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A Mutator Phenotype Promoting the Emergence of Spontaneous Oxidative Stress-Resistant Mutants in *Campylobacter jejuni*

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ABSTRACT *Campylobacter jejuni* is a leading cause of foodborne illnesses worldwide. As a microaerophilic organism, *C. jejuni* must be able to defend against oxidative stress encountered both in the host and in the environment. How *Campylobacter* utilizes a mutation-based mechanism for adaptation to oxidative stress is still unknown. Here we present a previously undescribed phenotypic and genetic mechanism that promotes the emergence of oxidative stress-resistant mutants. Specifically, we showed that a naturally occurring mutator phenotype, resulting from a loss of function mutation in the DNA repair enzyme MutY, increased oxidative stress resistance (OX^R) in *C. jejuni*. We further demonstrated that MutY malfunction did not directly contribute to the OX^R phenotype but increased the spontaneous mutation rate in the peroxide regulator gene *perR*, which functions as a repressor for multiple genes involved in oxidative stress resistance. Mutations in PerR resulted in loss of its DNA binding function and derepression of PerR-controlled oxidative stress defense genes, thereby conferring an OX^R phenotype and facilitating *Campylobacter* survival under oxidative stress. These findings reveal a new mechanism that promotes the emergence of spontaneous OX^R mutants in bacterial organisms.

IMPORTANCE Although a mutator phenotype has been shown to promote antibiotic resistance in many bacterial species, little is known about its contribution to the emergence of OX^R mutants. This work describes the link between a mutator phenotype and the enhanced emergence of OX^R mutants as well as its underlying mechanism involving DNA repair and mutations in PerR. Since DNA repair systems and PerR are well conserved in many bacterial species, especially in Gram positives, the same mechanism may operate in multiple bacterial species. Additionally, we developed a novel method that allows for rapid quantification of spontaneous OX^R mutants in a bacterial population. This method represents a technical innovation and may also be applied to other bacterial species. These findings significantly advance our understanding of bacterial mechanisms for survival under oxidative stress.

KEYWORDS *Campylobacter*, DNA repair, oxidative stress

Campylobacter jejuni is a major enteric pathogen, and it is considered to be the most common bacterial cause of human gastroenteritis in the world (1). The Centers for Disease Control and Prevention (CDC) of the United States estimated that it causes over 1.3 million cases of foodborne illnesses in the United States annually (2). As a microaerophilic organism and a pathogen transmitted mainly via the food chain, *C. jejuni* is exposed to highly variable oxygen concentrations. In order to survive, *C. jejuni* must be able to cope with high environmental oxygen tensions and resist the oxidative stresses encountered both in the host and in the environment (3). Reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals,

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are generated during aerobic metabolism by the stepwise one-electron reduction of molecular oxygen (4). Additionally, *C. jejuni* cells are also exposed to ROS produced by the host immune system (5, 6). ROS damages DNA and proteins and causes peroxidation of lipids (4, 7). To survive the stress from ROS, microorganisms, including *C. jejuni*, have developed various mechanisms to detoxify ROS (3, 8).

In many enteric Gram-negative bacteria such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, cells defend against oxidative stress by inducing two distinct stress responses, the peroxide stimulon and superoxide stimulon, which are regulated by the superoxide- and peroxide-sensing regulators, SoxRS and OxyR, respectively (7, 8). However, these regulators are not found in the sequenced *C. jejuni* genome (9), which suggests that oxidative stress defense in *Campylobacter* is regulated by different systems. van Vliet et al. identified a *C. jejuni* gene encoding a peroxide regulator (PerR) homolog, which was originally characterized in a Gram-positive bacterium, *Bacillus subtilis* (10). PerR was then found to regulate a number of oxidative stress-related genes in *C. jejuni* (10, 11). For example, catalase (KatA) and alkyl hydroperoxide reductase (AhpC), two of the most important factors in defending against oxidative stress, are negatively regulated by PerR in *C. jejuni* in an iron-dependent manner (10). Several other oxidative-stress-related genes are also regulated by PerR, including superoxide dismutase (SodB) and even *perR* itself (11, 12). It was later discovered that Fur (ferric uptake regulator), a PerR homolog, coregulates several oxidative stress defense genes (12, 13). Additionally, an OmpR-type response regulator, CosR, was also found to play an important role in oxidative stress defense in *C. jejuni* (14, 15). Thus, various regulons and enzymes function together in modulating oxidative stress defense in *C. jejuni*.

DNA base excision repair (BER) is also involved in defending against oxidative stress in living organisms. Specifically, the DNA glycosylase MutYH in humans locates and repairs 8-oxoguanine (8-oxoG) lesions, a common product of oxidative damage to DNA (16). Defects in the DNA glycosylase MutYH are shown to be directly associated with colorectal cancer, which underlines the importance of preventing mutations associated with 8-oxoG (17). The adenine glycosylase MutY in different bacterial species was also reported to be involved in the protection against oxidative stress (8, 18–20). Epsilonproteobacteria, including *Campylobacter* and *Helicobacter*, have a homolog of the MutY enzyme. A *Helicobacter pylori mutY* mutant exhibited a greater spontaneous mutation rate than its parent strain when incubated at 5% O_2. Interestingly, the mutation rate is further increased by exposing the *mutY* mutant to atmospheric levels of oxygen, which was not observed in an *E. coli mutY* mutant (19). Therefore, it is suggested that the *H. pylori* DNA repair system plays a significant role in defending against oxidative DNA damage.

Recently, we identified a *C. jejuni* mutator phenotype in the isolate named CMT, which carried a naturally occurring loss-of-function mutation (corresponding to an amino acid change) in the DNA repair gene *mutY* and showed enhanced emergence of spontaneous antibiotic-resistant mutants (21). Interestingly, we also observed that CMT demonstrated an increased oxidative stress resistance (OX^R) phenotype compared to the wild-type (WT) strain with a functional MutY system. This phenotype appeared to be contradictory to that in other bacteria such as *H. pylori*, since it was reported that *H. pylori* mutants lacking a functional DNA repair protein are more sensitive than WT cells to oxidative stress induced by agents such as H_2O_2 (22). Therefore, the unexpected OX^R phenotype in the *C. jejuni* CMT isolate prompted us to study the relationship between the DNA repair system and oxidative stress defense and understand how MutY affects *Campylobacter* survival under oxidative stress. By utilizing various genetic and biochemical methods, we found that MutY malfunction did not directly contribute to OX^R in *C. jejuni*; instead, it enhanced the spontaneous mutation rate in the *perR* gene, which consequently results in the loss of function of PerR and derepression of PerR-controlled oxidative stress defense genes, thereby conferring an OX^R phenotype in *Campylobacter*.

