ABSTRACT

KIM, JOO-SUNG. Natural Transformation-Mediated Transfer of Erythromycin Resistance in *Campylobacter coli* and *Campylobacter jejuni*. (Under the direction of Sophia Kathariou)

*Campylobacter* is the most common bacterial agent causing human gastroenteritis and is mainly transmitted through foods. *Campylobacter* is a zoonotic agent, and commonly colonizes poultry and other meat animals. Whereas erythromycin resistance in *Campylobacter jejuni* rarely occurs, erythromycin resistance in *Campylobacter coli* from meat animals is frequently encountered, and could represent a substantial barrier to antibiotic treatment of human infections. Erythromycin resistance in *C. coli* has been associated with a point mutation (A2075G) in the 23S rRNA gene, acting synergistically with the CmeABC efflux pump. However, the mechanisms responsible for possible dissemination of erythromycin resistance in *C. coli* remain poorly understood. In this study we investigated transformation-mediated acquisition of erythromycin resistance by genotypically diverse *C. coli* strains from turkeys and swine, with total genomic DNA from erythromycin-resistant *C. coli* used as donor. In addition, we studied the effects of environmental factors and species (*C. coli* vs. *C. jejuni*), and fitness costs of erythromycin resistance in transformants. Overall, transformation to erythromycin resistance was significantly more frequent in *C. coli* from turkeys than in swine-derived strains (P<0.01) with frequency of transformation $10^{-4}$ to $10^{-6}$ in turkey-derived strains, but $10^{-7}$ or less in *C. coli* from swine. Transformants harbored the point mutation, A2075G in the 23S rRNA gene. Erythromycin resistance was stable in transformants following serial transfers, and most transformants had high MIC values.
(>256µg/ml), as did the *C. coli* donor strains. In contrast to results obtained with transformation, spontaneous mutants had relatively low erythromycin MIC (32-64µg/ml) and lacked the A2075G mutation. Temperature profoundly affected frequency of transformation to erythromycin resistance in *C. coli* and transformation frequency at 42°C was significantly higher than at 25°C, 32°C and even 37°C. However, transformation to nalidixic acid resistance was not significantly affected by temperature. No significant difference in transformation frequency was detected between microaerobic (5-10% CO₂) and aerobic conditions. Starvation conditions did not affect transformation frequency to nalidixic acid resistance. Increasing incubation time from 3-4h to 15-17h significantly increased transformation frequency to erythromycin resistance (P<0.05). Transformation of *C. jejuni* using genomic DNA from erythromycin resistant *C. coli* revealed that transformation frequency of *C. jejuni* to erythromycin resistance was lower than *C. coli*, suggesting that erythromycin resistance in *C. coli* may not be disseminated via transformation in *C. jejuni* as frequently as in *C. coli*. Transformants derived from *C. jejuni*, however, had high erythromycin MIC values (>256µg/ml) and harbored the A2075G transition, similarly to *C. coli* transformants. When grown separately at 42°C, an erythromycin-resistant transformant derived from *C. coli* strain 961 had a similar growth rate as its erythromycin-sensitive parental strain, whereas an erythromycin-resistant transformant derived from *C. jejuni* strain SC49 had a significantly longer generation time compared to its parental strain. In competitive growth studies, however, the *C. coli* transformant was at competitive disadvantage in relation to its parental strain in stationary phase, whereas the *C. jejuni* transformant was at a slight fitness advantage after 14days. Furthermore, in the mixed
culture the generation time of the *C. jejuni* transformant was not significantly different from that of the parental strain. In conclusion, natural transformation has the potential to contribute to dissemination of high-level resistance to erythromycin among *C. coli* strains colonizing meat animals and temperature can greatly affect transformation to erythromycin resistance, but not to nalidixic acid resistance. These findings suggest that ecological attributes may play an important role and exert differential impact on the potential of the organism to acquire antimicrobial resistance determinants via natural transformation. However, further study is necessary to characterize the fitness of erythromycin resistant transformants in *Campylobacter* and identify possible mechanisms underlying the relatively low frequency of erythromycin resistance in *C. jejuni*. 
NATURAL TRANSFORMATION-MEDIATED TRANSFER OF ERYTHROMYCIN RESISTANCE IN *Campylobacter coli* AND *Campylobacter jejuni*

by

JOO-SUNG KIM

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

FOOD SCIENCE

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APPROVED BY:

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Chair of Advisory Committee
Biography

Joo-Sung (Paul) Kim was born in Seoul on May 26 in 1974 and is the first son of Kil-Yong Kim and Bok-Nyon Kim. He received his elementary, middle, and high school education in Seoul, South Korea. He graduated from On-Soo high school in February, 1993. He then entered Korea University at Seoul in March, 1993 and majored in food technology. He served in military at Uijeongbu, Gyeonggido from August, 1995 to October, 1997. He graduated from Korea University with a B. S. in food technology in division of life science in February, 2000. Then, he moved to United States and started his graduate study in department of food science and technology under the direction of Dr. F. Ann Draughon at the University of Tennessee, Knoxville. He was awarded his Master of Science degree in July, 2002 with the research of evaluation of enrichment methods for Yersinia enterocolitica O:3 and O:8. He has been pursuing Ph. D. in food science department under the direction of Dr. Sophia Kathariou at North Carolina State University from August, 2002.
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CHAPTER 1

Literature Review
Overview of *Campylobacter*

Since *Campylobacter jejuni* and *C. coli* were recognized as important agents for human gastroenteritis in the late 1970s (Engberg et al., 2001), *Campylobacter* species have emerged as one of the major causes of bacterial diarrhea in humans worldwide (Adak et al., 2005; Yan and Taylor, 1991). It is estimated that more than 2 million illnesses are due to *Campylobacter* infections annually in the USA (Mead et al., 1999).

*Campylobacter* is gram negative and microaerophilic. It is spiral or S-shaped in its normal state (Buck et al., 1983; Taylor, 1992), but the cells become spherical or coccoid upon prolonged exposure to air or in old cultures (Buck et al., 1983; Nachamkin et al., 2000b). The coccoid form is considered to be a degenerative state for this organism. Spiral cells are 0.5 to 5 µm long, and 0.2 to 0.8 µm wide (Vandamme, 2000). The colonies of *Campylobacter* spp. are normally grey, flat, irregular and spreading, particularly on freshly made media (Nachamkin et al., 2000b). Hippurate hydrolysis is a test which can differentiate between *C. jejuni* and *C. coli*, with *C. jejuni* being positive (Skirrow and Benjamin, 1980). A PCR method employing species-specific primers based on the hippuricase gene (*hip*) and the *ceuE* gene can be also used to specifically identify *C. jejuni* and *C. coli* (Gonzalez et al., 1997). Two serotyping schemes have been used for *Campylobacter*. The Penner scheme is based on heat-stable antigens (Penner and Hennessy, 1980) whereas the Lior scheme is based on heat-labile antigens (Lior et al., 1982). The heat-stable O-antigen in the Penner serotyping scheme is composed of capsular polysaccharide (Karlyshev et al., 2000).

*Campylobacter* has a single polar flagellum at one or both ends of the cell (Karmali and Skirrow, 1984). The flagellin subunits are encoded by two genes, *flaA* and *flaB* (Guerry et al.,
Genetic rearrangement within the genome or recombination by taking up exogenous DNA can occur, and this could be advantageous in pathogenesis and environmental adaptation (Wassenaar et al., 1995). Intraspecies recombination is frequent in *C. jejuni* (Suerbaum et al., 2001). Some *C. jejuni* and *C. coli* strains have methylated adenine at GATC sequences of their chromosomal DNA, suggesting that they may be able to also digest unmethylated foreign DNA (Edmonds et al., 1992).

In industrialized countries such as European nations and the Unites States, the incidence of *Campylobacter* infections is as high as 1% of the population per year (Wassenaar and Newell, 2000). *C. jejuni* is responsible for 80 to 90% of *Campylobacter* infection and *C. coli* for 5 to 10% (Nachamkin et al., 2000b). However, *C. coli* strains can comprise up to almost 20% among human clinical *Campylobacter* isolates (Luber et al., 2003) with symptoms typically as severe as those of infection due to *C. jejuni* (Skirrow and Blaser, 2000). *C. upsaliensis*, *C. lari*, and *C. fetus* can infrequently cause human illness (Vandamme, 2000). Fever, abdominal cramping, and diarrhea (with or without blood) are major symptoms of the illness (Nachamkin et al., 2000b). Especially serious is the life-threatening diarrhea that can occur among children (Crushell et al., 2004). The symptoms of *Campylobacter* infection are more severe in developed countries than in developing countries, possibly due to reduced exposure and low infection rates early in life in human populations in industrialized nations, compared to developing countries (Oberhelman and Taylor, 2000).

Consumption of undercooked poultry harboring *Campylobacter* is an important cause of *Campylobacter* infections in humans (Corry and Atabay, 2001; Eberhart-Phillips et al., 1997; Harris et al., 1986; Kapperud et al., 1992; Rosenfield et al., 1985; Tauxe, 1992). Cross-
contamination during food preparation can be also a substantial source for *Campylobacter* infection (Redmond and Griffith, 2003). Foreign travel, unchlorinated or environmental water, and unpasteurized or improperly pasteurized milk can be other sources of infection (Endtz et al., 2003; Leatherbarrow et al., 2004; Pebody et al., 1997; Smith et al., 1999). The lack of accurate information on the relative contributions of different sources to human infection is a barrier to effective disease-control measures (Dingle et al., 2002). Difficulties in epidemiological investigation are mainly due to the typically sporadic nature of human campylobacteriosis and frequent horizontal genetic exchanges between strains (Dingle et al., 2001; Dingle et al., 2002; Wassenaar et al., 1995; Wassenaar et al., 1998).

A seasonal variation in the level of *Campylobacter* can be significant in raw retail chickens (Meldrum et al., 2004). A peak occurred in June and the lowest level occurred in January, March, and December. Human infections appear to peak between spring and summer (Kovats et al., 2004; Nichols, 2005; Skirrow, 1987).

*Campylobacter* is normally commensal in poultry, swine and cattle (Miller and Mandrell, 2004). *Campylobacter* can typically not be isolated from broiler flocks during the first 10 days of life (Newell and Wagenaar, 2000), and flocks normally become infected when they are about 3 weeks old (Corry and Atabay, 2001). More than half of the chicken flocks can harbor *Campylobacter* spp. at slaughter (Cardinale et al., 2004), and the *Campylobacter* strains tend to be genotypically diverse (Hook et al., 2005). Cecal samples were more likely to have *C. jejuni* than samples from any other sites in the gastrointestinal tract (Cox et al., 2005). Raw chickens can frequently harbor *Campylobacter* (Corry and Atabay, 2001). Vertical transmission from breeders may take place, but does not appear to
be the primary transmission route (Corry and Atabay, 2001). The growth of Campylobacter in the chicken intestine can be greatly impaired by probiotic bacteria such as Lactobacillus (Chaveerach et al., 2004). Significant increases in the prevalence of C. coli was found in turkeys at the abattoir before slaughter, compared to prevalence before transport to abattoir (Wesley et al., 2005).

Pigs, cattle, and sheep are also frequently colonized with Campylobacter spp. (Wassenaar and Newell, 2000). C. jejuni is more common than C. coli among poultry and cattle, whereas the opposite is the case for swine (Aarestrup et al., 1997; Bae et al., 2005; Brown et al., 2004; Sáenz et al., 2000; Van Looveren et al., 2001). Significant variation in prevalence of C. coli was found among different pig farms (Payot et al., 2004b). Even though maternal C. coli strains initially infect the piglets, strains from other sources largely constitute the final profile of C. coli strains in swine (Alter et al., 2005). C. jejuni bacteriophage was isolated from broiler flocks and offers potential as a biological control agent. However, many C. jejuni strains were resistant to the bacteriophage (Connerton et al., 2004).

**Genome of Campylobacter**

The genome sizes of C. jejuni and C. coli are around 1.7 megabases (Mb) (Fouts et al., 2005). Major structural differences in the genomes of Campylobacter species are involved with inserted phage or genomic islands, including those harboring lipopolysaccharide biosynthesis genes (Fouts et al., 2005). The genomes of C. jejuni NCTC 11168 (human clinical isolate) and C. coli RM2228 (poultry isolate) do not harbor insertion sequences or
phage-associated sequences, whereas *C. jejuni* RM1221 (from chicken carcass) does (Fouts et al., 2005; Parkhill et al., 2000). Each genome contains about 1,600 to 1,800 open reading frames (ORFs) (Fouts et al., 2005). The two *C. jejuni* strains, NCTC 11168 and RM1221 are more closely related to each other than either is to *C. coli* RM2228 (Fouts et al., 2005). Average protein identity between *C. jejuni* RM1221 and *C. coli* RM2228 was 86% (Fouts et al., 2005). *C. upsaliensis* (75%) and *C. lari* (69%) are more distantly related to *C. jejuni* than *C. coli* is (Fouts et al., 2005). Of c.a. 1650 annotated genes, about 1300 genes appear to be common to all *C. jejuni* strains and these core genes encode mainly housekeeping functions such as metabolic, biosynthetic, cellular, and regulatory processes (Dorrell et al., 2001).

Genetic diversity is high in both *C. coli* and *C. jejuni*, which may be a key survival or pathogenesis mechanism for this microorganism in diverse host environments (Bereswill and Kist, 2002; Dingle et al., 2002; Dorrell et al., 2001; Leatherbarrow et al., 2004; Siemer et al., 2005). Extensive surface antigenic (flagella, lipopolysaccharide, and capsule) variability is especially found in *C. jejuni* (Dorrell et al., 2001). Frequent genetic exchange or rearrangements in the lipooligosaccharide genes seems to occur (Parker et al., 2005). Hypervariable homopolymeric repeat sequences consisting of polyG:C tracts were found in the *C. jejuni* NCTC11168 genome, especially in association with genes encoding surface antigens (Parkhill et al., 2000). They were found to be more common in *C. upsaliensis* RM3195 (Fouts et al., 2005). Genes responsible for iron acquisition, DNA restriction/modification, and sialylation were also diverse (Dorrell et al., 2001; Miller et al., 2005b). Type I restriction-modification (*hsd*) systems in *C. jejuni* could be assigned to one of three families (‘IAB’, ‘IC’, and ‘IF’), and considerable genetic diversity was detected in
‘IAB’, specifically in *hsdS* gene (Miller et al., 2005b). High variability in gene expression of *C. jejuni* NCTC 11168 was found in bacteria derived from different experimentally infected rabbits, indicating that it may be another mechanism to avoid host immune system (Stintzi et al., 2005). *C. jejuni* NCTC 11168 and its clonal variant showed large differences in phenotypes regarding motility, colonization, and invasion, including gene expression of respiration and metabolism genes (Gaynor et al., 2004).

The G+C content of the *Campylobacter* genus ranges from 29 to 35 % and it belongs to the lower end of the G+C content range among bacteria (Fouts et al., 2005; Neill et al., 1979). *C. jejuni* NCTC 11168 has constant G+C content of 30.6% over most of the genome, but interestingly it deviated from the average level in those loci where high strain-specific variations occur, which implies horizontal genetic exchange (Dorrell et al., 2001).

*Campylobacter jejuni* and *C. coli* possess three copies of the 23S and 16S rRNA genes (Kim et al., 1993; 1995; Taylor et al., 1992). Differentiation between *C. jejuni* and *C. coli* by sequencing 16S rRNA gene is impossible due to highly similar sequences, even though most of other species under *Campylobacter* genus can be discriminated with the method (Gorkiewicz et al., 2003).

The occurrence rate of plasmids in *Campylobacter* seems to be higher among chicken isolates (91%) than clinical isolates (44%) (Lee et al., 1994), and their size varies from 3.4 to 208 Kbp (Aquino et al., 2002; Bacon et al., 2000; Lee et al., 1994). Not all plasmids are associated with virulence (Schmidt-Ott et al., 2005).
Ecology of *Campylobacter*

It is unlikely that campylobacters grow in foods because they can grow only under unusual conditions, including increased carbon dioxide, reduced oxygen concentrations, and increased temperature. Dry conditions are especially hostile to *Campylobacter* (Corry and Atabay, 2001). Temperatures above 30°C and below 47°C allow the growth of *C. jejuni* and *C. coli* (Park, 2000), and 42°C is the optimal growth temperature of this species (Nachamkin et al., 2000b). In a study evaluating the susceptibility of *Campylobacter* to different temperatures, including freezer temperature (-20°C), refrigerator temperature (4°C), and room temperature (25°C), both *C. jejuni* and *C. coli* were most susceptible to -20°C and least susceptible to 4°C, both under microaerobic and aerobic conditions (Solow et al., 2003). Temperature significantly affects expression of many genes in *Campylobacter* (Stintzi, 2003). Significant up- or down-regulation of gene expression was found in 20% of the *C. jejuni* genes upon temperature upshift from 37°C to 42°C, but most of these expression level changes were transient (Stintzi, 2003). A significant increase in gene expression of *flaB* at 42°C compared to 37°C was also found in *Campylobacter* (Alm et al., 1993). Differentially expressed patterns of some genes involved in protein-glycosylation between 37°C and 42°C suggest a different membrane protein makeup at these two temperatures (Stintzi, 2003). Several heat shock proteins in *Campylobacter* have been found upon thermal stress (Konkel et al., 1998). DnaJ was one of proteins coping with thermal stress and was also found to be involved in colonization of chickens (Konkel et al., 1998). HtrA protease is required for tolerance to heat (44°C) and oxygen stress in *C. jejuni* (Brøndsted et al., 2005). Superoxide dismutase (SOD) encoded by gene *sodB* was found to be important for *C. coli* to survive
aerobically, to colonize chickens (Purdy et al., 1999), and to survive oxidative stress under freeze-thaw condition (Stead and Park, 2000). The change of fatty acid composition in the membrane of *C. jejuni* occurs when it enters stationary phase (Martinez-Rodriguez and Mackey, 2005).

**Antibiotic resistance**

Enormous amounts (18 million pounds annually) of antibiotics are estimated to be used for animal production (disease prevention and growth promotion) in the US and they may play a role in prevalence of antibiotic resistant bacteria (Roe and Pillai, 2003). Fluoroquinolones are commonly used to treat infections (e.g., *E. coli*) in poultry, whereas tylosin, tetracyclines, and sulfamethazine are used most commonly in swine production (McEwen and Fedorka-Cray, 2002).

*C. jejuni* and *C. coli* isolates are intrinsically resistant to several antibiotics such as bacitracin, novobiocin, rifampin, and vancomycin, but resistances to quinolones, tetracycline, and kanamycin are acquired (Taylor and Courvalin, 1988). Tetracyclines are used therapeutically or subtherapeutically in poultry (Smith et al., 2000). The resistance determinant *tet*(O) mediates tetracycline resistance in *Campylobacter* (Gibreel et al., 2004) through ribosomal protection (Taylor and Chau, 1996). The *tet*(O) gene can exist on plasmids or on the chromosome (Pratt and Korolik, 2005; Taylor et al., 1987). All three Kanamycin resistance determinants are also harbored by plasmids in *Campylobacter* spp. (Taylor, 1992).

