6-25-1990

Preparation of a bank of cloned genes from the chromosome of Agrobacterium tumefaciens and the isolation of genes involved in DNA repair and genetic recombination

Geoffrey A. Bartholomeusz

Rochester Institute of Technology

College of Science

Preparation of a bank of Cloned Genes from the Chromosome of *Agrobacterium Tumefaciens* and the Isolation of Genes involved in DNA Repair and Genetic Recombination

by

Geoffrey A. Bartholomeusz

A master's thesis, submitted to
The Faculty of the College of Science,
in partial fulfillment of the requirements for the degree of
Master of Science in Clinical Chemistry

25th June 1990

Thesis Committee:

Dr. Robert Rothman

Dr. Howard Harrison

Mr. James Aumer

Form 10
M.S. Clinical Chemistry

ROCHESTER INSTITUTE OF TECHNOLOGY
Rochester, New York 14623
Department of Clinical Sciences

STATEMENT FOR GRANTING OR DENYING PERMISSION TO REPRODUCE THESIS

The author of a thesis or formal research report should complete one of the following statements and include this statement as the page of the document following the title page. If the author does not wish to give permission to reproduce the document, then the first page will be both statements typed but not filled in.

Title of Thesis or Research Report Preparation of a bank of Cloned Genes from the Chromosome of Agrobacterium tumefaciens and the Isolation of Genes involved in DNA Repair and Genetic Recombination.

I, Geoffrey A. Bartholomeusz, hereby (grant, ~~deny~~) permission to the Wallace Memorial Library of R.I.T., to reproduce the document titled above in whole or in part. Any reproduction will not be for commercial use or profit.

OR

I, Geoffrey A. Bartholomeusz, prefer to be contacted each time a request for reproduction is made. I can be reached at the following address:

_____

_____

Date 25th June 1990

ABSTRACT

The virulent property of the *Agrobacterium tumefaciens* is associated with its tumor inducing (Ti) plasmid. Recent studies on the virulence of this bacterium has shown that genes located on its chromosome also contribute to this property. One such gene is thought to produce a protein that has properties similar to that of the recA protein of *E. coli*.

This thesis outlines the techniques that were used to try and isolate the recA-like gene from the chromosome of the *Agrobacterium tumefaciens*. All the techniques used in this study are outlined in detail and an explanation given for the choice of each technique. For reasons not completely understood, we were unable to isolate the recA-like gene even though a gene bank was successfully constructed and appropriately sized fragments extracted. Attempts were made to explain some of the unexpected results and appropriate steps were proposed to further understand them.

ACKNOWLEDGEMENTS

To begin with I record my deep appreciation to my supervisor Dr. Robert Rothman for granting me the opportunity to work on this project. I thank him for the unfailing courtesy and generosity with which he extended to me the benefits of his guidance, experience, and advice.

I owe Dr. Joseph Devine my thanks for granting me permission to conduct this research project in the area of Molecular Biology.

I also record my special thanks to Frank Pendola for his ready helpfulness, and for the many profitable discussions we had on matters connected with my work.

I would also like to express my thanks to Beverly Frisell-Fleig for the willing assistance she gave me in the early stages of this project.

I owe a special expression of thanks to my brother Brian for his generosity in granting me his time and expertise in the final presentation of this research project.

My wife Chandra was a great support to me by her confidence and understanding. I owe her more than I can express.

Finally, to my parents who always had faith in me I say thank you and may God bless you both.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

CHAPTER 1
INTRODUCTION

(1.1) Background.

Since 1907, *Agrobacterium tumefaciens*, a gram negative soil organism has been recognized as the etiological agent of Crown gall. Crown gall is a neoplastic disease induced in a wide variety of gymnosperms and dicotyledonous angiosperms by the inoculation of wound sites with the bacterium. The virulence trait of this bacterium is carried on one of a number of tumor inducing (Ti) plasmids.[23] This Ti plasmid is large, and ranges in size from 90×10^6 to 150×10^6 daltons.[23] It is essential for tumorigenesity.[10] The Ti plasmid of *Agrobacterium tumefaciens* contains two major groups of genes that are important for the establishment of neoplastic growth of infected tissue in many diverse plants.[3] The first group of genes is contained within the T-region of the Ti plasmid. This is the segment of the Ti plasmid that is transferred to the host plant and becomes integrated in the genome of the host plant during tumorigenesis.[3,25] These genes direct the production of opines, namely octopine and nopaline, as well as the production of growth regulating hormones.[3] They also determine the morphology of the tumor that results from the infection.[3] The T-DNA is flanked on the Ti plasmid by short, imperfect repeats that comprise the structural and functional termini of the T-DNA.[25] The second group of genes on the Ti plasmid is contained in the virulence (vir) region. This region is 35 kilobases long and is situated outside the T-DNA region.[21] The vir region is expressed in the bacterium particularly during the interaction between the bacterium and the plant cell.[3] Mutations in the vir region result in the loss of virulence.[3] The vir genes are thought to encode functions that are necessary for the transfer of the Ti plasmids into plant cells, although, they themselves, have not been found in tumor cells.[7]

Most Ti plasmids fall into one of two groups depending on whether they code for octopine or nopaline catabolism.[23] Strains harboring an octopine-type Ti plasmid generally induce unorganized tumors that produce octopine, while strains harboring the nopaline-type Ti plasmid usually induce teratomas, although they too can produce unorganized tumors. Ornithine is an important intermediate of octopine and nopaline degradation.[20] Ornothine is degraded to glutamic semialdehyde which in turn is converted to glutamate. This glutamate provides the carbon source for the bacterium.[20]

Although the Ti plasmid is clearly a primary determinant of virulence,[16] there is now growing evidence to suggest that there are a number of genes located on the chromosome of the bacterium that are involved in the initiation of the tumor by either direct or indirect involvement. An early step in tumor formation is the site specific attachment of the bacterium

to the plant cells.[17] Most of the evidence suggests that chromosomally encoded functions are primarily responsible for the attachment ability of *Agrobacterium tumefaciens.*[7] As is the case in *E. coli*, there may also be chromosomally encoded genes in *Agrobacterium tumefaciens* that code for the function of DNA repair and genetic recombination. If the *Agrobacterium tumafaciens* are exposed to U.V. light or mitomycin C prior to their infection of plants they acquire an enhanced tumor forming ability.[11]

The precise mechanism of this enhanced tumor forming ability in *Agrobacterium tumefaciens* is not clearly understood, but it is thought to be very similar to the inducible DNA repair system of *E. coli*, namely the S.O.S. repair system.[29] A discription of some well studied DNA repair systems of *E. coli* is necessary at this point, since it will give me a basis for comparison in my endeavour to try and explain the mechanisms involved in genetic recombination and ultraviolet light resistance by the *Agrobacterium tumefaciens.* Since an *E. coli* cell can often regulate the expression of a gene according to a need of its products it is not surprising that many DNA repair enzymes are induced by DNA damage. The most important and extensive group consists of the S.O.S. genes, that are induced by damage severe enough to stop DNA synthesis.[27] For example, when exposed to U.V. light pyrimidine dimers are formed in a DNA strand. These dimers cannot base pair. When a replicating fork meets such a dimer, replication stops and is re-initiated a short distance past the dimer.[27] The result is a gap in the newly synthesized strand and an exposed single stranded DNA template. RecA,one of the more important *E. coli* SOS proteins recognizes this single stranded DNA and binds onto it. Besides initiating DNA strand exchange and thus recombinational repair, the recA protein has a third enzymatic function entirely separate from recombination. It acquires a proteolytic function and destroys the lex A repressor. When present, the lexA protein maintains the S.O.S genes in a state of repression.[27] In this manner the recA protein promotes DNA repair both by recombination and by initiating the induction of about fifteen S.O.S. genes. By destroying the repressor action of lexA, recA also increases the synthesis of itself (see Fig. 1).[27] The increase concentration of recA in the cell will enhance the recombination of the bacterial genes with that of the plant genome. This could be the reason behind the enhanced tumor forming ability of the bacteria after exposure to ultraviolet light. Although the response to U.V. light is considerably more effective in *E. coli* as compared with *Agrobacterium tumafaciens,*[19] it has been clearly demonstrated that like *E. coli* the genes responding to U.V. light and mitomycin C in *Agrobacterium tumafaciens* are located in the chromosome.[19] Such genes are very important for the survival of the bacterium. In *E. coli* these genes have been very extensively studied.[8,18,26]

Among the better known of these *E. coli* gene products are photolyase, the excision repair genes, uvrABC endonucleases, the uracil DNA glycosylase, the rec proteins, mismatch

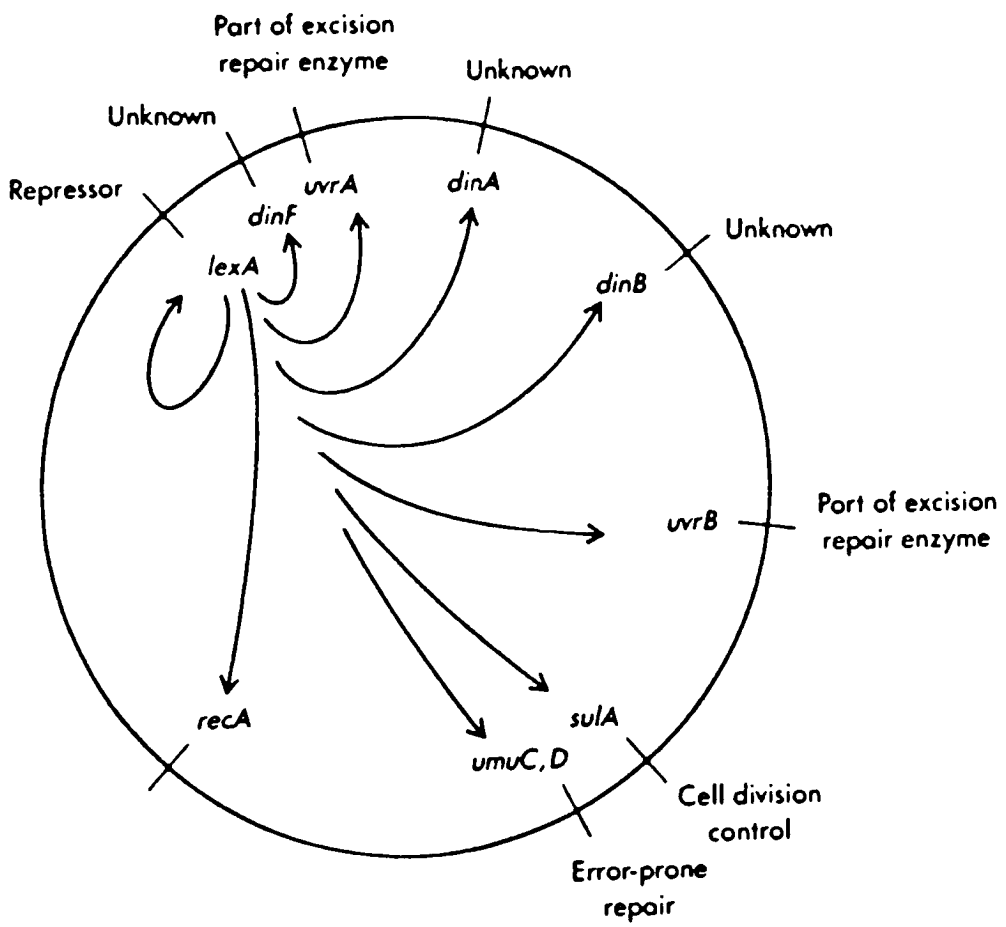

Figure 1. *The LexA control system.*

correction enzymes and the S.O.S. repair proteins such as umuC and umuD.[8,26] Among this collection of gene products it is the rec proteins that play an important role in DNA recombination and repair. To date 13 rec protein encoding genes involved in DNA repair and recombination have been identified.[18] These are rec A, B, C, D, E, F, J, N, O, ruv, sbcb and csb.[18] The recA protein plays perhaps the most important role in DNA repair and recombination in this group.

The recA gene was first isolated by Clark and his colleagues in 1965.[8] The normal level of this protein in the cells is about 2000 molecules per cell. This level increases to as high as 50,000 molecules per cell following the exposure of the cell to DNA damaging agents.[18] Some of these agents are ultraviolet light, nalidixic acid, bleomycin and mitomycin C.[18] Before trying to understand how the recA protein functions in DNA repair it is important to understand its mode of action during DNA recombination. DNA recombination is a fundamental reaction in providing diversity to genes. In basic terms, DNA recombination involves the making of a base-pair hybrid by joining two DNA molecules.[26] In order for the recA protein to be able to function effectively the DNA has to be single stranded. This was shown to be the case when it was observed that no reaction occurred when two homologous, but completely base-paired helixes were mixed with recA protein.[26]

When the recA protein encounters a single stranded piece of DNA it binds to it.The ratio of binding is approximately one polypeptide to five nucleotides.[26] The recA proteins completely cover the DNA strand. This single stranded DNA with the bound recA proteins is referred to as the recA filament.[26] As every recA protein binds onto the single stranded DNA it simultaneously binds a molecule of ATP. At this stage of the reaction the ATP attached onto the recA protein is not hydrolyzed but is instead carried along unchanged.[26] The ATP bound recA filament next wraps around a homologous strand of double helical DNA. The bonds holding the recA filament onto the double stranded DNA are formed between the nucleotides of the single stranded DNA and the nitrogen and oxygen atoms of the bases of the double stranded DNA that extend into its groves.[26] Thus the recA filament and the double stranded DNA are not held together by the usual Watson/Crick base pairing. Once binding has occurred the recA filament then moves along the double helix until it reaches a region of base complementation. When this has been achieved the recA protein melts the double helix at the point on complementation. This leads to the separation of the strands of the double helix. The recA protein then initiates the annealing of the single stranded DNA onto its complementary sequence of the melted double helix. Once a small segment of the single stranded DNA is properly annealed onto the double helix, the energy generated from the hydrolysis of the ATP bound to the recA protein drives the pairing reaction to completion, moving in the 5' to 3' direction relative to the single strand.[26] The recA protein is displaced as the new DNA hybrid

is formed. While the hybrid is being formed, the non-complementary strand is displaced such that when the new single strand is completely annealed to its complementary strand the displaced strand forms a loop around the hybrid.[26] This loop is referred to as the D-loop. This D-loop is cut out by the action of nucleases and the gaps formed by its removal are filled by the DNA polymerase. The free ends of the newly inserted single stranded DNA are joined by DNA ligase, completing the process of recombination (see Fig. 2).[27]

This same reaction can form a bridge between two separate duplex DNA's if one of them contains a single stranded region. Breaks as well as gaps in DNA initiate recombination because they provide sites for nucleases to degrade one strand of the duplex. These gaps also enable unwinding enzymes to enter the duplex and start to unwind the duplex, giving rise to single stranded DNA, which the recA protein can bind onto. A good initiator of recA recombinational repair is damage caused to duplex DNA on being exposed to ultraviolet light. This leads to the production of pyrimidine dimers in the DNA. Most of these dimers are removed by the excision repair enzymes.[8,26] This is usually the case in a DNA strand that has not yet been replicated. Post replication repair of DNA on the other hand is carried out by the recA protein.[8,26] The mechanism of recA controlled post replication repair is diagrammatically presented in Figure 3.[8]

Post replication repair deals with a pyrimidine dimer (A) that interferes with replication during which the parental strands are synthesized..1. Other dimers (B and C) are handled by excision repair enzymes. Dimer(A) prevents base-pairing along a stretch of one parental strand, producing a post replication gap opposite a stretch of single stranded DNA..2. RecA protein binds to this single stranded region..3., and aligns it with a homologous region of the sister duplex. When homologous pairing is achieved, an enzyme nicks the duplex..4. RecA protein switches the free end of the duplex's parental strand into the gap, producing a crossed strand exchange..5. The upper heteroduplex can now be repaired by DNA polymerase 1. With the correct sequence in place opposite dimer (A), and with recA released, dimer (A) is dealt with by excision repair enzymes..6. Finally, two cuts are made by an enzyme at the site of the crossed strand exchange..7., resolving the recombination process and producing two intact heteroduplex molecules..8.

