Chapter 4
Fucntional analysis of tailoring enzymes

Introduction:

Polyketides form a very versatile group of antibiotics. Many natural products from this family require post-polyketide synthase modifications including glycosylation, alkylation and oxidation/reduction to be fully active (Rix et.al., 2002). The attachment of the saccharide moieties to the macrocyclic and aromatic backbone adds up to the versatility and potency of these molecules, changes in the nature and extent of the sugar decoration alters the activity or even the specificity of these antimicrobial agents (Fu, et.al., 2003). Glycan alteration is one of the most common resistance mechanisms in the intracellular inactivation of polyketide antibiotics and is used by the Gram-positive *Streptomycetes* that produce these molecules as a protective mechanism from the action of their endogenous antibiotics (Quiros et. al., 1998). Moreover, it has been suggested that for macrolide antibiotics, in particular, glycosylation state may have an impact on resistance induction (Douthwaite, 2001). A fuller understanding of these resistance processes is thus vital.

Furthermore, antibiotic glycan alteration or iteration (so-called glycorandomization), which includes decoration of antibiotics with non-natural sugar variants, is also a potentially powerful strategy in combating emerging bacterial resistance (Barton et. al., 2001; Barton et. al., 2002; Fu, et.al., 2003). For example, the aminosugar desosamine plays a critical role in the activities of the antibiotic like Pikromycin, oleandomycin, tylosin and erythormycin. It promotes the ribosomal binding through a combination of hydrogen bonding and electrostatic interaction (Tu et.al., 2005). Similarly, in doxorubicin they aid in the binding of the molecule to the minor groove of DNA (Méndez and Salas, 2001). The biosynthesis of Thymidine Diphosphate desosamine requires the enzymes, TDP-D-glucose synthetase and TDP-glucose-4,6-dehydratase (He and Liu 2002).

The sequencing of 8.0 kb *EcoR*I DNA and its analysis indicated the presence of the complex sugar modifying genes like TDP-glucose-4,6-dehydratase and sugar O-methyltransferase along with the ketosynthase (KSα) and chain length factor (KSβ). This particular cluster showed a different kind of structural organization than the other aromatic polyketide cluster in terms of absence of Acyl carrier protein in the same region

as that for minimal PKS, a feature common to most of the aromatic PKS genes, however with the exception of the daunomycin cluster. Moreover, the cluster also showed the transcriptional coupling between the TDP-glucose-4,6-dehydratase and Ketosynthase which is a rarely observed phenomenon among the aromatic polyketide clusters (See Chapter 3 for details).

TDP-glucose-4,6-dehydratase is involved in the dehydratation of the TDP -D- glucose once it's synthesised from D- glucose -Phosphate to TDP-D-glucose with the help of the enzyme TDP-D-glucose synthetase during the synthesis of the deoxy sugars like desosamine (He and Liu 2002). Similarly the O-methyltransferase is the enzyme responsible to carry out methylation in the glycan molecule; it can methylate O, N and C atoms of the deoxysugar moiety. This enzyme is SAM (S-adenosyl methionine) dependent involved in the methylation of the deoxysugar L-Rhamnose in the Elloramycin biosynthesis (Patallo et.al., 2001) and Mithramycin Biosynthesis (Salas et. al., 2000).

In the work described in this chapter we have constructed the disruption and deletion mutant for the TDP-glucose-4,6-dehydratase gene, and disruption mutant for the O-methyltransferase. The disruption and deletion of these genes were done with an intention to assess the function of each of the genes and generate the derivative of the biomolecule to compare it to the wild type product in the latter case. This would give us an insight in to understanding the role of these genes in polyketide synthesis in *S. flaviscleroticus*.

4.1 General strategy used for construction of a gene deletion mutant

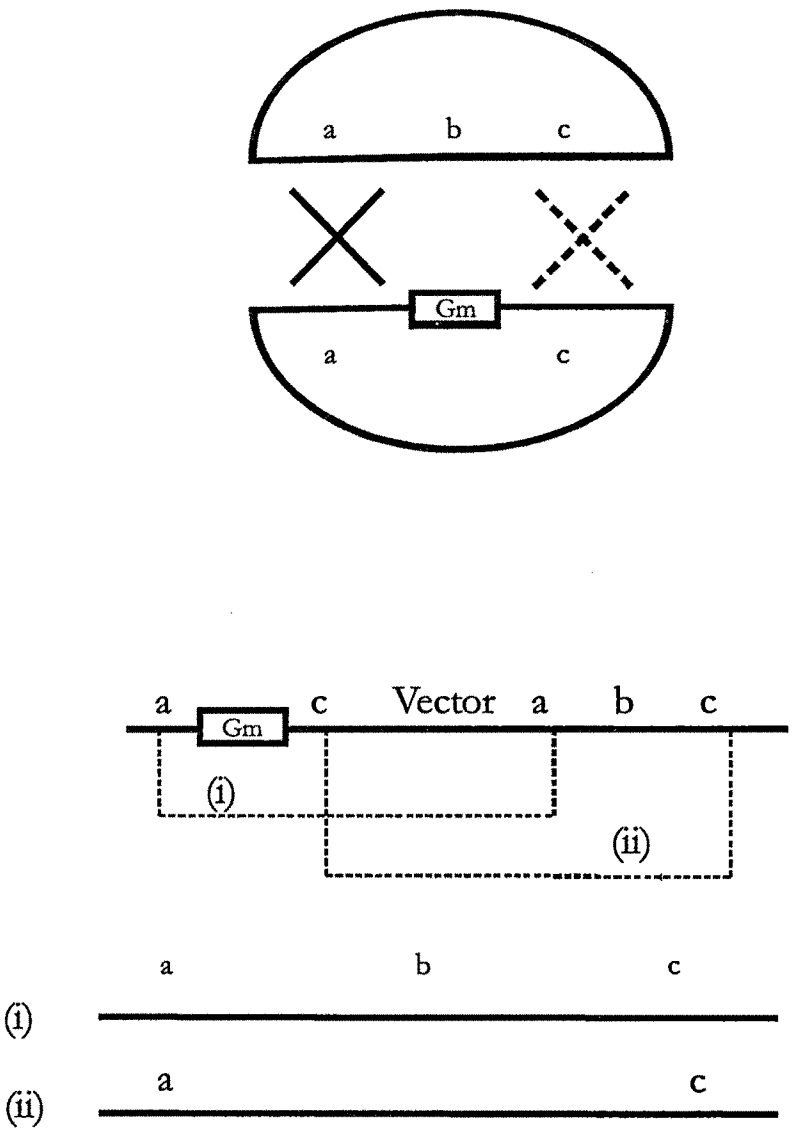


Figure 4.1.1: Schematic representation of homology dependent two-crossover recombination event that causes plasmid borne deletion of a gene to cross into the chromosome of wild type.

