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Mutation of the *Bacillus subtilis* Alkyl Hydroperoxide Reductase (*ahpCF*) Operon Reveals Compensatory Interactions among Hydrogen Peroxide Stress Genes

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In *Bacillus subtilis*, hydrogen peroxide induces the synthesis of catalase (KatA), alkyl hydroperoxide reductase (AhpCF), and a DNA-binding protein of the Dps family (MrgA). KatA, AhpCF, heme biosynthesis enzymes, and MrgA are also induced upon entry into stationary phase under conditions of iron and manganese limitation. In an effort to define the peroxide regulon repressor, PerR, we used mini-Tn*10* mutagenesis to identify loci affecting the regulation of *mrgA*. From this screen, we isolated two mini-Tn*10* insertions in *ahpC*, the gene encoding the small subunit of AhpCF, that increase the transcription of *mrgA-lacZ* even in iron-supplemented minimal medium. Indeed, these *ahpC*::Tn*10* insertions lead to elevated expression from all peroxide regulon promoters, including those for *mrgA*, *katA*, *hemAXCDBL*, and *ahpCF*. As a result, the *ahpC*::Tn*10* mutants display an increased resistance to H_2O_2. The *ahpCF* promoter region contains three sequences similar to the peroxide regulon consensus operator (per box). We demonstrate that the ability of *ahpC*::Tn*10* mutations to derepress *mrgA* requires aerobic growth. In contrast, a second distinct *trans*-acting regulatory mutation bypasses this requirement for aerobic growth. Since the peroxide regulon is activated in the absence of AhpCF, which degrades alkyl hydroperoxides, we propose that organic hydroperoxides may be physiologically relevant inducers in vivo.

We have investigated the effects of starvation for essential metal ions on gene expression in *Bacillus subtilis*. In earlier studies, we identified two genes with distinct patterns of regulation, *mrgC* and *mrgA* (11). The *mrgC* gene is induced by starvation for iron, and this regulation requires a *cis*-acting sequence resembling the binding site for the ferric uptake regulator (Fur) protein of *Escherichia coli* (fur box). Indeed, similar fur box elements in *B. subtilis* are important for the iron-mediated repression of iron uptake and siderophore biosynthesis genes (32, 35). In all studied cases, the expression of these iron-regulated genes can be repressed by iron but not by manganese or other transition metals.

In contrast to *mrgC*, *mrgA* is induced in stationary-phase cells, but this induction is prevented by manganese, iron, copper, or cobalt, with manganese being the most potent effector (11). The *mrgA* gene encodes a homolog of the *E. coli* Dps protein, an abundant DNA-binding protein produced in stationary phase which protects cells against H_2O_2 (1, 10). Like Dps, synthesis of MrgA is induced either by H_2O_2 or by entry into stationary phase (3, 10). The peroxide stimulon also includes KatA (major vegetative catalase), AhpCF (alkyl hydroperoxide reductase), and the heme biosynthesis enzymes (encoded by *hemAXCDBL*) (7, 12, 16, 24).

To define the mechanisms by which metal ions affect the transcription of *mrgA*, we have previously isolated *cis*- and *trans*-acting mutations which allow high-level expression of *mrgA* in the presence of Mn(II) (12). The *cis*-acting mutations revealed that metalloregulation and peroxide induction both require an inverted-repeat element located just upstream of the *mrgA* promoter. Sequence comparisons identified similar inverted repeats overlapping the promoters for *katA* and *hem AXCDBL*. These operators are designated per boxes to denote their role in controlling genes inducible by H_2O_2 (12).

Two classes of spontaneous *trans*-acting mutations were also found (12). Class I mutants are H_2O_2 resistant and constitutively transcribe the genes of the peroxide regulon, while class II mutants lack catalase activity and are H_2O_2 sensitive. We hypothesize that the class I mutants are altered in the level or activity of the putative per box binding protein, PerR, while the mutations of the class II mutants are in *katA*. Since deletions within the per box sequence result in constitutive expression of *mrgA*, we postulate that PerR is a repressor (12).

We now report the analysis of a third and distinct *trans*-acting factor affecting the regulation of *mrgA*. Analysis of mini-Tn*10* insertions which allow *mrgA* expression in rich medium led to the cloning and sequencing of the *B. subtilis ahpCF* operon. Indeed, the *ahpC*::Tn*10* insertion leads to elevated expression from *perR*-regulated promoters, including *katA*, *mrgA*, and *ahpC* itself. Cells lacking both AhpCF and KatA are severely impaired in aerobic growth, especially in minimal media, suggesting that these enzymes play complementary roles in protection against oxidative stress. Like the other members of the *perR* regulon, the *ahpCF* promoter contains sequences resembling the per box consensus, and transcription is derepressed by a class I regulatory mutation and is regulated by both growth phase and metal ions. Derepression of *mrgA-lacZ* by the class I regulatory mutation is observed in both aerobically and microaerobically grown cells, consistent with the hypothesis that this mutation affects the activity of PerR. In contrast, derepression of *mrgA-lacZ* by *ahpC*::Tn*10* is only observed in aerobically grown cells. This suggests that the inducer of the peroxide regulon is a by-product of oxidative metabolism, possibly an organic hydroperoxide which can be removed by AhpCF.

