CHAPTER 1

General Introduction

Microorganisms must maintain the intracellular environment for their survival and optimal growth, in spite of being in continuously changing and sometimes adverse external environment. Microorganisms have evolved many mechanisms through which they adapt to the perturbations in external environment for their successful survival. One of the most important internal conditions, which need to be regulated is redox status of the cell, especially conditions like oxidative stress and disulphide stress. For example in yeast, many genes involved in the maintenance of intracellular redox potential are found to be upregulated as a consequence of various environment stress responses (Gasch et al., 2000). Several types of redox chemicals like benzoquinone, ferricyanide, dithiothreitol (DTT) were shown to cause a behavioral response in *E. coli* (Bespalov, 1996). Thus, when environmental conditions change abruptly, the microorganisms respond by changing its genomic expression patterns to adapt to the new conditions.

There are several examples in literature on bacterial responses to oxidative stress. It is one of the most widely studied stress responses. Oxidative stress can be exerted by various external sources like high oxygen pressure, various oxidizing chemicals and UV irradiation or it can be a consequence of imbalance in the intracellular redox regulation. Responses to oxidative stress have been studied in a wide range of microorganisms and found that all aerobic organisms have evolved complex defense systems to overcome the damaging effects of reactive oxygen species (ROS) (McCord and Fridovich, 1988). In *E. coli* and *S. typhimurium* aerobic conditions lead to generation of endogenous ROS, largely through electron transport chain (ETC) ultimately leading to redox imbalance, known as oxidative stress. The oxidative stress response is very well studied in these organisms and found to induce many proteins like catalase, superoxide dismutase, chaparones (GroES, GroEL), DnaK and RecA which help to counteract the damage at various levels (Farr and Kogoma, 1991). The effects of oxidative stress, various mechanisms employed by microbes to overcome the stress, the proteins and enzymes induced by oxidative stress are discussed in the subsequent sections of this chapter. Besides, the chapter throws light on

reductive and thiol stresses, their consequences. Finally, the chapter presents the rationale behind the work along with the scheme of organization of results achieved.

1.1. Oxidative stress in bacteria

Aerobically grown organisms use molecular oxygen as an ultimate acceptor of electrons in respiration, the process in which nutrients are oxidized to obtain energy. As a consequence, several byproducts like superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) are generated. In *E. coli* respiratory chain accounts for around 87% of the total intracellular H_2O_2 generated (González-Flecha and Boveris, 1995). It is found to be the major source of ROS, mainly by mediating stepwise one electron reduction of the molecular oxygen (Equation 1.1).

$$O_2 \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow HO\cdot \rightarrow H_2O \qquad (1.1)$$

Similar to mitochondria of eukaryotic cells, the major electron leakage sites in bacteria were found to be NADH dehydrogenase and ubiquinone (González-Flecha and Boveris, 1995). Flavoproteins present in the cells are the major contributors to the generation of ROS, mainly through their auto-oxidation followed by their interaction with oxygen leading to generation of O_2^- and H_2O_2 (Figure 1.1) (Massey, 1994; Imlay, 2003). Besides this, certain environmental factors like UV irradiation and other oxidizing chemicals like paraquat, plumbagin or menadione, which generate O_2^- may lead to oxidative stress. H_2O_2, when reduced by iron through Fenton reaction, leads to formation of highly reactive hydroxyl (·OH) radical and causes major damage to the biological system. The major biological targets of ROS are DNA, RNA, lipids, and proteins.

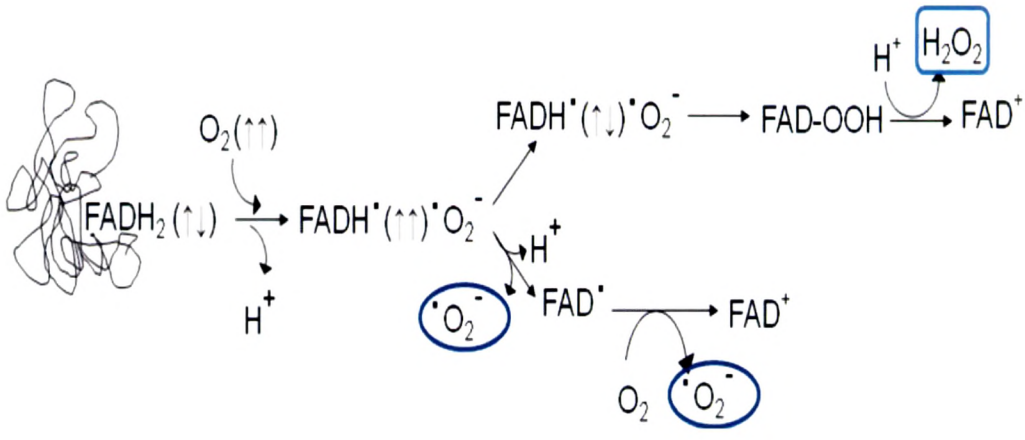

Figure 1.1: H_2O_2 **and** O_2^- **generation through autooxidation of flavoproteins.** (Adapted from Imlay, 2003)

1.1.1. DNA damage

ROS generated through above and many other routes result in numerous types of DNA lesions. H_2O_2 and O_2^- attack the nucleotide base producing 8-hydroxyguanine, hydroxymethyl urea, urea and thymine glycol (Halliwell and Aruoma, 1991; Imlay and Linn, 1988). Hydroxyl radical reacts with the sugar moiety of DNA leading to fragmentation and production of strand breaks with 3'-phosphate or 3'-phosphoglycolate termini. Thymine residues can be hydroxylated to produce 5-hydroxymethyl uracil or it can undergo oxidative degradation to produce thymine glycol and a urea residue (Demple and Linn, 1982). In addition, these alterations in the nucleotide bases cause unusual base pairing in the DNA resulting in mutations and thus affecting the function of many genes.

