Feedback control of *Campylobacter jejuni* flagellin levels through reciprocal binding of FliW to flagellin and the global regulator CsrA

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Summary

Bacterial flagella assembly is tightly regulated to ensure a timely and sequential production of the various flagellum constituents. In the pathogen *Campylobacter jejuni* the hierarchy in flagella biosynthesis is largely determined at the transcriptional level through the activity of the alternative sigma factors sigma[54] and sigma[28]. Here, we report that *C. jejuni* flagellin levels are also controlled at the post-transcriptional level via the thus far poorly-characterized flagellar assembly factor FliW. Analysis of flagellin synthesis in *C. jejuni* 81116 and a ∆*fliW* knock-out mutant showed reduced flagellin protein levels in the mutant strain while ectopic expression of FliW resulted in enhanced levels. Real-time RT-PCR revealed relatively minor changes in *flaA* and *flaB* mRNA levels for the recombinant and parent strain consistent with post-transcriptional regulation. Purified FliW was found to bind to FlaA and FlaB flagellin as well as to the global post-transcriptional regulator CsrA. Inactivation of CsrA resulted in increased levels of flagellin translation. An *in vitro* translation assay confirmed the regulatory role of CsrA in flagellin biosynthesis. We propose that competitive reciprocal binding of FliW to flagellins and the RNA binding protein CsrA serves as a feedback mechanism to control the number of cytosolic flagellin copies at the protein level.

Introduction

Bacterial flagella confer bacterial motility and contribute to bacterial virulence. In *Gammaproteobacteria* like *Escherichia coli* and *Salmonella enterica*, the flagellar apparatus is typically composed of thousands flagellin subunits built on a basal body complex (C-ring, MS-ring, L-ring, P-ring and rod) and a hook structure (Auvray *et al.*, 2001; Evans *et al.*, 2014). The basal body complex serves as a rotary motor which together with ion translocating stators distributed around the motor generates the torque that propels the flagella-mediated bacterial movement (Blair and Berg, 1990; Che *et al.*, 2014).

In *Epsilonproteobacteria*, the flagellar apparatus has a slightly different molecular architecture. In the bacterial pathogen *Campylobacter jejuni*, the basal body complex is larger than its counterpart in enteric bacteria, has a wider C-ring, and a L/P-ring complex attached to a large periplasmic basal disc (Chen *et al.*, 2011; Minamino and Imada, 2015). Moreover, the basal body has 17 stator complexes (Beeby, 2015) compared to 12 in *E. coli* (Reid *et al.*, 2006). The unusually large motor complex likely generates a higher torque which enables *C. jejuni* to swim in high-viscosity environments which immobilize *E. coli* and *S. enterica* (Ferrero and Lee, 1988; Beeby, 2015). The flagellar filament of *C. jejuni* consists of the structural flagellins FlaA or FlaB that polymerize into a fiber composed of seven protofilaments rather than the eleven present in *S. enterica* (Galkin *et al.*, 2008). Moreover, *C. jejuni* FlaA and FlaB are heavily *O*-glycosylated (Thibault *et al.*, 2001; Ewing *et al.*, 2009). Glycosylation of *C. jejuni* flagellins is essential for flagellar assembly and motility (Goon *et al.*, 2003; Logan, 2006). *C. jejuni* also encodes FlaC, a non-structural flagellin that is secreted into the environment and has been implicated in host cell invasion (Song *et al.*, 2004).

Besides the structural differences between the lateral flagella of *E. coli* and *S. enterica* and the polar flagella of *C. jejuni*, the regulation of the flagella biosynthesis differs between the species. In *E. coli* and *S. enterica*, the master *flhDC* operon (class 1) is at the top of the assembly hierarchy. FlhC and FlhD are the regulatory proteins that directly activate the genes that encode the structural components of the basal-

body hook structure as well as the alternative sigma[28] factor and the anti-sigma-factor FlgM (class 2). Upon secretion of FlgM, which occurs as soon as the basal body secretion system is functional, the sigma[28] protein is released to activate the genes that encode the filament, motor force generators and the chemosensory machinery (class 3) (Aldridge and Hughes, 2002; Macnab, 2004). Regulation of the flagellar genes in *C. jejuni* starts with the production of the flagellar export apparatus, which probably activates the two-component system FlgR/FlgS. This system together with the FlhF GTPase and alternative sigma factors sigma[28] and sigma[54] drives the transcription of genes encoding the basal body, hook and filament structural units (Hendrixson and DiRita, 2003; Wösten *et al.*, 2004; Balaban *et al.*, 2009). The flagellins FlaA, FlaB and FlaC are each regulated by a different sigma factor: sigma[28], sigma[54] and sigma[70] respectively. FlgM limits the length of the filament by suppression the transcription of both the sigma[28]- and the sigma[54]-dependent flagellins (Wösten *et al.*, 2010).

Next to the transcriptional regulation, bacterial protein synthesis may be influenced by post-transcriptional factors including the carbon starvation regulator CsrA (Timmermans and Melderen, 2010; Vakulskas *et al.*, 2015). In *E. coli*, CsrA is involved in regulating stationary-phase metabolism, biofilm formation (Jackson *et al.*, 2002) and motility (Wei *et al.*, 2001). *C. jejuni* also carries a CsrA homolog. Its inactivation results in a mutant phenotype that is altered in motility, biofilm formation, adherence to and invasion of INT407 cells and resistance to oxidative stress (Fields and Thompson, 2008). In a yeast two-hybrid screen interaction map for *C. jejuni* (Parrish *et al.*, 2007), CsrA was predicted to interact with a putative flagellar assembly factor FliW, which has been implicated in bacterial motility in *Treponema pallidum* (Titz *et al.*, 2006), *Borrelia burgdorferi* (Karna *et al.*, 2013) and *Bacillus subtilis* (Mukherjee *et al.*, 2011). In *C. jejuni*, the *fliW* gene has been identified to be important for motility in random transposon mutagenesis screen (Golden and Acheson, 2002).

In the present study, we investigated the function of the putative flagellar assembly factor FliW in *C. jejuni* motility. We provide evidence of that *C. jejuni* FliW is a central element in the post-transcriptional control of flagellin biosynthesis. We propose a feedback control mechanism of *C. jejuni* flagellin synthesis that controls the number of flagellin copies via cross-talk between FliW and CsrA that suppresses flagellin translation.

Results

C. jejuni *FliW influences flagella formation and function*

In order to investigate the biological role of FliW in *C. jejuni*, the central part of the putative *fliW* gene (C8J_1016) in strain 81116 was deleted and replaced by a *cat* cassette conferring chloramphenicol resistance. The selected mutant *C. jejuni* Δ*fliW* grew considerably faster than the parent strain (Fig. 1A). As a higher growth rate is often noted for non-motile *C. jejuni* strains, including a Δ*flaAB* mutant that lacks FlaA/B flagellin (Fig. 1A) (Wösten *et al.*, 2004), bacterial motility was tested using a swarming assay. Inactivation of *fliW* reduced *C. jejuni* swarming in soft agar (Fig. 1B–D). This phenotype was confirmed for eight independent *C. jejuni* Δ*fliW* clones and was more apparent when the soft agar plate was incubated a shorter time (Supporting Information Fig. S1); on average *C. jejuni* Δ*fliW* was 55% less motile compared to the wild type strain (Fig. 1E). Transmission electron microscopy on the parent and Δ*fliW* mutant strain revealed severely truncated flagella for the mutant strain (Fig. 1F–H). The average filament length of the wild type strain was 2.82 ± 0.86 μm (mean $\pm$ SD), whereas filaments produced by the Δ*fliW* mutant were 0.13 ± 0.13 μm long (Supporting Information Fig. S1). These findings resemble the mutant phenotype in several other *C. jejuni* strains (Golden and Acheson, 2002; Barrero-Tobon and Hendrixson, 2014; de Vries *et al.*, 2015) and points to role of FliW in gene regulation, flagella assembly and/or motility.

