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Regulation of the *Bacillus subtilis fur* and *perR* Genes by PerR: Not All Members of the PerR Regulon Are Peroxide Inducible

Mayuree Fuangthong, Andrew F. Herbig,† Nada Bsat,‡ and John D. Helmann*

Department of Microbiology, Cornell University, Ithaca, New York 14853-8101

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PerR is a ferric uptake repressor (Fur) homolog that functions as the central regulator of the inducible peroxide stress response in *Bacillus subtilis*. PerR has been previously demonstrated to regulate the *mrgA*, *katA*, *ahpCF*, *hemAXCDBL*, and *zosA* genes. We now demonstrate that PerR also mediates both the repression of its own gene and that of *fur*. Whereas PerR-mediated repression of most target genes can be elicited by either manganese or iron, repression of *perR* and *fur* is selective for manganese. Genetic studies indicate that repression of PerR regulon genes by either manganese or iron requires PerR and is generally independent of Fur. Indeed, in a *fur* mutant, iron-mediated repression is enhanced. Unexpectedly, repression of the *fur* gene by manganese appears to require both PerR and Fur, but only PerR binds to the *fur* regulatory region in vitro. The *fur* mutation appears to act indirectly by affecting cellular metal ion pools and thereby affecting PerR-mediated repression. While many components of the *perR* regulon are strongly induced by hydrogen peroxide, little, if any, induction of *fur* and *perR* could be demonstrated. Thus, not all components of the PerR regulon are components of the peroxide stimulon. We suggest that PerR exists in distinct metallated forms that differ in DNA target selectivity and in sensitivity to oxidation. This model is supported by the observation that the metal ion composition of the growth medium can greatly influence the transcriptional response of the various PerR regulon genes to hydrogen peroxide.

Metal ions participate in a myriad of cellular functions, including respiration, enzyme catalysis, and stabilization of protein structure. Intracellular metal ion homeostasis must be maintained not only to reap the benefits of these nutrients but also to protect against toxic effects when metals are in excess (25, 28). A detrimental characteristic of some metal ions, particularly Fe(II), is the ability to react with hydrogen peroxide (H_2O_2) to produce the damaging hydroxyl radical ($^{\cdot}OH$). It is therefore necessary that intracellular levels of both reactive oxygen species (ROS) and Fe(II) be tightly regulated (30, 31).

In *Bacillus subtilis*, iron uptake is regulated by Fur, a metalloregulatory protein that binds Fe(II) as a corepressor (2, 3). Under iron-replete conditions, Fur represses iron uptake functions, including siderophore biosynthesis and transport genes. In bacteria such as *Escherichia coli* and *Vibrio cholerae*, Fur can also bind manganese and repress iron uptake functions even when iron is limiting (15, 22). In contrast, repression of the Fur regulon in *B. subtilis* is highly selective for iron (3, 5).

The *B. subtilis* H_2O_2 stress response is regulated by PerR, one of three Fur homologs in this organism (3). Increased levels of H_2O_2, either exogenously or endogenously derived, induce *katA* (catalase), *ahpCF* (alkylhydroperoxide reductase), *hemAXCDBL* (heme biosynthesis), *zosA* (zinc uptake), and *mrgA* (DNA-binding protein) (1, 6, 8; A. Gaballa and J. D. Helmann, submitted for publication). Like that by other Fur homologs, repression by PerR requires a divalent metal ion. Addition of either Mn(II) or Fe(III) to cultures is sufficient to repress expression of *mrgA* and *katA*, two components of the PerR regulon (5, 6). Note that although the form of iron added to cultures is Fe(III), in vivo this is likely to be reduced to Fe(II), which is thought to be the form that interacts with PerR to effect repression. In vitro, purified PerR binds to operator sites overlapping target promoters. The ability of PerR to sense peroxide stress appears to be affected by the identity of the metal cofactor: PerR-Fe dissociates more readily from target operators than does PerR-Mn following exposure to H_2O_2 (18).

Bacteria have evolved complex mechanisms by which to coordinately regulate metal uptake and oxidative stress responses (30, 31). In *E. coli*, *fur* is controlled by the OxyR and the SoxR/S systems and induced by oxidants (34). Induction of Fur may allow the cell to repress iron uptake under conditions of oxidative stress, and the abundant Fur protein may also serve to scavenge free iron inside the cell (34). In contrast to iron, manganous and zinc ions can protect the cell against oxidative stress. We have recently demonstrated that ZosA (YkvW), a Zn(II) uptake ATPase, is controlled by PerR and facilitates increased accumulation of zinc under conditions of peroxide stress (Gaballa and Helmann, submitted).

In this study, we demonstrated that PerR functions as a transcriptional repressor both for its own gene and for *fur*. Both *perR* and *fur* are repressed by Mn(II) in a PerR-dependent fashion but are not repressed by iron. Unexpectedly, neither *fur* nor *perR* was significantly induced by H_2O_2. Since these results differ from those reported previously for other components of the PerR regulon, we herein present a comprehensive comparison of regulation of all PerR regulon genes. We demonstrate (i) that the metal selectivity of repression differs among the components of the PerR regulon, (ii) that

* Corresponding author. Mailing address: Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853-8101. Phone: (607) 255-6570. Fax: (607) 255-3904. E-mail: jdh9@cornell.edu.

† Present address: Department of Nutritional Sciences, University of Missouri—Columbia, Columbia, MO 65211.

