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Regulation of *perR* Expression by Iron and PerR in *Campylobacter jejuni*▽†

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Campylobacter jejuni **is a leading food-borne pathogen causing gastroenteritis in humans. Although OxyR is a widespread oxidative stress regulator in many Gram-negative bacteria, *C. jejuni* lacks OxyR and instead possesses the metalloregulator PerR. Despite the important role played by PerR in oxidative stress defense, little is known about the factors influencing *perR* expression in *C. jejuni*. In this study, a *perR* promoter-*lacZ* fusion assay demonstrated that iron significantly reduced the level of *perR* transcription, whereas other metal ions, such as copper, cobalt, manganese, and zinc, did not affect *perR* transcription. Notably, a *perR* mutation substantially increased the level of *perR* transcription and in *trans* complementation restored the transcriptional changes, suggesting *perR* is transcriptionally autoregulated in *C. jejuni*. In the *perR* mutant, iron did not repress *perR* transcription, indicating the iron dependence of *perR* expression results from *perR* autoregulation. Electrophoretic mobility shift assays showed that PerR binds to the *perR* promoter, and DNase I footprinting assays identified a PerR binding site overlapping the −35 region of the two *perR* promoters, further supporting *perR* autoregulation at the transcriptional level. Alignment of the PerR binding sequence in the *perR* promoter with the regulatory region of other PerR regulon genes of *C. jejuni* revealed a 16-bp consensus PerR binding sequence, which shares high similarities to the *Bacillus subtilis* PerR box. The results of this study demonstrated that PerR directly interacts with the *perR* promoter and regulates *perR* transcription and that *perR* autoregulation is responsible for the repression of *perR* transcription by iron in *C. jejuni*.**

Defense against reactive oxygen species is crucial to the survival of bacteria possessing aerobic metabolisms. Reactive oxygen species, such as the superoxide anion, hydrogen peroxide, and hydroxyl radical, are inevitably generated as by-products of aerobiosis, causing damage to biological molecules (16). In the generation of reactive oxygen species, intracellular free iron also plays a central role through the Fenton reaction (17). To achieve prompt protection from the damaging effects of reactive oxygen species, bacteria produce various oxidative stress defense proteins, and their expressions are controlled by elaborate regulatory mechanisms. In *Escherichia coli* and *Salmonella*, for example, SoxRS and OxyR are well-known regulators to facilitate bacterial resistance to superoxide and hydrogen peroxide stresses, respectively (16). Particularly, OxyR is a hydrogen peroxide-sensing regulator activating a number of genes highly induced by hydrogen peroxide (39). Although OxyR is a widespread oxidative stress regulator, some bacterial species, including *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Campylobacter jejuni*, lack OxyR and instead harbor PerR to regulate oxidative stress genes (24).

Campylobacter is a Gram-negative food-borne pathogen, causing estimated 400 to 500 million infections in humans worldwide per year (30, 38). Among *Campylobacter* species, *Campylobacter jejuni* is most frequently implicated in human campylobacteriosis, causing fever, diarrhea, and in some cases Guillain-Barré syndrome as a postinfection complication (1). As a microaerophile, *C. jejuni* requires 5 to 10% oxygen for its optimal growth and is sensitive to atmospheric oxygen levels. Due to the oxygen requirement for growth, *C. jejuni* is subject to oxidative stress and produces various oxidative resistance proteins, such as superoxide dismutase (SodB), catalase (KatA), and alkyl hydroperoxide reductase (AhpC) (2). While SodB detoxifies the superoxide anion, KatA and AhpC are involved in the peroxide stress resistance of *C. jejuni* (3, 11, 28). *C. jejuni* PerR regulates peroxide stress genes, including *katA* and *ahpC*, although PerR and PerR-like proteins have usually been reported in Gram-positive bacteria (34). In fact, *C. jejuni* is the first Gram-negative bacterium where a non-OxyR-dependent regulation system is reported to control peroxide stress genes (34). Since PerR represses peroxide resistance genes, a *perR* mutation renders *C. jejuni* hyper-resistant to hydrogen peroxide (34). In addition to the role of PerR in oxidative stress resistance, recent studies have demonstrated that *perR* expression is upregulated by acid shock (29), and a *perR* mutation significantly impairs *C. jejuni*'s ability to colonize the intestines of chickens (25), suggesting that PerR mediates various pathobiological functions of *C. jejuni*. Although a couple of studies have thus far investigated the PerR regulon

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
DH5α	F′ ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1* Δ(*lacZYA-argF*)*U169 deoR* λ$^-$	Invitrogen
BL21(DE3)	F′ *dcm ompT hsdS*(r_B^- m_B^-) *galDE3*(*lacI lacUV5-T7* gene 1 *ind-1 sam-7 nin-5*)	Novagen
C. jejuni		
NCTC11168	Wild type, a human isolate	27
FMB3001	pMW10::P_{perR}/NCTC11168	This study
FMB3003	*perR* mutant (*perR*::*cat*)	This study
FMB3007	pMW10::P_{perR}/FMB3003	This study
FMB3008	*perR* complementation	This study
Plasmids		
pUC19	Cloning and suicide vector; Amp^r	New England Biolabs
pUC19-*perR*	pUC19 carrying *perR*	This study
pUC19-*perR*::*cat*	pUC19 carrying *perR*::*cat*	This study
pET15b	Amp^r; N-terminal His tag, T7 promoter, pBR322 ori	Novagen
pET15b-*perR*	pET15b carrying *perR*	This study
pMW10	*E. coli-C. jejuni* shuttle plasmid; *lacZ mob repB*; Kan^r	36
pMW10::P_{perR}	pMW10 carrying *perR* promoter	This study
pMW10::P_{perR}-*perR*	pMW10 carrying *perR* promoter and *perR*	This study
pRY112	*E. coli-C. jejuni* shuttle plasmid; Cm^r	37

[a] Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

of *C. jejuni*, little is known about factors influencing *perR* expression in this bacterium despite its importance.