The strategy shown above is used for the deletion of the gene of an organism. In the above diagram. a, b and c are the three genes in the chromosome of which 'b' is the gene

is to be deleted. To achieve that, the DNAs flanking the 'b' gene were cloned into a suicide vector with an antibiotic marker. The DNA was introduced in the WT organism either by conjugation or transformation. The crossover can take place from the either side owing to the homology of the genes allowing the vector to insert in the chromosomal DNA by homologous recombination. The resultant strain will now have two copies of the genes flanking the 'to be' deleted gene 'b' along with the single copy of the vector. The insertion mutants were selected on plates supplemented with the antibiotic which is present as the selection marker on the vector. The second crossover can take place either from (i) or from (ii) again by homologous recombination, as shown in fig 4.1.1. If it takes place from (i) then the resultant strain will have all the three genes intact but the plasmid and plasmid marker will be lost. If the crossover takes place from (ii), it will give rise to the deletion mutant (fig. 4.1.1).

4.2: General strategy used for the disruption of the gene function

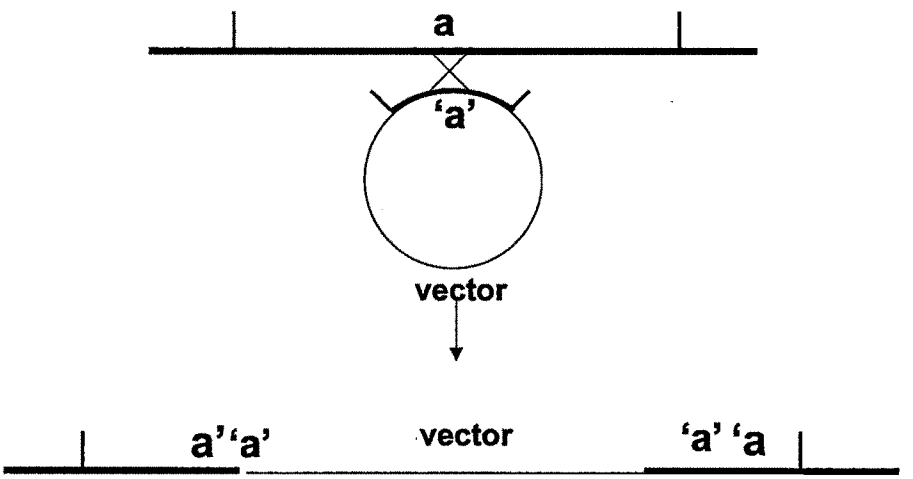


Figure 4.2.1: Homologous recombination between the cloned internal segment `*a*' of the gene a and the corresponding a sequence on the chromosome causes disruption of the gene.

The above diagram (fig. 4.2.1) represents the strategy used for the disruption of a gene in an organism. Here the middle region of the gene to be disrupted 'a' is cloned in to a

vector and then introduced in the WT organism by conjugation or transformation. The disruption of the gene occurs in one step as the single crossover homologous recombination between 'a' on the plasmid and a on the chromosome adds the plasmid sequences within the gene disrupting the integrity of the gene as a consequence. The disruptants are then selected on the same antibiotic for which the vector is carrying the marker gene.

4.3 Results and Discussion:

The sequences of 8.0 kb *Eco*RI fragment revealed that the genes for sugar biosynthesis and polyketide backbone synthesis are transcriptionally coupled. For example unlike in several other polyketide clusters, the TDP-glucose-4,6-dehydratase, the sugar modifying enzyme is in operon with the genes for polyketide condensing enzymes namely Ketosythase α and ketosynthase β. The genes like O-methyltransferase and TDP-glucose-4,6-dehydratase modify the sugar backbone and are known as the tailoring enzymes in the polyketide biosynthesis. In order to understand the role of these genes in the polyketide biosynthesis and their importance in the gene organization in the PKS cluster, we tried disruption of O- methyltransferase gene and disruption as well as deletion of the TDP-glucose-4,6-dehydratase gene.

4.3.1: Deletion of TDP-glucose-4,6-dehydratase gene:

The deletion of TDP-glucose-4,6-dehydratase gene was done using the flanking region of the TDP-glucose-4,6-dehydratase gene. This was achieved by the homologous recombination strategy.

4.3.1.1 Construction of the vector, 2Δ*Bam*HI for mutagenesis:

To cause deletion the gene TDP-glucose-4,6-dehydratase, the DNA flanking the gene was first cloned in to suicide vector. The 4.0 kb *Bgl*II DNA contains the transcriptionally coupled KS and TDP-glucose-4,6-dehydratase open reading frame. The 2Δ*Bam*HI plasmid constructed for the purpose of mutagenesis is derived as follows: The 4.0 kb *Bgl*II fragment was cloned at the *Bam*HI site in the pBR322 plasmid. The resulting Tet^S recombinant was digested with *Bam*HI to remove the 1.7kb and 0.2 kb of *Bam*HI

fragments from the 4.0 kb insert. Nucleotide sequence analysis reveals that the 1.7 kb *Bam*HI DNA contains the TDP-glucose-4,6-dehydratase gene. The DNA homology flanking the 1.7 kb and 0.2kb *Bam*HI DNA deleted from the vector is thus 0.15 kb and 1.9 kb. The 1.7 kb *Hind*III DNA from pGM160 (Muth et. al., 1989) which contains the gene *aac3AI* for resistance for gentamycin was further cloned at the *Hind*III site of the 2Δ*Bam*HI plasmid for neither the Tet^r nor the Amp^r genes present on pBR322, colE1 derivative plasmid of *E. coli*, is expressed in *Streptomyces*.

The construction of the clone has been shown in the figure 4.3.1.1 and the confirmation of whether the Gm^r has been cloned in the 2Δ*Bam*HI clones is shown in figure 4.3.1.2 where the 2Δ*Bam*HI has been digested with the *Hind*III enzyme to release the 1.7 kb Gm^r fragment and 6.5kb of the remaining DNA [(pBR322 4.3 +2.1 kb (4.0-1.7-0.2) of *Bgl*II DNA].