TABLE 1 *C. jejuni* strains used in this study

Strain	Relevant genotype or phenotype[a]	Source
11168	*C. jejuni* WT isolate	9
CMT	A naturally occurring mutant of *C. jejuni* 11168 with the G199→W change in MutY	21
81-176	*C. jejuni* WT isolate	52
11168Δ*mutY*	11168 derivative; Δ*mutY*::*cat* insertional mutation	This study
11168Δ*mutY*C	11168Δ*mutY* complement; 11168 Δ*mutY*::*cat 16S*::*mutY*	This study
81-176Δ*mutY*	81-176 derivative; Δ*mutY*::*cat* insertional mutation	This study
81-176Δ*mutY*C	81-176Δ*mutY* complement; 81-176 Δ*mutY*::*cat 16S*::*mutY*	This study
CMT $perR_{C143A}$	CMT spontaneous OX^R mutant; C143→A substitution in *perR*	This study
CMT $perR_{C250A}$	CMT spontaneous OX^R mutant; C250→A substitution in *perR*	This study
CMT $perR_{G319T}$	CMT spontaneous OX^R mutant; G319→T substitution in *perR*	This study
11168 $perR_{A338Del}$	11168 spontaneous OX^R mutant; A338 deletion in *perR*	This study
11168Δ*perR*	11168 derivative; Δ*perR*::*cat* insertional mutation	This study
11168Δ*perRCperR*$_{11168}$	11168Δ*perR* complement; 11168 Δ*perR*::*cat 16S*::$perR_{11168}$	This study
11168Δ*perRCperR*$_{C143A}$	11168Δ*perR* complement; 11168 Δ*perR*::*cat 16S*::$perR_{C143A}$	This study
11168Δ*perRCperR*$_{C250A}$	11168Δ*perR* complement; 11168 Δ*perR*::*cat 16S*::$perR_{C250A}$	This study
11168Δ*perRCperR*$_{G319T}$	11168Δ*perR* complement; 11168 Δ*perR*::*cat 16S*::$perR_{G319T}$	This study
11168Δ*perRCperR*$_{A338Del}$	11168Δ*perR* complement; 11168 Δ*perR*::*cat 16S*::$perR_{A338Del}$	This study
CMT $perR_{C143A}$RV	CMT $perR_{C143A}$ derivative, $perR_{C143A}$ reverted to $perR_{11168}$	This study
CMT $perR_{C250A}$RV	CMT $perR_{C250A}$ derivative, $perR_{C250A}$ reverted to $perR_{11168}$	This study
CMT $perR_{G319T}$RV	CMT $perR_{G319T}$ derivative, $perR_{G319T}$ reverted to $perR_{11168}$	This study
11168 $perR_{A338Del}$RV	11168 $perR_{A338Del}$ derivative, $perR_{A338Del}$ reverted to $perR_{11168}$	This study
CMT $perR_{C250A}$CT	CMT $perR_{C250A}$ derivative, control for *perR* mutant reversion	This study
11168 P_{katA}-*cat*	11168 fusion construct; 11168 *16S*::P_{katA}-*cat*	This study
CMT P_{katA}-*cat*	CMT fusion construct; CMT *16S*::P_{katA}-*cat*	This study

[a]*16S*, 16S rRNA gene.

RESULTS

Loss-of-function mutation in MutY leads to an oxidative stress resistance phenotype. All bacterial strains used in this study are listed in Table 1. *C. jejuni* 11168 (9) and its MutY mutant CMT (21) were tested for the susceptibility to a mixture of paraquat (0.2 mM) and H_2O_2 (2 mM). Interestingly, the CMT isolate was more resistant to the killing by the mixture of oxidants than *C. jejuni* 11168 under the same experimental conditions (Fig. 1). This finding suggested that the loss-of-function mutation (an amino acid change) in MutY of CMT might have contributed to the enhanced resistance to oxidative stress. To further investigate the role of MutY in the OX^R phenotype, we constructed insertional *mutY* mutants in *C. jejuni* 11168 and 81-176 as well as their complements. Using the same oxidative stress susceptibility assay, it was shown that the insertional *mutY* mutants of *C. jejuni* 11168 and 81-176 were indeed more resistant to the killing by paraquat and H_2O_2 than their parent (WT) strains (Fig. 1). Complementation of the *mutY* mutants fully restored their peroxide susceptibility to the WT level (Fig. 1).

Detection of spontaneous OX^R mutants after peroxide treatment. Although the oxidative stress susceptibility assay (Fig. 1) clearly showed that strain CMT was more resistant to the peroxide treatment than strain 11168, the disk inhibition assay did not show a significant difference between the diameter of zone inhibition for the tested oxidants of *C. jejuni* 11168 and that of the CMT isolate (Table 2). This discrepancy prompted us to hypothesize that the mutator isolate CMT might generate more spontaneous mutants that somehow survive the oxidant treatment better than do WT isolates. To evaluate this possibility, single colonies were randomly picked from the two strains grown on Mueller-Hinton (MH) plates with or without peroxide treatment (see "Oxidative stress sensitivity assay and selection of spontaneous OX^R mutants" in Materials and Methods). All picked colonies from MH plates without peroxide treatment (control group) showed similar susceptibility to their parent *C. jejuni* strains 11168 and CMT (data not shown). However, all three picked colonies from the CMT isolate grown on MH plates after the peroxide treatment were highly resistant to the tested oxidants in the disk inhibition assay, especially to H_2O_2 (Table 2). For strain 11168, few colonies grew on MH plates after the peroxide treatment, and it was possible to pick only three

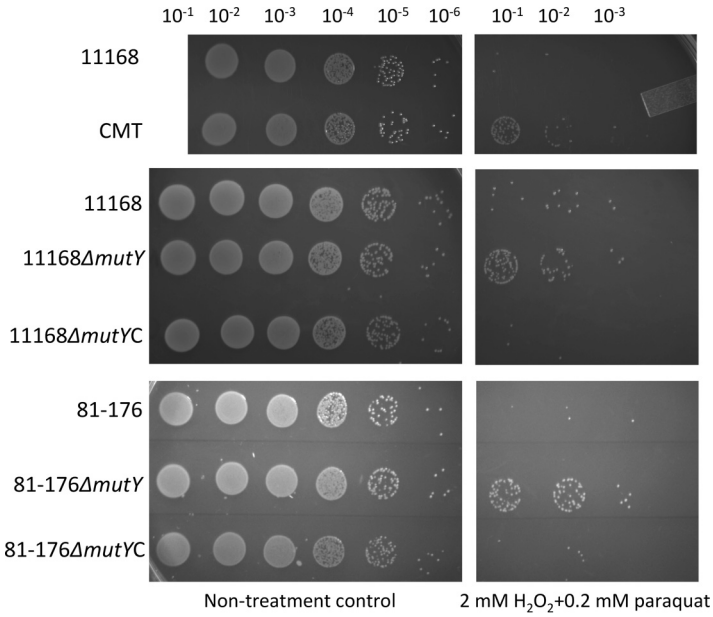

FIG 1 Enhanced resistance of the MutY mutants to oxidative stress as determined by a plate assay. The strains used in the test are labeled on the left. Their 10-fold serial dilutions are indicated at the top. The treatment was done with a mixture of 2 mM H_2O_2 and 0.2 mM paraquat for 30 min. Each set of assays was carried out in triplicate, and representative results are shown.

colonies in total from multiple plates. One of the three colonies was confirmed to have increased peroxide resistance by disk diffusion assay, while the other two remained susceptible to peroxide (Table 2). These results revealed the existence of spontaneous OX^R mutants in *C. jejuni*, which were observed after treating *C. jejuni* cultures with oxidants. This prompted us to further characterize the OX^R phenotype in *Campylobacter*.