FliW acts at the post-transcriptional level

To explain the observed Δ*fliW* phenotype, we first determined flagellin protein expression levels in the WT and Δ*fliW* mutant. Western blot analysis on cell lysates of the strains using a FlaA/B-specific antiserum as a probe demonstrated reduced flagellin levels in the Δ*fliW* strain compared to the WT. Conversely, overexpression of FliW as established by introduction of the plasmid pfliW (WT/pfliW) enhanced the amount of flagellin (Fig. 2A). As FlaB is barely expressed at the used growth temperature of 42°C, the reactive band consists of mainly FlaA (Wösten *et al.*, 2010). Analysis of the levels of the FlaC flagellin, which is not incorporated into the filament structure but secreted through the flagellum into the environment (Song *et al.*, 2004), revealed similar values for all strains (Fig. 2B) indicating that FliW does not influence the protein levels of all types of *C. jejuni* flagellin.

To further define the site of action of FliW, flagellin gene transcript levels in the parent, the Δ*fliW* mutant and WT/pfliW strains were compared (Fig. 2C). Real-time RT-PCR on isolated RNA using flagellin specific primer sets demonstrated a threefold increase in *flaA* transcript and a fourfold decrease in *flaB* transcript level in the Δ*fliW* mutant. Deletion of *fliW* did not affect *flaC* transcript levels. Overproduction of FliW did not influence neither *flaB* nor *flaC* transcription, although the level of *flaA* transcript was slightly decreased (Fig. 2C). The overall relatively minor differences in transcript
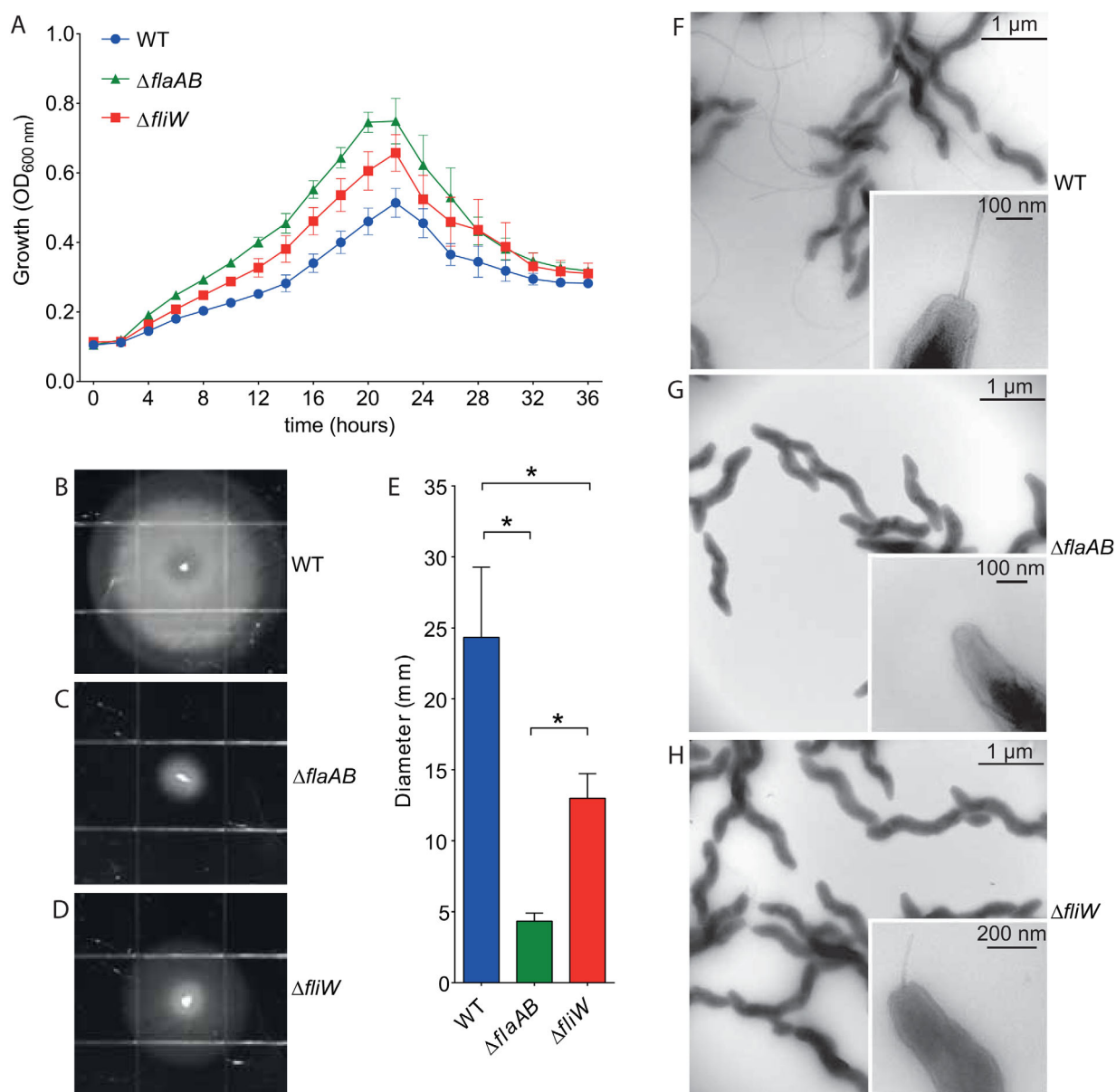
Fig. 1. Phenotypical analysis of a *C. jejuni* Δ*fliW* mutant.
A. Growth curves of *C. jejuni* WT, Δ*flaAB* and Δ*fliW* strains. Bacteria were grown at 42°C for 36 h in HI medium, under microaerophilic conditions. The graph represents the mean $OD_{600} \pm$ SD from three independent experiments.
B–D. Motility of the above mentioned strains tested in HI broth supplemented with 0.4% agar in plates with 1×1 cm grid. Bacteria were inoculated onto the plate and photographed after 18 h incubation at 42°C.
E. The diameter (mm) of the motility zones formed by the strains in soft agar. Data represent the mean $\pm$ SD of three independent experiments and were analysed by ANOVA with Bonferroni multiple comparison test.
F–H. Transmission electron microscopy images of different *C. jejuni* strains: (F) wild type, (G) Δ*flaAB* and (H) Δ*fliW*. Bacteria were grown in HI broth at 42°C under microaerophilic conditions and stained with uranyl acetate.

levels between the strains suggest that FliW likely exerts its effects at the post-transcriptional level.

Interaction of FliW with native and recombinant C. jejuni *FlaA/B flagellin*

To investigate whether the effect of FliW on flagellin protein levels was perhaps caused by a flagellar chaperone-like function or an effect on translation efficiency, we first expressed recombinant FliW fused to a polyhistidine-tag (His-tag). The resulting FliW-His protein was purified from *E. coli*, coupled to Ni^{2+}-NTA beads and used in pull-down experiments to assess the ability of FliW to bind native *C. jejuni* flagellins. As a source of flagellins, we used a culture supernatant of *C. jejuni* Δ*flgKM* (BC7) (Bleumink-Pluym *et al.*, 1999) which lacks
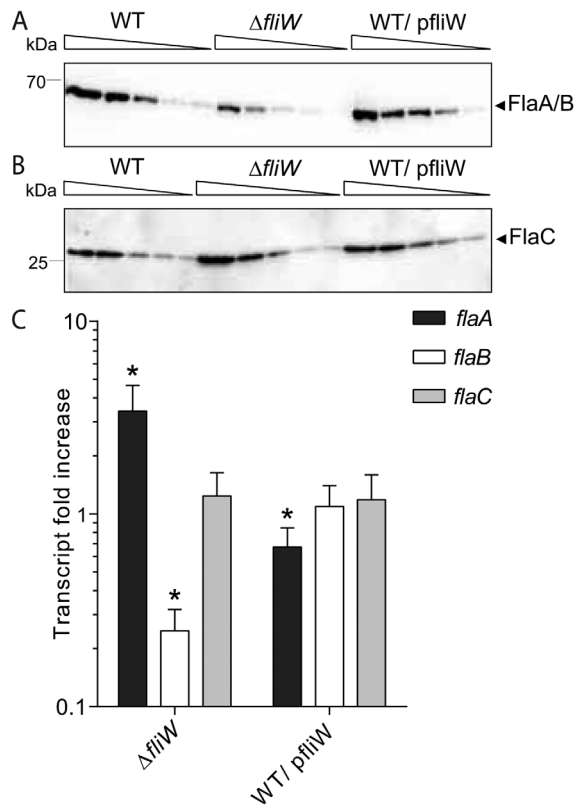
Fig. 2. Effect of FliW on *C. jejuni* flagellin protein and transcript levels.
A. Western blot analysis used to quantify the FlaA/B flagellin levels in the wild type, Δ*fliW* mutant and FliW overexpressing strain (WT/pfliW). Serially diluted samples (2×, 4×, 8×, 16×, 32×) of bacterial lysates were probed with polyclonal anti-FlaA/B antibodies. The depicted figure is a representative of three independent observations.
B. Western blot analysis of secreted FlaC levels. Serially diluted samples (1×, 2×, 4×, 8×, 16×) of culture supernatants of the same strains were probed with polyclonal anti-FlaC antibodies. The image is a representative of three independent experiments.
C. Real-time RT-PCR analysis of *flaA, flaB* and *flaC* transcripts. Transcript levels in Δ*fliW* and WT/pfliW are expressed as relative to the wild type strain. Each sample was examined in four replicates and was repeated with two independent batches of RNA. Data are presented as mean ± SD. Log transformed data were analysed by one-sample *t*-test (The asterisk indicates $P < 0.05$) to estimate the significance of transcript fold change between WT strain and Δ*fliW* or WT/pfliW.

