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Analysis of the Dual Promoters and the H_2O_2-responsive Element of the *catA* Gene Encoding Catalase A in *Streptomyces coelicolor*

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The *catA* gene encodes the major catalase in *Streptomyces coelicolor*, whose production increases upon H_2O_2 treatment. Besides the previously identified primary promoter (*catA*p1), a minor promoter (*catA*p2) was newly assigned by S1 nuclease mapping. The *catA*p2 transcript was observed transiently upon entry into the stationary phase in liquid culture and upon differentiation on solid plates, whereas the level of *catA*p1 transcription did not change significantly during this growth transition. The *catA*p1 promoter was transcribed by the major vegetative RNA polymerase holoenzyme containing σ^{HrdB}, whereas the *catA*p2 was transcribed *in vitro* by the holoenzyme containing σ^R that is activated under oxidative conditions. The *cis*-element regulating the H_2O_2-inducibility of *catA*p1 was identified within the 23 bp inverted repeat sequence located between -65 and -43 of the *catA*p1 promoter. We named this sequence HRE (H_2O_2-responsive element). The distal half of the inverted repeat was more crucial for H_2O_2-dependent induction of the *catA*p1 transcript than the proximal half. HRE most likely serves as a binding site for the H_2O_2-responsive repressor CatR.

Key words: catA, CatR, H_2O_2, dual regulation, H_2O_2-responsive elements, *Streptomyces coelicolor*

Reactive oxygen species (ROS) are produced from the incomplete reduction of oxygen during aerobic respiration, from exposure to radiation or redox-cycling drugs which undergo autooxidation or from macrophages in response to bacterial invasion. They can lead to damage of almost all cellular components such as DNA, membrane lipids, and proteins, causing cell death and many degenerative diseases (15). Both prokaryotic and eukaryotic cells are equipped with inducible defense systems that counter oxidative damage, but the mechanisms by which cells receive and respond to oxidative stress signals have not been thoroughly elucidated.

The responses induced by oxidative stress in bacteria have been studied predominantly in *Escherichia coli*. These studies have revealed two main oxidative regulons: OxyR regulon against H_2O_2 and SoxRS regulon against superoxide and nitric oxide (30). The induced expression of the OxyR regulon by H_2O_2 results in increased resistance of the cells to H_2O_2 compared with non-induced cells (10). Similarly, the constitutive overexpression of the genes in this regulon results in enhanced resistance to H_2O_2 (9). OxyR has been shown to be a positive regulator that binds to specific regions in the genes of this regulon such as *ahpCF* (encoding an NADPH-dependent alkylhydroperoxidase) and *katG* (encoding hydroperoxidase I) (31). It has been proposed that the redox sensing mechanism of OxyR involves thiol-disulfide exchange (32).

The response of *Bacillus subtilis* to H_2O_2 during exponential growth appears to be similar to that in *E. coli* (1, 11). Upon exposure to H_2O_2, *B. subtilis* induces DNA damage-responsive genes and a resident prophage (SOS regulon) as well as the *katA* gene encoding vegetative catalase (2). Additionally, exponentially growing *B. subtilis* cells demonstrate enhanced protection from killing by H_2O_2 when pretreated with sublethal concentrations of H_2O_2 (1, 3). Chen *et al.* (5) have postulated that there might exist a negative regulator (PerR), which acts on the conserved element (Per box) initially identified in *katA* and *mrgA* genes. PerR has been identified as one of the three *E. coli* Fur homologues predicted from *B. subtilis* genome sequence data (4). However, the importance of Per box and the molecular mechanism governed by PerR are currently unknown.

