frontiers in Microbiology

ORIGINAL RESEARCH
published: 25 April 2017
doi: 10.3389/fmicb.2017.00729

Cj0440c Affects Flagella Formation and *In Vivo* Colonization of Erythromycin-Susceptible and -Resistant *Campylobacter jejuni*

Haihong Hao[1,2†], Xia Fang[1,2†], Jing Han[3], Steven L. Foley[3], Yulian Wang[1,2], Guyue Cheng[1,2], Xu Wang[1,2], Lingli Huang[1,2], Menghong Dai[1,2], Zhenli Liu[1,2] and Zonghui Yuan[1,2]*

[1] National Reference Laboratory of Veterinary Drug Residues and MOA Key Laboratory for Detection of Veterinary Drug Residues, Huazhong Agricultural University, Wuhan, China, [2] MOA Laboratory for Risk Assessment of Quality and Safety of Livestock and Poultry Products, Huazhong Agricultural University, Wuhan, China, [3] Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR, USA

OPEN ACCESS

Edited by:
Avelino Alvarez-Ordóñez,
Universidad de León, Spain

Reviewed by:
Hongxia Wang,
University of Alabama at Birmingham, USA
Odile Tresse,
Oniris, France

***Correspondence:**
Zonghui Yuan
yuan5802@mail.hzau.edu.cn

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 01 August 2016
Accepted: 07 April 2017
Published: 25 April 2017

Citation:
Hao H, Fang X, Han J, Foley SL, Wang Y, Cheng G, Wang X, Huang L, Dai M, Liu Z and Yuan Z (2017) Cj0440c Affects Flagella Formation and In Vivo Colonization of Erythromycin-Susceptible and -Resistant Campylobacter jejuni. Front. Microbiol. 8:729. doi: 10.3389/fmicb.2017.00729

Campylobacter jejuni is one of the most common foodborne pathogen worldwide. A putative transcriptional regulator, *Cj0440c*, was up-regulated in the erythromycin-resistant *C. jejuni*, however, the precise role of *Cj0440c* is yet to be determined. The aim of this study was to determine the biological functions of *Cj0440c*. The *Cj0440c* isogenic mutants were constructed from erythromycin-susceptible *C. jejuni* NCTC 11168 (S) and -resistant *C. jejuni* 68-ER (R), designating as SM and RM, respectively. The isogenic Cj0440c mutants (SM and RM) and parental strains (S and R) were subjected to microarray and qRT-PCR analysis to examine the transcriptional profile changes contributed by *Cj0440c*. The antimicrobial susceptibility, flagellar morphology, *in vitro* growth and *in vivo* colonization in chickens were carried out to analyze the biological function of *Cj0440c*. The results showed that 17 genes were down-regulated in SM compared to S, while 9 genes were down-regulated in RM compared to R. The genes with transcriptional change were mainly involved in flagella biosynthesis and assembly. Using transmission electron microscopy, we found that the filaments were impaired in SM and lost in RM. The chicken colonization experiments showed that *Cj0440c* mutants (SM and RM) had reduced colonization ability in chickens when compared with corresponding parental strains (S and R). In conclusion, *Cj0440c* regulates flagella biosynthesis and assembly, and consequently affect the *in vivo* colonization of erythromycin-susceptible and -resistant *C. jejuni*.

Keywords: *Campylobacter jejuni, Cj0440c*, flagella, colonization, erythromycin resistance

INTRODUCTION

Campylobacter jejuni has been recognized as one of the most important pathogens, which can cause infectious diarrhea and severe forms of disease such as Guillain-Barre Syndrome or Miller Fischer Syndrome (Samuel et al., 2004; Hughes and Cornblath, 2005; Riddle et al., 2006). The CDC estimated that in 2009 the number of *Campylobacter* infection was 13.02 per 100,000 people (Silva et al., 2011). The cost of human Campylobacteriosis in the United States is estimated at $1.3 to 6.8 billion dollars annually (Scharff, 2012; Epps et al., 2013). Macrolides (e.g., erythromycin) are

the most important drugs of choice for clinical treatment of *Campylobacter* infections (Gibreel et al., 2005). Unfortunately, macrolides-resistant *Campylobacter* have emerged and impose a global public health concerns (Gibreel and Taylor, 2006; ECDC et al., 2009). In earlier study we demonstrated that the transcription level of *Cj0440c* was increased in high-level erythromycin-resistant *C. jejuni* (Hao et al., 2013).

Bioinformatic analyses suggested that *Cj0440c* is a putative transcriptional regulator and encodes a TENA/THI-4 family protein, however, the molecular function of this family is yet to be determined. The gene *Cj0440c* is located downstream of the *Cj0437–Cj0439* operon *(mfr,* methylmenaquinol:fumarate reductase) which plays an important role in the susceptibility to hydrogen peroxide (H_2O_2) (Parkhill et al., 2000; Weingarten et al., 2009; Kassem et al., 2012) and upstream of *Cj0441* (*acpP*, acyl carrier protein) which is a universal and highly conserved acyl donor for synthesis of fatty acid, endotoxin and acylated homoserine lactones for the quorum sensing in *C. jejuni* (Byers and Gong, 2007). Both the downstream and upstream genes of *Cj0440c* were essential for the growth, survival, colonization and pathogenesis in *Campylobacter*. Although *Cj0440c* is located on the opposite DNA coding strand, it may divergently transcribed with its up-and-downstream genes, and likely to act as a transcriptional regulator and play an important role in gene regulation and the biological function in *C. jejuni*. The biological functions of *Cj0440c* in *C. jejuni* are largely unknown.

In the present study, *Cj0440c*-inactivated mutation was constructed in both erythromycin-susceptible (S) and -resistant *C. jejuni* (R), the transcriptional profile and relative *in vitro* and *in vivo* phenotype determination were implemented to decipher the function and regulation mechanism of *Cj0440c*.

MATERIALS AND METHODS

Plasmids, Bacterial Strains, and Growth Conditions

The *C. jejuni* NCTC11168 (designated as **S**) was kindly provided by Chinese Center for Disease Control and Prevention. *C. jejuni* strains were routinely cultured in Mueller-Hinton (MH) medium at 42°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) in the anaerobic incubator (YQX-II, Shanghai, China) (Mace et al., 2015). The *Escherichia coli* DH5α was grown aerobically in Luria-Bertani medium at 37°C. The erythromycin-resistant *C. jejuni* strain 68-ER (designated as **R**) was descendant of *C. jejuni* NCTC11168 resulting from *in vitro* step-wise selection by erythromycin. Plasmids pGEM-T (Promega, Madison, WI, USA) and pMW10 was kindly provided by China Agricultural University and used for mutant vector construction.

Construction of Isogenic Δ*Cj0440c* Mutants

The DNA fragment containing *Cj0440c* gene and its flanking regions was amplified from *C. jejuni* NCTC 11168 genome using *Pfu* polymerase (Promega) with primers of $Cj0440cF_2$ and $Cj0440cR_2$ (**Table 1**) and was cloned into pGEM-T easy vector (Promega,) to generate plasmid pCJ0440c. Primers pCj0440cU and pCj0440cL (**Table 1**) carrying endonuclease restriction sites of KpnI and XbaI were used to inversely amplify DNA fragment from the vector of pCJ0440c using Taq and Pfu polymerase (8:1). A kanamycin resistance cassette (*kan*) was amplified from pMW10 plasmid with Pfu polymerase (Promega) using primers KanF and KanR (**Table 1**) which have the same restriction sites of KpnI and XbaI. The amplified DNA fragments of inverse PCR and *kan* were digested with KpnI and XbaI and purified with a PCR clean-up kit (Generay, Shanghai, China). The digested inverse PCR product was ligated to the *kan* cassette using T4 DNA ligase (Takara, Dalian, China) to obtain the construct plasmid pCJ0440c-Kan, which was then transformed into *E. coli* DH5α. The purified plasmid of pCJ0440c-Kan was introduced into S and R via electroporation according to the method described previously (Jeon et al., 2011). Insertional mutants, named SM and RM, respectively, were selected on MH agar plates with 25 μg/ml kanamycin and 50 μg/ml ampicillin. Both PCR and sequencing analysis of the *Cj0440c* mutants (SM and RM) confirmed that the mutation resulted in deletion of 200 bp of coding sequence in *Cj0440c* and simultaneous insertion of the *kan* gene into the same location.

