Divergent Distribution of the Sensor Kinase CosS in Non-Thermotolerant Campylobacter Species and Its Functional Incompatibility with the Response Regulator CosR of Campylobacter jejuni

Sunyoung Hwang\textsuperscript{2a}, William G. Miller\textsuperscript{3}, Sangryeo Ryu\textsuperscript{2a}, Byeonghwa Jeon\textsuperscript{1*}

\textsuperscript{1} School of Public Health, University of Alberta, Edmonton, Alberta, Canada, \textsuperscript{2} Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, and Center for Food and Bioconvergence, Seoul National University, Seoul, Korea, \textsuperscript{3} U.S. Department of Agriculture, Agricultural Research Service, Western Regional Research Center, Albany, California, United States of America

Abstract

Two-component signal transduction systems are commonly composed of a sensor histidine kinase and a cognate response regulator, modulating gene expression in response to environmental changes through a phosphorylation-dependent process. CosR is an OmpR-type response regulator essential for the viability of Campylobacter jejuni, a major foodborne pathogenic species causing human gastroenteritis. Although CosR is a response regulator, its cognate sensor kinase has not been identified in \textit{C. jejuni}. In this study, DNA sequence analysis of the \textit{cosR} flanking regions revealed that a gene encoding a putative sensor kinase, which we named \textit{cosS}, is prevalent in non-thermotolerant \textit{Campylobacter} \textit{spp.}, but not in thermotolerant campylobacters. Phosphorylation assays indicated that \textit{C. fetus} \textit{cosS} rapidly autophosphorylates and then phosphorylates \textit{C. fetus} CosR, suggesting that the CosRS system constitutes a paired two-component signal transduction system in \textit{C. fetus}. However, \textit{C. fetus} \textit{cosS} does not phosphorylate \textit{C. jejuni} CosR, suggesting that CosR may have different regulatory cascades between thermotolerant and non-thermotolerant \textit{Campylobacter} species. Comparison of CosR homolog amino acid sequences showed that the conserved phosphorylation residue (DS1), which is present in all non-thermotolerant \textit{Campylobacter} \textit{spp.}, is absent from the CosR homologs of thermotolerant \textit{Campylobacter} species. However, \textit{C. jejuni} CosR was not phosphorylated by \textit{C. fetus} CosS even after site-directed mutagenesis of NS1D, implying that \textit{C. jejuni} CosR may possibly function phosphorylation-independently. In addition, the results of \textit{cosS} mutational analysis indicated that CosS is not associated with the temperature dependence of the \textit{Campylobacter} \textit{spp.} despite its unique divergent distribution only in non-thermotolerant campylobacters. The findings in this study strongly suggest that thermotolerant and non-thermotolerant \textit{Campylobacter} \textit{spp.} have different signal sensing mechanisms associated with the CosR regulation.

Introduction

Campylobacter \textit{spp.} are associated with various forms of infectious diseases in animals and humans (e.g., infectious infertility and abortion in cattle and gastroenteritis in humans) \cite{1}. Within the \textit{Campylobacter} genus, most species are microaerophilic and grow at \(\sim 35-37\)\(^{\circ}\)C; however, thermotolerant species, such as \textit{Campylobacter jejuni}, \textit{Campylobacter coli}, \textit{Campylobacter lari} and \textit{Campylobacter upsaliensis}, are able to grow at \(42^{\circ}\)C and constitute a distinct assemblage in the phylogenetic tree of \textit{Campylobacter} \cite{2}. Among multiple \textit{Campylobacter} \textit{spp.}, thermotolerant \textit{C. jejuni} account for >\textit{90}\% of human campylobacteriosis, resulting in fever, diarrhea, and in some cases Guillain-Barré syndrome as a post-infection complication \cite{3}. Since the optimal growth temperature of \textit{C. jejuni} (i.e., \(42^{\circ}\)C) is close to the body temperature of avian species \cite{4}, \textit{C. jejuni} colonizes the gastrointestinal tracts of poultry, but as a commensal organism without causing any clinical symptoms \cite{5}. Due to this, most human infections with \textit{Campylobacter} are caused by the consumption of contaminated poultry \cite{6}.

