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OhrR Is a Repressor of *ohrA*, a Key Organic Hydroperoxide Resistance Determinant in *Bacillus subtilis*

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Bacillus subtilis **displays a complex adaptive response to the presence of reactive oxygen species. To date, most proteins that protect against reactive oxygen species are members of the peroxide-inducible PerR and σ^B regulons. We investigated the function of two *B. subtilis* homologs of the *Xanthomonas campestris* organic hydroperoxide resistance (*ohr*) gene. Mutational analyses indicate that both *ohrA* and *ohrB* contribute to organic peroxide resistance in *B. subtilis*, with the OhrA protein playing the more important role in growing cells. Expression of *ohrA*, but not *ohrB*, is strongly and specifically induced by organic peroxides. Regulation of *ohrA* requires the convergently transcribed gene, *ohrR*, which encodes a member of the MarR family of transcriptional repressors. In an *ohrR* mutant, *ohrA* expression is constitutive, whereas expression of the neighboring *ohrB* gene is unaffected. Selection for mutant strains that are derepressed for *ohrA* transcription identifies a perfect inverted repeat sequence that is required for OhrR-mediated regulation and likely defines an OhrR binding site. Thus, *B. subtilis* contains at least three regulons (σ^B, PerR, and OhrR) that contribute to peroxide stress responses.**

Elevated levels of reactive oxygen species (ROS) can damage proteins, DNA, and lipids and eventually lead to cell death. These ROS include hydrogen peroxide, superoxide anion, hydroxyl radical, and organic hydroperoxides. Bacteria have numerous enzymes to detoxify ROS (36), including catalases, superoxide dismutases, alkyl hydroperoxide reductase, and related peroxidases of the AhpC/thiol-specific antioxidant (TSA) family.

In *Bacillus subtilis*, there are several well-characterized systems that defend the cell against oxidants. Oxidatively stressed cells induce the synthesis of KatA, the major vegetative catalase (5, 15). A second catalase, KatB, is induced upon starvation or as part of the σ^B-dependent general stress response (17). A third catalase, KatX, is found in endospores (4, 30). *B. subtilis* also encodes a peroxide-inducible alkyl hydroperoxide reductase, encoded by the *ahpCF* operon (1, 7). Superoxide dismutase is encoded by the *sodA* gene (22, 23), which affects resistance to superoxide generating compounds and also participates in the maturation of the spore coat (21).

Alkyl hydroperoxide reductase (AhpCF) is the best-studied enzyme that can detoxify organic hydroperoxides (24) and is the founding member of the large AhpC/TSA family of peroxidases (11). The AhpC subunit reduces peroxides to the corresponding alcohols and it, in turn, is reduced by the AhpF flavoprotein (16, 25, 31, 32). Other members of the AhpC/TSA protein family can be reduced by thioredoxin and are referred to as thioredoxin-dependent peroxidases (TPx) (9, 10, 33). While most members of the AhpC/TSA family have two active site cysteine residues that are oxidized to a disulfide during each catalytic cycle, some related proteins have a single redox active cysteine (1 Cys peroxiredoxin proteins) and are reduced by an unknown electron donor. In addition to *ahpC, B. subtilis* contains three additional genes (*ytgI*, *ygaF*, and *ykuU*) that encode members of the AhpC/TSA family, but the functions of these genes have not yet been studied. A similar set of paralogs is found in yeast, which expresses five distinct members of the AhpC/TSA protein family which vary in subcellular localization (29).

Recently, a new type of organic hydroperoxide resistance (*ohr*) gene has been isolated from *Xanthomonas campestris* (27). The *ohr* mutant is more sensitive to organic hydroperoxides than is the wild type; however, it does not display sensitivity to hydrogen peroxide and superoxide generators (27). The Ohr protein is a member of a conserved family of proteins of largely uncharacterized function (OsmC/Ohr family [3]). Consistent with a role in organic peroxide detoxification, Ohr proteins have two conserved cysteine residues that are catalytically important, but Ohr proteins are not obviously homologous to the AhpC/TSA family of enzymes (3). There are two homologs of Ohr in *B. subtilis*; these homologs are encoded by the *yklA* and *ykzA* genes, but mutations in these genes have not been reported to have an effect on resistance to ROS (38).

In general, most enzymes that function in resistance to ROS are either inducible by oxidative stress or synthesized as part of a stationary-phase adaptative response. For example, *Escherichia coli* OxyR is a global peroxide regulator that can activate the expression of hydroperoxidase I (KatG), alkyl hydroperoxide reductase (AhpCF), a DNA-binding protein (Dps), and other resistance proteins (36). In *B. subtilis*, a similar peroxide stress response is regulated by PerR, a hydrogen peroxide- and metal ion-sensing repressor of the genes encoding KatA, AhpCF, MrgA (a Dps homolog), and heme biosynthesis enzymes (8). Interestingly, in both organisms, resistance to ROS

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is upregulated upon starvation. This stationary-phase induction of oxidant defenses is regulated by σ^S in *Escherichia coli* and by the general stress response regulator, σ^B, in *B. subtilis*.

We demonstrate here that the two *B. subtilis ohr* homologs, *yklA* and *ykzA*, are both involved in organic hydroperoxide resistance, and we therefore rename these genes *ohrA* and *ohrB*, respectively. In addition, we show that the intervening gene, *ohrR* (formerly *ykmA*), encodes an organic peroxide-sensing repressor (OhrR) for *ohrA*. In contrast, expression of *ohrB* is part of the σ^B-dependent general stress regulon (38).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All *E. coli* and *B. subtilis* strains were grown in Luria-Bertani (LB) medium with appropriate antibiotics (100 μg of ampicillin, 100 μg of spectinomycin, 10 μg of chloramphenicol, 8 μg of neomycin, and 1 μg of erythromycin per ml and 25 μg of lincomycin per ml for macrolide-lincosamine-streptogramin B [MLS] resistance) at 37°C with vigorous shaking.

Construction of *ohrA* and *ohrB* mutant strains. Previously, *yklA*::pMUTIN and *ykzA*::pMUTIN strains (BFS1816 and BFS1818) were described that contain insertional disruptions in each gene that result in transcriptional fusions to *lacZ* (38). Chromosomal DNA from BFS1816 *(ohrA-lacZ)* or BFS1818 *(ohrB-lacZ)* was transformed into CU1065 with selection for MLS resistance to generate strains HB574 and HB575, respectively. The presence of *lacZ* at the desired site was confirmed by PCR.

The *ohrB* gene was cloned into *Bam*HI and *Eco*RV-digested pBCSK (Stratagene) as a 593-bp PCR product extending 162 bp upstream and 21 bp downstream of the *ohrB* reading frame, generating plasmid pBC-zA. To create an *ohrA ohrB* double mutant, plasmid pMF2 was constructed by subcloning a 189-bp *Sph*I-*Eco*RI fragment of *ohrB* from pBC-zA into pGEM-cat at the *Sph*I-*Eco*RI sites. pMF2 was transformed into HB574 with selection for chloramphenicol resistance to generate HB2003. The presence of the *ohrB*::pMF2 disruption was confirmed by PCR of chromosomal DNA.

