Chapter 3

Characterization of chromomycin deletion mutant - JP1

Streptomyces flaviscleroticus was selected as the organism of this study majorly on the basis that there was no information in the literature about the bioactive compounds it produces (1). Genetic and chemical characterization of the polyketide of *S. flaviscleroticus* established it to be chromomycin, a yellow colored antibiotic, which is an aureolic class of antitumor molecule, aromatic polyketide in nature, and synthesized by polyketide synthase cluster of genes (PKSII). It is used *in vitro* as G/C specific fluorescent DNA binding dye, as an antibiotic and *in vitro* as anti cancer drug that inhibits RNA binding. It is toxic *in vivo* for growth of all types of cells including bacteria. Since the target of binding of the molecule is DNA, it strongly inhibits replication and transcription and thus growth. *S. flaviscleroticus* was identified as being synonymous to *S. minutiscleroticus* in 2007 (2). Incidentally, *S. minutiscleroticus* is known to produce chromomycin.

3.1 Introduction

actI and *actIII* have been used as probes to establish correlation between presence of DNA sequence in Actinomycetes and their ability to produce functional polyketides. Though the correlation facilitated cloning of polyketide producing genes from several organisms, often uncharacterized as in the case of the present work, for cryptic or silent gene clusters, the correlation may not apply (3). Thus, hybridization to these probes doesn't necessarily indicate that polyketide molecules will be produced. The earlier work presented in thesis entitled 'Functional and chemical characterization and heterologous expression of typeII polyketide synthase PKS gene of presumptive producer strain of *Streptomyces – S. flaviscleroticus* by Dr. Namita Kumari provides insight into genetic and biochemical potential of the then uncharacterized strain, *S. flaviscleroticus* with the generation of polyketide synthase knock-out mutant. The thesis work described the utility of the *actI* probe hybridization in identifying polyketide synthase (PKS II) from the genomic DNA of *S. flaviscleroticus*. The details of the mutant construction are briefly provided below. The unusual phenotypes of the mutant are the basis of the study of this Ph. D dissertation.

In order to understand the role of biosynthetic genes, 'gene disruption' approach is widely used. In *Streptomyces,* the null-mutant alleles of the target gene are constructed via a plasmid through homologous recombination. Depending on number

and positions of homologous sites on plasmid, single or double cross-over occurs as a result the targeted gene is either interrupted by insertion of foreign fragment or is partly or totally replaced by selection marker. Both the type of cross-over methods involve cloning of internal segment/s of the gene in the suicide vector, transformation of the host by the vector DNA, selection of the antibiotic resistance encoded in the plasmid, ensuring the integration of the suicide vector DNA into the chromosome by homologous recombination (4). These strategies are quite tedious as screening of several of clones is involved for rare recombination event of double cross-over especially when short homologous segments are employed. Currently, faster and efficient gene replacement method 'REDIRECT' is being employed. The method is based on replacement of a gene on a cosmid by a PCR fragment in an *E. coli* strain engineered for better λ recombination and low degradation of linear DNA strands. The mutated cosmid is extracted from *E. coli* and introduced in *Streptomyces* where by homologous recombination it replaces the gene of interest. The mutant cosmid construction is fast and success of recombination increases due to large homologous regions in cosmid. The resistance cassette can be looped out without disturbing the transcription of surrounding genes. This strategy allows study of effect of single gene deletion even if the gene is part of an operon (5).

3.2 Results

3.2.1 Construction of PKS deletion mutant

Genomic DNA library was prepared using chromosomal DNA of *S. flavisclerotic*us, which was partially digested by the restriction enzyme *Sau*3*A*I to generate DNA fragments with approximate size of ~25 kb and ligated to the *BamH*I digested cosmid vector pKC505 (6). The ligated cosmid DNA was packaged *in vitro* using λ packaging extract (Promega Packagene® Lambda DNA Packaging System) and subsequently used for infecting *E. coli* MC1061 cells. About 10,000 apramycin resistant (Ap^r) colonies were screened by colony hybridization for presence of aromatic polyketide synthase (PKS) genes using highly conserved typeII polyketide probe, *actI* DNA (7) from *S. coelicolor* (a kind gift from Prof. David Hopwood, JIC, U.K.). The insert in each of the eight PKS probe-positive cosmids contained 20-25kb of overlapping PKS DNA. A restriction map was constructed with respect to restriction enzymes *BamH*I, *Pst*I, *EcoR*I and *Bgl*II (Fig. 3.1). The most conserved *actI*

homologous PKS genes were targeted for deletion for assessing their functionality. We observed that the pKC505 cloning vector, though designed to be a bifunctional vector containing SCP2* replicon function for *Streptomyces*, did not exist stably and episomally in *S. flavisclerotic*us. This feature was exploited for the cloned insert mediated integration of the gene targeting vector into the chromosome by homologous recombination. Briefly, the scheme of the mutant construction is the standard method of engineering deletion of the portion of the PKS DNA present in the cloning vector, inserting an antibiotic resistance gene at the joint point of deletion, and crossing-in the deletion mutation on the chromosome by reciprocal recombination. For the construction of the gene targeting vector #2.19Δ*Bgl*pGMΔ*Pst*, the insert derived *Bgl*II fragments were deleted from the cosmid #2.19 by *Bgl*II restriction enzyme digestion followed by intramolecular ligation. It is pertinent to note that the vector backbone of pKC505 does not have a site for restriction enzyme, *Bgl*II. The resultant 2.19Δ*Bgl* plasmid (Fig. 3.1) is devoid of 12.641 kb insert DNA but retains 3-5 kb DNA on either side of the deletion. Inserting an antibiotic resistance gene at the point of deletion facilitates easy segregation of the deletion- and wild-type allele. We inserted the thiostrepton resistance gene (Tsr^r) present in the vector pGM160 (8) along with the vector sequences at the deletion joint point because the nature of the deletion can be verified easily with the retrieval in *E. coli* of the pGM160 vector along with the flanking sequences from the genomic DNA of the deletion mutant. The Tsr^r gene and the vector sequence of pGM160 were inserted at the unique *Bgl*II site of the 2.19Δ*Bgl* plasmid in two steps. First, the DNA between the *Pst*I restriction enzyme sites was removed from the plasmid pGM160 to rid of *Streptomyces* replicon function and to create the 5.66 kb derivative, pGMΔ*Pst*. In the second step, Tsr^r gene and the vector DNA of pGMΔ*Pst* was inserted at the unique *Bgl*II site of #2.19Δ*Bgl*II to generate the gene knock-out vector, #2.19Δ*Bgl*pGMΔ*Pst*. Though this vector contained two copies of pBR*ori* (colE1), the construct was stable in *recA*$^-$ host, DH5α. Reciprocal crossovers (occurring in two-steps), involving the homology flanking the deletion (borne on the plasmid), and the corresponding segment on the chromosome, places deletion mutation into the genome. In the first step, single crossover recombination integrates the gene targeting plasmid #2.19Δ*Bgl*IIpGMΔ*Pst* in the chromosome, generating the plasmid integrant (INT). The spore inoculum of one Tsr^rAp^r integrant was cultivated in the absence of antibiotic for three plate subcultures, and then was spread for the isolation of single