Another important role played by the recA protein is in the S.O.S. repair system.[8,26] This is a system that is found in cells and used as a final effort by a cell to remain viable after all the other repair systems have been overpowered by the DNA damage. These S.O.S. enzymes have the ability to alter the function of the DNA polymerase 111..so that it bypasses the dimer site. It does this by placing bases at random opposite the dimer.[26] Although this procedure leads to the formation of mutations at the site of damage, it protects the cell from inevitable destruction. A second important property of recA when bound to single stranded

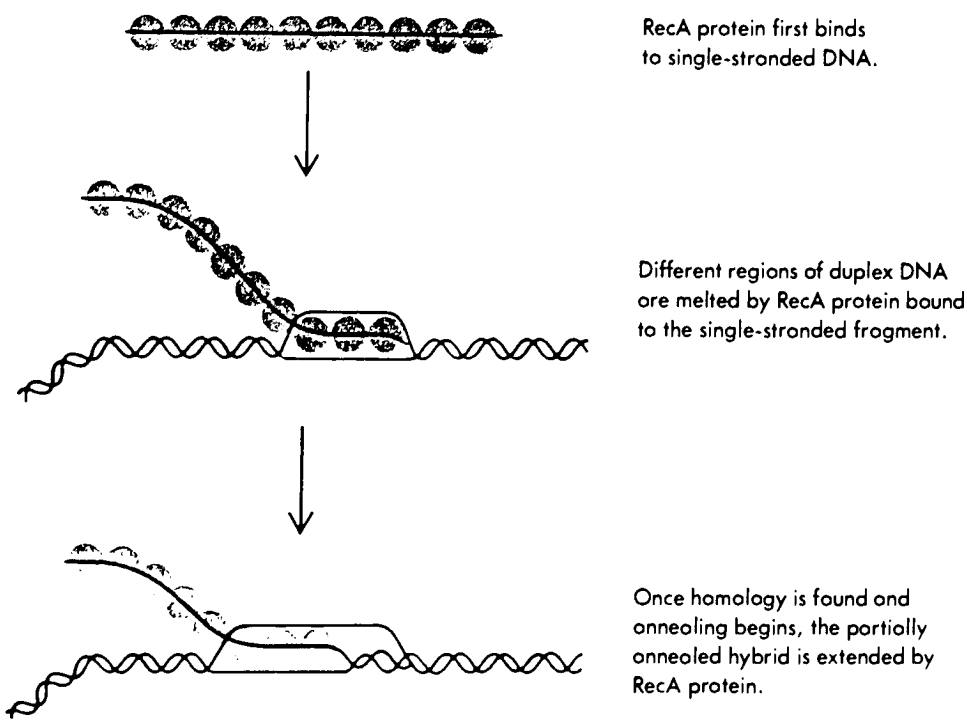

Figure 2. *RecA controlled recombination.*

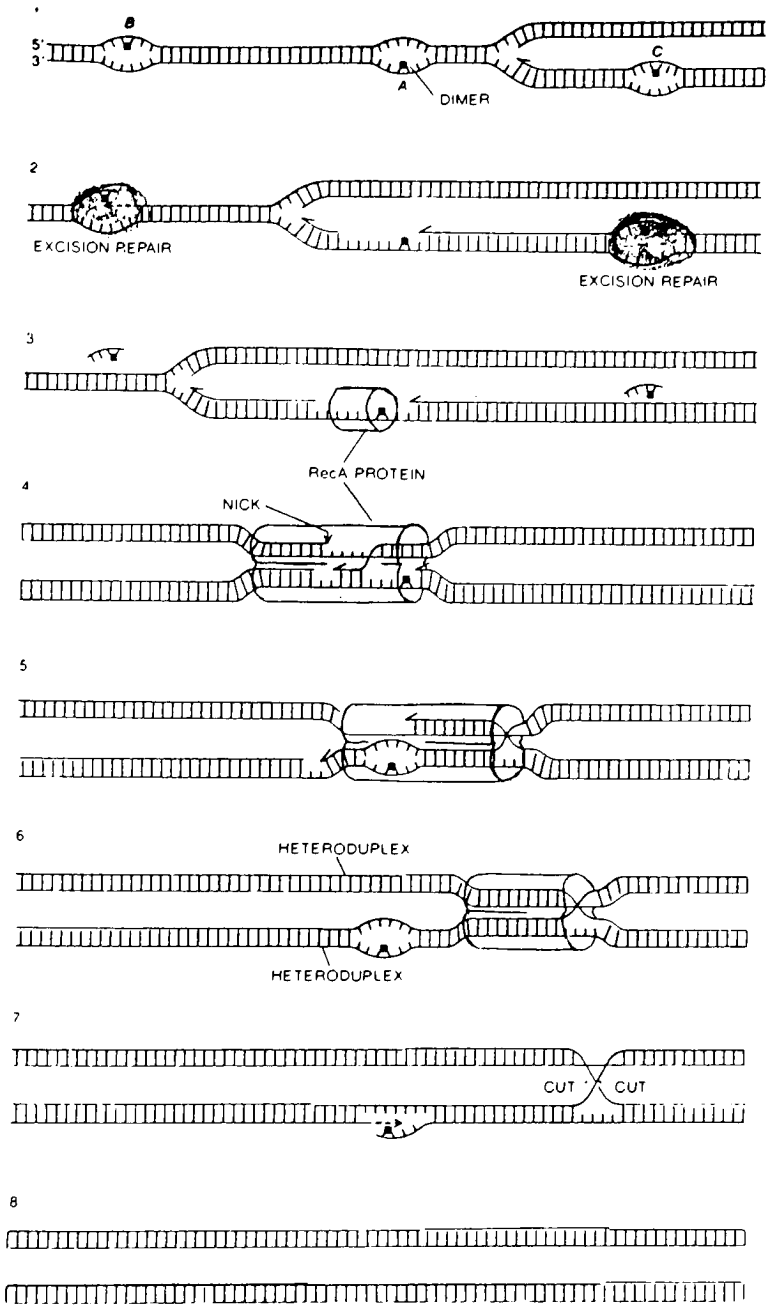

Figure 3. *RecA controlled repair of DNA dimer caused by UV exposure.*

DNA is that it now functions as a protease. In this form it then binds to the lexA repressor of the S.O.S. system and inactivates it. This enables the S.O.S. system to be turned on.[8,26]

In order to be able to identify the recA-like gene from among the very large collection of chromosomal fragments, each fragment has to be inserted into a plasmid (see Fig. 13). The vector with its inserted fragment can now be introduced into the host bacteria by the technique of in-vitro transformation. The vector used in this experiment is a plasmid. The plasmid is capable of autonomous extrachromosomal replication within the cell thereby ensuring the introduction of the recombinant plasmid into every daughter cell during cell division. Thus, a single plasmid introduced into a bacterium will after a few generations of bacterial growth be multiplied into a large number of copies.

In deciding upon which vector to use for the experiment two important criterian had to be taken into account. First, the bacteria containing the plasmid has to be selected from those that do not. This is done by introducing a gene coding for the resistance of a specific antibiotic into the vector. Thus, by growing the bacteria on a medium containing this specific antibiotic it will be noticed that only the bacteria containing the vector will grow in this medium. The second criterion is to be able to identify the bacterial cells containing the recombinant plasmid from those cells that only contain the plasmid. That is, the plasmid without the inserted fragment. A plasmid that fits these two criteria is the plasmid pUC9 (see Fig. 4). A good account on the geneology of the pUC plasmids is given in reference 22.

The pUC9 is a plasmid containing 2768 base pairs.[5] This plasmid is a modified pBR 322 vector containing the ampicillin gene and an added polylinker site similar to that of the M13 MP vector.[5] The plasmid also contains the α peptide of the lac Z gene. This peptide is 276 base pairs long,[2] and is situated about 90 base pairs down stream from the start codon of the Lac Z gene. When pUC9 is introduced into a host *E. coli* that lacks the alpha peptide that is a strain that produces a mutated lacZ protein and is thus unable to produce β galactosidace, the α peptide of the pUC9 complements with the deleted gene of the host chromosome enabling it to produce β galactosidase. This α complementation occurs between the plasmid and the deleted host lacZ gene causing the latter to become functional. β galactosidase producing colonies can be easily identified by one of a few color tests used to identify the presence of the enzyme. In this experiment, the bacterial cells were grown on MacConkey and ampicillin plates [see Sec. (2.12)]. Colonies producing the enzyme were able to break down the lactose present in the media into glucose and galactose. This reaction reduces the acidity of the medium, activating a pH indicator which is responsible for the red coloration of the colonies. When a fragment is inserted into the vector it does so in the lacZ gene rendering this gene inactive. Thus, insert bearing plasmids will fail to complement the lac^- genotype of an α complementary *E. coli* host. The plasmid transformants remain lac^- and the colonies fail to

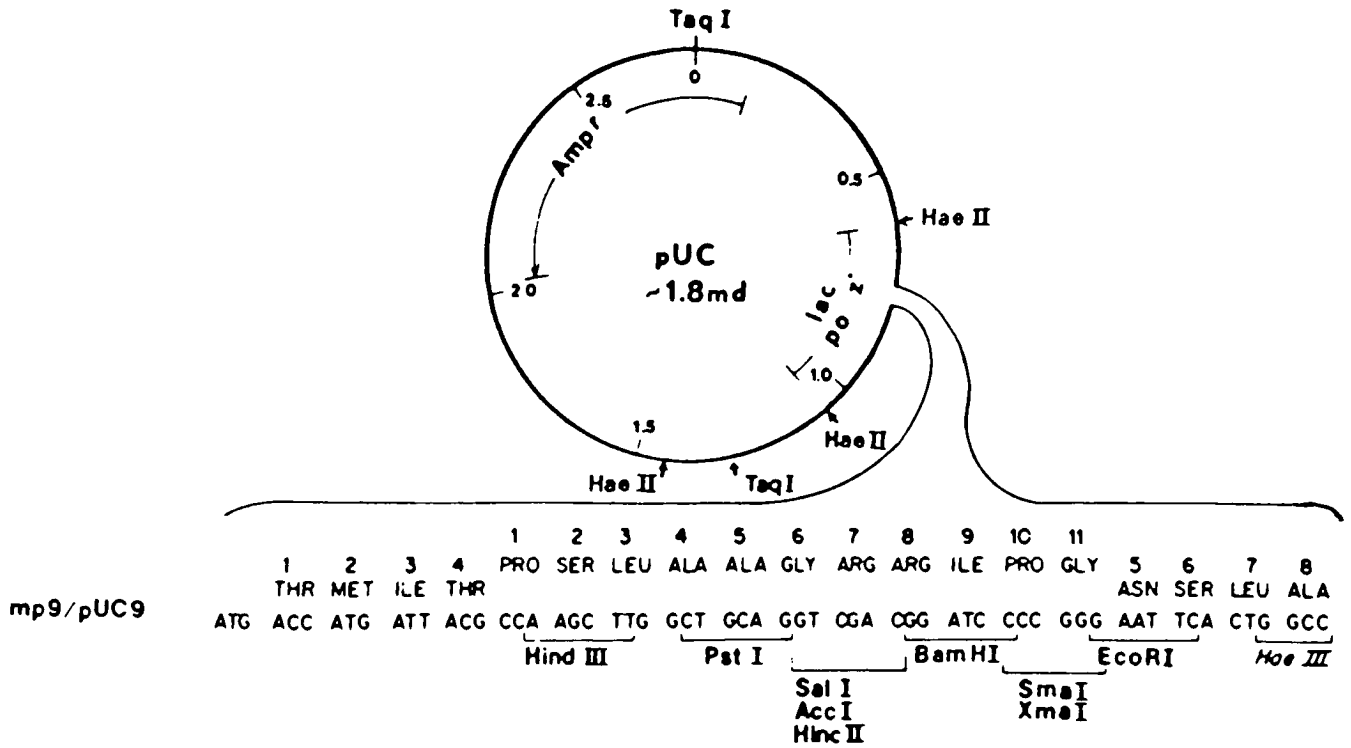

Figure 4. *Genomic construction of pUC9A.*

take on the red coloration on the MacConkey and lactose plates[4] and appear as white colonies on the medium. Other tests that can be used to identify the presence of β galactosidase are the X-gal test[13,14] and the lacZ assay.[14]

(1.2) PURPOSE OF RESEARCH.

The aim of this project is to prepare a genomic library from the chromosome of the *Agrobacterium tumefaciens*, and isolate from it a recA-like gene.

CHAPTER 2

MATERIALS AND METHODS

(2.1) Table of bacterial strains.

(Bethesda Research Laboratories Catalog and Reference Guide 1988).

JM 83: F$^-$ *ara* Δ(*lac* – *pro*AB) *rps*L ϕ80d *lac*ZΔM15 $r_K^+ m_K^+$

HB 101: F$^-$ *hsd*S20 $r_B^- m_B^-$ *rec*A13 *leu*B6 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 ' *rps*L20(str$^+$)*xyl*-5 *mtl*1 *sup*E44λ^-

DH 5 α: F$^-$ ϕ80d *lac*ZΔm15 *end*A1 *rec*A1 *hsd*R17($r_K^- m_K^-$) *sup*E44 *thi*-2λ^- *gyr*A96 *rel*A1 Δ(*lac*ZYA *ara*F) U169

BRC 49: recA$^-$, amp$^-$, isoleucine$^-$, valine$^-$, meth$^-$, pro$^-$

JC 8563: amp$^-$, arg, pro, leu, thr, xylene is an Hfr strain with the f'ori situated near the pro gene.

(2.2) Properties of the restriction enzymes.

