Molecular mechanisms of *Campylobacter jejuni* induced transmigration and invasion of host target cells

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Abbreviations

A, C, G, T	Adenine, Cytosine, Guanine, Thymine
AIDS	Acquired Immunodeficiency Syndrome
Amp^R, Kan^R	Resistance to ampicillin, kanamycin
Arp2/3 complex	Actin-related protein 2/3 complex
ATP	Adenosine-5'-tri-phosphate
BHI	Brain Heart Infusion
bp	Base pair
BSA	Bovine serum albumin
CadF	*Campylobacter* adhesion to fibronectin
CapA	*Campylobacter* autotransporter protein A
Cdc42	Cell division cycle 42
cDNA	Complementary deoxyribo nucleic acid
CDT	Cytolethal distending toxin
CFU	Colony forming unit
Cia	*Campylobacter* invasion antigens
CPS	Capsular polysaccharide
CRIB	Cdc42/Rac1 Interactive Binding
ceu	*campylobacter*-enterochelin-uptake
DCs	Dendritic cells
Dlg1	Drosophila disc large tumor suppressor
D-MEM	Dulbecco's-Modified Eagle Medium
DOCK180	Dedicator of cytokinesis 180
dsRNA, mRNA, rRNA, siRNA	Double stranded, messenger, ribosomal, small interfering ribonucleic acid
e.g.	For example (*exempli gratia*)
ECM	Extracellular matrix
EDTA	Ethylene diamine tetracetic acid
EGFR	Epidermal growth factor receptor
EPEC	Enteropathogenic *Escherichia coli*
et al.,	And others (*et alii*)
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FESEM	Field Emission Scanning Electron Microscopy
FlpA	Fibronectin like protein A
Fn	Fibronectin
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBS	Guillain-Barré syndrome
GEF	Guanine nucleotide exchange factor
Grb2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
GDP, GTP	Guanosine 5' di-, tri-phosphate
GTPase	Guanosine triphosphatase
HIV	Human immunodeficiency virus
HtrA	High temperature requirement A
IgG	Immunoglobulin Class G
IL-8	Interleukin 8
INT-407	Intestinal epithelial cells
IP	Immunoprecipitation
IPTG	Isopropyl β-D-thiogalactopyranoside
JlpA	*jejuni* lipoprotein A

List of abbreviations

kDa	Kilodalton
LB	Luria-Bertani
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
MEM	Eagle's Minimum Essential Medium
MH	Mueller-Hinton
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
NF-κB	Nuclear factor κB
NOD	Nucleotide-binding oligomerization domain
OD_{600}	Optical density at λ=600 nm
ori	Origin of replication
P130 Cas	Crk-associated substrate
PAF	Population attributable fraction
PAK	p21 activated kinase
PBD	p21 Binding Domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PSD95	post synaptic density protein
PEB1	Periplasmic binding protein
PFA	Paraformaldehyde
PKC	Protein Kinase C
PI3 kinase	Phosphatidylinositol 3 kinase
PolyA	Poly-adenylate
PTK	Protein-tyrosine kinase
PY	Phospho-tyrosine
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RISC	RNA-induced silencing complex
RNAi	RNA interference
Rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute Media
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2, SH3	Src homology 2, 3
TBS	Tris buffer saline
TCA cycle	Tricarboxylic acid cycle
TER	Transepithelial electrical resistance
Tiam1	T-cell lymphoma invasion and metastasis 1
TLR	Toll-like receptor
T3SS, T4SS	Type III, IV secretion system
U	Unit of enzyme activity (μmol min^{-1} mg $protein^{-1}$)
v/v	Volume per volume
w/v	Weight per volume
wt	Wild-type
x *g*	x-times acceleration due to gravity (9,81m/s^2)
ZO-1	zonula occludens-1 protein

ABSTRACT

Campylobacter jejuni is one of the most important bacterial pathogens causing food-borne illness worldwide. In industrialized countries *Campylobacter* are found to cause gastroenteritis more than 2-7 times as frequently as compared to *Salmonella* and *Shigella* species. Crossing the host epithelial barrier and invasion by *C. jejuni* is considered as one of the primary reasons of gut tissue damage, but the molecular mechanisms and the major factors involved in this process are widely unclear.

In the present study, I characterized the serine protease HtrA (high-temperature requirement A) of *C. jejuni* and its function in secretion and cleavage of the host junctional protein and tumor suppressor E-cadherin. *In vitro* cleavage assays and infection experiments showed that secreted HtrA triggers E-cadherin ectodomain shedding from MKN-28 polarized epithelial cells. A deletion of the *htrA* gene in *C. jejuni* led to severe defects in E-cadherin cleavage and transmigration of the bacteria. These results suggest that HtrA-mediated E-cadherin cleavage is involved in *C. jejuni* crossing of the epithelial barrier via the paracellular route between cells and represents a novel mechanism in pathogenesis.

Having established how *C. jejuni* reaches basolateral surfaces, the next aim was to investigate how this pathogen can enter the host cells. Using different molecular biological methods and various knockout cell lines derived from fibronectin$^{-/-}$, integrin-β1$^{-/-}$ and focal adhesion kinase (FAK)$^{-/-}$ deficient mice and corresponding wild-type controls it could be shown, that these host cell factors play a role in the activation of small Rho GTPases, such as Rac1 and Cdc42. Furthermore, membrane ruffling, filopodia formation, tight engulfment of the bacteria and invasion were only seen during infection of wild-type control cells. It was also demonstrated that *C. jejuni* activates FAK autophosphorylation activity which is required for stimulation of the guanine exchange factors DOCK180, Tiam-1 and Vav2. siRNA studies show that DOCK180 and Tiam-1 act cooperatively to trigger Rac1 activation, while siRNA and the use of Vav-1/2 $^{-/-}$ knockout cells showed that Vav2 is required for Cdc42 activation and *C. jejuni* invasion. Moreover, evidence is presented that activation of Rac1 and Cdc42 involves the bacterial fibronectin-binding protein CadF and the flagella apparatus. CadF appears to be a bi-functional protein enabling bacterial binding to host cells and stimulating signalling, which leads to the activation of downstream factors inducing GTPase signalling and host cell invasion by *C. jejuni*. Collectively, the results of this work suggest that *C. jejuni* can invade host cells by a unique mechanism and that fibronectin, integrins, FAK and the small Rho GTPases Rac1 and Cdc42 play crucial roles in the invasion process.

2. INTRODUCTION

2.1 Invasion of the gastrointestinal tract: an infection strategy by bacteria

The gastrointestinal tract in humans is one of its largest and most important organs. In an adult male, the gastrointestinal tract can be up to 6.5 m in size and is covered by the intestinal epithelium giving rise to a total surface of approximately 400-500 m^2. This epithelium exhibits not only crucial absorptive and digestive properties, but also represents an efficient barrier against the existing commensal microbial flora as well as intruding food-borne microbial pathogens. The normal gut flora consists of more than 1,000 microbial species, which represent a highly complex and dynamic community (Hooper and Gordon 2001; Eckburg *et al*., 2005). Exclusion of these microbes is not only a result of the continuous physical barrier formed by the tightly associated epithelial cells; the intestinal epithelium also has important host immune functions in order to recognise and tolerate commensals, and to eliminate pathogens (Backert and König, 2005; Tegtmeyer *et al*., 2012). The human immune system controls the resident microflora and defends against microbial infections through two crucial functional arms, the innate and adaptive immunity (Sansonetti 2004; Tsolis *et al*., 2008).

An important feature of several foodborne pathogens is their ability to bind to and invade eukaryotic cells. For many pathogenic bacteria the invasion into eukaryotic cells is an important initial step during infection and provide the bacteria with different advantages, such as avoiding the attacks of the host immune system or the effects by antibiotic treatments (Falkow *et al*., 1992; Oelschlaeger *et al*., 1999; Dersch, 2002). To access deeper tissues and cause short- or longterm infections, the pathogenic bacteria must overcome the epithelial barrier (Kazmierczak *et al*., 2001). Bacterial pathogens are able to enter host epithelial cells by different routes, known as the paracellular route and the transcellular route. The transcellular route means, that bacteria entering apical cell surfaces and leave the cell at the basolateral surface. In contrast, bacteria using the paracellular route cross the epithelial barrier by entering between epithelial cells and overcome the tight junctions and adherens junctions (Balkovetz and Katz, 2003).Several bacteria are even able to invade deeper tissues during their invasion process, as well as some other bacteria are able to strongly increase their numbers and thus triggering the apoptosis of these cells or other responses. Many but not all invasive bacteria are also able to enter non-phagocytic cells and/or phagocytes, such as neutrophil granulocytes or macrophages and survive in these cells (Moss *et al*., 1999).

2. Introduction

Bacterial pathogens can be basically divided into two main categories, invasive and non-invasive bacteria. On the one hand invasive bacteria are able to induce their own uptake and on the other hand bacteria play a passive role during phagocytosis by phagocytes.

The success of an infection depends on both, the bacteria and the cell and the "signals" that they send to each other (Finlay and Cossart, 1997).
After binding to the cell, bacteria activate different signalling cascades, which are also important for other functions of the cells, such as cell migration, cell division, cell adhesion and endocytosis (Finlay and Cossart, 1997).
In contrast to passive phagocytosis several pathogenic bacteria can cause their internalization directly, activated by specific virulence factors. This kind of invasion process begins with the binding of specific bacterial surface proteins to certain host cell receptors. By these interactions different intracellular signal transduction pathways can be induced, resulting in various rearrangements of the cytoskeleton and changes in the cell membrane structure at the position of bacterial adhesion and trigger by this way the uptake of the bacteria (Finlay and Cossart, 1997; Galan, 1994). In general, two different strategies for bacteria uptake are distinguished, the "zipper"- and the "trigger"- mechanism (Fig.1A, B).
The receptor-mediated invasion process of pathogenic bacteria is characterized by actin polymerization and formation of membrane ruffles. These changes inducing the uptake of the bacteria into the host cell. Several well-known pathogens, such as *Listeria monocytogenes*, *Yersinia pseudotuberculosis* and *Staphylococcus aureus* use this "zipper" mechanism to invade host target cells. By binding of the bacteria, different host signalling pathways are activated without additional involvement of the pathogen (Cossart and Sansonetti, 2004). Figure 1A shows the several proposed steps of invasion by the "zipper" mechanism.

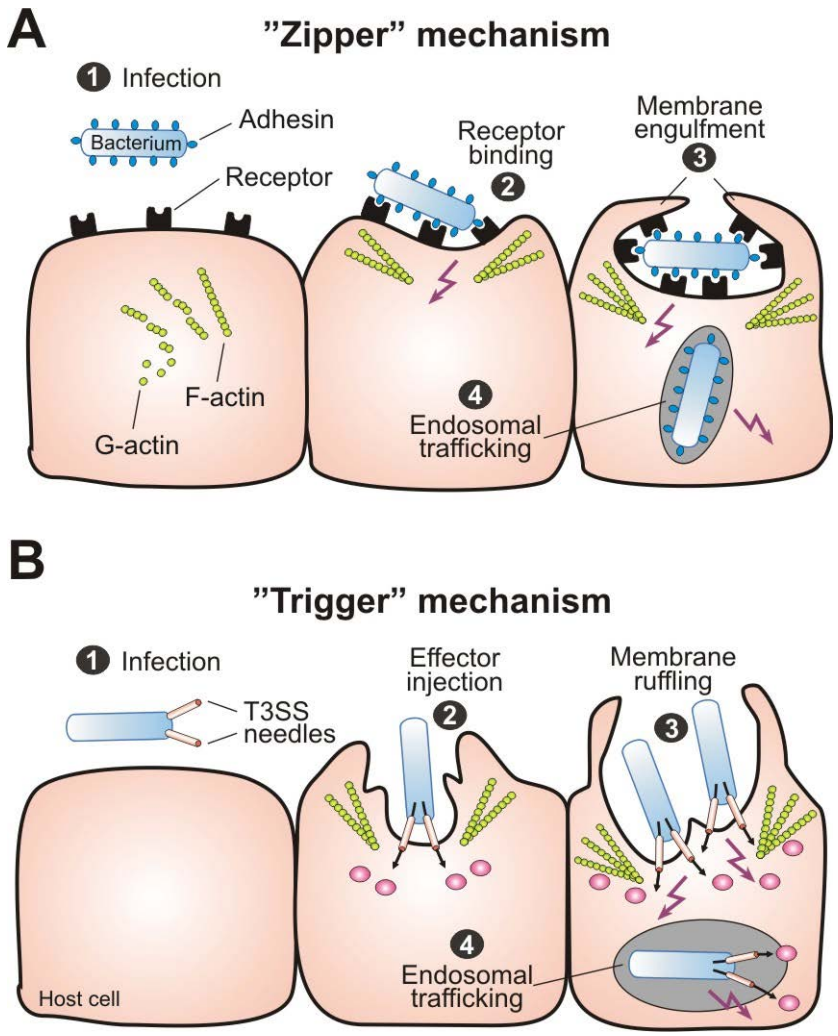

Figure 1:Primary mechanisms of bacterial invasion into non-phagocytic host epithelial cells. Schematic representation of the two different routes of entry by intracellular bacterial pathogens. The pathogens induce their own uptake into target cells by subversion of host cell signaling pathways using the "zipper" and "trigger" invasion mechanism, respectively. (A) Bacterial gastrointestinal pathogens commonly colonize the gastric epithelium [step 1]. The "zipper" mechanism of invasion involves the high-affinity binding of bacterial surface adhesins to their cognate receptors on mammalian cells [step 2], which is required to initiate cytoskeleton-mediated zippering of the host cell plasma membrane around the bacterium [step 3]. Subsequently the bacterium is internalized into a vacuole. Some bacteria developed strategies to survive within or to escape from this compartment [step 4]. (B) The "trigger" mechanism is used by *Shigella* or *Salmonella* spp. which also colonize the intestinal epithelium [step 1]. These pathogens use sophisticated type III or type IV secretion system (T3SS or T4SS) to inject various effector proteins into the host cell cytoplasm [step 2]. These factors manipulate a variety of signalling events including the activation of small Rho GTPases and cytoskeletal reorganization to induce membrane ruffling and subsequently bacterial uptake [step 3]. As a consequence of this signalling, the bacteria are internalized into a vacuole [step 4], followed by the induction of different signaling pathways for intracellular survival and trafficking. This figure was adapted from Tegtmeyer *et al.* (2012).

During the "trigger" mechanism the pathogen injects different effector proteins into the host target cell. This invasion process is induced by highly invasive bacteria, such as *Salmonella* or *Shigella*. The pathogens injects the effector proteins by sophisticated type III- or type IV secretion systems (T3SS or T4SS) into the host cell and this can induce actin polymerisation

events and membrane ruffles. These cellular changes are local and trigger the uptake of the bacteria into a given epithelial target cell (Cossart and Sansonetti, 2004). The process of the "trigger"-mechanism is schematically depicted in Figure1B.

As some bacteria can enter host epithelial cells by the paracellular route, they must disrupt epithelial intercellular and cell matrix junctions, such as tight junctions, adherens junctions or focal adhesions (Fig. 2A).

Tight junctions are important structures to lock the intercellular space between neighboring epithelial cells (Balda and Matta, 1998) and for maintaining the epithelial cell polarity. Tight junctions are narrow bands of membrane proteins which completely surround epithelial cells. In this way, tight junctions completely close the spaces between associated epithelial cells and serve as a diffusion barrier. This barrier controls effectively the flow of molecules over the epithelium. Tight junctions inhibit that membrane components pass from the apical surface to the lateral part and reverse. The probably best described tight junction proteins are the family of claudins, occludins and junctional adhesion molecules (JAMs). These integral membrane proteins are reticular arranged and bring the membrane of two adjacent cells directly in contact with each other (Fig. 2A). They form pores through which certain molecules can pass. Specificity of this diffusion barrier is adjusted to the epithelium by a different composition of claudins. Claudins and occludins are associated with zonula occludens proteins which build the linkage to the intracellular actin cytoskeleton (Alberts *et al*., 2002). JAMs are members of the immunoglobulin superfamily and as part of the tight junctions, they are also connected with the actin cytoskeleton (Praetor *et al*., 2009). Formation of tight junctions between epithelial cells and their ability to form a lock is dependent on the interaction of the tight junction protein complex with actin filaments (Fanning *et al*., 1999) and are also controlled by E-cadherin-mediated cell-to-cell adhesions in the adherens junctions (Gumbiner and Simons, 1986).

Adherens junctions represent a group of adhesion connections, such as zonula adherens, fascia adherens and punctum adherens. The adherens junctions establish the linkage between actin-filaments of two neighboring cells. Typical adherens junctions exist in epithelial cells, directly below tight junctions (Fig. 2A). The most important proteins of adherens junctions are the family of cadherins. Cadherins are Ca^{2+} dependent proteins which interact over several anchor proteins, such as catenins, vinculin or α–actinin with the actin cytoskeleton (Geiger *et al*., 1985). The best described cadherin family member is E-cadherin, which interacts by its cytoplasmic domain with the intracellular protein catenin and by this way catenin connects E-

cadherin to the cytoskeleton. This interaction is essential for the complete adhesive activity of cadherins and the formation of adherens junctions (Aberle *et al*., 1996).

Focal adhesions are cell junctions which link the actin cytoskeleton of the cell to the extracellular matrix (ECM) at basolateral surfaces. They serve as anchoring structures with the cell and as signalling carrier. Focal adhesions consist of more than 50 different proteins. The contact to the ECM is mediated by transmembrane integrin receptors. Integrins are heterodimers consisting of α– and β–subunits, which bind to the different ECM proteins. The β–subunit is linked to the actin filaments by adaptor proteins, such as talin, α-actinin, filamin or vinculin. In focal adhesions not only structural proteins could be found, but also signalling proteins, such as tyrosine kinases including c-Src or focal adhesion kinase (FAK) (Zamir and Geiger, 2001).

Figure 2B-D shows the different cell-cell and cell-matrix complexes of a normal polarized epithelial cell layer and the proposed signalling pathways in the different intercellular junctions during the infection with the model pathogenic organism *Helicobacter pylori* (Wessler and Backert, 2008).

As mentioned above, the human mucosa and especially the gastric mucosal epithelium is an important cell layer which forms the first barrier against several bacterial pathogens, including *Helicobacter pylori*. This human pathogen can colonize the gastric epithelium as a unique niche in the stomach and has developed different remarkable strategies to modify epithelial cell-cell and cell-matrix adhesions which induce to inflammation, proliferation, cell migration and invasive growth (Fig. 2B-D). *Helicobacter pylori* secrete a toxin (VacA) into the supernatant and inject CagA into cells by a T4SS. Both proteins interfere with tight junctional proteins (Fig. 2B). Injected CagA has also been shown to interact with the cytoplasmic domain of E-cadherin and disturbs cell polarity be inactivating the kinase Par1b (Fig. 2C). It has also been shown that a commonly known periplasmic protease of *H. pylori*, called HtrA (high-temperature requirement A), is secreted into the extracellular space, where it can cleave-off the ectodomain of E-cadherin directly (Hoy *et al*., 2010). HtrA-mediated cleavage of E-cadherin resulted in disruption of epithelial barrier functions and entry of bacteria between two adjacent cells. This mechanism could explain how *H. pylori* can make contact with the basolateral receptor of the T4SS, which is known as the integrin member α5β1 (Kwok et al., 2007; Wessler and Backert, 2008), to inject the CagA effector proteins, probably from the basolateral side of infected epithelial cells. Injected CagA is then phosphorylated by host kinases and interferes with various signalling pathways as indicated (Fig. 2D).

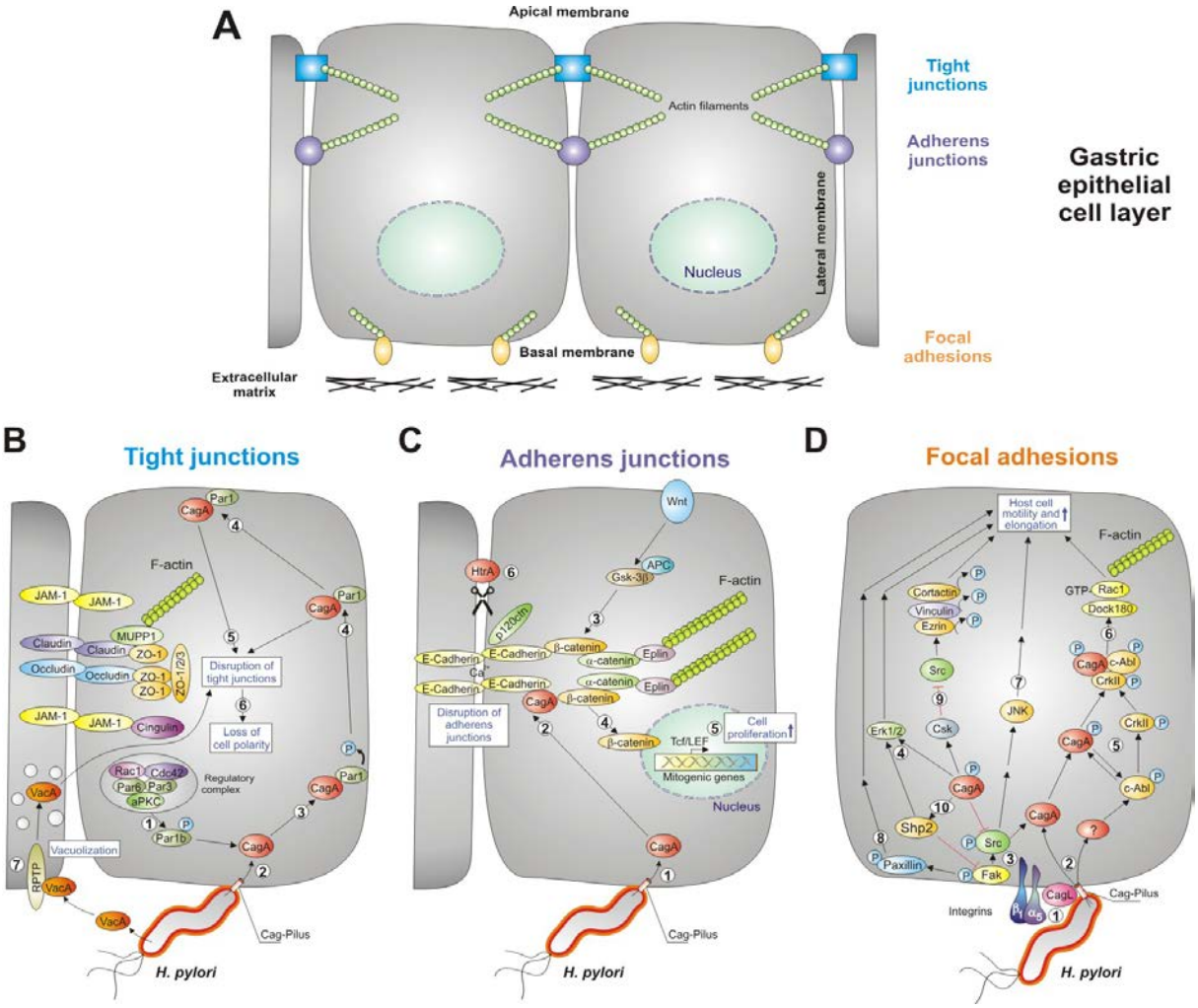

Figure 2: Epithelial intercellular junctions of the host and interactions with *Helicobacter pylori*. (A) A schematic presentation of a polarized cell layer in healthy epithelium. The different types of intercellular junctions are shown, including the tight junctions (TJs, blue), adherens junctions (AJs, violet) and focal adhesions (FAs, orange), which exhibit specific localization in the lateral or basal membranes as indicated. (B-D) Schematic presentation of specific junctional complexes and particular signalling pathways that are induced during infection with *H. pylori*. (B) TJs comprise at least four types of transmembrane proteins: occludin, claudins, junctional adhesion molecules (JAMs) and several cytoplasmic peripheral proteins. Whereas the transmembrane proteins mediate cell–cell adhesion, the cytosolic TJ plaque contains various types of proteins [e.g. PSD-95/Drosophila disc large/ZO-1 homology (PDZ) proteins such as the ZO protein-1, -2 and -3, cingulin and multi-PDZ protein-1 (MUPP1)] that link TJ transmembrane proteins to the underlying actin cytoskeleton. These adapters also recruit regulatory proteins, such as protein kinases, phosphatases, small GTPases and transcription factors, to the TJ. The integrity of TJs is maintained by a regulatory complex, for example, atypical PKC (aPKC), Rac1, Cdc42, Par6 and Par3. At TJs, aPKC phosphorylates Par1b kinase on Thr595. Activated Par1b specifically localizes to the basal and lateral membranes to regulate cell polarity [step 1]. *H. pylori* injects CagA proteins into the host-cell cytoplasm via a T4SS pilus [step 2]. Injected CagA binds Par1b and thereby inhibits PKC-mediated phosphorylation of Par1b on Thr595 [step 3]. The CagA–Par1b complex mislocalizes to TJs and apical membranes [step 4]. This signaling results in the disruption of TJs [step 5] and loss of cell polarity [step 6]. *H. pylori* secretes a second factor, the VacA toxin, which is internalized by RPTP receptors, followed by opening TJs using an unknown mechanism [step 7]. (C) The calcium-dependent integrity of AJs is stabilized by binding of E-cadherin to catenins. The proximal C-terminal domain of E-cadherin binds to the cytoplasmic protein β–catenin.The p120 catenin (p120ctn) binds to the juxtamembrane part of E-

cadherin and stabilizes the entire adherence complex. The E-cadherin–β-catenin complex is connected to the actin cytoskeleton via binding to α-catenin and EPLIN (epithelial protein lost in neoplasm). If not bound to the E-cadherin complex, β-catenin can translocate into the nucleus where it plays a part in nuclear gene transcription driven by transcription factors of the Tcf/LEF family to promote the expression of a wide range of target genes, including the proto-oncogenes c-myc and cyclinD1. CagA is injected by *H. pylori* [step 1] and can be recruited to E-cadherin in the AJs [step 2]. Binding of CagA to E-cadherin induces the release of β-catenin from the AJ complex, causing its cytoplasmic accumulation in a deregulated Wnt pathway comprising GSK-3b and adenomatous polyposis coli (APC) [step 3] and, subsequently, nuclear localization [step 4]. Nuclear β-catenin activates Tcf/LEF-mediated transcription, leading to cell proliferation [step 5]. Recent data also showed that the protease HtrA is secreted by *H. pylori* [step 6], and can cleave E-cadherin directly (Hoy et al., 2010). (D) In FAs, basal integrin α5β1 binds *H. pylori* CagL [step 1], a specialized adhesin on the T4SS pilus that is necessary for the injection of CagA [step 2]. CagL-dependent stimulation of integrin and the FAK kinase also activates Src, the tyrosine kinase of tyrosine-phosphorylated CagA ($CagA^{PY}$) [step 3]. CagAPY can stimulate multiple motility-inducing signaling cascades such as the activation of ERK kinase [step 4] and c-Abl and CrkII [step 5]. The CagAPY–Abl–Crk complex stimulates Dock180, a guanine-nucleotide-exchange factor for the GTPase Rac1, which can control F-actin polymerization necessary for host-cell motility [step 6]. CagL also activates the FAK→Src→JNK pathway [step 7] and paxillin [step 8]. Binding of CagAPY to Csk inactivates Src followed by dephosphorylation of ezrin, vinculin and cortactin [step 9]. Finally, $CagA^{PY}$ binds the tyrosine phosphatase Shp-2, which activates ERK (4) and negatively regulates FAK signaling [step 10], all of which contribute to host-cell motility and elongation. This figure was adapted from Wessler and Backert, (2008).

2.2 Epidemiology and clinical aspects of *Campylobacter*

The group of Prof. Backert is especially interested in molecular mechanisms of *Campylobacter* infections. The importance and appearance of *Campylobacter* infections in public health and food safety topics take a rapidly development in the last years.

Campylobacter-like organisms were first observed in microscopic studies by Theodor Escherich in 1886. He noted some non-culturable spiral-shaped bacteria that were later typically described as *Campylobacter* (Vandamme, 2000; King and Adams, 2008; Vandamme *et al*., 2010). *Campylobacter* was then also identified by two British veterinarians in 1906. They found typical bacteria in the uterine mucus of a pregnant sheep and described the presence of “large numbers of a peculiar organisms” (Skirrow, 2006; Zilbauer *et al*., 2008). First successful isolation of *Campylobacter* as *Vibrio fetus* was established in 1913 from aborted sheep by McFadyean and Stockman. In 1957, King and co-workers described the isolation from blood samples of children with diarrhoea and in 1963 the genus *Campylobacter* was first proposed by Sebald and Véron (On, 2001). In 1972 a Belgian clinical microbiologist first isolated *Campylobacter* from stool samples of patient with diarrhoea (Kist, 1985). A study of Butzler *et al*., in 1973 raised the interest in *Campylobacter*, because doctors and scientists noted their high incidence in human diarrhoea samples (On, 2001).

2. Introduction

With the development of selective growth media as common method in diagnostics, *Campylobacter* enteritis was recognized and *Campylobacter spp*. were established as human pathogens. In the last years the taxonomic structure of the genus *Campylobacter* has extensivly changed, is still controverse discussed and requires further investigations (On, 2001; Debruyne *et al*., 2005). At present some research groups proposed 16 species with six subspecies in the genus *Campylobacter* (On, 2001; Foster *et al*., 2004). The best characterized member of the genus is *C. jejuni*, which is responsible for as much as 80-85% of all *Campylobacter* infections in humans, but there exists a number of other *Campylobacter* species which can also cause human diseases (Moore *et al*., 2005). Together, three *Campylobacter* species (*C. jejuni, Campylobacter coli* and *Campylobacter fetus*) are most frequently found in infected humans (Konkel *et al*., 2001). Futhermore several other members of the *Campylobacter* spp such as *Campylobacter lari*, *Campylobacter upsaliensis* or *Campylobacter hyointestinalis* are suspected to cause infections in various animals (Butzler, 2004, Bourke *et al*., 1998; Engberg *et al*., 2000).

Today, infections with *Campylobacter* are the most common cause of acute diarrhoeal disease in developed countries and is one of the leading cause of enteric bacterial infections worldwide (Young *et al*., 2007; Nachamkin *et al*., 2008). Observations by the WHO suggest that worldwide about 4.5 billion incidences of diarrhoea every year, causing approximately 1.8 million deaths (WHO, 2004). Among those, *Campylobacter* infections may be responsible for as many as 400-500 million gastroenteritis cases annually worldwide (Friedmann *et al*., 2000). Statistical data showed that infections with *Campylobacter* cause very high use of medication and health service burden. For example, some reports indicated 2.5 million cases of campylobacteriosis per year with more than 200 deaths in the USA (Tauxe *et. al*, 1992; Forsythe, 2000).

In the USA, *Campylobacter*–associated illnesses cost up to 6.2 billion dollar per year (Forsythe, 2000). In the United Kingdom infections with *C. jejuni* are responsible for about 34% of all gastroenteritis cases and statistical data shows a new infection rate of 1% per year (Skirrow and Blaser, 1992). According to Skirrow (1991) the infection rates increase about four fold in one decade. In different studies it was found, that *Campylobacter* can cause acute diarrhoea more than 2-7 times as frequently as *Salmonella* and *Shigella* species or pathogenic *Escherichia coli*, thus underlining their overall importance among other foodborne microbial pathogens (Allos, 2001; Tam, 2001).

A statistical report from the Robert Koch Institute showed increasing *Campylobacter* cases in Germany. In 2006, 63/100,000 *Campylobacter* cases with a total amount of 52,000 cases and

in 2011 from January to September 40,000 cases of acute diarrhoea caused by *C. jejuni* were reported (Robert Koch Institut, Berlin/Germany; http://www.rki.de). This implicates that *Campylobacter* is on third position of pathogens, which cause acute diarrhoea, behind *Norovirus* and *Rotavirus*, but ranking before *Salmonella*.

Campylobacter is a Gram-negative, spiral shaped bacterium. It is 0.2 to 0.8 µm wide and 0.5 to 5.0 µm long and has non-spore-forming rods. *Campylobacter* possess bipolar flagella, which provide a high degree of bacterial motility.

Campylobacter neither ferments nor oxidizes carbohydrates, because it lacks the glycolytic enzyme phosphofructokinase. Thus, *Campylobacter* uses amino acids and intermediate metabolites of the tricarboxylic acid cycle as a source of energy (Vandamme *et al.*, 2000). It seems that *Campylobacter* is dependent on amino acids provided by its hosts or the associated intestinal microbial flora (Dasti *et al.*, 2010). The optimal growth temperature for *Campylobacter* varies from 37°C to 42°C, the latter corresponding to the approximate body temperature of poultry species (41°C–42°C) (Nachamkin, 1995). Thus, *Campylobacters* are very often found in the gastrointestinal tract of multiple avian species. *Campylobacter* prefers growing in a microaerophilic environment, requiring 5–10% O_2 and 10% CO_2. The bacterium is sensitive to salinity, acid conditions (pH $\leq$ 5.0), drying and freezing (Altekruse *et al.*, 1999).

Campylobacter jejuni has a small genome with a size of 1.6 – 1.7 Mb. The singular, circular chromosome has a G + C ratio of 30.3 to 30.6% and a high gene content of 94 to 94.3%. These features make it to one of the most dense bacterial genomes sequenced to date (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006). Until now, the complete genome sequences from seven different *Campylobacter* strains are available, including the four *C. jejuni* strains, 81-176 (Hofreuter *et al.*, 2006), NCTC 111-68 (Parkhill *et al.*, 2000), RM1221 (Fouts *et al.*, 2005) and CG8486 (Poly *et al.*, 2007). Furthermore, the genome sequences of *C. coli* strain RM2228, *C. lari* strain RM2100 and *C. upsaliensis* strain RM3195 (Fouts *et al.*, 2005) are also available.

Different *Campylobacter* species live predominantly as commensals in a wide range of different wild and domestic mammals and birds, including poultry. *Campylobacter jejuni* is a classical zoonotic pathogen. *Campylobacter jejuni* and *C. coli* mainly colonize birds, poultry, dairy cows and domestic pets. *Campylobacter jejuni* is a part of the intestinal flora in various birds and mammals and is also found in many foods of animal origin. These facts implicated, that poultry, meat of porks, beef, raw milk and milk products sources of human infections (Oyarzabal and Backert, 2012).

2. Introduction

It is well-known that most retail poultry is contaminated with *C. jejuni*. In a study by Stern and Line (1992), an isolation rate of 98% for chicken meat was reported. A further study showed, that 12% of raw milk samples in the USA are contaminated with *Campylobacter jejuni* (Rohrbach *et al*., 1992). *Campylobacter jejuni* is also found in various meat products. It was detected in 5% of raw ground beef and in 40% of veal specimens (Lammerding *et al*., 1988). Other *Campylobacter* species such as *C. coli* mainly colonize swines (Kist and Bereswill, 2001; Newell, 2001), as well as *C. fetus* which colonize cattle and sheep (Blaser, 1993).

Large outbreaks of human campylobacteriosis are rather sporadic and have different epidemiologic characteristics. There are often outbreaks reported during spring and autum (Tauxe *et al*., 1992), which are linked to the consumption of contaminated water (Mentzing, 1981; Jones and Roworth, 1996; Koenraad *et al*., 1997; Kuusi *et al*., 2005; Schuster *et al*., 2005) and raw milk (Korlath *et al*., 1985; Evans *et al*., 1996; Frost *et al*., 2002). Raw milk was the cause of infection in 30 of the 80 outbreaks of human campylobacteriosis between 1973 and 1992 in the USA (CDC, 1983 and 2002). Sporadic cases of human campylobacteriosis are more common (Friedman *et al*., 2004). Risk factors for sporadic infections are handling of raw poultry (Norkrans *et al*., 1982; Hopkins and Scott, 1983) and eating unsufficiently cooked poultry and meat (Kapperud *et al*., 1992; Oosterum *et al*., 1984; Hopkins *et al*., 1984; Harris *et al*., 1986; Deming *et al*., 1987). Carcasses contaminated with *Campylobacter* are the main cause of human sporadic infections (Adak *et al*., 1995; 2005). Friedman and co-workers showed in one study, that consumption of unproperly prepared chicken in restaurants is a major risk factor for *Campylobacter* infections in the USA (Friedman *et al*., 2004).

Other risk factors are probably not so important for the outcome of a sporadic illness, for example the contact with pets including cats (Hopkins *et al*., 1984; Deming *et al*., 1987) and dogs (Kapperud *et al*., 1992), as well as a person to person transmission is rather uncommon (Norkrans *et al*., 1982; Schmid *et al*., 1987).

Campylobacter is also an important problem in developing countries. The main causes for human campylobacteriosis in these countries are chickens, the poor sanitations and the close contact with the poultry and other animals in households (Coker *et al*., 2002). In these countries infection is very common in early childhood, mostly occuring in the first two life years (Skirrow, 1991, 1994; Taylor *et al*., 1993; Lindblom *et al*., 1995; Coker *et al*., 2002).

In industrial countries campylobacteriosis appears in all age groups, but infants and young adults at the age of 15 to 29 years exhibit the highest *Campylobacter* isolation rates (Allos and

Blaser, 1995; Friedman *et al*., 2000; Kist, 2002). Some statistic studies showed that in all age groups the incidence rate in men is 30% higher than in women (Friedman *et al*., 2000; Kist, 2002).

The major disease symptom for an *Campylobacter* infection in industrialized countries is the acute inflammatory gastroenteritis, whereas in developing countries the typical symptom is a watery, non-inflammatory diarrhoea. These observations show that symptoms which can be triggered by *Campylobacter* are dependent on the geographic location and socioeconomic circumstances (Taylor, 1992; Oberhelman and Taylor, 2000).

Until today it is widely unknown which particular mechanisms are responsibly for the different clinical outcomes of *Campylobacter* infections in humans in industrialized and developing countries. It seems that several host- and pathogen-specific factors are involved in the pathogenesis and the different clinical manifestation of *Campylobacter* infections. It was proposed that different levels of immunity and different natural immune stimulants in the environment play arole (Blaser *et al*., 1980). Furthermore, humans which are immunocompromised, such as patients with AIDS have a higher risk of acquiring *C. jejuni* than the non-HIV population (Butzler *et al*., 2004).

As described above, infections with *Campylobacters* are one of the main causes for acute diarrhoea worldwide. *C. jejuni* and *C. coli* are typical pathogens which cause acute gastroenteritis. To manifest an infection, *C. jejuni* must bypass the mechanical and immunological barriers of the human gastrointestinal tract. First line of defence is the mucus layer of the gastrointestinal tract. To interact with the epithelial cells *Campylobacter* must pass through the mucus layer (McSweegan and Walker, 1986). The bacteria colonize the distal small intestine and the colon and induce by this way mucosal edema, cellular infiltrates, small abscesses and focal ulcerations (Colgan *et al*., 1980). Clinical features of gastroenteritis due to *Campylobacter* are indistinguishable from disease symptoms induced by other enteric pathogens such as *Salmonella* or *Shigella*. Clinical manifestations are characterized by inflammation, abdominal cramps, fever and watery or bloody diarrhoea (Allos and Blaser, 1995). The average incubation time is three days, it ranges between one to seven days, with an infectious dose as low as 500-800 bacteria (Robinson, 1981). To develop symptomatic disease, ingestion of as few as 500 bacteria is sufficient (Black *et al*., 1988). Early symptoms of a *Campylobacter* infection are headache, back pain, myalgia and low fever, including abrupt onset of abdominal pain and diarrhoea (Kist and Bereswill, 2001). Acute illness is characterized by cramping abdominal pain, high fever and diarrhoea. In about 15% of all cases bloody stool was observed on the second or third day (Skirrow and Blaser, 2000). The

disease is typically resolved in one week and generally considered as a self limiting illness. Despite these facts *C. jejuni* infection is a significant cause for morbidity and mortality in humans, especially in developing nations (Mead *et al*., 1999).

