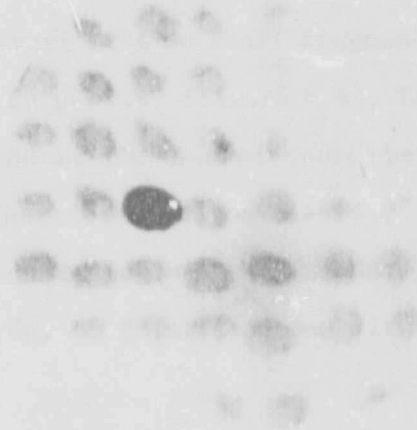

Figure 4.2. .*in situ* colony hybridisation of D286 wt genomic library using a ^{32}P labelled probe made from the D286 DNA insert isolated from pCD1253. Colony in row 5, column 3 (from left to right), is *E. coli* DH1 (pCD1253).

enting about 25% of the library were no longer able to grow and could not be recovered. Therefore the genomic library became incomplete and it was decided to construct a new library.

To determine if it was possible to continue using the same D286 wt DNA fragment in pCD1253 as a probe in further hybridizations it was necessary to establish categorically that such fragment had in fact originated from strain D286 wt DNA. DNA homology experiments showed that this was not the case.Undigested samples of pCD1253, pLAFRI and D286 wt DNA as well as a partially digested (EcoRI) sample of D286 wt DNA were electrophoresed (Figure 4.3), Southern blotted and then hybridized with a ^{32}P labelled probe of the putative D286 wt EcoRI fragment present in pCD1253. The probe hybridized only to pCD1253 (Figure 4.4). The lack of homology between the probe DNA and D286 wt DNA showed that the insert DNA in pCD1253 is not D286 wt DNA. Therefore it was decided to utilize pCDTn5-3, a hybrid pSUP106 cosmid containing a D286 wt DNA:: Tn5 insert to probe the new gene bank, in order to detect a clone with which chromosome walking could be started.

4.2 CONSTRUCTION OF A D286 WT CELLULAR DNA GENE BANK IN THE COSMID VECTOR PLAFRI

Partial EcoRI digestion of D286 wt DNA and total EcoRI digestion of pLAFRI

A calibration digestion was carried out to determine the conditions that would result in an abundance of 20-30 kb D286 wt DNA fragments, a prerequisite of the chromosome walking technique (Hadfield, 1985). The digested samples were electrophoresed (Figure 4.5). Analysis of the electrophoretogram indicated that digestion of D286 wt DNA for 1 h at 37° C with 0.39 U EcoRI/µg DNA produced large fragments in abundance (figure 4.5, lanes 3). Therefore D286 wt DNA was digested under these conditions and used in ligations to pLAFRI.

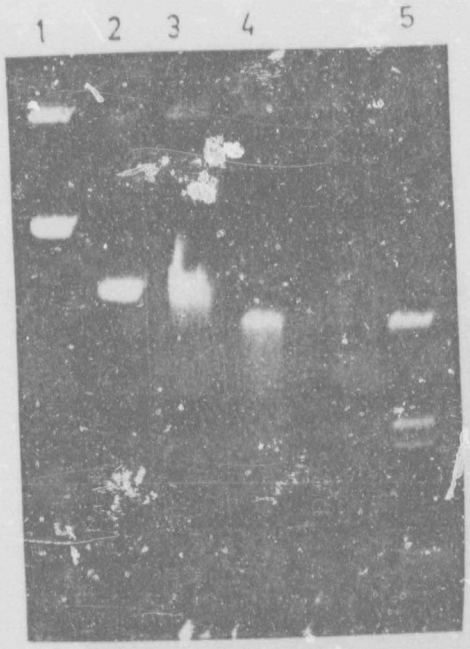

Figure 4.3. Agarose gel electrophoresis of pCD1253, pLAFRI and D286 DNAs. Lane 1, pCD1253; 2, pLAFRI digested with EcoRI; 3, D286 total DNA; 4, D286 total DNA partially digested with EcoRI; 5, λ HE (λ III).

