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UNIVERSITY OF ALBERTA

Transformation and Antibiotic Resistance in *Campylobacter* species

BY

Ying Wang

A THESIS

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

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED TRANSFORMATION AND ANTIBIOTIC RESISTANCE IN *CAMPYLOBACTER* SPECIES SUBMITTED BY YING WANG IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

Growing cells of *Campylobacter coli* and *C. jejuni* were naturally transformed by naked DNA without a requirement for any special treatment. Transformation frequencies for homologous chromosomal DNA were approximately 1 x 10^{-3} transformants per viable cell in *C. coli* and 1 x 10^{-4} in *C. jejuni*. *C. coli* UA585 is constitutively competent, and the competence level can be stimulated by DNA molecules. Maximum competence was found in the early exponential phase of growth. Campylobacters preferentially took up their own DNA in comparison with *Escherichia coli* chromosomal DNA which was taken up very poorly. Plasmid DNAs were taken up by campylobacters much less efficiently than homologous chromosomal DNA, and transformation into plasmid-free cells was very rare. However, with the use of recipients containing a homologous plasmid, approximately 1 x 10^{-4} transformants per cell were obtained. A *tet*(M) determinant, originally obtained from *Streptococcus*, and a staphylococcal kanamycin resistance determinant were transformed and expressed in *C. coli*.

A series of new *Campylobacter* spp. to *E. coli* shuttle plasmids, which contained additional cloning sites and selectable markers, were constructed from the shuttle vector pIL550A. Two campylobacter cloning vectors and two suicide vectors were also constructed. These plasmids were used throughout the studies in this thesis.

A chloramphenicol resistance determinant, originally cloned from a *C. coli* plasmid pNR9589 in Japan, was isolated, and the nucleotide sequence determined. It contains an ORF of 621 bp, and the gene product was identified as a CAT (Cm acetyltransferase). The deduced amino acid sequence shows 43% to 57% identity with other CAT proteins of both Gram-positive and Gram-negative origin. Although expression of the *cat* gene was constitutive in both *C. coli* and *E. coli*, results of primer extension experiments indicated that transcription was initiated at different sites in these two species. A kanamycin resistance determinant, identified as the *aphA-3* gene, was located downstream from the *cat* gene. The codon usage of the *cat* gene is very different from that used in *E. coli;* however, the CAT polypeptide was synthesized in large amounts in *E. coli* maxicells. Therefore, the codon usage bias is not one of the obstacles which affects *Campylobacter* spp. gene expression in *E. coli*.

The homologous DNA sequences upstream of the *tet*(O) and *tet*(M) ORF's were identified, and the nucleotide sequences determined. These two DNA sequences share a much higher degree of homology than the *tet*(O) and *tet*(M) ORF's themselves, and do not appear to encode any polypeptides. Deletion experiments showed that the DNA sequence upstream of *tet*(O) was required for high level tetracycline resistance, as well as the stability of the *tet*(O) ORF in the host cell. Complementation analysis suggested that this DNA sequence functioned *in cis* only. The transcription start point for *tet*(O) mRNA was located. Translation of the Tet(O) protein was studied using a T7 promoter vector and a wheat germ in vitro translation system. It appeared that the expression of Tet(O) is regulated at the translational level.

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Ap	ampicillin
AphA-3	aminoglycoside phosphotransferase specified by the *aphA*-3 gene
bp	base pair(s)
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cat	gene coding for CAT
Cm	chloramphenicol
DTT	dithiothreitol
Em	erythromycin
ExoIII	exonuclease III
h	hour(s)
Inc	Incompatibility group
IPTG	Isopropyl-β-D-thiogalactopyranoside
Km	kanamycin
kb	kilobase(s) or 1000 bp
KDa	kilodalton(s)
MH	Mueller-Hinton
MIC	minimal inhibitory concentration
min	minute(s)
mm	millimeter
Nal	nalidixic acid
ORF	open reading frame
rpm	revolutions per min

SD	Shine-Dalgarno sequence
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSC	standard saline citrate
ssDNA	single-stranded DNA
Str	streptomycin
Tc	tetracycline
TE	Tris-EDTA buffer

CHAPTER 1

INTRODUCTION

1.1 Relevant properties of *Campylobacter* species

Campylobacters have been recognized as a major cause of gastroenteritis in humans since 1977 (Skirrow, 1977; Finch and Riley, 1984). Organisms belonging to the genus *Campylobacter* were originally designated as "microaerophilic vibrios" by MacFadyean and Stockman (1913). The genus *Campylobacter*, in the family *Spirillaceae*, was created in the 1960's based on cell morphology, growth conditions, and G + C content (Sebald and Veron, 1963; Veron and Chatelain, 1973).

Campylobacters are small (0.5 - 5 µm long and 0.2 - 0.5 µm wide), Gram-negative, nonsporulating bacteria that grow optimally under microaerobic conditions (5 - 10% O_2, 3 - 10% CO_2). They are typically spiral in shape in young cultures and undergo a degenerative change to coccoid forms in older cultures (Ng et al., 1985). Usually they have a single polar flagellum at either one or both ends of the cell which accounts for their characteristic corkscrew-like motility (Smibert, 1978). Carbohydrates are neither fermented nor oxidized. Energy is obtained from amino acids or tricarboxylic acid cycle intermediates (Alexander, 1957; Lecce, 1958). Some species are pathogenic for man and animals. The majority of cases of *Campylobacter* gastroenteritis in humans are caused by *C. jejuni* (59-98% depending on the geographic region involved), whereas the closely related species *C. coli* is responsible for a much lower proportion of cases (1-41%) (Karmali et al., 1983; Ho and Wong, 1985). The mol% G + C of the DNA is ca. 32% (30 - 36%, Sebald and Veron, 1963). The genome sizes of *C. jejuni* and *C. coli* were estimated to be 1.7 Mbases by pulsed-field electrophoresis (Chang and Taylor, 1990; Nuijten et al., 1990; Yan and Taylor, 1991).

1.2 Genetic exchange in *Campylobacter*.

1.2a Natural genetic exchange in campylobacters. Conjugative plasmids encoding resistance to tetracycline, kanamycin, or chloramphenicol have been reported in campylobacters (Taylor and Courvalin, 1988). Most of these plasmids are ca. 50 kb in size, and can only replicate in *Campylobacter* species (Taylor and Courvalin, 1988). The transfer frequency was ca. 1 x 10^{-5} to 1 x 10^{-3} per recipient in a 24 h mating period (Taylor et al., 1981). Sex pili were not found on plasmid-containing *C. jejuni* cells (Taylor and Sherburne, unpublished data). Mechanisms of conjugative transfer and replication remain unknown in *Campylobacter* plasmids. *E. coli* plasmids such as pBR322 could not replicate in *Campylobacter* (Labigne-Roussel, 1987; Walker et al., 1986).

Bacteriophages have been isolated for *C. jejuni* and *C. coli* (Ritchie et al., 1983; Grajewski et al., 1985; Salama et al., 1989). However, phage-mediated transduction has not been reported in *C. jejuni* and *C. coli*.

Transposons have never been found in campylobacters, although a kanamycin resistance gene has been found to integrate into the chromosome from a plasmid at low frequency (Kotarski et al., 1986).

Both Tn5, a typical Gram-negative transposon (Berg, 1989), and Tn917, a Gram-positive transposable element (Shaw and Clewell, 1985) have been reported not to function in *Campylobacter* (Labigne-Roussel et al., 1988).

In a preliminary report several *C. coli* strains were found to be naturally competent for taking up DNA from their environment (Fraser and Riche, 1988).

1.2b Artificial gene transfer systems. In genetic studies, gene transfer is often initiated by artificial means; e.g., calcium chloride treatment for *E.coli* and some other Gram-negative bacterial species (Mandel and Higa, 1970), polyethylene glycol(PEG)-mediated transformation for protoplasts of Gram-positive bacteria (Chang and Cohen, 1979) or the whole cells of Gram-negative bacteria (Chung et al., 1989), or electrotransformation using high-voltage discharge to introduce DNA into a cell (Miller et al., 1988). These artificial methods are exploited probably because many bacterial species are not naturally transformable, and because plasmid and phage DNAs are believed to remain intact after entering into a cell by these artificial pathways.

Until recently, molecular studies of the biology and pathogenesis of campylobacters had been hampered by the lack of well-defined genetic exchange systems for DNA manipulation in *Campylobacter* (Walker et al., 1986). $CaCl_2$ or PEG treatment, which have been successfully applied in many Gram-negative bacteria, failed to render *Campylobacter* capable of taking up DNA (Ng and Taylor, unpublished results). The only artificial method used successfully to transform *C. jejuni* cells with plasmid DNA was electrotransformation (Miller et al., 1988).

1.2c Recent advances. Shuttle vectors which can be transfered from *E. coli* to *C. jejuni* by conjugation were the first successful attempt at *Campylobacter* gene manipulation (Labigne-Roussel et al., 1987). The transfer frequency of a shuttle vector pILL550 was 1 x 10^{-4} transconjugants per donor. The system requires a bifunctional vector and a helper plasmid, and also involves mixing cultures of *E. coli* and *C. jejuni*. Subsequently electrotransformation of *C. jejuni* with plasmid DNA was demonstrated by Miller et al.(1988). The transformation efficiency of a shuttle plasmid pILL512 into *C. jejuni* C31 was reported as high as 1.2 x 10^6 transformants per µg of DNA. Difficulties were encountered in electrotransformation of plasmid DNA isolated from *E. coli* into *Campylobacter* spp. due to restriction (Miller et al., 1988). However, a maximum transformation efficiency of 5 x 10^3 transformants per µg of DNA was obtained using the commercially available Gene Pulsar apparatus (Bio-Rad)(Yan, 1990).

1.2d Natural transformation in bacteria. Bacteria have evolved different mechanisms for the exchange of genetic material, including transformation, transduction, and conjugation. These may provide a potential advantage for the bacterium, because they enable an individual cell to accumulate advantageous mutations originating from separate individuals or even to acquire genetic material from other species. Natural transformation was the first genetic exchange mechanism discovered in bacteria (Griffith, 1928). Later the identification of the transforming factor as deoxyribonucleic acid (Avery et al., 1944; McCarty and Avery, 1946) provided the foundation for subsequent studies which demonstrated that DNA is the heritable material for all organisms. Since then the process of transformation has been extensively studied. It has also been successfully used in genetic analysis of bacteria, such as gene cloning (Gryczan et al., 1981; Macrina et al., 1980), chromosome mapping (Kunkel et al., 1990; Yan and Taylor, 1991), and gene replacement mutagenesis (Sharetzsky et al., 1991).

Most transformable bacteria only become competent under certain conditions of growth, thus competence appears to be an induced state. For example, if exponentially growing cells of *Haemophilus influenzae* are transfered into a defined medium which does not support growth, the entire culture becomes competent for DNA uptake. In *Neisseria gonorrhoeae,* however, it was reported that competence is constitutive (Smith et al., 1981).

Mechanisms of DNA binding and uptake appear to fall into two groups, one typical of Gram-positive organisms and the other found in some Gram-negative bacteria (Smith et al., 1981). There is a marked difference in the process of binding and transport of DNA through the envelope of the recipient cell. In *Bacillus subtilis, Streptococcus pneumoniae,* and *S. sanguis,* a single competent cell can bind and take up a large number of DNA molecules regardless of their source. During the uptake process one strand of the DNA is degraded while the complementary strand is transported into the cell (Smith et al., 1981;

Stewart and Carlson, 1986). Of the Gram-negative bacteria that possess natural transformation systems, *Haemophilus* species bind and take up DNA possessing a specific 11-base pair sequence (Danner et al., 1980). DNA uptake by *N. gonorrhoeae* also involves recognition of a specific 10-bp sequence (Goodman and Scocca, 1988). In both cases, only a few molecules of homologous DNA can be taken up by a competent cell, and heterologous DNA can only be taken up at much lower frequency (Goodgal, 1982).

Natural transformation of plasmid DNA is normally rare in both Gram-positive and Gram-negative bacteria, because the duplex DNA is nicked and partially degraded during both binding to and entering into the cell (Stewart and Carlson, 1986). The frequency of plasmid transformation can be increased by using a recipient containing a homologous plasmid. Thus the incoming plasmid can be rescued by the resident plasmid through homologous recombination (Gryczan et al., 1981; Macrina et al., 1980).

As previously mentioned, *C. coli* was found to be naturally competent (Fraser and Riche, 1988). Studies on this phenomenon might lead to the development of a simple system for gene transfer in *Campylobacter* spp.

1.3 Vectors constructed for *Campylobacter* species.

As previously mentioned, Km resistant shuttle cloning vectors have been constructed by Labigne-Roussel et al.(1987), and have been successfully used in cloning *Campylobacter* DNA sequences in *E. coli* and returning them to *Campylobacter* for expression. A similar suicide vector was also constructed and used to generate mutations in 16S ribosomal RNA in *C. jejuni* using a gene disruption and gene replacement technique (Labigne-Roussel et al., 1988). These constructions represent the first generation of vectors. They did not offer the researcher a good choice of restriction sites and nor did they have suitable markers for selection of recombinants.

1.4 Antibiotic resistance in *Campylobacter* species.

1.4a Mechanisms of antibiotic resistance in bacteria.

Antibiotics have been used extensively in the last few decades in the treatment of bacterial infections. They have also been used as growth promotional agents in animal feeds. Probably because of these enormous selective pressures, bacteria have evolved various mechanisms of antimicrobial resistance (Shafran, 1990), including:

(i) Reduction of drug concentration at the target site, which is achieved by a) reduction of drug uptake into the cell, e.g., chromosomal mutation of cadmium resistance; b) energy dependent efflux, e.g., tetracycline resistance in *E. coli*; c) enzymatic inactivation, e.g., ampicillin resistance; or d) chelation, e.g., copper binding protein;

(ii) Alteration of the target site by a) chromosomal mutation, e.g., rifampicin resistance; or b) enzymatic modification, e.g., erythromycin resistance in *Staphylococcus*;

(iii) Substitution of the target protein with a less sensitive enzyme, e.g., sulfonamide resistance;

(iv) Compensation of the effect of antibiotic, e.g., chromosomal mutation of streptomycin resistance, which reduces the tanslational error so that the cell can survive in the presence of low concentrations of streptomycin.

1.4b General introduction to antibiotic resistance in *Campylobacter* species. *C. jejuni* and *C. coli* are intrinsically resistant to a number of antibiotics, including bacitracin, novobiocin, rifampin, streptogramin B, and vancomycin (Taylor and Courvalin, 1988).

Tetracycline resistance was reported to be 0% in Sweden in 1978 (Vanhoof et al., 1978), 55% in Japan in 1987 (Sagara et al., 1987), and 43% in United States in 1989 (Tenover, 1991). The resistance gene is often encoded on conjugative plasmids of size 45 to 50 kb (Taylor and Courvalin, 1988). Tc^R determinants from a *C. jejuni* plasmid pUA466 and a *C. coli* plasmid pIP1433 have been cloned and nucleotide sequences determined (Taylor et al., 1987; Manavathu et al., 1988; Sougakoff et al., 1987). They are almost identical at the nucleotide level, and have been designated *tet*(O). Homology with *tet*(O) was detected in 98 out of 100 Tc^R *Campylobacter* strains isolated from humans and animals in various geographical areas (Sougakoff et al., 1987).

Kanamycin resistance is usually mediated by plasmids, and it appears to be associated more often with *C. coli* than with *C. jejuni* (Kotarski et al., 1986; Sagara et al., 1987). A Km^R determinant from *C. coli* plasmid pIP1433 was cloned and sequenced (Trieu-Cuot et al., 1985). It was identified as *aphA-3* which specifies a 3'-aminoglycoside phosphotransferase of type III. This gene was found previously only in Gram-positive cocci, in which it is widely distributed. On the other hand, Ouellette et al.(1987) cloned and partially sequenced a 2.2 kb fragment from a *Campylobacter*-like organism which confers kanamycin resistance in *E. coli*. It was identified as *aphA-1*, and was found to be almost identical to that of Tn*903*, which was originally derived from *E. coli* (Oka et al., 1981). Therefore, resistance to kanamycin in *Campylobacter* is probably due to in vivo acquisition of a gene from either a Gram-positive coccus or a Gram-negative bacterium. However, Tenover and co-workers (1989) cloned and sequenced a novel kanamycin resistance gene (*aphA*-7) from a *C. jejuni* plasmid, which shares 55% identity at the nucleotide level with the *aphA-3* gene. It was suggested that the *aphA-7* gene may be indigenous to campylobacters, because the 32.8% G + C ratio in the open reading frame (ORF) of *aphA-7* is similar to that of the *C. jejuni* chromosome (32%).

Erythromycin resistance also occurs more often in *C. coli* than in *C. jejuni* (Yan, 1990). Resistance appears to be due to a chromosomal mutation or mutations which results in alteration of the target site (Yan, 1990; Wang and Taylor, unpublished results).

Campylobacter strains resistant to nalidixic acid and other DNA gyrase inhibitors such as the fluoroquinolones have been reported (Taylor and Courvalin, 1985). Chromosomal mutations of Nal^R of *C. jejuni* and *C. coli* could also be selected in the laboratory at frequencies of about 5×10^{-9} (Taylor et al., 1985). Gootz and Martin (1991) demonstrated that the DNA gyrases from Nal^R mutants were 100-fold less susceptible than the wild-type enzyme to inhibition by quinolones in the DNA supercoiling reaction. Subunit switching experiments with purified A and B subunits from the wild type and one of the quinolone resistant mutants indicated that an alteration in the A subunit was responsible for resistance.

Resistance to high concentration of streptomycin and spectinomycin has been reported in campylobacters (Taylor and Courvalin, 1988). Pinto-Alphandary et al.(1990) demonstrated that the resistance in six out of eight strains was due to the production of aminoglycoside adenylyltransferase encoded by *aadE* which was thought to be specific for Gram-positive cocci (Ounissi and Courvalin, 1987). Moreover, one of these resistant strains hybridized with the *aadA* gene, a resistance determinant commonly found in Gram-negative bacteria (Hollingshead and Vapnek, 1985). These results constitute further evidence that *Campylobacter* spp. can acquire genes from both Gram-positive and Gram-negative bacteria.

1.4c Chloramphenicol resistance. Resistance to Cm in bacteria is most often due to the inactivation of the antibiotic by the enzyme chloramphenicol acetyltransferase (CAT), which is a trimer of identical subunits of ca. 25 kDa (Shaw, 1983). A number of *cat* genes have been cloned and sequenced from a diverse range of bacterial spp., luding *E. coli* Tn*9 cat* (type I) (Shaw et al., 1979), *E. coli cat* type III (Murray et al., 1988), *Proteus mirabilis cat* (Charles et al., 1985), *Streptomyces acrimycini cat* (Murray et al., 1989), *Bacillus pumilus cat-86* (Harwood et al., 1983), *Staphylococcus aureus* pC221 and pUB112 *cats* (Bruckner and Matzura, 1985; Shaw et al., 1985), *S. aureus* pC194 *cat* (Horinouchi and Weisblum, 1982), *Clostridium perfringens catQ* (Bannam and Rood, 1991), *Clostridium catP* and *catD* (Steffen and Matzura, 1989; Wren et al., 1989).

The *cat* genes have been located on plasmids, transposons, and bacterial chromosomes (Shaw, 1983). The expression of *cat* genes from Gram-negative organisms is frequently constitutive. Alternatively, in Gram-positive bacteria, they are usually inducible by subinhibitory concentrations of Cm (Shaw, 1983). The mechanism for the inducible phenotype is known as translational attenuation (Bruckner and Matzura, 1985; Lovett, 1990). It involves a DNA sequence that codes for a small leader peptide and an inverted repeat sequence, both of which are located directly upstream of the *cat* gene. The RNA transcript will form a stem-loop structure which sequesters the *cat* gene SD site. In the presence of Cm, the ribosome translating the leader peptide stalls, which leads to destabilizaton of the RNA stem and loop, thereby making the ribosome binding site of the *cat* gene available for the initiation of translation (Lovett, 1990).

Resistance to Cm is very rare among *Campylobacter* spp. There has been only a single report in which Sagara et al.(1987) cloned and expressed in *E. coli* a Cm^R determinant from a *C. coli* plasmid isolated in Japan.

1.4d Tetracycline resistance in bacteria. It is believed that Tc inhibits the growth of bacteria by binding to a single high affinity site on the 30S subunit of the 70S ribosome, and preventing the aminoacyl-tRNA binding to the A site of the bacterial ribosome (Goldman et al., 1983). Tetracycline resistance(Tc^R) is probably the most common antibiotic resistance encountered in bacteria. Three types of resistance mechanisms have been reported (Salyers et al., 1990):

1) the prevention of intracellular accumulation of Tc, which is mediated by a cytoplasmic membrane protein that actively pumps Tc out of the cell, such as classes A-F determinants in Gram-negative bacteria, classes K and L in Gram-positive cocci and *Bacillus;*
2) the inactivation of Tc, which is mediated by a class X determinant in *Bacteroides fragilis*. The resistance to Tc has been found to be expressed in *E. coli* under aerobic condition only, but not in the original host species;
3) ribosomal protection, a poorly understood process which acts at the level of protein synthesis. This type of resistance includes the *otr*A gene from *Streptomyces* (Ohnuki et al., 1985), *tet*(M) (Burdett, 1986) and *tet*(O) (Taylor, 1986). Both the *tet*(M) and *tet*(O) genes are widely distributed in Gram-positive and Gram-negative bacteria, including *Streptococcus* (Burdett et al., 1982; LeBlanc et al., 1988), *Staphylococcus* (Levy, 1984), *Mycoplasma* (Roberts et al., 1985), *Ureaplasma* (Roberts and Kenny, 1986), *Gardnerella* (Roberts et al., 1986), *Neisseria* (Morse et al., 1986), and *Enterococcus* (Bentorcha et al., 1991).

1.4e Tetracycline resistance mediated at the level of protein synthesis. It has been shown that ribosomes isolated from bacteria containing *otr*A, *tet*(M), or *tet*(O) were resistant to Tc in in vitro translation systems (Ohnuki et al., 1985; Burdett, 1986; Manavathu and Taylor, 1990). However, Ohnuki et al.(1985) showed that the Tc resistant ribosomes could become sensitive to Tc if washed with high salt solution. Also, Manavathu et al.(1990) found that ribosomes from Tc^S cells could be rendered less sensitive when mixed with a S100 fraction from Tc^R cells. Furthermore, neither the modification of ribosomal components nor the presence of the resistance gene products were ever detected from ribosomal preparations by SDS-PAGE or reversed phase high performance liquid chromatography (RP-HPLC) (Manavathu et al., 1990). These results suggest that a cytoplasmic protein is acting on ribosomes to make them less sensitive to the inhibitory action of Tc, and probably the protein is the product of the resistance gene. However, since the Tet(O) protein was never detected in the ribosomal preparation or in the S100 fraction from Tc^R cells (Manavathu et al., 1990), it may be present in a very small amount and probably acts in a catalytic fashion.

1.4f Genetics of *tet*(O) from *Campylobacter*. A Tc^R determinant from *C. jejuni* was cloned and expressed in *Escherichia coli* (Taylor et al., 1987). Nucleotide sequence analysis revealed an 1911-bp ORF [designated as *tet*(O)] which could encode a polypeptide of 72.3 kilodaltons(kDa) (Manavathu et al., 1988). It is very similar to the *tet*(M) gene with 76% identity at both nucleotide and deduced amino acid sequences. In an in vitro transcription/translation system, a polypeptide of about 70 kDa was detected along with many other smaller bands (Taylor et al., 1987; Manavathu et al., 1988). Similar results were also obtained in the studies of the *tet*(M) gene (Martin et al., 1986).

Taylor et al.(1987) demonstrated that the insertion of a transposon in the *tet*(O) ORF abolished Tc^R, however, attempts to remove the flanking region of *tet*(O) were unsuccessful. Two almost identical *tet*(O) genes were cloned and sequenced from *C. coli* (Sougakoff et al., 1987) and *Streptococcus mutans* (LeBlanc et al., 1988). The subcloning studies also placed the resistance determinant in a much larger fragment than the *tet*(O) ORF. Moreover, Hill et al.(1988) found that Tc MIC's decreased when only a 4.2-kb cloned fragment containing the *tet*(M) ORF(ca.1.9-kb) was present compared with the full transposon Tn*919* (16 kb) in *E. coli*, *Bacillus subtilis*, and *S. lactis*. Previous DNA hybridization experiments with the *tet*(O) and *tet*(M) determinants suggested that some DNA sequences flanking the *tet*(O) and *tet*(M) ORF's share a higher degree of homology than the gene coding sequences themselves (Taylor, 1986, Martin et al., 1986). By comparing the sequencing data of the *tet*(O) and *tet*(M) genes (Martin et al., 1986; Manavathu et al., 1988) with the results of Southern hybridization, the homologous sequence was located upstream of the *tet*(O) ORF. These results suggested that the Tet(O) protein specified by the *tet*(O) ORF may not be the only factor required for Tc resistance.

1.4g Homology of Tet(O) and Tet(M) with GTP-binding proteins. The amino acid sequences of Tet(M) and Tet(O) proteins were found to be homologous to several GTP-binding proteins such as elongation factors Tu (EF-Tu) and G (EF-G), especially at the N-terminal regions (Sanchez-

Pescador et al., 1988; Manavathu et al., 1990; Burdett, 1991). X-ray crystallographic studies have revealed that the regions of EF-Tu that show similarity with Tet(O) are involved in the binding of GTP and GDP (Jurnak, 1985). Burdett (1991) also showed that Tet(M) has an associated ribosome-dependent GTPase activity, and suggested that Tet(M) might function as an analog of EF-G based on extent of homology and similarity in size of the two proteins. However, the *tet*(M) gene was not able to replace EF-G or EF-Tu in mutants temperature sensitive for either of these proteins (Burdett, 1991).

