Identification and characterization of *Campylobacter jejuni* factors relevant for the infection process

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To my parents

and

all those individuals working for the peace and prosperity of mankind.

I hereby declare that this thesis entitled "Identification and characterization of *Campylobacter jejuni* factors relevant for the infection process" is my own work and that, to the best of my knowledge, it contains no material previously published or written by another individual or any material which to a substantial extent has been accepted for the award of any other degree or diploma of this institution or any other institute of higher education, except where due acknowledgment has been made in the text.

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ABBREVIATIONS

Ab.	Absorbance
ADP	Adenosine dinucleotide
AIDS	Acquired immunodifficieny syndrome
Amp	Ampicillin
AP	Alkaline phosphatase
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BSA	Bovine serum albumin
DNA	Desoxyribonucleic acid
dNTP	Dinucleotide phosphate
EDTA	Ethylenediaminetetraacetic acid
g	force of gravity
gDNA	Genomic DNA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria broth
mRNA	Messenger ribonucleic acid
ORF	Open reading frame
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
RE	Restriction enzyme
RNA	Ribonucleic acid
Spp.	Species
TAE	Tris Acetate EDTA
TEMED	N,N,N,N –Tetramethyl-Ethylenediamine
Tet	Tetracyclin
tRNA	Transport RNA
U	Unit
UV	Ultraviolet

Summary

Campylobacter jejuni is the most frequent bacterial cause of foodborne-illness in the developed world including Germany, where the reported number is 60,000 cases in a year. Despite the recent completion of its genome sequence, little is known about the pathogenesis of the disease caused by this bacterium. Different factors are reported to be contributing in this lack of understanding of the pathogenesis of campylobacteriosis, likewise, the unavailability of an efficient system of experimental genetics, the lack of appropriate animal models for the disease, and the genetic diversity of *Campylobacter* strains. Epidemiological and phenotypical studies suggest that strains of *C. jejuni* vary in their colonization and invasion abilities and most likely in their virulence potential.

By considering all these factors we collected lab strains, NCTC11168, NCTC11828, 81-176 and eighty-three clinical isolates and more precisely determined them by combining biochemical and molecular markers; 74 isolates (89.2%) were identified as *C. jejuni*, including 7 atypical *C. jejuni* isolates that failed to hydrolyse hippurate, and 9 isolates (10.8%) as *C. coli*. The prevalence of tetracycline resistance (Tcr), tetracycline minimal inhibitory concentration (MIC), and *tet*(O) gene localization were also investigated in 83 *Campylobacter* isolates. Tcr was detected in 6 out of 9 *Campylobacter coli* isolates (67%) and 13 out of 74 *C. jejuni* isolates (18%). Tcr was low levelled for *C. coli* (MIC: 16 µg/ml for all strains) and high levelled for *C. jejuni* (MIC: >256 for all strains). Both, low levelled and high levelled Tcr was associated with the presence of the *tet*(O) gene. In *C. jejuni, tet*(O) was plasmid-encoded in 54%, whereas in *C. coli, tet*(O) seems to be located on the chromosome.

Transposon mutagenesis of *C. jejuni* chromosomal DNA was performed by using the *in-vivo* transposition method, which produced a random transposon mutant library consisting of 660 individual mutants. The BALB/c mouse model was optimized for an *in-vivo* genetic screen of the random mutants. The first genetic screen of *C. jejuni* mutant library identified 3 mutants defective for their flagellar motility, an important virulence determinant of *C. jejuni.* Chromosomal DNA sequencing of these mutants revealed a single insertion in each of the two genes *cj0793* and *cj0955c*, respectively. Furthermore, the analysis of sequenced DNA proved one of these genes, *cj0955c,* coding for unknown functions and the second gene, *cj0793,* was identified as the two component sensor, which is known as a central factor for flagellar motility. In addition, electron microscope analysis revealed flagellated and non-flagellated

non-motile mutants of *C. jejuni*. The second genetic screen of *C. jejuni* revealed an osmo-sensitive mutant with an insertion in the gene *cj0009* encoding the NADPH-dependent glutamate synthase small subunit.

Overall, this study proved that the PCR based *in-vivo* transposon mutagenesis is an effective method to generate large number of mutants of *C. jejuni* and can be successfully applied to investigate virulence-associated apparatus of this important bacterial pathogen.

Introduction

1.1 *Campylobacteraceae*

1.1.1 History and taxonomy of the family *Campylobacteraceae*

Campylobacter was first described in 1880 by Theodor Escherich (Friedman et al., 2000). In 1886, Escherich published a series of articles in the Münchener Medizinische Wochenschrift in which he described spiral bacteria in the colons of children who had died of what he called 'cholera infantum'. Unfortunately, these articles, published in German, remained unrecognized for many decades until his findings were presented by Kist at the Third International Workshop held in Ottawa in 1985. In 1909, two veterinarians, McFadyean and Stockman described the association of a microorganism with epizootic abortion in ewes (cited by Franco, 1988). Few years later, it was shown that the same vibrio can be found in infectious abortions in sheep and pregnant cows (Fox, 1982; Franco, 1988), and the same microaerophilic spirillum bacterium was isolated by Smith in 1919 from aborted calf tissues (Smith and Taylor, 1919). Due to its comma–shaped morphology, Smith and Taylor proposed the name "Vibrio fetus" and the disease was called vibrionic abortion (Fox, 1982; Butzler, 1984; Franco, 1988; Skirrow, 1977). In 1931, a new "vibrio" was reported that caused dysentery in calves during the winter and its name was proposed as *Vibrio jejuni* (cited by Butzler, 1984; Franco, 1988).

The three identified vibrio microorganisms—*V. jejuni*, *V. coli*, and *V. fetus*—were named in association with specific diseases in animals (Butzler, 1984). However, *Campylobacter* were not generally recognized as fecal pathogens in humans until the late 1970s, occasional isolation of "*V. fetus*" had been obtained from blood or other normally sterile body fluids. In 1947, in France the organism was isolated from a woman who had suffered from septic abortion (Vinzent et al., 1947), but an event that took place in Illinois in May 1938 is now regarded as the first well documented incident of the human *Campylobacter* infections. It was a model investigation of a milk-borne outbreak of diarrhea that affected 355 inmates of two adjacent state institutions (Levy, 1946).

Recent reports showed that *Campylobacter jejuni* is the most common bacterial cause of food-borne disease in most of the industrialized countries with an estimated 2.5 million cases per year in the United States alone (Friedman et al, 2000).

For many years, the taxonomic study of *Campylobacter* was fraught with difficulty because it mainly relied on phenotypic character, which is difficult to standardize. In 1957, King was grouping the Vibrio bacteria and the clinical entities of bovine vibrionic abortions started to be better understood. In the same year, the first taxonomic differentiation of Vibrio started when he demonstrated that catalase–positive microaerophilic Vibrios could be differentiated by their ability to grow at different temperatures (Butzler, 1984). At this time, a sporadic abortion was very common among pregnant cows and these abortions probably resulted from the establishment of *V. fetus* in the intestinal tract of the cows. A second type of sporadic abortion was reported with reduced conception rates, which indicated a case of infectious infertility. The strains of "vibrio" causing this infectious infertility were identified in 1959 by Florent in Belgium (Florent, 1959). He named these strains, *V. fetus* var. venerealis, after showing that this type of abortion was transmitted venereally from symptomless bulls to cows and that infertility was the major consequence of the infection (Butzler, 1984). In 1963, Sebald and Véron classified these microaerophilic bacteria as a new genus called *Campylobacter* (Hébert et al., 1982). Ten years later, the first comprehensive taxonomy of *Campylobacter* was published by Véron and Chatelain and they recognized the microorganisms *Vibrio jejuni* and *Vibrio coli* in their classification system of the genus, later these names were accepted by the International Committee of Systematic Bacteriology (Skerman et al., 1980; Skirrow and Benjamin, 1980).

For a long time, it was not possible to distinguish between *C. jejuni* and *C. coli*, both bacteria were grouped together as *C. fetus ssp. jejuni* and nobody really knew about the difference of *C. jejuni* and *C. coli*. However, now it is possible to distinguish *C. jejuni* from *C. coli*, and even also two biotypes of *C. jejuni* (Harvey, 1980). In the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974), the genera *Spirillum* and *Campylobacter* constituted the family *Spirillaceae*. This grouping of different taxa in one family was based on a number of morphological features. Since the phylogenetic relationship of these organisms was still unknown, the use of the family name *Spirillaceae* was abolished and the genus *Campylobacter* was considered to belong to the class of *Proteobacteria* (Vandamme and Ley, 1991). Goodwin et al. (1989) purposed the first step in the revision of *Campylobacter* taxonomy. They outlined genotypic and phenotypic arguments to exclude *C. pylori* from the genera *Campylobacter* and *Wolinella*

and proposed a novel genus called *Helicobacter* to accommodate both *C. pylori* and *C. mustelae* and later, Vandamme et al. (1991) completely revised the taxonomy and nomenclature of the genus *Campylobacter* and related bacteria. *Campylobacter*, together with *Wolinella* and "*Flexispira*" and two generically misnamed Bacteroides species, were found to represent a separate, sixth rRNA superfamily sensu De Ley (De Ley, 1978), within the group of the gram-negative bacteria. This lineage is now better known as the epsilon sub-division of the proteobacteria. On the bases of enormous genotypic and phenotypic differences, Vandamme and Ley proposed that the genera *Campylobacter* and *Arcobacter* should be included in a separate family, named *Campylobacteraceae* (Vandamme and Ley, 1991).

1.1.2 Morphology and biochemical properties of *Campylobacter*

The name *Campylobacter is* derived from the Greek word "Kampylos," which means curved. *Campylobacter spp.* are non–spore–forming and gram–negative bacteria. They can be spiral, curved or occasionally straight rods, with size ranging from 0.2 to 0.8 μm wide and 0.5 to 5 μm long. *Campylobacter* may appear as spiral, S—, V—, or comma–shaped forms and may also be found in short or occasionally long chains. As *Campylobacter* cells begin to age, they become coccoid in shape (Moran and Upton, 1987). The cells are highly motile by means of single or occasionally multiple flagella at one or both ends (Ursing et al., 1994). Extremely rapid, darting motility of comma-shaped cells can be seen with a phase contrast microscope. According to On et al. (1995), the number of flagella should not be considered as an important taxonomic criterion for the *Campylobacter* genus because of high variation in the flagellar arrangement of certain species or strains. Both mono- and biflagellated cells of the same general shape and size within the same culture of strain have been observed (On et al., 1995). It has been speculated that genetic changes as a result of spontaneous mutation and other mechanism such as natural or plasmid–borne transformation may cause considerable phylogenetic diversity which is observed within the genus. These microaerophilic organisms grow best in an atmosphere containing 5 to 10% oxygen and an optimum temperature for their growth ranges from 30 to 42°C. Colony morphology should not be used as an important distinguishing factor because several factors including bacterial strain, basal medium, level of moisture on the surface of the

agar, incubation temperature and incubation time may affect colony morphology of this organism. Colony morphology is quite variable, from a thick translucent white growth to spreading film-like transparent growth which can be visible on the plating media within 24 to 48 hours of incubation (Franco, 1988). It is difficult to isolate this organism from fecal specimens without using selective techniques because campylobacters tend to multiply slower than other enteric bacteria (Franco, 1988).

Campylobacter do not ferment carbohydrates and usually obtain energy from amino acids or tricarboxylic acid cycle intermediates. Typical biochemical reactions include the reduction of fumerate to succinate, negative methyl red, acetoin, and indole production. Most species reduce nitrate and are oxidase positive but only *C. jejuni* is hippurate positive. *C. jejuni* is quite sensitive to drying and storage at room temperature, but at refrigeration temperatures and appropriate humidity, large number of bacteria may survive. Enzymes expressed by *C. jejuni* such as superoxide dismutase (SOD), catalase, peroxidase, glutathione synthetsae, and glutathione reductase may have a vital role in providing protection against oxygen toxicity (cited by Crushell et al., 2004). *Campylobacter* can be distinguished from *Arcobacter* due to its key features which include; *Arcobacter* grows at 15°C but not at 42°C, its optimal temperature for aerobic growth is 30°C, and its G+C content of the DNA ranges from 27 to 30 mol% (Ursing et al., 1994), while in *Campylobacter* it ranges from 28 to 46 mol%.

1.1.3 Culturing and identification of *Campylobacter*

There is lack of consensus on the issue of the standard culturing medium for the growth of *Campylobacter* in the laboratory. Special requirements for growth temperature, gaseous environment and nutrient-rich basal medium are major obstacles to develop an optimum medium for this fastidious organism. Another difficulty is over-growth of coliform bacteria, Proteus spp., yeasts and molds within a *Campylobacter* culture (Goossens and Butzler, 1992; Stern et al., 1992; Jeffrey et al., 2000). When setting the formulation of selective medium, the rate of the recovery of the desired organism is a crucial parameter. An ideal *Campylobacter* medium should provide excellent recovery and substantial selectivity against background flora with an easy and quick differentiation of bacterial species. It should be cost effective, easy to prepare and it must have good shelf life with a

possibility to minimize the risk of contamination of the medium, especially, when adding supplements following heat-steam sterilization.

Most importantly, the medium should allow the organism to grow into isolated and distinct colonies without inducing swarming. By different researchers and laboratories a wide variety of media have been used and modified for isolation of *Campylobacter;* such as Skirrow formulation, Butzler's Agar, *Campylobacter* Blaser Agar, Preston, Semisolid Blood-free Selective Medium (SSM), *Campylobacter* Thioglycollate medium, Campy-Brucella Agar Plate (Campy-BAP), *Campylobacter* Cefoperazone Desoxycholate Agar (CCDA), Abeyta-Hunt-Bark Agar (Campy-FDA), Brucella Broth with 0.16% Agar, Semi-Solid *Campylobacter* Medium, Skirrow formulation, Butzler's Agar, Preston, Semisolid Blood-free Selective Medium (SSM), and Campy-Cefex (Wang et al., 1980; Bolton and Coates, 1983; Goossens and Butzler, 1992; Stern et al., 1992; Hunt and Abeyta, 1995; Hunt et al., 1998; Jeffrey et al., 2000). Above mentioned media differ in several aspects, for example, the amount of basal medium, concentration of antibiotics, presence or absence of growth enhancers such as horse or sheep blood and presence of special components like charcoal. All these nutrient-rich media support growth of a large number of fastidious organisms without much selectivity towards *Campylobacter spp*. Skirrow formulation was the first selective medium developed specifically for *Campylobacter spp*. which contained a nutrient base, 7% lysed horse blood and a combination of antimicrobials such as trimethoprim, polymyxin B and vancomycin. *Camyplobacter* was able to grow well on this media but predominated growth of fecal background flora and *Proteus spp*. became a problem (Goossens and Butzler, 1992). After the development of Skirrow's medium, Campy-BAP was developed which contains a Brucella agar base, instead of the blood agar base found in the Skirrow medium, and 10% sheep blood. To suppress the growth of some gram-negative organisms present in fecal flora, especially *Pseudomonas spp*., trimethoprim is added to the medium and other antimicrobial agents such as cefoperazone, and polymyxin B. Vancomycin and rifampin can be used to inhibit growth of gram-positive bacteria. Moreover, the use of cycloheximide and amphotericin B is recommended to suppress the growth of yeast. Another *Campylobacter* medium is Butzler's Agar, which contain Columbia agar base instead of the Brucella agar. It is necessary to add 5% defibrinated sheep blood, cefoperazone, rifampicin, amphotericin B and colistin to the

Butzler's agar.The United States Department of Agriculture (USDA) developed the Campy-Cefex medium. The major differences in the medium, as compared to others like CCDA and Campy-BAP is the lack of blood, the incorporation of sodium metabisulfite, sodium pyruvate and the addition of lysed horse blood with FBP a mix of ferrous sulfate ($FeSO_4.7H_2O$). This medium is more effective in recovering *C. jejuni* (Stern et al., 1992). It has been known that the incorporation of FBP to solid media can enhance the growth of *Campylobacter*.

In one study, five different selective media Skirrow's, Butzler's, Blaser's, Campy-BAP and Preston's were compared for the isolation of *Campylobacter* (Bolton et al., 1983). It was reported that Preston medium preceded by enrichment on modified Preston Enrichment Broth was found to be the most selective medium for *Campylobacter* while Butzler was the least effective. *Campylobacter* are very sensitive to superoxide anions (Goossens and Butzler, 1992; Corry et al., 1995; Blais and Phillippe, 1999), and it is believed that the chemical mixture (FBP) quenches superoxide anions generated in the medium which might improve the aerotolerance of *Campylobacter* (Smibert, 1978; Goossens and Butzler, 1992). According to Smibert (1978), pyruvate can destroy hydrogen peroxide whereas iron and bisulfite together act non-enzymatically to destroy superoxide radicals. High-energy radicals such as superoxide and peroxides are generated by photochemical reduction of medium components and inhibit the growth of *Campylobacter,* thus incorporation of FBP mix into media has special importance. It has been described that the addition of FBP to the medium can cause ten times reduction in the hydrogen peroxide toxicity (Smibert, 1978). Glutathione and pyruvate are believed to protect the cells against self-destruction by reducing the oxygen tension of the media. They also can stimulate the expression of genes associated with the stationary phase, which allows the cells to adapt to stress responses protecting them against denaturation (Dodd, 1997). Often glutathione is added to the *Campylobacter* media to assist in the recovery of stress-damaged bacteria. Moreover, both FBP and blood are also added to the medium to minimize toxic oxygen products and to promote growth. Enzymes such as catalase, peroxidase, and peroxide dismutase, are present in the blood and have the ability to decompose toxic oxygen derivatives (Goossens and Butzler, 1992).

Incorporation of charcoal to the medium can be used as an alternative of blood and it has been reported that charcoal could effectively replace the blood in the media formulations (Bolton and Coates, 1983). Instead of the 5% of blood in other *Campylobacter* media CCDA contains 4 % charcoal. Although this media provides best selectivity and good recovery of *Campylobacter* (Stern et al., 1992), there are certain disadvantages using this medium, for example, the need for constant shaking of the medium during plate pouring to avoid precipitation of the charcoal, and the difficulties in the identification of the translucent colonies of *Campylobacter* on the dark opaque color of the medium. In short, there is no consensus on a single standard method for isolating *Campylobacter* from different food or environmental samples. However, several protocols have been published from different sources including the International Standards Organisation (ISO), Public Health Laboratory Services (PHLS) from the United Kingdom and the Food and Drug Administration of the United States of America. Recently, the UK Microbiological Safety of Food Funders Group (MSFFG) has commissioned publicly-funded research on *Campylobacter*. They recommended methods for highly contaminated samples as well as for food surveillance. In addition, MSFFG endorsed a WHO recommendation that there is a need for collaborative efforts on international level to develop techniques for isolation of *Campylobacter* from food and environment (MSFFG, 2001)

1.1.4 Epidemiology of *Campylobacter*

In developed countries, *Campylobacter* is one of the most frequently reported causes of acute bacterial gastroenteritis. Significant variations in incidence rates have been observed between different countries (Brieseman, 1990; O'Brien et al., 1999). Several factors, including differences in infection rates in food animals, food production system, or different patterns of food consumption can be responsible for these variations. These differences in incidence rates can also occur because of differences in diagnosis, reporting systems, or case definitions used in each country's surveillance systems (Brieseman et al., 1990). In the United States, it is estimated that 2.5 million cases of campylobacteriosis occur annually. Although accounting for only 5% of estimated food-related deaths, *Campylobacters* are responsible for approximately 17% of hospitalizations resulting from foodborne infections (Mead et al., 1999). Annual economic cost of *Camyplobacter*

associated illnesses is estimated up to $8 billion in the United States alone (Buzby et al., 1997). In a recent study in the United Kingdom, it was found that for every isolate of *Campylobacter* reported to the National surveillance Scheme, 7.6 were unreported. By extrapolating, it was estimated that the total number of cases of *C. jejuni* in 1999 in the United Kingdom was 450,000. This figure agrees closely with other community-based studies in both, the United Kingdom and United States that estimate a population-based incidence of approximately 1% (Kendall and Tanner, 1982; Tauxe et al., 1992).

The *Campylobacter* enteritis causes significant morbidity and mortality in developed countries. A report from the U.S. Centers for Disease Control and Prevention estimated that each year *Campylobacter* infection causes 124 deaths in the United States (Mead et al., 1999). In an epidemiological survey conducted in the European Union, it was reported that fifteen out of 18 EU countries reported 134,971 *Campylobacter* infections in 1999 alone (Takkinen et al., 2003). This study showed great variation in the notifications/100,000 inhabitants of different EU countries which ranged from 2.9 to 166.8 during the year 1999. In 1998, the mean number of notifications per100, 000 inhabitants in the EU was 61 and in 1999 it was 71. These results showed that the increase in the notifications was about 16% between 1998 and 1999. During the period from 1995 to 1999, 11 countries reported 154 outbreaks and the highest number of the reported outbreaks was in 1997. The reporting of outbreaks varied greatly by country however, these numbers reflect a rough estimate of the true situation. Forty-eight percent of the outbreaks were linked with food, which served as a vehicle for transmission. Use of unpasteurised milk was responsible for 15% and another 15% were water-borne. In every fifth outbreak (21%), the cause was not clear and remained unknown (Takkinen et al., 2003). During the year 2005, the number of reported cases of *Campylobacter* in Germany was highest than ever before, surpassing the number of 60,000, which made *Campylobacter* a number one bacterial pathogen responsible for food poisoning in Germany. Total numbers of cases of campylobacteriosis during the year 2005 were higher than the total number of cases of salmonellosis reported in Germany (RKI, 2006). *Campylobacter* species occur in the animal food chain of humans from the most to the least economically developed cultures. Although, *Campylobacter* species are recognized as among the most common causes of diarrhea worldwide, the epidemiology of

Campylobacter infections in the developing world differs markedly from that in the developed world.

1.1.5 Antibiotic resistance and sensitivity

Campylobacter jejuni and *C. coli* are susceptible to nalidixic acid, ciprofloxacin, norfloxacin, and ofloxacin (Taylor and Courvalin, 1988). Furazolidone is another drug that has been shown to be effective against *Campylobacter* species (Shane, 1997). All *C. jejuni* and *C. coli* isolates are intrinsically resistant to a number of antibiotics, including bacitracin, novobiocin, rifampin, streptogramin B, trimethoprim, vancomycin, and usually cephalothin. According to Taylor and Courvalin (1988), the *Campylobacter* genus, has apparently been able to acquire resistance determinants from both gram–positive and gram–negative organisms, although the former seem to be the more common source. *Campylobacter spp*. and *Enterococcus spp*. occupy a common niche (the human and animal gastrointestinal tracts) and DNA exchange between these two species within this environment is very likely to occur. *Campylobacter spp*. might acquire resistance against tetracycline, minocycline, kanamycin, chloramphenicol, streptomycin, spectinomycin, erythromycin, ampicillin and nalidixic acid. Erythromycin resistance is accompanied by cross–resistance to spiramycin, tylosin, and clindamycin (Blaser *et al.*, 1982; Taylor and Courvalin, 1988; Shane, 1997).

1.2 Pathogenesis

1.2.1 *Campylobacter* in the postgenomic era

The virulence machinery of many enteric prokaryotic pathogens consists of adhesion, invasion, toxin production, and subversion of host cell processes. The role of these virulence processes is well described in other enteric pathogens but the exact mechanism of *Campylobacter* pathogenesis is yet to be elucidated. In the past decade, *in-vitro* assays have permitted the identification and characterization of a limited number of *C. jejuni* virulence determinants. Some of the corresponding genes have been mutated in order to assess the functional roles of these proteins in *C. jejuni* enteritis by using *in-vivo* infection models (Crushell et al, 2004). However, the complete analysis of *Campylobacter* pathogenesis is hampered by difficulties in genetic manipulations, lack of an effective animal model of

human enteric infection and variability in virulence of different strains. In the year 2000, the completion of the *C. jejuni* NCTC11168 genome sequence was reported (Parkhill et al., 2000). The completion of the 1.6 megabase sequence and its deposition in the public domain has significant importance in *Campylobacter* research, and allows the identification of putative virulence factors. For example, it became evident that the only toxin genes present in *Campylobacter* are cytolethal distending toxin (CDTs) genes and there are no pilus structures encoded on the chromosome (Parkhill et al., 2000). Moreover, unique information from the completed *C. jejuni* genome sequence has prompted research endeavours into new directions. Especially, the identification of a substantial number of homopolymeric tracts indicated the potential importance of slipped-strand mispairing phenomena and consequently, lead to the idea of phase variation which plays a vital role in the virulence and survival of this organism (Bourke, 2002).

