

Testing the genetic potential of polyketide production by construction of knock-out mutant of *S. flaviscleroticus*.

Introduction

A widely used approach to characterize the biosynthetic genes is gene disruption by vector insertion using phage (Malpartida, 1987) or unstably maintained plasmid vectors called suicide vectors. Another approach is the replacement of chromosomal DNA in the producing organism by cloned DNA mutated *in vitro*.

The *actI* and *actIII* probes are being used to establish a strong correlation between the presence of DNA sequences that cross hybridizes with either or both the probes in *actinomycetes* and their ability to produce functional polyketide. The correlation does breakdown in some cases because of the genes being either cryptic/silent or nonfunctional. Thus, hybridization to these probes does not necessarily mean that polyketide molecule would be produced.

Genomic DNA of *S. flaviscleroticus* hybridizes to the *actI* probe. The genetic and biochemical potential of the uncharacterized strain, *S. flaviscleroticus* was ascertained in the work presented in this chapter. For this gene disruption of putative genes for the polyketide production was attempted. Hybridization to *actI* probe and production of multiple bioactivities was an indication of its potential to produce bioactive compound, one of them probably polyketide in nature. If the PKS genes are functional, the mutant constructed by deletion of the PKS genes would not produce one or more of the components on the bioautogram. If the PKS genes are functional it would help in expanding the number of genetically characterized cluster of PKS. The new cluster would be an important addition to the set of clusters like actinorhodin from *S. coelicolor*, granaticin from *S. violaceoruber*, tetrocenomycin from *S. glaucesenes*, oxytetracyclin from *S. rimosus*, frenolicin and nanomycin from *S. roseofulvus* (Hopwood, 1997) to name a few well characterized lists of compounds. The list is long and discussed in detail in review of literature.

Construction of deletion vector: The genomic DNA library had been constructed in the cosmid, pKC505, a vector that may be shuttled between *E. coli* and *Streptomyces*. This was the one of the easily available vector at the time the library was constructed. One of the drawbacks of this vector however, the instability of the cosmid backbone - a result of absence of stability function(s), (described in detail in chapter III) was used to our advantage as the vector was unstable in *S. flaviscleroticus* too. The instability was observed in terms of vector losing the apramycin resistance marker when amplified in absence of antibiotic; the insert was also lost during propagation.

One of the *act* hybridizing clones of the genomic DNA library mentioned above, #2.19, was selected for construction of the deletion vector. This and the rest of the *act* hybridizing clones (mentioned in chapter III), for sure contains the most conserved KS and CLF genes. Construction of the vector is schematically shown in Fig.2.1. The cosmid #2.19 contains four *Bgl*II sites in the insert DNA whereas the vector backbone does not contain any site for the same. Digestion by *Bgl*II, intramolecular religation, transformation into *E. coli* and selection for cosmid marker apramycin (Am^r) was carried out according to the protocols described in materials and methods. The resultant plasmid, called 2.19 Δ Bgl has been removed of 12-15 kb of insert DNA, ensures removal of minimal PKS. The structure of the plasmid was authenticated by restriction digestion to contain single *Bgl*II site (Fig.2.2). The unique *Bgl*II site is now flanked on either side by 4.5 kb and 5 kb DNA. This cosmid clone was selected on the basis of the fact that almost an equal amount of insert DNA flanks the deletion, a feature desirable for double crossover reciprocal recombination between deletion mutation engineered on the plasmid and the corresponding sequence on the chromosome, which places the deletion from the plasmid into the chromosome.

Further, the gene for thiostrepton-resistance (*tsr*), from the plasmid pGM160 was cloned at the unique *Bgl*II site in the following way (Fig 2.1). pGM160 (Muth et.al.,1989) has replicon for *Streptomyces* as well as for *E. coli* (Muth et.al.,1989). Plasmid pGM160 Δ Pst is constructed by deleting *Pst*I DNA from pGM160 (by intramolecular ligation following

digestion by restriction enzyme *Pst*I). The resultant plasmid is devoid of *Streptomyces* replicon, a feature responsible for converting the plasmid into a suicide vector for transformation of *Streptomyces*. Additionally, the gene knockout vector also contains thioestreptone resistance (*tsr*), ampicillin resistance (*bla*), and gentamycin resistance (*aacC1*) genes and pMB1 replicon for its propagation in *E. coli*. The *Bgl*III restriction enzyme linearized pGM160ΔPst DNA was ligated to 2.19ΔBgl at *Bgl*III site. The gene for gentamycin resistance is disrupted due to this cloning. The vector designated #2.19ΔBgl pGMΔPst (Fig. 2.1), was proved to be correct by restriction enzyme digestion analysis (Fig. 2.2). The strategy that engineers the deletion from the plasmid into the chromosome of *S. flaviscleroticus* is schematically described in Fig. 2.3.