flagella but secretes large amounts of flagellins into the medium (Barrero-Tobon and Hendrixson, 2014). SDS-PAGE analysis and Western blotting of the eluted fractions showed that the FlaA/B flagellins co-eluted with FliW-His from the Ni²⁺-affinity column (Fig. 3A). FlaC flagellin, which was present in supernatant of Δ*flgKM* strain, did not co-elute with FliW as verified by Western blot with polyclonal anti-FlaC serum (data not shown). These findings are in line with the results shown in Fig. 2 and corroborate that FliW regulates the structural flagellin levels. The co-elution of the FlaA/B flagellins was not observed when the motility-unrelated RacR-His protein
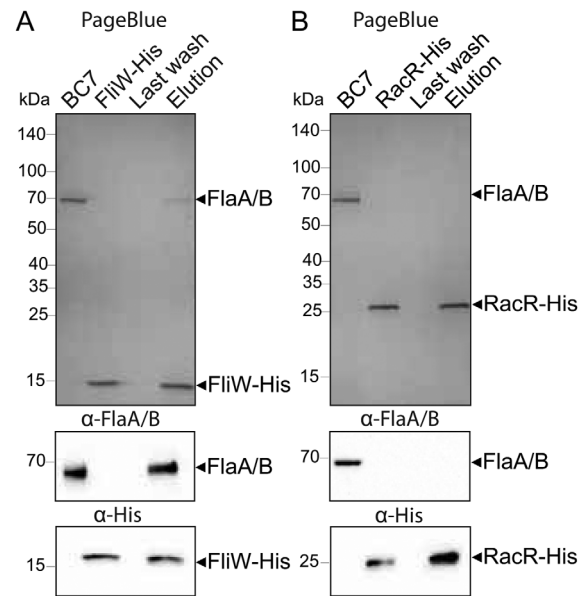

Fig. 3. FliW interacts with native FlaA/B flagellin.
A and B. Interaction of proteins was analysed by pull-down assay. The culture supernatant of *C. jejuni* Δ*flgKM* strain (BC7) secreting a large amount of flagellins was incubated with (A) FliW-His or (B) RacR-His. Mixtures were loaded onto Ni²⁺-NTA agarose beads. Non-specifically bound proteins were removed during the washing procedure. His-tagged proteins with all interacting partners were specifically eluted with the use of imidazole. Samples collected during the procedure were analysed by SDS-PAGE with PageBlue Protein Staining, Western blot with anti-His-HRP antibodies and Western blot with polyclonal anti-FlaA/B flagellin serum. Presented pictures are representative of three independent experiments.

(van der Stel *et al.*, 2015) rather than FliW-His was used as bait in the pull-down experiment (Fig. 3B), suggesting specific interaction of the structural flagellins with FliW.

To ascertain that FliW directly interacts with FlaA and FlaB, the corresponding genes and the FlaC-encoding gene were cloned with a C-terminal His-tag and expressed in *E. coli*. To be able to distinguish FliW and the His-tagged flagellins, the *fliW* gene was cloned with a glutathione S-transferase tag (GST-tag). Interaction of FliW with the flagellins was tested in Far Western blots of total cell lysates of *E. coli* expressing the His-tagged flagellins (Fig. 4A and B). Probing of the blot with purified GST-tagged FliW and subsequently with anti-GST antibodies (Fig. 4C) clearly demonstrated that FliW interacts with both FlaA and FlaB flagellin but not with FlaC. None of the proteins present in *E. coli* cell lysates were detected when the membrane was probed with GST instead of GST–FliW (data not shown). Similarly, the motility-unrelated RacR-His protein was not recognized by the GST-FliW protein (Fig. 4C), confirming the specificity of the interaction of FliW with the FlaA/B flagellins.

To investigate whether FliW is able to bind both non-glycosylated and glycosylated *C. jejuni* flagellins, the *E. coli* lysate containing His-tagged non-glycosylated
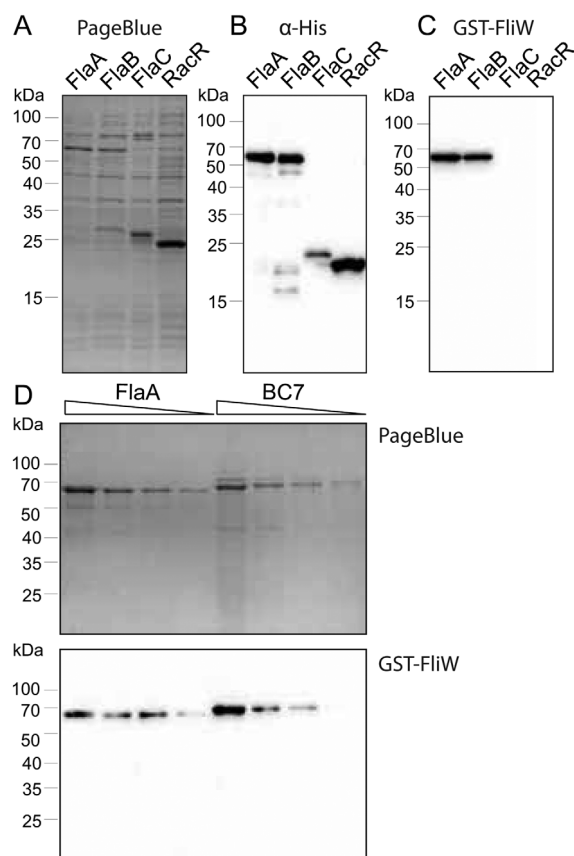
Fig. 4. Interaction of FliW with flagellins.
A-C. *E. coli* lysates containing His-tagged proteins were separated on SDS-PAGE and (A) stained with PageBlue Protein Staining or transferred onto nitrocellulose membrane and probed with (B) antibodies recognising His-tag, conjugated with HRP or (C) GST-FliW protein.
D. *E.coli* lysate containing His-tagged FlaA and the culture supernatant of strain BC7 containing native flagellins were serially diluted, separated on SDS-PAGE and stained with PageBlue Protein Staining or transferred onto nitrocellulose membrane and probed with GST-FliW. Binding of GST-FliW was detected with use of anti-GST mouse antibodies, followed by anti-mouse IgG, conjugated with HRP. Presented pictures are representative of three independent experiments.
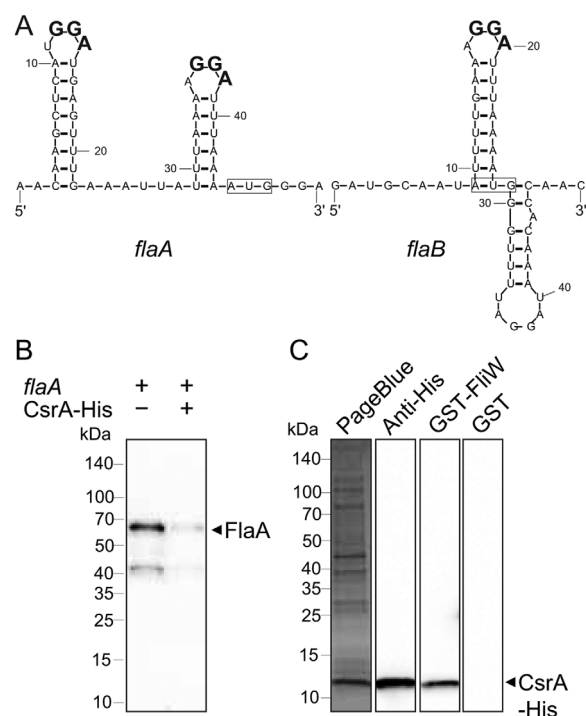
Fig. 5. FliW acts via the post-transcriptional regulator CsrA.
A. Analysis of GGA-motifs in flagellin transcripts. Each structure shows first 50 nucleotides (nt) of the transcript, what includes: 44 nt of 5′-untranslated sequence (5′-UTR) and 2 codons of *flaA* and 26 nt of 5′-UTR and 8 codons of *flaB*. Start codons are boxed. The conserved GGA-motifs present in the head of hairpins are indicated in bold.
B. Inhibition of translation of *flaA* mRNA in the *in vitro* translation assay. FlaA protein was expressed from its native 5′-UTR with or without the presence of purified CsrA-His. The synthetized flagellin was detected with a polyclonal FlaA/B-specific antiserum.
C. Total cell lysate of the *E. coli* expressing CsrA-His was subjected to Far Western blot assay. Proteins were separated on SDS-PAGE and stained with PageBlue Protein Staining or transferred onto nitrocellulose. Membranes were probed with anti-His antibodies (HRP-conjugated), GST-FliW protein or GST. Binding of GST-FliW and GST was detected with use of anti-GST mouse antibodies, followed by anti-mouse IgG, conjugated with HRP. Presented pictures are representative of three independent experiments.