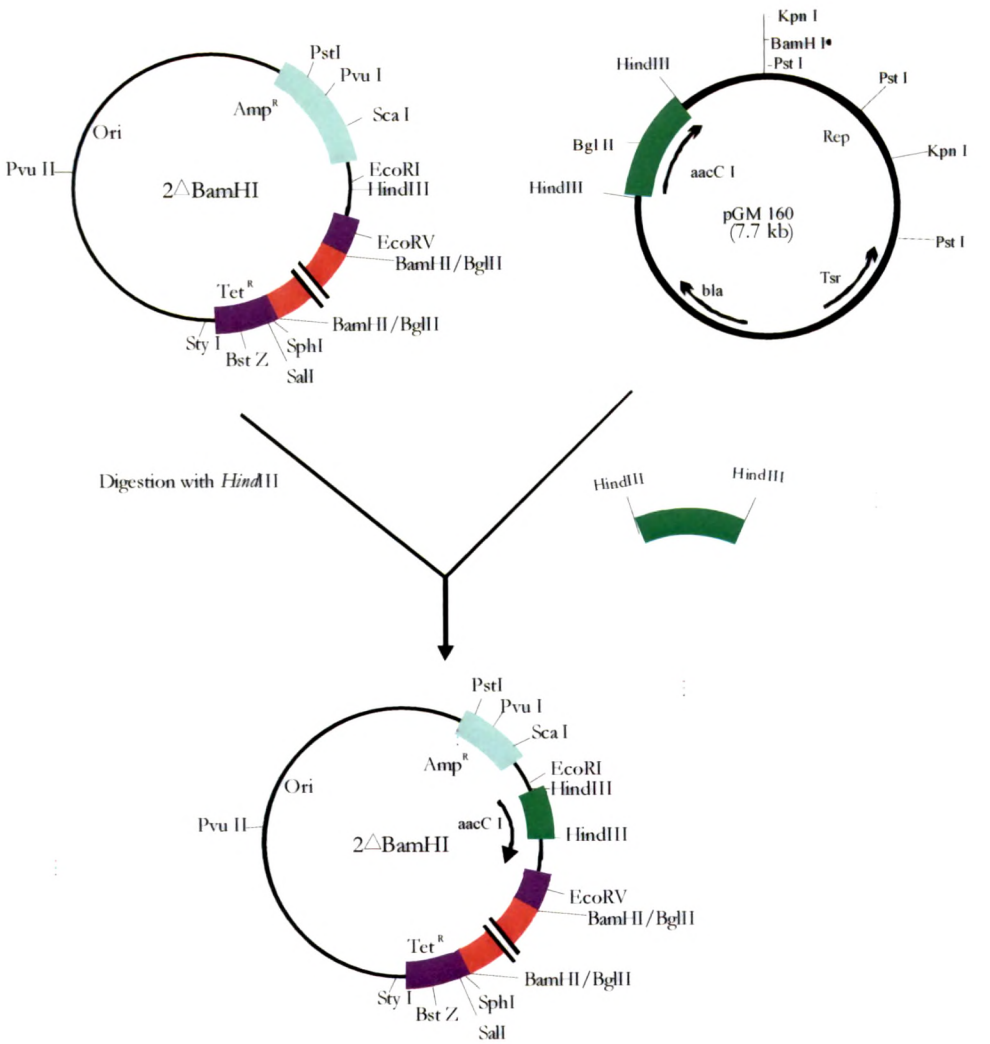


Figure 4.3.1.1: Construction of vector for mutagenesis

Figure 4.3.1.2: *Hind*III digestion of the 2Δ*Bam*HI Gm^r (I)

4.3.1.2:Construction of TDP-glucose-4,6-dehydratase (DH) mutant using homologous recombination strategy

The vector construct, 2Δ*Bam*HI Gm^r, which has *E. coli* replicon functions as suicide replicon for *Streptomyces*, as it cannot be maintained episomally as a plasmid. If a DNA sequence homologous to that in the chromosome is provided in the suicide plasmid, the same can promote homologous recombination with the chromosome and the vector can be now maintained as integrating vector. The first cross over as mentioned in the (Figure 4.3.1.2) allows the integration of the vector backbone on the WT chromosome with one of the regions of homology. We strongly feel that the homology of 1.2 kb must have been responsible for the formation of the integrant.

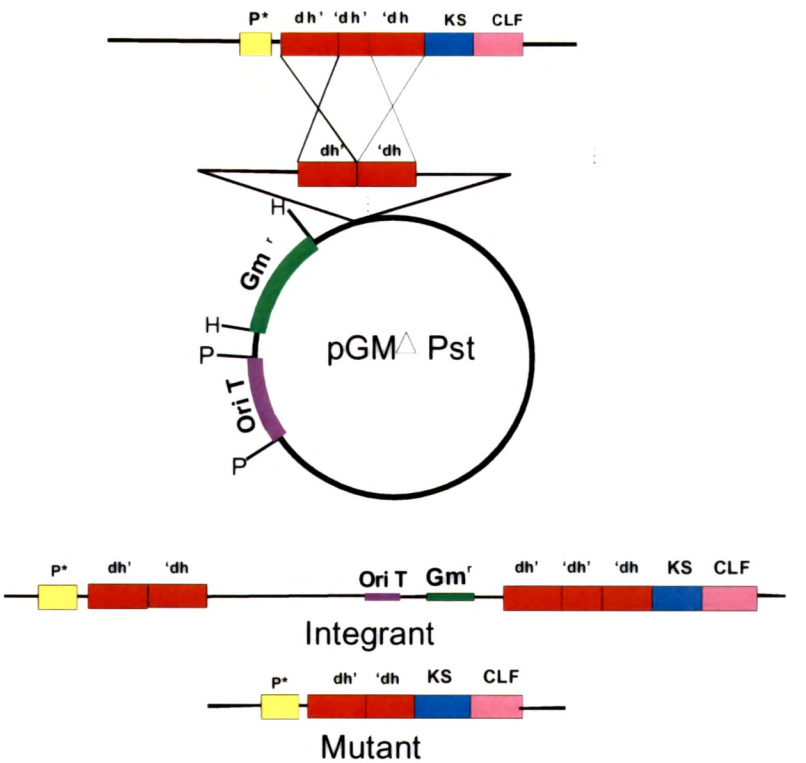


Figure 4.3.1.3: Construction of TDP-glucose-4,6-dehydratase deletion mutant using the homologous recombination strategy.

