Chapter 1

Genomic DNA Library Construction and deducing the overlap

Introduction:

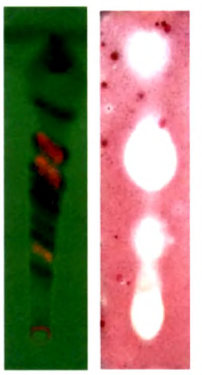
With the advent of molecular biology of *Streptomycetes* in the 1980s by Professor Hopwood (John Innes Centre, Norwich, UK), several biosynthetic gene clusters for different antibiotic classes have been cloned and characterized. Realization of the polyketides being the most abundant medicinal agents among natural products, genetic potential of several *Streptomyces* species has been tapped, aided largely also by the almost complete linkage between the biosynthetic and the resistance genes. About 30 gene clusters have been either completely or partially cloned for aromatic polyketides.

Complementing the classical screening for natural compounds, genome driven approaches like combinatorial biosynthesis are permanently gaining relevance for the generation of new structures. This technology utilizes the combination of genes from different biosynthesis pathways resulting in the production of novel or modified metabolites. The basis for this strategy is the access to a significant number of genes and the knowledge about the *act*ivity and specificity of the enzymes encoded by them.

Studies on characterization of new cluster of PKS genes were undertaken with the aim to further the potential of combinatorial biosynthesis and to understand the rules of PKS programming. The identification of the organism, *S. flavisclerotícus* for this purpose was based on certain criteria like (i) brevity of knowledge in the public domain of the literature of its production potential, (ii) the genetic potential being revealed in terms of production of polyketide compound in possessing the genes for aromatic type II polyketide compound, a finding borne by the genome hybridization to conserved PKS genes (see below), and (iii) that *S. flavisclerotícus* also produces multiple bio*act*ivities; thus the happenstance that the polyketide compound is produced is real.

The organism, *S. flavisclerotícus* produces yellow chromophore(s), which we found to be the antimicrobial in nature. The antibiotic principle can be extracted in the organic solvents like methanol, chloroform and ethyl acetate and separated by Thin Layer Chromatography in to five different spots, all of them biologically *act*ive against the gram positive bacteria like *S. aureus, M. luteus,* and *B. subtilis*. To investigate if the polyketide contributes to the bio*act*ivity profile of *S. flavisclerotícus*, we undertook

cloning the polyketide biosynthesis genes of this organism, alongside the chemical characterization studies.



A **B**

Figure 1 : A : Thin Layer Chromatography(TLC) of the crude extract of *S.flavisclerotícus* in Chloroform:Methanol solvent mix (9.3:0.7), B: Bioautogram of the TLC developed using *Micrococcus luteus.*

1.Results and Discussion:

1.1 Construction of genomic DNA library and colony hybridization:

1.1.1. Partial digestion and size fractionation of the genomic DNA of *S. flavisclerotícus*:

Following pilot experiment of standardization of partial digestion of the genomic DNA with 4 bp cutter restriction enzyme *Sau3A*I (see detailed protocol in Materials and Methods), 5-10 µg of chromosomal DNA of *S. flavisclerotícus* was partially digested with the *Sau3A*I. The samples were then size fractionated on continuous sucrose gradient of 10% and 40% (w/v). The digested DNA sample heated at 68 °C for 10 mins and then cooled at 20 °C was then layered gently on the top of the sucrose gradient. The tubes were centrifuged at 25,000 rpm for 22 hrs at 20 °C, punctured at the bottom and 500 µl fractions were collected. From 13 fractions 5µl of sample was loaded on the gel to

monitor the size and amount of DNA recovered after the centrifugation. The fractions in the range of 20-40 kb were pooled, ligated to *Bam*HI-*Hpa*I treated pKC505 cosmid,

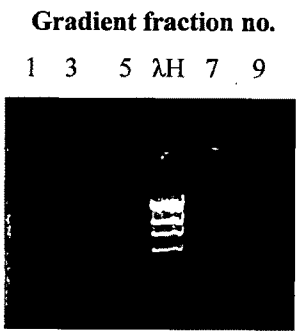


Figure 1.1: Size fractionation of the partially digested genomic DNA (by *Sau*3AI) of *S.flavisclerotícus.*

Characteristics of the cloning vector, pKC505:

- pKC505 is an *E.coli- Streptomyces* shuttle vector with containing *E.coli* specific *colEI* origin of replication and *Streptomyces* specific SCP2* origin of replication. The instability of the vector was realized due to lack of stability function of the SCP2* replicon (Muth et. al., 1989).

- The vector contains three *cos* sites - one between the unique *Hpa* I and the unique *Bam* HI site, the other two *cos* sites are present between *Hpa* I and unique *Eco*RI site, making it a suitable candidate for the packaging the cloned DNA in the λ particle.