colonies to screen for second crossover segregants in which the yellow diffusible pigment is not produced. However, the more effective screen turned out to be lack of sporulation. At about 1% frequency, the variants were apigmented and sporulation defective. For the syncytial mycelial inoculum that does not sporulate well, the problem of segregation of the mutant versus wild-type chromosomes requires extensive purification. With successive subculturing, the pure mutant cell also grew quite slowly and exhibited complete absence of sporulation; as anticipated the mutant was, thiostrepton-resistant and apramycin-sensitive (12 out of 12). It is important to note here that the homologous recombination between two copies of pBR*ori* DNA, presuming them to be in direct orientation in the chromosome of INT, would cause loss of sequences of pGMΔPst without affecting the thiostrepton-, apramycin-resistance or chromomycin synthesis. One representative mutant **JP1** was utilized for further studies (Fig. 3.2).

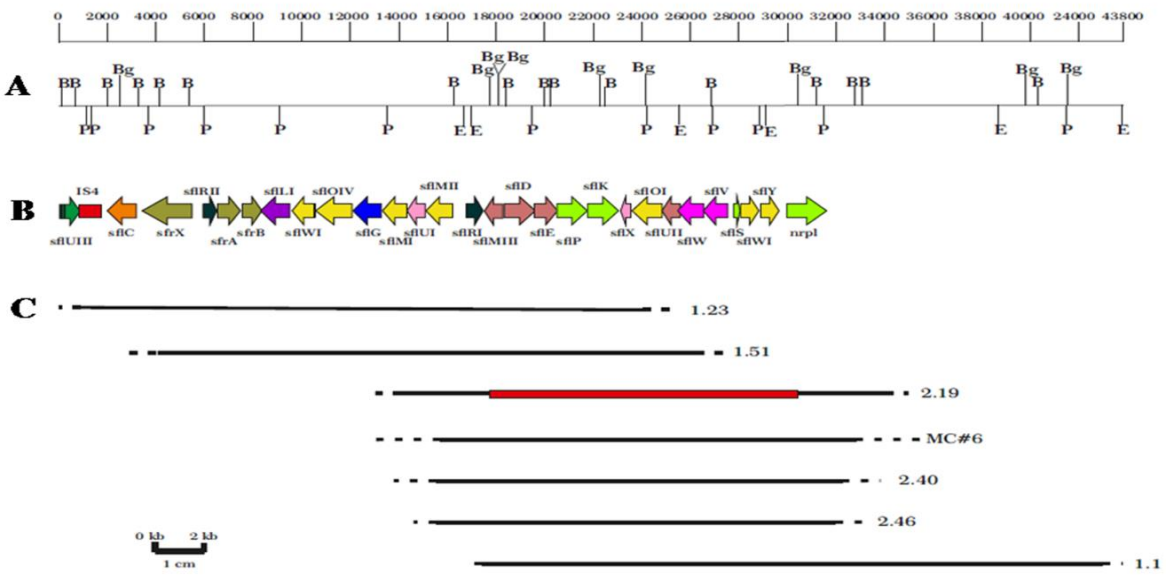

Figure 3.1 Restriction map and genetic organization of the partial biosynthetic gene cluster of Chromomycin A) Restriction map of the cluster with respect to enzymes *BamH*I, *Bgl*II and *Eco*RI.; B) The open reading frames, the direction of transcription are represented as arrows and their putative functions are described in Appendix I; C) The overlapping DNA present in different cosmid clones is indicated by bold line, the dotted lines indicate the tentative limits of insert DNA in each clone. The DNA deleted from the genome of the mutant JP1 is indicated in box in the cosmid 2.19.

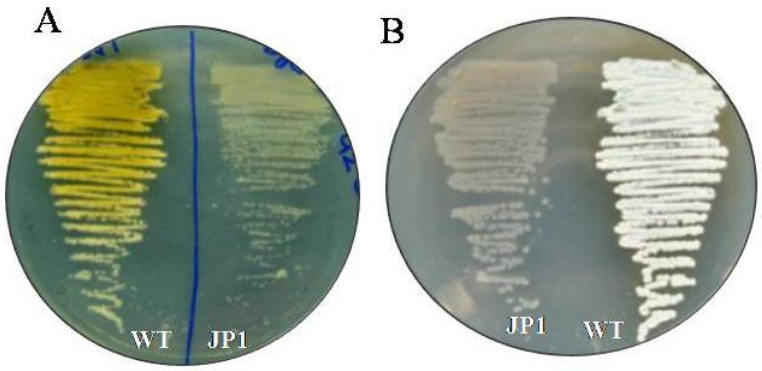

Figure 3.2 Chromomycin non-producer mutant JP1 lost ability to produce the yellow colored antibiotic – chromomycin and doesn't sporulate (R2YE agar plates) (A) Back view & (B) front view of the R2YE plate incubated at 30°C for 4-5 days.

3.2.2 Genetic evidence for the deletion of PKS genes

i) **Complementation of the non-producer phenotype of JP1 mutant by the #2.19 cosmid:** As indicated earlier, the cloning vector, pKC505 could not stably be maintained in *S. flavisclerotic*us. Thus, apramycin resistant #2.19 cosmid DNA transformants of JP1 (INT) contained the #2.19 cosmid integrated into the genome. Importantly, the integrants were reverted for all mutant phenotypes and were indistinguishable from wild-type. The complementation results convincingly established that the phenotypes (see below) of the non- producer mutant JP1 are the result of lack of chromomycin synthesis and/or deletion of the chromomycin biosynthesis genes, ruling out the possibility of a secondary mutation being its cause.

ii) **Retrieval of pGMΔ*pst* plasmid from the chromosome of JP1 mutant into *E. coli* DH5α**: The genomic DNA of JP1 was digested each by restriction enzymes *BamH*I and by *Bgl*II. Following intramolecular ligation and transformation of *E. coli* DH5α cells, the plasmid DNA was extracted and analyzed by *BamH*I and *Bgl*II restriction enzymes. As shown in Fig. 3.3, *BamH*I enzyme cleaves the chromosomal DNA flanking the deletion joint point as 2.284 kb unique *BamH*I DNA which in continuation with the 5.66 kb pGMΔ*pst* DNA fragment generates a 7.944 kb plasmid DNA (B) during intramolecular ligation and transformation in *E. coli* DH5α cells. The 2.284 kb fragment encompassing the deletion joint point is released from the vector plasmid as 2.284 kb *Bgl*II fragment upon restriction digestion (Fig 3.3 I; II; III). Vector pGMΔ*pst* (A) (5.66 kb) is reconstituted for gentamycin resistance (Gm^r) gene