Important late complications of *C. jejuni* infections are rheumatic and neurological complications, such as post infectious arthritis (Reiter´s syndrome) (Peterson, 1994b) or Guillain-Barré- and Miller-Fisher syndrome (Nachamkin, 2002; Yuki *et al*., 2004; 2005; Yuki and Koga, 2006). The Guillian-Barré syndrome (GBS) is an acute neurological disease affecting the peripheral nervous system and is characterized by loss of reflexes and symmetric paralysis. To date different observations suggest that GBS is mediated by an immune response to *C. jejuni* infection and that both the *C. jejuni* genotype and host differences are determinants for the development of GBS (Nachamkin *et al*., 1999; 2002; Blaser and Engberg, 2008). Recent studies suggest that carbohydrate mimicry of *C. jejuni* lipooligosaccharide (LOS) by human ganglioside can be a cause of GBS (Yuki *et al*., 2004; 2005; Yuki and Koga, 2006). The association between *C. jejuni* infections and the irritable bowel syndrome (Spiller, 2007) and immunoproliferative intestinal lymphomas (IPSID) (Lecuit *et al*., 2004) are not yet clear and still under investigation.

2.3 Pathogenesis and virulence mechanisms of *Campylobacter jejuni*

Host cell invasion of *C. jejuni* has been reported as one of the primary reasons of tissue damage in humans but the molecular mechanisms are widely unclear. To estimate the risk potential of *Campylobacter* it is important to know much more about the pathogenicity and involved bacterial virulence factors. By comparison to other neteric pathogens such as *Salmonella* and others, very little is known about specific virulence mechanisms of *C. jejuni*.

The adhesion of *Campylobacter* to the intestinal epithelium and the followed host cell entry are features which correlate with the pathogenesis of *Campylobacter* strains (Kist, 2002). In the *Campylobacter* spp. different virulence mechanisms have been proposed and it could be shown, that a combination of different potential virulence factors exhibit different pathogenicity mechanisms (Hänel *et al*., 1999).

Several *C. jejuni* pathogenicity-associated factors have proposed roles during host infection, but many of them were not yet clearly shown to play a major role (Ketley *et al*., 1997). In addition, it should be noted that significant genome variation exists among different strains which might play a role for the outcome of infections. *Campylobacter jejuni* infections are most frequently caused by contaminated food products. These infections are often harmless in

animals, but can make humans ill. Futhermore different observations have shown that the specific interaction between host and bacterium play a crucial role for an infection (Wassenaar and Blaser, 1999).

Among the potential pathogenicity and virulence properties, bacterial motility, chemotaxis, adhesion, invasion, toxins, iron acquisition, surface polysaccharide structures, oxidative stress response, heat shock response, and the flagella are the best characterized features and shared by all known *C. jejuni* strains (Dasti *et al*., 2010). In contrast, toxin production e.g. the cytolethal distending toxin (CDT) and structure of lipopolysaccharide (LPS) vary between different *C. jejuni* strains (Wassenaar and Blaser, 1999). The major known bacterial factors which play a crucial role in *C. jejuni* infection are presented and summarized in Table 1.

Campylobacter jejuni exhibit very high motility through its spiral shape and a single flagellum present at each pole (Ferrero and Lee, 1988). Flagellar-mediated motility is important for *C. jejuni* colonization of the host and its ability to enter the intestine (Lee *et al*., 1986). More than 40 genes are involved in the flagella biosynthesis and assembly (Wösten *et al*., 2004, 2008). The flagellum is composed of a couple of major structures including the basal body, the hook and the flagellar filament. The basal body is embedded in the membrane and serves as a motor for flagella rotation and subsequent bacterial movement (Wassenaar and Blaser, 1999).

The filament consists of two different flagellin subunits, the major FlaA and the minor one FlaB proteins (Guerry *et al*., 1990; Nuijten *et al*., 1990). Both are combined in the flagellum, but expression of FlaA is higher than that of FlaB. The flagellar filament protein expression is controlled by different transcription factors. The *flaA* gene is regulated by promotor σ^{28} (Guerry *et al*., 1990) and the *flaB* gene is regulated by the promoter σ^{54} (Alm *et al*., 1992; Wassenaar *et al*., 1994; Hendrixson *et al*., 2001). Recently, it was reported that the *flaA* gene is essential for invasion of intestinal epithelial cells, because it could be shown that deletion of the *flaA* gene leads to a trunced flagellar filament and a strong reduction in bacterial motility. In contrast, a mutation in the *flaB* gene had no effect on the motility (Guerry, 2007). These and other observations showed that the *flaA* gene is responsible for adherence, colonization of the gastrointestinaltract and invasion of host cells by *C. jejuni* (Jain *et al*., 2008). If this is mediated by its role in bacterial motility or another function is not yet clear.

Glycosylation of flagellin is an important feature in export and/or polymerization of flagellin. These glyco–modifications are encoded by an *O*–glycosylation locus (Guerry *et al*., 2006). The *C. jejuni* flagellum is composed of O–linked glycosylated flagellin; a two component system of the sensor FlgS and the regulator FlgR and this FlgS–FlgR–system is essential for

regulation of the flagellum and the flagellar biosynthesis (Hendrixson and DiRita, 2003; Wösten *et al*., 2004; Dasti *et al*., 2010). Furthermore, it seems that attached glycans can influence the flagella-mediated auto–agglutination and colony formation (Guerry, 2007; Guerry *et al*., 2006; van Alphen *et al*., 2008). The flagella have been shown as the first determinants identified to be involved in cellular attachment and this adhesion is dependent on motility and flagellar expression (Wassenaar *et al*., 1991; Grant *et al*., 1993). In studies with different animal models it could be shown that different *C. jejuni* flagellar deletion mutants have a reduced motility and adherence and are widely deficient in invasion (Yao *et al*., 1994). Additionally it has been reported, that the flagellum is essential for colonization in various animal models (Morroka *et al*., 1985; Pavlovskis *et al*., 1991; Nachamkin *et al*., 1993; Wassenaar *et al*., 1993; Hendrixson and DiRita, 2004). These findings indicate that the *C. jejuni* flagellum maybe involved in host cell adhesion, but other bacterial determinants must be involved in invasion, too.

C. jejuni is able to enter cells in the epithelium of the gastrointestinal tract. To invade, the bacteria first attach to the intestinal epithelial cells (Wassenaar and Blaser, 1999) and this adherence seems to be an essential prerequisite for subsequent *C. jejuni* colonization and pathogenesis.

Bacterial adherence involves several binding factors and/or their respective receptors on the host side. The binding of these factors is proposed to be fundamental for an efficient interaction with host cells. The molecular mechanisms by which *C. jejuni* adhere to the host cells are still not fully understood, but different proteins are proposed to act as bacterial adhesins and to be involved in the invasion process. Currently, the proteins CadF (Campylobacter adhesin to fibronectin), JlpA (jejuni lipoprotein A), PEB1 (periplasmic binding protein), FlpA (Fibronectin like protein A), MOMP (major outer membrane protein), CapA (*Campylobacter* autotransporter protein A) and P95 are proposed to act as *C. jejuni* adhesins.

Table 1: Bacterial factors and proposed roles in *Campylobacter jejuni* infection [a]

Bacterial factor	Proposed function	Applied experimental methods	Strains used	Cell system used	References
AspA, AspB	Aspartate ammonia lyase and amino transferase	Infection *in vitro*, GPA, ISA	81-176	T84	Novik *et al.*, 2010
CadF	Adhesin to fibronectin	Infection *in vitro* and chickens, GPA, FBA, CBA, ELISA	F38011, 11168, 81-176	INT-407, T84, LMH	Konkel *et al*., 1997; Ziprin *et al.* 1999; Monteville *et al*., 2002; Scott *et al*., 2010
CapA	Adhesin/Invasin	Infection *in vitro* and chickens, GPA	11168, F38011	Caco-2, LMH	Ashgar *et al*., 2007; Flanagan e*t al*., 2009
CDT	Cytolethal distending toxin	Treatment of cells *in vitro*, MI, FACS, IFM, DNAse assays	81-176	COS-1, REF52, Henle-407	Lara-Tejero and Galan, 2000
CiaB, CiaC	Invasin	Infection *in vitro*, GPA, MLA, T3SS assays	F38011, 11168	INT407	Konkel, 1999b; Christensen, 2009
CiaI	Intracellular survival	Infection *in vitro*, GPA, MLA, IFM	F38011	INT407, HeLa	Buelow *et al*., 2011
CJ0977 [b]	Invasion	Infection *in vitro* and ferrets, EM, MA, GPA	81-176	INT407	Goon *et al*., 2006
CstII	LOS sialylation	Infection *in vitro*, GPA	GB2, GB11, GB19	CACO-2, T84	Louwen *et al*., 2008
FlaC	Invasin	Infection *in vitro*, MA, EM, cell fractionation, GPA, IFM	TGH9011	HEp-2	Song *et al*., 2004
FlpA	Adhesin to fibronectin	Infection *in vitro* and chickens, FBA, ABB, GPA, IFM, ELISA	F38011	INT-407, Hela, LMH	Flanagan *et al.*, 2009; Konkel *et al.*, 2010, Eucker and Konkel, 2011
GGT	Gamma-glutamyl transpeptidase	Infection *in vitro*, mice and chickens, cell fractionation, MA, GPA, HPS, AA	RM1221, 81-176, 81116, 11168	INT-407, CCD841 CoN	Hofreuter *et al.*, 2006; 2008; Barnes *et al*., 2007
HtrA	Periplasmic protease and chaperone	Infection *in vitro*, GPA	11168	INT-407	Baek *et al*., 2011

JlpA [c]	Adhesin to HSP90-α, proinflammatory responses	Infection *in vitro*, GPA, BRP, ABB, ligand overlays, geldanamycin inhibitor, p38/NF-κB activation using AABs	TGH9011	HEp-2	Jin *et al*., 2001; Jin *et al*., 2003
KpsE, KpsM, KpsT	Capsule proteins, invasion	Infection *in vitro*, infection of chicken and ferrets, GPA	81-176, 81116	INT-407	Bacon *et al*., 2001; Bachtiar *et al*., 2007
Peb1, Peb3 and Peb4 [d]	Transport proteins and Chaperones	Infection *in vitro* and mice, BRP, GPA	81-176, 11168	Hela , INT407	Leon-Kempis *et al*., 2006; Min *et al*., 2009, Asakura *et al*., 2007; Kale *et al*., 2011
PflA	Motility	Infection *in vitro*, GPA	81-176	INT407	Yao *et al*., 1994
PorA (MOMP)	Major outer membrane protein	Infection *in vitro*, BRP	K22, 1767	INT407	Schroeder & Moser, 1997; Moser *et al*., 1997
SodB	Superoxide dismutase	Infection *in vitro* and mice, GPA, ISA	81-176	T84	Novik *et al*., 2010
VirK	Intracellular survival	Infection *in vitro* and mice, GPA, IFM	81-176	T84, COS-7	Novik *et al*., 2009

[a] Abbreviations: AA (apoptosis assay); AB (antibody); AAB (activation-specific antibodies); ABB (antibody blocking); BRP (binding assays using recombinant or purified protein); CBA (competitive binding assay); EM (electron mciroscopy); FACS (Fluorescence-activated cell sorting); FBA (fibronectin binding assay); GPA (gentamicin protection assay); HPS (hydrogen peroxide susceptibility test); IFM (immunofluorescence microscopy); ISA (intracellular survival assay); MI (microinjection of proteins); MA (motility assay on agar); MLA (^{35}S-methionine labelling assay); T3SS assays (transolaction assay using the *Yersinia* type III secretion apparatus);

[b] Another report indicated that the Cj0097 mutant has a deficiency in motility in liquid broth (Novik *et al*., 2010)

[c] Identified as an adhesin in strain TGH9011, but no effect observed with *jlpA* mutants in either 11168 or 81-176 strains(Van Alpen *et al*., 2008; Novik *et al*., 2010)

[d] These structural studies along with other assays suggest primary roles in protein transport. Originally, the Peb's were identified as putative adhesins (Pei *et al*., 1991).

(this table was adapted from O'Croinin and Backert, 2012)

The most extensively investigated adhesive protein is CadF, a 37 kDa outer membrane protein that mediate the binding of *C. jejuni* to the ECM protein fibronectin (Konkel *et al*., 1997, 1999a, 2005). A Δ*cadF* mutant showed a reduced binding to and invasion into INT-407 cells (Montville and Konkel, 2002; Montville *et al*., 2003; Krause-Gruszczynska *et al*., 2007a, 2007b) and was unable to colonize chickens (Ziprin *et al*. 1999). FlpA is a 46 kDa protein that also contributes to the binding of *Campylobacter* to epithelial cells and fibronectin. Konkel and co–workers reported that the binding of a Δ*flpA* mutant to INT407–cells is significantly reduced compared to the wild type strain. Futhermore they showed a dose dependent binding of FlpA to fibronectin (Konkel *et al*., 2010; Euker and Konkel, 2011). These results suggest that both CadF and FlpA may mediate *C. jeuni* adherence to the host cells via fibronectin.

JlpA is a 43.2 kDa protein. Mutation in the *jlpA* gene leads to a 18 – 19.4% reduction in adhesion of *C. jejuni* to HEp-2 cells (Jin *et al*., 2001, 2003). Furthermore, Jin and co-workers showed that a pre-incubation of HEp-2 cells with recombinant JlpA results in a dose–dependent reduction of adherence of *C. jejuni* (Jin *et al*., 2001). Moreover, PEB1 is a 28 kDa protein and a disruption of *peb1A* reduced the binding of *C. jejuni* to HeLa cells by 50–to 100–fold and is unable to colonize mice (Pei *et al*., 1998). MOMP (Moser *et al*., 1997), CapA (Ashgar *et al*., 2007) and P95 (Kelle *et al*., 1998) are also proposed to function as adhesins, but are still not well investigated.

The HtrA protease is another protein, which was identified to influence the adherence and invasion levels of *C. jejuni*. HtrA and its homologs constitute a group of heat shock induced serine proteases, similar to its counterpart observed in *H. pylori* (Hoy *et al*., 2010). Most bacterial HtrAs are described to localize in the periplasm and are involved in various aspects of protein quality control (Clausen *et al*., 2002). HtrA proteases were first described in *Escherichia coli* (Lipinska *et al*., 1989, Strauch *et al*., 1989), it are widely conserved in its protease domain and the involved in heat shock response. Several investigations in the last few years, shows that *C. jejuni* HtrA is a bifunctional protein, which has, in addition to its proteolytic function, a chaperone activity that can be switched on and off (reviewed in Clausen *et al*., 2011). Furthermore, it appears that the chaperone activity of *C. jejuni* HtrA may play a crucial role at stress tolerance and it has a regulatory function by its protease activity (Bæk *et al*., 2011). If *C. jejuni* HtrA, like its *H. pylori* counterpart, can be secreted into the cell culture supernatant, where it could possibly cleave host cell factors, has not been investigated yet.

In general, the ability of *C. jejuni* to enter non-phagocytic epithelial cells during infection is thought to be very important for its pathogenesis. There are some reports proposing that, similar to other foodborne pathogens including *Salmonella* and *Shigella*, *C. jejuni* can actively secrete a cocktail of potential virulence factors into the culture supernatant (Konkel *et al*., 2004). In fact, *C. jejuni* secretes a set of proteins called the *Campylobacter* invasion antigens or Cia proteins (Konkel *et al*., 1999b). The secretion mechanism and the function of the secreted proteins, however, are poorly characterized. One of the best characterized Cia proteins is the 73 kDa protein CiaB. CiaB appears to be required for the secretion process itself and is necessary for efficient entry of *C. jejuni* into the host cells (Konkel *et al*., 1999b). Internalization assays revealed a significant reduction in invasion of a Δ*ciaB* deletion mutant as compared to wild-type *C. jejuni*, and the Δ*ciaB* mutant also exhibited reduced chicken colonization (Ziprin *et al*., 2001). Interestingly, *Campylobacter jejuni* does not encode a

classical T3SS or T4SS (Parkhill *et al*., 2000; Fouts *et al*., 2005; Hofreuter *et al*., 2006). Instead, Konkel and co–workers showed that the flagellar apparatus serves as a T3SS for the export of Cia proteins (Konkel *et al*., 2001). These findings indicate that the flagellar export system may secrete both flagellar and non–flagellar proteins (Konkel *et al*., 2004; Song *et al*., 2004). FlaC, another recently identified *C. jejuni* protein, was also described to be secreted from the flagellar export apparatus. Δ*flaC* mutants are motile and show a functional flagellum, but they are defective in invasion of epithelial cells (Song *et al*., 2004). All these findings indicate that the flagellar export apparatus could be an important secretion device, explaining its requirement for *C. jejuni* host cell invasion. Novel findings, however, raised substantial doubt on the above CiaB importance in secretion of virulence factors and cell invasion (Novik *et al*., 2010). Thus, it is still unclear whether the flagellar structure is directly involved in secretion of Cia proteins triggering bacterial internalization or whether the seen entry defect by flagellar deletion mutants is due to loss of flagella-driven motility and subsequently less bacterial contact with its host target cell.

Another important pathogenicity-associated mechanism in early states of infection of *C. jejuni* is the production of toxins. Different research groups reported about their findings of certain cytotoxins, enterotoxins or both (Wassenaar 1997; Pickett, 2000). It is well known, that *C. jejuni* produce three different kinds of toxins including a thermo-labile protein cytotoxin (Misawa *et al*., 1994), an enterotoxin with antigenetic features to heat-labile enterotoxin (Klipstein and Engert, 1984a, 1984b; Ruiz-Palacios *et al*., 1983) and the cytolethal distending toxin CDT (Whitehouse *et al*., 1998). The best characterized toxin is CDT which was first discovered in *Escherichia coli, Shigella flexneri* and *Salmonella enterica*. In 2000, it was reported that CDT is also produced by most *C. jejuni* strains (Lara-Tejero and Galan, 2000).

CDT consists of three subunits, CdtA (30 kDa), CdtB (29 kDa) and CdtC (21 kDa) (Heywood, 2005). Different investigations showed that CdtA and CdtC are essential for binding to host cells and the subunit CdtB is the toxic part of the Cdt-ABC complex (Pickett and Whitehouse, 1999). It seems that CdtA and CdtC interact with each other and form with CdtB the CDT holotoxin, and this holotoxin is essential for delivery of CdtB into host cells (Lara-Tejero and Galan, 2001). In particular, the entry of CdtB into the nucleus is necessary for the cytotoxic activity (McSweeney and Dreyfus, 2004). The enzymatically active part the subunit CdtB leads to the cytotoxic effect, and biochemical assays have shown that CdtB is the first described toxin having DNAse activity and has sequence similarity with the family of DNAseI-like proteins (Lara-Tejero and Galan, 2000). The entry of CdtB to the nucleus of host cells leads to chromosomal DNA damage by causing a cell cycle arrest in the G2/M transition

phase (Pickett and Whitehouse, 1999). In several reports, it was shown that cytotoxicity and cell cycle arrest was caused by adding a combination of all three toxin subunits to cultured epithelial cells or microinjection of CdtB alone (Lee *et al*., 2003; Lara-Tejero and Galan, 2001). Thus, CdtA and CdtC may bind specifically to one or more yet unknown host cell surface receptor(s), and mediate the delivery of CdtB into target cells (Lara-Tejero and Galan, 2001; Lee *et al*., 2003).

It was also observed that CDT is able to interfer with the activity of different cells of the immune system, such as B- or T-cells (Lara-Tejero and Galan, 2002). CDT in *C. jejuni* might play a role in modulation of the immune response and invasion (Purdy *et al*., 2000). Furthermore, it could be shown, that *C. jejuni* Δ*cdt* mutants are able to colonize NF-κB-deficient mice, but are unable to induce gastroenteritis (Fox *et al*., 2004). However, CDT caused IL-8 production in humans, which in turn recruits dendritic cells, macrophages and neutrophils to the infection site and induced inflammation of the intestine (Hickey *et al*., 1999). It was further reported that *C. jejuni* CDT can cause apoptosis in monocytic cells *in vitro* (Hickey *et al*., 2005).

In contrast to the host response reported in humans, *C. jejuni* CDT obviously does not induce inflammation of the intestinal epithelium in chickens (reviewed in Young *et al*., 2007). CDT promotes the production of neutralizing antibodies only in humans, not in chicken. These findings indicate a host specific recognition of *C. jejuni* antigens. Difficulties to explain the specific role of CDT during campylobacteriosis are further documented by the finding of CDT-negative strains in humans with enteric diseases (AbuOun *et al*., 2005). Thus, the benefit of CDT toxins for the bacteria and associated infection cycle is not yet fully clear and needs further investigation.

Campylobacter jejuni is a classical zoonotic pathogen and can be found at the intestinal flora of bird and mammals. In this way *C. jejuni* can contaminate food and water. In association with contaminated water or food products *C. jejuni* enters the human intestine crossing the stomach acid barrier and colonizes the ileum and the colon. Chemotaxis is an effective feature of *C. jejuni* for human colonization and stimulus to respond to different environmental conditions. It is known, that *C. jejuni* are able to move along chemical gradients. Chemotaxis is necessary for *C. jejuni* colonization, because it could be shown that non-chemotactic mutants are unable to colonize host cells (Takata *et al*., 1992). At the genome sequence level, several chemotaxis genes have been identified in *C. jejuni* including *cheA, cheW, cheV, cheY, cheR* and *cheB*. Mutagenesis studies have shown that several of these genes are also involved

in colonization of the chicken intestine (Hendrixson and DiRita, 2004). Yao and co-workers reported that CheY plays a special role in flagellar rotation and is essential for bacterial gastroenteritis in the ferret animal model (Yao *et al*., 1994; Yao *et al*., 1997). They showed that a *C. jejuni* Δ*cheY* mutant is more adherent and invasive as compared to the corresponding wild-type strain (Yao *et al*., 1997). The observations that *cheY* and *cheA* mutants are unable to colonize the intestine of mice and chicken suggested that chemotaxis is essential for colonization in different hosts (Takara *et al*., 1992; Yao *et al*., 1997; Hendrixson and DiRita, 2004).

Another important pathogenicity feature of *C. jejuni* is the acquisition of iron from its host. Iron acquisition is important for the bacteria, because they must survive in low iron environments in the host, especially in the gastrointestinal tract (Braun and Killmann, 1999). *Campylobacter* possess several uptake systems for iron acquisition and is able to use iron of several sources, such as ferric iron bound to glycoproteins and siderophores, ferric iron in heme or ferrous iron (in *"Campylobacter"*, ed. I. Nachamkin, C.M. Szymanski and M. Blaser, 2008). *Campylobacter jejuni* can only use low amounts of iron in compounds (Field *et al*., 1986) and is unable to produce siderophores itself, but it can use exogenous siderophores of the host and several haem-based compounds, which are produced at the site of inflammation (Pickett *et al*., 1992).

Campylobacter jejuni uses a transport system for iron which is encoded by the *ceu* (campylobacter-enterochelin-uptake) operon (Richardson and Park, 1995). The observations of Crawthraw and co-workers suggested that *ceu* is not the only iron uptake system in *C. jejuni*, because they could show that a *C. jejuni* Δ*ceu* mutant is still able to colonize chicken (Cawthraw *et al*., 1996). Therefore, more studies are necessary to unravel the various colonization-associated iron uptake systems in *C. jejuni*.

Additionally, *C. jejuni* and other bacteria are able to store iron intracellularly. This ability is important for the growth of the bacteria especially in low iron environments and it also protects the bacteria against an iron overload. This protection is necessary, because too much iron results in an oxidative damage of several cellular components (reviewed in van Vliet *et al*., 2002). Wai and co-workers reported that *C. jejuni* is able to produce ferritin (Wai *et al*., 1995). Ferritin is an iron storage protein which helps *C. jejuni* to colonize the host and to protect it especially under conditions of varying O_2 levels (Ketley, 1997).

2.4 Host factors involved in the interaction of *C. jejuni* with target cells

Multiple microbial pathogens have evolved complex functional interfaces to affect the host-cell signalling pathways, to enter host cells, and to replicate and survive within host cells (Pizarro-Cerda and Cossart, 2006). Thus, different pathogens have developed several strategies to manipulate host cell functions and initiate disease. Very often, manipulations of host cell factors involved specific bacterial factors which can hijack host cell factors for bacterial advantage and spread. Increasing amounts of data accumulated in the last decade showing that *Campylobacter* damages intestinal epithelial cells and functions directly by cell invasion and production of toxins. Futhermore, *Campylobacter* is able to trigger inflammatory responses (Ketley, 1997; Wooldridge and Ketley, 1997). Thus, the entry of human intestinal epithelial tissue results in a loss of crucial cellular functions and is an important pathogenic mechanism of *C. jejuni*.

Previous reports and examinations of intestinal biopsies from patients, primates and other experimental animal models showed that *C. jejuni* is able to enter gut tissue cells *in vivo*. The same results were indicated by infections of cultured human intestinal epithelial cell lines *in vitro* (Russel *et al*., 1993; Babakhani *et al*., 1993).

Histological studies showed that *C. jejuni* can adhere to colonic epithelial cells after the passage through the gastrointestinal tract. This indicates that pathology basically is in the colon, but also in the intestinal crypts (van Speeuwel *et al*., 1985). During intestinal epithelial cell penetration specific bacteria–host interactions can cause diarrhoea and gastroenteritis. These observations indicated that invasion of host cells are the main cause of tissue damage by *C. jejuni in vivo* (Kopecko *et al*., 2001). To study *C. jejuni* invasion levels, the model strain 81-176 and gentamicin protection assays are commonly used in worldwide studies. The strain 81-176 is a clinical isolate of a patient with gastroenteritis. It has a very high invasion efficiency of host cells and the genome sequence is well known (Hu and Kopecko 1999; Hu *et al*., 2006a; Hofreuter *et al*., 2006; Krause-Gruszczynska *et al*., 2007a, 2007b).

We only know very little about host cell factors playing role in the adherence and invasion processes of *C. jejuni.* Most of our knowledge is based on studies using pharmacological inhibitors and gentamicin protection assays. For example, inhibition of protein tyrosine kinases reduces the amount of viable intracellular *C. jejuni* colony-forming units (CFU) (Wooldridge *et al*., 1996; Biswas *et al*., 2000, 2004; Hu *et al*., 2006a) and *C. jejuni* infection induces tyrosine phosphorylation of several yet unidentified host cell proteins (Biswas *et al*., 2004; Hu *et al*., 2006a).

Host cell surface receptor proteins comprise three main families: ion–channel–linked receptors, G–protein–coupled receptors and enzyme–linked receptors. Inhibition of heterodimeric G–proteins seems to donwregulate *C. jejuni* uptake as judged by gentamicin protection assays (Wooldridge *et al*., 1996). In addition, the host cells respond to an increase of intracellular Ca^{2+} level by rearranging the cytoskeleton and the Ca^{2+} release from intracellular host stores are necessary for the uptake of *C. jejuni* (Hu *et al*., 2005). In general, it has been proposed that *C. jejuni* induces Ca^{2+} release and triggers the activation of G - proteins, calmodulin, PKC (protein kinase C), PI–3–kinase (phosphatidyl-inositol–3–kinase), MAPKs (mitogen–activated protein kinases) and caveolae, which seem to be required for efficient invasion of host cells and requires polymerized microtubules (Nachamkin *et al*., 2008; Watson and Galan, 2008).

However, bacterial host cell entry by *C. jejuni* can proceed by microtubule–dependent (actin–filament–independent) and/or actin–dependent–pathways (Oelschlaeger *et al*., 1993; Hu and Kopecko 1999; Biswas *et al*., 2000, 2003; Monteville *et al*., 2003).

In 1992, Konkel and co-workers first reported a perinuclear migration of several internalized *C. jejuni* strains (Konkel *et al*., 1992b). Different studies showed that *C. jejuni* can survive within the endosome during passage through the host cell (Konkel *et al*., 1992b; Oelschlaeger *et al*., 1993; Watson and Galan, 2008; Buelow *et al*., 2011).

It was also proposed that the microtubule-associated motor proteins dynein and kinesin may play a major role for movement of the endosomes containing *Campylobacter jejuni*. Hu and Kopecko could show by immunofluorescence microscopy that internalised *C. jejuni* strain 81–176 co-localized with dynein and microtubules during the invasion process (Hu and Kopecko, 1999).

Once *C. jejuni* is internalized it can survive within epithelial cells (Konkel, *et al*., 1992b; Day *et al*., 2000). It is also able to evade the host immune system and cause an acute infection or a long–term persistend infection (Lastovica *et al*., 1996; Day *et al*., 2000).

The various roles of certain bacterial factors in host cell invasion and the mechanism by which *C. jejuni* triggers its eukaryotic cell entry is not well characterized to date. Only few results are currently reported about bacterial factors as well as host cell factors which are involved in the uptake of *C. jejuni* (Biswas *et al*., 2004). Our current knowledge on the potential role of several host cell factors in *C. jejuni* infections as well as the experimental methods and used strains is summarised in Table 2.

Table 2: Host factors and their proposed roles in *Campylobacter jejuni* infection

Host factor	Proposed function	Applied experimental methods	Strains used	Cell system used	References
Actin filaments	Invasion	Infection *in vitro*, Cytochalasin D and mycalolide B inhibitors, GPA, IFM	81116, HP5100, CCUG7800, F38011, 81-176	INT-407	Biswas *et al*., 2003; Monteville *et al*., 2003
Calcium	Invasion	Infection *in vitro*, BAPTA inhibitors, GPA	81-176	INT-407	Hu *et al*., 2005
Cdc42	Invasion	Infection *in vitro*, CA and DN constructs, GMT, CRIB-PD, GPA, IF, FESEM	81-176, 84-25, F38011	INT-407	Krause-Gruszczynska *et al*., 2007b
Caveolae	Invasion	Infection *in vitro*, filipin-III and MβCD inhibitors, DN constructs, GPA, IFM	N82, 81-176	Caco-2, INT-407, Cos-1	Wooldridge *et al*., 1996; Hu *et al*., 2006a; Watson and Galan, 2008
Dynein	Invasion, intracellular trafficking	Infection *in vitro*, nocodazole and o-Van inhibitor, GPA, IFM	81-176	INT-407	Hu and Kopecko, 1999
EGF receptor	Invasion	Infection *in vitro*, ABB, PD168393 and erlotinib inhibitors, GPA	F38011	INT-407	Eucker and Konkel, 2011
Fibronectin	Adhesion, invasion	Binding and infection *in vitro*, CBA, TWA, ABB, use of *cadF* mutant, GPA	F38011, 81-176	INT-407, T84	Monteville *et al*., 2003, Monteville and Konkel, 2002
G proteins	Invasion	Infection *in vitro*, pertussis and cholera toxin treatments, GPA	N82, 81-176	Caco-2, INT-407	Wooldridge *et al*., 1996; Hu *et al*., 2006a
Lysosomes	Intracellular trafficking	Infection *in vitro*, IFM with EEA-1, Lamp-1, Rab4 and Rab5	81-176	Cos-1	Watson and Galan, 2008
MAPK	Inflamma-tory signalling, invasion	Infection *in vitro*, binding of GST-JlpA *in vitro;* AABs for Erk, JNK and p38; MAPK inhibitors	THG9011, 81-176, 11168	HEp-2, T84, Caco-2, human colonic explants, INT-407	Jin *et al*., 2003; MacCallum *et al*., 2005; Chen *et al*., 2006; Hu *et al*., 2006a
Microtubule filaments	Invasion	Infection *in vitro*; nocodazole inhibitor	81-176, VC84	INT-407	Oelschlaeger *et al*., 1993
Mucin (chicken)	Inhibition of bacterial virulence	Binding studies, GPA	81-176	HCT-8	Alemka *et al*., 2010
Myd88	Colonisation controlled by TLRs	Colonisation of Myd88$^{-/-}$ but not wt control mice	81-176	Myd88$^{-/-}$ mice	Watson *et al*., 2007
NF-κB	Inflamma-tory signaling	Binding of GST-JlpA *in vitro*, AABs, cytokine release	THG9011	HEp-2	Jin *et al*., 2003
Nramp1	Colonisation of mice	Colonisation enhanced in Nramp1$^{-/-}$ mice	81-176	Nramp1$^{-/-}$ mice	Watson *et al*., 2007
Occludin	Impaired epithelial barrier functions	infection *in vitro*, TER, hyperphosphorylation of occludin, NF-κB activation, AABs, GPA	81-176, 11168	T84	Chen *et al*. 2006
Paxillin	Invasion	Phosphorylation of	F38011	INT-407	Monteville *et al*.,

		paxillin, infection *in vitro*, IP, AAB, GPA			2003
PI-3 kinase	Invasion	infection *in vitro*, LY294002 and wortmannin inhibitors, GPA	N82, 81-176; 27 clinical strains	INT-407	Wooldridge *et al*., 1996; Hu *et al*., 2006a; Biswas *et al*., 2000
PKC	Invasion	infection *in vitro*, Calphostin C inhibitor, GPA	81-176; 27 clinical strains	INT-407	Hu *et al*., 2005; Biswas *et al*. 2000
Rac-1	Invasion	CA and DN constructs, GMT, CRIB-PD, GPA, IFM, FESEM	81-176, F38011, 84-25	INT-407	Krause-Gruszczynska *et al*., 2007b
Src kinase	Invasion	Infection *in vitro*, PP2 inhibitor, GPA	F38011	INT-407	Eucker and Konkel, 2011

[a] Abbreviations: AB (antibody); AAB (activation-specific antibody), ABB (antibody blocking), CBA (competitive binding assay); MβCD (methyl-beta cyclodextrin); CA constructs (constitutive-active constructs), DN constructs (dominant-negative constructs), IP (immunoprecipitation), CRIB-PD (pull-down experiments to quantify GTPase-GTP levels), EEA-1 (early endosomal marker 1); FESEM (field emission scanning electron microscopy); G-lisa (ELISA-based GTPase-GTP quantification system); GPA (gentamicin protection assay), GST-JlpA (glutathione-S- transferase-tagged JlpA); GMT (GTPase-modifying toxins such as toxin B or CNF, which either inhibit or activate GTPases); IFM (immunofluorescence microscopy), IP (immunoprecipiation); MAPK (Mitogen-activated protein kinases); MyD88 (myeloid differentiation factor 88), o-Van (ortho-Vanadate inhibitor); PKC (protein kinase C); TER (transepithelial resistance); TWA (Transwell assays with polarised cells); wt (wild-type).

(this table was adapted from O'Croinin and Backert, 2012)

2.5 Host cellular responses associated with *C. jejuni* infections

Campylobacter jejuni is one of the leading causes of acute gastroenteritis in humans. Despite its major clinical relevance, the molecular basis of these gastrointestinal infections and the human immune responses to *C. jejuni* is still poorly investigated and understood. Healthy immunocompetent adult humans can usually resolve an infection with *C. jejuni* quickly, before an adaptive immune response appears, but can cause severe complications in children, elderly or immunocompromised people (Allos and Blaser, 1995). *Campylobacter jejuni* is able to stimulate the production of proinflammatory cytokines *in vivo* and *in vitro*. At first, *C. jejuni* must pass the acidic enviroment of the stomach and the intestinal epithelium, which forms an effective physical barrier. In the colon *C. jejuni* induces secretion of interleukin–8 (IL-8) when intestinal epithelial cells are infected. IL-8 is a proinflammatory cytokine which is well-known for its potential to recruit phagocytic cells to the site of infection (Hickey *et al*., 1999).

Additionaly, in the intestine, *C. jejuni* induces innate immune responses (Doorduyn *et al*., 2007; Neal *et al*., 1996), for example through the stimulation of Toll–like receptors (TLRs). These TLRs are the first immunological challenge that *C. jejuni* must overcome during infection. One of the most important TLRs are the members TLR-5, the pattern recognition receptor for bacterial flagellin (Andersen–Nissen *et al*., 2005; Grimbacher *et al*., 2003) and

TLR-9, which is a receptor for CpG disnucleotides, but it seems that both are not profoundly stimulated during infection and are rather avoided by *C. jejuni* (Dalpke *et al*., 2006).

However, an important signaling molecule downstream of TLRs is MyD88, an adaptor protein myeloid differentiation factor, which is essential for signaling through the TLRs and is important for an efficient colonization by *C. jejuni*. In different experiments with MyD88 deficient mice (Akira *et al*., 2001) it could be shown that these mice are susceptible to *C. jejuni* infection and can be easily colonised while wild-type mice clear the infection (Watson *et al*., 2007). These facts indicate that at least one of the TLR pathways has an important role in the control and the defense of *C. jejuni* infections. Different research groups could show that *C. jejuni* lipopolysaccharide and flagellin are not potential TLR–ligands (Hu and Hickey, 2005; Watson and Galan, 2005; Johanesen and Dwinell, 2006). Thus, what *C. jejuni* factor(s) is/are recognised by TLRs is yet unclear and needs to be investigated in future studies.

Recently, Zilbauer and co–workers showed that the innate immune response to *C. jejuni* is also dependent on the nucleotide–binding oligomerization domain (NOD) proteins. This intracellular pathogen recognition receptor is normally able to react with conserved microbial components and plays an important role in immune stimulation, but the actually involved *C. jejuni* factor(s) are also unknown (Akira and Taked 2004; Chen *et al*., 2006; Johanesen and Dwinell, 2006; Zilbauer *et al*., 2007).

Furthermore, the resistance–associated macrophage protein appears to be important to the defence of *C. jejuni* because it is involved in the activation of macrophages (Watson *et al*., 2007).

During *C. jejuni* infection of human intestinal cells they produce proinflammatory cytokines and chemokines. The most important is the neutrophile chemoattractant IL–8, but also IL–1α, IL–1β, IL–6 and TNFα (Hickey *et al*., 1999; Jones *et al*., 2003; Hu and Hickey, 2005; Bakhiet *et al*., 2004; Johanesen and Dwinell 2006). Production of these cytokines and chemokines cause the recruitment of neutrophils and monocytes and promote a potent activity against *C. jejuni* (Zilbauer *et al*., 2005). The exact role of monocytes and macrophages in campylobacteriosis, however, is still controverse discussed in the literature.

Several groups have shown that NF-κB and IL–1β are induced in monocytes. Futhermore, these monocytes are able to undergo apoptosis if they are infected with some *C. jejuni* strains (Szymanski *et al*., 2003; Wassenaar *et al*., 1997, Hickey *et al*., 2005). However, the role of macrophages in all these interactions is not fully clear because, on one hand, it is reported that *C. jejuni* is killed by macrophages (Kiehlbach *et al*., 1985) while other research groups could show that *C. jejuni* can survive for several days within murine macrophages (Hickey *et al*.,

2005; Day *et al*., 2000; Nachamkin and Yang, 1989). Nevertheless, these findings are in agreement with the idea that *C. jejuni* infections are very often self–limiting. Furthermore, an important finding is that *C. jejuni* can induce the production of IL–8 from intestinal epithelial cells by different stimuli (Hickey *et al*., 1999). The most important factor is probably CDT, a major antigen for antibody production, which was required for NF-κB stimulation and IL–8 secretion in infected polarised intestinal epithelial cells (Zheng *et al*., 2008). But Hickey and co–workers have shown in a different study that CDT is not required for IL–8 induction in infected non-polarised INT-407 cells (Hickey *et al*., 2000). These observations indicate that other stimuli must exist which can induce CDT-independent IL–8 release. Which *C. jejuni* factors can trigger these innate immune responses must be further investigated. Taken together, our knowledge about the interplay between the innate immunity and *Campylobacter* infections is still incomplete and requires more work in future studies.