1.1.2. Lipid peroxidation

Lipid peroxidation is a chain reaction, which is initiated by abstraction of hydrogen from unsaturated fatty acids forming lipid radicals. Lipid radical reacts with molecular oxygen to form lipid peroxyradical (ROO·), which can be reduced by transition metal ions, NADH or O_2^- to form lipid peroxide. Lipid peroxide

further propagates the chain leading to peroxidation of other lipid molecules. Another group of radicals formed in peroxidation, the lipid alkoxy radicals can disintegrate into smaller unsaturated fatty acids such as aldehydes, alkyl radicals, alkanes, ketones and epoxides resulting in increased membrane fluidity, thus leading to loss of structural integrity, which is of utmost importance for cell survival (Sevanian and Hochstein, 1985). These Lipid peroxidation intermediates and end products are also found to react with DNA leading to mutations and to some extent they also react with and inactivate proteins as well.

1.1.3. Protein damage

In *E. coli* several enzymes have been shown to be quite sensitive to oxidative damage, including dihydroxyacid dehydratase, glutamine synthetase, ribosomal protein L12, quinolate synthetase, aconitase, and 6-phosphogluconate dehydratase (Gardner and Fridovich, 1991). ROS have diverse effects on different proteins by reacting with specific amino acids or cofactors. Oxygen radicals convert proline and arginine residues into their carbonyl derivatives. Methionine and cysteinyl residues are oxidized to form methionine sulfoxide and disulfide derivatives, respectively. Iron sulfur proteins are the soft targets for superoxide radicals, which are attracted towards the iron present in iron sulfur [4Fe-4S] cluster and cause oxidation and loss of iron leading to degradation of the cluster rendering the enzyme inactive (Flint et al., 1993). For example, dihydroxy acid dehydratase, quinolate synthetase, aconitase, and 6-phosphogluconate dehydratase have [4Fe-4S] clusters and it is likely that these are the most common sites of O_2^- attack. Besides this, oxygen radicals oxidize thiols, catecholamines, ascorbate and tocopherol, which play a very significant role in the catalytic activity of their respective enzymes. These alterations generally inactivate the enzymes leading to their degradation.

1.2. Reductive stress

Concept of reductive stress is less studied as reductive stress is very less likely to occur mainly due to omnipresence of oxygen in the atmosphere of the earth.

However, there are instances where excess of thiols and other conditions like oxygen limitations which lead to reductive stress. Thiols are known to be potent reducing agents. Thiol groups possess high reactivity and varied redox potential depending on the structure of the compound bearing the thiol group. Thiols exert diverse effects on the biological systems depending on its redox status and the local environmental conditions. It was observed that catalase activity was reduced in *E.coli,* when treated with homocysteine (a thiol containing amino acid) (Fraser et al., 2006). Genes like *sodA, fldB* and *katG,* which are involved in protecting *E. coli* against oxidative stress were also found to be repressed, suggesting that the cells experience a reductive stress when exposed to high levels of extracellular homocysteine (Fraser et al., 2006). In *Salmonella typhimurium,* homocysteine is known to counteract the inhibitory effects of reactive nitrogen species (de Groote et al., 1996). The thiol-containing compound DTT can prevent induction of a heat shock response in HeLa cells and this is attributed to its reducing activity (Huang et al., 1994). In different eukaryotes reductive stress is known to cause deleterious effects including reduced life span of yeast and *C. elegans* and age-related protein aggregation disease in mice (Ralser et al., 2007). Reductive stress in the form of increased NADH/NAD^+ and GSH/GSSG ratio has been genetically and causally linked to dysregulation of antioxidative pathways in mice (Ghyczy and Boros, 2001; Rajasekaran et al., 2007).

In contrast to this, a number of studies on homocysteine toxicity in eukaryotic systems suggest that exogenous homocysteine treatment triggers oxidative stress through the increased production of hydrogen peroxide (Loscalzo, 1996; Stamler et al., 1993; Starkebaum and Harlan, 1986). In prokaryotes increased level of cysteine in the cell induces vulnerability of cells to H_2O_2 mediated oxidative damage (Berglin et al., 1982; Park and Imlay, 2003). In *E. coli,* inhibition of respiration leads to increase in NADH and reduced flavins, which promote oxidative DNA damage by reducing the intracellular iron (Woodmansee and Imlay, 2002). The results of both experimental lines indicate that the rate of DNA

damage is typically amplified by excess availability of reductants to recycle iron after it reacts with H_2O_2 by means of Fenton reaction.