Macrolides and fluoroquinolones are the first and second choice of drugs to treat *Campylobacter* enteritis in humans (Engberg et al., 2001). Upon introduction, the fluoroquinolones were recognized as a new approach to treat *Campylobacter* enteritis, but the
emergence of resistance to nalidixic acid and fluoroquinolones among clinical

*Campylobacter* isolates has been significantly increasing since the 1990s (Hoge et al., 1998; Luber et al., 2003; Lucey et al., 2002; Nachamkin et al., 2000b; Nachamkin et al., 2002; Payot et al., 2004b; Perez-Trallero et al., 1993; Rautelin et al., 1991; Reina et al., 1992; Smith et al., 2000). The use of quinolones in human and/or veterinary medicine appears to be responsible for the rapid increase of quinolone resistance in *Campylobacter* (Adler-Mosca et al., 1991; Endtz et al., 1991; Smith et al., 2000). The extensive use of these antibiotics in animal husbandry is of special concern (Li et al., 1998; Smith et al., 2000). Emergence of fluoroquinolone-resistant *Campylobacter* mutants was promoted by the enrofloxacin treatment in chickens infected with fluoroquinolone-sensitive *Campylobacter* (Luo et al., 2003). Furthermore, the mutants were highly resistant to ciprofloxacin (MICs > 32 µg/ml). In another study, fluoroquinolone resistant *C. jejuni* strains outcompeted fluoroquinolone sensitive parental strains or clonally related strains in the absence of antibiotic selection pressure, when the fluoroquinolone resistant and sensitive pairs were introduced into chickens (Luo et al., 2005). The enhanced fitness of fluoroquinolone resistant strains compared to sensitive strains was due to the point mutation on *gyrA* gene, responsible for the fluoroquinolone resistance. The rate of nalidixic acid resistance in *Campylobacter* isolated from broilers, swine, and cattle is substantial (10-80%) and tends to be higher in *C. coli* than *C. jejuni* isolates (Avrain et al., 2003; Bae et al., 2005; Li et al., 1998; Payot et al., 2004b; Pedersen and Wedderkopp, 2003; Van Looveren et al., 2001). Antibiotic resistant *Campylobacter* in food animals, resulting from the use of antimicrobials in animal husbandry can pass to humans through foods, contributing to the development or increase of antibiotic
resistance in clinical isolates (Perez-Trallero et al., 1993; Smith et al., 1999; Smith et al., 2000). Infections with quinolone or erythromycin (macrolide) resistant *Campylobacter* lead to much higher chance of invasive illness or death than quinolone or erythromycin sensitive *Campylobacter* (Helms et al., 2005).

A point mutation at codon 86 of *gyrA*, from ACT (Thr) to ATT (Ile) is responsible for high-level nalidixic acid and ciprofloxacin resistance in *Campylobacter* (Carattoli et al., 2002; Payot et al., 2004b; Wang et al., 1993). Asp-90-Asn and Thr-86-Lys point mutations can also be involved in resistance (Luo et al., 2003). The mutation at codon 86 can also act in combination with a single mutation, Arg-139-Gln in *parC* in *C. jejuni* (Gibreel et al., 1998).

Significant increases in ampicillin and tetracycline resistance as well as fluoroquinolone resistance among clinical isolates have been described (Luber et al., 2003). Multidrug resistance (ex. coresistance to nalidixic acid, erythromycin, and tetracycline) in *Campylobacter* spp. also appears to have become more common (Nachamkin et al., 2000b; Payot et al., 2004b). Multidrug resistance is more common in *C. coli* than *C. jejuni* (Bae et al., 2005; Burgess et al., 2005; Van Looveren et al., 2001). Such resistance was more likely to occur in farms with continuous-flow systems than a strict all-in-all-out system (Schuppers et al., 2005). For antimicrobial susceptibility, the outer membrane of gram-negative cells can be an important barrier to antibiotics, and the porin size of *C. coli* is smaller than *C. jejuni* (Page et al., 1989).

A multidrug efflux system prohibits the antimicrobial agents from reaching the targets by pumping out the antibiotics (Kohler et al., 1999). In *C. jejuni* and *C. coli*, this is another
mechanism for antimicrobial resistance, and two efflux systems are known, CmeABC and CmeDEF (Lin et al., 2002; Payot et al., 2004a; Pumbwe et al., 2005). The system is involved with the resistance of *Campylobacter* to various kinds of antimicrobials, including antibiotics, detergents and ethidium bromide (Lin et al., 2002; Pumbwe et al., 2005). The CmeABC is essential for resistance to bile salts and intestinal colonization of chickens (Lin et al., 2003). CmeB mutants showed significantly reduced MIC of ciprofloxacin and enrofloxacin (Luo et al., 2003), as well as erythromycin and other macrolides (Cagliero et al., 2005). The CmeABC efflux pump system plays a significant role in erythromycin resistance among *C. coli* isolates and tends to be more effective for erythromycin, than for fluoroquinolones (Ge et al., 2005; Payot et al., 2004a). However, fluoroquinolones or erythromycin are not substrates for CmeDEF, which instead has been implicated in resistance to ampicillin, ethidium bromide, acridine, sodium dodecyl sulfate (SDS), deoxycholate, and triclosan (Pumbwe et al., 2005). Efflux pump systems other than CmeABC and CmeDEF appear to exist in *Campylobacter* (Pumbwe et al., 2005), and analysis of eight other putative efflux pumps indicated that none was involved in antibiotic resistance (Ge et al., 2005).

**Erythromycin resistance**

*Campylobacter* has three copies of the ribosomal RNA operon (6 kilobases [kb]) (Parkhill et al., 2000). Changes in rRNA genes play an important role in the emergence of antibiotic resistant strains (Weisblum, 1995).

Erythromycin (macrolide) is used in treatment of diarrhea caused by *Campylobacter*. It inhibits protein synthesis at the 50S subunit of the ribosome (Madigan et al., 1997). The frequency of erythromycin resistance among *C. coli* strains is much higher than among *C.*
jejuni strains (Chuma et al., 2001; Engberg et al., 2001; Ge et al., 2003; Luber et al., 2003; Thwaites and Frost, 1999). The frequency of erythromycin resistance among C. coli strains isolated from swine (30 to 80%) and chickens (up to 60%) is substantial (Avrain et al., 2003; Bywater et al., 2004; Ishihara et al., 2004; Payot et al., 2004b; Sáenz et al., 2000; Van Looveren et al., 2001). Erythromycin resistance in C. coli strains from turkeys at retail is also significant (Ge et al., 2003; Luber et al., 2003). Both C. coli and C. jejuni isolates from turkey meat at retail showed higher rates of resistance to ciprofloxacin and erythromycin than those from chicken meat (Ge et al., 2003). However, the frequency of erythromycin resistance among human clinical isolates appears to remain low in most areas (Engberg et al., 2001; Gupta et al., 2004), even though increased frequency of erythromycin resistance among clinical isolates is reported in some areas (Rao et al., 2005).

The mechanism of erythromycin resistance in Campylobacter is associated with reduced affinity of erythromycin to ribosomes (Yan and Taylor, 1991). Erythromycin resistance in both C. coli and C. jejuni is chromosomally mediated (Yan and Taylor, 1991). A point mutation (A2075G) (based on 23S rRNA gene of C. jejuni NCTC 11168, equivalent to A2059G in Escherichia coli) in the 23S rRNA gene is associated with erythromycin resistance in C. coli and C. jejuni (Gibreel et al., 2005; Harrow et al., 2004; Jensen and Aarestrup, 2001; Vacher et al., 2003). In C. coli, only the A2075G transition has been found to be involved with erythromycin resistance. In C. jejuni, however, not only the A2075G transition, but also either a A2074C transversion or a A2074G transition in the 23S rRNA gene can be associated with erythromycin resistance, even though A2075G appears to be most common (Gibreel et al., 2005; Vacher et al., 2003). The A2075G transition in at least
two out of 3 copies of 23S rRNA genes appears to be necessary to confer macrolide resistance (Gibreel et al., 2005). Two point mutations, A2075G and A2074C conferring macrolide resistances were stable through repeated subcultures (Gibreel et al., 2005). The A2075G transition resulted in a wide range of MIC (from 32 to >1,024 µg/ml) (Gibreel et al., 2005; Jensen and Aarestrup, 2001; Vacher et al., 2003) whereas A2074C appears to give a narrower range of MIC (>512 µg/ml) (Gibreel et al., 2005). Erythromycin resistance in some, but not all, isolates, can be also associated with a multidrug efflux pump system (Gibreel et al., 2005; Mamelli et al., 2003). Both point mutation (A2075G) and CmeABC efflux pump were required to have high-level erythromycin resistance (Cagliero et al., 2005). Relatively low MIC (<128 µg/ml) values of strains with the A2075G transition may be due to the absence of the CmeABC efflux pump (Cagliero et al., 2005). Another efflux pump involved in macrolide resistance, independent of CmeABC appears to exist (Mamelli et al., 2005).

**Fitness cost of erythromycin (macrolide) resistance**

Antibiotic resistance can result in reduced fitness of bacteria in the absence of selective pressure in some, but not all, bacterial species. A macrolide-resistant *H. pylori* strain with point mutation (A to G) in the 23S rRNA gene showed similar growth rate as a clonally-related macrolide-sensitive strain when cultured separately, but had reduced fitness in competitive growth with the sensitive strain (Kanai et al., 2004). In *Mycobacterium smegmatis*, however, macrolide-resistant point mutations, 23S rRNA 2058A→G, or 23S rRNA 2059A→G did not affect fitness (Sander et al., 2002). In *Campylobacter*, naturally transformed *C. jejuni* 81116 with 300bp PCR product containing the point mutation A2059G
or A2058C (numbered based on *E. coli*) in 23S rRNA gene showed similar growth rates with its parental strain 81116 (Gibreel et al., 2005).

The burden of fitness cost caused by chromosomal mutations can be ameliorated through compensatory mutations, rather than via higher-fitness, drug-sensitive revertants (Levin et al., 2000). This is in agreement with the observation of higher compensatory mutation rate than the rate for revertants. Because relatively small number of cells are subcultured during serial passage- so called bottleneck effect, compensatory mutants are more likely to be transferred than drug-sensitive revertants due to higher number of organisms.

**Pathogenesis**

Colonization of the lower gastrointestinal tract by *Campylobacter* induces symptoms ranging from mild discomfort to acute gastroenteritis and bloody diarrhea in humans (Konkel et al., 2000). Diarrhea can last from five to seven days (Svedhem and Kaijser, 1980). In severe cases, septicemia can occur (Konkel et al., 2000). *Campylobacter* infection can occur in all age groups but is especially prevalent in children younger than 1 year and young adults between 15 and 44 years (Friedman et al., 2000; Svedhem and Kaijser, 1980). It shows relatively low fatality rate compared to other bacterial enteric pathogens (Friedman et al., 2000). It is self-limiting, even though reoccurrence of *Campylobacter* is possible (Svedhem and Kaijser, 1980). The infection normally stops in less than 2 weeks (Konkel et al., 2000). Incubation time normally ranges between two and five days (Karmali and Fleming, 1979) and the average onset time is 3.2 days (Konkel et al., 2003). The infectious dose can be as low as 500 organisms (Robinson, 1981).
In spite of the high prevalence of Campylobacter in human bacterial gastroenteritis, the pathogenesis of this microorganism is relatively poorly understood (Dorrell et al., 2001). To initiate infection, Campylobacter must penetrate the gastrointestinal mucus, and adhere to the gut enterocytes (Wallis, 1994).

The adherence of Campylobacter to the intestinal cells seems to be an important factor for virulence and C. jejuni strains from patients showed greater efficiency in binding to cultured cells (HeLa cells) than strains from asymptomatic individuals (Fauchere et al., 1986). C. jejuni swims straight with high velocity in a viscous environment, which may be important for the interaction with host epithelial cells (Szymanski et al., 1995). C. jejuni and C. coli have surface-exposed molecules, called adhesins, which facilitate binding to host cells (Pei and Blaser, 1993; Konkel et al., 2003), but these molecules seem to be expressed constitutively regardless of interaction with host cells (Konkel and Cieplak, 1992). The adhesins include PEB1, CadF, and JlpA (Konkel et al., 2003). A C. jejuni mutant of peb1A, a putative binding component of ABC transport, showed 50 to 100 fold less adherence to and about 15 fold less invasion of epithelial cells (Pei et al., 1998). The CadF proteins (37kDa) in C. jejuni and C. coli promote the binding of these pathogens to fibronectin, a component of the extracellular matrix in intestinal epithelial cells (Konkel et al., 1997; Monteville et al., 2003). The flagellum (Wassenaar et al., 1993), lipopolysaccharides (McSweegan and Walker, 1986), and the major outer membrane protein (MOMP) (Schröder and Moser, 1997) are also considered to be adhesins. The flagellum is an important motility and colonization factor in the chicken intestine (Nuijten et al., 2000). C. jejuni galE gene is involved in lipopolysaccharide biosynthesis and the galE mutant showed a reduction in adhering to and
invading INT407 cells (Fry et al., 2000). In addition, protein glycosylation is important in *C. jejuni* pathogenesis (Szymanski et al., 2002). Bile resistance appears to be required for *Campylobacter* to colonize animals (Lin et al., 2003; Raphael et al., 2005), and it was found to be mediated by the response regulator, CbrR (Raphael et al., 2005), as well as the efflux pump CmeABC (Lin et al., 2003; Pumbwe et al., 2005). Iron is an important factor for the pathogenesis of many human bacteria and the iron homeostasis may play a significant role in *Campylobacter* pathogenesis, including colonization (Palyada et al., 2004). In addition to chromosomal genes, plasmids can be involved in pathogenesis, including adherence and invasion (Bacon et al., 2000).

The attachment of *Campylobacter* on human epithelial cells can lead to internalization (de Melo et al., 1989). Internalization of *C. jejuni* is reduced at low temperature (Konkel et al., 1992). Cell invasion by *C. jejuni* is responsible for colon damage and diarrheal disease (Russell et al., 1993). Release of toxins, including enterotoxin and cytotoxin by *Campylobacter* adhering on epithelial cells can also induce diarrhea (Wallis, 1994).

Host cell signaling pathways are common in many invasive enteric bacteria such as *Salmonella typhimurium* and *Shigella flexneri* (Hu and Kopecko, 2000). These pathogens exploit the host cell machinery for invasion. Upon association with human intestinal epithelial cells, they secrete effector proteins into the host cells. This initiates host signal transduction events resulting in engulfment of the bacteria (Hu and Kopecko, 2000). Upon contact with host cells, *C. jejuni* F38011 was found to secrete eight proteins, including *Campylobacter* invasion antigen B (CiaB protein, ~73 kDa) (Konkel et al., 1999). CiaB protein has similarity with type III secreted proteins associated with host invasion in other
bacterial pathogens and is required for host internalization, but not for binding (Konkel et al., 1999). It is translocated into host cells and is also essential for the secretion of eight other proteins (Konkel et al., 1999). Flagella export apparatus is required for Cia protein secretion (Grant et al., 1993; Konkel et al., 2003; Konkel et al., 2004). Non-motile strains lose invasion capacity (Yao et al., 1994). The activation of protein tyrosine kinases and calcium ion release in host cells is a common signal transduction pathway and it was observed upon *C. jejuni* infection (Bereswill and Kist, 2002; Hu and Kopecko, 2000). It is still unclear, however, whether this is a major virulence mechanism among *Campylobacter* strains. Parasite-directed endocytosis process is used by *Campylobacter* for internalization (de Melo et al., 1989; Hu and Kopecko, 2000; Konkel et al., 2003). Most *C. jejuni* strains require host cytoskeletal structures composed of actin microfilaments (MFs), or microtubules (MTs), or both MTs and MFs to enter host cells (Hu and Kopecko, 2000).

Translocation is an important virulence aspect because *Campylobacter* can gain access to underlying tissues and spread throughout a host (Konkel et al., 2003). The ability of translocation does not correlate with the ability of invasiveness among *Campylobacter* strains (Harvey et al., 1999). The major route of *C. jejuni* translocation in polarized cell monolayers is still unclear (Konkel et al., 2003). *In vivo* translocation of *C. jejuni* through the intestinal epithelial cells has not been described (Konkel et al., 2003). *C. jejuni* can reach the lamina propria, and then reach different cellular receptors and professional phagocytic cells (Konkel et al., 2003). The occurrence of bacteremia in *Campylobacter* infection is low, ca. 0.4 % (Allos and Blaser, 1995), suggesting that the host’s immune system is effective in preventing the spread of the infection.
Intestinal pathogens can stay unaffected inside macrophages, acquire nutrients and be protected from immune surveillance (Konkel et al., 2003). If the pathogens survive in these cells, they can propagate in the host and eventually result in a carrier state, which can infect other individuals (Konkel et al., 2003). Catalase is important for the survival of *C. jejuni* in macrophages (Day et al., 2000). *C. jejuni* can be resistant to human monocytes and cause a bacteremia (Wassenaar et al., 1997). *C. jejuni* was resistant to guinea-pig resident peritoneal macrophages whereas *C. coli* was phagocytosed and killed (Banfi et al., 1986).

The clinical manifestations by *C. jejuni* depend on the susceptibility of host and the virulence of the infecting strains (Wallis, 1994). Both the cellular and humoral immune response occurs upon infection with *C. jejuni* (Konkel et al., 2000). *Campylobacter* outer membrane proteins and lipopolysaccharides (LPS) are strongly immunogenic and stimulate the production of antibodies (Konkel et al., 2000). Professional phagocytes are critical for blocking *C. jejuni* infection, as are T-cell dependent antibody responses (Konkel et al., 2000). *C. jejuni*-specific secretory immunoglobulin A (sIgA) is important to prevent the immediate spread of infection (Konkel et al., 2000). Lack of sIgA production limits the resolution of *Campylobacter* infection (Konkel et al., 2000).

**Strain variation in virulence**

The ability of *C. jejuni* to attach on and invade cultured cells is strain-dependent (Konkel et al., 2003; Konkel and Joens, 1989). Clinical strains were more invasive in Hep-2 cells than nonclinical strains (Konkel and Joens, 1989). In another study, clinical strains tend to be more invasive and cytotoxic than environmental strains isolated from water (Newell et al., 1985). Intracellular survival of *C. jejuni* varies depending on the macrophage cell types.
and *C. jejuni* strains (Konkel et al., 2003). In the bloodstream of chicken embryos, *C. jejuni* virulent strains were cleared significantly more slowly than avirulent strains and this was correlated with the lower rate of phagocytosis of virulent strains compared to avirulent strains *in vitro* (Field et al., 1991).

**Toxins**

Cytolethal distending toxin (CDT) is one of the *Campylobacter* cytotoxins and its mechanism involves cell cycle arrest in the G2 phase of the eukaryotic cells (Pickett, 2000). It causes HeLa, Chinese hamster ovary (CHO), and Hep-2 cells to slowly distend and then die (Johnson and Lior, 1988). About 40% of *C. jejuni* and *C. coli* appear to produce CDT (Johnson and Lior, 1988). Almost all *C. jejuni* strains isolated from turkeys, however, produced CDT in another study (Bang et al., 2004). The toxigenic effect of CDT increased upon pre-exposure of *Campylobacter* to subinhibitory concentration of erythromycin (Ismaeel et al., 2005). CDT consists of three protein subunits, CdtA, CdtB, and CdtC (Pickett, 2000). CdtB has deoxyribonuclease activity and is at least partially responsible for the activity of the CDT toxin (Lara-Tejero and Galan, 2000). Gastrointestinal lesions caused by a *C. jejuni* mutant lacking CDT were less severe than those caused by the isogenic wild-type strain, suggesting that CDT may have proinflammatory activity *in vivo* (Fox et al., 2004). *C. jejuni cdtdB* mutant strains showed reduced invasiveness into blood, spleen, and liver tissues in mice (Purdy et al., 2000). LPS, also called endotoxin, contains toxic phosphorylated glycolipids found in gram-negative bacteria, including *Campylobacter* (Moran et al., 2000).
Sequelae of infection

It has been recognized that *C. jejuni* infection is the most common antecedent to Guillain-Barré syndrome (GBS), an autoimmune-mediated disorder of the peripheral nervous system (Nachamkin et al., 2000a). GBS-associated *C. jejuni* isolates may share specific components of LPS. In *Campylobacter* LPS, a terminal core trisaccharide of Neu5Ac-Neu5Ac-Gal mimics the structure of human gangliosides (Penner and Aspinall, 1997). GBS involves the production of antibodies induced by *C. jejuni* surface lipopolysaccharide and lipoooligosaccharides and active against host ganglioside (Bereswill and Kist, 2002). In addition, arthritis and Reiter syndrome are postinfectious complications of campylobacteriosis (Bereswill and Kist, 2002).