(New England Biolab. Catalog 1988-1989)

BamH 1 isolated from *Bacillus amyloliquefaciens.*

enzyme recognition site

5'.....G↓GATCC.....3'
3'.....CCTAG↑G.....5'

the enzyme cleaves the recognition site to leave a 5' GATC extension which can be efficiently ligated to DNA fragments generated by BamH1, Bcl1, Mbo1, Sau3A 1, and Xho11.

Sau3A 1 isolated from *Staphylococcus aureus 3A.*

enzyme recognition site

5'.....↓GATC.....3'
3'.....CTAG↑.....5'

buffers containing 6mM of 2-mercaptoethanol have been observed to decrease the enzyme activity by as much as 50%.

Table 1.

Restriction digestions of the Agrobacterium chromosome.

	SAMPLES			
	1	2	3	4
chromosomal DNA (μl)	25	25	25	25
medium salt buffer(μl)	10	10	10	10
water (μl)	65	65	65	65
undiluted enzyme (μl)	5		-	
1/10 dilution of enzyme (μl)		5		
1/20 dilution of enzyme (μl)			5	
1/30 dilution of enzyme (μl)				5
sample total (μl)	105	105	105	105

* The concentration of the chromosomal DNA in the 25 μl was approximately 2.5 μg (Stock of chromosomal concentration: 10 μl =1 μg of DNA)

Hind $\overline{111}$ isolated from *Haemophilus influenza Rd.*

enzyme recognition site

5'.....A↓AGCTT.....3'

3'.....TTCGA↑A.....5'

(2.3) Restriction digestions of the Agrobacterium chromosome.

The restriction digestions of the Agrobacterium chromosome are tabulated in table 1. The enzyme used in this restriction digest was Sau 3A 1 (see section 3.3) The digestion in column 1 is a complete restriction digestion.In this case the enzyme is undiluted and cuts all the available restriction sites on the DNA.

The digestion in columns 2 to 4 are partial digestions. In this case the enzymes are diluted so that their concentrations are low and cut only a few of the restriction sites on the DNA.

(2.4) Sucrose Gradient.

10% solution - 20g of sucrose dissolved in 200ml of the sucrose gradient buffer

40% solution 80g of sucrose dissolved in 200ml of the sucrose gradient buffer

Preparation of the sucrose gradient buffer:

1M NaCl	100ml
20mM Tris pH 7.6	10ml
5mM EDTA	5ml
water	385ml
total volume	500ml

- prepare a $35ml$ sucrose gradient(10-40% w/v) in a polypropylene tube(Beckman S.W. 25).
- carefully load the sample beneath the meniscus of the gradient taking care not to disturb the gradient.

  load the gradient and DNA into a S.W. 25 rotor together with two other similar tubes containing $17ml$ of the 40 % sucrose solution and $18ml$ of the 10 % sucrose solution. The latter two tubes serve as balances.
- centrifuge in a Beckman LS-50 at 40,000 rpm for 24 hrs at 15°C.

  collect approximately $0.5ml$ samples starting from the bottom of the tube and moving upwards.

(2.5) Restriction digestion of pUC9

medium salt buffer	2.5 μl
water	9.5 μl
pUC9	10 μl
undiluted enzyme (Bam)	3.0 μl
Total	25.0 μl

The mixture was incubated at 37° C for two hours.

(2.6) Bacterial Alkaline phosphatase reaction of restricted pUC9.

Two different alkaline phosphatases are available for this reaction. These are bacterial alkaline phosphatase (BAP), and calf intestinal alkaline phosphatase (CIP). CIP is usually the preferred enzyme because it is thermolabile. Upon completion of a Phosphatase reaction, it is necessary to remove or inactivate the enzyme. CIP can be inactivated by heating at $68°C$ for thirty minutes in the presence of sodium dedocylsulfate. BAP on the other hand is resistant to this treatment and can be removed only by several phenol extractions. In this experiment BAP was used to treat the cut vector and the protocol followed is outlined below.

1. Add 5-10 units of BAP per μg of restricted DNA
2. Incubate at 65^oC for 45 minutes.
3. Extract once with phenol, once with a 1:1 mixture of phenol chloroform and once with chloroform (chloroform/isoamyl alcohol 24:1).
4. Adjust the salt concentration to 0.1 M NaCl.
5. Add twice the volume of ice cold ethanol and place the mixture on ice for 10 minutes.
6. Centrifuge the mixture for 5 minutes in an eppendorf centrifuge. After the centrifugation, remove the supernatant and allow the pellet to dry.
7. Resuspend the dried pellet in 50 μl of TE buffer.

The vector is now ready for the ligation reaction with the fragment.

(2.7) Ligation Mix.

The final step in forming the recombinant plasmid is the insertion of the fragment into the Bam/BAP treated pUC9 vector plasmid. This reaction is carried out in the presence of ATP and ligase, which are important in assuring a tight fix of the free ends of the fragment and vector to form a stable recombinant plasmid.The ligation mix was prepared as outlined below.

vector(pUC9)	34 μl
target(fragment)	34 μl
ligation buffer	6 μl
ATP	9 μl
T4 ligase	2 μl
sample total	85 μl

The ligation mix was allowed to react at refrigerator temperature overnight. On the next day the ligation mix was heated at 65^oC for 20 minutes. This was done in order to denature the remaining T4 ligase. The recombinant plasmid is now ready to be introduced into the host *E. coli* cell.

(2.8) Transformation of E. coli.

- $4ml$ of LB broth was inoculated with cells taken from a single colony of *E. coli* and incubated at 37°C overnight.
- $0.2ml$ of the overnight culture was mixed into $20ml$ of fresh LB broth and ampicillin (50 μg /ml) and incubated at 37°C until the optical density of the cells reached about 0.2.

- spin down the cells at 7000 rpm for 10 minutes.
- resuspend the pellet in 0.05 M calcium chloride and place on ice for one hour.
- spin down the cells at 7000 rpm for 10 minutes.
- resuspend the pellet in 1.5ml 0.05 M calcium chloride.

  mix 100 μl of competent cells and 10 μl of the recombinant plasmid and place the mixture on ice for 30 minutes.

  heat shock the mixture for 2 minutes at 37^{o}C.
- mix 100 μl of transformed *E. coli* into 2ml of fresh LB and ampicillin and incubate at 37^{o}C for 90 minutes.

  prepare 10-1, 10-2, 10-3 dilutions of cells and plate each dilution on a MacConkey ampicillin plate.

  incubate the plates at 37^{o}C for 48 hours.

(2.9) Plasmid Miniprep.

- inoculate 4ml of LB and ampicillin with a single transformed colony and incubate the culture at 37^{o}C overnight.
- add 1.5ml of the overnight culture into an eppendorf tube and spin down the cells in a mini centrifuge for 5 minutes.
- resuspend the pellet in 100 μl of GET buffer and let the mixture stand at room temperature for 5 minutes.
- add 200 μl of freshly prepared SDS+NaOH solution. Mix by gently inverting the tubes a few times and place the tubes in ice for 5 minutes.
- add 150 μl of cold potassium acetate pH 4.8. Mix by vortexing the tube upside and place in ice for 5 minutes.
- centrifuge for 10 minutes and transfer the supernatant into a new eppendorf tube.
- add an equal volume of a 1:1 phenol/chloroform extraction . Mix by vortexing and centrifuge for 5 minutes.
- transfer the upper aqueous layer into a new eppendorf tube and repeat the phenol/chloroform extraction.

- transfer the upper aqueous layer into a new eppendorf tube and add 1ml of 95% ethanol. Vortex to mix and let the mixture stand at room temperature for 5 minutes.

- spin down the cells for 5 minutes.

- pour off the supernatant and dry the pellet.

  resuspend the pellet in 50 μl of TE buffer and RNase.

(2.10) Isolation of Plasmid DNA.

- spin down the overnight culture of 200ml in a Sorvall GS-A rotor at 7000 rpm for 10 minutes.

- resuspend the pellet in ice cold LB broth and transfer the mixture to a clean 50ml polypropylene centrifuge tube.

  spin the cells down in a SS-34 rotor at 10,000 rpm for 15 minutes resuspend the cells in 1ml ice-cold 25 % sucrose and keep on ice for 30 minutes.

- add 0.2ml lysozyme, mix well and place on ice for 10 minutes. add 0.4ml EDTA mix well and place the mixture on ice for 10 minutes.

  add 1.6ml of Triton-100 lytic mix. Mix carefully by gently inverting the tube a few times and then place the tube on ice for 20 minutes.

- centrifuge at 17,000 rpm for 30 minutes.

- transfer the supernatant to a clean plastic disposable tube. - add 2ml TES and 4.9 g of cesium chloride and shake the mixture gently to dissolve the cesium chloride.

  transfer the sample into a Beckman "Ouick-Seal" tube and then add 200 μl of ethidium bromide.

  fill the remaining space of the tube with mineral oil.

  seal the top of the tube and place the tube in a Beckman ultracentrifuge rotor

- centrifuge at 40,000 rpm for 48 hours at 15^{o}C.

  at the end of the centrifugation, carefully remove the tube from the rotor and carefully clamp it onto a ring stand.

- using a hypodermic needle, puncture the top of the tube in order to let air enter the tube.

- in the presence of ultraviolet light, identify the plasmid band. Then using a 21-22 gauge needle attached onto a $5ml$ syringe, carefully penetrate the tube a centimeter below the plasmid band.

  angle the needle up into the plasmid band and carefully withdraw the band.

  extract the ethidium bromide three or four times with isopropanol saturated with cesium chloride

  dialize the sample in TE buffer overnight with 2 to 3 buffer changes.

(2.11) Conjugation:

donor strain:- J.C. 8563
recipient strain:- BRC 49

procedure.

Transform BRC 49 with recombinant plasmid.

- $2ml$ of the transformed cells are diluted into $8ml$ of fresh LB and ampicillin broth. At the same time inoculate $4ml$ of LB broth without ampicillin with JC 8653.
- incubate both cultures at 37^oC for 24 hrs.

  transfer $1ml$ of the overnight culture of JC 8653 into $20ml$ of fresh LB broth and grow the cells to mid-log phase.

  centrifuge $10ml$ of overnight BRC 49 and resuspend the pellet in $10ml$ of fresh LB without ampicillin.
- mix $5ml$ of BRC 49 prepared in step six with 10ml of JC 8563 prepared in step 5.

  incubate the mixture without shaking at 37^oC for 60 minutes.

  streak the mixture of cells on minimal selective agar plates and incubate the plates at 37^oC for 24 hours.

(2.12) Replica Plating [see Fig. 5].

Place the bottom of the wooden block which is covered by a piece of sterilized velvet firmly wrapped around it, onto the surface of the parent agar plate. Apply sufficient pressure to ensure that a significant amount of each colony on the surface of the agar adheres onto the velvet. Carefully lift the block away from the parent plate taking care not to change the orientation of the print. Maintaining this orientation, carefully place the velvet on the surface

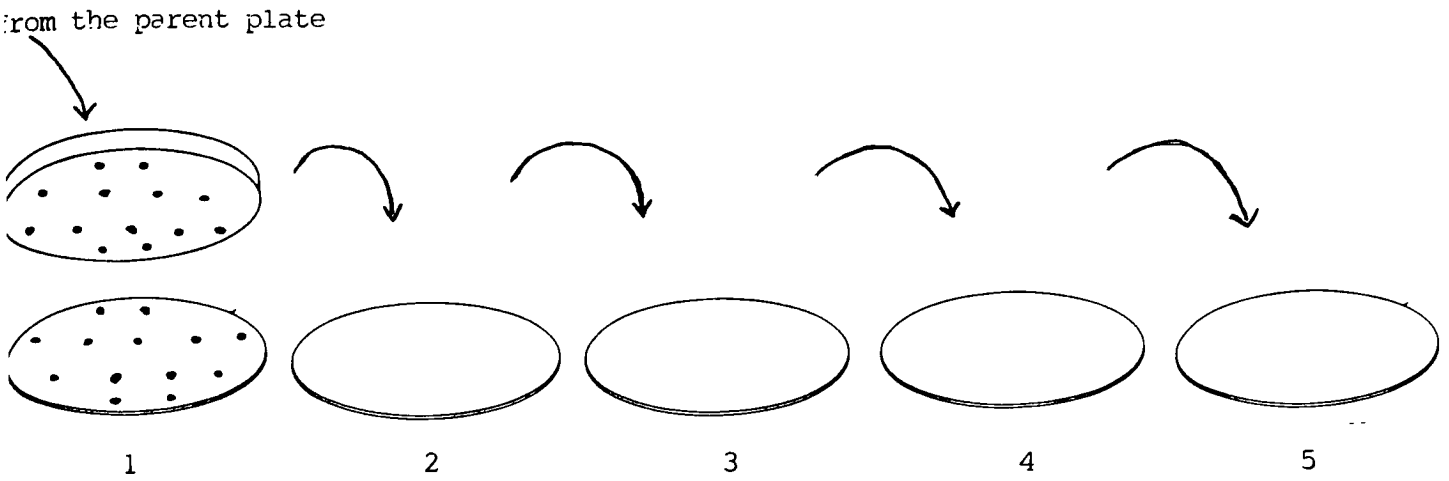

Figure 5. *Technique of Replica Plating*

of the fresh plates.Apply just enough pressure to make sure that the colony print on the velvet is transferred to each plate. The five plates are printed in turn without replacing the block on the parent plate. By maintaining the same orientation of the block during each print ensures that the pattern of colony distribution in each plate is identical to that of the parent plate. Incubate the five plates at 37°C for 24 hours.

(2.13) Preparation of the Ingredients.

MacConkey and Ampicillin agar plates.

peptone	17g
proteose peptone	03g
bile salts no.3	1.5g
sodium chloride	05g
agar	13.5g
neutral red	0.003g
crystal violet	0.001g

N.B. All these ingredients were premixed into a dehydrated powder by the manufacturer. 40 g of the mix is rehydrated in a liter of distilled water and autoclaved. When the mixture is cool ampicillin is added so that its final concentration is 50 μl /ml. Pour into plates before the agar solidifies.

LB Broth

tryptone	10g
yeast extract	05g
sodium chloride	05g
water	1 liter

Mix and autoclave. If LB agar has to be prepared, add 15 g of agar into the above mix before autoclaving. Pour into plates before the agar hardens.

TE Buffer

Tris 1M (pH 7.6)	$2ml$
EDTA 0.5M (pH 8)	$0.4ml$
water	$197.6ml$

GET Buffer

glucose	0.8g
Tris 1M (pH 8)	$5ml$
EDTA 0.5M (pH 8)	$4ml$
water	$191ml$

Minimum-Selective medium

preparation of the medium:

$1000ml$ distilled water mix and autoclave for 20 minutes

$50ml$ 10x56 buffer

20 g agar

When the agar mix is sufficiently cool, but warm enough to prevent the agar from solidifying add the following into the mixture.

$10ml$	20 % glucose
$0.2ml$	0.1 % vit B1
$10ml$	1 % threonine
$10ml$	1 % leucine
$10ml$	2 % argenine
$02ml$	2.5 % thymidine
$02ml$	ampicillin (25 mg/ml)

mix well and pour into sterilized plates.