(*aacCI*) when retrieved by *Bgl*II restriction enzyme from the genomic DNA of JP1 followed by intramolecular ligation, which could transform *E. coli* DH5α to both ampicillin resistance and gentamycin resistance.

iii) **Southern hybridization of the chromosomal DNA of JP1 mutant:** Genomic DNA of JP1 was digested each by *BamH*I and *Bgl*II and probed with P^{32} radiolabelled 5.66 kb pGMΔ*pst* vector. As expected, the probe hybridizes to 5.66 kb *Bgl*II DNA of the vector pGMΔ*pst*, and to 7.944 kb *BamH*I fragment consisting of 5.66 kb vector plus 2.284 kb *BamH*I insert encompassing the deletion joint point (Fig. 3.3 (IV)).

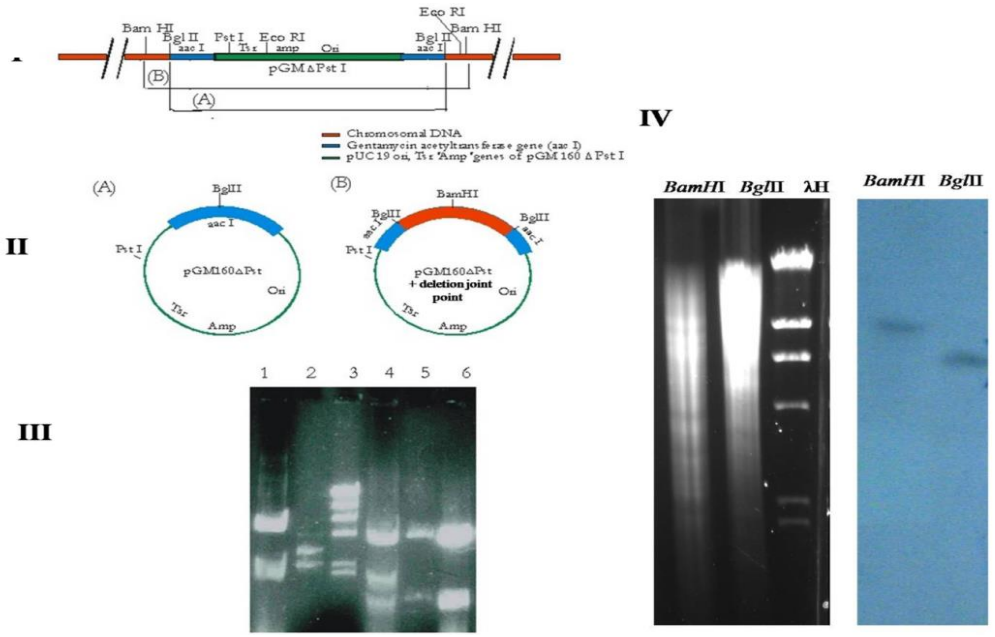

Figure 3.3 Genetic evidences for correctness of the deletion of PKS DNA in JP1 (I) RE map of deletion region of JPI; (II) Predicted structure of the retrieved plasmid following *Bgl*II (A) and *BamH*I (B) digestion of genomic DNA of JPI. (III) RE analysis of the retrieved plasmid; Lane 1- *Bgl*II digestion of plasmid retrieved (from JP1 mutant following *BamH*I digestion and religation) releases 2.284 Kb DNA encompassing deletion joint point.; Lane 2 - The *BamH*I fragment including the deletion joint point is cloned in pBKS vector and released with *BamH*I restriction digestion; Lane 3 - Lambda *Hind*III marker; Lane 4 - *Eco*RI+*Pst*I digestion of pGMΔpst plasmid recovered from JP1 (following *Bgl*II digestion and intramolecular religation) and Lane 5 - The *Eco*RI+*Pst*I digestion original pGMΔ*pst* vector DNA (IV) Southern hybridization of JP1 chromosomal DNA using pGMΔpst as a probe.

Left: agarose gel of the *BamH*I and *Bgl*II digestion of JP1 genomic DNA; Right: Southern blot of the same.

iv) **HPLC analysis of extracts of WT, INT and JP1:** Ethyl acetate concentrate of culture filtrates of WT are resolved into 3 peaks corresponding to chromomycin A1, A2, A3 at respective retention times (RT) of 22 min., 25 min. and 28 min. that are clearly absent in the extract of JP1. Importantly the same three peaks at the indicated RTs reappear in the extracts of INT (Integrant) upon being complemented by insert in the #2.19 cosmid (Fig. 3.4).

Each of the predictions made for the correctness of the deletion mutation was met with indicating that the deletion of the PKS DNA is precise and that the phenotypes are the result of the removal of chromomycin biosynthetic genes and/or inability to synthesize chromomycin.