Transformation of *S. flaviscleroticus* and selection of PKS⁻ mutant:

S. flaviscleroticus was transformed by vector #2.19ΔBglpGMΔPst by PEG mediated protoplast transformation (described in materials and methods). The transformants were selected on plates supplemented with apramycin. The am-resistant integrants represent a result of single crossover recombination between the deletion vector and the chromosome of host using either of 4.5/ 5kb insert homology, depicted according to Fig.2.3, as region **(i)** or **(ii)**. Twenty five integrants were obtained using 1μg of suicide plasmid DNA and 12 were checked for stable inheritance of plasmid resistance markers. Each one of the apramycin resistant transformant was thioestrepton resistant, unselected. The integrants were streaked in absence of antibiotic pressure and tested for stability of apramycin and thioestrepton marker. More than 99% of the cells were found to have stably inherited these two markers. One of the integrant was chosen for further work and amplified in absence of antibiotics for plasmid free cell to arise. The plasmid free cells which, represent the second crossover event, arise by one of the two mechanisms (A) and (B). The second crossover may take place by (B) using the homology other than the one used for integration of the plasmid and gives rise to apramycin-sensitive and thioestrepton-resistant mutants. In the schematic diagram Fig. 2.3, **(i)** is used for integration and **(ii)**

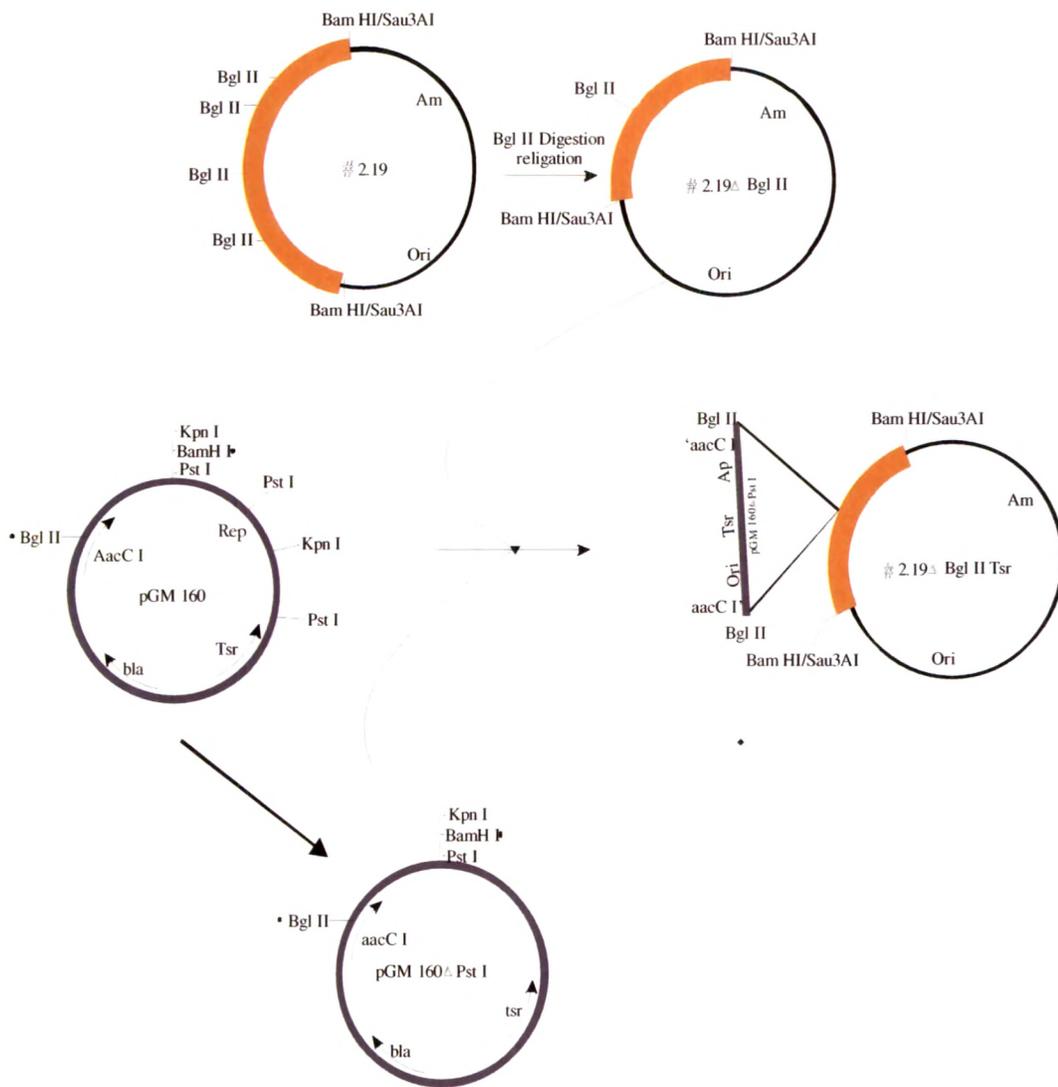


Fig. 2.1: Construction of gene disruption vector.



