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Dual Role of OhrR as a Repressor and an Activator in Response to Organic Hydroperoxides in *Streptomyces coelicolor*▽

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Organic hydroperoxide resistance in bacteria is achieved primarily through reducing oxidized membrane lipids. The soil-inhabiting aerobic bacterium *Streptomyces coelicolor* contains three paralogous genes for organic hydroperoxide resistance: *ohrA*, *ohrB*, and *ohrC*. The *ohrA* gene is transcribed divergently from *ohrR*, which encodes a putative regulator of MarR family. Both the *ohrA* and *ohrR* genes were induced highly by various organic hydroperoxides. The *ohrA* gene was induced through removal of repression by OhrR, whereas the *ohrR* gene was induced through activation by OhrR. Reduced OhrR bound to the *ohrA-ohrR* intergenic region, which contains a central (primary) and two adjacent (secondary) inverted-repeat motifs that overlap with promoter elements. Organic peroxide decreased the binding affinity of OhrR for the primary site, with a concomitant decrease in cooperative binding to the adjacent secondary sites. The single cysteine C28 in OhrR was involved in sensing oxidants, as determined by substitution mutagenesis. The C28S mutant of OhrR bound to the intergenic region without any change in binding affinity in response to organic peroxides. These results lead us to propose a model for the dual action of OhrR as a repressor and an activator in *S. coelicolor*. Under reduced conditions, OhrR binds cooperatively to the intergenic region, repressing transcription from both genes. Upon oxidation, the binding affinity of OhrR decreases, with a concomitant loss of cooperative binding, which allows RNA polymerase to bind to both the *ohrA* and *ohrR* promoters. The loosely bound oxidized OhrR can further activate transcription from the *ohrR* promoter.

Lipid hydroperoxide is a prominent nonradical product generated in the process of unsaturated fatty acid-initiated lipid peroxidation, triggered by both enzymatic and nonenzymatic processes under oxidative stress (14, 24). Lipid hydroperoxide can promote the generation of reactive lipid radicals, oxidize macromolecules, and affect the physical properties and structural organization of membrane components. To prevent the toxic effect of lipid peroxidation, living organisms have developed adaptive systems that include its detoxification. The best-characterized bacterial system for the detoxification of organic hydroperoxides is the alkyl hydroperoxide reductase (AhpC), a member of the peroxiredoxin superfamily (39). AhpC catalyzes the reduction of organic peroxides to their corresponding alcohols and degrades hydrogen peroxides generated endogenously from aerobic respiration (38, 40). In many bacteria, the expression of *ahpC* is regulated positively by the transcriptional activator OxyR, as in *Escherichia coli* and *Streptomyces coelicolor*, or negatively by repressor PerR, as in *Bacillus subtilis* (19, 32).

A second system, designated *ohr* (for organic hydroperoxide resistance), was initially discovered in *Xanthomonas campestris* (33). Unlike peroxiredoxins, Ohr homologues have been found only in bacteria, but widely distributed in both gram-positive and gram-negative bacteria (1). The expression of *ohr* is specifically induced by organic hydroperoxides, and inactivation of this gene leads to increased sensitivity to organic peroxides (32). Recent structural and biochemical studies on Ohr have shown that this enzyme contains alkyl hydroperoxide reductase activity and detoxifies organic hydroperoxides by reducing these peroxides to alcohols in a thiol-dependent manner (8, 9, 29).

The regulation of the *ohr* gene has been demonstrated to be mediated through OhrR, a member of MarR/SlyA family. OhrR is the organic peroxide-sensing transcriptional repressor that binds to the *ohr* promoter region in the absence of organic hydroperoxides (7, 12, 35). However, the mechanism by which OhrR senses and is inactivated by organic hydroperoxide appears different depending on the number of critical cysteine residues. In *B. subtilis*, the single conserved cysteine in OhrR is oxidized by organic hydroperoxides to Cys-sulfenic acid, which rapidly forms sulfenamide with backbone amide or mixed disulfides in the absence or presence of small thiols, respectively, resulting in derepression of *ohrA* (13, 28). In contrast, OhrR from *X. campestris*, with multiple (three) cysteine residues, forms intersubunit disulfide bonds when oxidized by organic hydroperoxides (36). Regardless of oxidation status, all OhrR proteins that have been experimentally studied so far act as repressors, as most MarR family members are.

The soil-inhabiting bacterium *Streptomyces coelicolor* is a model organism for studying morphological differentiation and antibiotic production. It contains a large linear genome encoding more than 7,800 protein products, about 1,000 of which are predicted to be transcriptional regulators (3). Through its life cycle it experiences various oxidants generated from aerobic metabolism or from the soil environment, including oxidative antibiotic compounds as well as plant exudates rich in polyunsaturated fatty acids. In order to cope with oxidative stresses, especially those generated through peroxides, *S. coelicolor* exploits transcriptional regulators such as OxyR, CatR (a PerR

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homologue), and σ^R, which induce the alkyl hydroperoxide reductase (AhpCD), catalase (CatA), and thioredoxin systems, respectively (17, 19, 34). Induction of catalase-peroxidase and a differentiation-related catalase is mediated through FurA and σ^B, respectively (6, 16). In this paper, we describe the regulation of the *ohr* genes encoding organic hydroperoxide resistance by OhrR in *S. coelicolor*, which exhibits specificity toward organic peroxides and uniqueness as a dual-function regulator serving as a repressor and an activator.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. coelicolor* A3(2) M145 was used as a wild-type strain and grown in YEME medium containing 10.3% sucrose (26). To apply oxidative stress in liquid culture, various concentrations of oxidants were added to exponentially growing cells (optical density at 600 nm, 0.3 to 0.5). For PCR-targeted mutagenesis, *E. coli* BW25113 with plasmid pIJ790 was used as recommended (15). The nonmethylating *E. coli* donor ET12567 with plasmid pUZ8002 was used for conjugal transfer (11).

Plasmids. The 0.73-kb fragment that contained the *ohrA-ohrR* intergenic region and the *ohrR* coding region was amplified from *S. coelicolor* cosmid SCE50 (from the John Innes Centre) with primers ohrAS1 (5′-GCCGTCGCGGCCG TGGGTGGC-3′; +31 to +50 from the *ohrA* start codon) and ohrROC (5′-GG TAGCCAGGATCCGTCATCGCGG [BamHI site underlined]) and cloned into the HincII site of pUC18 to yield pSO41. The 0.24-kb intergenic region of *ohrA* and *ohrR* was amplified from pSO41 with primers ohrAS1 and ohrRS2 (5′-CG GGCTCGGCTGCGGGCTCGGCTGCGGGCTCGGT-3′; +16 to +49 from the *ohrR* start codon) and cloned into the HincII site of pUC18 to yield pSO43. The pSET152 plasmid, which can be integrated into the chromosome (4), was modified to contain a hygromycin resistance cassette at the SphI site, resulting in pSET152H (a kind gift from Min-Sik Kim, Seoul National University).

