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Role of OxyR as a Peroxide-Sensing Positive Regulator in *Streptomyces coelicolor* A3(2)

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Genes encoding a homolog of *Escherichia coli* OxyR (*oxyR*) and an alkyl hydroperoxide reductase system (*ahpC* and *ahpD*) have been isolated from *Streptomyces coelicolor* A3(2). The *ahpC* and *ahpD* genes constitute an operon transcribed divergently from the *oxyR* gene. Expression of both *ahpCD* and *oxyR* genes was maximal at early exponential phase and decreased rapidly as cells entered mid-exponential phase. Overproduction of OxyR in *Streptomyces lividans* conferred resistance against cumene hydroperoxide and H_2O_2. The *oxyR* mutant produced fewer *ahpCD* and *oxyR* transcripts than the wild type, suggesting that OxyR acts as a positive regulator for their expression. Both *oxyR* and *ahpCD* transcripts increased more than fivefold within 10 min of H_2O_2 treatment and decreased to the normal level in 50 min, with kinetics similar to those of the CatR-mediated induction of the catalase A gene (*catA*) by H_2O_2. The *oxyR* mutant failed to induce *oxyR* and *ahpCD* genes in response to H_2O_2, indicating that OxyR is the modulator for the H_2O_2-dependent induction of these genes. Purified OxyR protein bound specifically to the intergenic region between *ahpC* and *oxyR*, suggesting its direct role in regulating these genes. These results demonstrate that in *S. coelicolor* OxyR mediates H_2O_2 induction of its own gene and genes for alkyl hydroperoxide reductase system, but not the catalase gene (*catA*), unlike in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium.

OxyR is an H_2O_2-sensing transcriptional regulator inducing more than 10 genes in response to H_2O_2 in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (15, 43). It is activated by a disulfide bond formation between two cysteine residues and induces the expression of *oxyS* (which encodes a small, nontranslated regulatory RNA), *katG* (which encodes hydrogen peroxidase I), *ahpC* (which encodes alkyl hydroperoxide reductase), *gorA* (which encodes glutathione reductase), *dps* (which encodes DNA binding protein), and *grxA* (which encodes glutaredoxin 1). Glutaredoxin 1 deactivates OxyR by reducing the disulfide bond, forming an autoregulatory feedback loop (49). Irrespective of its redox state, OxyR also acts as a repressor of its own expression like other LysR family of transcriptional regulators do. Several *oxyR* genes have been identified in other organisms, such as *Haemophilus influenzae* (35), various *Mycobacterium* species (20, 22, 36), *Xanthomonas* species (34), and even the anaerobic bacterium *Bacteroides fragilis* (40).

In *Mycobacterium* and *Xanthomonas* species, the *oxyR* gene is tightly linked to the genes for alkyl hydroperoxide reductase system. In *E. coli* and *S. enterica* serovar Typhimurium, the alkyl hydroperoxide reductase system is composed of two components, AhpC (22 kDa) and AhpF (54 kDa) (30). The reduced form of AhpC converts alkyl hydroperoxides to the corresponding alcohols with concomitant oxidation of the two sulfhydryls to a disulfide bond between two subunits. It has recently been reported that AhpC is the main defense system against endogenously generated hydrogen peroxide (18, 19). The oxidized AhpC contains two intersubunit disulfide bonds per dimer (39). AhpF, which shows homology to the thioredoxin reductase family, reduces the oxidized AhpC by transferring reducing equivalents from NAD(P)H to the disulfide of AhpC. The reduction of AhpC is mediated by two cysteine disulfide centers in AhpF (7). Likewise, in *Bacillus subtilis* (2, 5) and *Xanthomonas* species (34), AhpF is involved in the reduction of AhpC. In *Xanthomonas* species, the *ahpF*, *oxyR*, and *orfX* genes are arranged in an operon, and the *ahpC* gene is located upstream of *ahpF* as a monocistronic transcription unit. On the other hand, in *Mycobacterium* species, the *ahpC* gene is divergently transcribed from the *oxyR* gene, whereas the *ahpF* homologue has not been identified. Instead, the *ahpD* gene, encoding a protein with a thioredoxin fold, is located downstream of *ahpC* (48). It has recently been reported that AhpD in complex with Lpd (dihydrolipoamide dehydrogenase) and SucB (dihydrolipoamide succinyltransferase) reduces AhpC in an NADH-dependent manner (4).

AhpC homologues, named thiol-specific antioxidant or thioredoxin peroxidase (TPx), are also distributed among eukaryotic organisms (8, 10). In *Saccharomyces cerevisiae*, two AhpC homologous proteins, Tsa1p and Ahp1p, have been identified (9, 11, 32). Both proteins form intermolecular disulfide bonds, which can be specifically reduced by thioredoxin. Therefore, thioredoxin and the thioredoxin reductase system have the function of AhpF in this organism. In spite of the similarity in structure and activation mechanism between Tsa1p and Ahp1p, their activity is known to be specific for H_2O_2 and organic peroxide, respectively, in budding yeast.

Streptomyces is a gram-positive soil bacterium with high GC content which undergoes a complex cycle of morphological and physiological differentiation during growth. In previous stud-

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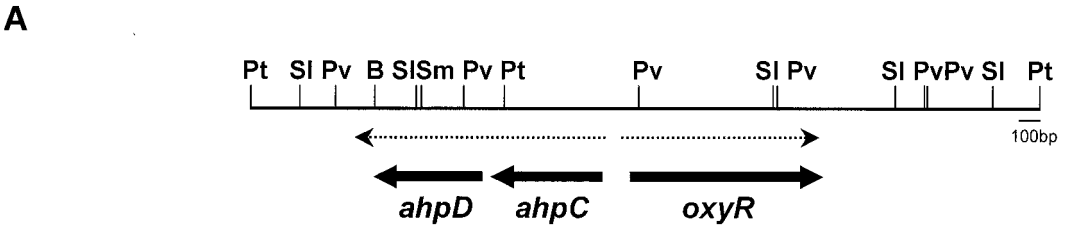