TABLE 1 | Primers used for construction of *Cj0440c* mutant and for real-time qPCR.

Primer name	Primer Sequence (from 5′ to 3′)	Product size (bp)
Primers used for construction and confirmation of *Cj0440c* mutant		
Cj0440c-F2	AATACCAGAAGCCGAAAC	2315
Cj0440c-R2	GAGGGTGAAATAGAAGGG	
pCj0440c-U	GGGGTACCAGATCATCCTTACAAGGAAT *Kpn*I site	5100
pCj0440c-L	GCTCTAGATTCATAGCAAAACGAAGT *Xba*I site	
Kan-F	GCTCTAGAAATGGGCAAAGCAT *Xba*I site	1203
Kan-R	GGGGTACCATAATGCTAAGACAAT *Kpn*I site	
Primers used for real-time qPCR		
Cj1339cF	TCCATTAAACGGTTGATATCTGCTT	125
Cj1339cR	AAGGCTATGGATGAGCAACTTAAAAT	
Cj1328F	CTTTTAGCGATGCTTTTGAAGACTTA	126
Cj1328R	CGCCACATAAATGCACTAAAGG	
Cj1294F	GGCGTAAAACACGCTTGTGTATT	79
Cj1294R	TTTCTTGGACACCTAGTGCTGTGTA	
Cj1338cF	TTACCATTGTTGATAGCTTGACCTAAA	75
Cj1338cR	TGCTTCAGGGATGGCGATA	
Cj0043F	GGGTTCTCCTGTTGCAAGTGA	75
Cj0043R	GCCCCTAAAACCCCAAAAAAT	
Cj0697F	TGGTTCAGACCAAAGATGGA	138
Cj0697R	TGCCAGCATTCTGAGGATTA	
Cj1242F	AAGACATTGATCTTGGTGCTG	143
Cj1242R	ATTGTTTGTGGCATTTCCTG	
Cj1385F	GGAAACTGGGACTTGGTAGGAA	83
Cj1385R	TGAGTATGGATGAAATCAGGGAATT	
Cj1464F	CGAGTAAAATCGCAGAGCAG	69
Cj1464R	TCGCAGCAGCTGTAGCTTT	
q16SF	GCTCGTGTCGTGAGATGTTG	199
q16SR	GCGGTATTGCGTCTCATTGTAT	

TABLE 2 | Transcriptional difference in the mutant SM comparing to its parental strain S determined by microarray.

Function class	Gene name	Gene function	Fold change
Target gene	*Cj0440c*	Putative transcriptional regulator	−24.3
Cell motility/signal transduction	*Cj1339c/flaA*	Flagellin	2.1
	Cj1338c/flaB	Flagellin	−3.5
	Cj1729c/flgE2	Flagellar hook protein FlgE	−2.2
	Cj0887c/flgL	Flagellar hook-associated protein FlgL	−2.4
	Cj1466/flgK	Flagellar hook-associated protein FlgK	−3.3
	Cj0043/flgE	Flagellar hook protein	−3.5
	Cj1462/flgI	Flagellar basal body P-ring protein	−3.0
	Cj0698/flgG	Flagellar basal body rod protein FlgG	−3.2
	Cj0687c/flgH	Flagellar basal body L-ring protein	−3.2
	Cj0697/flgG2	Flagellar basal-body rod protein	−3.6
	Cj0041/fliK	Putative flagellar hook-length control protein	−4.5
Carbohydrate metabolism	*Cj1327/neuB2*	*N*-acetylneuraminic acid synthetase	−3.1
	Cj1328/neuC2	UDP-*N*-acetylglucosamine 2-epimerase	−2.4
Amino acid/energy metabolism	*Cj1293/pseB*	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	−2.2
	Cj1294/pseC	C4 aminotransferase specific for PseB product	−2.5
Hypothetical proteins	*Cj1026c*	Putative lipoprotein	−2.1
	Cj1242	Hypothetical protein	−2.3
	Cj1632c	Putative periplasmic protein	5.7

Only flaA and Cj1632c were up-regulated.

TABLE 3 | Transcriptional difference in the mutant RM comparing to its parental strain R determined by microarray.

Function class	Gene name	Gene function	Foldchange
Target gene	*Cj0440c*	Putative transcriptional regulator	−35.2
Cell motility/signal transduction	*Cj1338c/flaB*	Flagellin	−2.3
	Cj0547/flaG	Flagellar protein FlaG	−2.3
	Cj0548/fliD	Flagellar capping protein	−2.1
	Cj0042/flgD	Flagellar basal body rod modification protein	−2.7
Energy metabolism	*Cj1385/katA*	Catalase	−2.5
Hypothetical proteins	*Cj1464/flgM*	Hypothetical protein	−4.4
	Cj1465	Hypothetical protein	−3.8
	Cj1242	Hypothetical protein	−2.0
	Cj0391c	Hypothetical protein	−2.1

RNA Microarray and Data Analysis

The transcriptional difference between *Cj0440c* mutants and their parental strains (SM&S and RM&R) was examined by microarray (CapitalBio Corporation, Shanghai, China). Briefly, the strains were separately grown in MH broth for 24 h at 42°C under microaerophilic conditions with shaking. Immediately after the incubation, twice volume of RNA protective reagent (Qiagen, Germantown, MD, USA) was added to the culture (with same OD_{600} of 0.3) to stabilize mRNA. The total RNA from each sample was extracted using RNeasy Protect Mini Kit (Qiagen) and purified using Nucleo®Spin RNA clean-up kit (Macherey-nagel, Germany). The RNA quality and quantity was determined by formaldehyde denatured gel electrophoresis and Nanodrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized from the extracted RNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was labeled by Cy5 or Cy3 dye and co-hybridized onto one microarray slide (NimbleGen 4 K × 72K), scanned by Axon Instruments Gene Pix 4000B (Union City, CA, USA) and read by Gene Pix Pro 6.0 (Axon Instruments). Microarray data were analyzed using Array Star software. The genes with False Discovery Rate (FDR)-corrected q-values < 0.01 and fold change >2 were selected as differentially expressed genes and subjected to NCBI gene annotation, KEGG pathway analysis and STRING 9.05 protein network analysis.

Microarray Data Accession Number

The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus database[1] and assigned accession number GSE49255 and GSE49256.