To introduce an *ahpC* mutation into the *ohrA* (HB574), *ohrB* (HB575), and *ohrA ohrB* (HB2003) mutant backgrounds, chromosomal DNA containing *ahpC*::Tn*10* (*ahpC1603*) (from strain HB6506 [7]) was transformed into HB574, HB575, and HB2003 to create HB2008, HB2009, and HB2010, respectively.

Construction of an *ohrR* (*ykmA*) mutant. The region of the *B. subtilis* chromosome containing the *ohrA, ohrR*, and *ohrB* genes was amplified by PCR to generate plasmid pYK15. A region extending from the *Pst*I site internal to *ohrA* to the *Sph*I site internal to *ohrB*, and therefore containing the entire *ohrR* gene, into pGEM-3zf to generate pGEM-mA. To construct an *ohrR* mutant, a kanamycin cassette from pDG792 (19) was subcloned into the *Bcl*I site internal to *ohrR* in pGEM-mA, generating pMF1. An *ohrR* mutant, HB2000, was constructed by transformation of linearized-pMF1 into CU1065 with selection for kanamycin resistance. HB2001 and HB2002 were generated by transforming *ohrR*::*kan* into HB574 and HB575, respectively. All strains were checked by PCR.

Construction of *ohrA-cat-lacZ* and *ohrR-cat-lacZ* fusions in SPβ. To construct an *ohrA-cat-lacZ* fusion, the *ohrA* promoter was amplified by PCR with primers 495 and 529. A *Bam*HI site was introduced into primer 529, and this PCR fragment contains internal *Hin*dIII sites. After *Bam*HI-*Hin*dIII digestion, this fragment was cloned into pJPM122 after digestion with *Bam*HI-*Hin*dIII to generate pMF3. To generate pMF4 containing an *ohrR-cat-lacZ* operon fusion, the *ohrR* promoter was amplified by PCR with primers 497 and 530 and cloned into pJPM122 as described above. pMF3 and pMF4 were transformed into strain ZB307A to transfer the promoter-*cat-lacZ* fusions into the SPβ*c2*Δ2::Tn*917*::pBSK10Δ6 prophage by double cross over recombination. Using phage transduction, the operon fusions were transferred to CU1065 to generate HB2012 (SPβ *ohrA-cat-lacZ*) and HB2011 (SPβ *ohrR-cat-lacZ*) and into the *ohrR* mutant strain to generate HB2014 and HB2013.

RNA isolation and Northern hybridization. Cells were grown to mid log phase (optical density at 600 nm of [OD_{600}] = 0.4). Oxidants and chemicals used for induction were 100 μM cumene hydroperoxide (CHP), 100 μM *tert*-butyl hydroperoxide, 100 μM H_2O_2, 4% ethanol, or 4% NaCl. After 15 min of treatment, the cells were placed immediately on ice and centrifuged at 10,000 rpm at 4°C. Total RNA was isolated using RNAwiz RNA isolation kit (Ambion). Then, 10 μg of total RNA was loaded onto a 1% formaldehyde gel. The separated RNA was then transferred to a nylon membrane and hybridized with radiolabeled probe at 42°C overnight in ULTRAhyb solution (Ambion). The *ohrA* probe was prepared by *Hin*fI digestion of the PCR product generated from primers 531 and 496. A 314-bp *Hin*fI fragment containing the *ohrA* coding region was purified from an agarose gel and labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase. The *ohrB* probe was prepared from an internal 200-bp *Sph*I-to-*Eco*RI fragment isolated from pBC-zA. The *ohrR* probe was prepared from *Hin*fI digestion products of the PCR fragment generated from primers 527 and 536. This PCR product contains the coding region of *ohrR*, which has two internal *Hin*fI restriction sites. *Hin*fI fragments were labeled by the fill-in method with [α-^{32}P]dATP. Membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) for 5 min at 42°C, followed by two washes with 0.1× SSC–0.1% SDS for 15 min at 42°C.

Primer extension. RNA was prepared using a hot phenol extraction protocol. A total of 10 μg of RNA was annealed with the ^{32}P-labeled oligonucleotide PE (Table 1). Primer extension reactions were performed using the Ready-To-Go You-Prime First-Strand Beads Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

β-Galactosidase assays. Cells were grown overnight in LB medium containing appropriate antibiotic(s) and then diluted 1:100 in the same medium. Samples of 1 ml were harvested at an OD_{600} of ca. 0.4 and assayed for β-galactosidase essentially as described earlier (26).

Disk diffusion assay. Cell were grown overnight in LB medium containing appropriate antibiotic(s) and then diluted 1:100 in the same medium. Then, 100 μl of cells at an OD_{600} of ca. 0.4 were mixed with 3 ml of LB containing 0.75% agar and poured onto plates containing 15 ml of LB agar with appropriate antibiotic(s). Next, 6-mm paper disks containing 10 μl of the indicated chemical were placed on top. Plates were incubated overnight at 37°C, and the clear zones were measured. The chemicals used included 0.4 M CHP, 0.2 M *tert*-butyl hydroperoxide, 1.6 M hydrogen peroxide, or 0.5 M paraquat.

Selection and characterization of mutants derepressed for *ohrA-cat-lacZ*. Approximately 10^4 cells of log-phase HB2012 were plated on LB agar containing 8 μg of neomycin, 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and between 2 and 5 μg of choramphenicol per ml. Blue colonies were recovered, and elevated expression of β-galactosidase activity was confirmed after growth in liquid medium. For each resulting strain, a transducing lysate was prepared and the SPβ *ohrA*-cat-lacZ* fusions were transferred to CU1065. Transductants that retained elevated β-galactosidase activity (4 of 12) were judged to contain *cis*-acting mutations. The *ohrA* promoter region was amplified from each transductant using a primer specific to the 5′ region of the *cat* gene (primer 366) and a primer annealing upstream of the insert (primer 535). The resulting PCR products were used directly as templates for sequencing. One strain chosen for further characterization was designated HB2031. HB2031 chromosomal DNA was transformed into the *ohrR* mutant HB2000 to generate HB2044.

RESULTS

The *B. subtilis* OhrA (formerly YklA) and OhrB (formerly YkzA) proteins are homologs of *E. coli* OsmC (38), an osmotically inducible envelope protein of unknown function (6, 18, 20). However, they are much more similar to *X. campestris* Ohr, a protein that protects cells against organic hydroperoxides (27). Previously, *ohrB* was shown to be under σ^B control and respond to general stresses, whereas *ohrA* transcription was found to be elevated in minimal medium (38).

