FliD.

Exploring the flagellar tip protein as a target against *Campylobacter jejuni*

Claudia Freitag

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Front cover: *Campylobacter jejuni* attaching to the cell surface via its flagellum. The picture is a combination of microscopy and artistic addition of the author, the scale is therefore not realistic.
Back cover: Altered graphical representation of the FliD protein of *Salmonella enterica* serovar Typhimurium, reprinted with permission of Keiichi Nambam, Osaka University.

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FliD.

Exploring the flagellar tip protein as a target against *Campylobacter jejuni*

FliD.

Het bestuderen van het flagellum tip eiwit ter bestrijding van *Campylobacter jejuni*

(met een samenvatting in het Nederlands)

FliD.

Das Erkunden der Eigenschaften der Flagellenspitze zur Bekämpfung von *Campylobacter jejuni*

(mit Zusammenfassung in deutscher Sprache)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 24 november 2017 des ochtends te 10.30 uur

door

Claudia Marianne Freitag
geboren op 29 april 1984
te Berlijn, Duitsland

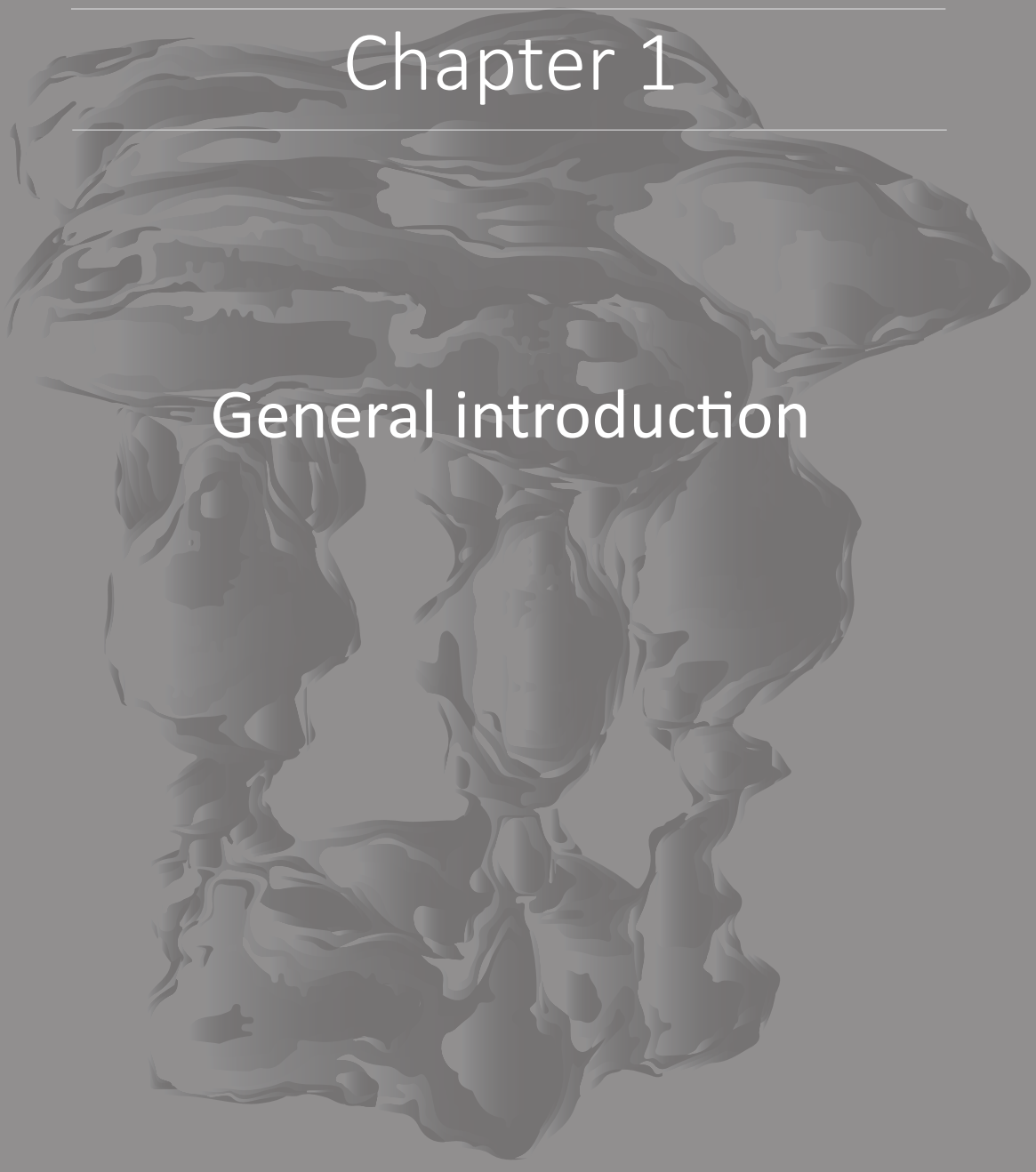
Promotor: Prof. Dr. J.P.M. van Putten

Für meine Großeltern:
Ruth & Friedrich
Ruth & Peter

Ick sitze hier und esse Klops,
Uff eenma kloppt's.
Ick kieke, staune, wundre mir,
Uff eenma jeht se uff, die Tür.
Nanu, denk ick, ick denk: nanu,
Jetzt isse uff, erst war se zu.
Ick jehe raus und kieke,
Und wer steht draußen? - Icke!

CONTENT

Chapter 1



General introduction

Campylobacter jejuni

Campylobacter jejuni (*C. jejuni*) is a Gram-negative bacterium belonging to the class of *Epsilonproteobacteria*. *C. jejuni* causes human diarrheal disease worldwide. After ingestion of contaminated meat products, the pathogen reaches its favorable niche in the intestine with help of its flagella and taxis systems. Subsequently, *C. jejuni* breaches the epithelial cell lining and induces intestinal inflammation. *C. jejuni* resides as a commensal in the chicken caeca. Therefore, measures as vaccination are under development to reduce the bacterial load in the animals before slaughter. However, in order to obtain a successful vaccine against *C. jejuni*, bacterial components critical for the interaction with the host should be identified.

Campylobacter infection in humans

Campylobacter represents the most frequently reported gastrointestinal bacterial pathogen in humans. Nowadays, the incidence of Campylobacteriosis is 2-3 times higher than infections with *Salmonella*[1]. Disease symptoms range from mild enterocolitis to watery or bloody diarrhea with fever, abdominal cramps and nausea. Additionally, there is a risk of post-infection sequelae, including peripheral neuropathies such as Guillain-Barré and Miller Fisher syndrome, and reactive arthritis[2]. Despite low hospitalization rates, infections with *Campylobacter* are often associated with high costs due to medical expenses and lost wages. The economical burden is estimated at several billions of Dollars in the US and millions of Euro in European countries per year[3].

Campylobacter disease represents an even greater health burden in developing countries where cases are underdiagnosed due to incomplete population-based surveillances[4]. Especially children suffer from *Campylobacter* infections. The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) collected stool samples from 8 sites (Bangladesh, India, Nepal, Pakistan, South Africa, Tanzania, Brazil and Peru) and found 85% of children *Campylobacter* positive within the first 12 months of life[5].

C. jejuni pathogenesis

Chicken meat represents the major source of human *C. jejuni* infection[1] and ingestion of a low number of bacteria is already sufficient to cause disease[6]. Symptoms appear after 2-5 days after the ingestion of *C. jejuni*. During this incubation time, the bacteria travel through the gastrointestinal tract, driven by chemotaxis and flagellar motility. After reaching the small bowel, *C. jejuni* efficiently penetrates the mucus layer and colonizes the crypts. Bacteria can be observed in strong association with and inside intestinal epithelial cells[7] but the detailed molecular interactions remain under investigation. At some point, the mucosal barrier is breached and bacteria can be found on the basolateral site of the epithelial monolayer[7]. Several mechanisms of translocation have been proposed, bacteria can translocate through the cells (transcellular), between the cells (paracellular) or enter via specialized sampling cells termed M-cells[8]. A newly discovered invasion pathway with subsequent cell invasion involves bacterial migration into the subcellular space in an actin- and microtubulin-independent manner[9].

Once the pathogen has crossed the epithelial cell barrier, immune cells are recruited and high amounts of leukocytes can be found in the faeces[6]. Important receptors involved in the interaction of bacteria with the immune cells are members of the Toll-like

receptor (TLR) and Nod-like receptor (NLR) families. They recognize conserved microbial molecular patterns, such as lipoproteins (TLR2 complex), LPS (TLR4, NLRP3), DNA (TLR9) and flagellin (TLR5, NLRC4, NAIP). The activation of most of these TLRs and NLRs results in the activation of the transcription factor NF-κB and the production of pro-inflammatory cytokines and chemokines, driving a strong immune response. Interestingly, *Campylobacter* manages to evade some of these innate immune receptors. For instance, the flagellin of *C. jejuni* lacks the TLR5 binding sites and is therefore not recognized by human (and chicken) TLR5. Exchange of the responsible domains with sequences from flagellin of *Salmonella* restored this activity[10]. Intact *C. jejuni* is unable to activate human TLR2 and TLR4, which suggests that these TLR ligands are efficiently shielded on the bacterial surface[11].

For a long time it remained a challenge to investigate *C. jejuni* pathogenesis *in vivo*, because mice are colonization resistant. The colonization resistance is probably due to the conventionally raised microbiota in mice as antibiotic treatment enabled *C. jejuni* colonization[12]. In line with this finding, gnotobiotic mice or mice with a complete human intestinal microflora are stably colonized and exhibit a pro-inflammatory immune response[13]. This observation has led to investigation of *Campylobacter* interaction with the human host microbiome for therapeutic purposes[14].

C. jejuni virulence factors

The repertoire of virulence factors of *C. jejuni* seems limited compared to other studied enteropathogens. Only one secretion system (T6SS) exists in *C. jejuni* but only some strains carry the corresponding genes in their genome, suggesting that it does not play a crucial role in pathogenesis[15]. Moreover, *C. jejuni* secretes a cytolethal distending toxin (CDT), which causes cell cycle arrest at the G2/M phase leading to cell death[16], but this factor is not required to establish disease. Motility and chemotaxis are the most important features of *C. jejuni* to efficiently colonize the intestine. Locomotion provided by one flagellum at each pole and the linked chemotaxis system enable the bacteria to efficiently reach their favorable niche.

In general, the flagellar and chemotaxis system of *C. jejuni* resemble the systems in other prokaryotes. Methyl-accepting chemotaxis proteins (MCPs) are bacterial chemoreceptors sensing specific environmental cues. Upon ligand binding, MCPs undergo conformational changes allowing the autophosporylation of the histidine kinase CheA. The phosphor-group is consequently transferred to the response regulator CheY, which interacts with the flagellar motor/switch components of the cytoplasmic C ring. The presence or absence of MCP ligands determines the flagellar rotation in a clockwise or counter-clockwise direction, which results in tumbling or forward movement of the bacteria, respectively. Interestingly, *C. jejuni* has several chemotaxis proteins with additional response-regulator containing domains, but their precise roles remain under investigation (reviewed in[17]).
Whereas most bacteria are able to utilize sugars as a carbon source, *C. jejuni* uses the amino acids serine, aspartate, glutamate and proline as their main energy source[18]. Therefore, these amino acids together with other TCA cycle intermediates such as succinate, pyruvate and lactate are potent chemoattractants for *C. jejuni*[19].

C. jejuni is well known for its extensive repertoire of enzymes involved in carbohydrate biosynthesis and the formation of membrane glycoproteins and glycolipids. Many of its surface structures are modified with glycans and this likely contributes to bacterial virulence.

The genes encoding the *N*-glycosylation pathway are located in the protein glycosylation locus (*pgl*) and pglB seems to be mainly responsible for the surface glycosylation patterns[20]. Strains deficient in this type of glycosylation show reduced colonization potential *in vivo*[21]. *C. jejuni* also produces a polysaccharide capsule which is loosely attached to the bacterium[22]. The high variability of capsule glycans between different *C. jejuni* strains forms the basis of the Penner serotyping[23]. The capsule is important for bacterial serum resistance and a non-capsulated mutant showed reduced colonization in mice, suggesting a potential role *in vivo*[24].

The surface membrane of *C. jejuni* is mostly composed of lipooligosaccharides (LOS). These glycolipids are also highly variable due to different organization of the *LOS* loci in the genome. Some strains are able to incorporate sialic acids into the LOS, which then resembles human gangliosides. Antibodies directed against these LOS forms can cross-react with neuronal structures of the host, resulting in the development of Guillain-Barré and Miller Fisher syndrome[25].

Finally, the *Campylobacter* flagellum is highly *O*-glycosylated at serine or threonine residues, resulting in an increase of 10% mass of the flagellin subunits. The genes involved in *O*-glycosylation reside in a hypervariable region of the genome[26]. The variable presence of these genes results in variable glycosylation patterns among strains[27]. The role of flagellar glycosylation in pathogenesis remains to be resolved.

C. jejuni adhesins

The molecular details as to how *C. jejuni* interacts with host cells remain largely unknown. Only several adhesins and their cell receptors have been identified so far **(Table 1)**. *C. jejuni* has two fibronectin-binding proteins termed CadF[28–30] and FlpA[30,31] which are confirmed to be required for efficient host cell adherence *in vitro* and *in vivo*. The major outer membrane protein (MOMP) is also involved in adhesion. Isolated from bacteria under native conditions, the protein blocks the attachment of bacteria to INT407 cells[32]. Further studies revealed that glycosylation on Thr268 of MOMP is crucial for the interaction with blood group antigens (Le^b, H-I and H-II) and that only glycosylated MOMP is able to block bacterial attachment. Bacteria with glycosylated MOMP also show reduced colonization in chickens in opposite to the generated unglycosylated mutant[33]. The surface exposed lipoprotein JlpA has also been proposed to contribute to bacterial adhesion based on the findings that genetic inactivation of *jlpA* results in reduced bacterial binding to cells and purified JlpA blocks adhesion of wild type *C. jejuni*[34]. The corresponding cell receptor has been identified as heat shock protein 90. Targeting of this receptor leads to activation of NF-κB[35]. Nevertheless, the role of JlpA *in vivo* remains controversial[30].

The relevance of several other *C. jejuni* proteins that have been proposed to contribute to early stages of cellular infection is still under debate due to incomplete or contradictory data. For instance, one study reported the autotransporter protein CapA (located in the outer membrane fraction) as being involved in *C. jejuni* adherence to human Caco-cells and colonization ability in chickens[36]. Another study did not observe this effect *in vivo*[30]. Similarly, the PEB proteins of *C. jejuni* were thought to play a role in adhesion. The inactivation of *peb1A* in *C. jejuni* resulted in 50-100 fold less adhesion to HeLa cells and 15-fold less invasion in INT407 cells[37]. Moreover, the mutant failed to colonize mice and chickens[30,37]. However, later studies showed that PEB1 is an aspartate/glutamate-binding protein that is mostly present in the bacterial periplasm and required for microbial growth[38].

The observed impaired colonization *in vivo* may thus be a secondary effect rather than due to adhesive properties of the protein. The related protein PEB3 was isolated by lectin-pulldowns of whole-cell lysate[39]. This protein shows resemblance to the *E. coli* adhesin Paa and the colonization factor AcfC from *V. chlorae*[40]. However, experimental evidence of a role in *C. jejuni* adhesion is entirely lacking. Mutagenesis of PEB4, a periplasmic protein of the same family, resulted in reduced bacterial adhesion to INT407 cells *in vitro* and reduced levels of mouse colonization[41]. As for PEB1, this can be a secondary effect, since PEB4 seems to be influence the outer membrane protein composition of *C. jejuni*[42]. Finally, many more putative gene products have been claimed to be involved in bacterial adhesion (**Table 1**) but in most cases it is not evident if their effects are direct or indirect due to secondary effects on the bacterial metabolism or membrane composition.

Table 1. Adhesion-related proteins of *C. jejuni* (strain 11168)

Gene number or product name	Receptor	Data summary	Remarks
CadF	Fibronectin	Mutagenesis showed reduced adhesion *in vivo* and *in vitro*[29,30] Purified CadF confirms adhesive role[29]	
FlpA	Fibronectin	Mutagenesis showed reduced adhesion *in vivo* and *in vitro*[30,31]	
MOMP	Blood group antigens	Protein blocked bacterial adhesion to INT407 cells[32] Mutagenesis showed reduced adhesion *in vivo* and *in vitro*[33]	Glycosylation of Thr268 is crucial for the effect[33]
JlpA	Heat shock protein 90	Mutant shows decreased adherence to Hep-2 cells, purified JlpA blocks bacterial binding[35] JlpA binding leads to proinflammatory signaling[35]	Controversial, no reduced colonization *in vivo*[30]
CapA	Unknown	Mutagenesis showed reduced adhesion *in vivo* and *in vitro*[36]	Controversial, no effect i*n vivo*[30]
PEB1a	Unknown	Mutagenesis showed reduced adhesion *in vivo* and *in vitro*[30,37]	Periplasmic protein important for glutamate/aspartate utilization[38]
PEB3	Unknown		Surface expression, binds lectin[39] No experimental evidence
PEB4	Unknown	Mutagenesis showed reduced adhesion *in vivo* and *in vitro*[41]	Potential secondary effect due to function in membrane composition[42]
CJ1349	Fibronectin/ fibrinogen	Mutagenesis showed reduced adhesion *in vitro*[28]	Not important for chicken colonization No complementation, no protein studies
CJ0091	Unknown	Outer-membrane lipoprotein Mutagenesis showed reduced adhesion *in vivo* and *in vitro*[43]	No further characterization
CJ0268c	Unknown	Mutagenesis showed reduced adhesion *in vitro* and heterologous expression in *E. coli* increased adhesion[44]	Periplasmic protein, probably involved in membrane stabilization[44]
CapB	Unknown		High amino acid resemblance to CapA

Motility and flagellar structure

As mentioned above, *C. jejuni* motility is crucial to reach its favorable niche in the intestinal crypts[6,45]. Additionally, the corkscrew shape of *C. jejuni* is thought to help the movement through the thick intestinal mucus layer[46,47]. Motility is conferred by a single flagellum situated on one or both poles in *C. jejuni*. In other enteropathogens such as *E. coli* and *Salmonella*, many more flagella are present per bacterium. Each flagellum is composed of three structural subunits: the basal body (motor), the hook (joint) and the filament (propeller). The basal body forms the anchoring basis of the flagellum by spanning the inner and outer bacterial membranes. It is composed of a rod and four ring structures termed C- (cytoplasmic), MS- (membrane-supramembrane), P- (peptidoglycan), and L- (lipopolysaccharide) ring (**Fig. 1**). A number of MotAB stator complexes together with the C-ring represent the rotor generating torque by (Na^+ or H^+) ion-flux forces, which drives the flagellar rotation. Despite gross similarities, the motor structures differ between bacteria, resulting in different torques and flagellar strengths. High-torque flagellar motors such as *C. jejuni* exhibit a larger C-ring and have additional periplasmic disk complexes[48]. This feature probably explains the higher flagellar force observed in *Epsilonproteobacteria* especially in high viscosity environments[49]. The rod connects the MS ring with the hook that transmits the motoric torque to the flagella fiber. This results in the helical propelling of the filament. The P and L rings function as molecular bushing[50].

The flagellar filament consists of thousands of subunits (flagellins) that form a hollow fiber. In *C. jejuni*, FlaA and FlaB represent the major building blocks and their transcription is controlled by different sigma factors (σ^{28} and σ^{54} respectively) and the two-component system FlgS/FlgR[51]. The anti-sigma factor FlgM controls the flagellar length[52]. In opposite to many other bacteria, the flagellin of *C. jejuni* is heavily *O*-glycosylated with final monosaccharides such as pseudaminic acid and its derivatives[53,54]. Glycosylation is crucial for flagellin incorporation into the filament[55]. Chaperone and regulatory proteins such as FliS and FliW are important for efficient filament assembly[56].

The flagellum is capped at its distal end by the flagellar tip protein FliD, also termed HAP2. This protein belongs to the family of hook-associated proteins (HAP)[57,58] (**Fig. 1**). The *fliD* gene in *C. jejuni* is located in the flagellin (*fla*) regulon and its transcription is regulated by σ^{28} and σ^{54} [51]. In other bacterial species, FliT has been characterized to chaperone FliD to the export gate[59]. FliT adopts a multimeric state and interacts with the 40 C-terminal amino acids of FliD and the export gate protein FlhA[60]. How *C. jejuni* FliD is guided to the flagellar secretion machinery is unknown as *C. jejuni* appears to lack a *fliT* gene. Studies on *Salmonella* indicate that FliD forms a multimeric cap on top of the hook-filament junction complex preceding flagellin export[58,61]. Next, flagellin subunits pass through the channel and polymerize to form the flagellar fiber. The exact mechanism of flagellar assembly and filament growth remain unsolved, since several studies suggest different concepts. One study proposes that the refolding force of N- and C-terminal connected flagellins at the flagellar tip is sufficient to pull the next flagellin subunit further resulting in a constant rate of flagellar growth[62]. A more recent study contradicts this concept by showing a discontinuous flagellin incorporation which depends on the proton motor force, suggesting a more simple injection-diffusion mechanism[63]. All above-mentioned concepts are based on *Salmonella* and it is assumed that the same principles hold true for *Campylobacter*, but no experiments on flagellar growth in *Campylobacter* have been conducted so far.

It should be noted that, despite similarity in function, the structure of the flagellar fiber differs among different bacterial classes. In *Gammaproteobacteria* such as

Salmonella the filament consists helices of 11 protofilaments[58] whereas the filament of Epsilonproteobacteria such as *Campylobacter* contains 7 protofilaments[64]. The reason for this difference remains to be found.

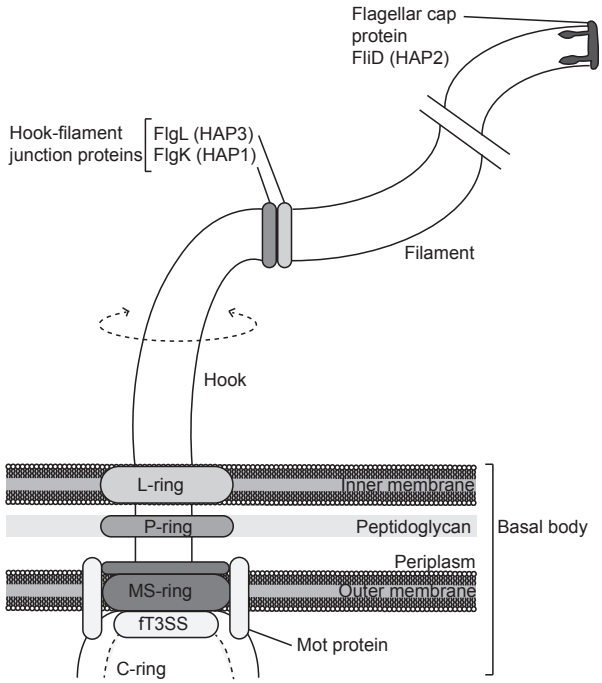


Figure 1: The structure of the bacterial flagellum.
The basal body is composed of four rings (the L- (lipopolysaccharide), the P- (peptidoglycan), the MS- (membrane-supramembrane) and the C (cytoplasmic)-ring). The export apparatus (flagellar Type III secretion system, fT3SS) is located within the C-ring. The Mot proteins drive the flagellar rotation. The hook extends from the bacterial surface and connects the filament to the basal body by the hook-filament junction proteins (FlgK/FlgL). The flagellar filament is composed of thousands of flagellins, which are capped by the FliD protein complex at the distal end.

Additional roles of the flagellum

Additional to motility, bacterial flagella have been implied to play a role in adhesion and virulence[65]. Flagella are long filaments protruding from the bacterial surface, thereby making it possible to reach out and attach to contact surfaces, acting as an early colonization factor[66]. However, the exact interacting partners often remain unknown. Flagellar adhesion has been described for multiple human pathogens, such as *Pseudomonas aeruginosa*[67], *Clostridium difficile*[68], *Bordetella pertussis*[69], *Escherichia coli*[70] and commensal species[71]. In *C. jejuni*, the flagellum has been shown to be essential for initial microcolony formation in human ileal tissue[72]. The related *C. concisus* has been shown to interact with microvilli of Caco-2 cells via its flagellum. This was accompanied with induced membrane ruffling and bacterial internalization[73]. Shearing flagella from *Campylobacter* or using aflagellated bacteria reduced adherence to INT407 cells *in vitro*[74]. Interestingly, immobilization of flagellar rotation increased bacterial adhesion, suggesting that the interaction is reversible with flagellar motor force[75]. In addition to a role in bacterial adhesion, the flagellum may secrete effector molecules[76,77], due to the ancestral resemblance with the bacterial type III secretion system (T3SS)[78]. In *C. jejuni*, the flagellum serves as secretor of Campylobacter

invasion antigen (Cia) proteins[79]. CiaC is secreted from the flagellum and induces host cell cytoskeleton rearrangements by delivery into the cytosol[80]. Similarly, CiaD has been shown to induce pro-inflammatory cell signaling and maximal *C. jejuni* invasion[81]. The cell receptor and detailed delivery mechanisms however remain to be defined.

Campylobacter colonization in chicken

Chickens represent the main reservoir for human infection with *C. jejuni*. In the European Union, up to 70% of retail broiler meat was tested positive for *Campylobacter*[1]. *C. jejuni* resides as a commensal in the chicken gut and colonized broilers show high numbers of bacteria in their caeca[82]. A very low infection dose is sufficient for successful colonization[83] but the used colonization strategies by the bacteria are still under investigation. Despite an initial immune response against *C. jejuni*, chickens don't display pathological traits or symptoms[84]. The infection usually rapidly spreads in the flock and broilers stay colonized until slaughter[85]. Interestingly, newly hatched chickens are usually resistant to colonization[85,86]. It is assumed that this resistance relies on the presence of maternally derived *Campylobacter*-specific antibodies[87,88]. The antibodies (termed IgYs) are transported first into the yolk and then into the bloodstream of the progeny. The transfer begins at embryonic day 7 and reaches highest rates at embryonic days 19-21 just prior hatching[89]. Increased permeability of the gut in early life likely allows IgY transport from the serum into the gut lumen[90,91], where the maternal antibodies protect the hatchling against infections with *C. jejuni*[87,92]. *In vitro* experiments showed IgY-dependent complement dependent killing of bacteria, clarifying the functional protective effect *in vivo*[91]. Further analyses of the antigen specificity show recognition of many different *Campylobacter* components and virulence factors by maternal IgYs. Additionally to reactivity against chemotaxis proteins or few periplasmic proteins, most antibodies are directed against surface-exposed or membrane-associated components. More precisely, they recognize in particular flagellar structures such as the filament proteins FlaA, FlaB and the hook protein FlgE, or other surface components such as MOMP, LPS, CadF and PEB[93,94]. The latter are of special interest because they are assumed to be involved in bacteria-host interactions and therefore represent interesting potential targets for interventions.

Prevention and treatment of *C. jejuni* infection

Human Campylobacteriosis is in most cases self-limiting, but macrolides and fluoroquinolones are the antibiotics classes of choice if treatment is needed. Unfortunately, *C. jejuni* is gaining increased resistance against doxycycline, trimethoprim-sulfamethoxazole and fluoroquinolones[95], therefore preventive measures to limit *C. jejuni* transmission are under investigation. Since *C. jejuni* is mostly acquired by humans via undercooked chicken meat, reduction of bacterial loads in animals is considered an efficient approach to avoid human infection.

Currently, chicken vaccination is the most promising strategy, but so far there is no *Campylobacter* vaccine on the market despite several promising efforts. Most studies focus on the surface-exposed or membrane-bound *C. jejuni* components, which are often also involved in bacterial adhesion. Potential antigenic candidates such as the PEB proteins are considered as vaccine antigens in humans[96] and vaccine studies in mice with MOMP showed protection against *C. jejuni* infection[97], but their potential as chicken vaccines has not been investigated. Another outer-membrane component CmeC is highly immunogenic

in chicken, but showed no protection possibly due to a non-optimized vaccination regiment[98]. Immunization of chicken with recombinant peptides derived from surface-exposed colonization proteins, such CadF, FlaA and FlpA is promising with a reduction in caecal bacterial counts by 2 $\log_{10}$ [99]. Flagellin is another potential vaccine candidate, since it is highly immunogenic in chickens[100] and humans[101]. Immunization with heat-killed *C. jejuni* and purified native flagellin resulted also in lower bacterial caecal loads[100]. Despite these hopeful findings, results are notoriously variable and difficult to reproduce. This makes the development of a chicken vaccine against *C. jejuni* still one of the biggest challenges in the field.

Scope and outline of this thesis

C. jejuni is the most prevalent bacterial cause of human diarrheal disease worldwide[1]. After consumption of contaminated chicken meat products, humans develop mild to severe gastroenteritis, which can lead to the development of peripheral neuropathies such as Guillain-Barré or reactive arthritis[2]. In order to develop a successful chicken vaccine to reduce the incidence of human disease, it is essential to understand the interactions between the bacterium and its host. For *C. jejuni*, one important virulence factor is its flagellum, which confers motility and is also involved in adhesion and secretion of virulence proteins necessary for successful infection[79]. However the underlying molecular mechanisms for the flagellar interaction with mucosal cells are poorly understood. Since the flagellum protrudes from the bacterial cell membrane, the flagellar tip protein FliD represents an attractive candidate for primary contact with the host.

The aim of the work described in this thesis was to explore the putative function of *C. jejuni* FliD protein as flagellar tip adhesin and its potential as a target for colonization or infection inhibitory compounds.

In **Chapter 2** of this thesis we investigated the potential of the flagellar tip protein FliD as new attachment factor for *C. jejuni* using native bacteria, recombinant FliD protein, and discovered the corresponding the host cell surface receptor. In **Chapter 3**, we focused on the presence and role of maternal chicken antibodies directed against FliD protein. These antibodies may provide protection by blocking the initial FliD-dependent colonization of the hatchling. In search for natural infection inhibitors, we aimed in **Chapter 4** to identify potential blocking activity in cow milk, which may carry different types of anti-infective compounds. In **Chapter 5**, we investigated structural features of *C. jejuni* FliD and compared them to the flagellar tip proteins of other species. We also determined whether the identified blocking antibodies and natural inhibitors displayed species-specific inhibition of FliD binding or pointed to a common mechanism of FliD host cell interaction. In **Chapter 6** the main findings are summarized and discussed in the context of the *Campylobacter* problem.

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Chapter 2

Host cell binding of the flagellar tip protein of *Campylobacter jejuni*

Claudia M. Freitag, Karin Strijbis, Jos P.M. van Putten

Department of Infectious Diseases & Immunology, Utrecht University, The Netherlands

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ABSTRACT

Flagella are nanofibers that drive bacterial movement. The filaments are generally composed of thousands of tightly packed flagellin subunits with a terminal cap protein, named FliD. Here we report that the FliD protein of the bacterial pathogen *Campylobacter jejuni* binds to host cells. Live-cell imaging and confocal microscopy showed initial contact of the bacteria with epithelial cells via the flagella tip. Recombinant FliD protein bound to the surface of intestinal epithelial cells in a dose-dependent fashion. Search for the FliD binding site on the host cell using cells with defined glycosylation defects indicated glycosaminoglycans as putative target. Heparinase treatment of wild type cells and an excess of soluble heparin abolished FliD binding. Binding assays showed direct and specific binding of FliD to heparin. Addition of an excess of purified FliD or heparin reduced the attachment of viable *C. jejuni* to the host cells. The host cell-binding domain of FliD was mapped to the central region of the protein. Overall, our results indicate that the *C. jejuni* flagellar tip protein FliD acts as an attachment factor that interacts with cell surface heparan sulfate glycosaminoglycan receptors.

INTRODUCTION

Bacterial pathogens need to interact with eukaryotic cells to establish an infection. Initial host cell contact is often conferred by hair-like appendages (termed pili or fimbriae) that protrude from the bacteria and that can bridge the electrostatic barrier with the host cell surface. The pilus filaments consist of polymers of thousands of protein subunits (pilins) and usually carry one or more tip proteins that bind to host cell surface receptors[1]. Another bacterial structure that extends from the bacterial surface and often contributes to bacterial pathogenesis is the flagellum. Like a pilus, the flagellum is composed of thousands of subunits (flagellins) that are assembled into a hollow fiber that is capped at its distal end by the flagellar cap protein, termed FliD (or HAP2). The flagellar fiber is anchored via a short hook filament structure to the flagellar motor complex in the bacterial membrane. This nanomachine drives the rotation of the flagella enabling movement of the bacteria. Besides its role in bacterial motility, the flagellar apparatus may act as secretion machinery that exports proteins into the environment or the host cell cytosol[2,3]. This function is consistent with the ancestral resemblance between the bacterial flagellar apparatus and type III secretion systems (T3SS) present in many Gram-negative bacterial pathogens[4]. Type III secretion systems form a needle-like structure that may inject effector molecules into eukaryotic cells to modulate host cell function[5,6].

The bacterial pathogen *Campylobacter jejuni* (*C. jejuni*) is the most frequent cause of bacterial foodborne disease[7]. In the natural infection, *C. jejuni* causes severe diarrheal infection and can be observed in strong association with and inside intestinal epithelial cells[8]. The molecular pathogenesis of the infection is still largely unknown. *C. jejuni* lacks pili for initial adherence to host cells and does not show T3SS-dependent bacterial invasion of host cells. However, the pathogen does carry a single flagellum at one or both of its polar ends. The major building blocks of the *C. jejuni* flagellum are the flagellin subunits FlaA and FlaB. The filament is supposedly capped by a FliD protein complex as reported for other bacterial species[10]. Typically, the *C. jejuni* flagellum is heavily glycosylated and its motor appears larger and more complex than in most enteric bacteria[11.] These traits likely enable *C. jejuni* to penetrate the viscous mucous barrier and to colonize the intestinal crypts[12]. Infection of human volunteers and animals with a mixture of motile and immotile *C. jejuni* showed only recovery of flagellated bacteria, indicating that the flagella are crucial for colonization[13,14].

A unique trait of *C. jejuni* appears to be the secretion of effector molecules through the flagellar apparatus rather than via a typical type III secretion system[3,15,16]. Some of those exported proteins, collectively called Campylobacter invasion antigens (Cia), modulate host cell biology[17,18]. How the proteins that are secreted via the flagellar apparatus of *C. jejuni* are delivered into the cells is still unresolved. Several other *C. jejuni* proteins have been proposed to contribute to early stages of cellular infection including the aspartate/glutamate binding protein Peb1A[19,20], the surface exposed lipoprotein JlpA[21], the fibronectin binding protein CadF[17,22,23], the major outer membrane protein MOMP[24], the FlpA protein[25] and the autotransporter protein CapA[26]. Which factor(s) confer(s) the initial contact with the host is still unknown.

Considering the central role of the flagellar apparatus in different steps in *C. jejuni* pathogenesis and microscopy observations on infected host cells, we hypothesized that the *C. jejuni* flagella and, in particular, the FliD tip protein may directly interact with the host cells. Here we provide evidence that the *C. jejuni* flagellar tip protein FliD binds to host

cells through specific interaction of the central region of the FliD protein with cell surface heparan sulfate glycosaminoglycan receptors.

RESULTS

Flagella-dependent anchoring of *C. jejuni* to host cells

Live imaging of cultured epithelial cells inoculated with *C. jejuni* strain 81116 and confocal fluorescent microscopy on cells with mCherry-expressing bacteria and differentially stained flagella repeatedly showed low numbers of free-floating bacteria that seemed to be tethered to the cells via the tip of the flagella (**Fig. 1A and Fig. 1B, movie S1**). These observations led us to hypothesize that the flagellar cap protein FliD of *C. jejuni* may interact with host cells. To test this scenario, we first constructed a Δ*fliD* mutant strain by allelic replacement of the *C. jejuni fliD* gene with an antibiotic resistance cassette. As FliD is important for flagella assembly[27] and thus bacterial motility, FliD was inactivated in the wild type strain 81116 as well as its immotile derivatives 81116Δ*flaAB* that is unable to produce the flagellin subunits, and 81116Δ*motAB* that lacks the *motAB* genes essential for the rotation of the flagella. Motility testing of the strains confirmed that inactivation of *flaAB*, *motAB* and/or *fliD* resulted in immotile bacterial phenotypes (**Fig. 1C and Fig. 1D**). Since FliD forms the flagellar cap protein complex important for filament growth and stabilization[28,29], we also verified flagellin expression in the various strains. The mutants 81116Δ*fliD* and 81116Δ*motAB*Δ*fliD* still expressed bacteria-associated flagellins, although levels were slightly reduced compared to the parent strain (**Fig. 1E**). Infection of epithelial cells with strain 81116Δ*motAB*Δ*fliD* and its parent 81116Δ*motAB* showed 70-95% reduction in *C. jejuni* adhesion for the FliD-deficient strain as determined by microscopy (**Fig. 1F**) and viability counting of cell-associated bacteria (**Fig. 1G**). Together, these data support the notion that FliD is important for the initial interaction of *C. jejuni* with epithelial cells.

Cloning and purification the *C. jejuni* flagellar tip protein FliD

In order to better assess the putative cell binding properties of FliD, the gene was cloned with a C-terminal TEV cleavage site followed by a His-tag in the expression vector PCP1. After removal of the His-tag with TEV protease, the purified recombinant FliD migrated on SDS-PAGE as a single band with the expected molecular mass of approximately 70 kDa (**Fig. 2A and Fig. 2B**). Immunization of rabbits with the recombinant protein yielded antibodies that specifically recognized the 70 kDa band in the Western blot (**Fig. 2C**). Reactivity with this protein was observed for all tested *C. jejuni* wild type strains (11168, 81116, 108, 81-176) but not for the FliD-negative strains (81116Δ*fliD*, 81116Δ*motAB*Δ*fliD*) (**Fig. 2D**), indicating that the antisera recognized *C. jejuni* FliD of multiple strains consistent with the conserved nature of the protein.

