

Campylobacter jejuni Contains Two Fur Homologs: Characterization of Iron-Responsive Regulation of Peroxide Stress Defense Genes by the PerR Repressor

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Expression of the peroxide stress genes alkyl hydroperoxide reductase (*ahpC*) and catalase (*katA*) of the microaerophile *Campylobacter jejuni* is repressed by iron. Whereas iron repression in gram-negative bacteria is usually carried out by the Fur protein, previous work showed that this is not the case in *C. jejuni*, as these genes are still iron repressed in a *C. jejuni fur* mutant. An open reading frame encoding a Fur homolog (designated PerR for “peroxide stress regulator”) was identified in the genome sequence of *C. jejuni*. The *perR* gene was disrupted by a kanamycin resistance cassette in *C. jejuni* wild-type and *fur* mutant strains. Subsequent characterization of the *C. jejuni perR* mutants showed derepressed expression of both AhpC and KatA at a much higher level than that obtained by iron limitation, suggesting that expression of these genes is controlled by other regulatory factors in addition to the iron level. Other iron-regulated proteins were not affected by the *perR* mutation. The *fur perR* double mutant showed derepressed expression of known iron-repressed genes. Further phenotypic analysis of the *perR* mutant, *fur* mutant, and the *fur perR* double mutant showed that the *perR* mutation made *C. jejuni* hyperresistant to peroxide stress caused by hydrogen peroxide and cumene hydroperoxide, a finding consistent with the high levels of KatA and AhpC expression, and showed that these enzymes were functional. Quantitative analysis of KatA expression showed that both the *perR* mutation and the *fur* mutation had profound effects on catalase activity, suggesting additional non-iron-dependent regulation of KatA and, by inference, AhpC. The PerR protein is a functional but nonhomologous substitution for the OxyR protein, which regulates peroxide stress genes in other gram-negative bacteria. Regulation of peroxide stress genes by a Fur homolog has recently been described for the gram-positive bacterium *Bacillus subtilis*. *C. jejuni* is the first gram-negative bacterium where non-OxyR regulation of peroxide stress genes has been described and characterized.

Campylobacter jejuni is a gram-negative, microaerophilic enteric pathogen of humans, causing gastroenteritis. The bacterium is one of the most frequently isolated causes of bacterial diarrhea and can therefore be considered a major public health and economic problem (16). As a microaerophile, *C. jejuni* needs to protect itself from reactive oxygen species that are the result of aerobic metabolism. A coordinated response of oxidative-stress genes is a necessity of the (micro)aerobic lifestyle.

Two of the most important bacterial oxidative-stress genes in the defense against peroxide stress inducers such as hydrogen peroxide and alkyl hydroperoxides are catalase (KatA) and alkyl hydroperoxide reductase (AhpC). Both of these enzymes have been identified in *C. jejuni* (3, 15). In most bacteria including *Escherichia coli*, *Salmonella typhimurium* (10), *Haemophilus influenzae* (18), and *Mycobacterium leprae* (12), KatA and/or AhpC expression is regulated by the OxyR regulator in response to oxidative stress. In *C. jejuni*, however, AhpC and KatA expression is transcriptionally repressed in response to

increasing environmental iron concentration (3, 31). The Fur protein usually mediates bacterial iron-responsive gene regulation. When the intracellular Fe²⁺ concentration is high, a complex consisting of a Fur dimer and Fe²⁺ binds to control sequences (Fur boxes) overlapping Fur-regulated promoters (11, 25). The presence of Fur box-like sequences in the promoters of both *ahpC* and *katA* indicated that iron regulation of these genes could be carried out by Fur. Unexpectedly, we found that in *C. jejuni* iron regulation of AhpC and KatA is Fur independent (31), and therefore we hypothesized that iron regulation of AhpC and KatA is mediated by another regulator.

In the gram-positive bacterium *Bacillus subtilis*, expression of AhpC and KatA is regulated by iron (6), and the presence of a repressor regulating AhpC and KatA has been predicted (9). Analysis of the *B. subtilis* genome sequence revealed the presence of three open reading frames (ORFs) encoding Fur homologs. One homolog was shown to function as the iron uptake regulator (Fur) (7), one as the zinc uptake regulator (Zur) (14), and the third, designated PerR, was responsible for the regulation of AhpC and KatA expression (7). In *B. subtilis*, therefore, PerR is a functional analog of the OxyR regulator. It was predicted that in other gram-positive bacteria peroxide stress genes and iron uptake might also be regulated by separate Fur homologs, as multiple Fur homologs have been described for several other gram-positive bacteria (7).

