Chapter 6

In vivo relevance of antioxidant properties of chromomycin

The unusual phenotypic manifestations of PKS deletion mutant such as slow growth, absence of sporulation, early loss of viability during stationary phase and lack of growth on minimal media containing glucose as sole carbon source as being due to absence of chromomycin biosynthesis, requires an explanation. In the present chapter we tried to explore the root cause of all the phenotypes and address the possibility that antibiotic – chromomycin might play essential role in the physiology of its producer organism. We also tried to establish the growth stage where antibiotic production initiates and verify whether chromomycin biosynthesis is inducible by oxidative stress.

6.1 Introduction

All natural products which are rich source of bioactive compounds produced by plants, bacteria, and fungi are considered as secondary metabolites. Major classes of natural products are terpenoids (including steroids), alkaloids, polyketides (PKs), nonribosomal peptides (NRPs), fatty acids, specialized aminoacids and peptides, phenylpropanoids, alkaloids and specialized carbohydrates. Antibiotics produced by microbes like bacteria and fungi are low molecular weight organic molecules having biological activities therefore used in various infectious diseases and as therapeutics. There are very few studies on the potential roles of these secondary metabolites in nature. The ‘secondary metabolites’ are sorted out by microbiologist as broad class of molecules being produced at tail end of microbial growth in lab condition and they are not required for growth of the producing organisms. This notion that these molecules are less important than others to its producer organism is derogatory. Why would an organism depleted of nutrients begin excreting such complex organic molecules? The common answer is they function as ‘antibiotic’ which is thought to be produced for biological warfare. Research on secondary metabolites was ignored as compared to primary metabolites. Recent developments in the field focus on concentration dependent effects of antibiotics which are produced by ‘so called’ non essential (luxurious) metabolism.

6.1.1 ‘Secondary’ roles of secondary metabolites

Since their discovery, antibiotics, were thought as ‘secondary metabolites’ having no vital role to play in the physiology of their producer organism. The notion

of antibiotics as toxic molecules secreted by an organism for competition advantage prevailed for long and therefore its other potential roles have remained obscure (1). However, since 1990s the concept of antibiotics having additional biological effects beyond biological warfare is gaining grounds (2, 3). Antibiotics at sub-inhibitory concentrations (sub-MIC) can alter global bacterial transcription patterns and modulate specific bacterial promoters (4, 5). For example thiostreptone shows induction of transcription from set of promoters at lower concentration (6), lincomycin stabilizes certain mRNA, puromycin affects nucleic acid synthesis, gramicidin modulates RNA polymerase activity, erythromycin and rifampicin at low concentration alter global transcription in *E. coli* and *S. typhimurium* (7). Davies *et. al.*, (8) have argued that majority of low-molecular-weight organic compounds (mainly antibiotics) made and secreted by microbes play roles as cell-signaling molecules in the environment. This argument is substantiated by following examples - virulence functions can be upregulated at sub-MICs of aminoglycosides and also induction of biofilm formation (9). Interestingly, studies illustrating striking effects of antibiotics on cellular functions like cell signaling (inter and intracellular) (10-12), induction of virulence, gene transfer, phage and motility related genes induction (13) and biofilm formation (14-16) elucidate that antibiotics have diverse roles beyond their bactericidal effects. Moreover, antibiotics such as macrolides, clindamycin, and piperacillin/tazobactam have been observed to decrease alginic acid production, biofilm formation, and virulence factor production in *P. aeruginosa* (17).

6.1.2 Antibiotics show hormesis

The majority of secondary effects of antibiotics are observed at sub-inhibitory concentrations, hence it is recognized that antibiotics exhibits concentration-dependent phenotypic response i.e. hormetic effect (12, 13). Carabrese (18) critically argued that most of the antibiotics show hormetic response i.e. at sub inhibitory concentrations (sub-MIC) these compounds may modulate the transcription of some 5%–10% of bacterial genes in the cell, often inducing 10- to 100-fold up- or downregulatory responses, with only limited effects on growth. At higher concentrations the compounds exhibit their well characterized inhibitory or cidal activities through target-related responses, with few transcription changes. MSL (macrolide-lincosamide-streptogramin) antibiotic have dual effect of transcription

modulation and interaction with ribosomes (19). Other studies have shown that sub-inhibitory concentrations of antibiotics induce mutation or lead to a hypermutable state, promote gene transfer processes such as transposition and conjugation (20).

6.1.3 Physiological roles of antibiotic to its producer organism

The aforementioned studies measure effects of antibiotics on other sensitive organisms, however it is still unrecognized if they have any function in physiology of the producer organism. There are two studies that document the function of antibiotics for producer organism. Newman's group in a series of papers demonstrated the 'colorful' antibiotics – phenazines synthesized by *Pseudomonas aeruginosa* possess beneficial roles for the producer's physiology such as intercellular signaling, electron acceptors in cellular energy generation or maintenance of the intracellular redox homeostasis, affecting central carbon metabolism (21) and iron acquisition (22). The phenazine knockout mutant forms wrinkled colony for better access to oxygen. Phenazines can modulate number of *Pseudomonas* genes including signaling genes which ultimately lead cells to excrete biofilm related polymers. Phenazines can also improve microbe's ability to access iron by transferring electrons to metal (23). The Newman group have showed (2007) that pyocynin (antibiotic phenazine) can alter redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa.* Recent papers studying physiological benefit of phenazine shows that redox cycling of endogenous phenazines assist the anaerobic survival of *P. aeruginosa* by oxidizing glucose and pyruvate to acetate, the two sugars otherwise are non fermentable by *Psuedomonas* spp. Phenazines do the trade by promoting redox homeostasis and ATP generation enabling maintenance of proton-motive force thus involved in energy generation (24).