FlaA and the culture supernatant of BC7 strain containing glycosylated flagellin were serially diluted, separated on SDS-PAGE and either stained with PageBlue Protein Staining or transferred onto nitrocellulose membrane and probed with GST-FliW. FliW recognized both non-glycosylated and glycosylated flagellins (Fig. 4D), indicating that the glycosylation event does not abolish the flagellin-FliW interaction.

Post-transcriptional regulation of C. jejuni *flagellin biosynthesis*

To explore the alternative option that FliW alters flagellin levels by influencing translation efficiency, we first analysed the FlaA and FlaB untranslated regions for putative regulatory elements. Analysis of the predicted RNA secondary structure of this region with the mFold software (Zuker, 2003) revealed the presence of the hairpin structures with a GAA motif in the central hexa-loop at the 5′-end of both the *flaA* and *flaB* (Fig. 5A) but not *flaC* sequence (data not shown). As a similar motif has been shown to interact with proteins belonging to CsrA/Rsm family of post-transcriptional regulators (Dubey *et al.*, 2005; Lapouge *et al.*, 2013), we next tested whether the CsrA protein of *C. jejuni* influences flagellin biosynthesis. Hereto we generated a CsrA mutant in strain 81116 by allelic replacement with a *cat* cassette. Analysis of flagellin levels in the Δ*csrA* strain
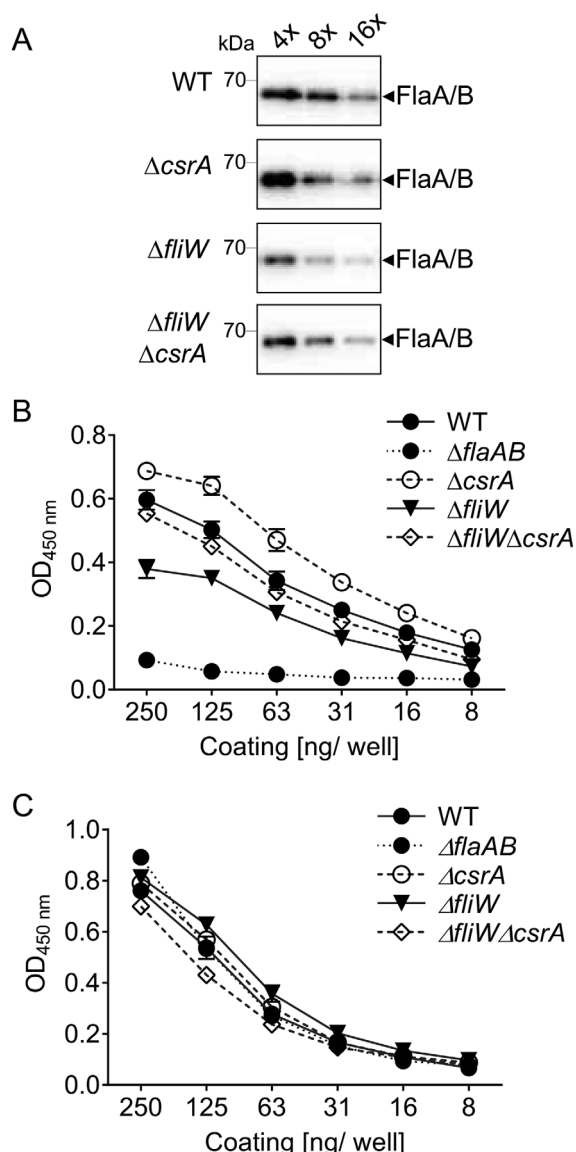
A

B

C

Fig. 6. FliW regulates CsrA-controlled translation of flagellin transcripts. FlaA/B flagellin levels in the *C. jejuni* WT strain and the Δ*csrA*, Δ*fliW* and Δ*fliW* Δ*csrA* derivatives were analysed.
A. Serially diluted samples of bacterial lysates were probed with a polyclonal anti-FlaA/FlaB serum and analysed by Western blot. The result is representative of three independent observations.
B. Analysis of flagellin levels in the *C. jejuni* strains as determined by ELISA with anti-FlaA/FlaB serum.
C. Detection of *N*-linked glycans in the same bacterial lysates using SBA-HRP as a probe. ELISA experiments were performed in triplicate from independently grown cultures. Data are represented as mean ± SD.

revealed increased amounts of FlaA/B flagellin compared to the parent strain (Fig. 6), suggesting that indeed CsrA acts as a negative regulator of FlaA/B translation. To verify this finding, we cloned, expressed and purified recombinant *C. jejuni* CsrA fused to a His-tag and used this protein in an *in vitro* translation assay.

The CsrA protein inhibited the translation of FlaA (Fig. 5B), indicating that CsrA acts as a post-transcriptional regulator of *flaA* mRNA translation initiation.

FliW regulates CsrA-controlled translation of flagellin transcripts

In order to link the CsrA-mediated regulation of flagellin biosynthesis to the effects of FliW, we investigated the possible interaction between FliW and CsrA as reported for *B. subtilis* (Mukherjee *et al.*, 2011). Hence, we tested a lysate of *E. coli* expressing CsrA-His for GST-FliW binding activity using Far Western blot analysis. As shown in Fig. 5C, GST-FliW clearly recognized CsrA-His but no other proteins present in the lysate, indicating that FliW not only binds to *C. jejuni* FlaA/B flagellin but also to CsrA.

To verify the modulating effect of FliW on the activity of the post-transcriptional regulator CsrA in *C. jejuni*, a double mutant Δ*fliW* Δ*csrA* in strain 81116 was constructed. Western blot analysis and enzyme-linked immunosorbent assay (ELISA) using total lysates of WT, Δ*csrA*, Δ*fliW* and Δ*fliW* Δ*csrA* strains as antigens and probed with the FlaA/FlaB-specific antiserum indeed revealed an increased amount of flagellins for the Δ*csrA* mutant and reduced levels for the Δ*fliW* mutant (Fig. 6A and B). In the double mutant Δ*fliW* Δ*csrA* that lacks the FliW-CsrA post-transcriptional regulation machinery, intermediate levels of flagellin were detected. When the SBA-HRP lectin, which binds to *N*-glycans of *C. jejuni* was used a probe, similar levels *N*-linked glycans were detected for tested *C. jejuni* lysates. Together these results confirm that FliW influences *C. jejuni* flagellin levels in a CsrA-dependent fashion.