Here we report the identification of a *C. jejuni* PerR homolog and describe the effects of a *perR* mutation. This is the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
<i>C. jejuni</i>		
NCTC 11168	Parental strain	National Collection of Type Cultures
AV63	11168 <i>perR</i> ::Kan ^r	This study
AV42	11168 <i>fur</i> ::Cm ^r	31
AV67	11168 <i>perR</i> ::Kan ^r <i>fur</i> ::Cm ^r	This study
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> Δ M15	Gibco BRL
Plasmids		
pBluescript II SK-	Cloning vector, Ap ^r	Stratagene
pJMK30	<i>C. coli</i> kanamycin (Kan ^r) resistance cassette in pUC19	31
pAV205	1.4-kb PCR-amplified fragment of <i>C. jejuni</i> NCTC 11168 containing the <i>perR</i> region in pBluescript	This study
pAV214	<i>Sma</i> I-cut Kan ^r cassette of pJMK30 cloned in the <i>EcoRV</i> site in <i>perR</i> in pAV205; the Kan ^r cassette is in the reverse orientation with respect to <i>perR</i>	This study

first gram-negative organism where PerR-like regulation has been described. A *C. jejuni perR fur* double mutant is also characterized where all iron regulation was abolished, and we show that both the *perR* and *fur* mutations influence expression of KatA.

MATERIALS AND METHODS

Media and growth conditions. *C. jejuni* strains were maintained on Mueller-Hinton (MH) media (Unipath) under microaerophilic conditions in a Variable Atmosphere Incubator (Don Whitley) containing 85% N₂, 10% CO₂, and 5% O₂. Media were routinely supplemented with 10 μ g of vancomycin and 5 μ g of trimethoprim per ml. Iron-restricted conditions were achieved by supplementing MH media with the iron chelator deferoxamine mesylate (desferal; Sigma Chemical Co.) to a final concentration of 20 μ M. Iron-replete conditions were achieved by adding Fe(III)SO₄ to MH media at a final concentration of 40 μ M. *E. coli* was grown aerobically in Luria-Bertani medium (23) at 37°C. When antibiotic selection was necessary, growth media were supplemented with ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), or chloramphenicol (20 μ g/ml). *C. jejuni* strains were tested for resistance to the peroxide stress inducers cumene hydroperoxide (CHP) and hydrogen peroxide by methods described previously (3).

Bacterial strains and plasmids. *C. jejuni* and *E. coli* strains and plasmids used in this study are listed in Table 1. The region encoding *perR* and upstream and downstream sequences was amplified with primers 5'-CGC-GGTACC-TAT-TGC-TTT-GCG-TTA-TCC-TAG-A and 5'-CGC-GGATCC-ATT-GGA-ACT-ATC-CAA-AGT-TGG-AA. These primers contain a 5' *Kpn*I or *Bam*HI restriction enzyme site (underlined) for cloning in pBluescript; thus, the polylinker *Hind*III and *EcoRV* sites were removed during cloning, allowing subsequent insertional mutagenesis of *perR*.

Recombinant DNA techniques. Restriction enzymes and T4 DNA ligase were purchased from Gibco BRL. All enzymes were used according to the manufacturer's instructions. Standard protocols were used for manipulation of DNA and transformation of *E. coli* (2, 23) and *C. jejuni* (30). Genomic DNA of *C. jejuni* was prepared by the method described by Ausubel et al. (2). Plasmid DNA was prepared with affinity columns (Qiagen). PCR was carried out with Expand Polymerase Mix (Boehringer). DNA sequencing was performed with an Applied Biosystems model 377 DNA sequencing system and a *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Protein manipulation and catalase assays. *C. jejuni* were fractionated by a technique described previously (31). Briefly, *C. jejuni* cells were subjected to osmotic shock to release the periplasm. Subsequently, the spheroplasts were disrupted by sonication, and cytoplasm and crude membranes were separated by ultracentrifugation. Inner membranes were solubilized in Sarkosyl, and outer membranes were pelleted by ultracentrifugation. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently stained with Coomassie brilliant blue. N-terminal amino acid sequences were determined from proteins transferred to Fluorotrans membranes (Flowgen Laboratories) by using Edman degradation on an ABI 476 sequencer (Applied Biosystems).

