Identification and characterization of virulence associated factors of *C. jejuni*

Dissertation

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2010

To my family, especially my wife

To my teachers

With the exception of references to the work of other scientists, I hereby declare that this thesis entitled "Identification and characterization of virulence associated factors of *C. jejuni*" all the work described in this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor submitted, neither in whole or in part, elsewhere to any other institution for degree, diploma other qualification.

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Göttingen, 20.09.2010 **Abdul Malik**

List of Publications

Tareen A.M., Dasti J.I., Zautner A.E., Groß U. and Lugert R. (2010). *Campylobacter jejuni* proteins Cj0952c and Cj0951c affect the chemotactical behavior towords formic acid and are important for the invasion of the host cells. *Microbiology.* [Epub ahead of print]

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List of Abbreviations

AMAN	Acute motor axonal neuropathy
AMSAN	Acute motor axonal and sensory neuropathy
ATP	Adenosine tri-phosphate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
^0C	Centigrade
Caco2	Human colon carcinogenic
$CaCl_2$	Calcium chloride
Cam^r	Chloramphenicol resistance
cDNA	Complementary DNA
CDT	Cytolethal distending toxin
CHO	Chinese hamster ovary
cfu	Colony forming unit
cm	Centimeter
Cp	Crossing point
ddH_2O	Double distilled water
DMEM	Dulbecco minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyrinucleotide triphosphate
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
Fig.	Figure
g	Force of gravity
GBS	Guillain-Barre syndrome

gDNA	Genomic DNA
GIT	Gastrointestinal tract
HCl	Hydrogen chloride
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
Hrs	Hours
kan^R	Kanamycin resistance
kb	Kilo base pair
KCl	Potassium chloride
kDa	Kilo Dalton
kV	Kilo volt
LB	Luria broth
M	Molar
Mbp	Mega base pair
MCPs	Methyl-accepting chemotaxis proteins
MH	Mueller Hinton
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimole
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese chloride
MOI	Multiplicity of infection
µg	Microgram e
µl	Microliter
mRNA	Messenger ribonucleic acid
NAD	Nicotineamide dinucleotide
$NaCl_2$	Sodium chloride
NADPH	Reduced nicotinamide adenine dinucletide and phosphate
NBT	Nitroblue tetrazolium chloride

NEAA	Non essential amino acid
ND	NanoDrop
Ni-NTA	Nickel-nitrilotriacetic acid
ng	Nanogram
OD	Optical density
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phosphate buffered saline
pmol	Pico mole
PCR	Polymerase chain reaction
$RbCl_2$	Rubidium Chloride
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-Polymerase chain reaction
s	Seconds
SDS	Sodiumdodecylsulfate
SOR	Sulphite:cytochrome c oxidoreductase
spp.	Species
TEMED	N,N,N,N-Tetramethyl-Ethylenediamine
TFB	Transformation buffer
Tlp	Transducer-like protein
Trp	Tryptophan
U	Unit
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume

SUMMARY

Campylobacter jejuni, an important food-borne human bacterial pathogen in industrialized countries and in the developing world, the leading cause of bacterial diarrhoea. The infection may be accompanied by fever and abdominal cramps and, as a post-infection complication, in rare cases Guillain-Barré syndrome might emerge. In contrast, *C. jejuni* is only a commensal in poultry, where the pathogen predominantly resides in the cecum which contains many anaerobic fermentative bacteria. Within these natural habitats, *C. jejuni* is able to metabolize products like free amino and keto acids that have been generated by these bacterial species or from the host himself.

In order to identify genes which are related to the invasion of host cells by the pathogen, 660 clones of a transposon mutant library generated in the clinical *C. jejuni* isolate B2 were screened. Thereby, a clone with a transposon insertion in gene *cj0952c* was identified. As in the sequenced *C. jejuni* strain NCTC 11168, the respective protein together with the gene product of adjacent gene *cj0951c* consists of two transmembrane domains, a HAMP domain followed by C-terminal putative MCP domain together believed to act as a chemoreceptor, designated as Tlp7. This thesis describes that genes *cj0952c* and *cj0951c* are important for the invasion of host cells by the pathogen. In addition, it is clearly shown that both genes are not translated as one protein in *C. jejuni* isolate B2 contradicting the idea of a postulated read-through mechanism. Moreover, these genes are responsible for reduced motility of *C. jejuni* and alter the chemotactical behavior of the pathogen towards formic acid. Finally, growth curve analysis indicates that these genes are not related to the utilization of formic acid by formate dehydrogenase.

A second gene investigated in this thesis with regard to reduced infectivity of *C. jejuni* isolate B2 is *cj0005c,* which encodes a molybdopterin oxidoreductase. Together with the monohaem cytochrome c oxidoreductase subunit encoded by *cj0004c*, both subunits constitute a sulphite: cytochrome c oxidoreductase (SOR).

Since *C. jejuni* lacks the glycolytic enzyme phosphofructokinase, glucose cannot be catabolized and other products serve as electron donors, e.g. succinate, malate, formate, D-lactate, hydrogen and NAD(P)H. The possession of a sulphite oxidation system is believed to be important for *C. jejuni* to survive in food which has been treated with sulphite for preservative reasons, as well as in humans since it has been shown that sulphite is also released by neutrophils as part of the host defense. In this thesis SOR is documented to be an important virulence factor of *C. jejuni* by reducing the motility of the pathogen and by the downregulation of *C. jejuni* genes known to belong to the virulence repertoire of the pathogen.

1 INTRODUCTION

1.1 *Campylobacter jejuni*

The genus *Campylobacter* belongs to the epsilon proteobacteria class, order *Campylobacterales* and to the family *Campylobacteriaceae*, which consists of 16 species where *Campylobacter jejuni* is known as the most common causative agent of gastroenteritis. *C. jejuni* is Gram negative, curved or rod shaped with a size of 0.2 to 0.8 µm and a single flagellum at one or both ends. An electron microscopy image of *C. jejuni* cells is shown in Fig 1.1.

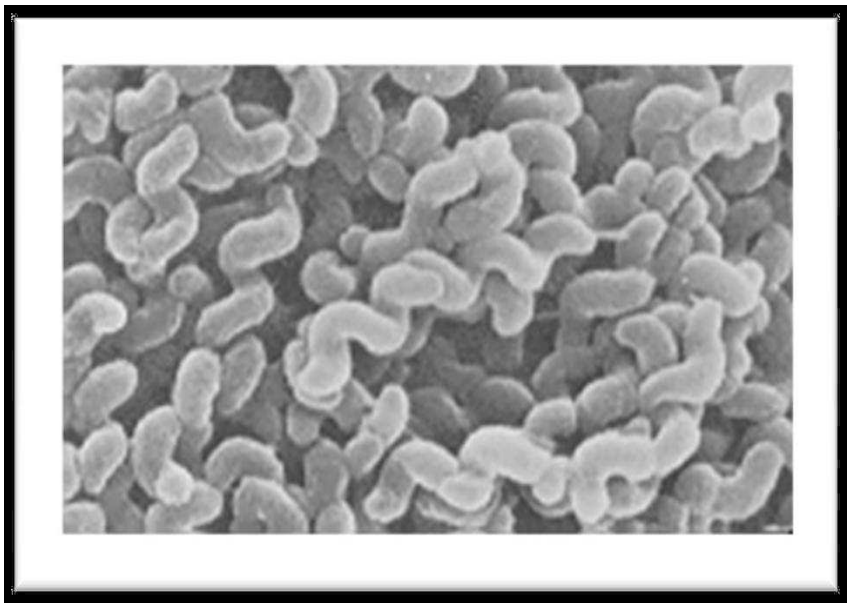

Figure 1.1 Electron microscopy image of *C. jejuni* cells

The figure reflects the typical spiral shape of *C. jejuni* bacteria (Courtesy: Prof. D. E. Taylor, University of Alberta, Canada and Adopted with permission from Lai-King N.G. et al., 1985; *J. of Bacteriology* 338-343)

The genome of *C. jejuni* has a size of approximately 1.6-1.7 Mbp and an AT ratio of about 70 % (Parkhill et al., 2000). *C. jejuni* needs a special microaerophilic environment at 37-42 ^{0}C for its growth. Poultry is the most prominent reservoir for

Campylobacter with microaerophilic conditions and a body temperature between 41 and 44 ^{0}C. There are numerous transmission routes for *Campylobacter* spp. to infect humans. Most infections usually account through the consumption of undercooked contaminated poultry and, to a lesser extent, through the ingestion of contaminated meat from cattle, sheep, pigs, goats or seafood (Yamazaki et al., 2009; Whyte et al., 2004). The ecological cycle of *C. jejuni* is shown in Fig 1.2.

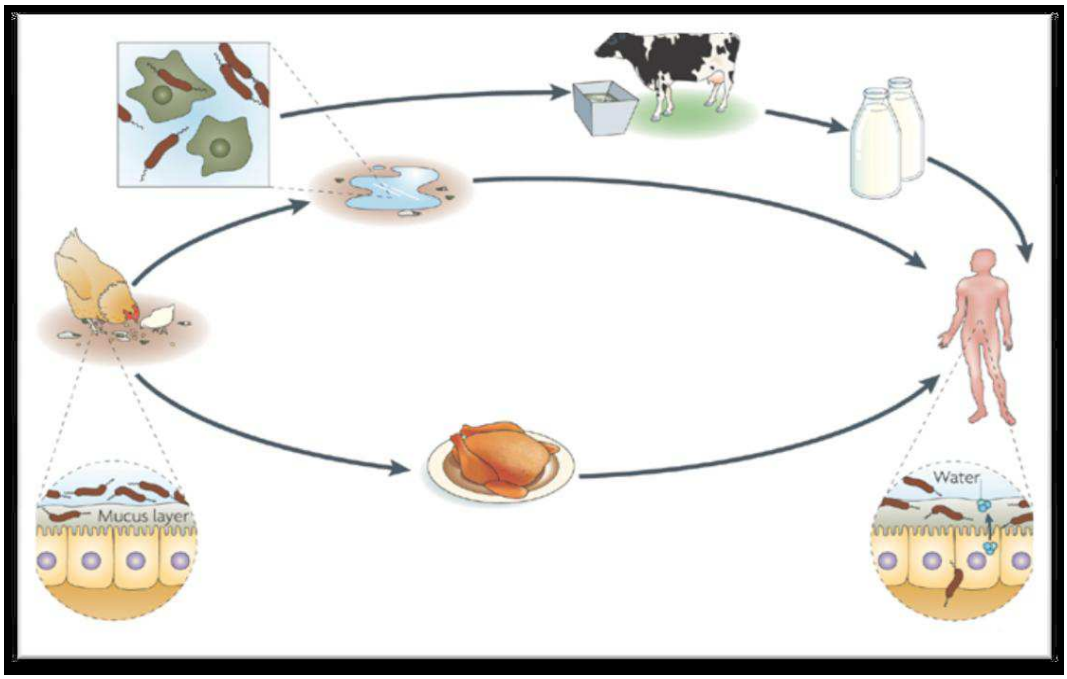

Figure 1.2 The diagram of *C. jejuni* ecological cycle

Ecological cycle shows that livestock, wild birds and chicken constitute the major reservoirs of *C. jejuni*. It colonizes the gastrointestinal mucosa of chicken; from there it is transferred between chicks within a flock through the faecal–oral route or enters the water supply. Humans are directly infected by *C. jejuni* through the consumption of contaminated animal products such as meat, particularly poultry or drinking of contaminated water or unpasteurized milk. In humans, *C. jejuni* invades the intestinal epithelial layer, which can result in inflammatory diarrhoea [Adopted with permission from Young et al*.,* 2007; *Nat Rev Microbiol* **5**:665-679).

1.1.1 *C. jejuni* caused diseases

Campylobacter jejuni is one of the most common causes of human diarrheal diseases. The incubation period can range from one to eight days with a typical time frame of three to four days (Skirrow et al., 1995). Clinical manifestation can range from watery to bloody diarrhoea often associated with acute abdominal cramps and fever. The peak of illness usually lasts for 24–48 hrs before it gradually resolves over a week, but convalescence may also be prolonged. The incidences of campylobacteriosis are increasing worldwide. Meanwhile, the number of cases often exceeding those of salmonellosis and shigellosis (Altekruse, 1999; Bryan and Doyle, 1995) with incidence rates in the United States of estimated 2.5 million cases and in Germany of more than 60,000 cases per year (Friedman et al., 2000). Age also determines the pattern of infection, the incidence under an age of four years is significantly higher than it is observed in the 19-29 year old patient group. There are peak incidences during the summer months indicating maximum replication rate of *C. jejuni* at higher temperatures (Asrat et al., 1999; Banmali et al., 2006).

The infection becomes subclinical, when patients frequently exposed to *Campylobacter* evolve immunity. This is most observed in children in developing countries, which are repeatedly infected, but also in certain western countries among people that are working in poultry abattoirs. In certain diseases e.g. diabetes, HIV infection, cancer in which the immune system is impaired, the risk of *Campylobacter* infection increases. In HIV positive patients, e.g. the risk of disease is 40 times higher than compared to normal patients (Sorvillo at al., 1990).

Like other enteropathogenic bacteria, *C. jejuni* can cause post-infectious manifestations like urticaria, erythemanodosum and reactive arthritis. In addition, *C. jejuni* has been recently described to be associated with immunoproliferative small intestinal diseases (Lecuit, 2004). The Guillain-Barré syndrome (GBS) is considered to be the most important post-infectious manifestation with reported

incidences of 1.2 to 1.9 per 100,000 in Europe alone (Rees, 1998; Govoni, 2001). Increasing rates of GBS are reported with advancing age of 75 years of about four per 100,000 compared to less than one per 100,000 in patients younger than 18 to 30 years. The most common subtypes are acute motor axonal neuropathy (AMAN), acute inflammatory demyelinating polyradiculoneuropathy (AIDP) and when sensory fibers are affected, acute motor and sensory axonal neuropathy (AMSAN) (Asbury et al., 1969; McKhann et al., 1991; Griffin, 1996). The main symptoms of GBS are rapidly progressing bilateral and relative symmetrical weakness of the limbs and impairment of the respiratory muscles. Frequently, pain occurs from mild to severe (Asbury and Cornblath, 1990).

A reduction of the infection rate of *C. jejuni* in humans will be related to the knowledge of biological aspects and, in particular, to the knowledge of virulence factors, which are directly responsible to the pathogenesis of the disease. However, less is known about the pathogenesis of the disease at the molecular level. Meanwhile, genome sequences of different *Campylobacter* strains are available, which open new directions in *Campylobacter* research (Parkhill et al., 2000).

1.2 General features of virulence associated factors of *C. jajuni*

1.2.1 Flagellum

Campylobacter jejuni possesses a polar flagellum which mediates motility that, in turn, plays a very vital role for host colonization and host-cell invasion (Yao et al., 1994; Ormonde et al., 2000). Early studies indicated that aflagellated *C. jejuni* mutants illustrate clearly reduced internalization into the host cells *in-vitro* (Yao et al., 1994; Wassenaar and Blaser, 1999). The flagellum of *Campylobacter jejuni* is composed of O-linked glycosylated flagellin. It was observed that for the expression of a functional flagellum different strains of *C. jejuni* are possessing different numbers of genes, e.g. 50 (*cj1293 to cj1342*) adjacent to *flaA* and *flaB*, in case of *C. jejuni* strain NCTC 11168 (Parkhill, 2000). In contrast, in *C. jejuni* strain

81-176, the flagellin O-linked glycosylation locus comprises only 26 genes (Guerry, 2006; Goon et al., 2003). Recent studies have suggested a two-component system comprised of the sensor FlgS and the response regulator FlgR to be the major control on flagella expression in *C. jejuni*. The composition of the cell wall and the flagellum of *C. jejuni* is shown in Fig. 1.3.

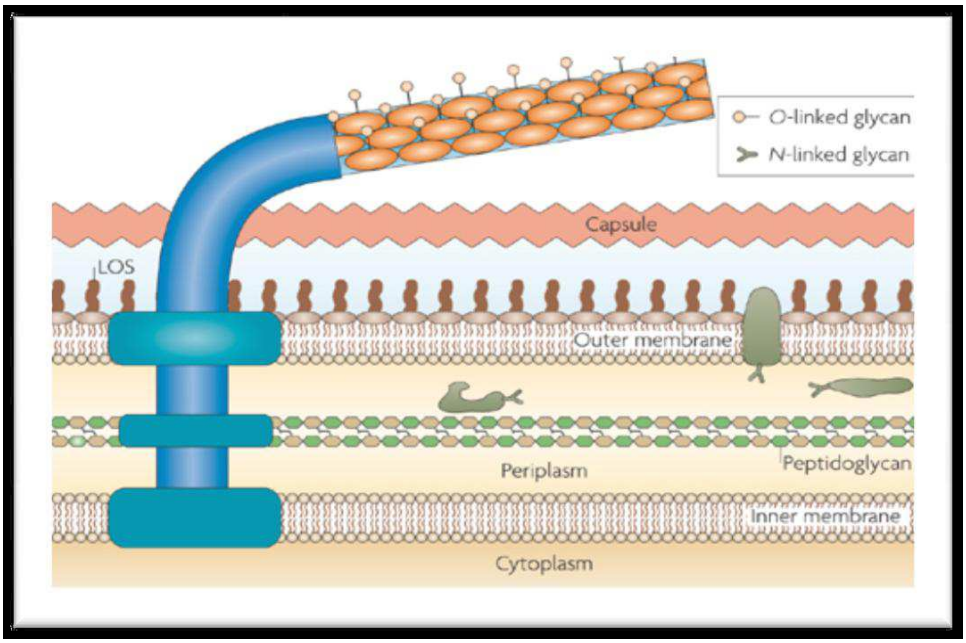

Figure 1.3 A cross-sectional appearance of the *C. jejuni* cell wall

The figure illustrates the flagellum, outer membrane protein and its associated lipooligosaccharides, capsule and the *O*- and *N*-linked glycans [Adopted with permission from Young et al., 2007; *Nat Rev Microbiol* **5**:665-679].

Specialized type-III-secretion systems have been shown to be important for virulence of many Gram-negative enteric pathogens. However, genomic studies have indicated that *C. jejuni* strains lack these systems (Galan and Collmer, 1999). However, there are several reports that the flagellum can function to secrete virulence associated proteins. One of these proteins that were identified along with seven other proteins is CiaB (Konkel et al., 1999). A *ciaB* mutant of *C.*

jejuni strain F38011 has no obvious motility defects, but is unable to secrete any of the Cia proteins. Moreover, the mutant has a 50-fold reduced invasion capacity compared to the parental strain F38011 (Konkel et al., 1999). In contrast, a mutation of *ciaB* in *C. jejuni* strain 81–176 had no significant effect on the invasion of INT407 cells (Goon et al., 2006). These different observations might be due to variable invasion mechanisms among strains, cell lines used or methodologies (Goon et al., 2006). At least, a minimum flagella structure is required for the secretion of Cia proteins. In an *flhB* mutant secretion of the Cia proteins was not detected.

Recent studies have suggested that in both, in a *flaA* mutant and in a *flaB* mutant secretion occurs, but not in a double mutant that lacks all filament structure (Konkel et al., 2004). Recently, secreted proteins FlgP and FlgQ, have been described to be important for flagellar motility in *C. jejuni* but their functional mechanisms remain unclear. Furthermore, they show no similarity with already characterized proteins (Sommerlad and Hendrixson, 2007).

