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Could FlhF be a key element that controls *Campylobacter jejuni* flagella biosynthesis in the initial assembly stage?

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ABSTRACT

The disordered arrangement of flagella biosynthetic genes, combined with a simplified regulatory mechanism, has made elucidating the process of *Campylobacter jejuni* flagellation difficult. FlhF is a recently identified element that controls the assembly of the flagella, although its function mechanism and regulatory preference are not well defined at present. In this study, we found that inactivation of FlhF caused the transcription of most flagella genes down-regulated. The importance of FlhF was systematically evaluated by analyzing changes in the transcription profiles between wild-type and *flhF* mutant strains, which showed that FlhF affects late flagella genes obviously. FlhF is constitutively expressed during *C. jejuni* growth, demonstrating that it is a class I flagella element that participates in early flagella assembly. In addition, the early flagella component FlhB was not localized to the cell pole in the *flhF* mutant. Thus, flagella assembly was impeded at the initial stage. We propose a model in which FlhF helps target the early flagella components to the cell pole, functioning prior to the formation of the flagella export apparatus, and thus places FlhF at the top of the flagella regulatory cascade hierarchy. Inactivation of FlhF impeded flagella assembly at the initial stage and decreased transcription of flagella genes through a feed-back control mechanism, leading to FlhF having a significant influence on the expression of late flagella components and resulting in the aflagellate *C. jejuni* phenotype. Our present study has uncovered how FlhF influences *C. jejuni* flagella biosynthesis, which will be helpful in understanding the *C. jejuni* flagella biosynthetic pathway and bacterial flagellation in general.

1. Introduction

The flagellum is the major motility organelle of many bacteria. Traditionally, it is regarded to be an important virulence factor since the synthesized flagellum is not only required for bacterial motility and chemotaxis and helps the bacterium move towards favorable environments and away from hostile surroundings (Lux and Shi, 2004), but also assists in bacterial adherence, host cell invasion and subsequent colonization (Josenhans and Suerbaum, 2002; Haiko and Westerlund-Wikstrom, 2013). *Campylobacter jejuni* is a microaerophilic, Gram-negative bacterium that causes bacterial gastroenteritis worldwide (Dasti et al., 2010; Silva et al., 2011; Yeh et al., 2013). *C. jejuni* produces a single flagellum at one or both poles. The role of the flagellum in *C. jejuni* is multifunctional since the polar flagella not only confers motility, which is necessary for infection (Van Vliet and Ketley, 2001), but also participates in secreting virulence proteins, avoidance of the innate immune response, mediating autoagglutination (AAG), and microcolony and biofilm formation (Guerry, 2007; Barrero-Tobon and Hendrixson, 2014).

Considering the significance of the flagellum, a comprehensive understanding of its function and assembly is necessary. The assembly of the flagella is a complex, ordered and is a high energy expenditure process, as more than 50 genes are predicted to be involved that are transcribed under precise control (Hendrixson and Dirita, 2003). The flagellation patterns among bacteria primarily includes 2 types (peritrichous and polar flagellation) (Kazmierczak and Hendrixson, 2013), although the flagella system of some peritrichous species (*Escherichia* and *Salmonella* species) are well characterized and have been used as

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paragons in subsequent research (Soutourina and Bertin, 2003), many questions remain regarding polar flagella systems (Gao et al., 2014). *C. jejuni* is a typical representative of a polarly flagellated bacteria, but compared with *Escherichia* and *Salmonella* species, the flagella genes of *C. jejuni* are scattered throughout the genome and exhibit little clustering (Wosten et al., 2004). Many of these genes are contained within the operons of other pathways (Hendrixson, 2008). In addition, an alternative sigma factor (σ54) was found to be important in the *C. jejuni* flagella gene transcription hierarchy, whereas the typical master regulator that initiates flagellar components transcription seems to be absent (Jagannathan et al., 2001; Soutourina and Bertin, 2003). The relatively complex gene arrangement, combined with a simplified regulatory mechanism, has made elucidating the process of *C. jejuni* flagella synthesis difficult. In addition, our understanding of this process remains rudimentary since the function of many flagella related genes are unknown or little-understood, especially regulatory candidates (Carrillo et al., 2004; Gao et al., 2014).

FlhF is a recently identified protein that regulates flagella biosynthesis and is mainly found in polarly flagellated bacteria. The inactivation of FlhF in various species leads to different phenotype (Pandza et al., 2000; Kusumoto et al., 2006; Salvetti et al., 2007; Kusumoto et al., 2008; Kusumoto et al., 2009; Schniederberend et al., 2013), and although it seems that FlhF is required for the spatial and numerical control of the synthesized flagella, how it functions is not well defined at present, and the intrinsic characteristics of FlhF have not formed an unified understanding (Kim et al., 2012; Kazmierczak and Hendrixson, 2013). For example, many studies of FlhF have focused on its influence on flagella gene transcription, however, the reported results are not always the same (Niehus et al., 2004; Correa et al., 2005; Murray and Kazmierczak, 2006; Balaban et al., 2009; Kim et al., 2012). And some other researchers also studied its role in determining the position of flagella substructure, considering that FlhF is a member of the signal recognition particle (SRP) associated GTPase family (Green et al., 2009; Guttenplan et al., 2013). Thus, the aim of this study was to gain insight into the role of FlhF with respect to the *C. jejuni* flagella system. The importance of FlhF for flagella generation was determined here, and its effect within the flagella hierarchy was systematically analyzed by analyzing the transcription profile of all the flagella genes. The expression kinetics of FlhF and its ability to recruit flagellar components to the cell pole were also assayed to further verify its function. Our collective results explain how the assembly of flagella in *C. jejuni* is blocked by the inactivation of FlhF.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

