

Transcriptional Regulation of the CmeABC Multidrug Efflux Pump and the KatA Catalase by CosR in *Campylobacter jejuni*

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CosR is an essential response regulator in *Campylobacter jejuni*, a major food-borne pathogen causing enteritis worldwide. A transcriptomic analysis performed in this study discovered 93 genes whose transcriptional levels were changed >2-fold due to the repression of CosR expression by antisense peptide nucleic acid. The identified CosR-regulated genes are involved in various cellular functions, such as energy production, protein synthesis and folding, flagellum biogenesis, and lipid metabolism. Interestingly, 17 of the 93 CosR-regulated genes (18.3%) are predicted essential genes, indicating that CosR may participate in the regulation of vital biological processes in *C. jejuni*. In particular, CosR knockdown increased the transcriptional levels of *cmeA*, *cmeB*, and *cmeC* genes, whose protein product (CmeABC) is an important determinant conferring multidrug resistance in *Campylobacter*. Negative regulation of *cmeABC* by CosR was verified by quantitative real-time PCR (qRT-PCR) and P_{*cmeABC*::lacZ} assay. The results of electrophoretic mobility shift assays (EMSAs) and DNase I footprinting assays demonstrated that CosR directly binds to the *cmeABC* promoter. Another notable finding is that CosR regulates the transcription of *kata*, the sole catalase gene in *C. jejuni*. Further characterization with qRT-PCR, the catalase enzyme assay, EMSA, and DNase I footprinting assays successfully demonstrated that CosR affects the *kata* transcription and the catalase activity by direct interactions with the *kata* promoter. The findings in this study clearly demonstrated that CosR regulates resistance mechanisms in *C. jejuni* by controlling the expression of genes involved in oxidative stress defense and extrusion of toxic compounds out of the cell.

Campylobacter jejuni is a leading bacterial cause of human gastroenteritis worldwide. The symptoms of campylobacteriosis include severe abdominal cramp, watery or bloody diarrhea, and in some rare cases Guillain-Barré syndrome as a postinfection complication (21). Although *C. jejuni* infection is usually self-limiting and doesn't require antibiotic treatment, antibiotics are warranted for severe cases of campylobacteriosis (30). However, the increasing prevalence of *Campylobacter* resistant to medically important antibiotics threatens public health. Among multiple mechanisms of antibiotic resistance, drug efflux pumps are considered the major cause of multidrug resistance in pathogenic bacteria (23). In *Campylobacter*, a few multidrug efflux pumps have been identified (1, 17, 27), and CmeABC is considered the most important multidrug efflux pump in this pathogenic bacterium, giving resistance to different classes of antibiotics and bile salts (27).

Aerobiosis inevitably produces reactive oxygen species (ROS), such as the superoxide anion (O₂^{•−}), hydrogen peroxide (H₂O₂) and hydroxyl radicals (HO[•]) as by-products, and ROS can damage intracellular materials (16). Various defense and regulatory mechanisms exist to protect the cell from oxidative stress. *C. jejuni* contains only a single type of catalase (KatA) and superoxide dismutase (SodB) (2), whereas *Escherichia coli* harbors two types of catalase (KatG and KatE) and three kinds of superoxide dismutase (SodA, SodB, and SodC) (6). In enterobacteria, such as *E. coli* and *Salmonella*, oxidative stress response is regulated by the SoxRS and OxyR transcription factors, which sense superoxide and peroxide stresses, respectively (6, 16). Exposure to redox-cycling drugs activates SoxR by the oxidation of [2Fe-2S] centers, and the activated SoxR stimulates the expression of SoxS, which then upregulates oxidative stress defense genes (13, 24, 46). OxyR controls the expression of about 40 genes mostly involved in peroxide stress

resistance (16). Neither the *oxyR* nor the *soxRS* homologue has been found in the genome sequence of *C. jejuni* (34). Notably, *C. jejuni* is the first Gram-negative bacterium which has been reported to harbor PerR, a non-OxyR-dependent regulatory system to control peroxide stress genes (42). The *C. jejuni* PerR regulates the peroxide stress genes including *kata* (catalase) and *ahpC* (alkyl hydroperoxide reductase); thus, inactivation of PerR renders *C. jejuni* hyper-resistant to hydrogen peroxide due to derepression of peroxide stress genes (42).

A recent proteomics study done by Garénaux et al. demonstrated that the protein level of CosR (Cj0355c) is significantly reduced by exposure to paraquat, a superoxide-generating drug, suggesting that CosR may be associated with the regulation of oxidative stress response in *C. jejuni* (9). CosR is an OmpR-type response regulator and essential for the viability of *C. jejuni* (9, 35). CosR homologues are found mostly in epsilonproteobacteria, such as *Campylobacter*, *Helicobacter*, and *Wolinella* (15), and the CosR homologue in *Helicobacter pylori* (HP1043) is also essential for bacterial viability (4). This suggests a common critical role played by CosR and its homologues in sustaining bacterial viability. In our previous study, we overcame the lethality problem resulting from a knockout mutation of the essential gene *cosR* by using antisense peptide nucleic acid (PNA), which was designed to

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specifically inhibit CosR, and demonstrated that CosR regulates the expression of oxidative stress proteins, such as SodB, Dps, and AhpC (15).

To obtain a better understanding of the CosR regulon at the transcriptomic level, in the present study, we performed an extensive transcriptomic analysis with a DNA microarray and report that CosR regulates expression of key determinants of stress resistance in *C. jejuni*, including the CmeABC multidrug efflux pump and the KatA catalase.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. jejuni* NCTC 11168 was used in the present study. A CosR-overexpression strain was constructed by integrating *cosR* and a chloramphenicol cassette into the chromosome according to the method described in our previous study (15). *C. jejuni* strains were grown at 42°C on Mueller-Hinton (MH) media (Difco) in a microaerobic condition generated by Anoxomat (Mart Microbiology BV, Lichtenvoorde, Netherlands). Kanamycin (50 µg ml⁻¹) or chloramphenicol (10 µg ml⁻¹) was occasionally added to culture media where required. Broth cultures were microaerobically grown with shaking at 180 rpm.