Overlapping roles of *ohrA* and *ohrB* in organic hydroperoxide resistance. Alkyl hydroperoxide reductase (AhpCF) reduces organic hydroperoxides to their corresponding alcohols. However, in previous studies we were unable to demonstrate an organic hydroperoxide-sensitive phenotype for an *ahpC*::Tn*10* mutant strain (7). Indeed, the most striking phenotype of this disruption mutant was an elevated resistance to H_2O_2 due to derepression of the PerR regulated *katA* gene. These results suggest that other gene products may also contribute to organic peroxide resistance.

Disk diffusion assays were used to determine if OhrA and OhrB protect cells against ROS and to determine if these functions are redundant with AhpCF. Mutation of *ohrA*, but

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics	Relevant mutation(s)	Reporter	Reference, source, or derivation
Strains				
B. subtilis				
CU1065	W168 *att*SPβ *trpC2*			37
ZB307A	W168 SPβ2Δ2::Tn*917*::pBSK10Δ6			40
BFS1816	168 wild-type *ohrA-lacZ*	*ohrA*	*ohrA*	38
BFS1818	168 wild-type *ohrB-lacZ*	*ohrB*	*ohrB*	38
HB574	CU1065 *ohrA-lacZ*	*ohrA*	*ohrA*	This work
HB575	CU1065 *ohrB-lacZ*	*ohrB*	*ohrB*	This work
HB1703	CU1065 *ahpC*::Tn*10* (1603)	*ahpC*		7
HB2000	CU1065 *ohrR*::*kan*	*ohrR*		This work
HB2001	HB574 *ohrR*::*kan*	*ohrR, ohrA*	*ohrA*	This work
HB2002	HB575 *ohrR*::*kan*	*ohrR, ohrB*	*ohrB*	This work
HB2003	HB574 *ohrB*::pGEMCAT	*ohrA, ohrB*	*ohrA*	This work
HB2006	ZB307A SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrR′-cat-lacZ)*		*ohrR*	This work
HB2007	ZB307A SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrA′-cat-lacZ)*		*ohrA*	This work
HB2008	HB574 *ahpC*::Tn*10*	*ohrA, ahpC*	*ohrA*	This work
HB2009	HB575 *ahpC*::Tn*10*	*ohrB, ahpC*	*ohrB*	This work
HB2010	HB2003 *ahpC*::Tn*10*	*ohrA, ohrB, ahpC*	*ohrA*	This work
HB2011	CU1065 SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrR′-cat-lacZ)*		*ohrR*	This work
HB2012	CU1065 SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrA′-cat-lacZ)*		*ohrA*	This work
HB2013	HB2000 SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrR′-cat-lacZ)*	*ohrR*	*ohrR*	This work
HB2014	HB2000 SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrA′-cat-lacZ)*	*ohrR*	*ohrA*	This work
HB2031	CU1065 SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrA*-cat-lacZ)*		*ohrA*	This work
HB2044	HB2000 SPβ*c2*Δ*2*::Tn*917*::ϕ*(ohrA*-cat-lacZ)*	*ohrR*	*ohrA*	This work
HB6506	HB1000 *ahpC*::Tn*10*(1603)	*ahpC*		7
E. coli				
DH5α	ϕ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^-, m_K^+) *supE44 relA1 deoR* Δ*(lacZYA-argF)*U169			Lab stock
GM 2163	F^- *ara-14 leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 supE44 hisG4 rpsL1* (Str^r) *xyl-5 mtl-1 dam*13::Tn*9* (Cm^r) *dcm-6 mcrB1 hsdR2* (r_K^-, m_K^+) *mcrA*			NEB
Plasmids				
pGEM-cat	pGEM-3zf(+)-cat-1 (carrying Cm^r gene)			39
pGEM-mA	pGEM-3zf(+) with *Pst*I-*Sph*I containing *ohrA′-ohrR-ohrB*			This work
pBC-zA	pBCSK (Stratagene) containing *ohrB*			This work
pJPM122	*cat-lacZ* operon fusion vector for SPβ			34
pDG792	pMTL23 containing Kan^r cassette			19
pMF1	pGEM-mA containing the *Bam*HI-*Bgl*II Kan^r cassette (1.6 kb) from pDG792 at *Bcl*I site in *ohrR*	*ohrR*		This work
pMF2	pGEM-cat containing intergenic *Sph*I-*Eco*RI fragment of *ohrB*			This work
pMF3	pJPM122 with *ohrA* promoter			This work
pMF4	pJPM122 with *ohrR* promoter			This work
Primers				
366	5′-ACTCTCCGTCGCTATTGTAACCAG-3′			Lab stock
495 (forward)	5′-CGGGATCCTAGCGGGGTAATGTTCAATG-3′			This work
496 (reverse)	5′-CCGAATTCAAAAGCGGTTGACATTCCAG-3′			This work
497 (forward)	5′-CGGGATCCTGTATTGCTTTGTCATCTCC-3′			This work
519 (reverse)	5′-CGGGATCCAAATCAAGAACACCGTCATC-3′			This work
527 (forward)	5′-GGTGAACACCATGGAAAATAAATT-3′			This work
528 (reverse)	5′-CCGGATCCGTTGCTGAATAAATAAA-3′			This work
529 (reverse)	5′-CGGGATCCAATGACCTTTCCTTCTCTTC-3′			This work
530 (reverse)	5′-CCCAAGCTTAAATCAAGAACACCGTCATC-3′			This work
531 (forward)	5′-CGGGATCCTATATTGGGGGAATGAAAAA-3′			This work
535	5′-GTACATATTGTCGTTAGAAC-3′			This work
536 (reverse)	5′-AATGTCAACCGCTTTTTCT-3′			This work
PE	5′-AACGCGGTCTGATCAAATGA-3′			This work

not *ohrB* or *ahpC*, leads to significantly increased sensitivity to CHP (Fig. 1A) and *tert*-butyl hydroperoxide (data not shown). The *ohrA ohrB* double mutant displays much greater sensitivity to CHP than either single mutant, suggesting that both proteins are involved in CHP detoxification and that lack of one can be partially compensated for by the presence of the other. In contrast, AhpCF does not appear to play a significant role in CHP resistance, a finding consistent with our previous studies.

