# *Chapter 2*

# *Construction of Restriction Map*

# Introduction:

For tapping the genetic potential of the lesser known strain *S. flavisclerotícus*, in terms of production of bioactive polyketide compound, an approach to studying the antibiotic gene cluster included construction of the genomic DNA library of the strain (described in chapter 3). The $PKS^{+}$ clones' were isolated by colony hybridization of the genomic DNA library. Construction of the overlap by restriction digestion analysis of different clones is also described in the chapter. Several presumptions regarding derivation of the overlap and reasoning used were tried and tested rigorously in fine mapping studies of the 45 kb of the PKS DNA. As against the working restriction map constructed for the PKS DNA by derivation of the overlap, the fine mapping studies involved sub-cloning different sized fragments from the various cosmids and ordering them. Cloning of smaller fragments not only made the restriction mapping experiments easier and manageable but the restriction digestion analysis also became simpler. This also turned out to be cost effective when the clones were sent for the sequencing.

The fine restriction mapping studies were initiated by the cloning of different *Eco* RI fragments from the collection of cosmid clones isolated in this work. As shown in the previous chapter, the ~45 kb region was divided into five *Eco*RI fragments, six *Bgl*II fragments and seven *Pst*I fragments. Thus to construct the restriction map, all the five *Eco*RI fragments were sub-cloned in pBluescriptKS vector and subjected to restriction digestion with four enzymes namely, *Eco*RI, *Bgl*II, *Pst*I and *Bam*HI, singly and in pairwise combination.


**Figure 1a: Placement of five *Eco*RI fragments constituting ~45 kb region.**

(i) The first 5.0 kb *Eco*RI fragment (from the left as shown in the figure.1a) which is the unique to 1.23 cosmid was cloned into pBKS.

(ii) The second fragment, the 11 kb *Eco*RI DNA which is common to both 1.51 and 1.23 cosmids was cloned from both sources and the one from the 1.23 was cloned in both the

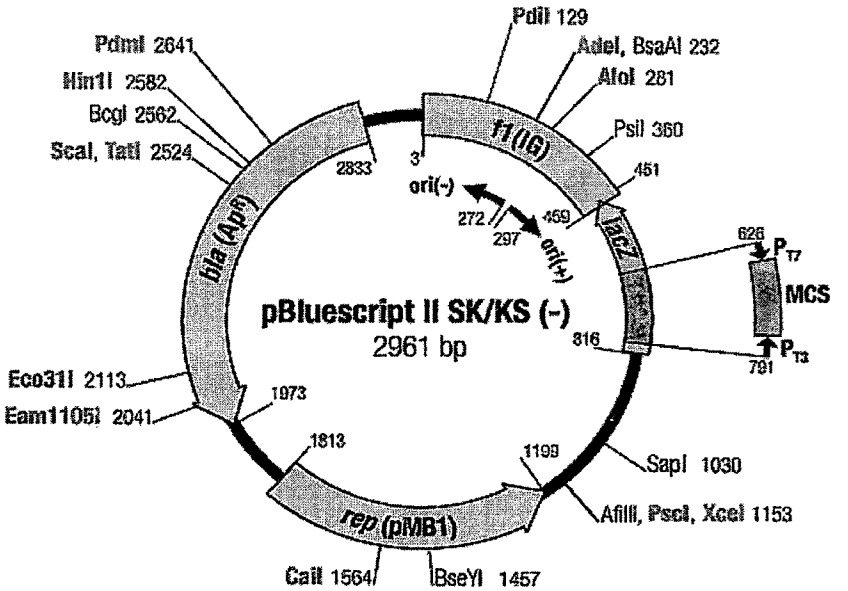
orientation. Importantly, the 11.0 kb DNA is indeed the same as is evident by the restriction enzyme digestion analysis of this insert DNA isolated from each of the two clones, reaffirming their origin without the cloning artifact, a fact that is repeatedly proved in this work.

(iii) The third and fourth fragment, 4.0 kb *Eco*RI and 8.0 kb *Eco*RI respectively were cloned from the cosmid 1.51. The last 17.0 kb fragment, unique to 1.1 cosmid, was cloned from the 1.1 cosmid. 4.0 kb- and 8.0 kb *Eco*RI inserts were also cloned in the opposite orientation during the screening of 17 kb insert from 1.1 cosmid's total digest. Once again, the same insert from different cosmids source was found to be identical for disposition of the restriction enzyme sites.

pBluescriptKS used for the cloning purpose is an important cloning vector, and is characterized for several aspects which are helpful in the cloning and maintenance of the foreign genes in the vector. Following are the characteristics:

- It is a high copy no. plasmid with *E. coli* origin of replication,
- Lac promoter for expression of the insert DNA in *E. coli* host,
- Ampicillin-resistance marker,
- Multiple cloning sites,
- The *lacZ* gene for α- complementation.

The cloning of all the *Eco* RI fragments was done in the *Eco* RI site in the MCS of the pBKS vector as shown in the



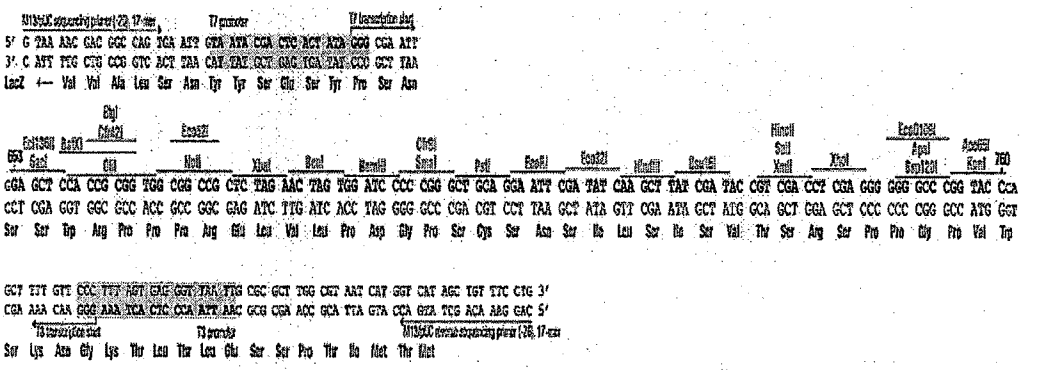



**Figure 2: plasmid BlueScript vector**

The cloning efficiency varied from 20% to 1% depending upon the size of the insert. For example, 17 kb insert was obtained in one out of 52 clones analyzed as against 4 kb *Eco*RI DNA present in 1 out of 5 clones analysed in an experiment attempting to clone 17 kb DNA from EcoRI digest of 1.1cosmid. The cloning of the 5 kb *Eco*RI fragment (from 1.23 cosmid clone), 11.0 kb *Eco*RI fragment (from 1.23 and the 1.51 cosmid), and 8.0 kb *Eco*RI fragment (from 1.51 clone) was done by digesting the respective cosmid DNA with *Eco*RI enzymes and then eluting the fragments from the agarose gel with help of glass wool. To clone the 4.0 kb *Eco*RI fragment (from 1.51 cosmid) and 17.0 kb

*Eco*RI fragment (from 1.1 cosmid clone), their respective parent cosmid clones were digested with the *Eco*RI and then the complete digestion mix was ligated with the *Eco*RI digested pBKS vector. This complete ligation mix was then transformed and from this experiment, the 4.0 kb and 17 kb *Eco*RI containing clones were screened. In this very experiment, clones with 11.0 kb and 8.0 kb fragments cloned in another orientation were also obtained. These clones were then subjected to single and double digestion with respect to four enzymes *Eco*RI, *Bgl*II, *Bam*HI and *Pst*I. These single and double digestions analysis of the different clones helped in constructing the restriction map. This analysis of the clones and the restriction map helped in further subcloning of these fragments. These subcloned fragments were sent for the sequencing the results of which will be explained in the next chapter.

## 2.1: Results and discussion:

### 2.1.1 Restriction Map of 5.0 kb *Eco*RI fragment:

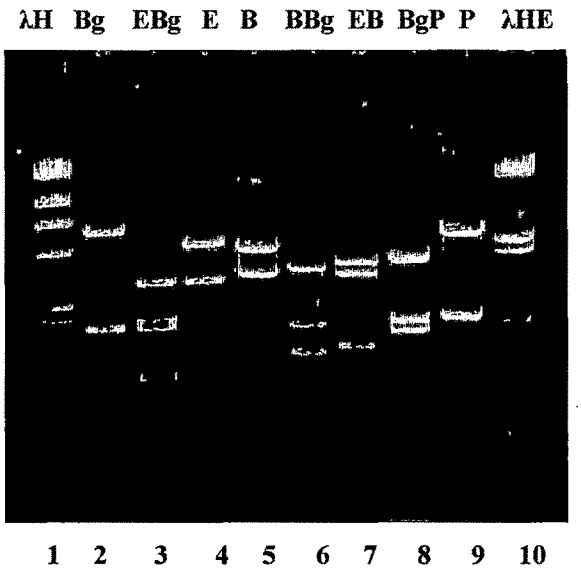



**Figure 2.1.1: Restriction digestion pattern of the 5.0 kb *EcoRI* fragment with respect to four enzymes E: *Eco*RI, Bg: *Bgl*II, B: *Bam*HI, P: *Pst*I.**

| Enzyme (single digestion) | No of fragments generated | Enzymes (double digestion) | No of fragments generated |
|---|---|---|---|
| *Eco*RI | Two (5.0kb, 3.0kb) | *Eco*RI –*Bgl*II (1.7kb,1.9kb,3.0kb,1.0kb) | Four |
| *Bgl*II | Two (1.7kb, 6.3kb) | *Bam*HI- *Bgl*II (4.0kb,1.9kb,1.2kb,0.6kb) | Four |
| *Bam*HI | Two (3.5kb, 4.5 kb) | *Eco*RI-*Bam*HI (3.0kb,3.5kb,1.5kb) | Three |
| *Pst*I | Two (2.4kb,5.6kb) | *Bgl*II- *Pst*I (1.6kb,1.5kb and 4.8kb) | Three |

**Table 2.1.1: The sizes of the fragments generated by the 5.0 kb *Eco*RI fragment by different enzymes digestion as shown in figure 2.1.1**

The 5.0 kb *Eco*RI fragment located at the extreme left of the ~45 kb region and unique to 1.23 cosmid was cloned in to pBKS vector. The cloning was done at the *Eco*RI site of the vector. For this purpose, the 5.0 kb fragment was gel eluted by the glass wool method as described in the (Sambrook et al.,2001). The eluted 5.0 kb DNA and the vector (pBKS) were used in the ratio of 6:1. The ligated product was transformed in the DH5α; transformants were selected on ampicillin –X gal plates and the white colonies checked for presence of insert. The gel shown above (Fig: 2.1.1) aided in generation of the restriction map of the 5.0 kb clone with respect to the four enzymes *Eco*RI, *Bgl*II, *Bam*HI and *Pst*I. All the four enzymes generate two bands each.

Upon digesting it with *Eco*RI enzyme, the cloned 5.0 kb fragment as expected, released from the 2.9 kb vector (lane no. 4). The vector backbone does not contain any *Bgl*II sites suggesting that two bands (1.7 kb and 6.3 kb ) generated in the *Bgl*II digestion (lane no.2) are because of the two internal sites for *Bgl*II (1.7 kb fragment is same as that in 1.23 cosmid which produces a doublet of 1.7 kb when digested with *Bgl*II (Fig. 1.4.2 Chapter 1). The *Eco*RI-*Bgl*II double digestion of this fragment shows the invariant 1.7 *Bgl*II fragment (lane no 3) and the pBKS vector backbone generated by the *Eco*RI digestion. The *EcoRI* (vector) - *Bgl*II (insert) fragment sizes from both the ends are 1.8 kb and 1.0 kb.

The exact placement of *Bgl*II will be clear from the *Bam*HI- *Bgl*II double digestion as the *Bam*HI is placed at one end of the vector and one in the insert. The *Bam*HI in the insert is present at the 3.5 kb distance from the *Bam*HI (vector) site (lane no. 5) which also remains the invariant fragment in the *Eco*RI- *Bam*HI double digestion in lane no 7. The 1.5 kb band in this lane suggests that internal *Bam*HI site is 1.5 kb away from the *Eco*RI of the vector as it also shows the presence of a 3.0 kb fragment which is the vector backbone generated by the *Eco*RI digestion. This internal *Bam*HI site is present in the 1.7 kb *Bgl*II fragment, generates two 1.2 kb and 0.6 kb (lane no.6) fragments in *BamHI-Bgl*II double digestion. In the same double digestion, one more band of the 1. kb size is generated which corresponds to the DNA between the *Bam*HI of the vector to the *Bgl*II in the insert. The rest of the DNA from second *Bgl*II to the *Eco*RI in the vector is part of the largest fragment of the size 4.0 kb including the vector backbone.

Similarly the *Pst*I digestion generates two fragments of which the insert derived *Pst*I is present at 2.4 kb distance from the *Pst*I of the vector which is present on the same side as *Bam*HI site (lane no.9). Since the 2.4 kb *Pst*I fragment is reduced to ~2.3 + 0.1 kb in the *Bgl*II –*Pst*I double digestion (lane no. 8), one of the two *Bgl*II site is present between the two *Pst*I sites at a distance of 0.1 kb and 2.3 kb (Fig. 2.1.1); the *Pst*I site in turn also reduces the 1.7 kb *Bgl*II fragment to ~1.6 kb + 0.1 kb .