Site-specific mutagenesis of *ohrR*. Cys-28 of OhrR was replaced with serine by use of the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid pSO41 DNA was used as a template with two complementary mutagenic primers, ohrR-C28SN (5′-CCAGCAGATCAGCTTCTCGCTGAG-3′ [mutated nucleotide underlined]) and ohrR-C28SC (5′-CTCAGCGAGAAGCTGATCTGCTG G-3′), resulting in pSO42 with the mutated *ohrR* gene. The mutation was confirmed by DNA sequencing.

Disruption of the *ohrR* gene and complementation. The Δ*ohrR* mutant was generated by replacing the coding sequence (from the 20th codon to the stop codon) with an apramycin resistance cassette using PCR-targeted mutagenesis (15). The remaining *ohrR* sequence in the mutant allows detection of *ohrR* transcripts by S1 mapping. The expected disruption was confirmed by PCR and Southern hybridization. To complement the Δ*ohrR* mutant, either the wild-type or the C28S mutant *ohrR* gene was recovered from pSO41 or pSO42 as PvuII fragments and introduced into pSET152H via the EcoRV site, followed by conjugal transfer to the Δ*ohrR* strain.

S1 nuclease protection assay. RNA was isolated from *S. coelicolor* cells grown in YEME medium using a standard protocol (26). The probes for *ohrA* and *ohrR* were amplified by PCR from pSO43 using ohrAS1 and M13 reverse primers for *ohrA* and ohrRS2 and M13 forward primers for *ohrR*. The probes for *ohrB* and *ohrC* were prepared by PCR from M145 genomic DNA using primers ohrBN (5′-TCCGGCGAGGAAGGAACGGG-3′) and ohrBS2 (5′-GGTGTAGAGGA CTTCGGACTGC-3′) for *ohrB* and primers ohrCN (5′-GGCGTCACAACAA CGGGCGC-3′) and ohrCS2 (5′-TCGGCCGAGCGTGCGTGGCC-3′) for *ohrC*. PCR products were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The probes for the *catA* and *ahpC* transcripts were prepared as described previously (18, 19). For high-resolution mapping, the protected DNA fragments were loaded onto a 6% (wt/vol) polyacrylamide gel containing 7 M urea, along with sequencing ladders generated with the pSO43 plasmid and primers ohrAS1 and ohrRS2. Following electrophoresis, gels were dried and exposed to X-ray films or phosphor screens for quantification with an image analyzer (BAS-2500; Fuji).

Purification of recombinant OhrR. The coding region of the wild-type or C28S mutant *ohrR* gene was amplified by PCR from pSO41 or pSO42 using the mutagenic primer OhrRON (5′-ACCCTGGAGCATATGACCACGCCC [the NdeI site is underlined]) and OhrROC. The PCR product was digested and cloned into pET15b, resulting in pSO44 and pSO45 for overproducing wild-type and C28S mutant OhrR, respectively. *E. coli* BL21(DE3)pLysS cells harboring these recombinant plasmids were grown in 200 ml LB to an optical density at 600 nm of 0.5 and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. After harvest, cells were resuspended in binding buffer (20 mM Tris-HCl [pH 7.9], 0.5 M NaCl, 5 mM imidazole) and disrupted by sonication. The cleared lysate was applied to a nickel-nitrilotriacetic acid column (Novagen). The His-tagged OhrR protein eluted with 200 mM imidazole was desalted through a PD-10 column. The N-terminal His tag was cleaved off by thrombin and purified through a nickel-nitrilotriacetic acid column. The OhrR protein was dialyzed twice against the storage buffer (20 mM Tris-HCl [pH 7.9], 100 mM NaCl, 0.1 mM EDTA, and 50% glycerol) at 4°C.

Gel mobility shift assay for OhrR binding. To generate series D probes (see Fig. 4A) that span different lengths of the promoter region, PCR was performed on plasmid pSO43 using forward primers ohrRD1 (5′-cccaagcTTCGGGAGGG GGCT GTGTG-3′ [capital letters, sequence matching nucleotides {nt} −115 to −97]) for D1, ohrRD2 (5′-cccaagctTAGAGCACGCCATTTGATCG-3′ [capital letters, sequence matching nt −81 to −62]) for D2, ohrRD3 (5′-cccaagcttCGC GCAACTAAATTGCACAC-3′ [capital letters, sequence matching nt −61 to −42]) for D3, and ohrRD4 (5′-cccaagcttAACTAAATCGCGGACAAGGC-3′ [capital letters, sequence matching nt −41 to −22]) for D4 and the reverse primer ohrRS2 (5′ end at +49). For the D1C1 probe, forward primer ohrRD1 and reverse primer ohrRC1 (5′-cgaattcGCCTTGTCC GCGATTTAG-3′ [capital letters, sequence matching nt −39 to −22]) were used. (Lowercase letters represent the unrelated sequences attached to the primers.) The D1, D2, D3, D4, and D1C1 PCR products were cloned to pUC18, generating pSO431, -432, -433, -434, and -435, respectively, and from these plasmids the final probe DNA was prepared by PCR using an M13 forward primer and reverse primer ohrRS2 (D1 to D4) or ohrRC1 (D1C1). To prepare series B probes of 60 bp, two complementary 60-mer oligonucleotides (−81 to −22 from the *ohrR* transcription start site), either nonmutated (B0) or with an unrelated 10-bp sequence (ATCGGT GTAC) substituted consecutively from −77 to −28 (B1 to B5), were synthesized and annealed in 0.25 M NaCl, 50 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Fragments B1 to B5 were used as competitors for an OhrR binding assay (see Fig. 4B). The probes were end labeled with [γ-^{32}P]ATP and incubated with OhrR protein in 20 μl binding buffer [20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μg/ml bovine serum albumin, 5 μg/ml calf thymus DNA, 50 μg/ml poly(dI-dC)] at room temperature for 10 min. To oxidize OhrR protein, organic hydroperoxides were added to the binding buffer at the indicated concentrations. Binding mixtures were run on a 5% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. Gel images were obtained by a phosphor image analyzer (BAS-2500; Fuji). For the B10 probe (−81 to −22), the flanking repeat motifs (−73 to −61 and −43 to −31) were replaced with random sequences (CGACCGACTGGCT).

