Insights into the impact of *flhF* inactivation on *Campylobacter jejuni* colonization of chick and mice gut

Fangzhe Ren[1], Xiaofei Li[2], Haiyan Tang[2], Qidong Jiang[2], Xi Yun[3], Lin Fang[3], Pingyu Huang[3], Yuanyue Tang[1,2,3], Qiuchun Li[1,2,3], Jinlin Huang[1,2,3*] and Xin-an Jiao[1,2,3*]

Abstract

Background: *Campylobacter jejuni* (*C. jejuni*) is a leading cause of foodborne gastroenteritis worldwide. This bacterium lacks many of the classical virulence factors, and flagellum-associated persistent colonization has been shown to be crucial for its pathogenesis. The flagellum plays a multifunctional role in *C. jejuni* pathogenesis, and different flagellar elements make diverse contributions. The *flhF* gene encodes the flagellar biosynthesis regulator, which is important for flagellar biosynthesis. In this study, the influence of *flhF* on *C. jejuni* colonization was systematically studied, and the possible mechanisms were also analyzed.

Results: The *flhF* gene has a significant influence on *C. jejuni* colonization, and its inactivation resulted in severe defects in the commensal colonization of chicks, with approximately 10^4- to 10^7-fold reductions (for NCTC 11168 and a *C. jejuni* isolate respectively) observed in the bacterial caecal loads. Similar effects were observed in mice where the *flhF* mutant strain completely lost the ability to continuously colonize mice, which cleared the isolate at 7 days post inoculation. Characterization of the phenotypic properties of *C. jejuni* that influence colonization showed that the adhesion and invasion abilities of the *C. jejuni flhF* mutant were reduced to approximately 52 and 27% of that of the wild-type strain, respectively. The autoagglutination and biofilm-formation abilities of the *flhF* mutant strain were also significantly decreased. Further genetic investigation revealed that *flhF* is continuously upregulated during the infection process, which indicates a close association of this gene with *C. jejuni* pathogenesis. The transcription of some other infection-related genes that are not directly involved in flagellar assembly were also influenced by its inactivation, with the flagellar coexpressed determinants (Feds) being apparently affected.

Conclusions: Inactivation of *flhF* has a significant influence on *C. jejuni* colonization in both birds and mammals. This defect may be caused by the decreased adhesion, invasion, autoagglutination and biofilm-formation abilities of the *flhF* mutant strain, as well as the influence on the transcription of other infection related genes, which provides insights into this virulence factor and the flagellum mediated co-regulation of *C. jejuni* pathogenesis.

Keywords: *flhF*, Colonization, *Campylobacter jejuni*

Background

Campylobacter jejuni is the most common cause of foodborne gastroenteritis in humans worldwide, and infections caused by this microbe often leads to diarrhea and sometimes to a severe inflammatory response with clinical symptoms of fever, abdominal cramping, bloody stools and other symptoms [1, 2]. *C. jejuni* is prevalent in the environment and can infect humans in a variety of ways [3, 4]. Although the incidence of *C. jejuni* infection is very high at present, causing it to receive increased attention [5], a complete understanding of the mechanisms of *C. jejuni*-associated disease is still an ongoing effort [3]. An analysis of the genome sequence of *C. jejuni* revealed that the cytolethal distending toxin (CDT) is the only toxin produced by this bacterium. Many other classical virulence factors that are common

Ren *et al. BMC Microbiology* (2018) 18:149

Page 2 of 11

to other pathogens are missing in the *C. jejuni* genome [6]. It has now been shown that colonization in the host intestinal tract and persistence to a sufficient load is crucial for *C. jejuni* pathogenesis [3, 7, 8].

Intestinal colonization by *C. jejuni* is influenced by multiple factors, including the capsule, glycosylation system, catabolism of L-serine, cytochrome c peroxidase, transport systems, putative adhesins, chemotaxis and flagellar motility [9–16]. The flagellum is particularly important among these factors [17]. *C. jejuni* produces a single flagellum at one or both poles, which not only provides chemotactic motility for migration to replicative niches but also helps the bacteria penetrate the mucus that covers the epithelial cells. In addition, the flagellum is involved in interactions with and invasion of the host epithelium, the secretion of invasion antigens, and the evasion of the innate immune system, is the essential virulence and colonization factor of *C. jejuni* [6, 15, 18–20]. The flagellum is a multicomponent organelle composed of a basal body, motor, rod, hook and flagellin filament. It appears that the *C. jejuni* flagellar substructures, flagellin glycosylation, motility and secreted invasion antigens are all required for host colonization but are not the only determinants [9, 16, 19, 21–23]. There are over 50 flagellum-related genes in the *C. jejuni* genome, and their corresponding contributions to colonization are also diverse [15, 16, 18, 22–25]. Considering the multifunctional role played by flagella during *C. jejuni* pathogenesis [26], a comprehensive understanding of their virulence potential is urgently needed.

The importance of FlhF for flagellar biosynthesis has been observed for several pathogens (primarily for the polarly flagellated bacteria) in recent years [27–33]. In *C. jejuni*, the inactivation of *flhF* has been observed to inhibit the synthesis of flagella and motility in previous studies [34, 35], but its contribution to *C. jejuni* colonization has not yet been characterized. In this study, the influence of *flhF* on *C. jejuni* colonization was systematically evaluated both in the reference strain and in an isolate. The results showed the significant effect this gene has in the host colonization process. The possible factors that may contribute to the colonization defect were assayed, including the flagella-associated abilities such as adherence and invasion to the host cells, autoagglutination (AAG) and biofilm formation. The mechanism of by which FlhF influences host colonization was also explored at the gene expression level. We observed that *flhF* is continuously upregulated during the cell infection process, and in addition to its major role in the flagellar system, some other infection-related genes were observed to be downregulated in the *flhF* mutant strain. The results of this study provide a reference for the further analysis of this virulence factor and investigations of the mechanism of flagella mediated co-regulation of pathogenesis in this bacterium.

Methods

Bacteria and cell culture

C. jejuni strains were grown on *Campylobacter* blood-free selective agar containing charcoal cefoperazone deoxycholate (CCDA) (Oxoid, Basingstoke, UK) or in Mueller-Hinton (MH) broth (BD Biosciences, Sparks, MD, USA) in a microaerophilic environment (5% O_2, 10% CO_2, and 85% N_2) and incubated at 42 °C. The *C. jejuni* isolate used in this study was isolated from the anal swabs of a monkey with diarrhea and was further identified using multiplex PCR (polymerase chain reaction) (Additional file 1: Figure S1) [36]. Appropriate antibiotics (50 μg/ml kanamycin, 20 μg/ml chloramphenicol, 10 μg/ml rifampicin, 6 μg/ml polymyxin B, 10 μg/ml trimethoprim, 100 μg/ml cycloheximide, 40 μg/ml vancomycin, 20 μg/ml amphotericin B or 60 μg/ml cefoperazone) were added to the medium as required. Inactivation of *flhF* and complementation of the mutant in *C. jejuni* NCTC 11168 and in the isolate were performed as previously described [34]. Human colon cancer cell line (Caco-2) was acquired from American Type Cell Culture (ATCC) and routinely cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified 37 °C atmosphere containing 5% CO_2.

Colonization assay

Chick and mice colonization assays were largely performed as described in previous studies [16, 24, 37]. Briefly, *C. jejuni* strains were harvested in phosphate buffered saline (PBS), and 1×10^8 colony-forming units (cfu) of bacteria were subsequently orally inoculated into 1-day-old *Campylobacter*-free Leghorn chicks obtained from the Jiangsu Institute of Poultry Science. Chicks were randomly allocated into three groups and sacrificed by CO_2 asphyxiation at 1 and 7 days post inoculation. For each chick, the intestinal tract was collected, separated following gentle extrusion of the lumenal contents, weighed, homogenized, serially diluted and plated on selective CCDA agar (containing rifampicin, polymyxin B, trimethoprim, cycloheximide, vancomycin, amphotericin B and cefoperazone) to count the bacteria load. Six-week-old C57/BL6 mice were acquired from the Comparative Medicine Center of Yangzhou University. The mice initially received 200 μl of a sodium bicarbonate solution (50 mg/ml), after which 1×10^8 cfu of bacteria were used to infect mice by orogastric inoculation 15–30 min later. The mice were sacrificed by cervical dislocation at the designated time point, and the colonization levels were evaluated using selective media containing amphotericin B and cycloheximide as described above.