The restriction map for the 5.0kb *Eco*RI fragment inferred from the analysis is as shown in Fig. 2.1.1a:

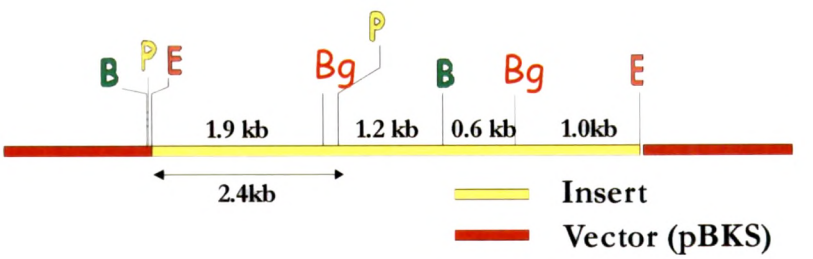



**Figure 2.1.1a : Restriction map for the 5.0 kb *Eco*RI fragment with respect to E: *Eco*RI, Bg: *Bgl*II, P: *Pst*I and B: *Bam*HI**

## 2.2 Restriction digestion analysis of 11 kb *Eco*RI fragment:

The 11.0 kb *Eco*RI DNA common to the cosmid clones, 1.51 and 1.23, was cloned from both the cosmids and in both the orientations in the pBKS vector referred to here as 11(+) and 11(-) respectively; +/- denoting the two opposite orientations.

### 2.2.1 *Eco* RI, *Bgl* II single and *Eco* RI – *Bgl* II double digestion of the 11.0 kb (+) *Eco* RI fragment derived from 1.23 and 1.51 cosmid clone.

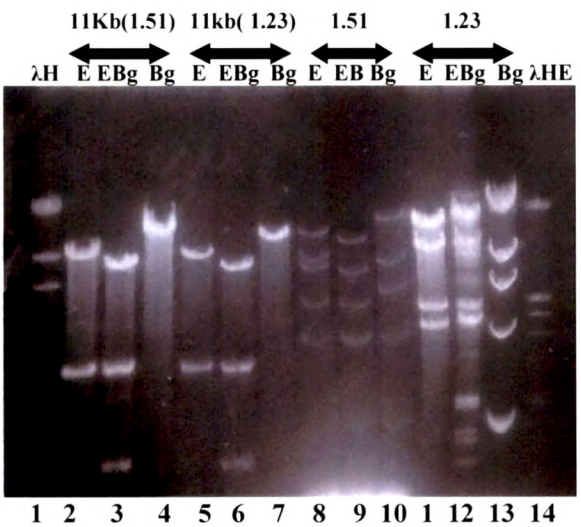



**Figure 2.2.1: Restriction digestion pattern of the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23) with respect to two enzymes E: *Eco*RI, Bg: *Bgl*II.**

| Enzymes | No. of fragments (From 1.23 and 1.51; both) |
|---|---|
| *Eco*RI | Two (11.0 kb, 3.0 kb) |
| *Bgl*II | One (14.0kb) |
| *Eco*RI-*Bgl*II | Three (1.2kb.3.0kb and ~10.0kb) |

**Table 2.2.1: The sizes of the fragments generated by the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23) by *Eco*RI and *Bgl*II digestion as shown in figure 2.2.1**

The gel shows the results of different digestion of 11 Kb *Eco* RI fragment cloned in the pBKS backbone from both 1.23 and 1.51 parent clones. The *Eco* RI digestion generates two bands; the first band corresponds to the 11 kb fragment and the second band is the pBKS vector corresponding to the 3 kb size confirming the cloning (lane no.2: 11 kb from 1.51 and lane no 5: 11 kb from 1.23).

Both the DNAs become linear upon digesting with *Bgl*II suggesting the presence of single *Bgl*II site in the insert (lane no.4: 11(+) kb from 1.51 and lane no 7: 11(+) kb from 1.23). The *Eco* RI – *Bgl* II digestion of both the clones generates three fragments of the size 1.2 kb, 3.0 kb and ~10.0 kb. The 3.0 kb is the vector backbone generated by the *Eco*RI digestion. DNA fragment of 1.2 kb band implies the distance between the *Eco*RI from the vector to the internal *Bgl*II, (lane no.3: 11(+) kb from 1.51 and lane no 6: 11(+) kb from 1.23). The direction of the *Bgl* II could be confirmed from the 11 kb (-) gel (to be explained later).

Placements of enzyme sites for *Eco*RI and *Bgl*II in 11.0kb *Eco*RI DNA is as shown:

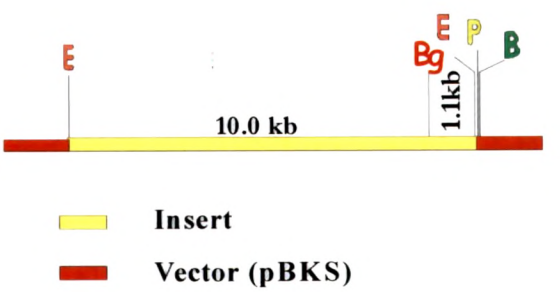



**Figure 2.2.1a : Restriction map for the 11.0 kb *Eco*RI fragment with respect to E: *Eco*RI, Bg: *Bgl*II.**

**2.2.2 *Eco* RI, *Pst*I single and *Eco* RI – *Pst*I double digestion of the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23).**

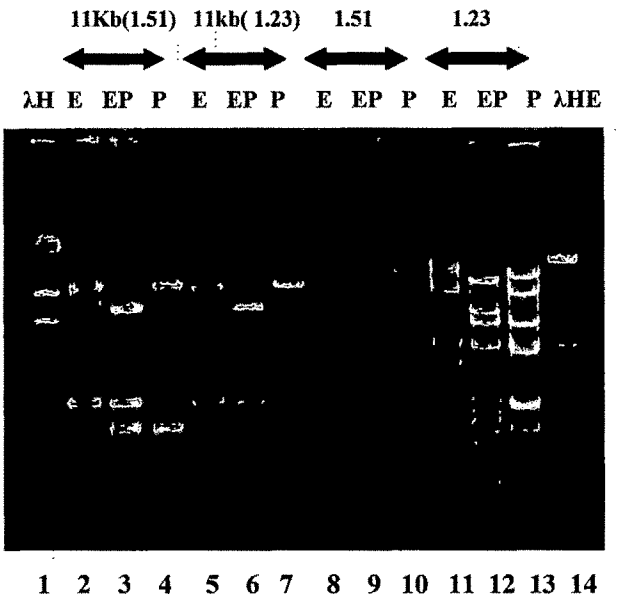



**Figure 2.2.2: Restriction digestion pattern of the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23) with respect to two enzymes E: *Eco*RI, P: *Pst*II.**

| Enzymes | No. of fragments From 1.23 and 1.51; both) |
|---|---|
| *Eco*RI | Two (11kb, 3.0kb) |
| *Pst*I | Two (2.5kb, 11.5kb) |
| *Eco*RI-*Pst*I | Three (2.5kb, 3.0kb, 8.5kb) |

**Table 2.2.2: The sizes of the fragments generated by the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23) by *Eco*RI and *Pst*II digestion as shown in figure 2.2.2**

The *Pst*I single digestion pattern in both the clones (from 1.23 and 1.51) shows that there is only one internal *Pst*I site in the the 11 kb fragment which is placed at 2.4 kb from the

*Eco*RI site(Lane no 4 and lane no 7). This is confirmed by the *Eco*RI –*Pst*I double digestion pattern which shows the release of same size *Pst*I fragment by *Pst*I/*Eco*RI (vector)- *Pst*I (insert); also generating the 3 kb vector backbone in the *Eco*RI digestion. This could be seen in the clones obtained from both the cosmid clones 1.23 and 1.51. Knowing the orientation of this fragment is important to understand how this fragment is joined with the adjoining *Eco*RI fragment.

Since the 11 kb insert cloned from cosmids 1.23 and 1.51 represents the same orientation, the clone containing the 11 kb insert representing the oppsosite orientation was constructed. This is explained in the subsequent section.

*Eco*RI and *Pst*I restriction enzyme analysis of the 11.0 kb *Eco*RI fragment can be diagrammed as below:

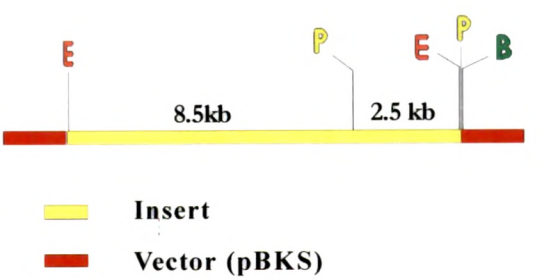



**Figure 2.2.2a : Restriction map for the 11.0 kb *Eco*RI fragment with respect to E: *Eco*RI, P: *Pst*I.**

**2.2.3 *Eco*RI, *Bam*HI single and *Eco*RI – *Bam*HI double digestion of the 11.0 *Eco* RI fragment derived from 1.23 and 1.51 cosmid clone.**

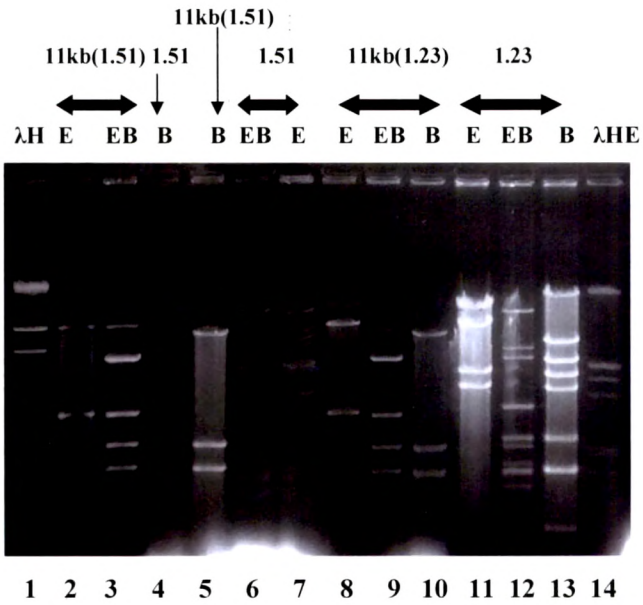



**Figure 2.2.3: Restriction digestion pattern of the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23) with respect to two enzymes E: *Eco*RI, B: *Bam*HI.**

| Enzymes | No of fragments |
|---|---|
| *Eco*RI | Two<br>11kb, 3.0kb |
| *Bam*HI | Four<br>0.2kb, 2.2kb,1.7kb, 10.1kb |
| *Eco*RI-*Bam*HI | Four<br>2.2 kb, 1.7kb, 3.0 kb, 7.0kb |

**Table 2.2.3: The sizes of the fragments generated by the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23) by *Eco*RI and *Bam*HI digestion as shown in figure 2.2.2**

11.0 kb fragment contains three insert borne *Bam*HI sites generating two fragments of the size 2.2 kb and 1.7 kb (Lane no 5 : 11kb clone from 1.51 and lane no.10: 11 kb from

1.23). These 2.2 kb and 0.2kb (this is not visible in this gel which is shown in the figure 2.2.4; BamHI digestion of the 11.0kbΔ*Pst*I (+) and (-) orientation) are the invariant fragment internal to 11 kb *Eco*RI fragment. The same sized *Bam*HI fragments are also present in the *BamH*I digestion of 8.0 kb *Eco*RI fragment as a result, a doublet is thus visible in the *BamH*I digestion of 1.51 (lane no. 4) and also in the *Eco*RI-*Bam*HI double digestion (lane no 6). However, expectedly neither *BamH*I (lane no 13) nor the *EcoR*I-*BamH*I digestions (lane no 12) of 1.23 cosmid produces the same doublet of 2.2 kb DNA as 1.23 cosmid lacks 11 kb DNA. The 2.2 kb DNA present in each of the digestions is same as the *actI* homologous DNA present in all the cosmid clones.

The *Eco*RI-*Bam*HI double digestion of the 11 kb fragment generates four bands of which one is invariable 2.2 kb fragment, second is 3.0 kb generated by the pBKS vector. The other two, 1.7 kb and 7.0 kb are generated by the *EcoR*I in the vector to the *BamH*I in the insert from both the ends (Lane no 3: 11kb clone form 1.51 and lane no.9: 11 kb from 1.23). This pattern matches with the 1.51 *Bam*HI single digestion (Lane no 4) pattern.

The 11 kb DNA cloned from both 1.51 and 1.23 cosmids behaved identically in various restriction enzyme digestions indicates that the insert in each has also been cloned in the same orientation which we designated as (+).

