Chapter 4

Chromomycin non-producer mutant JP1 exhibits enhanced oxidative stress

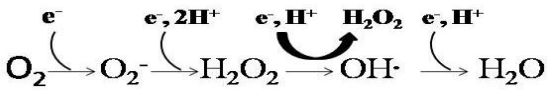
The genetically verified PKS deletion mutant of *S. flavisclerotícus* as expected failed to produce antibiotic chromomycin. Surprisingly, the mutant also exhibits a bewildering but related range of phenotypes that are suggestive of abnormal stationary phase. The unusual phenotypes are (I) slow growth, (II) absence of sporulation, (III) early loss of viability during stationary phase and (IV) lack of growth on minimal media. We undertook the study of the mechanistic defects of the chromomycin deletion mutant which led to these unusual phenotypes. In the present chapter (4) we attempted to address the rationale behind early loss of viability in the mutant.

4.1 Introduction

4.1.1 Generation of intracellular oxidative stress

For aerobic bacteria, oxygen acts as 'double edged sword', on one edge oxygen is utilized during metabolism which assists combustion of carbohydrate, organic acids and other sources of CO_2 and H_2O to generate energy, on the other edge the stable allotropic molecule owning two unpaired electrons generates reactive oxygen species (ROS) (1). Oxidative stress can be defined as interference in balance between productions of ROS and the ability of the biological system to detect presence of it and further detoxify and repair the damage. As the name implies reactive oxygen species are highly reactive in nature and thus damages lipids, proteins and nucleic acids within the cells (2). The reactive oxygen species generated due to oxidative stress are hydrogen peroxide (H_2O_2, formed by dismutation of O_2^- or by reduction of O_2), superoxide anions (O_2^-, formed by autooxidation reactions and by electron transport chain), hydroxyl radical ($OH^\bullet$, formed by Fenton reaction and decomposition of peroxynitrite), hypochlorous acid (HOCl, formed by myeloperoxidase), nitric oxide (NO, formed by nitric oxide synthase from arginine and oxygen and reduction of inorganic nitrate), Peroxynitrite anion ($ONOO^-$, formed by rapid reaction between O_2^- and $NO^\bullet$) and organic hydroperoxide (ROOH, formed by radical reactions with cellular components like lipids and nucleobases).

The univalent reduction series of oxygen occurs as described below



4.1.2 Damage caused by ROS in cells

The notion that reactive oxygen species is toxic to cell came into existence when it was observed that growth defects arises in cells after exposure to superoxide stress. Brown *et. al.,* in 1976, first time reported that hyperbaric oxygen can rapidly but reversibly inhibit growth and respiration of *E. coli* in minimal salt medium. They further found that mixture of 20 amino acids supplementation can rescue the growth defect on minimal medium, of *E. coli* because of oxygen toxicity (3). The first clear cut demonstration that oxygen is toxic came into existence when superoxide dismutase mutants of *E. coli* failed to grow aerobically on minimal glucose media. The growth defect can be averted by 20 amino acids (4). Thus these studies concluded that hyperoxia accelerates O_2^- formation which in turn affects amino acid biosynthesis pathways.

The mononuclear metalloenzymes are primary targets of oxidative stress (5). The most vulnerable enzymes to oxidative stress are those possessing iron-sulphur clusters or mononuclear iron which are disrupted by O_2^- (superoxide radical) and by H_2O_2 (6, 7). After protonation $O_2^{-\cdot}$ becomes strong univalent oxidant which abstracts single electron from [4Fe-4S] cluster. The oxidized cluster becomes unstable, further the iron atom which is coordinating substrate dissociates from the cluster which renders enzyme inactive (8). The family of Fe-S cluster cofactored dehydratase containing Fe-S cluster are highly sensitive to O_2^- such as dihydroxy acid dehydratase (8). The TCA cycle enzymes such as aconitase and fumarase are also equally sensitive to O_2^-. Hence the SOD mutants can't grow on TCA cycle substrates such as succinate and acetate (9-12).

Similar observation has been made in yeast *Saccharomyces cerevisiae*, mutants lacking CuZn-SOD exhibited aerobic lysine and leucine auxotrophy. The grounds for the auxotrophy are the biosynthetic pathways for both the amino acids contain 4Fe-4S cluster enzyme homologous to aconitase and hence sensitive to superoxide stress (13). Interestingly, the yeast mutants lacking mitochondrial SOD (MnSOD) exhibited short stationary phase survival. The reason for this phenotype is superoxide stress rendered the 4Fe-4S containing mitochondrial enzymes inactive (14). The Mn-SOD mutants can't grow on glycerol, a carbon source requiring robust TCA cycle (14, 15).

H_2O_2 also inactivates 4Fe-4S containing enzymes by Fenton reaction. The labile iron released, following destruction of iron–sulphur center by ROS reacts with H_2O_2 generating highly reactive $OH^{\bullet}$ (hydroxyl radical) (16). At high concentration, the H_2O_2 oxidizes sulphur containing amino acids (17, 18). Protein structure and function is deformed by oxidation of proteins by $OH^{\bullet}$ (hydroxyl radical) which generates amino acid radical and further initiating oxidative cascade (19). Constitutive stress of H_2O_2 inactivates many metalloenzymes. H_2O_2 exposure inactivates the Fe-S cluster synthesis system itself (20).

The superoxide radical and hydrogen peroxide also inactivates mononuclear iron enzymes such as epimerase, dehydrogenase, deformylases and deaminases (5). In case of epimerase the oxidized enzyme can be reactivated by addition of Fe^{+2}. Repeated cycles of inactivation and activation cause loss of activity of enzymes. Interestingly, the superoxide radical causes oxidation and release of iron atoms from mononuclear enzymes but the reaction doesn't form strong oxidant thus polypeptide remain undamaged. The repeated cycle in cells lead to mismetallation of enzyme with zinc. But the zinc containing enzymes are not as efficient as iron so there is progressive decline in function of these enzymes (21). In *E. coli* iron atom is replaced by manganese during H_2O_2 stress (22).

Mutagenesis results due to substantial damage caused by ROS. This is proved by studies where it is seen that the mutants lacking SOD or catalase/peroxidases exhibited high mutation rates (23, 24). The $OH^{\bullet}$ radical generated through Fenton reaction equally damages base and ribose moiety of DNA. Because of lower reduction potential of guanine, it is adversely affected. The end product 8-hydroxyguanine formed is highly mutagenic and it pairs with adenine in such a way that intrinsic repair system can't recognize the mismatch (25). Thymine oxidation is lethal as it blocks polymerase progression thus it is less mutagenic (26). The oxidation of ribose generates single stranded breaks. ROS causes lipid peroxidation in higher system where there is presence of polyunsaturated fatty acids unlike in bacteria where there are unsaturated and monounsaturated fatty acids which are not prone to peroxidation (27-29).

