NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

UNIVERSITY OF ALBERTA

CHARACTERIZATION OF ERYTHROMYCIN RESISTANCE IN *CAMPYLOBACTER SP.*

BY

© WILLIAM YAN

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

IN

MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA
FALL, 1990

ISBN 0-315-64767-1

UNIVERSITY OF ALBERTA

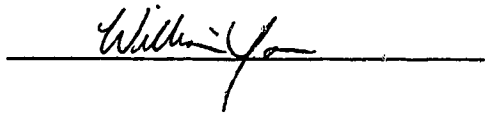
RELEASE FORM

NAME OF AUTHOR: WILLIAM YAN

TITLE OF THESIS: CHARACTERIZATION OF ERYTHROMYCIN RESISTANCE IN *CAMPYLOBACTER SP.*

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: FALL, 1990



6127-137 Avenue,
Edmonton, Alberta
T5A OP2

Date: August 28, 1990

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

THE UNDERSIGNED CERTIFY THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED CHARACTERIZATION OF ERYTHROMYCIN RESISTANCE IN *CAMPYLOBACTER SP.* SUBMITTED BY WILLIAM YAN IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES.

Diane E. Taylor

Dr. Diane E. Taylor

(Supervisor)

Glen D. Armstrong

Dr. Glen D. Armstrong

Mark S. Peppler

Dr. Mark S. Peppler

Kenneth L. Roy

Dr. Ken L. Roy

L. E. Bryan

Dr. Lawrence E. Bryan

(External Examinar)

Date: August 27, 1990

Abstract

The mechanism of resistance to erythromycin, the drug of choice in the treatment of gastroenteritis caused by *C. jejuni* and *C. coli*, is analyzed in these studies. Erythromycin resistances in three clinical isolates of *C. jejuni* and one *C. coli* isolate were found to be constitutive and chromosomally mediated. A high concentration of erythromycin failed to inhibit *in vivo* protein synthesis in these strains. Biological assays showed that extracellular degradation of erythromycin was not responsible for erythromycin resistance. The rates and amounts of uptake of [^{14}C]erythromycin by resistant and sensitive Campylobacter cells were determined to be similar. Binding assays indicated that [^{14}C]erythromycin binds significantly less to erythromycin-resistant Campylobacter 70S ribosomes and 50S ribosomal subunits than to those of sensitive strains. Therefore, it is likely that mutation in the Campylobacter 50S ribosomal subunit resulted in lower binding and constitutive resistance to erythromycin.

DNA from the nalidixic acid resistant *C. coli* UA417 and its streptomycin resistant derivative, UA417R, were digested with *Sal*I and *Sma*I and the sizes of the resulting fragments were determined using pulsed field gel electrophoresis. Similar restriction patterns of 7 and 13 fragments for the two respective enzymes were obtained and

the total genome size was determined to be approximately 1,700 kb. A physical map of the *C. coli* UA417R genome was constructed and the locations of the genes coding for 16S rRNA, the ß subunit of RNA polymerase, as well as genes involved in erythomycin and streptomycin resistance were determined. *Sma*I restriction patterns of 12 *C. jejuni* and 10 *C. coli* isolates were analyzed by PFGE and 16S rRNA hybridization. Although the two *Campylobacter* species displayed distinct restriction and hybridization patterns, significant intra-species differences were observed. Differentiation between the two species based on their restriction patterns fully coincided with results from hippurate hydrolysis and DNA dot blot hybridizations. Therefore, PFGE of Campylobacter genomic DNA digested with *Sma*I provides a reliable means of differentiating *C. jejuni* from *C. coli*. Furthermore, the distinct restriction patterns observed among many of the unrelated *C. jejuni* and *C. coli* strains indicates that PFGE may be a more practical approach to epidemiological studies than combining conventional electrophoresis with RNA hybridization procedures.

Acknowledgement

I would like to express my sincere gratitude to my supervisor, Dr. Diane Taylor, who provided me with the opportunity to carry out my PhD. studies in her laboratory. I have benefited immensely from her scientific expertise and patient guidance throughout my research training. The advice and assistance from the members of my supervisory committee, Dr. Glen Armstrong, Dr. Bill Paranchych, Dr. Mark Peppler and Dr. Ken Roy is also greatly appreciated. I have been extremely fortunate to have had the opportunity to work with and learn from all the students and staff members of the Department of Medical Microbiology and Infectious Diseases. In particular, my thanks go out to Ben Bablitz, Elisa Brose, Nick Chang, Andrew Chau, Koji Hiratsuka, Declan Maher, Elias Manavathu, Peter Newnham, Richard Sherburne, Michelle Simons, Emily Walter and Ying Wang for their advice, assistance, friendships and above all for their contribution toward a stimulating, productive and yet never boring working atmosphere in the lab over the past four years. I would also like to acknowledge San Vinh for his assistance with photography. The special friendships of Carol Ewanowich and Sharee Kuny, who were always there to listen, are deeply appreciated and will always be treasured. I also thank Liane Kim, whose unique and charming personality was a breath of fresh air that was much needed during the final

stretch of my thesis writing. I would like to acknowledge the Alberta Heritage Foundation for Medical Research and the Department of Medical Microbiology and Infectious Diseases for their financial support throughout my PhD program. Above all, I am grateful to my parents for their love, support and understanding without which my PhD studies would not have been possible.

Table of Contents

3. Results

List of Tables

List of Figures

List of Abbreviations

AMP	adenosine-monophosphate
ATCC	American Type Culture Collection
BAP	blood agar plate
BSA	bovine serum albumin
CFU	colony forming units
CHO	Chinese Hamster Ovary
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dal	daltons
EDTA	ethylenediaminetetraacetic acid
erm	erythromycin-resistance-methylase
G + C	Guanine + Cytosine
kb	kilobases
kdal	kilodaltons
LMP	low melting point
MH	Mueller Hinton
MIC	minimal inhibitory concentration
MLS	macrolide-lincosamide-streptogramin B
PEG	polyethylene glycol
PFGE	pulsed-field gel electrophoresis
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

SSC	standard sodium citrate
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet

1. Introduction

1.1a Relevant properties of *Campylobacter* spp.

Bacteria belonging to the genus *Campylobacter* are small (0.5 to 8 µm long and 0.2 to 0.5 µm wide), nonsporulating, gram-negative rods that grow optimally under microaerophilic conditions (5 - 10% O_2, 3 - 10% CO_2). They are typically spiral or S-shaped in young cultures and undergo a degenerative change to coccoid forms in older cultures (Ng *et al.*, 1985). Most species of *Campylobacter* have a single polar unsheathed flagellum at either one or both ends of the cell which accounts for their characteristic darting motility (Smibert, 1978). *Campylobacter* spp. was originally designated as "microaerophilic vibrios" (MacFadyean and Stockman, 1913). The group was later divided into *Vibrio fetus*, *V. jejuni* and *V. coli* in the family *Vibrionaceae* (Smith and Taylor, 1919; Jones and Little, 1931; Doyle, 1944). A major breakthrough occurred in 1947 when these organisms were cultured from human blood for the first time (Vinzent *et al.*, 1947). In 1957, it was observed that some members of the group which grew at the higher temperature of 42°C were associated with diarrheal diseases (King, 1957). By the early sixties, it became apparent that despite the similarity in morphology, this group of organisms was unrelated to other vibrios due to their different base

composition (Guanine + Cytosine content) and their varying ability to utilize sugars as energy sources (Alexander, 1957; Lecce, 1958; Sebald and Veron, 1963). Accordingly, a new genus, *Campylobacter*, in the family *Spirillaceae*, was created to include *V. fetus* and the other related organisms (Sebald and Veron, 1963; Veron and Chatelain, 1973).

1.1b Clinical significance of Campylobacter gastroenteritis. The importance of Campylobacter as the causative agent in human enteritis became apparent with the development of a direct plating method for the isolation of these organisms from clinical samples (Skirrow, 1977). By 1984, *Campylobacter* spp. ranked with or next to *Salmonella* spp. as the leading cause of human gastroenteritis (Finch and Riley, 1984). Of all the *Campylobacter* species, *C. jejuni*, *C. coli*, *C. fetus* and *C. laridis* are considered to be the major human pathogens. They were found to share less than 35% nucleotide similarity under stringent hybridization conditions (Belland and Trust, 1982; Harvey and Greenwood, 1983; Leaper and Owen, 1982; Owen, 1983; Ursing *et al.*, 1983). Of these, *C. jejuni* and *C. coli* are by far the most frequently isolated from patients suffering from Campylobacter gastroenteritis.

Following the recognition of Campylobacter gastroenteritis as an important clinical problem, there has been much interest in studying the pathophysiology of

this disease. Although Campylobacter enteritis is an important diarrheal disease worldwide, many aspects of Campylobacter infection show significant differences between developing and developed countries. The incidence of outbreaks of Campylobacter enteritis is highest during the summer months in developed countries whereas no apparent seasonal trend has been observed in developing countries (Blaser and Reller, 1981). Although Campylobacter enteritis can affect all age groups, the incidence appears to be highest among young children (Karmali and Fleming, 1979). It has been observed that in developing countries asymptomatic infection occurs in approximately 40% of young children whereas the isolation rate is much lower in adults (Blaser et al., 1983). In developed countries, on the other hand, asymptomatic carriers have not been reported. Symptoms associated with Campylobacter enteritis are milder and without inflammatory diarrhea in developing countries (Blaser *et al.*, 1983). The disease is usually gastrointestinal with abdominal pain, fever and possible complications such as Reiter's syndrome, reactive arthritis and Guillain-Barre syndrome (Jhonsen *et al.*, 1983; Ebright and Ryay, 1984; Kaldor and Speed, 1984). The incubation time varies between 1 to 7 days with diarrhea being self limited from 2 to 7 days in most cases (Blaser *et al.*, 1983). In addition to these common symptoms, bloody stools with mucus and fecal

leukocytes are frequently found in patients with Campylobacter enteritis in developed countries (Blaser *et al.*, 1983). The infectious dose has been reported to range from 500 to 10^6 organisms, depending on individual susceptibility or on the relative virulence of the Campylobacter strain (Steele and McDermott, 1978; Robinson, 1981). *C. jejuni* and *C. coli* are believed to be transmitted by consuming contaminated food and water, unpasteurized milk as well as by contact with fecal material from infected animals or humans (Blaser and Reller, 1981; Karmali and Fleming, 1979). *C. jejuni* is commonly found as a commensal in poultry, cattle, sheep and dogs whereas *C. coli* is mainly found in hogs. In developed countries such as Canada and the United Kingdom, the majority of Campylobacter gastroenteritis cases (95 - 98%) is caused by *C. jejuni* with *C. coli* being responsible for only 2 to 5% of the cases (Karmali *et al.*, 1983; Karmali and Skirrow, 1984; Thompson *et al.*, 1986). Other studies have shown a higher incidence of *C. coli* being isolated from patients with Campylobacter enteritis. One such report from Hong Kong showed 41% of Campylobacter isolates from outbreaks were *C. coli* (Ho and Wong, 1985). Nevertheless, it is clear from these studies that *C. jejuni* and *C. coli* are important causative agents of human gastroenteritis. As a result, extensive research over the past fifteen years has focused on studying (i) the

phenotypic markers useful for serotyping, biotyping and to investigate epidemiology; (ii) the possible virulence factors associated with these organisms in the establishment of infection; and (iii) the mechanisms by which they can mediate resistance to various groups of antibiotics.

1.1c Epidemiological markers of *C. jejuni* and *C. coli*. *C. jejuni* and *C. coli* are catalase positive, grow at 37°C and 42°C but not at 25°C, are susceptible to nalidixic acid (40µg/ml), and resistant to cephalothin (64 µg/ml) (Karmali and Skirrow, 1984). The two species are phenotypically very similar and have 53-96% DNA homology within species and less than 49% between species (Hebert *et al.*, 1984; Owen and Leaper, 1981; Roop *et al.*, 1984; Ursing *et al.*, 1983). Besides DNA homology studies, the most reliable test for differentiating *C. jejuni* from *C. coli* is the hippurate hydrolysis test (Harvey, 1980). *C. jejuni* was found to produce an enzyme, hippuricase, which hydrolyzes hippurate to give the end products benzoic acid and glycine. The glycine is in turn detected using ninhydrin reagent. *C. coli* strains, on the other hand, lack this enzyme and are therefore negative for hippurate hydrolysis. Unfortunately, a few hippurate-negative *C. jejuni* strains have been isolated thus raising questions as to the usefulness of the hippurate hydrolysis test (Hebert *et al.*, 1984; Totten *et al.*, 1985). Using gas-liquid

chromatography to detect small amounts of benzoic acid, Morris *et al.* (1985) showed that the traditional tube test lacked sensitivity. This might account for weak hippuricase producing *C. jejuni* strains being incorrectly labelled as hippurate-negative.

Like other *Campylobacter* spp., *C. jejuni* and *C. coli* cannot utilize sugars as energy sources and are inert in most biochemical tests. This poses problems in attempting to develop selective and/or differential media, screening for epidemiological markers as well as for determining the degree of relatedness of various *Campylobacter* spp. Various antibiotics such as cephalosporins, trimethoprim, vancomycin, rifampicin, polymyxins, bacitracin, novobiocin and fungicides, have been used as selective agents in media for the isolation of *C. jejuni* and *C. coli*. Two different serotyping schemes, developed by Lior and co-workers and Penner and co-workers, which are based on heat-labile and heat-resistant antigens respectively, are now commonly used to serotype strains of *C. jejuni* and *C. coli* (Lior *et al.*, 1982; Penner and Hennessy, 1980). These two schemes have since been compared and found to be equally effective (Jones *et al.*, 1985; Kaijser and Sjogren, 1985; Patton *et al.*, 1985). The first biotyping scheme for *Campylobacter sp.* involved testing for the production of H_2S in an iron medium containing 0.05% each of ferrous sulfate, sodium metabisulfite and sodium pyruvate (Skirrow and Benjamin,

1980). Based on this test, in which H_2S is derived from cysteine and thiosulfate by the action of cysteine desulfhydrase and thiosulfate reductase respectively, *C. jejuni* was differentiated into two biotypes. Since then, another biotyping scheme with the inclusion of a DNA hydrolysis test was developed which divided *C. jejuni* into four biotypes and *C. coli* into 2 biotypes (Lior, 1984). Other less common markers that have been proposed for epidemiological studies are phage typing (Grajewski *et al.*, 1985), plasmid profiles (Bopp *et al.*, 1985; Tenover *et al.*, 1984), chromosomal DNA restriction patterns (Bradbury *et al.*, 1984), auxotyping (Tenover *et al.*, 1985) lectin interaction (Wong *et al.*, 1986), detection of polymorphism in DNA encoding rRNA (Moureau *et al.*, 1989) and outer membrane gel electrophoresis profiles (Derclaye *et al.*, 1989). However, the relevance and overall effectiveness of these epidemiological markers has yet to be clearly established.

1.1d Virulence factors associated with *C. jejuni* and *C. coli*. Although *C. jejuni* and *C. coli* are now recognized as important human pathogens, the precise mechanism by which these organisms bring about gastroenteritis remains controversial. Two exotoxins, a heat-labile enterotoxin and a cytotoxin, are found to be produced by *C. jejuni*. The heat-labile enterotoxin, a 60,000 to 70,000 dalton protein which is functionally and

immunologically related to cholera toxin and heat-labile toxin of *Escherichia coli*, functions by increasing the intracellular concentration of cyclic-AMP following stimulation of the enzyme adenylate cyclase (Ruiz-Palacios *et al.*, 1983; McCardell *et al.*, 1984)). It has been found to cause elongation of Chinese Hamster Ovary (CHO) cells as well as rounding of Y-1 mouse adrenal cells (Ruiz-Palacios *et al.*, 1983; McCardell *et al.*, 1984). Less is known about the cytotoxin of *C. jejuni* which was found to have toxic effects on Vero (Johnson and Lior, 1984), CHO (Guerrant *et al.*, 1985; Johnson and Lior, 1984) and HeLa cells (Guerrant *et al.*, 1985; Yeen *et al.*, 1983). Although one or both of these *C. jejuni* exotoxins may be responsible for human clinical symptoms ranging from watery diarrhea to dysentery with bloody mucus and leukocytes in stool, their precise pathogenic significance has not been established.

Similarly, it is not clear whether the invasiveness of *C. jejuni* and *C. coli* plays a major role in the overall infective process. It has been shown that certain strains of *C. jejuni* and *C. coli* can invade the intestinal epithelium of infant mice (Newell and Pearson, 1984). Other studies have shown that *C. jejuni* and *C. coli* can associate with and invade HeLa cells (Manninen et al., 1982) and HEp-2 cells (Konkel and Joens, 1989). The observation that *C. jejuni* can survive for up to 7 days intracellularly in peripheral blood monocytes *in vitro* led

to the postulation that this may be a virulence factor in infection (Kiehlbauch *et al.*, 1985). However, neither the relationship between clinical presentation and invasiveness in *in vitro* systems nor the mechanism by which *C. jejuni* and *C. coli* invade different cells have been determined.

The best established virulence factor in *C. jejuni* and *C. coli* infections is the flagellum produced by these organisms. The flagellum of *C. jejuni* is unsheathed, consists of a 20 nm filament, a hook and a large disk associated with the basal body (Morooka *et al.*, 1983). The flagellin subunit ranges from 57,000 to 66,000 in molecular weight. It has been shown that *C. jejuni* is rapidly killed at pH 2.3 thus suggesting its susceptibility to gastric acid (Blaser *et al.*, 1980). Therefore, it would appear essential for *C. jejuni* to move through the mucus layer in order to attach and colonize epithelial cells. The role the flagellum plays in motility and as a possible adhesin established this surface protein structure as an important virulence factor. Caldwell and co-workers first reported the spontaneous bidirectional phase transition between flagellated and aflagellated phenotypes in *C. jejuni* (Caldwell *et al.*, 1985). It was shown that when *C. jejuni* was grown in culture, emergence of the aflagellated phenotype occurred at a frequency of 10^{-3} per cell per generation, whereas cells grown in rabbit intestine stongly favored the emergence of flagellated phenotype. In another

study, motile, flagellated *C. jejuni* strains were found to colonize the gastrointestinal tract of experimentally infected mice more successfully than aflagellated strains (Newell and McBride, 1985). In addition to phase variation, antigenic variation in *C. coli* flagella associated with programmed, reversible genomic arrangement has also been reported (Harris *et al.*, 1987). This antigenic variation may play an important role in protecting the bacteria from the host immune system. All these findings lend further support to the Campylobacter flagellum being an essential factor in the virulence of these organisms. Recently, a gene coding for the production of flagellin protein in *C. coli* was cloned and sequenced (Logan *et al.*, 1989). Furthermore, detailed analysis of the genomic organization and expression of flagellin genes have indicated that flagellin antigenic variation may involve rearrangement of sequences of two adjacent full-length flagellin genes, *flaA* and *flaB* (Guerry *et al.*, 1990). The elucidation of the molecular basis of flagellin antigenic variation will be invaluable for future attempts to use Campylobacter flagella as a possible candidate for vaccine development.

1.1e Mechanisms of antibiotic resistance in *C. jejuni* and *C. coli*. Although enteritis due to campylobacters is usually a self-limiting disease, treatment is often required in septicemic patients and

patients with prolonged illness. As a result, a substantial amount of interest has been shown over the years in determining the biochemical and genetic basis of resistance to various antibacterial agents in Campylobacter as well as the means by which these organisms acquired the specific resistance determinants. Various modes of resistance to different groups of antibiotics have evolved in microorganisms, including (i) exclusion of the drug at membrane barriers; (ii) chemical inactivation by extracellular enzymes (eg. ß-lactamases); (iii) efflux mechanisms for removal of intracellular antibiotics (eg. tetracycline); (iv) inactivation by intracellular enzymes (eg. aminoglycosides) and (v) modification of the target sites to render them unavailable to antibiotics. Research on antibiotic resistance in Campylobacter has mainly focused on *C. jejuni* and to a lesser extent, *C. coli*.

As previously mentioned, *C. jejuni* and *C. coli* are generally susceptible to nalidixic acid (MIC 2 - 16 µg/ml)(Karmali *et al.*, 1981). However, nalidixic acid resistant *C. jejuni* strains (MIC > 40µg/ml) occuring in 3% of 95 strains tested has been reported (Vanhoof *et al.*, 1978). Spontaneous mutations from nalidixic acid sensitivity to resistance in *C. jejuni* and *C. coli* occurs at a frequency of 10^{-8} (Taylor *et al.*, 1985). Furthermore, these mutants showed cross resistance to other quinolones such as enoxacin and ciprofloxacin. The mechanisms of

resistance against these DNA gyrase subunit A inhibitors have not been determined, although it is likely to involve either a change in the DNA gyrase enzyme or impermeability to the drugs.

Resistance to the aminoglycoside kanamycin is often plasmid mediated and is more frequently associated with *C. coli* than *C. jejuni* (Kotarski *et al.*, 1986; Lambert *et al.*, 1985; Sagara *et al.*, 1987). A 1,427 bp resistance determinant was cloned from the plasmid pIP1433 originally harbored in *C. coli* BM2509 and was found to contain the gene for the enzyme 3'-aminoglycoside phosphotransferase type III (Trieu-Cuot *et al.*, 1985). This gene, designated *aphA-3*, was previously found to be present only in gram positive cocci. Recently, DNA hybridization studies showed the *aphA-3* gene to be present in the chromosome of another kanamycin resistant isolate, *C. coli* UA696, which was originally isolated in Alberta, Canada (Taylor *et al.*, 1988). Therefore, it would appear that kanamycin resistant *C. coli* has acquired its resistance determinant from gram-positive cocci and that the gene *aphA-3* may be located on a transposable element.

Since the first report of plasmid-mediated tetracycline resistance in *C. jejuni* (Taylor *et al.*, 1980), plasmids coding for tetracycline resistance have been found in *C. jejuni* and *C. coli* isolates from Canada (Bradbury and Munroe, 1985; Ng *et al.*, 1987; Taylor *et al.*, 1986; Taylor

et al., 1983), France (Lambert *et al.*, 1985), the United States (Kotarski *et al.*, 1986; Tenover *et al.*, 1983; Tenover *et al.*, 1985) and Japan (Sagara *et al.*, 1987). Two tetracycline resistance determinants, both designated *tetO*, have been cloned and sequenced from *C. jejuni* pUA466 and *C. coli* pIP143 and were found to show 98% nucleotide homology with each other and 75-76% homology with the *tetM* gene in *Streptococcus pneumoniae* (Manavathu *et al.*, 1988; Martin *et al.*, 1986; Sougakoff *et al.*, 1987). No homology was found between *tetO* and other well characterized tetracycline resistance genes such as *tetA, B, C* and *D* from *Enterobacteriaceae* (Levy, 1984; Taylor *et al.*, 1983; Tenover *et al.*, 1987) and *tetK, L* and *N* from gram-positive cocci (Ng *et al.*, 1987; Sougakoff *et al.*, 1987; Tenover *et al.*, 1987). It has been shown that the protein encoded by *tetM* acts at the level of protein synthesis (Burdett, 1986;) which differs from the efflux mechanism previously reported for *Enterobacteriaceae* in which tetracycline is actively pumped out of resistant cells by a cytoplasmic membrane protein (Levy, 1984). Manavathu and co-workers (1990) found that the TetO protein also acts at the level of protein synthesis. Since TetO and TetM proteins have similar hydrophilicity profiles, it is not surprising that they also share a common mechanism of resistance. Therefore, as in the case of kanamycin resistance, *C.*

jejuni and *C. coli* appear to have acquired a tetracycline-resistance determinant from gram-positive cocci.

The Macrolide-Lincosamide-Streptogramin B (MLS) group of antibiotics include erythromycin which is the drug of choice for the treatment of Campylobacter gastroenteritis. The incidence of erythromycin resistance in *C. jejuni* ranges from 1% or fewer in the United Kingdom and Canada (Brunton *et al.*, 1978; Karmali *et al.*, 1982; Taylor *et al.*, 1986) to much higher frequencies in other European countries as well as in Japan (Michel *et al.*, 1983; Sagara *et al.*, 1987; Taylor *et al.*, 1987; Vanhoof *et al.*, 1978; Walder, 1979). In general, erythromycin resistance is more common in *C. coli* than *C. jejuni* (Karmali *et al.*, 1982; Sagara *et al.*, 1987; Taylor *et al.*, 1987; Burridge *et al.*, 1986). Macrolide resistance has been shown to be widespread in *C. coli* isolated from pigs in the United Kingdom and has been attributed to that country's policy of using tylosin and virginiamycin as growth promoters in agriculture (Burridge *et al.*, 1987). All erythromycin-resistant *C. jejuni* and *C. coli* strains are cross-resistant to spiramycin, tylosin and clindamycin. Furthermore, all *C. jejuni* and *C.coli* are intrinsically resistant to streptogramins. Aside from some evidence that erythromycin resistance in *C. jejuni* and *C. coli* are chromosomally mediated, little is known about the mechanism of resistance.

1.1f Future of Campylobacter research. In conclusion, members of the genus *Campylobacter* are a diverse group of organisms whose roles as animal and human pathogens have taken on great significance over the past twenty years. Research in *Campylobacter* has been hampered by difficulties with the development of appropriate isolation media, the lack of reliable epidemiological and taxonomic markers and above all the lack of efficient molecular genetic techniques for detailed genetic analysis of various aspects concerning this organism. The understanding of the mechanisms involved in virulence of *Campylobacter* sp. as well as their resistance to various antibiotics are currently in a state of flux. Recent advances in construction of shuttle cloning vectors for gene transfer from *E. coli* to *Campylobacter* spp. (Labigne-Roussel *et al.*, 1987), electroporation for the transformation of DNA into *C. jejuni* and *C. coli* (Miller *et al.*, 1988), natural transformation in *C. coli* (Wang and Taylor, 1990), 16S rRNA sequencing for the classification of *Campylobacter* spp. (Romaniuk *et al.*, 1987) and pulsed-field gel electrophoresis for the analysis of the *Campylobacter* genome (Yan and Taylor, in preparation) have provided the tools for finding answers to many questions that still remain concerning this microaerophilic enteric pathogen.

1.2a Mechanism of action and clinical application of MLS antibiotics. Although macrolides, lincosamides and streptogramin B are chemically distinct, they are closely related in their mode of action, their spectrum of activity and bacterial resistance mechanisms. Thus the term "MLS antibiotics" has been coined for this group of antibiotics (Weisblum, 1975). Erythromycin, the drug of choice for the treatment of Campylobacter gastroenteritis, is a member of the macrolide family of antibiotics. Like other MLS antibiotics, erythromycin inhibits bacterial protein synthesis at the chain elongation step by interfering with the peptidyltransfer reaction. Studies have shown that erythromycin binds to the large (50S) ribosomal subunit and stimulates the dissociation of peptidyl-tRNA from ribosomes (Menninger and Otto, 1982). In general, erythromycin is more effective against gram-positive than gram-negative bacteria. Most gram-negative bacteria are intrinsically resistant to low concentrations of MLS antibiotics due to (i) impermeability of the outer membrane to this group of hydrophobic antibiotics; (ii) natural methylation of certain adenine residues of 23S rRNA (Taubeneck, 1962; Mao and Putterman, 1968). Nevertheless, erythromycin therapy is still recommended for treatment of gastroenteritis in some cases since the accumulated local concentration of MLS antibiotics in the lumen of the intestinal tract is higher than the MICs of gram-negative

intrinsic resistances. Shortly after the introduction of erythromycin into clinical practice (McGuire *et al.*, 1952), erythromycin-resistant *Staphylococcus aureus* isolates were reported from France, the United Kingdom and the United States (Chabbert, 1956; Jones *et al.*, 1956; Garrod, 1957). Today, resistance to erythromycin as well as other MLS antibiotics is widespread among both gram-positive and gram-negative bacteria. Over the past twenty years, results from extensive research have demonstrated that alteration of the 50S ribosomal subunit is responsible for MLS resistance in microorganisms such as *Staphylococcus, Streptococcus, Bacillus, Bacteroides, Arthrobacter, Corynebacterium,* enterobacteria as well as the natural producers of MLS antibiotics, *Streptomyces*. Other studies have shown that drug modification by specific extracellular enzymes also plays an important role in bacterial resistance to some members of this group of antibiotics. The mechanisms and regulation of MLS resistance have been the subject of a number of reviews over the past several years (Arthur et al., 1987b; Cundliffe, 1989; Dubnau and Monod, 1986; Weisblum, 1985)

1.2b Role of erythromycin-resistance-methylases (*erm*) genes in MLS resistance. Resistance to MLS antibiotics, particularly in gram-positive cocci and *Streptomyces*, has been well characterized. Resistance to various MLS antibiotics can be constitutive or inducible.

Induction is usually specific (eg. MLS resistance in *S. aureus* is only induced by the 14-member macrolides erythromycin and oleandomycin (Weaver and Pattee, 1964) and the lincosamide celestricetin (Allen, 1977). All known inducers of MLS resistance, however, have been shown to be protein synthesis inhibitors (Pestka *et al.*, 1976). The importance of rRNA in the overall resistance to MLS antibiotics is now well documented. It was shown that ribosomes from *S. aureus*, which are normally highly sensitive to erythromycin, can be induced to become resistant due to reduced ribosomal affinity for the antibiotic (Kono *et al.*, 1966). Furthermore, it was determined that erythromycin was not inactivated by resistant *S. aureus* strains (Nakajima *et al.*, 1968). Instead, studies have shown that N^6-dimethylation of a specific adenine residue in the 23S rRNA was responsible for the decreased ribosomal affinity for MLS antibiotics (Lai and Weisblum, 1971; Lai *et al.*, 1973a). The resistant determinant codes for a *S*-adenosylmethionine-dependent 23S rRNA transmethylase which acts on an unpaired adenine (position 2058 of 23S rRNA)(Lai *et al.*, 1973b; Shivakumar and Dubnau, 1981; Skinner and Cundliffe, 1982) This adenine residue is located in a highly conserved region of the 3' half of prokaryotic 23S rRNA and has been postulated to be directly involved in the formation of the peptidyl transferase centre (Sigmund *et al.*, 1988). Since the

initial discovery, genes coding for methylases involved in MLS resistance have been found to be widespread among different organisms. Some of these genes (coined *erm* for erythromycin resistance methylase) were found to be located in plasmids and transposons (Foster, 1983; Clewell, 1981). Table 1.1 represents a list of *erm* genes which have been extensively characterized. Most of these *erm* genes have now been sequenced and their amino acid sequences compared. Extensive homologies shared by many of these *erm* genes have led investigators to attempt to establish evolutionary relationships between these resistance genes from various organisms.

1.2c Evolutionary relationship of *erm* genes. Comparisons of *erm* gene sequences at the protein level as well as in their codon usage have shed some light on their evolutionary relationships. The *ksgA* gene from *E. coli*, which codes for a 16S rRNA methylase responsible for susceptibility to the antibiotic kasugamycin, was also used in these comparisons. These rRNA methylases showed clusters of homology suggesting they are likely to be derived from a common ancestor (Dubnau and Monod, 1986; Arthur *et al.*, 1987b). Phylogenetic tree analysis showed that the non-erythromycin producers (*ermA, B, C, D, F, G* and *P*), the erythromycin producers (erm*E, A'*) and *ksgA* are located on 3 separate lines of descent (Arthur *et al.*,

Table 1.1 Comparison of MLS resistance determinants

Gene	Origin	Expression[1]	%G+C of erm	Reference
ermA	*Staphylococcus aureus* Tn554	IN	32.5	Murphy, 1985
ermB	*Streptococcus sanguis* pAM77	IN	32.0	Horinouchi *et al.*, 80
ermC	*Staphylococcus aureus* pE194	IN	26.0	Gryczan *et al.*, 1980
ermD	*Bacillus licheniformis*	IN	39.0	Gryczan *et al.*, 1984
ermE	*Streptomyces erythraeus*	CON	72.0	Uchiyama & Weisblum, 85
ermF	*Bacteroides fragilis* pBF4	CON	34.0	Rasmussen *et al.*, 86
ermA'	*Arthrobacter* spp.	ND	76.0	Roberts *et al.*, 1985

---

[1]Expression: IN=inducible; CON=constitutive; ND=not determined

1987b). Based on analysis of codon usage, it was determined that the presence of *erm* genes in gram-positive cocci is ancient since the preferential codon usage in these genes reflect the G + C content of the host chromosome. On the other hand, recent characterization of a methylase gene found in enterobacteria showed significant homology with the *ermB* gene from *Streptococcus* and demonstrated gram-positive codon usage (Trieu-Cuot *et al.*, 1987). This strongly suggests that the acquisition of this rRNA methylase gene from a gram-positive coccus was a recent evolutionary event. Two different theories have been proposed to explain how a common ancestor *erm* gene evolved into the diverse classes of *erm* genes found in various organisms today. The first theory favors the MLS producing bacteria such as *Streptomyces* and *Arthrobacter* as the original source of *erm* determinants (Walker and Walker, 1970; Benveniste and Davies, 1973). Based on homology as well as the fact that producer organisms are the first to be faced with selective pressure of the antibiotic, it is quite feasible that MLS resistance originated in these producer organisms before being disseminated among other bacteria. The transfer of genes within and between genera can be by transformation, self-transferable plasmids as well as transposable elements (Trieu-Cuot *et al.*, 1987; Arthur *et al.*, 1987a; Courvalin and Carlier, 1987). Divergent evolution in various hosts would then be

responsible for the present diversity of rRNA methylase sequences. The second theory, on the other hand, suggests convergent evolution as the basis of *erm* genes being present in non-producer bacteria. This was supported by the observation that homology between *ksgA* gene and *erm* genes was similar to that between producer and nonproducer *erm* genes (18-24%)(Arthur *et al.*, 1987b). The *ksgA* gene of *E. coli* dimethylates 2 adjacent adenines near the 3' end of 16S rRNA and is believed to play an important role in ribosome synthesis (Helser *et al.*, 1972). Therefore, it is possible that *erm* genes could have been derived from a homologous methylase gene originally required for cellular functions. These resistance genes, in turn, could be desseminated into other bacteria. It is not clear at the present time which of these theories, or whether a combination of the two, is the correct explanation of how *erm* genes evolved in MLS resistant organisms.