DNase I footprinting. The probe DNA was prepared by PCR using 5′-^{32}P-labeled primers for either the top or the bottom strand. The top-strand probe (221 bp) was generated with labeled ohrRD1 and M13 reverse primers. The bottom-strand probe (292 bp) was generated with labeled ohrRS2 and M13 forward primers. The amplified products were purified from the native polyacrylamide gel by a standard crush-and-soak method. Binding reactions were performed as in the gel mobility shift assay using 20,000 cps of the labeled probe in a 40-μl reaction volume. After 10 min of incubation at room temperature, DNase I treatment and gel electrophoresis were carried out as described previously (41).

In vitro transcription assay. The in vitro transcription assay was performed as described previously (25). The template DNA (294 bp) was generated by PCR from pSO431 using an M13 forward primer and an M13 reverse primer, encompassing the *ohrR-ohrA* intergenic region from −115 to +49 relative to the *ohrR* transcription start site (corresponding to +25 to −139 relative to the *ohrA* start site) and 130 bp (upstream and downstream) of unrelated vector sequence. From this template, transcripts of 93 nt and 111 nt are expected to be synthesized from the *ohrA* and *ohrR* promoters, respectively. As a control, transcripts from *rrnD* promoters were examined by using as a template a 438-bp AccI and AvaI fragment of an *rrnD* clone (provided by Mi-Young Hahn). The RNA polymerase (RNAP) holoenzyme was prepared from *S. coelicolor* as described previously (20). OhrR protein (3 and 6 pmol) was incubated with 0.15 pmol of template DNA at 30°C for 10 min in 13 μl of transcription buffer (40 mM Tris-HCl [pH 7.9], 10 mM $MgCl_2$, 0.6 mM EDTA, 0.4 mM potassium phosphate, 1.5 mM dithiothreitol [DTT], 0.25 mg/ml bovine serum albumin, and 33% [vol/vol]) glycerol. RNAP (1.5 pmol) was added and incubated at 30°C for 20 min before the start of RNA synthesis by addition of labeled nucleoside triphosphates. For single-round transcription, heparin (final concentration, 0.1 mg/ml) was added at 2 min after RNA synthesis. Transcripts were analyzed on 6% polyacrylamide gels containing 8 M urea, followed by autoradiography.

Synthesis of linoleic acid hydroperoxide. Linoleic acid hydroperoxide (LaOOH) was generated in vitro as described previously (44), by incubating 0.5 mM linoleic acid (L1012; Sigma) with soybean lipoxygenase IV (4,000 U; L3004; Sigma). The enzyme catalyzes abstraction of the H-11 hydrogen, which leads to

the specific formation of La-13-OOH (42). The reaction mixture was loaded onto an end-capped C_{18} reverse-phase column (Sepak cartridge; Waters), and the LaOOH was eluted with 1.5 ml of methanol. The solution was stored at −20°C in the dark.

RESULTS

Three paralogous genes for Ohr in *S. coelicolor*. The BLAST search of the complete genome sequence of *S. coelicolor* for homologues of *ohr* from *Xanthomonas campestris* pv. phaseoli revealed three paralogues with high degrees of similarity: SCO2986 (*ohrA*), SCO2396 (*ohrB*), and SCO7111 (*ohrC*), sharing 55, 55, and 47% identity with the corresponding genes in *X. campestris* pv. phaseoli. OhrA, OhrB, and OhrC all contain the conserved active-site cysteines determined for Ohr homologues from *X. campestris* and *B. subtilis* (1). The *ohrA* gene is divergently located from the *ohrR* gene, encoding a putative regulator of the MarR family that contains the critical cysteine conserved in OhrR orthologues (Fig. 1A). The *ohrB* gene resides near another putative regulator (SCO2398) of the MarR family, which lacks the critical cysteine conserved in OhrR orthologues. The *ohrC* gene is flanked by *fdxA* (encoding a putative ferredoxin) and a gene (SCO7112) for an ECF (extracytoplasmic function) sigma factor.

The *ohrA* gene is induced in response to organic peroxides. The expression behavior of the three *ohr* genes was examined by S1 mapping for cells treated with hydrogen peroxide, *tert*-butyl hydroperoxide (tBHP), KCl, or ethanol (Fig. 1B). We found that only the *ohrA* transcripts were induced dramatically (about 50-fold) by tBHP. The *ohrB* gene was expressed at a low level and was slightly induced by ethanol. The *ohrC* gene maintained a constitutive level of expression under all the conditions we examined. We then compared the induction behavior of *ohrA* with those of the alkyl hydroperoxide reductase system (AhpCD) regulated by OxyR and a major catalase (CatA) regulated by CatR in response to different types of peroxides (18, 19). *ohrA* was induced by organic peroxides (tBHP and linoleic hydroperoxide) and not by hydrogen peroxide, as expected for the regulation by organic-peroxide-specific OhrR orthologues (Fig. 1C). The *ahpC* and *catA* genes, however, were induced by both hydrogen peroxide and short-chain organic peroxides, suggesting that OxyR and CatR respond to both types of peroxides in *S. coelicolor*, in agreement with the behavior of known OxyR and PerR orthologues (5, 21, 27). Linoleic acid did not induce any gene expression, confirming the peroxide specificity of the response.

In order to estimate the contribution of Ohr paralogues and AhpCD to the protection of *S. coelicolor* cells against organic hydroperoxide, we compared the sensitivity phenotypes of *ohrA*, *ohrB*, and *ahpCD* disruption mutants. The *ohrA* mutant exhibited increased sensitivity to cumene hydroperoxide but not to hydrogen peroxide, whereas the *ohrB* and *ahpCD* mutants showed no change in sensitivity (data not shown). These results suggest that OhrA is the primary protection system against organic hydroperoxides.