Despite \textit{C. jejuni}'s fastidious nature, increasing numbers of human campylobacteriosis cases around the world suggest that this pathogenic bacterium may have many, but yet-unidentified, adaptation mechanisms to survive under harsh environmental conditions during its transmission from animal reservoirs, particularly poultry, to humans. To sense and respond to environmental changes by altering gene expression, bacteria possess efficient regulatory mechanisms, such as two-component regulatory systems (TCRSs) \cite{7,8}. TCRSs are typically composed of a sensor histidine kinase and a cognate response regulator \cite{7,8}. In response to appropriate environmental stimulus, a sensor kinase
auto-phosphorylates at a histidine residue and subsequently transfers the phosphoryl group to an aspartic acid residue in its cognate response regulator. The phosphorylation status of a response regulator is associated with its conformational change and affects its DNA-binding properties, which ultimately affects gene expression [9]. This helps bacteria adapt to environmental changes. The genome sequence of C. jejuni NCTC 11168 identified the presence of seven histidine kinases and 12 response regulators [10]. Interestingly, most TCRSs in C. jejuni are known to be involved in various pathogenic characteristics of C. jejuni, including bacterial motility, animal colonization, biofilm formation and bile acid resistance [11–16].

CosR is an OmpR-type response regulator essential for the viability of C. jejuni [14,17], and its homologs are found predominantly in ε-proteobacteria, such as Campylobacter, Helicobacter and Weilella [18]. In our previous studies, we revealed that CosR plays an important role in C. jejuni’s stress resistance by regulating the expression of key determinants of oxidative stress response and antibiotic resistance [18,19]. Based on the genome sequence of C. jejuni, there is no sensor kinase gene in the vicinity of cosR in C. jejuni, leaving a question on whether CosR is an orphan regulator or functionally linked to an unknown histidine kinase. In this study, we report that CosS, the cognate histidine kinase of CosR, is well conserved and present in non-thermotolerant Campylobacter spp., but absent from thermotolerant Campylobacter species. However, CosS from non-thermotolerant Campylobacter spp. does not phosphorylate C. jejuni CosR, suggesting that CosS in non-thermotolerant Campylobacter spp. is not functionally compatible with the response regulator CosR in C. jejuni despite its unique genetic organization.

Materials and Methods

Bacterial strains and culture conditions

C. jejuni subsp. jejuni NCTC 11168 and C. fetus subsp. fetus 82-40 are genome-sequenced strains and were used in this study. C. jejuni NCTC 11168 was routinely grown at 42°C on Mueller-Hinton (MH; Difco) media microaerobically (6% O2, 7% CO2, 10% H2, and 83% N2), and C. fetus 82-40 was cultured at 37°C on Brain Heart Infusion (BHI; Difco) media in a gas condition (10% CO2, 10% H2, and 80% N2). The different gas compositions were approximately 0.5 at 600 nm at 37°C.

Mutation and complementation of cosS in C. fetus, and heterogeneous expression of cosS in C. jejuni

A cosS knockout mutant was constructed in C. fetus 82-40 by using a suicide plasmid as described previously [20]. Briefly, cosS and its flanking region were amplified with the primers fetus_cosS_F (Xba): GGA GCT TCT AGA TGC TAT TGG G and fetus_cosS_R (Xba): AGA CAT CTA GAA CCT TTC AGT AC, and was cloned into an XbaI site on pUC19. The chloramphenicol resistance cassette (cat) amplified from pRY112 [21] was inserted into cosS on pUC19 to generate pUC19-cosS-cat, and the orientation of the antibiotic marker was confirmed by sequencing. After introducing the constructed suicide plasmid by electroporation, the cosS mutant was selected by growing on MH agar plates supplemented with chloramphenicol (10 μg ml⁻¹). For the cosS complementation of the C. fetus cosS mutant and the heterogeneous expression of cosS in C. jejuni, the cosS gene was amplified from C. fetus and integrated into a non-coding spacer region of rRNA gene clusters in the chromosome of the C. fetus cosS mutant and C. jejuni using a methodology reported previously [22]. Briefly, amplified DNA fragments of cosS and its flanking region was cloned into an XbaI site of pEMB that carries an RNA gene cluster and a kanamycin resistance cassette [16]. The plasmids were delivered to the C. fetus cosS mutant or C. jejuni strains by electroporation.

