CHAPTER 5

Role of CatR in DTT mediated catalaseA induction

5.1. Introduction

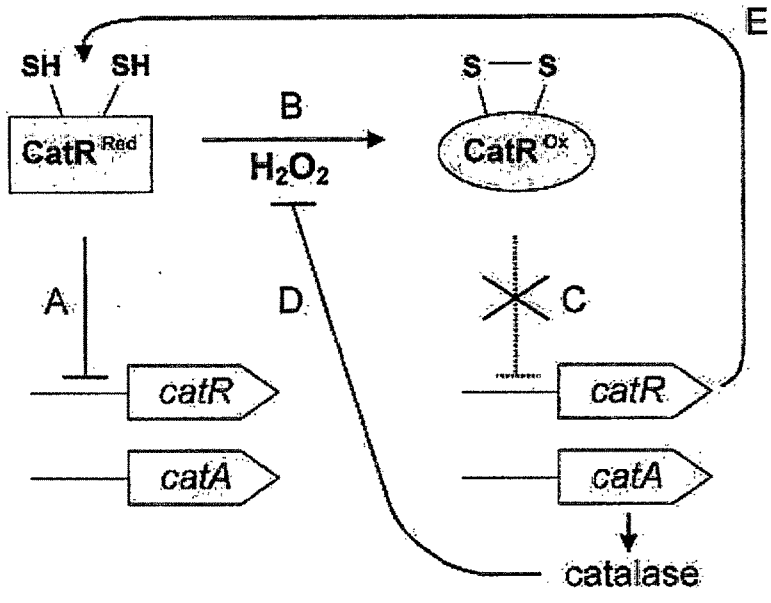
Redox dependent regulation is one of the most important aspects for the adjustment and survival of bacteria in the altered conditions like oxidative stress. The adaptation to oxidative stress in *Escherichia coli* and *Salmonella typhimurium* exert distinct responses against H_2O_2 and O_2^- controled by OxyR and SoxR/SoxS regulators respectively (Farr and Kogoma, 1991). OxyR is an H_2O_2 sensing transcriptional regulator, regulating the expression of at least nine genes including *oxyS* (a small, nontranslated regulatory RNA), *katG* (hydrogen peroxidase I), *ahpC* (alkyl hydroperoxide reductase), *gorA* (glutathione reductase), *dps* (DNA binding protein), and *grxA*(glutaredoxin 1) genes. OxyR is regulated by breaking and formation of disulphide bonds between two cysteine residues (Cys199 and Cys208) in response to changes in redox status of the cell (Zheng et al., 1998). Oxidized form of OxyR, with a disulphide bond formed in response to oxidative stress, binds to promoters of the above genes. Glutaredoxin 1 deactivates OxyR by reducing disulfide bond, forming an autoregulatory loop (Christman et al.,, 1985). 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 (Hidalgo et al., 1997). 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) (Koh et al., 1996).

In Gram-positive *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) (Bsat et al., 1998). It is proposed 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.

In *Streptomyces coelicolor* also adaptive response to H_2O_2 has been reported. Two-dimensional protein gel analyses revealed that synthesis of more than 100 proteins were induced when *S. coelicolor* cells were exposed to H_2O_2 (Chung and Roe, 1993) and the *catA* gene encoding catalaseA was identified as one of the H_2O_2 inducible genes (Cho and Roe, 1997; Kim et al., 1994). In *S. coelicolor*, the peroxide stress is sensed by at least three different regulators. RsrA, a peroxide-sensing transcriptional regulator is an antisigma factor for σ^R, which directs the expression of thioredoxin genes (Kang et al., 1999; Paget et al., 1998). OxyR regulates the expression of alkyl hydroperoxide reductase and its own gene product but do not affect catalaseA expression as in *E. coli.* (Hahn et al., 2002). The production of catalase A, which is the major vegetative catalase, is negatively regulated by a peroxide-sensing repressor, CatR (Hahn et al., 2000). CatR negatively regulates its own expression as well.

CatR is closely related to *B. subtilis* PerR in H_2O_2-dependent regulation of downstream target genes and share similar characteristics of peroxide sensing. Figure 5.1 represents the model proposed by Hahn et al. (2000), for the rapid adaptation response against H_2O_2 in *S. coelicolor*; according to which, under a normal reducing intracellular environment, CatR reamains bound to promoters of *catA* and *catR* genes and represses their transcription. Upon exposure to H_2O_2, the free cysteine thiols of CatR are oxidized to form disulfide bonds, causing loss of DNA binding activity and thus derepression of *catA* and *catR*. Induced catalaseA efficiently removes H_2O_2, whereas overproduced CatR represses both genes as soon as peroxide is removed, thus forming an efficient negative feedback loop.