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TABLE 1. Bacterial strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Characteristics	Source or reference
B. subtilis strains		
CU1065	W168 *attSP*β *trpC2*	46
MA991	Spontaneous H_2O_2-resistant YB886 derivative	18
ZB307A	W168 SPβ*c2*Δ2::Tn*917*::pSK10Δ6 (MLS^r)	52
YB2003	YB886 *katA*::Tn*917-lacZ*::pTV21Δ2 (Cm^r)	6
HB1000	ZB307A *attSP*β	11
HB1022	CU1065 *mrgA*::Tn*917-lacZ* (MLS^r)	11
HB1041	CU1065 *hemA*::pLC400 (Cm^r)	12
HB1122	ZB307A SPβ*c2*Δ2::Tn*917*::φ(*mrgA′-cat-lacZ*) (MLS^r Neo^r)	12
HB1202	Spontaneous *trans*-acting mutant derivative of HB1122(MLS^r Neo^r Cm^r)	12
HB1208	Spontaneous *trans*-acting mutant derivative of HB1122(MLS^r Neo^r Cm^r)	12
HB1302	HB1202 cured of SPβ	12
HB1308	HB1208 cured of SPβ	12
HB1408	HB1308 *mrgA*::Tn*917-lacZ* (MLS^r)	12
HB1603[a]	HB1022 *ahpC*::Tn*10* (MLS^r Spc^r)	This work
HB1613[a]	HB1022 *ahpC*::Tn*10* (MLS^r Spc^r)	This work
HB1620	ZB307A SPβ*c2*Δ2::Tn*917*::φ(*ahpC′-cat-lacZ*) (MLS^r Neo^r)	This work
HB6507	HB1000 *ahpC-1613* (Spc^r)	This work
HB6514	HB6507 SPβ1620 (MLS^r Neo^r Spc^r)	This work
HB6530	HB1000 *mrgA*::Tn*917-lacZ* (MLS^r)	This work
HB6532	HB1000 *katA*::Tn*917-lacZ*::pTV21Δ2 (Cm^r)	This work
HB6534	HB1302 *katA*::Tn*917-lacZ*::pTV21Δ2 (Cm^r)	This work
HB6538	MA991 SPβ1122 (MLS^r Neo^r)	This work
HB6540	HB1302 SPβ1122 (MLS^r Neo^r)	This work
HB6544	HB6507 SPβ1122 (MLS^r Neo^r Spc^r)	This work
HB6545	HB1302 *ahpC-1613* (Spc^r)	This work
HB6552	HB6534 *ahpC-1613* (Cm^r Spc^r)	This work
HB6562	HB1000 *hemA*::pLC400 (Cm^r)	This work
HB6564	HB6507 *katA*::Tn*917-lacZ*::pTV21Δ2 (Cm^r Spc^r)	This work
HB6567	HB1308 *ahpC-1613* (Spc^r)	This work
HB6575	HB6545 SPβ1620 (MLS^r Neo^r Spc^r)	This work
HB6576	HB6545 SPβ1122 (MLS^r Neo^r Spc^r)	This work
HB6590	HB1302 SPβ1620 (MLS^r Neo^r)	This work
HB6596	HB6507 *hemA*::pLC400 (Cm^r Spc^r)	This work
HB6599	HB6567 *mrgA*::Tn*917-lacZ* (MLS^r Spc^r)	This work
HB6601	HB6507 *mrgA*::Tn*917-lacZ* (MLS^r Spc^r)	This work
E. coli strains		
JM101	*supE thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15*)	33
JM2r$^-$	*mcrA*$^-$*B*$^-$ *hsdR*$^-$ *M*$^+$ *recA1* Δ(*lac-proAB*) *thi gyrA96 relA1 srl*::Tn*10* F′(*proAB lacZ* Δ*M15*)	S. Zahler
Phages		
SPβ1122	SPβ*c2*Δ2::Tn*917*::φ(*mrgA′-cat-lacZ*)	12
SPβ1620	SPβ*c2*Δ2::Tn*917*::φ(*ahpC′-cat-lacZ*)	This work
Plasmids		
pBluescript SK(+)	pBR322 replicon (Ap^r)	Stratagene
pGEM3Zf(+)cat-1	pBR322 replicon (Ap^r Cm^r)	50
pHEMA2	*B. subtilis* replicon (Cm^r Em^r)	34
pIC333	pUC replicon, shuttle vector, thermosensitive replicon for gram-positive hosts (MLS^r Spc^r)	40
pJPM122	pBR322 replicon (Ap^r Neo^r)	36
pTKlac	pBR322 replicon (Ap^r Cm^r)	23
pLC400	pTKlac with a 834-bp *Hin*dIII fragment from pHEMA containing the *hemA* promoter	12
pLC1603-RI	Plasmid rescue from *Eco*RI-digested HB1603 DNA (Ap^r)	This work
pLC1603-H3	Plasmid rescue from *Hin*dIII-digested HB1603 DNA (Ap^r)	This work
pLC1613-RI	Plasmid rescue from *Eco*RI-digested HB1613 DNA (Ap^r)	This work
pLC1613-H3	Plasmid rescue from *Hin*dIII-digested HB1613 DNA (Ap^r)	This work
pLC1620	pJPM122 with *Hin*dIII-*Bgl*II fragment from pLC1613-H3	This work
pLC1630	pBluescript SK(+) with *Sac*II-*Hin*dIII fragment from pLC1613-H3	This work

[a] Strains HB1603 and HB1613 differ only in the site of mini-Tn*10* insertion into the *ahpC* gene. The *ahpC* gene carrying the Tn*10* insertion in the 22nd codon is referred to as *ahpC-1603*, while that carrying the Tn*10* insertion in the 68th codon is referred to as *ahpC-1613.*