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Sco MSSKKRQPSLAQLRAFDAVAEHLHFRDAAAAIGMSQPALSGAVSALEEALGVTLLERTTRKVLLSPAGARLAVRTKAVLAEVGALVEEAEAVRAPFTGALRLGVIPTV
Mle MSDKSYQPTIAGLRAFVAVVEKGHFSAAASFLGVRQSTLSQALAALESGLGVQLIERSTRRVFVTTQGRHLLPRAQAAIEAMDAFTAAAAGESDPLRGGMRLGLIPTV
Xca        MNLRDLKYLVALADHKHFGRAASACFVSQPTLSTQIKKLEDELGVSLVERAPRKVMLTPAGREAAVRARSIVAEVEQMKEAARRSQDPEAGTVRLGIFPTL
Eco        MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLFTQAGMLLVDQARTVLREVKVLKEMASQQGETMSGPLHIGLIPTV
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Sco APYLLPTVLRLVHERYPDLDLQVHEEQTASLLEGLTTGRLDLLLLAVPLGVPGVTELPLFDEDFVLVTPLPHRLGGREGLERSVLRELKLLLLDEGHCLRDQALDICR
Mle APYVLPTMLTGLTHRLPTLTLRVVEDQTEHLLTALREGALDAAMIALPVETAGIAEIPIYDEDFVLALPPGHPLSGKRRVPTTALAQLPLLLLDEGHCLRDQTLDICR
Xca APYLLPHVVPRIRQRFPRLELLLIEEKSDQLMHQLREGRLDAALLALPLQDDQLHAEFLFEEPFVLAVPEGHPLSRHDSMTLDDLSEQRLLLLEDGHCLRDQALDVCH
Eco GPYLLPHIIPMLHQTFPKLEMYLHEAQTHQLLAQLDSGKLDCVILALVKESEAFIEVPLFDEPMLLAIYEDHPWANRECVPMADLAGEKLLMLEDGHCLRDQAMGFCF
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Sco EAGRAGVPATTTTAAGLSTLVQLVAGGLGVTLLPRTAVRVETSRSSQLLTASFTDP-APTRRIALAMRTGAARSAEYGELAAAVREAMA-DLPVRTVHD
Mle KSG-VQAELANTRAASLATAVQCVTGGLGVTLLPQSAAPVESVR-SKLGLAQFAAP-CPGRRIGLAFRSASGRSASYRQIAKIIGELISTEHHVRLVA
Xca LAG--ALEKSEFQATSLETLRQMVAANVGVTLLPLLAVKPPVARSENIRLIRFREDKQPSRRIAMAWRRSSAMTAFLEQLSQLFKELPESLFTLDQPATGPKAVAA
Eco EAG--ADEDTHFRATSLETLRNMVAAGSGITLLPALAVPPERKRDGVVYLPCIKPE--PRRTIGLVYRPGSPLRSRYEQLAEAIRARMDGHF----DKVLKQAV
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FIG. 1. (A) Restriction map and organization of the *ahpC*, *ahpD*, and *oxyR* genes. A restriction map of the 3.8-kb *Pst*I fragment containing the *ahpCD* and *oxyR* genes is presented. Thick arrows indicate the positions and directions of the three genes. The two divergent transcripts are shown as dashed arrows. Abbreviations: B, *Bam*HI; Pt, *Pst*I; Pv, *Pvu*II; SI, *Sal*I; Sm, *Sma*I. (B) Comparison of the predicted amino acid sequence of OxyR with its homologues. The amino acid sequence of OxyR from *S. coelicolor* (Sco) (AF186371) was aligned with those from *M. leprae* (Mle) (Al035300), *E. coli* (Eco) (J04553), and *X. campestris* (Xca) (U94336). The position of two cysteine residues involved in disulfide bond formation and activation of OxyR in *E. coli* are shaded and presented in boldface type.

ies, adaptive response to H_2O_2 was observed in *Streptomyces coelicolor* (33). Two-dimensional protein gel analysis revealed that *S. coelicolor* induced synthesis of more than 100 proteins when exposed to H_2O_2, and the *catA* gene encoding catalase A was identified as one of the H_2O_2-inducible genes (13, 17). The production of catalase A, which is the major vegetative catalase, is regulated by a peroxide-sensing repressor, CatR (26). Another peroxide-sensing transcriptional regulator found in *S. coelicolor*, RsrA, is an antisigma factor for σ^R, which directs the expression of thioredoxin genes (31, 37, 38). In this study, we present our finding of a third peroxide sensor in this organism, OxyR, and its role in regulating the expression of alkyl hydroperoxide reductase and its own gene product.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. coelicolor* M145 and *Streptomyces lividans* TK24 cells were grown as described previously (29). *E. coli* DH5α and BL21(DE3)pLysS were used for cloning and overexpression, respectively. *E. coli* ET12567 was used to prepare unmethylated DNA to transform *S. coelicolor*. XL1-Blue MRA was used as a host for the λEMBL3 genomic library of *S. coelicolor*.

Cloning of *ahpC*, *ahpD*, and *oxyR* genes. An internal *ahpC* gene fragment of 407 bp was generated from *S. coelicolor* by PCR using primers ACN (5′TTCT TCTGGCC[C/G]AAGGACTTCAC3′) and ACC (5′TTCAGAGT[C/G]GGGT CGCCGTT[C/G]C3′) designed from the conserved regions among known bacterial AhpC proteins. The PCR product was used as a probe to screen the λEMBL3 genomic library of *S. coelicolor* M145. The common 3.8-kb *Pst*I fragment in positive clones was sequenced and found to contain *ahpC*, *ahpD*, and *oxyR* genes (Fig. 1A). The nucleotide sequence information was deposited in GenBank under accession number AF186371.

RNA isolation. RNA was isolated from M145 cells grown in YEME (29). Cells were resuspended in modified Kirby mixture (1% sodium-triisopropyl naphthalene sulfonate, 6% sodium 4-amino salicylate, 6% phenol equilibrated with 10 mM Tris-HCl buffer [pH 8.3]) and disrupted by sonication with a microtip (Sonics and Materials Inc.) at 25% of the maximum amplitude (600 W, 20 kHz).

Northern blot analysis. Northern blot analysis of the *ahpC* transcript was performed according to standard procedures (41). Fifty micrograms of RNA was electrophoresed on a 1.2% agarose gel containing formamide. The probe used for detecting *ahpC* transcript was a 597-bp *ahpC* gene fragment, produced by PCR using primers ACON (5′TTGGAGAGCATATGCTCACTGTCG3′) and ACOC (5′CGGACTTCAGGGATCCGAGGGAC3′), and was labeled with [α-^{32}P]dATP.

S1 nuclease protection analysis. To generate the probe for S1 mapping the 5′ end of the *ahpCD* transcript, a 261-bp fragment was amplified by PCR using primers ACS1 (5′GGCGGTCAGGTCGAACTCGGGG3′) and OXYS1 (5′CA CGGAAGTGCAGGTGCTCGG3′) from pJH101 containing a 3.7-kb *Sma*I fragment in pUC18 (Fig. 2D). The amplified fragment was end labeled and digested with *Pvu*II, and the 218-bp probe was uniquely labeled at the ACS1 5′ end 45 nucleotides (nt) downstream from the start codon and was eluted from the agarose gel. For the *oxyR* probe, an 802-bp fragment was generated by PCR using primers OXYS1 and ACOC from pJH101. Since the 5′ end of the OXYS1 primer is located upstream of the *Nar*I site used to disrupt the *oxyR* gene, this probe can detect transcripts generated from the *oxyR* promoter in *oxyR* mutants as well. The amplified fragment was end labeled and digested with *Pst*I to prepare a 691-bp probe uniquely labeled at the OXYS1 5′ end, which is 79 nt downstream from the start codon. S1 mapping analysis was carried out with 5 to 50 μg of RNA as described previously (42). The hybridization products were analyzed on sequencing gels with the sequencing ladder generated from the primers ACS1 or OXYS1 and pJH101 as a template. To S1 map the *catA* gene, a 0.6-kb *Sal*I/*Bgl*II fragment uniquely labeled at the *Bgl*II site was used as a probe as previously described (26).