1.5 Objectives of this study.

As previously mentioned, molecular studies in *Campylobacter* spp. had been slow due to lack of well characterized systems for in vivo DNA manipulation. Therefore, a major goal of this thesis was to develop a simple and efficient system for gene transfer in campylobacters, as well as the construction of cloning vectors.

In this study, I demonstrated that most *C. coli* and some *C. jejuni* strains are naturally competent during the exponential phase of growth, and that they show strong selectivity for taking up their own DNA. A plasmid transformation system was developed, and a series of shuttle vectors, *Campylobacter* vectors, and integratable vectors were constructed. They were designed to have more selectable markers, multicloning sites from pUC13 or pUC19, and *lacZ'* blue/white color selection for recombinants.

Antibiotic resistance in campylobacters was also studied in this thesis with a focus on both chloramphenicol resistance and tetracycline resistance. Because of the potential use of the Cm^R determinant as a genetic marker in constructing *Campylobacter* vectors, as well as in studies of *Campylobacter* gene expression, I determined its nucleotide sequence. The gene was identified as *cat*, and was found to be a very useful marker because of its small size and its ability to express in both *E. coli* and *C. coli*. The expression of the *cat* gene was also investigated in this thesis.

The mechanism of tetracycline resistance mediated by *tet*(M) and *tet*(O) is still poorly understood (Salyers et al., 1990). Overproduction of the Tet(O) protein using procaryotic expression vectors with the *tac*, λP_L, or T7 promoter failed to yield amounts of Tet(O) protein detectable by Coomassie blue staining despite the presence of the Tc^R phenotype (Manavathu et al., 1990). In this thesis, transcription and translation of *tet*(O) was studied. A DNA sequence upstream of the *tet*(O) ORF was found to be homologous to a sequence at the 5'-end of the *tet*(M) ORF. Both nucleotide sequences from upstream of the *tet*(O) and *tet*(M) ORF's were determined, and their possible function investigated.

CHAPTER II

MATERIALS AND METHODS

2.1 Bacterial Strains, plasmids and phages. Strains of *Campylobacter* used in this study are listed in Table 2-1, and strains of *E. coli* listed in Table 2-2. Plasmids and phages employed are listed in Table 2-3, and the Campylobacter cloning vectors constructed in this study are listed in Table 2-4.

2.2 Growth conditions. Unless otherwise specified, *C. coli* and *C. jejuni* strains were routinely cultured in Mueller-Hinton (MH) broth or on MH agar (Oxoid Ltd., Basingstoke, England) at 37°C with 7% CO_2, and *E. coli* strains were grown in 2 x YT broth (1.4% tryptone, 1% yeast extract, 0.3% NaCl) or on LB agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) at 37°C. When necessary, the medium was supplemented with antibiotics (Sigma Chemical Co., St. Louis, Mo.) listed in Table 2-5.

Table 2-1. *Campylobacter* strains used in this thesis.

Strain	Chromosomal resistance phenotype	Plasmid content (size [kb])	Plasmid phenotype	Source or reference
C. coli				
UA417	Nal^R	-[a]	-	H.Lior (Canada)
UA417R	$Nal^R Str^R$	-	-	This thesis
UA420	-	-	-	M.A.Karmali (Canada)
UA585	Em^R	-	-	C.D.Ribeiro (Wales)
UA724	$Em^R Gm^R Km^R$	pUA724 (~30)	-	R.Gomez-Lus (Spain)
BM2509	Em^R	pIP1433 (47.2)	$Km^R Tc^R$	P.Courvalin
		pIP1445 (4.57)	-	(Trieu-Cuot et al., 1985)
C. jejuni				
UA67[b]	Nal^R	-	-	Taylor et al. (1986)
UA466	-	pUA466 (45)	Tc^R	Taylor (1986)
UA466R	$Nal^R Str^R$	pUA466 (45)	Tc^R	This thesis
UA649	-	pUA649 (40.8)	-	pUA466Δ*tet*(O)
UA650	-	-	-	plasmid-cured UA466 Taylor (1986)
UA697	Em^R	-	-	I.Phillips (U.K.)
C31	-	-	-	J.Miller (1988)

a -, Not detected, or not appliable.

b This strain was formerly called *C. jejuni* SD2.

Table 2-2. *E. coli* strains used in this thesis.

Strain	Genotype	Source or reference (description)
JM107	F' *lacI*^{q}*Z* ΔM15	Yanisch-Perron et al. (1985) (a *recA*$^+$ host for most plasmids and M13 phages)
HB101	*recA13, hsdS20, ara-14, proA2, lacY1, galK2, rpsL20* (*Str*^{R}), *xyl-5, mtl-1, supE44*	Maniatis et al. (1982) (a *recA*$^-$ host for plasmids)
CSR603	*recA1, uvrA6, phr-1*	Sancar et al. (1979) (a maxicell producing strain)
K38	HfrC (λ)	Russel and Model (1984) (a host for T7 promoter vectors)
BW313	*dut, ung, thi-1, relA, spoT1*/F' *lysA*	Kunkel (1985) (a host for producing uracil-containing DNA)
MV1191	(Tc^R)	(a host for producing phagemid ssDNA)
S1192	HfrH, *lacI*^{q}, *relA1, spoT1, ffs::kan-591* [λ*imm*^{434}, *c*$^+$, *nin5*, *Xho*I::Φ(P_{tac}-*ffs*)]	Brown (1987) (a strain in which the gene for 4.5S RNA is regulated by the *lac* operator, and requires inducers of *lac* such as IPTG for growth)

Table 2-3. Plasmids and phages used in this thesis.

Plasmid (size, kb)	Marker	Description (Source or reference)
pUC13 & pUC19 (2.7)	*bla, lacZ'*[a]	Plasmid cloning vectors (Vieira and Messing, 1982)
pUC118 (3.2)	*bla, lacZ'*	pUC18 contains a M13 replication origin (phagemid) (Vieira and Messing, 1987)
M13mp19 (7.3)	*lacZ'*	A M13 phage vector (Yanisch-Perron et al., 1985)
M13K07 (8.7)	*kan*	A helper M13 phage for producing phagemid ssDNA (Vieira and Messing, 1987)
pACYC177 (3.8)	*bla, kan*	A plasmid which is compatible with ColE1 derivatives and has ca.20 copies per cell (Chang and Cohen, 1978)
pACYC184 (4.0)	*cat, tet*	same as pACYC177 (Chang and Cohen, 1978)
pT7-5 (2.4)	*bla*	A T7 promoter vector which contains a T7 RNA polymerase promoter (Φ10), a polylinker, and a fragment from pBR322. (Tabor and Richardson)
pGP1-2 (7.2)	*kan*	A helper plasmid for pT7 series. It contains the T7 RNA polymerase gene and the λ repressor gene in pACYC177 (Tabor and Richardson, 1985)
pUA466 (45)	*tet*(O)	A *C. jejuni* Tc^R plasmid (Taylor, 1986)
pUOA2 (8.0)	*bla, tet*(O)	pUC8 + *tet*(O) (Taylor et al., 1987)
pJI3 (8.8)	*tet*(M)	pACYC177 + *tet*(M) (Burdett et al., 1982)

[a] The ability to complement a defective ß-galactosidase in *E. coli*.

Table 2-3. (continued)

Plasmid (size,kb)	Marker	Description (Source or reference)
pNR9013 (7.1)	*aphA-3,bla,cat*	pUC13 + *aphA-3* and *cat* from *C. coli* (Sagara et al.,1987)
pILL550A (8.6)	*aphA-3*	A shuttle plasmid which replicates in both *E. coli* and *C. coli*. (Labigne-Roussel et al.,1987)
pUB110 (4.4)	*kan*	A staphylococcal plasmid which is widely used as a cloning vector in *B. subtilis*. (Gryczan et al., 1978)
pE194 (3.5)	*ery*	A staphylococcal plasmid which is used as a source of Em^R gene in *Bacillus* and *Staphylococcus*. (Gryczan et al., 1978)

Table 2-4. *Campylobacter* cloning vectors constructed in this thesis.

		Replication origin		
Plasmid(size)	Marker	*E.coli*	*Campylobacter*	*oriT*[a]
pUOA13 (8.7)	*aphA-3,bla,lacZ'* [b]	+	+	+
pUOA14 (8.2)	*aphA-3,bla,cat*	+	+	-
pUOA15 (11.1)	*bla,lacZ',tet*(O)	+	+	+
pUOA17 (8.2)	*aphA-3,lacZ'*	+	+	+
pUOA18 (7.4)	*cat*	+	+	+
pUOA19 (5.0)	*aphA-3*	-	+	-
pUOA20 (4.8)	*cat*	-	+	-
pUOA22 (4.1)	*aphA-3,bla*	+	-	-
pUOA23 (3.8)	*cat,bla*	+	-	-

[a] *oriT* is the origin of transfer from a broad-host-range IncP plasmid (Guiney and Yakobson, 1983).

[b] The markers *bla* and *lacZ'* are not expressed in *Campylobacter*.

Table 2-5. Antibiotics.

Antibiotic	(Abbreviation)	Concentration in media (μg/ml) for *Campylobacter*	for *E. coli*
Ampicillin	(Ap)	-[a]	100
Chloramphenicol	(Cm)	15-25	15-25
Erythromycin	(Em)	32	15
Gentamycin	(Gm)	20	10
Kanamycin	(Km)	40	15
Nalidixic acid	(Nal)	24	24
Streptomycin	(Str)	10	300
Tetracycline	(Tc)	8-15	8-15

[a] -, not used.

2.3 MIC determination. Antibiotic MIC was determined by either broth or plate method. For *E. coli* strains, fresh cells were inoculated into MH broth and incubated for 4 h. Cultures were diluted to 10^4 cells/ml into MH broth containing different concentrations of antibiotic. The lowest concentration of antibiotic which inhibited any visible growth after 18 h incubation was taken as the MIC. For campylobacters, the inoculant was prepared by suspending one loop of overnight plate culture into MH broth to ca.10^7 cells/ml. It was then streaked onto MH agar containing different concentrations of antibiotic. The lowest concentration of antibiotic which inhibited any visible growth after 2 to 3 days incubation in a CO_2 incubator was recorded as the MIC.

2.4 Isolation of plasmid and chromosomal DNA. Plasmids from both campylobacters and *E. coli* were extracted by a modified method of Birnboim and Doly(1979). Briefly, cells (from 1 ml overnight broth culture or a toothpick from a plate culture) were resuspended in 115 µl of solution I (40mM Tris, pH7.9, 2.5mM EDTA, 15% sucrose), mixed with 230 µl of solution II (0.2N NaOH, 1% SDS), and put on ice for 5 min. The sample was mixed with 170 µl of solution III (3M Na^-, 5M acetate), and stored on ice for 5 min. Chromosomal DNA and protein were removed by centrifugation for 5 min at 4°C. The supernatant was mixed with 1 ml of ethanol, and stored on ice for 30 min or longer. DNA was collected by centrifugation for 8 min, and redissolved in 40 to 80 µl of TE buffer containing 20 µg/ml of DNase-free pancreatic RNase (prepared by the method of Maniatis et al., 1982). This DNA sample was used for examination of plasmid content and transformation. For restriction endonuclease digestion or DNA sequencing, one volume of water was added to the DNA sample, and the mixture was extracted with phenol/chloroform once. DNA was precipitated with sodium acetate and ethanol, rinsed with 70% ethanol, vacuum dried, and dissolved in TE. The procedure was sometimes scaled up 20 - 100 times, and the DNA obtained was further purified by cesium chloride-ethidium bromide density gradient centrifugation.

The *Bacillus* plasmid pUB110 was isolated following the procedure described above. The method was originally modified for the isolation of *Bacillus* plasmids. It was found to be suitable for Gram-negative bacteria as well.

The staphylococcal plasmid pE194 was also extracted as described above except that the cells were incubated in a modified solution 1 (40mM Tris, pH7.9, 2.5mM EDTA, 15% sucrose, 8% glucose, 100 µg of lysostaphin/ml) at 37°C for 10 min.

Chromosomal DNA was prepared from one or two agar plates of fresh culture. Cells were suspended in 11 ml of TES (10 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl) without washing, and 1 ml of 10% SDS was added. The suspension was shaken gently until clear (ca. 30 sec.), and then mixed with 3 ml of 5 M NaCl. Protein was removed by phenol/chloroform (20ml) extraction twice. Centrifugation was carried out at 8,000 rpm for 2 min. DNA was precipitated by adding two volumes of cold ethanol, collected with a glass rod, and redissolved in 1.5ml of TE buffer. RNA was removed by adding RNase to 20 µg/ml and incubating at 37°C for 15 min. The solution was extracted with phenol/chloroform once. DNA was precipitated with 0.3M sodium acetate and 2 volumes of ethanol, rinsed with 70% ethanol, and redissolved in 1ml of TE.

2.5 Isolation of phage ssDNA and total RNA. Single-stranded M13 or phagemid DNA was isolated according to Vieira and Messing(1987).

Total cellular RNA was extracted according to Aiba et al.(1982). Cells were harvested from 1 ml of fresh *E. coli* broth culture or a loopful of overnight *Campylobacter* plate culture in 300 µl of RNA buffer (10mM Tris, 100mM NaOAc, 5mM $MgCl_2$, pH5.3). The suspension was placed at 65°C and 30 µl of 10% SDS was added and mixed. Immediately an equal volume of 65°C phenol equilibrated with RNA buffer was added and shaken. The sample was mixed with 300 µl of chloroform and centrifuged. The aqueous phase was extracted with phenol/chloroform 2 or 3 times, then precipitated with 1M ammonium acetate and ethanol. RNA was dried and stored at -20°C, or redissolved in 150 µl of water and used immediately. When necessary, DNA was removed by addition of RNase-free DNase in the RNA buffer. The concentration of RNA was estimated by measuring its absorbance at 260 nm (1 absorbance unit = 40 µg/ml), or by electrophoresis through an 1% agarose gel in 10mM sodium phosphate buffer, pH 7.0, and comparison with known concentrations of M13 ssDNA after staining with ethidium bromide.

2.6 Transformation. *Campylobacter* spp. transformation was performed either on an agar surface or in a biphasic system.

a) Transformation on MH agar: fresh recipient cells (24 h growth on MH agar) were spread on MH agar at about 5 x 10^7 cells per plate and incubated for 6 h. Aliquots of DNA (ca. 0.2 μg in 5 μl MH broth, TE buffer, or ligation buffer) were spotted directly onto the inoculated agar without additional mixing or spreading, and incubation was continued for 5 h. b) Transformation in a biphasic system: cell suspensions (1 - 5 x 10^7 cells / ml MH) were added (0.2 ml / tube) to 10 x 120 mm test tubes containing 1.5 ml MH agar, and incubated for 2 to 6 h. DNA samples were added and incubation was continued for 3 to 5 h. DNase I and $MgCl_2$ were added to a final concentration of 25 μg /ml and 5 mM respectively at various times as required. *E. coli* was transformed by the $CaCl_2$ procedure (Cohen et al., 1972). Transformants were selected on MH or LB agar containing appropriate antibiotics.

2.7 DNA labeling. [^{32}P]-Labeled transforming DNA was prepared in vitro by either nick translation, end-labeling, or the random primer labeling method. Nick translation was performed according to Maniatis et al.(1982), except that only 1/50 amounts of DNase I were used, and DNA was preincubated with DNase I at 15°C for 30 min before DNA polymerase I, [α-^{32}P]dATP, dCTP, dGTP, and dTTP were added. End-labeled DNA was prepared by filling in with DNA polymerase I (Klenow fragment) in the presence of [α-^{32}P]dATP, dCTP, dGTP, and dTTP. Plasmid DNA was linearized by the restriction enzyme *Xba*I for end-filling. The reaction (typically 0.5 μg DNA in 20 μl mixture) was carried out at room temperature for 20 mins. Random primer labeling was performed as described (Feinberg and Vogelstein, 1983) except that, following the labeling, 0.1 mM of all four deoxyribonucleotide triphosphates and 3 units of Klenow fragment of DNA polymerase I were added, and the reaction was continued at room temperature for 30 mins. All the labeled DNA samples were collected by salt and ethanol precipitation with 1 µg of tRNA. The DNA precipitates were redissolved in MH broth and used immediately for DNA uptake experiments.

For DNA or RNA hybridization, the probe DNA was labeled with [α-^{32}P]dATP by a standard random primer labeling method (Feinberg and Vogelstein, 1983), or by primer extension using M13 ssDNA as template.

2.8 Labeled DNA uptake experiments. [^{32}P]-DNA uptake by competent *C. coli* cells was performed using a biphasic system. Typically, the cells were incubated with [^{32}P]-DNA (0.1 µg / ml). At various times, DNase I and $MgCl_2$ were added for 1 min. The cells were centrifuged, washed twice with cold TE buffer, dissolved in a lysing solution (TE, 1% SDS), and then counted in a liquid scintillation counter. Competition for DNA uptake was carried out using a procedure similar to that described by Scocca et al.(1974). Briefly, [^{32}P]-DNA was mixed with various concentration of competing DNA and added to the competent cells. After 15 min incubation at 37°C, DNase I and $MgCl_2$ were added. The cells were washed with cold TE and counted.

2.9 Detection of DNA hydrolysis. The production of DNase by campylobacters was detected as described (Lior and Patel, 1987). Briefly, a loopful of 24 h culture was inoculated onto a toluidine blue-DNA agar plate, which was incubated for 48 h. A clear colorless or pinkish zone around the inoculum was considered a positive reaction.

The results were recorded as follows: ++, a clear colorless zone of at lease 2 mm wide around the inoculum; +, a clear or pinkish zone of 1 - 2 mm; ±, a pinkish zone less than 1 mm; -, no reaction.

2.10 DNA sequencing. Sequencing deletions were generated by digestion with appropriate restriction enzymes and exonuclease III. The template used was either single-stranded M13 DNA (Yanisch-Perron et al., 1985) or double-stranded plasmid DNA (Chen and Seeburg, 1985; Wang, 1989). Synthetic oligonucleotides were used as primers. The nucleotide sequence was determined following the dideoxy method of Sanger et al.(1977) except that modified bacteriophage T7 DNA polymerase (Sequenase) (Tabor and Richardson, 1987) was used. DNA was labeled with [α-^{35}S]dATP and separated in 7% polyacrylamide gels.

2.11 RNA primer extension analysis. Oligonucleotide primers were labeled with [γ-^{32}P]ATP (Maniatis et al., 1982) or [α-^{35}S]dATP. For ^{35}S-labeling, the reaction was carried out as follows: Approximately 3 µg of total RNA was heated with 20 ng of primer in 10 µl of AMV reverse transcriptase buffer (50 mM Tris, pH8.3, 50 mM KCl, 7 mM $MgCl_2$) at 65°C for 2 min, and slowly cooled to 30°C for 20 min. AMV reverse transcriptase(3 units), [α-^{35}S]dATP(30 µCi), dCTP, dGTP, dTTP(0.1 mM each), DTT(5 mM) and buffer were added to the mixture. The labeling reaction was carried out at 42°C for 2 min. The concentration of dATP was then adjusted to 0.1 mM and the reaction was continued for 20 min before adding the sequencing stop solution (20 µl). A similar reaction without the primer was usually performed as control, and a dideoxy-sequencing reaction using the same primer was also performed to generate a marker ladder.

When ^{32}P-labeled primer was used, the same procedure described above was followed except that the labeling step was omitted.

2.12 Hybridization of nucleic acids. Southern transfers (Southern, 1975) were carried out with pure nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.). After a transfer, the filter was irradiated by UV (300 nm), and preincubated with buffer (2 x SSC, 50% formamide, 50 µg/ml salmon sperm ssDNA, 5 x Denhardt's solution) at 42°C for 1 h or longer. ^{32}P-Labeled DNA was added to ca. 5 x 10^6 cpm per ml, and incubated overnight. The filter was washed four times in 2 x SSC, 0.05% SDS at 42°C for 30 min each.

RNA was denatured with glyoxal before separating in a 1.4% agarose gel. Northern blot transfer was performed as described (Maniatis et al., 1982). Hybridization and washings were carried out the same as described above.

Colony hybridizations were performed as described (Maniatis et al., 1982). Hybridization was carried out at 37°C overnight in a solution (5 x SSC, 30% formamide, 25 µg/ml ssDNA) containing ca.1 x 10^6 cpm of probe DNA per ml, followed by four washings as described above.

2.13 Oligonucleotide-directed mutagenesis. Restriction enzyme cutting-sites were generated by site-directed mutagenesis using synthetic oligonucleotides and pUC118 ssDNA essentially as described (Kunkel, 1985; Vieira and Messing, 1982). A restriction fragment of

*Eco*RI and *Eco*RV containing *tet*(O) from pUOA2 was inserted into pUC118 between *Sma*I and *Eco*RI to generate pUOA2A. *E. coli* BW313(pUOA2A) was infected with M13K07, and uracil-containing ssDNA was isolated according to Vieira and Messing (1982). Single-stranded DNA was also prepared from MV1191(pUOA2A). The yield of single-stranded pUOA2A was much lower using BW313 than using MV1191.

The oligonucleotides were phosphorylated (Maniatis et al., 1982), annealed with pUOA2A ssDNA, and the complementary strand was synthesized using *E. coli* DNA polymerase (large fragment) and sealed with T4 DNA ligase. The samples were transformed into JM107, and Tc^R transformants were selected. The desired plasmid mutants were identified by either restriction mapping (when using uracil-containing DNA) or colony hybridization (when using DNA isolated from MV1191).

2.14 Maxicell labeling. *E. coli* host strain CSR603 was used, and the plasmid-coded proteins were labeled with [^{35}S]methionine essentially as described (Sancar et al., 1979). The late-log cells (0.3 ml) were spread onto LB agar and UV-irradiated. The plates were incubated in the dark at 37°C overnight. The cells were washed with sulphur-free M9 medium (Maniatis et al., 1982), incubated in the same medium supplemented with the required amino acids at 37°C with shaking for 2 h, and [^{35}S]methionine added (5 µCi) for 30 min. Labeled proteins were separated in SDS-PAGE gels (Laemmli, 1970).

2.15 Protein labeling with a T7 promoter vector. The *tet*(O) determinant and its derivatives were inserted into pT7-5, and transformed into *E. coli* K38(pGP1-2) (the heat shock for transformation was performed at 30°C for 2 min to prevent the induction of the T7 RNA polymerase gene). Plasmid-coded proteins were labeled with [^{35}S]methionine as described (Tabor and Richardson, 1985). Briefly, cells containing both pGP1-2 and the pT7 recombinant plasmid were grown in 2 x YT at 30°C for 5 h. The cells were washed and resuspended in sulphur-free M9 media supplemented with 20 µg/ml of thiamine and 18 amino acids (minus cysteine and methionine), and incubated at 30°C for 60 min. The temperature was shifted to 42°C for 15 min. Rifampicin was added to a final concentration of 200 µg/ml, and cells were left at 42°C for 10 additional min. The temperature was shifted down to 30°C for 20 min. Samples were then pulsed with 5 µCi of [^{35}S]methionine for 10 min. Labeled proteins were extracted and separated in SDS-PAGE gels.

2.16 In vitro protein labeling with *E. coli* S30 extract. An *E. coli* in vitro transcription/translation kit (Amersham) was employed. Proteins were labeled according to the manufacturer's direction. The control reaction using the DNA provided by the manufacturer was also included.

2.17 In vitro protein labeling with wheat germ extract. The template RNA was prepared by in vitro transcription using T7 RNA polymerase and pT7-UOA2B2 DNA as described (Contreras et al., 1982). The reaction (in 50 µl) contained 10 units of T7 RNA polymerase, 2 µg DNA, 100 µg/ml BSA, 10 mM DTT, 4 mM spermidine, 0.2 mM each of ATP, CTP, and UTP, 0.02 mM GTP, 0.2 mM $m^7G(5')ppp(5')G$, and buffer (40 mM Tris, pH7.5, 6 mM $MgCl_2$, 5 mM NaCl). The sample was incubated at 37°C for 30 min. RNA was precipitated with ammonium acetate and ethanol, and dissolved in water.

Proteins were labeled with $[^{35}S]$methionine using a wheat germ translation system (Promega) as directed by the manufacturer.

2.18 Primer extension inhibition by tetracycline. This was carried out essentially as the primer extension experiment (see 2.11). The oligonucleotide was heated with RNA or ssDNA in appropriate polymerase buffer at 65°C for 2 min. The temperature was shifted to 42°C. Tc was added to the primer-template mixtures to 40 or 100 μg/ml, and the samples were incubated for 20 min. The reaction was continued as described for the primer extension analysis.

CHAPTER III

NATURAL TRANSFORMATION IN *CAMPYLOBACTER* SPECIES

3.1 Isolation of mutants. To obtain more markers for the transformation study, a spontaneous Str^R mutant (designated UA417R) of the Nal^R *C.coli* UA417 strain was isolated by plating fresh UA417 cells onto MH agar containing streptomycin (10 µg/ml). The mutation frequency for Str^R in this strain was less than 10^{-9} per cell. The Str MIC for UA417R was 16 µg/ml. Spontaneous Str^R mutants were never obtained from *C.jejuni* UA466. Therefore, some UA466Str^R mutants were first isolated by transformation using UA417R DNA as the donor. The UA466R ($Nal^R Str^R$) strain was then isolated from one of the $Nal^S Str^R$ transformants by spontaneous mutation. Mutation frequencies for Nal^R in most *C. coli* and *C. jejuni* strains were about 5 x 10^{-9} (Taylor et al.,1985).

3.2 Natural competence of *C.coli* and *C.jejuni*, and transformation of chromosomal DNA. All five *C.coli* strains and three out of six *C.jejuni* strains tested were naturally competent for DNA uptake (Table 3-1). Transformation frequencies of two chromosomal markers (Nal^R and Str^R) were about 1 x 10^{-3} per viable cell in *C.coli* and 1 x 10^{-4} per viable cell in *C.jejuni* with saturating DNA concentrations. *C.coli* UA420 and BM2509 could not be transformed to Nal^R by UA417R DNA. *C.coli* UA585 was found to be highly competent in this preliminary study, and was chosen for most of the transformation experiments.