2.6 Role of other *C. jejuni* surface structures

Many microbiological pathogens encode specific cell–surface–structures, such as capsules, flagellins or surface–layer proteins. In addition, the outer membrane of Gram-negative bacteria, such as *C. jejuni*, commonly consists lipopolysaccharides (LPS) and lipooligosaccharides (LOS). LPS and LOS are often involved in serum resistance, endotoxicity and cell adhesion (Backert and König, 2005). The varying composition of these cell surface molecules may play an important role in the virulence of various bacteria and can probably help *C. jejuni* to evade antibody response (Moran *et al*., 1996). It has been reported that *C. jejuni* has a variety of different cell surface carbohydrates (Guerry *et al*., 2002; Karlyshev *et al*., 2005b; Moran *et al*., 2000; Prendergast *et al*., 2004). LPS and LOS are phosphorylated lipoglycans and glycolipids. LOS are composed of a lipid A molecule and a core oligosaccharide. The majority of the cell wall oligosaccharides of *C. jejuni* is attached to the lipid A molecule. The core oligosaccharide of *C. jejuni* is variable in structure (Parker *et al*., 2008; Gilbert *et al*., 2002), but the biological importance is yet unknown. LOS of *C. jejuni* contains sialic acids (NeuNAc) (Godschalk *et al*., 2004; Guerry *et al*., 2002; St.Michael *et al*., 2002). These acids can affect the immunogenicity and serum resistance of *C. jejuni* and they are important for the formation of ganglioside–like structures which are known and similar to that of *H. pylori*, *Haemophilus influenzae*, *Haemophilus ducreyi* and several *Neisseria* species. These ganglioside–like mimicking structures have been implicated in the induction of the paralytic disorders Guillian–Barré–syndrome as well as Miller–Fisher syndrome

(Komagamine and Yuki, 2006; Prendergast and Moran, 2000). Several research groups have shown that these neurological disorders are induced by an autoimmune response to ganglioside–like–structures and are also involved in induction of cross–reacting antibodies (Gilbert *et al.*, 2002; Godschalk *et al*., 2004; Yuki *et al*., 2004, 2005; Yuki and Koga 2006). To date it seems that the ability to generate several LOS structures may help *C. jejuni* to survive in the host.

It has been also shown that LPS of *C. jejuni* may play a role as adhesion molecule (McSweegan and Walker, 1986) and it can vary between different strains (Wassenaar and Blaser, 1999). Different analyses showed that some wild–type strains produce LPS with an O–specific chain and other produce LPS with a short polysaccharide and lipid A (Szymanski *et al*., 2003; Karlyshev *et al*., 2005a; Szymanski and Wren, 2005) The endotoxic properties of LPS is mediated by the lipid A component (Moran, 1995). Investigation of the biochemical features showed that *C. jejuni* LPS consists of a low–molecular–mass fraction that is similar to that in *Neisseria* and *Haemophilus* LPS (Conrad and Galanos, 1990; Moran *et al*., 1991; Aspinall *et al*., 1992, 1993a, 1993b; Mills *et al*., 1992) and a high–molecular–mass fraction (Mills *et al*., 1992). The crucial role of LPS in colonization, invasion and inflammation of *C. jejuni* is still not fully clear and should be investigated in more detail.

The biological function of capsules is diverse and varies between different bacteria. Many Gram-negative bacteria contain a class of cell surface molecules that are called capsular polysaccharides (CPS) (Karlyshev *et al*., 2005a). CPS has different functions, such as protecting the bacteria of adverse environments and plays a crucial role in pathogenesis (Roberts, 1996; Taylor and Roberts, 2005). CPS is loosely associated with the bacterial cell surface and can be released under mild conditions (Withfield and Roberts, 1999). The CPS is attached to the bacterial surface *via* phospholipids (Schmidt and Jann, 1982), which is in contrast to LPS and LOS, that binds to the outer membrane lipid A (Moran, 1995; Moran *et al*., 1991). CPS exhibits high biochemical significance for the bacteria and is the major antigen of the Penner serotyping system, that distinguishes *C. jejuni* isolates and based on its differences in capsule structure (Moran and Penner, 1999; Karlyshev *et al*., 2000). These serotyping schemes of *C. jejuni* strains showed that the variable structure of capsular polysaccharides may play an important role in host colonization, as well as evasion of host immune response. Karlyshev and co-workers could show a strong immune response against Penner-antisera in rabbits (Karlyshev *et al*., 2000; Kilcoyne *et al*., 2006), but this is in contrast to a impaired immune response in chickens (Jeurissen *et al*., 1998). These could be a reason

for the ability of *C. jejuni* to colonize and persist in chicken. To date the role of CPS in chicken colonization is controverse discussed in the literature, because CPS could prevent several adhesion molecules from interacting with host cell receptors, but in contrast non-capsulated bacteria exhibit a reduced ability in colonization of chickens (Bachtiar *et al*., 2007; Grant *et al*., 2005).

Furthermore, it seems that *C. jejuni* CPS maybe also involved in biofilm formation of the bacetria. This feature could contribute to the virulence of *C. jejuni* and may lead to an increase of resistance to antibacterial drugs (Moran and Ljungh, 2003; Otto, 2006). It has also been reported that *C. jejuni* CPS can induce an increasing surface hydrophilicity (Bacon *et al*., 2001). However, the role of CPS and CPS structures in host-bacteria interactions and development of gastroenteritis need more investigations in future.

2.7 Role of small RhoGTPases in *C. jejuni* invasion

The internalization of *Campylobacter jejuni* and other bacterial pathogens has been reported to be induced by rearrangements of the actin cytoskeleton caused by activation of small Rho GTPases (Hardt *et al*., 1998; Criss *et al*., 2001; Kazmierczak *et al*., 2001; Cossart and Sansonetti, 2004; Rottner *et al*., 2004; Pizzaro-Cerda and Cossart, 2006). Small Rho GTPases are monomeric guanine nucleotide-binding proteins with a molecular mass of about 20-25 kDa. To date the family consists of ~20 members. GTPases act as molecular switches that regulate many fundamental processes in healthy cells including growth, morphogenesis, cell mobility, axonal guidence, cytokine production and trafficking. Small Rho GTPases appear either in an inactive or active form, as they cycle between GDP-bound and GTP-bound conformations. In the GDP-bound state they are inactive and in the GTP-bound state they are active. In the active state GTPases transmitt extracellular signals to several downstream effector molecules (reviewed in Schmidt and Hall, 2002).

Small Rho GTPase family consist three subfamilies, Rho, Rac and Cdc42 and these have a distinct effect on the actin cytoskeleton. The most and best characterized members are RhoA, Rac1 and Cdc42 (Jaffe and Hall, 2005). RhoA proteins are involved in formation of stress fibers and focal adhesion complexes, while Rac1 proteins trigger lamellipodia and membrane ruffling, and Cdc42 proteins can induce filopodia formation (Scita *et al*., 2000). A growing number of known bacterial virulence factors acting on small Rho family GTPases comprises GEFs (guanine exchange factors) and GAPs (GTPase activating proteins). GEFs and GAPs are capable of regulating localized signalling to actin rearrangements at the site of bacterial

invasion. In addition many bacterial protein toxins are specialized to activate, inhibit or modify small Rho GTPases.

Recently, our group has reported that small Rho GTPases are involved in *C. jejuni* invasion (Krause-Gruszczynska *et al*., 2007b). In particular, the internalization of *C. jejuni* into INT-407 cells is accompanied by a time-dependent activation of both Rac-1 and Cdc42 (Krause-Gruszczynska *et al*., 2007b). Using specific GTPase-modifying toxins, inhibitors and GTPase expression constructs it was shown by immunofluorescence, gentamicin protection assay and other studies that Rac-1 and Cdc42, but not RhoA, play a role in *C. jejuni* invasion (Krause-Gruszczynska *et al*., 2007b). Maximal GTPase-GTP levels induced by *C. jejuni* also involved several host kinase activities as supported by inhibitor studies, and the bacterial fibronectin-binding protein CadF (Krause-Gruszczynska *et al*., 2007b). However, the molecular pathways involved in *C. jejuni*-triggered GTPase activation remained widely unclear.

2.8 Aim of the study

Foodborne infections with pathogenic bacteria have been reported to be one of the primary reasons of morbidity and death in humans worldwide. Host cell invasion of *C. jejuni* represents one of the leading causes of tissue damage in infected humans. However, the molecular mechanisms and factors which play a crucial role in this process are widely unclear.

The present work was performed to investigate and characterize bacterial pathogenicity factors and host cell signalling pathways, which are involved in infection, transmigration and invasion of epithelial cells by *C. jejuni*.

1.) To study the function and importance of *C. jejuni* HtrA in secretion, cleavage of E-cadherin and transmigration across polarised epithelial cells.
2.) To study the role of fibronectin, integrin-β1 receptor, focal adhesion kinase (FAK) and Src kinases for the induction of GTPase signalling and uptake of *C. jejuni.*
3.) To study the role of the above host cell components during the invasion process of *C. jejuni* using high-resolution electron microscopy.
4.) To identify crucial guanine-exchange factors (GEFs) involved in the activation of Rac1 and Cdc42.
5.) To investigate the role of the bacterial fibronectin-binding protein CadF and the flagellum for the induction of GTPase signalling and bacterial invasion.

3. MATERIALS

3.1 Bacterial strains

3.1.1 *Campylobacter* strains

Campylobacter jejuni wild type isolates and their isogenic mutants used in this work were provided by different cooperation partners. They are listed in Table 3 and 4.

Table 3: *Campylobacter jejuni* wild type strains.

***Campylobacter jejuni* wild type isolates**	**origin**
81-176, NCTC11168	Dr. William G. Miller, USDA, ARS, WRRC, Produce Safety and Microbiology Research Unit, Albany, USA
F38011	Prof. Dr. Michael E. Konkel, School of Molecular Biosciences, Center for Biotechnology, Washington State University, Pullman, USA
NCTC81116	Prof. Dr. Jos P. M. van Putten, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands
84-25	Prof. Martin J. Blaser, Department of Medicine, New York University School of Medicine, New York, USA
0097, C130, 128/94	Dr. Ingrid Haenel, Federal Research Institute for Animal Health, Jena, Germany
ST3046, 1543/01, RM1221, 2703/01	Institute of Medical Microbiology, Magdeburg, Germany

Table 4. *Campylobacter jejuni* isogenic mutants.

***Campylobacter jejuni* wild type isolates**	**origin**
81-176Δ*htrA*	Prof. Steffen Backert, University College Dublin, School of Biomolecular and Biomedical Science, Ireland
81-176Δ*flaA/B* 81-176Δ*flhA*	Prof. Martin J. Blaser, Department of Medicine, New York University School of Medicine, New York, USA Prof. Patricia Guerry, Enteric Diseases Department, Naval Medical Research Center, Silver Spring, Maryland, USA
F38011Δ*cadF*, F38011Δ*flpA*, F38011Δ*cadF*/Δ*flpA*	Prof. Dr. Michael E. Konkel, School of Molecular Biosciences, Center for Biotechnology, Washington State University, Pullman, USA
81116Δ*cadF*	Prof. Dr. Jos P. M. van Putten, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands
84-25Δ*kps*	Prof. Martin J. Blaser, Department of Medicine, New York University School of Medicine, New York, USA

3.1.2 *Escherichia coli (E.coli)* strains

E. coli strains used in this work are presented in Table 5.

Table 5: *Escherichia coli* strains.

***E.coli* strain**	**Genotype**
TOP10	F^{-} *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC*)* Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rsp*L (Str^{R}) *end*A1 *nup*G
BL21	F^{-} *ompT hsdS*$_{B}$(r^{B-} m^{B-}) *gal dcm*

3.2 Eukaryotic cell culture

Different cell lines used in this work are listed in Table 6.

Table 6: Eukaryotic cells

Cell line	**Characteristics**	**Origin / References**
Human cell lines		
INT-407	embryonic intestinal epithelial cells, adherent	ATCC CCL-6
MKN28	gastric epithelial cells, adherent	JCRB0253
Mouse cell lines		
GD25	Integrin subunit ß$_1$-deficient fibroblasts, adherent	Prof. Johansson; Wennerberg *et al.*, 1996
GD25-ß$_1$A	GD25 stably re-expressing wild-type ß$_1$A	Prof. Johansson; Wennerberg *et al.*, 1996
GD25-ß$_1$A$_{TT788-9AA}$	GD25 stably re-expressing mutated integrin subunit ß$_1$A	Prof. Johansson; Wennerberg *et al.*, 1998
GD25-ß$_1$A$_{Y783/795F}$	GD25 stably re-expressing mutated integrin subunit ß$_1$A	Prof. Johansson; Wennerberg *et al.*, 2000
FAK$^{-/-}$	fibroblasts derived from FAK-deficient mouse embryos	Prof. Ch. Hauck; Sieg *et al.*, 1999
FAK$^{+/+}$	FAK (-) stably re-expressing HA-epitope-tagged FAK	Prof. Ch. Hauck; Sieg *et al.*, 1999
Fn$^{-/-}$	fibroblasts derived from Fn-deficient mouse embryos	Prof. Takahashi; Nyberg *et al.*, 2004, Schroeder *et al.*, 2006
Fn$^{+/+}$	fibroblasts derived from mouse embryos	Prof. Takahashi; Nyberg *et al.*, 2004, Schroeder *et al.*, 2006
SYF	src$^{-/-}$, yes$^{-/-}$, fyn$^{-/-}$ *triple knockout*-Zellen	ATCC CRL-2498, Klinghoffer *et al.*, 1999
SYF + c-src	src, yes, fyn *knockout*-Zellen + c-src	ATCC CRL-2459, Klinghoffer *et al.*, 1999
Vav-1/2$^{-/-}$	fibroblasts derived from Vav-deficient mouse embryos	Prof. Ch. Hauck; Schmitter *et al.*, 2007

3.3 Growing medium and plates

Cultivation of *Campylobacter* strains was carried out on *Campylobacter* blood free selective agar base (Oxoid Basingstoke, UK) containing *Campylobacter* selective supplement. In addition Mueller-Hinton (MH) agar-plates with corresponding antibiotics were used or the bacteria were cultivated in BHI-medium.

***Campylobacter*-plates:**

***Campylobacter* Blood Free Selective Agar Base**			**Mueller-Hinton-Agar**	
per liter			**per liter**	
Nutrient Broth No. 2	25.0 g		Beef infusion solids	4.0 g
Bacteriological charcoal	4.0 g		Casein hydrolysate	17.5 g
Casein hydrolysate	3.0 g		Starch	1.5 g
Sodium desoxycholate	1.0 g		Agar	15.0 g
Ferrous sulphate	0.25 g			
Sodium pyruvate	0.25 g			
Agar	12.0 g			

***Campylobacter* Selective Supplement**	
per liter	
Cefoperazone	32.0 mg
Amphotericin B	10.0 mg

Antibiotics	**Final concentration**
Chloramphenicol	4 µg/ml
Tetracycline	10 µg/ml
Kanamycin	20 µg/ml

BHI medium	
per liter	
BHI	37.0 g

Cultivation of *E.coli* was carried out on Luria-Bertani (LB) agar plates or LB medium.

LB medium	
per liter	
tryptone	10.0 g
yeast extract	5.0 g
NaCl	10.0 g

For preparation of LB agar plates 18 g/l of agar was added to the LB medium.
For selection of antibiotic resistant *E. coli* antibiotics were added to the LB medium.

Antibiotics	**Final concentration**
Ampicillin	100 µg/ml
Kanamycin	30 µg/ml

3.4 Chemicals

If not noted otherwise, chemicals in analytical pure grade (p.a.) were used from the companies Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany) and Fisher Scientific (Dublin, Ireland).

3. 5 Enzymes and Proteins

*Bam*HI [20U/µl]	New England Biolabs (Frankfurt / Main, Germany)
*Bgl*II [10U/µl]	New England Biolabs (Frankfurt / Main, Germany)
Bovine serum albumin (BSA)	Sigma (Taufkirchen, Germany)
*Eco*RI [20U/µl]	New England Biolabs (Frankfurt / Main, Germany)
Fetal calf serum (FCS)	Gibco/ Invitrogen (Karlsruhe, Germany)
*Nde*I [20U/µl]	New England Biolabs (Frankfurt / Main, Germany)
*Not*I [20U/µl]	New England Biolabs (Frankfurt / Main, Germany)
Platinum® *Taq* DNA-Polymerase High Fidelity [5U/µl]	Invitrogen (Karlsruhe, Germany)
*Pst*I [20U/µl]	New England Biolabs (Frankfurt / Main, Germany)
*Sca*I [6U/µl]	New England Biolabs (Frankfurt / Main, Germany)

Taq-DNA-Polymerase	Quiagen (Hilden, Germany)
T4-DNA-Ligase	Fermentas (St.Leon-Roth, Germany)
Trypsin-EDTA	Sigma (Taufkirchen, Germany)
*Xho*I [20U/µl]	New England Biolabs (Frankfurt / Main, Germany)

3.6 Antibodies

3.6.1 Primary antibodies

Table 7: Antibodies used in this work for Western blotting analysis.

Antibody	Origin	Purchased from
Monoclonal		
α-DOCK180 (H-4)	mouse	Santa Cruz Biotechnology
α-E-Cadherin (HECD-1)	mouse	Calbiochem
α-Rac (clone 23A8)	mouse	Upstate Biotechnology
Polyclonal		
α-CadF-1	rabbit	BioGenes
α-Cdc42 (P1)	rabbit	Santa Cruz Biotechnology
α-E-Cadherin (H108)	rabbit	Santa Cruz Biotechnology
α-E-Cadherin (K20)	goat	Santa Cruz Biotechnology
α-FAK (A17)	rabbit	Santa Cruz Biotechnology
α-FAK-PY-925	rabbit	Biomol
α-GAPDH (V-18)	goat	Santa Cruz Biotechnology
α-HtrA	rabbit	Prof. Lone Broenstred, Denmark; BioGenes,
α-Integrin ß1	rabbit	Cell Signaling
α-MOMP	rabbit	BioGenes
α-EGFR	rabbit	Cell Signaling
α-PDGFR ß	rabbit	Cell Signaling
α-EGFR-PY-845	rabbit	Cell Signaling
α-PDGFR ß-PY-754	rabbit	Cell Signaling
α-Tiam1 (C-16)	rabbit	Santa Cruz Biotechnology
α-Vav-2 (H-200)	rabbit	Santa Cruz Biotechnology

3.6.2 Secondary antibodies

Secondary antibodies used in this work for Western blotting

Polyclonal-rabbit-anti-mouse Immunoglobulin / HRP	Dako (Hamburg, Germany)
Polyclonal-rabbit-anti-goat Immunoglobulin / HRP	Dako (Hamburg, Germany)
Polyclonal-swine-anti-rabbit Immunoglobulin / HRP	Dako (Hamburg, Germany)

3.7 DNA standards

As DNA-markers 1kb- or 100 bp-ladders from Fermentas were used (St.Leon-Roth, Germany). As standards for the molecular weight of proteins used prestained (10-250 kDa) - and prestained plus-protein ladders (Fermentas, St.Leon-Roth, Germany).

3.8 Oligonucleotids

Oligonucleotides used by cooperation partner Lieke B. van Alphen (Department of Infectious Diseases and Immunology, Utrecht University, The Netherlands) for amplification, cloning and sequencing of CadF protein gene *cadF* and its flanking regions:

CadF1 Fwd: 5'-TTGCTCTAAAGGATAACCTATGA-3'

CadF1 Rev: 5'-TATGGACGCCGCAAAGCAAG-3'

CadF2 Fwd: 5'-CCACTCTTCTATTATCCGCTCTACC-3'

CadF2 Rev: 5'-GGTGCTGATAACAATGTAAAATTTG-3'

3.9 Plasmids

Table 8: Plasmids used in this work.

Plasmid	**Marker/Characteristics**	**Origin/References**
pRK5	ColE ori, CMV Promoter, MCS, SV40 PolyA, SV40 ori, f1 ori, Amp^R, c-Myc	BD Biosciences, San Diego, USA
pcDNA3.1-FAK, , pcDNA3.1-FAK K454R, pcDNA3.1-FAK Pro^-, pcDNA3.1-FAK Y925F	pcDNA3.1 constructs containing cDNAs of HA-tagged wild-type FAK or different FAK mutants	Prof. Christof Hauck, Chair of Cell Biology, University of Konstanz, Konstanz, Germany; Sieg *et al.*, 1999

3.10 Inhibitors

Inhibitors used in this study.

Table 9: Pharmacological inhibitors used in this work.

Name	Final concentration	Function	Origin/References
NSC23766	50 µM	Inhibits Rac1 GDP/GTP exchange activity by interfering with the interaction between Rac1 and its GEFs Tiam1 and Trio	Calbiochem; Gao *et al.*, 2004
MβCD	1mM-10mM	Sequester cholesterol	Sigma-Aldrich; Watson and Galan, 2008
AG1478	10 µM	Inhibits EGFR	Sigma
AG370	10 µM	Inhibits PDGFR	Sigma
Wortmannin	1 µM	Inhibits PI3- kinase	Calbiochem; Biswas *et al.*, 2000; Hu *et al.*, 2006a
PF-573228	10 µM	Inhibits FAK	Tecris

4. METHODS

4.1 Molecularbiological methods

4.1.1 Digestion of DNA with restriction enzymes

For analytical restriction digests 1-3 µg DNA and for preparative digests 6-10 µg DNA were used. Restriction enzymes (2-5 U/µg) and their corresponding buffers were used according to the manufactors instruction (New England Biolabs, Frankfurt/Main, Germany). The reaction mix was incubated for 2 hours at 37°C, followed by separation and size determination of DNA fragments by agarose gel electrophoresis. DNA fragments of preparative restriction digests were isolated from the agarose gel by using the Jetsorb Gel Extraction Kit (Genomed, Löhne, Germany).

4.1.2 Ligation of DNA fragments

For the ligation of DNA fragments the Rapid DNA-Ligation Kit (Fermentas, St.Leon-Roth, Germany) was used according to the manufactors instruction.

For a 20 µl reaction mix used 1/5 Volume of 5x Rapid Ligation buffer, 5U T4-DNA-ligase, 50-100 ng vector DNA and insert DNA were used. The molar ratio of insert DNA to vector DNA was 3:1. The ligation reaction mix was incubated for 30 min at room temperature and directly used for transformation of competent *E.coli* cells.

4.1.3 Isolation of plasmid and genomic DNA

Isolation of plasmid DNA from bacteria was carried out with the Jetstar Plasmid Purification-Maxi-Kit according to the manufactors instruction (Genomed, Löhne, Germany).

The method is based on a modified alkaline/SDS lysis (Birnboim and Doly, 1979) and an anion exchange adsorption. The precipitated DNA was redissolved in water.

Genomic DNA was isolated as described by Wilson *et al*. (1989). In brief, *C. jejuni* growing on *Campylobacter* selective agar plates was resuspended in PBS and centrifuged (5000 x g, 5 min, RT). The pellet were mixed with 200µl of lysis buffer (50 mM EDTA, pH8.0.,1%SDS, 0.1 mg/ml proteinase K) and kept at 55°C for 1-2 hours to complete cell lysis. Subsequently a phenol-chloroform-extraction of the DNA was carried out. DNA was dried at RT and resuspended in water. Purified DNA was used as template for PCR amplification.

4.1.4 Polymerase chain reaction (PCR)

PCR was used to amplify DNA fragments and to verify the identity of bacterial strains.

Reactions were carried out in PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, USA) and in Perkin Elmer GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer, Waltham, USA).

DNA was amplified with specific primers (MWG-Biotech, Ebersberg, Germany) and *Taq*-DNA-polymerase (Quiagen) in PCR reaction mix as listed below. Platinum®-*Taq*-Polymerase (Invitrogen, Karlsruhe, Germany) was used for high fidelity amplification.

Reagents	**Final concentration**
dNTP-Mix	0.2 mM
each primer	100 pmol
DNA (template)	50 ng
10 x PCR buffer	5 µl
with $MgCl_2$	3 mM
Taq-polymerase	2.5 U
H_2O	Until 50 µl

PCR included a denaturation step at 94°C for 5 min and 35 times repeated cycles consisting of denaturation, primer annealing and DNA synthesis. The elongation times was dependent on the length of the DNA fragments which were to be amplified and the elongation temperature on which *Taq*-Polymerase was used.

4.1.5 Determination of DNA concentration

Concentrations of DNA were determined spectro-photometrically by using a biophotometer and UV cuvettes (Eppendorf, Hamburg, Germany). The optical density was determined at a wave length of 260 nm.

By an additional measurement at wave length of 280 nm, the purification of DNA can be estimated.

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4.1.6 DNA extraction from agarose gel

Isolation of DNA fragments from agarose gels was performed by using the Jetsorb Gel Extraction Kit (Genomed, Löhne, Germany) according to the manufactures instruction.
Agarose gels in TBE buffer were used. To visualize the DNA bands, the gel was shaked in a 0.02% methylenblue solution for 15 min.
Extracted DNA was verified by standard agarose gel electrophoresis.

4.1.7 Agarose gel electrophoresis

The analytical separation of DNA fragments was performed by using horizontal gel electrophoresis. The principle of gel electrophoretical separation is, that the nucleid acids migrate through the electrical field to the anode because they have a negative charge. The migration-speed is dependent of the molecule size, so that smaller molecules are faster than larger molecules. By staining the gel with ethidiumbromide, DNA fragments become visible under ultraviolet fluorescence light. By using this method it is possible to determine the size of DNA fragments in comparison to a DNA standard ladder.
To prepare the agarose gels (size 8 x 7cm), the agarose (Biozym, Hessisch Oldendorf; Germany) was dissolved in TBE buffer (44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA, pH 8.0) by cooking and addition of 0.1 µg/ml ethidiumbromide (Roth, Karlsruhe, Germany). The concentration of agarose range between 0.7 to 1.5 %, depending of the size of the DNA fragments. As running buffer 0.5 x TBE–buffer was used. To the DNA samples 1/6 volume of 6 x loading dye (Fermentas, St.Leon–Rot, Germany) was added. To determine the molecular size of the DNA fragments a GeneRuler^{TM} 1 kb DNA Ladder (Fermentas, St. Leon–Rot, Germany) was used. The electrophoresis was carried out at room temperature and constant voltage (10 V/cm). The DNA gels were documentated by using Lumi Imager F1 (Roche Diagnostics GmbH, Mannheim, Germany).

4.1.8 HtrA expression plasmids and purification

C.jejuni htrA gene (corresponding to *Cj*HtrA aa17-aa472) was amplified from genomic DNA. PCR fragments flanked by restriction sites for *Bam*HI / *Xma*I were cloned into pGEX-6P-1 (GE Healthcare). Generation of inactive HtrA S→A mutant (*Cj*HtrA S194A) was performed by S→A mutations in the active center using a site-directed mutagenesis kit (Stratagene). The mutated constructs were transformed into *E. coli* BL21 (NEB). For protein purification of GST-*Cj*HtrA , transformed *E. coli* was grown in 500 ml TB medium to an OD_{550} of 0.6 and the expression was induced by the addition of 0.1 mM isopropylthiogalactosid (IPTG). The

bacterial culture was pelleted at 4000×g for 30 min and lysed in 25 ml PBS by sonification. The lysate was cleared by centrifugation and the supernatant was incubated with glutathione sepharose (GE Healthcare Life Sciences) at 4°C overnight. The fusion protein was either eluted with 10 mM reduced glutathione for 10 min at room temperature or cleaved with 180 U Prescission Protease for 16 hours at 4°C (GE Healthcare Life Sciences). Elution and cleavage products were analyzed by SDS PAGE and zymography. Cleavage assays of purified HtrA with recombinant human full-length E-cadherin (R&D Systems), recombinant human His-tagged N-terminal NTF domain (Sino Biological) or human fibronectin (Calbiochem) were perfomed as described by Hoy *et al*., 2010.

4.1.9 Zymography

Bacterial lysates, cell culture supernatants (filtered through a 0.21 μM sterile filter, Roth) or recombinant HtrA were loaded onto 0.1 % casein containing gels and separated under non-denaturing conditions. After separation, the gel was re-naturated in 2.5% Triton X-100 solution at room temperature for 60 min with gentle agitation, equilibrated in developing buffer (50 mM Tris-HCl, pH 7.4; 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij35) at room temperature for 30 min with gentle agitation, and incubated overnight at 37°C in fresh developing buffer. Transparent bands of caseinolytic activity were visualized by staining with 0.5% Coomassie Blue R250.

4.2 Proteinbiochemical methods

4.2.1 Generation of polyclonal antibodies

The polyclonal antisera were produced by BioGenes (Berlin, Germany). The peptides for immunisation of αCadF (amino acid 293-306: QDNPRSSNDTKEGR), αFlpA (amino acids 365–378: NAVFKGIKEKRLKD), α-FlaA (amino acids 93-106: KTKATQAAQDGQSL) and αMOMP (amino acids 400-413: NLDQGVNTNESADH) were produced by BioGenes, too and conjugated to *Limulus polyphemus* haemocyanin carrier protein. For this purpose, it was immunized in two rabbits each time including one boost according to standard protocols (BioGenes). The antibodies were tested for its specificity in the lab of Prof. St. Backert and were available for me.

4.2.2 Immunoprecipitation

The immunoprecipitation (IP) is a technique to determine protein–protein interactions *in vitro.* This process can be used to separate specific antigens and their interaction partners from whole cell lysates or cell culture supernatants by using specific antibodies. After IP the protein can be analysed by using immunoblotting.

For a typical IP, 1 x 10^7 INT407–cells were harvested by centrifugation (5 min, 5000 rpm, 4°C). The cell–pellet was washed with ice-cold DPBS (Lonza, BioWhittaker, Basel Switzerland), centrifuged (5 min, 5000 rpm, 4°C) and lysated for 30 min on ice in lysis buffer [20 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, protease inhibitor cocktail (Roche)]. To lower the amount of non–specifically bind proteins it is necessary to pre-clear the lysates. For the pre-clearing the protein-G-sepharose (Amersham Bioscience, Freiburg, Germany) was washed three times with DPBS and one time with lysis buffer, each time centrifuged 5 min, 10000 rpm, 4°C. Following, the lysates were incubated with the protein-G-sepharose for 2 hours at 4°C to eliminate unspecific proteins, which bind to the protein-G-sepharose. Then the protein-G-sepharose and cell suspension were centrifuged (5 min, 3000 rpm, 4°C). The supernatant was incubated with specific antibody overnight at 4°C.

The IP-complexes were incubated with washed protein-G-sepharose for 2 hours at 4°C and precipitated by centrifugation (5 min, 5000 rpm, 4°C). Precipitated proteins were analysed by SDS-PAGE and immunoblotting.

4.2.3 Rac1- and Cdc42- activation assay (G-LISA™)

The Rho family of small GTPases consists of about 20 members. The most and best characterized members are Rac1, RhoA and Cdc42 proteins (Jaffe and Hall, 2005). GTPases switch between an active GTP-bound and an inactive GDP-bound state. The G-LISA™ assays use the fact, that Rho GTPase proteins recognized the GTP-bound state (Kazuko *et al*., 1998) to measure the Rac1- and Cdc42-activation.

Rac1 and Cdc42 activation were determined by the G-LISA™ Rac1- and Cdc42-activation assays (Cytoskeleton, Denver, USA).

For these assays, host cells were grown to 70% confluency in tissue culture petri dishes and then the cells were starved overnight. The cells were infected with *C. jejuni* for indicated periods of time. Subsequently, cells were washed with DPBS (Lonza, BioWhittaker, Basel Switzerland), resuspended in lysis buffer of the kit and harvested from the petri dishes with a cell scraper on ice. Total protein concentration in each lysate was determined by using the

protein assay reagent of the kit and measuring the OD_{600} nm of the samples with an Eppendorf-biophotometer *plus.* Cell lysates were equalized with lysis buffer to give identical protein concentrations.

The G-LISA™ Rac1 activation assay contains a Rac-GTP-binding protein immobilised on provided microplates. The corresponding amount of cell lysates were added to respective wells and incubated for 30 min under shaking (400 rpm) at 4°C. After washing the wells for three times with wash buffer the antigen presenting buffer was added to each well and incubated at room temperature for exactly 2 min. Followed by washing for three times and addition of the primary antibody for 45 min under shaking (400 rpm, RT). Each well was washed for three times with wash buffer again and subsequently the HRP-conjugated secondary antibody was added for 45 min under shaking (400 rpm, RT). Unbound antibody was removed by washing three times with wash buffer. To detect the luminescence signal, an HRP detection reagent was added to each well and the luminescence signal was quantified immediately by using a microplate reader (SpectraFluor Plus, Tecan).

The G-LISA™ Cdc42 activation assay contains a Cdc42-GTP-binding protein immobilised on provided microplates. The corresponding amount of cell lysates were added to respective wells and incubated for 15 min under shaking (400 rpm) at 4°C, followed by washing each well with wash buffer for three times. Immediately after washing the antigen presenting buffer was added for exactly 2 min at room temperature. After washing for three times again each well was incubated with the primary antibody for 30 min at room temperature under shaking (400 rpm). Unbound antibody was removed by three times washing with wash buffer, followed by incubation with an HRP-conjugated secondary antibody for 30 min under shaking (400 rpm, RT). After washing for three times again, HRP detection reagent was added to each well and incubated for 10 min at 37°C. After the incubation, stop buffer was added into each well and colorimetric signals were quantified by using a microplate reader (SpectraFluor Plus, Tecan).

4.2.4 CRIB-GST pulldown assay for Rac1-GTP and Cdc42-GTP

Rac1- and Cdc42 activation in infected cells was determined with the Rac1- and Cdc42 activation assay kit (Cytoskeleton), based on a pulldown assay using the Cdc42–Rac1 interactive binding domain of PAK1 fused to glutathione S-transferase, GST–CRIB (Sander *et al.*, 1998). Briefly, host cells were grown to 70 % confluency and serum-starved overnight.

Subsequently, cells were incubated in PBS (pH 7.4) as a control or infected with *C. jejuni* (MOI of 100) in a time course. Uninfected and infected host cells were washed with PBS, resuspended in the assay buffer of the kit, and detached from dishes with a cell scraper. For a positive and negative control, a portion of the uninfected cell lysate was mixed with GTPγ-S and GDP for 15 min, respectively. Cell lysates (treated with bacteria, GTPγ-S, GDP or untreated) were mixed with the PAK-RBD slurry (1 hour, 4°C). Finally, the beads were collected by centrifugation and washed three times with assay buffer. Activated Rac was then visualized by immunoblotting as described below. To confirm equal amounts of protein for each sample, aliquots of the lysates from different time points were also analyzed by immunoblotting. The GTPase activities were quantitated as band intensities representing the relative amount of active Rac1-GTP and active Cdc42-GTP using the Lumi-Imager F1 software program (Roche).

4.2.5 Cellular fractionation

To identify the different subcellular localizations of proteins in infected and non-infected cells a gradually extraction of three different protein fractions (cytosol, membrane and unsoluble fraction) was performed according Kenny and Finlay (Kenny and Finlay, 1997).

For fractionation, *Campylobacter jejuni* infected and uninfected INT-407 cells ($5x10^6$) were harvested by centrifugation (5 min, 5000 rpm, 4°C). The cell pellet was washed with ice-cold DPBS (Lonza, BioWhittake, Basel/Switzerland), centrifuged (5 min, 5000 rpm, 4°C) and resuspended in 100 µl saponin buffer [50 mM Tris-HCL (pH 7.5), 0.4 mM Na_3VO_4, 1mM NaF, 0.2 % Saponin, COMPLETE™ (Roche, Mannheim, Germany)] to permeabilise the cells. After 5 min of incubation and centrifugation (5 min, 10000 rpm, 4°C) the supernatant with the cytosolic protein fraction was resuspended in 100 µl 2 x SDS buffer [125mM Tris-HCL (pH 6.8), 4% SDS, 0.02% bromophenol blue, 20% glycerine, 100mM DTT]. To dissolve the membrane protein fraction the pellet was resuspended in 50 µl Triton X-100 buffer (Saponin buffer, 1% TritonX-100) and centrifuged (5min, 10000 rpm, 4°C). The supernatant with the membrane fraction was resuspended in 50 µl 2 x SDS buffer and the pellet, which is the insoluble protein fraction was dissolved in 100 µl 2 x SDS buffer. Different protein fractions were analysed by SDS-PAGE and Western blotting.

4.2.6 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic separation of proteins is based on their molecular size was performed by using denaturating, discontinuous polyacrylamide gels according to the method described by

Laemmli (Laemmli *et al*., 1970). With this technique proteins are loaded onto a gel that act as a molecular filter and retain larger proteins more than smaller ones. Proteins are negative charged and in an electrical field they migrate to the anode. For SDS-PAGE the vertical gel electrophoresis-Mini-Protean-III^{TM} of Bio-Rad (Munich, Germany) was used. The concentration of the stacking gel was 5% and the resolving-gel range between 6 to 12% (Sambrook *et al*., 1989). Lysates were resuspended in SDS-buffer and denatureted by heating for 5 min at 95°C, followed by loading 10 to 30 µl onto the gel. The electrophoretical separation was carried out at 120V. To determine the protein size a prestained protein ladder was used (Fermentas, St.Leon-Rot, Germany).
Following electrophoresis, the proteins were stained with Coomassie-Brilliant-Blue solution or transferred to a polyvinylidendifluorid membrane (PVDF, Millipor, Billerica, MA, USA).

Gel running buffer pH 8.3			**Transfer buffer pH 8.4**			**10xTBS buffer pH 7.4**	
per liter			per liter			per liter	
Tris	3.2 g		Tris	3.0 g		Tris	24.2 g
Glycine	18.8 g		Glycine	14.5 g		NaCl	80.0 g
10% (v/v) SDS	10 ml		10% (v/v) SDS	10 ml			

TBS-T buffer pH 7.4			**Coomassie stain**			**Coomassie de-stain sol.**	
per liter			per liter			per liter	
10xTBS pH 7.4	100 ml		Coomassie	2.5 g		Methanol	300 ml
Tween 20	1ml		Briliant Blue			Acetic acid	100 ml
			Methanol	450 ml			
			Acetic acid	100 ml			

4.2.7 Coomassie staining

By using the Coomassie-Brilliant-Blue solution the separated proteins can be directly stained on the SDS–PAGE protein gel. The sensibility of this method ranges between 1 µg to 0.1 µg protein. To stain the proteins, gels were shaked 30 min in Coomassie-Brilliant-Blue solution at room temperature. By this method the proteins are fixed by acetic acid and methanol

dissolves the Coomassie dye. The Coomassie dye accumulates to alkaline and aromatic side chains of amino acids and therefor the proteins become visible. After that, the gel was destained with corresponding solution. Gels and protein bands were documented and quantified by Lumi Imager F1 (Roche diagnostics GmbH, Mannheim, Germany).

