7-10^9/ml in 0.2 ml saline) were given intravenously. The rabbits were bled by cardiac puncture under anesthesia 10 days after the last immunization. The blood was allowed to clot and the sera separated, filtered (pore size, 0.22 µm; Millipore Corp, Bedford, Mass.), and stored at 4°C or -20°C in aliquots.

2.19 Examination of bacteria by electron microscopy

For the screening of *Campylobacter* strains for flagella, cells were grown for 16 h and harvested in saline. Cells were stained with phosphotungtstic acid on Formvar-

coated grids, followed by examination in a Jeol JEM 100S transmission electron microscope.

CHAPTER 3

CHARACTERIZATION OF THE OUTER MEMBRANE COMPONENTS OF *C. HYOINTESTINALIS*

3.1 Introduction

C. hyointestinalis has been associated with proliferative enteritis in swine (Gebhart *et al.*, 1983, 1985; Lambert *et al.*, 1984) and cattle (Diker *et al.*, 1990). Recent isolations from patients with proctitis and diarrhoea suggest that *C. hyointestinalis* may be an opportunistic enteropathogen of humans (Edmonds *et al.*, 1987; Fennell *et al.*, 1986; Minet *et al.*, 1988). It was most closely related to *C. fetus* among *Campylobacter* strains, and most distantly related to *C. jejuni* and *C. coli* (Gebhart *et al.*, 1985; Thompson *et al.*, 1988; Vandamme *et al.*, 1991). *C. hyointestinalis* has been shown to contain a novel menaquinone (Moss *et al.*, 1990); no other reports on its chemical composition have yet appeared.

The outer membrane of a number of pathogenic Gram-negative bacteria has been demonstrated to contain surface structures involved in the adherence of the bacteria to the host cell surface, in invasion of the host cells, in resistance to phagocytosis and killing by phagocytic cells of the host, in resistance to the bactericidal activity of serum, and in the acquisition of iron. Some of these outer membrane structures include pili, flagella, capsules, proteins, and LPS. The outer membranes of a variety of bacteria pathogenic for humans, including *E. coli, S. typhimurium, V. cholerae, C. jejuni*, and *C. coli*, have been well characterized.

In this chapter the composition of the outer membrane from 10 strains of *C. hyointestinalis* is examined. The outer membrane proteins, flagellins, and LPS were

characterized by SDS-PAGE and their immunological properties and cross-reactivity between *C. hyointestinalis* isolates on the one hand, and *C. jejuni* and *C. coli*, on the other, were explored. This examination was performed to determine if there are any surface antigens present in *C. hyointestinalis* which might prove to be useful as components of a *C. hyointestinalis* vaccine after further analysis at the molecular level.

3.2 Results

3.2.1 Membrane proteins of *C. hyointestinalis*

Total membrane material was prepared from the type strains of *C. jejuni*, *C. coli*, *C. hyointestinalis* and nine other strains of *C. hyointestinalis*, and examined by SDS-PAGE (Figure 3.1). The protein profiles of *C. jejuni* and *C. coli* were indistinguishable, as expected, and the same as described previously, with both species exhibiting a major outer membrane protein of 43 kDa and a flagellin protein of 62 kDa (Blaser *et al.*, 1983a; Newell *et al.*, 1984). The protein profiles exhibited by membranes from *C. hyointestinalis* strains were easily distinguished from those of *C. jejuni* and *C. coli*. Seven of the ten *C. hyointestinalis* strains examined, including the type strain NCTC 11608, produced protein profiles which were very similar, with two major polypeptides of 47 and 50 kDa. Three other strains of *C. hyointestinalis* had unique patterns with the M_r values of their two major polypeptides differing from those of the majority of the *C. hyointestinalis* strains examined. Strains 49226, NCTC 11562, and NCTC 11563 exhibited major proteins of 43 and 46 kDa, 44 and 48 kDa, and 45 and 50 kDa, respectively. These patterns shown by *C. hyointestinalis* are similar to those reported for *C. fetus* which displayed a protein profile with two major bands of apparent M_r values of 45 and 47 kDa (Logan and Trust, 1982; Blaser *et al.*, 1983a). Virulent strains of *C. fetus*, however, produced a major surface-exposed glycoprotein which varied in M_r from 98 kDa (Winter *et al.*, 1978) to 127 kDa (Fujimoto *et al.*, 1991), or 131 kDa (Dubreil *et al.*, 1988). No major protein band of this size was observed in the protein profiles of any of the *C. hyointestinalis* strains. Of the three atypical *C. hyointestinalis* strains, one (49226) was an Australian isolate, while the other two were from England.

FIGURE 3.1 The membrane proteins of *C. hyointestinalis* isolates.

Total membrane proteins from *C. jejuni*, *C. coli*, and ten strains of *C. hyointestinalis* were analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. The strains examined in each track are as follows:

(A) Protein size markers (Pharmacia)

(B) *C. jejuni* NCTC 11168

(C) *C. coli* NCTC 11366

(D) *C. hyointestinalis* NCTC 11608 (type)

(E) *C. hyointestinalis* 45104

(F) *C. hyointestinalis* 49905

(G) *C. hyointestinalis* 48869

(H) *C. hyointestinalis* 48318

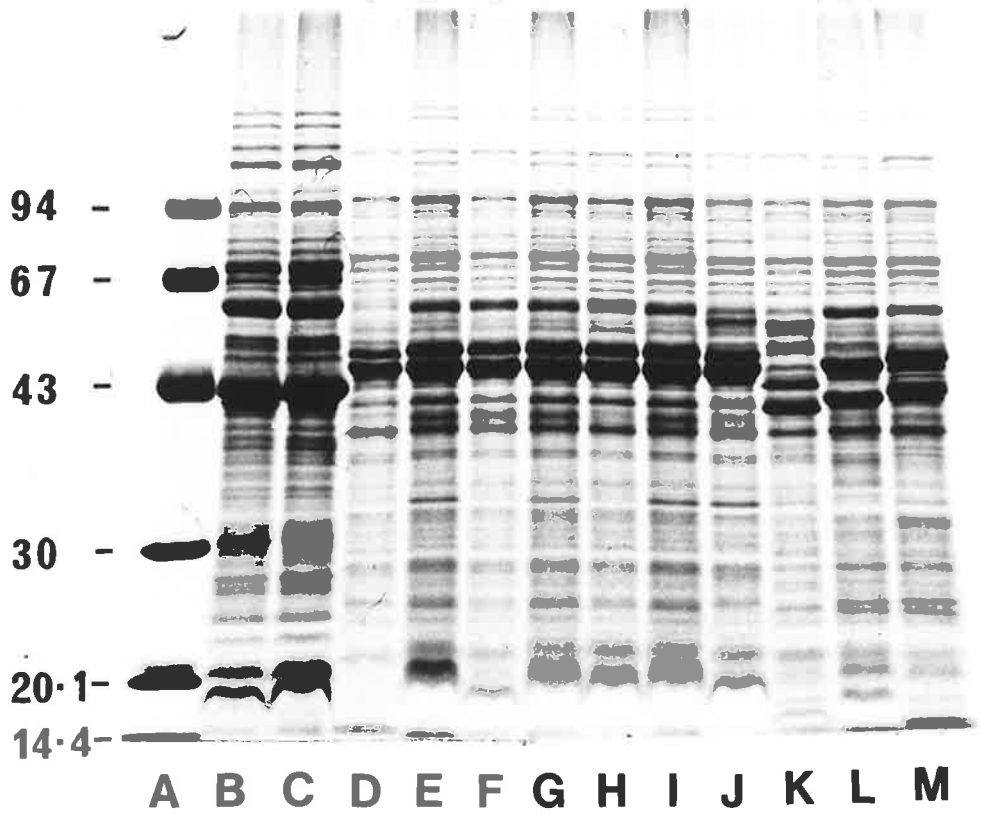
(I) *C. hyointestinalis* NCTC 11609

(J) *C. hyointestinalis* NCTC 11610

(K) *C. hyointestinalis* 49226

(L) *C. hyointestinalis* NCTC 11562

(M) *C. hyointestinalis* NCTC 11563


94
67
43
30
20·1
14·4
A B C D E F G H I J K L M

3.2.2 Outer membrane proteins of *C. hyointestinalis*

Total membrane material from the *C. hyointestinalis* type strain, the three isolates with unique profiles in Figure 3.1, *C. jejuni*, and *C. coli* was extracted with Sarkosyl and residual material examined by SDS-PAGE (Figure 3.2 A). The extraction removed the majority of the additional minor proteins but the major proteins remained; they may therefore be assigned to the outer membrane (Blaser *et al.*, 1983a).

3.2.3 Immunogenic proteins of *C. hyointestinalis*

Sarkosyl-extracted outer membrane material from the type strains of *C. jejuni*, *C. coli*, *C. hyointestinalis*, and of the three *C. hyointestinalis* strains with unique protein profiles, was immunoblotted with antiserum raised against live *C. hyointestinalis* strain NCTC 11608 (type strain) (Figure 3.2 B). Among the four *C. hyointestinalis* strains examined the serum detected four proteins of apparent M_r of 43 to 50 kDa in all four strains, but did not detect the major outer membrane proteins. A small protein of 23 kDa was also detected in all the *C. hyointestinalis* strains. Some cross-reactivity with *C. jejuni* and *C. coli* outer membrane proteins was observed. This reactivity with *C. coli* was principally with two minor outer membrane proteins of approximate M_r values of 23 and 41 kDa, and with the flagellin (62 kDa). In *C. jejuni* the reactivity was with a 39 kDa minor outer membrane protein and the flagellin (62 kDa). This indicates some antigenic relatedness between the three species.

3.2.4 Cross-reactivity with *C. jejuni* and *C. coli*

Antiserum raised against *C. jejuni* and *C. coli* detected several proteins of either strain by immunoblotting (Figure 3.2 C, D). The antisera raised against *C. coli* detected two proteins; one of an approximate M_r of 23 kDa in the four strains of *C. hyointestinalis* examined, and the other of an approximate M_r of 43 kDa in three of the four strains examined. The antisera raised against *C. jejuni* detected only the 23 kDa protein from these strains. This material was protein in nature as reactivity was lost following treatment of the membrane material with Proteinase K (Figure 3.3).

FIGURE 3.2 Immunoblotting of the outer membrane proteins of four *C. hyointestinalis* strains with antisera raised against *C. hyointestinalis*, *C. coli*, and *C. jejuni*.

Outer membrane samples from four *C. hyointestinalis* strains, *C. jejuni*, and *C. coli* were obtained by Sarkosyl extraction of total membrane material and analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (panel A) or Western blotting with antiserum to live *C. hyointestinalis* NCTC 11608 (panel B), live *C. coli* (panel C), or live *C. jejuni* (panel D). Protein size markers (Pharmacia) were used as standards and the protein sizes (kDa) are shown on the right. The strains examined in each track are as follows:

(A) *C. jejuni* NCTC 11168

(B) *C. coli* NCTC 11366

(C) *C. hyointestinalis* NCTC 11608

(D) *C. hyointestinalis* 49226

(E) *C. hyointestinalis* NCTC 11562

(F) *C. hyointestinalis* NCTC 11563

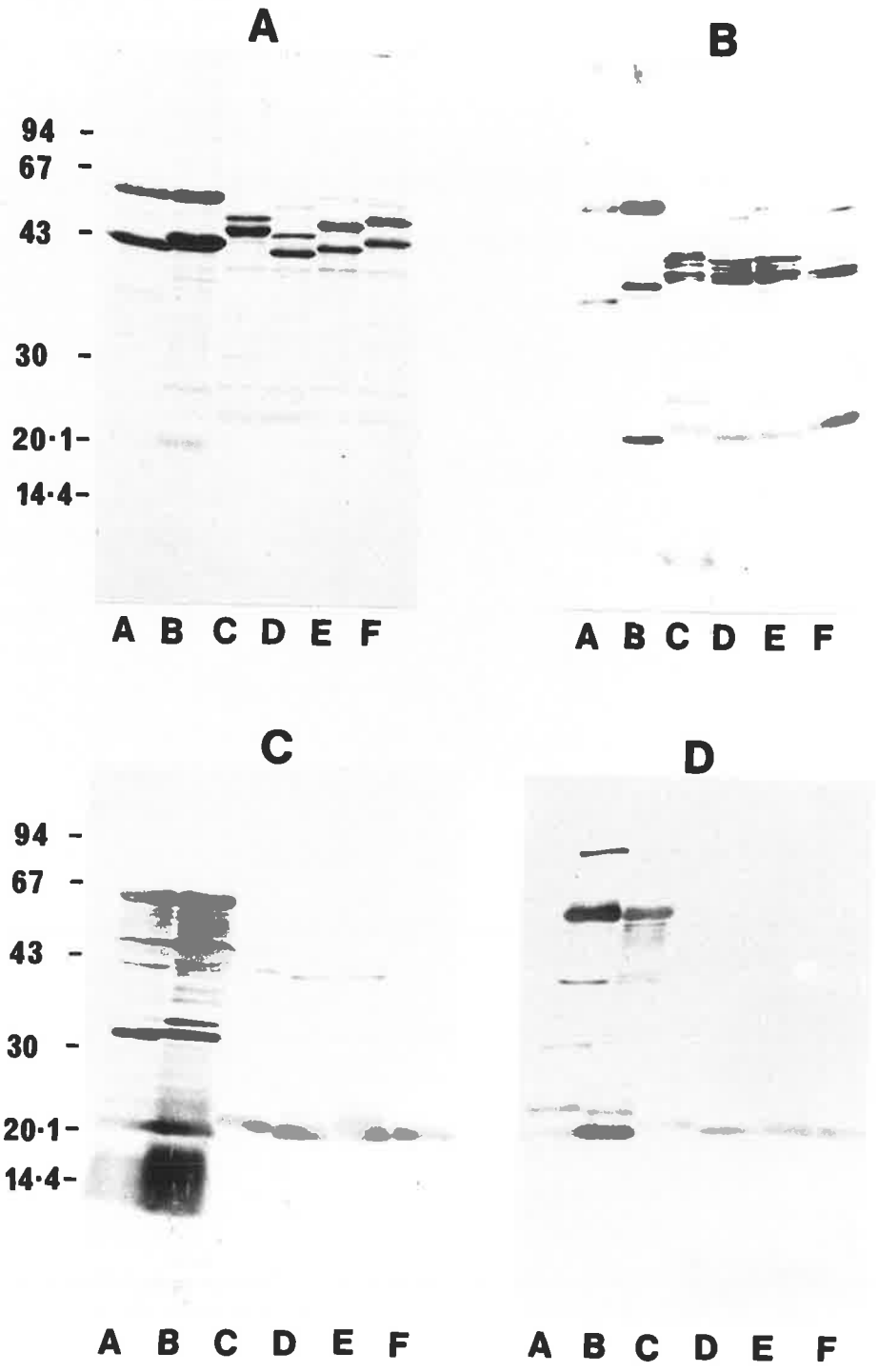

A
B
C
D
94
67
43
30
20·1
14·4
A B C D E F

FIGURE 3.3 Immunoblotting of outer membrane material showing the sensitivity of the *C. hyointestinalis* 23 kDa and 43 kDa outer membrane proteins to Proteinase K.

Outer membrane samples from four strains of *C. hyointestinalis* were obtained by Sarkosyl extraction of whole membrane material and treated with Proteinase K (2.5 mg/ml) at 60°C for 1h. Both Proteinase K treated and untreated samples from each *C. hyointestinalis* strain were then analysed by SDS-PAGE followed by Western blotting with antiserum against live *C. coli*. The 23 kDa protein of *C. hyointestinalis* is indicated by an arrow. The strains, and their treatment with Proteinase K, examined in each track are as follows:

(A) *C. jejuni* NCTC 11168

(B) *C. coli* NCTC 11366

(C) *C. hyointestinalis* NCTC 11608

(D) *C. hyointestinalis* NCTC 11608 (Proteinase K)

(E) *C. hyointestinalis* 49226

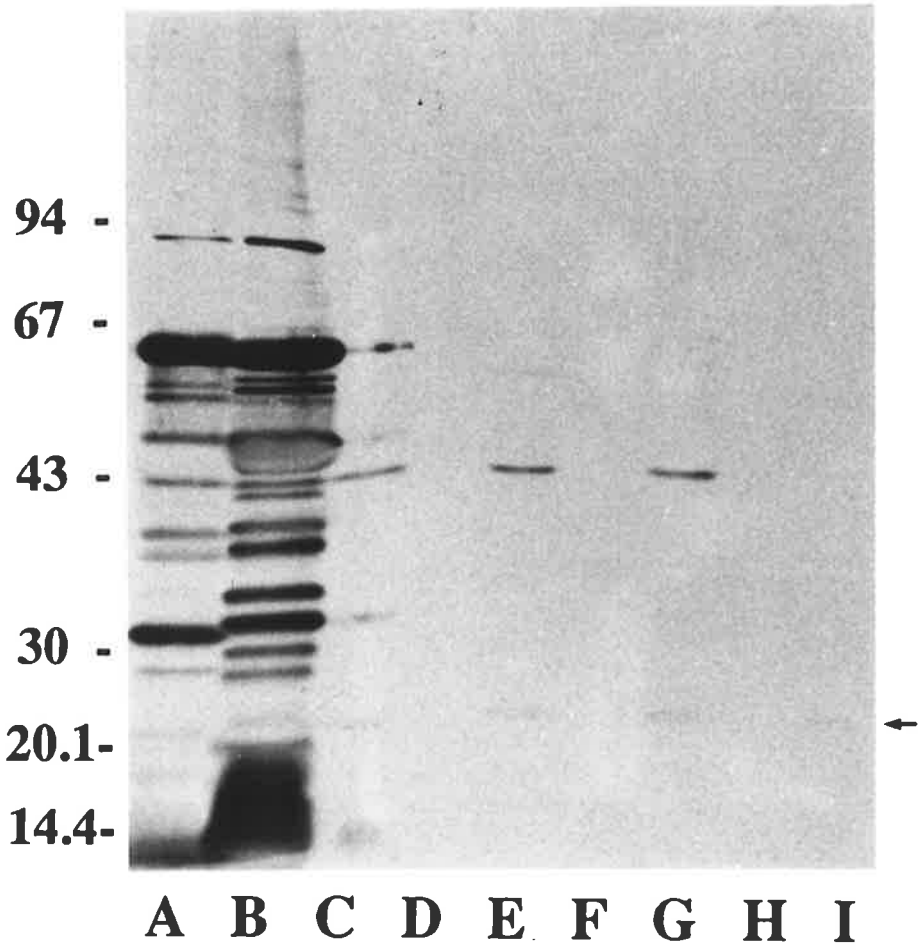
(F) *C. hyointestinalis* 49226 (Proteinase K)

(G) *C. hyointestinalis* NCTC 11562

(H) *C. hyointestinalis* NCTC 11562 (Proteinase K)

(I) *C. hyointestinalis* NCTC 11563

(J) *C. hyointestinalis* NCTC 11563 (Proteinase K)


94 -
67 -
43 -
30 -
20.1-
14.4-
A B C D E F G H I

3.2.5 Heat-modifiable protein

The major outer membrane protein (45 kDa) of *C. jejuni* and *C. coli* has been shown to be heat-modifiable (Logan and Trust, 1982; Huyer *et al.*, 1986; Page *et al.*, 1989). The major outer membrane protein in both species is solubilized at 37°C and migrates with an apparent M_r of 31 kDa when examined by SDS-PAGE (Huyer *et al.*, 1986; Page *et al.*, 1989). At temperatures of 55°C and higher, the protein shifts to an apparent M_r of 43 and 44 kDa in *C. jejuni* and *C. coli*, respectively (Huyer *et al.*, 1986; Page *et al.*, 1989). To investigate whether any of the major outer membrane proteins of *C. hyointestinalis* were heat-modifiable, outer membrane material, was prepared by Sarkosyl extraction from *C. jejuni*, *C. coli*, and four strains of *C. hyointestinalis*, and solubilized at 37°C, 56°C, and 100°C (Figure 3.4). The major outer membrane protein of *C. jejuni* and *C. coli* was observed to migrate at a lower M_r at 37°C, but migrated at a higher M_r at temperatures of 56°C and higher, reflecting the results of previous reports. The larger outer membrane protein of *C. hyointestinalis* was inadequately solubilized for SDS-PAGE at temperatures of 56°C or below (Figure 3.4). The fast-migrating major outer membrane protein of *C. hyointestinalis*, however, was adequately solubilized at 37°C, except possibly in the case of isolate NCTC 11562. The major outer membrane protein of *C. jejuni* and *C. coli* has been shown to be a porin (Huyer *et al.*, 1986; Page *et al.*, 1989), and by implication, it may be suggested that the larger outer membrane protein of *C. hyointestinalis* is a porin also.

3.2.6 Flagellin of *C. hyointestinalis*

C. hyointestinalis has been reported to express a polar flagellum at one or both ends of the cell (Gebhart *et al.*, 1985). Examination of the *C. hyointestinalis* type strain under the electron microscope revealed that only 1 to 2% of cells were flagellated, and processing of even large amounts of bacteria did not yield detectable flagellin (Figure 3.5). Flagella were however observed, under the electron microscope, in other strains of *C. hyointestinalis*. Material was extracted from these strains, and from *C. jejuni* and *C. coli*, using a method known to enrich for flagellin (see Section 2.14.8). This extracted material was examined by SDS-PAGE and a major band with an approximate M_r of 62 kDa was observed in the

FIGURE 3.4. Heat-modifiability of a major outer membrane protein of *Campylobacter* species.

Outer membrane material was prepared from *C. jejuni*, *C. coli*, and four strains of *C. hyointestinalis* and solubilized at 37°C (tracks A), 56°C (tracks B), or 100°C (tracks C), prior to analysis by SDS-PAGE. The outer membrane proteins which are ineffectively solubilized at <100°C, are indicated by small dark squares above the protein bands in the 100°C tracks. The strains examined in each track are as follows:

(1) *C. jejuni* NCTC 11168

(2) *C. coli* NCTC 11366

(3) *C. hyointestinalis* NCTC 11608

(4) *C. hyointestinalis* 49226

(5) *C. hyointestinalis* NCTC 11562

(6) *C. hyointestinalis* NCTC 11563

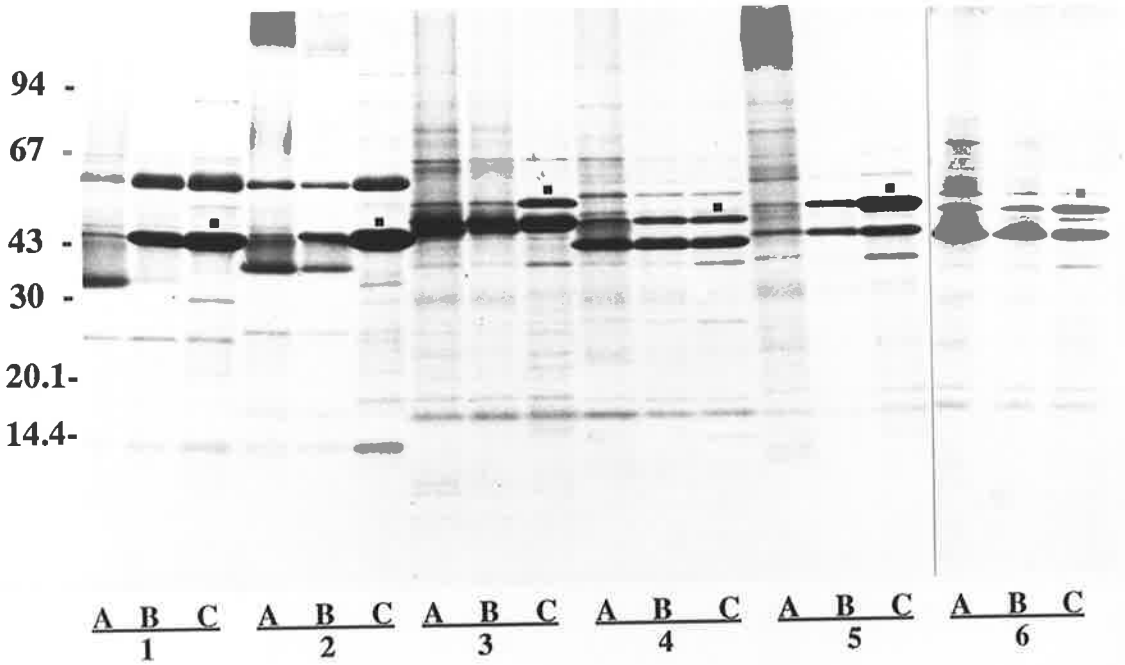

94 -
67 -
43 -
30 -
20.1-
14.4-
A B C
1
A B C
2
A B C
3
A B C
4
A B C
5
A B C
6

FIGURE 3.5 Examination of the flagellins of *C. hyointestinalis* strains.

Material was prepared from *C. jejuni*, *C. coli*, and four strains of *C. hyointestinalis* using a method which enriches for flagella (Section 2.14.8), and was analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (panel A) or Western blotting with antiserum to live *C. hyointestinalis* NCTC 11608 (panel B). No detectable flagella were isolated from *C. hyointestinalis* NCTC 11608. The strains examined in each track are as follows:

(1) *C. jejuni* NCTC 11168

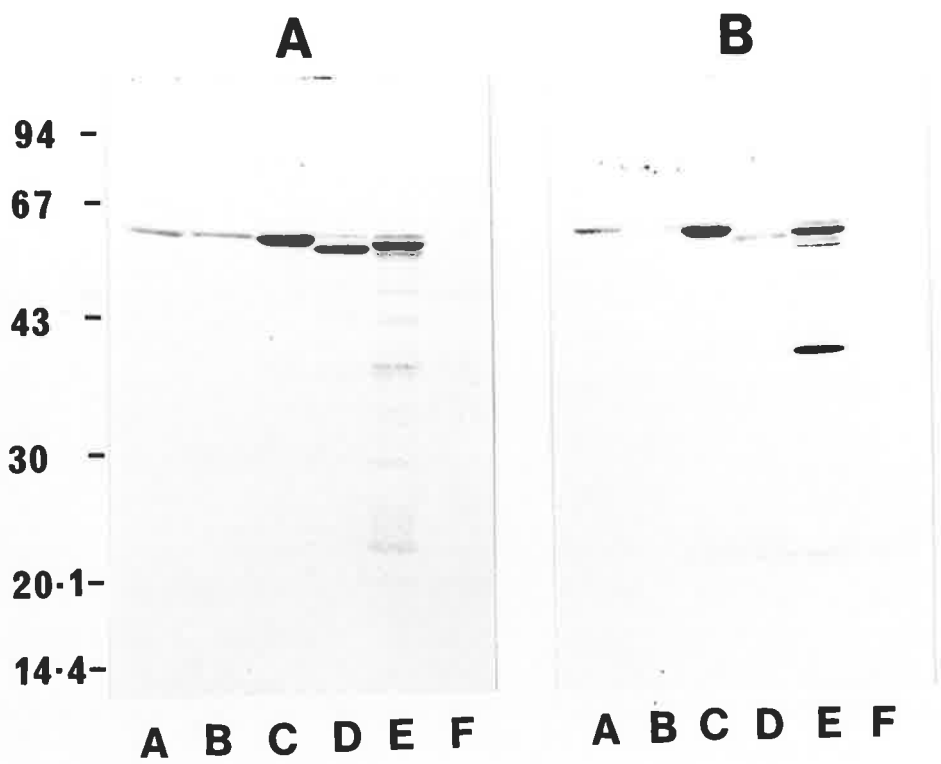
(2) *C. coli* NCTC 11366

(3) *C. hyointestinalis* NCTC 11563

(4) *C. hyointestinalis* NCTC 11562

(5) *C. hyointestinalis* 49226

(6) *C. hyointestinalis* NCTC 11608


A
B
94
67
43
30
20·1
14·4
A B C D E F
A B C D E F

extracted material from *C. jejuni*, *C. coli*, and three of the *C. hyointestinalis* strains examined (Figure 3.5). The major band present in the extracts from the three *C. hyointestinalis* strains was assumed to be the flagellin as it was derived using a flagellin enrichment procedure and had a similar M_r to that of the flagellins of *C. jejuni* and *C. coli*. Antiserum raised against the live *C. hyointestinalis* type strain reacted with the flagellins of the other *C. hyointestinalis* strains, and those of *C. jejuni* and *C. coli*, on immunoblotting (Figure 3.5). Even here, flagellin production by the *C. hyointestinalis* type strain could not be detected; it is possible that flagella expression by live bacteria in rabbits is better than observed here *in vitro*.

Material extracted from *C. hyointestinalis* strains 49226 and NCTC 11563 (Figure 3.5) appeared to contain major proteins of M_r values slightly less than 62 kDa. The major proteins had approximate M_r values of 60.5 kDa and 61 kDa in 49226 and NCTC 11563, respectively. A minor 62 kDa protein band was also observed in the flagellin extracts from either of these strains. It has been reported that flagellar expression in some strains of *C. coli* is subject to reversible antigenic variation, corresponding to the production of flagellar filaments containing antigenically distinguishable flagellin monomers of apparent M_r of 61.5 kDa in one antigenic phase and 59.5 kDa in the other (Harris *et al.*, 1987). A similar situation could occur in these two strains of *C. hyointestinalis* as there is a possibility that the 62 kDa protein band observed in these flagellin extracts may also be a flagellin monomer of a different apparent M_r.

3.2.7 Lipopolysaccharides of *C. hyointestinalis*

Previous work has shown that *C. jejuni* and *C. coli* synthesise LPS of small apparent M_r, as detected by silver staining of SDS-PAGE gels of Proteinase K-treated cell membrane material (Logan and Trust, 1984; Perez-Perez and Blaser, 1985), while some *C. jejuni* strains also make material of high M_r, reminiscent of the lipopolysaccharides of *Salmonella* strains. This higher M_r LPS, however, was detectable only by immunoblotting (Preston and Penner, 1987). Examination, by SDS-PAGE and staining, of material from *C. jejuni*, showed LPS entities of varying apparent M_r, but these represented aggregates of a low M_r material (Logan and Trust, 1984). The LPS of *C. fetus*, however, ran as a mixture of both low and higher M_r

materials, and material of higher M_r did not appear to be an aggregate of the lower M_r species, as isolation of the material from a gel, followed by re-electrophoresis, did not result in a change in apparent M_r (Perez-Perez *et al.*, 1986). The properties of the LPS from the type strains of *C. jejuni*, *C. coli*, *C. hyointestinalis* and the nine other strains of *C. hyointestinalis* were examined on SDS-PAGE by silver staining (Figure 3.6).

The type strains of *C. jejuni* and *C. coli* showed a low M_r LPS species, as did the *C. hyointestinalis* type strain. A number of the *C. hyointestinalis* strains examined expressed LPS of more than one M_r value and among the ten strains there was a great degree of heterogeneity with most strains appearing to express unique LPS profiles.

The LPS extracted from each of the ten *C. hyointestinalis* strains was examined on an immunoblot using a mixture of antisera which had been raised separately against purified LPS from all of the *C. hyointestinalis* strains (Figure 3.7). This was performed to determine if any of the *C. hyointestinalis* strains produced a series of high M_r LPS components characteristic of core-attached O-side chains of various lengths (as has been shown, by immunoblotting, with some strains of *C. jejuni* (Preston and Penner, 1987)). The immunoblot should also demonstrate whether the LPS of individual *C. hyointestinalis* strains was immunogenic. The LPS from strains NCTC 11608, 48869, and 48138 (tracks A, E, and F) reacted very weakly on immunoblotting; this could imply that the LPS from these strains is poorly immunogenic. The low M_r LPS materials from strains 45104 and 49905 (tracks B and D) were preferentially detected by the antiserum; high M_r LPS components, detected in these strains by silver staining (Figure 3.6), was not reactive upon immunoblotting. Again, the high M_r LPS components produced by these strains may be poorly immunogenic. Immunoblotting of the LPS from strains 49226, NCTC 11563, and NCTC 11609 (tracks C, H, and I) detected some higher M_r LPS material which was not present in the silver-stained gel in Figure 3.6. The LPS profiles on the immunoblot of strains NCTC 11562 and NCTC 11610 (tracks G and J), were very similar to their corresponding silver stain profiles present in Figure 3.6. These results show that previously noted heterogeneity in *C. hyointestinalis* LPS structure is also reflected in the reactivity of the LPS of various strains with homologous antisera.

FIGURE 3.6 Silver stained polyacrylamide gel analysis of the lipopolysaccharides of ten strains of *C. hyointestinalis*.

Samples of whole cell lysates were treated with Proteinase K and the residual material was electrophoresed in a 20% SDS-polyacrylamide gel which was then subjected to silver staining. The arrow marks a point of reference for Figure 3.7. The strains examined in each track are as follows:

(A) *S. typhimurium* C5

(B) *E. coli* K-12 DH1

(C) *C. jejuni* NCTC 11168

(D) *C. coli* NCTC 11366

(E) *C. hyointestinalis* NCTC 11608

(F) *C. hyointestinalis* 45104

(G) *C. hyointestinalis* 49226

(H) *C. hyointestinalis* 49905

(I) *C. hyointestinalis* 48869

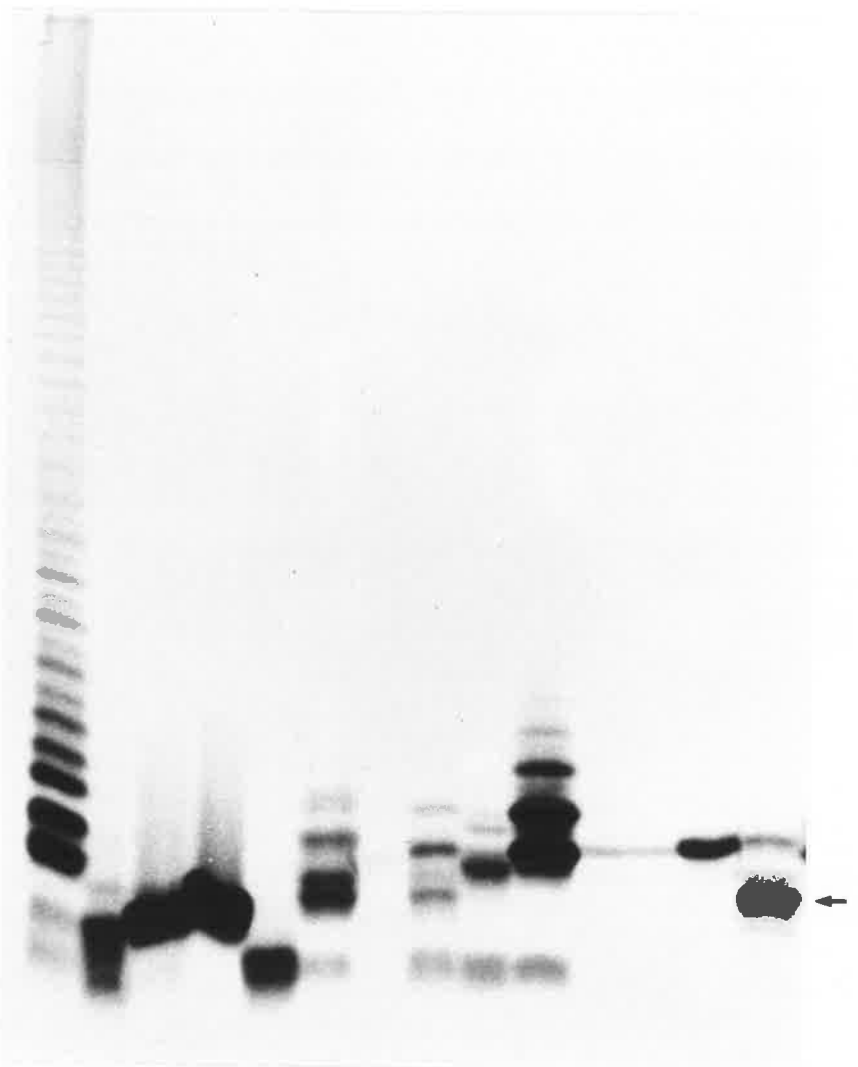
(J) *C. hyointestinalis* 48318

(K) *C. hyointestinalis* NCTC 11562

(L) *C. hyointestinalis* NCTC 11563

(M) *C. hyointestinalis* NCTC 11609

(N) *C. hyointestinalis* NCTC 11610



A B C D E F G H I J K L M N

FIGURE 3.7 Immunoblot of LPS from ten *C. hyointestinalis* strains.

Material from Proteinase K-treated cells was separated by SDS-PAGE was electroblotted to nitrocellulose and immunoblotted, using as a primary antibody a mixture of antisera, composed of equal volumes of sera raised against each individual live *C. hyointestinalis* strain. The arrow marks a point of reference for Figure 3.6. The strains examined in each track were as follows:

(A) *C. hyointestinalis* NCTC 11608

(B) *C. hyointestinalis* 45104

(C) *C. hyointestinalis* 49226

(D) *C. hyointestinalis* 49905

(E) *C. hyointestinalis* 48869

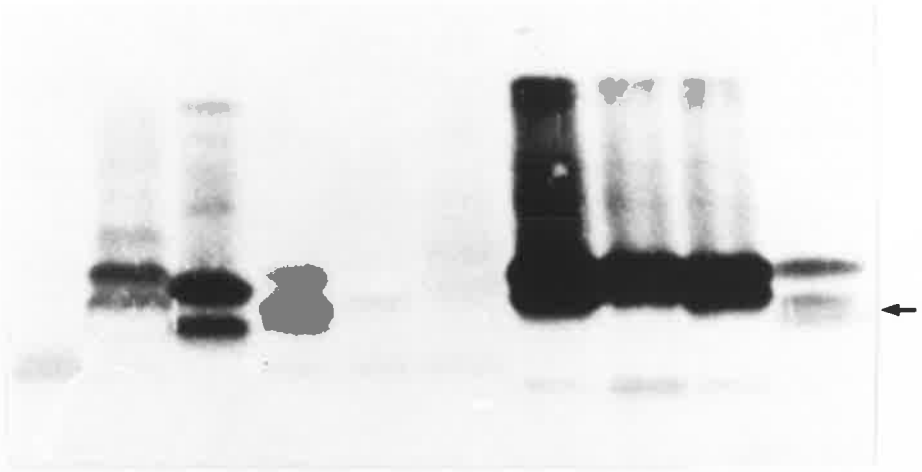
(F) *C. hyointestinalis* 48138

(G) *C. hyointestinalis* NCTC 11562

(H) *C. hyointestinalis* NCTC 11563

(I) *C. hyointestinalis* NCTC 11609

(J) *C. hyointestinalis* NCTC 11610



A B C D E F G H I J

3.2.8 Aggregation of LPS

The LPS of *C. hyointestinalis* strain 48138 was run on a gel, and the individual bands excised and re-electrophoresed (Figure 3.8). The fact that the apparent M_r's of individual bands did not change during this procedure may indicate that the bands in the stained gel represent molecular species of different M_r, and not aggregates of a material of low M_r. The high M_r LPS from *C. fetus* has also been reported not to consist of aggregates of lower M_r material (Logan and Trust, 1984).