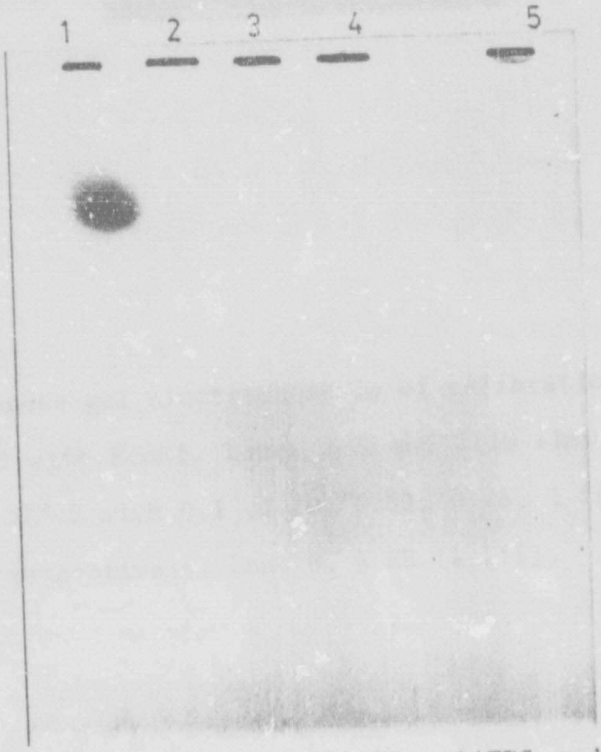

Figure 4.4. Hybridisation of pCD1253, pLAFRI and D286 wt DNAs with a ^{32}P labelled probe made from the DNA insert isolated from pCD1253. The DNAs were Southern blotted from the agarose gel seen in figure 4.3. Lane 1, pCD1253; 2, pLAFRI digested with EcoRI; 3, D286 wt total DNA; 4, D286 wt DNA partially digested with EcoRI; 5, λ HE (λ III).

Figure 4.5. Agarose gel electrophoresis of calibration digestion of D286 wt DNA digested with EcoRI. Lanes 1-5 and 7-10 show digestions of D286 DNA for 1 h at 37° C with 0.1 ,0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 U EcoRI respectively. Lane 6, λ HE (λ III).

Ligation and *in vitro* packaging

Agarose gel electrophoreses of EcoRI digested D286 wt and pLAFRI DNA ligations and control ligations can be seen in Figure 4.6. Lane 2 shows undigested pLAFRI whereas lanes 3 and 4 show pLAFR1 digested with EcoRI and pLAFRI after ligation to itself respectively. Lanes 5, 6 and 7 show D286 wt DNA, partially digested with EcoRI, undigested, and after T4 DNA ligase ligation to itself. In both cases it can be seen that after ligation the migration pattern of undigested DNA was restored to an extent indicative of successful ligation. It is difficult to explain, however, the appearance of additional DNA bands in lanes 7, 8 and 9, which in turn made difficult to conclude if insert-vector DNA ligation had taken place (lanes 8 and 9). However, since vector and insert DNAs ligated to themselves it was assumed that they had ligated to each other as well. The remaining ligated DNA was therefore used for *in vitro* packaging with λ DNA and subsequent infection of E. coli DH1.

A number of attempts to *in vitro* package ligated DNA did not meet with success. Since the system was proven to work efficiently when the packaging of standard λ DNA (provided by the manufacturer) was tested, it was suspected that the amount of DNA used was insufficient, therefore in the ligations described above, the amount of insert DNA was increased from about 200 ng to about 1 µg and insert to vector ratios of 2:1 and 5:1 were used. The *in vitro* packaging system used requires that the ligated DNA be added in a volume of 4 µl, to which end the samples in this work had to be concentrated by ethanol precipitation. Very low yield in the DNA precipitation step was shown to be the likely cause of of unsuccessful ligations. The DNA concentration of a 1 µl aliquote of the "concentrated" ligated DNA was determined (using a concentration gel, Figure 4.7) to be only 25 ng/µl. With such low yield in previous unsuccessful packagings, insignificant amounts of DNA were being added.

