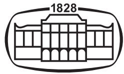

AKADÉMIAI KIADÓ

Motility related gene expression of *Campylobacter jejuni* NCTC 11168 derived from high viscous media

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ORIGINAL RESEARCH PAPER

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ABSTRACT

Flagellation is one of the major virulence factors of *Campylobacter jejuni* (*C. jejuni*), enabling bacterial cells to swarm in rather high viscous fluids. The aim of this study was to determine the impact of the surrounding viscosity on the expression of motility related genes of *C. jejuni*. Therefore, bacterial RNA was extracted from liquid cultures as well as from bacterial cells recovered from the edge and the center of a swarming halo from high viscous media. The expression pattern of selected flagellar and chemotaxis related genes was investigated by RT-PCR. Higher mRNA levels of class 1 and lower levels of class 2 and 3 flagellar assembly genes were detected in cells derived from the edge of a swarming halo than in cells from the center. This indicates different growth states at both locations within the swarming halo. Furthermore, higher mRNA levels for energy taxis and motor complex monomer genes were detected in high viscous media compared to liquid culture, indicating higher demand of energy if *C. jejuni* cells were cultivated in high viscous media. The impact of the surrounding viscosity should be considered in future studies regarding motility related questions.

KEYWORDS

C. jejuni, motility, flagellar assembly process, swarming, chemotaxis, qRT-PCR

INTRODUCTION

Campylobacter jejuni is one of the major foodborne pathogens world-wide, with 120,946 cases of illness reported to European Food Safety Authority (EFSA) in 2020 [1]. Motility is one of the factors needed for successful host colonization by *C. jejuni* [2].

The capability of *C. jejuni* to swarm in high viscous fluids depends on several factors. First, it has been hypothesized that the helical cell shape of *C. jejuni* contributes to “drilling” movement through mucus fluids in the gastrointestinal tract [3]. Second, flagellation is generally constructed at each pole which facilitate propeller motion to promote cell movement [4]. Third, proton flux passing through flagellar motors generates high torque to facilitate bacterial cells propulsion in viscous fluids [5]. The flagellar motor complex of *Campylobacter* is composed of a higher number and larger spatial arrangement of the stator complexes (MotAB) compared to those in *Escherichia coli* (*E. coli*), thereby generating high torque levels for flagellar rotation even in high viscous surroundings [6].

A flagellum could be divided in several structural parts, namely the motor complex, the rod, hook and the filament. To build a full flagellum a temporal coordinated expression of approximately 40–50 genes is required [7]. The flagellar assembly (FA) processing cascade has been divided into 3 classes [8, 9]. The class 1 genes of the flagellar apparatus mainly encode for the Type III Secretion System (T3SS), MS ring, and C ring which are regulated by σ^{70} [10]. Once T3SS, composed of FlhA, FlhB, FliP, FliQ and FliR, is initially formed, the MS ring proteins (FliF) and C ring proteins (FliG, FliM, and FliN) are recruited to surround the T3SS apparatus to form a “signal checkpoint”. The completed form of this checkpoint is sensed by the two-component sensor protein FlgS, which in turn activates the response

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regulator FlgR via phosphotransfer and thereby triggering the subsequent expression of σ^{54} dependent genes [10]. The proteins, encoded by σ^{54} dependent genes (class 2), are exported by the T3SS apparatus and construct rod, rings and hook orderly. Once the hook is completed, the anti-sigma factor FlgM is exported through the flagellum and the inhibited σ^{28} is released to regulate the class 3 flagellar genes expression. The respective proteins are synthesized and released through the flagellum apparatus to form the filament (FlaA and FlaB) and cap (FliD) [8, 11].

Rotation of the flagellum is driven by a motor-complex. The flagellar motor complex is composed of the stator complex (MotA and MotB) and the rotor (C-ring). The stator complex forms a proton flow channel to generate the torque needed for the rotation of the rotor [12], while the C-ring complex transmits the torque to the flagellum, resulting in propulsion of *C. jejuni* cells [4, 5]. Genes encoding for the C ring complex belong to the class 1 of the FA processing cascade, however, it is reported that the expression of the stator-complex genes *motA* and *motB* are independent of the FA processing cascade in *C. jejuni* [2]. The two stator monomers MotA and MotB facilitate bacterial cell swarming in the viscous environment [4], and the loading of them surrounding the rotor is highly dynamic [12].