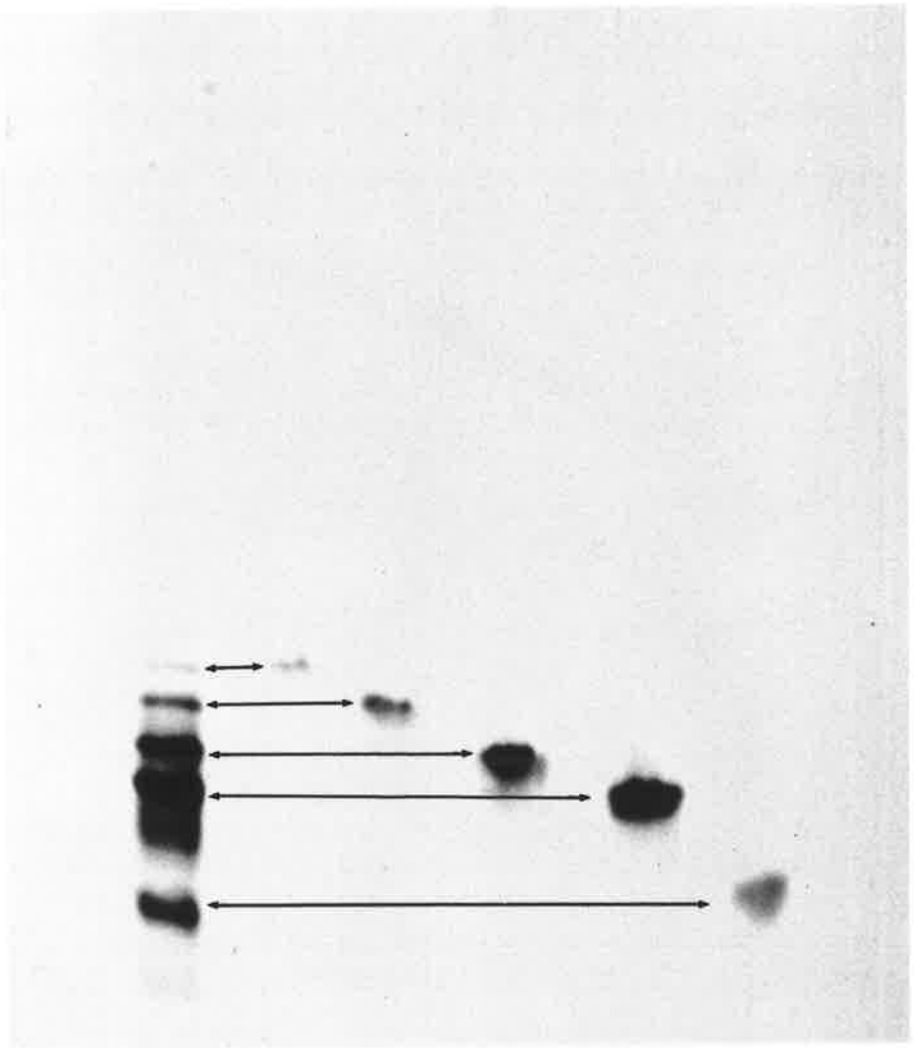
3.3 Summary and conclusions

Previous work has shown that *C. jejuni* and *C. coli* contain only a single major outer membrane protein of apparent M_r 41 to 45 kDa (Blaser *et al.*, 1983a). The outer membrane of *C. fetus*, however, contained two major proteins, both of higher apparent M_r than the *C. jejuni* or *C. coli* protein (Blaser *et al.*, 1983). As shown here, the situation with *C. hyointestinalis* was similar, insofar as all strains examined expressed two major proteins. Inter-strain variation in the apparent M_rs of the major outer membrane proteins was detected, with three of the ten strains differing from the norm. Of the seven of ten strains that appeared similar, four were from Australia, one (type) was from the U.S.A., and two were from England. In these strains, each of the two outer membrane proteins was of a higher apparent M_r than the *C. jejuni* or *C. coli* protein. Exposure to 100°C in SDS was necessary for the effective solubilisation of only the higher M_r protein, both in the type strain, and in the strains with unique outer membrane profiles. This characteristic of heat-modifiability has been observed with the porin proteins of *C. jejuni* and *C. coli* (Huyer *et al.*, 1986; Page *et al.*, 1989).

The type strain of *C. hyointestinalis* was poorly flagellated, but caused the generation, in rabbits, of anti-flagellar antibodies that detected material in flagella extracts from other *C. hyointestinalis* isolates, and *C. jejuni* and *C. coli*, in an immunoblot. This fact, and the observation that the apparent M_rs of the various major protein bands present in the solubilised flagella extracts were similar, suggests that these proteins are indeed flagellin monomers and that structural relationships between the flagellin of *C. hyointestinalis*, on the one hand, and

FIGURE 3.8 Examination of the lipopolysaccharide of *C. hyointestinalis* 48318 for aggregative behaviour.

A whole cell lysate from *C. hyointestinalis* 48318 was treated with Proteinase K and the material was initially separated by SDS-PAGE on duplicate gels. One gel was fixed and rapidly silver-stained for LPS, and then aligned with the duplicate unfixed gel. Areas containing the bands of interest were excised from the unfixed gel, placed in the wells of a fresh gel, and re-electrophoresed. The first track (A) shows LPS from *C. hyointestinalis* 48318, arrows indicate bands excised from an unstained equivalent gel and subjected to a second SDS-PAGE separation (tracks B to F).


A
B
C
D
E
F

flagellins of *C. jejuni* and *C. coli*, on the other, exist. An antigenic determinant common to denatured flagellin of *C. jejuni* and *C. coli* has been reported (Mills *et al.*, 1986), as has a similar determinant on the flagellins of *C. jejuni* and *C. fetus* (Blaser *et al.*, 1986a).

The observation that two of the *C. hyointestinalis* strains may produce two flagellin molecules could suggest that strains of *C. hyointestinalis* may be able to antigenically vary their flagella as has been seen in some strains of *C. coli* (Harris *et al.*, 1987). If this is so, a future line of work may be concerned with the purification of both flagellin molecules and the subsequent generation of monoclonal antibodies specific for each flagellin. By employing these monoclonal antibodies to detect clones which harbour the genes encoding for the flagellin, an understanding of the genetics responsible for this antigenic variation could be obtained, as has been done with strains of *C. coli* and *C. jejuni* (Harris *et al.*, 1987; Guerry *et al.*, 1988; Logan *et al.*, 1989; Thornton *et al.*, 1990; Wassenaar *et al.*, 1991).

The LPS of *C. hyointestinalis* showed considerable heterogeneity between strains. Some strains displayed LPS of only low M_r, while others synthesised LPS materials of higher M_r. In at least one instance, this higher M_r material did not represent artifactual aggregation of lower M_r material, as observed in LPS preparations from *C. jejuni* (Logan and Trust, 1984). *C. hyointestinalis* can therefore synthesise non-aggregative high M_r LPS material; a feature observed in its most closely related species, *C. fetus* (Thompson *et al.*, 1988; Vandamme *et al.*, 1991).

CHAPTER 4

DEVELOPMENT OF A SHUTTLE VECTOR FOR THE GENETIC ANALYSIS OF *C. HYOINTESTINALIS*

4.1 Introduction

Little is known about the molecular biology of pathogenesis in *Campylobacter*. Attempts to express cloned genes from *Campylobacter* species in *E. coli* K-12 have met with limited success (Walker *et al.*, 1986; Chan *et al.*, 1988; Nuijten *et al.*, 1989). To facilitate the analysis of gene expression of *Campylobacter* species, a plasmid shuttle vector, pILL550, has been constructed which can be mobilized from *E. coli* K-12 to *C. jejuni*, *C. coli*, and *C. fetus* (Labigne-Roussel *et al.*, 1987).

No genetic studies of *C. hyointestinalis* have been reported, and it is not known whether the *Campylobacter* shuttle vector pILL550 can replicate in *C. hyointestinalis* and consequently be used for cloning and expressing *C. hyointestinalis* genes. Most strains of *C. jejuni* and *C. coli* can take up DNA from their environment or receive plasmid DNA by electroporation (Miller *et al.*, 1988; Wang and Taylor, 1990b), however, this has not been demonstrated with *C. hyointestinalis*.

In this chapter the suitability of pILL550 as a shuttle vector for *C. hyointestinalis* is investigated and the subsequent construction of candidate *C. hyointestinalis*-specific shuttle vectors, based upon a cryptic plasmid isolated from a *C. hyointestinalis* strain, is described. The ability of *C. hyointestinalis* to undergo natural transformation and receive plasmid DNA by electroporation is also examined.

4.2 Results

4.2.1 Conjugal transfer of pILL550 into *C. hyointestinalis*

Attempts to mobilize pILL550 into several strains of *C. hyointestinalis* were unsuccessful in experiments where transfer was readily achieved using *C. coli* NCTC 11366 as a recipient. In agreement with a previous report (Labigne-Roussel *et al.*, 1987), *C. coli* transconjugants were obtained with a frequency of 10^{-4} transconjugants per donor present at the beginning of the mating (Table 4.1).

4.2.2 Conjugation of broad-host-range vectors into *C. hyointestinalis*

As *C. hyointestinalis* did not serve as a recipient in the conjugal transfer of pILL550, an investigation was made to determine if *C. hyointestinalis* could act as a recipient for some of the conjugative broad-host-range plasmids. Attempts to mobilize the broad-host-range-plasmid RP4 (from *E. coli* K-12 strain S17-1) and the broad-host-range cloning vectors pKT230 and pKT231 (derived from the broad-host-range plasmid RSF1010 and mobilized from the *E. coli* K-12 donor strain CB617; Bagdasarian *et al.*, 1981) were unsuccessful although the plasmids could be transferred into *S. typhimurium* strain LT2 SL2981 with high efficiency (Table 4.2). To determine whether or not the lack of conjugal transfer might be due to non-expression of the antibiotic-resistance determinants of these plasmids in *Campylobacter*, the kanamycin-resistance gene carried on the 1.427 kb *Cla*I-*Hin*dIII DNA restriction fragment from pILL550 was cloned between the *Cla*I-*Hin*dIII sites of pKT231, replacing the original kanamycin-resistance gene (Figure 4.1). This plasmid construct could also not be transferred by conjugation into *C. hyointestinalis* NCTC 11608 but could be transferred into *S. typhimurium* at a high efficiency (Table 4.2). The conjugative streptococcal transposon Tn*916*, which can be transferred naturally between Gram-positive and Gram-negative bacteria (Bertram *et al.*, 1991), was also examined for its ability to transfer to *C. hyointestinalis*. Mating experiments between *Enterococcus faecalis* CG110 harbouring the transposon and *C. hyointestinalis* did not, however, result in any transconjugants, whereas the transposon could be transferred into *S. typhimurium* with high efficiency (Table 4.2).

TABLE 4.1 Conjugal transfer of pILL550 from *E. coli* K-12 S17-1 into *Campylobacter* species

Recipient species	No. transconjugants per donor
C. coli NCTC 11366	10^{-4}
C. hyointestinalis NCTC 11608	$<10^{-9}$*
C. hyointestinalis NCTC 11562	$<10^{-9}$*
C. hyointestinalis NCTC 11563	$<10^{-9}$*
C. hyointestinalis NCTC 11609	$<10^{-9}$*
C. hyointestinalis NCTC 11610	$<10^{-9}$*
C. hyointestinalis 45104	$<10^{-9}$*
C. hyointestinalis 49226	$<10^{-9}$*
C. hyointestinalis 49905	$<10^{-9}$*
C. hyointestinalis 48869	$<10^{-9}$*
C. hyointestinalis 48138	$<10^{-9}$*

* Repeated attempts to obtain transconjugants with *C. hyointestinalis* in mating tests using, as donor, *E. coli* K-12 S17-1 harboring pILL550 were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

TABLE 4.2 Conjugal transfer of broad host-range vectors into *C. hyointestinalis* NCTC 11608

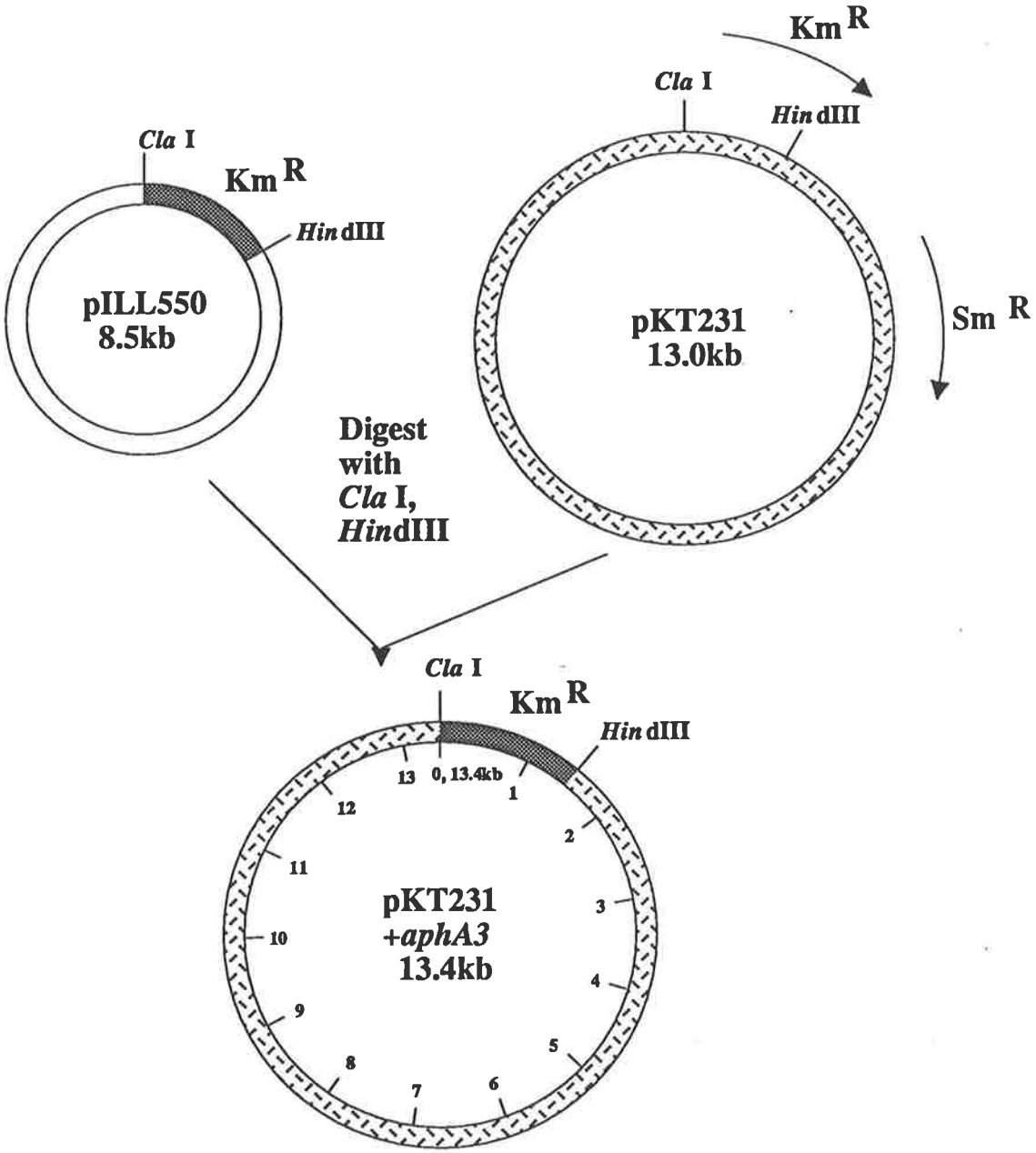
Broad host-range vector	No. of transconjugants per donor into recipient species:	
	S. typhimurium LT2 SL2981*	*C. hyointestinalis* NCTC 11608
RP4	10^{-4}	$<10^{-9}$**
pKT230	10^{-4}	$<10^{-9}$**
pKT231	10^{-4}	$<10^{-9}$**
pKT231+*aphA-3*	10^{-4}	$<10^{-9}$**
Tn*916*	10^{-4}	$<10^{-9}$**

* The *S. typhimurium* LT2 strain SL2981 was used as a control recipient in mating tests using, as donors, *E. coli* K-12 strains harbouring the plasmids under test. Donor counterselection was with tetracycline.

** Repeated attempts to obtain transconjugants with *C. hyointestinalis* NCTC 11608 in mating tests using, as donors, *E. coli* K-12 strains harbouring the various broad host-range vectors, or Tn*916*, were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

FIGURE 4.1 Construction of plasmid pKT231 harbouring the *aphA-3* gene.

The *Cla*I-*Hin*dIII DNA restriction fragment containing the *aphA-3* gene (Km^R) was cloned from pILL550 into pKT231 digested with *Cla*I.


Km R
Cla I
Hin dIII
pKT231
13.0kb
Sm R
Cla I
Km R
Hin dIII
pILL550
8.5kb
Digest
with
Cla I,
HindIII
Cla I
Km R
Hin dIII
0, 13.4kb
1
2
3
4
5
6
7
8
9
10
11
12
13
pKT231
+aphA3
13.4kb

It seemed possible that the replicons of these broad-host-range plasmids and the *C. coli* plasmid replicon of pILL550 were non-functional in *C. hyointestinalis*; attempts were therefore made to construct a *C. hyointestinalis*-specific cosmid shuttle vector. As a basis for constructing a *C. hyointestinalis*-specific plasmid vector, the ten *C. hyointestinalis* strains examined in Chapter 3 were screened for plasmid content with the aim of using an endogenous plasmid as the basis for the vector.

4.2.3 Plasmids of *C. hyointestinalis*

All ten *C. hyointestinalis* strains were screened for plasmid content (Figure 4.2). Of the ten isolates examined, four (all Australian) contained a single plasmid of the same size of 2.5 kb. No other plasmids were detected in any of the ten strains examined.

4.2.4 *C. hyointestinalis* 45104 cryptic plasmid restriction map

A *C. hyointestinalis* replicon was required for inclusion in a *C. hyointestinalis* shuttle vector. For this purpose the cryptic plasmid of *C. hyointestinalis* 45104 was chosen largely because of its small size (2.5 kb). Restriction analysis revealed that the plasmid contained very few common restriction endonuclease cleavage sites, with the most notable features being two *Hin*dIII sites and single sites for *Not*I, *Cla*I and *Eco*RV (Figure 4.3). No sites were detected for *Acc*I, *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Pst*I, *Pvu*I, *Pvu*II, *Sph*I, *Xba*I, or *Xho*I. As the *Cla*I site and one of the *Hin*dIII sites lay diametrically opposite on the plasmid map (Figure 4.3), and replication regions are generally small, there was a good possibility that cloning into *E. coli* K-12 of the whole cryptic plasmid, using one or the other of these sites, might result in a clone which contained the intact *C. hyointestinalis* plasmid replicon.

4.2.5 Construction of *C. hyointestinalis* shuttle vectors

The plasmid pPM2101 (Sharma *et al.*, 1989) was used to provide the *E. coli* K-12 replicon and the mobilization functions necessary for a shuttle vector. pPM2101 consists of the cosmid pHC79 and a fragment containing the broad-host-range RP4 *oriT* (Sharma *et al.*, 1989). pPM2101 was digested with *Cla*I and *Hin*dIII and the 1.427 kb *Cla*I-*Hin*dIII DNA

FIGURE 4.2 Plasmid isolation from four Australian *C. hyointestinalis* isolates.

Ten *C. hyointestinalis* strains were subjected to the plasmid isolation procedure described in Materials and Methods (Section 2.11; Method 1) and the material examined by agarose gel electrophoresis. Fragments of bacteriophage SPP1 DNA digested with *Eco*R1 were used as size standards. The fragment sizes (kb) were 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, and 0.36. The strains examined in each track were as follows:

(A) SPP1

(B) *C. hyointestinalis* NCTC 11608

(C) *C. hyointestinalis* 45104

(D) *C. hyointestinalis* 49905

(E) *C. hyointestinalis* 48869

(F) *C. hyointestinalis* 48318

(G) *C. hyointestinalis* 49226

(H) *C. hyointestinalis* NCTC 11562

(I) *C. hyointestinalis* NCTC 11563

(J) *C. hyointestinalis* NCTC 11609

(K) *C. hyointestinalis* NCTC 11610

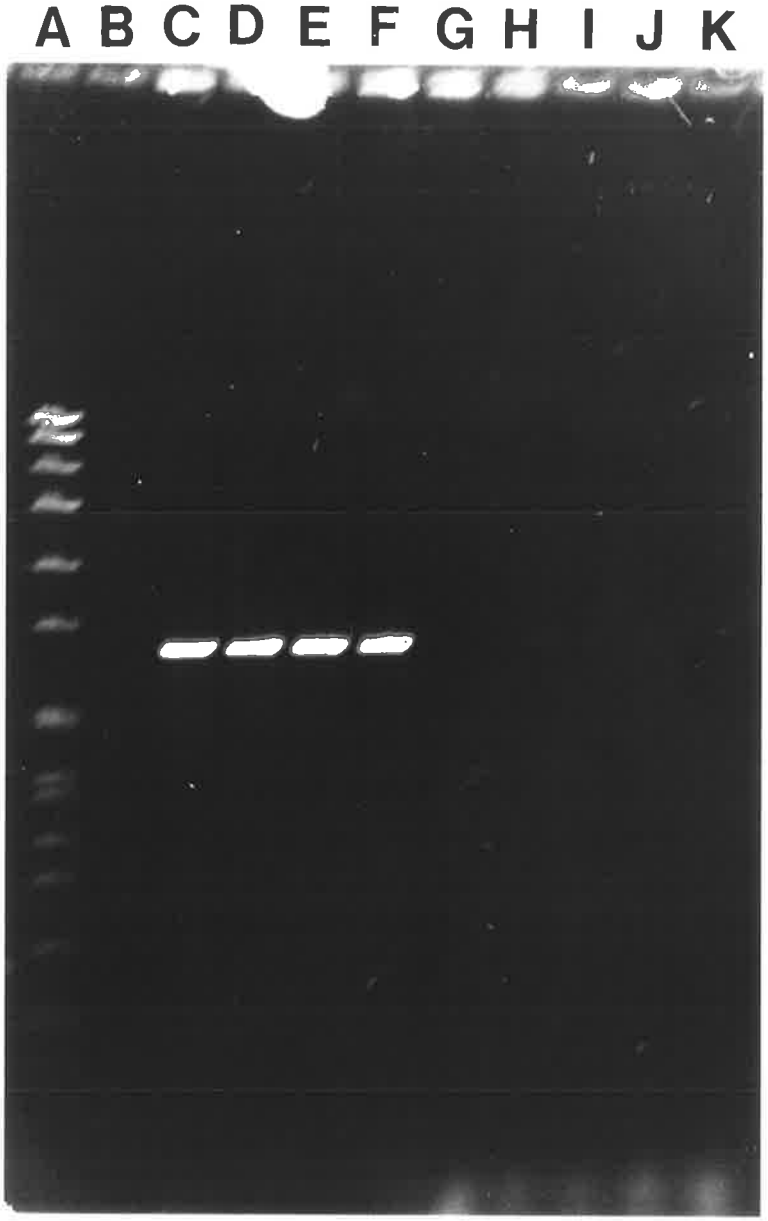
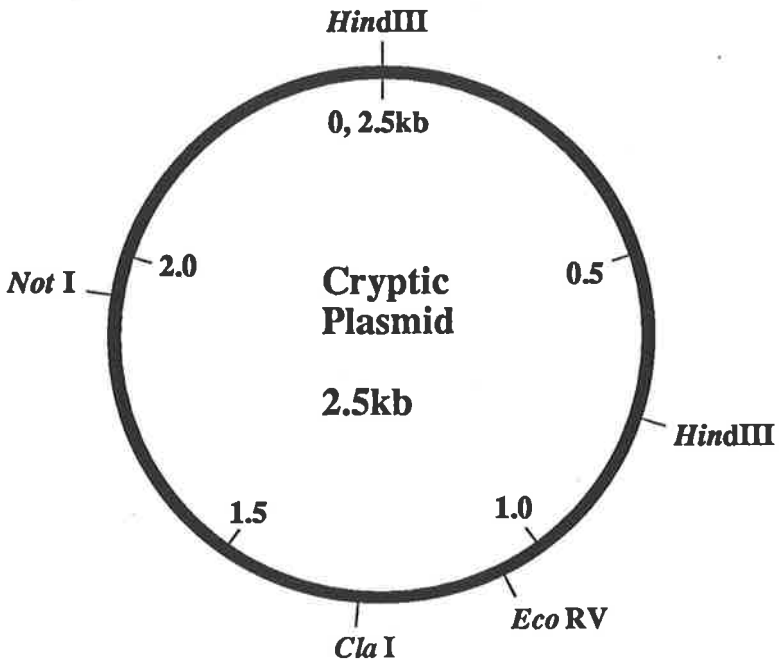

A B C D E F G H I J K

FIGURE 4.3 Restriction endonuclease-generated map of the 2.5 kb cryptic plasmid of *C. hyointestinalis* 45104.


*Hin*dIII
0, 2.5kb
Not I
2.0
Cryptic
Plasmid
2.5kb
0.5
*Hin*dIII
1.5
1.0
Eco RV
Cla I

restriction fragment of pILL550 containing the kanamycin resistance gene was cloned into pPM2101, generating the plasmid pCHI1 (Figure 4.4). The kanamycin resistance gene of pILL550 was chosen as a selectable marker because it was expressed in both *E. coli* K-12 and *Campylobacter* species (Labigne-Roussel *et al.*, 1987). The cryptic plasmid from *C. hyointestinalis* 45104 was partially digested with *Hin*dIII and ligated into the *Hin*dIII site of pCHI1. The resulting clones were screened for one which contained the entire cryptic plasmid cloned *via* the cryptic plasmid *Hin*dIII site opposite the single *Cla*I site. This plasmid was named pCHI2 (Figure 4.4). The cryptic plasmid was also digested with *Cla*I and ligated into the *Cla*I site of pCHI1. The resulting plasmid was designated pCHI3 (Figure 4.4).

4.2.6 Conjugation of pCHI2 and pCHI3 into *C. hyointestinalis*

pCHI2 and pCHI3 were transformed into the *E. coli* K-12 donor strain S17-1, which contains the transfer genes of RP4 integrated in the chromosome (Simon *et al.*, 1983), and the strains thus constructed were mated with *C. hyointestinalis* NCTC 11608 (type strain). Despite repeated attempts, no transconjugant colonies could be obtained (Table 4.3), even though pCHI2 and pCHI3 could be mobilized into *S. typhimurium* LT2 SL2981 at high efficiency. This indicated that the *oriT* and kanamycin resistance gene were both functioning in these plasmids. Attempts to mobilize pCHI2 and pCHI3 into *C. coli* and *C. jejuni* were also unsuccessful (Table 4.3). Because neither of these two plasmids could be conjugated into *C. hyointestinalis* the possibility existed that neither of them contained, intact, the entire replication region of the cryptic plasmid. This would be the case if the replication region were large enough (greater than approximately 1.25 kb) to cover both cryptic plasmid restriction sites used here for the shuttle constructions.

4.2.7 Construction of pCHI4

If the replicon of the cryptic plasmid did span the restriction sites then there was a possibility that a clone *via* the *Not*I site might contain the replicon in an undisrupted form, as the *Not*I site lay roughly halfway between the single *Cla*I site and the *Hin*dIII site cut in the

FIGURE 4.4 Construction of plasmids pCHI2 and pCHI3.

The *Cla*I-*Hin*dIII DNA restriction fragment containing the *aphA-3* gene (Km^R) was cloned from pILL550 into pPM2101 digested with *Cla*I and *Hin*dIII, yielding pCHI1. The cryptic plasmid from *C. hyointestinalis* 45104 was partially digested with *Hin*dIII and ligated into the *Hin*dIII site of pCHI1. The resulting clones were screened for those in which the entire cryptic plasmid was cloned *via* the cryptic plasmid *Hin*dIII site opposite the single *Cla*I site. The resulting plasmid was named pCHI2. The cryptic plasmid was also digested with *Cla*I and ligated into the *Cla*I site of pCHI1, yielding pCHI3.

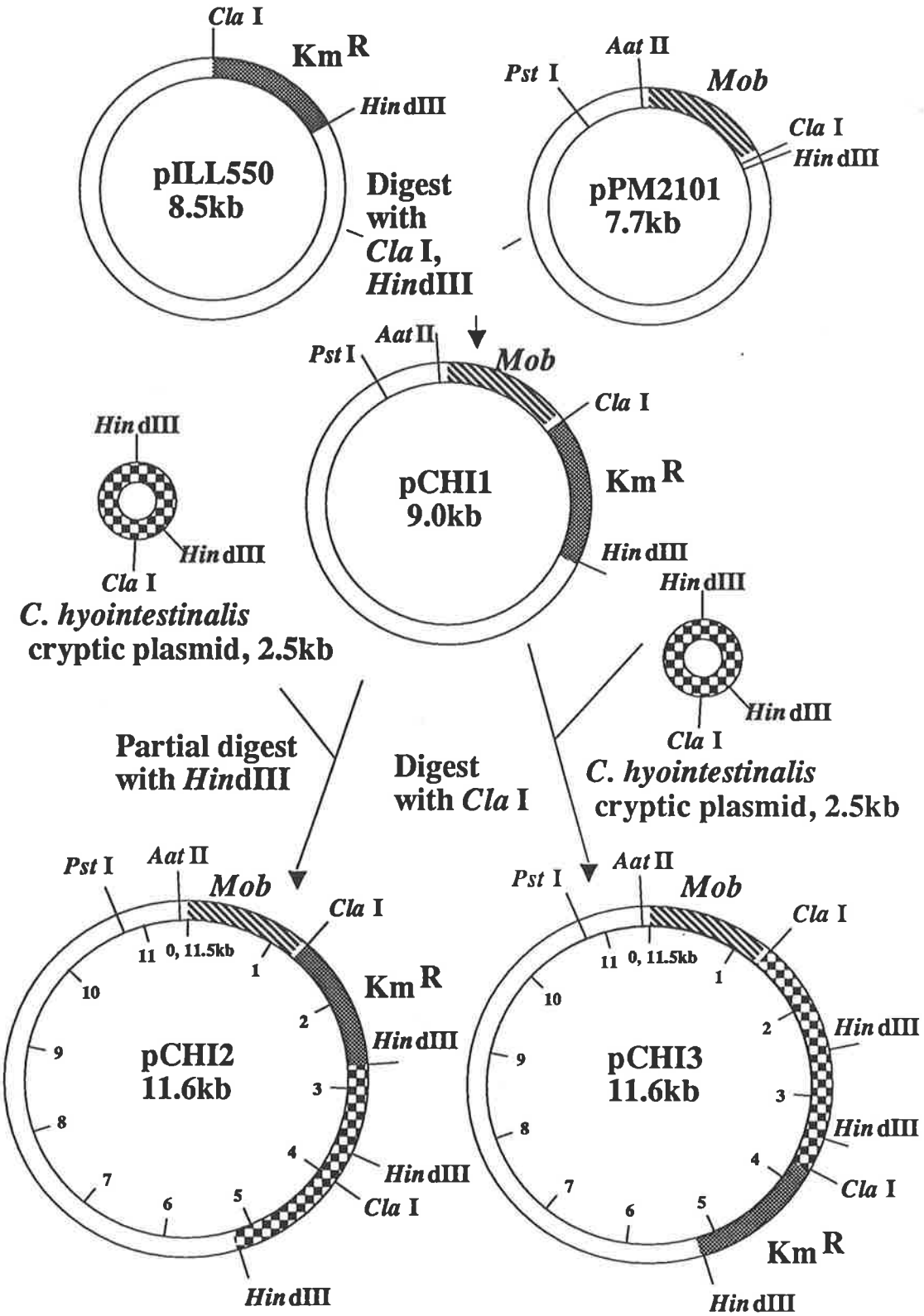

Cla I
Km R
Hin dIII
pILL550
8.5kb
Digest
with
Cla I,
HindIII
Aat II
Pst I
Mob
Cla I
Hin dIII
pPM2101
7.7kb
Aat II
Pst I
Mob
Cla I
pCHI1
9.0kb
Km R
Hin dIII
Hin dIII
Hin dIII
Cla I
C. hyointestinalis
cryptic plasmid, 2.5kb
Hin dIII
Hin dIII
Cla I
C. hyointestinalis
cryptic plasmid, 2.5kb
Partial digest
with HindIII
Digest
with Cla I
Pst I
Aat II
Mob
Cla I
11
0, 11.5kb
1
10
2
Km R
Hin dIII
9
pCHI2
11.6kb
3
8
4
Hin dIII
7
6
5
Cla I
Hin dIII
Pst I
Aat II
Mob
Cla I
11
0, 11.5kb
1
10
2
Hin dIII
9
pCHI3
11.6kb
3
Hin dIII
8
4
Cla I
7
6
5
Km R
Hin dIII

TABLE 4.3 Conjugal transfer of candidate shuttle vectors from *E. coli* K-12 S17-1 into *Campylobacter* species and *S. typhimurium* LT2 SL2981

Plasmid	No. of transconjugants per donor into the recipient species:			
	S. typhimurium LT2 SL2981	*C. hyointestinalis* NCTC 11608	*C. coli* NCTC 11366	*C. jejuni* NCTC 11168
pCHI2	10^{-4}	$<10^{-9}$*	$<10^{-9}$*	$<10^{-9}$*
pCHI3	10^{-4}	$<10^{-9}$*	$<10^{-9}$*	$<10^{-9}$*
pCHI4	10^{-4}	10^{-9}**	$<10^{-9}$*	$<10^{-9}$*
pCHI6	$<10^{-9}$#	ND	ND	ND
pCHI7	10^{-4}	$<10^{-9}$*	ND	ND
pCHI8	10^{-4}	$<10^{-9}$*	ND	ND
pCHI9	10^{-4}	$<10^{-9}$*	ND	ND
pCHI10	10^{-4}	$<10^{-9}$*	ND	ND

* Repeated attempts to obtain transconjugants with *Campylobacter* in mating tests using, as donors, *E. coli* K-12 S17-1 strains harbouring the various plasmids, were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

** Repeated attempts to obtain transconjugants with *C. hyointestinalis* NCTC 11608 in mating tests using, as donor, an *E. coli* K-12 S17-1 strain harbouring pCHI4, resulted in the isolation of one transconjugant in one particular experiment.

Repeated attempts to obtain transconjugants with *S. typhimurium* LT2 SL2981 in mating tests using, as donor, an *E. coli* K-12 S17-1 strain harbouring pCHI6, were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

ND: not determined

formation of pCHI2 (Figure 4.3). pPM2101 did not contain any *Not*I sites, but pCHI2 contained the *Not*I site of the cryptic plasmid. Rather than assume that a *Not*I clone might contain the replicon in an undisrupted form, an alternative construction, using pCHI2, ensured that the replicon was undisrupted. Here, the cryptic plasmid was digested with *Not*I and was cloned into the *Not*I site of pCHI2. The orientation of insertion was such that any open reading frame in the cryptic plasmid, even if the *Hin*dIII site was contained therein, must be present uninterrupted, in the new construct named pCHI4 (Figure 4.5).

4.2.8 Conjugation of pCHI4 into *C. hyointestinalis*

Repeated attempts to mobilize pCHI4 from *E. coli* K-12 S17-1 into *C. hyointestinalis*, *C. coli*, and *C. jejuni* were unsuccessful in tests where the plasmid could be transferred into *S. typhimurium* with high efficiency. After one mating with *C. hyointestinalis*, however, a single kanamycin resistant colony was obtained (Table 4.3). This colony was screened for plasmid content and was found to harbour a small plasmid of approximately 3.7 kb (Figure 4.6). This plasmid was named pCHI5. It appeared that approximately 10.3 kb of DNA had been lost from pCHI4 in the generation of pCHI5.

4.2.9 Restriction analysis of pCHI5

As pCHI5 was the only plasmid stable and selectable to date in *C. hyointestinalis*, the plasmid was examined in restriction enzyme digests with the aim of localizing the origin of the retained DNA. Cleavage of pCHI5 with *Cla*I generated two fragments of approximately 2.6 kb and 1.1 kb in size, but pCHI5 did not appear to be cleaved by *Hin*dIII (Figure 4.6). Only the 2.6 kb *Cla*I restriction fragment was identical in size to any *Cla*I fragment of pCHI4. This 2.6 kb *Cla*I fragment from pCHI4 contained the kanamycin-resistance gene, and approximately 1.2 kb of the cryptic plasmid, joined together by a *Hin*dIII site (Figure 4.5). The 2.6 kb *Cla*I fragment from pCHI5 could not, however, be cleaved by *Hin*dIII. The possibility existed that pCHI5 resulted from between one and several recombination events in pCHI4. An explanation of the nature and origin of pCHI5 was afforded by determining the nucleotide sequence of the plasmid (described in Chapter 5).

FIGURE 4.5 Construction of plasmid pCHI4.

The cryptic plasmid from *C. hyointestinalis* 45104 was digested with *Not*I and cloned into the *Not*I site of pCHI2. This generated a plasmid named pCHI4 which harboured two copies of the cryptic plasmid. The orientation of insertion of the cryptic plasmid in pCHI4 was such that any ORF in the cryptic plasmid, even if the *Hin*dIII site was contained therein, must be present uninterrupted.

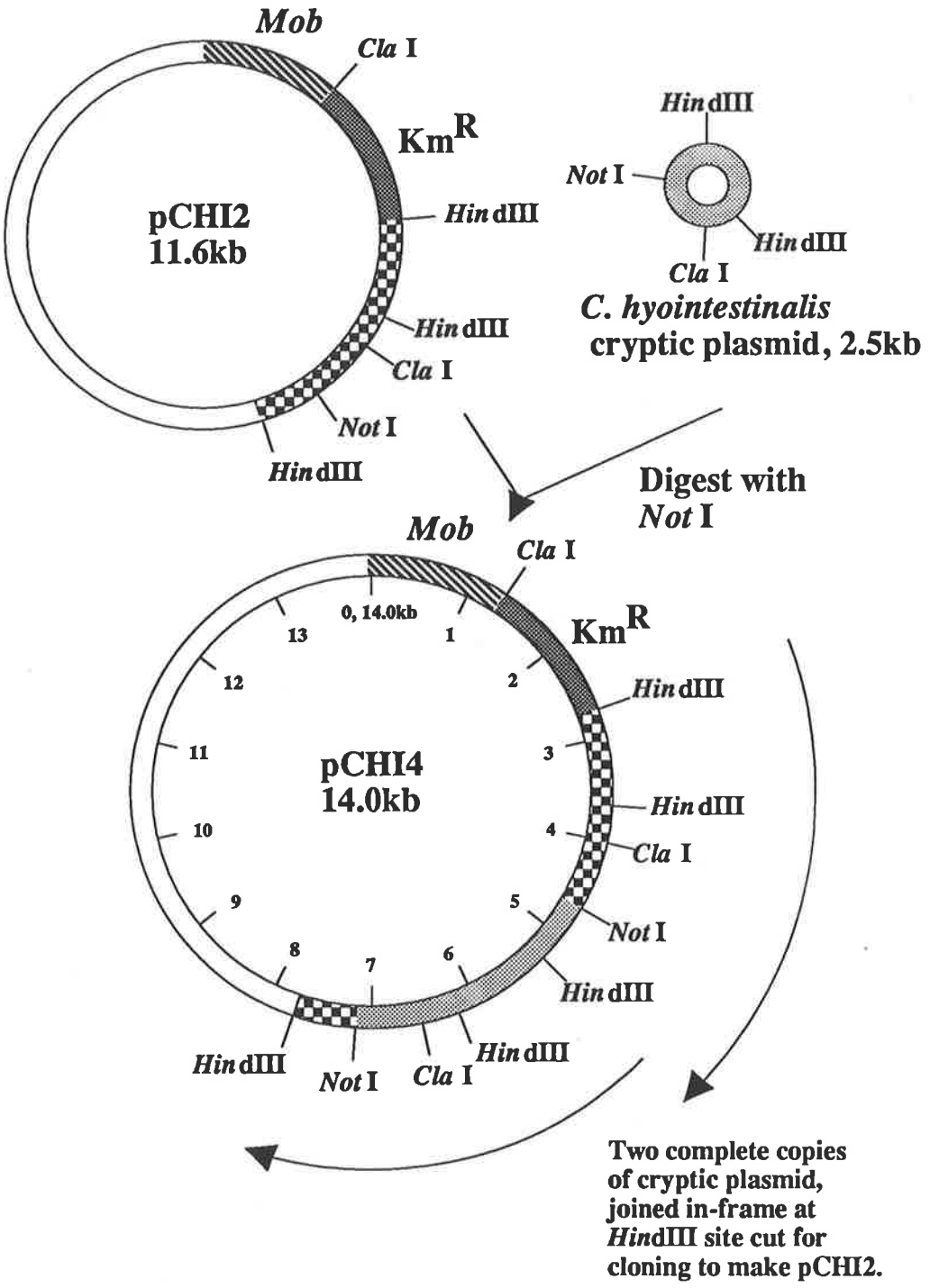

Mob
Cla I
KmR
pCHI2
11.6kb
Hin dIII
Hin dIII
Cla I
Not I
Hin dIII
Hin dIII
Not I
Cla I
C. hyointestinalis
cryptic plasmid, 2.5kb
Digest with
Not I
Mob
Cla I
0, 14.0kb
1
2
3
4
5
6
7
8
9
10
11
12
13
KmR
Hin dIII
Hin dIII
Cla I
Not I
Hin dIII
Hin dIII
Not I
Cla I
Hin dIII
pCHI4
14.0kb
Two complete copies
of cryptic plasmid,
joined in-frame at
HindIII site cut for
cloning to make pCHI2.

