

INTRODUCTION

A large number of pathogenic bacteria have become resistant to antibiotics in common use. This antibacterial resistance is presently an urgent focus of research and new antibiotics are necessary to combat these pathogens. Filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important microorganisms because of their ability to produce many kinds of secondary metabolites such as antibiotics (Williams et.al, 1983). Indeed, the Gram-positive *Streptomyces* species produce about 75% of commercially and medically useful antibiotics. In the course of screening for new antibiotics, several research studies are currently oriented towards isolation of new *Streptomyces* species from different soil and water samples. The genus *Streptomyces*, aerobic and spore-forming *actinomycetes*, possess DNA with a high guanine-plus-cytosine content and form extensive branching substrates and aerial mycelium.

Also discovery and development of new drugs by multidisciplinary science is a major source of new bioactive compounds. In this regard again systematic search for new compounds from natural source is a major supply of new antibiotic structure. An alternative to new compound from natural isolate is combinatorial biosynthesis. Construction of genetically engineered organisms, though new as compared to other conventional approaches, promises novel metabolites. Polyketides are a class of secondary metabolites, produced by *Streptomyces*, it has a huge range of structures, some of which are used as medical or veterinary agents. For example, erythromycin and tetracyclines are used as antibiotics, daunorubicin is used as an anti-cancer agent, and avermectin is used as an anti-helminthic. In fact 70% of antibiotics which are in use today are produced by *Streptomyces* and $\frac{2}{3}^{\text{rd}}$ of them are polyketide in nature. In view of the fact that major amount of drugs are sourced from *Streptomyces*, engineering gene cluster encoding bioactive compounds in these organism was a logical and rational thought. To

add to the advantage the genes encoding both typeI and typeII polyketides (described in detail in Review of literature), are conserved among *Streptomyces*.

A good amount of genetic engineering work was done using *S. coelicolor*. Heterologous expression of actinorhodin, a typeII polyketide, from *S. coelicolor* was done in *Streptomyces* sp AM-7161, producer of mederamicin and in *S. violaceoruber*, producer of granaticin. In both the cases the heterologous host produced actinorhodin and hybrid molecules apart from their endogenous antibiotic (Hopwood et.al., 1986). With these studies, genetic toolkit for the manipulation of *Streptomyces* was reasonably well developed and polyketide and oligopeptide antibiotics were being manipulated and heterologous organisms carrying recombinant genes producing novel compounds were being developed.

The biochemical pathway of quite a few polyketides like actinorhodin, tetracenomycin, oxytetracycline, etc. was characterized and entire set of biosynthetic genes was cloned and sequenced. Each type II PKS consists of several discrete polypeptides which resemble the subunits of fatty acid synthase (FAS) of *E. coli* and plants. This system also forms noncovalent association, where same catalytic centers are repeatedly used for all intermediates in the assembly of polyketide chain.

High degree of conservation among PKS reflects an evolutionary relationship among specific differences in the starter unit, chain length and pattern of reduction of its product. The molecular basis of PKS programming has been studied using two approaches. The first involves the construction of cell free system, in part involving the expression of individual components of PKS in *E. coli* or in their native hosts.

The second approach involves use of different combinations of PKS genes to generate hybrid PKS and then analysis of their metabolic capabilities. These approaches led to the identification of role of CLF, aspects of control of product structures exerted by KR, identification of factors that specify polyketide starter unit and control of cyclization. It has potential for further analysis of programming.

The work of the thesis was to further explore the combinatorial potential of type II aromatic PKS. The choice of type II PKS genes was two fold. First the PKS II genes were relatively smaller in size (~30-40kb) unlike type I PKS genes, which routinely span ~ 90-100 Kb and thus manipulation of small size DNA was expected to be less difficult in terms of resources required. Paraphernalia of strain/vectors required to begin work on PKS I genes, cloning often requires high capacity cloning vectors. The choice of the strain used in the study was based on the chromosome of the organism containing genes of type II polyketide and lack of literature on the chemical potential of this organism.

Second the state of the art of literature on type II PKS genes was more impressive when compared to type I PKS genes. When this work was undertaken only one compound erythromycin was characterized in type I group as contrast to genes for actinorhodin, tetracenomycin, daunamycin in case of type II PKS.