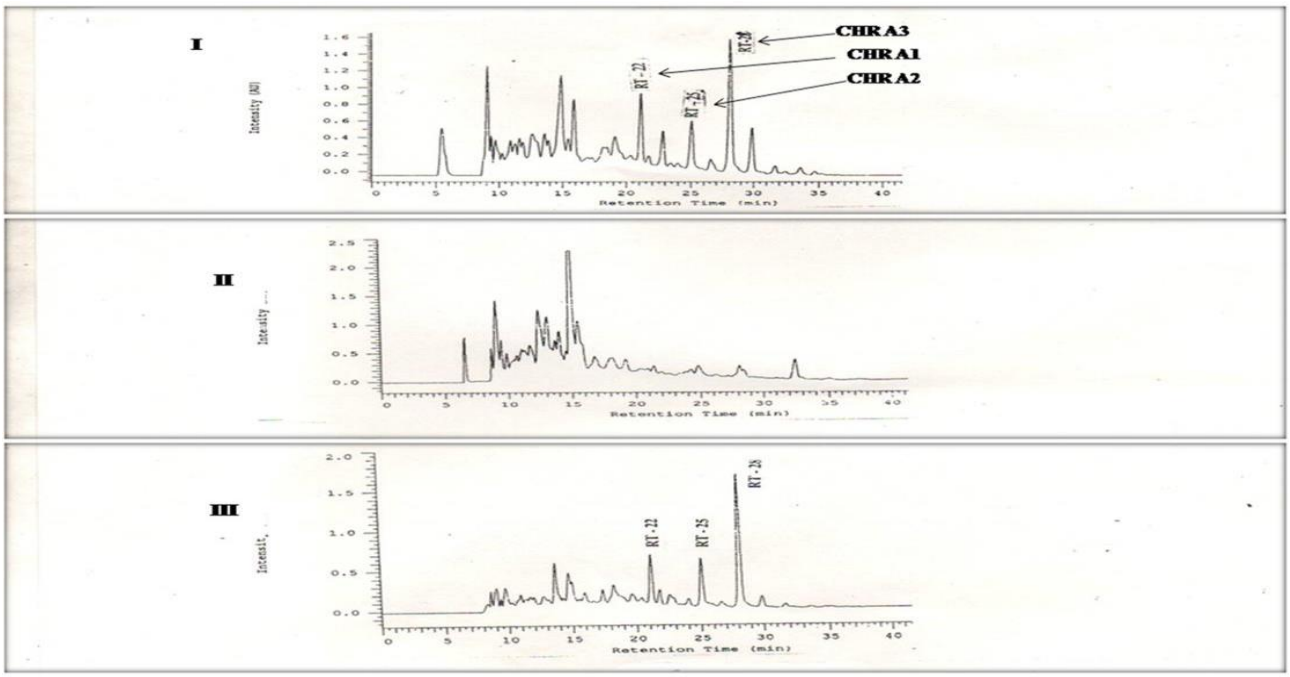

Figure 3.4 HPLC analysis of extracts for chromomycin production - HPLC analysis of extract of wild type (I), JP1 (II) and integrant (JP1+ #2.19) (III).The retention time are indicated for chromomycin A1, A2 and A3.

3.2.3 Structural characterization of the aromatic polyketide produced by *S. flaviscleroticus.*

Dr. Namita Kumari's thesis describes how the aromatic polyketide was characterized. The yellow bioactive compound of *S. flaviscleroticus* is produced in optimum amount on soyabean mannitol Agar. Ethly acetate was found to be best solvent for extraction of the compound from cells grown on solid agar media. The compound exhibited absorbance maxima at 420 nm (yellow chromophore) and contained an active fluorophore (Exitation: 435, Emission : 520). HPLC, TLC, LC-MS and NMR data suggested that the antibacterial compound produced by PKS cluster of *S. flaviscleroticus* was chromomycin. Similar to other chromomycin producers, *S. griseus* and *S. reseiscleroticus, S. flaviscleroticus* also produces all the three forms of chromomycin, A_1, A_2 and A_3. The major form is chromomycin A_3 in *S. flaviscleroticus* (Fig. 3.5).

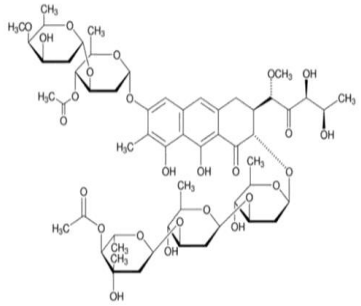

Figure 3.5 Structure of chromomycin A3

3.2.4 Unexpected phenotypes exhibited by chromomycin null-mutant JP1

The overt phenotype(s) exhibited by JP1 mutant were quite revealing. Not only does the deletion of 12 kb DNA abolished the strain's ability to produce antibiotic, chromomycin, it was also associated with interrelated phenotypes which include the following: i) reduction in the growth rate of the JP1 mutant by 2-fold (Fig. 3.6A; 3.7); ii) complete absence of sporulation (Fig. 3.6B); iii) early loss of viability (Fig. 3.8; 3.9); and iv) lack of growth on minimal medium with glucose as the sole carbon source (Fig. 3.6 C). None of these phenotypes are in accordance with the gene information residing in the 12 kb DNA region (Gene Bank Accession No.KC249518.1) deleted from the mutant strain, which harbors genes for minimal

PKS and modifying enzymes (Appendix I). The homologous genes from other clusters have been shown to be involved in antibiotic biosynthesis. Bioinformatic analysis of the 12 kb DNA deleted in the mutant revealed a close match of all the genes to chromomycin biosynthetic gene cluster of *S. griseus* subsp. *griseus* (Appendix I). However, a second function for some of the gene products can be a possibility. The fact that the #2.19 cosmid integrant of JP1 mutant (INT) was reverted for all the phenotypes of the mutant with respect to chromomycin synthesis, slow growth, sporulation and growth defect on minimal glucose medium rules out the possibility of a second mutation.

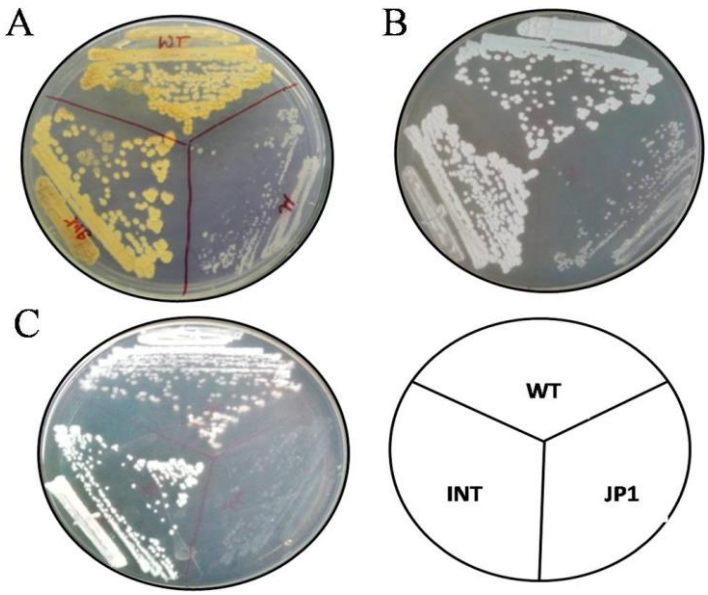

Figure 3.6 Phenotypes of chromomycin deletion mutant JP1: (A) Deletion mutant (JP1) does not produce yellow fluorescent antibiotic chromomycin on R2YE and grows slowly; (B) exhibits complete absence of sporulation; (C) does not grow on minimal media (R4) containing glucose as carbon source; in contrast INT (integrant) and wild type (WT) are indistinguishable with respect to all the phenotypes.