Increased catalase activity in the spontaneous OX^R mutants. To understand how the OX^R mutants were resistant to peroxides, we analyzed cellular catalase activities in the mutants and their parent strains. Whole-protein extracts were prepared from

TABLE 2 Oxidative stress sensitivity of various *C. jejuni* strains and constructs as measured by disk diffusion assay[a]

Strain	Mean diam (mm) of zone of inhibition		
	H_2O_2	Cumene hydroperoxide	Paraquat dichloride
11168	22	32	11
CMT	23.5	32	10.5
11168 *perR*$_{A338Del}$	6	25.5	6
CMT *perR*$_{C143A}$	6	25.5	6
CMT *perR*$_{C250A}$	6	26	6
CMT *perR*$_{G319T}$	6	25	6
11168Δ*perR*	6	ND[b]	ND
11168Δ*perRCperR*$_{11168}$	19.5	ND	ND
11168Δ*perRCperR*$_{C143A}$	6	ND	ND
11168Δ*perRCperR*$_{C250A}$	6	ND	ND
11168Δ*perRCperR*$_{G319T}$	6	ND	ND
11168Δ*perRCperR*$_{A338Del}$	6	ND	ND
CMT *perR*$_{C143A}$RV	24	ND	ND
CMT *perR*$_{C250A}$RV	24	ND	ND
CMT *perR*$_{G319T}$RV	26	ND	ND
11168 *perR*$_{A338Del}$RV	23.5	ND	ND
CMT *perR*$_{C250A}$CT	6	ND	ND

[a]Data are means from triplicate plates. The diameter of the disk itself is 6 mm; therefore, 6 mm indicates no obvious inhibition.
[b]ND, not determined.

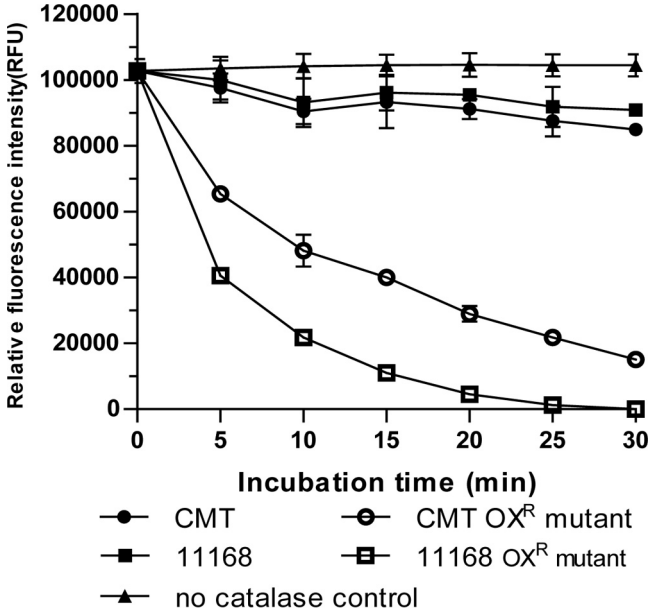

FIG 2 Catalase activities measured by the Amplex Red-based assay. PBS control (filled triangle), 11168 (filled square), CMT (filled circle), 11168 OX^R mutant 11168 $perR_{A338Del}$ (open square), and CMT OX^R mutant CMT $perR_{C143A}$ (open circle) were used in the assay. Amplex Red reagent was added to the reaction mixtures at 5-min intervals, and fluorescence signals from the oxidation indicator product resorufin were measured in triplicate wells. The experiments were carried out at least three times, and a representative result is shown.

C. jejuni 11168, CMT, and their corresponding spontaneous OX^R mutants and then tested for catalase activity. As depicted in Fig. 2, extracts from the spontaneous OX^R mutants exhibited significantly more rapid depletion of H_2O_2 than their parent strains (*C. jejuni* 11168 and CMT). Additionally, no obvious difference between *C. jejuni* 11168 and CMT was observed, indicating the MutY mutation itself did not contribute to the increased catalase activity in *Campylobacter*. Together, these results showed that the spontaneous OX^R mutants exhibited significantly elevated catalase activity compared with their parent isolates.

Elevated transcription of oxidative stress resistance genes in the spontaneous OX^R mutants. It was previously reported that catalase (KatA) and alkyl hydroperoxide reductase (AhpC) are two of the most important enzymes in the defense against oxidative stress in *Campylobacter* (10, 12, 23). In *C. jejuni*, PerR was shown to mediate iron-dependent regulation of *katA* and *ahpC* (10). In this study, the spontaneous OX^R mutants showed markedly elevated catalase activity compared with their parent isolates (Fig. 2), suggesting an increased expression of the catalase-producing genes. Therefore, transcription levels of the PerR regulon, including the *perR*, *katA*, and *ahpC* genes in the spontaneous OX^R mutants and their parent strains, were compared by utilizing real-time quantitative reverse transcription (RT)-PCR. Results revealed that the transcription of *katA* and *ahpC* were significantly upregulated in the OX^R mutants compared to 11168 or CMT (Table 3). Particularly, a >500-fold increase in the tran-

TABLE 3 qRT-PCR analysis of *katA*, *perR*, and *ahpC* transcription

Gene	Fold change ± SD ($2^{-\Delta\Delta CT}$)[a]		
	11168/CMT	CMT $perR_{C143A}$/CMT	11168 $perR_{A338Del}$/11168
katA	0.91 ± 0.14	564.93 ± 28.99	833.24 ± 151.17
perR	0.89 ± 0.09	2.32 ± 0.55	4.12 ± 0.53
ahpC	0.96 ± 0.21	12.16 ± 1.49	15.30 ± 3.32

[a]Fold change of gene transcription for the strain on the left of the slash relative to that on the right of the slash (e.g., the values for 11168/CMT represent fold change for 11168 gene transcription relative to CMT gene transcription).

scription of *katA* was detected in the OX^R mutants, consistent with their increased catalase production (Fig. 2). Transcription of the *perR* gene was also upregulated in the spontaneous OX^R mutants (Table 3), consistent with the previous finding that PerR is transcriptionally autoregulated in *C. jejuni* (11). There was no difference in the transcription of the three genes in *C. jejuni* 11168 and in the CMT isolate (Table 3), indicating that the MutY mutation itself did not affect gene expression of the PerR regulon. Taken together, the results strongly suggest that the hyperresistance to peroxide in the OX^R mutants is due to the upregulation of the PerR regulon in *Campylobacter*.