4.1.3 Role of iron in generating oxidative stress

Under physiological condition iron exists in Fe^{+3} forms which is generally insoluble form. The reduced form of iron i.e. Fe^{+2} is also present in biological system but because of high oxidative potential of it is tightly regulated and controlled. Iron is present as prosthetic component of many enzymes. Under aerobic condition ferrous ions frequently get oxidized which led to generation of oxidative stress via ROS. Molecular oxygen reacts with ferrous ion resulting in ferric ion formation and yielding O_2^- (Reaction 1). Upon dismutation of O_2^-, H_2O_2 is formed (Reaction 2). In Fenton reaction Fe^{2+} reacts with H_2O_2 to form hydroxyl radical (Reaction 3). Via Haber-Weiss reaction H_2O_2 reacts with O_2^- generating hydroxyl radicals (Reaction 4) (30).

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$ (Reaction 1)

$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ (Reaction 2)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$ (Reaction 3)

$O_2^{\bullet-} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$ (Reaction 4)

Thus in Fe^{2+} triggered Fenton/Haber-Weiss reaction produces harmful reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the highly destructive hydroxyl radical ($OH^{\bullet}$). The ROS generated causes damage to [Fe–S] clusters, protein carbonylation, Cys/Met-residue oxidation, membrane lipid peroxidation, and DNA damage.

4.1.4 Combating oxidative stress

In order to prevent the damage caused by ROS, living organisms maintain reducing environment inside the cells. In order to do so, cells employ enzymatic and non enzymatic ways to detoxify and repair the damage.

Enzymes involved in the detoxification of ROS

(i) Superoxide dismutases (SOD) - The superoxide anions (O_2^-) are highly dangerous for cells which inactivates [Fe-S] cluster of proteins and releasing free ion in the cells. Superoxide dismutases are the first line of defense of the cells in all aerobic organisms. It catalyzes dismutation of O_2^- to H_2O_2 and O_2 [$2O_2^- + 2H+ \rightarrow O_2 + H_2O_2$].

SODs have different cofactors namely Fe-, Cu-/Zn-, Ni- and Mn-. H_2O_2 can freely move through the membrane and oxidize certain biomolecules like [Fe-S] cluster, methionine and cystein residues present at active site of proteins.

(ii) Catalases and Peroxidases - The second line of defense is catalases and peroxidases. These enzymes convert H_2O_2 to H_2O and O_2 (30). Catalases contain heme as a prosthetic group and are homotetramers, while peroxidases use H_2O_2 to oxidize a number of compounds. The bifunctional catalase-peroxidases comprise varying ratios of these two enzymatic activities.

$$2H_2O_2 \rightarrow 2H_2O+O_2 \text{ (catalase)}$$

$$ROOR + \text{electron donor } (2e^-) + 2H^+ \rightarrow ROH + ROH \text{ (peroxidase)}$$

(iii) Alkyl hydroperoxidase reductase (AHP) - It is a two-component (AhpC–AhpF) thiol-based peroxidase system that transfers electrons from NADH to H_2O_2, thereby reducing H_2O_2 to water. Under routine growth condition AHP is primary scavenging enzyme. Detoxification of lower amounts of H_2O_2 by catalases is more problematic as the two step catalytic cycle of catalase halts with heme in intermediate that is ferryl/radical form which is potent oxidant, thus abstract electrons from surroundings. In order to avoid any further oxidation cycle the catalases bind to reducing reductant. For AHP such problem doesn't arise as it doesn't form dangerous oxidizing species thus it is more efficacious scavenger under low H_2O_2 concentration (8).

4.1.5 Link between oxidative stress and viability of an organism

Viability/life span of any organism is intimately linked with the management of oxidative stress. According to the free radical theory of aging, organisms age because of oxidative damage caused by ROS. Thus ROS play important role in determining longevity of cells. Better management of ROS generated during various cellular metabolic processes, more particularly in stationary phase, which is also called 'Achilles Heel' of bacterial life cycle, endow cells with better longevity. Any alteration in expression of antioxidant defense system during stationary phase can lead to rapid aging and untimely death of an organism. Physiology of stationary phase is studied in detail in Gram negative bacteria, *E. coli*. Less information is available for Gram positive bacteria. The detailed physiological responses of bacteria in stationary phase are described below.

4.1.5.1 Stationary phase physiology in Gram negative *E. coli* bacteria

4.1.5.1.1 Different phases of growth of bacteria

The growth of bacterial culture represents different phases (i) Lag phase – cells enter in the nutrient medium and there is no obvious growth because cells are adapting the cellular metabolism to new condition. The duration of the phase varies depending upon the species and for how long bacteria stayed starved; (ii) Logarithmic growth phase – cells starts dividing once they get adapted to new condition. The growth rate of the culture in logarithmic phase is determined based on the doubling time; (iii) Stationary phase – Once the nutrient is exhausted the culture enters stationary phase. This phase is characterized by plateau in the growth curve representing equilibrium between number of dividing and dying cells. Stationary phase not only begins due to nutrient limitation but also because of other stress factors; (iv) Death/decline phase – over the time in stationary phase, cells accumulate toxic products of catabolism leading to decline in number of viable cells. Either programmed cell death or stochastic cell death occurs in this phase.