1.2.1. Thiols in biology

Thiol is a compound that contains the functional group -SH. Being the sulfur analogue of an alcohol group -OH, the functional group is referred to either as a thiol group or a sulfhydryl group. Thiols play an important role in biology by various ways. Thiol containing amino acid cysteine plays an important role in determining the tertiary and quaternary structure of a protein by formation of intra-chain or inter-chain disulfide bonds respectively, mainly through the oxidation of two cysteines to form a cystine. Thiol group can be present at the active sites of an enzyme and can under go non-covalent interaction with the substrates to catalyze the reaction. For example in cysteine protease, cysteine thiol acts as a catalytic centre of the reaction. Besides their structural and catalytic roles in proteins, thiols are important constituents of many cofactors like CoenzymeA and other biomolecules such as glutathione and mycothiol. Cysteine has very high affinity for heavy metals like Pb^{2+}, Cd^{2+}, Hg^{2+} and causes inactivation of many enzymes leading to toxicity. Thiols are also well known intracellular reductive buffers, for the maintenance of redox homeostasis of a cell.

1.2.2. Role of thiols in redox regulation

As stated earlier, bacteria have evolved many sensors which monitor different redox signals such as availability of oxygen, redox state of the cell and presence of various ROS. Adapting to the alterations in the environment, these sensors convert redox signals into regulatory output mainly through transcriptional regulation. The major sensors are redox active cofactors like flavins, haem, iron-sulfur clusters, pyridine nucleotides or part of amino acid side chains like cysteine.

Cysteine thiol is very sensitive to oxygen radicals and in some proteins cysteines can undergo reversible oxidoreduction to form various redox intermediates like cystine (R-S-S-R'), sulfenic acid (R-SOH), sulfinic acid (R-S O_2H), sulfonic acid (R-

SO_3H) and *S*-nitrosothiol (R-SNO) (Figure 1.2). The rates and reaction products are determined by the local environment of cysteine in the protein. Depending on the redox environment, many regulatory proteins with these intermediate groups undergo conformational changes and show remarkable differences in their activity, like DNA binding seen with transcriptional regulators, and thus they act as molecular switches or redox sensors in order to sense the altered environmental conditions. Microbes use distinct sensing mechanisms to detect various forms of oxidative stress (Green and Paget, 2004).

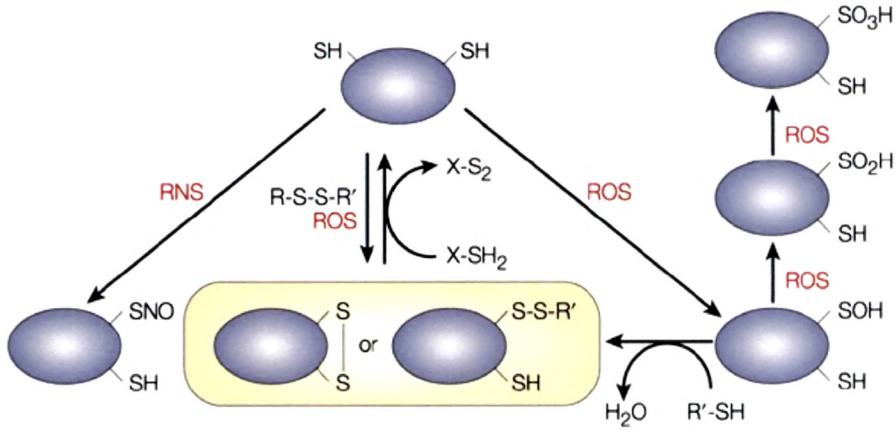

Figure 1.2: Role of cysteines in sensing the redox stress by formation of various derivatives of cysteines in the presence of ROS or RNS (Reactive nitrogen species).

1.3. Thiol based redox regulation

1.3.1. Thioredoxin and glutathione based reducing pathways

The cytoplasm of all organisms offers highly reducing environment. Most intracellular proteins maintain their cysteine thiols in the reduced state in contrast to extracellular secretory proteins in which thiol disulfide bond formation is a common event (Fahey et al, 1977). In *E. coli* thioredoxin and

glutaredoxin systems play an important role in the maintenance of these thiols in reduced state and prevent their oxidative damage (Figure. 1.3) (Holmgren, 1989).

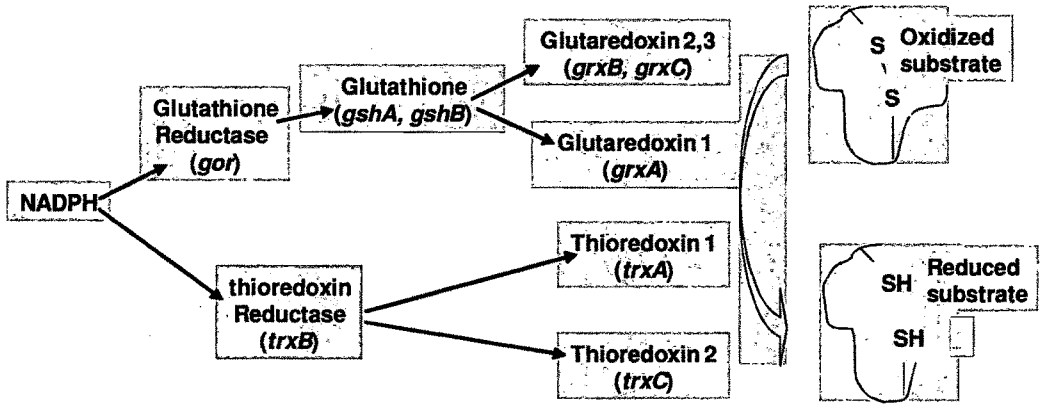

Figure 1.3: The disulfide-reducing pathways in the *E. coli* cytoplasm. Arrows represent the path of reduction of disulfide bonds. Gene names are given in parentheses.