A total of 12 Gm^r transformants were obtained with about 1 µgm of plasmid DNA. The colonies were amplified on the Gentamycin (Gm) plates. As expected, the phenotype of the transformant was unaffected with the integration of the vector sequences in to the chromosome with respect to the antibiotic production because integrants have two copies for flanking genes as shown in the (Figure: 4.3.1.2) which becomes the subject for the second cross over. The integrants were restreaked on the plates lacking the selection pressure at 30°C to allow the segregants of second cross over to grow. With the removal of the antibiotic pressure, in a small number of cells, the plasmid backbone gets cured as a result either of reversal of the integration (by I) or by (II), using the homology opposite to the one used in integration (Figure 4.3.1.3). The second crossover event gives rise to Gm sensitive mutants which are either DH^+ or DH^-, wild type or mutant for TDP-glucose-4,6-dehydratase gene respectively. The inoculum was amplified in the absence of selection pressure for five subcultures at 30°C. The culture was then diluted 10^6 fold and then plated on 12 plates. Out of around 12000 colonies, the four non pigmented colonies were observed which were also showing the non sporulating phenotype (Figure 4.3.1.4). Of which one of the mutant here is compared with the Gm resistant integrant. The mutant is sensitive to the antibiotic Gm, non sporulating and does not show the production of characteristic *S. flavisclerotícus* yellow pigment.

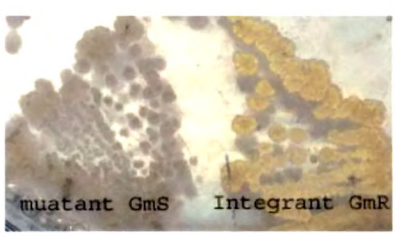


Figure 4.3.1.4: Phenotypic comparison between the Mutant GmS (gentamycin sensitive) and the integrant Gm^r (gentamycin resistant)

An important characteristic shown by this mutant is that it showed a very low vitality. It was becoming unculturable if kept for longer than 8-10 days. The experiments in the lab are on to find out the reason, as same was the case with the 2.19 deletion mutant (Namita Gupta Ph.D. thesis, 2007) in which almost all the essential gene for the polyketide synthesis were deleted. One of the assumptions being made is; impaired synthesis of polyketide antibiotic causes oxidative stress due to gradual reduction of the catalase

activity in stationary phase, hence reducing the ROS scavenging activity leading to the high mortality rate in the mutants.

4.3.1.3: *trans* complementation of the DH⁻ mutant

The 8.0 kb *Eco*RI fragment containing the TDP-glucose-4,6-dehydratase gene cloned in the integrating vector pSET152 and transformed in to the DH⁻ mutant. The comparative pigmentation of the mutant and the transformed mutant is shown in figure 4.3.1.5. The restoration of the pigment production is indicative of the trans complementation of the mutation in TDP-glucose-4,6-dehydratase, as the association between pigment production and antibiotic activity is absolute.

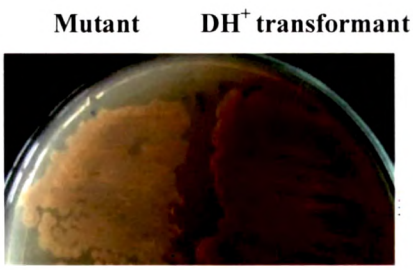


Figure 4.3.1.5: *Trans* complementation of the DH⁻ genotype by the 8.0 kb *Eco*RI DNA.

4.3.1.4.: TLC and HPLC analysis of the culture extract of the deletion mutant

The TLC, bioautogram and HPLC analysis of the mutant extract was carried out. Figure 4.3.1.6 represents the TLC separation of the ethyl acetate crude extract of the WT and the mutant in the 93:7 methanol:chloroform solvent system. Figure 4.3.1.6 A is the comparison of the TLC separation of the WT and mutant crude extract. The WT crude extract shows the TWO yellow spots on the plate, these two spots correspond to the polyketide antibiotic of our interest since these TWO spots (present in the WT) are clearly absent in the mutant's crude extract in the adjacent lane suggesting that the deletion of the TDP-glucose-4,6-dehydratase gene causes the complete impairment in synthesis of the polyketide antibiotic by *S.flavisclerotícus*. The bioautogram of the same plate was carried out by overlaying it with the test organism, the gram positive

Micrococcus luteus (Figure 4.3.1.6 B). The zone of inhibition corresponding to the TWO yellow spots in the WT is clearly seen missing in the mutant implying that the antibiotic production in the mutant is impaired.

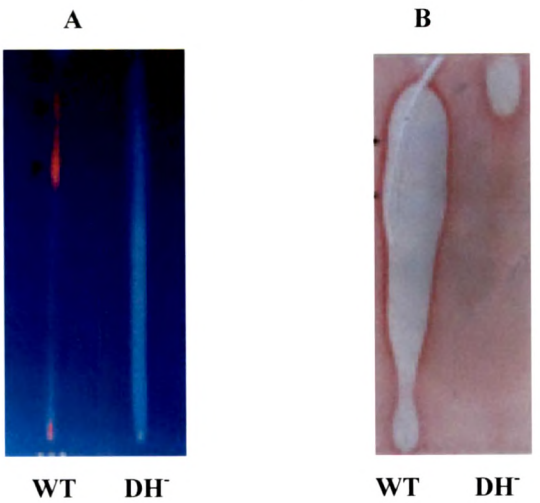


Figure 4.3.1.6: A: Represents the TLC separation of the crude extract of WT and mutant on a flurophore containing plate, B: bioautogram of the TLC developed using *Micrococcus luteus*

The HPLC profile of the crude extract of one of the mutants #3 was compared with the HPLC profile of the WT *S.flaviscleroticus*. The Polyketide antibiotic synthesized by the *S.flaviscleroticus* PKS gene cluster is separated at 22 min and 28 min RT (Figure 4.3.1.7) in the gradient solvent system of Solvent A – H_20 + 0.1% TFA (Tri Fluoro Acetic Acid), Solvent B- ACN (Acetonitrile) +0.1% TFA. The peaks which are visible in the WT at those two time points are clearly absent in the mutant (Figure 4.3.1.8). The HPLC was carried out in the Shimadzu gradient HPLC machine.