The restriction enzyme sites *Eco*RI and *Bam*HI can be placed in the 11 kb DNA as under:

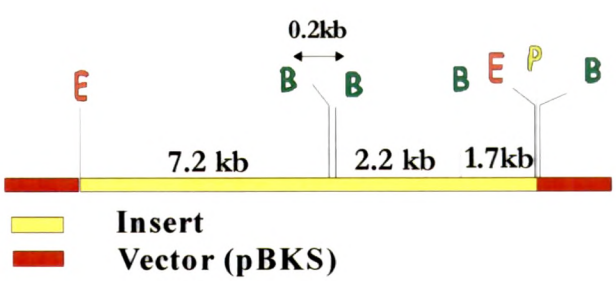



**Figure 2.2.3a : Restriction map for the 11.0 kb *Eco*RI fragment with respect to E: *Eco*RI, B: *Bam*HI.**

**2.2.4: *Pst*I, *Bgl*II single and *Pst*I – *Bgl*II double digestion of the 11.0 (+) *Eco* RI fragment derived from 1.23 and 1.51 cosmid clone.**

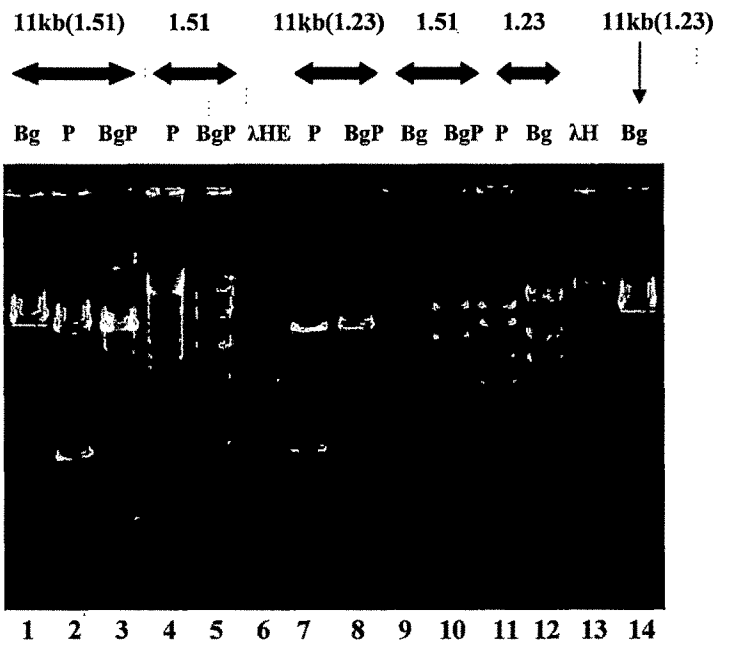



**Figure 2.2.4: Restriction digestion pattern of the 11.0 kb (+) *Eco*RI fragment (derived from the cosmid clones, 1.51 and 1.23) with respect to two enzymes Bg: *Bgl*II, P: *Pst*I.**

| Enzymes | No of fragments |
|---|---|
| *Bgl*II | One<br>15kb |
| *Pst*I | Two<br>11kb, 3.0kb |
| *Pst*I-*Bgl*II | Three<br>11.5kb, 1.5kb, 1.0 kb |

**Table 2.2.4: The sizes of the fragments generated by the 11.0 kb(+) *Eco*RI fragment (derived from both the cosmid clones, 1.51 and 1.23) by *Bgl*II and *Pst*I digestion as shown in figure 2.2.4.**

As explained earlier (Fig 2.2.1 ), the *Bgl*II single digestion of the 11 kb *Eco*RI fragment cloned from 1.51 and 1.23 cosmid show only one linear band suggesting that the 11 kb contains only one *Bgl*II site as this site is not there in the pBSK vector backbone (lane no 1: 11kb from 1.51, lane no14: 11kb from 1.23). *Pst*I digestion of the 11 kb fragment shows two bands suggesting one *Pst*I site internal to the fragment at the distance of 2.5 kb from the *Pst*I/*Eco*RI (lane no 2 for 1.51 clone and lane no 7 for 1.23 derived clone) site in the vector backbone.

The *Bgl*II- *Pst*I double digestion of the 11 kb fragment generates three fragments. The two fragments of 1.5kb and 1.0 kb are the ones which are generated by the *Bgl*II and *Pst*I sites present internal to the 11 kb fragments. The 2.5 kb *Pst*I fragment as shown in the lane no 2 is broken into two fragments implying the presence of *Bgl*II at 1.5 kb distance from the *Pst*I of the vector.

The *Pst*I pattern of the 11 kb does not match with that of the 1.51 because there is only one *Pst*I internally and the same in 1.51 it will be continuous with the *Pst*I of the adjacent 4.0 kb *Eco*RI fragment. Since the *Bgl*II to *Pst*I distance in this fragment is ~1.5 kb so this fragment is also seen in the *Bgl*II-*Pst*I double digestion of the 1.51 and 1.23 cosmid clones (lane no 5, 1.51 cosmid clone and lane no 10, 1.23 cosmid clone). The other 1.0 kb fragment in the *Bgl*II – *Pst*I double digestion of 11 kb clone is not seen in any of the parent cosmid clones as this is the fragment generated from the *Pst*I of pBSK vector to the internal *Bgl*II site.

Disposition of *Bgl*II and *Pst*I restriction enzyme sites in the 11.0kb *Eco*RI fragment:

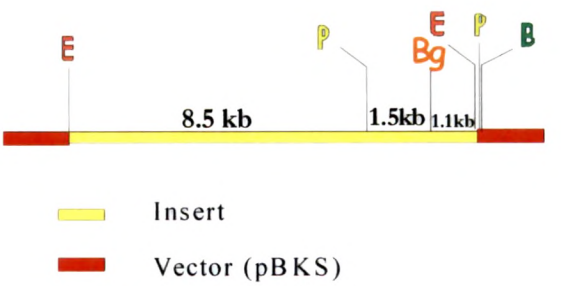



**Figure 2.2.4a : Restriction map for the 11.0 kb *Eco*RI fragment with respect to Bg: *Bgl*II, P: *Pst*I.**

### 2.2.5 *Bam*HI digestion of the 11.0 Δ*Pst*I (in both +/- orientation).

As explained in the *Eco*RI - *Bam*HI analysis (Fig. 2.2.3), one *Bam*HI site is present around ~ 2.3 kb away from the *Bam*HI of vector backbone and that *Pst*I digestion of the insert produces the fragment of the size ~ 2.5 kb same as that in BamHI digestion, given that the *Pst*I and the *Bam*HI in the vector are present on the same side of *EcoR*I, it becomes important to understand the location of *BamH*I i.e whether the *Bam*HI is present within the *Pst*I's limit or outside the limit in the fragment, the Δ *Pst*I of the 11 kb fragment has been constructed and the *Bam*HI digestion was carried out of the same. These results will be able to explain the exact location of *Bam*HI site with respect to the *Pst*I site in the 11 kb fragment solve the ambiguity in mapping these closely spaced sites.

The Δ *Pst*I clones for both the orientation of 11 kb were constructed by digestion by *Pst*I followed by intramolecular ligation of 11kb DNAs present in pBluescriptKS in both (+) and (-) orientations [see below Section 2.2.6 for the 11 kb DNA in (-) orientation]. *Bam*HI digestion of the clones is described in figure 2.2.5.


**Figure 2.2.5: *Bam*HI digestion of the 11.0 *Eco*RI fragment (in both +/- orientation).**

In the above gel the first two clone namely #11 (1) and (2) are the 11 kb clones in (+) orientation and the clone #7 (1) is another 11 kb clone in (-) orientation. The pictorial presentation of the clones in both the orientation is shown below. The orientation of the insert in the figures below is shown in two different direction. The *Bam*HI pattern of the 11 kb clone, as discussed earlier shows the presence of one ~2.5 kb fragment, generated because of the *Bam*HI from the vector and the last *Bam*HI from the insert apart from the internal 0.2 kb *Bam*HI fragment.

Now analysing the above gel, the first two lanes show three bands each suggesting that here *Bam*HI is present towards the left end as shown in Fig. 2.2.5, 2a. Following the loss of *Pst*I DNA, one *Bam*HI and one *Bgl*II is lost along with one *Eco*RI. This will generate three bands in *Bam*HI digestion, one which is internal 0.2 kb, another ~ 2.0 kb band corresponds to DNA between internal *Bam*HI to the *Bam*HI in the vector and then the rest of the DNA which includes around 6.0 kb DNA from the insert and the 3.0 kb vector.

The third lane represents result of the *BamH*I digestion of insert present in the orientation that is shown in the Fig.2.2.5, 2b. Here the *Pst*I digestion and religation cause the loss of internal 0.2 kb *Bam*HI fragment. The 11.0 kb Δ *Pst*I (-) clone will generate a 0.2 kb fragment because of the remaining *Bam*HI (insert) to the *Bam*HI (vector) after the two *Pst*I spanning region (one from the vector and one unique internal *Pst*I) is removed in Δ *Pst*I clone.

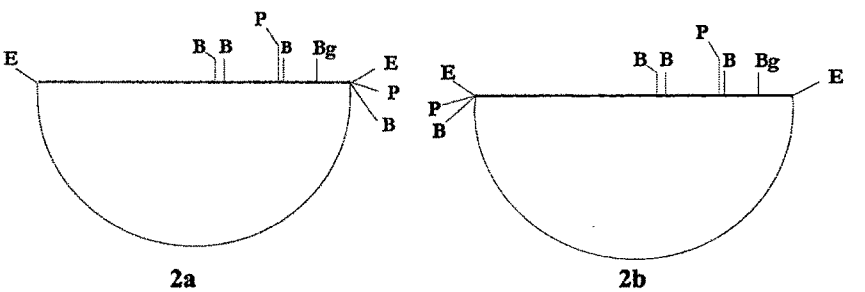



**Figure. 2.2.5a:Diagrammatic representation of the 11kbΔPstI clone in 2a (+) and 2b(-) orientation.**

**2.2.6: Single and double digestion of the 11.0 kb (-) orientation clone with respect to four enzymes *Bam*HI, *Bgl*II, *Pst*I and *Eco*RI.**

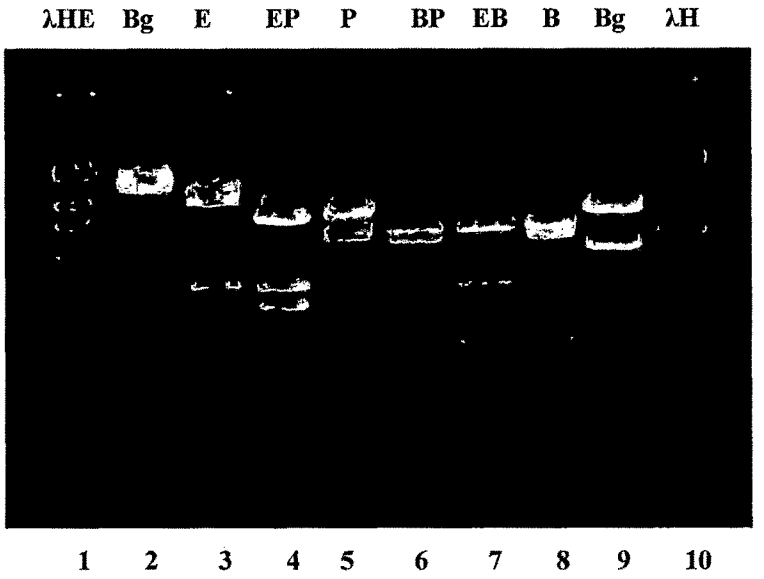



**Figure 2.2.6: Single and double digestion of the 11.0 kb (-) orientation clone with respect to four enzymes *Bam*HI, *Bgl*II, *Pst*I and *Eco*RI.**

| Enzymes | No. of Fragments |
|---|---|
| *Bgl*II | One<br>14kb |
| *Eco*RI | Two<br>11kb, 3.0 kb |
| *Pst*I | Two<br>8.5kb, 5.5kb |
| *Bam*HI | Four<br>6.3kb, 5.3kb, 2.2kb, 0.2kb |
| *Eco*RI-*Bam*HI | Five<br>7.0kb, 3.0kb, 2.2kb,1.7kb,0.2kb |
| *Bgl*II-*Pst*I | Three<br>8.5kb, 4.1 kb, 1.5 kb |
| *Bam*HI-*Pst*I | Four<br>7.2 kb, 4.7kb, 2.1kb, 0.2kb |
| *Eco*RI-*Pst*I | Three<br>8.5kb, 3.0 kb, 2.5kb |

**Table 2.2.6: The sizes of the fragments generated by the single and double digestion of 11.0 kb(-) orientation clone with respect to four enzymes *Bam*HI, *Bgl*II, *Pst*I and *Eco*RI.**

The above gel is the representation of the single and double digestion of the 11 kb *Eco*RI fragment cloned in to pBSK in orientation opposite to that used in the analysis described in Section 2.2; this will be called as 11 kb (-) orientation clone.

Since it contains only one *Bgl*II site in the insert and no *Bgl*II in the vector, the *Bgl*II digestion of the 11 kb(-) fragment (lane no 2) is exactly same as the one shown in the earlier gel (figure no2.2.3, lane no. 1) in the opposite direction.

(i) As discussed earlier in the 11 kb (+) clones (of 1.51 and 1.23 csomid clones) that there is only one internal *Pst*I site and the *Pst*I present at 2.5 b distance in he insert from the PstI site of the vector. In 11 kb (-) this 2.5 kb fragment continues with the pBKS vector resulting in to a 5.5kb fragment (figure2.2.6, lane no 5). The other 8.5 kb band is generated by the *Pst*I from the insert to the *Pst*I (vector).

(ii) The differnce in *Pst*I digestion is also reflected in the The *Eco*RI - *Pst*I double digestion as well of the 11 kb (-) orientation clone. The 5.5 kb *Pst*I fragment is split into 2.5 kb *Pst*I-*EcoR*I and 3.0 kb *EcoR*I vector DNA. The 8.5 kb fragment generated remains

as such representing the DNA between the *Pst*I (insert) - *Eco*RI (vector) of the cloning site (lane no 4).