Discussion

Flagellar biogenesis in bacteria is a highly-ordered process that involves the transcription of dozens of structural and regulatory genes. This tight regulation is needed, as the flagella biosynthesis is a complex and highly energy demanding process. The filament alone already consist of 20,000 flagellin subunits which are assembled into a filament as soon as the hook-basal body complex formation is completed (Auvray *et al.*, 2001; Evans *et al.*, 2014). Here, we provide evidence that the flagellar protein FliW of *C. jejuni* contributes to healthy flagella assembly by controlling the level of cytosolic flagellins. Our results indicate that FliW binds to both *C. jejuni* structural flagellin proteins (FlaA and FlaB) but also to the post-transcriptional regulator CsrA, which we found to regulate flagellin biosynthesis at the translational level. Our findings are consistent with a
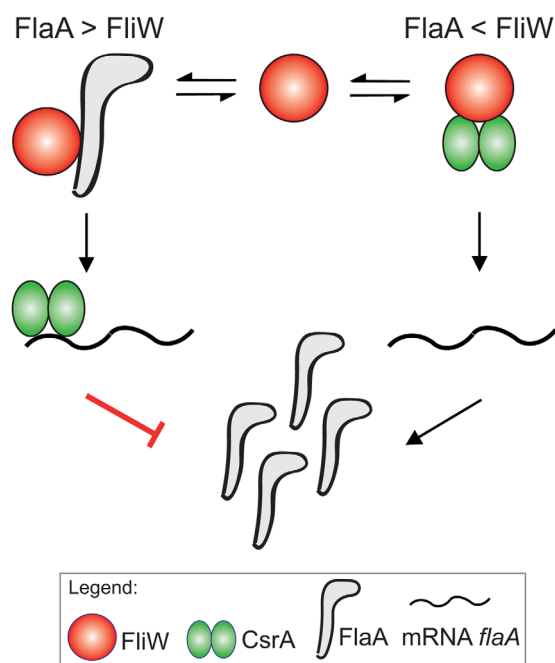
Fig. 7. Proposed mechanism of post-transcriptional regulation of flagellins by FliW in *C. jejuni*.
FliW binds either to CsrA or flagellin. When CsrA is sequestered by FliW, flagellin transcripts are translated resulting in an increase in flagellin protein level. When the flagellin level is high, FliW binds to flagellin enabling CsrA to exert its repressive function on flagellin translation initiation, resulting in a reduction of flagellin biosynthesis. The red T bar indicates inhibition.

mechanism of reciprocal binding of FliW to FlaA/B flagellin and to the CsrA protein that controls the level of cytosolic flagellin (Fig. 7). The translation of flagellin transcripts is suppressed by CsrA when FliW is complexed to cytosolic flagellin. When the level of free flagellin decreases as a result of the export through the flagellar apparatus, the available FliW captures CsrA to resolve the inhibition of translation initiation. This post-transcriptional mechanism of regulation of flagellin biosynthesis adds a new layer of complexity above the well-documented transcriptional regulatory system of flagellar biosynthesis in *C. jejuni*.

The biological function of *C. jejuni* FliW as a regulator of cytosolic flagellin levels, that acts by adjusting CsrA controlled translation efficiency, explains the reported Δ*fliW* mutant phenotype of truncated flagella and reduced motility (Golden and Acheson, 2002; Barrero-Tobon and Hendrixson, 2014). In the Δ*fliW* mutant, the intracellular levels of the global post-transcriptional regulator CsrA appear to determine the length of the flagellum. In the mutant strain, the CsrA protein cannot be captured by FliW when cytosolic flagellins levels become low. This results in persistence of the CsrA-mediated suppression of flagellin translation and thus in a shortage of flagellins required for

full assembly of the filament and bacterial motility. The noted apparent heterogeneity in flagella length and motility of the mutant (Fig. 1 and Supporting Information Fig. S1) likely reflects variation in CsrA levels in bacteria that differ in metabolic state. This may be exemplified by the motility zones of the Δ*fliW* mutant that were not only smaller in size but also much less dense compared to the parent strain (Fig. 1 and Supporting Information Fig. S1). In *E. coli*, such a difference in appearance results from a monolayer of motile bacteria at the edge of the swarming colony and bacteria that are differentiated to a vegetative morphology at the interior of the colony (Harshey, 2003; Swiecicki *et al.*, 2013). The *C. jejuni* Δ*fliW* mutant phenotype has previously been speculated to result from a lack of stabilisation of the flagellins in the absence of FliW (Barrero-Tobon and Hendrixson, 2014). We cannot exclude this additional function, although the ability of FliW to bind CsrA as well as FlaA and FlaB (but not FlaC) and the increased flagellin biosynthesis in the Δ*fliW* Δ*csrA* versus the Δ*fliW* mutant rather points to the regulatory effect of FliW on CsrA function.

The observed control in *C. jejuni* of flagellin biosynthesis directly at the level of translation enables a prompt tuning of the balance between flagellin transcription and flagellin assembly. This type of regulation optimizes energy expenditure and prevents the accumulation of cytosolic flagellins, which may become toxic to the bacterium. In addition, the FliW regulation of CsrA activity may provide coordinated regulation of flagellin biosynthesis and other CsrA regulated processes such as bacterial adherence, resistance to oxidative stress and virulence, although no target genes have thus far been identified (Fields and Thompson, 2008; Fields and Thompson, 2012). Theoretically, mechanisms of regulated binding of CsrA to other transcripts via additional, unidentified CsrA binding proteins may in turn affect flagellin biosynthesis by influencing the level of free CsrA. However, at this time, FliW is the first and only protein that complexes to CsrA that has been identified in *C. jejuni*.

The specific binding of *C. jejuni* FliW to FlaA and FlaB but not FlaC suggests that the CsrA-mediated post-transcriptional regulation is limited to the structural flagellins (FlaA and FlaB) and does not involve FlaC. This is conceivable as FlaA and FlaB are basic building blocks of the flagella fiber. The FlaC protein has not been demonstrated as a flagellum constituent but is rather secreted via the flagellar apparatus into the environment and is assumed to play a role in *C. jejuni* invasion of host cells (Song *et al.*, 2004; Christensen *et al.*, 2009). The secretion of FlaC may limit its accumulation and the need for tight control of the protein level in the cytosol. The differential regulation of the flagellins is also apparent at the transcriptional level. Here *flaA* and *flaB* are subject to transcriptional regulation via the alternative sigma factors (sigma[28] and sigma[54] respectively),

whereas the *flaC* gene is under the control of a sigma[70] promoter (Wösten *et al.*, 2010). Additionally, FlaA and FlaB but not FlaC are glycosylated before flagellar export, providing an additional layer of complexity to the assembly process. *C. jejuni* FliW binds to both non-glycosylated and glycosylated flagellins and thus appears to control the total pool of cytoplasmic structural flagellins (Fig. 4D).

Inactivation of *fliW* was accompanied by minor changes in *flaA* and *flaB* transcript levels suggesting limited feedback between cytosolic flagellin levels and flagellin transcription. It should be noted that in *C. jejuni* flagellin transcription is already tightly regulated via different factors including the two-component FlgR/FlgS system (Wösten *et al.*, 2004), the alternative transcription factors sigma[28] and sigma[54] (Hendrixson and DiRita, 2003), the anti-σ factor FlgM (Wösten *et al.*, 2010) and FlhF GTPase (Balaban *et al.*, 2009). The FlgR/FlgS system regulates sigma[54]-dependent transcription of mainly the basal body and hook structure while after completion of the hook the intracellular concentration of FlgM links flagella length with the activity of sigma[28] that controls amongst others the transcription of *flaA*. It has been shown that the secretion of FlgM decreases while the filament length elongates (Wösten et al., 2010). It can be imagined that the noted reduced amount of flagellin biosynthesis in the Δ*fliW* mutant that results in short flagellar filaments keeps intracellular FlgM levels low and thus indirectly stimulates *flaA* transcription by decreased scavenging of sigma[28]. This scenario may explain the observed increase in *flaA* but not *flaC* transcript in the Δ*fliW* mutant.