Catalase activity was measured by a technique described by Beers and Sizer (4). Briefly, *C. jejuni* cells were disrupted by sonication, and insoluble particles were pelleted by ultracentrifugation for 10 min at 100,000 \times *g*. The soluble fraction was subsequently used for quantitative analyses. The protein concentra-

tion was measured as described by Bradford (5). Catalase activity was measured by monitoring the enzymatic breakdown of hydrogen peroxide at 240 nm, by using 50 mM phosphate buffer with a hydrogen peroxide concentration of 19.6 mM.

RESULTS

***C. jejuni* contains a second Fur homolog.** To identify putative regulators of oxidative-stress genes, we screened the *C. jejuni* genome sequence (24) for OxyR and Fur homologs. This analysis did not reveal the presence of an OxyR homolog but did reveal the presence of an ORF encoding a second Fur homolog approximately 70 kb from *fur*. The region containing this second Fur homolog was amplified from NCTC 11168 by PCR, and the nucleotide sequence was confirmed to be identical to the sequence determined by the *C. jejuni* genome project. The 1.4-kb region amplified contained three ORFs (Fig. 1). The product of the one complete ORF, designated PerR, showed significant homology with bacterial Fur homologs. An alignment of the *C. jejuni* PerR protein with the *B. subtilis* PerR and the *C. jejuni* and *B. subtilis* Fur proteins is shown in Fig. 2. The identity between all Fur and PerR sequences was 17%. However, the identity between the two Fur proteins was 34%, and that between the two PerR proteins was 32%. The ORF upstream of *perR* encodes a putative transketolase B (*tktB*) homolog, and the downstream ORF (*orf3*) did

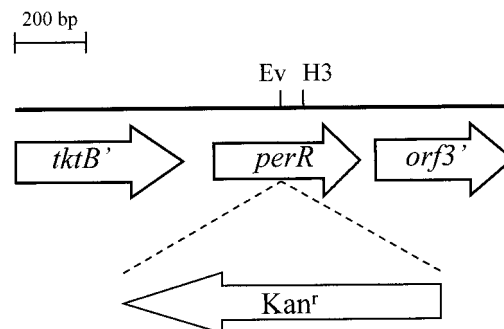


FIG. 1. Schematic diagram representing the genomic region containing the *perR* gene. The position and orientation of the inserted antibiotic resistance cassette in *perR* are shown. Only partial ORFs of *tktB* and *orf3* are indicated. Ev, *EcoRV*; H3, *Hind*III.



FIG. 2. Alignment of *C. jejuni* (Cj) and *B. subtilis* (Bs) PerR and Fur proteins. Boxed residues are identical in the PerR and Fur proteins. Asterisks and dots indicate identical residues and conservative substitutions, respectively, in all four proteins.

not have any significant homology with sequences deposited in the GenBank and EMBL databases (Fig. 1). The predicted *perR* gene is 408 bp long, encoding a protein of 15,926 Da with seven cysteine residues and eight histidine residues (Fig. 2). A putative ribosome binding site (AAGGA) was identified 10 bp upstream of the *perR* gene. There is 86 bp between *tktB* and *perR*, and this intergenic region has a very high A+T content of 90.6% and includes a putative stem-loop structure overlapping with the stop codon of *tktB*. This putative stem-loop structure has a stem of 11 bp and a loop of 5 bp and has a free energy of -8.6 kcal. Downstream of *perR* a small putative stem-loop structure is found with a stem of 5 bp, a loop of 3 bp, and a free energy of -2.2 kcal. The predicted transcriptional organization of the *perR* genomic region and the putative stem-loop structures present suggest that *perR* is transcribed as a monocistronic messenger, unlike *C. jejuni fur* (8, 29) and thus probably has its own promoter. The PCR primers used to amplify the *perR* region from *C. jejuni* NCTC 11168 gave similar-sized products with four other *C. jejuni* strains, indicating that the organization of the *perR* region is conserved in other *C. jejuni* strains (data not shown).