‡ Present address: Influx Inc., Chicago Technology Park, Chicago, IL 60612-3547.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
B. subtilis		
CU1065	W168 *att*SPβ *trpC2*	Laboratory stock
ZB307A	W168 SPβ*c2*Δ*2*::Tn*917*::pSK10Δ6	35
HB1000	ZB307A *att*SPβ	Laboratory stock
HB1122	ZB307A SPβ*c2*Δ*2*::Tn*917*::Φ(*mrgA-cat-lacZ*)	6
HB0518	ZB307A SPβ*c2*Δ*2*::Tn*917*::Φ(*katA-cat-lacZ*)	3
HB8206	ZB307A SPβ*c2*Δ*2*::Tn*917*::Φ(*zosA-cat-lacZ*)	A. Gaballa
HB1602	ZB307A SPβ*c2*Δ*2*::Tn*917*::Φ(*ahpC-cat-lacZ*)	1
HB0539	ZB307A SPβ*c2*Δ*2*::Tn*917*::Φ(*perR-cat-lacZ*)	This work
HB6560	ZB307A SPβ*c2*Δ*2*::Tn*917*::Φ(*fur-cat-lacZ*)	This work
HB0568	CU1065 SPβ*c2*Δ*2*::Tn*917*::Φ(*mrgA-cat-lacZ*)	This work
HB0567	CU1065 SPβ*c2*Δ*2*::Tn*917*::Φ(*katA-cat-lacZ*)	This work
HB8108	CU1065 SPβ*c2*Δ*2*::Tn*917*::Φ(*zosA-cat-lacZ*)	A. Gaballa
HB2083	CU1065 SPβ*c2*Δ*2*::Tn*917*::Φ(*ahpC-cat-lacZ*)	This work
HB1041	CU1065 *hemA-lacZ*	6
HB2062	CU1065 SPβ*c2*Δ*2*::Tn*917*::Φ(*perR-cat-lacZ*)	This work
HB2076	CU1065 SPβ*c2*Δ*2*::Tn*917*::Φ(*fur-cat-lacZ*)	This work
HB0509	HB1000 *perR*::*spc*	3
HB2078	CU1065 *perR*::*kan*	This work
HB2116	CU1065 *perR*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*mrgA-cat-lacZ*)	This work
HB2117	CU1065 *perR*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*katA-cat-lacZ*)	This work
HB2118	CU1065 *perR*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*zosA-cat-lacZ*)	This work
HB2120	CU1065 *perR*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*ahpC-cat-lacZ*)	This work
HB2121	CU1065 *perR*::*kan hemA-lacZ*	This work
HB2119	CU1065 *perR*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*perR-cat-lacZ*)	This work
HB2115	CU1065 *perR*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*fur-cat-lacZ*)	This work
HB2501	CU1065 *fur*::*kan*	This work
HB2082	CU1065 *fur*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*mrgA-cat-lacZ*)	This work
HB2110	CU1065 *fur*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*katA-cat-lacZ*)	This work
HB2111	CU1065 *fur*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*zosA-cat-lacZ*)	This work
HB2113	CU1065 *fur*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*ahpC-cat-lacZ*)	This work
HB2114	CU1065 *fur*::*kan hemA-lacZ*	This work
HB2112	CU1065 *fur*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*perR-cat-lacZ*)	This work
HB2081	CU1065 *fur*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*fur-cat-lacZ*)	This work
HB7503	CU1065 *mntR*::*kan*	27
HB2079	CU1065 *mntR*::*kan* SPβ*c2*Δ*2*::Tn*917*::Φ(*fur-cat-lacZ*)	This work
HB6507	HB1000 *ahpC*::Tn*10* (*spc*)	1
HB6566	HB1000 *ahpC*::Tn*10* (*spc*) SPβ*c2*Δ*2*::Tn*917*::Φ(*fur-cat-lacZ*)	This work
HB1308	Spontaneous *katA* mutant; cured of SPβ	6
HB6570	HB1308 SPβ*c2*Δ*2*::Tn*917*::Φ(*fur-cat-lacZ*)	This work
E. coli DH5α		Laboratory stock
Plasmids		
pBSKII$^+$	pBR322 replicon	Stratagene
pJPM122	*cat-lacZ* operon fusion vector for SPβ	29
pGEM-cat	pGEM-3zf(+)-cat-1	33
pDG780	pBSKII$^+$ containing kanamycin resistance cassette	13
pHB6518	pJPM122 with *fur* promoter	This work
pSK1	pGEM-cat with 542-bp fragment containing *perR*	3
pAFH3	pBSKII$^+$ with 542-bp fragment containing *perR*	This work
pAFH5	pJPM122 with *perR* promoter	This work
pMF20	pGEM-cat with 776-bp fragment containing *perR*	This work
pMF21	pGEM-cat with *perR*::*kan*	This work

both iron-mediated repression and manganese-mediated repression require PerR, and (iii) that the extent of induction by H_2O_2 is highly variable and is influenced by the metal ion composition of the growth medium. These results are supportive of a model in which PerR can exist in various metallated forms that differ in both DNA target selectivity and sensitivity to H_2O_2.

MATERIALS AND METHODS

Media and growth conditions. *B. subtilis* strains were grown at 37°C in Luria broth (LB) or minimal medium containing 40 mM potassium morpholinepropanesulfonic acid (MOPS) (adjusted to pH 7.4 with KOH), 2 mM potassium phosphate buffer (pH 7.0), glucose (2%, wt/vol), $(NH_4)_2SO_4$ (2 g/liter), $MgSO_4 \cdot 7H_2O$ (0.2 g/liter), trisodium citrate $\cdot 2H_2O$ (1 g/liter), potassium glutamate (1 g/liter), tryptophan (10 mg/liter), 3 nM $(NH_4)_6Mo_7O_{24}$, 400 nM H_3BO_3, 30 nM $CoCl_2$, 10 nM $CuSO_4$, 10 nM $ZnSO_4$, and $MnCl_2$ and $FeCl_3$ added to the concentrations indicated (5). Ampicillin (100 μg ml^{-1}), spectinomycin (150 μg ml^{-1}), or kanamycin (40 μg ml^{-1}) was used for selection of *E. coli* strains. Erythromycin (1 μg ml^{-1}) and lincomycin (25 μg ml^{-1}; for testing of macrolide-lincosamide-streptogramin B resistance), spectinomycin (100 μg ml^{-1}), kanamycin (10 μg ml^{-1}), neomycin (10 μg ml^{-1}), and chloramphenicol (5 μg ml^{-1}) were used for the selection of various *B. subtilis* strains.

Bacterial strains, phage, and plasmids. The *B. subtilis* strains and phage, *E. coli* strain, and plasmids used in this study are described in Table 1. SPβ phage

are derivatives of SPβ*c2*Δ*2* (35) and were constructed by integration of a promoter region-*cat-lacZ* operon fusion constructed in pJPM122 into strain ZB307A as described previously (29). SPβ-transducing lysates were produced by heat induction from the indicated lysogens.

Construction of *perR*::*kan* and *fur*::*kan* mutant strains. A fragment containing *perR* was amplified from *B. subtilis* CU1065 DNA with forward primer 5′-GCAAGCTTGAGTATATGGGAAT-3′ and reverse primer 5′-GGAATTCGGAAAAGAATTTGATGAGTC-3′ to introduced *Hin*dIII and *Eco*RI sites (underlined). The *Hin*dIII-*Eco*RI-digested fragment was cloned into pGEM-cat, generating plasmid pMF20. The *Sac*I-*Hin*cII fragment from pDG780 containing a kanamycin resistance cassette was inserted between *Sac*I and *Xma*I sites within *perR* to generate pMF21. CU1065 was transformed with *Sca*I-digested pMF21 with selection for kanamycin resistance, and transformants were screened for loss of plasmid-borne chloramphenicol resistance to distinguish single-crossover from double-crossover recombination. The resulting CU1065 *perR*::*kan* strain was designated HB2078. The presence of *perR*::*kan* in the strain was confirmed by PCR and Southern analysis. The isogenic *fur* mutant was constructed by transformation of CU1065 with chromosomal DNA from HB6543 (*fur*::*kan*) (3).

DNA manipulations and sequencing. Isolation of *B. subtilis* chromosomal DNA, transformation, and specialized SPβ transductions were done by standard procedures (7). *E. coli* plasmid DNA and restriction enzyme fragments were isolated with the QIAprep spin miniprep and PCR purification kits, respectively (Qiagen Inc., Chatsworth, Calif.). Restriction endonucleases, DNA ligase, Vent DNA polymerase (New England Biolabs, Beverly, Mass.), *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), RNase-free DNase, and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.) were used in accordance with the manufacturers' instructions. DNA sequencing was performed with AmpliTaq-FS DNA polymerase and dye terminator chemistry by the DNA services facility of the Cornell New York State Center for Advanced Technology-Biotechnology.

DNase I footprinting assays. Purification of *B. subtilis* Fur and PerR and DNase I protection assays were performed as previously described (2, 18). G+A sequencing ladders were generated as previously described (23), with incubation at 104°C for 20 min. PCRs were used to amplify templates for the footprinting experiments. The primer pairs used were as follows: for *perR*, 5′-GCAAGCTTGAGTATATGGGAAT-3′ and 5′-GGGGATCCGAGCCATAGAGTTAAC-3′; for *fur*, 5′-GCGCTGATTTCATCTCTCTTT-3′ and 5′-GGATGAGTGCAGTTGTTTCTTA-3′. Fragments were purified and digested with the appropriate restriction enzymes, and the ends were filled in with [α-^{32}P]dATP and the *E. coli* polymerase I Klenow fragment (exo$^-$; New England Biolabs).

