Isolation of the Regulator Gene Responsible for Overproduction of Catalase A in H₂O₂-resistant Mutant of *Streptomyces coelicolor*

Ji-Sook Hahn, So-Young Oh, Keith F. Chater[1], and Jung-Hye Roe*

Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea;
[1]Department of Genetics, John Innes Centre, Colney, Norwich NR4 7UH, U.K.

Streptomyces coelicolor produces three kinds of catalases to cope with oxidative stress and to allow normal differentiation. Catalase A is the major vegetative catalase which functions in removing hydrogen peroxide generated during the process of aerobic metabolism. To understand the regulatory mechanism of response against oxidative stress, hydrogen peroxide-resistant mutant (HR40) was isolated from *S. coelicolor* J1501 following UV mutagenesis. The mutant overproduced catalase A more than 50-fold compared with the wild type. The mutation locus *catR1* was mapped closed to the *mthB2* locus by genetic crossings. An ordered cosmid library of *S. coelicolor* encompassing the *mthB2* locus was used to isolate the regulator gene (*catR*) which represses catalase overproduction when introduced into HR40. A candidate *catR* gene was found to encode a Fur-like protein of 138 amino acids (15319 Da).

Key words: catA, hydrogen peroxide-resistant mutant, Fur homologue, repressor gene, *Streptomyces coelicolor*

All aerobically growing organisms come into contact with reactive oxygen species (ROS) generated as a result of normal metabolic processes. To counter the destructive nature of ROS, cells have evolved antioxidant defense mechanisms. Cells possess regulators that sense oxidant signals, and transduce the signals into gene expression.

The adaptive responses to oxidative stress have been best characterized in *Escherichia coli* and *Salmonella typhimurium*. These bacteria exert distinct responses against H_2O_2 and O_2^-, and OxyR and SoxR/SoxS regulators are involved in each process (10, 18). OxyR is an H_2O_2-sensing transcriptional regulator inducing at least nine genes in response to H_2O_2 (7). OxyR is activated by a disulfide bond formation between two cysteine residues (Cys[199] and Cys[208]), and induces the expression of *oxyS* (a small, non-translated regulatory RNA), *katG* (hydrogen peroxidase I), *ahpC* (alkyl hydroperoxide reductase), *gorA* (glutathione reductase), *dps* (DNA binding protein), and *grxA* (glutaredoxin 1) genes. Glutaredoxin 1 deactivates OxyR by reducing disulfide bond, forming an autoregulatory loop (19). Irrespective of its oxidation state, OxyR also acts as a repressor of its own expression similar to other LysR family of transcriptional regulators.

The SoxRS system is involved in O_2^--mediated stress responses. Upon O_2^- or NO attack, SoxR is suggested to

be activated by oxidation of [2Fe-2S] cluster, present in a pair per dimer in reduced SoxR (11). Activated SoxR induces expression of the *soxS* gene, and the SoxS protein activates various target genes including *sodA* (Mn-SOD), *nfo* (endonuclease IV), *zwf* (glucose-6-phosphate dehydrogenase), *fumC* (fumarase), *acn* (aconitase), and *fpr* (ferredoxin:NADPH oxidoreductase).

In *Bacillus subtilis* where *oxyR* homologue has not been identified, PerR, a Fur-homologous repressor, is known to be responsible for H_2O_2 induction of genes like *katA* (catalase), *ahpCF* (alkyl hydroperoxide reductase), *mrgA* (nonspecific DNA binding protein), and *hemAXCDBL* (heme biosynthesis operon) (3, 4). It is postulated that PerR activity might be regulated by metal-catalyzed oxidation of the protein or by a change in the oxidation state of the bound metal ion.