1.2.2 Invasion

The invasion capability of *C. jejuni* is an important virulence factor. Intracellular bacterial survival and replication has been observed extensively using mammalian cell tissue. Also, the results of intestinal biopsies of patients have demonstrated that *C. jejuni* invades the cells of the gut. Thereby, the ability of *C. jejuni* to invade host cell has been shown to be strain depended (Newell et al., 1985). Statistically, significant differences were observed in the ability of host cell invasion of *C. jejuni* strains from individuals with non inflammatory diarrhea versus individuals with colitis (Everest, 1992). In addition, it was observed that some *C. jejuni* strains show a very low level of invasion (Konkel et al., 1992), suggesting the use of different mechanisms for cell entry similar to those observed for different *Chlamydia* serovars (Byrne and Moulder, 1978; Clausen et al., 1997). In order to characterize the interaction of *C. jejuni* with host cells in vitro, invasion assays, largely based upon gentamicin protection, have been used to study extensively

the invasiveness of *C. jejuni* in various cell lines including HeLa, HEp2, INT 407 and Caco-2 cells (Fauche`re et al., 1986; Konkel and Joens, 1989; Wassenaar et al., 1991; Everest et al., 1992; Russell and Blake, 1994). Furthermore, Russell et al., (1993) reported based on an experimental primate model of *Macaca mulatta* - the ability of *C. jejuni* to invade colonic epithelial cells and to cause diarrhea. Moreover, mucosal damage as a result of a *C. jejuni* invasion of colonic epithelial cells has been reported in different animal models.

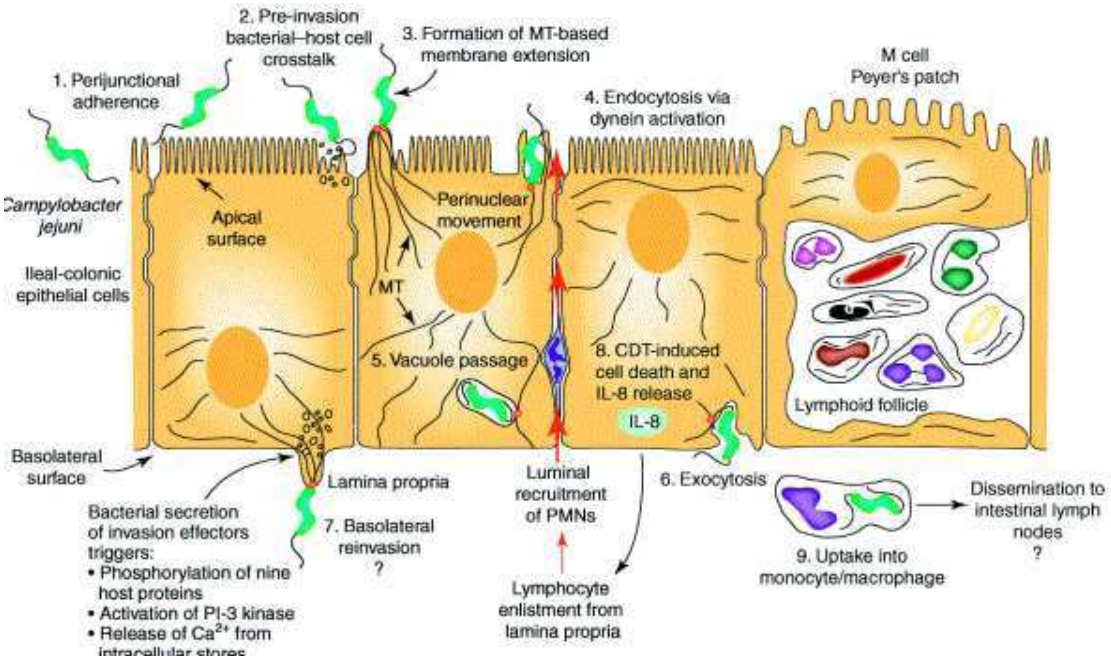

Figure 1.4 Pathogenesis model of *C. jejuni*.

(1) Initially, *C. jejuni* adheres at the apical cell surface at the peri-junctional region. (2) Where it secretes putative invasion effectors into the host cell. (3) Disruption of cortical actin filaments and an extension of microtubules (MTs) are mediated by host signalling cascades to form a membrane protrusion. (4) Endocytosis of the bacterium via membrane invagination. (5) Membrane bound *C. jejuni* vacuole moves to the basolateral surface through dynein along MTs. (6) For exocytosis (7) Again re-enter the epithelium basolaterally. (8) Interleukin (IL)-8 secretions of infected cells, and subsequent proliferation of lymphocytes from the lamina propria. (9) Uptake of *C. jejuni* into macrophages and local dissemination [Adopted with permission from Kopecko, et al. 2001; *TRENDS in Microbiology* **9**:389-396].

1.2.3 Chemotaxis

Chemotaxis is the ability of an organism to move toward or away from a chemical stimulus. Thereby, chemotaxis and cellular motility contribute to the colonization and virulence of pathogenic bacteria (Josenhans and Suerbaum, 2002). It is also reported that chemotactic mediated motility plays an important role in disease progression of virulent organisms, e.g. *Vibrio cholerae* (Lee et al., 2001). Several studies demonstrated the ability of *C. jejuni* to sense its external environment through chemical gradients, indicating that chemotaxis is an important virulence factor which plays a crucial role in the colonization and pathogenicity (Yao et al., 1997). *C. jejuni* was found to exhibit a number of chemoreceptors for the detection of chemoattractants and chemorepellents such as the amino acids L-glutamate, L-aspartate, L-cysteine, and L-serine, the carbohydrate L-fucose, and the organic acids pyruvate, fumarate, succinate, malate, citrate and ketoglutarate (Hugdahl et al., 1988).

The attraction and repulsion of chemicals is sensed by trans-membrane methyl-accepting chemotaxis proteins (MCPs) which transmit the information to the flagellum motor via the histidine kinase CheA and the response regulator CheY. *C. jejuni* strain NCTC 11168 possesses altogether two aerotaxis genes and ten genes for putative chemoreceptor molecules, designated Tlps for transducer-like proteins (Marchant et al., 2002). It has been reported that colonization in chickens and the invasion of human epithelial and chicken embryo cells are severely impaired in *C*. *jejuni* strains with mutations in *tlp1*, *tlp3*, *docB*, and *docC* (Christina et al., 2009). The importance of the chemosensory receptors of *C. jejuni* for virulence has also been described with a mutant lacking Tlp9 (Cj1189c or CetB), which shows invasion deficiency of human tissue culture cells (Golden and Acheson, 2002). Furthermore, Tlp10 (Cj0019) and Tlp4 (Cj0262c) were found to be necessary for wild-type colonization of the chick gastrointestinal tract (Hendrixson and DiRita, 2004). Altogether, although chemotaxis has been demonstrated to be important for *C. jejuni* (Hugdahl et al., 1988; Takata et al., 1992), the mechanistic source of the sensory control of motility is yet to be

elucidated.

1.2.4 Cytolethal distending toxin

Protein toxins are a well defined source of bacterial virulence with specific effects on normal host cell function. Mostly, they act on membranes or interfere with intracellular signaling by binding with specific signaling proteins thereby facilitating the process of infection. Although the genome of *C. jejuni* has been sequenced, yet only a few potential virulence factors produced are known (Parkhill et al., 2000). One of these is the cytolethal distending toxin (CDT), which is produced by a number of *Campylobacter* species, including *C. jejuni, C. lari, C. coli, C. fetus,* and *C. upsaliensis*, (Johnson and Lior, 1988; Mooney et al., 2001). This toxin was observed to induce cell distension in different mammalian cells such as HeLa, Chinese hamster ovary (CHO), Caco2 cells and other, which is characterized by elongation, swelling and eventually cell death (Whitehouse et al., 1998; Shenker et al., 1999). However, it was observed that not all cell types were sensitive to CDT because NIH 3T3 fibroblasts and mouse Y-1 adrenal cells are not affected by toxin treatment (Cortes-Bratti et al., 1999; Johnson and Lior, 1988). CDT is a holotoxin complex, which comprises of three polypeptide subunits encoded by the adjacent linked genes *cdtA*, *cdtB*, and *cdtC* (Smith and Bayles, 2006). The entire complex is required to show a maximal activity (Heywood et al., 2005). It has been documented that CdtA, CdtB, or CdtC have no toxic activity when applied to cells separately, but when these three proteins are combined, they interact together to form an active tripartite holotoxin which produces maximum cell toxicity (Lara-Tejero and Galan, 2000). How CDT causes pathogenesis in *C. jejuni* is still unclear, but the mechanism of action is known. The toxic component CdtB (Lara-Tejero and Galán, 2001) shows activity similar to the enzyme deoxyribonuclease (DNaseI) (Lara-Tejero and Galan, 2000) which causes cell cycle arrest in the G2/M transition phase through blocking of CDC2 kinase, an enzyme known to be involved in mitosis entry (Pickett and Whitehouse, 1999). The entry of CdtB in the nucleus, which depends on an atypical nuclear localization signal, is important for the cytotoxic activity (McSweeney and Dreyfus,

2004). It was observed that CDT acts as nuclease and cuts the double-stranded DNA of host cells (Hassane et al., 2001).

Incubation of HeLa cells with lysates from *cdtB*-deficient *C. jejuni* strains 81-176 and NCTC 11168 showed that the NCTC 11168 lysate was greatly attenuated, while 81-176 lysates retained toxic activity, suggesting CDT to be the principal, but not the only toxin of *C. jejuni* (Purdy et al., 2000). It was also observed that *C. jejuni* mutants lacking CDT are possessing the competence to colonize NF-kB-deficient mice, but are unable to cause gastroenteritis as it was observed for the wild type (Fox et al., 2004). Similar studies were also performed in *Helicobacter hepaticus,* where CDT mutants are showing the same results regarding to the colonization of the mouse gut (Young et al., 2004).

1.2.5 Translocation

Translocation is defined as the ability of bacteria to translocate from the gastrointestinal tract (GIT) to other internal organs (Berg, 1983). The competence to translocate across the cell barrier greatly differs among different pathogens. For instance, *S. Typhi* is able to migrate across a polarized monolayer which leads to cellular destruction and a complete loss of the monolayer integrity, whereas *S. typhimurium* causes significant less damage in the initial stage of the infection process (Kops et al., 1996). In order to infect the host, *C. jejuni* must cross the mechanical and immunological barriers of the GIT. In most organisms, the mucus membrane serves as the first line of defense which is 30 to 50 µm thick and is considered to be the first point of contact between the host cell and *C. jejuni* (Rozee et al., 1982). However, *C. jejuni* utilizes several virulence factors such as motility, adhesion, as well as capsule synthesis to penetrate these barriers (McSweegan and Walker, 1986). In addition, translocation was also observed in a few strains of *C. jejuni* across an intact polarized Caco-2 cell monolayer (Everes et al., 1992**)**. The exact mechanism by which *C. jejuni* translocates across polarized cells is still unclear, however, electron microscopic studies suggested that both, transcellular and paracellular routes exist to cross polarized epithelial

cells (Konkel et al., 1992a). Translocation also occurs by disruption of the tight junction of adjacent cells which was achieved by disturbing the cellular cytoskeleton or by altering the specific proteins of tight junctions which are claudins, occludin, and junctional adhesion molecules (Schneeberger and Lynch, 2004; MacCallum et al., 2005). Recently, it was also observed that *Campylobacter* induces translocation of non-invasive gastrointestinal bacteria (Lisa et al., 2009). The migration of *Campylobacter spp*. to the mesenteric lymph nodes is important for the pathogenesis of enteric infections which was observed in C3H or athymic and euthymic BALB/c germfree mice (Levine et al., 1983; FauchEre et al., 1985; Yrios and Balish, 1986).

Translocation is inhibited by chloramphenicol, which indicates that this process is linked to active bacteria (Konkel et al., 1992a). In addition, it was observed that butyrate, which is an bacterial metabolite found in the lumen of the large intestine protects Caco-2 monolayers from *C. jejuni* infection (Rechkemmer et al., 1988; Von et. al., 2008)

1.3 Response to stress

Campylobacter species lack many adaptive responses as compared to other food-borne pathogens and therefore are more prone to environmental stress. The genomic study of *C. jejuni* might be a sound tool to insight into pathogenicity, growth and survival mechanisms under different stressful conditions (Tauxe et al., 1992). Many studies have been attempted to focus on the involved mechanisms of *C. jejuni* survival at thermal stress i.e. above 42 ^{0}C. Konkel and coworkers (1998) have found that at least twenty four proteins were upregulated when heat shocked was provided to the cell in the temperature range from 43 ^{0}C to 48 ^{0}C. Under normal environmental conditions, some proteins behave as chaperones to ensure proper protein folding and also help to eliminate misfolded proteins (Konkel et al., 1998). Seventeen proteins that are either heat shock or chaperone homologues have been investigated in *C. jejuni* (Stintzi, 2003). Among these heat-shock proteins, some were molecular chaperones including GroELS, DnaJ and

DnaK (Murphy et al., 2006). DnaJ has been further characterized by Konkel et al., (1998). Thereby, it has been established that the growth of DnaJ mutants was drastically reduced when incubated at 46 ^{0}C. Furthermore, they have lost the ability to colonize chicken cells, which documents the fact that heat shock proteins have a key role in both, thermotolerance and colonization. Most prokaryotes possess two-component regulators, which act as a survival mechanism by affecting gene expression in response to an environmental change (Murphy et al., 2006). They are constituted by a cytoplasmic sensory histidine kinase and a response regulator in the cytoplasmic membrane.

There are nine response regulators and six histidine kinases that have been reported in *C. jejuni* (Murphy et al., 2006). A signal transduction system called RacR–RacS was described by Bras and coworkers (1999). To define temperature as the function of the two-component regulator, *C. jejuni* mutants were generated that do not produce RacR. RacR mutant bacterial cells show a growth rate as the normal strain at 37 ^{0}C, but have declined growth at 42 ^{0}C. The study concluded that RacR has a temperature-dependent influence on the growth rate. It has been demonstrated that *C. jejuni* cells in a stationary phase are less prone to heat stress as compared to the exponential-growth phase, suggesting that thermotolerance is not responsible by changes accompanying stationary phase entry (Kelly, 2001). This finding is further supported by the lack of the *rpoS* gene in the genome of *C. jejuni* (Parkhill et al., 2000).

The pH-value is also a determining factor of bacterial growth. The optimum pH for *C. jejuni* growth and survival is in the range between 6.5 and 7.5, with a maximum pH tolerance of about 9.0 (Chaveerach et al., 2003). *C. jejuni* cell numbers are significantly reduced when the pH is in a range above 9.0 or below 4.0 (Chaveerach et al., 2003). The survival and infectivity of *C. jejuni* depends how rapidly it adapts to the acidic environment in the gastrointestinal tract of the host (Reid et al., 2008). However, the mechanism of how *C. jejuni* responds to low pH-values has not been well documented. *C. jejuni* strain CI120 has an adaptive

tolerance response (ATR) for survival in the acidic environment that is induced by acid and oxygen (Reid et al., 2008). Protein synthesis is essential to augment ATR, indicating that pH-stimulating proteins are involved (Reid et al., 2008). However, a particular protein of *C. jejuni* responsible for protection against low pH has yet to be determined. (Reid et al., 2008, Ma et al., 2009).

Campylobacter spp. is less tolerant to a sudden change in solute concentrations (osmotic stress) than other food-borne pathogens (Doyle and Roman, 1982). Birk and coworkers (2004) used chicken juice to study *C. jejuni* survival at different sodium chloride concentrations. They reported that *C. jejuni* strains incubated at 42 ^{0}C were unable to grow in the presence of 2.0% sodium chloride. In addition, *Campylobacter* uses aerotaxis and chemotaxis as a defense mechanism to fight against various environmental responses (Hazeleger et al., 1998).

Objective of the study

Campylobacter jejuni has emerged as an important food-born gastrointestinal pathogen for the last few decades in developing and industrialized countries. Furthermore, post infectious manifestations e.g. reactive arthritis or Guillain-barre syndrome might occur. In Germany approximately sixty thousand cases of *Campylobacter* mediated enteritis are reported annually. Although, a few factors that are essential for our understanding of the pathogenesis have been described, the mechanism of *Campylobacter* infection in humans is still not fully understood.

In order to identify pathogenesis-related genes that have not been described so far, an existing transposon-generated mutant library of *Campylobacter jejuni* should be screened to detect mutants with reduced capacity to invade host cells. Corresponding genes, identified by this method, should furthermore be characterized with regard to their biological function to explain the particular effect of these genes during the procedure of host cell infection. For this, specific assays to answer the question which step in the complicated process of attachment, entry and maintenance of intracellular survival is affected by these new virulence associated factors will be performed. Taken together, this study is expected to give new insights in *Campylobacter* pathogenesis by the identification and characterization of to date unfamiliar infection-related factors.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Instrument Model and Manufacturer	
Agarose gel electrophoresis cells	Keutz Labortechnik, Reiskirchen
Bio safety cabin	BDK and Luft Reinraumtechnik, Gmbh
Balances	BP 221 S, Sartorius, Göttingen
	LP6200 S, Sartorius, Göttingen
Cell culture microscope	Modell DM IL, Leica, Heidelberg
Centrifuges	Megafuge 2.0 RS, Heraeus, Hanau
	Megafuge 2.0, Heraeus, Hanau
	Modell 5417 R, Eppendorf, Hamburg
	Modell 5417 C, Eppendorf, Hamburg
	RC-26 Plus, Sorvall-Kendro, Hanau
Electro Cell Manipulator	BTX ECM600, San Deigo, California
Incubators	Heraeus, Hanau
Gel documentation system	BioDoc II, Biometra, Göttingen
Gel electrophoresis cells for PAGE Modell	Mini Protean II, BioRad, München
Lightcycler 1.5	Roche, Basel, Schweiz
Magnet bars	RCT basic, IKA Labortechnik, Staufen
pH-Meter	Modell 766 Calimatic, Knick, Berlin
Power supply	EPS 600, Pharmacia Biotech
	Freiburg Standard Power Pack P25, Biometra,Göttigen
Pipet aid	Hirschmann Laborgeräte, Eberstadt
Pipettes	Modell Research, Eppendorf, Hamburg

Photometer	Ultraspec 1000, Pharmacia Biotech, Freiburg
Shaking Incubater	SM-30 Control, Johanna Otto GmbH,
Thermomixer	Modell Compact, Eppendorf, Hamburg
Thermocycler	Modell T3, Biometra, Göttingen
UV-Crosslinker	UVC-500, Hoefer, San Francisco, CA, USA
UV-Table	TFX-20M, Gibco BRL, Eggenstein
Water bath	Laborte Chink, Hannover Vinnhorst
NanoDrop ND 1000 spectrophotometer	Peqlab, Erlangen

2.1.2 Cell culture media and additives

2.1.2.1 Antibiotics (disc)

Ampicillin	Oxoid Hampshire, England
Kanamycin	Oxoid Hampshire, England
Tetracycline	Oxoid Hampshire, England
Ciprofloxacin	Oxoid Hampshire, England
Gentamicin	Oxoid Hampshire, England
Chloramphenicol	Oxoid Hampshire, England

2.1.2.2 Antibiotics (powder)

Chloramphenicol	Sigma Steinheim Germany
Kanamycin	Sigma Steinheim Germany
Penicillin	Sigma Steinheim Germany

2.1.2.3 Media and additives

Dulbecco's MEM (DMEM) Medium supplemented with 580 mg/l L–glutamine und 3.7 g/l $NaHCO_3$.