Fig. 2.2: RE digestion analysis of gene disruption vector: *Bgl*III digestion of lane1, 2.19Δ*Bgl*pGMΔ*pst*; lane2, 2.19Δ*Bgl*; lane3, *Pst*I digestion of pGMΔ*Pst*; lane4, λH marker DNA.

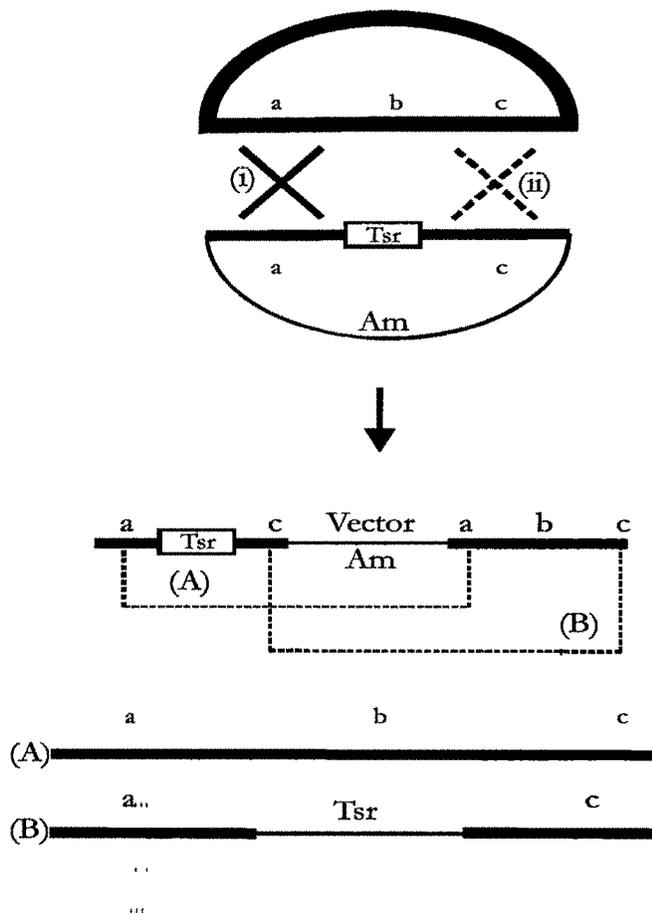


Fig. 2.3: Schematic representation of homology dependent two-crossover recombination event that causes plasmid borne deletion of PKS genes to cross into the chromosome of wild type.

for excision or vice-versa. It is evident from the diagram that the deletion mutant is constructed in this scheme of events. (A) If the second crossover were to occur using the same homology as that used for integration, the resulting plasmid free cells are both apramycin- and thiostrepton sensitive i.e., same as wild type. In the schematic diagram Fig. 2.3, (i) is used for integration and (ii) excision. After growing the integrant in absence of antibiotic selection and plating at suitable dilution, the non-sporulating variant were picked up. These were pigmented differently from wild type (Fig.2.4) and the bioactivity profile of the crude organic extract was also different (Fig.2.5). On further analysis the variants were not only thiostrepton-resistant and apramycin-sensitive (Fig. 2.6), but varied in different characteristics as described below. Approximately 8000 colonies were screened out of which 20 colonies were picked up as variant and 18 (90%) were putative mutants. One of the mutants would be referred to as JPI.

Phenotypic Characterization of Putative mutant (JPI):

- 1. Growth characteristics:** The putative mutant JPI and wild type *S. flaviscleroticus* were streaked on different media like R₂YE, MBA, and R₄. The mutant JPI is slow growing as compared to wild type, is non-sporulating on all the media tested, whereas wild type produces varying amount of spores on different media, maximum on SM, followed by R₄, MBA and R₂YE. *S. flaviscleroticus* produces yellow colored compound by third day of incubation followed by brown color by seventh day. Mutant JPI doesn't become yellow, it directly turns brown (Fig. 2.8), also the bioactivity profile is different from the wild type in each of the medium (Fig. 2.9).
- 2. Production of *S. flaviscleroticus* specific polyketide:** The wild type strain and mutant are differently colored and shows difference in bioactivity. The wild type produces a yellow diffusible color (Fig.2.4 & 2.8) and five zones of bioactivity on TLC after being resolved and developed for bioautography using *M. luteus* as test organism (Fig. 2.5). On the other hand, mutants are colorless in some media and brown colored in others (Fig.2.4& 2.8). Furthermore, TLC separation of the crude extract reveals complete absence of the yellow fluorescent spots, and absence of two of the zones of bioactivity (Fig. 2.7).

