Synopsis of the thesis on

Physiological Role of Chromomycin In *Streptomyces flaviscleroticus*

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"*The existence of microbes that have the capacity to produce antibiotics in artificial culture cannot be interpreted as signifying that such phenomena are important in controlling microbial populations in nature. Unless one accepts the argument that laboratory environments are natural, one is forced to conclude that antibiotics play no part in modifying or influencing living processes that normally occur in nature.''*

Selman Waksman (1961)

Introduction:

Rethinking secondary metabolism:

Natural products and their derivatives play crucial role in modern healthcare as frontline treatments for many diseases. Natural products are produced by plants, bacteria, and fungi. They have been a rich source of bioactive compounds for drug discovery and development. All natural products are considered as secondary metabolites. Major classes of natural products (Secondary metabolites) are terpenoids (including steroids), alkaloids, polyketides (PKs), nonribosomal peptides (NRPs), fatty acids, specialized aminoacids and peptides, phenylpropanoids, alkaloids and specialized carbohydrates. The 'secondary metabolites' are sorted out by microbiologist as broad class of molecules being produced at late stage 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. Study of this 'so called' non essential (luxurious) metabolism is currently being taken up by industrial scientists, academics and pharmacognosists.

Microbes like bacteria and fungi produces bewildering array of low molecular weight organic molecules having many biological activities but their roles in nature are unknown. Antibiotics are one of most widely studied among these small molecules, because of its enormous use in various infectious diseases and as therapeutics. However there are very few studies of the potential roles of these secondary metabolites in nature.

Streptomyces is recognized as industrially important microorganism as *Streptomyces* is prolific source of novel secondary metabolites with array of biological activities which ultimately find applications like anti-infectives, antibiotics, immunosuppresants, anticancerous compounds and other pharmaceutically important compounds. It arguably produces majority of antibiotics used in human and veterinary medicine and agriculture, and other valuable active metabolites like immunosuppresants, anti-cancer drugs and pesticides. It is for these benefits that there is large scale commercial/industrial exploitation of this organism for production of antibiotics and other biologically active compounds for human health applications.

Secondary roles of secondary metabolites:

In recent years the conception that secondary metabolites have other functions are highly compelling. Antibiotics have many other biological effects apart from their so called role of antibiosis. For example, Thiostreptone shows induction of transcription from set of promoters at lower concentration (Murakam *et. al.,* 1989), Lincomycin stabilizes certain mRNA (Maskusita *et. al.,* 1989), Puromycin affects nucleic acid synthesis. (Haba 1959), Gramicidin modulates RNA polymerase activity (Sarkar and Paulus 1972). Macrolides, clindamycin, and piperacillin/tazobactam have been observed to decrease alginic acid production, biofilm formation, and virulence factor production in *P. aeruginosa* (Tanaka et al., 2000)., erythromycin and rifampicin at low concentration alter global transcription in *E. coli* and *S. typhimurium* (Davies et al 2002).

Antibiotics show hormesis:

Carabrese (2002) critically argued that most of the antibiotics show hormetic response i.e. at subminimal 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) have dual effect of transcription modulation and interaction with ribosomes (Davies 2004). Other studies have shown that subinhibitory concentrations of antibiotics induce mutation or lead to a hypermutable state, promote gene transfer processes such as transposition and conjugation (Wang et al., 2005).

Antibiotics as cell signaling Agent and also induce biofilm formation:

Davies *et. al.,* (2007) 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 examples like - virulence functions can be upregulated; at sub-MICs of aminoglycosides and also induce biofilm formation (Hoffman et al. 2005).

Antibiotics as redox active molecule:

Pseudomonas bacteria make array of colorful phenazines that are toxic to other cells but play roles in their producer's metabolism like handling electrons during unavailability of oxygen. The phenazine knockout mutant forms wrinkled colony for better access to oxygen. Newman has put forward the notion that antibiotics could function as electron shuttles, not just as weapons. 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 (Newman et al 2004). The Newman group have showed (2007) that pyocynin (antibiotic Phenazine) can alter redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa*.

Dietrich et al., (2011), have enlightened the phenomena of 'eustress' (positive stress promoting suvival) 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.

In 2011, Wall *et al.,* proved that antibiotic from *Myxobacteria* helps in predation.

Jadomycin an angucyline antibiotic produced by *Streptomyces venezuelae* at sub MIC found to induce complex survival response in *Streptomyces ceolicolor* (Wang et al., 2014).