Purification of recombinant proteins of C. fetus CosR, C. jejuni CosR, CosR_N51D and the receiver domain of C. fetus CosS

To prepare CosR mutant in which an asparagine residue at position 51 was substituted with an aspartate residue (CosR_N51D), pET15b-cosR_N51D was generated by site-directed mutagenesis (QuickChange, Agilent Technologies), using CosR overexpressing plasmid pET15b-CosR constructed in our previous research as a template [18] and the appropriate primers, i151_g_c153_F: ATG GCC ATT AGA CAT TAT GAT TTA GGT AGA GAT TGG ACT TTA CCT GAT GG and i151_g_c153_R: GCC TCA GGT AAA GTC CAA TCT GCT AAA ACT AAA TGA TTA TGT CTA ATG CCG AT. For the purification of C. fetus CosR (CosR_F), the cosR gene C. fetus was PCR-amplified using primer pairs of CosR_F_His(Nde)-F: TTA AAG GAA CAT CTA GAT ATG AGA ATT TGG ATA G & CosR_F_His(Nco): GAT TGG ACT TTA CCT GAT GG and CosR_F_purification of CosR, CosR_J mutant (CosRJ_N51D) and C. fetus CosR (CosR_F) proteins were overexpressed and purified under the native conditions using Ni²⁺ affinity chromatography as previously described [18]. To purify the histidine kinase domain of CosS (trCosS), the kinase active domain of the cosS gene in C. fetus was amplified by PCR using the primers trCosSF_MBP_F (Nco): TGC TTT TAC CTA TAA and trCosSF_MBP_R (Xba): AAA GCC AC. After digestion with NdeI and BamHI, the PCR product was cloned into pET15b, which had been digested with the same enzymes, to generate pET15b-cosR. Histidine-tagged recombinant C. jejuni CosR (rCosR_F), CosR_J mutant (CosRJ_N51D) and C. fetus CosR (rCosR_F) proteins overexpressed and purified under the native conditions using Ni²⁺ affinity chromatography as previously described [18]. To purify the histidine kinase domain of CosS (trCosS), the kinase active domain of the cosS gene in C. fetus was amplified by PCR using the primers TrCosSF_MBP_F (Nco): TGC TTT TAC CTA TAA CCA TGG TTA CCT GAT GG and TrCosSF_MBP_R (Xba): AAA GCC ACT CTA GAC ATT TTG ATC. The resulting products were digested with NdeI and BamHI, and cloned into NotI and XhoI sites of pMBP-parallel [23], generating pMBP-CosS. E. coli BL21 (DE3) carrying plasmid pMBP-CosS was grown to an optical density of approximately 0.5 at 600 nm at 37°C. After induction with 0.1 mM IPTG at 30°C for 5 h, MBP (maltose binding protein) tagged trCosS (MBP-trCosS) was purified under a native condition using an amylose resin.

Autophosphorylation of trCosS

MBP-trCosS (2 μM) was incubated with 10 μCi of [γ⁻³²P]ATP in 20 μl of a buffer containing 50 mM Tris-Cl (pH 8.0), 75 mM KCl, 2 mM MgCl₂, and 1 mM DTT at 37°C for 5 h. At each time point, the reaction was stopped by adding SDS-loading buffer. Proteins were resolved by 10% SDS-PAGE, and the gels were dried and exposed to an imaging plate. The status of autophosphorylation was analyzed with the BAS2500 system (Fuji Film).

Phosphorylation assays of rCosR_F, rCosR_J and CosRJ_N51D by trCosS

In vitro phosphorylation transfer from MBP-trCosS to rCosR_F, rCosR_J or CosRJ_N51D was monitored as described previously [25,26]. Phosphorylation of 2 μM of rCosR_F, rCosR_J and
CosRS in *Campylobacter* Species

CosR\_N51D was achieved by adding the same amount of MBP-trCosS which had been autophosphorylated for 5 min in 20 μL of phosphorylation buffer as described above. The reaction was stopped with SDS-loading buffer after incubation at 37°C for 0.5, 1, 2, 5, 10, 20, or 30 min, and reaction samples were analyzed by SDS-PAGE. After electrophoresis, the gels were dried and autoradiographed.