Hahn J et al. J. Biol. Chem. 2000;275:38254-38260

Figure 5.1: Regulation of CatA by CatR under oxidative stress by H_2O_2 in *S. coelicolor*. Reduced CatR binds to *catA* and *catR* promoters and represses their transcription (*path A*). Upon exposure to H_2O_2, the free cysteine thiols of CatR are oxidized to form disulfide bonds (*B*), causing loss of DNA binding activity and thus de-repressing *catA* and *catR* (*C*). The induced catalase removes H_2O_2 (*D*), whereas the induced CatR (coupled with an increase in the proportion of the reduced form as peroxide is removed) represses both genes (*E*), forming a negative feedback loop.

According to the above mechanism of *catA* induction by H_2O_2, under reducing conditions *CatR* should go into a conformation with free cysteine thiols and thus restore its DNA binding ability. Therefore as per the proposed mechanism, it can be envisaged that under thiol stress CatR should remain bound to *catA* and *catR* promoters, consequently suppressing the expression of *catA*.

In contrast to the above mechanism, instead of suppression, *catA* was found to be induced under thiol stress (chapters 3 and 4). Thus, it is important to know whether *catA* induction under thiol stress is mediated by CatR regulon or not. In order to investigate the role of catR, its expression under thiol stress was

monitored indirectly by expression of a reporter protein EGFP (Enhanced Green Fluorescence Protein) under catR promoter and also by mRNA expression using RT-PCR (Reverse transcriptase-PCR).

5.2. Materials and methods

5.2.1. Expression of Enhanced Green Fluorescence Protein (EGFP) under catR promoter

In order to study *catR* expression, *catR* promoter was cloned immidiately upstream of EGFP using a shuttle vector pIJ8660 (Figure 5.2). pIJ8660 is an integrating plasmid vector, which is maintained as a single copy by insertion into the genome through *att* site. It contains MCS (multiple cloning site) prior to EGFP open reading frame. It has apramycin resistance marker *aac(3)IV*. It contains *oriC* for replication in *E. coli* and *oriT* for the conjugal transfer from *E. coli* to *S. coelicolor.*

The promoter sequence of *catR* (-111 to +74) was PCR amplified (30 cycles, 62 °C annealing temperature) from *S. coelicolor* genomic DNA using primers, CatRP(Forward) 5'TAT GGA TCC CTC TTG GCC AAT GCC GCC CCG G3' and CatRP(reverse) 5' AAT GGT ACC TCG GCC ACG ACC CGC CGC TGC G3' containing *Bam*HI and *KpnI* sites (underlined sequence) respectively. The PCR product of 197 bp and the plasmid pIJ8660 were digested with *Bam*HI and *Kpn*I in order to favour directional cloning of the DNA fragments. The ligation mixture was transformed into *E. coli* DH5α strain and the colonies obtained on LA plates containing 25 μM apramycin were screened for the presence of recombinant clones. All requisite molecular biology techniques were performed using standard protocols (Sambrook and Russell, 2001). The plasmids isolated from the transformants were confirmed for the presence of insert by analyzing relevant restriction digestion patterns. The confirmed recombinant plasmid was subsequently transformed into *E. coli* S17.1 strain to facilitate conjugal transfer

into *S. coelicolor.* Resultant *S. coelicolor* conjugants were screened on 10 μg/ml apramycin containing SMA (2% Soyabean, 2% mannitol and 2% agaragar) plates. EGFP expression was quantified fluorimetrically. *S. coelicolor* cells were sonicated after two hours of DTT treatment and the cell debris was removed by centrifugation (10,000 g X 10 minutes) and the protein in the supernatant was estimated by Folin's method (Lowry et al., 1951). Protein concentrations were normalized in supernatant fractions and the fluorescence was measured at λ_{ex} 490 nm and λ_{em} 512 nm using Hitachi F7000 spectrofluorimeter.