1.2.2 Flagella.

In *C. jejuni,* motility is achieved by a single flagellum at one or both ends of the bacteria, and it has an important role in virulence because it is required for the bacteria to reach the attachment sites and penetrate into the intestinal cells. It is also reported that the flagella of *C. jejuni* appeared to have an essential role in the causation of diarrheal disease. The importance of motility as a virulence factor is best demonstrated by true isogenic non-flagellated mutants, which are unable to colonize the intestine of experimental animals (Guerry et al., 1992). The role of flagella in the colonization of the mucous lining of the gastrointestinal tract has already been studied (Yao et al., 1994). Flagella are also important for invasion of host cells, as aflagellate organisms show markedly reduced internalization into host cells *in-vitro* (Wassenaar and Blaser, 1999). The flagella of *C. jejuni* are composed of proteins, encoded by two genes *flaA* and *flaB* sharing a high degree of sequence homology (Wassenaar and Blaser, 1999). It has been shown that defined mutations in the *flaA* gene results in truncated flagella and diminished motility which are unable to invade intestinal epithelial cells *in-vitro* (Wassenaar et al., 1991; Yao et al., 1994). Furthermore, a mutation in another gene encoding pyruvate formate lyase activating enzyme 1 (*pflA*) resulted in bacteria with paralyzed flagella that lead to a mutant which is still able to adhere, but is not capable of invasion *in-vitro* (Yao et al., 1994). Therefore, it

was proven that FlaA is not the only determinant that is critical for invasion of this pathogen *in-vitro*. The genome sequence analysis of *C. jejuni* strain 11168 led to the prediction of the involvement of more than 50 genes in the assembly of the flagella. The regulation of the *Campylobacter* FlaA-regulon seems to be more complex because the respective genes are located in more than 32 individual loci, while in *E. coli* they are located in only six loci. Despite the fact that the phenotypes of many non-motile mutants are known, the exact mechanism of regulation of the flagellar genes in *C. jejuni* is still not understood. Recently, some flagellar transcription activators like RpoN, FliA and FlgR were identified, but how exactly these proteins are regulated and their role in the assembly of *C. jejuni* flagellum remains to be elucidated (Wösten et al., 2004).

1.2.3 Chemotaxis

Chemotaxisis defined as the movement of an organism towards or away from a chemical stimulus. Chemotaxis has been noted to be an important factor in the colonization of pathogenic bacteria including *V. cholerae*, *S. typhimurium*, and *E. coli.* Several studies demonstrated that chemotaxis is an important virulence determinant in *C. jejuni* and plays an important role in the colonization of mice. The *cheY* null mutant of *C. jejuni* was generated (Yao et al., 1997) and found to display a nonchemotactic but motile phenotype. A three-fold increase in the adherence and invasion of INT 407 cells was noticed as compared to the wild type with the *che*Y null mutant, which was unable to colonize mice or cause symptoms in infected ferrets. In the same study, it was shown that *cheY* diploid isolates (having two copies of *cheY*) showed a chemotactic behaviour and a decrease in their *in-vitro* adherence and invasion capabilities. Although, this isolate was able to colonize mice, it was unable to cause disease in the ferret model. It was suggested that these bacteria migrated towards the mucus within the crypts, but were unable to penetrate the mucus (Konkel et al., 2001).

1.2.4 Adhesion and invasion

There are several reports exploiting *in-vitro* adherence assays to characterize the interaction of *C. jejuni* with host cells. It has been reported that C. *jejuni* is capable to bind to several cell lines of human (INT 407, HEp-2, and HeLa) and non-human origin (Vero, CHO-K1,

and MDCK) with equal efficiency. Human intestinal epithelial (INT407) and human colon (Caco-2) cell lines were thought to be good models to mimic those cells encountered by *C. jejuni in-vivo* and therefore, these cell lines were extensively used to study adhesion of *C. jejuni*. The concept that adhesion of *C. jejuni* to host cells is mediated by constitutively synthesized products is supported by the finding that metabolically inactive *C. jejuni* organisms can bind to cultured cells at levels equal to or greater than those at which metabolically active, untreated *C. jejuni* cells bind. A variety of putative adhesion factors of *C. jejuni* have been already identified which include the fibronectinbinding protein CadF (Konkel et al., 1997), a homologue of the gram-negative ABC transport system PEB1 (Pei and Blaser, 1993) and a major outer membrane protein (Moser et al., 1997). Recently, it has been suggested that a novel surface-exposed lipoprotein specific to *C. jejuni* plays a role in host cell adherence (Jin et al., 2001). The lack of a suitable and accessible animal model of infection is a major hindrance to determine the precise role of these potential virulence factors to human disease. A significant variation has been observed in the degree of invasiveness between different strains of *C. jejuni* (Newell *et al.*, 1985; Konkel and Joens, 1989). Invasion of *C. jejuni* has been shown in colonic epithelial cells taken from infected humans and macaque monkeys (Woolridge and Ketley, 1997). Different experiments on a variety of cell lines including human intestine-derived Caco-2 and INT 407 cells showed invasiveness (Woolridge and Ketley, 1997; Kopecko et al., 2001). Some isolates of *C. jejuni* such as the well characterized strain 81-176 are proven to be highly invasive in these experimental models, however, many other isolates show low levels of host cell entry *in-vitro* (Kopecko et al., 2001). Recently, entry of *C. jejuni* in polarized epithelial cells via the basolateral membrane has been demonstrated as well as there is evidence for paracellular passage and M-cell transcytosis (cited by Woolridge and Ketley, 1997). Some studies demonstrated microtubule-dependent invasion of *C. jejuni* 81-176 and its reliance on microtubule motors for uptake and intracellular motility (Bourke, 2002; Hu and Kopecko, 1999). Likewise, most strains of *C. jejuni* showed microfilament or microtubule-dependent invasiveness (Biswas et al., 2000).

1.2.5 Toxin production

Campylobacter jejuni-induced clinical symptoms, which often include a transient watery diarrhea that progress to a bloody diarrhea, are consistent with the idea that toxins play a role in this disease. Indeed, a variety of toxic activities has been reported in *C. jejuni*. However, cytolethal distending toxin (CDT) is the only verified *Campylobacter* toxin identified to date. CDT production by *Campylobacter* was first reported in 1988 (Johnson and lior, 1988). During the last few years, significant progress has been made to understand the cellular effect of CDT (Karlyshev and Wren, 2001; Hickey, 1999). In 1996, the isolation and characterization of the *cdt* genes from *C. jejuni* 81-176 was reported and it is now well known that CdtB is the active moiety of the Cdt ABC complex (Pickett, et al 1996). It appears that CdtA and CdtC interact with CdtB to form a tripartite CDT holotoxin necessary for the delivery of the enzymatically active subunit, CdtB (Lara-Tejero and Galan, 2001). It has also been shown by Whitehouse et al. (1998) that affected epithelial cells undergo cytodistension and cell cycle arrest in the G2/M phase. T lymphocytes exposed to CDT-mediating sonicates from *C. upsaliensis* showed cell cycle arrest. The role of CDT in *C. jejuni* pathogenesis has not been determined yet, however, it might play a role in modulation of immune response and invasiveness (Purdy et al., 2000).

Another increasingly recognized prokaryotic virulence mechanism is to subvert host cell processes by targeting bacterial products directly to the cytoplasm of the host. Konkel et al. (1999), reported a *C. jejuni* protein called CiaB that seems to enter host cells during the invasion process. It has been shown that isogenic CiaB mutants were deficient in secretion of a number of bacterial proteins. A flagellar export system encoded by the genome of *C. jejuni* 11168 has been reported, but there is no evidence for the presence of a typical type III secretory apparatus. Recently, Bacon et al identified homologues of a type IV secretory apparatus on a large plasmid of *C. jejuni* 81-176. The 37kb the pVir plasmid of *C. jejuni* harbours 54 predicted open reading frames (Bacon et al., 2002). In some studies, it has been shown that mutations in some of the plasmid-encoded genes might reduce invasion compared with the parental strain *in-vitro*. However, transfer of the plasmid to the sequenced strain, NCTC11168, did not show any difference in the invasiveness of this isolate (Bacon et al., 2000).

1.2.6 Responses to stress

Compared to other food-borne bacterial pathogens, *C. jejuni* and *C. coli* have limited capacity for growth in the environment. Accordingly, the organisms are microaerophilic, have minimum growth temperatures between 32 and 36°C, and have complex nutritional requirements. *Campylobacter* is unusually sensitive to different environmental stresses because unlike other enteric pathogens it lacks many adaptive responses. Genome analysis of *Campylobacter* proved that it does not possess the global regulator RpoS (Parkhill et al., 2000). In a number of gram-negative organisms, this global regulator is the basis for the survival of the bacterial cell during exposure to many types of environmental stresses. In some recent studies, it has been shown that *Campylobacter* can exhibit adaptive responses to both acidic and aerobic conditions (Murphy et al., 2003). It has also been recognized that *Campylobacter* is more resistant to stress than had previously been thought (MSFFG, 2001). *Campylobacter* can be inactivated by heat or normal pasteurization treatments. However it can exhibit a heat-shock response immediately after exposure to temperatures above the optimal range for growth (Konkel et al., 1998) and 24 proteins are preferentially synthesized by *C. jejuni* as heat-shock response. Some of these proteins such as GroELS, DnaJ, and Lon protease, have been characterized previously (Konkel, et al., 1998; Thies et al., 1999; Thies et al., 1998; Wu et al., 1994). In one study, it has been proven that mutants deficient in one of the heat-shock proteins have drastically reduced their growth at 46°C and are unable to colonize chickens. This finding suggests a role of heat-shock-proteins in both thermotolerance and colonization. There are potentially three regulatory systems that control the induction of the heat-shock response in *C. jejuni*. The RacRS regulation, previously characterized as a two-component regulatory system, is required for the differential expression of proteins at 37°C and 42°C and is therefore likely to play a role in the regulation of the heat shock response (Bras et al., 1999). The identification of CIRCE-like consensus sequences upstream of the groESl and dnaK operons of *C. jejuni* provides further support that the expression of these and maybe other heat shock genes is regulated by the HrcA homologue (Thies et al., 1999). Since *Campylobacter* can be isolated from a wide variety of aqueous environmental sources and refrigerated foods (Rollins et al., 1986), they must be able to survive exposure to low temperatures for considerable periods.

From analysis of the *C. jejuni* genome sequence, it appears that *Campylobacter* does not seem to produce cold-shock proteins which may be a factor for no growth below 30°C. Contrary to other bacteria, *C. jejuni* shows a rapid decline in its growth near its minimal growth temperature (Hazeleger et al., 1998). However, at temperatures as low as 4°C, *Campylobacter* still can perform respiration, generate ATP, but is unable to replicate (Hazeleger et al., 1998). Although at lower temperature viability of the organism is lost rapidly, it can still be isolated from frozen meats and poultry products (Fernandez et al., 1996). Most of *Campylobacter* species are microaerophilic, including the important species directly linked to foodborne infections and are sensitive towards oxygen and its reduction products (Stead et al., 2000). Exposure to oxygen, while inevitable for most bacterial pathogens, leads to the formation of reactive oxygen intermediates as harmful byproducts that are capable of damaging nucleic acids, proteins and membranes. Many bacteria utilize their osmoregulatory mechanism to cope with osmotic stress but in *Campylobacter* such system is known to be absent (Parkhill et al., 2000).

1.2.7 Bile tolerance

In comparison to gram-positive bacteria, gram-negative bacteria express lipopolysaccharide (LPS) which confers resistance against hydrophobic compounds such as bile salts. Bile salts can act as detergents by solublizing the membrane proteins and lead to the destruction of the cell. Although LPS can provide advantage to bacterial cells during their passage through the small intestine of warm-blooded animals, they can be destroyed in the presence of high concentration of bile. According to Rhoades and Tanner (1995), a normal human liver secretes 600 to 1,200 ml of bile per day into the duodenum. Bile is composed of bile salts, bile pigments, cholesterol, phospholipids and proteins and it is synthesized in the liver from cholesterol and bile acids. There are several reports on bacterial tolerance to the bile salts including adaptation to lethal concentrations of bile salts leading to a significant cross–protection towards heat shock (Christopher et al., 1982; Flahaut et al., 1996). Flahaut et al. (1996) described that pretreatment with bile salts can result in the induction of a subset of heat-shock proteins. These proteins may have a role in the observed cross–protection effect. Heat and bile salt shock can lead to the formation of proteins that help to protect the bacterial cell against external stress factors. Pace et al. (1997) described that low

levels of bile might be important in regulating bacterial physiology and it may facilitate the host–pathogen interaction. It has been shown that bile can expedite growth of nutrient–deprived *Vibrio parahaemolyticus* and enhance its virulence, size of capsule and adherence to epithelial cells. Culturing of *Campylobacter* with bile may increase its capability to adhere and invade epithelial cells (Pace et al., 1997). Comparing the incidence of *C. jejuni* in the viscera, gallbladder and bile of broiler chickens, the liver was the organ of choice for *Campylobacter* infection (Carvalho et al. 1997). Although, the frequency of *C. jejuni* in the bile was low (6.9%), it may indicate that *Campylobacter* can survive and maybe multiply in high concentrations of bile. In line with this suggestion is the fact that 21% of the bile samples from chickens contained *Campylobacter spp.* (cited by Carvalho et al., 1997).

1.3 Animal models

The mechanisms by which *Campylobacter jejuni* induces disease in human beings remain unknown. Therefore, there is a need of an appropriate animal model to identify virulence factors of *Campylobacter jejuni.* Laboratory mice are not naturally colonized with *C. jejuni*. This may be due to competition with enteropathogenic *Helicobacter* species, which have been recognized as murine commensals and pathogens (Fox et al., 1997). However, mice and rats have been used in various experiments involving *C. jejuni*, because of their defined microbial flora, and prescribed genetic traits (Migaki and Capen, 1984). Also, genetically manipulated mice offer a unique opportunity to study the influence of host genotype on the expression of the disease (Kaufmann, 1994; Kaufmann and Ladel, 1994). With a few exceptions, oral infection of mice with *C. jejuni* results in intestinal colonization and in some cases bacteremia, but usually does not cause clinical diarrhea (Abimiku and Dolby. 1988; Abimiku and Borriello, 1989; Blaser et al., 1983; Field et al., 1981). Pretreatment with oral antibiotics can prolong colonization. The mouse model also has highlighted the essential role of the bacterial flagella for colonization of the intestinal mucosa (Morooka and amako, 1985; Newwell et al., 1985). More recently, mutation in the *C. jejuni* cell-binding factor, PEB1a, was shown to significantly reduce the rate and duration of colonization of mice by *C. jejuni* 81- 176. Since most of the strains of mice can be experimentally colonized by *C. jejuni*, they have been used in a number of studies (Abimiku and Dolby, 1988, Abimiko et al., 1989). Identification of bacterial factors

important for the colonization of chickens may lead to the development of targeted intervention strategies to reduce contamination of the food chain by this pathogen. However, so far, the value of models of avian colonization to investigate human infection is not established. Comparison of animal models of the two related pathogens *C. jejuni* and *Helicobacter pylori* has been quite informative. Although faced with similar problems, acceptable animal models of several disease manifestations of *H. pylori* infection are now available. This experience suggests that appropriate models of campylobacteriosis can in principle be developed in the future.

1.4 Genetics of *C. jejuni* and gene manipulation.

It has been recently reported that *C. jejuni* has a circular chromosome of 1,641,481 base pairs (30.6% G+C) which is predicted to encode 1,654 proteins and 54 stable RNA species. The genome of *C. jejuni* was found to be unusual because there are virtually no insertional sequences and very few repeated sequences with an additional surprising feature of harbouring hypervariable sequences. These short homopolymeric runs of nucleotides are present in genes which are responsible for encoding the biosynthesis or modification of surface structures or in closely linked genes of unknown function. Availability of sequence data of the *Campylobacter jejuni* NCTC11168 genome now offer enormous opportunities to understand the molecular basis of virulence of this important pathogen. Post-genome analysis proposes that 24 hypervariable sequences mostly found in genes encoding surface structures may play a key role in enabling *C. jejuni* to evade the host immune response. Additionally three sialylation pathways were identified which may have a significant role in pathogenesis (Taylor, 1992).

Conjugative plasmids encoding Cm^r, Km^r, and/or Tc^r are found more frequently in *C. coli* strains than in *C. jejuni* strains. These plasmids usually range in size from 45 to 50 kb with a G+C content of 31-33 mol%, or being approximately equivalent to those of the host species. Some of these plasmids were sequenced and analysed for their role in virulence but in most of the cases only restriction maps of Tc^r and Km^r plasmids have been constructed. It has been described that their host range was strictly restricted to closely related *Campylobacter* species and that plasmid transfer frequencies can range from about 1×10^{-5} to 1×10^{-3} transconjugants per recipient cell in a 24 h mating period. Wang and Taylor

(1990) observed that both *C. coli* and *C. jejuni* strains could take up DNA without any special treatment. Previously reported natural transformation frequencies were approximately 1×10^{-3} transformants per recipient cell for *C. coli* and 1×10^{-4} transformants per recipient cell for *C. jejuni* (Konkel et al., 2001). The incubation of DNA with DNaseI prevents transformation. However, no corelation between extracellular DNase production by *Campylobacter* recipient strains and its capacity to take-up foreign DNA has been found. Reported data suggest that cross-species transformation can occur between *C. jejuni* and *C. coli.* However, transformation of *Campylobacter* with plasmid DNA is much less efficient than with chromosomal DNA. There is evidence that small plasmids transform *C. coli* UA585 at a frequency 1,000-fold lower than that of chromosomal DNA markers (Wang and Taylor, 1990). *Campylobacter spp*. appears to tolerate exposure to high-voltage electric fields without any difficulty. Although *C. jejuni* NCTC1168 and other strains have been successfully electro-transformed, some other strains of *C. jejuni* and *C. coli* are not proven to be efficient strains in electroporation studies (Yan et al., 1990).

The first shuttle vector to be constructed for *E. coli* to *Campylobacter* transfer was pILL550, which conferred resistance to kanamycin in both *Campylobacter* and *E. coli* (Labigne-Roussel, 1987). The vector pILL550 contains an origin of replication derived from the *C. coli* plasmid piP445 that functions in both *Campylobacter* species and in *E. coli*. The presence of an oriT sequence from the IncPa plasmid RK2 facilitates the vector to be mobilized by a transfer-competent P-group plasmid into *Campylobacter* species. After pILL550 several more shuttle vectors were constructed and the similar strategy devised by Labigne-Roussel has been used (Vieira and Messing, 1982).

Various antibiotic resistance determinants, Cm^r, Km^r, and Tc^r which consist of the *cat, aphA-3*, and *tet*(O) genes, respectively, are used as plasmid selection markers in *Campylobacter*. Previously, *C. jejuni* were inactivated by shuttle transposon mutagenesis (Labigne-Roussel et al., 1988; Wang et al., 1991). The same strategy has been used to inactivate flagellar genes in both *C. jejuni* and *C. coli* (Guerry et al., 1991; Wassenaar et al., 1991). Some plasmids that replicate only in *Campylobacter* have also been constructed (Wang and Taylor, 1990).

Aims of the study

Despite its well known importance as a human pathogen and the recent completion of its genome sequence, the pathogenesis of campylobacteriosis is poorly understood. This relative paucity of the understanding of *Campylobacter* pathogenesis is partly due to the fact that systems of experimental genetics are still in the phase of development for *Campylobacter jejuni* and *C. coli.* Standard genetic techniques developed for the *Enterobacteriaceae* have faced considerable obstacles to be successfully adapted for campylobacters. *E. coli* plasmids, even from broad-host range compatibility groups, fail to replicate in *Campylobacter.* Another problem is the unavailability of established high throughput methods for the efficient generation of random mutants. Signature-tagged mutagenesis (STM) is a high throughput method and has been used to identify genes involved in pathogenicity of different bacteria (Hensel and Holden, 1996). However, the lack of an *in-vivo* transposon mutagenesis method for the efficient generation of random mutants of *Campylobacter* spp. has restricted molecular genetic studies. Previous efforts to generate mutants have mainly relied on shuttle mutagenesis and homologous recombination **(**Bleumink-Pluym et al., 1999; Labigne-Roussel et al., 1988; Yao et al., 1994). An *in-vivo* transposition system based on the *Himar1* transposable element has also been reported (Golden et al., 2000). However, the weakness of this system is unavoidable restriction of the suicide vector, which severely affects its efficiency and raises questions about its use as a high throughput method to generate random mutants of *C. jejuni.*

Considering the limitations of previously described methods for the identification of the putative virulence factors of *Campylobacter jejuni*, we sought to develop an efficient system for the *in-vivo* transposition of the *C. jejuni* genome, using an *aphA*-3 gene as an antibiotic marker. The Tn5 transposon has been used for the insertion mutagenesis of a variety of gram-negative bacteria and Tn*5* is one of the intensively studied transposons. Therefore, the EZ::TN transposase, a well characterized bacterial transposase for transposon delivery, was applied in this study as a tool to generate mutants of *C. jejuni.* The structure and mechanism of action for this enzyme have been well elaborated for both wild and mutant types. It is known that in contrast to the wild-type transposases, hyperactive Tn5 mutant transposase (Tn5p) exhibits high transposition efficiency *in-vitro*. The production of DNA: transposase complexes with the transposase protein that is able to bind

to both ends of transposon DNA has already been reported (Goryshin et al., 2003). Such complexes are known as transposomes and are formed by the protein binding to specific 19-bp recognition Mosaic End (ME) sequences of the transposon in the absence of Mg^{2+} ions. The activity of the transposome is dependent on Mg^{2+}, therefore, after the electroporation, the transposase becomes activated in the presence of cellular Mg^{2+} levels and ultimately integrates the transposon DNA into a random position in the bacterial chromosomes (Goryshin et al., 2003). We therefore, sought to develop a transposon mutagenesis system based on Tn5 transposons to generate randomly knocked-out mutants of *Campylobacter jejuni*. In addition, we evaluated the principle feasibility and effectiveness of this method to design a signature- tagged mutagenesis study for the identification of putative virulence genes of *C. jejuni.* Furthermore, for the *in-vivo* screening of mutants, we ought to optimize BALB/c mice as a screening model.

Taken together, the prime objective of this study was to develop an efficient method to generate random mutants of *C. jejuni* and to optimize an *in-vivo* screening model to initiate a signature tagged mutagenesis study for the identification of putative virulence genes of *C. jejuni.*

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Equipment	Model and manufacturer
Agarose gel electrophoresis chambers	Keutz Labortechnik, Reiskirchen
Balances	BP 221 S, Sartorius, Göttingen
	LP6200 S, Sartorius, Göttingen
Bio safety cabin	BDK und Luft Reinraumtechnik, GmbH
Centrifuges	Megafuge 2.0 RS, Heraeus, Hanau
	Modell 5417 R, Eppendorf, Hamburg
	Modell 5417 C, Eppendorf, Hamburg
Electro Cell Manipulator	BTX ECM600, San Diego, California
Zeiss energy filtering Cryo-elecron microscope	Zeiss EM 902 Göttingen
Gel documentation system	BioDoc II, Biometra, Göttingen
Homogenizer with glass pestle	Schütt Labortechnik GmbH, Göttingen
Hybridization oven	Modell OV5, Biometra, Göttingen
Light microscope	Olympus BH-2, Japan
Magnet bars	RCT basic, IKA Labortechnik, Staufen
pH-Meter	Modell 766 Calimatic, Knick, Berlin
Photometer	Ultraspec 1000, Pharmacia Biotech, Freiburg
Pipet-aid	Hirschmann Laborgeräte, Eberstadt
Pipettes	Modell Research, Eppendorf, Hamburg
Power supply	EPS 600, Pharmacia Biotech, Freiburg
	Standard Power Pack P25, Biometra, Göttingen
Semi-Dry blotter	Sartorius, Göttingen
Shaking incubator	SM-30 Control, Johanna Otto GmbH, Hechingen
Thermocycler	Modell T3, Biometra, Göttingen
Thermomixer	Modell Compact, Eppendorf, Hamburg
UV-Crosslinker	UVC-500, Hoefer, San Francisco, CA, USA
UV-Table	TFX-20M, Gibco BRL, Eggenstein
Water bath	Rottberg, Laborgeräte Glasapparatebau Göttingen

2.1.2 Bacterial cell culture media and additives

2.1.2.1 Bacterial cell culture media

Name	Source
Mueller Hinton Agar	Oxoid
Columbia Agar base	Merck
Brain Heart Infusion	Bacto™
Sheep blood	Oxoid

Luria Bertani Broth (LB)	**LB-Plates**
1% Bacto-Trypton	0.5% Bacto-yeast extract
0.5% Bacto-yeast extract	0.1% Bacto-Trypton
0.5% NaCl	1% NaCl
	1.5% Agar (Difco, Detroit, USA)

2.1.2.2 Antibiotics (Dics)

Antibiotic	Source
Ampicillin	Oxoid
Tetracycline	Oxoid
Kanamycin	Oxoid
Ciprofloxacin	Oxoid
Gentamicin	Oxoid
Chloramphenicol	Oxoid

2.1.2.3 Antibiotics (powder)

Antibiotic	Source
Chloramphenicol	Sigma
Kanamycin	Sigma
Tetracycline	Sigma

2.1.2.4 Chemicals and reagents

Unless otherwise specified, chemicals and solutions were purchased from Boehringer (Mannheim), Calbiochem (Bad Soden), Merck (Darmstadt), Roth (Karlsruhe), or Sigma (Deisenhofen). In routine, all solutions were prepared by using double distilled water.