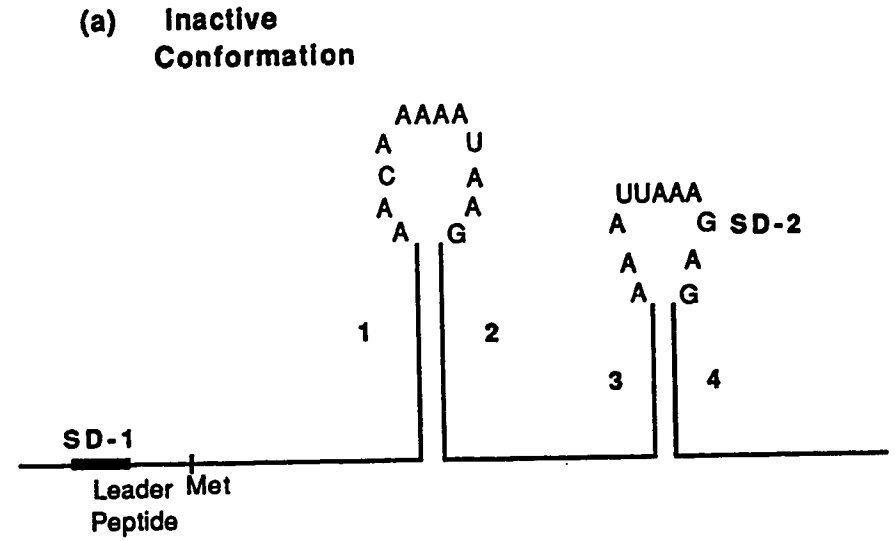
1.2d Regulation of methylase production - the translational attenuation model. Similar to the homology observed between different *erm* genes, the mechanism by which the expression of some of these genes is regulated is also highly conserved. The regulation of *ermC* expression has been characterized most extensively and results from these studies provide the basis for the translational attenuation model as the mechanism by which all inducible *erm* genes are regulated. It was shown that

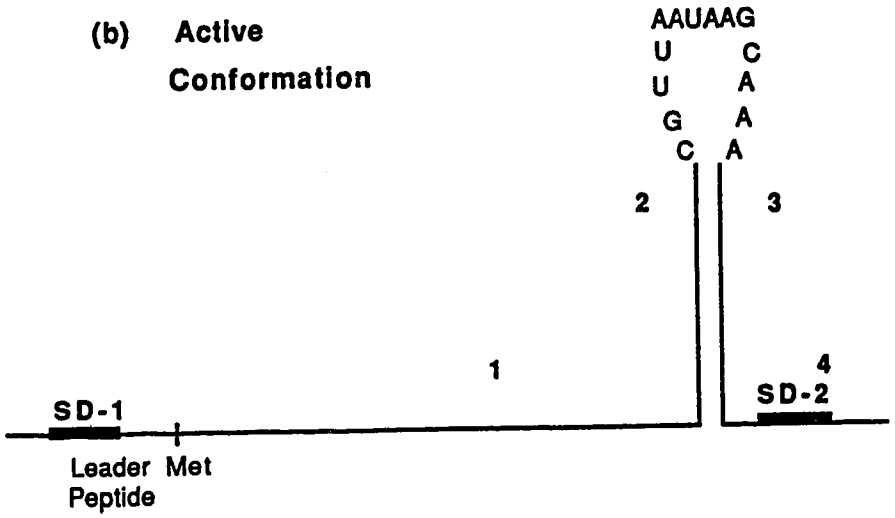
ermC mRNA is constitutively produced and that activation by an inducer takes place post-transcriptionally (Shivakumar *et al.*, 1980). The *ermC* gene has been sequenced and shown to have an open reading frame for a 29 kilodalton protein preceded by a 141 base leader sequence including an open reading frame for a 19 amino acid leader peptide (Gryczan *et al.*, 1980; Horinouchi and Weisblum, 1980). This leader sequence contains four complementary inverted repeats which are capable of forming stem-loop structures. Two similar models, with variation in some of the more detailed events, have been proposed for this complicated regulation mechanism (Gryczan *et al.*, 1980; Hahn *et al.*, 1982 and Horinouchi and Weisblum, 1980, 1981).

According to both models, the four inverted complementary repeat sequences (1, 2, 3 and 4) can base-pair to give two different conformations. In the first confomation, two stem-loop structures, 1 + 2 and 3 + 4 are formed (Fig. 1a). The base pairing of 3 + 4 stem-loop partially blocks the Shine-Delgarno 2 (SD-2) sequence, which is required for ribosome attachment preceding the translation of the 29 kdal methylase, thus results in an inactive mRNA. In the second conformation, an inducer such as erythromycin acts on sequence 1 during its translation into the 19 amino acid leader peptide. As the ribosome stops during translation, sequence 2 is physically displaced from sequence 1 and base pairs with sequence 3.

Fig.1.1 Regulation of *ermC* expression by translational attenuation. Fig. 1.1a and Fig. 1.1b represent the inactive and active conformation of the translation attenuation model. SD-1 and SD-2 represent the locations of the Shine-Delgarno sequences 1 and 2 respectively.

Regulation of *ermC* Expression





This 2 + 3 conformation unmasks SD-2 and results in the activation of the methylase mRNA (Fig. 1b). Thus *ermC* is regulated by translational attenuation. In addition, the *ermC* methylase autoregulates its own production by binding to *ermC* mRNA and prevent its use by the ribosomes. A similar translational attenuation mode of regulation has been proposed for other *erm* genes such as *ermA* of *S. aureus* (Murphy, 1985), *ermB* from *S. sanguis* (Horinouchi *et al.*, 1983) and *ermD* from *B. licheniformis* (Gryczan *et al.*, 1984). It should be noted, however, that other *erm* genes show different mechanisms of regulation. The *ermF* gene lacks a leader sequence and its expression in *B. fragilis* is constitutive (Ramussen *et al.*, 1986). Similarly, *ermE* and *ermA'* lack leader sequences and may be coordinately regulated with adjacent genes (Roberts *et al.*, 1985; Uchiyama and Weisblum, 1985). This led to the theory that the ancestral *erm* gene was either constitutively expressed or unregulated. Over time, the metabolic stress associated with the production of large amount of methylases led to the evolution of a complex regulatory apparatus.

1.2e Resistance to MLS antibiotics due to alteration in ribosomal proteins. Unlike the widespread MLS resistance due to methylation of 23S rRNA, resistance due to ribosomal protein alteration has been found exclusively in *E. coli* and is less well characterized. One study showed mutations in one or both

of ribosomal proteins L4 and L22 to be responsible for high levels of erythromycin resistance in *E. coli* (Wittmann *et al.*, 1973). Similarly, lincomycin resistant *E. coli* mutants have been shown to possess an alteration in either the L14 or L15 ribosomal proteins (Hummel *et al.*, 1979). The precise role these ribosomal proteins play in antibiotic binding, whether they are actually part of the antibiotic binding site or whether they play an indirect role in shaping the overall geometry of the binding site, has yet to be determined. Thus it is now apparent that methylation of 23SrRNA, and, to a certain extent, ribosomal protein modification, play a crucial role in bacterial resistance to different MLS antibiotics.

1.2f Ribosomal modification: rRNA vs. ribosomal proteins as target site. It has been a long held notion that resistance involving modification of ribosomes is mainly focused on alterations in ribosomal proteins. Table 1.2 lists some examples of ribosomal protein alterations which have resulted in prokaryotic resistance to different antibiotics. This has been challenged by evidence stemming from recent studies, including those involving the *erm* mode of resistance discussed above, which has led to the current consensus that ribosomal RNA may also be an important target for protein synthesis inhibitors (Cundliffe, 1986). Recent rRNA sequencing and mutant studies of various organisms have revealed the

Table 1.2 Alterations in ribosomal proteins associated with antibiotic resistance

Organisms[1]	Ribosomal Proteins	Antibiotic Resistance[2]	References
E. coli	L4	Em,Sp	Wittmann *et al.*, 1973
E. coli	L3 or L4	Ti	Bock *et al.*, 1982
E. coli	L14 or L15	Lm	Hummel *et al.*, 1979
E. coli	L6	Gm	Buckel *et al.*, 1977
E. coli	S12	Sm	Ozaki *et al.*, 1969
E. coli	S5	Spm	Bollen *et al.*, 1969
E. coli	S17	Ne	Bollen *et al.*, 1975
B. subtilis	L11 lacking	Tsp	Wienen *et al.*, 1979
B. subtilis	L11	Mi	Spedding and Cundliffe, 1984
S. cerevisiae	L24	Cy	Stocklein and Piepersberg, 80
S. cerevisiae	L3	Tr	Fried and Warner, 1981

---

[1]Organisms: *E.=Escherichia; B.=Bacillus; S.=Saccharomyces*

[2]Antibiotic Resistance: Em=erythromycin; Sp=spiramycin; Ti=tiamulin; Lm=lincomycin; Gm=gentamicin; Sm=streptomycin; Spm=spectinomycin; Ne=neamine; Tsp=thiostrepton; Mi=micrococcin; Cy=cycloheximide; Tr=trichodermin

primary and secondary structures involved in different antibiotic resistances. Mutations or modifications in rRNA have been found to be involved in the binding of different antibiotics to ribosomes (Moazed and Noller, 1987). Some examples of alterations in either the 16S or 23S rRNA which have resuled in specific resistance to various antibiotics are listed in Table 1.3. It is easy to appreciate how ribosomal protein modifications as well as methylation of specific rRNA residues by plasmid or chromosomally-encoded methylases can affect the binding of various antibiotics to ribosomes. Single mutations within rRNA (*rrn*) operons, on the other hand, are recessive since they alter only a small portion of the total cellular rRNA. For example, it has been shown that there are seven rRNA operons in *E. coli* . As a result, mutations are difficult to detect due to the masking effect of other normal *rrn* operons within the bacterial cell. In the four examples given in Table 1.3 in which mutations involving either single nucleotide conversions or deletion of specific residues resulting in antibiotic resistance, the mutated rRNA operons were all cloned into multicopy plasmids. This led to the isolations of dominant or co-dominant resistance mutations. The precise mechanism by which these recessive mutations overcome other wildtype rRNAs in the cell is not clearly understood. Previous investigations have shown that relatively small amounts of resistant RNA structures

Table 1.3

Alterations in rRNA associated with antibiotic resistance

Organisms	Resistance Phenotype[1]	Site of rRNA Modification[2]	References
Staphylococcus aureus	MLS	methylation of 23S:2058 (plasmid)	Lai *et al.*, 1973
Streptomyces erythraeus	MLS	methylation of 23S:2058	Skinner *et al.*, 1983
Streptomyces tenjimariensis	Km	methylation of 16S:1408	Beauclerk & Cundliffe, 87
Streptomyces azureus	Tsp	methylation of 23S:1067	Cundliffe, 1978
Escherichia coli	MLS	23S:2058 A to U	Sigmund *et al.*, 1984
Escherichia coli	Em,Olm,Cm	23S:2057 G to A	Ettayebi *et al.*, 1985
Escherichia coli	Em	deletion of 23S:1219-30	Steige *et al.*, 1983
Archaebacteria	Sm	16S:912 U to C	Schmid *et al.*, 1982

---

[1]Resistance: Cm=chloramphenicol; Em=erythromycin; Km=kanamycin; MLS=macrolide-lincosamide-streptogramin B; Olm=oleandomycin; Sm=stretomycin; Tsp=thiostrepton

[2]Site of modification: 16S=16SrRNA; 23S=23SrRNA

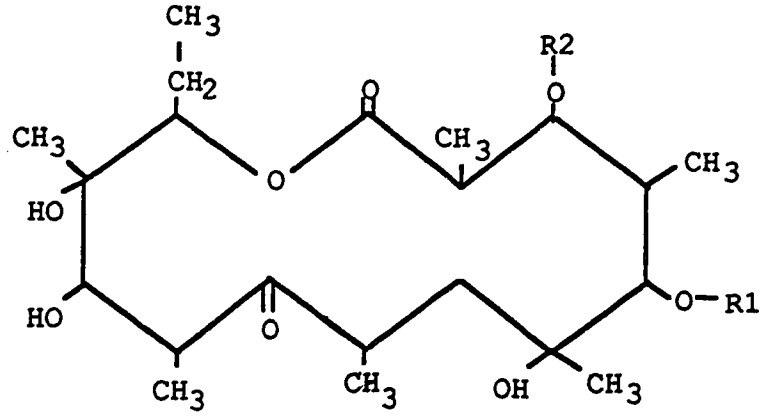
can lead to high level resistance against spectinomycin, chloramphenicol as well as MLS-antibiotics (Mark *et al.*, 1983; Sigmund *et al.*, 1984, Sigmund and Morgan, 1982). It was also demonstrated that an increased proportion ofresistant-type *rrn* operons resulted in an increased degree of resistance (Ettayebi *et al.*, 1985). Therefore, it has been suggested that resistance to these bacteriostatic antibiotics may be co-dominant with sensitivity.

1.2g Extracellular enzymatic inactivation of MLS antibiotics. In addition to ribosomal modification, resistance has also been attributed to extracellular inactivation of some MLS antibiotics involving specific enzymes produced by both gram-positive and gram-negative microorganisms. In general, this mechanism of resistance is less common and not as well characterized as the modification of the 50S ribosomal subunits. Resistance to streptogramins involving streptogramin A *O*-acetyltransferase (De Meester and Rondelet, 1976; LeGoffic *et al.*, 1977b) and streptogramin B hydrolase (LeGoffic *et al.*, 1977a) has been reported. Similarly, the lincosamides lincomycin and clindamycin have been found to be inactivated by either phosphorylation or nucleotidylation of the hydroxyl group in position 3 of these antibiotics (Marshall *et al.*, 1986). Two related Stapylococcal genes, *linA* and *linA'* (91% homology), have been shown to

inactivate lincomycin by nucleotidylation in position 4 of the antibiotic (Leclercq *et al.*, 1987). Finally, although macrolide modifying activities have been detected in *Lactobacillus* spp. (Devriese and Dutta, 1984) and *Streptomyces* spp. (Feldman *et al.*, 1964), neither the mechanism nor the genes involved have been characterized. Inactivation of erythromycin by the enzyme erythromycin esterase, which catalyzes the hydrolysis of the lactone rings of 14-membered macrolides such as erythromycin and oleandomycin (Fig. 1.2), is relatively widespread in enterobacteria (Andremont *et al*, 1986b; Arthur *et al.*, 1987a; Barthelemy *et al.*, 1984; Arthur *et al.*, 1986). Two unrelated genes, *ereA* and *ereB*, which code for type I (349 amino acids) and type II (419 amino acids) esterases, have been sequenced (Ounissi and Courvalin, 1985; Arthur *et al.*, 1987a). While the *ereA* gene has a G + C content of 50% which is similar to the host *E. coli* chromosome, *ereB* has a significantly different G + C content of 36%. Therefore, it has been suggested that *ereB* may have originated from an excgenous source such as gram-positive cocci (Arthur *et al.*, 1986; Trieu-Cuot *et al.*, 1987). Recently, a new intracellular enzyme, macrolide 2'-phosphotransferase, has been purified and characterized from an *E. coli* strain and was found to be highly active against erythromycin (O'Hara *et al.*, 1989). Therefore, unlike the situation with methylases where a single alteration in 23S rRNA leads to

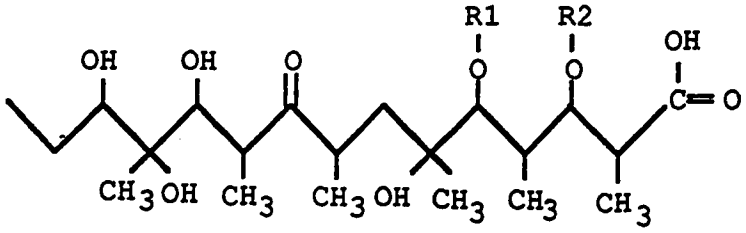
Fig. 1.2 Mechanism of action of erythromycin esterase. Inactivation of erythromycin by the enzyme erythromycin esterase involving the hydrolysis of lactone rings are shown. The structures of the side chain groups R1 and R2 are also shown.

Mechanism of Action of Erthromycin Esterase

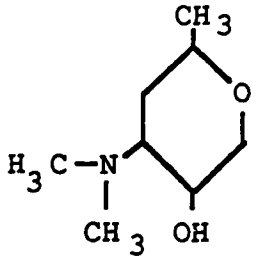


Erythromycin

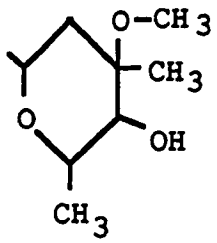
Erythromycin Esterase



Hydrolysed Erythromycin



R1=desosamine



R2=cladinose

cross-resistance to different MLS antibiotics, enzymatic inactivation of these drugs appears to involve a wide range of enzymes with varying activities against specific antibiotics.

1.3a Development and application of PFGE technology. The development of pulsed-field gel electrophoresis (PFGE) has been a major breakthrough in genomic analysis of different microorganisms. Since its invention (Schwartz *et al.*, 1984), the first major application in bacteriology has been the construction of a low-resolution restriction map of the *E. coli* chromosome (Smith *et al.*, 1987). Although conventional agarose gel electrophoresis has been widely used in molecular studies of plasmid DNA, this technique proves to be unsuitable for analysis of large molecular weight DNA such as genomic DNA. While small DNA molecules migrate through agarose matrices in a size-dependent manner due to sieving effects, large DNA molecules (>30 kb) move at similar rates through the matrix due to lack of sieving. In PFGE, DNA molecules are subjected to alternating electrical fields causing the molecules to undergo directional changes during electrophoresis. In general, large DNA molecules change direction at a slower rate than smaller DNA molecules. Therefore, separation is based on the varying degree in which the migration rates of these large DNA molecules are

retarded throughout the gel matrix. The pulse time, which corresponds to the amount of time each directional field is turned on, can be varied to obtain optimal resolution for different sizes of DNA molecules. Another important development in PFGE was the preparation of chromosomal DNA in agarose blocks. This effectively prevented the DNA from shearing thus providing intact genomic DNA for restriction analysis (Smith and Cantor, 1987; Smith *et al.*, 1988). The overall strategy employed in restriction mapping of plasmids can be applied to the construction of genomic restriction maps. The selection of appropriate restriction endonucleases capable of generating small numbers of restriction fragments is essential for the successful construction of genomic maps. Two restriction endonucleases with 8-base-pair recognition sequences, *Not*I and *Sfi*I, as well as several other enzymes with 6-base pair recognition sequences have been found to be suitable for the analysis of genomes of *E. coli* and other organisms (Smith and Condemine, 1990). Enzymes found to be ideal for analysis of one particular organism, however, may not necessarily generate suitable restriction patterns with DNA from a different organism. Therefore, as a rule, extensive screening using a large number of restriction endonucleases is required whenever PFGE is to be applied to a new microorganism. After digestion with the appropriate restriction enzymes, locations of different genes can then

be determined by hybridization studies in which the cloned genes are used as radioactively labelled probes (Smith *et al.*, 1987). Sufficient nicking of the large DNA fragments by UV irradiation before transfer from gels to nitrocellulose membranes appears to be an essential requirement for these hybridization studies (Smith *et al.*, 1988). Furthermore, the genome sizes of different organisms can be determined by the summation of restriction fragment sizes generated by a series of restriction endonucleases. Based on these techniques, the genomic maps of *Mycoplasma pneumoniae* (Wenzel and Herrmann, 1988), *Caulobacter crescentus* (Ely and Gerardot, 1988), *Haemophilus influenzae* (Kauc *et al.*, 1989), *Haemophilus parainfluenzae* (Kauc and Goodgal, 1989) and the thermophilic archaebacterium *Thermococcus celer* (Noll, 1989) have been constructed. Over the years, genetic analysis of *C. jejuni* and *C. coli* has been hampered by the lack of a genomic map for determining the locations of mutations and cloned gene segments. Therefore, we believe that construction of *Campylobacter* species genomic maps using PFGE technology would be invaluable in the genetic characterization of these enteric pathogens.

1.3b PFGE as an alternative tool in epidemiological studies of *Campylobacter* sp. As previously mentioned, bacteria belonging to the genus *Campylobacter* are biochemically inert organisms. This lack of biochemical

markers has been a problem in conducting epidemiological studies in both human and veterinary diseases. Outbreaks of human Campylobacter enteritis attributed to the consumption of unpasteurized milk (Blaser *et al.*, 1979; Robinson *et al.*, 1981) and improperly cooked meat products contaminated with *C. jejuni and C. coli* have been reported. This re-emphasizing the need for epidemiological tools to determine the source of the infectious agent and its route of transmission to the infected patients. Detection of phenotypic variations by serotyping, which involves surface antigens, as well as antibiotic sensitivities has been widely used in epidemiological studies. The influence of environmental factors on the stability of most of these phenotypic properties, however, is the major weakness of this approach. Although the use of plasmid restriction profile analysis has been useful in epidemic studies involving *Salmonella typhimurium* (O'Brien *et al.*, 1982; Olsvik *et al.*, 1985; McDonough *et al.*, 1989), *Borrelia coriaceae* (LeFebvre and Perng, 1989) and *Staphylococcus aureus* (Harstein *et al.*, 1989), its usefulness in Campylobacter epidemiology is limited since human isolates of *C. jejuni* possess plasmids at a low frequency (Austen and Trust, 1980; Bradbury *et al.*, 1983). Recently, direct genomic comparisons between *C. jejuni* isolates obtained from an outbreak of human enteritis and those from cattle and a milk source were carried out using

chromosomal restriction endonuclease digestion analysis and separation of the DNA fragments by conventional agarose gel electrophoresis (Bradbury *et al.*, 1984). Unfortunately, the large number of chromosomal DNA fragments generated by most restriction enzymes resulted in complex DNA banding patterns which were often difficult to interpret. A related approach involving rRNA hybridization has been shown to be valuable for typing strains of *Campylobacter* species (Moureau *et al.*, 1989). Differentiation of *C. jejuni* from *C. coli* based on different electrophoretic profiles of the outer membrane proteins has also been reported (Declaye *et al.*, 1989). With the small number of restriction fragments generated by PFGE analysis of bacterial chromosomes, we believe that this approach, either alone or in combination with 16S rRNA hybridization studies, may be a suitable alternative in epidemiological studies of Campylobacter infection. This is supported by the finding that PFGE restriction profiles can be used to resolve recent evolutionary divergence within lineages of *E. coli* isolated from the urinary tract of patients (Arbeit et al., 1990). Therefore, the advent of PFGE may play an important future role in both genetic as well as epidemiological characterization of the enteric pathogen Campylobacter.

2. Materials and Methods

2.1 Bacterial strains and plasmids. Erythromycin resistant and sensitive strains of *C. jejuni* and *C. coli* strains used in the study of the mechanism of erythromycin resistance and their relevant properties are listed in Table 2.1. The *C. jejuni and C. coli* strains used in pulsed-field gel electrophoresis studies are listed in Table 2.2 and Table 2.3 respectively. Other bacterial strains used in natural transformation as well as those harboring plasmids used in hybridization studies are listed in Table 2.4.

2.2 Bacterial growth conditions. All Campylobacter strains were grown in Mueller-Hinton (MH) broth or on MH agar at 37°C in the presence of 7% CO_2. Strains of *Campylobacter* spp. used in serotyping and biotyping experiments were grown on Blood agar plates (BAP) at 42°C in the presence of 7% CO_2. An erythromycin concentration of 32 μg/ml was used for maintenance and selection of erythromycin resistant *C. jejuni* and *C. coli* strains. All *Escherichia coli* and *Staphylococcus aureus* strains were cultured on MH agar containing the appropriate antibiotics at 37°C.

2.3 Determination of Minimal Inhibitory Concentrations (MICs) of Antibiotics. MICs of erythromycin, oleandomycin and lincomycin against *C. jejuni*

Table 2.1

Bacterial strains used in erythromycin resistance studies

Strain	Plasmid	Resistance[1]	Source
C. jejuni UA67	-	Nal	Canada[2]
C. jejuni UA695	Tet^R plasmid	Em, Tet	Canada[3]
C. jejuni UA697	-	Em	England[4]
C. jejuni UA709	-	Em	Netherlands[5]
C. jejuni UA736	-	-	Netherlands[5]
C. coli UA585	-	Em	Wales[6]
C. coli UA586	-	-	Wales[6]
C. coli UA417	-	Nal	Canada[7]
E. coli BM2571	pIP1527	Em, Cm, Tet	France[8]
S. aureus 25923	-	-	ATCC

---

1 Resistance phenotypes: Cm=chloramphenicol; Em=erythromycin; Nal=nalidixic acid; Tet=tetracycline.

2 D. E. Taylor, University of Alberta
3 Provincial Laboratory of Northern Alberta, Canada
4 I. Phillips, St. Thomas's Hospital, London, England
5 H. Endtz, Academish Zekenhuis, Leiden, Netherlands
6 Public Health Lab Service, University Hospital, Wales, U.K.
7 H. Lior, Laboratory Center for Disease Control (LCDC), Ottawa, Canada
8 P. Courvalin, Insitut Pasteur, Paris, France

Table 2.2

Campylobacter jejuni strains used in PFGE studies

Strain	Resistance[1]	Origin	Source
UA53	Tc	Human	Canada[2]
UA67	Nal	Human	Canada[3]
UA268	-	Human	U.K.[4]
UA365	-	Cattle	U.K.[4]
UA580	-	Unknown	Canada[5]
UA709	Em	Human	Netherlands[6]
UA695	Em,Tc	Human	Alberta[7]
UA697	Em	Human	U.K.[8]
UA736	-	Human	Netherlands[6]
UA336	-	Cattle	U.K.[4]
UA344	-	Cattle	U.K.[4]
UA347	-	Milk	U.K.[4]

---

[1] Resistance phenotypes: Em=erythromycin; Nal=nalidixic acid; Tc=tetracycline

[2] J. M. S. Dixon, Provincial Laboratory of Northern Alberta, Canada

[3] D. E. Taylor, University of Alberta, Canada

[4] A. D. Pearson, Southampton, U.K.

[5] H. Lior, LCDC, Ottawa, Canada

[6] H. Endtz, Netherlands

[7] Provincial Laboratory of Northern Alberta, Canada

[8] I. Philips, St. Thomas's Hospital, London, England

Table 2.3

Campylobacter coli strains used in PFGE studies

Strain	Resistance[1]	Origin	Source
UA37	Em, Tc	Human	Belgium[2]
UA40	Em	Human	Belgium[2]
UA261	-	Human	U.K.[3]
UA417	Nal	Human	Canada[4]
UA578	-	Unknown	Canada[4]
UA585	Em	Human	Wales[5]
UA586	-	Human	Wales[5]
UA748	Em	Human	Belgium[6]
UA749	Em	Human	Belgium[6]
UA757	-	Unknown	U.S.A.[7]

---

[1] Resistance: Em, erythromycin; Nal, nalidixic acid; Tc, tetracycline
[2] R. Vanhoof, Brussels, Belgium
[3] A. D. Pearson, Southampton, U.K.
[4] H. Lior, LCDC, Ottawa, Canada
[5] Public Heath Lab Services, University Hospital, Wales, U.K.
[6] H. Goosens, Brussel, Belgium
[7] J. Miller, Stanford University, U.S.A.

Table 2.4

Bacterial strains and plasmids used in genomic mapping studies of *Campylobacter*

Strain	Plasmid	Resistance[1]	Source
C. coli UA417	None	Nal	Canada[2]
C. coli UA417R	None	Sm, Nal	Canada[3]
C. jejuni UA723	None	-	U. S. A.[4]
E. coli DT1276	pAR140	Ap, Tc	U. S. A.[5]
E. coli DT2042	pKK3535	Ap	Canada[6]
-	pRIF1	Ap, Cm	Canada[7]

---

[1] Resistance: Ap=ampillicin; Nal=nalidixic acid; Sm=streptomycin; Tc=tetracycline.
[2] H. Lior, LCDC, Ottawa, Canada
[3] D. E. Taylor, University of Alberta, Canada
[4] J. Miller, Stanford University, U. S. A.
[5] A. Rashtchian, Integrated Genetics, Framingham, Massachusetts, U. S. A.
[6] L.-K. Ng, LCDC, Ottawa, Canada
[7] B. Albritton, Privincial Laboratory of Northern Alberta

and *C. coli* strains were determined using the method previously described (Steers *et al.*, 1959). Single colonies of different Campylobacter strains were used to inoculate 2 ml of MH broth followed by overnight incubation at 37°C in the presence of 7% CO_2. Cultures were then diluted with MH broth to a density of approximately 10^7 CFU/ml. Equal amounts of each culture were then inoculated onto MH agar plates containing increasing concentrations of the appropriate antibiotic using a Steers replicator. Reference strains other than *Campylobacter* were grown in nutrient broth at 37°C, diluted and treated in the similar manner as the *Campylobacter* strains. Erythromycin was purchased from Ayerst, Mckenna & Harrison Inc. whereas oleandomycin and lincomycin as well as antibiotic discs containing 15 µg of erythromycin were purchased from Sigma Chemical Co., St. Louis, Mo.

2.4 Serotyping of *C. jejuni* and *C. coli* strains. Serotyping was carried out based on the Lior serotyping scheme. Cultures were grown on MH blood agar plates at 37°C for 48 hours under 7% CO_2. A heavy loopful of cells was suspended in phosphate-buffered-saline 0.01 M, pH7.2 containing 0.025% bovine pancreatic DNase (Boehringer Mannheim, Germany). One drop of 2% NaCl was added and checked for autoagglutinability to ensure smooth culture was present. Slide agglutinations were then performed by using polyvalent pooled antisera; then identification of

serogroup was accomplished using monovalent, unabsorbed antisera and finally, confirmation of serogroups with absorbed antisera. Culture suspensions and antisera were mixed well and the results were recorded within 1 minute to eliminate the possibility of detecting agglutinations due to cross-reacting antibodies.

2.5 Biotyping of *C. jejuni* and *C. coli* strains. Biotyping of the Campylobacter strains based on rapid hippurate hydrolysis test, rapid H_2S test and DNA hydrolysis test was carried out. Fresh cultures of Campylobacter incubated at 42°C in 7% CO_2 were used in all three tests.

Rapid hippurate hydrolysis test. The rapid hippurate hydrolysis test was carried out by resuspending 1 small loopful of *Campylobacter* culture in 0.4 ml of 1% sodium hippurate solution. After thorough mixing, the suspension was incubated for 2 hours at 37°C. This was then overlayed with 0.2 ml of ninhydrin solution (3.5% ninhydrin in butanol-acetone 1:1) and incubated for another 10 minutes. A deep purple color represented a positive reaction whereas a colorless or light purple color was indicative of a negative hippurate hydrolysis test.

Rapid H_2S test. The rapid H_2S test was performed using FBP agar medium supplemented with ferrous sulphate·$7H_2O$, sodium metabisulphite and sodium pyruvate (see Appendix 1). A large ball-like inoculum of fresh overnight *Campylobacter* cells was gently suspended in the upper third of the medium

Table 2.5

Biotyping scheme for *C. jejuni* and *C. coli*

	C. jejuni Biotype				*C. coli* Biotype	
Tests	I	II	III	IV	I	II
Hippurate	+	+	+	+	-	-
Rapid H_2S	-	-	+	+	-	-
DNA Hydrolysis	-	+	-	+	-	+

without mixing and incubated at 37°C for 2 hours. A blackening around the bacterial mass represented a positive reaction and usually begins to appear within 30-45 minutes.

DNA hydrolysis test. The DNA hydrolysis test was carried out using a modified toluidine blue-DNA agar (see Appendix 2). A small loopful of a 24-48 hour old culture was inoculated in a circular area on a toluidine blue-DNA agar plate and incubated at 42°C for 24-48 hours. A clear-colorless or pinkish zone around the inoculum was considered a positive reaction. The current biotyping scheme for *C. jejuni* and *C. coli* is shown in Table 2.5. Both serotyping and biotyping were performed at the Laboratory Centre for Disease Control, Ottawa, Ontario.

2.6 Chromosomal DNA isolation for cloning and natural transformation. Preparations of Campylobacter chromosomal DNA were isolated from 20-25 MH plates of cells grown overnight at 37°C in the presence of 7% CO_2. Cells were harvested with 2 ml of TE buffer (50 mM Tris, 5 mM EDTA, pH 8.0) per plate to a volume of 20-25 ml per centrifuge tube. The suspensions were centrifuged for 15 minutes at 8,500 rpm (Beckman JA-14 rotor) at 4°C to sediment cells. The cell pellet was washed with 25 ml of TES buffer (10 mM Tris, 25 mM EDTA, 0.15 M NaCl, pH 8.0) and the centrifugation procedure was repeated as before. The pellet was suspended in 5.0 ml of TES buffer and 0.5 ml of lysozyme solution (2 mg/ml in TE buffer), mixed gently

and incubated for 15 minutes at 37°C. A 0.6 ml volume of Sarcosyl/Pronase solution (10% Sarcosyl and 5 mg of Pronase per ml in TE buffer) was added and the mixture was further incubated for 1 hour at 37°C. The sample was then extracted 3 times with 5 ml of phenol and 5 ml of chloroform. Centrifugation was carried out at 17,000 x g for 15 minutes and the aqueous phase was removed and saved. DNA was precipitated by slowly adding two volumes of cold 95% ethanol and carefully mixing the two phases. White DNA strands were found to formed rapidly. When mixing and precipitation was completed, the DNA was carefully removed with a glass rod and suspended in 2-5 ml of TE buffer and stored at 4°C.

2.7 Attempts at molecular cloning of the erythromycin resistance determinant. Molecular cloning experiments were carried out using the cloning vector pBR322. Chromosomal DNA from *C. jejuni* UA695, UA697, UA709 and *C. coli* UA585 was either randomly sheared in a syringe followed by sucrose gradient fractionation or digested using the restriction endonucleases *Pst*I and *Bam*HI. Chromosomal DNA fragments were ligated with vector DNA (*Eco*RI linkers were used for the ligation of sheared DNA fragments) before transformation into *E. coli* DB11 or *E. coli* HB101. Clones containing insert DNA were isolated based on antibiotic resistance (*Pst*I-tetracycline resistant, ampicillin sensitive; *Bam*HI-tetracycline sensitive, ampicillin resistant). Positive clones were

spotted onto MH agar plates containing erythromycin (32 µg/ml) for the selection of erythromycin resistant clones. Similarly, cloning was also carried out using the cosmid vector pHC79. *Pst*I and *Bam*HI erythromycin-resistant transfectants were screened for as described above.

2.8 Transformation of shuttle vector DNA into *C. jejuni* by electroporation. Transformation of the shuttle vector pILL550 by electroporation was carried out as previously described (Miller et al., 1988). The *C. jejuni* strain C31 previously found to be suitable for electroporation was used as the host. Cuvettes with gap distances of 0.2 cm and 0.4 cm were used. Viability controls were included in each electroporation study in which treated organisms were plated to confirm viability relative to untreated organisms.

2.9 Natural transformation of *C. coli* with chromosomal DNA. *Campylobacter coli* transformation was performed either on the surface of MH agar plates or in a biphasic system (Wang and Taylor, 1990). For transformation on MH agar, fresh recipient cells, either *C. coli* UA417R or UA585, were spread on MH agar to a cell density of approximately 5 x 10^7 cells per plate and incubated for 6 hours at 37°C in the presence of 7% CO_2. Aliquots of the appropriate chromosomal DNA sample (approximately 0.2 µg in 10-20 µl of TE buffer) were applied to each plate and incubated for a further 5 hours. For transformation in a biphasic system, fresh *C. coli*

recipient cells were suspended in MH broth to a density of 1-5 x 10^7 cells per ml and a 0.2 ml volume was transferred to a test tube (10 by 120 mm) containing 1.5 ml of MH agar. The tube was incubated for 6 hours at 37°C in 7% CO_2 at which time aliquots of DNA samples were applied to each tube and incubation was continued for 5 hours. Potential transformants from both transformation procedures were selected by spreading transformed cells on the appropriate MH selective agar plates containing either erythromycin (32 µg/ml), nalidixic acid (48 µg/ml) or streptomycin (10 µg/ml) and incubated for 48 hours at 37°C in the presence of 7% CO_2.

2.10 Determination of spontaneous mutation rates to streptomycin and erythromycin resistance in *Campylobacter* spp. The rates at which spontaneous streptomycin and erythromycin resistance occur in *C. jejuni* UA67 were determined by plating individual colonies of one week old cultures on MH agar plates containing 25 µg/ml and 32 µg/ml of streptomycin and erythromycin respectively. The stability of erythromycin resistance in *C. jejuni* UA695, UA697, UA709 and *C. coli* UA585 was also determined. Individual colonies from one week old cultures of these resistant strains were plated on a MH agar plate as well as a MH agar plate containing 32 µg/ml of erythromycin.

2.11 Detection of erythromycin-modifying activity. Tests for the detection of extracellular erythromycin-modifying enzymes were carried out as

described previously (Andremont et al., 1986). *S. aureus* strain ATCC 25923 was used as the indicator strain and *E. coli* BM2571 was used as a positive control. Antibiotic filter discs containing 15µg of erythromycin were used in the assays. Briefly, a suspension of the indicator strain was spread on a MH agar plate at a concentration to give a confluent lawn of growth. An erythromycin disc was applied to the center of the plate after which a loopful of the test bacteria was streaked from the center of the plate. An arrow-shape distortion in the circular zone of inhibition of *S. aureus* ATCC 25923 was an indication of destruction of erythromycin by extracellular modifying enzyme released from the test culture.