[1] http://www.ncbi.nlm.nih.gov/geo/database

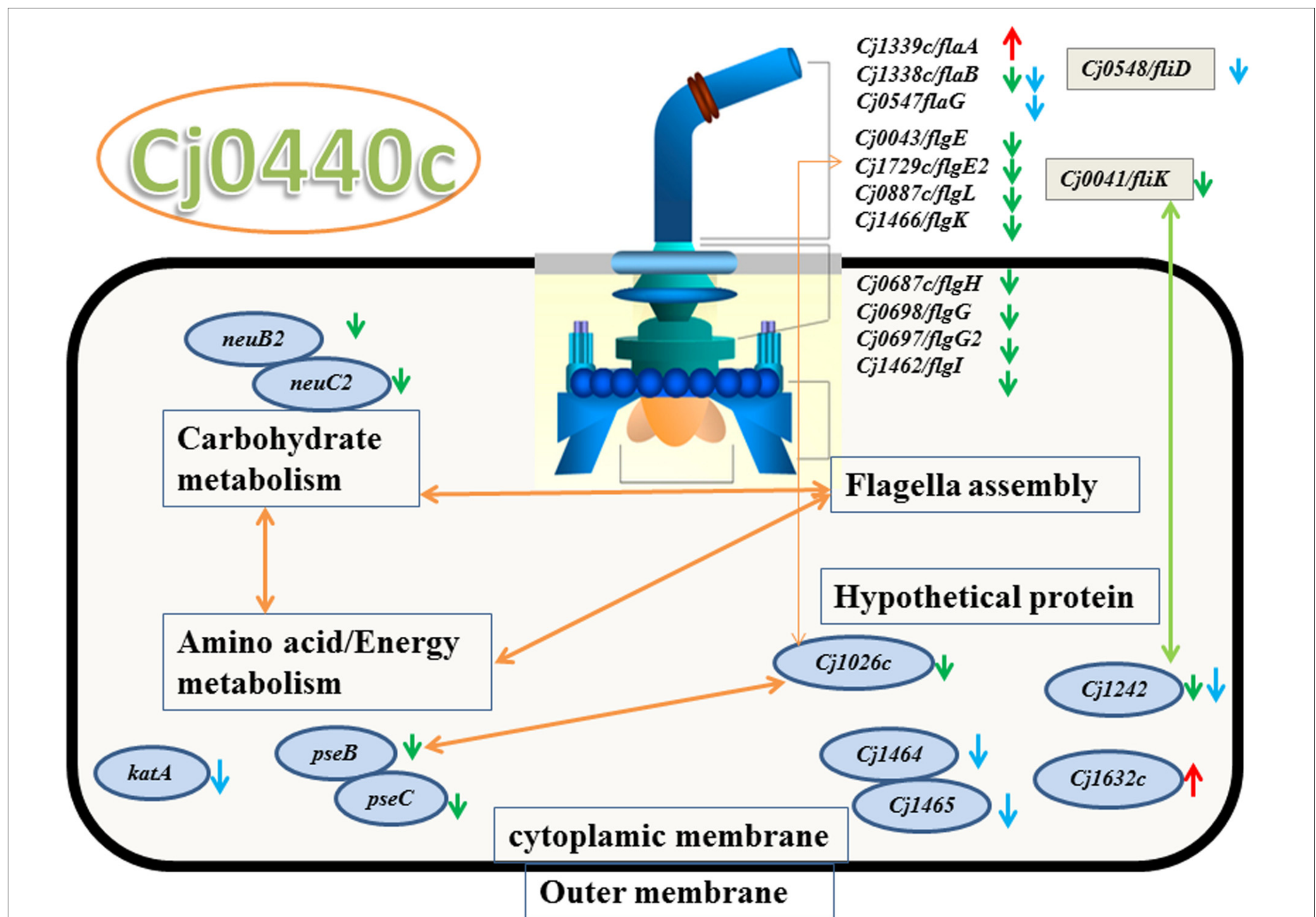

FIGURE 1 | Significant genes in *Cj0440c*-inactivated mutants and their relationship based on KEGG and STRING protein network analysis. Green arrow and red arrow were genes down-regulated and up-regulated in SM, respectively. Genes with blue arrow were down-regulated in RM. The yellow double sided arrow means positive relationship between these genes and green double sided arrow means negative relationship between these genes.

qRT-PCR

The same batches cDNA of *Cj0440c* mutants (SM and RM) and their parental strains (S and R) used in microarray were subjected to qRT-PCR analysis to confirm the transcriptional difference of some respective genes identified by microarray following method described in previous study (Hao et al., 2010). Briefly, the PCR amplification was performed in IQ5 Multicolor Real-time PCR Detection System (Bio-Rad). The cycling conditions were as follows: 3 min of pre-incubation at 95°C, followed by 30 cycles of 10 s at 95°C and 40 s at 60°C. The primer sets used for specific genes are listed in **Table 1**. 16S rDNA was used as an internal control for normalization. The experiment was done in triplicate to obtain the average value of fold change. The student's *t*-test was performed to analyze the significant difference between mutants and their parental stains.

Antimicrobial Susceptibility Test

Minimum inhibitory concentrations (MICs) of nine antimicrobial agents (azithromycin, erythromycin, tylosin, ciprofloxacin, olaquindox, chloromycetin, tetracycline, gentamicin, and ceftriaxone) were determined using agar dilution method recommended by Clinical and Laboratory Standards Institute (CLSI). *C. jejuni* ATCC 33560 was used as a quality control strain.

Transmission Electron Microscopy

The presence and length of flagella on the four *C. jejuni* strains (S, SM, R and RM) were examined using transmission electron microscopy according to a previously described method (Barrero-Tobon and Hendrixson, 2014; Matsunami et al., 2016). Briefly, bacterial suspensions were obtained after washing plate with 2 ml sterile phosphate-buffered saline and spotted on carbon-coated copper grids. The cells were stained with 2% phosphotungstic acid (pH 6.7) for 1 min. Samples were observed employing a HITACHI H-7650 transmission electron microscope (Hitachi, Japan).

In Vitro Growth Determination

To compare the growth kinetics of the mutants with that of the parental strains, a fresh culture (100 μL) of each *C. jejuni* strain

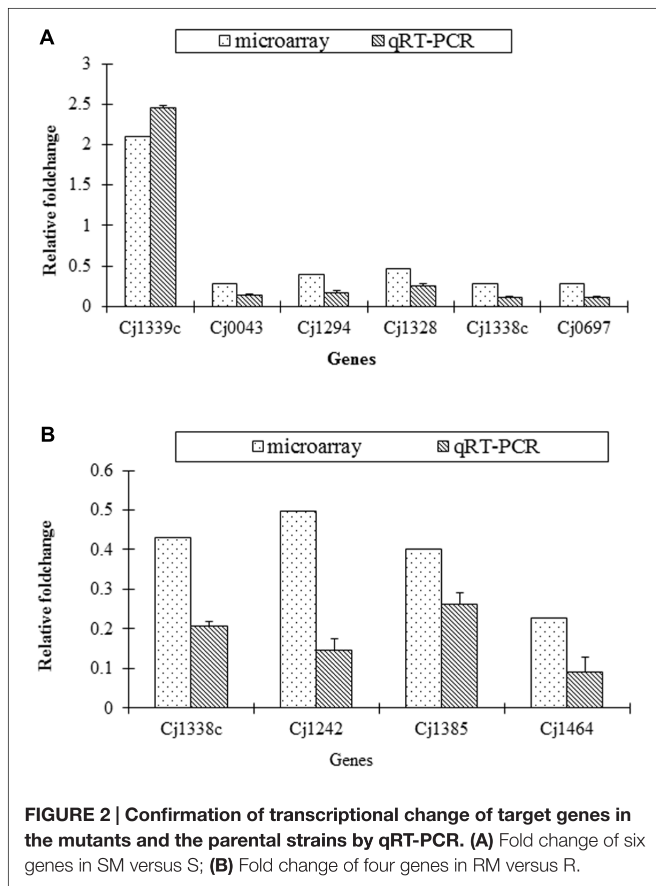

FIGURE 2 | Confirmation of transcriptional change of target genes in the mutants and the parental strains by qRT-PCR. (A) Fold change of six genes in SM versus S; **(B)** Fold change of four genes in RM versus R.