Kits and reagents

DNA Cloning Kits

TOPO TA Cloning Kit	Invitrogen, Karlsruhe
QIAGEN PCR Cloning Kit	Qiagen, Hilden

Plasmid-DNA Isolation Kits

GenElute Plasmid Mini Prep Kit	Sigma-Aldrich, Deisenhofen
GeneElute Plasmid Maxi Prep Kit	Sigma-Aldrich, Deisenhofen

DNA Isolation from Agarose Gels

QIAEX II Gel Extraction Kit	Qiagen, Hilden

DNA Purification

QIAquick PCR Purification Kit	Qiagen, Hilden

Genomic DNA Isolation

Qiagen genomic DNA isolation Kit	Qiagen, Hilden

2.1.2.5 DNA Standards

100 bp-DNA Ladder	MBI Fermentas, St. Leon-Rot 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80 bp
1kb Ladder	MBI MBI Fermentas, St. Leon-Rot 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 bp

2.1.2.6 Membranes and filters

Nitrocellulose membrane	Hybond P (Amersham Biosciences)
Nylon membrane	Hybond P (Amersham Biosciences)

2.1.2.7 Enzymes

Quick T4 DNA Ligase	New England Biolabs, Schwalbach
Proteinase K	Roth, Karlsruhe
KOD DNA-polymerase	Novagen, Darmstadt
Taq DNA-Polymerase	Roche, Mannheim
Restriction endonucleases	New England Biolabs, Schwalbach
Shrimp Alkaline Posphatase	Boehringer Mannheim
RNase A	Sigma-Aldrich, Deisenhofen

2.1.2.8 Bacterial strains

Bacterial Strains	**Specification**	**Source**
Campylobacter jejuni	NCTC11168	Dr. A. Karlyshev
	NCTC11828	Prof. M. Kist, Freiburg
	81-176	Prof. M. Kist, Freiburg
	480	Prof. M. Kist, Freiburg
	B2	University Hospital, Göttingen
E. coli	DH5α	Invitrogen
	DH10B	Invitrogen
	DH10BpACYC184	Prof. Jorge E. Galan, USA

2.1.3 Vectors

Vector	Type	Selection	Source	Reference
pBluescript II KS(+)	Phagemid Vector	Amp®	Stratagene	Short et al, 1988
Kan®PBluescript II	Phagemid Vector	Amp®, Kan®	This study	This study
*pUOA18	Shuttle Vector	Cm®	Dr.R. Schmitt--Ott	Vieira & Messing, 1982
Kan®pUOA18	Shuttle Vector	Cm®, Kan®	This study	This study
pSB1699	Shuttle Vector	Kan®	Prof. J.E Galan	Galan et al, 2001
pMOD™	Tn5 construction Vector	Amp®,	Epicentre Biotech	Epicentre Biotechnologies
**pMOD™ Kan®	Tn5 construction Vector	Amp®, Kan®	This study	This study

* This plasmid contains an origin of replication derived from the *C. coli* plasmid piP445 and functions both in *Campylobacter* species and *E. coli.*

** Transposon Construction Vectors pMOD™-3<MCS> replicates in standard *E. coli* strains using a colE1 origin of replication for rescue cloning applications. Transposons made with the pMOD™-3<R6Kγori/MCS> vector also have an R6Kγori within the transposon. After cloning of *aphA-3*gene in this vector it was named as pMOD™ Kan®.

2.1.4 Oligonucleotide Primers

Table 2.1: Oligonucleotide Kan® primers were used as reverse primers to amplify a DNA fragment sized 1.8kb including the kanamycin resistance gene (*aphA-3*) tagged with 20 to 25 bp variable DNA sequences (underlined in the table) and at the 5´site of each tag, a restriction digestion site *GGGGTAC* for *Kpn*I restriction endonuclease was added to facilitate cloning into the pMOD™ vector. Primer Kan®FT3 (5´-AGGGGAGAGAGAGAGGAAGG-3´) was used as a forward primer and PBS_Kan® vector as a template for the polymerase chain reaction.

Kan® Primers	**Sequence 5´ to 3´**
Kan® T97	*GGGGTACC*AGCGTACGCGGCGTCATGGGAACCCAGCGAACCATTTGAG
Kan® T106	*GGGGTACC*GTCGCACGTTCTGCTGGTATGAACCCAGCGAACCATTTGAG
Kan® T108	*GGGGTACC*TCGCATCTTGTCATATAGGCAACCCAGCGAACCATTTGAGG
Kan® T111	*GGGGTACC*GATAGGCTTATATGCGTGCTAACCCAGCGAACCATTTGAGG
Kan®T112	*GGGGTACC*TACGGATCTCTGTGAAGTTAGTGCAACCCAGCGAACCATTTGAGG
Kan® T113	*GGGGTACC*TACGGCCAGATTCGAGAGGTCTATAACCCAGCGAACCATTTGAGG
Kan® T116	*GGGGTACC*TACGGTCGCCTATTCTCATGTCAGAACCCAGCGAACCATTTGAGG
Kan® T117	*GGGGTACC*TACGGATAACGATGGGTGCAATCTAACCCAGCGAACCATTTGAGG
Kan® T119	*GGGGTACC*TACGGCCTGTAATGGTGGATCTCAAACCCAGCGAACCATTTGAGG
Kan® T124	*GGGGTACC*GTACGCTGTCAACTGTACTGTCATCACCCAGCGAACCATTTGAGG
Kan® T126	*GGGGTACC*GTACGGTGCCGTATGTGTTAATGCAACCCAGCGAACCATTTGAGG
Kan® T127	*GGGGTACC*GTACGCGGATCTATGGCATCTACTGACCCAGCGAACCATTTGAGG
Kan® T129	*GGGGTACC*GTACGTCGTGCAATTTAGAGGTGTCACCCAGCGAACCATTTGAGG
Kan® T133	*GGGGTACC*TACGCCAATAGGTGCTCACGTCATAACCCAGCGAACCATTTGAGG
Kan® T135	*GGGGTACC*TACGTAGCAGTCTTGGCATACATGAACCCAGCGAACCATTTGAGG
Kan® T136	*GGGGTACC*TACGCTGTGACTGACTGTAGCTCTAACCCAGCGAACCATTTGAGG
Kan® T139	*GGGGTACC*TACGGATATGCGTTACGTGAGTCTAACCCAGCGAACCATTTGAGG
Kan® T141	*GGGGTACC*TACGTCCCAGAATTGTCAGCGATTAACCCAGCGAACCATTTGAGG
Kan® T142	*GGGGTACC*TACGCCAATAGTGATGAGTCGCCTAACCCAGCGAACCATTTGAGG
Kan® T143	*GGGGTACC*TACGGATCTCGATTATGCTCAAGGAACCCAGCGAACCATTTGAGG
Kan® T144	*GGGGTACC*TACGCTGTCAGATTAGTGAGCATGAACCCAGCGAACCATTTGAGG
Kan® T145	*GGGGTACC*TACGCTGGCTCTGGATCTAGTCTCAACCCAGCGAACCATTTGAGG
Kan® T147	*GGGGTACC*TACGTTGGCAGATTTGGTATGCACAACCCAGCGAACCATTTGAGG
Kan® T150	*GGGGTACC*TACGATCCAGCATCTCGGGTACTGAACCCAGCGAACCATTTGAGG
Kan® T152	*GGGGTACC*TACGCATGAGAGTGCGGCTAACTAAACCCAGCGAACCATTTGAGG
Kan® T155	*GGGGTACC*TACGAGCAGAGATGGACAGACCTCAACCCAGCGAACCATTTGAGG

Kan® T156	*GGGGTACC*TACGTATAGCAATTCGGTATGCGGAACCCAGCGAACCATTTGAGG
Kan® T165	*GGGGTACC*TACGCACACGGCTGGAGAGCATATAACCCAGCGAACCATTTGAGG
Kan® T167	*GGGGTACC*TACGCGCATACGTGTGGACTGATAAACCCAGCGAACCATTTGAGG
Kan® T169	*GGGGTACC*TACGATCTGGCACTGACTCTCAATAACCCAGCGAACCATTTGAGG
Kan® T171	*GGGGTACC*TACGTGCCAGAGTTTCAGCTTCTCAACCCAGCGAACCATTTGAGG
Kan® T175	*GGGGTACC*TACGGTGCGTCCTTCTGCAATCTGAACCCAGCGAACCATTTGAGG
Kan® T181	*GGGGTACC*TACGAAGAGAGCTGAATCACGTCTAACCCAGCGAACCATTTGAGG
Kan® T182	*GGGGTACC*TACGATCATGTCAAGATCAGTGGGAACCCAGCGAACCATTTGAGG
Kan® T183	*GGGGTACC*TACGGAAACACTTATCAGATCGCGAACCCAGCGAACCATTTGAGG
Kan® T184	*GGGGTACC*TACGGCTGCATATTCGTAACCATGAACCCAGCGAACCATTTGAGG
Kan® T186	*GGGGTACC*TACGATCTCCACTGCATCAGGTGAAACCCAGCGAACCATTTGAGG
Kan® T189	*GGGGTACC*TACGTATATTAAGTGCTGTGGCGCAACCCAGCGAACCATTTGAGG
Kan® T190	*GGGGTACC*TACGCAGACGTATCTCTTGTTGCAAACCCAGCGAACCATTTGAGG
Kan®T192	*GGGGTACC*TACGCTGTCATCTCTGTCAATGGAAACCCAGCGAACCATTTGAGG

Table 2.2: Oligonucleotide tag-specific (TS) primers for PCR amplification and screening of random mutants of *C. jejuni* in the pool. Each primer sequence written in the table corresponds to a specific variable tag attached with the kanamycin resistance gene *aphA*-3.

Primer	**Sequence 5´ to 3´**
Kan®TS97	AGCGTACGCGGCGTCATGGGCAT
Kan®TS106	GTCGCACGTTCTGCTGGTATGCAT
Kan®TS108	TCGCATCTTGTCATATAGGC
Kan®TS111	GATAGGCTTATATGCGTGCT
Kan®TS112	TACGGATCTCTGTGAAGTTAGTGC
Kan®TS113	TACGGCCAGATTCGAGAGGTCTAT
Kan®TS116	TACGGTCGCCTATTCTCATGTCAG
Kan®TS117	TACGGATAACGATGGGTGCAATCT
Kan®TS119	TACGGCCTGTAATGGTGGATCTCA
Kan®TS124	GTACGCTGTCAACTGTACTGTCATC
Kan®TS126	GTACGGTGCCGTATGTGTTAATGCA
Kan®TS127	GTACGCGGATCTATGGCATCTACTG
Kan®TS129	GTACGTCGTGCAATTTAGAGGTGTC
Kan®TS133	TACGCCAATAGGTGCTCACGTCAT
Kan®TS135	TACGTAGCAGTCTTGGCATACATG
Kan®TS137	TACGCTGTGACTGACTGTAGCTCT

Kan®TS139	TACGGATATGCGTTACGTGAGTCT
Kan®TS141	TACGTCCCAGAATTGTCAGCGATT
Kan®TS142	TACGCCAATAGTGATGAGTCGCCT
Kan®TS143	TACGGATCTCGATTATGCTCAAGG
Kan®TS144	TACGCTGTCAGATTAGTGAGCATG
Kan®TS145	TACGCTGGCTCTGGATCTAGTCTC
Kan®TS147	TACGTTGGCAGATTTGGTATGCAC
Kan®TS150	TACGATCCAGCATCTCGGGTACTG
Kan®TS152	TACGCATGAGAGTGCGGCTAACTA
Kan®TS155	TACGAGCAGAGATGGACAGACCTC
Kan®TS156	TACGTATAGCAATTCGGTATGCGG
Kan®TS165	TACGCACACGGCTGGAGAGCATAT
Kan®TS167	TACGCGCATACGTGTGGACTGATA
Kan®TS169	TACGATCTGGCACTGACTCTCAAT
Kan®TS171	TACGTGCCAGAGTTTCAGCTTCTC
Kan®TS175	TACGGTGCGTCCTTCTGCAATCTG
Kan®TS181	TACGAAGAGAGCTGAATCACGTCT
Kan®TS182	TACGATCATGTCAAGATCAGTGGG
Kan®TS183	TACGGAAACACTTATCAGATCGCG
Kan®TS184	TACGGCTGCATATTCGTAACCATG
Kan®TS186	TACGATCTCCACTGCATCAGGTGA
Kan®TS189	TACGTATATTAAGTGCTGTGGCGC
Kan®TS190	TACGCAGACGTATCTCTTGTTGCA
Kan®TS192	TACGCTGTCATCTCTGTCAATGGA

Table 2.3: Oligonucleotide primers used for the amplification and sequencing of particular genes mentioned in the table.

Name	**Location**	**Sequence 5´ to 3´**	**Size**
SBF	Tn55 in pBS1699 plasmid	ACCGAGGTATGAAAACGAG	1.7kb
SBR	Tn55 in pBS1699 plasmid	TATATCAAGTAAAGTAACAAA	1.7kb
JA4	*aphA*-3 Kan® gene	TAATAAATGGCTAAAATGAGAATA	574bp
JA5	*aphA*-3 Kan® gene	TCGCCGTGGGAAAAGACAA	574bp
TetF	*tet*O gene	TTGCCAATGGTATATCGGGAA	530bp
TetR	*tet*O gene	TGCGGGGGTACTTACACGACT	530bp
HIPF	*hipO* gene of *C. jejuni*	GAA GAG GGT TTG GGTGGT	735bp

HIPR	*hipO* gene of *C. jejuni*	AGC TAGCTTCGCATAATAACTTG	735bp
CCF	*aspA* gene of *C. jejuni*	GGT ATG ATT TCT ACAAAGCGA	583bp
CCR	*aspA* gene of *C. jejuni*	ATA AAA GAC TAT CGT CGC GTG	583bp
SekanF	*aphA*-3 Kan® gene	TATCACCTCAAATGGTTCGCTGGG	200bp
SekanR	*aphA*-3 Kan® gene	GGGGATCAAGCCTGATTGGGAGA	200bp

Table 2.4 Oligonucleotide primers used to confirm a recovered pool of mutants during the *in-vivo* screen in BALB/c mice. Each primer sequence can bind specifically with a variable DNA tag present in the transposon.

Primer Name	**Sequence 5´ to 3´**
Kan®TS106	GTCGCACGTTCTGCTGGTATGCAT
Kan®TS108	TCGCATCTTGTCATATAGGC
Kan®TS111	GATAGGCTTATATGCGTGCT
Kan®TS112	TACGGATCTCTGTGAAGTTAGTGC
Kan®TS113	TACGGCCAGATTCGAGAGGTCTAT
Kan®TS116	TACGGTCGCCTATTCTCATGTCAG
Kan®TS117	TACGGATAACGATGGGTGCAATCT
Kan®TS141	TACGTCCCAGAATTGTCAGCGATT
Kan®TS142	TACGCCAATAGTGATGAGTCGCCT
Kan®TS144	TACGCTGTCAGATTAGTGAGCATG
Kan®TS145	TACGCTGGCTCTGGATCTAGTCTC
Kan®TS150	TACGATCCAGCATCTCGGGTACTG

2.2 Methods

2.2.1 Media and growth conditions

Campylobacter strains were cultured on Columbia agar base (Merck) supplemented with 5% sheep blood (BA), polymyxin B (2.5 IU/ml), trimethoprim (5µg/ml), and vancomycin 10µg/ml and incubated at 42°C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) for 48 hours. For broth culturing of *C. jejuni*, Mueller Hinton (MH) and Brain heart infusion (BHI) broth have been used throughout this study. Microaerophilic conditions were generated using the BBL CampyPak Plus (Becton Dickinson), gas pack in an enclosed chamber or by inflating plastic sealed bags with a gas mixture containing 85% N_2, 10% CO_2 and 5% O_2. For the selection of *C. jejuni* mutants, antibiotics were used in the following concentrations: kanamycin, 50µg/ml; chloramphenicol, 20µg/ml. All *C. jejuni* strains were stored at −80°C in MH broth containing 15% glycerol. *E. coli* DH5α was grown in Luria–Bertani (LB) agar or broth. For *E. coli*, antibiotics were used in the following concentrations: ampicillin, 100µg/ml; kanamycin, 50µg/ml; and chloramphenicol, 30µg/ml. All *E. coli* strains were stored at −80°C in LB containing 20% glycerol. Mueller Hinton agar supplemented with 5% sheep blood (BA) with different tetracycline concentrations was used for determination of MICs.

2.2.2 Biochemical and molecular characterization of strains

2.2.2.1 Biochemical characterization

All *Campylobacter* strains used in this study were biochemically differentiated at species level by gram stain, oxidase and catalase activities, hippurate hydrolysis, hydrogen sulfide production and susceptibility to nalidixic acid by using a commercially available species differentiation kit (API CAMPY, bioMérieux, Marcy-l'Etoile, France).

2.2.2.2 Genomic and plasmid DNA preparation

Small-scale genomic DNA preparation was done by the CTAB (hexadecyltrimethyl ammonium bromide) genomic DNA isolation method. A lawn culture on Columbia blood agar plates was flooded with 3 ml of physiological 0.9% NaCl and 1.5ml of cells was centrifuged for 2.5 min at 14,000 g (Eppendorf 5417R) at room temperature. The pellet was resuspended by vortexing in 567µl of TE buffer (pH 7.4), with 30µl of 10% SDS and 6µl

of 10mg/ml proteinase K (QIAGEN), mixed, and incubated for an hour at 37°C. Subsequently, 100µl of 5M NaCl and 80µl CTAB/NaCl solution were added and mixed thoroughly by vortexing, and incubated for an hour at 65°C. DNA was extracted with chloroform, isoamylalcohol (24:1). DNA precipitation was done by an equal volume of ice cold isopropanol and pelleted by centrifugation for 10 min at 14,000 g at room temperature. DNA pellets were washed with 1ml 70% ethanol and resuspended in 50 µl of sterile water and stored at -20°C. Plasmid DNA from *Campylobacter* isolates was purified from an overnight culture on blood agar by using mini-Qiagen columns (Qiagen, Hilden, Germany) as proposed by the manufacturer.

2.2.2.3 Quantification of DNA

DNA concentrations were determined by measuring the absorbance at 260nm (A_{260}) in an Eppendorf spectrophotometer. DNA concentrations were quantified based on the formula that an absorbance of 1 unit at 260nm corresponds to 50µg DNA per ml. To confirm the concentration of the DNA, 5µl of each sample was migrated on agarose gel with 10µl of 1 kb DNA ladder, the concentration of the DNA was estimated by comparing intensities of the bands on agarose between the sample and the bands of the ladder.

2.2.2.4 PCR and Southern blot analysis of the *hipO* gene

Campylobacter lab strains and clinical isolates were analyzed for the presence of the *hipO* gene. The primer pair used for detection of the *hipO* gene consisted of HIP400F and HIP1134R (Table 2.3). The expected amplified product size was 735bp. The PCR reaction mixture consisted of 1x PCR buffer for Taq polymerase (Roche Diagnostics, Mannheim, Germany); 1ng of *C. jejuni* genomic DNA, 200 µM each of dATP, dCTP, dGTP and dTTP; 0.4 µM each primer and 1U of Taq polymerase. PCR parameters were initial denaturation at 94°C for 30s, annealing at 58°C for 1 min and extension at 72°C for 2min for 40 cycles. Final extension was done at 72°C for 10min. The presence of the *hipO* gene in isolates lacking biochemical hippurate hydrolase activity was confirmed by Southern blot analysis. By using the above mentioned primer pair, an amplified PCR product was labelled with digoxigenin 11-UTP (Roche Diagnostics, Mannheim, Germany) and was used as a probe to hybridize with *Bgl*II-digested genomic DNA of *Campylobacter* isolates. DNA was blotted onto a nitrocellulose membrane (Optitran BA-S 85, Schleicher and Schuell, Dassel,

Germany). Solutions and conditions were used according to the standard protocol (Sambrook and Russell, 2001). Hybridization was performed at 42°C for 18h. Washings for membranes were carried out at 65°C twice in 2x SSC, 0.5% SDS for 15 min at 37°C and in 0.1x SSC, 0.5% SDS for 30 min. Digoxigenin was detected with specific peroxidase-labelled antibodies using the ECL™ analysis system (Amersham Pharmacia Biotech, Freiburg, Germany) according to the recommendations of the supplier. Hippurate hydrolase negative isolates were confirmed as *Campylobacter coli* by a PCR method (data not shown). Genomic DNA was isolated by the CTAB (hexadecyltrimethyl ammonium bromide) method and a set of a previously reported primer pair was used to amplify a 583bp fragment of the aspartokinase gene (Linton et al., 1997).

2.2.3 Drug resistance of *Campylobacter* isolates

2.2.3.1 Antibiotic resistance and tetracycline MICs determination.

For the drug resistance study, *Campylobacter* strains were initially tested for resistance to ampicillin, ciprofloxacin, erythromycin, gentamicin and tetracycline by a disc-diffusion method. A slightly turbid bacterial suspension by emulsifying colonial growth in 5 ml sterile water was prepeared. A swab was charged with the water suspension and this was dispersed over the surface of a blood agar plate to produce a lawn of confluent bacteria. Sterile tweezers were used to place a disc on the surface of the agar plate after ensuring that the discs were widely spaced. The plates were incubated in a microaerophilic atmosphere at 37°C for 24 - 48 h. The bacterial growth zones of inhibition were examined around the antibiotic discs. A zone of inhibition indicated either sensitivity (S) or resistance (R) of the isolate to a particular antibiotic (Gaudreau and Gilbert, 1997). The minimal inhibitory concentration of tetracycline was subsequently determined by an agar dilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (NCCLS, 1997; CLSI, 2006). Mueller-Hinton blood agar supplemented with tetracycline concentrations of 4, 8, 16, 32, 64, 128 and 256µg/ml were inoculated with 1µl of (BHI) containing 1-2 × 10^7 bacteria and incubated at 37°C under microaerophilic conditions for 40-48h. Two *C. jejuni* tetracycline resistant and one *C. jejuni* strain known to be tetracycline susceptible (MIC 4µg/ml) were used as controls.

Each experiment was conducted in triplicate. The lowest concentration of the antimicrobial agent that produced no visible growth was considered as MIC for the relative isolate.

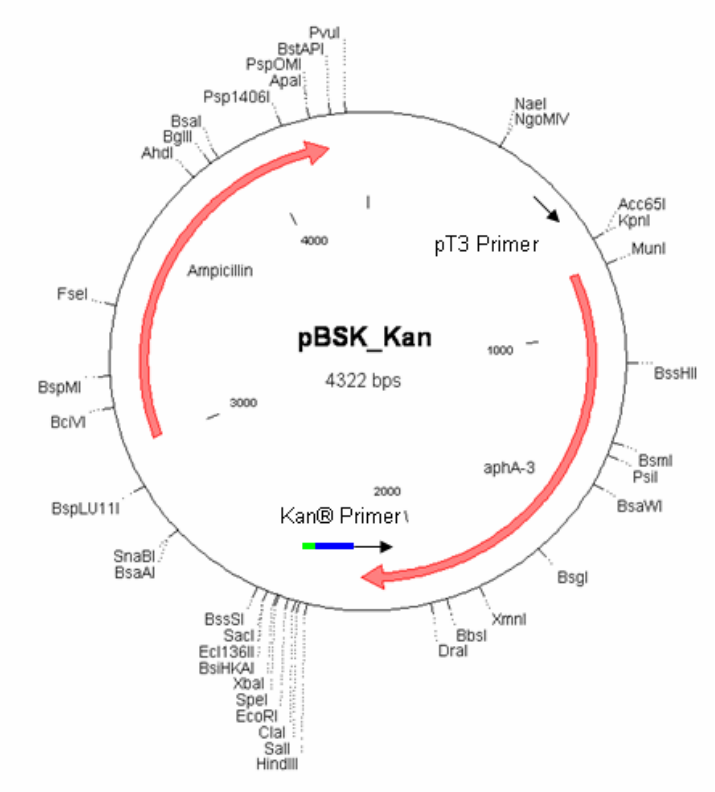
2.2.3.2 Detection and localisation of the *tet*(O) gene in *C. coli* isolates

The presence of the *tet*(O) gene in tetracycline-resistant *C. coli* isolates was confirmed by a *tet*(O)-specific PCR. The primer pair used for amplification of the *tet*(O) gene was TetF and TetR (Table 2.3). The PCR conditions were as follows: an initial melting temperature 94 °C for 1 min, annealing at 55 °C for 30s , extension at 72°C for 2 min, and a final extension at 72 °C for 10 min. Southern blot analysis was performed to determine the *tet*(O) localisation. DNA probes were generated and labelled with digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) by PCR using the same primer pair. Plasmid pCjA13 carrying the *tet*(O) gene was used as a template for this PCR reaction The generated probes were hybridised with the *Hind*III-digested *C. coli* plasmids. DNA was then blotted onto nitrocellulose membranes (Optitran BA-S 85, Schleicher and Schuell, Dassel, Germany) and detection of the *tet*(O) gene was performed with a specific peroxidase-labelled antibody using the ECL™ analysis system (Amersham Pharmacia Biotech, Freiburg, Germany). Hybridization was performed at 42°C for 18h. Washings of the membranes were carried out at 65°C twice in 2x SSC, 0.5% SDS for 15 min at 37°C and in 0.1x SSC, 0.5% SDS for 30 min.

