NUCLEOTIDE SEQUENCING AND FUNCTIONAL ANALYSIS OF SOME GENES IN PKS CLUSTER OF *S. flavisclerotícus* AND STUDIES ON MODE OF ACTION OF THE POLYKETIDE.

SUMMARY

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Introduction

Polyketides are remarkable class of natural products. In addition to an enormous range of structural and functional diversity, they also exhibit a wide range of biological diversity applicable to agrochemistry and pharma industry. This made the scientists world over to carry out the research aimed at molecular understanding of their biosynthesis. As a family the PKS is presumed to have evolved from the fatty acid synthetases and thus, the two are related at a mechanistic and architectural level. Despite their similarity with the FAS, a more or less systemic approach has been taken to classify the different types of PKSs based on their source or products as well as biochemical and genetic data. Two major type of PKSs have been identified form the microorganisms with characteristic architectural differences. Type I PKSs have multiple active sites on each Polypeptide responsible for producing complex polyketides like erythromycin and Type II contains the single active site on each polypeptide responsible for producing aromatic polyketide like Daunomycin.

The use of genetic engineering turned out to be very handy in understanding the molecular mechanism involved in the polyketide biosynthesis. One such organism *S. coelicolor* whose genetics is well studied is used as a reference to target new polyketide producing organism. The well understood genetics of this organism also helped in decoding the mechanism of the pathways involved in the biosynthesis of polyketides from putative polyketide producing strains. The DNA sequencing information of some of the polyketide producing strains helped in getting the insight of the polyketide synthesizing machinery. The information was used in not only exploring the potential of an organism to produce the compound but also helped in generation of "unnatural natural compounds" giving rise to hybrid antibiotics. Granitorrhodin is one such example.

The present work is an attempt of studying vaious aspects involved in new drug discovery namely, Product - the bioactive polyketide molecule (Chapter -3 and Chapter- 4), Producer – *S. flavisclerotícus* (Chapter- 5), and Production Machinery – Polyketide Synthase Cluster (Chapter-6 and Chapter- 7).

In Chapter-3, **activity of the polyketide** was tested for spectrum, minimal inhibitory concentration, growth curve, post-antibiotic effects and against pathological isolates from human samples. **Mode of action** of the antibiotic was cracked fluorimetrically by exploiting the self-fluorescing nature of the molecule and was found to be DNA binding. **Mode of resistance** acquired against this molecule was studied in adaptive mutants of *M. luteus* were studied in Chapter- 4. DPH probe mediated membrane fluidity changes, pigment overproduction studies, multidrug resistance and efflux essay were done. Cloning and expression of genomic DNA library of this mutant in E. coli was attempted. SDS-PAGE of the mutant against wild-type was carried out.

Chemical characterization of the molecule (by a colleague) later revealed the polyketide to be Chromomycin A3, a well characterized drug obtained from *S. griseus* sub. *griseus.* Based on these results and known ambiguity of *Streptomyces* classification, a comparative **Morphological, Cultural, Biochemical** and **Molecular taxonomy** (16S-rDNA and KS gene) studies were done to ascertain that the two producers are different, if not the product. The results of the study are compiled and analyzed in Chapter-5.

Different **Sequencing strategies** including transposon mediated sequencing, sub-cloning and primer walking were employed for ~40 kb PKS gene cluster library. **Sequence analysis** of raw data was done to find ORFs, conserved domains and designate putative proteins, using computational tools available on web. **Functional analysis** of genes, namely *sfr*A, *sfr*B and *sfr*X involved in imparting self-resistance was studied in heterologous host. Functional analysis of a key regulatory gene (*sfl*RI) along its promoter (*act*Rp), presumed to be involved in activation of chromomycin biosynthesis, was also attempted.

Activity and mode of action of the polyketide:

The molecule, which is a product of type II polyketide synthase gene cluster, is aromatic in nature. The secondary metabolites from *S. flavisclerotic*us have been tested for its antimicrobial activity over a range of microorganisms. The crude extract has been found to be effective on broad range of microbes like, gram-positive and gram-negative bacteria and fungi.