CHAPTER 3.
RESULTS

(3.1) Preparation of the Chromosomal fragment.

In order that the genes bearing the recA-like property are not cut during digestion,it becomes essential to cut the chromosome of the *Agrobacterium tumefaciens* into fragments large enough so that they contain a complete gene. If the gene is cut into separate fragments it becomes inactive. Thus, in order to prevent the cutting of the gene, fragment sizes ranging from between 6 kilobases to 14 kilobases were chosen.

The chromosomal DNA was digested with the restriction enzyme Sau3A 1 [see Sec. (2.3)]. In setting up the restriction digestion it becomes important to cut the chromosome into fragments of varying sizes so that eventually the appropriate fragment range can be isolated. In order to do this, two important variables have to be taken into account. The first variable is the concentration of the enzyme. Before deciding upon the concentration of the enzyme to be used it is important that one is aware of the property of the particular enzyme.

Sau3A 1 isolated from *Staphylococcus aureus 3a*, is a four base cutter whose recognition site is 5'....GATC.....3'. One unit of the enzyme is defined as the amount of enzyme required to digest one microgram of DNA in one hour at thirty seven degrees centigrade in fifty μl of assay buffer (New England Biolab.). Since Sau3A 1 is a four base cutter, it becomes obvious that if the concentration of the restriction enzyme is too high, the chromosomal DNA will be cut into a very large number of small fragments being of little use to the experiment.

The concentration of the enzyme used was eight units of enzyme per μl . In order that the chromosome be cut into appropriately sized fragments, samples of chromosomes were mixed with varying concentrations of the enzyme. The concentrations of the enzyme used in this experiment are outlined below:

5 μl of undiluted enzyme	enzyme concentration = 40 units
5 μl of 1/10 dilution	enzyme concentration = 4 units
5 μl of 1/20 dilution	enzyme concentration = 2 units
5 μl of 1/30 dilution	enzyme concentration = 1.33 units

The complete restriction digestion is tabulated in table 1 (see page 12).

The second variable is the duration of incubation of the restriction digest at 37°C. An appropriate time span has to be determined so that the chromosome will be cut into fragments of the desired size range.(*i.e:* 6Kb-14kb)

All 4 samples outlined in table 2 were placed in a $37°C$ water bath together. After one minute of incubation, 20 μl of each sample was taken out and introduced into a clean

eppendorf tube. The restriction digestion reaction in each tube was terminated by adding 40 μl of 1:1 phenol chloroform mixture. This same procedure was carried out after 3, 5, 10, and 20 minutes of incubation.The end result was twenty samples divided into five groups.

Group 1. samples 1-4 where the reaction was stopped after 1 minute
Group 2. samples 5-8 where the reaction was stopped after 3 minutes
Group 3. samples 9-12 where the reaction was stopped after 5 minutes
Group 4. samples 13-16 where the reaction was stopped after 10 minutes
Group 5. samples 17-20 where the reaction was stopped after 20 minutes

Having collected the samples in each group, the next step was the separation of the fragments in each of the twenty samples. This was achieved by running the samples on an 0.8% agarose gel electrophoretic plate. The result of this gel is shown in Figure 6. (Figure 6b is a continuation of Figure 6a).

By observing the fragment distribution on the electrophorized gel it becomes very evident that the fragment distributions of wells 4, 5 and 17 cover a very wide range of fragment size. In well 4 almost all the fragments are large while in well 17 almost all the fragments were small. Well 5 on the other hand contained fragments of intermediate size. The reason why the samples in these three wells were chosen is that there is an overlap of fragment size within these wells. Thus, pooling these samples prevents the possibility of leaving out a sample containing the required fragment size.

From the results of this initial digestion it now becomes possible to choose the appropriate reaction conditions that will produce fragments of uniform size distribution ranging from small to large fragments. These reaction conditions can be determined by going back to the samples that were loaded into wells 4, 5 and 17 of the agarose gel.

(a) Well 4 contained sample 4 (see table 1.) after one minute of incubation.
(b) Well 5 contained sample 1 (see table 1.) after three minutes of incubation
(c) Well 17 contained sample 1 (see table 1.) after twenty minutes of incubation.

The above three reaction conditions were now repeated but this time the restriction digests were increased twenty fold so as to contain about 50 μg of chromosomal DNA in each sample (see table 2).

After the appropriate periods of incubation the reactions in each samples were terminated by adding 4200 μl of a 1:1 phenol chloroform mixture. The three samples were then pooled.

The next step in the experiment was to separate the fragments of appropriate size contained in the new mixture. A precise separation of these fragments was achieved by the sucrose gradient sedimentation procedure [see Sec. (2.4)].

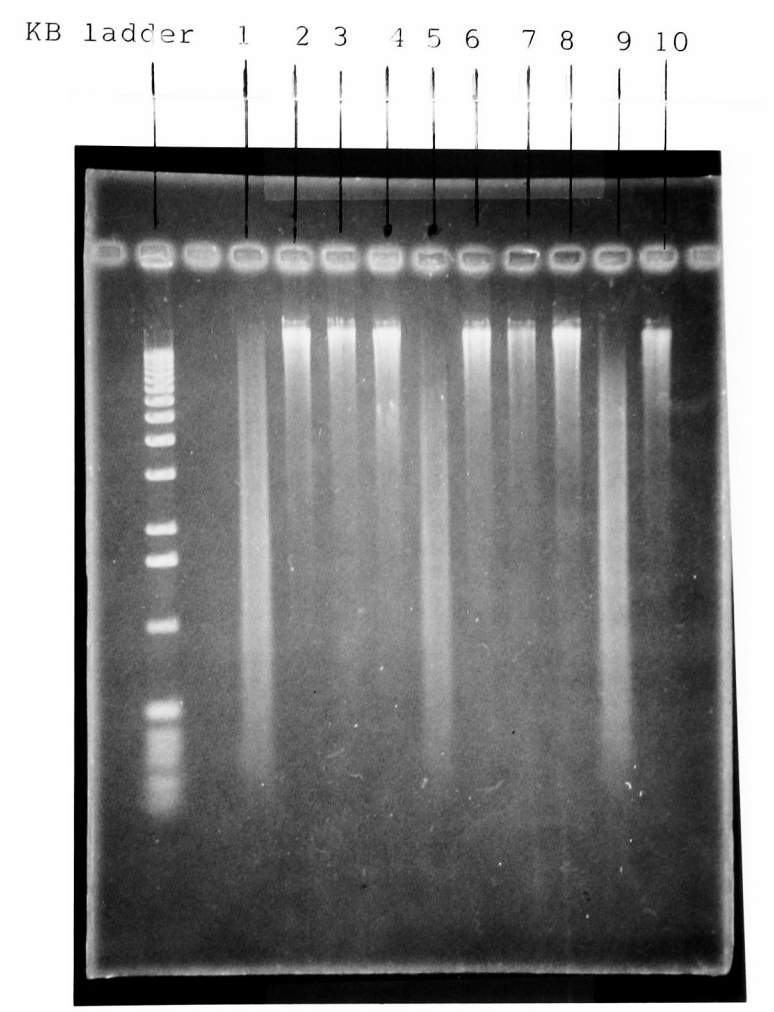

Figure 6a. *An* 0.8% *agarose gel showing fragment distribution of samples with varying enzyme concentrations and time of incubation.*

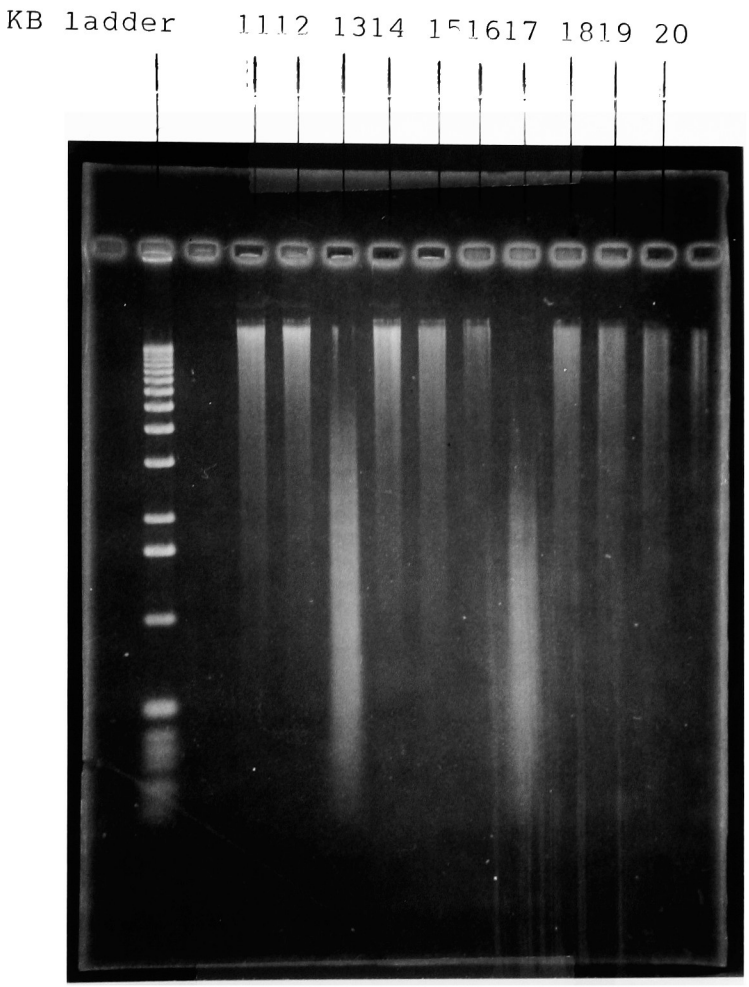

Figure 6b. *An 0.8% agarose gel showing fragment distribution of samples with varying enzyme concentrations and time of incubation. (contd.)*

Table 2.

Amplified restriction digestion

	SAMPLES		
	1	2	3
chromosomal DNA (μl)	500	500	500
medium salt buffer (μl)	200	200	200
water (μl)	1300	1300	1300
undiluted enzyme (μl)	-	100	100
1/30 dilution of the enzyme (μl)	100		
sample total (μl)	2100	2100	2100

sample 1. was incubated for one minute
sample 2. was incubated for three minutes
sample 3. was incubated for twenty minutes

(3.2) The Sucrose Gradient.

The two concentrations of sucrose used for the gradient were a 10% solution and a 40% solution of sucrose.

Preparation of the sucrose solutions:

When the sucrose gradient was prepared, the sample of chromosomal fragments was carefully loaded onto the gradient. This was done by gently introducing the chromosomal fragments just beneath the meniscus of the sucrose gradient. If the loading step is correctly executed, the DNA can be seen as a concentrated mass resting on top of the gradient (see Fig. 7).

At the end of the period of centrifugation the sucrose gradient was carefully taken out of the ultra centrifuge. This step has to be carried out with utmost care in order to prevent mixing of the fragments dispersed within the gradient. Using the narrow gauged tube which is once again passed through the peristaltic pump, the sucrose gradient and its contents are emptied (commencing from the bottom and moving upwards), into a collecting tray containing 120 wells. Twelve to thirteen drops of the extracted gradient were introduced into each well.

The first well of the collecting tray contained a 40% sucrose solution. The concentration of the sucrose solution then decreased in a gradient ending at a 10% sucrose solution in the final well of the collecting tray. A total of 120 wells were loaded from this sucrose gradient.

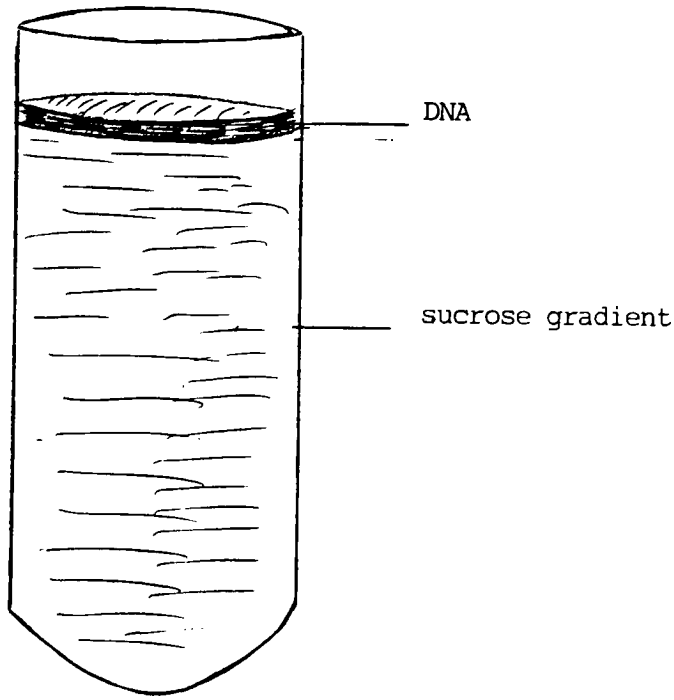

Figure 7. *The loading of the Sucrose Gradient.*

The next step was to determine the range of the fragment sizes in each well. To be able to detect a change in fragment size, samples were taken from every fifth well starting with the first well.

20 μl samples were extracted from the chosen wells and introduced into an eppendorf tube. Into each tube was also added 5 μl of tracking dye. The twenty samples were then loaded onto an 0.8% agarose gel. The gel was run at 120 volts until the tracking dye left the gel. The result of the gel is shown in Figure 8.

As seen in the gel, the fragments contained in sample 16 and beyond were so small that they most likely moved off the gel. Using the Kb ladder as a size marker it is noted that the fragments contained in sample 10 ranged from about 6kb to 14kb.

In order to determine a more precise fragment range the well corresponding to sample 10 was identified on the collecting tray. Next, samples were taken from the five wells immediately preceding this well and immediately following this well. These eleven new samples were now loaded onto a gel in the same way as was outlined earlier. The results of this second gel is shown in Figure 9.