Animal models in study of virulence properties

Mice and chickens are most commonly used in the study of *C. jejuni* colonization (Konkel et al., 2003). The mouse model is relatively inexpensive to maintain and operate, and allows for application of numerous reagents (such as monoclonal antibodies) to study the host response after colonization. The chicken model is ideal for the study of *Campylobacter* adherence. To study *Campylobacter* infection, young weanling ferret has been most commonly used. It is a good model to study adherence of *Campylobacter* (Konkel et al., 2003). The piglet model is adequate to study symptoms and disease because it more readily mimics those in humans (Konkel et al., 2003).

Natural transformation

Natural transformation is one of three key mechanisms of DNA transfer in bacteria (Prescott et al., 1999). Transformation is the first discovered mechanism for genetic
exchange in bacteria (Snyder and Champness, 1997). In 1928, Griffith could obtain pathogenic *Streptococcus pneumoniae* cells when the live nonpathogenic cells were mixed with dead pathogenic cells. Later, the transformation of the pneumococci was ascribed to DNA (Avery et al., 1944). Transformation requires several proteins and is highly regulated (Dubnau, 1999). Bacteria may take up foreign DNA as a carbon and nitrogen source or for repairing damaged DNA of their own (Snyder and Champness, 1997). Also, recombination following transformation increases genetic diversity and speeds up evolution (Snyder and Champness, 1997). For example, *Helicobacter pylori* is one of the most genetically diverse bacterial species (Suerbaum et al., 1998) and transformation is the only known mechanism of genetic exchange for this species (Dubnau, 1999). In *Neisseria gonorrhoeae*, the antigenic variations by natural transformation may enable the bacteria to avoid host immune responses (Meyer et al., 1990; Seifert et al., 1988).

Natural competence is a physiological state, which permits the efficient uptake of macromolecular DNA by bacteria (Dubnau, 1999). Competence is common and found in more than 40 bacterial species (Lorenz and Wackernagel, 1994).

**The development of competence in *Bacillus subtilis***

The mechanism for development of competence in *Bacillus subtilis* is well studied. In *B. subtilis*, competence is achieved at the end of the exponential growth phase (Albano et al., 1989; Provvedi and Dubnau, 1999). Competence development is initiated by two extracellular signaling peptides, the ComX pheromone and competence stimulating factor (CSF) which are secreted by bacteria during growth (Snyder and Champness, 1997; Solomon et al., 1995). Cells become competent only in the presence of high concentration of the
pheromone. Two different sensing pathways exist for the responses to the ComX pheromone and CSF (Solomon et al., 1995). In the pathway of ComX pheromone, the information related to high population or low nutrients is registered in ComP, a sensor protein (Snyder and Champness, 1997). Then, the ComP is phosphorylated and the phosphate is transferred to ComA, a transcription factor which allows transcription of competence genes. In the case of CSF, the Spo0K oligopeptide permease is required for transport of CSF into the cell (Magnuson et al., 1994). It is required for both competence and sporulation (Snyder and Champness, 1997) and the activity of CSF is concentration-dependent (Lazazzera et al., 1999). It stimulates the development of competence at low concentrations, and it inhibits competence but stimulates sporulation at high concentrations.

**Binding and uptake of DNA in Gram-Positive bacteria**

The binding of double-stranded DNA to the cell surface is the first step in transformation (Dubnau, 1999). In *B. subtilis*, no nucleotide sequence preference occurs during the binding events (Dubnau, 1999). There are a limited number of uptake sites per cell. In *B. subtilis* and *Streptococcus pneumoniae*, the bound double-stranded DNA are fragmented, and the estimated size of the fragmented DNA is 13.5 -18 kilobases and 6 kilobases, respectively (Dubnau, 1999). In *S. pneumoniae*, single-strand nicks occur before double-strand breaks (Lacks, 1979). Single-stranded donor DNA is internalized into recipient cells (Bodmer and Ganesan, 1964). The internalized strand replaces the strand of the same sequence in the recipient chromosome, in the process of homologous recombination (Snyder and Champness, 1997), thus it forms a heteroduplex of base-paired donor and recipient strands (Strauss, 1970). The non-transported strand is degraded.
In *B. subtilis*, the responsible nuclease for the degradation of the non-transported strand is probably localized outside the membrane or within an aqueous channel (Dubnau, 1999). The entry process of bound donor DNA into recipient cells is very quick and it takes a short lag period of about 1 min at 37°C (Levine and Strauss, 1965). The donor DNA is taken up in the linear form (Dubnau, 1999), and the average size of the integrated donor DNA is between 8.5 and 10kb (Dubnau and Cirigliano, 1972). The donor DNA is estimated to pass into the cytosol at the rate of about 180 nucleotides/s at 28°C (Dubnau, 1999). *B. subtilis* does not have a preference between the two strands in the uptake (Vagner et al., 1990). In *B. subtilis*, the proton driving force, especially ΔpH is responsible for DNA uptake (van Nieuwenhoven et al., 1982).

In *S. pneumoniae*, the transport mechanism of donor DNA is similar to *B. subtilis*. *S. pneumoniae* does not seem to have base sequence preference for binding (Dubnau, 1999), and only a single strand of donor DNA is inserted into the cells (Méjean and Claverys, 1984). The entry of donor DNA starts from the newly formed 3’ end by double strand breaks and proceeds to 5’ end (Méjean and Claverys, 1988). On the other hand, the degradation of the non-transported strand starts from the 5’ end and proceeds to 3’ end and its rate is similar to the rate of uptake of the inserted strand, estimated as 90-100 nucleotides/s at 31°C (Méjean and Claverys, 1993).

**Binding and uptake of DNA in Gram-Negative bacteria**

*Haemophilus influenzae* and *Neisseria gonorrhoeae* are naturally competent and transformable bacteria and have been well studied (Snyder and Champness, 1997). In *H. influenzae*, competence is optimal in late log phase (Redfield, 1991), and transformation
efficiency is enhanced in nutrient-depleted medium (Herriott et al., 1970). Both *H. influenzae* and *N. gonorrhoeae* require species-specific uptake sequences in the donor DNA for transformation (Danner et al., 1980). The recognition sequences for *H. influenzae* and *N. gonorrhoeae* are the 11bp sequence 5’-AAGTGCGGTCA-3’ and the 10bp sequence GCCGTCTGAA, respectively (Danner et al., 1980; Elkins et al., 1991). These uptake sequences appear much more frequently in the genome than would be expected (Dubnau, 1999). The uptake sequences for *H. influenzae* are often found as inverted repeats between genes and may be capable of forming stem-loop structures in mRNA for transcription termination (Smith et al., 1995). The uptake rate in *Haemophilus* was estimated between 500 and 1000 nucleotides/s (Kahn and Smith, 1984), and the cells have 4 to 8 active sites for uptake of donor DNA (Deich and Smith, 1980). *H. influenzae* has a periplasmic compartment, called ‘transformasomes’ in which double-stranded donor DNA is sequestered early in the transformation pathway (Snyder and Champness, 1997). The double-stranded DNA in ‘transformasomes’ is inaccessible by external DNase and internal restriction and modification enzymes (Barany and Kahn, 1985). In *H. influenzae*, DNA uptake may be driven by both components, ΔpH and electrical potential of the proton motive force in a proton-DNA symport mechanism (Bremer et al., 1984).

The entry of donor DNA to transformasomes involves double-stranded DNA, but the integration to recipient chromosomes involves only single-stranded DNA (Notani and Goodgal, 1966) and it accompanies the degradation of the other strand upon exiting transformasomes (Barany et al., 1983; Dubnau, 1999). In *N. gonorrhoeae*, circular plasmid DNA from donor is fragmented to linear double-stranded DNA during uptake (Biswas et al., 1999).
However, not all gram-negative bacteria show uptake sequence specificity. For instance, *Acinetobacter calcoaceticus* can take up DNA from any source (Palmen et al., 1993).

**Competence proteins**

Several proteins required for DNA binding and uptake have been characterized. *B. subtilis* is well studied in this area. In *B. subtilis*, DNA binding to the cell surface and subsequent internalization are mediated by several proteins, and the proteins are regulated by competence transcription factor ComK (Dubnau, 1999; van Sinderen et al., 1995). Mutants lacking these proteins had significantly reduced transformation (Hahn et al., 1987). *comE* is required for DNA binding to the competent cells and for DNA uptake (Hahn et al., 1993). The *comE* operon contains four open reading frames and the *comE* transcript is at high level in the stationary phase (Hahn et al., 1993). In the *comE* operon, *comEA* and *comEC* are required for transformability (Inamine and Dubnau, 1995). *comEA* is required for the binding of DNA to the competent cell surface (the receptor for DNA binding) and for internalization, and it encodes an integral membrane protein (Inamine and Dubnau, 1995). The *comEC* is only required for internalization (Inamine and Dubnau, 1995). ComEA has high-affinity for DNA binding and ComEA lacking the helix-turn-helix motif shows no detectable DNA-binding activity, implying that this motif is a DNA receptor for transformation (Provvedi and Dubnau, 1999). It prefers double-stranded to single-stranded DNA (Provvedi and Dubnau, 1999). ComG (Albano et al., 1989) and ComC (Mohan et al., 1989) are also essential for DNA binding to cell surface. All seven ComG proteins are membrane-associated (Chung et al., 1998) and required for DNA binding (Chung and Dubnau, 1998). These proteins may
provide donor DNA with easy access to complex and thick cell walls containing plenty of teichoic acid polyanions (Dubnau, 1999). They may increase porosity and shield negative charges on the cell wall. These competence proteins resemble proteins for the assembly of type-4 pili and for protein secretion in *Pseudomonas aeruginosa* (Hobbs and Mattick, 1993) and other bacteria, and are thus designated PSTC proteins (Pilus, Secretion, Twitching motility, and Competence) (Dubnau, 1999). The ComF1 protein is located on the cell membrane and is required for DNA uptake during transformation, but not for DNA binding (Londoño-Vallejo and Dubnau, 1994).

In *N. gonorrhoeae*, a natural transformation is functionally related to the processes of type-4 pilus biogenesis (Fussenegger et al., 1997). The components of pilus and its formation machinery are directly or indirectly related to DNA transport across the outer membrane (Fussenegger et al., 1997). PilE, the major pilus subunit and PilC, a pilus biogenesis factor, are essential for DNA uptake and act at the cell surface (Rudel et al., 1995). PilQ, an outer membrane secretin which is a pore-forming protein, is required for competence because it enables DNA to efficiently enter the cells (Long et al., 2003). PilG (Tonjum et al., 1995) and PilT (a cytoplasmic protein involved in pilus retraction) (Wolfgang et al., 1998) are also involved in pilus biogenesis and competence development. PilT is unnecessary for DNA binding, and is specifically involved with DNA uptake (Aas et al., 2002). There are also other competence proteins unrelated to pilus biogenesis. They are involved in steps such as DNA translocation across the peptidoglycan layer or the inner membrane after DNA uptake (Fussenegger et al., 1997). They are ComL (Fussenegger et al., 1996a), ComA (Facius and Meyer, 1993) and Tpc (Fussenegger et al., 1996b). ComL is a peptidoglycan-linked
lipoprotein, and ComA is associated with the inner membrane and with DNA translocation into the cytoplasm. ComA is similar to ComEC in *B. subtilis* (Fussenegger et al., 1997). The Tpc mutant shows not only cell separation defects but also reduced transformation, and is also deficient in epithelial cell invasion (Fussenegger et al., 1996b).

Several competence proteins essential for transformation were found also in *S. pneumoniae* and they shared homology with competence proteins in other bacteria (Campbell et al., 1998). CilA is similar to the single-stranded DNA binding protein (SSB) of *B. subtilis* (Campbell et al., 1998), and one type of SSB is required for optimal transformation (Lindner et al., 2004). CilB is similar to the competence protein DprA in *H. influenzae*, and CilC is similar to ComC in *B. subtilis* (Campbell et al., 1998). The CilD operon encodes at least two proteins which are similar as ComGA and ComGB in *B. subtilis* (Campbell et al., 1998). CilE refers to ComEA and ComEC in *B. subtilis* (Campbell et al., 1998). The EndA protein is located in the cell membrane (Lacks and Neuberger, 1975) and has a signal sequence to anchor the protein to the membrane (Puyet et al., 1990). It has deoxyribonuclease and endonuclease activity and is required for degradation of the non-transported strand and for the insertion of complementary strand (Lacks et al., 1975; Puyet et al., 1990). No competence protein with nuclease activity required for the entry of donor DNA was found in other bacteria (Dubnau, 1999).

In *H. influenzae*, DprA may be an inner membrane protein and may be involved in the insertion and recombination of donor chromosomal DNA (Karudapuram et al., 1995). The *dprABC* genes are transcriptionally coregulated and competence inducible, and require the product of the *tfoX* (*sxy*) gene for transcriptional activation (Karudapuram and Barcak, 1997).
Por (a periplasmic protein disulfide oxidoreductase) is required for the correct assembly or folding of disulfide-containing cell envelope proteins involved in competence development or DNA binding (Tomb, 1992). The adenylate cyclase gene (*cya*) (Dorocicz et al., 1993) and the gene encoding the cAMP-receptor protein, *crp* (Chandler, 1992) are also required for competence development in *H. influenzae*.

**Transformation in Campylobacter**

Some *C. coli* and *C. jejuni* strains can be naturally transformed by naked DNA without any treatments whereas some strains remain non-competent (Wang and Taylor, 1990; Wassenaar et al., 1993). All of five *C. coli* strains and three of six *C. jejuni* strains were naturally transformable to nalidixic acid and/or streptomycin resistances in one study (Wang and Taylor, 1990). In another study, three of four *C. jejuni* strains were transformable with isogenic chromosomal DNA to fluoroquinolone resistance (Wilson et al., 2003). Transformation frequencies for chromosomal DNA can reach up to $10^{-3}$ per recipient cell for both *C. coli* and *C. jejuni* (Wang and Taylor, 1990; Wilson et al., 2003). *C. jejuni* has a preference for DNA of its own species (Wilson et al., 2003), but can still be naturally transformed by DNA from *C. coli* (Alm et al., 1991). Transformation frequency of *C. jejuni* still greatly varies depending on *C. jejuni* donor strains (Wassenaar et al., 1993; Wilson et al., 2003). Transformation frequency can vary depending on CO$_2$ concentration (Wilson et al., 2003). Frequency of transformation of *C. jejuni* 81-176 to chloramphenicol resistance was significantly higher in an atmosphere with 0.7% CO$_2$ than 10% CO$_2$. Transformation frequencies seem to be highest during early log phase (Wang and Taylor, 1990; Wilson et al.,
2003), and a significant decrease of transformation frequency at high cell density (\( >10^8 \) c.f.u./ml) was observed in the presence of 5 -10% CO\(_2\) (Wilson et al., 2003).

Transformation of *Campylobacter* with chromosomal DNA is much more efficient than with plasmid (Taylor, 1992). Sequence length requirement for homologous recombination during natural transformation is as short as 200 to 300 bp in both *C. jejuni* and *C. coli* and the increased length of homologous sequences tends to increase transformation efficiency (Richardson and Park, 1997; Wassenaar et al., 1993). DNA on introduced plasmids with little or no DNA homology to chromosomal sequences can still be integrated at random into the *C. coli* genome (Richardson and Park, 1997).

Natural competence in *C. jejuni* was influenced by a gene, *galE*, involved in lipopolysaccharide biosynthesis (Fry et al., 2000), and the plasmid-borne gene *comB3* (*virB10*) (Bacon et al., 2000). VirB10 is a glycoprotein, and the N97-linked glycosylation of VirB10 is necessary for full competence (Larsen et al., 2004). *comB3* (*virB10*) showed homology to the component of type IV secretion systems which is essential for natural transformation in *H. pylori* (Bacon et al., 2000; Hofreuter et al., 1998; Hofreuter et al., 2001). The *comB3* is also related to invasiveness (Bacon et al., 2000).

Other competence genes on the chromosome in *C. jejuni* are likely to be involved in steps such as DNA uptake, DNA transport across inner membrane and recombination into the chromosome. Such genes were identified by a genetic approach, which identified the insertionally inactivated genes in *C. jejuni* transposon insertion mutants with impaired transformation ability in *C. jejuni* (Wiesner et al., 2003). *C. jejuni* seems to use certain components similar to those of other transformation systems as well as certain novel
components. The mutants in 11 genes showed about $10^3$-fold reduced transformation frequency. Some of these genes encode proteins homologous to those of type II secretion systems, biogenesis of type IV pili and competence (PSTC) in other bacteria (Fouts et al., 2005; Wiesner et al., 2003) even though *Campylobacter* does not seem to produce a pilus (Gaynor et al., 2001). The type II secretion system is involved in the extracellular secretion of toxins and hydrolytic enzymes, and in the biogenesis of type IV pilus (Sandkvist, 2001). Among proteins required for transformation, CtsE shares homology with ComGA of *B. subtilis* and PilT of *N. gonorrhoeae*, and CtsF is similar to PilG of *N. gonorrhoeae* (Wiesner et al., 2003). One ORF, Cj0011c, is homologous to ComEA of *B. subtilis* (Wiesner et al., 2003).

In *Helicobacter pylori* which is closely related to *Campylobacter*, strains which have natural competence tend to have higher MIC or resistance to metronidazole compared to noncompetent strains, suggesting that natural transformation may play a significant role in acquiring antibiotic resistance in *H. pylori* and possibly in *Campylobacter*. (Yeh et al., 2002).

**Conjugation**

The conjugal transfer of antibiotic resistant determinants for tetracycline, erythromycin, kanamycin, streptomycin, and neomycin from *C. jejuni* to *C. fetus* was observed with transfer frequencies per donor strain ranging from $10^{-8}$ to $10^{-4}$ (Ansary and Radu, 1992).
Genotyping methods

Serotyping has been used for a long time to discriminate strains, but problems exist in association with standardization of antisera, expense, and the existence of untypable strains (Dorrell et al., 2001; Nakari et al., 2005).

The flagellin gene and lipooligosaccharide biosynthesis gene sequences in *Campylobacter* are heterogeneous enough to be used in genotyping schemes such as Restriction Fragment Length Polymorphism (RFLP) (Nakari et al., 2005; Parker et al., 2005; Wassenaar et al., 2000). *fla* typing demonstrated higher discrimination than random amplified polymorphic DNA (RAPD) (Ertas et al., 2004). However, the association between *fla* typing pattern and fluoroquinolone resistant strains in *C. jejuni* was not found (Nachamkin et al., 2002).

Pulsed-field gel electrophoresis (PFGE) is one of the most commonly used genotyping methods for *Campylobacter* species due to its high discriminatory power (Fitzgerald et al., 2001; Gibson et al., 1995; Hume et al., 2002; Leatherbarrow et al., 2004; Ribot et al., 2001). Strains belonging to same *fla* type can be differentiated by PFGE analysis (Leatherbarrow et al., 2004). Typically the enzymes SmaI and KpnI are used for PFGE analysis (Michaud et al., 2001; Ribot et al., 2001).

Amplified fragment length polymorphism (AFLP) is another fingerprinting method which has a high discriminatory power for *Campylobacter* strains (Duim et al., 1999). Among *Campylobacter* strains isolated from humans and poultry, genetically unrelated *C. jejuni* strains produced heterogeneous patterns while related strains produced similar AFLP patterns (Duim et al., 1999). AFLP and *fla* typing were found to be complementary in
genotyping (Wittwer et al., 2005). Selective amplification of restriction fragments from the use of restriction endonuclease HindIII and HhaI gives informative band patterns with 40 to 70 bands (Duim et al., 1999).

Multilocus sequence typing (MLST) method takes advantage of nucleotide sequences of seven housekeeping loci exhibiting sufficient diversity not only in C. jejuni (Dingle et al., 2001; Suerbaum et al., 2001), but also in C. coli, C. lari, and C. upsaliensis (Miller et al., 2005a). It can be also used to identify mixed cultures and lateral gene transfer (Miller et al., 2005a). No relationship between strain origins of C. jejuni and ST lineages was noted, even though human isolates were predominantly in the ST-21 complex (Dingle et al., 2001). Multilocus sequence typing accompanied with analysis of heat-stable serotyping antigen and the sequence analysis of the short variable region (SVR) of the flagellin gene flaA showed that 92% of 814 C. jejuni isolates belonged to one of 17 clonal complexes (Dingle et al., 2002). Most clonal complexes contained multiple heat-stable serotypes and flaA SVR variants (Dingle et al., 2002).

