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

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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 (H2O2) (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 
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% O2, 10% CO2, and 85% N2) 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 ΔCj0440c 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 Cj0440cF2 and Cj0440cR2 (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 Kpnl 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 Kpnl and XbaI. The amplified DNA fragments of inverse PCR and kan were digested with Kpnl 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. 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 mutations, 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.

| Primer name | Primer Sequence (5’ to 3’) | Product size (bp) |
|-------------|-----------------------------|------------------|
| Cj0440c-F2  | AATACCAGAAAGCCGAAC         | 2315             |
| Cj0440c-R2  | CAGGGTTGAAATAGAAGGG          |                  |
| pCJ0440c-U | GGGGTACGATCATCTTCAAAGGAAT Kpnl site | 5100 |
| pCJ0440c-L | GCCTGATAGTTGACAAAAAGAAGAGT XbaI site | 1203 |
| Kan-F       | GCCTGAAATTAGGGCAGACAGCAT |                  |
| Kan-R       | GCCTGAAATTAGGGCAGACAGCAT |                  |

Primers used for real-time qPCR

| Primer name | Primer Sequence (5’ to 3’) | Product size (bp) |
|-------------|-----------------------------|------------------|
| Cj1399cF    | TCCATAAACCGGTGATATCTGCTTT | 125              |
| Cj1399cR    | AAGGCTATTGATGACGCACTAAATAT | 126              |
| Cj1328F     | CTTTATGCGATCTTTGAGAAGCTTA | 79               |
| Cj1328R     | GCCACATAAAAGCACTAAGGG       |                  |
| Cj1294F     | GCCTGAAAACACGCTTGTGATTT    | 75               |
| Cj1294R     | TTTCATGGACACTAATGTGCTTGTA |                  |
| Cj1338cF    | TTACACTTGGTGATACCTGACCTAA | 75               |
| Cj1338cR    | TGCTCAGGAGGCGGCTGA          |                  |
| Cj0043F     | GGGTTCCTATGTCGGAAAGTGA     | 75               |
| Cj0043R     | GCCCTTAAACACCCAAAAAAAT      | 138              |
| Cj0697F     | TTGCTCAGAACCACAGTGAGTGA    |                  |
| Cj0697R     | TGCTGACATTGCTTCGAGATTA     |                  |
| Cj1242F     | AAGACAGTTGATCTTGCTGTC      | 143              |
| Cj1242R     | ATGGTTCTGCGATTTCTCTG        |                  |
| Cj1385F     | GGAAGACTGCGAGGCTTGGAGA     | 83               |
| Cj1385R     | TGAGTATGAGAGAATCAGGGAAATT |                  |
| Cj1464F     | CGAGTAAATCGCAGACAGC        | 69               |
| Cj1464R     | TGCGACAGGCGTGTCAGTGGTGTTTT |                  |
| q16SR       | GCGGTATTGCGGCTTCTGAGATT    | 199              |
| q16SF       | GCGGTATTGCGGCTTCTGAGATT    |                  |
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/blaA  | Flagellin                                          | 2.1         |
|                                 | Cj1338c/blaB  | Flagellin                                          | -3.5        |
|                                 | Cj1729c/fgE2  | Flagellar hook protein FgE                         | -2.2        |
|                                 | Cj0687c/fgL   | Flagellar hook-associated protein FgL              | -2.4        |
|                                 | Cj1466/fgK    | Flagellar hook-associated protein FgK              | -3.3        |
|                                 | Cj0043/fgE    | Flagellar hook protein                             | -3.5        |
|                                 | Cj1462/fgf    | Flagellar basal body P-ring protein                | -3.0        |
|                                 | Cj0698/fgG    | Flagellar basal body rod protein FgG               | -3.2        |
|                                 | Cj0687c/fgH   | Flagellar basal body L-ring protein                | -3.2        |
|                                 | Cj0697/fgG2   | Flagellar basal-body rod protein                   | -3.6        |
|                                 | Cj0041/flK    | Putative flagellar hook-length control protein     | -4.5        |
| Carbohydrate metabolism         | Cj1327/neuB2  | N-acetylmuraminic acid synthetase                  | -3.1        |
|                                 | Cj1228/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                                      | Fold change |
|---------------------------------|---------------|----------------------------------------------------|-------------|
| Target gene                     | Cj0440c       | Putative transcriptional regulator                 | -35.2       |
| Cell motility/signal transduction| Cj1338c/blaB  | Flagellin                                          | -2.3        |
|                                 | Cj0647/fgA    | Flagellar protein FiaG                             | -2.3        |
|                                 | Cj0648/fgD    | Flagellar hook-associated protein FgD              | -2.4        |
|                                 | Cj0042/fgD    | Flagellar hook-associated protein FgD              | -2.7        |
| Energy metabolism               | Cj1385/katA   | Catalase                                           | -2.5        |
| Hypothetical proteins           | Cj1464/fgM    | 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 RNasey Protect Mini Kit (Qiagen) and purified using NucleoSpin 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 database¹ and assigned accession number GSE49255 and GSE49256.

¹http://www.ncbi.nlm.nih.gov/geo/database
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, chloramphenicol, 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
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 10⁹ 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 10⁹ 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.