Binding of recombinant FliD to mucosal epithelial cells

The cell binding properties of the recombinant FliD protein were investigated in a cell-based ELISA that involved incubation of purified protein with cultured eukaryotic cells followed by quantification of FliD binding using the generated anti-FliD antibodies in combination with a HRP-labeled conjugate. Epithelial cells were fixed to prevent detachment of cells and

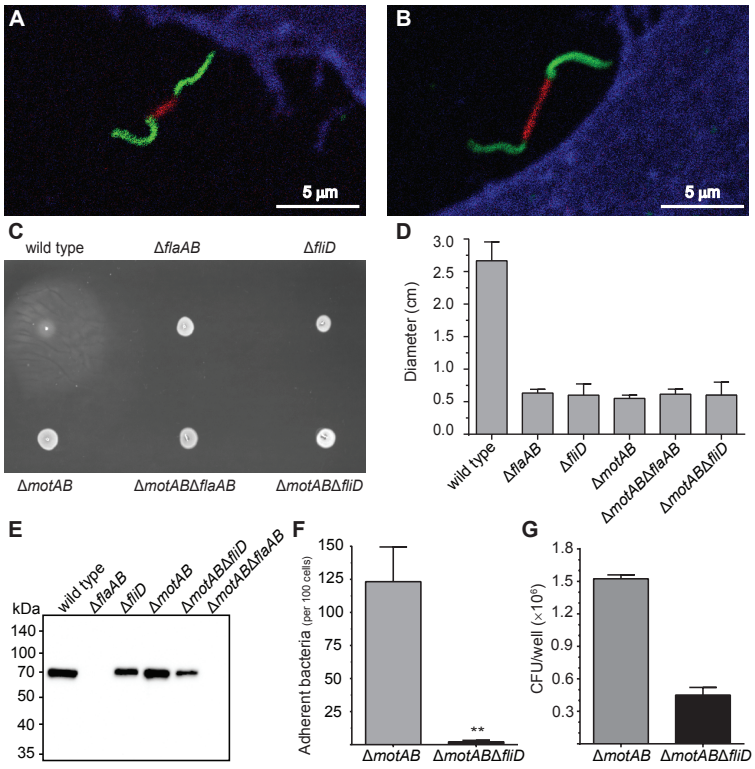

Figure 1: Flagella tip mediated attachment of *C. jejuni* to epithelial cells.
(A-B) Immunofluorescence microscopy on *C. jejuni* 81116 wild-type infected Chang epithelial cells. After 2 hours infection, cells were washed and fixed. The cell surface was stained with WGA-Alexa Fluor 633 (blue). The flagella of mCherry-positive bacteria (red) were visualized using anti-*C. jejuni* antibodies, followed by goat-anti-rabbit-IgG Alexa Fluor 488 (green). **(C-D)** Effect of inactivation of *flid* and *flaAB* on the motility of strain 81116 and its *motAB* derivatives in soft agar plates (C), as quantified by growth zone diameter (D). **(E)** Flagellin levels in bacterial pellets of strain 81116 and the indicated mutants as determined by Western blot. Bacterial lysates were separated by SDS-PAGE, blotted, and probed with anti-flagellin antiserum followed by goat-anti-rabbit-IgG-HRP. **(F)** FliD-dependent attachment of *C. jejuni* attachment to epithelial cells as determined by microscopy. Cells were incubated with the indicated strains for 2 h, fixed, and stained with 0.01% crystal violet. Bacterial adhesion was quantified by light microscopy. Each bar represents the mean ± SEM of three different experiments. Data were analyzed by Student's *t*-test (***P*=0.01). **(G)** FliD-dependent attachment of *C. jejuni* attachment to epithelial cells as determined by viability counting. After 2 h of infection, the number of attached bacteria as recovered as colony forming units (CFU) on blood agar plates was counted. Each bar represents the mean ± SD of duplicate values of one representative of three experiments.

potential internalization of FliD during the assay. This approach demonstrated binding of FliD to the epithelial cells in a dose-dependent fashion reaching a maximum of binding at a protein concentration of 10 µg/ml (**Fig. 3A**). Fluorescence microscopy of non-fixed FliD-exposed (**Fig. 3B upper panel**) and control cells (**Fig. 3B lower panel**) using a red-fluorescent labeled conjugate to visualize the protein, confirmed the presence of discrete clusters of FliD at the apical surface of the cells. Similar assays with the intestinal cell lines HRT-18 and HT-29 cells revealed that FliD also bound to these cells in a dose dependent manner (**Fig. 3C**). Immunofluorescence microscopy confirmed binding of FliD to both types of intestinal cells, with a trend towards increased binding to HT-29 cells (**Fig. 3D and Fig. 3E upper panels**) probably due to a different differentiation status of the cell lines[30]. The staining was not observed for non-FliD exposed control HRT-18 and HT-29 cells (**Fig. 3D and Fig. 3E lower**

panels).

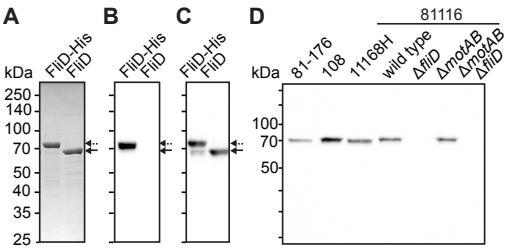

Figure 2: Isolation of recombinant FliD and reactivity of the generated antiserum.
(A) SDS-PAGE of purified recombinant *C. jejuni* FliD protein before (dotted arrow) and after (solid arrow) removal of the His-tag with TEV protease. **(B-C)** Western blot of the same samples showing the reactivity of the FliD protein with (B) antibodies directed against the His-tag and (C) the raised FliD antisera. **(D)** Western blot showing the reactivity of the FliD antiserum with FliD in lysates of the *C. jejuni* wild type strains (81-176, 108, 11168H and 81116) and the FliD-negative strains 81116Δ*fliD* and 81116Δ*motAB*Δ*fliD*.

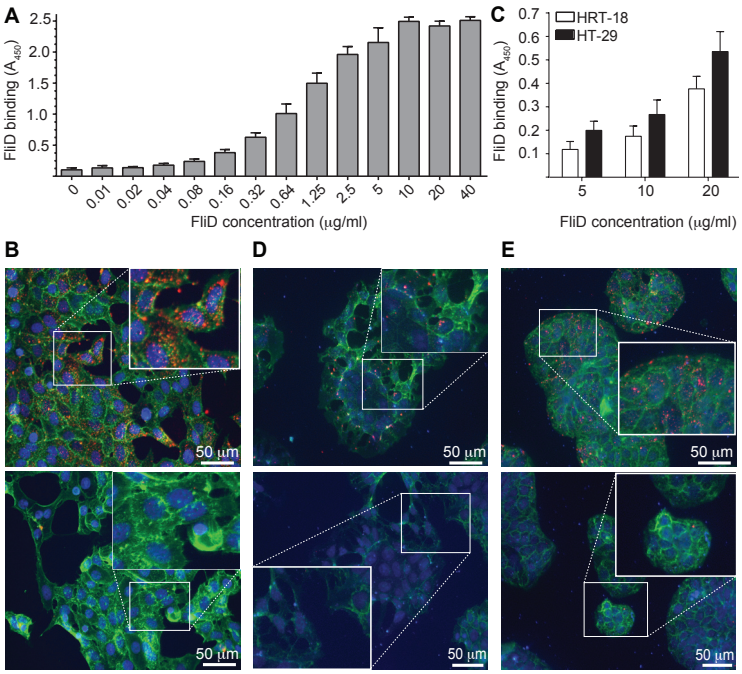

Figure 3: FliD binding to different types of epithelial cells.
(A) Cell-based ELISA results showing the binding of recombinant *C. jejuni* FliD protein to fixed Chang epithelial cells after incubation (1 h, 20°C) with the indicated protein concentrations. Binding was quantified using anti-FliD antibodies in combination with HRP-conjugated secondary antibodies. Results represent the mean ± SEM of three different experiments. **(B)** Immunofluorescence microscopy on unfixed Chang cells incubated with (upper panel) or without (lower panel) FliD protein (2 μg/well, 1 h). Nuclei were stained with DAPI (blue). The cell surface was stained with WGA-Alexa 488 (green). FliD was detected using anti-FliD antibodies followed by goat-anti-rabbit-IgG Alexa 568 (red). The inserts show a magnified view of the marked areas, demonstrating clusters of FliD bound to the cell surface. **(C)** Binding of the indicated concentrations of FliD protein to HRT-18 and HT-29 intestinal epithelial cells as determined by cell-based ELISA. Each bar represents the mean ± SEM of three different experiments after background subtraction. **(D-E)** Immunofluorescence microscopy on (D) HRT-18 and (E) HT-29 intestinal epithelial cells incubated with (upper panel) or without (lower panel) FliD protein as described in B

Characterization of the FliD binding site on the eukaryotic cells

As bacterial pathogens often exploit cell surface (glyco)proteins as host cell receptors[31], we first tested the binding of FliD to Chinese hamster ovary (CHO) cells with defects in their *N*- or *O*-glycosylation machinery using the cell-based ELISA. FliD binding to CHO-lec2 and CHO-lec8 cells that lack terminal sialic acids and terminal galactose residues on their respective sugar chains[32], was unaltered compared to the binding of FliD to wild type CHO-K1 cells (**Fig. 4A**). However, FliD bound significantly less to CHO-745 cells defective in glycosaminoglycan (GAG) synthesis[33] (**Fig. 4A**). In line with this finding, pre-incubation of cells with sodium chlorate which inhibits sulfation of proteins and carbohydrates including glycosaminoglycans[34], reduced FliD binding significantly (**Fig. 4B**).

To investigate the class of GAGs responsible for the binding of FliD, the recombinant protein was incubated with various types of GAGs prior to addition to the cells. Effective dose-dependent inhibition of binding was obtained with heparin in the concentration range between 1-100 ng/ml (**Fig. 4C**). The addition of hyaluronic acid, chondroitin sulfate A, or dermatan sulfate did not reduce FliD binding, even at concentrations of up to 1,000 ng/ml (**Fig. 4C**).

To confirm heparan sulfate containing molecules as putative cell surface binding partner of FliD, epithelial cells were treated with the enzyme Heparinase-I, which removes heparan sulfate from the cells surface[54]. Heparinase treatment reduced the binding of FliD to the cells in an enzyme activity-dependent fashion, whereas treatment of the cells with chondroitinase ABC had no effect on the binding of FliD (**Fig. 4D**).

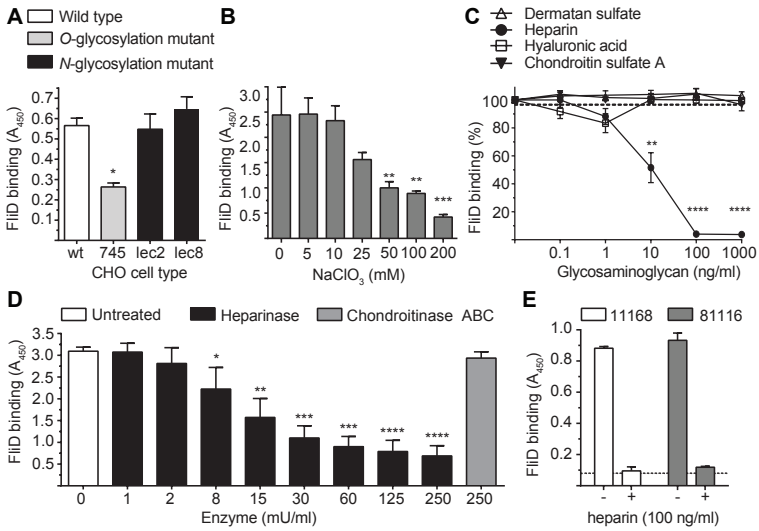

Figure 4: Binding of FliD to heparan sulfate moieties on the host cell surface.
(A) FliD-specific cell-based ELISA showing the binding (1 h) of FliD protein (20 µg/ml) to CHO-K1 wild type cells, the *N*-glycosylation mutants CHO-lec2 and CHO-lec8 cells, and the glycosaminoglycan deficient CHO-745 cells. **(B)** FliD-binding cell-based ELISA showing the binding (1 h) of FliD to Chang cells pre-treated (16 h) with the indicated concentrations of the sulfation inhibitor sodium chlorate ($NaClO_3$). **(C)** Competitive effect of the indicated types of glycosaminoglycans on FliD binding to Chang cells. FliD protein was preincubated with the indicated concentrations of different compounds and then subjected to the cell-based ELISA procedure. **(D)** FliD-specific cell binding after treatment (1 h, 37°C) of Chang cells with the indicated concentrations of Heparinase or Chondroitinase ABC. The binding assay was performed as described under A. **(E)** FliD protein of *C. jejuni* strains 81116 and 11168 was incubated with heparin prior addition to Chang cells as described under C. Results are the mean ± SEM of three independent experiments. Statistical significance was calculated using One-way ANOVA (**P*<0.05,***P*<0.01, ****P*<0.001m*****P*<0.0001)

We also cloned and expressed the FliD protein of the frequently studied isolate *C. jejuni* strain 11168 (94% amino acid sequence similarity). As expected, this protein showed similar host cell binding characteristics as noted for FliD protein of strain 81116 (**Fig. 4E**). Overall, our findings strongly suggest that *C. jejuni* FliD binds to heparan sulfate moieties at the epithelial cell surface.

Direct interaction of FliD with heparin

To demonstrate direct interaction of FliD with heparan sulfate, recombinant FliD was coated in an ELISA set-up and binding of biotinylated heparin was measured using HRP-streptavidin as conjugate. In line with the results above, heparin bound to FliD in a dose-dependent manner (**Fig. 5A**). The specificity of the interaction was demonstrated by the inability of chondroitin sulfate A, dermatan sulfate, or hyaluronic acids to outcompete the FliD binding of biotinylated heparin, whereas heparin strongly reduced the binding of the biotinylated molecule (**Fig. 5B**).

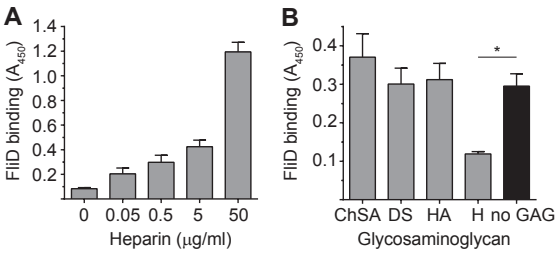

Figure 5: Direct and specific interaction of FliD with heparin.
(A) Binding assay showing the dose-dependent binding of the indicated concentrations of biotinylated heparin to recombinant *C. jejuni* FliD. Heparin binding was measured using Streptavidin-HRP. **(B)** Binding assay showing the competitive effect of unbiotinylated chondroitin sulfate A (ChSA), dermatan sulfate (DS), hyaluronic acid (HA) and heparin (H) on the binding of biotinylated heparin to immobilized *C. jejuni* FliD. Biotinylated heparin binding was detected as in A. Statistical significance was assessed by Students *t*-test (*P<0.05).

Native *C. jejuni* FliD binds to heparan sulfate glycan receptors

To ensure that the observed FliD binding properties obtained with recombinant protein mimicked those of natural *C. jejuni* FliD, we tested the binding characteristics of native FliD protein. The native protein is present in high amounts in the culture supernatant of the *C. jejuni* 81116Δ*flgKM* mutant which has a defect in flagella filament formation[35] (**Fig. 6A**). Incubation of epithelial cells with concentrated culture supernatant of this mutant resulted in a dose-dependent binding of FliD to the cells (**Fig. 6B**), as was noted earlier for recombinant FliD. To ascertain that the observed binding required cell surface GAGs, FliD containing supernatant was incubated with different concentrations of heparin or dermatan sulfate prior to addition to cells. Consistently, heparin inhibited the binding of native FliD to the cells at the low dose of 10-100 ng/ml of heparin, whereas dermatan sulfate had no effect (**Fig. 6C**).

FliD and heparin reduce adhesion of *C. jejuni* to host cells

To assess whether the binding of FliD to cell surface heparan sulfate receptors could be exploited to inhibit *C. jejuni* adhesion to host cells, we investigated the role of soluble FliD protein and heparin in an infection setting. Epithelial cells were incubated with recombinant FliD (25 μg/well) and subsequently infected with *C. jejuni* strain 81116Δ*motAB*, which displays higher adhesive potential than the wild type probably due to its defect in bacterial motility[36]. Quantification of adherent bacteria showed that pre-incubation of the cells with

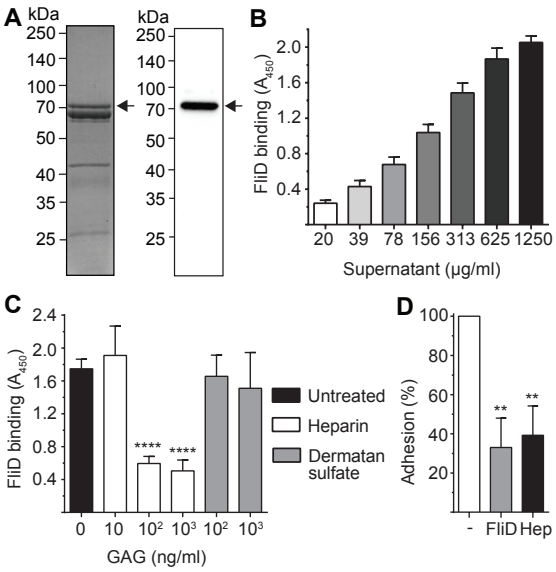

Figure 6: Binding of C. jejuni-derived FliD and intact *C. jejuni* to glycosaminoglycans and its inhibition by soluble FliD protein and heparin. (A) SDS-PAGE (left panel) and Western blot probed with the FliD antiserum (right panel) illustrating the presence of FliD in the culture supernatant of strain 81116Δ*flgKM*. (B) Cell-based ELISA demonstrating the binding of *C. jejuni*-derived FliD to Chang cells detected using FliD antiserum and HRP-conjugated goat-anti-rabbit IgG. **(C)** Cell-based ELISA showing the effect of the indicated concentrations of heparin and dermatan sulfate on the binding of FliD present in *C. jejuni* culture supernatant (625 µg protein/ml, black bar) to Chang cells. Binding of FliD was detected as in B. Statistical significance was determined by One-way ANOVA in comparison to the untreated control (****P<0.0001). **(D)** Binding of viable *C. jejuni* (strain 81116Δ*motAB*) to Chang cells after pre-incubation of the cells with buffer (white bar) or recombinant FliD (25 µg, grey bar), or incubation of bacteria with soluble heparin (0.5 mg, black bar). Bacterial attachment was determined by counting the number of attached bacteria expressed as relative adhesion compared to the non-treated control. Results are the mean ± SD of three experiments. Statistical significance was calculated using Student's t-test in comparison to the untreated control (**P<0.01).

FliD reduced *C. jejuni* adhesion by approximately 65% (**Fig. 6D**). A similar strong reduction in *C. jejuni* attachment was observed when the bacteria were pre-incubated with heparin prior to their addition to the cells (**Fig. 6D**). These results strongly suggest that *C. jejuni* FliD is a major attachment factor and that the *C. jejuni* adhesin to host cells can be inhibited by the use of competing purified FliD protein as well as a soluble receptor analogue.

Mapping of the host cell binding region in FliD

In the absence of any three-dimensional structural information of the *C. jejuni* FliD protein, we used the protein information of the FliD protein of *Salmonella* Typhimurium as starting point to map the host cell binding domain in FliD. The FliD protein of *S*. Typhimurium forms a pentameric complex with a central pentagonal plate and five leg domains that are buried within the flagellar structure. The terminal regions of the different FliD proteins in the complex likely form a coiled-coil structure that interacts with the flagellin subunits at the filament end[10,28]. Based on this model, we first constructed a *C. jejuni* FliD protein that lacked the first N-terminal 70 amino acids of the protein and the last 87 residues at the C-terminal end (**Fig. 7A**). The purified protein (named FliDΔNC) migrated on SDS-PAGE with the expected molecular mass of 60 kDa (**Fig. 7B**). Cell binding assays with the recombinant FliDΔNC protein using the polyclonal anti-FliD antiserum as a probe showed similar cell binding activity as observed for the full length protein (**Fig. 7C**), indicating that the N- and C-terminal regions are not required for cell binding.

To further map the potential binding region, we fragmented the FliDΔNC protein by cloning three DNA segments, named A, B and C (**Fig. 7A**). In order to ensure equal detection of the different protein fragments in the binding assay, the constructs were cloned with a C-terminal sortase-binding motif which enabled site-specific biotinylation and detection with Streptavidin-HRP[37]. Expression and purification of the corresponding proteins resulted in

three protein fragments with apparent molecular masses of 29, 35 and 32 kDa, respectively (**Fig. 7D**) which after the sortase labeling procedure migrated on SDS-PAGE with apparent molecular masses of approximately 13, 24 and 14 kDa, respectively (**Fig. 7D**). Western blotting confirmed the successful biotinylation of the protein fragments (**Fig. 7E**). Incubation of the protein fragments A, B and C with cells showed that only fragment B displayed strong cell binding activity (**Fig. 7F**), suggesting that the cell binding site encompasses the central part (amino acids 210-418) of the FliD protein.

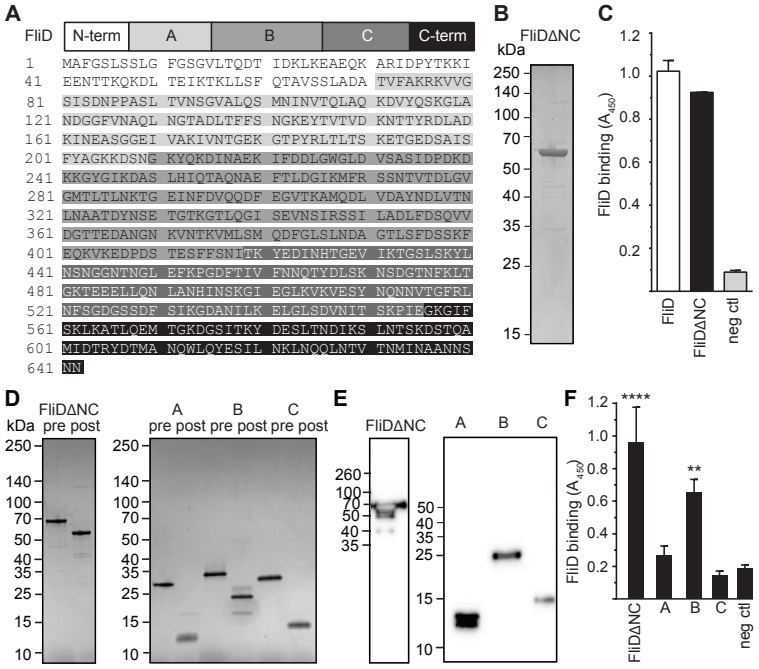

Figure 7: The central region of FliD is important for cell binding.
(A) Graphical representation of the FliD protein sequence of strain 11168 indicating the N-terminal and C-terminal domains and the cloned FliD fragments (A-C). The grey shades of the FliD fragments correspond to the highlighted protein sequences respectively. **(B)** SDS-PAGE demonstrating the migration of pure FliD protein lacking its N- and C-terminal ends (FliDΔNC). **(C)** Cell-based ELISA results after the addition of full length recombinant *C. jejuni* FliD (40 µg/ml), FliDΔNC or buffer only (neg ctl) to fixed Chang epithelial cells. Binding was quantified using anti-FliD antibodies in combination with HRP-conjugated secondary antibodies. Results represent the mean ± SD of duplicates of one representative of three independent experiments. **(D)** SDS-PAGE of isolated FliDΔNC protein and the FliD protein fragments A, B and C before and after the sortase reaction. **(E)** Western blot of the biotinylated FliDΔNC protein and the FliD protein fragments A, B and C probed with Streptavidin HRP. **(F)** Cell-based ELISA showing the binding of the biotinylated FliDΔNC protein and the FliD protein fragments A, B and C (75 pmoles) to Chang cells. Binding was detected using Streptavidin-HRP. Incubation of the cells with buffer only served as control (neg ctl). Results are the mean ± SD of three experiments. Statistical significance was calculated using One-way ANOVA in comparison to the negative control (***P*<0.01, *****P*<0.0001).

DISCUSSION

Bacterial movement and firm attachment to host cells are important virulence traits of many pathogens. Here we provide evidence that the flagellar tip protein FliD of *C. jejuni* is not only required for bacterial motility but also acts as an early attachment factor. FliD-deficient strains displayed strongly reduced bacterial adhesion to epithelial cells and recombinant

FliD as well as native soluble FliD bound to the surface of cells. We identified heparan sulfate glycosaminoglycans as the binding partner of FliD on the host cells and found that both soluble FliD and the receptor analogue heparin can inhibit *C. jejuni* attachment to the cells. The dual function of FliD as capping protein important for flagella assembly and as flagellar tip attachment factor underscores the proposed key role of the flagella in the natural *C. jejuni* infection[13].

The hypothesis that *C. jejuni* FliD may act as an attachment factor was based on live imaging and fluorescence microscopy on infected epithelial cells that showed the marked presence of bacteria that seemed anchored to the host cells via their flagellar tip. This feature did not require flagellar rotation as bacterial attachment was even stronger in a *motAB* mutant that lacks the proteins that drive the rotation of the flagella[36]. Genetic inactivation of FliD largely abolished this initial *C. jejuni* attachment. It remains a major challenge to investigate FliD-mediated attachment *in vivo* due to the lack of appropriated animal models and the necessity of motility for efficient *C. jejuni* colonization[36].

As FliD is essential for the polymerization of the flagellin subunits into the filament, the lack of bacterial adhesion to the host cells may theoretically reflect the absence of flagellins rather than FliD per se. This was excluded after investigation of the binding properties of purified recombinant FliD. This demonstrated direct and dose-dependent binding of *C. jejuni* FliD to the epithelial cells.

A role of flagella in bacterial attachment to host cells has previously been suggested for various bacterial species[38] including *C. jejun*[16,39], *Pseudomonas aeruginosa*[40], *Clostridium difficile*[41], *Bordetella pertussis*[42] and commensal species[43]. In most cases this concept was based on investigation of the adhesive potential of the entire flagella (comprising both flagellins and flagellar cap) and hence the specific role of FliD was not further clarified. For pathogenic *E. coli*, both flagella and isolated flagellins have been proposed as adhesion factors[44,45]. This adhesion may be mediated by the soluble EtpA adhesin that anchors to distal end of the *E. coli* flagella probably after loss of the FliD cap complex[46]. To our knowledge the only identified FliD proteins with adhesive properties thus far are the FliD of *P. aeruginosa*[47] and of *C. difficile*[48]. *P. aeruginosa* FliD confers binding to Lewis(x) or sialyl-Lewis(x) determinants present on the surface on cells and on mucins[49]. For *C. difficile* FliD no cellular binding partner has thus far been identified.

Our successful search for the *C. jejuni* FliD receptor(s) on the host cells was important to validate the specificity of the binding of *C. jejuni* FliD to the cells. We identified heparan sulfate glycosaminoglycans as main binding partner of FliD. This discovery is based on the reduced binding of FliD to glycosaminoglycan-deficient host cells, the inhibition of FliD binding by soluble heparin but not by other glycosaminoglycans, the reduced FliD binding after treatment of the cells with heparinase-I, and the direct binding of FliD to heparin. The reduced FliD binding after treatment of the cells with sodium chlorate, which prevents the sulfation of glycoproteins and carbohydrates[34] further indicates that sulfate moieties are critical for binding. The degree of sulfation differs between glycosaminoglycans, with heparan sulfate being the most extensively sulfated class[50]. The interaction of FliD with the host cell receptors was observed for several *C. jejuni* isolates, consistent with the conserved nature of the protein. Bacterial attachment to heparin sulfate proteoglycans (HSPG) has been reported for a large array of pathogens[51,52], in particular *Borrelia burgdorferi* 53 and the pathogenic *Neisseria* species[54]. The binding of neisserial Opa/Opc adhesins to HSPG is followed by internalization of the bacteria[55]. For *C. jejuni* FliD-dependent bacterial invasion

was not observed (data not shown). This may be explained by the large density of neisserial surface adhesins compared to the presence of a single FliD complex at the flagella tip. A large density of adhesins may more easily induce the clustering of host receptors that is often needed to trigger the receptor-mediated internalization[56].

In the flagellum of *S*. Typhimurium, the flagellar tip protein complex consists of pentamers of FliD that are assumed to form a surface-exposed plate structure with five leg domains that are buried within the flagellar structure[10,28]. In search for the host cell binding region in *C. jejuni* FliD, we demonstrated that the N-terminal and C-terminal domains of the protein (which may form a leg domain in *S*. Typhimurium FliD) do not carry the host cell binding domain. Instead our results locate the binding site in the central region of the protein between the amino acids 210-418. Heparan sulfate has a high negative charge and interacts with diverse proteins including extracellular matrix proteins, growth factors, cytokines and coagulation factors. The ability of proteins to bind heparin is largely determined by the spatial orientation of basic amino acids residues[57]. The *C. jejuni* FliD protein including the region between amino acids 210-418, contains a large number of basic amino acid residues. Elucidation of the three-dimensional structure of the *C. jejuni* FliD complex may enable a more precise mapping of the amino acid residues in FliD that are critical for the contact with the cell surface heparin sulfate bearing receptors.

The function of FliD as early cell attachment factor resembles in some aspects the pilus-mediated adhesin mechanism that is present in many other pathogens. These hair-like appendages extend from the bacterial cell surface to confer initial anchoring of the bacteria to the host cells. Furthermore, the *E. coli* fimbrial tip protein FimH is conserved throughout the family *Enterobacteriaceae* and binds to carbohydrates (mannose residues) on the cell surface of host cells to promote bacterial attachment[58] similarly to *C. jejuni* FliD. *C. jejuni* does not express pili but instead appears to exploit the flagellar tip as cellular adhesin to bind to host cell glycosaminoglycans. A major difference between the two mechanisms however is that *C. jejuni* has only two single polar flagella which contrasts with the usually large number of pili that is expressed by a single bacterium.

C. jejuni flagella have also been implicated to act as a secretion apparatus that delivers bacterial effector molecules into the environment including the cellular cytosol[3]. This mechanism resembles to some extent the classical type III secretion system present in other bacterial pathogens such as *Yersinia*, *Shigella*, and *Salmonella*. This system encodes a needle-like structure that injects proteins into the cytosol to modulate host cell functions[5,6.] *C. jejuni* does not have a classical type III secretion but appears to exploit the flagella secretion apparatus to achieve the same goal[3,18,59,60]. The mechanism that underlies the translocation of the *C. jejuni* proteins into the cells is still elusive, but the secretion event itself seems to require neither flagellin subunits nor FliD[3]. Interestingly, in *Yersiniae* the deployment of the Yop T3SS machinery appears to be triggered via binding of the bacterial LrcG protein to heparan sulfate glycosaminoglycans at the cell surface[61]. It is tempting to speculate that binding of *C. jejuni* FliD to the same type of glycosaminoglycans has a similar effect on the flagella secretion machinery but this awaits future study.

Finally, the finding that both preincubation of host cells with FliD and of *C. jejuni* with the soluble receptor analogue heparin inhibit the initial bacterial attachment strongly suggests that native flagella-associated FliD is able to interact with the identified receptor molecules on the host cells. This finding together with the high sequence conservation of

FliD among *C. jejuni* strains may indicate the flagellar tip protein as a putative target for infection intervention.

MATERIAL AND METHODS

Cell culture

Human Chang epithelial cells (ATCC CCL20.2) were routinely propagated in 25 cm^2 flask in RPMI 1640 tissue culture medium supplemented with 5% FCS at 37°C and 5% CO_2. The human colon cell lines HT-29 (ATCC HTB-38) and HRT-18 (ATCC CCL-244) were cultured in DMEM with 10% FBS. CHO-K1 and CHO-745 cells[33] were grown in RPMI+10% FCS. CHO-lec2 and CHO-lec8 cells[32] were kept in RPMI+Ham's-F12 (1:1) supplemented with 5% FCS.

Bacterial culture and construction of mutants

C. jejuni strains 11168H, 81116, 108 and 81-176 were routinely grown as previously described[62]. In order to generate the strain 81116Δ*motAB*Δ*fliD*, the *fliD* gene and its flanking regions were amplified by PCR using primers fliD-1F (5'-AGCAGGACTTGATGCTTGTG-3') and fliD-1R (5'-GCAGCAAGAAGGGATGGTTA-3') using genomic DNA from strain 480 as a template. The PCR product was ligated into the pGEM-T Easy vector (Promega) and introduced into *E. coli* DH5α. Reverse PCR on this vector, using the outward oriented primers fliD-2F (5'-GTGGATCCGCAAGAGATGACTGGAAAAG-3') and fliD-2R (5'-GCGGATCCTGAGTTAAAACCCCAGAACC-3') was used to delete 1,650 bp from *fliD* and to introduce a unique BamHI restriction site that served to insert the Cm^r cassette from pAV35[63], yielding pGEM*fliD:cm*. The plasmid was verified by sequencing. This knockout construct was then transformed into *C. jejuni* 81116Δ*motAB* as previously described[64] to generate 81116Δ*motAB*Δ*fliD*. The correct disruption of *fliD* was verified by PCR. The 81116Δ*flgKM* strain was constructed as described[35].

Motility assay

Motility was assessed as previously described[65]. Shortly, *C. jejuni* strains 81116 wild type and its mutant strains Δ*motAB*, Δf*laAB*, Δ*fliD,* Δ*motAB*Δ*flaAB* and Δ*motAB*Δ*fliD* were grown overnight in HI broth. One microliter overnight culture equal to OD=1.5 was pipetted into semi-solid medium (HI medium with 0.4% agar) and bacterial migration was measured after incubation (24 h) under microaerophilic conditions at 37°C.

Infection assays

Infection experiments were performed as previously described[54]. Cells were seeded onto 12-mm or 25-mm circular glass coverslips (Menzel) in a 24-well or 6-well tissue culture plate respectively, and grown to 70% confluence in 36 h. Prior to the addition of bacteria, cells were rinsed twice with Dulbecco's PBS (DPBS) and left in Hepes buffer (20 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH7.4) supplemented with 30 mM L-serine and 5 mM glucose. *C. jejuni* (20 h of growth at 37°C under microaerophilic conditions) was added to the cells at a bacterium/host cell ratio (MOI) of 100:1 and incubated for two hours at 37°C at 5% CO_2. Infection was stopped by carefully rinsing the cells three times with DPBS. Live imaging was performed using a Leica SP-II confocal microscope (video). The number of

attached bacteria was determined as described[54]. Bacterial adherence was scored blindly by examining at least 300 cells on 10 different fields using a light microscope. For viability counting of cell-associated bacteria, cells were lysed cells with 1% saponin in Hepes buffer prior to plating serial dilutions on blood agar plates by 10 µl running droplets. The number of colony forming units was determined after two days of growth under microaerophilic conditions.

For infection assays in the presence of recombinant FliD, the cultured cells were incubated with 25 µg of recombinant FliD for 1 h at 20°C and washed three times with Hepes buffer before addition of *C. jejuni*. When heparin was added to bacteria prior infection, the respective MOI was incubated for 30 min at 20°C with heparin before addition to the cells.

Purification of recombinant FliD and generation of anti-FliD antibodies

The *fliD* gene (Cj0548) from *C. jejuni* 11168 was amplified with PacI and NotI overhangs (underlined) using primers 5'-CCTTAATTAAATGGCATTTGGTAGTCTATCTAGTTTAG-3' (fwd) and 5'-CCGCGGCCGCATTATTAGAATTGTTTGCCGCATTAATCATATTAG-3' (rev) and directly ligated into the PCP1 vector (in house derivative of pET101) in frame with a C-terminal tobacco etch virus cleavage site (TEV) and a 6xHis-tag. The plasmid (named PCP1*fliD-TEV-His*) was transformed to *E. coli* BL21+. Gene expression was induced by addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to 1.5 h log phase grown cultures. After 4 h of induction (37°C) FliD protein was isolated under native conditions. Bacteria (of 50 ml culture) were collected by centrifugation and resuspended in 6 ml of buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 7.4) and incubated with lysozyme (1 mg/ml) for 30 min on ice. Lysed bacteria were spun (14,000 x g, 20 min, 4°C) and the supernatants were incubated (4°C) with 4 ml of nickel-coated beads (Ni-NTA Agarose, Thermo Scientific) for 1 h with end-of-end rotation. NTA beads were washed with 10 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.4) followed by 40 ml of washing buffer + 0.1% Triton-X114, and another 40 ml of washing buffer. Bound protein was eluted from the beads with 4 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 7.4) and cleaved with 2,000 U of TEV protease (T4455, Sigma) (16 h, 4°C, end-over-end rotation). The protein/TEV mixture was dialysed overnight at 4°C against DPBS. The His-tagged protease was removed using Ni-NTA agarose beads. Protein concentration was measured and samples (0.3-1 mg/ml) were stored in DPBS at -20°C.

The polyclonal antiserum reactive against *C. jejuni* FliD was generated by Eurogentec. Rabbits were immunized four times with 100 µg/ml of recombinant FliD from *C. jejuni* 11168 (without adjuvant). Titration curves were made to determine the optimal dilutions to be used in ELISA, Western blot and immuno-microscopy.

SDS-PAGE and Western blotting

Quality assessment of purified FliD was done by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by PageBlue staining as previously described[64]. To determine if rabbit anti-FliD antibodies recognized FliD derived from *C. jejuni*, 10 µl of an overnight culture (OD=1.5) from different strains was separated by SDS-PAGE and proteins transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad). FliD protein was detected using the anti-FliD antiserum (dilution 1:1,000) in Tris-buffered saline with 0.05% Tween (TBS-T) + 2% milk followed by secondary goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) (dilution 1:10,000, Sigma). The blots were developed with ECL

SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific).

To quantify bacteria-associated flagellin levels, bacteria were washed with PBS prior to separation on SDS-PAGE as described above. Flagellin was detected using in-house rabbit anti-FlaA antiserum (dilution 1:10,000) followed by goat anti-rabbit-HRP.

Cell-based ELISA

Cells were grown to confluence in a 96-well plate. All incubations steps (20°C, 1 h, with shaking) were performed in the presence of 2% bovine serum albumin (BSA, A7030, Sigma) in DPBS and followed by three washing steps with DPBS. When appropriate, cells were grown in a 96-well plate and incubated overnight with the indicated concentrations of sodium chlorate as previously described[54]. Cells were fixed for 1 h with 1% paraformaldehyde in DPBS, blocked with 4% BSA, and incubated with 20 µg/ml FliD in DPBS (unless indicated otherwise). Cell-bound FliD was detected with anti-FliD antiserum (dilution 1:10,000) and secondary goat anti-rabbit IgG-HRP (dilution 1:10,000; A4914, Sigma) using HRP-substrate (555214, BD). Absorbance was measured in a plate reader at 450 nm (Fluostar Omega, BMG Labtech).

For competition assays with soluble receptor, 20 µg/ml of FliD was pre-incubated with different concentrations of glycosaminoglycans using the following reagents: hyaluronic acid sodium salt (53747, Sigma), chondroitin sulfate A (C9819, Sigma), dermatan sulfate (also called chondroitin sulfate B, C3788, Sigma) or heparin sodium salt (H4784, Sigma). Cleavage of cell surface glycosaminoglycan chains was established by treatment of the cells (1 h, 37°C) directly prior to the fixation step with the indicated concentrations of Heparinase-I (H2519, Sigma) and Chondroitinase ABC (C2905, Sigma) according to the manufactures recommendations.

