Summary

Rethinking on secondary status of secondary metabolite

It is perceived since long that secondary metabolites, mainly antibiotics, secreted at the end of the growth of an organism play no vital role to the growth or survival of the producer organism. The notion that antibiotics are secreted only for self defense and that they are 'secondary' is pejorative as it raises a valid question as to why would an organism harbor genes for complex molecules and begin excreting in large amounts in nutrient starvation condition. There are very few studies contesting the idea that antibiotics have just weaponry roles. Reports on second roles of antibiotics have emerged only during last couple of decades, clearly warranting re-examination of secondary status of these molecules. Several of these studies infer that the world of sub-inhibitory concentration of antibiotics is indeed different from the concentration required for killing. This applies to both - organisms being affected and the organism that produces it. However, the latter effect is not obvious and thus the direct benefit to the producer organism is not fully appreciated. The results of the present work indicate that chromomycin synthesized by *Streptomyces flavisclerotic*us benefits the physiology, metabolism and survival of its producer at concentration permissive for growth.

The important physiological role of chromomycin in metabolism of *S. flavisclerotic*us was realized with an insightful observation that the genetically verified PKS null mutant unable to synthesize chromomycin exhibited a range of unexpected overt phenotypes apart from expected phenotype of loss of antibiotic production. The overt phenotypes exhibited by the chromomycin null mutant included – slow growth on rich media, absence of sporulation, early loss of viability and inability of mutant to grow on synthetic media with glucose as sole carbon source. We undertook this study to uncover potential role of chromomycin in repairing majority of the defects. The observation that chromomycin null mutant loses viability early evidently indicated imbalance in redox status of cell. As oxidative stress is intimately linked to longevity of bacterial system, any alteration in expression of antioxidant enzymes favoring oxidative environment in the cell could lead to untimely death of an organism. Based on this premise it became apparent that there might be alteration in expression of antioxidant enzymes in chromomycin null mutant which leads to early loss of viability in relation to wild type. The precocious decline in the expression of

catalase in the stationary phase with unaltered levels of SOD at any stage of growth of chromomycin non-producer mutant resulted in oxidative stress. The enhanced oxidative stress is evident as an increase in quantifiable DCFDA fluorescence, a measure of intracellular ROS and increased extracellular production of H_2O_2. Furthermore, the metabolic defect in chromomycin deletion mutant is congruent with cellular adaptation to oxidative stress. The mutant fails to grow on chemically defined media with glucose as sole carbon source due firstly to, a complex amino acid requirement of the oxidatively stressed cells and secondly to an inherent preference of *S. flavisclerotic*us for amino acids as the carbon source over glucose. Increase in the flux of the carbon through pentose phosphate pathway and at the expense of glycolysis is all too evident in the non-producer mutant. This alteration is extremely important for maintaining reducing environment under oxidative stress condition. As glycolysis is not fully functional in chromomycin non-producer, there is a deficient level of acetyl Co-A, a precursor for TCA cycle. Along with constitutive oxidative stress which disrupts iron sulphur centers of many enzymes, limitation of acetyl Co-A impedes functioning of TCA cycle in chromomycin null mutant which is reflected in the poor activities of key TCA cycle enzymes such as aconitase and isocitrate dehydrogenase and metabolite α-ketoglutrate. Moreover, along with deficient TCA cycle, oxidative stress also hampers biosynthesis of many amino acids rendering chromomycin null mutant fully dependent on casamino acids as sole carbon source instead of glucose.

In order to explain how deletion of chromomycin biosynthesis genes led to oxidative stress and metabolic readjustments in the mutant, antioxidant property of chromomycin was tested *in vitro* using different assays and indeed chromomycin scored better as an antioxidant than tertacyclin – a yellow colored polyketide antibiotic, an *in vitro* antioxidant itself. The most persuasive result of this dissertation is the *in vivo* reparative function of chromomycin, possibly due to its antioxidant nature. Supplementation of chromomycin at sub-inhibitory concentration to the growth medium of the non-producer mutant rescues its viability loss and also repairs metabolic deficiencies. Activity and expression of glycolytic enzyme phosphofructokinase, activities of TCA cycle enzymes and pentose phosphate pathway were found to be repaired along with reduction in oxidative stress. The *in vivo* reparative effects of chromomycin clearly demonstrates that the loss of production of chromomycin led to bewildering range of unexpected phenotypes which

are not due to deletion of the function(s) of gene(s) for its biosynthesis. These results pose an important question - if the secondary metabolites are produced only in stationary phase then why absence of its synthesis led to defect in the exponential growth phase. The answer to this question is explained by the result that chromomycin is produced in small amounts even in growth phase and this way it may play a role in maintaining redox homeostasis and physiology of the cell. This proposal is proved to be valid in the experiment analyzing growth phase dependent expression of representative early and late genes of the chromomycin biosynthesis cluster - the structural and regulatory genes' are expressed as early as 12 hours post inoculation of the spores implying the potential to produce chromomycin during the early stages of the growth of the producer. Moreover, the aforesaid inference is apposite in the context that extracellular oxidative stress enhances chromomycin production in the growth phase of the producer wild type.

Till date a more direct role of antibiotic to the producer organism is revealed only in two reports - (i) Phenazines, an antibiotic produced by *P. aerugenosa* have been reported to play important role in maintaining cellular homeostasis and metabolism of the producer and (ii) myxoveriscin, an antibiotic produced by *Myxococcus* has a causal role for the virulence of the producer bacteria. The present study too widens our appreciation of potential roles of the secondary metabolites' beneficial functions for their producers. An interesting concept of 'eustress' has been proposed as positive stress promoting survival. The redox active molecules such as phenazines and chromomycin produced by *P. aerugenosa* and *S. flaviscleroticus* respectively benefit their producers under active growth condition at concentration permissive for growth (eustress); the higher concentrations inhibit the growth of the producers (distress) unless in non-growing stationary phase. Recent observations reveal pro- and anti-oxidant nature of antibiotics for instance; minocyclin is reported to have antioxidant and neuroprotection effect.

The lead obtained in the study about the novel mutation causing overproduction of chromomycin and simultaneous resistance to different aminoglycosides is worth exploration. The overproducer mutant is different from the mutants described in the literature so far. The uniqueness lies in the fact that the enhancement in chromomycin production equals (~176 fold) to 180 fold oversynthesis of actinorhodin by octuple mutant of *S. coelicolor* without any effect on growth. The effect of streptomycin resistant mutation on antibiotic synthesis is unique

as well. In contrast to the enhancement of endogenous antibiotic synthesis, as in other species, the mutation isolated here dramatically decreases antibiotic production. This is ample justification for its characterization.

Recent genome sequencing projects have revealed that antibiotics produced by *Streptomyces* are always underestimated. Bioinformatics analysis of their genomes indicates that majority of the antibiotic clusters found in the genome are not expressed under laboratory conditions and such clusters are known as cryptic/silent clusters. These gene clusters remain treasure trove for discovery of novel compounds. The importance of present study lies in the context of unexpressed/silent gene clusters. Given a large repertoire of unexpressed genes for secondary metabolites in soil organisms, there is strong possibility that the potential of second function of antibiotic unrelated to its grown inhibitory effect is real. Moreover, the novel functions of chromomycin and its ilk for their producers may be required for shaping the bacterial ecosystem.