Growth and biochemical tests

Overnight cultures of *C. jejuni* were harvested and diluted in MH broth to a starting optical density at

Ren *et al. BMC Microbiology*   (2018) 18:149

Page 3 of 11

600 nm (OD600) of 0.08. Two-milliliter aliquots of the suspension were transferred into the wells of a 12-well plate and incubated at 42 °C and 120 rpm under a micro-aerophilic atmosphere. Samples were collected at indicated intervals and the OD600 values were determined to compare the growth of the different strains. Biochemical testing was performed as described by Valenza et al. [38]. Bacteria were harvested in a 0.45% NaCl solution and the turbidities were adjusted to an equivalent of a McFarland standard of 3. The suspensions were inoculated into a Neisseria-Haemophilus (NH) identification card, which was automatically analyzed by a VITEK 2 microbiology system (bioMérieux, France).

Cell adhesion and invasion assay
Adhesion and invasion assays were performed as described previously [39]. Caco-2 cells were first seeded into a 24-well plate at a density of 1×10^5. After the cells incubated for 18 h, *C. jejuni* that had grown for 20 h were harvested and resuspended in DMEM, and 1 ml suspensions containing 1×10^7 cfu were then inoculated into each well. The plates were centrifuged at 600×*g* for 5 min to enhance the contact between bacteria and eukaryotic cells. For bacterial adhesion assays, cells were incubated for 2 h, washed three times with PBS to remove the unattached bacteria, and then were lysed in 1 ml of PBS containing 0.1% TritonX-100, with serial dilutions of the suspensions subsequently spread onto CCDA plates to quantify the number of bacteria. To assess the number of invaded bacteria, the inoculated cells were first incubated for 2 h, washed once with DMEM and then were incubated in DMEM containing gentamicin (100 μg/ml) for another 1 h. The extracellular bacteria were killed during this period, and the number of invaded bacteria was determined as described above.

Autoagglutination assay
The autoagglutination assay was performed as previously described [23]. *C. jejuni* strains were grown for 20 h prior to use, after which each strain was suspended in PBS and adjusted to an OD600 of 1.0. Five-milliliter aliquots of the suspension were transferred into a series of sterile glass tubes (13 × 100 mm), which were incubated under microaerophilic conditions for a predetermined time. After incubation, 200 μl of the bacterial suspension was carefully removed from the top of each tube, and the OD600 was measured to evaluate the degree of AAG. Bacterial cells that strongly agglutinate will fall to the bottom of the tube, leading to a decreased OD600 value.

Biofilm-formation assay
The ability of *C. jejuni* strains to form biofilms was measured by crystal violet staining, using a method described by Naito et al. with some modifications [40]. Briefly, bacteria cultured for 20 h were harvested and diluted in MH broth to an OD600 of 0.1, after which 200 μl of each suspension was inoculated into 96-well polypropylene plates and incubated at 42 °C under microaerobic conditions. After incubating for 48, 60, 72, 84 and 90 h, the plates were washed with PBS and dried at 60 °C for 30 min. Next, 200 μl of a 1% crystal violet solution was added to each well to stain the biofilms that had formed. After staining at room temperature for 30 min, the plates were rinsed thoroughly with PBS three times to remove the unbound crystal violet. The plates were subsequently dried at 60 °C for 15 min, and the biofilms were quantified by determining the OD570 values after the remaining crystal violet was solubilized in a solution composed of 30% methanol and 10% acetic acid.

RNA isolation and quantitative real-time PCR
Quantitative real-time PCR (qRT-PCR) was performed as described previously [34]. *C. jejuni* was cultured for designated time, after which the RNA was extracted using an RNeasy plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated RNA was then used for cDNA synthesis using an RT reagent Kit (TaKaRa, Dalian, China). qRT-PCR was performed with a ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics, GmbH, Germany). The primers used for qRT-PCR are listed in the Additional file 1: Table S1. The *cj0402* gene (*glyA*) was used as an endogenous control to evaluate the relative expression of the target genes.

Gene collection and microarray analysis
The genes required for *C. jejuni* adhesion/invasion and colonization that were not directly involved in flagellar assembly were identified and are listed in Additional file 1: Table S2, and this information was primarily obtained from previously published reports. The relative mRNA expression of the selected genes in the *C. jejuni flhF* mutant strain versus the wild-type strain were acquired and analyzed using our previous whole transcriptome microarray data [34], which has been submitted to the NCBI Gene Expression Omnibus (GEO) database with the accession number GSE 87852. The fold change in gene expression was graphically presented in a heatmap that was generated using the Hemi software, with different colors representing the degree of variation in expression [41].

Statistical analysis
The data are presented as the means ± standard deviation (SD) from three independent experiments. Student's t-test was used to assess the significance of the observed differences ($P < 0.05$ *, and $P < 0.01$ **).
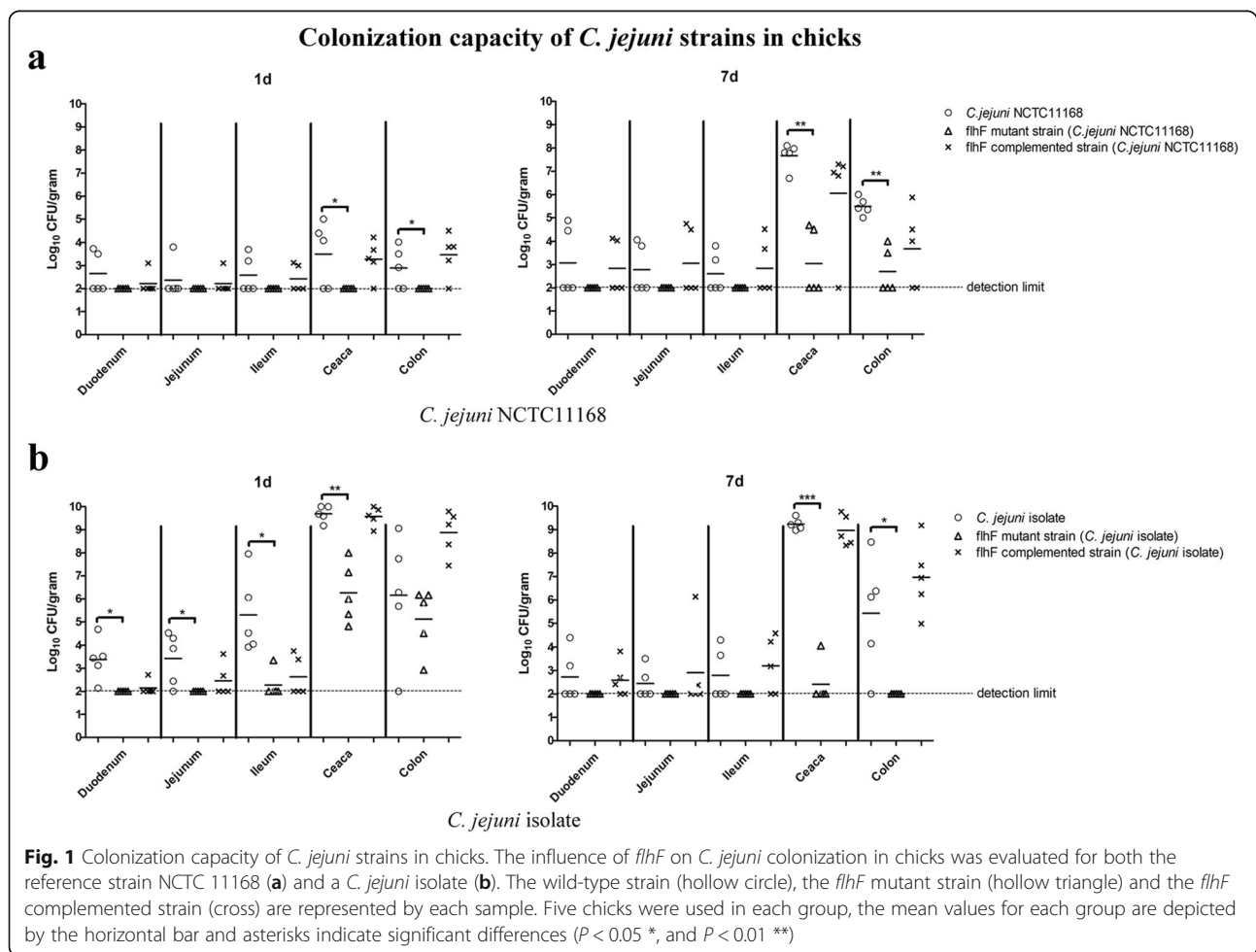
Results