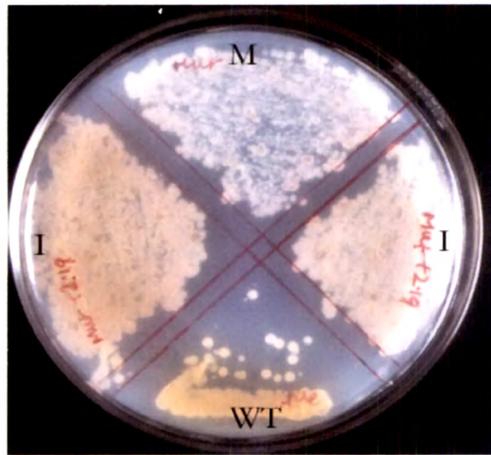
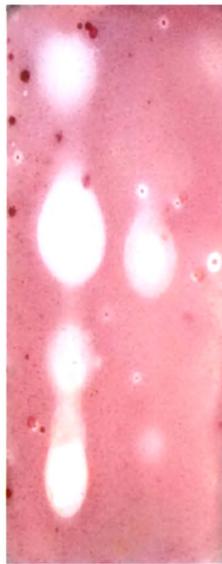
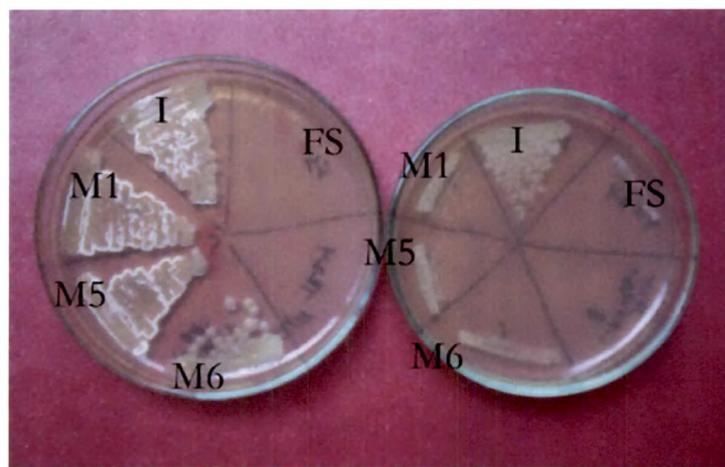


Fig. 2.4: Diffusible color production by *S. flaviscleroticus* (WT), PKS⁻ mutant JP1 (M), and, JP1/2.19 (I)



WT M

Fig. 2.5: Bioautogram of wildtype and mutant extract, following one dimensional TLC separation, using *M. luteus* as test organism.



a

b

Fig. 2.6: The phenotype of deletion mutants (M1, M5, M6) scored on plates supplemented with a) Thiostrepton, b) Apramycin. I, integrant; and FS, wildtype *S. flaviscleroticus*.

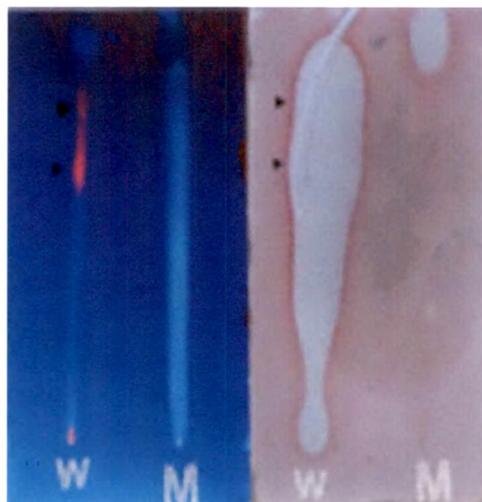


Fig.2.7: ID TLC profile and bioautogram of extract of wild type and JP1 mutant (M). Yellow colored spots and associated bioactivity is indicated by arrow.

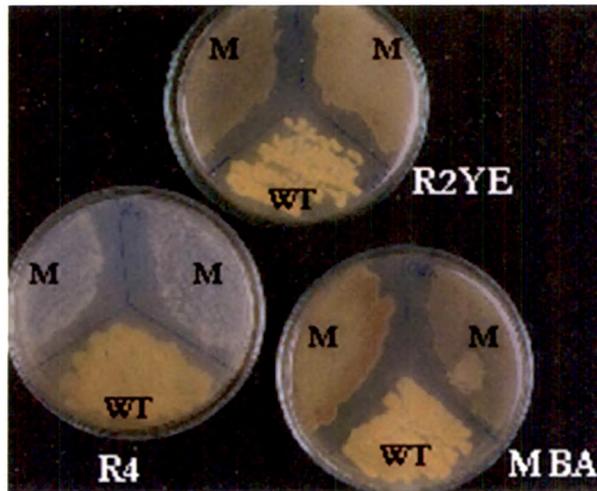


Fig.2.8: Growth features and diffusible color produced by JP1 mutant (M) and wildtype (WT) on different media.