Occurrence of point mutations in *perR* of the spontaneous OX^R mutants. The spontaneous OX^R mutants from *C. jejuni* 11168 or CMT were sequenced for mutations in oxidative stress defense-related genes, including *katA*, *ahpC*, *sodB*, *perR*, and *fur*. The three OX^R mutants originated from CMT were found to carry a single substitution in the *perR* gene, namely, a C143→A, C250→A, or G319→T transversion, which led to a 48Ser→Tyr, 86Thr→Lys, or 107Glu→Stop codon change, respectively, in the PerR protein (Table 1). Except for the point mutations in *perR*, no additional mutations were detected in other sequenced genes, including their promoter regions in the OX^R mutants of the CMT isolate. The only OX^R mutant from 11168 was found to carry a 1-bp deletion (338A) in the *perR* encoding sequence, resulting in a frameshift after 112 amino acids and truncation of PerR. No mutations were detected in the other analyzed genes. Therefore, all of the analyzed spontaneous OX^R mutants carried point mutations in *perR*, suggesting that these point mutations might have affected the function of PerR, which consequently led to the peroxide resistance phenotype in *Campylobacter*.

The point mutations in *perR* of the spontaneous OX^R mutants abolished PerR function. In order to investigate whether PerR from the spontaneous OX^R mutants is still functional in *Campylobacter*, the *perR* gene sequences from the spontaneous OX^R mutants were cloned into a *C. jejuni perR* knockout strain (Δ*perR*::*cat*). These constructs were assayed for H_2O_2 susceptibility using the disk inhibition assay. The Δ*perR*::*cat* mutant complemented with the wild-type 11168 *perR* sequence served as a control. All the constructs were confirmed with the desired *perR* insertions in the chromosomal location by DNA sequencing. As shown in Table 2, 11168Δ*perR* exhibited significantly reduced susceptibility to exposure to H_2O_2 compared with the wild-type strain 11168. Complementation of the mutant with *perR* from 11168 (11168Δ*perRCperR*$_{11168}$) restored its susceptibility to H_2O_2. In contrast, the complementation constructs 11168Δ*perRCperR*$_{C143A}$, 11168Δ*perRCperR*$_{C250A}$, 11168Δ*perRCperR*$_{G319T}$, and 11168Δ*perRCperR*$_{A338Del}$ (Table 1) with a *perR* containing either a *perR*$_{C143A}$, *perR*$_{C250A}$, *perR*$_{G319T}$, or *perR*$_{A338Del}$ mutation failed to restore the susceptibility to H_2O_2 (Table 2). These results indicated that the mutated perR sequences failed to restore the H_2O_2 susceptibility, suggesting that the point mutations in PerR compromised its function.

Reversion of the point mutations in *perR* of the OX^R mutants restored their susceptibility to H_2O_2. To further define the roles of the *perR* point mutations in oxidative stress resistance in *C. jejuni*, the point mutations were reverted to the wild-type *perR* sequence by homologous recombination, creating constructs CMT *perR*$_{C143A}$RV, CMT *perR*$_{C250A}$RV, CMT *perR*$_{G319T}$RV, and 11168Δ*perR*$_{A338Del}$RV (Table 1), in which the reversion was accompanied by an insertion of a kanamycin resistance cassette in the adjacent gene *cj0323*. To ensure that insertion of a kanamycin resistance cassette in *cj0323* itself did not affect the susceptibility to oxidative stress, a control construct CMT *perR*$_{C250A}$CT (Table 1) was also made, in which the kanamycin resistance cassette was inserted into *cj0323* without reversion of the mutation in *perR*. As shown in Table 2, the control construct did not show any change in H_2O_2 susceptibility compared with that of the OX^R mutants, while all the revertants showed a drastic increase in H_2O_2 susceptibility, to a level comparable to that of wild-type *C. jejuni* 11168. Together, these results clearly indicated that point mutations in *perR* were responsible for the oxidative stress resistance phenotype.

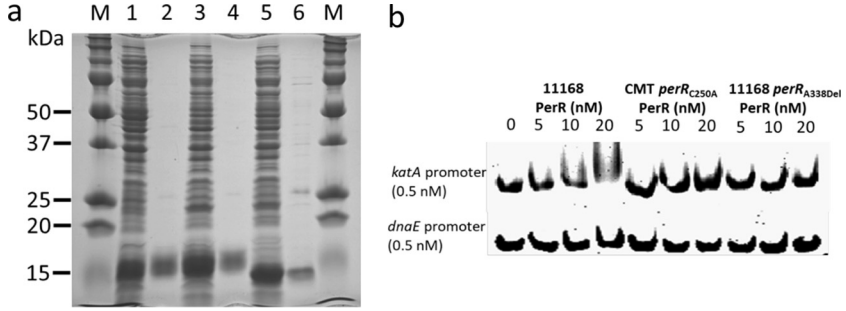

FIG 3 Production of rPerR proteins in *E. coli* and analysis of their binding to the promoter DNA of *katA*. (a) SDS-PAGE analysis of rPerR produced in *E. coli*. Lane M, prestained molecular mass markers (Bio-Rad); lanes 1, 3, and 5, whole-cell lysates of *E. coli* constructs (induced with 1 mM IPTG) expressing PerR of 11168, CMT $perR_{C250A}$, and 11168 $perR_{A338Del}$, respectively; lanes 2, 4, and 6, purified rPerR of 11168, CMT $perR_{C250A}$, and 11168 $perR_{A338Del}$, respectively, purified by Ni-nitrilotriacetic acid affinity chromatography. (b) EMSAs using purified rPerR and promoter DNA of *katA*. The various rPerR proteins and their concentrations are indicated above the panel. The *dnaE* promoter DNA is used as a negative control.