Chemotaxis is crucial for directed motility [2, 13, 14]. The bacteria sense molecules in the environment around them and transduce signals by chemotaxis system to the down-stream flagellar rotation system. The methyl-accepting-domain containing chemoreceptor proteins (also called transducer-like proteins, TLP) of *C. jejuni* could be classified into three groups: Group A TLPs sense signals from the outer environment and Group C from the cytoplasm [14]. The only TLP member of Group B (CetA) builds a bipartite energy taxis system together with CetB [13]. CetB contains a PAS domain, which can sense light, oxygen, proton motive force and the redox state of compounds from electron transport system [15, 16]. CetA conveys the signal obtained from CetB to the core chemotaxis system to control bacterial flagella rotation. Tlp8, belonging to group C TLPs, was also reported to function as energy taxis sensor. *C. jejuni* is attracted by compounds involved in oxidative phosphorylation metabolism through energy taxis rather than chemotaxis as suggested by Vegge et al. [17]. CetAB navigates *C. jejuni* towards electron acceptors or donors, while Tlp8 navigates cells away from high redox potentials. By the combination of both systems *C. jejuni* can maintain an optimal energy and redox balance [14, 16].

The core signal transducing system is a two-component system comprising of the histidine autokinase CheA and the response regulator CheY. CheW functions as a coupling scaffold protein connecting TLPs and CheAY. CheV, a CheW-like protein with an additional response regulator motif, specifically exists in *Campylobacter*, and might be involved in chemoeffector adaptation [14]. The phosphorylation status of CheY direct the flagellar motor switch proteins FliM or FliM-FliN to counter-clockwise or clockwise rotation respectively.

Bacteria are usually studied as planktonic cells in liquid culture of low viscosity, however bacterial gene expression and behavior might be greatly affected by the surrounding viscosity [18]. Detailed descriptions of the gene expression pattern of *C. jejuni* swarming in high viscous media are still missing. In general, reports of bacterial RNA extraction from high viscous media remains limited. In this study, an effective method of RNA extraction from *C. jejuni* within high viscous media has been established. The motility dependent gene expression, derived from *C. jejuni* of the edge and the center of the swarming halo (SH) in high viscous media and from low viscous culture was determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions

C. jejuni NCTC 11168 were cultivated on Mueller-Hinton agar supplemented with 5% sheep blood (MHB; both Oxoid, Hampshire, UK). The plates were incubated at 37 °C under microaerobic conditions (5% O_2, 10% CO_2) generated by Anoxomat (Omni Life Science, Bremen, Germany) for 72 h. Overnight culture was obtained by culturing colonies from MHB plates in 5 mL of Brucella broth (BD, Heidelberg, Germany) and incubation at 37 °C under microaerobic conditions for 24 h to enter early stationary phase.

Swarming assay

The overnight culture was 1:10 diluted in Brucella broth to obtain a concentration of approximately 10^7 colony-forming units (CFU)/mL. 1 μL of the diluted culture was dropped on high viscous media, which was made up of Brucella broth and 0.4% agar. The plates were incubated at 37 °C for 24 h under microaerobic conditions before RNA extraction. The image of bacterial swarming halo in Brucella semisolid agar was captured under Vilber imaging system (Eberhardzell, Germany) with exposure time of 3s.

RNA extraction

For the RNA extraction of the cells derived from liquid culture (low viscous media), 10 mL of the overnight culture was centrifuged at 7,000*g for 5 min. The supernatant was discarded and the bacteria were lysed by the addition of 1 mL of QIAzol (Qiagen, Hilden, Germany). The solution was vortexed for 5 min and incubated at room temperature for 5 min prior to storage at −80 °C. For the RNA extraction of the cells derived from high viscous media, pieces of approximately 1 cm^3, including bacterial cells and surrounding agar, was cut out and collected in a sterile Falcon tube. The agar pieces derived from several plates were combined in one Falcon tube to obtain high concentration of bacterial cells. A comparable volume of QIAzol as the volume of the agar pieces was added into the Falcon tube. The solution was vortexed for 5 min and incubated at room temperature for 5 min prior to storage at −80 °C.