Pyocyanin (PYO) has 'double edge sword' effect throughout on the life cycle of *Pseudomonas aeroginosa* briefly, positive effect is seen during exponential phase of cell growth when cells are resistant to PYO and it can turn on energy dependent mechanism resulting in survival and a negative effect - that is poisoning and death in stationary phase when environmental conditions are unfavorable to power defense mechanism. This nuanced role of PYO has net beneficial role to the producer organism. Thus PYO promote survival within biofilms where cells are oxidant-limited (25).

Dietrich et al., (26) have enlightened the phenomena of 'eustress' (positive stress promoting survival) in context of redox active metabolite - phenazines and *Pseudomonas.* They have argued that phenazine can cause eustress in their producer organism. Phenazines can increase the bioavailability of iron, facilitate maintenance of intracellular redox homeostasis, and transmit intercellular signals to coordinate gene expression across cell populations. These roles imply that phenazines play primary roles in biological functions of the producer itself.

Wall, *et al.* (27) reported that myxovirescin, an antibiotic produced by *Myxococcus xanthus,* can function as a predatory weapon and therefore directly involved in virulence. Jadomycin, an angucyline antibiotic produced by *Streptomyces venezuelae* at sub-MIC is found to induce complex survival response in *Streptomyces ceolicolor* (28). Lincomycin at sub-MIC found to induce expression of genes of secondary metabolism (Actinorhodin overproduction) in *Streptomyces coelicolor* (29).

Recent studies demonstrate concentration-dependent pro- and antioxidant effects of antibiotics; the latter effect is potentially pertinent to human health applications. Tetracyclines are known as antibiotics acting against a wide range of aerobic and anaerobic Gram-positive and Gram-negative bacteria on 30S ribosomal subunit of bacterial RNA, inhibits loading of aminoacyl-tRNA to ribosomes ultimately blocking the bacterial protein synthesis, and also inhibit metalloproteinases. The former activity of tetracyclin requires Mg^{2+} and the latter one requires Zn^{+2}. Novel derivatives of tetracyclin and minocyclin (an antibiotic belonging to the tetracycline family) inhibit binding of Mg^{2+} and Zn^{+2} required for their antibiotic activity and have improved antioxidant activities. Thus it could be used in treatment of complications related to oxidative stress (30).

Minocyclin is reported as direct antioxidant that may contribute to its neuroprotective property by mitigating oxidative stress, a common cause of neurodegenerative diseases. Sequence selective DNA binding drugs such as mithramycin A3 and chromomycin A3 effectively inhibit apoptosis and DNA damage in cortical neurons caused by oxidative stress induced by DNA-damaging agents and thus possess neuroprotection properties. The neuroprotection property is attributed to

inhibiting gene expression by displacing transcriptional activators that bind to G-C rich regions of promoters of apoptatic pathway proteins (31, 32).

Several important functions of secondary metabolites have been discovered in studies designed to isolate metabolites possessing human medicinal value besides their antibiotic function which also includes antioxidant function of some well-known antibiotics. Quinone anhydroexfoliamycin belonging to group of quinolone antibiotic and the red pyrrole-type pigment undecylprodigiosin synthesized by *Streptomyces coelicolor* displayed complete inhibition of neuronal cytotoxicity and reduces ROS. These compounds provide complete protection against oxidative stress with mitochondrial function, inhibit ROS production and amplify antioxidant enzyme levels glutathione and catalase. These results demonstrate that *Streptomyces* metabolites could be useful for the development of new drugs for prevention of neurodegenerative disorders such as Parkinson's, Alzheimer's diseases and cerebral ischemia (33). Therefore, functions of antibiotics besides their growth inhibitory effect are a distinct possibility.

6.1.4 Cross talk between oxidative stress and secondary metabolism

The secondary metabolism in *Streptomyces* is aerobic thus affected by oxygen availability. As noticed earlier in chapter 4 and 5, the reactive oxygen species formed during oxygen consumption generates reactive oxygen species like hydrogen peroxide, superoxide radicals and hydroxyl radicals which ultimately damage cellular components like nucleic acid, protein and lipids. Adaptive response is triggered to thwart toxic effects of ROS which includes modulation of gene expression to changes in enzymatic and non enzymatic activities. Thus the activated molecular machinery senses and scavenges ROS and eventually repairs the molecular damage. Alterations in the secondary metabolic profile are observed in quite a few studies as a consequence of adaptive response to oxidative insult. Although the molecular mechanisms behind the effect of oxidative stress on production of secondary metabolism alterations remain unclear. (i) *catR* (repressor of *catA*) mutant of *S. coelicolor* A3(2) overproduced catalase but produced reduced amounts of blue antibiotic actinorhodin (34); (ii) Oxidative challenge induced by redox cycling agents like phenazine methosulfate (PMS) (generator of intracellular superoxide) led to increased expression of *cas2* (clavulanic acid synthase, gene for clavulanic acid

biosynthesis enzyme), *cefF* (gene for cephamycin C biosynthesis) and *ccaR* (regulatory gene required for biosynthesis of both clavulanic acid and cephamycin C via protein phosphorylation) (35); (iii) In *Streptomyces natalensis* producer of pimaricin, a 26-member tetraene antifungal macrolide antibiotic, the superoxide dismutase mutant produces low levels of pimaricin compared to wild type and mutant defective of H_2O_2 detoxifying enzymes showed pimaricin overproduction. ROS homeostasis imbalance triggered redox based regulation network which eventually modulated pimaricin biosynthesis in *S. natalensis* (36); (iv) Validamycin A production was enhanced by *S. hygroscopicus* in response to increased ROS which is induced by H_2O_2 treatment (37).