Mutated PerR failed to bind to promoter DNA of *katA*. To determine if the point mutations in *perR* affect its direct interactions with target promoter DNA, a gel mobility shift assay was performed using purified rPerR and the promoter region of *katA*. Purification was successful with only three of the five rPerRs made in *E. coli*, including rPerR from 11168, CMT $perR_{C250A}$, and 11168 $perR_{A338Del}$ (Fig. 3a). Multiple trials for purifying rPerR from CMT $perR_{C143A}$ and CMT $perR_{G319T}$ under the same conditions failed to yield any purified rPerR (see Discussion). Therefore, only three rPerRs were further evaluated by electrophoretic mobility shift assays (EMSAs). As shown in Fig. 3b, the wild-type 11168 rPerR bound to the *katA* promoter, shown by shift of bands, but not to the control *dnaE* promoter. However, rPerR from both CMT $perR_{C250A}$ and 11168 $perR_{A338Del}$ did not bind to either *katA* or *dnaE* promoter DNA. Together, these findings indicate that the point mutations in rPerR proteins of CMT $perR_{C250A}$ and 11168 $perR_{A338Del}$ resulted in loss of binding of PerR to the *katA* promoter, explaining the derepression of the PerR regulon.

Elevated spontaneous OX^R mutation frequency in the CMT isolate. It was not possible to determine the spontaneous OX^R mutation frequency directly by using H_2O_2 as a selection agent, since it is not stable and tends to be degraded in the culture medium. To quantify the differences in the frequencies of emergence of spontaneous OX^R mutants between *C. jejuni* 11168 and the CMT isolates, we developed a reporter system by fusing the promoter of *katA* with the chloramphenicol resistance gene *cat*, yielding constructs 11168 P_{katA}-*cat* and CMT P_{katA}-*cat*. The Cm^r mutation frequency for the CMT P_{katA}-*cat* isolate was 5.90×10^{-7}, >100-fold higher than that of the *C. jejuni* 11168 P_{katA}-*cat* isolate (5.14×10^{-9}). This difference is statistically significant ($P < 0.0001$; Student's *t* test) and indicates that the CMT isolate is much more mutable to oxidative stress.

For each of 11168 P_{katA}-*cat* and CMT P_{katA}-*cat*, 15 or 16 Cm^r colonies grown on the selective plates were randomly picked and subsequently sequenced for the *perR* gene sequence and tested for H_2O_2 susceptibility. As expected, all sequenced Cm^r colonies carried point mutations in the *perR* gene (see Table S1 in the supplemental material). For strain 11168 P_{katA}-*cat*, most (except one) of its Cm^r mutants had a 1- to 2-bp insertion or deletion in *perR*, which resulted in frameshift and truncation of the PerR protein. However, all mutant colonies sequenced for strain CMT P_{katA}-*cat* carried a G→T or C→A transversion in the *perR* encoding region. Interestingly, 13 of the 16 G→T or C→A transversions led to codon changes from an amino acid to termination codons (TAA or TAG), resulting in early translational termination of the PerR protein. The fact that all OX^R mutants from the *C. jejuni* CMT isolate carried a C→A or G→T transversion in *perR* (Table S1) is consistent with the previous finding that the MutY mutation in CMT promoted G→T and C→A transversions (21). These results revealed that the Cm^r

mutants generated from strain CMT had distinct mutation patterns compared with those of 11168, but the end outcomes of these mutations in the two strains were the same, i.e., loss of function for PerR. The disk diffusion assay showed that all the Cm^r mutants carrying *perR* mutations from either 11168 P_{katA}-*cat* or CMT P_{katA}-*cat* were also highly resistant to H_2O_2 (data not shown), which indicated that the promoter fusions (Cm^r) allowed for accurate selection of OX^R mutants. Together, these results revealed that spontaneous OX^R mutants occurred much more frequently in the CMT isolate than in 11168 and the OX^R mutants carried loss-of-function mutations in PerR.

DISCUSSION

Unlike the spontaneous mutants resistant to antibiotics, spontaneous OX^R mutants have been rarely reported in bacteria. It was previously reported that a point mutation in the *oxyR* gene, which encodes the peroxide sensor OxyR, led to the activation of the oxidative stress defense genes and peroxide resistance in a plant pathogen, *Xanthomonas campestris* (24). With Gram-positive *Bacillus subtilis*, Chen et al. discovered that spontaneous PerR mutants overproduced KatA and AhpC and displayed an H_2O_2 resistance phenotype (25). Interestingly, *C. jejuni*, a Gram-negative bacterium, lacks OxyR and instead possesses the metalloregulator PerR, which is normally found in Gram-positive bacteria such as *B. subtilis* (26), *Staphylococcus aureus* (27), *Enterococcus faecalis* (28), and *Streptococcus pyogenes* (29). Although the insertional inactivation of PerR has been linked to increased aerotolerance and hyperresistance to H_2O_2 in *C. jejuni* (10, 12, 30), spontaneous mutations conferring peroxide resistance in *Campylobacter* have not been described. In this study, the MutY mutation in the mutator isolate CMT was found to increase the spontaneous mutation frequency in *perR*, leading to malfunction of PerR, derepression of the PerR regulon, and consequently the increased emergence of OX^R mutants in *Campylobacter*. These findings reveal a new role for a mutator phenotype and the underlying mechanism in promoting the emergence of OX^R mutants.

Previously, we found that the MutY mutation in *C. jejuni* increased the frequencies of G-T or C-A mutation, which resulted in an increase of the spontaneous mutation rate in the *gyrA* gene and consequently the enhanced occurrence of fluoroquinolone-resistant mutants in *C. jejuni* (21). The MutY mutation also elevated the mutation rate for β-lactam resistance in *C. jejuni* (21). In this study, the same MutY mutant isolate was found to increase the emergence of spontaneous OX^R mutants, and this was due to the increased G-T or C-A mutation rate in the *perR* gene. Since the antibiotic-resistant mutants and OX^R mutants were developed from spontaneous mutations in different target genes, the two phenotypes did not overlap, and the OX^R mutants analyzed in this study did not show enhanced resistance to several tested antibiotics, including fluoroquinolones and β-lactams (data not shown). However, this does not necessarily exclude the possibility that a mutant isolate may harbor mutations in both *perR* and other genes (e.g., *gyrA*) targeted by antibiotics, which is expected to occur less frequently and requires the use of both antibiotics and oxidants for selection. Additional studies are needed to assess this possibility.

PerR plays an important role in defense against oxidative stress in *C. jejuni* (3, 12, 30). PerR represses the expression of *katA* and *ahpC*, and insertional mutation of *perR* results in overexpression of the KatA and AhpC proteins, which makes *C. jejuni* hyperresistant to peroxide stresses such as cumene hydroperoxide and hydrogen peroxide (10). The oxidative-stress-sensing mechanism by PerR has not yet been fully investigated in *Campylobacter*, but it was suggested that PerR senses peroxide stress by oxidation of the metal cofactors, including iron and manganese and subsequent oxidation of histidine residues in PerR protein (31). Interestingly, the expression and activity of KatA are still partially regulated by iron in the *perR* mutant but not in the *fur* and *perR* double mutant background (10). Additionally, inactivation of either *perR* or *fur* led to a partial reduction in *C. jejuni* colonization of chicken, but colonization was fully compromised in the PerR and Fur double mutant (12), suggesting that Fur and PerR have overlapping functions in modulating *Campylobacter* iron regulation and colonization in animals (31).