The oxidative response of *Streptomyces coelicolor*, a gram-positive antibiotic-producing bacterium, has been revealed to some extent. *S. coelicolor* produces two kinds of superoxide dismutases whose levels are relatively high to effectively counteract the harmful effects of superoxide anion (22, 23). It also produces multiple catalases (24) encoded by *catA*, *catB*, and *catC* genes (7, 8, 12). The

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vegetative catalase (CatA) is believed to play a major role in reducing the amount of H_2O_2 inside the cell as HPI (KatG) does in *E. coli*. CatB is produced late in the growth phase and is required for proper differentiation and osmotic resistance of *S. coelicolor* (8). CatC is produced transiently at the late exponential phase (12). CatA is induced by H_2O_2 in liquid culture at the level of transcription. The expression level on surface culture is much higher than that in liquid culture, suggesting more effective oxidative induction on surface culture. Recently we identified an H_2O_2-responsive repressor, CatR, which induces the *catA* gene in response to H_2O_2 (13, 14). In this paper, we present the evidence on the dual promoter activities of the *catA* gene and the *cis*-acting H_2O_2-responsive element (HRE). We propose that this element might be the binding site for CatR repressor.

Materials and Methods

Bacterial strains and culture conditions

Growth and maintenance of *S. coelicolor* A3(2) strains (J1501 and its derivatives) were done essentially as described by Hopwood *et al.* (16) and Cho (6). Pre-germinated spores (about 10^8~10^9 spores/100 ml broth) or 5% seed culture of *S. coelicolor* cells were inoculated and grown in YEME liquid medium (1% glucose, 0.5% Bacto-peptone, 0.3% malt extract [Difco], 0.3% yeast extract [Difco]) containing 34% sucrose at 30°C with vigorous shaking. The growth rates and phases were determined as described by Cho and Roe (7) by measuring optical density at 640 nm. For plate cultures, 10^7 pre-germinated spores or patches of mycelia were inoculated on the R2YE agar (10.3% sucrose, 1% glucose, 1.01% $MgCl_2$, 0.024% K_2SO_4, 0.001% casamino acid [Difco], 0.5% yeast extract, 1.43% [~20 mM] TES [N-tris {hydroxymethyl}methyl-2-aminoethanesulfonic acid], pH 7.0, 20 mM $CaCl_2$, 0.005% K_2HPO_4, 0.3% proline, and 2% agar) media either overlaid with a cellophane disc or not. The growth rate on solid media was assessed by measuring the amount of mycelium (dry or wet cell weight). The recombinant DNAs were introduced into either *E. coli* DH5α (27) or into *S. lividans* TK24 protoplasts (16). To obtain methylation-negative DNA, *E. coli* ET12567 (25) was used. *E. coli* cells were grown at 37°C in LB (1% tryptone [Difco], 0.5% yeast extract, and 1% NaCl) supplemented with appropriate antibiotics. TK24 and J1501 cells were grown in YEME liquid medium containing 0.5% and 1.0% glycine, respectively, and protoplasted as described by Hopwood *et al.* (16).

Preparation of cell extract and protein analyses

Harvested mycelium was suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication with Sonicator ultrasonic liquid processor (Misonix Inc.). The suspension was clarified by centrifugation at 4°C to obtain the supernatant as cell extract. The concentrations of total proteins in cell extracts or partially purified proteins were quantified using Protein assay kit (BioRad). For Western immunoblot analysis, anti-CatA antiserum was raised in mice against the partially purified CatA protein from the CatA-overproducing mutant (HR40) as described previously (8). Catechol dioxygenase XylE activity was assayed in 100 mM potassium phosphate buffer (pH 6.8) containing 2 mM catechol by detecting the increase in absorbance at 375 nm. Changes in optical density per minute were converted directly to enzyme units (17).

Recombinant DNA techniques

pUC18 was used as the general-purpose cloning vector in *E. coli*. For measuring the promoter activity of various promoter mutants in *Streptomyces*, pXE4, a promoter-less *xylE* reporter plasmid (17), and pYC1, a promoter-less *lacZ* reporter plasmid (6), were used. pYC1 was generated by the fusion of pRS415 (28) and SCP2* *rep/stb* region from pXE4 by *Eco*RI/*Bam*HI digestion. Plasmid DNAs from *E. coli* or from *S. lividans* TK24 were prepared by alkaline lysis. DNA fragments were purified from agarose gels using GeneClean Kit II (BIO101) or by the freeze-squeeze method.