After observing the second gel, samples 1, 2, 3, 6, 8, and 11 were chosen for the remaining

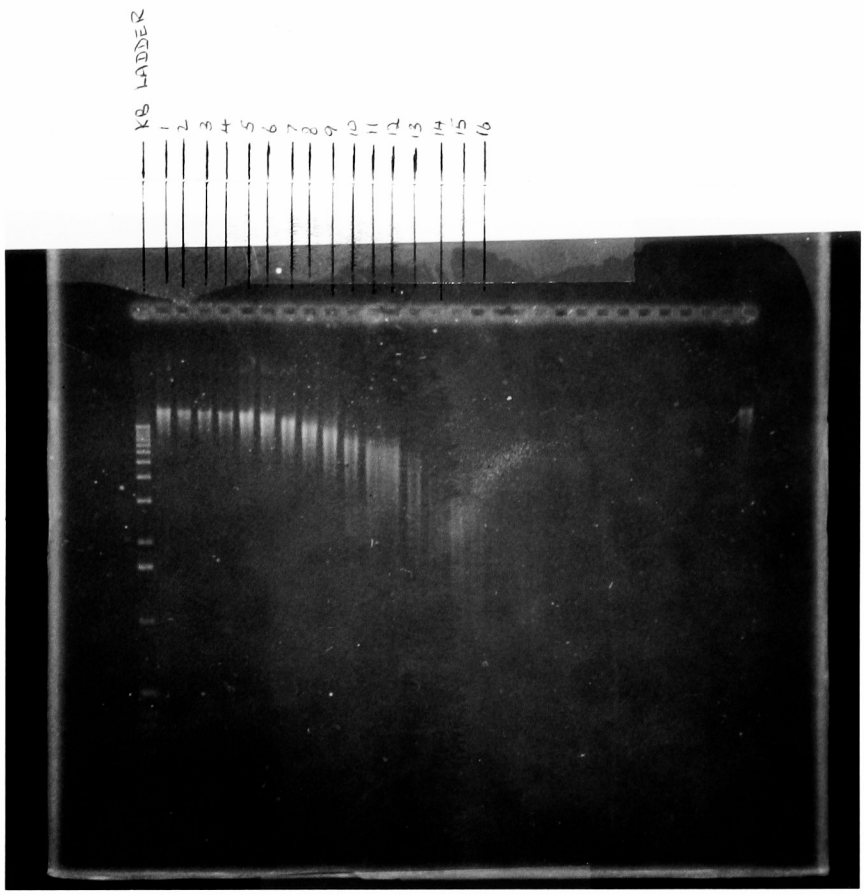

Figure 8. *An* 0.8% *gel of sucrose gradient.*

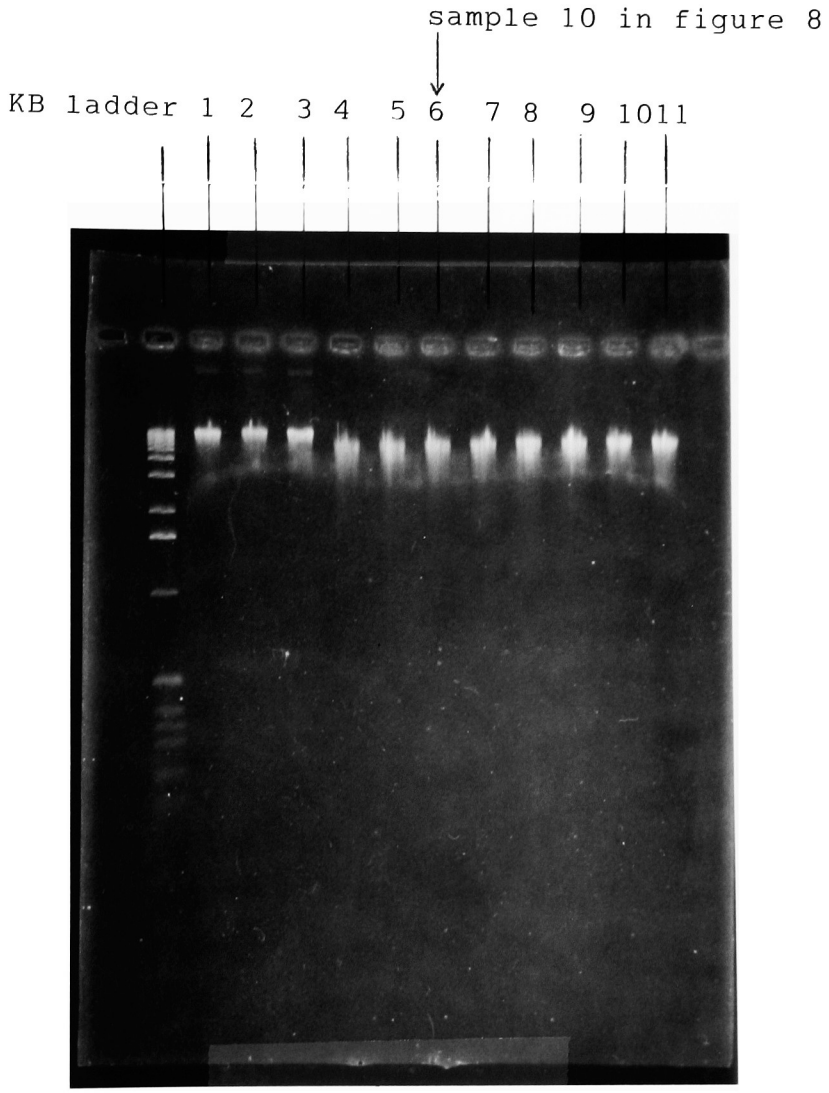

Figure 9. *An 0.8% gel of closely related samples.*

steps in the isolation of fragments covering a size range from 6kb to 14kb. The wells in the collecting tray from which these samples were taken was next identified. The entire content of each well was extracted, giving 6 samples each containing about 300 μl of the gradient. Each sample was then dialyzed in dialysis buffer for 24 hours in order to remove the sucrose. After the 24 hour period of dialysis,during which time the dialyzing buffer was changed once, each sample contained clean DNA (see Figure 10).

The 6 samples containing the clean DNA were now pooled. The fragments in this pooled sample were then separated on a second sucrose gradient. The results of the gel containing samples from every fifth well from samples of the second gradient is shown in Figure 11.

By careful analysis of this gel it became evident that sample 10 contained the fragment size range that was closest to the desired 6kb to 14kb range. As was done earlier the well corresponding to sample 10 was identified, and a gel run containing the five samples immediately preceding and following this sample. Since the fragment ranges in all these samples were very similar, all 11 samples were pooled and dialyzed for 24 hours in order to remove the sucrose (Figure 12).

The dialyzed sample was now introduced into an eppendorf tube and stored until further use. This sample contained chromosomal fragments that ranged from 6kb to 14kb.

(3.3) Restriction Digestion of pUC9.

The restriction digestion of pUC9 is outlined in section (2.5). In order to be able to insert the fragment into the plasmid the circular plasmid has to be cut at a single spot in order to make it linear having two free ends (see Figure 13). Cutting of the plasmid is achieved by using restriction enzymes that cut the plasmid at specific base sequences. The enzyme used in this experiment was BamH 1 [see Sec. (2.2)]. This enzyme produces cut ends in the plasmid that are complementary to the free ends of the fragments produced by cutting the chromosome with Sau3A 1 (see Fig. 13). The complementary free ends of the plasmid and fragment can now be joined together. At the same time the complementary free end of the plasmid can rejoin closing the plasmid and preventing the fragment from being inserted. Thus to favor the forming of recombinant plasmids over the closing up of the vector, the cut vector is treated with alkaline phosphatase prior to its ligation to the fragment [see sec. (2.6)].

(3.4) Transformation of *E. coli*.

The bacteria used for the transformation was *E. coli* K12 strain DH5 α. This strain has a lac deletion, undergoes α complementation with the recombinant plasmid and lacks the restriction enzymes that destroys the incoming foreign DNA. *E. coli* double their generation