In the present study, we investigated the role of various metal ions in the expression of the metalloregulator PerR and presented the PerR binding sequence, the first described in Gram-negative bacteria. In addition, we demonstrate that *perR* is transcriptionally autoregulated and that *perR* autoregulation is responsible for the iron-responsive repression of *perR* in *C. jejuni*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. jejuni* NCTC11168 was used (27). *C. jejuni* NCTC11168 and its derivatives were grown at 42°C in Mueller-Hinton (MH) media (Oxoid) or MEMα (minimum essential medium alpha; Gibco, catalog no. 41061) under microaerobic conditions generated by Anoxomat (Mart Microbiology B.V, Netherlands). Culture media were occasionally supplemented with kanamycin (50 μg ml^{-1}) or chloramphenicol (10 μg ml^{-1}) where required. Broth cultures were microaerobically grown with shaking at 180 rpm. All of the strains and plasmids used in the present study are listed in Table 1.

Construction of a *perR* mutant and a complementation strain. A DNA region containing *perR* and its flanking area was amplified by PCR with perR_F_EcoRI and perR_R_PstI primers (Table 2). The PCR product was ligated into pUC19 after digestion with EcoRI and PstI. A chloramphenicol resistance cassette (*cat*) was PCR amplified from pRY112 (37) with *Vent* DNA polymerase (New England Biolabs) using the catF(SmaI) and catR(SmaI) primers and inserted into an EcoRV site of the pUC19::*perR* plasmid. The orientation of the *cat* cassette was confirmed by sequencing. The constructed plasmid (pUC19-*perR*::*cat*) was electroporated into *C. jejuni*, and a *perR* mutant was screened by growing on MH agar plates supplemented with chloramphenicol (10 μg ml^{-1}). The allelic exchange in the *perR* mutant was confirmed by PCR (data not shown). A *perR* flanking region was PCR amplified with c_perR_F(SmaI) and c_perR_R(SmaI) primers and cloned into a noncoding region of the *E. coli-C. jejuni* shuttle plasmid pMW10, which had been digested with SmiI. The pMW10 harboring *perR* was introduced into the *perR* mutant by conjugation, as described previously (23).

Determination of the *perR* transcriptional level with reporter gene assays. The promoter and partial coding region of *perR* were amplified with perR_F(BamHI) and perR_R(BamHI) primers and cloned into a BamHI site of pMW10 that contains the promoterless *lacZ* gene. The plasmid was mobilized to *C. jejuni* strains by conjugation. β-Galactosidase assays were performed with *C. jejuni* strains harboring the *perR* promoter (P_{perR})-*lacZ* transcriptional fusion construct, as described previously (36). To examine the effect of metal ions on *perR* transcription, the defined culture medium MEMα was used in the assay after supplementation with $CoCl_2$, $CuCl_2$,$FeSO_4$, $Fe_2(SO_4)_3$, $MnCl_2$, or $ZnCl_2$.

Purification of rPerR. Recombinant PerR (rPerR) was expressed in *E. coli* BL21(DE3) using the pET15b vector (Novagen). The *perR* gene was PCR amplified with perR_his_F(NdeI) and perR_his_R(BamHI) primers. After digestion with NdeI and BamHI, the PCR products were cloned into pET15b that had been digested with the same enzymes. The constructed plasmids were transformed into *E. coli* BL21(DE3). *E. coli* harboring pET15b::*perR* was grown to an optical density at 600 nm of 1.0 at 37°C, and the expression of rPerR was induced by 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 6 h at 20°C. Bacterial cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl). Cells were lysed by sonication and the cell extracts were purified under native conditions using Ni^{2+} affinity chromatography (Qiagen). The His tag of rPerR was removed with a thrombin cleavage capture kit (Novagen). Briefly, rPerR was treated with biotinylated thrombin,

TABLE 2. Primers used in this study

Primer	DNA sequence (5′–3′)
catF(SmaI)	GTGTTCCTTTCCCGGGTAATTGCG
catR(SmaI)	CATAAAAACCCGGGGGAACTAAAGGG
perR_F_EcoRI	TCTAGAAAAAGAATTCAATAGTTTGTTG
perR_R_PstI	ACACTATGATCTGCAGTTATTTGAG
c_perR_F(SmaI)	AATAGTTTGTTGTCTAGAGTTTTAAAAT
c_perR_R(SmaI)	ATTTCATAAACCTCTAGAATAACCC
perR_F(BamHI)	ATGATTTGGATCCAAAAGTTGTTAGCTTTG
perR_R(BamHI)	CGCTAAAGGGATCCAAGGGTATTC
perR_his_F(NdeI)	AAGCAAGGAATAAATCATATGGAATTAC
perR_his_R(BamHI)	CATTAAAAGGATCCTTAAAATATATGGG
perR_F	GATTTGCATGTAAAAGTTGTTAGCTTTGAA TATGAA
perR_R	CGTCGCTAAAGAGATTGAAGGGTATTCTT
perR_in_F	AAAAGACATGAGCATCCTAATATTGATG
perR_in_R	GACACTTTTTGCAATTATCAACATAAGC
ahpC_F	ATCATCAACGATAGCATTCACTGGACAC
ahpC_R	AATACTACCGCTCCTTTTGGACCTATG
ahpC_in_F	ACTCCAGTAAATCAAGGTGGTATTGGTC
ahpC_in_R	TTTTTGCCAAGATATTCAGCCACGCC
perR_FP_F	AGCCTTGCAAGAAATGAATAATAATGC
perR_FP_R	ATTCATCAATATTAGGATGCTCATGTC
perR_PE_F	AGCCTTGCAAGAAATGAATAATAATGC
perR_PE_R	GCCTATCTTTTTTTCTAAATGCTCTTG
PE_R	ATTCATCAATATTAGGATGCTCATGTC