To detect the binding of the biotinylated FliD proteins, HRP-labeled Streptavidin (016-030-084, Jackson ImmunoResearch) was used diluted 1:50,000.

Immunofluorescence microscopy

Cells were seeded onto 12 mm circular glass coverslips in a 24-well tissue culture plate and grown to 70% confluence in 36 h. Cells were rinsed, incubated (1 h, 20°C) with 4% BSA in DPBS and then with 20 µg/ml FliD (1 h, 20°C) in DPBS. To visualize FliD binding to the cell surface, the cells were subsequently stained with WGA-Alexa Fluor 488 (Invitrogen) and the FliD protein with the anti-FliD antiserum (dilution 1:1,000), and goat anti-rabbit-IgG conjugated to Alexa Fluor568 (A11036, Invitrogen). Samples were viewed in an Olympus fluorescence microscope.

For visualization of bacterial adherence via the flagellar tip, cells were infected as described above with mCherry-positive *C. jejuni*. Hereto plasmid pMA5-metK-mCherry66 was introduced into the relevant strains. After 1 h of incubation, the infected cells were fixed and blocked as mentioned above. Subsequently, cells were stained with WGA-Alexa Fluor 633 (Invitrogen) and flagella with polyclonal anti-*C. jejuni* antiserum (R624, 1:1000) in combination with goat anti-rabbit-IgG conjugated to Alexa Fluor 488 (A11034, Invitrogen). Pictures were taken at 100x magnification with 2x optical zoom using the Leica SP-II confocal microscope. Brightness and contrast were adjusted using ImageJ software.

ELISA

To show direct interaction between FliD and different classes of glycosaminoglycans, FliD protein in bicarbonate buffer (C3041, Sigma) was coated (2 µg of protein/well, 16 h, 4°C,) onto a 96-well ELISA plates (E Bioscience, 44-2404-21). Wells were washed three times with DPBS and blocked (1 h) with 4% BSA in DPBS. Biotinylated heparin (B9806, Sigma) was added at the indicated concentrations. After 1 h of incubation (20°C), HRP-streptavidin (016-030-084, Jackson ImmunoResearch) diluted (1:10,000) in 2% BSA in DPBS was added. After an additional hour of incubation, wells were washed and incubated with HRP-substrate (555214, BD) for 15 min. The reaction was stopped with 1 M H_2SO_4. Absorbance was measured in a plate reader at 450 nm (Fluorstar Omega, BMG Labtech). For the competition experiment, 50 µg/ml of glycosaminoglycans were pre-incubated with 5 µg/ml of biotinylated heparin (B9806, Sigma) prior to the addition to the FliD-coated wells.

Construction and sortagging of FliD fragments

The plasmid PCP1f*liD-TEV-His* was cleaved with NotI and AscI (Thermo Scientific) to remove the TEV cleavage site. DNA sequences encoding the sortase recognition motif LPETG with a GGGS linker and cleaved NotI and AscI overhangs (underlined) with a phosphorylated 5'-end (5'-P-GGCCGCCGGTGGTGGTGGTTCTCTGCCGGAAACCGGTGG-3' and 3'-CGGCCACCACCACCAAGAGACGGCCTTTGGCCACCGCGC-P-5') were hybridized by heating at a one-to-one ratio (100 µM = 100 pmol/µl) for 5 min at 95°C and then gradually (1°C/min) cooled to 20°C in a PCR thermocycler (Bio-Rad). The generated double stranded DNA was ligated into PCP1, yielding PCP1*fliD-sort-His*. In order to generate FliD fragments, the vector PCP1*fliD-sort-His* was digested with NotI and PacI in order to remove the *fliD* gene. Fragments were constructed by amplifying parts of the *fliD* gene by PCR by using following primer sets with NotI and PacI restriction sites (underlined): FliDΔNC: FliDΔNC-fwd 5'-CCCTTAATTAAATGACGGTTTTTGCGAAAAG-3' and FliDΔNC-rev 5'-AAAGCGGCCGCTTCTATAGGCTTAGAGGTTATATTTAC-3'; Fragment A: FliD-A-fwd 5'-CCCTTAATTAAATGACGGTTTTTGCGAAAAG-3' and FliD-A-rev 5'-AAAGCGGCCGCATTCGAATCTTTTTTTCCCG-3'; Fragment B: FliD-B-fwd 5'-CCCTTAATTAAATGGGTAAATATCAAAAAGATATAAATG-3' and FliD-B-rev 5'-AAAGCGGCCGCAATATTTGAAAAAAAACTTTCAG-3'; Fragment C: FliD-C-fwd 5'-CCCTTAATTAAATGACTAAATACGAAGACATTAATCAC-3' and FliD-C-rev 5'-AAAGCGGCCGCTTCTATAGGCTTAGAGGTTATATTTAC-3'. The PCR products were then digested with PacI and NotI and ligated into the open PCP1*sort-His* vector. After gene expression and protein purification as described above, the products were dialyzed against TBS and biotinylated using the sortase reaction[37]. FliD proteins (6.5 nmoles) were incubated (16 h, 20°C) with equal amounts of *S. aureus* sortase enzyme (pet30b-7M SrtA was a gift from Hidde Ploegh, Addgene plasmid #51141) and 50x molar excess of GGGK-biotin (synthesized as described in[67] to site-specifically biotinylate the C-terminus. Free biotin was removed by dialysis against PBS (16 h, 4°C). The His-tagged sortase was removed using Ni-NTA beads. Biotinylation was confirmed by Western blot using Streptavidin-HRP.

Statistical analysis

Experiments were performed at least three times in duplicate. Results were analyzed for statistical differences using the Prism GraphPad Software (version 6.05) by Student's

unpaired *t*-test, one-way or two-way ANOVA where appropriate. Significance was accepted at $P<0.05$.

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Chapter 3

Maternal chicken antibodies inhibit binding of the *Campylobacter jejuni* flagellar cap protein FliD to host cells

Claudia M. Freitag*, Yaro Laenen*, Katarzyna A. Radomska, Jos P.M. van Putten

Department of Infectious Diseases & Immunology, Utrecht University, The Netherlands

In preparation

*contributed equally

ABSTRACT

Campylobacter jejuni is the leading bacterial cause of human gastroenteritis. Chickens are the main reservoir of the pathogen, however colonization of chicks with *C. jejuni* is delayed due to protective maternal anti-*Campylobacter* antibodies. Previously we showed that the flagellar cap protein FliD of *C. jejuni* is an important host attachment factor during infection. Interestingly FliD is highly immunogenic in chickens and therefore we investigated the possible role of maternally derived FliD-specific antibodies as colonization inhibiting factor. Western blots and ELISAs demonstrated high amounts of maternal IgY antibodies against the flagellar cap protein in the egg yolk. Isolation of anti-FliD antibodies by affinity chromatography showed that they are species specific, but cross-react with different *C. jejuni* isolates, suggesting protection against several *C. jejuni* strains. Pre-incubation of FliD protein with anti-FliD antibodies strongly inhibited FliD binding to cells. We could locate the potential epitopes in the central part of the protein, which also contains the potential cell-binding site(s). Finally, we observed that the antibody titers were highest after hatch and remained detectable during the first two weeks of life, which correlates with the delayed colonization of chicks by *C. jejuni*. In conclusion, our results suggest a role for FliD specific antibodies in the protection against *C. jejuni*. FliD could thus be an interesting target for preventing *C. jejuni* colonization in chicken and infection in humans.

INTRODUCTION

The human pathogen *Campylobacter jejuni* (*C. jejuni*) is the leading cause of bacterial enterocolitis[1,2]. Once acquired, *C. jejuni* penetrates the intestinal mucus and induces a potent mucosal inflammatory response[3]. Disease symptoms include acute diarrhea, fever, and abdominal pain but infection may be followed by serious sequelae such as the neuroparalytic Guillain-Barré syndrome. Chickens are a main reservoir of *C. jejuni* and contaminated poultry meat products are considered an important source of human infection[2]. Despite interventions which are in place to reduce the bacterial load, commercial broiler flocks are often colonized with *C. jejuni*[4,5]. *C. jejuni* colonizes the lower intestinal tract of chickens in high numbers but usually without apparent pathology[3,5]. Nevertheless, colonization of the chicken gut by *C. jejuni* elicits a strong immune response as evidenced by high serum antibody titers[6–9]. Interestingly, newly hatched chickens between the age of 3 days and 2-3 weeks are usually resistant to colonization[10,11]. This resistance at least partially relies on the presence of maternally derived *Campylobacter*-specific antibodies[3,9]. During chick embryonic development, IgY antibodies are transferred from the blood circulation of the hen into the egg yolk of the maturing oocyte[12]. The IgY is then transported across the yolk sac membrane into the bloodstream of the progeny via the yolk sac IgY receptor (FcRY) starting at embryonic day 7[13,14]. The rate of IgY transfer varies during embryogenesis and reaches highest rates at embryonic days 19-21 just prior to hatching[13]. The titer of maternally derived IgY in the serum of the progeny remains high for the first two weeks of life and then declines. Increased permeability of the chicken gut in early life likely allows the IgY to be transported into the gut lumen[6,15]. This way maternal IgY can protect against infections with intestinal parasites[16,17], viruses[18,19] and bacteria[15], including *C. jejuni*[6,7,9].

The mechanism via which maternal antibodies delay intestinal colonization by *C. jejuni* is not fully understood. Analyses of the antigen specificity of the IgY indicates recognition of a large array of *Campylobacter* components[3,6,20]. One major class of immunogenic antigens that have been identified are components of the bacterial flagella[8,20–22]. The flagella of *C. jejuni* (one at each pole) confer motility especially in viscous environments and are vital for colonization of the avian intestine[23,24]. Each flagellum consists of a basal body complex that is linked via a flexible hook filament to the flagellar fiber. This polymeric structure consists of thousands of flagellin (FlaA and FlaB) subunits[25] and carries the flagellar cap protein FliD (also called HAP2) at its distal end. The FliD protein is essential for flagellum polymerization[26] but may also facilitate adhesion of *C. jejuni* to heparan sulfate containing receptors on the eukaryotic cell surface[27]. The FliD-mediated attachment of *C. jejuni* to host cells is inhibited with recombinant FliD protein as well as a soluble receptor analogue (heparin)[27]. Despite its low abundance compared to the flagellin subunit protein, the FliD of *C. jejuni* is an immunodominant protein and antibodies can be detected in colonized birds[28].

Considering the apparent function of FliD in the attachment of *C. jejuni* to host cells and its immunogenicity, we investigated the presence and possible role of maternally derived FliD-specific antibodies as colonization inhibiting factor. In the present study, we successfully isolated maternal FliD-specific antibodies from egg yolk and provide evidence that the antibodies cross-react with FliD from different *C. jejuni* strains but not with FliD of other bacterial enteropathogens. Moreover, the anti-FliD IgYs inhibit binding of *C. jejuni* FliD to eukaryotic cells, and gradually disappear from the animals after the first two weeks of life.

RESULTS

Presence of maternal FliD-specific antibodies

As a first step to investigate the potential protective function of maternal anti-FliD immunoglobulins, we screened egg yolk for the presence of such antibodies. Total IgY was purified from the yolk of four eggs by PEG-precipitation[29] with an average yield of approximately 15 mg/ml protein per egg (**Fig.1A**). The presence of FliD-specific antibodies in these samples was assessed by Western blot. Total lysates of *C. jejuni* strain 81116 and its corresponding Δ*fliD* mutant[27] were separated by SDS-PAGE, blotted, and probed with the isolated IgY. This revealed for all four IgY batches several reactive bands, one of which with the apparent molecular mass of FliD (70 kDa). This band was absent in the FliD-deficient strain (**Fig. 1B**, **arrow**). The FliD reactivity of the IgY samples was further confirmed when recombinant *C. jejuni* FliD protein purified from *E. coli* was used as antigen (**Fig. 1B**). Of note, the isolated IgY barely showed reactivity with recombinant *C. jejuni* flagellin (FlaA) (**Fig. 1B**). This lack of reactivity may be attributed to the well-documented protein sequence variability between strains or the lack of glycosylation of the *E. coli* derived recombinant flagellin used as antigen. Control antisera confirmed the presence of FliD in the wild type and of FlaA in both the wild type and Δ*fliD* mutant (**Fig.1C**).

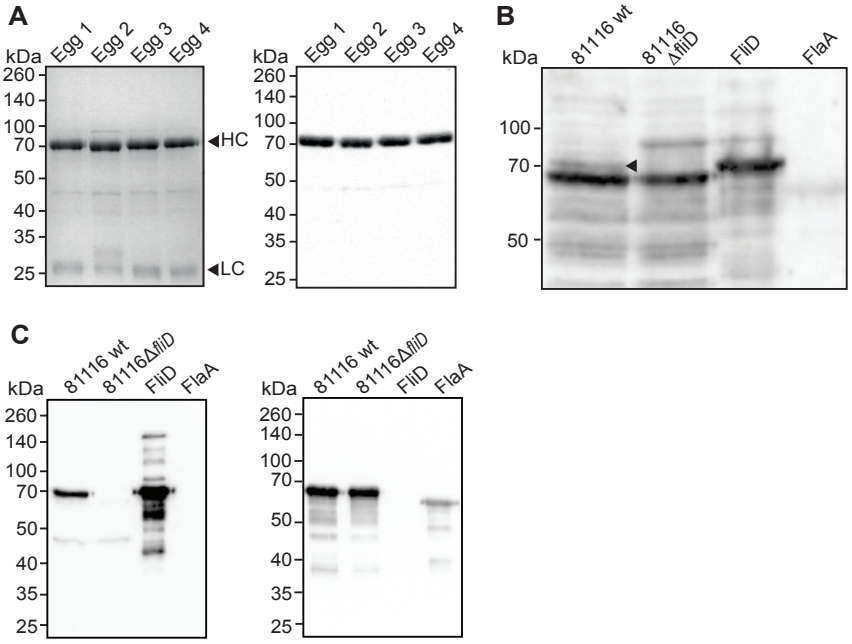

Fig. 1: Purification and FliD reactivity of chicken IgY.
(A) SDS-PAGE (left) of total IgY purified from egg yolk stained with PageBlue. Migration of the 68-kDa heavy chain (HC) and 25-kDa light chain (LC) is indicated. Western blot (right) of the same samples confirming the presence of IgYs by incubation with goat anti-chicken-IgY HRP-conjugated antibodies recognizing only the heavy chain (HC). **(B)** Western blot showing the reactivity of the pooled batches of isolated IgY with bacterial lysates of *C. jejuni* strain 81116, its FliD-negative derivative, and with recombinant *C. jejuni* FliD and FlaA proteins. The arrow indicates the FliD protein that is lacking in the mutant strain. **(C)** Control Western blot with the same samples as in B showing the migration of the FliD and FlaA protein as detected with specific anti-FliD (left panel) and anti-FlaA (right panel) rabbit antisera.

Isolation of *C. jejuni* FliD antibodies

To be able to better characterize the maternal anti-FliD antibodies, we isolated them from the total IgY pool by affinity chromatography. Hereto, recombinant FliD protein was produced with an additional C-terminal LPXTG motif enabling biotinylation by site-specific sortagging (**Fig. 2A**)[27]. The biotinylated FliD was immobilized on streptavidin beads and used as an affinity handle to isolate the FliD-specific antibodies. Analysis of the fractions obtained before and after passage of the IgY over the column using ELISA with purified recombinant FliD or FlaA as antigens confirmed the presence of FliD- and FlaA-specific antibodies in the total IgY pool (**Fig. 2B**) and showed a reduction in anti-FliD titer after column passage. Elution and functional analysis of the retained antibodies demonstrated reactivity with FliD protein but not with FlaA (**Fig. 2C**), indicating successful isolation of FliD-specific antibodies from the FliD coated beads.

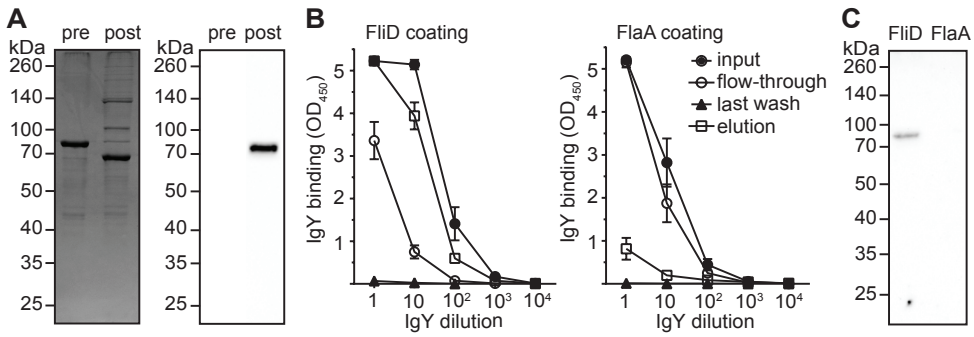

Fig. 2: Isolation and reactivity of *C. jejuni* FliD-specific antibodies.
(A) SDS-PAGE (left panel) and Western blot (right panel) showing the migration of *C. jejuni* FliD before (pre) and after (post) successful biotinylation via the sortagging technique. Biotinylation of FliD was verified with Streptavidin-HRP (right panel). **(B)** Fractions collected during FliD-specific affinity chromatography (input, flow-through, last wash, elution) were analyzed by ELISA. The fractions were titrated against FliD (left panel) or FlaA (right panel) as coating antigens. The concentration of the undiluted IgY was 200 µg/ml for the input and flow through fraction, and 10 µg/ml for the eluted (FliD-specific) IgY fraction. Results are the mean ± SD of three independent experiments. **(C)** Western blot showing the reactivity of the isolated FliD-specific antibodies with recombinant FliD and FlaA.

Species specificity of anti-FliD antibodies

To investigate the cross-reactivity of the isolated maternal FliD-specific IgY between different *C. jejuni* isolates, we cloned the *fliD* genes from the *C. jejuni* strains 108, 81116 and 81-176. All genes were successfully expressed with a C-terminal his-tag and the respective recombinant FliD proteins were purified using NTA (Ni+)-affinity chromatography (**Fig. 3A**). Both Western blotting and ELISA with the different purified FliD protein as antigens demonstrated that IgY antibodies that had been isolated using FliD derived from strain 11168 cross-reacted with the FliD proteins of the other *C. jejuni* strains (**Fig. 3B, Fig. 3C**). These results are consistent with the high level (94-98%) of amino acid sequence conservation of the *C. jejuni* FliD proteins[27]. To ensure that we did not omit antibodies reacting against non-conserved epitopes during the isolation process, we also tested reactivity of total IgY pools against FliD of the different *C. jejuni* strains. All tested eggs contained anti-FliD antibodies against each of the four strains in comparable amounts (data not shown). This suggests the presence of specific antibodies, which are most likely directed against the conserved structures of different *C. jejuni* FliD proteins.

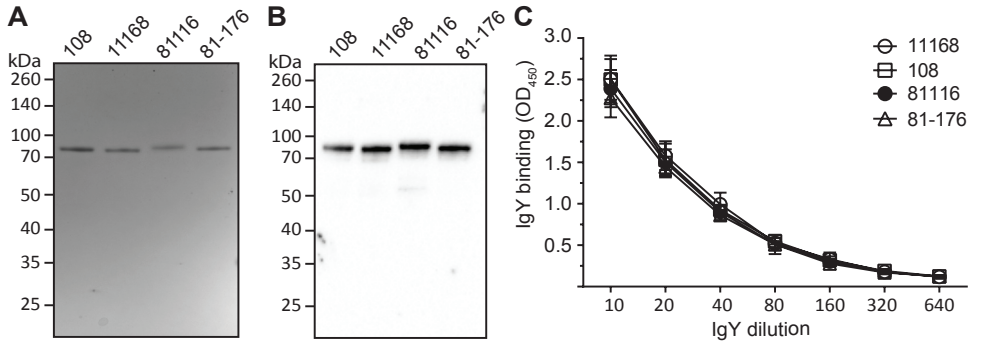

Fig. 3: Cross-reactivity of anti-FliD antibodies among different *C. jejuni* strains.
(A) Electrophoretic mobility (SDS-PAGE) of recombinant *C. jejuni* FliD from *C. jejuni* strains: 11168, 108, 81116 and 81-176 isolated from *E. coli*. Proteins were stained with PageBlue. **(B-C)** Western blot (B) and ELISA (C) showing the reactivity of FliD-specific antibodies purified using FliD of *C. jejuni* strain 11168 with recombinant FliD of above mentioned strains. IgY binding was detected using goat-anti-chicken-IgY-horse reddish peroxidase (HRP). ELISA data represent the mean ± SD of three independent experiments.

As FliD has a conserved function as flagellar cap protein among all flagellated species, we next determined the cross-reactivity of the isolated anti-FliD antibodies using a diverse set of bacterial species. Therefore, we expressed and purified FliD proteins from *Erwinia amylovora*, *Pseudomonas aeruginosa*, *Salmonella* Enteriditis, *Salmonella* Typhimurium, and *Campylobacter iguaniorum* (**Fig. 4A**). Analysis of the reactivity of the isolated FliD-specific antibodies with these FliD proteins by Western blotting revealed specific recognition of the (control) FliD protein of *C. jejuni* but not of the FliD proteins of the other species (**Fig. 4B**). ELISA assays with the set of recombinant FliD proteins as antigens confirmed these results, perhaps except for the very weak reactivity with FliD protein of *Campylobacter iguaniorum* (**Fig. 4C**), which represents the closest relative belonging to the same species as *C. jejuni*. Together, the results suggest that the maternal FliD-specific antibodies in the egg yolk display clear species specificity but are cross-reactive among different *C. jejuni* strains.

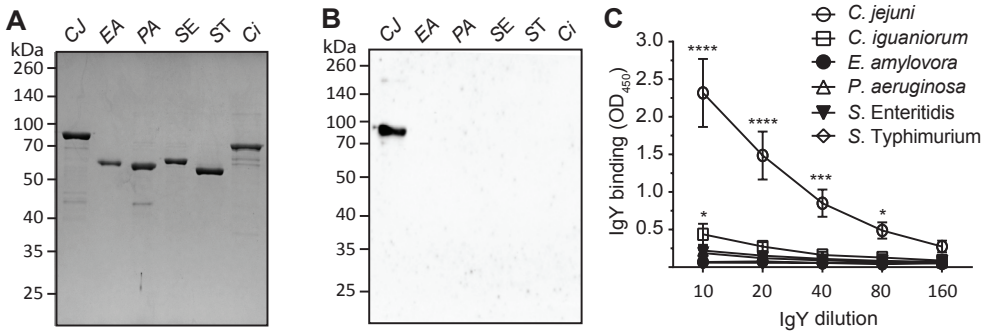

Fig. 4: Species specificity of FliD-specific antibodies.
(A) Electrophoretic mobility of purified recombinant FliD proteins from *C. jejuni* (Cj), *E. amylovora* (EA), *P. aeruginosa* (PA), *S. enterica* serovar Enteritidis (SE), *S. enterica* serovar Typhimurium (ST) and *C. iguaniorum* (Ci) isolated from *E. coli*. Proteins were stained with PageBlue. **(B-C)** Western blot (B) and ELISA (C) showing the reactivity of *C. jejuni*-FliD-specific antibodies with recombinant FliD of the indicated species. IgY binding was detected using goat-anti-chicken-IgY-horse reddish peroxidase (HRP). ELISA data represent the mean ± SD of three independent experiments. Data were analyzed by two-way ANOVA in comparison to the background, (****P<0 .0001, ***P<0.001, *P<0.05).

FliD maternal antibodies inhibit FliD binding to cells

The *C. jejuni* FliD protein can act as a bacterial attachment factor through binding to heparan sulfate containing receptors at the surface of eukaryotic cells[27]. To assess whether the maternal FliD-antibodies in egg yolk are capable to inhibit this process, we incubated purified recombinant biotinylated FliD protein of *C. jejuni* with either the isolated FliD-specific IgY antibodies or, as a control, similarly isolated chicken interferon-specific IgY and added these mixtures to cultured mucosal cells. Quantification of FliD binding after 1 hour of incubation using a cell-based ELISA approach[27] showed that the FliD-specific antibodies inhibited the attachment of FliD to the cells up to 75%. The degree of inhibition correlated with the FliD-antibody concentration (**Fig. 5**). Similarly, rabbit antibodies in anti-FliD antiserum also inhibited FliD binding in a dose-dependent manner, whereas the interferon-specific antibodies did not inhibit the FliD attachment (**Fig. 5**), suggesting that FliD-specific antibodies were required for this inhibition of FliD attachment.

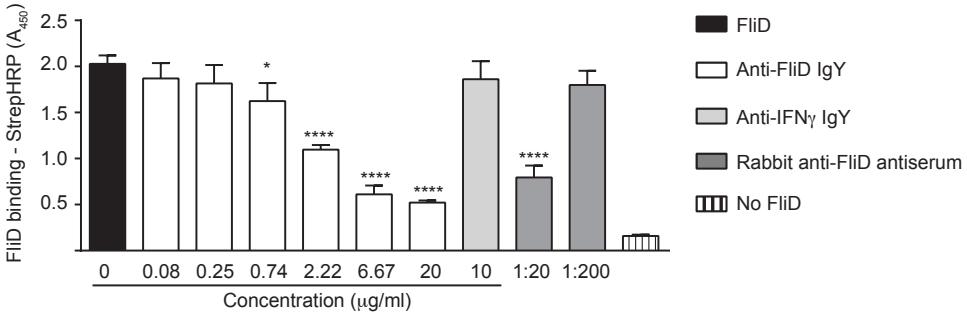

Fig. 5: Inhibition of FliD attachment to eukaryotic cells by FliD-specific IgY.
Purified recombinant biotinylated *C. jejuni* FliD was added to Chang epithelial cells in the absence (black bar) and presence of the indicated concentrations of FliD-specific IgY (white bars), or with control anti-IFNγ-specific IgY antibodies (light grey bars). Different dilutions of anti-FliD antiserum raised in rabbits (dark grey bars) were used as an additional control. Cell attachment of FliD was measured after 1 h of incubation using Streptavidin-HRP. Binding is expressed as absorbance and represents the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA in comparison to the FliD control (****P<0 .0001, *P<0.05).

Epitope characterization

To further substantiate the blocking of the FliD-host cell interaction, we determined the immunogenic region(s) of FliD recognized by the maternal antibodies. As structural data on the FliD of *C. jejuni* does not exist, we first compared the binding of the isolated FliD-specific antibodies to the full length protein (amino acids 1-642), a fragment containing the central region (amino acids 71-555, FliDΔNC), and a protein fragment that lacked the central region and consisted of a fusion of the amino-terminal (amino acids 1-70) and carboxy-terminal (amino acids 566-642) protein parts (FliDΔC) (**Fig. 6A**). All fragments were expressed and purified from *E. coli* using an N-terminal his-tag (**Fig. 6B**). Probing of the blotted protein fragments with the FliD-specific antibodies revealed strong reactivity with the full length FliD and the central part of the protein but not with the fragment lacking this region (**Fig. 6C**). This pointed to the central part of FliD as immunodominant region. As antigen used in an ELISA setting may contain more conformational protein epitopes, we also tested the reactivity of the antibodies towards the proteins coated onto ELISA plates. Again, the FliD-specific antibodies reacted strongly with the full-length protein and FliDΔNC but only weakly

with the FliDΔC construct lacking the central protein domain (**Fig. 6D**). This effect was not due to a coating differences as detected with an anti-His antibody (**Fig. 6D**).

To further pinpoint the antibody recognition sites in FliD, we tested the reactivity of the FliD-specific IgY towards the separate amino- and carboxy-terminal regions and three protein fragments (A, B and C) that together cover the central part of the FliD protein (**Fig. 6A** and **Fig. 6E**). Western blotting demonstrated that the antibodies recognized fragment B (amino acids 210-418) (**Fig. 6F**). ELISA results showed variable reactivity of the antibodies for all protein fragments but strongest reactivity towards fragment B (**Fig. 6G**). We previously demonstrated that this fragment harbors host cell attachment activity[27].

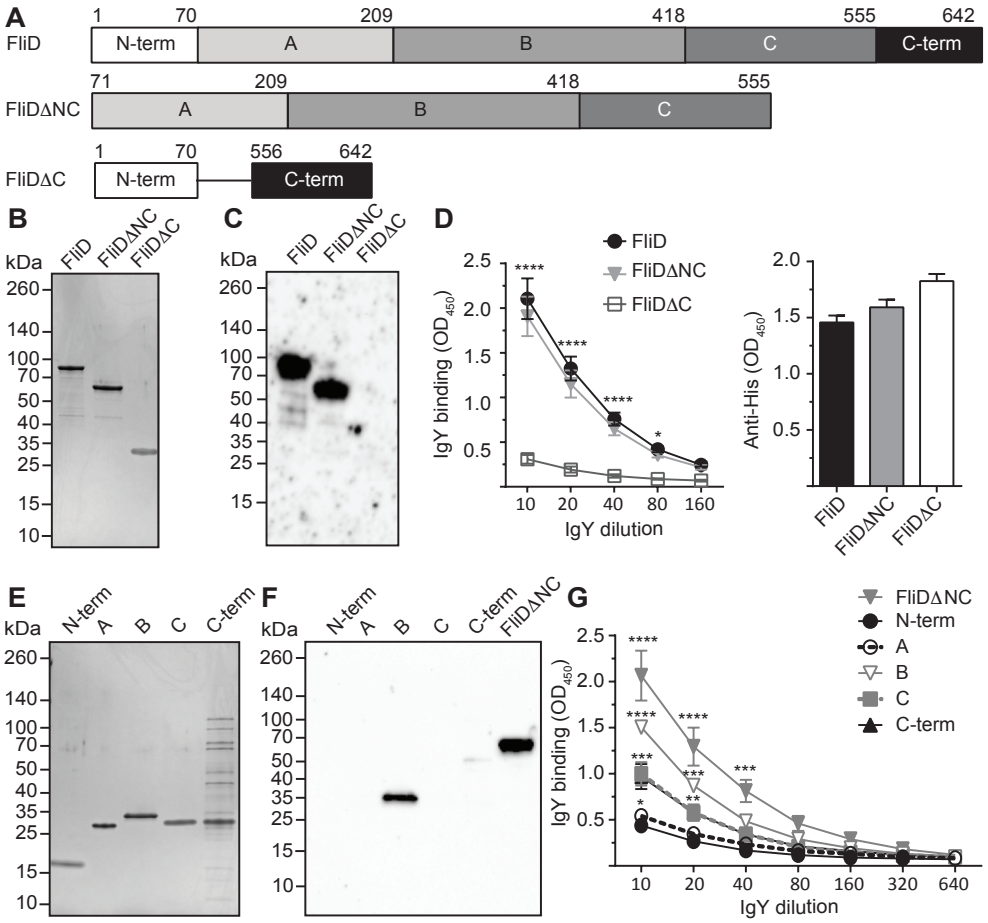

Fig 6. FliD-specific antibodies are directed against the central domain of FliD.
(A) Graphical representation of full length FliD protein of *C. jejuni* and the truncated constructs with the corresponding amino acid numbers. **(B)** SDS-PAGE of purified recombinant full length FliD and the FliD constructs lacking the N- and C-terminal ends (FliDΔNC), or the central region of the protein (FliDΔC). **(C)** Western blot showing the reactivity of the isolated FliD-specific antibodies with full length FliD and the FliD constructs lacking the N- and C-terminal ends (FliDΔNC), or the central region of the protein (FliDΔC). Antibody binding was detected using secondary goat-anti-chicken-IgY conjugated to HRP. **(D)** ELISA showing the binding of the indicated dilutions of the isolated FliD-specific antibodies to FliD, FliDΔNC and FliDΔC (left panel). Antibody reactivity was determined using secondary goat-anti-chicken-IgY conjugated to HRP, and expressed as absorbance values. The right panel shows the coating control as detected with anti-His antibody. Results are the mean ± SD of three independent experiments. Data were analyzed by two-way ANOVA in comparison to the background, differences between FliD full length and

FliDΔNC were not significant. **(E-F)** SDS-PAGE (E) and Western blotting (F) of the indicated purified FliD fragments as described in A. Protein staining and immunodetection were performed as described above (B and C). **(G)** ELISA showing reactivity of isolated anti-FliD IgYs with the indicated purified FliD fragments. Data were analyzed by two-way ANOVA in comparison to the background (****P<0 .0001, ***P<0 .001, **P<0 .01*P<0.05).

Kinetics of FliD-specific maternal antibody reactivity

To investigate the time period at which maternal FliD-specific antibodies may inhibit the attachment of FliD to host cells, we determined the presence of these antibodies in chicken sera of embryos and hatchlings over time. Hereto, chicken sera of 3-7 chickens were collected on embryonic days 18 and 20 and after hatching on days 1, 5, 9, 10 and 14 and subjected to a FliD-specific ELISA. As depicted in **Fig. 7**, FliD-antibodies were barely detected at embryonic day 18, but had increased at embryonic day 20. From the first day after hatch until day 5, the maternal FliD antibody reactivity reached the highest titers, which started to decline after the first week of life. At two weeks post-hatch, maternal anti-FliD IgYs were barely detectable (**Fig. 7**). These results suggest that most effective inhibition of FliD attachment to the host can be expected during the first two week of life.

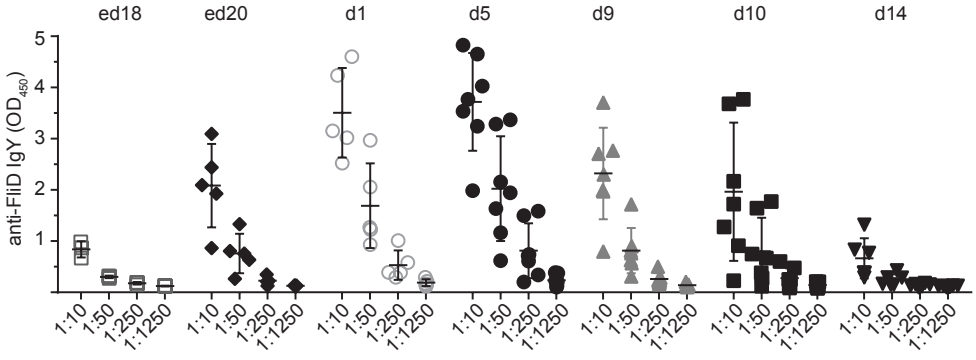

Fig. 7: Maternal FliD-specific antibodies persist during the first two weeks of life.
ELISA showing the presence of FliD-specific antibodies in the sera of 3-7 chicken collected on embryonic days 18 and 20 and after hatching on days 1, 5, 9, 10 and 14. FliD-specific antibodies were detected with secondary goat-anti-chicken-IgY conjugated to HRP. Each dot represents the average of two replicates of one individual chicken, expressed as absorbance values.

DISCUSSION

The flagellar tip protein FliD is essential for *C. jejuni* flagella assembly and bacterial motility[27,30]. Recent work indicates that FliD may also function as an early attachment factor conferring flagella-mediated adhesion to mucosal cells[27]. This perspective led us to examine the presence, role, and species-specificity of maternally derived FliD-specific antibodies. Our results show that maternal IgY isolated from egg yolk contains antibodies directed against the flagellar tip protein FliD of *C. jejuni* and that isolated FliD-specific antibodies inhibit the binding of FliD to eukaryotic cells. The antibodies are predominantly directed against the central part of the FliD protein that interacts with the host cell receptor. The IgY antibodies cross-react with FliD from different *C. jejuni* strains but not with FliD proteins from several other bacterial species. The maternal FliD-specific antibody titers were highest in the first two weeks after hatching and then rapidly declined. These kinetic changes match the

maternal immunity-mediated period of resistance of young chicken to *C. jejuni* colonization. Our results support the concept that the maternal FliD-specific antibodies contribute to protection against colonization by *C. jejuni* in early life.

Specific antibodies directed against the *C. jejuni* FliD protein were initially identified when the total IgY pool purified from egg yolk was tested for reactivity against whole cell lysates of the *C. jejuni* strain 81116 and its FliD-negative derivative. Immunoblotting revealed several reactive bands including a protein of with a molecular mass of approximately 70 kDa that was absent in the Δ*fliD* mutant strain. The reactivity was confirmed when purified recombinant FliD was used as an antigen in ELISA and Western blot. Thus far, maternal antibodies have been reported to recognize a variety of *C. jejuni* antigens, in particular lipopolysaccharide, the major outer membrane protein (MOMP), CadF, and flagellar proteins[20–22,31,32]. To our knowledge the presence of maternal FliD-specific antibodies has not been reported, although such antibodies have been detected in sera of >4 week-old birds[28]. The generation of FliD-specific antibodies during *C. jejuni* colonization may be seen as remarkable considering the very low amount of antigen that is part of the flagellum. Bacterial flagella may consist of up to 20,000 flagellin subunit proteins in comparison to only a few FliD proteins per flagellum[33]. Yet, the maternal IgY did not recognize purified recombinant flagellin. This may be explained by the variation in immunodominant flagellin regions among circulating *C. jejuni* strains and/or by the absence of glycan moieties that are naturally present on *C. jejuni* flagellins but absent in the *E. coli*-derived recombinant protein.

A relevant issue to address in determining the origin of the observed immunoreactivity against FliD was to define the species-specificity of the maternal FliD-antibodies. For this purpose, we isolated *C. jejuni* FliD-specific antibodies from the total pool of IgY using biotinylated recombinant FliD as affinity tag. This showed that the isolated antibodies cross-reacted with recombinant FliD proteins from different *C. jejuni* strains. This finding is in line with the conserved nature (94-99% identity) of the FliD protein among *C. jejuni* strains. Our approach does not exclude that the egg yolk contains additional FliD antibodies directed against the variable region of FliD of the strain that colonized the hens. Most important is that the isolated FliD-specific antibodies did not recognize recombinant FliD proteins from *e.g. S. enterica* or *P. aeruginosa* and reacted only very weakly with FliD of *C. iguaniorum* (35% identity). This suggests that the isolated antibodies likely recognized *C. jejuni*-specific protein epitopes that were absent in the FliD of the other species. The natural *in vivo* immunogenicity of *C. jejuni* FliD resulting in maternal antibody reactivity provides a biological basis for efforts to develop FliD-based *Campylobacter* vaccine[34].