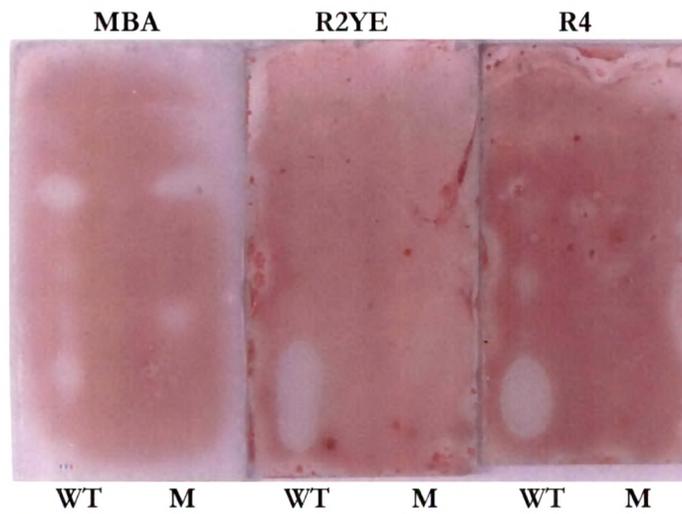
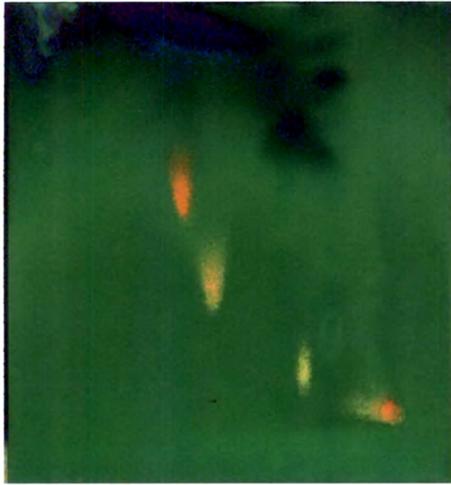


Fig. 2.9: Bioautogram of the extract of *S. flavisclerotius* (WT) and JP1 (M) grown on different media. *M. luteus* was indicator organism.

Mutant JP1 was checked for its antibiotic production profile in media like R₂YE, R₄ and MBA. In each of the media it could be seen that same bioactive spots were cleanly missing when compared to bioautogram of wild type (Fig.2.9). For further probing this phenotype, both *S. flaviscleroticus* and putative mutants were grown on SM media and the crude extract was prepared in ethyl acetate. The concentrated extract was separated on 1D and 2D TLC and bioautogram was developed as described in materials and methods. The wild type extract shows two yellow fluorescent spots, II and III on 1D TLC (Fig.2.7); these resolve further into three spots on 2D TLC viz., II, IIIA and IIIB (Fig. 2.10). Bioautogram developed using *M. luteus* as the test organism showed that all fluorescent spots are bioactive. Both fluorescence and bioactivity are clearly missing in mutant extract (Fig.2.7 & 2.10).

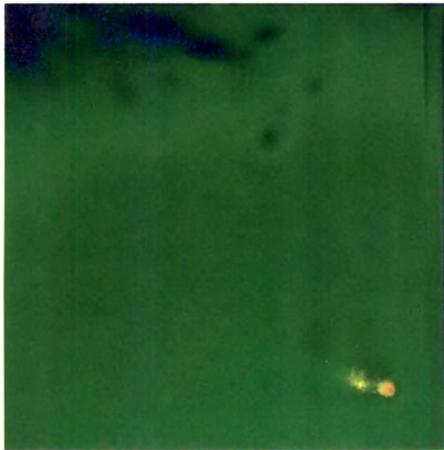
3. **HPLC of wild type and Putative mutant extracts:** The extract of wild type/mutant was resolved by HPLC according to the program described in materials and methods. In the program water + trifluoroacetic acid (TFA) and acetonitrile (ACN) + 1% TFA was used to form a gradient from 0% ACN to 100% ACN. The peaks at RT 22', 25', and 28' present in wild type extract were completely absent from mutant extract (Fig.2.11). These three peaks correspond with II, IIIA and IIIB respectively.
4. **Complementation of the deletion mutation by #2.19 DNA:** If the phenotype of loss of fluorescence and bioactivity in the mutants is associated with the deleted DNA, introduction of the missing DNA back into the mutant should restore the wild type phenotype, viz., production of yellow colored fluorescent compound and bioactivity. Indeed #2.19 transformants produced yellow colored, UV fluorescent, bioactive II, III as expected (Fig.2.4 and 2.12). The HPLC peaks at RT 22', 25', and 28' were also restored in the reintegrants (JP1/#2.19), (Fig.2.11).
5. **Genetic correctness of the deletion mutation:** The putative JP1 mutant was obtained as a result of double crossover between chromosome of wild type, *S. flaviscleroticus* and the gene disruption plasmid. If this is true, the deletion structure should be