HRE mutant construction by PCR

A series of the deletion and point mutations in *catA* promoter were generated by PCR. PCR reactions were performed in a thermal cycler (Perkin-Elmer Cetus) for 30 cycles with the following conditions: 94°C for 1 min for denaturation, 50°C for 1 min for annealing, and 72°C for 45 sec for extension. The forward primers are 18~24 nt in length with their 5′ boundaries designated in Fig. 2. The reverse primer is 5′-TCG GAG AAG ATC TTC GCG CTG G-3′ containing the unique *Bgl*II site at +271 of the *catA* gene. The PCR products were made blunt and initially cloned into the *Hinc*II-digested pUC18. The insert was recovered by digestion with *Hind*III and *Bgl*II and was further cloned into the *Hind*III/*Bam*HI-cut pXE4 or pYC1. pXE42 and pXE45 are pXE4 derivatives containing promoter fragments D2 and D5, whose 5′ boundaries are -84 and -38, respectively. TK24 cells containing pXE42 and pXE45 were grown on R2YE plates for 40 h and subjected to XylE enzyme assay (17). pYC43 and pYC44 are pYC1 derivatives containing promoter fragments D3 and D4, whose 5′ boundaries are -69 and -54, respectively. pYC43M1 (62AGA to CTC) and pYC43M4 (47CTAGΔ) contained the indicated base substitutions and deletions within HRE in the pYC43 background. TK24 cells containing pYC1 derivatives were grown in YEME liquid media for 28 h, and subjected to treatment with 100 μM H_2O_2 as described elsewhere (7). The transcripts from the plasmids were assessed by S1 nuclease

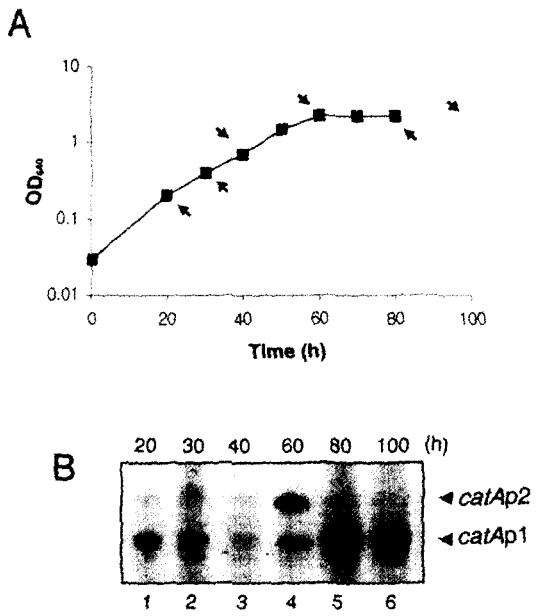


Fig. 1. Production of transcripts from two promoters of the *catA* gene. (A) *S. coelicolor* A3(2) cells were grown in YEME media. The growth was monitored by measuring absorbance at 640 nm. At the indicated time, samples were taken for RNA analysis. Germinated spores were used to inoculate YEME containing 34% sucrose. The doubling time was estimated to be about 11 h. The 60 h culture corresponded to the transition from exponential to stationary phase. (B) The presence and the level of *catA* transcripts were monitored by S1 nuclease mapping analysis.

mapping as described below.