Lincomycin at sub MIC found to induce expression of genes of secondary metabolism (Actinorhodin overproduction) in *Streptomyces coelicolor* (Imait,2015*).*

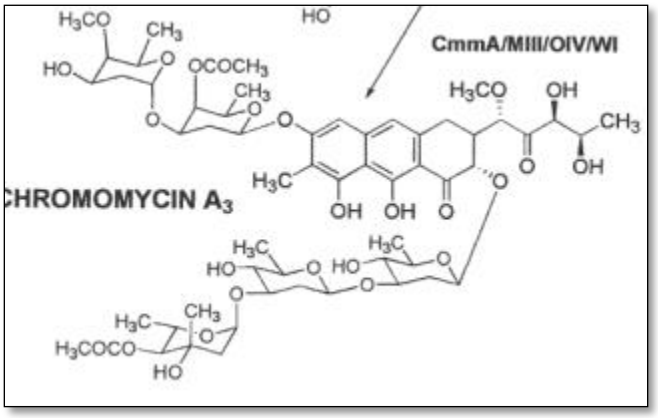
Antibiotic Overproduction:

As the production of the secondary metabolites mainly antibiotics is metabolically costly to the cell so the amount produced by the cell are far lower than those necessary for industrial-scale production. Some cryptic pathways have been discovered that do not actively produce any metabolites which can only be activated under certain conditions, many of which are unknown. These factors, however, necessitate that producing organisms undergo significant strain improvement to yield the desired metabolite at an industrial scale. *Streptomyces* are exploited for production of a wide range of secondary metabolites. Great deal of work is being carried out for the improvement in efficiency of antibiotic production by the producer strain *Streptomyces*. The traditional convention for improvement of secondary metabolites is altering various physico-

chemical factors. Random mutagenesis approach has been used in industry for strain improvement.

Metabolic engineering is in demand to maximize product yields. It includes: alteration of metabolic flux, deregulation of specific biosynthetic pathway, inducing resistance to several antibiotics, overexpression of structural genes, genome shuffling, and expressing biosynthetic gene cluster in heterologous host. These are labor and cost intensive approaches. Ribosome engineering is an innovative approach to increase antibiotic production. This approach involves engineering drug resistant mutations in rRNA or ribosomal proteins, several of them have been shown to cause hyperproduction of various antibiotics. As proposed by Ochi *et. al.*, increased stability of the ribosome in ribosome targeting antibiotic resistant mutants is responsible for enhancement of antibiotic overproduction in *Streptomyces* spp. Introduction of several drug resistant mutations targeting ribosomes have cumulative effect on antibiotic production. Octuple mutants resistant to antibiotics Streptomycin, Gentamycin, Rifampicin, Geneticin, paromomycin , fusidic acid, lincomycin , and thiostrepton produces large amounts of the polyketide antibiotic - actinorhodin, 180-fold higher than the level produced by the wild type.

Background: Our lab is working with the strain *Streptomyces flaviscleroticus* which contains type-(II) PKS (polyketide synthase) cluster for production of anticancerous antibiotic, Chromomycin. Chromomycin had been purified, characterized and confirmed by NMR analysis in our lab. The premise of the project is nonacceptance of the traditional view that the antibiotics, relegated to secondary metabolite status, are less important to the producer organism than are other metabolites. The structure of Chromomycin is shown below:



PKS deletion mutant of *S. flavisclerotícus* was constructed which failed to produce Chromomycin. The expected phenotype resulting from deletion is lack of antibiotic production. 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) increased permeability to different compounds (IV) early loss of viability during stationary phase and (V) no growth on minimal media.

The phenotypic manifestations of the defect in Chromomycin genes reinforce the question how relevant is the pronouncement that antibiotics are just secondary metabolites. We undertook the study regarding mechanistic defects in Chromomycin deletion mutant. We attempted to find out in this work, how deletion of chromomycin synthesis cluster genes resulted in such unusual phenotypes by addressing the question if Chromomycin has some other role to play in physiology of its producer organism apart from antibiosis?

The chapters of thesis will be as follow:

(1) Study of oxidative stress parameters in Chromomycin deletion mutant, complementation of the mutant with genes for Chromomycin biosynthesis and generation of single gene deletion mutant

(a) **Study biomarkers of oxidative stress:**

. In the antibiotic deletion mutant the phenotypes like early loss of viability, loss of sporulation, increased sensitivity to antibiotics and large clumpy growth clearly indicates defective stationary phase. The accelerated aging, reflected in rapid loss of viability can be correlated with enhanced ROS production in mutant. In order to study oxidative stress activity of catalase and SOD were measured by spectophotometric method and Native PAGE, during growth phase, early stationary phase and late stationary phase. The results obtained shows that in Chromomycin deletion mutant there is drastic drop in catalase activity in stationary phase which is the ‘Achilles Heel’ of bacterial life cycle. The levels of SOD were found be unperturbed. Decline in the levels of catalase in the mutant during stationary phase assisted by unaltered

activity of SOD result in oxidative stress, as evidenced by high levels of DCFDA fluorescence (reactive oxygens species). This also explains early loss of viability.