2.12 Determination of inducibility of erythromycin resistance in *C. jejuni* and *C. coli*. Overnight cultures (1.0 ml) of erythromycin resistant *C. jejuni* and *C. coli* strains were used to inoculate 100 ml of fresh MH broth either with or without sub-inhibitory levels of erythromycin (32 µg/ml), lincomycin (13.6 units/ml) or oleandomycin (32 µg/ml). After 8 hours of incubation at 37°C under 7% CO_2, 100 mg of erythromycin was added to each culture. A growth control culture without any antibiotics added was also included in each experiment as a control. Cultures were incubated for 72 hours with OD_{600} readings taken at various time points (0, 8.0, 24.0, 32.0, 48.0, 56.0 and 72.0 hours) using a Phillips Pye Unicam PU8800 spectrophotometer.

2.13 *In Vivo* Uptake of [^{14}C]erythromycin. Cultures of erythromycin resistant and sensitive *C. jejuni* and *C. coli* were grown overnight in MH broth, centrifuged and suspended to OD_{600} of 0.4. Cells were then pre-incubated at 37°C for 15 minutes followed by the addition of ~1.5 µCi of [^{14}C]erythromycin (DuPont, NEN Research Products, Boston, Mass.). The cells were further incubated at 37°C for 3 hours with 0.5 ml samples removed at various time intervals (15, 30, 60, 90, 120, 180 minutes). Samples were applied to Millipore filters (HAWP; 0.45µm pore size; pre-washed with 2.0 mg/ml of unlabeled erythromycin to reduce background non-specific binding of [^{14}C]erythromycin to filters) under vacuum, washed 3 times with ice cold 10 mM Tris-HCl, pH 7.6, dried at 65°C and the radioactivity measured using a Beckman LS6800 scintillation counter. Experiments were also repeated with 0.5 M NH_4Cl included in the washing buffer and the resulting counts compared.

2.14 Effect of erythromycin on *in vivo* protein synthesis. Fresh overnight cultures of *C. jejuni* UA695, UA697, UA709 and UA736 as well as *C. coli* UA585 and UA586 were harvested, washed and suspended in fresh MH broth to a cell density of 0.25 at OD_{600}. Portions (1.0 ml) of the cell suspensions were incubated at 37°C for 4 hours with 20 µCi/ml of [^{35}S]methionine (Dupont, NEN Research Products, Boston, Mass.) and various concentrations of erythromycin (0, 5.0, 30.0 and 100.0 µg/ml). The radiolabelled cells were washed with ice cold 10 mM Tris-HCl, pH 7.6 and lysed

in cracking buffer (Dougan and Kehoe, 1984) by boiling for 5 minutes. The polypeptides were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using the Biorad Protein I electrophoresis apparatus and subjected to autoradiography as described previously (Bonner and Laskey, 1974). The extent of [^{35}S]methionine incorporation into polypeptides was taken as a measure of *in vivo* protein synthesis in the Campylobacter cells.

2.15 Isolation and purification of Campylobacter ribosomes by Sephacryl S-200 column chromatography. Ribosomes from *C. jejuni* and *C. coli* were isolated using a modified version of a previously described method (Jelenc, 1980). Large scale preparations of overnight *C. jejuni* and *C. coli* cultures (5 x 750 ml of MH broth) were used to harvest ~3.0 g wet weight of cells. Cell pellets were frozen overnight at -70°C then subjected to grinding for 20 minutes at 0°C in the presence of 2 x their weight of Alumina and collected by the addition of Buffer I (5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 5 mM NH_4Cl, 95 mM KCl, 1 mM dithioerythritol, 20 mM Tricine, pH7.5) containing DNase (2 µg/ml). The cell debris was removed by centrifugation twice at 13,000 rpm (Beckman JA-21 rotor). The supernatant solution was decanted and ammonium sulfate was added with stirring to a final concentration of 210 mg/ml. The pH of the solution was adjusted to 7.5 with ammonium hydroxide, stirred for 30 minutes and the protein precipitate was removed by

centrifugation as before. The opalescent supernatant solution containing 70S ribosomes was introduced at 25 ml/hour into a 3 X 50-cm Sephacryl S-200 column pre-equilibrated in buffer II (buffer I made 1 M in NH_4Cl) and was eluted at the same rate in buffer II. Fractions (1.0 ml) were collected and diluted (1/100) for determination of absorbance at 260 nm. After pooling peak A_{260} fractions equal to or higher than half the maximum peak absorbance, 70S ribosomes were precipitated by the addition of polyethylene glycol 8000 (PEG) to a final concentration of 100 mg/ml. The mixture was stirred for 30 minutes and collected by centrifugation at 10,000 rpm (Beckman JA-21 rotor) for 15 minutes. The ribosome pellet was then resuspended in Buffer I and applied to a Sephadex G-75 column to remove PEG from the ribosomal preparation. The A_{260} of each fractions (0.5 ml) was determined, the peak fractions pooled and 200 µl aliquots of the purified 70S ribosomes were stored at -20°C. Subunits, 30S and 50S, were isolated by sucrose gradient centrifugation (7.5 to 30% sucrose gradients) in polymix buffer (same as buffer I, but with 5 mM sodium phosphate instead of Tricine) containing 0.4 M NaCl using the SW40 Beckman rotor for 18 hours at 19,000 rpm. The subunits were collected and stored in the same manner as intact 70S ribosomes.

2.16 Isolation and purification of Campylobacter ribosomes by high speed centrifugation. Fresh cultures of *Campylobacter* spp.

were grown and harvested as described above and washed with Standard buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 60 mM KCl, 6mM ß-mercaptoethanol, pH 7.4). The cells were sedimented by centrifugation at 5,000 rpm (Beckman JA-14 rotor) and frozen overnight at -70°C. The cells were then thawed, ground with twice their weight of Alumina and suspended in Standard buffer containing DNase (2.0 µg/ml). The cell debris was removed by centrifugation twice at 10,000 rpm (Beckman JA-21 rotor) for 30 minutes each. The clear supernatant solution was then centrifuged at 50,000 rpm (Beckman Ti70.1 rotor) for 2 hours at 4°C. A hard, transparent ribosomal pellet corresponding to 70S ribosomes was obtained which was thoroughly suspended in a small volume of Standard buffer (0.5 - 1.0 ml depending on size of pellet). Small aggregates were removed by centrifugation at 5,000 rpm in a microfuge for 5 minutes. Dilutions were made (1/100) before the absorbance at 260 nm was measured to determine the concentration of the ribosomal preparation. Alternatively, 30S and 50S ribosomal subunits were prepared by suspending the ribosomal pellet in low [$MgCl_2$] Standard buffer containing 0.5 mM instead of 10 mM $MgCl_2$. This was applied to 7 to 30% sucrose gradients and centrifuged for 6 hours at 35,000 rpm in a SW40 Beckman rotor. Fractions (0.5 ml) were collected and the absorbance at 260 nm was measured to determine the peak fractions corresponding to 30S and 50S subunits. All purified 70S, 50S and 30S ribosome samples

were stored at -20°C. The concentration of ribosomes is determined based on the equation that 1 A_{260} unit = 2.6 µmol of 70S ribosomes.

2.17 Binding of [^{14}C]erythromycin to Campylobacter 70S ribosomes, 50S and 30S ribosomal subunits. Erythromycin-resistant and sensitive *Campylobacter* 70S ribosomes (130 pmol) were incubated in 70 µl of reaction buffer (50 mM Tris hydrochloride, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 6 mM ß-mercaptoethanol, 0.1 mM EDTA) and various concentrations (20 to 100 µM) of [^{14}C]erythromycin for 30 minutes at 37°C. The ribosomes were applied to prewashed Millipore filters (GSWP; 0.22 µm pore size) under vacuum and washed 3 times (5 ml each) with ice cold reaction buffer. The filters were then dried for 30 minutes at 65°C and the radioactivity associated with the filters was determined. Similar conditions were used to carry out binding assays using 50S and 30S ribosomal subunits. To obtain the background count, reaction mixtures without ribosomes were treated identically, collected on filters, washed, and the radioactivity associated with the filters was determined for each concentration of [^{14}C]erythromycin used.

2.18 Restriction endonuclease digestion and PFGE analysis of Campylobacter genomic DNA. Fresh overnight cultures (one plate each) of *C. jejuni* and *C. coli* were harvested using 1.0 ml of TE buffer (50 mM Tris, 5mM EDTA, pH8.0). Agarose blocks were prepared by adding

200 µl of cell suspension to 1.0 ml of 1.0% ultra pure low melting point agarose (Bethesda Research Laboratories Canada, Burlington, Ontario, Canada), mixed thoroughly, and 100 µl was used per agarose block. The solidified agarose blocks were then incubated in ESP lysis buffer (0.25mM EDTA, 0.5% lauroyl sarcosine, 0.5 mg/ml proteinase K) at 50°C for 48 hours. To prepare blocks for restriction endonuclease digestion, thin slices of the DNA blocks were cut out and washed thoroughly in phenyl methyl sulfonyl fluoride (PMSF) solution (0.175 mg/ml of TE buffer). This is essential for the removal of any proteinase activity associated with the ESP buffer which would interfere with the action of the restriction enzymes. After further thorough washing with TE buffer, the blocks were incubated in 90 µl of the appropriate digestion buffer and 10 units of restriction enzyme. The restriction endonucleases *Hind*III, *Sal*I and *Sma*I were purchased from Boehringer Mannheim Canada Ltd., Dorval, Quebec, Canada and used under the conditions recommended by the manufacturer. PFGE was carried out using the LKB 2015 Pulsaphor Electrophoresis unit equipped with a hexagonal electrode. The agarose gels (1.0%) were prepared in 0.5 x TBE running buffer (50 mM Tris Base, 50 mM boric acid, 1 mM EDTA, pH 8.5) were subjected to electrophoresis for 24 hours at different voltage and pulse time settings at a constant temperature of 12.0°C. The gels were stained with ethidium bromide and photographed under UV illumination using a

Pentax 35mm camera equipped with a Kenko R2 filter. Phage λ DNA concatamers (Biorad Laboratories Canada Ltd., Mississauga, Ontario, Canada) were used as molecular weight standards.

2.19 Transfer of DNA by Southern blotting. Chromosomal DNA fragments from ethidium bromide-stained agarose gels were transferred onto nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N. H.) by the method of Southern, 1975. After photography, the agarose gels were incubated with shaking in denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for 1 hour. The denaturing buffer was replaced by neutralizing solution (1 M Tris-HCl, 2 M NaCl, pH 5.5) and further incubated with shaking for 1 hour. A piece of nitrocellulose filter, slightly larger in size than the agarose gel, was cut and thoroughly wetted with distilled water followed by soaking in 20 x SSC solution. The neutralized agarose gel was then placed on a transfer apparatus made up of a large glass tray containing 20 x SSC on which a glass plate and a piece of Whatman 3 mm filter paper acting as a wick were placed. The nitrocellulose filter was placed on the agarose gel while ensuring that all air bubbles were removed. Five pieces of Whatman 3 mm filter paper the size of the nitrocellulose filter were cut (the bottom two pieces were soaked in 20 x SSC) and placed on top of the nitrocellulose filter. This was covered with a stack of paper towels (2-3 inches thick) to which a glass plate and a weight was placed on top. The apparatus was

then covered with plastic wrap to prevent drying. After overnight blotting was completed, the nitrocellulose filter was removed and well positions were marked with a ball point pen. The filter was then quickly washed with 2x SSC for 15 minutes, air dried and incubated overnight in a 65°C oven. The filter was stored at room temperature until the hybridization experiments were carried out.

2.20 DNA Hybridization. Hybridization of ^{32}P-labelled DNA to nitrocellulose filters was carried out in Phillips Seal-a-bags using the method described by Portnoy *et al.* (1981). Nitrocellulose filters were pre-incubated with 5 ml of pre-incubation buffer (50% formamide, 5 x SSC, 0.1% SDS, 1 mM EDTA, 1 x Denhardt's solution consisting of 0.02% Ficoll, 0.02% BSA and 0.02% polyvinylpyrrolidone in 1 x SSC) for at least 3 hours at 42°C. After pre-incubation, excess buffer was removed from the bags. The hybridization solution (2.5 ml pre-incubation buffer, 0.25 mg sonicated herring sperm DNA and radioactive probe heat denatured for 10 minutes in boiling water) was added and the bags were resealed and incubated overnight at 42°C. Filters were then washed with 5x SSC, 0.1% SDS and 1 mM EDTA for 1 hour at 65°C. They were then rinsed quickly with 2x SSC solution, air dried and exposed to x-ray film (Kodak Xar-5) for the appropriate period of time at -70°C before developing.

2.21 DNA extraction from low melting point agarose for probe preparation and natural

transformation. Different plasmids containing regions of DNA coding for various genes were used as probes in the hybridization studies as well as donor DNA in natural transformation experiments. In most cases, a restriction fragment of the plasmid was extracted from low melting point (LMP) agarose gels and used in order to obtain increased specificity. Plasmid DNA was treated with the appropriate restriction endonuclease and subjected to agarose gel electrophoresis using 0.8% LMP agarose. After staining with ethidium bromide, the required DNA fragments were excised from the gel, excess agarose was trimmed off and the desired portion was put into a 1.5 ml Eppendorf tube containing 200 µl of TE buffer. This was then incubated at 65°C for 10 minutes to melt the agarose block. The DNA was extracted with an equal volume of phenol and the aqueous phase was removed. Another 200 µl of TE buffer was added to the tube containing the LMP agarose and the phenol extraction was repeated. The combined aqueous phase samples were extracted once with an equal volume of butanol. The aqueous phase from this procedure was then collected and extracted once with chloroform, and the extracted DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of cold 95% ethanol for 1 hour at -20°C. The DNA was then washed with cold 70% ethanol, dried under vacuum and suspended in the appropriate volume of TE buffer. DNA samples extracted for natural transformation studies were cut out of LMP agarose

gels, melted at 65°C for 5 minutes and used in the biphasic transformation procedure described above.

2.22 Preparation of radioactively-labelled DNA by nick translation. A 5 x nucleotide buffer solution was prepared by adding 1 µl each of 10 mM dCTP, 10 mM dGTP and 10 mM dTTP, 50 µl of 10x buffer (250 mM Tris HCl, 25 mM $MgCl_2$, 50 mM β-mercaptoethanol, pH 7.5) and 47 µl distilled water. A nick translation reaction mixture was prepared with approximately 0.5 µg of the DNA to be labelled (total volume 20 µl), 20 µl of 5 x nucleotide buffer, 2.0 µl of DNase solution (1 x 10^{-7} mg/ml), 51 µl of distilled water and 5.0 µl of [α-^{32}P]dATP. The reaction mixture was incubated at 15°C for 15 minutes at which time 2.0 µl of DNA polymerase I (0.5 units) was added. The mixture was further incubated overnight at 15°C. The mixture was then precipitated by the addition of: 20 µg yeast tRNA, 1/10 volume 3 M sodium acetate and 2.5 volumes of cold 95% ethanol and incubated at -20°C for 1 hour. The DNA pellet was obtained by centrifugation for 5 minutes and then washed with cold 70% ethanol, recentrifuged, air dried and redissolved in 400 µl of TE buffer. The amount of labelling was determined by measuring the radioactivity in 10 µl of the DNA sample using a scintillation counter. The remaining portion of the DNA probe was stored at -70°C.

2.23 Rapid chromosomal DNA isolation for dot blot hybridization. One loopful of fresh overnight culture was suspended in 1.0 ml of MH broth followed by

centrifugation for 30 seconds. The cell pellet was suspended in 200 µl of isolation buffer (0.15 M NaCl, 0.1 M EDTA) to which 10 µl of RNase (10 mg/ml) and 5 µl of SDS (20% w/v) were added. The mixture was incubated at 65°C for 15 minutes followed by the addition of 500 µl of phenol:chloroform:iso-amyl-alcohol extraction solution (25:24:1). The aqueous phase was removed to a new tube and the organic phase was washed with 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The pooled aqueous phases were extracted once with chloroform, ethanol precipitated at -20°C for 1 hour, dried and the DNA redissolved in 150 µl of TE buffer.

2.24 Dot blot hybridization. Purified chromosomal DNA samples (20 µl) were applied to nitrocellulose filters, denatured in 0.5 M NaOH; 0.15 M NaCl ; neutralized in 10 mM Tris-HCl; 0.15 M NaCl; pH 7.5, air-dried and incubated overnight at 65°C. Two different DNA probes were used in these dot blot studies. pDT1720, a DNA probe containing a 1475 bp *Eco*RI fragment, hybridized specifically with *C. jejuni* DNA under both high (42°C) and moderate (37°C) stringency conditions. The second DNA probe, pDT1719, contained a 1845 bp *EcoRI* fragment and hybridized to *C. jejuni* and *C. coli* DNA under moderate stringency conditions. The amount of hybridization with *C. coli* DNA was found to be reduced under high stringency conditions (Taylor and Hiratsuka, in press). In the present dot blot experiment, both DNA probes were labelled

with [α-^{32}P]dATP by nick translation and hybridizations were carried out under high (pDT1720) and moderate (pDT1719) stringency conditions.

3. RESULTS

3.1 Serotyping, biotyping and plasmid content of *C. jejuni* and *C. coli* strains. The serotypes and biotypes of all strains of *Campylobacter* spp. (based on the Lior serotyping and biotyping schemes) used in the characterization of erythromycin resistance are listed in Table 3.1. The serotype of *C. jejuni* UA697 could not be determined because of its inability to produce flagella. Despite culturing in MH broth and semi-solid agar, motility could not be restored. As expected, the erythromycin-resistant *C. coli* strain UA585 and its erythromycin-sensitive derivative shared the same serotype and biotype. On the other hand, *C. jejuni* UA709 and UA736, which were initially considered to be an isogenic pair isolated from a single patient, were found to have a similar biotype but different serotypes. When a plasmid isolation procedure (Birnboim and Doly, 1979) was used to screen for the presence of plasmids, all erythromycin-resistant strains of Campylobacter were found to be plasmid-free except for *C. jejuni* UA695 which contained a 45 kb plasmid encoding tetracycline resistance. The tetracycline resistance was found to transfer at a frequency of 1.2×10^{-5} transconjugants per recipient in overnight mating whereas transfer of erythromycin resistance was not detected.

Table 3.1

Serotype and biotype of Campylobacter strains

Strains	Serotype	Biotype
C. jejuni UA67	38	III
C. jejuni UA695	2	II
C. jejuni UA697	NT[1]	I
C. jejuni UA709	53	II
C. jejuni UA736	1	II
C. coli UA417	29	II
C. coli UA585	8	II
C. coli UA586	8	II

---

[1]NT=nontypable

3.2 MICs of erythromycin, lincomycin and oleandomycin against *C. jejuni* and *C. coli* strains. The MICs of the three MLS antibiotics erythromycin, lincomycin and oleandomycin against the different strains of *Campylobacter* spp. are shown in Table 3.2. All the resistant strains (*C. jejuni* UA695, UA697, UA709 and *C. coli* UA585) were found to be resistant to high levels of the antibiotics as compared to sensitive strains. Spontaneous mutation rates for streptomycin and erythromycin resistance were 1.79×10^{-8} and $<1.0 \times 10^{-9}$ respectively. No spontaneous mutation to erythromycin sensitivity was obtained from any of the erythromycin resistant Campylobacter strains.

3.3 Attempts to clone the erythromycin resistance determinant. In total, 856 pBR322 clones and 355 pHC79 cosmid clones containing Campylobacter insert DNA were obtained. Of the pBR322 clones (using either *E. coli* DB11 or *E. coli* HB101 as host), 12 were found to be erythromycin-resistant upon initial selection. When plasmid DNA from these clones was isolated and retransformed into *E. coli* DB11 or *E. coli* HB101, all 12 were found to be erythromycin-sensitive. Therefore the initially observed erythromycin resistance may have been due to spontaneous mutations in the *E. coli* host. No erythromycin-resistant clones were detected among the pHC79 cosmid clones.

Table 3.2

MICs of erythromycin, lincomycin and oleandomycin against Campylobacter strains

	MICs[1]		
	Em	Lm	Olm
Strains	(µg/ml)	(units/ml)	(µg/ml)
C. jejuni UA67	2	13.6	8
C. jejuni UA695	>1024	217.6	1024
C. jejuni UA697	>1024	217.6	2048
C. jejuni UA709	>1024	217.6	1024
C. jejuni UA736	4	13.6	8
C. coli UA585	>1024	217.6	1024
C. coli UA586	2	13.6	4
C. coli UA417	2	13.6	4

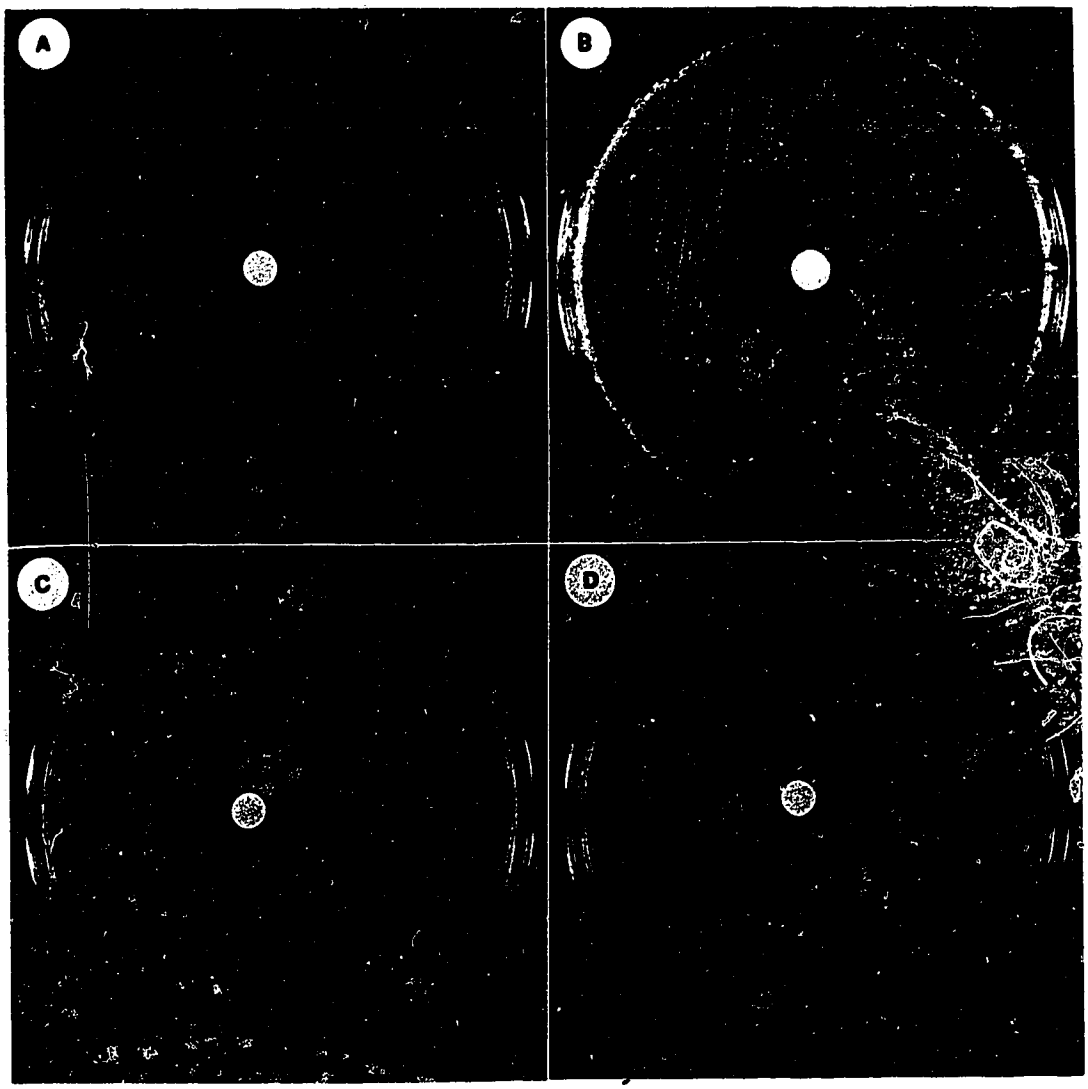
---

[1]Antibiotic Resistance: Em=erythromycin; Lm=lincomycin; Olm=oleandomycin

3.4 Transformation of shuttle vector into Campylobacter by electroporation. The shuttle vector pILL550 DNA was isolated from *C. jejuni* UA723 and used in electroporation studies. Kanamycin-resistant transformants were selected. A transformation frequency of approximatley 1.0×10^3 transformants/µg DNA was obtained using 0.4 cm gap cuvettes. A frequency of 5.0×10^3 transconjugants/µg DNA was obtained when 0.2 cm gap cuvettes were used. Results showed multiple pulsing (up to five cycles of pulses) had no effect on the transformation frequency. Pulsing at maximum voltage (12.5 kV/cm) for five cycles did not reduce cell viability.

3.5 Absence of erythromycin-modifying activity in Campylobacter. Biological assay for the detection of erythromycin-modifying activity was performed on *C. jejuni* UA67 and UA695, *C. coli* UA585 and *E. coli* BM2571 (Fig. 3.1). The latter is a positive control for erythromycin-modifying activity encoded by the *ereA* gene. The erythromycin-sensitive *C. jejuni* UA67 (A) did not grow beyond nor distort the zone of inhibition on the confluent lawn of *S. aureus* indicator culture. In (B), a clearly observable arrow-shaped distortion in the zone of inhibition was produced by the growth of *E. coli* BM2571. With the erythromycin-resistant *C. jejuni* UA695 and *C. coli* UA585 (C and D respectively), growth was observed within the zone of inhibition. Distortion in the shape of the zone, however, was not detected. Similar results were

Fig. 3.1 Biological assay for the detection of erythromycin-modifying activities in Campylobacter. (A) negative control *C. jejuni* UA67; (B) positive control *E. coli* BM2571; (C) *C. jejuni* UA695; (D) *C. coli* UA585. Distortion in the zone of inhibition of growth of the *S. aureus* 25923 indicator strain represents the presence of erythromycin-modifying activity.


A
B
C
D

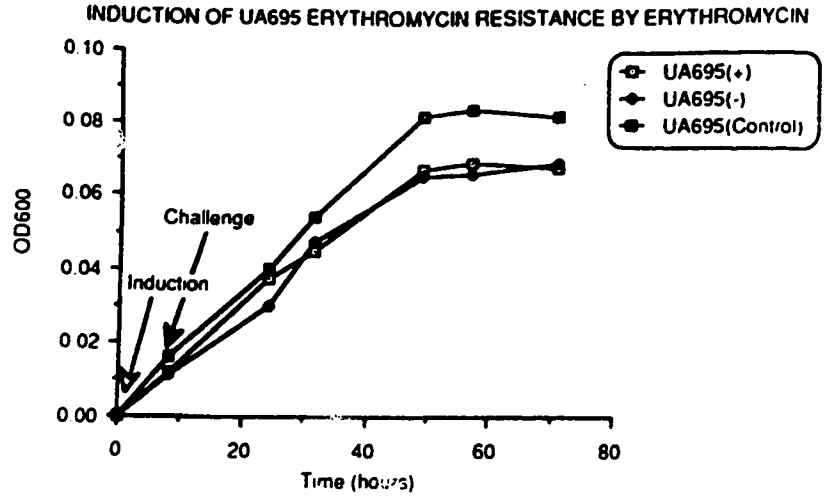
obtained for *C. jejuni* UA697 and UA709 (data not shown). Therefore, results from these biological assays demonstrated that erythromycin-resistant *C. jejuni* UA695, UA697, UA709 and *C. coli* UA585 do not produce extracellular enzymes capable of modifying the antibiotic.

3.6 Absence of induction of erythromycin resistance in Campylobacter. Erythromycin-resistant *C. jejuni* and *C.coli* strains were pre-incubated with sub-inhibitory levels of erythromycin, lincomycin or oleandomycin followed by incubation with high concentrations of erythromycin and the growth rates of the cultures were monitored over a 72 hour period and compared with uninduced and control cultures. The resulting growth curves of *C. jejuni* UA695, UA697 and UA709 as well as *C. coli* UA585 are shown in Fig. 3.2, Fig. 3.3, Fig. 3.4 and Fig. 3.5 respectively. In each case, the growth rate was highest in control cultures in which no erythromycin was added. No obvious difference in growth rate was detected, however, between induced and uninduced cultures regardless of the inducing antibiotic used. The absence of induction by erythromycin, lincomycin and oleandomycin demonstrates that erythromycin resistance in *C. jejuni* and *C. coli* is constitutive.

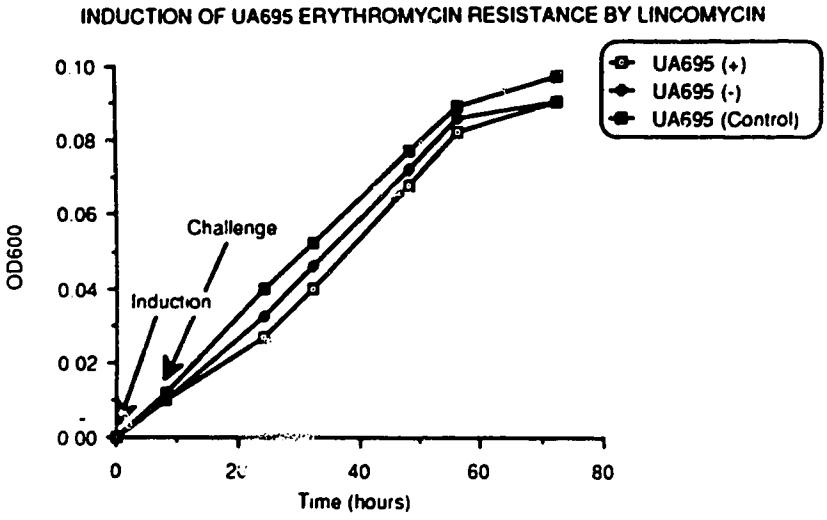
3.7 *In vivo* uptake of [^{14}C]erythromycin. The rates of *in vivo* uptake and accumulation of [^{14}C]erythromycin by *C. jejuni* UA67, UA695 and UA697 are

Fig. 3.2 Induction study of *C. jejuni* UA695 erythromycin resistance. *C. jejuni* UA695 cultures were pre-incubated with (A) erythromycin; (B) lincomycin and (C) oleandomycin before being challenged with a high concentration of erythromycin. A legend for the lines representing cultures with pre-incubation, without pre-incubation and growth curve control is shown for each graph.

A


INDUCTION OF UA695 ERYTHROMYCIN RESISTANCE BY ERYTHROMYCIN
UA695(+)
UA695(-)
UA695(Control)
OD600
0.10
0.08
0.06
0.04
0.02
0.00
Challenge
Induction
0
20
40
60
80
Time (hours)

B


INDUCTION OF UA695 ERYTHROMYCIN RESISTANCE BY LINCOMYCIN
UA695 (+)
UA695 (-)
UA695 (Control)
OD600
0.10
0.08
0.06
0.04
0.02
0.00
Challenge
Induction
0
40
60
80
Time (hours)

C

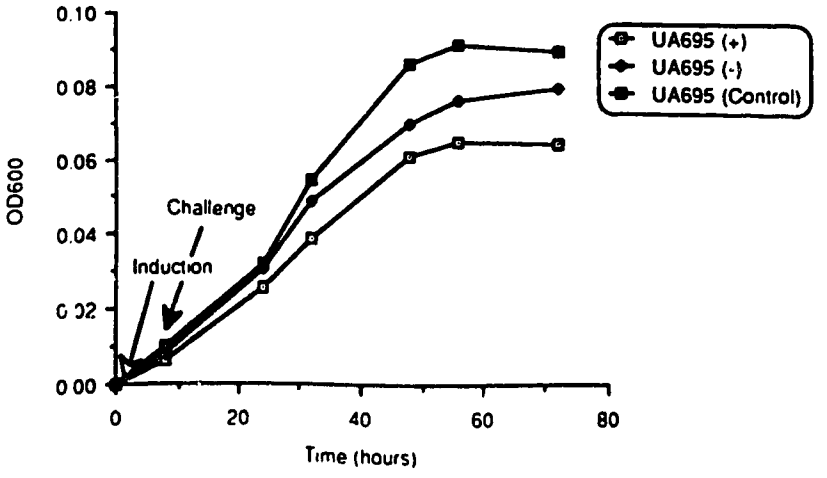
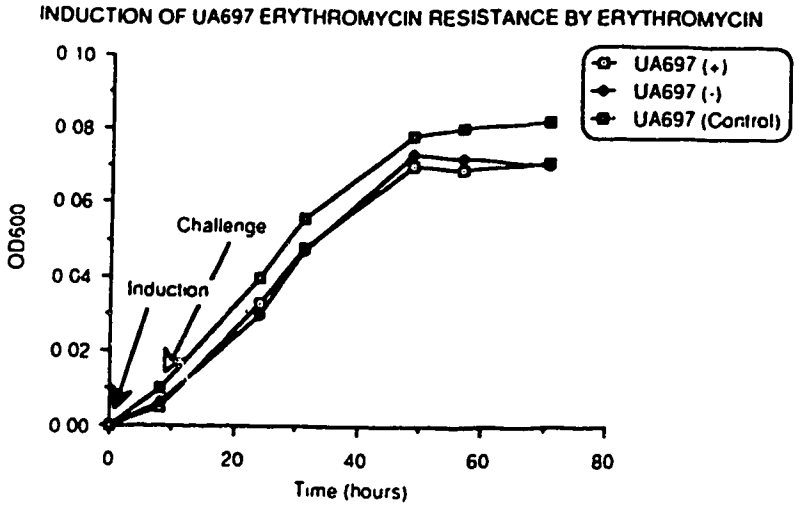
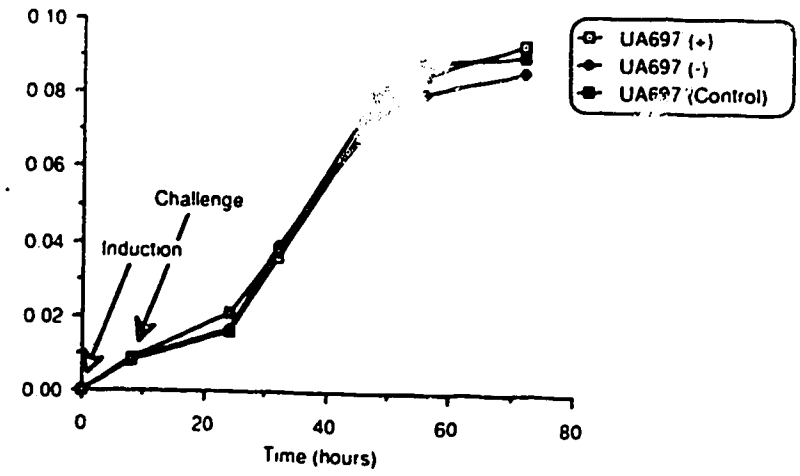

INDUCTION OF UA695 ERYTHROMYCIN RESISTANCE BY OLEANDOMYCIN
UA695 (+)
UA695 (-)
UA695 (Control)
OD600
0.10
0.08
0.06
0.04
0.02
0.00
Challenge
Induction
0
20
40
60
80
Time (hours)

Fig. 3.3 Induction study of *C. jejuni* UA697 erythromycin resistance. *C. jejuni* UA697 cultures were pre-incubated with (A) erythromycin; (B) lincomycin and (C) oleandomycin before being challenged with high concentration of erythromycin. A legend for the lines representing cultures with pre-incubation, without pre-incubation and growth curve control is shown for each graph.

