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Thiol stress induces catalaseA in Streptomyces coelicolor

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Streptomyces coelicolor, a gram positive bacterium, lacking glutathione was selected as a model organism for characterizing the enzymes involved in the maintenance of thiol-disulphide balance and inter-conversion by subjecting it to thiol stress. Protein profiles of samples subjected to thiol stress were compared with controls. One of the induced proteins was 'CatalaseA' as identified by MALDI-TOF and further confirmed by partial sequencing using LC/ESI-MS/MS. Measurement of catalase activity in stressed samples showed large increase supporting the structural identification. Disturbance in redox balance induces CatalaseA, to protect the bacterium from impending DNA damage mediated by hydrogen peroxide and hydroxyl radicals. Catalase seems to be a point of convergence for oxidative, reductive and thiol stresses.

Keywords: *Streptomyces coelicolor*; MALDI-TOF; LC/MS; thiol stress; catalase.

1. Introduction

Disulphides play very important role in maintaining the tertiary structure of many proteins and quartenary structure in some oligomeric proteins. In certain proteins like lysozyme, disulphide formation is the first step in the folding, followed by formation of secondary structure [1] and removal of disulphides results in total loss of structure [2]. Thiols also have an equally indispensable role as active sites in enzymes or coenzymes and in the detoxification of certain toxic chemicals. In addition thiols act as reducing buffers intracellularly. Thiol-disulphide ratio plays a key role in the maintenance of intracellular redox balance. In many regulatory proteins thiol-disulphide interconversion is used as an on-off mechanism for intracellular regulation.

In vivo, there are several enzymes meant for maintenance of thiol-disulphide balance. Thioredoxin, glutaredoxin, glutathione peroxidase belong to this group. Additionally, there are enzymes which assist in the formation of disulphides and their isomerization, facilitating folding of proteins to their native state. Protein disulphide isomerase (PDI) in eukaryotes was found to accelerate refolding of disulphide scrambled proteins [3] and was GSH/GSSG ratio dependent for this activity [4].

In gram negative bacteria disulphide formation occurs in the periplasmic space [5]. DsbA, a thiol-disulfide oxidoreductase was the first enzyme to be identified in the periplasm of *Escherichia coli* [6]. Later another five enzymes were identified, which either complement or assist DsbA in disulphide bond formation or isomerization [7].

Thiols being intracellular reducing buffers, thiol stress has two pronged effect on biological systems. Primarily, being reductive in nature, they change the redox balance and also donate electrons to intracellular electron acceptors [8, 9]. These intracellular reductants reduce Fe^{3+} to Fe^{2+}. Subsequently, Fe^{2+} catalyses the breakdown of intracellular hydrogen peroxide to hydroxide ion and hydroxyl radical, while getting oxidized to Fe^{3+}. The hydroxyl radicals cause DNA damage (Fenton reaction).

In eukaryotes thiols were reported to cause reductive stress [8]. Thiols were shown to induce the expression of catalase, Mn-superoxide dismutase [10] and monooxygenase [11] in certain eukaryotic

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systems. Secondly, they reduce disulphide bonds in proteins, accumulating unfolded proteins, which in turn give a stress signal to the cell causing expression of stress responsive proteins. This is referred to as unfolded protein response (UPR) [12].

In this work *Streptomyces coelicolor*, a gram positive, aerobic bacterium belonging to actinomycetes, was selected as a model organism, to study the effects of thiol stress for the following reasons: Actinomycetes possess mycothiol in place of glutathione, the most common thiol buffer. The effect of disulphide stress in the cytoplasm of *Streptomyces coelicolor* has been well studied. The –SH/ -S-S- levels are maintained in this organism by thioredoxin and thioredoxin reductase. This thioredoxin system is modulated by the sigma factor σ^R [13-15]. However, there are no studies on the effects of thiol stress in *Streptomyces coelicolor*. Further, there is not much information available about the enzymes involved directly or indirectly in thiol-disulphide balance and interconversion, in this organism.