2.4 Genetic modification of *Campylobacter jejuni*

2.4.1 Construction of EZ::TN transposon elements

The kanamycin resistance gene *aphA*-3 originated from *Campylobacter coli* is able to confer the respective resistance in *C. jejuni*. The EZ::TN transposon construction system (Epicenter) was used to construct a EZ::TNkan® transposon element. The plasmid PSB1699, bearing kanamycin resistance gene *aphA*-3, was kindly provided by Jorge E. Galan from Yale University. The *aphA*-3 gene was sub-cloned into *Bam*HI digested pBluescript SKII plasmid (Stratagene), which resulted in the pBSK_Kan plasmid (Fig. 2.1).



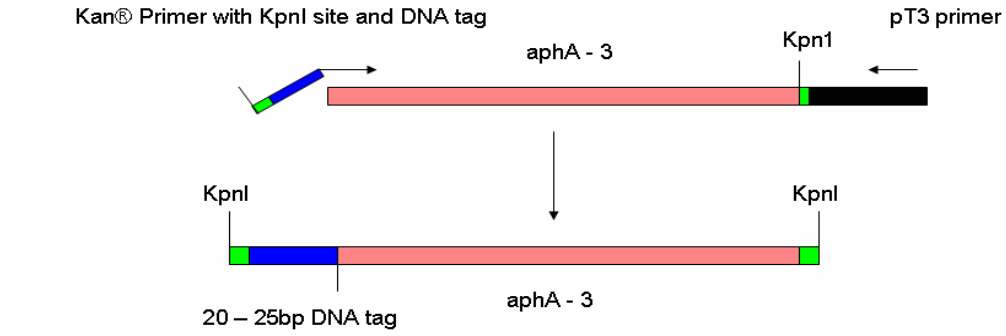


Figure 2.1: pBSK_Kan contains the T3 and T7 bacteriophage promoters. Kanamycin resistance cassette *aphA-3* was subcloned into *a Bam*HI restriction site of the vector and pT3 and Kan® primers were used to amplify a 1.8kb tagged *aphA*-3 gene.

The plasmid pBSK_kan served as a template for a PCR reaction designed to tag the *aphA*-3 gene by using Kan® primers (Table. 2.1). Each Kan® primer contained a 20 to 25 bp variable DNA tag named "signature tag", a primer binding site for the *aphA-3* gene and a

*Kpn*1 restriction. The tag sequences were chosen from those previously reported to work well in similar experiments (http://www.lshtm.ac.uk/itd/units/pmbbu/karlyshev/tags.htm accessed on October 21, 2003). By using the plasmid pBSK_Kan as a template, a PCR was carried out to amplify a 1.8kb DNA fragment by using the T7 primer of pBluescript SKII plasmid and a Kan® primer (Fig. 2.1). The PCR conditions were as follows: an initial melting temperature of 95°C for 1 min, annealing at 58 °C for 30s, extension at 72°C for 2 min and a final extension at 72°C for 10 min for 40 cycles.

The kanamycin resistance gene (*aphA*-3) was tagged with 40 variable DNA signatures and the DNA fragments of the *aphA*-3 gene were subcloned into *Kpn*I-digested transposon construction vector EZ::TN pMOD™. Prior to ligation, EZ::TN pMOD™ was dephosphorylated by using shrimp alkaline phosphatase and gel-purified with the QIAGEN Gel purification kit. DNA concentrations were estimated by measuring the absorbance at 260 nm (A_{260}) in an eppendorf spectrophotometer and also verified by loading 5µl from each sample DNA on agarose gel. The concentrations and sizes of DNAs were estimated by comparing intensities of bands of known DNA concentrations and sizes, DNA fragments were ligated into EZ::TN pMOD™ vector which resulted inEZ::TN pMOD™ Kan® (Fig. 2.2).

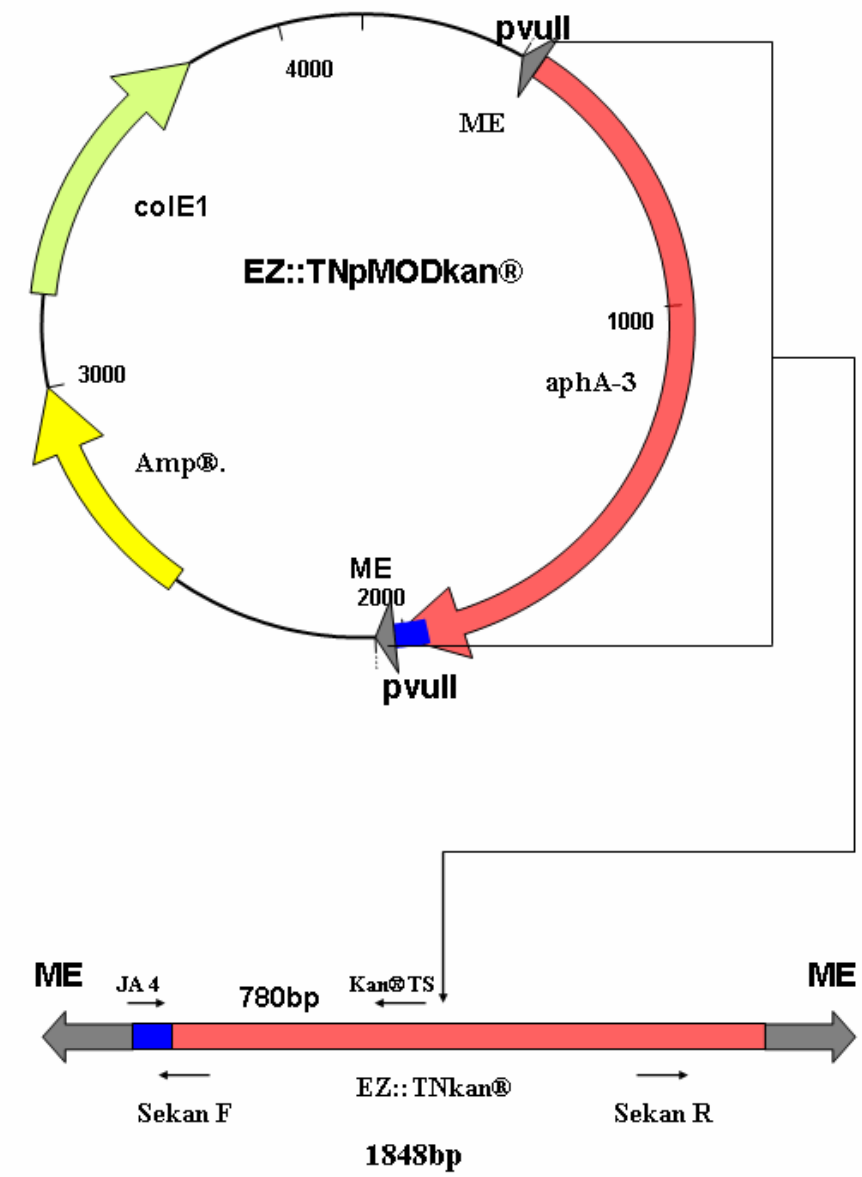


Figure 2.2: EZ::TN™ Transposon Construction Vector pMOD™-2<MCS> replicates in standard *E. coli* strains using a colE1 origin of replication. EZ::TNkan® transposons were constructed by subcloning the tagged *aphA*-3 gene (shown in red) in pMOD™-2. Also shown are the primers used for tag-specific PCR (JA4 and kan®TS see table 2.2) and those used for sequencing of the regions flanking transposon insertion sites (SekanF and SekanR). ME represent mosaic ends of the transposon.

2.4.2 Competent cells of *E. coli* and *C. jejuni* for electro-transformation

A single fresh colony of *E. coli* DH5α was inoculated into 20ml LB medium and placed at 37°C with a shaking speed 250 rpm. Then, 10ml of the culture was diluted in one liter of pre-warmed LB medium and was left on the shaker at 37°C until O.D. 600 = 0.5-0.8 was reached. The culture was transferred into 50 ml Falcon centrifuge tubes and was chilled for 15 minutes. After chilling, the tubes were centrifuged for 15 minutes at 4000 × g at 4°C in a pre-cooled centrifuge (Megafuge 2.0 RS, Heraeus, Hanau). Cell pellets were gently resuspended in ice-cooled water and centrifuged for 15 minutes. The same step was repeated twice. Cell pellets were again gently resuspended in 20 ml 10% glycerol and centrifuged as in previous steps. Finally, all pellets were carefully resuspended to a final volume of 2-3 ml in 10% glycerol. Cells were stored as aliquots (50µl/tube) at -80°C. For the production of competent cells of *C. jejuni,* a lawn culture on Columbia blood agar plates was flooded with 1ml MH broth per plate. Subsequently, this was centrifuged for 5min at >10,000 × g at 4°C in a tabletop centrifuge (Eppendorf 5417R). Pellets were gently resuspended in 1ml of ice cold wash buffer (272mM Sucrose, 15% Glycerol) and washed thrice by centrifuging at >10,000 × g at 4°C. After the last washing, the pellet was resuspended in 500µl of ice cold washing buffer and 50µl aliquots of cells were stored at -80°C. All the steps of this procedure were performed on ice.

2.4.3 Electroporation

Freshly made competent cells were used for all electroporations. Forty microliter of competent cells were pipetted into ice cold sterile 0.5ml microfuge tubes. An appropriate number of bacterial electroporation cuvettes were kept with the cells on ice. 10pg to 20ng of the DNA needed to be electroporated was added in a volume of 1-2µl to each microfuge tube and the tube was incubated on ice for 30-60 seconds. All appropriate positive and negative controls were included. The electroporation apparatus was set to deliver an electrical pulse of 25uF capacitance, 2.5 KV and 200 Ohm resistance. The DNA cell mixture was pipetted into a cold electroporation cuvette and the cuvette was placed in the electroporation device. A pulse of electricity was delivered to the cells at the setting described above. A time constant of 4-5 milliseconds was registered each time. After the pulse, the electroporation cuvette was immediately removed and 1 ml of SOC medium was

added. The cells were transferred to a 17 x150 mm polypropylene tube and were incubated with gentle rotation for 1 hour at 37°C. Then, different volumes (up to 200µl) of the electroporated cells were plated onto LB agar containing kanamycin (50µg/ml). Finally, the plates were incubated at 37°C in inverted positions. After 16 hours, transformed colonies were observed.

2.4.4 Amplification of the transposons

For screening purpose, a single colony was picked from each plate by using a sterile pipette tip. All picked colonies were inoculated into 5ml LB broth with 50µg/ml kanamycin in a 15 ml tube. The cultures were kept on a shaker for overnight at 37°C and were used for plasmid preparation by using mini-Qiagen columns (Qiagen, Hilden, Germany). The isolated plasmids (EZ::TN pMODkan®) were checked for purity and DNA concentrations. Subsequently, restriction digestions were carried out to liberate the transposons by exploiting *Pvu*II restriction sites of the plasmids (EZ::TN pMODkan®). Digested plasmids were analyzed for correct sizes of the transposons by visualizing on agarose gel. Five µl of the digested DNA from each reaction were loaded on 0.9% agarose gel including a positive control and 1 Kb ladder. One µl of the DNA (EZ::TN pMODkan®) from each plasmid preparation was diluted and 5pg of the DNA was used as a template for PCR by using the primer pair FP1 and RP1 provided from epicentre with plasmid EZ::TN pMOD™. The PCR conditions used for this reaction were an initial melting temperature of 95°C for 1 min, annealing at 62°C for 30s, extension at 72°C for 2 min and a final extension at 72°C for 10 min. Finally, the transposons were amplified by KOD DNA polymerase under the PCR conditions mentioned above. The PCR products were digested with *Pvu*II restriction endonuclease to liberate EZ::TNkan® transposons from the over hanging DNA sequence of the EZ::TN pMOD™ vector. All PCR products were analyzed for their sizes on 0.9% agarose gels by loading 2µl of each sample DNA. The rest of the DNA was loaded on the 0.9% agarose gel in the absence of ethidium bromide and transposons were gel purified. DNA concentrations of transposon DNAs were quantified and stored at -20 °C.

2.4.5 Cross hybridization of tags

The presence of 40 “signature tags” in the transposons was verified by a PCR method. Tag-specific primers named as “TS primers” were used to amplify a 780bp DNA fragment. The PCR assay was performed by using a mixture of 39 templates that was prepeared by mixing 1µl (100ng) of each plasmid preparation (EZ::TNpMODKan®). Forty different mixtures of templates were prepared and in each mix a template corresponding to a particular tag to be verified for cross hybridization was excluded (Fig. 2.3). The final concentration of the DNA in each mix was adjusted as 1ng/µl. In principle, each template mix could be used for the amplification of one particular signature tag. One micro-litre of each mix was used as a DNA template and forty different PCRs were set to verify cross-hybridization of the “signature tags”. This PCR assay provided a chance to every tag-specific primer “TS” to bind with any DNA template but excluded the possibility for binding to its original corresponding DNA template. All the tag-specific primers were designed to have the same annealing temperatures. Therefore, PCR conditions given below were possible for all reactions: 35 cycles of an initial melting temperature of 95°C for 1 min, annealing at 58°C for 30s, extension at 72°C for 2 min and a final extension at 72°C for 10 min. No cross hybridization of `signature tags´ was detected in this experiment.

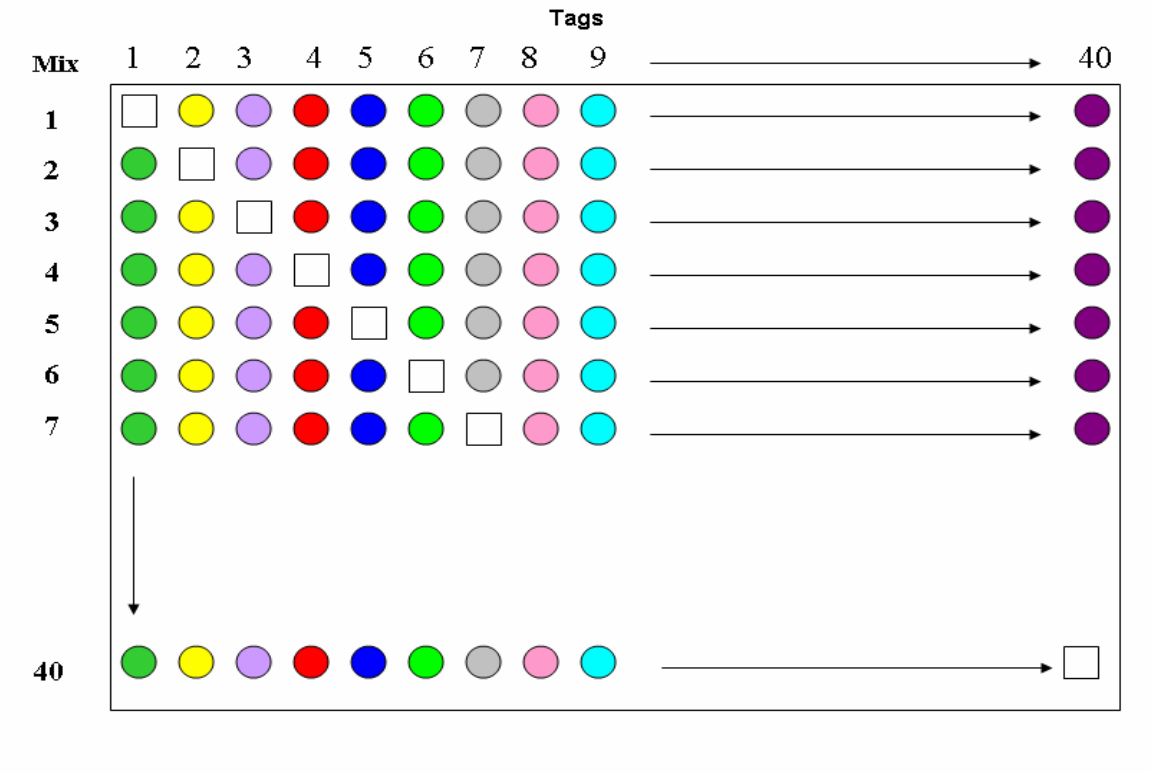


Figure 2.3: Each coloured circle denotes a unique DNA tag, whereas, an entire row represents one DNA template mix used for PCR analysis. Each DNA tag was checked for cross-hybridization by excluding one particular tag in each mix that is represented as white box in the diagram.

2.4.6 *In-vivo* transposition

The transposition reaction was carried out by mixing 100ng agarose gel-purified EZ::TNKan® transposon DNA, 2µl of EZ::TN transposase, 2µl of 100% glycerol and subsequent incubation for 30 min at room temperature. The reaction mixture was electroporated into *E. coli* DH5α and transposon insertional mutants were selected by plating the transformants on LB agar plates containing 50µg/ml of kanamycin. For generating random insertion mutants of *C. jejuni*, the transposition reaction was carried out as described above. The reaction mixture was electroporated into *C. jejuni* B2 strain as previously described and kanamycin-resistant colonies were selected on Columbia blood agar plates containing 50µg of kanamycin per ml. All selected colonies were stored in BHI broth with 15% glycerol in -80°C

2.4.7 Verification of randomness of transposon insertions

The random insertion of transposon Tn5Kan® throughout the *C. jejuni* genome was confirmed by Southern blot analysis of 18 randomly chosen kanamycin insertion mutants. Genomic DNA was prepeared by the CTAB (hexadecyltrimethyl ammonium bromide) genomic DNA isolation method and digested with *Bsp*h1 restriction endonuclease. Then, DNA was blotted onto a nitrocellulose membrane (Optitran BA-S 85, Schleicher and Schuell, Dassel, Germany). Solutions and conditions were used according to the standard protocol (Sambrook and Russell, 2001) and were as mentioned before (2.2.2.4).

2.5 Screening of *C. jejuni* mutants

2.5.1 Sensitivity to sodium chloride and sodium deoxycholate

Minimum inhibitory concentration of NaCl for the *C. jejuni* B2 strain was determined by using the agar dilution method. Bacteria of the B2 and NCTC11168 strains were cultured in Mueller Hinton Broth (0.5% NaCl) for 24 hours. OD was set as 1.0 at 550nm wavelengths. After serial dilutions, 100µl of each cell suspension was plated on Columbia blood agar plates that supplemented with 0, 0.5, 1.0, 1.5, 2.0, 4.0, or 6.0% (wt/vol) NaCl. The plates were incubated at 42°C in a microaerophilic atmosphere. Duplicate determinations were done for each treatment studied. The same procedure was adopted to determine the MICs and to test the sensitivity of *C. jejuni* mutants for sodium deoxycholate.

2.5.2 Motility assay

Transposon mutants were screened for motility by measuring swarming on motility agar plates. Transformants were grown in Mueller Hinton broth and then diluted to an optical density at 600 nm (OD_{600}) of 0.025. Approximately 1 µl of this suspension was stabbed into a 0.4% MH agar plate. Plates were incubated in micro-aerobic conditions at 37°C for approximately eighteen hours. Control strain NCTC11168 was grown on each plate to monitor plate-to-plate variation.

2.5.3 Transmission Electron Microscopy

Staining with a 3% (wt/vol) aqueous solution of phosphotungstic acid (pH 7.0) or a 4% (wt/vol) aqueous solution of uranyl acetate (pH 4.5) was performed according to the method of Valentine et al (Valentine et al., 1968). Electron micrographs were taken, at calibrated magnifications, with a Zeiss energy filtering Cryo-elecron microscope (Zeiss EM 902) (Zeiss, Germany), equipped with a TRS 1K camera and was operated in the conventional bright-field mode. Digital images were acquired by scanning photographs or petri dishes (UMAX Power Look). Image processing, which was limited to cropping, conversion to grayscale, and adjustment of brightness and/or contrast, was done with Photoshop (Adobe, Inc.) software. Image enhancement of flagella was performed by translational analysis (Hoppert and Holzenburg, 1999).

2.6. *In-vivo* screening of *C. jejuni* mutants

2.6.1 Mice infection assay

Campylobacter jejuni strains NCTC11168, 81-176, NCTC11828 and the clinical isolate B2 were cultured on Columbia blood agar plates. After 24 hours, the bacteria were harvested in separate culture tubes by using 5ml of sterile saline and diluted to an OD at 550 nm (OD_{500}) of 0.05. Subsequently, the suspensions were serially diluted ten-fold and plated onto Columbia blood agar plates. The number of cfu was counted after 24 hours of incubation at 42°C in a micro-areophilic atmosphere. Group of three 5 to 6 weeks old female BALB/c mice were infected intra-peritoneally with a bacterial suspension containing approximately, 1×10^8, 1×10^9 and 1×10^{10}cfu/ 0.2ml. Mice were sacrificed after 7 days post infection. The liver was put into in a 50ml sterile Falcon tube that contained 5ml of saline. All tubes containing tissues and saline were weighed and registered. The tissue was homogenized and the mixtures were shifted to a 50ml sterile Falcon tube and kept on ice for five minutes. From each homogenized mixtures, 1ml was carefully transferred to another sterile tube and centrifuged for 2 minutes at 10,000 rpm. The pellets were resuspended in 1ml of sterile distilled water. Dilution series were made in sterile distilled water and 100µl of each dilution was plated on Columbia blood agar plates that were kept under microaerophilic conditions. A few colonies were picked and sub-

cultured on a fresh blood agar plate to perform biochemical and molecular identification for the particular mutant strain. For *in-vivo* screening of the signature-tagged transposon mutagenized and pooled bacteria, the same procedure was followed. However, in this case, mutants recovered from the liver were plated on Columbia blood agar plates that contained 50µg/ml of kanamycin. For histopathological examination, fixed liver tissue samples from mice were processed, embedded in paraffin, and sectioned at 5–6 mm for hematoxylin and eosin (HE) staining. Replicate sections from spleen and liver were Giemsa stained to identify bacteria.

2.6. 2 Analysis of Tn5 Kan®-Campy insertions

To determine the precise sites of insertion of selected mutants, genomic DNA from the different *C. jejuni* insertion mutants were prepared by using a genomic DNA preparation kit. Genomic DNA from the mutants was then used as a template in a inverse PCR. Two primers, SekanF and SekanR, that are directed outwards within the *aphA*-3 cassette, were used to determine the point of insertion of the EZ::TNKan®.

3 Results

3.1 Species identification of thermophilic *Campylobacter* strains

Infections caused by *C. jejuni* are highly prevalent in humans and the mechanisms of pathogenesis of the disease are poorly understood. In addition, it is not clear whether certain types of *C. jejuni* strains show a specific preference for particular hosts. Therefore, before initiating a study to explore virulence factor of *C. jejuni*, strain variation was taken into consideration and proper characterization of strains used in the study was carried out. Hippurate hydrolase is an important biochemical test used to differentiate between *C. jejuni* and other *Campylobacter* species. We collected eighty-three thermophilic *Campylobacter* isolates from patients of the University Hospital of Göttingen who suffered from gastroenteritis. The isolates were identified by testing for the presence of the *hipO* gene by using phenotypic and molecular methods. On the basis of their hippurate hydrolase activity, 67 isolates (81%) were identified as *C. jejuni* (Table 3.1).