- Apramycin resistance gene [*aac(3)IV*] expressible in both *Streptomyces* and *E. coli* makes it suitable for the selection of cloned DNA in both the species - *E.coli* and *Streptomyces*.

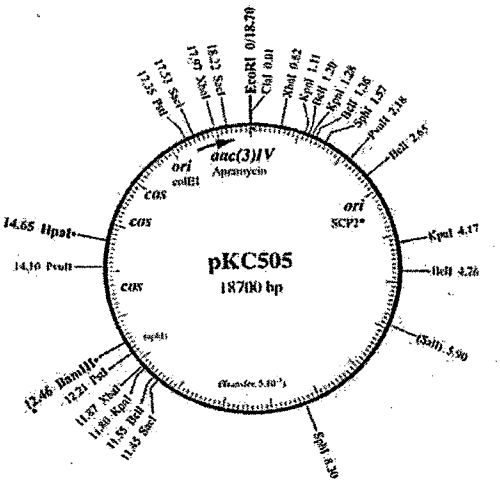


Figure 1.2: The *E.coli- Streptomyces* shuttle vector pKC505 used in construction of genomic DNA library.

The scheme depicting the cloning of genomic DNA insert in the cosmid vector is shown below. (Fig. 1.3) Briefly, 5 µg pKC505 was digested with restriction enzyme *Hpa*I, dephosphorylated using calf intestinal alkaline phosphatase (CIP) to prevent self ligation of the vector fragments and subsequently restriction digested with *BamHI*. Genomic DNA fractionated by density gradient was ligated to vector DNA at stoichiometry of 2:1. 1.0µg of the ligated DNA was added to *in vitro* packaging extract (Promega), incubated at 22^{0}C for 2.5 hrs, clarified with chloroform and added to 5 ml of host MC1061 cells grown in the presence of 0.2% maltose + 10mM $MgCl_2$ to an OD of 0.5. After 30 min at room temperature, 20 ml of Luria broth was added to the infected cells and incubation continued at 30^{0}C for another 1.5 hrs. 0.1 ml of cells was plated on LA supplemented with 25 µg/ml of apramycin.