Transcription of the *ohrA* and *ohrR* genes. The time course of induction of the *ohrA* and *ohrR* genes was examined further. Both genes were induced rapidly by tBHP with similar kinetics (Fig. 2A). The extents of induction for *ohrA* and *ohrR* were more than 50- and 20-fold, respectively, at 20 min of exposure

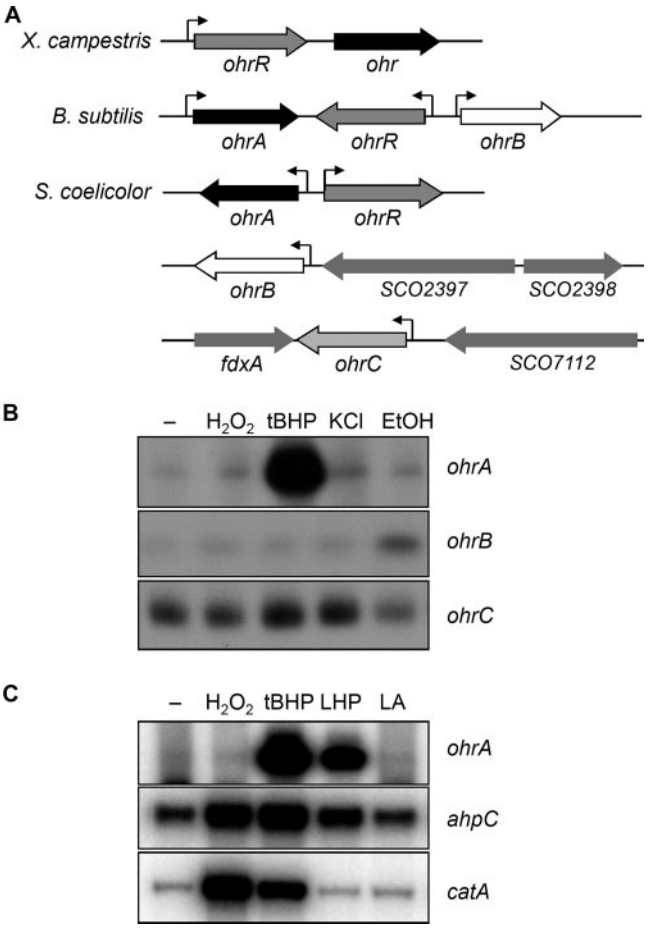

FIG. 1. Expresson of the *ohr* genes. (A) Arrangements of the *ohr* genes in *S. coelicolor*, *X. campestris*, and *B. subtilis*. Bent arrows indicate the position and the direction of transcription. (B) Induction of three *ohr* paralogues in *S. coelicolor* in response to various stressors. M145 cells were grown to early-exponential phase in YEME medium and either left untreated or treated with 0.1 mM H_2O_2, 0.1 mM tBHP, or 4% ethanol (EtOH) for 10 min or with 200 mM KCl for 30 min. S1 mapping analysis was performed to detect transcripts from the *ohrA*, *ohrB*, and *ohrC* genes. (C) Organic peroxide-specific induction of *ohrA* in comparison with other peroxide-inducible genes. Cells were grown and treated with oxidants as in the experiment for which results are shown in panel B. Linoleic acid hydroperoxide (LHP) and linoleic acid (LA) were treated for 10 min at 25 μM final concentrations. Transcripts from the *ohrA*, *ahpC*, and *catA* genes were analyzed by S1 mapping.

to 0.1 mM tBHP, but transcripts returned to the prestimulus level after about an hour. The transcription start (+1) sites were determined by high-resolution S1 mapping (Fig. 2B). The +1 site of *ohrA* was located at the G residue 48 nt upstream from the translational start codon, whereas that of *ohrR* was located at the A residue coinciding with the initiating nucleotide of the start codon. The putative promoter elements were predicted (Fig. 2C). The *ohrA* promoter elements (TCTACT for −10 and TTGCGC for −35 with 17-bp spacing) match quite well with the consensus sequence recognized by the primary vegetative sigma factor σ^{HrdB} (25), whereas the predicted *ohrR* promoter sequences (TACCCT for −10 and AATCGC for −35 with 17-bp spacing) show much less similarity. This

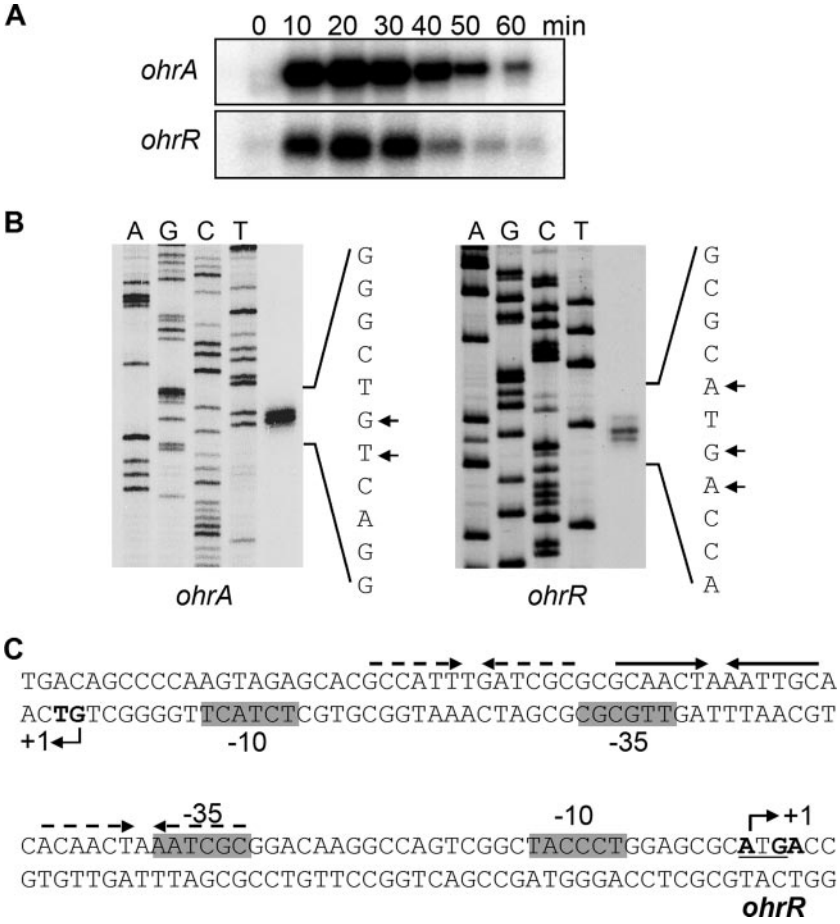

FIG. 2. Organic peroxide induction of the *ohrA* and *ohrR* genes. (A) Time course of induction. M145 cells were grown in YEME liquid medium to mid-exponential phase and treated with 0.1 mM tBHP for various lengths of time (0 to 60 min) before harvest. Transcripts from the *ohrA* and *ohrR* genes were analyzed by S1 mapping. (B) Determination of transcriptional start sites for *ohrA* and *ohrR*. High-resolution S1 mapping was carried out for RNAs prepared from cells treated with tBHP for 10 min. (C) Sequence information in the divergent intergenic region of *ohrA* and *ohrR*. Transcriptional initiation sites of *ohrA* and *ohrR* are boldfaced and are indicated by bent arrows. The +1 site was assigned from the longest protected signal. Putative promoter elements (−10 and −35 boxes) for both genes are shaded. The translational start codon for *ohrR* is underlined. Inverted-repeat motifs are indicated, the primary motif with solid-line arrows and the flanking secondary motifs with dashed arrows.

suggests that *ohrA* could be recognized by the major sigma factor σ^{HrdB}, whereas *ohrR* could be recognized very weakly by σ^{HrdB} or by an uncharacterized alternative sigma factor (20).