Preparation and treatment of CosR-specific PNA (CosR-PNA). PNAs used in this study were commercially synthesized by Panagene (Daejeon, Korea). The design and use of CosR-PNA was reported in our previous study (15). Based on the genome sequence of *C. jejuni* NCTC 11168 (34), briefly, a 16-mer PNA (CATTTGTTCTATCCTT) was designed to reverse complementarily bind to the leader sequence spanning the ribosomal binding site and the start codon of *cosR*, and conjugated with the permeabilizing oligopeptide (KFFKFFKFFK) (10). The optical density of bacterial culture was adjusted to ~0.07 at 600 nm (~10⁷ CFU ml⁻¹), and CosR-PNA (1.5 µM) was added to the bacterial cultures. *C. jejuni* was grown for 8 h in the culture conditions described above.

Transcriptomic analysis. (i) Preparation of total RNA for a DNA microarray. *C. jejuni* cells were grown at 42°C in MH broth to the mid-exponential phase for ~8 h with shaking, and the culture media were supplemented with 1.5 µM CosR-PNA for CosR-knockdown. The total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions from three independent bacterial cultures. After DNase treatment with a Turbo DNA-free kit (Ambion), the total RNA was purified using acid-phenol solution. The integrity of purified total RNA was measured with a Bioanalyzer 2100 RNA Nano kit (Agilent Technologies, USA).

(ii) cDNA synthesis and microarray hybridization. The synthesis of target cDNA probes and hybridization were performed as described previously (47). RNA samples (30 µg) were reverse transcribed using random primers and Superscript II kit (Invitrogen, USA) according to the manufacturer's instructions. After purification, labeling reactions were performed with a Bioprime labeling kit (Invitrogen) in a volume of 50 µl with a modified deoxynucleoside triphosphate pool containing 120 µM (each) dATP, dGTP, and dCTP; 60 µM dTTP; and 60 µM Cy5-dUTP (for CosR knockdown) or Cy3-dUTP (for the wild type). Labeled targets were subsequently purified by using a Qiaquick PCR cleanup kit (Qiagen). Labeled cDNAs were mixed together and hybridized to an assembled *C. jejuni* subsp. *jejuni* NCTC 11168 custom 3 × 15K microarray slide with 97% coverage of the genome (MYcroarray, Ann Arbor, MI) at 50°C for 16 h using a hybridization oven (Agilent). The hybridized microarrays were washed according to the MYcroarray washing protocol. Finally, microarrays were spin dried and stored in the dark until scanned.

(iii) Image acquisition and analysis. The hybridization images were analyzed by GenePix Pro 6.0 (Axon Instruments, CA). The average fluorescence intensity for each spot was calculated, and the local background was subtracted. All data normalization and selection of fold-changed genes were performed using GeneSpring 7.3.1 (Agilent). Intensity-dependent normalization (Lowess) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity-versus-ratio curve. The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control

TABLE 1 Primers used in this study

Primer	Sequence (5'–3') ^a
Amplification and EMSA	
cmeA_PF_F(BamHI)	AAATGTTTTTCTAAATGGATCCAATAGCTCC
cmeA_PF_R(Xba)	AGTAAAGCACACATCTAGAGCTAAAATAG
katA_PF_F(Xba)	TAAAACAGCTCTAGAAGGAGTGATTTC
katA_PF_R(Xba)	TGAATTTTGGTTATCATCTAGAATGTTTCC
katA_ESIC_F	CGCAGGCGCAAAAGGACCTTTAC
katA_ESIC_R	TCAGGGAATTTATAAGCATCGCGGATG
cmeA_ES_F	ATGAAAAAAATGCAGAAAACTTGCTGTTTC
cmeA_ES_R	TATTTTGGTGCTTCTCTTTGCTGTC
cmeA_ESIC_F	CTGTAAACAACCATGAGTGCTAAATCTGAAG
cmeA_ESIC_R	TAGACTAGCTTTGAATTGTTAAATGTAGCAAG
qRT-PCR	
katA_F	GCTTGAAAACTTGCTCATC
katA_R	TTTGGTATAAGCACTTAAGTC
cmeABC_F	GCTTTAGGTGTTGTGCTTTT
cmeABC_R	ATGGTTGTTACAGGTTGAGG
Cjr01_F	TCGAACGATGAAGCTTTTAG
Cjr01_R	TTGCTCTCTGTGTAGGG

^a Underlining indicates the enzyme recognition sites.

channel intensity. The entire set of microarray data was deposited to Gene Expression Omnibus (GEO) with the accession numbers GSE40201 and GPL15955.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated with the RNeasy minikit (Qiagen) from *C. jejuni* cultures incubated as mentioned above in transcriptional analysis. After the removal of residual DNA contamination from the total RNA solution using the Turbo DNA-free kit (Ambion), cDNA was synthesized using the Omniscript RT kit (Qiagen) and random hexamers (Invitrogen). Quantification of cDNA was carried out using IQ SYBR green PCR Supermix (Bio-Rad), and real-time amplification of PCR product was analyzed using an iCycler optical module (Bio-Rad). The amplification program consisted of one cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The mRNA levels of each gene were normalized to the expression level of *Cjr01* encoding 16S rRNA. The relative amount of cDNA was calculated according to the 2^{-ΔΔCT} method (29). The sequences of primers for the expression of *katA*, *cmeABC*, and *Cjr01* are presented in Table 1.

Measurement of catalase activity. The catalase activity was measured with a method based on the development of colorimetric precipitation as described previously (44). After separation of protein samples on an 8% polyacrylamide gel under native conditions, the gel was washed in three changes of distilled water for a total of about 45 min. The gel was then transferred and immersed in 0.003% H₂O₂ solution on a rotating turntable for 10 min at room temperature in the dark. The H₂O₂ solution was removed, and the gel was briefly rinsed with distilled water and placed in the negative staining solution containing 2% ferric chloride and 2% potassium ferric cyanide with gentle rocking over a light until the gel turned uniformly greenish blue due to the precipitation of ferric chloride and potassium ferric cyanide mediated by H₂O₂.

P_{cmeABC}::lacZ fusion assay. The promoter and partial coding region of *cmeABC* were amplified with the primers cmeA_PF_F(BamHI) and cmeA_PF_R(XbaI) (Table 1) and cloned into pMW10 (45), which contains the promoterless *lacZ* gene. The plasmid was mobilized to *C. jejuni* by conjugation, and β-galactosidase assays were performed as described previously (45).