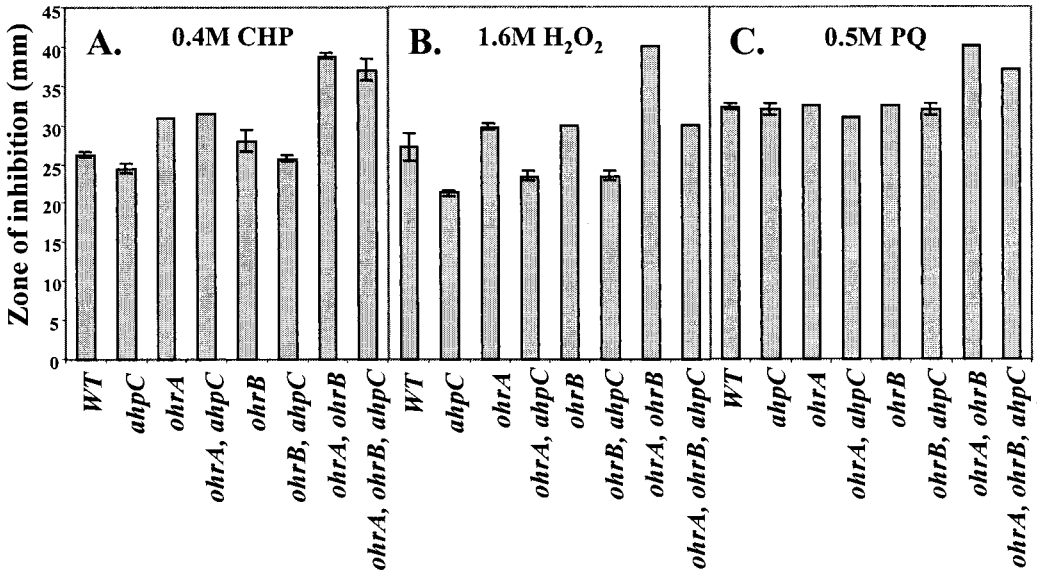


FIG. 1. Roles of OhrA, OhrB, and AhpCF in protection against ROS. The sensitivity of each indicated strain was measured as a zone of growth inhibition in a disk diffusion assay. Filters contained either 0.4 M CHP (A), 1.6 M H_2O_2 (B), or 0.5 M paraquat (C). The data shown are representative of three experiments. The error bars indicate the standard deviations from duplicate samples. PQ, paraquat.

In all four strains containing an *ahpC* mutation, resistance to CHP is not significantly altered relative to the control strain (Fig. 1A). Thus, even in the absence of both OhrA and OhrB, AhpCF still does not play a measurable role in CHP resistance. These strains all lack AhpCF function since, as reported previously (7), mutation of *ahpC* leads to derepression of catalase and a consequent increase in H_2O_2 resistance (Fig. 1B). In addition to greatly increased sensitivity to CHP, the *ohrA ohrB* double mutant also displays a striking sensitivity to both H_2O_2 (Fig. 1B) and the superoxide-generating compound, paraquat (Fig. 1C).

Transcriptional regulation of *ohrA* and *ohrB*. Northern blot analysis of CU1065 RNA isolated after exposure to various stresses demonstrates that *ohrA* is strongly induced by *tert*-butyl hydroperoxide and CHP, but not by H_2O_2, ethanol, or salt (Fig. 2A). In contrast, *ohrB* is strongly induced by ethanol or salt (Fig. 2B), a result consistent with the data of Volker et al. (38). It is also weakly inducible by *tert*-butyl hydroperoxide and CHP (Fig. 2B).

The regulation of *ohrA* by organic peroxides was also confirmed in primer extension experiments. A major *ohrA* transcript was found in cells induced with *tert*-butyl hydroperoxide and corresponds to a candidate σ^A-dependent promoter (Fig. 3). This inducible transcript corresponds to the transcript previously described for the *ohrA* gene (38). The constitutive signal corresponding to an apparent start site further upstream may be due to readthrough transcripts from the upstream *proBA* operon: this signal may result from reverse transcriptase pausing or termination at the base of the *proBA* terminator stem-loop. Readthrough from this upstream operon is consistent with the observation that *ohrA* expression is enhanced in minimal medium (38).

The induction of *ohrA* by organic peroxides was also confirmed using transcriptional reporter fusions (Table 2 and Fig. 4). With the pMUTIN derived transcriptional fusion, *ohrA-lacZ* expression can be induced ~100-fold by either CHP or *tert*-butyl hydroperoxide (Fig. 4). Similar regulation is also seen when a 219-bp region containing the *ohrA* promoter is used to generate a *lacZ* fusion inserted ectopically in SPβ (Table 2). This suggests that all necessary *cis*-regulatory elements are present within this DNA fragment.

Although AhpCF, at the levels present under these growth conditions, does not contribute significantly to protection against the killing action of CHP (Fig. 1A) or *tert*-butyl hydroperoxide (data not shown), AhpCF can reduce these compounds in vivo. This is apparent since the *ohrA* promoter can be induced by CHP and *tert*-butyl hydroperoxide at lower concentrations in strains carrying an *ahpC* mutation (Fig. 4). Note that these experiments were performed using the pMUTIN derived *ohrA-lacZ* fusion, so all strains are also mutant for *ohrA*.

OhrR is a repressor of *ohrA*. The *ohrA* and *ohrB* genes are transcribed in the same direction and are separated by *ohrR* (formerly *ykmA*), which is transcribed in the opposite direction and encodes a member of the MarR family of transcriptional repressors (Fig. 5). This proximity makes OhrR a good candidate for a regulator of *ohrA* and/or *ohrB*. In addition, an OhrR family member is known to repress *ohr* expression in *X. campestris* (S.M., unpublished data).

To determine if OhrR is a transcriptional regulator of *ohrA* and/or *ohrB*, β-galactosidase activity was measured in wild-type (HB2012) and *ohrR* mutant (HB2014) cells harboring an *ohrA-cat-lacZ* transcriptional fusion carried at SPβ (Table 2). The >100-fold upregulation of *ohrA* in the *ohrR* mutant was also confirmed in strains constructed using the pMUTIN integrational vector (which are additionally mutant for *ohrA*). The β-galactosidase activity in cells harboring *ohrA-lacZ* and an *ohrR* mutation (HB2001) was very high (~2,500 U) compared