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The samples were thawed and mixed with 0.2 times volume of chloroform before vortexing for 10 s. The solution was centrifuged at 4 °C and 7,000*g for 15 min. The aqueous phase was collected and mixed with the same volume of 70% ethanol. The solution was vigorously vortexed and immediately loaded onto a RNeasy Midi column (Qiagen) for total RNA extraction according to the manufacturer's instructions. Briefly, samples were centrifuged at 5,000*g for 5 min and the flow through was discarded. Each column was washed once with 4 mL of Buffer RW1 and twice with 2.5 mL of RPE buffer. The RNA was eluted in 100 μL RNase-free water. To obtain a higher total RNA concentration, a second elution step was performed by the first eluate, as recommended by the instruction. The RNA concentration was measured by Nanodrop 2000 (Life Technologies, Darmstadt, Germany).

DNase treatment

Potential residual DNA was destroyed by DNase I (Life Technologies) treatment. Briefly, a total volume of 40 μL working solution was composed of 1 μg of RNA, 1 × reaction buffer with $MgCl_2$, 4 U DNase I and 40 U RiboLock RNase Inhibitor. The solution was incubated at 37 °C for 15 min. EDTA with a final concentration of 5 mM was subsequently added prior to heating at 65 °C for 10 min. The mixture was stored on ice to perform cDNA synthesis subsequently.

cDNA synthesis

The Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher, Vilnius, Lithuania) was used for reverse-transcription of RNA sample. Briefly, a total volume of 15 μL working solution containing 300 ng RNA, 5 μM Random Hexamer Primer and 0.5 mM dNTP Mix was incubated at 65 °C for 5 min. Subsequently 4 μL RT Buffer and 1 μL Maxima H Minus Enzyme Mix was added prior to a second incubation step at 25 °C for 10 min followed by 50 °C for 30 min, and a final heating step at 85 °C for 5 min. The cDNA samples were diluted 1:10 with RNase-free water. A negative control without the addition of Maxima H Minus Enzyme Mix was tested at the same time of cDNA synthesis.

Quantitative real-time PCR

The cDNA products were analyzed by qPCR assay using SsoFast™ EvaGreen Supermix (Bio-Rad, Munich, Germany). The primers listed in Table 1 were designed by previous researchers [19–26] or in this study and synthesized by Metabion International AG (Planegg, Germany). A final volume of 15 μL working mix included 1 × SsoFast Supermix, forward/reverse primers with proper concentrations (mentioned in Table 1) and 1 μL of cDNA. The 2-step-amplification program used was composed of an initial heating step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 15 s, and was finalized by the melting curve analysis. The qPCR assays were carried out by the CFX96™ Real-Time System (Bio-Rad). Ct values was obtained from CFX Manager software (Bio-Rad). ΔCt was calculated by normalization to *rpoA*. Expression changes between different conditions were calculated as fold change by $2^{-\Delta\Delta Ct}$ method [27]. Changes in the gene expression level >1.5-fold were considered as up-regulated, whereas <0.67-fold as down-regulated.

RESULTS

Flagellar associated gene expression pattern of *C. jejuni* swarming in high viscous media

The expression of selected genes belonging to the flagellar apparatus were investigated during cultivation in viscous media. After 24 h incubation a bacterial swarming halo (SH) was formed due to bacterial growth and swarming starting from the center spot (Fig. 1). Agar pieces, containing bacterial cells, were collected from the SH Center or SH Edge to extract RNA. The gene expression pattern of *C. jejuni* cells swarming in high viscous media was compared between the SH Center and the SH Edge (Fig. 2).

In total, the expression of 6 out of 18 flagellar assembly (FA) processing genes were up-regulated and of 9 genes were down-regulated by comparing the expression pattern of the SH Edge with the SH Center. The up-regulated genes belong to the class 1 of the FA process, which include the T3SS components *flhA*, *flhB*, the C ring component *fliM*, and the transcriptional regulators *rpoD* (σ^{70}), *rpoN* (σ^{54}) and *flgR* (two-component response regulator). The investigated genes which were down-regulated mostly belong to the class 2 genes. These include the rings and rod associated genes *flgI*, *flgH* and *flgG2*, the hook associated genes *flgD*, *flgE*, *flgK*, as well as the minor flagellin *flaB*. Furthermore, the expression of the gene *flgM,* encoding the anti-σ^{28} factor, was down-regulated. In contrast, the expression of *fliA*, encoding the σ^{28} factor, was only slightly enhanced, but was closely below the threshold for being labelled as up-regulated. While the expression of the flagellin gene *flaA* was down-regulated, the expression of the genes *fliD* (encoding the filament capping protein) and *fliS* (encoding an export chaperon), also belonging to class 3, did not differ in their expression levels. The expression of both motor complex encoding genes (*motA* and *motB*) were slightly enhanced, but only *motA* reached the threshold being considered as up-regulated.