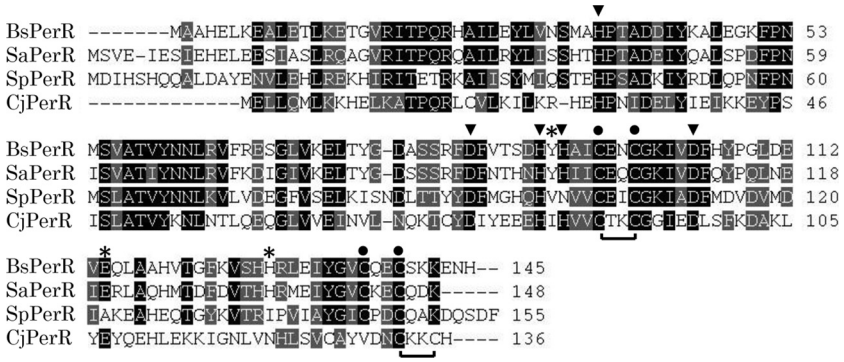

FIG. 1. Sequence alignment of the PerR proteins. The amino acid residues required for binding to a regulatory metal ion (Mn^{2+} or Fe^{2+}) in PerR (21, 33) are indicated with an arrowhead. Two zinc-binding Cys-XX-Cys motifs reported in *B. subtilis* (18, 21, 33) are marked with a dot, and Cys-XX-Cys motifs in *C. jejuni* are indicated with a bracket. Amino acid residues involved in metal ion selectivity (22) are indicated with an asterisk. The sequences for *B. subtilis* PerR (BsPerR; GenBank accession number NP_388753.1), *S. aureus* PerR (SaPerR; NP_646618), *S. pyogenes* (SpPerR; YP_595895.1), and *C. jejuni* PerR (CjPerR; YP_002343760) are shown.

and His tag-free rPerR was separated with streptavidin agarose and spin filters. Purified rPerR protein was visualized by SDS-PAGE.

EMSA. An electrophoretic mobility shift assay (EMSA) was performed as described elsewhere, with some modifications (19). The promoter regions of *perR* and *ahpC* and the internal coding regions of each gene for competition were amplified by PCR with the primer pairs of perR_F and perR_R (product size, 373 bp), ahpC_F and ahpC_R (403 bp), perR_in_F and perR_in_R (331 bp), and ahpC_in_F and ahpC_in_R (323 bp), respectively (Table 2). The PCR products were purified from an agarose gel using Wizard SV Gel and the PCR Clean-Up System (Promega) and labeled with [γ-^{32}P]ATP (GE Healthcare). Unincorporated radioisotope was removed with a MicroSpinG-25 column (GE Healthcare). The 0.2 nM ^{32}P-labeled DNA probe was incubated with rPerR at different concentrations 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 $(NH_4)_2SO_4$, 5 mM dithiothreitol (DTT), 0.2% Tween 20, 30 mM KCl, and 0.1 μg of poly(dI-dC)]. The reaction mixtures were resolved in a 6% polyacrylamide gel, and the radioactivity of the DNA probes was visualized with the BAS2500 system (Fuji Film).

Primer extension assays. Primer extension assays were performed as described previously (31). Briefly, *C. jejuni* was grown to the mid-exponential phase for approximately 8 h in MH broth with shaking and harvested by centrifugation at 10,000 × *g* for 5 min. The total RNA was purified with TRIzol (Invitrogen) according to the manufacturer's instructions. Purified RNA was resuspended in sterile distilled RNase-free water, and the RNA concentration was determined by measuring the optical density of the solution at 260 and 280 nm using NanoVue (GE Healthcare). A portion (10 pmol) of the PE_R primer was labeled with ^{32}P at the 5′ end by 10 U of T4 polynucleotide kinase (Invitrogen) and 80 μCi of [γ-^{32}P]dATP for 30 min at 37°C. The labeling mixture was heated at 70°C for 10 min and purified with MicroSpin G-25 columns (GE Healthcare). The γ-^{32}P-end-labeled primer (0.5 pmol) was coprecipitated with 15 μg of total RNA by the addition of sodium acetate and absolute ethanol. The pellet was washed with 75% ethanol, dried at room temperature, and resuspended in 20 μl of 250 mM KCl, 2 mM Tris (pH 7.9), and 0.2 mM EDTA. The mixture was heated to 65°C and then was allowed to cool to room temperature for 1 h. After annealing, 50 μl of reaction solution containing 5 μg of actinomycin D, 700 μM deoxynucleoside triphosphates, 10 mM $MgCl_2$, 5 mM DTT, 20 mM Tris (pH 7.6), 30 U of RNasin (Promega), and 150 U of Superscript III reverse transcriptase (Invitrogen) was added. The mixture was incubated at 42°C for 70 min and treated with 100 U of RNase T_1 (Invitrogen) at 37°C for 15 min. The sample was ethanol precipitated after addition of 1.4 μl of 5 M NaCl with 2.5 volumes of absolute ethanol and then washed with 75% ethanol. Each sample was resuspended with 6 μl of formamide dye and 4 μl of Tris-EDTA (pH 8.0) buffer and then denatured at 90°C for 3 min. The samples were resolved on 6% polyacrylamide–8 M urea gels, and the reverse transcription signals were analyzed by using BAS 2500 (Fuji Film). The PE_R primer (Table 2) was used for sequencing the *perR* promoter region with a SequiTherm EXCELII DNA sequencing system (Epicentre).

DNase I footprinting assays. DNase I footprinting assays were performed as reported elsewhere (7). A DNA region containing the *perR* promoter was PCR amplified with a ^{32}P-labeled primer perR_FP_R and a nonradiolabeled primer perR_FP_F, and the PCR product was purified from an agarose gel with a Wizard SV Gel and PCR Clean-Up System (Promega). rPerR was incubated with the ^{32}P-labeled *perR* promoter at 37°C for 15 min in 40 μl of the DNA-binding buffer [20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 5 mM DTT, 0.2% Tween 20, 30 mM KCl, 10 mM $MgCl_2$, and 0.1 μg of poly(dI-dC)], and the reaction mixture was treated with 0.1 U of DNase I (Takara). The reactions were stopped by adding 200 μl of ice-cold stop solution (0.4 M sodium acetate, 2.5 mM EDTA), and the DNA products were purified by phenol extraction and ethanol precipitation. The digested DNA fragments were separated by electrophoresis on 6% polyacrylamide–8 M urea gels alongside sequencing ladders that had been generated with the same ^{32}P-labeled PerR_FP_R primer used to amplify DNA fragments for the DNase I digestion.