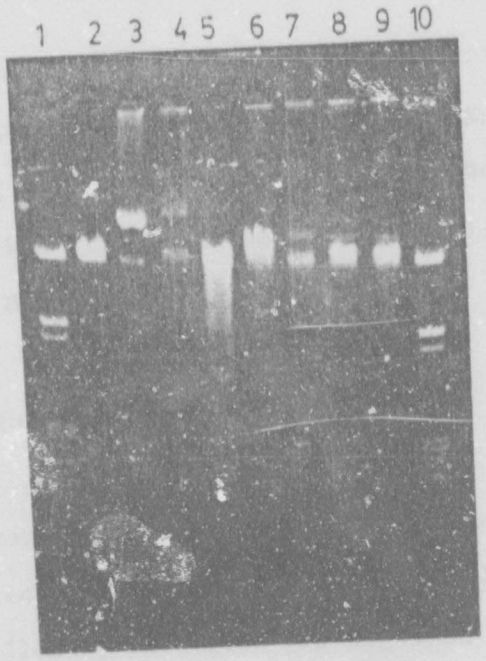

Figure 4.6.Agarose gel electrophoresis of ligation of pLAFRI digested with EcoRI and D286 wt total DNA partially digested with EcoRI, and control ligations. Lane 1, λ HE (λ III); 2, pLAFRI digested with EcoRI; 3, pLAFRI undigested; 4, pLAFRI ligated to itself; 5, D286 wt total DNA partially digested with EcoRI; 6, D286 wt total DNA undigested; 7, D286 wt total DNA partially digested with EcoRI and ligated to itself; 8. 5:1 ratio D286-pLAFRI DNAs ligation; 9, 2:1 D286-pLAFRI DNAs ligation; 10, λHE (λ III).

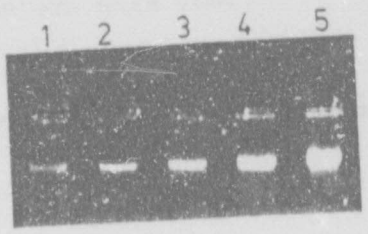

Figure 4.7. Determination of concentration of pLAFRI-D286 wt ligation DNA used for *in vitro* packaging. Lane 1, ligated DNA; 2, 3, 4 and 5, λ DNA 25, 50, 100 and 200 ng respectively.

Following infection strains harboring recombinant pLAFR1 were selected by plating in LB agar supplemented with Tc (20µg/ml). From the number of colonies arising and from the knowledge of the amount of DNA used in the packaging reaction (100 ng) the packaging efficiency was determined to be 1.1 x 10^6/µg DNA.

Determination of library size

In order to determine the number of members necessary for the genomic library to represent the entire D286 cellular DNA, plasmid DNA from 9 clones selected following infection was digested to completion with EcoRI and the samples electrophoresed (Figure 4.8). This yielded a number of fragments, in addition to the open vector, the sizes of which were added in order to determine the insert size of each of the recombinant cosmids digested (Table 4.1). The average insert size (27.7 kb) was used for the calculation of the number of members needed in the library using the formula published by Maniatis et al. (1982). It was calculated that the library should have at least 497 members, 1750 colonies were then propagated in order to work with a complete library even if a considerable percentage of the members were lost.

Corroboration that the insert DNA in the recombinant cosmids originated from strain D286 wt DNA

In order to confirm that the insert in the newly constructed library had indeed originated from D286 wt, the recombinant cosmids of 4 randomly selected clones of the gene bank were digested with EcoRI and Southern blotted agarose gel electrophoreses of such digestions (Figure 4.9) hybridised with a ^{32}P labelled D286 wt DNA probe (Figure 4.10). As expected the results show that the insert DNA was D286 wt DNA. Since the characteristics of this new library satisfied our expectations it was used through the rest of this study. From here onwards, when reference is made

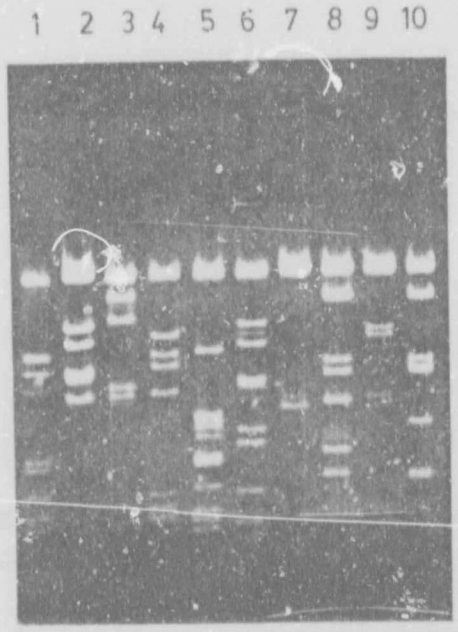

Figure 4.8. Agarose gel electrophoresis of total EcoRI digestions of 9 recombinant cosmids from the D286 wt gene library used for the determination of average size of inserts. Lane 1, λ HE (λ III); 2-10 , pCD2775. pCD0175, pCD0531, pCD0211, pCD0463, pCD0151, pCD0412, and pCD0411 respectively.