Streptomycetes are Gram-positive soil bacteria that undergo complex cycle of morphological and physiological differentiation. Adaptive response to H_2O_2 has been reported in *Streptomyces coelicolor* (13). Two-dimensional protein gel analyses revealed that synthesis of more than 100 proteins are induced when *S. coelicolor* cells are exposed to H_2O_2 (8). *S. coelicolor* produces two monofunctional catalases, CatA and CatB, and a catalase-peroxidase CatC. CatA is the major vegetative catalase inducible by H_2O_2, and CatB is a stationary phase-specific catalase inducible by osmotic stress (5, 6). CatC is expressed transiently at late exponential to early stationary phase. CatA enables

* To whom correspondence should be addressed.
(Tel) 82-2-880-6706; (Fax) 82-2-888-4911
(E-mail) jhroe@plaza.snu.ac.kr

cells to cope with toxic hydrogen peroxide generated during aerobic growth, whereas CatB enables cells to cope with osmotic stress and allow normal differentiation. Regulatory factors for the expression of these enzymes have not been identified yet. In this study we report the isolation of a candidate repressor gene (*catR*) which encodes a negative regulator for the expression of the *catA* gene.

Materials and Methods

Bacterial strains and culture conditions

Strains used in this study were listed in Table 1. *S. coelicolor* A3(2) J1501 was used for mutagenesis and isolation of H_2O_2-resistant mutants. J699, J650 (16), and J801 were used for genetic mapping. *Streptomyces* cells were either grown in YEME medium for liquid culture, or on R2YE, nutrient agar (NA), or minimal medium plates for surface culture as described previously (12). *E. coli* ET12567 (14), a non-methylating strain, was used to prepare DNA to transform into *S. coelicolor*.

Mutagenesis and isolation of H_2O_2- resistant mutants from S. coelicolor

S. coelicolor J1501 spores were illuminated with UV light to a survival rate of 0.1 to 0.5%. About 5×10^4 spores were incubated on NA medium at 30°C for 18 h, and treated with 10 ml of 3 to 5 mM H_2O_2 for 1 h with rocking. After removing H_2O_2 solution, plates were further incubated to isolate surviving colonies. Approximately 1×10^3 spores of surviving colonies were further grown on NA plate for 18 h, and then treated with 1 mM H_2O_2 for 1 h for a second round of screening. Five clones showing 100% survival were selected as H_2O_2-resistant mutants.

Genetic mapping of the mutated locus catR1 in HR40

Genetic crosses and data analyses were carried out as described previously (12). On R2YE media, an H_2O_2-resistant mutant HR40 (SCP1⁻) was crossed with J699 (NF), a J650 derivative containing *bldB*43 mutation. The *his*⁺ *strA*1 recombinants were selected on minimal media containing streptomycin (10 μg/ml) and appropriate growth factors except histidine to exclude parental genomes. Two hundred recombinants were analyzed for their aux-

otrophic markers and bald phenotype forming no aerial mycelium. Recombinants were transferred to NA plates by tooth-picking and incubated 30°C for 18 h. To detect the overproduction of catalase in mutants, the extent of O_2 bubbling was monitored by dropping 30% H_2O_2 on each colony. To isolate NF derivative of HR40, HR40 was crossed with J801, a wild type NF strain. Selection was carried out for *strA*1, and among the recombinants, *his*⁻, *ura*⁻, and *catR*1 stains were selected and named as HR402. HR402 (NF) was crossed with J650 (NF), and selection was done for *ura*⁺ and *cys*⁺ markers. One hundred recombinants were analyzed for their genetic markers.