RNA isolation and S1 nuclease mapping

Mycelial cells at various growth phases were harvested by centrifugation (6000 × *g*) at 4°C for 5 min. RNA was isolated using Kirby mix (1% [w/v] sodium triisopropylnaphthalene sulfonic acid, 6% sodium 4-amino salicylic acid, 6% [v/v] phenol in 50 mM Tris-HCl [pH 8.0]) as described by Hopwood *et al.* (16). Following extraction with phenol/$CHCl_3$, the aqueous phase was precipitated with isopropanol and stored at -70°C as precipitates. The probe used for S1 nuclease mapping was prepared by *SalI*/*BglII* digestion of the cloned *catA* gene and radiolabeled uniquely at the 5' end of the *BglII* site (7). The S1 mapping was done as described by Smith and Chater (29). The S1 probe was hybridized with 50 μg of RNA at 50~55°C for more than 6 h. The protected DNA fragments were resolved on 6% polyacrylamide gel containing 7 M urea.

In vitro transcription assay

In vitro transcription assays were done essentially as described by Kang *et al.* (20) with minor modifications. For *in vitro* transcription of *catA*p1 and *catA*p2, 1.5 pmole of purified core polymerase and 3 to 4 pmole of σ^{HrdB} or σ^{R} protein overproduced and purified from *E. coli* were used to transcribe 0.2 pmole of 635 bp *SalI*/*BglII* fragment of *catA* promoter in 20 μl standard transcription mixture. Following initiation of transcription, heparin (100 μg/ml) was added to ensure single-round transcription. Transcripts were analyzed in 5% polyacrylamide gel containing 7 M urea.

Results

The transition phase-specific promoter, catAp2

The *catA* gene transcripts are primarily derived from the *catA*p1 promoter at all growth phases. An additional transcript (*catA*p2) which is 68 nt larger than *catA*p1 transcript, was observed from cells at a specific growth phase. In liquid culture, it appeared transiently at the late exponential phase (about 60 h following inoculation) and disappeared as cells progressed into the stationary phase (Fig. 1). It appeared also transiently at the onset of differentiation on surface culture (data not shown).

The functional significance of this transient expression of *catA*p2 in both liquid and surface cultures is not known yet. However, it seems that the transient increase of *catA*p2 transcript, especially on surface culture, may not cause a significant increase of CatA protein, since the level of CatA protein remains rather constitutive during this phase (data not shown).

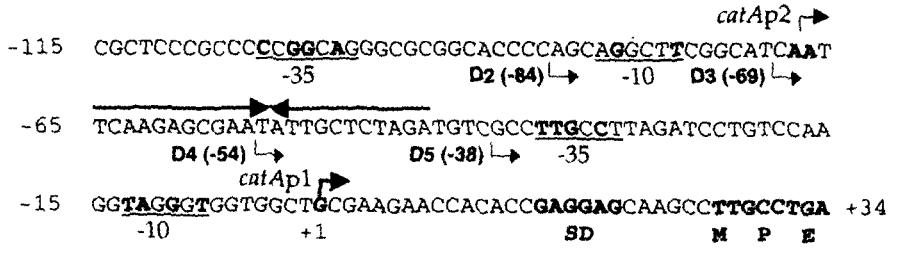
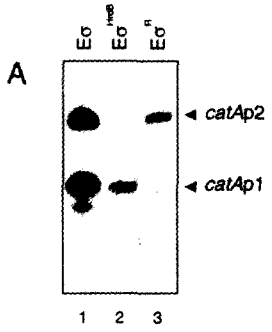


Fig. 2. Nucleotide sequences of the promoter region of *catA* gene. Putative promoter elements (-35 and -10), ribosome binding site (SD), and the N-terminal codons are presented as well as the transcription start sites (bent arrows) for *catA*p1 and *catA*p2 promoters. An inverted repeat sequence of 23 bp (from -65 to -43 nucleotide relative to the *catA*p1 trahscription start site) is observed immediately upstream of the -35 region of the *catA*p1 promoter. The 5' boundaries of PCR-generated deletion mutants (D2, D3, D4, and D5 from -84, -69, -54, and -38, respectively) for the *catA*p1 promoter analysis are indicated with their 5' end positions marked.