(0.5 McFarland) was inoculated into 100 mL MH broth and the cultures were incubated at 42°C under microaerobic conditions for 36 h with shaking. The growth kinetics was determined by measuring the absorbance in 600 nm (OD_{600}) at 0, 4, 8, 12, 20, 22, 24, 27, 31, 33, and 36 h post-inoculation.

Single and Competitive Colonization in Chicken

Newly hatched broiler chickens (White Leghorns) were purchased from Zhengda Limited Company (Wuhan, China). All the broiler chickens were examined for *C. jejuni* to ascertain that birds are free of *C. jejuni* prior to infection all the chickens (Hao et al., 2015).

These chickens were randomly assigned to seven groups with 6 to 10 chickens per group. One group was used as a control. Four groups were used for single colonization test in which 10^9 CFU *C. jejuni* strains (S, SM, R and RM) were individually inoculated via oral gavage into each group. Another two groups were used for pairwise competition test in which 10^9 CFU *C. jejuni* pairwise mixtures (S&SM or R&RM) were inoculated via oral gavage to each group. Fecal samples were collected from each bird at 3, 6, 9, and 12 days' post-infection. The CFU of S, SM, R and RM were determined using *Campylobacter* selective CCDA agar (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) with or without 25 μg/ml Kanamycin or 50 μg/ml erythromycin. Each sample was spread onto three respective selective plates to obtain the average CFU.

The significance of differences between mutant and parental strain in colonization at each sampling time point was determined by using Student's *t*-test, Welch's *t*-test to allow for non-constant variation across treatment groups, and the Wilcoxon rank-sum test to allow for non-normality (Guo et al., 2008; Luangtongkum et al., 2012; Xia et al., 2013). Differences were considered significant at a *P*-value of <0.01.

TABLE 4 | Minimum inhibitory concentration (MIC) of *Cj0440c* mutant strains and parental strains to different drugs.

Strains	MIC to different drugs (μg/mL)								
	ERY	TYL	AZI	TET	CIP	CHL	GEN	CRO	OLA
S	1	4	0.0625	0.5	0.125	2	0.5	16	2
SM	1	4	0.0625	0.5	0.125	2	0.5	16	1
R	256	256	32	0.5	0.125	2	0.5	16	2
RM	256	256	32	0.5	0.125	2	0.5	16	1

S was C. jejuni NCTC 11168; SM was Cj0440c deletion mutant of S; R was Erythromycin resistant strain selected from C. jejuni NCTC 11168; RM was Cj0440c deletion mutant of R. The drugs included erythromycin (ERY), tylosin (TYL), azithromycin (AZM), tetracydine (TET), ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN), ceftriaxone (CRO) and olaquindox (OLA).

Ethics Statement

The animal study was approved by Animal Ethics Committee of Huazhong Agricultural University (HZAUCH 2013-002) and the Animal Care Center, Hubei Science and Technology Agency in China (SYXK 2013-0044). All experimental procedures in this study were performed according to the guidelines of the committee on the use and care of the laboratory animals in Hubei Province, China. All the animals were monitored throughout the study for any signs of adverse effects.

RESULTS

Differentially Expressed Genes in SM and RM

The target gene *Cj0440c* was down-regulated in the *Cj0440c*-inactivated mutants (SM and RM). The other differentially expressed genes in *Cj0440c* mutants (SM and RM) compared to their parental strains (S and R) were shown in **Tables 2**, **3**. The relationship of these different genes was summarized in **Figure 1**.

A flagellin gene (*flaA*) and a gene (*Cj1632c*) encoding a putative periplasmic protein were up-regulated in SM as compared to S (indicated by red arrows in **Figure 1**). Among the down-regulated genes in SM (indicated by green arrows in **Figure 1**), 10 genes (*flaB*, *flgE*, *flgE2*, *flgG*, *flgG2*, *flgH*, *flgI*, *flgK*, *flgL*, and *fliK*) are possible involve in flagellar assembly; 2 genes (*pseB* and *pseC*) in carbohydrate metabolism; 2 genes ($neuB_2$ and *neuC*) in surface glycoprotein metabolism.

None of the genes were up-regulated genes were found in RM when compared the expression of different genes with R. Eleven down regulated genes in RM (indicated by blue arrows in **Figure 1**) are flagellar associated genes (*flgD*, *fliD*, *flaG* and *flaB*),

A B C D

FIGURE 3 | Flagella morphology of S (A), SM **(B)**, R **(C)**, and RM **(D)** under transmission electron microscope. The magnification used for TEM images in the captionis are 1 μm.

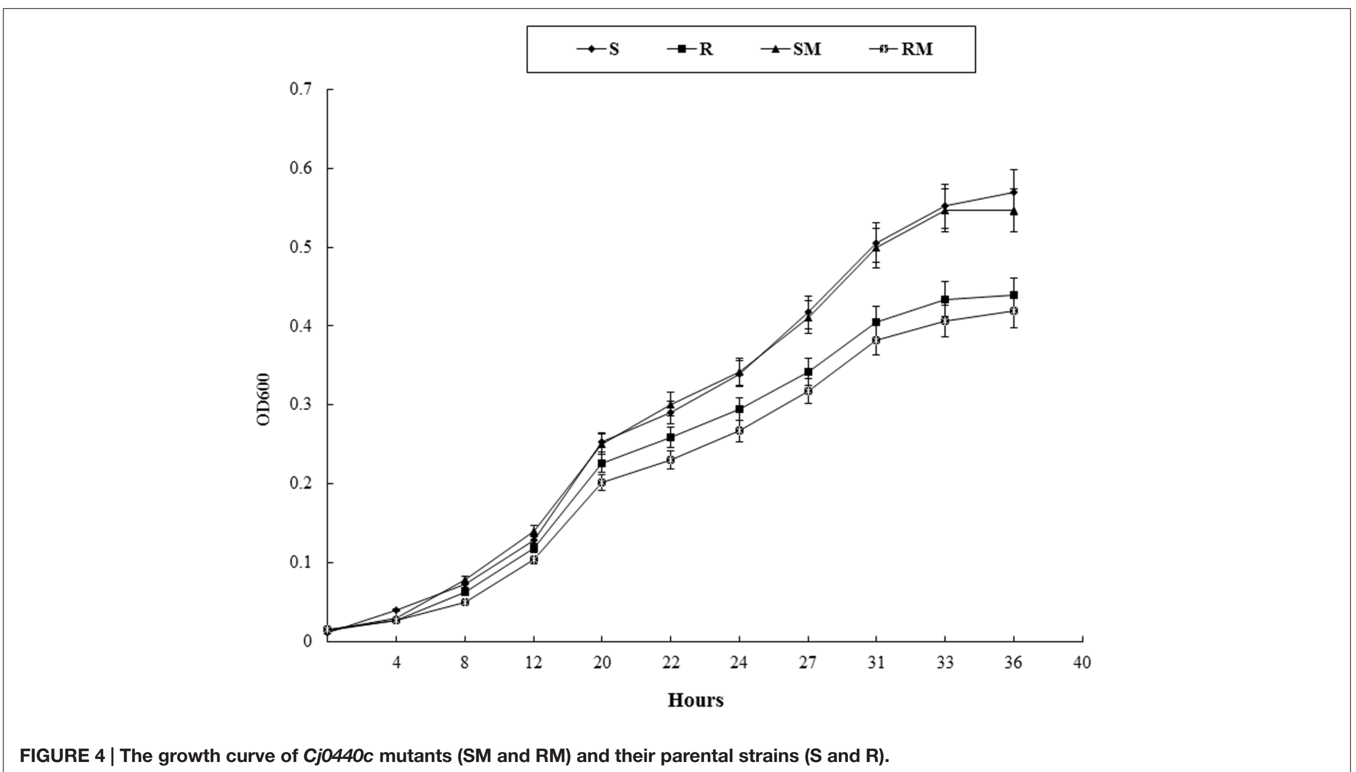

FIGURE 4 | The growth curve of *Cj0440c* mutants (SM and RM) and their parental strains (S and R).