Primer extension analysis. For mapping of the *fur* promoter, HB0509 (*perR* mutant) cells were grown in LB and total RNA was isolated at the end of logarithmic growth essentially as previously described (10). For the *perR* promoter, total RNA was isolated from mid-logarithmic-phase cells with an RNAWIZ kit (Ambion). Primer extension reactions were set up as follows. Thirty micrograms of RNA was hybridized to ~2 pmol of the appropriate end-labeled primer in buffer containing 60 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM dithiothreitol (DTT), and 40 U of RNasin (Promega). Following hybridization, extension buffer (72 mM NaCl, 50 mM Tris-HCl [pH 7.9], 10 mM DTT, 20 mM $MgCl_2$), deoxynucleoside triphosphates, and avian myeloblastosis virus reverse transcriptase were added to the mixture, which was incubated at 37°C for 30 min. The primer extension products were precipitated, resuspended in sequence loading buffer, and loaded onto a 6% acrylamide sequencing gel. A PCR cycle sequencing kit (Epicenter) was used to generate sequencing ladders corresponding to the *perR* and *fur* promoter-operator regions.

Northern analysis of *fur*. Samples for Northern analysis were prepared as described for the resuspension experiment and collected at 3 h after resuspension. Total RNA was isolated with RNAWIZ reagent (Ambion). A 5-μg sample of total RNA was then separated with a 1% formaldehyde gel, transferred to nylon membrane, and hybridized with radiolabeled probe at 50°C overnight in ULTRAhyb solution (Ambion). Membranes were then washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS), followed by two washes with 0.5× SSC plus 0.1% SDS for 15 min at 50°C. The *fur* probe was prepared by *Sau*3AI digestion of the PCR product containing the complete coding region of *fur*, which contains three internal *Sau*3AI sites. The digested products were purified and labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase.

Construction of *cat-lacZ* reporter fusions. For the *fur-cat-lacZ* fusion, the promoter region of *fur* was amplified from HB1000 chromosomal DNA by a PCR with 5′-GATCCTCTAAGCTTTTTTAAAATC-3′ as the forward primer and 5′-ATCAACGGATCCGAACTC-3′ as the reverse primer. The PCR mixture contained 50 μM deoxynucleoside triphosphates, 100 pmol each of the forward and reverse primers, 2 U of Vent DNA polymerase, and Vent buffer in a total volume of 100 μl. The reaction mixtures were subjected to denaturation for 2 min at 94°C, followed by 30 cycles of 10 s at 95°C, 30 s at 50°C, 30 s at 72°C, and a final extension of 5 min at 72°C. The resulting PCR product was cloned into pJPM122 as a *Hin*dIII-to-*Bam*HI fragment (underlined sites) to generate pHB6518. The insert was verified by sequencing. pHB6518 was linearized and used to transform ZB307A with selection for neomycin resistance to generate strain HB6560. Phage generated from this strain (SPβ6560) was used to move the *fur-cat-lacZ* operon fusion into various strain backgrounds as indicated.

For the *perR-cat-lacZ* fusion, an *Eco*RV-*Hin*cII fragment from pSK1 containing the *perR* promoter was cloned into the *Eco*RV site of pBSK, generating pAFH3. The *Bam*HI-*Hin*dIII fragment from pAFH3 was cloned into pJPM122 to generate reporter fusions as described above. The *mrgA*, *katA*, *ahpC*, and *zosA-cat-lacZ* fusions are described elsewhere (1, 3, 6; Gaballa and Helmann, submitted). The DNA fragments used for *cat-lacZ* fusions contained all of the putative promoters and Per boxes. The fragments used to generate promoter fusions extended from −464 to +47 for *mrgA*, from −304 to +265 for *katA*, from −332 to +32 for *zosA*, from −1326 to +159 for *ahpC*, from −90 to +97 for *perR*, and from −120 to +312 for *fur* (all relative to the start codon). The *hemA-lacZ* fusion was constructed by Campbell integration of a plasmid containing *hemA-lacZ* (6). The *hemA-lacZ* fusion was moved to different backgrounds by transformation with HB1041 (CU1065 *hemA-lacZ*) chromosomal DNA and selection for chloramphenicol resistance.

β-Gal assays. For resuspension experiments, overnight cultures of cells grown in LB containing appropriate antibiotics were transferred at a 1:100 dilution into fresh MOPS-buffered minimal medium with 10 μM $FeCl_3$ and 5 μM $MnCl_2$. The overnight cultures were transferred again at a 1:100 dilution into the same minimal medium. The cells were incubated until the optical density at 600 nm was about 0.2. The cells were then washed once with minimal medium with no added $FeCl_3$ or $MnCl_2$, collected by centrifugation, and resuspended in minimal medium either with no added $FeCl_3$ or $MnCl_2$ or with 10 μM $FeCl_3$, 5 μM $MnCl_2$, or both. This time point was designated time zero. Samples were removed for β-galactosidase assay (β-Gal) at the indicated times by the method of Miller as described previously (5, 24). All assays were performed on duplicate samples, and the values were averaged. Glassware was acid washed when possible.

For experiments with the *fur* and *mntR* mutant strains (see Fig. 5), overnight cultures of cells grown in LB containing appropriate antibiotics were transferred at a 1:100 dilution into fresh MOPS-buffered minimal medium with 10 μM Fe(III). The overnight cultures were transferred again at a 1:100 dilution into minimal medium with either 1 or 10 μM Fe(III) (as indicated) and the indicated concentration of Mn(II). Cells in mid-log phase were collected for β-Gal assay.

Peroxide induction experiments. Cell samples at 2 h after resuspension (see resuspension experiment protocol described above) were transferred into new tubes (prewarmed at 37°C). H_2O_2 (100 μM) was added, and samples were taken for β-Gal assay after 30 and 60 min.

Polyclonal anti-PerR Ab production and immunoblotting experiments. Purified PerR was submitted to the Cornell University Animal Research Laboratory for production of rabbit polyclonal antibodies (Ab). Immunodetection was performed with crude extracts by SDS-polyacrylamide gel electrophoresis, followed by electroblotting onto polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 50 mg of nonfat dried milk per ml at 4°C. Anti-PerR Ab was added at a dilution of 1:500 in Tris-buffered saline (TBS) with 0.05% Tween 20. After incubation for 1 h at room temperature, the membrane was washed three times in TBS-Tween 20. Goat anti-rabbit Ab conjugated to alkaline phosphatase (Bio-Rad) was used as the secondary Ab at a concentration of 1:3,000. After incubation for 1 h, the membrane was washed three times in TBS-Tween 20 and once in TBS. The colorimetric signal was visualized by incubation with alkaline phosphatase substrate (Bio-Rad) in development buffer in accordance with the manufacturer's protocols.

RESULTS

Identification of PerR-binding sites in the *perR* and *fur* promoter regions. We have previously identified five operons within the PerR regulon (*mrgA*, *katA*, *ahpCF*, *hemAXCDBL*, and *zosA*) (1, 3, 4, 6; Gaballa and Helmann, submitted). To identify additional candidate components of this regulon, we searched the *B. subtilis* genome with the 15-bp Per box consensus sequence (TTATAATnATTATAA; reference 17) and