Transcription of catAp1 and catAp2 promoters in vitro

The transcription start sites for *cat*Ap1 and *cat*Ap2 transcripts determined by high-resolution S1 nuclease mapping (7, data not shown) are shown in Fig. 2. Putative promoter elements for each promoter were marked (see Fig. legend). To identify the sigma factors recognizing each promoter, we performed *in vitro* transcription analysis using different sigma factors. As demonstrated in Fig. 3A, we observed that *cat*Ap1 transcript was generated by $E\sigma^{HrdB}$, whereas *cat*Ap2 transcript was generated by $E\sigma^{R}$ (Fig. 3A). The *cat*Ap1 promoter elements resemble the consensus sequence recognized by the major sigma factor (σ^{HrdB}), consistent with this result (Fig. 3B). The promoter elements of *cat*Ap2 partially match the consensus sequence recognized by σ^{R} (19) as shown in Fig. 3B. Whether the transcription of *cat*Ap2 is dependent on σ^{R} *in vivo* was tested using a *sigR* deletion mutant J2139. The *cat*Ap2 transcript was still observed in *sigR* mutant (data not shown), suggesting that the *in vitro* recognition by σ^{R} could result from the relaxed specificity of binding to the *cat*Ap2 promoter and/or that some other σ^{R}-like factor(s) could compensate for the absence of σ^{R} *in vivo*. Since there are about 15 σ^{R}-like sigma factors in *S. coelicolor* (M.-Y. Hahn, personal communications), we think that the latter possibility is more likely.



B

catAp1	CC**TTGCCT**TAGATCCTGT CCAAGG**TAGGGT**GGTGGCT**G**
rrnDp2	CG**TTGACA**CGGAGCGAGCGGGGAGG**TAGATT**CGAACA
σ^{HrdB} consensus	**TTGACA** (N16-17) **TAGRRT**
catAp2	CC**CCGGCAG**GGCG CGGCACCCCAGC**AGGCTT**CGGCATC**AA**
glnRp2	GC**TGGGCAG**AG GACTCGGGCAACGT**AGCCCC**CGGGTCCT
sigRp2	CC**TGGGAAT**GACCGGCGGACGGCCAC**TGTTGT**GCCGTT**C**
hrdDp2	GT**TGGGAAT**TCTGTCCGGATTCCAGT**CGTTGT**TTCCA**T**
trxBp	CT**CGGGAAT**TCTGTCCCGATTCCAGT**CGTTGT**TTCCTTC
σ^{R} consensus	YGGGAAT (N16-17) YGTTGY

Fig. 3. Transcription of *cat*Ap1 and *cat*Ap2 in vitro. (A) *catA* promoter fragment was incubated with purified *S. coelicolor* RNA polymerase holoenzyme containing mixtures of various sigma factors (Es; lane 1), purified core enzyme plus σ^{HrdB} ($E\sigma^{HrdB}$; lane 2), or core enzyme plus σ^{R} ($E\sigma^{R}$; lane 3). *In vitro* transcription assay was done as described in Materials and Methods. (B) The putative promoter elements of *cat*Ap1 and *cat*Ap2 promoters were compared with the consensus and representative promoter elements recognized by σ^{HrdB} and σ^{R}, respectively (20).

HRE, the cis-acting element for H_2O_2-induction of catAp1 promoter

It has been speculated that an inverted repeat structure immediately upstream of the -35 box of *cat*Ap1 is the site responsible for H_2O_2-induction of the *cat*Ap1 promoter by binding H_2O_2-responsive CatR repressor (14). We tested this proposal by using *xylE* reporter plasmids containing various lengths of *cat*Ap1 promoter. Catechol dioxygenase activity derived from pXE42 and pXE45 plasmids containing promoter regions up to -84 and -38 nucle-