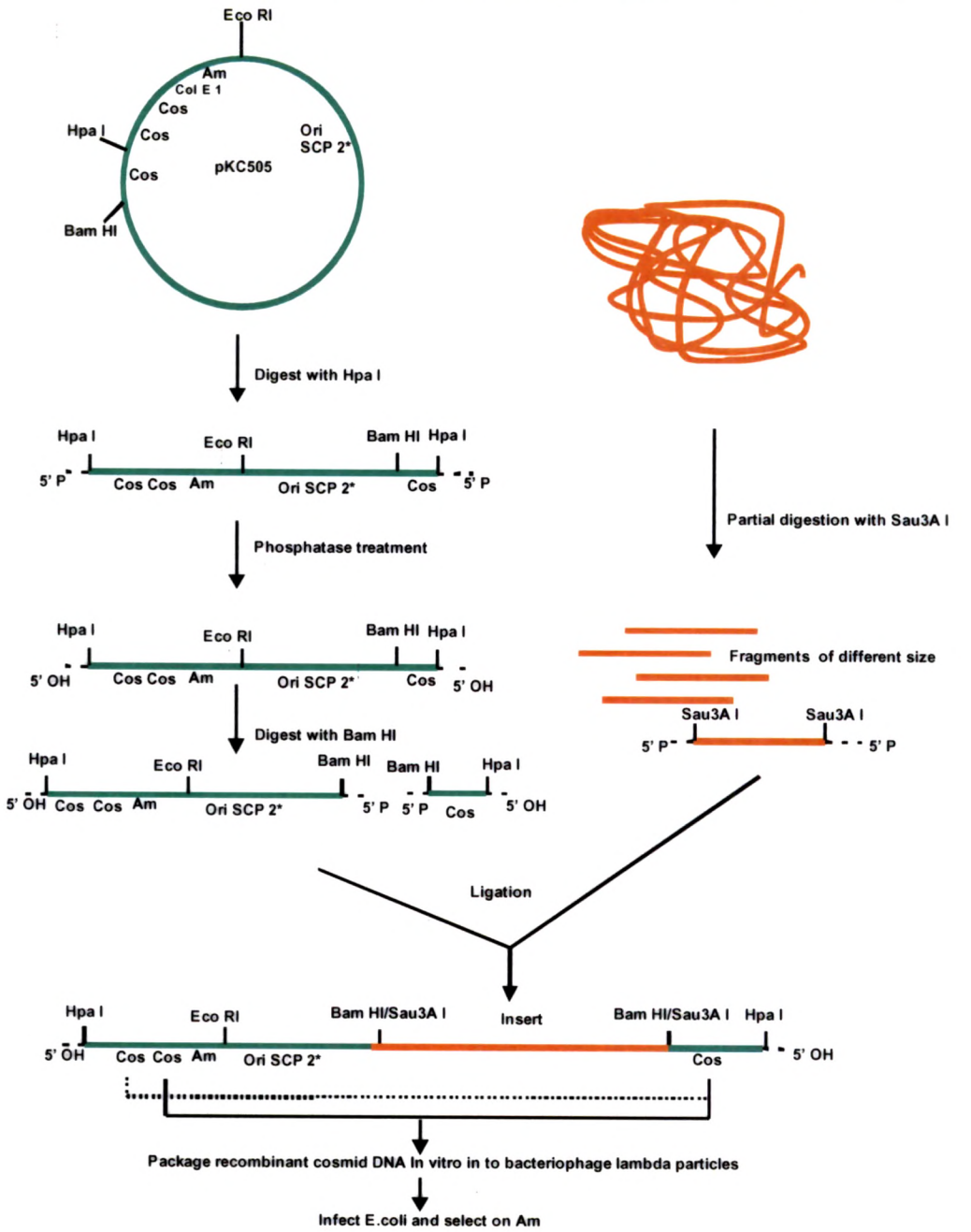


Figure 1.3: Schematic presentation of *in vitro* packaging of cosmid + genomic DNA

As a result of the cosmid backbone packaging along with the insert in the λ phage particles (the DNA between two successive *cos* sites), the *Hpa* I site was lost thus

shortening the vector by ~ 2.5 kb and bringing the unique *Eco*RI site in the cosmid that much nearer to the *Bam*HI site used for cloning (See Fig. 1.2).

Estimation of efficiency of cloning of genomic DNA insert:

Given that 0.1 ml of *in vitro* packaged λ infected cells yielded ~ 800 transductants, the total number of transductants per μg DNA (cloning efficiency) was equal to 800 x 10 x 20 = 1.6 x 10^5 /μg DNA.

Estimating the average insert size:

Restriction digestion of DNA from four independent clones (Fig.1.4) indicated that the average size of the insert DNA is in the range ~ 25-35 kb. This was estimated by adding up the sizes of all the other bands in the restriction digestion of a cosmid clone, and summing up the size of all DNA bands. Also more than 90% of the clones contained the insert (Fig. 1.4).

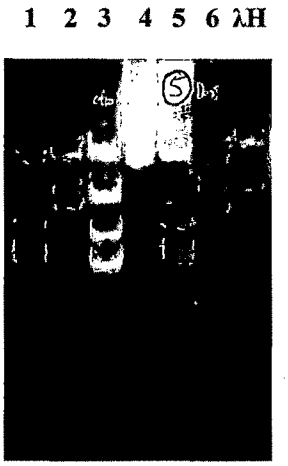


Figure 1.4 : *Pst*I digestion of the randomly chosen clones from the library: The first 13.7 kb band is derived purely from the cosmid backbone and thus common to all the clones.

The probability that the library is representative and complete is calculated using Clark and Carbon formula that relates the probability (*P*) of including any DNA sequence in a random library of *N* independent recombinants.

$$N = \frac{\ln(1-P)}{\ln\left(1-\frac{1}{n}\right)}$$

Thus, $1.6 \times 10^5 = \ln(1\text{-}P)/\ln(1-1/20) = \ln(1\text{-}P)/\ln(0.95) = \sim 1$

Thus the probability that the library is representative of the complete genome is more than 99.99%.

1.2: Preliminary characterization of library:

Characterization of the library was done in two ways.

(i) Genomic DNA library is representative:

After establishing that the insert DNA in each of the four randomly chosen clones was approximately 20-25 kb in size (Fig. 1.4), the library being representative was found out as follows.

DNA from collection of library clones was digested with restriction enzyme *Pst*I. The smear of *Pst*I digest begins at ~ 30 kb and continues up to 2 kb with the major vector derived 13.7 kb *Pst*I fragment present in the digest of the library pool, whereas, the *Pst*I digest of the genomic DNA is same as that of the cosmid library pool minus the vector derived fragment (Fig.1.5). The result indicates that at least with respect to *Pst*I, the library is representative and complete.

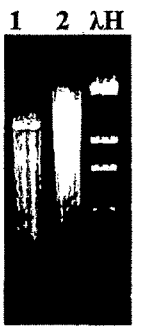


Figure 1.5: *Pst*I digestion of library pool and the *S.flaviscleroticus* genomic DNA. Lane 1 represents the library pool in which pKC505 derived 13.7 kb fragment is visible. Lane 2 is the *Pst*I digested *S.flaviscleroticus* genomic DNA

(ii) Demonstration of absence of cloning artifact:

The colinearity of insert DNA in one of the cosmid clone #MC 6 with that with chromosome was established. Here the chromosomal DNA of *S. flaviscleroticus* and the cosmid #MC6 was each digested with restriction enzyme *Pst*I and *BamH*I, electrophoresed, transferred onto hybond nylon membrane and probed with the uniformly labeled total DNA of MC 6 clone (Fig. 1.6).