Notably, all *C. jejuni* spontaneous OX^R mutants analyzed in this study carried mutations in *perR*, not in *fur*, suggesting a more prominent role of PerR in modulating gene expression associated with oxidative stress defense in *C. jejuni*.

Analysis of the *perR* gene sequences in spontaneous *C. jejuni* OX^R mutants revealed amino acid changes and frameshift mutations in PerR (Table 1; see also Table S1 in the supplemental material). The crystal structure of PerR from *B. subtilis* revealed the structural basis for peroxide sensing by PerR (32). Interestingly, alignment of *C. jejuni* PerR with the *B. subtilis* homolog indicated that 48Ser and 84His are conserved in both PerR proteins, with the 48Ser located in the DNA binding helix and 84His as an identified important metal binding site in PerR (see Fig. S1 in the supplemental material), suggesting that mutation of the two amino acids may affect the function of the PerR regulator. Other mutations produced frameshifts and truncation of the PerR protein (136 amino acids [aa] in total). Results of EMSAs indicated that the rPerR protein with either the 84His→Asp mutation or the truncation after the N-terminal 112 aa failed to bind to the *katA* promoter, indicating that these two mutated forms of PerR lacked DNA binding activity, which implies the derepression of the PerR regulon in *C. jejuni*. Indeed, real-time PCR data (Table 3) revealed a drastic overexpression of the *katA* gene in the OX^R mutants, which is further correlated with the increased production of catalase activities in OX^R mutants (Fig. 2), explaining the enhanced resistance to peroxides. The specific role of the PerR mutations in oxidative stress resistance was further demonstrated by reverting the changes back to the wild-type sequences (Table 1). Together, these results convincingly established the molecular mechanisms involved in the OX^R phenotype.

Several attempts to purify the rPerR proteins with the 48Ser→Tyr mutation or the truncated protein with the N-terminal 107 aa from the *E. coli* host were not successful. IPTG (isopropyl-β-D-thiogalactopyranoside) induction indicated that the two mutant PerR proteins were expressed as determined by SDS-PAGE (see Fig. S2 in the supplemental material). It is unknown whether the two mutations affected the folding or solubility of rPerR in *E. coli*. Although they could not be purified for the EMSA, it is very likely that they lost the ability to bind to DNA, as the OX^R mutants carrying the PerR mutations showed drastically increased expression of *katA* and *ahpC*.

The MutY protein specifically repairs the G→T or C→A transversion (33). The *C. jejuni* CMT isolate has lost the repair function due to a mutation in MutY and consequently shows an elevated mutation rate with the G→T or C→A transversion (21). This was also seen in this study, as all OX^R mutants from strain 11168 carried a 1- to 2-bp deletion or insertion in *perR*, but all the OX^R mutants from the CMT isolate carried either a G→T or a C→A transversion (Tables 1 and S1). Reversion of the point mutations in *perR* to the WT 11168 sequence fully restored their susceptibility to H_2O_2 (Table 2), indicating that these mutations are responsible for the peroxide resistance of *C. jejuni* OX^R mutants. Thus, it can be concluded that the MutY mutation in CMT promotes oxidative stress resistance via enhancing the spontaneous loss of function mutation rates in *perR* with a G→T or C→A transversion, leading to overexpression of oxidative defense genes and consequently the increased emergence of OX^R mutants in *Campylobacter*.

In summary, the results in this study revealed how a mutator phenotype elevates the spontaneous mutation rate in *perR* and consequently promotes the emergence of OX^R mutants in *C. jejuni*. Given the importance of PerR in *Campylobacter* physiology and colonization of an animal host, permanent loss of PerR function is likely to be detrimental for the long-term adaptation of *Campylobacter* to various environments. However, elevated mutations in *perR* may facilitate *Campylobacter* survival under certain conditions, such as the food production environment, where *Campylobacter* is exposed to high-level oxidative stress. Multiple studies showed that *C. jejuni perR* mutants displayed significantly increased peroxide resistance and aerotolerance (10, 12, 30). For example, there were 2 to 3 log more surviving *C. jejuni perR* insertional mutants than there were wild-type cells after 9 h of incubation under aerobic conditions (30). Recently, aerotolerant *C. jejuni* and *Campylobacter coli* have been increasingly isolated from various sources, including chicken or retail meat samples (34–36). However, the

mechanisms underlying the aerotolerant phenotype remain to be deciphered. Thus, it would be interesting to investigate if *perR* mutations are involved in aerotolerance under natural conditions. Since PerR is well conserved (26–29, 37) and a mutator phenotype due to defects in the DNA repair system has been widely reported in many bacterial species (38–42), it would also be intriguing to determine whether mutator isolates also promote the emergence of spontaneous OX^R mutants in other bacterial species. Further in-depth examination of the relation between mutators and spontaneous OX^R mutants should provide novel insights into the adaptive mechanisms against oxidative stress in bacterial organisms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* was cultured using Mueller-Hinton (MH) broth or agar (Difco) at 42°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2). *E. coli* was grown on Luria-Bertani (LB) agar or in LB broth at 37°C for 24 h under aerobic conditions.

Oxidative stress sensitivity assay and selection of spontaneous OX^R mutants. The oxidative stress sensitivity assay was carried out as previously described with some modification (22). Fresh overnight cultures of *C. jejuni* strains under microaerobic conditions were adjusted to a final optical density at 600 nm (OD_{600}) of 0.5 (10^9 CFU/ml) in MH broth. Cultures for each isolate were divided into two groups, with one group containing the addition of a final concentration of a mixture of 0.2 mM paraquat dichloride and freshly prepared 2 mM H_2O_2 and the other group with no treatment, as control. All cultures were incubated at room temperature for 30 min and then serially diluted and plated on MH agar plates, which were incubated for 2 to 4 days under microaerobic conditions. Colonies on the plates were counted for CFU calculation, and single colonies were randomly picked for detection of spontaneous OX^R mutants. The picked colonies were subsequently subjected to disk inhibition assay with oxidants as described below. Those colonies that were highly resistant to the tested oxidants in the disk inhibition assay were stocked for further analysis and characterization.

Disk inhibition assay. In order to quantitatively compare the oxidative stress sensitivities of different *C. jejuni* isolates, a disk inhibition assay was performed as described previously (12). Each *C. jejuni* isolate was tested for susceptibility to 3% H_2O_2 and 3% cumene hydroperoxide in dimethyl sulfoxide (DMSO) and 100 mM paraquat dichloride in H_2O, respectively. DMSO, which was used for preparing the cumene hydroperoxide solution, was used as a control.