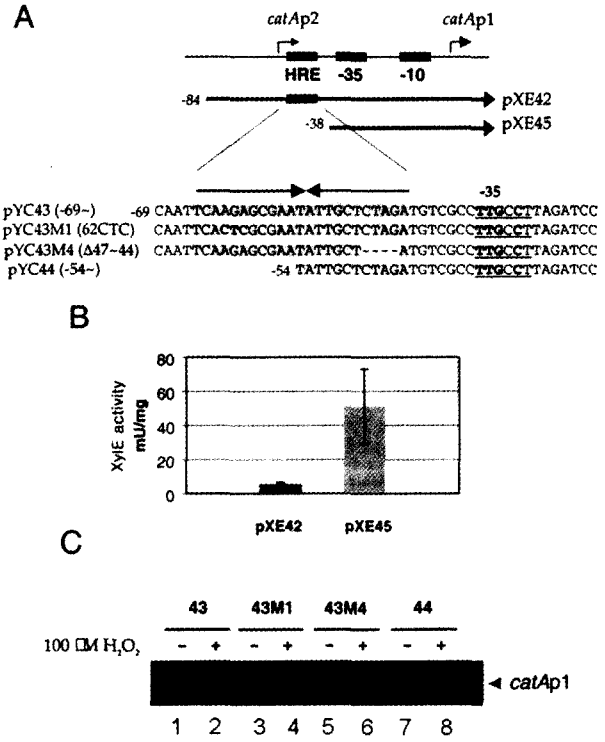


Fig. 4. Effect of HRE mutations *in vivo*. (A) Schematic presentation of various *catA* promoter variants cloned in reporter plasmids. As indicated in Fig. 2, four deletion mutants (D2, D3, D4, and D5) were created by PCR. D2 (from -84 to +271) and D5 (from -38 to +271) were fused to the promoter-less *xylE* gene in pXE4 plasmid to create pXE42 and pXE45, respectively. D3 and D4 were fused to the promoter-less *lacZ* gene in pYC1 plasmid to create pYC43 and pYC44. From pYC43, a triplet transversion converting AGA to CTC at -62 nt position and a 4-nucleotide deletion from -47 to -44 within the inverted repeat sequence were further created, resulting in pYC43M1 and pYC43M4, respectively. (B) Catechol dioxygenase activity from pXE42 and pXE45 plasmids. *S. lividans* TK24 cells transformed with pXE42 or pXE45 were grown on R2YE plates containing 50 μg/ml thiostrepton for 40 h. Cell extracts were assayed for catechol dioxygenase activity. Data are an average of three independent experiments with standard error bars. (C) Effect of HRE mutations on the H_2O_2-inducibility of *cat*Ap1 expression. pYC1-derived plasmids (pYC43, pYC43M1, pYC43M4, or pYC44) were introduced into TK24 cells. Transformants grown to the mid-exponential growth phase in YEME liquid media were treated with either 100 μM H_2O_2 for 1 h (lanes 2, 4, 6, and 8) or nothing (lanes 1, 3, 5, and 7). Transcripts were analyzed by S1 nuclease mapping.

otides, respectively, was measured (Fig. 4A, B). The observation that the deletion of the region between -84 and -39 increased the promoter activity suggested that the region contains a negative regulatory element. Assuming that the 23 bp inverted repeat structure (HRE, H_2O_2-responsive element) within this region might be a putative repressor binding site, we created several deletion or transversion mutants within HRE as shown in Fig. 4A. pYC1 derivatives, *lacZ*-reporter plasmids containing various *catA* promoter variants, were generated. *S. lividans* TK24 cells transformed with these plasmids were grown in liquid culture. Prior to cell harvesting, the culture was treated with 100 μM H_2O_2 for 1 h for induction of *catA*p1 transcription. The transcript level was analyzed by S1 mapping. As demonstrated in Fig. 4C, deletion up to -69 (pYC43) had no effect on *catA* transcription, whereas the introduction of triplet transversion (pYC43M1) caused derepression of *catA* transcription in the absence of H_2O_2 treatment (lane 3). Unexpectedly, an internal 4-bp deletion in the proximal half of the inverted repeat (pYC43M4) did not produce any effect. The entire deletion of the distal half (pYC44) caused dramatic derepression (lane 7). More than 10-fold increase in the amount of derepression in pYC44 compared with pYC43M1 suggests that CatR may bind partially to the HRE site of the M1 mutation, having lost its ability to respond to H_2O_2. These results suggest that the distal half of the inverted repeat plays a critical role in the repression via HRE.