TABLE 4.1 Insert sizes of 9 recombinant cosmids of the D286 wt gene bank

Hybrid cosmid designation	Number of EcoRI fragments of D286 insert DNA	Total insert size
pCD0451	7	30.19 kb
pCD2727	8	30.87 kb
pCD0155	8	26.39 kb
pCD0531	11	25.89 kb
pCD0211	9	30.97 kb
pCD0463	2	27.57 kb
pCD0151	5	26.92 kb
pCD0412	4	21.32 kb
pCD0411	6	29.17 kb

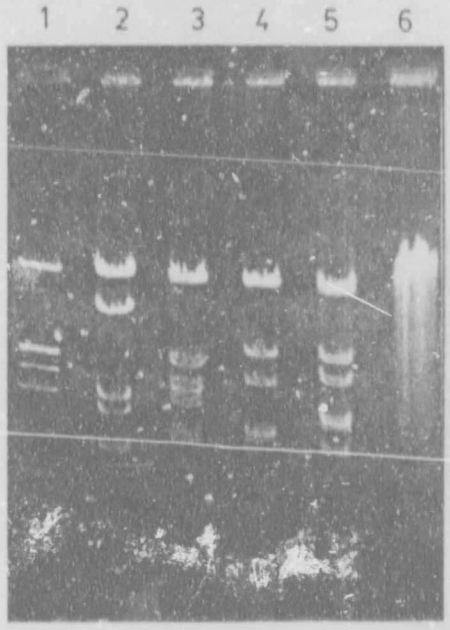

Figure 4.9. Agarose gel electrophresis of total EcoRI digestions of four recombinant cosmids from the D286 wt gene library. Lane 1, λ HE (λ III); 2-5, pCD0151, pCD0155, pCD0211, and pCD0451 respectively; 6, D286 DNA partially digested with EcoRI.

Figure 4.10. Hybridisation of recombinant cosmids subjected to EcoRI total digestion with a ^{32}P labelled probe made from D286 wt total DNA partially digested with EcoRI. DNA samples were Southern blotted from the gel seen in figure 4.9. Lane 1, λHE (λIII); 2-5, pCD00151, pCD0155, pCD0211 and pCD0451 respectively; 6, D286 wt DNA partially digested with EcoRI.

to the D286 wt DNA gene bank, we refer to the library described in this section.

4.3 DETECTION OF THE FIRST CLONE CARRYING PUTATIVE AGROCIN D286 GENES, E.COLI DH1 (PCD0932), IN THE D286 WT GENE BANK

The recombinant cosmid pCDTn5-3, which consists of pSUP106 (Priefer et al.,1985) carrying a D286 wt DNA:: Tn5 insert, was labelled with [α-^{32}P]dCTP to a specific activity of 1 x 10^8 cpm/µg DNA and used to probe the D286 wt gene library in search for clones carrying putative agrocin genes. *In situ* colony hybridisation of the library resulted in the detection of 1 clone, *E. coli* DH1 (pCD0932) that hybridised strongly with the ^{32}P labelled probe (Figure 4.1).

In order to characterise the recombinant cosmid pCD0932 it was subjected to EcoRI total digestion and the digest electrophoresed (Figure 4.12). To corroborate that the insert DNA was in fact D286 wt DNA, the gel was Southern blotted and hybridized with a ^{32}P labelled D286 wt DNA probe. To obtain reasonable [α-^{32}P dCTP incorporation during nick translation as well as detectable hybridisation it was necessary to use D286 wt DNA partially digested with EcoRI. This improves incorporation of label as well as hybridisation results markedly, apparently because it relaxes the DNA.

The insert DNA of pCD0932 was shown to consist of 4 EcoRI fragments (Figure 4.12, lane 4 and Figure 4.14 lanes 2-5 and 6) of 14.3, 3.55, 1.99 and 1.95 kb, totaling 21.8 kb (sizes were calculated from electrophoretograms shown in Figure 4.19). Results of DNA hybridizations (Figure 4.13) showed the insert to be D286 wt DNA. Overloading (in order to visualise the lower molecular weight fragments) resulted in lack of discrimination between the vector DNA and the larger EcoRI D286 DNA fragment. Therefore, the individual D286 DNA fragments (that had been