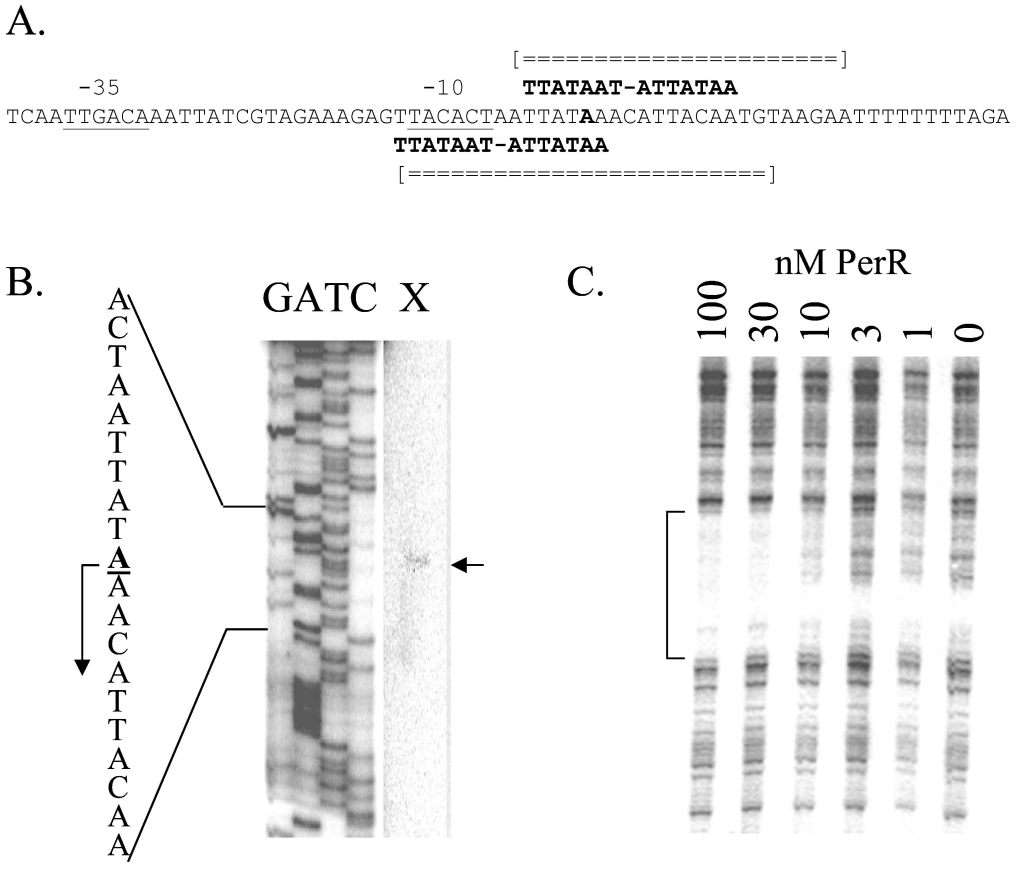

FIG. 1. Interaction of PerR with the *perR* regulatory region. (A) The −35 and −10 regions of the *perR* promoter are underlined, and the two overlapping Per box elements are indicated. The A residue start site for transcription is in bold. Regions of both DNA strands protected against DNase I digestion by bound PerR protein are indicated by broken double lines. (B) Primer extension mapping of the *perR* transcriptional start site (arrow) indicates transcript initiation with the A residue indicated in the sequence to the left. (C) DNase I footprint of purified PerR binding to the *perR* promoter. PerR was added at the concentrations (nanomolar) indicated in the presence of 10 μM Mn(II). Shown are results from footprinting on the bottom strand; however, top-strand analysis was also performed (data not shown). The regions protected against digestion, as determined by alignment with G+A sequencing ladders (data not shown), are summarized in panel A.

found additional candidate Per boxes upstream of the *perR* and *fur* genes.

Two overlapping Per boxes are present upstream of the *perR* gene (Fig. 1A), which is suggestive of a role in autoregulation. By primer extension start site mapping, we determined that transcription initiates from a σ^A-type promoter at an A residue 45 bp upstream of the start codon (Fig. 1B). The upstream Per box overlaps the −10 consensus sequence. In DNase I footprinting experiments, as little as 10 nM PerR protected an ~25-bp region surrounding the transcription start site against DNase I digestion (Fig. 1C). This is comparable to the affinity of PerR for other operator regions that have been studied (18).

Genome searches also revealed a consensus Per box in the *fur* regulatory region. The DNA sequence of the *fur* gene (Fig. 2A) contains a candidate σ^A-dependent promoter with a 12-of-14 match to the −35 and extended −10 consensus sequences (16). Primer extension analysis, performed with RNA isolated from late-logarithmic-phase (transition phase) cells, identified two transcripts that initiated at A and G residues located 25 and 27 nucleotides upstream from the *fur* start codon (Fig. 2B). PerR bound tightly to the *fur* operator; strong protection was again observed with as little as 10 nM PerR (Fig. 2C). These results are consistent with a direct role of PerR as a repressor of the *fur* gene. However, since Per and Fur boxes are similar in sequence, this region can also be interpreted as a weak (12-of-19) match to the Fur box, raising the possibility that *fur* is autoregulated. However, no Fur binding was observed, even at a concentration of 100 nM (data not shown). At known Fur-regulated promoters, 10 nM Fur is usually sufficient to saturate binding (N. Baichoo and J. D. Helmann, submitted for publication). These results indicate that Fur does not interact directly with its own promoter and suggest, instead, that Fur is regulated directly by PerR (see below).

Patterns of metalloregulation within the PerR regulon. All of the components of the PerR regulon that have been described are repressed by addition of manganese to the growth medium, and in some cases, repression by iron has also been observed. We sought to directly compare the metalloregulation of all of the components of the PerR regulon under identical conditions. To monitor expression of *perR* and *fur*, we constructed transcriptional fusions between the corresponding regulatory regions and *lacZ* and integrated the resulting *cat-lacZ* operon fusions into the SPβ prophage. Similar reporter

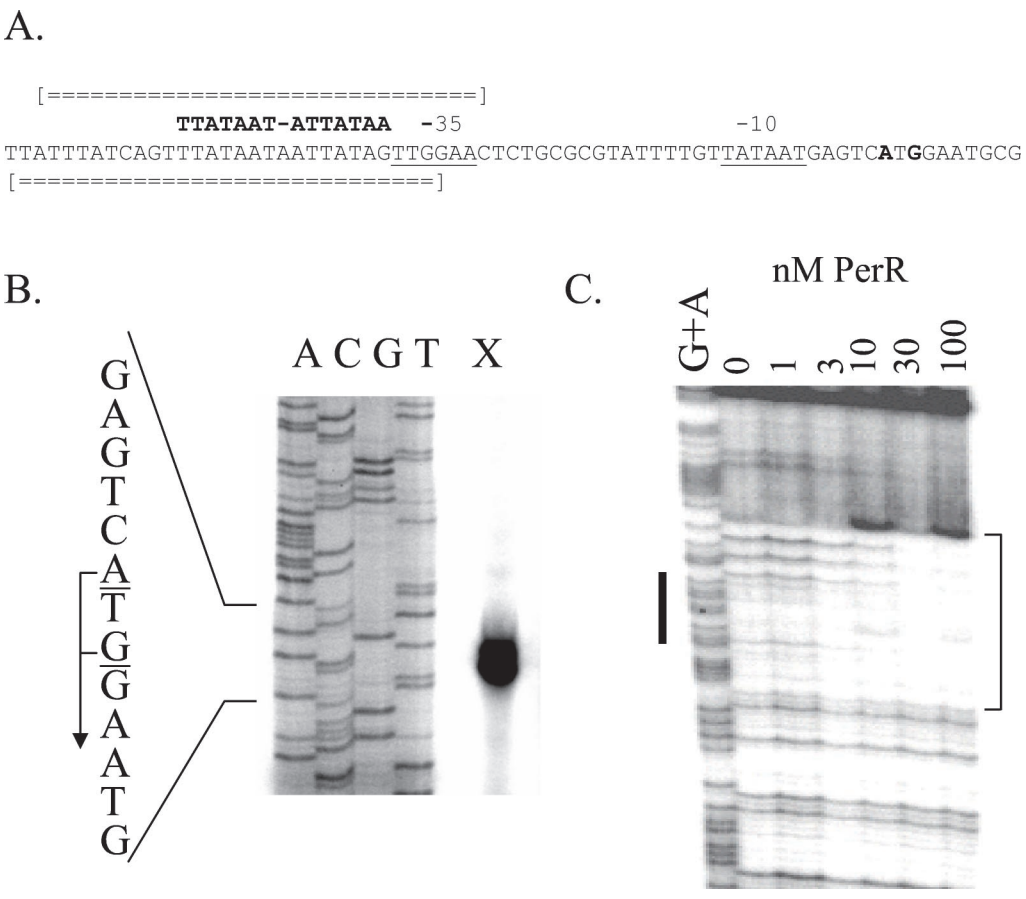

FIG. 2. Interaction of PerR with the *fur* regulatory region. (A) The −35 and −10 elements of the *fur* promoter are underlined, and the transcriptional start sites are in bold. The Per box upstream of the −35 element is indicated. Regions of both DNA strands protected against DNase I digestion by bound PerR protein are indicated by broken double lines. (B) Primer extension mapping of the transcription start sites of the *fur* gene. Transcription initiates at the indicated A and G residues. (C) DNase I footprint of PerR binding to the *fur* operator region. The results shown are for the bottom strand. Purified PerR was added at the concentrations indicated in the presence of 10 μM Mn(II). The bold line adjacent to the G+A ladder indicates the position of the Per box, and the protected region is bracketed.