Table 3.1: Characterization of hippurate hydrolase positive *C. jejuni* isolates

[a]Isolate	plasmid	*tet*O PCR & blot		ERY	AMP	TET	CIP	GEN
CjA5	+	+	+	[b]**S**	[c]**R**	**R**	**R**	**S**
CjB2	+	+	+	**S**	**R**	**R**	**R**	**S**
CjA9	+	+	+	**S**	**R**	**R**	**S**	**S**
CjA13	+	+	+	**S**	**R**	**R**	**S**	**S**
CjA14	+	+	+	**S**	**S**	**R**	**R**	**S**
CjA6	+	+	+	**S**	**S**	**R**	**S**	**S**
CjA7	+	+	+	**S**	**S**	**R**	**S**	**S**
CjA8	+	-	-	**S**	**R**	**S**	**S**	**S**
CjB3	+	-	-	**S**	**R**	**S**	**S**	**S**
CjB5	+	-	-	**S**	**R**	**S**	**S**	**S**
CjB4	+	-	-	**S**	**S**	**S**	**R**	**S**
CjA4	+	-	-	**S**	**R**	**S**	**S**	**S**
CjA22	+	-	-	**S**	**S**	**S**	**R**	**S**
CjB1	+	-	-	**S**	**S**	**S**	**R**	**S**
CjA15	-	-	-	**S**	**S**	**R**	**R**	**S**
CjA31	-	-	-	**S**	**R**	**S**	**R**	**S**

CjA34	**-**	**-**	**-**	**S**	**R**	**S**	**R**	**S**
CjB10	**-**	**-**	**-**	**S**	**R**	**S**	**R**	**S**
CjC1	**-**	**-**	**-**	**S**	**R**	**S**	**R**	**S**
CjA33	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjA3	**-**	**-**	**-**	**S**	**R**	**S**	**R**	**S**
CjA11	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjA18	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjA25	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjA26	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjA28	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjA29	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjC8	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjA2	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjB6	**-**	**-**	**-**	**S**	**R**	**S**	**S**	**S**
CjB19	**-**	**-**	**-**	**S**	**S**	**R**	**S**	**S**
CjA27	**-**	**-**	**-**	**S**	**S**	**R**	**S**	**S**
CjB8	**-**	**-**	**-**	**S**	**S**	**R**	**S**	**S**
CjC2	**-**	**-**	**-**	**S**	**S**	**R**	**S**	**S**
CjB9	**-**	**-**	**-**	**S**	**S**	**S**	**R**	**S**
CjC4	**-**	**-**	**-**	**S**	**S**	**S**	**R**	**S**
CjC13	**-**	**-**	**-**	**S**	**S**	**S**	**R**	**S**
CjB20	**-**	**-**	**-**	**S**	**S**	**S**	**R**	**S**
CjA17	**-**	**-**	**-**	**S**	**S**	**S**	**R**	**S**
CjA16	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA19	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA20	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA21	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA23	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA24	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA30	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA32	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA35	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB11	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB12	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB13	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB14	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB15	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**

CjB16	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB7	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjA10	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC3	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC6	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC7	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC9	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC10	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC11	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC12	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC14	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjC15	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB18	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**
CjB21	**-**	**-**	**-**	**S**	**S**	**S**	**S**	**S**

Table 3.1: Presence of the tetracycline resistance gene *tet*(O) was determined by PCR and Southern blot. Antibiotic resistance frequencies of clinical isolates of *C. jejuni* were determined by agar dilution test. ERY, erythromycin; GEN, gentamicin; AMP ampicillin; CIP, ciprofloxacin; TET, tetracycline. [a]Cj = *C. jejuni,* [b]S,= sensitive [c]R,= resistant.

Our data showed that sixteen *Campylobacter* isolates (19%) did not show any hippurate hydrolase activity. However, in seven of these hippurate hydrolase-negative isolates, the *hipO* gene was detected by probing genomic DNA with a digoxigenin-11-dUTP-labelled *hipO*-probe (Fig 3.1a); these isolates were identified as atypical *C. jejuni.* In addition, *C. coli*-specific PCR was used to confirm these isolates. The remaining nine hippurate hydrolase negative isolates were confirmed as *C. coli*, by a specific PCR (Fig. 3.1b).

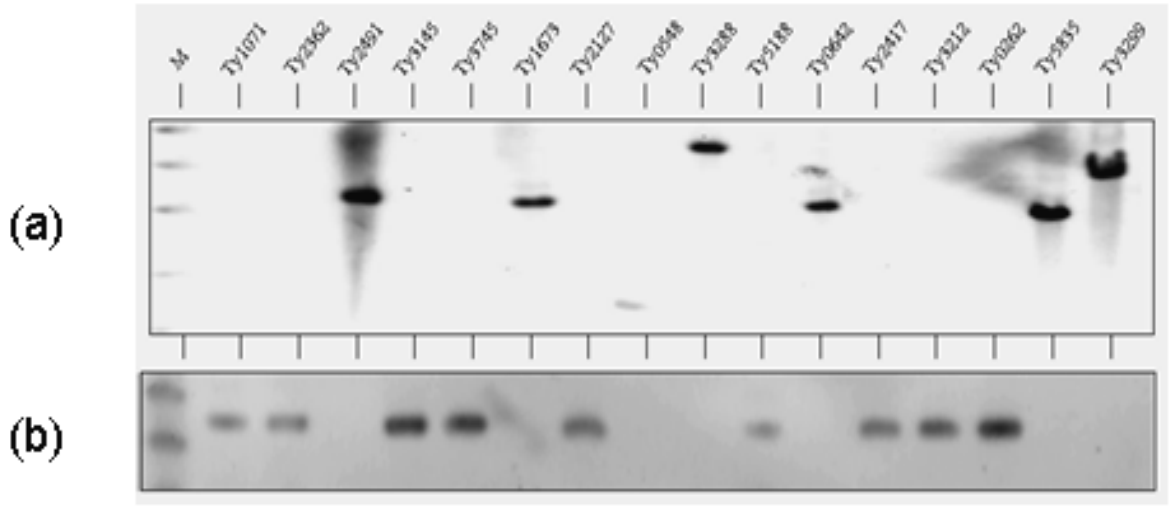


Figure 3.1: Genotypic species differentiation of hippurate hydrolase-negative thermophilic *Campylobacter* strains. (a) *Bgl*II-digested genomic DNA probed with digoxigenin-labelled *hipO*. (b) *C. coli*-specific PCR amplification of genomic DNA.

3.2 Antibiotic resistance and the role of plasmids in *C. jejuni* and *C. coli*

Tetracycline MICs were determined for those 19 *Campylobacter* clinical isolates that were initially identified to be tetracycline resistant by the disk diffusion test. Tetracycline MICs ranged from 16 to 256mg/L. In case of thirteen *C. jejuni* isolates, MIC was determined as >256mg/L and 6 *C. coli* isolates had MIC of 16mg/L (Table 3.2).

Table 3.2: Characterization of tetracycline-resistant *Campylobacter* isolates

Isolate	*hipO*	‡*aspA*	†Detectable plasmids	TET ® (MIC)	PCR	*tet*(O) Hybridization
***CjA5**	+	§Nd	+	R (>256µg/ml)	+	Plasmid
CjB2	+	Nd	+	R (>256µg/ml)	+	Plasmid
CjA9	+	Nd	+	R (>256µg/ml)	+	Plasmid
CjA13	+	Nd	+	R (>256µg/ml)	+	Plasmid
CjA14	+	Nd	+	R (>256µg/ml)	+	Plasmid
CjA6	+	Nd	+	R (>256µg/ml)	+	Plasmid
CjA7	+	Nd	+	R (>256µg/ml)	+	Plasmid
CjA15	+	Nd	-	R (>256µg/ml)	+	Genomic

CjB19	+	Nd	-	R (>256µg/ml)	+	Genomic
CjA27	+	Nd	-	R (>256µg/ml)	+	Genomic
CjB8	+	Nd	-	R (>256µg/ml)	+	Genomic
CjC2	+	Nd	-	R (>256µg/ml)	+	Genomic
Cj3288	+	-	-	R (>256µg/ml)	+	Genomic
#Cc1071	-	+	+	R (16µg/ml)	+	Genomic
Cc3745	-	+	+	R (16µg/ml)	+	Genomic
Cc2127	-	+	+	R (16µg/ml)	+	Genomic
Cc3145	-	+	-	R (16µg/ml)	+	Genomic
Cc3212	-	+	-	R (16µg/ml)	+	Genomic
Cc0262	-	+	-	R (16µg/ml)	+	Genomic

†The method applied in this study allowed the detection of plasmid sized 1-66kb. *Cj = *C. jejuni*, # Cc = *C. Coli*, *§Nd.= Data not available,* ‡ Aspartokinase gene of *C. coli*

The frequency of tetracycline resistance was significantly higher (χ^2 test: $p < 0.001$) in *C. coli* (67%) than in *C. jejuni* (18%). In both *Campylobacter* species, the tetracycline resistance was associated with the presence of the *tet*(O) gene. Plasmids were detected in 23% (n = 19) of the all *Campylobacter* isolates.(Fig. 3.2, Fig. 3.4a).

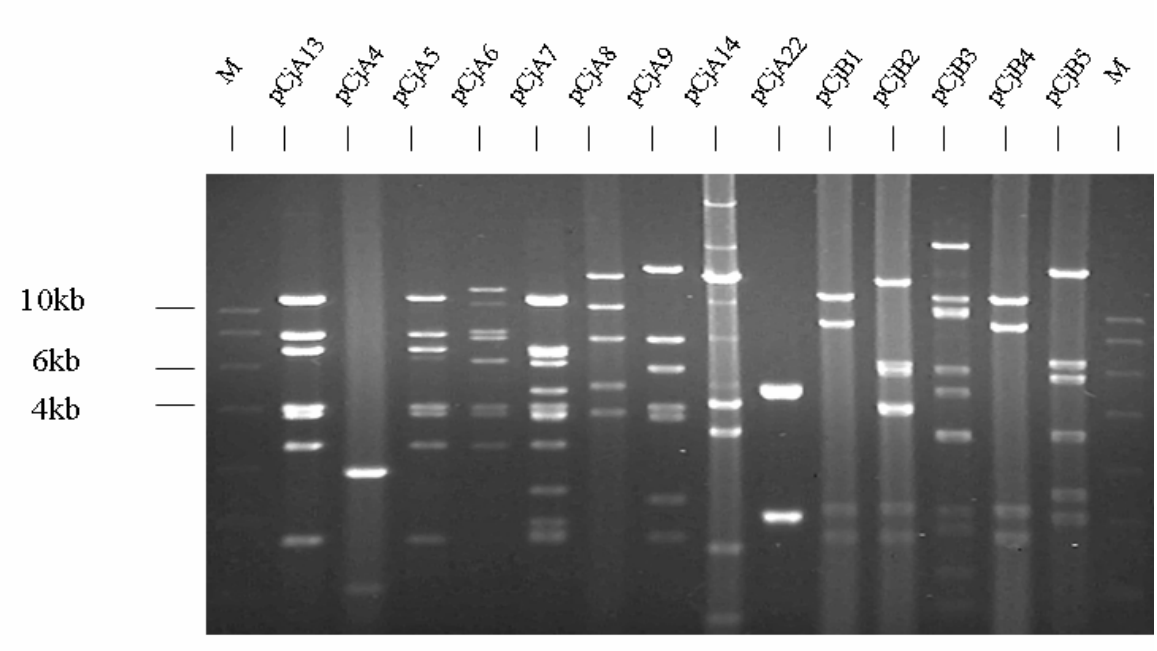


Figure 3.2: Figure shows diversity of *C. jejuni* strains. *Bgl*II-digested plasmid DNA of *Campylobacter jejuni* isolates

On species level, 19% (n = 14) of the *C. jejuni* and 56% (n = 5) of the *C. coli* isolates harboured plasmids. The size of plasmids ranged between 6 and 66 kb for *C. jejuni* and between 5 and 9 kb for *C. coli* (Data not shown). Fifty percent (n = 7) of *C. jejuni* and 60% (n = 3) of the plasmid-positive *C. coli* isolates were resistant to tetracycline (Table 3.2). PCR amplification of the *tet*(O) gene from total bacterial DNA revealed that high-level tetracycline resistance (MIC >256µg/ml) is associated in all cases with the presence of the *tet*(O) gene in *C. jejuni* (Table 3.2). In *C. coli* isolates, tetracycline resistance is significantly lower (minimal inhibitory concentration 16µg/ml) than in *C. jejuni*. To determine the localization of the *tet*(O) gene, plasmid DNA from *C. jejuni* and *C. coli* strains was probed with the digoxigenin-11-dUTP labelled *tet*(O) probe (Fig. 3.3 and Fig. 3.4b).

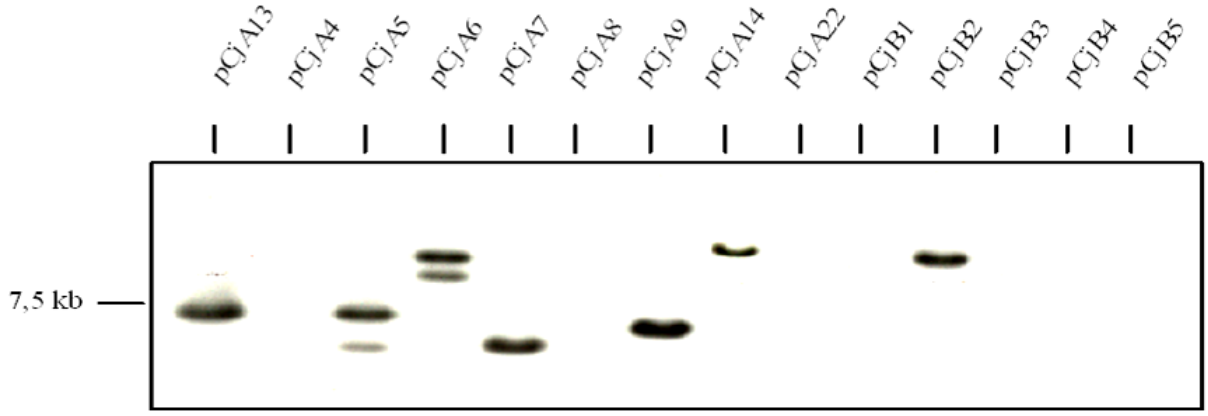


Figure 3.3: Correlation of tetracycline resistance of *C. jejuni* isolates and the presence of the *tet*(O) gene. *Bgl*II-digested *C. jejuni* plasmid DNA was probed with the digoxigenin-labelled *tet*(O) gene. For pCjA5 and pCjA6, two bands are visible that most probably are due to incomplete digestion of plasmid DNA.

Our results revealed that 54% (n = 7) of the tetracycline resistant *C. jejuni* isolates carried the *tet*(O) gene on their plasmids (Fig. 3.3), whereas in *C. coli*, none of the isolates carried the *tet*(O) gene on the plasmid (Fig. 3.4b). However, the presence of the *tet*(O) gene in the chromosomal DNA was confirmed by PCR from genomic DNA of *C. coli* (data not shown).

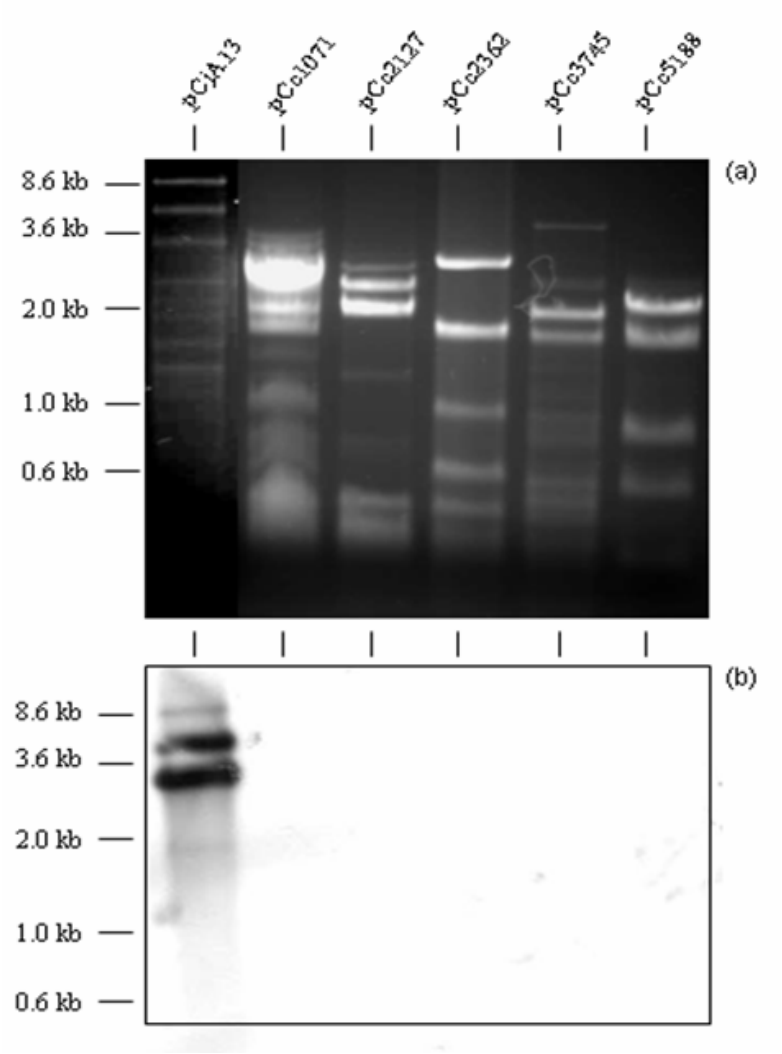


Figure 3.4: *Hind*III-digested *C. coli* plasmid DNA, (a) agarose gel electrophoresis of ethidium bromide stained plasmid DNA. (b) Probed with digoxigenin labelled *tet(O)*.pCjA13 served as a positive control and two bands are visible that are most probably the result of incomplete digestion of plasmid DNA.

3.3. *In-vivo* transposition of *E .coli* and *C. jejuni*

By using the scheme illustrated in chapter 2, the kanamycin resistance gene (*aphA*-3) was tagged with 40 variable DNA signatures (tags) which resulted into 1.8kb DNA fragments (Fig. 3.5).

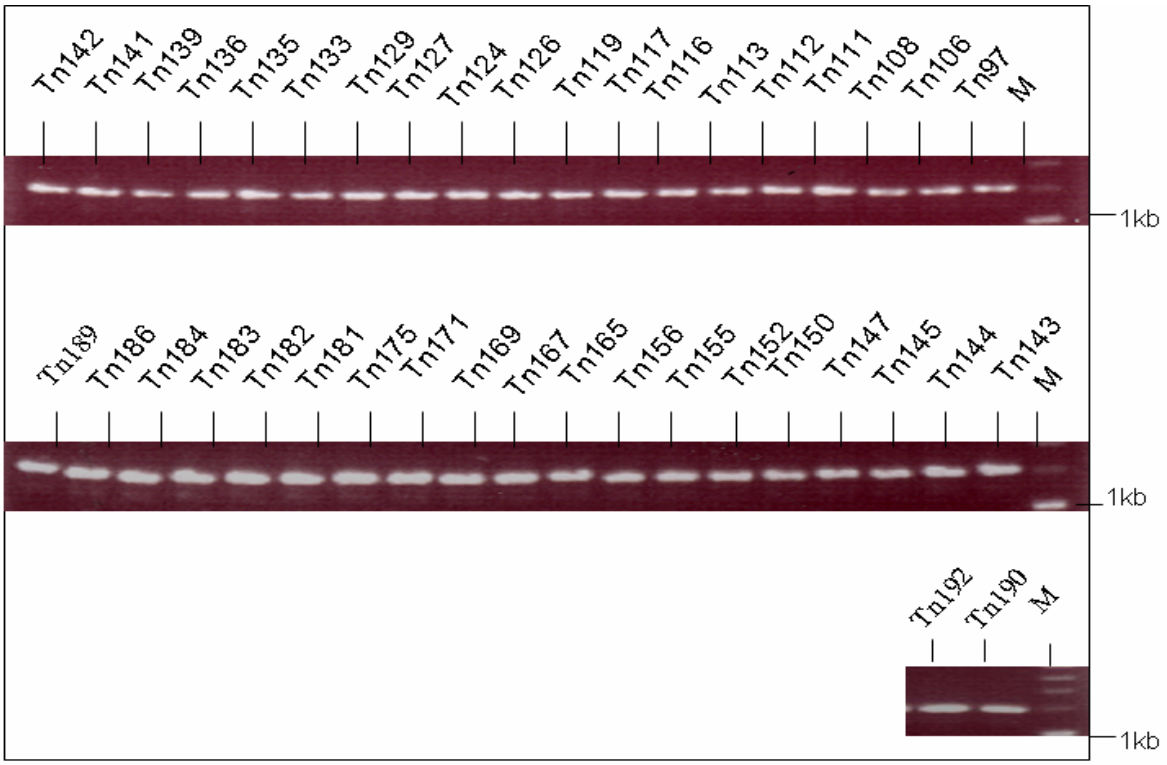


Figure 3.5 Each band labelled as "Tn" represents an EZ::TN5kan® transposon tagged with a unique DNA sequence of 20 to 25bp. In the figure "M" represents 1kb DNA ladder. The size of each fragment was estimated as 1.8kb.

Prior to the *in-vivo* transposition, all the tagged transposon fragments were analyzed for the cross hybridization by using a PCR strategy (See chapter 2). This analysis revealed no cross hybridization of the tags (data not shown). *E. coli* DH5α electrocompetent cells were used to test the efficiency of the EZ::TN5kan® transposon for *in-vivo* transposition. A total of 1×10^4 transformants/µg of transposon DNA were obtained, indicating that EZ::Tnkan® can be successfully used for *in-vivo* transposition. Several repetitions of this experiment produced comparable number of transformants. In all cases, no antibiotic-resistant colonies were obtained in the absence of the EZ::TN transposase in the reaction mixture.

To generate random insertional mutants of *C. jejuni,* EZ::TN transposons:transposase complexes were prepared according to the above mentioned procedure: one µl of the transposons:transposase complex was electroporated into *C. jejuni* strain B2. Kanamycin-resistant colonies were selected on Columbia blood agar plates containing 50µg of kanamycin per ml. One electroporation of *C. jejuni* strain B2 resulted in 3.5×10^2 to 5.8×10^3 transformants/µg of transposon DNA. During all the electroporation experiments, 1µl of transposon DNA (in the absence of transposase), and glycerol mix was electroporated as a negative control which produced no visible colonies on blood agar plates. Our experiments confirmed the functionality of the constructed transposons and it was clearly demonstrated that they can be used to produce insertional mutagenesis in *C. jejuni.* Approximately 55 random mutants of *C. jejuni* were generated reaching a total number of 660 by utilizing 12 transposons each tagged with a unique DNA tag (Fig. 3.6).

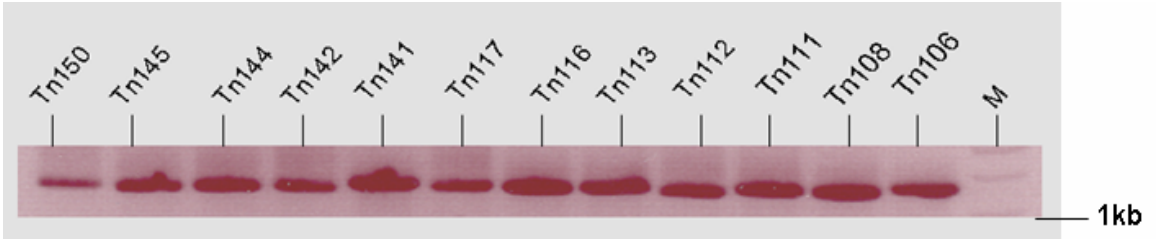


Figure 3.6 Transposons used to generate STM mutants. Each band represents 1.8kb EZ::TN5kan® transposon used for the *in-vivo* transposition of *C. jejuni* chromosome. Transposons shown in the figure were used to generate a random library of the 660 mutants. "M" represents 1kb DNA ladder

3.4 Random insertions of EZ::TNkan® in the chromosome of *C. jejuni.*

To determine whether EZ::TNkan® is inserted randomly in the *C. jejuni* genome, Southern blot analysis of 18 randomly chosen kanamycin insertion mutants was performed. Chromosomal DNAs were isolated from the different mutants of *C. jejuni,* subsequently digested with *Bsp*HI and separated on a 0.9% agarose gel. Restriction endonuclease *Bsp*HI was chosen because it cuts in the middle of the transposon. Therefore, when probed with digoxigenin-11-dUTP labelled EZ::TNkan® transposon DNA; two unique bands of different sizes were expected from digested chromosomes on single insertion of the

transposon. However, as is shown in Figure 3.7, a few samples (lane 3, 9, 19) produced a single band. Strong labelling of these bands compared to other ones, however, indicate that they most likely are the result of an overlap of two fragments of similar sizes. In hybridization experiments, the *Bsp*HI-digested chromosomal DNA of wild-type *C. jejuni* did not show any hybridization signal (Lane 7). Taken together, this analysis demonstrated random insertion of EZ::TNkan® transposon which proofs the usefulness of this transposon in generating pools of random mutants of *C. jejuni.*

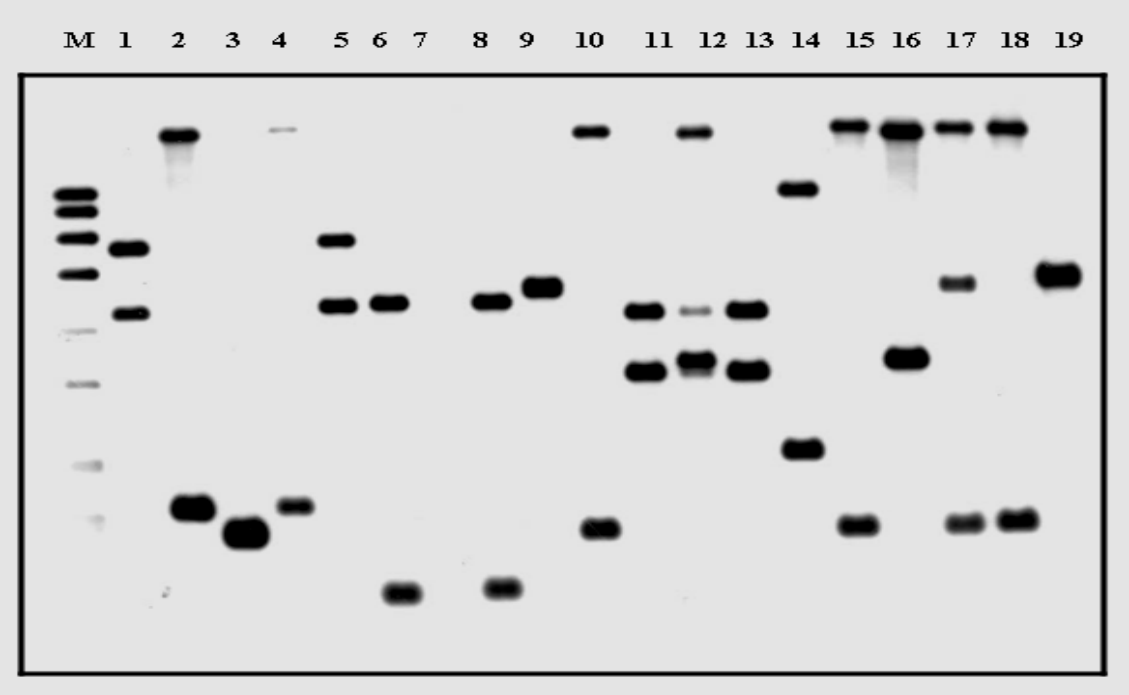


Figure 3.7: Southern blot analysis of randomly inserted Tn5kan® transposons in *C. jejuni.* Chromosomal DNAs from 18 randomly chosen insertion mutants (lane 1-6 to 8-19) and wild type B2 (Lane No.7) were digested with *Bsp*HI and probed with the digoxigenin labelled *aphA*-3 gene.