(iii) In the *Bam*HI digestion of the 11 kb (-) insert, 6.5 kb (generated by the *Bam*HI from the vector to the first *Bam*HI in the insert) and 5.5 kb (generated by the last *Bam*HI in the insert constituting around 2.5 kb insert till the *Bam*HI in the vector spanning the complete 3.0 kb vector (lane no. 9) DNAs are the diagnostic fragements of the (-) orientation in contrast to the 1.7 kb, 10.1 kb DNA formed for the 11 kb in (+) orientation.

(iv) The *Bam*HI - *Pst*I double digestion (-) orientation clone generates four bands, of which the 7.2 kb and 4.7 kb *Bam*HI fragment are the differentiating fragment from the (+) orientation. The 7.2 kb fragment of the (-) orientation otherwise continues in to the pBKS vector in the (+) orientation generating a ~10.2 kb fragment and 4.7 is broken down in 1.7 and 3.0 kb because of the present of *Bam*HI/*Pst*I of the vector from that end.

(v) The *Eco*RI- *Bam*HI double digestion of the 11 kb (-) orietation and (+) orientation will generate the same sized fragment because of the the cloning has been done at the *Eco*RI site.

(vi) The *Bgl*II-*Pst*I double digestion of the 11 kb (-) orientation clone generates three bands. Of which the first ~8.5 kb is the diagnsotic band generated by the *Pst*I from the vector to the internal *Pst*I which makes the 11.5 kb band in the (+) orientation clone inclusive of the 3.0 kb vector backbone. The second differentiating band is the ~ 4.1 kb which makes the 1.1(insert)+3.0 kb vector in (-) orientation, the same band in the (+) orientation is split in to 1.1 kb and 3.0 kb in size.

## 2.3: *BamHI*, *PstI* and *BamHI-PstI* double digestion of 4.0 kb *EcoRI* fragment

4.0 kb EcoRI fragment generated from 1.51 cosmid clone was the gel eluted and cloned into the EcoRI digested pBKS vector. Out of 80 colonies, 14 white colonies were obtained containing the insert.

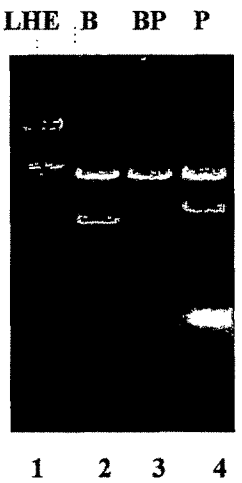



**Figure 2.3.1: Single and double digestion of the 4.0kb *Eco*RI clone with respect to two enzymes B: *Bam*HI, and P : *Pst*I .**

As shown in chapter 1 (Fig.1.4.2), the *Bgl*II from the 11.0kb *Eco*RI fragment extends up to 8.0 kb *Eco*RI as 9.5 kb *Bgl*II DNA, spanning complete 4.0 kb *Eco*RI fragment suggesting that there is no *Bgl*II site in the 4.0 kb *Eco*RI fragment. The *Bam*HI- and *Pst*I digestion suggest that there are two sites of *Bam*HI and one of *Pst*I within the DNA. The *Pst*I generates two fragments - the 2.0 kb fragment generated from *Pst*I of the vector to the one in the insert. This DNA is part of the 3.8 kb *Pst*I fragment (*Pst*I in the 8.0kb *Eco*RI to the *Pst*I in 4.0kb *Eco*RI fragment) produced by the digestion of 1.51 cosmid. The *Bam*HI digestion of the 4.0 kb fragement generates three fragments of which 1.5 kb fragment is the internal fragement. On the other hand, 1.0 kb *Bam*HI fragment is generated by the *Bam*HI from the vector and the *Bam*HI from the insert (Fig. 2.3.1 lane no. 2) suggesting that the internal 1.5 kb *Bam*HI fragment is also present towards the

8.0kb *Eco*RI fragment. Presence of *Pst*I at 2.0 kb distance from the same end breaks the internal 1.5 *Bam*HI fragment into 1.0 kb and 0.5 kb fragment.

The figure depicts the *Bam*HI and *Pst*I placements in the 4.0 kb *Eco*RI frragment

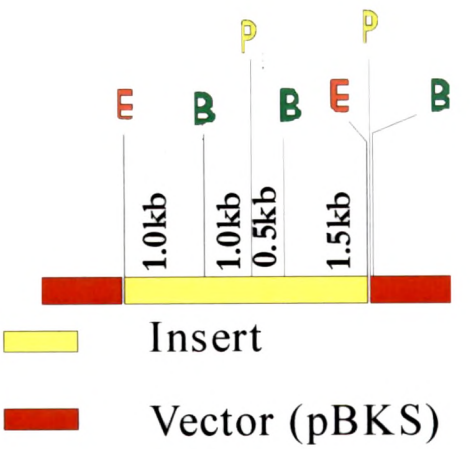
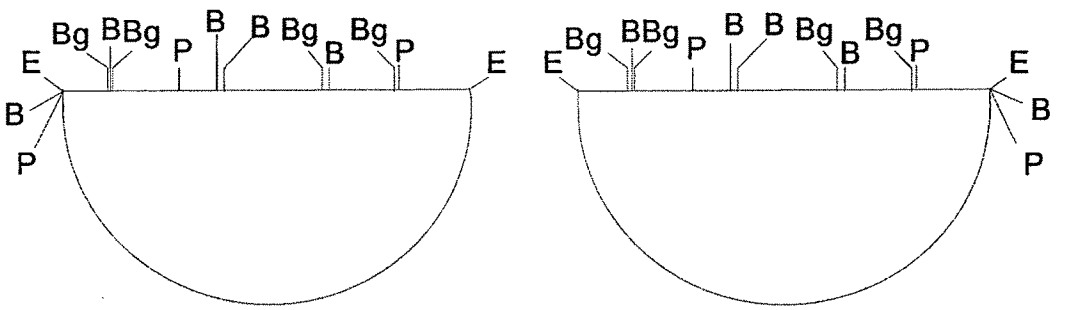



**Figure 2.3.1a: Restriction map for the 4.0 kb *Eco*RI fragment with respect to B: *Bam*HI, P: *Pst*I.**

## 2.4. Digestion analysis of 8.0 kb Clone:

This 8.0 kb *Eco*RI fragment was cloned from the 1.51 cosmid. This is the fragment which hybridises with the *actI* porbe. The *actI* hybridizing 4.0kb *Pst*I and 4.0 kb *Bgl*II fragments are also the part of this fragment only. To clone this fragment, the 1.51 cosmid was digested with the *Eco*RI enzyme, electrophoresed in 0.8% agarose gel, gel eluted by the glaswool method and cloned in the pBKS vector. Out of 80 colonies obtained four were showing the white phenotype, containing the 8.0 kb fragment. The 8.0 kb clone was subjected to various single and double resticiton enzyme digestions using the four enzymes, *Eco*RI, *Bgl*II, *Bam*HI and *Pst*I. In the Figure. 2.4, 3a and 3b shown below, the orientation of the 8.0 kb fragment is according to the figure 2.4, 3a .




3a 3b

**Figure 2.4 : Digrammatic representation of the 8.0 kb clone in 3a (+) and 3b (-) orientaion.**

**2.4.1: Single and Double digestion of the 8.0 kb *Eco*RI fragment with respect to three enzymes *Pst*I, *Bgl*II and *Bam*HI.**

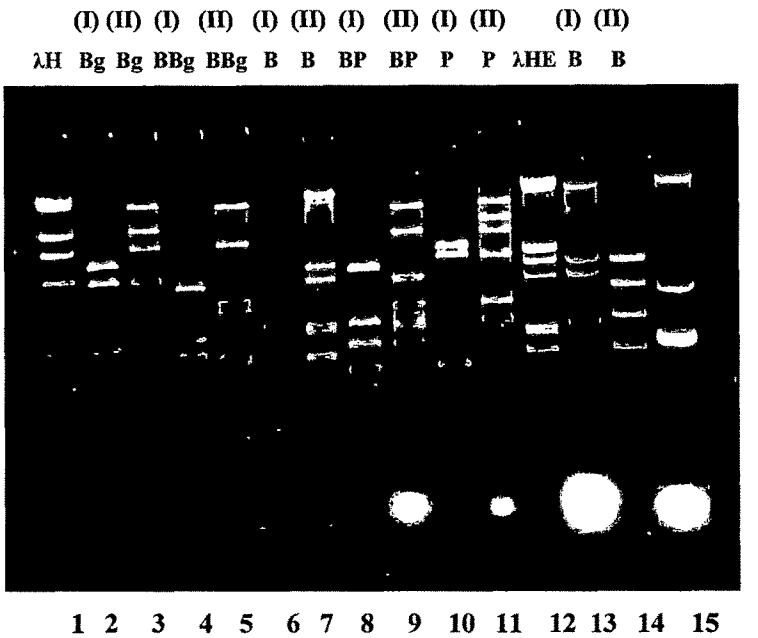



**Figure 2.4.1: Single and double digestion of the 8.0kb *Eco*RI clone with respect to three enzymes B: *Bam*HI, P: *Pst*I and Bg : *Bgl*II . (I) represent the 8.0 kb *Eco*RI fragment cloned in pBKS, and (II) represents 1.51 cosmid clones**

| Enzymes | No. of fragments |
|---|---|
| *Bgl*II | Four<br>5.0 kb, 4.0kb, 1.9 kb, 0.2kb |
| *Bam*HI | Five<br>4.0kb, 3.4kb, 2.2kb, 1.7kb, 0.2kb |
| *Pst*I | Three<br>5.5kb, 4.5kb, 1.0kb |
| *Bam*HI- *Bgl*II | Nine<br>3.9kb,1.4kb,0.1kb,1.6kb,2.1 kb,0.2kb,0.15kb 0.15kb,1.55kb |
| *Bam*HI-*Pst*I | Seven<br>2.2kb,1.2kb,2.5 kb,1.1kb,0.6kb.0.2kb, 4.0 |

**Table 2.4.1: The sizes of the fragments generated by the single and double digestion of the 8.0kb *Eco*RI clone with respect to three enzymes B: *Bam*HI, P : *Pst*I and**

Single enzyme digestion analysis (i) *Bgl*II:

The *Bgl*II digestion of the 8.0 kb *Eco*RI fragment produces four bands, suggesting that the 8.0 kb clone contains four *Bgl*II sites as there are no *Bgl*II sites in the pBSK backbone. The 4.0 kb *Bgl*II, 1.9kb *Bgl*II and the 0.2 kb *Bgl*II are the internal fragments of the 8.0 kb *Eco*RI fragment. The 4.0 kb *Bgl*II fragment is the *actI* hybridizing fragment.

Figure showing placement of *Bgl*II enzymes in the 8.0 kb *Eco*RI fragment.

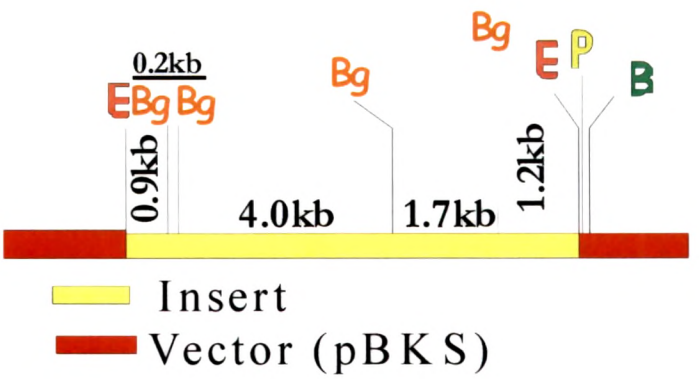



**Figure 2.4.1a: Restriction map of the 8.0 kb *Eco*RI fragment with respect to *Bgl*II**

Single enzyme digestion analysis (ii) *Bam*HI:

The *Bam*HI digestion of 8.0 kb fragment generates five bands, of which 4.0 kb and 3.5 kb are the ones which are generated by the *Bam*HI present at the two extreme ends. The 4.0 kb *Bam*HI fragment includes ~3 kb vector back bone and 1.0 kb of insert DNA. 3.5 kb DNA is the insert DNA which is generated by the last *Bam*HI site in the insert to the *Bam*HI site in the vector (fig.2.4 3a). The remaining two fragments are the 2.2 kb and 1.7 kb internal *Bam*HI fragments of which 1.7 kb *Bam*HI is the unique fragment. These two fragments 2.2 kb and 1.7 kb fragments are separated by 0.2kb *Bam*HI fragment (lane no 6).