4.2.8 Western blotting

Western or immunoblotting was used for the specific detection of proteins (Towbin *et al*., 1992). Proteins which were separated by SDS-PAGE and transferred to a PDVF membrane (Millipore, Billerica, MA, USA) were detected by using specific antibodies. For the transfer a semidry-blot-apparatus (Roth, Karlsruhe, Germany) was used, which consists of two graphite electrodes. The PVDF membrane was activated by methanol, followed by aquilibration in transfer buffer. The whatman papers were also aquilibrated in transfer buffer. For the blot assembling three whatman papers were added to the graphite anode, followed by the membrane, the resolving gel, three further whatman papers and the graphite cathode. The protein transfer was carried out for 2 hours at 0.8 mA/cm^2. During the protein transfer the negative charged proteins migrate towards the anode and are immobilised on the PVDF membrane. After blotting the PVDF membrane was washed in TBST and incubated in blocking buffer for 2 hours at room temperature or overnight at 4°C to saturate unspecific binding sites. As blocking buffer used 3 % bovine serum albumine [(BSA), Sigma, Taufkirchen, Germany] in TBST or 5 % milk powder (Roth, Karlsruhe, Germany) in TBST was used. Following blocking, the membrane was incubated with the primary antibody for 2 hours at room temperature or overnight at 4°C. The antibodies were used to manufactores instructions. Unbound antibodies were removed by washing the membrane three times for 15 min each with TBST. As secondary antibody horseradish peroxidase conjugated IgG (DakoCytomation, Hamburg, Germany) was added for 1 hour at room temperature in dark, followed by washing three times for 15 min each with TBST. The protein-antibody-complex was visualized by the ECL Plus Western Blotting Detection System (Amersham, Bioscience, Freiburg, Germany). For detection and documentation and quantification of bands the Lumi Imager F1 (Roche Diagnostics GmbH, Mannheim, Germany) was used.

To re-probe the membrane with other primary antibodies, the membrane was incubated in stripping buffer for 45 min at 60°C. The membrane was washed several times with TBST, blocked and probed again as described above.

Stripping buffer pH 6.7	
per 100 ml	
1M Tris	6.25 ml
10% (v/v) SDS	20.0 ml
ß-mercaptoethanol	0.833 ml

4.3 Microbiological methods

4.3.1 Cultivation of *Campylobacter jejuni*

Campylobacter jejuni was cultivated on *Campylobacter* blood-free selective Agar Base (Oxoid Basingstoke, UK) containing *Campylobacter* growth selective supplement (Oxioid). For selection of antibiotic resistant *C. jejuni* strains, sterile filtered antibiotics were added to the media. The bacteria were streaked out with a blue loop (Greiner-Bio-One, Frickenhausen, Germany) from a frozen stock (-80°C) and incubated at 37°C under microaerophilic conditions, generated by CampyGen (Oxoid) in an Anaero-Jar (Oxoid). After 2 days the bacteria were diluted and streaked out on three *Campylobacter* plates. For the dilution a sterile cotton swob (Raucotupf, Lohmann and Rauscher, Rengsdorf, Deutschland) was used with which the bacteria were harvested and resuspended in 500 µl BHI medium (brain heart infusion, Fluka BioChemika, Buchs, Switzerland) and streaked out on the first *Campylobacter* plate. For the second and third plate the same cotton swob was used, resuspended in BHI-medium and streaked out on the plates. Following, the plates were incubated for 16 hours at 37°C under microaerophilic conditions in an Anaero-Jar with CampyGen. For *C. jejuni* infection experiments the third dilution was used.

For preservation of strains, bacterial cells from an abundantly covered plate were re-suspended in BHI-media with 20% glycerine and stored at -80°C.

4.3.2 Infection experiments with *Campylobacter jejuni*

4.3.2.1 Infection of host cells

For infection experiments *C. jejuni* was harvested from the third agar plate with a sterile cotton swob (Raucotupf, Lohmann and Rauscher, Rengsdorf, Deutschland), re-suspended in BHI-medium and the optical density was determined by measuring at $\lambda = 600$ nm (OD_{600}) in a UV/Vis spectrometer Lambda 2 (Perkin Elmer Waltham, USA). Subsequently, the bacteria

suspension was added to the incubated cells. At the time point of infection the confluence of the cells was ~ 70%. The cells were infected with a multiplicity of infection (MOI) of 100 for indicated periods of time at 37°C in 5 % CO_2.

4.3.2.2 Infection of host cells in transwell-filter-system

Infection experiments in transwell-filter-system were used to determine the dependent of contact as well as the effect of the co-infected bacteria to release specific *C. jejuni* determinants. For this the transwell-filter-systems (Corning B.V. Lifescience, Schiphol, Niederlande) with poresize of 3.0 µm or 0.4 µm were used.

The transwell-filter-system is a two component system, in a 12- well plate and separate filters. Before use, the system was pre-incubated with cell culture medium for 1 hr. Subsequently MKN28 cells were seeded on the filter and incubated for 14 days at 37°C in 5% CO_2. During incubation, it is important to change the cell culture medium all 2 days and 16 hrs before infection the cells were washed with DPBS (Lonza, BioWhittaker, Basel, Switzerland) and the medium was changed again. To determine, the cells growth it is necessary to measure the TER (transepithelial resistance) of the cells. Infections were carried out with different *C. jejuni*-wt strains and mutants with a MOI of 50 for indicated periods of time (30 min to 24 hrs). At each time point a particular amount of the basolateral medium of the transwell-filter-system was plated on Mueller-Hinton-agar (MH)-plates and incubated for 4 days under microaerophilic conditions.

4.3.3 Motility assay

Motility phenotypes of strains were tested in MH media containing 0.4 % agar. Bacterial cells were harvested from a 36 hour culture on conventional agar plates and resuspended in PBS to obtain an optical density at 600 nm of 0.45 (approximately 1×10^9 cfu/ml). The bacteria were incubated for 30 min in the presence or absence of 20 µg/ml α-FlaA antibody or pre-immune serum as control. To ensure that equal amounts of antibody were present on the entire agar surface in the α-FlaA sample, 50 µl of the antibody solutions were plated onto the corresponding plates. Subsequently, 2 µl of a bacterial suspension of 2×10^8 cfu/ml (+/- antibody) were stabbed into motility agar. Plates were incubated at 37°C under microaerophilic conditions for 24 hours, followed by measuring the diameter of the resulting swarms. The results were the mean of at least five separate measurements from three experiments.

4.3.4 Gentamycin protection assay

Gentamicin protection assay is a method which was used to analyse and quantify cellular invasion and number of intracellular bacteria (Kopecko *et al.*, 2001). During infection gentamicin was added, followed by cell lysis and bacterial cultivation on plates. The antibiotic gentamicin was used because it is not able to cross the eukatyotic cell membrane, thus the bacterial counts of gentamicin-treated cells present internalized bacteria, whereas extracellular bacteria were killed.

Corresponded eukaryotic cells were seeded to give 4 x 10^5 at 12 well tissue culture plates and infected as described above. After 6 hrs of infection cells were washed three times with pre-warmed cell culture medium to remove non-adherend bacteria. To determine the CFUs (colony forming units) corresponding to intracellular bacteria, the eukaryotic cell monolayers were treated with 250µg/ml gentamicin (Sigma-Aldrich, Steinheim, Germany) at 37°C for 2 hours, washed three times with medium and incubated with 0.1 % saponin (Sigma-Aldrich, Steinheim, Germany) in PBS at 37°C for 15 min. The treated monolayers were resuspended, diluted and plated on MH-agar plates. To determine the CFUs of host associated bacteria, the infected monolayers were incubated with 1 ml of 0.1 % saponin in PBS at 37°C for 15 min without prior treatment with gentamicin. The resulting suspensions were diluted and plated as described above. For each strain, the level of bacterial adhesion and invasion was determined by calculating the number of CFU. In control experiments, 250 µg/ml gentamicin killed all extracellular bacteria (data not shown) and all experiments were performed in triplicates.

4.3.5 Inhibitor studies

For performance of inhibiotion and activation studies, host cells in 1 ml cell culture medium were pre-treated for 30 min with corresponding inhibitors presented in Table 9 (chapter 3.10), followed by infection with *C. jejuni*.

4.3.6 Cultivation and storage of *Escherichia coli*

For cultivation of *E. coli* Luria Broth (LB) plates or LB medium with corresponding antibiotics were used. Bacteria were incubated overnight at 37°C in an incubator or shaker.

For preservation of strains 20 % glycerine were added to *E. coli* overnight cultures and stored at -80°C.

4.3.7 Preparation and transformation of competent *E. coli* cells

Transformation is called the adsorption of free DNA into competent bacteria cells. This method is used to transfer and amplify recombinant DNA fragments into bacteria. Most bacteria such as *E.coli* don´t exhibit natural competence, therefore they must be treated physically or chemically before transformation. By this way they are able to absorb DNA fragments.

4.3.7.1 Preparation of chemical competent *E. coli* cells

Competent cells for transformation with DNA fragments were made by the calciumchlorid method (Mandel and Higa, 1970). *E.coli* overnight cultures were diluted to a OD_{600} of 0.1 in LB-medium and were grown to a OD_{600} of 0.3 to 0.5. By centrifugation (10 min, 3.000 rpm) bacteria of 100 ml culture were harvested and resuspended in 50 ml ice-cold $CaCl_2$ (100 mM). After centrifugation (5 min, 1.500 rpm) the pellet was resuspended in 10 ml $CaCl_2$ (100 mM) and incubated for 30 min on ice. Then, the cells were centrifuged (5 min, 1.500 rpm) and the pellet was resuspended in 5 ml 100 mM $CaCl_2$ with 10 % glycerine. The competent cells were divided into 100 µl aliquots and stored at -80°C.

4.3.7.2 Chemically transformation of *E.coli* cells

100 µl competent *E. coli*-TOP-10 or BL21-cells were thawed on ice and incubated with 0.25 µg plasmid DNA or 2-5 µl ligation preparation for 20 min on ice. Subsequently cells were heatshocked for 1 min at 42°C and incubated for 10 min on ice again. 1ml of SOC-medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and cells were incubated for 1 hour at 37°C under shaking. Following adequate amounts were plated on corresponding LB selective plates.

4.4 Cell biological methods

4.4.1 Cultivation of eukaryotic cell lines

Different eukaryotic cell lines were used in this work (see Table 7 below). A adherend cell lines were cultivated at the indicated medium with 10% heat inactivated (30 min at 56°C) fetal calf serum [(FCS), Gibco/Invitrogen, Karlsruhe, Germany] and 1 % antibiotic-antimycotic-solution (Sigma-Aldrich, Taufkirchen, Germany) at 37°C and 5 % CO_2. Confluent growth cultures were washed two times with DPBS (Lonza, BioWhittaker, Basel, Switzerland) and trypsinized by trypsin-EDTA incubation (Sigma-Aldrich, Taufkirchen,

Germany) for 5 min at 37°C and 5% CO_2. Subsequently the cells were resuspended in indicated medium and dispensed into new culture flasks or wells (Greiner-Bio-One, Frickenhausen, Germany).

Table 7: Eukaryotic cell lines used in this work.

Cell line	Medium
INT-407	Eagle's Minimum Essential Medium (MEM) containing 2 mM L-glutamine and Earle's salts, 100 units (U)/ml penicillin, 100 µg/ml streptomycin, 10% FBS (Invitrogen)
MKN28	RPMI 1640 containing 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B,10%FBS(Invitrogen)
GD25	Dulbecco's Modified Eagle Medium (D-MEM) containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
GD25-ß$_1$A, GD25-ß$_1$A$_{TT788-9AA}$, GD25-ß$_1$A$_{Y783/795F}$	See GD25 cells, with 10 µg/ml puromycin
Fn (-/-)	D-MEM containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
Fn (+/+)	D-MEM containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
FAK (-/-)	D-MEM containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
FAK (+/+)	See FAK (-) cells, with 200 µg/ml hygromycin
SYF	D-MEM containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
SYF + c-src	D-MEM containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
Vav-1/2 $^{-/-}$	D-MEM containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)

4.4.2 Freezing and thawing of cells

For preservation of the cell-lines, cells were trypsinized at described in chapter 4.4.1 and centrifuged for 10 min, 1.000 rpm. The cell pellet was resuspended in freezing medium (90% FCS, 10% DMSO) and aliquoted into cryotubes. Cells were stored for 24 hours at -80°C and for long time preservation at liquid nitrogen.

For thawing, cells were quickly (40-60 sec) warmed to 37°C, mixed with indicated culture medium and centrifuged at 1.000 rpm for 10 min. The cell pellet was resuspended in culture

medium and transfered in cell culture flasks. DMSO [(Dimethylsulfoxide), Sigma, Taufkirchen, Germany] rests were removed after 24 hrs by changing the culture medium.

4.4.3 Transient transfection of plasmid DNA

Transfection is the insertion of DNA, such as plasmids into eukaryotic cells. Two different methods are exsisting, the temporary insertion of DNA into host cells (transient transfection) and the durable insertion of DNA into the genome (stabile transfection).
By transfection of expression plasmids, which contains an open reading frame for a particular protein, eukaryotic cells were decided to synthesize exogenous proteins.

The plasmids were transfected with the transfection reagent *GeneJammer* (Stratagene, Amsterdam, Netherlands) to manufactors instruction. By mixing the DNA with the polyamine transfection reagent DNA-containing complexes were formed. These complexes are adsorbed to the cell surface and mediate the adsorption of the DNA into the cells by endocytosis. An amount of 3 µl/1 µg DNA was added to cells and incubated for 36 hrs. The efficiency of transfection was verified by Western blotting with corresponding antibodies.

4.4.4 Transient transfection of siRNA

RNA interference (RNAi) is a method to silence genes on posttranscriptional level (Cullen, 2002). Double stranded RNA (dsRNA) is introduced into a cell resulting in sequence specific degradation of homologous messengerRNA (mRNA) (Hannon, 2002). The dsRNA is cut by a cell specific ribonuclease (*dicer*) at 21-25-mere ribonucleotids (Bernstein *et al*., 2001). These RNA fragments, called small interfering RNA (siRNA), bind to different proteins and generate the RNA-induced silencing complex (RISC) (Hammond *et al*., 2000). By an ATP-dependent cleavage of the dsRNA complex is formed, which is able to bind to the complementary mRNA and induce its degradation.
However, the insertion of dsRNA with more than 30 nucleotides into mammal cells is difficult and leads to unspecific effects in the cell or apoptosis. To avoid these effects, siRNAs were used for the specific reduction of gene expression in mammal cells (Elbashir *et al*., 2001; Martinez *et al*., 2002).
In this work commercial siRNAs (DOCK180, Vav-2, Tiam-1) from Santa Cruz Biotechnology (Heidelberg, Germany) were used and transfections were carried out at manufactors instructions. Rac1- and Cdc42-siRNA were synthesized and obtained from MWG-Biotech (Ebersberg, Germany) with the following target sequences Rac1: (5'-

AAAACTTGCCTACTGATCAGT-3') and Cdc42: (5'-TTCAGCAATGCAGACAATTAA-3'). For downregulation of Tiam-1 both siRNA from Santa Cruz Biotechnology and MWG-Biotech were used. The Tiam-1 target sequence is: (5'-ACAGCTTCAGAAGCCTGAC-3'). For transfection corresponding cells were seeded to reach 70-80% confluency. For the transfection two different solutions (A and B) were prepared. For solution A, 80µl transfection medium (serumfree cell culture medium) with 1 µg siRNA was used and for solution B, 80 µl transfection medium was mixed with 4.8 µl transfection reagent. Both solutions were incubated for 5 min, following solution A and B were mixed together, incubated for 20 min at room temperature and 640 µl transfection medium was added. The medium from the cells was removed, changed with the transfection reagent solution and incubated for 5-7 hours at 37°C and 5% CO_2. After incubation 1ml growth medium (cell culture medium + 20 % FCS + 2 % PS) was added and the cells were incubated for 36 hrs at 37°C and 5% CO_2. The efficiency of transfection was verified by Western blotting with appropriate antibodies.

4.5 Microscopic methods

4.5.1 Phase contrast microscopy

For phase contrast microscopy the cells were directly analyzed on cell culture plate with a phase contrast microscope (Olympus, Hamburg, Germany). For documentation a CCD camera MP focus 5000 (Intas, Göttingen, Germany) and the ImageProPlus software (MediaCybernetics, Wokingham Berkshire, UK) were used.

4.5.2 Field Emmision Scanning Electron Microscopy (FESEM)

For FESEM corresponding cells were seeded to coverslips or apical membran of transwell-filter-system and infected for an indicated period of time with *C. jejuni* or mutants, then fixed with specific fixation solution containig 5 % formaldehyde and 2 % glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose; pH 6.9) and subsequently washed several times with cacodylate buffer. Samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90 and 100 %) on ice for 15min for each step. Samples in the 100% acetone step were allowed to reach room temperature before another change of 100% acetone. Samples were then subjected to critical-point drying with liquid CO_2 (CPD030, Bal-Tec, Liechtenstein). Dried samples were covered with a 10 nm thick gold film by sputter coating (SCD040, Bal-Tec, Liechtenstein) before examination in a field emission scanning electron microscope (Zeiss DSM-982-Gemini) using the Everhart

Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

4.6 Statistical analysis

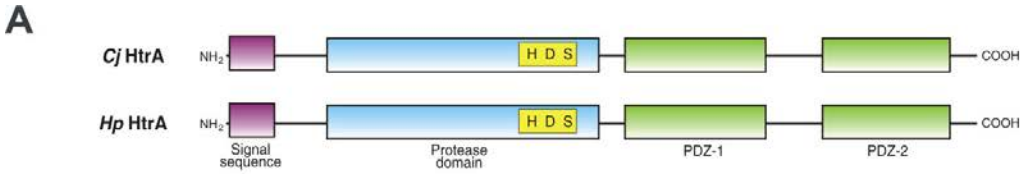
All data were evaluated using Student t-test with SigmaStat statistical software (version 2.0). Statistical significance was defined by $P \leq 0.05$ (*) and $P \leq 0.005$ (**). All error bars shown in figures and those quoted following the +/- signs represent standard deviation.

5. RESULTS

5.1 Part 1: Role of the protease HtrA during *C. jejuni* infection

5.1.1 HtrA protease is conserved in *H. pylori* and *C. jejuni*

The high temperature requirement A (HtrA) proteins constitute a group of heat shock induced serine proteases, which were identified to influence the adhesion and invasion properties of different bacterial pathogens. Many bacterial HtrAs have been described to be localized in the periplasm and are involved in various aspects of intracellular quality protein control (Clausen *et al*., 2002). The group of HtrA proteins typically consists of a signal peptide, a trypsin-like serine protease domain and one or two so-called PDZ domains (Fig. 3A). PDZ is an acronym which combines the first letters of three proteins, post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (ZO-1), that were first discovered to share this domain (Kennedy, 1995). The PDZ domains typically act as protein binding modules which interact with given target proteins (Kim *et al*., 2005). In addition, by binding of the PDZ domain in one HtrA molecule to that in other HtrA molecules, HtrA can build-up to highly proteolytically active oligomers (Krojer *et al*., 2008). The HtrA protease domain consists of an active site, called the catalytic triad, which is formed by the conserved amino acid residues histidine, asparagine and serine (Kim *et al*., 2005).
Recently, the groups of Prof. S. Wessler (University Salzburg) and Prof. S. Backert (University Magdeburg and University College Dublin) reported the surprising finding that HtrA of the gastric pathogen *H. pylori* is specifically secreted in the cell culture supernatant, where it can cleave the ectodomain of the host cell adhesion protein and tumor-suppressor E-cadherin (Hoy *et al*., 2010). Shedding of the E-cadherin ectodomain disrupted epithelial barrier functions in the infected epithelium allowing *H. pylori* to access the intercellular space and reach basolateral surfaces. Since we are interested in another gastrointestinal pathogen, *Campylobacter jejuni,* where it is completely unknown how they cross the epithelial barrier, we proposed in this project that the latter pathogen may also use its HtrA protease to breach the barrier of polarised epithelial cells. A sequence alignment of HtrAs from different *C. jejuni* and *H. pylori* strains was performed and revealed a high degree of similarity between the HtrA sequences of different species (Fig. 3B). I also found that the amino acids in the catalytic triad (histidine, asparagine and serine) are conserved and at the right position among these proteins (Fig. 3B, red, shaded with yellow). This suggests that HtrA's are very conserved in various *C. jejuni* isolates and represent a possible candidate factor to investigate how this pathogen could cross the epithelial barrier.