EMSA. Electrophoretic mobility shift assay (EMSA) was performed as described previously (15). rCosR was produced in *E. coli* BL21(DE3) with pET15b::*cosR* and purified under the native conditions using Ni²⁺ affinity chromatography (15). The DNA fragments containing the promoter region of *katA* and *cmeA* were amplified by PCR with the primer pairs

*katA*_PF_F(Xba)/*katA*_PF_R(Xba) and *cmeA*_ES_F/*cmeA*_ES_R, respectively (Table 1). The PCR products were purified from an agarose gel using a gel extraction kit (Qiagen) and labeled with [γ - 32 P]ATP (GE Healthcare). After the elimination of the unincorporated radioisotope using a MicroSpinTMG-25 column (GE Healthcare), the 0.2 nM 32 P-labeled DNA probe was incubated with the purified rCosR protein at different concentrations (0, 0.8, 1.6, 2.4, and 3.2 nM) at 37°C for 15 min in 10 μ l of the gel-shift assay buffer [20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM (NH₄)₂SO₄, 5 mM dithiothreitol, 0.2% Tween 20, 30 mM KCl, 0.1 μ g of poly(dI-dC)]. For the competitive EMSA, unlabeled target DNA probes and internal coding regions of each gene were prepared by gel extraction after PCR amplification with the specific primer pairs listed in Table 1. The reaction mixtures were resolved in a 6% polyacrylamide gel, and the radiolabeled DNA fragments were visualized using the BAS2500 system (Fuji Film).

DNase I footprinting assay. DNase I footprinting assays were performed as described elsewhere (8). Briefly, DNA fragments containing the *katA* and *cmeABC* promoter region were amplified by PCR using a 32 P-labeled primer [*katA*_PF_R(Xba) and *cmeA*_PF_R(Xba)] and an unlabeled primer [*katA*_PF_F(Xba) and *cmeA*_ES_F], respectively (Table 1), and were purified from the agarose gel with a gel extraction kit (Qiagen). Binding reaction of rCosR to the *katA* and *cmeABC* promoters was performed at 37°C for 10 min in 40 μ l of the gel-shift assay buffer (see above) containing 10 mM MgCl₂. The reaction mixture was treated either with or without 0.1 or 0.5 U of DNase I (TaKaRa), and the reactions were stopped by the addition of 200 μ l of ice-cold stop solution (0.4 M sodium acetate, 2.5 mM EDTA). After the purification with phenol extraction and ethanol precipitation, the digested DNA fragments were separated by electrophoresis in 6% polyacrylamide–8 M urea gels alongside sequencing ladders that were generated with the same 32 P-labeled primer used to amplify DNA fragments for DNase I digestion.

RESULTS

Effects of CosR knockdown on transcriptomic profiles. Transcriptomic profile changes under the CosR-knockdown condition were analyzed with DNA microarrays. Because of the cell death problem resulting from a knockout mutation in the essential gene *cosR*, microarray analysis was performed with RNA samples collected under the CosR-knockdown condition where the protein level of CosR was significantly reduced by gene knockdown with CosR-PNA as described in our previous study (15). The results of microarray analysis revealed that 480 genes were regulated either positively or negatively by CosR knockdown >1.5-fold with statistical significance ($P < 0.05$) (see Tables S1 and S2 in the supplemental material), and among these genes >2-fold changes were observed in 93 genes involved in various cellular processes, such as energy production, transcription, protein synthesis, motility, secondary metabolite biosynthesis, and stress defense (Tables 2 and 3). Interestingly, CosR knockdown affected the transcription of 17 genes which were reported to be essential in *C. jejuni* (31, 38), suggesting that CosR is associated with regulation of vital cellular processes sustaining *C. jejuni*'s viability (Tables 2 and 3; essential genes are indicated in boldface and by underlining). qRT-PCR also confirmed that the effect of CosR knockdown on the expression of the essential genes (see Fig. S1 in the supplemental material). Also, CosR knockdown affected the expression of several genes associated with flagellar biogenesis (Table 3); CosR knockdown upregulated *flgD* (encoding flagellum basal body rod modification protein), *flgE* (flagellum hook protein), *flgL* (flagellum hook-associated protein), and *fliK* (putative flagellum hook-length control protein). Based on the genome sequence of *C. jejuni* (34), the *flgD*, *flgE*, and *flgK* genes are encoded in a single polycistronic operon (data not shown). The transcriptional level changes

in *flgL* and *fliK* were confirmed by qRT-PCR (see Fig. S2A in the supplemental material). CosR knockdown increased bacterial motility compared to the wild type, whereas CosR overexpression reduced motility (see Fig. S2B in the supplemental material).

Negative regulation of *cmeABC* by CosR. CmeABC is a key determinant in conferring resistance to a broad range of antimicrobials and toxic compounds in *Campylobacter* (18, 27). The microarray results showed that the transcriptional levels of all *cmeA*, *cmeB*, and *cmeC* genes were increased by CosR knockdown >2-fold (Table 3). qRT-PCR and *P*_{*cmeABC*}::*lacZ* fusion assay showed that the transcriptional level of *cmeABC* was upregulated by CosR knockdown and reduced in the CosR-overexpressing strain (Fig. 1A and B), demonstrating the negative regulation of *cmeABC* expression by CosR. Since the binding of bile salts to CmeR, a repressor of *cmeABC*, derepresses *cmeABC* expression due to conformational changes in CmeR (26), the *P*_{*cmeABC*}::*lacZ* fusion assay carried out in the presence of cholic acid to examine whether the regulation of *cmeABC* by CosR would be affected by bile salts (Fig. 1B). Cholate (1 mg/ml) significantly increased the transcriptional level of *cmeABC*; however, the changes in the Miller units at different CosR levels were not affected by cholic acid compared to those without cholic acid (Fig. 1B). The results suggest that the regulation of *cmeABC* by CosR is independent of bile salts and presumably of CmeR as well, although the effect of bile salts on CosR expression is not yet known. Interaction between CosR and the *cmeABC* promoter was examined by performing EMSA, and the results showed that CosR bound to the *cmeABC* promoter (Fig. 1C). The PCR fragment from an internal coding region did not compete with the labeled probe of the *cmeABC* promoter; however, unlabeled *cmeABC* probes effectively competed with the labeled *cmeABC* probes (Fig. 1C). These results showed that CosR directly interacts with the *cmeABC* promoter and regulates *cmeABC* expression as a repressor.