MATERIALS AND METHODS

Bacterial strains and plasmids. All *B. subtilis* strains, plasmids, and phages used in this study are listed in Table 1. Plasmids pLC1603-RI, pLC1603-H3, pLC1613-RI, and pLC1613-H3 were recovered into *E. coli* from HB1603 and HB1613 by vector rescue experiments. Plasmid pLC1630 was made by cloning a 632-bp *Sac*II-*Hin*dIII fragment from pLC1613-H3 into pBluescript SK(+). An operon fusion linking the *ahpCF* promoter (4) to the *cat-lacZ* genes was made by cloning a 1,485-bp *Hin*dIII-*Bgl*II fragment from pLC1613-H3 into pJPM122 (36) cut with *Hin*dIII and *Bam*HI. This fragment extends from within *gntZ* (17) to the 53rd codon of *ahpC*. All phages used in this study were SPβ derivatives. The parental SPβ prophage (SPβ*c2*Δ2::Tn*917*::pSK10Δ6) residing in ZB307A carries the *c2* mutation, which allows heat induction of the prophage (52). SPβ trans-

ducing lysates were prepared by heat induction from strains HB1122 and HB1620 and were designated SPβ1122 and SPβ1620, respectively. SPβ1122 carries the wild-type *mrgA′-cat-lacZ* operon fusion (12), while SPβ1620 carries the wild-type *ahpC′-cat-lacZ* operon fusion.

Media and growth conditions. Manganese- and iron-limited minimal media (11) contained 40 mM potassium morpholinopropanesulfonic 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 · $2H_2O$ (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 20 nM $MnCl_2$. Manganese- or iron-replete media were made by adding $MnCl_2$ or $FeCl_3$ to 5 μM (final concentration) each. Iron was added from a stock of 10 mM $FeCl_3$ dissolved in 100 mM HCl. Glucose was included to inhibit sporulation during stationary phase. Minimal media were prepared with high-purity (Milli-Q) water to control the level of adventitious iron contamination. Minimal media and Luria-Bertani (LB) medium were supplemented with KNO_3 (0.2%) for microaerobic, anaerobic, and corresponding aerobic growth experiments. Aerobically growing cultures were incubated at 37°C with shaking, while microaerobic incubations were all performed at 37°C in maximally filled, tightly sealed tubes without shaking. For cell viability measurements, cultures were grown overnight in LB with KNO_3 (0.2%) aerobically or microaerobically. CFUs for aerobic or microaerobic cultures were determined on LB plates with KNO_3 (0.2%) aerobically or using anaerobic jars, respectively. Plates contained 1.5% Bacto Agar (Difco, Detroit, Mich.) and 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; United States Biochemical Co., Cleveland, Ohio) per ml. Ampicillin (200 μg/ml) or spectinomycin (200 μg/ml) was used for the selection of *E. coli* strains. Erythromycin (1 μg/ml) and lincomycin (25 μg/ml) (for testing macrolide-lincosamide-streptogramin B [MLS] resistance), spectinomycin (100 μg/ml), chloramphenicol (5 μg/ml), or neomycin (10 μg/ml) was used for the selection of various *B. subtilis* strains.

DNA manipulations and sequencing. Isolation of *B. subtilis* chromosomal DNA, transformation, and specialized SPβ transductions were done by standard procedures (15). *E. coli* plasmid DNA was isolated by the alkaline lysis method (33) or by using the SpinBind Mini-Prep System (FMC BioProducts, Rockland, Maine). Restriction endonucleases and DNA ligase were used according to the manufacturer's instructions (New England Biolabs, Inc., Beverly, Mass.). Plasmid rescue experiments were performed as previously described (11, 20). 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.

Construction of a chromosomal mini-Tn*10* library. We constructed a *B. subtilis* chromosomal DNA library containing random mini-Tn*10* insertions by using pIC333 (40), a delivery vector for mini-Tn*10*. This plasmid contains a ColE1 origin and a thermosensitive origin of replication for gram-positive hosts (inactive at temperatures higher than 35°C). Plasmid pIC333 was transformed into CU1065 (Table 1), and transformants were selected for MLS resistance on LB plates at 28°C. A less than 2-day-old transformant was used to inoculate 2× YT (33) containing spectinomycin (Spc); the culture was grown overnight at 28 to 30°C and then diluted 1:100 in the same medium. After growth at 28 to 30°C for 3 h, the culture was then shifted to 37°C and grown for 4 h or more before diluted aliquots were plated on LB, LB with Spc, and LB with Spc and erythromycin (2 μg/ml), followed by overnight incubation at 37°C. The rest of the culture was collected by centrifugation, resuspended in 2× YT with 15% glycerol, and stored at −80°C. The transposition efficiency was estimated by the ratio of the number of colonies on LB plus Spc to that on LB and was within the general range (0.01 to 1%) of transposition efficiency reported for this system. Aliquots of the Tn*10* library were plated onto LB plus Spc plates and incubated overnight at 37°C. Approximately 10^4 transposants were collected and grown to saturation in 2× YT with Spc for chromosomal DNA isolation.