A striking result of our work was the inhibition by the maternally-derived FliD-specific antibodies of the attachment of FliD to eukaryotic cells. *C. jejuni* FliD has recently been proposed to facilitate the early attachment of *C. jejuni* through binding to heparan sulfate containing host cell receptors[27]. This interaction is inhibited by an excess of purified FliD protein or soluble receptor analogue. Mapping of the domain in FliD that is recognized by the inhibitory maternal *C. jejuni* FliD-specific antibodies identified the central domain (amino acids 210-418) of the protein as main antibody target. This domain also contains the host cell binding site[27]. FliD epitope mapping using synthetic peptides in combination with field sera of chicken of 44-52 of age failed to identify shared epitopes[35]. Whether this relates to the use of synthetic peptides rather than (large fragments of) FliD protein and/or

the use of sera instead of purified immunoglobulins remains to be elucidated. Our finding that maternally-derived antibodies can inhibit the attachment of FliD to host cells indicates that this event may contribute to the resistance of young chicks against colonization by *C. jejuni*. At this time the molecular mechanism(s) via which maternal immunity protects new hatchlings against colonization by *C. jejuni* are largely unknown. It has been proposed that maternal antibodies stimulate complement-mediated killing, bacterial agglutination and clearance, inhibition of bacterial motility and/or the blocking of bacteria-host cell interaction[6,20,31]. Our results indicate inhibition of the attachment of the flagellar tip protein FliD to host receptors by maternal antibodies may be a natural protective element. This finding supports the classification of *C. jejuni* FliD as promising candidate vaccine antigen.

Monitoring of FliD-specific antibody titers in chicken sera during the first weeks of life revealed high titers during the first ten days followed by a rapid drop in reactivity at two weeks of age. This antibody kinetics nicely correlates with the well-established resistance of young chicks against colonization by *C. jejuni* during this period[10,11]. The importance of maternal antibodies in the protection against colonization in early life was nicely demonstrated in a study using specific-pathogen-free chicken flocks. This work showed that the progeny of chicken that lacked *C. jejuni* antibodies was more susceptible to colonization by *C. jejuni* at 3 days of age than the offspring of chicken that had been colonized with *C. jejuni* and carried *Campylobacter*-specific antibodies[9]. FliD-specific antibodies were not identified in the sera but whether this is due the minor quantities of FliD present in bacterial flagella has not been investigated.

On the basis of our work, FliD may be considered as an attractive candidate antigen for use in passive or active immunization to reduce *Campylobacter* colonization and transmission. Feeding of 6-days-old chicken with IgY isolated from hyperimmune egg yolk has yielded variable results with regard to protection against colonization[29,36], perhaps related to differences in antibody specificity. Alternatively, FliD may have potential as a vaccine component. Purified protein is highly immunogenic and, as our results indicate, antibodies can inhibit the interaction of the protein with host cells. Active subcutaneous immunization of chicken with a GST-FliD fusion protein has been demonstrated to generate antibodies but provided only transient antibody-independent protection against challenge with *C. jejuni*[34]. To our knowledge, oral immunization with soluble non-GST tagged FliD protein has not been performed.

Overall, our study provides evidence that the egg yolk and young chickens carry maternally-derived *C. jejuni* FliD-specific antibodies that display cross-reactivity among *Campylobacter* and that inhibit the attachment of the protein to eukaryotic cells. On the basis of our results, FliD may be considered an attractive target to reduce bacterial colonization and the chicken as a source of human infection.

MATERIAL AND METHODS

Cell culture and bacteria

Chang human conjunctiva epithelial cells (ATCC CCL20.2) were routinely cultured in 25 cm^2 flask in RPMI 1640 tissue culture medium supplemented with 5% FCS (Gibco) at 37°C and 5% CO2. *Campylobacter jejuni* wild type strain 81116 and 81116Δ*fliD*[27] were grown overnight in heart-infusion (HI) medium (Biotrading) under microaerophilic conditions. One microliter of

culture (OD_{550} 1.5) was used to load onto a 10% SDS-PAGE to test for the presence of anti-*C. jejuni* antibodies in the egg yolk.

IgY purification

IgYs were extracted from egg yolk of biological chicken eggs (purchased at the local grocery store). IgY was isolated by PEG-precipitation as described[29]. Protein concentration was measured using BCA kit (23225, ThermoScientific) and samples stored at 4°C. Purity of isolated IgYs was visualized by SDS-Page protein staining using PageBlue (ThermoScientific) and by Western blot using goat anti-chicken IgY-FC HRP-conjugated antibodies (AAI29P, AbD Serotec, 1:10,000).

Western blot

Proteins (1 µg) were separated by SDS-PAGE and transferred onto polyvinylidene fluoride or nitrocellulose membrane. The membrane was blocked with 4% skimmed milk (ELK, Campina) in Tris buffered saline + 0.05% tween (TBST) for 1 h at room temperature (RT). All further incubation steps were performed in 2% milk in TBST for 1 hour at RT. Membranes were incubated with IgYs (20 µg/ml) and binding was detected using secondary goat anti-chicken IgY-FC HRP-conjugated antibodies (AAI29P, AbD Serotec, 1:10,000).

Isolation of specific anti-FliD IgY with biotinylated FliD

Specific antibodies were isolated from extracted IgY using affinity chromatography. Recombinant FliD was biotinylated by the sortagging technique as described[27]. Then 7 mg of the total IgY pool was incubated with 1 ml streptavidin-agarose beads (20347, ThermoScientific) for 1 h at RT. Beads were washed with 50 ml Dulbecco's PBS (DPBS) and specific IgYs eluted with 900 µl low pH solution (0.1 M glycine, pH 2.8). The eluate was immediately pH neutralized with 100 µl 100 mM Tris (pH 8.5). Protein concentration was measured using BCA kit (5-40 µg/ml) and samples were directly used due to limited stability.

ELISA

Proteins were diluted to 10 µg/ml in bicarbonate buffer (C3041, Sigma) and coated overnight at 4°C onto a 96-well Maxisorp ELISA plate (M9410-1CS, Sigma-Aldrich). Wells were blocked for 1 h with 4% skimmed milk in TBST at RT. IgYs were serially diluted as described in 2% skimmed milk in TBST. IgY binding was subsequently detected using goat anti chicken HRP-conjugated antibodies. Wells were washed 3x with TBST after each step. ELISA was developed using HRP-substrate (555214, BD) for 15 minutes until the reaction was stopped with 1 M H_2SO_4. Color development was measured using a plate reader at 450 nm (FLUOstar Omega, BMG Labtech). The sera from healthy chickens (Ross 308 broilers) were a generous gift from Dr. T. Cuperus and Prof. Dr. H. Haagsman. Sera were taken post mortem at embryonic day 18, 20, and after hatch on days 1, 5, 9, 10, and 14[37,38].

Cloning and purification of proteins

FliD protein and its truncations were constructed and isolated as previously described[27]. In a similar approach, primers were designed to amplify *fliD* genes from *C. jejuni*, *E. amylovora*, *P. aeruginosa*, *S. enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium and *C.*

iguaniorum (Table 1). The genes with NotI and PacI overhangs were cloned into the PCP1-sort expression vector which contains a His-tag for purification[27]. The recombinant FlaA of *C. jejuni* was constructed as described elsewhere[39]. Proteins were expressed in *E. coli* BL21+ with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 hrs. FliD proteins from *C. jejuni*, *P. aeruginosa*, *S*. serovar Enteritidis, *S*. serovar Typhimurium and *C. iguaniorum* were purified under native conditions as previously described[27]. Shortly, pellets were lysed in resuspension buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 1 mg/ml lysozyme, pH 7.4), spun, and supernatant incubated with nickel-coated beads (Ni-NTA Agarose, ThermoScientific) for 1 hr. NTA beads were washed extensively with 40 ml washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.4) and with 40 ml washing buffer + 0.1% Triton-X114. Proteins were eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 400 mM imidazole, pH 7.4 and dialyzed twice against Dulbecco's Phosphate Buffered Saline (DPBS) at 4°C. FlaA from *C. jejuni* and FliD from *E. amylovora* were purified under denaturing conditions. Bacteria were first lysed by sonication, then the pellet was incubated overnight at room temperature in a denaturing buffer (8 M urea, 10 mM imidazole, PBS, pH 7.4) to dissolve inclusion bodies. After incubation with nickel beads and extensive washing with denaturing buffer, proteins were eluted with 8 M urea, 500 mM imidazole, PBS, pH 7.4 and dialyzed twice against DPBS. Protein concentrations were measured using the BCA kit and stored at -20°C.

Table 1: Primers used in this study

Campylobacter jejuni strain 11168 (GI:112359906), 108 (internal database), 81116 (GI:157385793) and 81-176 (GI:87250070	fwd	5'-CCTTAATTAAATGGCATTTGGTAGTCTATCTAGTTTAG-3’
	rev	5'-CCGCGGCCGCATTATTAGAATTGTTTGCCGCATTAATCATATTAG-3'
Erwinia amylovora (GI:490260894)	fwd	5'-CCTTAATTAAATGGCTAGTATTTCTACTTTAGGCATC-3'
	rev	5'-CCGCGGCCGC CTTACTGGAACTGCTTGAGGTTGAAAAC -3'
Pseudomonas aeruginosa (GI:2276418)	fwd	5'-CCTTAATTAAATGGCGAACAGTACGACG-3'
	rev	5'-CCGCGGCCGCGCTTTTCTTCACAAGGCCAGGC -3’
Salmonella enterica serovar Enteritidis (GI:206708330)	fwd	5'-CCTTAATTAAATGGCTTCAATTTCATCATTAGGTG-3'
	rev	5'-CCGCGGCCGC GGACTTGTTCATAGCATTAAATTGCTGGG -3’
Salmonella enterica serovar Typhimurium (GI:301158474)	fwd	5'-CCTTAATTAAATGGCTTCAATTTCATCATTAGGTG-3'
	rev	5'-CCGCGGCCGC GGACTTGTTCATAGCTGTAAATTGCTGG-3’
Campylobacter iguaniorum (GI:669187914)	fwd	5'-CCTTAATTAAATGGCAGTAGGTAGCGTAAC-3
	rev	5'-CCGCGGCCGC GTTGCTATTACTTGCTTGTTCGATCATTG-3’

Cell-based ELISA with IgY based inhibition of FliD binding

FliD binding to cells was performed as described with three washing steps after each incubation[27]. Shortly, Chang cells were grown to full confluence in a 96-wells plate, fixed, and blocked with 4% bovine serum albumin (BSA, Sigma) in DPBS. Biotinylated FliD was pre-incubated with serially diluted anti-FliD IgYs for 45 min and added to the cells for 1 h at RT to allow binding. Next, bound FliD was detected with HRP conjugated Streptavidin (Jackson ImmunoResearch) for 1 h at RT. The reaction using HRP-substrate was stopped after 30 min and color development was measured using a plate reader at 450 nm.

Statistical analysis

Experiments were performed at least three times in duplicate. Results were analyzed for statistical differences using the Prism GraphPad Software (version 6.05) by one-way ANOVA or two-way ANOVA. Significance was accepted at $P<0.05$.

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Chapter 4

Milk caseins block *C. jejuni* FliD binding to cells

Claudia M. Freitag[1], Dominique Thies-Weesie[2], Jos P.M. van Putten[1]

[1]Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

[2]Van 't Hoff Laboratory for Physical and Colloid Chemistry, Debye Institute for Nanomaterials Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

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ABSTRACT

Campylobacter jejuni (*C. jejuni*) is the most prevalent bacterial foodborne pathogen. Breast-feeding is associated with reduced *C. jejuni* infections in infants. This has been attributed to protective milk oligosaccharides and/or maternal antibodies. We recently identified the flagellar cap protein FliD of *C. jejuni* as early bacterial attachment factor. Hence we investigated whether milk was able to block this interaction. Pre-incubation of FliD with milk blocked FliD attachment to host epithelial cells in a dose-dependent manner. Fractionation and characterization of the milk identified caseins rather than oligosaccharides or secretory antibodies as inhibitory compounds. Strong inhibition of FliD attachment to cells was obtained with isolated α-, β- as well as κ-caseins. Dephosphorylated caseins were unable to block FliD binding, suggesting that the phosphoryl serine groups in caseins, which sequester calcium and other metal ions, are critical for inhibition of FliD binding. Indeed, inhibition of FliD binding was mimicked by the presence of the calcium chelator EDTA and enhanced in the presence of high concentrations of calcium and magnesium. Analytical ultracentrifugation of recombinant FliD protein indicated similar sedimentation velocity profiles in presence of EDTA or extra calcium, suggesting that the interaction with the host cells rather than the multimerization of the FliD protein complex was affected by the treatment. Adhesion assays with intact *C. jejuni* rather than purified FliD protein showed that caseins reduced *C. jejuni* attachment to cells by 50%. Together, our data indicate that milk caseins can block the binding of the flagellar tip protein FliD of *C. jejuni* to host heparan sulfate receptors and that this event is likely mediated via the sequestering of critical divalent cations.

INTRODUCTION

Bacterial foodborne infections pose a major health risk, especially to infants in developing countries. *Campylobacter jejuni* (*C. jejuni*) is the most prevalent foodborne pathogen causing severe human diarrheal disease[1]. The bacterium uses the chicken gut as main reservoir. Human infection often follows the consumption of contaminated poultry meat products or tainted water. Theoretically, hygienic manners and good cooking practice should reduce the infection incidence but the estimated hundreds of millions of cases of *Campylobacter*iosis worldwide proof this to be insufficient. There is still no effective vaccine against *C. jejuni* for use in chicken or humans. However, at very young age, infants may at least partially be protected against *C. jejuni* infection via maternal immunity and breast feeding[2–4], indicating potential for milk as source of anti-infective compounds.

Milk is a complex fluid that contains carbohydrates, fat, proteins, and minerals. Milk oligosaccharides and glycoproteins are able to modulate the newborn's immune system by reducing acute phase cytokine levels stimulated by pathogen-associated molecular patterns[5] and shape the developing microbiome in the infant[6]. Milk glycans may also act as soluble decoy receptors preventing bacterial adhesion to host cells[2]. Milk oligosaccharides have been demonstrated to inhibit the binding of *C. jejuni* to fucosyl-lactose expressing CHO cells[7]. Milk proteins can be classified into two major groups on the basis of their solubility: the water-soluble whey proteins and the casein phosphoproteins, which form colloidal particles termed casein micelles. The whey proteins are quite diverse and include immunoglobulins, serum albumin, α-lactalbumin[8] and, in non-human species, β-lactoglobulin[9]. Some of the whey proteins such as the iron-scavengers lactoferrin have antimicrobial activity[10].

Casein micelles are composed of α_{S1}-, α_{S2}-, β- and κ-caseins. These proteins self-assemble due to their disordered structure into gel-like micelles with particle sizes of 50-250 nm[11,12]. The most recent model describes that the nanogel particles are comprised of non-defined aggregates of caseins which interact weakly with each other and form a more open and porous protein matrix embedding calcium phosphate[13,14]. Although all types of caseins can be found throughout the micelle, the common idea is that the outer micelle layer is enriched with κ-caseins, which stabilize the casein micelle structure and solubility due to their glycosylation pattern[15,16]. The primary function of the casein micelles is the transport of calcium phosphate. The delivery of high amounts of the water-insoluble calcium phosphate to the newborn is crucial for the development of bones and teeth[17]. Caseins retain calcium through the presence of clusters of phosphoseryl residues. Dephosphorylation of caseins results in impaired calcium-binding capacity[18]. Besides the casein-bound calcium, the micelles also contain inorganic calcium phosphate representing 50% of all calcium, 30% magnesium and 10% citrate present in milk, which can diffuse between the micelle and the soluble whey fraction[19–21].

In the present study, we searched for milk components that may inhibit *C. jejuni* adhesion to host cells. In this work, we focused on the inhibition of the cellular binding of the flagellar cap protein FliD, which we recently identified as early *C. jejuni* adhesion[22]. The FliD protein of *C. jejuni* is located at the tip of the flagella and binds to host cell glycosaminoglycan receptors. The binding of FliD and *C. jejuni* is inhibited by removal of the receptors and by the soluble receptor analogue heparin. Here we provide evidence that milk proteins, and more specifically, the casein proteins can act as inhibitor of FliD binding and *C. jejuni* adhesion to host cells, thereby possibly contributing to the prevention of *C. jejuni* infection in breast-fed infants.

RESULTS

Milk blocks FliD binding to cells

In order to assess whether milk may provide protection against *C. jejuni* infection by inhibiting the attachment of the flagellar tip protein FliD to host cells, we tested the binding of recombinant FliD protein to cultured epithelial cells in the absence and presence of different concentrations of milk. Cell-bound FliD was quantified using an ELISA-like experimental setup that employs anti-FliD antibodies to determine the attachment of FliD to the eukaryotic cells[22]. Pre-incubation of FliD (2 µg) with fresh cow milk or commercially available skim milk indeed blocked subsequent FliD binding to cells in a dose-dependent manner (**Fig. 1A** and **Fig. 1B**). Half-maximal inhibition of binding was observed at the low concentrations of 0.1% of fresh milk and 0.05% of skim milk. Maximal inhibition of FliD binding was obtained with 2.5-5% of milk. In humans, protection against *C. jejuni* has been associated with specific anti-*Campylobacter* antibodies in milk[4,23]. Hence we searched for the presence of anti-FliD antibodies in bovine milk. ELISA using recombinant FliD as antigen detected low amounts of anti-FliD antibodies in 10% fresh milk, but not in 1% milk. Skim milk (1-10%), which showed even stronger blocking activity than fresh milk, entirely lacked detectable amounts of FliD antibodies at all dilutions (1-10%) tested (**Fig. 1C**). These results indicate that other milk constituents than FliD-antibodies are responsible for the inhibition of FliD binding to cells.

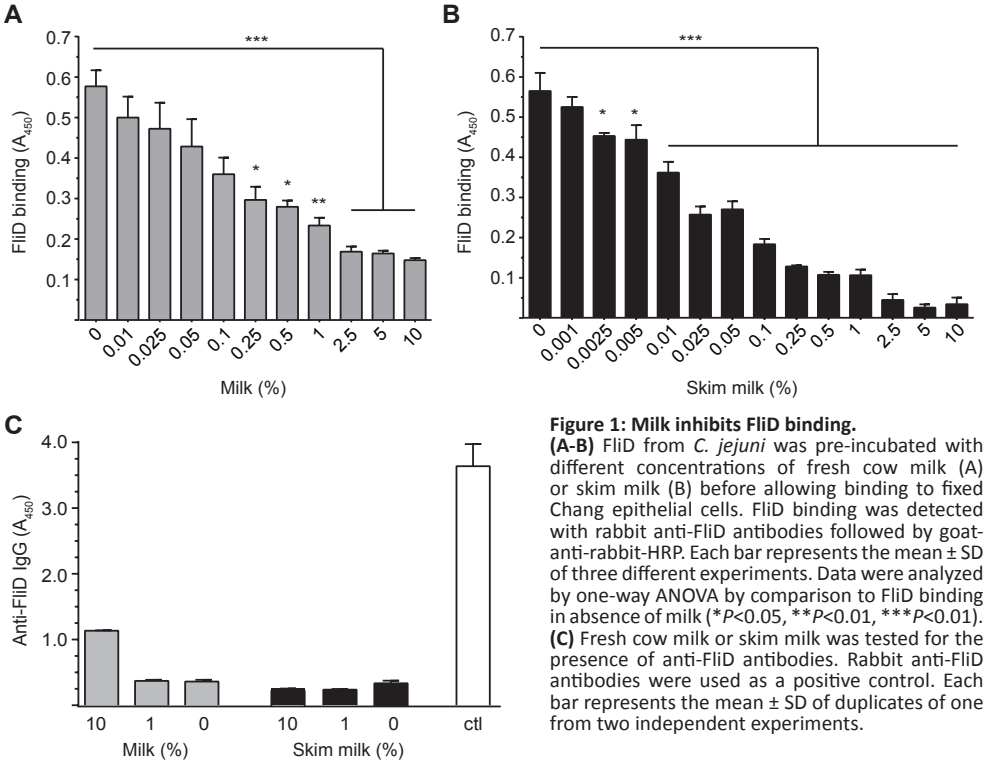

Figure 1: Milk inhibits FliD binding.
(A-B) FliD from *C. jejuni* was pre-incubated with different concentrations of fresh cow milk (A) or skim milk (B) before allowing binding to fixed Chang epithelial cells. FliD binding was detected with rabbit anti-FliD antibodies followed by goat-anti-rabbit-HRP. Each bar represents the mean ± SD of three different experiments. Data were analyzed by one-way ANOVA by comparison to FliD binding in absence of milk (**P*<0.05, ***P*<0.01, ****P*<0.01). **(C)** Fresh cow milk or skim milk was tested for the presence of anti-FliD antibodies. Rabbit anti-FliD antibodies were used as a positive control. Each bar represents the mean ± SD of duplicates of one from two independent experiments.

Characterization of the inhibitory fraction in milk

As a first step to identify the inhibitor of FliD binding in milk, we attempted to clarify the nature of the blocking agent. Heating (100°C, 15 min) of the (skimmed) milk or size exclusion by dialysis (10 kDa cut-off) did not abolish the blocking activity. Similarly, treatment with periodate (10 mM, 30 min) to oxidase carbohydrates had no effect (data not shown). These results suggested that the inhibitory factor was larger than 10 kDa in size, relatively heat stable, and not of glycan structure.

Milk proteins can be divided in two main groups: the caseins and the whey proteins. To determine whether the inhibitor belongs to one of these groups of proteins we separated the milk caseins from the whey proteins by pH precipitation[24]. Lowering the pH of fresh cow milk to 4.6 resulted in precipitation of mainly caseins, whereas the whey proteins remained in the soluble fraction (**Fig. 2A**). Incubation of FliD with each fraction reconstituted to their original volume and pH, showed that the FliD-inhibitory activity in the milk was retained in the casein fraction (**Fig. 2B**). Pre-incubation of FliD with the whey protein fraction yielded similar FliD binding activity as observed in the absence of milk (**Fig. 2B**). Similar fractionation experiments with skim milk confirmed that the casein fraction but not the whey proteins blocked FliD attachment to host cells in a dose-dependent fashion (**Fig. 2D**).

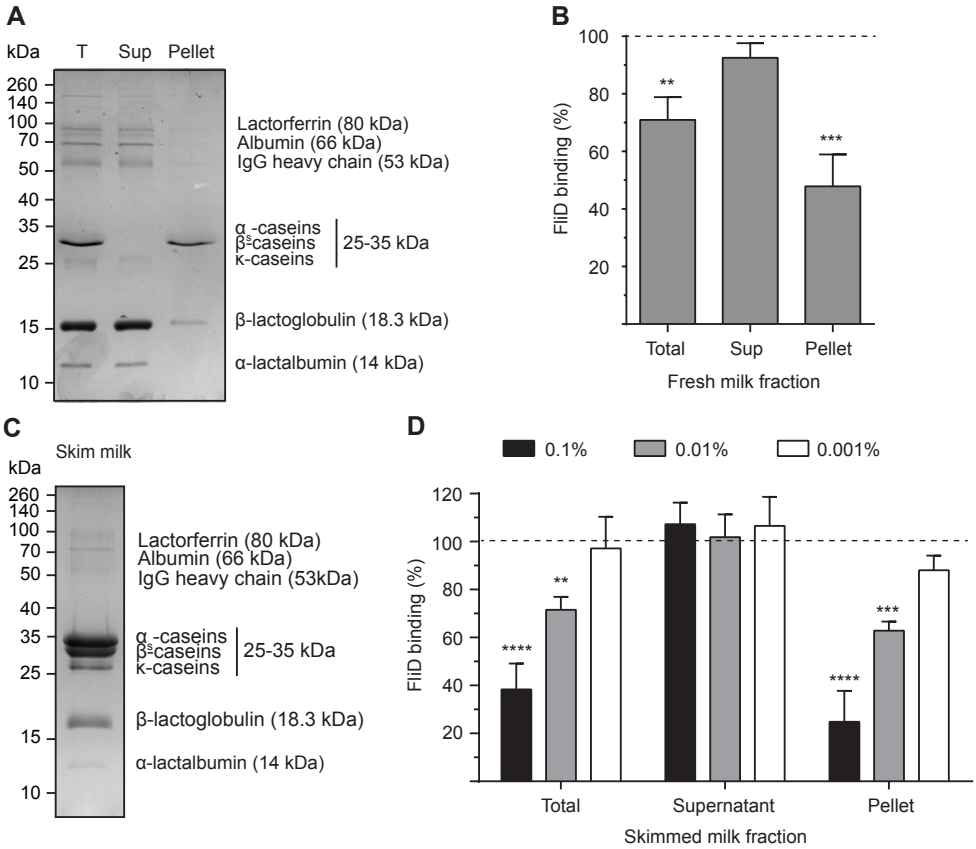

Figure 2: Characterization of inhibitory fraction in fresh cow milk and skim milk.
(A) Fresh cow milk was precipitated by lowering the pH to 4.6. The pellet was resuspended to the same volume with water and the pH of both fractions readjusted to 6.8. All fractions were separated by SDS-PAGE and

proteins were stained with PageBlue. **(B)** FliD was pre-incubated (1 h, RT) with each fraction (derived from 1% milk) and FliD binding to cells was detected with anti-FliD antiserum. The casein-containing pellet fraction but not the whey fraction (supernatant) inhibited FliD binding to cells. Each bar represents the mean ± SD of three different experiments. Data were analyzed by one-way ANOVA by comparison to FliD binding in absence of milk (***P*<0.01, ****P*<0.01). **(C)** Skim milk (12%) was separated on SDS-PAGE and proteins were stained with PageBlue. **(D)** Skim milk (12%) was treated as described in A and all fractions tested for FliD inhibition as described in B. Only the casein fraction (pellet) contained the inhibitor. Each bar represents the mean ± SD of three different experiments. Data were analyzed by two-way ANOVA by comparison to FliD binding in absence of milk (***P*<0.01, ****P*<0.01*****P*<0.001)

Casein phosphoserine-dependent inhibition of FliD binding

To seek direct evidence that caseins are capable to block FliD attachment to host cells, we tested the inhibitory activity of three types of milk caseins, termed α-, β- and κ-caseins. Incubation of FliD with different concentrations (25-2,500 μg/ml) of each of the caseins demonstrated that all proteins efficiently blocked FliD binding in a dose-dependent manner (**Fig. 3A**). Control experiments with the whey protein lactoglobulin showed no effect on FliD binding (**Fig. 3B**), suggesting casein-specific inhibitory activity. Amino acid sequence alignment of all bovine caseins shows no conserved motif shared between the caseins that

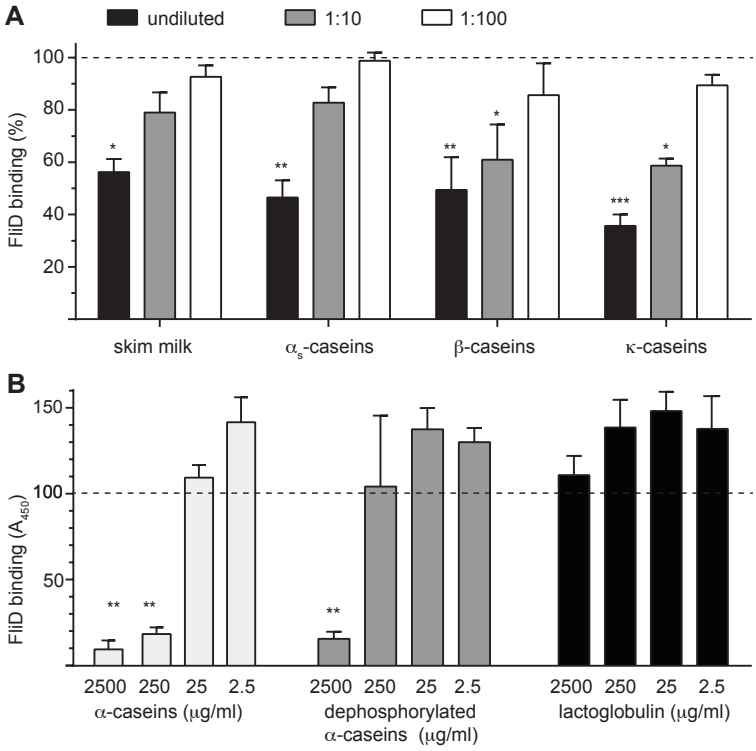

Figure 3: Caseins block FliD binding to cells due to their phosphorylated serines.
(A) FliD was pre-incubated (1 h, RT) with different concentrations of skim milk (starting at 1%) or caseins (starting at 2.5 mg/ml) prior allowing binding to cells. FliD binding was detected with anti-FliD antiserum and goat anti-rabbit-HRP. All classes of caseins block FliD binding. Each bar represents the mean ± SEM of three different experiments. Data were analyzed by two-way ANOVA by comparison to FliD binding in absence of milk (**P*<0.05, ***P*<0.01, ****P*<0.01). **(B)** FliD was pre-incubated (1 h, RT) with different concentrations of α-caseins (light grey bars), dephosphorylated α-caseins (dark grey bars) or lactoglobulin (black bars) prior allowing binding to cells. FliD binding was detected with anti-FliD antiserum. Each bar represents the mean ± SD of at least two different experiments. Data were analyzed by Student's *t*-test by comparison to FliD only control (100%) (***P*<0.01).

could explain the general inhibition of FliD binding by the different types of caseins (**Suppl. Fig. 1**).

Caseins are rich in phosphoserine groups that sequester and transport high amounts of calcium and magnesium[19] and thereby increase the uptake of calcium in the intestine[18,25]. To investigate the possible involvement of the casein phosphoserine clusters in the inhibition of FliD binding, we tested the blocking effect of dephosphorylated caseins. Dephosphorylated α-caseins failed to abolish the binding of FliD to eukaryotic cells at concentrations of 25 and 250 µg/ml (**Fig. 3B**) in opposite to the phosphorylated counterparts. At very high concentrations (2,500 µg/ml), non-phosphorylated casein did inhibit FliD binding which may be attributed to the presence of residual phosphoserine groups. Together, the results suggest that phosphoserine groups on the caseins are of major importance for inhibition of FliD binding to host cells (**Fig. 3B**).

Cation-sensitive binding of FliD to host cells

As the main function of phosphoserines on caseins is to bind and transport cations like calcium and magnesium, we tested whether the cation-chelating activity of the phosphoserines was responsible for the inhibition of the FliD binding properties. Binding of FliD to cells in DPBS containing 0.9 mM of $CaCl_2$ and 0.5 mM of $MgCl_2$ was much higher than in PBS lacking these cations (**Fig. 4A**). Similarly, the addition of 1 mM of EDTA to Ca/Mg containing PBS nullified the positive effect of the cations on FliD binding (**Fig. 4A**). The presence of 1 mM of $CaCl_2$ and 1 mM of $MgCl_2$ increased FliD binding in PBS in an agonistic manner (**Fig. 4B**). These results demonstrate that FliD binding is at least partly dependent on the presence of cations.

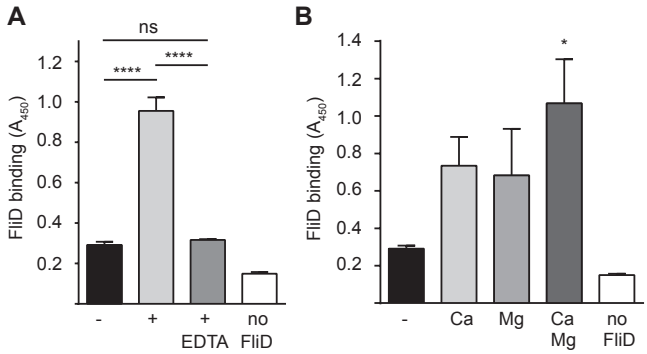

Figure 4: FliD binding increases in the presence of calcium and magnesium.
(A) FliD was diluted in PBS without Ca/Mg ("-", black bar) or in DPBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in the absence ("+", light grey bar) or presence of 2 mM of EDTA (dark grey bar) while incubation with Chang cells. FliD binding was detected with rabbit anti-FliD antibodies followed by goat-anti-rabbit-HRP. **(B)** FliD was incubated in PBS without Ca/Mg ("-", black bar), or with additional 1 mM of mM $CaCl_2$ (light grey bar), 1 mM of $MgCl_2$ (middle grey bar), or both (dark grey bar), while allowing FliD binding as described in (A). All bars represent mean data from at least three independent experiments with SEM. Statistical significance was calculated using one-way ANOVA (*P<0.05, ****P<0.0001).

Calcium-insensitive FliD multimer formation

One hypothesis that explains the observed inhibition of FliD attachment to host cells is that the phosphorylated caseins as well as EDTA chelate cations required for FliD binding activity. Another reason could be that FliD structure changes upon cation removal. To test the latter hypothesis, we investigated whether calcium influenced FliD multimer formation. Hereto a

truncated form of FliD lacking the disordered N and C-terminal domain (FliDΔNC) but still capable to bind to host cells[22] was subjected to analytical ultracentrifugation (AUC). FliDΔNC was diluted in DPBS in the absence or presence of additional calcium (1.5 mM) or EDTA (10 mM). As shown in **Fig. 5**, these molecules barely influenced the apparent multimeric state of the protein, suggesting that the cation-sensitivity of FliD binding is probably not caused by a change in FliD multimer formation.

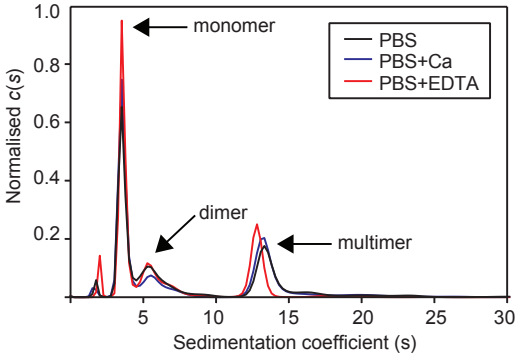

Figure 5: Cation-insensitivity of the FliD protein complex.
Sedimentation velocity (SV) profiles measured by analytical ultracentrifugation (AUC) of FliDΔNC protein (1.88 mg/ml) in presence of calcium (1.5 mM) or EDTA (10 mM).

Caseins block calcium dependent *C. jejuni* attachment

Finally, we investigated whether caseins could block the attachment of intact *C. jejuni* to cultured host cells. Hereto, epithelial cells were incubated with *C. jejuni* strain 81116 (10^8 CFU ml^{-1}) in the absence or presence of caseins (20 mg/ml). After 2 h of incubation, the cells were rinsed and the number of adherent bacteria determined by plating and counting of colony forming units. The presence of physiological concentration of caseins inhibited *C. jejuni* adhesion by 50% (**Fig. 6A**). A similar inhibition (60%) of bacterial attachment was observed after the addition of EDTA (**Fig. 6B**). When additional calcium and magnesium was added to the adhesion assay, a 3-fold increase in *C. jejuni* adhesion to the cells was measured in comparison to PBS alone (**Fig. 6C**). These results indicate that caseins as well as EDTA at least partially reduce and that cations enhance *C. jejuni* adhesion to host cells. These findings resemble the adhesion characteristics of purified recombinant FliD.

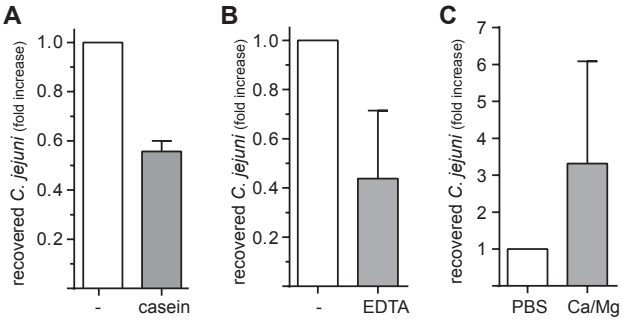

Figure 6: Caseins block calcium- dependent *C. jejuni* infection of host cells.
(A) Epithelial cells were incubated with *C. jejuni* strain 81116 in the absence (white bar) or the presence of mixed caseins (30 mg/ml, grey bar). The number of cell-associated bacteria after 2 h of incubation was recovered as colony forming units (CFU after 48 h) on saponin blood agar plates and counted. Each bar represents mean ± SEM of two experiments expressed as the relative fold increase in comparison to the control (-). **(B)** Infection assay as described in A performed in DPBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in the absence (white bar)

or the presence of 5mM EDTA (grey bars). Each bar represents mean ± SEM of three experiments expressed as the relative fold increase in comparison to the control (-). **(C)** *C. jejuni* strain 81116 was incubated with epithelial cells in PBS without Ca/Mg (white bar) or PBS supplemented with 1mM of $CaCl_2$ and 1mM of $MgCl_2$ (grey bar). Cell associated bacteria were recovered as described in A. Each bar represents mean ± SEM of three experiments expressed as the relative fold increase in comparison to the control (PBS).

DISCUSSION

Milk is a complex bodily fluid with a variety of biological functions[26,27]. Besides a source of nutrients, minerals and vitamins, milk contributes to shaping of the immature immune system of the newborn and can protect against enteropathogens. Here we provide evidence that milk inhibits the attachment of the principal bacterial foodborne pathogen *C. jejuni* to eukaryotic cells and blocks the binding of the flagellar cap protein FliD of *C. jejuni* to host cell heparan sulfate receptors. We identified milk caseins rather than secretory anti-*C. jejuni* antibodies as inhibitory factor. Caseins appeared to exert their effect though their calcium and magnesium chelating properties rather than via glycan moieties that inhibit e.g. *Helicobacter pylori* adhesion[28]. Our findings imply that milk caseins can inhibit bacterial adhesion via different mechanisms.

Breast-feeding has been associated with a lower incidence of *C. jejuni* infections in infants[4]. This protective effect of human milk has been linked to the presence of secretory anti-*C. jejuni* antibodies[29]. As we recently identified the flagellar tip protein FliD of *C. jejuni* as early attachment factor[22], we tested the effect of skim milk and fresh cow milk on the attachment of FliD and *C. jejuni* to host cells. Indeed milk was capable to inhibit the interaction of FliD and the intact bacteria with the host cells. However, this effect was unlikely caused by the presence of *C. jejuni*-FliD specific maternal antibodies as none or only low amounts of both contained FliD-antibodies were detected in milk powder and fresh cow milk, respectively. The limited amount of FliD antibodies is consistent with the general low level of anti-*C. jejuni* antibodies in milk of non-immunized cows[30].