PerR is the repressor of *C. jejuni* AhpC and KatA peroxide stress genes. The *perR* gene was mutated to determine whether it was the repressor of the *ahpC* and *katA* genes in *C. jejuni*. By using the unique internal *EcoRV* site (Fig. 1), a kanamycin resistance cassette was inserted into the *perR* gene, and the disrupted *perR* gene was subsequently introduced into the *C. jejuni* NCTC 11168 genome by allelic exchange (17). Mutants were obtained with the antibiotic resistance cassette inserted in either orientation at the same frequency. This is in contrast with the *C. jejuni fur* gene (31). Correct allelic exchange of the wild-type *perR* allele with the mutated copy was confirmed by PCR (data not shown). The *C. jejuni perR* mutant was designated AV63. In addition to the *perR* mutant, we also constructed a *perR fur* double mutant by mutating *perR* in *C. jejuni* AV42, which is a *fur* mutant with a chloramphenicol resistance cassette inserted in the same orientation as *fur* (31). The presence of mutated *fur* and *perR* alleles was confirmed by PCR (data not shown). The *perR fur* double mutant, named AV67, was viable and showed the same growth characteristics as a *fur* mutant (31). The *perR* mutant showed the same growth characteristics as the wild-type strain (data not shown).

Protein profiles and subsequent protein sequencing were used to determine whether PerR was indeed the iron-respon-

sive repressor of *ahpC* and *katA* expression. Bacteria were grown under iron-restricted and iron-replete conditions and fractionated into periplasm, cytoplasm, and outer membranes. This approach has been used previously to identify members of the *fur* regulon of *C. jejuni* (31). These protein profiles following separation by SDS-PAGE are shown in Fig. 3; proteins previously identified as Fur repressed are indicated, and these are all still iron repressed in the *perR* mutant but not in the *fur* mutant or the double mutant. This shows that the *perR* mutation does not notably affect Fur-regulated protein expression. Three highly expressed proteins with molecular sizes of approximately 25, 26, and 55 kDa can be seen in the periplasmic and cytoplasmic fractions of the *perR* mutant and *perR fur* mutant. Expression of these three proteins is iron repressed in the wild type and *fur* mutant (Fig. 3). All three proteins were identified by N-terminal amino acid sequencing. The N-terminal amino acid sequence of the 55-kDa protein (MKKLT NDFG) was identical to that of *C. jejuni* KatA (15). Surprisingly, the N-terminal amino acid sequence of both the 25- and the 26-kDa proteins (MIVTKKALDF) was identical to that of AhpC (3).

The *perR* mutation makes *C. jejuni* hyperresistant to peroxide stress. The *perR* mutation derepressed the *katA* and *ahpC* promoters, leading to a very high level of expression of KatA and AhpC. In order to test whether this led to expression of functional enzymes, the level of resistance of wild-type *C. jejuni* to peroxide stress was compared to those of the *perR*, *fur*, and *perR fur* mutants. The results obtained with two levels of the peroxide stress inducers hydrogen peroxide (cleared by KatA) and CHP (cleared by AhpC) are shown in Fig. 4. The *perR* mutation induces hyperresistance to both of these peroxide stress inducers, showing that in a *perR* background both overexpressed enzymes are functional. The *fur* mutation alone did not have a major effect on resistance of *C. jejuni* to the peroxide stress inducers.

The derepression of KatA expression by the *perR* mutation was determined by direct enzyme assay. KatA synthesis was measured directly by spectrophotometric detection of the rate at which hydrogen peroxide was utilized. The results of this assay are shown in Table 2. In the wild-type strain, KatA activity was almost completely absent under iron-replete conditions. The *perR* mutation substantially increased KatA activity under iron-replete conditions but particularly under low-iron conditions, where KatA activity was 10-fold higher than that in the wild type. Interestingly, KatA activity was still 2-fold iron repressed in the *perR* mutant, and in the *fur* mutant KatA activity was 4-fold lower than that in the wild type under low-iron conditions but higher than that in the wild type under high-iron conditions. Such an effect of the *fur* mutation on iron-repressed expression of *katA* suggests that Fur coregulates *katA* expression with PerR. This hypothesis is supported by the observation that in the *perR fur* double mutant KatA levels were almost as high as those in the *perR* mutant, but regulation in response to iron levels is no longer evident (Table 2).