FIGURE 4.6 Analysis of pCHI5 by restriction endonuclease digestion with *Cla*I and *Hin*dIII.

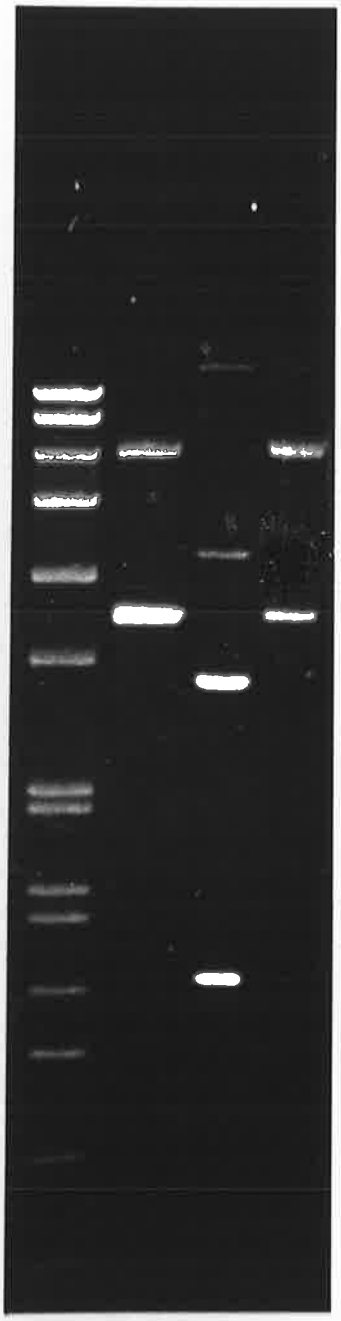
pCHI5 was digested separately with *Cla*I and *Hin*dIII and examined by agarose gel electrophoresis. Fragments of bacteriophage SPP1 DNA, digested with *Eco*R1, were used as size standards. The fragment sizes (kb) were 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, and 0.36. The plasmids, and the enzymes used for digestion with in each track, are as follows:

(A) SPP1

(B) pCHI5 (uncut)

(C) pCHI5 (*Cla*I)

(D) pCHI5 (*Hin*dIII)



A B C D

4.2.10 Transformation of pCHI5 into *E. coli* K-12 and *C. hyointestinalis*

pCHI5 could not be transformed into *E. coli* K-12 DH1 when transformants were selected for resistance to kanamycin, and had therefore lost the ability to replicate in *E. coli* K-12. An attempt was made to transform pCHI5 into $CaCl_2$-treated *C. hyointestinalis* cells by the heat-shock method. Cells from *C. hyointestinalis* NCTC 11608 were grown to mid-exponential phase (16 h) and treated with $CaCl_2$ as described in Section 2.7. After heat shocking of the cells in the presence of pCHI5, cells were plated onto kanamycin-selection plates. Thirteen kanamycin resistant colonies of *C. hyointestinalis* were obtained (Table 4.4). All of the colonies were screened for plasmid content and each contained pCHI5.

An attempt was also made to electroporate pCHI5 into *C. hyointestinalis* NCTC 11608 by the method described in Section 2.8. Approximately 5 x 10^3 kanamycin-resistant colonies of *C. hyointestinalis* could be obtained per μg of pCHI5 DNA (Table 4.4). Individual colonies were screened for plasmid content and found to contain pCHI5. These results indicated that pCHI5 contained all the genetic information necessary for autonomous replication in *C. hyointestinalis*. Also, *C. hyointestinalis* could be transformed, under the electroporation conditions used, with *C. hyointestinalis*-modified plasmid DNA. It was clear that the kanamycin-resistance gene was present on pCHI5 and was efficiently expressed in *C. hyointestinalis*. As pCHI5 represented a selectable cloning vector for *C. hyointestinalis* (but not a shuttle vector), a more detailed examination of the functions encoded by the plasmid was warranted.

4.2.11 Identifying DNA regions of pCHI5 by Southern hybridization

Probing a series of plasmids with nick-translated pCHI5 revealed that pCHI5 did not hybridize with pHC79 but did with pPM2101 (Figure 4.7). The only difference between the two probed plasmids was the *oriT* DNA from RP4 present in pPM2101, thus some *oriT* DNA was present in pCHI5. The absence of hybridization with pHC79 agreed with the data which indicated that pCHI5 could not be transformed into *E. coli* K-12 and had therefore probably lost the pHC79 DNA necessary for replication in *E. coli* K-12. pCHI5 also hybridized with the 1.427 kb *Cla*I-*Hin*dIII kanamycin-resistance DNA restriction fragment of pCHI1, in line

TABLE 4.4 Transformation of pCHI5 into *C. hyointestinalis* NCTC 11608 by electroporation or $CaCl_2$ treatment

Transformation procedure	No. of transformants/μg DNA
$CaCl_2$	13
Electroporation	5×10^3

FIGURE 4.7 Southern hybridization analysis of pCHI5.

Various plasmid constructs were digested with *Cla*I, or *Cla*I and *Hin*dIII, and electrophoresed on a 0.8% agarose gel (panel A). After transfer to nitrocellulose, the filter was probed with α-[^{32}P]-dCTP nick-translated pCHI5, washed, and subjected to autoradiography (panel B).

pCHI5 hybridized to itself and pPM2101 but not to pHC79. pCHI5 also hybridized with the 1.427 kb *Cla*I-*Hin*dIII fragments of pCHI1 and pCHI4 carrying the *aphA-3* gene (indicated by arrow 1). Hybridization (but not as strong as with the fragments mentioned earlier) was also observed with the 1.2 kb *Cla*I-*Hin*dIII and 0.9 kb *Hin*dIII DNA restriction fragments of the cryptic plasmid (indicated by arrows 2 and 3, respectively). Fragments of bacteriophage SPP1, digested with *Eco*R1, were used as size standards. The fragment sizes (kb) were 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, and 0.36. The plasmids and the enzymes used for digestion in each track are as follows:

(A) SPP1

(B) pCHI5 (uncut)

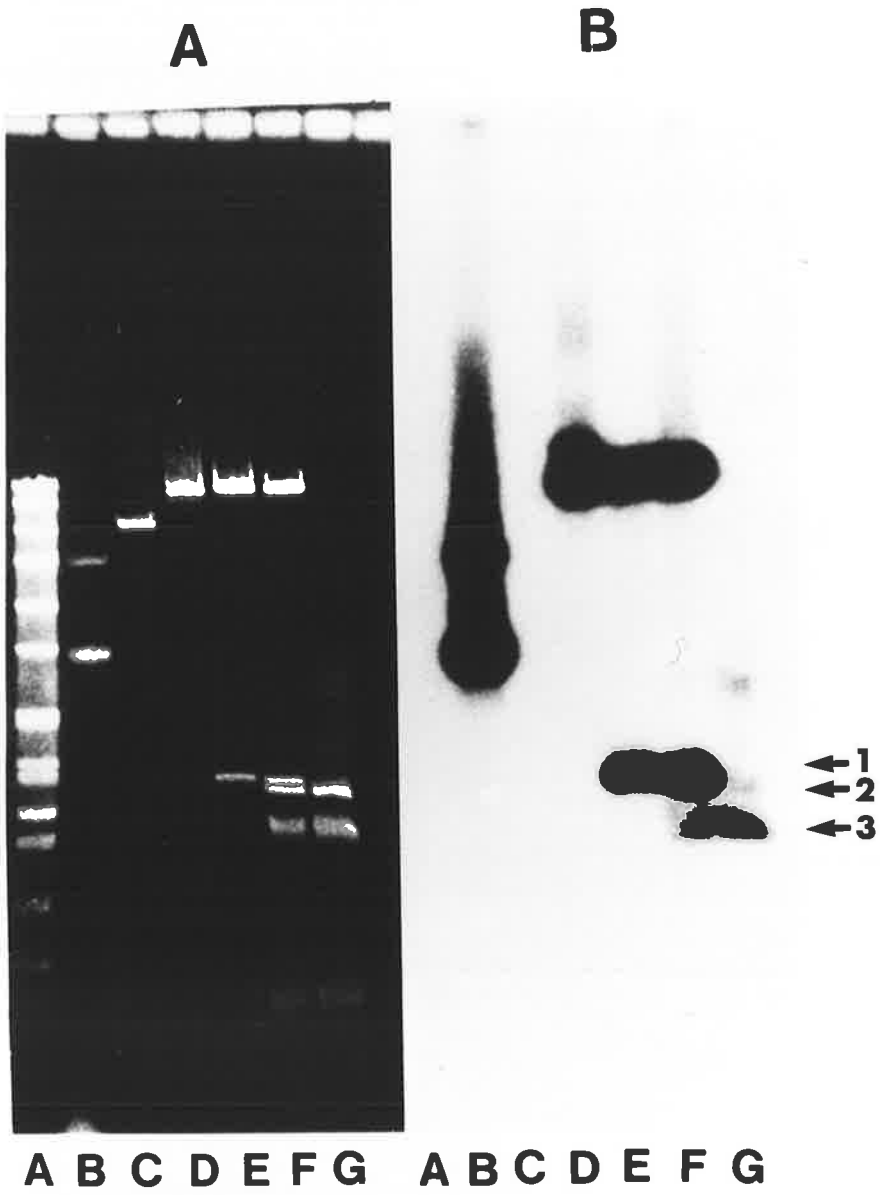
(C) pHC79 (*Cla*I)

(D) pPM2101 (*Cla*I)

(E) pCHI1 (*Cla*I and *Hin*dIII)

(F) pCHI4 (*Cla*I and *Hin*dIII)

(G) cryptic plasmid of *C. hyointestinalis* 45104 (*Cla*I and *Hin*dIII)


A
B
1
2
3
A B C D E F G A B C D E F G

with the electroporation data indicating that pCHI5 carried the kanamycin resistance gene. pCHI5 hybridized with the 1.2 kb *Cla*I-*Hin*dIII and 0.9 kb *Hin*dIII DNA restriction fragments of the cryptic plasmid indicating that regions from these fragments might be involved in the replication of the cryptic plasmid. In summary, pCHI5 appeared to contain DNA from the *oriT* region of RP4, the kanamycin-resistance gene, and some of the cryptic plasmid of *C. hyointestinalis* 45104, but not any DNA of pHC79.

4.2.12 *OriT* function in pCHI5

To determine whether the *oriT* DNA contained in pCHI5 was functional, pCHI5 was partially digested and cloned *via* one of its *Cla*I sites into the 3.0 kb *Cla*I fragment of the plasmid pcos2EMBL generating plasmid pCHI6 (Figure 4.8). This 3.0 kb *Cla*I fragment contained the replicon of pcos2EMBL and pCHI6 therefore replicated in *E. coli* K-12. There are no *Cla*I sites present in the *oriT* of RP4 (Fürste *et al.*, 1989) and so cloning *via* this site should not interfere with *oriT* function. pCHI6 was transformed into *E. coli* K-12 S17-1 and mated with *S. typhimurium* LT2 SL2981. No transconjugants were obtained (Table 4.3) which indicated that the *oriT* DNA present on pCHI5 was non-functional, and was probably not present in its complete form. The appropriate positive control (a plasmid incorporating the replicon from pcos2EMBL with a funtional *oriT*) was not examined. However, the DNA sequence of pCHI5 revealed that the residual *oriT* DNA could not have facilitated conjugal transfer (discussed in Chapter 5).

4.2.13 Attempts to construct shuttle vectors using pCHI5

As pCHI5 had been shown to replicate stably in *C. hyointestinalis*, attempts were made to utilize it in the construction of a *C. hyointestinalis* shuttle vector. pCHI5 was partially digested with *Cla*I and the whole plasmid was cloned into pCHI1 *via* either *Cla*I site separately to generate plasmids pCHI7 and pCHI8 (Figure 4.9). As the Southern hybridization data described previously had indicated that pCHI5 contained some DNA from the *oriT* and the kanamycin-resistance gene of pCHI4, there was then a possibility that one of the *Cla*I sites present in pCHI5 was the original *Cla*I site between the *oriT* and the

FIGURE 4.8 Construction of plasmid pCHI6.

The 3.0 kb *Cla*I DNA restriction fragment of pcos2EMBL, harbouring an *E. coli* K-12 replicon, was cloned into pCHI5 partially digested with *Cla*I. Clones were selected, in *E. coli* K-12, for resistance to kanamycin as pCHI5 harbours the *aphA-3* gene (Km^R) but cannot replicate in *E. coli* K-12. The resulting plasmid was named pCHI6.

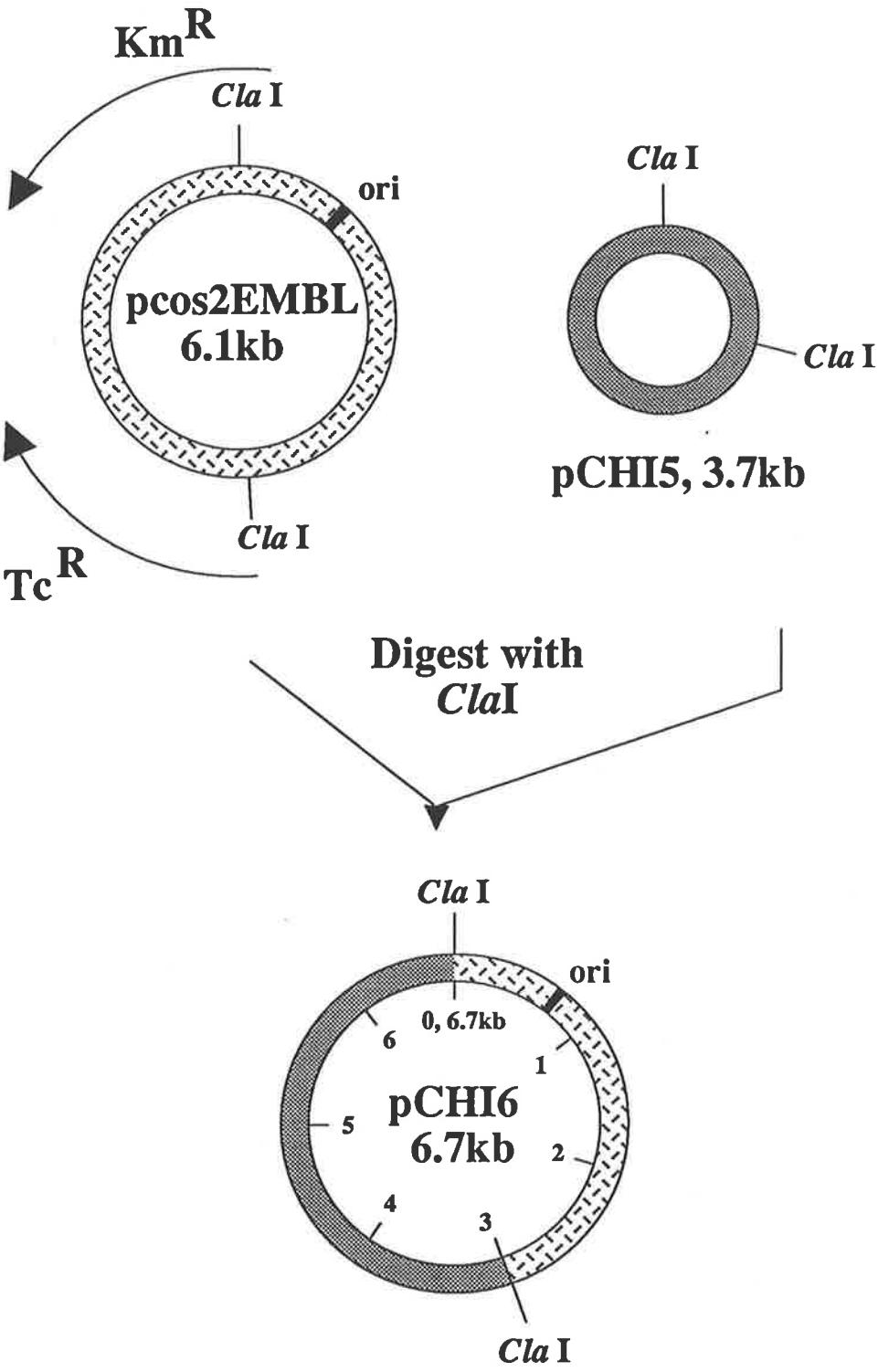
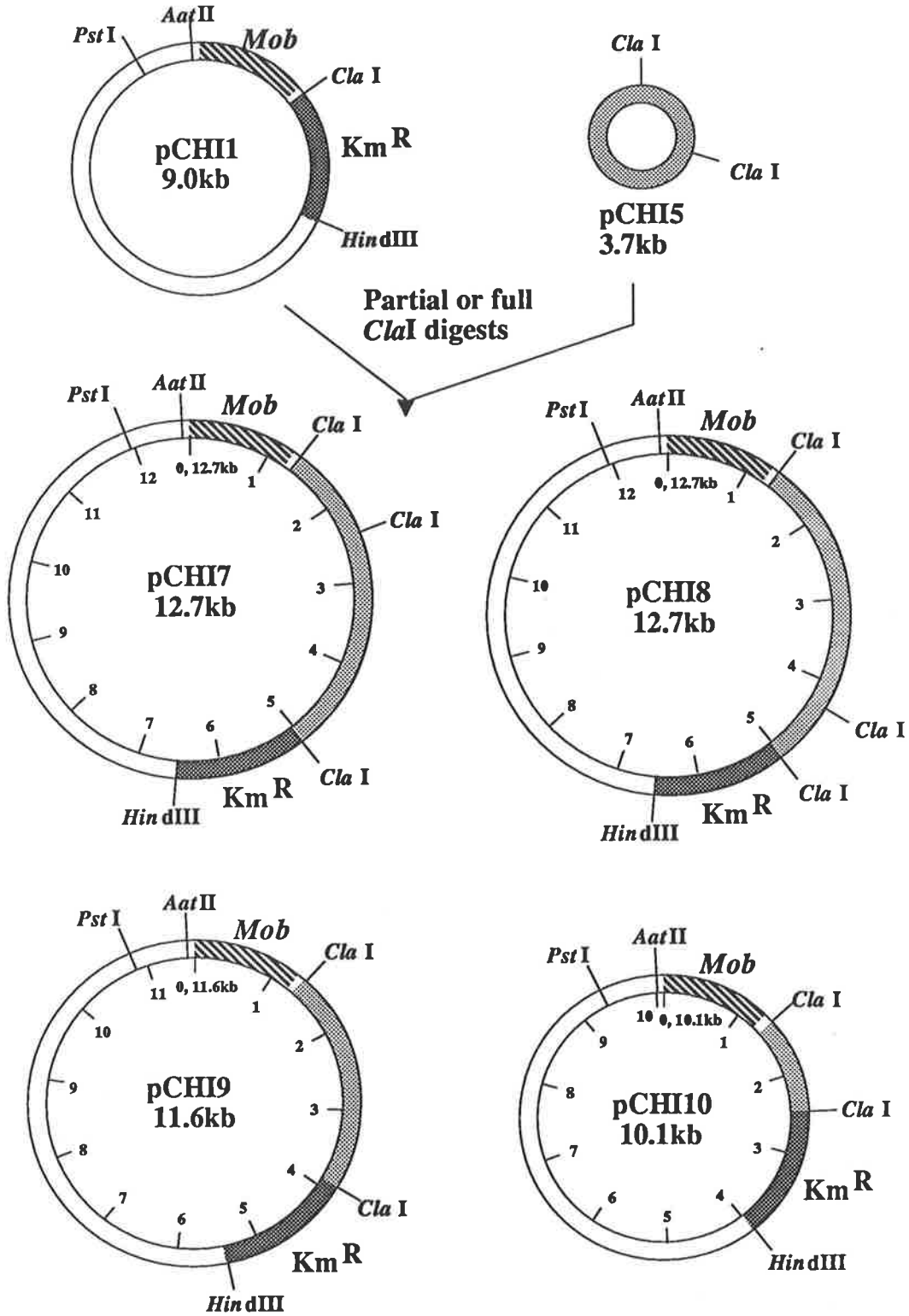

Km^R
Cla I
ori
pcos2EMBL
6.1kb
Cla I
Tc^R
Cla I
Cla I
pCHI5, 3.7kb
Digest with
ClaI
Cla I
ori
0, 6.7kb
1
2
3
4
5
6
pCHI6
6.7kb
Cla I

FIGURE 4.9 Construction of plasmids pCHI7, pCHI8, pCHI9, and pCHI10.

pCHI5 was fully or partially digested with *Cla*I in separate experiments, and the resulting fragments were cloned into the *Cla*I site of pCHI1. The whole of pCHI5 was cloned separately by either of its *Cla*I sites into pCHI1, and the resulting plasmids were named pCHI7 and pCHI8. The 2.6 and 1.0 kb *Cla*I DNA restriction fragments were cloned separately into pCHI1 and the resulting plasmids were named pCHI9 and pCHI10, respectively.


Pst I
Aat II
Mob
Cla I
pCHI1
9.0kb
Km R
Hin dIII
Cla I
Cla I
pCHI5
3.7kb
Partial or full
ClaI digests
pCHI7
12.7kb
0, 12.7kb
pCHI8
12.7kb
pCHI9
11.6kb
0, 11.6kb
pCHI10
10.1kb
0, 10.1kb

kanamycin-resistance gene (Figure 4.5). Therefore there was a strong possibility that at least one of these plasmids contained the uninterrupted cryptic plasmid replicon present in pCHI5. Also, the 2.6 kb and 1.1 kb *Cla*I fragments of pCHI5 were cloned separately into pCHI1 to generate pCHI9 and pCHI10 respectively (Figure 4.9). These plasmids could be mobilized efficiently into *S. typhimurium*, but no transconjugants could be obtained in *C. hyointestinalis* with any of the constructs (Table 4.3).

These data, in conjunction with the pCHI4 results, suggested that the inability of the shuttle vector constructs to be mobilized into *C. hyointestinalis* was probably not because they lacked an uninterrupted replicon. Since pCHI4 and either pCHI7 or pCHI8 contain an uninterrupted replicon, this suggested that *C. hyointestinalis* might possess a barrier which prevented foreign DNA from entering the cell. The apparent inability of the conjugative broad-host-range vectors and subsequent constructs to replicate in *C. hyointestinalis* also gave credence to this theory. The most likely barrier *C. hyointestinalis* might possess was a restriction system which destroyed incoming unmodified DNA. If this was the case then the possibility existed that the original isolate of *C. hyointestinalis* harbouring pCHI5, named W64, was a mutant for this restriction system since later experiments (Section 4.2.15) demonstrated that pCHI5 had most likely entered *C. hyointestinalis via* conjugation and not by natural transformation. Because of this, W64 was examined in more detail to determine whether or not it was a restriction mutant and whether it could act as an efficient recipient in conjugation.

4.2.14 Conjugation into a cured strain of *C. hyointestinalis* W64

To determine if W64 was in fact a restriction mutant, which might act as an efficient recipient in conjugation, pCHI5 had to be removed so that it would not exclude, by incompatibility, any candidate shuttle vectors from entering the cell. W64 was subcultured repeatedly without kanamycin selection until a cured derivative lacking pCHI5, named W173, was obtained. Attempts to conjugate pILL550, pCHI2, pCHI3, pCHI4, pCHI7, pCHI8, pCHI9, and pCHI10 into W173 were unsuccessful in tests where all plasmids were

readily transferable to *S. typhimurium* (Table 4.5), indicating that W173 appeared to be no more efficient as a recipient in conjugation than the wild-type *C. hyointestinalis* type strain.

Attempts to electroporate pILL550, pCHI2, pCHI3, pCHI4, pCHI7, pCHI8, pCHI9, and pCHI10 extracted from an *E. coli* K-12 background, into W173, were unsuccessful (Table 4.6). pCHI5, however, could be electroporated back into W173 at an efficiency which was as high, but no better than that seen with the wild-type *C. hyointestinalis* type strain (Table 4.4).

These results suggested that W173 was not a restriction mutant of the wild-type *C. hyointestinalis* type strain. This did not mean that a restriction system did not play a role in preventing DNA being mobilized into *C. hyointestinalis*, but implied that the generation of W64 was not the consequence of inactivating a restriction system. As pCHI5 could be electroporated efficiently into the wild-type *C. hyointestinalis* type strain (in contrast to a variety of other plasmids, from *E. coli* K-12, with intact *C. hyointestinalis* replicons) it was presumably *C. hyointestinalis*-modified. Thus, W64 still expressed a restriction/modification system akin to the wild-type strain. It is likely that the conjugation event which gave rise to pCHI5 within W64 involved fortuitous modification of the incoming DNA prior to the action of the restriction system (discussed in Chapter 7).

4.2.15 Natural transformation of *C. hyointestinalis*

Due to the lack of success in detecting conjugation of plasmid DNA into *C. hyointestinalis*, an investigation was made to determine whether plasmid DNA could be introduced into *C. hyointestinalis* by natural transformation. This was performed either on an agar surface or in a biphasic system. 10 µg of pILL550, pCHI5, pCHI2, pCHI3 and pILL550 plasmid DNA extracted from *C. coli* (i.e.: *C. coli*-modified) were used separately in an attempt to naturally transform *C. coli*, *C. hyointestinalis* NCTC 11608 and *C. hyointestinalis* 45104 (which harbours the cryptic plasmid) (Table 4.7). No kanamycin-resistant transformants were obtained with either *C. hyointestinalis* NCTC 11608 or 45104 using any of the plasmids, including the *C. hyointestinalis*-modified pCHI5. Natural transformation on an agar surface with pILL550, which had been modified by *C. coli*, yielded some 10

TABLE 4.5 Conjugal transfer of candidate shuttle vectors into *C. hyointestinalis* strain W173

Plasmid	No. of transconjugants per donor into the recipient species:	
	S. typhimurium LT2 SL2981	*C. hyointestinalis* W173
pILL550	10^{-4}	$<10^{-9}$*
pCHI2	10^{-4}	$<10^{-9}$*
pCHI3	10^{-4}	$<10^{-9}$*
pCHI4	10^{-4}	$<10^{-9}$*
pCHI7	10^{-4}	$<10^{-9}$*
pCHI8	10^{-4}	$<10^{-9}$*
pCHI9	10^{-4}	$<10^{-9}$*
pCHI10	10^{-4}	$<10^{-9}$*

* Repeated attempts to obtain transconjugants with *C. hyointestinalis* strain W173 in mating tests using, as donors, *E. coli* K-12 S17-1 strains harbouring the various plasmids, were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

TABLE 4.6 Electroporation of candidate shuttle vectors, isolated from *E. coli* K-12 DH1, or *C. hyointestinalis* W64 (pCHI5), into *C. hyointestinalis* strain W173

Plasmid	No. of transformants/μg DNA
pILL550	<10*
pCHI2	<10*
pCHI3	<10*
pCHI5**	5×10^3
pCHI7	<10*
pCHI8	<10*
pCHI9	<10*
pCHI10	<10*

* Repeated attempts to obtain transformants of *C. hyointestinalis* strain W173 with plasmid DNA isolated from *E. coli* K-12 DH1 were unsuccessful. 10 transformants/μg DNA was the lower limit of transformant detection in these experiments.

** pCHI5 was isolated from *C. hyointestinalis* W64.

TABLE 4.7 Natural transformation of plasmid DNA into *Campylobacter* strains on an agar surface or in a biphasic system

Donor DNA	No. of transformants into the recipient species:		
	C. coli NCTC 11366	*C. hyointestinalis* NCTC 11608	*C. hyointestinalis* 45104
pILL550*	10, 0##	0, 0	0, 0
pILL550**	0, 0	0, 0	0, 0
pCHI2**	0, 0	0, 0	0, 0
pCHI3**	0, 0	0, 0	0, 0
pCHI5#	0, 0	0, 0	0, 0

* Plasmid pILL550 was isolated from *C. coli* NCTC 11366.

** These plasmids were isolated from *E. coli* K-12 DH1.

Plasmid pCHI5 was isolated from *C. hyointestinalis* W64.

The first figure refers to the agar surface data, the second to the results from the biphasic system tests.

kanamycin-resistant colonies of *C. coli*, but pILL550 extracted from an *E. coli* K-12 background produced no transformants. The low number of transformants with the *C. coli*-modified pILL550 was not completely unexpected since the *C. coli* type strain produces an extracellular DNase which could reduce the transformation efficiency of the strain (Wang and Taylor, 1990b). The two *C. hyointestinalis* strains used here do not produce an extracellular DNase (data not shown). The strain 45104 was chosen for examination here as it has been reported that strains of *C. jejuni* and *C. coli* harbouring homologous plasmids take up plasmid DNA at a greater frequency than those lacking plasmids (Wang and Taylor, 1990b).

These results indicated that at least two strains of *C. hyointestinalis* could either not be naturally transformed, or that such transformation occured at a very poor efficiency. Also, the results suggested that pCHI5 probably did not originate from some extracellular pCHI4 plasmid DNA taken up during the mating experiment between *C. hyointestinalis* and the *E. coli* K-12 strain harbouring pCHI4, and that pCHI5 was most probably introduced to *C. hyointestinalis via* conjugation.

4.3 Summary and conclusions

It was initially shown that pILL550 appeared to be an unsuitable shuttle vector for genetic work with *C. hyointestinalis*. The inability of pILL550 to be mobilized into *C. hyointestinalis* may be due to the fact that it contained a replicon which originated from a *C. coli* cryptic plasmid, and may therefore be inoperative in a *C. hyointestinalis* host. A single plasmid of 2.5 kb was isolated from four Australian strains of the ten strains screened. The plasmid of each isolate was identical based on restriction enzyme mapping (unpublished data). It has been reported that few porcine *C. hyointestinalis* isolates contain plasmids (Gebhart *et al.*, 1989). Some *C. hyointestinalis* isolates (4/30) screened by Edmonds *et al.* (1987) contained plasmids of 1.4-4.8 kb and 1/8 isolates examined by Boosinger *et al.* (1990) contained a plasmid of 1.6 Mdal (2.45 kb) while 2/8 contained a 38 Mdal plasmid.

In an attempt to determine whether the inability of pILL550 to be mobilized into *C. hyointestinalis* was because its replicon originated from *C. coli*, two candidate shuttle

vectors, pCHI2 and pCHI3, based upon the cryptic plasmid from *C. hyointestinalis* 45104, were constructed. Neither of these plasmids could be mobilized into *C. hyointestinalis*. This may have been due to the fact that neither of them may have contained an intact replicon of the cryptic plasmid. Another candidate shuttle vector, pCHI4, was constructed where this was not so, but this could not be readily mobilized into *C. hyointestinalis*. After one mating, using an *E. coli* K-12 conjugative strain harbouring pCHI4 as a donor, a single kanamycin-resistant colony of *C. hyointestinalis* was obtained. This strain was screened for plasmid content and contained a small plasmid, pCHI5, of approximately 3.7 kb. pCHI5 had lost the ability to replicate in *E. coli* K-12 but could be electroporated efficiently into *C. hyointestinalis*. Southern hybridization data showed that pCHI5 contained some *oriT* DNA, but this was shown to be non-functional in mobilization as the plasmid pCHI6, consisting of the *E. coli* K-12 replicon of pcos2EMBL cloned into a *Cla*I site of pCHI5, could not be mobilized into a *S. typhimurium* recipient. Some regions of the *C. hyointestinalis* cryptic plasmid, presumably involved in replication, were also present. A series of candidate shuttle vectors utilizing pCHI5 were constructed, but none of them could be mobilized into *C. hyointestinalis*, despite the fact that at least one of these must have contained an undisrupted form of the replicon of the cryptic plasmid. Attempts to mobilize some conjugative broad-host-range vectors into *C. hyointestinalis* were also unsuccessful. A cured derivative of the original *C. hyointestinalis* isolate containing pCHI5 was obtained to determine whether the original isolate harbouring pCHI5 was a restriction mutant, but this, like its wild-type parent, was ineffective as a recipient in conjugation. It was also demonstrated that some strains of *C. hyointestinalis*, unlike *C. jejuni* and *C. coli*, could not be transformed naturally, which suggested that pCHI5 was most probably introduced to *C. hyointestinalis via* conjugation.

The isolation of a kanamycin-resistant colony of *C. hyointestinalis* harbouring pCHI5 was important as it showed that a genetically engineered plasmid could be stably maintained in *C. hyointestinalis*. It also demonstrated that the kanamycin-resistance gene was functional in *C. hyointestinalis* and that this was not a factor in explaining the lack of success with conjugation. Further analysis of pCHI5 could help provide the information as to which

region of the cryptic plasmid is involved in replication. The observation that pCHI5 could be efficiently electroporated into *C. hyointestinalis* was particularly important as it demonstrated at least one method for introducing (modified) plasmid DNA into *C. hyointestinalis.*

The evidence presented in this Chapter suggests that *C. hyointestinalis* might possess a feature which acts as a barrier to the introduction of foreign DNA. The most likely barrier is a restriction system. *C. jejuni* has been shown to possess a strong restriction system capable of significantly decreasing the efficiency of transformation of unmodified plasmid DNA (Miller *et al.*, 1988). If *C. hyointestinalis* did possess a restriction system then it should be possible to obtain a restriction mutant by mutagenesis; this work is described in Chapter 6.

CHAPTER 5

GENETIC ORGANIZATION OF THE CRYPTIC PLASMID OF *C. HYOINTESTINALIS*

5.1 Introduction

The nucleotide sequences of very few *Campylobacter* genes have been determined. These genes include the tetracycline-resistance gene (*tetO*) (Sougakoff *et al.*, 1987; Manavathu *et al.*, 1988), the kanamycin-resistance gene (*aphA-7*) (Tenover *et al.*, 1989), the lysyl-tRNA synthetase gene (*lysS*) (Chan and Bingham, 1992), the serine hydroxymethyltransferase gene (*glyA*) (Chan and Bingham, 1990), the 16S rRNA (Rashtchian *et al.*, 1987; Kim and Chan, 1989), and the flagellin genes (*flaA* and *flaB*), all from *C. jejuni* (Nuijten *et al.*, 1990b). The kanamycin-resistance gene (*aphA-3*) (Trieu-Cuot *et al.*, 1985), the chloramphenicol-resistance gene (*cat*) (Wang and Taylor, 1990a), and the flagellin genes (*flaA* and *flaB*) (Guerry *et al.*, 1990), all from *C. coli*, have also been sequenced. The analysis at the primary nucleotide sequence of genes, provides an insight into the expression and regulation of these genes and enables protein sequence prediction.

The nucleotide sequence of a *Campylobacter* plasmid replicon has not been reported, but the replicons from a number of bacterial plasmids have been sequenced and it has been shown that the essential functions for the autonomous replication of plasmid DNA reside in a relatively small stretch (1 to 5 kb) of the plasmid genome (Nordström, 1984).

In this chapter, the nucleotide sequence and genetic organization of the 2.5 kb cryptic plasmid isolated from *C. hyointestinalis* strain 45104 is examined. The replication origin region was identified by virtue of its similarity in structure and organization to the origins of

other bacterial plasmids. The gene and regulatory region within the replicon were analysed. The nucleotide sequence of the cryptic plasmid-derived region of plasmid pCHI5 was also obtained. This has permitted the boundaries of the cryptic-plasmid-derived DNA in pCHI5 to be determined and provide a basis for understanding the mechanism by which this plasmid was generated.

5.2 Results

5.2.1 DNA sequencing

The cryptic plasmid from strain 45104 was digested with *Cla*I, *Hin*dIII, and *Cla*I and *Hin*dIII together, and the resulting fragments were cloned into the multiple cloning site region of M13mp18 (Vieira and Messing, 1982). Sequencing was performed by the chain termination method (Sanger *et al.*, 1977, 1980) using Sequenase™. Universal primer was employed to sequence cloned DNA from within the polylinker. Synthetic oligonucleotides were used to extend the sequence. The sequencing strategy is shown in Figure 5.1. The entire nucleotide sequence of the 2.5 kb cryptic plasmid of *C. hyointestinalis* 45104 was determined from both strands by the chain termination method and is shown in Figure 5.2.

5.2.1.1 Nucleotide sequence of the cryptic plasmid

Analysis of the determined sequence revealed that the plasmid contained 2,512 bp and had an overall A+T content of 71.8%. The sequence revealed only one open reading frame (ORF), named ORF1, of 1011 bp, beginning at nt 401 and terminating at nt 1412. ORF1 could encode a 337 amino acid protein of 39,667 Da, designated RepA (gene: *repA*).

An initiator region is required for the efficient initiation of protein translation and consists of the required initiation codon (AUG) and also a sequence, known as a ribosome binding site, situated just upstream (5-8 nt) of this codon, that displays homology with a sequence, the Shine-Dalgarno sequence, on the free 3′ end of the 16S rRNA (Shine and Dalgarno, 1974). The sequence complementary to the Shine-Dalgarno sequence is AAGGAGGU and mutations leading to divergence from this sequence drastically reduce the level of translation initiation (Gold *et al.*, 1981; Kozak, 1983). The putative ribosome

FIGURE 5.1 Strategy used for dideoxy sequencing of the cryptic plasmid of *C. hyointestinalis* 45104.

The cryptic plasmid of *C. hyointestinalis* 45104 was digested with *Cla*I, *Hin*dIII, or *Cla*I and *Hin*dIII together, and the resulting fragments were cloned into M13mp18. Sequence information from these fragments was first obtained using universal primer. Synthetic oligonucleotide primers were then used to extend the sequence in either direction. For accurate sequence determination at the polylinker junctions, the -40 sequencing primer (5′ GTTTTCCCAGTCACGAC 3′) was used. Sequences indicated with an asterisk were generated using a specifically synthesized oligodeoxynucleotide primers.