4.1.5.1.2 Alterations in physiology and metabolism in stationary phase

The physiology of the stationary phase is best understood in the prototype Gram negative bacterium, *E. coli*. Viability in stationary phase is a direct measure of the ability of any organism to combat oxidative stress generated during the nongrowing phase. Stationary phase is characterized by many physiological, morphological and metabolic changes (31). The alterations in the physiology and metabolism occurring during conditional senescence of stationary phase of *E. coli* exhibit striking similarity with the aging process of higher organisms. Genes expressed during stationary phase/ starvation are mainly involved in survival as mutants affected in these genes exhibit increased or decreased survival (31). These genes encode proteins for protection of cells against external stresses like heat, oxidant and osmotic, thus stress cross protection occurs. The stress cross protection depends on the regulator σ^S, transcription factor sigma that regulates expression of proteins related to stress management, central carbon metabolism and morphology. Sigma factors like σ^H, σ^E, response regulator of oxidative stress - OxyR and SoxRS work along with σ^S during stasis. σ^H and σ^E are required for protection against oxidative damage. In *E. coli* or any other gram negative bacteria the starvation response triggers the alternative sigma factor RpoS (σ^S), which controls upto 10% of

E. coli genes, genes for survival under stress (32, 33). In aerobic organisms, oxygen toxicity arises as it forms 'free radicals' during various physiological and metabolic processes. Free radicals are atoms or molecules containing one or more unpaired electron, it is highly unstable, short lived and highly reactive. In order to attain stability, free radicals abstract electrons from other compounds causing it to oxidize. The oxidized molecule than becomes free radical itself and thus chain reaction cascade begins ultimately damaging living cell (34). Oxidative damage is 'Achilles heel' of stationary phase (31). In *E. coli* it is reported that during starvation condition oxidative modification of protein such as carbonylation and illegitimate disulphide bond formation increases. The proteins which are affected due to stasis and stress induced oxidation are the Hsp70 chaperone DnaK, the histone-like protein HNS, the universal stress protein UspA, elongation factors EF-Tu and EF-G, glutamine synthetase, glutamate synthase, aconitase, malate dehydrogenase, and pyruvate kinase (31). Ultimately, cellular damage occurs including peptide chain elongation, protein folding, large scale DNA damage, gene expression and central carbon metabolism (glycolysis, TCA cycle, PPP and ETC). Proteins containing metal center are more vulnerable to oxidation. Stasis induced protein oxidation also affects ribosomal proofreading leading to erroneous incorporation of amino acids into the protein generating aberrant protein isoforms seen as protein stuttering on 2D-PAGE. In nonproliferating cells, increase in frameshifting, missence errors and stop codon readthrough occurs along with augmentation of oxidation of aberrant proteins leading to increased mistranslation. In the line with this fact, in *rpsL141* mutants having intrinsically hyperaccurate ribosomes, the protein carbonylation is attenuated in early stages of stationary phase (35). Because of protein carbonylation, the targeted protein becomes susceptible to proteolysis. Life expectancy of an organism is closely linked to levels of oxidatively damaged proteins. This notion is supported by study of *sod* mutants, in stationary phase *sodA* and *sodB* mutants lose culturability at increased rate (36, 37) .

Time dependent corruption in flow of information from DNA to RNA to protein leads to molecular and cellular aging. DNA is the most sensitive target for corruption. Defects in DNA like chromosome fragmentation, deletions, point mutations and transposition are most serious than defects in RNA or proteins. During aging, the repair system doesn't work adequately leading to accumulation of damaged

DNA. This fact is supported by high genomic mutation rates in stationary phase of *E. coli* cells. These mutations are important for adaptation but it equally limits the life span of organism (31).

4.1.5.1.3 Alteration in regulation of fatty acid degradative (*fad*) genes in stationary phase

Gene expression of FadR regulon is amplified during stationary phase, the main role of *fad* genes are in growth on long chain exogenous fatty acids (38). Moreover, these genes are also required to harness endogenous carbon and energy during stasis condition from digestion of membrane constituents. In *E. coli*, the FadR acts as repressor of genes encoding proteins involved in long-chain fatty acid transport, activation and β-oxidation. Acyl Co-A thioesters bind to FadR and release repression of *fad* genes. During stasis, the *fad* gene product acyl-CoA synthetase, encoded by *fadD*, generates acyl-CoA which is further catabolized to generate acetyl-CoA a source of carbon and energy (39). This conception is supported by generation of *fadF* (acyl-CoA dehydrogenase) mutants in which β-oxidation is hindered and thus there is poor survival during carbon starvation (40). The depression of *fad* genes during stationary phase are observed both in *E. coli* and *Salmonella typhimurium* (38, 40). There is an interesting and essential regulatory mechanism which is operated during severe stress condition termed as 'emergency derepression' wherein the promoter *uspA* encoding universal stress protein is under dual control (both positive and negative). During starvation stress the *uspA* promoter is induced by activating pathway when FadR is active repressor. The positive regulatory pathway involves ppGpp (guanosine 3′, 5′-bisphosphate) dependent activation exerted through β-subunit of RNA polymerase. ppGpp is found to be major effector molecule of stringent response pathway of Eubacteria and is accumulated in response to stress condition such as heat shock, oxidative stress and during starvation of amino acids, carbon, phosphate and nitrogen. During emergency derepression ppGpp dependent activation of promoter of *uspA* occurs by overriding FadR repression during severe stress. The emergency derepression is also operative on other FadR regulated genes which are induced by starvation (41).

4.1.5.1.4 Catabolic modulation during starvation

It is well studied in *E. coli* that many physiological adjustments are required to survive in absence of carbon and energy source. In carbon/energy starved condition

E. coli cells increases synthesis of glycolytic enzymes, and pyruvate formate lyase, phosphor-trans-acetylase and acetate kinase with concomitant reduction of TCA cycle enzymes (42). The modulation during aerobic carbon/energy starvation favoring synthesis of catabolic enzymes is astonishingly similar to responses of cells which are being shifted to anaerobic condition. Under anaerobic condition *E. coli* can use variety of electron acceptors, in absence of electron acceptors cells rely on fermentation to generate energy. Thus variety of genetic regulatory systems of *E. coli* directs the cells to select the most efficient metabolic system to sustain growth in any given environment. Examples of some common global regulatory systems involved in metabolic regulation are ArcA-ArcB, NarL-NarX, Fnr and FurR. ArcA–ArcB is a two component regulatory system being activated in absence of any of the electron acceptor. In carbon starved stationary phase cells the two component response regulator ArcA is required to reduce synthesis of TCA cycle enzymes which generates $NADH/FADH_2$ resulting in reduced respiratory activity. If the cells fail to modulate the catabolic activities it loses viability early in stationary phase as seen in case of ArcA mutants (43). ArcA is activated in stationary phase by its sensor component ArcB via redox control of quinone pool. The ArcB interacts with membrane quinone pool and senses the oxidation/reduction status of the cells (44). There are several roles of ArcB/ArcA system in stasis survival such as reduction in production and activity of aerobic respiratory apparatus during starvation prevents uncontrolled drainage of endogenous reserves during autophagy. Secondly, reduced production of respiratory components act as defense mechanism, as by doing so cells protect itself from the damaging effects of ROS generated by electron transport chain. This is supported by evidence, if superoxide dismutase is overproduced in Δ*arcA* mutant the accelerated dying off of the cells can be impeded under glucose starvation condition (43).