3.2.5 Construction of single gene mutant for chromomycin biosynthesis

In order to prove that the phenotypes of the JP1 mutant are not due to inordinate expression of the remaining genes of the chromomycin biosynthesis cluster, and that the single gene mutation results in the phenotypes same as JP1 deletion mutant, *sflE*, the gene involved in deoxysugar biosynthesis was targeted for

disruption. The thiostreptone resistance gene casette' was inserted in *sflE* gene at the unique restriction site (*Kpn*I) in the plasmid pSETΔ*pst*8kb*EcoR*I (Fig. 3.7). The plasmid pSETΔ*pst*8kb*EcoRI* contains 8 kb fragment of PKS cluster and includes sequence of genes for ketosynthase, chain length factor, O-methyl transferase, NDP-4,6 dehydtratease and partial sequence of cyclase (Appendix I). *sflE* is the promoter proximal gene of the operon consisting of downstream promoter distal genes *sflP* and *sflK*. The insertion of Tsr^r cassette within *sflE* gene may exert polarity effect on the expression of distal genes, *sflP* and *sflK*, encoding heterodimeric ketosynthase. The plasmid maps of the vectors used for *sflE* mutant construction are shown in Fig. 3.8. The 4 kb insert DNA flanking the *sflE::tsr* mutation promoted recombination of the plasmid with the genome of wild type to generate Tsr^r Ap^r single cross over integrant or Tsr^r Ap^s double cross over mutants (Fig. 3.9). Since the methylated DNA of the plasmid from *E.coli* is restricted by *S. flavisclerotic*us because of methyl specific restriction system, the DNA was propagated through non-methylaing *E.coli* host ET12567 strain. To promote efficient recombination, preformed single strand form of the plasmid DNA was generated by alkaline treatment of the plasmid as described in materials and methods. Out of 24 transformant colonies tested, 8 colonies were Tsr^r and Ap^s indicating the generation of *sflE* mutant (Fig. 3.10). Since the protoplasts are multinucleate, the transformed cells required a thorough purification for segregation of mutant and wild type chromosomes. The Ap^s and Tsr^r *sflE* mutant colony formed from protoplast cells were invariably yellow in color (indicating chromomycin production) because of multinucleate protoplasts, and were purified on thiostreptone containing plates lacking apramycin. Following 4-5 subcultures on plates same as above, white colonies (indicating no chromomycin production) were observed which were Tsr^r and Ap^s. The white colonies were distinctly non-sporulating as well and astonishingly, were losing viability earlier than JP1 (Fig. 3.10). After a round or two of purifications, the chromomycin non-producer *sflE*::*tsr* mutants, couldn't be cultured at all.

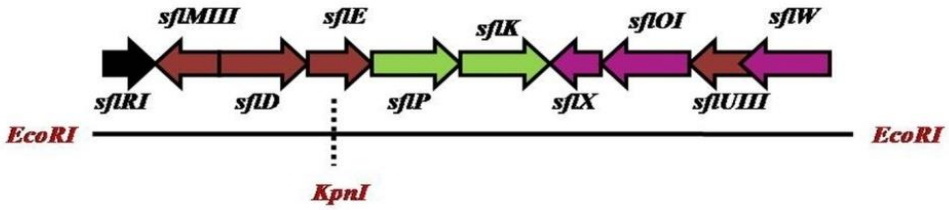

Figure 3.7 Genetic map of the 8 kb *Eco*RI fragment of chromomycin biosynthetic gene cluster - The *Eco*RI fragment consisting of a portion of chromomycin biosynthesis genes including *sflE* is shown and the unique restriction site of *Kpn*I is indicated. The thiostreptone resistance cassette is cloned at this *Kpn*I site to cause disruption of *sflE*. The 8 kb *Eco*RI is cloned in suicide vector pSET152Δ*pst* at the *Eco*RI site.

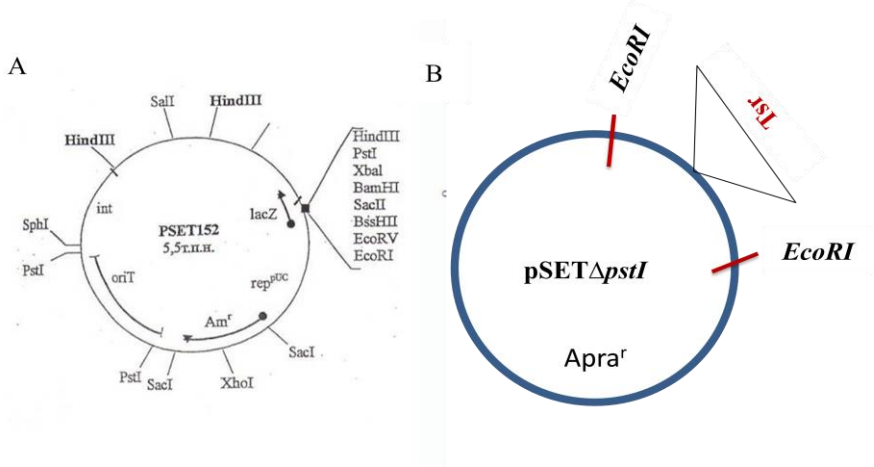

Figure 3.8 Plasmid maps of vectors used for *sflE* mutant construction (A) pSET152 plasmid (B) pSET152Δ*pst*. The 8 kb partial chromomycin biosynthetic cluster was cloned at the *Eco*RI site of the vector pSET152Δ*Pst*, *sflE*::*tsr*^{r} mutation was created by inserting thiostrepton resistance cassette in the *sflE* ORF at unique *Kpn*I site.

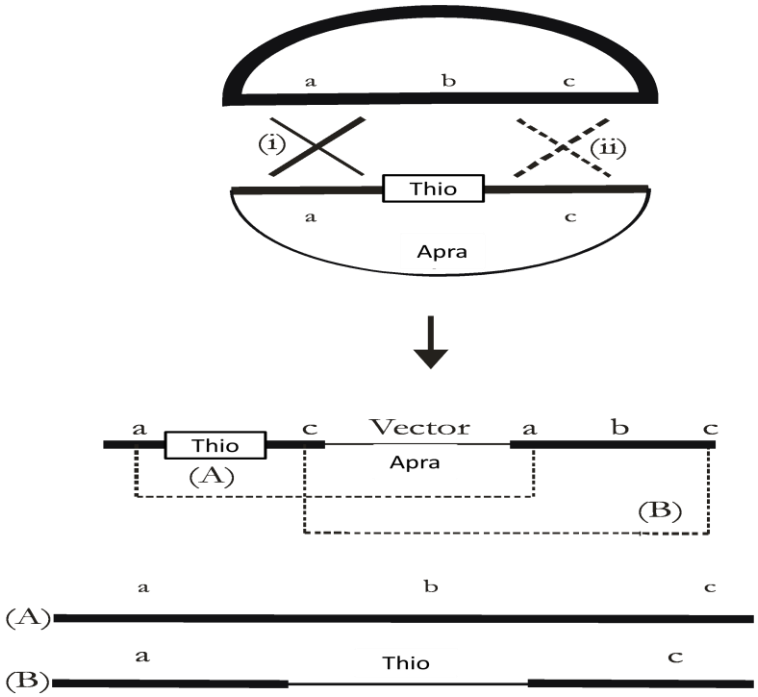

Figure 3.9 Diagrammatic representation of double cross over homologous recombination event.