DMEM Medium (10x) with 4.5 g/l D-glucose, 8 mg/l Phenol Red, without $NaHCO_3$, Na-pyruvate and L-glutamine.

Penicillin / Streptomycin, 10,000 U / 10,000 µg/ml.
Fetal Calf Serum (FCS); heat inactivated by incubation for 45 min at 56 ^{0}C.
EDTA (Versen) 1% in PBS without Ca $^{2+}$, Mg^{2+}
Trypsin (1:250) 0.25% in PBS without Ca^{2+}, Mg^{2+}
PBS (phosphate buffered saline)
All the product mentioned above were obtain from Biochrom, Berlin

2.1.2.4 Disposable materials and plastic ware

Disposable materials and plasticware used in the cell culture were purchased from Nunc,

Roskilde, Denmark; Falcon, Becton – Dickinson, Heidelberg; Corning Costar, Bodenheim; Greiner, Frickenhausen; Braun, Braun-Melsungen, Melsungen.

2.1.2.5 Cell lines

Human colon carcinoma Caco2

2.1.2.6 Bacterial strains and mutants

Bacterial Strains	**Specification**	**Source**
Campylobacter jejuni	NCTC11168	Dr. A. Karlyshev
	81-176	Prof. M. Kist, Freiburg
	B2	University Medical Center, Göttingen
E. coli	DH5α	Invitrogen
	BL21	CodonPlus-RIL Stratagene
Transposone-generated mutants Library	B2 mutants	Dr. J. I. Dasti, Gottingen Institute of Medical. Microbiology

2.1.2.7 Bacterial culture media

Luria Bertani (LB) Broth

1% Bacto-Trypton
0.5% Bacto-yeast extract
0.5% NaCl

LB-Plates	0.5% Bacto-yeast extract 0.1% Bacto-Trypton 0.5% NaCl 1.5% Agar (Difco, Detroit, USA)
Brain Heart Infusion	Bacto™
Columbia Agar Base	Merck, Darmstadt
Muller Hinton Agar	Oxoid, Wesel
Sheep Blood	Oxoid Wesel

2.1.3 Chemicals and reagents

Chemicals and solutions were purchased from Roche (Mannheim), Merck (Darmstadt), Roth (Karlsruhe), Calbiochem (Bad Soden) or Sigma (Deisenhofen). In general, solutions were prepared using double distilled water.

2.1.3.1 Antibodies

Monoclonal Mouse anti His Tag primary antibody (Qiagen) Hilden
Horseradish-Peroxidase-Conjugated anti Mouse secondary antibody (Dianova)

2.1.3.2 Membranes and filters

Nitrocellulose membrane	Hybond N (Amersham Biosciences)
Nylon membrane	Hybond P (Amersham Biosciences)
Filter discs	Oxoid, Wesel

Corning 26 mm Syringe Filter, 0.20 µm Micron Membrane, 0.45 µm Micron Membrane

2.1.3.3 Enzymes

Quick T4 DNA Ligase	New England Biolabs
Proteinase K	Roth, Karlsruhe
Pfu DNA-Polymerase	Promega, Mannheim
Taq DNA-Polymerase	Roche, Mannheim

Restriction endonucleases	New England Biolabs
Alkaline antarctic Phosphatase	New England Biolabs
KOD DNA-Polymerase	Novagen
RNase A	Sigma-Aldrich, Steinheim
DNase 1	Sigma-Aldrich, Steinheim
RQI Rnase Free DNA	Promega, Mannheim

2.1.3.4 Standard Buffers

Alkaline Phosphatase (AP) staining solution	0.05% (v/v) BCIP 0.5% (v/v) NBT
BCIP	5% (w/v) 5-bromo-4-chloro-3-indolyl-phosphate in ddH_2O
Blocking solution	5% (w/v) dry skimmed milk power 0.2% (v/v) Tween 20 0.2% (w/v) NaN_3 in 1x PBS (pH 7.4)
Coomassie staining solution	0.025% (w/v) Coomassie brilliant blue G 30% (v/v) methanol 10% (v/v) acetic acid in ddH_2O
Destaining solution	30% (v/v) methanol 10% (v/v) acetic acid in ddH_2O
10x DNA loading dye	40% (v/v) glycerol 1% (w/v) bromophenol blue in TE buffer (pH 8.0)
NBT	1%(w/v) nitotetrazolium blue chloride in ddH_2O
Separating gel (12%)	0.940 ml 2 M Tris-HCl (pH 8.8) 0.1 ml 10% (w/v) SDS

	1.94 ml 30% acrylamide
	0.02 ml10% (w/v) APS
	0.01ml TEMED
	2.0 ml ddH_2O
Stocking gel (4.4%)	0.625 ml 0.5 M Tris-HCl (pH 6.8)
	0.05 ml 10% (w/v) SDS
	0.36 ml 30% acrylamide/
	0,125 ml 10% (w/v) APS
	0.01ml TEMED
	0.01 ml Bromphenol Blue
	in ddH_2O
2x Sample buffer	20 % (v/v) glycerol
	1 % (w/v) SDS
	125 mM Tris-HCl (pH 6.8)
	0.002 % (w/v) bromophenol blue
	65 mM DTT
	in 10 ml ddH_2O
Substrate solution	200 ml 10% (v/v) diethamolamine
	(pH 9.6)
	20 ul 5 mM $MgCl_2$
	800 ml physiological NaCl (pH 7.4)
50x TAE (pH 8.0)	242 g Tris
	57.1 ml 98% acetic acid
	100 ml 0.5 M EDTA (pH 8.0)
	in 1 L ddH_2O
10x TE buffer (pH 8.0)	100mM Tris
	100 mM EDTA
	in ddH_2O
Washing solution	0.05% (v/v) Tween 20
	in 1xTBS (pH7.4)
1% Triton X-100/PBS	1% (v/v) Triton X-100

	in 1x PBS
TFB1 (pH 5.8)	100 mM RbCl
	50 mM $MnCl_2$
	30 mM KAc
	10 mM $CaCl_2$
	15% (v/v) glycerol
	in ddH_2O
TFB2 (pH 6.8)	10 mM MOPS
	10 mM RbCl
	75 mM $CaCl_2$
	15% (v/v) glycerol
	in ddH_2O

2.1.3.5 Kits and reagents

RNA Isolation

Ribo-Pure Bacteria Kit	Ambion

Reverse Transcription

One Step RT-PCR Kit	Qiagen, Hilden

DNA Cloning Kit

QIAGEN PCR Cloning Kit	Qiagen, Hilden

Plasmid-DNA Isolation

GenElute Plasmid Mini Prep Kit	Sigma-Aldrich, Deisenhofen

DNA Isolation from Agarose Gels

QIAEX II Gel Extraction Kit	Qiagen, Hilden

DNA and PCR Purification

QIAquick PCR Purification Kit	Qiagen, Hilden

Genomic DNA Isolation

QIAamp. DNA Mini Kit	Qiagen, Hilden

Protein-His Tag Expression and Purification

QIAexpress Kit	Qiagen, Hilden

Quantification of Proteins

Pierce BCA Protein Assay Kit	Pierce, Rockford, IL, USA

Molecular weight markers

GeneRuler 100 bp DNA Ladder	MBI Fermentas
GeneRuler 1kb DNA Ladder	MBI Fermentas
Prestained Protein Marker, Broad Range	New England Biolabs

2.1.3.6 Oligonucleotide

Table 2.1 HPLC-purified oligonucleotide primers used for sequencing

Gene	Primer name	Sequence (5' to 3')
aphA-3	KanF	TATCACCTCAAATGGTTCGCT
cj0952c-51c	Cj0952c-51cF	GCCAATTCATTTTCATTTTGAGA
	Cj0952c-51cR	TTTTAATGCTTAAAGTTGTTGTTGG

Table 2.2 HPLC-purified oligonucleotide primers used for cloning in pRRC and pBluescript II

Gene	Primer name	Sequence (5' to 3')
cj0952c	Cj0952cF	GCTCTAGAT*AGGA*AACT**ATG**ATGTTTAAAACTA
	Cj0952cR	GCTCTAGAAATTAATTGATATATCCACA
cj0952c/	Cj0952cF	GCTCTAGAT*AGGA*AACT**ATG**ATGTTTAAAACTA
cj0951c	Cj0951cR	NNNTCTAGACCTTTAAATTTGAAATTGGTTAAGTTCGC
cj0952c/	Cj0952cF	GCTCTAGAT*AGGA*AACT**ATG**ATGTTTAAAACTA
cj0951cHis	Cj0951His	GGCTCTAGATTAGTGATGGTGATGGTGATGAATTTG AAATTGGTTAAGTTCGC
cj0952c/	Cj0952F	GCTCTAGAT*AGGA*AACT**ATG**ATGTTTAAAACTA
cj0950c	Cj0950R	NNNTCTAGAGGGCATTATTTAAAAAAGATTTTGATTTC
cj0005c	Cj00005cF	GCTCTAGATTA*AGGA*TATTTCA**ATG**AAACAGAAC
	Cj00005cR	GCTCTAGAACTTAAACTCCATAAACATTAACTC
kanR	Kan1	**P**-GTAAGATTATACCGAGGTATGAAAACG
	Kan2	**P**-AATCTAGGTACTAAAACAATTCATCCA

XbaI restriction sites for cloning in pRRC and pBluescript II are underlined, the start codon of *cj0952c* and *cj0005c* are shown in bold and the putative SD sequence is illustrated in *italic*. Primers Kan1 and Kan2 are 5'-phosphorylated. Gene numbering according to *C. jejuni* strain NCTC 11168.

Table 2.3 Oligonucleotide primers used for semiquantitative real-time RT-PCR analysis

Gene	Primer name	Sequence (5' to 3')
23S	Cj23SF	GTTCGCCATTTAAAGCGGTA
	Cj23SR	TGCTCTTGGCAGAACAACAG
cj0951c	Cj0951fF	GAGTTACCAAAGCCCTAGCATC
	Cj0951R	CATGGTCAATCAAGCAGG
cj0952c	Cj0952F	GCCACTTCCTTGAGC
	Cj0952R	CGCCCTTTGATTGCAGATG
cj0953c	Cj0953F	GGCTGAAATGATTACTTCTAC
	Cj0953R	CTAACCGATATTAACGCAGC
groEL (cj1221)	GroELF	ATGGGGCCAAGAGGACGCAA
	GroELR	GCAGTAGTTGTTCCATCGCCTGCT
dnaJ (cj1260c)	DnaJF	GGCTTTGGCTCATCGCGTCG
	DnaJR	ACCTTGAGAAACCCCAACCTGTCC
dnaK (cj0759)	DnaKF	TGCTGTGTATGAACGCGGCGA
	DnaKR	ACCGCTTGGCGTTTTGCACT
cheY (cj1118c)	CheYF	GCTGAGCATGGCGTTGAAGC
	CheYR	ACTCCAAGCCATTCATTTCTGGCA
tlp7 (cj0952c)	Tlp7F	GAGTTACCAAAGCCCTAGCATC
	Tlp7R	CATGGTCAATCAAGCAGG
cadF (cj1478c)	CadFF	TGGACATTATGGCGCGGGTGT
	CadFR	TGTGGAGTTGCACGAGTATCAGCA
peb1a (cj0921c)	Peb1aF	ACAAGAGGCCCTTTGCTTGATAATGGT
	Peb1aR	AGTTGCAGCTTGAGCCACTCCA
jlpA (cj0983)	JlpAF	AGCACACAGGGAATCGACAGCA
	JlpAR	AAATGACGCTCCGCCCATTAACA
ciaB (cj0914c)	CiaBF	TCATGCGGTGGCATTAGAATGGG
	CiaBR	CATCATTTGGAACGACTTGAGCTGAGA
cdtB (cj0078c)	CdtBF	TGCAAGGCTCATCCGCAGCC
	CdtBR	TGGCGTCCTGTTGGAGTGGC

flaA (cj1339c)	FlaAF	AGGCGCTATGGCTGTGATGGA
	FlaAR	TGCACTCTCGGCTGCAAAGTCT
ptmC (cj1327)	PtmCF	ACAGGCATGGAAATTTAGGCGA
	PtmCR	ACAAGCTCCCAAGGCAACCGC
ptmD (cj1328)	PtmDF	ACCAAAGCAAATGCCGATGAAAATGG
	PtmDR	AACGCCCTTTTTGTCTATCGCCT
ptmE (cj1329)	PtmEF	ACTAGGTACAGCAGGGGCTTTAAGC
	PtmER	CTCACGCACGCAAACGCTCA
ptmF (cj1330)	PtmFF	GCTAGGTGGTGGGGTTTTACTCGA
	PtmFR	GCCAAAAAGGCAAAATCATCGCTTGT
ptmB (cj1331)	PtmBF	GCTAGGGGTGGTAGCAAAGGCG
	PtmBR	GCAAGGCATCACGCATGACAGGA
ptmA (cj1332)	PtmAF	CGCGTTAATACTCTAGCAAGCGGAGG
	PtmAR	AAGCCCCATCCATCATCTACCACT
pseD (cj1333)	PseDF	CCCATGGCAATGATCCTCTTGATGC
	PseDR	TCCAAAGAAAGCACATAATCGGGCT
pseE (cj1337)	PseEF	TGTTGGAACGCGATGAAATAGTAGCT
	PseER	GCATCTCGCAAAAGGCTGGCC
pseB (cj1293)	PseBF	CGGGCGGAACAGGCTCGTTT
	PseBR	CGTCGATGACATTTTGCGCACCG
pseC (cj1294)	PseCF	GCCGCTACAGCTAATGCGGC
	PseCR	GCTGGTTTATCCCTAAAGCACAAGCA
pglE (cj1121c)	PglEF	AGGAGCTATTGGCGTAGCGCA
	PglER	TCATCGCCGTGCCACTTGC
pseA (cj1316)	PseAF	ATGGCGGTCCTGCGAGTAGT
	PseAR	TGCTCTAACCAAAGCCGTATTTGCA
porA (cj1259)	PorAF	TGGCTGCAGAGCAAGGTGCA
	PorAR	AACCTACAGCAGCAGCACCG
flap (cj1279c)	FlpAF	GGTTCTGAACGAAGCCCGGCT
	FlpAR	AGACGCTATGGCGGGGGAGCA

ptmG (cj1324)	PtmGF	TGTGATCACTGCGGTGATGCCAA
	PtmGR	ACCTCCACTTACGGCAATAGCACA
ptmH (cj1325)	PtmHF	TGGCAATGGTGTACATTCAGCG
	PtmHR	TCCACCTGTGTTCAAAAGTTCGT

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Bacterial strains and culture conditions

The *Campylobacter jejuni* strains used in this study were B2, isolated in the University Medical Center Göttingen from a patient suffering from gastroenteritis (Schmidt-Ott et al., 2005; Dasti et al., 2007), NCTC 11168 and strain 81-176. All strains were cultured on Columbia blood agar supplemented with 5 % defibrinated sheep blood under microaerophilic conditions (85 % N_2, 10 % CO_2, and 5 % O_2) at 42 ^{0}C for 24 hours. As per requirement, appropriate antibiotics were added at the following concentrations: kanamycin (50 µg/ml) or chloramphenicol (30 µg/ml). For growth experiments, bacteria were grown at 42 ^{0}C in MH broth under microaerophilic conditions in the presence or absence of 10 mM formic acid or 10 mM Na_2SO_3. Every growth experiment was carried out three times. *Escherichia coli* strain DH5α which was used for cloning experiments was grown on Luria-Bertani (LB) agar or in Luria-Bertani broth at 37 ^{0}C. When necessary, the medium was supplemented with ampicillin (100 µg/ml).

2.2.1.2 Preparation of chemical competent *E.coli* cells

A single colony of *E.coli* DH5α was inoculated into 10 ml of LB broth and kept overnight at 37 ^{0}C under shaking. 2 ml of the over night culture was added into 100 ml of pre-warmed LB broth and left on the shaker until an $OD_{(600nm)}$ of 0.5 was reached. The culture was transferred into a 50 ml Falcon tube and cooled on ice for 10 min. Cells were centrifugated at 4000 x g for 10 min at 4 ^{0}C in a pre-cooled centrifuge. Then the cell pellet was gently resuspended in 30 ml ice cold TFB1 buffer and incubated on ice for 30 min. Cells were pelleted by centrifugation as described above, resuspended in 2 ml ice-cold TFB2 buffer and incubated on ice for another 30 min. After incubation aliquots of 100 µl were stored at -80 ^{0}C.

2.2.1.3 Generation of electrocompetent bacterial cells

10 ml of LB-broth were inoculated with a single *E. coli* DH5α colony and incubated overnight at 37 ^{0}C under shaking. Afterward, three ml of the overnight culture were grown in 500 ml LB-broth at 37 ^{0}C until an $OD_{(600nm)}$ of 0.35-0.45 was reached. After placing the culture on ice for 10 min it was centrifuged for 15 min at 4,000 × g at 4 ^{0}C. The pellet was gently resuspended in 50 ml cold water and again centrifuged for 15 min at 4,000 × g. After repeating this step three times, the cell pellet was resuspended in 15 ml 10 % glycerol and centrifuged again. Finally, the cell pellet was carefully dissolved in a final volume of 1 ml 10 % glycerol and 100 µl aliquots were used for each electroporation. In order to prepare competent cells of *C. jejuni,* cells were collected from Columbia blood agar plates and centrifuged at 5,000 × g at 4 ^{0}C for 10 min. The cells so obtained were resuspended in 1 ml ice-cold washing buffer containing 272 mM sucrose and 15 % glycerol at 4 ^{0}C. After repeating this step three times, the pellet was re-suspended in 400 µl washing buffer and 100 µl aliquots were used for each transformation.

2.2.1.4 Electroporation

After the addition of 0.5 to 3 µg of plasmid DNA to the electrocompetent cells, the mixture was transferred into an ice-cold electroporation cuvette and the cuvette was incubated on ice for 30 sec. Electroporation was performed at 2.5 kV, 25 µF and 200 Ω using a BTX Electro Cell Manipulator. Then, 500 µl of SOC medium was added to the cuvette and in case of *C. jejuni* the suspension was transferred onto a non selective Columbia blood agar plates and incubated overnight at 37 ^{0}C under microaerophilic conditions. Finally, the cells were transferred onto selective plates and incubated at 42 ^{0}C under microaerophilic conditions for further 2-3 days. After electroporation of *E.coli* cells, the suspension was transferred to a polypropylene tube, incubated with gentle rotation for 1 hour at 37 ^{0}C and plated on LB agar containing the appropriate antibiotic.

2.2.1.5 Cultivation of eukaryotic cells

Human colon carcinoma 2 (Caco2) cells were maintained in 175 cm^2 and 25 cm^2 cell culture flasks as well as in 6 well plates in Dulbecco minimal essential medium (DMEM) supplemented with 10 % heat inactivated fetal calf serum, 1x non-essential amino acids, with/without 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were routinely cultured in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 ^{0}C. The Caco2 cells were grown in 175 cm^2 flask to form a confluent monolayer for 2 days. Afterwards, the cells were split by removing the old media, washing the monolayer with 5 ml EDTA and detaching the cells with 5 ml of trypsin. Then 10 ml of DMEM medium (10 % FCS and 1x non-essential amino acids) were added, the cells were centrifugated at 1380 rpm for 5 min. and recultivated in 175 cm^2 cell culture flasks (10 % FCS, 1 % NEAA, 1 % Penicillin/streptomycin).