The *Bam*HI location in the 8.0 kb *Eco*RI fragment is represented in the figure below

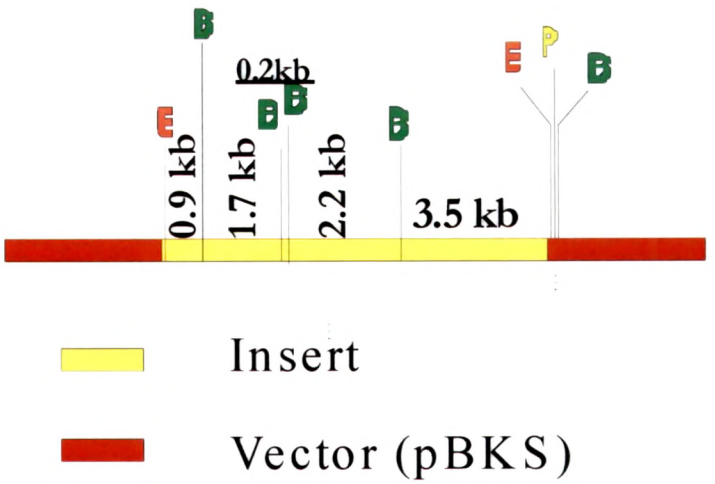



**Figure 2.4.1b: Restriction map of the 8.0 kb *Eco*RI fragment with respect to B: *Bam*HI**

Double enzyme digestion analysis (iii) *Bam*HI+ *Bgl*II:

The invariant 2.2 kb *Bam*HI DNA (the *actI* homologous DNA) is placed completely inside the 4.0 kb *Bgl*II fragment; the 4.0 kb *Bgl*II DNA limits extends into 1.7 kb *Bam*HI fragment and includes 1.5 kb out of 1.7 kb *Bam*HI DNA, thus 1.7 kb *Bam*HI is split in to 1.5 kb and 0.15 kb fragment in the *Bam*HI-*Bgl*II double digestion (lane no. 4). This suggests that 1.7 kb- and 2.2 kb *Bam*HI fragments are present adjacent to each other and it also indicates that either *Bam*HI is placed 0.15 kb inside the 4.0kb *Bgl*II fragment or as much out side it. Splitting 0.2 kb *Bgl*II fragment in to two, 0.15 and 0.1 kb, suggests otherwise and that *Bam*HI end of the 1.7 kb *Bam*HI fragment is present just outside the 4.0 kb *Bgl*II in the 0.2 kb *Bgl*II fragment (refer 2.43a and lane no 4 in the fig 2.4.1). The second *Bam*HI end of the 2.2 kb *Bam*HI is present just out side the 4.0 kb *Bgl*II fragment, breaking the 2.2 kb *Bam*HI fragment in to 2.1 and 0.1 kb fragments.The third fragment is also the invariant 1.9 kb *Bgl*II fragment which is present adjoining the 4.0 kb *Bgl*II fragment and the contains the *Bam*HI site from the 2.2 kb *Bam*HI fragment extending 0.3 kb into it. This accounts for fragment of 1.6 kb present in the *Bam*HI-*Bgl*II

double digestion (figure 2.4,3a and lane no 4). From the last *Bgl*II of 1.9 kb *Bgl*II fragment to the *Bam*HI in the vector, one more fragment of the size 1.4 kb is generated.

The restriction map generated by the *Bam*HI and *Bgl*II double digestion for the 8.0 kb *Eco*RI fragment

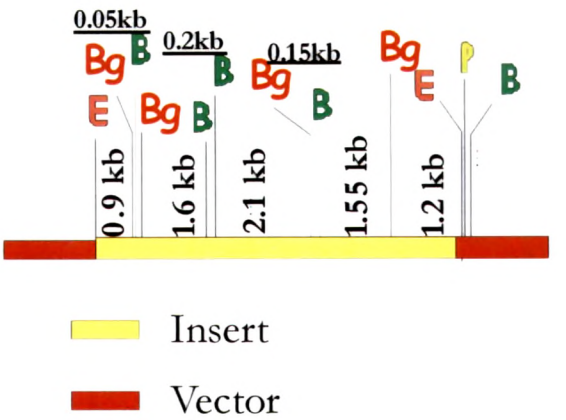



**Figure 2.4.1c: Restriction map of the 8.0 kb *Eco*RI fragment with respect to B: *Bam*HI and Bg: *Bgl*II**

Double enzyme digestion analysis (i) *Bam*HI-*Pst*I:

The *Pst*I digestion of the 8.0 kb fragment generates three fragments (2 insert derived and one from the vector). The two internal *Pst*I sites generates the unique 4.5 kb fragment (Fig 2.4.1.lane no 10) which hybridizes with the *actI* probe. The third band is the one generated by the internal *Pst*I to the *Pst*I from the vector which is around 1.2 kb in size. This fragment is also seen in the *Bam*HI- *Pst*I double digestion since this is the end fragment generated by the *Pst*I in the insert to the *Bam*HI/*Pst*I in the vector (Fig 2.4.3a, lane no 8). The internal 4.5 kb *Pst*I fragment thus includes the complete 0.2 kb- and 2.2 kb *Bam*HI. Since the 0.2 kb *Bam*HI separates 1.7 kb- and 2.2kb *Bam*HI fragments, the 1.7 kb *Bam*HI is broken in to 0.6 kb and 1.1 kb fragment by the *Pst*I in the insert ( Fig 2.4.3A, and lane no. 8). The 2.5 kb fragment is generated by the *Bam*HI of 2.2 kb *Bam*HI fragment to the *Pst*I of the 4.5 kb *Pst*I fragment.

The *Bam*HI - *Pst*I location inside the 8.0 kb EcoRI fragment as inferred from the above gel analysis

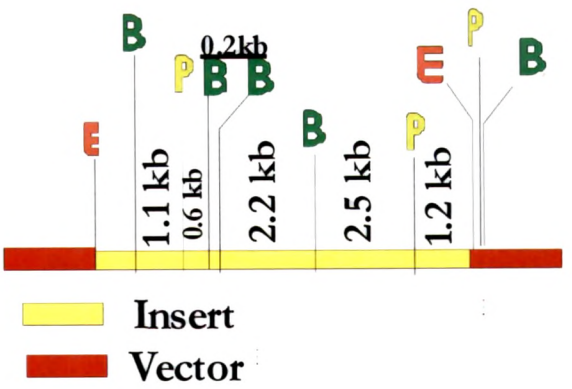



**Figure 2.4.1d: Restriction map of the 8.0 kb *Eco*RI fragment with respect to B: *Bam*HI and P: *Pst*I**

**2.4.2: Single and Double digestion of the 8.0 kb EcoRI fragment (in both (+) /(-) orienatation) with respect to three enzymes EcoRI, *Bgl*II and *Bam*HI.**

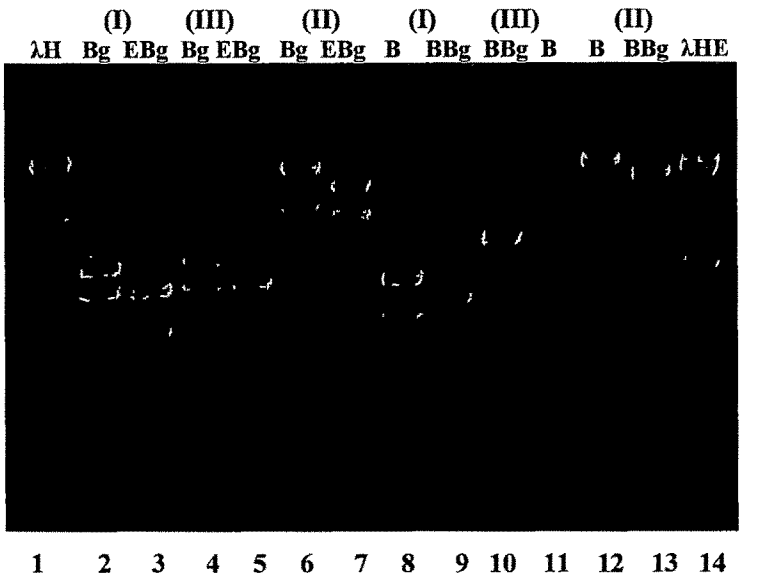



**Figure 2.4.2: Single and double digestion of the 8.0kb(+) *Eco*RI clone and 8.0 kb (-) *Eco*RI clone with respect to three enzymes B: *Bam*HI, E : *Eco*RI and Bg : *Bgl*II . (I) represents the 8.0 kb(+) *Eco*RI fragment cloned in pBKS, (II) represents 1.51 cosmid clone and (III) represents 8.0 kb(-) *Eco*RI fragment cloned in pBKS**

| Enzymes | No of fragments |
|---|---|
| *Bgl*II | Three<br>5.1kb,4.0kb,1.9kb |
| *Eco*RI-*Bgl*II | Five<br>4.0kb,1.9kb, 3.0kb, 1.2 kb , 0.9 kb |
| *Bam*HI | Five<br>4.0kb, 3.4kb, 2.2kb, 1.7kb, 0.2kb |
| *Bam*HI-*Bgl*II | Nine<br>3.9kb,1.4kb,0.1kb,1.5kb,2.1 kb,0.2kb,0.15kb 0.15kb,1.55kb, (0.9 kb and 4.0kb instead of one 1.5 and 3.9 kb in other orientation). |

**Table 2.4.2: The sizes of the fragments generated by the single and double digestion of the 8.0kb(+) *Eco*RI clone and 8.0 kb (-) *Eco*RI clone with respect to three enzymes B: *Bam*HI, E : *Eco*RI and Bg : *Bgl*II .**

Double enzyme digestion analysis (i) *Eco*RI- *Bgl*II:

The *Bgl*II digestion of the 8.0 kb fragment in either orientation generates three bands of which 4.0 kb and 1.9 kb are the internal invariant fragments (lane no 2). These two fragments also remain unchanged in the *Eco*RI- *Bgl*II double digestion (lane no 3). The second band in this *Eco*RI-*Bgl*II double digestion is the pBSK vector back bone. The fourth fragment is the 1.2 kb fragments which is generated by the *Eco*RI (vector) to the last internal *Bgl*II representing the right hand side of the 8.0 kb region (Fig.2.4,3a). The 0.9 kb fragment is the one which is generated by the *Eco*RI (vector) to the first *Bgl*II site in the insert, which means it continues with the *Bgl*II in the 11.0 kb fragment spanning complete 4.0 kb *Eco*RI fragment. The 0.2 kb *Bgl*II (lane no.2) fragment is invariant and internal to the 8.0kb *Eco*RI is not clearly visible here. Lane no 4 and 5 also represents the *Bgl*II and *Eco*RI - *Bgl*II digestion of the 8.0 kb fragment cloned in the (-) orientation (Fig.2.4,3b). Since there are no *Bgl*II in the vector backbone, the *Bgl*II and *Eco*RI-*Bgl*II pattern of both the orientations (+) and (-) are exactly the same. Except for the 3 kb vector band, the other bands are same as in the *Bgl*II and *Eco*RI- *Bgl*II digestions of the complete cosmid clone 1.51 (lane no 6 and 7).

Double enzyme digestion analysis (ii) *Bam*HI - *Bgl*II:

The lane no 8 and 9 represents the *Bam*HI and *Bam*HI – *Bgl*II digestion of the 8.0 kb clone. This pattern represents the cloning done according the Fig. 2.4, 3a which is explained in the earlier section. The *Bam*HI digestion pattern of the 8.0 kb fragment cloned in the other orientation (-) shows 2.2 and 1.7 kb invariant fragments present internally. The change in the orientation changes the bands generated by the two extreme ends of the 8.0kb fragment. The 4.0 kb fragment of (+) orientation changes to 0.9 kb in this (-) orientation, and the 3.5 kb fragment generated by the *BamH*I (vector) – *BamH*I (insert) in the (+) orientation continues with the vector and makes the 6.5kb fragment in the (-) orientation (lane no 10 and fig. 2.4, 3a and 3b).

The *Bam*HI- *Bgl*II double digestion of the 8.0(-) kb fragment generates nine (lane no 10) common fragments for the internal *Bam*HI and *Bgl*II sites. The number of bands generated here are same as the one for the 8.0 kb fragment cloned in the (+) orientation (Fig. 2.4, 3a, lane no 9). The doublet generated by the internal *Bam*HI - *Bgl*II digestion of 1.5 kb, 1.55 kb is same, like wise the 2.1 kb fragment generated in (+) orientation clone is seen in (-) orientation clone as well (lane no 9). Here, the first *Bgl*II of the clone (lane no 9) is present towards the *Bam*HI of the insert hence 0.9 kb fragment between *Bgl*II (insert) to the *Bam*HI (vector) is generated. The 1.5 kb fragment in the lane no 9 generated by the last *Bgl*II of the insert to the *Bam*HI in the vector backbone is now the part of the large fragments present along with the vector backbone making 4.5 kb size fragment. Here also all the fragments < 0.2 kb in size are not visible in this gel. Importantly, all the fragments generated and explained in the double digest can be accounted for in the *Bam*HI- *Bgl*II double digestion of 1.51 cosmid (lane no 14).