OhrR mediates organic-peroxide-responsive induction of *ohrA* and *ohrR* through derepression and activation, respectively. In order to verify the role of OhrR as a putative regulator for *ohrA* and *ohrR*, we created a mutant strain that lacks the majority of the *ohrR* coding region from the 20th codon. The *ohrA* and *ohrR* transcripts were analyzed by S1 mapping in the Δ*ohrR* mutant. The *ohrR*-specific probe whose 5′ end corresponds to nt +49 from the start site is capable of detecting transcripts from the truncated *ohrR* gene, which retains the coding sequence up to nt +57. The effects of mutations of other peroxide-sensing regulators (Δ*oxyR* and Δ*catR*) were examined in parallel for comparison. The results presented in Fig. 3 demonstrate that the expression pattern of *ohrA* was not affected by Δ*oxyR* or Δ*catR* mutations but became constitutive in the Δ*ohrR* mutant, suggesting that OhrR modulates *ohrA* expression as a repressor. To our surprise, however, in the Δ*ohrR* mutant, *ohrR* gene expression was not induced by tBHP, in contrast to about 12-fold induction in the wild type. This suggests that OhrR could act as a positive regulator to induce its own gene in response to oxidants. The uninduced basal level was slightly elevated, about 3.5-fold, relative to the wild type level. We examined the half-lives of the *ohrR* and *ohrA* tran-

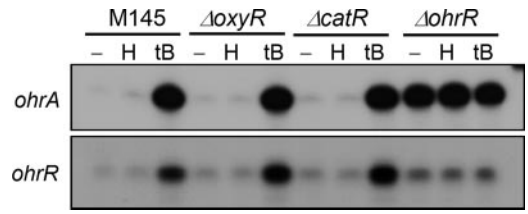

FIG. 3. Dual role of OhrR as a repressor for *ohrA* and an activator for *ohrR*. Transcripts from *ohrA* and *ohrR* were analyzed by S1 mapping in different genetic backgrounds: M145 cells (wild type) and the Δ*oxyR*, Δ*catR*, and Δ*ohrR* mutants. Cells were grown in YEME medium to early-exponential phase and either left untreated or treated with 0.1 mM H_2O_2 (H) or tBHP (tB) for 10 min.

scripts in the wild type and the Δ*ohrR* mutant. The half-life of *ohrA* transcripts was not affected by Δ*ohrR* mutation, being about 30 min. The half-life of *ohrR* transcripts was about 4 min in the wild type and increased more than 10-fold in the mutant (data not shown). This may partly account for the elevated uninduced level of *ohrR* transcripts in the Δ*ohrR* mutant. Taking this effect on mRNA stability into consideration, we can safely propose that OhrR is required for the induction of its own gene at the transcriptional level and does not act by modulating its stability. In other words, OhrR acts as a positive regulator of transcription for its own synthesis in the presence of an organic peroxide.

Binding of OhrR to the intergenic region of *ohrA* and *ohrR*. In order to disclose the binding behavior of OhrR, we purified His-tagged recombinant OhrR from *E. coli* and performed a gel mobility shift assay with a DNA fragment (D1) encompassing the entire intergenic region from nt −115 to +49 relative to the transcription start site of the *ohrR* gene (Fig. 4A). The boundary of the specific binding region in the D1 probe was estimated by competing the bound complex with various competitor DNAs (D2, D3, D4, and D1C1) encompassing different subregions of the long fragment D1. The results in Fig. 4A demonstrate clearly that the specific competition occurred with fragments D2 and D3 but not with D4, suggesting that the region between −61 and −42 contains the specific binding determinant for OhrR. The binding determinant was further narrowed down by using a 60-mer DNA probe (B0; from −81 to −22) and competitors that replaced 10 bp consecutively from position −77 to −28 with an unrelated sequence (ATCGGTGTAC) (B1 to B5) (Fig. 4B). The OhrR-bound complex in the nonmutated fragment (B0) was competed out entirely by the B1 and B2 fragments, suggesting that sequences from −77 to −58 do not contribute to OhrR binding. However, the dramatic decrease in competition by the B3 fragment suggests that a critical binding determinant resides in the 10 bp between −57 and −48. B4 and B5 caused partial and full competition, respectively. Inspection of the nucleotide sequence within the critical binding region determined from the results shown in Fig. 4A and B allowed us to propose a near-perfect inverted-repeat motif (GCAACT-N-AATTGC from −58 to −46) as the primary binding signature for OhrR. Two related motifs with three nucleotide deviations were found to flank the central core repeat (Fig. 4C). As judged from the location of the inverted-repeat motifs, it appears likely that the central core motif is the primary binding site for OhrR and that the adjacent motifs serve for cooperative multimeric binding. This coincides with the presence of three shifted bands for complex formation in the gel.

In order to find the effect of OhrR oxidation, we performed a gel mobility shift assay with OhrR treated with tBHP or cumene hydroperoxide. Two kinds of 60-mer DNA probes were used: one with three inverted repeats from −81 to −22 (B0, as used for Fig. 4B) and one with only the central core repeat (B10, in which the flanking −73-to-−61 and −43-to-−31 regions are replaced with random sequences and which hence retains only the central 17 bp from −60 to −44 nonmutated). Surprisingly, treatment of OhrR with 0.1 mM tBHP or cumene hydroperoxide only slightly decreased the binding of OhrR on the B0 fragment (Fig. 5A). When we used a higher concentration of tBHP (1 mM) or 20 μM linoleic acid hydroperoxide to oxidize OhrR, similar results were obtained (data not shown). Using the B10 fragment, where only a single species of OhrR-bound complex was observed, as expected,