B

```
Cj81-176        -MKK-IFLSLSLASALFAASINFNESTATANRVN-PAAGNAVLSYHDSIKDAKKSVVNIST 58
CjRM1221        -MKK-IFLSLSLASALFAASINFNESTATANRVN-PAAGNAVLSYHDSIKDAKKSVVNIST 58
CjNCTC11168     -MKK-IFLSLSLASALFAASINFNESTATANRVN-PAAGNAVLSYHDSIKDAKKSVVNIST 58
Hp26695         MMKKTLFISLALALSLNAGNIQIQSMPKVKERVSVPSKDDTIYSYHDSIKDSIKAVVNIST 61
HpP12           MMKKTLFISLALALSLNAGNIQIQSMPKVKERVSVPSKDDTIYSYHDSIKDSIKAVVNIST 61
Hp35A           MMKKTLFISLALALSLNAGNIQIQSMPKVKERVSVPSKDDTIYSYHDSIKDSIKAVVNIST 61
                 *** :*:**:** :* *..*:::. . . :**. *: .::: ********: *:******

Cj81-176        SKTITRANRPSPLDDFFNDPYFKQFFDFDFPQRKGKNDKEVVSSLGSGVIISKDGYIVTNN 119
CjRM1221        SKTITRANRPSPLDDFFNDPYFKQFFDFDFPQRKGKNDKEVVSSLGSGVIISKDGYIVTNN 119
CjNCTC11168     SKTITRANRPSPLDDFFNDPYFKQFFDFDFSQRKGKNDKEVVSSLGSGVIISKDGYIVTNN 119
Hp26695         EKKIKNNFIGG---GVFNDPFFQQFFG-DLGGMIPK--ERMERALGSGVIISKDGYIVTNN 116
HpP12           EKKIKNNFIGG---GVFNDPFFQQFFG-DLGGMIPK--ERMERALGSGVIISKDGYIVTNN 116
Hp35A           EKKIKNNFIGG---GVFNDPFFQQFFG-DLGGMIPK--ERMERALGSGVIISKDGYIVTNN 116
                .*.*..    .   ..****:*:***. *:     *  :.:  :*****************

Cj81-176        HVVDDADTITVNLPGSDIEYKAKLIGKDPKTDLAVIKIEANNLSAITFTNSDDLMEGDVVF 180
CjRM1221        HVVDDADTITVNLPGSDIEYKAKLIGKDPKTDLAVIKIEANNLSAITFTNSDDLMEGDVVF 180
CjNCTC11168     HVVDDADTITVNLPGSDIEYKAKLIGKDPKTDLAVIKIEANNLSAITFTNSDDLMEGDVVF 180
Hp26695         HVIDGADKIKVTIPGSNKEYSATLVGTDSESDLAVIRITKDNLPTIKFSDSNDISVGDLVF 177
HpP12           HVIDGADKIKVTIPGSNKEYSATLVGTDSESDLAVIRITKDNLPTIKFSDSNDISVGDLVF 177
Hp35A           HVIDGADKIKVTIPGSNKEYSATLVGTDSESDLAVIRITKDNLPTIKFSDSNDILVGDLVF 177
                **:*.**.*.*.:***: **.*.*:*.*.::*****:*  :**.:*.*::*:*:  **:**

Cj81-176        ALGNPFGVGFSVTSGIISALNKDNIGLNQYENFIQTDASINPGNSGGALVDSRGYLVGINS 241
CjRM1221        ALGNPFGVGFSVTSGIISALNKDNIGLNQYENFIQTDASINPGNSGGALVDSRGYLVGINS 241
CjNCTC11168     ALGNPFGVGFSVTSGIISALNKDNIGLNQYENFIQTDASINPGNSGGALVDSRGYLVGINS 241
Hp26695         AIGNPFGVGESVTQGIVSALNKSGIGINSYENFIQTDASINPGNSGGALIDSRGGLVGINT 238
HpP12           AIGNPFGVGESVTQGIVSALNKSGIGINSYENFIQTDASINPGNSGGALIDSRGGLVGINT 238
Hp35A           AIGNPFGVGESVTQGIVSALNKSGIGINSYENFIQTDASINPGNSGGALIDSRGGLVGINT 238
                *:******* ***.**:*****..**:*.********************:**** *****:

Cj81-176        AILSRGGGNNGIGFAIPSNMVKDIAKKLIEKGKIDRGFLGVTILALQGDTKKAYKNQEGAL 302
CjRM1221        AILSRGGGNNGIGFAIPSNMVKDIAKKLIEKGKIDRGFLGVTILALQGDTKKAYKNQEGAL 302
CjNCTC11168     AILSRGGGNNGIGFAIPSNMVKDIAKKLIEKGKIDRGFLGVTILALQGDTKKAYKNQEGAL 302
Hp26695         AIISKTGGNHGIGFAIPSNMVKDTVTQLIKTGKIERGYLGVGLQDLSGDLQNSYDNKEGAV 299
HpP12           AIISKTGGNHGIGFAIPSNMVKDIVTQLIKTGKIERGYLGVGLQDLSGDLQNSYDNKEGAV 299
Hp35A           AIISKTGGNHGIGFAIPSNMVKDIVTQLIKTGKIERGYLGVGLQDLSGDLQNSYDNKEGAV 299
                **:*: ***:************** ..:**:.***:**:*** :  *.** :::*.*:***:

Cj81-176        ITDVQKGSSADEAGLKRGDLVTKVNNKVIKSPIDLKNYIGTLEIGQKISLSYERDGENKQA 362
CjRM1221        ITDVQKGSSADEAGLKRGDLVTKVNDKVIKSPIDLKNYIGTLEIGQKISLSYERDGENKQA 362
CjNCTC11168     ITDVQKGSSADEAGLKRGDLVTKVNDKVIKSPIDLKNYIGTLEIGQKISLSYERDGENKQA 362
Hp26695         VISVEKDSPAKKAGILVWDLITEVNGKKVKNTNELRNLIGSMLPNQRVTLKVIRDKKERAF 359
HpP12           VISVEKDSPAKKAGILVWDLITEVNGKKVKNTNELRNLIGSMLPNQRVTLKVIRDKKERAF 359
Hp35A           VISVEKDSPAKKVGILVWDLITEVNGKKVKNTNELRNLIGSMLPNQRVTLKVIRDKKERTF 359
                : .*:*.*.*.:.*:   **:*:**.* :*.. :*:* **::  .*:::*.  ** :::

Cj81-176        SFILKGEKE-NPKGVQSD------LIDGLSLRNLDPRLKDRLQIPKDVNGVLVDSVKEKSK 417
CjRM1221        SFILKGEKE-NPKGVQSD------LIDGLSLRNLDPRLKDRLQIPKDVNGVLVDSVKEKSK 417
CjNCTC11168     SFILKGEKE-NPKGVQSD------LIDGLSLRNLDPRLKDRLQIPKDVNGVLVDSVKEKSK 417
Hp26695         TLTLAERKNPNKKETISAQNGAQGQLNGLQVEDLTQETKRSMRLSDDVQGVLVSQVNENSP 421
HpP12           TLTLAERKNPNKKETISAQNGAQGQLNGLQVEDLTQKTKRSMRLSDDVQGVLVSQVNENSP 421
Hp35A           TLTLAERKNPNKKETISAQNGSQGQLNGLQVEDLTQKTKRSMRLSDDVQGVLVSQVNENSP 421
                :: *  .*: * * . *        ::**.:.:*  . *  :::..**:****. .*:*:*

Cj81-176        GKNSGFQEGDIIIGVGQSEIKNLKDLEQALKQVN-KKEFTKVWVYRNGFATLLVLK 472
CjRM1221        GKNSGFQEGDIIIGVGQSEIKNLKDLEQALKQVN-KKEFTKVWVYRNGFATLLVLK 472
CjNCTC11168     GKNSGFQEGDIIIGVGQSEIKNLKDLEQALKQVN-KKEFTKVWVYRNGFATLLVLK 472
Hp26695         AEQAGFRQGNIITKIEEVEVKSVADFNHALEKYKGKPKRFLVLDLNQGYRIILVK- 476
HpP12           AEQAGFRQGNIITKIEEVEVKSVADFNHALEKYKGKPKRFLVLDLNQGYRIILVK- 476
Hp35A           AEQAGFRQGNIITKIEEVEVKSIADFNHALEKYKGKPKRFLVLDLNQGYRIVLVK- 476
                .:::**::*:**  : : *:*.: *:::**:: : * :   *   .:*:  :**
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Figure 3: **Comparision of *C. jejuni* and *H. pylori* HtrA proteins.** (A) Schematic diagram of the domain arrangement of HtrAs from *C. jejuni* (*Cj*) and *H. pylori* (*Hp*). (B) Multiple sequence alignment of different *C. jejuni* and *H. pylori* HtrAs. The protein sequences of *Cj*81-176, *Cj*RM1221, *Cj* NCTC11168, *Hp*26695, *Hp*P12 and *Hp*35A are aligned. The conserved amino acids of the catalytic triad are indicated in red (H: Histidine; D: Asparagine; S: Serine).

5.1.2 Analysis of *C. jejuni* wild-type strain and isogenic *ΔhtrA* deletion mutants by scanning electron microscopy

To first aim was to generate and investigate *htrA* mutants in *C. jejuni*. We obtained from Prof. Brønsted (University Copenhagen) *C. jejuni* wild-type strain NCTC11168, the isogenic NCTC11168*ΔhtrA* deletion mutant and NCTC11168*ΔhtrA/htrA* S197A, a strain with complemented *htrA* carrying a point mutation S197A in the active centre, rendering the protein to be catalytically inactive (Baek *et al*., 2011). The nomenclature S194 was chosen by the authors due to the full sequence of the protein minus the cleaved-off signal peptide (Baek *et al*., 2011). In addition, we produced another *ΔhtrA* deletion mutant in *C. jejuni* wild-type strain 81-176. Next, I aimed to investigate the morphology of the produced *C. jejuni* strains. For this purpose single bacterial colonies were grown for two days on *Campylobacter* blood free selective agar base (Oxoid) containing *Campylobacter* growth selective supplement (Oxoid) and corresponding antibiotics in agar plates and the different strains were visualized by scanning electron microscopy (SEM). SEM revealed that the *C. jejuni* wild-type strains 81-176 and NCTC11168 revealed no obvious phenoptypical differences as compared to their *htrA* mutants. All mutants were viable and produce intact bipolar flagella as compared to their wild-type counterparts (Fig. 4, blue arrows), and are therefore available for subsequent functional studies.

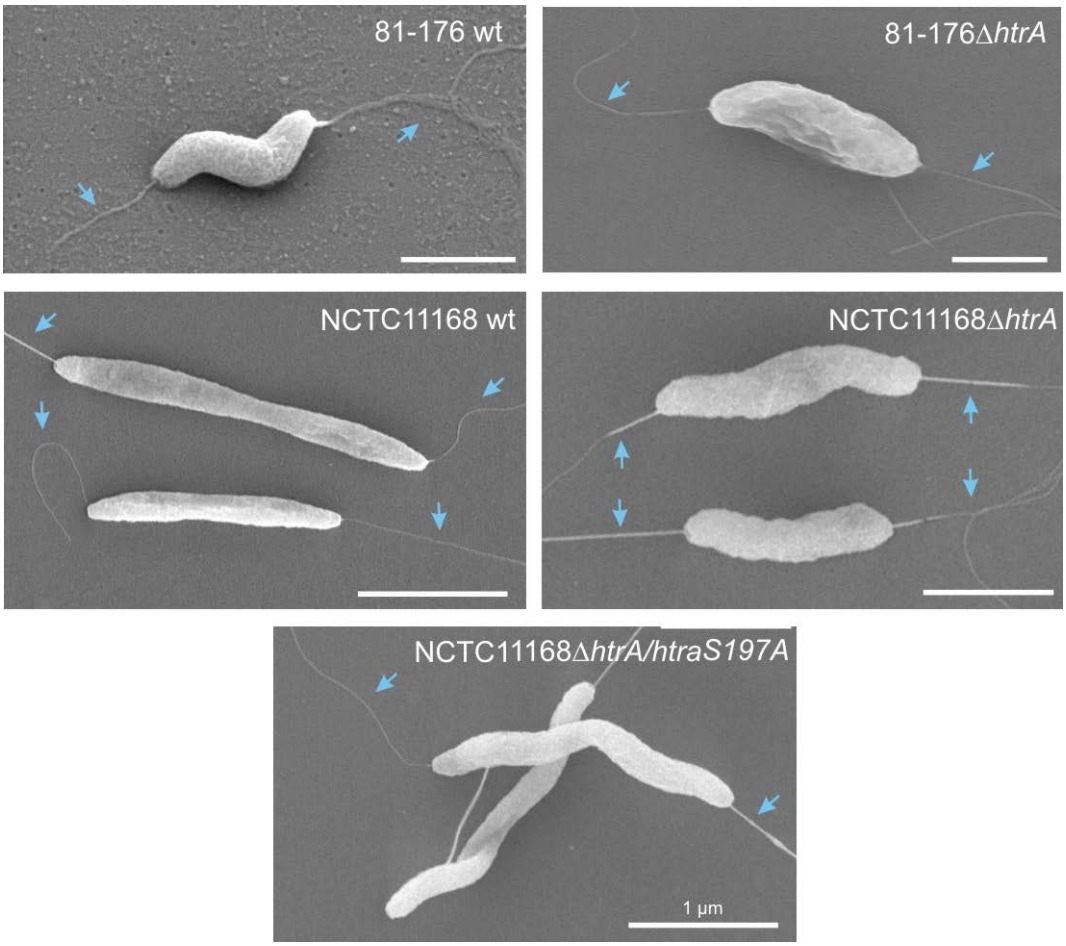


Figure 4: Analysis of *C. jejuni* and *C. jejuni ΔhtrA* mutants by scanning electron microscopy. Scanning electron microscopy revealed that *C. jejuni* and different *C. jejuni ΔhtrA* mutants produce intact bipolar flagella (blue arrows). Each barr corresponds to 1µm.

5.1.3 *C. jejuni* secretes HtrA into the culture supernatant

The remarkable sequence homology between HtrA's from *H. pylori* and *C. jejuni* (Fig. 3) led us to propose that *C. jejuni* HtrA maybe secreted into the cell culture supernatant, similar to its *H. pylori* counterpart. To test this hypothesis, *C. jejuni* wild-type strain 81-176 and 81-176*ΔhtrA* deletion mutant were grown in BHI broth medium for 12 hours to an OD_{600nm}=1. The supernatant and the cell pellets were separated by centrifugation, and the supernatant was further purified from remaining bacterial cells by passage through a 0.21 µm sterile filter. The resulting bacterial pellets and supernatants were analysed by immunoblot analysis. The results confirmed that the HtrA protein was not synthesized by the *ΔhtrA* mutant, whereas HtrA is produced by the wild-type isolate and is secreted into the supernatant (Fig. 5). As control, the different fractions were reprobed with an antibody against *C. jejuni* MOMP. The results verify that equal amounts of protein were loaded in each pellet sample and exhibit the absence of

MOMP in the supernatants. Thus, the presence of HtrA in the bacterial supernatant is specific and not due to artificial lysis of the bacteria. Similar results were obtained with the NCTC11168 strains (data not shown).

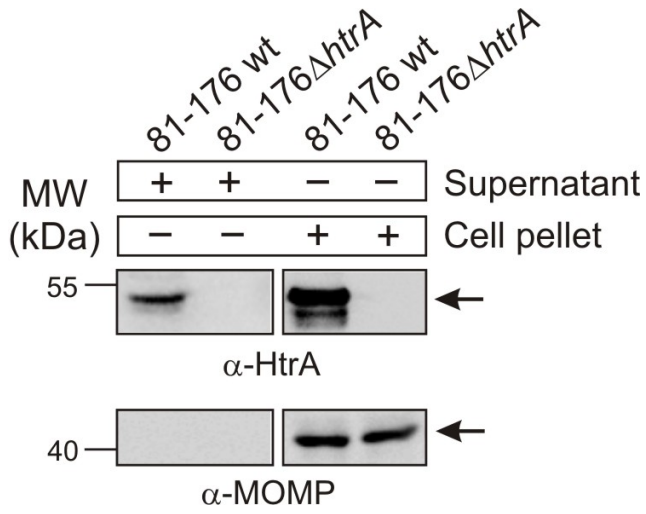


Figure 5: HtrA secretion into the supernatant of cultured *C. jejuni.* Wild-type (wt) strain 81-176 and 81-176*ΔhtrA* mutant were analysed. The bacteria were grown for 12 hours in BHI liquid broth. The secretion of HtrA proteins during culturing was verified by immunoblotting using the α-HtrA antibody. As control, the blots were stripped and reprobed with an α-MOMP antibody.

5.1.4 Analysis of HtrAs in different *C. jejuni* wild-type strains and *htrA* mutants

The next aim was to investigate if secreted HtrA from the two *C. jejuni* strains are proteolytically active. For this purpose, wild-type strains, *ΔhtrA* deletion and point mutants as control (81-176 wt, 81-176*ΔhtrA*, NCTC11168 wt, NCTC11168*ΔhtrA* and NCTC11168*ΔhtrA*S197A) were grown as described and purified bacterial culture supernatants were prepared. The caseinolytic activity of HtrA protease was then detected by casein zymography (Hoy *et al*., 2010). For this purpose bacterial lysates were loaded onto a 0.1% casein containing gel and separated under non-denaturating conditions. The results show that *C. jejuni* HtrA's from both wild-type strains gave rise to active multimers with a molecular weight of more than 200 kDa, while bands for the active monomers (ca. 53 kDa) were hardly detectable (Fig. 6). Corresponding signals for active HtrA's were completely absent in the two *ΔhtrA* deletion mutants. Interestingly, the NCTC11168*ΔhtrA*S197A mutant also formed a multimer at about 200 kDa (red asterisk), but this multimer was not active as expected (Fig. 6). The identity of multimeric HtrA proteins on the gels was further verified by mass spectrometry of the excised bands (data not shown). In addition, the casein zymography

gels exhibited two other *C. jejuni* proteins (at 63 kDa and 42 kDa, respectively) with proteoltyic activity on casein, but their identity is yet unknown (Fig. 6, yellow asterisks).

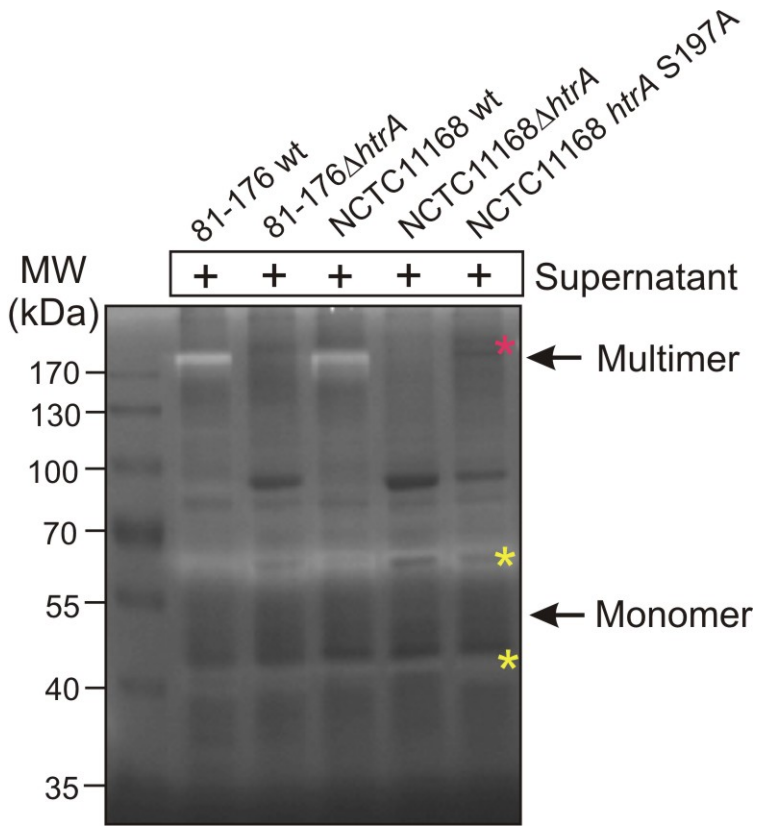


Figure 6: Detection of proteolytically active HtrA proteins in different *C. jejuni* strains. Supernatants of *C. jejuni* 81-176 wild-type (wt), 81-176*ΔhtrA*, NCTC11168 wt, NCTC11168*ΔhtrA* and NCTC11168*ΔhtrA*S197A were tested for protease activities by casein zymography. Active (wt) and inactive (S197A) HtrAs were separated on non-denaturing gels and multimeric HtrA complexes were detected as described (Hoy *et al*., 2010). Active monomeric HtrA was hardly visible as indicated. Zymography of HtrA was performed with the kind help of Prof. Wessler and B. Hoy (University Salzburg).

5.1.5 Multiple *C. jejuni* strains express active HtrA

As next, we cloned and overexpressed *C. jejuni* HtrA of strain 81-176 in *E. coli* BL-21 with the help of our collaborator, Prof. S. Wessler (University Salzburg). HtrA was expressed and purified as GST fusion protein (Fig. 7). The GST tag was cleaved-off and HtrA was purified to almost homogeneity (Fig. 7, lane 8). As expected, HtrA from *C. jejuni* had slightly smaller molecular weight as compared to its *H. pylori* counterpart, due to the smaller size of the expressed protein (472 vs. 476 amino acids) (see Fig. 3B, lanes 8 and 9).

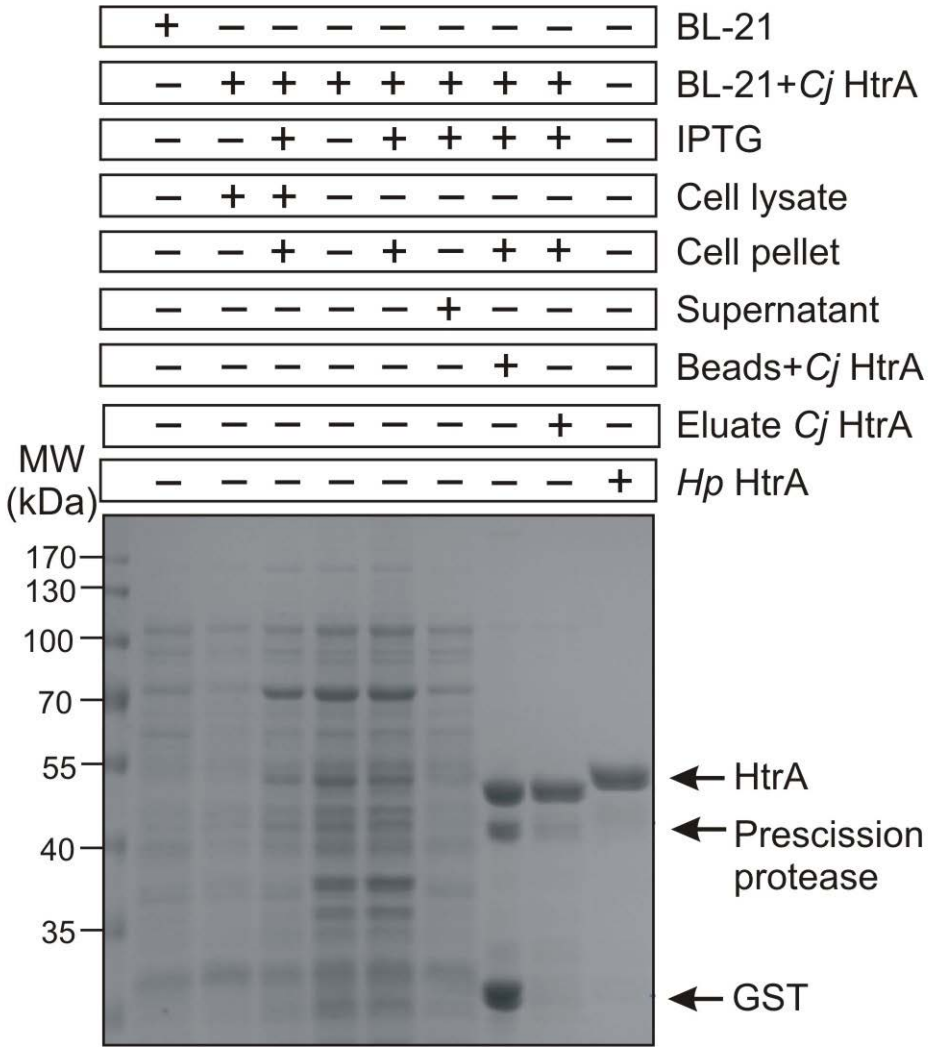


Figure 7: Overexpression and purification of *C. jejuni* HtrA. A Coomassie-stained gel of different fractions and purified HtrA proteins during the purification procedure is shown. Purification of HtrA was performed with the kind help of Prof. Wessler and B. Hoy. For details see Materials and Methods and text.

Recombinant HtrA was then taken as a control to test if a larger collection of wild-type *C. jejuni* strains also express active HtrA proteins. For this purpose *C. jejuni* wild-type strains, including RM1221, ATCC43430, TGH-9011, NCTC11168, 1849, 81-176, 1543/01, 2703/01 and ST3046 were grown as described above. The corresponding cell pellets were harvested and analyzed for HtrA protease activities by casein zymography. All tested strains expressed the active HtrA multimer with a molecular size of >200 kDa (Fig. 8). Interestingly, I could confirm that recombinant *C. jejuni* HtrA also formed highly active multimers at the same size of >200 kDa (Fig. 8A, lane 1). In addition, recombinant monomeric HtrA showed a faint band for monomeric protease activity at 53 kDa, while this band is only hardly visible for any wild-type *C. jejuni* strain. However, these findings confirm our knowledge from HtrA in other bacteria such as *E. coli* where the HtrA multimers are highly proteolytically active rather than the monomer (Krojer *et al*., 2008).

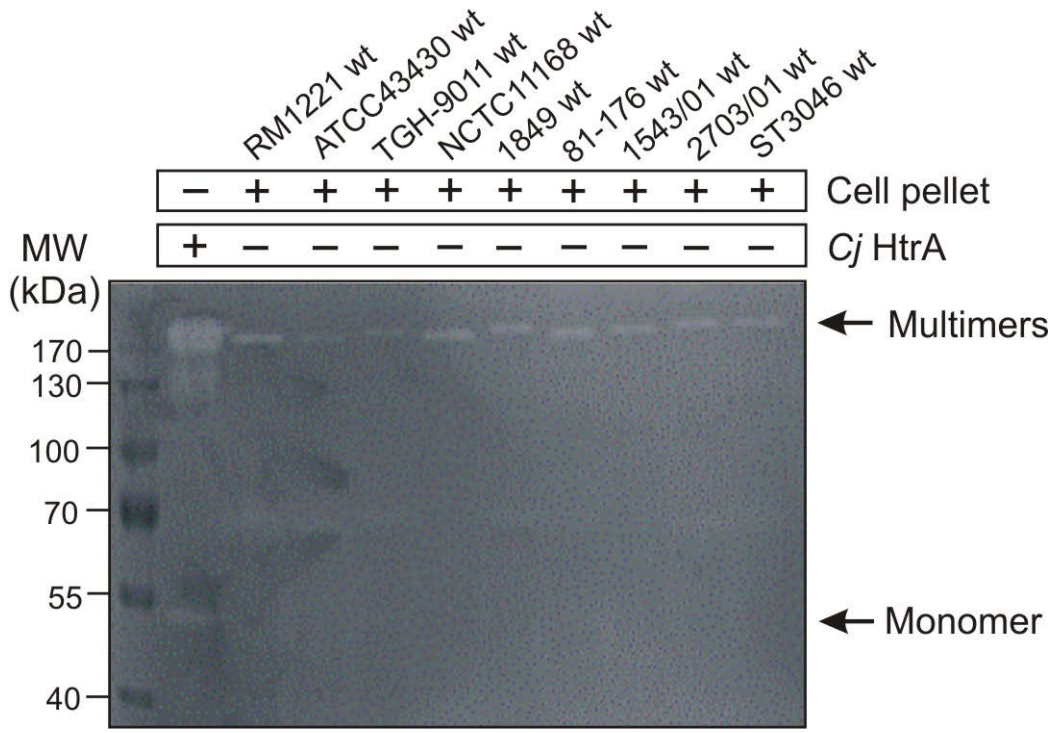


Figure 8: A large variety of *C. jejuni* strains express active HtrA proteins. The bacteria were grown as described and HtrAs were separated on non-denaturing gels. Proteolytically active HtrA complexes were detected by casein zymography as described (Hoy *et al*., 2010). The position of active monomeric and multimeric HtrA proteins is indicated with arrows.

5.1.6 *In vitro* cleavage with purified *C. jejuni* and *H. pylori* HtrAs

As described above, our groups have recently shown that *H. pylori* HtrA is secreted into cell culture supernatant and can cleave the cell adhesion protein E-cadherin during infection *in vivo* and as recombinant protein *in vitro* (Hoy *et al*., 2010). Full length E-cadherin has a molecular weight of about 130 kDa and it can be cleaved by HtrA giving rise to a 90 kDa extracellular domain amino-terminal fragment (NTF) and a 40 kDa carboxy-terminal fragment (CTF1) (Fig. 9A). To determine whether *C. jejuni* HtrA can also cleave E-cadherin, recombinant HtrA was incubated with recombinant E-cadherin for 16 hrs at 37°C. The ectodomain shedding of E-cadherin was detected by loss of full length E-cadherin using α-E-cadherin antibodies H-108 or HECD1 recognising the EC5 subunit in the NTF domain. It was shown that *C. jejuni* HtrA efficiently cleaved E-cadherin as monitored by the disappearance of full length E-cadherin protein band and increased amount of the 90 kDa NTF domain. Recombinant *H. pylori* HtrA also cleaved E-cadherin as monitored under identical conditions

as expected (Fig. 9B). As control, it could be shown that *C. jejuni* HtrA does not cleave purified fibronectin *in vitro* (Fig. 9C).

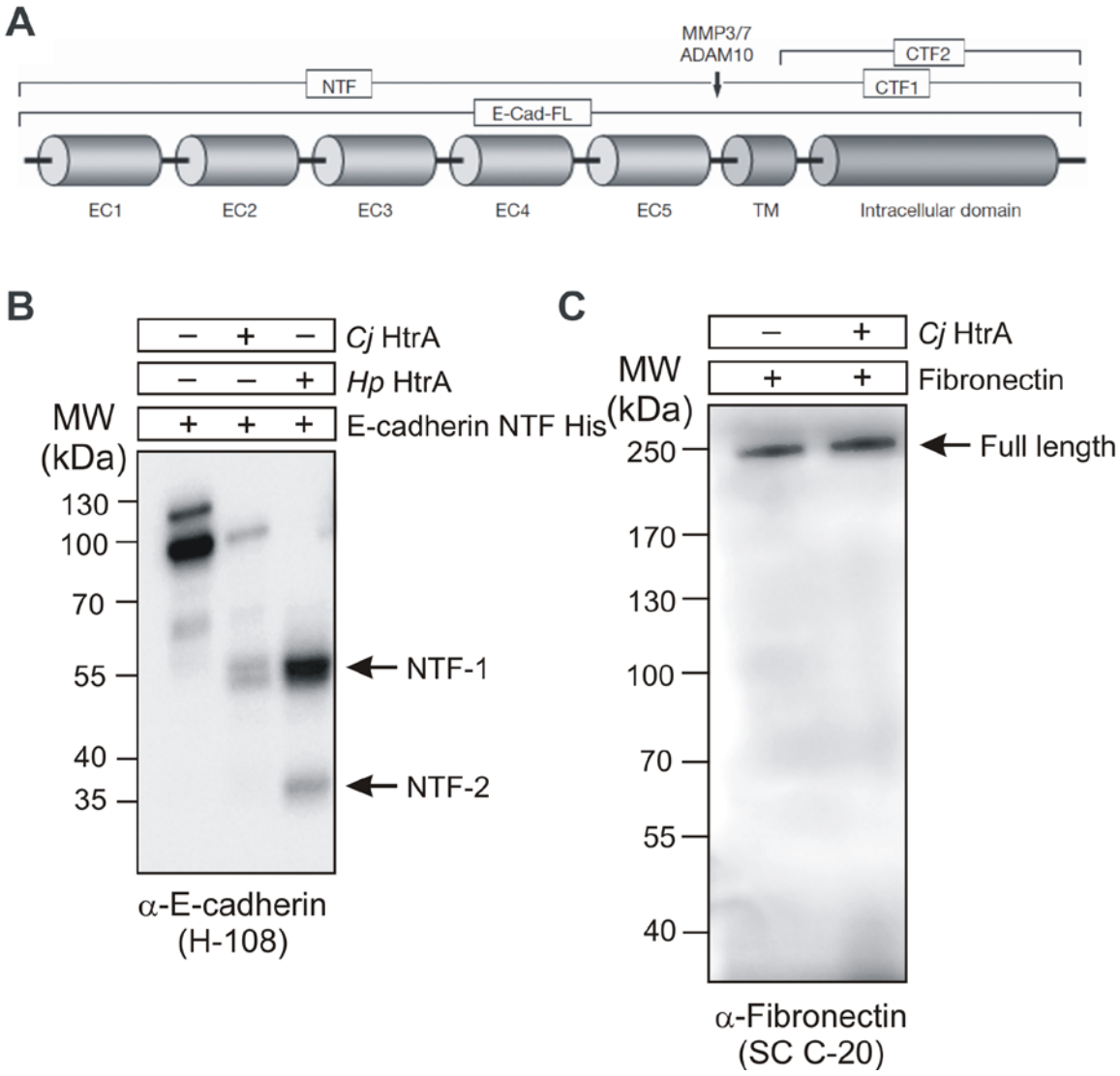


Figure 9: Recombinant *C. jejuni* HtrA cleaves E-cadherin but not fibronectin. (A) Schematic presentation of the E-cadherin domain structure. E-cadherin consists five extracellular domains (EC1-EC5), a transmembrane (TM) domain and an intracellular domain (CTF) at the C-terminus of the protein. Cleavage by HtrA proteases generates an extracellular N-terminal fragment (NTF). (B) *In vitro* cleavage of E-cadherin incubated with purified *C. jejuni* HtrA or *H. pylori* HtrA. (C) *In vitro* cleavage of fibronectin incubated with purified *C. jejuni* HtrA. All reactions were incubated for 16 hrs at 37°C.

5.1.7 *In vivo* cleavage of E-cadherin in *C. jejuni* infected INT-407 cells

The next aim was to investigate if *C. jejuni* HtrA can cleave E-cadherin during infection *in vivo*. For this purpose, E-cadherin-expressing INT-407 cells were infected with *C. jejuni* strain NCTC11168 wt in a time course for an indicated periods of time (up to 6 hours) and the cleavage of E-cadherin was determined by immunoblotting using an α-E-cadherin antibody (HECD-1; Calbiochem). The results show that the overall amount of full length E-cadherin decreased over time but was not eliminated (Fig. 10). In addition the signals of the entire 90 kDa NTF increased over time up to 4 hrs and then dropped somewhat as detected in the supernatant of infected cells. In contrast, no E-cadherin cleavage was observed during infection with the isogenic Δ*htrA* mutant (data not shown).

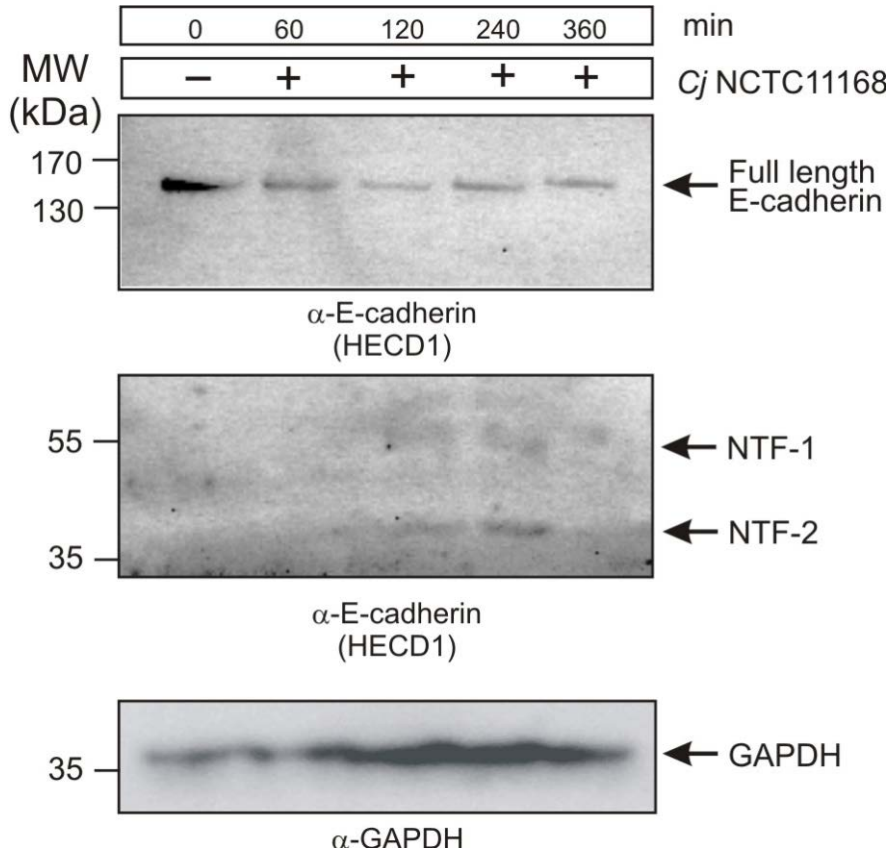
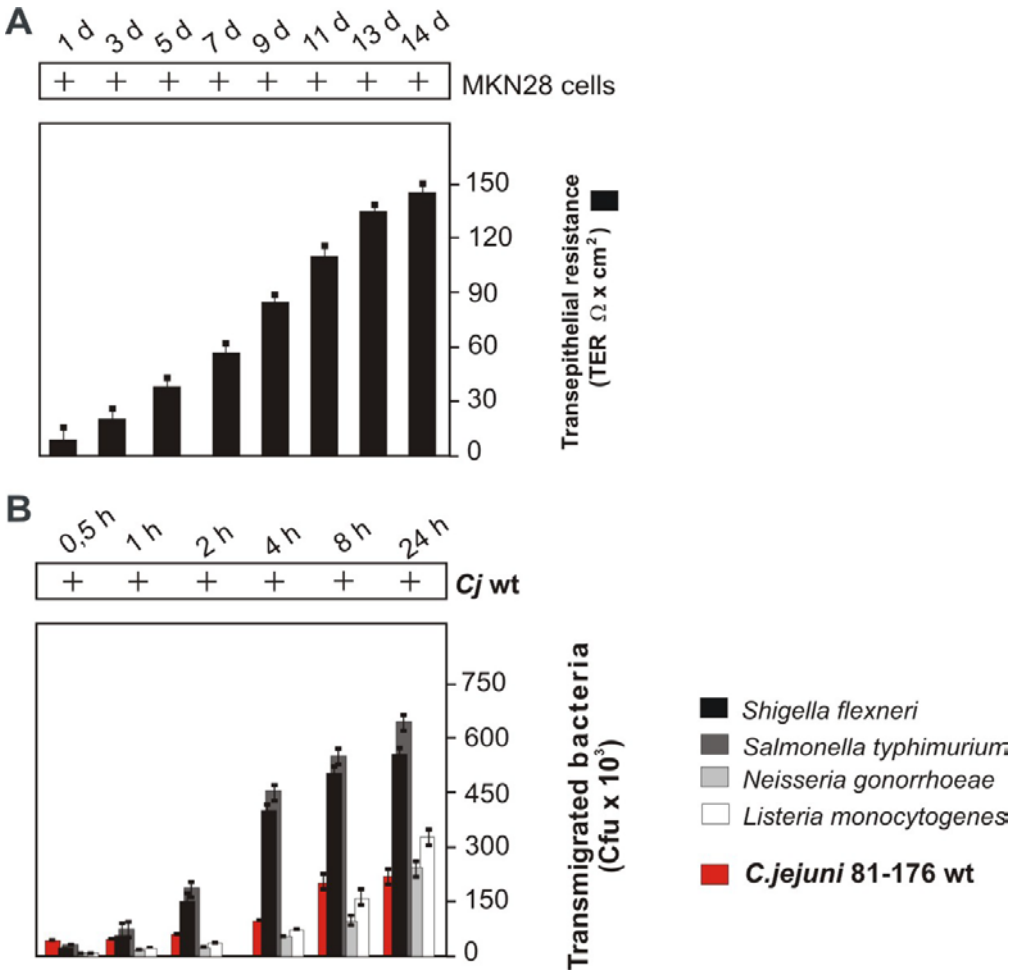


Figure 10: *In vivo* cleavage of E-cadherin in infected INT-407 cells. INT-407 cells were infected with *C.jejuni* NCTC11168 wt strain for 0 to 360 min. Full length E-cadherin and the NTF- fragment were detected using the indiacted antibody. GAPDH expression levels were determined as loading control of total cellular protein.

5.1.8 *C. jejuni* wt strains transmigrate efficiently through polarised MKN-28 cell layers

Next it was investigated, if *C. jejuni* can transmigrate through polarised epithelial cells and compared this capability with well-known invasive bacterial pathogens under identical settings. For this purpose, MKN-28 cells were seeded and differentiated on a transwell-filter system. The growth of the cells was followed by measuring the transepithelial resistance (TER) every 3 days of culture (Fig. 11A). After differentiation for 14 days and a TER between 140-150 Ω/cm^2 as reported previously for effective polarisation of this cell line (Wroblewski *et al*., 2009), proper cell monolayers and junction formation were confirmed by E-cadherin staining in immunofluorescence microscopy (not shown). MKN-28 cells were then infected with different *C. jejuni* strains and other pathogens as controls including *Salmonella typhimurium*, *Shigella flexneri*, *Neisseria gonorrhoea* and *Listeria monocytogenes* for indicated periods of time (0.5-24 hours). The transmigrated bacteria in the lower chamber were collected and the CFUs determined. The CFUs showed that *C. jejuni* wild-type transmigrated quickly, even much faster than the other pathogens during the first 30 min (Fig. 11B). As control, non-pathogenic *Escherichia coli* strain TOP-10 didn't transmigrate under the same conditions as expected (data not shown).



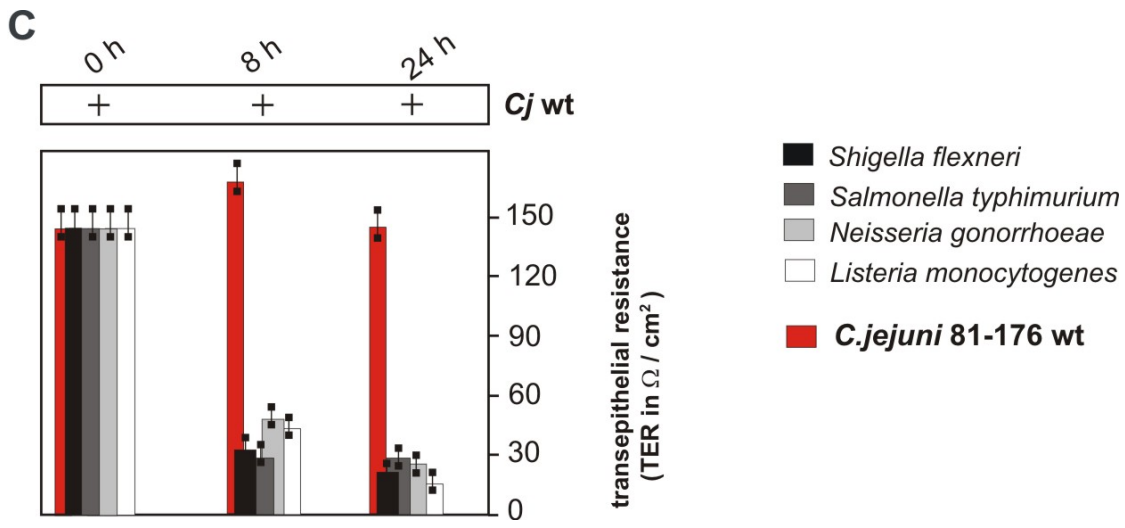


Figure 11: **Infection of polarised MKN-28 cells with different bacterial pathogens in a transwell filter system in a time course (0.5-24 hours).** (A) Differentiation and TER establishing of MKN-28 cells over 14 days culturing (without infection). (B) CFUs of transmigrated bacterial pathogens and *C. jejuni* wt strain 81-176 in the indicated time course. (C) TER measurement of infected MKN-28 cells during the indicated time course.

Interestingly, the measurement of TER during infection revealed that while all pathogens including *S. typhimurium, S. flexneri, N. gonorrhoea* and *L. monocytogenes* reduced TER substantially, infection with *C. jejuni* did not influence TER (Fig. 11C). This indicates that *C. jejuni,* in contrast to the other pathogens, does induce a permanent opening of the cell-to-cell junctions in order to induce its transmigration.

5.1.9 *C. jejuni* Δ*htrA* deletion mutant have a strong defect in transmigration

To investigate if the expression of HtrA is important for triggering transmigration of the bacteria across a polarised epithelium, MKN-28 cells were grown and differentiated as described above, followed by infection with *C. jejuni* wild-type strains 81-176 or NCTC11168 and their isogenic *ΔhtrA* deletion mutants. The numbers of transmigrated bacteria were also quantified as CFU. It could be shown that both *ΔhtrA* mutants exhibited a strong defect in transmigration as compared to wild-type *C. jejuni* (Fig. 12). In addition, *C. jejuni* expressing the HtrA S197A point mutant and a flagellar mutant (Δ*flaA/B*) were also widely deficient in transmigration, while a Δ*cadF* mutant showed similar transmigration rates at the 4 hour time point (Fig. 12). These observations suggest that secreted HtrA of *C. jejuni* and its protease activity, but also the flagellar-driven motility play crucial roles in breaching the epithelial barrier by this pathogen.

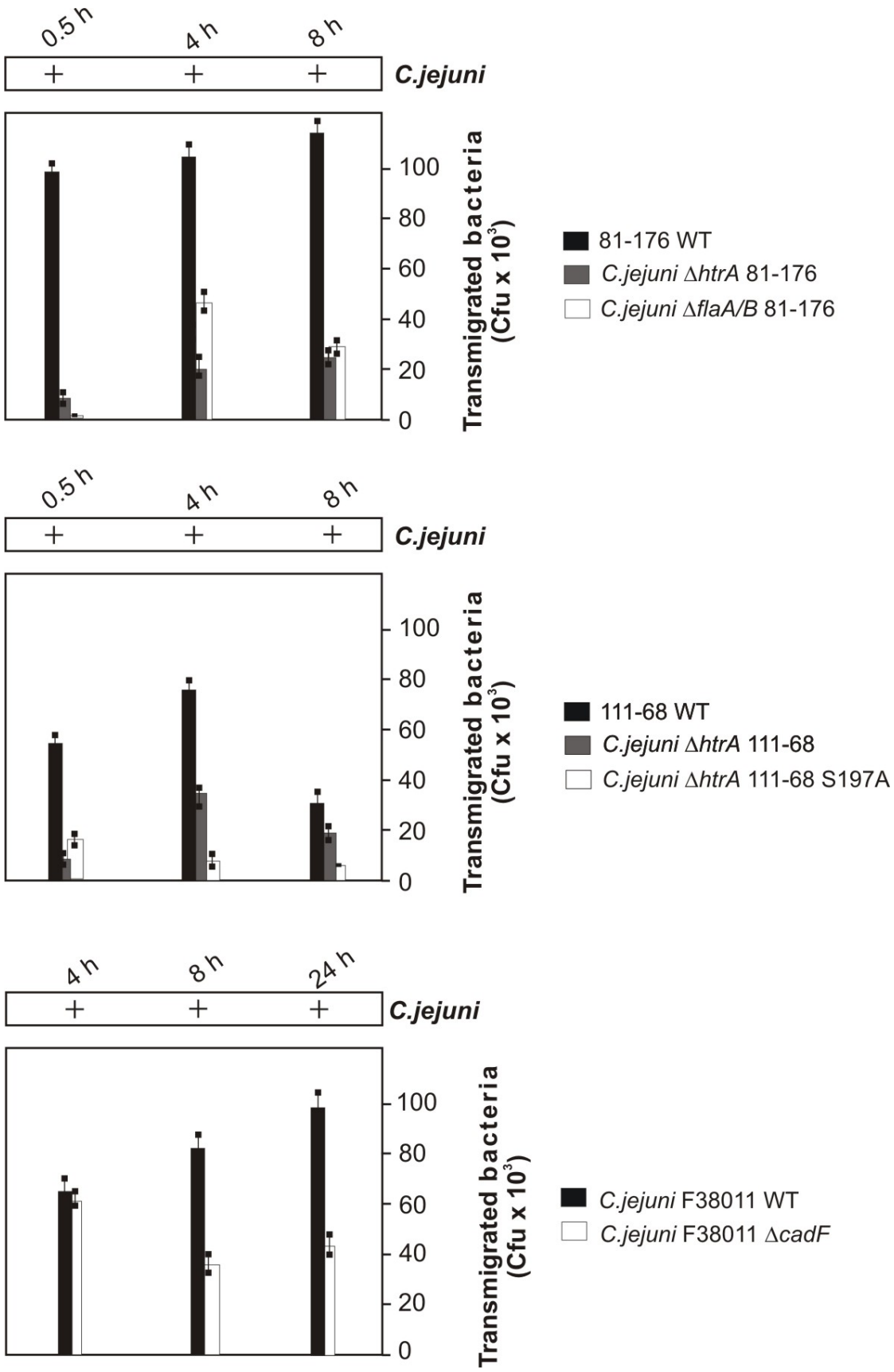


Figure 12: **Transmigration of wild-type (WT) and isogenic *C. jejuni* mutants across polarised MKN-28 cells.** A transwell system with differentiated MKN-28 cells (14 days) was infected with the indicated strains during a time course**.** Transmigrated bacteria were harvested from the bottom chambers, grown on MH plates, and CFUs were determined in triplicates.

5.2 Part 2: Signalling to small RhoGTPases and *C. jejuni* host cell entry

5.2.1 *Campylobacter jejuni* invasion of epithelial cells is time-dependent and associated with accumulating levels of Rac1-GTP and Cdc42-GTP

Having established that *C. jejuni* can breach the epithelial barrier rapidly via the paracellular route between cells, with secreted HtrA cleaving E-cadherin playing a role, the next aim was to investigate how the foodborne pathogen can enter the host cells. Host cell invasion by *C. jejuni* has been reported as one of the primary reasons of gut tissue damage in infected humans, but molecular invasion mechanisms and cellular factors involved in this process are widely unknown. Our recent data indicated that small Rho GTPases, including Rac1 and Cdc42 but not RhoA, are activated during infection and play a role for the invasion process (Krause-Gruszczynska *et al*., 2007b). Pharmacological inhibitor studies indicated that Rac1 and Cdc42 are activated via two different pathways, a PI3-kinase dependent pathway leading to activation of Cdc42 and a PI3-kinase independent pathway leading to activation of Rac1 (Krause-Gruszczynska *et al*., 2007b), but the involved signalling cascades remained to be established in more detail.

Aim of the following set of experiments was therefore to continue previous work done in the group by Dr. Krause-Gruszczynska and to pinpoint novel signalling components involved in *C. jejuni*-mediated GTPase activation and host cell entry. First, I aimed to confirm the time frame required for *C. jejuni* invasion in INT-407 intestinal epithelial cells. For this purpose, the cells were infected for different time periods (ranging from 0 min to 480 min) and the number of intracellular bacteria was then determined by gentamicin protection assay. The results show that invasion of *C. jejuni* into non-phagocytic cells occurred as early as 30 min after infection and that the number of intracellular cells increased rapidly between 4 to 8 hours (Fig. 13A).

Previous studies have used GST-CRIB pulldown assays to quantify the amounts of GTP-bound GTPase during infection (Krause-Gruszczynska *et al*., 2007b). The next aim therefore was to confirm that Rac1 and Cdc42 are activated in infected INT-407 cells using a novel commercial kit called G-Lisa assay. The results show that infection with *C. jejuni* wild-type strain 81-176 induced the generation of active Rac1-GTP (Fig. 13B) as well as active Cdc42-GTP in a time dependent manner during the infection (Fig. 13C). These results are in good agreement with the previously reported data from GST-CRIB pulldown assays (Krause-Gruszczynska *et al*., 2007b) and confirmed by an independent new approach that invasion of *C. jejuni* into cultured host cells is associated with the generation of active small Rho GTPases Rac1 and Cdc42.

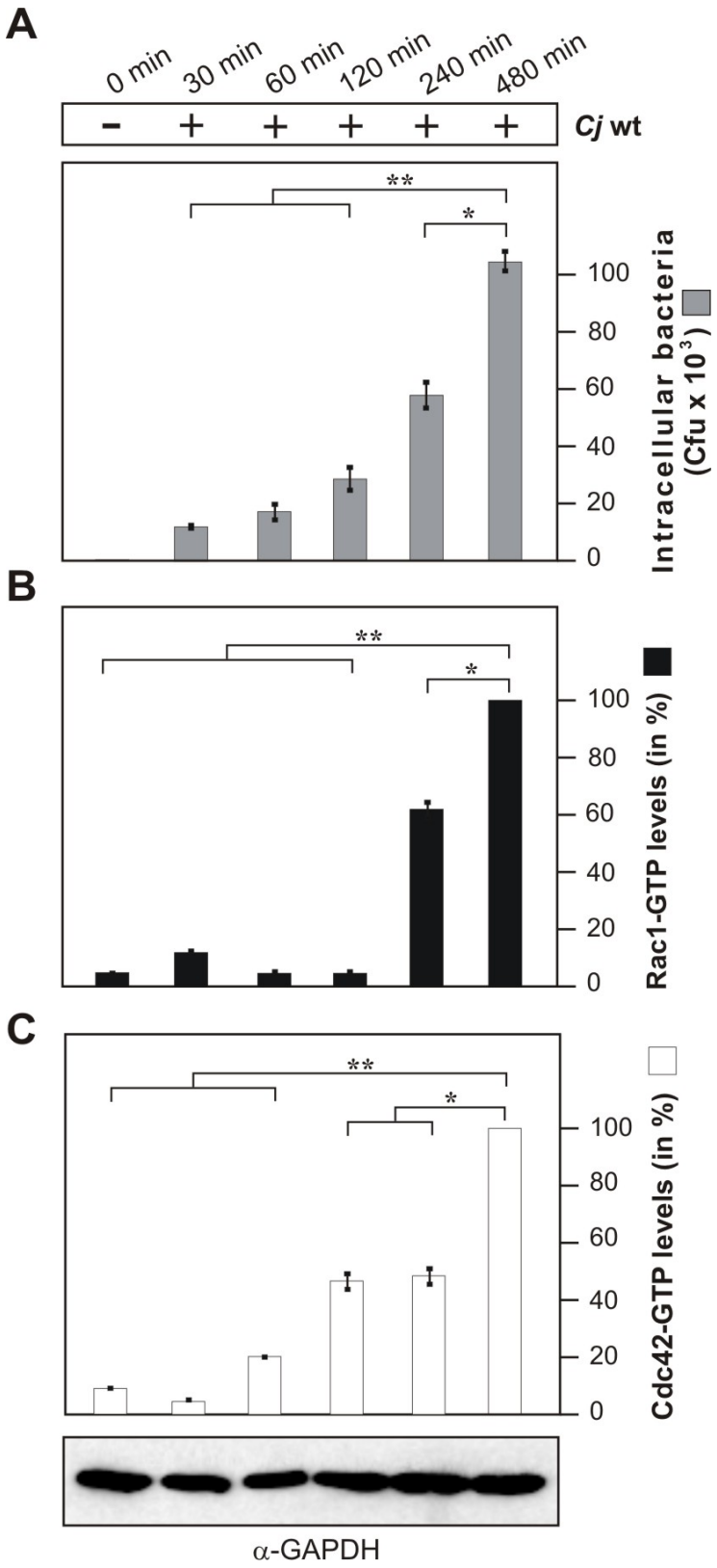


Figure 13: *C. jejuni* wt strain 81-176 enter INT-407 cells over time and this is associated with increasing levels of Rac1-GTP and Cdc42-GTP. (A) INT-407 cells were infected for indicated periods of time with *C. jejuni* and the number of intracellular bacteria was quantified by gentamicin protection assay. (B and C) Quantification of Rac1 and Cdc42 activity during the course of infection. The presence of active Rac1-GTP and Cdc42-GTP levels was quantified by G-Lisa. One hundred % of activity corresponds to the highest amount of the detected GTPase level. Similar amount of cells at every time point was confirmed by α-GAPDH Western blotting as control.

5.2.2 Lipid rafts are essential for *C. jejuni* host cell invasion and activation of Rac1- and Cdc42-GTP