A


INDUCTION OF UA697 ERYTHROMYCIN RESISTANCE BY ERYTHROMYCIN
UA697 (+)
UA697 (-)
UA697 (Control)
OD600
0 10
0 08
0 06
0 04
0 02
0 00
Challenge
Induction
0
20
40
60
80
Time (hours)

B


INDUCTION OF UA697 ERYTHROM RESISTANCE BY LINCOMYCIN
UA697 (+)
UA697 (-)
UA697 (Control)
0 10
0 08
0 06
0 04
0 02
0 00
Challenge
Induction
0
20
40
60
80
Time (hours)

C

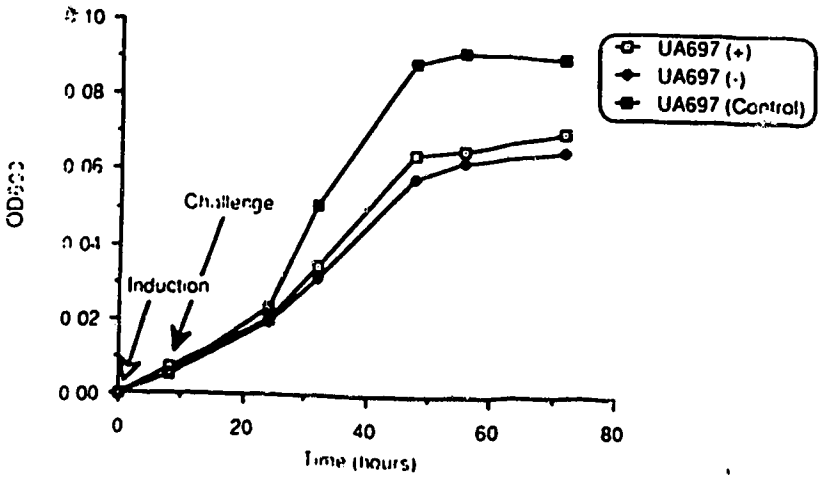
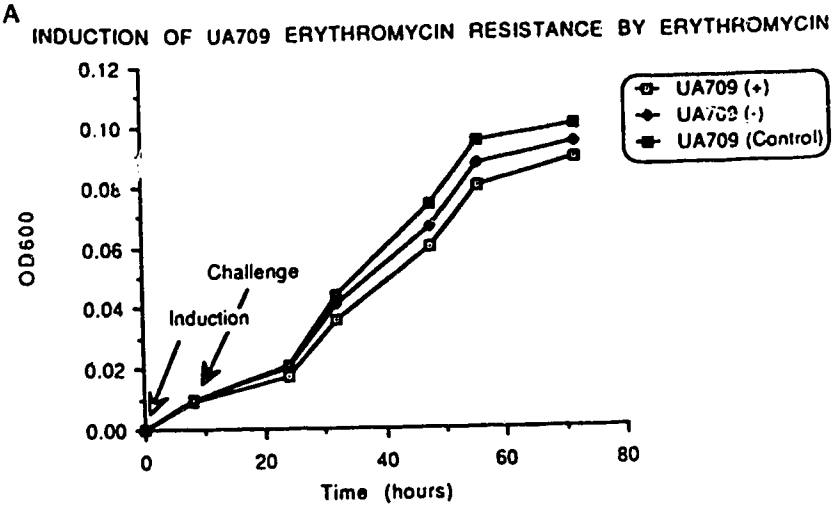
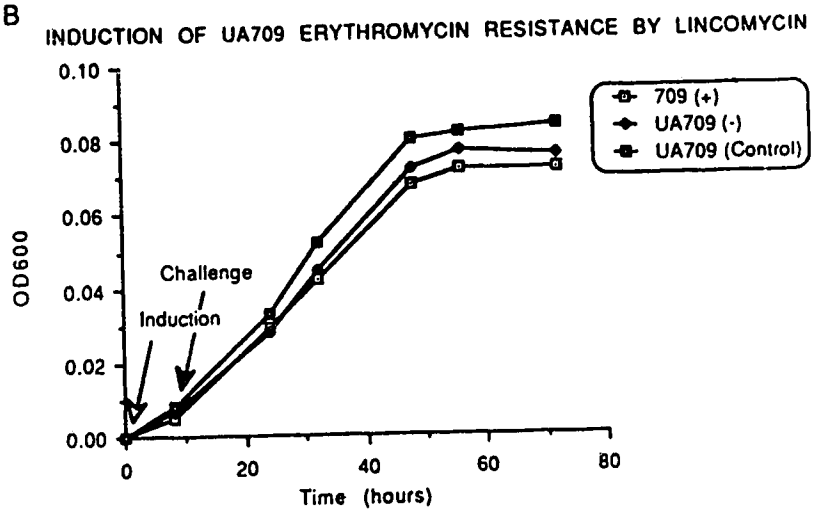

INDUCTION OF UA697 ERYTHROMYCIN RESISTANCE BY OLEANDOMYCIN
UA697 (+)
UA697 (-)
UA697 (Control)
0 10
0 08
0 06
0 04
0 02
0 00
Challenge
Induction
0
20
40
60
80

Fig. 3.4 Induction study of *C. jejuni* UA709 erythromycin resistance. *C. jejuni* UA709 cultures were pre-incubated with (A) erythromycin; (B) lincomycin and (C) oleandomycin before being challenged with high concentration of erythromycin. A legend for the lines representing cultures with pre-incubation, without pre-incubation and growth curve control is shown for each graph.

A


INDUCTION OF UA709 ERYTHROMYCIN RESISTANCE BY ERYTHROMYCIN
0.12
0.10
0.08
0.06
0.04
0.02
0.00
OD600
Challenge
Induction
0
20
40
60
80
Time (hours)
UA709 (+)
UA709 (-)
UA709 (Control)

B


INDUCTION OF UA709 ERYTHROMYCIN RESISTANCE BY LINCOMYCIN
0.10
0.08
0.06
0.04
0.02
0.00
OD600
Challenge
Induction
0
20
40
60
80
Time (hours)
709 (+)
UA709 (-)
UA709 (Control)

C

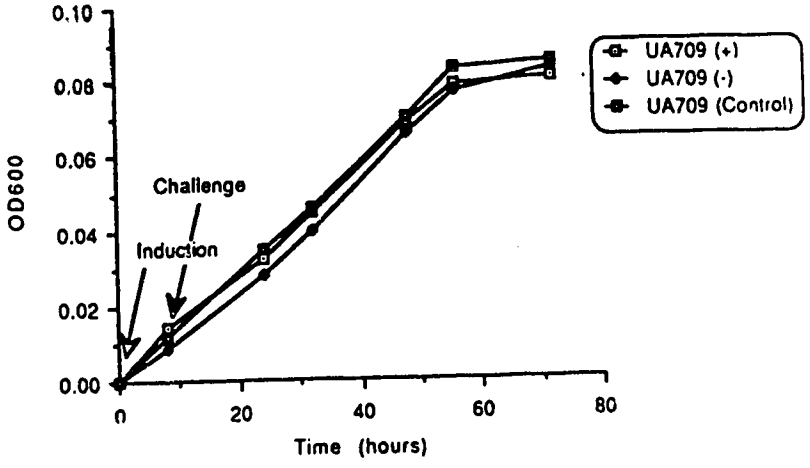
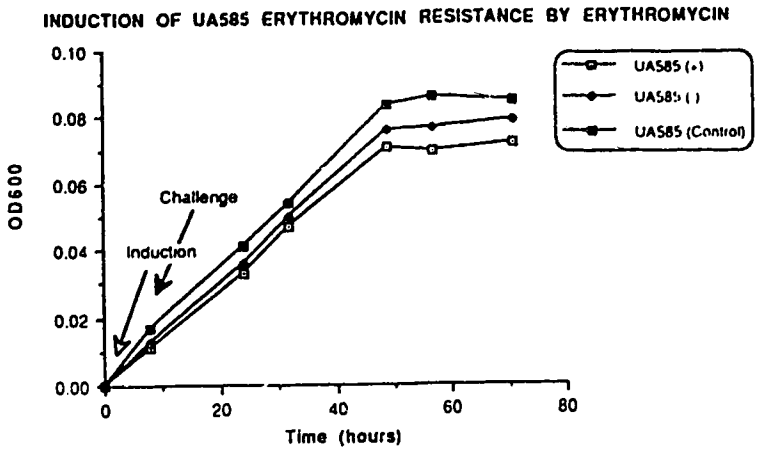
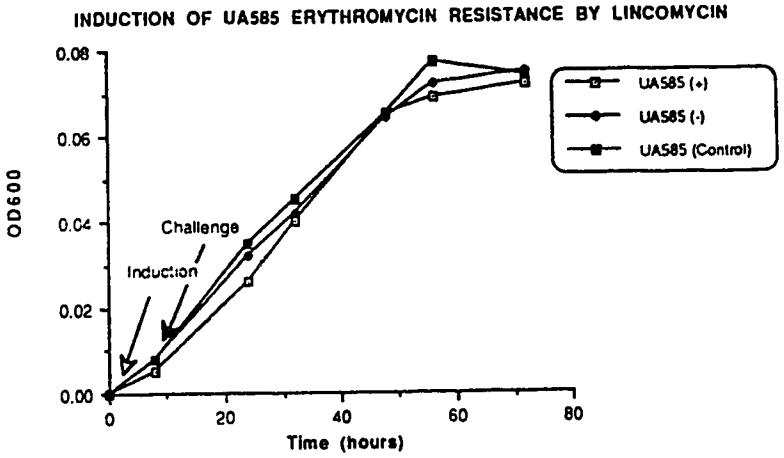

INDUCTION OF UA709 ERYTHROMYCIN RESISTANCE BY OLEANDOMYCIN
0.10
0.08
0.06
0.04
0.02
0.00
OD600
Challenge
Induction
0
20
40
60
80
Time (hours)
UA709 (+)
UA709 (-)
UA709 (Control)

Fig. 3.5 Induction study of *C. coli* UA585 erythromycin resistance. *C. coli* UA585 cultures were pre-incubated with (A) erythromycin; (B) lincomycin and (C) oleandomycin before being challenged with high concentration of erythromycin. A legend for the lines representing cultures with pre-incubation, without pre-incubation and growth curve control is shown for each graph.

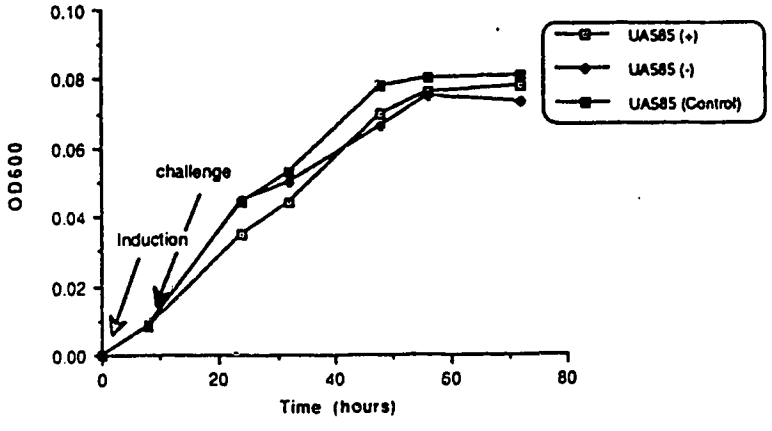
A


INDUCTION OF UA585 ERYTHROMYCIN RESISTANCE BY ERYTHROMYCIN
OD600
0.10
0.08
0.06
0.04
0.02
0.00
0
20
40
60
80
Time (hours)
Challenge
Induction
UA585 (+)
UA585 (-)
UA585 (Control)

B


INDUCTION OF UA585 ERYTHROMYCIN RESISTANCE BY LINCOMYCIN
OD600
0.08
0.06
0.04
0.02
0.00
0
20
40
60
80
Time (hours)
Challenge
Induction
UA585 (+)
UA585 (-)
UA585 (Control)

C


INDUCTION OF UA585 ERYTHROMYCIN RESISTANCE BY OLEANDOMYCIN
OD600
0.10
0.08
0.06
0.04
0.02
0.00
0
20
40
60
80
Time (hours)
challenge
Induction
UA585 (+)
UA585 (-)
UA585 (Control)

shown in Fig. 3.6. There was no obvious difference in [^{14}C]erythromycin uptake between the two resistant strains and the sensitive UA67. Maximal uptake was reached by 60 minutes followed by decrease in accumulation over the next 120 minutes. Similar results were observed for the resistant *C. jejuni* strain UA709 when compared with the rate of uptake by the sensitive strain UA736 (data not shown). Fig. 3.7 shows similar rates of [^{14}C]erythromycin uptake and accumulation by *C. coli* UA585 and UA586. The decrease in accumulation over the final 60 minutes is less pronounced than that observed with the *C. jejuni* strains. Washing with 0.5M NH_4Cl had little effect on the radioactivity measured on the filters. Therefore, although different strains of *C. jejuni* and *C. coli* varied in their sensitivities to erythromycin, their abilities to transport the antibiotic into the cell and the subsequent accumulation of the drug were similar.

3.8 Effect of erythromycin on protein synthesis in *Campylobacter* spp. The amount of [^{35}S]methionine incorporation into Campylobacter cell protein was taken as an indication of *in vivo* protein synthesis. *C. coli* UA585 and UA586 were incubated with different concentrations of erythromycin (0, 5.0, 30.0 and 100.0 µg/ml) and equal amounts of [^{35}S]methionine. The autoradiogram of SDS-PAGE profiles from each culture is shown in Fig. 3.8. Both *C. coli* UA585 and UA586 in the

Fig. 3.6 ***In vivo*** **uptake of [^{14}C]erythromycin by** ***C. jejuni*** **UA695 and UA697 and UA67.** The amounts of radioactivity associated with each culture at different time periods are taken as the amount of uptake of [^{14}C]erythromycin. Each point represents the mean value of two independent determinations. The experiment was performed twice and similar results were observed (The standard deviation values were too small to show on graph). The legend for the lines representing the uptake of [^{14}C]erythromycin by *C. jejuni* UA695, UA697 and UA67 is shown in the figure.

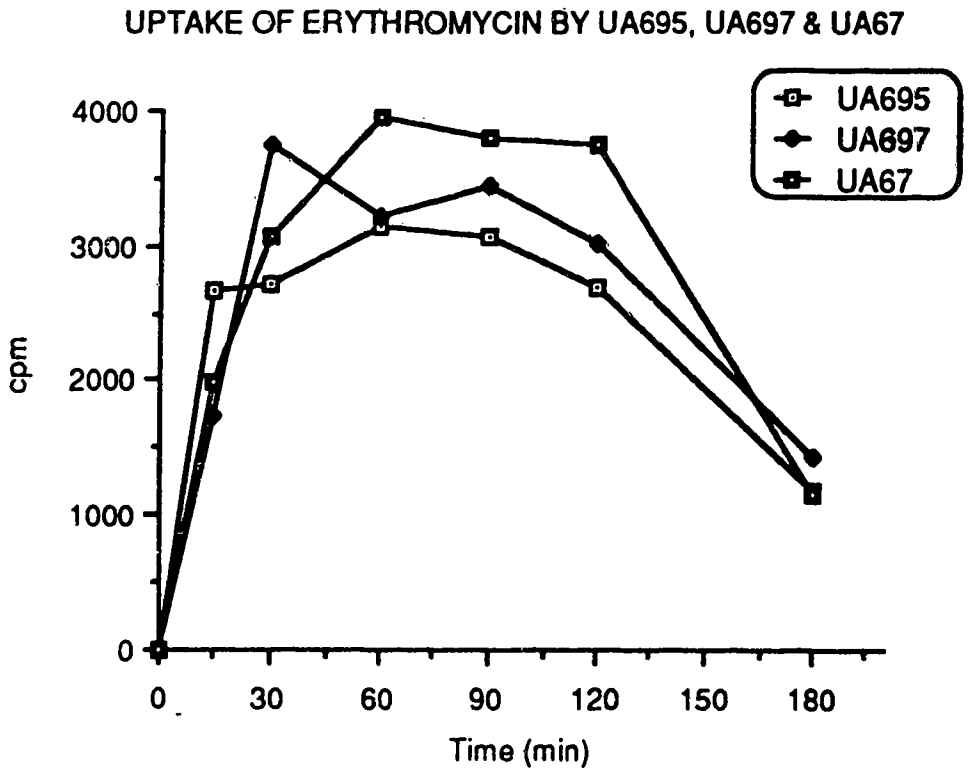

UPTAKE OF ERYTHROMYCIN BY UA695, UA697 & UA67
UA695
UA697
UA67
4000
3000
2000
1000
0
cpm
0
30
60
90
120
150
180
Time (min)

Fig. 3.7 ***In vivo*** **Uptake of [^{14}C]erythromycin by *C. coli* UA585 and UA586.** The amounts of radioactivity associated with each culture at different time periods are taken as the amount of uptake of [^{14}C]erythromycin. Each point represents the mean value of two independent determinations. The experiment was performed twice and similar results were observed (The standard deviation values were too small to show on graph). The legend for the lines representing the uptake of [^{14}C]erythromycin by *C. coli* UA585 and UA586 is shown in the figure.

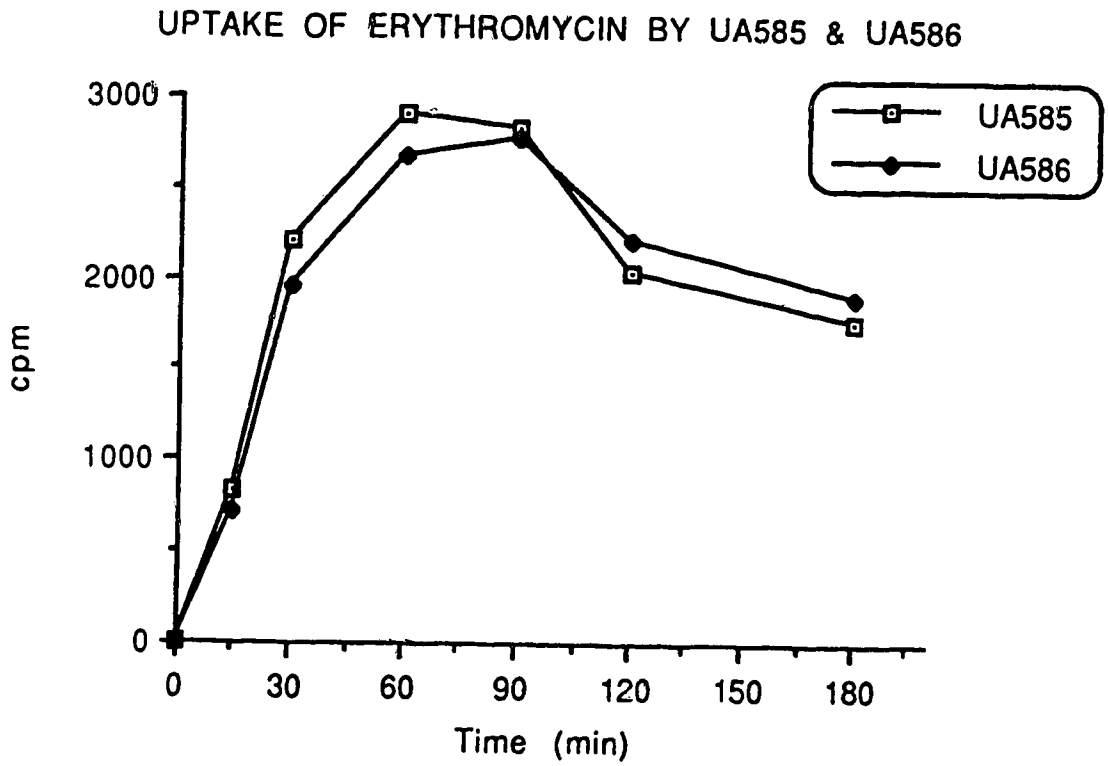
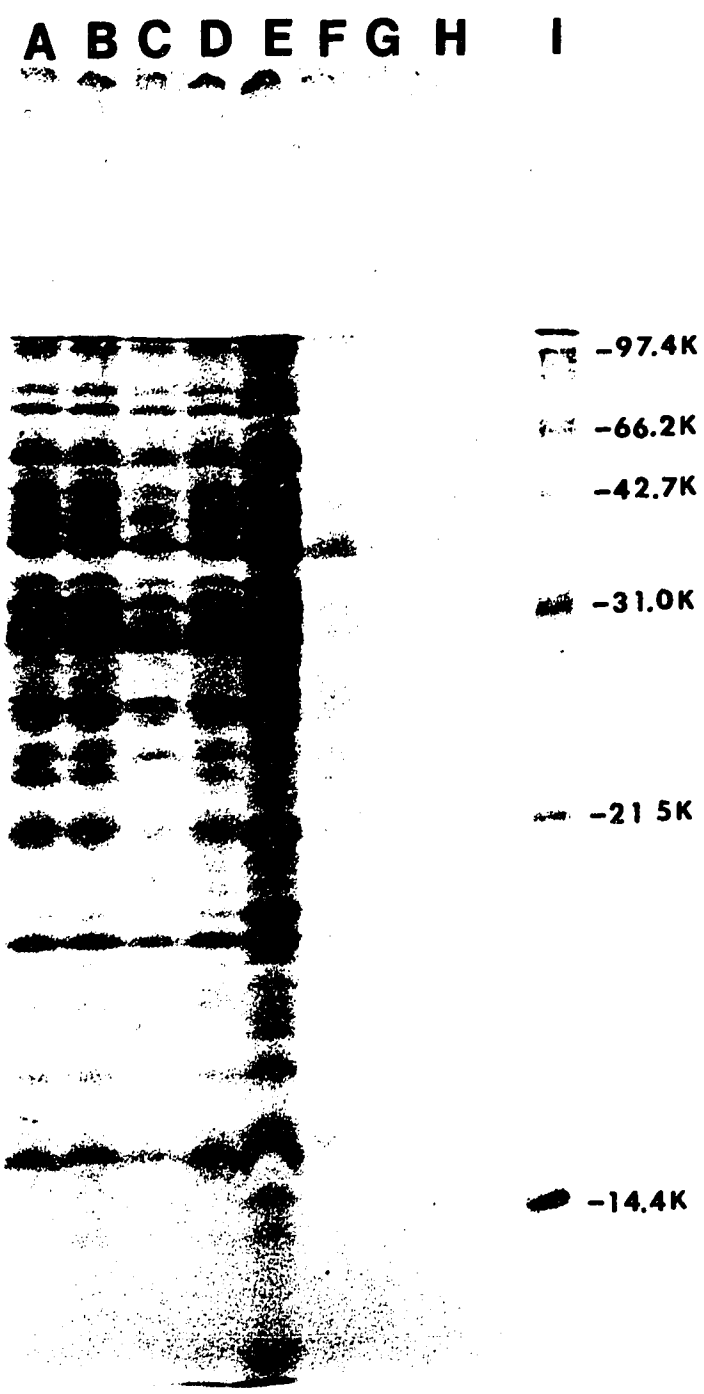
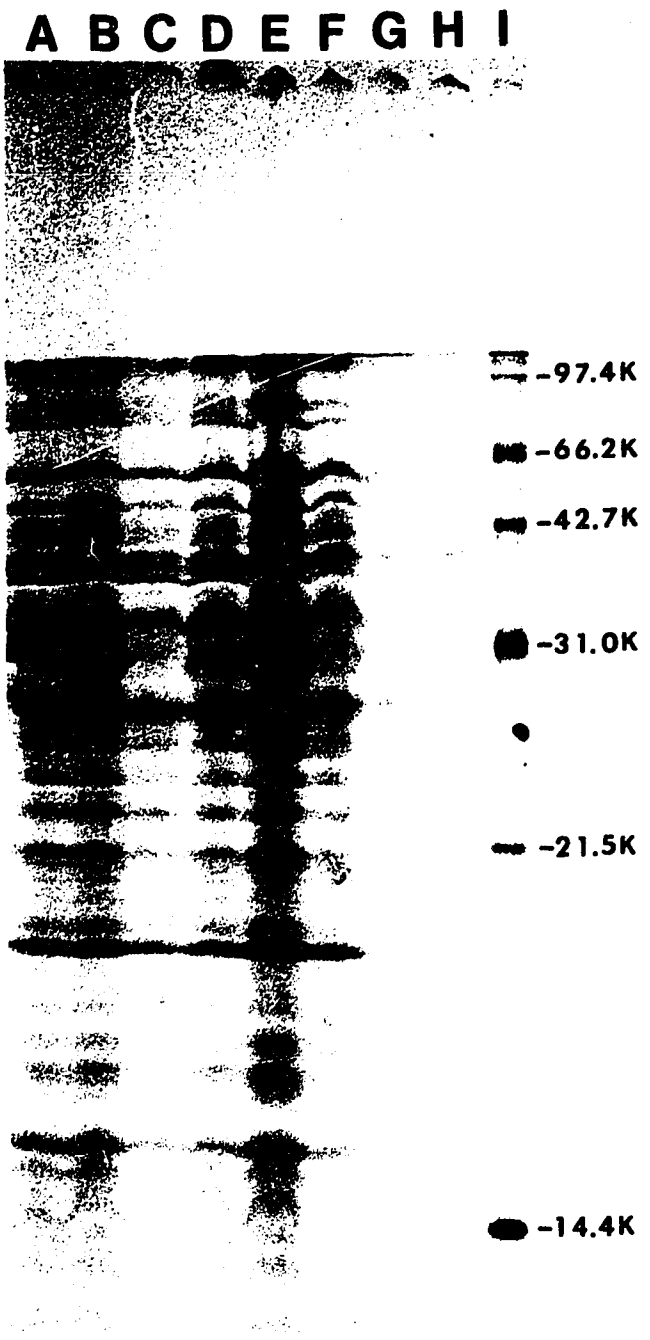

UPTAKE OF ERYTHROMYCIN BY UA585 & UA586
3000
2000
1000
0
cpm
0
30
60
90
120
150
180
Time (min)
UA585
UA586

Fig. 3.8 [^{35}S]methionine incorporation by *C. coli* UA585 and UA586 in the presence of increasing erythromycin concentrations. The extent of [^{35}S]methionine incorporation into the polypeptides separated by SDS-PAGE represents a measure of *in vivo* protein synthesis. Lanes A through D represent the amount of incorporation in UA585 cells incubated with 0, 5.0, 30.0 and 100.0 µg/ml of erythromycin respectively. Lanes E through H show the amount of incorporation in UA586 cells incubated with 0, 5.0, 30.0 and 100.0 µg/ml of erythromycin. Lane I represents [^{14}C]protein molecular weight standards: Methylated phosphorylase b (97.4 K); Methylated bovine serum albumin (66.2 K); Methylated ovalbumin (42.7 K); Methylated carbonic anhydrase (31.0 K); Methylated trypsin inhibitor (21.5 K); Methylated lysozyme (14.4 K).


A B C D E F G H I
-97.4K
-66.2K
-42.7K
-31.0K
-21.5K
-14.4K

absence of erythromycin incorporated significant amounts of [^{35}S]methionine (Lanes B and E) thus demonstrating that *in vivo* protein synthesis occured during the incubation period. However, the addition of 5 µg of erythromycin per ml to *C. coli* UA586 (Lane F), almost completely inhibited [^{35}S]methionine incorporation into proteins. No protein synthesis was detected when higher levels of erythromycin (30.0 and 100.0 µg/ml) were added (Lanes G and H). Protein synthesis in the erythromycin-resistant *C. coli* strain UA585, on the other hand, was unaffected by erythromycin concentrations as high as 100 µg/ml (Lane D). A similar study was carried out on *C. jejuni* strains UA709 and UA736 and the results are shown in Fig. 3.9. An erythromycin concentration of 100 µg/ml had no effect on [^{35}S]methionine incorporation in the resistant strain UA709. A lower concentration of erythromycin (5 µg/ml) resulted in decreased [^{35}S]methionine in *C. jejuni* UA736, although the degree of inhibition was less pronounced than that in *C. coli* UA586. [^{35}S]methionine incorporation was almost completely inhibited by an erythromycin concentration of 30 µg/ml. Similar effects of erythromycin on [^{35}S]methionine incorporation were observed for erythromycin-resistant *C. jejuni* UA695 and UA697 and erythromycin-sensitive *C. jejuni* UA67 (data not shown). Therefore, unlike the sensitive strains, *in vivo* protein synthesis in erythromycin-resistant *C. jejuni* and *C. coli* strains was unaffected by high levels of the antibiotic.

Fig. 3.9 [^{35}S]methionine incorporation by *C. jejuni* UA709 and UA736 in the presence of varying erythromycin concentrations. The extent of [^{35}S]methionine incorporation into the polypeptides separated by SDS-PAGE represents a measure of *in vivo* protein synthesis. Lanes A through D represent the amount of incorporation in UA709 cells incubated with 0, 5.0, 30.0 and 100.0 µg/ml of erythromycin respectively. Lanes E through H show the amount of incorporation in UA736 cells incubated with 0, 5.0, 30.0 and 100.0 µg/ml of erythromycin. Lane I represents [14C]protein molecular weight standards: Methylated phosphorylase b (97.4 K); Methylated bovine serum albumin (66.2 K); Methylated ovalbumin (42.7 K); Methylated carbonic anhydrase (31.0 K); Methylated trypsin inhibitor (21.5 K); Methylated lysozyme (14.4 K).


A B C D E F G H I
-97.4K
-66.2K
-42.7K
-31.0K
-21.5K
-14.4K

3.9 [^{14}C]erythromycin binding to purified ribosomes from *Campylobacter* spp. Ribosomes from *C. jejuni* and *C. coli* were isolated as described in section 2.15. Fig. 3.10 shows the A_{260} readings of *C. coli* UA586 ribosomal fractions collected from a Sephacryl S200 column with the major peak corresponding to 70S ribosomal subunits and minor peaks representing the 50S and 30S subunits. Fig. 3.11 shows the A_{260} readings of sucrose gradient fractions following centrifugation for the isolation of 50S and 30S ribosomal subunits. Although a small amount of intact 70S ribosomes were still present, the majority of the ribosomes were dissociated into 50S and 30S subunits.

The amount of [^{14}C]erythromycin binding to purified 70S ribosomes from erythromycin-resistant and sensitive *C. jejuni* and *C. coli* cells was determined as described in section 2.17. The binding studies demonstrated that under identical assay conditions, ribosomes from erythromycin-resistant *C. coli* UA585 bind less [^{14}C]erythromycin than those isolated from the erythromycin-sensitive derivative *C. coli* UA586 (Fig. 3.12). Similar binding properties were observed for 50S ribosomal subunits isolated from *C. coli* UA585 and UA586; resistant subunits bind significantly less [^{14}C]erythromycin than sensitive subunits (Fig. 3.13). As expected, [^{14}C]erythromycin did not bind to 30S ribosomal subunits from either *C. coli* UA585 or UA586 (data not shown). Similar results were obtained when the amount

Fig. 3.10 A_{260} elution profile of *C. coli* UA586 ribosomal fractions from a Sephacryl S200 column. Fractions eluted from a Sephacryl S200 column were diluted (1/100) before the A_{260} readings were recorded. The fraction numbers represent fractions collected after the sample has entered 15 cm into the column. The three distinct peaks corresponding to 70S ribosomes as well as the 50S and 30S ribosomal subunits of UA586 are indicated by arrows on the graph.

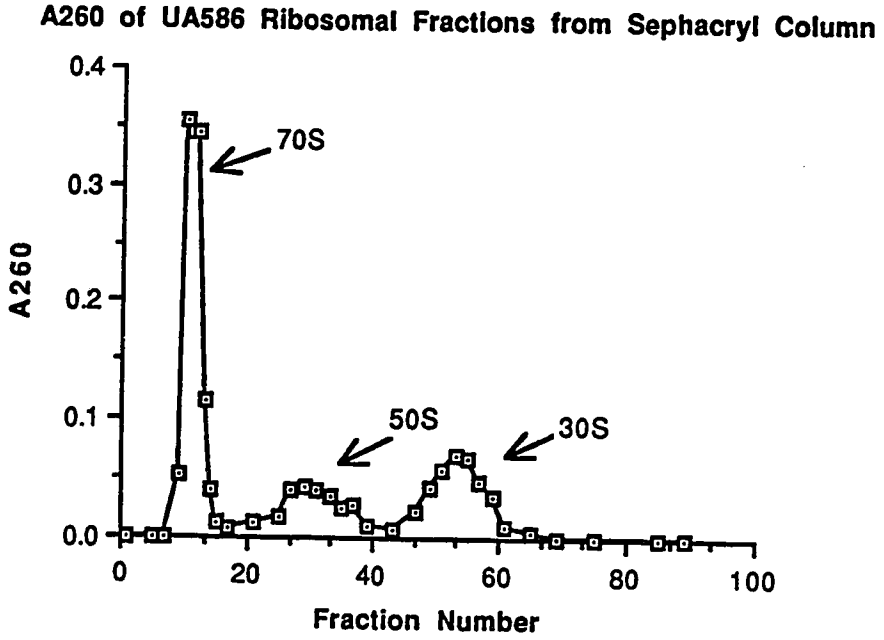

A260 of UA586 Ribosomal Fractions from Sephacryl Column
0.4
0.3
0.2
0.1
0.0
A260
70S
50S
30S
0
20
40
60
80
100
Fraction Number

Fig. 3.11 A_{260} elution profile of *C. coli* UA586 ribosomal subunits from sucrose gradient centrifugation. Fractions eluted from sucrose gradients prepared in low [$MgCl_2$] buffer were diluted (1/100) before the A_{260} readings were recorded. The major peaks corresponding to UA586 70S ribosomes as well as 50S and 30S ribosomal subunits are indicated by arrows on the graph.

A260 of UA586 Ribosomal Fractions from Sucrose Gradient

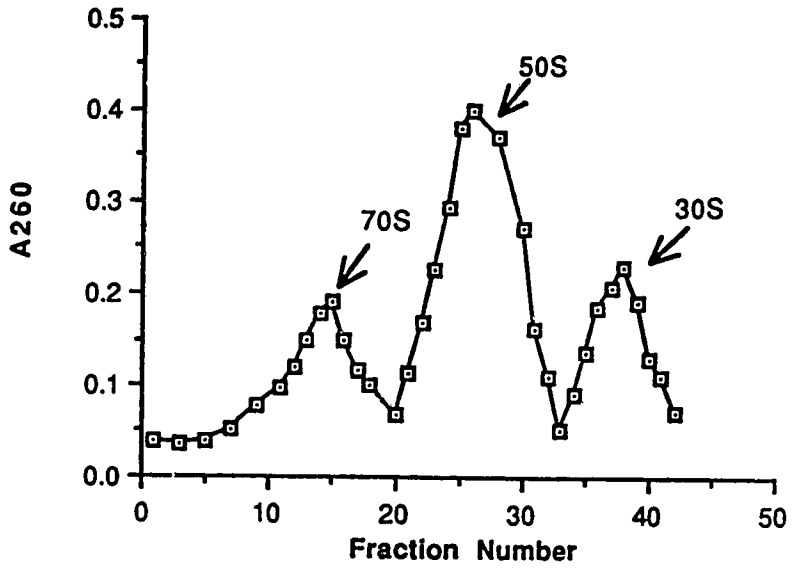


Fig. 3.12 Binding of [^{14}C]erythromycin to 70S ribosomes isolated from *C. coli* UA585 and UA586. The binding of [^{14}C]erythromycin to purified *C. coli* UA585 and UA586 70S ribosomes was determined as described in section 2.17. The legend for the lines representing the binding curves of UA585 and UA586 is shown on the graph. Each point represents the mean of two independent determinations. The experiment was performed twice and similar results were obtained (The standard deviation values were too small to be shown on the graph).