Thioredoxin system is ubiquitously present from bacteria and archea to humans and is composed of NADPH, thioredoxin (*TrxA*) and a flavoprotein thioredoxin reductase (*TrxB*). Thioredoxin is a low molecular weight protein (12kDa) containing a conserved dithiol motif (CXXC) and a characteristic thioredoxin fold. The redox potential of thioredoxins being very low (-270 mV for *E. coli* thioredoxin 1) they are kept in the reduced thiol state in the cell by thioredoxin reductase, making them efficient protein thiol-disulfide reductants. After the reduction of the specific protein, oxidized thioredoxin again gets reduced by thioredoxin reductase in NADPH dependent manner (Williams, 1995). The redox properties of thiol-disulfide reductases and oxidases depend largely on the nature of the two amino acids between the cysteines of the active site motif CXXC (Mossner et al, 1998). Similarly, most of the organisms including animals, plants, Gram negative bacteria and some Gram positive bacteria except actinomycetes possess glutathione-glutaredoxin reductase system, a parallel pathway to maintain the intracellular reducing environment. Glutathione (L-γ-glutamyl-L-

contain thioredoxin fold and active site motif CXXC, hence they are classified into thioredoxin family. In contrary to thioredoxin reductase pathway, disulphide oxidation pathway introduces new disulphide bonds in to the substrate proteins.

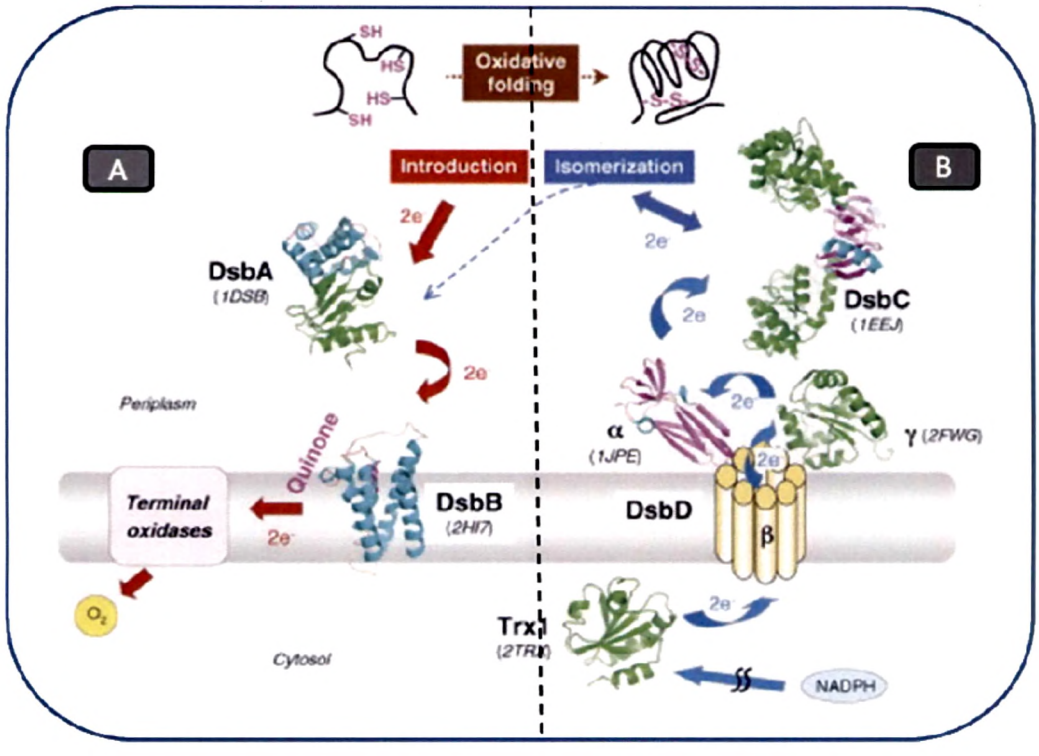

Figure 1.4: Pathways for disulphide bond formation. (A) Oxidative pathway introduces new disulphide bonds by transferring electrons from substrate protein to molecular oxygen via DsbA, DsbB, quinone and terminal oxidases. **(B)** Isomerization pathway corrects the disulphide bonds by DsbC which gets reduced by accepting the electrons from ultimate donor NADPH via thioredoxin, and DsbD.

DsbA is the major disulphide bond forming enzyme. The enzyme is highly oxidizing, having second highest redox potential among the known thioredoxin related proteins. Active site disulphide Cys30 and Cys34 get reduced and introduce disulphide bond in the substrate protein in an indiscriminate manner. Reduced DsbA is oxidized back and its disulphide bonds are restored by DsbB.