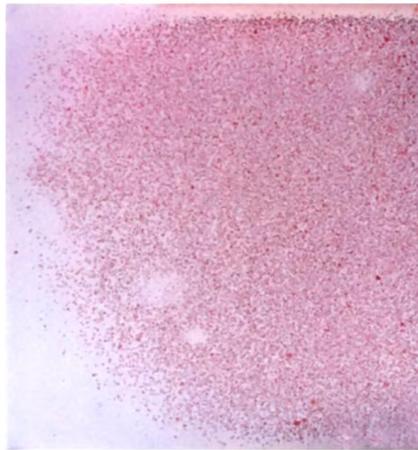
WT



WT



M



M

Fig. 2.10: Two dimensional TLC and bioautogram of extract of wild-type and PKS⁻ mutant. Left, visualization of the TLC under 300 nm UV light; Right, bioautogram using *M. luteus* as test organism.

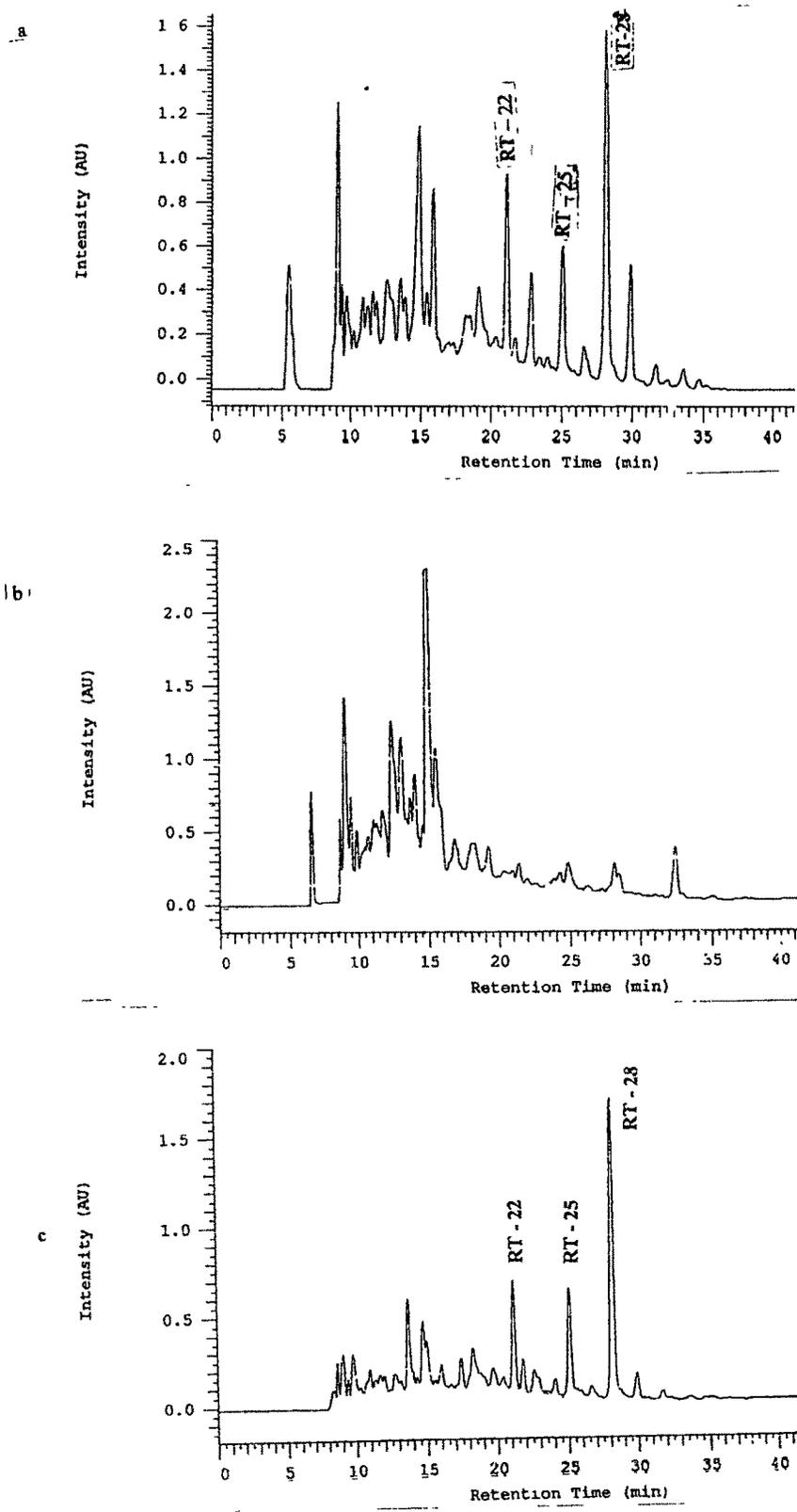
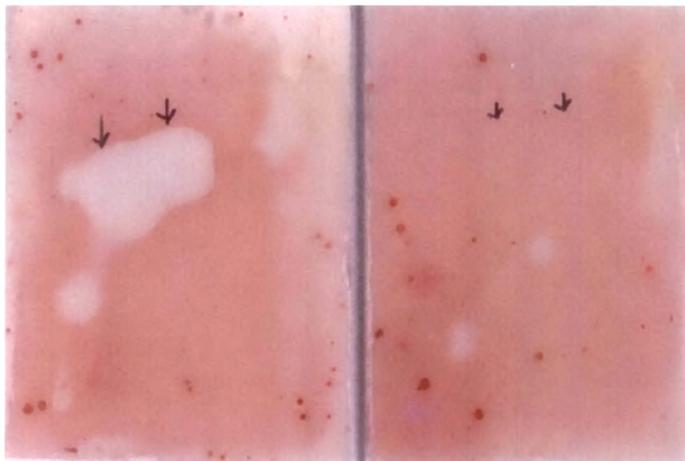