constructs were used for the other PerR regulon genes, with the exception of the *hemA* promoter, which was fused to a *lacZ* reporter gene by plasmid integration (Table 1).

To compare the abilities of Mn(II) and Fe(III) to repress PerR-regulated genes, we used resuspension experiments as previously described (3, 5). In these studies, cells were grown to mid-logarithmic phase, harvested, and resuspended in minimal medium containing various levels of Mn(II) and Fe(III). In confirmation of our previously reported studies (3, 5), expression of an *mrgA-cat-lacZ* fusion is very low when cells are resuspended in minimal medium containing either 5 μM Mn(II), 10 μM Fe(III), or both. Only when both metal ions are omitted from the medium does gene expression commence (Fig. 3A). This supports the suggestion that either Mn(II) or Fe(II) can function in vivo as a corepressor with PerR. Similar patterns of metalloregulation were observed for the *katA* (Fig. 3B) and *ahpC* (Fig. 3D) promoter fusions. Repression of *hemA* by iron was inefficient but still observable (Fig. 3E).

In contrast, expression of the *perR-cat-lacZ* (Fig. 3F) and *fur-cat-lacZ* (Fig. 3G) fusions increased after resuspension in medium lacking both metal ions or containing added Fe(III). Only when Mn(II) was present was expression efficiently repressed by PerR. Even addition of 100 μM Fe(III) did not repress gene expression nearly as efficiently as 5 μM Mn(II) (data not shown). A similar pattern was observed for the *zosA* gene: expression was repressed by Mn(II) but not by iron (Fig. 3C). The manganese selectivity of transcriptional repression was corroborated by additional studies: immunoblotting with anti-PerR Ab demonstrated that cells grown with 5 μM Mn(II) had 2.4-fold lower levels of PerR protein, whereas iron supplementation led to a slight (1.2-fold) increase in PerR (data not shown). Similarly, the effects of metal ions on *fur* transcription were measured by Northern analysis: Fe(III) supplementation slightly increased mRNA levels, while Mn(II) decreased mRNA levels (Fig. 3H). Repression of *zosA* is also selective for Mn(II), as judged by Northern analysis (data not shown).

Role of PerR in metalloregulation. The diverse responses of the various components of the PerR regulon to metal ion supplementation could be due to the combinatorial effects of multiple metalloregulatory proteins. For example, it is possible that PerR mediates Mn(II)-dependent repression while Fur might contribute to the iron-dependent effects. To determine whether the effects of manganese and iron require PerR, Fur, or both, we repeated these studies after transfer of the reporter

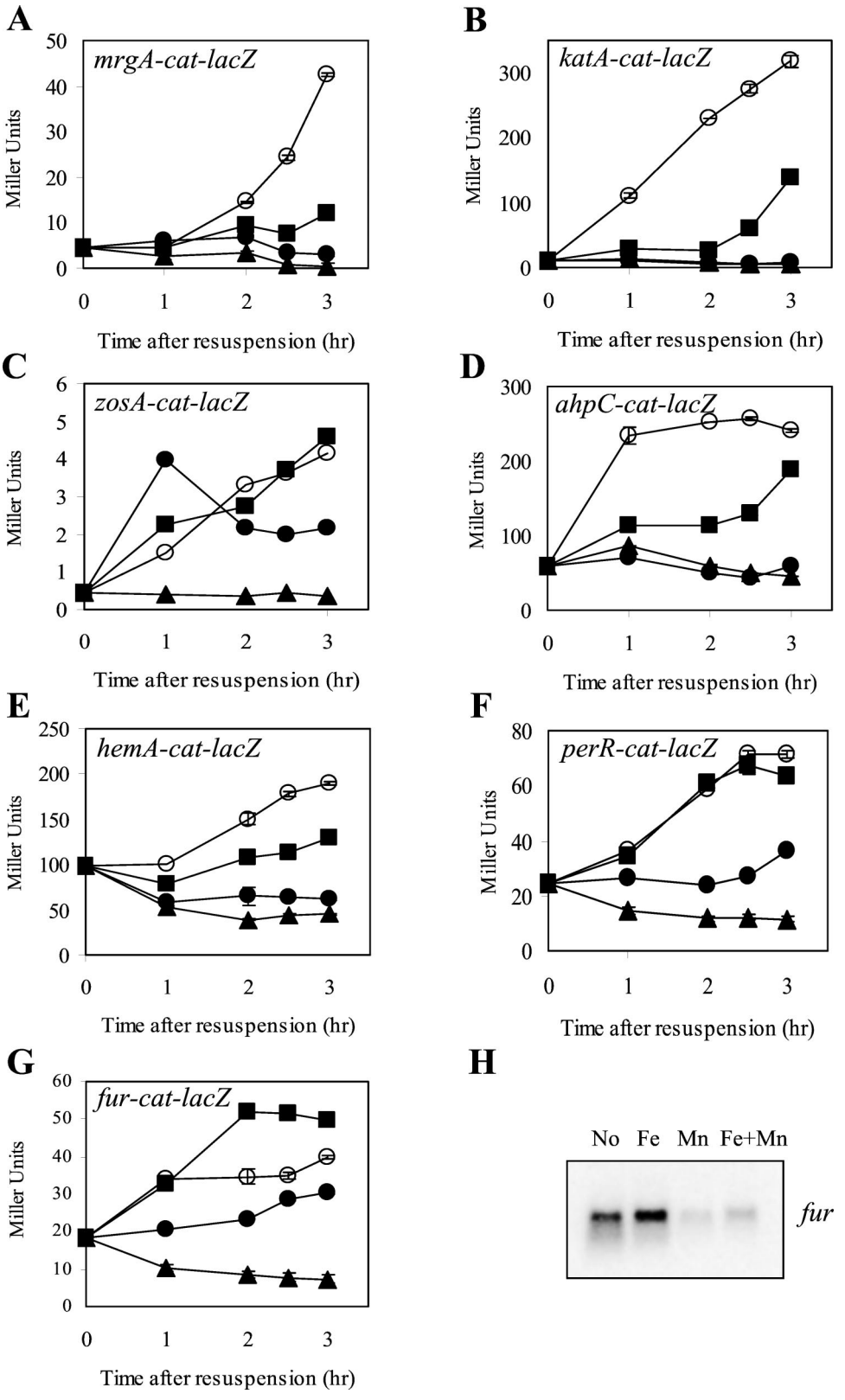

FIG. 3. Metal selectivity of gene regulation in resuspension experiments. Cells were resuspended in minimal medium either containing no added Mn(II) or Fe(III) (open circles) or containing 5 μM Mn(II) (filled triangles), 10 μM Fe(III) (filled squares), or both (filled circles). Strains contained *mrgA-cat-lacZ* (A), *katA-cat-lacZ* (B), *zosA-cat-lacZ* (C), *ahpC-cat-lacZ* (D), *hemA-cat-lac* (E), *perR-cat-lacZ* (F), or *fur-cat-lacZ* (G). Samples were taken at the times indicated and assayed for β-Gal activity. The results shown are representative of at least three independent experiments; error bars represent the standard error of the mean of duplicate samples. Panel H is a Northern blot analysis of the *fur* transcript from cultures collected 3 h after resuspension in minimal medium containing the indicated metal ion supplementation.