Oxidative stress susceptibility and aerotolerance tests

After pre-culturing on BHI agar for 16 h, *C. fetus* strains were harvested and the cell suspension was adjusted to an OD\textsubscript{600} of 0.1. Aliquots of bacterial cells were exposed to atmospheric conditions with shaking at 220 rpm for 12 h or at a final concentration of 20 mM paraquat and H\textsubscript{2}O\textsubscript{2} under microaerobic conditions for 2 h. After exposure, viability changes were determined by dotting serially diluted bacterial cultures on agar plates.

Figure 1. Genomic organization of cosR flanking regions of *Campylobacter* species and other bacterial species in ε-Proteobacteria.
Genomic organization of cosR homolog (black arrows) flanking regions shows: (A) the absence of cosS in thermotolerant *Campylobacter* spp.; *C. jejuni* NCTC11168 [GenBank accession number: AL111168.1], *C. coli* RM2228 (AAFL00000000.1), *C. lari* RM2100 (NC\_012039.1), (B) the presence of cosS (gray arrows) in non-thermotolerant *Campylobacter* spp.; *C. fetus* 82-40 (CP000487.1), *C. concisus* 13826 (CP000792.1), *C. curvus* 525.92 (CP000767.1), *C. hominis* ATCC BAA-381 (CP000776.1), (C) different prevalence in the other bacterial species of ε-Proteobacteria; *W. succinogenes* DSM174 (NC\_005090.1), *A. butzleri* RM4018 (CP000361.1), *S. deleyianum* DSM6946 (CP001816.1), *H. pylori* 26695 (NC\_000915.1).

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Figure 2. Amino acid sequence analysis of CosR homologs. (A) Multiple alignment of CosR homologs (GenBank accession number indicated in parentheses) in Campylobacter spp.: C. jejuni CosR (Cj0355c: YP_002343793.1), C. coli CosR (CC00443: WP_002778246.1), C. lari CosR (Cla_0175: YP_002574789.1), C. fetus CosR (CFF8240_0242: YP_891446.1), C. concisus CosR (CFC8240_0242: YP_001466303.1), C. curvus CosR (CCV52592_1693: YP_001408852.1), C. hominis CosR (CHAB381_0745: YP_001406322.1), H. pylori HP1043 (NP_223100.1), H. pullorum HPMG 439 (EEQ62982.1), and H. canadensis HCAN 1051 (EES89763.1). The highly conserved residues and a phosphate-accepting aspartate residue in the receiver domain are indicated by an arrowhead and a star, respectively [27,32,33]. (B) Phylogenetic tree of CosR homologs in campylobacters. The tree was generated by using the MegAlign program (DNASTAR) based on the Jotun-Hein alignment of amino acid sequences of CosR homologs. Other CosR homologs in e-Proteobacteria include Arcobacter butzleri Abu0375 (YP_001489319.1), Sulfurospirillum deleyianum Sde1_0235 (YP_003303308.1), and Wolinella succinogenes WS0306 (NP_906557.1). doi:10.1371/journal.pone.0089774.g002
Electrophoretic mobility shift assay (EMSA)

To perform EMSA, the DNA fragments containing the promoter region of sodB, katA, abpC and one1 in C. jejuni were amplified and labeled with \(\gamma^{32}\text{P}\) ATP (GE Healthcare) as described previously [18,19]. The 0.2 nM of \(\gamma^{32}\text{P}\)-labeled DNA probe was incubated with 3.2 nM concentration of the purified rCosR, rCosR or CosR_N51D protein at 37°C for 15 min in 10 µl of the gel-shift assay buffer (20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM \(\text{NH}_{4}\text{SO}_{4}\), 5 mM DTT, 0.2% Tween 20, 30 mM KCl, 0.1 µg poly (dI-dC)). The reaction mixtures were resolved in a 6% polyacrylamide gel, and the radiolabeled DNA fragments were visualized using the BAS2500 system (Fuji Film).