Disruption of the *oxyR* gene in *S. coelicolor*. A *Nar*I/*Sal*I fragment (0.4 kb) of the *oxyR* gene cloned in pUC18 (pJH110) was excised as a *Hin*dIII fragment using polylinker sites and cloned into pKC1139 (3), which contains a tempera-

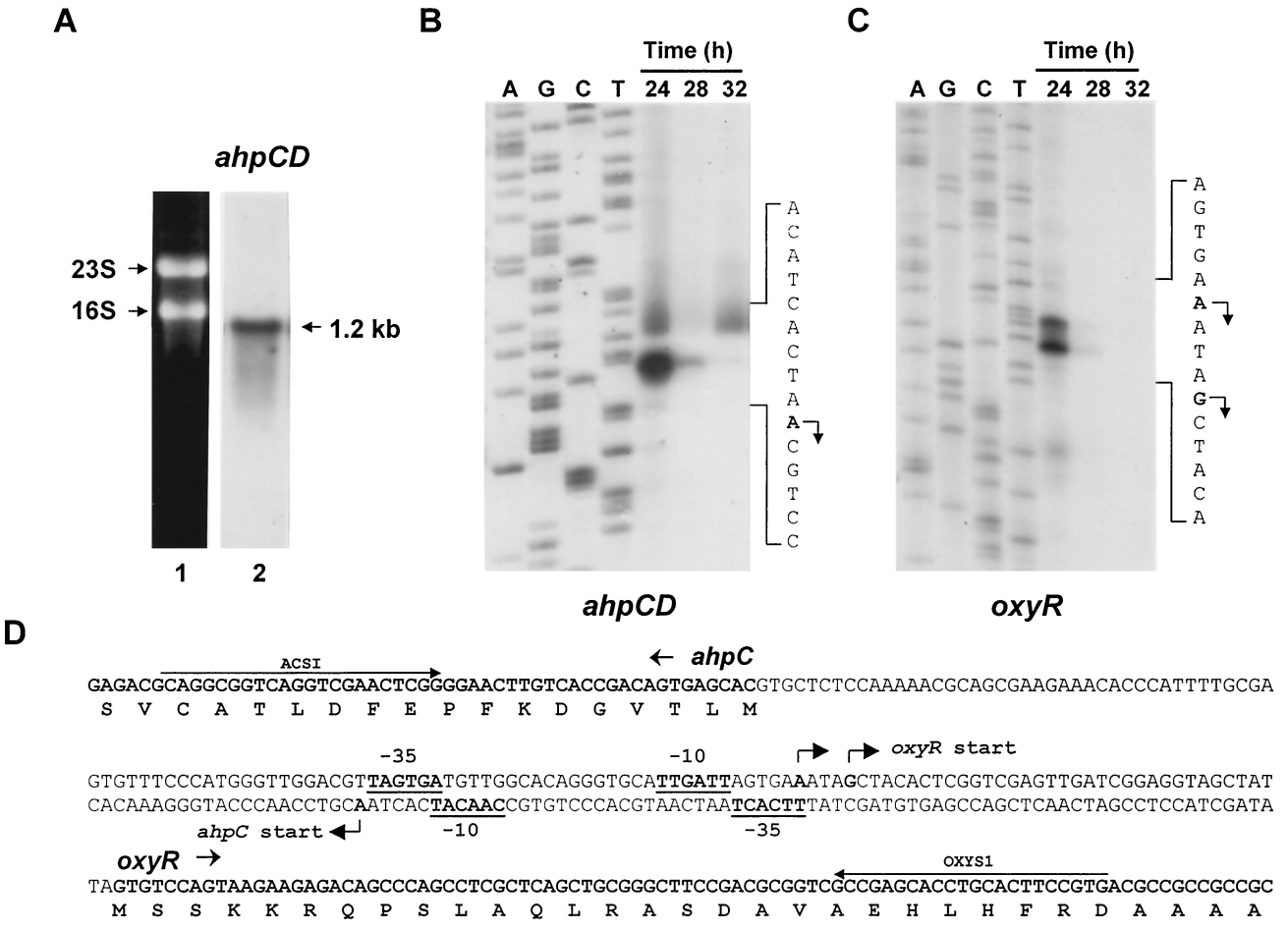

FIG. 2. Transcription of the *ahpCD* and *oxyR* genes. (A) Northern blot analysis of the *ahpC-ahpD* transcript. RNA was isolated from *S. coelicolor* M145 cells grown in YEME for 24 h. Fifty micrograms of RNA was loaded on 1.2% agarose gel containing formamide, and Northern blot analysis was carried out using a 0.6-kb *ahpC* DNA probe as described in Materials and Methods (lane 2). Lane 1 shows 23S and 16S rRNA bands stained with ethidium bromide. (B and C) High-resolution S1 mapping of the 5′ ends of *ahpCD* (B) and *oxyR* (C) mRNA. RNAs prepared from M145 cells grown in YEME at 30°C for 24, 28, and 32 h, were subjected to S1 mapping analysis as described in Materials and Methods. The protected fragments were analyzed on sequencing gels with sequencing ladders generated from the same primers and templates used for the preparation of the probes. The transcription start sites are shown in boldface type and designated by arrows on the sense sequence of each transcript. (D) Nucleotide sequence of the intergenic region of *oxyR* and *ahpCD*. The *oxyR* sense strand sequence is presented, except in the center line, where both strands are presented to show divergent promoter elements. Transcription start sites of the *ahpCD* (*ahpCp*) and *oxyR* (*oxyRp*) are indicated by bent arrows. The putative −10 and −35 elements of *ahpCD* and *oxyR* promoters are in boldface type and underlined. Primers used to generate S1 probes (ACS1 and OXYS1) are indicated by arrows.

ture-sensitive replication origin, resulting in pJH405. The pJH405 plasmid DNA was prepared from *E. coli* ET12567 and then introduced into *S. coelicolor* M145 protoplasts. The transformants were selected on plates containing apramycin (50 μg/ml) at 30°C. Spores of the transformants were plated on NA medium (29) containing apramycin and incubated at 37°C for 2 days to select plasmid-integrated clones. Disruption of the *oxyR* gene was confirmed by Southern hybridization.