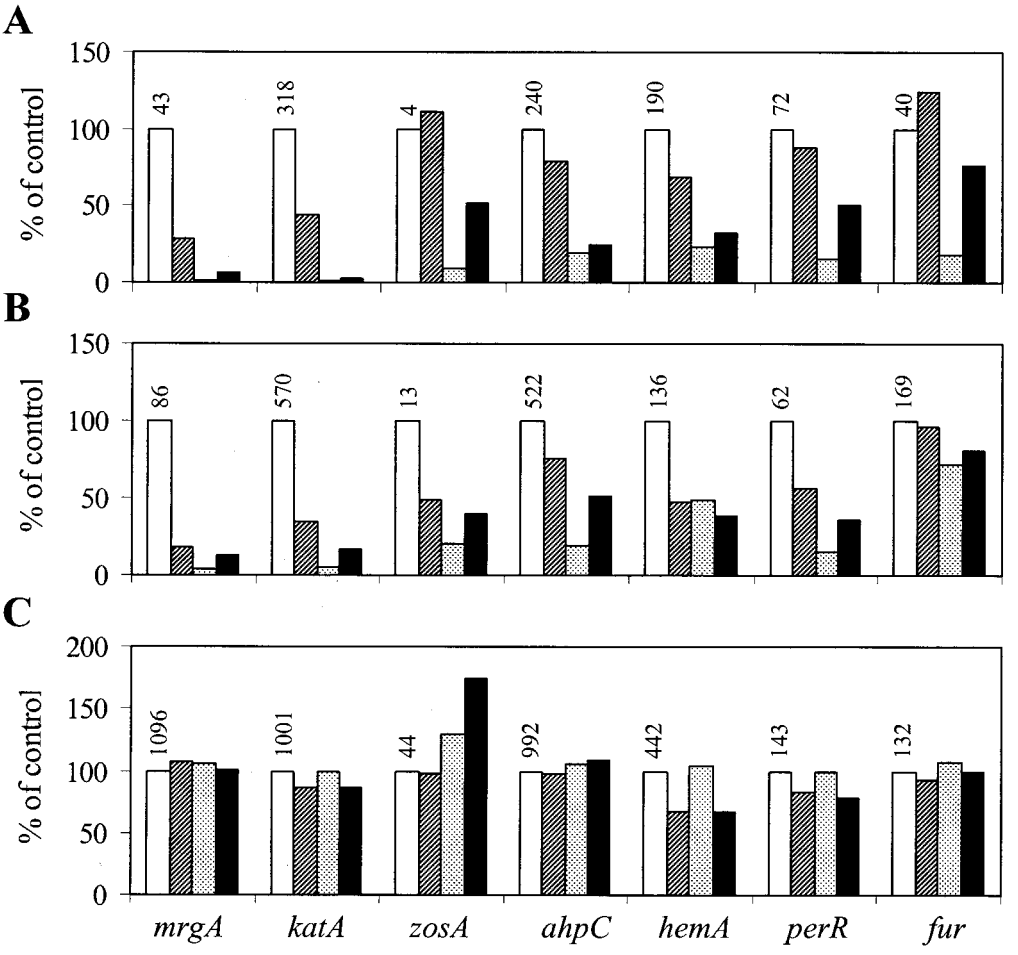

FIG. 4. Roles of PerR and Fur in metalloregulation of PerR regulon genes. Resuspension experiments were performed as described in the legend to Fig. 3 with all seven promoter fusions (indicated at the bottom of panel C) in either the wild-type (A) background or the *fur* (B) or *perR* (C) mutant strain background. All samples were measured 3 h. after resuspension and normalized to the level in medium lacking manganese and iron supplementation (white bars; absolute values are shown above the bars). The cells were resuspended in minimal medium either containing (from left to right) no added Mn(II) or Fe(III) (white bars) or containing 10 μM Fe(III) (hatched), 5 μM Mn(II) (stippled), or both (black). The data in panel A are the same as those shown for the 3-h time point in Fig. 3.

fusions to *perR* and *fur* mutant strains. For this analysis, we compared the levels of β-Gal 3 h after resuspension in minimal medium containing 5 μM Mn(II), 10 μM Fe(III), neither, or both. The results for the wild-type strain (from Fig. 3) are summarized in Fig. 4A. Note that Mn(II) addition led to decreased expression of all of the genes, while iron supplementation had a variable effect.

When this experiment was repeated with the *perR* mutant strain (Fig. 4C), there was no significant repression of any of the genes by either Mn(II) or Fe(III) addition. Furthermore, comparison of the actual expression levels indicated that all of the PerR-regulated genes are derepressed in the *perR* mutant background. This suggests that even after resuspension in the unsupplemented (metal-limited) minimal medium, there is still significant PerR-dependent repression of these genes. These data indicate that the effects of both Mn(II) and Fe(III) on expression of PerR regulon components are mediated by PerR.

Effects of perturbing metal ion homeostasis on metalloregulation. In contrast with *perR*, the *fur* mutation had relatively modest effects on the metalloregulation of PerR regulon genes (Fig. 4B). The overall patterns of metal-dependent repression are similar to that of the wild type for nearly all of the PerR regulon components. However, several interesting effects are worth noting. First, in several cases, Fe(III) elicits greater repression in the *fur* mutant than in the wild type. In the *fur* mutant, for example, resuspension in the iron-supplemented medium reduces expression of the *zosA* and *perR* promoters, which were not iron responsive in the wild type. Second, in most cases, the absolute levels of expression are somewhat higher in the *fur* mutant background, suggesting that there is a general, nonspecific derepression of these genes in this strain background. Third, the *fur* mutation also affected the Mn(II)-dependent repression of some genes. This is most apparent for the *fur-cat-lacZ* fusion, which was strongly repressed by Mn(II) in the wild-type strain (Fig. 4A) but not in either a *perR* (Fig. 4C) or a *fur* (Fig. 4B) mutant strain. These effects could all be explained by elevated intracellular iron pools in the *fur* mutant

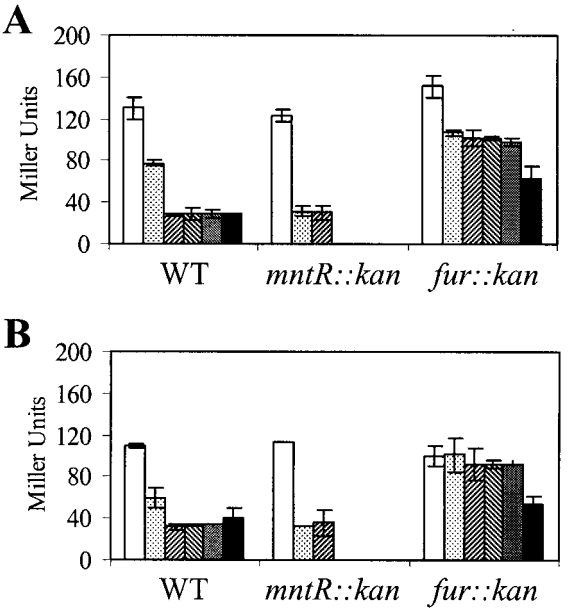

FIG. 5. Effects of *mntR* and *fur* mutations on metalloregulation of the *fur* gene. Overnight cultures of strains carrying the *fur-cat-lacZ* reporter fusion were diluted 1:100 into minimal medium with either 1 μM Fe(III) (A) or 10 μM Fe(III) (B) and Mn(II) at 0, 0.1, 1, 10, 100, or 1,000 μM (left to right). Cells were grown to mid-log phase and collected for β-Gal assay. Note that the *mntR* mutant strain does not grow in concentrations of Mn(II) of 10 μM and greater (27), so no data were obtained for these conditions. WT, wild type.

strain that shift PerR from the manganese-containing form to the iron-containing form, thereby leading to enhanced repression of iron-responsive genes and decreased repression of genes responsive only to Mn(II). Note that manganese and iron have been shown to compete for binding to PerR in in vitro studies (18).