Table 3-1. Transformation of different strains of *C.coli* and *C. jejuni* with homologous chromosomal DNA.

Recipient	No. of transformants per spot[a] (~ 5 X 10^6 cells) Nal[R]	Str[R]	DNA hydrolysis[b]
C.coli			
UA417	NA	3500	+
UA420	0	3000	+
UA585	6000	4000	±
UA724	1000	1000	±
BM2509	0	4000	±
C.jejuni			
UA67	NA	0	++
UA466	300	1000	+
UA649	200	800	+
UA580	150	600	-
UA697	0	0	-
C31	0	0	±

[a] Transformation was performed on MH agar (see Chapter II: Materials and Methods). The donor DNA (0.2 µg in 5 µl TE buffer) was from *C. coli* UA417R for transformation of *C. coli* strains, or from *C. jejuni* UA466R for transformation of C. *jejuni* strains. The cells within the DNA spot were scraped up and spread on selective media with or without dilution. All experiments were performed at least twice. NA, not applicable.

[b] ++, strong positive; +, weak positive; ±, very weak reaction after 2 to 3 days incubation; -, negative.

Campylobacter strains have been reported to produce DNase (Hebert et al., 1982), therefore, DNA hydrolysis was examined in these *Campylobacter* strains. There was no clear correlation between DNase activity of *Campylobacter* strains themselves and competence, and production of a small amount of extracellular DNase activity did not appear to interfere with chromosomal DNA transformation when the donor DNA was suspended in TE buffer (Table 3-1). Addition of 10 mM $MgCl_2$ to donor DNA did not affect the chromosomal transformation frequency in UA585 which had little DNase activity, but reduced the transformation frequencies in UA417 and UA466 to about 40%, both of which showed some DNase activity.

C.coli UA585 cells could be transformed to Nal^R by *C. jejuni* UA466R DNA at about 20% efficiency as compared to homologous DNA transformation, but these interspecies Nal^R transformants grew more slowly than either parent. *C. jejuni* UA466 could be transformed to Str^R by *C. coli* UA417R DNA at 1% efficiency; however, these transformants exhibited normal growth rates.

Transformation of both Nal^R and Str^R markers was also performed. The transformation frequencies of UA585 were approximately 1.2×10^{-3} transformants per viable cell for the Nal^R marker, and 4×10^{-4} for the Str^R marker, and about 2×10^{-7} for the Nal^RStr^R co-transformants. The results indicated that these two sites were unlinked in this transformation system.

Transformation frequency was increased as donor DNA concentration increased, and the saturation level of transforming DNA was about 1 μg/ml when cells were incubated with DNA for 30 min (Fig.3-1). However, the transformation efficiency obtained from the sample of 0.01 μg DNA/ml concentration (4×10^5 transformants/μg DNA) was much higher than that from 1 μg DNA/ml sample (8×10^3 transformants/μg DNA). Transformation frequency was also increased as incubation time with DNA increased, and no transformants were obtained when DNase I and $MgCl_2$ were added at 0 min (Fig.3-2).

3.3 Influence of growth phase on competence. In most transformable bacteria, competence only appears under certain growth conditions or in a specific growth phase (Smith et al., 1981). To study the development of competence, a biphasic transformation procedure was performed using different growth phases of *C. coli* UA585 cultures as recipients and UA417R DNA as the donor. UA585 cells were found to be competent constitutively throughout their growth cycle (Fig.3-3). A maximum number of transformants was obtained after 6 h incubation, but the transformation frequency (2×10^{-4} transformants per viable cell) was lower than that of 2 h samples (5×10^{-4} transformants per viable cell). This indicated that the early exponential phase bacteria were slightly more competent than the late exponential phase cells.

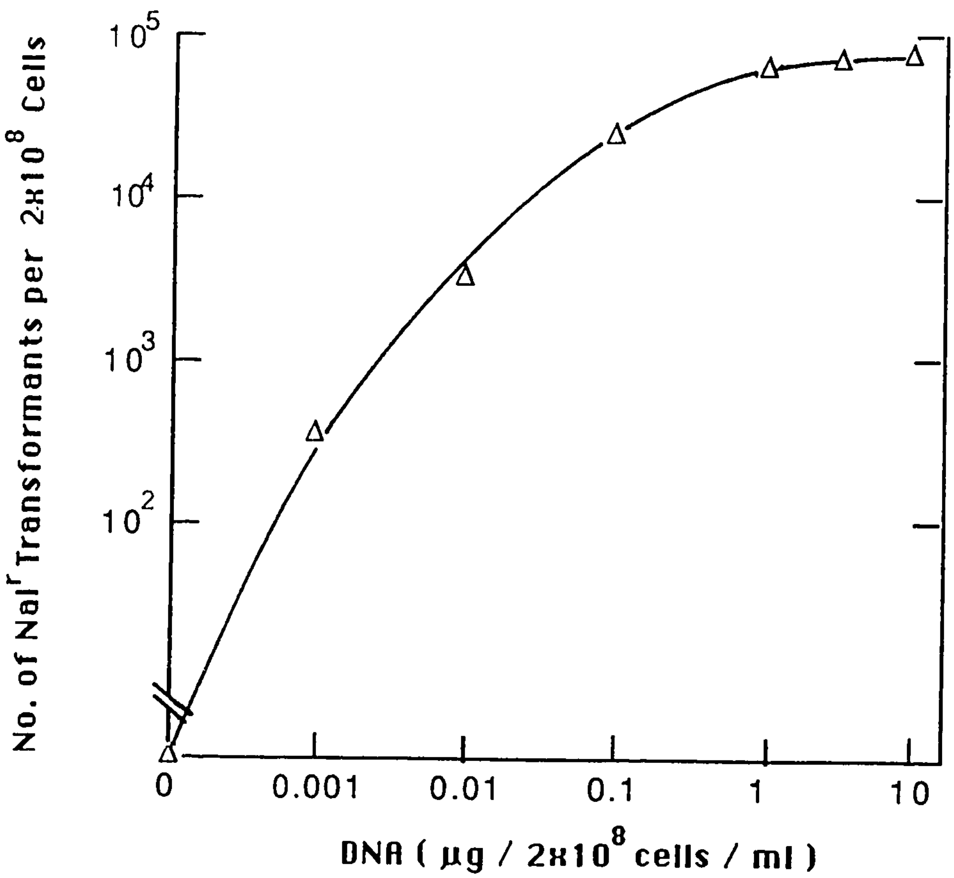


Fig.3-1. Dependence of transformation frequency on the concentration of donor DNA. The recipient was *C. coli* UA585, and the donor was *C. coli* UA417 DNA. Transformation was performed in a biphasic culture system. DNA was added at the indicated concentrations for 30 min before DNase I and $MgCl_2$ were added. Nal^R transformants were selected after 3 h incubation.

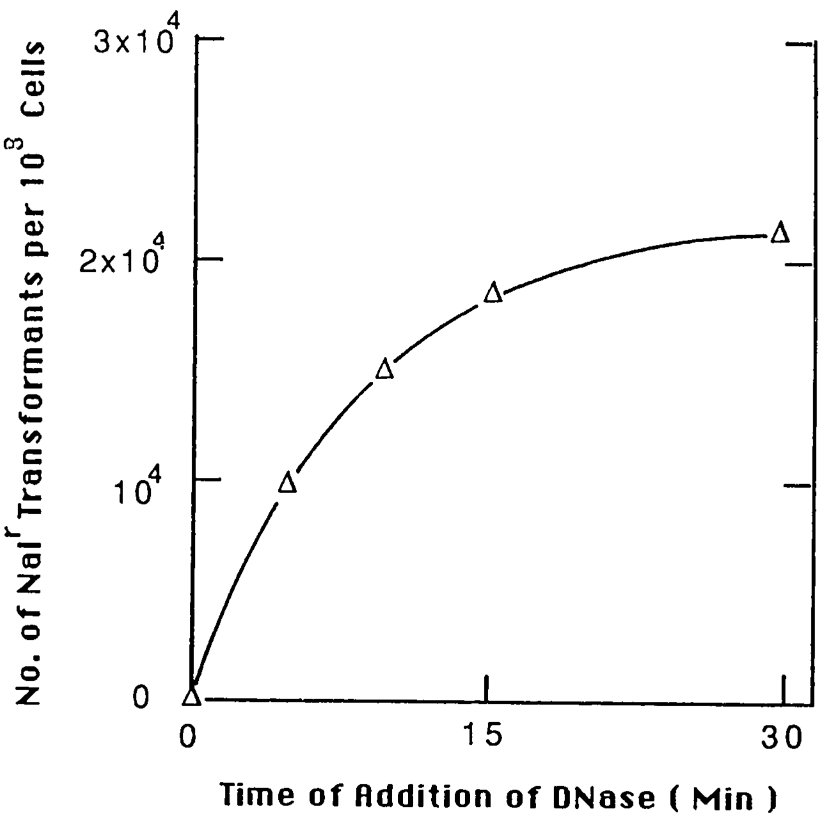


Fig.3-2. Dependence of transformation frequency on the incubation time with donor DNA. The recipient was UA585 (5×10^8/ml), and the donor was UA417 DNA (1 µg/ml). DNase I and $MgCl_2$ were added at the indicated time after DNA was added. Nal^R transformants were selected after 3 h incubation.

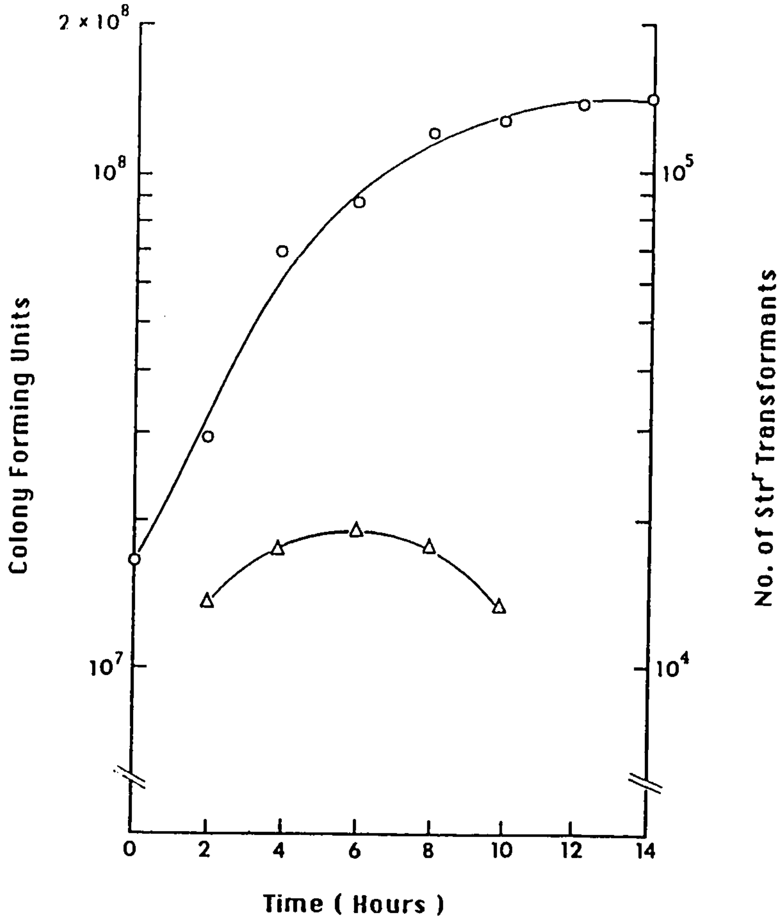


Fig.3-3. Effect of growth phase on competence of *C. coli* UA585. Transformation was performed in the biphasic system. UA417R DNA (in TE buffer) was added to 1 μg/ml at the indicated time intervals, and cell number was counted by plating onto MH agar. Str^R transformants were selected after 4 h incubation. o-o, colony forming units per ml; Δ-Δ, number of transformants per ml.

3.4 ^{32}P-DNA uptake and competition studies. Fig.3-4 shows the kinetics of irreversible uptake of homologous (*C. coli* UA417), heterologous (*E. coli*) or plasmid (pUOA13 isolated from *E. coli*) DNA by *C.coli* UA585 cells. Uptake of *C. coli* chromosomal DNA continued to increase up to 28 mins of incubation, whereas *E. coli* DNA and plasmid pUOA13 DNA were absorbed in barely detectable amounts. Similar results were obtained when DNA labeled by end-filling or the random primer labeling method was used as the donor (data not shown).

When unlabeled *C. coli*, *C. jejuni* and *E. coli* DNA were used to compete with ^{32}P-labeled DNA for uptake, I found that *C. coli* and *C. jejuni* DNA competed for uptake with approximately equal efficiency, whereas *E. coli* DNA did not interfere with the uptake of *C. coli* DNA (Fig.3-5).

Experiments on competition of DNA uptake showed that *C. coli* specifically took up DNA from the same or closely related species, and heterologous DNAs did not compete with homologous DNA for uptake. However, the data also suggested that *C. coli* absorbed more DNA in the presence of competing DNA (Fig.3-5). For example, addition of 5-fold more *E. coli* DNA resulted in an increase of radioactive *C. coli* DNA uptake by 1.3-fold in 15 min, and addition of 5-fold more *C. jejuni* DNA only decreased labeled DNA uptake by 2.5-fold. The result suggested that competence in *C. coli* may be inducible by binding of DNA molecules to the cell surface.

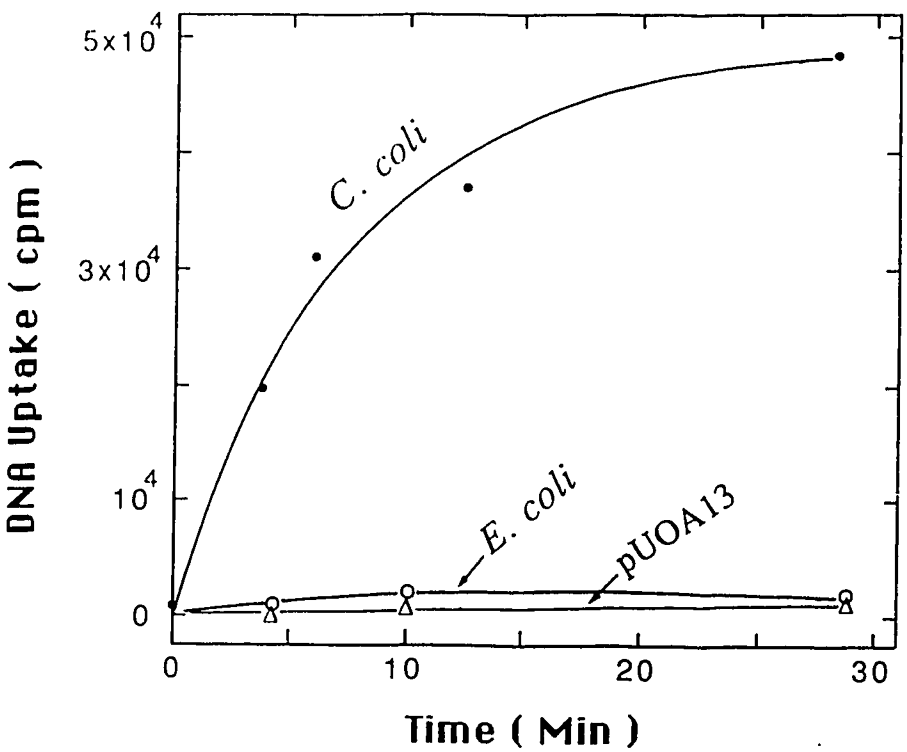


Fig.3-4. Kinetics of DNA uptake by *C. coli* UA585 cells. *C. coli* UA585 cells were incubated with ^{32}P-labeled DNA from *C. coli* UA417 (●-●), *E. coli* JM107 (o-o), or plasmid pUOA13 from *E. coli* (Δ-Δ). The uptake assay is described in Materials and Methods.

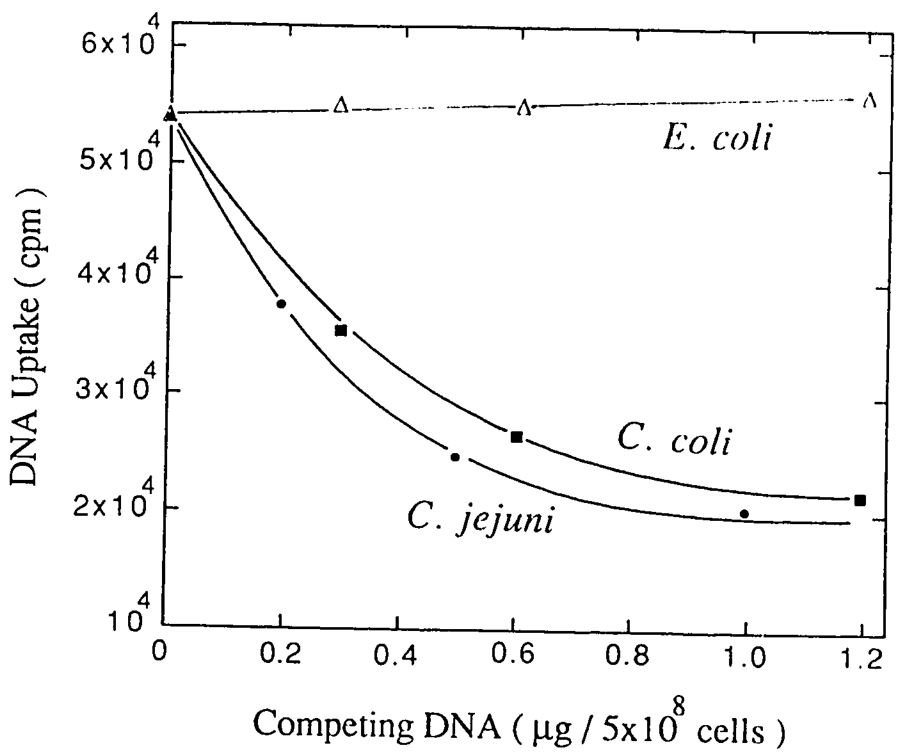


Fig.3-5. Competition by unlabeled DNA in the uptake of radioactive *C. coli* DNA. *C. coli* UA585 cells were incubated with ^{32}P-labeled *C. coli* UA417 DNA (0.2μg/ml) for 15 min. Unlabeled *C. coli* (■-■), *C. jejuni* (●-●) or *E. coli* (Δ-Δ) DNAs were present at the indicated concentrations.

3.5 Competence is stimulated by DNA molecules. A biphasic culture system was used to transform *C. coli* UA585 into Nal^R in the presence or absence of competing DNA. Table 3-2 shows that, when transforming DNA and nontransforming DNA were added at the same time, the transformation frequency of Nal^R UA585 increased 1.5-fold in the presence of 5-fold more *E. coli* DNA, and decreased about 3-fold in the presence of 5-fold more *C. jejuni* DNA. Fig 3-6 shows that addition of 2 µg/ml *E. coli* DNA stimulated the competence level of UA585 for about 90 min.

3.6 Transformation of plasmid DNA. The transformation frequencies of plasmid pUOA17 (8.3 kb) and pIL550A (8.6 kb) into plasmid-free UA585 cells (Table 3-3) were about 1000 times lower than that of chromosomal markers (Table 3-1). Plasmid transformants (shuttle vectors) using *C. coli* UA417 or *C. jejuni* UA649 as recipients were never obtained; probably these two strains have some DNase activity (Table 3-1). When strain UA585 containing a homologous plasmid was used as the recipient, transformation frequencies of the shuttle plasmids were increased by 100-fold, although they were still 10 times lower than that of chromosomal markers (Table 3-1). Transformation of the 45-kb plasmid pUA466 into plasmid-free *C. coli* or *C. jejuni* strains was not successful. However, Tc^R transformants could be obtained by transformation of pUA466 into *C. jejuni* UA649 which contains a 4.2-kb deletion of plasmid pUA466 and in which most of the *tet*(O) determinant has been deleted (Taylor, 1986). In this strain the transformation frequency of Tc^R was close to that of chromosomal markers.

Table 3-2. Transformation of *C. coli* UA585 in the presence of competing DNA.

Donor (0.4μg/ml)	Competing DNA (2μg/ml)	No. of Nal^R transformants per 10^8 cells[a]
C. coli UA417R	0	2×10^4
	E. coli JM107	3×10^4
	C. jejuni UA466	7×10^3

[a] Transformation was performed in a biphasic culture system. Donor DNA was mixed with competing DNA in 40 μl of MH broth, and added to the competent UA585 cells. DNase I and $MgCl_2$ were added after 30 min. Nal^R transformants were selected after 3 h of incubation.

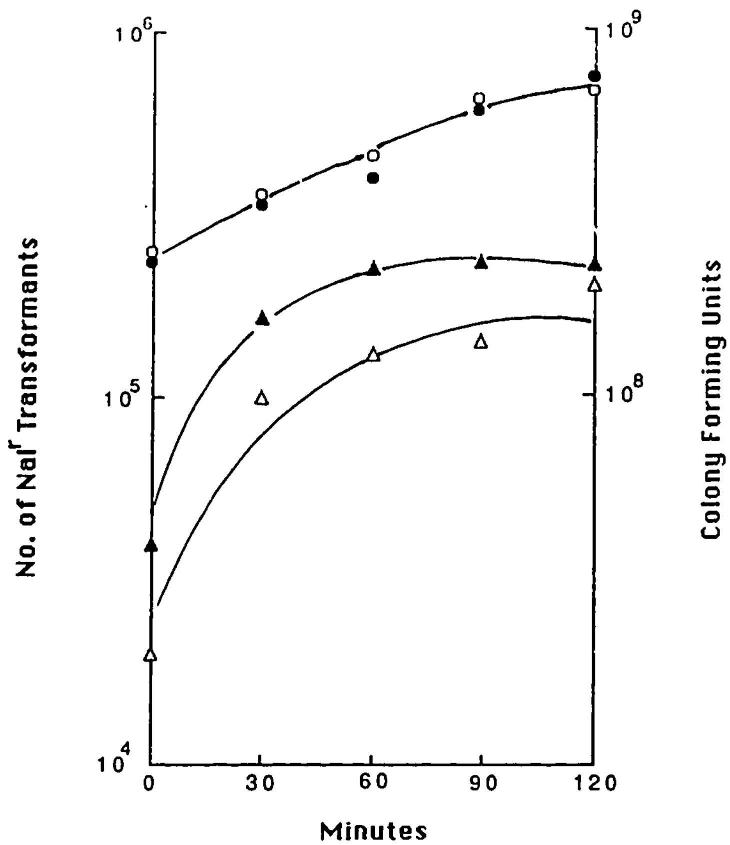


Fig.3-6. Stimulation of competence with *E. coli* DNA. The recipient was *C. coli* UA585, and the donor was UA417 DNA. Transformation was performed in a biphasic system. *E. coli* DNA (2µg/ml) was added to the competent cells 3 h after innoculation. The transforming DNA (0.4µg/ml) was then added at the indicated time intervals. The number of cells was counted by plating onto MH agar. Nal^R transformants were selected after 3 h incubation. o-o, colony forming units per ml without competing DNA, or with competing DNA (●-●); Δ-Δ, number of transformants per ml without competing DNA, or with competing DNA (▲-▲).

Table 3-3. Transformation of plasmid DNA into *C.coli* and *C. jejuni* strains

Donor[a]	Recipient	No. of transformants per spot[b] (marker selected)
	C. coli	
pIL550A(*C.jejuni*)	UA417	0 (Km)
pIL550A(*C.jejuni*)	UA585	8[c] (Km)
pUOA17(*E.coli*)	UA585	3[c] (Km)
pUOA17(*C.coli*)	UA585	6[c] (Km)
pUOA17(*E.coli*)	UA585(pUOA15)	100 (Km)
pUOA17(*C.coli*)	UA585(pUOA15)	640 (Km)
pUOA15(*C.coli*)	UA585(pUOA13)	1200 (Tc)
	C. jejuni	
pIL550A(*C.jejuni*)	UA649(pUA649)	0 (Km)
pUA466 (*C.jejuni*)	UA649(pUA649)	200 (Tc)
pUA466 (*C.jejuni*)	UA650	0 (Tc)

[a] Plasmid DNA was isolated from the bacterial species indicated in parentheses.

[b] Transformation was performed on MH agar. Approximately 5 x 10^6 cells within the DNA spot were scraped up and spread onto MH agar containing antibiotics. Number of transformants were the average number of two experiments.

[c] These transformants were confirmed by isolation of plasmid DNA and electrophoresis through 0.7 % agarose gel.

3.7 Cloning and expression of streptococcal *tet*(M) gene in *C. coli*. The *tet*(M) gene was originally cloned into the *E.coli* vector pACYC177 at the *Hinc*II site (pJI3)(Burdett et al., 1982). The gene has never been identified in *Campylobacter* spp.(Sougakoff et al., 1987). The 5-kb *Hinc*II fragment containing the *tet*(M) determinant is very unstable when cloned in a high copy number *E.coli* plasmid such as the pUC series of vectors (Hill et al., 1988). A stable *tet*(M)-pUC13 clone was obtained by cleaving pJI3 with *Hinc*II, partial digestion with *Bal*31, and insertion into the *Sma*I site of pUC13 (termed pUOA11, 6.9-kb). The plasmid pUOA11 (0.3 μg) was linearized with *Hinc*II and ligated with *Sma*I-cut pIL550A (0.3 μg), and the mixture was used directly to transform UA585(pUOA13) cells on MH agar. Two Tc^R colonies were obtained from some of the DNA-treated cells. Plasmid DNA was isolated from these two transformants, and shown to have the predicted size. The minimal inhibition concentration (MIC) of tetracycline for both clones was 256 μg/ml.