Another potential constituent of milk that may provide protection against *C. jejuni* infection are glycan structures. Human milk glycoproteins (HMGPs) have been shown to block the interaction of several pathogens to host cells[31,32]. For instance, mucin-1 and mucin-4 derived from milk inhibit the invasion of bacteria such as *S*. Typhimurium[33] or virus entry to intestinal cells [34,35]. Similarly, bile-salt-stimulated lipase in milk blocks the binding of capsids of Norwalk virus to its carbohydrate ligand on the host cells[36] . Inhibition of rotavirus infection is seen with the human but not bovine for the glycoprotein lactadherin. This variable inhibition is mostly due to different glycosylation in the two species[34]. Besides glycoproteins, free human milk oligosaccharides (HMO) with concentrations up to 60 g/L composed of glucose, galactose, N-acetylglucosamine, L-fucose and sialic acid[37] may directly interact with specific pathogens. These glycans may prevent microbial adhesion by mimicking the carbohydrate structures on host receptors[38]. *C. jejuni* is also known to interact with glycans on the host cells [39]. Studies with fucosylated H-2 antigen overexpressing cells suggest that the H-antigen (Fucα1, 2Galβ-1, 4GlcNAc) on intestinal cells is used as attachment site by *C. jejuni*[7]. Further glycan arrays showed that *C. jejuni* is able to bind different carbohydrate ligands depending on the environment and growth conditions. It has been hypothesized that mannose and sialic acids are required for initial contact with host cells, whereas colonization follows interaction with fucose and terminal galactose residues[40]. Particularly relevant here is that

milk has been reported to inhibit *C. jejuni* infection. Especially fucosyllactose (2'FL) which is highly abundant in human milk, did inhibit *C. jejuni* binding and subsequent infection *in vitro* and in a mouse model[7]. Clinical studies showed that *C. jejuni* diarrhea was reduced in infants breast-fed with milk containing high amounts 2'FL [41,42]. In the present study, we found that the inhibitory effect of milk on the attachment of the FliD protein and *C. jejuni* was not sensitive to carbohydrate oxidizing agent metaperiodate and resided in the casein fraction rather than whey fraction that contains most oligosaccharides.

Characterization of the blocking fraction and the use of purified proteins identified caseins as the primary inhibitor of the interaction of FliD and *C. jejuni* to eukaryotic cells. Caseins constitute up to 50% of total milk proteins and are able to sequester large amounts of calcium and magnesium due to presence of a large number phosphorylated serine structures[18]. Skim milk contains more caseins in comparison to the fresh cow milk[43], which correlates with the higher potential to block FliD binding to cells (**Fig. 1A**). Caseins have previously been implicated as attachment inhibitory factors. Phosphorylated forms of β-caseins isolated from human milk were shown to reduce the adhesion of *Haemophilus influenza* to pharyngeal cells[44] and the adhesion of Streptococci[45], probably via glycan moieties of caseins[46]. K-caseins have been reported to inhibit the adhesion of *H. pylori* to human gastric cells. This effect has been attributed to receptor-mimicking fucosyl-residues that are present on certain K-caseins[28]. In our case, inhibition of FliD and *C. jejuni* attachment was observed for all types of caseins and showed to be insensitive to treatment with metaperiodate, pointing to a different inhibitory mechanism.

Our findings suggest that caseins inhibit FliD binding to cells most likely due to their calcium-chelating activity. This hypothesis is based on the inability of dephosphorylated caseins, which bind much less divalent cations, to block the interaction. Additionally, we found that chelation of divalent actions by EDTA instead of caseins reduced FliD binding, whereas additional calcium and magnesium enhanced FliD binding. In search for a plausible explanation of these observations, we determined the effect of EDTA and divalent cations on the multimeric state of the FliD protein complex bearing in mind that the major outer membrane protein (MOMP) of *C. jejuni* requires the presence of calcium for its trimerization and function[47]. Analytical ultracentrifugation experiments with the functional *C. jejuni* FliDΔNC protein revealed no evidence of a cation-sensitive change in the protein complex, suggesting an alternative mechanism of inhibition.

As FliD from *C. jejuni* binds to heparan sulfate on the cell surface[22], it can be envisioned that caseins and divalent cations exert their inhibitory effect by altering the FliD receptor molecules. Studies with small heparin oligosaccharides show binding of calcium or other ions to the ion binding sites most likely situated between the glucosamine and iduronate residues of the glycosaminoglycan[48]. The interaction with metal ions such as Mg, Ca, Zn, Fe, Ni and Mn induces a conformational change, which stiffens and stabilizes the heparin molecule[49]. Consequently, cations play a functional role for the heparin/heparan sulfate interactions with surface receptors. In the case of fibroblast growth factor receptor, calcium and heparan sulfate are cooperating to modulate the activity of the receptor complex. In absence of fibroblast growth factor heparan sulfate glycosaminoglycans restrict receptor autophosphorylation, whereas in the absence of heparin, the divalent cations restrict ligand binding and subsequent receptor activation[50]. Whether caseins inhibit FliD and *C. jejuni* attachment through modulation of the status of the metal-ion dependent heparan sulfate

receptors awaits structural analysis of the FliD-heparan sulfate complex.

MATERIAL AND METHODS

Bacterial culture

E. coli BL21+ bacteria expressing recombinant FliD proteins were grown on LB plates containing ampicillin (Biotrading) at 37°C. After one day of culture, bacteria were overnight grown in LB medium without antibiotic and the next day diluted 1:20 in fresh medium and left to grow until OD_{550}=0.5.

C. jejuni strain 81116 was grown on blood agar plates (Biotrading) under microaerophilic conditions at 37°C for 48 h. For infection experiments, bacteria were diluted in DMEM (D5030, Sigma) supplemented with 25 mM aspartate (Sigma), 25 mM serine, 25 mM pyruvate and 25 mM fumarate (Sigma) and grown overnight at 37°C under microaerophilic conditions.

Cell culture

Human Chang epithelial cells (ATCC CCL20.2) were routinely propagated in 25-cm^2 tissue culture flasks (Corning) in RPMI 1640 medium (Gibco) supplemented with 5% FCS at 37°C and 5% CO_2. For use in FliD and *C. jejuni* adhesion assay, cells were respectively seeded onto 96-well or 24-wells plates at 48 h or 36 h prior to use.

Milk and caseins

Skim milk powder (Elk, FrieslandCampina) was solubilized in distilled water as instructed by the manufacturer. Fresh cow milk was purchased from a local farm (Zeist, The Netherlands). Whey and casein fractions were formed by adding 10% acetic acid to the skim milk or fresh milk until the pH dropped from 6.8 to 4.6[24]. The fractions were separated by centrifugation (15 min, 4,000 x *g*) at room temperature (RT). The supernatant was collected and the precipitate was resuspended in water. The pH of both fractions was adjusted to 6.8. Alpha-caseins (C6780), β-casein (C6905), κ-caseins (C0406), dephosphorylated α-caseins (C8032) and β-lactoglobulin (L3908) were purchased from Sigma, dissolved in distilled water, filter-sterilized, and stored at 4°C.

FliD protein preparation

Recombinant *C. jejuni* FliD and the truncated FliDΔNC protein were isolated as previously described[22]. Shortly, the expression of FliD was induced in exponentially growing BL21+ *E. coli* by the addition of 1 mM of IPTG. After 4 h of incubation at 37°C, bacteria were collected by centrifugation. The pellet was resuspended in 50 mM of NaH_2PO_4, 300 mM of NaCl, 5 mM of imidazole, and pH 7.4. After incubation with lysozyme (1 mg/ml) for 30 min on ice and removal of bacterial debris (14,000 *x g,* 20 min, 4 °C), the supernatants were incubated (4°C) with Ni–NTA agarose beads for 1 h with end-of-end rotation. After extensive washing, bound protein was eluted with 50 mM of NaH_2PO_4, 300 mM of NaCl, 500 mM of imidazole, pH 7.4. The C-terminal His-tag was cleaved with TEV protease overnight at 4°C. After dialysis of the protein/TEV mixture against DPBS, the His-tagged protease was removed using Ni-NTA agarose beads.

FliD binding assay

FliD binding to cells was carried out as previously described[22]. In brief, epithelial cells grown to confluence in a 96-well plate were fixed with 1% paraformaldehyde (30min, RT). After blocking with 4% BSA (1h) FliD protein (2 μg/well) was allowed to bind (1 h, RT). Unless mentioned otherwise, FliD was diluted in Dulbecco's Phosphate Buffer Saline supplemented with 0.9 mM calcium chloride (Ca) and 0.5 mM magnesium chloride (Mg) (D8662, Sigma). For the binding experiments without cations, DPBS without Ca/Mg (D8537, Sigma) was supplemented with 1 mM of $CaCl_2$ and/or 1 mM of $MgCl_2$. For blocking experiments, FliD protein was pre-incubated at RT with skim milk or fresh cow milk at the indicated final concentrations for 1 h prior to addition to the cells. FliD binding to cells was detected with anti-FliD antiserum (dilution 1:10,000) and secondary goat anti-rabbit IgG-HRP (dilution 1:10,000; A4914, Sigma) using HRP-substrate (555214, BD). Absorbance was measured in a plate reader at 450 nm (Fluostar Omega, BMG Labtech). Results were expressed relative to 100% binding in absence of inhibitor. Experiments were performed at least three times in duplicate.

Sedimentation velocity by analytical ultracentrifugation (SV-AUC)

The multimeric states of FliDΔNC in presence of cations or EDTA were investigated by sedimentation velocity experiments on XL-I and XL-A analytical ultracentrifuges (Proteomelab and Optima XL-A, Beckman Coulter) using absorbance optics. Samples were centrifuged in 12 mm path length double-sector aluminum centerpieces with sapphire windows in a 4-hole AN60Ti rotor at a speed of 40,000 rpm at 20°C. SednTerp (http://sednterp.unh.edu) was used to calculate protein partial specific volumes and solvent densities and viscosities from the protein amino acid sequences and buffer compositions. Analysis and fitting of the data was performed using the program Sedfit v 14.3 [51]. A continuous c(s) distribution model was fitted to the data to determine the number of species, their sedimentation coefficients, and their fractional contributions to the species populations. All sedimentation coefficients were corrected to $s_{20,w}$ values.

Recombinant FliDΔNC protein was isolated under native conditions as described above and dialysed against DPBS containing 1 mM of $CaCl_2$ and 1 mM $MgCl_2$ without tag-removal. Prior to AUC, FliDΔNC (6342.42 μg/ml) was diluted to 1.88 mg/ml (31.47 μM) in DPBS (with Ca/Mg), or DPBS (with Ca/Mg) with 1.5 mM of additional Ca or DPBS (with Ca/Mg) with 10 mM of EDTA. Four-hundred μL of sample was put in the above-mentioned centerpieces and 420 μL of its buffer was brought into the reference sector. Prior to the sedimentation velocity experiment the filled cell was equilibrated for at least 2 h at the desired experimental temperature of 20°C. Change in solute concentrations were detected by 180-200 absorbance scans measured at 280 nm.

Infection assays

Cells were seeded onto 12-mm cover slips and grown to 70% confluence in 36 h. Prior to the addition of bacteria, cells were rinsed twice with Dulbecco's PBS (D8537, Sigma) and left in DPBS supplemented with 2 mM aspartate, 2 mM serine, 2 mM pyruvate, 2 mM fumarate (Sigma) in the absence or presence of 5 mM EDTA or physiological casein concentrations in milk. Hereto, α-caseins (17 mg/ml), β-casein (10 mg/ml) and κ-caseins (3 mg/ml) were added to the medium. *C. jejuni* strain 81116 was added to the cells at a bacterium/host

cell ratio (MOI) of 100:1 and incubated for 2h at 37°C at 5% CO_2. Infection was stopped by carefully rinsing the cells three times with DPBS and cells were lysed cells with 1% saponin in DPBS prior to plating serial dilutions on blood agar plates by 10 µl running droplets. After two days of growth under microaerophilic conditions the number of colony forming units was determined. Results are expressed as fold increase in comparison to the control.
For infection assays in the presence or absence of cations, the assay was performed in DPBS without Ca/Mg (D8537, Sigma) and DPBS was supplemented with 1 mM of $CaCl_2$ and/or 1 mM of $MgCl_2$.

Statistical analysis

Results were analyzed for statistical differences using the Prism GraphPad Software (version 6.05) by Student's unpaired *t* test, one- or two-way analysis of variance where appropriate. Significance was accepted at $P<0.05$.

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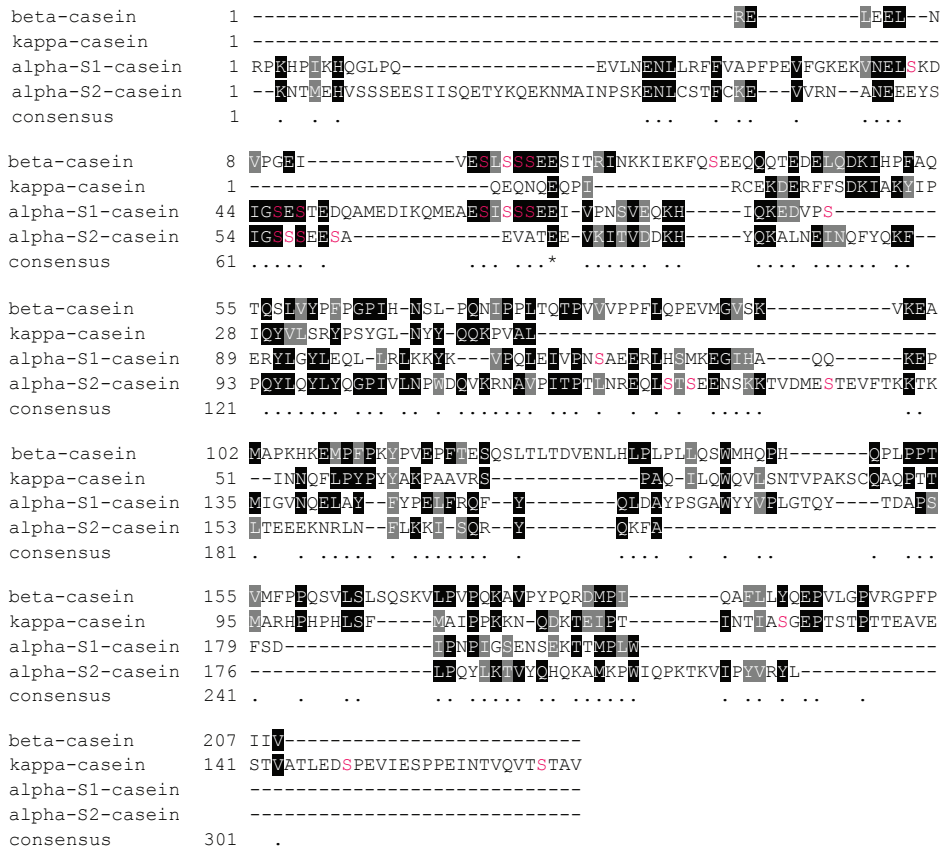
SUPPLEMENTARY FIGURE



Suppl. Fig. 1: Alignment of bovine caseins.
Multiple alignment of bovine caseins (α_{s1}-casein precursor GI: 30794348; α_{s2}-casein precursor GI:27806963; β-casein precursor GI:162805; κ-casein precursor GI:27881412) generated with Clustal Omega. The asterix represent entirely conserved residues and the dots represent at least 50% conservation. Phosphoserines are highlighted in red.

Chapter 5

Cell binding properties of the flagellar tip protein FliD

Claudia M. Freitag[1], Dominique Thies-Weesie[2], Jos P.M. van Putten[1]

[1]Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

[2]Van 't Hoff Laboratory for Physical and Colloid Chemistry, Debye Institute for Nanomaterials Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

In preparation

ABSTRACT

FliD proteins are flagellar cap proteins that facilitate correct assembly of the flagellin subunits. We recently showed that the FliD protein of *C. jejuni* also acts as an early attachment factor that confers binding to host cell heparan sulfate receptors. In the present study, we investigated the binding potential of FliD proteins of other species and searched for a common binding signature within the protein. Hereto, we cloned and expressed FliD proteins from *Campylobacter iguaniorum*, *Clostridium difficile*, *Pseudomonas aeruginosa, Salmonella* Typhimurium and *Salmonella* Enteritidis. All recombinant proteins formed multimeric complexes as shown by cross-linking experiments. Adhesion assays using sortagged-biotinylated FliD proteins revealed that all tested FliD species attached to cultured eukaryotic cells. Binding was inhibited by soluble heparin and strongly reduced when glycosaminoglycan-deficient cells were used, pointing to interaction with heparan sulfate glycosaminoglycans. Comparative structural analysis revealed a common architecture of the different FliD proteins and the presence of several lysines in the surface-exposed D2 and D3 domains representing putative heparin binding sites. Overall, our results suggest that FliD from different bacterial species share the ability to bind glycosaminoglycan receptors. The putative receptor-binding region in FliD may serve as a target of FliD-attachment inhibitory compounds.

INTRODUCTION

Flagella-driven motility is an important trait of many bacterial species. The fast rotation of the flagella propels the bacteria towards or away from favorable or harmful environments. Bacteria may carry one or more polar flagella as well as flagella that are laterally anchored in the cell wall. The flagellum is composed of three basic building blocks: the basal body, the hook filament and the flagella fiber. The basal body spans the bacterial membrane(s) and contains the inner motor complex that drives the rotation of the flagellum[1]. The hook filament serves as a flexible joint that connects the basal body and the flagellar filament and places the fiber under the right angle needed for functional rotation of the flagella. The flagellar filament is a hollow fiber and is composed of multiple helices of protofilaments of thousands of assembled subunits (flagellins). The growth of the filament is guided by the flagellar tip protein FliD (also termed HAP2)[2]. Flagella from different species share a common basic architecture and function but also display intrinsic differences. For example, in *Gammaproteobacteria* (such as *Salmonella*, *Escherichia* and *Pseudomonas*) the flagella fiber consists of 11 protofilaments[3], whereas the filament of *Epsilonproteobacteria* (e.g. *Campylobacter*) is composed of 7 protofilaments[4]. The reason for this evolutionary difference is unknown.

Besides conferring bacterial motility, flagella may interact with their host. Consistent with their ancestral resemblance to the type III secretion system (T3SS), the flagellar secretion machinery can release proteins into the environmental niche or the host cytosol[5,6]. For instance, the flagellar machinery of *Campylobacter jejuni* (*C. jejuni*) which is the cause of most bacterial diarrheal diseases in humans[7], is crucial for excretion of *Campylobacter* invasion antigens (Cia). These proteins promote bacterial internalization and pathogenesis[5]. The underlying interacting contacts between host and pathogen however are still unknown. In addition to being a secretion system, we recently provided evidence that the flagellar tip protein FliD of *C. jejuni* can act as an early attachment factor and binds specifically to heparan sulfate receptors on the surface of eukaryotic cells[8]. The potential relevance of this finding is illustrated by the presence of high amounts of anti-FliD antibodies in chicken eggs that block the binding of *C. jejuni* FliD to the cell surface (Chapter 3). These maternal antibodies are transferred to the new offspring and may protect it against *C. jejuni* in early life[9]. Thus, *C. jejuni* FliD may be an attractive target for an anti-*Campylobacter* vaccine.

The role of *C. jejuni* FliD in bacterial adhesion fits with previous reports of interaction of *Clostridium difficile* FliD to intestinal mucus and of *Pseudomonas* FliD with largely unidentified cell surface receptors[10–12]. These findings suggest a common function of FliD in bacterial adhesion. FliD protein structures have been (partially) resolved for FliD of *E. coli*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa* and *Serratia marcescens* [14–16]. The structures confirmed the existence of FliD as oligomeric complexes[2] but have thus far not been associated with the putative function of the proteins in bacteria-host interactions. In the present study we compared to putative adhesive role of FliD proteins in representatives of *Gamma-* en *Epsilonproteobacteria* and exploited the available structural information to predict host cell binding sites in *C. jejuni* FliD. Our results indicate that FliD attachment to host cell (glycosaminoglycan) receptors is a common feature of bacterial FliD proteins. The host cell binding region in *C. jejuni* FliD was predicted to reside in the D3 domain of the protein.

RESULTS

Expression of recombinant FliD protein from diverse species

As a first step to compare the functional and structural properties of bacterial FliD proteins, we cloned and expressed the corresponding genes from the bacterial enteropathogens; *Campylobacter iguaniorum, C. jejuni, Clostridium difficile, Pseudomonas aeruginosa* (strains PAK and PAO1), *Salmonella enterica* serovar Typhimurium and serovar Enteritidis in an *E. coli* expression system. His-tagged recombinant proteins were purified by Ni^{2+}-affinity chromatography and analyzed by SDS-PAGE. All proteins migrated according to their apparent molecular mass of 77 kDa, 73 kDa, 64 kDa, 59 kDa, 57 kDa, 58 kDa and 58 kDa, respectively (**Fig. 1**).

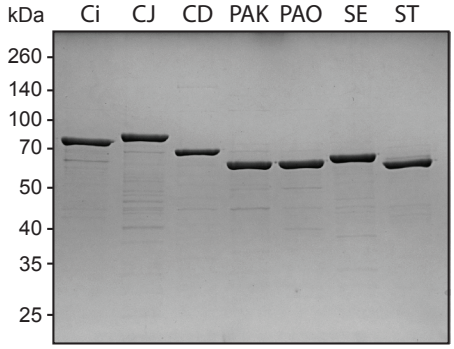
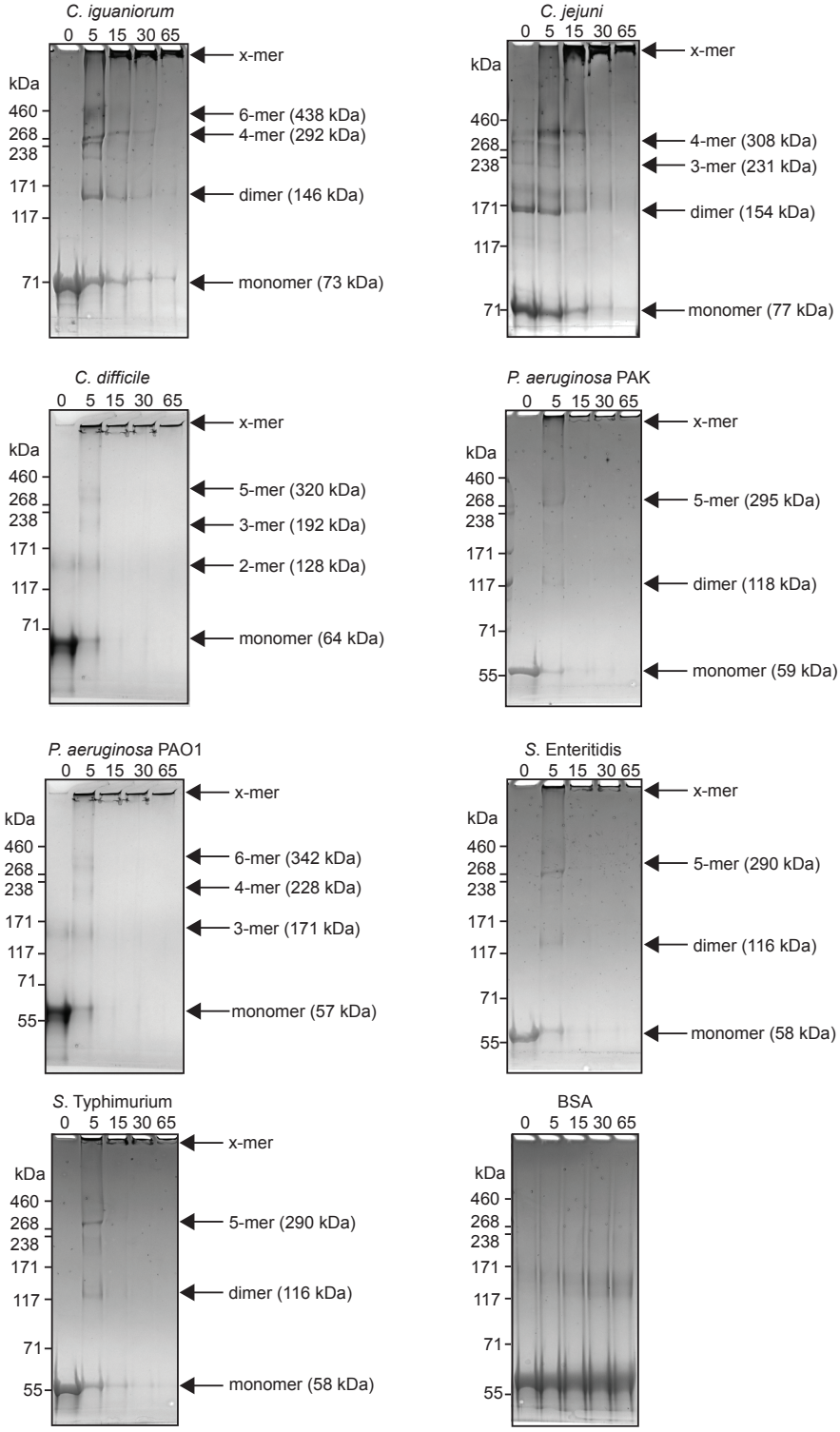


Figure 1: Purification of FliD protein of different species. His-tagged FliD of *C. iguaniorum* (Ci), *C. jejuni* (CJ), *C. difficile* (CD), *P. aeruginosa* PAK, *P. aeruginosa* PAO1, *S.* Enteritidis (SE) and *S.* Typhimurium (ST) were isolated from *E. coli*. Proteins (1 µg) were separated by denaturating SDS-PAGE and visualized with PageBlue. Molecular mass of markers is indicated in kilo Daltons (kDa).

To assess the ability of the different recombinant FliD proteins to form oligomers, we determined their electrophoretic mobility after crosslinking of the purified proteins (2.5 mg/ml) with the zero-crosslinking agent glutaraldehyde (0.05 %) for a period of 0-65 min. SDS-PAGE analysis of the protein samples showed that all proteins migrated as multimeric protein complexes under denaturating conditions (**Fig. 2**). The size of the FliD complexes increased dependent on the time of incubation with the cross-linking agent. Parallel treatment of bovine serum albumin (control) yielded no protein complexes (**Fig. 2**). The formation of multimeric FliD complexes is consistent with their native multimeric state as flagellar cap protein complex.

Figure 2: Multimerization of FliDs of different species.
FliD-His complexes (2.5 mg/ml) of the indicated species were crosslinked using 0.05% glutaraldehyde and incubated for the indicated time (min). BSA (2 mg/ml) was used as a negative control. The reaction was stopped with Tris buffer. Proteins were separated by denaturating SDS-Page and stained with PageBlue. The arrows indicated multimeric complexes and their apparent mass in kilo Daltons (kDa).


C. iguaniorum
0 5 15 30 65
kDa
460
268
238
171
117
71
x-mer
6-mer (438 kDa)
4-mer (292 kDa)
dimer (146 kDa)
monomer (73 kDa)
C. jejuni
0 5 15 30 65
kDa
460
268
238
171
117
71
x-mer
4-mer (308 kDa)
3-mer (231 kDa)
dimer (154 kDa)
monomer (77 kDa)
C. difficile
0 5 15 30 65
kDa
460
268
238
171
117
71
x-mer
5-mer (320 kDa)
3-mer (192 kDa)
2-mer (128 kDa)
monomer (64 kDa)
P. aeruginosa PAK
0 5 15 30 65
kDa
460
268
238
171
117
71
55
x-mer
5-mer (295 kDa)
dimer (118 kDa)
monomer (59 kDa)
P. aeruginosa PAO1
0 5 15 30 65
kDa
460
268
238
171
117
71
55
x-mer
6-mer (342 kDa)
4-mer (228 kDa)
3-mer (171 kDa)
monomer (57 kDa)
S. Enteritidis
0 5 15 30 65
kDa
460
268
238
171
117
71
55
x-mer
5-mer (290 kDa)
dimer (116 kDa)
monomer (58 kDa)
S. Typhimurium
0 5 15 30 65
kDa
460
268
238
171
117
71
55
x-mer
5-mer (290 kDa)
dimer (116 kDa)
monomer (58 kDa)
BSA
0 5 15 30 65
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460
268
238
171
117
71
55

Characterization and stability of the *C. jejuni* FliD complex.

Biochemical and structural analyses indicate that the FliD proteins of *E. coli*, *Salmonella, and Pseudomonas* form hexa- and pentameric FliD protein complexes[15,16]. In order to learn more about the composition of the *C. jejuni* flagellar cap complex, the recombinant (non-cross-linked) FliD multimers of *C. jejuni* were subjected to size-exclusion chromatography. Prior to this analysis, the full length His-tagged FliD protein was purified from *E. coli* and cleaved with TEV protease (48 kDa) to remove the tag, resulting in a non-tagged FliD protein of 69.8 kDa (**Fig. 3A**). Gel filtration chromatography using Superose® 10/300 and a set of protein standards showed that *C. jejun*i FliD mainly eluted as a multimer of approximately 450 kDa (**Fig. 3B**).

We also investigated if the truncated version of FliD (termed FliDΔNC) that lacks the hydrophobic N-terminal and C-terminal regions but still contains the potential cell binding site(s)[8] also exists as a multimer in solution. Crosslinking experiments showed that *C. jejuni* FliDΔNC still can form multimers in a time-dependent manner (**Fig. 3C**). To substantiate these observations, we determined sedimentation velocity profiles from analytical ultracentrifugation (SV-AUC) experiments with non-crosslinked FliDΔNC protein. This showed a protein concentration-independent distribution of FliDΔNC as monomers (60 kDa, 46%), dimers (126 kDa, 11%) and multimers (478 kDa, 26%) (**Fig. 3D**). These results suggest that at least part of the truncated FliD proteins favored the formation of relatively stable octamers (~478 kDa).

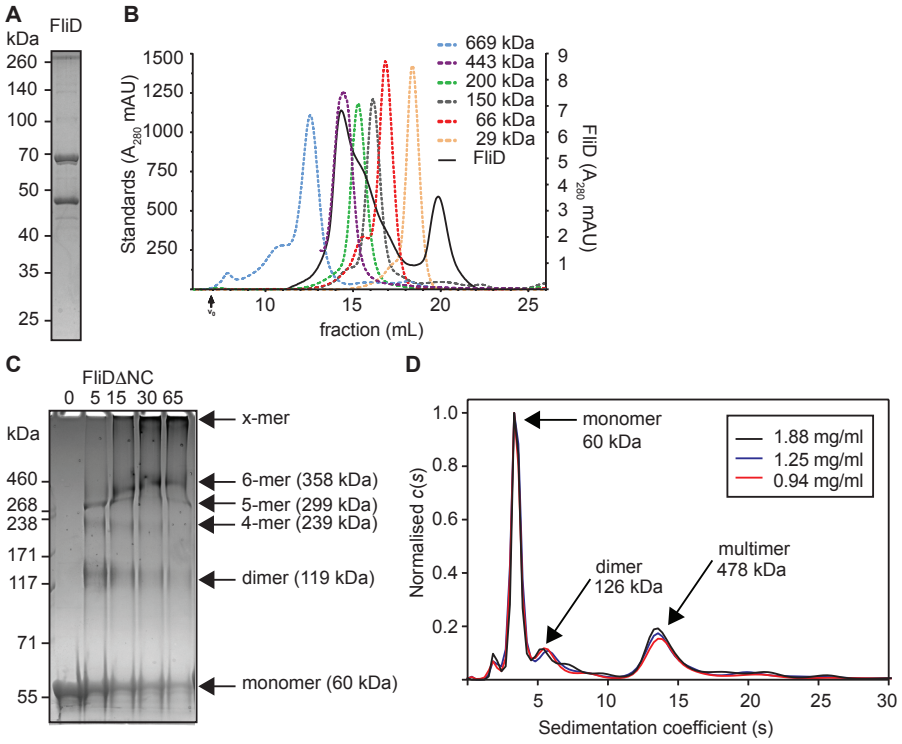

Figure 3: Characterization and stability of *C. jejuni* FliD multimers.
(A) Full length His-tagged FliD of *C. jejuni* was purified from *E. coli* and cleaved with TEV (48 kDa) to remove the tag resulting in FliD of 69.8 kDa visualized by SDS-PAGE. Proteins were stained with PageBlue. **(B)** Proteins as described in A were subsequently analyzed by gel filtration chromatography using a Superose® 10/300 column.

Standards of indicated molecular weights were run to estimate the size of the FliD multimer complex. **(C)** His-tagged FliDΔNC (FliD encompassing amino acids 71-555, 2.8 mg/ml) was purified from *E. coli* and crosslinked with 0.05% glutaraldehyde for the indicated times. The reaction was stopped with Tris buffer. Proteins were separated by denaturating SDS-PAGE and stained with PageBlue. **(D)** Sedimentation velocity profiles measured by analytical ultracentrifugation (SV-AUC) of FliDΔNC protein showing a concentration-independent distribution of monomers, dimers and multimers.

Common host cell binding properties of FliD of different species

The successful expression and multimer formation of the different FliD proteins led us to compare their host cell binding properties. For this purpose, we biotinylated the different recombinant FliD proteins site-specifically at their C-terminal end by the use of sortase[8]. Despite differences in sortagging efficiency (**Fig. 4A**), we obtained biotinylated FliD proteins for all species as evident from their reactivity with HRP-labeled Streptavidin (**Fig. 4B**).

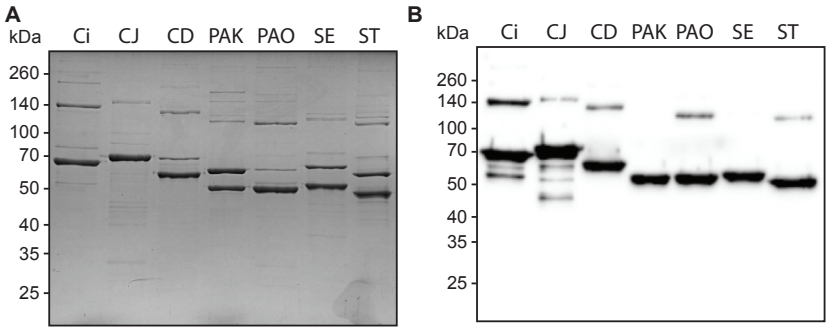

Figure 4: Site-specific sortagging of FliD protein of different species.
(A) His-tagged FliD of *C. iguaniorum* (Ci), *C. jejuni* (CJ), *C. difficile* (CD), *P. aeruginosa* PAK, *P. aeruginosa* PAO1, *S.* Enteritidis (SE) and *S.* Typhimurium (ST) were isolated from *E. coli*. Proteins were site-specifically biotinylated at their C-terminal end by sortase for 4 h at 20°C. Proteins were separated on SDS-PAGE and stained with PageBlue. **(B)** Streptavidin-HRP Western blot of the above-mentioned proteins confirming the successful biotinylation of the FliD proteins.

The host cell binding potential of the FliD proteins (25 pmol) was tested using HeLa cells as well as intestinal INT407, HRT18 and mucus-producing HT29-MTX cells. FliD binding was determined after 1 hour of incubation using Streptavidin-HRP to detect bound proteins. FliD protein from all species bound to the surface of HeLa cells, whereas more variation in binding of the proteins was observed with the other cell lines (**Fig. 5**). The binding of *C. jejuni* and *Salmonella* FliD appeared to display the least cell type specificity, whereas *Pseudomonas* PAK FliD poorly bound to all types of intestinal cells (**Fig. 5**).

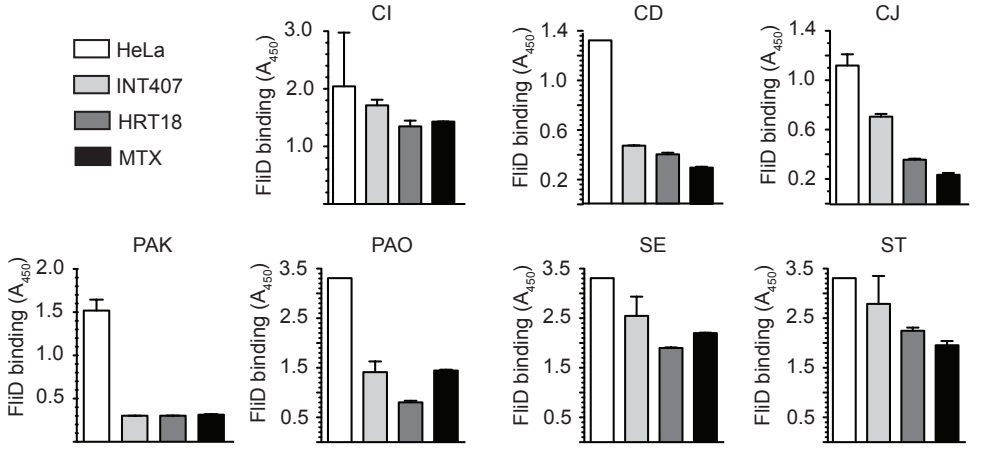

Figure 5: Cell binding of FliD of different species.
Biotinylated FliD (25 pmol) of *C. iguaniorum* (Ci), *C. jejuni* (CJ), *C. difficile* (CD), *P. aeruginosa* PAK, *P. aeruginosa* PAO1, *S*. Enteritidis and *S*. Typhimurium was incubated with HeLa cells (white bar) or INT407 and HRT18 intestinal cells (grey bars) or with mucus-producing HT29-MTX cells (black bar). The binding was detected with Strep-HRP. Bars represent the mean ±SD from one representative of three independent experiments after background subtraction.

FliD proteins bind heparan sulfate glycosaminoglycans

We recently identified heparan sulfate glycosaminoglycans as host cell receptors for *C. jejuni* FliD (Freitag et al., 2017). To assess with this binding event is specific for *C. jejuni* FliD or a more common feature among this class of proteins, we tested the binding properties of the FliD proteins from the other bacterial species using wild type CHO cells and CHO-745 mutant cells that lack glycosaminoglycans. All of the tested FliD proteins showed considerable lower binding to the mutant CHO-745 cells compared to wild type cells (**Fig. 6A**), suggesting interaction with glycosaminoglycans on the host cell surface. The ability of the FliD proteins to bind this class of molecules was further substantiated by inhibition of the binding by soluble heparin (100 ng/ml) but not by hyaluronic acid (100 ng/ml) (**Fig. 6B**). Similarly, the addition of caseins (250 µg/ml) which we identified as natural *C. jejuni* FliD binding inhibitors in milk (Chapter 4), reduced the interaction of all other tested FliD proteins with the eukaryotic cells (**Fig. 6B**). Together, these results suggest the presence of common host cell binding characteristics among the FliD proteins from *C. iguaniorum*, *C. jejuni*, *C. difficile*, *P. aeruginosa* PAK, *P. aeruginosa* PAO1, *S*. Enteritidis and *S*. Typhimurium.