The results described below convincingly demonstrate that most of the pleiotropic phenotypes and metabolic defects of the chromomycin non-producer mutant are reversible and reparable by *ex vivo* supplementation chromomycin by its action as an antioxidant both *in vitro* and *in vivo*. A more direct role of a secondary metabolite for its producer than that reported here is an uncommon finding.

6.2 Results

6.2.1 Chromomycin *in vitro* can act as antioxidant

In order to explain the cause of generation of oxidative stress in the chromomycin non-producer mutant JP1, we tested the possibility if chromomycin is endowed with antioxidant properties. The precedence to this notion are examples of antibiotics acting both as pro- and antioxidants (30). We compared antioxidant potential of chromomycin using a panel of three different methods to the standard ascorbic acid and to tetracycline as the latter is yellow colored and a polyketide same as chromomycin; also it has been shown to be an antioxidant by the *in vitro* tests.

6.2.1.1 DPPH assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay measures the hydrogen donating ability of the antioxidant to stable DPPH radical. Comparison of IC50 values of different compounds showed radical scavenging activity of chromomycin was at an intermediate level, 3.6 times less than the standard ascorbic acid but 5 times more than the tetracyclin (Table 6.1).

6.2.1.2 FRAP assay

In the FRAP assay, the total reducing power of electron donating antioxidant in the reaction is related to change in color of ferrictripyridyltriazine (Fe III TPTZ, straw color) complex to its ferrous form (blue color) that is monitored at 593nm. Chromomycin was 10 times more effective than tetracycline in reducing Fe^{3+} to Fe^{2+} (Table 6.1).

6.2.1.3 Ferric ferrozine assay

The ferric ferrozine assay, unlike the FRAP is performed under physiological pH (38). Ferrozine (FZ) is ferrous stabilizing ligand. Ferric ion in the presence of ferrozine easily oxidizes antioxidants and is itself reduced to Fe(II)-FZ, yielding a very high molar absorptivity and thus enhanced sensitivity for most antioxidants. In this assay, chromomycin scored better as an antioxidant than tetracycline (Table 6.1) indicating that the antioxidant effect of chromomycin is valid even under physiological pH.

Table 6.1 Assessing *in-vitro* antioxidant and iron reducing property of chromomycin

	Ascorbic acid	**Chromomycin**	**Tetracyclin**
DPPH assay IC50 (50%Scavenging activity) in µg	**7.7**	**28**	**141**
FRAP assay FRAP Value in µM	**2**	**0.7166 ± 0.05**	**0.0766 ± 0.02**
Ferric Ferrozine Assay Total Antioxidant Capacities (TAC) mM trolox-equivalent	**$1.1X10^{-2}$**	**$2.8X10^{-3}$**	**$4.1X10^{-4}$**

Besides being an antioxidant *in vitro*, chromomycin has been shown to bind iron, magnesium, manganese and copper (39). The metal binding and the *in vitro* antioxidant and iron binding/reducing activity of chromomycin may possibly have *in vivo* repercussions.

6.2.2 Rescue functions of *ex vivo* supplementation of chromomycin

6.2.2.1 Chromomycin significantly enhances the viability of the JP1 mutant in the stationary phase

The *in vitro* properties of chromomycin as an antioxidant may explain some of the pleiotropic phenotypes of the chromomycin non-producer mutant. The *in vivo* relevance of the chromomycin was tested by its ability to reverse/correct the phenotypes of JP1. We individually incorporated chromomycin at sub-inhibitory concentrations (Chapter 2) and the antioxidant ascorbic acid in the medium at the start of the growth and monitored viability. As indicated earlier, there was an almost complete loss of viability of JP1 in 96 hours, while the viability of the WT was reduced by only 10-fold during the corresponding time (Fig. 6.1). Significantly, the rapid early loss of the viability of the mutant was reversed to a significant extent by the addition of chromomycin at sub-inhibitory concentrations (Fig. 6.1A; 6.1B), as evidenced by a >50-fold greater number of colonies in the culture treated with chromomycin (Fig. 6.1A) and the significant number of cells stained green (Fig. 6.1B). In addition, supplementation by ascorbic acid, a proven antioxidant of nonbacterial origin, can also reverse the loss of viability of the mutant in the stationary phase to a significant extent (Fig. 6.1).