3.8 Transformation of staphylococcal DNA into *C. coli*. Campylobacters have been known to acquire genes such as Km^R determinant (Trieu-Cuot et al., 1985) and Sm^R determinant (Pinto-Alphandary et al., 1980) from Gram-positive cocci. The *tet*(M) gene which is commonly found in Gram-positive cocci was also cloned and expressed in *C. coli*. Therefore, the staphylococcal plasmids pUB110 (Km^R, isolated from *Bacillus subtilis*, Gryczan et al., 1978) and pE194 (Em^R, isolated from *Staphylococcus aureas*, Weisblum et al., 1979) were tested to transform *C. coli* UA585 and UA417. The results showed (Table 3-4) that both plasmids could not transform *C. coli* by themselves; and that the staphylococcal Km^R gene, but not the Em^R gene, was able to be expressed in *C. coli*.

Table 3-4. Transformation of staphylococcal DNA in *C. coli*.

Recipient	Donor[a]	Transformants per 10^8 cells[b] (marker selected)
UA585	pUB110	0 (Km)
	pUB110(*Bam*HI)[c] + UA417[d](*Bgl*II)	3×10^3 (Km)[e]
	pUB110(*Bam*HI) + pUOA20[f](*Bam*HI)	1×10^2 (Km)
		6×10^2 (Cm)
UA417	pUB110	0 (Km)
	pUB110(*Bam*HI) + UA417(*Bgl*II)	2×10^3 (Km)[e]
	pE194	0 (Em)
	pE194(*Pst*I) + UA417(*Pst*I)	0 (Em)
	pE194(*Pst*I) + pUOA20(*Pst*I)	0 (Em)
		0.2×10^2 (Cm)

[a] Donor DNA was in either TE or ligation buffer and was added to ca.1 µg/ml.

[b] Transformation was performed in a biphasic system. Transformants were selected after 4 h incubation.

[c] DNA was digested with the enzyme indicated in parentheses, and ligated.

[d] Chromosomal DNA of UA417.

[e] pUB110 was transformed into *C. coli* probably by integrating into the chromosome.

[f] pUOA20 is a Cm^R campylobacter plasmid (see chapter iv).

CHAPTER IV

CONSTRUCTION OF CLONING VECTORS

4.1 Construction of shuttle vectors. To study plasmid transformation and to improve techniques for molecular cloning in *Campylobacter* spp., some shuttle vectors were constructed using pIL550A as the parent. Plasmid pUOA13 (Fig. 4-1) consisted of the entire pUC13 plasmid (cut at the unique *Aat*II site) and an *Eco*RI-*Sal*I fragment of pIL550A which contains a replication origin of *C.coli* plasmid pIP1445, a Km resistance gene (*aphA-3*), and an origin of transfer (*ori*T) of the promiscuous plasmid RK2 (Labigne-Roussel et al., 1987). This plasmid encodes Ap and Km resistance, and the ability to complement a defective ß-galactosidase in *E.coli*, whereas only Km resistance is expressed in *Campylobacter* spp. Plasmid pUOA15 was derived from pUOA13 by replacing the Km resistance gene with the *tet*(O) gene from pUOA3 (Taylor et al., 1987). The *tet*(O) determinant is expressed in both *E.coli* and *Campylobacter* spp. Plasmid pUOA17 was obtained by deleting the *Cla*I-*Sca*I fragment from pUOA13, thus removing part of the *E.coli* Ap^R gene and one of the two *Eco*RI sites.

Two shuttle vectors which use the *cat* gene as one of the genetic markers were also constructed. Plasmid pUOA14 (Fig. 4-2) was made by inserting a *cat* fragment from pYW70 into pUOA13 between the *Sma*I sites, thus replacing the *lac*Z' gene and conjugative transfer origin(*ori*T). The resulting plasmid contains Ap^R, Cm^R and Km^R genes. All these markers are expressed in *E. coli*, whereas only Cm^R and Km^R are expressed in *C. coli*. Plasmid pUOA18 (Fig. 4-2) was obtained by replacing the Ap^R

and Km^R genes of pUOA13 with the *cat* gene between the *Eco*RV and *Sca*I sites. The plasmid still contains *lacZ'* and *oriT* sequences.

These plasmids have been transfered into *Campylobacter* spp. by natural transformation and electrotransformation (data not shown). All of them, except pUOA14, can be transfered into *Campylobacter* from *E. coli* by conjugation provided that the *E. coli* donor cells contain an IncP helper plasmid (Labigne-Roussel et al., 1987).

4.2 Construction of *Campylobacter* cloning vectors. Two *Campylobacter* cloning vectors were constructed (Fig.4-3). Plasmid pUOA19 was derived from pUOA13 by *Eco*RI and *Pst*I digestion and self-ligation, to delete the pUC13 DNA portion and the origin of transfer sequence (*oriT*). The resulting plasmid, pUOA19, was introduced into competent *C. coli* UA585(pUOA15) cells by natural transformation, and Km^{R}Tc^S transformants were selected. The plasmid was isolated and its structure confirmed by restriction mapping. Plasmid pUOA20 was obtained from pUOA19 by replacing the *aphA-3* gene with the *cat* sequence from pYW70 (see chapter V) between the *Eco*RV and *Sac*I sites. These two plasmids cannot replicate in *E. coli* JM107. These vectors have been used for subcloning *Campylobacter* DNA fragments from *E. coli* into *Campylobacter* spp., as well as in transformation studies and in the construction of shuttle plasmids.

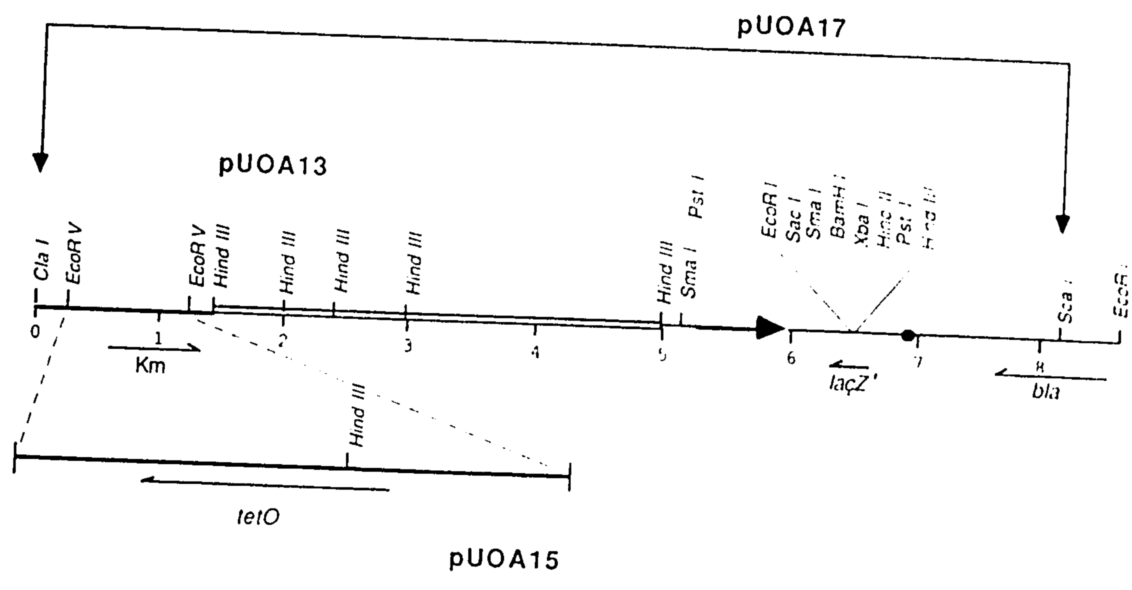
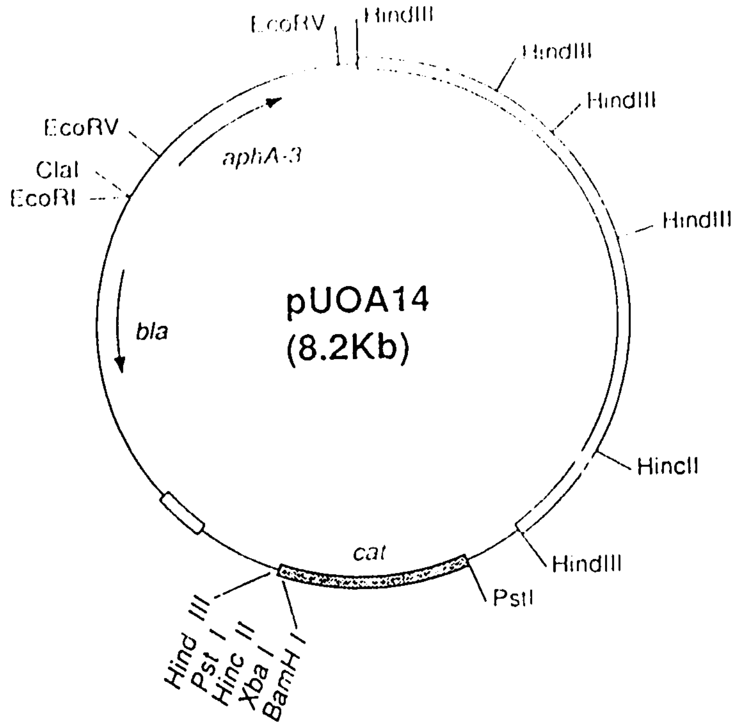


Fig.4-1. Restriction maps of the shuttle vectors pUOA13, pUOA15, and pUOA17. ———, fragment containing *aphA-3* gene or *tet*(O) gene; ═══, DNA sequence from the *Campylobacter* plasmid pIP1455; ⟶, oriT DNA; ____, pUC13 DNA. Numbers represent kilobase pairs.



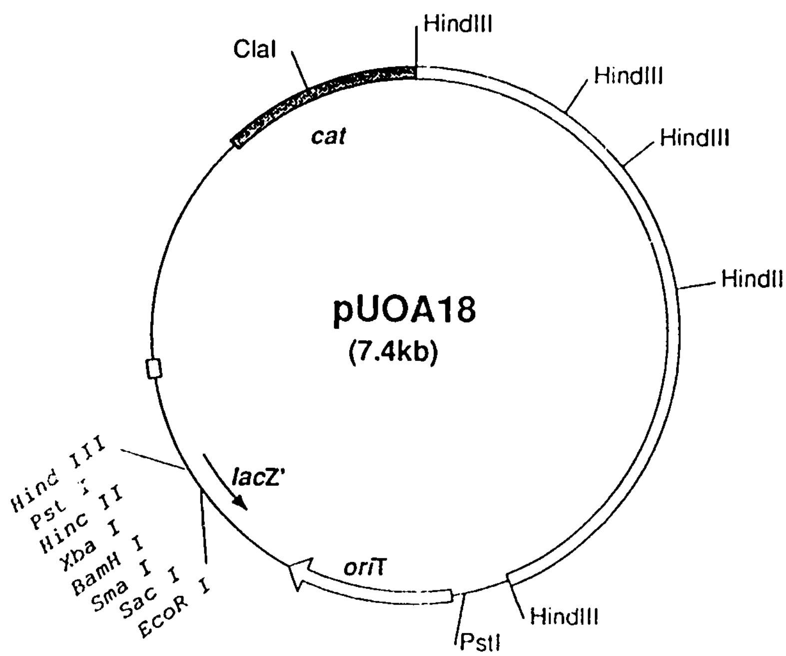
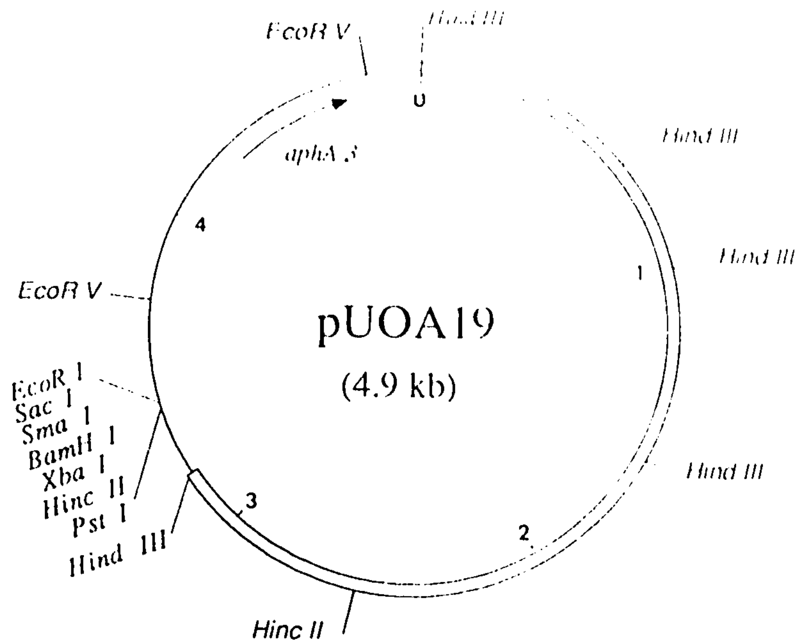


Fig.4-2. Restriction maps of the shuttle vectors pUOA14 and pUOA18. The shaded box is the DNA fragment containing the *cat* gene. The double lines represent the replication origin of *Campylobacter* or *E. coli*.



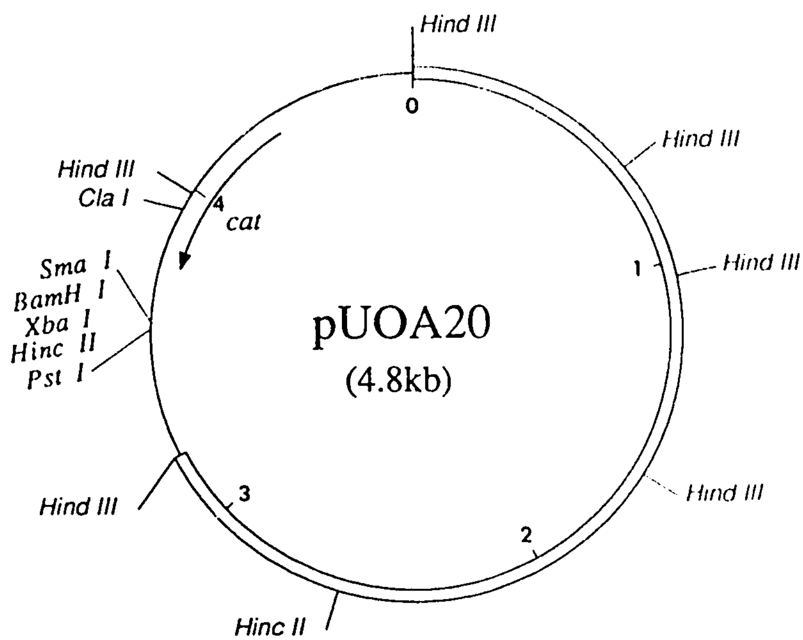


Fig.4-3. Restriction maps of the *Campylobacter* cloning vectors pUOA19 and pUOA20. The double lines are the DNA fragment from *C. coli* cryptic plasmid pIP1455 (Labigne-Roussel et al., 1987), which contains the *Campylobacter* replication origin. Numbers are in kb.

4.3 Suicide vectors. Two suicide vectors were also constructed (Fig.4-4). Plasmid pUOA22 was obtained by inserting a *ClaI-HindIII* fragment containing the *aphA-3* gene from pUOA13 into the *AatII* site of pUC19. Plasmid pUOA23 was constructed by inserting a *BssHII-EcoRI* fragment containing the *cat* gene from pYW70 into the *AatII* site of pUC19. These two plasmids cannot replicate in *Campylobacter* spp., however, they can integrate into the *Campylobacter* chromosome by homologous recombination if they contain a portion of *Campylobacter* chromosomal DNA. These vectors should be useful in cloning, chromosome mapping, and insertion mutagenesis.

To test these possible applications, *C. coli* UA417 DNA was digested by *BglII* or *XbaI*, and inserted into pUOA23 at the *BamHI* and *XbaI* sites respectively. The ligation mixtures were used directly to transform competent UA585 cells. Cm^R transformants were selected at ca. 2×10^3/ml from the *BamHI-BglII* ligation mixture, and 1×10^3/ml from the *XbaI* sample. Chromosomal DNA was isolated from one of the transformants (from the *BamHI-BglII* ligation mixture), cleaved with *BglII*, *PstI*, and *XbaI* respectively, and transformed into *E. coli* JM107 after self-ligation. About 2×10^2/ml Ap^R transformants were obtained from the *PstI* and *XbaI* digestion samples, but not from the *BglII* sample. Plasmid DNAs isolated from these transformants contained a ca. 6-kb insertion (the *PstI* sample) or a 1.6-kb insertion (*XbaI* sample). These DNAs were able to transform Cm^R into *C. coli* UA585. Probably they integrated into the chromosome at homologous sites via Campbell-type insertion.

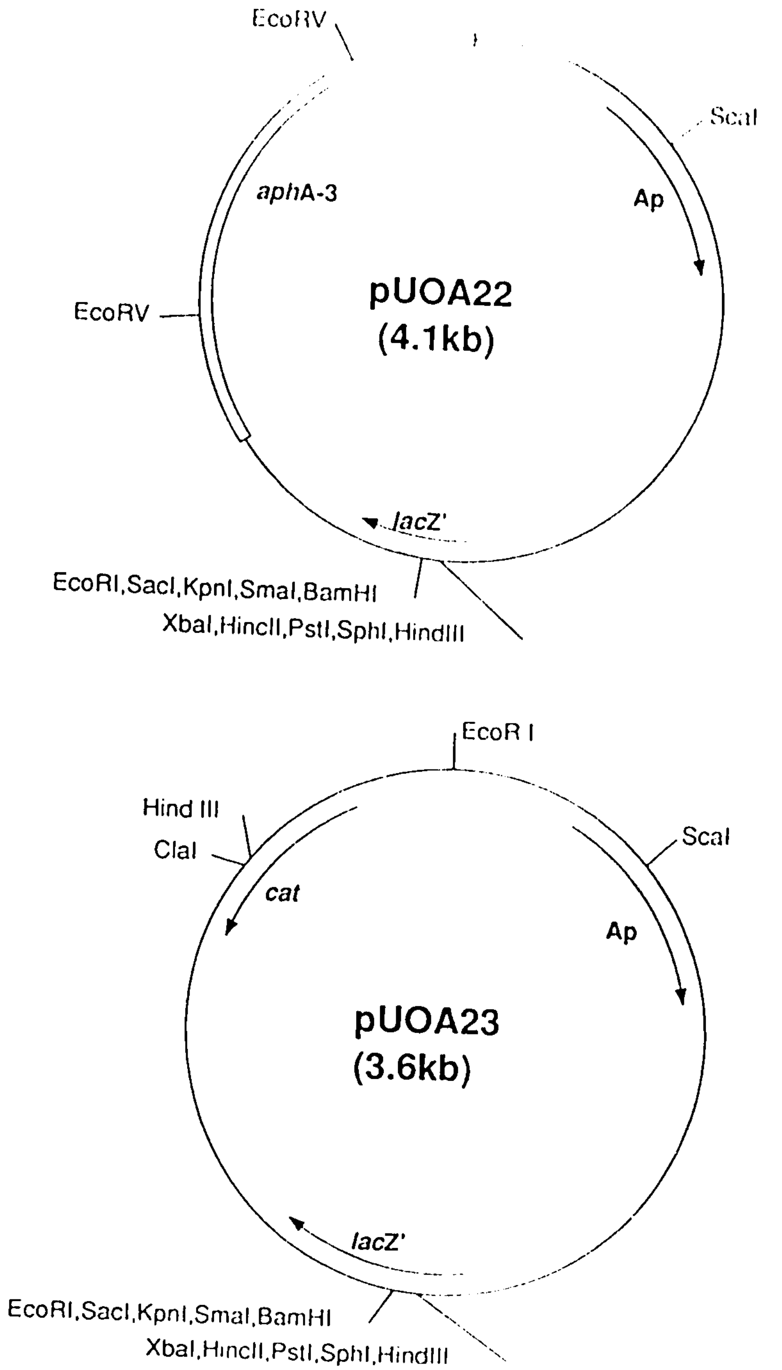


Fig. 4-4. Restriction maps of the suicide vectors pUOA22 and pUOA23.

CHAPTER V

CHLORAMPHENICOL RESISTANCE IN *CAMPYLOBACTER COLI*

5.1 Nucleotide sequence of the *cat* gene. A DNA fragment containing Cm^R and Km^R determinants from a *C. coli* plasmid pNR9589 was cloned previously by Sagara et al.(1987) into the *E. coli* vector pUC13 (named pNR9013). This DNA fragment, kindly provided by Dr. R. Nakaya, was mapped with various restriction enzymes shown in Fig.5-1. Deletion of either *Hin*dIII fragment abolished Cm resistance, which was consistent with previous findings (Sagara et al., 1987). Serial deletions in both directions were generated close to the *Hin*dIII site in the cloned DNA fragment by digestion with appropriate restriction enzymes and ExoIII (Fig.5-1). Sequencing of these deletions revealed a potential SD site, followed by an ATG start codon and an ORF of 621 bp, which could code for a polypeptide of 207 aa with a calculated M_r of 24,294 (Fig.2). After the TAA stop codon, there were two short inverted repeats which may function as the transcriptional terminator (Rosenberg and Court, 1979). Several direct repeats and inverted repeats were also identified upstream of the ORF (Fig.5-2).

This ORF exhibited substantial homology with other *cat* genes. No potential leader sequence coding for a short peptide was identified preceding the structural gene, which suggested that this *cat* gene may be expressed constitutively (Bruckner and Matzura, 1985; Ambulos et al., 1986). The G + C content in the coding sequence is 37.5%, which is slightly higher than that of the *C. coli* genome (32%, Taylor et al., 1983).

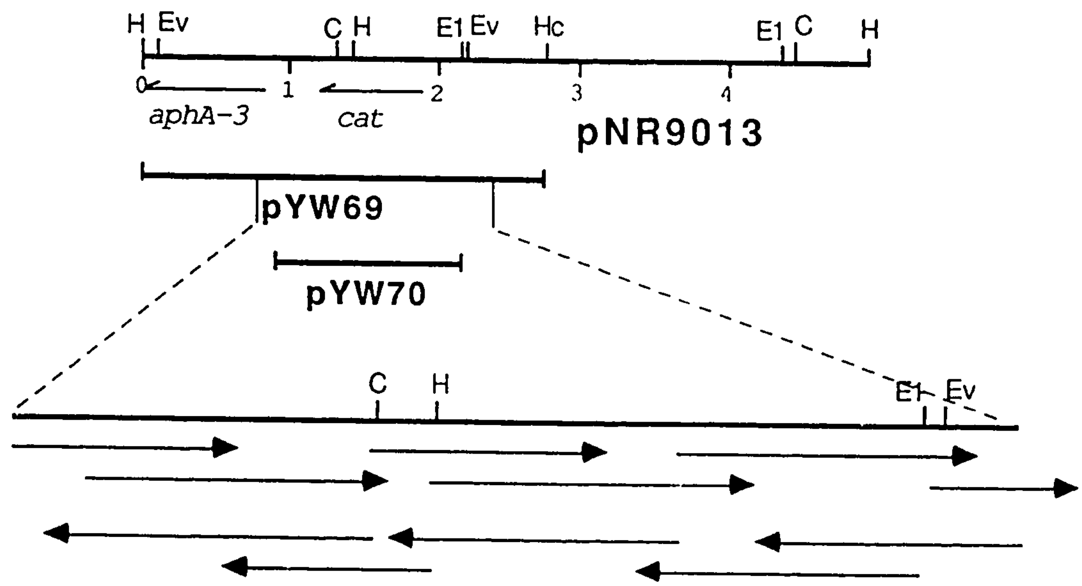


Fig. 5-1. Restriction map of the cloned *C. coli* DNA fragment from pNR9013 and it derivatives pYW69 and pYW70, and the sequencing strategy employed for the determination of the nucleotide sequences of the *cat* gene. pYW69 was obtained by deleting the *Hinc*II fragment from pNR9013. pYW70 was obtained as one of the deletions generated by *Exo*III digestion. The restriction sites are: C, *Cla*I; E1, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hc, *Hinc*II. Numbers are in kb, and arrows specify the extent and direction of sequences.

A Km^R determinant identified as the *aphA-3* gene was located downstream of the *cat* gene. The nucleotide sequence beginning from number 726 (Fig. 2) was found to be identical to that of the *aphA-3* gene from *C. coli* plasmid pIP1433 (Trieu-Cuot et al., 1985), except that an A was missing at the SD site. This mutated SD site may constitute a stronger ribosome binding site (Trieu-Cuot et al., 1985). The *aphA-3* gene in pNR9013 does not have the promoter sequence found in the *aphA-3* gene in pIP1433 (Trieu-Cuot et al., 1985). Probably the transcription of the *aphA-3* gene in pNR9013 is read through the *cat* gene from the *cat* promoter.

A sequence of 11 nucleotides downstream of the *aphA-3* gene in pIP1433 was found to be homologous to two 12-base direct repeats, which are in the middle of the 20-base direct repeats upstream of the *cat* gene (Fig.5-2). Therefore, the 3'-end of the *aphA-3* gene of pNR9013 (the *Eco*RV-*Hin*dIII fragment) was sequenced. The nucleotide sequence of this fragment was found to be identical to that from pIP1433 (Fig. 5-2). The biological function or the evolutionary significance of these direct repeats is unknown. It appeared that the *cat* and/or *aphA-3* gene(s) were not located on a transposon, because pNR9013 DNA could not transform *C. coli* UA585 into Cm^R or Km^R, i.e., the DNA fragment containing *cat* and *aphA-3* could not insert into the *C. coli* chromosome when introduced into *C. coli* cells.