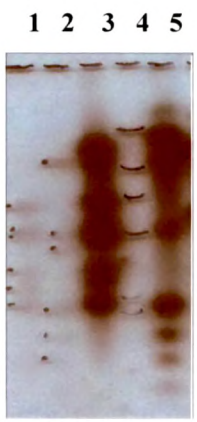


Figure 1.6: Insert DNA in # MC6 being colinear with the chromosomal DNA: Southern hybridization of MC 6 cosmid DNA digested with *Pst*I (lane 3) and *Bam*HI (lane 5), and corresponding enzyme [*Pst*I (lane 1); *Bam*HI, (lane 2)] digest of genomic DNA of *S. flaviscleroticus* using total MC 6 DNA as a probe. Lane 4 contains the λ DNA digested with *Hind* III.

Almost all of the insert derived fragments, whether arising from *Pst*I or *BamH*I digestion of cosmid MC6 are same as that from corresponding enzyme digest of chromosomal DNA. Evidently, the cloning artifacts are at their minimum in the library constructed in this work, a problem that accounts for spurious linkage between unrelated sequences. For example, as could be seen in the lane no 1 and 3, the 13.7 kb *Pst*I fragment containing only vector DNA, present in *Pst*I digestion of MC6, is conspicuously absent in the *Pst*I digest of genomic DNA (lane 1); rest of the DNA fragments are absolutely matching between lanes 1 and 3.

1.3 Colony hybridization of the genomic DNA library:

About 10,000 clones from the library were analyzed for possessing the PKS II genes. For this purpose, ~ 800-1000 cells were plated on 10 different plates supplemented with 25µg of apramycin. The DNA released from the lysed cells was transferred to nylon membrane, immobilized, fixed by baking under vacuum at 80^0C and hybridized with *actI* DNA radiolabeled uniformly with P^{32}. The stringency of hybridization and subsequent washings are as described in Materials and Methods. The point to take note of here is that the signal of hybridization could be retained even after extensive washings upto 0.2 X SSC and 65^0C, indicating the heterologous *actI* sequences being highly similar to PKS genes of *S. flavisclerotícus,* a fact borne by sequencing the DNA (see Chapter 3). From the first screen, about 200 hybridization-positive colonies were selected for the second screen which was done by manually gridding the inoculum on LA agar supplemented with 25µg of apramycin and subjecting them to hybridization under the same conditions as mentioned above for the first screen. The results of the second screen are shown in Fig.1.7. The clones taken from the plate no 1 were given the prefix 1 followed by their position on the plate. Similarly, with clones from the plate no 2 were given the prefix 2 followed by their position on the plate.

Ten clones namely, 1.51, 1.23, 1.1, 2.19, MC 6, 2.40, 2.46, 2.34, 2.30 etc. were selected from this experiment as putative PKS^+ clones.

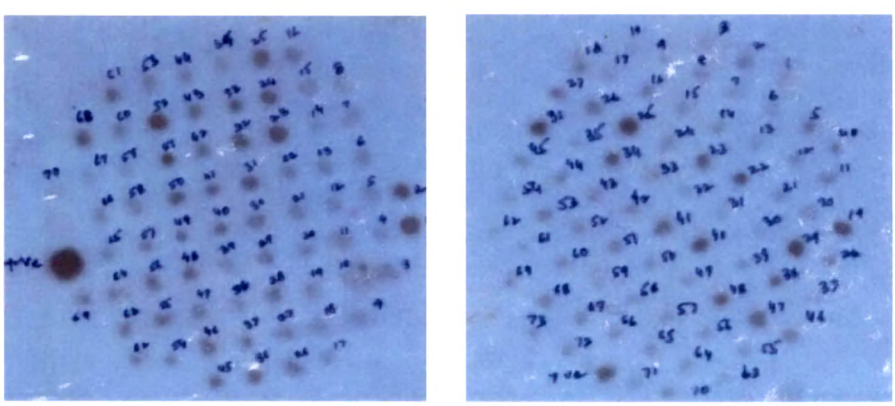


Figure 1.7: Colony hybridization of the few hundred gridded colonies selected from the first screen of 10,000 clones. Almost 100 clones from each plate were probed along with a positive control (pIJ2345, *actI*$^+$ plasmid) with *actI* DNA.