Overproduction and partial purification of OxyR. Mutagenic primers OXYON (5′AGGTAGCTACATATGTCCAGTAAG3′; the *Nde*I site is underlined) and OXYOB (5′GGGTGGTCGCCGGATCCCTCA3′; the *Bam*HI site is underlined) were used to amplify the *oxyR* coding region by PCR. The 972-bp PCR product digested with *Nde*I and *Bam*HI was cloned into pET21c (Novagen) to generate pJH4. *E. coli* BL21(DE3)pLysS cells harboring pJH4 were grown in 200 ml of Luria-Bertani medium to an A_{600} of 0.5 and treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Following harvest, cells were resuspended in lysis buffer (20 mM Tris-HCl [pH 7.9], 0.15 M NaCl, 5 mM EDTA, 0.1 mM dithiothreitol [DTT], 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) and disrupted by sonication. The lysate was centrifuged at 16,000 × *g* for 10 min, and the pellet was washed with lysis buffer containing 2% sodium deoxycholate and recentrifuged. The washed pellet was dissolved in lysis buffer containing 8 M urea, and the insoluble residue was removed by centrifugation at 16,000 × *g* for 10 min. The dissolved protein was dialyzed twice for 8 h against 10 volumes of lysis buffer at 4°C. The dialyzed extract was centrifuged at 16,000 × *g* for 20 min to remove any precipitated material, and the clarified solution was loaded onto 10 ml of heparin-Sepharose (CL 6B column). Proteins were eluted using a gradient of 0.2 to 1.0 M NaCl in TGED buffer (10 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol). OxyR was eluted with NaCl at a concentration between 0.4 and 0.6 M NaCl. The fractions enriched in OxyR were pooled and dialyzed against the storage buffer (10 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 M KCl, 50% glycerol).

Gel mobility shift assay. The DNA fragment spanning the *ahpC-oxyR* intergenic region was generated by PCR using primers ACS1 (5′ end at position −182 relative to the *oxyR* start codon) and OXYS1 (5′ end at position +79). The promoter fragment of the *furA-catC* operon spanning from nt −99 to +92 relative to the *furA* start site was also generated by PCR as described previously (27). The PCR product was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. A 0.6-kb *Sal*I/*Bgl*II fragment containing the *catA* promoter up to nt −364

was end labeled with [α-^{32}P]dATP and Klenow enzymes according to the method of Hahn et al. (26). The unlabeled isotope was removed by centrifugation through a Sephadex G-50 spin column. The labeled probe was incubated with 200 ng of purified OxyR in 20 μl of binding buffer [25 mM Tris-HCl (pH 7.8), 0.5 mM EDTA, 6 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 100 μg of poly(dI-dC) per ml, 5% glycerol] at 30°C for 10 min. The DNA-protein mixture was electrophoresed on a 4% native polyacrylamide gel in 20 mM Tris-borate buffer. The gels were dried and analyzed by autoradiography.

Expression of the *ahpC*, *ahpD*, and *oxyR* genes in *S. lividans*. The recombinant plasmid pJHOxyR was constructed by cloning a 2.6-kb *Pst*I fragment containing the *oxyR* gene into the *Pst*I site of pIJ702, a *Streptomyces* multicopy plasmid vector. To generate pJHAhpCD containing the complete *ahpC* and *ahpD* genes, a 1.9-kb *Pst*I/*Pvu*II fragment was cloned into pUC18 and then into the *Eco*RI/*Hin*dIII sites of pIJ718, a pIJ702-derivative containing modified polycloning sites (donated by Y.-H. Cho). To generate pJHAhpC carrying the complete *ahpC* gene, a 0.8-kb *Pvu*II fragment was cloned into pUC18 and then into pIJ718 using the *Eco*RI and *Hin*dIII sites in the polylinker region. Preparation of protoplast and transformation were done as described elsewhere (29). The transformants were selected and maintained in the presence of thiostrepton (50 μg/ml).

Western blot analysis. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol) for 10 min and then transferred to nitrocellulose membrane (BA79; Schleicher & Schuell) at 60 V for 60 min using Trans-Blot Cell (Bio-Rad). The membrane was blocked for 1 h in Tris-buffered saline containing 0.1% Triton X-100 (TBST) supplemented with 0.5% bovine serum albumin. The membrane was then incubated with a 1:10,000 dilution of polyclonal mouse antibodies raised against AhpC and AhpD for 1 h and then washed twice with TBST for 10 min each. The reacting signal was detected by goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase using a Western ECL detection system (Amersham Biosciences, Ltd.).

RESULTS

Cloning and sequence analysis of the *oxyR* and *ahpCD* genes in *S. coelicolor*. A gene fragment containing an *ahpC* gene homologue was isolated from the λEMBL3 genomic library of *S. coelicolor* A3(2) M145. The nucleotide sequence analysis of the common 3.8-kb *Pst*I fragment from positive clones revealed that this region contains three open reading frames (ORFs), one showing high homology to known *ahpC* genes and another showing high homology to known *oxyR* genes (Fig. 1A).

The *ahpC* gene encodes a protein of 184 amino acids with a calculated molecular mass of 20,679 Da. This protein is highly homologous to other known bacterial alkyl hydroperoxide reductases (AhpC; about 60% identity with those from *Mycobacterium* spp.) and its eukaryotic homologues known as thiol-specific antioxidants or thioredoxin peroxidases. Two active-site cysteine residues are conserved among these AhpC homologues. The *ahpD* gene is located 7 nt downstream of the *ahpC* gene, encoding a protein of 178 amino acids (19,000 Da) with significant homology (about 55 to 80% identity) to *ahpD* genes also located downstream of the *ahpC* gene in *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Streptomyces viridosporus*.

The *oxyR* gene is located 138 nt upstream from the *ahpC* gene in a divergent orientation. It encodes a protein of 313 amino acids (33,096 Da) showing homology to other known OxyR proteins from *E. coli* (16), *Mycobacterium leprae*, *H. influenza* (35), *Xanthomonas campestris*, and *S. viridosporus* (Fig. 1B). Two cysteine residues (C^{199} and C^{208}) known to be involved in the disulfide bond formation and activation of OxyR in *E. coli* are also conserved in the *S. coelicolor* OxyR protein (C^{206} and C^{215}).

The organization of the *ahpC*, *ahpD*, and *oxyR* genes in *S. coelicolor* is the same as those found in *S. viridosporus* and several *Mycobacterium* species such as *M. leprae*, *M. tuberculosis*, and *M. bovis* (36, 48). In *M. tuberculosis*, *oxyR* is naturally inactivated by multiple mutations (20). In *M. leprae*, translation of the region downstream of *ahpC* gene revealed the presence of an AhpD homologue. However, this ORF contains a frameshift and hence is interrupted by stop codons. It is not certain whether this truncation is due to a sequencing error.