Fig. 5-2. Nucleotide sequence of the DNA fragment containing the *cat* gene from *C. coli* plasmid pNR9589 (GenBank, accession # M35190). Part of the sequence from pIP1433 (Trieu-Cuot et al., 1985) is also presented and compared. Identical nucleotides are indicated by plus. Dashes represent the sequence not shown or not determined. Inverted repeats are depicted by underlined arrows, and four groups of direct repeats are depicted by numbered arrows above letters. The deduced amino acid sequence of the CAT monomer is also presented.

```
                              -308  ATTCCCACAACGCCGGAA ACAAGCCGTG -281

         1                                                      EcoRV
CCACGAGCTTATAATAAAAG AGGGAAGAGAAGCGTATTTT TCCTCACTTCCGGTGAAGGA TATCGAGAAA -211

           EcoRI                                     1
AATCTAAATGATAACGGAAT TCCGTCGTCGGTATCGTATG GAGCGGACAACGAGTAAAAG AGTGACCGCC -141

                                                                 2    3
GAGATAACCCATTGCTCGGC GGTGTTCCTTTCCAAGTTAA TTGCGTGATATAGATTGAAA AGTGGATAGA  -71

          2     3                        start cat RNA
TTTATGATATAGTGGATAGA TTTATGATATAATGAGTTAT CAACAAATCGGAATTTACGG AGGATAAATG -1
   -35                       -10                                SD

ATGCAATTCACAAAGATTGA TATAAATAATTGGACACGAA AAGAGTATTTCGACCACTAT TTTGGCAATA   70
 M  Q  F  T  K  I  D   I  N  N  W  T  R   K  E  Y  F  D  H  Y   F  G  N

CGCCCTGCACATATAGTATG ACGGTAAAACTCGATATTTC TAAGTTGAAAAAGGATGGAA AAAAGTTATA  140
T  P  C  T  Y  S  M   T  V  K  L  D  I  S   K  L  K  K  D  G   K  K  L  Y

CCCAACTCTTTTATATGGAG TTACAACGATCATCAATCGA CATGAAGAGTTCAGGACCGC ATTAGATGAA  210
 P  T  L  L  Y  G   V  T  T  I  I  N  R   H  E  E  F  R  T  A   L  D  E

AACGGACAGGTAGGCGTTTT TTCAGAAATGCTGCCTTGCT ACACAGTTTTTCATAAGGAA ACTGAAACCT  280
 N  G  Q  V  G  V  F   S  E  M  L  P  C   Y  T  V  F  H  K  E   T  E  T

TTTCGAGTATTTGGACTGAG TTTACAGCAGACTATACTGA GTTTCTTCAGAACTATCAAA AGGATATAGA  350
F  S  S  I  W  T  E   F  T  A  D  Y  T  E   F  L  Q  N  Y  Q   K  D  I  D

CGCTTTTGGTGAACGAATGG GAATGTCCGCAAAGCCTAAT CCTCCGGAAAACACTTTCCC TGTTTCTATG  420
  A  F  G  E  R  M   G  M  S  A  K  P  N   P  P  E  N  T  F  P   V  S  M
             HindIII
ATACCGTGGACAAGCTTTGA AGGCTTTAACTTAAATCTAA AAAAAGGATATGACTATCTA CTGCCGATAT  490
 I  P  W  T  S  F  E   G  F  N  L  N  L   K  K  G  Y  D  Y  L   L  P  I
                                                   ClaI
TTACGTTTGGGAAGTATTAT GAGGAGGGCGGAAAATACTA TATTCCCTTATCGATTCAAG TGCATCATGC  560
F  T  F  G  K  Y  Y   E  E  G  G  K  Y  Y   I  P  L  S  I  Q   V  H  H  A

CGTTTGTGACGGCTTTCATG TTTGCCGTTTTTTGGATGAA TTACAAGACTTGCTGAATAA ATAAAATCCC  630
 V  C  D  G  F  H   V  C  R  F  L  D  E   L  Q  D  L  L  N  K   U

AGTTTGTCGCACTGATAAAA ACCCTTTAGGAACTAAAGGG CGCACTTCTATACTCTCTGT CGAGAGTAGT  700

                           BssHII
GCGTCCTGCGGAGCTTCATT CCCGGTCAGCGCGCTTATCA ATATATCTATAGAATGGGCA AAGCATAAAA  770
[pIP1433]   ----GGCA+GG +ATA+G++++++++++++++ ++++++++++++++++++++ ++++++++++

ACTTGCATGGACTAATGCTT GAAACCCAGGACAATAACCT TATAGCTTGTAAATTCTATC ATAATTGTGG  840
++++++++++++++++++++ ++++++++++++++++++++ ++++++++++++++++++++ ++++++++++
                                        4          4
TTTCAAAATCGGCTCCGTCG ATACTATGTTATACGCCAAC TTTGAAAACAACTTTGAAAA ACCTGTTTTC  910
++++++++++++++++++++ ++++++++++++++++++++ ++++++++++++++++++++ ++++++++++


TGGTATTTAAGGTTTTAGAA TGCAAGGAACAGTGAATTGG AGTTCGTCTTGTTATTAATT AGCTTCTTGG  980
++++++++++++++++++++ ++++++++++++++++++++ ++++++++++++++++++++ ++++++++++
                         SD                  start aphA-3          EcoRV
GGTATCTTTAAATACTGTAG AAA-GAGGAAGGAAATAATA AATGGCT------//----- GATATCGGGG
++++++++++++++++++++ +++-++++++++++++++++++++++++++------//----- ++++++++++

AAGAACAGTATGTCGAGCTA TTTTTTGACTTACTGGGGAT CAAGCCTGATTGGGAGAAAA TAAAATATTA
++++++++++++++++++++ ++++++++++++++++++++ ++++++++++++++++++++ ++++++++++
             end of aphA-3    3                     HindIII
TATTTTACTGGATGAATTGT TTTAGTACCTAGATTTAGAT GTCTAAAAAGCTT
++++++++++++++++++++ ++++++++++++++++++++ +++++++++++++
```

5.2 Comparisons of nucleotide and amino acid sequences. The nucleotide sequence in the *C. coli cat* coding region exhibits 57 ± 1% identity with all other known *cat* sequences except the *Clostridium perfringens catP* sequence, which shares 67% identity with the *C. coli cat* gene.

The comparison of the deduced amino acid sequence of the CAT monomer with other CAT sequences also revealed similar results (Fig. 5-3): 44 ± 1% identity with all other CAT amino acid sequences, except the *C. perfringens* CATP sequence (57%). If amino acids of similar chemical structure are considered, the *C. coli* CAT also exhibited greatest homology with the *C. perfringens* CATP (73%). The sequence consisting of IPLSIQVHHAVCDGFH close to the C-terminus (boxed in Fig.5-3) is highly conserved. This conserved sequence is believed to be part of the active center of the enzyme (Shaw, 1983; Leslie et al., 1988).

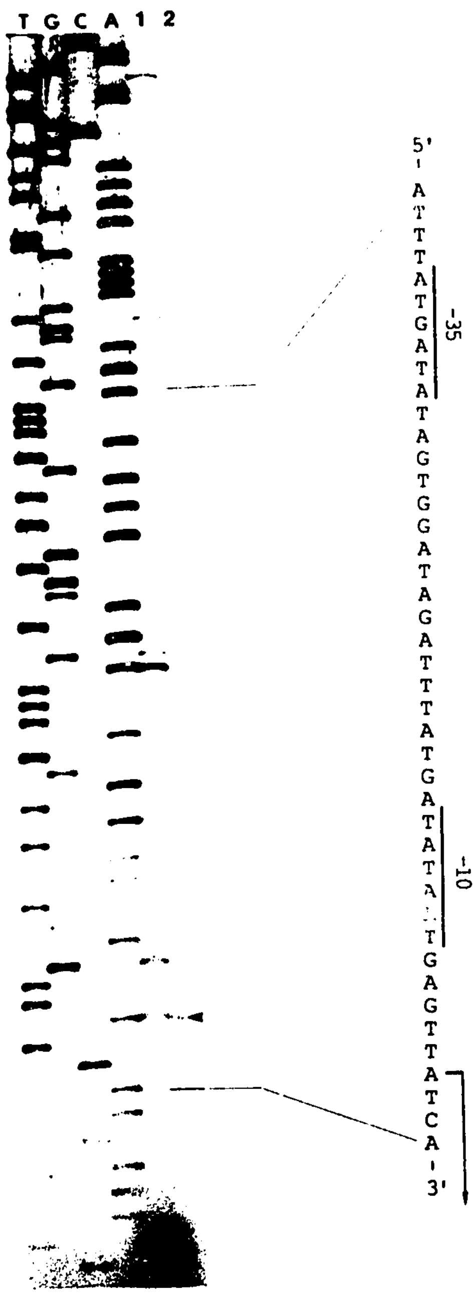
Fig. 5-3. Comparison of amino acid sequences of seven CAT monomers. Data are taken from: *C. coli* (Fig. 5-2), *E. coli* Tn*9* (Alton and Vapnek, 1979), *Proteus mirabilis* (Charles et al., 1985), *Bacillus pumilus cat86* (Harwood et al., 1983), *C. perfringens* (Steffen and Matzura, 1989), *Staphylococcus aureus* pC194 (Horinouchi and Weisblum, 1982), and *S. aureus* pC221 (Shaw et al., 1985). The sequences boxed are highly conserved in all the CAT monomers. Designations: i, identical amino acid; o, identical amino acid in six out of the seven sequences; s, similar amino acids in all CAT monomers.

```
C. coli            MQFTKIDINNWTRKEYFDHYFGNTPCTYSMTVKLDISKLKKDGKK    LYPTLLYGVTTIINRHEEFRTAL
E. coli Tn9      MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFIHILARLMNAHPEFRMAM
P. mirabilis     MDTKRVGILVVDLSQWGRKEHFEAFQSFAQCTFSQTVQLDITSLLKTVKQNGYKFYPTFIYIISLLVNKHAEFRMAM
B. pumilus cat86   M FKQID ENYLRKEHFHHYMTLTRCSYSLVINLDITKLHAILKEKKLKVYPVQIYLLARAVQKIPEFRMDQ
C. perfringens     MVFEKIDKNSWNRKEYFDHYFASVPCTYSMSLKVDITQ     IKEKGMKLYPAMLYYIAMIVNRHSEFRTAI
S. aureus pC194    MNFNKIDLDNWKRKEIFNHYL NQQTTFSITTEIDISVLYRNIKQEGYLFYPAFIFLVTRVINSNTAFRTGY
S. aureus pC221    MTFNIIKLENWDRKEYFEHYF NQQTTYSITKEIDITLFKDMIKKKGYEIYPSLIYAIMEVVNKNKVFRTGI
                     so   o iii i  s      oso    siis     i     ii  s     so   ii

C. coli          DENGQVGVFSEMLPCYTVFHKETETFSSIWTEFTADYTEFLQNYQKDIDAFGERMGMSAKPNPPENTFPVSMIPW
E. coli Tn9       KDGELVIWDS VHPCYTVFHEQTETFSSLWSEYHDDFRQFLHIYSQDVACYGENLAYFPKGFI ENMFFVSANPW
P. mirabilis      KDGELVIWDS VNPGYNIFHEQTETFSSLWSYYHKDINRFLKTYSEDIAQYGDDLAYFPKEFI ENMFFVSANPW
B. pumilus cat86 VND ELGYWEI LHPSYTILNKETKTFSSIWTPFDENFAQFYKSCVADIETFSKSSNLFPKPHMPENMFNISSLPW
C. perfringens   NQDGELGIYDE MIPSYTIFHNDTETFSSLWTECKSDFKSFLADYESDTQRYGNNHRMEGKPNAPENIFNVSMIPW
S. aureus pC194  NSDGDLGYWDK LEPLYTIFDGVSKTFSGIWTPVKNDFKEFYDLYLSDVDKYNGSGKLFPKTPIPENAFSLSIIPW
S. aureus pC221  NSENKLGYWDK LNPLYTVFNKQTEKFTNIWTESDNNFTSFYNNYKNDLLEYKDKEEMFPKKPIPENTIPISMIPW
                   s  o      s i ioso  o oio sis        i  o  i   s      i    ii o si  ii

C. coli          ISFEGFNLNLKKGYDYLLPIFTFGKYYEEGGKYY IPLSIQVHHAVCDGFH VCRFLDELQDLLNK          (207)
E. coli Tn9      VSFTSFDLNVANMDNFFAPVFTMGKYYTQGDKVL MPLAIQVHHAVCDGFH VGRMLNELQQYCDEWQGGA     (219)
P. mirabilis     VSFTSFNLNMANINNFFAPVFTIGKYYTQGDKVL MPLAIQVHHAVCDGFH VGRLLNEIQQYCDEGCK       (217)
B. pumilus cat86 IDFTSFNLNVSTDEAYLLPIFTIGKFKVEEGKII LPVAIQVHHAVCDGYH AGQYVEYLRWLIEHCDEWLNDSLHIT (220)
C. perfringens   STFDGFNLNLQKGYDYLIPIFTMGKIIKKDNKII LPLAIQVHHAVCDGFH ICRFVNELQELIIVTQVCL     (212)
S. aureus pC194  TSFTGFNLNINNNSNYLLPIITAGKFINKGNSIY LPLSLQVHHSVCDGYH AGLFMNSIQDLSDRPNDWLL    (216)
S. aureus pC221  IDFSSFNLNIGNNSNFLLPIITIGKFYSENNKIY IPVALQLHHAVCDGYH ASLFMNEFQDIIHKVDDWI     (215)
                   i  ioiis    s  is i ii     o     sisssioiioiiiisi    s  o
```

5.3 Expression of the *cat* gene in *E. coli* and *C. coli*. To study *cat* gene expression in *C. coli*, a shuttle plasmid (pYW69C) was constructed by inserting the entire pYW69 plasmid into the *Campylobacter* vector pUOA19 (see chapter iv) at the unique *Bam*HI site, and introducing it into *C. coli* UA585(pUOA15) cells by natural transformation. Induction experiments were carried out with *E. coli* JM107(pYW69) and *C. coli* UA585(pYW69C). The growth rates of both strains were not affected by the exposure of cultures to Cm at 25 µg/ml with or without induction (1 µg Cm/ml for 2 h). The spectrophotometric CAT assay (Shaw, 1975) was also performed in *E. coli*. There was no significant difference in CAT activity in both induced and uninduced JM107(pYW69) cells. Therefore, the *Campylobacter cat* gene is expressed constitutively in both *E. coli* and *C. coli*.

Primer extension experiments were performed to analyze the *cat* transcripts in both *E. coli* and *C. coli*. Two oligonucleotides were synthesized: (5'-GCATCATTTATCCTCC) which consisted of nucleotide sequence from +4 to -12 relative to ATG start codon (Fig.5-2), and (5'-CACTTCGAGCTTTAAA) which was from -453 to -468 (sequence data not shown beyond nucleotide.-308). A major extension product from the first primer was obtained when RNA from *C. coli* UA585(pYW69C) was used as template (Fig.5-4), and no apparent bands could be identified from the second primer. It is likely that transcription is initiated at the 32nd nucleotide upstream of the ATG start codon in *C. coli*. The potential -10 and -35 sites with a space of 18 nucleotides in between were identified (Fig.5-4). This region is probably the promoter in *C. coli*. When *E. coli* JM107(pYW69) RNA was used as template, however, several bands were obtained from the first primer (Fig. [illegible]-4) and two bands from the second primer (data not shown). Some of these bands could be artifacts caused either by degradation of the *cat* mRNA or by some unknown secondary structure formation within the RNA molecules. However, the upstream sequence of the *cat* gene contains several regions similar to the canonical *E. coli* promoter sequence (Rosenberg and Court, 1979). It is more likely that the *E. coli* RNA polymerase recognized these sequences as promoters, and the heavy bands obtained are probably true extension products. Similar phenomena have been observed in other antibiotic-resistance genes. Ballester et al.(1980) demonstrated that pC194 *cat* mRNA was synthesized from different promoters in *Streptococcus pneumoniae* and *B. subtilis*. Hill et al. (1988) also showed that DNA fragment upstream of the *tet*(M) ORF contained at least seven regions with promoter activity.

Fig. 5-4. Primer extension analysis of the *cat* transcripts. Several extension products were obtained when *E. coli* JM107(pYW69) RNA was used as template (lane 1). Only one band (indicated by an arrowhead) was identified with the use of *C. coli* UA585(pYW69C) RNA as template (lane 2). (In a preliminary experiment, no bands smaller than 400 bp were detected when the primer was omitted from the reaction mixtures from both RNA samples.) A dideoxy sequencing reaction using the same primer was also performed. The deduced -10, -35, and the possible transcription initiation site are indicated.


T G C A 1 2
5'
A T T T A T G A T A T A G T G G A T A G A T T T A T G A T A T A T G A G T T A T C A
3'
-35
-10

5.4 Maxicell analysis of plasmid proteins. Plasmids pYW69, pYW70 and pUC19 were introduced into an *E. coli* maxicell strain CSR603, and the labeled proteins were examined by SDS-polyacrylamide gel electrophoresis. Fig.5-5 shows two novel proteins of approximately 23.5 and 31.5 kDa produced by pYW69. The former, also present in maxicells containing pYW70, has a molecular size in good agreement with the predicted size (M_r = 24,294) of the CAT polypeptide calculated from the deduced amino acid sequence. The latter was identified as the AphA-3 protein, since its apparent M_r of 31,500 is in good agreement with that of 31,047 predicted from the AphA-3 amino acid sequence (Trieu-Cuot et al., 1985). Since the *C. coli* CAT was synthesized in large amounts in *E. coli* maxicells (Fig. 6), the codon usage bias appears to have little effect in *Campylobacter* spp. gene expression in *E. coli*. The relatively weaker expression of the *aphA-3* gene (Fig. 6) supports the hypothesis that the *aphA-3* mRNA may be transcribed from the *cat* promoter.

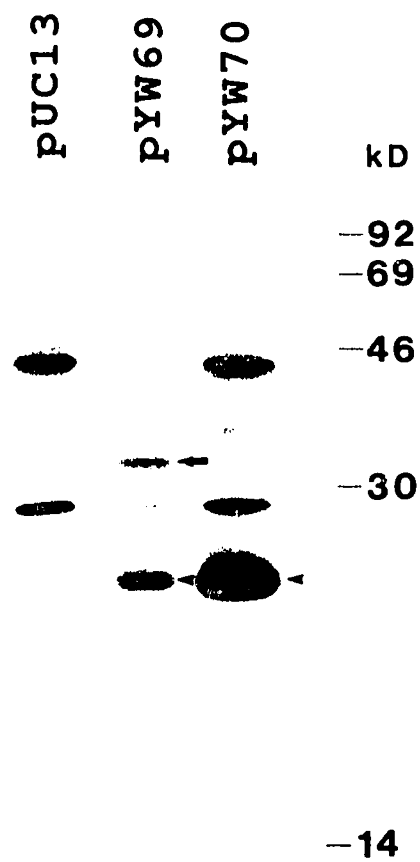


Fig. 5-5. Autoradiogram of a polyacrylamide gel showing [^{35}S]methionine-labeled polypeptides from *E. coli* maxicells containing pUC13, pYW69, or pYW70. The CAT monomer is indicated by arrowheads, and the AphA-3 protein is indicated by an arrow.

CHAPTER VI

TETRACYCLINE RESISTANCE MEDIATED BY *TET*(O) AND *TET*(M)

6.1 Determination of DNA sequences upstream of *tet*(O) and *tet*(M). Plasmid pUOA2 containing the *tet*(O) determinant (Taylor et al., 1987) and pUOA11 containing the *tet*(M) determinant (see chapter iii) were mapped with restriction enzymes as shown in Fig.6-1. For determining the DNA sequence upstream of *tet*(O), a series of deletions was generated by exonuclease III digestion from the unique *Eco*RV site and from the *Hin*cII site at the 5'-end of the *tet*(O) ORF. A DNA fragment from pUOA2 was also subcloned into M13mp19 and pUC118. Several oligonucleotides were synthesized and used as primers to complete the sequencing. More than 1.5-kb of nucleotide sequence upstream of the *tet*(O) ORF was determined (Fig.6-2). This sequence is relatively AT rich (74%), compared with the *tet*(O) ORF (60%) and *C. jejuni* chromosomal DNA (ca. 68%). No potential ORF starting from ATG and encoding more than 20 amino acids was found in either direction from this sequence. There is a possible ORF starting from an GTG codon at nucleotide -1497. However, no Shine-Dalgarno sequence precedes this possible ORF, and deletion of this sequence did not affect the Tc resistance level.

A sequence of approximately 500 nucleotides upstream of the *tet*(M) ORF was also determined (Fig.6-2) using the synthetic oligonucleotides as primers, and it was found to be highly homologous to the DNA sequence upstream of *tet*(O). The sequences from -131 to -174 and from -199 to -263 relative to the *tet*(M) ATG start codon were almost identical to the DNA sequence at the same position in *tet*(O).

It is notable that these two homologous DNA fragments upstream of the *tet*(O) and *tet*(M) ORF's have exactly the same number of nucleotides (266-bp) from the ATG start codon of both genes (boxed sequences in Fig.6-2).

Forty-one nucleotides upstream of *tet*(O) there are two inverted repeats followed by five T residues, which is similar to the classical transcriptional terminator (Rosenberg and Court, 1979). Upstream of *tet*(M) at the same position, a set of inverted repeats of 22 nucleotides and a string of T's are present (Fig.6-2), which may also function as a transcriptional terminator.

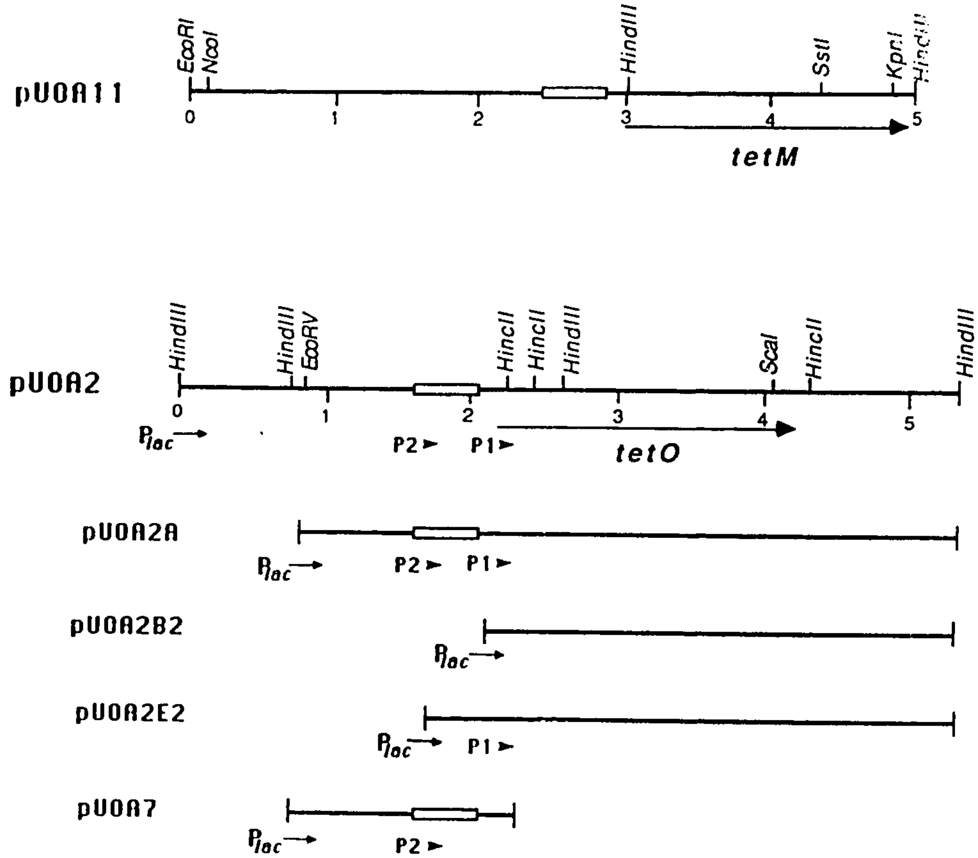


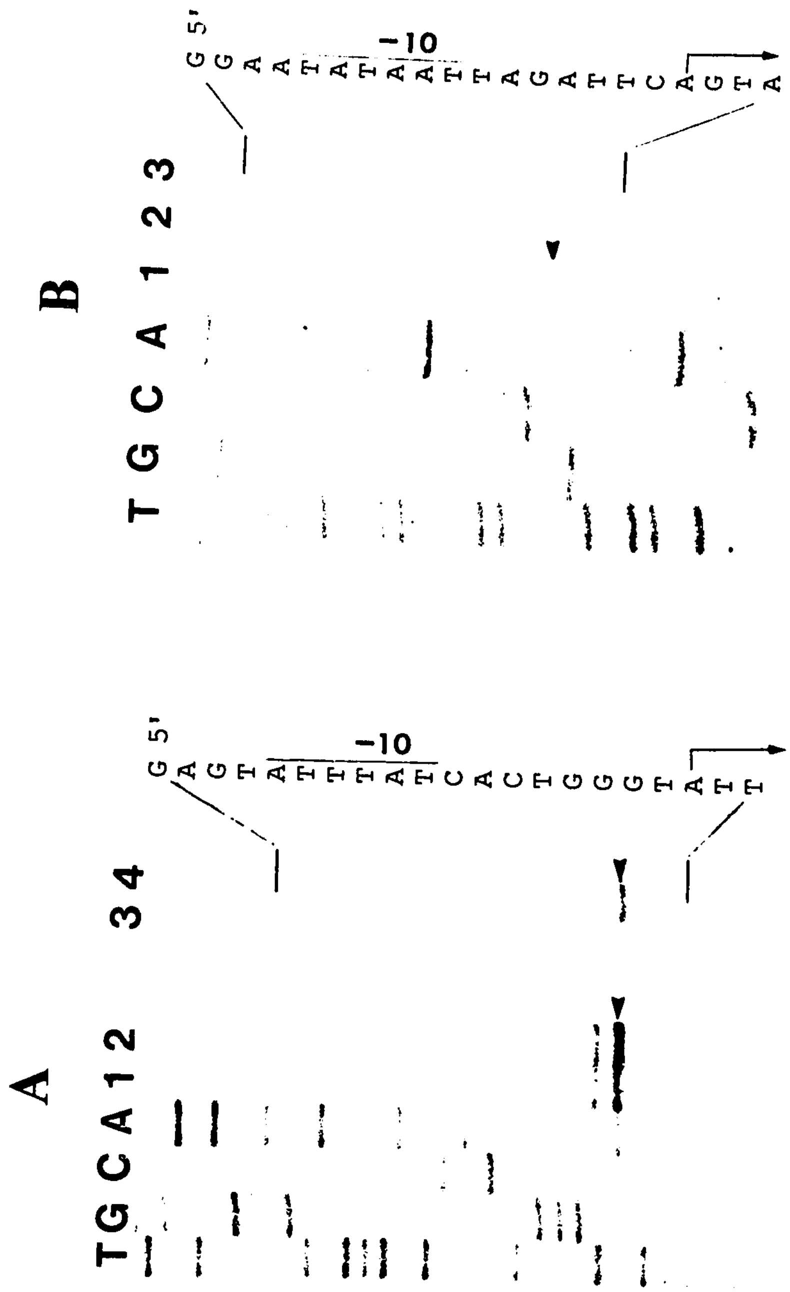
Fig.6-1. Restriction maps of DNA fragments from plasmids pUOA11, and pUOA2 and its derivatives. The highly conserved regions are boxed. Promoters for *tet*(O) and their direction are indicated. Numbers are kilobase pairs.