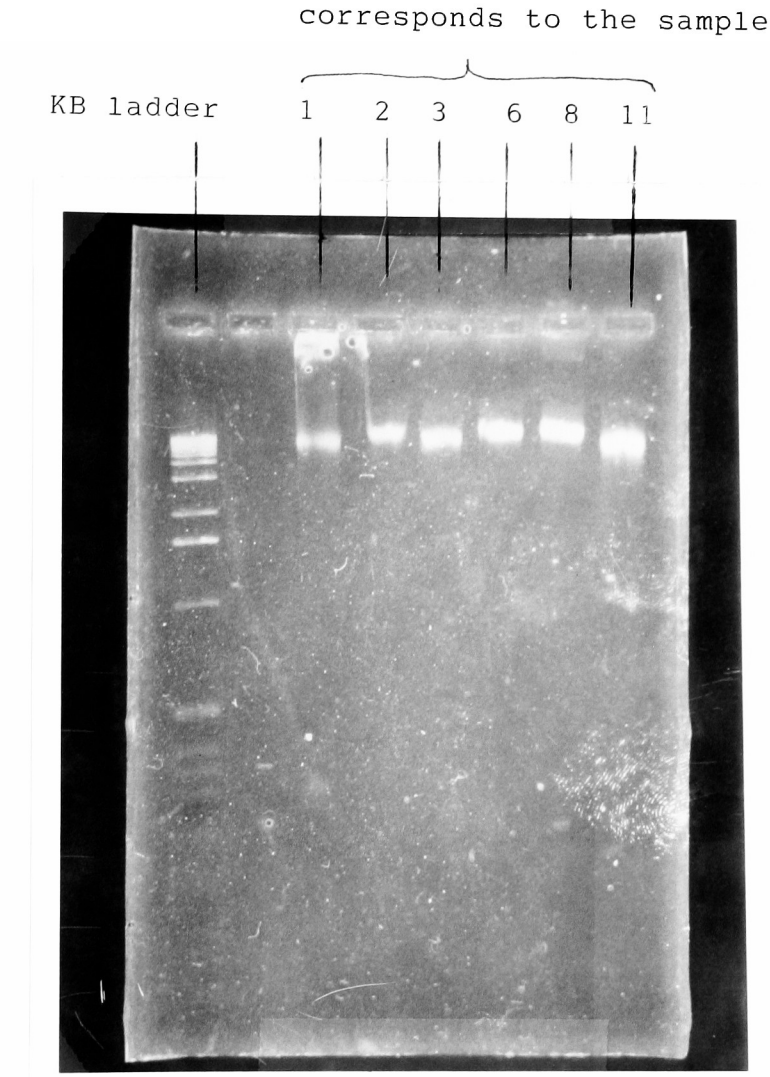

Figure 10. *An* 0.8% *gel of the dialyzed sample.*

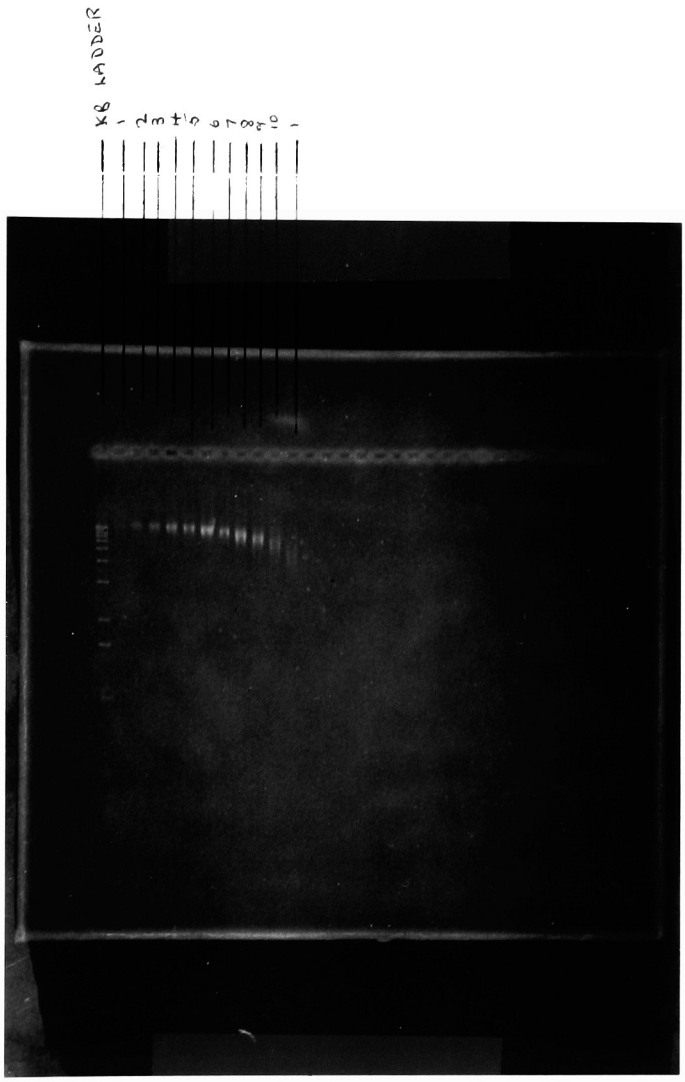

Figure 11. *An* 0.8% *gel of second sucrose gradient.*

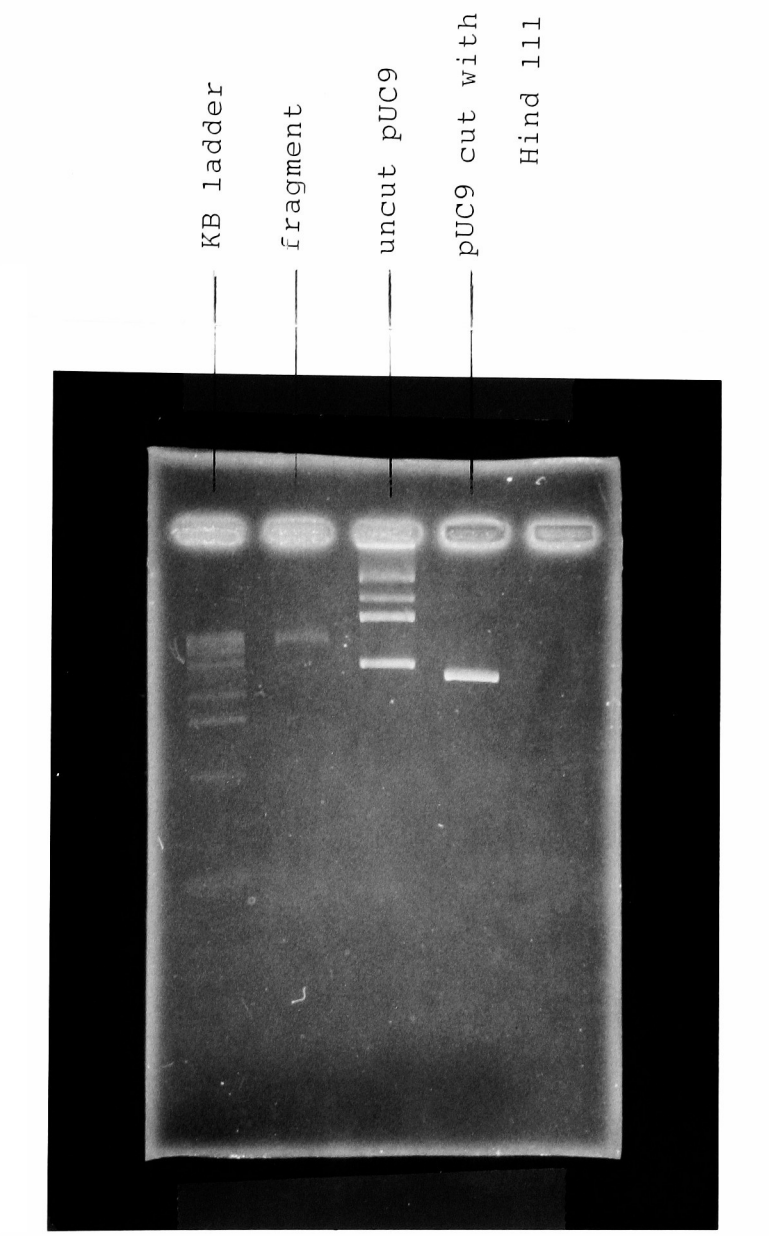

Figure 12. *An* 0.8% *gel of purified chromosomal fragment.*

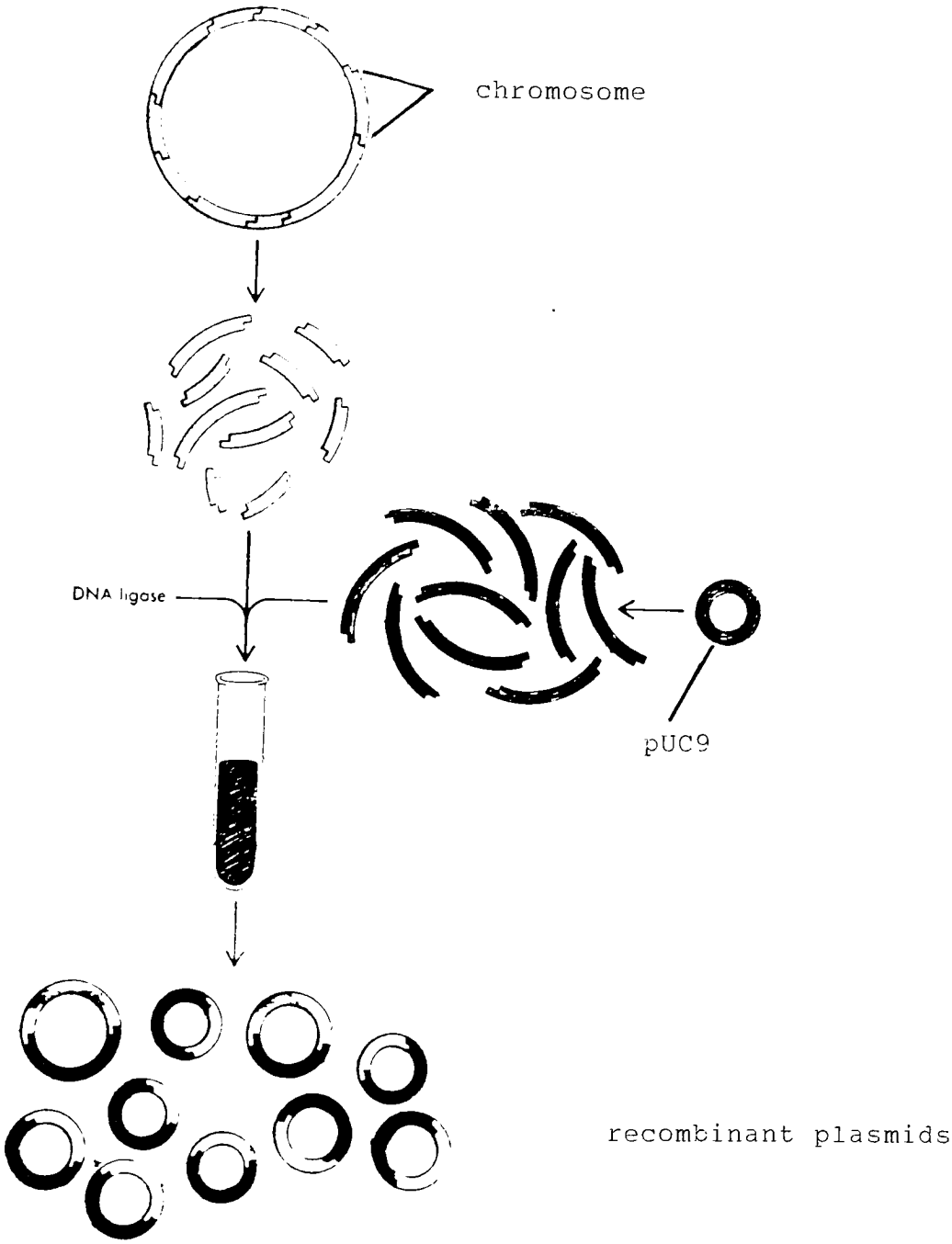

Figure 13. *Formation of a recombinant DNA library.*

approximately every twenty minutes and therefore it takes between 2 to 3 hours for the cells to reach their mid log growth phase under the outlined conditions.

The calcium chloride is hypotonic and causes the cells to swell and become spheroplasts. The volume of calcium chloride used to resuspend the pellet is not fixed,[4] nor is the time the calcium chloride treated cells are kept on ice.[1,5] It was found that resuspending the cells in $10ml$ of calcium chloride and placing the resuspended cells on ice for one hour produced competent cells. After the calcium chloride treated cells had been placed on ice for one hour, and centrifuged at 7000 rpm for 10 minutes, the cells which were now spheroplasts, produced a pellet whose appearance differed significantly from that of the pellet obtained when the overnight culture was centrifuged at 7000 rpm for 10 minutes. The first pellet was compact and dense while the second pellet was larger, more translucent and ring shaped. This latter appearance is indicative of competent cells. When the DNA is mixed with the competent cells, it forms a complex of hydroxyl-calcium phosphate, which adheres to the cell surface. This complex is DNase resistant. The heat shock treatment stimulates the cells so that they take in the DNA complex.

Two groups of colonies were identified based on colony color after the period of incubation.These were the white colonies and the red colonies. The white colonies were those that had been transformed with the recombinant plasmid while the red colonies were those that had been transformed by the pUC9 plasmid that had no fragment.

The white colonies were now stored. This was done by first picking out the white colonies from the plates and transferring them onto fresh MacConkey plates. One hundred different colonies were streaked onto each of the fresh MacConkey plates in the manner shown in Figure 14. The loaded plates were now incubated at 37^oC for 48 hours. After this period of incubation, the plates were taken out of the incubator and placed in the cold room at 4^oC until further use.

In order to make sure that the *E. coli* was transformed with the recombinant plasmid containing the fragment whose size ranged between 6 and 14 kilobases, a mini prep of randomly picked cells was carried out.

Three colonies from each master plate were chosen for the miniprep. The mini prep procedure described in section 2.9 was repeated in each case.

(3.5) Miniprep of transformed *E. coli*.

During the time the cells are sitting in the GET buffer, the lysozyme present in the buffer will strip away peptidoglycans in the cell wall of the bacterial cells while the glucose in the buffer creates an isotonic environment that prevents the resistant spheroplasts from rupturing as a result of osmotic shock. After the 5 minute period, 200 μl of a freshly prepared sodium

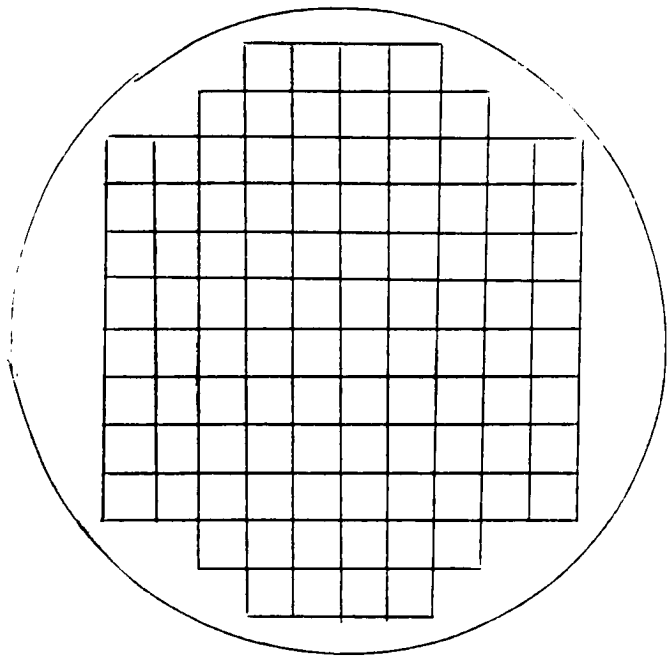

Figure 14. *Pattern of the parent plate containing transformed E. coli harboring the recombinant plasmid.*

hydroxide (NaOH) and sodium dedocylsulfate (SDS) mixture is added into the tube. The latter mixture is prepared by mixing $2ml$ of 0.5M NaOH, $0.5ml$ of 10% SDS solution and $2.5ml$ of water. The mixing of the SDS solution into the cells has to be carried out very carefully by inverting the tube a few times.This is done to prevent the DNA from being destroyed. The combination of alkali and detergent will dissolve the cell membrane and cause the cells to lyse. The addition of potassium acetate to the mixture produces a fluffy precipitate. This is because potassium acetate neutralizes the sodium hydroxide lowering the pH from about 9 to about 7. The lower pH and higher salt concentration aggregates the SDS and cell membranes into a fluff.

The nucleic acids in the potassium acetate precipitated supernatant (450 μl) has now got to be separated from the protein and cell debris also present in the sample. This separation is achieved by the phenol/chloroform extraction. The addition of the phenol/chloroform mix and the subsequent centrifugation, partitions the mixture in the eppendorf tube into an upper aqueous phase and a lower phenol phase. The nucleic acids settle in the aqueous phase while the protein and the rest of the cell debris settle in the phenol phase. Apart from purifying the nucleic acids the phenol chloroform extraction also destroys the activity of any nuclease present in the sample. The collected aqueous phase is subjected to a second phenol chloroform extraction. The double phenol chloroform extraction step ensures a relatively pure sample of nucleic acid with little or no nuclease contamination. The DNA isolated at this stage is mainly plasmid DNA. The chromosomal DNA of *E. coli* being considerably larger than the plasmid DNA becomes entangled in the cell debris and is washed away by the phenol extraction.

The 450 μl of aqueous phase is now mixed with $1ml$ of a 95% solution of ethanol and this mixture is left to stand at room temperature for 5 minutes. It is then centrifuged for 5 minutes. The ethanol precipitates the nucleic acid, which at times can be seen as a small white pellet at the bottom of the eppendorf tube.The ethanol is now poured out and the pellet allowed to dry. Drying of the pellet is speeded up by placing the opened eppendorf tube in a $37°C$ incubator for between 10 to 15 minutes. Finally, the dried pellet is resuspended in 50 μl TE buffer containing RNase at a final concentration of 20 μg /ml [see Sec. (2.12)]. The RNase present in the buffer destroys the RNA. Thus the final content of the sample is purified plasmid DNA.

Having isolated the plasmid, the next step was to run each isolated plasmid of an argarose gel in order to determine the size of the plasmid DNA. Between 8 to 12 different isolated plasmids were run together in each gel. Each run of the plasmids was accompanied with a pUC9 plasmid which served as the control and a KB ladder which enabled one to determine the size of the plasmid being analyzed. The plasmid samples run on the gel were divided into two groups. In one group the plasmid samples were run uncut, while in the other group the

plasmids were cut with a restriction enzyme before being run on the gel. The former group outlined the size of the inserted fragment while the latter group made it easy to determine the variability of the inserted fragments. The restriction enzyme used in this case was Hind $\overline{111}$ [see Sec. (2.2)]. There is only one restriction site for this enzyme on the plasmid pUC9. This site is situated in the polylinker region of the plasmid (see Fig. 4). Thus, when the recombinant plasmid is digested with Hind $\overline{111}$, all plasmids irrespective of the chromosomal fragment they carry will be cut at the same spot to produce a single linear fragment. There is no way, however, to predict how many restriction sites will be present on each inserted chromosomal fragment. The varying number of fragments produced by the digestion of the recombinant plasmids with Hind $\overline{111}$ suggests, therefore, that the chromosomal fragments inserted into the pUC9 vector are different (see Fig. 15).

The restriction digestion for the above reaction was set up as shown below.

recombinant plasmid	8 μl
medium salt buffer	2 μl
water	9 μl
Hind $\overline{111}$	1 μl
total of the restriction digest	20 μl

Another observation that should be mentioned here is that, if one looks at the sample of uncut recombinant plasmids in figure 15, it is clearly evident that the recombinant plasmids are significantly larger than the control pUC9 vector. This is an indication that the inserted chromosomal fragment is within the size range 6 to 14 kilobases. This is the chosen size range of the chromosomal fragments.

A formula is used to determine how many clones will be required in order to be sure of isolating the desired fragment. Let n be the size of the genome relative to a single cloned fragment.The number of independent recombinations required in the library must be greater than n, because sampling variations will lead to the inclusions of some sequences several times, and the exclusion of other sequences in the library of just N recombinants.[13] This formula which relates the probability (P) of including any DNA sequence in a random library of N independent recombinations[13] is

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

n is the size of the genome relative to a single cloned fragment (*i.e.* size of genome/size of fragment). The size of the genome $= 3.27 \times 10^4$ kb and the size of the fragment is 14 kb. Therefore,