Gene expression of *C. jejuni* swarming in high viscous media compared to liquid culture

The gene expression pattern of cells derived from liquid culture (low viscous media) was further compared to the patterns derived from either SH Edge or SH Center in high viscous media (Fig. 3). In the SH Center (Fig. 3A), most of the investigated genes associated with the FA process with up-regulated expression belong to class 2 and 3, except for *fliA* and *fliD,* while the genes with down-regulated expression belong to class 1. By comparing mRNA levels from the

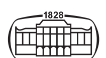

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Table 1. Primers used in this study

Category	Primer	Sequence	Target	Final concentration	Function	Reference
Inner reference	rpoA-F rpoA-R	CGAGCTTGCTTTGATGAGTG AGTTCCCACAGGAAAACCTA	cj1595	300 nM	DNA-directed RNA polymerase subunit alpha	[19]
Chemotaxis	cetA-F cetA-R	CCTACCATGCTCTCCTGCAC CGCGATATAGCCGATCAAACC	cj1190	300 nM	bipartite energy taxis response protein	[20]
	cetB-F cetB-R	GCCTTGTTGCTGTTCTGCTC TTCCGTTCGTCGTATGCCAA	cj1189	300 nM	bipartite energy taxis response protein	[20]
	cheA-F cheA-R	GCTTCAGGTAGTAATGCTA TAACGCTCTTCAACATCA	cj0284c	300 nM	two-component system, chemotaxis family, sensor kinase	[21]
	cheV-F cheV-R	TTCGTGGAGTGGTTATAC AAGTTGCTGGTTCTATATCT	cj0285c	300 nM	two-component system, chemotaxis family, chemotaxis protein	[21]
	cheW-F cheW-R	ATGGCTGGTCCTGATGTCG CCAGCATTTCCTCCAACTCC	cj0283c	300 nM	purine-binding chemotaxis protein CheW	[22]
	cheY-F cheY-R	GCTTCAGGTAGTAATGCTA TAACGCTCTTCAACATCA	cj1118c	300 nM	two-component system, chemotaxis family, chemotaxis protein	[21]
	tlp8-F tlp8-R	GAGGATGTTAATCAGAGTGT TTAGCAACTTCAGCAGAG	cj1110	300 nM	MCP-type signal transduction protein	[21]
Flagella assembly	flaA-F flaA-R	CAGCAGAATCGCAAATCCGT CCATGGCATAAGAGCCGCTT	cj1339c	300 nM	flagellin A	This study*
	flaB-F flaB-R	GTTAAAGCAGCTGAATCAACCA ACTCATAGCATAAGAACCTGATTG	cj1338c	300 nM 900 nM	flagellin B	This study
	flgD-F flgD-R	TATGCAAAAATGGCTGGACA TGAACCGCTTCCTCCAGTAG	cj0042	300 nM	flagellar hook assembly protein	This study
	flgE-F flgE-R	TGCGATGGATGTTGAAGGTA CCCAAAATCTGCACGAGAAT	cj0043	300 nM	flagellar hook protein	This study
	flgG2-F flgG2-R	GCGTTGATAAAAACGGAAGC CCCATCTTTTTGCAAAGCTC	cj0697	300 nM	flagellar basal-body rod protein	This study
	flgH-F flgH-R	TTTTTGGTTGTTCTGCAACG TTGCTTTGTTTTGGTGCAAG	cj0687c	300 nM	flagellar basal body L-ring protein	This study
	flgI-F flgI-R	TGCAGTGCAAATCAAGGATG CGCTTCCATTAAGCCCTACA	cj1462	300 nM	flagellar basal body P-ring protein	This study
	flgK-F flgK-R	GCGTTTTCCCGACTTACAAA TTTTCGTTGGGGTTAGATGC	cj1466	300 nM	flagellar basal body P-ring protein	This study
	flhA-F flhA-R	TAAGCGAAGGGCAAAACGG AATACAAAATACAATCACGCCAATG	cj0882c	300 nM	flagellar biosynthesis protein	[21]
	flhB-F flhB-R	GCAGGTGCGGATGTGGTG TTGTTTTATGCGAAGAGCGAGA	cj0335	300 nM	flagellar biosynthesis protein	[21]
	fliD-F fliD-R	GGATTTGGTTCTGGGGTTTT CGAGCTTTTTGCTCTGCTTC	cj0548	300 nM	flagellar hook-associated protein 2	This study
	fliM-F fliM-R	CAAACCGTGATATTATGATGGGTG ATACCACTTCAGCACGACCGA	cj0060c	300 nM	flagellar motor switch protein	[21]
	fliS-F fliS-R	CCCCGCAAAAACTTATTGAA CTCTTGCACAAAAACGCAAA	cj0549	300 nM	flagellar secretion chaperone	This study
	motA-F motA-R	CGGGTATTTCAGGTGCTT CCAAGGAGCAAAAAGTGC	cj0337c	300 nM	flagellar motor complex protein	This study
	motB-F motB-R	AATGCCCAGAATGTCCAGCA AGTCTGCATAAGGCACAGCC	cj0336c	300 nM	flagellar motor complex protein	[23]