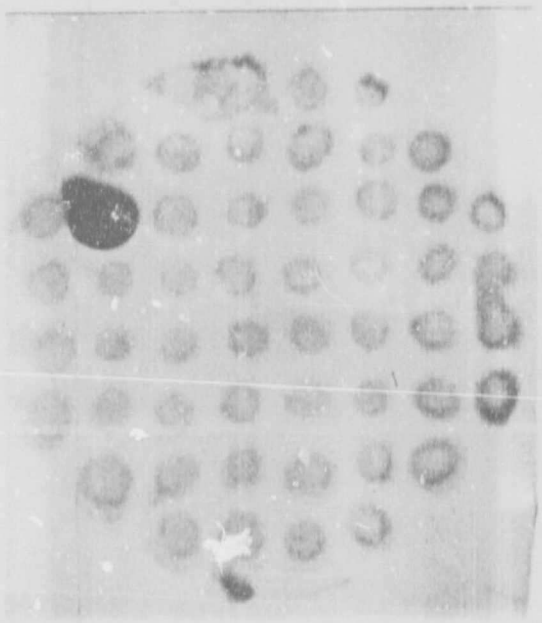

Figure 4.11. *in situ* colony hybridisation of the D286 wt gene library with a ^{32}P labelled pCDTn5-3 probe. The filter which contained *E. coli* DH1 (pCD0932), the only positive colony detected, is shown in row 3, column 2 (from left to right).

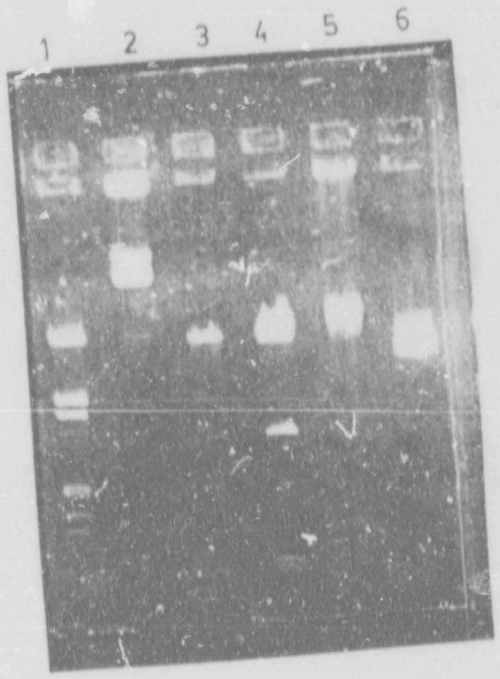

Figure 4.12. Gel electrophoresis of pCDTn53, pLAFRI, D286 and pSUP204 DNAs. Lane 1, λ HE (λ III); 2-4, pCDTn5-3, pLAFRI, pCD0932, subjected to EcoRI total digestion respectively; 5, D286 wt total DNA; 6, pSUP204.

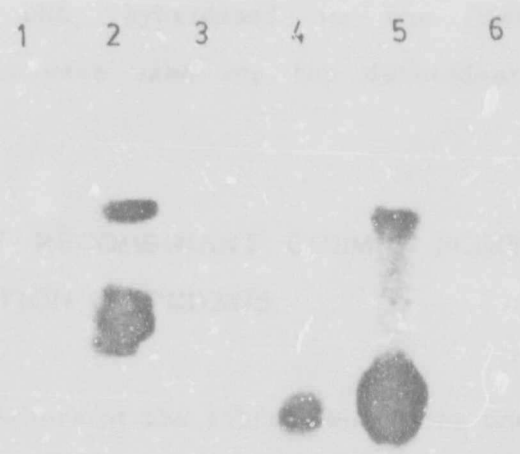

Figure 4.13. Hybridisation of Southern blotted pCDTn5-3, pLAFRI, pCD0932, D286 and pSUP204 DNAs with a ^{32}P labelled D286 wt DNA probe. DNAs were Southern blotted from the agarose gel seen in figure 4.12. Lane 1, λ HF (λ III); 2-4, pCDTn5-3, pLAFRI and pCD0932 subjected to EcoRI total digestion respectively; 5, D286 wt total DNA; 6, pSUP204.

purified from from electrophoresed EcoRI digestions of pCD0932 with the purpose of making a labelled probe) were electrophoresed (Figure 4.14) and the Southern blotted gels hybridized with a the ^{32}P labelled D286wt DNA probe. The results (Figure 4.15) further confirmed those obtained from figure 4.13 and helped to establish that the larger D286 wt DNA fragment, and not vector DNA, hybridized to the D286 wt DNA probe.The electrophoretograms were used for the determination of the fragment sizes.