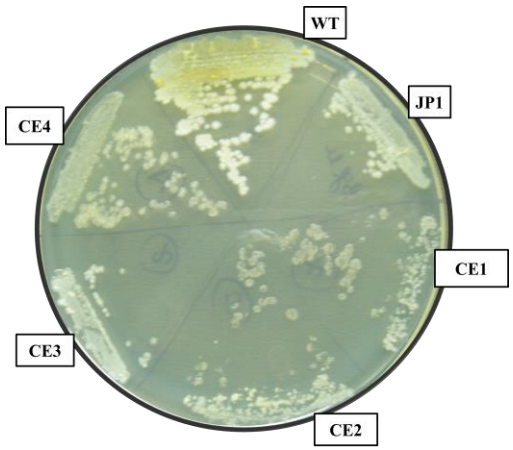

Figure 3.10 Slow growth and poor viability of *sflE* mutants – CE1, CE2, CE3 & CE4 on R2YE plate with wild type and JP1 as control.

Single gene insertion mutations in *sflP* (encoding ketosynthase) and *sflMIII* (encoding o-methyl transferase) were attempted. For this purpose, the internal fragments of *sflP* and *sflMIII* were PCR amplified (Appendix I) and cloned in suicide vector pGM160Δ*kpn*I. The homology of internal DNA fragment of the gene caused integration of the vector into the corresponding DNA *sflP/sflMIII* in the genome. There were many unsuccessful attempts to select for transformants. The most

probable reason for the inability to get the growth of the transformants is the physiological importance of chromomycin for the growth of the organism (Chapter 7).

3.2.6 Growth of the JP1 is slower than that of wild type

The growth of JP1 mutant is markedly slow even on rich R2YE agar medium (Fig. 3.6A; 3.6B). We quantitated and compared this growth with the wild type (WT) and INT in liquid medium tryptone soy broth. It is evident from the growth curve plot that the JP1 mutant grows 2-fold slow even in liquid broth. There is clearly a longer lag and a deficit of approximately 8-10 hours in the time taken by the JP1 mutant to exhibit equivalent increase in the DNA content in relation to both INT and WT (Fig. 3.11). The growth curve plot helped us define the mid-log phase and stationary phase of growth for each of the strains. The mid-log and stationary phases of growth for WT and INT are at 24-32 hours and 50-60 hours respectively and that for JP1 are 40-46 hours 70-80 hours respectively.

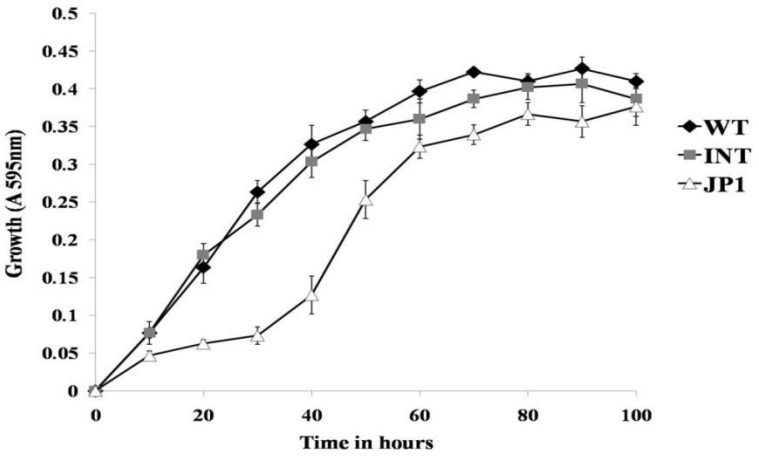

Figure 3.11 Growth rates of wild type, JP1 and INT cells grown in TSB were measured by DNA (A595nm) estimation. The results are representative of three independent experiments and the error bars represent mean ± SD

3.2.7 Chromomycin non-producer JP1 mutant loses viability early in stationary phase

Viability was monitored by two methods – (i) by plating on R2YE agar and enumerating colonies from equivalent volume (0.1 ml) of culture of each of WT (and INT) and JP1 mutant harvested at different hours of growth and (ii) by the live/dead stain (BacLight kit, Invitrogen) in which fluorescent nucleic acid dyes, Syto9 and

propidium iodide differentially stain live cells (green) and dead cells (red). We are aware that measuring viability by the plating method is only an approximate estimate for number of viable cells for mycelial growth of *Streptomyces;* nonetheless this method is useful for continuous assessment of colony forming ability of the culture over several days of incubation. Fig. 3.13 shows representative view of live/dead stained cells of the wild type and the mutant at 24-32 hours and 40-46 hours of incubation respectively containing about 80-90% viable cells. An aliquot of 0.1 ml culture of each strain when plated produced a lawn growth that we considered as 100% viability. The viability of the JP1 begins to diminish after 72 hours of growth. By 96-120 hours of growth, both WT and INT contain 50% each of red (dead) and green (live) staining cells, in striking contrast to JP1 in which all 100% cells stained red, indicating that they were completely non-viable (Fig. 3.13). The same pattern was reflected in the plating experiment- the loss of viability of the WT and INT was 10 fold as compared to the radical loss of viability (<0.01%) of JP1 culture post 96 hours of growth (Fig. 3.12).