Fig.6-2. Nucleotide sequences of the DNA fragments upstream of *tet*(O) from pUOA2 and *tet*(M) from pUOA11. Homologous nucleotides are indicated by plus; gaps are introduced for the missing nucleotides. Inverted repeats are depicted by underlined arrows. Transcriptional initiation sites were depicted by arrows above the letters. The sites where primers (mer2, 3, 4) attached are underlined. The possible promoters (-10 and -35 sites) are indicated. Restriction sites generated by site-directed mutagenesis are also underlined and indicated.

```
                                                   pUOA2 (tetO)   -1594 GACGAGC -1588

TAGGACAAAAAAGAGAAAAA AGGATAAATTATTATGTAAG AGACCTTGGAAAAAGCCTAT GTATGCAGCCTTGATAAGAT -1508

AAATGAAATCGTGCGGCAAA CAAGGTATTACAAAAGCATA AAATTTGCAGGAGATTTTCT AGAAAGCTATGATGCTAAAG -1428

                                                         HindIII
AATATGTCAAAGCCATAGGC AACGCCCTTAAGCAGGATAA ATTTGCCATATATGAAAGC TTTTAAGAATAGATTTTAAT -1348

GTTTTAATCAATTGTCATAG CGTTCAAGAAGTTATAGAAA AATCATTAAATACTAAAATT AATTTCAATCTAAATAAATT -1268

 EcoRV
TGATATCCACTTGGCTTTAT CTTTTGCAATTTCTTTAAAT TTTATTGCAAAAAACGAACA GAATAAATTGTATAAATTTG -1188

TTTTGGAGAATAACAAATTA ATATATGATTATATTGATTT TATTAATAATAATTTTGCCA ACGAGCATTTTATTAAAATA -1108

                                       mer4
AAATACAAAAGAAAAAAATA CAAAATTATTAATATTGCCT CATTTTTATTGTATCACAAA TTAAAACCACAGAAAGAAAG -1028

CTATCAAAATGAATTTCTTG AAATTTATATACTAATAAAT GATTATATTAAATTAAGCTA TGAAACAAATAATTTAATTA -948

ACCTTAACATTAATTCTATA AATAGAATAACAAACGAACA TAATGTTTTAACGATAGAAT TAGAAAAAAAACAAATTCCA -868

AAAAATAAAAAACTTAAAAT TAAAGAAGATTTTATCAATT TAAAACTTCCAGAAGAATTT AAACTAATAGAAACGCATAA -788

AGAACTTTACTTGCACGGAA TGGAGCAAAAAAATTGCGTT TATACAAGAAGAAGAGAAAT TGAAGACGGCTTAAGTGCCA -708

TTTATAGTTTAAATTACGAA GGAGGAGTTTATACATTGGA AATTTTCAAAAGAAAAAATA AATTTGCAATTAAGGAAATC -628

AAAGCAAAATATAATGAATT TGCAAATAAAGAAGTTATAA ATTTTGTTGAAAAAAGCCTG AAAGCTGTTTAAAAAATACA -548

                                                                       mer3
AGGAGTTAAAATGAATACAA ACAAAATTACAGATTTACAC ATACAAATTTCAAAGGAAGA CTTGAAAATTTTAGATTTAC -468
 pUOA11 (tetM)          +T+C+++ATCG++AGG +++ATC+AG++ +C++T+TC GC++ +C+G+GG++TA+AT+ -462

TACTTAAAGCCAATGAC    TCTAATAT TGTTTTTTATT GCGAAAACGATGAAGAACAT TTTATTTTTACAGAAGTAAA -389
G++CAGC++A+C+AAGC++C G+AGG+++C+CAA+++G+++ TG+T+CTT++ A++++++GG GAGTAA++GGA+++TTGT++ -383

ACTTGGAAATTATTTTTGCA TAAACAGATGATTAGTGGCA GGGGGAAATCCTG CCGCTT TTTCTGCTTTAGTTTGTCA  -311
+A+AAC+++ ++++GG+          ++  +++++++CA+AT+ CTTT+T++++A++TA+T+++ +++GATAAAA+A++G+AG+T -311

  (-35)      P2(E.coli)      (-10)                                               GAATTC (EcoRI)
 GCTTGACAAATAAAGGGTT AAGGAATATAATTAGATTCA GTATTATACAAGGAGTTAAT AAATATGCGGCAAGGTATTC -232
TG+++T+++++++TGCTC++ +C+TGC+++T+++TA+G+G+ C++++ ++A+++++++++++ +++++++++++++++++++ -232

                                                                            mer2
TTAAATAAACTGTCAATTTG ATAGTGGGAACAAA AAGTA GCAGTCCGTTTCACTTTTAA TATGGGGCTTAGTTTTTTGT -153
++++++++++++++++++++ ++++C+++++++++T++T++ +AT++++ ++      +++++G G+  +++++++++++++++ -159

ACCCAGTTTAAGAATACTTT TATCATGTAATTTTATA T  GCCCGAAAACATATAAGTGT TTTG  GGGCTATTG GAGT  -78
++++++++++++++++C++ ++++++++G+++C++A+G+A T++A++G++T++C+GTA++C ++++TATAC++++G+TT+TG  -79

-35          P1          -10                               GGAT CC (BamHI)       GATATC (EcoRV)
TATTTA   CCCAGTGATAG GAGTATTTATCACTGGGTAT TTTTATGCCCTTTTTTGGGT GTTGATAGGAAGAAAATCAC   -1
C++AA+AAT++++++++++A A++++++++++++++++ ++ ++++++++++ ++++G++T+ T++++AT+++G++++++++   -1
                                                                             SD
ATGAAA    +6     (start tetO ORF)
++++++    +6     (start tetM ORF)
```

6.2 Analysis of *tet*(O) transcripts in both *E. coli* and *C. coli*. The transcripts initiated from upstream of the *tet*(O) ORF were analyzed by the technique of primer extension. Two synthetic primers (mer1 and 2) were first employed: mer1(5'CGTCAACGTGAGCCAG) is complementary to a sequence in the *tet*(O) ORF from +25 to +40 (Manavathu et al., 1988); mer2 is complementary to a sequence indicated in Fig.6-2 (191 bases upstream of the mer1). One major extension product at -42 was obtained with the use of mer1 and RNA isolated from both *E. coli* JM107(pUOA2) and *C. coli* UA585(pUOA15) (Fig.6-3A). No bands were identified upstream of the two inverted repeats, which supported the hypothesis that this region of dyad symmetry is a transcriptional terminator. The -42 site is likely to be the start point of the *tet*(O) RNA, because most of the cDNA synthesis was able to pass the inverted repeats structure when the RNA transcribed from T7 polymerase was used as a template (see section 6.5, Fig.6-5). Another extension product was obtained from *E. coli* RNA using mer2, but not from *C. coli* RNA (Fig.6-3B). Therefore, promoters (-10 and -35 sites) for *E. coli* and *C. coli* were assigned (designated as P1 and P2, Fig.6-2 and 6-3) according to Hawley and McClure (1983). The P1 is apparently an extremely weak promoter, for it has only 5/12 (42%) nucleotides identities to the canonical *E. coli* promoter sequence. These data indicated that the transcription of the *tet*(O) mRNA is highly restricted by using a transcriptional terminator and a very weak promoter.

Fig.6-3. Primer extension analysis of transcripts of the *tet*(O) resistance determinant. Extension products are indicated by arrowheads. The template sequences are depicted, which were generated by dideoxy sequencing method with sequenase using the same primer. The predicted -10 sites are indicated. A) primer extension from mer1. The RNA template used was either from *E. coli* (lane 1 and 2) or from *C. coli* (lane 3 and 4). B) primer extension from mer2. lane 1, the template used was from *E. coli;* lane 2, same as lane 1, except that the primer was omitted from the reaction; lane 3, the template was from *C. coli*.


A
TGCA12
34
B
TGCA123
-10
-10

The possibility that RNA molecules transcribed from upstream of *tet*(O) ORF are involved in tetracycline resistance was further investigated by Northern blot analysis. Using mer2 as the probe, three bands (approximately 530, 2,000, and 2,700 nucleotides respectively) were detected from *C. coli* UA585(pUOA15) RNA, of which the 530 nucleotides RNA species was just barely detectable (Fig.6-4). A small RNA molecule was readily identified from *E. coli* JM107(pUOA2) RNA, and some faint bands of sizes from 1.8 to 3.5 kb were also detected. This small RNA is probably the product of the transcription initiated at P2 and terminated at the two inverted repeats.

To locate other possible transcriptional initiation site(s) upstream of *tet*(O), two more primers were synthesized (mer3 and 4), which are complementary to the sequences indicated in Fig.6-2. However, no extension products were detected using these two primers.

6.3 Construction of deletion derivatives of pUOA2, and determination of tetracycline MIC. Plasmid pUOA2 was linearized at the *Eco*RV site, digested with exonuclease III for 3 to 10 min at 37°C. After S1 nuclease treatment, the DNA was self-ligated and transformed into JM107 competent cells. Twenty Tc^R transformants were isolated, and plasmid sizes were examined. Four smaller plasmids were sequenced, and the smallest one was found to be deleted to the -334 site (25 nucleotides upstream of the P2's -35 site). Deletion experiments using *Bal* 31 failed to isolate any smaller plasmids when recombinants were selected on Tc containing media.

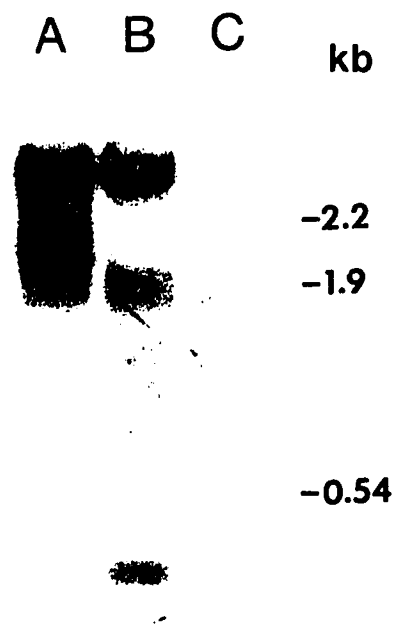


Fig.6-4. RNA transcripts from upstream of the *tet*(O) ORF. The probe was the ^{32}P-labeled mer2. Lanes: A, RNA from *C. coli* UA585(pUOA15); B, RNA from *E. coli* JM107(pUOA2); C, RNA from JM107(pUC8).

Because of the failure to isolate deletions missing the region upstream of the *tet*(O) ORF using exonuclease III and *Bal* 31, site directed mutagenesis was employed. Plasmid pUOA2 was cut by *Pst*I (at the cloning site of the vector) and *Eco*RV, and the 4.4-kb DNA fragment containing the whole Tc^R determinant was inserted into pUC118 between the *Pst*I and *Sma*I sites to produce pUOA2A (Fig.6-1). Two restriction enzyme-cutting sites (*Bam*HI and *Eco*RI) were generated upstream of the *tet*(O) ORF (see Fig.6-2) using the single stranded pUOA2A DNA, and the resulting plasmids were designated pUOA2B1 and pUOA2E1 respectively. The deletion plasmid generated at the *Bam*HI site was named pUOA2B2, and that deleted at *Eco*RI site, pUOA2E2 (Fig.6-1). Thus, the *tet*(O) ORF in pUOA2E2 is under the control of P1 and *lacZ* promoters(P_{lac}), whereas in pUOA2B2 it is controlled by the *lacZ* promoter only. The 4.4-kb DNA fragment from pUOA2 was also cloned in between the *Hinc*II sites of pACYC184, which has a much lower copy number than that of the pUC plasmid vectors. This plasmid was named pACYC-UOA2A. The 3.4-kb *Eco*RI-*Pst*I (at the cloning site of the vector) fragment from pUOA2E1 was cloned into pAC [illegible] between the *Hinc*II and *Pst*I sites, and it was called pACYC-UOA2E2.

The Tc MIC of *E. coli* JM107 containing these different recombinant plasmids were determined (Table 6-1). The results demonstrated that the removal of some of the sequence upstream of the *tet*(O) ORF greatly reduced the resistance level of Tc. For example, the MIC was 80 µg/ml for JM107(pUOA2E1) compared to 14 (-IPTG) or 16 (+IPTG) for JM107(pUOA2E2); and the MIC was 50 µg/ml for JM107(pACYC-UOA2A) compared to 6 for JM107(pACYC-UOA2E2). The decrease in Tc MIC did not appear to be due to the removal of the P2 promoter, since most transcripts from P2 may not pass the two inverted repeat sequences and therefore are unable to reach the *tet*(O) ORF. The finding that the Tc MIC's for JM107(pUOA2E2) were almost identical both in the presence or absence of IPTG indicated that the putative transcriptional terminator is very effective, which prevents *tet*(O) transcription from other upstream promoters.

The 3.4-kb *Eco*RI-*Pst*I fragment of pUOA2E2 was inserted into the campylobacter vector pUOA19, and introduced into *C. coli* UA585 by natural transformation. Tc^R transformants were obtained on Mueller-Hinton agar with 12 µg Tc per ml; whereas pUOA2E2 and pACYC-UOA2E2 could not transform *E. coli* to Tc^R if selected on LB with 12 µg Tc per ml, suggesting that the P1 is a campylobacter promoter, and the expression from P1 in *E. coli* is minimal. However, these Tc^R *C. coli* transformants were extremely unstable. Therefore, the Tc MIC specified by this deletion was not tested in *C. coli*. Furthermore, attempts to clone the deletion fragment from pUOA2B2 under the control of campylobacter promoters (from kanamycin or chloramphenicol resistance genes) failed. The recombinant DNAs appeared to be lethal to the *C. coli* host.

Table 6-1. Tetracycline resistance of plasmid variants and complementation tests.

Host	Plasmid [description]	MIC[a] (μg/ml)
JM107	pUC8 [vector]	2
($recA^+$)	pUOA2 or pUOA2A [*tet*(O) in pUC8]	70
	pUOA2B1 [no deletion]	70
	pUOA2B2 [deletion, P_{lac}-*tet*(O)]	32
		40 (IPTG)[b]
	pUOA2E1 [no deletion]	80
	pUOA2E2 [deletion, P_{lac}-P1-*tet*(O)]	14
		16 (IPTG)[b]
	pACYC-UOA2A [*tet*(O) in pACYC184]	50
	pACYC-UOA2E2 [deletion, P1-*tet*(O)]	6
HB101	pUOA7 [DNA upstream of *tet*(O)]	2
($recA^-$)	pACYC-UOA2E2 [deletion, P1-*tet*(O)]	4
	pACYC-UOA2E2, pUOA7	4

[a] At least two individual transformants were tested in each case.

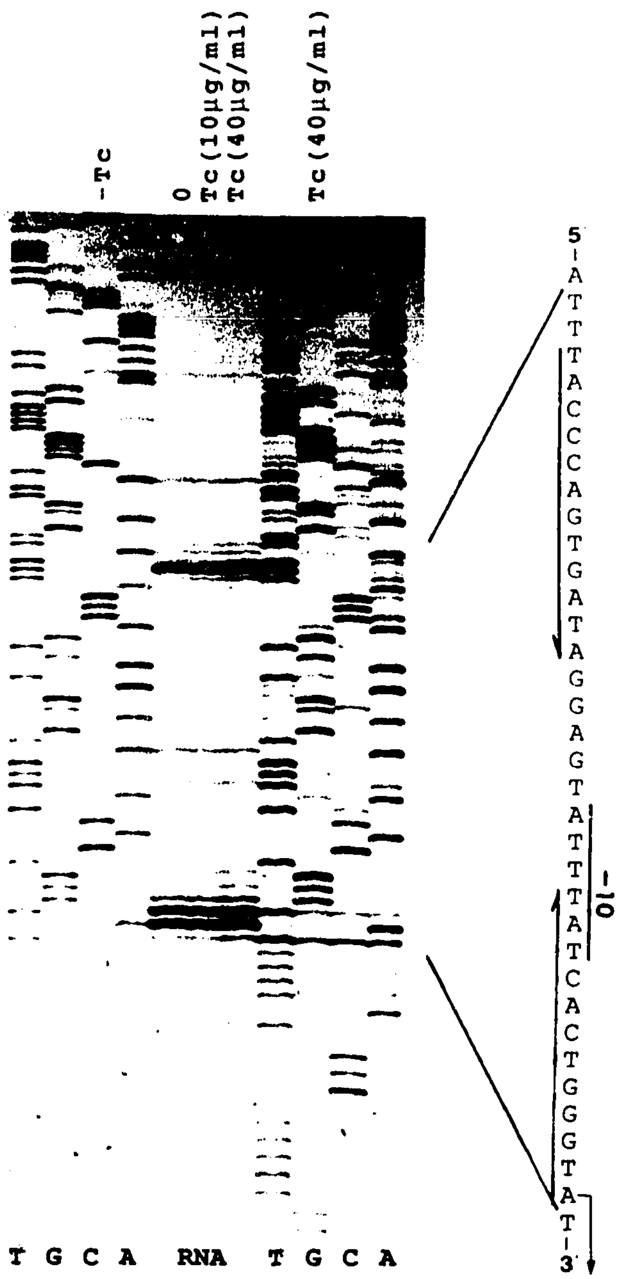
[b] MIC was tested in the presence of the P_{lac} inducer IPTG (0.03 mM final concentration).

6.4 Complementation analysis. Because the DNA sequence upstream of the *tet*(O) ORF is required for high level Tc resistance, and does not appear to encode any polypeptide, a hypothesis was proposed that this DNA sequence codes for an RNA molecule which is involved in Tc resistance. Therefore, complementation experiments were carried out. No Tc^R transformants were obtained when pUOA7 was introduced into HB101(pACYC-UOA2E2). Three Ap^R transformants were tested, and the tetracycline MIC were exactly the same as HB101(pACYC-UOA2E2) (Table 6-1). When a recA$^+$ strain JM107 was used as the host, Tc^R transformants were obtained at ca. 5% transformation efficiency of the Ap^R transformants. Four Tc^R transformants were examined for their plasmid content, and all contained a single recombinant plasmid. Four other pairs of plasmids constructed in a similar fashion were tested. None of these plasmids pairs increased the Tc MIC of the parent strains (data not shown). The results suggest that the DNA sequence upstream of *tet*(O) functions in cis only.

6.5 Primer extension inhibition by tetracycline.

Besides a high affinity site on the 30S subunit of the bacterial ribosome, tetracycline has been known to bind with low affinity to DNA, RNA, and many proteins (Kohn, 1961; Day, 1966). To test whether Tc binds to DNA or RNA at the 5'-end of the *tet*(O) ORF, primer extension inhibition was designed and performed. The primer merl was annealed with ssDNA or RNA containing the *tet*(O) determinant in the presence of Tc, and extended by the Sequenase or AMV reverse transcriptase. The result (Fig.6-5) showed that there were no Tc high affinity sites on these DNA and RNA molecules. However, the experiment could not rule out the possibility that Tc might bind to the RNA-protein or DNA-protein complex.

Fig.6-5. Primer extension inhibition of the RNA and DNA upstream of the *tet*(O) ORF. RNA primer extension was performed with the Tc concentration of 0, 10, and 40 μg/ml respectively. DNA sequencing was performed in the presence or absence of Tc (40μg/ml). Part of the template sequence was predicted with the possible -10 site, the palindrome sequence, and the mRNA start point.


-Tc
0
Tc(10μg/ml)
Tc(40μg/ml)
Tc(40μg/ml)
T G C A RNA T G C A
5'
A
T
T
T
A
C
C
C
A
G
T
G
A
T
A
G
G
A
G
T
A
T
T
T
A
T
C
A
C
T
G
G
T
A
T
3'
-10

6.6 Production of Tet(O) protein with a T7 promoter vector. The vector pT7-5 was used to construct the following plasmids (Fig.6-6): pT7-UOA2A (insertion of the 4.4-kb *Eco*RV-*Pst*I fragment of pUOA2A containing the entire *tet*(O) determinant into pT7-5 between the *Sma*I and *Pst*I sites); pT7-UOA2B2 (insert.on of the *Bam*HI-*Pst*I fragment of pUOA2B2 into pT7-5 between the *Bam*HI and *Pst*I sites); pT7-UOA2B2-*Hin*dIII (*Hin*dIII fragment deletion of pT7-UOA2B2, thus deleting most of the C-terminal end of the Tet(O) protein); pT7-UOA2B2-*cat* (insertion of the *C.coli cat* gene from pYW70 into pT7-UOA2B2 between the *Eco*RI and *Sma*I sites). After transforming these plasmids into *E. coli* K38, plasmid-coded proteins were labeled with [^{35}S]methionine and separated in SDS-PAGE gels.

Fig.6-7A shows that: 1) A 70 kDa polypeptide which is probably the Tet(O) protein (72.3 KDa based on the deduced amino acid sequence, Manavathu et al., 1988) was produced in all constructions containing the *tet*(O) ORF; 2) The presumed Tet(O) protein was produced in larger quantities in this system than in other systems such as *E. coli* in vitro transcription/translation (Taylor et al., 1987; Manavathu et al., 1988; section 6.7, Fig.6-8), expression vector pKK233-2 (Manavathu et al., 1990), *E. coli* minicells (Taylor et al., 1987), or maxicells (data not shown). However the production was still poor, and the yield was the same both in the presence or absence of Tc; 3) The truncated Tet(O) protein was synthesized in much larger quantities than the complete Tet(O) protein, even though it used the same translational initiation sequence; 4) The *C. coli* CAT protein was produced in much larger quantities than Tet(O) from the same mRNA in spite of the fact that it contains fewer methionine residues and has a higher percentage of *E. coli* rare codons than the *tet*(O) gene (see Chapter V), suggesting that Tet(O) synthesis was highly restricted at the translational level.

Fig.6-7B showed that no apparent degradation of the 70 kDa polypeptide [presumed the Tet(O) protein] was detected after 60 min incubation at 30°C with or without Tc, while some of unknown smaller polypeptides disappeared. Therefore, Tc probably did not affect the degradation rate of the Tet(O). In addition, the small polypeptides were apparently not the degradation products of the Tet(O) protein.

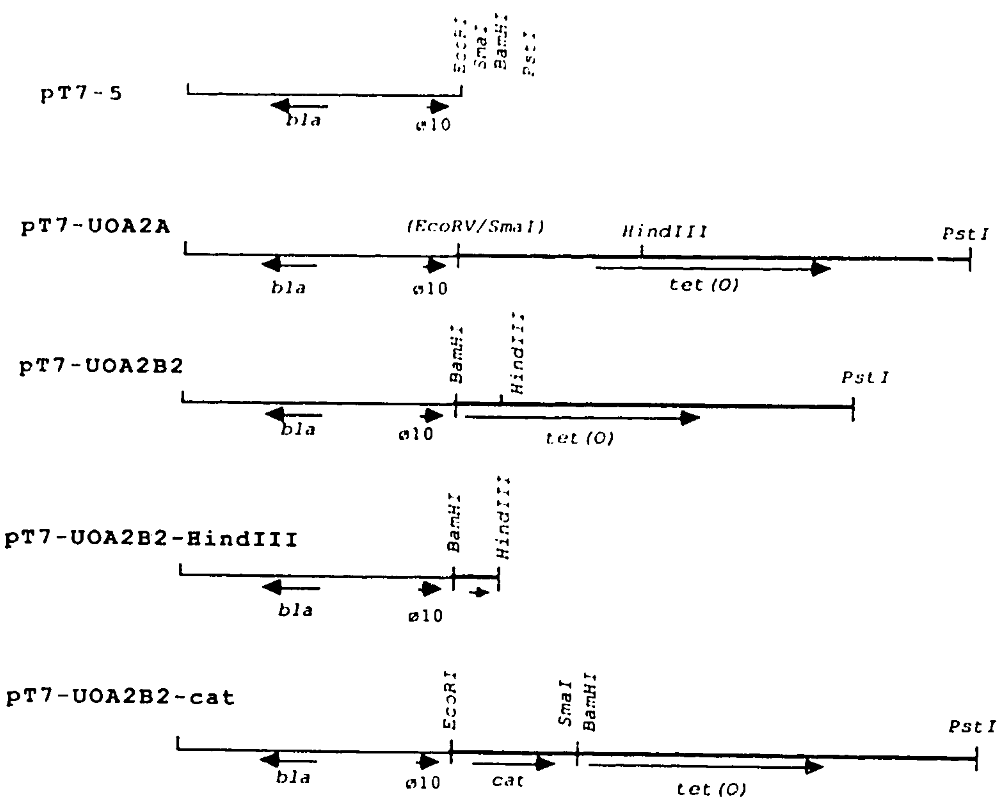


Fig.6-6. Restriction maps of the plasmid pT7-5 and its derivatives.