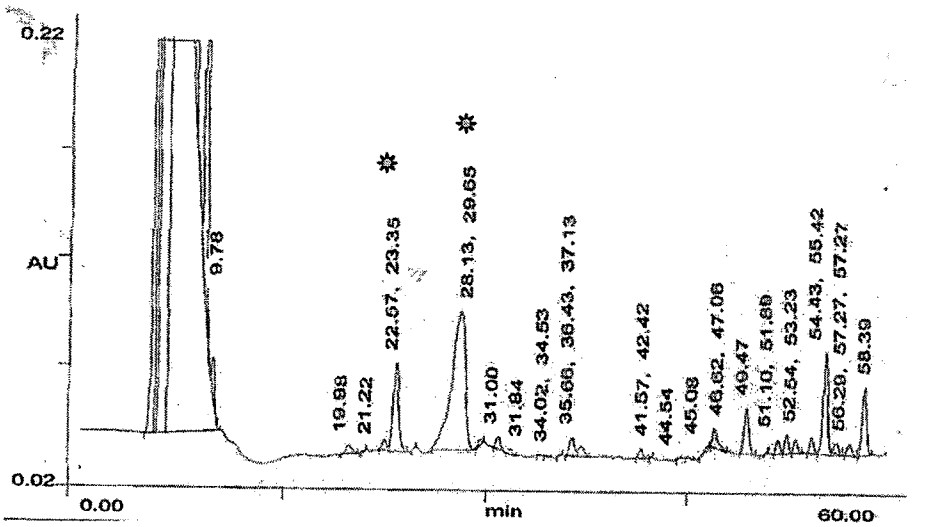


Figure : 4.3.1.7: HPLC profile of WT crude extract

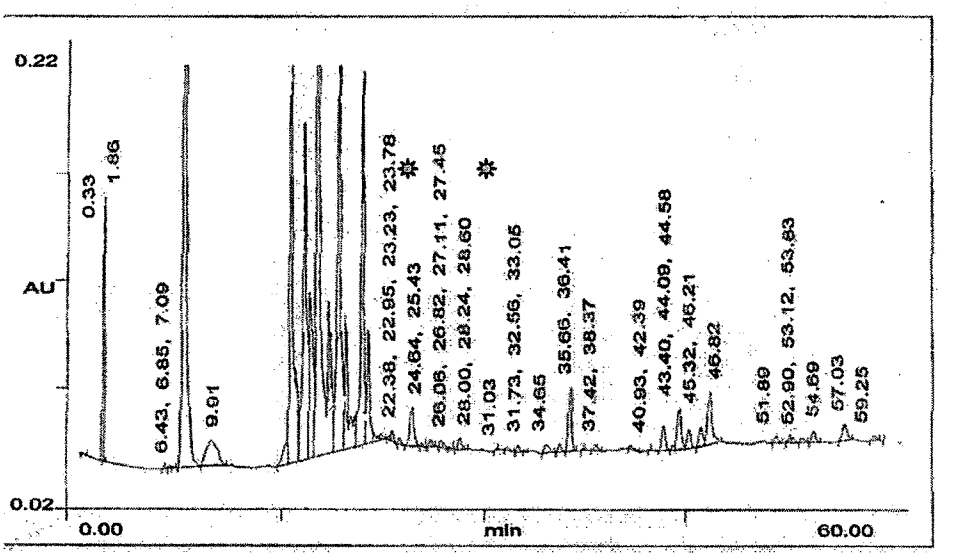


Figure: 4.3.1.8: HPLC profile of the TDP-glucose-4,6-dehydratase deletion mutant

4.3.2: Insertion Mutagenesis of the TDP-glucose-4,6-dehydratase gene:

The insertion mutagenesis of the TDP-glucose-4,6-dehydratase gene is a one step process in which the middle portion of the gene is used as source of the homologous DNA for insertion of a vector in the chromosome in WT organism in order to disrupt the gene.

4.3.2.1: Construction of the vector for insertion mutagenesis

As mentioned in the earlier chapter (Chapter 2) the 0.2 kb *Bam*HI fragment is present between the 2.2 kb and 1.7 kb *Bam*HI fragments in the 8.0 kb *Eco*RI fragments. The 0.2 kb *Bam*HI fragment which comprises of the middle portion of the TDP-glucose-4,6-dehydratase gene was cloned in to the suicide vector pGMΔ*Pst*I, an *E. coli* plasmid with the gene for thiostrepton resistance for selection in *Streptomyces* (Muth et al., 1989) The 0.2 kb gene was cloned in to the *Bgl*II site of the vector (Figure 4.3.2.1). The vector construction was confirmed by digesting it with *Hind*III (Figure 4.3.2.2) in which the vector where the cloning has occurred shows the difference of 0.2 kb (I) in the second band of *aac1* DNA of 1.7 kb as compared to the *Hind*III digestion of the vector alone (II). Transformants of *Streptomyces* strain in this experiment were selected on plates supplemented with thiostrepton.