1.4 Analysis of the digestion pattern of the cosmid clones and deducing the overlap between them:

To determine the relation between the different clones, overlap between the clones was studied. DNA of each of the clones was subjected to single digestion with various enzymes (Figs. 1.4.1 and 1.4.2).

The *actI* hybridizing region shared between different cosmids, determined from the Southern hybridization studies (see below), was used as the reference fragment in different restriction digests to compare different clones. The DNA flanking the hybridizing region was found out for each clone and compared to its being present in other clones. Out of a total of 8 independent clones, four clones, 1.51, 1.23, 2.19 and 1.1 were chosen as the representative clones for spanning the maximum amount of DNA between them. We estimated that, together they span ~ 45 kb of PKS DNA (see also below). To deduce the overlap between them, the digestion pattern of these four clones with respect to the enzymes *Eco*RI, *PstI*, *BglII* and *BamHI* was studied.

1.4.1 **Eco*RI fragments:

8.0 kb : Common to 1.1, 1.51 and 2.19.
4.0 kb : Common to1.23, 1.51 and 2.19.
11.0 kb : Common to 1.51 and 1.23.
5.0 kb and 17 kb : Unique to 1.23 and 1.1 respectively.

*The vector derived fragments have been eliminated from the list.

This *actI* hybridizing 8.0 kb fragment is common to all the clones except 1.23; this clone contains almost complete 8.0 kb fragment but one of the *Eco*RI sites of the 8.0 kb DNA. Thus this fragment is continuous with the *Eco*RI site in the vector making it ~13 kb in size. The only fragment which is common to all is the 4.0 kb fragment suggesting that 8.0 kb and 4.0 kb are 'linked' with each other. Similarly, the 11 kb fragment is shared by both 1.51 and 1.23 besides 4.0 kb fragment, the fragments can be ordered as 11 kb – 4.0 kb – 8.0 kb. The unique 5.0 kb and 17 kb must be located at the two extremes of the 45

kb since they are unique to 1.23 and 1.1 respectively, 5.0 kb unique fragment from the cosmid clone 1.23 being the extreme left and the 17 kb fragment from 1.1 being extreme right of the ~45 kb region. This was further proved in the *Pst*I digestion of the cosmid clones where the 3.0 kb *Pst*I fragment in 1.1 is not seen in any of the other clones but 2.19. 1.1 also shows unique *Bgl*II which is not present in any other clone proving that 2.19 clone extends beyond the 8.0 kb fragment in to 17 kb but up to the first *Pst*I site (present 3.0 kb away from the *Eco*RI site of the 17 kb fragment) in the 17 kb fragment. The 5.0 kb fragment seen in the clone 1.51 is the one which is generated by the 8.0 kb *Eco*RI site to the *Eco*RI in the vector back bone. This could not be the part of the of the ~45 kb region as the *Pst*I digestion of the 1.23 and 1.51 differs.

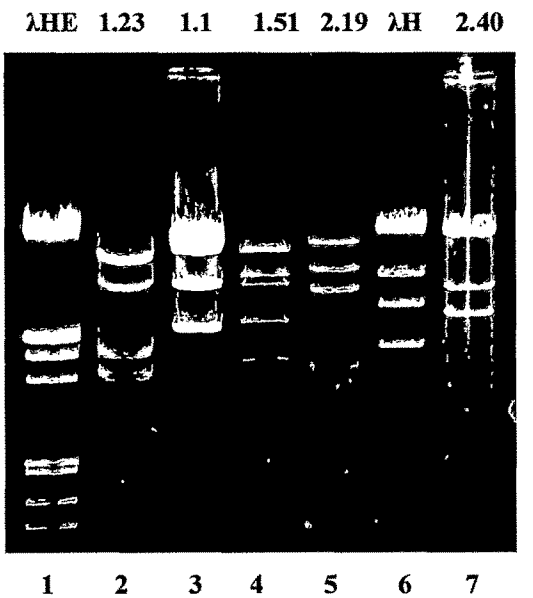


Figure 1.4.1: *Eco*RI digestion pattern of the cosmid clones. Lanes 2-5,7 represents the *Eco*RI digestion of different cosmid clones. Lane no. 1 and 6 contains the λHE (λ DNA digested with *Hind* III- *Eco*RI) and λH (*Hind* III) respectively.

_Pst_I digestion:

4.5 kb and 13.7 kb fragments : Common to all the clones

7.0 kb fragment : common to 1.51, 1.23 and 2.19.

3.5 kb fragment : common to 1.51,1.23 and 2.19.

6.0 kb fragment : common to 1.1 and 2.19

3.4 kb, 4.7kb, 4.2 kb and two 2.3 kb fragments : unique to 1.1 cosmid clone.

12.5 kb : Unique to 1.23 cosmid clone

10.5 kb :unique to1.51 cosmid clone

4.0 kb :unique to 2.19 cosmid clone

4.3 kb :unique to 1.23 cosmid clone.