Host cell membranes are composed of a classical lipid bilayer, containing proteins that span this bilayer and interact with these membrane-attached lipids. This lipid bilayer is composed of two apposing leaflets, forming a two-dimensional liquid with fascinating properties designed to perform the functions all cells basically require (Fessler and Parks, 2011; Simons and Sampaio, 2011). To coordinate these functions, the bilayer has evolved the propensity to segregate its constituents laterally. This capability is based on dynamic liquid-liquid immiscibility and underlies the "lipid raft" concept of membrane sub-compartmentalization (Fessler and Parks, 2011; Simons and Sampaio, 2011). By definition, lipid rafts are specific microdomains of the plasma membrane in eukaryotic cells which are enriched in cholesterol and sphingolipids. These domains seem to favour the interactions of particular membrane-associated proteins such as receptor molecules and the regulation of signalling pathways within cells (Fessler and Parks, 2011; Simons and Sampaio, 2011).

Previous studies have shown that addition of the pharmacological inhibitor methyl-beta cyclodextrin (MβCD), an agent that sequesters cholesterol and disrupts lipid rafts, decreased the ability of *C. jejuni* to enter several cultured epithelial cell lines, suggesting that lipid rafts may be required for efficient host cell invasion by the pathogen (Watson and Galan, 2008). Thus, we proposed that intact lipid rafts in target cells may be required for *C. jejuni* triggered Rac1 and Cdc42 activation. To investigate this question, increasing concentrations of MβCD (1-10 mM) were added to INT-407 cells 30 min prior to infection and kept throughout the entire time course. As control, the viability of cells was carefully checked in order to exclude any toxic effects resulting in a loss of host cells from the monolayer or other damage. Quantification of total intracellular *C. jejuni* was determined by gentamicin protection assay and effects on the generation of active Rac1-GTP and Cdc42-GTP were analyzed. Indeed, addition of MβCD blocked *C. jejuni*-induced internalization into INT-407 cells (Fig. 14A) as well as the activation of Rac1 and Cdc42 in a dose-dependent manner (Fig. 14B), suggesting that lipid rafts maybe targeted by *C. jejuni* to trigger downstream signalling leading to Rac1 activation.

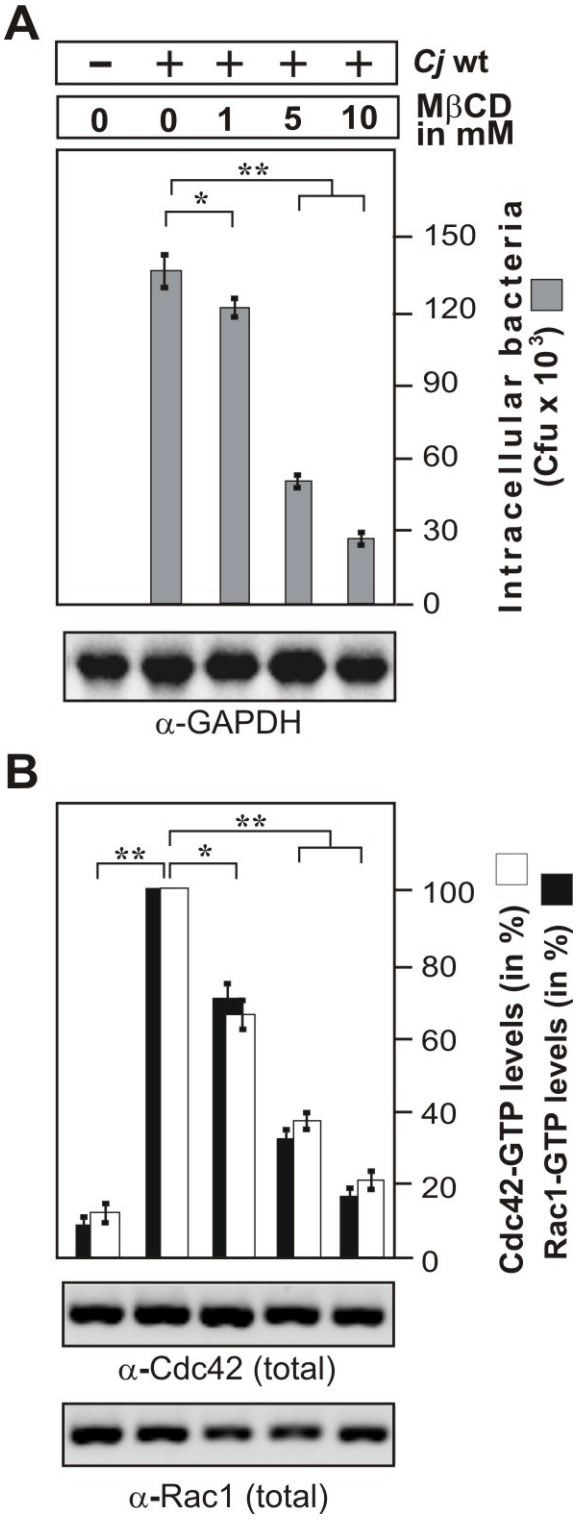


Figure 14: Lipid rafts are necessary for *C. jejuni* host cell entry and activation of Rac1-GTP and Cdc42-GTP. (A) Effects of MβCD targeting lipid rafts on host cell internalization of *C. jejuni*. INT-407 monolayers were pre-incubated with the indicated concentrations of MβCD for 30 min, followed by 6 hours infection with *C. jejuni*. Total intracellular *C. jejuni* were quantified by gentamicin protection assays. (B) The presence of active Rac1-GTP and Cdc42-GTP was analyzed by CRIB-GST pulldown assay and quantified. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP level. Similar quantities of total Rac1, Cdc42 and GAPDH were confirmed by Western blotting. (*) $P<0.05$ and (**) $P\leq0.005$ were considered as statistically significant as compared to the control.

5.2.3 *Campylobacter jejuni* invasion and GTPase activation require fibronectin, integrin, FAK and Src

Since *C. jejuni* encodes CadF, an outer membrane protein binding to the extracellular matrix protein fibronectin at the basolateral side of host cells (Konkel *et al*., 2001; Konkel *et al*., 2004; Euker and Konkel 2011), it was proposed that a classical fibronectin→integrin-β1→focal adhesion kinase (FAK) signalling pathway could be involved in *C. jejuni*-triggered activation of Rac1 and Cdc42 GTPase activity. To investigate this question, I utilized fibroblast cell lines derived from fibronectin$^{-/-}$ (Nyberg *et al*., 2004), integrin-β1$^{-/-}$ (so called GD25 cells) (Wennerberg *et al*., 1996), FAK$^{-/-}$ knockout mice (Sieg *et al*., 1999), and c-*src*-/-, c-*yes*-/-, and c-*fyn*-/- (SYF) triple knockout mice (Klinghoffer *et al*., 1999), which have the great advantage that the cells are completely devoid of expressing the proteins of interest. As controls, I utilised in parallel experiments floxed fibronectin$^{+/+}$ cells, GD25 cells stably re-expressing wild-type integrin-β1A (GD25β1A) and FAK$^{-/-}$ cells stably re-expressing wild-type FAK. The absence and presence of expression of the respective proteins was verified by immunoblotting using specific antibodies as indicated (Fig. 15A-D). The cell lines were infected with wild-type *C. jejuni*, followed by invasion assays. Quantification of the intracellular bacteria by gentamicin protection assay showed that wild-type *C. jejuni* can effectively enter the control cells, while the knockout cells exhibited significant deficiencies for bacterial uptake (Fig. 15A-D). This suggests that fibronectin, integrin-β1, FAK and Src play a crucial role in the invasion process of *C. jejuni.*

As next, the wild-type and mutant cell lines were infected with wild-type *C. jejuni* under identical conditions as described above followed by preparation of cell lysates and GST-CRIB pulldown assays to determine the Rac1- and Cdc42-GTP levels during infection. The results show that activation of Rac1 and Cdc42 are profoundly activated in the infected wild-type control cells, but this was strongly impaired in each of the infected knockout cell lines (Fig. 15E-H). These findings clearly correlate with the ability of *C. jejuni* to enter each of these cell lines, suggesting that the fibronectin→integrin-β1→FAK→Src signalling cascade is upstream of *C. jejuni*–triggered GTPase activation.

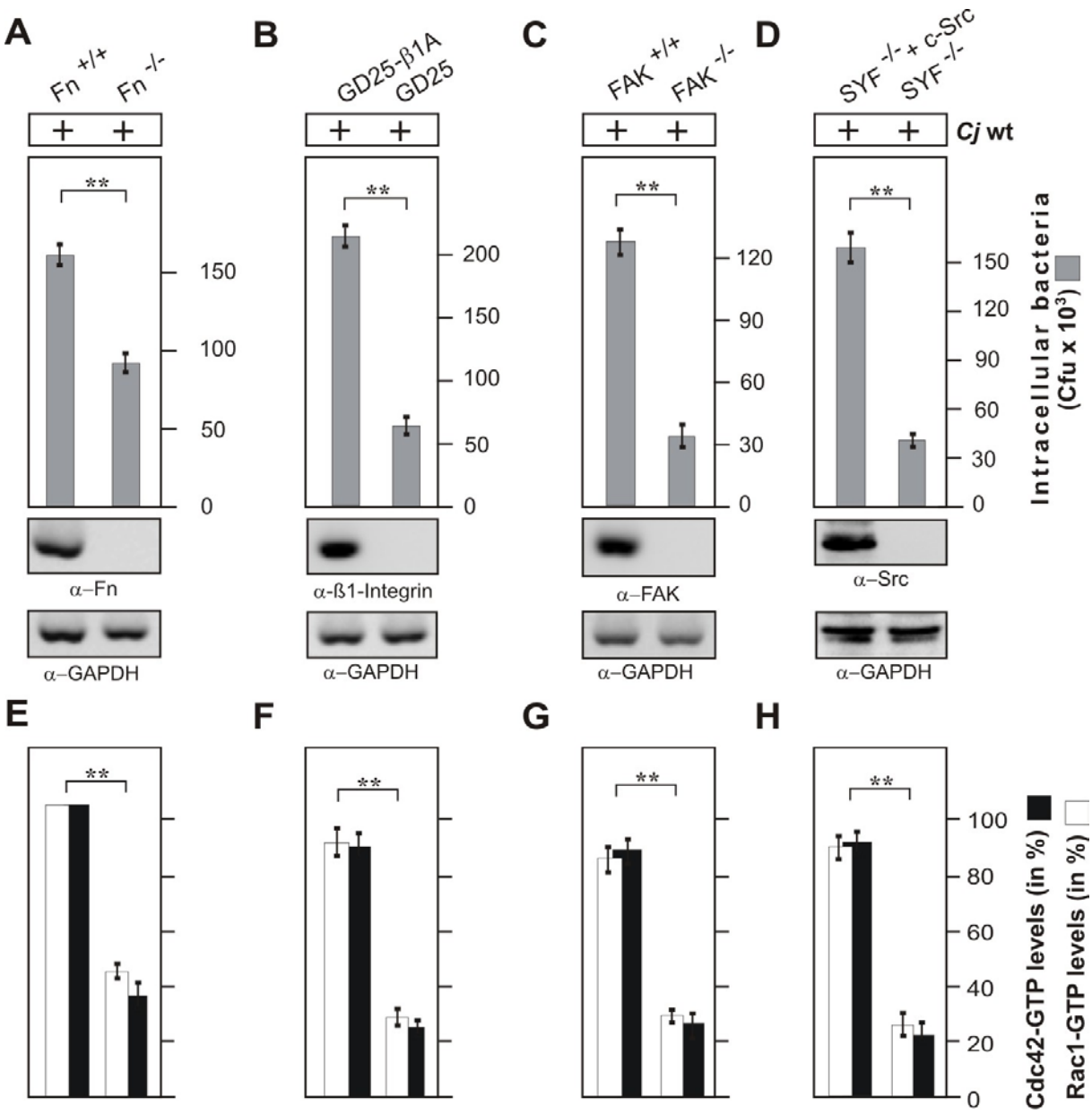


Figure 15: Fibronectin, integrin, FAK and Src are necessary for efficient *C. jejuni* invasion and activation of Rac1 and Cdc42 Rho GTPases. The following cells lines were infected with wild-type (wt) *C. jejuni* 81-176 for 6 hours. (A) Fibronectin deficient cells ($Fn^{-/-}$) and corresponding floxed wt cells ($Fn^{+/+}$), (B) integrin-β1-deficient cells (GD25) and GD25 stably re-expressing wt integrin-β1A (GD25-β1A) cells, (C) FAK-deficient cells ($FAK^{-/-}$) and $FAK^{-/-}$ cells stably re-expressing wt FAK and (D) Src kinase-deficient cells ($SYF^{-/-}$) and $SYF^{-/-}$ cells stably re-expressing wt c-*src*. Intracellular *C. jejuni* were quantified by gentamicin protection assays. Fibronectin, integrin-β1, FAK and Src protein expression was verified by Westernblotting using the indicated antibodies. GAPDH expression levels were determined as loading control. (E-H) Rac1 and Cdc42 activation was determined under identical conditions by CRIB-GST pulldowns. (**) $P \leq 0.005$ was considered as statistically significant..

5.2.4 *Campylobacter jejuni* induces filopodia formation in wild-type cells

The above results led us to propose that fibronectin, integrin-β1 and FAK may form a signalling complex to induce GTPase activity during infection. Classical features for the activation of Rac1 is membrane ruffling and lamellipodia formation, while Cdc42 activation is associated with the generation of filopodia (Schmidt and Hall, 2002). Thus, the next aim was to investigate if these features can be visualized by electron microscopy. First, I wanted to investigate if Cdc42-triggered filopodia can be seen on cells upon contact with the bacteria. To investigate this question, wild-type fibroblasts were infected with *C. jejuni* followed by analysis of host cells by high resolution field emission scanning electron microscopy (FESEM), performed by cooperation partner Prof. M. Rohde (Helmholtz Center for Infection Research, Braunschweig, Germany). FESEM analysis revealed that *C. jejuni* profoundly induced filopodia formation at the periphery and top of infected host cells (Fig. 16A, up to 7 µm long, blue arrows), while only very few of these structures could be seen in non-infected wild-type fibroblasts as control (Fig. 16B).

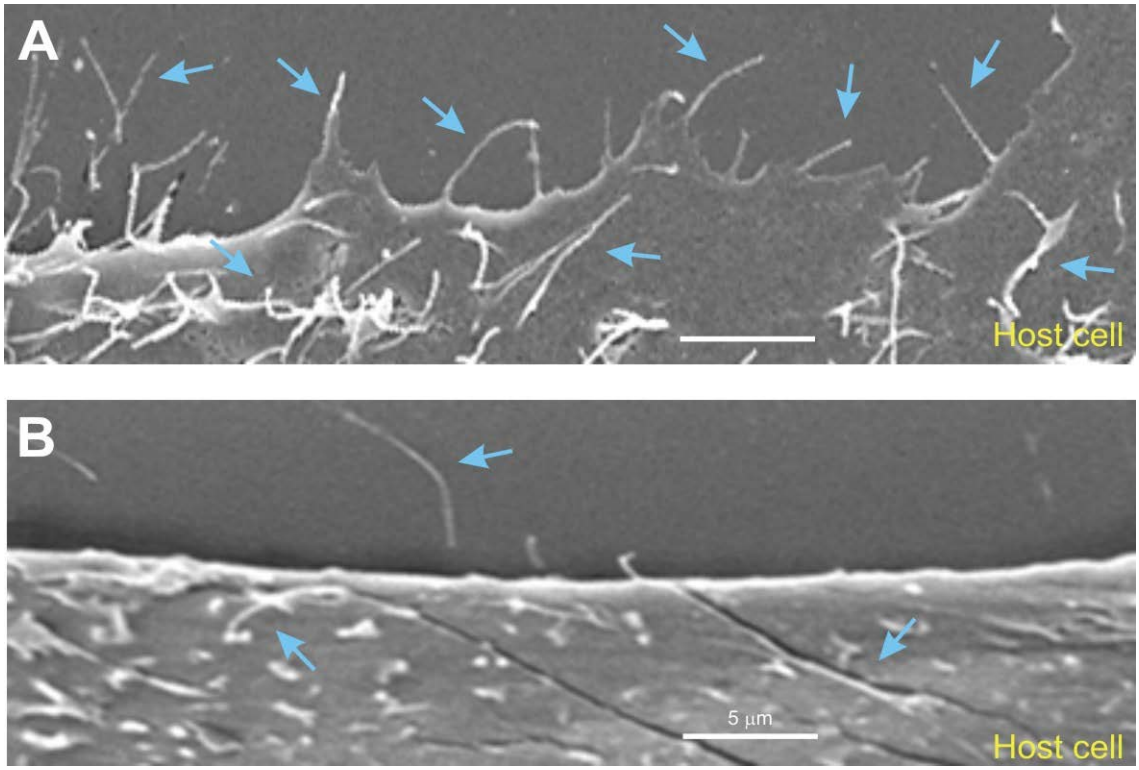


Figure 16: High resolution FESEM of *C. jejuni*-induced filopodia formation. Representative sections of wild-type fibroblasts incubated for 6 hours with wt *C. jejuni* 81-176 (A) and non-infected fibroblast control cells that were mock-treated (B) are shown. Infection revealed the occurrence of membrane protrusion events with long filopodia at the periphery and on top of cells which were only sporadically seen in the non-infected control cells (blue arrows).

5.2.5 *Campylobacter jejuni* induces membrane ruffling and invasion in wild-type cells but not in any of the investigated knockout cell lines

Next, wild-type fibroblasts and their corresponding fibronectin$^{-/-}$, GD25 and FAK$^{-/-}$ knockout cell lines were infected with *C. jejuni* and the interaction of *C. jejuni* with the surface of host cells were analyzed by resolution FESEM. After infection, the micrographs revealed that the bacteria were able to attach to the host cell surface, followed by cellular invasion which was observed predominantly after 4-6 hours of infection (Fig. 17). Tight engulfment of the bacteria and the occurrence of membrane ruffling as typical for Rac1 activation (red arrows), closely associated with filopodia structures (blue arrows) as well as the appearance of elongated microspikes (green arrowheads) were also regularly observed. Interestingly, similar to earlier observations in our group with infected INT-407 cells (Krause-Gruszczynska et al., 2007b), it was found that *C. jejuni* entered the wild-type fibroblast cells in a very specific fashion, first with its flagellar tip followed by the opposite flagellar end (Fig. 17, yellow arrows). The time frame of observing invading bacteria correlated with the above described results obtained from gentamicin protection assays (Fig. 13A) and GTPase activation assays in a corresponding time course (Fig. 13B,C).

Next, I infected fibronectin$^{-/-}$, GD25 and FAK$^{-/-}$ knockout cells with wild-type *C. jejuni* for 6 hours under identical conditions as described above, followed by preparation for FESEM. Close inspection of these infected cells also revealed bound bacteria (yellow arrows) at the surface of the cells with short microspikes (green arrowheads) present, but almost no membrane ruffles or invading *C. jejuni* could be detected (Fig. 18 and data not shown). The predominant observation of membrane ruffling and the generation of filopodia structures in wild-type cells confirms the typical occurrence of Rac1 and Cdc42 GTPase activation followed by dynamic membrane rearrangements during the *C. jejuni* invasion process, and these events appear to be dependent on the expression of three crucial host cell factors, which were identified as fibronectin, integrin-β1 and FAK.

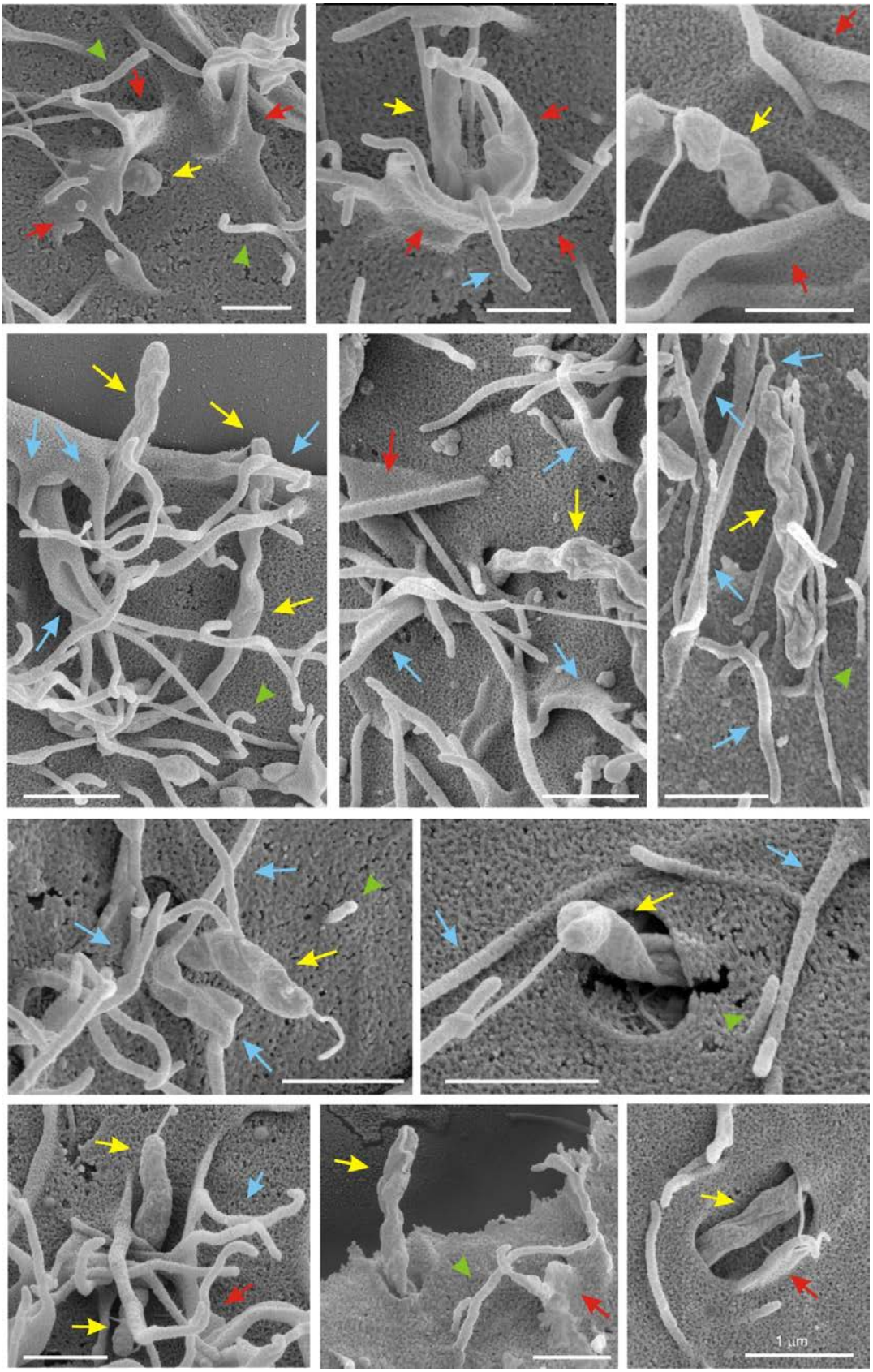


Figure 17: High resolution field emission scanning electron microscopy of *C. jejuni* invasion. *C. jejuni* 81- 176 infected for 4-6 h were able to induce their entry into the wild-type (wt) fibroblast target cells and were regularly associated with membrane ruffles (red arrows), filopodia-like structures (blue arrows) as well as elongated microspikes (green arrowheads). Invading bacteria were also marked (yellow arrows).

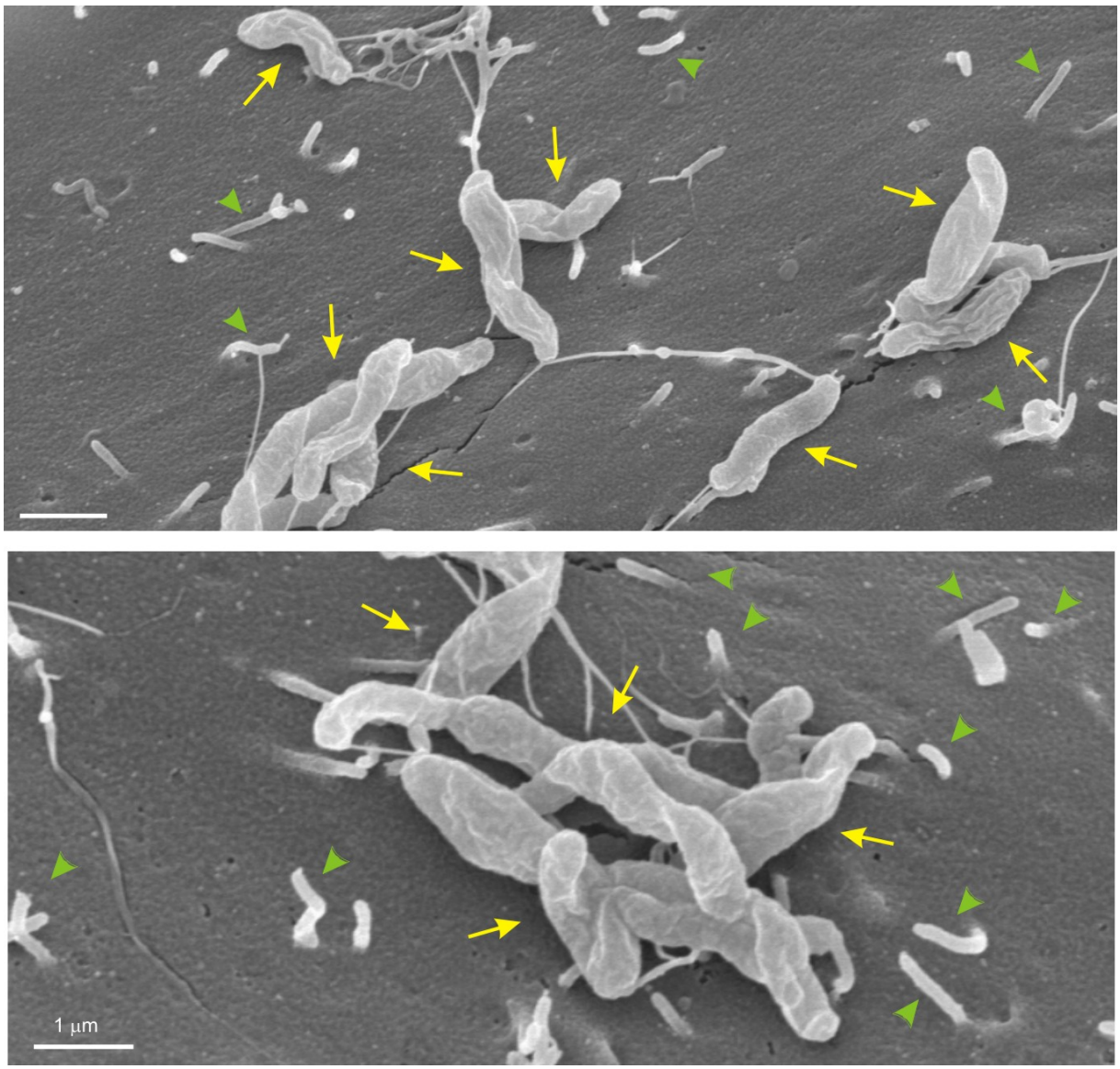


Figure 18: High resolution field emission scanning electron microscopy of *C. jejuni* invasion of knockout cell lines. Infection of GD25 knockout cells with wild-type (wt) *C. jejuni* 81-176 for 6 hours also revealed bacterial attachment, but membrane dynamics or invasion were not induced. Similar results were obtained during infection of fibronectin$^{-/-}$ and FAK$^{-/-}$ cell lines (data not shown).

5.2.6 Importance of FAK and CadF for *C. jejuni*- induced Rac1 and Cdc42 activation

The above results showed that FAK is important for *C. jejuni* invasion and GTPase activation. FAK is an intracellular non-receptor tyrosine kinase and important modulator of integrin-dependent focal adhesion cell contacts, thereby orchestrating well-known integrin-initiated outside-in signalling events (Sieg *et al*., 1999; Hauck *et al.,* 2002). It was therefore hypothesized that the well-known *C. jejuni* fibronectin binding protein CadF (Konkel *et al*., 2001; Konkel *et al*., 2004; Euker and Konkel 2011) could trigger fibronectin→integrin-β1→FAK signalling cascade leading to GTPase activation. To test this idea, FAK-expressing wild-type cells were infected with wild-type *C. jejuni* and an isogenic Δ*cadF* mutant for different time periods (30–240 min) and the amount of intracellular bacteria was quantified by gentamicin protection assay. Quantification data show an increasing amount of intracellular bacteria over the time for wild-type *C. jejuni* while significantly less invasion was observed for the Δ*cadF* mutant (Fig. 19A). This is in well agreement with earlier publications in the group using a different cell line, INT-407 (Krause-Gruszczynska *et al*., 2007a,b).

To investigate if CadF and FAK are required for *C. jejuni* induced Rac1 and Cdc42 activation, $FAK^{-/-}$ knockout cells and $FAK^{-/-}$ cells re-expressing FAK were infected under the same conditions as shown in Figure 16A, followed by CRIB-GST pull-down assays. While growing levels of activated Rac1 and Cdc42 were detected in FAK-positive cells infected with wild-type *C. jejuni* over time, only very small amounts of detectable activation of Rac1 (Fig. 19B) and Cdc42 (Fig. 19C) was found in $FAK^{-/-}$ cells during the entire course of infection. This again indicates the clear involvement of FAK in signalling upstream of Rac1 and Cdc42 activation during *C. jejuni* invasion. Furthermore, significantly reduced Rac1-GTP (Fig. 19B, right lanes) and Cdc42-GTP (Fig. 19C, right lanes) levels were observed in both FAK-positive and $FAK^{-/-}$ cells infected with the Δ*cadF* mutant under the same settings. These findings support the view that the CadF protein plays a role not only in sole binding of the bacteria to host cells but also in signalling leading to FAK-mediated activation of Rac1 and Cdc42. However, the Δ*cadF* mutant was still able to induce some Rac1- and Cdc42-GTPase activation in FAK-positive cells (Fig. 19B and C, right lanes), suggesting that CadF is not the sole signalling component in *C. jejuni*, but other bacterial factors are also implicated in this signalling cascade.

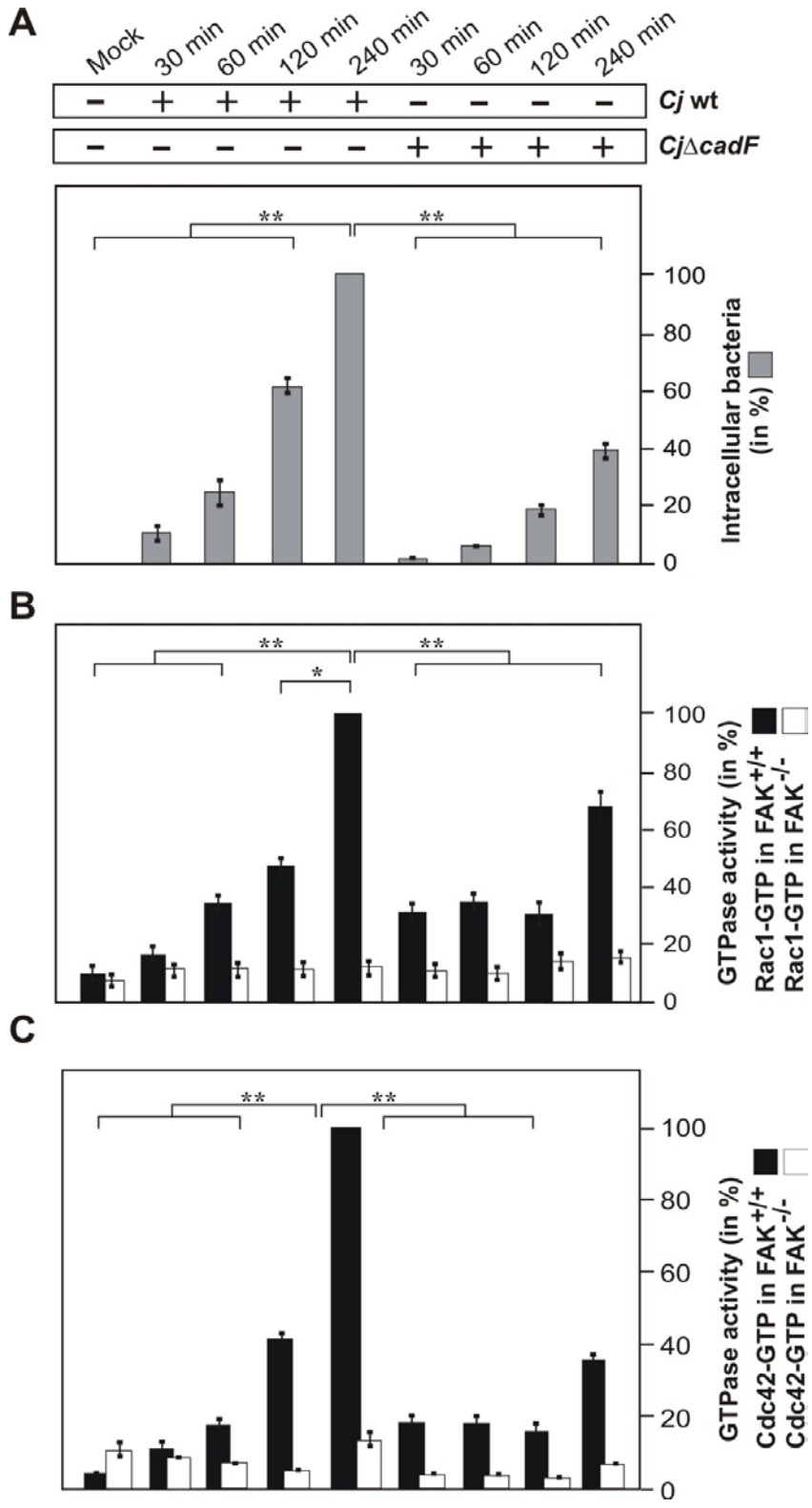


Figure 19: Importance of FAK for *C. jejuni*-induced Rac1 and Cdc42 activation and role of the bacterial CadF protein. (A) FAK-positive fibroblasts were infected with wild-type (wt) *C. jejuni* strain F38011 or isogenic F38011Δ*cadF* for indicated periods of time. Intracellular bacteria were quantified by gentamicin protection assay. (B, C) Quantification of Rac1 and Cdc42 GTPase activity during the course ofinfection. FAK-positive and FAK-deficient cells were infected with *C. jejuni* wt strain F38011 or F38011Δ*cadF* mutant for the indicated periods of time. The presence of bound, active Rac1- GTP or was analyzed in CRIB-GST pull-down assays. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP level. (*)P≤0.05 and (**)P≤0.005 were considered as statistically significant as compared to the control.

5.2.7 Wild-type *C. jejuni* but not *ΔcadF* mutant induces profound FAK, EGFR and PDGFR phosphorylation during infection

Having established that expression of FAK tyrosine kinase is crucial for *C. jejuni*-triggered signalling and invasion, the next aim was to investigate if *C. jejuni* infection activates FAK autophosphorylation and if this is associated with the activation of growth factor receptor tyrosine kinases including EGFR (epidermal growth factor receptor) and PDGFR (platelet-derived growth factor receptor), which are also present in membrane lipid rafts. For this purpose, host cells were infected with wild-type *C. jejuni* and an isogenic Δ*cadF* deletion mutant in a time course. Protein lysates from the infected cells were prepared and subjected to Western blotting using commercially available activation-specific phospho-antibodies for FAK, EGFR and PDGFR (Fig. 20A). The results shows that wild-type *C. jejuni* significantly induced the autophosphorylation of FAK at tyrosine residue Y-397 in the active centre (PhD thesis of M. Krause-Gruszczynska) and Y-925 at the carboxy-terminus of FAK, the phosphorylation of EGFR at Y-845 and the phosphorylation of PDGFR at Y-754 over time (Fig. 20A). This data indicated that maximal levels of kinase phosphorylation appeared after 4 hours of infection (Fig. 20B), which correlated with increasing Cdc42-GTP levels over time (Fig. 13C) and the invasion capabilities of wild-type *C. jejuni*, as determined by gentamicin protection assays (Fig. 19A).

Interestingly, infection with the Δ*cadF* mutant, as examined under identical conditions as described above, revealed that phosphorylation of FAK, EGFR and PDGFR were widely impaired (Fig. 20A, B) and this also correlated with the reduced invasiveness of this mutant (Fig. 19A). In agreement with this observation, Krause-Gruszczynska reported that Y-397 in FAK is also much less phosphorylated during infection with the same isogenic Δ*cadF* deletion mutant in a time course (PhD thesis of M. Krause-Gruszczynska). These findings collectively suggest that the bacterial CadF protein is involved in *C. jejuni*-induced FAK, EGFR and PDGFR kinase activities, as well as in host cell invasion of the bacteria.

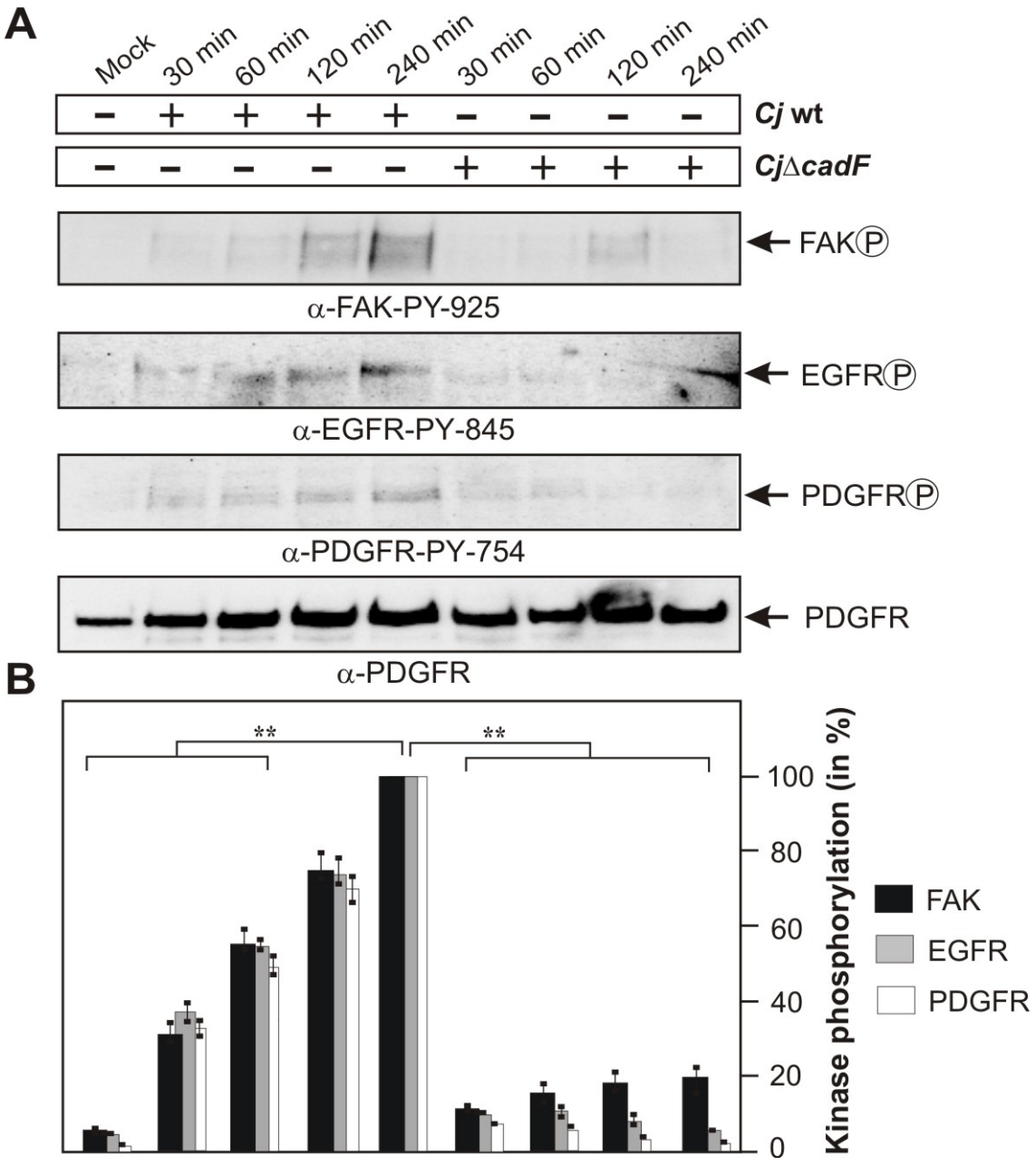


Figure 20: Importance of CadF for *C. jejuni*-induced FAK, EGFR and PDGFR activation. (A) FAK-positive fibroblasts were infected with wild-type (wt) *C. jejuni* strain F38011 or isogenic F38011Δ*cadF* mutant for the indicated periods of time. FAK, EGFR or PDGFR phosphorylation/activation was analysed by immunoblotting with the indicated phosphor-specific antibodies. Total PDGFR expression levels were determined as loading control. **(**B) Quantification of FAK, EGFR and PDGFR kinase phosphorylation during the course of infection was done using the Lumi-imager F1 (Roche). One hundred % of activity corresponds to the highest amount of phosphorylation detected per experiment and selected kinase (lane 5). (*) $P \leq 0.05$ and (**) $P \leq 0.005$ were considered as statistically significant.

5.2.8 Activities of FAK, EGFR, PDGFR and PI3-kinase are also important for *C. jejuni*-induced Cdc42-GTP levels and invasion

Previous results in the group have shown that pharmacological inhibition of EGFR, PDGFR and PI3-kinase did not downregulate the *C. jejuni*-mediated activation of Rac1-GTP (Krause-Gruszczynska *et al*., 2007b). It was therefore proposed that activated EGFR, PDGFR and PI3-kinase (and also FAK) could play an important role for the induction of Cdc42-GTP levels. To test this idea, INT-407 cells were pre-treated for 30 min with the pharmacological inhibitors AG1478 (EGFR inhibitor), AG370 (PDGFR inhibitor), wortmannin (PI3-kinase inhibitor) or PF-573228 (FAK inhibitor) followed by infection with wild-type *C. jejuni*. The results show that inhibition of each of these kinases had a profound suppressive effect on both Cdc42-GTP levels and bacterial invasion (Fig. 21). Complementary gentamicin protection assays exhibited a significant reduction of intracellular *C. jejuni*, confirming the involvement of PDGFR, EGFR and FAK in the uptake of *C. jejuni* (Fig. 21).

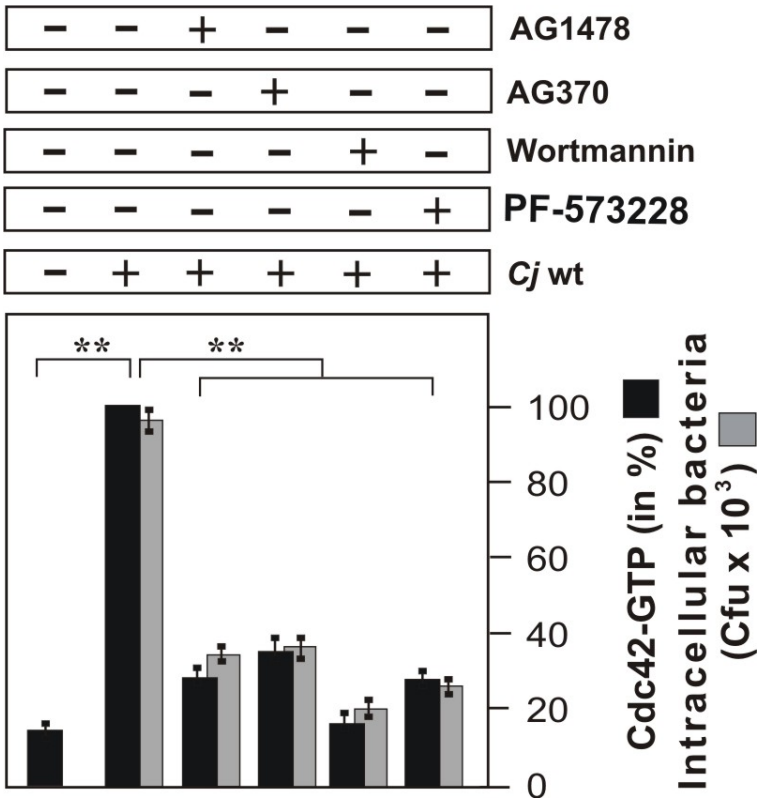


Figure 21: Importance of FAK, EGFR, PDGFR and PI3-kinase activities for *C. jejuni*-induced activation of Cdc42 and bacterial invasion. INT-407 monolayers were pre-incubated for 30 min with the indicated pharmacological inhibitors and infected with wild-type (wt) *C. jejuni* 81-176 for 6 hours. Intracellular *C. jejuni* were quantified by gentamicin protection assays. The presence of active Cdc42-GTP was quantified by CRIB-GST pull-downs. One hundred % of activity corresponds to the highest amount of detected Cdc42-GTP level (lane 2). (*) $P \leq 0.05$ and (**) $P \leq 0.005$ were considered as statistically significant.

5.2.9 The guanine exchange factors Tiam1, DOCK180 and Vav2 are required for Rac1 and Cdc42 activation and *C. jejuni* invasion

Cycling of small Rho GTPases between the inactive and active forms is commonly stimulated by a class of proteins called guanine nucleotide exchange factors (GEFs) and negatively regulated by GTPase activating proteins (GAPs). GEFs trigger the exchange of GDP for GTP to generate the active form of a given GTPase, which is then capable of recognizing downstream targets (Schmidt and Hall, 2002; Hsia *et al*., 2003; Tomar and Schlaepfer, 2009). Thus, it would be important to find out which GEFs are involved in the activation pathways of Cdc42 and Rac1. Previous results in the group have shown that downregulation of various typical GEFs including Vav2, Tiam-1, DOCK180, but not Trio, downregulated *C. jejuni* invasion as measured by gentamicin protection assays (PhD thesis of M. Krause-Gruszczynska). However, it remained unknown which of these GEFs is upstream of Cdc42 and Rac1, and if there are differences in GEF specificity for activating either GTPase during infection.

To identify which GEFs are involved in *C. jejuni*–induced Cdc42 or Rac1 activation, the expression of the three candidate GEFs including Vav2, DOCK180 and Tiam-1 was downregulated using target-specific siRNA, followed by infection with wild-type *C. jejuni* and CRIB-GST pulldowns. Western blotting controls verified that each of these GEFs is downregulated as expected but a scrambled non-targeting siRNA control did not influence the expression of any of the chosen GEFs. The results of subsequent CRIB-GST pulldown assays showed that, while the downregulation of Vav2 led to the predominant inhibition of Cdc42-GTP levels (Fig. 22A), both downregulation of Tiam-1 and DOCK180 (Fig. 22B, C) or transfection of scrambled siRNA control had no significant effect on *C. jejuni*-triggered Cdc42-GTP production (Fig. 22D-F). Importantly, downregulation of Vav2 did not significantly reduced Rac1-GTP levels in the same assay, while downregulation of Tiam-1 and DOCK180 led to the predominant inhibition of Rac1-GTP levels (Fig. 22D-F). These findings are in agreement with the hypothesis that different GEFs regulate different GTPases during *C. jejuni* infection. Vav2, but not Tiam-1 or DOCK180, plays a crucial role in *C. jejuni*-induced Cdc42 activation; while Tiam-1 and DOCK180, but not Vav2 are involved in *C. jejuni*-induced Rac1 activation.

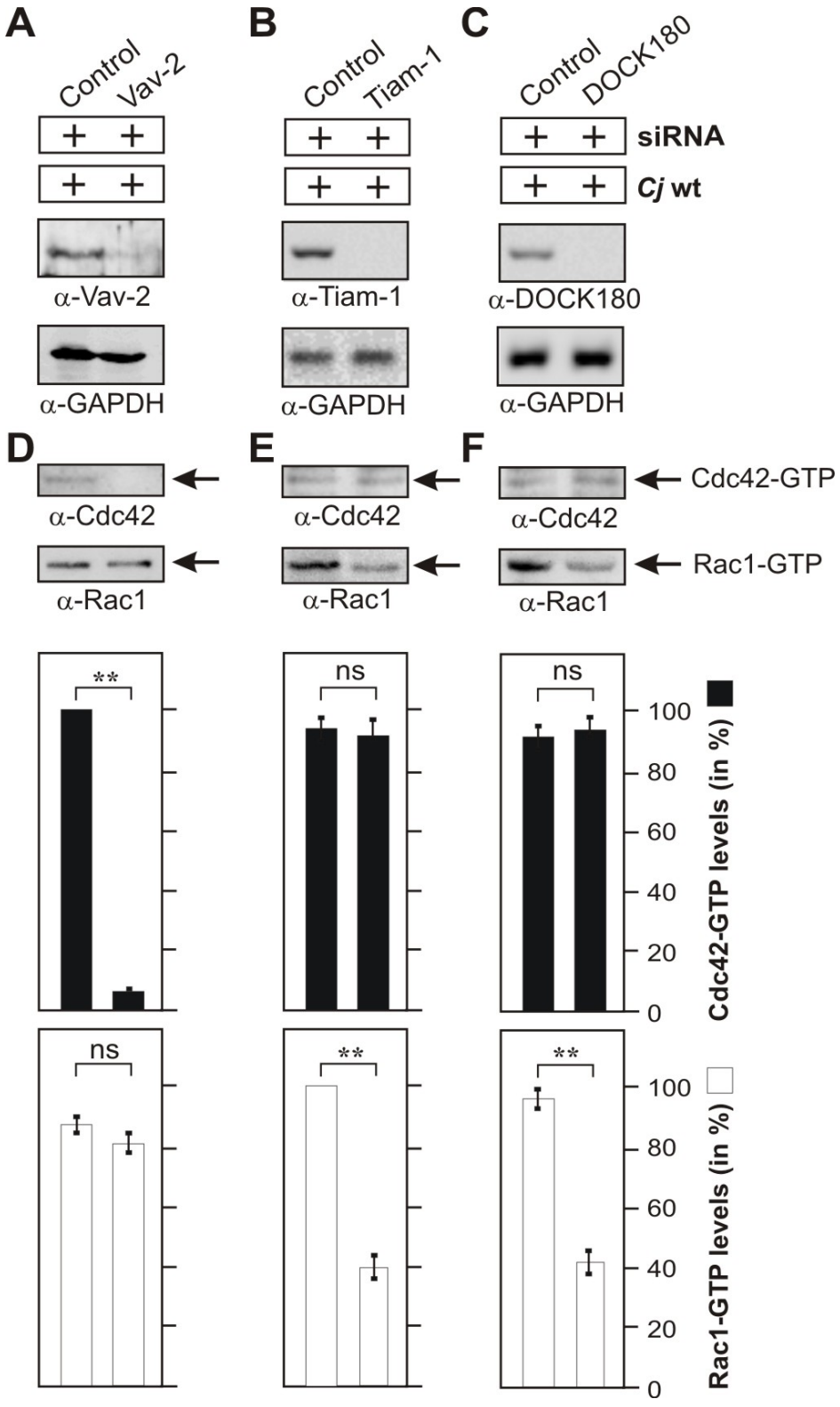


Figure 22: **Importance of guanine exchange factors for *C. jejuni*-induced Rac1- and Cdc42 activation.** INT-407 cells were transfected for 48 hrs with siRNA for Vav2 (A), Tiam-1 (B) or DOCK180 (C) as well as a scrambled siRNA as control for each experiment. Immunoblotting with the indicated antibodies confirmed knockdown of the respective proteins. GAPDH expression levels were determined as loading control. Quantification of Rac1- and Cdc42-GTPase activity after infection with *C. jejuni* wt 81-176 for 6 hrs. The presence of active Rac1-GTP and Cdc42-GTP was analyzed by CRIB-GST pulldown assays followed by Western blotting using α-Rac1 and α-Cdc42 antibodies (D-F). One hundred % of activity corresponds to the highest amount of detected Rac1- and Cdc42-GTP level.

5.2.10 Tiam-1 and DOCK180 act cooperatively to trigger Rac1 activation and *C. jejuni* invasion downstream of FAK

In the above experiments it was noted that downregulation of the GEFs Tiam-1 or DOCK180 did not lead to a complete blockade of Rac1 activity and *C. jejuni* uptake. Therefore, it was proposed that both GEFs may act together in *C. jejuni*-infected cells. To investigate this question, Tiam-1 and DOCK180 expression was downregulated by siRNA, either alone or simultaneously, followed infection with wild-type *C. jejuni* and G-Lisa to determine Rac1 activity. The results show that simultaneous downregulation of Tiam-1 and DOCK180 led to a profound block of *C. jejuni*-induced Rac1 activity (Fig. 23A, B). A similar strong blockade of Rac1 levels was achieved in infected $FAK^{-/-}$ cells (Fig. 19B) or by infection in the presence of the FAK kinase inhibitor PF-573228 (Fig. 23C). As expected, simultaneous downregulation of Tiam-1 and DOCK180 resulted not only in the profound inhibition of Rac1 activity but also profound blockade of *C. jejuni* invasion (Fig. 23D). These data suggest that the above experiments work identified an important pathway of *C. jejuni* host cell entry, proceeding by activation of a FAK→Tiam-1/DOCK180→Rac1 signalling cascade downstream of fibronectin and intergin-β1.

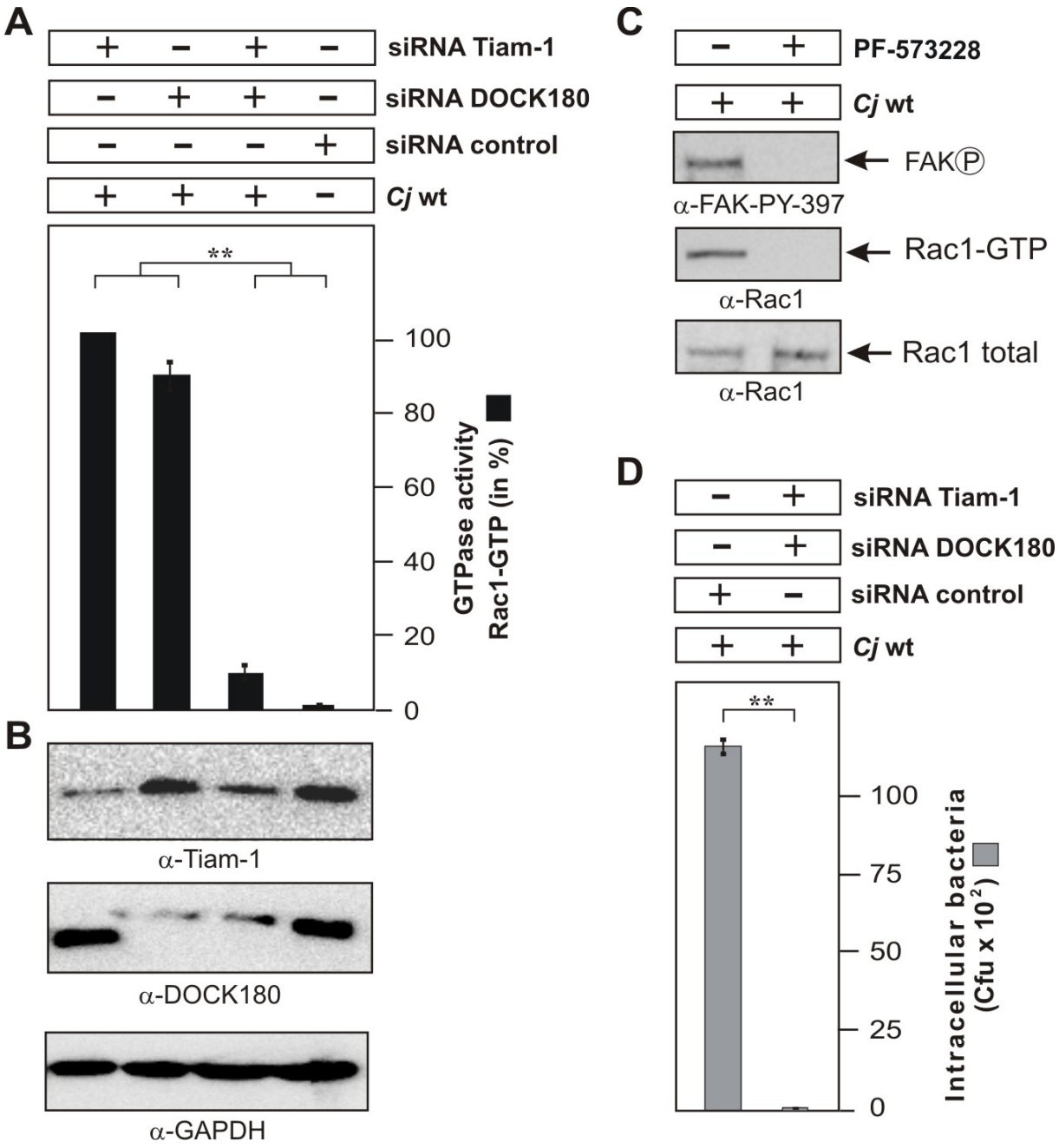