Regulation of *katA* by CosR. Consistent with our previous report, CosR affected the expression of oxidative stress genes. CosR-knockdown upregulated *rrc* (2.2-fold; Table 3), *dps* (1.5-fold; see Table S2 in the supplemental material), and *sodB* (1.7-fold; see Table S2 in the supplemental material) and downregulated *katA* (1.7-fold; see Table S1 in the supplemental material) and *p19* (2.7-fold; Table 2). KatA is the sole catalase in *C. jejuni* and plays an important role in *Campylobacter*'s resistance to H₂O₂ and persistence within macrophages (7). The results of qRT-PCR showed that the transcriptional level of *katA* was reduced by CosR knockdown and was increased by CosR overexpression compared to that of the wild type (Fig. 2A). To determine whether the transcriptional changes in *katA* would affect the enzyme activity, the catalase activity was measured with a catalase assay, which is based on the formation of colorimetric precipitate, ferrichloride-ferricyanide complex, via the reaction between H₂O₂ and ferricyanide (44). Consistent with the transcriptional changes, the catalase activity was decreased by CosR knockdown and increased in the CosR-overexpression strain (Fig. 2B). Interaction between CosR and the *katA* promoter was determined by EMSA, and the results showed that CosR bound to the *katA* promoter (Fig. 2C). These results successfully demonstrated that CosR binds to the *katA* promoter and positively regulates *katA* transcription and affects the catalase activity in *C. jejuni*.

Determination of CosR binding sites in the *katA* and *cmeABC* promoters. The results of microarray analysis, qRT-PCR, and EMSA demonstrated that CosR regulated *katA* and *cme*

TABLE 2 Genes downregulated by CosR knockdown^a

Functional category	Gene	Locus tag	Description	Fold change ^b	P
Translation, ribosomal structure, and biogenesis	<i>tgt</i>	Cj1010	Queuine tRNA-ribosyltransferase	-2.114	0.01636
	<i>valS</i>	Cj0775c	Valyl-tRNA synthetase	-2.732	0.017
Transcription	<i>greA</i>	Cj0287c	Transcription elongation factor GreA	-2.008	0.0053
	<i>ruvB</i>	Cj1362	Holliday junction DNA helicase RuvB	-2.222	0.00859
DNA replication, recombination, and repair					
Cell division and chromosome partitioning	<i>mvp</i>	Cj1606c	Putative ATP/GTP-binding protein	-4.975	0.015
Posttranslational modification, protein turnover, and chaperones	<i>groES</i>	Cj1220	Cochaperonin GroES	-13.138	0.00456
	<i>groEL</i>	Cj1221	Chaperonin GroEL	-7.583	0.00168
	<i>Cj0954c</i>	Cj0954c	Putative DnaJ-like protein	-2.921	0.00341
	<i>hypB</i>	Cj0623	Hydrogenase isoenzyme formation protein	-2.433	0.015
	<i>hypC</i>	Cj0624	Hydrogenase isoenzyme formation protein	-2.475	0.017
	<i>hypD</i>	Cj0625	Hydrogenase isoenzyme formation protein	-2.618	0.004
	<i>hypE</i>	Cj0626	Hydrogenase isoenzyme formation protein	-2.439	0.009
	<i>trxB</i>	Cj0146c	Thioredoxin reductase	-2.398	0.002
Inorganic ion transport and metabolism	<i>Cj1613c</i>	Cj1613c	Putative pyridoxamine 5'-phosphate oxidase	-2.410	0.01488
	<i>Cj1658</i>	Cj1658	Putative iron permease	-2.066	0.02772
	<i>p19</i>	Cj1659	Periplasmic protein p19	-2.747	0.025
Energy production and conversion	<i>Cj0037c</i>	Cj0037c	Putative cytochrome <i>c</i>	-2.790	0.01208
	<i>Cj0203</i>	Cj0203	Putative citrate transporter	-3.525	0.00698
	<i>Cj1153</i>	Cj1153	Putative periplasmic cytochrome <i>c</i>	-2.053	0.01988
	<i>napA</i>	Cj0780	Nitrate reductase catalytic subunit	-2.179	0.009
	<i>napB</i>	Cj0783	Periplasmic nitrate reductase small subunit	-2.262	0.01099
	<i>napG</i>	Cj0781	Quinol dehydrogenase periplasmic component	-2.326	0.024
	<i>napH</i>	Cj0782	Quinol dehydrogenase membrane component	-2.188	0.009
	<i>sucC</i>	Cj0533	Succinyl-CoA synthetase subunit beta	-2.146	0.027
	<i>sucD</i>	Cj0534	Succinyl-CoA synthetase alpha chain	-2.146	0.034
Amino acid transport and metabolism	<u><i>dapD</i></u>	<u>Cj1605c</u>	Putative 2,3,4,5-tetrahydropyridine-2-carboxylate <i>N</i> -succinyltransferase	-3.155	0.01173
	<i>ilvH</i>	Cj0575	Acetolactate synthase 3 regulatory subunit	-2.070	0.007
	<i>ilvI</i>	Cj0574	Acetolactate synthase 3 catalytic subunit	-2.110	0.009
Nucleotide transport and metabolism	<i>Cj0594c</i>	Cj0594c	Putative DNA/RNA nonspecific endonuclease	-2.410	0.02564
	<i>Cj0898</i>	Cj0898	Putative histidine triad (HIT) family protein	-2.163	0.00825
	<i>purH</i>	Cj0953c	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	-2.392	0.01
	<i>purL</i>	Cj0955c	Phosphoribosylformylglycinamide synthase II	-2.793	0.003
Coenzyme metabolism	<i>Cj0436</i>	Cj0436	Putative pyridoxamine 5'-phosphate oxidase	-2.456	0.01122
Lipid metabolism	<i>acpP</i>	Cj0441	Acyl carrier protein	-7.463	0.00875
	<u><i>fabF</i></u>	<u>Cj0442</u>	3-Oxoacyl-(acyl carrier protein) synthase II	-6.526	0.00383
	<u><i>accA</i></u>	<u>Cj0443</u>	Acetyl-CoA carboxylase carboxyltransferase subunit alpha	-4.065	0.00599
Secondary metabolites biosynthesis, transport, and catabolism	<u><i>fabG</i></u>	<u>Cj0435</u>	3-Ketoacyl-(acyl-carrier-protein) reductase	-2.567	0.01624
General function prediction only	<i>amaA</i>	Cj1363	Acid membrane antigen A	-2.156	0.00711
	<i>Cj0773c</i>	Cj0773c	Putative binding-protein dependent transport system permease	-3.072	0.00907
	<i>Cj0774c</i>	Cj0774c	ABC transporter ATP-binding protein	-2.990	0.00898
Function unknown	<i>Cj0573</i>	Cj0573	Putative GatB/Yqey family protein	-2.134	0.01623
	<i>Cj0593c</i>	Cj0593c	Putative integral membrane protein	-2.079	0.00487
	<i>Cj0776c</i>	Cj0776c	Putative periplasmic protein	-2.632	0.015
	<i>Cj0880c</i>	Cj0880c	Hypothetical protein	-2.070	0.02268
	<i>Cj1725</i>	Cj1725	Putative periplasmic protein	-2.252	0.01401
	<i>ctsR</i>	Cj1475c	Hypothetical protein	-2.008	0.01739
	<i>glpT</i>	Cj0292c	Pseudogene	-2.012	0.00746