Isolation of *ahpC*::Tn*10* insertion mutants derepressed for *mrgA-lacZ* expression (HB1603 and HB1613). The chromosomal DNA library containing random mini-Tn*10* insertions was used to transform HB1022 to Spc resistance on LB plates containing X-Gal. The majority of the transformants were light blue after 1 to 2 days of incubation at 37°C, similar to the parent strain, HB1022. However, some transformants exhibited enhanced β-galactosidase (β-gal) activity. This phenotype was linked to the Spc^r marker by transformation. DNA was subsequently recovered by plasmid rescue from several of the derepressed strains. Restriction analysis indicated that some of the clones were identical and presumably were derived from sibling colonies. Two strains (HB1603 and HB1613) with distinct insertions were chosen for more detailed characterization.

Cloning and sequencing of the *ahpCF* operon. Plasmids pLC1603-RI, pLC1603-H3, pLC1613-RI, and pLC1613-H3 (Table 1) contain the mini-Tn*10* element with a ColE1 origin, the ampicillin resistance gene, and flanking *B. subtilis* chromosomal DNA. To sequence the DNA upstream and downstream of the transposon, two synthetic primers corresponding to the left and right ends of mini-Tn*10*, 5′-GCCGATTCATTAATGCAG-3′ and 5′-CCCACTTATAAACAAAAG-3′, respectively, were used. The sequences of both strands in pLC1630 were also determined to obtain the sequence distal to the end of mini-Tn*10* in pLC1613-H3.

β-gal assays. For the determination of the β-gal activities of the various transcriptional fusions, cells were transferred at a 1/100 dilution from an overnight culture grown in minimal medium with Mn(II) (5 μM) into fresh minimal medium limiting for either iron, manganese, neither metal, or both metals. Samples were then collected from overnight or 48-h (for the microaerobic experiments) cultures and assayed for β-gal levels by the method of Miller as previously described (11, 26). All assays were performed on duplicate samples, and the values were then averaged.

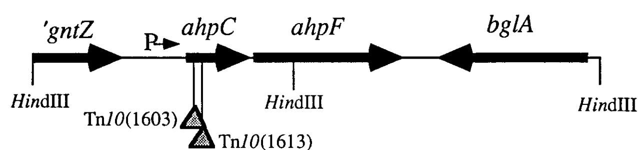

FIG. 1. Genetic organization of the chromosomal region containing the *ahpCF* operon. The *ahpCF* operon is downstream of the *gnt* operon (17), and transcription is convergent with the β-glucosidase gene, *bglA* (51). The DNA region sequenced in this study extends from the *Hin*dIII site in *gntZ* to the *Hin*dIII site in *ahpF*. P indicates the *ahpCF* promoter.

Computer analysis. Sequence comparisons and database searches were performed by using BLAST (2).

Nucleotide sequence accession number. The DNA sequence of *ahpCF* has been deposited in the GenBank and EMBL databases under accession number D78193 (22).

RESULTS

Isolation of mini-Tn*10* insertions which derepress *mrgA-lacZ* expression. HB1022 (*mrgA-lacZ*) was transformed with a *B. subtilis* chromosomal DNA library containing random mini-Tn*10* insertions, and colonies were screened for enhanced expression of β-gal under repressing conditions (LB plates) (11). From approximately 8,000 colonies, several transformants with smaller colony size and elevated β-gal expression were identified. HB1603 and HB1613 were further characterized, and both exhibited full derepression of *mrgA-lacZ* in iron-replete minimal medium (see below). This phenotype was found to be 100% linked to the transposon insertion.

Sequence analysis of the *ahpCF* operon. To identify the gene(s) into which the mini-Tn*10* had inserted, DNA upstream and downstream of the transposons was isolated by plasmid rescue. DNA sequencing revealed that the mini-Tn*10* had inserted into the same gene (*ahpC*) in both HB1603 and HB1613, after the 22nd and 68th codons, respectively (Fig. 1). The recovered *Hin*dIII fragment overlaps the end of the gluconate operon (*gntRKPZ* [17]) and contains the complete *ahpC* gene and the 5′ end (the first 136 codons) of *ahpF*. The predicted AhpC protein contains 187 amino acids and matches exactly the N-terminal sequence of a 23-kDa H_2O_2-inducible protein identified previously and postulated to be AhpC (18). The first 21 residues of the predicted AhpF protein are identical to the experimentally determined N-terminal sequence of a 54-kDa H_2O_2-inducible protein proposed to be AhpF (18). The complete sequence of *ahpF* was inferred by combining the sequence of this *Hin*dIII fragment with that of a *Hin*dIII fragment sequenced by Zhang and Aronson (51) and was later verified when the complete sequence of this region of the chromosome was determined as part of the *B. subtilis* genome project (22) and by Antelmann et al. (4). The *B. subtilis* AhpC and AhpF proteins are highly similar to related proteins from other organisms; both are over 50% identical to the corresponding homologs from *Salmonella typhimurium* (9, 44) and have substantially higher identity to homologs from other gram-positive organisms, including *Amphibacillus xylanus* (27), *Bacillus alcalophilus* (48), and *Staphylococcus aureus* (5).