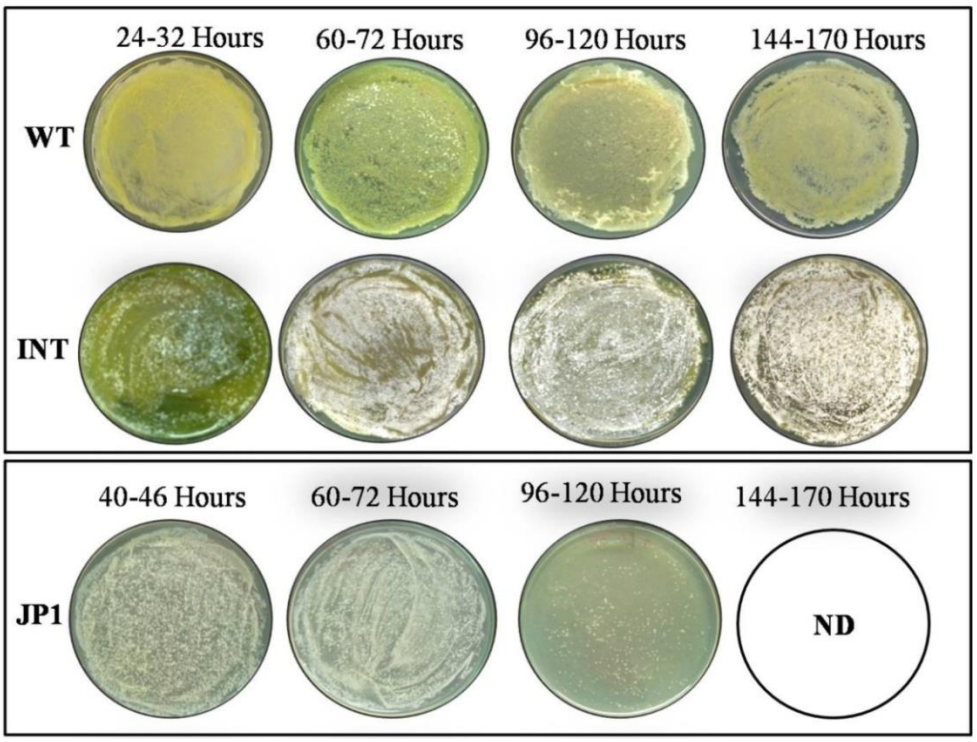

Figure 3.12 Viability of cells of WT, INT and JP1 is measured by plating on R2YE (undiluted)

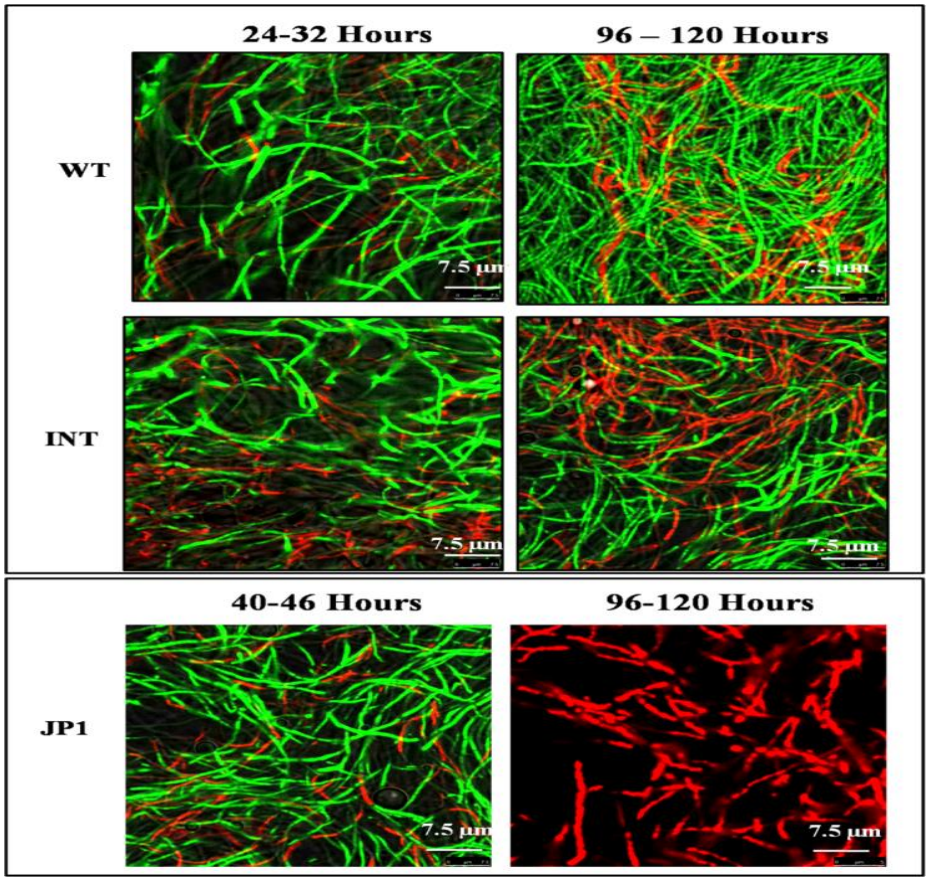

Figure 3.13 Representative view of staining of WT, INT and JP1 by Live / Dead Baclight Kit; and visualization under Leica TCSSP2-AOBS confocal laser-scanning microscope. The viability dyes stain dead cells red and live cells green. Scale bar 7.5 µm.

3.3 Discussion

To test essentiality of PKS genes in antibiotic biosynthesis, we constructed a genetic deletion mutant JP1. The chromomycin non-producer mutant exhibits the expected phenotype of loss of chromomycin production along with many unexpected and overt phenotypes such as reduced growth rate, complete absence of sporulation, early loss of viability and lack of growth on minimal medium with glucose as the sole carbon source. The pleiotropic phenotypes of JP1 mutant are inconsistent with functions encoded in the deleted region of the DNA (9). The mutation in chromomycin non-producer mutant was verified by multiple genetic evidence (Fig. 3.3). The DNA corresponding to the deletion in JP1 (present in the #2.19 cosmid) complemented all the phenotypes of the mutant such as chromomycin biosynthesis, slow growth, sporulation defect and growth defect on minimal media when returned

to the chromosome of the JP1 mutant (JP1/#2.19 OR INT), ruling out the possibility of a second mutation as a cause of pleiotropic phenotypes of JP1 mutant. This result is also reinforced in the inability of *sflE*::*tsr* mutant to grow/remain viable post purification and inability of construction of *sflP* and *sflMIII* knockout mutants.

The pleiotropic phenotypes of the non-producer mutant suggest a reevaluation of the traditional view that antibiotics have no important role for the producer organism. Investigation of two of the phenotypes of the non-producer mutant, namely, the striking early loss of viability of the non-producer mutant in stationary phase and inability of JP1 mutant to grow on minimal medium with glucose as sole carbon source has had been the basis of this thesis work.

There is a remarkable conservation of sequence and organization of the genes of the chromomycin biosynthesis gene cluster of *S. flavisclerotic*us with those of *S. griseus* sub spp. *griseus* (Acc. no. AJ578458) and *S. reseisclerotic*us (Acc. no. MG975976) (Fig. 3.14). It is important to note that *Streptomyces griseus* sub spp. *griseus* and *S. flavisclerotic*us are unrelated based on the ribotyping of the 16S rDNA sequence (Dr. Maulik Thaker's thesis). The phenotypes of chromomycin non-producer mutant of *S. flavisclerotic*us are indeed unique, since the chromomycin non-producer mutant of *S. griseus* doesn't resemble that of *S. flavisclerotic*us. The reason for the discrepancy is not yet clear as the strain-specific effects of mutational conditions are not uncommon in *Streptomyces*. For example, the mutations in *pfk2*, *zwf* and *relA* exhibit strain specific effects on antibiotic production. Alterations in genes of central carbon metabolism responsible for generation of intermediates also for secondary metabolite synthesis, exhibit strain specific effects - *pfk2* enhances actinorhodin synthesis by *S. coelicolor* but not in the closely related actinorhodin producer, *S. lividans* (10); *zwf* mutation, on the other hand, stimulated actinorhodin production in *S. lividans* (11) whereas the *zwf2* mutation in *S. coelicolor* apparently increases actinorhodin yield which is attributed to increased growth of the mutant (12). Mutation in *relA*, the (p)ppGpp synthetase, enhances antibiotic clavulanic acid and cephamycin C production by *S. clavuligerus* (13), whereas the same mutation abolished undecylprodigiosin and actinorhodin synthesis in *S. coelicolor* (14). Thus the strain specific effects of chromomycin is not unusual, however the implications are presently unsettled and may represent the variation in the adoption of secondary metabolites for functions other than antibiosis.