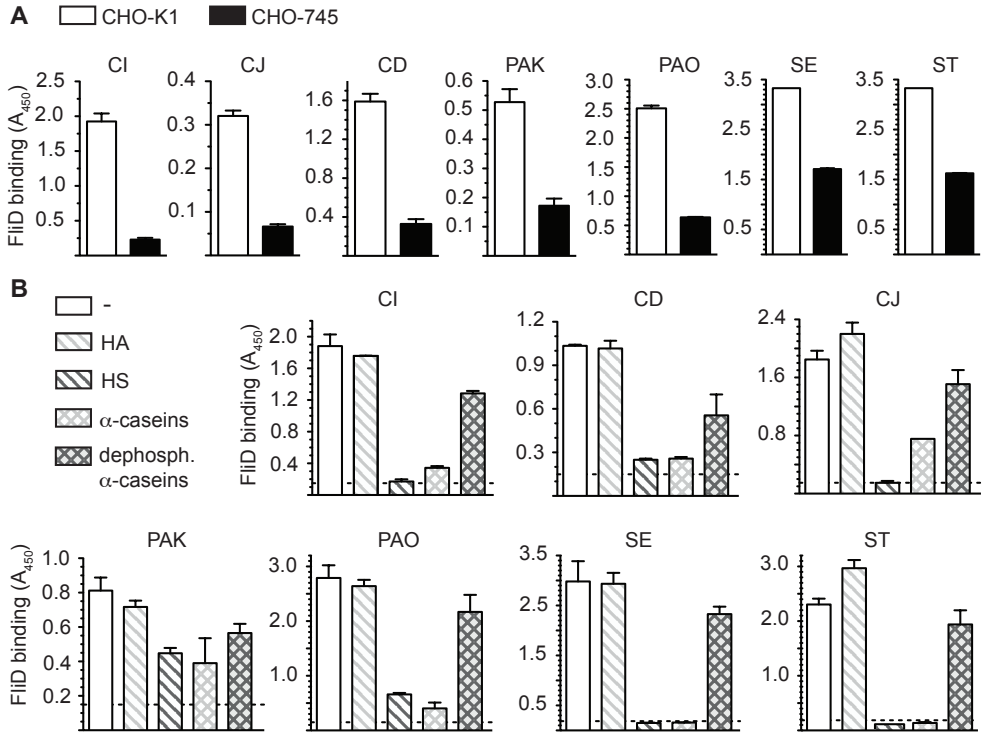

Figure 6: FliDs of different species bind heparan sulfate glycosaminoglycans.
(A) Biotinylated FliD (25 pmol) of *C. iguaniorum* (Ci), *C. jejuni* (CJ), *C. difficile* (CD), *P. aeruginosa* PAK, *P. aeruginosa* PAO1, *S.* Enteritidis and *S.* Typhimurium was allowed to bind to either CHO-K1 wild-type cells (white bar) or to CHO cells deficient in glycosaminoglycan synthesis (CHO-745 cells, black bar). Bars represent the mean ±SD from one representative of three independent experiments after background subtraction. **(B)** FliD proteins were preincubated with soluble hyaluronic acid (HA) or with heparin sulfate (HS) (both 100 ng/ml, hatched bars), or with phosphorylated or dephosphorylated caseins (both 250 µg/ml, crossed bars) prior allowing binding to INT407 cells. Bars represent the mean ±SD from one representative of two independent experiments; a dashed line indicates the background binding.

Common protein features of FliD proteins

In search for protein domains that may explain the seemingly common host cell binding characteristics of the different FliD proteins, we compared their amino acid sequences in more detail. The different FliD proteins differed considerable in total number of amino acids (**Table 1**). The FliD proteins from the *Epsilonproteobacteria C. jejuni* and *C. iguaniorum* consisted of 642 and 602 amino acids respectively, whereas the FliDs from the *Gammaproteobacteria E. coli* strain K-12, *S*. Enteritidis, *S*. Typhimurium, *P. aeruginosa* strain PAK and *P. aeruginosa* strain PAO1 showed to be much shorter and are composed of 467-478 amino acids. FliD from *C. difficile* was found to be 507 amino acids in length. Amino acid sequence alignment revealed a low degree of similarity (35-50%) between the different FliD proteins, perhaps with exception of the *E. coli* and *Salmonella* FliD proteins (70-90% similarity) (**Table 2**). Other characteristics such as isoelectric point (pI), amino acid composition, and number of charged amino acid residues appeared rather similar for the different FliD proteins (**Table 1**). All proteins contained 9-12% positively charged amino acids (Arg, His, Lys) which, when appropriately arranged, may confer binding to sulfated glycosaminoglycans[19].

Table 1: Comparison of FliD protein sequences of *C. difficile* (NCBI Reference Sequence: WP_003421095.1), *C. iguaniorum* (GI:669187914), *C. jejuni* strain 11168 (GI:218562200), *E. coli* (NCBI Reference Sequence: WP_000146784.1), *P. aeruginosa* strain PAK (GI:489211809), *P. aeruginosa* strain PAO1 (NCBI Reference Sequence: WP_003082190.1), *S*. Typhimurium (GI:301158474) and *S*. Enteritidis (GI:197940049)

Species	AA	MW	pI	- res			+ res		
C. difficile	507	65294.79	5.19	Asp (D) Glu (E)	38 37	7.5% 7.3%	Arg (R) His (H) Lys (K)	12 2 53	2.4% 0.4% 10.5%
C. iguaniorum	602	64490.1	4.63	Asp (D) Glu (E)	41 34	6.8% 5.6%	Arg (R) His (H Lys (K)	4 0 49	0.7% 0.0% 8.1%
C. jejuni	642	69775.2	4.81	Asp (D) Glu (E)	53 34	8.3% 5.3%	Arg (R) His (H) Lys (K)	8 3 58	1.2% 0.5% 9.0%
E. coli	468	48456.45	4.82	Asp (D) Glu (E)	34 7	7.3% 1.5%	Arg (R) His (H) Lys (K)	7 1 45	1.7% 0.2% 5.6%
P. aeruginosa PAK	478	50735.3	8.33	Asp (D) Glu (E)	38 19	7.9% 4.0%	Arg (R) His (H) Lys (K)	15 0 44	3.1% 0.0% 9.2%
P. aeruginosa PAO1	474	49449.56	6.52	Asp (D) Glu (E)	35 17	7.4% 3.6%	Arg (R) His (H) Lys (K)	13 0 39	2.7% 0.0% 8.2%
S. Enteritidis	468	49956.8	5.06	Asp (D) Glu (E)	37 24	7.9% 5.1%	Arg (R) His (H) Lys (K)	11 0 42	2.4% 0.0% 9.0%
S. Typhimurium	467	49834.8	5.27	Asp (D) Glu (E)	35 23	7.5% 4.9%	Arg (R) His (H) Lys (K)	9 0 44	1.9% 0.0% 9.4%

Table 2: Identity and similarity comparison of FliD protein sequences of *C. difficile* (CD), *C. iguaniorum* (Ci), *C. jejuni* (CJ), *E. coli* strain K-12 (EC), *P. aeruginosa* strain PAK, *P. aeruginosa* strain PAO1 *S*. Enteritidis (SE) and *S*. Typhimurium (ST).

Identity	CD	Ci	CJ	EC	PAK	PAO	SE	ST
CD	100							
Ci	11.94	100						
CJ	11.51	34.44	100					
EC	15.27	17.52	17.20	100				
PAK	14.39	13.45	14.78	29.28	100			
PAO	14.80	13.76	14.57	27.36	41.14	100		
SE	14.78	19.07	18.42	52.02	26.19	26.51	100	
ST1	15.33	18.91	18.44	50.53	26.79	26.51	78.42	100

Similarity	CD	Ci	CJ	EC	PAK	PAO	SE	ST
CD	100							
Ci	31.04	100						
CJ	29.97	55.99	100					
EC	36.73	40.35	37.60	100				
PAK	38.13	36.55	37.10	53.59	100			
PAO	37.55	36.96	36.12	51.72	62.12	100		
SE	36.50	39.42	36.83	70.06	50.99	49.60	100	
ST1	36.50	37.50	36.07	68.15	53.39	49.60	88.89	100

Structure prediction of the *C. jejuni* FliD and the central binding domain

Closer inspection of the *C. jejuni* FliD amino acid sequence predicted the presence of an alternating pattern of 12 helices and 23 beta-sheets (**Fig. 7**). The N- and C-terminus of the protein are composed of helices that likely interact with related structures of flagellins that form the flagella fiber[20]. The region of FliD previously implicated in the binding to host cells comprises the amino acids 210 and 418[8]. This region is predicted to form 6 beta-sheets and 4 alpha-helices and contains several positively charged lysine residues, which could contribute to the heparin-binding character (**Fig. 7**).

In order to learn more about the potential receptor binding domains within *C. jejuni* FliD, we took advantage of the crystal structures of the FliD proteins of *Serratia marcescens*, *S*. Typhimurium, *E. coli* and *P. aeruginosa*[14–16]. We modeled FliD of *C. jejuni* onto the protein structure using Phyre2 [21]. Although all known FliD structures are incompletely resolved, their structures resemble each other and show gross resemblance with features of bacterial flagellins (cf **Fig. 8A** vs. **Fig. 8B** and **C**). Both type of proteins are assumed to have a four-domains structure, although the structure of the D0 domain of FliD has not yet been resolved probably due to the predicted disordered structure[17] (**Fig. 8**). The predicted structure of the C-terminal D1 domain in *C. jejuni* has a low (<35%) prediction confidence due to lacking templates and the presence in *C. jejuni* FliD of an additional region of 130 amino acids (**Suppl. Fig. 1**). These residues are probably situated in the D1 domain. In contrast, the predicted structure of the D2 and D3 domains of *C. jejuni* FliD resemble the comparable domains in the resolved FliD structures (cf **Fig. 3F** vs. **Fig. 3B-E**) and were used in further identification of the host cell binding site.

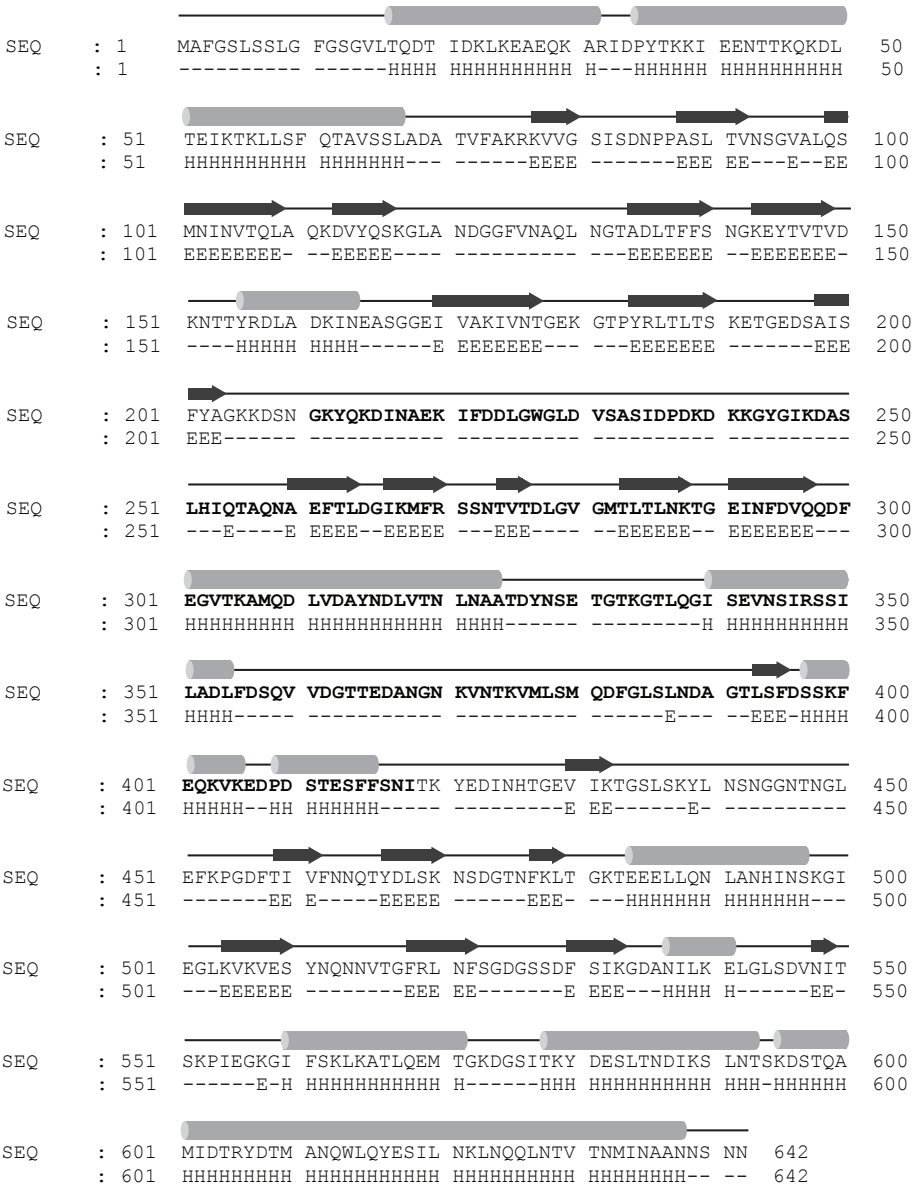

Figure 7: Secondary structural prediction of FliD of *C. jejuni.*
Secondary structure of FliD of *C. jejuni* strain 11168 predicted as described in the materials and methods. The region encompassing the binding sites important for the interaction of FliD to the cell surface is highlighted in bold. Predicted alpha-helices (H) are depicted as grey bars and predicted beta-sheets (E) are represented by black arrows.

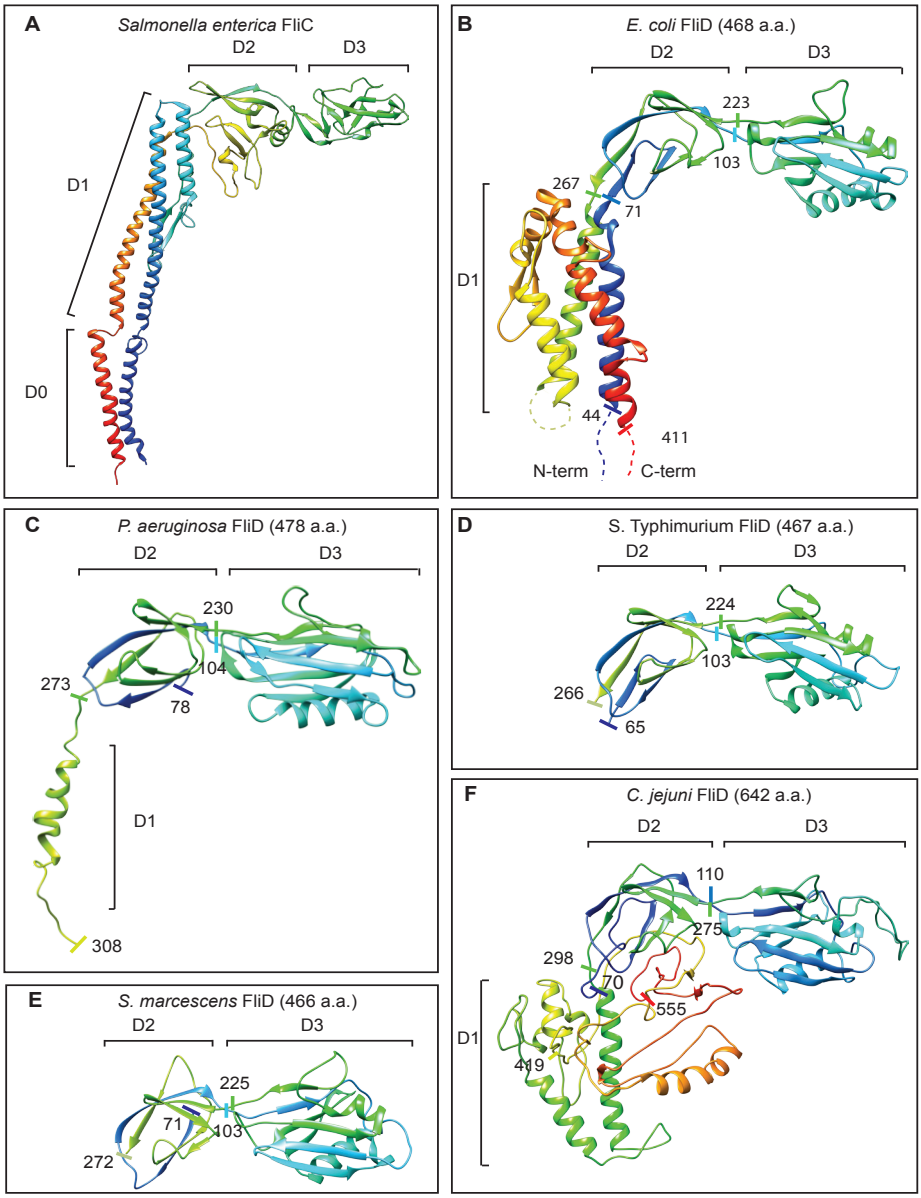

Figure 8: The structure of the FliD monomer.
(A) Cartoon display of the four-domain organization of flagellin of *S. enterica* (PDB: 3A5X). **(B-E)** Crystal structures of FliD of *E. coli* (PDB: 5H5V, B), FliD of *P. aeruginosa* PAO1 (PBD: 5FHY, C), FliD of *S.* Typhimurium (PDB: 5H5T, D) and FliD of *S. marcescens* (PDB: 5XLJ, E) show similar multidomain architecture. **(F)** Prediction of the structure of *C. jejuni* FliD strain 11168 lacking the N- and C-terminal ends using PHYRE2 prediction software[21]. The respective sequences lengths and specific amino acid numbers are indicated in the models.

Putative heparin binding site in *C. jejuni* FliD

In an attempt to pin-point the heparin-binding region in *C. jejuni* FliD, we focused on the central part of the protein previously implicated in heparin binding[8]. This region encompasses the amino acids 210-418 and spans the D1, D2 and D3 domains (**Fig. 9A**). Of these, the domains D2 and D3 are most likely responsible for the interaction with the host, as these regions are predicted to be exposed on the flagellar tip. *In silico* modeling of the three-dimensional structure of this region indicated several closely located lysines in the D3 domain (**Fig. 9B**). This is of interest as heparin binding is associated with a clustering of positively charged amino acids such as lysines and arginines[19]. On the basis of our analysis, the amino acids K247, K214 and K211 within *C. jejuni* FliD represent interesting candidates for interaction with the host cell. Similar analysis on the FliD proteins of *P. aeruginosa* and *S*. Typhimurium also revealed protruding lysine residues in the D2 and D3 domains. This suggests that FliD proteins share a common signature of host cell binding (**Fig. 9C** and **9D**).

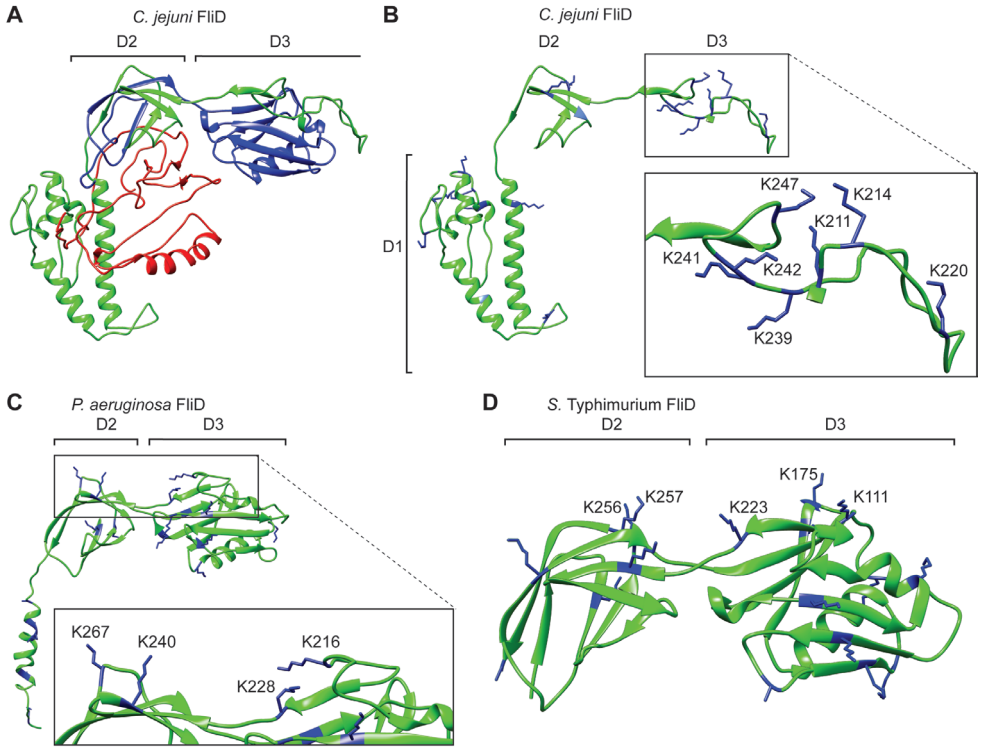

Figure 9: Shared structural characteristics of the putative cell-binding site in FliD
(A) Predicted model of FliDΔNC from *C. jejuni*. The central binding region containing the cell-binding sites is colored in green. The N- and C-terminal parts are indicated in blue and red, respectively. **(B)** Potential interacting lysines in *C. jejuni* FliD are indicated in blue. The small box shows the central cell-binding region of FliD of *C. jejuni* (green as in A), the large box shows a magnification of the D3 domain with annotated lysine residues (indicated in blue). **(C-D)** Lysine residues in the D2 and D3 domains in the FliD proteins of *P. aeruginosa* PAO1 (PBD: 5FHY, C) and *S*. Typhimurium (PDB: 5H5T) representing potential heparan sulfate binding sites, coloration as in B.

DISCUSSION

Awareness is growing that the flagellar tip protein FliD not only facilitates the assembly and stabilization of the flagella fiber but may also display adhesive properties. Several studies indicate that FliD proteins of *Clostridium difficile* and *Pseudomonas* bind to intestinal mucus and intestinal cells, respectively[10–12]. We recently demonstrated that FliD of the major foodborne pathogen *C. jejuni* interacts with host cell surface heparan sulfate receptors[8]. In the present study we provide evidence that adhesion of FliD to host cell glycosaminoglycans receptors is a common feature of FliD proteins of a diverse set of bacterial pathogens, i.e. *C. jejuni*, *C. iguaniorum, S*. Enteritidis, *S*. Typhimurium, *P. aeruginosa* and *C. difficile.* Available protein structure information on several FliD proteins allowed us to reconstruct the structure of the assumed host cell binding region in *C. jejuni* FliD and to predict the putative binding pocket in the D3 domain of the protein. These results underpin the adhesion potential of the flagellar tip protein FliD and may provide a basis for the development of specific inhibitors of flagella-host cell contact.

An essential step in assessing the putative adhesive properties of FliD was the analysis of the general characteristics and folding of the FliD proteins. Comparison of the FliD protein sequences of a number of *Gamma-* and *Epsilonproteobacteria* revealed relatively low amino acid conservation despite their common function as a flagellar cap protein. Additionally, the length of the different FliD proteins strongly varied between 468 (*E. coli* FliD) to 642 amino acids (*C. jejuni* FliD). These differences between FliD proteins may reflect or contribute to the existing variation in flagellar structures among bacterial taxa or species. It has been shown that the flagellar filament in *Gammaproteobacteria* (such as *Salmonella*, *Escherichia* and *Pseudomonas*) is composed of 11 protofilaments[3] in opposite to 7 protofilaments in case of the flagellum of *Epsilonproteobacteria* such as *Campylobacter*[4]. As the filament capping function of FliD plays a key role in the growth of the filament and correct assembly of the flagellar subunits, it is imaginable that the nature of the FliD complex influences the flagella architecture. Of note here is that the hook filament of *C. jejuni*, which connects the flagellar filament to the basal body, adopts a helical 11-protofilament structure. The hook filament is capped with the hook-filament junction protein HAP3. Thus the hook filament and flagellum capping proteins of *C. jejuni* may have evolved differently[22]. Which evolutionary pressure caused the difference between HAP3 and FliD and further between the variant FliD proteins in different bacterial species remains to be investigated.

A common principle of all FliD proteins is that they need to adopt an oligomeric state in order to efficiently allow flagellin subunit assembly[17,23]. We purified FliD proteins from *C. jejuni*, *C. iguaniorum, C. difficile*, *P. aeruginosa* strains PAK and PAO1, *S*. Enteritidis and *S*. Typhimurium. Chemical crosslinking experiments with the recombinant proteins indicated FliD multimerization for all species. FliD from S. Typhimurium has previously been shown to exhibit a pentameric state[16,17], whereas FliD from *P. aeruginosa* and *E. coli*[16] form hexamers[15,16]. Recently, FliD from *S. marcescens* was shown to adopt a tetrameric structure[14]. It has been proposed that the *C. jejuni* FliD adopts a trimeric state when correlating the number of protofilaments (7 in *C. jejuni*) and the equation "2 N + 1" where N is the number of FliD oligomers[14]. Our size-exclusion chromatography experiments with full length FliD rather suggest that *C. jejuni* FliD forms heptamers, whereas our analytical centrifugation experiments using truncated FliD lacking the N- and C-terminal ends points to the formation of FliD octamers. This difference in complex formation suggests that the amino-termini of

FliD influence the oligomerization of *C. jejuni* FliD as has been observed for FliD from *P. aeruginosa* strain PAO1[15]. Generally, the multimerization of FliD is assumed to be driven by the D2 and D3 domains. One D2 domain connects to the adjacent D3 domain generating a head-to tail 4-, 5- or 6-pointed star plate[14,16]. Attempts to prevent multimerization of *S*. Typhimurium FliD by site-specific mutagenesis suggested that residues in the D2 and D3 domains as well as both termini drive the oligomerization process[15,16]. However, the exact involved residues remain undefined. Important here is that the formation of the different FliD multimers indicated proper folding of the recombinant proteins, which was important to investigate the putative adhesive function of the proteins.

For testing the host cell binding qualities of the different FliD proteins, we biotinylated the proteins at their C-terminal ends using the sortagging technique. This was favored over random biotinylation, which may potentially influence the adhesin-receptor interactions. All purified FliD proteins were capable to bind to eukaryotic cells, although variation was observed between FliD proteins and different cell types. Typically, only very low levels of FliD attachment were measured for CHO-745 cells that are deficient in glycosaminoglycan biosynthesis[24]. Moreover, binding of all FliD types was inhibited in the presence of heparin but not by the soluble glycosaminoglycan hyaluronic acid. Together, these findings strongly suggest that the FliD proteins bound to host cell heparan sulfate glycosaminoglycan receptors, as we previously found for *C. jejuni* FliD[8]. Differences in adhesion between cell types may then be caused by variable availability of cell surface glycosaminoglycans. The apparent common function of FliD of different species as attachment factor seems at variance with their relatively low amino acid similarity but is consistent with their common function as multimeric flagellar capping protein. Until now, FliD from *E. coli* has been reported to attach to microvilli on intestinal cells[11] and *C. difficile* FliD to mucus[12]. *P. aeruginosa* FliD binds to Lewis(x) or sialyl-Lewis(x) determinants present on the surface on cells and on mucins[10,25]. We did not observe increased binding of the different FliD proteins to mucus secreting HT29-MTX cells. Nevertheless, mucus is a highly negatively charged component of the gut and FliD appears to bind to negatively charged heparan sulfate moieties on the cell surface. Therefore it is tempting to hypothesize that FliD binding to negatively charged determinants is a common feature of FliD proteins from different species.

In order to further unravel the molecular basis of the FliD-glycosaminoglycan interaction, we followed a comparative protein structure approach taking advantage of the resolved crystal structures of several FliD species[14–16]. Modeling of the FliD amino acid sequence of *C. jejuni* on monomeric crystal structures *P. aeruginosa* [15], *E. coli* and *S*. Typhimurium [16] predicted a conserved multi-domain architecture with two surface exposed D2 and D3 head domains. These domains have been suggested to form the plate of the flagellar tip[15,16]. We previously situated the region responsible for the cell binding activity in the central part of the protein (amino acids 210-418)[8], which encompasses the D2-D3 domain. Closer inspection of the predicted structure of this region revealed the presence of several lysines in close proximity in the D3 domain. It is tempting to speculate that basic residues K247, K214 and K211 form a binding site that interacts with heparan sulfate glycosaminoglycans at the cell surface. Comparative analysis of this region in FliD from *Salmonella* and *Pseudomonas* showed the presence of different non-conserved lysines residues that could be responsible for the interaction of FliD with the cell surface. The D2 and D3 domains of *E. coli* FliD contain no lysine residues but instead have many arginine residues which may results in similar charge

characteristics. Future crystal structure analysis of the *C. jejuni* FliD-heparin complex and site-specific mutagenesis are necessary to confirm the presence of a conserved heparin binding signature among FliD proteins. At this time, the identification of the flagellar tip protein FliD as attachment factor in multiple bacterial species substantiates the multifunctional nature of the proteins and opens perspectives for the development of inhibitory compounds.

MATERIAL AND METHODS

Cell culture

The cell lines HeLa-57A[26], HRT-18 (human colon cells, ATCC CCL-244), INT 407 (human embryonic intestine, ATCC CCL 6), and HT29-MTX (mucus-secreting intestinal cells, kindly provided by Dr. Thécla Lesuffleur, INSERM, Lille, France), were routinely propagated in 25-cm^2 tissue culture flasks in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% FCS at 37°C and 10% CO_2. CHO-K1 and CHO-745 cells[24] were grown in RPMI + 10% FCS under 5% CO_2.

Bacterial growth

Campylobacter jejuni strain 11168 and *Campylobacter iguaniorum*[27] were grown on saponin agar plates at 37°C under microaerophilic conditions (80% N_2, 10% CO_2, 5% O_2, 5% H_2). *Pseudomonas aeruginosa* strains PAO1[26] and PAK[26], *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) strain 90-13-706 and serovar Typhimurium (*S.* Typhimurium) strain SL1344, and *E. coli* DH5/ BL21+ were grown on LB plates with the appropriate antibiotics at 37°C in air. *Clostridium difficile* (clinical isolate #78 kindly provided by Mirjam Duijvestijn, Department of Infectious Diseases and Immunology, Utrecht University) was grown under anaerobic conditions at 37 °C.

Cloning of *fliD* genes of different species

FliD proteins and *C. jejuni* FliDΔNC which lack the distal N-terminal and C-terminal regions were obtained as previously described[8]. In a similar approach, primers (Table 3) were designed to PCR amplify FliD genes from *C. jejuni*, *P. aeruginosa* PAK and PAO, *S. enterica* serovar Enteritidis and serovar Typhimurium and *C. iguaniorum* using 1 µg of DNA template in a total volume of 50 µl according to the manufacturer's recommendations (Phusion High-Fidelity DNA Polymerase, ThermoFisher Scientific). PCR conditions were: 98°C for 30 sec (initial denaturation) followed 36 cycles at 98°C for 30 sec (denaturation), at 55°C for 30 sec (annealing), and at 72°C for 80 sec (extension). The PCR products with NotI and PacI overhangs were cloned into the *PCP1fliD-sort-His* expression vector behind an IPTG-inducible promoter and in front of the sortagging motif and the C-terminal His-tag[8]. Cloned products were verified by DNA sequencing (Macrogen).

Table 3: Primers used in this study

Clostridium difficile	fwd	5'-CCTTAATTAAATGTCAAGTATAAGTCCAGTAAGAGTTAC-3'
	rev	5'-CCGCGGCCGCATTACCTTGTGCTTGTGAGAAATAATTC-3'
Campylobacter iguaniorum (GI:669187914)	fwd	5'-CCTTAATTAAATGGCAGTAGGTAGCGTAAC-3'
	rev	5'-CCGCGGCCGC GTTGCTATTACTTGCTTGTTCGATCATTG-3'

Campylobacter jejuni strain 11168 (GI:112359906)	fwd	5'-CCTTAATTAAATGGCATTTGGTAGTCTATCTAGTTTAG-3'
	rev	5'-CCGCGGCCGCATTATTAGAATTGTTTGCCGCATTAATCATATTAG-3'
Pseudomonas aeruginosa PAK (GI:2276418)	fwd	5'-CCTTAATTAAATGGCGAACAGTACGACG-3'
	rev	5'-CCGCGGCCGCGCTTTTCTTCACAAGGCCAGGC -3'
Pseudomonas aeruginosa PAO1 (GI:15596291)	fwd	5'-CCTTAATTAAATGGCCGGTATCTCGATAG-3'
	rev	5'-CCGCGGCCGCGGTCTTCTTGCCGCTGCC-3'
Salmonella enterica serovar Enteritidis (GI:206708330)	fwd	5'-CCTTAATTAAATGGCTTCAATTTCATCATTAGGTG-3'
	rev	5'-CCGCGGCCGC GGACTTGTTCATAGCATTAAATTGCTGGG -3'
Salmonella enterica serovar Typhimurium (GI:301158474)	fwd	5'-CCTTAATTAAATGGCTTCAATTTCATCATTAGGTG-3'
	rev	5'-CCGCGGCCGC GGACTTGTTCATAGCTGTAAATTGCTGG-3'

Expression and purification of FliD proteins

Recombinant FliD proteins were purified under native conditions as previously described[8]. Proteins expression in *E. coli* BL21+ was induced by incubation with 1 mM of isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 hrs at 37°C. Bacterial pellets were lysed in resuspension buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 1 mg/ml lysozyme, pH 7.4) for 1 h at 4°C, centrifuged (14,000 *x g,* 20 min, 4°C), and the supernatants incubated with nickel-coated beads (Ni-NTA agarose, ThermoFisher Scientific) for 1 h at 4°C. NTA beads were washed extensively with 40 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.4) and with 40 ml of washing buffer containing 0.1% Triton-X114. Proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 400 mM imidazole, pH 7.4) and dialyzed (10 kDa cut-off, 68100 ThermoFisher Scientific) twice against 2 liters of Dulbecco's Phosphate Buffered Saline *(*DPBS) for 16 h at 4°C. Protein concentrations were determined using BCA Protein assay (23225, ThermoFisher Scientific). Proteins were stored in DPBS at -20°C.

Chemical crosslinking of FliD proteins

Six microliters of His-tagged FliD proteins (concentrations ranging from 2-2.5 mg/ml) were incubated in DPBS in presence of 500 mM of NaCl and 0.05% glutaraldehyde (01909, PolyScience) for the indicated times. The reaction was stopped by adding 3 µl of 0.5 M of Tris-HCl pH 6.8. Subsequently, proteins were boiled in Laemmli buffer and loaded on pre-casted 3-8% Tris-acetate gel (3450130, Bio-Rad) using the Criterion System (Bio-Rad). Proteins were separated for 100 min at 100 V in Tris-tricine buffer (161-0744, Bio-Rad) and visualized using PageBlue protein stain. BSA (2 mg/ml) was used as a negative control.

Gel filtration chromatography

C-terminal His-tagged C. jejuni FliD was isolated as described above, cleaved with 2000 units of TEV protease (T4455, Sigma) overnight at 4°C with end-over-end rotation, and dialyzed twice against PBS. Proteins were concentrated using 30 kDa cut off-filters (Pierce) by centrifugation (14,000 x *g*, 15 min) and then applied to a Superose® 10/300 column using the Akta Explorer system (GE Healthcare) equilibrated with DPBS (D8537, Sigma). The Gel filtration markers kit (MWGF1000, Sigma) containing thyroglobulin (669 kDa), ferritin (443 kDa), amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and anhydrase

(29 kDa), was run in advance to be able to estimate the size of the FliD multimer.

Sedimentation velocity profiling

Recombinant *C. jejuni* FliDΔNC protein was isolated under native conditions as described above and dialyzed against DPBS containing 1 mM of $CaCl_2$ and 1 mM $MgCl_2$. Several concentrations of FliDΔNC were diluted to 1.88 mg/ml (31.47 µM), 1.25 mg/ml (20.92 µM) and 0.94 mg/ml (15.73 µM) with DPBS containing Ca/Mg. Four-hundred µL of sample was put in 12 mm path length double-sector aluminum centerpieces with sapphire windows centerpieces and 420 µL of its buffer was brought into the reference sector. Prior to the sedimentation velocity experiment the filled cell was equilibrated for at least 2 h at the desired experimental temperature of 20°C. Change in solute concentrations were detected by 180-200 absorbance scans measured at 280 nm.
The oligomeric states of FliDΔNC were investigated by sedimentation velocity experiments on XL-I and XL-A analytical ultracentrifuges (Proteomelab and Optima XL-A, Beckman Coulter) using absorbance optics. Samples were centrifuged in the above-mentioned centerpieces in a 4-hole AN60Ti rotor at a speed of 40,000 rpm at 20°C. SednTerp (http://sednterp.unh.edu) was used to calculate protein partial specific volumes and solvent densities and viscosities from the protein amino acid sequences and buffer compositions. Analysis and fitting of the data was performed using the program Sedfit v 14.3. A continuous c(s) distribution model was fitted to the data to determine the number of species, their sedimentation coefficients, and their fractional contributions to the species populations. All sedimentation coefficients were corrected to $s_{20,w}$ values.

Biotinylation of FliD proteins using sortase-tagging

FliD proteins (13 nmoles) were incubated (4 h, room temperature (RT)) with 2x molar excess of *S. aureus* sortase enzyme (pet30b-7M SrtA was a gift from Hidde Ploegh, Addgene plasmid #51141) and 30x molar excess of GGGK-biotin (synthesized as described in[28] to site-specifically biotinylate the C-terminus. The His-tagged sortase was removed using 100 µl of Ni-NTA beads (1 h, RT). Free biotin was removed by dialysis against DPBS (16 h, 4°C). Biotinylation was confirmed by Western blot using Streptavidin-HRP.

SDS-PAGE and Western blotting

Purity of proteins was estimated by SDS-PAGE using 10% gels. Samples were boiled in Laemmli buffer before loading onto the gel (1 µg of protein/lane) placed in a Bio-Rad PROTEAN® system. Gels were runs at run at 150 V for 60 min. Proteins were visualized using PageBlue (24620, ThermoFisher Scientific). For Western blotting, proteins were transferred onto PVDF (0.22 µm pore size) in the Trans-Blot® Turbo™ system (Bio-Rad) at 25 V for 10 min. After blocking with 4% skim milk (Elk, Campina) in Tris-buffered saline with 0.05% tween (TBS-T) (4 h, RT), blots were incubated (1 h, RT) with the HRP-conjugated streptavidin (016-030-084, Jackson ImmunoResearch) diluted (1:10,000) in 2% skim milk in TBS-T. The blots were developed with ECL SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) and antibody reactivity was visualized in a Gel Doc™ XR system (Bio-Rad).

Cell-based ELISA

Cell-binding assays were performed as previously described[8]. Shortly, fully confluent cells in a 96-wells plate grown in tissue culture medium, were rinsed with DPBS, fixed with 1% paraformaldehyde (1 h, RT), and blocked with 4% bovine serum albumin (BSA, A7030, Sigma) in DPBS (D8662, Sigma). Biotinylated FliD proteins of the indicated species (25 pmol per well) diluted in 2% BSA-DPBS were allowed to bind for 1 h at RT. After extensive rinsing with DPBS, cell-bound FliD was detected with HRP-labeled streptavidin (1:50,000). HRP activity was measured by adding HRP-substrate (555214, BD Biosciences). The reaction was stopped after 15 min with 1 M H_2SO_4. Color development was measured using a plate reader at 450 nm (FLUOstar Omega, BMG Labtech}. When indicated, FliD proteins were preincubated with heparin (H4784, Sigma), hyaluronic acid (53747, Sigma), α-caseins (C6780, Sigma) or dephosphorylated α-caseins (C8032, Sigma) in the indicated concentrations for 1 h at RT prior allowing FliD binding to cells.