The availability of complete genome sequencing information for several Campylobacter strains affords the possibility of high-resolution strain subtyping or of identifying strain-specific unique genes by means of DNA microarrays (Poly et al., 2004; 2005). The disadvantages of microarray are the high cost of synthesis of gene-specific primers to amplify each gene in a genome, and the technically demanding aspects of the methodology (Dorrell et al., 2001).

In Campylobacter, some genotypes are found in diverse hosts whereas others are unique to a particular host (Fitzgerald et al., 2001). C. coli appears to be more host adapted than C.
jejuni (Hopkins et al., 2004; Leatherbarrow et al., 2004). In C. coli, poultry-derived strains clustered separately from swine-derived strains by Fluorescent AFLP-based genotyping (Hopkins et al., 2004). Also, AFLP genotyping implies that avian species (chicken, turkeys) rather than swine are likely to be sources of human C. coli infections (Siemer et al., 2005). A particular group of genotypes based on flaA typing was highly associated with C. coli from environmental water samples, implying that some C. coli strains may have adapted to this environment (Kemp et al., 2005; Leatherbarrow et al., 2004).
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CHAPTER 2

Natural Transformation-Mediated Transfer of Erythromycin Resistance in

*Campylobacter coli* from Turkeys and Swine
ABSTRACT

Erythromycin resistance in *Campylobacter coli* from meat animals is frequently encountered, and could represent a substantial barrier to antibiotic treatment of human infections. Erythromycin resistance in this organism has been associated with a point mutation (A2075G) in the 23S rRNA gene. However, the mechanisms responsible for possible dissemination of erythromycin resistance in *C. coli* remain poorly understood. In this study we investigated transformation-mediated acquisition of erythromycin resistance by genotypically diverse *C. coli* strains from turkeys and swine, with total genomic DNA from erythromycin resistant *C. coli* of either turkey or swine origin used as donor. Overall, transformation to erythromycin resistance was significantly more frequent in *C. coli* from turkeys than in swine-derived strains (P<0.01). Frequency of transformation to erythromycin resistance was $10^{-4}$ to $10^{-6}$ in turkey-derived strains, but was $10^{-7}$ or less in *C. coli* from swine. Transformants harbored the point mutation, A2075G in the 23S rRNA gene, as did the erythromycin-resistant strains used as donors of the DNA. Erythromycin resistance was stable in transformants following serial transfers in the absence of the antibiotic, and most transformants had high MIC values (>256µg/ml), as did the *C. coli* donor strains. In contrast to results obtained with transformation, spontaneous mutants had relatively low erythromycin MIC (32-64µg/ml) and lacked the A2075G mutation in the 23S rRNA gene. These findings suggest that natural transformation has the potential to contribute to dissemination of high-level resistance to erythromycin among *C. coli* strains colonizing meat animals.
INTRODUCTION

*Campylobacter* is the most common diarrhea-causing bacterial agent in humans in the United States and other industrialized nations (15,22). Most (80-90%) human infections involve *C. jejuni*, with *C. coli* accounting for the majority of the remainder (17).

*Campylobacter* is a zoonotic agent, and commonly colonizes poultry and other meat animals, including swine, cattle and sheep (13). Currently, the acquisition of antibiotic resistance by *C. jejuni* and *C. coli* is of great public health concern. Although most *Campylobacter* infections in humans are self-limited and do not require antibiotic treatment, severe infections and predisposing factors may necessitate antimicrobial treatment (22). The efficiency of fluoroquinolones has been compromised by increasing trends for fluoroquinolone resistance among human isolates (8, 18). Currently, erythromycin and other macrolides (e.g. azithromycin) are the leading choice for treatment of severe *Campylobacter* infections (17).

An issue of special concern is *C. coli*’s propensity to acquire resistance to macrolides, including erythromycin. In contrast to *C. jejuni*, which largely remains sensitive to erythromycin, *C. coli* has been found to be frequently resistant to erythromycin and other macrolides (1, 2, 3, 5, 6, 21). *C. coli* appears to have a predilection for swine (1, 21, 25), but recent data indicate that *C. coli* also commonly colonizes commercial turkeys in eastern N. Carolina, a region which is a major contributor to turkey production in the United States (15, 22). Erythromycin and other macrolides (e.g. tylosin) are extensively used therapeutically and as growth promoters in animal agriculture, possibly creating selection pressure for resistance to these antibiotics in *C. coli* (8), even though the reasons for the significantly greater prevalence of erythromycin resistance in *C. coli* than in *C. jejuni* remain unclear. A
point mutation, A2075G, in the 23S rRNA gene (position 2075 in the 23S rRNA gene of *C. jejuni* NCTC 11168, corresponding to position 2059 in the 23S rRNA gene of *Escherichia coli*) is associated with erythromycin resistance in *Campylobacter* (10, 11, 14, 24).

*C. jejuni* and *C. coli* are well known for the ability to acquire exogenous DNA by natural transformation (27, 29), with transformation frequency for chromosomal DNA markers (nalidixic acid and streptomycin resistance) as high as $10^{-3}$ per recipient cell in *C. coli* (27). Chromosomal and plasmid-borne competence genes have been identified in *C. jejuni* (4, 28). However, the role of transformation in dissemination of resistance to erythromycin and other macrolides in *C. coli* has not been described. Overall limited data exist on transformation in *Campylobacter* isolated from meat animals, and most investigations have involved *C. jejuni*. In this study, we investigated transformation-mediated transfer of erythromycin resistance in *C. coli* from farm animals (turkeys and swine) *in vitro*.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The *C. coli* strains used in this study are listed in Table 2.1. These strains are part of our laboratory’s *Campylobacter* strain collection, and were obtained from turkeys and swine at different farms in eastern North Carolina, South Carolina, and Virginia between 2001 and 2004. *Campylobacter* had been isolated from turkey fecal or cecal samples and from swine fecal samples using direct plating on charcoal cefoperazone deoxycholate agar (CCDA) (Oxoid, Basingstoke, Hampshire, England) at 42°C under microaerobic conditions, as described previously (24). Species determinations and
antibiotic susceptibility profiles were done as described (16, 23). Tetracycline (10 \( \mu \text{g/ml} \)), erythromycin (8 \( \mu \text{g/ml} \)), streptomycin (15 \( \mu \text{g/ml} \)), nalidixic acid (20 \( \mu \text{g/ml} \)), kanamycin (25 \( \mu \text{g/ml} \)), and ampicillin (100 \( \mu \text{g/ml} \)) were tested to determine antibiotic susceptibility profiles. 

*Clostridium jejuni* ATCC33560 (American Type Culture Collection, Manassas, VA) was used as a quality control. Bacteria were routinely grown on Mueller Hinton Agar (MHA) (Mueller Hinton Broth with 1.2% agar) (Becton Dickinson, Sparks, MD) for 20 to 24h at 42°C under microaerobic conditions generated by the CampyPak Microaerophilic System (BBL, Sparks, MD), and preserved in Brain Heart Infusion broth (Becton Dickinson) supplemented with glycerol (20%) at -70°C, as described (24).

**Strain fingerprinting.** *Campylobacter* isolates were subtyped with PCR restriction fragment length polymorphism analysis of *flaA* gene (*flaA* typing) and with pulsed field gel electrophoresis (PFGE) analysis as described (16, 24). Briefly, in *flaA* typing, *flaA* gene region (1.7 kb) was amplified with PCR and the PCR product was cut with enzyme DdeI (New England Biolabs, Beverly, Mass.). The restriction fragments were separated on 2% Tris borate-EDTA gels for 60 min at 70 V. PFGE analysis followed the previous protocol as described (22) with enzyme SmaI and KpnI. The lambda ladder PFG marker (New England Biolabs, Beverly, MA) was used as a molecular weight marker and *C. jejuni* NCTC11168 was used as a reference strain.

**Erythromycin MIC determinations.** Erythromycin MICs were determined by the agar dilution method following the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, 2002). Briefly, *Campylobacter* strains were streaked on Sheep Blood Agar Plates (Remel, Lenexa, KS) and incubated for 36 to 48h at 42°C under
microaerobic conditions. Isolated colonies were subcultured in Mueller Hinton Broth (MHB) (200 µl). Aliquots (2 µl were tested) in duplicate were tested for growth at erythromycin concentrations up to 256 µg/ml and monitored following 48h incubation at 42°C, microaerobically. Threshold for resistance was MIC greater than or equal to 8 µg/ml, as described (10). High-level resistance corresponded to growth of the bacteria in the presence of 128 µg/ml or 256 µg/ml erythromycin.

**Mueller Hinton Agar (MHA) transformation assay.** Erythromycin resistant and erythromycin sensitive strains were selected as donors and recipients, respectively, for the transformation assays. All recipients were tested with a panel of three donor strains (1705, 1800r, and 2901) (Table 2.1). In addition, some recipients were also tested with genomic DNA from additional donor strains, including swine-derived strain 1686 and turkey-derived strains 1420, 1702, 2562, and 2774. Genomic DNA of erythromycin resistant donors was extracted using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) as described (24). A loopful of the erythromycin sensitive recipient (from 20-24h old cultures on MHA, grown at 42°C microaerobically), was spotted on MHA plates in triplicate, then 4 µl genomic DNA of the donor strain was added to each spot and mixed, with diameter of the spot after mixing, ca. 0.5 cm. The plates were incubated overnight (15-17h) at 42°C under microaerobic conditions, and the entire material from each spot was spread-plated on a separate 12 cm-diameter MHA plate containing erythromycin (10 µg/ml) (EMHA). Each transformation included as negative control spots of the recipient without donor DNA, on the same MHA plate. The EMHA plates were incubated for 48h at 42°C under microaerobic conditions and examined for growth of *Campylobacter*. A quantitative estimate of the extent of growth on
these plates, termed transformation index, was made as follows: scores of 1.0, 0.5, and 0 were given to transformations yielding growth on more than 50% of the EMHA agar plate surface (ca. 100 colonies or more), less than 50% of the surface (less than 100 colonies), and no detectable growth, respectively. The scores were calculated based on the arithmetic mean from two independent experiments, each done in triplicate. Some of erythromycin-resistant transformants were tested for acquisition of additional antibiotic resistance markers from the donor. Erythromycin-resistant transformants grown on EMHA were subcultured on MHA plates containing antibiotics to which donor was resistant but the recipient was susceptible, and incubated for 48h at 42°C under microaerobic conditions. Antibiotics were tetracycline (10 µg/ml), streptomycin (15 µg/ml), nalidixic acid (20 µg/ml), kanamycin (25 µg/ml), and ampicillin (100 µg/ml).

**Determination of CFU/recipient before and after overnight (15-17h) incubation during MHA transformation.** To determine whether CFUs in the recipient culture changed significantly during the overnight incubation employed for the MHA transformation assays, CFUs were enumerated before and after the overnight incubations. Before transformation assay, a loopful of recipient from Sheep Blood Agar Plate was diluted in Mueller Hinton Broth (MHB) (10^{-7} and 10^{-8}). Aliquots (100 µl) were plated on MHA and incubated for 24-36h in order to determine CFUs in this amount of recipient before transformation. After overnight incubation in transformation, a loopful of recipient which was spotted on MHA with donor DNA was removed, diluted in MHB and plated on MHA in the same way as before transformation, in order to determine CFUs after transformation.

**Broth transformations and determination of transformation frequency.**
Broth transformations followed a previously described protocol (29) with modifications. Briefly, recipient strains were grown on Sheep Blood Agar plates at 42°C for 48h under microaerobic conditions. A single colony was transferred to 5 ml of MHB and incubated at 42°C for 24h under microaerobic conditions. 0.1ml of this culture was added to 50ml MHB preconditioned at 42°C, and incubated for 7h at 42°C under microaerobic conditions to reach exponential phase. For each transformation, 1.0ml of this culture was transferred to a sterile polypropylene round-bottom tube (14ml) (Becton Dickinson, Franklin Lakes, NJ) and 15 µl of total genomic DNA (c.a. 3 µg) from the donor was added. Negative controls were processed identically, except that no genomic DNA was added. After 5h incubation at 42°C under microaerobic conditions, 100 µl was spread-plated on EMHA in triplicate, the plates were incubated 36-60h at 42°C microaerobically, and colonies were enumerated. Dilutions (10⁻⁴, 10⁻⁶) at the end of the 5h transformation period were also plated on MHA and incubated for 24-36h at 42°C microaerobically, in order to determine the CFU/ml of the recipient. Transformation frequency was determined as the ratio of the number of transformants/ml to total CFU/ml of the recipient.

**Transformation with MHA-DNA assay.** Efficiencies of transformation to erythromycin and nalidixic acid resistances were compared utilizing the MHA-DNA assay as described previously with modifications (28). Briefly, erythromycin sensitive recipient was grown in 10ml MHB for overnight (c.a. 24h). Donor DNA (4 µl) was spread on MHA plates, and 100 µl of diluted (10⁻⁴) recipient culture was spread on the same plates. The MHA plates were incubated for 24 to 36h at 42°C microaerobically. Randomly chosen isolated colonies on MHA plates were transferred to EMHA and to MHA plates containing nalidixic acid (20
µg/ml) (NMHA). For each antibiotic, 70 colonies were tested. The EMHA and NMHA plates were incubated for 48h at 42°C microaerobically, and colonies were enumerated. A similar number of colonies from negative control plates (similarly diluted culture of the recipient, plated on MHA without donor DNA added onto the plates) were transferred to EMHA and NMHA, and the plates were similarly incubated and enumerated, to determine presence of spontaneous resistant mutants.

**Restriction of chromosomal DNAs from recipients with Sau3AI and MboI.**

To determine whether strains used as recipients harbored methylated adenines at GATC sites of their genome, approximately 3 µl of chromosomal DNAs from swine-derived recipients were digested with the restriction endonucleases Sau3AI and MboI, following the recommendations of the vendor (New England Biolabs, Beverly, MA). The digestions were incubated at 37°C overnight. After digestion, the samples, along with uncut genomic DNAs of the same strains, were electrophoresed (60V for 1h) in 1% agarose gels with ethidium bromide (5 µl/100ml). DNA Molecular Weight Marker II (Hind III-digested λDNA) (Roche, Indianapolis, IN) was used as molecular weight marker. The agarose gels were visualized under UV light and photographed by using KODAK 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY).

**Isolation of spontaneous mutants and determination of mutation frequency.**

On rare occasions, one or two colonies grew on the negative control plates during the transformation assay. These colonies, presumed to correspond to spontaneous erythromycin-resistant mutants, were subcultured onto EMHA, and then stored at -70°C. To further assess spontaneous mutation frequency for resistance to erythromycin, selected erythromycin
sensitive strains were subcultured on Sheep Blood Agar at 42°C under microaerobic conditions, a single colony was transferred to MHB (10ml), and incubated for 40-44h under the same conditions. Aliquots (1ml) were transferred to three sterile microcentrifuge tubes (ISC BioExpress, Kaysville, Utah), centrifuged at 5400 × g for 10min, resuspended in 100 µl of MHB and spread-plated on MHA containing 8 µg/ml or 10 µg/ml erythromycin. The plates were incubated 48h at 42°C microaerobically, and colonies were enumerated. The cultures were also serially diluted, plated on MHA, and incubated for 24-36h to enumerate total CFUs. Mutation frequency (ratio of erythromycin-resistant CFU/ml to total CFU/ml) was determined based on two independent experiments for each strain.

Sequence analysis of 23S rRNA gene. A 470 bp internal fragment of the 23S rRNA gene was amplified using forward primer, F2-campy-23S (5’-AATTGATGGGGTTAGCATTAGC-3’) and reverse primer, R1-campy-23S (5’-AACGATTCTCAACCGTTCTG-3’) (24). PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 34 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were electrophoresed (3% agarose), the band corresponding to the amplified product was excised, and the DNA was purified using the QIAquick gel extraction kit (Qiagen). The purified products were sequenced (Davis Sequencing, Davis, CA), and the sequences were analyzed by using the online multiple sequence alignment program ClustalW (www.ebi.ac.uk/clustalw/).

Stability of erythromycin resistance in transformants. Transformants 961-1705em and 1651-1705em were daily subcultured in 5ml MHB (1:10,000 dilution) for 14
days, in the absence of erythromycin. The cultures were diluted and plated on both MHA and EMHA on day 7 and 14, and CFUs on MHA and EMHA were compared.

**Statistical analysis.** The impact of host (turkeys vs. swine) of the recipient and donor strains on transformation was analyzed with split-plot ANOVA in glm, using SAS (SAS Institute Inc., Cary, NC). Transformation frequency data were transformed with arcsin for normal distribution.

**RESULTS**

**Transformation-mediated acquisition of erythromycin resistance in C. coli strains of turkey and swine origin**

A total of 26 C. coli strains of meat animal origin (13 each from swine and from turkeys) were tested for transformation to erythromycin resistance with the agar transformation assay, using as donor total genomic DNA from at least three different erythromycin-resistant C. coli strains (derived from turkeys and swine) (Table 2.1). The recipient organisms were chosen so as to include erythromycin-sensitive organisms with different genomic fingerprints, as determined by *flaA* typing (data not shown) and by PFGE with enzyme SmaI and KpnI (Fig. 2.1, 2.2, and 2.3). In addition, the recipients differed in terms of their overall antibiotic susceptibility profiles (Table 2.1). The donor organisms also had different genomic fingerprints (Fig. 2.4).

The majority (19/26) of the strains frequently yielded erythromycin-resistant transformants on the selective media (EMHA) (Table 2.1). Negative controls, which lacked donor DNA, yielded either very few (one or two colonies) or, most commonly, no colonies at all on EMHA (data not shown). CFU determinations of the recipients immediately prior to and at the completion of the overnight incubation showed that CFUs in the spots on MHA
were maintained at the same level (ca. $10^8$CFU), with no significant differences in CFUs between before and after the overnight incubations (Table 2.5). Bacteria from turkeys were overall more likely to become transformed to erythromycin resistance than those from swine (92.3% vs. 53.8%; $P<.01$). Of the seven strains which transformed poorly or not at all (transformation index <0.16), six were from swine, and only one (7580) was of turkey origin. The transformation index was not significantly influenced by the origin (swine vs. turkey) of the donor DNA ($P>.05$), since DNA from the turkey-derived strain 1705 was similar in its efficiency to transform recipients to erythromycin resistance as DNA from the swine-derived strains 1800r or 2901 (Table 2.1). Use of additional turkey and swine-derived erythromycin-resistant strains as sources of donor DNA in transformations of a subset of the recipients also failed to reveal significant impact of the source of the donors (data not shown). Most recipients per spot were maintained at $10^9$ CFU and no significant difference in CFU was found between before and after overnight (15-17h) incubation (Table 2.5).

Enumerations of transformed cells and accurate determinations of transformation frequency utilizing the agar assay were hampered by difficulty in obtaining discrete colonies on EMHA plates, possibly due to clumping of the recipient cells during the transformation period on the agar plates, and the highly mucoid colony morphology of the \textit{C. coli} strains. A liquid (MHB)-based transformation assay was therefore employed to accurately determine transformation frequency. A subset of nine strains (four from turkeys and five from swine), were studied with the MHB assay, using the same panel of donor DNAs employed with the agar assay. Transformation frequency of turkey-derived organisms was found to be in the range of $10^{-4}$ to $10^{-6}$, whereas it was typically $10^{-7}$ or lower in strains from swine (Table 2.2).
Transformation frequency of three of the swine-derived strains (P5, 2113, and 5980) was below the detection limit, with no transformants identified in any of the transformations using these strains as recipients, even though two of these strains (P5 and 2113) had average transformation index of 0.6 and 0.8, respectively, in the agar assay (Table 2.1). Similarly to the findings with the agar assay, transformation frequency was not significantly influenced by the source of the donor DNA (Table 2.2).