Regulation of *ahpC* expression. A possible σ^A-dependent promoter element (19) was identified upstream of *ahpCF*. Primer extension analysis by Antelmann et al. (4) mapped the 5′ end of the *ahpCF* transcript to an adenine 64 nucleotides upstream of the *ahpC* translational start site. Since AhpC and

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ACGAATTGGTCTGAATAACCTGTATTAAAAACACGGTCAGTTTCAACTGAACCGTGTTTTTTTCTTCTATCCCAAACAAC 80
T  N  W  S  E  * (gntZ)      ----------->  <-----------  gnt terminator

AGAAGACATAAGTTATGTTTTAAATATCATAAACAGTGATACAATAATAGCTAGAAACAATCCCCAAGATCATCATGTGA 160

CAGCAGATTGCTGTTCCGTTCTATTCTGCTTTTGGCCATTGTCCTTTGCCCTTGACCATGGTGTCATAAAATGTTCAAAT 240

GCCAAAACGGCCTGGCATTCTGATTTACAAAAGTCCCTGAAAACCTTATCTAATTGAGAATCAGCCTCTCATTTATTATA 320

                                                                -35
AAAGAAATGCCTAAATCCTACCTGTCACACCTTTATTAAGATGAAAAAAAGTAGGTTATGGCTTGACAAAAAATATATAT 400
                                                                          [=====
      -10                                                          r.b.s.
TAATTAATAATTCATATATAATTAGAATTATTATTGAAAGCGATTATGCTTTCTAATACATTTTTAGGAGGAATATACAT 480
=0======]            [======0======]
    [======0======]