DISCUSSION

Reactive oxygen species cause damage to DNA, proteins, and membranes. Bacteria have developed systems which include catalase, alkyl hydroperoxide reductase, and superoxide dismutase to clear these reactive oxygen species. In the *Enterobacteriaceae*, most oxidative-stress proteins have been identified as being induced by different kinds of oxidative stress and can be subdivided in two separately regulated classes; (i) the O_2^- (superoxide) stress proteins and (ii) the peroxide stress proteins. The O_2^- stress proteins include manganese-contain-

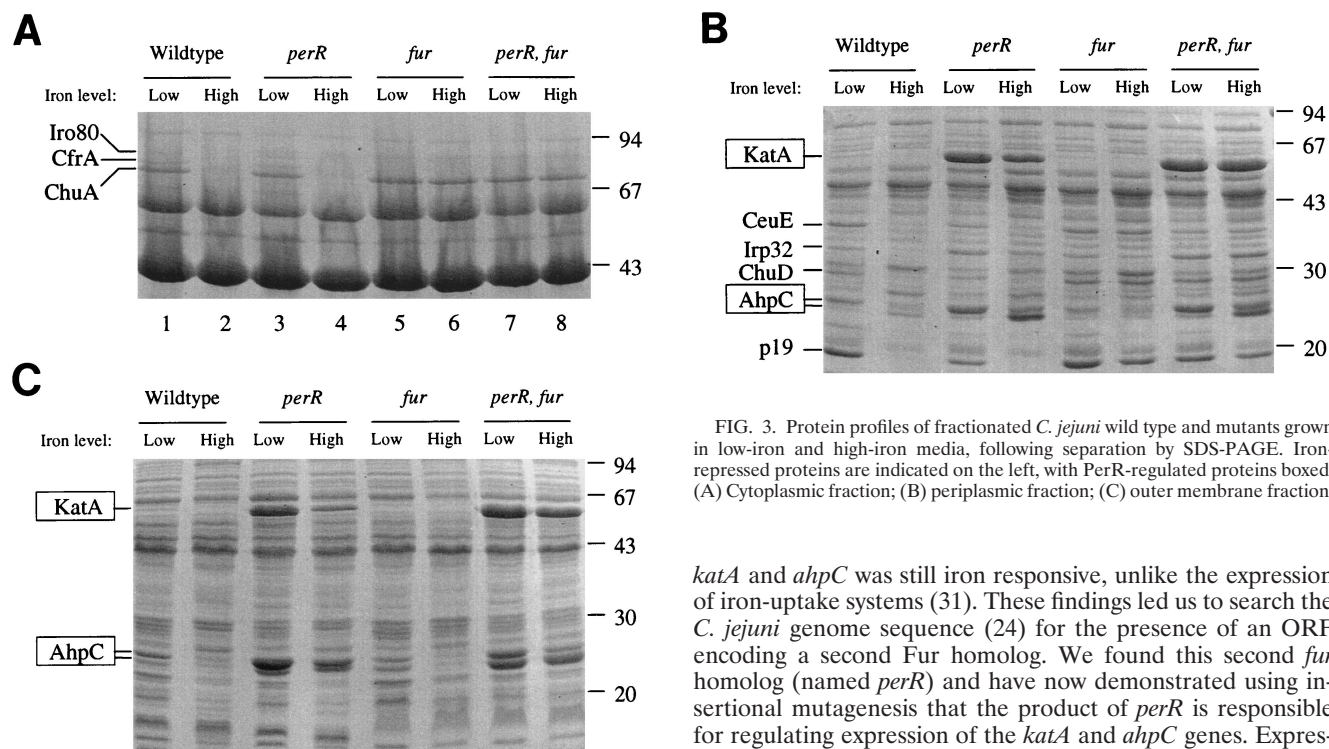


FIG. 3. Protein profiles of fractionated *C. jejuni* wild type and mutants grown in low-iron and high-iron media, following separation by SDS-PAGE. Iron-repressed proteins are indicated on the left, with PerR-regulated proteins boxed. (A) Cytoplasmic fraction; (B) periplasmic fraction; (C) outer membrane fraction.

ing superoxide dismutase (SodA) and endonuclease IV (13). Catalase and alkyl hydroperoxide reductase are members of the peroxide regulon (13).