^a Underlined and boldfaced genes are essential for the viability of *C. jejuni*, based on reports by Stahl and Stintzi (38) and Metris et al. (31), respectively. CoA, coenzyme A.^b The minus symbol indicates downregulation by CosR knockdown.

TABLE 3 Genes upregulated by CosR knockdown^a

Functional category	Gene	Locus tag	Description	Fold change	P
Translation, ribosomal structure and biogenesis	<u>Cj0722c</u>	<u>Cj0722c</u>	Putative DNA methylase	2.036	0.00677
Posttranslational modification, protein turnover, and chaperones	<u>Cj1365c</u>	Cj1365c	Putative secreted serine protease	2.168	0.00613
	<u>dsbA</u>	Cj0872	Putative protein disulphide isomerase	2.404	0.02011
	<u>htpG</u>	Cj0518	Heat shock protein 90	2.079	0.01
Cell envelope biogenesis, outer membrane	<u>dgkA</u>	Cj0257	Diacylglycerol kinase	2.186	0.00238
	<u>glmS</u>	<u>Cj1366c</u>	Glucosamine-fructose-6-phosphate aminotransferase	2.012	0.01338
	<u>pglF</u>	Cj1120c	UDP-GlcNAc C4,6 dehydratase	2.138	0.028
Cell motility and secretion	<u>flgD</u>	Cj0042	Flagellar basal body rod modification protein	2.159	0.01308
	<u>flgE</u>	Cj0043	Flagellar hook protein	2.156	0.01035
	<u>flgL</u>	Cj0887c	Flagellar hook-associated protein FlgL	2.228	0.00678
	<u>fliK</u>	Cj0041	Putative flagellar hook-length control protein	2.157	0.00755
	<u>lspA</u>	Cj0361	Lipoprotein signal peptidase	2.091	0.012
Inorganic ion transport and metabolism	<u>Cj0519</u>	Cj0519	Putative rhodanese-like domain protein	2.185	0.00455
	<u>Cj0522</u>	Cj0522	Putative Na ⁺ /P _i cotransporter protein	2.154	0.00264
	<u>pstS</u>	Cj0613	Putative periplasmic phosphate binding protein	2.067	0.02
Energy production and conversion	<u>rrc</u>	Cj0012c	Non-haem iron protein	2.242	0.002
	<u>sdhB</u>	Cj0438	Putative succinate dehydrogenase iron-sulfur protein	2.159	0.006
	<u>sdhC</u>	Cj0439	Putative succinate dehydrogenase subunit C	2.068	0.009
Amino acid transport and metabolism	<u>aroK</u>	<u>Cj0387</u>	Shikimate kinase	2.827	0.01923
	<u>putA</u>	Cj1503c	Putative proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase	2.028	0.008
	<u>putP</u>	Cj1502c	Putative sodium/proline symporter	2.442	0.009
Nucleotide transport and metabolism	<u>pyrC</u>	Cj0259	Dihydroorotase	2.075	0.0096
Coenzyme metabolism	<u>dfp</u>	Cj0822	Bifunctional phosphopantothienoylcysteine decarboxylase/phosphopantothenate synthase	2.191	0.03033
	<u>folD</u>	Cj0855	Bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase	2.121	0.01026
Secondary metabolites biosynthesis, transport, and catabolism	<u>cmeA</u>	Cj0367c	Periplasmic fusion protein CmeA	2.485	0.01579
	<u>cmeB</u>	Cj0366c	Inner membrane efflux transporter CmeB	2.294	0.01059
	<u>cmeC</u>	Cj0365c	Outer membrane channel protein CmeC	2.127	0.01653
General function prediction only	<u>Cj0236c</u>	Cj0236c	Putative integral membrane protein	2.089	0.0283
	<u>Cj0256</u>	Cj0256	Putative sulfatase family protein	2.078	0.00544
	<u>Cj0770c</u>	Cj0770c	Putative NLPA family lipoprotein	2.371	0.01032
	<u>Cj0771c</u>	Cj0771c	Putative NLPA family lipoprotein	2.408	0.01623
	<u>Cj0772c</u>	Cj0772c	Putative NLPA family lipoprotein	2.358	0.00361
	<u>engA</u>	Cj0386	GTP-binding protein EngA	2.080	0.00462
Function unknown	<u>Cj0040</u>	Cj0040	Hypothetical protein	2.306	0.04341
	<u>Cj0069</u>	Cj0069	Hypothetical protein	2.147	0.00597
	<u>Cj0070c</u>	Cj0070c	Hypothetical protein	2.156	0.04309
	<u>Cj0243c</u>	Cj0243c	Hypothetical protein	2.362	0.0106
	<u>Cj0249</u>	Cj0249	Hypothetical protein	2.012	0.03032
	<u>Cj0391c</u>	Cj0391c	Hypothetical protein	2.061	0.01112
	<u>Cj0428</u>	Cj0428	Hypothetical protein	2.190	0.00859
	<u>Cj0520</u>	Cj0520	Hypothetical protein	2.168	0.01622
	<u>Cj0742</u>	Cj0742	Pseudogene	2.454	0.04945
	<u>Cj0873c</u>	Cj0873c	Hypothetical protein	2.070	0.01804
	<u>Cj1242</u>	Cj1242	Hypothetical protein	2.419	0.00765
	<u>Cj1501</u>	Cj1501	Hypothetical protein	2.146	0.00568
	<u>Cj1650</u>	Cj1650	Hypothetical protein	2.948	0.00732

^a Underlined and boldfaced genes are essential for the viability of *C. jejuni*, based on reports by Stahl and Stintzi (38) and Metris et al. (31), respectively.