Transformation to erythromycin resistance was less frequent than transformation to nalidixic acid resistance in *C. coli*. Using the MHA-DNA assay for transformation with donor DNA from an erythromycin- and nalidixic acid-resistant strain, it was found that the recipients (which were sensitive to both antibiotics) hardly transformed to erythromycin resistance, whereas transformation to nalidixic acid resistance frequently occurred (Table 2.6).

During transformation to erythromycin resistance with the MHA assay, recipients which acquired erythromycin resistance frequently acquired other antibiotic resistances as well (Table 2.7). Transformation to tetracycline, streptomycin, or ampicillin resistance frequently occurred during transformation to erythromycin resistance, whereas transformation to nalidixic acid or kanamycin resistance rarely occurred. Acquisition of up to four antibiotic resistances including erythromycin resistance was found in one recipient, 426-9.

**Overall genomic profile stability following transformation to erythromycin resistance.** Erythromycin resistant transformants of four different *C. coli* strains were examined in terms of their PFGE fingerprints, in comparison to the fingerprints of the
parental isolates. One randomly chosen transformant was tested per strain. Three of the erythromycin-resistant transformants had PFGE patterns indistinguishable from those of their respective parental strains with both SmaI and KpnI. However, the PFGE pattern of the erythromycin-resistant transformant 1536-1705em was markedly different from that of the parental strain 1536, with both enzymes (Fig. 2.5 and 2.6).

**Erythromycin-resistant transformants can themselves serve as donors of erythromycin resistance in subsequent transformations.** An erythromycin resistant transformant of strain 3237, designated 3237-1705em (derived from transformation of 3237 by DNA from strain 1705) was found to be as effective in transforming the erythromycin sensitive strain 7474 as DNA from strain 1705 (Table 2.2). The ability of transformants to serve as donors of erythromycin resistance in transformations of sensitive recipients, including the original parental strain, was also confirmed with the agar transformation assay (data not shown).

**Restriction of chromosomal DNAs from recipients.** Chromosomal DNAs from six randomly chosen swine-derived recipients (426-9, 3175, P5, WP66, 1684 and WP145) were studied for the existence of methylated adenines or cytosines in GATC sequences (Fig. 2.7). Restriction enzymes Sau3AI can cleave unmethylated GATC and GATC containing methylated adenine while MboI can also cleave unmethylated GATC but can not cleave GATC containing methylated adenine (7). Four of the six swine-derived strains (426-9, P5, WP66, and WP145) had DNA that could be digested by Sau3AI but not by MboI digestion (Fig. 2.7), suggesting methylation of adenines at GATC sites. Turkey-derived recipient 3237
did not have methylation in GATC sequences (data not shown). DNA samples were not
cleaved in the absence of restriction enzymes (Fig. 2.7).

**Frequency of spontaneous mutations to erythromycin resistance in *C. coli*.**

Spontaneous erythromycin-resistant mutants could be only rarely detected in the negative
control plates of the transformation experiments. Frequency of spontaneous mutation to
erthyromycin resistance was determined in seven turkey-derived strains (961, 3325, 1536,
931g, 3237, 1787, and 44nec), and two strains from swine (426-9 and 614-3m). Spontaneous
mutants could be isolated from only three strains (3237, 1536, and 44nec) of the nine strains
that were tested, with frequency generally lower than $10^{-8}$.

**High-level resistance to erythromycin was acquired by transformation, in
contrast to relatively low levels of resistance in spontaneous mutants.** All tested
erthyromycin sensitive strains, including those used as recipients in transformation, had
uniformly low MIC to erythromycin ($<4 \mu g/ml$), in contrast to erythromycin resistant field
strains, including those used as sources of donor DNA in transformations, all of which had
high erythromycin MIC ($>256 \mu g/ml$) (Table 2.3). Erythromycin MICs were also determined
for thirty four transformants, derived from different recipient / donor combinations. Most
(30/34) had similarly high level of resistance (MIC>$256 \mu g/ml$) (Table 2.3). Only four
transformants (three derived from recipient 426-9 and one from WP19) had lower
erythromycin MICs (32-128 $\mu g/ml$). In contrast to the overall high MICs of field strains and
transformants, spontaneous erythromycin-resistant mutants had relatively low MIC (32-64
$\mu g/ml$) (Table 2.3).
Analysis of 23S rRNA gene sequences reveals the A2075G transition in transformants. The nucleotide sequence of a PCR-amplified internal fragment (470 bp) of the 23S rRNA gene that harbors position 2075 was determined for a panel of 30 strains, including nine erythromycin-sensitive recipients, nine erythromycin-resistant strains used as sources of DNA in transformations, 12 transformants from different recipient/donor combinations, and three spontaneous mutants. A portion of the nucleotide sequence is shown in Table 2.4. The A2075G transition was detected in all nine erythromycin resistant strains, whereas all nine erythromycin sensitive strains harbored adenine at position 2075. No other differences in nucleotide sequence were detected between erythromycin resistant and sensitive strains in the 470 bp region (data not shown).

The A2075G transition was detected in 11 of 12 tested transformants. Two transformants, 1536-1705em and 1702rnd-1702em were found to have both A and G upon visual examination of the sequencing chromatograms, suggesting that the transition was absent from at least one of the three copies of the 23S rRNA gene in these transformants. None of the three tested spontaneous mutants harbored the transition (Table 2.4).

A strong correlation between erythromycin MIC and presence of the A2075G transition was noted. All erythromycin resistant field strains and transformants with high erythromycin MICs (>128 µg/ml) harbored the transition, whereas the putative transformant WP19-1420em which had relatively low MIC (32 µg/ml), and spontaneous erythromycin resistant mutants which also had MIC of 32-64 µg/ml, lacked this transition (Table 2.4).

Erythromycin resistance is stable in transformants, in the absence of the antibiotic. Stability of erythromycin resistance in the absence of the antibiotic was
investigated in two transformants, 961-1705em and 1651-1705em. In both cases, resistance remained stable after 14 successive daily transfers in the absence of erythromycin, with similar CFU/ml of the cultures on plates with and without erythromycin (data not shown).

DISCUSSION

Even though thermophilic campylobacters have long been known to be naturally competent, limited data exist on the role of transformation in dissemination of high-level erythromycin resistance in *C. jejuni* and *C. coli* that colonize meat animals. Considering the prevalence of *C. coli* in animals such as swine and turkeys, the high incidence of resistance to erythromycin and other macrolides in these bacteria, and the rising status of these drugs as first line of treatment of human infections, it is important to investigate mechanisms that may underlie dissemination of erythromycin resistance in *C. coli*.

Our findings suggest that transformation can indeed mediate acquisition of high-level resistance to erythromycin in animal-derived *C. coli*, with the majority (12/13) of the strains from turkeys and several (7/13) of the *C. coli* strains from swine yielding transformants under laboratory conditions with the agar assay. Frequencies of transformation, determined using broth assays, were also significantly higher in turkey-derived than swine-derived strains. Transformation frequencies were below detection level in two of the swine strains that yielded transformants in the agar assay, suggesting that the agar assay may be preferable for determining whether a strain is capable of transformation to erythromycin resistance, even though it does not lend itself to accurate determination of transformation frequencies. To our knowledge, this is the first documentation that *C. coli* strains from meat animals can be naturally transformed to high-level erythromycin resistance with genomic DNA from
erythromycin resistant bacteria, and that host-related differences in transformation potential may exist.

In contrast to transformants, which had high-level resistance and harbored the A2075G transition in the 23S rRNA gene, spontaneous erythromycin-resistance mutants were resistant to relatively low levels of the antibiotic and lacked the A2075G transition, as has also been described by others (19). Thus, such spontaneous mutations alone are unlikely to be responsible for the frequent occurrence of resistance to erythromycin in *C. coli* from meat animals (1, 2, 3, 5, 6, and 21), which this study and others (10, 20) have found to be characterized by high erythromycin MIC and to be accompanied by the A2075G transition in the 23S rRNA gene. Our findings suggest that erythromycin resistance acquired by transformation was stable in the absence of the antibiotic. In addition, DNA from transformants could transform other strains to erythromycin resistance, with transformation frequency similar to that obtained with DNA from erythromycin resistant field strains. Taken together, the available data suggest that transformation may indeed contribute to the high prevalence of high-level resistance to erythromycin in *C. coli* colonizing turkeys and swine. In terms of other modes of transfer of this resistance, conjugation is unlikely, since high-level resistance is mediated by a chromosomal marker (the substitution in the 23S rRNA gene), and no evidence currently exists on phage-mediated transfer (transduction) in *Campylobacter*. However, we cannot exclude the possibility that conditions in swine and turkey production systems (especially the extensive use of the macrolide tylosin as growth promoter) may be such that high-level mutants (harboring the A2075G transition) are selected for, in contrast to the relatively low-level spontaneous mutants obtained in the
laboratory.

Considering the spontaneous mutants did not have the A2075G transition, the origin of the A2075G transition in erythromycin-resistant *C. coli* is unclear. It could have been transferred from other bacterial species such as *Helicobacter pylori*, an organism genetically closely related to *Campylobacter*, through horizontal gene transfer. In *H. pylori*, most common point mutation in 23S rRNA gene responsible for macrolide resistance corresponds to the position 2075 (cognate with position 2059 in *E. coli*) in 23S rRNA gene of *C. coli* (26). In addition, 23S rRNA genes of *C. coli* and *H. pylori* have about 85% identity based on DNA sequence alignment, implying high chance of homologous recombination. In other possibility, long exposure of *C. coli* to sublethal level of erythromycin in animal hosts could have resulted in the A2075G transition through spontaneous mutation which could not be demonstrated with selection for relatively higher MIC (8-10 µg/ml) in our laboratory.

The documentation of the A2075G transition in the 23S rRNA gene from erythromycin resistant *C. coli* from swine and turkeys confirms and extends previous detection of this mutation in erythromycin-resistant *C. coli* from humans, swine, broilers, and sheep (10, 11, 14, 20). Recently, the role of this transition in high-level erythromycin resistance was confirmed by transformation of *C. jejuni* 81116, derived from a human clinical case, to high-level resistance, using as donor an amplified fragment of the 23S rRNA gene that harbored the transition (10). Our data with transformants that had a mixture of the wildtype sequence and the sequence harboring the A2075G transition suggest that high-level resistance to erythromycin in *C. coli* can be conferred even when the transition may be absent from one of the three 23S rRNA genes, as also described recently with *C. jejuni* (10).
In our study, the efficiency of transformation of *C. coli* to erythromycin resistance could be as high as $10^{-4}$ in certain strains, but was overall lower than previously reported transformation frequency ($10^{-3}$) of this species to nalidixic acid and streptomycin resistance (27). Data from our laboratory, using *C. coli* sensitive to both erythromycin and nalidixic acid, also suggested that transformation to nalidixic acid resistance was generally more efficient than transformation of the same strain to erythromycin resistance. The reasons for the relatively low frequency of transformation to erythromycin resistance remain unclear, but may be due to the apparent requirement for the A2075G transition in at least two of the three copies of the 23S rRNA gene in bacteria with high-level resistance (10), thus necessitating multiple transformation events. However, in strain 1702rnd which had the highest transformation frequency ($10^{-4}$), some of transformants grew relatively slowly and required up to 60h incubation to form average-size colonies, suggesting that such a high transformation frequency for erythromycin resistance may be due to transition in one of the three copies of the 23S rRNA gene. In contrast, nalidixic acid resistance involves a single chromosomal locus (*gyrA* harboring a specific substitution) (8).

Transformation to multiple drug resistances indicates that *C. coli* may easily acquire other antibiotic resistances in the process of transformation to erythromycin resistance. *C. coli* strains isolated from poultry, swine, and cattle are frequently resistant to multiple drugs (3, 6, 21). Despite recombination possibly at multiple loci, most erythromycin-resistant transformants appear to be genetically similar as their parental strains after transformation to erythromycin resistance, based on PFGE analysis.

The reasons for the relatively low transformation frequency of swine-derived *C. coli*...
remain unclear. Molecular subtyping studies have shown that *C. coli* strains from swine are
genotypically distinct from those colonizing poultry (12), and this may also suggest
physiologic differences and specialized environmental requirements for transformation in
swine-derived strains. Alternatively, such strains may lack competence due to the existence
of barriers to gene flow, such as restriction/modification systems. Methylated adenine can be
frequently involved in restriction/modification among gram-negative bacteria and was also
previously found in GATC sequences of *Campylobacter* (7). Methylated adenine in GATC
sequences was also found in some swine-derived recipients in this study. However, there was
no difference in transformation frequency to nalidixic acid resistance of strain WP66 between
genomic DNA from nalidixic acid resistant isogenic mutant derived from itself and strain
1705 as donor (data not shown). In addition, strain WP145 could not be transformed to
nalidixic acid resistance with genomic DNA from nalidixic acid resistant isogenic mutant as
well as strain 1705. Taken together, it suggests that restriction/modification system may be
not responsible for such a relatively low transformation frequency or lack of transformation
in some swine-derived strains.

Some strains may lack competence due to the absence of proteins involved in the
transformation process (28). Preliminary data with the swine-derived strains showed that not
only WP145, but also strains 5980 and 1684 are unable to acquire resistance to nalidixic acid
by transformation (J. S. Kim and S. Kathariou, unpublished). Especially, strain 1684 could
not be transformed to nalidixic acid resistance even with genomic DNA of nalidixic acid
resistant isogenic mutant derived from itself, which is similar to strain WP145. It indicates
that these strains (WP145, 5980, and 1684) may lack protein components essential for natural
transformation process. Interestingly, many of genes (cj1343c, cj1076, cj1077, cj1028c, cj1473c, cj1475c, cj1470c, and cj1474c) involved in transformation process of C. jejuni were found not to exist in chicken-derived C. coli RM2228 based on a basic local alignment search tool (BLAST) (28). The existence of strains of Campylobacter that lacked competence has been noted before (27, 29). In H. pylori, non-competent strains were less likely to be resistant to metronidazole compared to competent strains, suggesting that transformation is important to disseminate antibiotic resistance in that organism (30). Further studies are needed to more accurately evaluate the role of transformation in dissemination of antibiotic resistance in C. coli.

In conclusion, our results indicate that, under laboratory conditions, transformation can be effective in acquisition of high-level resistance to erythromycin by C. coli from turkeys and swine, whereas spontaneous mutations result in a genetically distinct class of mutants, which lack the A2075G transition and are resistant to relatively low levels of the antibiotic. Further studies are needed to elucidate the mechanisms underlying the observed differences in the potential for transformation to erythromycin resistance in organisms derived from different hosts, and to characterize the potential for transformation under conditions that prevail in the turkey and swine production ecosystems.

ACKNOWLEDGMENTS

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REFERENCES:


TABLE 2.1. Transformation index of transformation-mediated acquisition of erythromycin resistance in *C. coli* strains isolated from turkeys and swine

<table>
<thead>
<tr>
<th>Source of recipient</th>
<th>Recipient</th>
<th>Donor</th>
<th>1705</th>
<th>1800r</th>
<th>2901</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>961 (TSNKA)</td>
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<td>1.0</td>
<td>1.0</td>
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<td>Turkey</td>
<td>3325 (TSNA)</td>
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<td>Turkey</td>
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<td>1.0</td>
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<tr>
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<td>1.0</td>
<td>1.0</td>
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<tr>
<td>Turkey</td>
<td>1651 (TKA)</td>
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<td>1702rnd (TSA)</td>
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*a* Antibiotics which the recipient is resistant to are listed: T- tetracycline, S- streptomycin, N- nalidixic acid, K- kanamycin, A- ampicillin.

*b* Susceptible to all antibiotics tested.

*c* Strain 1705 (TESNKA) was of turkey origin, and 1800r (TESNKA) and 2901 (TEA) were of swine origin. Additional donors when used included strain 1686 (TEKA) derived from swine and strains 1420 (TESNKA), 1702 (TESNKA), 2562 (TESNKA), and 2774 (TESNKA), derived from turkeys.

*d* Average of transformation index was calculated based on results obtained using donors 1705, 1800r, 2901 and additional donor strains (if any).
TABLE 2.1. (continued)

<table>
<thead>
<tr>
<th>Source of recipient</th>
<th>Recipient</th>
<th>Donor&lt;sup&gt;c&lt;/sup&gt;</th>
<th>1705</th>
<th>1800r</th>
<th>2901</th>
<th>Average&lt;sup&gt;d&lt;/sup&gt;</th>
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</table>

<sup>a</sup> Antibiotics which the recipient is resistant to are listed: T- tetracycline, S- streptomycin, N- nalidixic acid, K- kanamycin, A- ampicillin.

<sup>b</sup> Susceptible to all antibiotics tested.

<sup>c</sup> Strain 1705 (TESNKA) was of turkey origin, and 1800r (TESNKA) and 2901 (TEA) were of swine origin. Additional donors when used included strain 1686 (TEKA) derived from swine and strains 1420 (TESNKA), 1702 (TESNKA), 2562 (TESNKA), and 2774 (TESNKA), derived from turkeys.

<sup>d</sup> Average of transformation index was calculated based on results obtained using donors 1705, 1800r, 2901 and additional donor strains (if any).
TABLE 2.2. Frequency of transformation to erythromycin resistance in *C. coli* strains from turkeys and swine

<table>
<thead>
<tr>
<th>Recipient (Source)</th>
<th>Donor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1702rd (Turkey)</td>
<td>1800r</td>
<td>4.7×10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>961 (Turkey)</td>
<td>1705</td>
<td>7.7×10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>4.2×10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>5.0×10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>3237 (Turkey)</td>
<td>1705</td>
<td>1.5×10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>2.7×10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>9.2×10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>3325 (Turkey)</td>
<td>1705</td>
<td>8.1×10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>8.5×10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>8.4×10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>7474 (Turkey)</td>
<td>1705</td>
<td>2.3×10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>3237-1705em&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8×10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>9.5×10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>1.4×10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains were of turkey (1705) and swine (1800r and 2901) origin, as described in table 2.1.

<sup>b</sup> Erythromycin resistant transformant, derived from strain 3237 transformed with chromosomal DNA from strain 1705.
<table>
<thead>
<tr>
<th>Recipient (Source)</th>
<th>Donor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>614-3m (Swine)</td>
<td>1705</td>
<td>1.4×10⁻³</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>4.9×10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>2.1×10⁻⁷</td>
</tr>
<tr>
<td>426-9 (Swine)</td>
<td>1705</td>
<td>1.3×10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>&lt;1.3×10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>1.3×10⁻⁸</td>
</tr>
<tr>
<td>P5 (Swine)</td>
<td>1705</td>
<td>&lt;3.6×10⁻⁹</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>&lt;3.6×10⁻⁹</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>&lt;3.6×10⁻⁹</td>
</tr>
<tr>
<td>2113 (Swine)</td>
<td>1705</td>
<td>&lt;2.8×10⁻⁹</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>&lt;2.8×10⁻⁹</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>&lt;2.8×10⁻⁹</td>
</tr>
<tr>
<td>5980 (Swine)</td>
<td>1705</td>
<td>&lt;3.2×10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>1800r</td>
<td>&lt;3.2×10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>2901</td>
<td>&lt;3.2×10⁻⁸</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains were of turkey (1705) and swine (1800r and 2901) origin, as described in Table 2.1.
TABLE 2.3. Distribution of erythromycin MICs for *C. coli* field strains, erythromycin resistant transformants, and spontaneous mutants

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>&lt;4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>&gt;256</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin sensitive field isolates (n=15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Erythromycin resistant field isolates (n=16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Erythromycin resistant transformants (n=34)</td>
<td>1</td>
<td>3</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin resistant spontaneous mutants (n=3)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**TABLE 2.4.** Erythromycin MIC and sequences of internal fragment of the 23S rRNA gene of *C. coli* field strains, erythromycin resistant transformants, and spontaneous mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC (µg/ml)</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11969c</td>
<td>1</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>Recipients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1536</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1651</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>2775</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>3175</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>3237</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>426-9</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>931g</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1702rnd</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>WP19</td>
<td>&lt; 4</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>Mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1651</td>
<td>32</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCGGTTACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>3237</td>
<td>64</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>WP19</td>
<td>64</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>Transformants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WP19-1420em</td>
<td>32</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>426-9-2562em</td>
<td>128</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>426-9-2774em</td>
<td>128</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>426-9-2901em</td>
<td>128</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1536-1705em</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1651-1705em</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>2775-3174em</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>3175-1686em</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>3237-1420em</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>931g-2901em</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>931g-1800rem</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1702nd-1702em</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>Donors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1420</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1686</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1705</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1702</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>1800r</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>2562</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>2774</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>2901</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
<tr>
<td>3174</td>
<td>&gt;256</td>
<td>GAAAATTCCTCTACCCGCGGCAAGACGGGAAGACCCCCCGTGACCTTTACTACAGCTTGA</td>
</tr>
</tbody>
</table>

*a* Erythromycin-sensitive *C. coli* strain from raw chicken carcass (10), GenBank accession number AY249915.