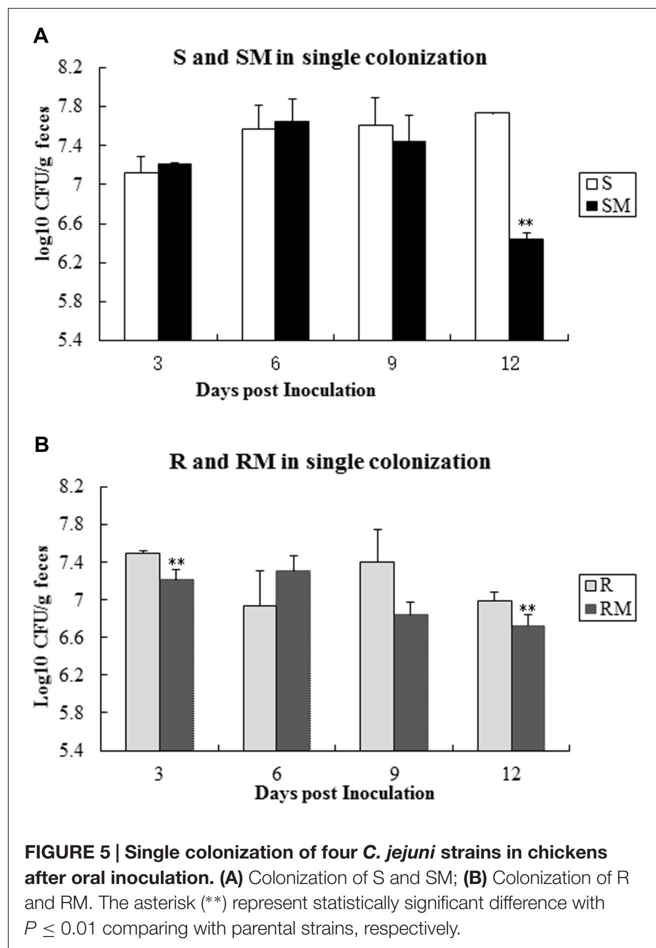

FIGURE 5 | Single colonization of four *C. jejuni* strains in chickens after oral inoculation. (A) Colonization of S and SM; **(B)** Colonization of R and RM. The asterisk (**) represent statistically significant difference with $P \leq 0.01$ comparing with parental strains, respectively.

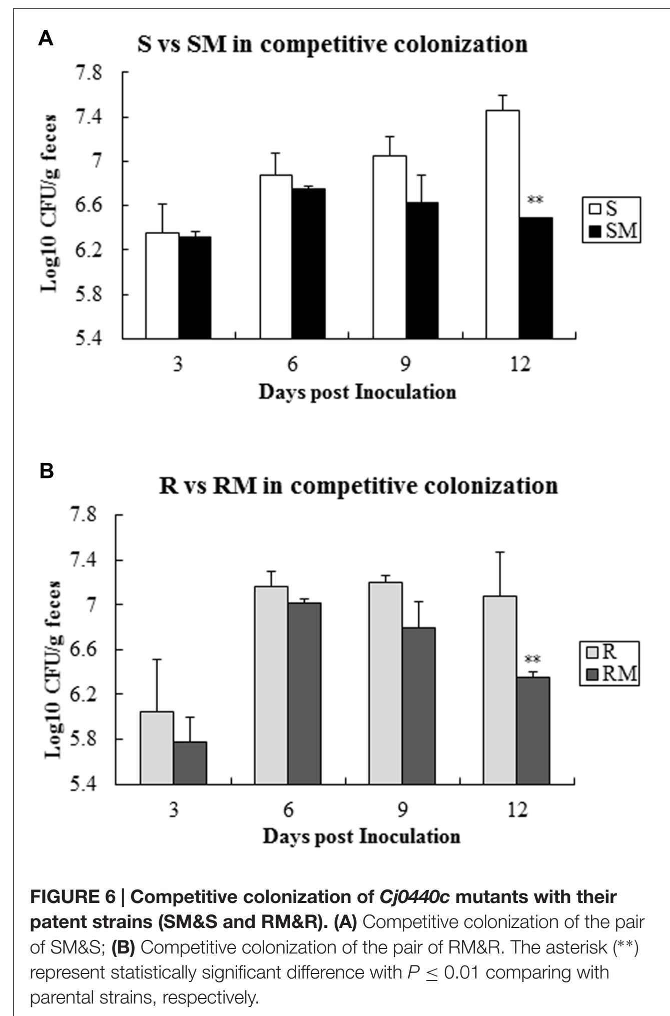

FIGURE 6 | Competitive colonization of *Cj0440c* mutants with their patent strains (SM&S and RM&R). (A) Competitive colonization of the pair of SM&S; **(B)** Competitive colonization of the pair of RM&R. The asterisk (**) represent statistically significant difference with $P \leq 0.01$ comparing with parental strains, respectively.

a catalase encoding gene (*katA*) and four genes with unknown function (*flgM*, *Cj1465*, *Cj0391*, and *Cj1242*).

When submitted to STRING 9.05 and KEGG pathway analysis, the result showed that 10 flagellar genes were interacted with other down-regulated genes (*pseB*/*C*, *neuB*$_2$/*C2* and *Cj1026c*) (**Figure 1**).

The transcriptional change of several representative genes detected in microarray was validated by qRT-PCR. The similar change of the selected genes was found both in microarray and qRT-PCR (**Figure 2**).

The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus database[2] and assigned accession numbers GSE49255 (RM&R) and GSE49255(SM&S).

Antimicrobial Susceptibility of *Cj0440c* Mutants

As shown in **Table 4**, there was no significant difference between MIC of nine antimicrobial agents in *Cj0440c* mutants (SM and RM) comparing to their parental strains (S and R). Inactivation of *Cj0440c* did not affect antimicrobial susceptibility of *C. jejuni*.

[2]https://www.ncbi.nlm.nih.gov/geo/

Flagella Presence and Length

The electron micrographs of all tested strains were shown in **Figure 3**. The results showed that parental strains (S and R) had long, spiral and complete flagella filaments in two sides (**Figures 3A,C**). However, SM had shorter filaments in only one side (**Figure 3B**). No filaments of RM were detected in RM (**Figure 3D**). These findings indicated that *Cj0440c* may affect the formation of flagella in *C. jejuni.*

In Vitro Growth of *Cj0440c* Mutants

Growth kinetics of *Cj0440c* mutants (SM and RM) and their parental strains (S and R) were determined in MH broth. No significant difference in growth rate was observed between SM and S. The RM exhibited slower growth rate compared to its parental R, however, the difference was not significant (**Figure 4**).

In Vivo Colonization of *Cj0440c* Mutants

To determine the colonization capacity, broiler chickens were infected individually with four *C. jejuni* strains (S, R, SM and RM). All the strains were able to colonize in chicken intestinal tract, albeit at different rate. Comparing with the parental strains

(S or R), the *Cj0440c* mutants (SM and RM) showed a significant reduction in colonization on 12 days' post-inoculation (**Figure 5**).