Bioinformatics analysis and structure predictions

Alignment of FliD amino acid sequences of *C. jejuni* strain 11168 (GI:218562200), *C. iguaniorum* (GI:669187914), *C. difficile* (NCBI Reference Sequence: WP_003421095.1), *E. coli* (NCBI Reference Sequence: WP_000146784.1), *P. aeruginosa* strain PAK (GI:489211809), *P. aeruginosa* strain PAO1 (NCBI Reference Sequence: WP_003082190.1), *S.* Typhimurium (GI:301158474) and *S*. Enteritidis (GI:197940049) was performed using Clustal Omega[29]. Data were further processed with Ident and Sim[30] with similarity set based on the side chains (polar vs non-polar) and charge (negative vs positive).

The prediction of the secondary structure of FliD of *C. jejuni* was performed with SPIDER2[31], APSSP2[32] and PsiPred[33] and structures predicted by at least two out of the three programs were depicted. The tertiary structure of FliD of *C. jejuni* was predicted using Phyre2 software[21]. The templates with most confidence were obtained using the FliD structure of *E. coli* (5H5V), *P. aeruginosa* PAO1 (5FHY) and *S.* Typhimurium (5H5T). UCSF Chimera version 1.10.1 was used to visualize the three-dimensional structures[34].

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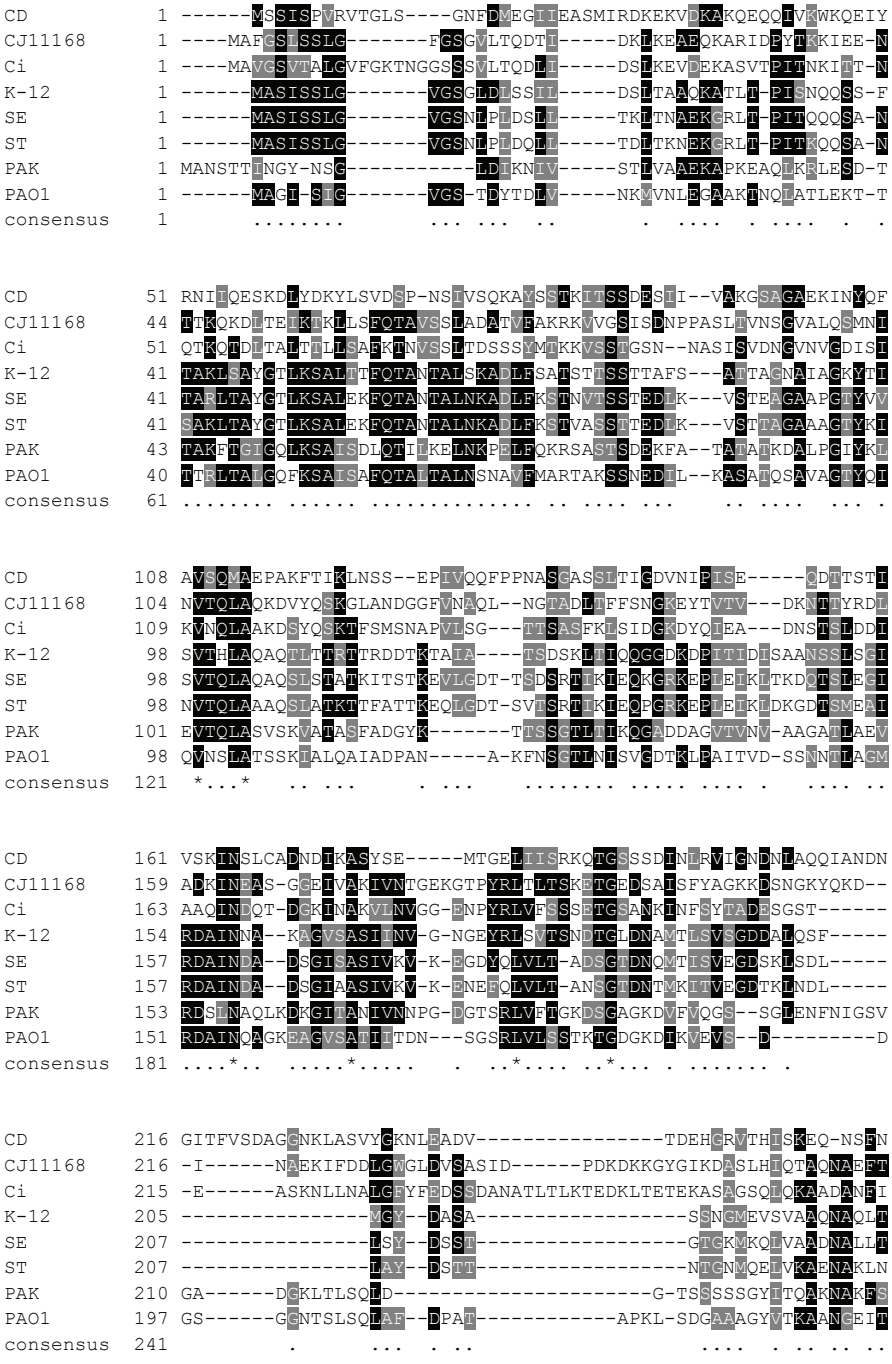
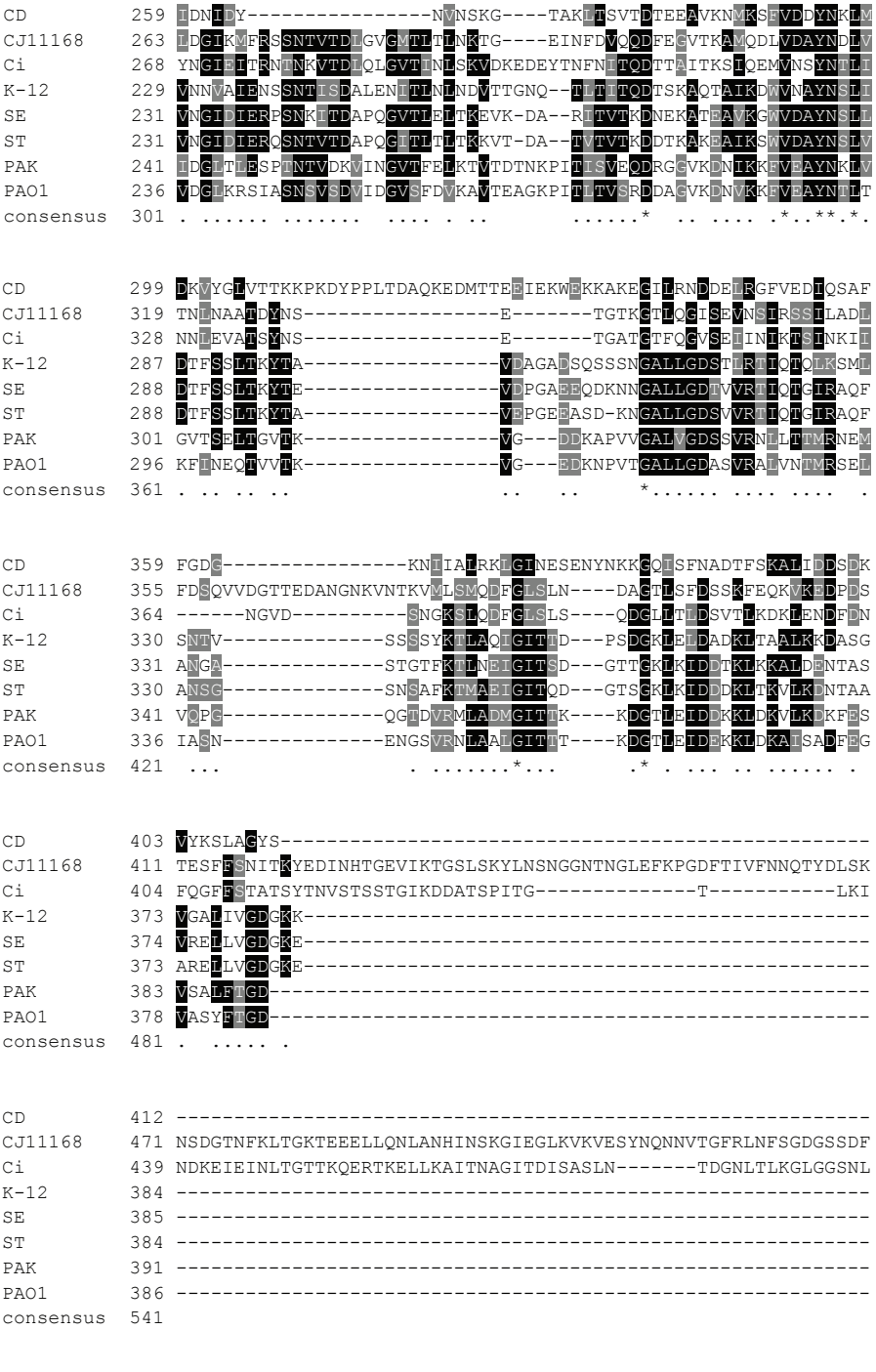
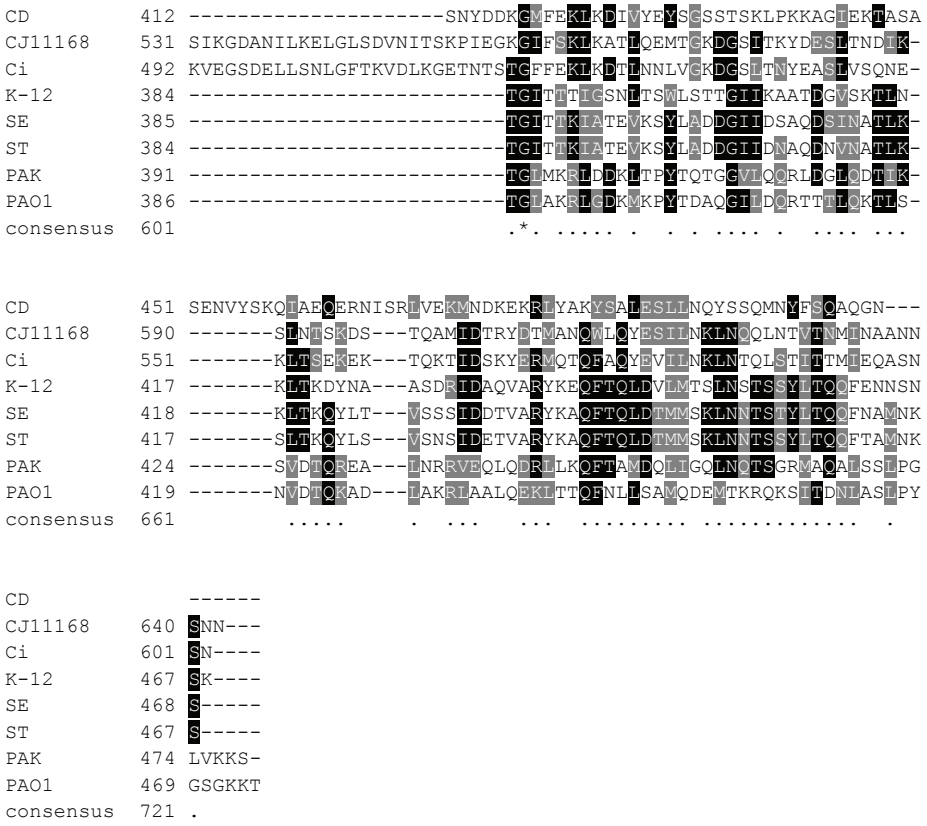
SUPPLEMENTARY FIGURE







Supplementary figure 1: Alignment of FliDs of different species
Alignment of FliD protein sequences of *C. difficile* (NCBI Reference Sequence: WP_003421095.1), *C. jejuni* strain 11168 (GI:218562200), *C. iguaniorum* (GI:669187914), *E. coli* strain K-12 (NCBI Reference Sequence: WP_000146784.1), *S*. Typhimurium (GI:301158474) and *S*. Enteritidis (GI:197940049) and *P. aeruginosa* strain PAK (GI:489211809) and strain PAO1 (NCBI Reference Sequence: WP_003082190.1) generated with Clustal Omega and Boxshade. Residues boxed in black correspond to 50% identity. Residues boxed in gray correspond to 50% similarity.

Chapter 6

General discussion

Aim of the thesis

Campylobacter jejuni is most common bacterial agent causing human diarrhea[1]. Symptoms can range from mild colitis to severe bloody enteritis with abdominal cramping. In some cases, autoimmune disorders such as *Guillain-Barré* syndrome can follow due to *C. jejuni* LOS-specific antibodies that cross-react with host surface structures[2]. Chickens represent the main reservoir of *C. jejuni* and contaminated meat products are an important source of human infection. One major strategy to reduce human infection is to limit *C. jejuni* colonization of the chicken gut. An alternative is to develop a vaccine that protects humans against infection. Both solutions require detailed knowledge of the behavior of the bacterium in the complex setting of the intestine. The present study was designed to understand the initial molecular interaction of *C. jejuni* with its host and to investigate natural infection intervention mechanisms.

At this time, only a few *C. jejuni* virulence factors have been identified and detailed understanding as to how *C. jejuni* interacts with the intestinal host cell lining and causes human but not chicken pathology, is lacking. Adhesion to host cells is often the first step during infection. Only a few *C. jejuni* adhesins and their corresponding receptors have been characterized but their role during infection is still disputed. However, it is generally accepted that the bacterial flagella are essential for bacterial colonization and infection. *C. jejuni* carries one single flagellum at each pole that can rotate a very high speed. This propeller-like function in combination with the highly complex chemotaxis system moves the bacterium to its preferred intestinal niche. Immotile bacteria are unable to colonize chickens[3]. Additionally, the flagellum of *C. jejuni* has been proposed to act as a protein secretion system, releasing invasion proteins into the host cytosol[4]. However, this needs to be confirmed for different *C. jejuni* strains.

The work described in this thesis focused on the initial interaction of *C. jejuni* flagella with host cells. In the studies described in **Chapter 2**, we discovered that the flagellar tip protein FliD binds to the cell surface, acting as an early attachment factor. Moreover, we identified heparan sulfate glycosaminoglycans as the binding partner of FliD at the host cell surface. The region in FliD that is responsible for the interaction was mapped to the central part of the FliD protein. The potential significance of this discovery became evident when we found that anti-FliD antibodies in the egg yolk can block the binding of FliD to cells (**Chapter 3**). These maternal antibodies may thus contribute to the documented protection of chickens against *C. jejuni* colonization within the first two weeks of age. The interaction of the *C. jejuni* FliD with host cells also led to the discovery that milk contains a natural inhibitor of FliD binding. We identified milk caseins as the inhibitory compound (**Chapter 4**). Lastly, by employing the availability of structural data of FliD proteins of several other bacterial species, we predicted the structure of FliD of *C. jejuni* in order to identify patches with cell binding potential. This work also revealed that the flagellar tip proteins of different species display bacterial adhesion properties as well (**Chapter 5**), suggesting a common mechanism of FliD-host cell interaction. Together, our findings shed light on how *C. jejuni* and perhaps other bacterial species exploit their flagella tip protein for initial contact with the host and that maternal antibodies and milk constituents may be valuable natural sources to prevent this interaction.

FliD as key factor in flagellum assembly and motility

The flagellar tip protein FliD (HAP2) is present in virtually all flagella bacterial species. The protein belongs to the family of hook-associated proteins (HAP). These proteins including the proteins FlgK (HAP1) and FlgL (HAP3) were originally though to connect the hook filament of the flagellum to the flagella fiber. Later, it became clear mainly from studies with *Salmonella* Typhimurium[5] that FliD (Hap2) did not stay at the tip of the hook, but moved forward from the hook-filament junction towards the distal end of the flagellum during growth of the flagella filament[6,7]. The force needed for flagella growth is supposed to be provided either by entropic folding of flagellin on the flagellar tip[8] or by the active flagellar motor force[9]. In both cases, the common concept is that flagellin can only travel in a linearized form through the flagellar channel and will fold one after another at the distal end pushing the flagellar tip protein FliD forward[7]. This means that on one hand FliD needs to be flexible enough to allow flagellin folding and on the other hand needs to stay firmly enough attached to allow flagellum growth.

Initially, the importance of FliD for bacterial motility was demonstrated in studies with *Salmonella* that showed that loss of FliD resulted in immotile bacteria[10,11]. In analogy, we observed a similar immotile phenotype when we introduced an antibiotic resistance cassette in the *C. jejuni fliD* gene, confirming that FliD is also essential for motility in *C. jejuni* (**Chapter 2**). In fact, FliD is involved in flagellar assembly by capping the filament as bacteria that lack FliD release high amounts of the flagellin subunits in their supernatant[11]. In the case of *Salmonella*, the addition of purified FliD can inhibit this leaking of flagellins, suggesting that external FliD can associate to the flagellar tip and close the hollow flagellar fiber[11].

FliD consist as a multimeric complex. Despite the important role of FliD in flagella formation, it is not known how *C. jejuni* prevents FliD self-polymerization and is processed inside the bacterium to the flagellar export gate. In *Salmonella*, the flagellar junction proteins HAP1 and HAP3, the FliC flagellin subunit, and the FliD cap are assisted by the chaperones FlgN, FliS and FliT, respectively[12,13]. For *C. jejuni*, however, the only known chaperone is FliS which interacts with FlaA[14]. In *S*. Typhimurium, the FliD chaperone FliT binds with 3 of its 4 helices to the 40 amino acids of the C-terminus of FliD[12,15]. The *Salmonella fliT* gene is located in the genome directly upstream of *fliS*. In *C. jejuni* the gene upstream of *fliS* is annotated to encode a hypothetical protein. Future research will determine if this gene product is the FliD chaperone in *C. jejuni*.

FliD multimerization

In order to efficiently cap the flagellum, FliD has to adopt a multimeric state. Electron microscopy on *Salmonella* FliD showed that the protein assembles into a ring shaped structure. Size exclusion chromatography revealed that FliD existed as a pentamer with a diameter of 10 nm[16]. On the basis of these observations, the FliD complex is considered to be arranged in a star-shaped structure and to be composed of “plate-” and “leg-” structures (**Fig. 1A**). The plate consists of the surface exposed part of the cap, whereas the legs are interacting with the flagellin subunits and buried within the flagellum[17]. Interestingly, the shape and size of the plate differs between species as FliD adopts different multimeric states depending on the species as shown by recently available crystal structures. While FliD from *Salmonella* exists as a pentamer[18], the FliD from *P. aeruginosa* and *E. coli* are hexamers[18,19] whereas FliD from *S. marcescens* assembles into a tetrameric structure[20] (**Fig. 1B**). Which

amino acids residues of FliD partake in the multimerization process is still unknown. Attempts to map the multimerization sites in *Salmonella* FliD showed that mutation of V172 and I234/I236 resulted in lost oligomerization potential[18]. This suggests that hydrophobic interactions might be involved. Additionally, a mutation of L443 in the C-terminus inhibited multimerization. This effect could be due to loss of the FliT binding site[21]. The identified amino acid residues are conserved in some, but not all species. This observation together with the high degree of dissimilarity between different FliD proteins (**Chapter 5**) suggests that specific charged residues or protein regions rather than conserved interacting regions are the basis of the multimerization.

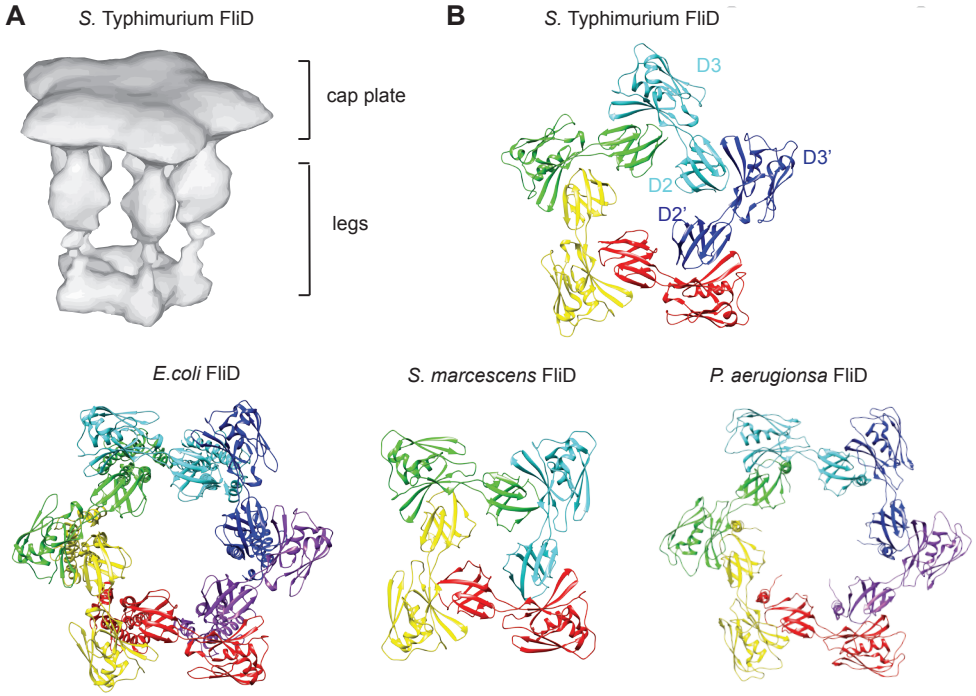


Figure 1: Multimerization of FliD of different species.
(A) 3D reconstruction of FliD of *S.* Typhimurium. The star-shaped pentamer consists of a cap plate and five legs. Reprinted with permission from[22] **(B)** Top view of the FliD multimer cap formation of *S.* Typhimurium (PDB 5H5T), *E. coli* (PDB 5H5V), *S. marcescens* (PDB 5XLJ) and *P. aeruginosa* (PDB 5FHY). Each monomer is depicted in a different color and the corresponding D2 and D3 domains are indicated in the respective color.

As described in thesis, the FliD protein of *C. jejuni* also adopts a multimeric state as demonstrated by chemical cross-linking experiments (**Chapter 5**). To identify the composition of the multimers, we employed several complementary approaches. Size exclusion chromatography (SEC) using the ÄKTA system, indicated that FliD complex had a mass range of 450-500 kDa, pointing to a heptameric structure. This was confirmed by analytical ultracentrifugation (AUC). However, using AUC, we observed a substantial proportion of FliD in a monomeric form, which was not apparent in the SEC experiments. This might be explained by the use of His-tagged truncated FliD for AUC whereas for SEC we used full length protein with enzymatically removed tag. This may indicate that the termini of the protein are somehow involved in proper formation of recombinant multimeric FliD, as has been shown for FliD of *P. aeruginosa*[19].

The difference in multimerization of FliD between different species may be related to the difference in flagellar structure. For instance, the flagellum of *Gammaproteobacteria* such as *Salmonella* consists of 11 protofilaments[23], whereas the flagella fiber of *Epsilonproteobacteria* like *Campylobacter* are composed 7 protofilaments[24] (**Fig. 2**). This difference may ask for a different flagellar cap structure.

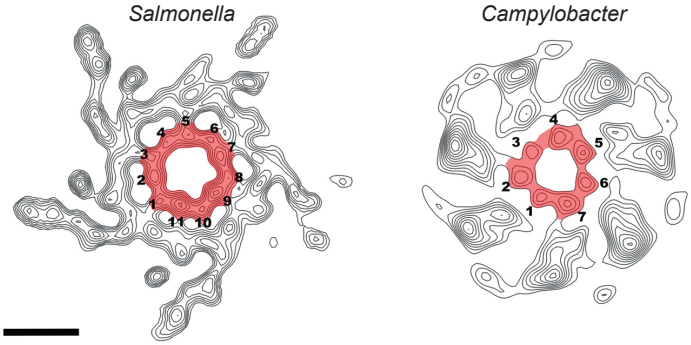


Figure 2: Cross-section of reconstituted flagellar structures of *Salmonella* and *Campylobacter*.
The flagellum of *Salmonella* is composed of 11 protofilaments, whereas de flagellum of *Campylobacter* has 7 protofilaments. The black bar indicated 50 Å. Reprinted with permission from[24].

In line with this hypothesis, the complementation of a *Pseudomonas* Δ*fliD* mutant with *fliD* from *Salmonella* failed and resulted in immotile bacteria, in opposite to complementation with the wild type *Pseudomonas fliD*[19]. Interestingly however, complementation of a *Salmonella* Δ*fliD* mutant with *fliD* from *E. coli*, but not with *fliD* from *P. aeruginosa*, restored motility. The complemented strain produced a similar number of flagella per bacterium as the wild type albeit with slightly reduced lengths[25]. This result seems rather surprising as the FliD proteins of *E. coli* and *Pseudomonas* both form hexamers in opposite to the pentameric FliD from *Salmonella*. During our comparative bioinformatics analysis on FliD of different species, phylogenetic tree analysis showed that the sequence conservation of FliD proteins reflects the familial genetic proximity, indicating that genetic relatedness of FliD is more important for FliD function than the identified differences in multimeric complexes between different species (data not shown). Complementation of the *C. jejuni ΔfliD* mutant with different FliD species have not been performed but may shed more light on the importance of the strict nature of the FliD complex for flagella assembly. To determine the actual shape and composition of FliD protein of *C. jejuni*, additional experiments using more precise techniques such as cryo-electron microscopy or crystal structure determination are required.

The function of *C. jejuni* FliD as bacterial adhesin

The first indication that *C. jejuni FliD* may act as an adhesin was the observation during live imaging of infection of cultured cells that bacteria attached to cells seemingly by their flagellar tip (**Chapter 2**). Since FliD is important for *C. jejuni* motility, we used the immotile Δ*motAB* background to construct a Δ*fliD* mutant to rule out the involvement of motility during infection. We also tested the Δ*flaAB* mutant, which lacks the flagellin subunit and thus cannot make flagella. This mutant also showed reduced adhesion capacities, despite the presence of FliD in this mutant (data not shown). This may indicate that the attachment of the flagellar tip protein to host cells requires a certain distance from the bacterial body,

which is conferred by the flagellin filament. Although our observations seem to implicate FliD in cellular adhesion, additional interactions of the flagellin subunit protein cannot be excluded. Moreover, we found that inactivation of the motor proteins (*motAB*) leads to increased adhesion (data not shown). This suggests that the rotation of the flagella may rescue the bacterial adhesion and perhaps that flagellar binding is reversible. What signals may reduce the flagellar movement in the vicinity of host cells is unknown, but may involve chemotactic signals.

Binding assay with purified recombinant FliD protein confirmed the binding of FliD to host cells. We mostly used recombinant FliD protein in these studies to exclude a possible role of contaminating flagellin proteins. However, similar results were obtained with native FliD proteins isolated from *C. jejuni* culture supernatants (**Chapter 2**). Immuno-fluorescence microscopy revealed that FliD was present at the cell surface as large protein clusters. Whether the protein adhered in aggregated form to the cells or whether FliD binding results in receptor clustering and/or uptake needs to be further investigated. Incubation of cells with FliD-coated beads did not result in bead internalization (data not shown).

C. jejuni FliD binding to heparan sulfate

In search for the host cell binding site that is targeted by *C. jejuni* FliD, we tested *C. jejuni* FliD binding to several cell types with different glycosylation deficiencies (**Chapter 2 and Chapter 5**). We choose this strategy as bacterial pathogens often use glycans as host cell receptors during adhesion[26]. We observed reduced binding to cells lacking glycosaminoglycans (GAG) on their surface. The use of several soluble GAG types as receptor analogs showed that FliD interacts with soluble heparin. This was confirmed by reduced binding to cells after enzymatic removal of heparan sulfate. Finally we could show direct binding of FliD to heparin in an ELISA setting (**Chapter 2**). Heparan sulfate glycosaminoglycans are ubiquitously present on eukaryotic cells and often serve as receptor for bacterial and viral pathogens. The binding to the receptors is mostly based on electrostatic interactions with spatially clustered sulfate residues on the glycosaminoglycans and may be just a first step to anchor the pathogen to the cell surface.

In a rather classical search for the region in FliD that confers binding to the receptor, we constructed several FliD protein fragments and tested them for their binding capabilities. This showed that the central region of the FliD protein encompassing amino acids 210-418 is responsible for the observed binding activity (**Chapter 2**). To further pinpoint the binding region, we looked for a potential common heparin-binding motif in the FliD protein sequence. Heparin binding sites may typically contain the XBBXBX or XBBBXXBX motifs in the protein sequence, where B represent a basic residue and X a hydropathic residue[27]. However, many proteins do not exhibit such a motif and yet bind heparan sulfate, questioning whether these motifs are the solely determinant of GAG binding. Nowadays, the motif is reduced to the presence of one polar residue flanked by two cationic residues whereby the structural topology of positively charged residues determines the heparin binding property[28]. Due to the increasing number of available crystal structures of FliD from other species[18,19], we were able to use a bioinformatical approach to build a homology model of the putative structure of *C. jejuni* FliD and identify the amino acid residues that may be involved in the interaction with heparan sulfate (**Chapter 5**). In **Chapter 2**, we could narrow the heparin-binding region of FliD to residues located between amino acids 210 and 418. Modeling *C. jejuni* FliD *in silico* showed several positively charged lysines head domains of the FliD protein, which

are supposed to be surface exposed. Zooming into the second head region showed the presence of three potential lysines in close proximity, resulting in a positively charged patch that could be a key region in the interaction with GAG. Mutagenesis studies are needed to determine whether these residues are indeed involved in flagellar cell attachment in *C. jejuni*.

Other functions of *C. jejuni* FliD

Role of FliD binding in protein secretion

The flagellar apparatus resembles in many aspects the type III secretion system (T3SS) that is essential for pathogenicity in many other bacterial species. T3SS inject bacterial effector molecules into the host cell cytoplasm to manipulate host cell biology. *C. jejuni* and several other species do not express a T3SS, but it does secrete bacterial proteins through the flagella fiber[29–31]. For *C. jejuni*, a functional flagellar structure is necessary for the export of *Campylobacter* invasion antigens (Cia) and the flagellin type C[4,32,33].These secreted proteins seem to be involved in cell invasion and apoptosis[34]. In *C. jejuni* strain strain 81-176, the CiaC protein is delivered into the host cytosol by flagellar secretion and induces membrane ruffling[35]. Although, a mutation in *fliD* in *C. jejuni* did not alter the secretion pattern of the Cia proteins in culture broth, the bacterial adhesion and invasion were reduced[4]. Since FliD is located at the flagellar tip, it likely represents the contact point of the flagellar secretion system with the host cell. Therefore, it would be interesting to study whether the absence of FliD on the flagellar tip results in a decreased translocation of Cia proteins into the host cytoplasm, thereby contributing to the reduced invasion phenotype. An even more revolutionary scenario would be when interaction of FliD with the host cell surface results in the opening of the flagella fiber enabling the local release of effector molecules. This would imply that FliD might also have a role in the secretion of virulence factors in *C. jejuni*.

FliD as mechanosensor

Besides a role in flagella assembly, bacterial adhesion and protein secretion, FliD may have a function in the mechanosensing of surfaces. One hypothesis is that the stators in the flagellar body are dynamic sensors, which change structure upon an external stimulus[37]. In *Bacillus subtilis*, the inactivation of the motor stator gene *motB* results in increased activation of the two-component system DegS/DegU, which is involved in biofilm formation[38]. This suggests that sensing surfaces elicits a response changing the behavior of motile bacteria into a less motile type to enable contact. However the stimuli and signal transfer are unknown so far. It is tempting to speculate that the flagellar tip, which protrudes far from the bacterial body, could be involved in this mechanism. This would provide a link between attachment and a stop of flagellar rotation and/or flagella assembly, which would be beneficial for colonization. In order to sense surfaces, some bacteria like *Caulobacter crescentus* have pili, which together with the flagellum confer adhesion. Upon pili contact with the surface, the flagellar rotation stops and the bacterial surface components are stimulated to adhere more firmly to the surface[39]. Since *Campylobacter* has no pilin genes encoded in the genome, it may be hypothesized that the flagellum itself operates as a mechanosensor, which represents an interesting field to investigate in the future.

Soluble FliD as decoy

Finally, we found considerable amounts of soluble FliD protein in the *C. jejuni* culture supernatant. This was unexpected as only a few bacterial FliD molecules are needed to form the FliD complex on the tip of the flagella. Whether the secretion of FliD in the medium has a functional role remains to be investigated. It can be hypothesized that the secreted FliD interacts with host cells. Preliminary studies suggest that recombinant FliD may activate the inflammasome without activating TLRs directly (data not shown). Alternatively, it can be imagined that the secreted FliD acts as a decoy mechanism to neutralize FliD-specific antibodies (**Chapter 3**) that may limit bacterial motility or flagella filament growth. The presence of large amounts of anti-FliD antibodies in egg yolk indicates that the protein is immunogenic. These antibodies have potential to inhibit *C. jejuni* colonization of the chicken gut (**Chapter 3**).

FliD in *C. jejuni* vaccine development

Since we implicated FliD as an important virulent factor promoting bacterial host attachment for *C. jejuni*, FliD could represent a valuable target for vaccine development. Previously, it has been demonstrated that *C. jejuni* resides as a commensal in the chicken gut. However, newly hatched chicks remain *Campylobacter*-free during the first two weeks of life due to protection by maternal antibodies. These antibodies (IgYs) are transferred from the hen through the egg yolk into the chick's blood system and to the intestines via its leaky gut[40]. We detected high amounts of anti-FliD antibodies in egg yolk and chicken blood. Interestingly, serum titers started declining in the second week of life, which correlates with the time frame of protection against *Campylobacter* (**Chapter 3**). This suggests that they may contribute to the *C. jejuni* colonization resistance of newly hatched chickens.

Several attempts were undertaken to develop a successful vaccine to combat *Campylobacter* colonization of the chicken gut. The search for antigenic structures of *C. jejuni* in chickens revealed that flagellin and other flagellar proteins are highly immunogenic[41–43]. However, the different glycosylation pattern of flagellin (and other surface exposed components) among different *C. jejuni* strains challenges heterologous protection[44]. We observed that anti-FliD antibodies reacted with FliD from different *C. jejuni* isolates, suggesting potential cross-protection against multiple strains (**Chapter 3**). The generation of cross-protective antibodies directed against *C. jejuni* flagellin is hampered by post-translational modification of the flagellin with variable type of *O*-glycans. There is no indication that the FliD protein undergoes a similar process (**Chapter 2**).

Despite the high seroprevalence of anti-FliD antibodies in chicken sera[45], only one vaccination study with FliD has been performed[46]. In this study, chickens were immunized twice with approximately 40 µg FliD per chicken at the day of hatch and at day 14, and challenged on day 28 with 10^7 bacteria. No protective effect was observed, which could be explained by the low vaccination dose and the high bacterial challenge dose[47]. Altogether, those non-optimized parameters could have overruled the protective effect. Clearly, more studies are needed to determine whether FliD is useful as a vaccine antigen. As FliD is only present on the bacterium in very small numbers but, perhaps as decoy, secreted into the environment it can be defended that a successful vaccine should likely be composed a combination with multiple antigens[48].

Prevention of *C. jejuni* infection

In addition to vaccination, a chicken colonization by *C. jejuni* may be reduced by the administration of feed supplements including IgYs. These antibodies can induce agglutination or immobilization of bacteria in the intestinal mucus or increase complement-mediated uptake by phagocytes[48,49]. Feeding chickens before slaughter with anti-FliD IgYs would represent a relatively cheap and simple method to reduce bacterial load. Orally administrated IgYs have been successfully applied to reduce the colonization chicken with *Salmonella*, *E. coli* and *Campylobacter* without affecting their health or behavior[50]. Whether anti-FliD antibodies contributed to this effect was not investigated. However, the use of anti-FliD IgYs as feed in a *C. difficile* infection hamster model resulted in significant increased survival[51], underpinning the potential of FliD as a target of protection. In **Chapter 5**, we found that FliD from *C. difficile* also binds to cells and that egg yolk contains a substantial amount of anti-FliD antibodies. This may suggest that similar binding and defense mechanisms may exist for *C. jejuni* and *C. difficile*.

Besides maternal antibodies, we discovered that milk blocks the interaction of FliD with host cells. This suggests that milk or milk constituents might serve as an alternative to prevent infection with *C. jejuni* (**Chapter 4**). Milk has previously been found to (partially) protect infants from *C. jejuni* infection. This protective effect was attributed to the presence of anti-*C. jejuni* antibodies in human breast milk[52]. We did not detect any *C. jejuni* FliD-specific antibodies in bovine milk. Instead, we identified milk caseins as blockers of the FliD-host cell interaction. The main function of caseins is to transport calcium and magnesium to the newborn. These cations are bound by phosphoserine moieties on the caseins. Since dephosphorylated caseins did not inhibit the binding of FliD to host cells, it can be hypothesized that the inhibition of FliD binding to the eukaryotic cells by milk may be due to interaction of FliD with the phosphoserine clusters on casein rather that the sulfated gags receptors on the host cells. Alternatively, phosphoserines may indirectly block FliD binding to the cells by capturing critical cations. The latter hypothesis is supported by the cation-sensitivity of the FliD binding. Our analytical centrifugation experiments showed that the removal of cations did not alter the multimeric state of FliD (**Chapter 4**). Thus, it can be hypothesized that cations enhance FliD adhesion to host cells by stabilizing heparan sulfate on the cell surface. Several studies report the importance of cations in the stabilization of the heparin molecule[53,54]. Unraveling of the exact mechanism of casein-mediated blocking of FliD binding and the specific role of cations in the interaction is important when milk or caseins are considered to be used as protective agents. This is of additional interest as soluble oligosaccharides in milk often act as a receptor decoys for bacteria and thereby protect humans from infections[55]. It has been shown that *C. jejuni* interact with glycans on the cell surface[56] and that especially the fucosylated blood antigens play an important role during human infection[57]. Overall, the data suggests that milk represents a multipotent inhibitor of the interaction of *C. jejuni* with host cells by providing inhibitory antibodies, glycans as well as caseins.

Conserved properties of FliD in other species

Given the importance of FliD in *C. jejuni* attachment and since all flagellated bacteria carry FliD as the flagellar cap protein, we investigated the binding properties of FliD of *C. difficile*, *C. iguaniorum*, *P. aeruginosa* strains PAK and PAO1, and *S. enterica* serovar Enteritidis and serovar Typhimurium (**Chapter 5**). We found that FliDs from all tested species exhibit similar

binding properties as demonstrated for FliD of *C. jejuni*, despite very low protein sequence conservation. FliD from *E. coli* has previously been shown to be involved in adherence to microvilli from enterocytes[58], whereas FliD from *C. difficile* binds to mucus[59] and FliD from *P. aeruginosa* strain PAO1 to sialyl-Lewis X antigens in mucus[60]. Interestingly, both heparin and milk caseins were able to inhibit the interaction of the various FliD proteins with the host cells. These results hint to a conserved role of FliD in bacterial adhesion to sulfated glycosaminoglycan-like receptors either on the cell surface or in the mucus. Analysis of the protein structures identified several putative regions in the different FliD proteins that could contribute to the interaction. However, more research is required to provide sufficient evidence whether these sites are indeed involved in the FliD-mediated bacterial attachment.