Cloning of the candidate catR gene from ordered cosmid library

Ordered cosmids (17) encompassing the genomic region around the *mthB*2 locus, from K13 to 2E1, were kindly provided by H. M. Keiser at John Innes Centre, UK. Cosmid DNAs were prepared from *E. coli* ET12567, and then introduced into HR40 protoplast by transformation. Cosmid-integrated clones were selected on R2YE medium containing 200 μg/ml kanamycin, and tested for the complementation of HR40 phenotype, i.e. repression of CatA overproduction. CatA expression was detected by H_2O_2 bubble test or by SDS-PAGE analysis. Two overlapping cosmids, 6F2 and 7E4, were selected as complementing cosmids. *Eco*RI and *Bam*HI restriction fragments in the overlapping region of the two cosmids were cloned into pSET152 (2), a conjugation vector containing viral attachment (*att*) site. Each plasmid was introduced into *E. coli* ET12567 containing mobilizing plasmid pUZ8002 (D. H. Figurski, personal communication), and then introduced into HR40 by conjugation by a modification of the method by Mazodier *et al.* (15). The exoconjugants were selected on R2YE plates containing 50 μg/ml apramycin, and tested for their ability to repress CatA overproduction in HR40.
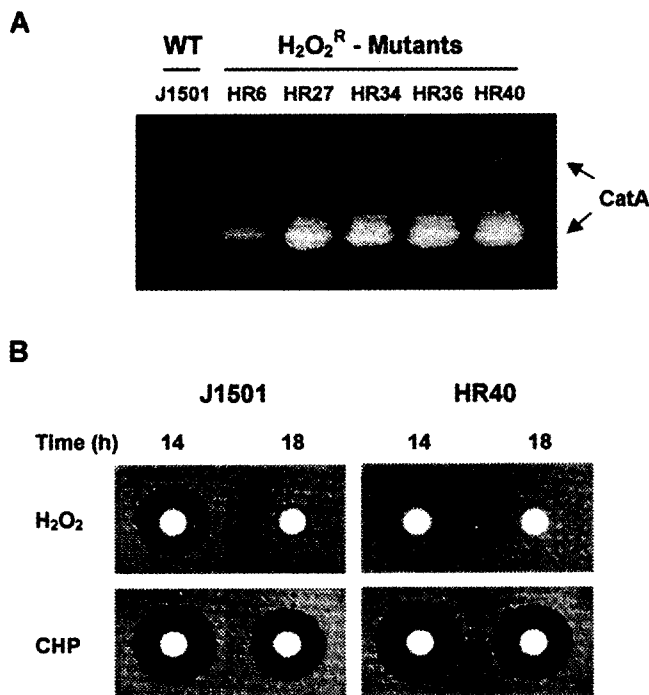
Results and Discussion

Isolation of H_2O_2-resistant mutants

H_2O_2-resistant mutants were isolated from UV-mutagenized spores of *S. coelicolor* J1501. Five mutants were iso-

Table 1. *Streptomyces coelicolor* strains used in this study

Strains	Genotype or description[a]	Source or reference
M145	Prototrophic SCP1⁻ SCP2⁻ Pgl⁺	Hopwood *et al.*, 1985
J1501	*hisA*1 *uraA*1 *strA*1 SCP1⁻ SCP2⁻ Pgl⁻	Hopwood *et al.*, 1985
J801	Prototrophic NF	Hopwood *et al.*, 1985
J650	*cycD*18 *mthB*2 NF	Merrick, 1976
J699	*cycD*18 *mthB*2 *bldB*43 NF	Merrick, 1976
HR40	*catR*1 *hisA*1 *uraA*1 SCP1⁻ SCP2⁻ Pgl⁻	This study
HR402	*catR*1 *hisA*1 *uraA*1 SCP1⁻ SCP2⁻ Pgl⁻ NF	This study

[a]SCP, *S. coelicolor* plasmid; Pgl⁻, φC31 sensitive; NF, SCP1 integrated into the chromosome at 9 o'clock; SLP *S. lividans* plasmid.