Analysis of transcripts from *ahpC*, *ahpD*, and *oxyR*. Since the start codon of the *ahpD* coding region is located just 7 nt downstream of the *ahpC* stop codon, the possibility of their cotranscription was examined by Northern hybridization analysis. The size of the mRNA hybridized with the randomly labeled *ahpC* gene fragment was about 1.2 kb (Fig. 2A), indicating that the *ahpC* and *ahpD* genes are cotranscribed from a single promoter.

To determine the transcription start site, S1 nuclease mapping analysis was done. The RNA samples were prepared from M145 cells grown for 24, 28, and 32 h in YEME. The examined time span corresponded to the growth from the early to the mid-exponential phases. When hybridized with the 168-bp S1 probe, end labeled at the 5′ end of primer ACS1, a single species of protected band was detected, suggesting that the transcription starts from the A residue 63 nt upstream of the *ahpC* start codon (Fig. 2B). The level of *ahpCD* transcript was high at the early exponential phase and decreased rapidly when cells entered the mid-exponential phase. Putative −35 (TTCA CT) and −10 (CAACAT) promoter elements resembling $E\sigma^{hrdB}$-type consensus sequences (TTGaCA-N_{17-18}-TAgaaT, lowercase letters indicate less conserved nucleotides) were identified upstream of the transcription start site (Fig. 2D).

The transcription start site of the *oxyR* gene was also determined using the same RNA samples (Fig. 2C). Two species of protected bands were detected with the 691-bp S1 probe end labeled at the 5′ end of primer OXYS1 (Fig. 2D). The transcription start sites of the *oxyR* gene were mapped to the A and G residues located at 40 and 36 nt upstream of the *oxyR* start codon, respectively (Fig. 2D). The expression pattern of *oxyR* mRNA during growth was similar to that of *ahpCD* mRNA. Putative −35 (TAGTGA) and −10 (TTGATT) promoter elements resembling $E\sigma^{hrdB}$-type consensus sequences were identified upstream of the transcription start site (Fig. 2D).

AhpC and AhpD proteins were overproduced in *E. coli* using the pET21c overexpression vector. The apparent molecular masses determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were a little higher (AhpC, 25 kDa; AhpD, 21 kDa) than those predicted form the nucleotide sequences. The overexpressed proteins were used to raise mouse antibodies. The change in the level of AhpC and AhpD proteins was then monitored during growth by immunoblot analysis (Fig. 3). The decrease in the level of AhpC and AhpD proteins during growth reflected their mRNA levels. The change in the level of AhpC protein was more dramatic than that in the AhpD protein levels, most likely reflecting their difference in stability.

Effect of OxyR and AhpCD overproduction. To investigate the role of AhpC, AhpD, and OxyR in defense against oxidative stress, we introduced these genes into *S. lividans* TK24 on multicopy plasmid pIJ702 and investigated the effect of their overproduction on the synthesis of various antiperoxide enzymes and resistance against oxidants.

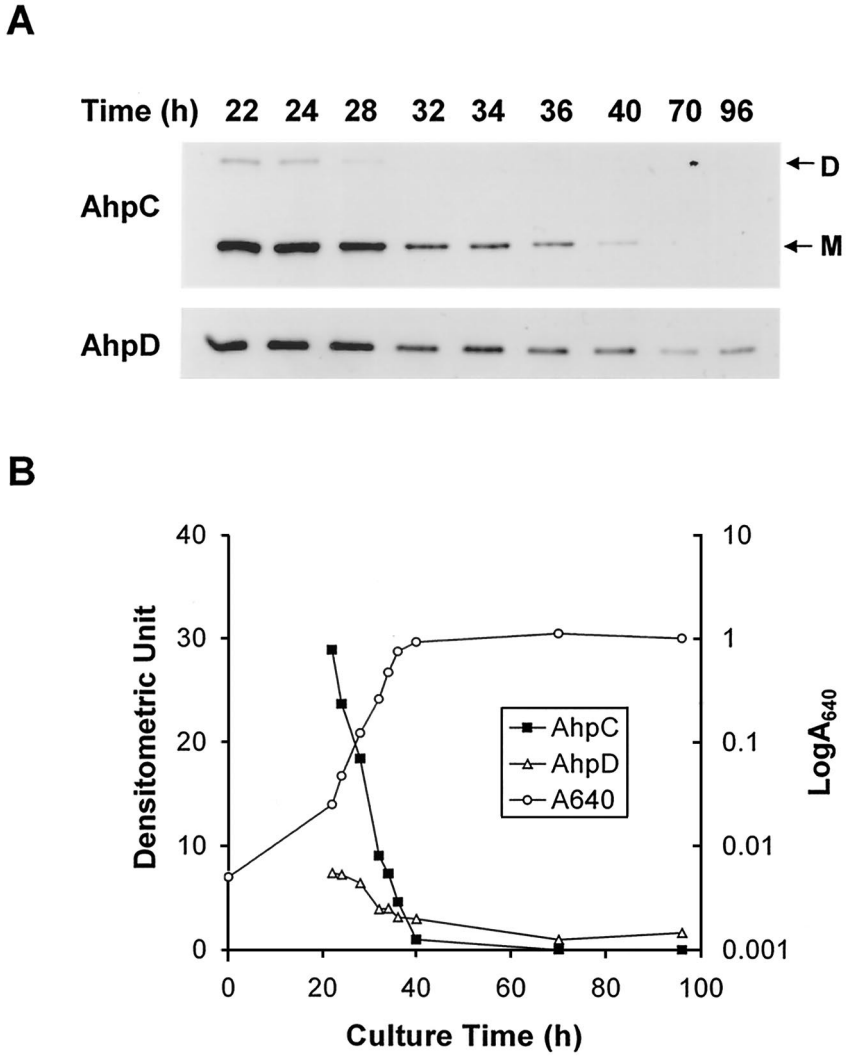

FIG. 3. Growth-dependent expression of AhpC and AhpD proteins. M145 cells were grown in YEME. At various time points aliquots were taken for measurement of optical density at 640 nm and lysed to prepare cell extracts. (A) The AhpC and AhpD proteins were detected by Western blot analysis as described in Materials and Methods. The positions of the monomer (M) and dimer (D) of AhpC are shown. (B) The growth curve and the relative band intensity of the densitometric tracing data in panel A are presented. The densitometric value was set to 1 for AhpC at 40 h (closed square) and for AhpD at 70 h (open triangle).