Inactivation of *flhF* in *C. jejuni* dramatically influences its colonization in both chicks and mice
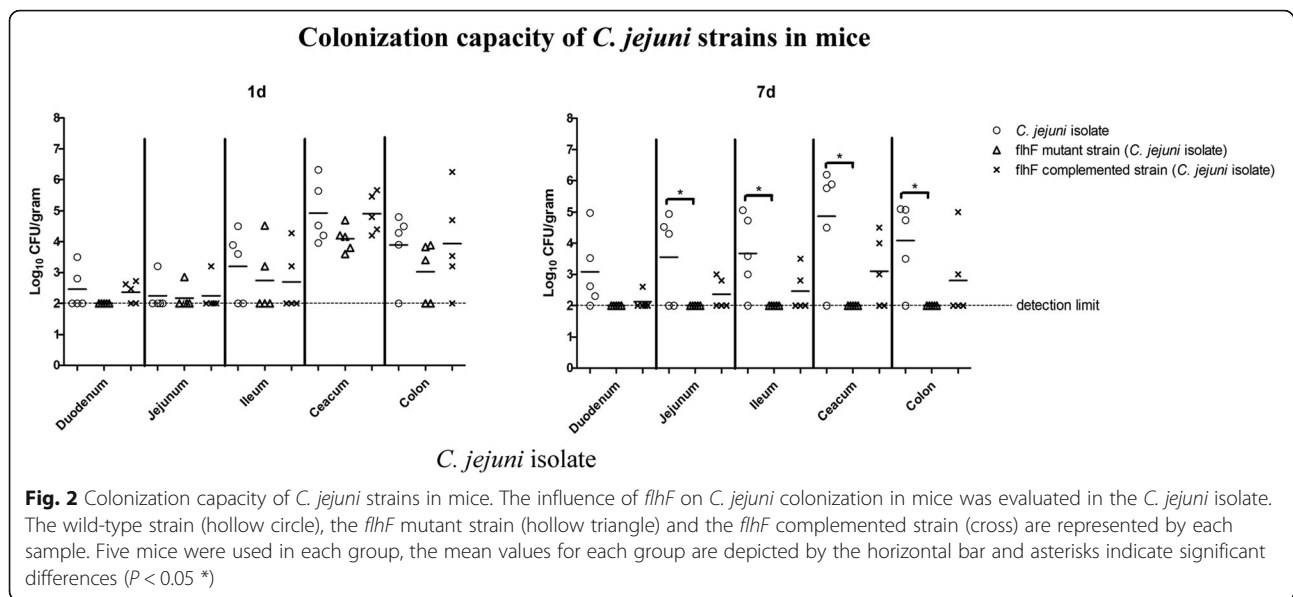
The importance of *flhF* on *C. jejuni* commensal colonization in chicks was systematically analyzed for both the reference strain NCTC 11168 and for a *C. jejuni* isolate. For NCTC 11168 (Fig. 1a), the *C. jejuni* bacterial load in the intestinal tract was relatively low 1 day after inoculation. However, we could not isolate the *flhF* mutant strain at this time point and a significant difference in colonization was observed compared to the wild-type strain in the large intestine (caeca and colon) ($P < 0.05$). On day 7, the *flhF* mutant strain still could not colonize the small intestines of chicks (duodenum, jejunum and ileum), and although it was present in the large intestines of some chicks, the bacterial load was significantly lower than that observed for NCTC 11168-infected group ($P < 0.01$).

A hyper-colonization phenotype was observed for the *C. jejuni* isolate, as the bacterial loads in the intestines of chicks infected with the same dose were notably higher than in those of NCTC 11168-infected chicks, thus the

effect of *flhF* was further determined in this *C. jejuni* isolate. As shown in Fig. 1b, the *flhF* gene was observed to be important in the initial establishment of colonization, since at 1 day post infection, the number of recovered isogenetic *flhF* mutant was significantly lower than that observed for chicks infected with the wild-type strain for both the small intestine ($P < 0.01$) and the caeca ($P < 0.05$). On day 7, the *flhF* mutant strain was almost completely cleared from the chicks and could be found only in the caeca of one chick, while the wild-type *C. jejuni* isolate consistently colonized the large intestines of chicks with a high load (approximately 10^9 cfu/g in the caeca). Complementation of *flhF* recovered this deficiency somewhat, both in *C. jejuni* NCTC 11168 and in the isolate, indicating that *flhF* significantly influences the commensal colonization of chicks.

The mice were less susceptible to *C. jejuni* colonization than the chicks. Although the *C. jejuni* isolate was observed to stably colonize both animals, the reference strain NCTC 11168 failed to colonize the mice in our study. For the *C. jejuni* isolate (Fig. 2), the bacteria recovered from the intestinal tract of mice were notably less abundant

Fig. 1 Colonization capacity of *C. jejuni* strains in chicks. The influence of *flhF* on *C. jejuni* colonization in chicks was evaluated for both the reference strain NCTC 11168 (**a**) and a *C. jejuni* isolate (**b**). The wild-type strain (hollow circle), the *flhF* mutant strain (hollow triangle) and the *flhF* complemented strain (cross) are represented by each sample. Five chicks were used in each group, the mean values for each group are depicted by the horizontal bar and asterisks indicate significant differences ($P < 0.05$ *, and $P < 0.01$ **)

Fig. 2 Colonization capacity of *C. jejuni* strains in mice. The influence of *flhF* on *C. jejuni* colonization in mice was evaluated in the *C. jejuni* isolate. The wild-type strain (hollow circle), the *flhF* mutant strain (hollow triangle) and the *flhF* complemented strain (cross) are represented by each sample. Five mice were used in each group, the mean values for each group are depicted by the horizontal bar and asterisks indicate significant differences ($P < 0.05$ *)

than those recovered from the chicks. However, the results showed that *flhF* does not greatly influence the initial colonization of mice. Although the average amount of the isolated *flhF* mutant was less than that of the wild-type strain, no significant differences were observed after 1 day post inoculation. On day 7, the *flhF* mutant strain could not be recovered from any regions of the intestines, while the wild-type strain continuously colonized the mice, with significant differences observed in the jejunum, ileum, caecum and colon ($P < 0.01$). These results suggested that inactivation of *flhF* resulted in the eventual elimination of the bacteria in mice.

flhF does not influence the growth or primary biochemical characteristics of *C. jejuni*

The observed growth curves indicated that the inactivation of *flhF* does not influence the normal growth of *C. jejuni*. When cultured in MH broth, the OD600 values for *C. jejuni* NCTC 11168, the *flhF* mutant and the complemented strains were nearly same at different time points and no significant differences were observed, suggesting that these strains have a similar growth rate (Fig. 3). The biochemical characteristics of the *C. jejuni* strains were analyzed using an NH card with a VITEK 2 compact instrument. The NH card contains 30 biochemical tests that includes five alkalization tests, 10 acidification tests, 11 glycosidase and peptidase tests and four miscellaneous tests. For the *C. jejuni flhF* mutant and the complemented strain, all the indexes tested were the same as the NCTC 11168 strain except for OPS (phenylphosphonate) (Additional file 1: Table S3), indicating that the primary biochemical characteristics of *C. jejuni* were unaffected in the *flhF* mutant.