DsbB is a transmembrane protein with four helices, containing two periplasmic loops, a short loop P1 and a long and flexible loop P2. Cys 104 and Cys 130 of P2 form disulphide bond which is donated directly to Cys30 and Cys34 of DsbA (Inaba, 2006). P2 cysteins are oxidized by P1. P1 also contains two cysteines in the form of CXXC motif Cys 41 and Cys 44, which further gets oxidized directly by donating its electrons to ubiquinone or menaquinone. Through these quinones electrons are led to terminal oxidases via electron transport chain.

Second pathway is isomerization pathway which reshuffles the already formed disulphide bonds to give native conformation to the protein (Kadokura et al., 2003). It is mainly comprised of DsbC and DsbD. DsbC exerts isomerase activity only in the reduced state, which is maintained through the activity of the cytoplasmic membrane protein DsbD. DsbC possesses chaperone activity which helps in the correct folding of the protein. DsbD is an integral membrane protein having eight transmembrane segments which forms the β domain flanked by periplasmic N-terminal and C-terminal domains, called α and γ, respectively. Although there is no loss or gain of electrons in isomerization, DsbC gets oxidized spontaneously due to oxidizing environment of periplasmic space. In order to maintain DsbC in the reduced state there should be continuous flow of electrons which is ultimately derived from NADPH. Electrons flow from NADPH in the cytosol, through thioredoxin1, β, γ and α domains of DsbD, to DsbC thus making DsbC active for reduction.

1.3.4. OxyR and PerR

OxyR and PerR are the regulators of the responses to hydrogen peroxide stress (Zheng et al., 1998; Imlay, 2008). OxyR was first discovered in H_2O_2 hypersensitive mutants of *salmonella*. OxyR is widely present in bacteria and belongs to LysR family of transcription factors. OxyR is present as a tetrameric protein which binds to -35 region of the promoter and regulates the expression of around twenty genes which employ different mechanisms and control the damage created to the cellular components by ROS or RNS under oxidative stress

(Table 1.1). OxyR contains six cysteines, out of which cys 199 and cys 208 play important role and are sufficient in regulating the activity of OxyR under oxidative stress. Cys 199 gets oxidized by H_2O_2 to form sulphenic acid or condenses with cys 208 to form disulphide bond. This oxidized form of OxyR is active and induce the expression of various genes listed in Table 1.1.

Table 1.1: H_2O_2 **response of *E. coli, B. Subtilis* and *Staphylococcus aureus* genes directly under the regulation of OxyR and PerR regulators and their role in redox homeostasis.** (Adapted from Imlay, 2008)

Role	***Escherichia coli* (OxyR response)**	***Bacillus subtilis* (B.s.) and/or *Staphylococcus aureus* (S.a.) (PerR response)**
H_2O_2 **scavenging**	AhpCF	AhpCF (*B.s., S.a.*)
	CatalaseG	CatalaseA (*B.s., S.a.*) Bcp (thiol peroxidase) (*S.a.*)
Heme synthesis	Ferrochelatase	HemAXCDBL (*B.s.*)
FeS cluster assembly	SufABCDE	
Iron scavanging	Dps	MrgA (Dps) (*B.s., S.a.*) Ferritin (*S.a.*)
Iron-import control	Fur	Fur (*B.s., S.a.*)
Divalent cation import	MntH	MntABC (*S.a.*) ZosA (*B.s.*)
Disulfide reduction	Thioredoxin C Glutaredoxin A Glutathione reductase DsbG (periplasmic reductase)	Thioredoxin reductase (*S.a.*)
Unknown function	Several	Several

After oxidative stress response, when levels of H_2O_2 start declining, the disulphide bond between cys 199 and cys 208 of OxyR gets reduced by glutaredoxin, which in turn is under the control of OxyR, forming a negative feedback loop limiting the induction and magnitude of oxidative stress response. PerR is another H_2O_2 sensor present in many Gram positive bacteria like *Bacillus subtilis* and *Staphylococcus aureus* (Bsat et al., 1998). Under normal conditions it binds to the Fe atom and acts as a repressor. During H_2O_2 stress bound Fe gets oxidized directly through Fenton reaction and generates ferryl and/or hydroxyl radicals. These radicals cause the oxidation of metal coordinating histidine leading to dissociation of Fe. The Fe deficient PerR loses the affinity for DNA and thus releasing the repression of many genes involved in management of oxidative stress. Most of these genes are homologous to those of OxyR controlled genes and are having similar metabolic effects indicating the common roles of OxyR and PerR in combating the oxidative stress in different organisms.