**Results and Discussion**

Selective prevalence of cosS in non-thermotolerant Campylobacter spp.

CosR is an OmpR-type response regulator encoded by cosR, whose homologs are prevalent in all genome-sequenced Campylobacter species. In our previous study, no sensor kinase gene was found near cosR in the C. jejuni genome, raising a question that CosR may be an orphan response regulator [18]. *H. pylori* HP1043, a CosR homolog, is an orphan response regulator and functions in a phosphorylation-independent manner [27,28]. Currently, nothing is known about the cognate sensor kinase and phosphorylation of CosR in C. jejuni. In this study, DNA sequence analysis of cosS and their flanking regions in Campylobacter spp. revealed that several Campylobacter spp. have a gene downstream of a cosS homolog which encodes a histidine kinase with several highly-conserved and well-known motifs in the cytoplasmic portion, such as the histidine phosphotransfer domain containing the histidine phosphorylation site (at His-190 in C. fetus) and the C-terminal catalytic and ATP-binding domain (Fig. S1). Interestingly, this sensor kinase gene, which we named cosS, is prevalent only in non-thermotolerant Campylobacter spp., including C. fetus, C. hominis, C. curvus, and C. concisus, but not in thermotolerant Campylobacter spp., such as C. jejuni, C. coli and C. lari (Fig. 1A and B). Other members of \(\varepsilon\)-Proteobacteria, such as Wolinella succinogenes, Arcobacter butzleri and Sulfoviridans deleyianum, possess the cosS homologs (Fig. 1C). Like campylobacters, interestingly, the prevalence of the cosS ortholog is dependent on the species in helicobacters. For example, Helicobacter pullorum possesses a cosS ortholog (HPMG440) whereas Helicobacter pylori does not [29]. Furthermore, preliminary genomic sequencing data suggest that cosR is encoded by all campylobacters, whereas cosS is encoded by all validly-described taxa only within the non-thermotolerant group of campylobacters, including C. hyointestinalis, C. lari, C. mucosalis, C. sputorum and C. ureolyticus (unpublished data). The clear difference in cosS prevalence between thermotolerant and non-thermotolerant Campylobacter spp. raised two research questions, whether: (i) CosS is responsible, in part, for the temperature dependence of the two Campylobacter groups; and (ii) CosS in non-thermotolerant Campylobacter spp. might be functionally linked to CosR in C. jejuni.

**Analysis of amino acid sequence of CosR homologs**

Comparison of the CosR homolog amino acid sequences in Campylobacter spp. revealed that the C-terminal DNA-binding domain was highly conserved, but the N-terminal receiver domain was more variable (Fig. 2A). The results of BLAST analysis strongly supported the divergent difference in the amino acid sequences of CosR between thermotolerant and non-thermotolerant campylobacters. CosR homologs shared higher similarity in all thermotolerant clades, depending on the amino acid sequences of

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**Figure 3. Autophosphorylation and phosphotransfer of C. fetus CosS.** (A) Analysis of autophosphorylation of MBP-tagged cytoplasmic domain of the sensor histidine kinase C. fetus CosS (MBP-trCosS). The status of the MBP-trCosS autophosphorylation was analyzed over time after incubation with \(\gamma^{32}\text{P}\) ATP by SDS-gel electrophoresis and autoradiography. The C. fetus rCosR (rCosR_F) and C. jejuni rCosR (rCosR_J) proteins were incubated for 30 min with \(\gamma^{32}\text{P}\) ATP. (B) Phosphorylation of rCosR_F by MBP-trCosS. Autophosphorylation of MBP-trCosS (2 µM) was accomplished by incubation of the protein with \(\gamma^{32}\text{P}\) ATP for 2 min. Time course of phosphotransfer from \(\gamma^{32}\text{P}\)-labeled MBP-trCosS is indicated on top. (C) Non-phosphorylation of C. jejuni rCosR_J and CosR_J mutant (CosRJ_N51D) by MBP-trCosS. doi:10.1371/journal.pone.0009774.g003
the CosR homologs (Fig. 2B). These results show that the similarities of CosR homologs vary between thermotolerant and non-thermotolerant Campylobacter groups despite their ubiquitous presence in all Campylobacter species.