A

WT   H₂O₂ᴿ - Mutants

J1501   HR6 HR27 HR34 HR36 HR40

CatA

B

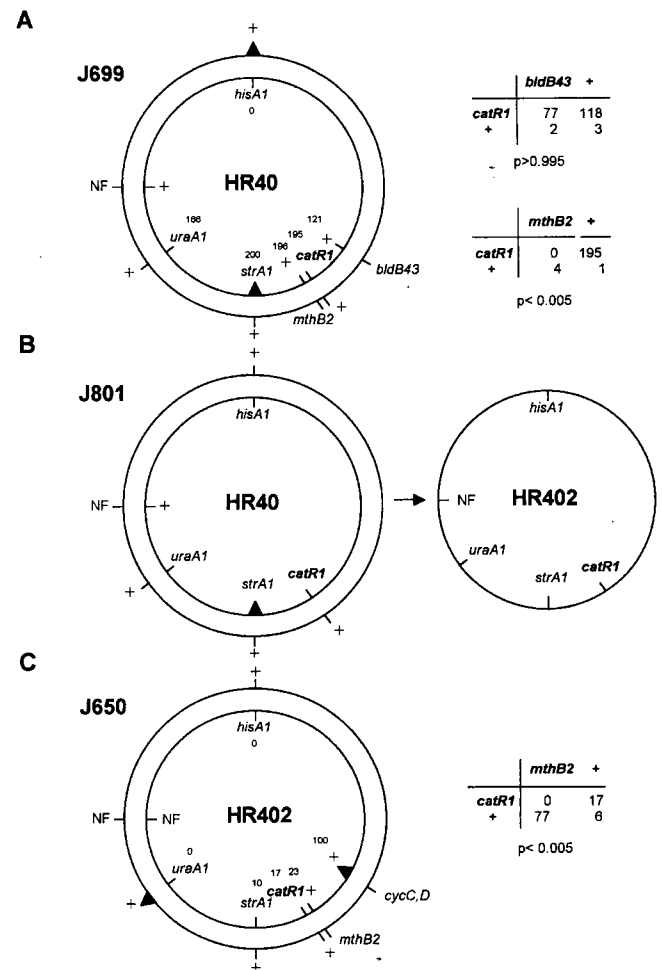J1501   HR40

Time (h)   14   18   14   18

H₂O₂

CHP

Fig. 1. Overproduction of catalase A (Cat A) in H_2O_2-resistant mutant HR40. (A) Detection of CatA activity in wild type and H_2O_2-resistant mutants. *S. coelicolor* J1501 and five mutants (HR6, HR27, HR34, HR36, and HR40) were grown in YEME medium for 40 h. Cell extracts were electrophoresed on 7% native polyacrylamide gel, and then stained for catalase activity. The two catalase bands were derived from CatA. (B) Resistance of HR40 to H_2O_2. Lawns of J1501 and HR40 were generated by overlaying NA (nutrient agar) plates with soft NA containing about 10^7 spores. Following 14 or 18 h incubation at 30°C, 30 μl of 30% H_2O_2 or 10 μl of 80% cumene hydroperoxide (CHP) were spotted on paper discs placed at the center of the plate. Photographs were taken after further incubation at 30°C for 24 h.

A

J699

bldB43 +
catR1   77   118
+   2   3

p>0.995

mthB2 +
catR1   0   195
+   4   1

p< 0.005

B

J801

C

J650

mthB2 +
catR1   0   17
+   77   6

p< 0.005

Fig. 2. Genetic mapping of the *catR1* locus, the mutated gene in HR40. (A) Crossing of HR40 with J699. (B) Scheme to generate HR402, an NF strain derivative of HR40. (C) Crossing of HR402 with J650. Selections used to exclude parental genomes were indicated as solid triangles. Numbers around the circles indicate allele frequencies among the recombinants. The segregation of *catR1* with respect to other markers is tabulated. The probability of independence (p) is presented.

lated at a frequency of 10^{-5} per survivor following mutagenesis. All the mutants overproduced major vegetative catalase CatA as judged by vigorous O_2 bubbling on H_2O_2 drop test and by activity staining (Fig. 1A). One of the mutants, HR40, was subjected to further characterization.

HR40 exhibited almost the same growth rate as the wild type strain J1501. It differentiated normally, even though with less amount of blue antibiotic production than the wild type. It produced large quantity of CatA protein as clearly visible on Coomassie staining of the SDS-PAGE gel. However, no other significant change in protein profile was detected as compared to the wild type strain (data not shown). HR40 overproduced CatA about 50-fold more compared with the wild type as judged by activity staining (Fig. 1A).

Although HR40 was more resistant to H_2O_2 than the wild type, it was slightly more sensitive to cumene hydroperoxide, reflecting the reduced expression of alkyl hydroperoxidase system (AhpC, D) (Fig. 1B). Sensitivity of HR40 against superoxide generating reagents such as

paraquat, plumbagin, and menadione was similar to that of the wild type (data not shown).

Genetic mapping of HR40

For genetic mapping of the mutated locus *catR1*, HR40 and J699 (NF strain) cultures were crossed by co-inoculation on plates. (Fig. 2A). Chromosome transfer is mediated primarily by plasmid SCP1 through the designated site of NF, which represents the integrated state of SCP1 at 9 o'clock on the genetic map. The *strA1 his⁺* recombinants were selected and analyzed for various genetic markers as described in Materials and Methods. The frequency at which the catalase-overproducing phenotype was recovered among recombinants suggested that the mutated locus was closely linked to *mthB2* locus. To reduce the bias caused by unidirectional chromosomal

transfer from an NF to non-NF strain, an NF derivative of HR40, HR402, was constructed by genetic crossing between HR40 and J801 (Fig. 2B). HR402 (*cat*R1, NF) was then crossed with J650 (Fig. 2C). Recombinants were selected for *ura*+ and *cys*+ markers and the frequency of catalase overproduction (*cat*R1) and methionine auxotrophism (*mth*B2) was assessed. The result clearly indicated that the mutated locus lies very close to *mth*B2. It also confirmed that the phenotype of HR40 resulted from a mutation at a single locus.