1.3.5. σ^R-RsrA

In *S. coelicolor* oxidative stress is sensed by a different mechanism. It involves σ^R-RsrA system. σ^R is an ECF (extracellular function) factor synthesized continuously by the cell and is a key regulator of the oxidative stress. This was proved by the fact that σ^R null mutant was found to be sensitive to oxidizing agents such as the superoxide-generating, redox cycling compounds menadione and plumbagin, and was particularly sensitive to a thiol-specific oxidant called diamide (Åslund & Beckwith, 1999). Normally it remains inactive by binding with antisigma factor RsrA (Kang et al., 1999). RsrA contains seven cysteines out of which three bind to a zinc cofactor and increase its affinity for σ^R binding. Under thiol disulphide stress, disulphide bonds are formed leading to structural change in RsrA and release of σ^R. Released σ^R binds to RNA polymerase and switches on the expression of specific genes like *trxBA* through binding to one of the two promoters, namely trxBp1, which codes for the operon containing thioredoxin and thioredoxin reductase (Paget et al., 1998) as well as those genes involved in synthesis of small molecular weight thiols like Cysteine (*cysM*),

CoenzymeA (*coaE*) and molybdopterin (*moeB*), compounds which get oxidized under diamide stress. Mycothiol is also under the control of σ^R comes from the fact that sigR mutant has very low level of mycothiol as compared to wild type *S. coelicolor*. Some of the targets of σ^R involve ribosome associated products like relA, ssrA and the ribosomal protein gene rpmE, suggesting that ribosome composition and function are modified in response to disulfide stress (Paget et al., 2001). The induction of relA and ssrA may then provide pathways to rescue stalled ribosomes and to slow ribosome production and growth, respectively, thereby focusing available resources on stress survival. Like oxyR, sigR is also autoregulated by binding to its specific promoter sigRp2 shutting down its own expression under normal conditions.

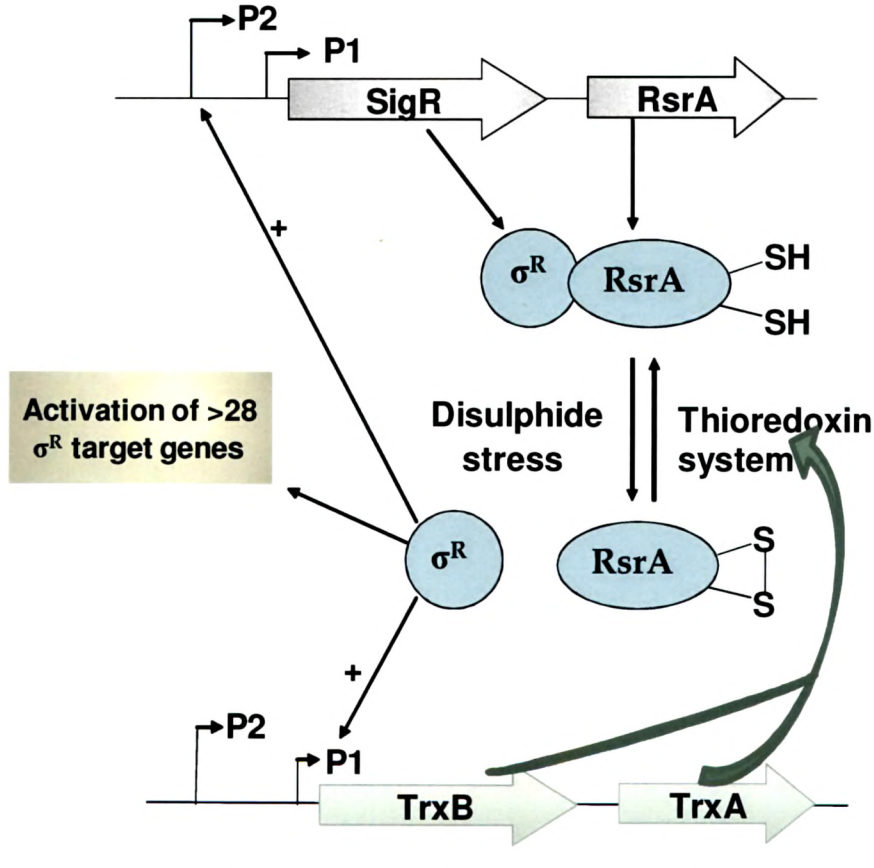

Figure 1.5: Sensing disulphide stress in *S. coelicolor* through σ^R-RsrA system.

1.3.6. CatR

CatR is found to be the major regulator of CatalaseA in *S. coelicolor*. *S. coelicolor* contains three catalases, which are expressed during different phases of the growth cycle. CatalaseA is generally expressed in the log phase and is the major catalase induced under oxidative stress so it is essential for the aerobic growth of the organism and resistance to H_2O_2. (Cho and Roe, 1997) CatR was first discovered in the CatalaseA over expressing mutant. Sequence comparison with other regulatory proteins showed that CatR is PerR like repressor, a member of Fur superfamily (Hahn et al, 2000). CatR mutant shows over expression of both catA and catR genes, establishing that the promoters of these two genes are under CatR regulation. Hence, CatR is autoregulatory in its function, similar to σ^R. DTT-treated CatR binds specifically to the catA and catR promoters, and this binding is abolished when exposed to H_2O_2. It is suggested that CatR regulation may possibly be mediated by formation and breaking of disulphide bonds similar to OxyR. However, PerR a homologue of both CatR and OxyR, is regulated through oxidation of its amino acids, His37 and His91. As both of these His residues are conserved in CatR, there are chances that CatR may employ the mechanism observed with PerR.