We chose to subject *Streptomyces coelicolor* to thiol stress by exposing it to dithiothreitol (DTT) and compare the protein profiles in treated and untreated cells, to identify proteins which show altered expression. We report here that a 55kDa band was prominently induced under thiol stress and using MALDI-TOF and nano-LC-ESI-MS/MS, we have identified it to be catalaseA. Identity of the induced band was further confirmed by measuring the catalase activity in cellular extract of *S. coelicolor* exposed to thiol stress. Our results also show a change in the thiol content in the cytoplasm of *S. coelicolor* under DTT stress.

Catalase hydrolyzes intracellular hydrogen peroxide to water, preventing free radical production through Fenton reaction. Induction of catalase expression appears to be a protective mechanism against free radicals produced under thiol stress.

2. Experiments

2.1.a Culture growth and standardization of the concentration of DTT necessary for inducing stress.

Streptomyces coelicolor cultures were grown to log phase in YEME media at 30°C and exposed to 2-20mM DTT for four hours. The mycelia were harvested by centrifugation and washed twice with sonication buffer (20mM Tris, pH 7.5, 100mM $MgCl_2$, 0.1% Triton X-100). The pellets were harvested after centrifugation and incubated at 4°C for 30min after resuspending in sonication buffer containing 1mg/ml lysozyme, 1mM PMSF. The mycelia were sonicated thrice for 30 seconds in continuous mode. The lysates were centrifuged at 60,000g for 30min and the supernatant was collected. The protein concentration of the supernatant was estimated using Folin Lowry method.

2.1.b Total thiol estimation by 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB)

In order to check the intracellular redox status of *S.coelicolor* under DTT stress, the total thiol content was estimated. Actively growing culture under log phase was treated with DTT and lysed. Total intracellular thiol content was estimated using the method given by Sedlak and Lindsay [16]. The thiol content was expressed as the equivalent of thiol content of DTT from the standard curve of DTT.

2.2 Protein profiling by gel electrophoresis

For analysis by 1D and 2D gel electrophoresis, protein from supernatant of lysate was precipitated by adding three volumes of cold acetone and left overnight at -20°C. The pellet collected by centrifugation was washed twice with cold acetone and allowed to dry at room temperature and analyzed by 1D SDS-PAGE [17]. Gels were stained with Coomassie brilliant blue. Samples were also analyzed by 2D gel electrophoresis using Bio-Rad Protein IEF (isoelectric focusing) Cell. Briefly ~200 µg of cell extract protein was dissolved in 185 µl of IEF buffer supplied by Bio-Rad. An 11 cm IPG strip pH 4-7 was rehydrated with the above sample in IEF buffer for 14h at 20°C. Following rehydration, IEF was

performed on a Biorad 2-D gel electrophoresis system with the stepped program until the focusing reaches a voltage of 42 kVh and then the voltage was allowed to decrease from 8000 to 500 V in 1h. The proteins in IPG strip were reduced and alkylated with iodoacetamide. Second dimension electrophoresis was performed using Laemmli's method.

2.3 In-gel digestion

Protein bands of interest were excised from the gel. Trypsin digestion and extraction of the peptides was carried out using standard protocol [18-21].

2.4 MALDI-TOF analysis of the peptides

Tryptic digests of specific gel bands were analysed by mass spectrometry. MALDI-TOF mass spectra were recorded in reflectron mode on Tof-Spec 2E (Micromass, UK), fitted with a 337-nm laser. 1.0 µl of peptide digest in 70% acetonitrile, 0.1 % trifluoroacetic acid (TFA) was mixed with equal volume of a solution of α-cyano (10 mg/ml solution in 70% acetonitrile/ H_2O containing 0.1 % TFA was used. Peptide mass fingerprint (PMF) of the digested protein was obtained in the reflectron mode at the operating voltage of 20 kV. 30-40 spectra were summed, smoothened and used for database search. Database search of the PMF was carried out on the website www.matrixscience.com using the mascot program in the MSDB database.