RESULTS

Amino acid sequence analysis of *C. jejuni* PerR. Comparison of amino acid sequences revealed that *C. jejuni* PerR shares 32, 31, and 31% identity to the PerR proteins from the Gram-positive *B. subtilis*, *S. aureus*, and *S. pyogenes*, respectively (Fig. 1). In *B. subtilis*, Mn^{2+} and Fe^{2+} are metal ions involved in the regulatory function of PerR, and five amino acid residues (H37, D85, H91, H93, and D104) form a square pyramid to mediate the binding of PerR either to Mn^{2+} or Fe^{2+} (18, 21). These five amino acid residues are also highly conserved in *C. jejuni* PerR, suggesting that *C. jejuni* PerR may require Mn^{2+} or Fe^{2+} for gene expression regulation as does *B. subtilis* PerR. The metal ion Zn^{2+} has a structural role in *B. subtilis* PerR (20), and four cysteine residues (C96-XX-C99 and C136-XX-C139 of *B. subtilis* PerR; "X" represents any amino acid residue) are associated with Zn^{2+} binding in a tetrahedral fashion (18, 21, 33). The cysteine pair C96-XX-C99 of *B. subtilis* PerR is highly conserved among the PerR proteins, including *C. jejuni* PerR (C89-XX-C92 of *C. jejuni* PerR), and another cysteine pair (C132-XX-C135) is also present in *C. jejuni* PerR near the conserved *B. subtilis* C136-XX-C139 motif (Fig. 1). Amino acid residues of the less conserved metal binding site, such as Y92, E114, and H128 of *B. subtilis* PerR, are known to influence metal ion selectivity between Mn^{2+} and Fe^{2+} (22). Although these three amino acid residues are relatively well conserved in Gram-positive bacteria, they are not conserved in *C. jejuni* except E114 (E107 of *C. jejuni* PerR) (Fig. 1). These results suggest that *C. jejuni* PerR may bind to Zn^{2+} and either Mn^{2+} or Fe^{2+} as does *B. subtilis* PerR; however, poor conservation of the amino acid residues facilitating metal-ion selec-

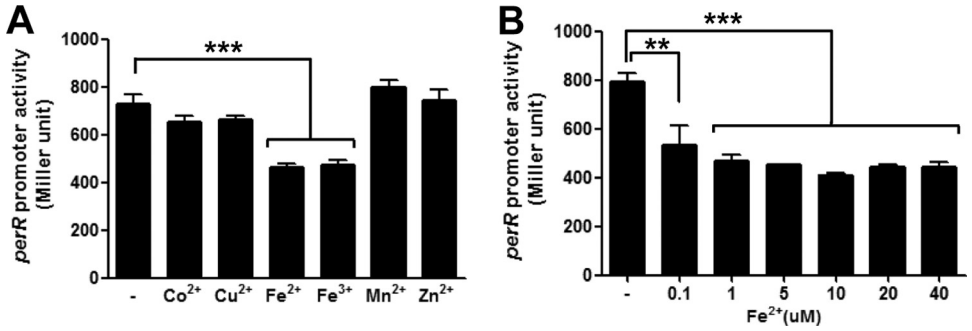

FIG. 2. Effects of metal ions on *perR* transcription. (A) P_{perR}-*lacZ* fusion assays in the presence of 20 μM $CoCl_2$, 20 μM $CuCl_2$,40 μM $FeSO_4$, 40 μM $Fe_2(SO_4)_3$, 40 μM $MnCl_2$, or 10 μM $ZnCl_2$. The "–" indicates the negative control without any metal ions tested. (B) Concentration-dependent reduction of *perR* transcription by iron. The "–" indicates the negative control without iron. Metal ions were added to MEMα, and *C. jejuni* cultures were grown for 8 h before carrying out the assay. The results show the means and standard deviations of three independent experiments. **: $P < 0.01$; ***, $P < 0.001$. The significance of the results was statistically analyzed by one-way analysis of variance (ANOVA) with Dunnett's post tests at a 95% confidence interval using Prism software (version 5.01; GraphPad Software, Inc.).

tivity suggests that *C. jejuni* PerR may have different metal-ion selectivity from *B. subtilis* PerR.

Effect of metal ions on *perR* expression. To investigate the role of metal ions in *perR* expression, the level of *perR* transcription was measured with β-galactosidase assays after the addition of various metal ions, such as Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, and Zn^{2+}, to the defined medium MEMα, which does not contain any of these metal ions. The P_{perR}-*lacZ* fusion assay showed that *perR* is constitutively expressed independent of the growth phase of *C. jejuni* (date not shown). The tested metal ions exhibited differential effects on *perR* transcription. The presence of iron, either ferrous (Fe^{2+}) or ferric (Fe^{3+}), significantly repressed *perR* transcription (Fig. 2A), whereas Co^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+} did not alter the level of *perR* transcription (Fig. 2A). When different concentrations of iron were used in the assay, as low as 0.1 μM iron significantly decreased *perR* expression, and the reduction of *perR* transcription by iron was saturated at concentrations greater than 5 μM (Fig. 2B). These findings demonstrate that, in *C. jejuni*, *perR* expression is primarily regulated in response specifically to iron even at low concentrations. To address whether hydrogen peroxide influences the responsiveness of *perR* transcription to metal ions, the promoter fusion assay was conducted in the presence of hydrogen peroxide; however, hydrogen peroxide did not make any significant changes (see Fig. S1 in the supplemental material). Regardless of the presence or absence of hydrogen peroxide, iron was the major metal ion affecting *perR* transcription.