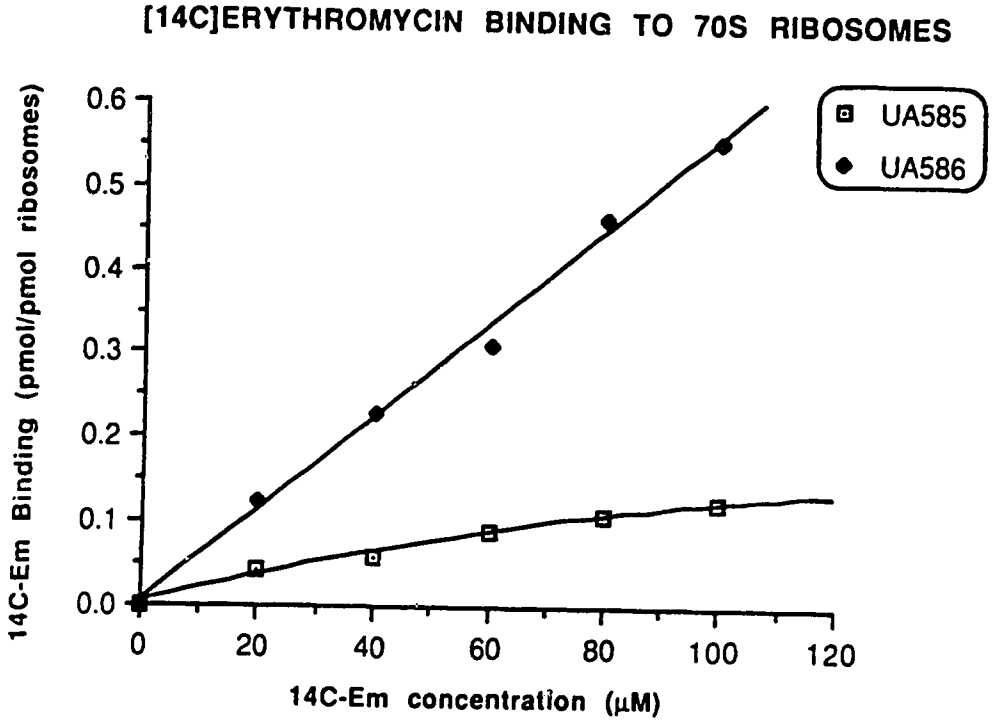
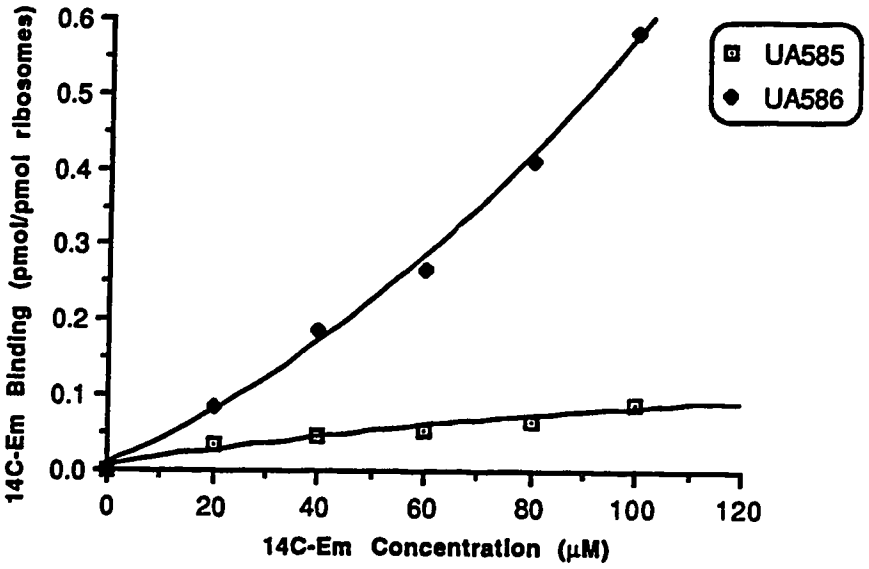

[14C]ERYTHROMYCIN BINDING TO 70S RIBOSOMES
14C-Em Binding (pmol/pmol ribosomes)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
120
14C-Em concentration (μM)
UA585
UA586

Fig. 3.13 Binding of [^{14}C]erythromycin to 50S ribosomal subunits isolated from *C. coli* UA585 and UA586. The binding of [^{14}C]erythromycin to purified *C. coli* UA585 and UA586 50S ribosomal subunits was determined as described in section 2.17. The legend for the lines representing the binding curves of UA585 and UA586 is shown on the graph. Each point represents the mean of two independent determinations. The experiment was performed twice and similar results were obtained (The standard deviation values were too small to be shown on the graph).

[14C]ERYTHROMYCIN BINDING TO 50S RIBOSOMAL SUBUNIT



of [^{14}C]erythromycin which bound to ribosomes and ribosomal subunits of *C. jejuni* UA709 and UA736 as well as resistant strains UA695/UA697 and the sensitive strain UA67 were compared. Therefore, these results suggest that some alterations in the 50S ribosomal subunits of *Campylobacter* spp. can inhibit the binding of erythromycin which leads to resistance.

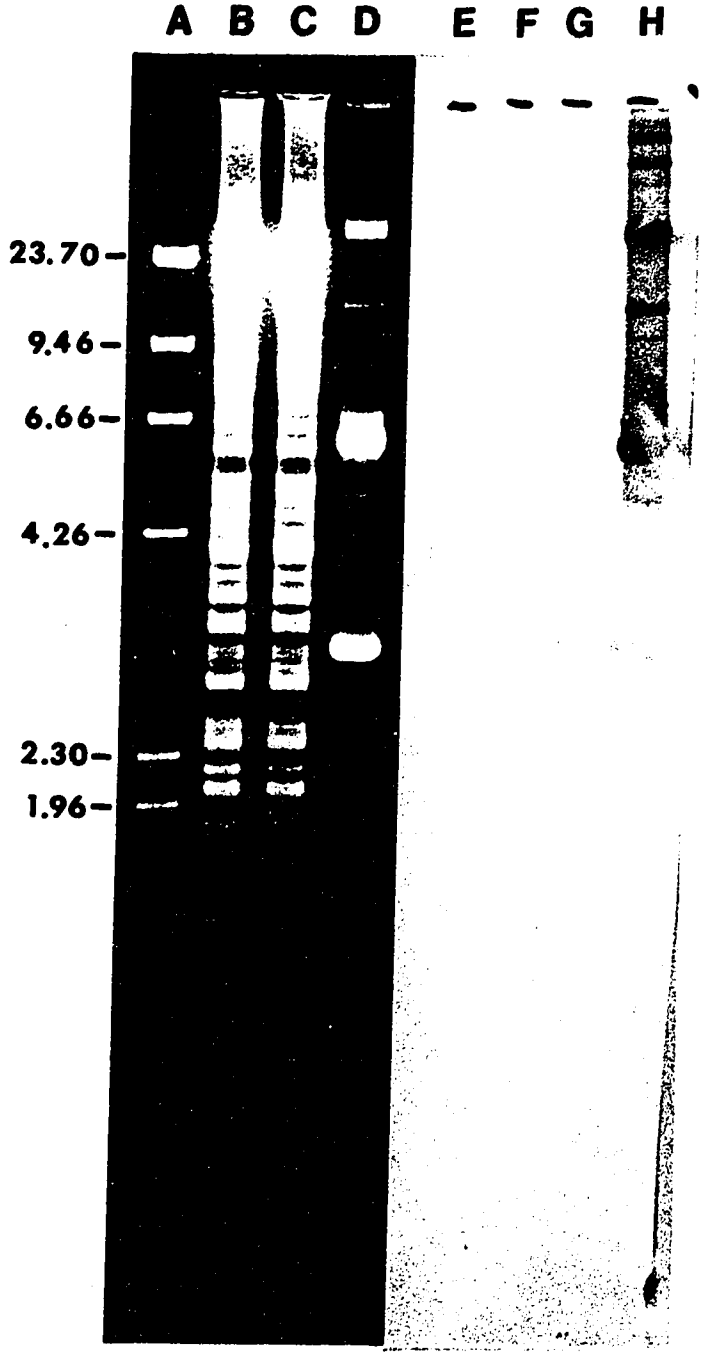
3.10 Natural transformation of erythromycin resistance. In order to confirm that erythromycin resistance is chromosomally mediated, genomic DNA isolated from *C. coli* UA585 was used to transform *C. coli* UA417 cells by the plate transformation method and erythromycin-resistant transformants were selected. A transformation frequency of approximately 50 transformants/spot (corresponding to 1 x 10^{-5} transformants/viable cell) was obtained. Since *C. coli* UA585 does not contain any plasmid DNA, the success of the natural transformation procedure demonstrates that erythromycin resistance in *C. coli* is chromosomally mediated. However, chromosomal DNA from *C. jejuni* UA695, UA697 and UA709 was unable to transform *C. coli* UA417 to erythromycin resistance.

3.11 Hybridization of erythromycin-resistant chromosomal DNA with *ermE* probe. DNA containing the *ermE* gene of *S. erythraeus* was labelled by nick translation as described in section 2.22 and used as a probe in hybridization studies under moderate stringency conditions (37°C). Chromosomal DNA isolated from *C. jejuni* UA695,

UA697 and UA709 as well as *C. coli* UA585 was digested with the restriction endonuclease *Pst*I, subjected to agarose gel electrophoresis and transferred onto nitrocellulose filters as described. Fig. 3.14 is a photograph of an agarose gel showing the *Pst*I digestion patterns of UA695 and UA697 chromosomal DNA (Lanes B and C) as well as its corresponding autoradiogram after hybridization with the *ermE* probe. Bacteriophage λ DNA digested with *Hind*III and DNA containing the *ermE* gene were used as negative and positive controls respectively (Lanes A and D). Neither the λ DNA (lane E) nor the chromosomal DNA from *C. jejuni* UA695 and UA697 (lanes F and G) hybridized with the *ermE* probe. Strong hybridization, however, was observed between the probe and the positive control DNA (lane H). Similar results were obtained when hybridization studies were carried out using chromosomal DNA from *C. jejuni* UA709 and *C. coli* UA585. Since hybridizations were performed under relatively low stringency conditions, these results indicate that erythromycin-resistant *C. jejuni* and *C. coli* chromosomes do not contain any methylase genes related to the *ermE* gene of *S. erythraeus*.

3.12 Selection of appropriate restriction endonucleases for PFGE analysis of Campylobacter chromosome. PFGE analysis of the Campylobacter genome was used as a means of locating the region involved in erythromycin resistance. A large number of restriction endonucleases were used in the digestion of *C. jejuni* and

Fig. 3.14 Hybridization of *C. jejuni* UA695 and UA697 DNA with *ermE* probe. A radioactively labelled *ermE* probe was used to hybridize *C. jejuni* UA695 and UA697 chromosomal DNA digested with *Pst*I and separated by agarose gel electrophoresis. Lanes A through D represents the photograph of an ethidium bromide-stained agarose gel. Lane (A) λ DNA digested with *Hind*III; (B) *C. jejuni* UA695 chromosome digested with *Pst*I; (C) *C. jejuni* UA697 chromosome digested with *Pst*I; (D) *ermE* probe. Lanes E through H show the corresponding hybridization patterns.


A B C D E F G H
23.70
9.46
6.66
4.26
2.30
1.96

C. coli genomic DNA prepared in agarose blocks as described in Materials and Methods. The restriction endonucleases used along with their specific recognition sequences are listed in Table 3.3. The number and sizes of DNA fragments generated by these enzymes show tremendous variation. For instance, *Not*I, which has an eight-basepair recognition sequence, failed to digest *C. jejuni* and *C. coli* genomic DNA. On the other hand, digestion with the restriction endonuclease *Hind*III resulted in the production of fragments too numerous to count. Fig. 3.15 shows a sample of the restriction patterns of *C. jejuni* UA580 DNA produced by some of the enzymes used in an initial screening experiment. Of the nine restriction endonucleases tested, only *Sma*I (Lane i) produced a restriction pattern suitable for genomic mapping analysis. Further screening with other enzymes demonstrated that three endonucleases, *Sal*I, *Sma*I and *Kpn*I, were the most suitable for PFGE analysis of *C. jejuni* and *C. coli* genomic DNA. Subsequent studies using *Kpn* I, however, showed the digestion patterns generated by this enzyme were not reproducible. Therefore, only the enzymes *Sal*I and *Sma*I were utlilized in all subsequent PFGE studies.

3.13 PFGE of *Sal*I and *Sma*I restriction fragments of *C. coli* UA417 and UA417R genomic DNA. The nalidixic acid-resistant *C. coli* strain UA417 and its spontaneous streptomycin-resistant mutant UA417R were

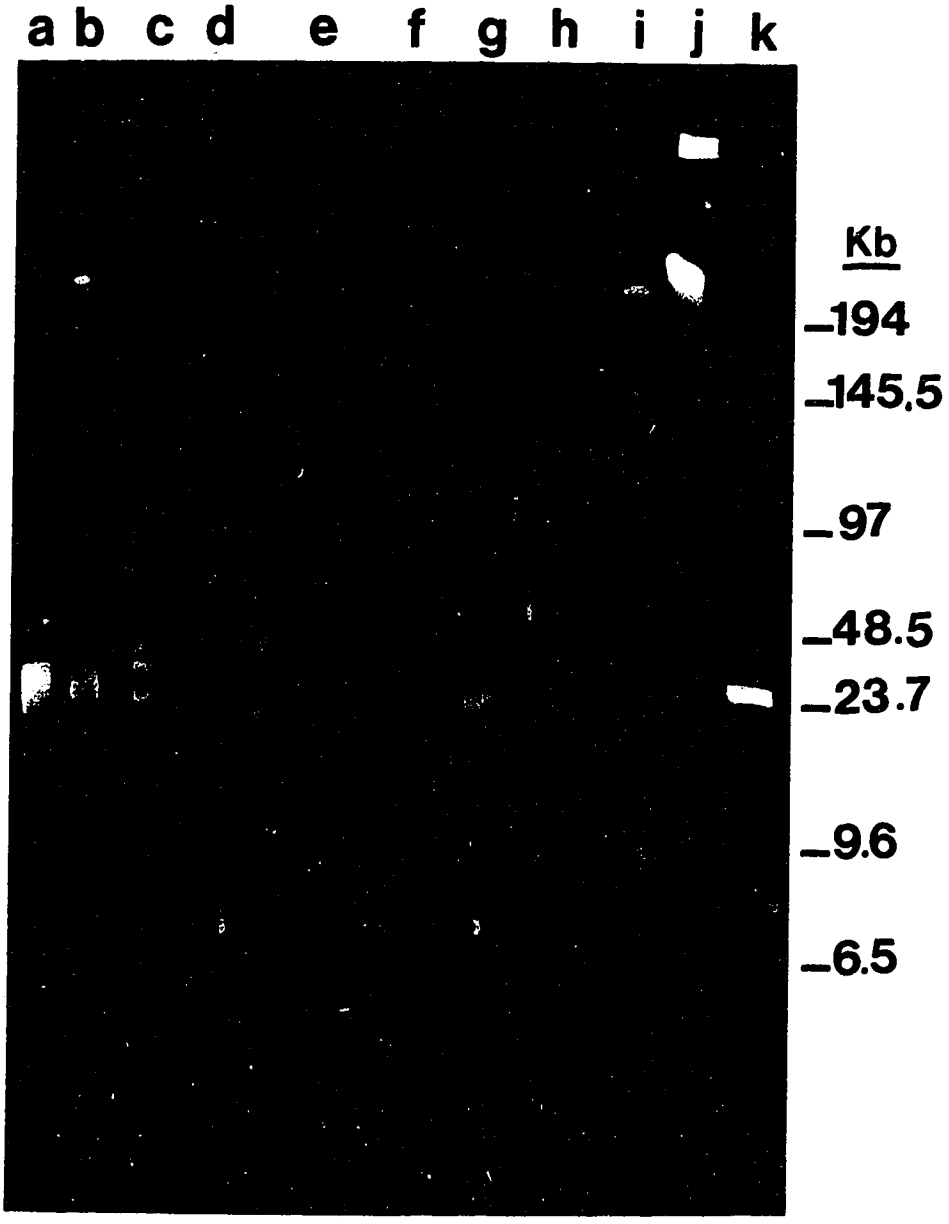
Table 3.3
Restriction endonucleases used in PFGE

Restriction endonuclease	Recognition sequence[1]	Number of fragments
*Not*I	GC/GGCCGC	0
*Sfi*I	GGCCNNN/NGGCC	0
*Dra*I	TTT/AAA	many
*Sma*I	CCC/GGG	few
*Sal*I	G/TCGAC	few
*Kpn*I	GGTAC/C	few
*Sca*I	AGT/ACT	many
*Bam*HI	G/GATCC	> 10
*Eco*RV	GAT/ATC	many
*Hind*III	A/AGCTT	many
*Mlu*I	A/CGCGT	many
*Nae*I	GCC/GGC	smear
*Nar*I	GG/CGCC	many
*Nde*I	CA/TATG	many
*Pst*I	CTGCAG	many
*Pvu*II	CAG/CTG	many
*Msp*I	C/CGG	many

---

[1]Nucleotides: N=any nucleotide; /=cleavage site of each enzyme

Fig. 3.15 PFGE analysis of *C. jejuni* UA580 genomic DNA digested with various restriction endonucleases. Chromosomal DNA from *C. jejuni* UA580 was prepared in agarose blocks and digested with different restriction endonucleases as described in section 2.18. PFGE was then performed in 1% agarose at 175V and 12 second pulse time for 24 hours in 0.5x TBE buffer. The restriction endonucleases used are represented in the photograph of the ethidium bromide-stained agarose gel by: Lane (a) *Mlu*I; Lane (b) *Msp*I; Lane (c) *Nde*I; Lane (d) *Ava*I; Lane (e) *Cfo*I; Lane (f) *Pst*I; Lane (g) *Pvu*II; Lane (h) *Sca*I; Lane (i) *Sma*I; Lane (j) λ DNA concatamers; Lane (k) λ DNA digested with *Hind*III.


a b c d e f g h i j k
Kb
194
145.5
97
48.5
23.7
9.6
6.5

chosen for *Sal*I and *Sma*I digestion as well as for PFGE. In addition, *C. coli* UA417 and UA417R were found to be highly efficient hosts for natural transformation of C. coli DNA. Genomic DNA from *C. coli* UA417 and UA417R was isolated in agarose blocks and subjected to *Sal*I and *Sma*I digestions. The *Sal*I restriction patterns of UA417 and UA417R are shown in Fig. 3.16. Optimal conditions for the resolution of larger molecular weight DNA fragments, that is 200V and 35 second pulse time, were used. Digestion of both UA417 (Lane B) and UA417R (Lane C) genomic DNA gave identical patterns of 7 distinct restriction fragments. The largest fragment located near the top of the gel represents a partial digestion fragment. Lane A shows the separation of bacteriophage λ concatamers which were used as standards for the size determination of *Sal*I restriction fragments. *Sma*I restriction patterns of UA417 and UA417R are shown in Fig. 3.17. In this experiment, PFGE was performed at 175V and 12 second pulse time in order to obtain optimal resolution of restriction fragments in this size range. *Sma*I digestion resulted in 12 restriction fragments each for UA417 (Lane A) and UA417R (Lane B) respectively. Lane C shows bacteriophage λ concatamers as molecular weight standards. Subsequent studies using extended electrophoresis time showed that the third largest *Sma*I restriction fragment represents a doublet of 2 similar sized fragments (data not shown). Therefore, the UA417 and

Fig. 3.16 PFGE of *SalI* restriction fragments of *C. coli* UA417 and UA417R genomic DNA. Chromosomal DNA from *C. coli* UA417 and UA417R was digested with *SalI* and subjected to PFGE in 1% agarose at 200V and 35 second pulse time for 24 hours in 0.5 x TBE buffer. Lanes A through C represent an ethidium bromide-stained gel: Lane (A) λ DNA concatamers; Lane (B) *C. coli* 417 chromosome digested with *SalI*; Lane (C) *C. coli* 417R chromosome digested with *SalI*. The molecular weights of the λ concatamers as well as the locations of the 7 *SalI* fragments are indicated on the figure.

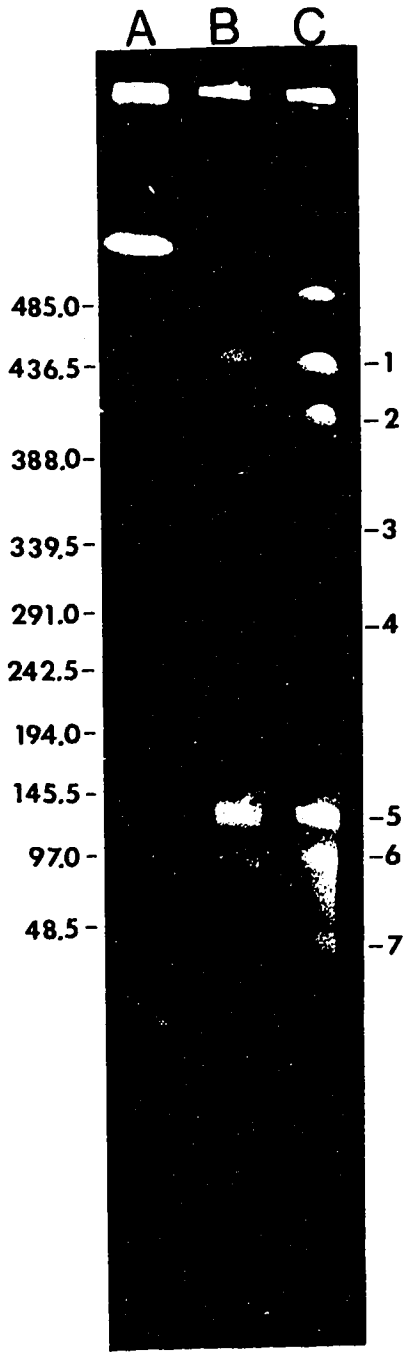
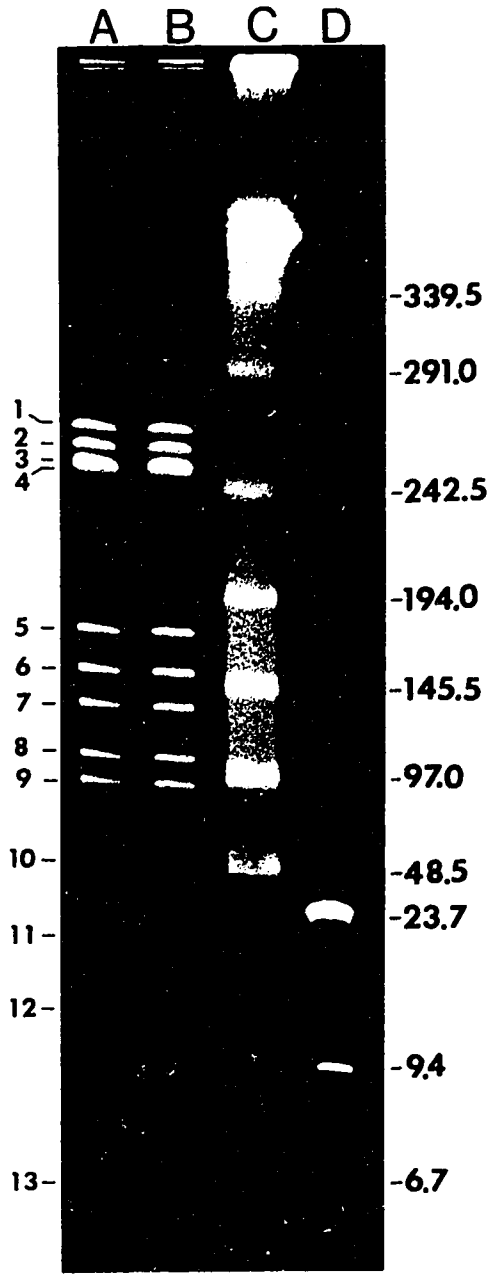

A
B
C
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5
1
2
3
4
5
6
7

Fig. 3.17 PFGE of *Sma*I restriction fragments of *C. coli* UA417 and UA417R genomic DNA. Chromosomal DNA from *C. coli* UA417 and UA417R was digested with *Sma*I and subjected to PFGE in 1% agarose at 175V and 12 second pulse time for 24 hours in 0.5x TBE buffer. Lanes A through D represent an ethidium bromide-stained gel: Lane (A) *C. coli* UA417 digested with *Sma*I; Lane (B) *C. coli* 417R chromosome digested with *Sma*I; Lane (C) λ DNA concatamers; (D) λ DNA digested with *Hind*III. The molecular weights of the λ concatamers as well as the locations of the 13 *Sma*I fragments are indicated on the figure.


A
B
C
D
339.5
291.0
242.5
194.0
145.5
97.0
48.5
23.7
9.4
6.7
1
2
3
4
5
6
7
8
9
10
11
12
13

UA417R genomes were each determined to contain 13 *Sma*I restriction fragments.

3.14 Determination of genome sizes of *C. coli* UA417 and UA417R. The molecular weights of *Sal*I and *Sma*I restriction fragments were determined based on standard curves generated by plotting the relative mobilities of bacteriophage λ concatamers vs their molecular weights. Fig. 3.18 represents a typical standard curve in which the migration distances were plotted against the molecular weights of the corresponding concatamers. Minor variations in mobilities, particularly with the larger molecular weight fragments, were observed from gel to gel. Therefore, average values for each *Sal*I and *Sma*I restriction fragment were obtained from five independent PFGE studies for each restriction enzyme. The molecular weights of the 7 *Sal*I fragments and the 13 *Sma*I fragments are listed in Table 3.4. The genome sizes of UA417 and UA417R, as determined by the summation of restriction fragment sizes, were 1,697 kb and 1,679 kb for *Sal*I and *Sma*I respectively.

3.15 Hybridization of UA417R genomic DNA with 16S rRNA and pRif probes. The plasmid pAR140 (Rashtchian *et al.*, 1987) contains a 1.7 kb *Hind*III fragment coding for the 16S rRNA of *C. jejuni* (Fig. 3.19). Since *C. coli* UA417 and UA417R are identical in their restriction patterns, hybridization studies were carried out using only UA417

Fig. 3.18 Standard curve of migration distances plotted against molecular weights of bacteriophage λ DNA concatamers. The graph shows a typical standard curve obtained when λ DNA concatamers are separated by PFGE performed using 1 % agarose at 200V and 35 seconds pulse time for 24 hours in 0.5x TBE buffer.

Standard Curve for PFGE Molecular Weight Determination

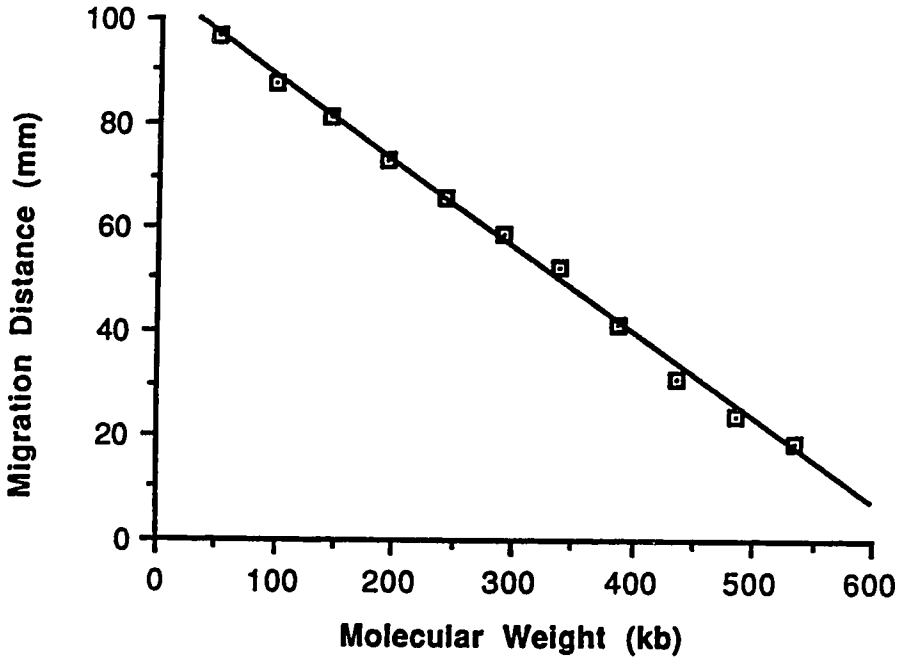


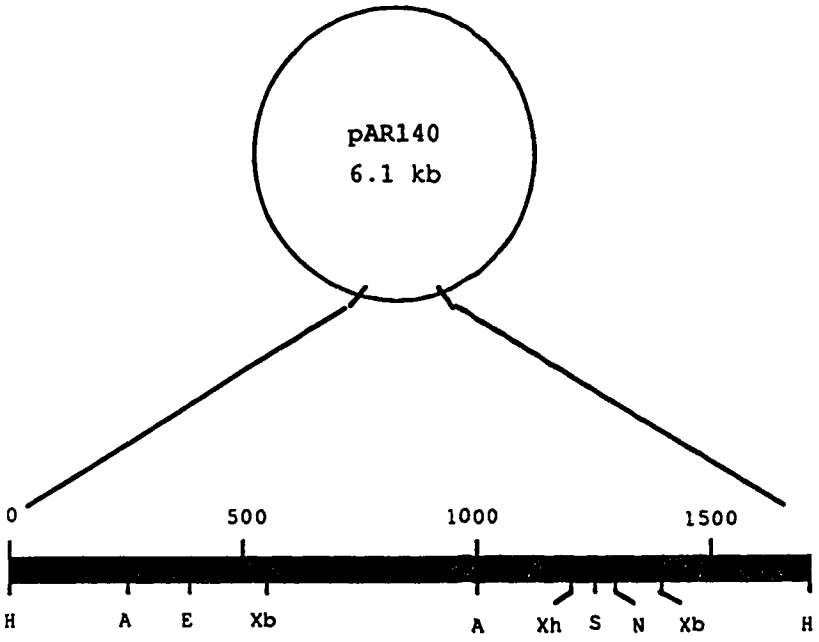
Table 3.4

Campylobacter coli UA417/UA417R chromosomal DNA fragment sizes as determined by PFGE

Fragment	Fragment size (kb) after digestion with: *SalI*	*SmaI*
1	430 ± 7.3	255 ± 5.8
2	400 ± 5.2	250 ± 2.4
3	344 ± 3.7	240 ± 5.8
4	276 ± 4.3	238 ± 2.4
5	125 ± 3.1	163 ± 4.6
6	97 ± 3.1	143 ± 2.8
7	25 ± 0.7	124 ± 2.3
8		97 ± 2.1
9		85 ± 2.9
10		42 ± 2.1
11		23 ± 1.9
12		13 ± 0.7
13		6 ± 0
Total:	1697 ± 6.8	1679 ± 8.1

Fig. 3.19 Restriction map of pAR140 containing the 16S rRNA gene of *C. jejuni*. The plasmid pAR140 was constructed by the insertion of a 1.7 kb *Hind*III fragment coding for *C. jejuni* 16S rRNA into the cloning vector pUN121. The restriction sites located in this 1.7 *Hind*III fragment is shown in Fig. 3.19.

RESTRICTION MAP OF pAR140



Restriction Sites

A = *Ava* II

E = *Eco* I

H = *Hind* III

N = *Nco* I

S = *Sma* I

Xb = *Xba* I

Xh = *Xho* I

genomic DNA. The *Hind*III fragment from pAR140 containing the *C. jejuni* 16S rRNA gene was isolated from LMP agarose gel and radioactively labelled by nick translation. Fig. 3.20 shows the hybridization pattern of this probe with UA417 DNA digested with *Sal*I. The 16S rRNA probe hybridized strongly with UA417 *Sal*I fragments 2 and 5. Weak hybridization was also detected in the region of *Sal*I fragment 4. This weak hybridization was absent when identical hybridization studies were carried out on two other occasions. This suggests that the probe may be reacting weakly with some partially digested or degraded DNA migrating in the same region as *Sal*I fragment 4. In hybridization studies using UA417 DNA digested with *Sma*I, the 16S rRNA probe hybridized stongly with *Sma*I fragments 2, 5 and 8 (Fig. 3.21). In another hybridization study, plasmid DNA containing the pRif gene coding for the *B*-subunit of RNA polymerase of *Haemophilus influenzae* (W. Albritton, personal communication) was radioactively labelled and used as a probe. Results from this study showed that the pRif probe hybridized with UA417 *Sal*I fragments 4 and 7 as well as *Sma*I fragments 1 and 11.

3.16 Natural transformation of erythromycin, streptomycin and nalidixic acid resistance in *Campylobacter coli*. After PFGE was performed using LMP agarose, individual *Sal*I and *Sma*I fragments were cut out, melted and used as donor DNA in biphasic natural

Fig. 3.20 PFGE analysis of *C. coli* UA417 *SalI* restriction fragments and hybridization patterns with the 16S rRNA probe. Chromosomal DNA from *C. coli* UA417 was digested with *SalI* and subjected to PFGE in 1% agarose at 200V and 35 second pulse time for 24 hours in 0.5 x TBE buffer. Lanes A and B represent the ethidium bromide-stained gel: (A) λ DNA concatamers; (B) *C. coli* UA417 chromosome digested with *SalI*. Lanes C and D represent the corresponding hybridization patterns with the radioactively labelled 16S rRNA probe. The fragments showing hybridization with the probe are indicated on the figure.

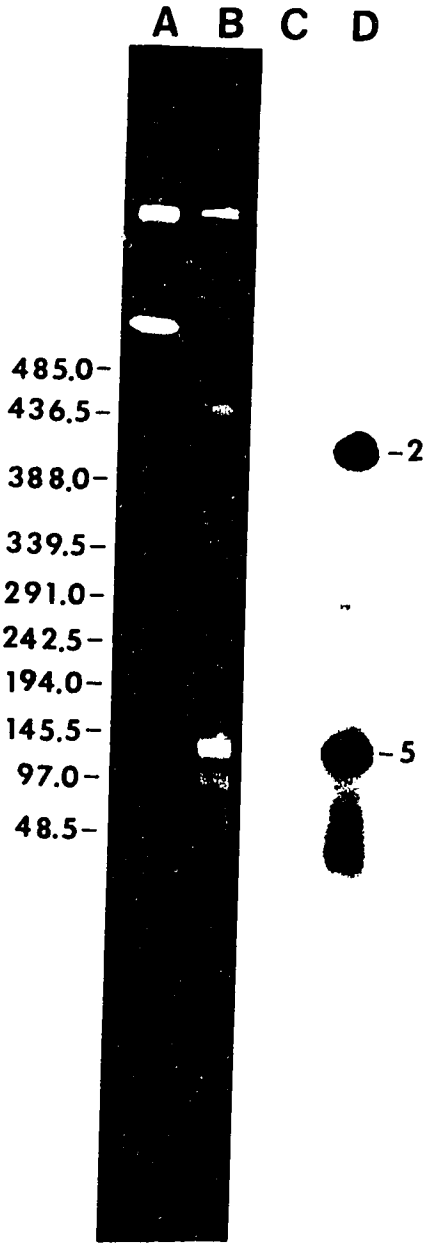

A
B
C
D
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5
2
5

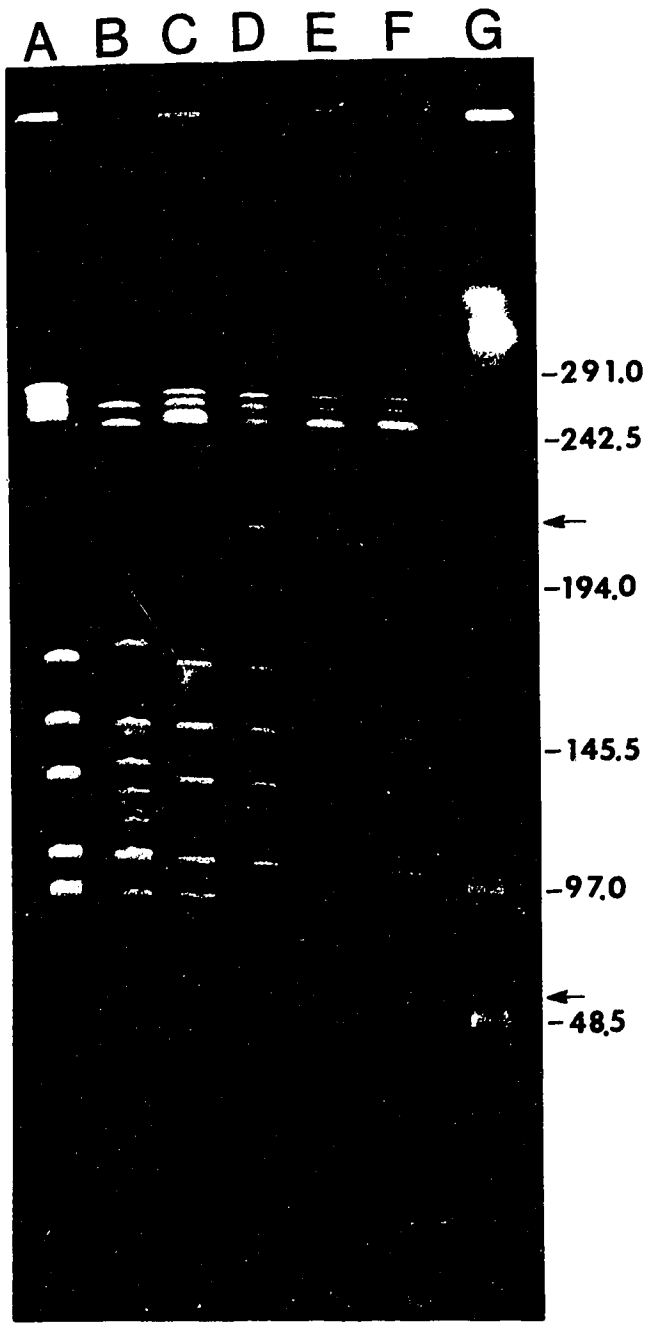
Fig. 3.21 PFGE analysis of *C. coli* UA417 *Sma*I restriction fragments and hybridization patterns with the 16S rRNA probe. Chromosomal DNA from *C. coli* UA417 was digested with *Sma*I and subjected to PFGE in 1% agarose at 175V and 12 second pulse time for 24 hours in 0.5 x TBE buffer. Lanes A and B represent the ethidium bromide-stained gel: (A) λ DNA concatamers; (B) *C. coli* UA417 chromosome digested with *Sma*I. Lanes C and D represent the corresponding hybridization patterns with the radioactively labelled 16S rRNA probe. The fragments showing hybridization with the probe are indicated on the figure.