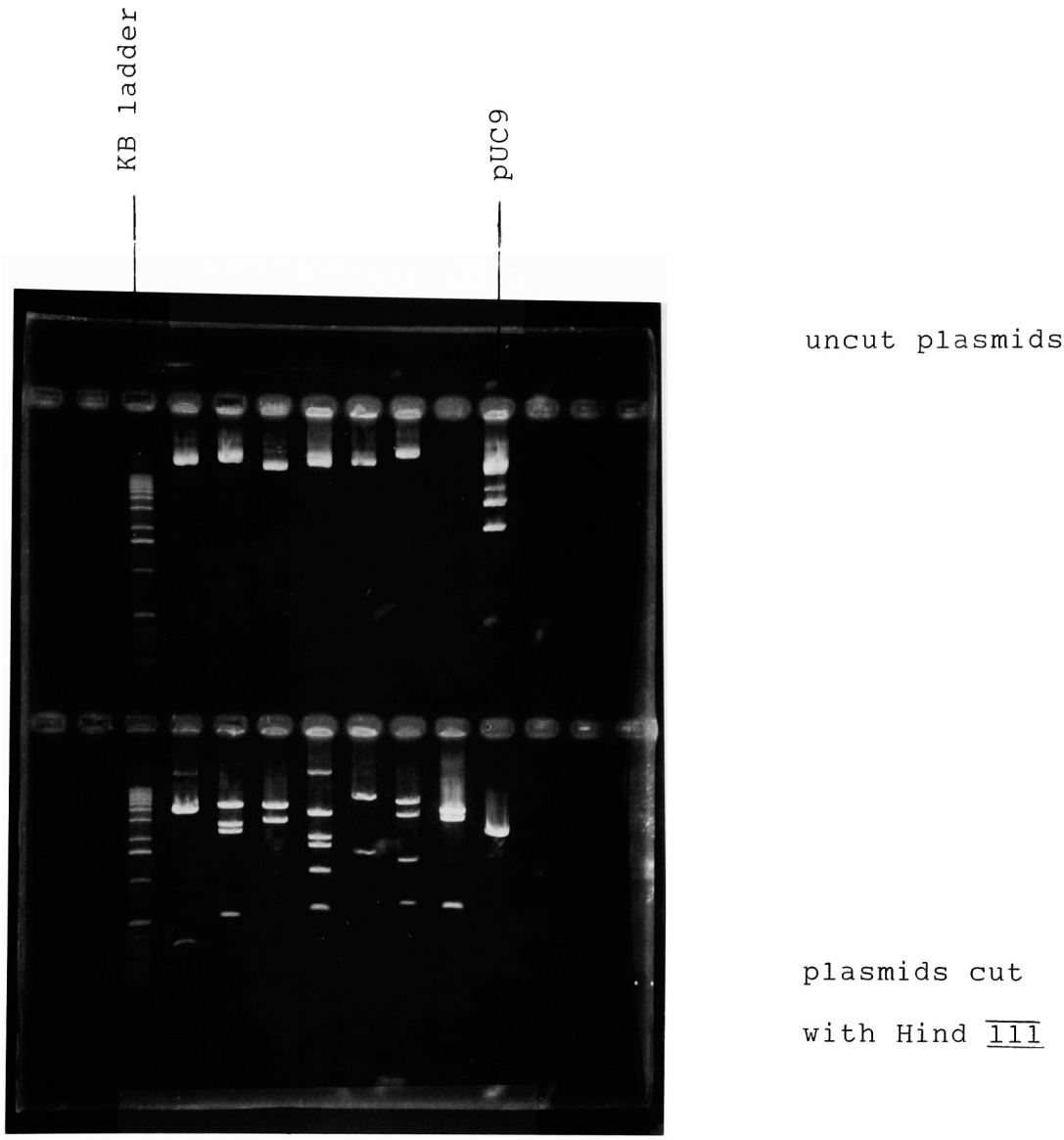

Figure 15a. *An 0.8% gel of recombinant plasmids.*

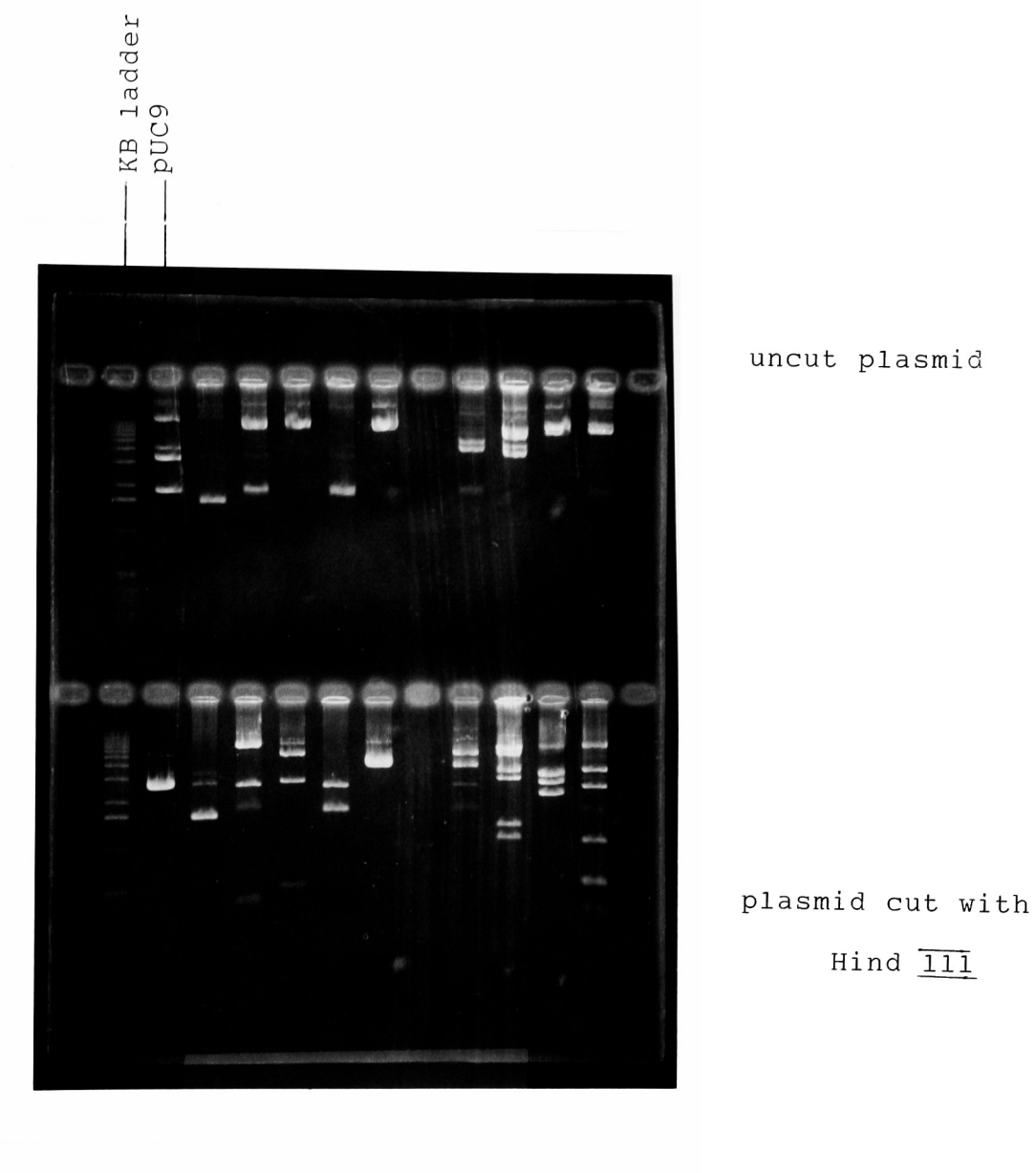

Figure 15b. *An 0.8% gel of recombinant plasmids.*

$$n = \frac{3.27 \times 10^4}{14} = 2337.2 \tag{2}$$

If $P = 0.95$, then, from expression (1) we have

$$N = \frac{\ln(1 - 0.95)}{\ln\left(1 - \frac{1}{2337.2}\right)} = 7000 \tag{3}$$

Therefore, to achieve a 95 % probability (P = 0.95) of including the recA-like gene in a random Agrobacterium chromosomal DNA library of 14 kb fragment size, approximately 7000 bacterial colonies containing the recombinant plasmid were picked and stored on the master plates. Once these 7000 bacterial colonies had been isolated, the final step in the construction of the bacterial gene bank was straightforward. All the 7000 transformed *E. coli* colonies were pooled together in $25ml$ of LB broth and centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in $20ml$ of fresh LB broth containing ampicillin. This $20ml$ of mixed cells was divided into twenty $1ml$ vials and stored at -70^oC. The twenty vials at -70^oC constituted the entire gene bank.

Having constructed the gene bank, the last step was to screen the large collection of chromosomal fragments for the desired gene. The first step was to purify the collection of recombinant plasmids from the bank. This was done by a complete plasmid preparation [see sec. 2.10].

(3.6) Isolation of the Plasmid DNA.

After the cells had been spun down they were resuspended in 25% sucrose solution. The sucrose creates an isotonic solution and prevents the cells from rupturing violently when treated with lysozyme and Triton-X. The lysozyme added into the mixture removes the cell walls by breaking down the glycosydic bonds in the polysaccharide backbone of peptidoglycans. Such an event converts the cells into protoplasts. Then, EDTA added together with the lysozyme, acts as a chelating agent, absorbing divalent cations. This is important because when cells are lysed, the nucleases released by these cell are not regulated and can destroy DNA indiscriminately when activated by divalent cations. Thus, by binding onto the divalent cations the EDTA deprives the nucleases of these ions. The result is inactive nucleases.

The Triton-X is a detergent and lyses the cell by dissolving the lipids in its cell membrane. Since the Triton-X is gently mixed into the mixture the much larger chromosomes do not rupture and become entangled in the cell debris. Therefore, after the centrifugation step,

most of the chromosomal DNA remains in the pellet. The plasmid DNA, on the other hand being considerably smaller, diffuses out into the surrounding solution.

After the cesium chloride density centrifugation, the plasmid DNA was easily distinguished from the chromosomal DNA as it was the lower of the two bands seen in the gradient when viewed in the presence of ultraviolet light. The result of this procedure was the isolation of very pure plasmid DNA.

(3.7) Conjugation.

Next, the purified recombinant plasmids were transformed into the strain BRC 49, and conjugated with the strain JC8563. This step is carried out to isoalate the recombinant plasmid containing the RecA-like gene. The plasmids were first introduced into a RecA$^-$ strain of *E. coli* (BRC49). The tranformation procedure of BRC49 with the recombinant plasmid is carried out as described in sec. (2.8). The transformed BRC49 is then conjugated with JC8563, an Hfr strain of *E. coli* and the evidence of genetic recombination looked for. The occurence of genetic recombination indicates the presence of the recombinant plasmid containing the RecA like gene in BRC49.

The genotype for the donor and recipient strains are given in section (2.1). The donor strain, JC8563, is a high frequency of recombination strain (Hfr). It contains the F plasmid, a small high copy number plasmid, incorporated into its chromosome. This F plasmid gives the cell the ability to produce sex pili which it can use to conjugate with female cells (lacks F plasmid) changing them into male cells containing the F plasmid. Since the F plasmid is incorporated into the chromosome of JC 8563, the chromosome now behaves as a large F plasmid. These cells are males and thus have the ability to initiate the formation of sex pili on the surface of the cells. The BRC 49, lacking the F plasmid, is a female and thus the recipient of the DNA from the male. When the sex pili of JC 8563 come in contact with the surface of BRC 49 its chromosome begins to replicate. The origin of replication of the chromosome is at the origin of the F plasmid incorporated in it. By the method of rolling circle replication a copy of the donor DNA is passed into the recipient. The time of conjugation is usually long enough to enable an entire F plasmid to be transferred from donor to recipient but is not long enough to enable the transfer of the entire chromosome. During the conjugation of JC 8563 and BRC 49 only a few chromosomal genes of the Hfr strain will be transferred into BRC 49. Thus when the cells separate after conjugation the BRC 49 will contain the front portion of the F plasmid and a small portion of the JC 8563 chromosome immediately following the F plasmid origin of replication (see Fig. 16). Since the entire F plasmid is not transferred into BRC 49 from JC 8563, the former still remains female. In this conjugation experiment the separation of the donor chromosome occurs after the desired chromosomal genes of JC8563

have been transferred. Following the leading F plasmid are the chromosomal genes for proline, leucine, threonine and argenine. The proline gene is of real interest because the recipient cells lack proline, and will incorporate the incoming proline gene into its own chromosome by the process of recombination only if it contains the recombinant plasmid having the recA-like gene. Such cells will now produce proline and can be easily identified on a minimal selective media lacking proline, but containing ampicillin. The ampicillin in the medium prevents the donor cells from growing since these cells are sensitive to ampicillin. Thus, the only cells that will grow in this selective media are those recipient cells that contain a recombinant plasmid containing the recA-like gene, or the proline gene of the *Agrobacterium tumefaciens.*

An easy experiment that will enable one to identify the cells containing the recA-like gene from the collection of cells is to expose the cells to ultraviolet light.

The total amount of cells that grew on the minimal selective agar was a strong function of the dilution. At a dilution of 10^{-1} the number of colonies obtained on the five plates were 15, 18, 12, 12, and 10, respectively. At a dilution of 10^{-2} all the plates were contaminated and at a dilution of 10^{-3} no growth was discernible. Sixty seven colonies in total were obtained from the five plates.

These colonies were streaked onto a fresh minimal media agar plate so that the colonies were evenly distributed over the surface of the agar and clearly separated from one another. The agar was incubated at 37°C for twenty four hours.

The print of the parent plate was transferred onto 5 other plates by the process of replica plating [see sec. 2.12]. Thus, the first plate had the highest concentration of cells in its print while the last plate had the lowest concentration of cells in its print. The 5 plates were now incubated at 37°C for twenty four hours.

The five plates were next exposed to ultraviolet light as outlined below.

plate no.	U.V. (joules)	time of exposure (s)
1	100	74
2	50	37
3	25	18.5
4	05	3.7
5	00	0

The five plates were then covered in foil and incubated at 37°C for forty eight hours. No cell growth was observed in any of these cultures.

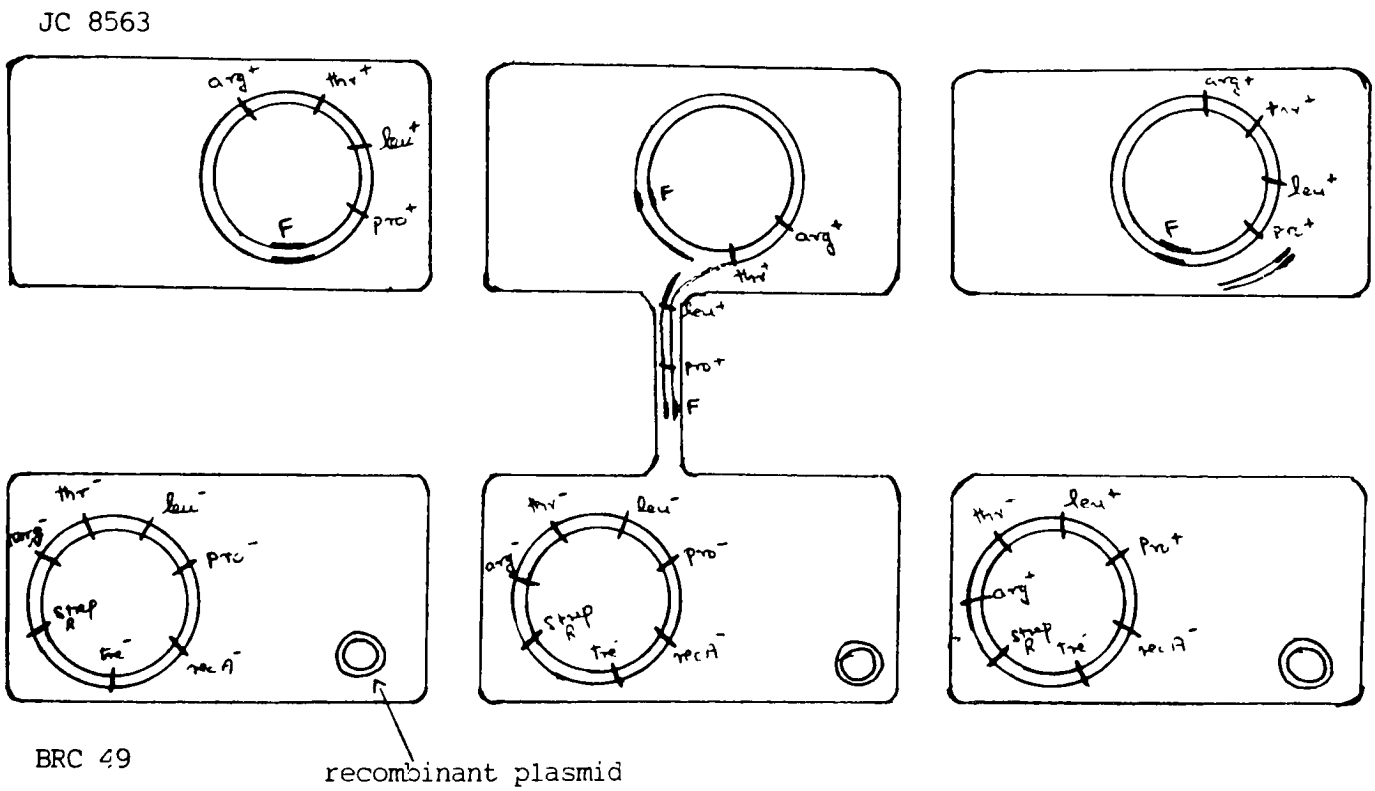

Figure 16. Conjugation of JC 8563 and BRC 49

CHAPTER 4
DISCUSSION AND CONCLUSIONS

(4.1) Discussion.