4.1.5.1.5 Role of autophagy during starvation

Under starvation condition in absence of external carbon source *de novo* protein synthesis is obligatory. The amino acids required for this are provided by peptidase dependent autophagy. This is evident by a study where mutant having reduced peptidase activity showed reduced stationary phase survival (45). Thus protein turnover provides amino acids required for starvation survival and protein synthesis (46).

4.1.5.2 Response to oxidative stress in Gram positive – *Streptomyces*

4.1.5.2.1 Development program in Gram positive - *Streptomyces* is linked to oxidative stress

The developmental program in *Streptomyces* involves passage from vegetative to sporulating stage accompanied with shift in primary metabolism to secondary metabolism along with aerial mycelium formation. The aerial mycelium formation occurs under high oxygen pressure leading to ROS generation. It is reported that ROS play role in development of different organism but not reported so far in *Streptomyces* species (47). In *Streptomyces,* the development program is also triggered by environmental stressful condition such as nutrient limitation which is convoyed with oxidative stress. As the *Streptomyces* is aerobic organism they have developed fine tuned response to oxidative stress.

4.1.5.2.2 Adaptation to oxidative stress in gram positive bacteria

Streptomyces is obligatory aerobic organism. Partial reduction of oxygen into water give rise to reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\bullet$). To thwart the toxic effects of ROS and in order to maintain redox homeostasis in cellular environment microorganisms have developed highly regulated response mechanisms to sense and detoxify ROS and repair the damage caused by ROS (48). In *Streptomyces* the transcriptional regulators like catR, Nut, OxyR and sigma factors governs the response of expression of antioxidant systems like catalase, SOD and alkyl hydro peroxidase. Any imbalance between oxidants and antioxidants in favor of oxidants leads to a physiologiacal condition called oxidative stress (49).

4.1.5.2.3 Oxidative stress regulatory sensor system

Bacteria have evolved regulatory sensory systems to stimulate expression of proteins involved in anti-oxidant defense. They consist of either single transcriptional regulator or two component system. In the earlier case the sensory and the response domains are present in the single protein itself (eg. oxyR and furS) while in the latter case there are two different proteins for each of the function (chrS-chrA or VicR-VicK) where an external stimulus is sensed by membrane protein kinase which activates and inactivates its cognate response regulator.

4.1.5.2.4 Single transcriptional regulator (OxyR and FurS)

OxyR is H_2O_2 sensing transcriptional activator activated by intramolecular disulphide bond formation between cysteine residues. The S-S form of oxyR acts as transcriptional activator of antioxidant defense genes. In *Streptomyces coelicolor*, OxyR is involved in regulation of alkyl hydroperoxide reductase system (AhpC and AhpD). These genes are located downstream of OxyR.

FurS is zinc containing redox regulator which represses transcription of catalase peroxidase (*cpeB* gene) in *Streptomyces reticulii* in its thiol reduced form upon binding of an operator located upstream of *furS-cpeB* operon. FurS contain 6 terminally located cysteine residues. In oxidative stress condition S-S bridge is formed within FurS, that causes conformational change which is not conducive for binding to the operator thus releases transcriptional blockage of *furS-cpeB* eventually leading to higher production of CpeB under oxidative stress condition. FurA regulates *katG* transcription.

4.1.5.2.5 Two component signal transduction systems (TCS)

By TCS bacteria sense, respond and adapt to changes in the environment or the intracellular state. The system consists of ROS signal activating a histidine kinase (SK) causing its autophosphorylation and one or more response regulators (RR) which upon phosphorylation are altered for DNA binding (50). Certain bacteria harbor upto 200 such TCS signaling system. This pathway is useful for bacteria to respond to various stress including nutrient stress, cellular redox state, osmolarity, quorum signals, antibiotics and more (51). The analysis of whole genome of *S. coelicolor* revealed presence of 67 sensor kinase genes, 64 of which lie adjacent to genes encoding response regulators (52). 67 sensor kinases and 68 response regulators are found in the genome of *S. avermitilis,* whereas in *E. coli* there are only 23 SK and 32 RR (53). Thus *Streptomyces* species is well equipped with TCS as compared to gram negative *E. coli* to respond readily to wide number of external stimuli.

4.1.5.2.6 Three component system

The three component system consists of third protein component, acts as accessory proteins which sense environmental conditions and in turn influences the kinase activity of its SK. The majority of three component systems are not involved in sensing oxidative stress. Eg. YycFG is best studied TCS with accessory protein which

is highly conserved and specific to low GC gram positive bacteria (54). The YycG SK of *Bacillus subtilis* contains two transmembrane helices subject to complicated circuit involving two other proteins with N-terminal transmembrane helices, YycH and YycI. All four genes are located in same operon (55). MtrB is subgroup of sensor kinase widely found to be conserved in Actinobacteria along with RR MtrA and the lipoprotein lpqB. The genes *MtrA*, *MtrB* and *LpqB* are clustered as single transcriptional unit (56).

HbpS-SenS-SenR is the three component regulatory system involved in detection of heme or iron mediated oxidative stress signals found in gram positive bacteria. Binding of iron atom induces conformational changes in HbpS which is recognized by histidine sensor kinase SenS causing its activation by autophosphorylation. The activated HbpS transfers the phosphate to RR further activating transcription of genes *hbpS* and *cpeB* which are involved in anti-oxidative response. In coupled oxidation HbpS binds to heme and degrade it in H_2O_2 dependent manner. Assembly mechanism in HbpS is imperative for correct functioning of HbpS-SenS-SenR signaling. HbpS like protein are found in many gram positive bacteria like *Streptomyces coelicolor* A3(2), *Streptomyces kasugaensis*, *Streptomyce griseus* and are mainly involved in antibiotic production.

In this chapter the chromomycin non-producer mutant has been characterized for increased oxidative stress as a cause of early death of the organism in stationary phase.