The *cat*R1 locus thus determined is distant from the *cat*A locus which has been physically mapped to overlapping cosmids F18 and F62 corresponding to *Ase*I F fragment of *S. coelicolor* chromosome at around 3 o'clock position (17) (E.-J. Kim, personal communication). We therefore propose that CatA overproduction in HR40 is caused not by an up-mutation in the *cat*A gene but by a mutation in another gene, most likely a regulator gene for *cat*A expression.

***Cloning of the putative repressor gene for*** catA ***expression***
In order to isolate the gene whose mutation caused overproduction of CatA in HR40, complementation experiment was carried out using ordered cosmids of *S. coelicolor* M145 genome (17). HR40 was transformed with 23 cosmids (from K15 to 2E1) around the *mth*B2 locus and screened for the decrease in CatA production. Integration of two overlapping cosmids, 6F2 and 7E4, caused reduction of CatA expression to the wild type level as judged by reduced O_2 bubbling on H_2O_2 drop test (Fig.

3A). Cosmid 6F2 and 7E4 share about 21 kb overlapping region. To map precisely the complementing locus, *Eco*RI and *Bam*HI fragments of the overlapping region were cloned into pSET152, a conjugation vector with viral integration site (*att*) (2). The plasmids were introduced into HR40 and tested for reduction in O_2 bubbling by H_2O_2 drop test. Two overlapping *Eco*RI (E1, 10 kb) and *Bam*HI (B1, 3 kb) fragments repressed the HR40 phenotype, indicating that a repressor gene resides within the 3 kb B1 fragment. The entire nucleotide sequence of the 3 kb *Bam*HI fragment was determined.

Nucleotide sequence analysis revealed that this fragment contained three ORFs (Fig. 3B). The deduced products of the *orf1* and *orf2* showed homology to inosine phosphate phosphatase and inosine phosphate dehydrogenase, respectively. The *orf3* product showed high homology to Fur (ferric uptake regulator) proteins. Since one of the Fur homologue in *B. subtilis*, PerR, has been identified as a repressor for peroxide regulon, we hypothesize that the *fur*-like *orf3* encodes CatR, a negative regulator of *cat*A expression in *S. coelicolor*.