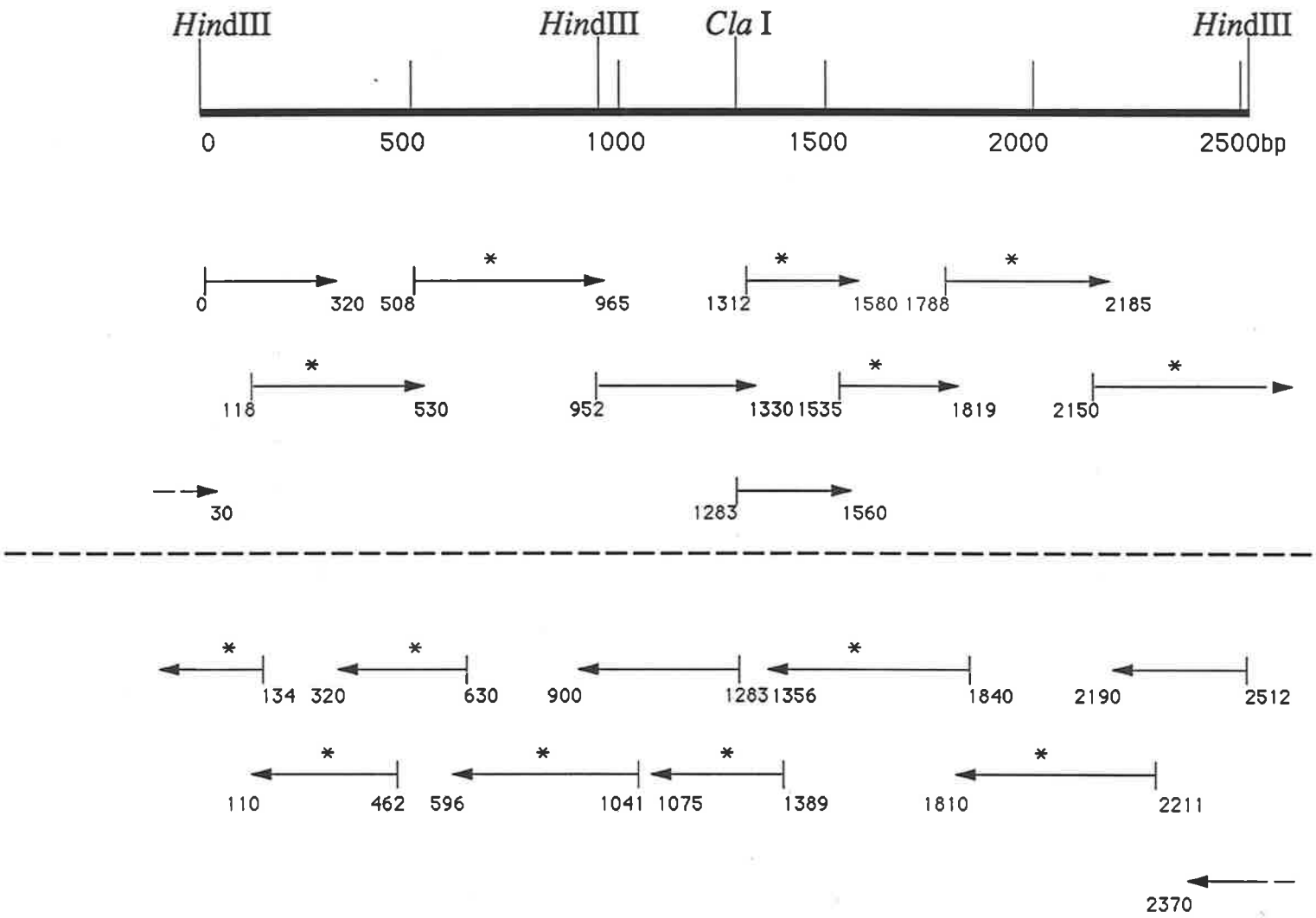

HindIII
HindIII
Cla I
HindIII
0
500
1000
1500
2000
2500bp
0
320
508
965
1312
1580
1788
2185
118
530
952
1330
1535
1819
2150
30
1283
1560
134
320
630
900
1283
1356
1840
2190
2512
110
462
596
1041
1075
1389
1810
2211
2370

FIGURE 5.2 Nucleotide sequence of the 2.5 kb cryptic plasmid of *C. hyointestinalis* 45104.

The nucleotide sequence is numbered from the *Hin*dIII site opposite the *Cla*I site in Figure 4.3, and the sequence reads "clockwise". The amino acids within the ORF that encodes RepA are numbered beginning at the initiation codon (ATG-Met). The amino acid sequence of the RepA protein is given and the putative ribosome binding site (RBS) (AAGG) is shown in boldface. The mRNA start site is also shown in boldface and indicated as +1 (see Section 5.2.12). On the basis of the mRNA start site, a putative σ^{70} promoter at -10 and -35 is shown in boldface. Four 19 bp direct repeat sequences are overlined and numbered from 1 to 4. A 99 bp A+T rich region is underlined and a site resembling the IHF-binding consensus (C/TAANNNNTTGATA/T) (Kur *et al.*, 1989) is marked. Inverted repeats are underlined with arrows.

```
1    A AGC TTT TAA ATA ATC AAT TTC AGC TTT TAA CTT TTC ATT TTT ACT CAT AAT AAG CCT TTT AGA TAT GCG CGT CTG CTT TTT      82

83   GAG TGT AGC GAT AAA AGC TAG GTA TCA AGC GCG CAC CGA ATT TAT TAA ATT ATA GCA TAA ATT AAA AAA AGT TTT TTT CTG TTA     166
                                  IHF
167  AGC TCT TTT TAT AAT ATA AAA AAA TGT TTC TTA ATG TTT AGC GAT TTT TTG ATG CCC TTA AAT AGG GCA TTT GAA AGG TGC TAT     250
          1                    2                                    3
251  TGT AAC TTT TTA TCC CTG AAT AGT AAC TTT TTA TCC CTA AAT AAT AAG AGA ATA GTA ACT TTT TAT CCC TAA ATA GTA ACT TAT     334
       -35                          -10          +1          4          RBS
335  ATT TTT AAA GTT ACT ATT TTA TGA TAT AAT TTT AGA ATA GTA ACT TTT TAT CCC TAA AGG CGA ATG ATG AAA AAA ACA GAA ATT     418
1                                                                                    Met Lys Lys Thr Glu Ile        6

419  AAA AAC ATA GCA AAT CGC CAA ATA ATG GCA CAA TCA AAT AAG GTT ATC ACA GCT AAA TAC GAA CTT ACA GAA GCG GAA CAA AAA     502
  7  Lys Asn Ile Ala Asn Arg Gln Ile Met Ala Gln Ser Asn Lys Val Ile Thr Ala Lys Tyr Glu Leu Thr Glu Ala Glu Gln Lys      34

503  ATC ATA CTA TTA GCA ATA GCA CAA GTG GAT AGC ATA AAA GAT AAA AAA TTT GGG ACG TAT AAA ATT ACA ATA CCA GAA TTA GAA     586
 35  Ile Ile Leu Leu Ala Ile Ala Gln Val Asp Ser Ile Lys Asp Lys Lys Phe Gly Thr Tyr Lys Ile Thr Ile Pro Glu Leu Glu      62

587  CAA AAA ATC GGA TCG AAA ATA AAA CAA GCT CAA TTA AAA GAA ACT TGC AGA AGA CTT ATG CAA AGG GTT GTT TAT ATA GAA AAT     670
 63  Gln Lys Ile Gly Ser Lys Ile Lys Gln Ala Gln Leu Lys Glu Thr Cys Arg Arg Leu Met Gln Arg Val Val Tyr Ile Glu Asn      90

671  GGT AAA AAT TGG AAA ATG TTT CAT TGG ATA AGC ACA GCC GAA TAC ATC GAC GGA GAA AAC ACA ATA AAA TTT AAA ATA AGC GAT     754
 91  Gly Lys Asn Trp Lys Met Phe His Trp Ile Ser Thr Ala Glu Tyr Ile Asp Gly Glu Asn Thr Ile Lys Phe Lys Ile Ser Asp     118

755  GAG ATG AAG CCA TTT TTA TTG CAA TTA AAA GGC AAT TTT ACA AAA ATA GAA TTA GAA AAT GCA TTA AAA TTT AAT GGC AAA TAT     838
119  Glu Met Lys Pro Phe Leu Leu Gln Leu Lys Gly Asn Phe Thr Lys Ile Glu Leu Glu Asn Ala Leu Lys Phe Asn Gly Lys Tyr     146

839  ACG CTT AGA TTT TAT CAA TTT TGT ATG CAA ATG CAA AAT CAA GCA ACT AAA AAA AGA ACT TTT GAA TTA TCA AAA CTT TAT GAA     922
147  Thr Leu Arg Phe Tyr Gln Phe Cys Met Gln Met Gln Asn Gln Ala Thr Lys Lys Arg Thr Phe Glu Leu Ser Lys Leu Tyr Glu     174

923  ATT TTA CAA TTA CCA GAG AGT TTA ACA ACA AGC TTT GCA AGA TTT AAA TTA AAA GTC ATA GAG CCA AGT ATA AAC GAG ATA AAC    1006
175  Ile Leu Gln Leu Pro Glu Ser Leu Thr Thr Ser Phe Ala Arg Phe Lys Leu Lys Val Ile Glu Pro Ser Ile Asn Glu Ile Asn     202

1007 ACC AAA TCA GAT ATA AAG GCT AAT TGG GAA ATT TCA AAG AAA ATC GGT AAA AAA ATA GTA GAA ATT GAG CTA AAT TTT AAA AGC    1090
203  Thr Lys Ser Asp Ile Lys Ala Asn Trp Glu Ile Ser Lys Lys Ile Gly Lys Lys Ile Val Glu Ile Glu Leu Asn Phe Lys Ser     230

1091 AAA GAA AGA CTA CAA GAA CAA ACT AAA CAA GCA AGA GAA GTT AAA AGC CTA AAA AAG TAT ATA GGC AAA CAA TGT TTA TAT TTT    1174
231  Lys Glu Arg Leu Gln Glu Gln Thr Lys Gln Ala Arg Glu Val Lys Ser Leu Lys Lys Tyr Ile Gly Lys Gln Cys Leu Tyr Phe     258
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1175  GAT TAT TTA ATA ATT ATC GAG CAG ATA TCT TAT AAC GCT GAA CAA AGC AGA TAC GAA GTA ATT TAC AAA GAC GGC ACA GGA GAT   1258
 259  Asp Tyr Leu Ile Ile Ile Glu Gln Ile Ser Tyr Asn Ala Glu Gln Ser Arg Tyr Glu Val Ile Tyr Lys Asp Gly Thr Gly Asp    286

1259  TTA TGT AGA GCA GAC TTT GAT AGT ATC GAT ATG CTA GAA ATT GCA ATA AAA AAA GGG AAA ATA GAA GCG AAC TTT AGA AAA GCA   1342
 287  Leu Cys Arg Ala Asp Phe Asp Ser Ile Asp Met Leu Glu Ile Ala Ile Lys Lys Gly Lys Ile Glu Ala Asn Phe Arg Lys Ala    314

1343  AAT CCG GAA CTT TTC AAA AAG ATT GAT AGT AAA GAA GAA ATA GCA AAT TTA TTT AGA GAT ATG ATG AAA TAA AAA ACA ATA AAT   1426
 315  Asn Pro Glu Leu Phe Lys Lys Ile Asp Ser Lys Glu Glu Ile Ala Asn Leu Phe Arg Asp Met Met Lys ***                  337

1427  CAG TTT TTT TGA TAT GAA AGT TCA TCT TTT GCT TGA GCT AGT GTA AGA AGT TTC ATT TTT TAA AAA AAT CCT TTC AGT ATT TTT   1510

1511  TAG GGG GGG GGT TAT GAG CGT AGT AGC CTA TAT TGT TAA TCA GGG CGT TGG CAA AGT TTA AAA GCC CTA ATT TTC AAT ATC TCA   1594

1595  AAG TGT TTT TAC CAA ACT TAA AAA TAG ATA AAG AGA TAA ACG GAA AAA AAT TAA ATA AAC AAT AGC CAA GTT ATA TAA GAA AAA   1678

1679  TAG AGC ATT TGA CAA TAA TAA ATT AAA AAA CAA ATA AAC AAT ACC AAT TTT AAT AAG TTG AGC CTC GCA GAG AAC TTA CCC AAA   1762

1763  ATG GCT AAA AAA GTT AAT AAC TTT TGT GCC TTT TGG CTA TCC TGC AAT ACT ACT AAA TTA ACT AAC TTT ATG CAA TTG AAT GAT   1846

1847  ACA AAA AAA TAT CAA TAT GGA ATA ATT TAT TTT TCA AAT ACC CTT AAA AAC GGC GTT TAG TTA AAG TAA GTA GTA AAC AAG TCA   1930

1931  AAA GGA GAA AAG CGG CCG CAG TGC TAC TGC GCG TAG AAC GCT TGT AAG CAC TTG CAG CTA AAA GAA GTG AAA TAT ATT TTT TCA   2014

2015  GTA CGG CTA AAA AGT TTT TAA ACG TTT ATG CGT TTT TAA GAG CAT ATA TGG GGC AAA TTT TAG CTT TGT AAA GTT TTT TAG TAG   2098

2099  TTT GAG TAA GGT TTT GCC TTT TAA ACG TTT AAA TCC GCT CTA AAT TAA TTT ATG CAC GGC TCA CTA AAT TTA ATT TCA CGA TAA   2182

2183  ATA TTG TTT TTC GCC TTT TAT AGG GGA AAC CCC CTA TAA CCC CCA AAT TGC CAA CTA AGC ACC TTT TAG GAA AGT ATA AGA GAT   2266

2267  AAT TCC AAG AGC TAA TAA AAT ACA AGC AAA TAA AAA CAT CAT TGC AAA AGT CAT TTT ATT TCC TTT CTT TTA GTT CTG ATA ATT   2350

2351  CAT CAA ATT TAT TCG TTA TTT TTC TAT GCA AGA AAA AAA CTA ATA GAG ATA ATA TTA CAA TAG CGA AGC CAA CTA AGA ATA AAT   2434

2435  CAT AAC TAT CAG CTT TGT TTT TAA TAG TGA CTA TCC AAC CAA TAA CAG CAA ACA AAG CAG TAA GCA AAA ATA TTA TTA           2512
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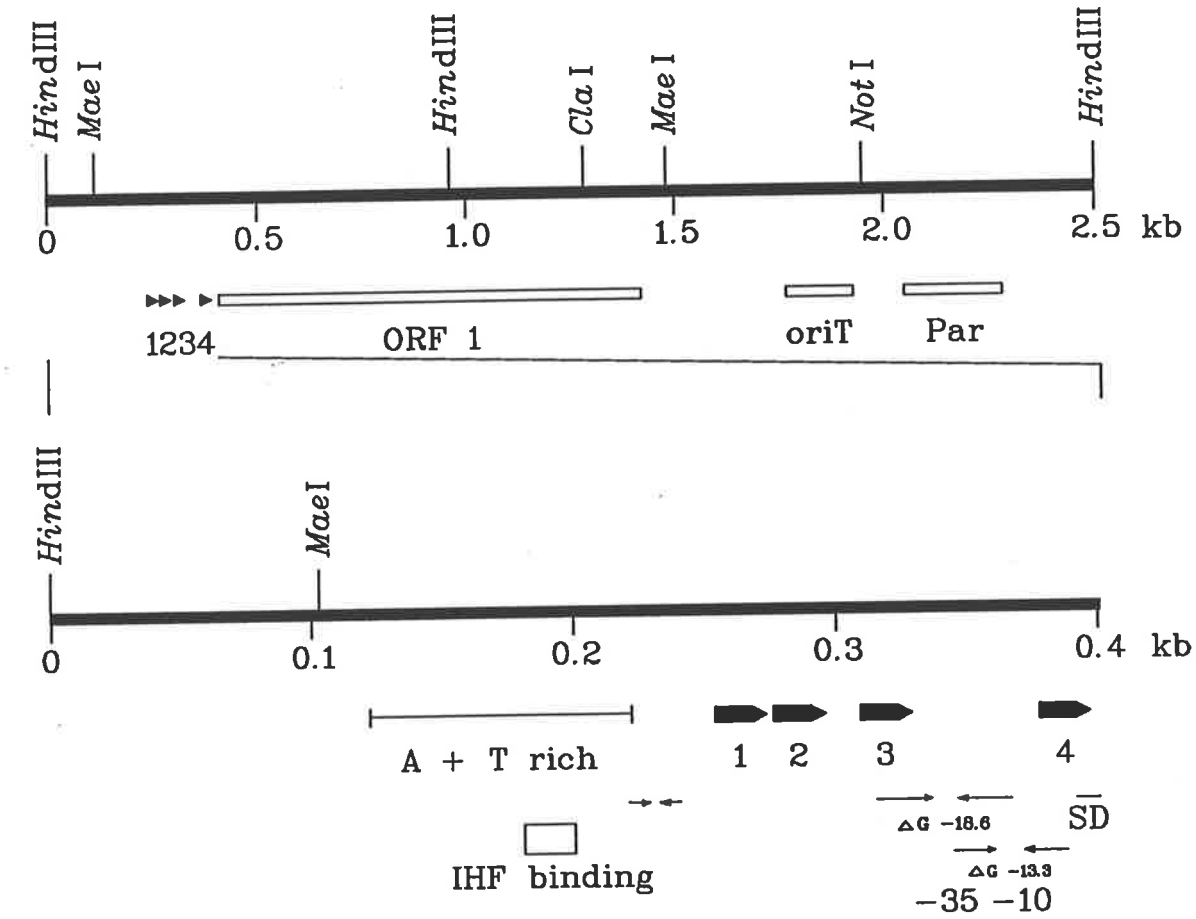
binding site located 6 nt before the AUG start codon of *repA* is AAGG. This spacing was optimal with the average being 7 nt (spacings of less than 5 and greater than 9 nt were rare) (Itoh *et al.*, 1984; Shepard *et al.*, 1982).

The nucleotide sequence upstream from ORF1 contains four direct repeats of a 19 bp sequence. Near the first repeat sequence is a short sequence of dyad symmetry which is located between nt 218 and 236 and is G+C rich (indicated by inverted arrows in Figure 5.3). Adjacent to this is a 99 bp region between nt 121 and 219 which is 83% A+T rich. Within this region lies a putative Integration Host Factor (IHF) binding site (Kur *et al.*, 1989) between nt 183 and 195. The organization of each region of the cryptic plasmid is shown in Figure 5.3.

This organization of the origin of replication in the cryptic plasmid is similar to those seen in a number of plasmids which use a series of direct DNA repeats, called iterons, as the main incompatibility determinant. The iterons are found near the origin of replication and near a *rep* gene encoding a replication initiation protein. The four 19 bp repeats of the cryptic plasmid fall well within the range for the size of the repeats (17 to 22 bp) and the number of repeats (4 to 8) in these plasmids (Sriprakash and Macavoy, 1987). The iterons show no nucleotide sequence homology with those of other plasmids, but may have similar functions as binding sites for a plasmid-encoded replication protein (Vocke and Bastia, 1983a; Yamaguchi and Masamune, 1985). ORF1 of the cryptic plasmid has the capacity to encode for a 39.7 kDa protein which corresponds well with the sizes of plasmid-encoded replication initiation proteins (29 to 40 kDa; Scott, 1984). In addition, the 99 bp A+T rich region adjacent to the repeats is similarly located to those in the replicons of other plasmids which utilize iteron-mediated replication (Scott, 1984). This A+T rich region has been predicted to facilitate melting of the DNA strands during the initiation of replication (Wada and Suyama, 1986). No binding sites (TTATA/CCAA/CA) for the DnaA protein, present in the A+T rich region of pSC101 (Fuller *et al.*, 1984), are found in the nucleotide sequence of the cryptic plasmid. DnaA binding sites, however, may be species-specific like those for IHF, the putative binding site which shows a strong homology with the *E. coli* K-12 consensus sequence but was not identical. The IHF binding site in pSC101 has been

FIGURE 5.3 Genetic organization of the 2.5 kb cryptic plasmid of *C. hyointestinalis* 45104.

The upper line shows restriction endonuclease sites in the cryptic plasmid. The position of ORF1, encoding RepA, and the putative *par* and *oriT*, regions are indicated by open boxes, and the four 19 bp repeats are shown as triangles. The 400 bp region upstream from the translational start of ORF1 has been expanded (bottom) to show the organization of the origin of replication. Regions of dyad symmetry and potential hairpin loop structures are indicated by arrows. SD: Shine-Dalgarno sequence (RBS).


HindIII
MaeI
HindIII
ClaI
MaeI
NotI
HindIII
0
0.5
1.0
1.5
2.0
2.5 kb
1234
ORF 1
oriT
Par
HindIII
MaeI
0
0.1
0.2
0.3
0.4 kb
A + T rich
1
2
3
4
ΔG −18.6
SD
ΔG −13.3
IHF binding
−35 −10

demonstrated to be essential for replication (Stenzel *et al.*, 1987) and it has been shown that the IHF can induce DNA bending of pSC101 *in vitro* and promote the interaction of the DnaA protein with its binding sites at the origin of replication (Stenzel *et al.*, 1991).

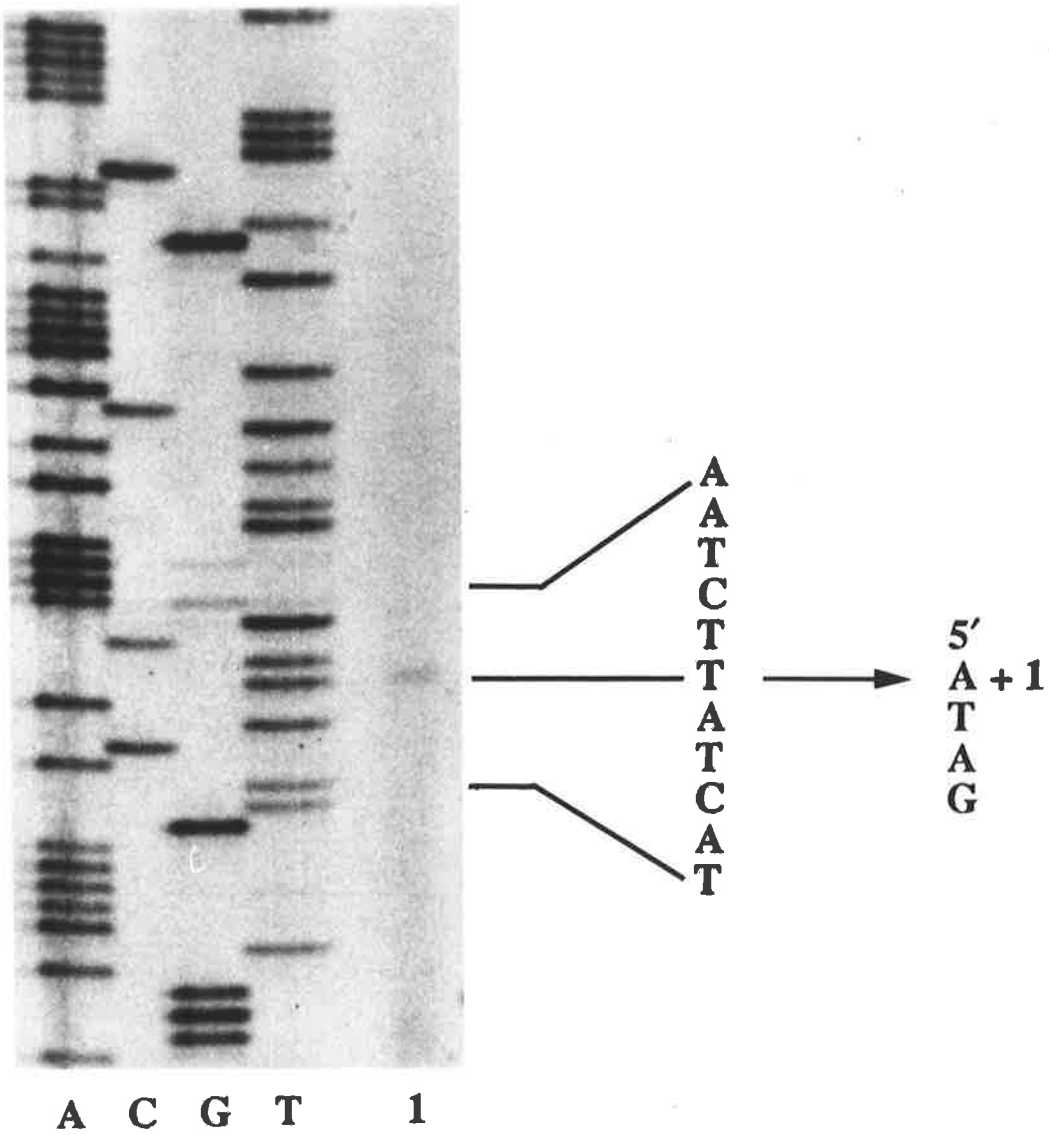
5.2.1.2 Analysis of the promoter region of *repA*

Analysis of a large number of *E. coli* K-12 promoter sequences, which use σ^{70}, has defined two consensus hexamer regions (Pribnow, 1975a, 1975b; Schaller *et al.*, 1975; Takanami *et al.*, 1976; Seeburg *et al.*, 1977; Hawley and McClure, 1983). The "Pribnow box" is located 10 bases upstream from the initiation site. The "Pribnow box" consensus sequence is T_{80} A_{95} T_{45} A_{60} A_{50} T_{96} where the subscript represents the percentage occurence of the base most frequently found at that position (Siebenlist *et al.*, 1980; Hawley and McClure, 1983). The other site is located 35 base pairs upstream of the transcription initiation site and is termed the "-35 region" or "recognition sequence" (Maniatis *et al.*, 1975; Pribnow, 1979; Hawley and McClure, 1983; Studnicka, 1987). The "-35 region" has been implicated in the initial recognition of the promoter site by the RNA polymerase. With the same notation as before, the "-35 region" consensus sequence is T_{82} T_{84} G_{78} A_{65} C_{54} A_{45} (Hawley and McClure, 1983). The importance of the individual bases within these hexamers in efficient promoter function has been well characterized by specific mutations which resulted in marked decreases/increases in promoter activity (Rosenberg and Court, 1979; Hawley and McClure, 1983).

A potential promoter was detected upstream from the *repA* gene by virtue of its strong homology to the consensus sequence for an *E. coli* σ^{70}-type promoter (Helmann and Chamberlin, 1988). The *E. coli* σ^{70} is associated with genes involved in the housekeeping functions of the cell (Hawley and McClure, 1983). Primer extension analysis using mRNA purified from *C. hyointestinalis* 45104 was performed to determine the transcription initiation site of the *repA* gene. The observed start point for the *repA* mRNA (Figure 5.4) is at nt 371, and is that predicted by the position of the σ^{70} promoter. The "-10 region", TATAAT, of this promoter is located between nt 359 and 364 (Figure 5.2). The corresponding "-35 region" located between nt 336 and 341 (Figure 5.2) is TTTTTA, where 3 of the 6 bases are identical

FIGURE 5.4 Determination of the *repA* transcription initiation site by primer extension.

A *repA*-specific synthetic 18-mer oligodeoxynucleotide primer (5′-ACCTTATTTGATTGTGCC-3′, which binds from nt 445 to 462 of the sequence of Figure 5.2), was labelled with [γ-^{32}P]-ATP at the 5′ end, hybridized to total RNA template extracted from *C. hyointestinalis* 45104, and extended. The extension products were separated on a 6% polyacrylamide/urea sequencing gel and visualized by autoradiography. The discrete product (track labelled 1) was compared with tracks from conventional sequencing reactions covering the initiation region. The 5′ end of the mRNA is indicated as +1.


A C G T 1
AATCTTATCAT
5′
A +1
T
A
G

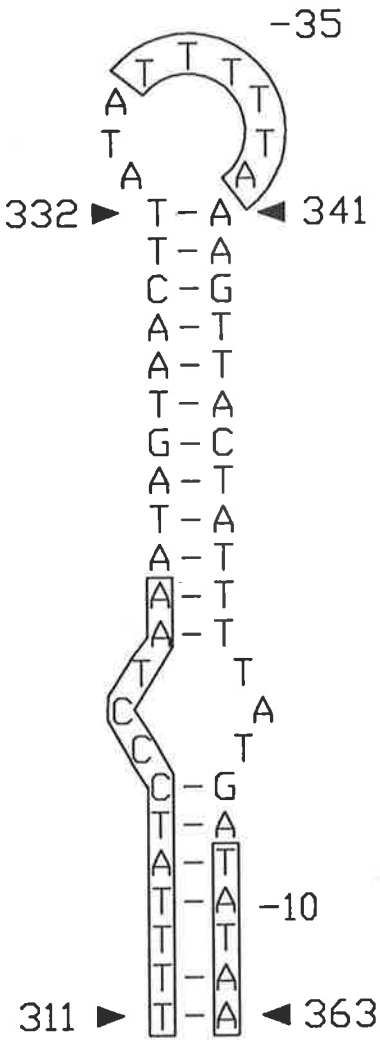
to the consensus. The spacing between the -10 and -35 regions has been implicated to play an important role in promoter strength. The limits of the spacing are 15 to 21 nt, with promoter strength being maximal at 17±1 nt (Rosenberg and Court, 1979; Hawley and McClure, 1983). Thus, the 17 nt spacing between the -10 and -35 regions of the promoter of *repA* is optimal.

The -10 and -35 regions are positioned within the loops of two potential hairpin structures which can form between nt 311 and 381 (Figure 5.5). These hairpin loop structures are flanked by two of the 19 bp repeats (nos. 3 and 4) and have free energies ($\Delta G°$) of -18.6 and -13.3 kCal/mol, respectively. Putative hairpin loop structures have been observed in the -35 region for the *repB* gene of pCI305 (Hayes *et al.*, 1991) and the -10 and -35 regions of the *repA* gene of pSC101 (Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985). Similarly, the -10 and -35 regions of the *repA* gene of mini-$R_{ts}1$ contain one inverted repeat with an 8 bp stem, and the -10 and translation initiation regions contain another inverted repeat of 6 bp (Itoh *et al.*, 1987). These data suggest that secondary structure plays an important role in the regulation of expression of these replication proteins, or in the initiation of replication.

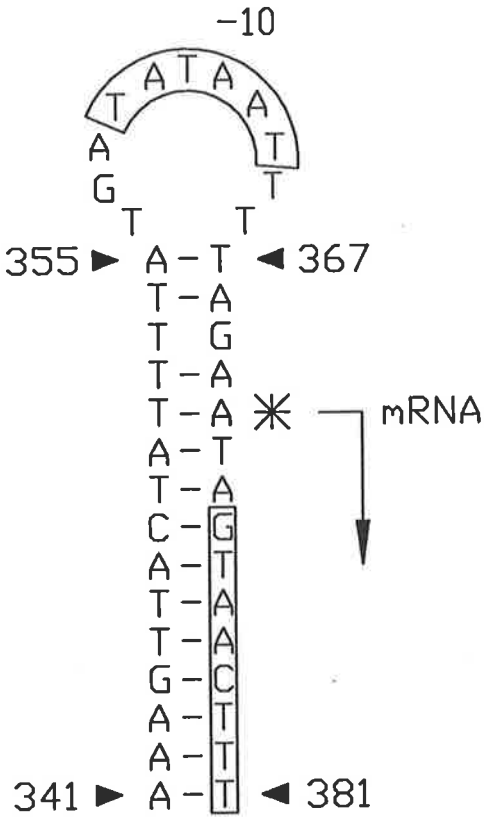
The two secondary structures overlapping the *repA* promoter utilize the same DNA sequence between nt 341 and 363 and so cannot exist independently. The stem of the first hairpin loop contains 12 bp of the 3′ end of repeat 3 and consequently, if these repeats are binding sites for RepA, the binding of RepA to repeat 3 may promote the formation of the second hairpin loop (Figure 5.5). Likewise, the stem of the second hairpin loop contains 8 bp at the 5′ end of repeat 4 suggesting that RepA binding to this site may promote the formation of the first hairpin loop. The possibility also exists that the stem of the first hairpin loop structure may in fact be an imperfect inverted repeat sequence which is also a binding site for RepA as has been observed in pSC101 (Linder *et al.*, 1985; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985). By binding to the inverted repeats, RepA of pSC101 competes with RNA polymerase for the *repA* promoter sequence and inhibits the *repA* transcription by autoregulation (Linder *et al.*, 1985; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985).

FIGURE 5.5 Two potential hairpin loop structures upstream from the translational start site for *repA* between nt 311 and 381.

The free energies (ΔG°, in kCal/mol) of each loop are indicated. The -35 and -10 boxes of the putative σ^{70} promoter of *repA* are shown. The *repA* mRNA start site is indicated by an asterisk on the second hairpin loop and the direction of transcription is shown by the arrow. The stem sequences containing the 3′ end of the third direct repeat and the 5′ end of the fourth direct repeat are boxed in the first hairpin loop and second hairpin loop, respectively.



△G −18.6



△G −13.3

5.2.1.3 Codon Usage

A summary of the codon usage within the coding region of the *repA* mRNA is shown in Table 5.1. Table 5.2 shows the relative *repA* codon usage as compared to the average usage in other sequenced *Campylobacter* genes. These genes include *tetO* (Sougakoff *et al.*, 1987; Manavathu *et al.*, 1988), *aphA-7* (Tenover *et al.*, 1989), *lysS* (Chan and Bingham, 1992), *glyA* (Chan and Bingham, 1990), and the *flaA* and *flaB* (Nuijten *et al.*, 1990b) genes from *C. jejuni*, together with the *aphA-3* (Trieu-Cuot *et al.*, 1985), *cat* (Wang and Taylor, 1990a), and the flagellin genes (*flaA* and *flaB*) from *C. coli* (Guerry *et al.*, 1990). It can be seen that the preferred codon usage within *repA* conforms well with other sequenced *Campylobacter* genes. The codon utilization in *repA* was very different from that used generally in *E. coli* K-12 and contained the so-called rare *E. coli* K-12 codons (AUA, UCG, CCU, CCC, ACG, CAA, AAU, and AGG; Konigsberg and Godson, 1983) at a frequency of 17.2% (largely through the use of AUA, CAA, and AAU).

5.2.1.4 Transcriptional Terminators

Analysis of the sequence after the UAA stop codon did not reveal the presence of any base-paired stem-loop structure that would be energetically favourable. However, two small stem loop structures were found at or downstream from the stop codon of *repA*. These occur between nt 1413 and 1435, and nt 1489 and 1511, but have free energy values of only -3.3 and -3.7 kCal/mol, respectively, and would therefore be unlikely to form readily.

5.2.2.5 The *par* region

A putative partition locus (*par*) is located between nt 2045 and 2250 (Figure 5.6). The *par* region contains information that is required for the accurate partitioning of plasmids during cell division (Austin and Abeles, 1983; Meacock and Cohen, 1980). The locus is characterized by a perfect 9 bp inverted repeat between nt 2200 and 2221. Adjacent to the left inverted repeat there is a putative IHF recognition site between nt 2180 and 2191. This region also contains six putative DNA gyrase binding sites. Four of these sites are in the coding strand and two are in the complementary strand. These sites share homology with the

TABLE 5.1 Codon usage within *repA* of *C. hyointestinalis*

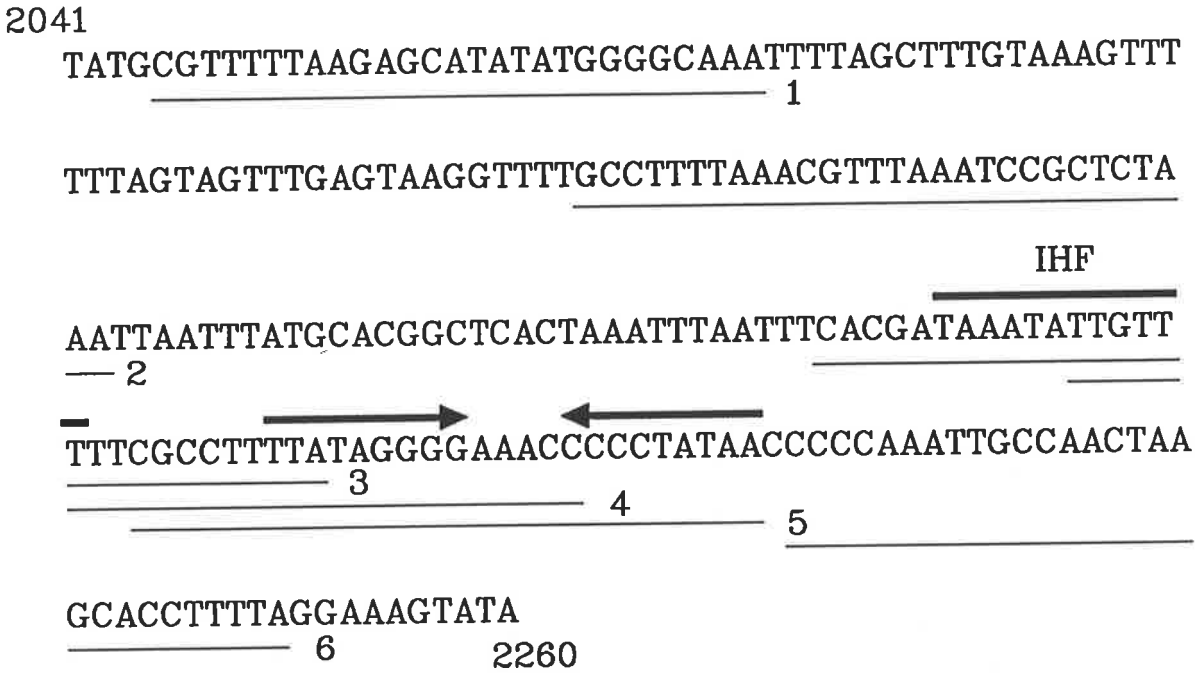
Codon	Number	Codon	Number
UUU-Phe	16	UAU-Tyr	9
UUC-Phe	1	UAC-Tyr	4
UUA-Leu	16	UAA-ochre	1
UUG-Leu	1	UAG-amber	0
CUU-Leu	5	CAU-His	1
CUC-Leu	0	CAC-His	0
CUA-Leu	5	CAA-Gln	19
CUG-Leu	0	CAG-Gln	1
AUU-Ile	9	AAU-Asn	12
AUC-Ile	7	AAC-Asn	6
AUA-Ile	23	AAA-Lys	44
AUG-Met	10	AAG-Lys	6
GUU-Val	4	GAU-Asp	10
GUC-Val	1	GAC-Asp	3
GUA-Val	2	GAA-Glu	26
GUG-Val	1	GAG-Glu	6
UCU-Ser	1	UGU-Cys	3
UCC-Ser	0	UGC-Cys	1
UCA-Ser	4	UGA-opal	0
UCG-Ser	1	UGG-Trp	3
CCU-Pro	0	CGU-Arg	0
CCC-Pro	0	CGC-Arg	1
CCA-Pro	4	CGA-Arg	0
CCG-Pro	1	CGG-Arg	0
ACU-Thr	4	AGU-Ser	4
ACC-Thr	1	AGC-Ser	7
ACA-Thr	10	AGA-Arg	11
ACG-Thr	2	AGG-Arg	1
GCU-Ala	4	GGU-Gly	2
GCC-Ala	1	GGC-Gly	4
GCA-Ala	12	GGA-Gly	3
GCG-Ala	2	GGA-Gly	2

TABLE 5.2 Comparison of *repA* gene codon usage with the codon usage amongst ten sequenced *Campylobacter* genes

Codon	% in *repA*	% in *Campylobacter*	Codon	% in *repA*	% in *Campylobacter*
UUU-Phe	94.1	81.3	GCU-Ala	21.1	44.6
UUC-Phe	5.9	18.7	GCC-Ala	5.3	9.3
			GCA-Ala	63.2	39.2
UUA-Leu	59.3	34.5	GCG-Ala	10.5	6.9
UUG-Leu	3.7	16.6			
CUU-Leu	18.5	30.9	UAU-Tyr	69.2	80.0
CUC-Leu	0.0	3.8	UAC-Tyr	30.8	20.0
CUA-Leu	18.5	8.0			
CUG-Leu	0.0	6.1	CAU-His	100.0	63.6
			CAC-His	0.0	36.4
AUU-Ile	23.1	36.5			
AUC-Ile	17.9	29.0	CAA-Gln	95.0	73.8
AUA-Ile	59.0	34.5	CAG-Gln	05.0	26.2
AUG-Met	100.0	100.0			
GUG-Met	0.0	0.0	AAU-Asn	66.7	72.6
			AAC-Asn	33.3	27.4
GUU-Val	50.0	43.7			
GUC-Val	12.5	6.1	AAA-Lys	88.0	76.7
GUA-Val	25.0	36.8	AAG-Lys	12.0	23.3
GUG-Val	12.5	13.3			
			GAA-Glu	81.3	68.6
UCU-Ser	5.9	30.6	GAG-Glu	18.7	31.4
UCC-Ser	0.0	2.5			
UCA-Ser	23.5	28.8	UGU-Cys	75.0	41.9
UCG-Ser	5.9	4.7	UGC-Cys	25.0	58.1
AGU-Ser	23.5	22.6			
AGC-Ser	41.2	10.9	UGG-Trp	100.0	100.0
CCU-Pro	0.0	32.5	CGU-Arg	0.0	16.0
CCC-Pro	0.0	14.5	CGC-Arg	7.7	6.8
CCA-Pro	80.0	27.7	CGA-Arg	0.0	6.4
CCG-Pro	20.0	25.3	CGG-Arg	0.0	7.8
			AGA-Arg	84.6	50.7
ACU-Thr	23.5	49.4	AGG-Arg	7.7	12.3
ACC-Thr	5.9	12.6			
ACA-Thr	58.9	31.1	UAA-ochre	100.0	40.0
ACG-Thr	11.8	06.8	UAG-amber	0.0	40.0
			UGA-opal	0.0	20.0
GGU-Gly	18.2	47.6			
GGC-Gly	36.4	14.3			
GGA-Gly	27.3	29.1			
GGG-Gly	18.2	9.0			

FIGURE 5.6 Nucleotide sequence of the putative *par* region between nt 2041 and 2260 of the cryptic plasmid of *C. hyointestinalis* 45104.

The arrows indicate the position of the 9 bp perfect inverted repeat. The positions of six putative DNA gyrase binding sites are underlined and numbered from 1 to 6. A site resembling the *E. coli* K-12 IHF-binding consensus is marked.


2041
TATGCGTTTTTAAGAGCATATATGGGGCAAATTTTAGCTTTGTAAAGTTT
1
TTTAGTAGTTTGAGTAAGGTTTTGCCTTTTAAACGTTTAAATCCGCTCTA
IHF
AATTAATTTATGCACGGCTCACTAAATTTAATTTCACGATAAATATTGTT
2
TTTCGCCTTTTATAGGGGAAACCCCCTATAACCCCCAAATTGCCAACTAA
3
4
5
GCACCTTTTAGGAAAGTATA
6
2260

E. coli K-12 DNA gyrase binding sites (Yang and Ames, 1988) of the REP consensus sequence (Figure 5.7). The putative *par* locus of the cryptic plasmid shows a number of features similar to the genetic organization of the *par* regions of bacteriophage P1 plasmid (Martin *et al.*, 1991) and pSC101 (Wahle and Kornberg, 1988). The inverted repeat sequence may be a binding site for a partitioning protein as has been demonstrated for the inverted repeat sequence at the P1 partitioning site (Martin *et al.*, 1991). With P1 plasmids, IHF has been shown to assist a partitioning protein in the assembly of a functional partition complex at the *par* locus (Funnell, 1988). The role played by DNA gyrase in plasmid partitioning is not certain. In pSC101, however, it is thought that DNA gyrase bound to the *par* region may associate with the cell membrane, in a process similar to that observed in chromosome segregation, and result in the efficient partitioning of the plasmid into each daughter cell during cell division (Wahle and Kornberg, 1988). To support this, it has been reported that membrane-binding of pSC101 is dependent upon the *par* locus (Gustafson *et al.*, 1983).

5.2.1.6 The *oriT* region

A putative origin of transfer (*oriT*) region is located between nt 1759 and 1886 (Figure 5.8). Mobilizable plasmids possess a DNA sequence, referred to as *oriT*, in which a site-specific nick is introduced into one of the two strands of duplex DNA; the nicked strand is then transferred to recipient cells beginning with the 5′ terminus (Willetts and Wilkins, 1984; Willetts and Skurray, 1987). The *oriT* regions of most mobilizable plasmids consist of an inverted repeat sequence adjacent to a "nick region" consensus sequence which contains a nick site. The sequence downstream from the nick site is usually A+T rich. In the cryptic plasmid the putative *oriT* region is characterized by an imperfect 19 bp inverted repeat between nt 1759 and 1798. Adjacent to the right arm of the inverted repeat is a sequence of 12 bp which is highly homologous to the consensus sequence of 12 bp located in the *oriT* "nick regions" of various plasmids including R64, RK2/RP4, R751, and pTF-FC2, and in the T-region border sequences of various Ti and Ri plasmids of *Agrobacterium* (Pansegrau and Lanka, 1991). This "nick region" of the cryptic plasmid shares total homology with the consensus sequence, and 9 of the 12 bases are identical to the nick region of RP4

FIGURE 5.7 Homology studies of the six sequences in the putative *par* region of the cryptic plasmid of *C. hyointestinalis* 45104 with the consensus for *E. coli* K-12 DNA gyrase binding sites.

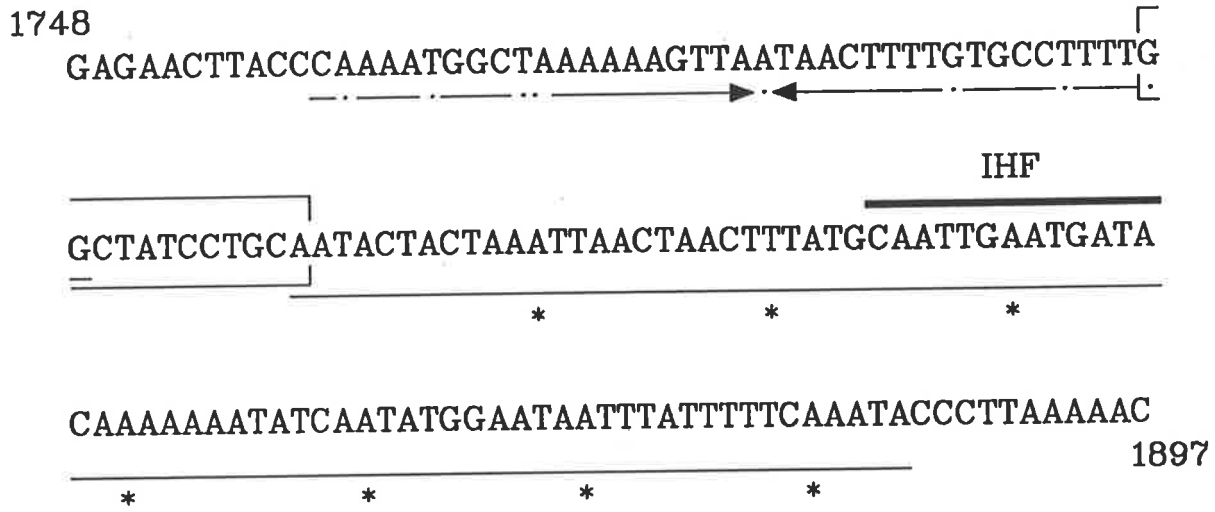
The six putative DNA gyrase binding sites of the cryptic plasmid are numbered from #1 to #6 and compared with the consensus of the *E. coli* K-12 DNA gyrase binding site (Yang and Ames, 1988). Consensus nucleotides marked with asterisks indicate conserved bases in at least 3 out of 6 of the putative *par* region sequences. Sequences #1 and #4 are in the complementary strand.