When the two pairs of *C. jejuni* strains (SM&S and RM&R) were infected chickens with one pair at a time, *Cj0440c* mutants (SM and RM) exhibited lower colonization level compared to their parental strains (S and R) after 9 days' post-inoculation (**Figure 6**).

DISCUSSION

Campylobacter jejuni is a very common foodborne pathogen in the developed world. Its biology and pathogenicity is largely unknown (Young et al., 2007). *Cj0440c* is a putative transcriptional regulator and an increased transcriptional level expression was detected in the erythromycin-resistant *C. jejuni* (Hao et al., 2013). The gene may encode a TENA/THI-4/PQQC family protein. TENA is one of a number of proteins that enhance the expression of extracellular enzymes (e.g., alkaline protease, neutral protease and levansucrase) (Pang et al., 1991). The extracellular enzymes may be regulated by the master regulator of flagellar genes (e.g., *flhDC*) (Cui et al., 2008). THI-4 protein is involved in thiamine biosynthesis (Akiyama and Nakashima, 1996). This family also includes bacterial coenzyme pyrroloquinoline quinone (PQQ) synthesis protein C (PQQC) proteins. PQQ is the prosthetic group of several bacterial enzymes, including methanol dehydrogenase of methylotrophs and the glucose dehydrogenase (Toyama et al., 2002, 2007). In *E. coli*, PQQ biosynthesis may be affected by *tldD* gene which encodes a peptidase involved in processing of small peptides (Holscher and Gorisch, 2006). The *tldD* may lead to chromosomal DNA relaxation and subsequent derepression of *cdtB* and *lgeR* which may regulate the expression of some flagellar genes (Haghjoo and Galan, 2007). Therefore, the TENA/THI-4/PQQC family may have some indirect relationship with flagellar genes.

The flagella formation plays an important role in the pathogenesis of *Campylobacter* including motility, microcolony formation, biofilm formation, autoagglutination, protein secretion, adherence to host cell, and host invasion (Guerry et al., 2006; Guerry, 2007). The major groups of down-regulated genes in *Cj0440c* mutants (SM and RM) were involved in flagellar assembly, including 11 genes (*flaB*, *flgE*/*E2*/L/K/H/*G*/*G2*/*I*, *flgK*, *flgL*, *fliK*) in SM and 4 genes (*flaB*/*G*, *flgD*, *fliD*) in RM (**Figure 1**). The down-regulation of those flagella-associated genes in *Cj0440c* mutants can reasonably explain why SM and RM lose one or two sides of filament. The reduced colonization of *Cj0440c* mutants may result from the down-regulation of flagella genes.

A second group of genes (*pseB, pseC, neuB2* and *neuC2*) down-regulated in SM were involved in *O*-linked glycosylation which was also essential for flagellin assembly. The *pseB/C* in *C. jejuni* contribute in glycosylation modifications of flagellin, often by non-specifically modifying the surface-exposed Thr, Ser, and Tyr residues of filament proteins FlaA and FlaB (Ewing et al., 2009). While *neuB2/C2* requires in *O*-linked glycosylation which may contribute to flagella antigen diversity of *Campylobacter* (Linton et al., 2000; Sundaram et al., 2004; Tabei et al., 2009). The down-regulation of these glycosylation-associated genes in SM suggested that *Cj0440c* may play an important role in flagella assembly.

Several hypothetical genes (*Cj1026c, Cj1242, Cj1464, Cj1465* and *Cj0391c*) were down-regulated in SM or RM. The Cj1026c (FlgP) was essential for motility of *C. jejuni* and possible encode the promoter of *flaG* (Sommerlad and Hendrixson, 2007). The Cj1464 (FlgM) may regulate temperature-dependent FlgM/FliA complex formation and flagella length of *C. jejuni* (Wösten et al., 2010). The *Cj0391c* generally co-expressed with flagella-associated genes and involved in biofilm formation of *Campylobacter* (Kalmokoff et al., 2006). The down-regulation of these genes suggested that *Cj0440c* may be closely associated with flagella biosynthesis and assembly.

All our data showed that *Cj0440c* may have close relationship with flagella biosynthesis and assembly, however, the precise role of *Cj0440c* in flagella formation pathway is yet to be determined. Flagellar biogenesis in *C. jejuni* requires three distinct sigma factors, including σ^{80}, σ^{54} (or RpoN) and σ^{28} (or FliA) (McCarter, 2006; Anderson et al., 2010). The FlgS/FlgR two-component system is required for transcription of the RpoN regulon (Joslin and Hendrixson, 2009). The FliK likely part of a negative feedback loop that turns off expression of σ^{54}-dependent genes (Ryan et al., 2005; Kamal et al., 2007). The FlgM (anti σ^{28}) may negatively regulate the class III motility genes (Wang et al., 2005). The present study showed that the transcription of *fliK* was down-regulated in SM and the transcription of *flgM* (*Cj1464*) was down-regulated in RM. The down-regulation of *fliK* and *flgM* can influence the down-regulation of class II and class III motility. The roles of *Cj0440c* on flagellar genes are complex and further investigations are required.

The transcriptional change of majority parts of the genes was similar in both SM and RM except for few differences. The *flaA* and *Cj1632c* were up-regulated and *O*-linked glycosylation was down-regulated only in SM, while *katA*, encoding a sole catalase, was down-regulated in RM but not in SM. The flagellar filaments of *Campylobacter* spp. were composed primarily by FlaA and FlaB flagellin (Guerry et al., 1991). The *flaA* was merely up-regulated in SM but *flaB* was down-regulated in both SM and RM. Findings of our study suggested that that the role *Cj0440c* on transcription of FlaA and FlaB flagellin are different in Ery^s and in Ery^r *C. jejuni*. The *katA* involves in oxidative stress and ROS defense which was essential for intra-macrophage persistence and environmental stress survival of *Campylobacter* (Farr and Kogoma, 1991; Day et al., 2000; Vliet et al., 2002; Flint et al., 2012). The down-regulation of *katA* in RM suggested that *Cj0440c* may interact with *katA* to improve their survival capacity in environmental stress.

The macrolide-resistance in *C. jejuni* generally suffered a fitness cost, however, several other factors may compensate the adaptation weakness (Björkman and Andersson, 2000; Kugelberg et al., 2006; Nilsson et al., 2006; Hao et al., 2010, 2013; Luangtongkum et al., 2012). Our previous study showed that *Cj0440c* was over-expressed in the Ery^r *C. jejuni* (Hao et al., 2013). The result from the present study suggests that *Cj0440c* plays a role in compensating the fitness cost of erythromycin resistance

through the positive relationship with flagellar and other related genes.

CONCLUSION

Cj0440c regulates expression of genes involved in flagella biosynthesis and assembly which consequently affects the growth or colonization of *C. jejuni in vitro* and *in vivo* environment.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XF, HH, YW, XW, and ZY. Performed the experiments: XF and HH. Analyzed the data: XF, HH, JH, SF, GC, LH, and ZY. Contributed reagents/materials/analysis tools: ZY, ZL, MD, and HH. Wrote the paper: HH, XF, JH, SF, and ZY.