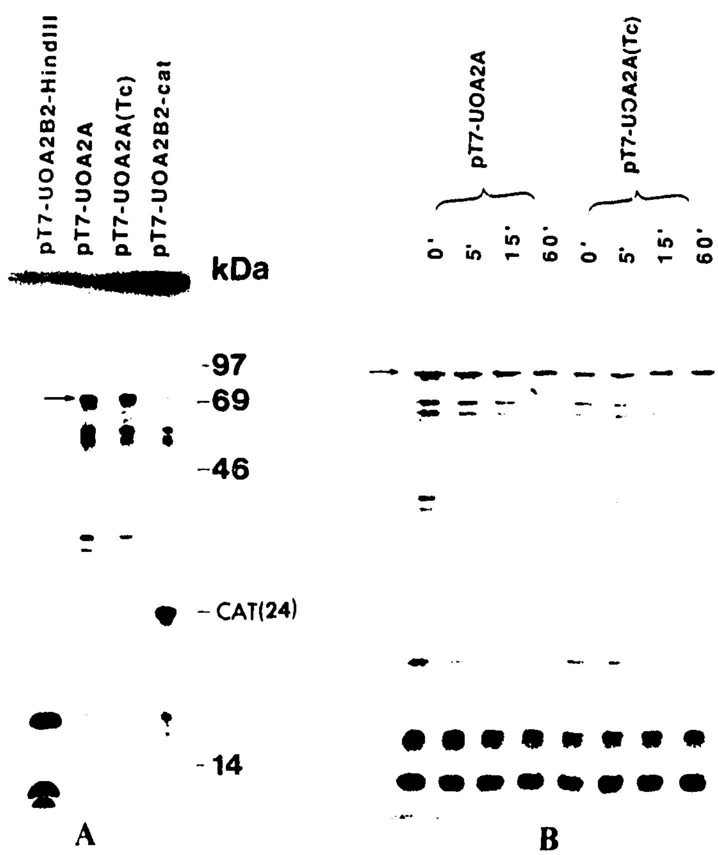


Fig.6-7. Autoradiogram of polyacrylamide gels showing [^{35}S]methionine-labeled polypeptides using a T7 promoter vector. The 70 KDa band corresponding to the Tet(O) protein is indicated by arrows. (Tc), *E. coli* cells were cultured and labeled in the presence of Tc (12µg/ml). A) Samples were labeled with [^{35}S]methionine for 5 min at 30°C. B) Samples were labeled for 1 min, followed with a chase using nonradioactive methionine. Aliquots were removed at 0, 5, 15, 60 min.

6.7 Tet(O) protein labeling with a wheat germ in vitro translation system. Because Tc resistance determined by *tet*(O) is mediated at the level of protein synthesis (Manavathu et al., 1990), Tet(O) probably acts directly on ribosomes, and makes them less sensitive to Tc. At the same time, it might have a second function in limiting its own production. If this is the case, the hyperproduction of Tet(O) could be achieved by using an eukaryotic expression system.

To test this, a wheat germ in vitro translation system was used. Fig.6-8 showed that in this system Tet(O) was produced from in vitro transcribed pT7-UOA2B2 RNA in similar amounts as the control from the eukaryotic BMV RNA, while the production of Tet(O) was not detectable using the same RNA and an *E. coli* transcription/translation system. The result supports the hypothesis that Tet(O) may act on the bacterial ribosome and terminate its own synthesis during translation.

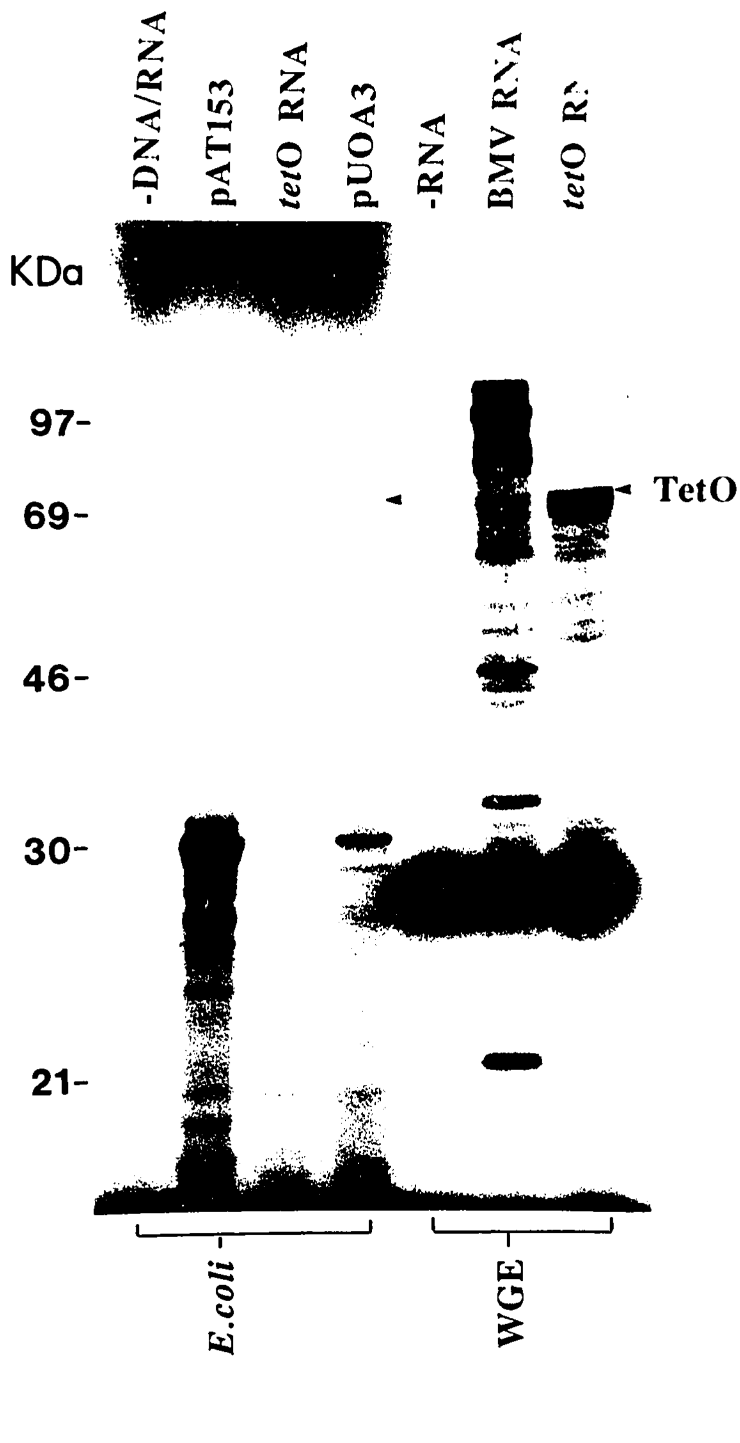


Fig.6-8. Autoradiogram of a polyacrylamide gel showing [^{35}S]methionine-labeled polypeptides from *E. coli* in vitro tanscription/translation and wheat germ extract systems. "*E. coli*", *E. coli* in vitro transcription/translation system; "WGE", wheat germ extract translation system.

6.8 Comparison of the amino acid sequence of Tet(O) with some homologous proteins. The deduced N-terminal amino acid sequence of Tet(O) has been found to be homologous to GTP-binding proteins involved in protein synthesis, especially elongation factors Tu and G (EF-Tu, EF-G) (Manavathu et al., 1990). The amino acid sequence of Tet(O) was compared with that of Tet(M) (Appendix 1), OtrA (a Tc^R determinant cloned from the tetracycline producer *Streptomyces rimosus*), EF-G (Appendix 2), LepA (a membrane-bound GTP-binding protein which is cotranscribed with the signal peptidase I gene and which is believed to be required for the elongation of nascent polypeptides of secreting proteins after the signal peptide was synthesized and elongation was stopped, March and Inouye, 1985a; March and Inouye, 1985b) (Appendix 3), EF-Tu, and SELB (an elongation factor necessary for the incorporation of selenocysteine into protein, Forchhammer et al., 1989) (Appendix 4).

The results of these amino acid sequences comparisons were summarized in Table 6-2. Tet(O) shares the greatest homology with Tet(M) (76% identities, or 85% similarities if amino acids of similar chemical structures are considered). Tet(O) also displays substantial homology to OtrA (38% identities), EF-G (28% identities) and LepA (26% identities) throughout its length. The N-terminal regions of these proteins share more homology than the C-terminals. Probably they are all involved in the binding of GTP and GDP (Jurnak, 1985), and in GTPase activity (Van Noort et al., 1986).

Table 6-2. Amino acid homology of Tet(O) to Tet(M), OtrA, and translation factors

Protein	Length (amino acid)	Identity (%)	Similarity (%)
Tet(O)	637	100	100
Tet(M)	639	76	85
OtrA	663	36	48
EF-G	701	28	46
LepA	598	26	33
EF-Tu	394	23[a]	33[a]
SELB	614	22	31

[a] EF-Tu is compared with 394 amino acids of the Tet(O) N-terminal sequence, whereas others are compared with the full length of Tet(O).

6.9 Complementation test for *E. coli* 4.5S RNA. A 4.5S RNA is essential for the growth of *E. coli* (Brown and Fournier, 1984), and is known to act on translating ribosomes (Brown, 1989). Suppressors reducing the requirement for 4.5S RNA often reside in the gene for EF-G (Brown, 1987). Interestingly, in eukaryotes, a 7S RNA was found to be an essential component of the signal recognition particle that participates in protein secretion (Poritz et al., 1988). Furthermore, genes for 7S RNAs were found to be able to replace the gene for 4.5S RNA in growth of *E. coli* (Brown, 1991). Therefore, the DNA sequence upstream of the *tet*(O) ORF was tested for its ability to replace the requirement of *ffs*, the structural gene for 4.5S RNA. The tester strain was S1192 (Brown and Fournier, 1984), in which the gene for 4.5S RNA is regulated by the *lac* operator. This strain requires an inducer of *lac* to grow. Plasmid pSB832 (ffs^+ in pBR327) permitted its growth in the absence of IPTG, whereas pUOA7 [a DNA fragment upstream of *tet*(O) in pUC8] did not (Table 6-3). Note that *lacO* carried on a multicopy plasmid can titrate *lac* repressor (Johnsrud, 1978), and reduce the requirement for the inducer IPTG.

The DNA sequence upstream of *tet*(O) was also compared with that of 4.5S RNA, and no similar motif was identified.

Table 6-3. Complementation test for 4.5S RNA.

Plasmid	Recipient	Efficiency of plating[a] (-IPTG / +IPTG)
pBR327	S1192	<0.001
pSB832		1
pUC8		0.05
pUOA7		0.04

[a] Transformants were selected directly on LB agar with 100 µg/ml Ap and 0.05 mM IPTG.

CHAPTER VII

DISCUSSION AND CONCLUSION

7.1 Natural transformation in *Campylobacter* species.

In this thesis I have demonstrated that campylobacters are naturally competent for DNA uptake. Chromosomal mutations to nalidixic acid resistance and streptomycin resistance were found to be good markers for transformation studies. High level streptomycin resistance in campylobacters has been shown to be due to in vivo acquisition of genes from Gram-positive and Gram-negative bacteria (MIC's range from 256 to >2,048, Pinto-Alphandary et al., 1990). Some *Campylobacter* strains can also mutate to low level streptomycin resistance (the MIC was 16 µg/ml for UA417R).

All five *C. coli* strains tested were highly competent, whereas only 3 out of 6 *C. jejuni* strains were naturally transformable with the transformation frequencies about one order less than those of *C. coli* (Table 3-1). This may be the reason for the increased incidence of antibiotic resistance seen in *C. coli* compared with *C. jejuni*, especially Em^R and Km^R (Kotarski et al., 1986; Sagara et al., 1987; Yan, 1990), since *C. coli* is more proficient in its ability to acquire and maintain foreign DNA and possibly also to spread it to other strains of *C. coli*.

C. coli was found to be constitutively competent for transformation (Fig.3-3), which appears similar to transformation in *N. gonorrhoeae* (Sparling, 1966; Biswas et al., 1977). In competition studies, however, the competence in *C. coli* was found to be stimulated by the addition of DNA molecules (Fig.3-5, Fig.3-6, Table 3-2), and the competence was not abolished by incubation with homologous DNA (data not shown). These findings suggest that the competence regime and DNA uptake mechanism are different from other well-studied systems. In *N. gonorrhoeae*, competence undergoes a rapid decay if the cells are incubated with homologous DNA (Dougherty et al., 1979), and in *Haemophilus*, it has been suggested that each cell has 4-8 receptors, each of which can be used only once to take up one molecule of DNA (Deich and Smith, 1980).

Transformation with both Nal^R and Str^R markers indicates that *C. coli* is able to take up only a very limited number of DNA molecules at any given time. The frequency of $Nal^R Str^R$ co-transformants (2×10^{-7} per viable cell) is only 0.017% of the frequency of Nal^R transformants (1.2×10^{-3}) or 0.05% of the frequency of Str^R transformants (4×10^{-4}). In *B. subtilis*, in which each competent cell takes up a large number of DNA molecules (Smith et al., 1981), the frequency of co-transformants for two unlinked markers can be as high as 2% of the frequencies of transformants for each marker (Wilson and Bott, 1968).

The results obtained from both ^{32}P-labeled DNA uptake and competition experiments demonstrate that the DNA uptake system of *C. coli* is specific for DNA derived from this and closely related species. Such a mechanism is very similar to that seen in *Haemopnilus* (Scocca et al., 1974) and *Neisseria* (Dougherty et al., 1979) species. Although direct evidence for a recognition sequence is still lacking, my results favor the view that a specific recognition sequence is present in both *C. coli* and *C. jejuni*. The possibility that DNA recognition involves interaction with modified residues (Scocca et al., 1974) is also likely, because the transformation efficiency of plasmid DNA isolated from *E. coli* was ca.10-fold lower than that from *C. coli* plasmid DNA (Table 3-3); also a ^{32}P-labeled shuttle plasmid DNA (pUOA15) from *E. coli* was absorbed by *C. coli* at much lower frequency than the same plasmid from *C. coli* (data not shown). However, the possibility that homologous recombination is required during the DNA uptake process (Stewart and Carlson, 1986) is unlikely, because *C. coli* cells take up *C. jejuni* DNA as efficiently as *C. coli* DNA (Fig.3-5), yet both DNAs share only a limited amount of homology (approximately 32-48%, Belland and Trust, 1982). Furthermore, the transformation frequency of the shuttle plasmid into *C. coli* cells containing a homologous plasmid was still much lower than that of homologous chromosomal DNA.

It has been demonstrated that a restriction/modification system exists in *C. jejuni* C31 (Labigne-Roussel et al., 1987; Miller et al., 1988) which acts on the *Eco*RI recognition site. DNA isolated from *Campylobacter* spp. was resistant to *Eco*RI digestion, and became susceptible if the same DNA was transfered to and isolated from *E. coli*. Similar results were obtained in electroporation experiments in which pILL550 DNA isolated from *E. coli* transformed *C. jejuni* poorly (Miller et al., 1988). In contrast, in natural transformation system the plasmid DNA isolated from *E. coli* could be transformed into *C. coli* at a considerably high frequency. This result suggests that the incoming DNA was protected from the action of restriction endonucleases, and is consistent with the protection of transforming DNA from cellular nucleases observed in other natural transformation systems (Smith et al., 1981).

The frequencies of plasmid transformation were about 1000 times lower than chromosomal markers with the use of the highly competent UA585 strain and small plasmids (e.g. pUOA17, 8.3 kb). Transformation of UA417 or UA466 with plasmid DNA's was not successful, probably because these two strains have some extracellular DNase activity (Table 3-1). Such low transformation frequencies may be due both to very inefficient uptake of the shuttle plasmid by *Campylobacter* spp. (Fig.3-4), combined with partial digestion of the donor DNA during uptake, which has been noted in all other well-characterized natural transformation systems (Smith et al., 1981). The damage to transforming plasmid DNA can be compensated by the use of recipients containing a homologous plasmid, thus the incoming plasmid can be rescued by the resident plasmid through homologous recombination (Smith et al., 1981). The results (Table 3-3) showed that transformation frequencies of the shuttle plasmids increased about 100-fold in this *C. coli* system.

Natural transformation in *Campylobacter* spp. has been used in transferring DNA from *E. coli* or other species into campylobacters, chromosome mapping (Yan and Taylor, 1991), as well as in the development of improved methods for gene replacement mutagenesis. It is so far the simplest and most efficient genetic method for studying these microorganisms.

7.2 Cloning vectors for *Campylobacter* species. A series of new shuttle plasmids was constructed in this thesis (pUOA13-

18, Fig.4-1, Fig.4-2) based on the first bifunctional cloning vector pILL550 (Labigne-Roussel et al., 1987). They encode *Campylobacter* Km^R, Tc^R, and/or Cm^R determinants (all of which are expressed in *E. coli*), as well as an *E. coli* Ap^R gene and the *lacZ'* gene. The latter contains a multiple-cloning site. Neither β-lactamase nor β-galactosidase are expressed in campylobacters. These plasmids also contain an *oriT* sequence from pRK212 (except pUOA14), and therefore, can be mobilized from *E. coli* to *Campylobacter* with the help of an IncP plasmid. The sizes of these plasmids range from 7.4 to 12.2-kb. These plasmids were primarily constructed for studies of gene transfer in campylobacters. In practice they are not as useful as the smaller ones which bear the same markers but replicate in *E. coli* or *Campylobacter* only.

With the development of a simple transformation system, construction of small *Campylobacter* cloning vectors was readily achieved. Two such plasmids pUOA19 and pUOA20 were made which carry Km^R or Cm^R genes as well as a polylinker (Fig.4-3). They are especially useful in transferring DNA from *E. coli* to *Campylobacter* spp. For example, an *E. coli* plasmid containing a cloned DNA sequence can be ligated directly with either one of these *Campylobacter* plasmids, and the recombinants transformed into both *E. coli* and *C. coli* cells.

Plasmids that contain a *Campylobacter* Km^R or Cm^R gene but replicate in *E. coli* only were also constructed (suicide vectors or integrative vectors, Fig.4-4). These vectors, when ligated to or inserted into a campylobacter chromosomal DNA fragment, can integrate into the Campylobacter chromosome by Campbell-type insertion or by homologous recombination. The potential uses of these plasmids are:

1) site-specific mutagenesis by gene disruption and replacement; 2) cloning of the DNA sequences adjacent to the vector's insertion site. 3) precise mapping of the cloned DNA on the chromosome by restriction digestions with enzymes that cut chromosome into a limit number of fragments and cut the vector once, followed by pulsed-field electrophoresis. 4) Cloning of the replication origin of *Campylobacter* plasmids.

7.3 Chloramphenicol resistance in *Campylobacter coli*.

The nucleotide sequence of a Cm^R gene cloned from a *C. coli* plasmid was determined (Fig.5-2). The gene was identified as a *cat* by comparison with other known *cat* sequences. The G + C content of the ORF is 37.5%, which is slightly higher than that of *C. coli* genome (30-36%). Whether this *cat* determinant is an indigenous campylobacter gene or is acquired from some other species remains unknown. It has not been identified in other species, and the codon usage bias in *Campylobacter* spp. genes is still unavailable. Comparison of nucleotide and amino acid sequences with other known *cat* genes showed that it is most closely related to the *catP* from *Clostridium perfringens* (Steffen and Matzura, 1989) and *catD* from *Clostridium difficile* (Wren et al., 1989), with which it shares 67% identities at the nucleotide level and 57% at the deduced amino acid level.

A Km^R determinant was found downstream of the *cat* gene. It is almost identical to the *aphA-3* gene from *C. coli* plasmid pIP1433 (Trieu-Cuot et al., 1985). However, part of the DNA sequence upstream of *aphA-3* including the promoter site is missing. It appears that this *cat* sequence was acquired by *C. coli* C-589 from some other source and inserted into the region upstream of *aphA-3* where four pairs of direct repeats were present (Trieu-Cuot et al., 1985). Although several direct and inverted repeats have been found flanking the *cat* and *aphA-3* ORF's, and the Km^R determinant has been shown to integrate into *C. coli* chromosome at low frequencies (Kotarski et al., 1986), there is no direct evidence that this *cat* and/or *aphA-3* are encoded on a transposon.

The *cat* gene is expressed constitutively in *C. coli* and in *E. coli*, which is similar to all other *cat* genes of gram-negative origin (Shaw, 1983). There is also no potential leader peptide or palindrome sequence upstream of the *cat* ORF. Such structures have been found to be involved in the inducibility of the *B. pumilus cat86* gene and the *S. aureus cat* gene (Bruckner and Matzura, 1985; Lovett, 1990).

Since the recent development of chemical synthesis of deoxyribopolynucleotides, primer extension is readily accessible, and was chosen for locating the *cat* promoter. The 5'-end of *cat* mRNA from *C. coli* was located at the 32nd nucleotide upstream of the ATG start codon. The possible -35 and -10 promoter sites were identified with an interval gap of 18 nucleotides (Fig.5-4). These sequences (-35, ATGATA, and -10, TATAAT) display 10 out of 12 identities with the *E. coli* consensus sequences (Rosenberg and Court, 1979). Trieu-Cuot et al.(1985) showed that the promoter for Km^R gene in *C. coli* (which appears to have been acquired recently from *Streptococcus*) is identical to the *E. coli* consensus sequences. These findings suggest that campylobacters may use promoter sequences similar to those used by *E.*

coli. However, results from primer extension experiments showed that several other canonical sequences were used by *E. coli* but not by *C. coli* as promoters for the transcription of this *cat* gene (Fig.5-4). Therefore, DNA sequences in addition to the -35 and -10 sites appear to be more important for the initiation of transcription in *C. coli*. Ballester et al.(1980) also found that the pC194 *cat* mRNA starts from different sites in *Streptococcus pneumoniae* and *Bacillu subtilis*.

The codon utilization of the *C. coli cat* gene is very different from that used in *E. coli*. It contains the so-called *E. coli* rare codons (ATA, TCG, CCT, CCC, ACG, CAA, AAT, and AGG; Konigsberg and Godson, 1983) at a frequency of 13.5%. However, protein labeling using *E. coli* maxicells showed that the CAT polypeptide was produced in large quantities (Fig.5-5). Therefore the codon usage bias is not one of the obstacles that affect *Campylobacter* gene expression in *E. coli*. This observation is supported by some recent reports. Sorenson et al. (1989) examined the effect of rare codons on the in vivo translation of a β-galactosidase fusion protein, and showed that rare codons only decreased the translation rate slightly, and the final yield of the protein was almost the same as the one which was coded with much less rare codons. Similar results were also obtained by Dix and Thompson (1989).

7.4 Tetracycline resistance mediated by *tet*(O) and *tet*(M). A DNA sequence upstream of the *tet*(O) ORF was found to be homologous to a sequence upstream of the *tet*(M) ORF by Southern hybridization (data not shown). Attempts to remove this sequence from *tet*(O) failed when transformants of Tc^R phenotype were selected, which suggested that this sequence may be involved in Tc resistance. Therefore, I determined the nucleotide sequences upstream of *tet*(O) and *tet*(M) (Fig. 6-2). They were found to share a higher degree of homology than the *tet*(O) and *tet*(M) ORF's themselves. The sequence upstream of *tet*(O) does not appear to encode any polypeptides judging from the nucleotide sequence analysis and protein labeling experiments (Fig.6-7).

A transcriptional terminator-like structure was identified directly upstream of *tet*(O) and *tet*(M) (Fig.6-2). This may prevent transcription from upstream promoters or serve as a transcriptional attenuator. Such a structure often indicates that the gene product is required in a very small amounts and/or is toxic to the normal cellular metabolism.

The 5'-end of the *tet*(O) mRNA was located at the 42nd nucleotide upstream of the ATG start codon in both *E. coli* and *C. coli* (Fig.6-3). The possible -35 and -10 sites (named P1) were assigned, which have only 5/12 identities to the *E. coli* consensus sequences. The P1 is apparently not an *E. coli* promoter, but likely an indigenous campylobacter promoter. The transcription from P1 is apparently very poor in *E. coli*. For example, pUOA2E2 [P1-*tet*(O) cloned in a high copy number plasmid pUC118] only confers low level Tc resistance, and pACYC-UOA2E2 which has a lower copy number does not even confer Tc resistance (Table 6-1). The same DNA fragment can render *C. coli* resistant to high levels of Tc.

Another potential promoter sequence (P2) was identified at 281 nucleotide upstream of the ATG start codon, of which the -35 and -10 sites are 100% identical to the *E. coli* consensus sequences. Transcription from P2 was demonstrated in *E. coli* but not in *C. coli* by primer extension experiments (Fig.6-3). A small RNA from *E. coli* was identified by Northern blot which probably starts from the P2 and terminates after the two dyad symmetry sequences. The same RNA species was not detected in *C. coli* (Fig.6-4).

These findings suggest that some nucleotide sequences other than the -35 and -10 sites appear to be more important in the ability of campylobacter RNA polymerase to recognize the promoter region. Therefore, the sequences of P1 and P2 were compared with that of the *C. coli cat* promoter, which is the only known campylobacter promoter up to now (Fig.5-4). It was found that the *cat* promoter shares extensive homology with the P1 (59% identity, Fig.7-1A) but not the P2 except at the -10 region (31% identity, Fig.7-1B). The promoter for the *aphA-3* gene, which appears to originate from *Streptococcus* and which has been shown to function in *C. coli* (Trieu-Cuot et al., 1985), was also compared and found to have substantial similarities with both the *cat* promoter and the P1 (53% identity, Fig.7-1A). These results provide further evidence that campylobacters use different promoter sequences from that in *E. coli*, and that the P1 is the promoter for *tet*(O) expression.