4.4 USE OF THE RECOMBINANT COSMID PCD0932 IN CHROMOSOME WALKING. DETECTION OF PCD2375

Since one of the members of the library was being used to probe the entire library it was not possible to use the entire recombinant cosmid pCD0932 as the probe. Therefore the individual D286 wt DNA fragments , isolated from an electrophoresed EcoRI digestion of pCD0932, were pooled and nick translated. The resulting probe (specific activity about 0.5×10^7 cpm/µg) DNA was used for the *in situ* colony hybridization of the library. One positive clone was detected, *E. coli* DH1 (pCD2375), (Figure 4.16).

Preliminary characterization of pCD2375 was carried out following an approach similar to that used in the characterization of pCD0932. EcoRI digested pCD2375 was electrophoresed (Figure 4.17). The resulting agarose gel was Southern blotted and hybridized with a pCD0932 insert-DNA ^{32}P labelled probe (Figure 4.18).

The DNA insert of pCD2375 consists of 4 EcoRI fragments (Fig 4.17) of 10.5, 7.3, 3.5 and 3.4 kb, totalling 24.8 kb. The third largest EcoRI insert fragment (3.5 kb) of pCD2375 coincides in size with the second largest EcoRI fragment of pCD0932 (Figure 4.17; lanes 2-3 vs lanes 4-5). That pCD2375 and pCD0932 have such 3.5 kb EcoRI fragment in common was shown by hybridization of Southern blotted EcoRI digestions of both

recombinant cosmids with a $^{32}p_p$ labelled probe made from the pCD0932 insert DNA (Figure 4.18). The 3.5 kb EcoRI fragment of pCD2375 hybridized to the ^{32}P labelled pCD0932 3.5 kb EcoRI fragment corroborating, as expected, that the 2 fragments are homologous D286 wt DNA. From the foregoing it follows that the D286 wt DNA inserts of pCD0932 and pCD2375 represent 2 contiguous DNA fragments with an overlapping 3.5 kb region.

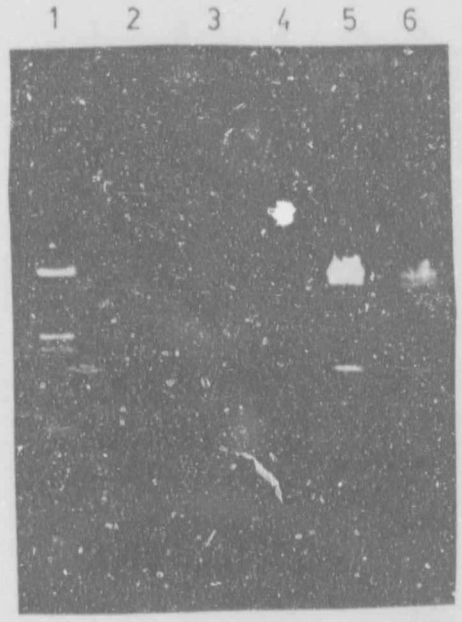

Figure 4.14. Agarose gel electrophoresis of pCD0932 subjected to EcoRI total digestion, and of isolated EcoR1 fragments of the D286 wt DNA insert in pCD0932. Lane 1, λ HE; 2-3 isolated isolated EcoRI D286 wt DNA insert fragments of pCD0932; 5-6, pCD0932 subjected to EcoRI total digestion.