The apparent common function of the flagellar tip protein of different species in the early contact with host cells supports the functional relatedness of the flagella and T3SS[61,62]. In both cases, the tip proteins of the flagellum or the syringe-like injection machinery of T3SS may confer the initial contact with the host cells to deliver effector molecules. The T3SS has two conserved translocator proteins and one needle tip protein forming a temporary assembled tip complex. Despite detailed research on the T3SS assembly and structure, the detailed underlying mechanisms of triggering and the consequent signal transduction to initiate effector protein translocation remain under investigation[62]. In *Pseudomonas*, the two hydrophobic translocator proteins PopD and PopB form a pore in the host cell membrane, after which they undergo conformational changes by a yet unknown trigger. Consequently, this change is transmitted to the needle-tip protein PcrV, which induces effector protein secretion. These findings suggests that the tip complex is not only required for pore formation, but is also involved in sensing the contact to the host[63], a feature shared with the mechanosensing ability of the flagellum. Similar to the charge-mediated FliD adherence (**Chapter 2 and 5**), the interaction of the T3SS complex with the host cell is most likely mediated by charge and hydrophobicity of the tip proteins and not by engaging a unique protein receptor. This may not be surprising since the T3SS needle should be able to efficiently inject effector proteins into different host cells under various conditions. Moreover, it may seem logical to engage a reversible interaction, as is the case for the FliD-mediated attachment of the flagellum. It is thus tempting to speculate that the shared ability of FliD from different species to bind to heparan sulfate moieties on cells may be a shared character with the T3SS due to the ancestral resemblance. At this point, it should be noted that heparin can interfere with the translocation of effector proteins in *Yersinia* by binding the regulatory protein LcrG. It remains a major challenge to observe this phenomenon *in vivo*, since many enteropathogens have additional virulence factors, which may hide or overrule FliD-mediated interaction with the cells.

Concluding remarks

C. jejuni is main cause of bacterial diarrheal disease in humans worldwide, however the detailed mechanisms underlying the interaction between the bacteria and the host remain poorly defined. Here we provide the first evidence that the flagellar tip protein FliD of *C. jejuni* acts as an attachment factor during infection and that FliD binds to heparan sulfate on the cell surface via its central domain. We also made first attempts to gain insights into its multimeric structure. By employing a bioinformatical approach, we propose that several lysine residues may be important determinants of heparan sulfate binding and cell

attachment. However more research is needed to fully unravel the FliD structure in *C. jejuni* and to precisely determine the residues involved in the interaction.

So far, insufficient measures are available to reduce human *C. jejuni* infection. Given its importance as attachment factor and its immunogenic potential, we propose FliD as an attractive vaccine antigen candidate to reduce bacterial loads in chickens. The presence of anti-FliD antibodies in newly hatched chicks that are resistant to colonization further underpins the importance of FliD to be considered in future vaccination strategies. In addition to supplementing chickens with IgYs, we also propose milk consumption as a possible strategy to fight *C. jejuni* infection as milk caseins efficiently inhibit FliD binding to cells.

Overall, we identified the flagellar cap protein FliD as a new attachment factor for *C. jejuni*. In addition, we shed first light on the molecular mechanism underlying the binding of FliD to host cell heparan sulfate. Caseins or antibodies can block this interaction. These findings will hopefully contribute to the development of the much needed prevention strategies against *C. jejuni* induced diseases.

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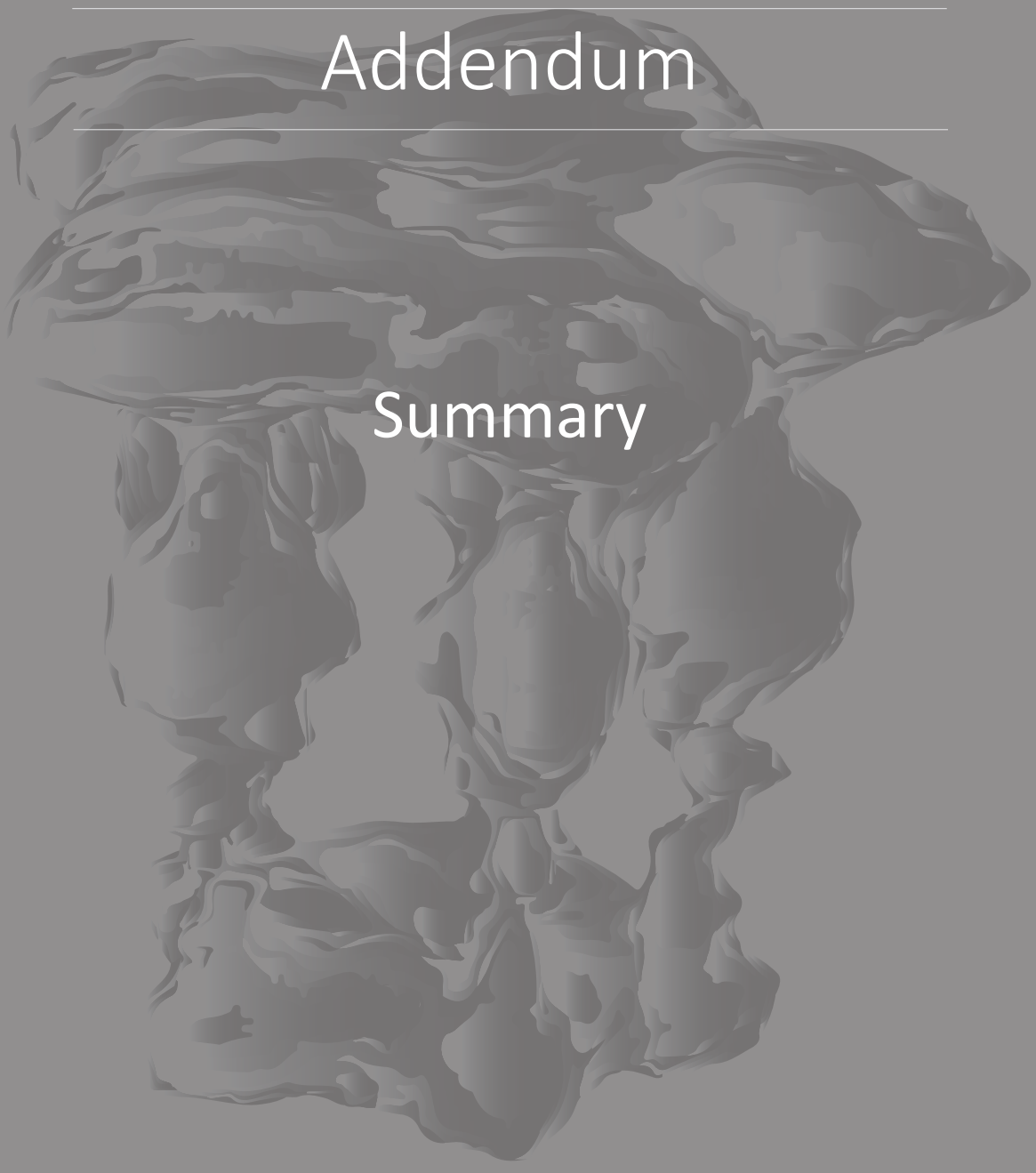
Addendum



Summary

Introduction

Campylobacter jejuni accounts for most bacterial diarrheal disease in humans, outnumbering infections caused by *Salmonella*. Most of the time humans get infected by consuming undercooked poultry meat. Once ingested, the bacteria reach their favorite niche deep in the intestinal crypts. Consequently, the epithelial intestinal barrier is breached, which causes a strong inflammatory response and results in clinical symptoms, such as bloody diarrhea, nausea, fever and abdominal pain. In most cases, the disease resolves after 5-7 days, however in some patients post-clinical complications develop, resulting in neuronal damage such as Guillain-Barré syndrome or other gastro-intestinal diseases.

C. jejuni is a Gram-negative bacterium, which resides as a commensal in the chicken gut. The animals do not display pathology or symptoms and why humans but not chickens are susceptible for disease remains still a mystery. Once a single chicken encounters the bacteria, the entire flock will be colonized in a very short time. The chicken caeca contain a lot of pyruvate, serine, aspartate and fumarate, which are the preferred energy sources for *C. jejuni*.

Surprisingly, in opposite to other pathogens such as *Salmonella*, *C. jejuni* exhibits very few virulence factors and its pathogenicity is largely unknown on the molecular basis. It is widely accepted that motility and chemotaxis are the most important features necessary for successful colonization and infection. Motility is conferred by two flagella, one situated on each pole, which allow the bacterium to move with high speed through liquid and viscous environments such as the mucus in the gut. The flagellum is composed of several important segments. The flagellar motor is found in the bacterial membrane and generates torque. It rotates the flagellar filament, which is connected to the motor via a hook structure. At the end of the filament, we can find the flagellar tip protein, termed FliD, which is necessary for correct filament formation. Additionally, the flagellum protrudes far from the bacterial body and is also involved in adhesion to the cell surface, but the detailed mechanisms remain unsolved.

In order to be able to prevent *C. jejuni* infection in humans, one must first understand the full repertoire of bacterial adhesion and invasion factors. This thesis describes the interaction of the flagellar tip protein with the host and identifies components that interfere with this interaction. This information is important in the development of anti-infective agents and an effective vaccine.

This thesis

A possible role of the flagellum in bacterial attachment to mucosal cells was initially observed during infection experiments, in which *C. jejuni* seemingly attached to the cell surface via its flagellar tip (front cover). The flagellar tip consists of the flagellar cap protein FliD, which aids flagella assembly and stabilizes the flagellar structure. In search for an additional function in bacterial attachment, we purified recombinant FliD protein from an *E. coli* expression system and tested its binding capabilities to different cell types (**chapter 2**). Reduced binding to glycosylation-deficient cell lines pointed to a role of glycosaminoglycans (GAGs) as a

potential binding partner of FliD. GAG moieties are long chains of repetitive, heavily sulfated diplo-sugars present on the cell surface making them an attractive target for the bacterium. Neutralization experiments with different soluble receptors and enzymatically removing these receptors identified heparan sulfate as the class of GAGs that are responsible for FliD binding. Importantly, we also confirmed that FliD derived from *C. jejuni* supernatant exhibits similar binding characteristics to the recombinant protein. Finally, we mapped the heparan binding site in the central region of FliD protein. Overall, we identified an additional role for FliD during early attachment of *C. jejuni* to cells and showed that the flagellar tip protein FliD is necessary for more than solely maintaining flagellum stability.

Chickens represent the main reservoir for human infection with *C. jejuni* and reducing the bacterial load in poultry would consequently result in reduced infections in humans. Interestingly, chicken remain *Campylobacter*-free in the first two weeks of their lives. This colonization resistance is widely assumed to be related to the maternal immunity conferred by the mother hen. Unlike mammals, which receive antibodies via breast milk, the maternal antibodies in birds (termed IgYs) are deposited in the yolk during the making of the egg. During embryonic growth, they are transported via specific receptors into the blood and mucosal surfaces of the progeny and protect it from infection in early life. We therefore hypothesized that antibodies directed against FliD of *C. jejuni* might play a role in the colonization resistance in the first two weeks of age. In **chapter 3**, we detected high amounts of anti-FliD antibodies in the egg yolk. The isolated anti-FliD IgYs were cross-reactive against *C. jejuni* from different strains, suggesting protection against infection with several *C. jejuni* strains. Most importantly, the IgYs block the binding of FliD protein to cells. Further epitope determination revealed that they are directed mostly against the receptor binding site (identified in chapter 2) in the center of the FliD protein, which could explain their blocking mechanism. This information opens new perspectives to reduce *C. jejuni* colonization in chickens to prevent human disease.

In **chapter 4**, we continued our search for agents that block the interaction of FliD and the cell surface and discovered the presence of an inhibitor in cow milk. After excluding the presence of anti-FliD antibodies, we identified milk caseins as the inhibitory agent. Caseins are milk proteins that give milk the white color. They represent the major protein fraction in milk. The main function of caseins is the binding and sequestering of calcium and magnesium. These divalent cations bind to specific phosphoserine residues present in the casein proteins. The cations are transported in high amounts to the newborn, where they are important for bone and teeth formation. We found that dephosphorylated caseins, which are unable to bind cations, are also unable to block FliD binding to cells. This suggested that either the phosphoserines themselves or the cation-binding characteristic is important for the inhibition. We next showed that FliD binding is partially cation dependent, as the cation chelator EDTA could also block the interaction, however the precise role of calcium and magnesium in FliD binding remains to be elucidated. As caseins also inhibit *C. jejuni* infection, milk might represent an anti-infective agent, preventing human disease.

Since all flagellated bacteria have FliD as their flagellar tip protein, we next investigated whether the cell binding ability of FliD is unique for *C. jejuni* or whether this feature is shared with other flagellated bacteria. In **chapter 5**, we report on the binding capabilities of FliD of other species. All tested FliD proteins were able to bind heparan sulfate on the cell surface

and displayed similar binding characteristics as found for *C. jejuni* FliD. Recent protein crystal structures of FliD from other bacteria (namely *Salmonella*, *Escherichia* and *Pseudomonas*) allowed us to model *C. jejuni* FliD and identify potential cell binding sites in the FliD protein. Several positively charged lysine residues in close proximity to each other could represent a possible binding site, since the interaction with heparan sulfate is most likely charge based. A similar putative binding region can be found in FliD from other flagellated bacteria such as *Salmonella.* This could explain the shared binding characteristic of FliD from several species.

Overall, this thesis sheds light on the interaction of bacteria with host cells and unravels how the flagellum is important for bacterial adhesion. More precisely, it describes the binding of the flagellar tip protein FliD of several species to host cells. This suggests that the role of the flagellum is not solely restricted to providing motility but additionally might bridge the bacterial body and the cell surface during infection. In this process, FliD represents the bacterial attachment factor and heparan sulfate its cognate cell receptor. In line with this, we identified several blocking agents, which prevent the interaction between FliD and the cell surface. This indicates that the host has evolved mechanisms to avoid FliD binding and indirectly that FliD may be an attractive target to prevent bacterial attachment and human infection of different pathogens. High-resolution 3D structures of FliD in complex with its receptor may further pinpoint the exact interacting residues in the FliD protein.

Nederlandse samenvatting

Introductie

Campylobacter jejuni is de voornaamste oorzaak van bacteriële diarree bij de mens en overtreft het aantal infecties veroorzaakt door Salmonella. Meestal worden mensen besmet door de consumptie van ongebakken pluimveevlees. Eenmaal in het lichaam, bereiken de bacteriën hun favoriete niche, diep in de darm crypten. Vervolgens wordt de epitheel-darmbarrière aangetast, met een sterke ontstekingsrespons tot gevolg wat resulteert in klinische symptomen, zoals bloedige diarree, misselijkheid, koorts en buikpijn. In de meeste gevallen verdwijnen deze symptomen na 5 tot 7 dagen, maar in sommige patiënten kunnen post-klinische complicaties optreden, die kunnen resulteren in andere gastro-intestinale aandoeningen of in neuronale schade zoals het Guillain-Barré syndroom.

C. jejuni is een Gram-negatieve bacterie, die als commensaal pathogeen in de darm van de kip voorkomt. In tegenstelling tot bij de mens, vertonen de kippen geen pathologie noch symptomen. De reden waarom mensen, maar niet kippen vatbaar zijn voor ziekte, is nog steeds een mysterie. Zodra een enkele kip geïnfecteerd geraakt, wordt in een zeer korte tijd de hele toom gekoloniseerd. De kip caecum bevat veel pyruvaat, serine, aspartaat en fumaraat, die fungeren als belangrijkst energiebron voor *C. jejuni*.

Verrassend genoeg en in tegenstelling tot andere pathogenen zoals Salmonella, heeft *C. jejuni* zeer weinig virulentie factoren en is de moleculaire basis van de pathologie grotendeels onbekend. Het wordt algemeen aanvaard dat motiliteit en chemotaxis de belangrijkste determinanten zijn voor een succesvolle kolonisatie en infectie. Twee flagellen, één op elke pool, zijn de drijvende kracht achter de motiliteit van *C. jejuni* en laat de bacterie toe om met hoge snelheid te bewegen doorheen vloeibare en viskeuze omgevingen zoals de mucus in de darm. De flagel bestaat uit verschillende segmenten waaronder de flagellaire motor. Deze bevindt zich in het bacteriemembraan en genereert de rotatie kracht die via een haakstructuur doorgeven wordt aan het flagellaire filament waardoor deze roteert. Aan het einde van het filament bevindt zich het flagellair tip eiwit, genaamd FliD, dat nodig is voor een correcte filamentvorming. Daarnaast strekt het flagel zich ver uit van het bacteriële lichaam en is deze betrokken bij de adhesie aan het celoppervlak volgens een nog onbekend mechanisme.

Om infectie met *C. jejuni* bij mensen te voorkomen, moet men eerst meer te weten komen over het volledige repertoire van bacteriële adhesie- en invasiefactoren. Dit proefschrift beschrijft de interactie van het flagellair tip eiwit met de gastheer en identificeert componenten die interfereren met deze interactie. Deze informatie is belangrijk voor de ontwikkeling van anti-infectieuze middelen en een effectief vaccin.

Dit proefschrift

De mogelijke betrokkenheid van het flagel tijdens bacteriële aanhechting aan mucosale cellen werd initieel waargenomen tijdens infectie experimenten, waarbij *C. jejuni* schijnbaar verbonden was aan het celoppervlak via de tip van de flagel (voorblad). Deze flagellaire tip bestaat uit het flagellair tip eiwit FliD wat een rol speelt tijdens de assemblage van de flagel en de stabilisatie van de flagellaire structuur. Op zoek naar een bijkomende rol tijdens bacteriële adhesie, zuiverden we recombinant FliD eiwit op uit een E. coli expressiesysteem en testten we de bindingsmogelijkheden aan verschillende cel soorten (hoofdstuk 2). Verminderde

binding aan glycosylatie-deficiënte cellijnen wees op een rol van glycosaminoglycanen (GAGen) als een potentiële bindingspartner van FliD. Deze GAGen bestaan uit lange ketens van repetitieve, zwaar gesulfeerde diplo-suikers die aanwezig zijn op het celoppervlak, waardoor ze een aantrekkelijk doelwit vormen voor de bacterie. Neutralisatie experimenten met verschillende oplosbare receptoren en enzymatisch verwijderen van deze receptoren leidde tot de identificatie van heparaan sulfaat als de klasse GAGen die verantwoordelijk zijn voor FliD binding. Daarnaast bevestigden we ook dat FliD afkomstig van *C. jejuni* supernatant dezelfde bindingskarakteristieken heeft als recombinant opgezuiverd eiwit. Tenslotte hebben we de heparaan sulfaat bindingsplaats in het centrale gebied van FliD-eiwit weten te lokaliseren. We toonden dus aan dat FliD niet enkel belangrijk is voor het handhaven van de stabiliteit van het flagel, maar ook een bijkomende rol heeft tijdens de vroege adhesie van *C. jejuni* aan cellen.

Kippen zijn de voornaamste bron van menselijke infecties door *C. jejuni*. Het verminderen dus van de bacteriële kolonisatie bij pluimvee zal bijgevolg resulteren in een verminderd aantal infecties bij mensen. Interessant genoeg blijven kippen Campylobacter-vrij gedurende de eerste twee weken van hun leven. Het wordt algemeen aangenomen dat deze kolonisatieweerstand te wijten is aan de materiële immuniteit die wordt overgedragen door de moederhen. In tegenstelling tot bij zoogdieren, die antilichamen binnen krijgen via de moedermelk, komen bij vogels de antilichamen van de moeder (zogenaamde IgY's) terecht in de dooier tijdens de productie van het ei. Tijdens de embryonale groei worden vervolgens deze IgY's via specifieke receptoren naar het bloed en mucosale oppervlakten van de nakomelingen getransporteerd wat vervolgens bescherming biedt tegen infecties gedurende het prille begin van hun leven. We veronderstelden dus daarom dat antilichamen gericht tegen FliD van *C. jejuni* mogelijks een rol zouden kunnen spelen in de kolonisatieweerstand tijdens de eerste twee weken. In hoofdstuk 3, hebben we grote hoeveelheden anti-FliD antilichamen in de eidooier gedetecteerd. De geïsoleerde anti-FliD IgY's waren kruis-reactief tegen verschillende *C. jejuni* stammen, wat dan ook suggereert dat deze bescherming bieden tegen infectie door verschillende *C. jejuni* stammen. Belangrijker nog, deze IgY's waren in staat de binding van FliD eiwit aan cellen te blokkeren. Verdere epitoop bepaling toonde aan dat deze antilichamen voornamelijk gericht waren tegen de receptorbindingsplaats (geïdentificeerd in hoofdstuk 2) in het centrum van het FliD-eiwit, wat een mogelijke verklaring zou kunnen zijn voor het mechanisme waarmee IgY's infectie verhinderen. Deze informatie opent nieuwe perspectieven om kolonisatie van *C. jejuni* in kippen te verminderen en daarmee gepaard ook eventueel menselijke ziekte te voorkomen.

In hoofdstuk 4 hebben we verder gezocht naar componenten die de interactie van FliD en het celoppervlak zouden kunnen blokkeren. Dit leidde tot de identificatie van de aanwezigheid van een remmer in koemelk. Na het uitsluiten van de aanwezigheid van anti-FliD antilichamen, vonden we dat melk caseïnes fungeerden als het actieve bestanddeel. Caseïnes vertegenwoordigen de belangrijkste eiwitfractie in melk en geven melk zijn karakteristieke witte kleur. De belangrijkste functie van caseïne is het binden en verzamelen van calcium en magnesium. Deze tweewaardige kationen binden aan specifieke fosfoserine residuen die aanwezig zijn in de caseïne eiwitten. Deze kationen worden in grote hoeveelheden vervoerd naar de pasgeborene, waar ze belangrijk zijn voor de vorming van bot en tanden. We vonden dat gedefosforyleerde caseïnes, die dus niet in staat zijn kationen te binden, ook niet in staat zijn FliD binding aan cellen te blokkeren. Dit suggereerde dat

ofwel de gefosforyleerde serines zelf of de kationbindende eigenschappen belangrijk zijn voor de remming. Vervolgens bleek dat FliD-binding gedeeltelijk kationafhankelijk is, aangezien de kation chelator EDTA ook de interactie kan blokkeren. Momenteel echter is de exacte rol van calcium en magnesium tijdens FliD-binding nog steeds onduidelijk. Aangezien caseïnes ook *C. jejuni* infectie remmen, zou dus ook melk als een anti-infectieus middel kunnen beschouwd worden in de strijd om menselijke ziekte te voorkomen.

Aangezien alle geflagelleerde bacteriën FliD bevatten als flagellair tip eiwit, hebben we vervolgens onderzocht of het adhesie vermogen van FliD uniek is voor *C. jejuni* of dat deze functie gedeeld wordt met andere geflagelleerde bacteriën. In hoofdstuk 5 rapporteren en vergeleken we de bindingsmogelijkheden van FliD van andere soorten. Alle geteste FliD-eiwitten waren in staat om heparaan sulfaat op het celoppervlak te binden en etaleerden dezelfde bindingseigenschappen als deze van *C. jejuni* FliD. Recente eiwit kristalstructuren van FliD van andere bacteriën (namelijk Salmonella, Escherichia en Pseudomonas) stelden ons in staat om *C. jejuni* FliD te modelleren om op deze manier potentiële cellulaire bindingsplaatsen te identificeren in het FliD-eiwit. Verscheidene positief geladen dichtbij elkaar gelegen lysines zouden een mogelijke bindingsplaats kunnen vormen, aangezien de interactie met heparaan sulfaat vermoedelijk gebaseerd is op basis van ladingen. Een soortgelijk mogelijke bindingsplaats kan gevonden worden in FliD van andere geflagelleerde bacteriën zoals Salmonella en zou een verklaring kunnen bieden voor de gelijkaardige bindingseigenschap van FliD van verschillende soorten.

Samengevat werpt dit proefschrift een eerste licht op de interactie van bacteriën met gastheercellen en ontrafelt het mogelijks belang van de flagel tijdens bacteriële adhesie. Meer precies beschrijft dit proefschrift de binding van het flagellair tip eiwit FliD van verschillende soorten aan gastheercellen. Dit suggereert dat de rol van het flagel niet enkel beperkt is tot het verschaffen van motiliteit, maar bovendien ook dienst zou kunnen doen als overbrugging van het bacteriële lichaam tot het celoppervlak tijdens de infectie. In dit proces vertegenwoordigt FliD de bacteriële bindingsfactor en heparaan sulfaat de herkende cel receptor. In lijn hiermee hebben we verschillende blokkerende componenten geïdentificeerd, die de interactie tussen FliD en het celoppervlak verhinderen. Dit geeft aan dat de gastheer mechanismen heeft ontwikkeld om FliD-binding te vermijden en indirect dat FliD een aantrekkelijk doelwit zou kunnen zijn om bacteriële binding en menselijke infectie door verschillende pathogenen te verhinderen. Hoge-resolutie 3D-structuren van FliD in complex met de receptor zouden ons in staat moeten stellen de nauwkeurige interactie-residuen in het FliD-eiwit verder te bepalen.

Zusammenfassung in deutscher Sprache

Einleitung

Campylobacter jejuni ist die Hauptursache für bakterielle Diarrhöe beim Menschen und die Anzahl der Infektionen überschreitet selbst die Anzahl durch Salmonellen verursachter Infektionen. In der Regel infizieren sich Menschen durch den Verzehr von rohem Geflügelfleisch. Einmal im Körper erreichen die Bakterien ihre Lieblingsnische tief im Darm. Anschließend wird die Epithelbarriere durchbrochen, und dadurch eine starke Entzündungsreaktion hervorgerufen, die zu klinischen Symptomen wie blutigem Durchfall, Übelkeit, Fieber und Bauchschmerzen führt. In den meisten Fällen verschwinden diese Symptome nach 5 bis 7 Tagen, aber bei einigen Patienten können postklinische Komplikationen auftreten, die zu anderen gastrointestinalen Störungen oder zu neuronalen Schäden, z. B. dem Guillain-Barré-Syndrom, führen können.

C. jejuni ist ein Gram-negatives Bakterium, das als Kommensal im Darm von Hühnern auftritt. Im Gegensatz zu Menschen zeigen Hühner keine Pathologie oder Symptome. Es noch stets unbekannt, warum Menschen, aber Hühner nicht, anfällig für die Krankheit sind. Sobald ein einzelnes Huhn infiziert ist, wird die gesamte Schar in kürzester Zeit von *C. jejuni* kolonisiert. Der Blinddarm von Hühnern enthält viel Pyruvat, Serin, Aspartat und Fumarat, die als Hauptenergiequellen für *C. jejuni* fungieren.

Überraschenderweise und im Gegensatz zu anderen Pathogenen wie Salmonellen hat *C. jejuni* nur sehr wenige Virulenzfaktoren und die molekulare Basis der Pathologie ist weitgehend unbekannt. Es ist weithin akzeptiert, dass Motilität und Chemotaxis die wichtigsten Faktoren für eine erfolgreiche Kolonisation und Infektion sind. Zwei Geißeln, auch Flagellen genannt, die sich an je einem Pol befinden, sind die treibende motorische Kraft und erlauben es dem Bakterium, sich mit hoher Geschwindigkeit durch flüssige und visköse Umgebungen wie dem Darmschleim zu bewegen. Das Flagellum besteht aus mehreren Segmenten. Der Flagellenmotor befindet sich in der Bakterienmembran und erzeugt die Rotationskraft, die durch eine Hakenstruktur auf das Flagellum übertragen wird, wodurch es sich dreht. Am Ende des Filaments befindet sich die Flagellenspitze, ein Eiweiß genannt FliD, das für die richtige Filamentbildung benötigt wird. Außerdem erstreckt sich das Flagellum weit von dem Bakterienkörper weg und ist an der Zelloberflächenhaftung beteiligt.

Um eine Infektion mit *C. jejuni* beim Menschen zu verhindern, ist es notwendig mehr über das volle Repertoire an bakteriellen Adhäsions- und Invasionsfaktoren zu erfahren. Diese Dissertation beschreibt die Wechselwirkung der Flagellenspitze mit dem Wirt und befasst sich mit Faktoren, die diese Wechselwirkung stören. Diese Informationen sind wichtig für die Entwicklung von Antiinfektiva und wirksamen Impfstoffen.

Diese Dissertation

Die mögliche Beteiligung des Flagellums an der bakteriellen Bindung an Schleimhautzellen wurde anfangs mit Hilfe von Infektionsexperimenten beobachtet, wobei *C. jejuni* anscheinend mit der Zelloberfläche über die Flagellenspitze (Titelseite) verbunden war. Diese Flagellenspitze besteht aus dem Eiweiß FliD, das beim Aufbau und der Stabilisierung der Flagellenstruktur eine Rolle spielt. Auf der Suche nach einer zusätzlichen Funktion während der bakteriellen Adhäsion wurde das rekombinante FliD-Protein aus einem *E. coli*-Expressionssystem gereinigt und die Bindungsmöglichkeiten für verschiedene Zelltypen

getestet (**Kapitel 2**). Die reduzierte Bindung an Glykosylierungs-defizienten Zelllinien deutet auf eine Rolle von Glykosaminoglykanen (GAGs) als potentiellen Bindungspartner von FliD hin. Diese GAGs bestehen aus langen Ketten von sich wiederholenden, stark sulfatierten Diplo-Zuckern, die auf der Zelloberfläche vorhanden sind, und damit ein attraktives Ziel für das Bakterium sind. Neutralisationsversuche mit verschiedenen löslichen Rezeptoren und die enzymatische Entfernung dieser Rezeptoren führten zur Identifizierung von Heparansulfat als Klasse GAG, die für die FliD-Bindung verantwortlich sind. Darüber hinaus haben wir auch bestätigt, dass FliD aus *C. jejuni* Kulturüberstand die gleichen Bindungseigenschaften wie rekombinant gereinigtes Protein hat. Schließlich konnten wir die Heparansulfat-Bindungsstelle im zentralen Bereich des FliD-Proteins lokalisieren. So haben wir gezeigt, dass FliD nicht nur für die Aufrechterhaltung der Stabilität des Flagellums wichtig ist, sondern auch eine zusätzliche Rolle bei der frühen Adhärenz von *C. jejuni* an Zellen hat.

Hühner sind die Hauptquelle der menschlichen Infektionen mit *C. jejuni*. Deswegen würde eine Verringerung der Bakterienkolonisation bei Geflügel zu einer reduzierten Anzahl von menschlichen Infektionen führen. Interessanterweise bleiben die Hühner in den ersten zwei Wochen ihres Lebens *Campylobacter*-frei. Es wird allgemein angenommen, dass dieser Widerstand gegen die Kolonisation auf die maternale Immunität zurückzuführen ist, die von der Henne übertragen wird. Im Gegensatz zu Säugetieren, die Antikörper durch die Muttermilch bekommen, werden bei Vögeln die maternalen Antikörper (so genannte IgY‘s) während der Entstehung des Eies in das Eigelb deponiert. Während des embryonalen Kükenwachstums werden diese IgYs dann über spezifische Rezeptoren zu den Blut- und Schleimhautoberflächen des Kükens transportiert und schützen es vor frühen Infektionen. Wir haben daher angenommen, dass Antikörper, die gegen FliD von *C. jejuni* gerichtet sind, während der ersten zwei Wochen möglicherweise eine Rolle bei der Besiedlungsresistenz spielen könnten. In **Kapitel 3** haben wir große Mengen an Anti-FliD-Antikörpern im Eigelb gefunden. Die isolierten Anti-FliD-IgYs waren gegen verschiedene *C. jejuni*-Stämme kreuzreaktiv, was darauf hindeutet, dass sie Schutz gegen Infektionen mit verschiedenen *C. jejuni*-Stämme bieten. Am wichtigsten war, dass diese IgYs die Bindung von FliD-Protein an Zellen blockieren konnten. Ein weiterer Epitop-Assay zeigte, dass diese Antikörper primär gegen die in Kapitel 2 identifizierten Rezeptorbindungsstellen im Zentrum des FliD-Proteins gerichtet waren, was eine mögliche Erklärung für den Mechanismus sein könnte. Diese Information eröffnet neue Perspektiven, um die Kolonisation von *C. jejuni* bei Hühnern zu reduzieren und damit auch mögliche menschliche Erkrankungen zu verhindern.

In **Kapitel 4** haben wir nach weiteren Komponenten gesucht, die die Interaktion von FliD und der Zelloberfläche blockieren könnten. Dies führte zur Identifizierung eines Inhibitors in Kuhmilch. Nach dem Ausschluss der Anwesenheit von Anti-FliD-Antikörpern fanden wir, dass Milch-Kaseine als Wirkstoff wirkten. Kaseine stellen die Hauptproteinfraktion in Milch dar und geben der Milch ihre unverwechselbare weiße Farbe. Die Hauptfunktion von Kaseinen ist die Bindung und Sammlung von Kalzium und Magnesium. Diese zweiwertigen Kationen binden an spezifische Phosphoserinreste, die in den Kaseineiweißen vorhanden sind. Diese Kationen werden in großen Mengen zum Neugeborenen transportiert, wo sie für die Bildung von Knochen und Zähnen benötigt werden. Wir fanden heraus, dass dephosphorylierte Kaseine, die somit nicht in der Lage sind Kationen zu binden, auch nicht in der Lage sind, die FliD-Bindung an Zellen zu blockieren. Dies deutet darauf hin, dass entweder die phosphorylierten Serine selbst oder die Kationenbindungseigenschaften für die Hemmung verantwortlich

sind. Anschließend haben wir gesehen, dass die FliD-Bindung teilweise kationabhängig ist, da der Kation-Chelator EDTA auch die Bindung von FliD an Zellen blockieren kann. Derzeit ist jedoch die genaue Rolle von Kalzium und Magnesium während der FliD-Bindung noch unklar. Da Kaseine auch *C. jejuni*-Infektionen hemmen, könnte Milch als antiinfektiöses Mittel zur Verhütung von menschlichen Erkrankungen in Betracht gezogen werden.

Da alle begeißelten Bakterien FliD als Flagellenspitzenprotein enthalten, untersuchten wir weiter, ob die Adhäsionsfähigkeit von FliD für *C. jejuni* einzigartig ist oder ob diese Funktion mit anderen Bakterien geteilt wird. In **Kapitel 5** verglichen wir die FliD-Bindungsmöglichkeiten anderer FliD Typen. Alle getesteten FliD-Proteine waren in der Lage, Heparansulfat auf der Zelloberfläche zu binden und zeigten die gleichen Bindungseigenschaften wie die von *C. jejuni* FliD. Die jüngsten Proteinkristallstrukturen von FliD aus anderen Bakterien (*Salmonella*, *Escherichia* und *Pseudomonas*) ermöglichten es uns, *C. jejuni* FliD zu modellieren, um potentielle zelluläre Bindungsstellen im FliD-Protein zu finden. Mehrere positiv geladene benachbarte Lysine könnten eine mögliche Bindungsstelle bilden, da die Bindung an Heparansulfat vermutlich auf Ladungen basiert. Eine ähnliche mögliche Bindungsstelle kann in FliD von anderen begeißelten Bakterien wie Salmonellen gefunden werden und könnte eine Erklärung für die ähnliche Bindungseigenschaft von FliD verschiedener Spezies liefern.

Zusammenfassend lässt sich sagen, dass diese Arbeit über die Wechselwirkung von Bakterien mit Wirtszellen Aufschluss gibt und wie wichtig das Flagellum während der bakteriellen Adhäsion ist. Genauer gesagt, beschreibt diese Arbeit die Bindung des Flagellenspitzenproteins FliD von verschiedenen Bakterienarten an Wirtszellen. Dies deutet darauf hin, dass die Rolle des Flagellums nicht nur auf Motilität beschränkt ist, sondern auch als Überbrückung des Bakterienkörpers zur Zelloberfläche während der Infektion dienen könnte. Hierbei stellt FliD den bakteriellen Bindungsfaktor und Heparansulfat den Zellrezeptor dar. Im Einklang damit haben wir mehrere blockierende Komponenten identifiziert, die die Wechselwirkung zwischen FliD und der Zelloberfläche verhindern. Dies deutet darauf hin, dass der Wirt Mechanismen entwickelt hat, um FliD-Bindung zu vermeiden und indirekt, dass FliD ein attraktives Ziel sein könnte, um menschliche Infektion durch verschiedene Krankheitserreger zu verhindern. Hochauflösende 3D-Strukturen von FliD im Komplex mit dem Rezeptor werden es uns in der Zukunft ermöglichen, die genauen Bestandteile im FliD-Protein weiter zu bestimmen, die für die Binding verantwortlich sind.

Acknowledgement
Curriculum vitae

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CURRICULUM VITAE

Claudia Marianne Freitag was born on 29th of April 1984 in Berlin, Germany. After graduating from high school (Lycée français, Berlin), she started training as Medizinisch-technische Laboratoriumsassistentin (Lette Verein, Berlin). After the study she enrolled in a Leonardo da Vinci program, funding a 4-month internship at the Napier University in Edinburgh (Scotland). As she enjoyed living abroad, Claudia started her studies in Biomedical Sciences in Edinburgh and finished her B.Sc. (hons) in 2009 with a thesis about "Macrophage phenotype in response to nematode parasite products" under the supervision of Dr. Lorna Proudfout (School of Life Science, Napier University). She then moved to the Netherlands to enrol into the Master program Infection and Immunity at Utrecht University. During the studies she had the opportunity to perform an internship at the Department of Medical Microbiology (UMC Utrecht) under the supervision of Dr. Suzan Rooijacker and Dr. Ilse Jongerius, where she investigated the functions of the immune evasion protein extra-cellular fibrinogen-binding protein (efb) of *Staphylococcus aureus*. She continued working on other *S. aureus* surface proteins during a short internship in Houston (Center for Infectious and Inflammatory Diseases, Texas A&M University) under the supervision of Dr. Ya-Ping Ko. Additionally, Claudia also gained experience in industry during a 6-months internship at Genmab B.V. (Utrecht, the Netherlands), where she did applied research on antibody-drug conjugates under the supervision of Dr. Bart de Goeij. In October 2011, she started her PhD research on the flagellar tip protein FliD of *Campylobacter jejuni* at the Department of Infectious Diseases and Immunology (Utrecht University). The findings are described in this thesis. Claudia continued her international life-style by moving to Haasrode, Belgium, where she works as a clinical research assistant at Medpace BVBA since September 2017.