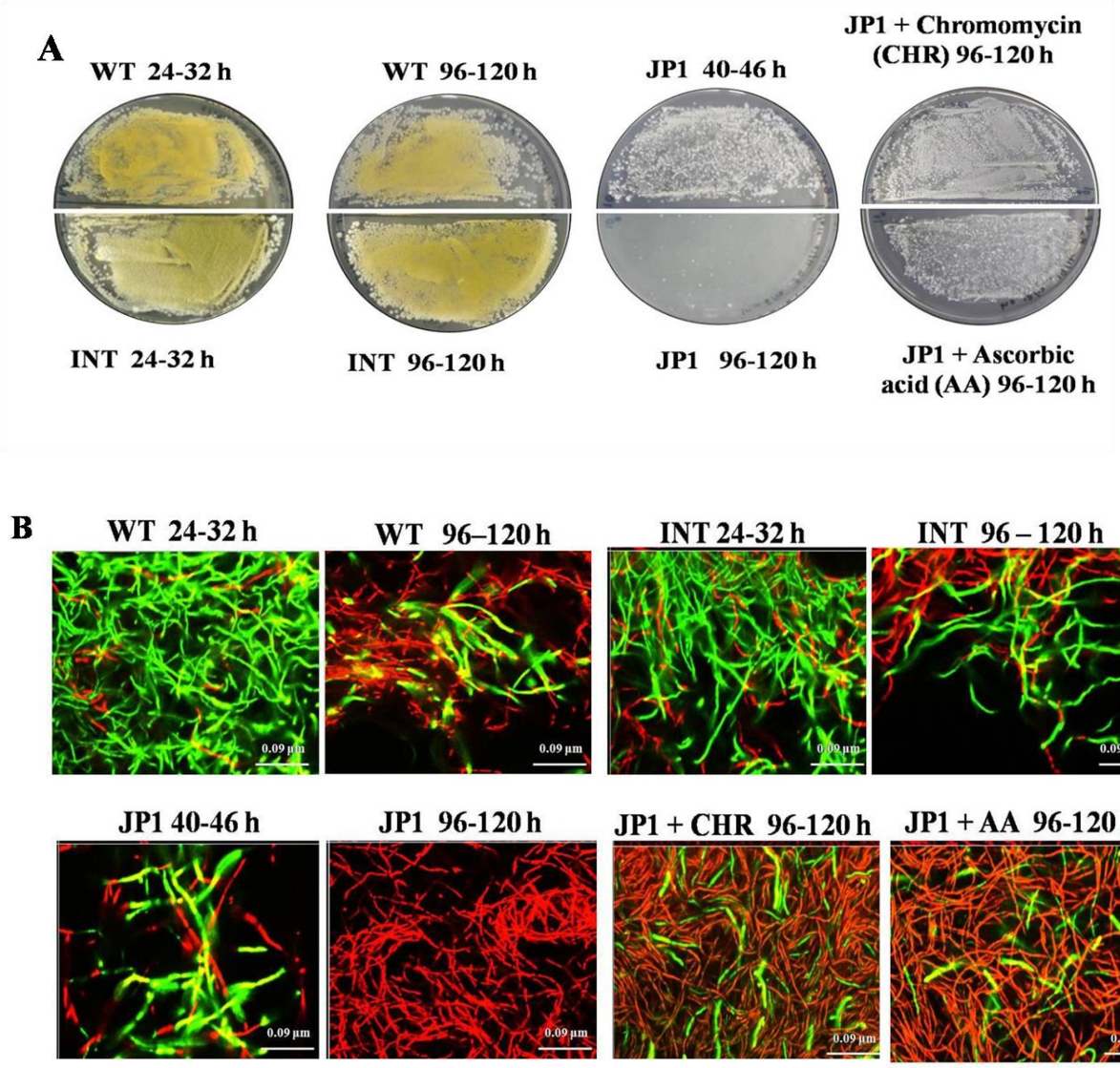


Figure 6.1 Restoration of viability of JP1 by *ex vivo* supplementation of chromomycin and ascorbic acid – (A) Survival in stationary phase (96-120 hours) of the JP1 mutant is enhanced in the presence of sub-inhibitory concentration of chromomycin and ascorbic acid as measured by plating on R2YE (undiluted). The plate pictures are representative of several plating experiments; (B) live/Dead staining of chromomycin and ascorbic acid treated culture of JP1 (Images are taken under LSM – 710 confocal microscope, Scale bar: 0.09 µm). Hours of growth are as indicated. The experiments were repeated more than three times.

6.2.2.2 Chromomycin reduces the intracellular levels of the reactive oxygen species, NADPH and extracellular production of H_2O_2.

External supplementation of chromomycin at sub-inhibitory concentration during the growth of JP1 reduced the total ROS as measured by DCFDA fluorescence (Fig. 6.2A), extracellular production of H_2O_2 as measured by Amplex red –HRP method (Fig. 6.2B) and intracellular NADPH (Fig. 6.2C) to levels similar to those of the wild type, indicating that the oxidative stress phenotype of the non-producer mutant is due to inability of the mutant to produce chromomycin and that the extracellular supplementation of chromomycin can largely reverse the overt effects of oxidative stress.