The discovery that CsrA regulates *flaA* and *flaB* translation initiation in *C. jejuni* was triggered by *in silico* analysis of the 5′-UTR of the flagellin transcripts. According to the previously established structural model, the proteins belonging to CsrA/Rsm family make optimal contacts with the RNA sequence 5′-(A)/(U)CANGGANG(U)/(A)−3′, in which the central ribonucleotides form a hexaloop (Dubey *et al.*, 2005; Lapouge *et al.*, 2013). The *in silico* analysis revealed the sequence (5′-UCANGGAUGA-3′) in a hexaloop at the 5′-end of *flaA* transcript and two hairpin-like structures in the vicinity the Shine Dalgarno sequence in *flaB* transcript, among them a hexaloop containing the GGA-motif. We did not find any similar motifs for the *flaC* transcript (data not shown). The sequence 5′-UCAUGGAUGA-3′ present in a canonical hexaloop at the 5′-end of *flaA* transcript is expected to make optimal contacts with CsrA. It can be argued that the less canonical motif present in the hexaloop at the 5′-end of *flaB* transcript results in less optimal recognition but this awaits detailed binding kinetics studies.

In *E. coli*, the CsrA protein is a global regulator which represses various metabolic pathways and processes that are induced in the stationary phase of growth while it activates certain exponential phase functions (Timmermans and Melderen, 2010; Vakulskas *et al.*, 2015). It is also involved in the flagellar biosynthesis as stabilizer of the transcript of master operon for flagellum biosynthesis in *E. coli*, *flhDC* (Wei *et al.*, 2001; Yakhnin *et al.*, 2013). A direct effect on the translation of flagellin mRNA as occurs in *C. jejuni*, however, has not been reported. The vast majority of regulators belonging to CsrA/Rsm family is coordinated by a set of two small non-coding RNAs, *csrB* and *csrC*. Regulation of *E. coli csrB* and *csrC* is directed by the BarA/UvrY two-component system (Romeo *et al.*, 2013; Yakhnin *et al.*, 2013). The *C. jejuni* genome lacks both the small non-coding RNAs and a two-component system of the similar function (Kulkarni *et al.*, 2006; Fields and Thompson, 2012). This makes FliW the only known regulator of CsrA activity in *C. jejuni*.

FliW orthologs are absent in *E. coli* but have been identified as a flagellar assembly factor in several bacterial species, including *T. pallidum* (Titz *et al.*, 2006), *B. burgdorferi* (Karna *et al.*, 2013) and *B. subtilis* (Mukherjee *et al.*, 2011). Bioinformatic analysis indicates that FliW orthologues are also abundant among *Epsilonproteobacteria* (Supporting Information Fig. S2). The role of these proteins in this phylum remains to be elucidated but may be related to the regulatory function in flagella biosynthesis described here. In *T. pallidum*, FliW has been proposed to act as a flagellin chaperone (Titz *et al.*, 2006). Interestingly, in the Gram-positive bacterium *B. subtilis* FliW binds to CsrA and, with a lower affinity, to flagellin, suggesting that partner switch occurs when cytoplasmic flagellin level exceeds a threshold what releases CsrA from the complex (Mukherjee *et al.*, 2011). This resemblance in regulatory mechanisms is striking as *B. subtilis* and *C. jejuni* are only distantly related and differ substantially in the composition and decoration of their flagella. The peritrichous flagella in *B. subtilis* are composed of a single flagellin subunit (Hag) (Mukherjee and Kearns, 2014) whereas the bipolar single flagella of *C. jejuni* consist of two structural flagellins that need to be glycosylated during the process of flagellar filament formation (Ewing *et al.*, 2009). Considering these substantial differences, we did not expect conservation of FliW-CsrA post-transcriptional regulation of flagellin biosynthesis among the species. This resemblance in regulatory mechanisms between the Gram-negative *C. jejuni* and the Gram-positive *B. subtilis* however, stresses the apparent importance of prompt control of the cytosolic flagellin levels during flagella biogenesis and the need for post-transcriptional regulation of this event.

In conclusion, our data demonstrate the existence of post-transcriptional regulation of flagellin biosynthesis in

C. jejuni. The mechanism involves the reciprocal binding of the FliW protein to cytosolic flagellin and the global post-transcriptional regulator CsrA which was found to regulate flagellin translation initiation.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in the study are listed in Supporting Information (Table S1). *E. coli* strains were grown at 37°C on Luria Bertani (LB) plates (Biotrading) or in LB broth (Biotrading). *C. jejuni* strain 81116 and derivatives were routinely cultured on plates with 5% saponin-lysed horse blood (Biotrading) at 37°C, under microaerophilic conditions (5% O_2, 7.5% CO_2, 7.5% H_2, 80% N_2). Liquid *C. jejuni* cultures were grown in Heart Infusion (HI) broth (Biotrading) at 42°C, under microaerophilic conditions. When appropriate, growth media were supplemented with ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or chloramphenicol (20 μg ml^{-1}).

Recombinant DNA techniques

Plasmids were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). Genomic DNA of *C. jejuni* strains was isolated with the High Pure PCR Template Preparation Kit (Roche Life Science). DNA fragments were extracted from agarose gels using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). Primers were obtained from Thermo Fisher Scientific and are listed in Supporting Information (Table S2). DNA polymerases, restriction endonucleases, T4 DNA ligase were purchased from Thermo Fisher Scientific. All kits and enzymes were used according to the manufacturers' instructions. All inserts of DNA vectors constructed in this study were verified by sequencing (Macrogen).

Construction of C. jejuni strains

The *C. jejuni* 81116 *fliW*-encoding gene (C8J_1016), was amplified with the KR72-KR73 primer pair and cloned into the pJet1.2 vector. The resulting vector pJet1.2-*fliW* was used as a template in an outward PCR (primer pair KR74-KR75). The PCR product was ligated to the *cat* cassette excised from the pAV35 vector with BamHI, yielding the knockout construct pJet1.2-*fliW*::*cat*. The vector pJet1.2-*fliW*::*aph* was constructed in the same manner, except that the *cat* cassette was replaced by the *aph* gene excised from pMW2 with BamHI. The *cat* and *aph* cassette confer chloramphenicol and kanamycin resistance respectively from their own promoters. In both cases, the cassette was inserted in the same orientation as the *fliW* ORF and replaced the central 284 bp of the 390 bp-sized *fliW* gene, leaving 54 bp of 5'-end of *fliW* ORF and 52 bp of 3'-end of *fliW* ORF intact. Possible effects on the downstream *proC* gene (C8J_1017) which is required for proline biosynthesis (Yura and Vogel, 1959; Gherardini *et al.*, 1990; King *et al.*,

2000) were ruled out as *C. jejuni* was always grown in the proline-rich HI medium.

To inactivate *csrA* (C8J_1044), the gene and its flanking regions were amplified from genomic DNA of *C. jejuni* 81116 with the NB280-NB281 primer pair and cloned into the pJet1.2 vector, yielding pJet1.2-*csrA*. This plasmid was used in an outward PCR using the primer pair NB290-NB291. The *cat* cassette of pAV35 isolated as a EcoRV and SacII fragment was ligated to the outward PCR product digested with same restriction enzymes. After ligation the resulting knockout construct pJet1.2-*csrA*::*cat* contained the *csrA* gene in the same orientation as the *csrA* ORF. The constructs were introduced into *C. jejuni* 81116 by natural transformation (Wassenaar *et al.*, 1993) to yield strain *C. jejuni* Δ*fliW*, *C. jejuni* Δ*csrA* and *C. jejuni* Δ*fliW*Δ*csrA*. Disruption of the genes was verified by PCR. To rule out the occurrence of a polar effect, the expression of the downstream genes (C8J_1017 downstream *fliW*, RT7-RT8 primer pair; C8J_1045 downstream *csrA*, RT11-RT12 primer pair) was confirmed by RT-qPCR analysis.