Fig. 2.11: HPLC Chromatogram of a) Wild type, b) Mutant, JPI and c) Reintegrant, JPI/#2.19.



JPI/2.19

Mutant



JPI/2.19

Mutant

Fig.2.12: Two dimensional TLC and bioactivity of extract of integrant (JPI/#2.19) and JP1 mutant. Top, visualization of the TLC plate under 300 nm UV light; Bottom, bioautogram using *M. luteus* as test organism.

recovered from the chromosome of mutant JPI. Since the plasmid pGM160 Δ Pst contains the *E. coli* replicon, appropriate digestion, intramolecular re-ligation and transformation into *E. coli* should generate the plasmid with structures described in Fig 2.13. Working according to this rationale, chromosomal DNA from the putative mutants was isolated, digested with i) *Bgl*III and ii) *Bam*HI, re-ligated and transformed into DH5 α and the recovered plasmids were analyzed.

i) Plasmid DNA retrieved from chromosome of the mutant following *Bgl*III digestion

and religation: The transformants of DH5 α were selected for resistance to ampicillin, marker of the pGM160 Δ Pst. The gene for gentamycin resistance *aacC1* in pGM160 Δ Pst is however disrupted due to ligation at the *Bgl*III site of #2.19 Δ BglIII. If the predicted structure of the deletion is correct, the plasmid retrieved in the case of *Bgl*III digestion and re-ligation should contain reconstituted *aacC1* gene for gentamycin resistance [Fig 2.13, (A)]. All the DH5 α transformants were indeed gentamycin resistant. The plasmid DNA from one representative transformant was indistinguishable from the pGM160 Δ Pst plasmid – *Eco*RI + *Pst*I double digestion pattern of the recovered plasmid and that of original plasmid is the same (Fig.2.13, III lane 5&6)

ii) Plasmid DNA recovered from chromosome by *Bam*HI digestion and religation:

In this case the DH5 α transformants selected on LA + ampicillin, were not Gm^r, as expected (Fig.2.13, IIB). The unique *Bgl*III site in 2.19 Δ Bgl is represented by 2.3 kb *Bam*HI DNA encompassing the deletion joint. The same 2.3 kb DNA fragment marking the deletion should be recovered from plasmid pGM Δ Pst retrieved (after *Bam*HI digestion and re-ligation) from the chromosome of the integrant [Fig. 2.13, II (B)]. This 2.3 kb DNA should be released from the recovered plasmid now by *Bgl*III treatment instead by *Bam*HI. This is most interesting and convincing observation of this retrieved plasmid and is possible only with the predicted deletion structure being shown schematically in Fig.2.13, I.