(continued)

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Table 1. Continued

Category	Primer	Sequence	Target	Final concentration	Function	Reference
Sigma factors & regulators	flgM-F	TGGCAAATACCGCATTAAATA	cj1464	300 nM	flagellar biosynthesis protein FlgM	[24]
	flgM-R	GCTGTAGCTTTTGTATCGATTTTATAAG				
	fliA-F	GCCTAAAGCTTATGCACAAATGC	cj0061c	300 nM	RNA polymerase sigma factor FliA	[24]
	fliA-R	CGTTCTTTTAGTCTAAAAGCCATAGCA				
	rpoD-F	GAACGAATTTGATTTAGCCAATGA	cj1001	300 nM	RNA polymerase primary sigma factor	[25]
	rpoD-R	CCCATTTCTCTTAAATACATACGAACAG				
	rpoN-F	ATCGGGCTCTTTGCTTGCTA	cj0670	300 nM	RNA polymerase sigma-54 factor	[26]
	rpoN-R	AATCGGCAACCAAGAGCGTA				
	flgR-F	CGGTTCGTTTGGGAGTAAAA	cj1024c	300 nM	two-component system response regulator; sigma-54 associated transcriptional activator	This study
	flgR-R	GCACGCTTAATAGCCTCGAC				

*Designed in this experiment using primer prism 3 software.

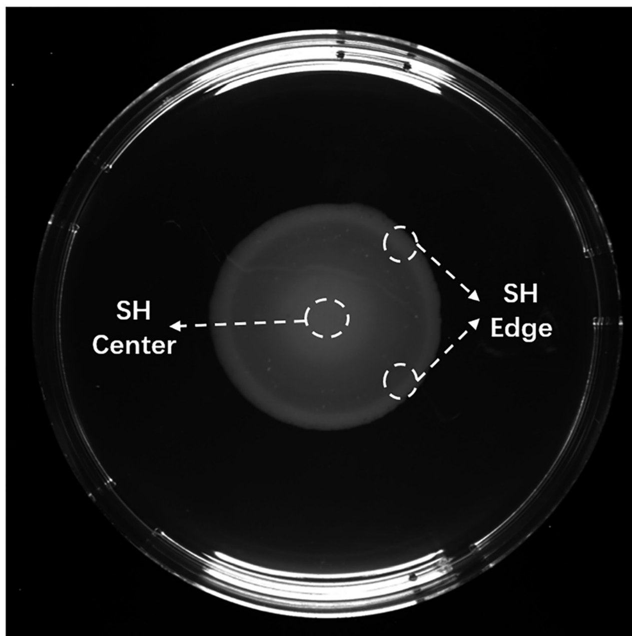


Fig. 1. **The image of *C. jejuni* swarming halo on the surface of BB semisolid agar.** The isolation spot was marked in dashed circles. SH: swarming halo

SH Edge with the liquid culture (Fig. 3B), differentially expressed genes associated with the FA process encode for regulatory proteins (*rpoD*, *flgR*, up-regulated; *fliA*, down-regulated) or structural proteins (*flhB*, *flgD*, *flaB*, *fliD*, down-regulated). The remaining 11 out of 18 genes were not differently expressed.