To overexpress *fliW*, the gene was cloned into the vector pMA5 resulting in plasmid pfliW. Hereto the *fliW* was amplified from the *C. jejuni* 81116 using the primers KR76 and KR77. The PCR product was digested with SacI and KpnI and subsequently ligated into pMA5 digested with the same restriction enzymes. The ectopic *fliW* gene was introduced into *C. jejuni* 81116 via conjugation (Labigne-Roussel *et al.*, 1987), yielding the overexpressing strain *C. jejuni*/pfliW.

Growth curves

Overnight cultures of *C. jejuni* strains were diluted to OD$_{600}$ of 0.05 in a 100-well honeycomb plate. Bacterial growth was monitored in a Bioscreen C MRB (Oy Growth Curves Ab) computer-controlled incubator, placed inside an anaerobic chamber (Coy Labs, Grass Lake, MI, USA). The Bioscreen system was set to measure the OD$_{600}$ every 15 min for 36 h. Strains were grown with continuous shaking in HI broth at 42°C, under microaerophilic conditions. Experiments were repeated three times in duplicate.

Motility assay

One microliter of *C. jejuni* culture, diluted to OD$_{600}$ of 1, was stabbed with a pipette into HI plates containing 0.4% agar. Swarming was assessed after incubation under microaerophilic conditions at 42°C for 18 h. Motility was scored by measuring the diameter of motility zones. The experiment was repeated three times.

Electron microscopy analysis

C. jejuni strains were grown until mid-exponential phase (OD$_{600}$ 0.4–0.7) and adjusted to a concentration of around 1×10^9 cells ml^{-1}. Bacteria were fixed with 4% glutaraldehyde, 5 mM $CaCl_2$, 10 mM $MgCl_2$, in 0.1 M Na-cacodylate buffer. Fixed samples were adhered to carbon-coated grids and stained with 0.5% uranylacetate. Imaging was done using a FEI Tecnai 12 electron microscope at 80 kV.

Representative images were taken after observation of more than 50 bacteria. The length of the flagellar filaments of 23 wild type and 31 Δ*fliW* mutant bacteria was estimated using ImageJ/Fiji software as described (http://fiji.sc/SpatialCalibration).

Real-time RT-PCR analysis

Real-time RT-PCR (RT-qPCR) analysis was performed as previously described (Wösten *et al.*, 2004). Primers used in the assay (*gyrA*: MW670-MW671, *flaA*: MW147-MW148, *flaB*: MW145-MW146, *flaC*: MW455-MW456) are listed in Supporting Information Table S2. RNA was isolated from mid-exponential phase *C. jejuni* cultures (OD$_{600}$ 0.4–0.7) using the RNA-Bee kit (Tel-Test) according to manufacturer's protocol. RNA was treated with 1 μg of DNAse per μg of RNA for 30 min at 37°C. The DNase was heat-inactivated for 10 min at 65°C in the presence of 2.5 mM EDTA. mRNA levels were determined in a LightCycler 480 Real-Time PCR System using the Brilliant III Ultra-Fast Sybr-Green qRT-PCR kit according to manufacturer's instructions. Per reaction 10 ng of DNase treated RNA was used as template. Real-time cycler conditions were 30 min at 48°C, followed by 5 min at 95°C and then for 45 cycles at 95°C for 5 s, 60°C for 30 s. Specificity was confirmed by inclusion of template- or reverse transcriptase-free controls. The calculated threshold cycle (Ct) for each gene amplification was normalized to the Ct value for *gyrA* gene, amplified of the corresponding sample, before calculating fold change using the arithmetic formula ($2^{-\Delta\Delta Ct}$), where $\Delta\Delta Ct = $ [(Ct target gene − Ct *gyrA*) mutant − (Ct target gene − Ct *gyrA*) wild type] (Livak and Schmittgen, 2001). Each sample was examined in four replicates and was repeated with two independent preparations of RNA.

Preparation of C. jejuni lysates

C. jejuni strains were grown until mid-exponential phase (OD$_{600}$ 0.4–0.7) at 42°C. The OD$_{600}$ of all cultures was adjusted to 0.5 and 1 ml of each culture was centrifuged (2 500 × g, 10 min), washed once with 1 ml of TBS (20 mM Tris, 150 mM NaCl, pH 7.4) and suspended in 1 ml of TBS. After sonication (Branson Sonifier, duty cycle 50%, output control 2, 30 s), the protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were prepared in triplicate from independently grown cultures.

Analysis of flagellin levels by Western blot

Western blot analysis was performed on *C. jejuni* lysates separated by SDS-PAGE (500 ng, 250 ng and 125 ng of each sample) and transferred onto nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membrane was incubated for 1 h with 5% skim milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4) in order to block non-specific binding. The membrane was probed with a polyclonal anti-FlaA/B (Nuijten *et al.*, 1989) or anti-FlaC serum (Wösten *et al.*, 2010), both diluted

at 1:10 000 in 2% skim milk in TBS-T. Incubation was continued for 1 h and followed by three five-minute washes with TBS-T. Reactive bands were detected with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP; Sigma) diluted 1:10 000 in 2% skim milk in TBS-T and visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Images were taken with ChemiDoc MP system (Bio-Rad). Three biological replicates were analysed.

Analysis of flagellin and N-linked glycans levels by ELISA

Flagellin levels in *C. jejuni* strains were determined by ELISA. Flat-bottom 96-well Maxisorp Nunc-Immuno plates (Thermo Fisher Scientific) were coated (16 h) with total cell lysates of *C. jejuni* strains (250–8 ng/well) in 0.05 M carbonate buffer, pH 9.6. Plates were washed five times with TBS-T and blocked for 1 h with 5% skim milk in TBS-T. After incubation (60 min) with anti-FlaA/B serum (Nuijten *et al.*, 1989) with 2% skim milk in TBS-T, the plates were rinsed (5 times), and incubated with 100 μl of goat anti-rabbit IgG antibody conjugated with HRP (Sigma) diluted 1:10 000 in 2% skim milk in TBS-T. After 1 h, plates were washed (5 times) and TMB substrate (BD OptEIA, BD Biosciences) was added. After 10 min, the reaction was stopped by the addition of 1 M H$_2$SO$_4$. Absorbance was measured at 450 nm in a microplate reader (FLUOstar Omega, BMG Labtech).

The lectin soybean agglutinin, conjugated with HRP (SBA-HRP), which has previously been used to demonstrate *C. jejuni* N-linked protein glycosylation (van Sorge *et al.*, 2009) was used as a coating control for ELISA experiment. Briefly, Maxisorp Nunc-Immuno plates were coated with total cell lysates of *C. jejuni* as described above. Plates were washed five times with TSM-T buffer (20 mM Tris, 150 mM NaCl, 1 mM CaCl$_2$, 2 mM MgCl$_2$, 0.05% Tween 20, pH 7.0) and blocked 2 h with 1% BSA in TSM-T. After 2 h of incubation with 0.5 μg ml^{-1} SBA-HRP (Sigma) in TSM-T with 1% BSA, the plates were washed (5 times) and TMB substrate was added. After 10 min the reaction was stopped by the addition of 1 M H$_2$SO$_4$. Absorbance was measured at 450 nm in a microplate reader (FLUOstar Omega, BMG Labtech). ELISA experiments were performed in triplicate, from independently grown cultures.

Cloning and expression of recombinant proteins

pSCODON1.2 expression vector was used to create a C-terminal His-tag fusion to FliW or RacR. The *fliW* gene was amplified with KR62-KR63 primer pair using pJet1.2-*fliW* as a template. The PCR fragment was directly cloned into the NdeI and XhoI sites of the expression vector, resulting in pSCODON1.2-FliW. The *racR* gene was amplified with the primers MW632-MW510 from genomic DNA of *C. jejuni* 81116 and cloned into pGEM-T Easy, generating the pGEM-*racR* vector. The insert was excised form pGEM-*racR* with restriction enzymes NdeI and XhoI and cloned into NdeI and XhoI sites of pSCODON1.2, yielding pSCODON1.2-RacR vector.