1.4. *Streptomyces coelicolor* as a model organism

Streptomyces are a group of Gram positive soil bacteria belonging to the actinomycetes. The features making them distinct from other bacteria are their high G+C (69-78%) rich genome, large and linear chromosome, filamentous growth and complex life cycle with morphological differentiation. Owing to broad range of metabolic processes and biotransformation ability, they play a crucial role in the degradation of insoluble remains of other organisms like lignocelluloses, chitin and other complex biopolymers, thus performing an important function in the maintenance of carbon cycle. The capacity for the production of a diverse range of complex secondary metabolites makes them the most useful organisms in biotechnology industry. They produce more than 50% of the known antibiotics and other microbial products like anticancer agents,

immunosuppressors and a variety of enzymes with commercial value (Kieser et al., 2000). Since *streptomycetes* are amongst the most ubiquitous and numerous soil bacteria, they have evolved the regulatory mechanisms to adapt to adverse conditions like nutritional deprivation, antibiotic stress, redox stress and others. They share many common features with pathogenic *Mycobacteria*, belonging to actinomycetes. Studies relating to various stress responses and their regulation in harmless *Streptomyces* help in understanding the physiology and metabolism of pathogenic actinomycetes such as *Mycobacterium tuberculosis.*

Streptomyces coelicolor A3(2) is the model organism, since its genetics is well studied (Hopwood, 1999) and genome is sequenced (Bentley et al., 2002). It is also well characterized at transcriptome (Huang et al., 2001; Bucca et al., 2003) and proteome (Hesketh et al., 2002; Novotna et al., 2003) levels. The rapidly accumulating information on genome wide analysis has given much insight into the metabolic and regulatory mechanisms of this species under different conditions. Amongst all the bacterial genomes sequenced, *S. coelicolor* has the largest genome having 8,667,507 base pairs and largest number of predicted genes (7,825 genes in all) with the large number of expressed proteins (Bentley et al., 2002). The life cycle of *S. coelicolor* involves a complex mechanism similar to that of fungi. It begins with the germination of a single spore into a vegetative mycelium, in this form it will penetrate and utilize a variety of nutrients through secretion of various hydrolytic enzymes. Once the nutrients get depleted mycelium develops aerial hyphae and undergoes synchronous septation to form a chain of unigenomic spores and characteristic grey pigment as they mature (mc Gregor, 1954) (Figure 1.6). Owing to its high versatility and morphological variation it has become one of the model organisms for the study of developmental regulation, secondary metabolic pathways and response to external stimuli. The genome wide analysis shows a strong emphasis on regulation, with 965 proteins (12.3%) predicted to have regulatory function thus adapting to a wide range of environmental stress/stimuli and to exploit a large variety of nutrient sources (Bentley et al., 2002).

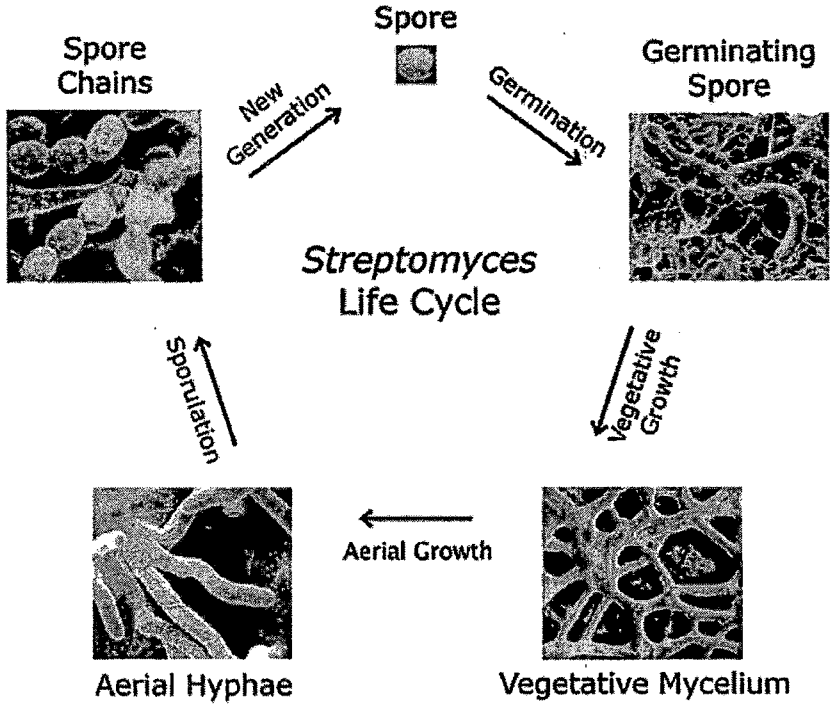

Figure 1.6: The life cycle of *Streptomyces coelicolor*. From a single spore a vegetative mycelium germinates, this is followed by production of aerial hyphae. These hyphae in turn will undergo synchronous septation to produce unigenomic spore compartments, which will disperse and thus commence a new cycle.

1.4.1. Redox control in *Streptomyces*

In order to combat the altered redox environment particularly oxidative stress *Streptomyces* activate many functions to overcome the detrimental effects of ROS. As a part of this response many transcriptional factors are activated or inactivated. As discussed earlier, like all other bacteria, *Streptomyces* also exploit the chemistry of the residues and cofactors for the maintenance of redox balance. In other words, the sensitivity of these molecules like Fe-S clusters and cysteine thiol groups, to ROS is exploited to create regulators that can switch from one state to another by means of conformational change in the regulatory protein.