**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 (SYKK 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*, *flgF*, *flgG*, *flgH*, *flgL*, *flgK*, *flgL*, and *flgI*) are possible involve in flagellar assembly; 2 genes (*pseB* and *pseC*) in carbohydrate metabolism; 2 genes (*neuB* 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*, *flfD*, *flaG* and *flaB*),

### Single and Competitive Colonization in Chicken

Newly hatched broiler chickens (White Leghorns) were purchased from Zhengda Limited Company (Wuhan, China).

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

| Strains | 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 (AZI), tetracycline (TET), ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN), ceftriaxone (CRO) and olaquindox (OLA).
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 caption is 1 µm.

FIGURE 4 | The growth curve of Cj0440c mutants (SM and RM) and their parental strains (S and R).
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, neuB2/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.

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 \textit{Cj0440c} mutants (SM and RM) showed a significant reduction in colonization on 12 days' post-inoculation (Figure 5).

When the two pairs of \textit{C. jejuni} strains (SMK& and RM&K) were infected chickens with one pair at a time, \textit{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**

\textit{Campylobacter jejuni} is a very common foodborne pathogen in the developed world. Its biology and pathogenicity is largely unknown (Young et al., 2007). \textit{Cj0440c} is a putative transcriptional regulator and an increased transcriptional level expression was detected in the erythromycin-resistant \textit{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., \textit{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 methyloptrophs and the glucose dehydrogenase (Toyama et al., 2002, 2007).

In \textit{E. coli}, PQQ biosynthesis may be affected by \textit{tdlD} gene which encodes a peptidase involved in processing of small peptides (Holscher and Gorisch, 2006). The \textit{tdlD} may lead to chromosomal DNA relaxation and subsequent derepression of \textit{cdtB} and \textit{legR} 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 \textit{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 \textit{Cj0440c} mutants (SM and RM) were involved in flagellar assembly, including 11 genes (\textit{flaB}, \textit{flgE}/\textit{E2}/\textit{L}/\textit{K}/\textit{H}/\textit{G}/\textit{G2}/\textit{I}, \textit{flgK}, \textit{flgL}, \textit{flIK}) in SM and 4 genes (\textit{flaB}/\textit{G}, \textit{flgD}, \textit{flID}) in RM (Figure 1). The down-regulation of these flagella-associated genes in \textit{Cj0440c} mutants can reasonably explain why SM and RM lose one or two sides of filament. The reduced colonization of \textit{Cj0440c} mutants may result from the down-regulation of flagella genes.

A second group of genes (\textit{pseB}, \textit{pseC}, \textit{neuB2} and \textit{neuC2}) down-regulated in SM were involved in O-linked glycosylation which was also essential for flagellin assembly. The \textit{pseB/C} in \textit{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 \textit{neuB2/C2} requires in O-linked glycosylation which may contribute to flagella antigen diversity of \textit{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 \textit{Cj0440c} may play an important role in flagella assembly.

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

All our data showed that \textit{Cj0440c} may have close relationship with flagella biosynthesis and assembly, however, the precise role of \textit{Cj0440c} in flagella formation pathway is yet to be determined. Flagellar biogenesis in \textit{C. jejuni} requires three distinct sigma factors, including $\sigma^{36}$, $\sigma^{54}$ (or RpoN) and $\sigma^{28}$ (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 FlIA likely part of a negative feedback loop that turns off expression of $\sigma^{54}$-dependent genes (Ryan et al., 2005; Kamal et al., 2004). The FlgM (anti $\sigma^{28}$) may negatively regulate the class III motility genes (Wang et al., 2005). The present study showed that the transcription of \textit{flk} was down-regulated in SM and the transcription of \textit{flgM} (\textit{Cj1464}) was down-regulated in RM. The down-regulation of \textit{flk} and \textit{flgM} can influence the down-regulation of class II and class III motility. The roles of \textit{Cj0440c} on flagellar genes are complex and further investigations are required.

The transcrional change of majority parts of the genes was similar in both SM and RM except for few differences. The \textit{flaA} and \textit{Cj1632c} were up-regulated and O-linked glycosylation was down-regulated only in SM, while \textit{kata}, encoding a sole catalase, was down-regulated in RM but not in SM. The flagellar filaments of \textit{Campylobacter} spp. were composed primarily by FlAA and FlAB flagellin (Guerry et al., 1991). The \textit{flaA} was merely up-regulated in SM but \textit{flaB} was down-regulated in both SM and RM. Findings of our study suggested that the role \textit{Cj0440c} on transcription of \textit{FlaA} and \textit{FlaB} flagellin are different in Ery and in Ery'. The \textit{kata} involves in oxidative stress and ROS defense which was essential for intra-macrophage persistence and environmental stress survival of \textit{Campylobacter} (Farr and Kogoma, 1991; Day et al., 2006; Vliet et al., 2002; Flint et al., 2012). The down-regulation of \textit{kata} in RM suggested that \textit{Cj0440c} may interact with \textit{kata} to improve their survival capacity in environmental stress.

The macrolide-resistance in \textit{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 \textit{Cj0440c} was over-expressed in the Ery' \textit{C. jejuni} (Hao et al., 2013). The result from the present study suggests that \textit{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.

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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.

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