To test the idea that the effects of the *fur* mutation on the Mn(II)-dependent repression of *fur-cat-lacZ* might be due to alterations to the intracellular metal ion pools, we measured the transcriptional response of the *fur* promoter region to various levels of Mn(II) (ranging from 100 nM to 1 mM). Consistent with previous studies of PerR regulon genes, 1 μM Mn(II) was sufficient for complete repression of *fur-cat-lacZ* in medium containing either 1 μM (Fig. 5A) or 10 μM Fe(III) (Fig 5B). The relatively inefficient repression elicited by 100 nM Mn(II) was enhanced in an *mntR* mutant strain that is derepressed for Mn(II) uptake (27). In contrast, there was little repression by Mn(II) in the *fur* mutant strain unless very high concentrations of Mn(II) were added. Since Mn(II) uptake is tightly regulated by MntR, it is difficult to perturb intracellular Mn(II) levels simply by manipulating concentrations in the medium. Experiments with a *fur mntR* double mutant might address this question, but we have not succeeded in constructing such a strain. Nevertheless, these results are consistent with the idea that PerR responds to intracellular pools of Mn(II) and Fe(II) and that these are affected by mutations that alter metal ion homeostasis systems.

Patterns of H_2O_2 induction within the PerR regulon. Many of the genes in the PerR regulon are known to be strongly induced by H_2O_2, and this transcriptional response has been shown to be modulated by the metal ion content of the growth medium: growth in Mn(II)-supplemented medium greatly reduced the extent of induction, while iron supplementation enhances induction (6, 18). Unexpectedly, we were unable to induce expression of the *fur* gene with H_2O_2, paraquat, or cumene hydroperoxide (data not shown). Furthermore, expression of the *fur* gene was not derepressed in either an *ahpC* or a *katA* mutant background (data not shown), conditions that lead to elevated expression of other peroxide-inducible genes (1, 6).

To compare the abilities of all of the PerR-regulated operons to be induced by H_2O_2 and to systematically investigate the effects of metal ions on induction, we have resuspended cells in minimal medium containing 5 μM Mn(II), 10 μM Fe(III), neither, or both, as shown in Fig. 3. After 2 h of growth, cultures were treated with 100 μM H_2O_2 (or left untreated) and gene expression was measured after 30 min (Fig. 6) or 60 min (data not shown). As expected, the level of gene expression was lowest in those cells growing in the Mn(II)-supplemented medium and these cells displayed the least response to H_2O_2 challenge. In contrast, in media supplemented with Fe(III), there was greater induction of the *mrgA*, *katA*, and *zosA* promoters. While qualitatively similar, the absolute level of induction in these resuspension experiments is not as great as that observed previously for the *mrgA* (4), *katA* (18), or *zosA* promoter (Gaballa and Helmann, submitted). Indeed, transcriptional profiling experiments performed with rich medium suggest that *mrgA*, *katA*, and *zosA* can each be fully derepressed by H_2O_2: the increase in mRNA levels in H_2O_2-induced cells is comparable to that in a *perR* mutant (J. D. Helmann et al., unpublished data).

In contrast with the *mrgA* and *katA* promoters, the *ahpC* and *hemA* promoters were only weakly induced by H_2O_2 treatment and little, if any, induction of the *perR* and *fur* promoters was observed. However, these promoters are all repressed by PerR in response to Mn(II). These results demonstrate that PerR regulon components differ both in susceptibility to repression by various metal ions and in the ability to be induced by H_2O_2. Since PerR-mediated repression can be elicited by manganese, iron, and perhaps other divalent metal ions (5), this leads to a model in which various metallated forms of PerR differ in both DNA target selectivity and reactivity with H_2O_2. Experiments testing these ideas by the in vitro reconstitution of different forms of PerR have been done (18), and additional studies are in progress.

DISCUSSION

The *E. coli* Fur protein is the prototype of a large family of metal-dependent repressor proteins. In *E. coli* and many other gram-negative bacteria, Fur regulates iron uptake functions by repressing gene expression in the presence of Fe(II), which acts as a corepressor (9). *B. subtilis* contains three Fur homologs that coordinate gene expression in response to iron (Fur), zinc (Zur), or H_2O_2 (PerR) (3, 11). All three proteins are dimeric, DNA-binding repressors that contain a single Zn(II) atom per monomer, which is thought to play a structural role, and a second regulatory metal ion that acts as a cofactor that is necessary for binding to the target operator sites (2, 18). However, they differ in metal selectivity: Fur is