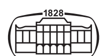
Among these selected genes, it is noticeable that the expression of a couple of genes is regulated in the same direction in both SH Center and SH Edge compared to liquid culture (Fig. 3). These included up-regulated expression of the motor complex genes (*motA* and *motB*) and chemotaxis related genes (*cetB*, *cheV*, and *tlp8*), as well as down-regulated expression of the genes *flhB*, *fliA* and *fliD*, whereas for three genes the direction of regulation was the opposite between SH Center and SH Edge compared to liquid culture. Of these genes, *rpoD* and *flgR* were down-regulated in the SH Center and up-regulated at the SH Edge compared to the liquid culture. The expression of *flaB* was regulated in the opposite way. Further, *cheA*, *cheW*, *cheY* and *flgK* did not show expression differences.

DISCUSSION

Differential expression of FA processing genes at the SH center and SH edge in high viscous media

While cultivating bacterial cells in liquid culture results in a quite homogenous cell population, cultivating bacteria in highly viscous media results in an inhomogenous cell population. For *E. coli* it has been reported that near the SH Edge highly motile cells are apparent while in the SH center less motile cells are stacked in a three-dimensional structure with many layers [28]. Additionally, growth of bacterial cells located at the SH center is limited by competition for nutrients. In contrast, the cells at the SH Edge remain rapidly growing [29]. Therefore, we expected different gene expression pattern in *C. jejuni* cells located at the SH Center and the SH Edge in high viscous media. Given that the flagellum is essential for bacterial cell motility, genes associated with the FA process were investigated.

Genes associated with the FA process are commonly divided into 3 classes, and the expression of each class is regulated by a different sigma factor, σ^{70} (RpoD), σ^{54} (RpoN) and σ^{28} (FliA), respectively. In this study, we observed that the genes with up-regulated expression at the SH Edge compared to the SH Center *(rpoD*, *flhA*, *flhB*, *fliM*, *rpoN* and *flgR)*, belong to class 1 of the FA process. Meanwhile, down-regulated genes *flgI*, *flgH*, *flgG2*, *flgD*, *flgE*, *flgK*, and *flaB*, which are σ^{54} dependent and encode for hook basal body



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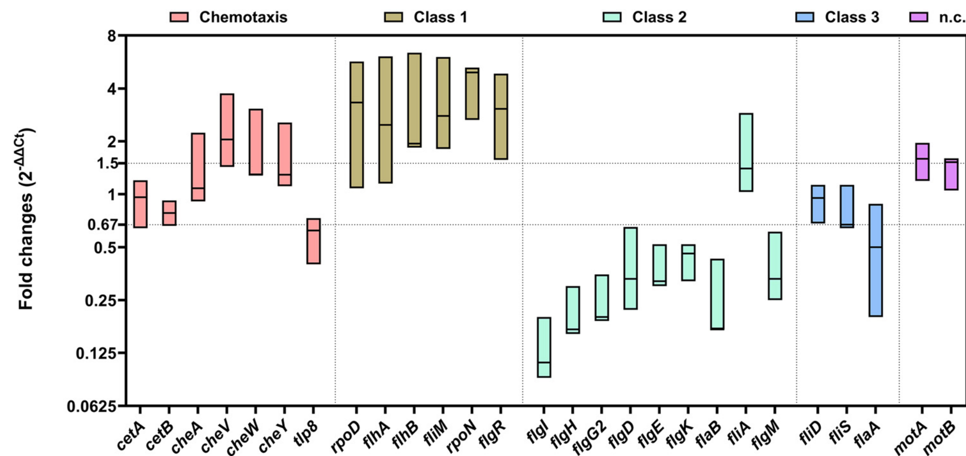