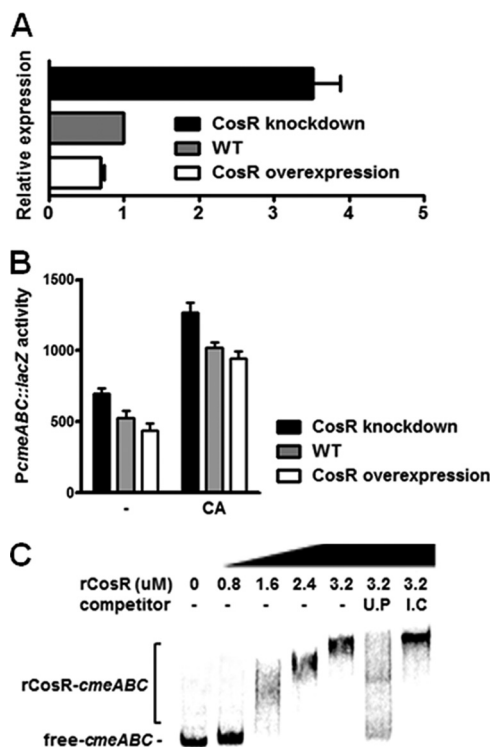


FIG 1 Negative regulation of *cmeABC* by CosR. (A) qRT-PCR analysis of *cmeABC* transcription. *C. jejuni* strains were cultured at different CosR levels at 42°C for 8 h under microaerobic conditions to extract total RNA. The CosR-knockdown condition was generated by supplementing 1.5 μM CosR-PNA to the medium. The results of three independent experiments are expressed as means and standard deviations. (B) *P_{cmeABC}::lacZ* fusion assay in the absence or presence of cholate (CA). Cholate (1 mg/ml) was added to the culture medium, and *C. jejuni* strains were grown for 6 h prior to the assay. The “–” symbol indicates the cultures without cholate. (C) Binding of rCosR on the *cmeABC* promoter. Radiolabeled DNA probes of the *cmeABC* promoter were incubated with rCosR at different concentrations, and unlabeled DNA probes (U.P) and internal coding regions (I.C) of *cmeABC* were used as a competitor.

ABC by binding to their promoters (Fig. 1 and 2). DNase I footprinting assays were carried out to further determine the CosR binding sites in the *katA* and *cmeABC* promoter regions (Fig. 3). The assay found a region (from –37 to –17) overlapping with the –35 element of the *katA* promoter was protected from DNase I treatment (Fig. 3A and C). The CosR binding site in the *katA* promoter doesn't overlap with the predicted PerR binding site (–104 to –86) (43). We identified two putative holo-Fur binding sites (–51 to –65 and –91 to –105) in the *katA* promoter based on the consensus holo-Fur binding site (TGATAAT-T-ATTA TCA) (5). Although one of the holo-Fur binding sites overlapped with the putative PerR binding site, the CosR binding site does not overlap with the holo-Fur binding sites (Fig. 3C). CosR also bound to an upstream region of the CmeR binding site in the *cmeABC* promoter (Fig. 3B and D) (25). Nucleotide sequences of the CosR binding sites in the *katA* and *cmeABC* promoters were aligned with the previously reported CosR binding sequences in the *sodB* and *ahpC* promoters (15), and the multiple alignment revealed a consensus CosR binding sequence (ttTaAanaAAAaTT AagaTTT; lowercase and capital letters indicate less and highly conserved residues, respectively, and “n” represents any nucleotide [Fig. 4]), which is almost identical to the consensus CosR

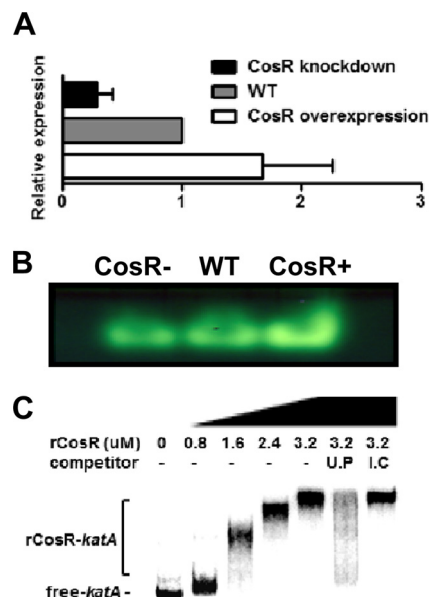


FIG 2 Positive regulation of *katA* by CosR. (A) qRT-PCR analysis of *katA* transcription. The results are expressed as the means and standard deviations of three independent experiments. (B) Catalase activity at different CosR levels. In these experiments, CosR-knockdown was achieved by adding 1.5 μM CosR-PNA to culture medium. The results show the representative of three independent experiments. The symbols “–” and “+” indicate knockdown and overexpression, respectively. (C) Binding of rCosR to the *katA* promoter. Radiolabeled DNA probes of the *katA* promoter were incubated with rCosR at different concentrations, and unlabeled DNA probes (U.P) and internal coding regions (I.C) of *katA* were used in the competition assay.

binding sequence reported in our previous study (tttaAanAaAAA TtAtgaTTt) (15).

DISCUSSION

CmeABC is the major multidrug efflux pump contributing to *Campylobacter*'s resistance to a variety of toxic compounds, antimicrobials, and bile salts (27, 28). The *cmeABC* mutant is highly susceptible to clinically important antibiotics and cannot colonize the chicken intestines due to elevated susceptibility to bile salts (28). CmeABC is a resistance-nodulation-cell division (RND)-type efflux pump consisting of the periplasmic protein CmeA, the inner membrane protein CmeB, and the outer membrane protein CmeC encoded in the polycistronic operon *cmeABC* (25, 27). The results of DNA microarray analysis in the CosR-knockdown condition demonstrated a significant change in the transcriptional levels of all three genes in the *cmeABC* operon (Table 3). The microarray results were further supported by qRT-PCR and *P_{cmeABC}::lacZ* assays (Fig. 1A and B). CmeR has thus far been the only transcriptional regulator involved in the control of *cmeABC* expression (26). Although bile salts are a substrate of the CmeABC efflux pump, bile salts can also affect the CmeR regulation (26). Interaction of bile salts with the CmeR protein causes conformational changes in CmeR and results in the derepression of *cmeABC* expression (26). To investigate whether CosR interferes with the function of CmeR on *cmeABC* expression, a *P_{cmeABC}::lacZ* assay was carried out in the presence of cholic acid. The results showed that cholic acid increased the transcriptional level of *cmeABC* (Fig. 1B), indicating that *cmeABC* expression was derepressed by the interaction between CmeR and bile salts; binding of bile salts to