Randomness of these mutants was also confirmed by DNA sequencing results of insertional mutants with defined phenotypes.

3.5 Isolation of non-motile mutants of *C. jejuni*

The flagellar motility is known as one of the important virulence factors and colonization determinants of *C. jejuni*. Therefore, to test the usefulness of our transposition system, we chose to screen 660 of the obtained mutants for motility defects on MH motility agar. Three mutants were found as completely non-motile or displayed reduced or altered motility phenotypes (Fig. 3.8).

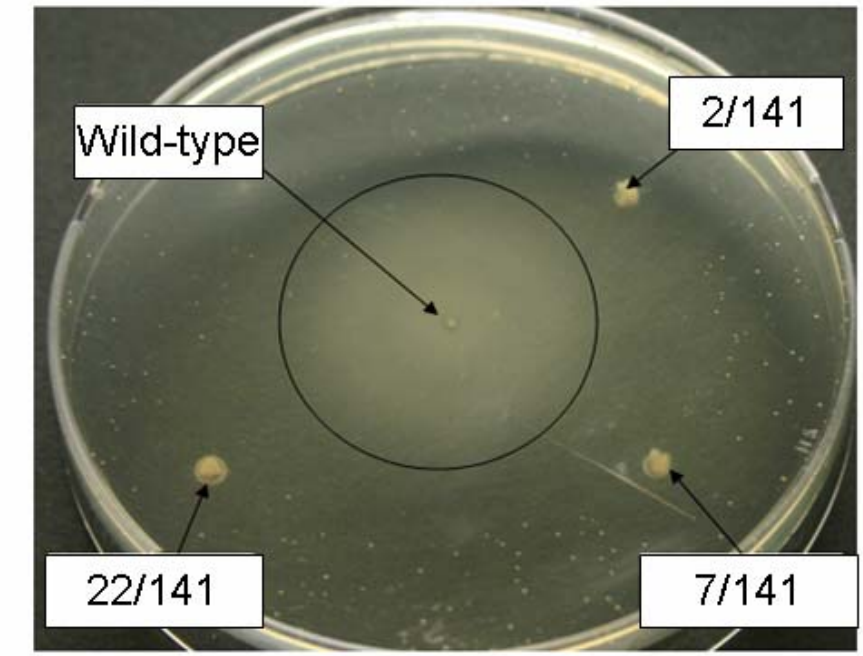


Figure 3.8: Non motile mutants of *C. jejuni*. Transposon mutagenesis produced three (2/141, 7/141, and 22/141) completely non-motile isolates. WT, wild type B2 strain of *C. jejuni* showed a swarming phenotype indicating full motility (in circle).

The chromosomal DNA from the two mutants was isolated and used for DNA sequence analysis. This revealed insertion of EZ::TNkan® transposon in the genes *cj0793* (encoding

the sensor recognition domain of FlgS) in mutant 2/141, and *cj0955c* (encoding probable phosphoribosylformylglycinamidine synthase subunit II) in mutant 7/141.

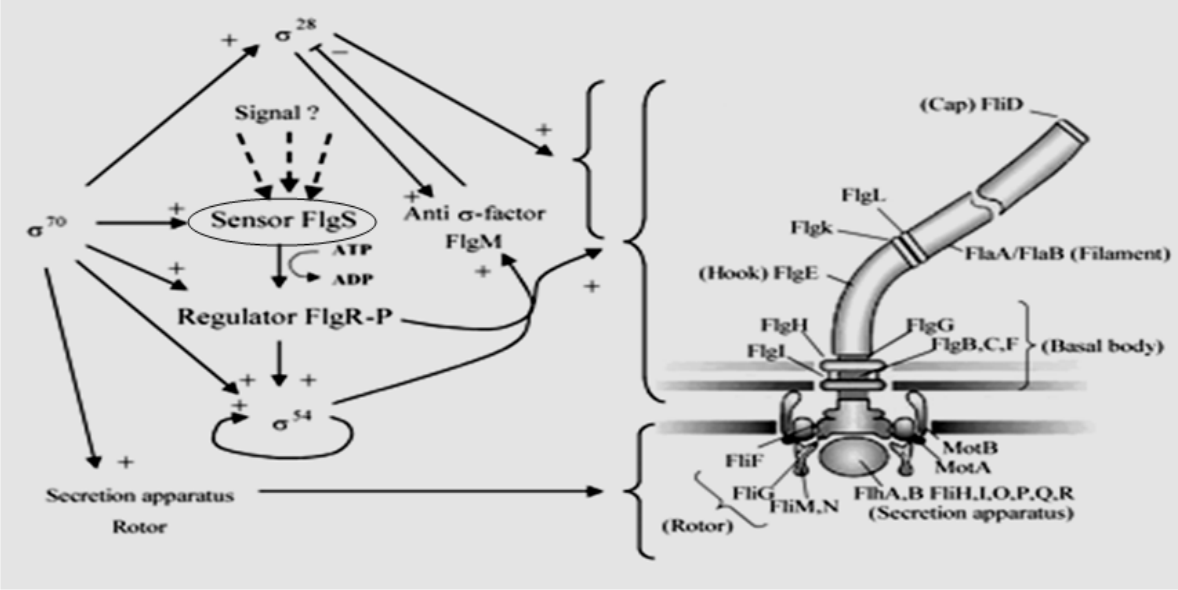


Figure 3.9: A model of the regulation of the flagellum of *C. jejuni.* In the presence of an unknown signal, sensor recognition domain of FlgS (encoded *by cj0793*), autophosphorylates which results in the transfer of the phosphate to the response regulator FlgR, and the alternative factor σ^{54}, stimulates the transcription of the genes needed for the activation of FliA (factor σ^{28}) which is required for the full assembly of the flagella. (Purposed by Wösten, 2004).

One of the insertion sites of these mutants was found in the two component sensor gene *cj0793* (Fig. 3. 9), which appeared to be a central factor for flagellar motility (Wösten et al., 2004). This non-motile mutant, however, showed normal flagella (Fig.3.10). Another non-motile mutant had the EZ::TNkan® insertion in a gene (*cj0955c*) of unknown function or not previously associated with motility or any other phenotype in *C. jejuni*. Electron microscopic analysis of the non-motile mutants revealed the absence of flagella in two mutants 22/141 and 7/141.

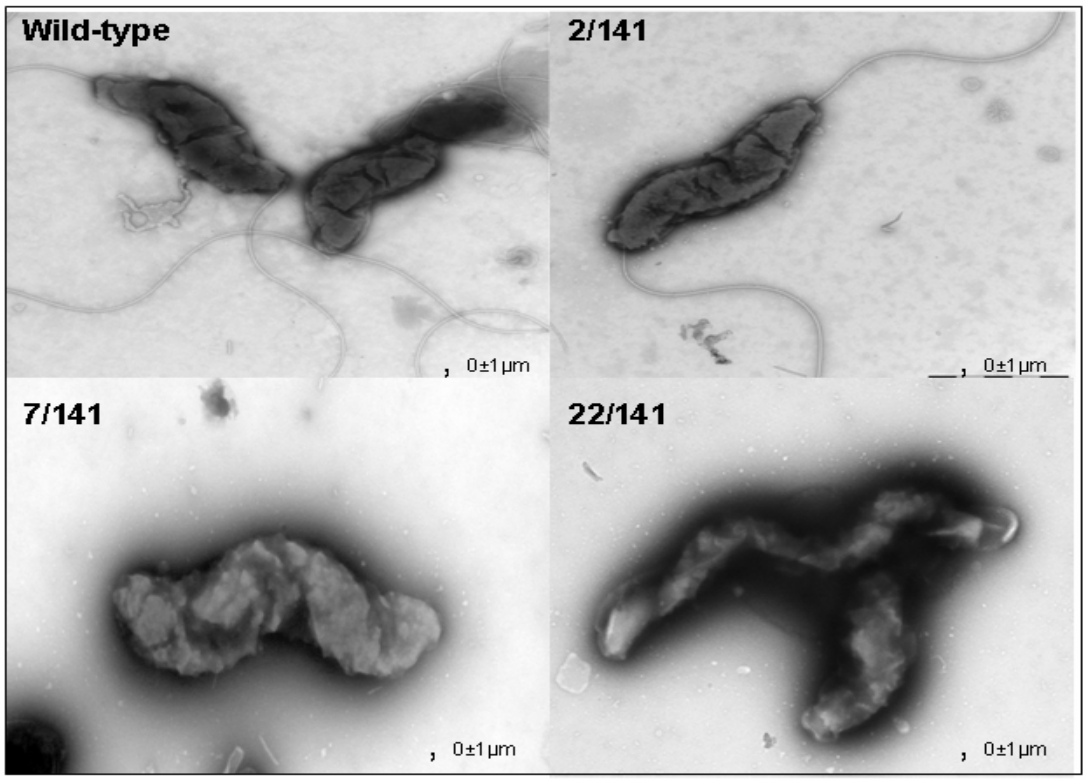


Figure 3.10 Electron micrographs of the *C. jejuni* strain B2 (wild-type) and non-motile mutants 2/141, 7/141and 22/141. Bars, 0±1µm.

3.6 Isolation of an osmo-sensitive mutant

Sodium chloride is known as one of the most important food adjuncts in food preservation. Most strains of *C. jejuni* can grow well in the presence of 0.5% NaCl. By using an agar dilution method, we tested various concentrations (0.5% to 4.5% wt/vol) of NaCl to determine the minimal inhibitory concentration of NaCl for selected strains of *C. jejuni*. Our results showed that the growth of all tested *C. jejuni* strains (NCTC11168, NCTC11828 and B2) was completely inhibited in the presence of 2% NaCl. At 42°C however, in the presence of 1.5% NaCl, a normal growth of the B2 strain was observed. All 660 random mutants of *C. jejuni* were screened for their sensitivity towards 1.5% salt in comparison to *C. jejuni* wild type strain B2. Our screen revealed that one mutant that had insertion of EZ::TNkan® in gene cj0009 (*gltD*), encoding the NADPH-dependent glutamate synthase small subunit showed reduced growth in the presence of 1.5% NaCl. The NADPH-dependent (NADPH-GltS) is mostly found in bacteria and is a

multicomponent ironsulfur flavoprotein belonging to the class of N-terminal nucleophile amidotransferases. It catalyzes the conversion of L-glutamine and α-ketoglutarate into two molecules of L-glutamate.

3.7 Screening for sodium deoxycholate sensitivity

Bile is primarily composed of bile salts and up to some extent of phospholipids and cholesterol (Crawford, 1999). It is reported that the average concentration of bile salts in the human intestine varies from 0.2 to 2%. The primary bile salts are cholic acid, glycocholic acid, deoxycholic acid, and taurocholic acid (Elliot, 1985). In chickens, a natural host for *C. jejuni*, the concentration of bile salts ranges from 0.01% in the cecum to 0.7% in the jejunum (Lin et al., 2005). The bile salts facilitate the digestion of fats and they also serve as an effective antimicrobial agent (Gunn, 2000). In gram-negative bacteria, bile salts can pass directly across the outer membrane or pass through porins (Thanassi et al., 1997), which indicates that enteric pathogens have developed some mechanisms to overcome the damaging effects of bile salts. We therefore screened 660 random mutants of *C. jejuni* for their sensitivity towards 1% (wt/vole) sodium deoxycholate known as bile salt. In this screen, no difference in growth of wild type and mutants was observed. These repetitive assays demonstrate that, in principle, the EZ::TNkan® transposon system is suitable for the random mutagenesis of *C. jejuni.*

3.8 Optimization of the BALB/c mouse model for *C. jejuni* infection

To determine the optimum infection dose and suitable strain for signature-tag mutagenesis (STM) experiments, we tested four different strains of *C. jejuni* (NCTC11168, NCTC11828, 81-176, B2) with infection doses, 1×10^8, 1×10^9 and 1×10^{10} cfu, respectively. Seven days after i.p. infection, all mice were sacrificed and analysed for the presence of bacteria within their livers. The infection dose of 1×10^8 and 1×10^9 bacteria resulted in low recovery of bacteria from the liver (Table 3.3). However, higher numbers of bacteria were recovered when mice were infected with 1×10^{10} bacteria.

Table 3.3 Female BALB/c mice were infected intraperitoneally with 1×10^8 and 1×10^9 viable bacteria. The numbers of recovered *C. jejuni* from the livers after 7 days of infection are shown in the right column. Each

value represents the number of colonies counted on blood agar recovered from the liver of the individual mouse (> 3 mice for each strain).

***C. jejuni* strain**	**Infection dose**	**Mouse No**	**Recovered cfu/mg**
NCTC11168	1×10^8	1	0
		2	0
		3	0
NCTC11828	1×10^8	1	900
		2	0
		3	0
B2	1×10^8	1	0
		2	0
		3	0
81-176	1×10^8	1	0
		2	0
		3	0
NCTC11168	1×10^9	1	3
		2	10
		3	5
NCTC11828	1×10^9	1	1
		2	1
		3	0
B2	1×10^9	1	4×10^2
		2	1×10^3
		3	3×10^2

Since, in preliminary experiments, infection with 1×10^9 bacteria of strain NCTC11168 and 81-176 showed lowest recovery of bacteria as compared to NCTC11828 and B2 in all experiments performed, these strains were excluded in further experiments. For the comparative analysis, three female BALB/c mice were tested for each infection dose 1×10^8, 1×10^9 of each strain respectively as shown in table 3.3. Infection dose of 1×10^{10} of strain B2 produced reproducible and higher numbers of recovered bacteria. In some of the

experiments, the same infection dose of NCTC11828 also resulted in higher numbers of bacteria from the livers. However, a lack of reproducibility and high variation in the numbers of recovered bacteria was a major obstacle in the optimization of a suitable infection dose for this particular strain. A comparison of two groups of seven female BALB/c mice with an infection dose of 1 $\times 10^{10}$ and respective numbers of recovered bacteria are shown in Table 3.4

Table 3.4 Female BALB/c mice were infected intraperitoneally with 1×10^{10} viable bacteria. The numbers of recovered *C. jejuni* from the livers after 7 days of infection are shown in the right column. Each value represents the number of colonies counted on blood agar recovered from the individual mouse (> 7 mice for each strain).

***C. jejuni* strain**	**Infection dose**	**Mouse No**	**Recovered cfu/mg**
B2	1×10^{10}	1	2×10^5
		2	1.5×10^4
		3	8.6×10^4
		4	1.3×10^4
		5	2×10^5
		6	1×10^3
		7	1×10^4
NCTC11828	1×10^{10}	1	3
		2	1
		3	1
		4	12.5×10^5
		5	1
		6	0
		7	0

Pathohistological examinations showed both macroscopic and microscopic changes in the liver and spleen of infected animals. Primary infection generally resulted in hepatosplenomegaly with inflammation. Furthermore, on the liver surface nodules indicated abcess like appearences (Fig. 3.11)

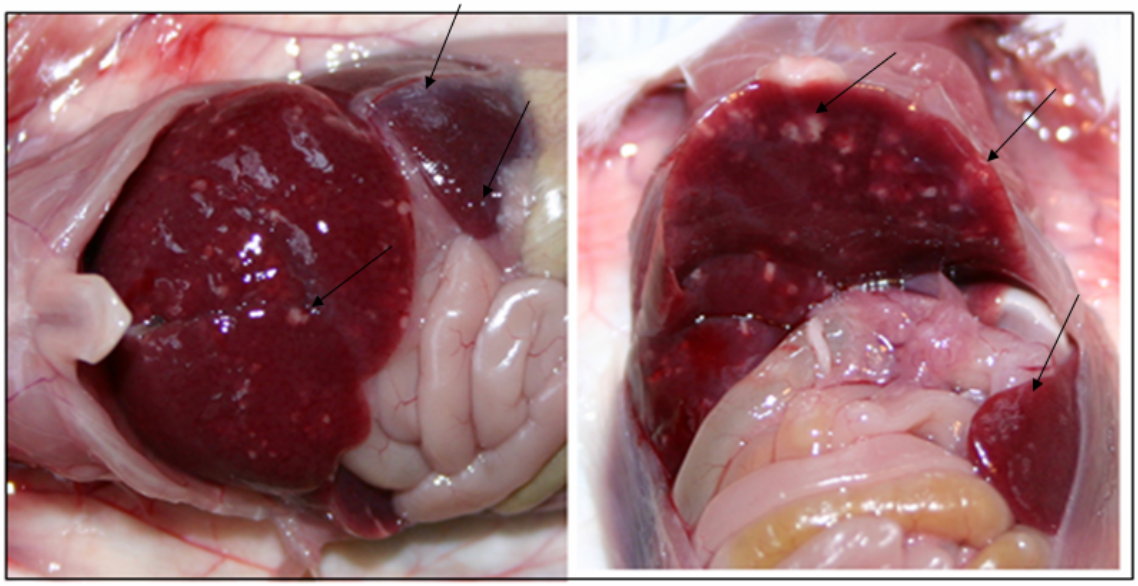


Fig 3.11 Gross pathology of *C. jejuni* infection in intraperitoneally infected female BALB/c mice at day 7 post infection. The livers were enlarged and contained numerous abcess-like nodes (accentuated by arrows). In addition, enlargement of the spleen is shown.

In histological sections, an inflammatory response could be seen, followed by tissue necrosis. The cell infiltrate consisted primarily of neutrophils and rarely lymphocytes (Fig 3.12). Although infection of mice with the *C. jejuni* strain B2 at an infection dose of 1×10^{10}cfu, resulted in a significantly higher number of recovered bacteria from the liver as compared to the other *C. jejuni* strains. A moderate variation in the number of recovered bacteria was observed within every single experiment. In repeated experiments, it was observed that 1×10^{11} cfu was a lethal infection dose for all mice tested in this study. Our data indicated that the optimum infection dose leading to reproducible results via the intraperitoneal infection route is 1×10^{10} cfu of *C. jejuni* strain B2. All other strains tested in this study showed lower and inconsistent recovery rates of bacteria from the livers of female BALB/c mice.

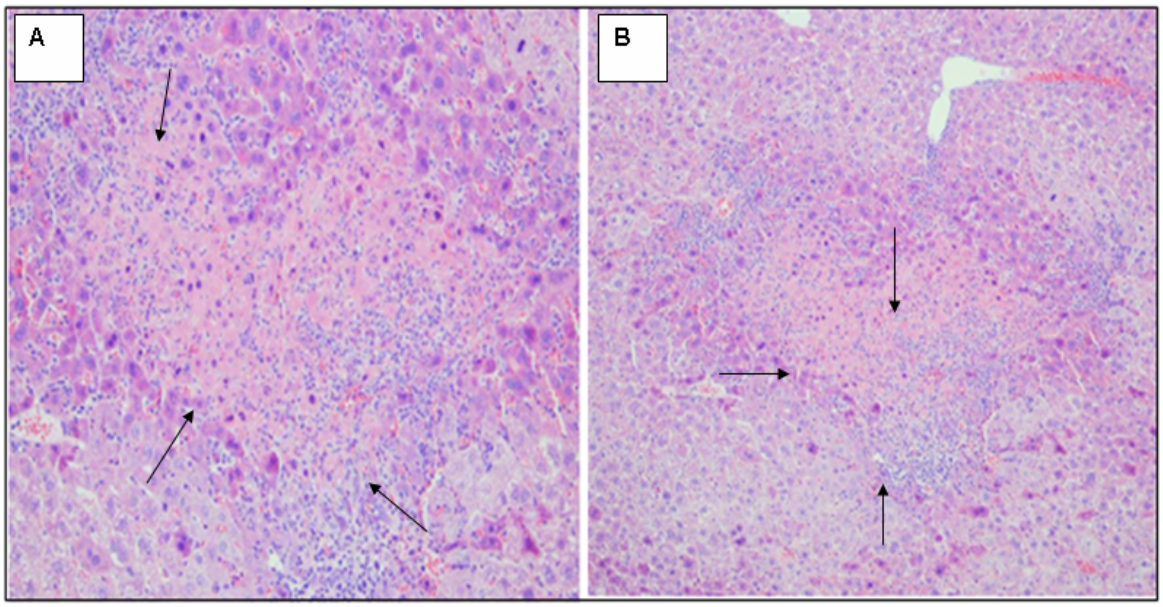


Figure 3.12 (A) Liver histology of a female BALB/c mouse 7 days after infection with 1×10^9 *C. jejuni* (B2 strain) showing lesions with infiltration of mononuclear cells. (B) Necrotic lesion (arrows) in the liver of a BALB/c mouse day 7 post infection with an infection dose 1×10^9 *C. jejuni. A+B*= Hematoxylin stained sections.

3.9 Application of signature tagged mutagenesis of *C. jejuni in vivo*

The *in-vivo* screening of signature tagged transposon mutants was done by using a pool of forty randomly selected mutants labelled with eight different signature tags. Mutants were subcultured on Columbia blood agar containing 50µg/ml kanamycin. Eight different pools, each containing five different mutants were prepared and six weeks old female BALB/c mice were challenged with an infection dose of 1×10^{10}cfu, respectively. Seven days post infection, the mutants were recovered from livers and plated on Columbia blood agar plates containing 50µg/ml of kanamycin for further analysis. A variable recovery rate was observed ranging between 1×10^3 to 1×10^6 cfu (Fig. 3.13).