 ahpC
TATGTCTTTAATCGGTAAAGAAGTACTTCCATTCGAAGCAAAAGCATTCAAAAACGGTGAATTCATCGATGTAACAAACG 560
 M  S  L  I  G  K  E  V  L  P  F  E  A  K  A  F  K  N  G  E  F  I  D  V  T  N  E
```

FIG. 2. Sequence of the *gnt-ahp* intergenic region. The promoter, transcription start site (4), and ribosome-binding site (r.b.s.) of *ahpCF* are underlined. Three putative per box sequences (12) are indicated in brackets. Only the most highly conserved 14 bp of the per box dyad are indicated. The asterisk indicates the *gntZ* stop condon.

AhpF were previously identified as major H_2O_2-inducible proteins (18), we inspected the *gnt-ahp* intergenic region for possible per box sequences (Fig. 2). Three regions with similarity to the per box (12) were identified overlapping the *ahpCF* promoter.

To study the regulation of *ahpCF* transcription, we constructed an *ahpC′-cat-lacZ* operon fusion in SPβ (HB1620) (Table 1). We then examined the expression of *ahpC′-cat-lacZ* as a function of growth phase in minimal medium lacking both Fe(III) and Mn(II). This fusion is expressed at a moderate level in early-logarithmic-phase cells (ca. 70 Miller units) and induced fivefold upon entry into stationary phase (Fig. 3A). When HB1620 was grown overnight in minimal medium, expression of the *ahpC′-cat-lacZ* fusion was decreased by the presence of Mn(II) and, to a lesser extent, Fe(III) (Fig. 3B).

Effect of *ahpC*::Tn*10* insertions on expression of the peroxide regulon. We next tested the effects of one of the *ahpC*::Tn*10* insertions (*ahpC-1613*) on the expression of PerR regulon genes in defined minimal media (Table 2). For these studies, we used *lacZ* transcriptional fusions to *mrgA*, *katA*, and *hemA* at their respective genomic loci. We also used SPβ-borne *cat-lacZ* operon fusions to *ahpC* and *mrgA*. All fusions were introduced by transformation or transduction into HB1000 and HB6507. Since Mn(II) or Fe(III) can repress many PerR regulon genes, we assayed gene expression after overnight growth in defined minimal medium with limiting Fe(III) and Mn(II) [no added Fe(III), 20 nM Mn(II)], 5 μM Mn(II), 5 μM Fe(III), or both Mn(II) and Fe(III) at 5 μM each.

As previously shown (11), *mrgA* (HB1122) is repressed in stationary phase by Fe(III) and more strongly by Mn(II) (Table 2). In the *ahpC-1613* background (HB6544), expression of *mrgA* in the unsupplemented minimal medium was elevated about twofold and Fe(III)-mediated repression was lost. In contrast, *ahpC-1613* did not derepress *mrgA* in cells grown in the presence of Mn(II) or both Mn(II) and Fe(III) (Table 2). Both the *mrgA′-cat-lacZ* fusion (in SPβ) and the *mrgA*::Tn*917-lacZ* fusion behaved similarly in these studies (data are shown for only *mrgA′-cat-lacZ*), indicating that the MrgA$^-$ phenotype in the latter strain did not affect *mrgA* regulation under the conditions tested.

We then examined the effects of *ahpC-1613* on catalase expression. In HB6532 (which is phenotypically KatA$^-$), *katA-lacZ* expression is high during post-exponential phase in the absence of Fe(III) or Mn(II) supplementation. Like *mrgA*, *katA* is metalloregulated: expression can be reduced nearly threefold by 5 μM Fe(III) or 80-fold by 5 μM Mn(II) (Table 2). The *ahpC-1613* mutation in HB6564 eliminates repression by either Fe(III) or Mn(II), although a modest effect is still

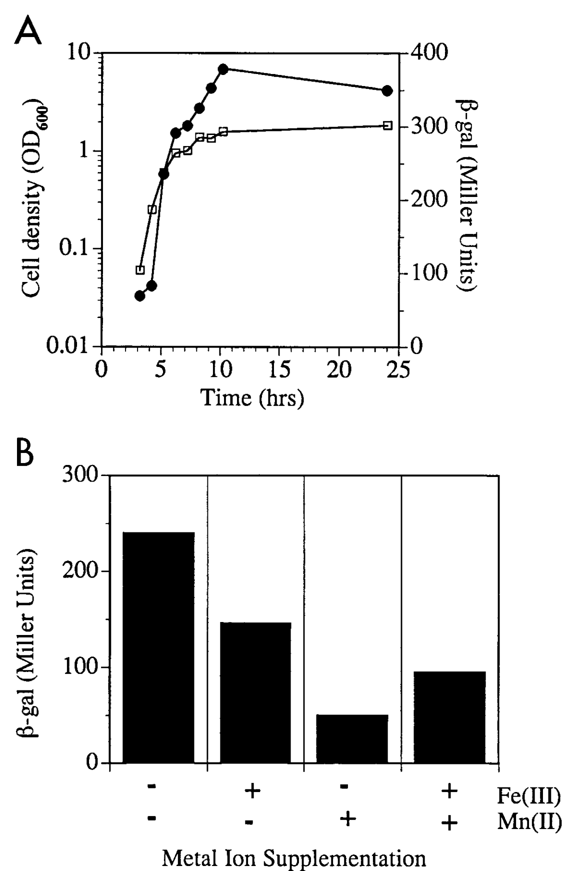

FIG. 3. Expression of *ahpC′-cat-lacZ* as a function of growth phase and medium metal ion composition. (A) HB1620 was grown in minimal medium limiting for Fe(III) and Mn(II), and the culture density (optical density at 600 nm) (□) and β-gal level (in Miller units) (●) were determined as functions of time. (B) The level of β-gal expression was determined after overnight growth in minimal medium with no added Fe(III) and low (20 nM) Mn(II) or supplemented (5 μM) with either or both metals as indicated.

TABLE 2. Effects of distinct *trans*-acting mutations on expression of *mrgA*, *katA*, and *ahpC* transcriptional fusions

Strain	*lacZ* fusion	Relevant phenotype	β-gal activity (Miller units) in MM supplemented with[a]:			
			No additions	Fe	Mn	Fe+Mn
HB1122	*mrgA* (SPβ)		188	18	2	2
HB6544	*mrgA* (SPβ)	AhpC⁻	299	325	5	8
HB6540	*mrgA* (SPβ)	1302[b]	805	860	906	1,374
HB6576	*mrgA* (SPβ)	1302 AhpC⁻	714	454	8	17
HB6532	*katA*	KatA⁻	488	169	6	3
HB6564	*katA*	KatA⁻ AhpC⁻	518	478	453	167
HB6534	*katA*	KatA⁻ 1302	617	144	5	6
HB6552	*katA*	KatA⁻ 1302 AhpC⁻	1,150	1,220	316	72
HB1620	*ahpC* (SPβ)		468	266	58	144
HB6514	*ahpC* (SPβ)	AhpC⁻	442	512	210	434
HB6590	*ahpC* (SPβ)	1302	709	798	1,418	4,744
HB6575	*ahpC* (SPβ)	1302 AhpC⁻	480	547	264	499

[a] Assays were conducted in duplicate after overnight growth in the indicated minimal medium (MM).
[b] 1302 represents the *trans*-acting mutation in strain HB1302, which is a cured derivative of the originally isolated mutant strain (HB1202).

observed when both metal ions are present (Table 2). As a consequence, the *ahpC-1613* mutation markedly increases resistance to H_2O_2 as judged by zone of inhibition assays on LB plates (4) (data not shown).

Expression of *hemA-lacZ* is also temporally regulated, and post-exponential-phase levels of β-gal are moderately reduced by Mn(II) or Fe(III) (12) (data not shown). The *ahpC-1613* mutation had little effect on the Mn(II)-mediated repression but led to generally elevated expression of *hemA-lacZ* in the other metal ion-supplemented media.

The *ahpC'-cat-lacZ* fusion is also affected by *ahpC-1613*. In HB6514, repression of *ahpC'-cat-lacZ* by Mn(II) is reduced fourfold while Fe(III) repression is lost (Table 2). Moreover, expression is elevated during logarithmic growth (data not shown).