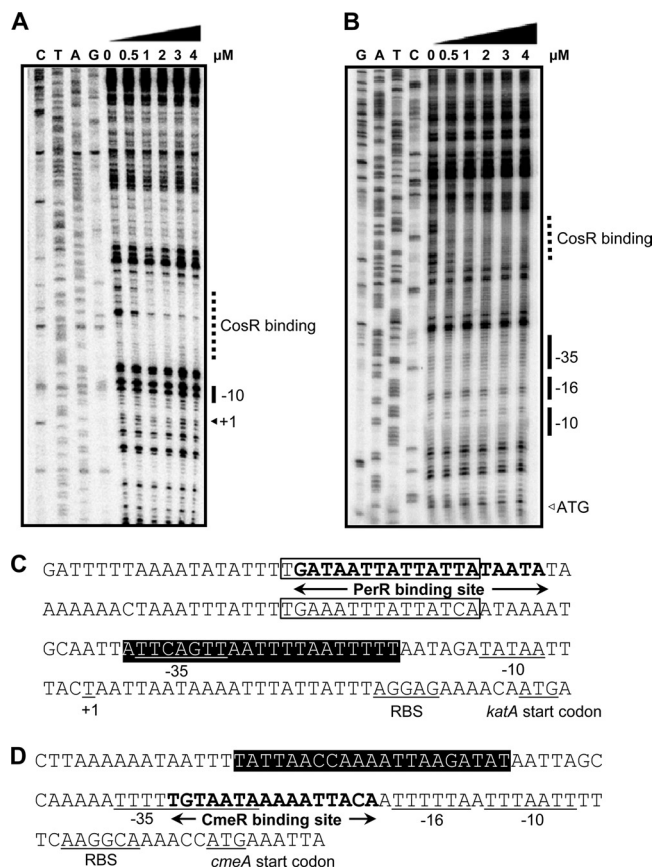


FIG 3 Determination of the CosR binding sites in *katA* and *cmeABC* promoters by DNase I footprinting. The CosR binding regions in the promoter regions of *katA* (A) and *cmeABC* (B) are indicated by dotted lines; the promoters are based on previous reports (11, 25). Fur binding sites are marked with open boxes based on the consensus sequence for holo-Fur binding site (5). The CmeR binding site was reported previously by Lin et al. (25). The PerR binding region (C) in the *katA* promoter and the CmeR binding region (D) in *cmeABC* promoter are marked with boldface letters and arrows. The start codon, the transcriptional start site (+1), and the -10, -16, and -35 elements are underlined. The regions protected by rCosR from DNase I cleavage are indicated by a black background.

CmeR reduces the binding affinity of CmeR to the *cmeABC* promoter (26). However, CosR knockdown or overexpression caused similar changes in the Miller units of the $P_{cmeABC}::lacZ$ assay regardless of the presence or absence of cholic acid, indicating that CosR did not affect the derepression of *cmeABC* by bile salts; however, it is unknown whether bile salts would affect the function or expression of CosR. Also, the CosR binding site discovered by DNase I footprinting assay was located 17 bp upstream of the CmeR binding site in the *cmeABC* promoter (Fig. 3B and D), suggesting that binding of CosR and CmeR to the *cmeABC* promoter may not spatially interfere with each other.

Lin et al. reported that a mutation of *cmeR* resulted in a >6-fold increase in the level of P_{cmeABC} transcription and increased the MICs of ciprofloxacin (2-fold), cefotaxime (2-fold), and erythromycin (4-fold) (25). Recently, it was reported that salicylate, a nonsteroidal anti-inflammatory compound, induces *cmeABC* transcription by inhibiting CmeR binding to the *cmeABC* promoter (37). An ~2-fold increase in the level of *cmeABC* transcription at 100 μ g of salicylate/ml did not change MICs of antibiotics

BsodB	+15	ataaaggagagaattatctttg	+35
BahpC1	-37	tttaaaaaataaagattt	-17
BahpC2	+1	tatcaaaataattattt	+21
BkatA	-37	attcagtttaattttaattttt	-17
BcmeABC	-73	tattaaccataaataagatat	-53
Consensus		ttTaAanaAAAaTTAagaTTT	

FIG 4 Alignment of CosR binding sequences. Nucleotide sequences of the CosR binding sites for the *katA* (BkatA) and *cmeABC* (BcmeABC) promoters were determined from Fig. 3 and compared to the CosR binding sequences in the *sodB* (BsodB) and *ahpC* (BahpC1 and BahpC2) promoters reported in our previous study (15). Highly conserved nucleotides are shaded on black background, and identical nucleotides are indicated in capital letters. “n” means any nucleotide.

except for only a moderate change in ciprofloxacin (2-fold) but enhanced bacterial growth in the presence of antibiotics at sub-MICs (37). Similarly, neither CosR knockdown nor overexpression produced noticeable changes in the MICs of several antibiotics tested here, including ciprofloxacin, erythromycin, and tetracycline (data not shown). Based on previous reports and our findings in the present study, both CmeR and CosR function as repressors for *cmeABC*, and it would be interesting to investigate whether CosR and CmeR would interplay in the regulation of *cmeABC* under certain conditions.

It is an interesting finding that CosR is involved in the regulation of both oxidative stress and drug efflux pump. A similar example can be found in SoxRS, a well-known oxidative stress regulator in *E. coli*. SoxRS not only controls oxidative stress defense but also affects antibiotic resistance by upregulating the AcrAB multidrug efflux pump (22). The *E. coli* AcrAB is functionally coupled to the outer membrane protein TolC and significantly contributes to *E. coli*'s resistance to a wide range of toxic compounds, including antibiotics, detergents, and dyes (32). Increased levels of AcrAB-TolC resulting from elevated *soxR* expression by mutations render *E. coli* and *Salmonella* resistant to antibiotics (12, 19). The removal of toxic compounds by efflux pumps may contribute to the detoxification of cells, and presumably this is why some oxidative stress regulators control the expression of oxidative stress genes and efflux pumps as well. To the best of our knowledge, CosR is the first reported transcriptional regulator that controls the expression of genes involved in oxidative stress resistance and a multidrug efflux pump in *C. jejuni*.