*Bam*HI-*Bgl*I double digestion analysis of the 8.0 kb EcoRI fragment in (+) and (-) , both orientation can be pictorially represented as below

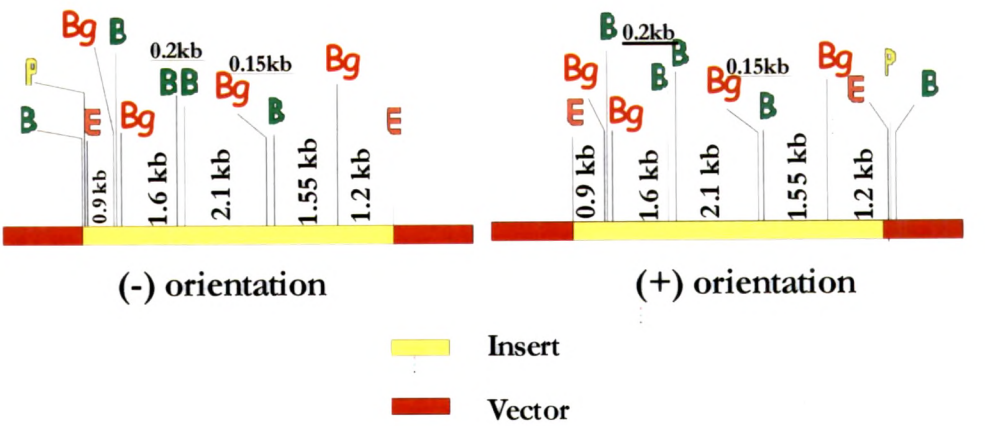



**Figure 2.4.2a: Restriction map of the 8.0 kb *Eco*RI fragment (+) and (-) orientation with respect to B: *Bam*HI and Bg: *Bgl*II**

**2.4.3: Single and Double digestion of the 8.0 kb *Eco*RI fragment with restriction enzymes *Eco*RI and *Pst*I**

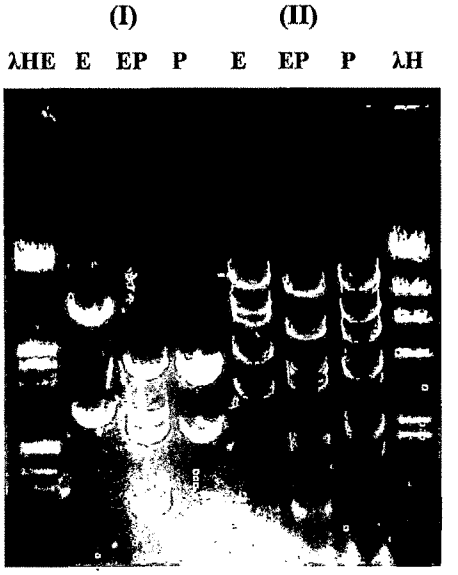



**Figure 2.4.3: Single and double digestion of the 8.0kb (+) *Eco*RI clone with respect to two enzymes E: *Eco*RI, and P : *Pst*I. (I) represents the 8.0 kb *Eco*RI fragment cloned in pBKS, and (II) represents 1.51 cosmid clone**

| Enzymes | No of fragments |
|---|---|
| *Eco*RI | Two<br>8.0kb, 3.0kb |
| *Pst*I | Three<br>4.5kb, 4.0 kb, 2.6kb |
| *Eco*RI-*Pst*I | Four<br>4.5kb,3.0kb,2.6kb,1.0kb |

**Table 2.4.3: The sizes of the fragments generated by the single and double digestion of the 8.0kb(+) *Eco*RI clone with respect to two enzymes E: *Eco*RI, and P : *Pst*I.**

Single and double digestion by REs *Eco*RI and *Pst*I:

This gel represents the *Eco*RI and *Eco*RI-*Pst*I double digestion of the 8.0 kb clone along with the 1.51 cosmid. The double digestion of the 8.0 kb clone generates four bands - the first band is *actI* hybridizing invariant 4.5 kb fragment (lane no 3). Thus this fragment is also seen in the single *Pst*I digested 8.0 kb fragment (lane no 4) as could be seen in the 1.51 clone digested with *Pst*I in lane no. 7. Second band in lane no 3 is the 3.0 kb pBSK vector. Third band is *Pst*I-*Pst*I fragment of about 2.6 kb in size which is generated by the *Eco*RI/*Pst*I of the vector to the first *Pst*I again from the left which is also seen in the single *Pst*I digestion of the 8.0 kb clone (lane no 4) and *Eco*RI – *Pst*I double digestion of the 1.51 cosmid, (lane no 6). The last band is the around 1.0 kb in size which is generated by the last internal *Pst*I (Insert)- *Eco*RI (in the vector) towards the right hand side in the (Fig.2.4, 3a). This is also seen in the *Eco*RI-*Pst*I double digestion of the 1.51 clone (lane no 6).

The single *Pst*I digestion of the 8.0 kb clone generates three bands (lane no 4). The first band is the 4.5 kb invariant insert derived *Pst*I band which is also seen in the *Eco*RI- *Pst*I double digestion of the 8.0 kb clone (lane no 3) as well as the *Eco*RI-*Pst*I double digestion of the 1.51 clone (lane no 6) and also the single *Pst*I digestion of the 1.51 clone (lane no 7). The second band is 4.0 kb in size generated by the *Pst*I from the vector to the first internal *Pst*I which is around 1.0kb away from the *Eco*RI. Thus all the bands in the double and single digestion of the 8.0 kb clone are accounted for in the corresponding RE digestions of the 1.51 clone.

Figurative display of the *Eco*RI – *Pst*I double digestion of the 8.0 kb *Eco*RI fragment

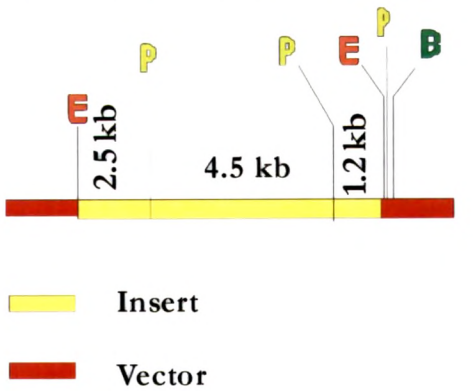



**Figure 2.4.3a: Restriction map of the 8.0 kb *Eco*RI fragment with respect to E: *Eco*RI and P: *Pst*I**

**2.4.4: Single and Double digestion of the 8.0 kb(+) *Eco*RI fragment with enzymes *Bgl*II and *Pst*I.**

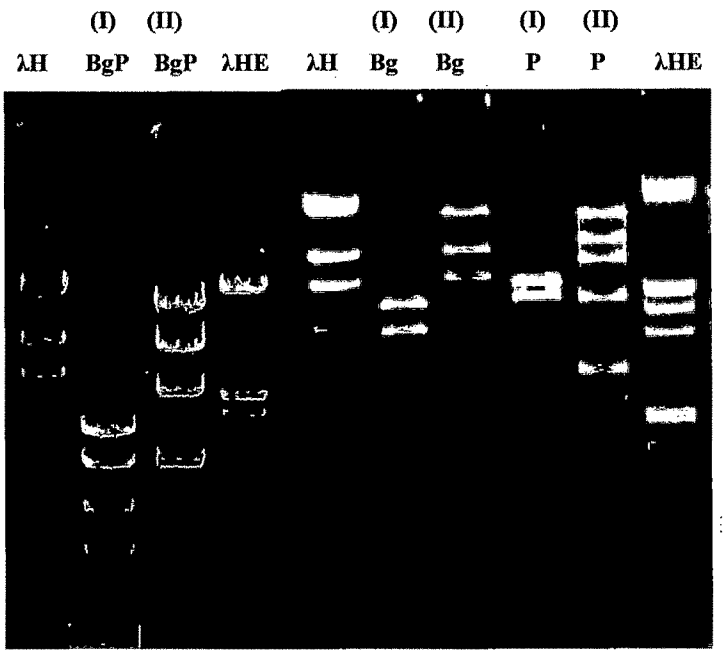



**Figure 2.4.4: Single and double digestion of the 8.0kb (+) *Eco*RI clone with respect to two enzymes Bg: *Bgl*II, and P : *Pst*I. (I) represents the 8.0 kb *Eco*RI fragment cloned in pBKS, and (II) represents 1.51 cosmid clone**

| Enzymes | No of fragments |
|---|---|
| *Bgl*II | Four<br>5.0 kb, 4.0kb, 1.9 kb, 0.2kb |
| *Pst*I | Three<br>4.5kb, 4.0 kb, 2.6kb |
| *Bgl*II- *Pst*I | Seven<br>3.8 kb, 1.9kb, 3.0kb,1.0kb,1.0kb, 0.2kb, 0.2kb |

**Table 2.4.4: The sizes of the fragments generated by the single and double digestion of the 8.0kb(+) *Eco*RI clone with respect to two enzymes Bg: *Bgl*II, and P : *Pst*I.**

Single and double digestion by REs *Bgl*II and *Pst*I:

The gel shown here is the *Bgl*II, *Pst*I single and *Bgl*II-*Pst*I double digestion of the 8.0 kb clone and 1.51 cosmid DNA. There are two sets of digestions shown here the first set represents the *Bgl*II *Pst*I double digestion of 8.0 kb clone and 1.51 cosmid. The second set represents the single *Bgl*II and *Pst*I digestion of the 8.0 kb and 1.51 clones. The *Bgl*II - *Pst*I double digestion of the 8.0 kb clone generates five bands. As seen earlier that 8.0 kb fragment contains internal 4.5 kb *Pst*I fragment. This 4.5 kb *Pst*I fragment is populated with three *Bam*HI sites and two *Bgl*II sites. The *Bam*HI-*Pst*I double digestion has been explained in the section 2.2.1.

The *Bgl*II – *Pst*I double digestion generates five bands, the first band is around 3.8 kb in size that is generated by the last *Pst*I in the insert (towards the left; Fig. 2.4.3a) upto the *Pst*I in the vector present at the opposite end thus spanning complete vector backbone within. The second band is the *Pst*I- *Bgl*II fragment in the insert present 1.0 kb internally in the 4.0 kb *Bgl*II fragment and breaking the 4.0 kb *Bgl*II fragment in to 3.0 kb and 1.0 kb (one of the bands in the doublet). The third fragment is the unique and invariant 1.9 kb *Bgl*II fragment present internally to the 8.0 kb insert and also within the 4.5kb *Pst*I fragment thus it is also seen in the *Bgl*II-*Pst*I double digestion of the 1.51 cosmid clone, the single *Bgl*II digestion of the 1.51 and 8.0 kb clone. The fourth fragment is the doublet of 1.0 kb generated respectively by the *Pst*I of the vector to the first *Bgl*II in the insert and the second *Bgl*II (i.e the *Bgl*II from the 0.2 kb fragment) to the first *Pst*I of the 4.5 kb internal *Pst*I fragment. Other than the first fragment rest all the fragments are accounted for in the *Bgl*II- *Pst*I double digestion of the 1.51 cosmid clone. The second set contains the single *Bgl*II and *Pst*I digestions of the 8.0 kb clone and 1.51 cosmid clones. This has been explained in the earlier gels (see Section 2.4.1, figure 2.4.1, lane no 2 and 3; 8.0kb and 1.51 respectively for *Bgl*II digestion and section 2.4.3 figure 2.4.3, lane no 3 and 7; 8.0kb and 1.51 respectively for *Pst*I digestion ).

The diagrammatic representation of the *Bgl*II and *Pst*I sites in the 8.0 kb *Eco*RI clone

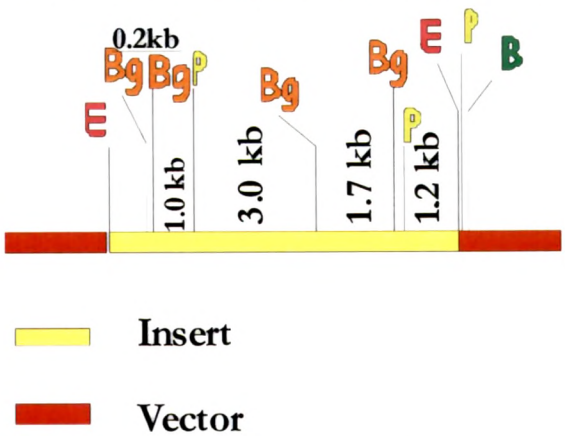



**Figure 2.4.4a: Restriction map of the 8.0 kb *Eco*RI fragment with respect to Bg: *Bgl*II and P: *Pst*I**

**2.4.5: Single and Double digestion of the 8.0 kb *Eco*RI fragment with enzymes *Eco*RI and *Bam*HI**

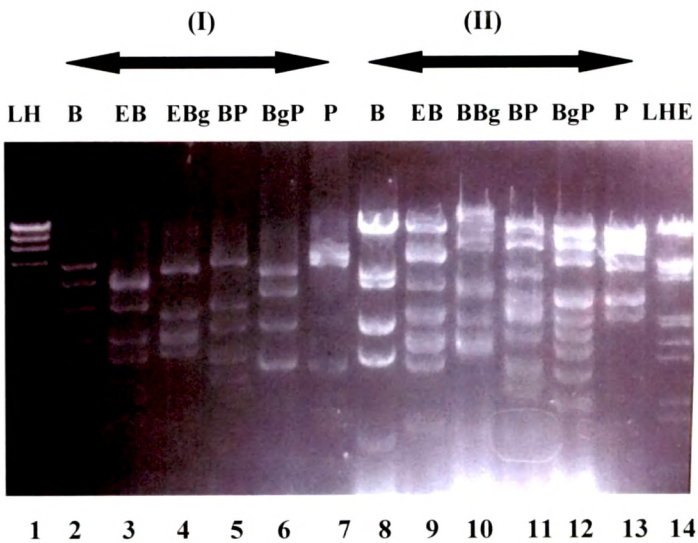



**Figure 2.4.5: Single and double digestion of the 8.0kb (+) *Eco*RI clone with respect to two enzymes B: *Bam*HI, and E : *Eco*RI. (I) represents the 8.0 kb *Eco*RI fragment cloned in pBKS, and (II) represents 1.51 cosmid clone**

| Enzymes | No of fragments |
|---|---|
| *Eco*RI | Two<br>8.0kb,3.0kb |
| *Bam*HI | Five<br>4.0kb, 3.4kb, 2.2kb, 1.7kb, 0.2kb |
| *Eco*RI- *Bam*HI | Six<br>3.0kb,3.0kb, 2.2kb, 1.7kb, 1.0 kb,0.2kb |

**Table 2.4.5: The sizes of the fragments generated by the single and double digestion of the 8.0kb(+) *Eco*RI clone with respect to two enzymes *Bam*HI, and *Eco*RI**

Analysis of Single and Double digestion with enzymes *Eco*RI and *Bam*HI:

Though the above gel represents the several double digestions of the 8.0 kb clone and the 1.51 cosmid, the analysis presented here is with respect digestion by REs *Eco*RI and *Bam*HI. Lane no 2 contains the single *Bam*HI digested 8.0 kb clone generating four bands, two of them invariant internal 2.2 kb and 1.7 kb fragments which are also present in the *Bam*HI digested 1.51 clone as well in lane no 8. The first fragment of ~ 4.0 kb is generated by the *Bam*HI from the vector to the first *Bam*HI (this includes the 3.0 kb vector) in the insert as seen in (Fig2.4, 3a). Second ~3.0 kb fragment generated is the last *Bam*HI in the insert to the *Bam*HI in the vector (Fig. 2.4, 3a). Thus the two fragments represent the fragments derived from the ends of the insert.