For the analysis of nature of PKS programming, number of available aromatic PKS gene sets needs to be expanded, that dictate the synthesis of more structurally diverse polyketides. The PKS cluster from *S. flaviscleroticus*, the organism of the proposed study carried out and presented in this thesis, would be an important addition to the set. Characterization of a new cluster would help in expanding the combinatorial potential of the PKS genes for production of new compounds.

S. flaviscleroticus, a member of genus *Streptomyces* was selected for the present study on the basis of following reasons.

1. Its genomic DNA hybridizes to *actI* DNA from *S. coelicolor* which encodes ketosynthase and chain length function, the most conserved region among different type II polyketide producers, indicating that this organism could be potential producer of polyketide.
2. The organism produces yellow diffusible color on growth media (Fig.II), and the organic solvent extract of the organism is bioactive against gram positive bacteria and fungi. The extract when resolved on TLC, shows number of fluorescent and

nonfluorescent spots, bioautogram of this TLC developed using *M. luteus* as test organism showed five distinct zones of inhibition (Fig. I2).

3. There was no information in the literature available regarding its potential to produce bioactive compound indicating that the cluster could be a new one.

Work done in the lab:

For the characterization of the cluster, genomic DNA library was constructed in the *E. coli*-*Streptomyces* shuttle cosmid vector, pKC505. The library of 1000 clones was screened with *actI* probe in colony hybridization experiment. A set of eight cosmid clones, which hybridized to the probe, were picked up as putative carrier of the PKS cluster. Each clone carried ~ 30 kb insert DNA. Southern hybridization of these eight cosmid clones, digested with different restriction enzymes and probed with *actI* probe indicated that *actI* hybridizing fragment in each RE digestion was same suggesting that there is only one set of PKS genes and the set of *act* hybridizing clones contain overlapping DNA. Insert in one of the clone, # MC6 was shown to be colinear with that of chromosomal DNA, suggesting that there was no cloning artifact.

In the context of the above information, the present work was proposed for the characterization of the cluster. For this the plan of work included assessing contribution of the PKS cluster in the bioactivity of the organism, determining the structure of the polyketide if produced and heterologous expression of the putative clones carrying the PKS cluster.

Reiterating the objectives of the present study:

1. Standardization of transformation and conjugation condition for *S. flaviscleroticus*.
2. Construction of knock-out mutant of *S. flaviscleroticus* using gene disruption strategy, to assess the role of Polyketide Synthase (PKS) gene for the 'bioactivity' spectrum of this organism.
3. Expression of polyketide synthase genes of *S. flaviscleroticus* in heterologous hosts and characterization of 'bioactive' compounds of the recombinant strains.

4. Characterization of the aromatic polyketide produced by *S flaviscleroticus*.

The work elements of the thesis were organized into three chapters. The results pertaining to first objectives are described in the first chapter which includes standardization of transformation and conjugation conditions for the new species of *Streptomyces*, *S flaviscleroticus*.

The second and third chapter describes respectively, testing the genetic potential of *S. flaviscleroticus* for polyketide production by gene disruption and heterologous expression studies. Also work on characterization of polyketide produced by *S flaviscleroticus* was carried out in the fourth chapter.



Fig.I1: Morphological features and diffusible color produced by *S. flaviscleroticus* grown for 3 days (left) and 7 days (right).

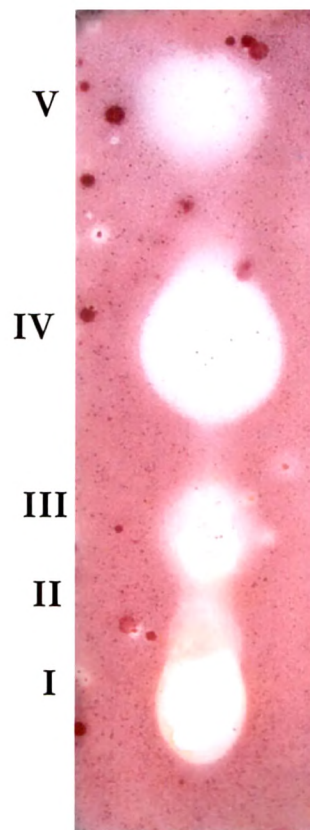


Fig.I2: Bioautogram of *S. flaviscleroticus* extract developed by using *Micrococcus luteus* as the test organism.