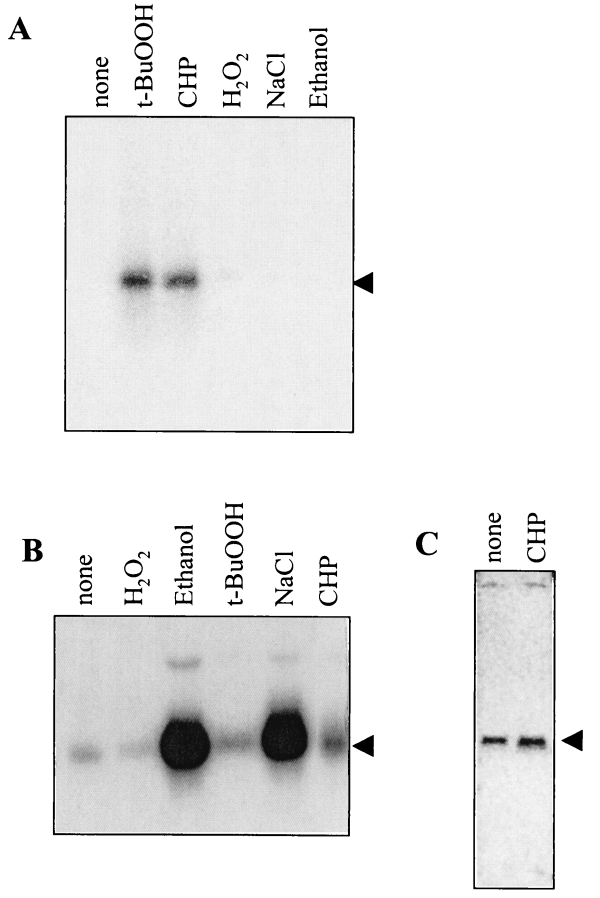


FIG. 2. Northern analysis *ohr* region genes. Expression of *ohrA* (A), *ohrB* (B), and *ohrR* (C) was measured using 10 μg of total RNA from each sample separated on a 1% formaldehyde gel. RNA was transferred to a nylon membrane and hybridized with a radiolabeled DNA fragment containing the coding region of each gene. Arrows indicate the major transcript of each gene. Cells were either uninduced (none) or were treated with 100 μM CHP, 100 μM *tert*-butyl hydroperoxide (t-BuOOH), 100 μM H_2O_2, 4% ethanol, or 4% NaCl for 15 min as indicated.

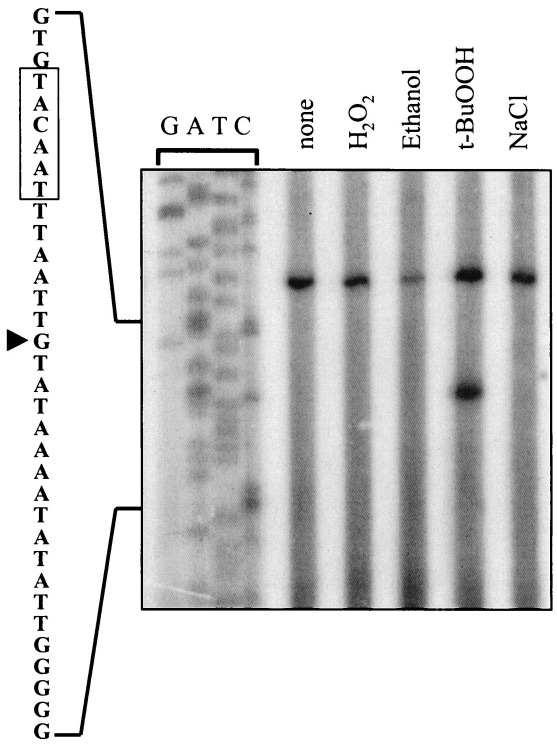


FIG. 3. Primer extension analysis of the *ohrA* promoter. Cells were grown and treated as described for Fig. 2 prior to RNA isolation. The major alkyl peroxide responsive transcriptional start point for the *ohrA* gene corresponds to position −27 relative to the start codon, in agreement with previously published start site mapping data (38). The origin of the larger band is not clear, but may be due to readthrough transcription from the upstream *proAB* operon.

to cells harboring *ohrA-lacZ* alone (HB574) (~6 U). In contrast, mutation of *ohrR* did not greatly affect the level of expression of the *ohrB-lacZ* fusion, which is very low in growing cells (1 to 2 U). These data demonstrate that mutation of *ohrR* is sufficient for derepression of *ohrA*, but not *ohrB*.

There is no significant increase in *ohrR-cat-lacZ* activity in *ohrR* versus wild-type cells (Table 2), suggesting that OhrR is not autoregulated. Moreover, expression of the *ohrR-cat-lacZ* fusion did not respond to CHP treatment (Table 2), a finding consistent with the slight response to CHP (1.3-fold induction) observed in the Northern analysis of *ohrR* mRNA (Fig. 2C).

Putative binding site of OhrR. Inspection of the *ohrA* promoter region reveals possible binding motifs for OhrR. The *ohrA* promoter region contains one perfect inverted repeat (TACAATT-AATTGTA) and an adjacent imperfect repeat with three mismatches (Fig. 6A). Alternatively, this region may be viewed as an 11-bp direct repeat.

To determine if these sequence motifs are important for OhrR-mediated repression, we selected for mutant strains that were derepressed for *ohrA-cat-lacZ* expression and characterized the resulting *cis*-acting mutants. Two independent mutants (*ohrA**) contained the identical 15-bp deletion (Fig. 6B). These mutations likely arose from unequal crossing over between the two 11-bp direct-repeat elements noted above. Remarkably, this deletion also removes the native −10 element of the *ohrA* promoter but replaces this region with another sequence that closely matches the −10 consensus, thereby likely generating a new σ^A-dependent promoter.

To determine if this altered promoter retains sequences that bind OhrR, the *ohrA*-cat-lacZ* fusion from one representative strain (HB2031) was transduced into the *ohrR* mutant to gen-

TABLE 2. β-Galactosidase activity of *ohrA* and *ohrR* transcription fusion in wild-type and *ohrR* backgrounds

Strain	Genotype		Mean β-Galactosidase activity ± SD (Miller unit)[a]	
	Mutation	Reporter	Uninduced	100 μM CHP
HB2012	None	*ohrA*	3.44 ± 0.09	90.47 ± 1.35
HB2014	*ohrR*	*ohrA*	513.04 ± 19.58	520.31 ± 16.43
HB2011	None	*ohrR*	3.65 ± 0.19	3.73 ± 0.12
HB2013	*ohrR*	*ohrR*	4.27 ± 0.14	4.46 ± 0.24
HB2031	None	*ohrA**	128.55 ± 4.12	ND
HB2044	*ohrR*	*ohrA**	230.37 ± 31.22	ND

[a] ND, not done.