In the *Eco*RI-*Bam*HI double digestion of the 8.0 kb clone, the first band (lane no 3) is the doublet of 3.0 kb. One of which is the pBSK vector backbone itself; second band in the doublet is the last *Bam*HI from the insert to the *Bam*HI in the vector (see Fig.2.4, 3a) which is also seen in the single *Bam*HI digestion of the 8.0 kb clone (lane no 2) and *Eco*RI-*Bam*HI double digestion of the 1.51 cosmid clone (lane no 9). The second and third bands are the invariant internal 2.2 kb and 1.7 kb *Bam*HI fragments (lane no 2 and lane no 8). These bands are seen in all the three digestions i.e single *Bam*HI digestions of 8.0 kb clone (lane no 2) and 1.51 clones (lane no 8) and the double digestions of the 1.51 cosmid (lane no 9). The fifth band is the one generated is around 1.0 kb in size generated

by the *Eco*RI from the vector to the first *Bam*HI in the insert. Rest all other double digestions have been discussed in the earlier gels separately.

Internal *Bam*HI presentation in the 8.0 kb *Eco*RI fragment in the figurative form

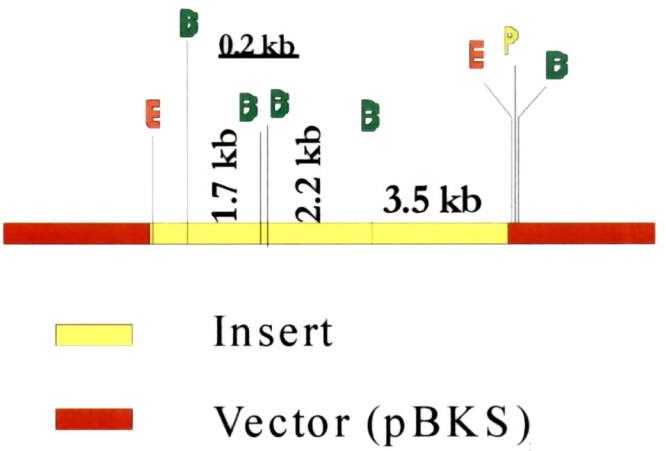



**Figure 2.4.5a: Restriction map of the 8.0 kb *Eco*RI fragment with respect to B: *Bam*HI**

**2.4.6: *Bgl*II ,*Bam*HI and *Pst*I digestion of the 4.5kb *Pst*I fragment cloned in pBluescriptKS:**

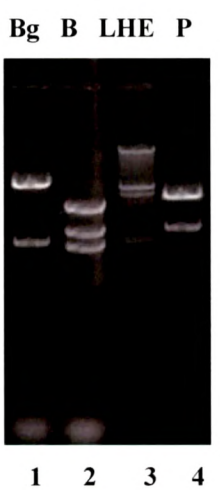



**Figure 2.4.6: Single and double digestion of the 4.5kb *Pst*I clone with respect to three enzymes B: *Bam*HI, Bg: *Bgl*IIand P: *Pst*I.**

| Enzymes | No of fragments |
|---|---|
| *Bam*HI | Four<br>2.2 kb, 0.2 kb , 1.5 kb. 4.0kb |
| *Bgl*II | Two<br>6.2kb,1.7 kb |
| *Pst*I | Two<br>4.8kb and 3.0 kb |

**Table 2.4.6: The sizes of the fragments generated by the single and double digestion of the 4.0kb *Eco*RI clone with respect to two enzymes B: *Bam*HI, and P: *Pst*I.**

The *actI* hybridizing 4.5 kb *Pst*I fragment is the unique fragment in the 8.0 kb *Eco*RI fragment. As explained in the earlier gels (Fig. 2.4.3 and 2.4.4) and the figures above, the ~4.5 kb *Pst*I fragment in the 8.0 kb *Eco*RI fragment spans the middle region of the 8.0 kb fragment. This region is highly populated with the *Bam*HI and *Bgl*II sites. Thus to understand how these sites are placed with respect to each other the ~4.5 kb *Pst*I was cloned in the pBKS vector. The orientation of the cloned DNA is in the direction as shown in the cartoon no 3a.

The above gel (Fig. 2.4.6) shows the single *Bam*HI, *Bgl*II and *Pst*I digestion of the ~ 4.5 kb fragment. The *Bgl*II digestion of the 4.5 kb *Pst*I fragment generates two bands indicating the presence of two *Bgl*II sites in the ~4.5 kb *Pst*I fragment the second band here is the unique 2.0 kb *Bgl*II fragment as seen in 1.51 cosmid clone ( Fig. 2.4.1, lane no. 3 ) and 8.0 kb clone (Fig 2.4.1, lane no 2). Since the first band generated is the around 6.2 kb in size, the *Bgl*II end of this 2.0 kb *Bgl*II fragment is present at around 2.5 kb distance from the *Pst*I site and that the 2.5 kb is the part of unique 4.0 kb *Bgl*II fragment. Thus 4.5 kb *Pst*I fragment and 4 kb *Bgl*II DNAs overlap by 2.5 kb amount of DNA. Since the *Bgl*II digestion shows the absence of 0.2 kb *Bgl*II fragment it implies that the unique 4.0 kb *Bgl*II fragment is continuous with the 2.0 kb *Bgl*II fragment.

The *Bam*HI digestion of the ~4.5 kb *Pst*I. clone generates four bands suggesting that there are three internal *Bam*HI sites in the 4.5 kb *Pst*I fragment, the unique *actI* hybridizing *Bam*HI 2.2 kb fragment, the 0.2 kb *Bam*HI fragment which is present as the spacer between the 1.7kb *Bam*HI and 2.2 kb *Bam*HI fragments and the third fragment is

the 1.7 kb *Bam*HI fragment generated by the *Bam*HI (internal) to *Pst*I./ *Bam*HI (vector). To explain this, double digestion was also set up for this 4.5 kb *Pst*I clone (explained in Section 2.4.7 below ). The first fragment is around 4.0 kb in size generated by the *Bam*HI from the vector to the first *Bam*HI present internally from the opposite end suggesting that the first *Bam*HI inside the *Pst*I fragment is approx. at a 1 kb distance, as this fragment contains the complete 2.9 kb pBKS vector. The *Pst*I digestion, which is the confirmation of the cloning (lane no 4), produces two bands, the first one is ~ 4.5 kb in size and the second one is the 2.9 kb in size i.e pBKS vector.

**2.4.7: *Bgl*II , *Bam*HI and *Pst*I digestion of the 4.5kb *Pst*I fragment cloned in pBKS:**

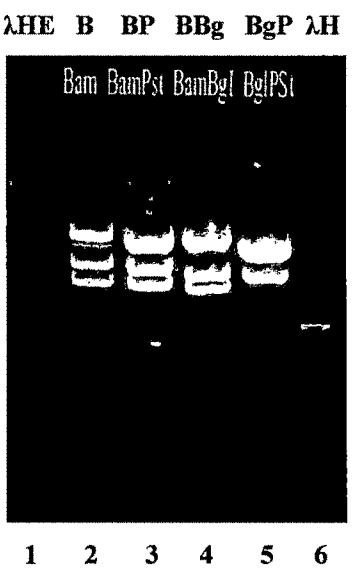



**Figure 2.4.7: Single and double digestion of the 4.5kb *Pst*I clone with respect to three enzymes B: *Bam*HI, Bg: *Bgl*II and P: *Pst*I.**

| Enzymes | No. of fragments |
|---|---|
| *Bam*HI | Four<br>2.2 kb, 0.2 kb , 1.5 kb. 4.0kb |
| *Bam*HI-*Pst*I | Five<br>2.2kb,1.5 kb, 0.8kb, 0.2kb |
| *Bam*HI-*Bgl*II | Six<br>3.8kb,2.0kb, 0.2 kb, 0.2 kb,1.7kb,0.1kb |
| *Bgl*II- *Pst*I | Four<br>3.1kb,3.0kb,1.9kb.0.1kb |

**Table 2.4.7: The sizes of the fragments generated by the single and double digestion of the 4.5kb *Pst*I clone with respect to three enzymes *Bam*HI, *Bgl*II and *Pst*I**

Since the orientation of the 8.0 kb *Eco*RI fragment and the 4.5 kb *Pst*I fragment after cloning in the pBKS vector was chosen to be same, the placements of the internal *Bam*HI and *Bgl*II sites are according to Fig. 2.4, 3a. The *Bam*HI digestion of the ~4.5kb *Pst*I as explained contains the three *BamH*I sites, *actI* hybridising 2.2 kb fragment, 0.2 kb internal *Bam*HI fragment and a 1.5 kb fragment generated by the internal *Bam*HI up to the *Pst*I from the vector (lane no 2).

The *Bam*HI-*Pst*I double digestion (lane no 3) of the 4.5 kb *Pst*I generates five fragments. The first band is the pBKS vector generated by the *Pst*I digestion. The second band is that of invariant internal 2.2 kb *Bam*HI fragment. The third band is also the same 1.5 kb *Bam*HI fragment seen in the single *Bam*HI digestion. The fourth band is the *Pst*I of the vector to first *Bam*HI in the insert (the orientation as shown in the Fig. 2.4, 3a above) which is around 0.8 kb in size. The last band is the unique 0.2 kb *Bam*HI, present internally. Since the 0.2 kb *Bam*HI fragment intervenes the 1.7 kb *Bam*HI and 2.2 kb *Bam*HI fragments, the 0.8 kb fragment seen in *Bam*HI-*Pst*I double digestion is the part of 1.7 *Bam*HI which is placed first in the 8.0 kb *Eco*RI fragment as shown in cartoon 2.

The *Bam*HI-*Bgl*II double digestion in the fourth lane of the 4.5 kb *Pst*I fragment generates six fragments. The first band is 3.8 kb in size generated by the *Bam*HI from the insert to the *Bam*HI present exactly at the opposite end in the vector consisting of 0.8 kb of DNA from the insert and 3.0 of the vector. The second band contains the *Bgl*II at ~ 2.0 kb distance from the *Bam*HI, in the *act I* hybridizing 2.2 kb *Bam*HI fragment, reducing it

by 0.2 kb. Thus 0.2 kb *Bam*HI-*Bgl*II along with the 0.2 kb *Bam*HI fragment is visible as the doublet in the above gel. The third band, 1.7 kb in size is the resultant of the presence of *Bam*HI of the 2.2 kb *Bam*HI site at 0.2 kb distance in the unique 1.9 kb *Bgl*II fragment. In addition, one ~ 0.1 kb fragment is also generated which can be seen in the gel as the last fragment. This band is formed ed by the *Bgl*II of the 1.9 kb *Bgl*II fragment to the *Bam*HI in the vector as this *Bgl*II is just 0.1 kb inside the ~4.5 kb fragment and the *Bam*HI is present on the same side of the *Pst*I in the vector (Fig. 2.4, 3a ), thus this band is also seen in the *Bgl*II-*Pst*I double digestion of the *Pst*I fragment.

Lane no 5 contains the *Bgl*II-*Pst*I double digestion of the 4.5 kb *Pst*I clone. The first two bands of 3.1 kb and 3.0 kb are a doublet, the first of which is *Pst*I from the left hand side (acc. to Fig. 2.4, 3b above) to the first *Bgl*II inside the 4.5 kb vector which is ~3.2 size. The second band in this doublet is the 2.9 pBKS vector generated because of the *Pst*I digestion. The third band is the unique 1.9 kb *Bgl*II fragment that is also the part of the ~4.5 kb *Pst*I fragment. The last fragment is around 0.1 kb generated by the last *Bgl*II of the 1.9 kb *Bgl*II and the *Pst*I (ref Fig. 2.4, 3a).