C. jejuni flhF mutant shows a decreased ability to infect cultured epithelial cells

To characterize the infectivity of *C. jejuni* after inactivation of *flhF*, the ability of the strains to invade cultured epithelial cells was assessed. As shown in Fig. 4, inactivation of *flhF* significantly influenced the ability of *C. jejuni* to infect intestinal epithelial cells. Despite the effort made to minimize motility-dependent effects via centrifuging cells, the relative percentage of the *flhF* mutant strain to adhere and invade Caco-2 cells was 52 and 27%, respectively, compared to the NCTC 11168 strain. Complementation with *flhF* restored this defect in the mutant, and the infection ability of the complemented strain was nearly same as that observed for the wild-type strain (129 and 97% for adherence and invasion, respectively). These results suggest that the adherence to the epithelium and the subsequent invasion during *C. jejuni* infection is hampered by the inactivation of *flhF*.

The autoagglutination and biofilm-formation abilities of *C. jejuni* are decreased by the inactivation of *flhF*

The AAG ability of *C. jejuni* strains was compared after they were statically incubated for a short (3, 6, and 9 h) and long (21, 24, and 27 h) time spans (Fig. 5a). The AAG phenotype of each tested strain became more prominent over time. During the first 6 h, no significant difference in the AAG ability was observed between the *C. jejuni* NCTC 11168 and *flhF* mutant strains. The influence of *flhF* on *C. jejuni* AAG became noticeable after the strains were incubated for 9 h. The measured OD600 of *C. jejuni* NCTC 11168 was significantly lower than that of the *flhF* mutant strain ($P < 0.05$), and this effect increased over time, as the observed difference was more significant at 21–27 h ($P < 0.01$).
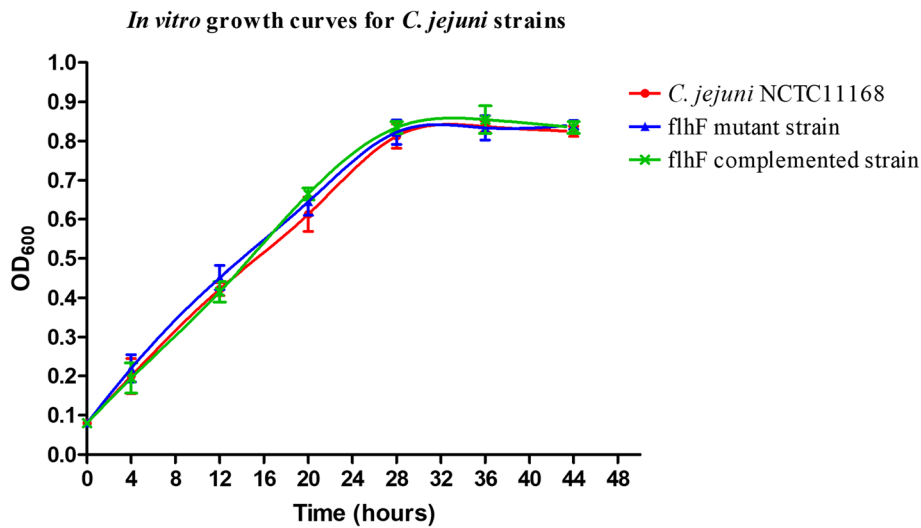
Ren et al. BMC Microbiology   (2018) 18:149

Page 6 of 11

Fig. 3 Growth curves of C. jejuni strains. C. jejuni NCTC 11168 and its derivatives were initially adjusted to an optical density at 600 nm (OD600) of 0.08, after which they were grown in MH broth under microaerophilic conditions with shaking at 42 °C, with 200 μl of samples were withdrawn at designated time point to examine growth. The wild-type strain (red circle), the flhF mutant strain (blue triangle) and the flhF complemented strain (green cross) are represented by each sample. The data are presented as the means ± standard deviation (SD) from three independent experiments

The biofilms formed by the strains were quantified after being statically cultured for 48–96 h. As shown in Fig. 5b, the biofilms formed for each strain continuously increased over time, but the biofilm-forming potential was different between the strains. The *flhF* mutant strain showed a defect in biofilm formation, as the OD570 values of the tested strains across all assayed time points were significantly decreased compared to those observed for the NCTC 11168 strain ($P < 0.01$).

The biofilm-formation defect was partially restored after complementation of *flhF*, the AAG ability was also increased somewhat in the complemented strain. The detected values for the biofilms formed and AAG ability of the complemented strain were all higher than the mutant at all the assayed time points, although these values did not reach the level of the wild-type strain.

flhF is continuously upregulated during *C. jejuni* infection and influences the transcription of other infection-related genes

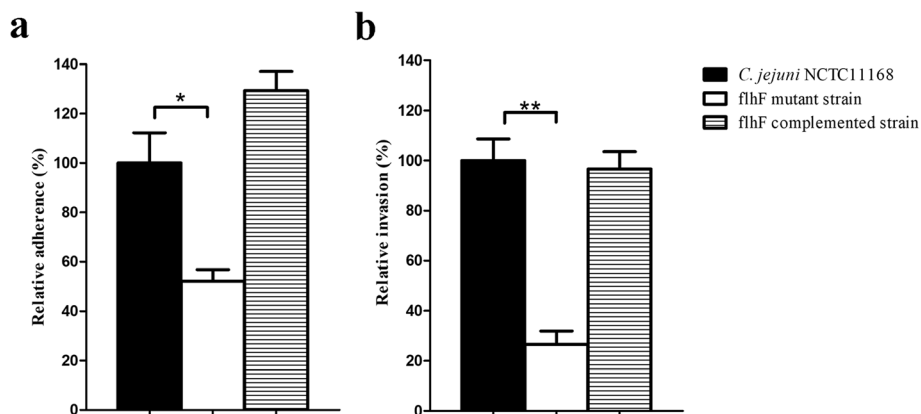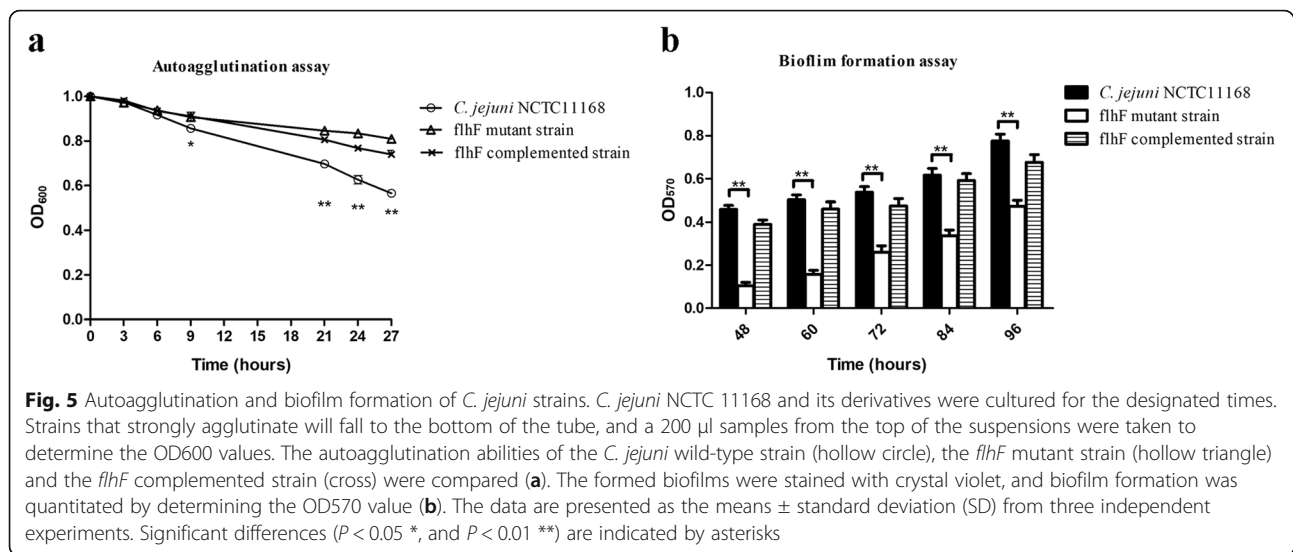To analyze whether *flhF* is associated with *C. jejuni* infection in vivo, the NCTC 11168 strain was cultured in