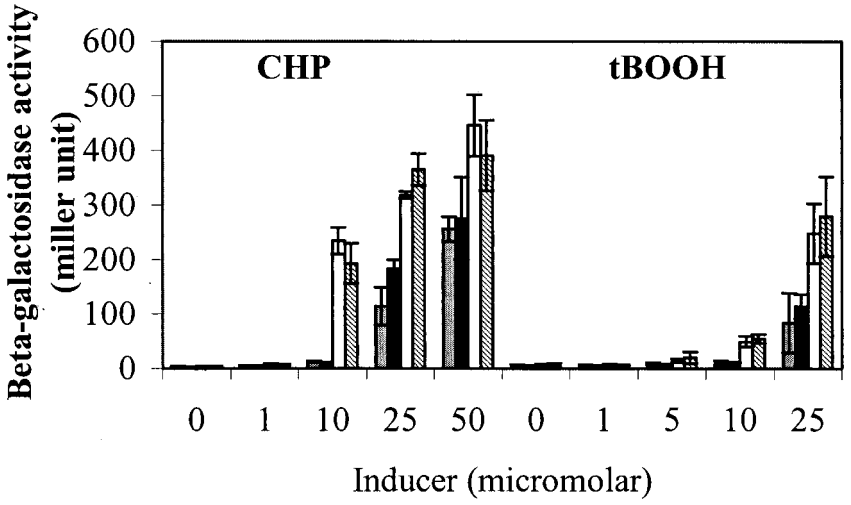
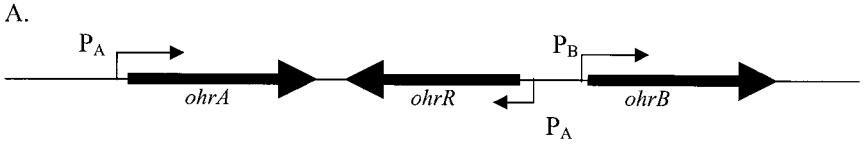


FIG. 4. Effect of an *ahpC* mutation on induction of *ohrA* by organic hydroperoxides. β-Galactosidase activities were assayed in various mutants (bars: gray, *ohrA*, HB574; black, *ohrA ohrB*, HB2003; white, *ohrA ahpC*, HB2008; cross-hatched, *ohrA ohrB ahpC*, HB2010). Cells were grown to mid-log phase, and various concentrations of CHP or *tert*-butyl hydroperoxide (tBOOH) were added to the cultures for 15 min at 37°C with shaking. The data shown are representative of triplicate determinations.

erate strain HB2044. Comparison of β-galactosidase activity in the wild-type and *ohrR* mutant cells indicates that OhrR still exerts a small, but reproducible, repressive effect on this promoter (Table 2). This result is consistent with models in which OhrR binds to the inverted repeat sequences noted above and suggests that the imperfect inverted repeat, which is retained in the mutant promoter region, may be sufficient for mediating some repression by OhrR.



B.

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OhrR Bs     ------------MENKFDHMKLENQLCFLLYASSREMTKQYKPLLDKLNITYPQYLALL 47
OhrRa Pa    ---------MSRLPTTPCEQLKLDNQLCFALYSTSLQMTKVYKPLLQALGLTYPQYIAML 51
OhrRb Pa    ----MSTRGKVARPDGVEESLLLDNQLCFKLYAASRAVIRGYRPLLEQIGLTYPQYLVML 56
OhrR Ac     ------------MQQDASILELDNQLCFLIYSTNLALHQLYRKLLSPLGLTYPQYLVML 47
OhrR Sc     MTTPATEPAAEPAAEPATDWLRLDQQICFSLSAASRAFGSVYRVVLKDLGLTYSQYLVLL 60
OhrR Vc     ----MPSP-QVSCQTPTHDPLLLENQVCFPLYSASNAVIRAYRPLLEQLDITYSQYLVLL 55
OhrR Stc    ----------------MDYNMKLANQVCFSAYNVSRLFAQFYEKELKSFGLTYSQYLVLL 44

OhrR Bs     LLWEHET-----LTVKKMGEQLYLDSGTLTPMLKRMEQQGLITRKRSEEDERSVLISLTE 102
OhrRa Pa    VLWERDG-----LTVGEISARMLTDPGSLTPLLKRLEGEGLITRTRSSEDERVVLLRLTD 106
OhrRb Pa    VLWEWHASPPEQPTVKALGDRLLLDSGTLTPLLKRLEQLGLVDRRRASHDEREVHLTLTV 116
OhrR Ac     VLWEKDQ-----LTVSEIGSRLFLESSTLTPLLKKLEHLGLLSRKRSTRDERQVIITLSS 102
OhrR Sc     VLWEHGELP-----VKRLGEHLRLDSGTLSPLLKRLEAAGLVRRERSTRDERSVQVRLTE 115
OhrR Vc     VLWQQNG-----INVKDLGIKLHLDSGTLTPLLKRLEAKGIVERRRSSSDERVRELFLTP 110
OhrR Stc    SLWEEILK-----HYIQLVINWNLSSNTLTPLLKRLEQSGWVTRQKSDQDKRQLIVSLTQ 99

OhrR Bs     DGALLKEKAVDIPGTILGLSKQSGEDLKQLKSALYTLLETLHQKN-------- 147
OhrRa Pa    KGRALQRQAESIPACILESTGLDLPQLVALKDELLALRGNLQAPD-------- 151
OhrRb Pa    PGITLRRERVIPLRQQLICSTGFDLNEMFDTHQRLGGLLSRFRLVVGG------ 163
OhrR Ac     QGQELKQQALDIPHQTAQASACNLDELMQLKEQLETLRTNISQ---------- 145
OhrR Sc     EGAALRGRAVEVPRRIAAAIGLDLAEVQDDRARLDRLTAALDAYGTAPAPEPS 168
OhrR Vc     AGFALQEQARSVPNEMLCKFDLSLEELISLKTLCEKILHTLD---------- 152
OhrR Stc    KGKDQKQPIYDAISRCVSED-MNLDLYKQTKDIMDQLEAILRKQLNK------ 145
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FIG. 5. *ohrR* encodes a MarR-like repressor of *ohrA*. (A) Schematic of the *ohrA ohrR ohrB* region. P_A indicates a σ^A-dependent promoter element; P_B indicates a σ^B-dependent promoter. (B) alignment of OhrR with other closely related MarR family members. The abbreviations used are as follows (strain; GenBank accession number): OhrR Bs (*B. subtilis*; E69857), OhrRa Pa (*P. aeruginosa* PAO1; D83290), OhrRb Pa (*P. aeruginosa* PAO1; G83292), OhrR Ac (*Acinetobacter* sp. strain ADP1; CAA70318), OhrR Sc (*Streptomyces coelicolor*; CAB87337); OhrR Vc (*Vibrio cholerae* group O1 strain N16961; B82389), and OhrR Stc (*Staphylococcus sciuri* strain ATCC 29062). The amino acid sequences were aligned (using CLUSTALW) and conserved residues highlighted using the BoxShade utility.