The level of antiperoxide enzymes in cells grown on NA plate for 24 and 36 h, corresponding to the substrate mycelium and aerial mycelium stage, was examined by immunoblotting (Fig. 4A). Control cells harboring pIJ702 vector only exhibited growth phase-dependent expression of AhpC and AhpD on NA plate as observed in liquid culture of *S. coelicolor*. Overproduction of OxyR led to prolonged synthesis of AhpC and reduced production of CatA as observed at 36 h. When AhpC and AhpD (pJHAhpCD) or AhpC (pJHAhpC) alone were overproduced, catalase A and catalase C expression decreased compared with that observed in the control cells. These results imply the existence of a mechanism balancing the expression of AhpC and catalases. Synthesis of other antioxidant enzymes such as superoxide dismutases (SodN and SodF) and glucose-6-phosphate dehydrogenase were not affected by the elevation of OxyR or AhpC levels (data not shown).

We then examined the effect of these genes on resistance against oxidative stress by plating spores on NA plates containing various oxidants (Fig. 4B). Overproduction of AhpC alone was enough to confer resistance to cumene hydroperoxide but not to H_2O_2. Cells containing pJHOxyR were more resistant to cumene hydroperoxide and to H_2O_2 than the cells containing pJHAhpC or pJHAhpCD. These results demonstrates that (i) AhpC can function in removing the toxic effect of organic peroxide as predicted and (ii) OxyR induces the defense system against H_2O_2 and organic peroxide, involving further antioxidant proteins in addition to AhpCD and catalases.

Positive regulation of *ahpCD* and *oxyR* genes by OxyR. To examine the role of OxyR in transcriptional regulation, *oxyR* gene was disrupted by integration of a pKC1139-derived recombinant plasmid containing an internal fragment of *oxyR* gene. The growth rate of the *oxyR* disruptant (JH10) was significantly reduced compared to the wild type, similar to the

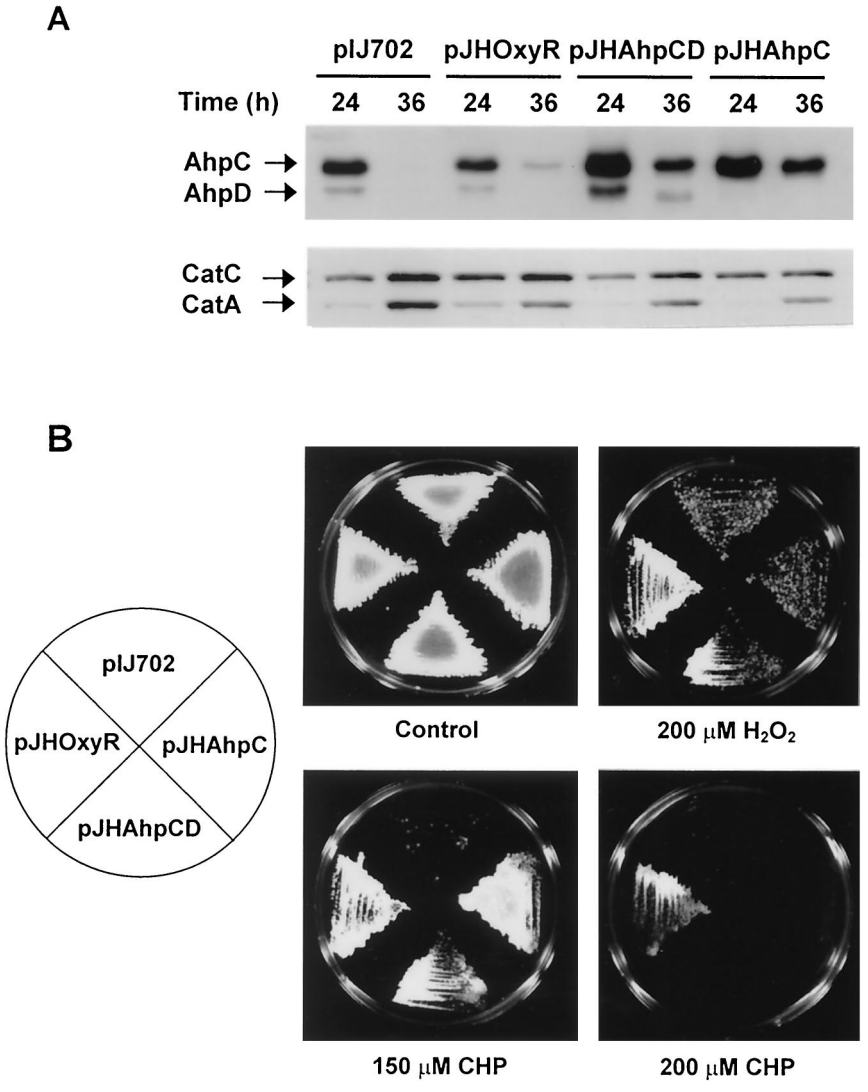

FIG. 4. Overproduction of AhpC, AhpD, and OxyR and their contribution to resistance against oxidants. (A) Overproduction of AhpC and AhpD proteins. *S. lividans* cells containing *oxyR* (pJHOxyR), *ahpCD* (pJHAhpCD), or *ahpC* (pJHAhpC) genes on multicopy plasmid pIJ702 were grown on NA plates containing thiostrepton (50 μg/ml) until they formed substrate mycelium (24 h) or aerial mycelium (36 h). The amount of CatA, CatC, AhpC, and AhpD proteins in cell extracts was determined by Western blot analysis. (B) Effect of overproduction on resistance against hydrogen peroxide and cumene hydroperoxide. About 10^6 spores of cells harboring plasmids were transferred to plates of NA medium containing 200 μM hydrogen peroxide, 150 to 200 μM cumene hydroperoxide (CHP), or no oxidants (control) with thiostrepton (50 μg/ml). Cells were incubated at 30°C for 3 days.

behavior of *E. coli oxyR* mutant (24). Furthermore, JH10 exhibited the bald phenotype with a failure in the formation of aerial mycelium on R2YE plates (29). It differentiated normally on SFM or minimal medium (29). When the level of *ahpCD* and *oxyR* transcripts in wild type (M145) and *oxyR* mutant (JH10) was examined by S1 mapping, we found that both transcripts were markedly decreased in the mutant, implying that OxyR acts as a positive regulator of both *ahpCD* and *oxyR* gene expression (Fig. 5). For *oxyR* mutant, the *oxyR*-specific probe detects nonfunctional RNA containing only the N-terminal part of *oxyR* gene and some vector sequence. Hence, there is a possibility that some probable change in RNA stability may interfere with accurate measurement. Expression of other genes such as *catC* (which codes for catalase-

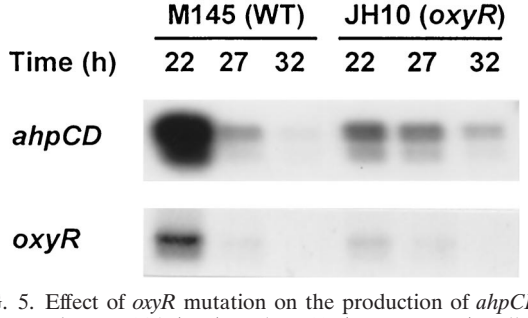

FIG. 5. Effect of *oxyR* mutation on the production of *ahpCD* and *oxyR* transcripts. M145 (WT) and JH10 (*oxyR* mutant) cells were grown in YEME for 22, 27, and 32 h. The amounts of *ahpCD* and *oxyR* transcripts were determined by S1 mapping as described in the text.