Fig. 4 The ability of C. jejuni strains to adhere to and invade Caco-2 cells. Cells were infected with C. jejuni NCTC 11168 and its derivatives for 2 h. A comparison of the levels of adherence is presented as the values of C. jejuni mutant and complemented strains relative to that of the wild-type strain, which was set at 100% (**a**). Gentamicin was added to another plate of cells and incubated for another 1 h to enumerate the invaded bacteria (**b**). The data are presented as the means ± standard deviation (SD) from three independent experiments. Significant differences ($P < 0.05$ *, and $P < 0.01$ **) are indicated by asterisks

Fig. 5 Autoagglutination and biofilm formation of *C. jejuni* strains. *C. jejuni* NCTC 11168 and its derivatives were cultured for the designated times. Strains that strongly agglutinate will fall to the bottom of the tube, and a 200 μl samples from the top of the suspensions were taken to determine the OD600 values. The autoagglutination abilities of the *C. jejuni* wild-type strain (hollow circle), the *flhF* mutant strain (hollow triangle) and the *flhF* complemented strain (cross) were compared (**a**). The formed biofilms were stained with crystal violet, and biofilm formation was quantitated by determining the OD570 value (**b**). The data are presented as the means ± standard deviation (SD) from three independent experiments. Significant differences ($P < 0.05$ *, and $P < 0.01$ **) are indicated by asterisks

vitro and used to infect Caco-2 cell for 4, 8, 16 or 24 h, respectively. The RNA was extracted from samples at each time point, and a qRT-PCR assay was performed to compare the relative expression of *flhF* at the different time points. Compared to the in vitro groups, *flhF* was observed to be upregulated during the infection process at all the assayed time points. The observed fold-changes in expression were all over 3, and specifically for the 4, 8, 16 and 24 h time points were increased by 3.19-, 5.10-, 3.98- and 4.74-fold, respectively (Fig. 6a). This result suggests that *flhF* is a virulence-associated gene that may have a continuous role during *C. jejuni* infection.

The genes not directly involved in flagellar assembly but that were reported to influence *C. jejuni* adhesion, invasion and colonization were summarized (Additional file 1: Table S2) and their relative expression after inactivation of *flhF* was analyzed using our previous microarray data. As shown in Fig. 6b, both the colonization-(40%, 20/50) and adhesion/invasion- (50%, 25/50) related genes were downregulated after the inactivation of *flhF*. The average influence on adhesion/invasion was slightly higher than that observed for colonization. Some genes that could influence both the adhesion/invasion and colonization ability of *C. jejuni* were identified (the overlapping genes are presented in the middle of the heatmap), and their average transcription was observed to be decreased more than that of the other two groups. Some genes were selected for further qRT-PCR verification. In addition to *cadF* and *docC*, which were slightly upregulated in the qRT-PCR assay (Fig. 6c), all the other assayed genes showed the same trends as the microarray, where the transcription of *fedA*, *fedB*, *fedC* and *fedD* (Feds) were apparently affected. Thus, besides the major role *flhF* plays in the flagellar system, it may also affect

C. jejuni infection in other ways, but the underlying mechanism still requires further investigation.

Discussion

The efficient establishment of colonization is a prerequisite for *C. jejuni* pathogenesis. It was originally hypothesized that motility plays an essential role in *C. jejuni* colonization, as only motile strains could be recovered from human volunteers [42]. Subsequent studies revealed that flagella are an important colonization factor, as intact and motile flagella are required for efficient colonization [43]. The flagellar filament accounts for the largest proportion of the synthesized flagella and was the earliest studied flagellar substructure. The *C. jejuni* flagellar filament is composed of two almost identical flagellin proteins. Flagellin A is predominantly responsible for the intact and fully motile flagellation phenotype and has a specific contribution to *C. jejuni* colonization regardless of the effects observed by a defect in motility or inactivation of *flaB* [44]. Although Wassenaar et al. suggested that flagellin A, rather than motility, is essential for optimal colonization [44]. However, the role of motility cannot be ignored, as the mutation of *pflA*, which results in nonmotile bacteria with paralyzed flagella, also influences *C. jejuni* colonization [16].

Subsequent studies focused on the role of other flagellar elements. For the flagellar biosynthesis regulators RpoN (σ54) and FliA (σ28), mutation of either of these regulators resulted in an apparent attenuation for caecal colonization in chicks, but the loss of *fliA* had a more obvious effect than that of *rpoN*, despite its lesser influence on *C. jejuni* motility [18]. FlgR is a σ54-dependent response regulator that was observed to decrease the *C. jejuni* colonization ability by 10- to 500-fold in chicks [45]. Although inactivation of the flagellar motor protein
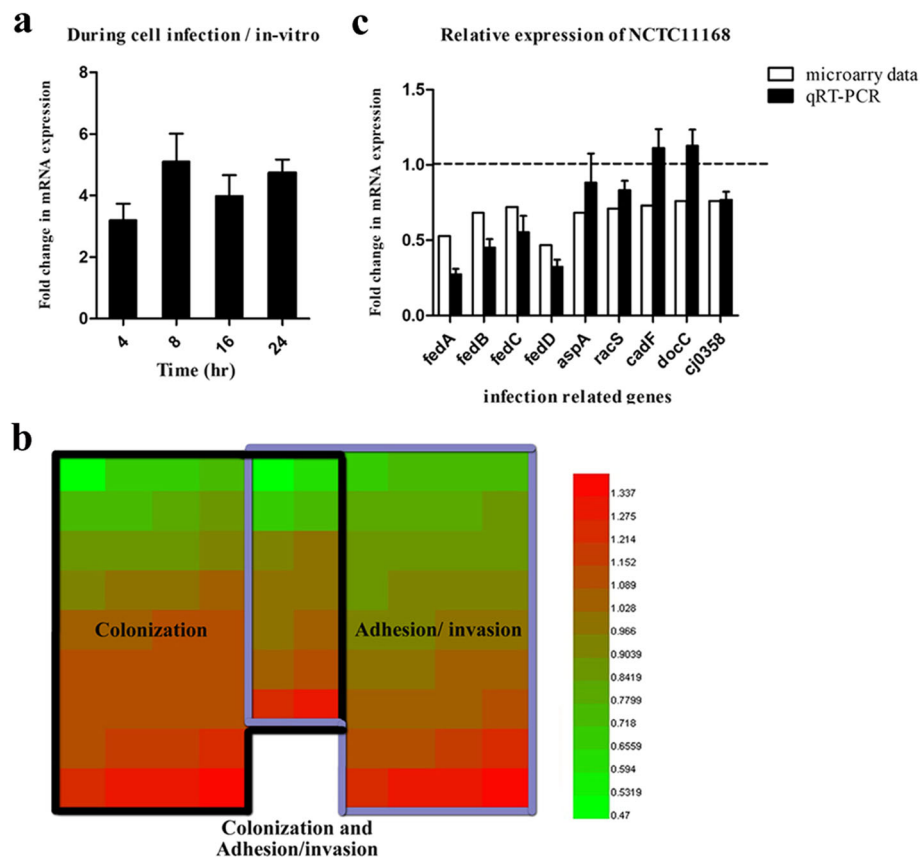
Fig. 6 Analysis of relative mRNA expression. *C. jejuni* NCTC 11168 was grown in vitro and in Caco-2 cells. The *flhF* gene was observed to be continuously upregulated during the Caco-2 cell infection process. Each column represents the relative mRNA expression of *flhF* during the cell infection process to the in vitro group. The data are presented as the means ± standard deviation (SD) from three independent experiments (**a**). Heatmap presentation of relative mRNA expression of other infection-related genes not directly involved in flagellar assembly. Red and green in the heatmap represent gene upregulation and downregulation, respectively, in the *flhF* mutant relative to the wild-type strain, with more saturated colors representing a greater difference as indicated in the bar (**b**). Some representatives were selected for further qRT-PCR verification. Each column represents the relative mRNA expression of the *flhF* mutant strain relative to the *C. jejuni* NCTC 11168 strain. The data are presented as the means ± standard deviation (SD) from three independent experiments (**c**)