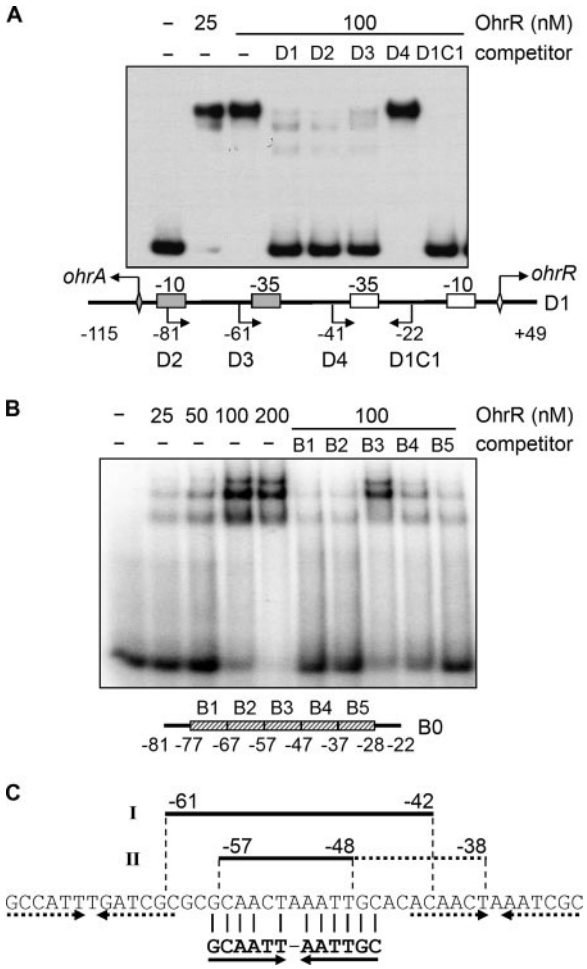

FIG. 4. Binding of purified OhrR to the intergenic region of *ohrA* and *ohrR*. (A) Gel mobility shift assay with an intergenic DNA fragment. An end-labeled D1 probe that spans the region from −115 to +49 nt relative to the *ohrR* transcriptional start site was bound to purified His-tagged OhrR in the absence or presence of cold competitors containing various subsets of D1: D2 from −81 to +49, D3 from −61 to +49, D4 from −41 to +49, and D1C1 from −115 to −22. (B) Fine mapping of the OhrR binding determinant with mutated competitors. The labeled DNA probe (B0) that spans the region from −81 to −22 was bound with OhrR in the absence or presence of various competitor DNAs (B1 to B5) of the same length but with 10-bp substitutions from −77 to −28. (C) The critical region for OhrR binding. Regions I and II, determined through series D and B competitors, respectively, are indicated. The presence of a near-perfect inverted repeat is indicated by solid arrows, along with the flanking repeats of similar sequence (dotted arrows).

we found that organic peroxides significantly weakened the binding of OhrR to the primary binding site. The dissociation constant of binding to the primary site was estimated to change from ~30 nM for reduced OhrR to ~75 nM for oxidized OhrR (data not shown).

The effect of OhrR oxidation on transcription was examined in vitro. Using the RNAP holoenzyme purified from *S. coelicolor*, we were able to detect only the *ohrA* transcripts in vitro. The inability to detect *ohrR* transcripts could be due to low affinity of the holoenzyme for the promoter and/or the scarcity of the specific sigma factor that recognizes the *ohrR* promoter

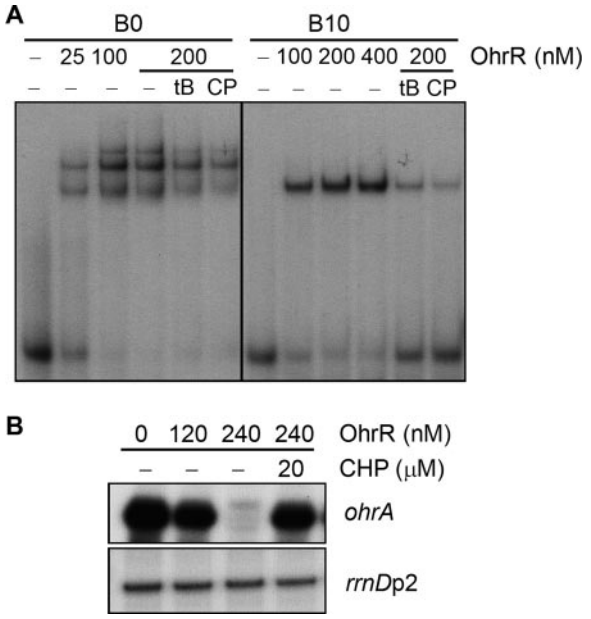

FIG. 5. Effects of organic hydroperoxides on the binding activity of OhrR. (A) Decrease in the binding affinity of OhrR toward DNA by organic peroxides. Gel mobility shift assays were performed with the B0 and B10 probes as described for Fig. 4B. The B10 probe is the same length as B0 (60 bp) but contains only the central inverted-repeat motif intact from −60 to −44, with unrelated flanking sequences. OhrR was either left untreated or treated with 0.1 mM tBHP (tB) or cumene hydroperoxide (CP). (B) Effect of cumene hydroperoxide (CHP) on the repression of *ohrA* transcription by OhrR in vitro. Transcription was performed in vitro with an *ohrA-ohrR* intergenic DNA template and the RNAP holoenzyme purified from *S. coelicolor* as described in the text. Only the transcript from the *ohrA* promoter was detected. OhrR was either left untreated or treated with 20 μM cumene hydroperoxide. As a negative control, transcription from $rrnDp_2$ was monitored.

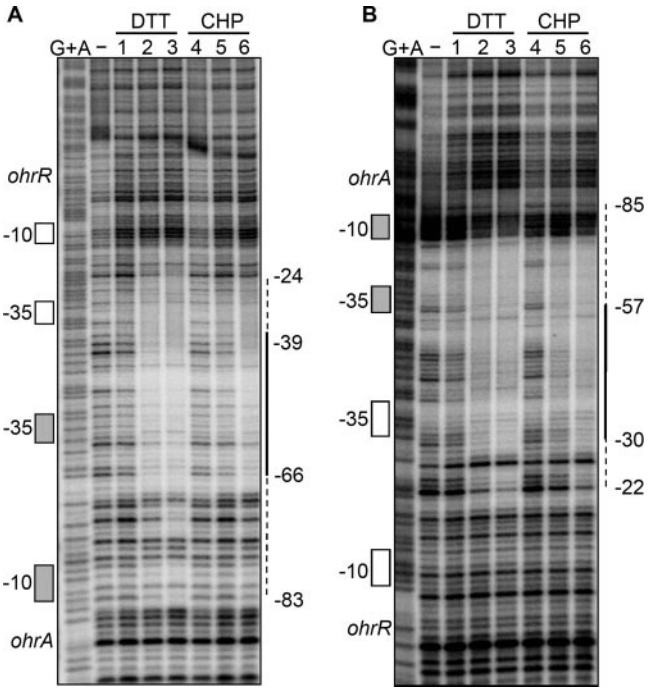

FIG. 6. DNase I footprinting analysis of OhrR binding in the presence and absence of organic peroxide. The DNA probes, labeled at the 5′ end of either the top (A) or the bottom (B) strand, were incubated with increasing concentrations of OhrR (62.5 nM for lanes 1 and 4, 125 nM for lanes 2 and 5, and 250 nM for lanes 3 and 6) treated with either 10 mM DTT (lanes 1 to 3) or 0.2 mM cumene hydroperoxide (CHP) (lanes 4 to 6), followed by DNase I treatment. The samples were run on a 6% polyacrylamide sequencing gel with a Maxam-Gilbert G+A sequencing reaction. The region protected by oxidized OhrR at 250 nM (lanes 6) is indicated by a thick solid line, whereas the extended region protected by reduced OhrR at 125 and 250 nM is indicated by a dashed line.

in the holoenzyme preparation. The results in Fig. 5B show that OhrR inhibits *ohrA* transcription in the absence of oxidants, an effect that can be reversed by treatment with 20 μM cumene hydroperoxide. Therefore, the decrease in the binding affinity of oxidized OhrR most likely allowed competitive binding of the RNAP holoenzyme to the *ohrA* promoter.