Phosphotransfer between CosS and CosR

To investigate if the sensor kinase CosS is able to autophosphorylate and transfer a phosphate group to its putative response regulator CosR, we chose and examined the CosRS system in C. fetus because the genetic organization of the cosR flanking region in C. fetus is highly similar to that in C. jejuni in comparison with other non-thermotolerant Campylobacter species (Fig. 1). The MBP-tagged cytoplasmic histidine kinase domain of C. fetus CosS was purified and incubated with [γ-32P]ATP. In the presence of radioactive ATP, C. fetus CosS was rapidly autophosphorylated (Fig. 3A), showing that C. fetus CosS possesses autokinase activity, which is a typical property of a two-component sensor kinase. As expected, both C. fetus CosR (CosR_F) and C. jejuni CosR (CosR_J) were not phosphorylated by ATP in the absence of CosS (Fig. 3A). Addition of autophosphorylated C. fetus CosS to CosR_F rapidly dephosphorylated C. fetus CosS and subsequently transmitted the phosphate to its cognate response regulator CosR_F, suggesting that CosS and CosR form a two-component signal transduction system in C. fetus (Fig. 3B). However, C. fetus CosS did not phosphorylate CosR_J (Fig. 3C), suggesting that C. fetus CosS is not functionally coupled to the CosR response regulator of C. jejuni. As shown in Fig. 2A, CosR_J and CosR_F share high similarities in amino acid sequence with highly conserved aspartate residues, with the exception of D51 (Fig. 2A). To examine the role of D51 in phosphorylation, a CosR_J_N51D mutant was generated and used in a phosphorylation assay; however, the introduction of D51 did not make C. jejuni CosR to be phosphorylated by C. fetus CosS (Fig. 3C). It has been reported that deletion of four amino acid residues (51st–54th amino acid residues corresponding to CosR_J; Fig. 2A) in the receiver domain of H. pylori HP1043 rendered HP1043 independent of phosphorylation [27]. Similarly, it would be possible that C. jejuni CosR may function in a phosphorylation-independent manner. Consistent with the phylogenetic analysis (Fig. 2B), the results suggest that the CosR proteins may have different signal transduction systems between thermotolerant and non-thermotolerant Campylobacter species. CosS and CosR constitute a TCRS in non-thermotolerant Campylobacter species, whereas CosR in thermotolerant campylobacters is not functionally coupled to CosS in non-thermotolerant Campylobacter species.

Role of CosS in thermotolerant growth

The distribution of cosS is clearly divergent between thermotolerant and non-thermotolerant Campylobacter groups. Based on the selective prevalence of CosS only in non-thermotolerant Campylobacter spp., we hypothesized that CosS may be associated with Campylobacter’s adaptation to different growth temperatures. To investigate this possibility, we constructed a cosS knockout mutant of C. fetus and a C. jejuni strain heterogenously expressing C. fetus...
cosS, and observed bacterial growth at 37°C and 42°C. However, neither the cosS deletion in C. fetus nor cosS expression in C. jejuni altered bacterial growth compared with each parental strain (Fig. 4A). This suggests that CosS may not contribute to the temperature dependencies of thermotolerant and non-thermotolerant Campylobacter species. The results of the heterogenous expression of cosS in C. jejuni are still consistent with the results of phosphorylation assays. Since CosR_J is not phosphorylated by C. fetus CosS (Fig. 3C), the heterogenous expression of C. fetus CosS did not affect C. jejuni’s regulation of gene expression via CosR. Nevertheless, at this point, we cannot completely exclude a possible role of CosR in the temperature dependent growth of C. jejuni under certain conditions, because the expression level of CosR is more than 4-fold higher at 37°C than at 42°C, suggesting that CosR may be involved in the growth temperature-associated regulation of gene expression [30].