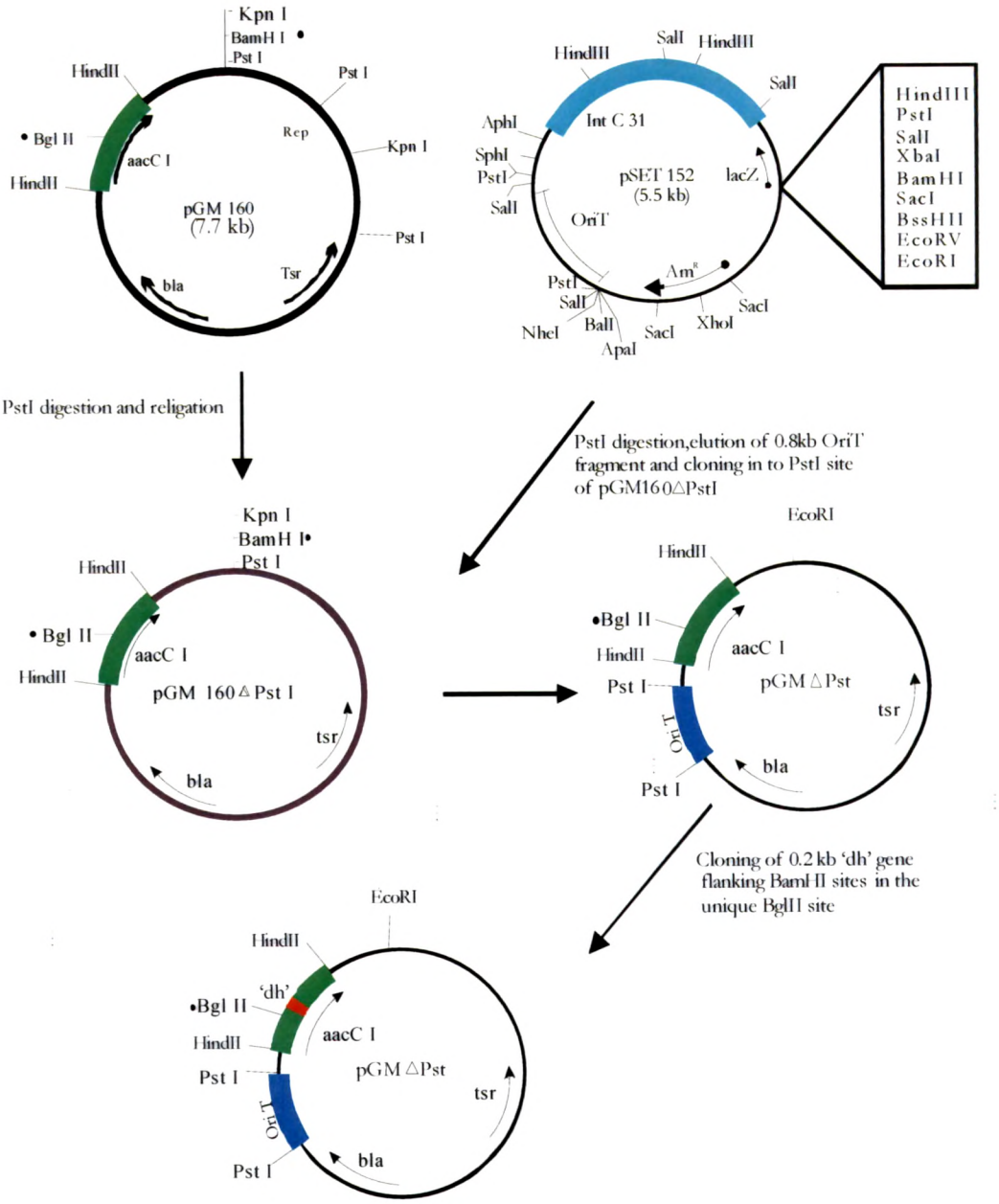


Figure 4.3.2.1: Construction of insertional inactivation vector.

Figure 4.3.2.2: *Hind*III digestion the pGMΔ*Pst*I vector (II) and pGMΔ*Pst*I containing the 0.2 kb *Bam*HI fragment in the unique *Bgl*II.

4.3.2.2: Construction of insertion mutant using homologous recombination strategy

For creation in one step of the disruption mutant, the suicide vector DNA was introduced in the WT *S.flavisclerotícus* by protoplast transformation. About 5 transformants were obtained using 1 μgm of DNA. The formation of the insertion mutant is shown in Figure 4.3.2.3.

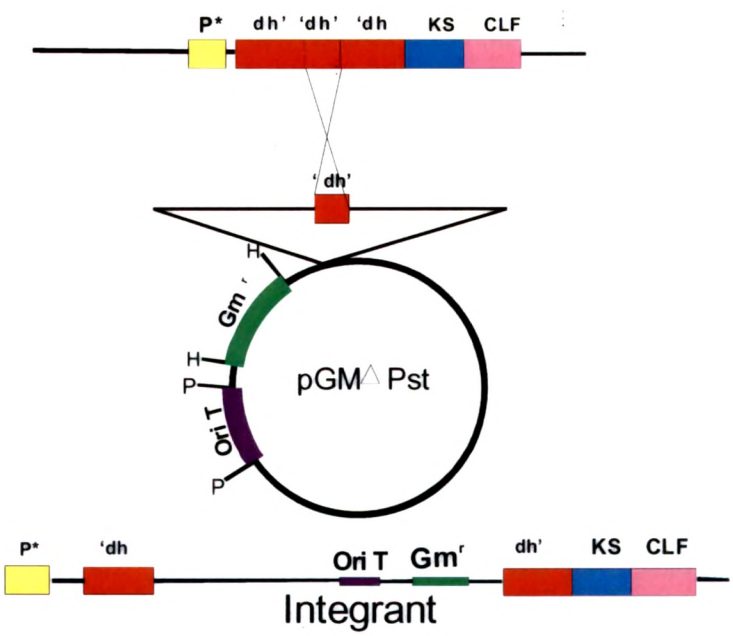
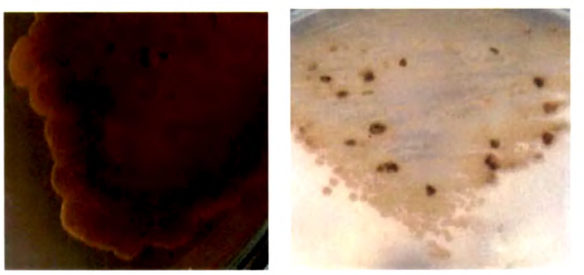


Figure: 4.3.2.3: Strategy used to construct the insertion mutant

4.3.2.3: Phenotypic characteristic of the insertion mutant

The mutant showed absence of yellow diffusing pigment which is seen in WT (Figure 4.3.2.4). It also did not show the intracellular accumulation of the pigments, show poor growth with very low viability, the phenotype that is reminiscent of the DH deletion mutant.



WT **Mutant**

Figure 4.3.2.4: Pigment production by the WT and the DH mutant.

4.3.2.4: Genetic confirmation of the mutant:

Since the mutant showed very distinctive, atypical characteristic phenotype when compared to the WT *S.flavisclerotícus*, its being disruption derivative of WT *S.flavisclerotícus* was confirmed by -

(i) Its ability to grow in the presence of polyketide produced by WT *S.flavisclerotícus*, the extract which otherwise is inhibitory to growth of other *Streptomyces* species.

(ii) The mutant was repeatedly purified on the R2YE medium without selection pressure to obtain revertants which regain the capacity to produce the polyketides as a result of plasmid excision from the genome, by the process of reversal of integration mediated by homologous recombination (Figure 4.3.2.5).

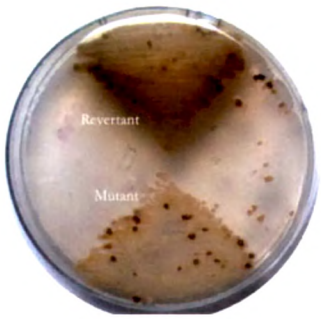


Figure 4.3.2.5: Wild type-like revertant and mutant phenotypes: Repeated streaking of the mutant on R_2YE plate causing the reversal of DH^- phenotype because of plasmid curing

(iii) The mutant was also transformed with the 8.0 kb *Eco*RI fragment containing TDP-glucose-4,6-dehydratase, KS and CLF genes cloned in to the integrating vector pSET 152 to rescue the WT phenotype of the mutant.

4.3.2.5: Bioautogram, TLC and HPLC analysis of the insertion mutant:

The crude extract from the mutant and the WT organism grown on the SM plates for four days at 30°C was prepared by extracting it in the ethyl acetate. Both these extracts were then separated on Thin Layer Chromatography using the solvent system Chloroform: Methanol in the ratio of 97:3. The TLC and bioautogram carried out for both mutant and WT is shown below.