2.2.1.6 Invasion and adhesion assays

Bacterial invasion of host cells was initially described by Everest et al., (1992). After achieving a semiconfluent layer cells were washed with PBS and 400µl *C. jejuni* suspension in DMEM, (10 % FCS and 1x NEAA) were added. To assure that the number of bacteria was identical in every assay performed, the solution was adjusted to an $OD_{(600nm)}$ of 0.5 representing a multiplicity of infection (MOI) of 100 and incubated at 37 ^{0}C, 5 % CO_2 to allow the bacteria to invade the host cell. In some experiments bacteria were brought in contact with the Caco2 cell by centrifugation on 6 well plates at 600 x g for 15 minutes. At 2 hrs post infection, the cells were washed three times with 1x PBS before further incubation with culture medium containing 100 µg/ml gentamicin. After treatment with gentamicin for 2 hrs the cells were washed again three times with 1x PBS. Then the cells were lysed with 1 % Triton X-100 for 10 min to release intracellular bacteria. Finally, the number of viable bacteria was determined by plating serial dilutions on Columbia blood agar and counting of the number of bacteria grown after incubation for 48 hrs at 42 ^{0}C under microaerophilic conditions. Thereby, the number of colonies obtained after the invasion assay with wild-type strain B2,

mutants and complemented mutants was defined as cfu in each experiment. In order to investigate bacterial adhesion, Caco2 cells were incubated with the bacteria for only 30 min. After the monolayer was washed with PBS, the cells were lysed and the bacteria were plated on Columbia blood agar plates in order to determine the number of recovered bacteria as described above.

2.2.1.7 Freezing and thawing of Caco2 cells

The detached Caco2 cells were suspended in 40 % DMEM and mixed with the same volume of 2x freezing solution (20 % DMSO, 40 % FCS in DMEM). One mililiter aliquotes were kept for one day at -80 ^{0}C and transferred into liquid nitrogen for long term storage. In order to thaw the frozen cells, a cryotube was taken from liquid nitrogen and incubated in a 37 ^{0}C water bath. After the cells were thawed they were cultured under the conditions described above.

2.2.2 Modification of nucleic acids

2.2.2.1 Preparation of genomic DNA

Genomic DNA of *C. jejuni* was isolated using the QIAamp DNA Mini Kit following the manufacturer's instructions. The bacteria were removed from culture plates and mixed with 180 µl of Buffer ATL by vigorous shirring. Then 20 µl of Proteinase K was added and the sample was incubated at 56 ^{0}C until the bacteria were completely lysed. After lysis 200 µl of AL buffer was added and the sample was incubated at 70 ^{0}C for 10 min. After mixing 200 µl of ethanol (100 %) to the sample the lysate was transferred to a QIAamp spin column and centrifuged at 6,000 g for 1 minute. The column was washed once with 500 µl of buffer AW1 and then with 500 µl buffer AW2. Finally, the QIAamp spin column was transferred in a clean 1.5 ml tube and the genomic DNA was eluted with 200 µl Buffer AE.

2.2.2.2 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* cells using a plasmid purification kit (Sigma-Aldrich) according to the manufacturer's instructions. Bacteria from a 2 ml

of overnight culture were centrifugated and resuspented in 200 µl resuspension solution. Then 350 µl of neutralization solution was added and the sample was transferred to a spin column and centrifugated at maximum speed for 1 min. After washing the column with 750 µl washing solution, the plasmid DNA eluted with 10 µl of the appropriate buffer.

2.2.2.3 Isolation of RNA

Bacterial RNA was extracted from C. *jejuni* cells with the RiboPure Bacter Kit (Ambion) following the recommendations of the protocol. After the over night culture was centrifugated, 350 µl RNAwiz solution was added and the sample was transferred to a tube containing 250 µl Zirconioa beads. After mixing for 10 min at maximum speed the sample was centrifugated for 5 min at 4 ^{0}C. The supernatant was transferred to a fresh 1.5 ml tube and mixed with 0.2 x volume of chloroform. Then the sample was centrifugated for 5 min at 4 ^{0}C and the aqueous phase was conveyed to a fresh 1.5 ml tube where 0.5 x volume of 100 % ethanol was added. The mixture was converted into filter cartilage for centrifugation at 13,000 rpm for 1 min. Afterwards the flow-through was discarded and the column was washed once with 700 µl wash solution 1 and twice with wash solution 2/3. Finally, the RNA was eluted by applying 30 µl of preheated (95 ^{0}C to 99 ^{0}C) elution solution to the centre of filter and centrifugation for 1 min. After RNA isolation, DNA contaminations were removed by DNase I treatment and incubation at 37 ^{0}C for 30 min followed by denaturation of the enzyme by for 15 min at 75 ^{0}C. To make sure that the RNA was free of remaining traces of DNA after DNase I treatment, PCR assays were performed after every preparation.

2.2.2.4 Quantification of DNA and RNA

Concentrations of DNA and RNA were determined with a NanoDrop ND 1000 spectrophotometer according to the instruction of the manufacturer.

2.2.2.5 Polymerase chain reaction (PCR)

PCR was used to amplify *C. jejuni* genes for subsequent cloning into vectors pRRC or pBluescript II. The genomic DNA of wild type B2 and mutant were used as template to amplify the desired sequences, using PCR primers with or without introduced endonuclease restriction sites which are summarized in Table 2.2. Each 50 µl of PCR mixture contained 40 ng genomic DNA, 10 mM TRIS-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, all four dNTPs (each 0.2 mM) and 2.5 U Taq DNA polymerase . After initial incubation at 95 ^{0}C for 1 min, 40 cycles at 95 ^{0}C for 30 s, 55 ^{0}C for 30 s and 72 ^{0}C for 1 min were carried out with a final incubation at 72 ^{0}C for 10 min. Different annealing temperatures were used based on the melting point of the respective primer pairs, while the length of the amplified region determined the elongation time (30 s for each 1 kb). All PCR products were analysed on 1-1.5 % agarose gels stained with ethedium bromide at a final concentration of 1 µg/ml. The size of PCR products was determined by using the 1 kb or 100 bp GeneRulers DNA ladder which was run along with the PCR sample.

2.2.2.6 Conventional reverse transcriptase-PCR

Conventional (RT)-PCR assays were carried out with the OneStep RT-PCR Kit following the manufacturer's recommendations. After reverse transcription of 10 ng for 30 min at 50 ^{0}C, 35 cycles of amplification were performed according to the following protocol: cDNA was denatured at 94 ^{0}C for 30 s, primer annealed at 55 ^{0}C for 30 s and extended at 72 ^{0}C for 1 min, with a final incubation at 72 ^{0}C for 10 min. The primers used for reverse transcriptase-PCR are listed in Table 2.3.

2.2.2.7 Real-Time reverse transcriptase-PCR analysis

Semi-quantitative real-time RT-PCR analysis was carried out with the LightCycler 1.5 and the QuantiFast SYBR Green RT-PCR Kit. LightCycler PCR assays were performed in glass capillaries in triplicate in a final volume of 20 µl with 10 pmol of each HPLC purified primer, 10 µl of master mix and 50 ng. Amplicons of the constitutively transcribed *23S* gene were applied to adjust the RNA samples under

investigation based on the crossing points obtained for this gene. *23S* RT-PCR was carried out according to this protocol: initial reverse transcription for 20 min at 50 ^{0}C and denaturation for 5 min at 95 ^{0}C was followed by 25 cycles of denaturation (95 ^{0}C; 10 s), annealing (50 ^{0}C; 10 s) and elongation (72 ^{0}C; 5 s). After adjustment of the RNA samples real time RT-PCR assays for the genes under investigation were run. Therefore, reverse transcription was followed by 25 cycles of denaturation (95 ^{0}C, 10 s), annealing (55 ^{0}C; 10 s) and elongation (72 ^{0}C; 10 s). Each PCR included a negative control comprising of all elements without RNA to monitor possible contamination. The primers used for both assays are listed in Table 2.3. Every assay was run in duplicate. The specifity of the signal was assured by melting curve analysis and agarose gel electrophoresis. Semi-quantification of transcription levels were calculated as follows: $x = 2^{\Delta Cp}$, where x represents the factor of altered transcription, and ΔCp illustrates the difference of crossing points (Cp1-Cp2) of the two samples to be compared.

2.2.2.8 Enzymatic digestion of DNA

Purified PCR products harbouring the required endonuclease restriction sites and cloning vectors were digested by 10 U restriction endonuclease in such a way that 10 µl of purified PCR product or 1 µg of cloning vector were incubated with 1x NEB buffer, 1x 10% BSA (optional) and dH_2O in a volume of 20 µl at 37 ^{0}C for 2 hrs.

2.2.2.9 Purification of PCR products

DNA extraction from agarose gels were performed using QIAGEN PCR Purification Kit according to manufacturer's protocol. DNA fragment was excised with a scalpel from the agarose gel, weighted and three volume of buffer QG to one volume of excised gel was added, respectively. The mixture was completely dissolved by incubating at 50 ^{0}C for 10 min and mixed thoroughly with 1 gel volume of isoproponal. Then the sample was transferred to a QIAquick spin column placed in 2 ml collection tube, centrifugated 13,000 rpm for one min and washed with 750 µl of buffer PE. After all, the DNA was eluted by adding 30 µl of

elution buffer in the middle of column and final centrifugation at 8,000 rpm for 1 min.

2.2.2.10 Ligation

The restricted DNA fragments were ligated to a vector in a final volume 20 µl containing 1:3 molar ratios of vector and insert, 1 µl of Quick T4 DNA ligase, 1x ligation buffer and ddH_2O. The ligation reaction mixture was kept at room temperature for 1 hour or incubated over night at 16 ^{0}C.

2.2.2.11 Direct sequencing of genomic DNA from *C. jejuni*

Each 10 µg of genomic DNA were sequenced directly by the SeqLab Company (Göttingen, Germany) applying 10 pmol of KanF primer which is listed in Table 2.1.

2.2.3 Experiments related to mutants of *C. jejuni* and their characterization

2.2.3.1 Knock-out inactivation of *cj0005c*

Primers Cj0005cF and Cj0005cR (Table 2.2) were used to amplify a 1239 bp DNA fragment corresponding to *C. jejuni* gene *cj0005c*. The PCR fragment was XbaI-digested and inserted into plasmid vector pBluescript II KS to obtain pBcj0005c. To insert a kanamycin resistance cassette containing the *aphA-3* gene, PCR was carried out with plasmid pSB1699 as a template and the 5'-phosphorylated primers Kan1 and Kan2 (Table 2.2). The PCR reaction contained 10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, all four dNTPs (each 0.2 mM), 10 pmol of primers and 1 U Pfu Polymerase to generate a blunt-end PCR product. After initial incubation at 95 ^{0}C for 1 min, 30 cycles at 95 ^{0}C for 30 s, 55 ^{0}C for 30 s and 72 ^{0}C for 1 min followed, with a final incubation at 72 ^{0}C for 10 min. Following purification of the PCR product using the QIAquick PCR Purification Kit (Qiagen), the kanamycin resistance cassette was cloned into plasmid pBcj0005c, which was BsaB1 blunt end restricted and dephosphorylated with Antarctic Phosphatase (New England Biolabs) to obtain pBcj0005cKan.

2.2.3.2 Cloning of *C. jejuni* genes into expression vector pRRC

The *C. jejuni* genes were PCR-amplified and subsequently cloned into expression vector pRRC using primers harboring restriction sites for XbaI, respectively. The primer sequences with XbaI restriction sites are mentioned in Table 2.2. The *Campylobacter* expression vector pRRC contains a *Cam*^{r} gene cassette which is bounded by a 16S sequence and tRNAs for alanine and isoleucine, respectively. Immediately downstream of the *Cam*^{r} gene cassette resides a single XbaI site to allow the expression of XbaI-cloned genes under the control of the constitutively expressed *Cam*^{r} gene promoter. After transformation of *C. jejuni* with the recombinant plasmid, the *Cam*^{r} gene cassette together with the XbaI-cloned gene is integrated into one of the *16S* loci of the recipient cell via highly efficient double recombination (Karlyshev and Wren, 2005).

2.2.3.3 Motility assay

One microliter of an overnight culture of *C. jejuni* adjusted to an $OD_{(600nm)}$ of 0.025 was sticked in the center of a 0.4 % Mueller-Hinton agar plate with the help of a suited normalized inoculation loop. Afterwards, the plates were incubated at 42 ^{0}C under microaerophilic conditions for 36 hrs. The low concentration of the agar allows the bacteria to swarm, and to form a visible halo within the agar. Motility of the bacteria under investigation was judged by the expansion of the halos which was examined by the measurement of the respective radii. Each experiment was performed seven times.

2.2.3.4 Chemotaxis assay

For chemotaxis assays, bacteria were grown on Columbia blood agar plates overnight at 42 ^{0}C under microaerophilic conditions. Then, the bacteria were suspended in PBS (pH 7.0), adjusted spectrophotometrically to an $OD_{(600nm)}$ of 1 and mixed (1:1) with temperate soft agar (0.8 %, BD, Heidelberg, Germany). Afterwards, 12 ml of the bacterial soft agar suspension were poured into a petri dish and 6 mm discs sodden with 20 µl of the respective test chemicals (0.1 M and pH 7.0) were placed on the solidified agar. Zones of bacterial attraction or

repulsion were measured after four hours of incubation at 42 ^{0}C under microaerophilic conditions. The chemotaxis assays have been carried out four times, respectively.

2.2.3.5 Autoagglutination assay

Autoagglutination assays were performed as described by Misawa and Blaser (2000). Bacteria inoculation from petri dishes were suspended in PBS and adjusted to an $OD_{(600)}$ of approximately 1.0. Bacterial suspensions of 2 ml were incubated under a microaerophilic condition for 24 hrs at 37 ^{0}C. Then, 1 ml of the suspensions were removed carefully and the $OD_{(600)}$ was measured. Bacterial strains under investigation were measured five times. The obtain data were normalized following the protocol of Howards and co-workers (2009).

2.2.4 Analysis of Protein Expression

2.2.4.1 Measurement of protein concentration

Protein concentrations were quantified by BCA protein assay kit (Pierce) according to the manufactures instructions.

2.2.4.2 Sodium dodecyl sulphate polyarcylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE. The acrylamide gel cast was set up by laying two spacer sandwiched between rectangular glass plates. Firstly, the separating gel (10 % or 12 %) was applied to the space. After the polymerization of the separating gel, the stocking gel (4.4 %) was pipetted on the top of the separating gel and a comb was inserted into the sandwiched plates. After the gel was solidified, the comb was removed and the gel was ready to use.

2.2.4.3 Protein analysis by SDS-PAGE

The bacteria were suspended in 8 M urea and boiled for 10 min at 95 ^{0}C in order to lyse the cells. Equal amounts of cell lysates were mixed with 2 x SDS-PAGE

sample buffer and heated at 95 ^{0}C for 10 min before gelelectrophoretic separation. The SDS gel was run at 32 mV for approximately 2hrs. After electropheresis, gel was stained with Coomassie staining solution for 1 hour. Unspecific staining was removed by incubating the gel in 30 % methanol and 10 % acetic acid. Finally, the Coomassie stained gel was sealed in cellophane and dried overnight.

2.2.4.4 Purification of a 6xHis-tagged Protein from *C. jejuni* under denaturing conditions

All steps were carried out according to the QIA expressionist protocol (Qiagen) with the solutions recommended for purification of 6x His-tagged proteins under denaturating conditions. The cell were suspended in lysis buffer (100 mM NaH_2PO_4, 8 M urea, 10 mM Tris-HCl, pH8.0) and stirred on a shaker for 60 min at room temperature. After centrifugation at 10,000 g the supernatant was mixed with Ni-NTA beads (1 ml Ni-NTA slurry to 4 ml cleared lysate) and gently mixed at 200 rpm on a rotary shaker for 90 min at room temperature. Then the lysate Ni-NTA was loaded carefully into an empty column and the flow through was collected. The column was washed twice with 4 ml buffer C (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3) and the his-tagged protein was eluted with elution buffers D (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 5.9) and E (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5) following the instruction of the manufacturer.

2.2.4.5 Western blotting

Proteins separated on 12 % SDS-PAGE were transferred to a PVDF membrane (GE Healthcare) using a semidry transport system (Sartorius). For this the blot sandwich was build up in the following arrangement. Anode (+): soaked 6 Whatman filter papers in 0.3 M Tris-HCl, pH 10.4 and 20 % methanol, soaked 3 Whatman filter papers in 25 mM Tris-HCl, pH 10.4 and 20 % methanol, nitrocellulose membrane gel, 9 Whatman filter papers soaked in 40 mM 6-aminocapronic acid, pH 7.6 and 20% methanol, Cathode (-) . The proteins were

transferred at 0.9 mA /cm^2 of gel size for 90 min. The membrane was blocked for 1 h with 5 % milk powder in PBS containing 0.05 % Tween 20. After incubation of the membrane with a 1:3,000 diluted monoclonal mouse anti His primary antibody over night at 4 ^{0}C, the immune complexes were labelled with a 1:3000 diluted horseradish-peroxidase-conjugated anti mouse secondary antibody for 1 h at room temperature. The membrane was washed 4 x for 20 min with 0.05 % Tween 20 and incubated with ECL reagent (GE Health Care). Finally the membrane was covered by plastic foil and visualized by ECL chemoluminiscence following the manufacturer's recommendations.

2.2.4.6 Statistical analysis

Significant differences between mean values were calculated by the Student's *t*-Test using MS-Excel software. *P*-values of less than 0.01 were considered to be significant.

3 RESULTS

3.1 Invasion capacity of *C. jejuni* isolate B2 and the *C. jejuni* strains NCTC 11168 and 81-176

Invasion experiments using Caco2 cells were performed in order to determine the infectivity of *C. jejuni* strain B2 compared to the well characterized *C. jejuni* strains NCTC 11168 and 81-176 by performing gentamicin protection assays. Thereby, it was observed that isolate B2 is more invasive than both reference strains. The result of this experiment is shown in Fig. 3.1. The mean value of colonies that were recovered from infections with the clinical isolate B2 was $1.2x10^6$ cfu/ml, whereas $0.85x10^5$ and $4.1x10^5$ cfu/ml were counted for, NCTC 11168 and 81-176, respectively.

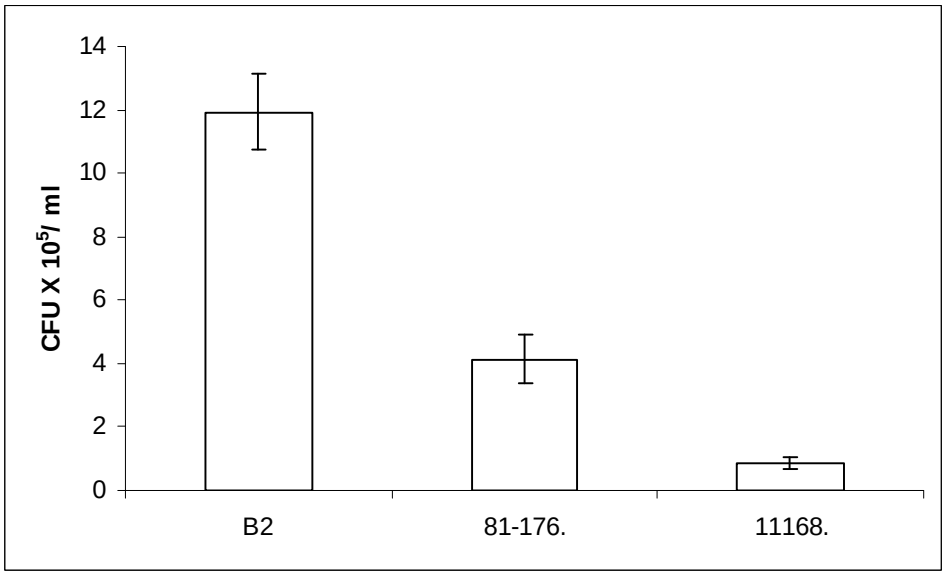

Figure. 3.1 Comparative analysis of the *C. jejuni* strains B2, NCTC 11168 and 81-176

Invasion assays using Caco2 cells showed increased infectivity of clinical isolate B2. The mean values of colonies recovered from NCTC 11168 and 81-176 were $0.85x10^5$ and $4.1x10^5$ cfu/ml. Compared to these two reference strains, the infectivity of the clinical isolate B2 was clearly higher with $1.2x10^6$ cfu/ml.