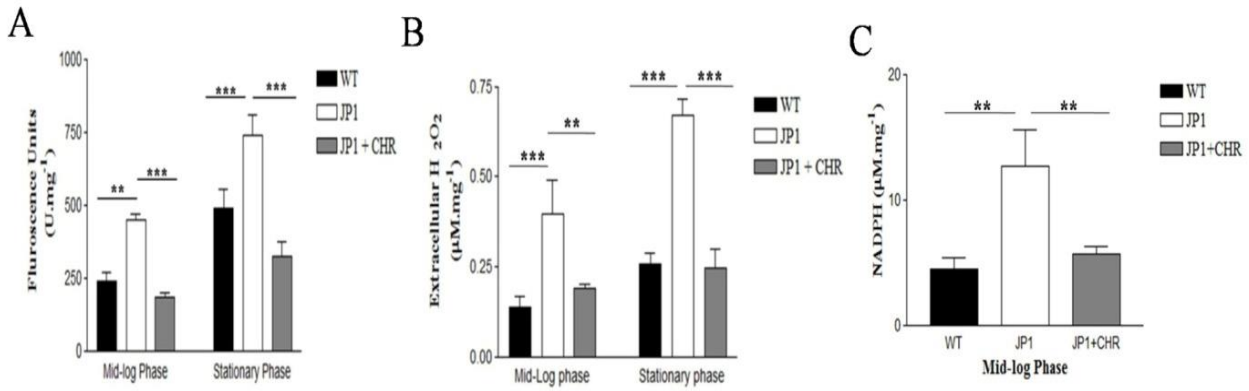
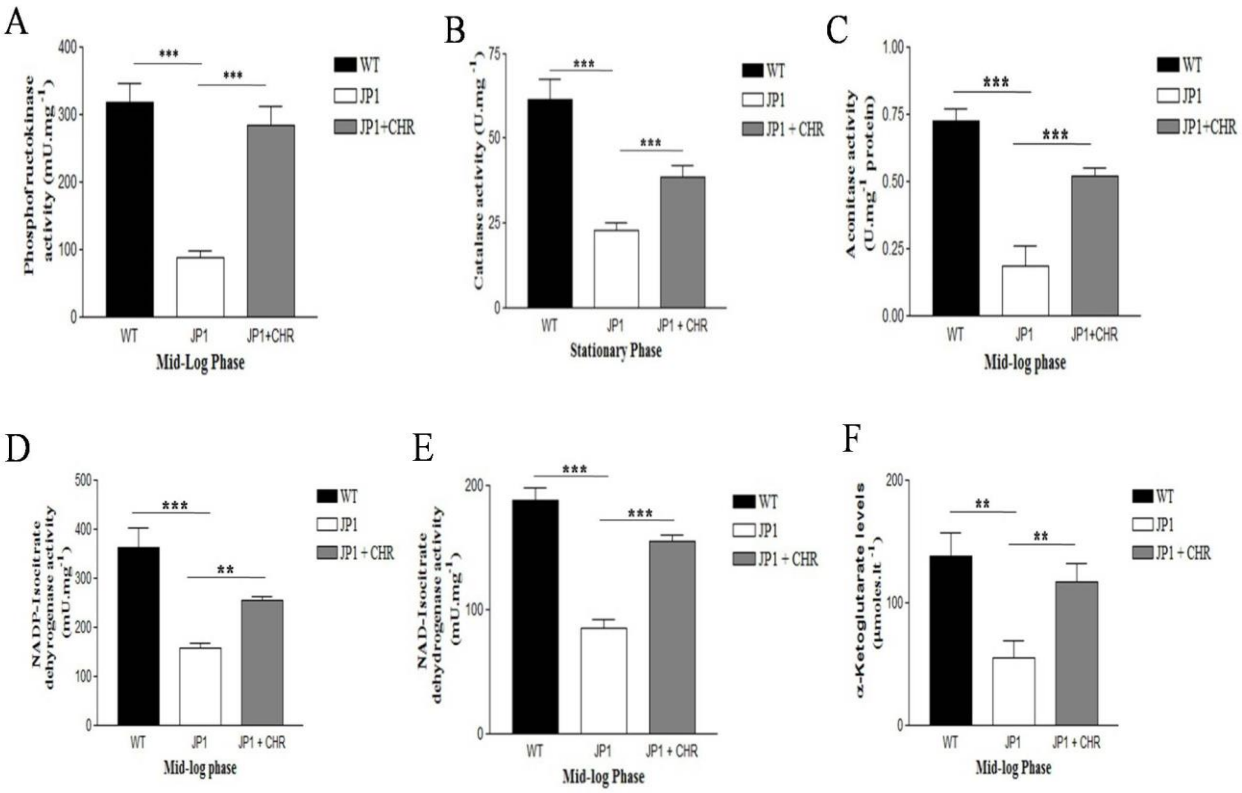


Figure 6.2 Restorative effects of chromomycin (A) Decrease in ROS levels is measured by DCFDA fluroescence; (B) reduction in extracellular production of H_2O_2 is measured by amplex red-HRP method; (C) decrease in intracellular levels of NADPH is measured by enzymatic cycling method. The results are representative of three independent experiments; error bars indicate 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 Prism3) for multiple comparisons and Student's t-test *** $p < 0.001$, **$p < 0.01$.

6.2.2.3 Chromomycin reverses the loss of activity/expression of PFK and catalase and activities of TCA cycle enzymes.

It is notable that the supplementation of sub-inhibitory concentrations of chromomycin during growth enhanced the ATP-PFK in mid-log phase (Fig. 6.3A) and catalase activities in stationary phase (Fig. 6.3B) by two- to three-fold relative to

the JP1 culture grown in its absence. The enhancement in activity was evidently due to augmented transcription. Semiquantitative RT-PCR analysis of the *pfk* genes indicated that the expression of *pfk3*, the presumptive ATP-PFK, was significantly improved in the cells of JP1 by chromomycin treatment, while the *pfk2* transcript was only marginally affected (Fig. 6.3G). There was a comparable effect of chromomycin on the activity and expression of different catalase genes. The total enzymatic activity was significantly improved (Fig. 6.3B). Similarly, the expression of *cat1* and *cat2* was restored in the stationary phase by the addition of chromomycin (Fig. 6.3G). Furthermore, the activities of TCA cycle enzymes (aconitase, NAD- and NADP-IDH) and the levels of the metabolite α- ketoglutarate were augmented in the cells of JP1 grown in the presence of sub-lethal concentration of chromomycin (Fig. 6.3 C; D; E; F). These effects could well be indirect as a result of the correction of oxidative stress parameters (Fig. 6.2) unless validated by purified cell-free extract *in vitro*.



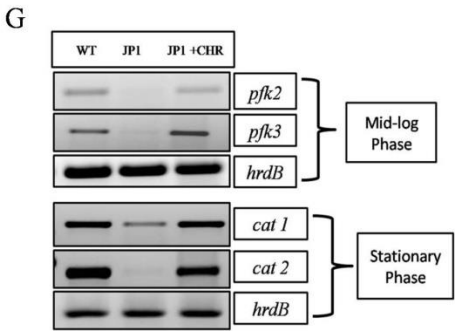


Figure 6.3 The restorative effects of sub-lethal concentration chromomycin on central carbon metabolism in JP1. Restoration of (A) ATP-PFK activity; (B) catalase activity; Kreb cycle enzyme activities for (C) aconitase; (D) NADP-IDH; (E) NAD-IDH and metabolites, (F) α-ketoglutarate levels; (G) *pfk* transcript in mid-log phase and *cat1* and *cat2* transcript in stationary phase. The results are representative of three independent experiments; error bars indicate 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 Prism3) for multiple comparisons and Student's t-test *** $p < 0.001$, **$p < 0.01$.