*b* Em indicates that this was an erythromycin resistant transformant obtained from a specific recipient-donor combination. For example, 1536-1705em was a transformant of strain 1536 by DNA from strain 1705.

*c* Letter in enclosure corresponds to position 2075; N indicates that a mixture of A and G was observed in this position.
TABLE 2.5. Impact of overnight (15-17h) incubation on survival of *C. coli* recipients in transformation with the MHA assay under microaerobic conditions at 42°C

<table>
<thead>
<tr>
<th>Recipient</th>
<th>CFU/recipient Before incubation</th>
<th>CFU/recipient After incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3325</td>
<td>7.5×10⁸</td>
<td>3.0×10⁸</td>
</tr>
<tr>
<td>931g</td>
<td>2.7×10⁹</td>
<td>2.3×10⁹</td>
</tr>
<tr>
<td>1651</td>
<td>1.2×10⁹</td>
<td>1.9×10⁹</td>
</tr>
<tr>
<td>1787</td>
<td>2.2×10⁹</td>
<td>1.5×10⁹</td>
</tr>
<tr>
<td>6034</td>
<td>1.3×10⁹</td>
<td>1.5×10⁹</td>
</tr>
<tr>
<td>37-2nec</td>
<td>1.7×10⁹</td>
<td>1.8×10⁹</td>
</tr>
<tr>
<td>WP14</td>
<td>1.6×10⁹</td>
<td>1.3×10⁹</td>
</tr>
</tbody>
</table>
TABLE 2.6. Comparison of transformation efficiency to erythromycin and nalidixic acid resistance in transformation of *C. coli* with the MHA-DNA assay

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Erythromycin</th>
<th>Nalidixic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3237 (TKA)</td>
<td>1420 (TESNKA)</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>1651 (TKA)</td>
<td>3174 (TESNKA)</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>1702rnd (TSA)</td>
<td>1702 (TESNKA)</td>
<td>3</td>
<td>67</td>
</tr>
</tbody>
</table>

*Antibiotics which the strain is resistant to are listed: T- tetracycline, E- erythromycin, S- streptomycin, N- nalidixic acid, K- kanamycin, A- ampicillin.*

*Numbers of colonies grown on EMHA and NMHA out of 70 colonies subcultured on each type of medium.*
TABLE 2.7. Acquisition of additional antibiotic resistance markers by *C. coli* erythromycin resistant transformants

<table>
<thead>
<tr>
<th>Erythromycin-resistant transformants</th>
<th>Other antibiotic resistance tested for co-transformation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Acquired antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>3325-1686em</td>
<td>K</td>
<td>None</td>
</tr>
<tr>
<td>1536-3174em</td>
<td>SK</td>
<td>S</td>
</tr>
<tr>
<td>931g-1800rem</td>
<td>SK</td>
<td>None</td>
</tr>
<tr>
<td>1651-1705em</td>
<td>SN</td>
<td>S</td>
</tr>
<tr>
<td>426-9-1686em</td>
<td>TA</td>
<td>TA</td>
</tr>
<tr>
<td>426-9-2562em</td>
<td>TSNA</td>
<td>TSA</td>
</tr>
<tr>
<td>426-9-2573em</td>
<td>TSNA</td>
<td>TA</td>
</tr>
<tr>
<td>426-9-2774em</td>
<td>TSNA</td>
<td>TA</td>
</tr>
<tr>
<td>426-9-2901em</td>
<td>A</td>
<td>None</td>
</tr>
<tr>
<td>426-9-2921em</td>
<td>TA</td>
<td>T</td>
</tr>
<tr>
<td>3175-1800rem</td>
<td>SN</td>
<td>SN</td>
</tr>
<tr>
<td>WP19-1420em</td>
<td>TSNK</td>
<td>None</td>
</tr>
</tbody>
</table>

<sup>a</sup> T- tetracycline, S- streptomycin, N- nalidixic acid, K- kanamycin, A- ampicillin.
FIG. 2.1. Smal-digested PFGE patterns of turkey-derived *C. coli* strains used as recipients in transformation. Lanes: 1, molecular weight marker; 2, strain 961; 3, strain 3325; 4, strain 1536; 5, strain 931g; 6, strain 3237; 7, strain 1651; 8, strain 1702md; 9, strain 1787; 10, strain 44nec; 11, strain 6034; 12, strain 37-2nec; 13, strain 7474; 14, strain 7580; 15, *C. jejuni* NCTC11168.
FIG. 2.2. KpnI-digested PFGE patterns of turkey-derived *C. coli* strains used as recipients in transformation. Lanes: 1, molecular weight marker; 2, strain 961; 3, strain 3325; 4, strain 1536; 5, strain 931g; 6, strain 3237; 7, strain 1651; 8, strain 1702md; 9, strain 1787; 10, strain 44nec; 11, strain 6034; 12, strain 37-2nec; 13, strain 7474; 14, strain 7580; 15, *C. jejuni* NCTC11168.
FIG. 2.3. Smal-digested PFGE patterns of swine-derived *C. coli* strains used as recipients in transformation. Lanes: 1, 13, molecular weight marker; 2, strain P5; 3, strain WP145; 4, strain WP66; 5, strain 1684; 6, strain 2113; 7, strain 3175; 8, strain 426-9; 9, strain 614-3m; 10, strain 5980; 11, strain WP14; 12, *C. jejuni* NCTC11168.
FIG. 2.4.  Smal-digested PFGE patterns of *C. coli* strains used as donors in transformation. Lanes: 1, molecular weight marker; 2, strain 1705; 3, strain 1800r; 4, strain 2901; 5, *C. jejuni* NCTC11168.
FIG. 2.5. Smal-digested PFGE patterns of *C. coli* erythromycin-resistant and erythromycin-sensitive field strains and erythromycin-resistant transformants derived from them in transformation. Lanes: 1, 12, molecular weight marker; 2, strain 1705; 3, strain 961; 4, transformant 961-1705em; 5, strain 1651; 6, transformant 1651-1705em; 7, strain 3237; 8, transformant 3237-1705em; 9, strain 1536; 10, transformant 1536-1705em; 11, *C. jejuni* NCTC11168.
FIG. 2.6. KpnI-digested PFGE patterns of *C. coli* erythromycin-resistant and erythromycin-sensitive field strains and erythromycin-resistant transformants derived from them in transformation. Lanes: 1, 11, molecular weight marker; 2, strain 1705; 3, strain 961; 4, transformant 961-1705em; 5, strain 1651; 6, transformant 1651-1705em; 7, strain 3237; 8, transformant 3237-1705em; 9, strain 1536; 10, transformant 1536-1705em.
FIG. 2.7. Sau3AI (A) and MboI (B) digestions of chromosomal DNAs from swine-derived *C. coli* used as recipients in transformation. Lanes: 1, 8, Hind III-digested λDNA as molecular weight marker (fragment sizes are 23, 9.4, 6.5, 4.3, 2.3, 2.0, and 0.5 from top to bottom); 2, strain 426-9; 3, strain 3175; 4, strain P5; 5, strain WP66; 6, strain 1684; 7, strain WP145. C represents negative controls.
CHAPTER 3

Impact of Environmental Factors and Incubation Time on Natural Transformation Frequency in *Campylobacter coli*
ABSTRACT

*Campylobacter* is naturally competent, but limited information exists on the impact of environmental conditions on transformation frequency. In this study, we investigated the impact of temperature, microaerobic vs. aerobic atmosphere, nutrient depletion, and incubation time on frequency of transformation to erythromycin and nalidixic acid resistance in *C. coli*. Transformation to erythromycin resistance at 42°C was 10-650 fold more frequent than transformation at 25°C. Noticeable reductions in transformation frequency was also noticed at 32°C and 37°C, in comparison to 42°C. However, difference in frequency of transformation to nalidixic acid resistance was less than eight fold between 42°C and 25°C. Transformation frequency to erythromycin or nalidixic acid resistance was not significantly different between microaerobic (5-10% CO₂) and aerobic conditions, at either 42°C or 25°C. Transformation frequency was furthermore not significantly different under conditions of nutrient depletions at 25°C. Transformation frequency to erythromycin resistance, but not to nalidixic resistance, was significantly (P<0.05) higher when cells were incubated in the presence of donor DNA for 15-17h than for 3-4h. In conclusion, frequency of transformation to erythromycin resistance greatly varies significantly depending on temperature, and transformation frequencies for different antibiotic resistance markers can be differently affected by environmental factors.
INTRODUCTION

*Campylobacter* is known to be naturally competent (Wang and Taylor, 1990), and natural transformation may make a significant contribution to genetic diversity (Wassenaar et al., 1995). Natural transformation may be also substantially relevant to dissemination of antibiotic resistance in *Campylobacter* (Chapter 2). Erythromycin and fluoroquinolones are first line drugs to treat human infections by *Campylobacter* in severe cases or immunocompromised people (Skirrow and Blaser, 2000), and resistance to these antimicrobial agents can seriously compromise treatment outcomes. A point mutation, A2075G in 23S rRNA gene (position 2075 in the 23S rRNA gene of *C. jejuni* NCTC 11168, corresponding to position 2059 in the 23S rRNA gene of *Escherichia coli*) is associated with erythromycin resistance in *Campylobacter* (Gibreel et al., 2005; Harrow et al., 2004; Jensen and Aarestrup, 2001; Vacher et al., 2003). A point mutation in codon 86 of the DNA gyrase gene *gyrA*, from ACT (Thr) to ATT (Ile) is responsible for high-level nalidixic acid and ciprofloxacin resistance in *Campylobacter* (Carattoli et al., 2002; Engberg et al., 2001; Wang et al., 1993). *C. jejuni* is responsible for the majority of human *Campylobacter* infections, with *C. coli* accounting for 5-10% of clinical cases of campylobacteriosis (Nachamkin et al., 2000), but resistance to antibiotics is more common in *C. coli* than *C. jejuni* (Avrain et al., 2003; Bae et al., 2005; Engberg et al., 2001). Pronounced differences have been noticed in prevalence of resistance to erythromycin and other macrolides. Such resistance remains rare in *C. jejuni*, but is often extensive in *C. coli* (Avrain et al., 2003; Bae et al., 2005; Van Looveren et al., 2001).
Campylobacter is an obligate microaerophile, and the thermophilic species C. jejuni and C. coli can only grow at temperatures above 30°C and below 47°C (Park, 2000). Campylobacter is commonly found in many kinds of animal hosts including poultry, swine, cattle, and wild birds, and in environmental samples such as water (Park, 2000), as well as foods. C. coli appears to be more host adapted than C. jejuni (Hopkins et al., 2004; Leatherbarrow et al., 2004). In spite of the potential of transformation to contribute to genetic diversity and dissemination of antibiotic resistance in Campylobacter, the effect of environmental factors on transformation is poorly understood. Understanding the impact of environmental factors is important, considering that Campylobacter is a fastidious microorganism that is also frequently isolated from many different sources, characterized by diverse environmental conditions. Identification of ecologically relevant environmental influences on transformation will lead to better understanding of the role of transformation in generation of genetic diversity and dissemination of antibiotic resistance in Campylobacter.

In this study, we investigated the effect of temperature, microaerobic vs. aerobic conditions, nutrient depletion and incubation time on frequency of transformation to erythromycin and nalidixic acid resistance.

MATERIALS AND METHODS

Bacterial strains.

C. coli strains used as recipients and donors in transformation are listed in Table 3.1. These strains are part of our laboratory’s Campylobacter strain collection, and were obtained from turkeys and swine fecal samples at different farms in eastern North Carolina, South
Carolina, and Virginia between 2001 and 2004. Strains were isolated using direct plating on charcoal cefoperazone deoxycholate agar (CCDA) (Oxoid, Basingstoke, Hampshire, England) at 42°C under microaerobic conditions generated by the CampyPak Microaerophilic System (BBL, Sparks, MD), and preserved in Brain Heart Infusion broth (Becton Dickinson) supplemented with glycerol (20%) at -70°C, as described (Smith et al., 2004). Species identity and antibiotic susceptibility profiles were determined as described (Lee et al., 2005; Smith et al., 2004).

**Erythromycin MIC determinations.** Erythromycin MICs were determined following the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, 2002). Growth was tested at erythromycin concentrations up to 256 µg/ml and monitored following 48h incubation at 42°C, microaerobically. Threshold for resistance was MIC of greater than or equal to 8 µg/ml, as described (Gibreel et al., 2005). High-level resistance corresponded to growth of the bacteria in the presence of 128 µg/ml or 256 µg/ml erythromycin.

**Transformation assays.**

Based on the antibiotic resistance profiles, erythromycin resistant and erythromycin sensitive strains were selected as donors and recipients, respectively, for the transformation assays. Genomic DNA of erythromycin resistant donors was extracted using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) as described (Smith et al., 2004).

Broth transformations followed a previously described protocol (Wilson et al., 2003) with modifications, as described in Chapter 2. Briefly, recipients (961, 3237, and 1702rnd), previously known to be competent (Chapter 2), were grown on Sheep Blood Agar plates
(Remel, Lenexa, KS) at 42°C for 36-48h under microaerobic conditions generated by the CampyPak Microaerophilic System (BBL). A single colony was transferred to 5 ml of Mueller Hinton Broth (MHB) and incubated at 42°C for 24h under microaerobic conditions. 0.1ml of this culture was added to 50ml MHB preconditioned at 42°C, and incubated for 7h at 42°C under microaerobic conditions to reach exponential phase. For each transformation, 1.0ml of this culture was transferred to a sterile polypropylene round-bottom tube (14ml) (Becton Dickinson, Franklin Lakes, NJ) and 15 µl of total genomic DNA (c.a. 3 µg) from strain 1800r was added. Negative controls were processed identically, except that no genomic DNA was added. Following 5h incubation at 42°C microaerobically and aerobically, and at 25°C microaerobically and aerobically, or at any other temperatures studied, 100 µl was spread-plated on MHA plate containing erythromycin (10 µg/ml) (EMHA) in triplicate to enumerate erythromycin-resistant transformants. To enumerate nalidixic acid resistant transformants, 100 µl was spread-plated on MHA plate containing nalidixic acid (16 µg/ml) (NAL) after ten and 100 fold dilutions in MHB. The plates were incubated 36-60h at 42°C microaerobically, and colonies were enumerated. Dilutions (10⁻⁴, 10⁻⁶) of the culture at the end of the 5h transformation period were also plated on MHA and incubated for 24-48h at 42°C microaerobically, in order to determine the CFU/ml of the recipient. Transformation frequency was indicated as the ratio of the number of transformants/ml to total CFU/ml of the recipient.

The agar assay was also used for several C. coli strains (3237, 44nec, 931g, 2775, 1651, 3325, 3175, 426-9, and P5) (Table 3.1). These strains were previously known to be competent, as described in Chapter 2. The agar assay was used as described before (Chapter
2) to determine transformation during overnight incubation (15-17h) at 42°C and microaerobically and aerobically, as well as at 25°C microaerobically and aerobically. To quantitatively describe transformation frequency with the MHA assay, transformation index was calculated also as described in Chapter 2.

**Effect of nutrient depletion on transformation.**

*C. coli* strain 3237 was used to study the effect of nutrient depletion on transformation frequency. The recipient (3237) was grown on Sheep Blood Agar plates (Remel, Lenexa, KS) at 42°C for 48h under microaerobic conditions generated by the CampyPak Microaerophilic System (BBL). A single colony was transferred to 5 ml of MHB and incubated at 42°C for 24h under microaerobic conditions. 0.1ml of this culture was added to 50ml MHB preconditioned at 42°C, and incubated for 12h at 42°C under microaerobic conditions. Aliquots (1ml) were transferred to two sterile microcentrifuge tubes (ISC BioExpress, Kaysville, Utah), centrifuged at 7000 × g, resuspended with Phosphate Buffered Saline (PBS) (Sigma, St. Louis, MO). After two more washes, the cells were resuspended in 1ml PBS and 1ml MHB, respectively, transferred to polypropylene round-bottom tubes (14ml) (Becton Dickinson, Franklin Lakes, NJ) and incubated for 3h at room temperature, to establish starvation conditions in the PBS-suspended cells. Upon 3h incubation, 15 µl of total genomic DNA (c.a. 3 µg) from *C. coli* strain 1800r was added to both cultures in PBS and MHB, and the suspensions were further incubated for 5h at room temperature under aerobic conditions. At that time, 100 µl was spread-plated on EMHA in triplicate to enumerate erythromycin resistant transformants. In addition, dilutions were spread-plated on NAL in triplicate to enumerate nalidixic acid resistant transformants, and on MHA to enumerate total
CFUs. The plates were incubated 36-48h at 42°C microaerobically, and colonies were enumerated. Transformation frequency to erythromycin and nalidixic acid resistance was determined as the ratio of the number of transformants/ml (enumerated on EMHA on NAL, respectively) to total CFU/ml of the recipient.

**Effect of incubation time on transformation to erythromycin resistance.**

*C. coli* strains 3237, 961, 1787, 1684, 1651, 3175, 2775, and 1536 were previously known to be competent (Chapter 2), and were studied to evaluate the effect of incubation time (3-4h and 15-17h) on transformation to erythromycin resistance. The agar assay was used as described in Chapter 2. Briefly, a loopful of the erythromycin sensitive recipient (from 20-24h old cultures on MHA, grown at 42°C microaerobically), was spotted on two sets of MHA plates in triplicate, then 4 µl genomic DNA of the donor strain was added to each spot and mixed, with diameter of the spot after mixing, ca. 0.5 cm. Two sets of plates were incubated 3-4h and overnight (15-17h), respectively, in separate jars at 42°C under microaerobic conditions, and the entire material from each spot was spread-plated on EMHA. Each transformation included as negative control spots of the recipient without donor DNA, on the same MHA plate. The EMHA plates were incubated for 48h at 42°C under microaerobic conditions, and transformation index was calculated as described in Chapter 2.

**Statistical analysis.** Data were analyzed with SAS (SAS Institute Inc., Cary, N.C.). ANOVA was used with F test to find a significant difference (P<0.05).

**RESULTS**

**Effect of temperature on frequency of transformation to erythromycin and nalidixic acid resistance.**
Three *C. coli* strains were investigated in terms of the impact of temperature on transformation to erythromycin resistance. Temperature had a profound effect, with frequency of transformation being significantly higher at 42°C than 25°C for all three strains (Table 3.2). Transformation to erythromycin resistance at 42°C was 10-650 fold more frequent than at 25°C. For two of the strains (961 and 3237), transformation frequency at 42°C ranged between $10^{-5}$ and $10^{-7}$ but was below detection level at 25°C (Table 3.2). Higher frequency of transformation to erythromycin resistance at 42°C compared to 25°C was also found with the MHA assay. Transformation index was significantly higher at 42°C compared to 25°C ($P<0.05$) (Table 3.3). Transformation to erythromycin resistance at temperatures below 42°C and above 25°C was studied with one strain, *C. coli* 961. A significant decrease in transformation frequency was observed as temperature decreased from 42°C to 37°C, and very few transformants were obtained at 32°C (Figure 3.1).