Some of the major switches are already discussed in the earlier sections, which include thioredioxin system, the major system in *S. coelicolor* for the maintenance of redox homeostasis offering protection against oxidative stress. The second line of thiol based defense is offered by mycothiol against disulfide stress. Thiol based transcriptional regulators like OxyR, PerR, σ^R–RsrA, and CatR have already been discussed in the above sections. Other non-thiol based redox regulators are also present in *Streptomyces* such as iron-sulfur clusters, OhrR, Rex, σ^B, DosS and DosT (sensors of NO, CO and O_2), The Fur superfamily of regulators (sensors of metals and peroxides) like FurA, CatR, Nur, Zur and the WhiB-like family of proteins (den Hengst and Buttner, 2008).

There is very little information in literature on the effects of thiol stress in *S. coelicolor*. The main objective of this work is to understand the redox regulation at biochemical and genetic levels in this organism, when challenged with thiols and subsequently understand the role of proteins which are up or down regulated in the process.

1.5. Brief introduction to chapters

Chapter 1 presents a general introduction to oxidative, reductive and thiol stresses, the importance of various proteins and enzymes in the maintenance of redox balance and the mechanisms of their action and regulation. The chapter also introduces the main objectives of the work and their chapter-wise presentation.

As mentioned earlier in section 1.4. of this chapter *Streptomyces coelicolor* has been chosen as a model organism, as it is a major producer of antibiotics and secondary metabolites, an actinomycete closely related to the dreaded pathogens *Mycobateria* with very high G+C content in the genome. Moreover, the organism lacks glutathione, the most common thiol buffer and instead has mycothiol like

Mycobacteria. All these above reasons led to choosing *Streptomyces coelicolor* as the model organism for this study.

Chapter 2 has been dedicated to standardization of conditions for thiol stress and establishing reductive nature of thiol stress. In order to understand the effects of thiol stress, *S. coelicolor* was subjected to different concentrations of DTT for varied time periods to find the right concentration and duration for inducing thiol stress. The effect of DTT on redox balance of the organism was monitored by measuring changes in total thiol content and observed that the intracellular thiol content increased as a result of thiol stress. Protein profiles of control and thiol stressed bacteria were compared on SDS-PAGE and found induction of ~55 kDa band. The effect of thiol stress was compared using protein profiles of other organisms like *E. coli, Streptomyces lividans* and *Streptomyces griseus. E.coli* did not show any striking differences in the intensity of bands between control and DTT stress. *S. lividans* and *S. griseus* showed induction of a band approximately 55kDa like *S. coelicolor.*

In Chapter 3 proteins showing differential expression under thiol stress have been observed using 2D-PAGE. Subsequently, the proteins were identified using MALDI-TOF. The proteins induced under thiol stress can be loosely grouped into transcriptional regulators, enzymes of primary and secondary metabolism, membrane proteins, proteins involved in nucleic acid metabolism, hypothetical proteins (without any known functions). Besides these the two proteins were shown to be down regulated under DTT stress. The major band reported in Chapter 2 was found to be CatalaseA, an enzyme known to convert hydrogen peroxide, a precursor for ROS, to harmless water.

In Chapter 4 the role of CatalaseA in DTT mediated thiol stress was investigated. From catalase assay the enzyme was found to be induced ~100 fold under thiol stress. Further studies using inhibitors and mutagenesis of catalaseA gene established the protective role played by the enzyme under thiol stress as the chief cause for its induction. Thiol stress, by forcing the organism towards more

reductive conditions accelerate Fenton reaction thereby increasing the production of hydrogen peroxide. Hydrogen peroxide undergoes natural degradation to produce hydroxyl radicals, which are hazardous to the integrity of DNA. Hence, the enzyme catalaseA is induced to remove the excess hydrogen peroxide.

CatR is a repressor which is known to regulate expression of *catA*. Under normal conditions, CatR remains bound to the promoter of *catA* gene. When there is oxidative stress, two cysteine residues in CatR protein undergo oxidation changing the conformation of the protein and releasing it from DNA. Thiol stress, being reductive in nature could not be regulating *catA* gene through same mechanism. Hence, the role of CatR was investigated in **Chapter 5** by monitoring the m-RNA levels of *catR* in DTT mediated catalaseA induction, using RT-PCR. Besides, the gene for fluorescent protein EGFP was cloned under *catR* promoter and its expression was monitored *in vivo* under thiol stress. The results of both these experiments established that CatR is not involved in thiol stress mediated induction of CatA.

In **Chapter 6** focussed on the influence of thiol stress on oxidative phosphorylation in *S. coelicolor*. Electron transport chain is a well known source of ROS in aerobic organisms. Hence, the respiratory rates were compared between control and DTT treated cells at different complexes of ETC. It was found that overall respiratory rate increased under thiol stress. However, there is no difference in the functionality of various complexes and also ROS levels remained unchanged due to induction of CatA. The levels of ROS were compared between control and CatA mutant under thiol stress. In CatA mutant it was observed that there was increased production of ROS due to failure of induction of *catA*. The summary of the research described in this thesis is presented at the end.