6.2.3 Chromomycin biosynthesis genes are expressed early in the exponential phase of growth and induced by H_2O_2 stress

The above results arguably indicate that chromomycin play an important physiological role for the producer *S. flavisclerotic*us. As these secondary molecules are produced at late-log /stationary phase of growth of their producer, the reparative effects of extracellular supplementation of sub-MIC concentration of chromomycin on different physiological and biochemical parameters during exponential phase of growth requires that chromomycin be produced intracellularly during early phases of growth of its producer. This proposal necessitated monitoring growth phase dependent expression of chromomycin biosynthesis genes.

The synthesis of secondary metabolites is temporal, limited to the stationary phase, and thus proposed to serve no function for their producers. However, the growth of the non-producer mutant, JP1, is retarded in comparison to the wild type, which is difficult to reconcile, given the temporal segregation of its synthesis. We therefore assessed the expression of a few representative genes of chromomycin

biosynthesis, (i) *sflD* (NDP- glucose synthase) being the promoter proximal gene, represents early gene of the four gene transcript consisting of *sflP* (promoter distal) that encodes the acetate condensing aglycone synthesis function, (ii) *sflMIII* (O-methyl transferase) represents the late step in chromomycin biosynthesis, (iii) *sflRI*, encodes a putative transcriptional activator and (iv) *sflRII*, encodes a putative repressor to regulate the synthesis of chromomycin. We observed that the expression of *sflRI*, *sflMIII* and *sflD* was detectable within 12 h of growth following the inoculation of spores that significantly increased in mid-log phase (27 h) and continued to remain high in the stationary phase (60 h / end of the experiment) (Fig. 6.4). In contrast, the expression of *sflRII* was detectable at all time points of growth at constant levels (Fig. 6.4).

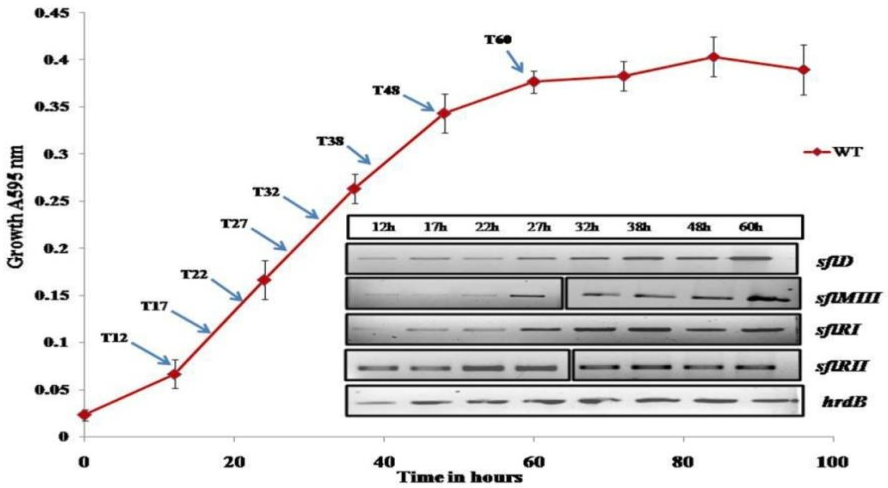


Figure 6.4 Early growth phase expression of chromomycin biosynthesis genes in the wild type - Growth by DNA estimation (A_{595} nm) of WT in liquid R2YE medium and expression analysis of representative chromomycin biosynthesis genes. The cells were harvested for the isolation of total RNA at the time points indicated. Stages of growth were defined by changes in DNA content of the cells. The expression analysis results are representative of three independent experiments.

6.2.4 Induction of chromomycin biosynthesis by external supplementation of H_2O_2

In order to prove that chromomycin plays a role in the fight against oxidative stress, the study of induction of chromomycin biosynthesis by external supplementation of H_2O_2 is obligatory. Treatment of wild type cells at mid-log phase

of growth in TSB broth (~ 28 – 32 hours) with 50 mM H_2O_2 for 1 h, induced expression of *sflD* and putative pathway specific activator *sflRI*, suggesting that expression of *sflD* and *sflRI* in mid-log phase is probably dependent on regulation by oxidative stress (Fig. 6.5). The synthesis of chromomycin in response to oxidative stress could be adaptive in nature to counter ROS stress owing to its anti-oxidant nature. The details of the mechanism of the regulation require further study.

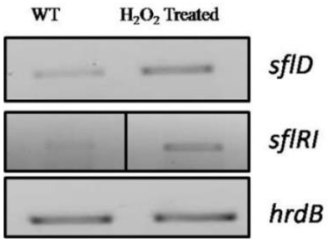


Figure 6.5 Induction of chromomycin biosynthesis genes in the wild type by extracellular H_2O_2 - Induction by 50mM H_2O_2 of early chromomycin biosynthesis gene, *sflD,* representing the transcript of *sflP* (encoding the chromomycin aglycone synthesizing function) and putative activator, *sflRI.* The expression analysis results are representative of three independent experiments.