3.2 Identification of invasion related *C. jejuni* genes

To identify candidate genes that mediate a less invasive activity of *C. jejuni* to invade Caco2 cells, altogether 660 clones of an existing transposon-based mutant library of the *C. jejuni* strain B2 which was kindly provided by Dr. J. I. Dasti, (University of Gottingen) were individually analysed by performing gentamicin protection assays. Out of these 660 clones, seven clones have been detected with a strongly decreased invasiveness (Fig 3.2). Direct genomic sequencing was applied in order to determine the transposon insertion site within the genome of the respective *C. jejuni* clones, using a primer that binds directly to the 5' region of the kanamycin resistance cassette and, therefore, allows the determination of the affected *C. jejuni* genes. In order to approve the reduced invasiveness, gentamicin protection assays with the respective clones and wild-type strain B2 were repeated five times. Thereby, the mean value of colonies recovered from clinical isolate B2 were $1.1x10^6$ cfu/ml whereas the cfu/ml obtained for the respective mutants were as follows: *cj0005c*: $2,8x10^5$ cfu/ml, *cj0078c*: $3.4x10^5$ cfu/ml, *cj0093*: $4.1x10^5$ cfu/ml, *cj0268c*: $3.3x10^5$ cfu/ml, *cj0721c*: $3.2x10^5$ cfu/ml, *cj1439c*: $3x10^5$ cfu/ml, and *cj0952c*: $2.3x10^5$ cfu/ml with P-values of <0.001, respectively. The results are summarized in Fig. 3.2 and table 3.1.

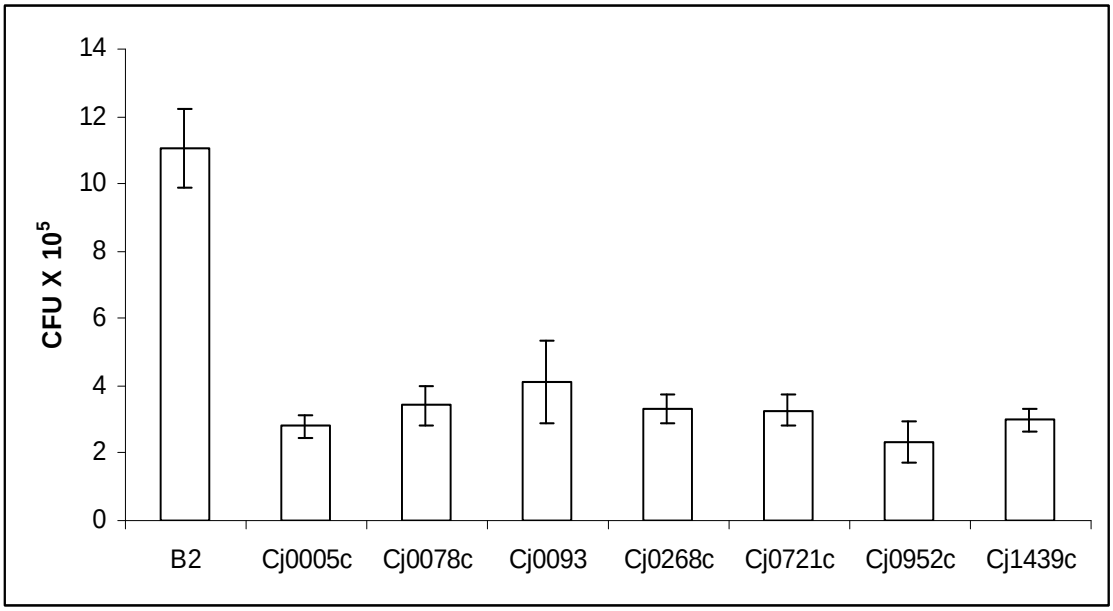

Figure 3.2 Invasion of the B2 wild-type strain and selected clones of a transposon based mutant library

The names of clones correspond to the mutated gene. See text for details.

Table 3.1 Specification of the detected genes with regard to function and localization as far as known

Gene	Putative Function
cj1439c	**UPD-galactopyranose mutase**
cj0952c	**N-terminal of a chemoreceptor**
cj0721c	**Integral membrane protein**
cj0268c	**Transmembrane protein**
cj0093	**Periplasmic protein**
cj0078c	**CdtB, cytolethal distending toxin**
cj0005c	**Sulphite:cytochrome c oxidoreductase**

Gene cj0952c represents the N-terminus of a putative chemoreceptor, whereas *cj0005c* encoding one subunit of a sulphite: cytochrome c oxidoreductase (SOR). Since to my knowledge neither a chemoreceptor nor an enzyme from the energy metabolism has been connected to the infectivity of *C. jejuni* so far, these two genes were selected for further investigation.

3.3 Characterization of *cj0952c*

3.3.1 Transposon insertion in gene *cj0952c* does not alter the transcription of both upstream gene *cj0953c* as well as downstream gene *cj0951c*

To determine the effect of the transposone insertion into gene *cj0952c* on the transcription of the upstream and downstream genes, reverse transcription PCR assays with the wild-type strain B2 and the mutant B2Δ*cj0952c*. was performed The non-coding sequence between *cj0953c* and *cj0952c* has a length of 96 bp, and the gap between *cj0952c* and *cj0951c* is 263 bp in the clinical isolate B2, indicating a transcription of these three genes in a polycistronic fashion. For this, an altered transcription of especially the downstream located gene *cj0951c* cannot be excluded in case of a transposon insertion within gene *cj0952c* (Fig. 3.3). As it is shown in Fig. 3.4, transcription of *cj0952c* including the transposon, but also of *cj0953c* and *cj0951c* could clearly be proven in the mutant B2Δ*cj0952c,* ensuring the invasion-deficient phenotype to be exclusively mediated by the insertion of the transposon into *cj0952c.*

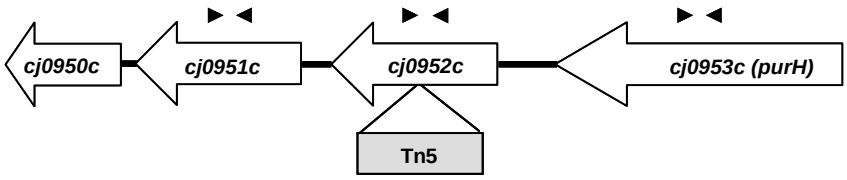

Figure. 3.3 Genomic composition of *cj0953c, cj0952c, cj0951c* and *cj0950c* in the clinical *C. jejuni* isolate B2

In the above vectorial diagram the directions of the arrows denotes the transcriptional orientation of the genes. Primers used for RT-PCR analysis are shown as arrowheads. The Insertion site of the transposon is indicated. (Genbank accession number GU799572).

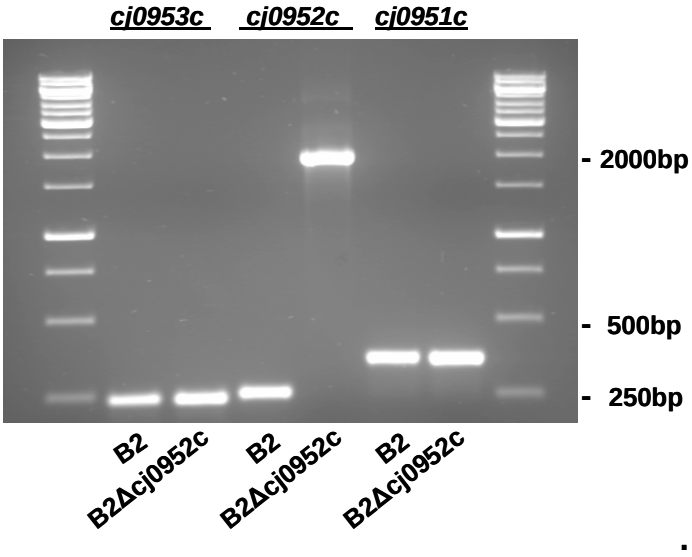

Figure 3.4 RT-PCR assays for the investigation of *C. jejuni* genes *cj0951c, cj0952c* and *cj0953c* in wild-type B2 strain and the mutant strain B2Δ*cj0952c*

The PCR amplicons are displaying the expected size for *cj0953c* (225 bp), *cj0952c* wild-type (267 bp), *cj0952c* with the integrated transposon (2091 bp) and *cj0951c* (356 bp). To confirm RNA purification, PCR analysis on RNA templates under investigation yielded no amplicons, reflecting that DNA contaminations were completely removed (not shown).

3.3.2 Functional restoration of *C. jejuni* B2Δ*cj0952c*

To restore the infectivity of the B2Δ*cj0952c* mutant, *cj0952c* and *cj0951c* alone and *cj0952c* combined with *cj0951c* have been cloned into vector pRRC and were

channelled into B2Δ*cj0952c*. Subsequent gentamicin protection assays on Caco2 cells restored the parental phenotype only in the presence of both genes. Neither *cj0952c* nor *cj0951c* alone increased the mutant infectivity towards the wild-type B2. A graphical representation of altogether five independent experiments is shown in Fig 3.5.

Unlike to wild-type strain B2, transcription of *cj0952c* in the complemented mutants is under control of a cam^R promoter. Real time RT PCR was performed in order to compare the transcription level of *cj0952c* in the wild-type and the complemented mutants. RNA samples adjustment was performed according to the crossing points of the *23S* transcripts, respectively. Then, the *cj0952c* transcription level of the wild type strain, the mutant B2Δ*cj0952c* complemented with *cj0952c* alone and complemented with *cj0952c-cj0951c* were determined. While the average crossing points of both complemented mutants were 20.49 (*cj0952c*) and 20.18 (*cj0952c-cj0951c*), a specific signal for *cj0952c* from wild type strain was primal obtained at a crossing point of 32.44, clearly demonstrating a more than 4000 fold upregulation of *cj0952c* in the complemented mutants compared to the wild type strain. Consequently, a low transcription level of *cj0952c* under the control of the cam^R promoter could be excluded as a reason for the incomplete invasion phenotype of the mutant which only habours the *cj0952c* gene.

In contrast to the clinical isolate B2 and *C. jejuni* strain NCTC 11168 where the putative chemoreceptor Cj0952c-Cj0951c is coated by two individual genes, the corresponding putative chemoreceptor in *C. jejuni* strain 81-176 is encoded by a single gene that covers the transmembrane domain, the HAMP domain and also the MCP domain. To further investigate whether the single gene of *C. jejuni* 81-176 is able to complement B2Δ*cj0952c*, was cloned it into pRRC and introduced the recombinant plasmid into the mutant. Thereby, the Caco2 cell infection was also restored up to the wild type level as summarized in Fig. 3.5.

In a next attempt to restore the invasiveness of the mutant B2*Δcj0952c* completely, a PCR fragment encompassing *cj0952c-cj0951c-cj0950c* was cloned into vector pRRC. Gene *cj0950c* is located downstream of *cj0951c* and the corresponding gene product is a putative lipoprotein of yet unknown function. It possesses a predicted signal peptide and shows homology to the heat shock protein HSLJ of *E. coli*. Since nothing is known about the role of this gene, a functional correlation of Cj0950c with Cj0952c and Cj0951c seemed to be possible. Gentamicin protection assays after introduction of the recombinant plasmid carrying all three genes obtained the recovery rates comparable to the mutant complemented with *cj0952c-cj0951c* indicating that Cj0950c is not in a functional correlation with Cj0952c and Cj0951c (Fig. 3.5 and Table 3.2).

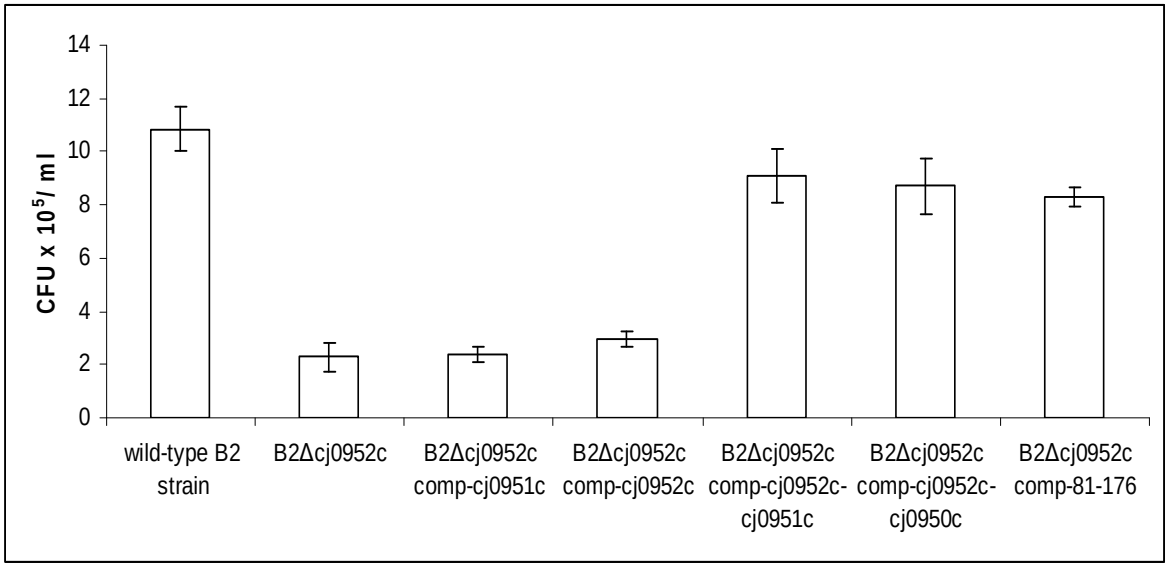

Figure 3.5 Infection of Caco2 cells by wild-type strain B2, the mutant and the complemented mutants

Investigation revealed that the reconstitution of *C. jejuni* wild-type B2 strain invasiveness was achieved by complementation of B2Δ*cj0952c* with *cj0952c-cj0951c* but not with *cj0952c* or *cj0951c* alone. The additional expression of Cj0950c had no influence on the recovery rate. Complementation of B2Δ*cj0952c* with the corresponding gene of *C. jejuni* strain 81-176 (B2Δ*cj0952c*-comp-*81-176*) also shifted the infectivity of the mutant back to wild-type level. The experiments were carried out in triplicate and have been repeated five times. The error bars represent the standard deviation.

Table 3.2 The mean values of colonies recovered after five independent invasion experiments

Strain	Cfu/ml (x 10^5)	Standard deviation (x10^5)
wild-type B2	10.86	± 0.86
B2Δ*cj0952c*	2.30	± 0.53
B2Δ*cj0952c* comp-*cj0952c*	2.98	± 0.28
B2Δ*cj0952c* comp-*cj0951c*	2.38	± 0.26
B2Δ*cj0952c* comp-*cj0952c-cj0951c*	9.08	± 1.00
B2Δ*cj0952c* comp-*cj0952c-cj0950c*	8.72	± 1.04
B2Δ*cj0952c* comp-81-176	8.30	± 0.37

The parental strain B2 was significantly more invasive as compared to the reduced invasiveness of the mutant *B2Δcj0952c* and the mutants complemented with *cj0952c* or *cj0951c* (P<0.001). The variation of recovered colony numbers after invasion assays with mutants complemented with *cj0952c-cj0951c*, *cj0952c-cj0950c* or 81-176 was subsidiary (P>0.001). The standard deviations are indicated. See text for details.

3.3.3 *C. jejuni* genes *cj0952c* and *cj0951c* of the clinical isolate B2 are translated into separate proteins

C. jejuni genes *cj0951c* and *cj0952c* in the clinical isolated B2 as well as in the strain NCTC 11168 have been annotated to be pseudogenes, postulated to be translated into one functional protein by a speculated read through mechanism. To prove this hypothesis, PCR and reverse transcription-PCR with DNA and RNA of the parental strain B2 was carried out to detect alterations of the synthesized RNA e.g. a stop codon replacement of *cj0952c* to create one open reading frame for both genes. PCR and reverse transcription-PCR assays have been carried out with the primers Cj0952c-51cF and Cj0952c-51cR (listed in Table 2.1) which are spanning the 3'-region of *cj0952c,* the intergenic region and the 5'-domain of *cj0951c*. After sequencing of the PCR and RT-PCR products no differences between both amplicons could be detected. Neither the stop codon of *cj0952c* at

position 891663 of the genome was converted, nor other changes within the RNA sequence could be found contradicting the idea of a postulated read-through mechanism (data not shown). However, the analysis of the RT-PCR amplicon could justify that *cj0951c* and *cj0952c* are co-transcribed on one mRNA. Afterwards, genes *cj0952c* and *cj0951c* together were cloned into vector pRRC to answer the question whether these genes are translated as one protein or separately. For the proof of the recombinant protein expression, the primer corresponding to the 3'-end of *cj0951c* was extended by nucleotides representing a 6x His-tag (Table 2.2). After introduction of the recombinant plasmid into mutant B2Δ*cj0952c*, the proteome was analysed by Western blotting. As a negative control, lysate of parental strain B2 was used. Thereby, a band corresponding to approximately 25 kDa was obtained, which is going along with the predicted protein size of Cj0951c but not with the expected size for Cj0952c-Cj0951c (58 kDa), confirming that no read-through mechanism takes place in *C. jejuni* strain B2 (Fig. 3.6). For the confirmation of the immunoblot results, the experiment with other randomly chosen clones which harboured the recombinant plasmid was repeated. While in most of the clones expression of the corresponding protein could not be detected, the same 25 kDa bands were observed in two other clones. No band was found for a recombinant protein with the size of approximately 58 kDa (data not shown).

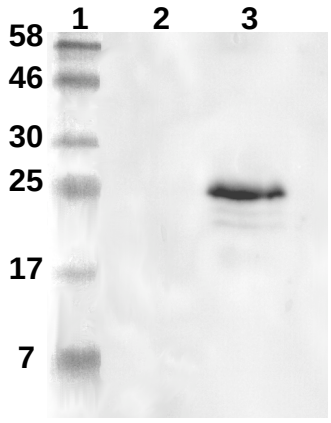

Figure 3.6 Detection of the recombinant protein in *C. jejuni* by Immunobloting

Lane 1: molecular mass marker (molecular masses are indicated in kDa), lane 2: wild-type strain B2, lane 3: mutant B2Δ*cj0952c*-comp-*cj0952c*-*cj0951c*His. The observed protein molecule exhibited a molecular mass of approximately 25 kDa, which resembled the size of Cj0951c. No protein of a molecular mass of 58 kDa which would indicate a Cj0952c-Cj0951c composition could be detected.