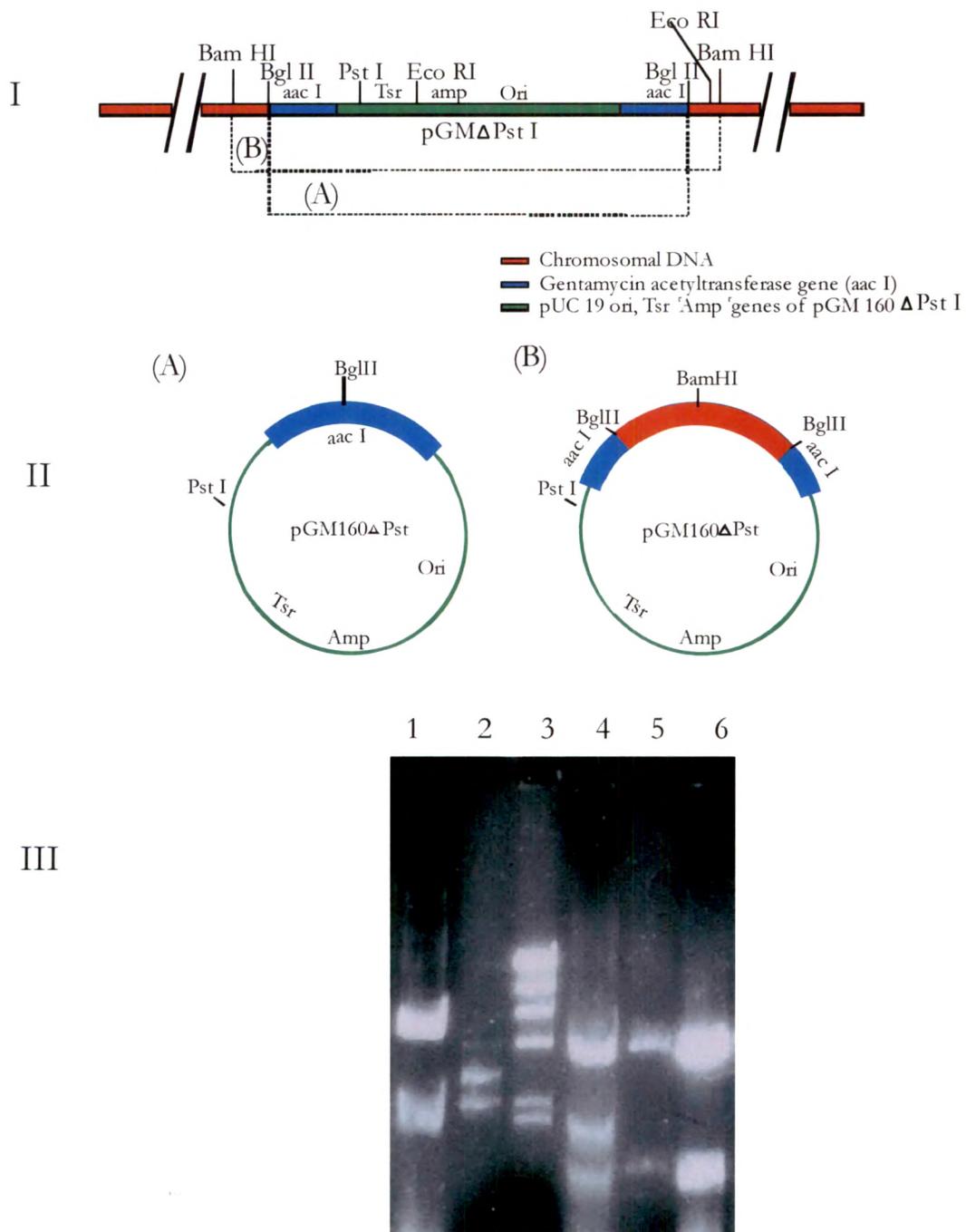


Fig.2.13: Proof of genetic correctness of predicted deletion structure of mutant JPI. I) RE map of deletion region of JPI. II) Predicted structure of the retrieved plasmid following *Bgl*II (A) and *Bam*HI (B) digestion of genomic DNA of JPI. III) RE analysis of the retrieved plasmid.

This 2.3 kb *Bam*HI DNA has been cloned in PBSK (Fig.2.13, III lane2), it is released by *Bgl*II digestion from the retrieved plasmid (Fig. 2.13,III lane 1) proving that this DNA was integrated into the chromosome and retrieving it from the same has resulted in this reversal.

Each of the two experiments above validated the predictions and confirmed the genetic correctness of the deletion in the PKS⁻ mutant.

6. Southern Hybridization of mutant JP1: Deletion in JP1 was also analysed by Southern hybridization. Genomic DNA was digested with *Bam*HI and *Bgl*II and was probed with pGMΔpst as probe DNA. The probe hybridizes to ~9 kb *Bam*HI and ~6.6 kb *Bgl*II DNA of mutant respectively (Fig.2.14), reinforcing the genetic correctness of the mutant.

Discussion & Conclusions: Confirmation that the *S. flaviscleroticus* DNA, cloned by homology to actinorhodin PKS genes, indeed encodes functional PKS for *S. flaviscleroticus* specific polyketide production was obtained by targeted deletion of equivalent region in the *S. flaviscleroticus* chromosome. Analysis of the culture extract obtained from mutant showed that the ability to produce *S. flaviscleroticus* specific polyketide had been lost in the mutant, demonstrating that the genetic potential of the organism is expressible- a polyketide compound is produced by this organism and that it is associated with bioactivity. The structure of the deletion was confirmed and found to be correct, by genetic evidences and Southern analysis. The results in this chapter unambiguously defined the major part of the bioactivity profile being due to the production of polyketide. Two other bioactivities produced by the deletion mutant indicate that the multiple bioactivity profile of *S. flaviscleroticus* is due to production of different types of bioactive compounds, besides polyketide component.

B Bgl λ H B Bgl



Fig. 2.14: Southern hybridization of mutant JP1s genomic DNA using pGM Δ pst as probe. B, *Bam*HI restriction digestion; Bgl, *Bgl*III restriction digestion of the genomic DNA.