Like many other polyketide antibiotics, the pure polyketide demonstrated effect against gram positive (*Micrococcus luteus, Bacillus subtilis, Staphylococcus aureus*) but could not inhibit growth of gram-negative bacteria (*E. coil, Salmonella typhimurium*) and did not exhibit any effect when tested against fungi (*Saccharomyces. cerevisiae, Aspergillus niger*). Usually the lipopolysaccharide layer of gram-negative bacteria forms a physical barrier and curtails the entry of the bulky molecules in the cell, but studies using Tris & EDTA treatment as well as LPS deficient mutant suggest it not to be so. Minimal inhibitory concentration as determined by agar well and broth assay methods show its effects at nanogram concentration. Growth curve studies propose the molecule to be bactericidal in nature. The concentration and time dependent effects as well as post antibiotic effect with respect to *S. aureus* have also been determined.

The activity studies on various pathogenic strains of clinical importance isolated from various human sources shows encouraging results and the molecule has been found to be active on several isolates showing multidrug resistance.

The polyketide acts on active cells to cause total lethality at nanogram concentration. Usually antibiotics act only on actively growing cells and do not show any effect on stationary phase cultures. The polyketide when studied on induced stationary phase cultures could exhibit potential bactericidal effect. The result was suggestive of the target being a very vital that can be acted upon even under not actively dividing conditions. The probable candidate targets hypothesized were membrane or nucleic acid. A change in membrane fluidity of cells on addition of the polyketide was studied, by adopting method used to study

mitochondrial membrane fluidity, using DPH fluorescence probe. No significant change was detected.

The self-fluorescing nature of the polyketide was exploited to check for nucleic acid binding assay. The molecule, in presence of DNA and a divalent cation, when excited at 470nm, showed a marked increase in fluorescence confirming the DNA binding nature of the polyketide. Inability of the same to bind, when RNA was used as source of nucleic acid suggests the binding to be very specific and not intercalating type.

Mechanism of adaptive resistance to chromomycin in *M. luteus*:

Acquiring resistance against antibiotics is a common phenomenon and microbes achieve it in different ways. The expected frequency of obtaining resistant mutant is between 10^{-7} and 10^{-8}. *M. luteus* cultures failed to develop resistant mutant even at 10^{-11} when grown in presence of chromomycin. This result and DNA binding mode of action is suggestive that point mutation(s) cannot confer resistance against chromomycin.

Adaptive mutants were developed that could resist supralethal concentrations of chromomycin. The mutants were found to be overproducing carotenoid. Its possible role in imparting resistance was studied using carotenoid inhibitor. A notable difference in membrane fluidity of wild type and the resistant mutant was detected, which can be attributed to elevated carotenoid levels. The mutant could also confer cross-resistance to other antimicrobials namely Daunomycin, Tetracycline and Erythromycin. The adaptive mutants generated against each of them conferred cross-resistance to same group, confirming the cross-resistance not to be chance event. The resistant mutant in presence of any of the above mentioned antimicrobials turned sensitive on addition of reserpine, an inhibitor of efflux pump, to it. This is suggestive of the role of efflux pump to be involved in imparting resistance.

At gene level, the revertant frequency of the mutant is suggestive of gene amplification occurring in the resistant mutant. To clone the gene for resistance the library of genomic DNA from resistant mutant was generated in pBlueScriptKS and the *E. coli* transformants were screened for the phenotype against erythromycin and tetracycline, but no positive clone could be achieved.

SDS-PAGE studies of resistant mutant suggested two highly overexpressing protein bands of nearly 40KDa and 50KDa sizes, when compared to wild type. It also showed a dose dependent induction of these bands.

Comparative taxonomy:

The similarity of region of cluster sequenced earlier suggested a very high similarity with *S. griseus*. Later, the structural determination revealed it to be the same molecule (chromomycin) produced by *S. griseus. Streptomyces* classification has been very wobbling and several genera and species have been reclassified from time to time. Thus, it became very important to check the relatedness of the two chromomycin producers that has very high conservation in their PKS clusters.