3.3.4 Decreased motility of *C. jejuni* B2Δ*cj0952c*

The chemotaxis machinery of a pathogen is known to be connected to motility (Hugdahl et al., 1988). For this, motility assays were used to investigate whether the mutant B2Δ*cj0952c* has an altered phenotype. Thereby, a significantly reduced motility of the mutant compared to the parental strain B2 was determined. While the average diameter of the motility zone on semi-solid agar plates for B2 was 47.4 mm ± 3.13, the mutant was clearly less motile with a mean diameter of 30.6 mm ± 2.4 ($P<0.0001$). After complementation of the mutant with *cj0952c* or *cj0951c*, the motility remained reduced compared to the parental strain (30.6 mm ± 2.9, 29.4 mm ± 2.3, $P<0.0001$). In contrast, when *cj0952c* together with *cj0951c* was introduced into the mutant, the motility of the wild-type was restored (46.6 mm ± 3.2). Also the introduction of the corresponding gene of *C. jejuni* 81-176 into the mutant was successful to restore the parental phenotype. The motility zone of this revertant was 46.2 mm ± 2.8. The results are shown in Fig. 3.7 and 3.8.

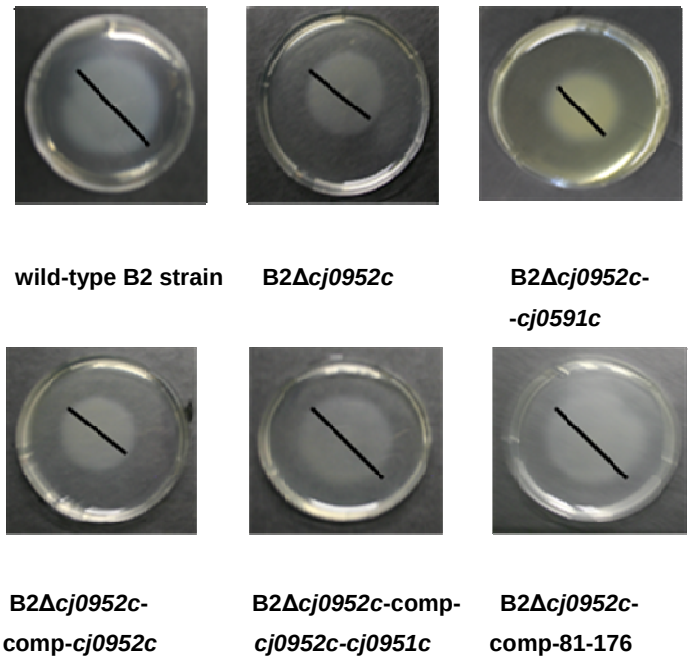

Figure 3.7 Motility of wild type strain, complemented mutants and *cj0952c*-mutant of *C. jejuni* strain B2

The motility of B2Δ*cj0952c* compared to wild-type strain B2 is not restored after introduction of *cj0952c* or *cj0951c* alone but only in combination of *cj0952c* and *cj0951c* or introduction of the corresponding gene from *C. jejuni* strain 81-176. Diagonal lines show the bacterial spread on the agar plates.

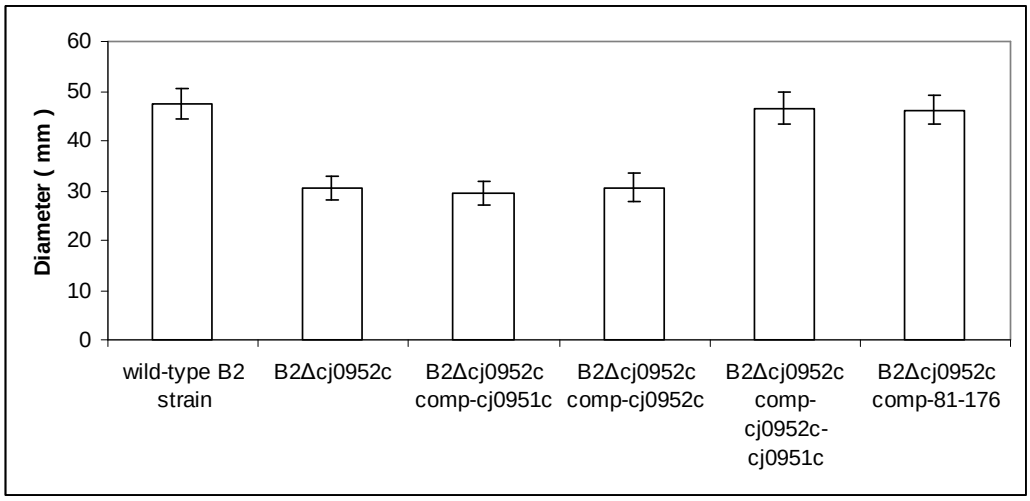

Figure 3.8 Motility zones of the wild-type strain B2, the mutant B2 Δ*cj0952c* and the complemented mutants

Motility assays were carried out on semisolid 0.4 % Mueller Hinton agar plates inoculated with the indicated strains and incubated at 42 ^{0}C under microaerophilic conditions. Each experiment was performed seven times. See text for details.

3.3.5 The *C. jejuni* proteins Cj0952c and Cj0951c alters the chemotactical behaviour of the pathogen in the presence of formic acid

Chemotaxis assays were performed to investigate the chemotactical behaviour of the B2Δ*cj0952c* mutant compared to that of the wild type strain B2 in order to investigate the function of Cj0952c-Cj0951c. For both strains, the accumulation of bacterial cells indicate chemoattraction around the plugs containing L-asparagine, L-aspartate, L-cysteine, fumarate, L-glutamate, D-lactate, L-(-)-malate, pyruvate, L-serine and succinate. Repulsion was demonstrated around the plugs containing cholic acid, deoxycholic acid, glycocholic acid and taucholic acid while no taxis response could be detected from PBS, L-fucose, and citrate. No significant differences in the extent of attraction or repulsion between wild type strain B2 and the mutant B2Δ*cj0952c* could be identified (not shown).

In contrast, in the case of formic acid a clear difference was observed. It was recently described that formic acid serves as chemoattractant for *C. jejuni* (Vegge

et al., 2009). In accordance with the results of this publication, we demonstrated a significant attraction halo for the parental strain B2 around the fomate-sodden paper disc, whereas for B2Δ*cj0952c*, a biphasic halo with an inner repulsion zone surrounded by a diminished ring was observed (Fig. 3.9). The chemotactical behaviour was restored by complementation of the mutant with *cj0952c-cj0951c* or the corresponding gene from *C. jejuni* 81-176, but no restoration occurred when the mutant was complemented with *cj0952c* or *cj0951c* alone (Fig 3.9). After four independent experiments, the attraction zones from B2 and the mutant complemented with genes *cj0952c* and *cj0951c* as well as the gene from *C. jejuni* 81-176 were 26.5 mm (± 2.6), 25 mm (± 2.5) and 25.5 mm (± 2.6), whereas the attraction zones of the mutant and the mutant complemented either with *cj0952c* or *cj0951c* alone were 10.2 mm (± 0.9), 10.0 mm (± 1.4) and 10.2 mm (± 1.7) as shown in Table 3.3. These results gave strong evidence that the genes *cj0952c* and *cj0951c* together are encoding a chemoreceptor which is involved in the chemotactical recognition of formic acid.

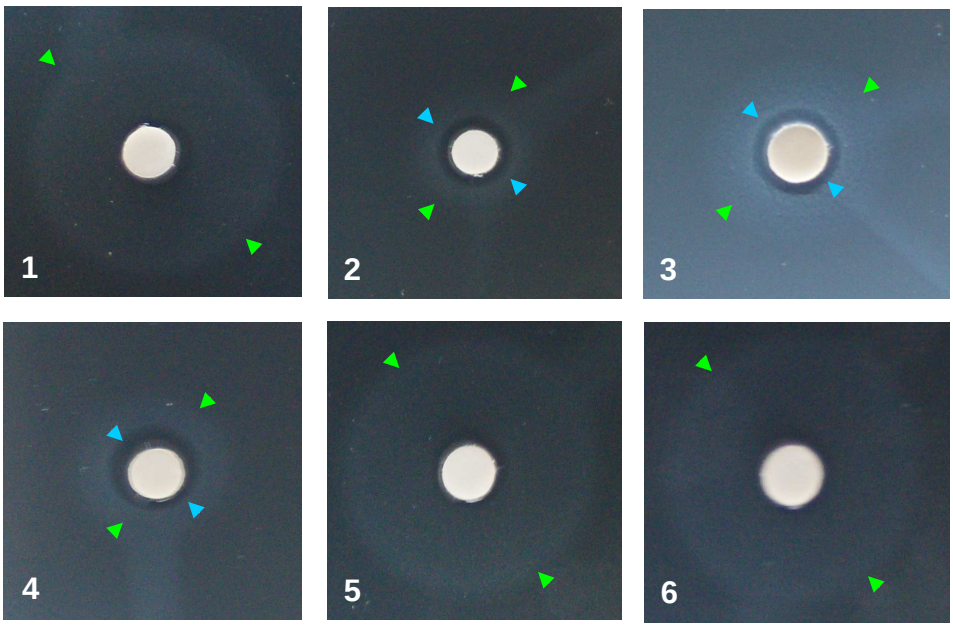

Figure 3.9 Photographical representation of the chemotactical behaviour of the investigated strains in the presence of formic acid

1. wild-type strain B2, 2. B2Δ*cj0952c*, 3. B2Δ*cj0952c*-comp-*cj0951c*, 4. B2Δ*cj0952c*-comp-*cj0952c* 5. B2Δ*cj0952c*-comp-*cj0952c-cj0951c*, 6. B2Δ*cj0952c*-comp-*81-176*. Whereas wild-type strain B2, the mutant strain B2Δ*cj0952c* complemented with *cj0952c-cj0951c* or the corresponding gene from *C. jejini* strain 81-176 clearly demonstrated chemoattraction around the formate sodden paper (green arrows), mutant B2Δ*cj0952c* as the mutant complemented with *cj0952c* or *cj0951c* alone demonstrated a diversified chemotactical response: an inner repulsion zone (blue arrow) could be observed surrounded by a dense chemo attractant ring (green arrow).

Table 3.3 Chemoattraction zones of *C. jejuni* wild type strain, Complemented mutants and mutant are indicated in mm

Strain	Chemoattraction zone (mm)	Standard deviation (mm)
wild-type B2	26.50	± 2.64
B2Δ*cj0952c*	10.25	± 0.95
B2Δ*cj0952c* comp-*cj0951c*	10.25	± 1.70
B2Δ*cj0952c* comp-*cj0952c*	10.00	± 1.41
B2Δ*cj0952c* comp-*cj0952c-cj0951c*	25.00	± 2.58
B2Δ*cj0952c* comp-81-176	25.5	± 2.64

Chemotactical response of wild-type B2 and the mutants B2Δ*cj0952c* complemented with *cj0952c-cj0951c* or the respective gene from strain 81-176 are identical. In contrast, neither *cj0952c* nor *cj0951c* did restore the parental phenotype and exhibited significantly reduced chemotaxis ($P<0.001$) towards formic acid.

3.3.6 The presence or absence of Cj0952c-Cj0951c does not affect the usage of formic acid

Both, immunoblot analysis and molecular genetic approaches indicate that both proteins are translated separately and not as a single chemoreceptor. For further investigation of this unusual composed chemoreceptor, Cj0952c-Cj0951c we examined if this composition is correlated with the utilization of formic acid. The growth of the wild-type strain B2, the mutant, the *cj0952c-cj0951c*-complemented mutant and the mutant harbouring the single gene from *C. jejuni* 81-176 was measured in the presence of 10 mM formic acid. As a negative control, a formate dehydrogenase-deficient mutant of the B2 strain (B2Δ*fdh*) was used. Thereby, no correlation of the chemoreceptor presence or composition and formic acid utilization was found. Wild type strain B2, the *cj0952c*-mutant and the complemented mutants showed exponentially identical proliferation while the growth of B2Δ*fdh* was clearly reduced (Fig 3.10a). Increased growth of bacterial

strains under investigation in the absence of formic acid demonstrated comparable results although the growth of B2Δ*fdh* was slightly shifted, eventually due to the presence of minor traces of formic acid in the MH broth which could not be metabolized by B2Δ*fdh* (Fig 3.10b).

a) b)

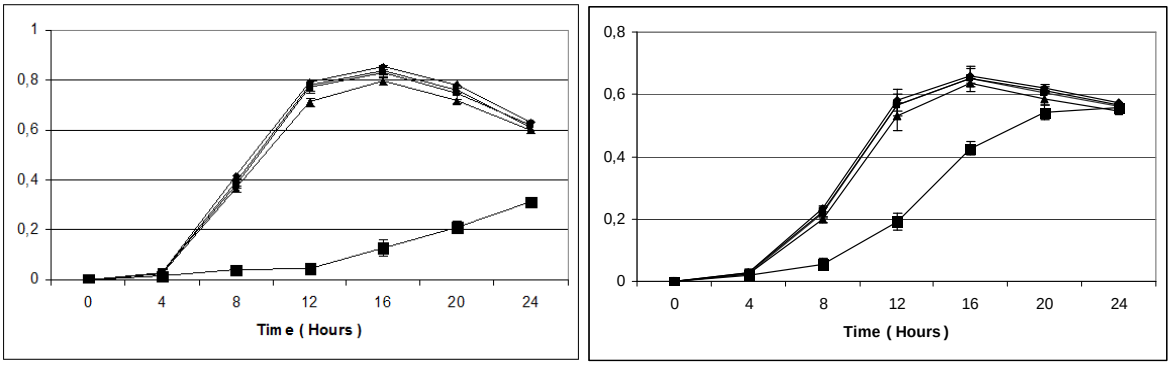

Figure 3.10 Growth curve with and without 10 mM formic acid

Growth curve of parental strain B2 (♦), B2Δ*cj0952c* (▲), B2Δ*cj0952c*-comp-*cj0952c-cj0951c* (■), B2Δ*cj0952c*-comp-81-176 (●) and (B2Δ*fdh* (■) in MH broth supplemented (a), or not supplemented (b) with 10 mM formic acid. The y axis shows the optical density measured at 600 nm. Error bars are indicated. See text for details.

3.4 Characterization of *cj0005c*

The gene product of *cj0005c* is encoding a molybdopterin oxidoreductase which, together with the monohaem cytochrome c oxidoreductase (*cj0004c),* constitutes a sulphite:cytochrome c oxidoreductase (SOR). Gentamicin protection assays on Caco2 cells with parental strain B2 and the mutant B2Δ*cj0005c* clearly demonstrated the declined infectivity of the mutant compared to the wild type strain (Fig. 3.12.) The number of colonies recovered from wild type strain B2 was represented as 100%. The recovery rate of the mutant varied between 22 and 28 %.

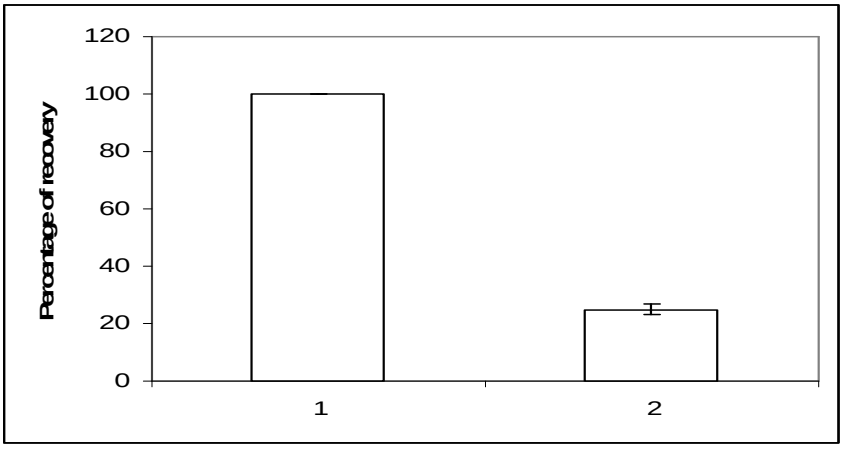

Figure 3.11 Recovery of *C. jejuni* bacteria after invasion assays on Caco2 cells

(1) Parental strain B2, (2) the mutant of B2 carrying a transposon insertion in gene *cj0005c* (B2Δ*cj0005c*). The recovery rate of bacteria from *B2Δcj0005c* is clearly reduced compared to the wild type strain. Given the number of colonies reclaimed from strain B2 is defined as 100 %, the number of colonies from *B2Δcj0005c* varied between 22 and 28 % with a mean value of 25 % (± 6.5) and a P-Value of <0.0001.

3.4.1 Construction of a *cj0005c* knock-out mutant and complementation of the mutant phenotype

For validation and confirmation of the result described above, homologous recombination was applied to knockout the *cj0005c* gene in order to verify, that the invasion-deficient phenotype of *C. jejuni* B2Δ*cj0005c* was due to the transposon insertion into gene *cj0005c* and not to other genetic rearrangements somewhere in the genome. A recombinant plasmid carrying the *cj0005c* gene interrupted by a kan^R gene cassette was introduced into strain B2. Subsequent PCR analysis were performed with genomic DNA from the obtained *C. jejuni* clones for the detection of an amplicon corresponding of the size of *cj0005c* including the *aphA*-3 cassette (B2::*cj0005c*) (Fig. 3.12b). Using gentamicin protection assays, the knock-out mutant B2::*cj0005c* confirmed the phenotype of *C. jejuni* strain B2 harbouring the transposon within *cj0005c*, which strengthens the finding that the mutation of *cj0005c* is responsible for the invasion-deficient phenotype (Fig. 3.12c). For the restoration of the phenotype of parental strain B2,

cj0005c was cloned into *C. jejuni* expression plasmid pRRC and introduced into B2::*cj0005c* to obtain B2::*cj0005c*-comp-*cj0005c* (Fig. 3.12a). Afterwards, the invasion assays were repeated with wild-type strain B2, both mutants and the complemented knock-out mutant. The number of B2 colonies recovered was defined as 100 %, the mean values of invasion capacities of B2Δ*cj0005c*, B2::*cj0005c* and B2::*cj0005c*-comp-*cj0005c* were 25 %, 29.8 % and 85.6 % respectively. Since the P-values of both mutants were less than 0.0001, the altered invasion capacities were strongly significant. In contrast, the P-value for the complemented knock-out mutant to be less invasive was only 0.16, which showed no significant difference compared to the wild-type strain B2. (Fig. 3.12c).