Figure 23: Importance of Tiam-1, DOCK180 and FAK activation in *C. jejuni*-induced Rac1 activation and bacterial invasion. (A) INT-407 cells were transfected for 48 hrs with siRNA for Tiam-1, DOCK180 or a scrambled siRNA as control. Quantification of Rac1 GTPase activity after infection with wild-type (wt) *C. jejuni* 81-176 for 6 hours. The presence of bound, active Rac1-GTP was analyzed by G-Lisa. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP level (lane 1). (B) Immunoblotting with the indicated antibodies confirmed knockdown of the proteins. GAPDH expression levels were determined as control. (C) Addition of FAK kinase inhibitor PF-573228 during a 6 hour infection led to the disappearance of Rac1-GTP and FAK phosphorylated at Y-397. Active Rac1-GTP levels were determined by CRIB-GST pulldowns. (D) Intracellular bacteria were quantified by gentamicin protection assays in the indicated siRNA-treated cells. (**) $P \leq 0.001$ were considered as statistically significant as compared to the control.

5.2.11 The flagellum is also involved in *C. jejuni*-induced Rac1 activation and invasion

The above experiments and previously published work (Krause-Gruszczynska *et al*., 2007b) indicated that CadF is not the sole bacterial gene involved in *C. jejuni*-induced GTPase activation. Thus, the following experiments were desgined to search for other bacterial factors playing a role in this signalling. The flagellar apparatus was reported to be one of the most intensively investigated pathogenicity determinant in *C. jejuni* (Konkel *et al*., 2004; Guerry, 2007). To test the hypothesis that the flagellar apparatus maybe also involved in GTPase activation, host cells were infected with the wild-type strain 81-176 and its isogenic mutants Δ*fla*A/B, lacking the two major flagella subunits FlaA and FlaB (Goon *et al*., 2006), and Δ*flhA*, a key element involved in the coordinate regulation of late flagellar genes and other factors in *C. jejuni* (Carillo *et al*., 2004). First, I confirmed the absence of flagella in both mutants (Fig. 24A), followed by infection assays. As expected activated Rac1 and Cdc42 was detected in FAK-positive cells after infection with wild-type *C. jejuni* (Fig. 24B). In contrast, no detectable Rac1 and Cdc42 activation was found in cells infected with Δ*fla*A/B or Δ*flhA* mutants during the entire course of infection (Fig. 24B), indicating an important role of the flagellum in activating Rac1 and Cdc42 by *C. jejuni*, in addition to the contribution by CadF as shown above.

There is some controversy in the literature about whether the *C. jejuni* flagellum-mediated bacterial motility is important for invasion or if the flagellum can secrete invasion-related bacterial factors in the supernatant (Konkel *et al*., 2004; Novik *et al*., 2010). To investigate if the flagellar effect on invasion is direct or indirect, we were searching for a condition in which the flagellar motility is not affected, but invasion can be impaired. An α-FlaA antibody against a conserved region at the amino-terminus of FlaA was generated, and this antibody recognises *C. jejuni* FlaA proteins in Westernblots (Fig. 25D). Wild-type *C. jejuni* was then pre-incubated with the α-FlaA antibody or pre-immune serum as control followed by motility assays in soft agar. Treatment of any of the used *C. jejuni* strains with the pre-immune serum revealed no significant differences in bacterial motility or invasion as compared to non-treated bacteria (Fig. 25A, C and data not shown). The results also showed that while the presence of α-FlaA antibody had a slight but no significant effect on motility of wild-type *C. jejuni* or Δ*cadF* mutant (Fig. 25A, B), bacterial invasion was significantly impaired as determined by gentamicin protection assay (Fig. 25C, D). This suggests that the flagellum of *C. jejuni* has a motility-independent activity which is involved in bacterial entry into host target cells.

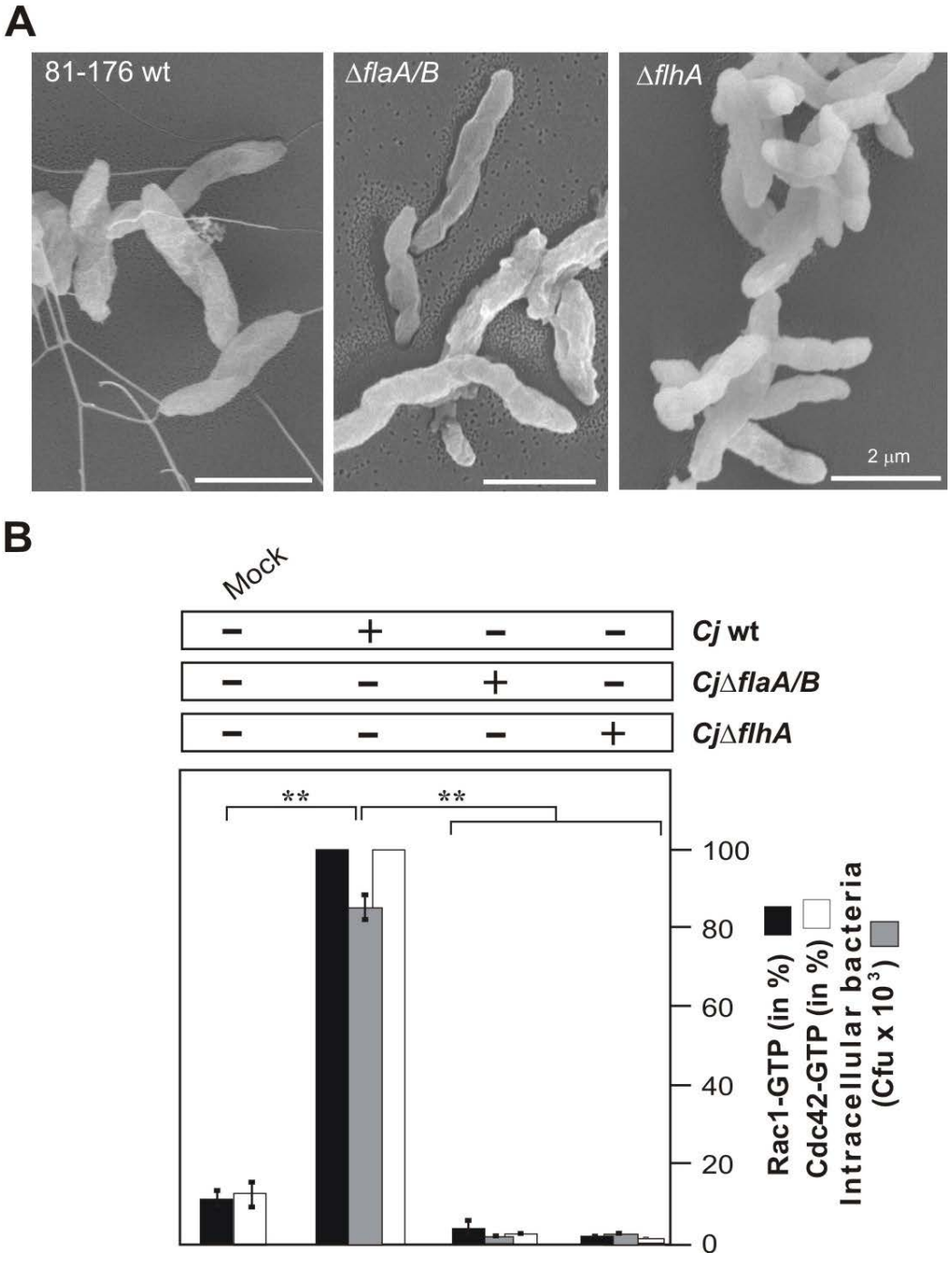


Figure 24: Importance of the flagellar apparatus for *C. jejuni*-induced activation of Rac1 and bacterial invasion. (A) High resolution field emission scanning electron microscopy of *C. jejuni* wild-type (wt) 81-176, 81-176Δ*flaA/B* and 81-176Δ*flhA* mutants. (B) FAK- positive cells were infected with the indicated strains in a time course. The presence of bound, active Rac1-GTP was analyzed in CRIB-GST pulldown assays. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP level. The amount of intracellular bacteria was quantified by gentamicin protection assays. (**) P≤0.001 were considered as statistically significant as compared to the control.

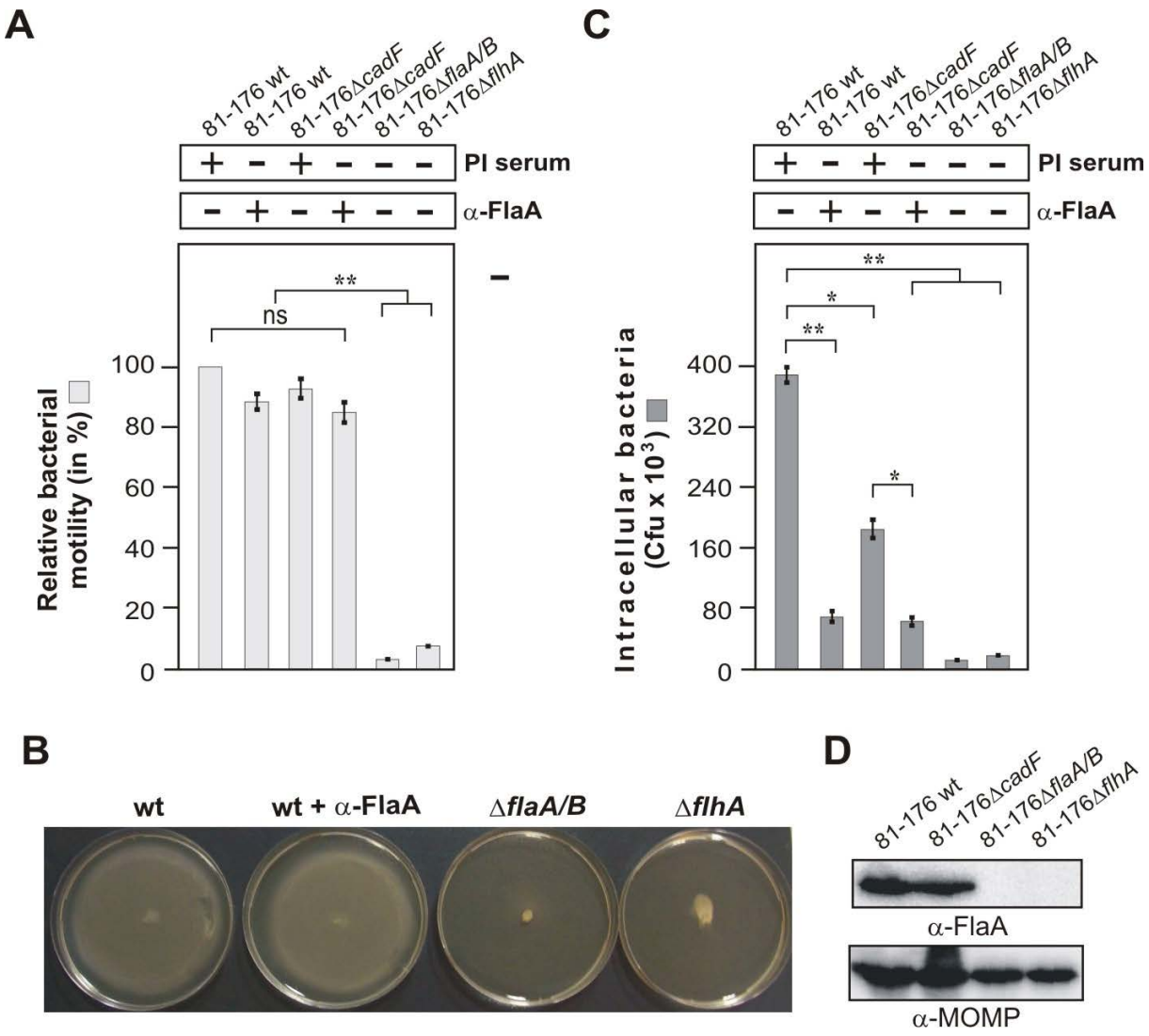


Figure 25: Different effects of an α-FlaA antibody on *C. jejuni* motility and host cell invasion. (A) Quantification of motility using the indicated wt and mutant strains in the presence of 20 µg/ml α-FlaA antibody or pre-immune (PI) serum as control. One hundred % of motility corresponds to the highest motility presented by *C. jejuni* wt strain 81-176. (B) Examples of motility phenotypes with indicated strains. (C) INT-407 cells were infected with the indicated wt and mutant strains for 6 hrs in presence or absence of 20 µg/ml α-FlaA antibody or PI serum as control. Intracellular *Campylobacter* cells were quantified by gentamicin protection assays. (D) The α-FlaA immunoblot shows that flagellin is expressed in wt and Δ*cadF C. jejuni*, but not in flagellar mutant bacteria. The α-MOMP immunoblot served as loading control. (*) P≤0.05 and (**) P≤0.005 were considered as statistically significant when compared to the control.

6. DISCUSSION

Campylobacter species are commensals in many birds and domestic mammals, and have been described as being the most important cause of bacteria-caused food poisoning worldwide. *Campylobacter jejuni* is the most predominant and best described species in this genus. Infection with *C. jejuni* is the major cause of human bacterial gastroenteritis worldwide and may be responsible for as many as 400–500 million cases annually (Friedman *et al*., 2000). The "U.S. Centers for Disease Control and Prevention" estimated that *C. jejuni* causes food-borne illness per year in about 1% of the entire population in the United States (Hu *et al*., 2008). Disease outcome varies from mild, non-inflammatory, self-limiting diarrhea to severe, inflammatory, bloody diarrhea lasting for several weeks, but is also associated with the development of the reactive arthritis and peripheral neuropathies, the Miller–Fisher and Guillain–Barrè syndromes (Wassenaar and Blaser, 1999; Young *et al*., 2007; Poly and Guerry, 2008; Blaser and Engberg, 2008). The availability of numerous complete genome sequences of different *C. jejuni* strains has unravelled an organism that exhibits a large degree of strain to strain variation. This natural heterogeneity has made studying the pathogenicity mechanisms of this pathogen particularly challenging. However, significant progress has been made in recent years in contributing to our understanding of the role of several key factors including the cytolethal distending toxin CDT (Lara-Tejero and Galan, 2000; Ge *et al*., 2008) as well as glycosylation and molecular mimicry processes (Guerry and Szymanski, 2008; Nothaft and Szymanski, 2010). However, one of the key differences between infection of humans and chickens by *C. jejuni* and other species is the apparently increased number of bacteria invading epithelial cells in the human host (Young *et al*., 2007; Nachamkin *et al*., 2008). This suggests that both bacterial adherence to and invasion into intestinal epithelial cells as well as breaching the epithelial barrier in the gut system may be critical virulence mechansims that are essential for disease development. Genome analyses revealed a notable absence of numerous classical pathogenicity factors in *C. jejuni*, making predictions very difficult. Since a suitable animal model system mimicking human infection is also not available, a wide variety of *in vitro* cell culture models have been applied to identify the *C. jejuni* factors that play a role in adherence and invasion (Table 1). Unfortunately, the use of different *C. jejuni* strains and various cell models of infection led to substantial confusion and controversies in the literature. Thus, the identification of novel bacterial and host factors as well as signalling pathways and other mechanisms involved in infection processes is a

pressing issue and the key for better understanding of the pathogenesis and developing therapeutics to treat corresponding infections in future.

6.1 Role of the HtrA protease in *C. jejuni* transmigration across polarised epithelial cells

The intestinal mucosa in the human gut forms a tight barrier which protects against invasion of the host by commensal non-pathogenic microbes residing in the intestinal lumen. Some enteric pathogenic bacteria, such as *Salmonella*, *Shigella*, or *Listeria*, have specific tissue-invading properties and can physically breach the intestinal mucosal barrier (Salyers and Whitt, 1994; Cossart and Sansonetti, 2004; Rottner *et al*., 2005; Backert and Koenig, 2005). In general, bacterial pathogens can translocate via a paracellular route (migration from the apical to the basolateral cell surface by passage between neighbouring cells) or a transcellular route (migration from the apical to the basolateral cell surface by host cell uptake, followed by intracellular trafficking). Pathogen-induced passage of the human intestinal mucosa may involve crossing of absorptive enterocytes or passage through the specialized microfold cells (so-called M-cells) in the gut epithelium. In addition to transcellular entry, some other bacterial pathogens can disrupt the tight junctions between enterocytes and cross *via* the paracellular mechanism (Cossart and Sansonetti, 2004; Rottner *et al*., 2005; van Alphen *et al*., 2008). A well studied example is *Salmonella enterica* serovar Typhimurium which can also cross the intestinal barrier. All known *Salmonella* are highly invasive, facultative intracellular pathogens that preferentially enter the M cells overlaying small intestinal Peyer's patches, although they can also enter and pass through epithelial cells of the intestinal tract *in vivo* and in cultured polarized epithelial cells *in vitro* (Gerlach and Hensel, 2007; Stecher and Hardt, 2008; Tsolis *et al*., 2008). In addition, *Salmonella* can penetrate the intestinal epithelial barrier by uptake into dendritic cells (DCs) that protrude into the intestinal lumen (Niess *et al*., 2005). Once the *Salmonella* have crossed the epithelium, they are present either inside the DCs or are quickly taken up by those cells or macrophages within the lamina propria. Once internalized, macrophages then transport the bacteria from the gastrointestinal tract to the bloodstream, ultimately leading to a systemic infection in the human body (Cossart and Sansonetti, 2004; Gerlach and Hensel, 2007; Tsolis *et al*., 2008). *Shigella flexneri* may also breach the intestinal barrier by transcytosis across M cells (Cossart and Sansonetti, 2004; Rottner *et al*., 2005). On the other hand, very little is known about *C. jejuni*. It has previously been reported to undergo transcellular translocation across polarised mucosa; but there have also been reports on the

paracellular translocation of this pathogen (Konkel *et al*., 1992a; Everest *et al*., 1992; Harvey *et al*., 1999; Monteville and Konkel, 2002; Hu *et al*., 2008; Rees *et al*., 2008; Kalischuk *et al*., 2009). The reason for these discrepancies is not fully clear, but is probably due to the lack of bacterial factors involved in transmigration and different methodologies, as will be discussed below and compared with the data obtained in the present study.

Cultured mammalian cells are commonly used as a simple model system in order to investigate the interactions between a given bacterial pathogen and the host epithelial cell, and this system is more easily controlled as compared to infection studies in whole animals. The MKN-28, Caco-2, HT29, and T84 human cell lines exhibit the ability to form polarized cell monolayers when grown under appropriate conditions, thereby affording a model to assess the ability of bacteria to translocate across an intact epithelial cell barrier (Monteville and Konkel, 2002; Wroblewski *et al*., 2009). Polarized cells are characterized by defined apical and basolateral cell surfaces separated by tight junctions (Fig. 26A), which limit the passage of solutes through the paracellular spaces (Balkovetz and Katz, 2003). Transepithelial electrical resistance (TER) is very often used as an index of tight junction permeability and cell monolayer integrity. Disruption of the intercellular tight junctions by certain damage or infections can result in a decrease in TER. Previous work has revealed that *C. jejuni* can translocate Caco-2 and other polarized cell monolayers without a concomitant loss in TER (Konkel *et al*., 1992a; Everest *et al*., 1992; Harvey *et al*., 1999; Bras and Ketley, 1999), indicating that *C. jejuni* can translocate across a given polarised cell monolayer whose integrity, however, remains intact. In contrast, other research groups reported on a time-dependent decrease of TER caused by *C. jejuni* infection while the bacterial factor(s) triggering a reduction in TER were not addressed (Chen *et al*., 2006; Wine *et al*., 2008; Pogacar *et al*., 2010). Thus, there are some conflicting data in the literature and a consensus is yet to be reached among investigators as to the mechanism of translocation.

One major aim of the present work was therefore to address some of the above questions directly. For example, we asked if the serine protease HtrA in *C. jejuni* could play a role in targeting host proteins and could be involved in bacterial crossing the polarised epithelial cell layer? The idea for this project came when the groups of Prof. S. Wessler (University Salzburg) and Prof. S. Backert (University Magdeburg and University College Dublin) discovered that HtrA from the related bacterial pathogen *Helicobacter pylori* is actively secreted into the culture supernatant (Hoy *et al.*, 2010). This was a very surprising finding at that time because it is well known that HtrA is a chaperone and protease in the periplasm of

many bacteria including *E. coli*, playing crucial roles in protein quality control, protein folding, and preventing host induced protein-denaturation and bacterial unfolded protein responses (Clausen *et al*., 2011). Moreover, Hoy and co-workers could show that *H. pylori* HtrA cleaves the ectodomain of the cell adhesion protein and tumor suppressor E-cadherin and in this way *H. pylori* disrupted the intercellular barrier between polarised MKN-28 host cells, allowing *H. pylori* to access the intercellular space and basolateral surfaces (Hoy *et al*., 2010). We therefore asked if the above new findings may represent a common phenomenon in bacterial pathogenesis including that of *C. jejuni* ?

Novel data presented in this work show that HtrA from *C. jejuni* can be indeed secreted into the cell culture supernatant during bacterial growth in BHI medium or during infection of MKN-28 cells. In addition, it was shown that *C. jejuni* can cross polarised MKN-28 monolayers very rapidly. The first viable transmigrated wild-type *C. jejuni* CFU were detected after 15-30 min (Fig. 11 and data not shown). In contrast, invasion of different types of host cells was observed at much later time points and was obvious between 4-6 hours of infection (Figs. 13, 17). These facts alone already indicate that transmigration of *C. jejuni* via the transcellular route would take much longer time until the first bacteria would reach basolateral surface as observed in the transwell assays. Instead, these findings strongly argue for the paracellular route mainly used by these bacteria. Moreover, it was found that the generated isogenic *C. jejuni ΔhtrA* mutants exhibited a strongly reduced transmigration potential, indicating that HtrA indeed plays a role in this process. In addition, evidence was presented that recombinant HtrA from *C. jejuni* can cleave E-cadherin *in vitro*, and during infection *in vivo*. the NTF domain from E-cadherin, while it leaves the receptor molecule fibronectin uncleaved. Thus, cleavage of E-cadherin may be involved in *C. jejuni* transmigration. (Figs. 9, 10). The exact cleavage sites are yet unknown, but this is under investigation by other lab members in our groups. In addition, the total amount of cell-based E-cadherin dropped down during the course of infection, but did not lead to a complete cleavage even at late time points of infection (6 hrs). We propose that cleavage of E-cadherin by HtrA during infection is a temporary and locally restricted process, possibly achieved by surface-exposed and/or secreted HtrA proteins when the bacteria enter the intercellular space. Host cells continuously translate large amounts of E-cadherin proteins, and therefore cleaved proteins could be rapidly replaced by the host cell machinery. This hypothesis could also explain why no significant reduction in TER was observed during infection with *C. jejuni*.

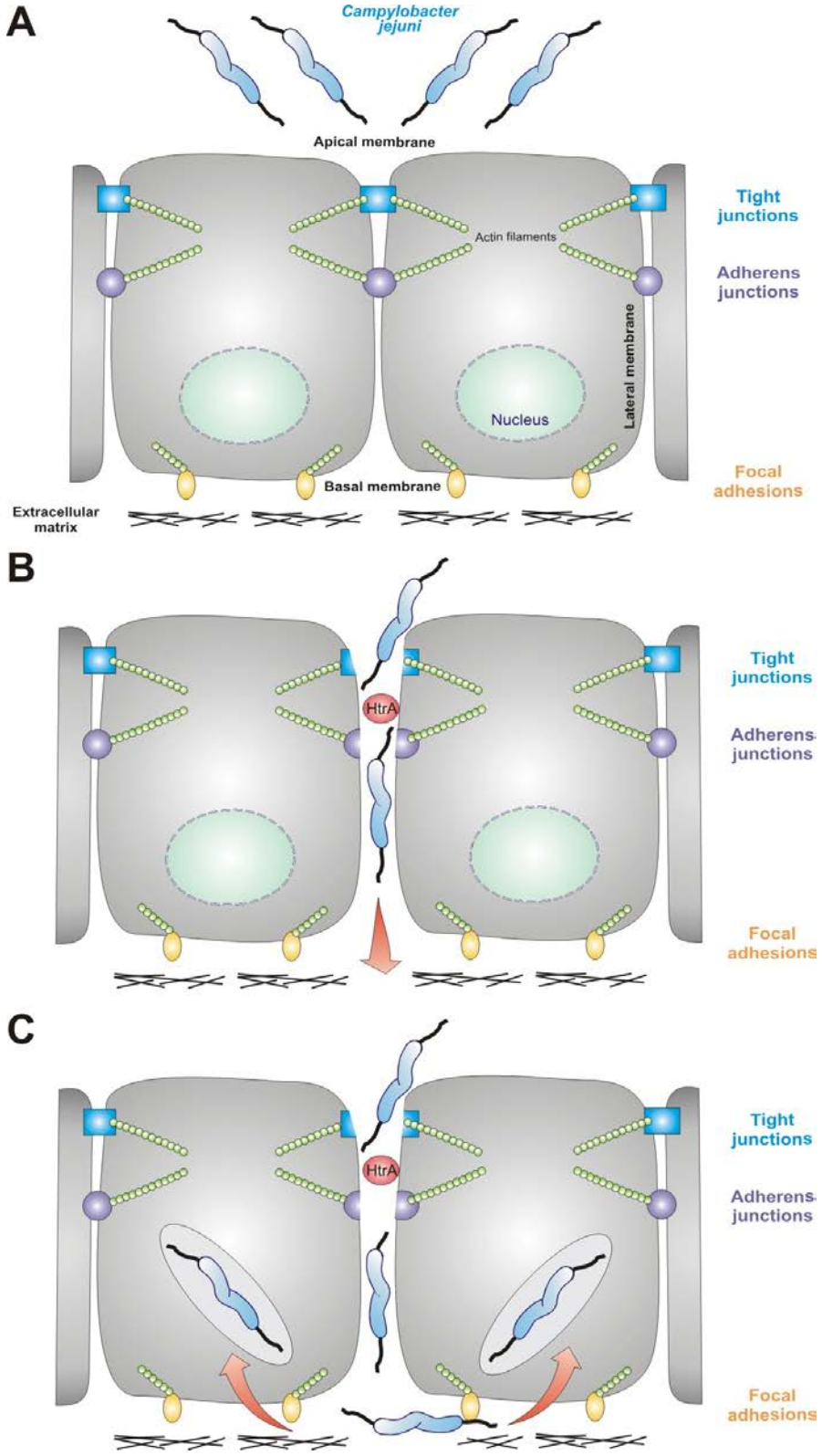


Figure 26: Proposed model of epithelial intercellular junctions and interactions with *C. jejuni.* (A) Schematic presentation of a polarized cell layer in epithelium. Different types of cell junctions are shown, including the tight junctions (TJs, blue), adherens junctions (AJs, violet) and focal adhesions (FAs, orange) before *C. jejuni* entry the host cell. (B) *Campylobacter jejuni* secretes the HtrA protease into the extracellular space where it can cleave the junctional protein E-cadherin, allowing *C. jejuni* to enter the cell monolayer by the paracellular route. (C) Once *C. jejuni* reaches basolateral surfaces, it can bind to the fibronectin/integrin host cell receptor complex and activate different signalling cascades which result in *C. jejuni* engulfment and uptake into the cells.

Although significant progress with respect to identification of the first *C. jejuni* factor and the first host cell target involved in transmigration of the pathogen has been clearly identified in this work, a couple of questions were not yet addressed and need to be answered in future studies. First, it is not yet clear how HtrA, both from *C. jejuni* and *H. pylori,* is secreted into the extracellular space. HtrA from both species have a conserved signal peptide at the amino-terminus (Fig. 3), which is used for its transport to the periplasm, explaining the commonly known periplasmic nature of HtrA. This explains how the protein can pass the inner bacterial membrane by a classical Sec-dependent transport pathway, but does not give a hint how the protein can pass the outer membrane of the Gram-negative bacterium. However, very recent data from our group and Swedish collaborators on the composition of shedded outer membrane vesicles (OMVs) from *H. pylori* showed that HtrA is predominantly present in these extracellular compartments (Olofsson *et al*., 2010). HtrA from *Chlamydia trachomatis* may also be present in OMVs as shown recently (Wu *et al*., 2011). Thus, this secretion route can certainly represent one possible mechanism how HtrA can be transported outside of the bacteria. However, the process of bacterial OMV generation in cell culture takes several hours (Olofsson *et al*., 2010), which does not explain the rapid transmigration of *C. jejuni* in the transwell assays. Therefore, we propose that another more rapid secretion mechanism must exist in these bacteria, which should be investigated in future studies.

Second, it is not yet clear how *C. jejuni* passes the tight junctions between polarised cells, which are located above the E-cadherin-based adherens junctions (Fig. 26A). An interesting hypothesis is that HtrA might also cleave other host cell surface proteins possibly including tight junction proteins. If this is the case is not yet clear. In this context, however, it should be also noted that I have identified two other *C. jejuni* protein candidates (at 63 kDa and 42 kDa) with proteolytic activity in casein assays (Fig. 6). The identity of these proteins should be identified and functional studies must be performed. Third, it would be also highly interesting to investigate if *C. jejuni* HtrA can cleave chicken E-cadherin, because the current work is restricted to human E-cadherin. Human and chicken E-cadherins differ by certain polymorphisms, and when chicken E-cadherin cannot be cleaved by HtrA this could explain why *C. jejuni* is not invasive in avian species and lives there as a commensal. Thus, the present work also provides a series of new ideas for future experiments.

6.2 The small Rho GTPases Rac1 and Cdc42 play a crucial role in *C. jejuni* host cell invasion

After having established that *C. jejuni* can mainly cross the epithelial barrier via the paracellular route between neighbouring cells, with secreted HtrA cleaving E-cadherin playing a role (Fig. 26), the next aim was to investigate how this pathogen can enter the host target cells directly. Host cell invasion is an important process during infection of many pathogenic bacterial species and involves numerous steps such as bacterial binding at specific receptor sites, signalling to the host cell, re-programming of intracellular host signal transduction pathways, membrane and cytoskeletal rearrangements, and eventual engulfment of the bacterium (Cossart and Sansonetti, 2004; Rottner *et al*., 2005; Backert and Koenig, 2005). The latter processes commonly involve the activity of one or more small Rho GTPases. Rho GTPase family members, including its major members Rac1, Cdc42 and RhoA, are small GTP-binding proteins that serve as guanine nucleotide-regulated switches which transmit external stimuli to modulate different cellular functions (Tran Van Nhieu *et al*., 1999; Cossart and Sansonetti, 2004; Schmidt and Hall, 2002; Rottner *et al.,* 2005; Tomar and Schlaepfer, 2009). Importantly, host cell entry of *C. jejuni* is considered as one of the primary reasons for bacterial-caused tissue damage of this pathogen, however, the molecular mechanism(s) of *C. jejuni* invasion is/are widely unclear. The group of Prof. S.Backert has previously shown that *C. jejuni* invasion of INT-407 cells is accompanied by time-dependent activation of small Rho GTPases Rac1 and Cdc42 (Krause-Gruszczynska *et al*., 2007b), but the specific signalling cascades remained to be investigated. The second main aim of this PhD thesis was therefore to continue this work and pinpoint more signalling factors in these Rho GTPase activation pathways. Combined with the data of the PhD thesis of M. Krause-Gruszczynska (2008), the present work leads to the completion of two independent GTPase cascades which are embedded in an overall model of *C. jejuni*-induced disease-associated signalling pathways as presented in Figure 27.

Our previous work using specific GTPase-modifying toxins, inhibitors and GTPase expression constructs showed that Rac1 and Cdc42 activity is clearly involved in *C. jejuni* invasion (Krause-Gruszczynska *et al*., 2007b). In this work, I started with confirming that infection with *C. jejuni* induces the activation of Cdc42-GTP and Rac1-GTP levels using a novel ELISA-based procedure, called G-Lisa. The obtained results were in good agreement with the previously published time-dependent activation of both small Rho GTPase members (Krause-Gruszczynska *et al*., 2007b). Further experiments using the lipid raft-disrupting