6.3 Discussion:

In the present chapter, the *in vitro* antioxidant properties of chromomycin (Table 6.1) have been established by different methods whose *in vivo* relevance may explain some of the pleiotropic phenotypes of the chromomycin non-producer mutant. The *in vitro* antioxidant nature of chromomycin is indeed relevant *in vivo* as the viability defect of the JP1 mutant is reversed by the nonbacterial antioxidant molecule, ascorbic acid (Fig. 6.1). This is also consistent with the concentration-dependent pro- and antioxidant effects of antibiotics (40). The evidence so far indicated that supplementation of chromomycin is likely to exhibit corrective effects only at sub-lethal concentration on viability and other physiological functions. However, the effect is limited to correcting the viability defect of JP1 (Fig. 6.1), enhancing the expression and activity of the ATP-PFK, Kreb cycle enzymes and catalase in the stationary phase (Fig. 6.3), resetting the levels of the reducing agent NADPH, and decreasing the intracellular levels of H_2O_2 and ROS (Fig. 6.2).

The inability to reverse the growth defect of the JP1 mutant on mineral media with glucose as the sole carbon source may be the result of (i) an inherent preference of *S. flavisclerotic*us for amino acids as the carbon source, (ii) reconfiguration of carbon metabolism due to constitutive oxidative stress in the JP1 mutant, and (iii) the inability of exogenous chromomycin provided during growth to mimic its intracellular regulated production. It must also be noted that chromomycin is a DNA-binding molecule that inhibits replication/transcription and severely inhibits the growth of the producer bacterium during the exponential phase.

The importance of chromomycin to its producer is evident in early expression of the genes of the chromomycin biosynthesis and possibly its production, which may benefit the physiology and metabolism during the log phase of growth of the wild type. Possible synthesis of chromomycin at the early time points (12-, 17-, 21 h) may represent its sub-MIC concentration found to affect the physiology but presumably insufficient to bind DNA and inhibit replication/transcription, the known mechanism of action of chromomycin for the inhibition of growth (Fig. 6.4). The analysis of growth phase dependent expression of complete cluster of antibiotic biosynthesis genes in the case of *S. coelicolor* demonstrated that actinorhodin gene expression is limited to late stationary phase whereas that of RED and CDA is evident in the mid-log phase (20 h) but not in the early growth phase (12 h) (41). The genes of the tylosin biosynthesis of *S. fradiae* have been analyzed at early log (18 h) and late stationary phase (40 h) of growth. Only about one third of the genes are expressed in the early log phase, suggesting the production of tylosin is unlikely to begin in the early phase of growth (42). Although the gene expression profile of *S. flavisclerotic*us is not exhaustive, comprising of one early- (*sflP*), two late acting (*sflD* and *sflMIII*) and two regulatory genes (*sflRI* and *sflRII*), the basal level expression of the genes at 12 h of growth (early log phase) which increases substantially at 21 h (mid-log phase) and remains high upto 60 h (late-log phase) is probably significant (Fig. 6.4). We believe that the basal level expression of the representative genes reflect the chromomycin biosynthesis potential of the organism in the early phase of growth and may mimic the growth beneficial effects of the sub-lethal concentration of chromomycin. A more detailed study of the growth phase expression of chromomycin biosynthesis genes is required to validate the proposal.

The results obtained so far indicate that deletion of chromomycin gene cluster lead to oxidative stress in JP1 mutant. As a corollary, the external imposition of oxidative stress should induce chromomycin biosynthesis genes if the antioxidant function of chromomycin is important in the defense response of the cell. Indeed, chromomycin biosynthesis genes are inducible by oxidative stress (Fig. 6.5). The synthesis of chromomycin in response to oxidative stress could be adaptive in nature to counter ROS stress owing to its anti-oxidant nature.

There are studies indicating correlation/ cross-talk between ROS homeostasis and antibiotic production both of them are characteristically important for stationary phase. For example the antibiotic pimaricin is affected by altered expression of antioxidant enzymes, however the purpose of this regulation is not apparent (36).

6.4 References

1. Vining LC (1990) Functions of secondary metabolites. *Annu Rev Microbiol* 44:395-427.
2. Demain AL & Fang A (2000) The natural functions of secondary metabolites. *Advances in biochemical engineering/biotechnology* 69:1-39.
3. Davies J (1990) What are antibiotics? Archaic functions for modern activities. *Mol Microbiol* 4(8):1227-1232.
4. Tsui WH, *et al.* (2004) Dual effects of MLS antibiotics: transcriptional modulation and interactions on the ribosome. *Chem Biol* 11(9):1307-1316.
5. Goh EB, *et al.* (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc Natl Acad Sci U S A* 99(26):17025-17030.
6. Murakami T, Holt T, & Thompson C (1989) Thiostrepton-induced gene expression in Streptomyces lividans. *Journal of bacteriology* 171(3):1459-1466.
7. Goh E-B, *et al.* (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proceedings of the National Academy of Sciences* 99(26):17025-17030.
8. Yim G, Wang HH, & Frs JD (2007) Antibiotics as signalling molecules. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 362(1483):1195-1200.
9. Hoffman LR, *et al.* (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436(7054):1171.
10. Yim G, Wang HH, & Davies J (2007) Antibiotics as signalling molecules. *Philos Trans R Soc Lond B Biol Sci* 362(1483):1195-1200.
11. Fajardo A & Martinez JL (2008) Antibiotics as signals that trigger specific bacterial responses. *Curr Opin Microbiol* 11(2):161-167.
12. Davies J, Spiegelman GB, & Yim G (2006) The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9(5):445-453.