2.5 nanoLC-ESI-MS /MS

Proteins in the gel slices were digested with trypsin and peptides extracted and dried. Dried mixture of tryptic peptides was dissolved in 20µl of 5% acetonitrile, 0.01% TFA. After injection (8µl) the peptides were concentrated on Trap C-18 enrichment column (0.3 x 5 mm, Agilent) and washed with 100 µl 5% acetonitrile, 0.01% TFA. The enrichment column was then switched into the nanoflow path (200 nl/min) and further separated on C-18 reversed phase ZORBAX 300SB-C-18 nanocolumn (0.075 x 150 mm; Agilent) coupled with the nanoelectrospray ionization (nESI) source of XCT Plus ion trap (Agilent 1100 Series LC/MSD Trap XCT Plus System). The nanoflow gradient started with 5% acetonitrile, 0.01% TFA and increased up to 55% acetonitrile, 0.01% TFA with a slope of 1%/min. Automated MS/MS spectra were acquired during the run in data-dependent acquisition mode with selection of three of the most abundant precursor ions (0.5 min active exclusion; 2+ ions preferred). Spectra were extracted using Spectrum Mill software extractor (Agilent) and searched against NCBI database.

2.6 Catalase assay

For enzyme activity assays, *S. coelicolor* lysates were prepared as described above. Catalase activity was assayed by monitoring the degradation of H_2O_2 at 240 nm every 10 seconds [22]. 1 ml of 10 mM H_2O_2 solution in 50mM potassium phosphate, pH 7.0 was used as assay solution and 5-50 µl of cell extract supernatant was added. Catalase specific activity was expressed as µmoles of H_2O_2 degraded per min per mg protein.

2.7 Effect of o-phenanthroline on catalase induction

Cultures of *S.coelicolor* growing in mid-log phase were incubated with 1mM O-phenanthroline for 30min. prior to DTT stress and catalase activity was assayed as earlier to estimate the level of induction.

The direct effect of O-phenanthroline on catalase enzyme was estimated *in vitro* by adding 1mM O-phenanthroline to lysate prior to catalase assay.

2.8 Superoxide dismutase assay

For superoxide dismutase activity assay, 200µl supernatant was added to 750µl of 0.2 mM potassium phosphate, pH 8.0 followed by 50µl of 0.5mg/ml of pyrogallol. Autooxidation of pyrogallol was monitored by recording the absorbance at 420nm every 10 sec for 3 minutes. Specific activity of SOD was estimated by units of enzyme required for 50% inhibition of auto-oxidation of pyrogallol per mg protein [23].

3. Results

The effect of DTT on the growth of *S. coelicolor* was measured by estimating the total protein content of the mycelia. With increasing concentrations of DTT, total protein content drops rapidly suggesting severe decrease in growth (Figure 1a). Mycelial lysis was observed at 15mM DTT. So for all subsequent experiments, a concentration of 10mM DTT was used with an exposure time of 4 hours. In addition, intracellular total thiol content was estimated. Under DTT stress total thiol content was found to be higher than that of control, showing that the environment in the bacterium was reducing (Figure1b).

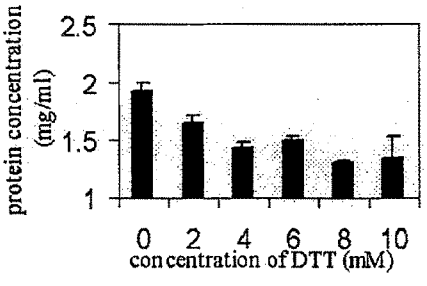

Figure 1a

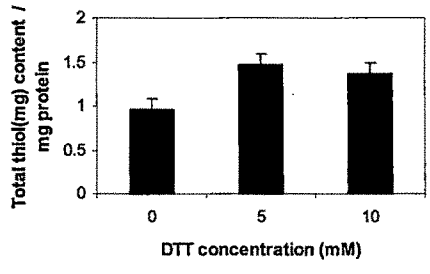

Figure 1b

Figure 1 a) Effect of DTT on the growth of *S. coelicolor*. Total protein content of culture was plotted as function of DTT concentration used for thiol stress. Cultures were grown to mid log phase and different amounts of DTT were added. Cultures were further allowed to grow for four hours and total cell protein content was measured. **b)** Estimation of intracellular total thiol content. Samples were prepared as above and total intracellular thiol content was estimated.