A

```
                                        TTG
tet(O) P1      ATATA AGTGTTTTGGGGCTA GAGTTATTTACCCAGT GATAGGAGTAT T
               ::::: : ::     : :: :: ::  :: : :   ::: :::::   :::
C. coli cat    ATATAGATTGAAAAGTGGATA GATTTA TGATATAGTGGATAGATTTATGA
               ::  ::   : : : ::  :  ::: :: ::: :     :::: :  ::  :
aphA-3         AT GAGGAGGCAGATTGCCTT GATATATTGACAATACTGATA AGATAATA
                                      A      -35

                               start of mRNA
tet(O) P1      TATCACTGGGTAT
               ::: :   : :::
C. coli cat    TATAATGAGTTAT
               ::::::   :  :
aphA-3         TATAATATATCTTTAC
                 -10
```

B

```
C. coli cat    ATATAGATTGAAAAGTGGATAGATTTATGATATAGTGGATAGATTTATGA
                     ::     : : : :     :     : :  :  : :      :
tet(O) P2      CCGCTTTTTCTGCTTTAGTTTGTCAGCTTGACAAATAAAGGGTTAAGGAA
                                             -35

                               start of mRNA
C. coli cat    TATAATGAGTTAT
               :::::: :  :
tet(O) P2      TATAATTAGATTCAG
                 -10
```

Fig. 7-1. (A) Comparison of nucleotide sequences of the *tet*(O) promoter P1, the *C. coli cat* promoter, and the *aphA-3* promoter. (B) Comparison of nucleotide sequences of the *C. coli cat* promoter and the *tet*(O) P2 sequence. The -35 and -10 sites are underlined and indicated. The transcription start points are indicated by darker lettering.

For studying the function of the conserved DNA sequence upstream of *tet*(O), and for later production of Tet(O) protein, a *Bam*HI site was generated downstream of the P1, and an *Eco*RI site downstream of the P2. Deletions were generated at these enzyme cutting sites, and Tc MIC's for these deletions were determined. Removal of some of the sequences upstream of *tet*(O) was found to reduce greatly the resistance level of Tc in *E. coli* (Table 6-1). However, attempts to transfer these deletion derivatives into *C. coli* failed. These plasmids were either lethal to the *C. coli* host or extremely unstable. Therefore, the Tc MIC could not be tested in *C. coli*.

Since this conserved DNA sequence does not appear to encode a promoter, a polypeptide, or to be involved in the regulation of gene expression, it might code for an RNA molecule which is involved in Tc resistance and/or protection of the ribosome from the toxic effect of the Tet(O) protein. This hypothesis is very attractive, by virtue of the fact that Tet(O) may bind to the ribosome (Manavathu et al., 1990) which is a protein-RNA complex. Furthermore, a 4.5S RNA has been known to interact with EF-G and the ribosome and also to participate in protein synthesis (Brown and Fournier, 1984; Brown, 1987). However, complementation tests showed that this conserved sequence cannot complement in trans the *tet*(O) ORF for high level Tc resistance (Table 6-1), and it cannot complement the *E. coli* 4.5S RNA for growth either (Table 6-3). Moreover, *E. coli* and *C. coli* apparently use different promoter sequences upstream of the *tet*(O) gene. RNA transcribed in vitro from this sequence was also shown not to bind Tc by itself (Fig.6-5).

High level production of the Tet(O) protein appears to be highly toxic to cells. The constructions in which the *tet*(O) ORF was put under the control of strong promoters, including the promoters from the *C. coli cat* gene and the *aphA-3* gene, appeared to be non-viable. Cloning of *tet*(O) into the secreting vector pIN-III-*ompA* (Takahara et al., 1985) was also unsuccessful. The appropriate recombinant plasmids were never obtained (data not shown). Labeling of the Tet(O) protein using all available *E. coli* protein labeling systems (including minicells, maxicells, in vitro transcription/translation system, and T7 promoter vectors) never resulted in a single band. The labeling patterns are very similar in all these systems: a polypeptide of ca. 70 KDa [Tet(O)] together with many other smaller protein bands (Taylor et al., 1987; Manavathu et al., 1988; Fig.6-7; Fig.6-8).

The highest yield of Tet(O) in *E. coli* was obtained by using a T7 promoter vector pT7-5 (Fig.6-7). The results obtained are summarized as follows:

1) The Tet(O) production was not affected by the presence or absence of tetracycline;
2) The truncated Tet(O) was hyperproduced, therefore, the initiation of translation for Tet(O) was efficient;
3) The *C. coli* CAT polypeptide was synthesized in much larger quantities than Tet(O) from the same mRNA in spite of the facts that it contains a higher percentage of *E. coli* rare codons and has fewer methionine residues than the *tet*(O) gene;
4) The labeling patterns were almost identical using the complete *tet*(O) determinant and the mutant that had the upstream DNA deleted,

therefore, the DNA sequence upstream of *tet*(O) is not likely to encode any polypeptides;

5) The small polypeptides were apparently not degradation products of Tet(O). They also did not appear to be products initiated from the internal ATG codons, because not all of the ATG triplets in the *tet*(O) ORF possess an SD site. Therefore, they might be products of premature termination of Tet(O).

All these findings suggest that Tet(O) may act on the bacterial ribosomes and prevent its own production. This hypothesis of translational regulation was supported by the successful production of the Tet(O) protein using a wheat germ in vitro translation system (Fig.6-8). A similar mechanism might be used in the coordination of rRNA and ribosomal protein synthesis in *E. coli* (Matin et al., 1989). Furthermore, Young and Bernlohr (1991) discovered that EF-Tu is methylated in response to nutrient deprivation in *E. coli*. It was proposed that the methylated EF-Tu might act at the level of translation, and could be responsible for shutting down most vegetative protein synthesis during starvation while allowing the synthesis of starvation-induced proteins to continue.

Tet(O) showed substantial homology to some GTP-binding proteins, especially EF-G, LepA, SELB, and EF-Tu. Tc is thought to block the A site of the bacterial ribosome (Goldman et al., 1983). Therefore, Tet(M) has been proposed to function as a Tc resistant elongation factor (Sanchez-Pescador et al., 1988). However, chlortetracycline has been found to stimulate the EF-Tu-dependent GTPase activity (Hamel et al., 1972). Moreover, Burdett (1991) showed that Tet(M) could not replace the EF-Tu in a temperature-sensitive mutant. The amino acid comparisons (see Appendices and Table 6-2) show that Tet(O) exhibits less homology to SELB and EF-Tu (both of which are responsible for delivering the aminoacyl-tRNA into the A site) than to EF-G (which is required for ribosome translocation during peptide synthesis) and LepA (which may be necessary for the elongation of nascent polypeptides of secreting proteins after the signal peptide has been synthesized and elongation is halted, March and Inouye, 1985).

These data suggest that Tet(O) and Tet(M) may function in a fashion similar to EF-G and LepA. Here I propose a model in which Tc inhibits protein synthesis, not by directly blocking the ribosomal A site as was postulated previously (Goldman et al., 1983), but by arresting the ribosome during its movement along the messenger RNA. The possibility that Tet(O) and Tet(M) mimic EF-G is unlikely, since the *tet*(M) gene can not complement an EF-G temperature-sensitive mutant (Burdett, 1991), and EF-G-associated GTPase activity is not inhibited by Tc (Hamel et al., 1972). It is possible that Tet(O) may function to complete the translocation process left unfinished by EF-G due to inhibition by Tc. This model accommodates all the available evidence concerning Tet(O) and Tet(M).

7.5 Future studies on *Campylobacter* genetics and tetracycline resistance mediated by *tet*(O). In conclusion, studies in this thesis have provided additional tools for future research on campylobacters. The following experiments could be done in the near future as a continuation of this thesis:

1) Identification of the probable recognition sequence for DNA uptake in transformation, which could be used for construction of high efficient plasmid vectors, as well as for studying the DNA receptor and other transformation processes.

2) Precise mapping of cloned *Campylobacter* genes on the chromosome by using the suicide vectors and pulsed-field electrophoresis.

3) Identification of the consensus sequence of *Campylobacter* promoters by sequencing and comparing some additional *Campylobacter* promoter sequences.

4) Hyperproduction and purification of the Tet(O) protein using eukaryotic expression systems, such as yeast promoter vectors, or the baculovirus expression system.

5) Effect of Tc on aminoacyl-tRNA binding to the A site and ribosomal translocation using an *E. coli* in vitro translation system.

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APPENDIX 1

```
Tet(O)  MKIINLGILAHVDAGKTTLTESLLYTSGAIAELGSVDEGTTRTDTMNLERQRGITIQTAV   60
        ||||| | ||||||||||||||||| |||| |||||| ||| ||   |||||||||||| 
Tet(M)  MKIINIGVLAHVDAGKTTLTESLLYNSGAITELGSVDRGTTKTDNTLLERQRGITIQTAI   60

Tet(O)  TSFQWEDVKVNIIDTPGHMDFLAEVYRSLSVLDGAVLLVSAKDGIQAQTRILFHALQIMK  120
        |||||   ||||||||||||||||||||||||||| || ||||| |||||||||||    
Tet(M)  TSFQWKNTKVNIIDTPGHMDFLAEVYRSLSVLDGAILLISAKDGVQAQTRILFHALRKIG  120

Tet(O)  IPTIFFINKIDQEGIDLPMVYREMKAKLSSEIIVKQKVGQHPHINVTDNDDMEQWDAVIM  180
        |||||||||||| ||||  ||   | ||| ||  ||||  ||   |      |||| || 
Tet(M)  IPTIFFINKIDQNGIDLSTVYQDIKEKLSAEIVIKQKVELHPNMRVMNFTESEQWDMVIE  180

Tet(O)  GNDELLEKYMSGKPFKMSELEQEENRRFQNGTLFPVYHGSAKNNLGTRQLIEVIASKFYS  240
        ||| ||||| |||     ||||||  || |  |||||||||||| |   |||||  ||||
Tet(M)  GNDYLLEKYTSGKLLEALELEQEESIRFHNCSLFPVYHGSAKNNIGIDNLIEVITNKFYS  240

Tet(O)  STPEGQSELCGQVFKIEYSEKRRRFVYVRIYSGTLHLRDVIRISEKEKIKITEMYVPTNG  300
        ||  ||||||| |||||||||| |  | | ||| |||||  ||||||||||||||   ||
Tet(M)  STHRGQSELCGKVFKIEYSEKRQRLAYIRLYSGVLHLRDPVRISEKEKIKITEMYTSING  300

Tet(O)  ELYSSDTACSGDIVILPNDVLQLNSILGNEILLPQRKFIENPLPMIQTTIAVKKSEQREI  360
        ||   | | || |||| |  | ||| ||   |||||  ||||||  |||    |  ||| 
Tet(M)  ELCKIDKAYSGEIVILQNEFLKLNSVLGDTKLLPQRERIENPLPLLQTTVEPSKPQQREM  360

Tet(O)  TTTHEIILSFLGNVQMEVICAILEEKYHVEAEIKEPTVIYMERPLRKAEYTIHIEVPPNP  440
          |||||||||| ||||| || | |||||| |||||||||||||| ||||||||||||||
Tet(M)  SATHEIILSFLGKVQMEVTCALLQEKYHVEIEIKEPTVIYMERPLKKAEYTIHIEVPPNP  440

Tet(O)  FWASVGLSIEPLPIGSGVQYESRVSLGYLNQSFQNAVMEGVLYGCEQGLYGWKVTDCKIC  500
        |||| |||  ||| |||||||| |||||||||||||||||  |||||||||| |||||||
Tet(M)  FWASIGLSVAPLPLGSGVQYESSVSLGYLNQSFQNAVMEGIRYGCEQGLYGWNVTDCKIC  500

Tet(O)  FEYGLYYSPVSTPADFRLLSPIVLEQALKKAGTELLEPYLHFEIYAPQEYLSRAYHDAPR  560
        | ||||||||||||||| | |||||| ||||||||||||| | |||||||||||| ||| 
Tet(M)  FKYGLYYSPVSTPADFRMLAPIVLEQVLKKAGTELLEPYLSFKIYAPQEYLSRAYNDAPK  560

Tet(O)  YCADIVSTQIKNDEVILKGEIPARCIQEYRTDLTYFTNGQGVCLTELKGYQPAIGKFICQ  620
        ||| || || || |||| |||||||||||| ||| ||||  |||||||||    |   ||
Tet(M)  YCANIVDTQLKNNEVILSGEIPARCIQEYRSDLTFFTNGRSVCLTELKGYHVTTGEPVCQ  620

Tet(O)  PRRPNSRIDKVRHMFTS       637
        |||||||||||| ||
Tet(M)  PRRPNSRIDKVRYMFNKIT     639
```

Matches = 490   Mismatches = 147   Unmatched = 2

Length = 639   Matches/length = 76.6 percent

Data are taken from: Tet(O) (Manavathu et al., 1988), Tet(M) (Martin et al., 1986)

APPENDIX 7

```
Tet(O)   MKII      NLGILAHVDAGKTTLTE SLLYTSGAIAELGSVDEGTTRTDTMNLERQRG  53
             |    | || || |||||| ||   | || |      | |  |    |    |  ||
EF-G     APTTPIARYRNIGISAHIDAGKTTTTERILFYT GVNHKIGEVHDGAATMDWMEQEQERG  59

Tet(O)   ITI QTAVTSF       QWEDVKVNIIDTPGHMDFLAEVYRSLSVLDGAVLLVSAKDGIQ 106
         |||  | | |        | |    ||||||| ||  || ||  |||||    |  | |
EF-G     ITITSAATTAFWSGMAKQYEPHRINIIDTPGHVDFTIEVERSMRVLDGAVMVYCAVGGVQ 119

Tet(O)   AQTRILFHALQIMKIPTIFFINKIDQEGIDLPMVYREMKAKLSSEIIVKQ KVGQHPH I 164
          |         | |   | || |  |     |    |  |        |   |   |
EF-G     PQSETVWRQANKYKVPRIAFVNKMDRMGANFLKVVNQIKTRLGANPVPLQLAIGAEEHFT 179

Tet(O)   NVTD          ND D     MEQWD  A  VIMGN              DELLEKYMSG 192
          | |          || |     |   |  |   |   |              || |||  |
EF-G     GVVDLVKMKAINWNDADQGVTFEYEDIPADMVELANEWHQNLIESAAEASEELMEKYLGG 239

Tet(O)   KPFKMSELEQEENRRFQNGTLFPVYHGSA KNNLGTRQLIEVI    AS        KFYS 240
                |        | |     |  ||| ||       |  ||      |
EF-G     EELTEAEIKGALRQRVLNNEIILVTCGSAFKNKGVQAMLDAVIDYLPSPVDVPAIDCILK 299

Tet(O)   STP E GQS  E LCGQVFKIEYSEKRRRFVYVRIYSGTLHLRDVIRISEKEKIKITEMY 295
          || |   |  |       |||             | |||     |    | |
EF-G     DTPAERHASDDEPFSALAFKIATDPFVGNLTFFRVYSGVVNSGDTVLNSVKAARERFGRI 259

Tet(O)   V PTNGELYSSDTACSGDI  VILPNDVLQLNSILGNEILLPQRKFIENPLPMIQTTIAV 352
         |                ||   |   ||       |             | | | |    |||
EF-G     VQMHANKREEIKEVRAGDIAAAIGLKDV TTGDCLCDPDAPIILERMEFPEPVI   SIAV 416

Tet(O)     K KSEQREILLGALTEISDCDPLLKYYVDTTTHEIILSFLGNVQMEVICAILEEKYHV 409
          | |  | |    ||       ||      |       |     |       |          |
EF-G     EPKTKADQ EKMGLALGRLAKEDPSFRVWTDEESNQTIIAGMGELHLDIIVDRMKREFNV 475

Tet(O)   EAEIKEPTVIYMERPLRK    AEYTIHIEVPPNPFWASVGLSIEPLPIGS GVQYE  SR 463
         ||     | | |    |    |                  |     ||  ||    ||
EF-G     EANVGKPQVAYRETIRQKVTDVEGKHAKQSGGRGQYGHVVIDMYPLEPGSNPKGYEFIND 535

Tet(O)   VSLGYLNQSFQNAVMEGVLYGCEQG LYGWKVTDCKICFEYGLYYSPVSTPADFRLLSPI 522
            |      || |       | | | |  | |        | |    |    | |   |
EF-G     IKGGVIPGEYIPAVDKGIQEQLKAGPLAGYPVVDMGVRLHFGSYKDVDSSELAFHLAASI 595

Tet(O)   VLEQALKKAGTELLEPYLHFEIYAPQEYLSRAYHDAPRYCADIVSTQIKNDEV ILKGEI 581
             |||  ||||   |    |          |               |     | |
EF-G     AFKEGFKKAKPVLLEPIMKVEVETPEQNTGDVIGDLSRRRGMLKGQQSEVTGVKIHAQVP 655
```

```
Tet(O)  PARCIQEYRTDLTYFTNGQGVCLTE LK GYQPAIGKFICQPRRPNSRIDKVRHMFTS     639
                  | | |     | |       | ||     |            |
EF-G    LSEQMFGYATQLRSLTKGRASYTMEFLKYDEAPSNVAQAVIEARGK                 [illegible]
```

Matches = 198   Mismatches = 422   Unmatched = 98

Length = 718   Matches/length = 27.5 percent

Data are taken from: Tet(O) (Manavathu et. al., 1988), Ef-G (GeneBank).

APPENDIX 3

```
Tet (O)   MK IINLGILAHVDAGKTTLTESLLYTSGAIAELGSVDE GTTRTDTMNLERQRGITI Q  57
          || | |  | || | || ||  |       |    |  |      | | ||| ||| |
LepA      MKNIRNFSIIAHIDHGKSTL  S  DRIIQICGGLSDREMEAQVLDSMDLERERGINIKA  56

Tet (O)   TAVT    SFQWEDVKVNIIDTPGHMDFLAEVYRSLSVLDGAVLLVSAKDGIQAQTRILF 113
            ||        |    | |||||| ||  || |||    || | | |  |  |||
LepA      QSVTLDYKASDGETYQLNFIDTPGHVDFSYEVSRSLAACEGALLVVDAGQGVEAQTLANC 116

Tet (O)   HALQIMKIPTIFFINKIDQEGIDLPMVYREMKAKLSSEIIVKQKVGQHPHINVTDNDDME 173
               |        ||||    |   |  |
LepA      YTAMEMDLEVVPVLNKIDLPAADPERVAEEIEDIVGIDAHRRGALFSENRRWCAGRSRTS 176

Tet (O)   QWDAVIMGND E LLEKYMSGKPFKMSELEQEENRRFQNGTLFPVYHGSAKNNLGTRQLI 231
                 | | |  |        |    |      |  :|||     |       | |
LepA      GARHSAAGSDPEGPLQALIIDSWF DNYLGVVSLIRIKNGTL   RKGDKVKVMSTGQ   230

Tet (C)   EVIASKFYSSTP  EGQSEL CGQVFKIEYSEKRRRFVYVRIYSGTLHLRDVIRISEKEK 288
             |      ||      || || |       |      |     || |    |    ||
LepA      TYNADRLGIFTPKQVDRTELKCGEVGWLVCAIK DIGAPV   GDTLTL   AR NPAEK 282

Tet (O)   IKITEMYVPTNGELYSSDTACSGDIVILPNDVLQLNSILGNEILLPQRKFIENPLPMIQT 348
                 |      |      | |      | |   | | |   |    | | |
LepA      ALPGFKKV  KPQVYAGLFPVSSDDYEAFRDALGKLS L NDASL    FYE P     ES 329

Tet (O)   TIAVKKSEQREILLGAL TEISDCDPLLKYYVDTTTHEIILSFLGNVQMEVICAILEEKY 407
            |      |   || |  ||        |  |  |          |  ||     |  |
LepA      SSA LGFGFRCGFLGLLHMEIIQERLEREYDLDLIT      TAPTVVYEVETTSREVIY 382

Tet (O)   HVEAEIKEPTV  IYMERPLRKAEYTIHIEVPPNPFWASVGLSIEPLPIGSGVQYESRVS 465
           |    | | |  || |  ||  |  |  |       |  |  |       |||    |
LepA       VDSPSKLPAVNNIY E  LR  E PI AECHMLLPQAYLGNVITLCVEKRGVQ TNMVY 433

Tet (O)   LGYLNQSFQNAVM EGVLYGCEQGLYGWKVT DCKICFEYGLYYSPVSTPADFRLLSPIV 523
           |          | | ||      |   | |        |       |       |
LepA      HGNQVALTYEIPMAEVVL DFFDRL   KSTSRGYASLDYNFKRFQASDMVRVDVLINGE 489

Tet (O)   LEQALKKAGTELLEPYLHFEIYAPQEYL SRAYHDAPRYCADIVSTQIKNDEV ILKGEI 581
             ||              |       |  |   |     | |    |    |  |
LepA      RVDALALITHRDNSQNRGRELVEKMKDLIPRQQFD IAIQAAIGTHIIARSTVKQLRKNV 548

Tet (O)   PARCIQEYRTDLTYFTNGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFTS     637
           | |   |  |             | |     ||     |      |  |
LepA      LAKC   YGGD ISRKKKLLQKQKEGKKRMKQIG NVELPQEAFLAILHVGKDNK      598
```

Matches = 169   Mismatches = 410   Unmatched = 77

Length = 657   Matches/length = 25.7 percent

Data are taken from: Tet(O) (Manavathu et al., 1988), LepA (March and Inouye, 1985).

APPENDIX 4

```
Tet (O)   MKIINLGILAHVDAGKTTLTESLLYTSGAIAELGSVDEGTTRTDTMNLERQRGITIQTAV  60
          | |   |   ||| ||||    ||    ||   | |       |    |  || ||
SELB      MIIATAG   HVDHGKTT    LL   QAI  TG V     NADRLPEEKKRGMTIDLGY  42

Tet (O)   TSFQWEDVKV NIIDTPGHMDFLAEVYRSLSVLDGAVLLVSA KDGIQAQTRILFHALQI 118
                |  |   || |||  ||          | | ||| |  ||  ||||     ||
SELB      AYWPQPDGRVPGFIDVPGHEKFLSNMLAGVGGIDHA LLVVACDDGVMAQTREHLAILQL 101

Tet (O)   MKIP TIFFINKID QEGIDLPMVYREMKAKLSSEIIVKQKVGQHPHINVTDNDDMEQWD 176
             |       | |         | |  |  |  |                   |
SELB      TGNPMLTVALTKADRVDEARVDEVERQVKEVL REYGFAEAKLFITAATEGRGMDALREH 160

Tet (O)   AVIMGNDELLEKYMSGKPFKMSELEQEENRRFQNGT L FPVYHGSAKNNLGTRQLIEVI 234
                 |      |                   || |   |  |      |      |
SELB      LLQLPERE HASQHSFRLAIDRAFTVKGAGLVVTGTALSGEVKVGDSLWLTGVNKPMRVR 219

Tet (O)   ASKFYSSTPEGQSELCGQVFKIEYSEKRRRFVYVRIYSGTLHLRDVIRISEKEKIKITEM 294
          |        |      ||   |             |  |   | ||        |
SELB      ALHAQNQPTE  TANAGQ  RI ALNIAGDAEKEQINRGDWLLADVPPEPFTRVIVELQT 274

Tet (O)   YVP TNGELYSSDTACS  GDIVILPNDVL QLNSILGNEILLPQRKFIENPLPMIQTTI 350
            | |         | |     | |  | |  |            |
SELB      HTPLTQWQPLHIHHAASHVTGRVSLLEDNLAELVFDTPLWLADNDRLVLRDISARNTLAG 334

Tet (O)   A VKKSEQREILLGALTEISDCDPLLKYYVDTTTHEIIL SFLGNVQMEVICAILEEKYH 408
          | |                     |     |       |     |         |
SELB      ARVVMLNPPRRGKRKPEYLQWLASLARAQSDADALSVHLERGAVNLADFAWARQLNGEGM 394

Tet (O)   VEAEIKEPTVIYMERPLRKAEYTIHIEVPPNPFWASVGLSIEPLPIGSGVQYESRVSLGY 468
           |     |  |     |  |              |         | | |     |  |
SELB      RE LLQQPGYIQAGYSLLNAPVAARWQRKILDTLATYHEQHRDEP GPGRERLRRMALPM 452

Tet (O)   LNQSFQNAVME GVLYGCEQGLYGW KVTDCKICF EYGLYYSPVSTPADFRLLSPIVLE 525
                    |     |      ||    | |  | |          |  |      |
SELB      EDEALVLLLIEKMRESGDIHSHHGWLHLPDHKAGFSEUQQAIWQKAEP LFGDEPWWVRD 511

Tet (O)   QALKKAGTELLEPYLHFEIYAPQEYLSRAYHDAPRYCAD IVSTQIKNDEVILKGEIPAR 584
           | |  ||      |     | |     |      |  | ||     |    |  |
SELB      LA KETGTDEQAMRL TLRQAAQQGIITAIVKDRYYRNDRIV  EFANMIRDLDQECGST 567

Tet (O)   CIQEYRTDLTYFTNGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFTS        637
          |    |  |     |       |  |   || |    || |         |
SELB      CAADFRDRL   GVGRKLAIQIL EYFDRIG F  TRRRGNDHLLRDALLFPEK       614
```

Matches = 141   Mismatches = 457   Unmatched = 56

Length = 654   Matches/length = 21.6 percent

Data are taken from: Tet(O) (Manavathu et al., 1988), SELB (Forchhammer et al., 1989).

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Genet 404	6	Microb 607	8