4.2 Results

4.2.1 Deletion of chromomycin biosynthesis genes is associated with oxidative stress in JP1 mutant

We rationalized that the early loss of viability of JP1 mutant is the result of generation of reactive oxygen species (ROS) causing oxidative stress. As mentioned earlier, it is well recognized fact that aging/death of any organism is closely related to redox status of cells, highly oxidized environment in the cell damages cellular constituents that can lead to early aging and untimely death of an organism including bacteria. Under oxidative stress condition antioxidant enzymes are upregulated to counter ROS stress. Preliminary experiment, 'H_2O_2 bubbling' was carried to check

status of antioxidant enzyme catalase in the cells. The agar plugs of the chromomycin deletion mutant, JP1 grown on R2YE solid agar medium for 2 days (mid-log phase) consistently produced more H_2O_2 bubbles and the bubbling reduced drastically on 7th day (stationary phase) upon hydrogen peroxide (H_2O_2) exposure in comparison to the WT cells (Fig. 4.1). The result indicates that the antioxidant response in JP1 mutant is altered in comparison to the wild type. We then quantitated and compared the antioxidant enzymes in WT, INT and JP1.

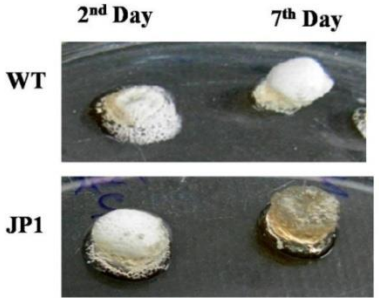


Figure 4.1 Bubbling by H_2O_2 of agar plugs of the wild type and JP1 grown on R2YE agar

4.2.2 Status of antioxidant enzymes catalase(s) in JP1 mutant (activity and expression):

4.2.2.1 Catalase enzyme activity:

As JP1 mutant loses viability in stationary phase, in order to decide the time point representing stationary phase where the viability is not substantially compromised, catalase activity was continuously monitored at interval of 24 hours after the start of the growth (Fig. 4.2). There was a remarkable pattern (Fig. 4.2) in the catalase enzyme activity in each of the strain - the activity slowly rose in the WT (and INT) from ~30 hours onwards exhibiting a peak at 72-80 hours and then gradually decreased in WT (INT) over next 48 hours (120 hrs). The pattern of catalase activity in the stationary phase was co-relatable to the viability as measured by plating (Fig. 3.12; 3.13). In contrast, the catalase activity in the JP1 was at its peak in the cells grown for 40-46 hours that deteriorated continuously over 60-80 hours. Analogously, the catalase activity pattern correlated with the viability of JP1 – number of viable cells being maximum between (40-72) hours of growth that reduced drastically post 96 hours of inoculation (Fig. 3.11). We thus chose 24-32 hours and 50-60 hours of

growth for WT and INT as the time points representing mid-log phase and stationary phase respectively whereas, the JP1 grown for 40-46 hours was selected to represent mid-log phase and the growth for 70-80 hours as stationary phase (Fig. 3.11; 3.12; 3.13). We next attempted to measure if the change in activity is at expression levels of four different catalase genes identified in the genome of *S. flavisclerotícus* by semi quantitative RT-PCR in order to identify the catalase(s) that contributed to the precipitous drop in its activity in the JP1.

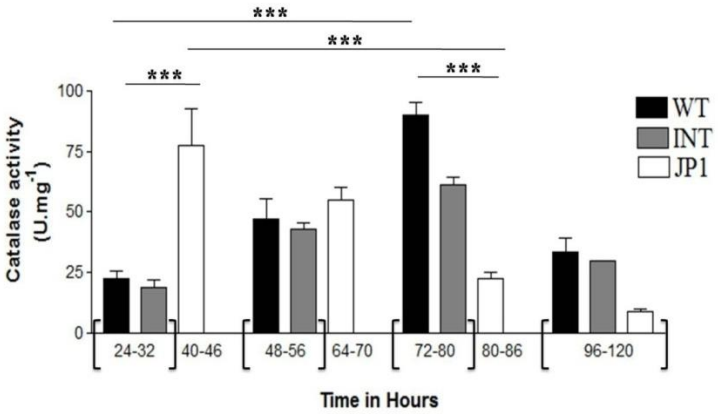


Figure 4.2 Total catalase activity monitored at different time points in WT, JP1 and INT. Results are representative of three independent experiments and error bars represent mean ± SD. Statistically significant differences between strains at each time point were assessed by one-way ANOVA followed by post hoc test (Tukey test; GraphPad Prism) ***$p < 0.001$.

4.2.2.2 Interpretation of functional equivalents of catalase genes in *S. flavisclerotícus* by comparing with catalases identified from other *Streptomyces*:

There are four different genes for catalase in the genome sequence of *S. flavisclerotícus* (Gene Bank Accession No.MAZZ00000000). We arbitrarily denoted them as *cat1* through *cat4*. In the literature the functionality of multiple catalases-' is addressed in greater detail in the three species of *Streptomyces* namely *S. ceolicolor*, *S. nataleinsis* and *S. avermitilis* (57-61). Comparison of amino acid sequence of each of the catalase genes of *S. flavisclerotícus* with those of the species above depict that

the monofunctional catalases are closely related and similarly the bifunctional catalases, catalase/peroxidases. On this basis, Cat1 and Cat3 are monofunctional catalase, Cat2 a bifunctional catalase/peroxidase and Cat4 is related to manganese-dependent catalase. However the conservation in the sequence is an insufficient basis for assuming function for each of them. For example, growth phase and stationary phase catalases are not adequately described in *S. avermitilis* (62), whereas they are identified in *S. coelicolor* (59); furthermore, there is no amino acid sequence similarity of stationary phase catalase of *S. coelicolor* with that of catalases-1, -2 or -3 of *Streptomyces flaviscleroticus*. Cat3 amino acid sequence matches poorly to either of the well defined CatA or CatB of *Streptomyces avermitilis, S. coelicolor* and *S. natalensis,* whereas Cat1 of *Streptomyces flaviscleroticus* matches closely with monofunctional Catalase - CatA1 of *S. avermitilis* (90% identity) and *S. natalensis* (87% identity). The regulator of CatA1 i.e. CatR is equally highly conserved. Similarly, Cat2 being a catalase/peroxidase matches to Cpx of *S. natalensis* (67% identity) and Cpe of *S. coelicolor* (83% Identity).