(b**) Complementation of the deletion genes:**

In order to check that the phenotypes are due to the deletion and there is no other mutation in the deletion mutant, genes of the Chromomycin biosynthesis were introduced back. The defective phenotypes of the antibiotic deletion mutant are restored to wild type with introduction of the genes of Chromomycin biosynthesis corresponding to the deletion.

(c) **Single gene mutant**

An insertion mutant disrupted for NDP-glucose 4, 6-dehydratase (*cmmE*, gene involved in deoxysugar biosynthesis) exhibits similar phenotypes as the deletion mutant. The disruption of gene for deoxysugar biosynthesis was carried out by introducing 'Thiostreptone resistance gene casette' at the unique retriction site (*KpnI*) of *cmmE* in the plasmid. The insertion cassette was recombined on the chromosome using double homologous recombination strategy. An insertion mutant disrupted for *cmmE*, gene exhibits similar phenotypes as the deletion mutant like slow growth and early loss of viability.

. (2) **Evaluating *in vitro* antioxidant property of Chromomycin:**

(a) **Evaluating *in vitro* Antioxidant & Iron reducing property of Chromomycin*:***

The results so far obtained indicate that Chromomycin deletion can leads to oxidative stress in the deletion mutant. In order to answer the question whether Chromomycin act as an antioxidant various in –vitro tests were conducted like

DPPH assay (1-Diphenyl-2-picryl hydrazyl) assay: Chromomycin has significant free radical scavenging activity as compared to tetracyclin – a yellow colored polyketide antibiotic.

FRAP assay (Ferric Iron Reducing Antioxidant property): Chromomycin can reduce iron and has antioxidant property.

Iron Scavenging activity

CAS assay: Chromomycin can act as siderophore as it can scavange iron from CAS –a blue colored dye which gets converted to yellow color upon loss of iron.

Ferric ferrozine based assay: The novel antioxidant assay of ferric reducing capacity measurement also indicates that Chromomycin has iron reducing and antioxidant property

Together the results indicate that Chromomycin is an antioxidant and has iron reducing activity.

(b) In Vitro Reversible Aconitase Activity:

Aconitase contains [4Fe-4S] cluster in which there is an unprotected labile Fe atom, this atom can be easily removed under oxidative and low iron conditions (J. A. Middaugh, 2004). In vitro the aconitase enzyme of wild type culture was inactivated by hydrogen peroxide treatment the resulting inactive aconitase can be reactivated by addition of chromomcin along with iron. The result proves that Chromomycin can reduce iron and can reactivate inactivated enzyme.

(c) In vivo role of Chromomycin in cell viability and catalase induction

Chromomycin when added in the deletion mutant culture in late log phase where resistance genes for Chromomycin are expressed the viability of the mutant is sustained and it also shows induction of catalase activity which is lost in mutant culture after mid log phase.. Thus this result shows that Chromomycin has important role in physiology of its producer organism like it is vital for growth and survival of its producer organism.

(3) Investigate Metabolic Defect in Chromomycin deletion mutant

The PPP pathway and Glycolysis:

The metabolic defect was investigated for the antibiotic deletion mutant failing to grow on minimal media because of its inability to utilize glucose as sole carbon source and instead require amino acid as sole carbon source. It was found that the mutant has no defect in uptake of glucose. The glucose taken in is diverted to PPP pathway in order to generate more reducing power to combat oxidative stress in deletion mutant. As a result there is complete reconfiguration of carbon flux in the mutant with upregulation of PPP pathway and concomitant decrease in flux through glycolysis. The former parameter is evaluated by assaying PPP pathway enzymes like glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and the latter parameter is monitored by enzymatic assays for glycolysis, key regulatory enzyme phosphofructokinase. As expected PPP upregulation increases amount of reducing metabolite – NADPH. The levels of NADPH were found to be high in case of deletion mutant as compared to wild type.