Whole cell protein extracts from control and 10mM DTT treated cultures were subjected to SDS-PAGE analysis to determine the changes in the protein expression profile (Figure 2a). An intense band of ~55kDa protein was over-expressed in the DTT treated sample. These extracts were also analyzed by 2-dimensional gel electrophoresis to identify the subtle changes in protein expression profile (Figure 2b). Apart from 55 kDa protein, several new low molecular weight (10-30 kDa) proteins appeared in DTT treated culture (marked with arrows). Differences in the levels of expression of several other proteins are also observed between sample and control.

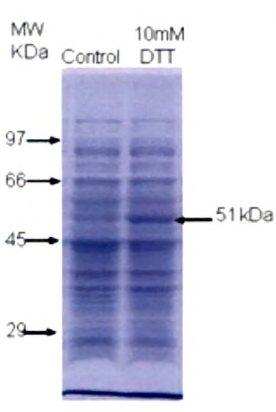

Figure 2a

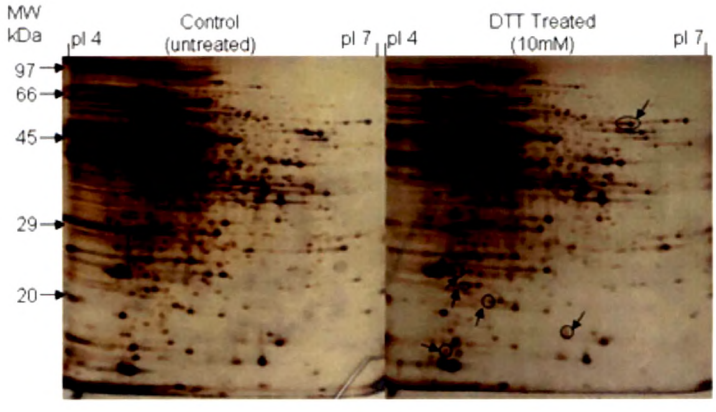

Figure 2b

Figure 2 a) 12% SDS-PAGE for whole cell lysates of proteins from *S. coelicolor* treated with 10 mM DTT and control. Over-expresion of a protein band at ~55kDa was observed for the treated culture lane. **b)** 2-Dimensional gel electrophoresis on control and 10mM DTT treated samples. Several new spots were observed in low molecular weight region (marked by arrows).

For characterizing the 55kDa protein, peptides from trypsin digested 55kDa band were extracted and subjected to MALDI analysis. Figure 3a) shows the peptide mass spectra. As expected, treated sample has several new peptides. From the peptide mass finger print of 55kDa band protein(s) of treated samples, out of 22 peptide masses submitted for identification, in MSDB database search, 10 peptide mass values were found to match within ±1.0Da error with high probability score of 63 and sequence coverage of 29% with 'CatalaseA' (NCBI: T42038; SwissProt: Q9RJK9) (EC 1.11.1.6).

For unequivocal identification of the induced protein(s), tryptic digests of the 55kDa band prepared from control and DTT treated samples, were subjected to nanoLC-ESI-MS/MS. Figure 3b) presents the nanoLC-ESI-MS/MS data set for a peptide sequence of the 55kDa band. After the search in the NCBI database using Spectrum Mill search engine, catalaseA with 42% sequence coverage was identified as unique and major component of treated sample. In contrast catalaseA was present in substantially lower concentration in the control sample and led to the conclusion that catalaseA is the major protein induced in *S. coelicolor* by DTT stress. Figure 3c) presents the complete sequence of *S. coelicolor* catalaseA with the highlighted regions of protein sequence covered in MALDI-TOF spectra and/or sequenced by nanoLC-ESI-MS/MS.