4.2.2.3 Transcription analysis of catalase genes in WT and JP1 mutant

Quantitation of RNA transcripts of each of the catalase genes from WT, INT and JP1 mutant in both mid log phase of growth and in stationary phase by semiquantitative RT-PCR demonstrated that the transcript amounts of *cat1* and *cat2* were higher in JP1 in mid-log phase that subsequently decreased in the stationary phase cells (Fig. 4.3); incidentally, they were also the H_2O_2-inducible catalases (Fig. 4.4). In contrast, *cat3* expression was undetectably low in JP1 in both the mid-log and stationary phases (Fig. 4.3). In the case of WT (and INT), the expression of all three catalases increased in the stationary phase when compared to the mid-log phase. This pattern closely paralleled the total enzymatic activity profile. Taken together, the results of the expression of different catalase genes may account for the characteristic pattern of the total enzymatic activity in both JP1 and WT (and INT). The expression of *cat4*, the putative manganese-dependent catalase, was not detectable during all phases of growth.

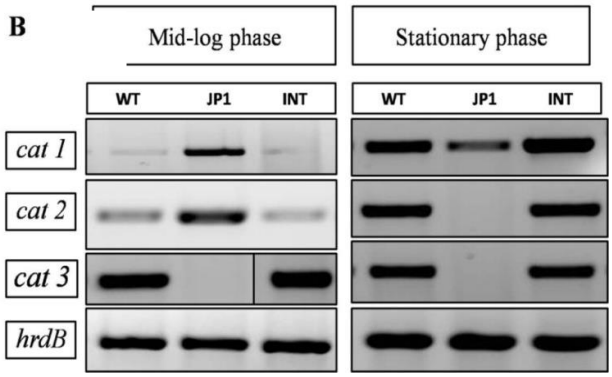


Figure 4.3 Transcription analysis of catalase genes, *cat1* through *cat3* by semi quantitative RT-PCR of RNA extracted from cells of WT, INT and JP1 mutant in mid-log and stationary phase of growth. Results are representative of three independent experiments. Agarose gel electrophoresis image processed by ImageJ software

4.2.2.4 Analysis of H_2O_2 inducible genes in *S. flavisclerotícus* wild type

Transient extracellular H_2O_2 (100mM) treatment for 2 hours to the wild type cells grown for mid-log phase induced *cat1* and *cat2* but not that of *cat3*. We observed no significant difference in the expression of AHP. This result is compatible with relative functions of catalase and AHP in *E. coli*. Catalases are important for detoxification of H_2O_2 at high concentration whereas AHP is required to quench micromolar amount of H_2O_2 inside the cell. To test the inducibility of different catalases, high concentration of H_2O_2 is used in the study (Fig. 4.4); it is possible that alkyl hydroperoxidase is not induced under this condition.

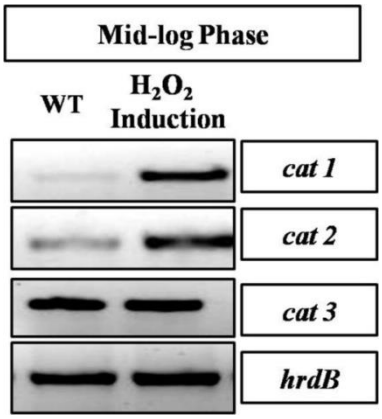


Figure 4.4 Transcription analysis of induction of *cat1* & *cat2* in mid-log phase culture of WT treated with 100 mM H_2O_2 for 2 hours. The transcription profiles are

representative of three independent experiments**.** Agarose gel electrophoresis image processed by ImageJ software.

4.2.2.5 Sensitivity of JP1 mutant to killing by H_2O_2 in mid-log and stationary phase

The fact that treatment with higher amounts of H_2O_2 can kill the cells if the cells are compromised for its antioxidant response is premise of the present experiment. Cells of both the wild type and JP1 were treated with 100 mM concentration of H_2O_2 both in mid-log phase and stationary phase and their viability was monitored. The JP1 cells were readily killed in the stationary phase when treated with 100 mM H_2O_2 for 1 hour, than when the cells were in the mid-log phase and when compared to cells of WT in both phases of growth (Fig. 4.5). The results are in agreement with altered status of catalase and hydroperoxidase activity.

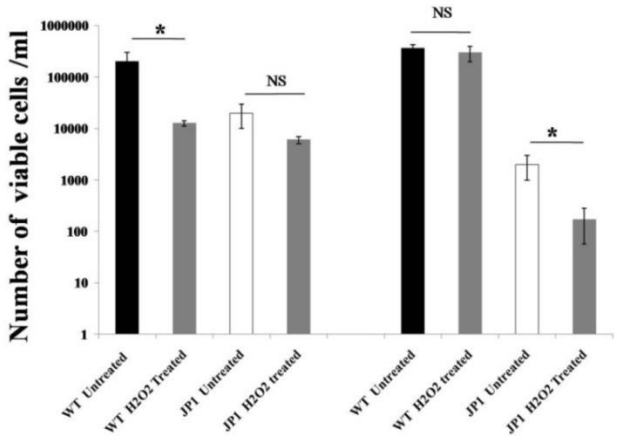


Figure 4.5 Sensitivity of JP1 mutant to killing by H_2O_2 in mid-log and stationary phase. Results are representative of three independent experiments and error bars represent mean ± SD. Statistically significant differences between strains at each time point were assessed by one-way ANOVA followed by post hoc test (Tukey test; GraphPad Prism) *$p < 0.05$.

4.2.2.6 Alkylhydroperoxidase enzyme activity

Alkylhydroperoxidase enzyme is also involved in detoxification of H_2O_2 inside the cell. We found the activity of alkyl hydroperoxidase enzyme was 2-fold more in the JP1 in relation to the WT and INT in the mid-log phase and diminished in the stationary phase (Fig. 4.6).

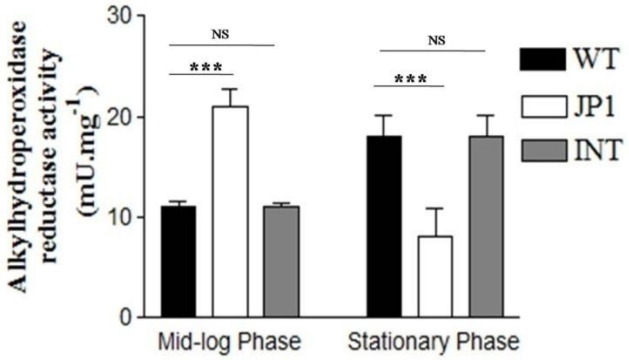


Figure 4.6 Alkyl hyroperoxidase reductase enzyme activity monitored at different phases of growth in WT, INT and JP1. Results are representative of three independent experiments and error bars represent mean ± SD; ***p < 0.001.