Discussion

Dual promoter regulation of catA

The induction by H_2O_2 and the persistent expression of *catA* in the stationary phase parallels the behavior of *E. coli katG* and *B. subtilis katA* gene expressions. The transcription of *B. subtilis katA* has been reported to increase in the stationary phase by more than 5-fold, being affected by *spo0A* locus (3). Likewise, *E. coli katG* is under the control of RpoS in the stationary phase (18). Analogous with *E. coli katG* being dependent on OxyR and RpoS and with *B. subtilis katA* being PerR- and Spo0A-dependent, *catA* may be subjected to the regulation by CatR in response to H_2O_2 and by unknown stationary phase-specific factors. In this study we found that the *catA* gene contains two promoters that are recognized by different sigma factors. The major promoter is *catA*p1 that is induced by H_2O_2 or hyperoxic conditions as encountered in the plate culture, and the minor one is *catA*p2 that is transiently expressed upon entry into the stationary phase.

The biological significance of this second promoter is currently unknown. It may reflect the condition where some transient disulfide stress is experienced during the transition period, activating the σ^R factor (21, 26). Further studies are necessary to elucidate this possibility.

HRE, a cis-acting negative regulatory element for H_2O_2 response

We previously identified the *catR* gene encoding an *E. coli* Fur-like protein and a functional homologue of *B. subtilis* PerR (13, 14). It represses the *catA*p1 transcription in the absence of H_2O_2. The purified CatR protein binds specifically to the *catA*p1 promoter fragment only under a reducing condition (14). Similar sequences of dyad symmetry have been identified in both *catA*p1 and *catR*p2 promoter regions, being proposed as the binding site of CatR. In this study, we experimentally verified the *cis*-acting element responsible for H_2O_2 inducibility within the *catA* promoter region. This dyad symmetry, called H_2O_2-responsive element (HRE), is located between -65 and -43 nucleotide from the *catA*p1 transcription start site. In *B. subtilis*, an AT-rich inverted repeat sequence was suggested to be the Per box, based on point mutation analyses and the compilation of several genes regulated by PerR. It has been shown to mediate the repression in reducing conditions (5). The position of Per box at *katA* and *mrgA* promoters is similar to that of HRE at *catA*p1 promoter. We further demonstrated that the distal half of the HRE symmetry is more important for correct binding than the proximal half, in contrast to the finding in *B. subtilis* where the proximal region of Per box is also important in the repression of *mrgA* gene (5). These results lead us to the conclusion that the CatR protein may regulate the *catA* gene through binding to HRE, whose distal half provides a more crucial binding site to interact with CatR.

Molecular mechanism for H_2O_2 response in S. coelicolor

In *E. coli*, the positive regulator OxyR acts in response to H_2O_2. The pre-existing reduced form of OxyR protein is directly activated by an oxidative signal, in a protein thiol-dependent manner, stimulating the transcription of target promoters. The signal involves a disulfide bond formation between cysteine 199 and cysteine 208 near the C-terminus, which can be reversibly reduced by glutaredoxin for homeostasis (32). The molecular mechanism behind the expression of *B. subtilis katA* in response to H_2O_2 has not been fully characterized, except that PerR is the key regulator. *S. coelicolor* responds to H_2O_2 by forming a disulfide bond in CatR which then loses its ability to bind to the target site (14). Our current study suggests that the HRE, especially the distal site, provides optimal binding sites for CatR. The different behavior of base substitutions compared with the deletions in the half site will provide clues to elucidate the molecular mechanism for CatR-HRE interaction.

Acknowledgments

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