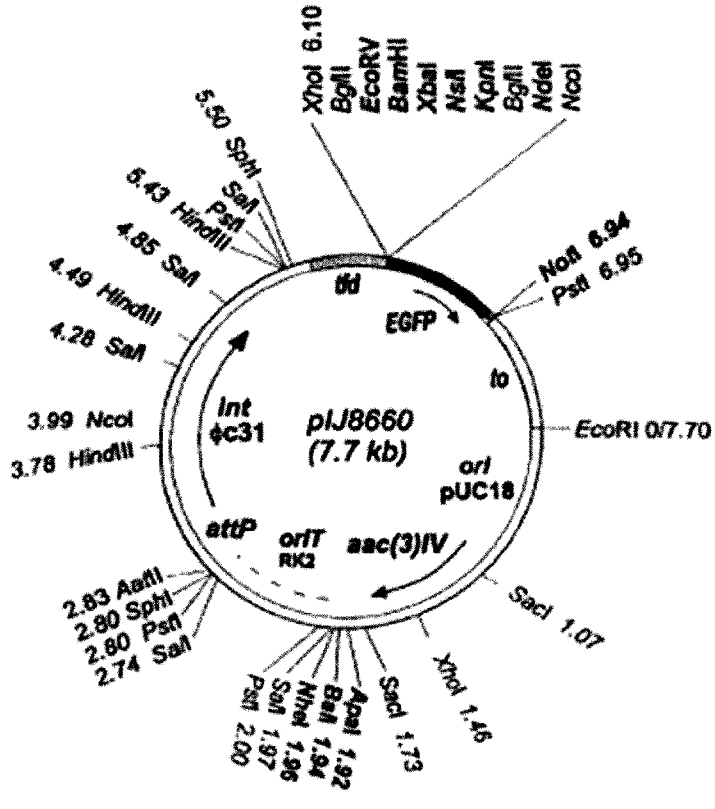


Figure 5.2: Plasmid restriction map of pIJ8660. *Bam*HI and *Kpn*I sites in the MCS were used for cloning of catR promoter. Open reading frame of EGFP (Enhanced Green Fluorescent Protein) without promoter; *ori* pUC18 (origin of replication of pUC18 plasmid in *E. coli);* Aac(3)IV (apramycin resistance gene for the selection of transformants); oriT RK2 (origin for conjugal transfer); *attP* (site for integration into the genome); intΦc31 (gene coding for integrase activity).

5.2.2. RNA isolation and reverse transcriptase PCR (RT-PCR)

RNA was isolated from growing cultures of *S. coelicolor* using TRIZOL reagent (Invitrogen, USA) (van Keulen et al., 2004) with some modifications as per manufacturer's instructions. *S. coelicolor* cultures were exposed to thiol stress as mentioned earlier. After two hours of treatment, 1.5 ml mycelium was pelleted and immediately resuspended in 1 ml TRIZOL reagent. The mycelium was sonicated (two pulses of 10 seconds each) in the TRIZOL reagent and the cell debris was removed by centrifugation at 1000 g for 10 minutes. Chloroform (200 µl) was added to the supernatant and the two phases were separated by centrifugation (1,000 g X 10 min). The upper aqueous phase was carefully transferred to another eppendorf tube. RNA was precipitated by 500 µl isopropanol and kept at 4 °C for 15 minutes and centrifuged at 10,000 g for 10 minutes. The pellet was washed with 500 µl 70% ethanol and air-dried till the smell of ethanol goes off and finally resuspended in 25 µl double distilled water. DNase treatment (1 U/µg for 45 minutes) was given to remove residual genomic DNA and DNase was inactivated by heating at 75 °C for 10 minutes. Integrity of RNA was confirmed by denaturing agarose gel electrophoresis. The purity and concentration of RNA was determined by $OD_{260/280}$ ratio.

Reverse transcription was performed using "First strand cDNA synthesis kit" (Fermentas Life Sciences) as per the manufacturer's protocol. The first strand cDNA was synthesized using random primers supplied with the kit and 5µg of total RNA as template. *catA* and *catR* specific cDNA was amplified by PCR (30 cycles, 60 °C annealing temperature). Specific primers used were *catA* (Forward)- 5′ CTG GGA TCC GGT CGG AAA C 3′ , *catA* (Reverse)- 5′ GGT GGA TCC GGT AGT TCT CCG-3′, *catR* (Forward) 5′ AGC GAA TTC AGG TCC TCG ACG GCG AAC-3′ and *catR* (Reverse) 5′ CGC GAA TTC CGC AGA CCA GGT GGT GGT 3′. The PCR amplified products were run on 1.5% agarose gel electrophoresis and the band instensities which are directly proportional to the DNA concnetration were quantified densitometricaly using "Image J" software.