```
# 1          gCaaaaA-T--tCt--CG--tataTaCcCCgTtTa
# 2         gcCTt--t-T---aaa-CGtttaaaTcCGCtcTAa
# 3          cacG--A-Taaatat-tGtt---tTtCGCCTTtTa
# 4         aaCaa-aA-a--GCGG-aa---aatatCcCCTTtgg
# 5        cgcCTtttA-T---aGG-gG----aaacCcCCTataa
# 6        cccCaa--AtT--GCcaaCt-----aaGCaCCTTtTa

Consensus:    CTG--A-T--GCGG-CG-----GTGCGCCTTAT
              *    * *   *   **       * * **** *
```

FIGURE 5.8 Nucleotide sequence of the putative *oriT* region between nt 1748 and 1897 of the cryptic plasmid of *C. hyointestinalis* 45104.

The arrows indicate the position of the 19 bp imperfect inverted repeat. The 12 bp putative nick region as defined from the consensus sequence (A/G T/C/G A/T/C T/C ATCCTG C/T C/A) (Pansegrau *et al.*, 1991) is boxed. A 78 bp A+T rich region is underlined and a site resembling the consensus of the IHF binding site is marked. Specific nucleotides of in-phase recurring runs of adenine or thymine residues that might contribute to static bending of the *oriT* region are marked by an asterisk. The distance between each asterisk depicts the helical repeat of B-form DNA, showing the "in-frame" spacing of bend-forming nucleotides.


1748
GAGAACTTACCCAAAATGGCTAAAAAAGTTAATAACTTTTGTGCCTTTTG
IHF
GCTATCCTGCAATACTACTAAATTAACTAACTTTATGCAATTGAATGATA
CAAAAAAATATCAATATGGAATAATTTATTTTTCAAATACCCTTAAAAAC
1897

(Figure 5.9). If this sequence was a nick region, then the most likely nick site may be between nt 1806 and 1807. This site is 8 bp from the end of the right arm of the 19 bp inverted repeat which is the same position as that observed for the nick sites of RP4, R751 (Pansegrau *et al.*, 1990b), and R64 (Furuya and Komano, 1991). The sequence of the imperfect inverted repeat of the cryptic plasmid is not, however, similar to those of the 19 bp imperfect inverted repeats of RP4 or R751 (Fürste *et al.*, 1989), nor to the 17 bp imperfect inverted repeat of R64 (Furuya *et al.*, 1991). The *oriT* of RP4 has also been shown to contain a 172 bp sequence neighbouring the nick site which is required for full *oriT* activity (Pansegrau *et al.*, 1990b). This region has the potential to form a bent structure as it contains in-phase recurring runs of adenine or thymine residues and it was thought that DNA bending in this region could favour a conformation that facilitates access of the relaxosome protein complex (bound to the right arm of the inverted repeat) to its recognition sites within the nick region (Pansegrau *et al.*, 1990b). Downstream from nt 1806 is an 80 bp region which is 83% A+T rich and contains a putative IHF recognition site between nt 1835 and 1848; this region may have a function similar to that of the 172 bp sequence of RP4.

It has not been demonstrated whether the cryptic plasmid can be mobilized from *C. hyointestinalis* 45104 *via* this transfer origin into other *C. hyointestinalis* recipients. If it can, then it cannot be self-transmissible as it does not encode the proteins necessary to mediate this transfer and they may therefore be chromosomally encoded (no other plasmids were seen in *C. hyointestinalis* 45104; Figure 4.2). The possibility might also exist that the cryptic plasmid is no longer able to be mobilized as this *oriT* sequence is all that remains of an original (more complex) plasmid transfer region.

5.2.2 Analysis of the RepA protein

Analysis of the plasmid-encoded proteins in *E. coli* K-12 minicells harbouring pCHI2 revealed a protein of approximately 40 kDa (Figure 5.10) which is in good agreement with the size predicted for the protein encoded by ORF1. No other non-vector proteins could be detected when comparing the protein products of pCHI2 to pCHI1 which produces the *aphA-3* gene product (kanamycin-resistance) originating from pILL550 and the *bla* gene

FIGURE 5.9 Alignment of the putative "nick region" of the cryptic plasmid of *C. hyointestinalis* 45104 with the defined and potential "nick regions" of various plasmids.

Conserved nucleotide positions are boxed. Locations of nick-sites are indicated by arrows. The plasmid sequences are taken from Pansegrau and Lanka (1991).

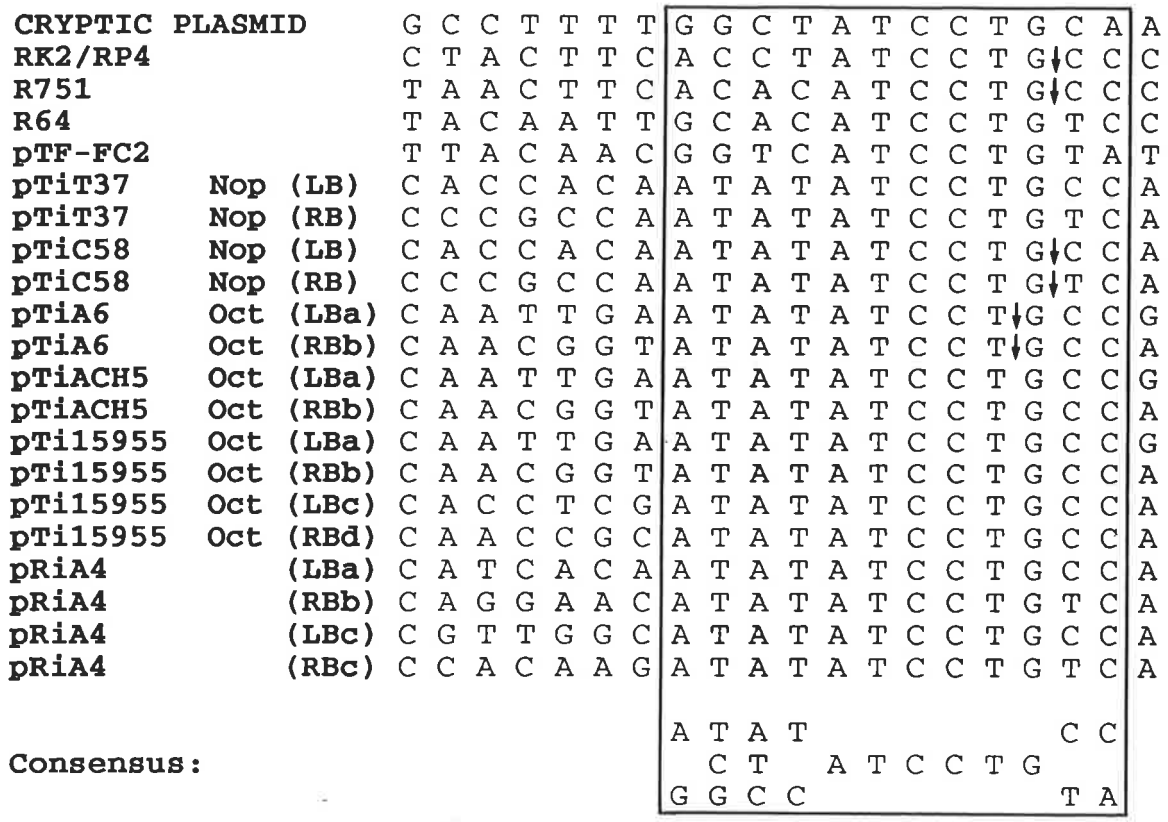

CRYPTIC PLASMID G C C T T T T G G C T A T C C T G C A A
RK2/RP4 C T A C T T C A C C T A T C C T G C C C
R751 T A A C T T C A C A C A T C C T G C C C
R64 T A C A A T T G C A C A T C C T G T C C
pTF-FC2 T T A C A A C G G T C A T C C T G T A T
pTiT37 Nop (LB) C A C C A C A A T A T A T C C T G C C A
pTiT37 Nop (RB) C C C G C C A A T A T A T C C T G T C A
pTiC58 Nop (LB) C A C C A C A A T A T A T C C T G C C A
pTiC58 Nop (RB) C C C G C C A A T A T A T C C T G T C A
pTiA6 Oct (LBa) C A A T T G A A T A T A T C C T G C C G
pTiA6 Oct (RBb) C A A C G G T A T A T A T C C T G C C A
pTiACH5 Oct (LBa) C A A T T G A A T A T A T C C T G C C G
pTiACH5 Oct (RBb) C A A C G G T A T A T A T C C T G C C A
pTi15955 Oct (LBa) C A A T T G A A T A T A T C C T G C C G
pTi15955 Oct (RBb) C A A C G G T A T A T A T C C T G C C A
pTi15955 Oct (LBc) C A C C T C G A T A T A T C C T G C C A
pTi15955 Oct (RBd) C A A C C G C A T A T A T C C T G C C A
pRiA4 (LBa) C A T C A C A A T A T A T C C T G C C A
pRiA4 (RBb) C A G G A A C A T A T A T C C T G T C A
pRiA4 (LBc) C G T T G G C A T A T A T C C T G C C A
pRiA4 (RBc) C C A C A A G A T A T A T C C T G T C A
A T A T C C
Consensus: C T A T C C T G
G G C C T A

FIGURE 5.10 Expression of plasmid-encoded proteins.

Plasmid-encoded proteins were analysed using the *E. coli* K-12 minicell producing strain DS410. Minicells harbouring the various plasmids were purified on sucrose gradients, labelled with [^{35}S]-methionine and solubilized in SDS sample buffer. The plasmid-encoded proteins were visualized by autoradiography after electrophoretic separation in SDS on an 11-20% polyacrylamide gradient gel. The RepA protein is indicated by the arrow. Protein size markers (Pharmacia) were used as standards and the protein sizes (kDa) are shown on the right. The plasmids harboured by DS410 in each track are as follows:

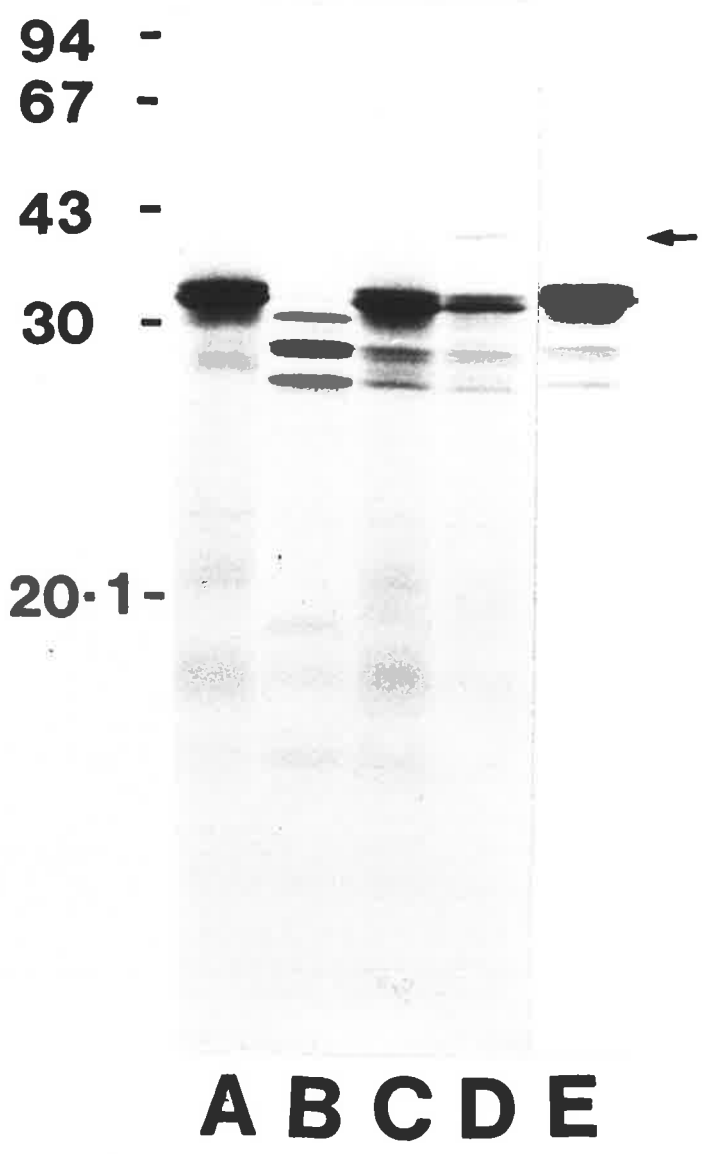
(A) pILL550

(B) pPM2101

(C) pCHI1

(D) pCHI2

(E) pCHI3


94
67
43
30
20·1
A B C D E

products (ampicillin-resistance) originating from pPM2101. The expression of a replication protein could not be observed in pILL550. The 1 _DNA from the *C. coli* plasmid pIP1445 present in pILL550 has not been fully characterized and may not encode a replication protein; the plasmid may replicate in a fashion similar to ColE1. Alternatively, the plasmid may encode a replication protein that contains a low number of methionine residues and is thus not detected here by autoradiography. In addition, minicell analysis of pCHI3 did not reveal any non-vector proteins, which confirms that the protein of interest is indeed the product of *repA*. pCHI3 has the cryptic plasmid cloned *via* the *Cla*I site, at nt 1285 (within the *repA* gene; Figure 5.2) and therefore can not express the RepA protein in minicells, but could express the products of any other genes present on the cryptic plasmid.

A plot of the hydropathic nature of the predicted RepA protein has been made according to Kyte and Doolittle (1982) (Figure 5.11). This plot suggests that overall the protein is hydrophilic with a mean hydropathic value of -0.54. This overall hydrophilicity is consistent with the intracellular cytoplasmic location expected for a replication initiation protein.

The rules of Chou and Fasman (1974a, 1974b, 1978) were applied to predict the secondary structure of RepA from the predicted amino acid sequence (Figure 5.12). Amino acid residues 119 to 138 and 295 to 314 show helix-turn-helix motifs, which have been observed in the replication initiation proteins of pSC101 (Vocke and Bastia, 1983a) and other DNA binding proteins (Pabo and Sauer, 1984).

The putative RepA protein shows homology of 23.1% identity and 72.4% similarity across the whole protein with the RepA replication protein of pFA3 (Gilbride and Brunton, 1990) of *Neisseria gonorrhoeae* (Figure 5.13). Amino acid residues 110 to 154 and 185 to 207 of RepA also show strong homologies with the replication proteins of pSC101 (Vocke and Bastia, 1983a), R6K (Stalker *et al.*, 1982), and pCI305 of *Lactococcus lactis* subsp. *lactis* (Hayes *et al.*, 1991) (Figure 5.14), suggesting that these residues may be part of some conserved structural region for the replication initiation functions of plasmid-encoded replication proteins.

FIGURE 5.11 Hydropathic nature of RepA.

The hydropathic nature of the amino acid sequence of the RepA protein was analysed for hydrophobicity according to Kyte and Doolittle (1982) using a window of nine amino acids.

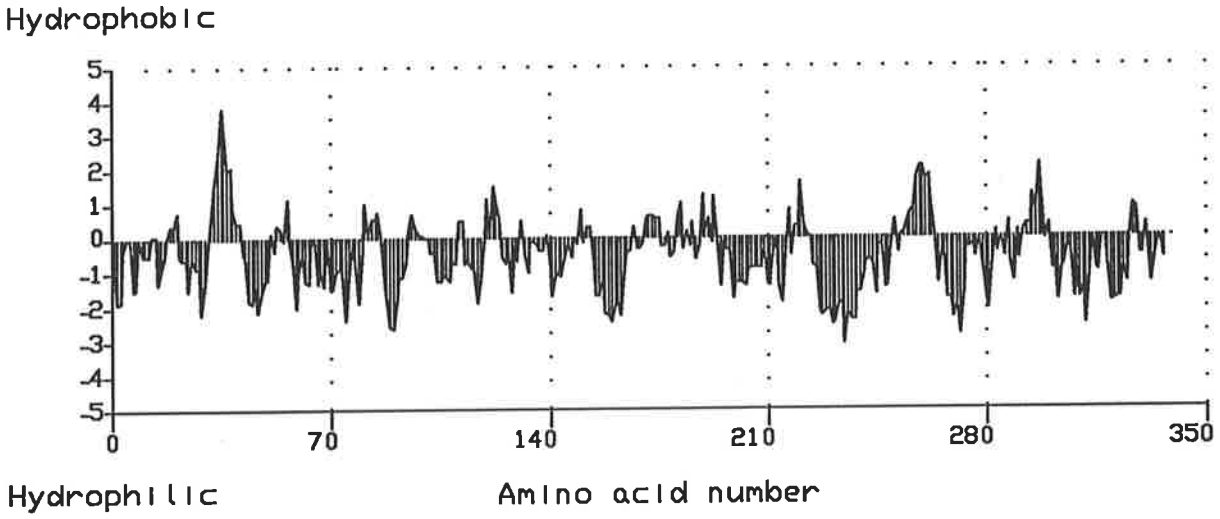

Hydrophobic
5
4
3
2
1
0
-1
-2
-3
-4
-5
0
70
140
210
280
350
Hydrophilic
Amino acid number

FIGURE 5.12 Predicted secondary structure of RepA.

The amino acid sequence of the RepA protein was subjected to analysis using the algorithm of Chou and Fasman (1978).

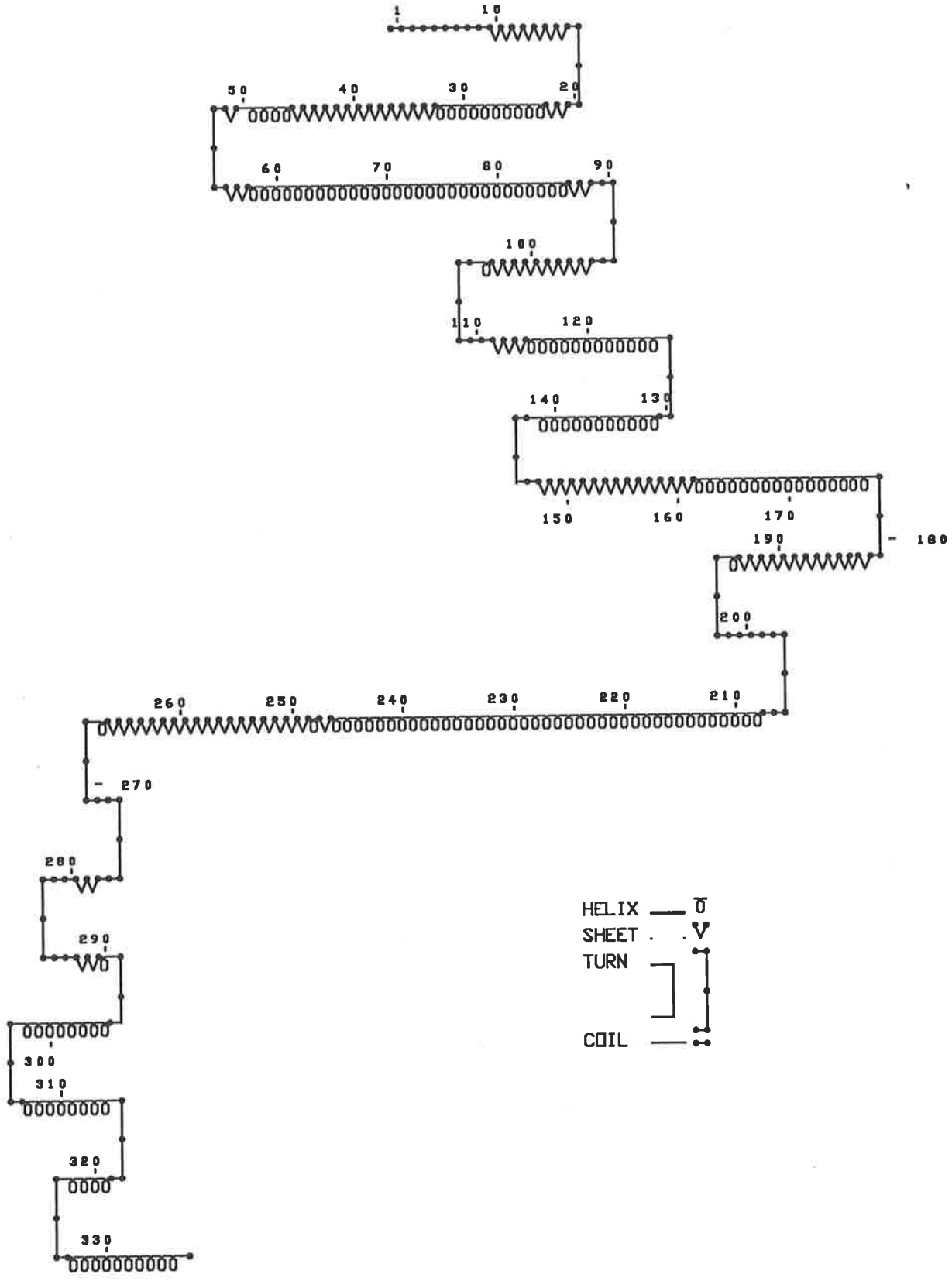

1
10
50
40
30
20
60
70
80
90
100
110
120
140
130
150
160
170
180
190
200
260
250
240
230
220
210
270
280
290
300
310
320
330
HELIX
SHEET
TURN
COIL

FIGURE 5.13 Homology studies of ChRepA and NgRepA.

The amino acid sequence of the RepA protein of *C. hyointestinalis* 45104 (ChRepA) was compared with data in the SWISSPROT bank, in a search for homologous sequences. The replication initiation protein of the *Neisseria gonorrhoeae* plasmid pFA3 (NgRepA) was found by this method to show significant homology to ChRepA. These two proteins were then analysed by the CLUSTAL programme for maximal alignment (Higgins and Sharp, 1988). Identical amino acids are indicated with an asterisk, similar amino acids are indicated by a dot.

```
C.h. RepA  MKKTEIKNIANRQIMAQSNKVITAKYELTEAEQKIILLAIAQVDSIKDKKFGTYKITIPE
N.g. RepA  M--------PNDLVVVKANSLIEANYRLSIDEIRILALTIGTMDPKSNQKI--FDFTVAD
           *        .*  .....*..*.*.* *. .* .*. *.*. .*. ...*.  ...*...

C.h. RepA  LEQKIGSKIKQA---QLKETCRRLMQRVVYIENGKNWKMFHWISTAEYIDGENTIKFKIS
N.g. RepA  FVREFPEISQDNAYKQIQAAIKRIYDRSVKTEDKDRVTEFRWVSSRTYFKKEGRFRIAMT
           . ... .  ...   *.... .*. .* * .*. .. . *.*.*. .*.. *. ... ..

C.h. RepA  DEMKPFLLQLKGNFTKIELENALKFNGKYTLRFYQFCMQMQNQATKKRTFELSKLYEILQ
N.g. RepA  DEVMPYLTQLKGQFTQYQLKHIAYFNSVHSIRIYE--LITQYRSVGSREITVEKLKEWLQ
           **..*.* ****.**. .*..   **. ...*.*.  .  * ... .*.....** * **

C.h. RepA  LPESLTTSFARFKLKVIEPSINEINTKSDIKANWEISKKIGKKIVEIELNFKSKERLQEQ
N.g. RepA  VENKYP-RFNSLNQRVLEPAITEINEKSDLVVEVEQIKR-GRTIHSLNFVIGSKKRTAQK
           . .. . .*.... .*.**.*.***.***. .. *  *. *..* .... . **.* ...

C.h. RepA  TKQAREVKSLKKYIGKQCLYFDYLIIIEQISYNAEQSRYEVIYKDGTGDLCRADFDSIDM
N.g. RepA  IE---EVAKRPVFPHKN--KYGKFVKLDKQNPKMSNHEYGLWARDCLKIL-EDHYTDITK
           ..   ** .   .  *.   .. .. ... . . ..  *.. .*    *  .....*..

C.h. RepA  L-EIAIKKGKIEANFRKANPELFKKIDSKEEIANLFR---------DMMK-
N.g. RepA  VTNEDLRNYWV---FLAGNDSNRSKLGSKSDFLNELKKRGYKLVDCELVKI
           . . ....  .   *  .* .  .*..**... * ..          ...*
```

FIGURE 5.14 Homology studies of ChRepA and the replication initiation proteins of pFA3, pSC101, R6K, and pCI305.

The amino acid sequence of ChRepA was compared with data in the SWISSPROT bank, in a search for homologous sequences. With amino acids 110 to 154, and 185 to 207 of ChRepA, there were strong homologies with the replication initiation proteins of the plasmids pFA3, pSC101, R6K, and pCI305. Amino acids that are identical with or similar to those of ChRepA are written in boldface. The alignment of these homologous regions were made using the MACAW program (Schuler *et al.*, 1991).

```
pChyo      110  NTIKFKISDEMKPFLLQLK---GNFTKIELENALKFNGKYTLRFYQFC  154
pFA3       103  GRFRIAMTDEVMPYLTQLK---GQFTQYQLKHIAYFNSVHSIRIYELI  147
pSC101     106  EKLELVFSEEILPYLFQLK----KFIKYNLEHVKSFENKYSMRIYEWL  149
R6K        131  GYLSLKFTRTIEPYISSLIgkkNKFTTQLLTASLRLSSQYSSSLYQLI  178
pCI305     127  DDVKIEFHREIMPYLINLK---QNFTQHALSDIAELNSKYSIILYRWL  171