In building up the gene bank 4 essential stages were involved. These stages were;

(1) a method for generating DNA fragments,
(2) a reaction which joins the foreign DNA to the vector,
(3) a means of introducing the artificial recombinant into the host cell in which it can replicate, and
(4) a method of selecting or screening for a clone of recipient cells that have acquired the recombinant plasmid.

As seen in the methodology described in the previous section, success was achieved in all the stages leading up to the construction of the gene bank using the strain of *E. coli* DH5 α. This result was obtained after unsuccessful attempts using other strains of *E. coli*.

In the first attempt I was unable to proceed beyond stage three. This is the stage involving the transformation step. The strain of *E. coli* used during this attempt was K12 JM 83. After transforming the bacteria with the recombinant plasmid and incubating the transformed cells at $37^{o}C$ for 24 hours the expected red and white colonies were noted. The transformation frequency was also very satisfactory. However, when the transformed cells were picked at random and their recombinant plasmids isolated and examined for the size of the insert the results were indeed very unexpected. The inserts in all the recombinant plasmids were so very small that it became difficult to clearly differentiate between the control pUC9 vector and the recombinant plasmid (see Fig. 17). This was surprising since the chromosomal fragments used were those chosen among the group known to be within the size range 6 to 14 kilobases. This observation suggested one of three possibilities. First, there may have been some degree of contamination within the sample containing the chromosomal fragments. One such contaminant may have been the presence of nuclease in the sample. This is highly unlikely since the phenol chloroform extraction carried out in the production of the sample would have destroyed all the nuclease. Another possible contaminant is smaller fragments that may have accidentally got into the sample. If this were the case, then one would have expected that at least half the recombinant plasmids contained the chromosomal fragment of the prepared size, that is between 6 to 14 kilobases.

The second possibility can be attributed to the host bacteria. The genotype of the *E. coli* K12 JM 83 is $r^{+}m^{+}$. That is, it contains restriction enzymes that destroy DNA, and also

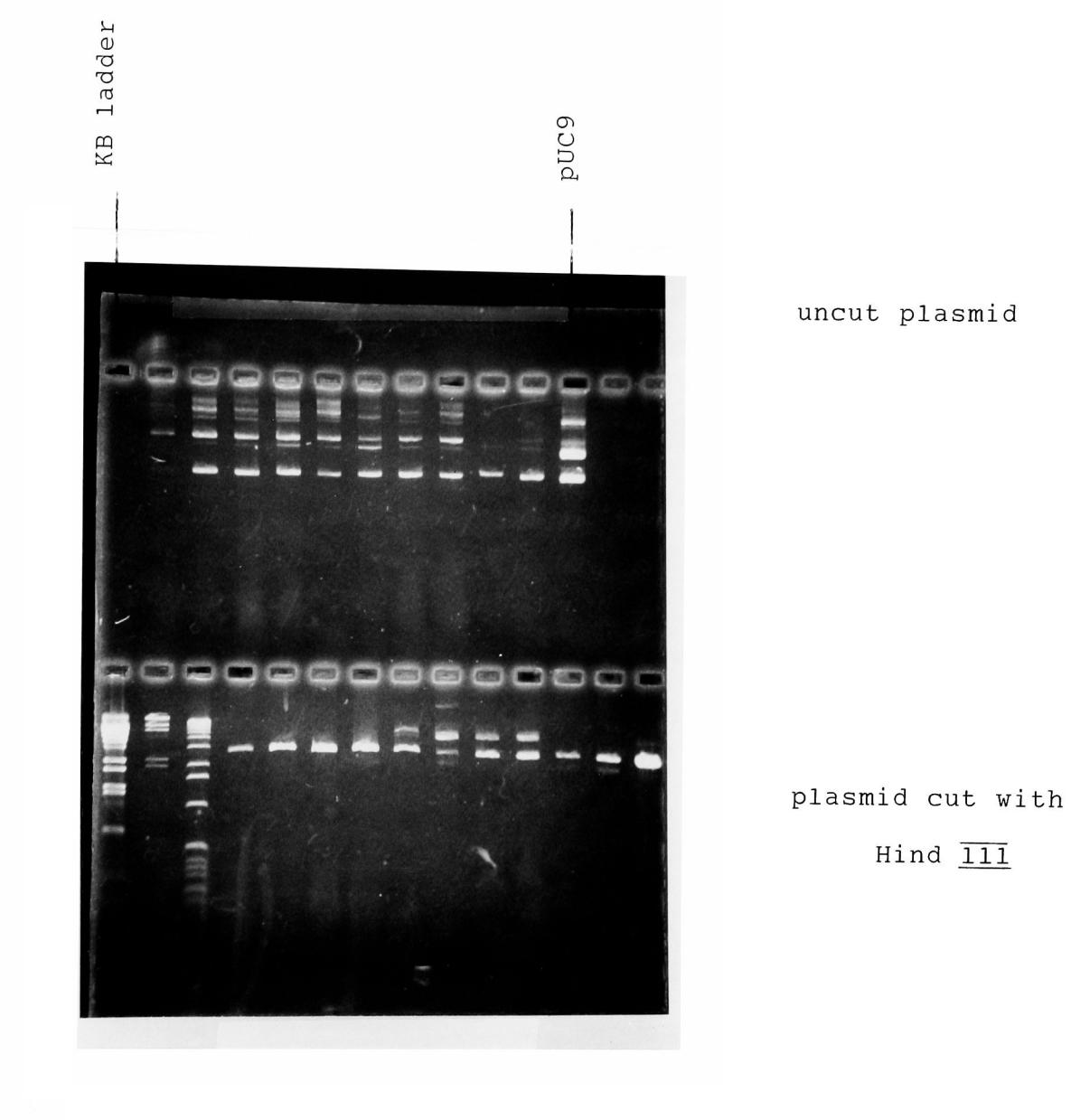

Figure 17. *An 0.8% gel of recombinant plasmids with inadequate fragment inserts.*

contains methylases that methylase its own DNA, preventing it from being destroyed by the restriction enzymes. The incoming foreign DNA is not methylated and thus is vulnerable to attack by the restriction enzymes of the bacteria. This might be an answer to the presence of the small chromosomal fragments contained in the recombinant plasmid. The weakness that this possibility has is that it cannot explain why the restriction enzymes of the bacteria did not destroy the plasmid vector.

The third possibility is derived from known observations.[26] These are that large inserts which slow down the multiplication rates of their host plasmids tend to be replaced by smaller derivative plasmids that have lost a section of their DNA inserts. A fourth possible explanation why no cells grew when exposed to ultraviolet light suggesting that no cell had the recA gene. This is quite surprising since 7000 cells were collected giving a 95 % probability of isolating at least one colony containing the recA-like gene.

Since the last two possibilities were related to fragment size, I repeated the purification of the chromosomal fragments and isolated a pure sample of chromosomal fragments ranging in size from 6 to 14 kilobases. At the same time in order to rule out the possibility of the restriction enzymes contained in the strain K12 Jm 83 destroying the fragments. I transformed the recombinant plasmids into a new strain of *E. coli* K 12 namely, HB101 (m^-r^-). In this attempt I failed to achieve a good transformation frequency. A very large proportion of the transformed bacteria did not grow and those that did took 48 hours to grow to an appreciable size. For my third and final attempt I chose the strain of *E. coli* K12 DH5 α. This strain had the genotype r^-m^+ and had alpha complementation with the lac gene of the plasmid pUC9. The results obtained in this attempt were successful and have been outlined in detail in the section on materials and methods. Due to time constraints the conjugation and ultraviolet exposure steps were not repeated.

(4.2) Summary and Conclusions.

The gene bank completed, contains approximately 7000 separate clones of bacteria. Contained in each clone is a single fragment of the chromosomal DNA. Among this large collection of genes I expect there to be present the gene that codes for the protein having recA-like properties. This gene bank also contains many other genes that may be the focus of other important studies. It is therefore my sincere hope that this gene bank will serve as a beneficial stepping stone for researchers who strive to further their understanding into the properties of the recA-like gene of *Agrobacterium tumafaciens* and the other important genes that may also be contained within this chromosome.

LIST OF REFERENCES

1. Berger, Shelby I., and Kimmel, Alan R., **Guide to Molecular Cloning Techniques**, Academic Press Inc. (1987).

2. Casdaban, Malcolm J., and Cohen, Stanley, N., *Analysis of Gene Control Signals by DNA fusion and cloning in Escherichia coli*, J. Mol. Biol., Vol. 138, pp. 179-207 (1980).

3. Close, T.J., Tait, R.C., and Kado, C.I., *Regulation of Ti plasmid virulence Genes by a Chromosomal Locus of Agrobacterium tumafaciens*, J. Bacteriol., Vol. 164, No.2, pp. 774-781 (November 1985).

4. Dagert, M., and Ehrlich, S.D., *Prolonged incubation in calcium chloride improves the competence of E. coli cells*, Gene, vol. 6, pp. 23-28 (1979).

5. Davis, L.G., Dibner, M.D., and Battey, J.F., **Basic methods in molecular biology**, Elesevier (1986).

6. Douglas, Carl J., Halperin, Walters, and Nester, Eugene W., *Agrobacterium tumefaciens mutants affected in attachment to plant cells.*, J. Bacteriol., Vol. 152, No. 3, pp. 1265-1275 (December 1982).

7. Douglas, Carl J., Staneloni, Roberto J., et al., *Identification and Genetic Analysis of an Agrobacterium tumefaciens Chromosomal Virulence Region*, J. Bacteriol. Vol. 161, No. 3, pp. 850-860 (March 1985).

8. Flanders, Paul Howard, *Inducible repair of DNA*, Scientific American, Vol. 245, No. 5, pp. 72-80 (November 1981).

9. Garfinkel, David J., and Nester, Eugene W., *Agrobacterium tumefaciens mutants affected in Crown Gall Tumorigenesis and Octopine Catabolism*, J. Bacteriol., Vol. 144, No. 2, pp. 732-743 (November 1980).

10. Hamada, Suzzane E., Luckey, John P., and Farrand, Stephen K., *R-plasmid-Mediated Chromosomal Gene Transfer in Agrobacterium tumefaciens*, J. Bacteriol., Vol. 139, No. 1, pp. 280-286 (July 1979).

11. Herberlein, G.T., and Lippincott, J.A., *Photoreversible ultra violet enhancement of infectivity in Agrobacterium tumefaciens*, J. Bacteriol., Vol. 87, pp. 1511-1514 (1965).

12. Low, K. Brooks, **The Recombination of Genetic Material**, Academic Press Inc. (1988).

13. Maniatis, T., Fritsch, E.F., and Sambrook, J., **Molecular Cloning.- A Laboratory Manual**, Cold Spring Harbor Labs. (1982).

14. Miller, Jeffrey H., **Experiments in Molecular Genetics**, Cold Spring Harbor Labs. (1982).

15. Old, R.W., and Primrose, S.B., **Principles of Gene Manipulations**, 3rd edition, Blackwell Scientific Publications (1985).

16. Pischl, D. L., and Farrand, S. K., *Characterization of Transposon Tn5-Facilitated Donor Strains and Development of a Chromosomal linkage map for Agrobacterium tumefaciens*, J. Bacteriol., Vol. 159, No. 1, pp. 1-8 (July 1984).

17. Robertson, Jennie L., Holliday, Teresa, and Matthysse, Ann G., *Mapping of Agrobacterium tumefaciens Chromosomal Genes Affecting Cellulose Synthesis and Bacterial Attachment to Host Cell*, J. Bacteriol., Vol. 170, No. 3, pp. 1408-1411 (March 1988).

18. Rossbach, S., Schell, J., and de Bruign, F.J.,*Cloning and Analysis of Agrobacterium tumefaciens C-58 loci involved in Glutamine biosynthesis. Neither the glnA (GSI) nor the glnll (GSLL) Gene plays a special role in virulence*, Mol. Gen., Vol. 212, pp. 38-47 (1988).

19. Rothman, Robert H., and Kelly, Elizabeth R., *Inducible DNA Repair in Agrobacterium tumefaciens*.

20. Schardl, C.L., and Kado, C.L., *Ti plasmid and Chromosomal Ornithine Catabolism Genes of Agrobacterium tumefaciens C58*, J. Bacteriol., Vol. 155, No. 1, pp. 196-202 (July 1983).

21. Setlow, Jane K., Spikes, Deborah, and Griffin, Kathleen, *Characterization of the resL gene of Haemophilus influenzae and behavior of the gene in Escherichia coli*, J. Bacteriol., Vol. 170, No. 9, pp. 3867-3881 (September 1988).

22. Stryer, Lubert, **Biochemistry**, 3rd edition, W.H. Freeman and Company (1988).

23. Thomashow, Michael F., Nutter, Robert, et al., *Integration and Organizational Ti plasmid sequences in Crown Gall Tumors*, Cell, Vol. 19, pp. 729-739 (March 1980).

24. Vieira, Jeffrey, and Messing, Joachim, **The pUC plasmids, an M13mp7-derived System for Insertion mutagenesis and Sequencing with Synthetic Universal Primers**, Gene, Vol. 19, pp. 259-268 (1982).

25. Ward, Eric R., and Barnes, Wayne M., *Integration of multiple copies of a foreign sequence into the Ti plasmid of Agrobacterium tumefaciens*, Gene, Vol. 75, pp. 305-314 (1989).

26. Watson, Bruce, Currier, Thomas C., et al.*Plasmid required for Virulence of agrobacterium tumefaciens*, J. Bacteriol., Vol. 123, No. 1, pp. 255-264 (1975).

27. Watson, James D., et al., **Molecular Biology of the Gene**, 4th edition, The Benjamin/Cummings Publishing Company Inc. (1987).

28. Watson, James D., Tooze, John, and Kurtz, David T., **Recombinant DNA- A Short Course**, W.H. Freeman and Company (1983).

29. Witkin, E.M., *Ultra violet mutagenesis and inducible repair in E. coli*, Bacteriol. Rev., Vol. 40, pp. 869-907 (1976).