A
B
C
D
242.5
194.0
145.5
97.0
48.5
-2
-5
-8

transformation studies. For the selection of streptomycin-resistant transformants, *Sal*I and *Sma*I fragments from *C. coli* UA417R were isolated and used to transform cultures of *C. coli* UA417. Only *Sal*I fragment 3 and *Sma*I fragment 6 were found to transform streptomycin resistance into *C. coli* UA417. In total, 12 streptomycin-resistant transformants were obtained. For the selection of erythromycin-resistant transformants, an initial plate transformation was performed using isolated *C. coli* UA585 genomic DNA as donor and *C. coli* UA417 as host. A total of 20 erythromycin-resistant UA417 transformants were obtained.

All of these erythromycin-resistant transformants as well as the 12 streptomycin-resistant transformants were subjected to *Sma*I digestion followed by PFGE analysis. Fig. 3.22 shows the *Sma*I restriction patterns of two erythromycin-resistant transformants (Lanes C and D), two streptomycin-resistant transformants (Lanes E and F) as well as the original UA417 (Lane A) and UA585 (Lane B) genomic DNA. With the exception of the erythromycin-resistant transformant in Lane D, all of the remaining transformants possessed identical restriction patterns to that of UA417. The unique erythromycin-resistant transformant (Lane D) lacks *Sma*I fragment 3. In its place, two new fragments were generated (indicated by arrows). Natural transformation studies showed that the larger of

Fig. 3.22 PFGE analysis of *Sma*I restriction fragments of *C. coli* UA417 erythromycin and streptomycin-resistant transformants. Lanes A through G represent an ethidium bromide-stained agarose gel for the separation of *Sma*I restriction fragments of different Campylobacter genomic DNA: (A) *C. coli* UA417; (B) *C. coli* UA585; (C) *C. coli* UA417 erythromycin-resistant transformant #1; (D) *C. coli* UA417 erythromycin-resistant transformant #2; (E) *C. coli* UA417 streptomycin-resistant transformant #1; (F) *C. coli* UA417 streptomycin-resistant transformant #2; (G) λ DNA concatamers.

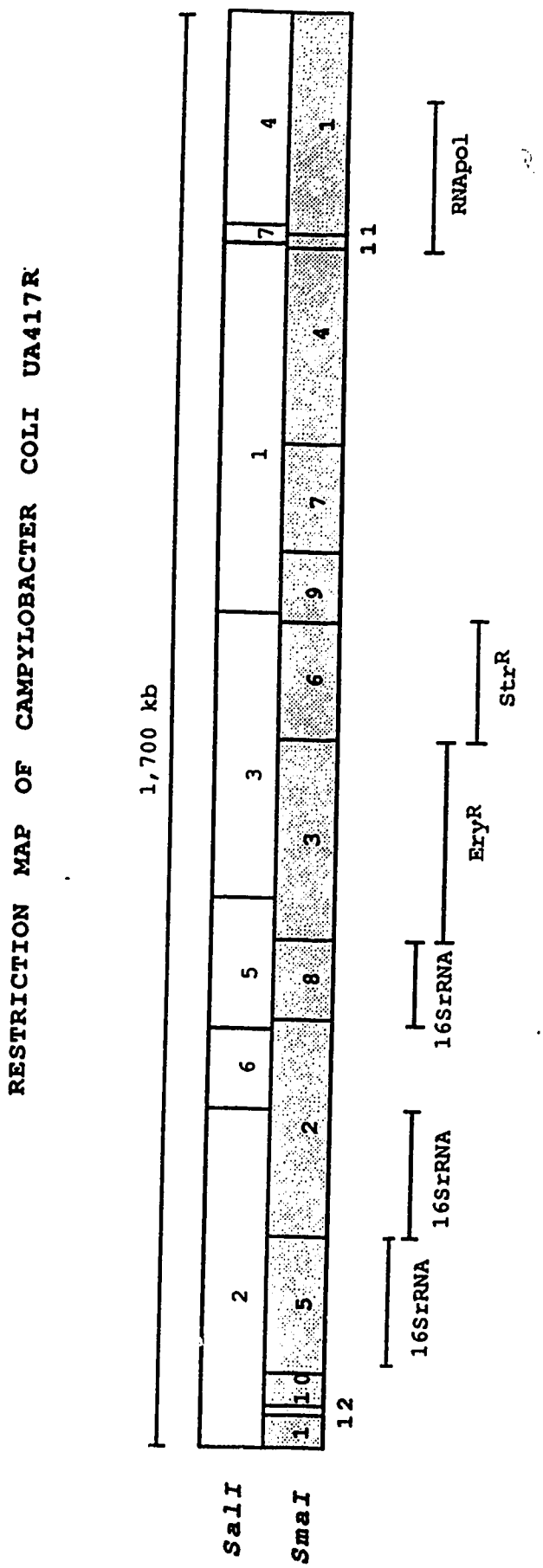

A B C D E F G
-291.0
-242.5
-194.0
-145.5
-97.0
-48.5

the two new *Sma*I fragments from this transformant as well as fragment 3 from the remaining erythromycin-resistant transformants were able to transform erythromycin resistance to UA417. All erythromycin and streptomycin-resistant transformants possessed similar MICs to the original resistant strains UA585 and UA417R.

C. coli UA585 had to be used as the host for the selection of nalidixic acid-resistant transformants since UA417 is naturally resistant to this antibiotic. When individual UA417 *Sma*I and *Sal*I fragments were used to transform UA585 cells, no nalidixic acid resistant transformants were detected. This is likely attributable to the fact that *C. coli* UA585 is a less suitable host for transformation than *C. coli* UA417.

3.17 Construction of a physical map of the UA417R genome. Results from PFGE analysis, hybridization studies using the 16S rRNA and RNA polymerase probes as well as natural transformation studies provided the starting points for the construction of the UA417 genomic map. Overlapping *Sma*I and *Sal*I fragments from these experiments as well as partial restriction fragments found in some of the pulsed-field gels were used for map construction. Location of the remaining fragments was determined by isolating and radioactively labelling individual *Sma*I and *Sal*I fragments for use as probes in hybridizing DNA digested with the other restriction enzyme. Results accumulated from all of these studies led to the construction of the UA417 genomic

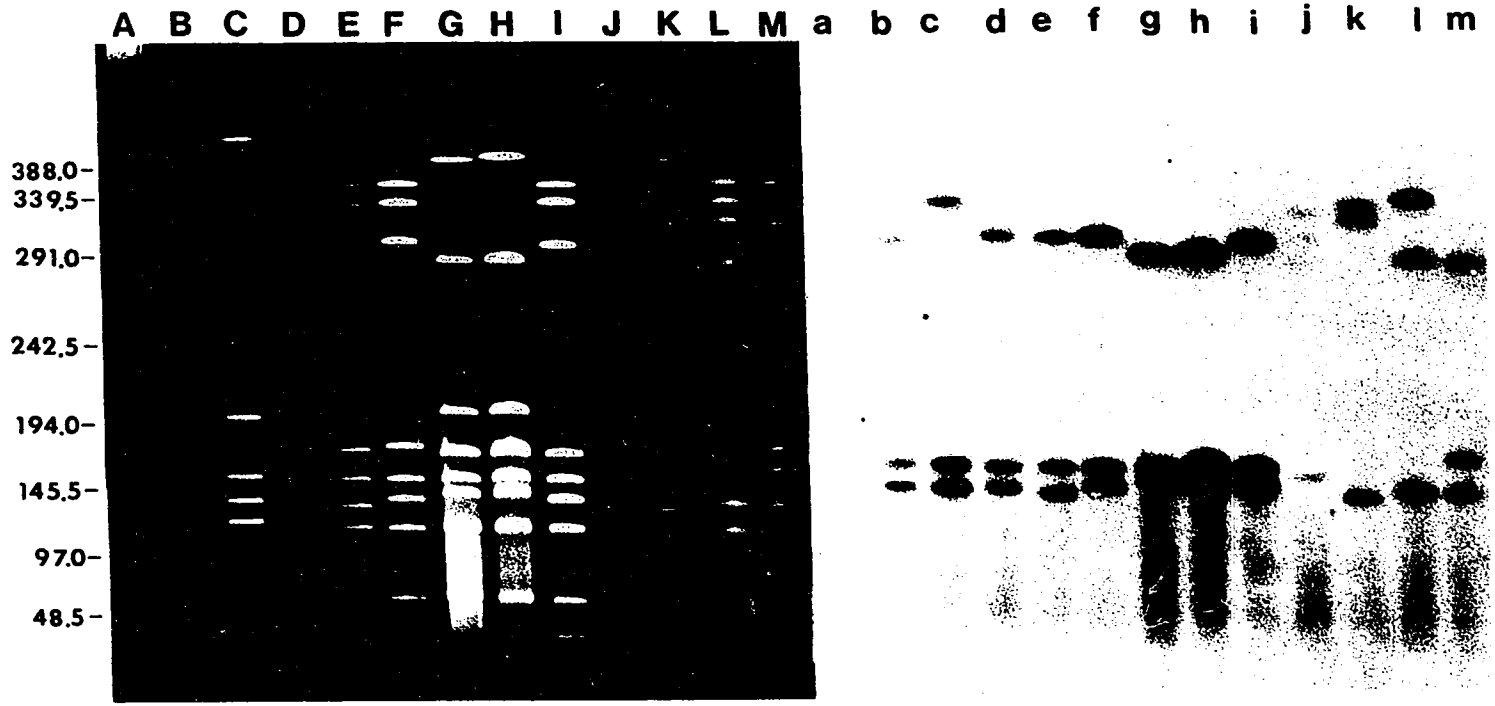
Fig. 3.23 Physical map of *C. coli* UA417R genome. The *Sal*I and *Sma*I fragments shown in the physical map of *C. coli* UA417R genome are numbered according to decreasing sizes with fragment 1 being the largest. The locations of genes coding for 16S rRNA and ß-subunit of RNA polymerase as well as the regions involved in erythromycin and streptomycin resistance are indicated on the map.


RESTRICTION MAP OF CAMPYLOBACTER COLI UA417R
1,700 kb
SalI
SmaI
12
11
16SrRNA
16SrRNA
16SrRNA
ErγR
StrR
RNApol

map as shown in Fig. 3.23. The low resolution locations of the 16S rRNA genes, the RNA polymerase gene as well as genes involved in erythromycin and streptomycin resistance are also indicated on the map.

3.18 *Sma*I PFGE restriction patterns and 16SrRNA hybridization patterns of various *C. jejuni* and *C. coli* genomic DNA. Genomic DNA from various *C. jejuni* and *C. coli* strains was digested with the restriction endonuclease *Sma*I and subjected to PFGE. Fig. 3.24 shows an ethidium bromide-stained gel representing the *Sma*I restriction patterns as well as the corresponding 16S rRNA hybridization patterns of the twelve *C. jejuni* strains tested. *C. jejuni* UA365, UA580 and UA709 (lanes E, F and I) had identical restriction patterns consisting of 9 distinct *Sma*I restriction fragments. Similarly, UA695 and UA697 (lanes G and H) shared a common restriction pattern of 8 *Sma*I fragments which differed from all the other 10 *C. jejuni* strains tested. All the remaining 7 *C. jejuni* strains, UA53, UA67, UA268, UA736, UA336 UA344 and UA347 had unique restriction patterns ranging from 7 to 9 *Sma*I fragments. It should be noted that UA709 and UA736, which were initially considered to be an isogenic pair isolated from a patient, displayed distinct restriction patterns. Similarly, the two cattle strains, UA336 and UA344, and the milk isolate UA347 (lanes K, L and M respectively) each showed unique restriction patterns despite the fact that they were all isolated during an outbreak of Campylobacter

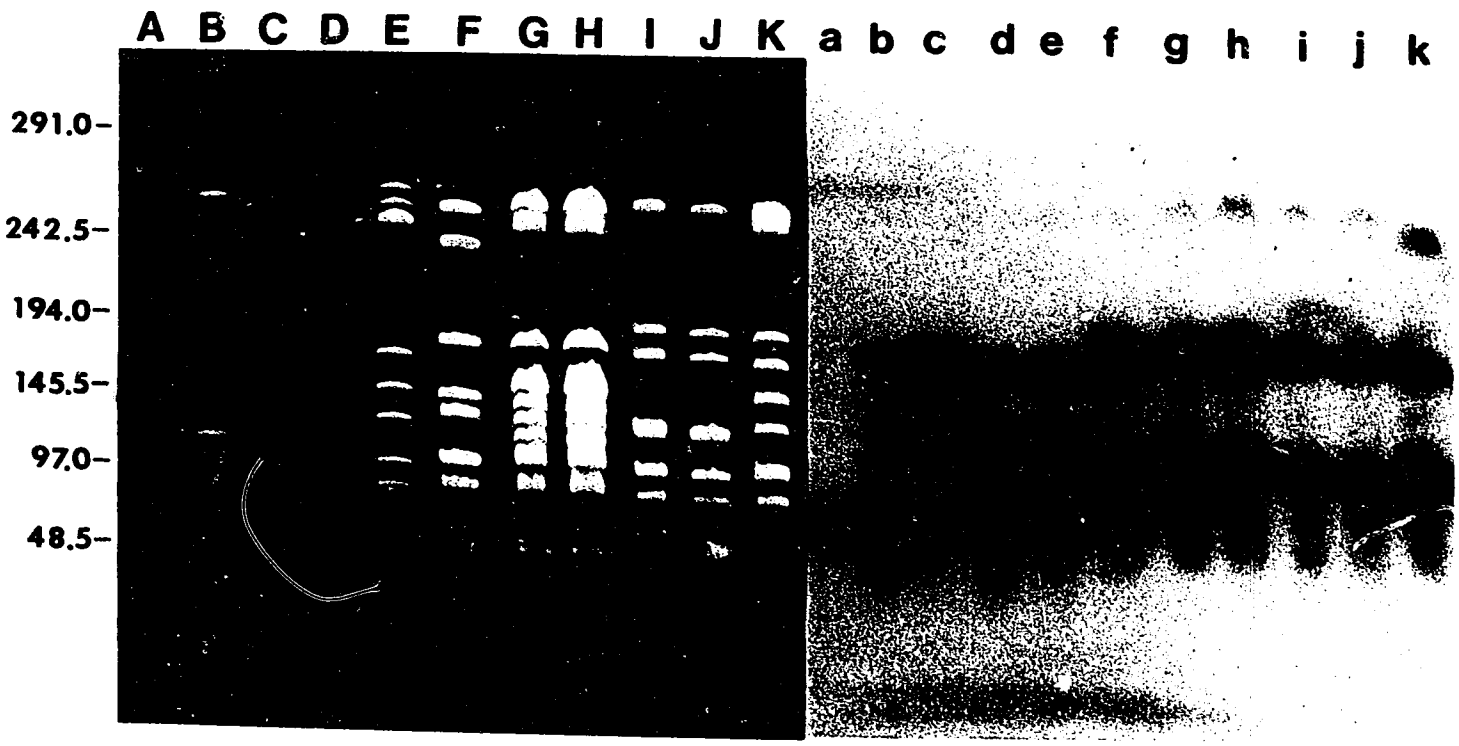
Fig. 3.24 PFGE and 16S rRNA fingerprinting analysis of twelve *C. jejuni* strains. Chromosomal DNA from 12 *C. jejuni* strains was digested with *Sma*I and subjected to PFGE in 1% agarose at 175V and 12 seconds pulse time for 24 hours in 0.5x TBE buffer. Lanes A through M represents the ethidium bromide-stained agarose gel: (A) λ DNA concatamers; (B) UA53; (C) UA67; (D) UA268; (E) UA365; (F) 580; (G) 695; (H) UA697; (I) UA709; (J) UA736; (K) UA336; (L) UA344; (M) UA347. Lanes a through m represents the corresponding 16S rRNA fingerprinting patterns.


A B C D E F G H I J K L M a b c d e f g h i j k l m
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

enteridis in Southhampton, U. K. With the exception of UA268 (lane D), the *Sma*I restriction pattern of these *C. jejuni* strains were consistent with their 16SrRNA fingerprinting patterns (ie. all the strains showing similar restriction patterns also showed similar hybridization patterns whereas those having unique restriction patterns showed different fingerprinting patterns). UA268, on the other hand, had unique restriction pattern but an identical finger- printing pattern to those of UA365, UA580 and UA709.

The *Sma*I restriction patterns of the ten *C. coli* strains tested as well as the corresponding hybridization pattern with 16SrRNA are shown in Fig. 3.25. The erythromycin-resistant strain *C. coli* UA585 (lane G) and its isogenic erythromycin-sensitive derivative UA586 (lane H) had an identical restriction profile consisting of 13 *Sma*I fragments. *C. coli* UA748 and UA749 (lanes I and J), both of which are erythromycin-resistant human isolates from Belgium, shared a common restriction profile of 11 *Sma*I fragments. The remaining 6 *C. coli* strains, UA37, UA40, UA261, UA417, UA578 and UA757 all possessed unique restriction patterns ranging from 10 to 12 *Sma*I fragments. It should be noted that UA261, which has a typical *C. coli* restriction pattern, was initially reported to be a *C. jejuni* isolate. Unlike the 16S rRNA hybridization patterns observed for the 12 *C. jejuni* strains, those observed for the 10 *C. coli* strains appeared to be highly conserved

Fig. 3.25 PFGE and 16S rRNA fingerprinting analysis of ten *C. coli* strains. Chromosomal DNA from 10 *C. coli* strains was digested with *Sma*I and subjected to PFGE in 1% agarose at 175V and 12 seconds pulse time for 24 hours in 0.5x TBE buffer. Lanes A through K represent the ethidium bromide-stained agarose gel: (A) λ DNA concatamers; (B) UA37; (C) UA40; (D) UA261; (E) UA417; (F) UA578; (G) UA585; (H) UA586; (I) UA748; (J) UA749; (K) UA757. Lanes a through k represent the corresponding 16S rRNA hybridization patterns.


A B C D E F G H I J K a b c d e f g h i j k
291.0–
242.5–
194.0–
145.5–
97.0–
48.5–

despite the variability in their *Sma*I restriction patterns. Two particular *Sma*I fragments (approximately 100 and 160 kb in size) hybridized stongly, whereas a larger molecular weight fragment in the range of 240 kb displayed weak hybridization.

3.19 Rapid hippurate hydrolysis test. In order to confirm the differntiation between *C. jejuni* and *C. coli* based on PFGE and 16S rRNA hybridization patterns, rapid hippurate hydrolysis tests were carried out on all strains of *Campylobacter* spp. used in this study (Table 3.5). With the exception of UA261, all strains described as *C. jejuni* were hippurate positive, whereas all *C. coli* strains were negative. UA261, which was originally reported to be a *C. jejuni* human isolate from Southampton, was unable to hydrolse hippurate.

3.20 Dot blot analysis of Campylobacter strains. Another approach for the differentiation of *C. jejuni* and *C. coli* strains is DNA hybridization with radioactively-labelled probes specific for one or both of these species. Dot blot analysis of chromosomal DNA isolated from the 22 strains of *Campylobacter* spp. used in this study was performed with the *C. jejuni*-specific DNA probe pDT1720 and the *C. jejuni/C. coli*-specific probe pDT1719. Dot blot analysis using pDT1720 as probe was carried out under high stringency conditions (Fig. 3.26 panel A). The probe pDT1720 hybridized with all the strains which possessed the

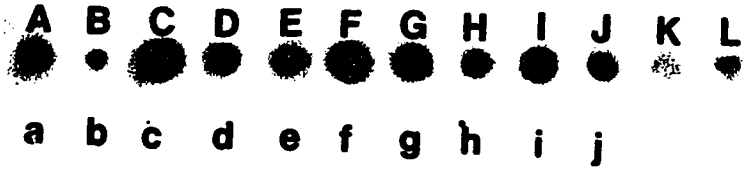
Table 3.5

Rapid hippurate hydrolysis test for Campylobacter strains

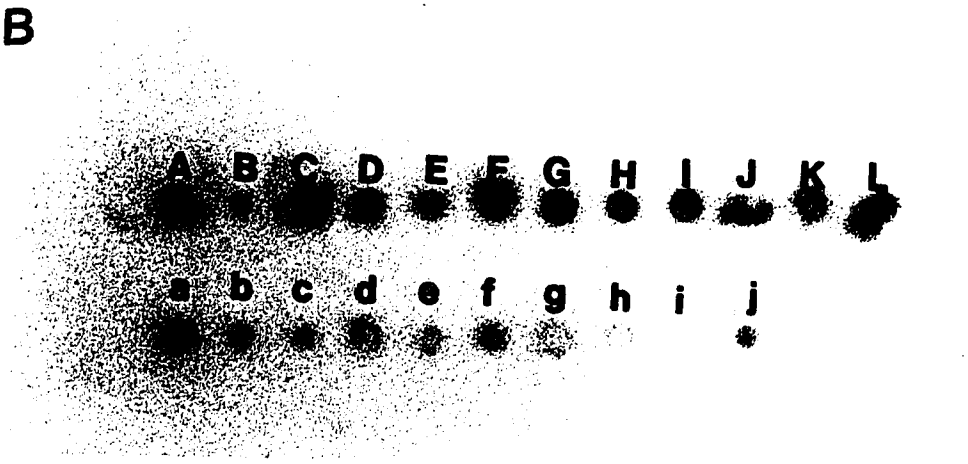
Bacterial strain	Hippurate hydrolysis
C. jejuni	
UA53	+
UA67	+
UA268	+
UA365	+
UA580	+
UA695	+
UA697	+
UA709	+
UA736	+
UA336	+
UA344	+
UA347	+
C. coli	
UA37	-
UA40	-
UA261	-
UA417	-
UA578	-
UA585	-
UA586	-
UA748	-
UA749	-
UA757	-

Fig. 3.26 Dot blot analysis of Campylobacter strains using the DNA probes pDT1719 and pDT1720. Chromosomal DNA was isolated from the 22 Campylobacter strains and spotted onto nitrocellulose membrane in the following order: (A) UA53; (B) UA67; (C) UA268; (D) UA365; (E) UA580; (F) UA695; (G) UA697; (H) UA709; (I) UA736; (J) UA336; (K) UA344; (L) UA347; (a) UA37; (b) UA40; (c) UA261; (d) UA417; (e) UA578; (f) UA585; (g) UA586; (h) UA748; (i) UA749; (j) UA757. Panel A represents hybridization carried out using pDT1720 under high stringency conditions. Panel B represents hybridization carried out using pDT1719 under moderate stringency conditions.

A



B



typical *C. jejuni* PFGE restriction patterns, and which were also hippurate positive. On the other hand, the probe failed to hybridize with strains having *C. coli* restriction patterns, and which were hippurate negative. Hybridization using pDT1719 under moderate stringency conditions hybridized with all 22 chromosomal samples (Fig. 3.26 panel B). These results indicate that the 10 strains which did not hybridize with the pDT1720 probe, are *C. coli*. Since UA261 hybridized with the pDT1719 probe but not with the pDT1720 probe, UA261 was confirmed to be a *C. coli* and not a *C. jejuni* isolate as initially reported.

4. DISCUSSION

Although erythromycin resistance in *Campylobacter* spp. emerged over ten years ago, little is known about the characteristics and mechanism of resistance to this macrolide antibiotic. With one exception, all of the erythromycin-resistant *C. jejuni* and *C. coli* strains were found to lack plasmid DNA. A combination of PFGE analysis of genomic restriction fragments and natural transformation studies was used to locate the genes responsible for erythromycin resistance to within a 240 kb region of the *C. coli* genome. Therefore, unlike the plasmid-mediated methlyases found in many erythromycin-resistant gram-positive bacteria and the erythromycin-modifying enzymes found in erythromycin-resistant gram-negative bacteria, erythromycin resistance in *C. jejuni and C. coli* was determined to be mediated by chromosomal gene products. Biological assays failed to detect any extracellular erythromycin-modifying activity in any of the erythromycin-resistant strains of *Campylobacter* spp. The possibility of intracellular modification of the antibiotic, however, cannot be excluded based on these results. As previously discussed, many of the well characterized erythromycin resistance systems involving different *erm* genes can be induced by various MLS antibiotics based on a translational attenuation mechanism. Erythromycin resistance in *C. jejuni* and *C. coli*, on the other hand, was

found to be constitutive when erythromycin, lincomycin and oleandomycin were used as inducing agents. Furthermore, this constitutive mode of resistance was found to be highly stable, with no spontaneous mutation to erythromycin sensitivity being detected. The constitutive nature, the stability of the resistance phenotype, as well as the high MICs associated with erythromycin resistance in *Campylobacter* spp. suggest that either modifications in the ribosomal target sites or barriers to entry of the antibiotic into resistant cells are likely to be the mechanism of resistance.

In order to gain insight into the mechanism of erythromycin resistance, isogenic pairs of resistant and sensitive *C. jejuni* and *C. coli* were sought to analyze various aspects of the resistance phenotype. Although *C. jejuni* UA709 and UA736 were clinical isolates obtained from a single patient and were initially thought to be isogenic, serotyping results showed that they belong to two different Lior serogroups. This lends support to the long recognized clinical concern that a patient can be colonized by more than one strain of *Campylobacter* species at a given time. *C. coli* UA586, on the other hand, was a rare laboratory-isolated, erythromycin-sensitive derivative of its resistant parent strain UA585. Since both strains were found to belong to Lior serogroup 8 and biogroup II, it is more than likely that these two strains are indeed isogenic. Therefore, although comparisons were made

between the various resistant and sensitive pairs of organisms, those results derived from *C. coli* UA585 and UA586 are likely to be more significant.

Using [^{14}C]erythromycin, the uptake and accumulation of the antibiotic was found to be identical in resistant and sensitive strains of *C. jejuni* and *C. coli*, thus indicating that a permeability barrier is unlikely to be the mechanism of resistance. Erythromycin is known to function by inhibiting bacterial protein synthesis at the chain elongation step (Menninger and Otto, 1982). Using [^{35}S]methionine incorporation as an indication of *in vivo* protein synthesis in resistant cells, it was found that while minute amounts of erythromycin completely inhibited growth in sensitive cells, high concentrations of the drug (100µg/ml) had little effect on the synthesis of proteins in resistant cells. It is of interest to note that two of the erythromycin susceptible strains used in these studies, *C. coli* UA586 and *C. jejuni* UA736, behaved slightly differently in the presence of erythromycin. While UA586 protein synthesis was completely abolished with 5 µg/ml of erythromycin, the same concentration of the antibiotic had much less pronounced effect on UA736 protein synthesis. This observation coincides with the fact that UA586 has a lower MIC (4 µg/ml) than that of UA736 (8 µg/ml). Results from these studies indicated that after normal entry of erythromycin into resistant cells, its inhibitory activity on bacterial protein synthesis was

somehow abrogated. Hence, ribosomes from erythromycin-resistant and sensitive *C. jejuni* and *C. coli* strains were isolated, purified and analyzed to determine if modifacation in these target sites is involved in the resistance mechanism.

Procedures previously used to isolate ribosomes from *E. coli* were modified to derive a suitable method for the isolation and purification of ribosomes from *Campylobacter* spp. Since the growth rate of Campylobacter is much slower than that of *E. coli*, scaled-down versions of earlier procedures were used. As a result, the yields of purified ribosomes from Campylobacter spp. were much lower than those obtained from *E. coli*. The first method employed, involving high-speed centrifugation, resulted in Campylobacter 70S ribosomal aggregates being isolated in the form of hard, transparent pellets. These ribosomal aggregates were extremely difficult to resuspend completely thus further limiting the final yield of purified ribosomes. An alternate method, in which samples containing 70S ribosomes were passed through a Sephacryl S-200 column, proved to be much more efficient for the isolation of ribosomes from Campylobacter. The eluted peak fractions corresponding to 70S ribosomes were pooled concentrated using PEG. This eliminated the high-speed rifugation step which in turn improved the final yield ribosomes. Furthermore, a sufficient yield of 50S and 30S subunits was obtained from these 70S ribosome samples

by sucrose gradient centrifugation in the presence of a low concentration of $MgCl_2$. The ability of [^{14}C]erythromycin to bind to resistant ribosomes was found to be significantly lower than binding to ribosomes isolated from sensitive cells. More importantly, similar binding properties were observed with purified 50S ribosomal subunits. These observations confirmed that some modification in the ribosomal structure corresponding to the binding site for erythromycin in the 50S subunit is likely to be responsible for inhibiting the action of the antibiotic thereby rendering these organisms resistant. Extensive research on other bacterial macrolide-resistant systems has demonstrated that modifications in either the protein or RNA component of the ribosomes could be the basis of resistance.

It has been reported previously that no homology was detected between total DNA isolated from erythromycin-resistant Campylobacter strains and probes specific for the genes encoding ribosomal methylases (*ermA*, *ermB*, *ermC*, *ermD* and *ermF*, Papadopoulou and Courvalin, 1988). In the present study, we have determined that the *ermE* gene from *Streptomyces erythraeus* also lacked homology with chromosomal DNA isolated from erythromycin-resistant Campylobacter strains. Based on these findings, it would appear unlikely that resistance in *C. jejuni* and *C. coli* strains is the result of a foreign methylase gene being introduced into these organisms. The possibility of a

methylase originating in Campylobacter which is unrelated to other previously characterized enzymes, however, cannot be excluded.

Modifications in the 23S rRNA other than methylation may also play a role in resistance. The recessive nature of single mutations in one of several rRNA (*rrn*) operons present within a given cell limits the degree by which the resistance is expressed. It has been demonstrated that the MIC of an antibiotic increased according to the proportion of resistant-type *rrn* operons. Since the resistant *C. jejuni* and *C. coli* strains characterized in these studies all showed high levels of resistance to erythromycin, this raised questions as to whether a single mutation in one *rrn* operon can account for the resistance phenotypes. One possible explanation may be the different number of copies of rRNA genes in Campylobacter compared to other organisms such as *E. coli*. In contrast to *E. coli*, which contains seven copies of rRNA genes, studies have demonstrated that *Campylobacter* spp. possess only three copies of rRNA genes (Kim and Chan, 1989). Results obtained from physical mapping of the *C. coli* UA417R genome here, in which the 16S rRNA probe hybridized with three different *Sma*I restriction fragments, lend further support to these findings. Furthermore, the lower copy number of rRNA genes in Campylobacter coincides with its much slower growth rate than *E. coli* (R. J. Redfield, personal communication). Therefore, with only three *rrn* operons, a single mutation

in one of the rRNA genes would result in approximately one-third of the total cellular rRNA being in the mutated form which may account for the high level of resistance.

It has been shown previously that mutations in ribosomal proteins L4 and L22 of *E. coli* confer high level resistance to erythromycin (Wittmann et al., 1973). Therefore, similar mutations in Campylobacter ribosomal proteins located in or near the binding site for erythromycin may be responsible for resistance. Since genes coding for ribosomal proteins in Campylobacter are likely to be single copy, a single mutation in one or more essential proteins could readily lead to high level resistance.

Results from our analysis of erythromycin-resistant *C. jejuni* and *C. coli* strains demonstrated that modification in the 50S ribosomal subunits of these bacteria, either involving the 23S rRNA or one or more ribosomal proteins, is the basis of high level resistance. Since resistance involves mutation in the Campylobacter chromosome and not the production of a methylase or erythromycin-modifying enzymes, the risk of rapid conjugative spread of resistance between organisms is minimal. However, as is evident with the much higher incidence of erythromycin-resistant isolates of *C. coli* compared with *C. jejuni* in various geographic locations, environmental factors such as the amount of erythromycin used in clinical practice as well as the use of macrolide and related antibiotics in

livestock feeds can result in the increased selection of erythromycin-resistant isolates. This could pose a significant clinical problem when treatment of Campylobacter gastroenteritis with erythromycin, the drug of choice in such cases, is necessary.

Attempts were made to clone and express the Campylobacter erythromycin resistance determinant in *E. coli*. A number of factors, such as differences in promoters, different codon usages as well as restriction modification systems, have made the cloning of Campylobacter genes extremely difficult. In the case of erythromycin resistance, this is further complicated by the recessive nature of the resistance phenotype. The resistance determinant, once cloned into an *E. coli* cell, must compete with the pre-existing sensitive ribosomes of the host. As a result, even if low level expression is achieved, detection of resistance can be difficult. Therefore, despite the high MICs associated with erythromycin-resistant *Campylobacter* sp., initial selection of resistant clones must be done using low levels of the antibiotic. This poses another problem since as previously mentioned, enterobacteria are naturally resistant to low levels of erythromycin. Hence, mutants of *E. coli*, such as the strains DB11 and HB101, which show sensitivity to low concentrations of erythromycin, must be used as hosts in cloning experiments. The instability in these mutants, however, resulted in problems with false positive resistant

clones in which the *E. coli* hosts had reverted to the low level resistance phenotype of the parental strain. The combination of these difficulties no doubt contributed to the inability to clone the Campylobacter erythromycin-resistance determinant into *E. coli*.

An alternative approach was used in which attempts were made to clone the resistance determinant into erythromycin-sensitive Campylobacter cells using the technique of electroporation. Before the actual cloning experiments were carried out, the efficiency at which the shuttle cloning vector pILL550 can transform Campylobacter host cells using electroporation was first determined. Miller and co-workers (1988) had previously published that an optimal transformation frequency of 1.0×10^5 transformants per µg DNA could be obtained. However, using the same plasmid and host strain, as well as electroporation conditions, we were unable to obtain similar transformation frequencies. The highest frequency we obtained was approximately 1×10^3 transformants per µg DNA. With this 100-fold lower frequency, it seemed unlikely that cloning using this approach could be successful. Instead, genomic DNA extracted from PFGE restriction fragments was used with natural transformation and subsequent selection of erythromycin-resistant transformants to locate the region of DNA involved in resistance. Initial mapping indicated that a 240 kb *Sma*I fragment from *C. coli* UA585 was capable of transforming erythromycin resistance into *C. coli*

UA417. Future studies involving further digestions of this extracted fragment with appropriate restriction enzymes followed by natural transformation studies using these smaller DNA fragments may be used to map more precisely the location of these resistance genes. Eventually, a restriction fragment of sufficiently small size could be obtained which could then be subjected to DNA sequencing and other detailed genetic analysis.