5.3. Results

5.3.1. Cloning of *catR* promoter in expression vector pIJ8660

The *catR* promoter was cloned as discussed in section 5.2 (Figure 5.3) and was named as pIJ8660catRP (*catR* promoter). Results were confirmed by the release of 190 bp *catRP* (*catR* promoter) insert upon digestion with *Bam*HI and *Kpn*I (Figure 5.4)

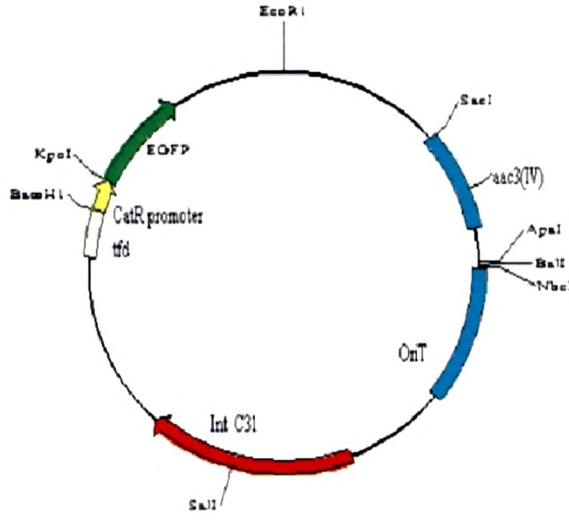


Figure 5.3: Cloning of *catR* promoter in pIJ8660. *catR* promoter sequence (-111 to +74) was cloned in pIJ8660. *CatR* promoter was placed immediately before EGFP open reading frame containing ribosome binding site to express EGFP under *catR* promoter.

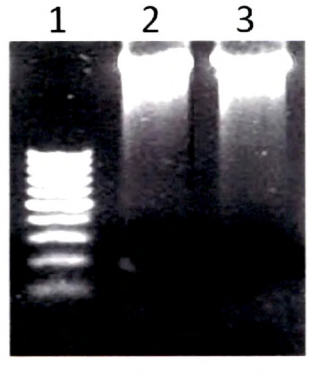
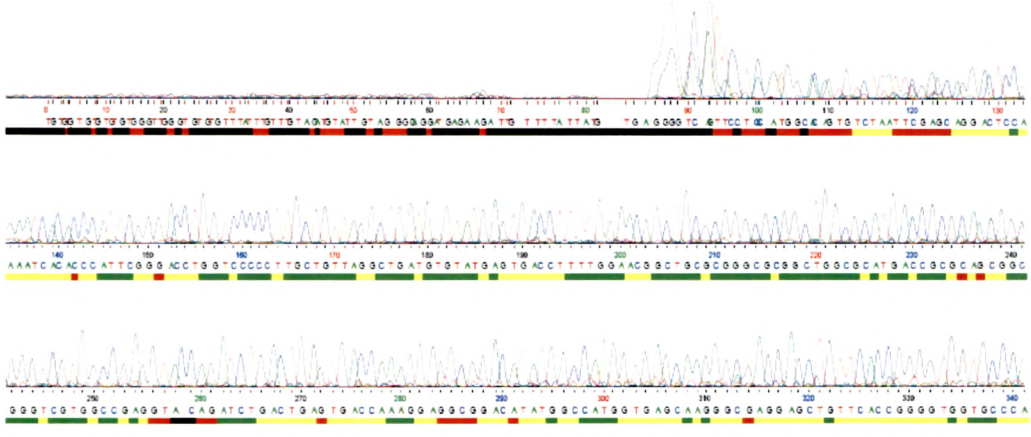


Figure 5.4: Restriction digestion of pIJ8660catRP. Lane 1: 100bp DNA ladder. Lane 2: Insert release of 190 bp observed upon digestion of pIJ8660catRP with *Bam*HI and *Kpn*I. Lane 3: Empty vector pIJ8660 digested with *Bam*HI and *Kpn*I.

Promoter sequence in the recombinant plasmid was confirmed by DNA sequencing from MWG DNA sequencing services (Figure 5.5) and found to be exactly matching to the Gene database (SCO5206) (http://www.genome.jp/dbget-bin/www_bget?sco:SCO5206).