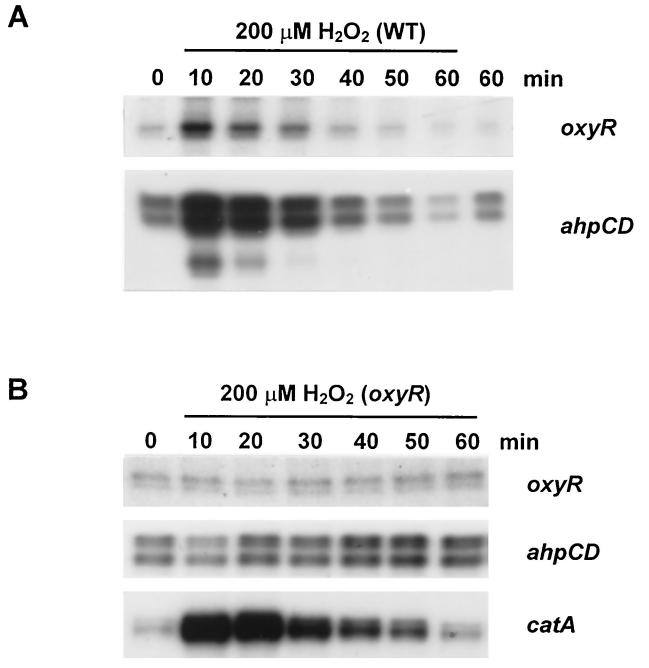

FIG. 6. OxyR-dependent induction of the *ahpCD* and *oxyR* genes by H_2O_2. Induction of *ahpCD* and *oxyR* by H_2O_2 in wild-type (A) and *oxyR* mutant (B) cells. *S. coelicolor* M145 and JH10 cells were grown in YEME to the early exponential phase and treated with 200 μM H_2O_2. Samples were taken at 10-min intervals over 1 h, and S1 mapping analysis was carried out for *oxyR* and *ahpCD* transcripts.

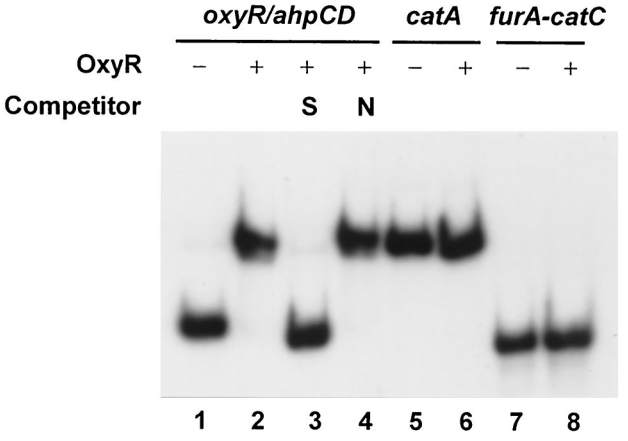

FIG. 7. Binding of OxyR to *ahpCD-oxyR* intergenic region. A gel mobility shift assay of OxyR binding to different promoter fragments was performed. Two hundred nanograms of purified OxyR (6 pmol) was incubated with ^{32}P-labeled promoter fragments of *oxyR-ahpCD*, *catA*, or *furA-catC* as described in Materials and Methods. OxyR binding was detected by electrophoresis on 4% polyacrylamide gel and autoradiography. Lanes 1, 5, and 7 contain only the radiolabeled probes. Probes were incubated with OxyR without any competitors except poly(dI-dC) (lanes 2, 6, and 8) or with a 200-fold molar excess of unlabeled probe DNA (S; lane 3) or nonspecific DNA fragments (N; lane 4).

peroxidase), *catA* (which codes for catalase A), *sodN* (which codes for Ni-superoxide dismutase [SOD]), *sodF* (which codes for Fe-SOD), and *zwf* (which codes glucose-6-phosphate dehydrogenase) were not affected by the loss of *oxyR* (data not shown).

H_2O_2 induction of *ahpCD* and *oxyR* genes mediated by OxyR. H_2O_2 inducibility of *ahpCD* and *oxyR* genes in *S. coelicolor* was then investigated. Wild-type *S. coelicolor* cells were grown to early exponential phase and treated with 200 μM H_2O_2 for different lengths of time up to 1 h. The levels of *ahpCD* and *oxyR* transcripts were analyzed by S1 mapping (Fig. 6A). The level of both *oxyR* and *ahpCD* transcripts increased more than fivefold within 10 min of H_2O_2 treatment, returning to the prestimulus level in 50 min. The level of AhpC and AhpD proteins increased about twofold within 30 min of H_2O_2 induction as monitored by immunoblot analysis (data not shown).

In the *oxyR* mutant (JH10), neither *ahpCD* nor *oxyR* was induced by H_2O_2 treatment, demonstrating that H_2O_2 induction of these genes is mediated by OxyR (Fig. 6B). The gene for catalase A (*catA*), known to be induced by H_2O_2 via inactivation of repressor CatR (26), was induced normally in *oxyR* mutant. These results clearly demonstrate that the two separate peroxide-sensing regulators independently control the expression of two different antiperoxide enzymes in *S. coelicolor*.

Binding of OxyR protein to the *oxyR-ahpCD* intergenic region. Participation of OxyR as a direct regulator of *ahpCD* and *oxyR* gene expression was further examined by DNA binding analysis. OxyR protein was purified from *E. coli* following overproduction using the pET system. The binding activity to the promoter region of *ahpCD-oxyR*, as well as the *catA* and *furA-catC* genes, was examined by gel mobility shift assay. As presented in Fig. 7, the purified OxyR bound specifically only to the *ahpCD-oxyR* intergenic region, but not to *catA* or *furA-catC* promoters. The result suggests that OxyR most likely regulates *ahpCD* and its own gene transcription via direct interaction with the binding site located in the intergenic promoter region.