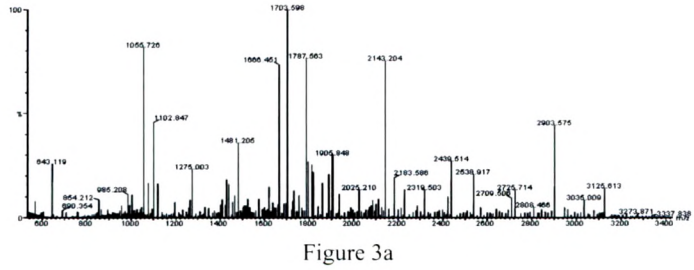

Figure 3a

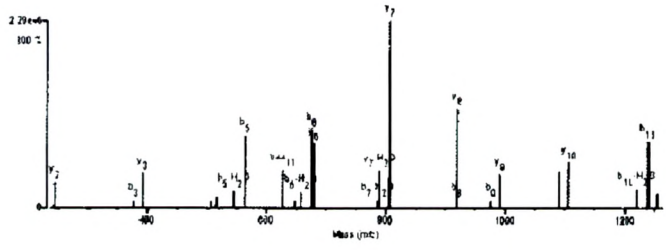

Figure 3b

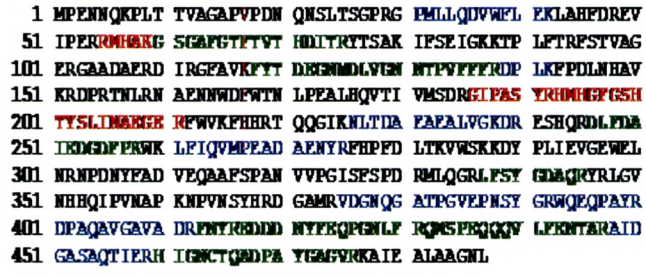

Figure 3c

Figure 3 a) MALDI-TOF spectra of the peptides extracted from 55kDa band of DTT treated *S. coelicolor* cultures. CatalaseA was identified in sample culture treated with 10 mM DTT. **b)** Identification of 55 kDa protein by nanoLC-ESI/MS/MS. MS/MS of m/z = 741.5 peak present in DTT treated sample, and absent in untreated sample, as identified by ion chromatogram leading to identification of catalaseA peptide 246DLFDAIEDGDFPK259. **c)** Sequence of *S. coelicolor* catalaseA (NCBI: T42038) (EC 1.11.1.6) (ENTREZ CAB58320). Peptides positively identified only by MALDI are shown in red, sequenced by nanoLC-ESI/MS/MS are shown in blue and the regions identified in both experiments are marked in green. There was 29% sequence coverage by peptides detected in MALDI and 42% of the protein was sequenced in MS/MS experiment.

To further confirm the identity of 55 kDa band protein as catalaseA, cellular extracts of *S. coelicolor* exposed to different concentrations of DTT were assayed for catalase enzyme activity. Results presented in Figure 4a) show that specific activity of catalase increased dramatically by a factor of >8 upon exposure to 15mM DTT. However, there was no significant change in the activity of superoxide dismutase (SOD) (Figure 4b).

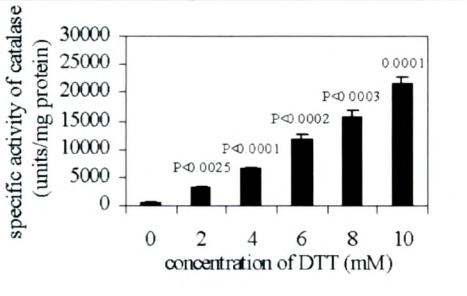

Figure 4a

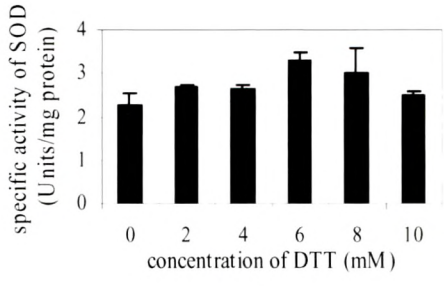

Figure 4b

Figure 4 Effect of DTT exposure of *S. coelicolor* on **a)** the specific activity of CatalaseA and **b)** Superoxide dismutase (SOD). Different amounts of DTT were added to log phase cultures of *S. coelicolor* and incubated for 4 hours. Lysates were prepared and assayed for catalase and SOD levels.