Derepression of *perR* transcription by a *perR* mutation. A sequence resembling the *B. subtilis* PerR box was found upstream of *perR* in *C. jejuni* (35); however, it has been unknown whether PerR is autoregulated in *C. jejuni*. To address whether PerR regulates *perR* expression in *C. jejuni*, a P_{perR}-*lacZ* fusion assay was performed with a *perR* mutant. Due to the effect of iron on *perR* transcription (Fig. 2), the assay was conducted with the defined medium MEMα in the presence or absence of iron. Interestingly, the P_{perR}-*lacZ* fusion assay demonstrated that *perR* expression was significantly increased in the *perR* mutant compared to the wild type (Fig. 3), suggesting that *perR* is autoregulated in *C. jejuni*. Compared to the wild type, in *trans* complementation of *perR* slightly reduced the level of *perR* transcription (Fig. 3), presumably because PerR produced from the *perR* gene in the multicopy shuttle plasmid further repressed *perR* expression. Interestingly, the *perR* mutation eliminated iron-dependent repression of *perR* (Fig. 3), indicating that the *perR* repression by iron is mediated by *perR* autoregulation. These results clearly demonstrated that *perR* is autoregulated at the transcriptional level in *C. jejuni* and that *perR* autoregulation accounts for the repression of *perR* transcription by iron.

Interaction of PerR with the *perR* promoter. Because the P_{perR}-*lacZ* fusion assay showed that *perR* is transcriptionally autoregulated (Fig. 3), an EMSA was performed to investigate whether PerR directly binds to the *perR* promoter. The rPerR protein was expressed in and purified from *E. coli* and was used in EMSA. The results of the EMSA demonstrated that PerR bound to the *perR* promoter, whereas DNA fragments amplified from the *perR* coding region did not compete with the DNA probe of the *perR* promoter region, confirming the specificity of PerR binding to the *perR* regulatory region (Fig. 4A).

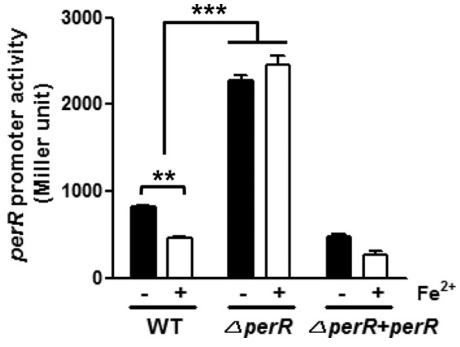

FIG. 3. Derepression of *perR* expression by a *perR* mutation. The results of P_{perR}-*lacZ* fusion assays show the level of *perR* transcription in the wild type (WT), the *perR* mutant (Δ*perR*), and the complementation strain (Δ*perR*+*perR*) in the presence (+) or absence (−) of iron. The results show the means and standard deviations of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$. The significance of results was statistically analyzed by two-way ANOVA of variance with Bonferroni's post tests at a 95% confidence interval using Prism software (version 5.01; GraphPad Software, Inc.).

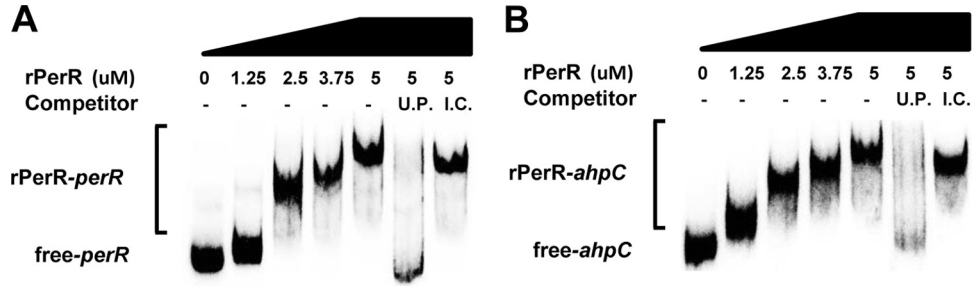

FIG. 4. Binding of PerR to the promoter regions of *perR* (A) and *ahpC* (B). The *ahpC* promoter was used as a positive control of EMSA. The concentrations of rPerR are indicated above the panel. "U.P." and "I.C." stand for "unlabeled probe" and "internal coding region" used as competitors in the assay.

To confirm the binding of PerR to a known PerR-regulated gene, the *ahpC* promoter was included as a positive control in EMSA (Fig. 4B), because PerR is known to regulate *ahpC* in *C. jejuni* (34). These findings show that PerR binds to the *perR* promoter, strongly indicating PerR directly regulates its own transcription.

Determination of the PerR binding site in the *perR* promoter. DNase I footprinting assays were performed to determine the PerR binding site in the *perR* promoter. Prior to DNase I footprinting analysis, primer extension assays were conducted to define the *perR* promoter. Two transcriptional start sites were found in the promoter region of *perR* (Fig. 5A). The transcriptional start site (TS2; Transcriptional Start site 2) located at 26-bp upstream of the *perR* start codon was sharp, whereas the other transcriptional start site (TS1; Transcriptional Start site 1) was broad; presumably, the repeated A and T around TS1 made the control of transcription initiation at TS1 loose. DNA sequences similar to the consensus RpoD promoter of *C. jejuni* were found upstream of each transcriptional start site (Fig. 5C and D). DNase I footprinting assay exhibited that a 26-bp AT-rich region from −36 to −61 was protected from DNase I cleavage (Fig. 5B). The protected region contained a sequence resembling the canonical PerR box of *B. subtilis*, and the PerR binding site determined by DNase I footprinting overlaps with the putative −35 region of the two *perR* promoters (Fig. 5C and D); thus, binding of PerR