pChyo      185  SFARFKLKVIEPSINEINTKSDI  207
pFA3       175  RFNSLNQRVLEPAITEINEKSDL  197
pSC101     182  EFKRLNQWVLKPISKDLNTYSNM  204
R6K        219  DFPIFKRDVLNKAIAEIKKKTEI  241
pCI305     217  RFDRLEHRVLKEPIEEINENTSF  239
```

These structural features and homologies with other replication proteins suggested that RepA is indeed a replication initiation protein.

5.2.3 Nucleotide sequence determination and analysis of various regions of pCHI5

By comparison with the minimal DNA regions essential for autonomous replication in other plasmids, it was determined (Section 5.2.1.1) that the replication region of the cryptic plasmid is most probably located in a region of approximately 1.4 kb beginning just before the A+T rich region of the origin and ending after the termination codon of *repA*. Sequence analysis of pCHI5 could help confirm this.

Specific regions of pCHI5 were sequenced to properly define the boundary limits of the cryptic plasmid replication region, and to determine how pCHI5 was generated from pCHI4. The 2.6 and 1.1 kb *Cla*I fragments of pCHI5 were cloned into the *Acc*I site of the multiple cloning site of M13mp18 (Vieira and Messing, 1982). Sequencing was performed using the chain termination method (Sanger *et al.*, 1977, 1980) using Sequenase^{TM}. Universal primer was employed to sequence cloned DNA from the polylinker. Regions of pCHI5 were sequenced by the double stranded sequencing method using oligonucleotides which had been made to determine the sequence of the cryptic plasmid. Sequence data was checked by comparison with the published sequences of the *oriT* of RP4 (Fürste *et al.*, 1989), *aphA-3* (Trieu-Cuot *et al.*, 1985), and the sequence of the cryptic plasmid obtained here (Figure 5.2). The sequencing strategy is shown (Figure 5.15). The genetic organization of pCHI5 is shown in Figure 5.16.

5.2.3.1 Genetic organization of pCHI5

Analysis of the regions of determined sequence reveal that pCHI5 is composed of three distinct DNA regions. These regions originate from the *oriT* of RP4, the *aphA-3* gene, and the cryptic plasmid, and agree with the Southern hybridization data obtained for pCHI5 described earlier (Section 4.2.11). It was determined that 654 bp of the *oriT* region is present in pCHI5. This region contains the sequence beginning from the 5′-terminal nucleotide at the *oriT* nick site (Pansegrau *et al.*, 1990b) to the *Cla*I site adjoining the *oriT* and the *aphA-3*

FIGURE 5.15 Strategy used for dideoxy sequencing of pCHI5.

Some of the regions of pCHI5 were sequenced by the double stranded sequencing method using oligodeoxynucleotides which had been made for sequence determination of the cryptic plasmid (Figure 5.1). DNA sequences obtained with these primers are indicated by the "#" symbol. The 2.6 and 1.1 kb *Cla*I fragments of pCHI5 were cloned into M13mp18 and sequenced using universal primer or the -40 primer (to accurately determine sequence at polylinker junction regions). Synthetic oligodeoxynucleotides were constructed to extend the sequence in either direction; sequences obtained with new primers are indicated by asterisks. Specific regions of pCHI5 were sequenced to properly define the boundary limits of the cryptic plasmid replication region, and to determine how pCHI5 was generated from pCHI4. Many regions were only sequenced in one direction but the sequence data obtained from these regions were checked by comparison with the published sequences of the *oriT* of RP4 (Fürste *et al.*, 1989), *aphA-3* (Trieu-Cuot *et al.*, 1985), and the sequence of the cryptic plasmid herein obtained (Figure 5.2).

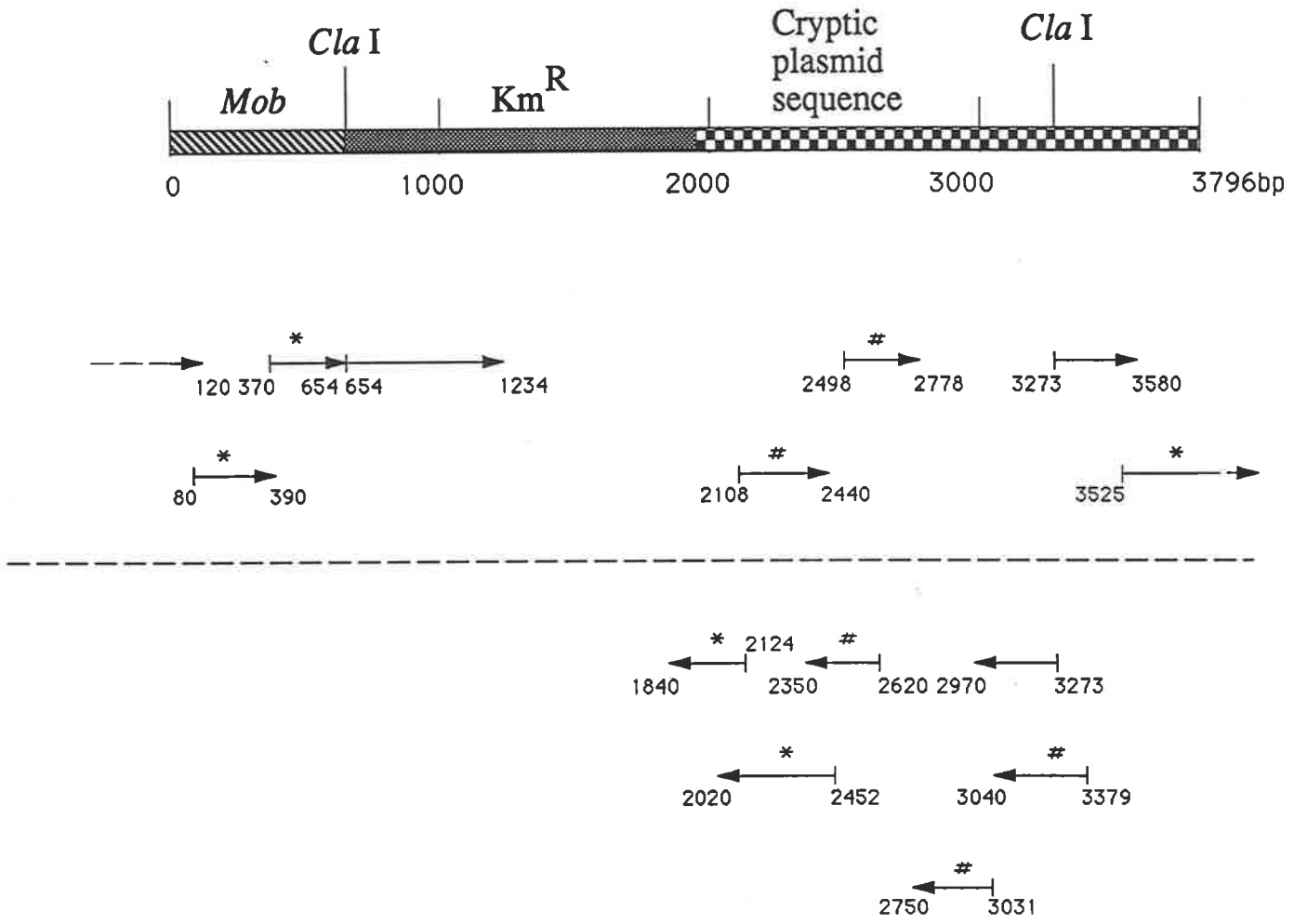
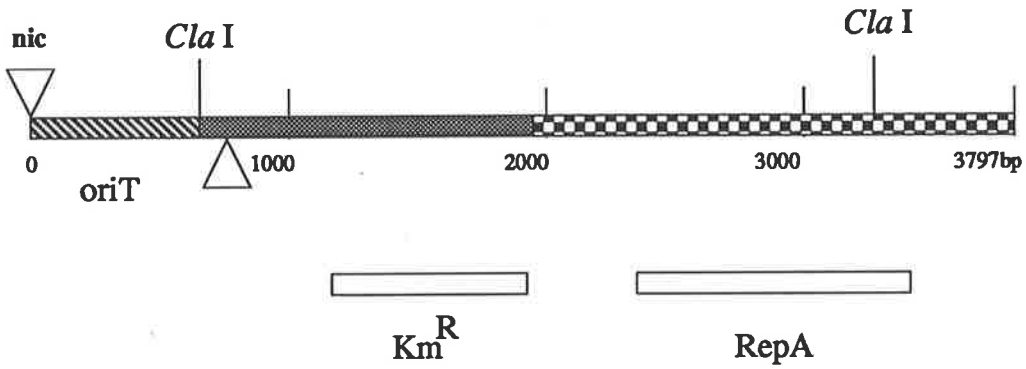

Cla I
Mob
Km^R
Cryptic plasmid sequence
Cla I
0
1000
2000
3000
3796bp
*
120 370
654 654
1234
#
2498
2778
3273
3580
*
80
390
#
2108
2440
*
3525
* 2124
#
1840
2350
2620 2970
3273
*
2020
2452
#
3040
3379
#
2750
3031

FIGURE 5.16 Genetic organization of pCHI5.

The positions of the ORF's encoding the *aphA-3* and *repA* genes are indicated by open boxes. The position of the 91 bp deletion present upstream from the *aphA-3* gene is indicated by a triangle. The position of the 5′-terminal nucleotide of the "nick region" of the *oriT* is indicated by a triangle labelled nic. pCHI5 consists of three distinct regions of DNA: a region of the *oriT* of RP4 (marked by diagonal lines), the *Cla*I-*Hin*dIII containing the *aphA-3* gene (marked in grey), and 1806 bp of the cryptic plasmid containing the *repA* gene (marked by chequered squares).

pCHI5, 3797bp



gene in pCHI4. pCHI5 does not possess the imperfect 19 bp inverted repeat sequences normally located 8 bp upstream from the nick site. The proximal arm of the inverted repeat has been demonstrated to be the assembly site for the relaxosome at *oriT* which is responsible for the initial cleavage at the nick site of the strand to be transferred to a recipient cell (Fürste *et al.*, 1989; Ziegelin *et al.*, 1989; Pansegrau *et al.*, 1990b). This site has been demonstrated to be essential for the efficient mobilization of *oriT* plasmids (Fürste *et al.*, 1989; Pansegrau *et al.*, 1990b; Waters *et al.*, 1991). The absence of this region would explain why pCHI6 can not be mobilized into *S. typhimurium* (Section 4.2.12)

Adjoining this region of *oriT* DNA, is a 1.336 kb *Cla*I-*Hin*dIII fragment containing the *aphA-3* kanamycin-resistance determinant. The sequence of this region is identical to that published (Trieu-Cuot *et al.*, 1985) except that there appears to be a 91 bp deletion from nt 107 to 198 in the published sequence of the DNA fragment containing *aphA-3* (Figure 5.17). This deletion occurrs within a region of direct repeat homology (Figure 5.17) and may have occurred by homologous recombination. The deleted sequence harbours the -35 and -10 promoter region of *aphA-3* (Trieu-Cuot *et al.*, 1985) but does not appear to have any deleterious effect on the expression of the gene. Further analysis demonstrated that this deletion is present in the original vector construct pCHI1 (and therefore pCHI4 as well) and is not a feature unique to pCHI5 (discussed in Chapter 6).

The sequence adjacent to this DNA fragment containing the *aphA-3* gene contains 1.8 kb of the cryptic plasmid DNA. These regions are joined by the original *Hin*dIII site into which the cryptic plasmid was first cloned (Section 4.2.5). This region spans the *Hin*dIII site at nt 1 to nt 1806 of the cryptic plasmid sequence given in Figure 5.2 and contains the A+T rich region, the four 19 bp direct repeats, and the *repA* gene. The deletion terminates at nt 1806 which was joined to the 5′-terminal nucleotide (nick site) of the *oriT* region (Figure 5.18). The sequence just upstream from nucleotide 1806 has already been shown to be homologous to the common sequence motif from a variety of plasmid DNA transfer origins (in particular RP4) (Figure 5.9). The observation that the cryptic plasmid sequence ends at position 1806 and is then coupled to the 5′-terminal nucleotide of the RP4 *oriT* sequence implies that this site is in fact a nick site in the wild-type cryptic plasmid. This region may

FIGURE 5.17 Nucleotide sequences of the DNA fragments upstream from the *aphA-3* genes from plasmids pILL550 and pCHI5.

The -35 recognition site, -10 Pribnow box, and the transcription start point are shown in bold face where known. Direct repeats are shown by numbered arrows. The 91 bp deleted sequence in pCHI5 between nt 107 to 198 is marked by the dashed lines. The nucleotide sequence of the region upstream from the *aphA-3* gene in pILL550, and the corresponding nucleotide numbers, are as given by Trieu-Cuot *et al.* (1985).

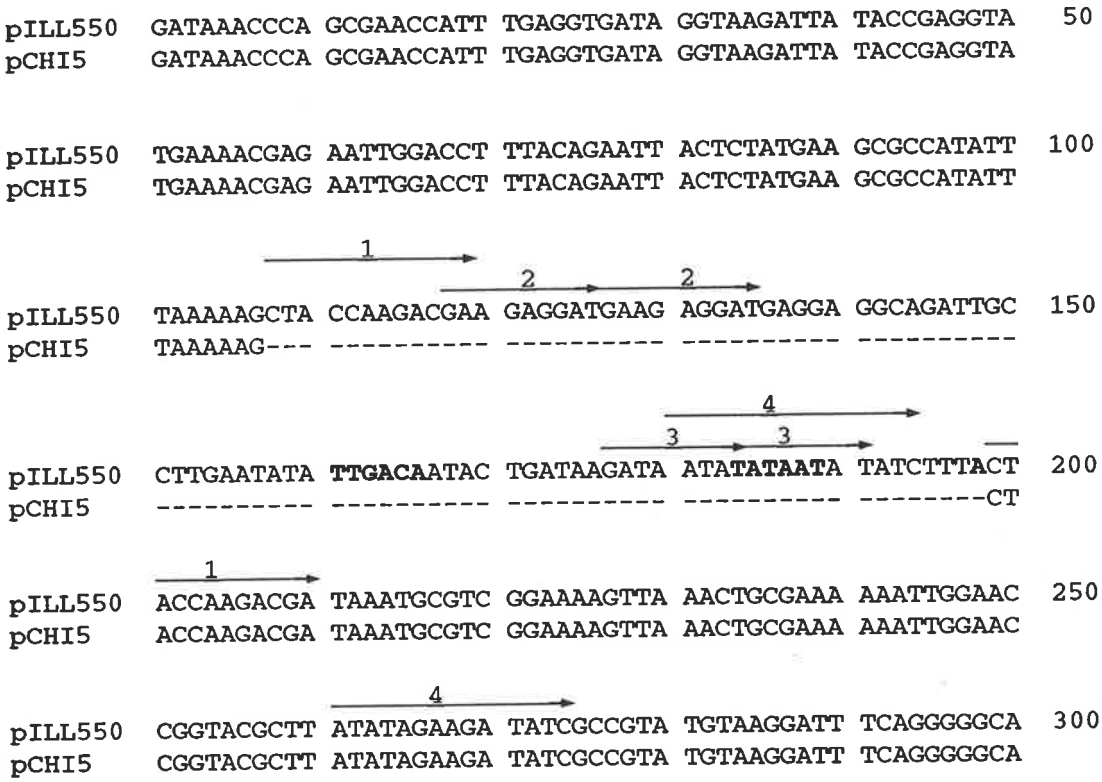
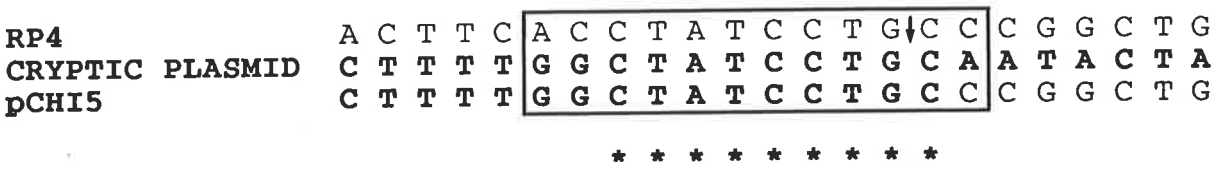

pILL550 GATAAACCCA GCGAACCATT TGAGGTGATA GGTAAGATTA TACCGAGGTA 50
pCHI5 GATAAACCCA GCGAACCATT TGAGGTGATA GGTAAGATTA TACCGAGGTA
pILL550 TGAAAACGAG AATTGGACCT TTACAGAATT ACTCTATGAA GCGCCATATT 100
pCHI5 TGAAAACGAG AATTGGACCT TTACAGAATT ACTCTATGAA GCGCCATATT
1
2
2
pILL550 TAAAAAGCTA CCAAGACGAA GAGGATGAAG AGGATGAGGA GGCAGATTGC 150
pCHI5 TAAAAAG--- ---------- ---------- ---------- ----------
4
3
3
pILL550 CTTGAATATA TTGACAATAC TGATAAGATA ATATATAATA TATCTTTACT 200
pCHI5 ---------- ---------- ---------- ---------- --------CT
1
pILL550 ACCAAGACGA TAAATGCGTC GGAAAAGTTA AACTGCGAAA AAATTGGAAC 250
pCHI5 ACCAAGACGA TAAATGCGTC GGAAAAGTTA AACTGCGAAA AAATTGGAAC
4
pILL550 CGGTACGCTT ATATAGAAGA TATCGCCGTA TGTAAGGATT TCAGGGGGCA 300
pCHI5 CGGTACGCTT ATATAGAAGA TATCGCCGTA TGTAAGGATT TCAGGGGGCA

FIGURE 5.18 Comparison of the nucleotide sequence of pCHI5, at the junction between nt 1806 of the cryptic plasmid and the 5′-terminal nucleotide of the *oriT* region, with the nick region of the *oriT* of RP4.

Nucleotides homologous to the nick region of RP4 in pCHI5 are indicated by an asterisk. The "nick region" of RP4 is boxed and the nick site is indicated by an arrow. The cryptic plasmid sequence given is from nt 1892 to nt 1813. The cryptic plasmid sequence, and the sequence homologous to it in pCHI5, are shown in bold.


RP4
A C T T C A C C T A T C C T G C C C G G C T G
CRYPTIC PLASMID
C T T T T G G C T A T C C T G C A A T A C T A
pCHI5
C T T T T G G C T A T C C T G C C C G G C T G
* * * * * * * * *

have been mistaken by the *E. coli* K-12 transfer proteins as being the 3′-terminal end of the RP4 *oriT* and the two ends were cleaved/ligated together to form pCHI5. This result implies that this region of the cryptic plasmid may indeed be involved in some mobilization function and that a natural nick site may be present between nt 1806 and 1807. pCHI5 appears to be identical to pCHI4 except that the transfer of DNA into the recipient cell starting at the 5′ nucleotide of *oriT* of RP4 has terminated at nucleotide 1806 of the cryptic plasmid DNA, and religated it back to the 5′ end, thus deleting approximately 3.2 kb of cryptic plasmid sequence, all of pHC79, and approximately 600 bp of the RP4 *oriT*. These data suggest that pCHI5 was introduced into *C. hyointestinalis via* a conjugation event, which is in agreement with earlier observations (Section 4.2.15).

The sequences of the *Hin*dIII sites adjacent to the *aphA-3* gene and the cryptic plasmid DNA, and at position 953 within the cryptic plasmid DNA are conserved in pCHI5. Earlier observations had demonstrated that pCHI5 could not be cleaved by *Hin*dIII (Section 4.2.9) which is in conflict with these findings. However, it has been subsequently discovered that if pCHI5 is incubated with *Hin*dIII for 16 h, some partial digestion at these sites can be observed (Figure 5.19). When pCHI6 (derived from pCHI5; Section 4.2.12) extracted from an *E. coli* K-12 host is incubated with *Hin*dIII, the sites are readily cleaved (Figure 5.19). The inability of *Hin*dIII to cleave pCHI5 after 2 h incubation could not be explained by contaminating DNA-binding proteins as cleavage did not occur after pCHI5 had been treated with Proteinase K (unpublished data). There is no obvious explanation for the inability of *Hin*dIII to cleave pCHI5 after 2 h of incubation.

The sequence analysis of pCHI5 confirmed that the region of cryptic plasmid DNA between nt 1 and 1806 (Figure 5.2) is sufficient for the autonomous replication of plasmid DNA within *C. hyointestinalis*. This confirms the fact that pCHI2 should contain an undisrupted form of the cryptic plasmid replicon.

5.3 Summary and conclusions

A summary of the genetic organization of the cryptic plasmid of *C. hyointestinalis* is shown in Figure 5.2. The nucleotide sequence of the cryptic plasmid was determined and

FIGURE 5.19 Restriction endonuclease digestion of pCHI5 with *Hin*dIII for 16 h.

pCHI5 was digested with *Hin*dIII for 16 h and examined by agarose gel electrophoresis. Four DNA bands were observed; the slowest migrating corresponds to uncut plasmid DNA, the second is linearized plasmid (approximately 3.7 kb), and the other two correspond to the two *Hin*dIII DNA restriction fragments of pCHI5 (approximately 2.8 and 0.9 kb). Bacteriophage SPP1 DNA digested with *Eco*R1 was used as a standard. The fragment sizes (kb) are 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, and 0.36. pCHI6 was digested with *Cla*I and *Hin*dIII for only 2 h. The plasmids and the enzymes they are digested with in each track are as follows:

(A) SPP1

(B) pCHI5 (uncut)

(C) pCHI5 (*Cla*I)

(D) pCHI5 (*Hin*dIII)

(E) pCHI6 (*Cla*I and *Hin*dIII)

A B C D E

contains a single ORF which, when translated, would give rise to a protein of 337 amino acid residues with a size of 39.7 kDa, designated RepA. A 280 bp region upstream from the ORF consists of an extremely A+T rich region, four 19 bp direct repeats, and a short sequence of dyad symmetry. This organization is similar to that seen in the replication regions of other plasmids. The promoter region of the *repA* gene shows homology to the consensus sequence for an *E. coli* K-12 σ^{70}-type promoter and the -10 and -35 regions of the promoter are positioned within the loops of two potential hairpin structures. These secondary structures are flanked by two of the 19 bp repeats and may play a role in the autoregulation of RepA.

Computer analysis of the RepA protein according to the program of Kyte and Doolittle (1982) suggests that overall the protein is hydrophilic in nature. The secondary structure predicted by Chou and Fasman (1974a, 1974b, 1978) shows that RepA contains two helix-turn-helix motifs, typical of many plasmid-encoded replication initiation proteins and DNA binding proteins. Computer homology searches of the amino acid sequence using the GenBank and EMBL Gene/Protein Sequence Database found that RepA shows extensive homology with the replication initiation protein encoded by the *Neisseria gonorrhoeae* plasmid pFA3, and that two stretches of amino acid residues in RepA show strong homology with the replication proteins of pSC101, R6K, and pCI305. This suggests that RepA is a replication initiation protein.

Two regions not associated with replication were also identified on the cryptic plasmid. A putative *par* locus, consisting of a 9 bp inverted repeat sequence and six putative 28 bp DNA gyrase binding sites, is located between nt 2045 and 2250. A putative *oriT* region, consisting of an imperfect 19 bp inverted repeat adjacent to 12 bp which shares a strong homology with the nick regions from a variety of DNA transfer origins of other plasmids, is located between nt 1759 and 1886. Downstream from nt 1806 is an 80 bp region which is 83% A+T rich and contains a putative IHF binding site; this site may be involved in DNA bending to allow the putative nick site to be made accessible and to be efficiently recognized by the transfer proteins. It has not been demonstrated, however, that the cryptic plasmid can be mobilized from this *oriT* region.

A summary of the genetic organization of pCHI5 is shown in Figure 5.16. The nucleotide sequence of various regions of pCHI5 reveal that the plasmid consists of three regions of DNA which have strong homology with the DNA sequences of the *oriT* of RP4, the *aphA-3* kanamycin-resistance gene, and the cryptic plasmid. It has been determined that pCHI5 is identical to pCHI4 except that all the DNA between nt 1806 of the cloned cryptic plasmid and the 5′-terminal nucleotide of the nick site of *oriT* has been deleted. The observation that nt 1806 of the cryptic plasmid sequence was coupled to the 5′ end of the *oriT* implies that pCHI4 has been cleaved/ligated at this site to generate pCHI5. These data suggest that pCHI5 was introduced into *C. hyointestinalis via* conjugation. The region of cryptic plasmid DNA between nt 1 and 1806, containing the *repA* gene and the 400 bp upstream, is sufficient to sustain autonomous replication of plasmid DNA in *C. hyointestinalis*. This implies that the original *C. hyointestinalis* shuttle vector, pCHI2, should contain an uninterrupted form of the cryptic plasmid replicon as it has the cryptic plasmid cloned *via* the *Hin*dIII site at nt 1. If this is true then the inability to conjugate pCHI2 into *C. hyointestinalis* would suggest that *C. hyointestinalis* possesses a barrier to conjugation, which is most probably a restriction system. It should then be possible to obtain a restriction mutant of *C. hyointestinalis* by mutagenesis. It should then be further possible to conjugate pCHI2 into this strain, and stable replication should also be observed. Work along these lines is described in Chapter 6.

CHAPTER 6

BARRIERS TO TRANSFORMATION AND CONJUGATION IN THE GENUS *CAMPYLOBACTER*

6.1 Introduction

DNA restriction systems have been demonstrated in a wide variety of taxonomically unrelated bacteria (Roberts, 1985) and have been shown to prevent the acquisition of plasmid DNA during conjugation in *E. coli* K-12 (Arber and Morse, 1965), *Legionella pneumophila* (Marra and Shuman, 1989), and *Neisseria gonorrhoeae* (Butler and Gotschlich, 1991).

The ability of *L. pneumophila* to act as a recipient of IncQ and IncP plasmids in matings with *E. coli* K-12 has been shown to vary from strain to strain (Marra and Shuman, 1989). These workers found that the low-efficiency mating of the Philadelphia-1 strain was due to a Type II restriction-modification system. They isolated a Philadelphia-1 mutant which had a high ability to act as a recipient in inter-species matings and found that it lacked the restriction activity (Marra and Shuman, 1989). Similarly, Guiney (1984), utilized *E. coli* K-12 recipients, both with and without the *Eco*RI restriction system, to demonstrate that the frequency of mobilization of RK2 derivatives (containing from one to four artificially inserted *Eco*RI sites) into the recipient containing R.*Eco*RI was reduced from one to five orders of magnitude respectively, compared with the mating frequencies to a recipient devoid of R.*Eco*RI. Butler and Gotschlich (1991) reported that RSF1010 could be mobilized into *N. gonorrhoeae* F62-RN at a very low frequency, but that this frequency was increased by greater than

four orders of magnitude when the plasmid was methylated *in vivo* by the methylase *Sss*I (M.*Sss*I), a ^{ME}CG methylase from *Spiroplasma* spp. They therefore demonstrated that protection of RSF1010 from gonococcal restriction systems *in vitro* correlated with an increase in mobilization frequency *in vivo*.

The implication of these investigations is that during mobilization, a single-stranded molecule enters the host cell and is not methylated. After synthesis of the complementary strand, the double-stranded molecule exists transiently as an unmethylated duplex subject to restriction. If the modification enzyme acts prior to restriction, the plasmid is protected and survives in the new host, assuming it can replicate (Guiney, 1984).

Restriction-modification systems have been identified in *Campylobacter* species (Miller *et al.*, 1988). *C. jejuni* has been shown to possess a strong restriction system capable of significantly decreasing the efficiency of transformation of unmodified plasmid DNA (Miller *et al.*, 1988).

In this chapter a restriction mutant of *C. hyointestinalis* strain NCTC 11608 is isolated and its ability to act as a recipient in matings with *E. coli* K-12 donors harbouring various *C. hyointestinalis* shuttle vectors is examined. The ability of the *C. hyointestinalis* shuttle vectors to be mobilized into, and replicate in *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, and a spontaneous restriction mutant of *C. coli* strain NCTC 11366 is also examined. An analysis of the ability of the type strains of *C. jejuni*, *C. coli*, *C. fetus* subsp. *fetus*, and *C. hyointestinalis* to be transformed by plasmid DNA modified by other *Campylobacter* species is performed. A study of the ability of a *C. hyointestinalis* shuttle vector to be mobilized between *Campylobacter* species is also investigated.

6.2 Results

6.2.1 Construction of candidate shuttle vector pCHI12

The DNA sequence of the cryptic plasmid, obtained in Section 5.2.1.1, revealed that the replication region from the cryptic plasmid could be cloned in an intact form *via* its *Not*I

site. The DNA sequence obtained from pCHI5 suggested that the "nick region" present in the putative *oriT* of the cryptic plasmid could be mistaken during conjugation for the "nick region" of the *oriT* of RP4 present in *E. coli* K-12 strain S17-1 following the initial cleavage and transfer of plasmid DNA. The possibility therefore existed that the conjugal transfer of the shuttle vector pCHI2, which had the "nick region" of the cryptic plasmid in the same position as pCHI4, could be accompanied by deletion of the vector portion downstream from the "nick region", as had apparently occurred during transfer of pCHI4, to form pCHI5. If any genetic material was cloned downstream from this "nick region" in pCHI2 the possibility thus existed that it could be deleted upon transfer. A new vector was therefore constructed in which the "nick region" of the putative *oriT* of the cryptic plasmid was placed closer to the "nick region" of the *oriT* of RP4. pCHI1 was digested with *Pst*I and *Aat*II and the 53 bp *Pst*I-*Aat*II fragment from the polylinker of the cloning vector pGEM5zf(+) (which contains a *Not*I restriction site) was cloned into pCHI1, generating the plasmid pCHI11 (Figure 6.1). The cryptic plasmid from *C. hyointestinalis* strain 45104 was digested with *Not*I and ligated into the *Not*I site of pCHI11; the resulting plasmid was designated pCHI12.

6.2.2 mobilization of pCHI12 into *C. hyointestinalis*

pCHI12 was transformed into the *E. coli* K-12 donor strain S17-1 and the strain thus constructed was mated with *C. hyointestinalis* NCTC 11608. Attempts to mobilize pCHI12 into *C. hyointestinalis* were unsuccessful although it was readily transferable to *S. typhimurium* (Table 6.1). This result reflected what had been observed with pCHI2, that a candidate shuttle vector known to harbour an intact copy of the replicon of the cryptic plasmid could not be mobilized into *C. hyointestinalis*. The fact that two independent candidate shuttle vectors, each with an intact replication region, could not be mobilized into *C. hyointestinalis* gave further support to the theory that *C. hyointestinalis* contained a restriction system which acted as a barrier to the introduction of foreign DNA *via* conjugation.

FIGURE 6.1 Construction of plasmid pCHI12.

The *Aat*II-*Pst*I DNA restriction fragment containing a *Not*I site was cloned from pGEM5zf(+) into pCHI1 digested with *Aat*II and *Pst*I, yielding pCHI11. The cryptic plasmid from *C. hyointestinalis* 45104 was digested with *Not*I and ligated into the *Not*I site of pCHI11, generating pCHI12.

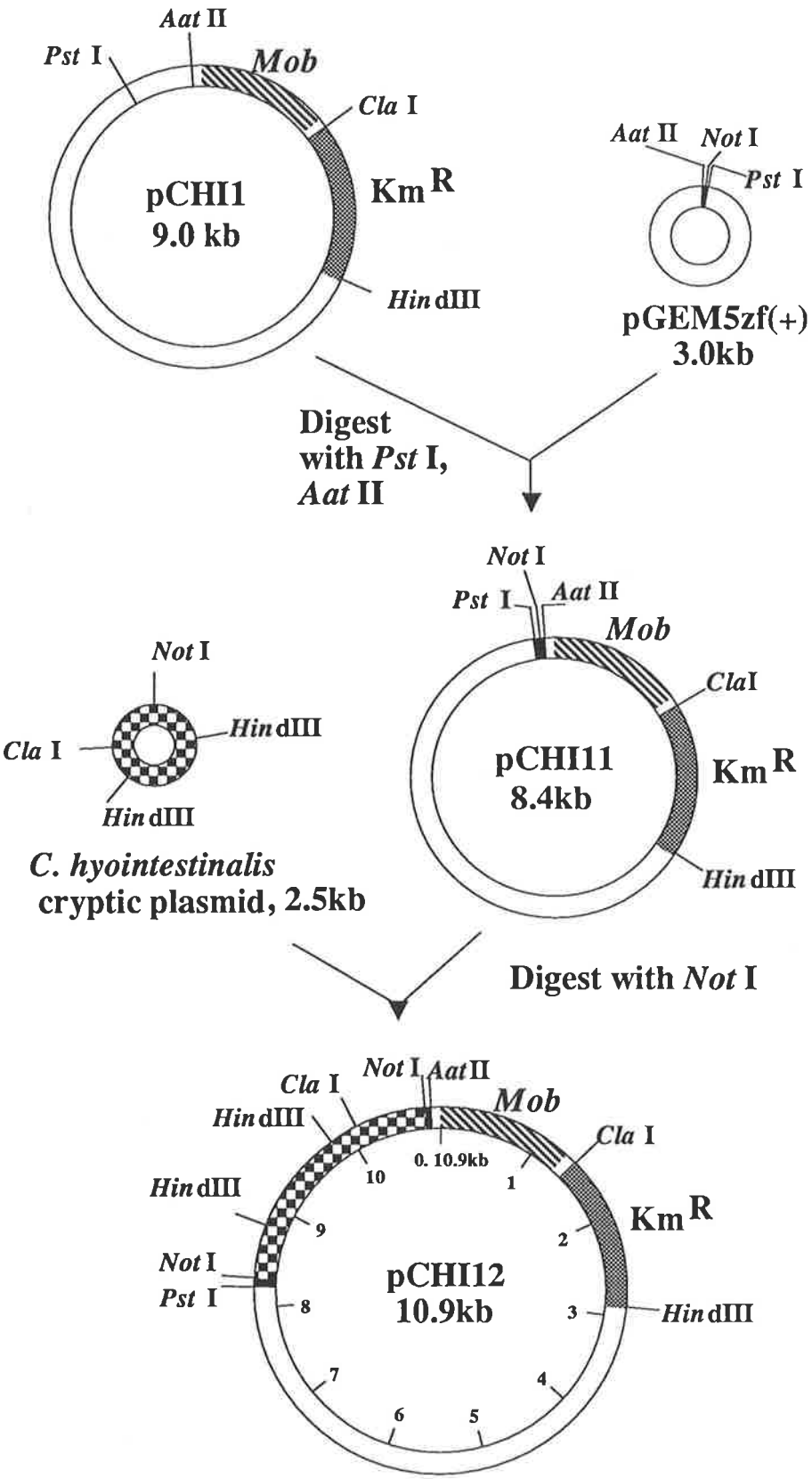

Aat II
Pst I
Mob
Cla I
pCHI1
9.0 kb
Km R
Hin dIII
Aat II
Not I
Pst I
pGEM5zf(+)
3.0kb
Digest
with Pst I,
Aat II
Not I
Pst I
Aat II
Mob
ClaI
pCHI11
8.4kb
Km R
Hin dIII
Not I
Cla I
Hin dIII
Hin dIII
C. hyointestinalis
cryptic plasmid, 2.5kb
Digest with Not I
Cla I
Not I
Aat II
Mob
Hin dIII
Cla I
0. 10.9kb
10
1
Hin dIII
9
2
Km R
Not I
Pst I
pCHI12
10.9kb
8
3
Hin dIII
7
4
6
5

TABLE 6.1 mobilization of pCHI12 and pCHI15 from *E. coli* K-12 S17-1 into *C. hyointestinalis* NCTC 11608

Plasmid	No. of transconjugants per donor into recipient species:	
	S. typhimurium LT2 SL2981	*C. hyointestinalis* NCTC 11608
pCHI12	10^{-4}	$<10^{-9}$*
pCHI15	10^{-4}	$<10^{-9}$*

* Repeated attempts to obtain transconjugants with *C. hyointestinalis* NCTC11608 in mating tests using, as donors, *E. coli* K-12 S17-1 harbouring pCHI12 or pCHI15 were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

6.2.3 Construction of pCHI15

The DNA sequence obtained from Chapter 5 revealed that there was a 91 bp deletion of the promoter region of the *aphA-3* gene present in pCHI5, and that this deletion was present in the original vector construct pCHI1 and therefore in all the other candidate *C. hyointestinalis* shuttle vectors constructed to this point (Figure 6.2). This deletion did not appear to have any deleterious effect on the expression of the *aphA-3* gene in *C. hyointestinalis* as pCHI5 provided a high level of resistance against kanamycin. However, it was felt that a candidate *C. hyointestinalis* shuttle vector which contained the complete 1.427 kb fragment harbouring the *aphA-3* gene from pILL550 should be made, so as to eliminate any possibility that this deletion could be responsible for the inability of *C. hyointestinalis* candidate shuttle vectors to be mobilized into *C. hyointestinalis*. To do this, a vector was constructed in a manner similar to that used for the generation of pCHI12. pPM2101 was digested with *Cla*I and *Hin*dIII and the 1.427 kb *Cla*I-*Hin*dIII DNA restriction fragment from pILL550 containing the *aphA-3* gene was cloned into pPM2101 (Figure 6.3). The resulting clones were screened for plasmid content and plasmids were isolated and digested with *Cla*I and *Hin*dIII. The difference between a *Cla*I-*Hin*dIII DNA restriction fragment harbouring the 91 bp deletion and the 1.427 kb *Cla*I-*Hin*dIII DNA restriction fragment from pILL550 is clearly visible on the gel (Figure 6.2). A plasmid which contained the 1.427 kb *Cla*I-*Hin*dIII DNA restriction fragment of pILL550 was isolated and designated pCHI13. The 53 bp *Pst*I-*Aat*II fragment from pGEM5zf(+) was cloned into pCHI13 as described earlier, to generate a plasmid named pCHI14. The cryptic plasmid was then ligated into the *Not*I site present on pCHI14 to generate the shuttle vector pCHI15. A *Cla*I-*Hin*dIII digest of pCHI15 revealing the presence of the 1.427 kb fragment is shown (Figure 6.2).

6.2.4 mobilization of pCHI15 into *C. hyointestinalis*

pCHI15 was transferred into the *E. coli* K-12 donor strain as described earlier and the resulting strain mated with *C. hyointestinalis* NCTC 11608. Attempts to mobilize pCHI15 into *C. hyointestinalis* were unsuccessful in tests where it was readily

FIGURE 6.2 Restriction endonunclease digestion of shuttle vector constructs with *Cla*I and *Hin*dIII.

Plasmid DNA was digested with *Cla*I and *Hin*dIII and examined by agarose gel electrophoresis. Bacteriophage SPP1 DNA digested with *Eco*R1 was used as a standard. The fragment sizes (kb) were 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, and 0.36. The 1.427 kb *Cla*I-*Hin*dIII DNA restriction fragment harbouring the *aphA-3* gene is indicated by arrow number 1, and the corresponding fragment harbouring the 91 bp deletion is indicated by arrow number 2. The DNA analysed in each track are as follows:

(A) SPP1

(B) pILL550

(C) pCHI1

(D) pCHI2

(E) pCHI4

(F) pCHI12

(G) pCHI15

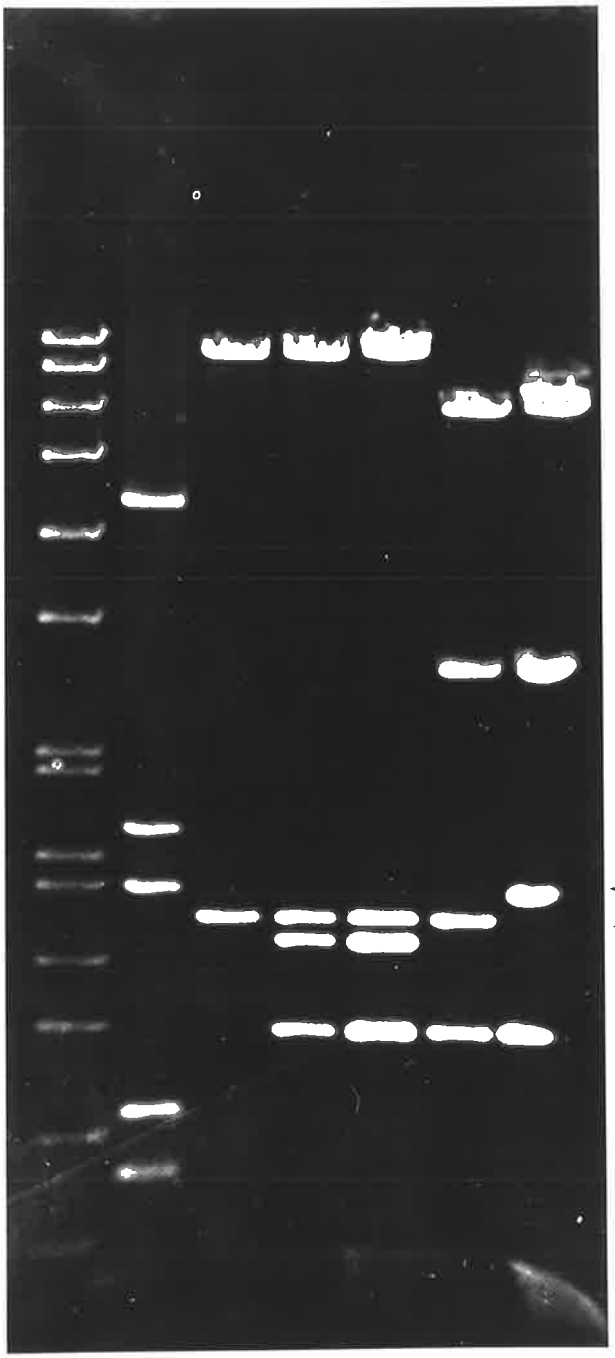
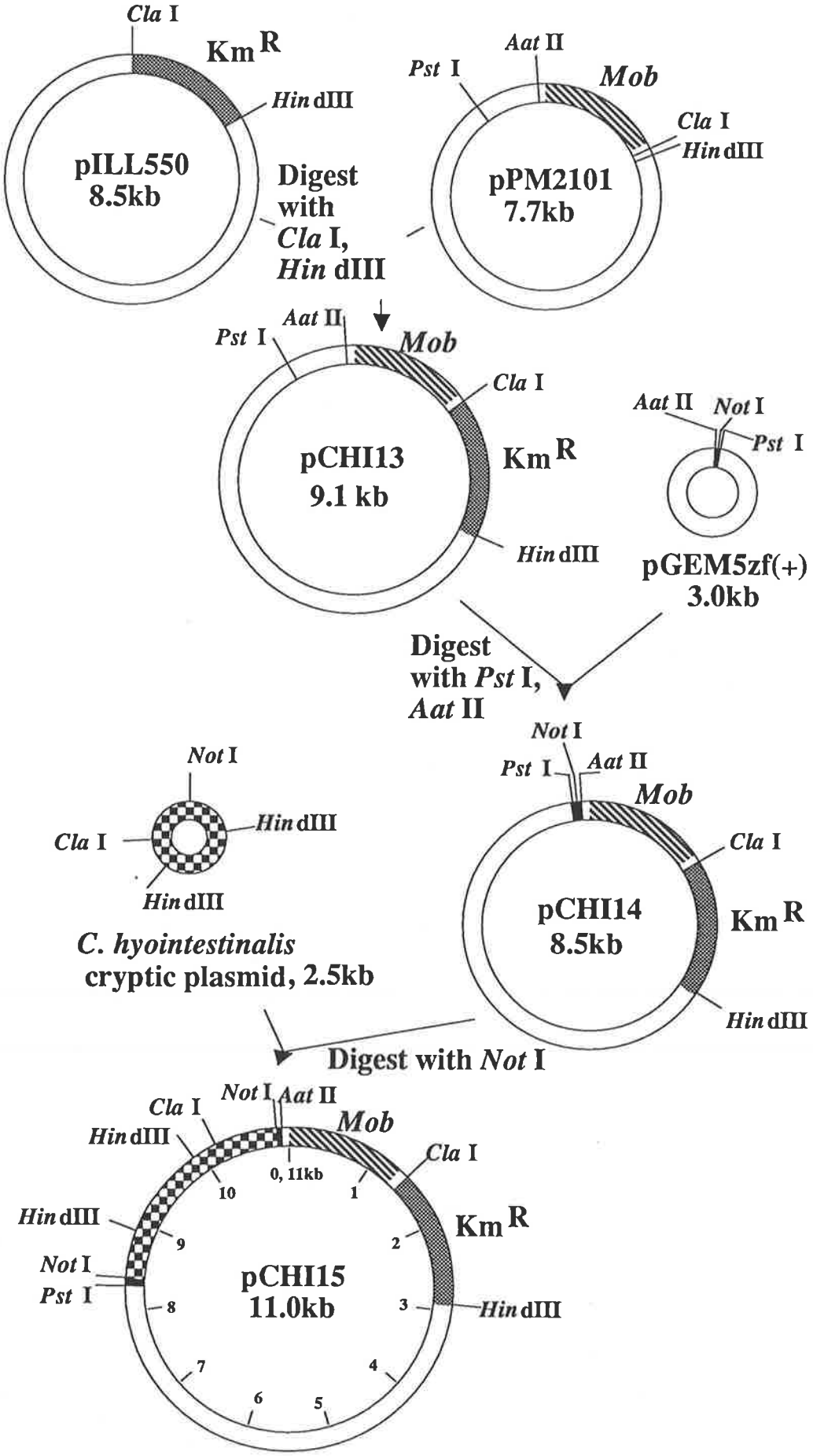

1
2
A B C D E F G

FIGURE 6.3 Construction of plasmid pCHI15.

The *Cla*I-*Hin*dIII DNA restriction fragment containing the *aphA-3* gene (Km^R) was cloned from pILL550 into pPM2101 digested with *Cla*I and *Hin*dIII. The resulting clones were screened until one was isolated which contained the entire 1.427 kb *Cla*I-*Hin*dIII fragment from pILL550 and was named pCHI13. The *Aat*II-*Pst*I DNA restriction fragment containing a *Not*I site was cloned from pGEM5zf(+) into pCHI13 digested with *Aat*II and *Pst*I, yielding pCHI14. The cryptic plasmid from *C. hyointestinalis* 45104 was digested with *Not*I and ligated into the *Not*I site of pCHI14, generating pCHI15.


Cla I
Km R
Hin dIII
pILL550
8.5kb
Digest
with
Cla I,
Hin dIII
Aat II
Pst I
Mob
Cla I
Hin dIII
pPM2101
7.7kb
Aat II
Pst I
Mob
Cla I
pCHI13
9.1 kb
Km R
Hin dIII
Aat II
Not I
Pst I
pGEM5zf(+)
3.0kb
Digest
with Pst I,
Aat II
Not I
Cla I
Hin dIII
Hin dIII
C. hyointestinalis
cryptic plasmid, 2.5kb
Not I
Pst I
Aat II
Mob
Cla I
pCHI14
8.5kb
Km R
Hin dIII
Digest with Not I
Not I
Aat II
Cla I
Hin dIII
Mob
0, 11kb
Cla I
Hin dIII
Not I
Pst I
pCHI15
11.0kb
Km R
Hin dIII
0
1
2
3
4
5
6
7
8
9
10

transferable to *S. typhimurium* (Table 6.1). This result confirmed that the 91 bp deletion present in the cloned DNA fragment, harbouring the *aphA-3* gene, of the earlier candidate shuttle vectors was not responsible for the inability of these shuttle vectors to be mobilized into *C. hyointestinalis*.

6.2.5 mobilization of *C. hyointestinalis* shuttle vectors into both subspecies of *C. fetus*

C. fetus subsp. *fetus* and *C. fetus* subsp. *venerealis* are the two campylobacters most closely related to *C. hyointestinalis* (Gebhart *et al.*, 1985; Thompson *et al.*, 1988; Vandamme *et al.*, 1991). Attempts to mobilize various *C. hyointestinalis* candidate shuttle vector constructs into these two subspecies produced some surprising results. Transconjugants were obtained with a frequency of 10^{-4} transconjugants per donor, with recipients *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, using plasmids pCHI2, pCHI12, pCHI15, and pILL550 (Table 6.2). Attempts to mobilize pCHI1 and pCHI4 into *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* were unsuccessful where the plasmids could be transferred into *S. typhimurium* with a high efficiency (Table 6.2). *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* transconjugants were screened for plasmid content and contained structurally intact pCHI2, pCHI12, pCHI15, and pILL550 as expected.

These results indicated that the candidate *C. hyointestinalis* shuttle vectors pCHI2, pCHI12, and pCHI15 are all capable of being efficiently mobilized, and replicated stably, in *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. The inability of pCHI1 to be mobilized and to replicate in these two *C. fetus* subspecies implies that the replication of these candidate *C. hyointestinalis* shuttle vectors in these two *C. fetus* subspecies is dependent upon the presence of the *C. hyointestinalis* replication region from the cryptic plasmid. This implies that the cryptic plasmid of *C. hyointestinalis* can replicate in *C. fetus*. The observation that pCHI2 and pCHI12 are efficiently mobilized into these two *C. fetus* subspecies again confirmed that the 91 bp deletion present in the promoter region of the *aphA-3* gene present on these plasmids did not affect the ability of the plasmids to

TABLE 6.2 Conjugal transfer of candidate shuttle vectors from *E. coli* K-12 S17-1 into *C. fetus* subsp. *fetus* NCTC 10842 and *C. fetus* subsp. *venerealis* NCTC 10342

Plasmid	No. of transconjugants per donor into the recipient species:		
	S. typhimurium LT2 SL2981	*C. fetus* subsp.*fetus* NCTC 10842	*C. fetus* subsp.*venerealis* NCTC 10342
pCHI1	10^{-4}	$<10^{-9}$*	$<10^{-9}$*
pCHI2	10^{-4}	10^{-4}	10^{-4}
pCHI4	10^{-4}	$<10^{-9}$*	$<10^{-9}$*
pCHI12	10^{-4}	10^{-4}	10^{-4}
pCHI15	10^{-4}	10^{-4}	10^{-4}
pILL550	10^{-4}	10^{-4}	10^{-4}

* Repeated attempts to obtain transconjugants with*C. fetus* subsp. *fetus* NCTC 10842 and *C. fetus* subsp. *venerealis* NCTC 10342 in mating tests using, as donor, *E. coli* K-12 S17-1 harbouring the various plasmids were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

replicate, or the provision of resistance against kanamycin. This deletion would therefore not account for the lack of success in mobilization of these plasmids into *C. hyointestinalis*. The fact that pCHI4 could not be mobilized into either *C. fetus* subspecies was not unexpected as sequence data (Section 5.2.1.1) showed that this plasmid contained two copies of the cryptic plasmid replicon and therefore two copies of the four 19 bp direct repeats (i.e.: eight repeats in all) which have been shown to be associated with the regulation of plasmid replication in other replicons (Novick, 1987). Therefore, it is possible that both origins can be folded back to each other as a result of the protein-protein interactions that can occur between the RepA proteins bound to each set of iterons. This folded structure would most likely prohibit replication from the origins by steric hindrance (Nordström, 1990). The steric hindrance model of replication is supported by the demonstration that replication of plasmid RK2 is inhibited *in vitro* as well as *in vivo* by the addition of iteron-containing DNA; this inhibition is not relieved by even drastic increases in the concentration of the Rep protein TrfA (Kittel and Helinski, in press, cited by Nordström, 1990). Similar results have been reported for plasmid R6K (McEachern *et al.*, 1989). The initiation of replication of pCHI4 might therefore proceed with difficulty due to the folding of the replicon around the origin regions. The efficient mobilization of pILL550 into both *C. fetus* subspecies was in agreement with a previous report (Labigne-Roussel *et al.*, 1987).

The ability to mobilize candidate shuttle vectors harbouring the *C. hyointestinalis* cryptic plasmid into the two subspecies of *C. fetus*, but not into *C. hyointestinalis*, suggests again that *C. hyointestinalis* contains a barrier to the introduction of foreign DNA *via* conjugation; this barrier is less severe in either of the *C. fetus* subspecies. This further suggests that *C. hyointestinalis* might possess a restriction system which acts as a barrier to conjugation; it should be possible to obtain a restriction mutant of *C. hyointestinalis*.

6.2.6 Heat attenuation of the *C. hyointestinalis* restriction system

It has been observed with strains of *Streptomyces clavuligerus* (Bailey and Winstanley, 1986) and *Streptomyces tendae* (Engel, 1987) that plasmid transformation is impeded by restriction systems, and that these restriction systems are heat-sensitive and could be partially inactivated by heat treatment so that incoming DNA is initially a substrate for modification. To determine whether *C. hyointestinalis* NCTC 11608 contained a restriction system which may be temporarily inactivated in this way, attempts were made to mobilize pCHI15 from the *E. coli* K-12 donor strain S17-1 into *C. hyointestinalis* NCTC 11608 cells which had been incubated at 50°C for 10, 20, and 30 minutes (Section 2.17). This treatment did not severely impair the viability of the cells (Table 6.3). No transconjugants were obtained in these experiments (Table 6.3). This showed that heat treatment of *C. hyointestinalis* cells was not sufficient to enable the mobilization of pCHI15 into *C. hyointestinalis*.

6.2.7 mobilization of pCHI15 into *C. hyointestinalis* cells mutagenized with MNNG

The first attempt to isolate a restriction mutant of *C. hyointestinalis* NCTC 11608 was made using a modification of the method described by Morooka *et al.* (1985), where *C. hyointestinalis* cells were harvested and incubated for one hour with different concentrations of MNNG ranging from 5 to 100 μg/ml. The percentage of cells surviving this treatment ranged from 30 to 90% depending on the concentration of MNNG used. These cells were harvested and outgrown for 48 hours and then used as recipients in mating experiments with *E. coli* K-12 donor cells harbouring pCHI15. Despite repeated attempts, no kanamycin-resistant transconjugants of *C. hyointestinalis* were obtained after treatment of *C. hyointestinalis* cells with MNNG at any concentration (Table 6.4).