TCA cycle:

As the flux through glycolysis is down there is impediment in TCA cycle. Enzyme activities of TCA cycle enzymes like Aconitase, Isocitrate dehydrogense and succinate dehydrogenase were found to be two fold less and the amount of TCA intermediates like alpha keto glutarate and Acetyl co A levels was found to be reduced in Chromomycin deletion mutant**.**

These results so far show that the deletion of biosynthetic cluster of secondary metabolite-chromomycin affects primary metabolism. This suggests a provocative correlation between primary and secondary metabolism. Restructuring primary metabolism does affect secondary metabolite production; however the converse seems to be the case in the Chromomycin non producer mutant of *Streptomyces flaviscleroticus.*

(4) Chromomycin overproducer mutant:

During course of our experiments we found a mutant strain, #OP of *Streptomyces flaviscleroticus* which overproduces Chromomycin.

(a) Phenotypes of Overproducer mutant and Quantitation of antibiotic chromomycin produced by #OP as compared to wild type

The overproducer mutant has all contrasting phenotypes

(1)Profuse Sporulation,

(2) Increased growth rate on solid media and

(3) Extended viability as compared to antibiotic deletion mutant

(4) Uselected apramycin resistance

Antibiotic Chromomycin was extracted and quantitated from wild type and #OP, the densitometric analysis of TLC shows that #OP synthesizes 20 fold more antibiotic - chromomycin as compared to wild type

(b) Levels of expression of transcriptional regulator of Chromomycin i.e. activator and repressor in #OP and wild type

The RT-PCR studies shows that expression levels of activator (*cmmRI*) is higher than repressor (*cmmRII*) in #OP as compared to wild type, 16srRNA was used as internal control for the study.

(c) Antibiotic resistance and cross resistance pattern of #OP versus Wild type:

It was observed that the overproducer mutant is resistant to very high levels of apramycin unselected, and shows cross-resistance to other aminoglycosides like kanamycin, geneticin, gentamycin, and tobramycin but not to amikacin, and neomycin and paramomycin.

(d) Trace apramycin resistance mutation in #OP

Till to date, mutants resistant to antibiotics like Streptomycin, Gentamycin, Rifampicin, Geneticin, paromomycin, fusidic acid, lincomycin, and thiostrepton are reported to cause overproduction. Here we describe isolation of a new mutation to apramycin resistance leading to

Chromomycin overproduction. Moreover, atypical features like no change in growth rate and increased viability is also observed for the mutant. This is in contrast to the finding that antibiotic resistant mutation is generally associated with compromised growth rate. We intend to characterize and evaluate genetic engineering potential of the mutation causing chromomycin overproduction in #OP by uncovering the mechanism of novel apramycin resistant mutation. Further we want to explore its industrial applicability.

In order to check whether *aac3(IV)* responsible for apramycin resistance generally found in the bifunctional plasmids of *Streptomyces* is responsible for apramycin resistance and leading to chromomycin overproduction, the plasmid pSET152 harboring *aac3(IV)* gene was transformed in wild type by conjugation. The trasformants didn't overproduce Chromomycin ruling out the possibility that *aac3(IV)* gene is responsible for Chromomycin overproduction in #OP. The apramycin mutation described here is unlikely to be defining any of the three mechanisms; a) rRNA methylase; b) Efflux function; c) rRNA mutations. Each of the three cannot effectively explain one step high level resistance to apramycin and faster growth of overproducer mutant.

Initially it was thought that mutation in ribosomal protein can account for improved growth and one step resistance. When the whole genome sequencing project for #OP was completed, the S12 protein (the target for apramycin) was matched at protein level to its nearest sequenced organisms and there was 100% similarity in the sequence indicating that ribosomal protein mutation is not the case for apramycin resistance.

The only possibility for the mutation is apramycin modification. In order to locate mutation genomic library construction has been attempted.

(d) Genomic DNA library construction:

In order to clone the apramycin resistance mutation, the genomic DNA library construction has been attempted for #OP in a bifunctional episomal vector pWHM3 (thiostreptone resistance). The library pool representing ~50,000 colonies with average insert size of 3 kb was transformed in DH5α. The DNA of the library was transformed further in non methylating host of *E.coli* ET12567, as the *Streptomyces flavisclerotic*us possesses restriction modification system. The library hence prepared has been used to transform *Streptomyces (*wild type). The scoring for apramycin resistant colony in wild type transformats is under process.

(e) De novo whole genome sequencing of #OP:

In order to know the genetics of this organism, we attempted whole genome sequencing by next generation de novo - whole genome sequencing project with a 'genome"IT" company – Bionivid' using Illumina NextSeq. The sequence is being assembled to submit the draft genome sequence to NCBI.

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