FUNDING

This work was supported by National Key research and development program (2016YFD0501302), National Natural Science Foundation of China (31101856), National Basic Research Program of China (2013CB127200), Morning program of Wuhan in China (2015070404010191), Fundamental Research Funds for the Central Universities (2662015PY035), and National Program for Risk Assessment of Quality and Safety of Livestock and Poultry Products (GJFP2016008). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. *Disclaimer***:** The opinions expressed in this manuscript are solely the responsibility of the authors and do not necessarily represent the official views and policy of the US Food and Drug Administration. Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

ACKNOWLEDGMENTS

We thank Qijing Zhang and Zhangqi Shen from Iowa State University in USA, Congming Wu and Yang Wang from China Agricultural University for kindly providing the vector plasmids including plasmids pGEM-T and pMW10; and we thank Maojun Zhang from Chinese Center for Disease Control and Prevention for kindly providing *C. jejuni* NCTC11168. We thank Drs. Kidon Sung and Bijay Khajanchi from US FDA National Center for Toxicological Research for their critical review of manuscript.

REFERENCES

Akiyama, M., and Nakashima, H. (1996). Molecular cloning of thi-4, a gene necessary for the biosynthesis of thiamine in *Neurospora crassa*. *Curr. Genet.* 30, 62–67. doi: 10.1007/s002940050101

Anderson, J. K., Smith, T. G., and Hoover, T. R. (2010). Sense and sensibility: flagellum-mediated gene regulation. *Trends Microbiol.* 18, 30–37. doi: 10.1016/j.tim.2009.11.001

Barrero-Tobon, A. M., and Hendrixson, D. R. (2014). Flagellar biosynthesis exerts temporal regulation of secretion of specific *Campylobacter jejuni* colonization and virulence determinants. *Mol. Microbiol.* 93, 957–974. doi: 10.1111/mmi.12711

Björkman, J., and Andersson, D. I. (2000). The cost of antibiotic resistance from a bacterial perspective. *Drug Resist. Updat.* 3, 237–245. doi: 10.1054/drup.2000.0147

Byers, D. M., and Gong, H. (2007). Acyl carrier protein: structure-function relationships in a conserved multifunctional protein family. *Biochem. Cell Biol.* 85, 649–662. doi: 10.1139/O07-109

Cui, Y., Chatterjee, A., Yang, H., and Chatterjee, A. K. (2008). Regulatory network controlling extracellular proteins in *Erwinia carotovora* subsp. carotovora: FlhDC, the master regulator of flagellar genes, activates *rsmB* regulatory RNA production by affecting gacA and hexA (*lrhA*) expression. *J. Bacteriol.* 190, 4610–4623. doi: 10.1128/JB.01828-07

Day, W. A., Sajecki, J. L., Pitts, T. M., and Joens, L. A. (2000). Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect. Immun.* 68, 6337–6345. doi: 10.1128/IAI.68.11.6337-6345.2000

ECDC, EFSA, EMEA and SCENIHR (2009). Joint opinion on antimicrobial resistance (AMR) focused on zoonotic infections. *EFSA J.* 7, 1372.

Epps, S. V., Harvey, R. B., Hume, M. E., Phillips, T. D., Anderson, R. C., and Nisbet, D. J. (2013). Foodborne *Campylobacter*: infections, metabolism, pathogenesis and reservoirs. *Int. J. Environ. Res. Public Health* 10, 6292–6304. doi: 10.3390/ijerph10126292

Ewing, C. P., Andreishcheva, E., and Guerry, P. (2009). Functional characterization of flagellin glycosylation in *Campylobacter jejuni* 81-176. *J. Bacteriol.* 191, 7086–7093. doi: 10.1128/JB.00378-09

Farr, S. B., and Kogoma, T. (1991). Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* 55, 561–585.

Flint, A., Sun, Y.-Q., and Stintzi, A. (2012). *Cj1386* is an ankyrin-containing protein involved in heme trafficking to catalase in *Campylobacter jejuni*. *J. Bacteriol.* 194, 334–345. doi: 10.1128/JB.05740-11

Gibreel, A., Kos, V. N., Keelan, M., Trieber, C. A., Levesque, S., Michaud, S., et al. (2005). Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*: molecular mechanism and stability of the resistance phenotype. *Antimicrob. Agents Chemother.* 49, 2753–2759. doi: 10.1128/AAC.49.7.2753-2759.2005

Gibreel, A., and Taylor, D. E. (2006). Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother.* 58, 243–255. doi: 10.1093/jac/dkl210

Guerry, P. (2007). *Campylobacter* flagella: not just for motility. *Trends Microbiol.* 15, 456–461. doi: 10.1016/j.tim.2007.09.006

Guerry, P., Alm, R., Power, M., Logan, S., and Trust, T. (1991). Role of two flagellin genes in Campylobacter motility. *J. Bacteriol.* 173, 4757–4764. doi: 10.1128/jb.173.15.4757-4764.1991

Guerry, P., Ewing, C. P., Schirm, M., Lorenzo, M., Kelly, J., Pattarini, D., et al. (2006). Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence. *Mol. Microbiol.* 60, 299–311. doi: 10.1111/j.1365-2958.2006.05100.x

Guo, B., Wang, Y., Shi, F., Barton, Y. W., Plummer, P., Reynolds, D. L., et al. (2008). CmeR functions as a pleiotropic regulator and is required for optimal colonization of *Campylobacter jejuni* in vivo. *J. Bacteriol.* 190, 1879–1890. doi: 10.1128/JB.01796-07

Haghjoo, E., and Galan, J. E. (2007). Identification of a transcriptional regulator that controls intracellular gene expression in *Salmonella* Typhi. *Mol. Microbiol.* 64, 1549–1561. doi: 10.1111/j.1365-2958.2007.05754.x

Hao, H., Dai, M., Wang, Y., Chen, D., and Yuan, Z. (2010). Quantification of mutated alleles of 23S rRNA in macrolide-resistant *Campylobacter* by TaqMan real-time polymerase chain reaction. *Foodborne Pathog. Dis.* 7, 43–49. doi: 10.1089/fpd.2009.0339

Hao, H., Liu, J., Kuang, X., Dai, M., Cheng, G., Wang, X., et al. (2015). Identification of *Campylobacter jejuni* and determination of point mutations associated with macrolide resistance using a multiplex TaqMan MGB real-time PCR. *J. Appl. Microbiol.* 118, 1418–1425. doi: 10.1111/jam.12793

Hao, H., Yuan, Z., Shen, Z., Han, J., Sahin, O., Liu, P., et al. (2013). Mutational and transcriptomic changes involved in the development of macrolide resistance in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* 57, 1369–1378. doi: 10.1128/AAC.01927-12

Holscher, T., and Gorisch, H. (2006). Knockout and overexpression of pyrroloquinoline quinone biosynthetic genes in *Gluconobacter oxydans* 621H. *J. Bacteriol.* 188, 7668–7676. doi: 10.1128/JB.01009-06

Hughes, R. A., and Cornblath, D. R. (2005). Guillain-Barré syndrome. *Lancet* 366, 1653–1666. doi: 10.1016/S0140-6736(05)67665-9

Jeon, B., Wang, Y., Hao, H., Barton, Y.-W., and Zhang, Q. (2011). Contribution of CmeG to antibiotic and oxidative stress resistance in *Campylobacter jejuni*. *J. Antimicrob. Chemother.* 66, 79–85. doi: 10.1093/jac/dkq418

Joslin, S. N., and Hendrixson, D. R. (2009). Activation of the *Campylobacter jejuni* FlgSR two-component system is linked to the flagellar export apparatus. *J. Bacteriol.* 191, 2656–2667. doi: 10.1128/JB.01689-08

Kalmokoff, M., Lanthier, P., Tremblay, T.-L., Foss, M., Lau, P. C., Sanders, G., et al. (2006). Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. *J. Bacteriol.* 188, 4312–4320. doi: 10.1128/JB.01975-05