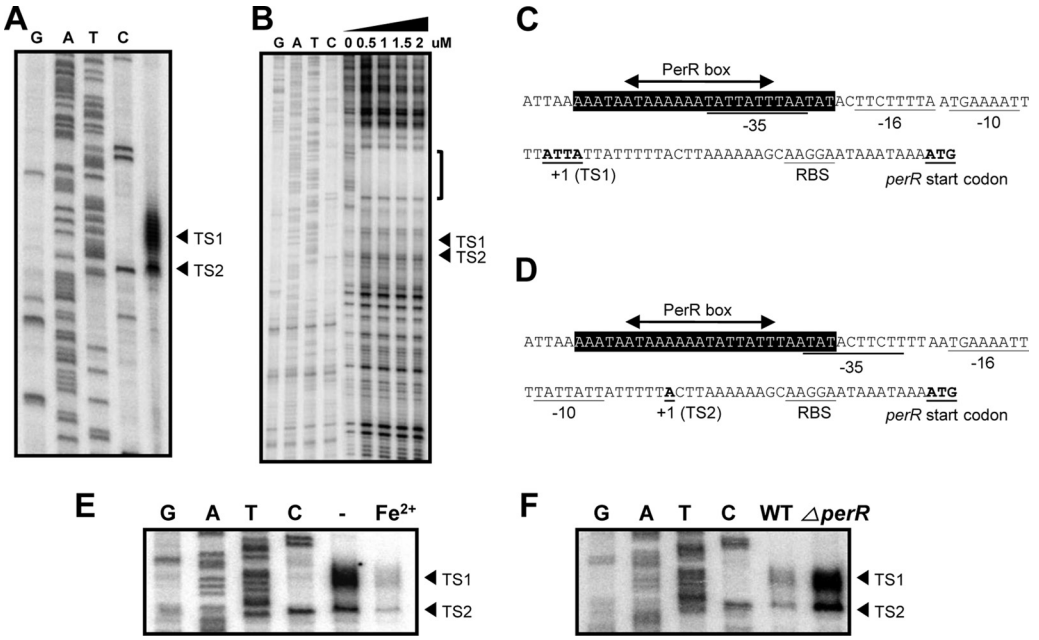

FIG. 5. Characterization of the *perR* transcriptional start site and identification of the PerR binding site. (A) Determination of the transcriptional start site of *perR* by a primer extension assay. Two transcriptional start sites of *perR* are designated TS1 (Transcriptional Start site 1) and TS2, which are indicated with an arrowhead on the right. (B) Determination of the PerR binding site in the *perR* promoter by DNase I footprinting. The PerR binding region is indicated with a bracket and the transcriptional start sites are marked with an arrowhead. The *perR* promoters initiating transcription at TS1 (C) and TS2 (D). The −10, −16, and −35 elements of the *perR* promoter are underlined. The region protected from DNase I cleavage is indicated with black background, and a sequence similar to the *B. subtilis* PerR box is marked with an arrowed line. (E) Repression of *perR* transcription by iron at the transcriptional start site TS1 and TS2. Primer extension assay was conducted with 10 μg of total RNA isolated from *C. jejuni* cultures grown in MEMα in the absence (–) or presence of 40 μM $FeSO_4$. (F) Derepression of *perR* transcription by the *perR* mutation. Primer extension assay was performed with 14 μg of total RNA from the wild type (WT) and the *perR* mutant (Δ*perR*) grown in MEMα in the presence of 40 μM $FeSO_4$.

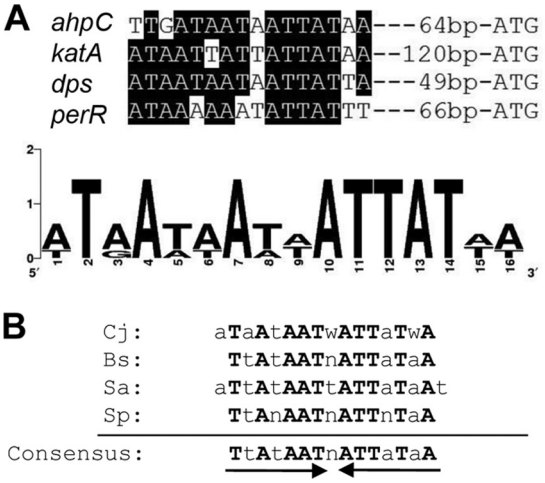

FIG. 6. Determination of the *perR* binding sequence in *C. jejuni*. (A) Multiple alignment of the PerR box present in the *perR* promoter and putative PerR binding sequences of the PerR-regulated genes of *C. jejuni*. The black background indicates identical or highly conserved residues. Sequence logo was generated with the WebLogo version 2.8.2 (5). (B) Comparison of consensus PerR binding sequences of *C. jejuni*, *B. subtilis* (9), *S. aureus* (15), and *S. pyogenes* (4). Identical residues are written in boldface capital letters.

would hinder access of RNA polymerase to the *perR* promoter and represses *perR* transcription. Consistent with the effects of iron and the *perR* mutation on *perR* transcription (Fig. 2 and 3), primer extension assays showed that iron significantly reduced the level of *perR* transcripts from the two transcriptional start sites (Fig. 5E), and the *perR* mutation notably increased the signal intensity of the two start sites (Fig. 5F), indicating that both iron and PerR affects the initiation of *perR* transcription at the two transcriptional start sites. The results demonstrated that PerR recognizes the PerR box present in the *perR* promoter, and its binding to the *perR* regulatory region may interfere with *perR* transcription.

Consensus PerR binding sequence in *C. jejuni*. Based on the PerR binding sequence present in the *perR* promoter, putative PerR binding sequences were identified in the promoter region of PerR regulon genes, such as *ahpC*, *katA*, and *dps* (25, 34). Sequence alignment revealed the PerR binding sequence of *C. jejuni* (ATaAtAATwATTaTwA; "w" means A or T, capital letters are identical residues, and lowercase letters indicate less-conserved residues) (Fig. 6A), which is quite similar to the canonical PerR boxes reported in other Gram-positive bacteria (Fig. 6B). The conserved PerR binding sequence determined by aligning the PerR binding sequences of several bacterial species is identical to the *B. subtilis* PerR box (Fig. 6B). These findings demonstrate that the *C. jejuni* PerR binding site shares high sequence similarities with the PerR boxes of Gram-positive bacteria.