compound methyl-beta cyclodextrin (MβCD), sequestering cholesterol, indicated that lipid rafts and possibly one or more lipid raft-associated host receptor(s) are involved both in bacterial invasion and GTPase activation. Using knockout cell lines of different host receptors (fibronectin $^{-/-}$, GD25 integrin-β1$^{-/-}$) and kinases (FAK$^{-/-}$), siRNA transfection, G-Lisa, CRIB pulldowns, gentamicin protection assays and electron microscopy I was able to demonstrate that *C. jejuni* exploits two pathways leading to Rac1 or Cdc42 activation. The first identified pathway involves a fibronectin→integrin-β1→FAK→DOCK180/Tiam1 signalling cascade, which is important for triggering Rac1 GTPase activity followed by bacterial entry of host target cells. Using the same knockout cells lines of several host receptors and kinases and additionally the SYF knockout cell line and similar methodology, it could be shown that *C. jejuni* exploits a fibronectin→integrin-β1→FAK/Src→EGFR/PDGFR→PI3-kinase→Vav2 signalling pathway, which is crucial for triggering Cdc42 GTPase activation and invasion.

Most of the recent studies in the literature investigating *C. jejuni* invasion processes utilized pharmacological inhibitors or dominant-negative constructs which may have side effects, and thus only provide very limited clarity on host factors playing crucial roles in the bacterial entry process. In this work, I was using a series of knockout cell lines for infection assays. These cell lines have the important advantage over other cell systems that the respective genes of interest were completely deleted in the chromosomes. Thus, not even small traces of protein are expressed, allowing clear answers if certain genes are involved in a given response or not. In addition, *C. jejuni* has two reported fibronectin-binding proteins, CadF and FlpA (Moser *et al*., 1997; Konkel *et al*., 2010; Euker and Konkel, 2011), and maximal host cell entry of *C. jejuni* shown here was widely dependent on the expression of fibronectin, integrin-β1 and FAK. Since integrin-β1 is the natural receptor of fibronectin (Hauck *et al*., 2002; Hsia *et al*., 2003), the data of this work suggest a cascade of signalling events in a fibronectin→integrin-β1→FAK dependent fashion. In addition, it was found that Rac1-GTP and Cdc42-GTP levels induced by *C. jejuni* were significantly elevated in infected FAK-expressing but not in FAK-deficient cells, and GTPase activation was confirmed conclusively by two independent approaches, GST-CRIB pulldown and G-Lisa. In line with these observations, high-resolution electron microscopy (FESEM) revealed membrane dynamics, ruffling, filopodia formation and tight engulfment of *C. jejuni* upon infection with wild-type control cells, but not in any of the infected knockout cell lines. These findings provided further clear evidence that fibronectin, integrin-β1 and FAK are major host factors playing not only a role in Rac1 and Cdc42 activation but also in host cell entry of the *C. jejuni*.

Interestingly, our electron microscopic observations are very similar to that reported for *Staphylococcus aureus*, a Gram-positive pathogen also expressing various fibronectin-binding proteins, because infected fibronectin$^{-/-}$ or FAK$^{-/-}$ cells were also severely impaired in their ability to internalize the latter bacteria (Agerer *et al*., 2005; Schroeder *et al*., 2006). Furthermore, integrin-mediated uptake of *S. aureus* depends on the integrity of membrane-associated lipid raft microdomains (Hoffmann *et al*., 2010), and this is in line with the finding that MβCD-treatment blocked internalization of *C. jejuni*.

By engaging fibronectin and integrin-β1, *C. jejuni* seems to exploit the ability of this receptor complex to dynamically associate with the intracellular cytoskeleton and to generate the necessary pulling forces to promote bacterial uptake by the host cell. In non-infected healthy tissues, integrin-β1-containing fibrillar cell adhesions are important for the organisation of the extracellular matrix, as they co-align with fibronectin fibrils, and genetic elimination of integrin-β1 in GD25 cells results in defects in the assembly of a fibrillar meshwork of extracellular fibronectin (Danen *et al*., 2001; Wennerberg *et al*., 1996, Leiss *et al*., 2008). Cellular pulling forces generated via an integrin-β1-mediated linkage to the actin-myosin network seem to be critical for fibronectin fibril formation, as force triggered conformational changes are essential to expose cryptic oligomerisation motifs within the fibronectin molecules (Sechler *et al*., 2001). Importantly, FAK has been shown to play a key role in the formation of a fibrillar fibronectin extracellular matrix. Cultured FAK$^{-/-}$ cells *in vitro*, as well as FAK$^{-/-}$ mouse embryos *in vivo*, fail to properly assemble fibronectin fibrils (Ilic *et al*., 2004; Leiss *et al*., 2008). In accordance with the fact that FAK$^{-/-}$ cells are unable to properly organise the extracellular fibronectin matrix, it could be found that these cells are deficient in the ability to internalise *C. jejuni*, suggesting that fibronectin/integrin-linkages to the actin myosin network are disrupted and pulling forces are not provided. As one would expect from these results, *C. jejuni* profoundly stimulated FAK phosphorylation linked to its kinase activity during infection (Fig. 20). The activation of FAK has also been described for other pathogens targeting integrins for bacterial invasion or other purposes, including *S. aureus* (Agerer *et al*., 2005), *Yersinia pseudotuberculosis* (Alrutz and Isberg, 1998; Eitel *et al*., 2005) and *Helicobacter pylori* (Kwok *et al*., 2007; Tegtmeyer *et al*., 2010), thus FAK seems a favourite target in bacterial pathogenesis and is not restricted to *C. jejuni* invasion.

The observation that FAK expression and activation is required for both *C. jejuni*-triggered Rac1 and Cdc42 activity and invasion, led us to investigate the involved downstream

signalling in more detail. A series of well-described GEFs downstream of FAK are DOCK180, Tiam1 and Vav2 (Hauck *et al.*, 2002; Hsia *et al.*, 2003). Using siRNA knockdown assays, the importance of GEFs including Tiam-1, DOCK180 or Vav2, was investigated for the production of Rac1-GTP and Cdc42-GTP levels in infected cells. Importantly, it was found that while siRNAs against DOCK180 or Tiam1 exhibited diverse downregulatory effects but no complete elimination of Rac1-GTP when applied separately, the double knockdown of both GEFs eliminated Rac1-GTP almost completely. Thus, DOCK180 and Tiam1 seem to act cooperatively to activate Rac1. Furthermore, Vav2 (but not Tiam-1 or DOCK180) was required for *C. jejuni*-induced Cdc42 activation as verified by siRNA knockdown. Thus, there are two separate signalling pathways downstream of FAK leading to activation of Rac1 and Cdc42.

The observation that FAK expression and activation is required for *C. jejuni*-triggered Rac1 activity and invasion represents a novel signalling pathway. In fact, *C. jejuni* induced the phosphorylation of FAK at Y-397 and Y-925, and expression of FAK point mutants including Y397F, K454R, Y925F or ΔPR1/2 (two proline-rich domains, required for protein-protein interactions) in $FAK^{-/-}$ cells did not restore bacterial uptake as compared to wt FAK (this work and PhD thesis of M. Krause-Gruszczynska, 2008). A well-described GEF downstream of this FAK signalling is DOCK180 (Hauck *et al.*, 2002; Hsia *et al.*, 2003). The signalling cascade involves p130Cas, an adapter molecule binding to proline-rich residues in the carboxyl-terminal domain of FAK. p130Cas then associates with the adapter protein Crk and this complex activates DOCK180. In addition, it has been shown that expression of phospho-mimetic FAK-Y925E enhanced cell protrusions together with activation of the same DOCK180-dependent Rac1 signalling pathway, thus, phosphorylation of FAK at Y-925 is also involved this scenario (Deramaudt *et al.*, 2011). These observations are in well agreement with our findings, suggesting that *C. jejuni* activates a classical FAK→p130CAS→Crk→DOCK180→Rac1 signalling pathway. However, as mentioned above, siRNA knockdown of DOCK180 expression was not sufficient to completely eliminate Rac1 activity and bacterial invasion. The other GEF required for *C. jejuni*-induced Rac1 activation was Tiam-1, but the molecular mechanism how FAK can target Tiam-1 is not yet clear. Interestingly, syndecan-2-mediated cell migration was diminished when cells were transfected with non-phosphorylatable FAK Y397F mutant or siRNA against Tiam-1, suggesting that a FAK→Tiam-1→Rac1 signalling pathway is activated (Park *et al.*, 2005). If

FAK can stimulate Tiam-1 directly or via another factor during infection with *C. jejuni*, however, needs to be investigated in future studies.

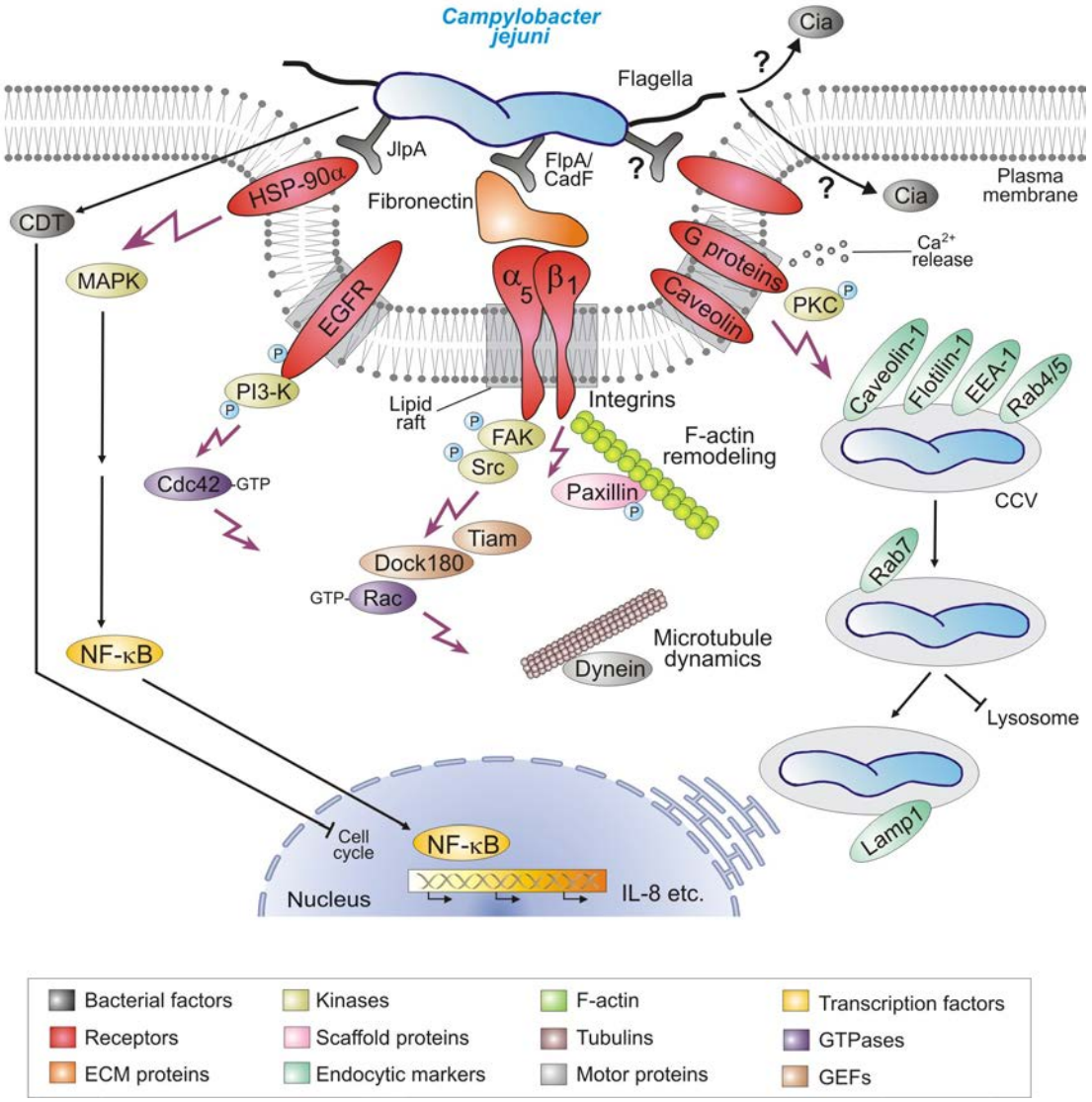


Figure 27: Hypothetical model for *C. jejuni*-induced signalling events leading to bacterial invasion and establishing infections. *C. jejuni* adheres to host cells via numerous reported and unknown factors. Several indicated host cell receptors have been proposed to play a role in the uptake of the bacteria, including two pathways via fibronectin, integrin and FAK leading to the activation of GTPases Rac1 and Cdc42, respectively. This potentially causes localized F-actin and/or microtubule rearrangements at the site of *C. jejuni* entry, resulting in engulfment and bacterial uptake. Several other indicated host cell signalling pathways such as Caveolin-dependent entry and intracellular survival in *Campylobacter*-containing vacuoles (CCVs) have been reported in *in vitro* infection models and may play a role during pathogenesis *in vivo*. Numerous other bacterial and host factors shown here are described in Tables 1 and 2. For more details, see text.

The above siRNA data indicate that Vav2 was required for *C. jejuni*-induced Cdc42 activation. The importance of Vav2 was then confirmed by the expression of dominant-negative plasmid constructs and the use of Vav1/2 knockout cells in infection assays (Fig. 23). Bacterial adhesion was also reduced in infected Vav1/2 knockout cells, which can be explained by reduced GTPase activation as compared to wild-type cells. This is in agreement with reports showing that Vav2 is also involved in the uptake of other pathogens including *Yersinia* and *Chlamydia* (Lane *et al*., 2008; McGee *et al*., 2003). Moreover, in our previous studies the expression of various point mutations in Vav2 clearly linked this signalling directly to growth factor receptors and PI3-kinase (PhD thesis of M. Krause-Gruszczynska, 2008). The application of selective inhibitors during *C. jejuni* infection showed then that the kinase activities of EGFR, PDGFR and PI3-kinase are also required for Cdc42 activation. This was also confirmed by the expression of dominant-negative versions of EGFR or PDGFR, which exhibited suppressive effects on *C. jejuni* uptake (PhD thesis of M. Krause-Gruszczynska, 2008). Extensive research on the regulation of growth factor receptor activation and signalling by integrin-mediated cell adhesion indicates that these two classes of receptors work cooperatively. Several studies showed that integrin ligation allows for the maximal activation of EGFR or PDGFR, thereby producing robust intracellular signals including small Rho GTPase activation (Cadobi *et al*., 2004; Alexi *et al*., 2011). These observations are in well agreement with our findings, suggesting that *C. jejuni* activates, *via* fibronectin and integrins, a FAK/Src→EGFR/PDGFR→PI3-kinase→Vav2→Cdc42 signalling pathway (Fig. 27). However, transfection with both dominant-negative PDGFR and dominant-negative EGFR constructs resulted in no additive reduction of *C. jejuni* invasion (PhD thesis of M. Krause-Gruszczynska, 2008). These latter finding suggests that besides EGFR and PDGFR other signalling pathway(s) are also implicated in *C. jejuni* internalization.

Previous studies in Prof. Backert's group indicated that *C. jejuni* pathogenicity factors such as cytolethal distending toxin CDT, plasmid pVir, the adhesin PEB1 or certain capsular genes are not required for *C. jejuni*-induced Rac1 and Cdc42 activation (Krause-Gruszczynska *et al*., 2007b). In the present work, I found that an isogenic Δ*cadF* mutant less efficiently induced activation of Rac1-GTP and Cdc42-GTP as compared to wild-type *C. jejuni*, suggesting that the fibronectin-binding protein CadF, probably in concert with another proposed novel fibronectin-binding protein FlpA (Eucker and Konkel, 2011), could be involved in GTPase activation. It therefore appears that CadF does not only act as a canonical adhesin for bacterial attachment to fibronectin, but could also stimulate integrins as well as

FAK, EGFR and PDGFR kinase activity, which subsequently may activate Vav2 and Cdc42, important for maximal *C. jejuni* invasion. Since Δ*flaA/B* or Δ*flhA* knockout mutants lacking the flagella induced only very little Rac1-GTP and Cdc42-GTP levels, another *C. jejuni* determinant playing a role in Rac1 and Cdc42 activation is the flagellar apparatus. The flagellum appears to be a major colonization determinant of *Campylobacter*, shown to be essential for successful infection of several animal models (Morooka *et al*., 1985; Wassenaar *et al*., 1993; Hendrixson and DiRita, 2004). In addition, FlaA/B proteins play a profound role in *C. jejuni* invasion of epithelial cells (Poly and Guerry, 2008; Wassenaar *et al*., 1991; Grant *et al*., 1993; Yao *et al*., 1994). However, the possible impact of flagellar proteins in host cell entry is controverse in the literature. One hypothesis is that the flagella, like their evolutionary related T3SS counterparts, can secrete invasion-associated factors such as CiaB and others into the culture supernatant (Konkel *et al*., 1999b, 2001, 2004; Eucker and Konkel, 2011). The other hypothesis is that flagella-mediated bacterial motility is the driving force to permit host cell entry, but deletion of *ciaB* has no impact (Novik *et al*., 2010). Thus, it is still not clear if the flagellum, unlike its well-known function in bacterial motility, may transport bacterial effectors into the medium or into the host cell. Alternatively, the flagellum itself may target a host cell receptor directly to trigger signalling involved in invasion (Fig. 27), which should be investigated in future studies.

6.3 *C. jejuni* host cell invasion by the "zipper" or the "trigger" mechanism ?

There are two general strategies by which the multitude of enteric bacterial pathogens can enter host target cells. According to specific characteristics of the invasion process, we can distinguish between the classical "zipper"- and "trigger"-mechanisms, respectively (Cossart and Sansonetti, 2004). The "zipper"-mechanism is initiated by one or more bacterial surface proteins (commonly comprising adhesins and invasins) which bind to one or more specific host cell receptors followed by internalization, as reported for *Staphylococcus, Yersinia* or *Listeria* species (Fig. 28A). On the other hand, the "trigger"-mechanism involves T3SSs and T4SSs injecting bacterial proteins which often mimic or hijack specific host cell factors to trigger the bacterial uptake process, as described for *Salmonella, Bartonella* and *Shigella* (Fig. 28B).

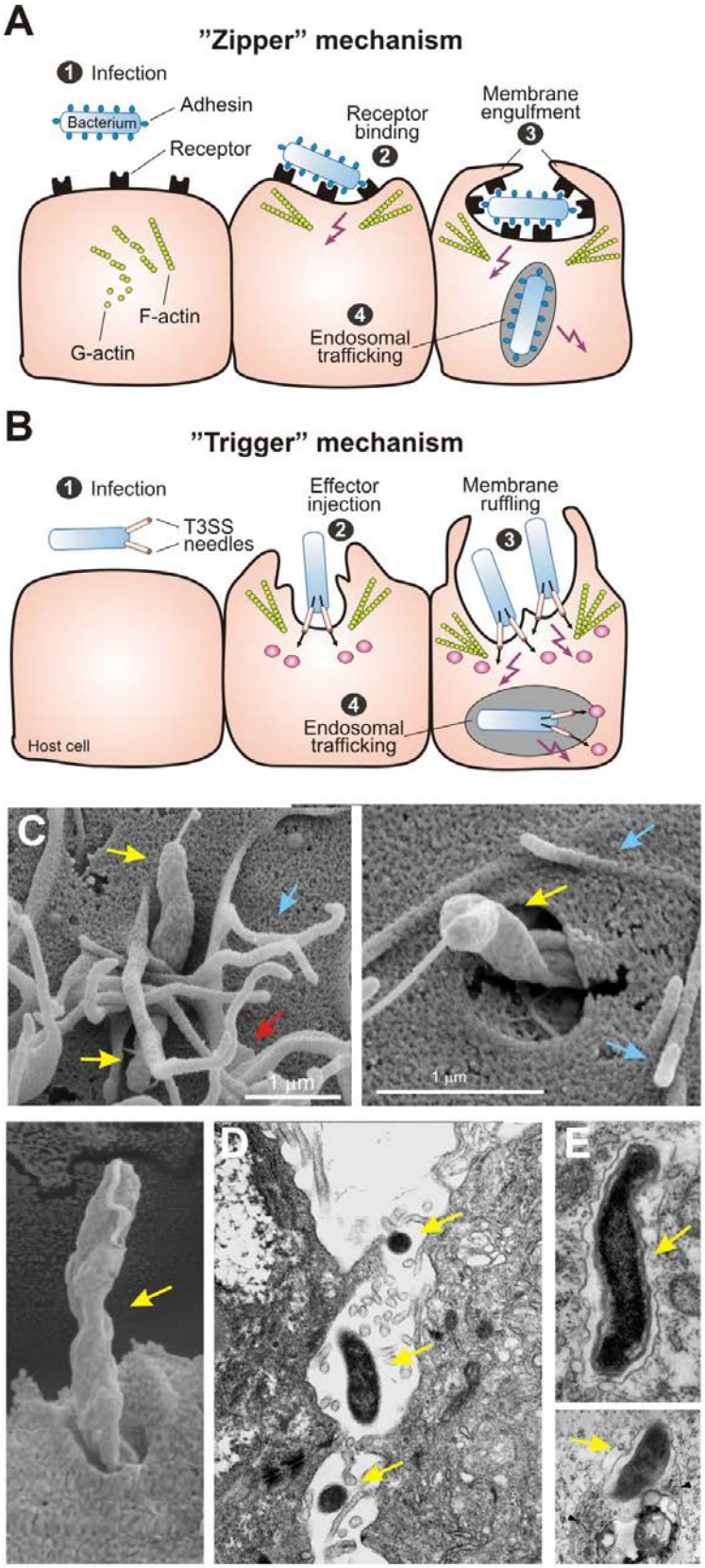


Figure 28: Primary mechanisms of bacterial invasion into non-phagocytic host epithelial cells. Schematic representation of the two different routes of entry by intracellular bacterial pathogens. The pathogens induce their own uptake into target cells by subversion of host cell signalling pathways using the "zipper" (A) and "trigger" (B) invasion mechanism, respectively, as described in Figure 1. (C) High resolution field emission scanning electron microscopy showing examples of invading *C.*

jejuni. *Campylobacter jejuni* 81- 176 infected for 4-6 hours were able to induce their entry into the wild-type fibroblast target cells and were regularly associated with membrane ruffles (red arrows) and filopodia structures (blue arrows). Invading bacteria were also marked (yellow arrows). (D) Electron micrograph of *C. jejuni* entering the intercellular space between two neighbouring epithelial cells. This picture was kindly provided by M. Konkel (Pullman University, USA). (E) Electron micrographs of *C. jejuni*–containing vacuoles (CCVs) that do not co-localize with BSA-gold (top) and CCVs that co-localize with BSA-Gold and resemble lysosomes (bottom, arrows) are shown. The two pictures were kindly provided by J. Galan (Yale University, USA).

It is well known that different pathogens such as *Salmonella* or *Shigella* inject bacterial GEFs directly into the host cells by a T3SS and induce intensive membrane ruffling by a "trigger" mechanism resulting in the uptake of the bacteria (Cossart and Sansonetti, 2004). *Campylobacter jejuni* does not encode a classical T3SS and T4SS (Konkel *et al*., 1999b; Hofreuter *et al*., 2006) nor bacterial GEFs as known from *Salmonella* or *Shigella*. In agreement with this observation, this work describes a set of three host cell GEFs playing an important role in activating Rac1 and Cdc42 GTPases involved in invasion. The model of Cia protein secretion through the flagellum (Euker and Konkel, 2011) is very tempting and would support the idea that *Campylobacter* uses a "trigger mechanism" of invasion involving the secretion of effector proteins directly into the cell to induce their uptake. Some electron microscopic evidence exists that would support this model (Fig. 28C), but it should be noted again that a recent study has suggested that CiaB plays a minimal or no role in invasion (Novik *et al*., 2010). Thus, much more work is required to confirm the role of the flagellum as a secretion system for effector proteins involved in invasion. On the other hand, the underlined importance of CadF and the fibronectin/integrin receptor complex as shown in this work might also give support to a "zipper"-like mechanism of invasion as used by *Listeria* or *Yersinia* species. Some electron microscopic pictures presented here may also support the latter model.

In conclusion, it is very difficult at present to conclusively state how *C. jejuni* facilitates its uptake into host epithelial cells. There is evidence presented in this work and specifically electron microscopic images which give support for both the "zipper" and "trigger" mechanisms of invasion, underlining the concept that *C. jejuni* enters epithelial cells by a unique mechanism. It may be that *C. jejuni* has developed during evolution a strategy which shares features of both of these mechanisms, but more work is clearly required to pinpoint the crosstalk of certain pathways used by this important pathogen to enter and survive in intestinal epithelial cells. At the moment, we favour a model where two major receptor-

involved pathways give rise to *C. jejuni* invasion, the fibronectin/integrin cascade leading to Rac1 or Cdc42 activation (this work), and possibly another entry route via caveolae structures as reported by another group (Watson and Galan, 2008) (Fig. 27). Future studies should investigate how this occurs exactly and if certain *C. jejuni* strains favour one or the other pathway to trigger its uptake. Although I included several strains in my studies, including the *C. jejuni* model strains 81-176, RM1221 and F38011, more strains from different origins should be investigated. It should be also studied what are the triggered pulling forces by the host cell that mediate engulfment, uptake and also membrane closure behind the entering bacteria. It will be also important to investigate the intracellular survival concept and spread of the bacteria in more detail as well as how they can infect even other organs such as the liver. It therefore appears that the major foodborne pathogen *C. jejuni* will continue to be a fascinating and rewarding research subject in the future.

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ZUSAMMENFASSUNG

Campylobacter jejuni ist ein Gram-negatives Bakterium der Gattung *Campylobacter*. Die Infektion mit diesem Pathogen ist eine der wichtigsten und häufigsten Ursachen für lebensmittelbedingte Durchfallerkrankungen und bestimmte Neuropathien beim Menschen. Durch *C. jejuni* verursachte Gastroenterititiden treten in Industrieländern 2-7 mal häufiger auf als bei Infektionen mit *Salmonella* and *Shigella*. Das Durchdringen der epithelialen Barrierefunktion von Wirtszellen im humanen Darmtrakt und die zelluläre Invasion von *C. jejuni* sind die wichtigsten Ursachen für Schäden im infizierten Gewebe, allerdings sind die molekularen Mechanismen und beteiligten Faktoren in diesen Prozessen noch nicht genau bekannt.

Im ersten Teil der vorliegenden Arbeit wurde gezeigt, das Wildtyp *C. jejuni* die Barrierefunktion von polarisierten MKN-28 Zellen im Transwell-System effizient überwindet. Ein bakterieller Faktor wurde identifiziert, die Serinprotease HtrA (*high-temperature requirement A*), welche hierbei eine entscheidende Rolle spielt. Es wurde gezeigt, dass *C. jejuni* HtrA aktiv in den Überstand des Mediums sekretiert, wo die Protease die Spaltung des bedeutenden „*adherens junction*“ Proteins und Tumorsuppressors E-Cadherin induziert. In *in vitro* Assays mit rekombinantem HtrA und in Infektionsexperimenten konnte gezeigt werden, dass HtrA zur Abspaltung der Ektodomäne von E-Cadherin in Wirtszellen führt. Außerdem konnte nachgewiesen werden, dass der Verlust des *htrA* Gens in *C. jejuni* zu einem Defekt in der Fähigkeit zur E-Cadherin Spaltung führt und damit auch zum weitgehenden Verlust der Fähigkeit der bakteriellen Transmigration in polarisierten MKN-28 Zellen. Diese Ergebnisse führten zu der Hypothese, dass die HtrA-vermittelte Abspaltung von E-Cadherin eine wichtige Rolle bei der Transmigration von *C. jejuni* über polarisierte Zellen mit Hilfe eines parazellulären Signalweges spielt.

Nachdem gezeigt werden konnte, dass *C. jejuni* zur Transmigration von polarisierten Zellen befähigt ist, sollte im zweiten Teil der Arbeit untersucht werden, wie *C. jejuni* in die Wirtszellen von der basolateralen Seite eindringt. Durch die Verwendung verschiedener molekularbiologischer Methoden und „*knockout*“ Zelllinien von Fibronektin, Integrin-β1 und fokaler Adhäsionskinase (FAK) aus defizienten Mäusen sowie den entsprechenden Wildtyp Zelllinien konnte gezeigt werden, dass diese Wirtszellfaktoren eine entscheidende Rolle für die Aktivierung von kleinen Rho GTPasen (Rac1 und Cdc42) und die bakterielle Invasion spielen. In Übereinstimmung mit diesen Befunden konnte weiterhin gezeigt werden, dass *C. jejuni* eine Kräuselung der Membranen (*ruffling*) und Filopodien-Bildung nur in den

infizierten Wildtyp Zellen verursacht. Außerdem konnte die durch *C. jejuni* aktivierte Autophosphorylierung von FAK und bestimmten Rezeptor-Tyrosinkinasen (EGFR und PDGRF) nachgewiesen werden. Diese Signalmoleküle stellen wiederum wichtige Schaltstellen für die Stimulierung von Guaninaustauschfaktoren dar, die hier als DOCK180, Tiam-1 und Vav2 identifiziert wurden. Durch siRNA Studien wurde anschliessend gezeigt, dass DOCK180 und Tiam-1 gemeinsam für die Aktivierung von Rac1 erforderlich sind, während Vav2 für die Aktivierung von Cdc42 verantwortlich ist. Weiterhin wurden Ergebnisse gewonnen, die zeigen, dass das Fibronektin-bindende Protein CadF und die Flagellen von *C. jejuni* in die Signaltransduktion zur Aktivierung von Rac1 und Cdc42 involviert sind. CadF ist somit ein bifunktionales Protein, welches nicht nur für die Bindung des Bakteriums an den Fibronektin/Integrin-Rezeptorkomplex der Wirtszelle verantwortlich ist, sondern trägt auch zur Aktivierung von *downstream* Faktoren und Invasion von *C. jejuni* in die Wirtszellen bei.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass *C. jejuni* fähig ist, durch einen besonderen Mechanismus die Barrierefunktion von Epithelzellen zu überwinden, wobei die Protease HtrA und E-Cadherin wichtige Signalfaktoren darstellen. Desweiteren wurde gezeigt, dass *C. jejuni* auch in die Wirtszellen eindringen kann und das Fibronektin, Integrin, FAK, verschiedene weitere Kinasen und die kleinen Rho GTPasen Rac1 und Cdc42 sowie bestimmte bakterielle Faktoren wie CadF und die Flagellen eine entscheidende Rolle im Invasionsprozess dieses bedeutenden Krankheitserregers spielen.

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Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zu dem Thema

selbstständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 19. Dezember 2011

Manja Böhm