Another important finding of the present study is that CosR positively regulates *katA* expression at the transcriptional level and affects the catalase activity in *C. jejuni* (Fig. 2). As the sole catalase in *Campylobacter*, KatA plays an important role in oxidative stress resistance and intracellular survival in the macrophage (7). Like Gram-positive bacteria, *C. jejuni* uses PerR as a repressor to control peroxide resistance genes including *katA* and *ahpC* (33, 42). Iron is also a regulatory factor of *katA* expression; iron represses *katA*, *ahpC*, and *perR* as well (3, 14). Although the effect of iron on *perR* expression results from negative *perR* autoregulation (20), the iron responsiveness of *katA* was eliminated not by inactivation of either *perR* or *fur* but only by the double mutation of *perR* and *fur*, indicating that both PerR and Fur participate in the control of *katA* expression (42). In fact, it was recently shown that holo-Fur binds to the *katA* promoter and negatively regulates *katA* expression (5). In contrast to the negative regulation of *katA* expression by PerR and holo-Fur, CosR activated *katA* expression

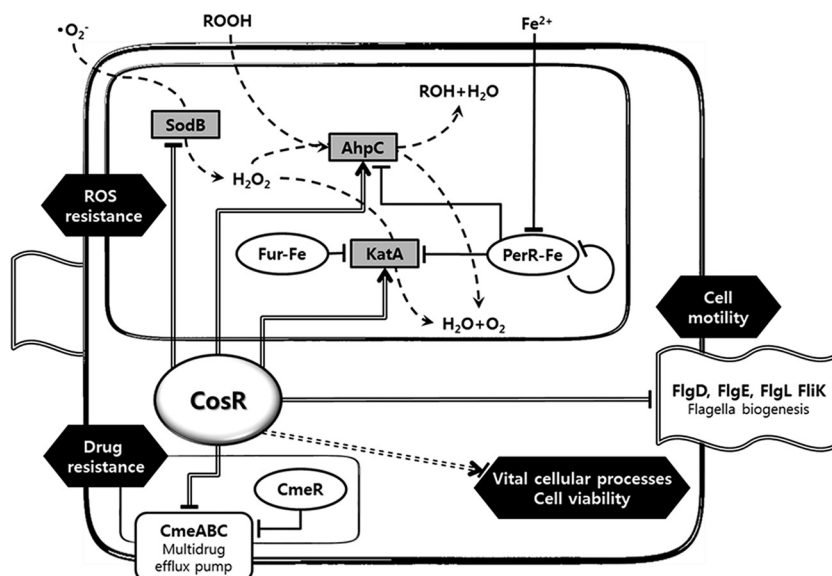


FIG 5 Schematic representation of CosR regulatory network in *C. jejuni*. The detoxification flow of oxidants by oxidative stress resistance enzymes (gray boxes) is drawn with dashed lines, and the transcriptional regulators (CosR, Fur, PerR, and CmeR) are boxed in ovals. The regulation by CosR is indicated with double-strand lines, and positive and negative regulations by CosR are marked with arrowheads and flat-line heads, respectively. CosR is indispensable to *Campylobacter*'s viability, although its regulation mechanism is not known. This is indicated with a dashed double-strand line.

like the *E. coli* OxyR. The CosR binding site is separated from the predicted PerR and Fur binding sites in the *kata* promoter (Fig. 3C), suggesting that CosR binding to the *kata* promoter may not interfere with PerR and Fur binding. The regulation of *kata* expression involves PerR, Fur, iron, and CosR, and further studies are required to explain how these multiple regulatory factors interplay to coordinate the expression of the sole catalase gene *kata*. The regulatory network of CosR is summarized in Fig. 5.

Although the *E. coli* OxyR is subject to negative autoregulation, OxyR acts mostly as an activator of the peroxide defense genes (39). The OxyR binding sites in the promoters of positively regulated genes typically overlap the -35 region and extend upstream from the promoter regions (41). This unique positioning of OxyR binding sites in the promoter region stimulates the interaction between OxyR and the α subunit of the RNA polymerase, activating gene expression (40). The region protected by CosR from DNase I treatment in the *kata* promoter overlaps with the -35 region of the *kata* promoter sequence (Fig. 3A and C). In a previous study, we showed that CosR positively regulates AhpC, and one of the CosR binding sites also overlaps the -35 region in the *ahpC* promoter (15). These findings suggest that CosR may interact with the RNA polymerase for activation similar to the *E. coli* OxyR. To elucidate the CosR regulation, in addition, further studies are required to determine whether CosR has its cognate sensor kinase or is an orphan response regulator and whether CosR is phosphorylated or not, since the phosphorylation status can affect the DNA binding affinity of a response regulator. It has been reported that HP1043, the CosR homologue in *H. pylori*, functions in a phosphorylation-independent manner (36); however, it remains unknown whether CosR is phosphorylated or not.

The transcriptomic analysis described here discovered 93 genes whose transcriptional levels were changed >2 -fold by CosR knockdown (Tables 2 and 3). The functions of the identified genes

are involved in a wide range of cellular processes, such as energy production, lipid metabolism, motility, amino acid transport, and drug efflux; however, 21.5% of the genes (20 of 93) have not been fully characterized. The DNA microarray analysis in the present study identified 53% of the genes that were reported in our previous proteomic study, such as *greA*, *groEL*, *rrc*, *p19*, and *htpG* (15). Consistent with our previous proteomic analysis (15), *sodB*, *rrc*, and *dps* were upregulated by CosR knockdown (Table 3 and see Table S2 in the supplemental material); however, *ahpC* was not downregulated by CosR knockdown in a DNA microarray analysis despite obvious expression changes at the protein level in our previous study (15), suggesting that posttranscriptional factors may also affect AhpC expression. Interestingly, ca. 18.3% of the CosR-regulated genes (17 of 93) are predicted essential genes in *C. jejuni* (31, 38). Although it is not certain whether CosR affects the expression of these essential genes directly or indirectly, CosR appears to be closely involved in the regulation of vital biological processes in *C. jejuni*. Given that CosR regulates genes related to resistance to oxidative stress and toxic compounds, CosR may participate in the overall stress resistance and survival of *Campylobacter*. Future studies will fill in the knowledge gaps regarding the environmental stimuli affecting the regulatory function of CosR and the coordination of regulatory factors of oxidative stress defense in *Campylobacter*.

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