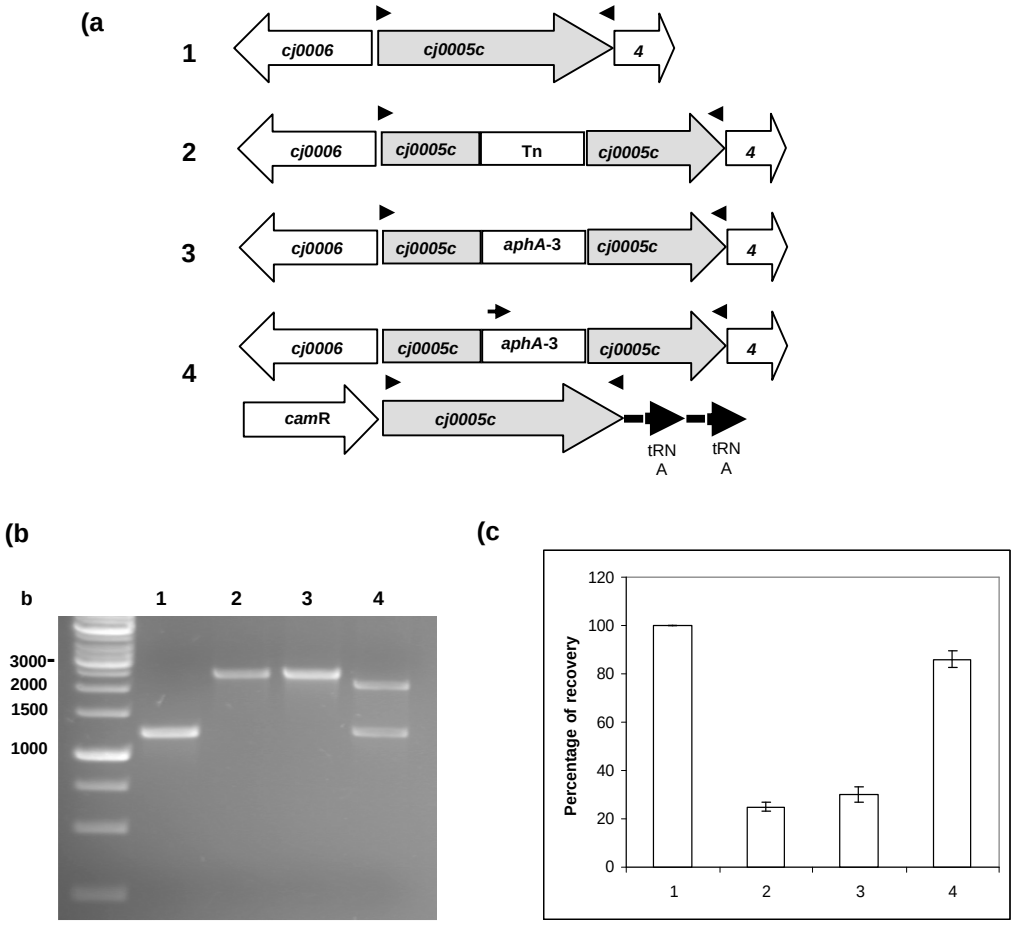

Figure. 3.12 Genome arrangements, verification of cloning procedures and invasion assays of the strains under investigation

(1) parental strain B2. (2) B2 with transposon insertion in *cj0005c* (B2Δ*cj0005c*), (3) *cj0005c* knock-out mutant (B2::*cj0005c*), (4) complemented knock-out mutant (B2::*cj0005c*-comp-*cj0005c*). (a) Genome disposals of the investigated bacterial strains. Primers Cj0005cF and Cj0005cR for the amplification of *cj0005c* are shown as arrowheads. The primer Kan1 that binds to the 5'-end of the kanamycin resistance cassette is illustrated as an arrow. (b) Detection of the native gene, the respective mutants and the complemented strain by PCR analysis. The native *cj0005c* gene has a size of 1239 bp (1). Insertion of the transposon and insertion of the kanamycin resistance gene yielded PCR-fragments of 2932 bp (2) and 2623 bp (3), respectively. PCR analysis of the complemented knock out mutant using the primers Cj0005cF and Cj0005cR revealed only the amplification of the wild type gene, probably because of its smaller size (not shown). To detect both, the gene with the kanamycin resistance cassette (2234 bp) and the native gene (1239 bp), PCR analysis with primers Cj0005cF and Cj0005cR and a third primer (Kan1) corresponding to the 5'-end of the *aphA*-3 gene were carried out (4). (c) Gentamicin protection assays. Invasion-

reduced phenotype of B2Δ*cj0005c* could be confirmed after specific knock-out of *cj0005c* (B2::*cj0005c*). Complementation of the knock-out mutant, in turn, reconstituted the parental phenotype. See text for details.

3.4.2 The loss of a functional SOR leads to reduced growth in the presence of sodium sulphite

In order to measure the effect of the SOR knock-out gene product on the proliferation of *C. jejuni*, growth experiments were carried out, both, in the presence and the absence of sodium sulphite. No distinguishable differences were found in the growth of the parental strain B2, the knock-out mutant B2::*cj0005c* and the complemented mutant in the absence of sodium sulphite. In contrast, in the presence of 10 mM sodium sulphite, the growth of the mutant exhibited a clear reduction compared to B2 and B2::*cj0005c*-comp-*cj0005c* (Fig. 3.13).

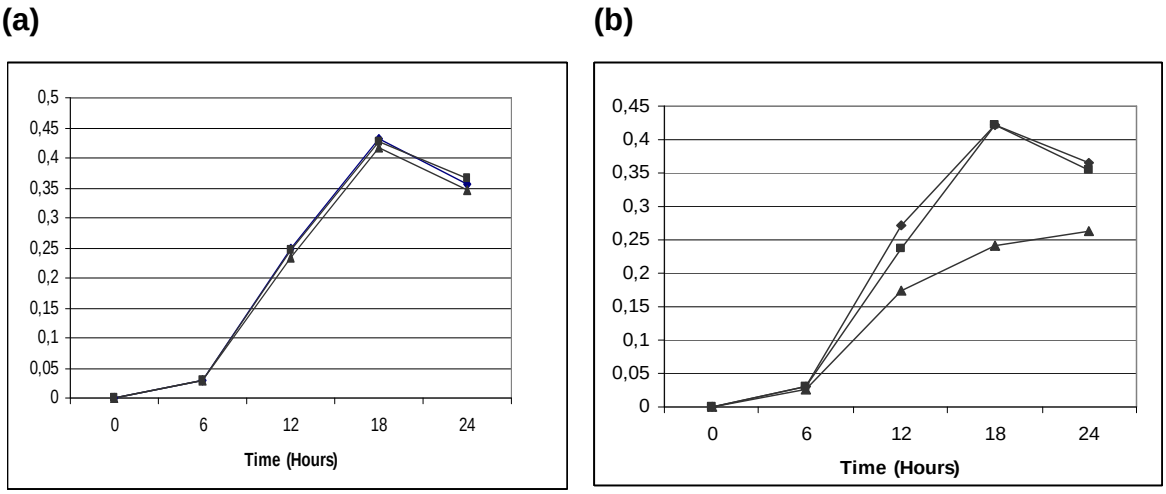

Figure. 3.13 Growth curve in the presence and absence of 10 mM Na_2SO_3

Parental strain B2 (♦), B2::*cj0005c* (▲) and complemented mutant B2::*cj0005c*-comp-*cj0005c* (■) not supplemented (a) and supplemented (b) with 10 mM Na_2SO_3. The optical density measured at 600 nm is shown on the y axis.

3.4.3 The absence of a functional SOR reduces the motility of *C. jejuni*

As reduced growth in case of an incomplete energy metabolism was detected, It was examined that if the motility of the pathogen was also diminished, since motility is known to be energy consuming. When the motility of the parental strain B2, the knock-out mutant and the complemented knock-out mutant was compared by using semi-solid motility medium, a loss of motility of the mutant B2::*cj0005c* could be clearly detected which could be reconstituted completely to the wild-type strain motility after introduction of a functional *cj0005c* gene into the mutant. The results of four independent motility assays performed are summarized in Fig. 3.14a. The mean diameter zone of motility for strain B2 was 40 mm ± 4.6, while the motility of the mutant B2::*cj0005c* was significantly reduced (18.2 mm ± 1.7, $P<0.01$) compared to B2. After introduction of a functional copy of gene *cj0005c*, the revertant returned to wild-type motility level with zone diameters of 38.7 mm ± 4.1 in average. These findings raised the idea that the reduced infectivity is first of all caused by a lack of motility as a result of a limited energy metabolism.

3.4.4 Reduced motility conduces to decreased invasiveness

To investigate whether the reduced motility is the sole reason for the strongly reduced invasion capacity of the *cj0005c*-deficient mutant or if any other factors are involved, motility was simulated by centrifugation of the bacteria onto the Caco2 host cells. Afterwards, gentamicin protection assays were performed again. Thereby, fixing the number of colonies recovered from wild-type strain B2 as 100 %, we detected 48.6 % of colonies ($P<0.01$) after centrifugation of the knock-out mutant B2::*cj0005c*, while the number of colonies recovered of the complemented mutant was 87 % in average which was not significantly different compared to the wild-type strain (Fig. 3.14b). Compared to the outcome of the gentamicin protection assays without centrifugation (Fig. 3.11) an increase of mutant colonies from 25 % up to 48.6 %.was clearly detected This finding was interesting, since one would have expected to shift infectivity of the mutant up to wild-type level after absorbing motility as a virulence factor in case that reduced motility is the only reason for diminished infectivity. But this result indicates that the reduced motility

is one, but not the only factor responsible for the clearly reduced invasiveness of the *cj0005c*-deficient mutant.

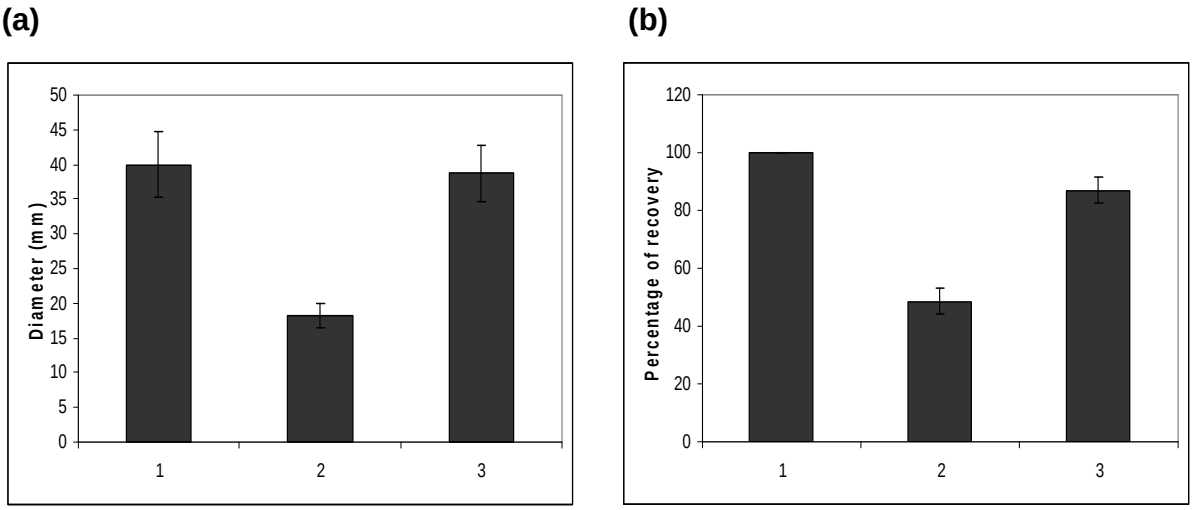

Figure. 3.14 Motility assays and gentamycine protection assays after centrifugation

1. Wild-type strain B2, 2. knock-out mutant B2::*cj0005c*, 3. complemented mutant B2::*cj0005c*-comp-*cj0005c.* (a) Motility assays. The diameter of the motility zones are indicated as bars. The *cj0005c*-deficient mutant possesses a clearly reduced motility compared to parental strain B2 which could be complemented after introduction of an intact copy of gene *cj0005c*. (b) Gentamicin protection assays after centrifugation of the bacteria onto Caco2 cells. Defining the number of isolate B2 colonies recovered as 100 %, the percentage of colonies of the knock-out mutant B::*cj0005c* colonies was 48.6 % in average, whereas complementation of the mutant restored the parental phenotype to 87 %. See text for details.

3.4.5 Diminished adherence contributes to reduced invasiveness

In order to characterize the process of invasion more in detail, It was investigated if already the adherence of the energy deficient mutant is hampered. Therefore, adhesion assays of wild-type isolate B2, the mutant B2::*cj0005c* and the complemented mutant were performed. To mimic the different motility of the bacteria under investigation, again, the bacteria were centrifuged onto the Caco2 cells. Thereby, an adhesion-deficient phenotype of the mutant could be clearly detected which could be restored to wild-type behaviour after complementation. Taken the number of colonies recovered from parental strain B2 as 100 %, the

mean value of colonies from the mutant B2::*cj0005c* was 67 % (± 5.1, P<0.0001) in contrast to B2::*cj0005c*-comp-*cj0005c* with a rate of recovery of 94 % (± 2.2) (Fig. 3.15).

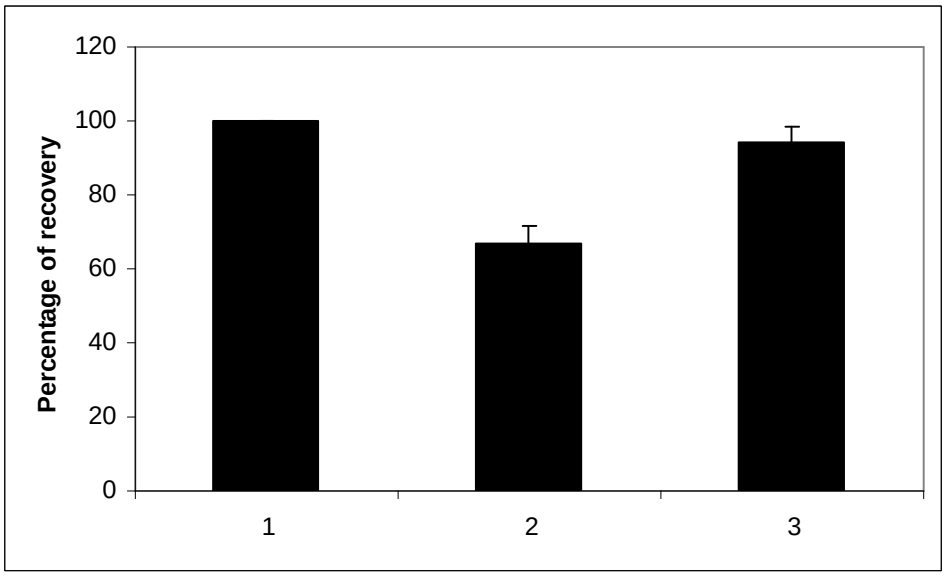

Figure 3.15 Adhesion assay

1. Wild-type strain B2, 2. knock-out mutant B2::*cj0005c*, 3. complemented mutant B2::*cj0005c*-comp-*cj0005c*. The adherence of the mutant B2::*cj0005c* onto Caco2 cells was incomplete compared to the wild-type strain. The percentage of adhered colonies of the mutant was only 67 % compared to wild-type isolate B2. See text for details.

3.4.6 The transcription of virulence associated genes is down-regulated in the absence of a functional SOR

After the detection that the reduced motility is one, but not the only reason for the decreased invasiveness of the mutant which is deficient in its energy metabolism, the transcription level of genes known to be involved in virulence of the pathogen was examined by semi-quantitative real time RT-PCR. The genes examined were the heat shock proteins *groEL* and *dnaJ* (Thies et al., 1999; Konkel et al., 1998),

the chemotaxis regulator gene *cheY* (Yao et al., 1997), and the putative chemoreceptor *tlp7* (Marchant et al., 2002), also genes known to be crucial for the process of binding and adhesion*,* like the gene for the fibronectin binding protein *cadF* (Konkel et al., 1997), as well as genes *peb1a* and *jlpA* known to encode adhesion-relevant proteins (Pei and Blaser, 1993; Jin et al., 2001). Furthermore, the transcription level of the *Campylobacter* invasive antigen *ciaB* (Rivera-Amill et al., 2001; Konkel et al., 2004), the cytothelial distending toxin *cdtB* (Pickett and Whitehouse, 1999) and *flaA* (Guerry et al., 1991) were compared. Furthermore the transcription of genes responsible for *O*-glycosylation of the flagellum by the synthesis of pseudaminic acid (PseAc) and legionaminic acid (LegAm) was examined (Logan et al., 2008). Thereby, a statistically significant 5.16 fold down-regulation of *dnaJ* in the mutant compared to the transcription level of this gene in wild-type strain B2 was clearly detected ($P<0.001$). Genes *ptmB, ptmC* and *ptmE*, which are involved in the synthesis of legionaminic acid (LegAm) were significantly down-regulated between factor 4.31 to 7.67 ($P<0.0001$) while other genes which are part of the legionaminic acid metabolism were downregulated significantly as well, but to a lesser extent (*ptmA, ptmF* or *ptmG)*. In contrast, the transcription level of all the other genes studied was not changed significantly irrespective of the presence or absence of SOR. The results are summarized in Table 3.4.The RNA level of mutant complemented with the intact version of *cj0005c* was examined, it was observed which that the crossing point for the respective genes which were almost identical with the wild type (Table 3.5)

Table 3.4. Transcription level of selected virulence-associated genes from *C. jejuni* in wild-type strain B2 and mutant B2::*cj0005c*

Gene	B2::*cj0005c*	B2 wild-type		Function
	Cp (mean value)	Cp (mean value)	Fold change (*t*-test P-value)	
GroEL	9.79 (± 0.002)	9.52 (± 0.02)	1.20 (0.03)	Heat-shock response
DnaJ	**23.34 (± 0.09)**	**20.97 (± 0.05)**	**5.16 (0.0001)**	**Heat shock response**
DnaK	12.8 (± 0.03)	12.64 (± 0.001)	1.11 (0.03)	Heat shock response
CheY	12.87 (±0.0002)	11.86 (± 0.87)	2.01 (0.13)	Chemotaxis response regulator
tlp7	6.36 (± 0.02)	6.66 (± 0.02)	1.23 (0.07)	Chemotaxis receptor
CadF	11.70 (± 0.008)	11.26 (± 1.15)	1.35 (0.51)	Fibronectin binding outer membrane protein
peb1a	9.66 (± 0.05)	9.78 (± 0.02)	1.08 (0.52)	Periplasmic binding protein
JlpA	13.84 (± 2.4)	13.44 (± 1.8)	1.31 (0.75)	Surface-exposed lipoprotein
CiaB	20.94 (± 0.08)	21.49 (± 0.09)	1.46 (0.08)	*Campylobacter* invasive antigen B
CdtB	13.55 (± 0.86)	13.84 (± 0.05)	1.22 (0.62)	Cytolethal distending toxin B
FlaA	11.52 (± 0.04)	11.98 (± 0.001)	1.37 (0.01)	Flagellum
FlpA	15.76 (± 0.27)	15.1 (± 0.10)	1.58 (0.10)	Adhesin
PorA	07.23 (± 0.50)	6.63 (± 0.66)	1.51 (0.058)	Adhesin
PglE	16.39 (± 0.71)	16.02 (± 1.03)	1.29 (0.64)	Protein glycosylation
PtmA	22.29 (± 0.55)	20.79 (± 0.17)	2.82 (0.0001)	LegAm synthesis
PtmB	**19.40 (± 0.77)**	**16.83 (± 0.01)**	**5.93 (0.0001)**	**LegAm synthesis**
PtmC	**18.31 (± 0.47)**	**16.20 (± 1.19)**	**4.31 (0.0001)**	**LegAm synthesis**
PtmD	20.53 (± 0.59)	20.16 (± 0.72)	1.29 (0.39)	LegAm synthesis
PtmE	**18.11 (± 0.06)**	**15.17 (± 0.38)**	**7.67 (0.0001)**	**LegAm synthesis**
PtmF	19.74 (± 0.50)	18.30 (± 0.89)	2.71 (0.0001)	LegAm synthesis
PtmG	19.16 (± 0.25)	17.64 (± 0.15)	2.86 (0.0001)	LegAm synthesis
PtmH	20.17 (± 0.26)	18.82 (±0.12)	2.54. (0.017)	LegAM synthesis
PseA	23.04 (± 0.48)	21.86 (± 0.19)	2.26 (0.016)	PseAc synthesis
PseB	20.71 (± 0.02)	21.29 (± 0.42)	1.49 (0.11)	PseAc synthesis
PseC	20.74 (± 0.05)	20.74 (± 0.016)	0.00 (0.80)	PseAc synthesis

PseD	23.30 (± 0.60)	21.64 (± 0.20)	3.16 (0.0001)	PseAc synthesis
PseE	21.35 (± 0.21)	19.8 (± 0.05)	2.92 (0.067)	PseAc synthesis

Strongly downregulated genes are indicated in bold. See text for details.