We attempted to place the sites for the restriction enzyme *Pst*I with reference to *Eco*RI. Two *Pst*I fragments, 4.5 kb and 13.7 kb in size were common to all the cosmid clones of which the 13.7 kb fragment is derived from the vector backbone. 4.5 kb fragment contains the *actI* homologous region suggesting that this is the part of the 8.0 kb *Eco*RI fragment, as this is confirmed by the restriction digestion analysis of the 8.0 kb subcloned DNA (see chapter 2). 7.0 kb fragment is common to three clones 1.51, 1.23 and 2.19 suggesting that this fragment is present towards the left side of the 8.0 kb *Eco*RI fragment as it is absent in the clone 1.1. Similarly 3.5 kb fragment is also common to the same three clones, hence this is also placed on the left hand side of the 8.0 kb *Eco*RI fragment in the ~45 kb cloned region. This also questions the placements of these two fragments, (7.0 kb and 3.5kb) with respect to each other. In the *Eco*RI digestion we have fixed the order of different *Eco*RI DNA fragments as 5.0 kb-11.0 kb-4.0 kb- 8.0 kb -17.0 kb. This order has also been confirmed from the restriction map constructed by individually cloning all the *Eco*RI fragments and analyzing their single and double digestions with respect to four enzymes. Out of these two fragments, the 3.5 kb *Pst*I fragment is generated by the *Pst*I present in the 8.0 kb *Eco*RI fragment to the *Pst*I in the 4.0 kb *Eco*RI, and 7.0 kb is generated by the *Pst*I from the 4.0 kb *Eco*RI fragment to the *Pst*I in the 11.0 kb *Eco*RI fragment.

The 6.0 kb fragment common to 1.1 and 2.19 has to be the part of the region towards the right hand side of the 8.0 kb *Eco*RI fragment generated by the *Pst*I from the 8.0 kb fragment to the *Pst*I from the 17 kb fragment. This suggests that clone 2.19 extends at

least up to 3.4 kb in the 17 kb fragment beyond the limits of 8.0 kb *Eco*RI fragment. 3.4 kb, 4.7 kb 4.2 kb and three 2.3 fragments are unique to 1.1 cosmid, of which 3.4 kb, 4.2 kb and two 2.2 kb fragments are internal fragments which are unique to 1.1 clone and are all part of the 17 kb *Eco*RI. The 5.2 kb fragment is generated by the last *Pst*I fragment in the 17 kb fragment to the *Pst*I in the vector which is 3.5 kb away from the cloning site, and one of the 2.2 kb fragment is generated by the *Pst*I which may be 0.2 kb away in the vector from the cloning site to the first *Pst*I site in the 1.1.

Another unique fragment seen in 1.23 clone is the 12.5 kb fragment, this is generated by the *Pst*I in the 11.0 kb *Eco*RI DNA to the *Pst*I in the 5.0 kb *Eco*RI fragment. Similarly the 10.5 kb unique DNA produced by 1.51, 4.0 kb by 2.19 and 4.3 kb by 1.23 are each produced by the last *Pst*I of the insert to the *Pst*I from the vector which is 3.5 kb away.

***Bgl*II Digestion:**

4.0 kb and 2.0 kb : common to all cosmids,
6.5 kb : common to 1.23, 2.19 and 1.51.
9.5 kb : common to 1.51 and 1.23.
16.0 kb : unique to 1.1.
1.9 kb : unique to 1.23.

*The restriction enzyme site for *Bgl*II is absent from the vector backbone.

The 4.0 kb *Bgl*II is the *actI* hybridizing fragment which is common to all the cosmid clones. 4.0 kb fragment, thus is the part of the 8.0 kb *EcoR*I fragment and also shares the same region with the 4.5 kb *Pst*I fragment. The 2.0 kb fragment is also common to all the cosmids so this also must be the part of the 8.0 kb *Eco*RI fragment and contiguous with 4.0 kb fragment. The 6.5 kb fragment is common to three cosmids 1.23, 2.19 and 1.51 so it must be present towards the left of the 8.0 kb *Eco*RI/4.0 +2.0 kb *BglII* fragment. This fragment could either be the part of the 8.0 kb fragment spanning 4.0 kb *Eco*RI fragment and extending up to the 11 kb, or could be the part of the 11 kb. Since 1.51 and 1.23 shows another unique fragment in 9.5 kb so this fragment should be the one emerging from the 11.0 kb *Eco*RI fragment to the 5.0 kb *Eco*RI fragment (as some amount of 5.0 kb *Eco*RI fragment is also present in 1.51 besides 11.0 kb *Eco*RI fragment). Thus the