MotAB caused *C. jejuni* to be nonmotile, these cells expressed a full-length flagellum yet completely lost the ability to colonize chicks [24, 46]. FlgK is a flagellar hook-associated protein, and the colonization of an *flgK* mutant was five orders of magnitude lower than that observed for wild-type strain in chicks [18]. The flagella secreted *Campylobacter* invasion antigen (Cia) proteins also play a role in colonization. Mutations in CiaI and CiaB lead to a defect in chick colonization compared to the wild-type strain [15, 22, 25]. Furthermore, genes associated with the flagellin O-linked glycosylation system also affect *C. jejuni* colonization and may be mediated by structural and surface changes in the glycan when interacting with the host [16, 23].

Although evaluation of bacterial colonization ability between studies may be difficult due to the diverse methods used by various laboratories, when assayed using the same method the flagellar elements were

shown to truly have a various contributions to *C. jejuni* colonization [16]. Thus, the role of FlhF on *C. jejuni* colonization was systematically analyzed in this study. *C. jejuni* is more commonly considered a commensal intestinal microbiota in chickens, where it has a persistent presence in the gut but does not lead to pathology [7]. The chick colonization assay was first performed using both the reference strain NCTC 11168 and an isolate from monkey diarrhea. Different *C. jejuni* strains shows diverse infectivity, and the colonization level of the NCTC 11168 strain was not ideal compared to that of the fecal isolate, which may be due to it being a poor colonizer [9]. In our study, inactivation of *flhF* in NCTC 11168 and the isolate both resulted in significantly decreased *C. jejuni* colonization at 1 and 7 days post inoculation, indicating that the commensal colonization of *C. jejuni* was severely hampered by the loss of *flhF*.

In contrast to chicks, *C. jejuni* infection in humans often leads to a series of illnesses, but the underlying mechanism is not fully understood [47]. To understand whether the importance of *flhF* on commensal colonization was consistent in mammals, mice were used for further assays. The reference strain NCTC 11168 failed to colonize the mice in our assay. Similar findings were also reported in a previous study, where NCTC 11168 was observed to colonize mice at very low efficiency, although after this strain was reisolated from mice it could then colonize mice with a high efficiency [48]. Thus, this phenomenon may be caused by the subculture of this genome-sequenced strain in the laboratory or the need for genetic adaptations in the intestinal tracts to promote its growth [48, 49]. The *C. jejuni* isolate used in our study could stably colonize the assayed mice. Compared to the chicks, the initial establishment of colonization in mice was not severely influenced by the inactivation of *flhF*, which may be due to the diverse characteristics of *C. jejuni* with respect to infecting different hosts. At 7-days post inoculation, all of the isogeneic *flhF* mutants were cleared from the mice, indicating its important role in promoting mice colonization.

The possible factors that may contribute to the colonization defect were investigated. *C. jejuni* growth and metabolism were observed to not be influenced by the mutation of *flhF*, eliminating the possible effects of the *flhF* mutation on bacterial multiplication. Previous studies showed that the motility and flagellar biosynthesis of *C. jejuni* were severely affected by the inactivation of *flhF* [34, 35]. The flagella of *C. jejuni* are involved in not just motility [26], so some of the flagella-associated phenotypes which might play a role in the bacterial colonization were subsequently analyzed. Adherence to the epithelium helps bacteria resist intestinal peristalsis and expulsion [17], and the subsequent invasion is also important. These factors are key determinants for *C. jejuni* colonization [50]. The potential infectivity of the assayed *C. jejuni* strains on Caco-2 cells was assessed. Despite efforts to minimize the influence caused by motility, both the adhered and invaded bacteria were observed to be significantly decreased after inactivation of *flhF*, indicating that the colonization was hampered at this step. Autoagglutination is the basis of bacterial aggregation, which is involved in biofilm formation [51]. Biofilm formation is essential for both environmental survival and successful infections [52, 53], and this mode of bacterial growth is also required for *C. jejuni* colonization [53]. The autoagglutination and biofilm-formation abilities of the *C. jejuni flhF* mutant strain were observed to be decreased in our study, contributing to the observed colonization defect of this strain. The complementation of *flhF* completely or partially restored the defects of adhesion, invasion and biofilm-formation abilities of the *flhF* mutant strain. However, limited restoration of the AAG ability was observed. Previous studies showed that the presence of the flagellin and its glycosylation modification is crucial for *C. jejuni* AAG [51]. We has observed the intact flagellin in the *flhF* complemented strain in our previous study [34], so flagellin glycosylation may not be fully restored after complementation of *flhF*.

In addition to the above mentioned phenotypic changes, the mechanism by which *flhF* affects *C. jejuni* colonization was also explored at the gene expression level. Although *flhF* is a class I flagellar gene that is stably expressed both in vitro and in vivo [34], when the two sets of expression data were compared, we observed that *flhF* is continuously upregulated during the infection process. This observation suggests a close relationship between this gene and *C. jejuni* infectivity, maybe an intact flagellum is required to maintain the secretion of virulence proteins during the infection process [6], or FlhF may function continuously to regulate other virulence factors in an unclarified-method. Inactivation of *flhF* globally affects flagellar gene transcription [34]. Unlike many other flagellated species whose flagellar genes are clustered into operons, the flagellar genes in *C. jejuni* are scattered throughout the genome [34, 54]. Many of these genes are grouped into operons that are involved in other pathways [54], and some genes are also co-expressed with flagellar genes [15]. Thus, we were interested in determining whether *flhF* could affect other infective factors not directly involved in flagellar assembly in this bacterium. Some representative genes were identified based on the literature, and using our previously obtained microarray data [34]. We observed that 45% (39/86) of the colonization and adhesion/invasion related genes were downregulated, among which the expression of flagellar coexpressed determinants (Feds) was apparently affected. This phenomenon may be caused by the Feds genes that belong to the flagellar σ28 regulon [15], which are significantly affected by the inactivation of *flhF* [34].