In contrast, when we compared the transcription level of the genes which are down-regulated in the complemented mutant with the transcription level of the respective genes in the parental strain, the values for the crossing points were almost identical. (Table 3.5).

Table 3.5. Restored transcription levels of genes in the complemented mutant compared to parental strain B2

Gene	B2::*cj0005c* *comp-cj0005c*	Fold change (Compared to wild-type B2
	Cp (mean value)	(*t*-test P-value)
ptmA	20.87 (± 0.05)	1.05 (0.571)
PtmB	17.45 (± 0.079)	1.53 (0.071)
PtmC	16.63 (± 0.87)	1.34 (0.407)
PtmE	15.6 (± 0.20)	1.34 (0.316)
PtmF	19.74 (± 0.50)	2.71 (0.014)
PtmG	17.62 (± 0.07)	1.01 (0.127))
PseD	22.39 (± 0.60)	1.68 (0.242)
DnaJ	21.11 (± 0.06)	1.01 (0.071)

3.4.7 Reduced autoagglutination of mutant B2::*cj0005c*

Recently, Howard and coworkers (2010) reported that *C. jejuni* mutants deficient in gene *cj1324 (ptmG)* or genes *cj1321-1325/6,* which are responsible for the legionaminic acid synthesis, showed a significantly decreased capacity for autoagglutination. When the mutant B2::*cj0005c* was compared with the wild-type strain B2, a significant down-regulation of *ptmG*-transcription and of other genes of the legionaminic acid metabolism was detected. For this, the competency of autoagglutination of mutant B2::*cj0005c* compared to wild-type strain B2 was investigated. As is shown in Fig 3.16, the autoagglutination ability of the mutant

strain is actually diminished. The percent autoagglutination of wild-type strain B2 and complemented mutant was 74.24 % (± 0.25) and 73.65 % (± 0.22), respectively, whereas the mutant B2::*cj0005c* had a remaining autoagglutination level of only 37.02 % (± 0.61) which was a significant reduction compared to B2 ($P<0.0001$).

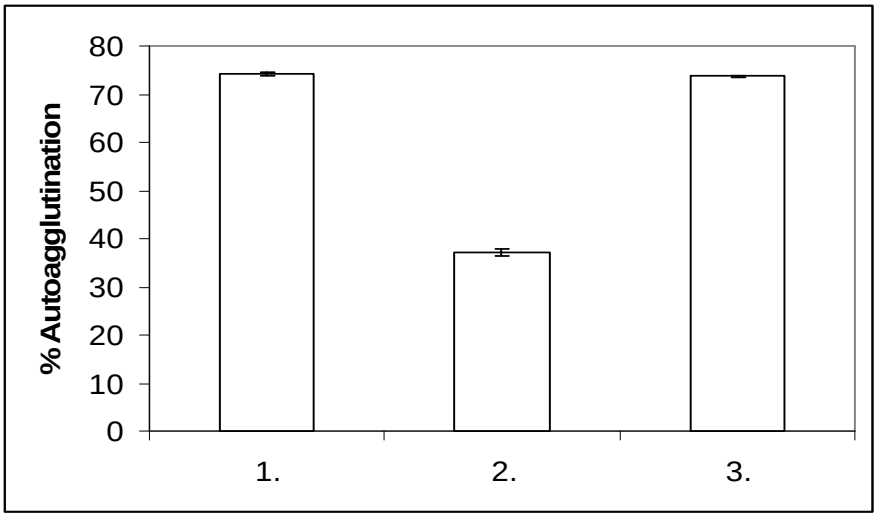

Figure. 3.16. Autoagglutination

Autoagglutination of mutant B2::*cj0005c* was compared to wild-type strain B2 and complemented mutant. 1. wild-type strain B2, 2. knockout mutant B2::*cj0005c*, 3. complemented mutant B2::*cj0005c*-comp-*cj0005c*. See text for details

4. DISCUSSION

Transposon insertions are interrupting the function of genes and are thus a tool for generating random mutations (Spradlingand and Rubin, 1982). For this, transposon mutagenesis techniques have also been used in *C. jejuni* research to detect genes which contribute to the pathogenicity of the bacterium (Colegio et al., 2001; Golden et al., 2000). Thereby, motility deficient mutants have been examined which carried the transposon in genes that have not been correlated with motility but in genes which belong to the chemotaxis machinery (Golden and Acheson, 2002; Hendrixson et al., 2001). Furthermore, transposon mutagenesis coupled with reporter assays have been used for the identification of genes involved in flageller regulation at the beginning of the flagellar transcription cascade (Hendrixson and DiRita, 2003) and were used to study the nature of *Campylobacter* colonization in the gastrointestinal tract of chickens (Grant et al., 2005), including the gene encoding the methyl-accepting chemotaxis protein *docB* (tlp 10) (Hendrixson and DiRita, 2004). By using transposon-based mutations several genes were identified that play an important role for host cell invasion and also for *C. jejuni* colonization (Novik et al., 2010). Javed and coworkers (2009) used transposon mutagenesis in a hyper-invasive clinical isolate of *C. jejuni* to detect 26 mutants with a clearly reduced invasion capacity. By applying transposon mutagenesis, *C. jejuni* genes which play an important role for the molecular basis of resistance to polymyxin and natural antimicrobial peptides could be identified (Lin et al., 2009).

The present study was undertaken to uncover additional genes of *C. jejuni* that are associated with the invasion process. A total of 660 clones of an existing transposon-based library of clinical *C. jejuni* isolate B2 were screened for reduced invasion of Caco2 cells. Thereby, a clone with a transposon insertion in gene *cj0952c*, which shows a strong homology to genes representing methyl-accepting chemotaxis proteins (MCPs), was detected. Like all other motile bacteria, *C. jejuni* has the competence to sense its external environment through chemical

gradients. Cellular motility and chemotaxis have been connected to virulence of pathogenic bacteria and play a vital role in colonization and invasion of the host intestinal tract (Josenhans and Suerbaum, 2002). Initially, *cj0952c* was thought to be a pseudogene, since the immediately downstream located gene *cj0951c* which is encoding a signaling domain, together with Cj0952c, might act as a complete chemoreceptor. Performing BLASTN analysis in the NCBI database, in the *C. jejuni* strains NCTC 11168, CF93-6 and 84-25, the mentioned chemoreceptors is composed of two single genes. Similarly, the genes *cj0951c and cj0952c* are present separately in the clinical isolate B2. On contrary, in strains 81-176 or 81116, the corresponding chemoreceptor is the product of only one single gene encompassing the MCP domain, the transmembrane domain and the signaling domain as well. To date, *cj0952c* has not been described functionally, and was only demonstrated to be up-regulated during the process of chicken colonization (Woodall et al., 2005). As in many other bacteria, *C. jejuni* resides genes indispensable for chemotaxis like *cheA, chew, cheY* and MCPs receptors. It is suggested that interaction of CheW and CheV is necessary to regulate CheA activity via the MCPs (Rosario et al., 1994). Moreover, it has been shown that *C. jejuni* colonization is only possible with the help of chemotactic-mediated motility (Takata et al., 1992; Yao et al*.,* 1994). Altogether ten genes encoding chemotaxis proteins known as transducer-like proteins (Tlps) were identified in the genome of *C. jejuni*. These ten Tlps can further be classified according to their predicted domain organization into three different groups (A-C) (Marchant et al., 2002). The Tlp group A is further divided in subgroups Tlp1, 2, 3, 4, 7 and 10. Thereby, Tlp1 has been demonstrated to be the aspartate chemoreceptor of *C. jejuni* and plays a role in the colonization of the intestinal tract (Hartley-Tassell et al., 2010).

Predicted structures of group A Tlps shows classical MCPs organization, identical to that of *E. coli* MCPs, with a periplasmic sensory domain, which is extremely variable between different receptors, two transmembrane domains, and the C-terminal cytoplasmic signaling domain (Marchant et al., 2002). Cj0952c-Cj0951c (Tlp7) belongs to group A chemoreceptors which are MCPs and having four

domains that consist of a N-terminal transmembrane domain, a periplasmic ligand-binding domain, a HAMP domain and a C-terminal cytoplasmic signaling domain at the C-terminal end, in which the HAMP domain converts the ligand-induced conformational changes into kinase controlling signals. (Aravind and Ponting, 1999; Butler and Falke, 1998; LeMoual and Koshland, 1996; Williams and Stewart, 1999). Cj0951c shows strong homologies with the cytoplasmic signaling domain of MCPs that possess an adaption region with internal methylation sites. This flexible region mediates the interaction with kinase CheA and chemotaxis protein CheW (Hazelbauer et al., 2008).

In this study, it was shown that transposon insertion in gene *cj0952c* does not affect the transcription of the adjacent gene *cj0951c*. In order to create a functional chemoreceptor both genes have probably to be transcribed on the same RNA to allow the aggregation of both subunits to obtain one functionally active chemoreceptor.

To answer the question whether both genes are translated as one protein, the corresponding genes *in trans* in *C. jejuni* was expressed. For the detection of the recombinant protein a 6x His-tag was added to the C-terminus of Cj0951c. Subsequent western blot analysis only detected a protein that corresponded to the size of Cj0951c but not to the size of a protein that would indicate a protein composed of Cj0952c and Cj0951c. For this, in *C. jejuni* strain B2, a non-covalent interaction of both subunits might be postulated for the creation of a functional chemoreceptor.

Host-pathogen interaction is a prerequisite for *C. jejuni* pathogenicity and is mediated by the chemotaxis system as it directs the pathogen towards the host cell at the site of entry. Gene alterations in the chemotaxis apparatus reduce the strength of *C. jejuni* to infect human and chicken cells as well. Mutant strains which are deficient in *cj0262* (Tlp4, docC) or *cj0019* (Tlp10, docB) revealed a decreased colonization of the chicken intestinal cells (Hendrixson and DiRita,

2004). In additional, the chemoreceptors Tlp1 (*cj1506*), Tlp2 (*cj0144*), Tlp3 (*cj1564*), Tlp4 and Tlp10 are important for the disease-causing ability of the invading pathogen (Golden and Archeson, 2002; Hendrixson and DiRita, 2004; Vegge et al., 2009). However, less is known about the chemoreceptors of *C. jejuni*, so far.

Recently, Cj1506c (Tlp1) of *C. jejuni* was identified as the aspartate chemosensory receptor and was the first chemoreceptor of this pathogen described with respect to its particular ligand (Hartley et al., 2010). In this study it has described that the inactivation of gene *cj0952c* changes the chemotactic behaviour of *C. jejuni* with regard to formic acid. While in the wild-type strain formic acid was clearly a chemoattractant, the mutant showed a biphasic halo together with an inner repulsion zone which was surrounded by a diminished ring. The reduced, but still measurable attraction of the mutant towards formic acid might be mediated by the energy taxis system of the pathogen which consists of the two proteins CetA and CetB that act together as energy taxis receptors. In this system, changes in the redox state of the electron transport chain are sensed by CetB and are subsequently relayed to CetA via direct interaction. CetA further transduces the signal to the chemotactic machinery and leads to changes in the direction of motility (Hendrixson et al., 2001; Elliot and DiRita, 2008). Energy taxis has been shown to direct *C. jejuni* towards attractants which serve as carbon sources, electron acceptors and, as in the case of formic acid, electron donors (Vegge et al., 2009). The formation of an inner repulsion zone might suggest that another chemosensory mechanism exists that recognizes formic acid as a chemorepellent.

The enzyme formate dehydrogenase catalizes the oxidation of formate to CO_2, protons and electrons which are injected into the respiratory chain and, thus, serves as an electron donor. The enzyme consists of four subunits which are encoded by the *fdh*ABCD operon of *C. jejuni* encompassing the genes *cj1511c-cj1508c* (Weerakoon et al., 2009). In this study it was shown that the *C. jejuni*

chemoreceptor for the detection formic acid is composed by the proteins Cj0952c and Cj0951c. To investigate whether this unusual composition of the chemoreceptor is going along with the utilization of its particular chemoattractant, the growth curves in the presence and absence of formic acid was carried out. Thereby any differences in the proliferation of wild-type strain B2, the mutant B2Δ*cj0952c* and the complemented mutant was not detected. Interestingly, the growth of the bacteria in general (except of B2Δ*fdh*), significantly promoted in the presence of formic acid in medium, indicating that the generation of electrons by the oxidation of formic acid is an important process in the context of the energy metabolism of *C. jejuni*.

Many prokaryotes possess a complex electron transport chain to cope their energy requirement. Similarly, *C. jejuni* has a highly complex respiratory chain that allows the pathogen to use a variety of electron donors like, succinate, malate, D-lactate, hydrogen, formate, NAD(P)H and sulphite (Kelly, 2001; Sellars et al., 2002; Myers and Kelly, 2005; Weerakoon et al., 2009). In *C. jejuni* resides an enzyme named molybdenum-containing sulphite: cytochrome c oxidoreductase (SOR) which is involved in the oxidation of sulphite to sulphate and the release of electrons which are used as fuel in the respiratory chain. SOR is the ultimate product of two genes including *cj0005c* and *cj0004c* and has similarity to the corresponding enzyme of *Starkeya novella* (Jonathan and David, 2005). Gene *cj0004c* is responsible for encoding the monohaem cytochrome c subunit, whereas *cj0005c* encodes the molybdopterin oxidoreductase. Both subunits of the SOR work in a unifunctional manner and catalyze the conversion of sulphite to sulphate in the presence of oxygen to generate electrons which are used in the respiratory chain after the bc_1 complex at the level of cytochrome c (Kappler et al., 2000; Kappler and Dahl, 2001; Myers and Kelly, 2005).

In this work, a *cj0005c*-deficient mutant of *C. jejuni* with strongly diminished motility function of the pathogen and, therefore, a reduced ability to invade the host cells was investigated, which, in contrast to other motility deficient strains, is

limited in its energy metabolism instead of the flagellar apparatus. *C. jejuni* motility function is the key regulator to infect the assigned host, so we can say that motility is the main player responsible for *C. jejuni* colonization. In the recent years, several studies have documented the impact of genes and gene products of the flagellar apparatus with particular reference to the colonization or invasion of host cells. Morooka and coworkers (1985) described that motility-deficient strains of *C. jejuni* were eliminated from the intestinal tract of suckling mice, while a motility-competent strain colonized the lower portion of the intestine, the caecum as well as the colon. Pathogen colonization in this animal was shown to depend on the flagella, because a non-flagellated mutant was not able to survive inside the rabbit intestine (Pavlovskis et al., 1991). In addition, the chicken intestinal tract was shown to be invaded only by sound motile strains of *C. jejuni,* reflecting an intact flagellum to be an important invading factor of the pathogen to cause disease (Nachamkin et al., 1993). Mutants can be generated by homologous recombination procedures which can be subsequently investigated in *in vitro* cell culture experiments for their ability to infect the host cells. Thereby, the role of motility in the invasiveness of *C. jejuni* could also be established (Wassenaar et al., 1991; Yao et al., 1994; Szymanski et al., 1995; Konkel et al., 2004). In addition, numbers of coding sequences and gene products have been described that attributed to the flagellum function for successful motility which is further responsible for motility-mediated pathogen infectivity (Hendrixson, 2008).In order to exclude motility as a virulence factor, the wild-type strain B2, the mutant and the complemented mutant were centrifuged directly onto the host cells. Interestingly, the infectivity of the mutant up to 48.6 % was only restore compared to the parental strain, which clearly indicated that motility was only one, but not the only reason for the incomplete infectivity of the mutant. For this, it was investigated whether the energy-deficient genotype of the mutant has some effect on the transcription level of other virulence-related genes. Semi-quantitative real time RT-PCR was used to investigate selected genes associated with stress response, motility, chemotaxis as well as binding and adhesion. In addition, genes essential for the invasion of host cells by *C. jejuni* were examined. As a result of

these investigations, a significant down-regulation of *dnaJ* and of genes for the synthesis of legionaminic acid in the mutant compared to parental strain B2 was detected.

In most prokaryotes and eukayotes the DnaJ (Hsp 40) proteins reside as co-chaperones of their corresponding heat shock protein 70 (Hsp 70) partners (DnaKs) (Genevaux et al., 2007). The DnaJ family proteins have an N-terminal J-domain of approximately 70 amino acids (Hennessy et al., 2005). The molecular function of DnaJ is the interaction of the substrate-bound cochaperone with its corresponding ATP-bound DnaK protein via J-domain and ATPase domain, which mediates a subsequent conformational change of DnaK. Conformational change of DnaK, in turn, stimulates ATP-hydrolysis and is leading to high affinity and low exchange rates for its particular substrate (Liberek et al., 1991; Karzai et al., 1996; Laufen et al., 1999). DnaJ co-chaperones in *C. jejuni* were shown to be an integral part of the heat shock response of the invader (Konkel et al., 1998). The DnaJ protein sequences of *C. jejuni* exhibit 60.8 % homology with the corresponding protein of *E. coli.* It is also established that the function of an *E. coli dnaJ* deletion mutant could be restored by complementing the corresponding gene from *C. jejuni* strain F38011 (Konkel et al., 1998).

The acetoamidino form of legionaminic acid (LegAm) together with pseudaminic acid (PseAc) are *O*-linked to the flagellin protein of most *C. jejuni* strains (Logan et al., 2008). Previously, eight genes have been reported to be involved in the legionaminic acid synthetic pathway including *cj1324 (ptmG), cj1325/6 (ptmH), cj1327-cj1330 (ptmC, ptmD, ptmE, ptmF), cj1331 (ptmB)* and *cj1332 (ptmA)* gene (McNally et al., 2007). Although the role of *O*-glycosylation by legionaminic acid is still not well defined, recent investigations have given a first insight into its biological relevance (Howard et al., 2010). In their report they describe a *cj1324*-deficient mutant, which is fully motile but has a decreased autoagglutination and biofilm formation due to the lack of two legionaminic acid glycan modifications. Furthermore, this mutant possesses a diminished activity to invade chicken cells.

When the transcription level of the LegAm genes in the *cj0005c* mutant was investigated, only a 2.86 fold down-regulation of *cj1324 (ptmG)* was documented. In this study a reduced autoagglutination was shown, but the formation of biofilm was not affected (data not shown). In contrast to the study from Howard et al., (2010) the transcription level of all genes known to be involved in the synthesis of legionaminic acid was examined. Thereby a strong down-regulation of *ptmB (cj1331*), *ptmC (cj1327)* and *ptmE (1329),* in the *cj0005c*-deficient mutant was detected and a weaker down-regulation of genes *ptmA (cj1332), ptmF (cj1330)*, and *ptmG (cj1324)* compared to wild-type strain B2. Although transcription analysis was not carried out that covered the whole genome status of the pathogen, alterations in the transcription level of other genes are likely. Nevertheless, the results were obtained so far might indicate a correlation of the affected biological properties of the mutant (e.g. diminished adhesion and autoagglutination) with the downregulation of genes of the LegAm metabolism.

Taken together, two factors of *C. jejuni* that contribute to host cell invasion by two different mechanisms was identified. In the case of *cj0952c,* a chemoreceptor subunit is deleted, which probably reduces the proper accession of *C. jejuni* to its host cell by a restricted response towards the chemoattractant formic acid and, consequently, by reduced motility. In contrast, the *cj0005c*-deficient mutant is limited in motility and adhesion probably due to its narrowed energy metabolism.

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