The impact of temperature on transformation to nalidixic acid resistance was evaluated with strains 3237 and 1702rnd, because strain 961 was resistant to this antibiotic. At 42°C, frequency of transformation to nalidixic acid resistance ($10^{-4}$) was significantly higher than to erythromycin resistance ($10^{-7}$) for strain 3237, whereas strain 1702rnd had similar transformation frequency to erythromycin and nalidixic acid resistance ($10^{-4}$) at this temperature. In contrast to transformation to erythromycin resistance, however, transformation to nalidixic acid resistance was not significantly different between 42°C and 25°C for either strain under either microaerobic or aerobic conditions (Table 3.2). There was also no significant difference in frequency of transformation to nalidixic acid resistance.
between 42°C ($1.0 \times 10^{-3}$) and 39°C ($7.6 \times 10^{-4}$) in strain 614-3m under microaerobic conditions.

Transformation to nalidixic acid resistance at 25°C was also readily observed with several recipients (3237, 41nec, 2760, and 910C1), upon 3-4h incubation with donor DNA in the MHA assay (data not shown).

**Lack of difference in transformation frequency between microaerobic and aerobic conditions.**

No significant differences in frequency of transformation to erythromycin resistance were observed between microaerobic (5 to 10% CO$_2$) and aerobic conditions at 42°C (Table 3.2). Erythromycin resistant transformant derived from strain 3237 under aerobic conditions had high MIC (>256 µg/ml), similar to transformants derived under microaerobic conditions (Chapter 2). Transformation index with the MHA assay was not significantly different (P>0.05) aerobically and microaerobically (Table 3.3). Similarly to results with transformation to erythromycin resistance, no significant differences were observed in frequency of transformation to nalidixic acid resistance between microaerobic and aerobic conditions, either at 42°C or at 25°C (Table 3.2).

**Effect of nutrient depletion on frequency of transformation to erythromycin and nalidixic acid resistances.**

Frequency of transformation of strain 3237 to nalidixic acid resistance under the stress of nutrient depletion caused by suspension of washed cells in buffer (PBS) was approximately $10^{-5}$, and was similar to transformation frequency of similarly washed cells
suspended in MHB. No erythromycin resistant transformants were obtained at room temperature with either the PBS or the MHB suspensions (data not shown).

**Effect of incubation time on transformation to erythromycin resistance.**

Transformation to erythromycin resistance was more frequent upon 15-17h incubation than upon 3-4h incubation at 42°C under microaerobic conditions (P<0.05) (Table 3.4). Eight recipients were tested for transformation to erythromycin resistance upon 3-4h vs. 15-17h incubation at 42°C under microaerobic conditions. Only one recipient (strain 3237) was transformed to erythromycin resistance upon 3-4h incubation, whereas six recipients including strain 3237 were transformed upon 15-17h incubation (Table 3.4). Increased transformation frequency was also found in 5h incubation time, compared to 2h with broth assay (data not shown).

**DISCUSSION**

It is intriguing that transformation to erythromycin resistance is significantly affected by temperature, whereas transformation to nalidixic acid resistance is not affected in a wide range of temperatures, between 25°C and 42°C (Tables 3.2 and 3.3; Fig. 3.1). Our findings suggest that 42°C may be the optimal temperature for transformation to erythromycin resistance since a significant drop in transformation frequency was noticed at 37°C (Fig. 3.1). At temperatures higher than 42-43°C, growth and survival of *Campylobacter* would be compromised. Optimal transformation frequency to erythromycin resistance at 42°C suggests that such transformation may take place efficiently in the gastrointestinal tract of poultry, whose body temperature is 42-43°C. Conversely, the noticeable decrease in transformation
frequency for erythromycin resistance at 37°C may suggest that such transformation may be less likely within the gastrointestinal tract of animals with lower body temperature such as humans (37°C) and swine (38-39°C). In fact, preliminary data with the swine-derived strain 614-3m showed noticeably reduced transformation to erythromycin resistance at 39°C in comparison to 42°C. We cannot exclude the possibility, however, that other factors which are not included in this in vitro study, but reside solely in swine may alleviate the impact of reduced temperature and enable frequent transformation to erythromycin resistance within these hosts.

Wilson et al. (2003) found that the efficiency of transformation to chloramphenicol resistance increased by ten to 100 fold as CO2 concentration declined from 10% to 0.7%. In our study we showed that transformation was equally effective under microaerobic (5-10% CO2) and aerobic (0.03% CO2) conditions, suggesting that these differences in CO2 concentration did not significantly affect transformation to nalidixic acid and erythromycin resistances. However, further studies are necessary to investigate the effect of CO2 on transformation to these two important antibiotic resistance determinants. Differences in the strains that were used, the antibiotic resistance markers that were transformed, and the experimental conditions including CO2 concentration range, may account for the different findings between this study and the study of Wilson et al. (2003).

Nalidixic acid resistant C. coli isolates are typically also resistant to fluoroquinolones (Lee et al., 2005). The results from transformation to nalidixic acid resistance suggest that acquisition of fluoroquinolone resistance through transformation may occur frequently and consistently in a wide range of CO2 concentrations and temperatures in animals or in the
environment. In contrast, transformation to erythromycin resistance is temperature-specific and occurs best at 42°C, typical of the avian body temperature. Thus, acquisition of fluoroquinolone resistance through transformation may occur frequently in various niches, but acquisition of erythromycin resistance may occur much more commonly inside animal (especially avian) hosts than in the environment. In addition, this findings imply that the use of one marker to study environmental factors affecting transformation frequency in Campylobacter may lead to pitfalls. For example, temperature would be considered an insignificant factor in transformation if transformation to only nalidixic acid resistance were studied.

The reason for temperature-sensitive transformation to erythromycin resistance, but temperature-insensitive transformation to nalidixic acid resistance remains elusive. The marked dependence of transformation to erythromycin resistance depending on temperature may be related to differentially regulated gene expression of the organism at 42°C compared to 37°C (Stintzi, 2003). Pronounced differences between the 42°C and 37°C transcriptome have been noted with C. jejuni. It is possible that chromosomal DNAs containing gyrA mutation (encoding nalidixic acid resistance) may use a different DNA uptake or transport system from chromosomal DNAs containing the 23S rRNA gene mutation. Proteins for uptake or transport of DNA containing gyrA mutation may be expressed at a constant level regardless of temperature change, but proteins for uptake or transport of DNA containing the 23S rRNA gene mutation may be expressed in a great variation on temperature change.

Extracellular DNA acquired by transformation can be not only recombined with genome in bacteria, but also serve as nutrients and biosynthesis precursors (Finkel and Kolter, 2001).
Nutritional downshift can lead to competence in *Haemophilus influenzae* and *Bacillus subtilis* (Redfield, 1993). Transformation frequency of *Campylobacter*, however, does not appear to be enhanced by nutrient depletion. Transformation to nalidixic acid was somewhat (about 10 fold) lower in the nutrient depletion experiments, both in the PBS and MHB suspensions. Stress caused by washes and centrifuge may have resulted in lower transformation frequency, compared to other experiments in which washes and centrifugation were not used. No transformation event for erythromycin resistance in nutritional downshift indicates that acquisition of erythromycin resistance in *C. coli* in environmental water is very unlikely to occur.

Our findings on the increased transformation frequency to erythromycin resistance at 15-17h compared to 3-4h are in agreement with a previous study showing that increase of incubation time with donor DNA also increased frequency of transformation to nalidixic acid resistance (Wang and Taylor, 1990). This study also shows the increased frequency of transformation to erythromycin resistance upon increased incubation time with donor DNA.

In conclusion, transformation of *C. coli* to erythromycin resistance appears to be regulated by temperature, in contrast to transformation to nalidixic acid resistance which appears to be temperature independent. These findings suggest that ecological attributes (ex. environmental temperature, host) may exert differential impact on the potential of the organism to acquire antimicrobial resistance determinants via natural transformation. Additional studies are needed to characterize the impact of temperature on transformation at the molecular level.
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of *Campylobacter* strains isolated from food animals in Belgium. J. Antimicrob.

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*Campylobacter jejuni* gyrA gene and characterization of quinolone resistance mutations.


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gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer.

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*Campylobacter jejuni* requires components of a type II secretion system. J. Bacteriol.
185:5408-5418.

Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake
TABLE 3.1. Strains used in natural transformation study for environmental factors and incubation time

<table>
<thead>
<tr>
<th>Strains</th>
<th>Recipient/Donor</th>
<th>Antibiotic resistance profile&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Animal source</th>
<th>Date isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>961</td>
<td>Recipient</td>
<td>TSNKA</td>
<td>Turkey, ceca</td>
<td>Jan, 2002</td>
</tr>
<tr>
<td>3237</td>
<td>Recipient</td>
<td>TKA</td>
<td>Turkey, feces</td>
<td>Aug, 2002</td>
</tr>
<tr>
<td>1702rnd</td>
<td>Recipient</td>
<td>TSA</td>
<td>Turkey, ceca</td>
<td>Apr, 2002</td>
</tr>
<tr>
<td>614-3m</td>
<td>Recipient</td>
<td>TSKA</td>
<td>Pig, feces</td>
<td>Jun, 2002</td>
</tr>
<tr>
<td>1536</td>
<td>Recipient</td>
<td>TNA</td>
<td>Turkey, ceca</td>
<td>Mar, 2002</td>
</tr>
<tr>
<td>1651</td>
<td>Recipient</td>
<td>TKA</td>
<td>Turkey, feces</td>
<td>Apr, 2002</td>
</tr>
<tr>
<td>1684</td>
<td>Recipient</td>
<td>TA</td>
<td>Pig, feces</td>
<td>Apr, 2002</td>
</tr>
<tr>
<td>1787</td>
<td>Recipient</td>
<td>TNKA</td>
<td>Turkey, feces</td>
<td>May, 2002</td>
</tr>
<tr>
<td>2775</td>
<td>Recipient</td>
<td>TNKA</td>
<td>Turkey, ceca</td>
<td>Jul, 2002</td>
</tr>
<tr>
<td>3175</td>
<td>Recipient</td>
<td>TKA</td>
<td>Pig, feces</td>
<td>Aug, 2002</td>
</tr>
<tr>
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<td>Recipient</td>
<td>TSNA</td>
<td>Turkey, feces</td>
<td>Aug, 2002</td>
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<td>426-9</td>
<td>Recipient</td>
<td>K</td>
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</tr>
<tr>
<td>P5</td>
<td>Recipient</td>
<td>TKA</td>
<td>Piglet, feces</td>
<td>Feb, 2002</td>
</tr>
</tbody>
</table>

<sup>a</sup> Antibiotics which the recipient is resistant to are listed: T- tetracycline, E- erythromycin, S- streptomycin, N- nalidixic acid, K- kanamycin, A- ampicillin.
### TABLE 3.1. (Continued)

<table>
<thead>
<tr>
<th>Strains</th>
<th>Recipient/Donor</th>
<th>Antibiotic resistance profile&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Animal source</th>
<th>Date isolated</th>
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<tr>
<td>1800r</td>
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<td>Turkey, ceca</td>
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<td></td>
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<td>Donor TEA</td>
<td>Pig, feces</td>
<td>Jul, 2002</td>
<td></td>
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<td>Donor TESNKA</td>
<td>Turkey, feces</td>
<td>Aug, 2002</td>
<td></td>
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<td>Donor E</td>
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<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Antibiotics which the recipient is resistant to are listed: T- tetracycline, E- erythromycin, S- streptomycin, N- nalidixic acid, K- kanamycin, A- ampicillin.
TABLE 3.2. Impact of temperature and microaerobic vs. aerobic atmosphere on transformation frequency of *C. coli* to erythromycin and nalidixic acid resistance

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Antibiotic resistance marker</th>
<th>Microaerobic</th>
<th>Aerobic</th>
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</thead>
<tbody>
<tr>
<td>961</td>
<td>Erythromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42°C</td>
<td>$1.3 \times 10^{-5} \pm 5 \times 10^{-7}$</td>
<td>$&lt;9.3 \times 10^{-8}$</td>
<td>$1.1 \times 10^{-5} \pm 9.1 \times 10^{-6}$</td>
</tr>
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<td>25°C</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>42°C</td>
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<td>25°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3237</td>
<td>Erythromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42°C</td>
<td>$8.0 \times 10^{-7} \pm 6.8 \times 10^{-7}$</td>
<td>$&lt;5.7 \times 10^{-8}$</td>
<td>$6.6 \times 10^{-7} \pm 9.2 \times 10^{-7}$</td>
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<tr>
<td>25°C</td>
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<td></td>
</tr>
<tr>
<td>42°C</td>
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<td></td>
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</tr>
<tr>
<td>25°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>$2.8 \times 10^{-4} \pm 9.0 \times 10^{-5}$</td>
<td>$2.5 \times 10^{-4} \pm 6.6 \times 10^{-5}$</td>
<td>$2.1 \times 10^{-4} \pm 1.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>1702rnd</td>
<td>Erythromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42°C</td>
<td>$2.6 \times 10^{-4} \pm 2.9 \times 10^{-4}$</td>
<td>$4.0 \times 10^{-7} \pm 5.7 \times 10^{-7}$</td>
<td>$1.5 \times 10^{-4} \pm 1.1 \times 10^{-4}$</td>
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<tr>
<td>25°C</td>
<td></td>
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<td>42°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>$9.3 \times 10^{-4} \pm 3.3 \times 10^{-4}$</td>
<td>$1.4 \times 10^{-4} \pm 9.8 \times 10^{-5}$</td>
<td>$5.4 \times 10^{-4} \pm 4.1 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

\(^a\) Data presented are averages ± 1 standard deviation, based on at least two independent experiments

\(^b\) A and B are statistically significant (P<0.05)
FIG. 3.1. Effect of temperature on transformation to erythromycin resistance in *C. coli* strain 961 under aerobic conditions. The error bars represent standard deviation.
TABLE 3.3. Transformation index of transformation-mediated acquisition of erythromycin resistance at different temperatures and microaerobic vs. aerobic atmosphere with the MHA assay

<table>
<thead>
<tr>
<th>Source</th>
<th>Recipient</th>
<th>Donor</th>
<th>MA&lt;sup&gt;a&lt;/sup&gt;, 42°C</th>
<th>MA&lt;sup&gt;b&lt;/sup&gt;, RT</th>
<th>AE&lt;sup&gt;c&lt;/sup&gt;, 42°C</th>
<th>AE, RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>3237</td>
<td>1800r</td>
<td>1.0</td>
<td>0.3</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1420</td>
<td>1.0</td>
<td>0.3</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3624</td>
<td>1.0</td>
<td>0.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Turkey</td>
<td>44nec</td>
<td>1800r</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Turkey</td>
<td>931g</td>
<td>1800r</td>
<td>0.7</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Turkey</td>
<td>2775</td>
<td>3174</td>
<td>1.0</td>
<td>0.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Turkey</td>
<td>1651</td>
<td>1705</td>
<td>1.0</td>
<td>0.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Turkey</td>
<td>3325</td>
<td>1686</td>
<td>1.0</td>
<td>0.0</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2562</td>
<td>0.8</td>
<td>0.0</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Swine</td>
<td>3175</td>
<td>1800r</td>
<td>0.7</td>
<td>0.0</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Swine</td>
<td>426-9</td>
<td>1800r</td>
<td>0.7</td>
<td>0.0</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2573</td>
<td>0.6</td>
<td>0.0</td>
<td>0.8</td>
<td>0.0</td>
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<tr>
<td>Swine</td>
<td>P5</td>
<td>2921</td>
<td>0.8</td>
<td>0.0</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
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</tbody>
</table>

<sup>a</sup> MA, microaerobic conditions
<sup>b</sup> RT, room temperature
<sup>c</sup> AE, aerobic conditions
<sup>d</sup> ND, not done
TABLE 3.4. Transformation index of transformation-mediated acquisition of erythromycin resistance on different incubation time

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Incubation time</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3-4h</td>
<td>Overnight</td>
</tr>
<tr>
<td>3237</td>
<td>3237-1705</td>
<td>1.0</td>
</tr>
<tr>
<td>961</td>
<td>1705</td>
<td>0.0</td>
</tr>
<tr>
<td>1787</td>
<td>1705</td>
<td>0.0</td>
</tr>
<tr>
<td>1684</td>
<td>1705</td>
<td>0.0</td>
</tr>
<tr>
<td>1651</td>
<td>1705</td>
<td>0.0</td>
</tr>
<tr>
<td>3175</td>
<td>1705</td>
<td>0.0</td>
</tr>
<tr>
<td>1800r</td>
<td>1705</td>
<td>0.0</td>
</tr>
<tr>
<td>3174</td>
<td>1705</td>
<td>0.7</td>
</tr>
<tr>
<td>3175</td>
<td>3174</td>
<td>0.0</td>
</tr>
<tr>
<td>2775</td>
<td>3174</td>
<td>0.0</td>
</tr>
<tr>
<td>1536</td>
<td>3174</td>
<td>1.0</td>
</tr>
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</table>
CHAPTER 4

Comparison of Natural Transformation to Erythromycin Resistance Between

Campylobacter coli and Campylobacter jejuni and Fitness of C. coli and C. jejuni

Erythromycin-Resistant Transformants
ABSTRACT

Erythromycin resistance has been repeatedly noted to be more common in *Campylobacter coli* than in *Campylobacter jejuni*, but the reasons for this difference remain unknown. We hypothesized that *C. jejuni* may be able to acquire erythromycin resistance *in vitro* via transformation, but transformants may be of reduced fitness, thus accounting for limited prevalence of erythromycin resistance in *C. jejuni* field isolates. We investigated transformation of *C. jejuni* strain SC 49 isolated from turkeys to erythromycin resistance, using as donor genomic DNA from erythromycin resistant field isolates of *C. coli*. Transformation of *C. jejuni* to erythromycin resistance was less likely to occur than transformation of *C. coli* when genomic DNA from *C. coli* was used as donor. *C. jejuni* erythromycin-resistant transformants, however, had high erythromycin MIC values and harbored the A2075G transition in the 23S rRNA gene, similarly to *C. coli* transformants. When grown in separate cultures, an erythromycin-resistant transformant derived from *C. coli* strain 961 had growth rate similar to that of the erythromycin-sensitive parental strain, whereas an erythromycin-resistant transformant derived from *C. jejuni* strain SC49 had significantly reduced growth rate (P<0.05) compared to its parental strain. Complex results were obtained from competitive growth studies involving batch cultures with 1:1 initial mixture of each erythromycin-resistant transformant and its parental strain. The *C. coli* erythromycin-resistant transformant was at a competitive disadvantage in relation to its parental strain in stationary phase, whereas the erythromycin-resistant *C. jejuni* appeared to lack any disadvantage during exponential growth, and was at a slight fitness advantage after 14 days. In conclusion, erythromycin resistance derived from *C. coli* may be less frequently
acquired by *C. jejuni* than by *C. coli* through transformation. Additional *in vitro* and *in vivo* models need to be developed to better characterize the possible effects of erythromycin resistance in the fitness of *C. coli* and *C. jejuni* from meat animals.

**INTRODUCTION**

A substantial number of *Campylobacter* isolates from meat animals such as poultry and swine are erythromycin resistant (Avrain et al., 2003; Bywater et al., 2004; Payot et al., 2004b; Sáenz et al., 2000; Van Looveren et al., 2001). Prevalence of erythromycin resistance among *C. coli* strains isolated from turkey meat and from turkeys at pre-harvest level was also significant (Ge et al., 2003; Lee et al., 2005; Luber et al., 2003). Frequency of erythromycin resistance among *C. coli* strains is higher than among *C. jejuni* strains in most cases (Engberg et al., 2001; Ge et al., 2003; Luber et al., 2003). However, the reason for such difference in prevalence of erythromycin resistance between *C. coli* and *C. jejuni* remains unknown. Transformation can be relevant to dissemination of erythromycin resistance in *Campylobacter* (Chapter 2). However, the relative ability of *C. jejuni* vs. *C. coli* to acquire erythromycin resistance via transformation has not yet been reported.