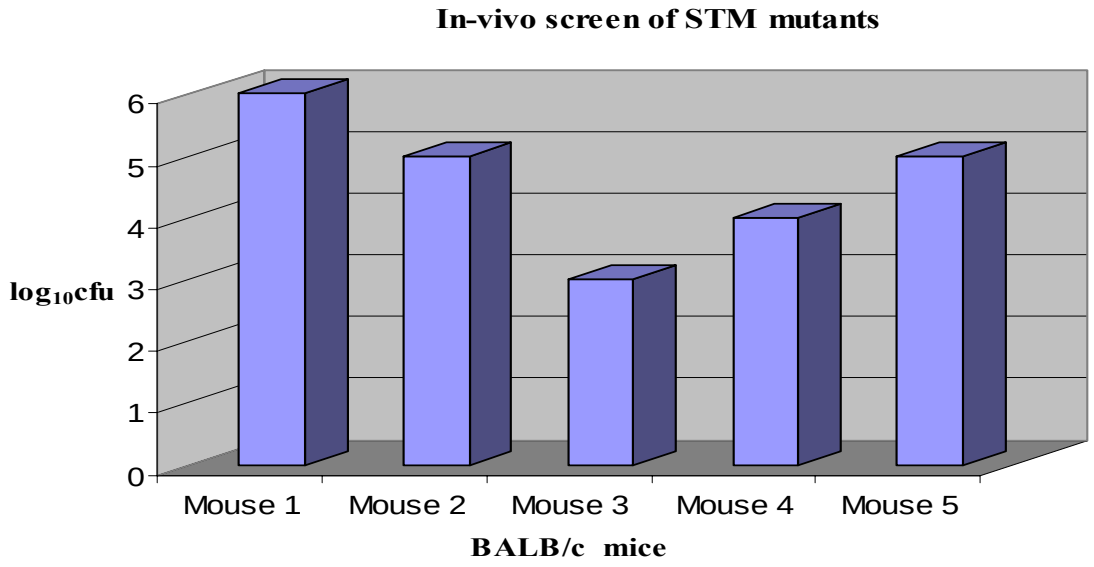


Figure 3.13: Numbers of *C. jejuni* mutants that were recovered from the liver of each female BALB/c mouse, 7 days after infection. Each BALB/c mouse was infected intraperitoneally with 1×10^{10} viable STM mutants of *C. jejuni*. Data are described as $\log_{10}$ cfu recovered from each liver.

To confirm possible attenuated mutants, a PCR analysis by using tag-specific primers (Table 2.4) and a generic primer JA4 (Table 2.3) was carried out. Genomic DNA from the recovered mutants was used as a template. A pool of 40 signature-tagged mutants of *C. jejuni* was tested in six weeks old female BALB/c mice. The fundamental methodology of our STM system, following the production of a mutant library, is separated into two phases, an input and an output phase (Hensel et al 1995). The two phases of the experiment are essentially identical except that, prior to the out-put phase, a round of selection was performed. The presence or absence of the mutant is detected in both phases by extracting genomic DNA from the pool of mutants and PCR amplification of the transposon by using a primer pair based on regions of variable tags (Fig. 3.14).

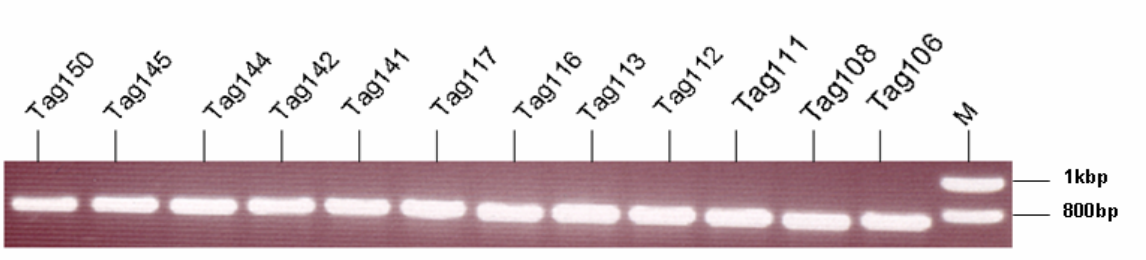


Figure 3.14. Using tag-specific primer (Table 2.4) and a generic primer (Table 2.3; JA4 primer), every single mutant can be distinguished from each other by amplifying a 780bp DNA fragment. "M" represents 1kb DNA ladder

The comparison of the input pool and the out-put pool can reveal attenuated mutants. If the mutant is absent from the out-put pool but is present in the input pool, then the mutant is presumed to be attenuated which can be the result of random insertion of the transposon in the virulence associated gene. The scheme for the experiment is illustrated in Figure 3.15. All the tested mutants and their relative pools are shown in Table 3.5.

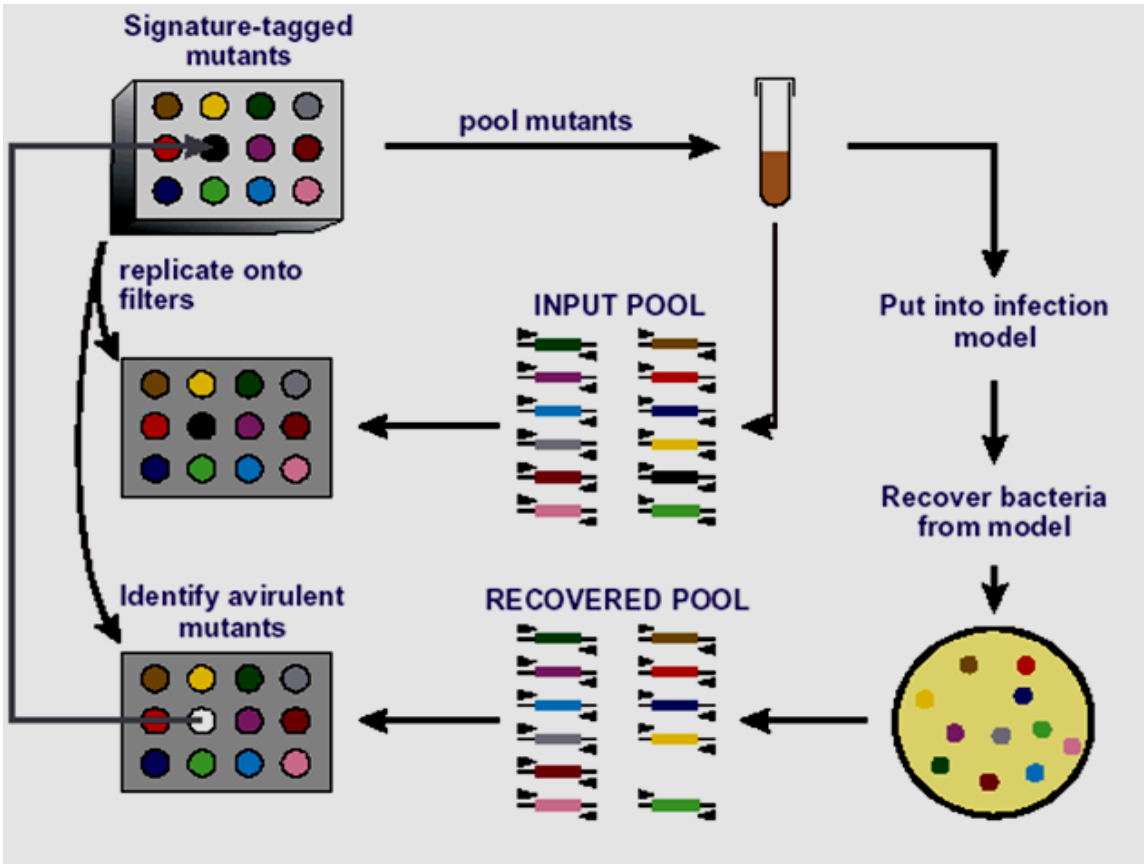


Figure 3.15: General scheme for the screening of a pool of STM mutants of *C. jejuni*

by comparing the in-put pool and the out-put pool of mutants. Female BALB/c mice were used as a screening model in this study.

Pool No	tag A	tag B	tag C	tag D	tag E	tag F	tag G	tag H
Pool 1	1	M	1	1	1	1	1	M
Pool 2	2	2	2	2	2	2	2	2
Pool 3	3	3	3	3	3	3	3	3
Pool 4	4	4	4	4	4	4	4	4
Pool 5	5	M	5	5	5	5	5	5

Table 3.5 Five different pools (in rows) prepeared by mixing five mutants from the each of the eight different tags A-H (in columns), were injected into six weeks old female BALB/c mice. In the Table boxes denoting "M" represent missing mutants from each recovered pool of the STM mutants.

Our results showed that 37 mutants were recovered and were verified by a tag-specific PCR. However, in repeated PCRs done by using total genomic DNA from the output pool as a template, three mutants were not detected (Table 3.5 denoted as "missing") during the first screening experiment. To verify their attenuation, we retested whether these mutants were reproducibly missing. Verification was done in three repeated experiments by following the same procedure. However, the missing mutants were recovered and confirmed by the tag-specific PCR. Therefore, the probability to achieve an avirulent mutant by screening a pool of small number of mutants is low. However, our results indicated that in principle female BALB/c mice can be utilized as a screening model for randomly generated signature- tagged mutants of *C. jejuni*.

4 Discussion

4.1 Genotypic and phenotypic diversity of *Campylobacter spp.*

Over the last few decades, *Campylobacter jejuni* has emerged as an important food-borne pathogen and is a major public health concern. *C. jejuni* causes gastro-intestinal infections as well as post infection manifestations, e.g Guillain-Barré syndrome or reactive arthritis (Schmidt-Ott et al., 2006). Annually, approximately 60,000 cases of *Campylobacter spp.* enteritis are reported in Germany (RKI, 2006). Complications of *C. jejuni* infection vary from mild, noninflammatory, self-limiting diarrhoea to severe, inflammatory, bloody diarrhoea (Wassenaar and Blaser, 1999). Consequently, the importance of *C. jejuni* as a major public health problem is well recognized which makes it important to understand the pathogenesis of this disease. The genome sequence of *C. jejuni* has been recently reported (Parkhill et al., 2000), and little is known about the mechanisms, contributing to the genetic diversity of this pathogen. The sequenced strain NCT 11168 contains no transposons, phage remnants, or insertion sequences in its genome. Several reports have pointed out a relative intraspecies genotypic and phenotypic diversity in comparison to other enteropathogens.

There are two serotyping schemes detecting a wide variety of serotypes (Lior et al., 1982). In addition, the genotypic diversity of this pathogen has been reported by ribotyping, pulsed-field gel electrophoresis, and several PCR-based techniques. However, due to the limitations of these methodologies, further characterization of the genetic basis is hindered. Diversity in *C. jejuni* strains has also been reported at the phenotypic level, particularly for characteristics implicated in pathogenicity, including adherence, invasion of epithelial cell lines, toxin production, serum resistance, polysaccharide production, sialylation of LOS and the ability to colonize chickens. Due to the direct association of the clinical isolates with disease, they can provide a valuable source for the selection of highly pathogenic strains. As for example, strain 81-176 was isolated from a patient during an outbreak of campylobacteriosis, and proven to be highly pathogenic in monkeys and humans (Russell et al., 1989). Therefore, with the major objective to later identify putative virulence factors of *C. jejuni,* we collected and characterized clinical isolates of *C. jeuni* to increase the overall probability for the selection of a suitable strain for further analysis.

Due to the lack of reliable specific phenotypical markers for species identification, many clinical laboratories identify *Campylobacter* isolates only on the genus level. Hippurate hydrolysis has been suggested as a key phenotypical test for differentiation of *C. coli* and *C. jejuni* (Harvey et al., 1980). On the basis of amino acid homology of the hippurate hydrolase, it was classified as a non-peptidase homologue of the M40 peptidase family within the MH peptidase by the Merops Protease Database (Merops, 2002). Other members of this family of peptidases are also reported in plants, archaebacteria and eubacteria. The hippurate hydrolase of *C. jejuni* can cleave the benzoyl group from *N*-benzoylglycine, therefore, it may be considered as an amidohydrolase. It is reported that other M40 peptidases were also proven to be amidohydrolases, including a thermostable carboxypeptidase from *Sulfolobus solfataricus*, an *N*-acyl-L-amino acid amidohydrolase from *Bacillus stearothermophilus*, a family of indole-3-acetic acid (IAA) amino acid hydrolases from the plant *Arabidopsis thaliana*, an IAA-aspartic acid hydrolase from *Enterobacter agglomerans* and an *N*-carbamylase from *Pseudomonas spp.* (Watabe *et al*., 1992). The enzymatic properties of the hippurate hydrolase enzyme suggest that it would be active under normal physiological conditions. It is speculated that carboxypeptidase activity of this enzyme might play a role in the nutrient acquisition by the bacterium during infection. Such phenomena would directly contribute to the virulence of *C. jejuni.* However, the *in-vivo* function of the hippurate hydrolase of *C. jejuni* remains to be understood (Steel et al., 2006).

Using commercially available test systems, a hippurate hydrolysis test can be performed rapidly but major difficulties remain to correctly identify any hippurate hydrolase-negative isolate of *C. jejuni.* The gene *hipO* is highly conserved among the strains of *C. jejuni* and does not exhibit any significant polymorphism, which makes it a reliable marker for the identification of *C. jejuni* strains (Steinhauserova et al., 2001). However, some atypical *C. jejuni* strains fail to express this enzyme. These atypical strains of *C. jejuni* are frequently reported in the literature (Linton *et al*. 1997). We therefore combined reliable biochemical and molecular markers for species differentiation of eighty-three clinical isolates. On the basis of their hippurate hydrolase activity, 67 isolates (81%) were identified as *C. jejuni*. Sixteen *Campylobacter* isolates (19%) did not show any hippurate hydrolase activity and

were further evaluated for the presence of *hip*O gene. In conclusion, in the present study, initially nine strains were biochemically typed as *C. coli,* but were proven to be *C. jejuni* after Southern blot analysis.

4.2 Antibiotic resistance in *C. jejuni* and *C. coli*

The current findings stress that empiric antimicrobial therapy of *Campylobacter* enteritis should relay on the locally assessed susceptibility profiles. Thus, a general knowledge of the expected susceptibility pattern of *Campylobacter* species causing infections in a given geographic region is a prerequisite to initiate the most appropriate antimicrobial treatment.

Furthermore, it is necessary to perform antimicrobial profiling of clinical strains, supposed to be used for further research involving genetic manipulation. Therefore, after precise identification at the species level, we analysed drug resistance in 83 clinical isolates of *Campylobacter* by using a disk diffusion method (Gaudreau and Gilbert, 1997).

Previously, a higher rate of ciprofloxacin resistance was observed in *C. jejuni* isolates collected from lifestock (45.8%) and human individuals (45.1%) in Germany (Luber et al., 2003). In our study, 25.3% of *C. jejuni* and 10% *of C. coli* isolates showed resistance to ciprofloxacin. Comparable findings have been observed in The Netherlands with 29% (Talsma et al., 1999) and Greece with 30.6% (Chatzipanagiotou et al., 2002). The resistance rate against ciprofloxacin in Germany is lower as compared to other countries like Spain with 82% (Prats et al., 2000) and Thailand with 84% (Hoge et al., 1998). However, since at least some of the isolates showed resistance, the use of ciprofloxacin as a first choice for treatment might be questionable.

Tetracyclines have been purposed as an alternative choice in the treatment of *C. jejuni* and *C. coli* enteritis. Large geographical variations in susceptibility pattern of *C. jejuni* and *C. coli* to tetracycline have been observed. The rate of resistance in Denmark ranges from 0 to 11% (Aarestrup et al., 1997), in Spain it is 25% (Gomez-Garces et al., 1995), and in the United States 48% (Nachamkin et al., 1994). In this study, tetracycline MICs were determined for 19 *Campylobacter* isolates that were identified to be tetracycline-resistant by the disk diffusion test. Tetracycline MICs ranged from 16 to 256µg/ml. High-level tetracycline resistance was found in *C. jejuni,* whereas in *C. coli* isolates, tetracycline resistance level was significantly lower. In case of 13 *C. jejuni* strains, MIC was

determined as >256µg/ml whereas six *C. coli* isolates had a MIC of 16µg/ml. The frequency of tetracycline resistance was significantly higher (χ^2 test: $p < 0.001$) in *C. coli* (67%) than in *C. jejuni* (18%).

C. coli is frequently found in pigs (Moore and Madden, 1998), and it is known that the regular use of antimicrobial agents for therapeutic and growth promotion can play a role in the prevalence of antimicrobial-resistant strains of *C. coli* in pigs (Payot et al, 2001). Therefore, a higher frequency of tetracycline resistance in clinical isolates of *C. coli* might be linked with the use of related antibiotics in the food chain. Bacterial resistance to tetracycline commonly arises through one of these four identified mechanisms: efflux of tetracycline, modification of tetracycline, ribosomal protection, or mutation of the 16s rRNA (Burdett et al., 1991; Ross et al., 1998; Schnappinger and Hillen, 1996). Of these, the plasmid-mediated *tet*(O)-encoded tetracycline resistance is reported frequently in *Campylobacter spp.* (Lee et al., 1994; Manavathu et al., 1988). The plasmids bearing the *tet*(O) determinant were also isolated from other bacteria, such as *Enterococcus faecalis* and *Streptococcus spp*. and the plasmids were shown to have similar sizes and restriction profiles compared to those isolated from *C. jejuni* and *C. coli* (Zilhao et al., 1988).

Previously, the isolation rate of plasmids from *Campylobacter* species has been reported variable with 44% to 91% for clinical and poultry isolates (Gaudreau and Gilbert, 1997). In this study, approximately 23% (n = 19) of *Campylobacter* isolates harboured plasmids, ranging in size from 5.0 to 66 kilobases (kb). Significant differences between plasmids were detected in both species of *Campylobacter*; 19% (n = 14) of the *C. jejuni* isolates and 56% of *C. coli (n = 5)* isolates harboured plasmids. Instead of 33 to 66 kilobases sized plasmids found in *C. jejuni*, *C. coli* isolates harboured plasmids sized 5 to 9 kilobases.

Fifty percent (n = 7) of plasmid-harbouring *C. jejuni* and 60% (n = 3) of the plasmid-positive *C. coli* isolates were resistant to tetracycline. To determine the localization of the *tet*(O) gene, plasmid DNA from *C. jejuni* and *C. coli* isolates was probed with the digoxigenin-11-dUTP labelled *tet*(O) gene. Our results reveal that 54% (n = 7) of the tetracycline-resistant *C. jejuni* isolates carried the *tet*(O) gene on their plasmids. Surprisingly, in *C. coli* none of the plasmids carried the *tet*(O) gene. Amplification of the *tet*(O) gene from genomic DNA of tetracycline-resistant *C. coli* isolates indicated a chromosomal localization of the *tet*(O) gene. However, considering the limitation of the

alkaline lysis method for plasmid isolation, the presence of low-copy number plasmids larger than 70kb can not be totally excluded. It has been suggested previously that recombination events between plasmids and the chromosome or integration of a plasmid might occur which could explain chromosomally mediated tetracycline resistance in these isolates (Boosinger et al., 1990). It is also known that illegitimate recombination can cause integration of a heterologous plasmid in *C. coli* (Richardson and Park, 1997) and this would ultimately lead to a higher frequency of chromosomally mediated tetracycline resistance in *C. coli.* Previously, conjugation in two isolates having plasmids sized 40.5 kb (pCjA9) and 41.9 kb (pCjA13) has been shown (Schmidt-Ott et al., 2005). In that study, Southern blot analysis showed that tetracycline resistance in these isolates was *tet*(O) encoded and plasmid mediated which ultimately confirms conjugation transfer of *tet*(O) in these *C. jejuni* isolates.

In conclusion, resistance against tetracycline in *C. jejuni* and *C. coli* isolates was associated with the *tet*(O) gene in all cases, and there was a strong correlation between tetracycline resistance and plasmid carriage in *C. jejuni* isolates. Although all plasmid-containing isolates of *C. coli* were resistant to tetracycline, none of the *C. coli* isolates carried the *tet*(O) gene on the plasmid. Instead, the *tet*(O) gene seems to be chromosomally encoded in all tetracycline-resistant *Campylobacter coli* isolates.

4.3 Construction of transposon for the *in-vivo* transposition of the *C. jejuni*

During the last decade, the development of new genetic tools provided enormous opportunities to identify putative virulence factors of microbial pathogens. One of the most powerful genetic approaches used for the identification of virulence associated genes is signature-tagged mutagenesis (STM), which was initially developed by David Holden and colleagues in 1995. This approach led to the identification of hundreds of new genes related to the virulence in a broad range of bacterial pathogens. Despite the efforts made to understand, so far little is known about the virulence factors of *C. jejuni*. One of the reasons in this lack of understanding is the deficiency of high through-put strategies to generate random mutants that can be tested in different biological environments. Several approaches have been previously used to generate mutants which mostly relied on shuttle mutagenesis and homologous recombination. A different approach called *in-vivo* transposition based on

the *Himar1* transposable element and a suicide vector has been used recently (Colegio et al., 2001). However, the reported weakness of this system is that restriction of the suicide vector cannot be avoided which severely affects its efficiency. Although the utility of this approach cannot be completely denied for some applications, low-efficiency of these systems makes it difficult to use them when the isolation of a large pool of mutants is intended.

There are several factors contributing to the difficulties to generate a high-throughput mutagenesis system for *C. jejuni* which is primarily due to the existence of powerful restriction barriers and inefficient expression of the appropriate transposase enzymes *in-vivo*, or a combination of these and some other factors (Colegio et al., 2001). In previous STM studies, composite transposons such as Tn5 elements have been used. These transposons are capable to encode two proteins, the transposase and a related protein, the transposition inhibitor, whose relative abundance determines, in part, the frequency of the Tn5 transposition event. The synthesis of these proteins found to be programmed by a complex set of genetic regulatory elements. For example, the host DNA methylation function, *dam,* inhibits transposase promoter recognition and indirectly enhances the transposition inhibitor promoter. The inhibitor lacks the N-terminal 55 amino acids of the transposase, suggesting that this sequence plays a key role in the transposition process. However, an intact N-terminal sequence is required for the transposase's recognition of the 19-bp end DNA sequences which is the first critical step in the transposition process for Tn5 elements. Transposase-end DNA interaction is itself regulated by an intricate series of reactions involving several host proteins: DnaA, Dam, and Fis. The unique function of this transposase is that it acts primarily *in cis* and inhibits its own activity *in trans.* Models to explain these properties are described and it is known that transposition occurs preferentially from newly replicated DNA, yet to be partitioned to progeny cells (Steiniger-White et al., 2004). Recent elucidation of the X-ray co-crystal structure of Tn5 transposase complexed with a DNA recognition end sequence provided the first three-dimensional picture (Fig. 4.1) of an intermediate in a transposition/retroviral integration pathway (Goryshin et al., 2000).

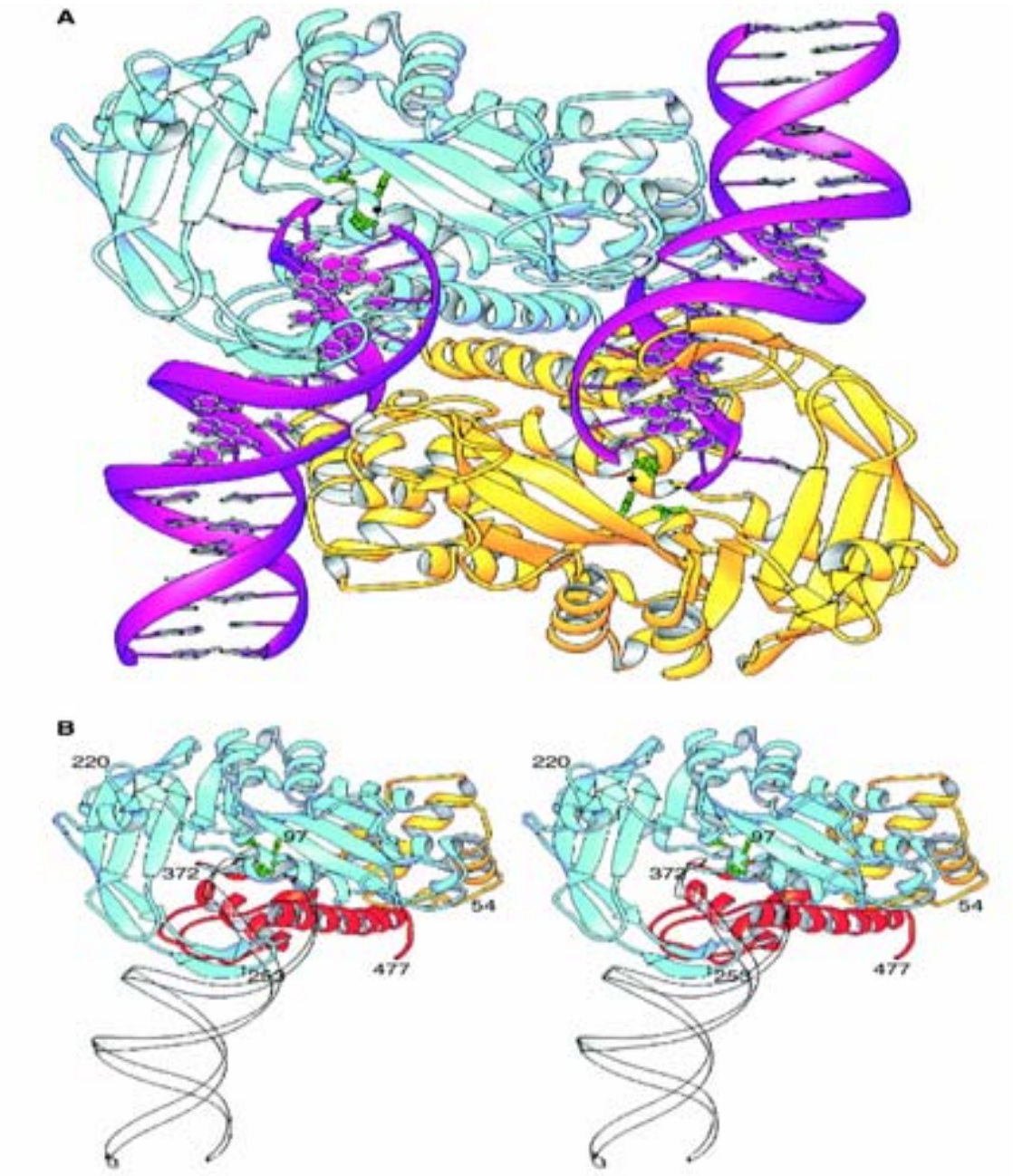


Figure 4.1: The structure of the Tn5 transposase/DNA complex. (**A**) Ribbon representation of the transposase/DNA dimer viewed along a crystallographic two-fold axis of symmetry. One protein subunit is colored yellow, the other is blue, and the two 19-bp DNA molecules are purple. The three catalytic residues are represented as green ball-and-stick structures, and the associated Mn^{2+} ion is black. (**B**) Stereoview of one monomer of transposase. The NH_2-terminal domain is yellow, the catalytic domain is blue, and the COOH-terminal domain is red. The active site residues Asp^{97}, Asp^{188}, and Glu^{326} and the associated Mn^{2+} ion are shown as green ball-and-stick structures. The backbone of a double-stranded DNA is represented by transparent ribbons. (from Goryshin et al, 2000).