Amino acid sequence analysis of the catR ***gene product***
The *cat*R gene encodes a protein of 138 amino acids with the deduced molecular mass of 15,319 Da. It shows high homology with other Fur proteins, especially with Fur homologous protein of *S. venezuelae* (FurV), whose gene is transcribed divergently from the *bca* gene encoding bromoperoxidase-catalase (9). The CatR protein shares several conserved histidine and cysteine residues with other
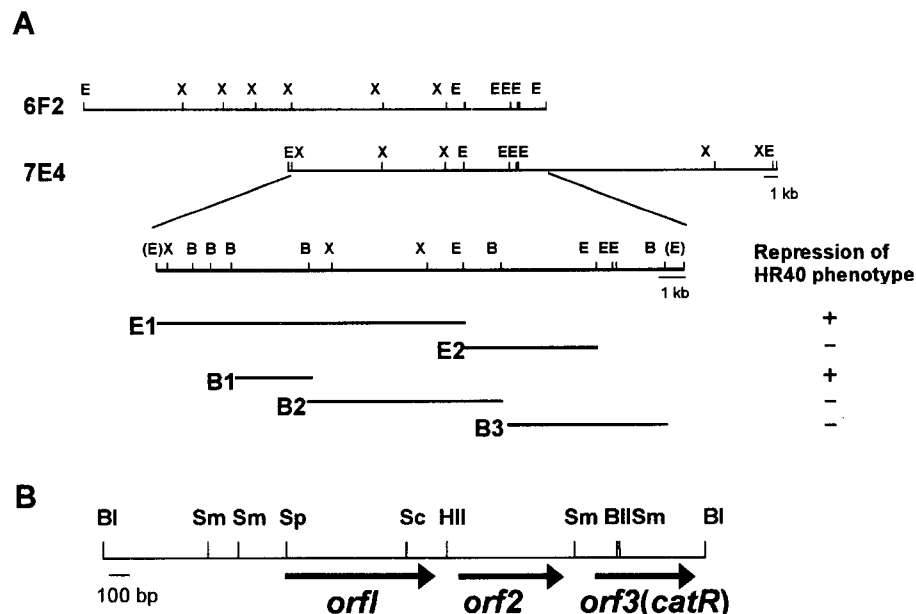
Fig. 3. Isolation of the candidate repressor gene for *cat*A expression. (A) Restriction map of cosmid clones 6F2 and 7E4. The *Eco*RI and *Bam*HI fragments in the overlapping region of the two cosmids were cloned into pSET152 and then introduced into HR40 by conjugation. The ability of exoconjugants to repress HR40 phenotype was assessed by bubbling test. B, *Bam*HI; E, *Eco*RI; X, *Xba*I. (B) Deduced open reading frames within B1 fragment. Restriction enzyme sites are shown on top. BI, *Bam*HI; BII, *Bgl*II; HII, *Hinc*II; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I.

```
Sco CatR   MSDLLERLRGRGWRMTAQRRVVAEVLDG-EHVHLTADEVHSRAVDKLPEISRATVYNTLGELVSLGEVLEVAT 72
Sve FurV   MSDLLERLRGRGWRMTAQRRVVAEVLDG-DHVHLTADEVHARAVTRLPEISRATVYNTLGEMVTLGEVIEVAT 72
Bsu PerR   MAAHELKEALETLKETGVRITPQRHAILEYLVN-SMAHPTADDIYKALEGKFPNMSVATVYNNLRVFRESGLVKELTY 77
Mtu FurA   MSSVSSIPDYAEQLRTADLRVTRPRVAVLEAVN--AHPHADTETIFGAVRFALPDVSRQAVYDVLHALTAAGLVRKIQP 77
Mtu FurB            MSAAGVRSTRQRAAISTLLET-LDDFRSAQELHDELRRRGENIGLTTVYRTLQSMASSGLVDTLHT 65
Eco Fur    MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEIGLATVYRVLNQFDDAGIVTRHNF 73
                :   . :  *    *   :     :         .  ::  :.           ::.  :**   *   :    *  *


Sco CatR   DKRAKRYDPNXHRPHHHLVCARCGAIRDVH-PTGNPLADLPDSERFGFTVSDVEVTYRGTC--PNCAAA 138
Sve FurV   DGRAKRYDPNAHRPHQHLVCGRCGAIRDVH-PGGDPLADLPDTERFGFTISNVEVTYRGIC--PSCAGS 138
Bsu PerR   GDASSRFD-FVTSDHYHAICENCGKIVDFHYPGLDEVEQL-ASDHNGFLLDEAEVIYWGLC--PDCSISDTSRSHP 145
Mtu FurA   SGSVARYESRVGDNHHHIVCRSCGVIADVD-CAVGEAPCLTAAHVTGFKVSHHRLEIYGVC--QECSKKE---NH 150
Mtu FurB   DTGESVYRRCSEHHHHHLVCRSCGGSTIEVG-DHEVEAWAAEVATKHGFSDVSHTIEIFGTC--SDCRS 130
Eco Fur    EGGKSVFELTQQHHHDHLICLDCGKVIEFS-DDSIEARQREIAAKHGIRLTNHSLYLYGHCAEGDCREDE--HAHEGK 148
                        * * :* **  :.           : *:    :    * *   .*
```

Fig. 4. Comparison of CatR with other Fur homologues. Predicted amino acid sequence of CatR from *S. coelicolor* (Sco) was aligned with those from other bacterial Fur homologues: *S. venezuelae* (Sve) FurV (X14792), *B. subtilis* (Bsu) PerR (Z99108), *M. tuberculosis* (Mtu) FurA (Z97193) and FurB (Z95208), and *E. coli* (Eco) Fur (D90708). Sequences of CatR, FurV, and PerR, which are thought to share similar function in peroxide regulation, are presented from the top. Conserved cyteine (C) or histidine (H) residues are shaded. Asterisks and dots indicate identical and similar matches, respectively.