DISCUSSION

As a metalloregulatory protein, PerR has exhibited differential metal ion dependency in multiple bacterial species. The *B. subtilis* PerR protein contains two metal ions; Zn^{2+} and either Fe^{2+} or Mn^{2+}, where the former is required for protein stability and the latter participates in regulation (13, 20). While Mn-bound PerR prevents full derepression of the PerR regulon genes by hydrogen peroxide, Fe-bound PerR demonstrates complete derepression of the PerR regulon genes by hydrogen peroxide (13). Although both Fe^{2+} and Mn^{2+} can bind to PerR and modulate the expression of the PerR regulon genes, only Mn^{2+} is involved in the regulation of *perR* expression in *B. subtilis* in a way that Mn^{2+} represses *perR* expression (10). Similarly, in *S. aureus*, *perR* expression is repressed by Mn^{2+}, and Fe^{2+} rather increases the level of *perR* transcription (15). However, the CatR regulator, a PerR homolog in *Streptomyces coelicolor*, does not appear to be responsive to metal ions (12). Previous proteomic and transcriptomic analyses have reported that the expression level of *perR* and the PerR regulon genes, such as *katA* and *ahpC*, are reduced by iron in *C. jejuni* (14, 26); however, details of the effect of iron on *perR* expression and the roles of other metal ions in *perR* expression have not been understood. In the present study, *perR* expression was significantly repressed by iron even at low (0.1 μM) concentrations (Fig. 2). Unlike *B. subtilis* and *S. aureus*, Mn^{2+} did not affect *perR* expression in *C. jejuni* (Fig. 2A), and other metal ions, including Co^{2+}, Cu^{2+}, and Zn^{2+}, did not change the level of *perR* transcription (Fig. 2A). Both Fe^{2+} and Fe^{3+} similarly repressed *perR* transcription (Fig. 2A), probably due to intracellular conversion between Fe^{2+} and Fe^{3+}.

Notably, a *perR* mutation eliminated iron-mediated repression of *perR* transcription (Fig. 3), suggesting the repression of *perR* by iron results from *perR* autoregulation. In *B. subtilis* and *S. aureus*, *perR* is autoregulated; however, in these bacteria, *perR* is repressed by Mn^{2+}, but not by Fe^{2+} (10, 15). Structural analysis of the *B. subtilis* PerR protein revealed that five amino acid residues (H37, D85, H91, H93, and D104) of the regulatory metal binding site form a square pyramidal environment to capture either Mn^{2+} or Fe^{2+} (21, 33). Amino acid sequence analysis of PerR proteins showed that these five amino acid residues are well conserved in most PerR proteins except H93 of *S. pyogenes* PerR, which is replaced with an asparagine (N101; Fig. 1). Recently, Ma et al. reported that amino acid residues in the less-conserved metal binding site of *B. subtilis* PerR, including Y92, E114, and H128, significantly affected the metal ion selectivity between Mn^{2+} and Fe^{2+}, possibly by influencing the coordination geometry of the PerR protein (22). The three amino acid residues are highly conserved in *S. aureus* PerR, whereas only E114 (E107 of *C. jejuni* PerR) is conserved in *C. jejuni* PerR (Fig. 1). Consistently, *perR* transcription is repressed by Mn^{2+} in *B. subtilis* and *S. aureus* (10, 15); however, in *C. jejuni*, *perR* expression was insensitive to Mn^{2+} but exhibited substantial responsiveness to Fe^{2+} (Fig. 2). Based on this report, the poor conservation of the amino acid residues mediating the metal ion selectivity may result in different metal ion dependence of *perR* expression in *C. jejuni* compared to *B. subtilis* and *S. aureus*.

It has been reported that PerR regulates the oxidative stress defense genes in *C. jejuni* (25, 34); however, interaction of PerR with the promoter of PerR-regulated genes has not yet been empirically proven. In the present study, we first demonstrated PerR binding to the promoter region of the *perR*-regulated genes in *C. jejuni* (Fig. 4). PerR bound to the regulatory regions of *perR* and *ahpC* (Fig. 4); *ahpC* was used as a positive control in EMSA, because *ahpC* is known to be reg-

ulated by PerR in *C. jejuni* (25, 34). Two adjacent transcriptional start sites of *perR* were identified by primer extension analysis (Fig. 5A). The same results were obtained in our multiple trials of primer extension analysis by using several different primers and RNA samples from different batches (data not shown). Two *perR* promoters resembling the consensus RpoD promoter sequence of *C. jejuni* were identified upstream of the transcriptional start sites (Fig. 5C and D). DNase I footprinting assays identified a 26-bp region overlapping with the −35 region of two *perR* promoters (Fig. 5B, C, and D), suggesting that PerR binding may interfere with *perR* transcription driven by the two *perR* promoters. In order to assess which promoter is primarily affected by iron and PerR, primer extension analysis was performed in the presence or absence of iron and with the *perR* mutant. The results demonstrated that the transcript levels of both TS1 and TS2 were affected by iron and the *perR* mutation (Fig. 5E and F). This is consistent with the finding that the PerR binding site overlaps with the −35 region of two *perR* promoters (Fig. 5C and D). Although both of the *perR* promoters were regulated by iron in the present study, it is highly possible that these two promoters can be differentially regulated under certain conditions. It would be an interesting future study to investigate the regulation of the two *perR* promoters in *C. jejuni*.