Kamal, N., Dorrell, N., Jagannathan, A., Turner, S. M., Constantinidou, C., Studholme, D. J., et al. (2007). Deletion of a previously uncharacterized flagellar-hook-length control gene fliK modulates the sigma54-dependent regulon in *Campylobacter jejuni*. *Microbiology* 153, 3099–3111. doi: 10.1099/mic.0.2007/007401-0

Kassem, I. I., Khatri, M., Esseili, M. A., Sanad, Y. M., Saif, Y. M., Olson, J. W., et al. (2012). Respiratory proteins contribute differentially to *Campylobacter jejuni*'s survival and *in vitro* interaction with hosts' intestinal cells. *BMC Microbiol.* 12:258. doi: 10.1186/1471-2180-12-258

Kugelberg, E., Kofoid, E., Reams, A. B., Andersson, D. I., and Roth, J. R. (2006). Multiple pathways of selected gene amplification during adaptive mutation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17319–17324. doi: 10.1073/pnas.0608309103

Linton, D., Karlyshev, A. V., Hitchen, P. G., Morris, H. R., Dell, A., Gregson, N. A., et al. (2000). Multiple N-acetyl neuraminic acid synthetase (neuB) genes in *Campylobacter jejuni*: identification and characterization of the gene involved in sialylation of lipo-oligosaccharide. *Mol. Microbiol.* 35, 1120–1134. doi: 10.1046/j.1365-2958.2000.01780.x

Luangtongkum, T., Shen, Z., Seng, V. W., Sahin, O., Jeon, B., Liu, P., et al. (2012). Impaired fitness and transmission of macrolide-resistant *Campylobacter jejuni* in its natural host. *Antimicrob. Agents Chemother.* 56, 1300–1308. doi: 10.1128/AAC.05516-11

Mace, S., Haddad, N., Zagorec, M., and Tresse, O. (2015). Influence of measurement and control of microaerobic gaseous atmospheres in methods for *Campylobacter* growth studies. *Food Microbiol.* 52, 169–176. doi: 10.1016/j.fm.2015.07.014

Matsunami, H., Barker, C. S., Yoon, Y. H., Wolf, M., and Samatey, F. A. (2016). Complete structure of the bacterial flagellar hook reveals extensive set of stabilizing interactions. *Nat. Commun.* 7:13425. doi: 10.1038/ncomms13425

McCarter, L. L. (2006). Regulation of flagella. *Curr. Opin. Microbiol.* 9, 180–186. doi: 10.1016/j.mib.2006.02.001

Nilsson, A. I., Zorzet, A., Kanth, A., Dahlström, S., Berg, O. G., and Andersson, D. I. (2006). Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6976–6981. doi: 10.1073/pnas.0602171103

Pang, A. S., Nathoo, S., and Wong, S. L. (1991). Cloning and characterization of a pair of novel genes that regulate production of extracellular enzymes in *Bacillus subtilis*. *J. Bacteriol.* 173, 46–54. doi: 10.1128/jb.173.1.46-54.1991

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Riddle, M. S., Sanders, J. W., Putnam, S. D., and Tribble, D. R. (2006). Incidence, etiology, and impact of diarrhea among long-term travelers (US military and similar populations): a systematic review. *Am. J. Trop. Med. Hyg.* 74, 891–900.

Ryan, K. A., Karim, N., Worku, M., Penn, C. W., and O'Toole, P. W. (2005). *Helicobacter pylori* flagellar hook-filament transition is controlled by a FliK functional homolog encoded by the gene HP0906. *J. Bacteriol.* 187, 5742–5750. doi: 10.1128/JB.187.16.5742-5750.2005

Samuel, M. C., Vugia, D. J., Shallow, S., Marcus, R., Segler, S., McGivern, T., et al. (2004). Epidemiology of sporadic *Campylobacter* infection in the United States and declining trend in incidence, FoodNet 1996-1999. *Clin. Infect. Dis.* 38(Suppl. 3), S165–S174. doi: 10.1086/381583

Scharff, R. L. (2012). Economic burden from health losses due to foodborne illness in the United States. *J. Food Prot.* 75, 123–131. doi: 10.4315/0362-028X.JFP-11-058

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Front. Microbiol.* 2:200. doi: 10.3389/fmicb.2011.00200

Sommerlad, S. M., and Hendrixson, D. R. (2007). Analysis of the roles of FlgP and FlgQ in flagellar motility of *Campylobacter jejuni*. *J. Bacteriol.* 189, 179–186. doi: 10.1128/JB.01199-06

Sundaram, A. K., Pitts, L., Muhammad, K., Wu, J., Betenbaugh, M., Woodard, R. W., et al. (2004). Characterization of N-acetylneuraminic acid synthase isoenzyme 1 from *Campylobacter jejuni*. *Biochem. J.* 383, 83–89. doi: 10.1042/BJ20040218

Tabei, S. M., Hitchen, P. G., Day-Williams, M. J., Merino, S., Vart, R., Pang, P. C., et al. (2009). An *Aeromonas caviae* genomic island is required for both O-antigen lipopolysaccharide biosynthesis and flagellin glycosylation. *J. Bacteriol.* 191, 2851–2863. doi: 10.1128/JB.01406-08

Toyama, H., Fukumoto, H., Saeki, M., Matsushita, K., Adachi, O., and Lidstrom, M. E. (2002). PqqC/D, which converts a biosynthetic intermediate to pyrroloquinoline quinone. *Biochem. Biophys. Res. Commun.* 299, 268–272. doi: 10.1016/S0006-291X(02)02603-7

Toyama, H., Nishibayashi, E., Saeki, M., Adachi, O., and Matsushita, K. (2007). Factors required for the catalytic reaction of PqqC/D which produces pyrroloquinoline quinone. *Biochem. Biophys. Res. Commun.* 354, 290–295. doi: 10.1016/j.bbrc.2007.01.001

Vliet, A. H., Ketley, J. M., Park, S. F., and Penn, C. W. (2002). The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. *FEMS Microbiol. Rev.* 26, 173–186. doi: 10.1111/j.1574-6976.2002.tb00609.x

Wang, Q., Suzuki, A., Mariconda, S., Porwollik, S., and Harshey, R. M. (2005). Sensing wetness: a new role for the bacterial flagellum. *EMBO J.* 24, 2034–2042. doi: 10.1038/sj.emboj.7600668

Weingarten, R. A., Taveirne, M. E., and Olson, J. W. (2009). The dual-functioning fumarate reductase is the sole succinate:quinone reductase in *Campylobacter jejuni* and is required for full host colonization. *J. Bacteriol.* 191, 5293–5300. doi: 10.1128/JB.00166-09

Wösten, M. M., Van Dijk, L., Veenendaal, A. K., De Zoete, M. R., Bleumink-Pluijm, N., and Van Putten, J. P. (2010). Temperature-dependent FlgM/FliA complex formation regulates *Campylobacter jejuni* flagella length. *Mol. Microbiol.* 75, 1577–1591. doi: 10.1111/j.1365-2958.2010.07079.x

Xia, Q., Muraoka, W. T., Shen, Z., Sahin, O., Wang, H., Wu, Z., et al. (2013). Adaptive mechanisms of *Campylobacter jejuni* to erythromycin treatment. *BMC Microbiol.* 13:133. doi: 10.1186/1471-2180-13-133

Young, K. T., Davis, L. M., and Dirita, V. J. (2007). *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat. Rev. Microbiol.* 5, 665–679. doi: 10.1038/nrmicro1718

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Hao, Fang, Han, Foley, Wang, Cheng, Wang, Huang, Dai, Liu and Yuan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.