A further attempt was made to obtain a restriction mutant of *C. hyointestinalis* NCTC 11608 using a modification of the method described by Eisenstark (1965). A suspension of *C. hyointestinalis* cells in mid-exponential phase was spread onto three blood agar plates and two separate drops of MNNG (50 μg/ml) were placed on the

TABLE 6.3 mobilization of pCHI15 into *C. hyointestinalis* NCTC 11608 cells incubated at 50°C prior to mating

Time of incubation at 50°C (min)*	No. transconjugants per donor
10	$<10^{-9}$**
20	$<10^{-9}$**
30	$<10^{-9}$**

* Cell viability after heat treatment ranged from 85-95%.

** Repeated attempts to obtain transconjugants with *C. hyointestinalis* NCTC 11608 in mating tests using, as donor, *E. coli* K-12 S17-1 harbouring pCHI15 were unsuccessful, even when the recipient cells had been incubated at 50°C for up to 30 min prior to mating. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

TABLE 6.4 mobilization of pCHI15 into *C. hyointestinalis* NCTC 11608 mutagenised with MNNG

Concentration of MNNG used to mutagenise *C. hyointestinalis* NCTC 11608 (μg/ml)*	No. transconjugants per donor
5	$<10^{-9}$**
10	$<10^{-9}$**
25	$<10^{-9}$**
50	$<10^{-9}$**
100	$<10^{-9}$**
50#	10^{-8}##

* The cell survival rates, with increasing MNNG concentrations, were 90, 80, 65, 45, and 30%, respectively.

** Repeated attempts to obtain transconjugants with *C. hyointestinalis* NCTC 11608 in mating tests using, as donor, *E. coli* K-12 S17-1 harbouring pCHI15 were unsuccessful, even when the recipient cells had been incubated with varying concentrations of MNNG prior to mating. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

\# Two drops of MNNG, at 50 μg/ml, were placed on the surfaces of three agar plates previously spread with *C. hyointestinalis* NCTC 11608, cells surrounding the zones of cell death were harvested, and mating into these cells (from *E. coli* K-12 S17-1 harboring pCHI15) performed by a modification of the method described by Eisenstark (1965).

10 transconjugants of *C. hyointestinalis* NCTC 11608 were isolated.

surface of each plate. The plates were then incubated for 48 hours and the zones of growth surrounding the areas of killing were harvested and used as recipients in a mating mixture with the *E. coli* K-12 donor strain harbouring pCHI15. Ten kanamycin-resistant transconjugant colonies of *C. hyointestinalis* were obtained (Table 6.4). Analysis of contained plasmid DNA revealed that pCHI15 had been transferred and remained intact in the *C. hyointestinalis* recipients. It therefore appears that these transconjugants had obtained pCHI15 *via* conjugation from the *E. coli* K-12 donor, and that they are most probably mutants in the restriction system which has been suggested earlier as responsible for the prevention of entry of foreign DNA into the wild-type *C. hyointestinalis* cell.

6.2.8 mobilization into *C. hyointestinalis* W186

To determine whether these isolates were in fact restriction mutants and could therefore act as efficient recipients in bacterial conjugation, one of the transconjugants was repeatedly subcultured without kanamycin selection until a cured derivative, named W186, was obtained. Attempts to mobilize pCHI1, pCHI4, pILL550, and pKT231 containing the *aphA-3* gene from pILL550, into W186 were unsuccessful (Table 6.5). However, *C. hyointestinalis* transconjugants were obtained at a frequency of 10^{-4} transconjugants per donor with plasmids pCHI2, pCHI12, and pCHI15. transconjugants obtained from the mating experiments with W186 involving these plasmids were screened for plasmid content and contained structurally intact pCHI2, pCHI12, and pCHI15 as expected.

These results indicate that W186 is an efficient recipient of *C. hyointestinalis* shuttle vectors harbouring an intact copy of the cryptic plasmid. The observation that pCHI1 could not be mobilized into W186 showed that the presence of the *C. hyointestinalis* from the cryptic plasmid is critical for plasmid replication in *C. hyointestinalis*. As was observed earlier with the two subspecies of *C. fetus*, pCHI4 could not be mobilized into W186. Again, this is most probably due to the fact that this construct contains two copies of the cryptic plasmid and therefore cannot

TABLE 6.5 mobilization of candidate shuttle vectors into *C. hyointestinalis* W186

Plasmid	No. of transconjugants per donor into the recipient species:	
	S. typhimurium LT2 SL2981	*C. hyointestinalis* W 186
pCHI1	10^{-4}	$<10^{-9}$*
pCHI2	10^{-4}	10^{-4}
pCHI4	10^{-4}	$<10^{-9}$*
pCHI12	10^{-4}	10^{-4}
pCHI15	10^{-4}	10^{-4}
pILL550	10^{-4}	$<10^{-9}$*
pKT231+*aphA-3*	10^{-4}	$<10^{-9}$*

* Repeated attempts to obtain transconjugants with *C. hyointestinalis* W186 in mating tests using, as donor, *E. coli* K-12 S17-1 harbouring the various plasmids were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these experiments.

replicate efficiently in *C. hyointestinalis* or *C. fetus* due to steric hindrance resulting from folding of the replicon around the origins. The 91 bp deletion present in the promoter region of the *aphA-3* gene contained in pCHI2 and pCHI12 does not appear to have any deleterious affect on the expression of the gene as W186 derivatives with these plasmids expressed high resistance to kanamycin. The *C. coli* shuttle vector, pILL550, cannot be mobilized into W186, indicating that the replication region from the *C. coli* cryptic plasmid pIP1445, incorporated into pILL550, cannot be maintained in a *C. hyointestinalis* host. The broad-host-range plasmid pKT231 harbouring the kanamycin-resistance determinant from pILL550 also could not be mobilized into W186, suggesting that the replicon is non-functional in a *C. hyointestinalis* host. These results suggest that a *C. hyointestinalis*-specific replicon is essential for plasmid replication in *C. hyointestinalis*.

6.2.9 Electroporation into *C. hyointestinalis* W186

To determine whether W186 is in fact a mutant in a restriction system, electroporation experiments into W186, using pCHI15 DNA isolated from *E. coli* K-12 DH1, were performed. W186 was transformed by *E. coli* K-12-modified pCHI15 at a frequency of 5 x 10^3 transformants per μg of DNA, whereas no transformants of the wild-type parent *C. hyointestinalis* NCTC 11608 could be isolated with the same plasmid DNA (Table 6.6). Transformant colonies were screened for plasmid content and found to contain structurally intact pCHI15. This result suggests that W186 is a restriction mutant. Attempts to electroporate pCHI15 DNA extracted from the W186 transformants into the wild-type parent *C. hyointestinalis* NCTC 11608 did not, however, produce any transformants (Table 6.6). This implied that W186, while a restriction mutant, was also defective in the modification gene(s) necessary to modify pCHI15 so that it would not be digested by the *C. hyointestinalis* restriction system when re-introduced into a wild-type *C. hyointestinalis* strain.

TABLE 6.6 Electroporation of pCHI15 into *C. hyointestinalis* W186 and *C. hyointestinalis* NCTC 11608

Plasmid	No. of transformants/μg DNA into the recipient species:	
	C. hyointestinalis NCTC 11608	*C. hyointestinalis* W186
pCHI15*	0	5×10^3
pCHI15**	0	5×10^3
pCHI5#	5×10^3	5×10^3

* Plasmid DNA was isolated from *E. coli* K-12 DH1.

** Plasmid DNA was isolated from *C. hyointestinalis* W186.

\# Plasmid DNA was isolated from *C. hyointestinalis* W64.

6.2.10 Examination of *C. hyointestinalis* W186 for other mutations

To compare the growth rates of *C. hyointestinalis* W186 and *C. hyointestinalis* NCTC 11608, both strains were plate grown to mid-exponential phase and the cells harvested and resuspended to a concentration of 5 x 10^9 cells/ml. 5 x 10^8 cells from each strain were cultured *in vitro* for 16 h under microaerophilic conditions (at which time the cells would be in the exponential phase of growth) and the cells harvested (1 ml saline) and enumerated by serial dilution. The number of cells/ml obtained for *C. hyointestinalis* W186 did not differ greatly from that observed for *C. hyointestinalis* NCTC 11608; each plate yielded approximately 3 x 10^9 cells/ml saline. This result implies that *C. hyointestinalis* W186 does not appear to be mutated for any genes required for optimal growth. An examination of the whole-cell protein profile of *C. hyointestinalis* W186 (by SDS-PAGE) was performed (Figure 6.4). *C. hyointestinalis* W186 did not appear to lack any of the major protein bands which were present in its parent: *C. hyointestinalis* NCTC 11608. This result implied that the mutagenesis procedure had not given rise to gross alterations in the genome of the strain encoding for these major proteins.

6.2.11 Electroporation of *Campylobacter*-modified plasmid DNA into *Campylobacter* species

To determine what restriction barriers existed between some of the type strains of *C. coli*, *C. jejuni*, *C. fetus* subsp. *fetus*, and *C. hyointestinalis*, electroporation experiments were performed, in which each of these species received plasmid DNA isolated from a variety of other *Campylobacter* species. Electroporation into *C. fetus* subsp. *venerealis* was not performed here because it was felt that the results obtained would be similar to those observed for *C. fetus* subsp. *fetus*, as the two subspecies are so closely related. The plasmids used were pILL550 (isolated from *C. coli*, *C. fetus* subsp. *fetus*, and *E. coli* K-12 DH1), pCHI15 (isolated from *C. fetus* subsp. *fetus*), and pCHI5 (isolated from *C. hyointestinalis* and *C. fetus* subsp. *fetus*). The results generally show that each of the *Campylobacter* species examined could be electroporated best by plasmid DNA which has been previously modified in the same species (Table 6.7).

FIGURE 6.4 Examination of whole cell proteins from *C. hyointestinalis* W186.

Whole cell samples from *C. hyointestinalis* W186 and *C. hyointestinalis* NCTC 11608 were suspended in 1 x sample buffer (Lugtenberg *et al.*, 1975) and heated at 100°C for 3 min prior to analysis by SDS-PAGE. Protein size markers (Pharmacia) were used as standards and the protein sizes (kDa) are shown on the right. The strains examined in each track are as follows:

(A) Protein size markers

(B) *C. hyointestinalis* NCTC 11608

(C) *C. hyointestinalis* W186

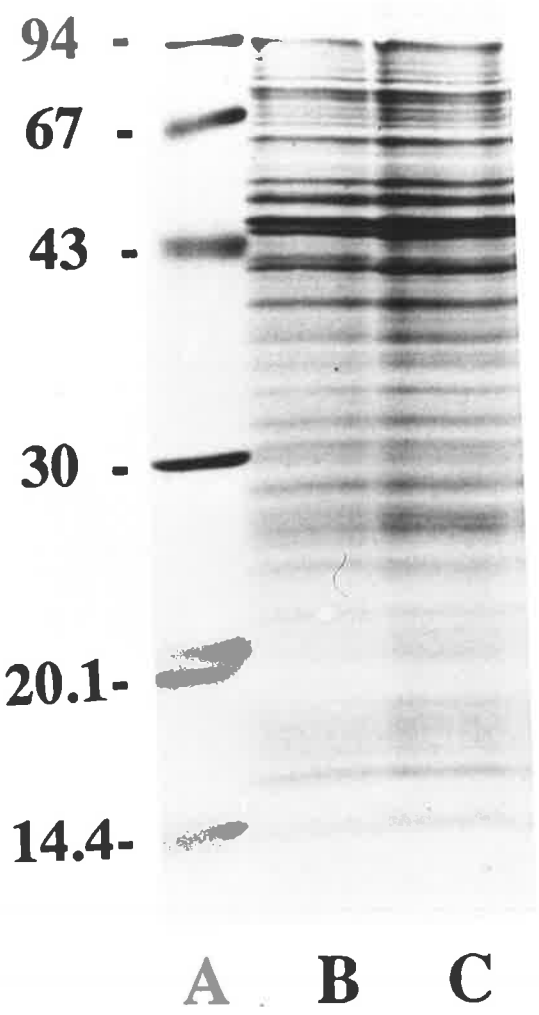

94 -
67 -
43 -
30 -
20.1-
14.4-
A
B
C

TABLE 6.7 Electroporation of *Campylobacter*-modified plasmid DNA into various strains of *Campylobacter*

Donor DNA	No. of transformants/μg DNA into the recipient species:			
	C. coli NCTC 11366	*C. hyointestinalis* NCTC 11608	*C. fetus* subsp. *fetus* NCTC 10842	*C. jejuni* NCTC 11168
pILL550*	5×10^3	0	0	0
pILL550**	50##	0	10^3	0
pCHI5***	50##	5×10^3	5×10^2	0
pCHI5**	50##	100	10^3	0
pCHI15**	50##	0	10^3	0
pILL550#	50##	0	0	0

* Plasmid was isolated from *C. coli* NCTC 11366

** Plasmid was isolated from *C. fetus* subsp. *fetus* NCTC 10842

*** Plasmid was isolated from *C. hyointestinalis* W64

\# Plasmid was isolated from *E. coli* K-12 DH1

Transformant numbers ranged from 20-70.

It was noted that:

(i) *C. hyointestinalis* and *C. fetus* subsp. *fetus* could both be electroporated by pCHI5 DNA which was modified in the other strain, but the efficiency of electroporation was generally about an order of magnitude lower than that observed when the plasmids were electroporated into the species from which they had been prepared (Table 6.7). Despite repeated attempts, no transformants could be obtained when *C. hyointestinalis* was electroporated with pCHI15 isolated from *C. fetus*.

(ii) A small number of *C. coli* NCTC 11366 transformants could be isolated after electroporation with all plasmids tested, regardless of the source of plasmid DNA (even when the DNA was obtained from *E. coli* K-12). This suggests that spontaneous restriction mutants of this *C. coli* strain appear readily at a low frequency.

The inability of *C. jejuni* NCTC 11168 to be transformed by pILL550 DNA which had been modified in *C. coli* NCTC 11366 was surprising as it has been previously reported that a strain of *C. coli* could be efficiently transformed by plasmid DNA which had been modified by *C. jejuni* (Miller *et al.*, 1988). It was therefore expected that transfer in the opposite direction would also be possible. However, different *C. jejuni* and *C. coli* strains were used in this previous report and the results that were obtained may be strain-specific.

6.2.12 Electroporation of pCHI15 into a restriction mutant of *C. coli*

The observation that *C. coli* NCTC 11366 could be transformed by pILL550 DNA isolated from *E. coli* K-12 DH1 was pursued to determine if these transformants were spontaneous *C. coli* restriction mutants. One of the *C. coli* transformants was repeatedly subcultured without kanamycin selection until a cured derivative, named W188, was obtained. When W188 was electroporated with pILL550 isolated from *E. coli* K-12 DH1 approximately 5 x 10^3 *C. coli* transformants were obtained per μg of DNA (Table 6.8). In an attempt to electroporate W188 with pCHI15 DNA isolated from *E. coli* K-12 DH1, approximately 10^3 *C. coli* transformants were obtained per μg of DNA (Table 6.8). Some transformants were screened for plasmid content and found to contain structurally intact

TABLE 6.8 Electroporation of plasmid DNA from *E. coli* K-12 DH1 into *C. coli* W188

Donor DNA	No. of transformants/μg DNA
pILL550	5×10^3
pCHI15	10^3
pCHI1	0

pCHI15. Attempts to electroporate pCHI1 DNA isolated from *E. coli* K-12 DH1 into W188 were unsuccessful (Table 6.8), indicating that the presence of the cryptic plasmid replicon contained in pCHI15 was critical for plasmid replication in *C. coli*. The pCHI15 DNA extracted from these transformants was used to electroporate the type strains of *C. coli* and *C. hyointestinalis*, and also W186 (Table 6.9). It was observed that this DNA could be electroporated into the wild-type parent *C. coli* NCTC 11366 at a frequency of 5×10^3 transformants per μg of DNA (Table 6.9). This implies that the pCHI15 DNA isolated from W188 is in fact modified for *C. coli* and therefore that W188 appears to be a restriction mutant that is still capable of producing the *C. coli* DNA modification enzyme(s). Attempts to electroporate this *C. coli*-modified pCHI15 into *C. hyointestinalis* NCTC 11608 were unsuccessful, but this plasmid DNA could be electroporated into the *C. hyointestinalis* restriction mutant, W186, at a frequency of 5×10^3 transformants per μg (Table 6.9). This implies that the wild-type *C. hyointestinalis* NCTC 11608 contains a restriction barrier which prevents *C. coli*-modified DNA from ready access to the cell. It should be noted that spontaneous restriction mutants of *C. hyointestinalis* NCTC 11608 could not be isolated, as was the case with *C. coli* NCTC 11366.

These results suggest that restriction mutants of the *C. coli* type strain appear spontaneously, at a low frequency, and that the electroporation method used is sufficiently sensitive to select these mutants from the wild-type cells. It was also shown that pCHI15 can replicate stably in a *C. coli* host, and that this replication is dependent upon the presence of the *C. hyointestinalis* cryptic plasmid. This further implies that the cryptic plasmid can replicate stably in *C. coli*. Also, the modification systems in both *C. hyointestinalis* NCTC 11608 and *C. coli* NCTC 11366 are host specific.

6.2.13 Mobilization of pCHI15 between *Campylobacter* species

To determine whether the putative *oriT* present on the cryptic plasmid (Section 5.2.1.6) is in fact functional (utilizable for conjugal transfer of DNA), attempts were

TABLE 6.9 Electroporation of *C. coli*-modified pCHI15 into *C. coli* NCTC 11366, *C. hyointestinalis* NCTC 11608, and *C. hyointestinalis* W186

Donor DNA	No. of transformants/μg DNA into the recipient species:		
	C. coli NCTC 11366	*C. hyointestinalis* NCTC 11608	*C. hyointestinalis* W186
pCHI15	5×10^3	0	5×10^3

made to mobilize pCHI15 between *Campylobacter* species. Attempts to mobilize pCHI15 from *C. fetus* subsp. *fetus* to *C. hyointestinalis* NCTC 11608, and the restriction mutant W186, were unsuccessful (Table 6.10). However, pCHI15 could be mobilized from *C. coli* NCTC 11366 into *C. fetus* subsp. *fetus* and W186 at a frequency of 3.0×10^{-4} and 2.5×10^{-3} transconjugants per donor, respectively (Table 6.10). No transconjugants were obtained when *C. coli* NCTC 11366 harbouring pCHI15 was mated with the wild-type *C. hyointestinalis* NCTC 11608. Therefore the restriction barrier present in *C. hyointestinalis* NCTC 11608, which inhibits transfer of *C. coli*-modified DNA by transformation (Section 6.2.12), also serves as a barrier to *C. coli*-modified DNA introduced *via* conjugation. As pCHI15 contains the *oriT* from RP4 as well as the putative *oriT* of the cryptic plasmid, the ability of pILL550 (which contains only the *oriT* from RK2) to be mobilized into *C. fetus* subsp. *fetus* was investigated. Attempts to mobilize pILL550 from *C. coli* to *C. fetus* subsp. *fetus* were unsuccessful (Table 6.10), implying that *C. coli* NCTC 11366 cannot mobilize plasmids *via* the *oriT* of RK2 but can mobilize plasmids which have the *oriT* from the *C. hyointestinalis* cryptic plasmid (It should be recalled that pILL550 is capable of replication in *C. fetus* subsp. *fetus*; Section 6.2.5). These results show that the putative *oriT* of the cryptic plasmid may indeed be functional in plasmid transfer, and that the cryptic plasmid itself may therefore be also mobilizable between *Campylobacter* cells. *C. coli* NCTC 11366 has been observed to harbour at least two small plasmids (of approximately 1.7 and 6.0 kb), and may possess a large plasmid of between 30 to 40 kb (unpublished data). The ability of *C. coli* NCTC 11366 to mobilize pCHI15 suggests that this strain harbours the genes (*tra*) encoding the necessary DNA transfer proteins, and that these genes may be located on one of these plasmids or on the chromosome.

6.3 Summary and conclusions

On the basis of the DNA sequence data of the cryptic plasmid obtained in Chapter 5, two shuttle vectors (pCHI12 and pCHI15) were constructed to contain an intact copy of the cryptic plasmid replicon (cloning of the entire cryptic plasmid *via* the

mobilization
TABLE 6.10 '. . of pCHI15, by conjugation, between *Campylobacter* strains

Donor	Plasmid	Recipient	No. of transconjugants per donor
C. fetus subsp. *fetus*	pCHI15	*C. hyointestinalis* NCTC 11608	$<10^{-9}$*
C. fetus subsp. *fetus*	pCHI15	*C. hyointestinalis* W186	$<10^{-9}$*
C. coli	pCHI15	*C. fetus* subsp. *fetus*	3×10^{-4}
C. coli	pCHI15	*C. hyointestinalis* NCTC 11608	$<10^{-9}$*
C. coli	pCHI15	*C. hyointestinalis* W186	2.5×10^{-3}
C. coli	pILL550	*C. fetus* subsp. *fetus*	$<10^{-9}$*

* Repeated attempts to obtain transconjugants from these mating experiments were unsuccessful. 10^{-9} transconjugants per donor cell was the lower limit of transconjugant detection in these tests.

single *Not*I site). pCHI15 was constructed to contain a complete copy of the *aphA-3* gene from pILL550. Neither of these two plasmids could be mobilized into *C. hyointestinalis*, which is in line with the results obtained with the *C. hyointestinalis* shuttle vector pCHI2. These data imply that *C. hyointestinalis* NCTC 11608 contains a restriction barrier against the introduction of foreign DNA *via* conjugation, and that the 91 bp deletion present in the *aphA-3* gene of the earlier *C. hyointestinalis* candidate shuttle vectors is not responsible for the absence of mobilizationof these plasmids into *C. hyointestinalis*.

It was observed that the *C. hyointestinalis* shuttle vectors pCHI2, pCHI12, and pCHI15 could be efficiently mobilized into both subspecies of *C. fetus*. However, pCHI1 and pCHI4 cannot replicate in either of the *C. fetus* subspecies. The replication of the shuttle vectors in *C. fetus* was thus found to be dependent upon the presence of the cryptic plasmid of *C. hyointestinalis*. The efficient mobilization and stable replication of these *C. hyointestinalis* shuttle vectors in *C. fetus* also suggests that *C. hyointestinalis* contains a barrier to the introduction of foreign DNA *via* conjugation which is not present in either of the *C. fetus* subspecies.

The restriction system of *C. hyointestinalis* appeared not to be attenuated by heat treatment, but a restriction mutant of *C. hyointestinalis*, named W186, was obtained by chemical mutagenesis with MNNG. W186 acts as a high-efficiency recipient in the mobilization of plasmids pCHI2, pCHI12, and pCHI15, but no transconjugants were obtained with pCHI1, pCHI4, pILL550, or pKT231 containing the *aphA-3* gene from pILL550. These data confirm that some candidate *C. hyointestinalis* shuttle vectors can indeed replicate in *C. hyointestinalis*, and that the presence of a *C. hyointestinalis*-specific replicon is essential for plasmid replication in *C. hyointestinalis*. W186 can be efficiently electroporated with pCHI15 DNA isolated from an *E. coli* K-12 strain; a property expected of a restriction mutant. Other data suggest, however, that W186 has also been mutated for its DNA modification system.

An examination of the restriction barriers that existed between the type strains of *C. jejuni*, *C. coli*, *C. fetus* subsp. *fetus*, and *C. hyointestinalis* was performed; it was generally observed that each species might contain a restriction system capable of

degrading plasmid DNA modified in the others. The exception to this general rule was between the closely related species *C. hyointestinalis* and *C. fetus* subsp. *fetus*; each can be transformed by heterologously-modified plasmid DNA (pCHI5), but at a transformation frequency that was usually an order of magnitude lower than seen in the positive controls. However, attempts to transform pCHI15 which had been modified by *C. fetus*, into *C. hyointestinalis*, were unsuccessful. These results may be explained by the presence of different restriction systems in both *C. hyointestinalis* and *C. fetus*. pCHI5, being only 3.7 kb in size, is smaller than pCHI15 and may harbour only one target site for the restriction enzyme of either species, whereas pCHI15, being 11.0 kb in size, may harbour several sites. pCHI5 resident in either of the two species would have the target site of the host restriction enzyme modified, but the target site of the restriction enzyme of the recipient would not be methylated and could be cleaved upon transformation. The cleavage of pCHI5 at one target site in the recipient would generate a linear molecule which would eventually become degraded by the DNases of the host. The possibility also exists, however, that this linear pCHI5 molecule could re-circularize to reform an intact plasmid, which could be methylated at its target site before it could be re-cleaved by the restriction enzyme of the host. This may explain the ten-fold drop (either way) in transformation efficiency of pCHI5 between the two species; the majority of cleaved pCHI5 molecules would not re-circularize. If pCHI15 harboured a greater number of target sites for each restriction enzyme then it would be impossible for it to re-circularize in the recipient and this would explain why no transformants could be obtained when pCHI15 extracted from *C. fetus* was transformed into *C. hyointestinalis*. This may also help explain how pCHI5 managed to bypass the restriction system of *C. hyointestinalis* during its formation following conjugation with *E. coli* K-12 S17-1 harbouring pCHI4. The pCHI5 molecule generated in the *C. hyointestinalis* recipient, following conjugation, would have been linearized by the restriction enzyme but may then have re-circularized and become methylated. This may help explain why the generation of pCHI5 from pCHI4 appears to occur at an extremely low frequency.

Spontaneous restriction mutants of the *C. coli* type strain NCTC 11366 could be isolated, by electroporation, at a low frequency, and one of these restriction mutants, named W188, was used to demonstrate that the *C. hyointestinalis* shuttle vector pCHI15 can replicate in a *C. coli* host. This replication is dependent upon the presence of the cryptic plasmid of *C. hyointestinalis*. W188 also appears to retain a *C. coli* modification sytem.

Mating experiments involving different *Campylobacter* species determined that pCHI15 can be mobilized between *Campylobacter* species, namely, from *C. coli* to *C. fetus* subsp. *fetus* and W186, but not into the wild-type *C. hyointestinalis* type strain. The ability of pCHI15 to be mobilized between *Campylobacter* species is most probably due to the presence of the putative *oriT* region present on the cryptic plasmid. Naturally-occuring conjugative tetracyline-resistance plasmids of *C. jejuni* have been reported to be efficiently mobilized from *C. jejuni* into *C. coli*, *C. fetus* and *C. lari* (Taylor *et al.*, 1981, 1986; Tenover *et al.*, 1985). This, however, is the first report of the mobilization of a recombinant plasmid between *Campylobacter* species. The restriction barrier in the *C. hyointestinalis* type strain, which can actively degrade foreign DNA introduced *via* conjugation, also prevents any conjugal transfer of pCHI15 from *C. coli*. These results imply that the putative *oriT* of the cryptic plasmid may in fact be functional, and that the *C. coli* type strain harbours the necessary (*tra*) genes, the products of which could recognize the cryptic plasmid *oriT* and transfer the plasmid from this region.

CHAPTER 7

DISCUSSION

7.1 Introduction

C. hyointestinalis has been associated with proliferative enteritis in swine (Gebhart *et al.*, 1983, 1985; Lambert *et al.*, 1984) and cattle (Diker *et al.*, 1990), but recent isolations from patients with proctitis and diarrhoea suggest that it may be also an opportunistic enteropathogen of humans (Edmonds *et al.*, 1987; Fennel *et al.*, 1986; Minet *et al.*, 1988). Indeed, five of the ten strains of *C. hyointestinalis* examined in this study were isolated from the stools of Aboriginal chidren with diarrhoea. Among *Campylobacter* species, *C. hyointestinalis* is most closely related to *C. fetus* and most distantly related to *C. jejuni* and *C. coli* (Gebhart *et al.*, 1985; Thompson *et al.*, 1988; Vandamme *et al.*, 1991).

Very little is known about the molecular biology of the pathogenesis of the *Campylobacter* species. Research effort has generally been concentrated on the recognized human pathogens *C. jejuni* and *C. coli*, and to a lesser extent on *C. fetus*. The genetics of these three species are under study and a shuttle vector has been constructed for the movement of DNA between these *Campylobacter* species (Labigne-Roussel *et al.*, 1987). Techniques for introducing DNA (by electroporation and natural transformation) into strains of *C. jejuni* and *C. coli* have also been developed (Miller *et al.*, 1988; Wang and Taylor, 1990b). However, no genetic study of *C. hyointestinalis* has been undertaken. Even the outer membrane of the organism, probably harbouring critical components for pathogenesis, remains poorly characterized. The membranes of *C. jejuni*, *C. coli*, and *C. fetus* have been examined in some detail (Blaser *et al.*, 1983a; Logan and

Trust, 1982). Initial work in this thesis involved the characterization of outer membrane components of ten strains of *C. hyointestinalis*. The ability of *C. hyointestinalis* to receive DNA *via* conjugation, electroporation, and natural transformation was then examined. A number of *C. hyointestinalis*-specific candidate shuttle vectors (incorporating a *C. hyointestinalis* cryptic plasmid) were then constructed and their ability to be mobilized into *C. hyointestinalis* was examined. Due to the lack of success, the complete genetic characterization of the *C. hyointestinalis* cryptic plasmid which formed part of the candidate shuttle vectors was performed. These data suggested improvements which were subsequently implemented in the construction of candidate shuttle vectors. When these vectors also appeared incapable of replication in *C. hyointestinalis*, it became apparent that the organism must possess a restriction system to prevent the entry and/or establishment of DNA *via* conjugation. By chemical mutagenesis, a restriction-less mutant of *C. hyointestinalis* was obtained. This strain, however, did not retain the ability to modify its own DNA. Several of the putative shuttle vectors could be shown to enter, and be stably maintained within, the strain.

7.2 Characterization of the components of the outer membrane of *C. hyointestinalis*

An examination of the composition of the outer membranes from ten strains of *C. hyointestinalis* was performed with methods used in studies of the membrane composition of *C. jejuni*, *C. coli*, and *C. fetus* (Blaser *et al.*, 1983a; Logan and Trust, 1982). The outer membrane protein profiles of the *C. hyointestinalis* strains were similar to those which had been reported for *C. fetus*, with each strain expressing two major outer membrane proteins. However, a major higher M_r protein (S-layer) such as that produced by virulent strains of *C. fetus*, was not observed in any of the *C. hyointestinalis* strains. Immunoblotting studies showed that antisera raised against *C. hyointestinalis* reacted with some *C. jejuni* and *C. coli* outer membrane proteins. The 23 and 43 kDa outer membrane proteins of *C. hyointestinalis* reacted on immunoblotting with antisera raised against

C. jejuni and *C. coli*, indicating an antigenic relatedness between the outer membrane proteins of the three species.

The type strain of *C. hyointestinalis* appeared to be poorly flagellated but flagella from other *C. hyointestinalis* strains were easily obtained. The flagellins isolated from these strains were of a similar M_r (62 kDa) to those produced by *C. coli* and *C. jejuni*, and flagellin of all strains reacted in immunoblots with antisera raised against *C. hyointestinalis*. This suggested structural and antigenic relationship(s) between the flagella of these three species.

The LPS of *C. hyointestinalis* showed considerable heterogeneity, with some strains displaying LPS of only low M_r, while others synthesized higher M_r LPS components. The synthesis of such higher M_r LPS material has been observed in *C. fetus* (Logan and Trust, 1984; Perez-Perez and Blaser, 1985).

The composition of the outer membrane of *C. hyointestinalis* was, therefore, similar in many respects to that of *C. fetus*. This conclusion was in agreement with the observation that, among *Campylobacter* species, *C. hyointestinalis* is most closely related to *C. fetus* (Gebhart *et al.*, 1985; Thompson *et al.*, 1988; Vandamme *et al.*, 1991).

7.3 mobilization of plasmid DNA into *C. hyointestinalis*

To facilitate the analysis of gene expression of *Campylobacter* species, a plasmid shuttle vector, pILL550, has been constructed which can be mobilized at a high efficiency from *E. coli* K-12 to *C. jejuni*, *C. coli*, and *C. fetus* (Labigne-Roussel *et al.*, 1987). It was shown here, however, that pILL550 appeared to be unsuitable for genetic work with *C. hyointestinalis* as attempts to mobilize pILL550 into several strains of *C. hyointestinalis* were unsuccessful. Further attempts to mobilize the broad-host-range plasmid RP4, the broad-host-range cloning vectors pKT230 and pKT231, and the conjugative streptococcal transposon Tn*916*, into *C. hyointestinalis*, were also unsuccessful. The inability of pILL550, which contains the replicon originating from the *C. coli* plasmid pIP1445, and the other broad-host-range vectors, to be mobilized into

C. hyointestinalis was initially thought to be due to the inability of these replicons to function in a *C. hyointestinalis* host.

As a basis for constructing a *C. hyointestinalis*-specific plasmid vector, the ten *C. hyointestinalis* strains examined earlier were screened for plasmid content with the aim of using an endogenous plasmid as the basis for the vector. It was observed that four (Australian) isolates of *C. hyointestinalis* harboured a small (2.5 kb) cryptic plasmid. As the replicons of pILL550 and the other broad-host-range vectors apparently could not function in a *C. hyointestinalis* host, a series of *C. hyointestinalis*-specific candidate shuttle vectors based upon the 2.5 kb cryptic plasmid (to provide the appropriate genetic material for replication in *C. hyointestinalis*) was constructed. Despite repeated attempts, none of these *C. hyointestinalis* candidate shuttle vector constructs could be efficiently mobilized into *C. hyointestinalis*. These results suggested that *C. hyointestinalis* might possess a barrier to the introduction of foreign DNA *via* conjugation. The most likely barrier was a restriction system.

In order to determine whether such a system was preventing plasmid DNA from entering the cell *via* conjugation, the location of the replication region of the cryptic plasmid needed to be identified in order to confirm that one or more of these candidate *C. hyointestinalis* shuttle vectors contained this replicon in an intact form. The DNA sequence of the cryptic plasmid revealed that one of the candidate shuttle vectors, pCHI2, should contain an intact replicon. Therefore, pCHI2 should have had the ability to replicate in *C. hyointestinalis* and so its inability to be mobilized provided further evidence that a restriction system was preventing it from becoming established. Consequently, a mutant in the restriction system was sought.

The treatment of *C. hyointestinalis* cells with the chemical mutagen MNNG eventually yielded a *C. hyointestinalis* restriction mutant, designated W186, with which *C. hyointestinalis* transconjugants could be obtained at a frequency of 10^{-4} transconjugants per donor for pCHI2, or with two other plasmids containing an intact form of the cryptic plasmid (pCHI12 and pCHI15). It was determined that W186 was a restriction mutant by virtue of the fact that it could be electroporated with

E. coli K-12-modified pCHI15 DNA to yield 5 x 10^3 transformants per μg of DNA. Plasmid pILL550, and the broad-host-range plasmid pKT231 harbouring the *aphA-3* gene from pILL550, could not be mobilized into W186, suggesting that these plasmids could not function in a *C. hyointestinalis* host. A *C. hyointestinalis*-specific replicon (like that of the cryptic plasmid), was essential for plasmid replication in *C. hyointestinalis* as a vector lacking the cryptic plasmid, pCHI1, could not be mobilized into W186. This vector was otherwise similar to the successful shuttle vectors.

Thus, *C. hyointestinalis* appeared to be unique among the *Campylobacter* species studied here insofar as it seemed to possess a restriction system that was capable of degrading foreign DNA entering the cell *via* conjugation. In *E. coli*, As DNA enters a cell *via* conjugation it does so in a single-stranded unmethylated form. The complementary strand is synthesised and if the modification enzyme acts prior to restriction, the plasmid is protected and survives in the host, assuming it can replicate. In *C. hyointestinalis*, therefore, it may be that the restriction system is more efficient than its associated modification system. As the *C. hyointestinalis* restriction system appears so efficient, the possibility also exists that the restriction enzyme can cleave single-stranded DNA. By way of precedent, it has been shown that R.*Hae*III can digest single-stranded DNA *in vitro* (Wells and Neuendorf, 1981). The observation that W186 could be electroporated with unmodified plasmid DNA suggests, however, that this strain has lost the ability to digest double-stranded DNA.