Effects of a *trans*-acting mutation (HB1302) on the expression of *mrgA*, *katA*, and *ahpC* in the absence and presence of AhpC. We next tested the effects of a class I (putative PerR) spontaneous regulatory mutation in HB1302 (12) on the expression of *mrgA*, *katA*, and *ahpC* (Table 2). As reported previously (12), expression of *mrgA* is increased severalfold in HB6540 and neither Mn(II) nor Fe(III) causes repression. Similar effects are seen here for *ahpC'-cat-lacZ* in HB6590. In the case of supplementation with both Fe(III) and Mn(II), we observed enhanced expression. In contrast, the mutation in HB1302 does not seem to have a dramatic effect on *katA* expression (HB6534).

Surprising epistatic interactions are observed between the mutation in HB1302 and *ahpC-1613*. As noted above, the *ahpC-1613* mutation overcomes the repressive effect of Fe(III) on *mrgA'-cat-lacZ* (HB6544), and this effect is also observed in HB6576 (Table 2). Unexpectedly, the additional presence of the *ahpC-1613* mutation in HB6576 restores Mn(II) repression of *mrgA'-cat-lacZ*. The general derepression of *ahpC'-cat-lacZ* in HB6590 is reduced by *ahpC-1613* in HB6575 (Table 2). In contrast, *katA-lacZ* is still repressed by Mn(II) in HB6534. Moreover, Mn(II) repression is greatly decreased by the presence of *ahpC-1613* in HB6552. In summary, these data indicate that the mutation in HB1302 increases expression of *mrgA* and *ahpC* in all tested media but that this effect is reduced or eliminated in the presence of *ahpC-1613*.

Post-exponential-phase induction of *mrgA* is abolished in microaerobically grown cells. To determine if the post-exponential-phase induction of the peroxide regulon genes requires oxygen, we compared levels of β-gal expression from the *ahpC* and *mrgA* promoters in cells grown aerobically or microaerobically in the presence of nitrate as the terminal electron acceptor (Fig. 4). The normal post-exponential-phase induction of *mrgA* (HB1122) was completely eliminated in the mi-

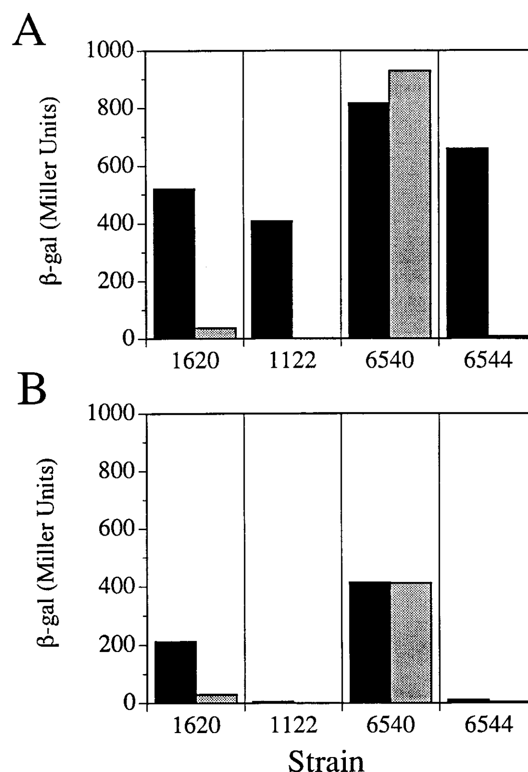

FIG. 4. Expression of *ahpC'-cat-lacZ* and *mrgA'-cat-lacZ* aerobically (A) and microaerobically (B). Strains were grown overnight in minimal medium with no added Fe(III) and low Mn(II) (dark bar) or with no added Fe(III) and 5 μM Mn(II) (stippled bar). The Mn(II) effect noted for both *ahpC'-cat-lacZ* (HB1620) and *mrgA'-cat-lacZ* (HB1122) reflects a lack of induction upon entry into stationary phase (Fig. 3) (11). When grown microaerobically, induction of *mrgA'-cat-lacZ* is no longer observed, and this requirement for oxygen cannot be bypassed by the *ahpC-1613* mutation (HB6544) but can be bypassed by the *trans*-acting mutation isolated previously (12), HB6540.

croaerobically grown cells. Moreover, the presence of either a spontaneous catalase mutation (HB1208) (data not shown) or *ahpC-1613* (HB6544) failed to restore *mrgA-lacZ* expression under microaerobic growth conditions (Fig. 4B). In contrast to *mrgA*, *ahpC* is still expressed microaerobically, albeit at a lower level, and Mn(II) repression is still observed. Thus, expression of *ahpC* is not dependent on oxidative metabolism, and this operon appears to have a significant basal level of expression, consistent with the growth phase dependence experiments (Fig. 3A). Of the strains shown, only HB6540 expressed *mrgA* under microaerobic conditions. This result supports the hypothesis (see the introduction) that the *trans*-acting class I mutation in HB6540 affects PerR.

Phenotypes of *katA ahpC* and *katA ahpC mrgA* mutants. In the course of the above-described experiments, we discovered striking growth phenotypes for the various double and triple mutants of *ahpC*, *mrgA*, and *katA*. For example, a *katA ahpC* double mutant (HB6567 and its derivatives) exhibits slow growth in liquid media and lyses on LB plates. These defects are aggravated by the *mrgA* mutation in the triple mutant, HB6599. This strain could not grow aerobically (and grew only poorly microaerobically) in our defined minimal medium. Cell viability measurements with rich media (data not shown) confirm that *katA ahpC* (HB6567) and *katA ahpC mrgA* (HB6599) mutants are most compromised in aerobic growth, but these strains grow as well as the wild type microaerobically. Further investigation of these growth defects is under way.

DISCUSSION

The ability to effectively manage reactive oxygen species is crucial to survival in an aerobic environment. Alkyl hydroperoxide reductase (AhpCF) was originally identified as an enzymatic activity responsible for the increased resistance of *Salmonella typhimurium oxyR1* mutant cells to various alkyl hydroperoxides (13, 21). Strains lacking AhpCF are much more sensitive to alkyl hydroperoxides and have an elevated rate of spontaneous mutagenesis (41). AhpCF activity requires two separable protein components, a 52-kDa flavoprotein disulfide oxidoreductase (AhpF) and a 22-kDa substrate-binding protein (AhpC), which together catalyze the reduction of hydroperoxides to their corresponding alcohols (21, 28). AhpF is a member of the thioredoxin reductase family of proteins and functions to reduce redox-active disulfides within AhpC (29, 30). AhpC is related to a family of eukaryotic antioxidant proteins now known to catalytically reduce peroxides in the presence of thioredoxin, thioredoxin reductase, and NADPH and christened as peroxiredoxin (9).

Expression of the *B. subtilis ahpCF* operon. MrgA, AhpC, AhpF, and KatA are overproduced in a spontaneous H_2O_2-resistant strain, MA991 (10, 18). In an effort to isolate regulatory factors controlling the expression of *mrgA* in *B. subtilis*, we screened for mini-Tn*10* insertions which led to elevated expression of *mrgA* under repressing conditions (rich medium). Unexpectedly, two such insertions were in *ahpC.*