Fig. 2. **Higher mRNA levels of class 1 and lower levels of class 2 genes in cells derived from the SH Edge compared to cells derived from the SH Center.** The expression level of selected genes was analyzed by semiquantitative real-time PCR, and differential expression pattern determined by the $2^{-\Delta\Delta CT}$ method. Each floating bar depicted the median of 3 independent biological replicates and the min to max of the data range. The dash line indicates the threshold for differential gene expression with >1.5-fold (up-regulation) or <0.67 (down-regulation). Genes are classified into different groups: chemotaxis (red), flagellar assembly processing cascade (class 1: gold; class 2: green; class 3: blue), as well as not classified genes (purple)

proteins and flagellin minor protein, belong to class 2. Similar to the expression pattern of the class 2 genes, the expression of the class 3 flagellar gene, the flagellin major monomer gene *flaA,* was down-regulated. In the FA processing, the class 2 genes are activated through interaction of FlgR and σ^{54} after the T3SS-MS-C ring complex construction is completed [10, 11, 30]. This clear pattern of opposite regulated expression between class 1 and class 2 & 3 genes suggested that different FA processing stages were determined from the majority of bacterial cells at the SH Center and the SH Edge in high viscous media. Wright and colleagues [31] performed a gene expression analysis throughout the growth curve of *C. jejuni* NCTC 11168 in liquid culture. They have shown a continuous down-regulated expression of class 1 genes and up-regulated expression of several class 2 and 3 genes from late exponential until late stationary phase. Even though these differences were not statistically significant, they have shown a trend of flagellar gene expression during the stationary phase. We suggest that the bacterial cell at the SH Center were older than the cells at the SH Edge. This hypothesis is corroborated as the cells at the SH Center also possessed down-regulated expression of class 1 and up-regulated expression of class 2 and 3 genes compared to cells derived from the SH Edge. This would suggest that the majority of the cells at the SH Center are in late stationary phase while a majority of cells at the SH Edge were in the late exponential/early stationary phase. These differences through the growth phase could explain the different gene expression patterns determined at the SH Edge and SH Center. Further proof is needed to verify this hypothesis.

In the FA processing model of *Salmonella* Typhimurium and *E. coli*, the expression of the σ^{28}-dependent flagellar assembly genes of class 3 are not activated until the hook is completed, due to the negative regulation by FlgM binding σ^{28} [32, 33]. Once the hook is constructed, FlgM is secreted in the environment, enabling unbound σ^{28} to activate the expression of *flaA* as well as of other class 3 genes [24]. However, in our study, only the expression of *flaA* was down-regulated at the SH Edge compared to the SH Center, while the other class 3 genes (*fliD* and *fliS*) did not show differential expression (Fig. 2). One hypothesis might be that *flaA* is partially transcribed before hook completion. Even though the expression of the *flaA* gene is commonly reported to be regulated by σ^{28} [2, 34], Hendrixson and colleagues have reported that *flaA* expression was only partially defective in the *C. jejuni* 81–176Δ*fliA* mutant [35]. Furthermore, Dugar et al. described continuous *flaA* expression in *C. jejuni*, with highest mRNA level reached in overnight cultures [36]. Therefore, it seems plausible that the observed *flaA* expression level at the SH center is σ^{28} independent.

Loading of the motor complex by the stators MotA and MotB is a highly dynamic process [12]. Given that high loading of motor stator monomers facilitates bacterial cell's swarming in viscous media, the slightly up-regulated expression of both genes *motA* and *motB* in our study might indicate higher demand of the stator complex within the cells at the SH Edge compared to cells located at the SH Center in high viscous media. This is in line with the description for *E. coli* SHs, in which cells located at the SH Center were less motile compared to cells located near the SH Edge [28].

Genes differentially expressed in high viscous media and liquid culture

As gene expression studies are mostly conducted from liquid cultures, we further analyzed the differences in motility related gene expression of cells derived from high and low viscous media. The expression pattern of genes belonging to FA processing cascade (class 1, 2 and 3) derived from the SH Center compared to liquid culture was shown in Fig. 3A.