DISCUSSION

In the present study, the *ahpCD* and *oxyR* genes were isolated from *S. coelicolor*, and OxyR was proposed as a positive regulator of these genes. In *S. coelicolor*, the *oxyR* gene is transcribed divergently from the *ahpCD* operon as observed in *S. viridosporus* and mycobacterial species examined, such as *M. leprae*, *Mycobacterium avium*, *M. tuberculosis*, *M. bovis*, and *Mycobacterium marinum* (20, 21, 36). It is an unexpected observation that *S. coelicolor* OxyR acts a transcriptional activator of its own gene. Most members of the LysR family of transcriptional regulators, including *E. coli* OxyR, autoregulate their own expression as negative regulators. In *E. coli*, *oxyR* expression decreases rather than increases during the first 10 min of H_2O_2 treatment (44). In *S. coelicolor*, by contrast, both *ahpCD* and *oxyR* are induced by H_2O_2 in an OxyR-dependent manner.

In *E. coli* and *B. subtilis*, the *ahpC* gene consists of an operon with *ahpF* encoding NAD(P)H-dependent AhpC reductase (2, 5, 46). However, the *ahpD* genes found in *S. coelicolor* and *Mycobacterium* spp. reveal little homology to the *ahpF* gene. The AhpD protein is about 19 kDa, much smaller than AhpF (52 kDa), and does not contain FAD or NAD(P)H binding domains conserved in AhpF proteins. However, the conserva-

tion of cysteine residues in the C-X-X-C motif among AhpD proteins from *S. coelicolor* and *Mycobacterium* spp. suggests their function as thioredoxin-like proteins involved in reducing AhpC (28). Since the overproduction of AhpC alone could render resistance to cumene hydroperoxide, it can be postulated that a catalytic amount of AhpD might be required. This idea is consistent with a recent finding in *M. tuberculosis* that AhpD acts as a thioredoxin-like molecule and reduces AhpC using electrons transferred from NADH through dihydrolipoamide dehydrogenase and dihydrolipoamide succinyltransferase (4).

The pattern of growth phase-dependent expression of the *oxyR* and *ahpCD* seems not uniform among organisms. In *E. coli*, *oxyR* mRNA showed biphasic expression with a peak at early exponential phase followed by a decline at the stationary phase. The induction of *oxyR* expression at exponential phase is dependent on the cyclic AMP receptor protein (CRP) regulator (25). We observed similar peak expression of *oxyR* (as well as *ahpC*) during early exponential growth of *S. coelicolor*. This growth phase-dependent expression of *oxyR* is modulated by OxyR itself, in contrast to the corresponding action of CRP in *E. coli*. This difference can be justified from the fact that the catabolite repression in *S. coelicolor* does not proceed through the glucose-phosphotransferase system and hence cAMP-CRP complex as occurs in *E. coli* (1). If the activity of OxyR is regulated by H_2O_2 produced by endogenous aerobic respiration as postulated for *E. coli* (24, 25), the positive regulation of the *oxyR* gene by OxyR may allow rapid amplification of response via positive feedback, ensuring a rapid production of alkyl hydroperoxide reductase and other *oxyR* regulon components in response to a subtle increase in H_2O_2 due to aerobic respiration.

The regulation of *ahpC* expression in *S. coelicolor* is still different from that of another gram-positive bacterium, *B. subtilis*. In *B. subtilis*, for which the *oxyR* homologue has not been identified, the *ahpCF* expression increases at the stationary phase. In this organism, a repressor Fur homologue, PerR, which is similar to CatR in *S. coelicolor*, is responsible for H_2O_2 induction and metal-dependent stationary-phase induction of genes like *katA* (which codes for catalase), *mrgA* (which codes for nonspecific DNA binding protein), and *hemAXCDBL* (which code for the heme biosynthesis operon) as well as *ahpCF* (6, 12).

Regulation of genes for the peroxide-removing system in *S. coelicolor* is achieved by at least four separate regulators. Alkyl hydroperoxide reductase (AhpC) is maximally produced during early exponential phase and is induced by exogenous H_2O_2, all under the control of OxyR (this study). Catalase A (CatA), a major monofunctional catalase, is produced maximally at the late exponential phase, maintaining its level throughout stationary phase, and is induced by exogenous H_2O_2 under the control by a Fur-type repressor, CatR (26). Catalase B, another monofunctional catalase, is produced only after stationary phase and upon differentiation of *S. coelicolor* under the control of a stationary phase-specific sigma factor σ^B (14). Catalase C, a catalase-peroxidase, is produced transiently during late exponential phase and is suggested to be regulated by another Fur-type repressor, FurA, in a metal-dependent manner (27). Therefore, in *S. coelicolor*, antioxidant genes are regulated by a wider variety of regulators than those observed in other organisms examined so far.

It can be postulated that in aerobically growing *S. coelicolor*, endogenous production of H_2O_2 from aerobic respiration during early phase of growth triggers rapid induction of the antioxidant system, including alkyl hydroperoxide reductase, to protect membrane lipids and genetic material, via increasing the synthesis and the activity of OxyR. In later growth phases when OxyR is no longer produced, the *catA* gene may be derepressed, via inactivation of repressor CatR by H_2O_2, to maintain the level of H_2O_2 under a certain limit. Therefore, the peroxide-sensitive regulators OxyR and CatR may divide their labor depending on the growth phase. Through the action of OxyR, CatR, and σ^B, *S. coelicolor* can be equipped with antiperoxide systems in all growth phases, whereas corresponding functions are exerted by PerR and SigB in *B. subtilis* and OxyR and σ^S in *E. coli*. A third peroxide-sensitive regulator in *S. coelicolor*, the anti-sigma factor RsrA, also plays a role in responding against a peroxide stress, by inducing thioredoxin and other thiol-reducing systems (31, 38), which may play some role in reducing AhpC. In *B. subtilis* and *X. campestris*, OhrR, a new regulator of the MarR family of transcription repressors, regulates the organic hydroperoxide resistance (*ohr*) gene in response to organic hydroperoxides (23, 45). Inspection of the *S. coelicolor* genome reveals three *ohr* homologs, and one of them neighbors a divergent ORF with moderate amino acid sequence homology to OhrR. This implies that there may exist yet another regulator of OhrR type involved in peroxide stress response in *S. coelicolor*.

In *S. coelicolor* OxyR did not regulate the production of other antioxidant enzymes such as Ni-containing SOD, Fe-containing SOD, or glucose-6-phosphate dehydrogenase. Nevertheless, cells overproducing OxyR were more resistant to H_2O_2 and cumene hydroperoxide than cells overproducing AhpC and AhpD, implying the presence of other components of the OxyR regulon having antioxidant function. Moreover, the *oxyR* disruptant showed a conditional bald phenotype, suggesting the role of *oxyR* in morphological differentiation as well (Hahn et al., unpublished data). An apparently related observation in *E. coli* has demonstrated the involvement of OxyR in the control of some surface properties, including colony morphology and auto-aggregation (47). Further studies on the genes regulated by OxyR as well as their regulation mechanism are expected to reveal the interesting function of OxyR in this organism.

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