In this study, we systematically analyzed the influence of FlhF on *C. jejuni* colonization and the possible mechanisms that may contribute to this defect, including the associated virulence phenotypes and a preliminary exploration of changes at the gene expression level. The pathogenesis of *C. jejuni* is not yet well elucidated compared to other analogous species that can also cause diarrheal diseases. *C. jejuni* lacks many of the classic virulence factors [1, 3, 6]. It is generally believed that *C. jejuni* has a regulatory mechanism that co-regulates its virulence. For example, the polar flagella are not only required for motility but also functions as a type III secretion system (T3SS) [26, 54]. The *flhF* gene is continuously upregulated during the infection process and

influences the expression of other infection-related factors, which indicates a close relationship between this gene and *C. jejuni* infectivity. It is unknown whether this is an isolated case or a common phenomenon for other flagellar genes or whether other aspects related to *C. jejuni* pathogenesis are affected. These questions still need to be further investigated, which will provide insights into the role of this gene as well as the mechanism of the co-regulation of virulence of *C. jejuni* flagella.

Conclusion

In this study, the influence of *flhF* on *C. jejuni* colonization was systematically evaluated. Inactivation of *flhF* resulted in severe defects in the commensal colonization of chicks and resulted in a complete loss in the ability of this bacterium to continuously colonize mice. A combination of phenotypic and genetic characterizations revealed that there is a decreased ability of *flhF* mutant strain to adhere and invade epithelial cells, and autoagglutination and biofilm formation, which may contribute to the colonization defect. The *flhF* gene was observed to be continuously upregulated during the infection process and affects the transcription of other infection-related genes that are not directly involved in flagellar assembly, such as the flagellar coexpressed determinants (Feds). This result makes sense, but their definite relationship with *flhF* and the underlying mechanisms that influence *C. jejuni* pathogenesis still requires further investigation.

Additional file

Additional file 1: Figure S1. Identification of the *C. jejuni* isolate. **Table S1.** Primers used in this study. **Table S2.** The relative mRNA expression of other infection-related genes in the microarray assay. **Table S3.** The biochemical characteristics of *C. jejuni* strains. (DOCX 243 kb)

Abbreviations
AAG: Autoagglutination; ATCC: American Type Cell Culture; CDT: Cytolethal distending toxin; CFU: Colony-forming units; Cia: *Campylobacter* invasion antigen; DMEM: Dulbecco's minimal essential medium; FBS: Fetal bovine serum; GEO: Gene Expression Omnibus; MH: Mueller-Hinton; NH: Neisseria-Haemophilus; OD: Optical density; PBS: Phosphate buffered saline; qRT-PCR: quantitative real-time polymerase chain reaction; SD: Standard deviation; T3SS: Type III secretion system

Authors' contributions
JH, XJ conceived and designed the experiments. FR, XL, HT, QJ and XY performed the experiments. LF, PH, YT and QL performed data analysis and interpretation of the results. FR, JH, YT drafted the manuscript. All authors have read and approved the final manuscript.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
[1]Jiangsu Key Lab of Zoonosis/Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, 48 East Wenhui Road, Yangzhou 225009, Jiangsu, China. [2]Key Laboratory of Prevention and Control of Biological Hazard Factors (Animal Origin) for Agrifood Safety and Quality, Ministry of Agriculture of China, Yangzhou 225009, Jiangsu, China. [3]Joint International Research Laboratory of Agriculture and Agri-product Safety, Ministry of Education of China, Yangzhou 225009, Jiangsu, China.

References
1. Du X, Wang N, Ren F, Tang H, Jiao X, Huang J. *cj0371*: A Novel Virulence-Associated Gene of *Campylobacter jejuni*. Front Microbiol. 2016;7:1094.
2. Zang XQ, Tang HY, Jiao XN, Huang JL. Can a visual loop-mediated isothermal amplification assay stand out in different detection methods when monitoring *Campylobacter jejuni* from diverse sources of samples? Food Control. 2017;75:220–7.
3. Dasti JI, Tareen AM, Lugert R, Zautner AE, Gross U. *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. Int J Med Microbiol. 2010;300(4):205–11.
4. Young KT, Davis LM, Dirita VJ. *Campylobacter jejuni*: molecular biology and pathogenesis. Nat Rev Microbiol. 2007;5(9):665–79.
5. McCarthy ND, Gillespie IA, Lawson AJ, Richardson J, Neal KR, Hawtin PR, Maiden MC, O'Brien SJ. Molecular epidemiology of human *Campylobacter jejuni* shows association between seasonal and international patterns of disease. Epidemiol Infect. 2012;140(12):2247–55.
6. Burnham PM, Hendrixson DR. *Campylobacter jejuni*: collective components promoting a successful enteric lifestyle. Nat Rev Microbiol. 2018;16(9):551–65.
7. Gao B, Vorwerk H, Huber C, Lara-Tejero M, Mohr J, Goodman AL, Eisenreich W, Galan JE, Hofreuter D. Metabolic and fitness determinants for in vitro growth and intestinal colonization of the bacterial pathogen *Campylobacter jejuni*. PLoS Biol. 2017;15(5):e2001390.
8. Silva J, Leite D, Fernandes M, Mena C, Gibbs PA, Teixeira P. *Campylobacter* spp. as a Foodborne Pathogen: A Review. Front Microbiol. 2011;2:200.
9. Jones MA, Marston KL, Woodall CA, Maskell DJ, Linton D, Karlyshev AV, Dorrell N, Wren BW, Barrow PA. Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract. Infect Immun. 2004;72(7):3769–76.

Ren *et al. BMC Microbiology*   (2018) 18:149

Page 11 of 11

10. Velayudhan J, Jones MA, Barrow PA, Kelly DJ. L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. Infect Immun. 2004;72(1):260–8.

11. Bingham-Ramos LK, Hendrixson DR. Characterization of two putative cytochrome c peroxidases of *Campylobacter jejuni* involved in promoting commensal colonization of poultry. Infect Immun. 2008;76(3):1105–14.

12. Davis LM, Kakuda T, DiRita VJ. A *Campylobacter jejuni* znuA orthologue is essential for growth in low-zinc environments and chick colonization. J Bacteriol. 2009;191(5):1631–40.

13. Flanagan RC, Neal-McKinney JM, Dhillon AS, Miller WG, Konkel ME. Examination of *Campylobacter jejuni* putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization. Infect Immun. 2009;77(6):2399–407.

14. Ribardo DA, Hendrixson DR. Analysis of the LIV system of *Campylobacter jejuni* reveals alternative roles for LivJ and LivK in commensalism beyond branched-chain amino acid transport. J Bacteriol. 2011;193(22):6233–43.

15. Barrero-Tobon AM, Hendrixson DR. Identification and analysis of flagellar coexpressed determinants (Feds) of *Campylobacter jejuni* involved in colonization. Mol Microbiol. 2012;84(2):352–69.

16. Hendrixson DR, DiRita VJ. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol. 2004;52(2):471–84.

17. Jeon B, Muraoka WT, Zhang Q. Advances in *Campylobacter* biology and implications for biotechnological applications. Microb Biotechnol. 2010;3(3):242–58.

18. Fernando U, Biswas D, Allan B, Willson P, Potter AA. Influence of *Campylobacter jejuni fliA, rpoN* and *flgK* genes on colonization of the chicken gut. Int J Food Microbiol. 2007;118(2):194–200.

19. Baldvinsson SB, Sorensen MC, Vegge CS, Clokie MR, Brondsted L. *Campylobacter jejuni* motility is required for infection of the flagellotropic bacteriophage F341. Appl Environ Microbiol. 2014;80(22):7096–106.

20. Barrero-Tobon AM, Hendrixson DR. Flagellar biosynthesis exerts temporal regulation of secretion of specific *Campylobacter jejuni* colonization and virulence determinants. Mol Microbiol. 2014;93(5):957–74.

21. Sommerlad SM, Hendrixson DR. Analysis of the roles of FlgP and FlgQ in flagellar motility of *Campylobacter jejuni*. J Bacteriol. 2007;189(1):179–86.

22. Biswas D, Fernando UM, Reiman CD, Willson PJ, Townsend HG, Potter AA, Allan BJ. Correlation between in vitro secretion of virulence-associated proteins of *Campylobacter jejuni* and colonization of chickens. Curr Microbiol. 2007;54(3):207–12.