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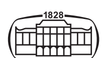
	Gene	center vs liquid (A)	edge vs liquid (B)
chemotaxis	*cetA*	1.96	1.49
	cetB	7.96	5.66
	cheA	0.89	1.17
	cheV	1.78	3.53
	cheW	0.84	1.08
	cheY	0.84	1.47
	tlp8	3.67	2.27
class 1	*rpoD*	0.42	1.64
	flhA	0.40	0.79
	flhB	0.33	0.63
	fliM	0.34	0.77
	rpoN	0.37	1.44
	flgR	0.63	1.73
class 2	*flgI*	4.25	0.86
	flgH	3.19	0.97
	flgG2	2.33	0.74
	flgD	1.26	0.61
	flgE	2.47	1.17
	flgK	1.48	0.68
	flaB	2.11	0.38
	fliA	0.42	0.47
	flgM	2.59	1.45
class 3	*fliD*	0.39	0.36
	fliS	1.81	1.33
	flaA	1.96	0.97
n.c	*motA*	1.59	1.89
	motB	1.89	1.99

up-regulation / none regulation / down-regulation

Fig. 3. **Heat map of gene expression pattern from high viscous media compared to low viscous media.** Fold changes of gene expression from the bacterial cells at the SH Center (A) or SH Edge (B) from high viscous media was compared to that from liquid culture, respectively. The expression level of selected genes was analyzed by semiquantitative real-time PCR, and differential expression pattern determined by the $2^{-\Delta\Delta CT}$ method. Each result represents the median from 3 independent biological replicates with 2 technical replicates for each. The significant results shown here were determined as > 1.5-fold (up-regulation, red color) or <0.67 (down-regulation, green color). Data range were shown in supplementary figures S1 and S2

In the SH Center, the expression of class 1 genes was down-regulated while an up-regulation was determined for most of the class 2 and 3 gene expressions. Meanwhile, the expression of *flgD* and *flgK* did not show significant regulation, whereas expression of *fliA* and *fliD* were down-regulated. Based on the data ranges of *flgD* and *flgK* (Fig. S1), both genes have shown similar trends as the remaining class 2 genes. Therefore, we deduced that the non-differential expression of both *flgD* and *flgK* were due to the systematic error. In contrast, in the gene expression pattern derived from the SH Edge compared to liquid culture (Fig. 3B), most of the genes related to the FA process have shown no differences (Fig. S2). Our data let us speculate that bacterial cells in the SH Center have mostly finished the FA process, while cells derived from liquid culture and the SH Edge are still in the process of flagellar assembly.



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Combining the expression patterns of genes sharing comparable regulation trends at SH Center and SH Edge compared to liquid culture (Fig. 3 AB), we observed that the gene expressions for σ^{28} (*fliA*), filament capping protein (*fliD*) and a T3SS component (*flhB*) were down-regulated in high viscous media. These genes play a central role in the FA process, however, their predominant expression in liquid culture compared to high viscous media remains unclear. Further experiments need to be conducted upon this topic.

However, the expression of the stator complex genes (*motA* and *motB*) as well as of genes involved in sensing energy taxis (*cheV*, *cetB*, *tlp8*) were up-regulated in high viscous media compared to liquid culture. As the result of competing with each other for access to nutrients, bacterial cells continue to rapidly grow after swarming to the SH Edge, while in the liquid culture, these cells would be eventually forced to balance growth and death [29]. We deduced that the high regulation level of energy taxis gene expressions was required for sensing favorable conditions to provide the higher energy needed for torque generation in high viscous media. Our results suggest that the movement of the cells to favorable conditions at the SH Edge needed higher energy in high viscous media.

CONCLUSION

In this study, differences in the *C. jejuni* gene expression pattern of the flagella apparatus were observed between cells derived from the SH Center and SH Edge in high viscous media. However, the expression pattern of these genes in liquid culture resembles that of cells derived from the SH Edge rather than cells derived from the SH Center in high viscous media. Nevertheless, genes belonging to energy taxis and energy conversion are higher expressed in high viscous media compared to liquid culture. Altogether this indicates that questions regarding the flagellar assembly process could be investigated from liquid cultures while questions regarding the swarming process itself should be investigated in high viscous media rather than in liquid cultures.

STATEMENTS

Ethics statement: Not applicable.

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Authors' contributions: GG and TA supervised the study; YS and GG designed experiments; YS performed experiments and wrote the paper; YS and GG analyzed data; YS, GG and TA made paper revisions.

Conflict of interest: The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1556/1886.2023.00006.

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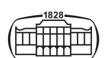

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