>s.coelicolor , carR promoter -111 to +74

CTCTTGGCCAATGCCGCCCCGGTTCCTACAATGGACAGTGTCTAATTCGAGCAGGACTCC
AAAATCACACCCATTCGGGACCTGGTCCCCCTTGCTGTTAGGCTGATGTGTATGAGTGAC
CTTTTGGAACGGCTGCGCGGGCGCGGCTGGCGCATGACCGCGCAGCGGCGGGTCGTGGC
CGAGGT

Figure 5.5: Sequence confirmation of *catR* promoter. The cloned catR promoter was sequenced from MWG DNA sequencing services using the same primers used for cloning. Black text indicates the promoter region of catR with initial coding region in blue text.

5.3.2. Effect of thiol stress on *catR* expression (by monitoring EGFP expression)

Compared to control (*S. coelicolor* containing pIJ8660 without catR promoter), there was increase in fluorescence in pIJ8660catRP containing cells suggesting the expression of EGFP under catR promoter (Figure 5.6). Fluorescence after 10mM DTT treatment remained unchanged as compared to untreated cells indicating that there is no change in the catR expression in the presence of DTT.

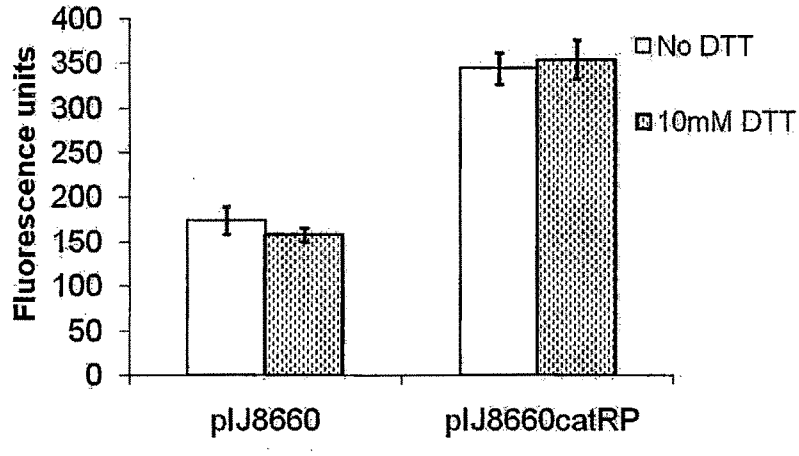


Figure 5.6: Effect of DTT on *catRP* expression using EGFP reporter protein. The EGFP expression was monitored fluorimetricaly in pIJ8660 and pIJ8660catRP cloned *S. coelicolor* cells treated with and without 10 mM DTT. There was no change in the EGFP expression in the presence of DTT in plasmid control pIJ8660 as well as pIJ8660catRP as compared to their respective controls (without DTT treatment).

5.3.3. Effect of thiol stress on *catR* expression (by monitoring mRNA expression)

To confirm the above *catRP* expression, *catR* mRNA expression was checked by RT-PCR (reverse transcriptase - PCR) and was compared with the *catA* expression. Figure 5.7 shows the total RNA extraction profile from wild type and *catA* mutant strains. RT-PCR results were in agreement with the EGFP reporter assay. As expected, in wild type *S. coelicolor*, *catA* expression was induced significantly in the presence of 10 mM DTT, where as catR expression remained unaltered (Figure 5.8). In catalaseA mutant, *catA* mRNA expression was down regulated in the presence of DTT which explains that the depleted catalase activity as established in chapter 4 is because of transcriptional down regulation in the presence of DTT. Besides in the mutant, there was no change in *catR* mRNA expression in the presence of 10 mM DTT (Figure 5.9) indicating that CatR does not play any role in the induction of catalaseA under thiol stress.