4.2.2.7 Superoxide Dismutase activity in WT and JP1

There was no significant difference in total SOD activity in the WT and the JP1 cultures in the mid-log phase and in the stationary phase (Fig. 4.7). This result suggests that the oxidative stress in the JP1 does not involve superoxide radicals. Since superoxide and hydrogen peroxide stress responses are exclusive, inducibility of SOD by H_2O_2 was not tested.

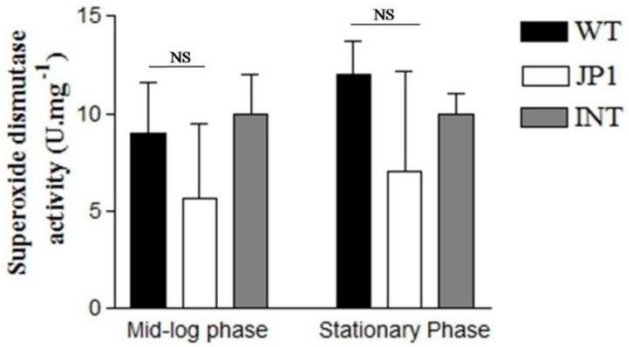


Figure 4.7 Total SOD levels in WT and JP1 mutant

4.2.3 Reactive Oxygen Species measurement in WT, INT & JP1

The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a chemically reduced form of fluorescein and most widely used to detect intracellular levels of H_2O_2 and other reactive oxygen species (ROS) generated by oxidative stress in cells. Upon cleavage of the acetate groups by intracellular esterases and by two-electron oxidation, the nonfluorescent H_2DCFDA is converted to the highly

fluorescent 2',7'-dichlorofluorescein (DCF) which is retained inside the cell which is monitored by spectroflourimeter.

The *in-vivo* (inside the intact cells) fluorescence units of the DCFDA dye were found to be almost two-fold higher in JP1 in both mid-log and stationary phase relative to the WT (and INT) indicating that the JP1 mutant is probably under constitutive oxidative stress (Fig. 4.8A; 4.9). The same result is reflected under confocal microscope where green fluorescence of DCFDA is observed in JP1 in mid-log phase whereas there is no fluorescence in the wild type control (Fig. 4.9). Reliability of the fluorophore- DCFDA is questioned in recent paper (16) because of its self oxidizing nature. Therefore *in vitro* (in cell-free extract) fluorescence of DCFDA was also monitored simultaneously and it was also found higher in JP1 in comparison to that of the WT when cell-free extracts were treated with DCFDA (Fig. 4.8B) (63). Interestingly, the proposal that DCFDA fluorescence could be used as a reporter of the redox reactions of intracellular iron (63) could be compatible with the role of chromomycin in iron homeostasis in the stationary phase, since chromomycin binds iron and other divalent metals (64).

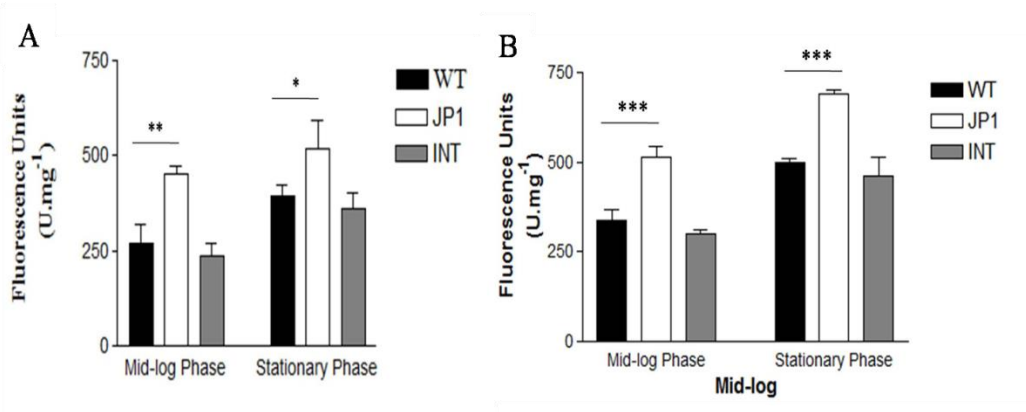


Figure 4.8 Amounts of ROS were compared between WT and JP1 mutant in mid-log phase by measuring fluorescence of DCFDA both (A) *in vivo* & (B) *in vitro* in mid-log and in stationary phase of growth. Results are representative of three independent experiments and error bars represent mean ± SD. Statistically significant differences between strains at each time point were assessed by one-way ANOVA followed by post hoc test (Tukey test; GraphPad Prism) *$p < 0.05$, ** $p < 0.01$,***$p < 0.001$.

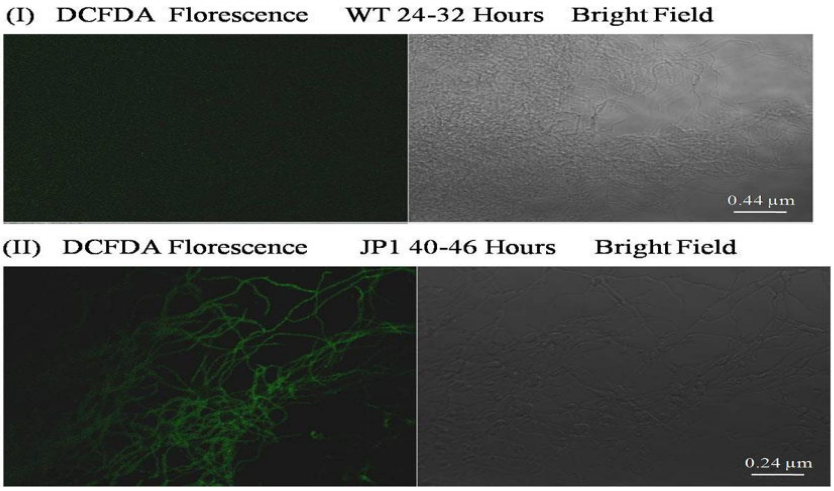


Figure 4.9 Visualization of DCFDA stained cells by confocal microscope in mid-log phase of growth

4.2.4 Detection of extracellular H_2O_2 production by JP1 mutant

High amounts of H_2O_2 accumulated inside the cells due to oxidative stress can diffuse out of the cells in the medium and can easily be detected. In the presence of H_2O_2, HRP (horseradish peroxidase) oxidizes Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) to resorufin, a chromophore with an absorption at 560 nm. Treating equal number of cells of each of the strain with Amplex red which reacts with the hydrogen peroxide secreted into the medium during that time unambiguously demonstrated that there was increased amount of extracellular H_2O_2 production in the JP1 culture in mid-log and stationary phase as compared to the wild type reflecting the decreased total catalase activity in JP1 mutant (Fig. 4.10).