Increased intracellular thiol content under DTT stress can cause accelerated reduction Fe^{3+} to Fe^{2+} increasing possibility for DNA damage by Fenton reaction. Catalase induction could be a mechanism for removal of hydrogen peroxide to prevent the damage. In order to test this possibility O-phenanthroline, a chelator of $Fe^{2+}$was added to cultures before DTT stress. In O-phenanthroline, the catalase activity of the DTT treated samples matched with the negative controls, which were not treated with DTT. This result showed that there was no further induction of catalase under DTT stress in O-phenathroline (Figure 5a).

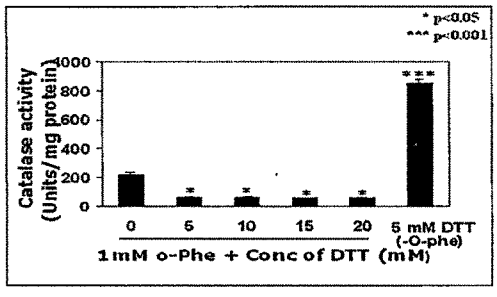

Figure 5a

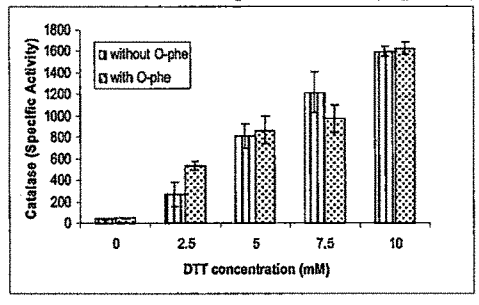

Figure 5b

Figure 5. Effect of O-phenanthroline on catalase activity in DTT treated samples of *S. coelicolor*. **a)** *S. coelicolor* cultures were incubated in 1mM O-phenanthroline for 30 min., prior to DTT treatment. After 4h of DTT treatment catalase activity was assayed. The sample treated with only 5mM DTT (last column) displays highly elevated catalase activity compared to 1mM O-phenanthroline and 5-20mM DTT treated samples. **b)** Direct effect of 1mM O-phenanthroline on catalase was monitored by adding it to lysates. The catalase assay showed no effect of O-phenanthroline on the enzyme.

Since, Catalase has Fe^{2+}as cofactor, there is a possibility of the cofactor being chelated by O-phenanthroline, inactivating the enzyme. To rule out this possibility the lysates of DTT treated cultures, were incubated with 1mM O-phenanthroline, before catalase assay. There was no change in activity of catalase at this concentration of O-phenanthroline (Figure 5b). This was confirmed by SDS-PAGE, where the intensity of catalase band of DTT treated sample in O-phenanthroline was equal to 0mM DTT control (results not shown).

4. Discussion

Our intention in subjecting *S. coelicolor* to thiol stress was to identify proteins which play a role in thiol, disulphide metabolism. The answer to the quest came from an interesting quarter. CatalaseA, an enzyme that converts hydrogen peroxide to water during oxidative stress, was found to be induced under thiol stress too.

Hydrogen peroxide is generated as a byproduct of redox reactions inside a cell. Hydrogen peroxide being an oxidizing agent is also a source of extremely potent hydroxyl radicals. Apart from damaging lipids and proteins, hydroxyl radicals can attack the bases and sugar phosphate backbone of DNA causing modifications and strand breaks [24]. Catalases, enzymes found in prokaryotes as well as eukaryotes are meant to convert endogenous hydrogen peroxide to omnipresent, harmless water.