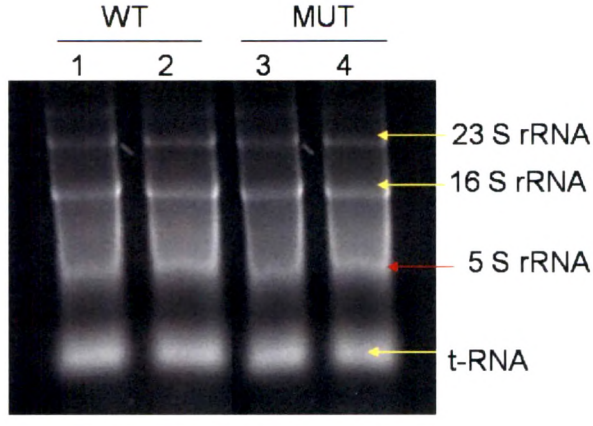
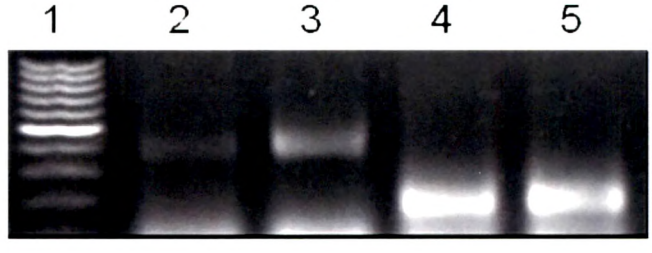
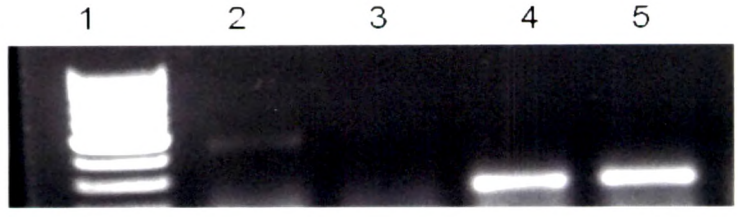


Figure 5.7: mRNA extraction profile from wild type (WT) and *catA* mutant (MUT). Lane 1: WT control, Lane 2: WT DTT, Lane 3: mutant control, Lane 4: mutant DTT.



CatA control	CatA DTT	CatR Control	CatR DTT
5.73 ± 0.15	13 ± 1.73 *	52.28 ± 0.52	62.39 ± 5.63

Figure 5.8 Effect of DTT on *catA* and *catR* mRNA expression in WT *S. coelicolor* by RT-PCR. Lane 1: 100 bp DNA ladder, Lane 2: CatalaseA control, Lane 3: CatalaseA 10 mM DTT, Lane 4: CatR control, Lane 5: CatR 10 mM DTT. Densitometric analysis table shows that there is a significant induction of *catA* but there is no change in the *catR* expression in the presence of DTT. Table shows normalized mean values ± SEM of densitometric analysis of three independent experiments. *$p<0.05$



CatA control	CatA DTT	CatR Control	CatR DTT
27.1± 1.24	16.3 ± 1.7	88.3 ± 4.6	93.7 ± 6.9

Figure 5.9: Effect of DTT on catA and catR mRNA expression in *catA* mutant *S. coelicolor* by RT-PCR. Lane 1: 100 bp DNA ladder, Lane 2: CatalaseA control, Lane 3: CatalaseA 10 mM DTT, Lane 4: CatR control, Lane 5: CatR 10 mM DTT. Densitometric analysis table shows that there is a decrease in *catA* but there is no change in the *catR* expression in the presence of DTT. Table shows normalized mean values ± SEM of densitometric analysis of three independent experiments.

5.4. Discussion

As discussed in the introduction, *catR* plays an important role in H_2O_2 mediated *catA* induction and its mechanism of regulation involves thiol oxidation and reduction of *catR*. The present study is completely different from oxidative stress mediated by H2O2, since here DTT is leading to reductive stress which is responsible for CatalaseA induction. This is the first study in bacteria, where catalase induction was observed as a result of reductive stress. We have shown here that it employs a different mechanism which is independent of CatR.

As per the proposed model of regulation, under normal condition CatR remains in reduced state which has high affinity to the promoter of *catA* and *catR* genes thus suppressing their expression (Hahn et al., 2000). In the presence of H_2O_2 the intracellular environment gets oxidized leading to formation of disulphide bonds in CatR and thus inducing their expression.

According to the above hypothesis it was assumed that under thiol stress (reducing conditions) *catR* cysteines should be maintained as free thiols and thus continuously suppresses the *catA* and *catR* expression. But in contrast to this hypothesis catA was induced in the presence of DTT. So the expression of *catR* was studied and was found to be uninduced, indicating that the catA induction under thiol stress must be through another mechanism.

Further if catA were to be regulated by catR even under thiol stress, then in case of *catA* mutant the catR induction should be more prominent because lack of catalaseA in the mutant will lead to accumulation of H_2O_2, which in turn oxidizes CatR, releasing it from CatR promoter and thus leading to more induction of *catR*. But from catR expression of *catA* mutant, this trend was not observed and catR levels remained same. The result, further, strengthens the absence of *catR* involvement in *catA* induction under thiol stress.