Antibiotic resistance can cause significant change in bacterial fitness. For instance, in *Helicobacter pylori* (a species closely related to *Campylobacter*), macrolide (erythromycin)-resistant strain showed reduced fitness compared to a clonally-related macrolide-sensitive strain in a competitive growth model (Kanai et al., 2004). In *Campylobacter*, fluoroquinolone-resistant *C. jejuni* had competitive advantage over fluoroquinolone-sensitive *C. jejuni* in chickens, even in the absence of fluoroquinolone selection pressure (Luo et al.,
On the other hand, the erythromycin resistance corresponding to the point mutation (A2075G) in 23S rRNA gene did not change growth rate of *C. jejuni* 81116 (Gibreel et al., 2005). Reports on fitness of erythromycin-resistant *C. coli* are not available, even though erythromycin resistance is much more frequent in *C. coli* than in *C. jejuni*. Study on fitness costs of erythromycin resistance in *C. coli* as well as *C. jejuni* is necessary to understand the significant differences in prevalence of erythromycin resistance between *C. coli* and *C. jejuni*. Furthermore, erythromycin resistance may be disseminated by natural transformation in *Campylobacter* (Chapter 2). Therefore, there is the need to evaluate fitness of erythromycin-resistant transformants.

In this study, we compared frequency of transformation to erythromycin resistance in *C. coli* vs. *C. jejuni*. Fitness of *C. jejuni* and *C. coli* erythromycin-resistant transformants and their respective wildtype erythromycin sensitive parental strains was evaluated during *in vitro* competitive growth assays, in order to further understand the mechanisms underlying dissemination of erythromycin resistance in *Campylobacter*.

**MATERIALS AND METHODS**

**Bacterial strains.** *C. coli* and *C. jejuni* strains used in transformation assay are listed in Table 4.1. These were isolated from turkeys, broilers, or swine at different farms located in North Carolina. *Campylobacter* was isolated from samples using direct plating on charcoal cefoperazone deoxycholate agar (CCDA) (Oxoid, Basingstoke, Hampshire, England) at 42°C under microaerobic conditions, as described previously (Smith et al., 2004). Species identity and antibiotic susceptibility profiles were determined as previously described (Lee et al.,
2005; Smith et al., 2004). To compare frequency of transformation to erythromycin resistance between *C. coli* and *C. jejuni*, erythromycin sensitive *C. coli* strains were selected and paired with *C. jejuni* strains, based on certain criteria, as follows, with the first strain of each pair being *C. coli* and the second being *C. jejuni*: Four pairs of strains were selected based on same antibiotic resistance profiles and were (1) 961 and 3240, (2) 3237 and SC49, (3) 37-2nec and 2146, and (4) WP14 and 2954; one pair (strains 1787 and 1531) were isolated from the same turkey flock (Flock JN) in North Carolina. One pair (strains 1702rnd and 3464MD) were selected based on both same antibiotic resistance profile and same flock (flock HW) in North Carolina; and one pair (strains 931g and 931c) were isolated from the same cecal sample derived from a turkey.

**Mueller Hinton Agar transformation assay and Erythromycin MIC determinations.** Transformation of one *C. coli* and one *C. jejuni* strain from each of the strain pairs listed above was done at the same time, using total genomic DNA of the erythromycin resistant *C. coli* strains as donor. Transformation to erythromycin resistance was studied with MHA and broth assays, as described in Chapter 2. Transformation index was calculated to quantitatively estimate the extent of growth on MHA plate containing erythromycin (10 µg/ml) (EMHA), as described in Chapter 2. Erythromycin MICs of *C. coli* and *C. jejuni* erythromycin-resistant transformants were determined based on the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, 2002).

**Pulsed-field gel electrophoresis analysis.** Pulsed-field gel electrophoresis (PFGE) of erythromycin-sensitive parental strains and their erythromycin-resistant transformants was done as described previously (Ribot et al., 2001). Enzyme SmaI and KpnI (New England
Biolabs, Beverly, MA) were used to digest genomic DNA. The lambda ladder PFG marker (New England Biolabs, Beverly, MA) was used as a molecular weight marker and *C. jejuni* NCTC11168 was used as a reference strain.

**Growth study in a separate culture.** *Campylobacter* strains were cultured separately to determine and compare growth rates. Bacteria were grown on Sheep Blood Agar plates (Remel, Lenexa, KS) at 42°C for 36-48h under microaerobic conditions. A single colony was transferred to 5ml of Mueller Hinton Broth (MHB) and incubated at 42°C for about 24h under microaerobic conditions generated by the CampyPak Microaerophilic System (BBL, Sparks, MD). After 100 fold dilutions, 0.1ml was transferred to 15ml of MHB and incubated at 42°C under microaerobic conditions. Sampling of 0.05ml from each culture was done at 0, 8, 20, 32, 44, 56, 68, and 75h for *C. coli* erythromycin-resistant transformant, 961-1705em (derived from transformation of 961 by donor DNA from strain 1705) and its parental strain 961. It was done similarly at 0, 8, 20, 32, 44, 56, 68, 80, and 92h for *C. jejuni* erythromycin-resistant transformant, SC49-910C1em (derived from transformation of SC49 by donor DNA from strain 910C1) and its parental strain SC49. Dilutions were plated on Mueller Hinton Agar (MHA) to determine CFU/ml.

**Competitive growth study.** Population dynamics in a mixed culture of erythromycin-resistant transformant and its parental strain were studied during 14 days of incubation at 42°C under microaerobic conditions. Cultures were inoculated and grown in 15ml of MHB as described above for the separate cultures. Briefly, erythromycin-sensitive strain and its erythromycin-resistant transformants were grown on Sheep Blood Agar Plates for 36-48h at 42°C under microaerobic conditions and a single colony was subcultured to
5ml MHB and incubated at 42°C under microaerobic conditions for about 24h. 0.1ml of cultures were inoculated to 15ml MHB after 100 fold dilution to make initial inoculum level at $10^4$ to $10^5$ CFU/ml with ratio 1:1 between erythromycin-sensitive strain and its erythromycin-resistant transformant. CFUs in 15ml MHB were determined immediately prior to incubation (time 0). Then, sampling of 0.05 ml from cultures was done at (in hours) 8, 20, 32, 44, 56, 68, 92, 144 (6 days), 192 (8 days), 240 (10 days), 288 (12 days), and 336h (14 days). The cultures were diluted and plated on both MHA and EMHA to enumerate total CFU and erythromycin-resistant transformants, respectively. CFUs of erythromycin-resistant transformants were subtracted from total CFUs to determine CFUs of the erythromycin-sensitive parental strain. Alternatively, the cultures were diluted and plated on MHA to enumerate total CFUs, and 50 to 100 randomly chosen isolated colonies from the MHA plates were picked with sterile toothpicks and subcultured on EMHA. Total CFUs multiplied by the ratio of number of colonies on EMHA to number of colonies subcultured from MHA to EMHA was CFUs of erythromycin-resistant transformants.

**Statistical analysis.** Data were analyzed with SAS (SAS Institute Inc., Cary, N.C.). F test with PROC GLM was used to study differences in generation times between erythromycin-sensitive strains and erythromycin-resistant transformants. The level of significance was set at P<0.05.

**RESULTS**

Transformation of *C. jejuni* to erythromycin resistance was less frequent than transformation of *C. coli.* Among seven *C. jejuni* strains tested for transformation to erythromycin resistance in parallel with *C. coli* strains, two were frequently transformed to
erythromycin resistance with high transformation index, 1.0, two were transformed less frequently compared to \textit{C. coli}, and three were not transformed to erythromycin resistance, whereas \textit{C. coli} strains in the parallel transformations were transformed with high transformation index (TI>0.8) (Table 4.2). \textit{C. jejuni} strain SC49 which was transformed at the intermediate level (TI=0.5) was also studied with the broth assay using erythromycin-resistant \textit{C. coli} DNA as donor, but no erythromycin-resistant transformants were obtained (<1.5x10^{-8}). However, the use of donor DNA from an erythromycin-resistant transformant derived from SC49 transformations using the MHA assay (SC49-910C1em) enhanced the transformation frequency (9.4x10^{-7}). All of erythromycin-resistant transformants derived from the four \textit{C. jejuni} strains, SC49, 1531, 931c, and 2954 had high erythromycin MICs (>256µg/ml) similar to transformants derived from \textit{C. coli} strains (Chapter 2). In addition, the A2075G transition in 23S rRNA gene was identified in the erythromycin-resistant transformant SC49-910C1em derived from strain SC49 (data not shown).

\textbf{PFGE patterns of erythromycin-sensitive parental strains and erythromycin-resistant transformants.} PFGE patterns of erythromycin-sensitive parental strains and erythromycin-resistant transformants were compared, after digestion of genomic DNA with enzymes Smal (Fig. 4.1) and KpnI (Fig. 4.2 and 4.3). The PFGE patterns of the \textit{C. coli} transformant 961-1705em and the \textit{C. jejuni} transformant SC49-910C1em were indistinguishable from those of their respective parental strains.

\textbf{Comparative growth of erythromycin-resistant transformants and parental strains in separate cultures.} Growth rates of the \textit{C. coli} transformant 961-1705em and the \textit{C. jejuni} transformant SC49-910C1em, along with the respective parental strains, were
determined in separate cultures. The initial number of viable cells was between $10^4$ and $10^5$ CFU/ml. When grown in separate cultures, the erythromycin-sensitive *C. coli* strain 961 and its erythromycin-resistant transformant 961-1705em had similar growth rates in a separate culture over the first 68h, with generation times of 1.6h and 1.7h, respectively (Fig. 4.4). The *C. jejuni* erythromycin-resistant transformant SC49-910C1em, however, had significantly longer generation time (1.5h) for the first 8h, compared to its parental strain SC49 (1.1h) ($P<0.05$) (Table 4.3; Fig. 4.5). After stationary phase, however, both had similar survival curves.

**Competitive fitness in mixed cultures.** The initial number of viable cells in mixed cultures used for competitive fitness studies was also between $10^4$ and $10^5$ CFU/ml for each strain. A competitive advantage of *C. coli* strain 961 over its erythromycin-resistant transformant 961-1705em was observed during stationary phase, whereas there was no significant difference in generation times during exponential growth phase (Fig. 4.6). Strain 961 represented about 94% of the population after 68h incubation period. The dominance of strain 961 increased during the 2 weeks of incubation, and the population of the transformant 961-1705em was below detection limit ($10^3$ CFU/ml) after 12 days. In *C. jejuni*, no significant difference was found in generation times between strain SC49 and erythromycin-resistant transformant, SC49-910C1em, in contrast to the findings from growth in separate cultures (Fig. 4.7). A slight competitive advantage was found in erythromycin-resistant transformant, SC49-910C1em over strain SC49 after 14 days (336h) of incubation time (Fig. 4.7).
DISCUSSION

*C. coli* and *C. jejuni* strains were selected in pairs, based on common antibiotic resistance profiles or common sources in order to compare species-dependent differences in transformation to erythromycin resistance.

It is not surprising that *C. jejuni* strains were less likely to be transformed to erythromycin resistance than *C. coli* strains by the use of donor DNA from *C. coli*. A previous study found that *C. jejuni* has a preference for donor DNA from its own species in transformation, and donor DNA from *C. coli* did not compete well with donor DNA from *C. jejuni* to transform other *C. jejuni* strains (Wilson et al., 2003). However, the 23S rRNA genes are extremely conserved, suggesting sufficient homology for transformation. The overall noticeably lower transformation index for erythromycin resistance in *C. jejuni* compared to in *C. coli* with donor DNA from *C. coli* indicates that erythromycin resistance in *C. coli* may be not frequently disseminated in *C. jejuni* through transformation. It correlates with the relatively low prevalence of erythromycin resistance in *C. jejuni* compared to *C. coli*, considering that transformation can be significantly related to dissemination of erythromycin resistance in *Campylobacter* (Chapter 2). However, some facts undermine this hypothesis: 1) certain *C. jejuni* strains could be frequently transformed to erythromycin resistance *in vitro*, 2) transformation of *C. jejuni* SC49 with DNA from an erythromycin-resistant transformant of the same strain resulted in increased transformation frequency, 3) *C. jejuni* transformants had high erythromycin MICs, and harbored the same 23S rRNA mutation found in *C. coli* transformants. Therefore, further study is required to better characterize acquisition of
erythromycin resistance in *C. jejuni* and identify the reasons for the low prevalence of erythromycin resistance in *C. jejuni*.

Fitness costs accompanying erythromycin resistance would be ideally studied by constructing erythromycin-resistant isogenic strains through transformation with a PCR product containing the 23S rRNA mutation, as was done for *C. jejuni* 81116 (Gibreel et al., 2005). However, such transformations were unsuccessful in our laboratory, for reasons that are not clear. Transformation with genomic DNA to erythromycin resistance, however, could at least result in transformants genetically similar to their parental strains, and makes such strains attractive to use for studying fitness changes upon acquisition of erythromycin resistance. In fact, PFGE fingerprints of transformants and parental strains were indistinguishable, with two enzymes, suggesting lack of major genomic rearrangements in these transformants.

It is interesting that the erythromycin-resistant transformant derived from *C. jejuni* had longer generation time than its parental strain in a separate culture. This was contrary to a previous study (Gibreel et al., 2005) which found no difference in growth rates between *C. jejuni* erythromycin-sensitive strains and erythromycin-resistant isogenic mutants. It is not clear why the *C. jejuni* transformant did not have any reduced growth rate compared to its parental strain in the competitive growth model, in contrast to the findings from growth in a separate culture. The available data do now allow any definite conclusions as to whether erythromycin resistance reduces fitness in *C. jejuni* and further research is necessary.

In mixed cultures, the *C. coli* transformant was at a competitive disadvantage in stationary phase. This was surprising, considering the higher prevalence of erythromycin
resistance in *C. coli* compared to *C. jejuni*, and the observed stability of erythromycin resistance in transformants in the absence of antibiotic pressure. However, it was noticeable that the *C. coli* transformant did not show any reduced growth rate during exponential growth in separate cultures or in the mixed culture competitive model. This suggests that erythromycin-resistant *C. coli* may compete well for growth in the initial stage of colonization of the intestine of young animals. In addition, antibiotic selection pressure may easily select for erythromycin-resistant strains, including transformants, and erythromycin-resistant *C. coli* may not be easily displaced under *in vivo* conditions. Finally, the fitness cost of erythromycin resistance could be ameliorated through compensatory mutation in other loci (Levin et al., 2000).

In conclusion, even though *C. jejuni* strains were less likely to be transformed to erythromycin resistance and erythromycin-resistant transformant of *C. jejuni* showed longer generation time than its parental strain in a separate culture, our overall results do not sufficiently explain the lower prevalence of erythromycin resistance in *C. jejuni* compared to *C. coli*. The reasons may reflect the limitation of *in vitro* model systems for analysis of fitness, and *in vivo* studies (e.g. involving colonization of chickens) may be suggested for the future. Further studies are necessary to understand the significant difference in prevalence of erythromycin resistance between *C. jejuni* and *C. coli*. 
REFERENCES:


TABLE 4.1. *C. coli* and *C. jejuni* strains used in the MHA transformation assay to compare transformation to erythromycin resistance

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Profile&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Animal source</th>
<th>Date isolated</th>
</tr>
</thead>
<tbody>
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<td>TSNKA</td>
<td>Turkey, ceca</td>
<td>Jan, 2002</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>3237</td>
<td>TKA</td>
<td>Turkey, feces</td>
<td>Aug, 2002</td>
</tr>
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<td><em>C. coli</em></td>
<td>1787</td>
<td>TNKA</td>
<td>Turkey, feces</td>
<td>May, 2002</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>1702rnd</td>
<td>TSA</td>
<td>Turkey, ceca</td>
<td>Apr, 2002</td>
</tr>
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<td>931g</td>
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<td>Turkey, ceca</td>
<td>Dec, 2001</td>
</tr>
<tr>
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<td>A</td>
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</tr>
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<td>Aug, 2002</td>
</tr>
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<td>TSNK</td>
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<td>Mar, 2002</td>
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<td>Turkey, meckle</td>
<td>Sep, 2002</td>
</tr>
<tr>
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</tr>
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<td><em>C. jejuni</em></td>
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<td>A</td>
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<td>Jun, 2002</td>
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<tr>
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<td>Broiler, ceca</td>
<td>Jul, 2002</td>
</tr>
<tr>
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<td>1420</td>
<td>TESNKA</td>
<td>Turkey, ceca</td>
<td>Mar, 2002</td>
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<td>910C1</td>
<td>TESKA</td>
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<td>Dec, 2001</td>
</tr>
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<td>TESNKA</td>
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<td><em>C. coli</em></td>
<td>2774</td>
<td>TESNKA</td>
<td>Turkey, ceca</td>
<td>Jul, 2002</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>3178</td>
<td>TESA</td>
<td>Pig, feces</td>
<td>Aug, 2002</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>2573</td>
<td>TESNKA</td>
<td>Turkey, ceca</td>
<td>Jul, 2002</td>
</tr>
</tbody>
</table>

<sup>a</sup>Antibiotics which the strain is resistant to are listed: T-tetracycline, E-erythromycin, S-streptomycin, N-nalidixic acid, K-kanamycin, A-ampicillin.
TABLE 4.2. Comparison of transformation to erythromycin resistance between *C. coli* and *C. jejuni*

<table>
<thead>
<tr>
<th>Species</th>
<th>Recipient</th>
<th>Donor</th>
<th>TI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Criteria in selecting recipients&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. coli</em></td>
<td>961</td>
<td>1420</td>
<td>1.0</td>
<td>Same AR profile</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>3240</td>
<td></td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>3237</td>
<td>910C1</td>
<td>1.0</td>
<td>Same AR profile</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>SC49</td>
<td></td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>1787</td>
<td>2562</td>
<td>1.0</td>
<td>Same farm (farm JN)</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>1531</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>1702rnd</td>
<td>1420</td>
<td>1.0</td>
<td>Same farm (farm HW)</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>3464MD</td>
<td></td>
<td>0.0</td>
<td>Same AR profile</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>931g</td>
<td>2774</td>
<td>1.0</td>
<td>Same cecum (turkey)</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>931c</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>37-2nec</td>
<td>3178</td>
<td>0.8</td>
<td>Same AR profile</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>2146</td>
<td></td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>WP14</td>
<td>2573</td>
<td>0.5</td>
<td>Same AR profile</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>2954</td>
<td></td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Transformation index

<sup>b</sup> AR, antibiotic resistance
FIG. 4.2. KpnI-digested PFGE patterns of \textit{C. coli} field strains and erythromycin-resistant transformant derived from strain 961. Lanes: 1, molecular weight marker; 2, strain 1705; 3, strain 961; 4, transformant 961-1705em.
FIG. 4.3. KpnI-digested PFGE patterns of *C. jejuni* strain SC49 and erythromycin-resistant transformant derived from strain SC49. Lanes: 1, molecular weight marker; 2, strain SC49; 3, transformant SC49-910C1em; 4, NCTC11168 (cut with KpnI).
FIG. 4.4. Growth curve of *C. coli* strain 961 and erythromycin-resistant transformant, 961-1705em in separate cultures.
FIG. 4.5. Growth curve of *C. jejuni* strain SC49 and erythromycin-resistant transformant, SC49-910C1em in separate cultures.
TABLE 4.3. Generation times of *C. jejuni* strain SC49 and erythromycin-resistant transformant, SC49-910C1em in separate cultures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean generation time (h)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC49</td>
<td>1.10 ± 0.17</td>
</tr>
<tr>
<td>SC49-910C1em</td>
<td>1.52 ± 0.29</td>
</tr>
</tbody>
</table>

$^a$ Data shown are averages from three independent experiments ± 1 standard deviation.
FIG. 4.6. Competitive growth study of *C. coli* strain 961 and erythromycin-resistant transformant, 961-1705em in a mixed culture. Data are from at least two independent experiments. CFUs of the transformant were below detection limit (c.a. 10^3 CFU/ml) after 250h.
FIG. 4.7. Competitive growth study of *C. jejuni* strain SC49 and erythromycin-resistant transformant, SC49-910C1em in a mixed culture. Data are from at least two independent experiments.