Role of CosS in oxidative stress response

In our previous studies, we demonstrated that CosR plays an important role in the oxidative stress response of C. jejuni [18,19]. Consistently, a very recent study showed that HP1043 mediates the oxidative stress resistance in H. pylori [31]. To examine the effect of a cosS mutation on the oxidative stress resistance and the acetotolerance of C. fetus, a cosS mutant and a cosS-complementation strain were exposed to oxidative stress reagents (20 mM of paraquat and H₂O₂) and atmospheric conditions. Although the acetotolerance of cosS mutant was slightly decreased compared with that of the wild type and the complementation strain (Fig. 4B), the cosS mutation did not affect the resistance of C. fetus against oxidative reagents (Fig. 4C). These results indicate that, unlike C. jejuni CosR, the C. fetus CosRS TCRS may not be involved in oxidative stress resistance. This would be because the two Campylobacter species have different oxidative stress response mechanisms. For example, C. fetus possesses two genes encoding superoxide dismutase (sodB and sodC) [data not shown], while C. jejuni harbors only sodB [10]. We also tried to investigate the impact of a cosR mutation on the oxidative stress resistance of C. fetus. However, cosR appears to be essential in C. fetus, because its knockout mutants were not generated despite our multiple attempts (data not shown). In addition, knockdown of cosR in C. fetus using peptide nucleic acids was not as effective as in C. jejuni (data not shown). Instead, a gel-shift assay was carried out to compare the binding affinity of C. jejuni CosR and C. fetus CosR to the promoters of oxidative stress genes that are regulated by C. jejuni CosR. In this assay, if C. jejuni CosR and C. fetus CosR recognizes similar DNA sequences, their binding efficiencies will be comparable to each other. C. jejuni CosR and CosR_J_N51D bound to the promoters effectively as reported previously [18,19]; however, the binding of C. fetus CosR to the tested promoters was extremely weak (Fig. 5). These findings suggest that the function of CosR may not be similar between C. jejuni and C. fetus. Future investigations are still required to determine the CosRS regulon for a better understanding of its regulatory functions in non-thermotolerant Campylobacter species.

In the present study, we revealed the different prevalence of CosS, the cognate histidine sensor kinase of CosR, between thermotolerant and non-thermotolerant Campylobacter species. The results of phosphorylation assays and amino acid sequence analysis showed that the CosRS system constitutes a paired TCRS in C. fetus, a non-thermotolerant species. However, C. fetus CosS does not phosphorylate C. jejuni CosR, suggesting that CosR may have different regulatory cascades between thermotolerant and non-thermotolerant Campylobacter spp. despite the closely related genetic organization in the cosRS region. In conclusion, the sensor kinase CosS in non-thermotolerant campylobacters is not functionally compatible with the response regulator CosR in C. jejuni, the thermotolerant Campylobacter species of highest public health importance. The results in this study strongly suggest the presence of different signal sensing mechanisms for the CosR regulation between thermotolerant and non-thermotolerant Campylobacter species.

Supporting Information

Figure S1 Amino acid sequence analysis of CosS homologs in non-thermotolerant Campylobacter species.

Figure 5. Binding of C. fetus CosR (rCosR_F), C. jejuni CosR (rCosR_J) and its mutant (CosRJ_N51D) to the promoter regions of genes of the C. jejuni CosR regulon. The binding efficiency of C. fetus CosR is significantly lower than that of C. jejuni CosR. The target genes are selected based on previous reports [18,19].
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Multiple alignment of CosS homologs (GenBank accession number indicated in parentheses) in non-thermotolerant Campylobacter spp.: C. fetus CosS (YP_091447.1), C. concisus CosS (YP_001466302.1), C. curvus CosS (YP_001408853.1), and C. hominis CosS (YP_001406323.1). The predicted conserved domains of histidine sensor kinases (histidine phosphotransfer domain and ATP-binding domain) and the histidine phosphorylation site are indicated by boxes and a star, respectively.

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Author Contributions
Conceived and designed the experiments: WM SR BJ. Performed the experiments: SH. Analyzed the data: SH WM SR BJ. Contributed reagents/materials/analysis tools: SR BJ. Wrote the paper: SH BJ.