In addition to being instrumental for determining the location of erythromycin-resistance genes within the *C. coli* genome, PFGE was also invaluable in the construction of a physical map of *C. coli* UA417R as well as for epidemiological analysis of *Campylobacter* spp. Although the use of PFGE in genomic analysis has been reported for a number of microorganisms, extensive screening for appropriate restriction enzymes as well as the determination of optimal electrophoresis conditions suitable for Campylobacter genomic DNA was required before PFGE analysis could be carried out. These preliminary screening procedures, which are normally required for each new organism to be analyzed, can be time consuming as well as expensive. After screening through a wide spectrum of restriction endonucleases, two enzymes, *Sal*I and *Sma*I, were found to consistently give reasonable numbers of restriction fragments suitable for PFGE analysis. The ranges of fragment sizes for the two enzymes, however, were significantly different. Therefore, different

electrophoresis conditions were employed for each enzyme in order to obtain optimal resolution of the restriction fragments. *SalI* restriction fragment sizes ranged from 25 to 430 kb whereas *SmaI* fragments ranged from 6 to 255 kb in size. Higher voltage and longer pulse time (200V and 35 second pulses) were found to be optimal for resolving *SalI* fragments whereas lower voltage and shorter pulse time (175V and 12 second pulses) were ideal for the separation of *SmaI* fragments. Under these electrophoresis conditions, commercially obtained λ DNA concatamer standards could be reproducibly resolved into 11 and 7 concatamers for *SalI* and *SmaI* conditions respectively. The standard curves derived from these molecular weight standards, as well as the resulting restriction fragment sizes, were highly reproducible.

PFGE of DNA fragments produced by the restriction enzymes *SalI* and *SmaI* was used to construct the first physical map of the *Campylobacter coli* chromosome. The total size of the *C. coli* genome was found to be approximately 1,700 kb, as determined by the summation of the sizes of fragments generated by the two enzymes used. This molecular weight is significantly smaller than a previous report in which the *C.jejuni* and *C. coli* genome sizes were determined to be between 2300 to 3460 kb based on spectrophotometric studies (Owen and Leaper, 1981). Similar discrepancies were previously reported for *H. influenzae* Rd. The genome size of this organism was

determined to be 2,560 kb based on DNA renaturation kinetics (Gillis et al., 1970) whereas its size by PFGE was determined to be 1,900 kb (Lee and Smith, 1988). With the agreement between the different restriction enzyme digests, the molecular weight obtained from pulse-field gel electrophoresis is likely to be a more accurate estimate of the *C. coli* genome size. The genome size of *C. coli* UA417R obtained in these studies is approximately equal to that of *C. jejuni* strain UA580 (approximately 1,700 kb) as previously determined in our laboratory (Chang and Taylor, in press) and also to that of *C. jejuni* 81116 (B. Van der Zeijst, personal communication). These genome sizes are comparable to those previously obtained for *Haemophilus influenzae* (1,980 kb) and *H. parainfluenzae* (2,340 kb) (Kauc *et al.*, 1989; Kauc and Goodgal, 1989). These findings are consistent with the fact that Campylobacter and Haemophilus are similar in their size, inability to utilize carbohydrates as energy source, G + C contents as well as their requirement for special atmospheric conditions for growth. This led to the hypothesis that some of the more highly conserved "housekeeping" genes of these two different groups of bacteria may share a significant amount of homology. One such gene, which was originally isolated from *H. influenzae* and codes for the production of RNA polymerase, hybridized to Campylobacter DNA under moderate stringency conditions. The location of this gene within the *C. coli* UA417R genome was subsequently determined.

Therefore, with the availability of the *C. coli* UA417R physical map, it is now possible to map other genes, including those coding for ribosomal RNA and ribosomal proteins, determinants required for the assembly of major outer membrane proteins and flagella, as well as genes coding for the production of *Campylobacter* cytotoxin.

The fact that *C. coli* UA417 and UA417R showed identical restriction patterns despite their different streptomycin resistance phenotypes suggested that significant deletion or rearrangement of the UA417 chromosome is not required for the expression of streptomycin resistance in *C. coli* UA417R. It is more likely that a point mutation or small deletion in a region outside any recognition sequences of the two enzymes used was responsible for resistance. Erythromycin-resistant transformant #2, on the other hand, was found to have an unique *Sma*I restriction pattern different from that of the sensitive host UA417. The loss of the *Sma*I fragment 3 and the subsequent generation of two new fragments in this transformant futher confirm that *Sma*I fragment 3 was involved in erythromycin resistance. Since chromosomal DNA from *C. coli* UA585 was randomly transformed into *C. coli* UA417, it was not surprising that various erythromycin-resistant transformants would have different regions of the UA585 genome surrounding the resistance determinant recombining with the host chromosome. The fact that both newly generated *Sma*I fragments in transformant #2 were able

to transform resistance suggests that this transformant may be useful in future detailed mapping of the erythromycin resistance determinant.

Another important application for PFGE analysis of Campylobacter genomic DNA is its role as an epidemiological tool. As previously mentioned, the biochemical inertness of *Campylobacter* spp. has resulted in a limited number of epidemiological markers being associated with these organisms. Because of this, direct comparisons of Campylobacter genomic DNA subjected to conventional agarose gel electrophoresis have been used in epidemiological studies of Campylobacter outbreaks. To overcome the problem of poor resolution of the large number of restriction fragments generated by conventional agarose gel electrophoresis, rRNA fingerprinting patterns have also been included to achieve easier differentiation of Campylobacter strains. Since PFGE analysis of genomic DNA produces far fewer fragments than the conventional method, better resolution of these fragments can be readily obtained. Therefore, I proceeded to carry out PFGE analysis on 13 different *C. jejuni* strains and 10 *C. coli* strains from various sources. Although minor variations in the overall restriction patterns are found in a majority of all these strains, distinct patterns unique for each of the two species were observed. *C. jejuni* genomic DNA from various strains digested with *Sma*I always possess at least one fragment in the range of between 400 and 500 kb. On

the contrary, *Sma*I did not produce restriction fragments larger than 250 kb from *C. coli* DNA. Therefore, these distinct restriction patterns can be used as criteria for preliminary identification of *C. jejuni* and *C. coli* isolates. The accuracy of species differentiation based on PFGE restriction patterns was determined by comparing these results with those obtained from rapid hippurate hydrolysis tests as well as DNA hybridization studies performed on the 22 Campylobacter strains. All 13 strains identified as *C. jejuni* according to their distinct PFGE restriction patterns were hippurate positive and hybridized with the *C. jejuni*-specific DNA probe pDT1720. On the other hand, the 10 strains determined to be *C. coli* were hippurate negative and only hybridized to the *C. jejuni/C. coli* probe pDT1719. However, UA261, which was initially reported to be a *C. jejuni* human strain isolated from a patient in Southampton, U. K., was found to have characteristic *C. coli* PFGE restriction patterns. Hippurate hydrolysis as well as DNA hybridization data confirmed that this strain was indeed a *C. coli* isolate. Therefore, PFGE analysis of *Sma*I restriction profiles of Campylobacter genomic DNA was shown to be a valuable alternative for species differentiation between *C. jejuni* and *C. coli*.

In addition to its application in species differentiation, the intra-species differences in PFGE restriction patterns observed between some of the strains suggest a possible role as an epidemiological tool. It is

of interest to note that three *C. jejuni* strains, UA365, UA 580 and UA709, isolated from widely separated geographic locations (United Kingdom, Canada and the Netherlands respectively), were found to have identical *Sma*I restriction patterns. Similarly, the erythromycin-resistant strains UA695 and UA697 shared identical restriction patterns despite being isolated in Alberta, Canada and the United Kingdom respectively. On the contrary, *C. jejuni* UA709 and UA736, initially thought to be an isogenic pair of strains, which had been isolated from the same patient, had significantly different *Sma*I restriction patterns. Therefore, this further strengthens the hypothesis that the patient was colonized by multiple strains of *C. jejuni* at the time.

PFGE analysis of *C. coli* strains also showed varying degrees of similarity in restriction patterns between strains. Erythromycin-resistant *C. coli* UA585 and its laboratory-derived erythromycin-sensitive derivative UA586 shared identical *Sma*I restriction patterns. This provides further proof of their isogenicity and hence their usefulness in the study of the mechanism of erythromycin resistance. *C. coli* UA748 and UA749, both erythromycin-resistant isolates from Belgium, were also found to have similar restriction patterns. On the other hand, the remaining *C. coli* strains, regardless of their origin, all possessed unique restriction patterns that were distinct from each other.

The usefulness of PFGE in epidemiology is further confirmed by the restriction patterns observed for *C. jejuni* UA336 (cattle isolate), UA344 (cattle isolate) and UA347 (milk isolate). These three strains were initially investigated by Bradbury and co-workers (1984) to determine if the cattle strain was the source of an outbreak of Campylobacter gastroenteridis with contaminated milk acting as the transmission agent. Our observations that the three strains showed distinctly different *Sma*I restriction patterns confirm their earlier findings that these strains were not related and therefore probably not the source of the outbreak.

In general, the number of strain-specific differences observed within *C. jejuni* is comparable with those observed among strains of *C. coli*. Different results were obtained, however, when 16S rRNA fingerprinting patterns of each species were analyzed. All the strains showing similar restriction patterns had identical hybridization patterns. Except for UA268, those *C. jejuni* strains with different restriction patterns all showed a high degree of variation in the hybridization pattern of the large molecular weight band. In the majority of these strains, hybridization patterns involving the two low molecular weight bands were conserved. Exceptions to this were observed in UA709, the two cattle isolates UA336 and UA344 as well as the milk isolate UA347. In these four strains, differences in hybridization patterns were observed in all three

fragments. With *C. jejuni* UA268, although it showed an unique restriction pattern that was different from all other *C. jejuni* strains tested, its rRNA hybridization pattern was identical to those observed for *C. jejuni* UA365, UA580 and UA709. Results similar to this were obtained for all the *C. coli* strains tested. Despite various unique restriction patterns observed among many of these *C. coli* strains, they all shared a common rRNA hybridization pattern. Thus while *C. jejuni* and *C. coli* strains with similar restriction patterns always shared similar hybridization patterns, those with different restriction patterns can have either similar or different rRNA hybridization patterns. It can be concluded from these observations that combined restriction and hybridization pattern analysis does not provide any more epidemiological information than that obtained from PFGE restriction profiles alone. Unlike conventional agarose electrophoresis analysis of Campylobacter chromosomal DNA in which rRNA fingerprinting was required to simplify the interpretation of complex restriction patterns, PFGE analysis on its own can achieve the same results due to high resolution of the small number of *Sma*I restriction fragments. Therefore, PFGE, performed under optimal conditions , represents a practical, reliable and improved alternative tool in Campylobacter epidemiology.

In conclusion, these studies have demonstrated that the chromosomally mediated, high level erythromycin

resistance in *C. jejuni* and *C. coli* is a result of target site modification. Modifications in the 50S ribosomal subunits of resistant Campylobacter cells by a mechanism distinct from those involving *erm* determinants found in other microorganisms may be responsible for the resistance to erythromycin. The erythromycin-resistant determinant was located within a 240 kb region of the *C. coli* UA417R genome based on physical mapping and natural transformation of restriction fragments generated by PFGE analysis. Furthermore, PFGE analysis of Campylobacter genomic DNA, without the need to perform rRNA hybridization, can be a practical and efficient alternative for the identification and epidemiological characterization of *C. jejuni* and *C. coli* clinical isolates.

Bibliography

Alexander, J. K. 1957. Energy sources utilized by *Vibrio fetus*. J. Bacteriol. **74**:168-170.

Allen, N. E. 1977. Macrolide resistance in *Staphylococcus aureus:* Inducers of macrolide resistance Antimicrob. Agents Chemother. **11**:669-674.

Andersson, S. and C. G. Kurland. 1987. Elongating ribosomes *in vivo* are refractory to erythromycin. Biochimie **69**:901-904

Andremont, A., G. Gerbaud and P. Courvalin. 1986a. Plasmid-mediated high-level resistance to erythromycin in *Escherichia coli*. Antimicrob. Agents Chemother. **29**:515-518.

Andremont, A., P. Raibaud and C. Tancrede. 1983. Effect of erythromycin on microbial antagonisms: a study in gnotobiotic mice associated with a human fecal flora. J. Infect. Dis. **148**:579-587.

Andremont, A., H. Sancho-Garnier and C. Tancrede. 1986b. Epidemiology of intestinal colonization by members of the family *Enterobacteriaceae* highly resistant to erythromycin in a hematology-oncology unit. Antimicrob. Agents and Chemother. **29**:1104-1107.

Andremont, A. and C. Tancrede. 1981. Reduction of the aerobic gram-negative bacterial flora of the gastrointestinal tract and prevention of traveller's diarrhea using oral erythromycin. Ann. Microbiol. (Paris) **132B**:419-427.

Arbeit, R. D., M. Arthur, R. Dunn, C. Kim, R. K. Selander and R. Goldstein. 1990. Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulsed field electrophoresis to molecular epidemiology. J. Infect. Dis. **161**:230-235.

Arevalo, M. A., F. Tejedor, F. Polo and J. P. G. Ballesta. 1988. Protein components of the erythromycin binding site in bacterial ribosomes. J. Biol. Chem. **263**:58-63.

Arthur, M. and P. Courvalin. 1986. Contribution of two different mechanisms to erythromycin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **30**:694-700.

Arthur, M., A. Andremont and P. Courvalin. 1986. Heterogeneity of genes conferring high-level resistance to erythromycin by inactivation in Enterobacteria. Ann. Inst. Pasteur/Microbiol. **137A**:125-134.

Arthur, M., A. Andremont and P. Courvalin. 1987a. Distribution of erythromycin esterase and rRNA methylase genes in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob. Agents Chemother. **31**:404-409.

Arthur, M., A. Brisson-Noel and P. Courvalin. 1987b. Origin and evolution of genes specifying resistance to macrolide, lincosamide and streptogramin antibiotics: data and hypothesis. J. Antimicrob. Chemother. **20**:783-802.

Austen, R. A. and T. J. Trust. 1980. Detection of plasmids in the related groups of the genus Campylobacter. FEMS Microbiol. Lett. **8**:201-204.

Barthelemy, P., D. Autisser, G. Gerbaud and P. Courvalin. 1984. Enzyme hydrolysis of erythromycin by a strain of *Escherichia coli*. A new mechanism of resistance. J. Antibiot. **37**:1692-1696.

Beauclerk, A. A. D. and E. Cundliffe. 1987. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. J. Mol. Biol. **193**:661-671.

Belland, R. J. and T. J. Trust. 1982. Deoxyribonucleic acid sequence relatedness between thermophilic members of the genus Campylobacter. J. Gen. Microbiol. **128**:2515-2522.

Benveniste, R. and J. Davies. 1973. Aminoglycoside antibiotic-inactivating enzymes in Actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. U.S.A. **70**:2276-2280.

Berryman, D. I. and J. I. Rood. 1989. Cloning and hybridization analysis of *ermP*, a Macrolide-Lincosamide-Streptogramin B resistance determinant from *Clostridium perfringens*. Antimicrob. Agents Chemother. **33**:1346-1353.

Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513-1523.

Blaser, M. J., D. J. Duncan, M. T. Osterholm, G. R. Istre and W. L. Wang. 1983. Serologic study of two clusters of infection due to *Campylobacter jejuni*. J. Infect. Dis. **147**:820-823.

Blaser, M. J., H. L. Hardesty, B. Powers and W. L. Wang. 1979. Survival of *Campylobacter fetus* subsp. *jejuni* in biological milieus. J. Clin. Microbiol. **11**:309-313.

Blaser, M. J. and L. B. Reller. 1981. Campylobacter enteritis. N. Engl. J. Med. **305**:1444-1452.

Bock, A., F. Turnowsky and G. Hogenauer. 1982. Tiamulin resistance mutations in *Escherichia coli*. J. Bacteriol. **151**:1253-1260.

Bollen, A., T. Cabezon, M. de Wilde, R. Villarroel and A. Herzog. 1975. Alteration of ribosomal protein S17 by mutation linked to neamine resistance in *Escherichia coli*. I. General properties of *neaA* mutants. J. Mol. Biol. **99**:795-806.

Bollen, A., J. Davies, M. Ozaki and S. Mizushima. 1969. Ribosomal protein conferring sensitivity to the antibiotic spectinomycin in *Escherichia coli*. Science **165**:85-86.

Bonner, W. M. and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83-88.

Bopp, C. A., K. A. Birkness, I. K. Wachsmuth and T. J. Barrett. 1985. In vitro antimicrobial susceptibility, plasmid analysis, and serotyping of epidemic-associated *Campylobacter jejuni*. J. Clin. Microbiol. **21**:4-7.

Bradbury, W. C., M. A. Marko, J. N. Hennessy and J. L. Penner. 1983. Occurrence of plasmid DNA in serologically defined strains of *Campylobacter jejuni* and *Campylobacter coli*. Infect. Immun. **40**:460-463.

Bradbury, W. C. and D. L. G. Munroe. 1985. Occurrence of plasmids and antibiotic resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from healthy and diarrheic animals. J. Clin. Microbiol. **22**:339-346.

Bradbury, W. C., A. D. Pearson, M. A. Marko, R. V. Congi and J. L. Penner. 1984. Investigation of a *Campylobacter jejuni* outbreak by serotyping and chromosomal restriction endonuclease analysis. J. Clin. Microbiol. **19**:342-346.

Brisson-Noel, A., M. Arthur and P. Courvalin. 1988. Evidence for natural gene transfer from gram-positive cocci to *Escherichia coli*. J. Bacteriol. **170**:1739-1745.

Brunton, W. A. T., A. A. M. Wilson and R. M Macrae. 1978. Erythromycin-resistant campylobacters. Lancet **ii**:1385.

Buckel, P., A. Buchberger, A. Bock and H. G. Wittmann. 1977. Alteration of ribosomal protein L6 in mutants of *Escherichia coli* resistant to gentamicin. Mol. Gen. Genet. **158**:47-54.

Burdett, V. 1986. Streptococcal tetracycline resistance mediated at the level of protein synthesis. J. Bacteriol. **165**:564-569.

Burridge, R., C. Warren and I. Phillips. 1986. Macrolide, lincosamide and streptogramin resistance in *Campylobacter jejuni/coli*. J. Antimicrob. Chemother. **17**:315-321.

Caldwell, M. B., P. Guerry, E. C. Lee, J. P. Burans and R. I. Walker. 1985. Reversible expressions of flagella in *Campylobacter jejuni*. Infect. Immun. **50**:941-943.

Catchpole, I., C. Thomas, A. Davies and K. G. H. Dyke. 1988. The nucleotide sequence of *Staphylococcus aureus* plasmid pT48 conferring inducible macrolide-lincosamide-streptogramin B resistance and comparison with similar plasmids expressing constitutive resistance. J. Gen. Microbiol. **134**:697-709.

Chang, N. and D. E. Taylor. 1990. Use of pulsed-field agarose gel electrophoresis to size *Campylobacter* spp. genomes and to construct a *Sal*I map of *Campylobacer jejuni* UA580. J. Bacteriol. (in press).

Chinali, G., E. Nyssen, B. Di Giambattista and C. Cocito. 1988. Action of erythromycin and virginiamycin S on polypeptide synthesis in cell-free systems. Biochim. Biophys. Acta **951**:42-52.

Christie, P.J., R. Z. Korman, S. A. Zahler J. C. Adsit and G. M. Dunny. 1987. Two conjugation systems associated with *Streptococcus faecalis* plasmid pCF10: identification of a conjugative transposon that transfers between *S. faecalis* and *Bacillus subtilis*. J. Bacteriol. **169**:2529-2536.

Clewell, D. B. 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409-436.

Clewell, D. B. and A. E. Franke. 1974. Characterization of a plasmid determining resistance to erythromycin, lincomycin and veramycin B in a strain of *Streptococcus pyogenes*. Antimicrob. Agents Chemother. **5**:534-537.

Clewell, D. B., Y. Yagi, G. M. Dunny and S. K. Schultz. 1974. Characterization of three plasmid deoxyribonuleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. J. Bacteriol. **117**:283-289.

Courvalin, P. and C. Carlier. 1987. *Tn1545*: A conjugative shuttle transposon. Mol. Gen. Genet. **206**:259-264.

Courvalin, P. M., C. Carlier, O. Croissant and D. Blangy. 1974. Identification of two plasmids determining resistance to tetracycline and to erythromycin in group D *Streptococcus*. Molec. Gen. Genet. **132**:181-192.

Courvalin, P., H. Ounissi and M. Arthur. 1985. Multiplicity of macrolide-lincosamide-streptogramin antibiotic resistance determinants. J. Antimicrob. Chemother. **16** *Suppl A*:91-100.

Covers, T. L. and M. J. Blaser. 1989. The pathobiology of *Campylobacter* infections in humans. Ann. Rev. Med. **40**:269-285.

Coyle, M. B., B. H. Minshew, J. A. Bland and P. C. Hsu. 1979. Erythromycin and clindamycin resistance in *Corynebacterium diphtheriae* from skin lesions. Antimicrob. Agents Chemother. **16**:525-527.

Cundliffe, E. 1969. Antibiotics and polyribosomes. II. Some effects of lincomycin, spiramycin, and streptogramin A *in Vivo*. Biochemistry **8**:2063-2066.

Cundliffe, E. 1978. Mechanism of resistance to thiostrepton in the producing-organism, *Streptomyces azureus*. Nature **272**:792-795.

Cundliffe, E. 1986. Involvement of specific portions of ribosomal RNA in defined ribosomal functions: a study utilizing antibiotics. p. 586. B. Hardesty and G. Kramer (eds.), Structure, function, and genetics of ribosomes. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

Cundliffe, E. 1987. On the nature of antibiotic binding sites in ribosomes. Biochimie **69**:863-869.

Cundliffe, E. 1989. Methylation of RNA and resistance to antibiotics. p. 227-247. In L. E. Bryan (ed.), Microbial resistance to drugs. Springer-Verlag, Berlin, Heidelberg, New YOrk, Toyko.

Cundliffe, E. and J. Thompson. 1979. Ribose methylation and resistance to thiostrepton. Nature **278**:859-861.

Datta, D. B., L.-M. Changdhien, C. R. Nierras, W. A. Strycharz and G. R. Craven. 1988. Identification of *Escherichia coli* ribosomal proteins by an alternative two-dimensional electrophoresis system. Anal. Biochem. **173**:241-245.

Deiko, S., R. Takata, S. Osawa K. Tanaka and M. Tanaki. 1970. Genetic studies of the ribosomal proteins in *Escherichia coli*. IV. Pattern of the alterations of ribosomal protein components in mutants resistant to spectinomycin or erythromycin in different strains of *Escherichia coli*. Mol. Gen. Genet. **107**:39-49.

De Meester, C. and J. Rondelet. 1976. Microbial acetylation of M factor of virginiamycin. J. Antibiotics. **29**:1297-1305.

Derclaye, I., I. Delor, M. Van Bouchaute, P. Moureau, G. Wauters and G. R. Cornelis. 1989. Identification of *Campylobacter jejuni* and *C. coli* by gel electrophoresis of the outer membrane proteins. J. Clin. Microbiol. **27**:1072-1076.

De Stasio, E. A., D. Moazed, H. F. Noller and A. E. Dahlberg. 1989. Mutations in 16S ribosomal RNA disrupt antibiotic-RNA interactions. EMBO Journal **8**:1213-1216.

Devriese, L. A. and G. N. Dutta. 1984. Effects of erythromycin-inactivating *Lactobacillus* crop flora on blood levels of erythromycin given orally to chicks. J. Vet. Pharmacol. and Ther. **7**:49-53.

Dougan, G. and M. Kehoe. 1984. The minicell system as a method for studying expression from plasmid DNA, p. 233-258 *In* P.M. Burdett and J. Grinsted (eds.), Methods in microbiology, vol. 17. Plasmid technology. Academic Press, Inc., New York.

Doyle, L. P. 1944. A vibrio associated with swine dysentery. Am. J. Vet. Res. **5**:3-5.

Dubnau, D. 1984. Translational attenuation: the regulation of bacterial resistance to the macrolide-lincosamide-streptogramin B antibiotics. Crit. Rev. Biochem. **16**:103-128.

Dubnau, D. and M. Monod. 1986. The regulation and evolution of MLS resistance. Banbury Rep. **24**:369-387.

Dutta, G. N. and L. A. Devriese. 1981. Macrolide-lincosamide-streptogramin resistance patterns in *Clostridium perfringens* from animals. Antimicrob. Agents Chemother. **19**:264-278.

Duval, J. 1985. Evolution and epidemiology of MLS resistance. J. Antimicrob. Chemother. **16** *Suppl A*:137-149.

Ebright, J. R. and L. M. Ryay. 1984. Acute erosive feative arthritis associated with *C. jejuni*-induced colitis. Am. J. Med. **76**:321-323.

Eisenstein, B. I. 1990. New molecular techniques for microbial epidemiology and the diagnosis of infectious diseases. J. Infect. Dis. **161**:595-602.

Ely, B. and C. J. Gerardot. 1988. Use of pulsed-field-gradient gel electrophoresis to construct a physical map of the *Caulobacter cresentus* genome. Gene **68**:323-333.

Ettayebi, M., S. M. Prasad and E. A. Morgan. 1985. Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of *Escherichia coli*. J. Bacteriol. **162**:551-557.

Feldman, L. I., I. K. Dill, C. E. Holmlund, H. A. Whaley, E. L. Paterson and N. Bohonos. 1964. Microbial transformations of macrolide antibiotics. Antimicrob. Agents and Chemother. 54-57.

Fernandes, P. B., W. R. Baker, L. A. Freiberg, D. J. Hardy and E. J. McDonald. 1989. New macrolide active against *Streptococcus pyogenes* with inducible or consititutive type of macrolide-lincosamide-streptogramin B resistance. Antimicrob. Agents Chemother. **33**:78-81.

Fierro, J. F., C. Vilches, C. Hardisson and J. A. Salas. 1989. Streptogramin-inactivating activity in three producer streptomycetes. FEMS Microbiol. Letters **58**:243-246.

Finch, M. J. and L. W. Riley. *Campylobacter* infections in the United States: Results of an 11-State surveillance. Arch. Intern. Med. **144**:1610-1612.

Foster, T. J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47**:361-409.

Fournet, M.-P., L. Deforges, R. Zini, J. Barre and J.-P. Tillement. 1987. Binding studies of macrolides, lincosamides and streptogramins to *Streptococcus* G group using ^{3}H-erythromycin. Biochem. Pharmacol. **36**:3495-3500.

Fried, H. M. and J. R. Warner. 1981. Cloning of yeast gene for trichodermin resistance and ribosomal protein L3. Proc. Natl. Acad. Sci. U.S.A. **78**:238-242.

Fujisawa, Y. and B. Weisblum. 1981. A family of r-determinants in *Streptomyces* spp. that specifies inducible resistance to macrolide, lincosamide and streptogramin type B antibiotics. J. Bacteriol. **146**:621-631.

Garrod, L. P. 1957. The erythromycin group of antibiotics. British Med. Journal. **2**:57-63.

Gillis, M., J. DeLey and M. DeCleene. 1970. The determination of molecular weight of bacterial genome DNA from renaturation rates. Eur. J. Biochem. **12**:143-153.

Goering R. V. and T. D. Duensing. 1990. Rapid field inversion gel electrophoresis in combination with an rRNA gene probe in the epidemiological evaluation of Staphylococci. J. Clin. Microb. **28**:426-2429.

Goldman, R. C. and S. K. Kadam. 1989. Binding of novel macrolide structures to Macrolides-Lincosamides-Streptogramin B-resistant ribosomes inhibits protein synthesis and bacterial growth. Antimicrob. Agents Chemother. **33**:1058-1066.

Graham, M. Y. and B. Weisblum. 1979. 23S ribosomal ribonucleic acid of macrolide-producing Streptomycetes contains methylated adenine. J. Bacteriol. **137**:1464-1467.

Grajewski, B. A., J. W. Kusek and H. M. Gelfand. 1985. Development of a bacteriophage typing system for *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. **22**:13-18.

Gryczan, T. J., G. Grandi, J. Hahn, R. Grandi and D. Dubnau. 1980. Conformational alteration of mRNA structure and the post-transcriptional regulation of erythromycin-induced drug resistance. Nucleic Acids Res. **8**:6081-6097.

Gryczan, T. J., M. Israeli-Reches, M. Del Bue and D. Dubnau. 1984. DNA sequence and regulation of *ermD*, a macrolide-lincosamide-streptogramin B resistance element from *Bacillus licheniformis*. Mol. Gen. Genet. **194**:349-356.

Guerrant, R. L., C. A. Wanke, R. A. Pennie, L. J. Barrett and A. A. Lima. 1987. Production of a unique cytotoxin by *Campylobacter jejuni*. Infect. Immun. **55**:2526-2530.

Guerry, P., S. M. Logan, S. Thornton and T. J. Trust. 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172**:1853-1860.

Hachler, H., B. Berger-Bachi and F. H. Kayser. 1987. Genetic characterization of a *Clostridium difficile* erythromycin-clindamycin resistance determinant that is transferable to *Staphylococcus aureus*. Antimicrob. Agents Chemother. **31**:1039-1045.

Hahn, J., G. Grandi, T. J. Gryczan and D. Dubnau. 1982. Translational attenuation of *ermC*: a deletion analysis. Mol. Gen. Genet. **186**:204-216.

Hardy, K. and C. Haefeli. 1982. Expression in *Escherichia coli* of a Staphylococcal gene for resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **152**:524-526.

Hardy, S. J. S., C. G. Kurland, P. Voynow and G. Mora. 1969. The ribosomal proteins of *Escherichia coli* I. Purification of 30S ribosomal proteins. Biochemistry **8**:2897-2905.

Harris, L. A., S. M. Logan, P. Guerry and T. J. Trust. 1987. Antigenic variation of *Campylobacter* flagella. J. Bacteriol. **169**:5066-5071.

Hartstein, A. I., V. H. Morthland, S. Eng, G. L. Archer, F. D. Schoenknecht and A. L. Rashad. 1989. Restriction enzyme analysis of plasmid DNA and Bacteriophage typing of paired *Staphylococcus aureus* blood culture isolates. J. Clin. Microbiol. **27**:1874-1879.

Harvey, S. M. 1980. Hippurate hydrolysis by *Campylobacter fetus*. J. Clin. Microbiol. **11**:435-437.

Harvey, S. M. and J. R. Greenwood. 1983. Relationships among catalase positive campylobacters determined by deoxyribonucleic acid-deoxyribonucleic acid hybridizations. Int. J. Syst. Bacteriol. **33**:275-284.

Hebert, G. A., P. Edmonds and D. J. Brenner. 1984. DNA relatedness among strains of *Campylobacter jejuni* and *Campylobacter coli* with divergent serogroup and hippurate reactions. J. Clin. Microbiol. **20**:138-140.

Helser, T. L., J. E. Davies and J. E. Dahlberg. 1972. Mechanism of kasugamycin resistance in *Escherichia coli*. Nature New Biol. **235**:6-9.

Ho, B. S. W. and W. T. Wong. 1985. A one-year survey of campylobacter enteritis and other forms of bacterial diarrhoea in Hong Kong. J. Hyg. (Camb.) **94**:55-60.

Horaud, T., C. Le Bouguenec and K. Pepper. 1985. Molecular genetics of resistance to macrolides, lincosamides and streptogramin B (MLS) in streptococci. J. Antimicrob. Chemother. **16** *Suppl A*:111-135.

Horinouchi, S., W. H. Byeon and B. Weisblum. 1983. A complex attenuator regulates inducible resistance to antibiotics in *Streptococcus sanguis*. J. Bacteriol. **154**:1252-1262.

Horinouchi, S. and B. Weisblum. 1980. Posttranscriptional modification of mRNA conformation: mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. U.S.A. **77**:7079-7083.

Horinouchi, S. and B. Weisblum. 1981. The control region for erythromycin resistance: free energy changes related to induction and mutation to constitutive expression. Mol. Gen. Genet. **182**:341-348.

Horinouchi, S. and B. Weisblum. 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150**:804-814

Hummel, H., W. Piepersberg and A. Bock. 1979. Analysis of lincomycin resistance mutations in *Escherichia coli*. Mol. Gen. Genet. **169**:345-347.

Hyder, S. L. and M. M. Streitfeld. 1973. Inducible and constitutive resistance to macrolide antibiotics and lincomycin in clinically isolated strains of *Streptococcus pyogenes*. Antimicrob. Agents Chemother. **4**:327-331.

Jablonski, P. E. and M. Mychajlonka. 1988. Oxacillin-induced inhibition of protein and RNA synthesis in a tolerant *Staphylococcus aureus* isolate. J. Bacteriol. **170**:1831-1836.

Jelenc, P. C. 1980. Rapid purification of highly active ribosomes from *Escherichia coli*. Anal. Biochem. **105**:369-374.

Jhonsen, K., M. Ostensen, A. Christine, S. Melby and K. Melby. 1983. HLA-B27 negative arthritis related to *Campylobacter jejuni* enteritis in three children and two adults. Acta Med. Scand. **214**:165-168.

Johnson, W. M. and H. Lior. 1984. Toxins produced by *Campylobacter jejuni* and *Campylobacter coli*. Lancet **i**:229-230.

Jones, D. M., E. M. Sutcliffe and J. D. Abbott. 1985. Serotyping of *Campylobacter* species by combined use of two methods. Eur. J. Clin. Microbiol. **4**:562-565.

Jones, F. S. and R. B. Little. 1931. Vibrionic enteritis in calves. J. Exp. Med. **53**:845-851.

Jones, W. F. Jr., R. L. Nichols and M. Finland. 1956. Development of resistance and cross resistance in vivo to erythromycin, carbomycin, oleandomycin and streptogramin. Proc. Soc. Exp. Biol. Med. **93**:388-393.

Kaijser, B. and E. Sjorgren. 1985. *Campylobacter* strains in Sweden. Acta Pathol. Microbiol. Immunol. Scand. Sect. B. **93**:315-322.

Kaldor, J. and B. R. Speed. 1984. Guillain-Barre syndrome and *Campylobacter jejuni*: a serological study. Br. Med. J. **288**:1867-1868.

Kamimiya, S. and B. Weisblum. 1988. Translational attenuation control of *ermSF*, an inducible resistance determinant encoding rRNA *N*-methyltransferase from *Streptomyces fradiae*. J. Bacteriol. **170**:1800-[illegible].

Karmali, M. A., S. A. DeGrandis and P. C. Fleming. [illegible]. Antimicrobial susceptibility of *Campylobacter jejuni* with special reference to resistance patterns of Canadian isolates. Antimicrob. Agents Chemother. **19**:593-597.

Karmali, M. A., S. A. DeGrandis, D. E. Taylor and P. C. Fleming. 1982. On the association between erythromycin resistance and failure to hydrolyse hippurate in *Campylobacter jejuni*, p.218-220. *In* D. G.

Newell (ed.), Campylobacter. Epidemiology, pathogenesis and biochemistry. MTP Press, Lancaster, United Kingdom.

Karmali, M. A. and P. C. Fleming. 1979. *Campylobacter* enteritis in children. J. Pediatr. **94**:527-533.

Karmali, M. A. , J. L. Penner, P. C. Fleming, A. Williams and J. N. Hennessy. 1983. The serotype and biotype distribution of clinical isolates of *Campylobacter jejuni* and *Campylobacter coli* over a three year period. J. Infect. Dis. **147**:243-246.

Karmali, M. A. and M. B. Skirrow. 1984. Taxonomy of the genus *Campylobacter*, p. 1-20. In J. P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc., Boca Raton, Fla.

Kauc, L. and S. H. Goodgal 1989. The size and a physical map of the chromosome of *Haemophilus parainfluenzae*. Gene **83**:377-380.

Kauc, L., M. Mitchell and S. H. Goodgal. 1989. Size and physical map of the chromosome of *Haemophilus influenzae*. J. Bacteriol. **171**:2474-2479.

Khan, S. A. and R. P. Novick. 1980. Terminal nucleotide sequences of Tn551, a transposon specifying erythromycin resistance in *Staphylococcus aureus*: homology with Tn3. Plasmid **4**:148-154.

Kiehlbauch, F. A., R. A. Albach, L. L. Baum and K. P. Chang. 1985. Phagocytosis of *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes. Infect. Immun. **48**:446-451.

Kim, N. W. and V. L. Chan. 1989. Isolation and characterization of the ribosomal RNA genes of *Campylobacter jejuni*. Current Microbiol. **19**:247-252.

King, E. O. 1957. Human infections with *Vibrio fetus* and a closely related vibrio. J. Infect. Dis. **101**:119-128.