Three explanations may be advanced. First, *C. hyointestinalis* may contain two restriction systems, one active against double-stranded DNA and the other against single-stranded DNA. Strain W186 would then carry a mutation(s) inactivating both systems. Second, *C. hyointestinalis* may have only one restriction system, active to cleave both single- and double-stranded DNA, and W186 is a mutant in this system. In the wild-type strain, the system would act to cleave DNA entering *C. hyointestinalis* both *via* conjugation (single-stranded), and *via* transformation (double-stranded) from *E. coli*. Finally, *C. hyointestinalis* may possess only one restriction system which is active only against double-stranded DNA, but with a modification enzyme much less efficient than the

corresponding enzymes in other *Campylobacter* species. If the methylation enzyme of the system were inefficient, then DNA entering *via* conjugation would be degraded not as a single-stranded form, but after at least some second strand synthesis had taken place.

The inability of plasmid DNA extracted from W186 to be electroporated into wild-type *C. hyointestinalis* suggests that this DNA has not been properly methylated to protect it from the *C. hyointestinalis* restriction system. It appears, then, that W186 is also mutated for its modification system and is unable to methylate this plasmid DNA. This is not unusual as mutation in the restriction-modification loci can result in two distinct phenotypes: either the loss of both restriction and modification functions or the loss of the restriction functions only (De Backer and Colson, 1991). Type I and Type III restriction enzymes exist as multifunctional enzyme complexes with separate subunits mediating restriction and modification activity (Bickle, 1987). In Type III restriction-modification systems, two adjacent genes called *mod* and *res* are transcribed from a promoter located before *mod*, and there is evidence for a second promoter between the genes which would lead to transcription of the *res* gene (Iida *et al.*, 1983). The close linkage between the restriction and modification genes in these systems implies that the mutation of one gene can affect the expression of the other. The mutations here observed in both the restriction and modification systems in *C. hyointestinalis* could be due to the clustering of induced mutations often observed after mutagenesis with MNNG (Miller, 1972). By screening a larger number of *C. hyointestinalis* restriction mutants, it might be possible to isolate a restriction mutant which still possessed modification activity.

The restriction system of *C. hyointestinalis* is very efficient against transformed unmodified double-stranded DNA, as electroporation of foreign DNA into wild-type *C. hyointestinalis* was invariably unsuccessful, except when DNA had been extracted from *C. fetus* subsp. *fetus*. The ability of pCHI5 DNA methylated by *C. fetus* to be introduced into *C. hyointestinalis* can be explained by the presence of different restriction enzymes in both species and only one target site on pCHI5 for each of these enzymes (see Section 6.3). Endonucleases show much greater variety than DNA methylating enzymes, and appear to have arisen independently of these modification systems in the course of

evolution (Chandraseguan and Smith, 1988). Approximately seven classes of methyltransferases have been distinguished (Klimasauskas *et al.*, 1989), implying that such enzymes in diverse organisms may be more closely related than endonucleases. However, plasmid DNA modification by *C. fetus* does not protect against restriction by *C. hyointestinalis*, since pCHI15 extracted from *C. fetus* could not be transformed into *C. hyointestinalis*. The electroporation of *Campylobacter* species with plasmid DNA modified by the same species has been shown here to be an efficient means of introducing DNA into *Campylobacter*.

Attempts to introduce plasmid DNA (both modified and unmodified) into two strains of *C. hyointestinalis* by natural transformation were unsuccessful. This might suggest that *C. hyointestinalis* cannot take up naked DNA from its surrounding environment, wheras, some strains of *C. jejuni* and *C. coli* do have this ability (Wang and Taylor, 1990b).

Future work in this area could involve the characterization and purification of the *C. hyointestinalis* restriction enzyme. A profitable line of research could be to attempt to methylate shuttle vector plasmid DNA *in vivo* in *E. coli* K-12 donors expressing various methylases, in order to protect the DNA from the *C. hyointestinalis* restriction system. Such an approach has been successful in the mobilization of plasmid DNA into *Neisseria gonorrhoeae* (Butler and Gotschlich, 1991). In this way, it might be possible to identify a protective methylase, which could provide information about the target site for the *C. hyointestinalis* restriction system.

7.4 The cryptic plasmid of *C. hyointestinalis*

From the few reports which have appeared, it seems likely that only a small percentage of *C. hyointestinalis* isolates contain plasmids (Boosinger *et al.*, 1990; Edmonds *et al.*, 1987). A single plasmid of 2.5 kb was identified here in four Australian isolates that were examined. The analysis of the DNA sequence of this cryptic plasmid revealed that it harbours only one ORF which encodes a 39.7 kDa replication initiation protein, named RepA. The presence of four 19 bp direct repeats upstream from the *repA*

gene suggests that the cryptic plasmid utilizes an iteron-mediated form of replication. The direct repeats are presumed to be binding sites for the RepA protein and therefore crucial for the initiation of plasmid replication. It is possible that Repeat #4, being downstream from the *repA* promoter and separated from the other repeats, may be a binding site for RepA which could play a role in the possible autoregulation of transcription of the *repA* gene. The RepA protein displays extensive homology with a plasmid-encoded replication initiation protein from *Neisseria gonorrhoeae*, and strong homology, in two regions of the protein, with the replication initiation proteins from other iteron-containing plasmids. The DNA sequence of pCHI5 shows that an 1806 bp fragment covering the *repA* and the four repeats is sufficient for autonomous replication in *C. hyointestinalis*. The cryptic plasmid was capable of autonomous replication in *C. coli* and *C. fetus* hosts implying that the cryptic plasmid itself should also be able to replicate in these species.

A putative *oriT* region was identified on the cryptic plasmid; this contained a putative "nick region" that was highly homologous to the consensus sequence of the *oriT* "nick regions" of many mobilizable plasmids. Mating experiments between various *Campylobacter* species demonstrated that the *C. hyointestinalis* shuttle vector, pCHI15, could be mobilized from *C. coli* to *C. fetus* and the *C. hyointestinalis* restriction mutant W186. The ability of pCHI15 to be mobilized was probably due to the presence of the putative *oriT* region of the cryptic plasmid and demonstrated that the cryptic plasmid itself may well be mobilizable. The cryptic plasmid cannot be self-transmissible as it does not encode the proteins necessary to mediate this transfer and they must therefore be encoded elsewhere in the *C. coli* donor. In future, it is possible that the *oriT* region may be useful as a component of other *Campylobacter* vectors.

As only one ORF was detected by sequence analysis the plasmid of *C. hyointestinalis* may be regarded as truly cryptic. There have been some reports of other plasmids which have contained only replicative regions. pADB from *Mycoplasma mycoides* subsp. *mycoides* is a 1.7 kb cryptic plasmid which has a single large ORF capable of coding for a polypeptide 198 amino acids long; the plasmid possessed three

21 bp repeat sequences (Bergeman *et al.*, 1989). pHD2 from *Bacillus thuringiensis* var. *kurstaki* strain HD1-DIPEL contained two ORFs, one of which encoded a 26.5 kDa polypeptide which may act as a site-specific topo-isomerase involved in plasmid replication, and the second encoded a 9.1 kDa polypeptide which exhibited considerable homology with the pLS1-encoded RepA polypeptide, thought to be involved in control of plasmid replication (McDowell and Mann, 1991).

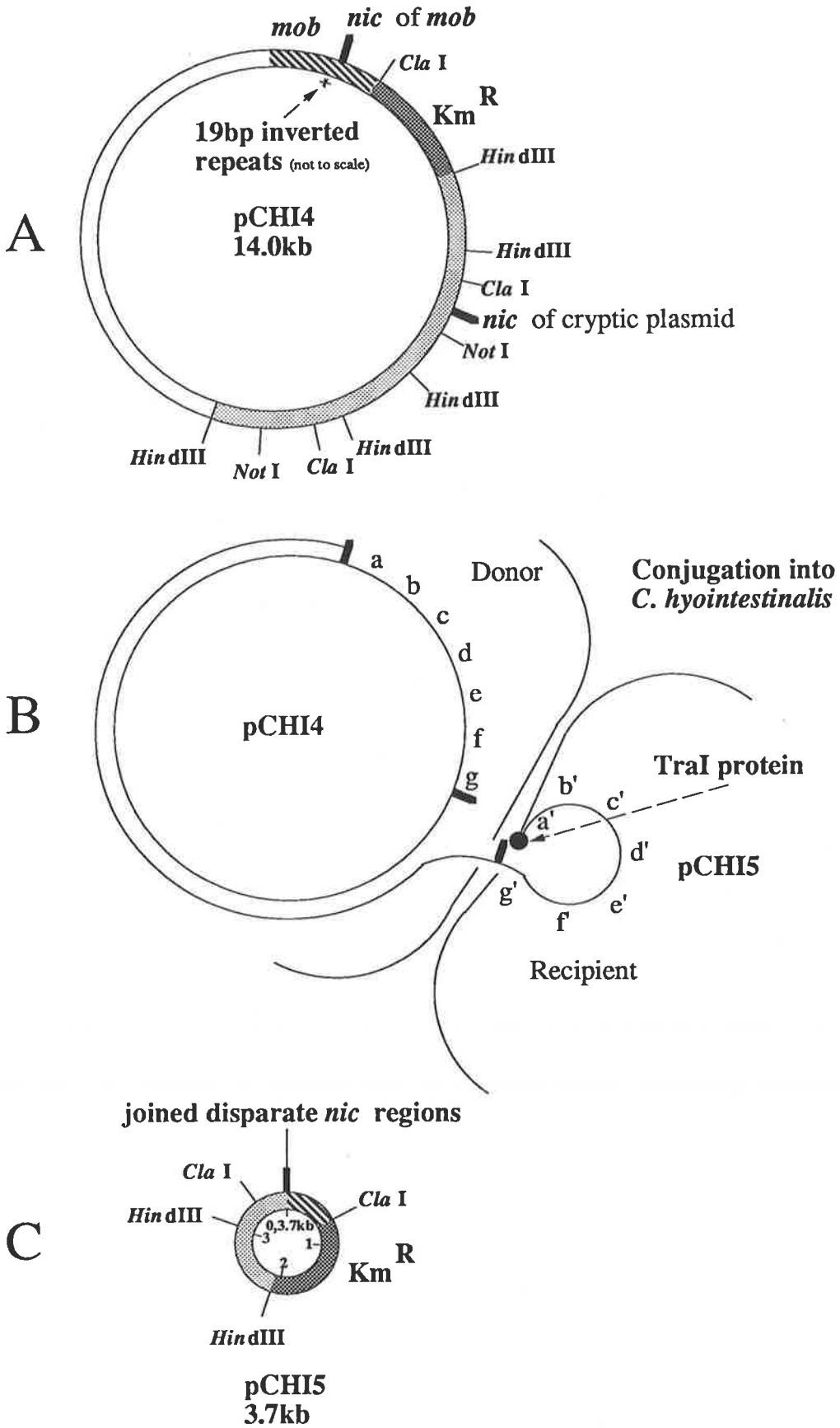
7.5 The generation of pCHI5 from pCHI4

In an early attempt to construct a *C. hyointestinalis* shuttle vector which contained an intact form of the cryptic plasmid, the cryptic plasmid was cloned inside a copy of itself in pCHI2 to form the plasmid pCHI4. It was later shown that pCHI4 contained two copies of the replicon (including associated iterons) and that it would therefore probably have difficulty replicating in a *C. hyointestinalis* host due to steric hindrance resulting from folding of the replicon around the origins (Nordström, 1990). This folding at the origins is due the protein-protein interactions that can occur between the RepA proteins bound to each set of iterons (Nordström, 1990). Such difficulty in replication was inferred by the inability of pCHI4 to be mobilized into W186.

Following one mating experiment into the type strain of *C. hyointestinalis*, however, a *C. hyointestinalis* transconjugant harbouring a small 3.7 kb plasmid, named pCHI5, was isolated. The DNA sequence obtained from pCHI5 determined that it was identical to pCHI4, except that there had been a deletion of DNA between the putative "nick region" of the *oriT* of the cryptic plasmid DNA and the "nick region" of the RP4 *oriT*. It had been shown that the *C. hyointestinalis* type strain did not appear to be transformed naturally with plasmid DNA (unlike strains of other *Campylobacter* species), and this implied that pCHI5 had been introduced into *C. hyointestinalis via* a conjugation event. This information, and the DNA sequence of pCHI5, suggests how pCHI5 may have been generated from pCHI4 (Figure 7.1). During the initiation of conjugal transfer of pCHI4, the *E. coli* K-12 S17-1-encoded DNA transfer proteins TraI, TraJ, and TraH

FIGURE 7.1 Model describing the generation of pCHI5 in *C. hyointestinalis* following the mobilization of pCHI4 from *E. coli* K-12 S17-1.

A map of pCHI4 showing the position of the "nick region" of the *oriT* from RP4 and the putative "nick region" of the cryptic plasmid is given in Panel A. During the initiation of conjugal transfer of pCHI4, the *E. coli* K-12 S17-1encoded DNA transfer proteins would assemble the relaxosome complex at the *oriT* of RP4, and the "nick region" is recognized by the TraI protein which cleaves one strand of the plasmid at the RP4 nick site. The TraI protein then becomes covalently attached to the 5′-terminal nucleotide of the nicked strand. After nicking of the strand destined for transfer, the two strands of the plasmid unwind, and TraI then associates with the cell membrane and pCHI4 is transferred to the *C. hyointestinalis* recipient cell in a single-stranded form with the 5′ terminus leading (Panel B). In the generation of pCHI5, it is believed that the DNA downstream from the 5′ terminus (designated a′ to g′) was transferred into the *C. hyointestinalis* recipient up to the position of the putative "nick region" of the cryptic plasmid. At this point the putative "nick region" of the cryptic plasmid was mistaken by the *E. coli* K-12 S17-1 transfer proteins as being the 3′ terminus of the RP4 *oriT*, such that it was subsequently cleaved/ligated back to the 5′ terminus. The joining of the disparate nick regions resulted in the formation of the plasmid pCHI5 which harbours all the genetic information necessary for autonomous replication and antibiotic resistance in *C. hyointestinalis* (Panel C).


A
mob
nic of mob
Cla I
Km R
19bp inverted
repeats (not to scale)
Hin dIII
pCHI4
14.0kb
Hin dIII
Cla I
nic of cryptic plasmid
Not I
Hin dIII
Hin dIII
Not I
Cla I
Hin dIII
B
a
b
c
d
e
f
g
pCHI4
Donor
Conjugation into
C. hyointestinalis
TraI protein
b'
c'
a'
d'
pCHI5
g'
e'
f'
Recipient
joined disparate nic regions
C
Cla I
Hin dIII
Cla I
0,3.7kb
3
1
2
Km R
Hin dIII
pCHI5
3.7kb

would have interacted and assembled the relaxosome at the *oriT* of RP4. TraI would then recognize the "nick region" of the *oriT* and cleave one strand of the plasmid at the RP4 nick site. The TraI protein would then become covalently attached to the 5′-terminal nucleotide of the nicked strand. In the normal situation, the 5′ terminus is then thought to become covalently linked to a membrane protein. Plasmid DNA transfer (in a single-stranded form) follows, until the protein to which the 5′ terminus is attached recognizes the 3′ terminus and ligates it to the original 5′ terminus (Willetts and Wilkins, 1984). In the formation of pCHI5, however, it is suggested that pCHI4 DNA transfer was normal, until the putative "nick region" of the cryptic plasmid came to the point of transfer across the membrane into the recipient cell (Figure 7.1). As the putative "nick region" of the cryptic plasmid is highly homologous to that of the "nick region" of RP4 (9 bp out of 12), it is suggested that, as a rare event, this "nick region" was mistaken by the *E. coli* K-12 S17-1 transfer proteins as being the 3′ terminus of the RP4 *oriT*, and that it was subsequently cleaved/ligated back to the 5′ terminus. Plasmid pCHI5, in the recipient *C. hyointestinalis* cell, was the result. This mistaken recognition of the putative "nick region" of the cryptic plasmid is thought to occur at an extremely low frequency, as there was no evidence of such recognition during any other mating experiments involving conjugation of plasmids harbouring a copy of this DNA region. It is also extremely unlikely that pCHI5 could have been "formed" inside the *E. coli* K-12 S17-1 donor and then transferred as a whole plasmid into *C. hyointestinalis*. This is due to the fact that pCHI5 does not contain the 19 bp inverted repeats upstream from the nick site in the RP4 *oriT*; the proximal arm of the inverted repeat is an essential binding site for TraJ, without which cleavage and transfer of the DNA cannot take place. It is also unlikely that pCHI5 could have been "formed" inside the *E. coli* K-12 S17-1 donor and then been transferred *via* the putative *oriT* of the cryptic plasmid, as the imperfect 19 bp repeats of this region share no homology with those of the *oriT* of RP4 and TraJ would therefore not bind to them. This was confirmed by the observation that pCHI6, which contains a complete copy of the putative *oriT* of the cryptic plasmid (but does not possess the *oriT* of RP4), could not be mobilized from *E. coli* K-12 S17-1 into *S. typhimurium* (Section 4.2.12). It

is not known how pCHI5 managed to evade the restriction system of the *C. hyointestinalis* type strain, although it is possible that pCHI5, being small, harbours only one target site for the *C. hyointestinalis* restriction system. The plasmid would then be linearized by the restriction enzyme, but might be subsequently re-circularized and methylated at its target site. This hypothesis is supported by the observation that pCHI5 could be transformed between *C. hyointestinalis* and *C. fetus*, but at an efficiency which was an order of magnitude lower than that observed when pCHI5 was transformed into the species from which it had been prepared (see Section 6.3). This may also explain why the isolation of pCHI5 in *C. hyointestinalis*, following conjugation with *E. coli* K-12 S17-1 harbouring pCHI4, was a rare event.

This result suggests that, in the course of normal conjugal DNA transfer, the 3′ terminus of the *oriT* of RP4 is recognized in some way, following the completion of DNA transfer, and is then ligated to the 5′ terminus. Future work to demonstrate this could include the construction of RP4 mobilization vectors which have a series of copies of the "nick region" of the *oriT* downstream from a complete RP4 *oriT* region (with a replicon and antibiotic resistancegene between the complete RP4 *oriT* and the extra "nick region(s)"). These vectors could then be mobilized into recipients; plasmids in transconjugants could then be examined by size and sequence analysis to determine if the transfer proteins had mistakenly recognized one of the downstream "nick region" copies as containing the 3′ terminus.

The *Hin*dIII sites present on pCHI5 could not be cleaved by the *Hin*dIII restriction enzyme under normal conditions (digestion for 2 h at 37°C with 2 units enzyme/μg of DNA). The sites were partially cleaved, however, when digestion proceeded for 16 h, or when pCHI5 had been cloned into a plasmid replicating in an *E. coli* K-12 background. This observation suggested that the *Hin*dIII sites of pCHI5 may have been methylated, or otherwise rendered resistant to restriction, when pCHI5 was present in *C. hyointestinalis*. The *Hin*dIII sites of the original cryptic plasmid (as isolated from *C. hyointestinalis* 45104) were, however, easily cleaved by *Hin*dIII, and chromosomal DNA from *C. hyointestinalis* NCTC 11608 (and W173) was also readily cleaved by this enzyme

(data not shown). The reason for the resistance of pCHI5 *Hin*dIII sites to digestion with the enzyme is therefore unclear. However, one possibility may be that these *Hin*dIII sites form part of some DNA secondary structure induced in this *C. hyointestinalis* strain which makes these sites not readily accessible to the enzyme *in vitro*.

7.6 Possible evolutionary significance of the restriction system in *C. hyointestinalis*

These studies have shown that *C. hyointestinalis* possesses a restriction barrier able to inhibit the introduction of foreign DNA *via* conjugation. *C. hyointestinalis* is then the first example of a *Campylobacter* species to possess such a system (*C. coli*, *C. jejuni*, and *C. fetus* do not appear to destroy DNA, entering *via* conjugation from *E. coli* K-12, as efficiently as observed here in *C. hyointestinalis*; Labigne-Roussel *et al.*, 1987). *C. fetus* and *C. hyointestinalis* have been shown to be closely related by DNA hybridization analysis (Gebhart *et al.*, 1985; Owen and Leaper, 1981; Roop *et al.*, 1984), and 16S rRNA sequence analysis has revealed that *C. fetus* subsp. *fetus* shares nearly 100% homology with the subspecies *venerealis* and 98% homology with *C. hyointestinalis* (Wesley *et al.*, 1991). These data verify the phylogenetic assignments of these species which have been proposed earlier on the basis of partial 16S rRNA sequence analysis (Thompson *et al.*, 1988) and DNA-rRNA (23S) hybridization experiments (Vandamme *et al.*, 1991). Therefore, the *C. fetus-C. hyointestinalis* cluster shows a spectrum of adaptation to host environments. It encompasses closely-related bacteria which are recovered from the intestinal tract (*C. hyointestinalis*), which colonize the intestine *en route* to the reproductive system (*C. fetus* subsp. *fetus*), or which inhabit exclusively the urogenital tract (*C. fetus* subsp. *venerealis*) (Wesley *et al.*, 1991). The studies described here have shown that neither of the two *C. fetus* subspecies appear to possess the restriction barrier to the introduction of foreign DNA *via* conjugation that is present in *C. hyointestinalis*. It follows, therefore, that they have either lost this ability or that *C. hyointestinalis* is the only one of these species to have obtained it. It is thought that *C. fetus* subsp. *venerealis* is a mutant of *C. fetus* subsp. *fetus* which has adapted to a

specific ecological niche in the bovine reproductive tract (Véron and Chatelain, 1973). It may be that in this branch of the *Campylobacter* phylogenetic tree, species have evolved from those which colonize the intestine to yield new species which colonize the reproductive tract. If this is the case then *C. hyointestinalis* may be the ancestral species of *C. fetus*. This would suggest that the ability to degrade foreign DNA introduced *via* conjugation may have been lost by *C. fetus*, rather than having been acquired by *C. hyointestinalis*. In all of the studies in this work, *C. fetus* behaves exactly as a *C. hyointestinalis* restriction mutant in its ability to receive foreign DNA *via* conjugation from an *E. coli* K-12 or *C. coli* donor. The loss of this restriction system may well have played an important role in the evolution of *C. fetus* from *C. hyointestinalis*. Such loss may have allowed *C. fetus* to take up foreign DNA *via* conjugation; the virulence of the organism could thereby have been affected (in degree, and in tissue tropism). In this manner, *C. fetus* could have acquired the ability to colonize the reproductive tract, a facility not present in *C. hyointestinalis*. The studies here have shown that *C. fetus* can act as a recipient in matings with *C. coli* as a donor (Section 6.2.12). *C. fetus*, therefore, has the potential to receive genetic material which is not available to *C. hyointestinalis*; this facility may also afford selective advantages.

7.7 Implications of this work for the future

7.7.1 The restriction-modification system of *C. hyointestinalis*

The restriction-modification systems of *Campylobacter* species have not been investigated at the molecular level. The restriction system present in *C. hyointestinalis* warrants further examination. The possibility may exist that the restriction enzyme is capable of cleaving single-stranded DNA. If this is the case, then the characterization and molecular cloning of the restriction system may provide some insight into the interaction of DNA-binding proteins with single-stranded DNA. Complete characterization would yield the genetic organization of this restriction-modification system, and allow its formal classification. Molecular cloning of the restriction-modification system would allow the re-introduction of the *res* and *mod* genes into W186, to determine if the barrier to the

introduction of foreign DNA *via* conjugation could be restored. Characterization of the restriction-modification system would enable an examination of the *C. fetus* genome to determine if it contains a mutated form of the *res* gene or if the gene is absent. It would be interesting to introduce the *res-mod* genes from *C. hyointestinalis* into *C. fetus* and then to ask if the genes could functionally act as a barrier to the introduction of foreign DNA *via* conjugation in this host.

7.7.2 The *oriT* and *par* regions of the cryptic plasmid

Although the cryptic plasmid is now well-characterized, the putative *oriT* and *par* regions of the plasmid are not. Work in the future may involve the subcloning of the *oriT* region to confirm that it can in fact act as a site to initiate the conjugal transfer of plasmid DNA. This could be useful in the construction of future *Campylobacter* vectors. It would be interesting to ask if *C. hyointestinalis* strains can mobilize plasmids *via* the *oriT*, or whether the ability to transfer plasmid DNA by conjugation is restricted to the other *Campylobacter* species. To determine if the putative *par* locus plays a role in partitioning, it would be instructive to delete this region from a shuttle vector construct, and then assess the stability of the resulting plasmid. One would predict (from the data obtained with pSC101) that the complete deletion of the *par* region would result in the loss of the plasmid in less than 100 generations (Meacock and Cohen, 1980). The DNase I protection (footprinting) technique, using purified DNA gyrase, could also be performed to confirm whether the sequences in the putative *par* region, homologous to the REP consensus sequence of *E. coli* K-12, are in fact binding sites for the protein.

7.7.3 Construction of *Campylobacter* Hfr strains

Little information concerning the genomes of *Campylobacter* species is available. *C. coli* NCTC 11366 has been shown to be able to mobilize pCHI15 into other *Campylobacter* species and it therefore harbours the genes encoding the Tra proteins responsible for plasmid transfer. These *tra* genes are likely to be plasmid-encoded (not on

although may be
pCHI15) the possibility exists that these genes encoded on the chromosome. If this is the case then it would be useful to determine if this strain is an Hfr, or if Hfr derivatives thereof can be constructed. If an Hfr *C. coli* strain could be made it would then be possible to move chromosomal DNA from *C. coli* into either *C. fetus* or a restriction mutant of *C. hyointestinalis*. Using animal models, or cell cultures *in vitro*, in which *C. coli*, as distinct from the other *Campylobacter* species, is thought to manifest pathogenicity, a beginning could be made on the mapping of genes encoding the *C. coli* virulence determinants. This would add to the preliminary genome maps of *C. jejuni* and *C. coli* strains, in which the relative positions of various house-keeping genes have been determined by pulse-field gel electrophoresis (Nuijten *et al.*, 1990a; Taylor *et al.*, 1992).

7.7.4 Cloning of *Campylobacter* virulence determinants

pILL550 has been shown to be a suitable cloning vector for *C. coli*, *C. jejuni*, and *C. fetus*, but not for *C. hyointestinalis*. The observation that pCHI15 can replicate in *C. fetus* and *C. coli* as well as *C. hyointestinalis* gives this vector an advantage over pILL550 in that it can replicate in a *C. hyointestinalis* host. The fact that pCHI15 is also a cosmid means larger fragments of genomic DNA may be cloned therein, than is the case with pILL550. It would therefore be possible to make a gene bank of any *Campylobacter* species in *E. coli* K-12 and move the entire bank into *C. coli*, *C. hyointestinalis* or *C. fetus*. Such an approach would allow the selection of various species-specific virulence determinants and surface antigens (Sharma *et al.*, 1989). An example of such work, suggested by the data described here which implies that *C. fetus* may be derived from *C. hyointestinalis*, would be to analyse *C. fetus* virulence determinants in a *C. hyointestinalis* host. *C. fetus* subsp. *fetus* causes infectious abortions and infertility in cattle and sheep (Morris and Patton, 1985), and is recognized as an opportunistic human pathogen causing systemic infections such as sepsis or meningitis in compromised hosts (Cover and Blaser, 1989; Penner, 1988). In *C. fetus* infections, the S-layer plays an important role in invasion and survival within the host (Blaser *et al.*, 1987; Fujimoto *et al.*, 1989; Pei and Blaser, 1990). The S-layer has been described as an

antiphagocytic antigen (McCoy *et al.*, 1975; Myers, 1971) and is associated with increased virulence in experimentally infected mice (Pei and Blaser, 1990) and serum resistance (Blaser *et al.*, 1985, 1987, 1988). The LPS of *C. fetus* shows a minimal core region and several high M_r complexes which were homogeneous in chain length (Logan and Trust, 1982, 1984; Perez-Perez and Blaser, 1985). This structural characteristic may be related to the ability of this species to assemble an S-layer (Belland and Trust, 1985). *C. fetus* has also been shown to be invasive for HEp-2 cells (Konkel and Joens, 1989). In contrast, it has been shown here that *C. hyointestinalis* does not possess an S-layer nor LPS of homogeneous chain length, and it is known that *C. hyointestinalis* is non-invasive for HEp-2 cells (Konkel and Joens, 1990). If a gene bank of *C. fetus* genomic DNA, in pCHI15, were moved into *C. hyointestinalis*, it would be possible to screen for an isolate which expressed the S-layer, or which synthesised the corresponding LPS. The *C. hyointestinalis* gene bank, harbouring cloned *C. fetus* DNA, could be added to HEp-2 cell monolayers, in an attempt to select for *C. hyointestinalis* isolates harbouring *C. fetus* DNA encoding the genes responsible for cell invasion. Work of this nature has been performed to isolate the genes encoding the invasin proteins of *Shigella* (Maurelli *et al.*, 1985), *Salmonella* (Galan and Curtiss, 1989), *Yersinia* (Miller and Falkow, 1988), and *Listeria* (Gaillard *et al.*, 1991). The same aim might also be achieved by introducing the bank into germ-free mice in which *C. fetus* is known to cause bacteremia (Pei and Blaser, 1990).

7.7.5 Examination of the pathogenicity and virulence determinants of *C. hyointestinalis*

A great deal of work remains to be done in the investigation of the pathogenicity of *C. hyointestinalis*. The role (if any) played by *C. hyointestinalis* in the aetiology of proliferative enteritis in swine needs to be elucidated. *C. hyointestinalis* may be opportunistic, and may only compromise an already established infection. Two degrees of severity of proliferative enteritis, the haemorrhagic condition and the non-haemorrhagic condition, may then reflect the presence or absence (respectively) of

C. hyointestinalis. The observation that *C. hyointestinalis* might be an opportunistic enteropathogen of humans could support the theory that *C. hyointestinalis* may be able to establish an infection, in pigs, following the initial infection of the intracellular CLOs.

C. hyointestinalis, as well as being associated with proliferative enteritis in pigs, has been reported to have caused watery diarrhoea in four compromised individuals, namely two homosexual men, an elderly woman, and an eight-month-old child (Edmonds *et al.*, 1987). *C. hyointestinalis* has been associated with diarrhoea in an immunodeficient patient with chronic myeloid leukeamia (Minet *et al.*, 1988), and with proctitis in a homosexual man (Fennel *et al.*, 1986). Five of the ten strains used in this study were isolated from the stools of Aboriginal children with diarrhoea. The opportunistic ability of these *C. hyointestinalis* strains may suggest that they possess some *C. coli* or *C. jejuni*-like determinants that the NCTC *C. hyointestinalis* strains do not have. By performing whole chromosome hybridizations, or by examination of the restriction enzyme patterns of these strains by pulse-field gel electrophoresis, it may be possible to identify any gross genomic differences between the strains; the differing DNA regions could then be analysed further.

It would be interesting to discover if these strains are in fact diarrhoeagenic in animal models. A variety of such models have been used to investigate the pathogenicity of *Campylobacter* organisms. However, no established animal models that mimic human disease, without surgical or antibiotic pretreatment of the animals, exist (Walker *et al.*, 1986). Caldwell *et al.* (1983) used the removable intestinal tie adult rabbit diarrhoea (RITARD) procedure to produce disease in 1 kg rabbits. Kazmi *et al.* (1984) have developed a model in which infant mice challenged intragastrically with strains of *C. jejuni* developed severe diarrhoea following virulence enhancement of the organisms by serial intraperitoneal passage in adult mice. If it could be established that some of the Australian strains of *C. hyointestinalis* (and not the NCTC strains) could cause diarrhoea in one of these systems, then it might be possible to test potential mutants of the Australian strains for loss of pathogenicity. The mutants could be generated through the use of subtractive hybridization, in which single-stranded DNA from a non-

diarrhoeagenic NCTC *C. hyointestinalis* strain absorbed single-stranded DNA from a diarrhoeagenic Australian *C. hyointestinalis* strain. Any residual DNA could then be cloned into pCHI15; it could then be determined if some such clones contained DNA unique to the Australian *C. hyointestinalis* strains, and whether the DNA was reactive with *C. coli* or *C. jejuni* DNA upon probing. The cloned DNA could then be mutated *in vitro* and the mutated "gene(s)" reintroduced into a diarrhoeagenic Australian *C. hyointestinalis* strain, or even into *C. coli* or *C. jejuni*. After allelic exchange, mutant strains could then be tested for diarrhoeagenic capacity in the animal models.

The use of *C. hyointestinalis* strains diarrhoeagenic in humans, and animal models for diarrhoeagenic bacteria, may be the method of choice for the study of *C. hyointestinalis* pathogenicity, at least until the possible role of *C. hyointestinalis* in proliferative enteritis is resolved. If *C. hyointestinalis* genes contributing to the diarrhoeagenic capacity of the bacterium were identified, it is likely that they would prove to be important in the understanding of the pathogenesis of proliferative enteritis.

The problems with restriction of incoming DNA, and replicon specificity, in *C. hyointestinalis* may not be unique to *C. hyointestinalis* among campylobacters. The work of this thesis provides a basis for the future genetic analysis of *C. hyointestinalis*. The shuttle vectors developed here, being cosmids, have the potential to be used in the cloning of large fragments of *C. hyointestinalis* DNA in *E. coli* K-12, and, being mobilizable, may also be used in the transfer of that DNA to *C. hyointestinalis* or other *Campylobacter* strains. This means that the technique of allelic exchange is now available for *C. hyointestinalis*. Such an approach to the molecular analysis of pathogenicity was previously confined to *C. jejuni*, *C. coli*, and *C. fetus*, among the campylobacters. It is true, however, that interesting genetic candidates for allelic exchange studies in *C. hyointestinalis* are not obvious as the virulence determinants of *C. hyointestinalis* are not understood. In the early work of this thesis, *C. hyointestinalis*-specific outer membrane proteins and LPS moieties (not present in *C. jejuni* or *C. coli*, as determined by immunoblotting), were identified. It may be that an understanding of the genetics of the synthesis of these materials would contribute to an understanding of the

unique features of *C. hyointestinalis* pathogenicity, and lead to the development of rational candidate vaccines against diseases caused by this organism.

APPENDIX

Certain material presented in this thesis has been published or are in preparation for publishing.

Published material:

Waterman, S.R., and Hackett, J. (1992) Outer membrane components of *Campylobacter hyointestinalis*. *FEMS Microbiol. Lett.* **92**: 279-284.

Manuscripts in preparation:

Waterman, S.R., Hackett, J., and Manning, P.A. (1992) Characterization of the replicative region of the small cryptic plasmid of *Campylobacter hyointestinalis*.

Waterman, S.R., Hackett, J., and Manning, P.A. (1992) Construction of a cloning shuttle vector in *Campylobacter hyointestinalis*.

Waterman, S.R., Hackett, J., and Manning, P.A. (1992) Recognition of a sequence at the nick region of the RP4 plasmid transfer origin that is essential for cleavage following transfer.

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