presence of 6.5 kb fragment in all the three clones 1.23, 2.19 and 1.51 clones suggests that this is located in the 8.0 kb *Eco*RI fragment and extending up to the *Bgl*II site in the 11.0 kb fragment. The 17 kb unique fragment seen in 1.1 is produced from the *Bgl*II in the 8.0 kb fragment to the *Bgl*II in the 17 kb suggesting that 17 kb contains only one *Bgl*II site and that too is present towards the extreme right spanning almost complete 17.0 kb *Eco*RI fragment. The unique 1.9 kb *Bgl*II fragment seen in the clone 1.23 has to be the part of the 5.0 kb *Eco*RI fragment as it is not seen in any other clones. Several of the predictions were tested in the fine mapping of the different DNA fragments common and unique to different cosmid clones and found to be true.

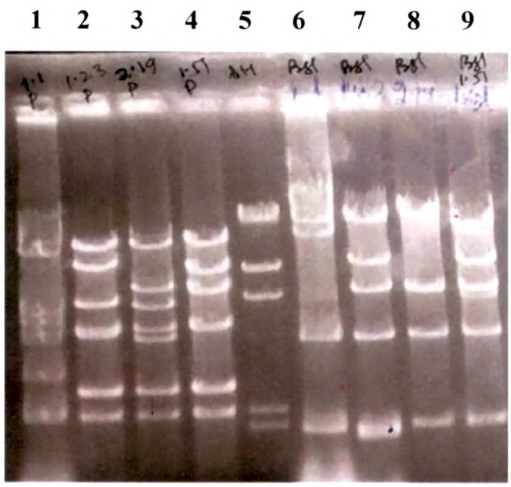


Figure 1.4.2: *Pst*I and *Bgl*II digestion pattern of the cosmid clones. Lane 1-4 represents the *Pst*I digestion of the cosmid clones indicated in the picture. Lane 6-9 represents the *Bgl*II digestion of the cosmids. Lane no 5 contains the λH DNA digested with *Hind* III.

1.5. Southern hybridization of the different cosmid clones:

The cosmids 1.1, 1.23, 1.51, 2.19 and MC 6 were digested with the restriction enzymes *Pst*I, *Eco*RI and *Bgl*II and probed with the *actI* DNA. The hybridization was carried out under high stringency conditions as described in Materials and Methods.

1.5.1. Southern hybridization of *Eco*RI digested cosmid clones 1.1, 1.23, 1.51, MC 6 and 2.19:

The *actI* hybridization signal in 1.1, MC 6, 1.51 and 2.19 (Fig.1.5.1) corresponds to the same 8.0 kb *Eco*RI fragment suggesting that the 8.0 kb *Eco*RI fragment contains the *actI* hybridizing genes. Cosmid clone 1.23 on the other hand, does not produce 8.0 kb *Eco*RI fragment, and exhibits the hybridizing signal with high molecular weight fragment. As discussed earlier, this clone does not have the second *Eco*RI site of the 8.0 kb *Eco*RI fragment and this band continues up to the *Eco*RI site in the cosmid back bone. Thus this band is larger than the 8.0 kb *Eco*RI fragment.

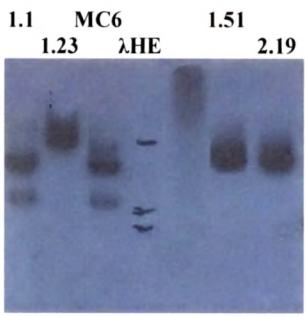


Figure 1.5.1: Southern hybridization of the *Eco*RI digested cosmid clones using *actI* DNA as the probe. Lane 4 contains the *Hind* III-*Eco*RI digested λ DNA

1.5.2. Southern hybridization of *Bgl*II digested cosmid clones 1.1, 1.23, 1.51, MC 6 and 2.19 :

In the previous section we discussed the southern hybridization pattern of the selected cosmid clones digested with the enzyme *Eco*RI. In this section the southern hybridization of the 1.1, 1.23, MC 6, 1.51 and 2.19 cosmids with respect to *Bgl*II digestion is discussed. The hybridization signal in all these clones including clone 1.23 corresponds to the 4.0 kb *Bgl*II fragment. The result is significant in that 4.0 kb *Bgl*II fragment not only contains the *actI* hybridizing genes or the conserved region but is also the part of the 8.0 kb *Eco*RI fragment. This result also testifies the earlier gel picture of the *Bgl*II digested cosmid clones, which shows the presence of complete 4.0 kb *Bgl*II fragment in all the cosmid clones including 1.23. This is explained in the next chapter in detail while constructing the restriction map of the PKS II cluster from this organism.