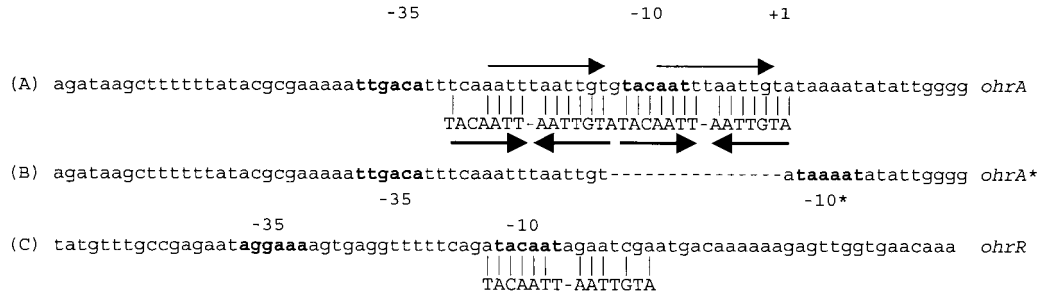

FIG. 6 Genetic identification of sequences required for OhrR-mediated repression. The perfect inverted repeat is indicated in capital letters with matching bases identified by a vertical line. (A) In the *ohrA* promoter, there are two adjacent inverted repeats. The first is imperfect; the second is a perfect inverted repeat (thick arrows). This region also contains two 11-bp direct repeats (thin arrows). The −10 and −35 regions are shown in boldface. (B) The sequence of the mutant promoter region (*ohrA*∗) is shown with a dashed line to indicate the 15-bp deletion. A new −10 element is created by the deletion. (C) A related, imperfect inverted repeat is found overlapping the *ohrR* promoter region.

DISCUSSION

Cells have evolved numerous overlapping mechanisms to protect against the ravages of ROS (35, 36). In the case of organic hydroperoxides, the best-studied defensive enzyme is alkyl hydroperoxide reductase, encoded by the *ahpCF* operon. However, bacterial cells contain additional activities that are important in protection against organic peroxides, including other peroxiredoxins and, as described here, members of the Ohr family. The role of Ohr in defense against oxidative stress was first described in *X. campestris* pv. phaseoli (27), and recent results indicate a similar function in *Pseudomonas aeruginosa* (28). Ohr proteins are not obviously homologous to known peroxidases, but it is reasonable to speculate that these proteins may enzymatically detoxify peroxides. Although Ohr expression is clearly regulated, the mechanisms controlling Ohr expression have yet to be described.

We have shown that the both OhrA and OhrB contribute to organic hydroperoxide resistance. Unlike PerR regulated genes, which can be induced by either organic hydroperoxides or H_2O_2 (7, 8, 12, 13), *ohrA* responds specifically to organic hydroperoxides, and this regulation requires OhrR. Consistent with previous studies, *ohrB* expression responds to heat, ethanol, and salt stress as part of the σ^B-dependent general stress response (Fig. 2A) (38). However, OhrB also has a role in organic hydroperoxide resistance, as shown by the increased CHP sensitivity of the *ohrA ohrB* double mutant (Fig. 1).

The relationship between the Ohr proteins and AhpCF is complex. Interestingly, only *ohrA* is under the control of OhrR. It is possible that OhrA plays the primary protective role when cells are exposed to organic hydroperoxides and OhrB is involved in detoxification of organic hydroperoxides produced during general stress. It is also possible that OhrA, OhrB, and the Ahp/TSA family members have distinct, albeit overlapping, substrate selectivities. Introduction of an *ahpC* mutation into the *ohrA, ohrB*, or *ohrA ohrB* strains did not increase sensitivity to organic hydroperoxides (Fig. 1), suggesting that AhpCF does not play a major role in protecting cells against the killing action of these organic hydroperoxides. The lack of a protective role for AhpCF in the present studies may result from the use of logarithmically growing cells (in which *ahpCF* is expressed at a low level) and the use of defined organic peroxides as the stressor. AhpCF and other genes repressed by PerR are known to be induced upon entry into stationary phase, upon starvation for iron and manganese, or in response to peroxides (7, 8, 14). In stationary-phase cells or under conditions in which both H_2O_2 and organic peroxides are generated, AhpCF levels would be elevated and could thereby contribute to oxidative defenses. Indeed, *perR* mutant cells have elevated resistance to CHP that depends on the *ahpC* gene (8). It is curious that AhpCF overproduction (in a *perR* mutant) leads to a CHP-resistant phenotype, whereas OhrA overproduction (in an *ohrR* mutant) does not, although OhrA is now sufficiently abundant as to be visible by Coomassie blue staining of whole-cell lysates (data not shown). Similarly, Ohr overproduction in *X. campestris* did not increase resistance to organic hydroperoxides (27).

The presence of two Ohr paralogs with distinct regulation is reminiscent of other genes involved in oxidative defense in *B. subtilis*. The *katA* gene is induced by ROS by virtue of its regulation by PerR, while the *katB* and *katX* genes are part of the σ^B regulon (4, 5, 8, 17, 30). Similarly, PerR represses expression of the Dps homolog encoded by *mrgA* (12), while a second Dps homolog encoded by the *dps* gene is regulated by σ^B (2).

Our genetic analysis defines a 15-bp region required for OhrR-mediated repression of the *ohrA* gene. This region includes a perfect inverted repeat, TACAATT-AATTGTA, which likely defines the OhrR binding site. Related imperfect inverted repeat sequences (three mismatches) are found in the *ohrA* and the *ohrR* promoter regions. Analysis of the *ohrA*∗ mutant suggests that an imperfect inverted repeat element may still allow some residual regulation by OhrR (Table 2). However, the imperfect inverted repeat overlapping the *ohrR* promoter does not appear to mediate repression, since we found no evidence for *ohrR* autoregulation (Table 2).

OhrA and OhrB are representative of a large family of conserved proteins found throughout the Bacterial domain (3). Our data lend further support to the suggestion that these proteins function in protecting cells against organic peroxides. Moreover, since *ohr* homologs are often found closely associated with an *ohrR*-like gene (3), the mechanism of regulation described here may also be conserved. Thus, OhrR is a novel type of organic peroxide-sensing transcription factor and rep-

resents a third regulator (together with PerR and σ^B) involved in oxidative stress responses in *B. subtilis*.