Konkel, M. E. and L A. Joens. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. **57**:2984-2990.

Kono, M. H. Hashimoto and S. Matsuhashi. 1966. Drug resistance of staphylococci. III. Resistance to some macrolide antibiotics and inducible system. Jpn. J. Microbiol. **10**:59-66.

Kotarski, S. F., T. L. Merriwether, G. T. Tkalcevic and P. Gemski. 1986. Genetic studies of kanamycin resistance

in *Campylobacter jejuni*. Antimicrob. Agents Chemother. **30**:225-230.

Labigne-Roussel, A., J. Harel and L. Tompkins. 1987. Gene transfer from *Escherichia coli* to *Campylobacter* Species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. J. Bacteriol. **169**:5320-5323.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.

Lai, C.-J. and B. Weisblum. 1971. Altered methylation of ribosomal RNA in an erythromycin-resistant strain of *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. **68**:856-860.

Lai, C.-J., B. Weisblum, S. R. Fahnestock and M. Nomura. 1973a. Alteration of 23 S ribosomal RNA and erythromycin-induced resistance to lincomycin and spiramycin in *Staphylococcus aureus*. J. Mol. Biol. **74**:67-72.

Lai, C.-J., J. E. Dahlberg and B. Weisblum. 1973b. Structure of an inducibly methylatable nucleotide sequence in 23S ribosomal ribonucleic acid from erythromycin-resistant *Staphylococcus aureus*. Biochemistry **12**:457-460.

Lambert, T., G. Gerbaud, P. Trieu-Cuot and P. Courvalin. 1985. Structrual relationship between the genes encoding 3'-aminoglycoside phosphotransferases in *Campylobacter* and gram-positive cocci. Ann. Inst. Pasteur (Paris) **136B**:135-150.

Lampson, B. C. and J. T. Parisi. 1986. Naturally occuring *Stpahylococcus epidermidis* plasmid expressing constitutive macrolide-lincosamide-streptogramin B resistance contains a deleted attenuator. J. Bacteriol. **166**:479-483.

Lampson, B. C. and J. T. Parisi. 1986. Nucleotide Sequence of the constitutive macarolide-lincosamide-streptogramin B resistance plasmid pNE131 from *Staphylococcus epidermidis* and homologies with *Staphylococcus aureus* plasmids pE194 and pSN2. **167**:888-892.

Lampson, B. C., W. Von David and J. T. Parisi. 1986. Novel mechanism for plasmid-mediated erythromycin resistance by pNE24 from *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **30**:653-658.

Leaper, S. and R. J. Owen. 1982. Differentiation between *C. jejuni* and allied hermophilic Campylobacters by hybridization of deoxyribonucleic acids. FEMS Microbiol. Lett. **15**:203-208.

Lecce, J. G. 1958. Some biochemical characteristics of *Vibrio fetus* and other related vibrios isolated from animals. J. Bacteriol. **76**:312-316.

Leclercq, R., A. Brisson-Noel, J. Duval and P. Courvalin. 1987. Phenotypic expression and genetic heterogeneity of lincosamide resistance determinants in Gram-positive cocci. Antimicrob. Agents and Chemother.

Lee, J. J. and O. Smith. 1988. Sizing of the *Haemophilus influenzae* Rd genome by pulsed-field agarose gel electrophoresis. J. Bacteriol. **170**:4402-4405.

LeFebvre, R. B. and G. C. Perng. 1989. Genetic and antigenic characterization of *Borrelia coriaceae*, putative agent of epizootic bovine abortion. J. Clin. Microbiol. **27**:389-3[illegible]

Le Goffic, F., M. L[illegible]u, J. Abbe C. Cerceau, A. Dublanchet and [illegible]l. 1977a. Plasmid mediated pristinamycin res[illegible]e: PH1A, a pristinamycin 1A hydrolase. Annale[illegible]e Microbio[illegible]. de L'Institut Pasteur. **128**:471-474.

Le Goffic, F., M. Capmau, D. Bonnet, C. Cerceau, C. Soussy, A. Dublanchet. 1977b. Plasmid-mediated pristinamycin resistance. PACIIA: a new enzyme which modifies pristinamycin IIA. J. of the Antibiotics. **30**:665-669.

Levy, S. B. 1984. Resistance to the tetracyclines, p.191-240. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc. New York.

Lior, H. 1984. New, extended biotyping scheme for *Campylobacter jejuni*, *Campylobacter coli*, and "*Campylobacter laridis*". J. Clin. Microbiol. **20**:636-640.

Lior, H., D. L. Woodward, J. A. Edgar, L. J. Laroche and P. Gill. 1982. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. J. Clin. Microbiol. **15**:761-768.

Logan, S. M., T. J. Trust and P. Guerry. 1989. Evidence for posttranslational modification and gene duplication of *Campylobacter* flagellin. J. Bacteriol. **171**:3031-3038.

Malke, H. 1974. Genetics of resistance to macrolide antibiotics and lincomycin in natural isolates of *Streptococcus pyogenes*. Mol. Gen. Genet. **135**:349-367.

Malke, H. and S. E. Holm. 1981. Expression of Streptococcal plasmid-determined resistance to erythromycin and lincomycin in *Escherichia coli*. Mol. Gen. Genet. **184**:283-285.

Manavathu, E. K., C. L. Fernandez, B. S. Cooperman and D. E. Taylor. 1990. Molecular studies on the mechanism of tetracycline resistance mediated by Tet(O). Antimicrob. Agents Chemother. **34**:71-77.

Manavathu, E. K., K. Hiratsuka and D. E. Taylor. 1988. Nucleotide sequence analysis and expression of a tetracycline resistance gene from *Campylobacter jejuni*. Gene **62**:17-26.

Maniatis, T., E. F. Fritsch and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Manninen, K. I., J. F. Prescott and I. R. Dohoo. 1982. Pathogenicity of *C. jejuni* isolates from animals and humans. Infect. Immun. **38**:46-52.

Mao, J. C.-H. and M. Putterman. 1968. Accumulation in Gram-positive and Gram-negative bacteria as a mechanism of resistance to erythromycin. J. Bacteriol. **95**:1111-1117.

Mark, L. G., C. D. Sigmund and E. A. Morgan. 1983. Spectinomycin resistance due to a mutation in an rRNA operon of *Escherichia coli*. J. Bacteriol. **155**:989-994.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208-218.

Marshall, V. P., T. E. Patt and A. D. Argoudelis. 1986. Enzymic nucleotidylation of lincosaminide antibiotics. J. Industrial. Microbiol. **1**:17-21.

Martin, P., G. Alloing, V. Mejean and J.-P. Claverys. 1987. Constitutive expression of erythromycin resistance mediated by the *ermAM* determinant of plasmid pAMβ1 results from deletion of 5' leader peptide sequences. Plasmid **18**:250-253.

Martin, P., P. Trieu-Cuot and P. Courvalin. 1986. Nucleotide sequence of the *tetM* tetracycline resistance determinant of the streptococcal conjugative shuttle transposon Tn*1545*. Nucleic Acids Res. **14**:7047-7058.

McCardell, B. A., J. M. Madden and E. C. Lee. 1984. *Campylobacter jejuni* and *Campylobacter coli* production of a cytotonic toxin immunologically similar to cholera toxin. J. Food Prod. **47**:943-949.

McDonough, P. L., J. F. Timoney, R. H. Jacobson and R. Khakhria. 1989. Clonal groups of *Salmonella typhimurium* in New York state. J. Clin. Microbiol. **27**:622-627.

McFadyean, J. and S. Stockman. 1913. Report of the Departmental Commitee appointed by the Board of Agriculture and Fisheries to enquire into epizootic abortion. Part III. Her Majesty's Stationary Office, London.

McGuire, J. M., R. L. Bunch, R. C. Anderson, H. E. Boaz, E. H. Flynn, H. M. Powell. 1952. 'Ilotycin', a new antibiotic. Antibiotics and Chemother. **2**:281-283.

Melancon, P., C. Lemieux and L. Brakier-Gingras. 1988. A mutation in the 530 loop of *Escherichia coli* 16S ribosomal RNA causes resistance to streptomycin. Nucleic Acid Res. **16**:9631-9639.

Menninger, J. R. 1985. Functional consequences of binding macrolides to ribosomes. J. Antimicrob. Chemother. **16 *Suppl* A**:23-34.

Menninger, J. R. and D. P. Otto. 1982. Erythromycin, carbomycin, and spiromycin inhibit protein synthesis by stimulating the dissociation of peptidyl-tRNA from ribosomes. Antimicrob. Agents Chemother. **21**:810-818.

Michel, J., M. Rogol and D. Dickman. 1983. Susceptibility of clinical isolates of *Campylobacter jejuni* to sixteen antimicrobial agents. Antimicrob. Agents Chemother. **23**:796-797.

Miller, J. F., W. J. Dower and L. S. Tompkins. 1988. High-voltage electroporation of bacteria: genetic transfromation of *Campylobacter jejuni* with plasmid DNA. Proc. Natl. Acad. Sci. USA **85**:856-860.

Moazed, D. and H. F. Noller. 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature **327**:389-394.

Monod, M. C. Denoya and D. Dubnau. 1986. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis*. J. Bacteriol. **167**:138-147.

Morooka, T., A. Umeda and K. Amako. 1983. Morphological differences in flagella in *Campylobacter fetus* subsp. *intestinalis* and *C. fetus* subsp. *jejuni*. Microbiol. Immunol. **27**:655-662.

Morris, G. K. and C. M. Patton. 1985. *Campylobacter*, p. 302-308. *In* E. H. Lennette, A. Balows, W. J. Hausler, H. J. Shadomy (ed.), Manual of Clinical Microbiology, 4th ed. American Society for Microbiology, Washington, D. C.

Moureau, P., I. Derclaye, D. Gregoire, M. Janssen and G. R. Cornelis. 1989. *Campylobacter* species identification based on polymorphism of DNA encoding rRNA. J. Clin. Microbiol. **27**:1514-1517.

Murphy, E. 1985. Nucleotide sequence of ermA, a macrolide-lincosamide-streptogramin B determinant in *Staphylococcus aureus*. J. Bacteriol. **162**:633-640.

Muto, Y., K. Bandoh, K. Watanabe, N. Katoh and K. Ueno. 1989. Macrolide accumulation by *Bacteroides fragilis* ATCC 25285. Antimicrob. Agents Chemother. **33**:242-244.

Nakajima, Y., M. Inoue, Y. Oka and S. Yamagishi. 1968. A mode of resistance to macrolide antibiotics in *Staphylococcus aureus*. Jpn. J. Microbiol. **12**:248-250.

Narayanan, C. S. and D. Dubnau. 1987. Demonstration of erythromycin-dependent stalling ribosomes on the *ermC* leader transcript. J. Biol. Chem. **262**:1766-1771.

Newell, D. G. and H. McBride. 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. J. Hyg. **95**:217-227.

Newell, D. G. and A. Pearson. 1984. The invasion of epithelial cell lines and the intestinal epithelium of infant mice by *Campylobacter jejuni/coli*. J. Diarrhoeal Dis. Res. **2**:19-26.

Ng, L.-K., R. Sherburne, D. E. Taylor and M. E. Stiles. 1985. Morphological forms and viability of *Campylobacter* species studied by electron microscopy. J. Bacteriol. **164**:338-343.

Ng, L.-K., M. E. Stiles and D. E. Taylor. 1987. DNA probes for identification of tetracycline resistance genes in *Campylobacter* species isolated from swine and cattle. Antimicrob. Agents Chemother. **31**:1669-1674.

Nirenberg, M. W. 1963. Cell-free protein synthesis directed by messenger RNA. Methods Enzymology **4**:17-23.

Noll, K. M. 1989. Chromosome map of the thermophilic Archaebacterium *Thermococcus celer*. J. Bacteriol. **171**:6720-6725.

Noll, M., B. Hapke, M. H. Schreier and H. Noll. 1973. Structural dynamics of bacterial ribosomes. J. Mol. Biol. **75**:281-294.

Novick, R. P. and E. Murphy. 1985. MLS-resistance determinants in *Staphylococcus aureus* and their molecular evolution. J. Antimicrob. Chemother. **16** ***Suppl A***:101-110.

Novick, R. P., E. Murphy, T. J. Gryczan, E. Baron and I. Edelman. 1979. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. Plasmid **2**:109-129.

O'Brien, T. F., J. D. Hopkins, E. S. Gilleece, A. A. Medeiros, R. L. Kent, B. O. Blackburn, M. B. Holmes, J. P. Reardon, J. M. Vergeront, W. L. Schell, E. Christenson, M. L. Bissett and E. V. Morse. 1982. Molecular epidemiology of antibiotic resistance in salmonella from animals and human beings in the United States. N. Engl. J. Med. **307**:1-6.

O'Hara, K., T. Kanada, K. Ohmiya, T. Ebisu, and M. Kono. 1989. Purification and characterization of macrolide 2'-phosphotransferase from a strain of *Escherichia coli* that is highly resistant to erythromycin. Antimicrob. Agents Chemother. **33**:1354-1357.

Ohnuki, T., T. Katoh, T. Imanaka and S. Aiba. 1985. Molecular cloning of tetracycline resistance genes from *Streptomyces rimosus* in *Streptomyces griseus* and characterization of the cloned genes. J. Bacteriol. **161**:1010-1016.

Olsvik, O., H. Sorum, K. Birkness, K. Wachsmuth, M. Figolstad, J. Lessen, K. Fossum and J. C. Feeley. 1985 Plasmid characterization of *Salmonella typhimurium* transmitted from animals to humans. J. Clin. Microbiol. **22**:336-338.

Osawa, S., E. Otaka, T. Itoh and T. Fukui. 1969. Biosynthesis of 50 S Ribosomal subunit in *Escherichia coli*. J. Mol. Biol. **40**:321-351.

Otaka, E., H. Teraoka, M. Tamaki, K. Tanaka and S. Osawa. 1970. Ribosomes from erythromycin-resistant mutants of *Escherichia coli* Q13. J. Mol. Biol. **48**:499-510.

Ounissi, H. and P. Courvalin. 1981. Classification of macrolide-lincosamide-streptogramin B type antibiotic resistance determinants. Ann. Inst. Pasteur Microbiol. **132B**:441-454.

Ounissi, H. and P. Courvalin. 1985. Nucleotide sequence of the gene *ereA* encoding the erythromycin esterase in *Escherichia coli*. Gene **35**:271-278.

Owen, R. J. 1983. Nucleic acids in the classification of Campylobacters. Eur. J. Clin. Microbiol. **2**:367-377.

Owen, R. J. and S. Leaper. 1981. Base composition, size and nucleotide sequence similarities of genome deoxyribonucleic acids from species of the genus *Campylobacter*. FEMS Microbiol. Letters **12**:395-400.

Ozaki, M., S. Mizushima and M. Nomura. 1969. Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in *E. coli*. Nature **222**:333-339.

Papadopoulou, B. and P. Courvalin. 1988. Dispersal in *Campylobacter* spp. of *aphA-3*, a kanamycin resistance determinant from gram-positive cocci. Antimicrob. Agents and Chemother. **32**:945-948.

Patton, C. M., T. J. Barrett and F. K. Morris. 1985. Comparison of the Penner and Lior methods for serotyping *Campylobacter* spp. J. Clin. Microbiol. **22**:558-565.

Penner, J. L. 1988. The Genus *Campylobacter*: a decade of progress. Clin. Microbiol. Rev. **1**:157-172.

Penner, J. L. and J. N. Hennessy. 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. J. Clin. Microbiol. 12:732-737.

Perkins, J. B. and P. J. Youngman. 1984. A physical and functional analysis of Tn917, a *Streptococcus* transposon in the Tn3 family that functions in *Bacillus*. Plasmid **12**:119-138.

Pestka, S. 1974. Antibiotics as probes of ribosome structure: Binding of chloramphenicol and erythromycin to polyribosomes; effects of other antibiotics. Antimicrob. Agents Chemother. **5**:255-267.

Pestka, S., R. Vince, R. Lemahieu, F. Weiss, L. Fern and J. Unowsky. 1976. Induction of erythromycin resistance in *Staphylococcus aureus* by erythromycin derivatives. Antimicrob. Agents Chemother. **9**:128-130.

Portnoy, D. A., S. L. Moseley and S. Falkow. 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775-782.

Ranzini, A. C. and D. T. Dubin. 1983. The "erythromycin-resistance" methylated sequence of *Staphylococcus aureus* ribosomal RNA. Plasmid **10**:293-295.

Rashtchian, A., M. A. Abbott and M. Shaffer. 1987. Cloning and characterization of genes coding for ribosomal RNA in *Campylobacter jejuni*. Current Micorbiol. **14**:311-317.

Rasmussen, J. L., D. A. Odelson and F. L. Macrina. 1986. Complete nucleotide sequence and transcription of *ermF*, a macrolide-lincosamide-streptogramin B resistance determinant from *Bacteroides fragilis*. J. Bacteriol. **168**:523-533.

Roberts, A. N., G. S. Hudson and S. Brenner. 1985. An erythromycin-resistance gene from an erythromycin-producing strain of *Arthrobacter* sp. Gene **35**:259-270.

Robinson, D. A. 1981. Infective dose of *Campylobacter jejuni* in milk. Br. Med. J. **282**:1584.

Romaniuk, P. J., B. Zoltowska, T. J. Trust D. J. Lane, G. J. Olsen, N. R. Pace and D. A. Stahl. 1987. *Campylobacter pylori*, the spiral bacterium associated with human gastritis, is not a true *Campylobacter* sp. J. Bacteriol. **169**:2137-2141.

Roop, R. M., R. M. Smibert and N. R. Krieg. 1984. Improved biotyping schemes for *Campylobacter jejuni* and *Campylobacter coli* . J. Clin. Microbiol. **20**:990-992.

Rubens, C. E. and L. M. Heggen. 1988. Tn*916* E: A Tn*916* transposon derivative expressing erythromycin resistance. Plasmid **20**:137-142.

Ruiz-Palacios, G. M., J. Torres, N. I. Escamilla, B. Ruiz-Palacios and J. Tamayo, 1983. Cholera-like

enterotoxin produced by *Campylobacter jejuni* characterization and clinical significance. Lancet **ii**:250-251.

Sagara, H., A. Mochizuki, N. Okamura and R. Nakaya. 1987. Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* with special reference to plasmid profiles of Japanese clinical isolates. Antimicrob. Agents. Chemother. **31**:713-719.

Schmid, G., T. Pecher and A. Bock. 1982. Properties of the translational apparatus of archaebacteria. Zentralbl. Bakteriol. Hyg. I. Abt. Orig. **C3**:209-217.

Schwartz, D. C. and C. R. Cantor. 1984. Separation of yeast chromosome-sized DNAs by pulsed-field gradient gel electrophoresis. Cell **37**:67-75.

Sebald, M. and M. Veron. 1963. Teneru en bases de l'ADN et classification des vibrions. Ann. Inst. Pasteur **105**:897-910.

Smibert, R. M. 1984. Genus *Campylobacter*. In Krieg, N. R. and H. G. Holt (Eds.) Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, pp. 111-118.

Serwold-Davis, T.M. and N. B. Groman. 1988. Identification of a methylase gene for erythromycin resistance within the sequence of a spontaneously deleting fragment of *Corynebacterium diphtheriae* plasmid pNG2. FEMS Microbiology Letters **56**:7-14

Shivakumar, A. G. and D. Dubnau. 1981. Characterization of a plasmid-specified ribosome methylase associated with macrolide resistance. Nucleic Acids Res. **9**:2549-2562.

Shivakumar, A. G., J. Hahn, G. Grandi, Y. Kozlov and D. Dubnau. 1980. Posttranscriptional regulation of an erythromycin resistance protein specified by plasmid pE194. Proc. Natl. Acad. Sci. U.S.A. **77**:3903-3907.

Sigmund, C. D., M. Ettayebi, A. Bordin and E. A. Morgan. 1988. Antibiotic resistance mutations in ribosomal RNA genes of *Escherichia coli*. Methods in Enzymology **164**:673-690.

Sigmund, C. D., M. Ettayebi and E. A. Morgan. 1984. Antibiotic resistance mutations in 16S and 23S ribosomal RNA genes of *Escherichia coli*. Nucleic Acids Res. **12**:4653-4663.

Sigmund, C. D. and E. A. Morgan. 1982. Erythromycin resistance due to mutation in a ribosomal RNA operon of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **79**:5602-5606.

Skinner, R. H. and E. Cundliffe. 1982. Dimethylation of adenine and the resistance of *Streptomyces erythraeus* to erythromycin. J. Gen. Microbiol. **128**:2411-2416.

Skinner, R. H., E. Cundliffe and F. J. Schmidt. 1983. Site of action of a ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. J. Biol. Chem. **258**:12702-12706.

Skirrow, M. B. 1977. Campylobacter enteritis: a "new" disease. Br. Med. J. **2**:9-11.

Skirrow, M. B. and J. Benjamin. 1980. Differentiation of enteropathogenic campylobacter. J. Clin. Pathol. **33**:1122.

Smibert, R. M. 1978. The genus *Campylobacter*. Annu. Rev. Microbiol. **32**:673-709.

Smith, C. L. and C. R. Cantor. 1987. Purification, specific fragmentation, and separation of large DNA molecules. Methods Enzymol. **155**:449-467.

Smith, C. L. and G. Condemine. 1990. New approaches for physical mapping of small genomes. J. Bacteriol. **172**:1167-1172.

Smith, C. L., J. G. Econome, A. Schutt, S. Klco and C. R. Cantor. 1987. A physical map of the *Escherichia coli* K12 genome. Science **236**:1448-1453.

Smith, C. L., S. Klco and C. R. Cantor. 1988. Pulsed field gel electrophoresis and the technology of large DNA molecules, 41-72. *In* K. Davies (ed.), Genome analysis: a practical approach. IRL Press, Oxford, England.

Smith, T. and M. S. Taylor. 1919. Some morphological and biological characters of the spirilla (*Vibrio fetus*, N. sp.) associated with disease of the fetal membranes in cattle. J. Exp. Med. **30**:299-311.

Sougakoff, W., B. Papadopoulou, P. Nordmann and P. Courvalin. 1987. Nucleotide sequence and distribution of gene *tetO* encoding tetracycline resistance in *Campylobacter coli*. FEMS Microbiol. Lett. **44**:153-159.

Southern, E. M. 1975. Detection of specific sequences among DNA fragmets separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

Spedding, G. and E. Cundliffe. 1984. Identification of the altered ribosomal component responsible for resistance to micrococcin in mutants of *Bacillus megaterium*. Eur. J. Biochem. **140**:453-459.

Steele, T. W. and S. McDermott. 1978. Campylobacter enteritis in South Australia. Med. J. Aust. **2**:404-406.

Steers, E., E. L. Foltz, B. S. Gravis and J. Riden 1955. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9**:307-311.

Steige, W. C. Glotz and R. Brimacombe. 1983. Localization of a series of intra-RNA cross-links in the secondary structure of 23S RNA induced by ultraviolet irradiation of *Escherichia coli* 50S ribosomal subunits. Nucleic Acids Res. **11**:1687-1706.

Stocklein, W. and W. Piepersberg. 1980. Altered ribosomal protein L24 in a cycloheximide-resistant strain of *Saccharomyces cerevisiae*. Curr. Genet. **1**:177-183.

Tanaka, T. and B. Weisblum. 1974. Mutants of *Staphylococcus aureus* with lincomycin- and carbomycin-inducible resistance to erythromycin. Antimicrob. Agents Chemother. **5**:538-540.

Tanaka, T. and B. Weisblum. 1975. Systematic difference in the methylation of ribosomal ribonucleic acid from gram-positive and gram-negative bacteria. J. Bacteriol. **123**:771-774.

Taubeneck, U. 1962. Susceptibility of *Proteus mirabilis* and its stable L-forms to erythromycin and other macrolides. Nature **196**:195-196.

Taylor, D. E., N. Chang, R. S. Garner, R. Sherburne and L. Mueller. 1986. Incidence of antibiotic resistance and characterization of plasmids in *Campylobacter jejuni* strains isolated from clinical sources in Alberta, Canada. Can. J. Microbiol. **32**:28-32.

Taylor, D. E. and P. Courvalin. 1988. Mechanisms of antibiotic resistance in *Campylobacter* species. Antimicrob. Agents Chemother. **32**:1107-1112.

Taylor, D. E., S. A. DeGrandis, M. A. Karmali and P. C. Fleming. 1980. Transmissible tetracycline resistance in *Campylobacter jejuni*. Lancet **ii**:797.

Taylor, D. E., R. S. Garner and B. J. Allan. 1983. Characterization of tetracycline resistance plasmids from *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. **24**:930-935.

Taylor, D. E. and K. Hiratsuka. 1990. Use of non-radioactive DNA probes for detection of *Campylobacter jejuni* and *Campylobacter coli* in stool specimens. Mol. and Cell. Probes (in press).

Taylor, D. E., L.-K. Ng and H. Lior. 1985. Susceptibility of *Campylobacter* species to nalidixic acid, enoxacin and other DNA gyrase inhibitors. Antimicrob. Agents Chemother. **28**:708-710.

Taylor, D. E., W. Yan, L.-K. Ng, E. K. Manavathu and P. Courvalin. 1988. Genetic characterization of kanamycin resistance in *Campylobacter coli*. Ann. Inst. Pasteur/Microbiol. **139**:665-676.

Taylor, D. N., M. J. Blaser, P. E. Echeverria, C. Pitarangsi, L. Bodhidatta and W.-L. L. Wang. 1987. Erythromycin-resistant *Campylobacter* infections in Thailand. Antimicrob. Agents Chemother. **31**:438-422.

Tejedor, F. and P. G. Ballesta. 1985. Components of the macrolide binding site on the ribosome. J. Antimicrob. Chemother. **16** *Suppl A*:53-62.

Tenover, F. C., M. A. Bronsdon, K. P. Gordon and J. J. Plorde. 1983. Isolation of plasmids encoding tetracycline resistance from *Campylobacter jejuni* strains isolated from simians. Antimicrob. Agents Chemother. **23**:320-322.

Tenover, F. C., J. S. Knapp, C. Patton and J. J. Plorde. 1985. Use of auxotyping for epidemiological studies of *Campylobacter jejuni* and *Campylobacter coli* infections. Infect. Immun. **48**:384-388.

Tenover, F. C., D. J. LeBlanc and P. Elvrum. 1987. Cloning and expression of a tetracycline resistance determinant from *Campylobacter jejuni* in *Escherichia coli*. Antimicrob. Agents Chemother. **31**:1301-1306.

Tenover, F. C., S. Williams, K. P. Gordon, N. Harris, C. Nolan and J. J. Plorde. 1984. Utility of plasmid fingerprinting for epidemiological studies of *Campylobacter jejuni* infections. J. Infect. Dis. **149**:279.

Tenover, F. C., S. Williams, K. P. Gordon, C. Nolan and J. J. Plorde. 1985. Survey of plasmids and resistance factors in *Campyloacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. **27**:37-41.

Teraoka, H. and K. Tanaka. 1971. Reaction of puromycin with *N*-acetylphenylalanyl-tRNA on ribosomes reassociated from *Escherichia coli* ribosomal subunits. Biochim. Biophys. Acta. **247**:304-309.

Thakker-Varia, S., A. C. Ranzini and D. T. Dubin. 1985. Ribosomal RNA methylation in *Staphylococcus aureus* and *Escherichia coli*: effect of the "MLS" (erythromycin resistance) methylase. Plasmid **14**:152-161.

Thakker-Varia, S., W. D. Jenssen, L. Moon-Mcdermott, M. P. Weinstein and D. T. Dubin. 1987. Molecular epidemiology of macrolides-lincosamides-streptogramin B resistance in *Staphylococcus aureus* and coagulase-negative Staphylococci. Antimicrob. Agents Chemother. **31**:735-743.

Thompson, J. S., F. E. Cahoon and D. S. Hodge. 1986. Rate of *Campylobacter* spp. isolation in three regions of Ontario, Canada, from 1978 to 1985. J. Clin. Microbiol. **24**:876-878.

Tobian, J. A., M. L. Cline and F. L. Macrina. 1984. Characterization and expression of a cloned tetracycline resistance determinant from the chromosome of *Streptococcus mutans*. J. Bacteriol. **160**:556-563.

Totten, P. A., F. C. Tenover, K. C. S. Chen, K. L. Bruch, K. K. Holmes and W. E. Stamm. 1985. Rapid genetic grouping test for identification of *Campylobacter* species. P. 234. *In* A. D. Pearson, M. B. Skirrow, H. Lior and B. Rowe (ed.), Campylobacter III: Proceedings of the third international workshop on campylobacter infections, Ottawa. Public Health Laboratory Service, London.

Trieu-Cuot, M. Arthur and P. Courvalin. 1987. Transfer of genetic information between gram-positive and gram-negative bacteria under natural conditions. p. 65-68. *In* R. Curtiss. and J. J. Ferretti (eds.). American Society for Microbiology, Washington, DC.

Trieu-Cuot, P., G. Gerbaud, T. Lambert and P. Courvalin. 1985. *In vivo* transfer of genetic information between gram-positive and gram-negative bacteria. EMBO J. **4**:3583-3587.

Uchiyama, H. and B. Weisblum. 1985. *N*-methyl transferase of *Streptomyces erythraeus* that confers resistance to the macrolide-lincosamide-streptogramin B antibiotics: amino acid sequence and its homology to cognate R-factor enzymes from pathogenic bacilli and cocci. Gene **38**:103-110.

Ursing, J., M. Walder and K. Sandstedt. 1983. Base composition and sequence homology of deoxyribonucleic acid of thermo-tolerant *Campylobacter* from human and animal sources. Curr. Microbiol. **8**:307-310.

Vanhoof, R., M. P. Vanderlinden, R. Dierickx. S. Lauwers, E. Yourassowsky and J. P. Butzler. 1978. Susceptibility of *Campylobacter fetus* subsp. *jejuni* to twenty-nine antimicrobial agents. Antimicrob. Agents Chemother. **14**:553-556.

Veron, M. and R. Chatelaine. 1973. Taxonomic study of the genus *Campylobacter* (Sebald and Veron) and designation of the new type strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Veron. Int. J. Syst. Bacteriol. **23**:122-134.

Vinzent, R., J. Dumas and N. Picard. 1947. Septicemie grave au cours de la grossesse due a un vibrion: avortement consecutif. Bull. Acad. Natl. Med. **131**:90-92.

Walder, M. and A. Forsgren. 1978. Erythromycin-resistant Campylobacters. Lancet **ii**:1201.

Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust and G. M. Ruiz-Palacios. 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol. Rev. **50**:81-94.

Walker, M. S. and J. B. Walker. 1970. Streptomycin biosynthesis and metabolism. J. Biol. Chem. **245**:6683-6689.

Wang Y. and D. E. Taylor. 1990. Natural transformation in *Campylobacter* species. J. Bacteriol. **172**:949-955.

Weaver, J. R. and P. A. Pattee. 1964. Inducible resistance to erythromycin in *Staphylococcus aureus*. J. Bacteriol. **88**:574-580.

Weisblum, B. 1975. Altered methylation of ribosomal ribonucleic acid in erythromycin-resistant *Staphylococcus aureus*, p.199-206. *In* D. Schlessinger (ed) Microbiology - 1974. American Society for Microbiology, Washington DC.

Weisblum, B. 1985. Inducible resistance to macrolides, lincosamides and streptogramin type B antibiotics: the resistance phenotype, its biological diversity, and structural elements that regulate expression - A review. J. Antimicrob. Chemother. **16***Suppl A*:63-90.

Weisblum, B. and V. Demohn. 1969. Erythromycin-inducible resistance in *Staphylococcus aureus*: survey of antibiotic classes involved. J. Bacteriol. **98**:447-452.

Weisblum, B., C. Siddhikol, C. J. Lai and V. Demohn. 1971. Erythromycin-inducible resistance in *Staphylococcus aureus*: requirements for induction. J. Bacteriol. **106**:835-847.

Weisblum, B., M. Y. Graham, T. Gryczan and D. Dubnau. 1979. Plasmid copy number control: isolation and characterization of high-copy-number mutants of plasmid pE194. J. Bacteriol. **137**:634-643.

Weisblum, B., S. B. Holder and S. M. Halling. 1979. Deoxyribonucleic acid sequence common to Staphylococcal and Streptococcal plasmids which specify erythromycin resistance. J. Bacteriol. **138**:990-998.

Welch, R. A., K. R. Jones and F. L. Macrina. 1979. Transferable lincosamide-macrolide resistance in *Bacteroides*. Plasmid **2**:261-268.

Wenzel, R. and R. Herrmann. 1988. Physical mapping of the *Mycoplasma pneumoniae* genome. Nucleic Acies Res. **16**:8323-8336.

Wienen, B., R. Ehrlich, M. Stoffler-Meilicke, G. Stoffler, I. Smit D. Weiss, R. Vince and S. Pestka. 1979. Ribosomal protein alterations in thiostrepton- and micrococcin-resistant mutants of *Bacillus subtilis*. J. Biol. Chem. **254**:8031-8041.

Wittmann, H. G., G. Stoffler, D. Apirion, L. Rosen, K. Tanaka, M. Tamaki, R. Takata, S. Dekio, E. Otaka and S. Osawa. 1973. Biochemical and genetic studies on two different types of erythromycin resistant mutants of *Escherichia coli* with altered ribosomal proteins. Mol. Gen. Genet. **127**:175-189.

Wong, K. H., S. K. Skelton and J. C. Feeley. 1986. Strain characterizaion and grouping of *Campylobacter jejuni* and *Campylobacter coli* by interaction with lectins. J. Clin. Microbiiol. **23**:407-410.

Yagi, Y., A. E. Franke and D. B. Clewell. 1975. Plasmid-determined resistance to erythromycin: comparison of strains of *Streptococcus faecalis* and *Streptococcus pyogenes* with regard to plasmid homology and resistance inducibility. Antimicrob. Agents Chemother. **7**:871-873.

Yagi, Y., T. S. Mclellan, W. A. Frez and D. B. Clewell. 1978. Characterization of a small plasmid determining resistance to erythromycin, lincomycin, and vernamycin B in a strain of *Streptococcus sanguis* isolated from dental plaque. Antimicrob. Agents Chemother. **13**:884-887.

Yeen, W. P., S. D. Puthucheary and T. Pang. 1983. Demonstration of a cytotoxin from *Campylobacter jejuni*. J. Clin. Pathol. **36**:1237-1240.

Appendix 1

Supplemented FBP agar medium for rapid H_2S Test

Medium A

Brucella broth (GIBCO)	2.9 g
Na_2HPO_4 (anhydrous)	0.118 g
KH_2PO_4 (anhydrous)	0.2 g
Agar (L28-OXOID)	0.2 g
H_2O	97 ml

This agar medium is autoclaved for 15 minutes at 15 lb. pressure then cooled to 45°C. The following solutions were then prepared separately in distilled water and filter sterilized:

Solution B	Ferrous sulphate $7 \cdot H_2O$	10%
Solution C	Sodium metabisulphite	10%
Solution D	Sodium pyruvate	10%

1 ml of solution B was aseptically added to 1 ml of solution C, mixed well, and the mixture was added to 1 ml of solution D. The entire mixture was then added to Medium A, adjusted to pH 7.3 and 3 ml portions were dispensed aseptically into 13 x 100 mm sterile screw capped tubes. Fresh media should be prepared every two weeks.

Appendix 2

Modified Toluidine Blue-DNA Agar for DNA Hydrolysis Test

Toluidine Blue-DNA Agar

Deoxyribonucleic Acid (DIFCO)	0.3 g
0.01 M $CaCl_2$	1.0 ml
NaCl	10.0 g
Agar (L28-OXOID)	6.5 g
0.05 M Tris Buffer pH 9.0	1.0 L

All ingredients were mixed and boiled until DNA and agar were completely dissolved. The mixture was cooled to 50°C before 2.5 ml of 3% Toluidine Blue "O" (Fisher Scientific Certified Stain) was added. After thorough mixing, the agar solution was dispensed into petri plates at approximately 25 ml/plate. (Sterilization not required). After solidifying, the plates were stored at room temperature in the dark before use.