The morphology and phenotypic characters of *S. flavisclerotícus* and *S. griseus* sub. *griseus* have been studied by growing them on various media and their tolerance against salt stress and varied pH conditions. Comparative studies of secondary metabolites by thin layer chromatography and spectrophotometry using the crude extracts of both species obtained from cultures grown on different media, have been recorded.

The conserved 16S r-DNA region, which is used in molecular taxonomy for studies at specific levels, was amplified from the genomic DNA using universal primers. The amplicon was cloned in pBlueScriptKS and sequencing for both the species were carried out using T3 and T7 universal primers. The sequencing reports have been analyzed using BLAST2SEQUENCES software.

From the morphological, cultural, biochemical and molecular studies it can be conclusively said that the two chromomycin producing species namely, *Streptomyces flavisclerotic*us and *Streptomyces griseus* significantly differ from each other owing to the differences observed in several aspects under study.

Nucelotide sequence analysis of chromomycin synthase cluster:

In earlier work the physical map of the PKS library was generated with respect to EcoR1 and BamH1. The 40kb cluster was divided into 5kb, 11kb, 4kb, 8kb and 17kb with respect to EcoR1. Of these 5kb and 8kb fragments were already sequenced.

Subcloning and primer walking strategy was used for 4kb, 8kb and 17kb fragments. For 11kb fragment, transposon (γδ) mediated sequencing strategy was employed. This was done with background information from end sequencing results of 5kb fragment suggesting absence of any PKS related gene, which ensured that the cluster ends in 11kb fragment.

Sequence alignment and BLAST analysis showed presence of 26 complete ORFs having high homology to PKS proteins from other clusters. These genes are organized in seven operons. Gene organization and orientation matches exactly to the Chromomycin cluster in *S. griseus* sub. *griseus*. A fresh attempt has been made to explain organization of genes in different operons, taking into consideration temporal requirement of proteins in molecule formation and maturation. Genes have been discussed, based on their putative role in biosynthesis, regulation or resistance.

Interestingly a transposon sequences has been detected in 17kb fragment which is located at a very strategic position. The truncated ORF of this transposon showed significant homology to ISSav4. There is no such transposon sequence present in Chromomycin cluster of *S. griseus* sub *griseus.* Albeit, this sequence does not have any direct role in chromomycin biosynthesis but might provide some evolutionary link.

Functional Analysis of regulatory gene:

Based on sequence analysis, three ORFs were assigned putative functions of resistance. Homology of SrfA and SfrB associates it with heterodimeric drug extrusion pump and SfrX is putative DNA repair protein. These genes were cloned and expressed in heterologous host (*S. lividans*) and their resistance determination in presence of chromomycin was conducted. SfrAB was found to resist chromomycin better as compared to SfrX, and therefore appears to be major resistance determinant, whereas SfrX appears to be playing supportive role. Preliminary results of earlier work in the lab suggesting, an non-polyketide gene from 11kb fragment, having role in resistance was ruled out in the present study.

Functional analysis of putative regulatory protein (SflRI) of SARP family was also attempted. Using computational tools, the promoter (actRp) for this protein was determined. It was found to be a complex region with direct and inverted repeats and another promoter on opposite strand. A transcriptional fusion with EGFP and subsequent expression studies in native and heterologous host was carried out. Thiostrepton mediated induction of tipAp-EGFP fusion was used to plot a standard graph. In experimental set, it was found that actRp was active in heterologous host, as marked by fluorescence, but not in native host. Use of different media, temperature and osmolarity also did not alter the results. This was indicative of a complex regulation being involved in chromomycin biosynthesis.

For functional analysis of sflRI, a modified strategy involving gene disruption and subsequent complementation in a single step was carried out. Though the strategy appeared promising, results could not be achieved due to poor transformation efficiency of *S. flaviscerloticus.*