We have used an *ahpC′-cat-lacZ* operon fusion to demonstrate that the *ahpCF* promoter is regulated, like *mrgA* and *katA* (7, 11, 12), by metal ions as well as by growth phase. Mn(II) is more potent in preventing the post-exponential-phase induction of *ahpCF*, *mrgA*, and *katA* than is iron (11, 12). Consequently, growth in the presence of Mn(II) greatly increases the sensitivity of stationary-phase cells to H_2O_2 (12), apparently by preventing the synthesis of protective functions (AhpCF, MrgA, and KatA) that are normally induced upon entry into stationary phase. Transcription of *ahpCF* is also increased by H_2O_2 and other stress conditions (4).

The promoter region of the *ahpCF* operon (4) contains three sequence elements similar to the per box regulatory element defined genetically for *mrgA* (12). PerR, the putative peroxide regulon repressor, is postulated to bind these operators. Under conditions of oxidative stress created either by addition of H_2O_2 to growing cells or by the elevated activity of the electron transport chain accompanying the transition to post-exponential phase (37, 43), PerR may dissociate from its operators, leading to gene induction. The ability of metal ions to modulate this regulatory process is not yet understood, but we have considered two distinct models. One model proposes that PerR itself binds metal ions, thus creating a redox center that can lead to autocatalytic oxidation and hence to derepression. Mn(II) may supplant Fe(II) as a corepressor for PerR and create a protein-DNA complex that is less susceptible to induction by reactive oxygen species. Alternatively, or in addition, the metal ion effects we observe could result from catalysis of transformations between various reactive oxygen species. Both Fe(II) and Mn(II) can catalyze the dismutation of H_2O_2 under appropriate conditions, and Fe(II) in particular is widely recognized as a catalyst of Fenton-type reactions leading to the formation of reactive hydroxyl radicals (8, 25, 38, 39, 45, 49). Although both Fe(II) and Mn(II) can efficiently quench peroxyl radicals, leading to the formation of more stable hydroperoxides, iron additionally catalyzes the heterolytic cleavage of alkyl hydroperoxides to form alkoxyl radicals and hydroxide ion (8, 14). Thus, Fe(II) acts to facilitate the formation of highly reactive oxygen species while Mn(II) can act as an antioxidant and quench radical chain reactions (14, 47). Further analysis is required to determine if the ability of metal ions to affect gene expression results from these types of chemical transformations.

Complexities of peroxide regulon control. The post-exponential-phase induction of the PerR regulon is controlled by multiple factors. The *ahpC-1613* mutation leads to elevated expression of all PerR regulon genes and thereby results in an increased resistance to killing by H_2O_2 (4) (data not shown). Mutations in either *katA* (12) or *ahpCF* lead to increased expression of the PerR regulon in both exponentially growing and post-exponential-phase cells. The simplest interpretation of these data is that catalase and AhpR act to remove endogenously generated reactive oxygen species that act as inducers of the regulon.

We have demonstrated that the derepression of *mrgA* observed upon entry into stationary phase does not occur in microaerobically grown cells and that this requirement for molecular oxygen cannot be bypassed by *katA* or *ahpC* mutations. This requirement could be bypassed, however, by another regulatory mutation which may affect the activity of PerR (Fig. 4). KatA is also under complex regulation: a *spo0A* mutation abolishes the growth phase-dependent regulation of *katA* but does not prevent induction by H_2O_2 in growing cells, and this effect can be suppressed by *abrB* (7). Finally, we note that *sigB* mutants alter expression of the peroxide regulon (4) (data not shown).

Nature of the peroxide regulon inducer. The pathway leading to the induction of the peroxide regulon is not well understood. Our previous genetic studies suggest that these genes are regulated by a repressor protein, PerR (12). Since exogenously added H_2O_2 induces the expression of the peroxide regulon, either H_2O_2 or a reaction product of H_2O_2 is likely to be the in vivo inducer. A similar pathway may operate in *E. coli*: H_2O_2 converts the enteric peroxide regulator, OxyR, from an inactive to an active state in vitro, thus activating transcription of OxyR-dependent genes (42). Our finding that the *ahpC-1613* insertion mutation derepresses the peroxide regulon sug-

gests that H_2O_2 may not be the only inducer. A similar inference can be drawn from the observation that *ahpC* mutations in *E. coli* lead to high-level transcription of the *oxyR*-regulated gene *oxyS*, and transcription is further elevated in the *katG ahpCF* double mutant (31). Although AhpCF is reported to reduce H_2O_2 in vitro (28, 30), our measurements of H_2O_2 consumption in wild-type and *katA* mutant strains suggest that KatA accounts for >95% of the H_2O_2-decomposing activity in vivo (data not shown). Moreover, the *ahpC-1613* mutation leads to an increased resistance to H_2O_2, which suggests that the capacity of the cell to remove H_2O_2 is increased. Nevertheless, expression of the peroxide regulon is increased. Thus, some other inducer must be accumulating under these conditions. It is likely that this inducer is a substrate of AhpCF, and we therefore postulate that an organic hydroperoxide may mediate induction of the peroxide regulon.

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