The chromomycin cluster of *S. reseiscleroticus* is indeed noteworthy. Fig. 3.15 shows that *S. flaviscleroticus* and *S. reseiscleroticus* are phylogenetically very close to each other. The IS (insertion sequence) sequence uniquely present in the chromomycin cluster of *S. flaviscleroticus* is preserved in *S. reseiscleroticus*. Additionally, the overall nucleotide BLAST score of the two clusters is 99% indicating the recent acquisition of the cluster by these two organisms whereas the IS sequence is absent in the chromomycin cluster of *S. griseus* and the nBLAST score is only in the range of 60-90% for different genes. It will be interesting to evaluate phenotypes of chromomycin non-producer mutant of *S. reseiscleroticus*.

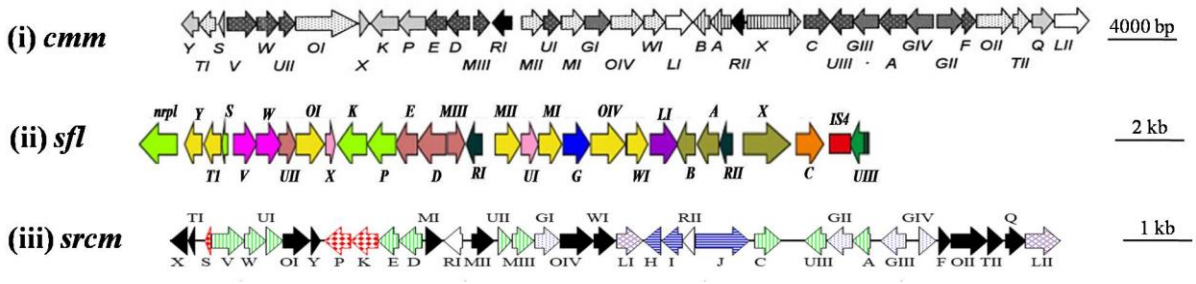

Figure 3.14 Comparison of organization of genes of chromomycin biosynthetic gene cluster of (i) *S. griseus* (*cmm*); (ii) *S. flaviscleroticus* (*sfl*) and (iii) *S. reseiscleroticus* (*srcm*).

Figure 3.15 Phylogenetic analysis of the 16S rRNA gene of *S. flaviscleroticus*, NCBI gene bank accession number - MAZZ00000000. The black arrows indicate positions of *S. flaviscleroticus* and *S. reseiscleroticus* (next page).


Streptomyces plicatus
Streptomyces enissocaesilis
Streptomyces rochei
Streptomyces mutabilis
Streptomyces ghanaensis
Streptomyces geysiriensis
Streptomyces enissocaesilis
Streptomyces fulvus
Streptomyces djakartensis
Streptomyces tricolorans
Streptomyces lomondensis
Streptomyces parvulus
Streptomyces endochromogenes
Streptomyces tendae
Streptomyces violascoruboides
Streptomyces glaucescens
Streptomyces paradoxus
Streptomyces ishyrus
Streptomyces violascochromogenes
Streptomyces humiferus
Streptomyces coelescens
Streptomyces fluvatilis
Streptomyces olivaceus
Streptomyces marokkonensis
Streptomyces thinghirensis
Streptomyces ambofaciens
Streptomyces hygroscopicus
Streptomyces albireticuli
Streptomyces spelbonae
Streptomyces malaysiensis
Streptomyces pilosus
Streptomyces fluvoviridis
Streptomyces malachitofluscus
Streptomyces endocoelicus
Streptomyces albogriseolus
Streptomyces albaduncus
Streptomyces griseoalbus
Streptomyces pseudogriseolus
Streptomyces ghanaensis
Streptomyces capillispiralis
Streptomyces cellulosae
Streptomyces werraensis
Streptomyces erythrogriseus
Streptomyces griseoincarnatus
Streptomyces variabilis
Streptomyces labedae
Streptomyces lusitanus
Streptomyces griseorubens
Streptomyces griseoflavus
Streptomyces malaysiensis 1
Streptomyces althioticus
Streptomyces violatus
Streptomyces purpurascens
Streptomyces janthinus
Streptomyces indiaensis
Streptomyces massasporeus
Streptomyces atratus
Streptomyces halealensis
Streptomyces nagaishii
Streptomyces anandii
Streptomyces coeruleofuscus
Streptomyces pactum
Streptomyces carpinensis
Streptomyces griseoalbamineus
Streptomyces griseomycini
Streptomyces endivialaceus
Streptomyces glaucus
Streptomyces limosus
Streptomyces caelestis
Streptomyces azureus
Streptomyces minutiscleroticus
Anonymous strain
Streptomyces africanus
Streptomyces afghaniensis
Streptomyces bacillaris
Streptomyces lavis
Streptomyces sphaeroviridis
Streptomyces aureoreticuli
Streptomyces virens
Streptomyces cavus
Streptomyces asterosporus
Streptomyces bullii
Streptomyces chilikensis
Streptomyces flagilis
Streptomyces mirabilis
Streptomyces griseoviridis
Streptomyces zinciresistens
Streptomyces lunglaiensis
Streptomyces spiralis
Streptomyces sioyaensis
Streptomyces roseoviolaceus
Streptomyces ruber
Streptomyces limbriatus
Streptomyces rubiginosus
Streptomyces thermocarboxydus
Streptomyces spectabilis
Streptomyces silaceus
Streptomyces auranticogriseus
Streptomyces intermedius
Streptomyces hoyaensis
Streptomyces daghestanicus
Streptomyces violascens
Streptomyces somaliensis
Streptomyces violatus
Streptomyces hebeiensis
Streptomyces griseiniger
Streptomyces microchaetacus
Streptomyces indonesiensis
Streptomyces sudanensis
Streptomyces diastatochromogenes
Streptomyces lincolnensis
Streptomyces venezuelae
Streptomyces fradiae
0.02
0.01
0.00

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