To monitor any change in the binding pattern of OhrR by oxidation, we further analyzed OhrR binding through DNase I footprinting. Increasing amounts of OhrR were incubated with the DNA template in either 10 mM DTT or 0.2 mM cumene hydroperoxide in the binding buffer. Either the top or the bottom strand of the DNA probe (−115 to +49) was labeled for detection. The results in Fig. 6 demonstrate that OhrR binding occurs at the same site regardless of oxidation. However, as predicted from the gel mobility shift assay, the extent of protection changed upon oxidation. Whereas the reduced OhrR at 125 nM protected a region from −83 to −24 as detected with the top strand (Fig. 6A, lane 2), weak protection occurred with oxidized OhrR at the same concentration (Fig. 6A, lane 5). The boundary of the protected region was narrower, from −66 to −39. Even at a higher concentration (250 nM), the boundary of protection did not expand to the extent observed with reduced OhrR. The protection pattern detected with the bottom strand (Fig. 6B) was similar to that with the top strand. However, the loss of protection around the −60-to-−80 region was not as pronounced as on the top strand, due to the scarcity of discrete bands. The primary protection site coincides nicely with the major binding site determined by the mobility shift assay and contains the central core inverted repeat. Therefore, the footprinting results demonstrate that the oxidation of OhrR results in a decrease in binding affinity for the primary binding site and a concomitant decrease in cooperative binding to the flanking sites.

Role of the conserved cysteine C28 in redox modulation of OhrR activity. In *S. coelicolor* OhrR, there is only a single cysteine (C28) that is conserved in other OhrR orthologues. To examine the contribution of C28 in modulating OhrR activity, we created a mutant *ohrR* gene (C28S) by replacing C28 with a serine codon. The mutant gene was introduced into the Δ*ohrR* strain via a pSET152-based vector to allow chromosomal integration of the gene through the *att* site. Either a parental vector or the wild-type *ohrR* gene was introduced in parallel as a control. The *ohrA* and *ohrR* transcripts were monitored by S1 mapping in the mutant. The results in Fig. 7 show that in the mutant provided with C28S OhrR, the *ohrA* and *ohrR* genes were not induced by 0.1 mM tBHP. This suggests that C28S OhrR no longer responds to an oxidant and thus stays as a repressor for the *ohrA* gene and does not serve as an activator for *ohrR*. The incomplete repression by wild-type and

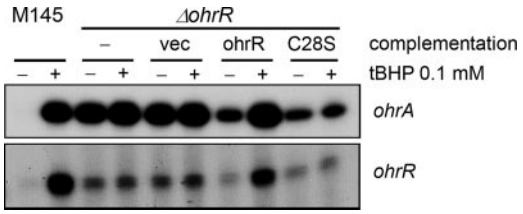

FIG. 7. The critical role of the single cysteine C28 in the organic peroxide sensing of OhrR. Shown are the induction patterns of the *ohrA* and *ohrR* transcripts in a Δ*ohrR* strain complemented with wild-type or C28S mutant *ohrR*. The wild type (M145), the Δ*ohrR* mutant, and the Δ*ohrR* mutant transformed with the parental pSET152 vector (vec) were examined in parallel for comparison. Cells were either left untreated or treated with 0.1 mM tBHP for 10 min to analyze RNAs by S1 mapping.

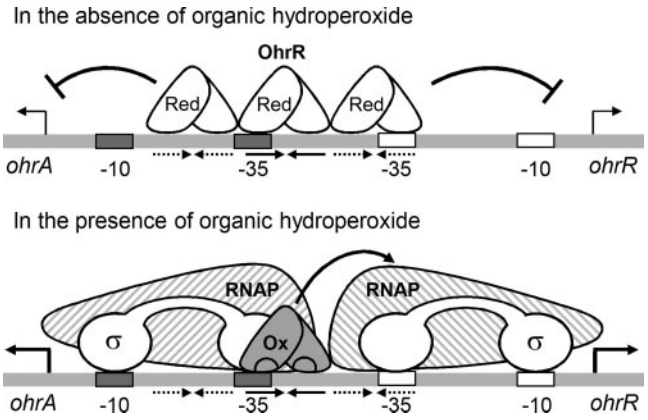

FIG. 8. Model of the dual action of OhrR for the *ohrA* and *ohrR* genes in response to oxidants. In the reduced (Red) form, OhrR binds cooperatively to the *ohrA-ohrR* intergenic region, hindering the binding of RNAP to promoters. In the oxidized (Ox) form, the binding affinity of OhrR decreases, with a concomitant decrease in cooperative binding. This allows competitive binding of RNAP holoenzymes to the *ohrA* and *ohrR* promoters. The loosely bound oxidized OhrR provides additional activation to RNA polymerase bound to the *ohrR* promoter. The two RNAPs at the *ohrA* and *ohrR* promoters could either contain different kinds of sigma factors or share the same sigma factor.

C28S OhrR in the absence of an oxidant is likely to have resulted from (i) a twofold increase in the number of OhrR binding sites due to the provision of a full binding site by the complementing genes and/or (ii) the fact that OhrR was not fully expressed at the *att* site where the complementing genes were integrated. We further examined the DNA binding behavior of the C28S mutant under reducing or oxidizing conditions. In contrast to wild-type OhrR, the mutant form bound to the DNA probe without any apparent change in binding affinity following treatment with tBHP or linoleic peroxide (data not shown).