Determination of catalase activities in *C. jejuni* WT and spontaneous OX^R mutants. Overnight cultures of *C. jejuni* WT strains and their corresponding spontaneous OX^R mutants grown on MH plates were washed twice with phosphate-buffered saline (PBS) buffer (pH 7.4) and then resuspended and normalized to an OD_{600} of 5 in PBS. One milliliter of the suspension was centrifuged at 6,000 $\times$ *g* for 5 min, and the supernatant was discarded. To the cell pellet was added 500 μl of B-PER II bacterial protein extraction reagent (Thermo Fisher Scientific). Whole-cell proteins were extracted using the protocol recommended by the manufacturer. Soluble lysates were then obtained by centrifugation at 10,000 $\times$ *g* for 20 min at 4°C. The catalase activity in the whole-cell extract was determined with the Amplex Red catalase assay kit from Molecular Probes using the methods indicated in the kit with some modifications. For the assay (Molecular Probes), catalase in a sample first reacts with H_2O_2 to produce water and oxygen (O_2). Then, the Amplex Red reagent is added to the reaction mixture and detects any unreacted H_2O_2 in the presence of horseradish peroxidase (HRP) to produce the fluorescent oxidation product resorufin. Therefore, as the concentration of H_2O_2 decreases in the reaction mixture due to catalase activity, the fluorescent signal from the oxidation indicator product, resorufin, decreases correspondingly. In this study, the experiment was carried out in a 96-well plate. First, 100 μl of 20 μM H_2O_2 in PBS buffer (pH 7.4) was aliquoted into each well. Subsequently, 10 μl of 1:4,000-diluted whole-cell extracts of *Campylobacter* or PBS buffer was quickly added into the wells containing H_2O_2. For each cell extract, three wells were used for each time point measurement. At 5-min intervals for 30 min, the Amplex Red reagent was added into the designated wells. The fluorescence of resorufin was measured using FLUOstar Omega (BMG Labtech, Offenburg, Germany) with excitation and emission wavelengths of 545 nm and 590 nm, respectively.

qRT-PCR analysis of transcription of oxidative-stress-related genes. Specific primers for the 16S rRNA gene, *katA*, *ahpC*, and *perR* in *Campylobacter* were designed using the Primer3 online interface (http://bioinfo.ut.ee/primer3/) and are listed in Table S2 in the supplemental material. *C. jejuni* 11168, MutY-deficient CMT, and their spontaneous OX^R mutants were grown in MH broth for 16 h under microaerobic conditions. Total RNA purification from the cultures and subsequent real-time quantitative RT-PCRs (qRT-PCRs) were carried out as previously described (43). The relative changes (*n*-fold) in gene transcriptions between the parent strains and their spontaneous OX^R mutants were calculated using the $2^{-\Delta\Delta CT}$ method (where C_T is threshold cycle) (44).

DNA sequence analysis of oxidative-stress-related genes. The total DNA from each *C. jejuni* strain was prepared by boiling the cells for 15 min. The supernatant was directly used as the template for PCRs. The encoding sequences and the promoter regions of *katA*, *ahpC*, *sodB*, *perR*, and *fur* were amplified using a DNA template prepared from the parent strains and their spontaneous OX^R mutants. The PCR products were sequenced in both directions with PCR primers used for the amplification (Table S2).

Construction of insertional *perR* and *mutY* mutants in *C. jejuni* isolates. Insertional mutants of *perR* and *mutY* were produced using natural transformation and homologous recombination. Primer pair perR-5F/perR-5R was used to amplify the 5′ part of *perR* and its upstream region (*perR*-5′ fragment), while

primer pair perR-3F/perR-3R was used to amplify the 3′ part of *perR* and its downstream region (*perR*-3′ fragment). The primer pair cat-F/cat-R was used to amplify the *cat* gene from the pRY112 plasmid, encoding chloramphenicol resistance (45). All PCRs were performed using the Phusion High-Fidelity DNA Polymerase (New England BioLabs, USA). The PCR-amplified *perR*-5′ fragment and the *perR*-3′ fragment were linked with the *cat* gene between them by overlap PCR using the primers listed in Table S2, resulting in the generation of the *perR*-5′-*cat*-*perR*-3′ construct, which was then purified and used to naturally transform *C. jejuni* 11168. Transformants were screened on MH agar plates containing 10 mg/liter chloramphenicol. The construct 11168Δ*perR* with the insertion of the *cat* cassette in the *perR* gene was confirmed by chromosomal DNA amplification using primers perR-5F/perR-3R. The same strategies were utilized to construct the insertional MutY mutants 11168Δ*mutY* and 81-176Δ*mutY* in *C. jejuni* 11168 and 81-176, respectively.

Complementation of the *perR* and *mutY* mutants. The 11168Δ*perR* mutant was complemented by inserting a *perR* gene from *C. jejuni* OX^R mutants in a chromosomal location between the 16S and 23S rRNAs as described by Muraoka and Zhang (46), with some modifications. Briefly, the pRRK plasmid, which contains an *aphA3* cassette in the orientation opposite to that of the ribosomal genes, was linearized by PCR using primers pRRK-lF and pRRK-lR. Primers *perR*-cF and *perR*-cR were used to amplify the intact *perR*, including its promoter region and ribosome binding site from *C. jejuni* 11168 and spontaneous OX^R mutants, i.e., 11168 $perR_{A338Del}$, CMT $perR_{C143A}$, CMT $perR_{C250A}$, and CMT $perR_{G319T}$ (Table 1). The *perR* amplicons and linearized pRRK plasmid were ligated utilizing the SLiCE cloning method (47), to obtain plasmid construct pRRK-*perR* with *perR* in the transcriptional direction opposite to that of the ribosomal genes. The nucleotide sequences of the inserted *perR* gene on pRRK plasmid were confirmed by sequencing using primers pRRK-seqF and pRRK-seqR. The pRRK-*perR* constructs were then used as suicide vectors to insert *perR* into the chromosome of the 11168Δ*perR* isolate. The complemented constructs 11168Δ$perRCperR_{11168}$, 11168Δ$perRCperR_{C143A}$, 11168Δ$perRCperR_{C250A}$, 11168Δ$perRCperR_{G319T}$, and 11168Δ$perRCperR_{A338Del}$ (Table 1) were selected on MH agar containing 30 μg/ml of kanamycin and 10 μg/ml of chloramphenicol. The same strategies were utilized to insert an intact *mutY* gene into the chromosome of 11168Δ*mutY* and 81-176Δ*mutY*, resulting in constructs 11168Δ*mutY*C and 81-176Δ*mutY*C (Table 1).