23. Howard SL, Jagannathan A, Soo EC, Hui JP, Aubry AJ, Ahmed I, Karlyshev A, Kelly JF, Jones MA, Stevens MP, et al. *Campylobacter jejuni* glycosylation island important in cell charge, legionaminic acid biosynthesis, and colonization of chickens. Infect Immun. 2009;77(6):2544–56.

24. Chang C, Miller JF. *Campylobacter jejuni* colonization of mice with limited enteric flora. Infect Immun. 2006;74(9):5261–71.

25. Ziprin RL, Young CR, Byrd JA, Stanker LH, Hume ME, Gray SA, Kim BJ, Konkel ME. Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. Avian Dis. 2001;45(3):549–57.

26. Guerry P. *Campylobacter* flagella: not just for motility. Trends Microbiol. 2007;15(10):456–61.

27. Pandza S, Baetens M, Park CH, Au T, Keyhan M, Matin A. The G-protein FlhF has a role in polar flagellar placement and general stress response induction in *Pseudomonas putida*. Mol Microbiol. 2000;36(2):414–23.

28. Kusumoto A, Kamisaka K, Yakushi T, Terashima H, Shinohara A, Homma M. Regulation of polar flagellar number by the *flhF* and *flhG* genes in *Vibrio alginolyticus*. J Biochem. 2006;139(1):113–21.

29. Salvetti S, Ghelardi E, Celandroni F, Ceragiol M, Gianness F, Senes S. FlhF, a signal recognition particle-like GTPase, is involved in the regulation of flagellar arrangement, motility behaviour and protein secretion in *Bacillus cereus*. Microbiology. 2007;153:2541–52.

30. Kusumoto A, Shinohara A, Terashima H, Kojima S, Yakushi T, Homma M. Collaboration of FlhF and FlhG to regulate polar-flagella number and localization in *Vibrio alginolyticus*. Microbiology. 2008;154:1390–9.

31. Kusumoto A, Nishioka N, Kojima S, Homma M. Mutational analysis of the GTP-binding motif of FlhF which regulates the number and placement of the polar flagellum in *Vibrio alginolyticus*. J Biochem. 2009;146(5):643–50.

32. Schniederberend M, Abdurachim K, Murray TS, Kazmierczak BI. The GTPase activity of FlhF is dispensable for flagellar localization, but not motility, in Pseudomonas aeruginosa. J Bacteriol. 2013;195(5):1051–60.

33. Kazmierczak BI, Hendrixson DR. Spatial and numerical regulation of flagellar biosynthesis in polarly flagellated bacteria. Mol Microbiol. 2013;88(4):655–63.

34. Ren F, Lei T, Song Z, Yu T, Li Q, Huang J, Jiao XA. Could FlhF be a key element that controls *Campylobacter jejuni* flagella biosynthesis in the initial assembly stage? Microbiol Res. 2018;207:240–8.

35. Balaban M, Joslin SN, Hendrixson DR. FlhF and its GTPase activity are required for distinct processes in flagellar gene regulation and biosynthesis in *Campylobacter jejuni*. J Bacteriol. 2009;191(21):6602–11.

36. Huang JL, Xu HY, Bao GY, Zhou XH, Ji DJ, Zhang G, Liu PH, Jiang F, Pan ZM, Liu XF, et al. Epidemiological surveillance of *Campylobacter jejuni* in chicken, dairy cattle and diarrhoea patients. Epidemiol Infect. 2009;137(8):1111–20.

37. Mansfield LS, Bell JA, Wilson DL, Murphy AJ, Elsheikha HM, Rathinam VA, Fierro BR, Linz JE, Young VB. C57BL/6 and congenic interleukin-10-deficient mice can serve as models of *Campylobacter jejuni* colonization and enteritis. Infect Immun. 2007;75(3):1099–115.

38. Valenza G, Ruoff C, Vogel U, Frosch M, Abele-Horn M. Microbiological evaluation of the new VITEK 2 Neisseria-Haemophilus identification card. J Clin Microbiol. 2007;45(11):3493–7.

39. Christensen JE, Pacheco SA, Konkel ME. Identification of a *Campylobacter jejuni*-secreted protein required for maximal invasion of host cells. Mol Microbiol. 2009;73(4):650–62.

40. Naito M, Frirdich E, Fields JA, Pryjma M, Li J, Cameron A, Gilbert M, Thompson SA, Gaynor EC. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. J Bacteriol. 2010;192(8):2182–92.

41. Deng W, Wang Y, Liu Z, Cheng H, Xue Y. HemI: a toolkit for illustrating heatmaps. PLoS One. 2014;9(11):e111988.

42. Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis. 1988;157(3):472–9.

43. Nachamkin I, Yang XH, Stern NJ. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. Appl Environ Microbiol. 1993;59(5):1269–73.

44. Wassenaar TM, van der Zeijst BA, Ayling R, Newell DG. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. J Gen Microbiol. 1993;139(Pt 6):1171–5.

45. Hendrixson DR. A phase-variable mechanism controlling the *Campylobacter jejuni* FlgR response regulator influences commensalism. Mol Microbiol. 2006;61(6):1646–59.

46. Mertins S, Allan BJ, Townsend HG, Koster W, Potter AA. Role of motAB in adherence and internalization in polarized Caco-2 cells and in cecal colonization of *Campylobacter jejuni*. Avian Dis. 2013;57(1):116–22.

47. Hermans D, Pasmans F, Heyndrickx M, Van Immerseel F, Martel A, Van Deun K, Haesebrouck F. A tolerogenic mucosal immune response leads to persistent *Campylobacter jejuni* colonization in the chicken gut. Crit Rev Microbiol. 2012;38(1):17–29.

48. Wilson DL, Rathinam VAK, Qi WH, Wick LM, Landgraf J, Bell JA, Plovanich-Jones A, Parrish J, Finley RL, Mansfield LS, et al. Genetic diversity in Campylobacter jejuni is associated with differential colonization of broiler chickens and C57BL/6J IL10-deficient mice. Microbiology. 2010;156:2046–57.

49. Gaynor EC, Cawthraw S, Manning G, MacKichan JK, Falkow S, Newell DG. The genome-sequenced variant of Campylobacter jejuni NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. J Bacteriol. 2004;186(2):503–17.

50. Hermans D, Van Deun K, Martel A, Van Immerseel F, Messens W, Heyndrickx M, Haesebrouck F, Pasmans F. Colonization factors of Campylobacter jejuni in the chicken gut. Vet Res. 2011;42:82.

51. Guerry P, Ewing CP, Schirm M, Lorenzo M, Kelly J, Pattarini D, Majam G, Thibault P, Logan S. Changes in flagellin glycosylation affect Campylobacter autoagglutination and virulence. Mol Microbiol. 2006;60(2):299–311.

52. Kalmokoff M, Lanthier P, Tremblay TL, Foss M, Lau PC, Sanders G, Austin J, Kelly J, Szymanski CM. Proteomic analysis of Campylobacter jejuni 11168 biofilms reveals a role for the motility complex in biofilm formation. J Bacteriol. 2006;188(12):4312–20.

53. Pascoe B, Lappin-Scott H, Sheppard SK, Asakura H. Does Biofilm Formation Aid Colonization and Infection in Campylobacter? In: Sheppard SK, Meric GUK, editors. Campylobacter Ecology and Evolution. Norwich: Caister Academic Press; 2014. p. 177–88. https://researchportal.bath.ac.uk/en/publications/doesbiofilm-formation-aid-colonization-and-infection-in-campylob

54. Hendrixson DR. Regulation of flagellar gene expression. In: Nachamkin I, Szymanski CM, Blaser MJ, editors. Campylobacter. 3rd ed. Washington, D.C.: ASM Press; 2008. p. 545–59.