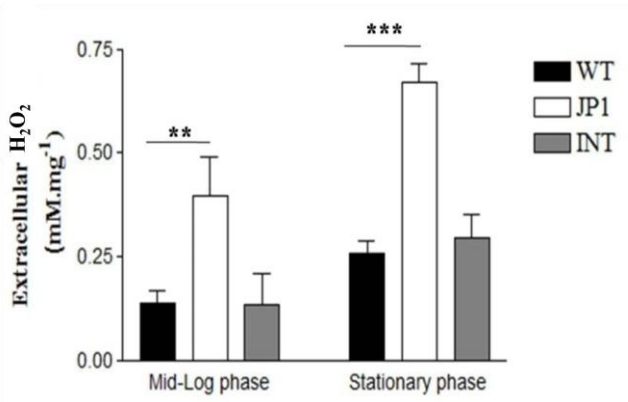


Figure 4.10 Extracellular production of H_2O_2, estimated by Amplex Red-HRP in cells of WT, INT and JP1 in mid-log and in stationary phase of growth. Results are representative of three independent experiments and error bars represent mean ± SD.

Statistically significant differences between strains at each time point were assessed by one-way ANOVA followed by post hoc test (Tukey test; GraphPad Prism) ** $p < 0.01$,***$p < 0.001$.

4.3 Discussion

The decreased viability/ life span is addressed in the present chapter of the chromomycin deletion mutant JP1. We reasoned that the early loss of viability of the JP1 in stationary phase may be the result of the generation of ROS causing oxidative stress. Bacterial growth can't proceed *ad infinitum*; due to nutrient depletion, they enter a phase of growth arrest termed as stationary phase. Loss in the plating efficiency of growth arrested stationary phase cells is closest the bacteria come to the mandatory death in aging organisms, and is purportedly due to increased oxidative damage to macromolecular machinery and proposed to be the cause limiting the life span of the model organisms *E. coli* and *S. cerevisiae* (65, 66). Oxidative stress is aptly called 'Achilles heel' determining viability and life span of any organism utilizing O_2 as a terminal electron acceptor. Any disturbance in the mechanism of management of oxidative stress is reflected in accelerated aging, decreased viability and effect on the life span (31). We based our experiments on this premise and demonstrated that the decrease in the viability in the stationary phase could be due to an increase in oxidative stress in the mutant.

All aerobic organisms are equipped with antioxidant enzymes catalase, peroxidase, alkyl hydroperoxide and superoxide dismutase to neutralize reactive oxygen species. Any imbalance in antioxidant enzymes could lead to oxidative stress. The average life span of the wild type *S. flavisclerotic*us is ~150 hours. The chromomycin deletion mutant JP1 loses viability in 96 – 120 hours. The possible reason of reduced life span / loss in plating efficiency is enhanced oxidative stress in JP1 mutant. The enhanced oxidative stress is result of altered levels of antioxidant enzymes like catalase and AHP (Fig. 4.2; 4.3; 4.6). The increased levels of catalase activity and its expression in mid-log phase of JP1 and its precocious decline in stationary phase with unaltered levels of SOD (Fig. 4.7) at any stage of growth resulted in oxidative stress. This result indicates that cells are under H_2O_2 stress and superoxide stress is not responsible for oxidative stress in JP1. Although total activity of AHP was found to be elevated in JP1 but it was not induced by extracellular H_2O_2,

consistent with the fact that catalases are important for detoxification of H_2O_2 at high concentration whereas AHP is required to quench micromolar amount of H_2O_2 inside the cell. The increased levels of oxidative stress is reflected in increased amounts of DCFDA fluorescence and the increased amounts of H_2O_2 secreted outside the cells as detected by Amplex red/horse peroxidase assay (Fig. 4.8; 4.9; 4.10). The result that JP1 is readily killed by external H_2O_2 treatment in stationary phase than the wild type is in agreement with the decrease in catalase and hydroperoxidase activity in stationary phase in JP1. Taken together, the results are indicative of JP1 mutant being under constitutive oxidative stress.

Oxidative stress play important role in development of *Streptomyces* mainly the transition between developmental stages. Moreover, the oxidative stress generated in response to nutrient limitation and other physiological stresses triggers development related programmed cell death in this organism. It is reported that oxidative stress is linked with impairment of morphological development. The genes associated with aerial mycelium formation and hydrophobic cover formation are downregulated in *katA1* (mono-functional catalase) and *catR* (Fur-like repressor of *katA1* expression) mutants of *S. natalensis*, thus resulted in highly proliferative and undifferentiated vegetative mycelia in former mutant and scarce aerial mycelium formation in later one (61). In *Streptomyces natalensis*, the programmed cell death and morphological differentiation are dependent on oxidative stress. The other phenotypes of mutant which includes defective sporulation can also be explained by the mechanism proposed for *katA1* and *catR* mutants of *S. natalensis*. There is clearly deficiency of catalases in JP1 in the stationary phase, which can't detoxify excess H_2O_2 generation in stationary phase resulting in early death in the stationary phase.

Stationary phase is affected by oxidative stress where antibiotic production also occurs. This raises important question whether oxidative stress is linked with antibiotic production or *vice versa*. This proposal has been tested in several studies. The reports described below demonstrate oxidative stress modifying secondary metabolite biosynthesis. The cross-talk between ROS homeostasis and antibiotic production has been established; in *S. coelicolor* the *catR* mutant overproduces catalase causing decrease in actinorhodin production (67). External supplementation of redox active molecule, phenazine in *S. clavurigerus* augmented clavulanic acid production and in *S. coelicolor* enhanced actinorhodin biosynthesis (68). Modulation

of intracellular H_2O_2, either by induction of oxidative stress by external supplementation of H_2O_2 or by generating knock out mutants of main H_2O_2 related enzyme alters pimaricin production in *S. natalensis.* The significance of this cross-talk for the organism's physiology/metabolism is however not clear. In contrast, the results reported in this chapter indicate clear role of the antibiotic, chromomycin in oxidative stress management in stationary phase, suggesting a putative antioxidant role of chromomycin.

4.4 References

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