To create the N-terminal GST-tag fusion to FliW, the *fliW* gene was amplified using primers KR100 and KR101 and pJet1.2-*fliW* as a template. The resulting PCR fragment was digested with EcoRI and XhoI and cloned into the EcoRI and XhoI sites of the pGEX4T-2 expression vector, generating pGEX4T-2-FliW.

Champion pET101 Directional TOPO Expression Kit (Thermo Fisher Scientific) was used to express the recombinant FlaA, FlaB, FlaC and CsrA proteins as a C-terminal fusion with a His-tag. Cloning was performed according to manufacturer's instructions. *C. jejuni* flagellin-encoding genes were amplified from genomic DNA of *C. jejuni* 81116 WT strain with the following primer pairs: (i) *flaA*: KR50-KR51, (ii) *flaB*: KR111-KR112 and (iii) *flaC*: FlaC_hisF-FlaC_hisR. The *csrA* gene was amplified with KR122 and KR123 primers and pJet1.2-*csrA* as a template.

The expression plasmids were transformed to *E. coli* BL21 Star (DE3). Pre-cultures were used to inoculate 25–50 ml of LB broth (1:50). Bacteria were grown at 32°C, until OD_{600} 0.5 then the protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 4 h of induction, the bacteria were collected by centrifugation (4000 × g, 15 min) and stored at −80°C.

Purification of recombinant proteins

Recombinant FliW-His and RacR-His were isolated from *E. coli* BL21 Star (DE3) under denaturing conditions. Bacterial pellets (collected from 25 ml culture) were suspended in 5 ml lysis buffer (20 mM Tris, 150 mM NaCl, pH 7.4, supplemented with EDTA-free complete protease inhibitor cocktail, Roche Diagnostics and 10 mg ml^{-1} lysozyme, Sigma) and incubated on ice for 1 h. Bacteria were disrupted by sonication (8 pulses of 15 s each with 20 s hold on ice). The insoluble fraction obtained after centrifugation (4 400 × g, 30 min, 4°C) was suspended in 5 ml solubilisation buffer (8 M urea, 20 mM Tris, 250 mM NaCl, 20 mM imidazole, pH 7.4). Sample was incubated overnight at RT with end-over-end rotation. To the solubilized proteins 1 ml of Ni^{2+}-NTA agarose beads (Thermo Fisher Scientific) was added and incubation continued for 2 h. The mixture was applied to a column. The beads were washed with a set of buffers with the decreasing urea concentration: 8 M, 4 M, 2 M, 1 M, 0.5 M, 0.25 M, 0.13 M, 0.06 M urea solution in 20 mM Tris, 250 mM NaCl, 20 mM imidazole pH 7.4 (5 ml of each buffer was used, 40 ml in total). The final washing step was performed with 5 ml of 20 mM Tris, 250 mM NaCl, 20 mM imidazole pH 7.4. His-tagged proteins were eluted from the nickel beads with 1 ml of 20 mM Tris, 250 mM NaCl, 300 mM imidazole, pH 7.4.

GST-tagged FliW was isolated using the Pierce Glutathione Agarose (Thermo Fisher Scientific) according to the gravity-flow column protocol provided by the manufacturer. To purify the protein, 1.5 ml of the resin was used per 50 ml of induced culture of *E. coli* BL21 Star (DE3) harboring pGEX4T-2-FliW. In the same manner GST protein was purified from *E. coli* BL21 Star (DE3) containing pGEX4T-2 vector. Purified proteins were dialysed overnight at 4°C with stirring, against 3 L of 50 mM Tris, 250 mM NaCl, pH 8.0.

Proteins were dialysed using SnakeSkin Dialysis Tubing, 10K MWCO (Thermo Fisher Scientific).

Pull-down experiments

His-tagged FliW or RacR was used as bait in the pull-down experiments. As a prey the supernatant of *C. jejuni* Δ*flgKM* (BC7) (Bleumink-Pluym *et al.*, 1999) culture was used. Pull-down experiment was repeated three times separately for each bait protein, immobilized and re-folded on the nickel beads. To the Ni^{2+}-NTA column containing either His-tagged FliW or RacR 5 ml of the supernatant of BC7 culture (grown at 42°C in 5 ml HI) was added. Column was closed and incubated with rotation end-over-end 1 h at RT. The beads were washed extensively with 45 ml of 20 mM Tris, 250 mM NaCl, 20 mM imidazole, pH 7.4. At this step, a control sample was collected (last wash) to confirm the removal of all non-specifically bound proteins. His-tagged proteins with all interacting partners were eluted from the column with 1 ml of 20 mM Tris, 250 mM NaCl, 300 mM imidazole, pH 7.4 (elution). Purified FliW-His and RacR-His, together with the input sample of the BC7 supernatant and the samples collected during the pull-down procedure (last wash and elution) were analysed by SDS-PAGE with the PageBlue Protein Staining solution (Thermo Fisher Scientific), by Western blot using anti-His-HRP antibody (Invitrogen) or polyclonal anti-FlaA/FlaB flagellin serum (Nuijten *et al.*, 1989).

Analysis of protein interactions by Far Western Blot

Total cell lysates of *E. coli* BL21 Star (DE3) harbouring the appropriate expression vectors were separated in SDS-PAGE and transferred onto nitrocellulose membrane. Non-specific binding was blocked by incubating the membrane 1 h with 5% skim milk in TBS-T. The membrane was probed with 50 μg GST-FliW or GST protein, diluted in 2% skim milk in TBS-T. Unbound proteins were removed by three five-minute washes with TBS-T. To detect the binding of GST-FliW or GST probe to the membrane the blot was incubated for 1 h with monoclonal anti-GST antibodies (Sigma), 1:10 000 diluted in 2% skim milk in TBS-T. After three 5-min washes with TBS-T the GST proteins were detected with anti-mouse IgG antibody conjugated with HRP (Sigma), diluted 1:8 000 in 2% skim milk in TBS-T. Reactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Images were taken with ChemiDoc MP system (Bio-Rad). The same protein samples were visualized with PageBlue Protein Staining (Thermo Fisher Scientific) and analysed by Western blot with anti-His-HRP antibody (Invitrogen).

To compare recognition of non-glycosylated and glycosylated flagellins by FliW, analogous experiment was performed. Briefly, *E. coli* lysate containing His-tagged FlaA and the culture supernatant of BC7 strain containing native flagellins were serially diluted (500 ng, 250 ng, 125 ng and 62.5 ng of each sample), separated on SDS-PAGE and stained with PageBlue Protein Staining or transferred onto nitrocellulose membrane and probed with GST-FliW and subsequently with anti-GST antibodies, followed by anti-mouse IgG antibody conjugated with HRP.

Analysis of CsrA-binding sites in flagellin transcripts

The prediction of RNA secondary structure was performed with mFold program (Zuker, 2003) with default settings. Transcriptional start sites of flagellins were based on (Dugar *et al.*, 2013).

In vitro translation

As a template for *in vitro* translation pET101-FlaA5UTR vector was used. The construct was created in outward PCR reaction with primers KR126 and KR127 and pET101-FlaA as a template. Native 5′UTR (44 bp) of *flaA* transcript was introduced to pET101-FlaA vector between T7 promoter and AUG codon of *flaA*.

Cell free protein synthesis was carried out using the PUREfrex System (Eurogentec). The reaction mixtures were prepared according to the manufacturer's instructions. As a template, 150 ng of pET101-FlaA5UTR was used. The reaction was performed in the absence and presence of purified CsrA-His (20 pmol). Flagellin synthesis was monitored by Western blot with polyclonal anti-FlaA/B antibodies. Experiment was performed in triplicate.

Statistical analysis

Prism software version 6.05 (GraphPad, San Diego, CA) was used for statistical analysis. Data were expressed as mean $\pm$ SD. Motility test results were analysed by one-way ANOVA followed by Bonferroni multiple comparisons test. The one-sample *t*-test on log-transformed data was used to estimate the significance of transcript fold change between WT strain and $\Delta fliW$ or WT/pfliW. $P < 0.05$ was considered statistically significant.

Acknowledgements

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