Therefore, prokaryotic transposon Tn5 is considered as a model system for studying the molecular mechanism of DNA transposition. There are several steps involved in the process of transposition: (a) binding of transposase monomers to the 19 bp end sequences; (b) oligomerization of the end-bound transposase monomers, forming a transposition synaptic complex; (c) blunt end cleavage of the transposition synaptic complex from adjoining DNA, resulting in formation of a released transposition complex or transposome (d) binding to target DNA; and (e) strand transfer of the transposon 3' ends into a staggered target sequence (Goryshin, and Reznikoff, 1998). It is reported that forming of a functional Tn*5* transposome complex is possible by incubating purified transposase with Tn*5* DNA. No Mg^{++} is necessary for the formation of these complexes. Upon addition of target DNA and Mg^{++}, Tn*5* transposomes can undergo efficient transposition *in-vitro*. Moreover Tn*5* transposomes are reported to be stable for over a month when stored at 4°C in the presence of 10% glycerol and for over a year at -20°C or -70°C without loss of activity. For *in-vivo* transposition, 1 µl of transposome complex can be electroporated to the target cells and in the presence of Mg^{++} of the target cells, an efficient transposition of Tn5 elements can be achieved (Goryshin et al., 2000).

Due to the well defined mechanism of these transposon elements, we chose to construct a Tn5 transposon using the transposon construction vector pMOD^{TM}. The pMOD^{TM} transposon construction vector is a high-copy number, pUC-based vector for the preparation of transposons. The vector contains a multiple cloning site (MCS) between the hyperactive 19bp mosaic ends that are specifically recognized by Tn5 transposase. The kanamycin resistance gene *aphA*-3 originated from *Campylobacter coli* (Trieu-Cuot et al., 1985) and was labelled with 40 different variable tags of 20-25bp. All the tagged *aphA*-3 genes were subcloned into the pMOD^{TM} transposon construction vector. Subsequent digestion with *Pvu*II restriction endonuclease released the Tn5 transposons tagged with 40 variables DNA tags.

This transposome-based *in-vivo* transposition approach was then used to generate random mutants of *C. jejuni* strain B2. B2 strain is a clinical isolate and was tested repeatedly in BALB/c mice for infection. In comparison to other strains, this isolate showed maximum numbers of bacterial recovery from the livers of BALB/c mice (up to 10^6cfu). In addition, electroporation efficiency of the B2 strain was tested by with the pUOA18 shuttle vector

which produced 1×10^4 transformants. Due to these important features of B2, we chose this isolate of *C. jejuni* to generate random mutants by *in-vivo* transposition. The electroporation of the transposome complex generated up to 5.8×10^3 transformants indicating that EZ::TNkan® can undergo efficient transposition *in-vivo*. For each tag, we selected 55 transformants and by using 12 tags, 660 random mutants were generated. To confirm randomness of our transposon approach, Southern blot analysis of 18 random mutants was performed. In comparison to the total number of 660 mutants which are assumed as random, the sample size of 18 insertional mutants is relatively small. Nevertheless, the obtained data support the notion that EZ::TNkan® elements can be inserted randomly within their target DNA.

4.4 Isolation of non-motile mutants of *C. jejuni*

In some pathogenic bacterial species, flagella play a crucial role in the establishment of infection. The biogenesis of a flagellum is carefully regulated and depends on the timed gene expression and synthesis of several flagellum components. In case of *C. jejuni,* the importance of motility as a virulence factor is best demonstrated by true isogenic non-flagellated mutants. These mutants are unable to colonize the intestine of experimental animals (Guerry et al., 1992).

In order to test the feasibility of our transposition model, we screened 660 random mutants for their flagellar motility and identified three non-motile mutants. Sequence analysis of the DNA of the non-motile mutants revealed two insertions in the following genes; *cj0793* (signal transduction histidine kinase), and *cj0955c* (probable phosphoribosylformylglycinamidine synthase subunit II). Transmission electron microscopic analysis showed normal flagella in the mutant having an insertion in the *cj0793* gene. An insertion in the *Cj 0955c* gene resulted in an aflagellated mutant.

In a previous report, the regulation of the *C. jejuni fla* regulon was reported which showed that the FlgS/FlgR two-component signal transduction system is essential for the biosynthesis of flagella (Wösten et al., 2004). In this study, non-motile mutant showed an insertion in the gene *cj0793* better known as FlgS, which acts as a sensor kinase (Wösten et

al., 2004). Due to an unidentified signal, the sensor kinase FlgS autophosphorylates and subsequently transfers its phosphate to its cognate response regulator FlgR. Phosphorylated FlgR and the sigma factor RpoN in turn trigger the expression of the genes needed for the assembly of the hook-basal body filament structure. This process is described as phase growth-dependent and very energy consuming. The autokinase activity, typical for two-component sensor kinase proteins, was confirmed by observing rapid phosphorylation of FlgS in the presence of ATP. Using a recombinant FlgS sensor protein, it was shown that FlgS was capable to maintain its phosphorylated status for several hours. However, phosphorylation of FlgR showed much less stability which appeared to be a characteristic shared with several other response regulators (Parkinson and Kofoid, 1992). In the absence of the putative signal recognition domain of the FlgS protein, phosphorylated FlgR was destabilized which indicated the dual function of complete FlgS sensor protein (i) to transfer its phosphate to FlgR and (ii) to stabilize the phosphorylated protein. FlgR is a member of the NtrC family of proteins and these proteins bind to enhancer-like sequences (>100 bp) upstream of sigma^{54}-dependent promoters to activate the transcription of these promoters (Kustu et al., 1991).

The signals that initiate the FlgS/FlgR two-component system to turn on the *fla* regulon are not known. *C. jejuni* showed less tolerance to the environmental stress as is present in the upper gastrointestinal tract than other foodborne pathogens and it cannot survive at a pH lower than 4.9, and is sensitive to osmotic stress (Park, 2002). Thus, suboptimal environmental conditions may act as a signal. Thus far, activation of the early transcriptional flagellar genes has only been extensively studied for species that carry the master operon *flhDC*. In *E. coli* and *Salmonella,* a large number of global regulatory proteins such as cAMP-CRP, DnaK, DnaJ, GrpE, OmpR H-NS, and adenylate cyclase have been implicated in the activation of these genes, indicating a very complex regulated system (Chilcott, and Hughes, 2000). In species, in which some parts of the flagellar biosynthesis machinery are under the control of a two-component system and sigma^{54}, like in *V. cholera, C. crescentus*, and *H. pylori*, the molecules that activate the sensors of these systems have not yet been identified (Wösten et al., 2004). In short, the role of the FlgS/FlgR two component systems in flagellar motility of *C. jejuni* is not fully elucidated. In our study,

insertion of EZ::TNkan® transposon in the *cj0793* gene resulted in a non-motile mutant of *C. jejuni* and our findings support the notion that the FlgS/FlgR two component systems has a role in flagellar motility. The other non-motile mutants had an insertion in the gene *cj0955c* (probable phosphoribosylformylglycinamidine synthase subunit II). The respective protein has not previously been associated with motility of this organism and the function of this proetin in flagellar biosynthesis is unclear. For the complete understanding of the role of these genes in the motility of *C. jejuni,* further analysis would be necessary.
Nevertheless, our results show that our transposon system can in principle be successfully used for the *in-vivo* transposition to generate random mutants of *C. jejuni* and to knock-out genes relevant to the infection process.

4.5 Isolation of an osmo-sensitive mutant of *C. jejuni*

In our second genetic screen, the mutant ΔGltS showed reduced growth in the presence of 1.5% (wt/vol) NaCl when compared with wild-type B2 strain of *C. jejuni*. DNA sequence analysis showed of EZ::TNkan® insertion in the gene *cj0009* (gltD) encoding NADPH-dependent glutamate synthase small subunit). The NADPH-dependent GltS (NADPH-GltS) is mostly found in bacteria and the enzyme is composed of two tightly bound dissimilar subunits, which form the *ab* holoenzyme containing one flavin adenine dinucleotide (FAD), one FMN cofactor, and three distinct iron-sulfur clusters: one [3Fe-4S]0,+1 center and two low potential [4Fe-4S]+1,+2 clusters. The larger (*a*) subunit (160 kDa) is known to catalyze the reductive synthesis of L-glutamate from L-glutamine and 2-oxoglutarate (Vanoni et al., 1998). The smaller (*b*) subunit (52 kDa) is a FAD-dependent NADPH oxidoreductase (Vanoni et al., 1996). Most of the information on this class of enzymes derives from work on the *Azospirillum brasilense* enzyme (Binda et al., 2000). Accumulation of glutamate in cells grown under osmotic stress is reported (Csonka, 1989, Csonka and Hanson, 1991). The response of bacteria to osmotic stress involves not only depression of functions to facilitate the adaptation of the cell to the environmental change but also influences the activity of cellular enzymes and regulatory proteins. In response to osmotic stress, the amount of unbound water decreases and the concentration of metabolic solutes increases (Cayley et al., 1992; Cayley et al., 1992b). The absolute amount of K^+ increases in nearly all cells and normally, the activity of intracellular water is altered by

these changes which affects the activity of many enzymes and regulatory proteins. Many bacteria accumulate organic compounds, including glutamate, in response to osmotic stress. Glutamate acts as an osmolyte to increase the intracellular concentration of solutes to reduce loss of water by osmosis (Imfoff et al., 1986). Glutamate also acts as a counter ion for K^+ that accumulates. It has been shown that K^+ glutamate stimulates protein-DNA interactions. Therefore, the accumulation of glutamate could be involved in regulation of the expression of many genes (Leirmo et al., 1987).

4.6 *In-vivo* screening model

The identification of putative virulence factors of *C. jejuni* has been been hampered by the lack of suitable genetic tools and subsequent *in-vivo* mutagensis system. Recently, several groups have developed methods for *in-vivo* and *in-vitro* transposon mutagenesis. Although being one of the most powerful tools used for *in-vivo* identification of virulence genes, the efficiency relies on several parameters, such as complexity of the infection dose, efficiency of hybridization, and variability in animal sensitivity to an infectious agent (Karlyshev et al., 2001)**.**

In humans, *C. jejuni* is able to colonize the small and large intestines thereby causing inflammatory diarrhea with fever. Non-specific defense mechanisms such as gastric acidity and intestinal transit time are considered as important factors However, these mechanisms are not sufficient to prevent colonization and subsequent inflammation. It has been reported that *C. jejuni* is able to invade different host cells and can persist and multiply within intestinal epithelial cells and macrophages *in-vitro*. This prolonged intra-cellular survival might be one of the important reasons for the development of bacteremia during *C. jejuni* infection (Gerritsen and Veringa, 1993).

An experimental model of murine campylobacteriosis has been previously reported and proven to be a useful *in-vivo* model to demonstrate bacterial dissemination and tissue invasion. Different strains of mice were tested for the establishment of an *in-vivo* model and BALB/c mice were the most sensitive and showed the most pronounced pathohistological changes in the examined organs. It has been clearly shown that intraperitoneal infection of BALB/c mice leads to systemic *C. jejuni* spread and

colonization of internal organs (Vucˇkovic´ et al., 1998). In this model, primary infection of the liver was found to be the most intense. We therefore, tried to optimize the BALB/c mice model for the screening of random transposon mutants of *C. jejuni.* First, four different wild type strains of *C. jejuni* (NCTC11168, NCTC11828, 81-176, B2) were with infection doses of 1×10^8, 1×10^9, 1×10^{10} cfu, respectively. For determining the recovery rates of bacteria, all mice were sacrificed seven days after infection. In our experiments, all the strains tested showed only low recovery of bacteria from the livers of BALB/c mice (10-100 cfu), when infected with 1×10^8 cfu of viable *C. jejuni* bacteria.

Using an infection dose of up to 1×10^9cfu increased the numbers of bacteria that were recovered from the livers up to 100 fold. However, strains NCTC11168 and 81-176 showed consistent lower recovery rates of bacteria when compared to NCTC11828 and B2. Hepatosplenomegaly and inflammation were consistently found and abcess-like nodes were usually observed on the surface of the livers spleens were abnormally enlarged and inflammed. Histopathology of the tissues showed an inflammatory response with a massive cell infiltrate consisting primarily of neutrophils and rarely lymphocytes. Previously, a similar histological finding was made in infected BALB/c mice (Vucˇkovic´ et al., 1998). However, we are not aware of any previous report suggesting the use of BALB/c mice as a screening model for randomly knocked-out mutants of *C. jejuni*.

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Appendix

ACCESSION M26832 aphA gene

```
   1 gataaaccca gcgaaccatt tgaggtgata ggtaagatta taccgaggta tgaaaacgag
  61 aattggacct ttacagaatt actctatgaa gcgccatatt taaaaagcta ccaagacgaa
 121 gaggatgaag aggatgagga ggcagattgc cttgaatata ttgacaatac tgataagata
 181 atatataata tatctttact accaagacga taaatgcgtc ggaaaagtta aactgcgaaa
 241 aaattggaac cggtacgctt atatagaaga tatcgccgta tgtaaggatt tcaggggca
 301 aggcataggc agcgcgctta tcaatatatc tatagaatgg gcaaagcata aaaacttgca
 361 tggactaatg cttgaaaccc aggacaataa ccttatagct tgtaaattct atcataattg
 421 tggtttcaaa atcggctccg tcgatactat gttatacgcc aactttgaaa acaactttga
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 961 aggctctttc actccatcga catatcggat tgtccctata cgaatagctt agacagccgc
1021 ttagccgaat tggattactt actgaataac gatctggccg atgtggattg cgaaaactgg
1081 gaagaagaca ctccatttaa agatccgcgc gagctgtatg atttttaaa gacggaaaag
1141 cccgaagagg aacttgtctt ttcccacggc gacctgggag acagcaacat ctttgtgaaa
1201 gatggcaaag taagtggctt tattgatctt gggagaagcg gcagggcgga caagtggtat
1261 gacattgcct tctgcgtccg gtcgatcagg gaggatatcg gggaagaaca gtatgtcgag
1321 ctatttttg acttactggg gatcaagcct gattgggaga aaataaaata ttatatttta
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ACCESSION AY190525 (*tet*O) gene,

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   1 atgaaaataa ttaacttagg cattctggct cacgttgacg caggaaagac aacattaacg
  61 gaaagtttat tgtataccag tggtgcaatt gcagaactag ggagcgtaga tgaaggcaca
 121 acaaggacag atacaatgaa tttggagcgt caaaggggaa tcactatcca gacagcagtg
 181 acatcttttc agtgggagga tgtaaaagtc aacattatag atacgccagg ccatatggat
 241 tttttggcgg aagtataccg ttctttatcc gcattagacg gagcagtatt attagtttct
 301 gcaaaggatg gcatacaggc acagacccgt atactgtttc atgcactaca gataatgaag
 361 attccgacaa ttttttttcat caataaaatt gaccaagagg ggattgattt gccaatggta
 421 tatcgggaaa tgaaagcaaa gctttcttcg gaaattatag tgaagcaaaa ggttgggcag
 481 catccccata taaatgtaac ggacaatgac gatatggaac agtgggatgc ggtaattatg
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 541 ggaaacgatg aactattaga gaaatatatg tcagggaaac cgtttaaaat gtcagaactg
 601 gaacaggaag aaaacaggag attccaaaac ggaacgttat ttcccgttta tcacggaagc
 661 gctaaaaaca atctggggat tcggcagctt atagaagtaa ttgccagtaa attttattca
 721 tcaacgcctg aaggtcaatc tgaactatgc gggcgggttt ttaagattga atattcaaag
 781 aaaaggcggc gttttgttta tgtgcgtata tatagcggaa cattgcattt gagggatgtt
 841 attagaatat ctgaaaaaga gaaaataaaa atcacagaga tgtgtgttcc gacaaacggt
 901 gaattatatt catccgatac agcctgctct ggtgatattg taattttacc aaatgatgtt
 961 ttgcagctaa acagtatttt ggggaacgaa atactgttgc cgcagagaaa atttattgaa
1021 aatcctctcc ctatgctcca aacaacgatt gcagtaaaga aatctgaaca gcgggaaata
1081 ttgcttgggg cacttacaga aatttcagat ggcgaccctc ttttaaaata ttatgtggat
1141 actacaacgc atgagattat actttctttt ttggggaatg tgcagatgga agtcatttgt
1201 gccatccttg aggaaaaata tcatgtggag gcagaaataa aagagcctac tgttatatat
1261 atggaaagac cgcttagaaa agcagaatat accatccaca tagaagtccc gccaaatcct
1321 ttctgggctt ctgtcgggtt gtccatagag ccgctcccta ttggaagcgg agtgcagtat
1381 gaaagcagag tttcacttgg atatttaaat caatcgttcc aaaatgcggt tatggagggg
1441 gttctttatg gctgcgagca ggggctgtat ggatggaaag tgacagactg taaaatctgt
1501 tttgaatatg gattgtatta tagtcctgta agtacccccg cagactttcg gctgctttcc
1561 cctatcgtat tggagcaggc tttaaaaaaa gcagggacag aactattaga gccatatctc
1621 cactttgaaa tttatgcacc gcaggaatat ctctcacggg cgtatcatga tgctccaagg
1681 tattgtgcag atattgtaag tactcagata aagaatgacg aggtcattct gaaaggagaa
1741 atccctgcta gatgtattca agaatacagg aacgatttaa cttgtttcac aaatgggcag
1801 ggagtctgct tgacagagtt aaaaggatac cagccagcta ttggtaaatt tatttgccaa
1861 ccccgccgcc cgaatagccg tatagataag gttcggcata tgttccacaa gttagcttaa
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ACCESSION AASY01000012 REGION: 48614...49633

Signal transduction histidine kinase Cj0793

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  61 ttagaaagtc ttatagaaca aacttatgta atagaaaatg aatacaaaac tttaaatgaa
 121 aactataatt ccttacgcgc aatggttgat gagattatag aggtattgcc tagtgcgctt
 181 tggattttag ataaagaaaa aaatattatt ttacaaaatc aagaagcctt gaaaaatcca
 241 aaattactta gtatcattag tcttgataaa attcgcgatg agcttgaatt tgaaggtagg
 301 ttttatgcgg taaaaatcat agcacataat gaaaaaacta tagtttcagc tacagatatt
 361 agcgatgaaa aacgcaatga aaggcttgca agcatgggta gtgtagcggc tcatttggca
 421 catgagataa gaaatcctat tggatctata tctttgcttg cttcaaccct ttttgctcgt
 481 agtgagctta aaaataagca tatagttctt gaaatccaaa aggctattgc tagagtagag
 541 cgtatcgtaa attcaacttt acttttact aagggtgtgc acatcaatgc tttaaatttt
 601 aatcttttag agttaaaaga agagtgtgaa agtgccatta actcttataa tttcacttct
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661 caaatagact ttgaaatatc ttttttggat aagcagattt gtgctgataa agctcttctt
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781 gaaaagccta tgattaaaat tttagcaagt tgtgataatg aaaaaatatg catcagagtg
841 tatgataatg gttgtgaaat aaaagatgag aaattggttt ttgaagcttt taaaacaaca
901 aaacttaaag gcaatggact tggtttgtct ctttctaaag aaatcataaa cgctcataag
961 ggagagttaa gttttcaaag tgatcctaag aatttttatt ttaccttacc tttggtgtga
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ACCESSION AASY01000003 REGION: 152963...153616

phosphoribosylformylglycinamidine synthase Cj0955c purL.

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  61 ggaaaacagg atttttaaa ctcagctaca agaggagcca aaggctttgc taagggtttt
 121 gctcgtgggg ttatggaaga aagaatggat gagtttaaaa ggcgtatgaa ttactatgtt
 181 atcgcacttt tggcaaaaat tgcaaaaagt gatggtaggg taagtgaaaa tgaagctgaa
 241 atgatcaaag atcttttaga tgcaaatgcc aaagatgaaa aagaaagagc ttttttaaaa
 301 gcaagtttta atgaacataa agaaaattta agtgatgctt tttatgtggc aaaagatttt
 361 ttaaaagaag tgcctttgcc taaaaatgag cgttttaatg tcttgcgtgt gcttgttttt
 421 atggctttaa tcgatgcaga ttttaatgct aaaaagcgtg aaattttaga gcaaatcgct
 481 aaagcctttg atatggcaaa aagcgaatta gacgctttta tagcaagtct ttcaaattta
 541 aaaagtacta aaaaagaatt aagccttgat gaagcttttg ctattttaga actttcaaat
 601 aatgcagatt taaatgcagt aaaaaaacaa taccgcaatt tagcaaaaaa ataa
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pBluescript II KS(+), 2961 bp

The following sequence has been verified for accuracy at the junctions.

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   1 ctaaattgta agcgttaata ttttgttaaa attcgcgtta aatttttgtt aaatcagctc
  61 atttttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga
 121 gatagggttg agtgttgttc cagtttggaa caagagtcca ctattaaaga acgtggactc
 181 caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc
 241 ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaagggag
 301 cccccgattt agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aagggaagaa
 361 agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac
 421 cacacccgcc gcgcttaatg cgccgctaca gggcgcgtcc cattcgccat tcaggctgcg
 481 caactgttgg gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg
 541 gggatgtgct gcaaggcgat taagttgggt aacgccaggg ttttcccagt cacgacgttg
 601 taaaacgacg gccagtgagc gcgcgtaata cgactcacta tagggcgaat tggagctcca
 661 ccgcggtggc ggccgctcta gaactagtgg atcccccggg ctgcaggaat tcgatatcaa
 721 gcttatcgat accgtcgacc tcgaggggg gcccggtacc cagcttttgt tccctttagt
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 781 gagggttaat tgcgcgcttg gcgtaatcat ggtcatagct gtttcctgtg tgaaattgtt
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 901 cctaatgagt gagctaactc acattaattg cgttgcgctc actgcccgct ttccagtcgg
 961 gaaacctgtc gtgccagctg cattaatgaa tcggccaacg cgcggggaga ggcggtttgc
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1441 aggtcgttcg ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg
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1981 gcttaatcag tgaggcacct atctcagcga tctgtctatt tcgttcatcc atagttgcct
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2281 ccattgctac aggcatcgtg gtgtcacgct cgtcgtttgg tatggcttca ttcagctccg
2341 gttcccaacg atcaaggcga gttacatgat cccccatgtt gtgcaaaaaa gcggttagct
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2461 tggcagcact gcataattct cttactgtca tgccatccgt aagatgcttt tctgtgactg
2521 gtgagtactc aaccaagtca ttctgagaat agtgtatgcg gcgaccgagt tgctcttgcc
2581 cggcgtcaat acgggataat accgcgccac atagcagaac tttaaaagtg ctcatcattg
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2881 tcatgagcgg atacatattt gaatgtattt agaaaaataa acaaataggg gttccgcgca
2941 catttccccg aaaagtgcca c
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Curriculum Vitae

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Research Interests

- Microbial pathogenesis
- Host-Pathogen interaction
- Virulence factors of *Campylobacter jejuni*

Dissertation title:

- Identification and characterization of *Campylobacter jejuni* factors relevant for the infection process.

Awards

- "PhD Research Scholarship" awarded from German Research Council (DFG).
- "M.Phil Research Scholarship" awarded from Centre of Excellence in Molecular Biology University of the Punjab Lahore Pakistan.

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Janurary 1997 - December 2001	BSc (Hon), Plant Breeding and Genetics, NWFP Agricultural University of Peshawar, Pakistan.