Oxidative-stress defense proteins are expressed at a basal level under normal nonstressed conditions, but all these systems need to be able to be upregulated in conditions of increased oxidative stress. The regulation of these systems has been extensively investigated in *E. coli*, where there are two regulatory systems for oxidative-stress genes. The SoxR-SoxS system regulates the superoxide regulon (1), and mutations in *soxR* or *soxS* fail to induce the members of their regulon (13). OxyR regulates the OxyR regulon, which is part of the peroxide stress regulon. The OxyR protein is a transcription factor that senses oxidative stress through disulfide bond formation, and, under conditions of oxidative stress, transcription of its regulon is induced following a change in OxyR conformation due to this disulfide bond formation (26, 27, 33). OxyR is considered a global regulator, and *H. influenzae oxyR* mutants are unable to respond to oxidative stress (18).

Homologs of the *oxyR* gene have been identified in many gram-negative bacteria, but no *oxyR* homologs have been identified in the genome sequence of *Helicobacter pylori* (28), and we did not find a homolog in the *C. jejuni* genome (24). This indicates that in *C. jejuni* as well as *H. pylori* regulation of oxidative-stress defense is organized differently. Three oxidative-stress defense genes—superoxide dismutase (*sodB*) (21, 22), catalase (*katA*) (15), and alkyl hydroperoxide reductase (*ahpC*) (3)—have been identified in *C. jejuni* and the closely related species *C. coli*. The transcription of the *katA* and *ahpC* genes in *C. jejuni* is responsive to the iron concentration in the growth medium (3). Iron-responsive regulation is usually mediated by the Fur protein, and putative Fur binding sequences were identified upstream of *sodB*, *katA*, and *ahpC*. However, it was demonstrated that in a *C. jejuni fur* mutant expression of

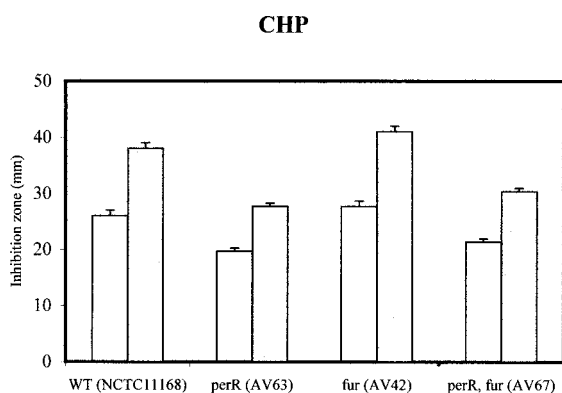
katA and *ahpC* was still iron responsive, unlike the expression of iron-uptake systems (31). These findings led us to search the *C. jejuni* genome sequence (24) for the presence of an ORF encoding a second Fur homolog. We found this second *fur* homolog (named *perR*) and have now demonstrated using insertional mutagenesis that the product of *perR* is responsible for regulating expression of the *katA* and *ahpC* genes. Expression of both KatA and AhpC was very high in the *perR* mutants, such that these proteins were now also detected in the periplasmic fraction. The increase in KatA and AhpC expression is higher than that observed under conditions of iron limitation, indicating that even under iron limitation transcription from the promoters of *katA* and *ahpC* is still not maximal. Thus, there are likely to be other environmental stimuli regulating expression of these oxidative-stress defense genes. This agrees with the observation that in *C. coli* KatA expression is induced under conditions of oxidative stress (19). As yet it is unknown whether other stimuli are important in oxidative-stress responses in *Campylobacter*.

The protein profiles showed only two proteins whose expression was affected by the *perR* mutation, and these proteins were identified as KatA and AhpC. In addition, unlike the *fur* mutant (31), the *C. jejuni perR* mutant showed growth characteristics similar to those of its parental strain, indicating that in *C. jejuni* PerR may not be a global regulator. In contrast, Fur regulates at least 13 proteins in *C. jejuni* (Fig. 3) (31) and also affects the expression of KatA and possibly AhpC (Fig. 3 and Table 2). The apparently small PerR regulon might explain why the *perR fur* double mutant was still viable. We also tested the influence of the *perR* and *fur* mutations on SodB activity. Although SOD activity was strongly induced under high-iron conditions, neither the *perR* or *fur* mutation influenced SOD enzyme activity (data not shown). However, given that SodB is

TABLE 2. Effects of *C. jejuni perR* and *fur* mutations on iron-regulated catalase activity