Figure 4.3.2.6 A shows the bioautogram of the DH (TDP-glucose-4,6-dehydratase) mutant and the WT. The crude extract of each of the strain was separated in the TLC plate using the above mentioned solvent system; it was then overlaid with the culture of *Micrococcus luteus* and stained with the dye tetrazolium chloride. The results shown here indicate that the yellow spot(s) present in WT extract and the associated bioactivity is totally absent in the extract of the TDP-glucose-4,6-dehydratase mutant.

The activity shown by *S.flaviscleroticus* PKS cluster is absent in TDP-glucose-4,6-dehydratase mutant as compared to the WT. Figure 4.3.2.7 B is the TLC result of the

partially purified crude extract of TDP-glucose-4,6-dehydratase mutant and the WT strain. The two yellow spots in the WT represent the antibiotic of our interest, which is clearly absent in the TDP-glucose-4,6-dehydratase mutant.

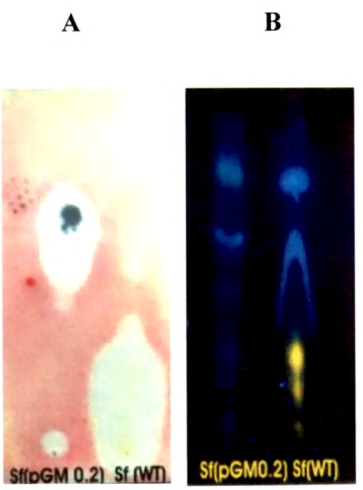


Figure 4.3.2.6: A: Bioautogram of the mutant and WT crude extract developed using *M.luteus*. B : TLC profile of TDP-glucose-4,6-dehydratase insertion mutant and *S. flavisclerotícus* WT.

The HPLC profile of the mutant and the WT crude extract were compared. The run condition for the HPLC are same the one described in the previous section. The results obtained clearly shown the absence 22' and 28' mins peaks in the mutant (Figure 4.3.2.7) as compared to the WT (Figure 4.3.1.7).

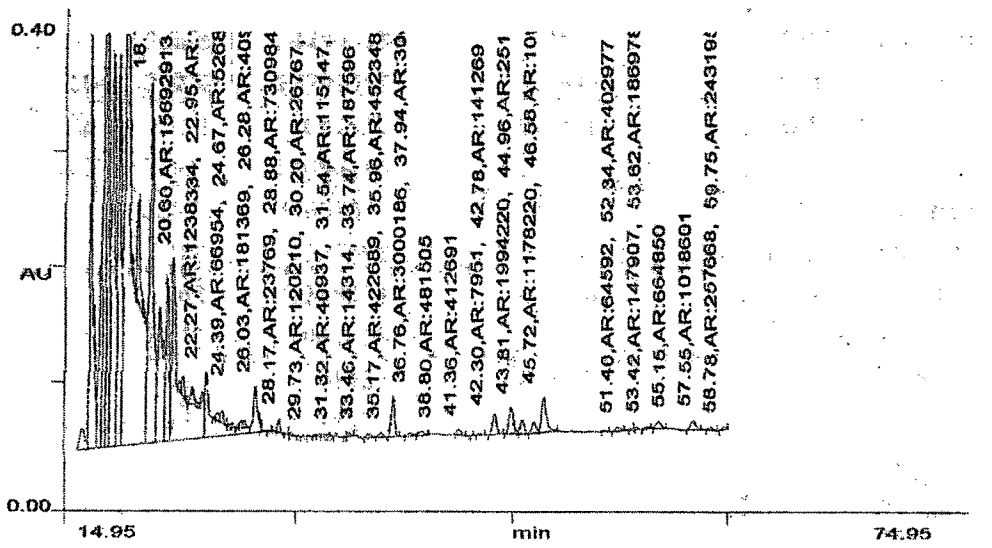


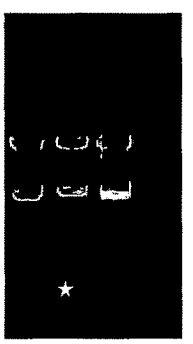
Figure 4.3.2.7: HPLC profile of the insertion mutant # 4a

4.3.3: Insertion Mutagenesis of O-Methyltransferase gene

O-methyltransferase (OMT) activity is proposed to be responsible for the methylation of the deoxysugar moiety in the polyketide backbone. This is present upstream of the TDP-glucose-4,6-dehydratase in the 8.0 kb *Eco*RI fragment however in opposite orientation.

4.3.3.1: Construction of the (OMT) disruption vector:

8.0 kb *Eco*RI fragment generates three *Bgl*II fragments of size 0.2 kb, 4.0 kb and 1.9 kb. The placement of these fragments is in the order 0.2-4.0-1.9 kb, of which the 0.2 kb *Bgl*II fragment constitutes the middle portion of the gene for the O- methyltransferase. The 8.0 kb *Eco*RI fragment was digested with the *Bgl*II enzyme and the 0.2 kb *Bgl*II fragment was gel eluted and cloned in to the suicide vector, a non integrating derivative pSET 152Δ*Hind*III vector. pSET152 is a specially designed vector in which the *IntC* 32 region from the broad host range *Streptomyces* bacteriophage allows for site specific integration of the vector into the chromosome at the *att* site (Bierman et al.,1992). The *int* DNA was removed by digestion of the vector with *Hind*III followed by the relegation to convert the integrating vector into a suicide vector in *Streptomyces*. Moreover this vector contains the *OriT* region responsible for the conjugal transfer of the genes. Presence of Apramycin marker helps in the selection of the plasmid in both the species, *E.coli* and *Streptomyces*.



1 2 3 λHE

Figure 4.3.3.1: *Eco*RI -*Pst*I digestion the vector for mutagenesis. 0.2 kb *Bgl*II DNA (internal fragment of the OMT gene) cloned in pSETΔ*Hind*III vector. The * shows the presence of the 0.2 kb fragment.

4.3.3.2: Construction of insertion mutant using homologous recombination strategy:

Once the middle region of the O- methyltransferase was cloned in to pSETΔ*Hind*III, the recombinant vector was transferred in to the *S.flavisclerotícus* by conjugation. About 150 ex conjugants were obtained for 10^8 donors used and were potential disruption mutants.