The restriction map of the 4.5 kb PstI fragment

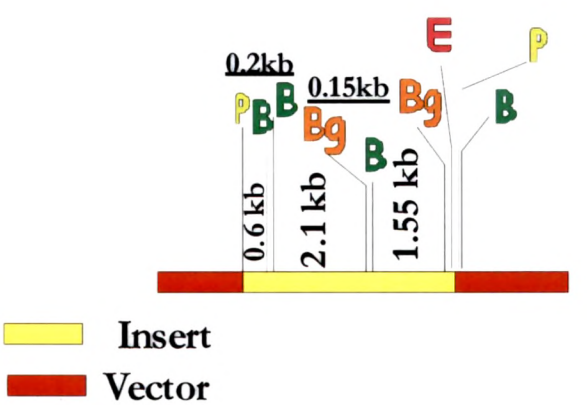



**Figure 2.4.7a: Restriction map of the 4.5 kb *Pst*RI fragment with respect to B: *Bam*HI, P: *Pst*I and Bg: *Bgl*II**

**2.4.8: Digestion of 8.0 kb Δ *Pst*I with *Pst*I, *Bgl*II and *Bam*HI**


**Figure 2.4.8: Single digestion of the 8.0 kb Δ *Pst*I (+) clone with respect to three enzymes B: *Bam*HI, Bg: *Bgl*II and P: *Pst*I.**

As explained in the earlier gel (figure 2.4.7, lane no. 2), 4.5 kb *Pst*I fragment accounts for the presence of three *Bam*HI and two *Bgl* II sites and remaining one site for *Bam*HI and two for *Bgl*II are present outside the limits of 4.5 kb PstI DNA in the 8 kb fragment (here the *Bam*HI from the vector is not considered). So the Δ *Pst*I clone construction for the 8.0 kb fragment in both the orientation was carried out by digestion with *Pst*I followed by re-ligation. If we consider the orientation of 8.0 kb as per Fig. 2.4,3a, then the two *Bgl*II sites and one *Bam*HI site will remain in the insert and rest all the sites will be lost and the *Bam*HI and *Pst*I of the insert will be towards the right end.

The above gel represents the 8.0 kb Δ *Pst*I in the same orientation as Fig. 2.4, 3a thus, the *Bgl*II digestion of the clone shows two fragments - one is 0.2kb *Bgl*II which remains out side the 4.5 *Pst*I suggesting the presence of this *Bgl*II site towards the opposite of the vector *Bam*HI and *Pst*I sites. The *Bam*HI digestion also generates two bands of which one site is provided by the vector and the other by the internal *Bam*HI. The *Bam*HI site as shown in the Fig. 2.4,3a A, the first *Bam*HI in the 8.0 kb fragment is present at 1.0 kb distance from the *Eco*RI site and first *Pst*I is present 2.5 kb from the *Eco*RI site. So here

in this gel we can see a *Bam*HI(vector) – *Bam*HI (insert) fragment of the 1.0 kb size is seen confirming the above placements with respect to these four enzymes *Eco*RI, *Bgl*II, *BamH*I and *Pst*I to be correct. This was further confirmed by the digestion pattern of the 8.0kbΔ PstI clone in other orientation.

**2.4.9: *Pst*I, *Bgl*II and *Bam*HI digestion of 8.0 kb Δ *Pst*I in (-) orientation and 8.0 kb Δ *Bgl*II**

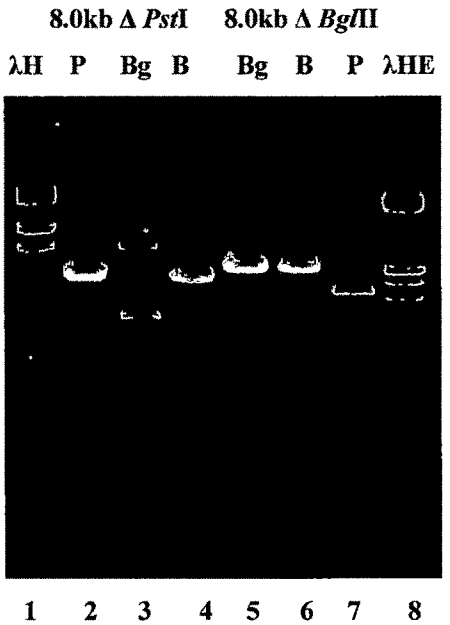



**Figure 2.4.9: Single digestion of the 8.0 kb Δ *Pst*I (-) clone and 8.0 kb Δ *Bgl*II clone with respect to three enzymes B: *Bam*HI, Bg: *Bgl*II and P: *Pst*I.**

The gel here represents the 8.0 kb Δ *Pst*I in other orientation according to Fig. 2.4, 3b and the 8.0kb Δ *Bgl*II in the direction according to Fig. 2.4, 3a. As shown in the Fig. 2.4, 3b, all the internal sites will be deleted if we construct the 8.0 kb Δ *Pst*I clone in this orientation. The gel shows linear band in *Bam*HI and *Pst*I digestions as only the *Bam*HI and *Pst*I from the backbone will remain (lane no 2 and 4). Also, digestion by *Bgl*II is not possible (lane no 3) since there is no *Bgl*II site in the vector back bone.

The second half represents the digestion of 8.0kb Δ *Bgl*II clone with *Bgl*II, *Bam*HI and *Pst*I. The orientation of this clone is according to the Fig. 2.4, 3a. The *Bgl*II and *Bam*HI

digestions show the formation of linearised DNA (lane no 5,6 ), the *Bam*HI here is from the vector backbone. The *Pst*I digestion shows two bands (lane no 7) as shown in the Fig. 2.4, 3a that the last *Bgl*II site is just 0.2 kb inside the 4.5kb *Pst*I fragment, this is also shown in the *Bgl*II- *Pst*I double digestion of the 4.5kb *Pst*I clone (Fig. 2.4.7, lane no 5). Thus after the removal of all the *Bgl*II sites, one band of the size 1.2 kb will be generated between the *Pst*I (vector ) – *Pst*I in the insert ( the second *Pst*I site of the 4.5 kb *Pst*I fragment) (lane no 7).

| Enzymes | No. of fragments |
|---|---|
| *Eco*RI | Two<br>(17.0kb, 3.0kb) |
| *Bgl*II | One<br>(20.0kb) |
| *Pst*I | Five<br>(4.4kb, 4.2kb,3.4kb, and two 2.3kb) |
| *Eco*RI-*Pst*I | Seven<br>(4.4kb,Two 3.4kb, 3.0 kb, two 2.3kb, 1.2kb) |
| *Bgl*II-*Pst*I | Seven<br>(4.4kb,4.2kb, two 3.4kb,2.3kb, two 1.2 kb) |

**Table 2.5.1: The sizes of the fragments generated by the single and double digestion of the 17.0 kb *Eco*RI clone with respect to three enzymes E: *Eco*RI, Bg: *Bgl*II and P: *Pst*I.**

This fragment contains the only one *Bgl*II site (lane no 6). This site is located at the extreme right end as 1.1 also generates a unique ~17 kb *Bgl*II fragment which is generated by the *Bgl*II from the 8.0kb *Eco*RI fragment to the *Bgl*II in the 17 kb fragment placing it at the extreme right end, i.e., the end of the cluster from the right. This *Bgl*II site is located approx. 2.5 kb inside the last *Eco*RI site . As could be visible in the *Eco*RI-*Bgl*II double digestion other than 3.0 kb vector and the first 14.5 kb band the third band is 2.5 kb of size (lane no. 4). 1.1 cosmid is unusual in that it contains maximum *Pst*I sites and these sites are mainly located in the 17 kb fragment - this fragment contains as many as five internal *Pst*I sites generating one 4.4 kb, one 3.4 kb and two 2.4 kb fragments (section 1.4.2). Since this 17 kb fragment is placed next to the 8.0 kb *Eco*RI fragment on its right, *Pst*I of the 4.5kb *Pst*I fragment (in the 8.0 fragment) extends up to ~3.4 kb in the 17kb DNA. Of the doublet generated at 3.4 kb, one is internal 3.4 kb which is invariant and another one is generated by the *Pst*I in the vector to the first *Pst*I which is the same 3.4 kb DNA linking 8.0 kb and 17.0 kb. Another ~4.2 kb fragment of *Pst*I is the one containing the 3.0 kb vector and 1.2 kb region of the 17 kb from the extreme right side. This could be seen in the *Eco*RI- *Pst*I digestion in which this fragment is broken down in to two fragments of 3.0 kb and 1.2kb. As discussed above the unique *Bgl*II is present 2.4 kb inside the 17kb fragment. Thus, as could be seen in the *Bgl*II- *Pst*I

digestion one of the 2.5 kb *Pst*I fragment is broken down in to two 1.2 kb fragments and rest all the fragments remains the same as in the *Pst*I single digestion.

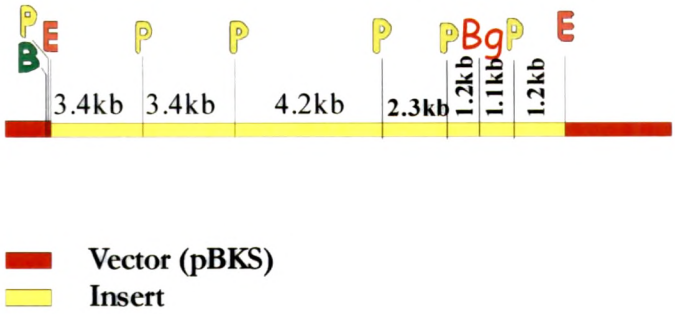



**Figure 2.5.1a: Restriction map of the 17.0 kb *Eco*RI clone with respect to three enzymes E: *Eco*RI, Bg: *Bgl*II and P: *Pst*I.**

## 2.6: Conclusion:

We discussed in the previous chapter that the four *actI* hybridizing clones isolated from the genomic DNA library of *S.flaviscleroticus* span ~45 kb region among each other. This region is supposed to contain the complete PKS cluster, to characterize it further the restriction map for this region was needed to be constructed.

This chapter discusses the construction of the restriction map of that 45 kb region. This region is divided in to five different sized *Eco*RI fragments in the sequence 5.0kb-11.0 kb-4.0 kb-8.0 kb-17kb. To construct the restriction map for this region all these fragments were subloned from the different parent cosmid clones. Fragment, 5.0 kb was cloned form 1.23 clone, 11.0 kb was cloned from the clone 1.23 and 1.51 in both orientation (+) and (-), 4.0 kb from 1.51, 8.0 in both the orientations (+)and (-) from 1.51 and 17 kb from 1.1. These subclones were then further subjected to single and double digestion with the enzyme *Bgl*II, *Eco*RI, *Bam*HI and *Pst*I.

Depending upon the *actI* hybridization pattern the restriction analysis of these subclones further helped in to narrowing down to the region containing the PKS cluster. To resolve some of the ambiguities generated by the restriction digestion analysis regarding the placement of come of the enzymes few more clones were constructed which include the

subcloning of 4.5 kb *Pst*I clone, construction of the 11kbΔ *Pst*I clone in (+) and (-) orientation, construction of 8.0 kbΔ*Pst*I clone and 8.0 kbΔ*Bgl*II clones. This restriction map eased our work to select out the region which could code for the functional PKS cluster. The 2.2 kb *Bam*HI fragment, 1.7 kb *Bam*HI fragment and 1.9 kb *Bgl*II fragment (from 8.0kb *Eco*RI fragment) were subcloned so that they could be sent for the sequencing. Subsequently the complete sequencing of this 45 kb region was carried out and it was found that the restriction which we had constructed could be exactly aligned with the one generated from the sequence except the one or two fragments which were <50 to 100 bp in size.

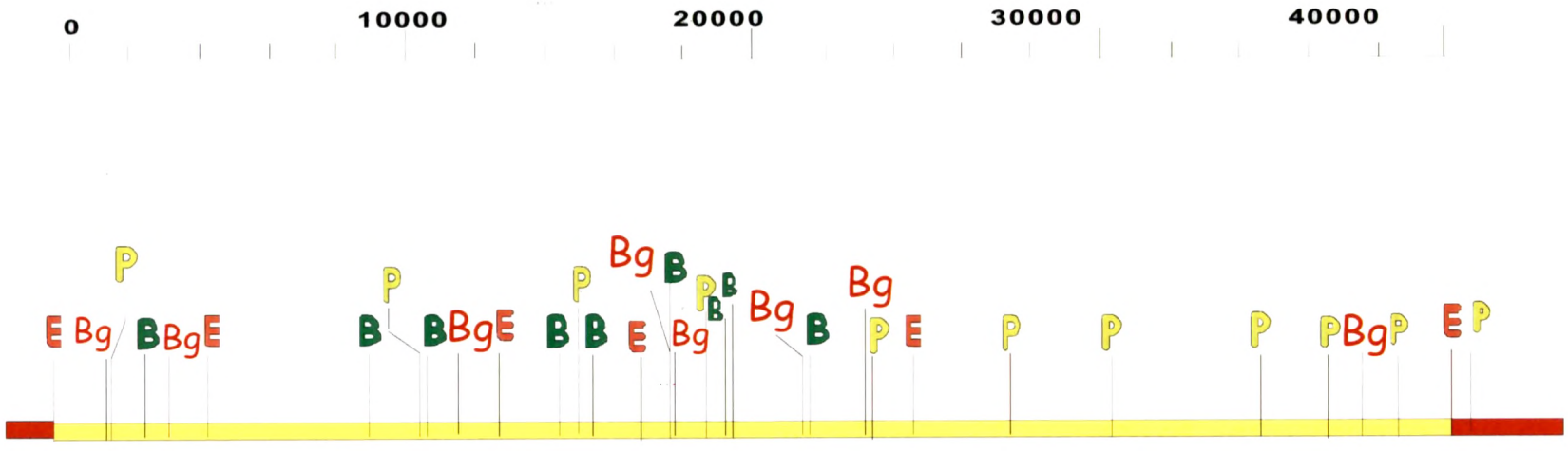



**Figure 3: Partial restriction map for the ~45 kb region with respect to four enzymes, E: *Eco*RI, Bg: *Bgl*II, P: *Pst*I, B: *Bam*HI**