<i>C. jejuni</i> strain	Catalase activity (U \pm SD)	
	Low iron	High iron
NCTC 11168 (wild type)	665.0 \pm 94.0	0.1 \pm 0.0
AV63 (<i>perR</i>)	7,101.4 \pm 1,038.9	3,205.8 \pm 134.6
AV42 (<i>fur</i>)	166.7 \pm 13.4	20.1 \pm 5.8
AV67 (<i>perR fur</i>)	6,435.6 \pm 285.6	6,902.7 \pm 1,215.1

(A)



(B)

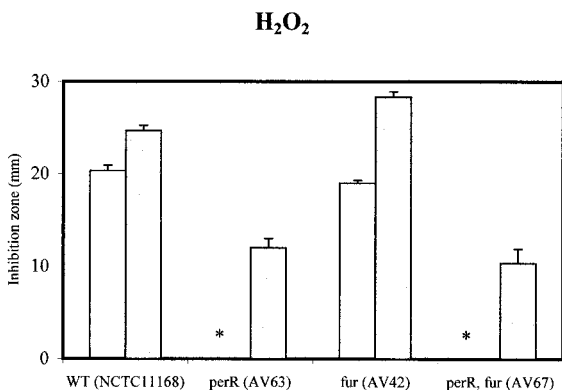


FIG. 4. Effects of *perR* and *fur* mutations on oxidative-stress resistance of *C. jejuni* wild type and mutants to CHP (A) and hydrogen peroxide (B). Resistance is expressed as the zone of growth inhibition after overnight growth on agar plates. Resistance was measured against 3% (open bars) and 10% (closed bars) CHP and H₂O₂. The error bars represent data from three separate plates. The *perR* mutation derepresses expression of AhpC and KatA and results in the *perR* mutants showing hyperresistance to CHP and H₂O₂. The *fur* mutation on its own slightly decreases resistance to both CHP and H₂O₂, whereas the *fur perR* double mutant is more resistant to CHP and H₂O₂. The *perR* mutants did not show an inhibition zone with 3% H₂O₂ (asterisk).

an iron-containing enzyme, under low-iron conditions *sodB* may be expressed but is not functional due to the iron limitation.

The *perR* system was first described for *B. subtilis*, where expression of KatA and AhpC is regulated by metal ions and oxidative stress (9). The PerR regulator was recently shown to be one of three Fur homologs (7), one other being the iron uptake regulator (designated Fur), and the third being the zinc uptake regulator (Zur) (14). Multiple Fur homologs have been described for several other gram-positive organisms and also for *P. aeruginosa* (32) and *E. coli* (20). The second *E. coli* Fur homolog regulates zinc uptake and has been designated Zur (20). The function of the second Fur homolog of *P. aeruginosa* is still unknown (32). We have now demonstrated the function of a second Fur homolog in *C. jejuni*, where it can be regarded as a functional analog for OxyR (Fig. 5). It is noteworthy that there is no second Fur or OxyR homolog in *H. pylori*, and thus, it remains unclear whether and how *H. pylori* regulates *kata* and *ahpC* expression.

Regulation of gene expression in *C. jejuni* is relatively poorly understood. This report is the first description of peroxide

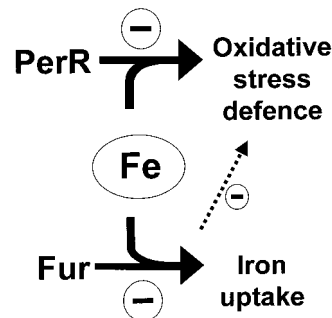


FIG. 5. Model illustrating Fur homolog-mediated negative regulation of oxidative-stress genes and iron transport systems in *Campylobacter*. The dotted arrow indicates a possible influence of Fur on oxidative-stress gene regulation involving either a direct interaction with oxidative-stress gene promoters or an indirect effect via transport systems on intracellular iron concentrations.

stress regulation by a second Fur homolog in a gram-negative bacterium. We also describe the first *C. jejuni* double regulatory mutant and show that both PerR and Fur have an effect on catalase expression, demonstrating regulatory cross-talk in *C. jejuni*. Future work will focus on the mechanisms of PerR regulation of peroxide stress resistance and further characterization of the *perR* and *perR fur* mutants using two-dimensional gel electrophoresis.

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