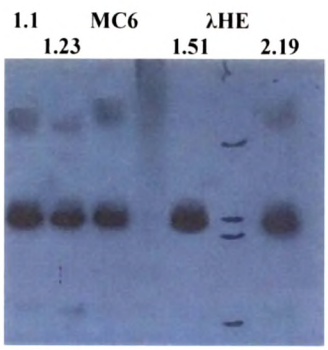


Figure 1.5.2 : Southern hybridization of the *Bgl*II digested cosmid clones using *actI* DNA as the probe. Lane no. 6 contains the *Hind* III- *Eco*RI digested λ DNA.

1.5.3. Southern hybridization of *Pst*I digested cosmid clones 1.1, 1.23, 1.51, MC 6 and 2.19 :

This section describes the southern hybridization of the *Pst*I digested cosmid clones 1.1, 1.23, 1.51, MC 6 and 2.19. All the clones show strong hybridizing signal corresponding to the ~4.5 kb DNA fragment. As seen in earlier in the section describing the *Pst*I restriction digestion of the cosmid clones, the ~4.5 kb *Pst*I fragment is present in all the cosmid clones including 1.23. Thus, this ~4.5 kb *Pst*I fragment is also internal part of 8.0 kb *Eco*RI fragment and 4.0 kb *Bgl*II fragment overlaps the ~4.5 kb *Pst*I fragment and shares the hybridizing region.

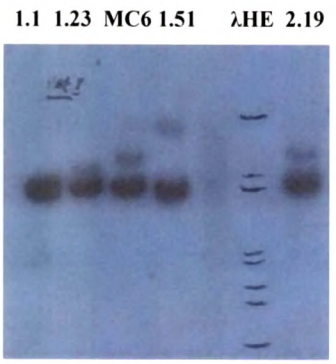


Figure 1.5.3: Southern hybridization pattern of the *Pst*I digested cosmid clones using *actI* DNA as the probe. Lane no. 6 contains the *Hind* III- *Eco*RI digested λ DNA.

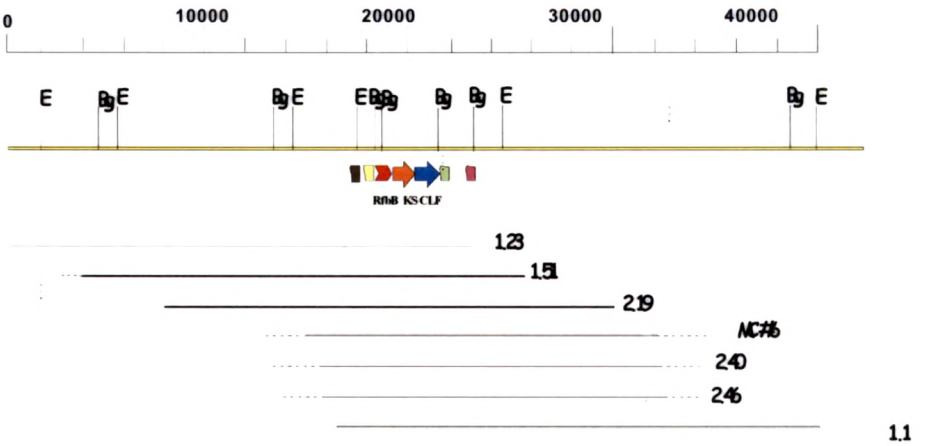


Figure. 1.6: Restriction map of the ~40Kb DNA with respect to E:*Eco* RI and Bg:*Bgl* and the limits of the insert DNA in the different cosmid clones. Analysis of the sequenced DNA indicates the presence of complete ORF (complete arrows) and incomplete ORFs (broken arrows) of

NDP Glucose Dehydratase, Beta Keto-acyl Synthase, Chain Length Factor O-methyl transferase, NDP-Glucose synthase, Cyclase, Monooxygenase

1.6: Conclusion:

This chapter discusses the successful construction of the *S. flavisclerotícus* library, its characterization, and isolation of ten certain clones containing the *actI* homologous region, the most conserved region in the aromatic polyketide synthase gene cluster.

- Statistical analysis of the number of clones of the library indicated that the library is complete and is also without cloning artifact, as indicated by the colinearity of the restriction fragments of the one of the clones with that of the genomic DNA.

- The library was representative as evidenced by the *Pst*I digestion of library pool DNA being the same as that of *S. flavisclerotícus* genomic DNA.

- The overlap among different clones was determined using restriction enzyme digestion analysis and Southern hybridization analysis. Four clones, namely, 1.23, 1.51, 2.19, and 1.1 together span a total of ~ 45 kb PKS DNA; remaining clones contained insert that was a ~20-25 kb part of this ~ 45 kb DNA.

- Using the information of the overlap between different cosmid clones, the working restriction map of the PKS region was constructed (Figure:1.6) This information was corroborated in the fine mapping by subcloning (Chapter 2).