Fur-like proteins from *B. subtilis* and *Mycobacterium tuberculosis* (Fig. 4). Fur was initially known as a transcriptional repressor of a large number of genes for iron uptake system in response to iron sufficiency in *E. coli* (1). More studies revealed that several Fur-like proteins functions in oxidative stress response as well as in metal metabolism. We propose that another Fur-like protein CatR in *S. coelicolor* regulates the expression of catA, derepressing its expression upon H_2O_2 induction. Further studies are required to clarify the regulatory mechanism of CatR in response to H_2O_2.

Acknowledgments

References

1. Bagg, A. and J.B. Neilands. 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiol. Rev.* 51, 509-518.
2. Bierman, M., R. Logan, K. O'Brien, E.T. Seno, R.N. Rao, and B.E. Schoner. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene.* 116, 43-49.
3. Bsat, N., A. Herbig, L. Casillas-Martinez, P. Setlow, and J.D. Helmann. 1998. *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Mol. Microbiol.* 29, 189-198.
4. Chen, L., L. Keramati, and J.D. Helmann. 1995. Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. *Proc. Natl. Acad. Sci. USA.* 92, 8190-8194.
5. Cho, Y.-H., E.-J. Lee, and J.-H. Roe. 2000. A developmentally regulated catalase required for proper differentiation and osmoprotection of *Streptomyces coelicolor. Mol. Microbiol.* 35, 150-160.
6. Cho, Y.-H., and J.-H. Roe. 1997. Isolation and expression of the catA gene encoding the major vegetative catalase in *Streptomyces coelicolor* Müller. *J. Bacteriol.* 179, 4049-4052.
7. Christman, M.F., R.W. Morgan, F.S. Jacobson, and B.N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium. Cell* 41, 753-762.
8. Chung, H.-J., and J.-H. Roe. 1993. Profile analysis of proteins related with hydrogen peroxide response in *Streptomyces coelicolor* (Müller). *Kor. J. Microbiol.* (currently *J. Micobiol.*) 31, 166-174.
9. Facey, S.J., F. Gross, L.C. Vining, K. Yang, and K.H. van Pee. 1996. Cloning, sequencing and disruption of a bromoperoxidase-catalase gene in *Streptomyces venezuelae*: evidence that it is not required for chlorination in chloramphenicol biosynthesis. *Microbiology* 142, 657-665.
10. Farr, S.B., and T. Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium. Microbiol. Rev.* 55, 561-585.
11. Hidalgo, E., H. Ding, and B. Demple. 1997. Redox signal transduction: mutations shifting [2Fe-2S] centers of the SoxR sensor-regulator to the oxidized form. *Cell* 88, 121-129.
12. Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward, and H. Schrempf. 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation, Norwich, UK.
13. Lee, J.-S., Y.-C. Hah, and J.-H. Roe. 1993. The induction of oxidative enzymes in *Streptomyces coelicolor* upon hydrogen peroxide treatment. *J. Gen. Microbiol.* 139, 1013-1018.
14. MacNeil, D.J., K.M. Gewain, C.L. Ruby, G. Dezeny, P.H. Gibbons, and T. MacNeil. 1992. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* 111, 61-68.

15. Mazodier, P., R. Petter, and C. Thompson. 1989. Intergeneric conjugation between Escherichia coli and Streptomyces species. J. Bacteriol. 171, 3583-3585.

16. Merrick, M.J. 1976. A morphological and genetic mapping study of bald colony mutants of Streptomyces coelicolor. J. Gen. Microbiol. 96, 299-315.

17. Redenbach, M., H.M. Kieser, D. Denapaite, A. Eichner, J. Cullum, H. Kinashi, and D.A. Hopwood. 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb Streptomyces coelicolor A3(2) chromosome. Mol. Microbiol. 21, 77-96.

18. Storz, G. and J.A. Imlay. 1999. Oxidative stress. Curr. Opin. Microbiol. 2, 188-194.

19. Zheng, M., F. Aslund, and G. Storz. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science 279, 1718-1721.