Site-specific reversion in PerR of *C. jejuni* OX^R mutant isolates. In order to investigate the role of the single amino acid change in PerR in mediating oxidative stress resistance, the *perR* mutations in four of the *C. jejuni* OX^R mutants were reverted to the WT *perR* sequence by using a previously reported method with some modifications (21, 48). *perR* (*cj0322*) and *cj0323* are tandemly positioned on the chromosome of *C. jejuni* and transcribed in the same direction. *cj0323* encodes a hypothetical protein with an unknown function. A *cat* cassette was inserted in the *cj0323* gene downstream of *perR* to facilitate the reversion of the specific mutation in *perR* by homologous recombination. Briefly, a 949-bp fragment containing the entire *perR* encoding sequence and part of its upstream gene *cj0321*, and another 802-bp fragment containing most of the *cj0323* encoding sequence, were amplified by primer pairs perR-rF/perR-rR and 0323-rF/0323-rR, respectively (Table S2), using *C. jejuni* 11168 DNA as the template. These two PCR fragments were then linked with the *cat* cassette by overlap PCR using the listed primers. The overlap PCR product was purified and used to naturally transform different *C. jejuni* OX^R mutants. Transformants were screened on MH agar plates containing 10 mg/liter chloramphenicol and confirmed by PCR amplification of the gene flanking the insertion site. This resulted in *C. jejuni* constructs 11168$perR_{A338Del}$RV, CMT $perR_{C143A}$RV, CMT $perR_{C250A}$RV, and CMT $perR_{G319T}$RV (Table 1), in which *perR* was reverted to the WT sequence at the original site in the genome of *C. jejuni* OX^R mutants through homologous recombination. The reversions were confirmed by DNA sequencing. As a control, CMT $perR_{C250A}$ was inserted with the *cat* cassette in *cj0323* without the *perR* reversion, and this construct, CMT $perR_{C250A}$CT, served to demonstrate that neither the presence nor the location of the *cat* cassette in *cj0323* had an effect on the peroxide susceptibility in *C. jejuni*.

Expression and purification of rPerR from WT and *C. jejuni* OX^R mutants. Full-length histidine-tagged recombinant PerR (rPerR) from the WT and its *C. jejuni* OX^R mutants was produced in the *E. coli* JM109 strain by using the pQE-30 vector (Qiagen). The complete coding sequences of *perR* in the isolates were amplified with primers perR-HisF and perR-HisR (Table S2). The amplified PCR products were ligated into the pQE-30 vector, which had previously been digested with BamHI and HindIII, utilizing the SLiCE cloning method as mentioned above. The plasmids in the *E. coli* clones producing different PerR proteins were sequenced, confirming the cloned sequences of *perR*. *E. coli* harboring pQE-30-*perR* was grown in LB broth at 37°C, with shaking at 180 rpm to an OD_{600} of 1.0. The expression of recombinant PerR was induced by addition of 1.0 mM IPTG for 5 h at 28°C. Purification of recombinant PerR proteins was performed by following procedures described previously (49, 50).

Electrophoretic mobility shift assays. In order to investigate the role of the single-nucleotide changes in affecting the binding of PerR to the promoter regions regulated by PerR, EMSAs were performed by a procedure described previously (30, 51), with some modifications. Briefly, primers KatAPromF/KatAPromR and DnaEPromF/DnaEPromR (30) were used to amplify the promoter regions of *katA* and *dnaE*, respectively. The purified PCR products were then labeled at the 3′ end with digoxigenin-11-ddUTP (DIG-11-ddUTP) by using the DIG oligonucleotide 3′-End Labeling kit (Roche Molecular Biochemicals). DIG-labeled PCR products were incubated in 0.5 nM aliquots with 5 to 20 nM purified recombinant PerR (rPerR) from either *C. jejuni* WT or OX^R mutant strains in 10 μl of binding buffer containing 50 μM $MnCl_2$, 20 mM Tris-Cl (pH 7.4), 50 mM KCl, 3 mM $MgCl_2$, 5% (vol/vol) glycerol, and 0.1% Triton X-100 (vol/vol). The reaction mixtures were incubated for 1 h at room temperature. The reaction mixtures were then subjected to electrophoresis on a nondenaturing 6% (wt/vol) polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE; 44 mM Tris, 44 mM boric acid, 1 mM EDTA [pH 8.0]) at 200 V for 45 min. The

DNA in the gel was transferred to a nylon membrane with a vacuum blotter. DIG-labeled DNA was detected and visualized by using alkaline phosphatase-conjugated anti-DIG antibody and the chemiluminescent substrate CDP-Star (Roche Molecular Biochemicals).

Determination of spontaneous OX^R mutation frequencies in *C. jejuni* isolates. The traditional method for measuring spontaneous mutation frequencies in *Campylobacter* (21) failed to determine spontaneous H_2O_2 resistance mutation frequencies, simply due to the rapid decomposition of H_2O_2 during medium preparation and culture incubation. Therefore, to quantify spontaneous OX^R mutation frequencies in *Campylobacter*, promoter-reporter fusion P_{katA}-*cat*, with the promoter region of the *katA* gene fused with promoterless chloramphenicol resistance gene *cat*, was constructed and cloned into the pRRK plasmid by SLiCE cloning using the listed primer pairs *cat*ORF-F/*cat*ORF-R and PkatA-F/PkatA-R (Table S2). The fusion gene P_{katA}-*cat* was then inserted into the genome of *C. jejuni* 11168 and CMT isolates, generating 11168 P_{katA}-*cat* and CMT P_{katA}-*cat* constructs. Both constructs showed chloramphenicol susceptibility (MIC = 1 to 2 μg/ml) similar to that of *C. jejuni* 11168 and CMT isolates, since transcription of the P_{katA}-*cat* gene is inhibited by the functional PerR protein in strains 11168 P_{katA}-*cat* and CMT P_{katA}-*cat*. However, those spontaneous *perR* mutations, which compromise PerR function, were expected to result in the derepression of the *katA* promoter of P_{katA}-*cat* and significantly increased expression of *cat*, reducing the susceptibility to chloramphenicol (MIC $\geq$ 16 μg/ml). Therefore, 4 μg/ml of chloramphenicol was used in this study to detect spontaneous *perR* loss-of-function mutations in *C. jejuni* 11168 P_{katA}-*cat* and CMT P_{katA}-*cat* constructs. The spontaneous chloramphenicol resistance (Cm^r) mutation frequencies were determined as previously described for spontaneous ciprofloxacin and ampicillin resistance mutations in *C. jejuni* (21). Several colonies growing on selective MH plates from the spontaneous Cm^r mutation frequency test were randomly picked to sequence the *perR* gene. Meanwhile, these colonies were subcultured and tested for their susceptibility to H_2O_2 using the disk diffusion assay, as described above.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01685-17.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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