In *B. subtilis* and *S. aureus*, the PerR binding sequences are known to be an AT-rich inverted repeat (9, 15). Particularly, in *B. subtilis*, the PerR target site consists of 15 bp with a core 7-1-7 inverted repeat (9). Similarly, the PerR binding site of *C. jejuni* is an inverted repeat consisting of only A and T (Fig. 6A). To the best of our knowledge, this is the first presentation of the PerR binding sequence in Gram-negative bacteria. Alignment of the PerR binding sequences of a few bacterial species revealed a consensus PerR binding sequence which is identical to that of *B. subtilis* (Fig. 6B). This suggests that the PerR binding sequences are highly conserved in both Gram-positive bacteria and the Gram-negative *C. jejuni*. In *B. subtilis*, two PerR boxes are present in the *perR* promoter, and one of the two PerR boxes overlaps with the −10 region of the *perR* promoter (10). A PerR box was identified by bioinformatics analysis in the downstream region of the *perR* transcriptional start site in *S. aureus* (15). Consistent with the presence of PerR box in the *perR* promoter regions of *B. subtilis* and *S. aureus*, *perR* is autoregulated in these bacteria, since a *perR* mutation derepresses *perR* transcription based on promoter fusion assays (10, 15). In contrast to *B. subtilis* and *S. aureus*, no PerR box was observed in the *perR* promoter of *S. pyogenes* (4), indicating that *perR* may not be autoregulated in this catalase-negative bacterium. A previous *in silico* analysis of the *C. jejuni* genome revealed the presence of a region in the *perR* promoter, whose DNA sequence is similar to the *B. subtilis* PerR box (35). In fact, the PerR binding region determined by DNase I footprinting in this study overlapped with the PerR box predicted by the *in silico* analysis due to its high sequence similarity to the PerR box of *B. subtilis*.

In *C. jejuni*, PerR represses *katA* and *ahpC*, encoding the major enzymes conferring resistance to peroxide stress (25, 34). As the sole catalase present in *C. jejuni*, KatA significantly contributes to the peroxide stress resistance and intracellular survival of *C. jejuni* (6, 11). The alkyl hydroperoxide reductase AhpC scavenges low levels of hydrogen peroxide in *E. coli* (32) and is involved in the defense against organic peroxide (e.g., cumene hydroperoxide) in *C. jejuni* (3). Reduced expression of *perR* will lead to derepression of *katA* and *ahpC*, and subsequently derepressed expression of *katA* and *ahpC* will render *C. jejuni* resistant to peroxide stress, whereas increased expression of *perR* will make *C. jejuni* susceptible to peroxide stress due to enhanced repression of *katA* and *ahpC* by PerR. By *perR* autoregulation, *C. jejuni* can tightly maintain the intracellular level of PerR and controls the expression of peroxide resistance genes. Therefore, *perR* autoregulation is important in the regulation of the oxidative stress defense of *C. jejuni*. In addition, iron is the metal ion involved in the generation of reactive oxygen species, particularly hydroxyl radicals, via the Fenton reaction. In fact, there is no enzyme capable of detoxifying hydroxyl radicals, although hydroxyl radicals can give damages to most biomolecules (16). Therefore, the iron dependence of *perR* autoregulation may enable *C. jejuni* to sense the level of intracellular free iron available for the Fenton reaction.

Multiple studies reported that both *perR* and *perR* regulon genes, including *katA* and *ahpC*, are repressed by iron (3, 14, 26), although the iron-dependent repression of *ahpC* and *katA* is mediated primarily by PerR (34). This cannot be simply explained by the role of PerR as a repressor of *katA* and *ahpC*, because reduced *perR* expression by iron should derepress *katA* and *ahpC*. It can be postulated that high concentrations of iron reduce *perR* expression but rather increase the level of iron-bound PerR, which in turn may further repress *katA* and *ahpC* at high iron concentrations. In order to prove this, however, we still need to understand the interaction of PerR with iron in *C. jejuni* and the contribution of iron to the DNA-binding activity of PerR. According to a report by van Vliet et al. (34), although the *perR* mutation substantially increased the KatA activity in *C. jejuni*, the KatA activity was still affected by iron in the *perR* mutant; the KatA activity was 2-fold higher in the low-iron condition than in the high-iron condition (34). However, the effect of iron on the KatA activity disappeared in the *perR* and *fur* double mutant, whereas a *fur* single mutation alone did not eliminate the iron-dependent regulation of the KatA activity (34). This suggests that the PerR regulon is perhaps regulated by the interplay between PerR and Fur in association with iron. In addition, we still cannot exclude the involvement of an unknown regulator, which may overcome the derepression of *katA* and *ahpC* by PerR and still can repress the PerR regulon genes in the presence of iron.

A transcriptomic analysis of the *perR* regulon performed by Palyada et al. showed that the microarray expression ratio of *perR* is not different between the *perR* mutant and the wild-type *C. jejuni* regardless of the presence of iron (25). This discrepancy can be attributed to the intrinsic differences between microarray analysis and promoter-fusion assay under different experimental settings. DNA microarray is best designed to analyze transcriptomic changes, but the results can be unavoidably affected by multiple factors, such as the high or low expression levels of transcripts (8). The *perR* transcription level was higher in the *perR* mutant than in the wild type by approximately 2- and 3-fold in the absence and presence of iron, respectively, as determined by the P_{perR}-*lacZ* fusion assay (Fig. 3). Such fold changes could not be fully reflected in the microarray analysis. In addition, the experimental conditions were also different. To assess the effect of excess iron, Palyada

et al. treated *C. jejuni* with iron for short time (15 min) prior to RNA extraction, whereas iron was added at the beginning of bacterial culture in the present study; microarray requires RNA samples that are produced right after condition changes, whereas the *lacZ* promoter fusion assay needs sufficient time to synthesize the β-galactosidase enzyme, whose expression is driven by the fused promoter.

In summary, we clearly demonstrated here that *perR* transcription is reduced by iron in *C. jejuni* and that *perR* autoregulation mediates the iron-responsive repression of *perR*. Based on the important roles played by PerR and iron in oxidative stress resistance, these findings provide new insights into the regulation of oxidative stress resistance in *C. jejuni*. In addition, we first reported the PerR box in Gram-negative bacteria, showing that the PerR box of *C. jejuni* is highly homologous to that of other Gram-positive bacteria, such as *B. subtilis* and *S. aureus*. Future studies will investigate how PerR senses peroxide stress and how PerR interplays with other regulatory elements in *C. jejuni*.

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