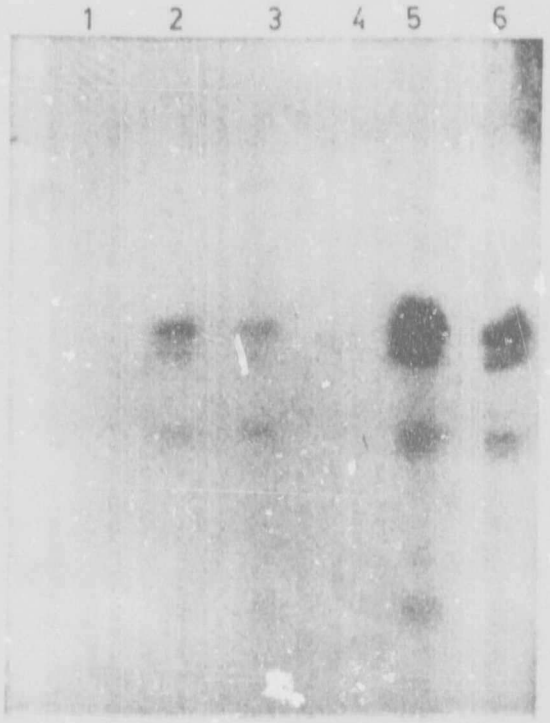

Figure 4.15. Southern blot hybridisation of pCD0932 subjected to EcoRI total digestion and of isolated EcoRl fragments of the D286 wt DNA insert of pCD0932 with a ^{32}P labelled D286 wt DNA probe. DNA was Southern blotted from the agarose gel shown in figure 4.14. Lane 1, λ HE (λ III); 2-3, isolated EcoRI fragments of the D286 DNA insert of pCD0932; 5-6, pCD0932 subjected to EcoRI total digestion.

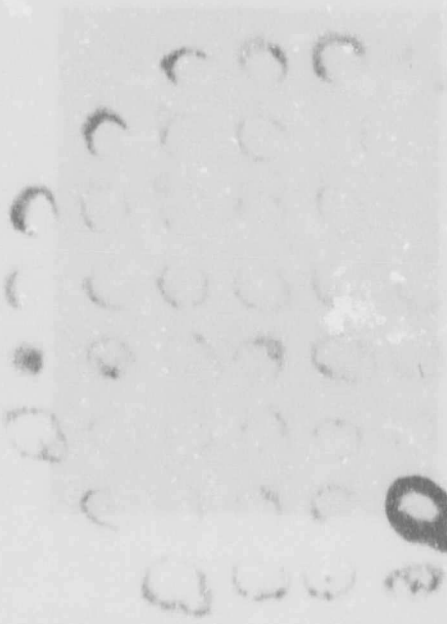

Figure 4.16. *in situ* colony hybridisation of the D286 wt gene library with a ^{32}P labelled probe made from the EcoRI D286 wt DNA fragments of pCD0932. The filter containing the only positive clone detected, *E. coli* DH1 (pCD2375) is in row 7, column 5 (from left to right).

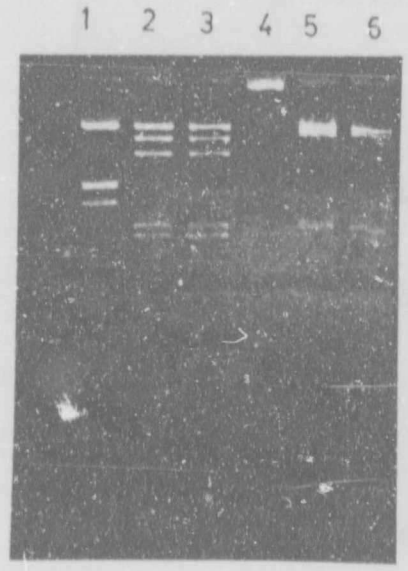

Figure 4.17. Agarose gel electrophoresis of EcoRI fragments of pCD0932 and pCD2375. Lane 1, λ HE (λ III); 2-3, pCD2375 EcoRI fragments; 4, pCD2375 undigested; 5-6, pCD0932 EcoRI fragments.

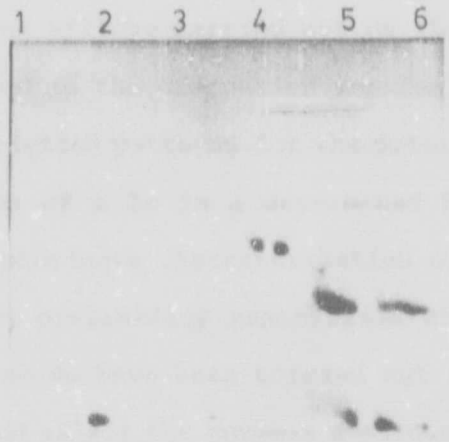

Figure 4.18. Southern blot hybridisation of pCD0932 and pCD2375 EcoRI fragments using a ^{32}P labelled probe made from the isolated D286 EcoRI DNA fragments of pCD0932. DNAs were Southern blotted from the gel seen in figure 4.17. Lane 1, λ HE (λ III); 2-3, pCD2375 EcoRI fragments; 4, pCD2375 undigested; 5-6, pCD0932 EcoRI fragments.