DISCUSSION

Among the three paralogues for organic hydroperoxide resistance (*ohr*) genes in *S. coelicolor*, *ohrA* is the only gene that is drastically induced by organic hydroperoxides and provides the primary protection against organic hydroperoxides. Even though alkyl hydroperoxide reductase (AhpCD), which is regulated by OxyR, is also induced by short-chain organic hydroperoxides, it plays a less prominent role in protecting *S. coelicolor* against organic hydroperoxides. The slight induction of *ohrB* by ethanol is comparable to the induction of *ohrB* by ethanol and salt in *B. subtilis* (12).

In *S. coelicolor*, OhrR represses the *ohrA* and *ohrR* genes under reducing conditions. Upon oxidation by organic hydroperoxides, the *ohrA* gene is induced through derepression, whereas the *ohrR* gene is induced through activation by OhrR. This regulatory behavior is different from those observed in other bacteria. In *B. subtilis*, *ohrR* expression is not affected by organic hydroperoxides and is not autoregulated (12). In *X. campestris* and *Agrobacterium tumefaciens*, *ohrR* as well as the *ohr* genes is repressed by OhrR and induced by organic peroxides through derepression (7, 43). In contrast to these OhrRs, which drastically lose their DNA-binding affinity upon oxidation by organic hydroperoxides, our study demonstrates that oxidized OhrR from *S. coelicolor* is weakened in binding affinity and thus still remains bound to the intergenic region between the divergent *ohrA* and *ohrR* genes. As described in the model presented in Fig. 8, the reduced form of *S. coelicolor* OhrR binds not only to the primary binding site that partially overlaps the −35 element of *ohrA* but also to the adjacent sites extending toward the −10 element of *ohrA* and the −35 element of *ohrR*. This binding pattern can result in repression for both the *ohrA* and *ohrR* promoters. The decrease in DNA binding affinity will loosen the binding not only to the central primary site but also to the adjacent sites, thus allowing RNAP to bind to the *ohrA* and *ohrR* promoters. Transcription from *ohrA* could be initiated by the RNAP holoenzyme alone, whereas that from *ohrR* appears to require additional activation by bound OhrR (Fig. 8). The position of oxidized OhrR binding to the central core region most likely allows activation via interaction with alpha and/or sigma subunits bound to the *ohrR* promoter without interfering with RNAP binding to the *ohrA* promoter. The possibility of additional involvement of the promoter-proximal site of *ohrR* in activation remains open for verification. The unique spatial arrangement of the two promoters and OhrR binding sites within this intergenic region, as well as the modulation in binding affinity, could have enabled the dual action of OhrR as a repressor when reduced and an activator when oxidized. Our model does not exclude the involvement of another activator whose activity is dependent on oxidized OhrR. However, considering the similar rapid kinetics of derepression (*ohrA*) and activation (*ohrR*) observed in Fig. 2A, it is not likely to involve any other regulator whose synthesis is dependent on OhrR. Whether the binding of RNAP to the *ohrR* promoter requires an alternate sigma factor other than σ^{HrdB} remains to be determined.

Comparison of the crystal structures of several MarR family regulators reveals significant similarity in overall structure: a triangular shape with winged HTH DNA-binding motifs at two corners. However, many local differences are present, revealing conformational plasticity (23, 30). There are reports that several MarR family regulators could function as both negative and positive regulators (2, 10). The mechanism of activation by these dual-function MarR family regulators most often involves competition with repressors and/or further allowance of activators to bind, as observed for SlyA in *Salmonella enterica* serovar Typhimurium, RovA in *Yersinia enterocolitica* and PecS

in *Erwinia chrysanthemi* (10). It has been suggested that RovA, a MarR-type virulence regulator of *Yersinia pseudotuberculosis*, can interact with RNAP and thus enhance transcription directly (46). Examples of switching from negative to positive regulators in response to oxidation, through binding to the same promoter region, have been reported for OxyR in regulating *ahpC* in *X. campestris* (31). Here reduced OxyR represses *ahpC* expression by completely blocking the −35 promoter element, whereas oxidized OxyR shifts the binding site to expose the −35 element and activates *ahpC* transcription in vivo and in vitro. This is consistent with the observation for *E. coli* that both the reduced and oxidized forms of OxyR bind to the same target sequence with different footprinting patterns (45). Similarly, SoxR simultaneously exerts constant repression of *soxR* and conditional activation of the neighboring divergent *soxS* gene without changing its occupancy of the single operator site (22). Even though the binding site is not shifted, activated (oxidized) SoxR bound in the spacer region between the −35 and the −10 element of the *soxS* promoter facilitates open (melted) promoter complex formation (37). Although all these examples share some common features in exhibiting the dual function of a single regulator, the detailed mechanism of switching from a negative to a positive regulatory mode is different with regard to target promoter configurations and the mode of conformational change upon oxidation. *S. coelicolor* OhrR provides yet another mode of dual action through modulation of binding affinity. Compared with that of *B. subtilis* OhrR, the amino acid sequence of the *S. coelicolor* OhrR polypeptide is longer by 13 N-terminal and 8 C-terminal residues. In the dimeric crystal structure of *B. subtilis* OhrR (23), the N terminus of one subunit and the C terminus of the other subunit are located close to each other in the exposed region, which could make contacts with neighboring RNAP. The activation domain and the possible role of C28 oxidation in triggering conformational change in *S. coelicolor* OhrR that enable it to act as an activator, in addition to affecting DNA affinity, remain to be determined.

The in vitro binding study with the full-length intergenic region revealed three OhrR-DNA complexes with different electrophoretic mobilities (Fig. 4B and 5A). One plausible interpretation is that one OhrR dimer binds to the central primary site (complex 1) and another one or two OhrR dimers bind cooperatively to the flanking sites. This is supported by the observation that the DNA probe of the same length containing only the central inverted-repeat sequence (probe B10) allows only one shifted band, which comigrates with the fastest-moving band (complex 1) (Fig. 5A). The sequence of the central primary motif (GCAACT-A-AAT TGC) shares similarity with the putative OhrR binding sequences from *B. subtilis* (TACAATT-T-AATTGTA), *Agrobacterium tumefaciens* (gcgTACAATT-T-AATTGTAcgc), and *X. campestris* (tTGCAATT-N_{17}-AATTGCAa), all sharing CAATT half-site sequences (7, 12, 43). Comparison of amino acid sequences reveals 9 identical residues out of 13 in the DNA recognition helix α4 of *B. subtilis* OhrR. In the *S. coelicolor* genome, only one site (the *ohrA* and *ohrR* intergenic region) matches the GCAANT-N-ANTTGC sequence perfectly. When one mismatch deviation is allowed, 16 sites are found within 350 bp upstream of the coding region. The downstream genes include those for acyl coenzyme A dehydrogenase, putative exporters, and several putative transcriptional regulators. Whether these are regulated by OhrR remains to be determined.

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