13. Yim G, Wang HH, & Davies J (2006) The truth about antibiotics. *Int J Med Microbiol* 296(2-3):163-170.
14. Hoffman LR, *et al.* (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436(7054):1171-1175.
15. Banin E, Vasil ML, & Greenberg EP (2005) Iron and Pseudomonas aeruginosa biofilm formation. *Proc Natl Acad Sci U S A* 102(31):11076-11081.
16. Lopez D, Fischbach MA, Chu F, Losick R, & Kolter R (2009) Structurally diverse natural products that cause potassium leakage trigger multicellularity in Bacillus subtilis. *Proc Natl Acad Sci U S A* 106(1):280-285.
17. Tanaka G, *et al.* (2000) Effect of clarithromycin on Pseudomonas aeruginosa biofilms. *Chemotherapy* 46(1):36-42.
18. Calabrese EJ (2004) Hormesis: a revolution in toxicology, risk assessment and medicine: Re-framing the dose–response relationship. *EMBO reports* 5(1S):S37-S40.
19. Tsui WH, *et al.* (2004) Dual effects of MLS antibiotics: transcriptional modulation and interactions on the ribosome. *Chemistry & biology* 11(9):1307-1316.
20. Wang Y, Rotman ER, Shoemaker NB, & Salyers AA (2005) Translational control of tetracycline resistance and conjugation in the Bacteroides conjugative transposon CTnDOT. *Journal of bacteriology* 187(8):2673-2680.
21. Price-Whelan A, Dietrich LE, & Newman DK (2007) Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in Pseudomonas aeruginosa PA14. *J Bacteriol* 189(17):6372-6381.
22. Price-Whelan A, Dietrich LE, & Newman DK (2006) Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol* 2(2):71-78.
23. Hernandez ME, Kappler A, & Newman DK (2004) Phenazines and other redox-active antibiotics promote microbial mineral reduction. *Applied and environmental microbiology* 70(2):921-928.
24. Glasser NR, Kern SE, & Newman DK (2014) Phenazine redox cycling enhances anaerobic survival in P seudomonas aeruginosa by facilitating generation of ATP and a proton-motive force. *Molecular microbiology* 92(2):399-412.
25. Meirelles LA & Newman DK (2018) Both toxic and beneficial effects of pyocyanin contribute to the lifecycle of Pseudomonas aeruginosa. *Molecular microbiology* 110(6):995-1010.
26. Okegbe C, Sakhtah H, Sekedat MD, Price-Whelan A, & Dietrich LE (2012) Redox eustress: roles for redox-active metabolites in bacterial signaling and behavior. *Antioxidants & redox signaling* 16(7):658-667.
27. Xiao Y, Wei X, Ebright R, & Wall D (2011) Antibiotic production by myxobacteria plays a role in predation. *J Bacteriol* 193(18):4626-4633.
28. Wang W, *et al.* (2014) Angucyclines as signals modulate the behaviors of Streptomyces coelicolor. *Proceedings of the National Academy of Sciences*:201324253.
29. Imai Y, Sato S, Tanaka Y, Ochi K, & Hosaka T (2015) Lincomycin at subinhibitory concentrations potentiates secondary metabolite production by Streptomyces spp. *Applied and environmental microbiology*:AEM. 04214-04214.
30. Lertvorachon J, *et al.* (2005) 1,12-substituted tetracyclines as antioxidant agents. *Bioorg Med Chem* 13(15):4627-4637.
31. Jain KK (2011) Neuroprotective agents. *The Handbook of Neuroprotection*, (Springer), pp 25-139.

32. Kraus RL, *et al.* (2005) Antioxidant properties of minocycline: neuroprotection in an oxidative stress assay and direct radical-scavenging activity. *Journal of neurochemistry* 94(3):819-827.
33. Leirós M, *et al.* (2013) Mitigation of ROS insults by Streptomyces secondary metabolites in primary cortical neurons. *ACS chemical neuroscience* 5(1):71-80.
34. Hahn J-S, Oh S-Y, Chater KF, Cho Y-H, & Roe J-H (2000) H2O2-sensitive fur-like repressor CatR regulating the major catalase gene in Streptomyces coelicolor. *Journal of Biological Chemistry*.
35. Kwon H-J & Kim S-U (1999) Molecular basis for enhanced biosynthesis of clavulanic acid by a redox-cycling agent, phenazine methosulfate, in Streptomyces clavuligerus. *Applied microbiology and biotechnology* 53(1):57-62.
36. Beites T, *et al.* (2011) Crosstalk between ROS homeostasis and secondary metabolism in S. natalensis ATCC 27448: modulation of pimaricin production by intracellular ROS. *PLoS One* 6(11):e27472.
37. Wei Z-H, Bai L, Deng Z, & Zhong J-J (2011) Enhanced production of validamycin A by H2O2-induced reactive oxygen species in fermentation of Streptomyces hygroscopicus 5008. *Bioresource technology* 102(2):1783-1787.
38. Berker KI, Güçlü K, Demirata B, & Apak R (2010) A novel antioxidant assay of ferric reducing capacity measurement using ferrozine as the colour forming complexation reagent. *Analytical Methods* 2(11):1770-1778.
39. Kamiyama M (1968) Mechanism of action of chromomycin A3. 3. On the binding of chromomycin A3 with DNA and physiochemical properties of the complex. *J Biochem* 63(5):566-572.
40. Schildknecht S, *et al.* (2011) Neuroprotection by minocycline caused by direct and specific scavenging of peroxynitrite. *J Biol Chem* 286(7):4991-5002.
41. Huang J, Lih C-J, Pan K-H, & Cohen SN (2001) Global analysis of growth phase responsive gene expression and regulation of antibiotic biosynthetic pathways in Streptomyces coelicolor using DNA microarrays. *Genes & development* 15(23):3183-3192.
42. Stratigopoulos G & Cundliffe E (2002) Expression analysis of the tylosin-biosynthetic gene cluster: pivotal regulatory role of the tylQ product. *Chemistry & biology* 9(1):71-78.