All bacterial strains and plasmids used in this study are listed in Table S1. *C. jejuni* NCTC11168 and its derivatives were routinely grown at 42 °C under microaerobic condition (5% O_2, 10% CO_2, and 85% N_2) 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). *E.coli* DH5α cells were grown on Luria-Bertani (LB) agar or in broth at 37 °C. Antibiotics for *C. jejuni* or *E. coli* growth were added to the medium when necessary at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml) or chloramphenicol (20 μg/ml). The plasmid pMD-19T (simple) (TaKaRa, Dalian, China) was used as cloning as well as a suicide vector in strain construction. The plasmid pRK2013 (Biomedal, Sevilla, Spain) is a helper plasmid used in triparental mating conjugations, while pRY107 and pUOA18 are *C. jejuni* shuttle vectors that were kindly supplied by Qijing Zhang (Iowa State University, Ames, USA).

2.2. Construction of C. jejuni mutant and complemented strains

The *C. jejuni* mutant was constructed by insertional mutagenesis as previously described (Akiba et al., 2006) and the primers used for strain construction are listed in Table S2. The target gene was amplified from the *C. jejuni* genome and was ligated into pMD-19T (simple) using conventional molecular genetic methods. Then a Kan^r cassette was amplified from pRY107 and inserted into this fragment to obtain the suicide plasmid. The suicide plasmid was electroporated into *C. jejuni* competent cells, and the resulting transformants were selected for on CCDA agar containing kanamycin 50 μg/ml. To complement the mutant, a constitutively active promoter region of the housekeeping gene *metK* was first introduced into the shuttle vector pUOA18 (Wosten et al., 1998; Miller et al., 2008). Then, the target gene was ligated immediately downstream of the promoter, generating a complementation plasmid via enzyme digestion and ligation. The plasmid was transferred into the mutant strain by triparental mating conjugation with the help of the plasmid pRK2013, by the method described by Miller et al. (2000). Colonies that appeared on CCDA agar containing chloramphenicol 20 μg/ml were picked and the resulting strains were confirmed by polymerase chain reaction (PCR).

2.3. Motility assay and transmission electron microscopy

The motility of the *C.jejuni* strains were compared as previously described (Gao et al., 2014). Bacteria were grown for 20 h prior to use, then each strain was suspended in MH broth and diluted to an optical density at 600 nm (OD600) of 0.8. Then, a sterilized inoculating needle was used to stab the bacteria into semisolid MH motility media containing 0.4% agar. The plates were incubated for 48 h and the motility phenotypes of the strains were assessed. For analyses of the presence of flagella on *C. jejuni* cells, bacteria were grown in MH broth at 30 rpm for 24 h, then 1 ml of culture was pelleted at 1000 × *g* for 1 min and cells were gently resuspended in phosphate buffered saline (PBS). Samples were subsequently used for microscopic observations. One drop of bacterial cell suspension was added on a copper grid for 10 min, the loaded grid was negatively stained with 1% (w/v) ammonium molybdate for 2 min and visualized with a Tecnai 12 transmission electron microscope (supplied by the Testing Center of Yangzhou University).

2.4. RNA isolation and quantitative real-time PCR

C. jejuni strains were grown for 20 h, then 1 ml of culture was centrifuged and the pelleted cells were lysed with lysozyme, from which total RNA was extracted using an RNeasy plus mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A total of 500 ng of RNA was used to synthesize cDNA using the RT reagent Kit (TaKaRa, Dalian, China), and the cDNA was then subjected to quantitative real-time PCR (qRT-PCR), using a FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics, GmbH, Germany) in an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qRT-PCR program used was as follows: 2 min at 50 °C, followed by 40 cycles of 30 s at 95 °C and 34 s at 60 °C. The specific primers used for qRT-PCR are listed in Table S2, with the *cj0402* gene *(glyA)* used as an endogenous control (Hu et al., 2013), and the relative expression of the target genes calculated using the 2-ΔΔCT method as previously described (Livak and Schmittgen, 2001).

2.5. Microarray

C. jejuni total RNA was extracted as described above. The quality of the RNA was assessed using an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US), and RIN ≥ 7 and 28S/18S ≥ 0.7 were considered usable. Custom-designed Agilent 15 k single color arrays were used for analyses of *C. jejuni* gene expression profiles, using three biological replicates for each sample. RNA amplification and