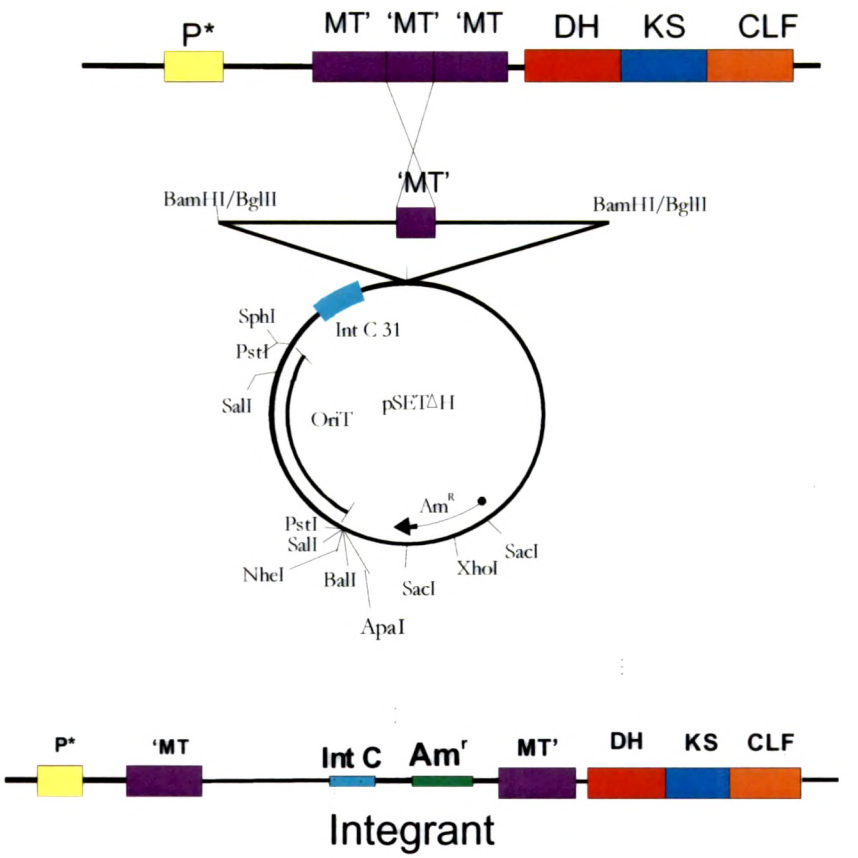
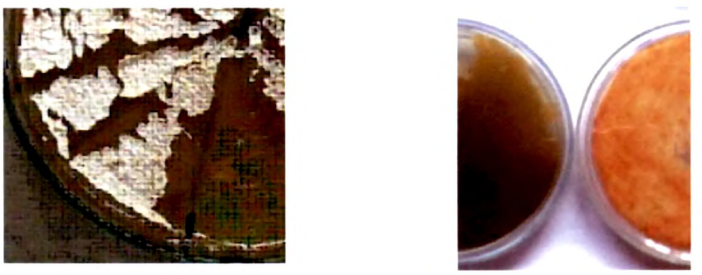


Figure: 4.3.3.2: Strategy used to construct the OMT insertion mutant

4.3.3.3: Phenotypic characteristics of the mutant:

The exconjugates growth on solid agar was quite unusual, a dense cottony growth with orange non diffusing pigments as shown in figure



A B

Figure 4.3.3.3: Phenotypic characterization of the mutant. A: shows the cottony growth of the mutant on apramycin supplemented plate B: comparison of the WT and O- methyl transferase mutant in terms of pigment production

4.3.3.4: Extraction of bio-active component, and TLC analysis of the insertion mutant

The mutant spores were plated on different media of varying pH, incubated at 30°C and the extraction was done using ethyl- acetate, the crude extract was partially purified using adsorption column chromatography. Culture grown on soyabean mannitol agar (acidic) showed intense pigmentation as compared to other media. Further the agar well assay and the TLC run was carried out for this extract. The agar well assay was carried out with this extract on gram positive *Micrococcus luteus* and gram-negative *DH5α* bacterial strain. O- methyltransferase mutant extract was active against both the strain where as WT was active only against gram-positive strain (data not shown).

Extract from the O- methyltransferase mutant subjected to thin layer chromatography (TLC) showed reduced amounts of yellow fluorescing pigment (and appearance of yellow-green fluorescent spot) in the extract from mutant and also that the Rf was different from that of three yellow fluorescent spots of the WT extract. This is expected, as the methyl group is lacking in the intermediate, which may affect polarity of the component. What remains to be found out are if this new spot is the intermediate in the O- methyltransferase mutant and if is bioactive against *M. luteus*.

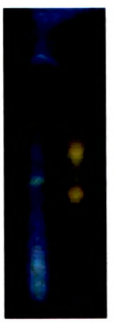


Figure 4.3.3.4 Comparison of TLC profile between O- methyltransferase mutant (M) and partially purified extract of *S.flavisclerotícus* (WT).

4.4: Conclusion:

Glycosylation of the Polyketide antibiotics is a very important phenomenon in giving the finishing touches the basic polyektide backbone. This modification often imparts the potency to the aglycon molecule. The extent of sugar decoration also provides the versatility and the specificity to the molecule which otherwise in the aglycon form is not even active many a times. The anti neoplastic drugs like daunomycin, mithramycin, chromomycin are the suitable example of this. Moreover the tailoring enzymes like O-methyltransferase which are involved in the methylation of the, N and C moieties in the polyketide as well as the sugar moiety plays a very vital role in the terminal modification of the nascent polyketide molecule.

In this chapter we undertook the study to find out the role of two enzymes TDP- 4,6-glucose dehydratase and O-methyltransferase, which were important in the glycation of the polyketide backbone produced by the *S.flavisclerotícus*' PKS gene cluster. The sequencing results of *S.flavisclerotícus* genome revealed that the enzyme TDP- 4,6-glucose dehydratase is present along side the KS and CLF region. The gene TDP- 4,6-glucose dehydratase was deleted as well as disrupted. The results established that the TDP- 4,6- glucose dehydratase was very much essential in the biosynthesis of the Polyketide molecule as the disruption and the deletion of the mutant gave to complete loss of phenotype and the bioactivity of the compound.

The presence of the O-metyltrasferase upstream of the KS, CLF and TDP- 4,6- glucose dehydratase gene also raised the question of its relevance in the PKS cluster so the disruption of this gene was also carried out. The results indicated that the disruption of this gene indeed made some difference in the phenotypic characteristics of the organism as well as the molecule but it also showed some activity against the Gram positive *Micrococcus luteus* as well Gram negative *DH5α*. Thus establishing the fact this was required for the terminal modification of the molecule.