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REFERENCES

1. **Antelmann, H., S. Engelmann, R. Schmid, and M. Hecker.** 1996. General and oxidative stress responses in *Bacillus subtilis*: cloning, expression, and mutation of the alkyl hydroperoxide reductase operon. J. Bacteriol. **178:** 6571–6578.
2. **Antelmann, H., S. Engelmann, R. Schmid, A. Sorokin, A. Lapidus, and M. Hecker.** 1997. Expression of a stress- and starvation-induced *dps*/*pexB*-homologous gene is controlled by the alternative sigma factor sigmaB in *Bacillus subtilis, J. Bacteriol.* **179:**7251–7256.
3. **Atichartpongkul, S., S. Lopraset, P. Vattanaviboon, W. Whangsuk, J. D. Helmann, and S. Mongkolsuk.** Bacterial Ohr and OsmC paralogs define two protein families with distinct functions and patterns of expression. Microbiology, in press.
4. **Bagyan, I., L. Casillas-Martinez, and P. Setlow.** 1998. The *katX* gene, which codes for the catalase in spores of *Bacillus subtilis*, is a forespore-specific gene controlled by sigmaF, and KatX is essential for hydrogen peroxide resistance of the germinating spore. J. Bacteriol. **180:**2057–2062.
5. **Bol, D. K., and R. E. Yasbin.** 1994. Analysis of the dual regulatory mechanisms controlling expression of the vegetative catalase gene of *Bacillus subtilis. J. Bacteriol.* **176:**6744–6748.
6. **Bouvier, J., S. Gordia, G. Kampmann, R. Lange, R. Hengge-Aronis, and C. Gutierrez.** 1998. Interplay between global regulators of *Escherichia coli*: effect of RpoS, Lrp and H-NS on transcription of the gene *osmC. Mol. Microbiol*. **28:**971–980.
7. **Bsat, N., L. Chen, and J. D. Helmann.** 1996. Mutation of the *Bacillus subtilis* alkyl hydroperoxide reductase (*ahpCF*) operon reveals compensatory interactions among hydrogen peroxide stress genes. J. Bacteriol. **178:**6579–6586.
8. **Bsat, N., A. Herbig, L. Casillas-Martinez, P. Setlow, and J. D. Helmann.** 1998. *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol. Microbiol. **29:**189–198.
9. **Carmel-Harel, O., and G. Storz.** 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annu. Rev. Microbiol. **54:**439–461.
10. **Cha, M. K., H. K. Kim, and I. H. Kim.** 1996. Mutation and mutagenesis of thiol peroxidase of *Escherichia coli* and a new type of thiol peroxidase family. J. Bacteriol. **178:**5610–5614.
11. **Chae, H. Z., K. Robison, L. B. Poole, G. Church, G. Storz, and S. G. Rhee.** 1994. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. Proc. Natl. Acad. Sci. USA **91:**7017–7021.
12. **Chen, L., and J. D. Helmann.** 1995. *Bacillus subtilis* MrgA is a Dps(PexB) homologue: evidence for metalloregulation of an oxidative-stress gene. Mol. Microbiol. **18:**295–300.
13. **Chen, L., L. Keramati, and J. D. Helmann.** 1995. Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. Proc. Natl. Acad. Sci. USA **92:**8190–8194.
14. **Chen, L., Q. W. Xie, and C. Nathan.** 1998. Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. Mol. Cell **1:**795–805.
15. **Dowds, B. C.** 1994. The oxidative stress response in *Bacillus subtilis*. FEMS Microbiol. Lett. **124:**255–263.
16. **Ellis, H. R., and L. B. Poole.** 1997. Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from *Salmonella typhimurium*. Biochemistry **36:**13349–13356.
17. **Engelmann, S., C. Lindner, and M. Hecker.** 1995. Cloning, nucleotide sequence, and regulation of katE encoding a sigma B-dependent catalase in *Bacillus subtilis*. J. Bacteriol. **177:**5598–5605.
18. **Gordia, S., and C. Gutierrez.** 1996. Growth-phase-dependent expression of the osmotically inducible gene *osmC* of *Escherichia coli* K-12. Mol. Microbiol. **19:**729–736.
19. **Guerout-Fleury, A. M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
20. **Gutierrez, C., and J. C. Devedjian.** 1991. Osmotic induction of gene *osmC* expression in *Escherichia coli* K12. J. Mol. Biol. **220:**959–973.
21. **Henriques, A. O., L. R. Melsen, and C. P. Moran, Jr.** 1998. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. J. Bacteriol. **180:**2285–2291.
22. **Inaoka, T., Y. Matsumura, and T. Tsuchido.** 1998. Molecular cloning and nucleotide sequence of the superoxide dismutase gene and characterization of its product from *Bacillus subtilis. J. Bacteriol*. **180:**3697–3703.
23. **Inaoka, T., Y. Matsumura, and T. Tsuchido.** 1999. SodA and manganese are essential for resistance to oxidative stress in growing and sporulating cells of *Bacillus subtilis. J. Bacteriol*. **181:**1939–1943.
24. **Jacobson, F. S., R. W. Morgan, M. F. Christman, and B. N. Ames.** 1989. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. J. Biol. Chem. **264:**1488–1496.
25. **Li Calzi, M., and L. B. Poole.** 1997. Requirement for the two AhpF cystine disulfide centers in catalysis of peroxide reduction by alkyl hydroperoxide reductase. Biochemistry **36:**13357–13364.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Mongkolsuk, S., W. Praituan, S. Loprasert, M. Fuangthong, and S. Chamnongpol.** 1998. Identification and characterization of a new organic hydroperoxide resistance (*ohr*) gene with a novel pattern of oxidative stress regulation from *Xanthomonas campestris* pv. phaseoli. *J. Bacteriol*. **180:**2636–2643.
28. **Ochsner, U., D. J. Hassett, and M. L. Vasil.** 2001. Genetic and physiological characterization of *ohr*, encoding a protein involved in organic hydroperoxide resistance in *Pseudomonas aeruginosa*. J. Bacteriol. **183:**773–778.
29. **Park, S. G., M. K. Cha, W. Jeong, and I. H. Kim.** 2000. Distinct physiological functions of thiol peroxidase isoenzymes in *Saccharomyces cerevisiae*. J. Biol. Chem. **275:**5723–5732.
30. **Petersohn, A., S. Engelmann, P. Setlow, and M. Hecker.** 1999. The *katX* gene of *Bacillus subtilis* is under dual control of sigmaB and sigmaF. Mol. Gen. Genet. **262:**173–179.
31. **Poole, L. B.** 1996. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 2. Cystine disulfides involved in catalysis of peroxide reduction. Biochemistry **35:**65–75.
32. **Poole, L. B., and H. R. Ellis.** 1996. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry* **35:**56–64.
33. **Rhee, S. G., S. W. Kang, L. E. Netto, M. S. Seo, and E. R. Stadtman.** 1999. A family of novel peroxidases, peroxiredoxins. Biofactors **10:**207–209.
34. **Slack, F. J., J. P. Mueller, and A. L. Sonenshein.** 1993. Mutations that relieve nutritional repression of the *Bacillus subtilis* dipeptide permease operon. J. Bacteriol. **175:**4605–4614.
35. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188–194.
36. **Storz, G., and M. Zheng.** 2000. Oxidative stress, p. 47–59. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington, D.C.
37. **Vander Horn, P. B., and S. A. Zahler.** 1992. Cloning and nucleotide sequence of the leucyl-tRNA synthetase gene of *Bacillus subtilis. J. Bacteriol.* **174:**3928–3935.
38. **Volker, U., K. K. Andersen, H. Antelmann, K. M. Devine, and M. Hecker.** 1998. One of two *osmC* homologs in *Bacillus subtilis* is part of the sigmaB-dependent general stress regulon. J. Bacteriol. **180:**4212–4218.
39. **Youngman, P.** 1990. Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus* species, p. 221–266. *In* C. R. A. C. Harwood (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester, England.
40. **Zuber, P., and R. Losick.** 1987. Role of AbrB in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis. J. Bacteriol*. **169:**2223–2230.