4.5 PRELIMINARY WORK ON THE RESTRICTION ENDONUCLEASE CHARACTERIZATION OF PCD0932 AND PCD2375.

Genetic manipulations will be carried out on the DNA inserts of pCD0932 and pCD2375 (outlined in the discussion section) that will require knowledge of their restriction patterns for the interpretation of results (for example the location of a Tn in a determined DNA fragment). Therefore, the restriction endonuclease characterization of pCD0932 and pCD2375 are underway. Thus far, preliminary restriction endonuclease digestions of both recombinant cosmids have been carried out in order to establish adequate conditions and select the enzymes most useful. A problem that arose is that it is not possible, at this point, to isolate the entire insert fragment of the hybrid cosmids, therefore digestions of the entire recombinant cosmids had to be carried out.

Preliminary digestions with a number of different enzymes (results not shown) indicated that in addition to EcoRI SalI and BglII were adequate choices because they cut the vector DNA only at 1 and 2 sites respectively and they produce a restriction pattern reasonably spread and with not too many fragments in both pCD2375 and pCD0932.

It was necessary to establish the conditions for obtaining a reasonable discrimination of sizes. This is because in single digests involving either SalI or BglII not all the insert DNA is separated from the vector DNA. The amount of insert DNA that remains with the vector can be estimated from the knowledge of the vector's size and that of the vector plus the insert DNA not removed by SalI or BglII. Since the DNA sizes involved in this estimation are in the range of the 20 kb, differences of less than 1-2 kb are difficult to detect with the agarose concentration used in the rest of this work (0.75%). Therefore, restrictions digestions were

electrophoresed in 0.45% agarose gels. The discrimination of large size DNA fragments improved greatly. Figures 4.19, 4.20 and 4.21 show the discrimination obtained using 0.45 % agarose gels electrophoresed for 24, 36 and 48 hs respectively in a large slab gel electrophoresis tank at 45V and 4° C. It can be seen that although good estimations of the larger size fragments can be made from these electrophoretograms, particularly from figure 4.21, the smaller fragments are very difficult to visualise because they tend to diffuse. Perhaps higher percentage agarose gels should be used for estimating the sizes of the smaller fragments.

In pCD0932 there are 5, 4 and 3 restriction sites for EcoRI, SalI and BglII respectively. In pCD2375 there are 5, 7 and 3 restriction sites for EcoRI, SalI and BglII respectively (Figure 4.19). Double digestions indicate the probable presence of doublets in pCD0932 digested with EcoRI-SalI and pCD2375 digested with EcoRI-SalI and BglII-SalI since the number of fragments that they give rise to is one less than the sum of the respective single digestions. Analysis of this information together with data not presented here are underway.

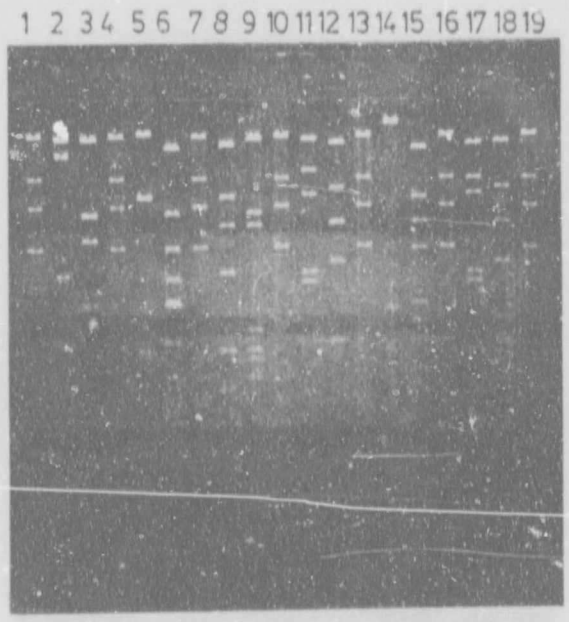

Figure 4.19. Gel electrophoresis (0.45 % agarose; run for 24 hs) of single and double digests of pCD0932 and pCD2375 with EcoRI, SalI and BglII. Lanes 1, 4, 7, 10, 13, 16 and 19, λ H (λ II). Lanes 2-3, 5-6, 8-9, pCD0932 digested with EcoRI, SalI, BglII, EcoRI + SalI, EcoRI + BglII and BglII + SalI respectively. Lanes 11-12, 14-15, 17-18, pCD2375 digested with EcoRI, SalI, BglII, EcoRI + SalI, EcoRI + BglII and BglII + Sal I respectively.