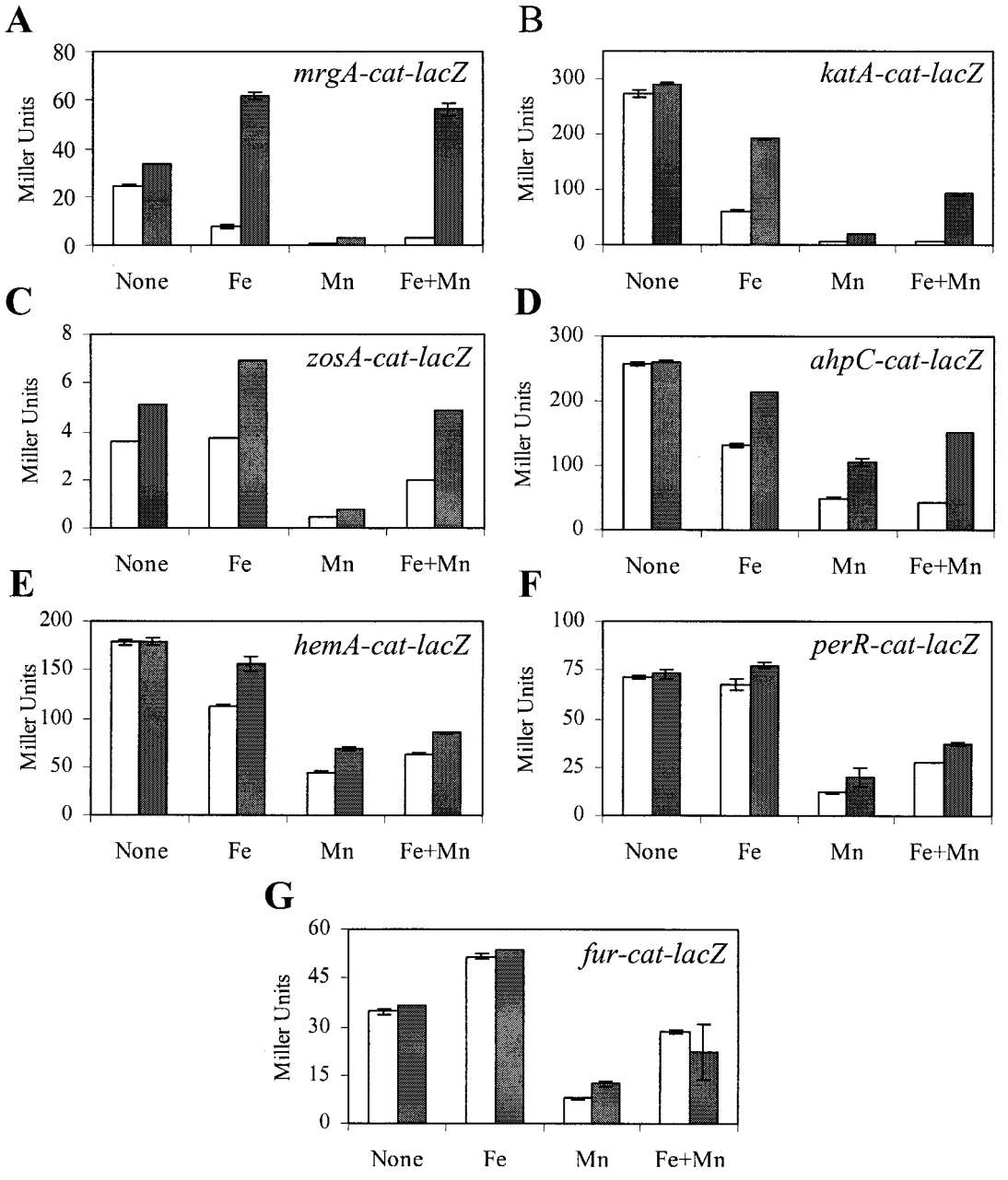

FIG. 6. Induction of PerR regulon genes by H_2O_2. Strains containing the indicated reporter fusions were grown in minimal medium with no added Mn(II) or Fe(III) (None) or with 10 μM Fe(III) (Fe), 5 μM Mn(II) (Mn), or both (Fe+Mn) as described in the legend to Fig. 3. At 2 h after resuspension, the cultures were split and 100 μM H_2O_2 was added to one sample. After growth for another 30 min, cells were harvested for β-Gal assay and induced expression (gray bars) was compared to expression in the absence of H_2O_2 addition (white bars). Note that the data represented by the white bars are the same as those for the 2.5-h time point in Fig. 3. Experiments were performed twice; error bars represent the standard error of the mean.

activated by Fe(II), Zur is activated by Zn(II), and PerR is activated by several different metal ions, including Mn(II) and Fe(II).

PerR negatively regulates expression of the peroxide stress stimulon (17). Genes shown to be under direct PerR control include *katA* (major vegetative catalase), *ahpCF* (alkyl hydroperoxide reductase), *mrgA* (Dps-like DNA-binding protein), *hemAXCDBL* (heme biosynthesis operon), and *zosA* (zinc uptake system). Here, we extend the PerR regulon to include both *perR* itself and *fur* (Fig. 1 and 2). Autoregulation of PerR has also been reported in *Staphylococcus aureus* (19) and in the homolog CatR from *Streptomyces coelicolor* (14). Unexpectedly, repression of *perR* and *fur* was elicited by addition of Mn(II), but not Fe(III), to the growth medium (Fig. 3). This is consistent with our suggestion that PerR forms different metallated species in the cell that differ in target selectivity (18).

We have systematically compared the metal selectivities of transcriptional repression of all of the PerR regulon components (Fig. 3 and 4). While all of the components of the

regulon are repressed by Mn(II), the transcriptional responses to iron vary from repression (*mrgA* and *katA*) to a slight induction (*fur*). Both the iron repression and the manganese repression of *mrgA* and *katA* require PerR and are independent of Fur (Fig. 4). This contrasts with the dual regulation of the *E. coli mntH* gene, which is repressed by Mn(II) through the MntR protein and by Fe(II) through Fur (26). Mutation of the *fur* gene also affects the metalloregulation of PerR regulon components. In the *fur* mutant strain, repression elicited by iron is enhanced and the Mn(II)-dependent repression of *fur* is greatly decreased (Fig. 4). Since Fur does not bind to the *fur* regulatory region, we suspect that derepression is an indirect effect of the *fur* mutation. Indeed, the observed derepression can be at least partially overcome with high levels of Mn(II) supplementation (Fig. 5). These findings are consistent with the observations that *fur* mutant cells accumulate elevated levels of intracellular iron (21; E. Guedon and J. D. Helmann, unpublished data) and that this may alter the distribution of PerR among its various metallated forms. For example, elevated intracellular Fe(II) may supplant Mn(II) in PerR, in effect causing derepression of *fur*.

The finding that *fur* is a direct target for PerR repression is reminiscent of the finding that *E. coli fur* is under the control of the peroxide-sensing transcription factor OxyR, as well as the superoxide response system SoxRS (34). However, unlike the situation in *E. coli*, transcription of *B. subtilis fur* is not peroxide inducible. Similar regulation has been observed in *S. aureus*: PerR represses *fur*, yet *fur* is not inducible by H_2O_2 (19). Interestingly, PerR repression in *S. aureus* is also elicited by Mn(II) but not by iron. The physiological relevance of regulating *fur* expression in response to intracellular Mn(II) levels is not clear.

The molecular mechanism by which PerR senses H_2O_2 is not clear, but we have proposed that it likely involves disulfide bond formation between two Cys residues postulated to serve as ligands for the regulatory metal ion (18). This model is supported by the observation that growth of cells with Mn(II) reduces the H_2O_2 induction of target genes, whereas growth with Fe(III) increases induction (Fig. 6). It is envisioned that these changes in medium composition affect the identity of the regulatory metal ion bound to PerR and thereby affect redox activity. Indeed, biochemical studies demonstrate that binding of PerR to its target operator regions in vitro is sensitive to H_2O_2 but can be restored by thiol-reducing agents such as DTT (18). Further, the identity of the regulatory metal ion cofactor influences the sensitivity of PerR to oxidants: the iron-containing form is quite sensitive to H_2O_2, while addition of Mn(II) reduces this sensitivity (18). Thus, we favor a model in which one or more of the redox-active cysteine residues also serves as ligand to the regulatory metal ion. An alternative model postulates that peroxide sensing involves disulfide bond formation between Cys residues that normally serve as ligands to the Zn(II) ion (14). This mechanism, analogous to the regulatory mechanism controlling Hsp33 activity (12, 20), was suggested for the *S. coelicolor* PerR ortholog CatR (14).

While it is tempting to speculate that the identity of the metal ion cofactor determines the relative sensitivity of different PerR species to H_2O_2, the correlation between metal specificity of repression and peroxide inducibility is imperfect. This model is supported by the observations that repression of both *mrgA* and *katA* by PerR can be elicited by iron and that both genes are strongly induced by H_2O_2. In contrast, repression of both *fur* and *perR* appears to be selective for Mn(II) and neither gene can be strongly induced by H_2O_2. However, the *zosA* gene is also selectively repressed by Mn(II) (although iron can serve as a repressor at least in a *fur* mutant strain) but this gene is strongly induced by H_2O_2 (Fig. 6 and data not shown).

The results reported here lead to two important conclusions about the PerR regulon. First, we demonstrated that not all components of the PerR regulon are inducible by peroxide. While the inability to induce *fur* with H_2O_2 was initially interpreted as resulting from the Mn(II) selectivity of gene repression, other factors may also be at play. Second, we demonstrated that the metalloregulation of different PerR regulon genes is distinct: some can be repressed by either manganese or iron, while others are manganese specific. It is interesting that the metal selectivity of PerR also varies between species: in *S. aureus*, PerR is selective for Mn(II) (19), while the *Campylobacter jejuni* ortholog responds to iron (32). In ongoing biochemical studies, we are attempting to generate different metallated forms of PerR for a direct comparison of DNA target selectivity and peroxide reactivity in vitro.

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