S. coelicolor has three different catalases. Two of these are monofunctional catalases, CatalaseA or CatA [25] and CatalaseB or CatB [26], while the third one is a catalase peroxidase, also referred to as CatalaseC or CatC [27]. Among these three catalases, CatA is the major catalase implicated in proper growth and resistance against H_2O_2.

Interestingly, in eukaryotes induction of catalase was observed under reductive stress. It was reported that disturbance caused to NADH/ NAD balance and release of non-heme Fe under reductive stress together lead to hydrogen peroxide generation with an associated increase in the level of catalase [28, 29]. Subsequently, catalase enzyme converts hydrogen peroxide to water and oxygen.

Studies on oxidative damage of DNA in vitro established that the mechanism of damage follows Fenton reaction, providing explanation for the above two observations [30]. Fenton reaction occurs in three steps. Fe^{3+} ions react with a reductant forming Fe^{2+} ions and oxidized reductant. In turn Fe^{2+} ions react with hydrogen peroxide to generate hydroxide ions and hydroxyl radicals, converting back into Fe^{3+}. Hydroxyl radical reacts with both sugar and base residues of DNA causing detrimental effects. In order to prevent accumulation of hydrogen peroxide and consequent formation of hydroxyl radicals organisms show induced expression of catalases, under oxidative as well as reductive stresses, as these two stresses represent two different halves of a cyclic reaction. In *E. coli* it was observed that NADH [30] and reduced flavins in nonrespiring bacteria cause oxidative DNA damage through Fenton reaction by transferring electrons to Fe^{3+} [31]. It was also observed in *E.coli* that abnormally high level of cysteine can cause oxidative DNA damage by acting as adventitious reductant of Fe^{3+} [32]. Hence, ready availability of reductant in the form of free thiols rendered the organism excessively sensitive to hydrogen peroxide. Upsetting Fenton reaction at any step mentioned above made *E.coli* more resistant to hydrogen peroxide. We presumed that even in *S. coelicolor* accelerated recycling of Fe^{3+}/ Fe^{2+} ions under thiol stress poses the threat of DNA damage and the bacterium tries to survive this challenge by catalaseA induction. The abolition of induction of catalase in O-phenanthroline under DTT stress confirms our hypothesis. This study indicates that catalase is a point of convergence for oxidative, reductive and/ or thiol stresses (Figure 6).

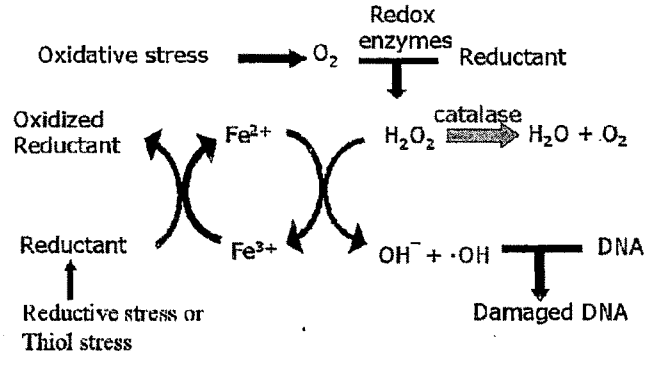

Figure 6. Mechanism of DNA damage under oxidative, reductive and or thiol stress. Removal of hydrogen peroxide by catalase averts DNA damage by hydroxyl free radicals.

In summary, under aerobic conditions DTT causes stress in *Streptomyces coelicor*, a gram positive bacterium, altering the expression of several proteins. We have identified one of the proteins that were induced under thiol stress to be catalaseA, using MALDI-TOF, and partial sequencing by LC/ESI-MS/MS. The cultures of *S. coelicor* under thiol stress showed increase in catalase activity. However, there was not much difference observed with superoxide dismutase activity. CatalseA removes hydrogen peroxide and prevents its decomposition to hydroxyl radicals by Fe^{2+} formed due to reductive stress caused by thiols.

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