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Cloning of the DNA of mycobacterium tuberculosis and analysis of the expressed protein antigens

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ii. The M.tuberculosis DNA is prepared for cloning by random fragmentation: the only method by which DNA can be fragmented in a truly random fashion, irrespective of its base composition and sequence, is mechanical shearing. The DNA is then methylated, ligated to Eco R1 linkers, and separated from excess linkers by size fractionation.

iii. The arms of the vector and the fragmented M.tuberculosis DNA are then ligated to form long concatemeric molecules that are subsequently packaged into λ heads using in vitro packaging systems. The recombinant phages are amplified by growth in E.coli and the resulting library can then be screened for the presence of recombinant genes/proteins.

2.9.2 Preparation of DNA for cloning

The method employed for the preparation of the mycobacterial DNA for cloning, and the construction of the genomic library, was essentially that described by Huynh, Young, and Davis (1985).

M.tuberculosis DNA was mechanically sheared to an average length of 5 kilobase pairs (kb) with 200 passages through a 27 gauge needle attached to a 1-ml syringe. The degree of

shear was monitored by electrophoresis through a 0.7% agarose gel (appendix 1). A positive control insert DNA (Promega Biotech, Madison, WI, USA) was used to test the performance of the system. The insert was a 3 kilobase plasmid DNA fragment with blunt ends.

2.5.3 Methylation of internal Eco R1 sites

2.5.3 i. Introduction

The Eco R1 methylation reaction protects any internal Eco R1 restriction sites that may occur in the DNA by subsequent digestion with Eco R1 when cohesive ends are generated on synthetic linkers. The reaction is carried out by Eco R1 methylase, which methylates the A residue in the Eco R1 recognition site (Perbal, 1984) - 5'-G AATT C-3'

3'-C TTAA G-5'

Eco R1 cannot digest at the methylated site, thus ensuring that the full foreign DNA sequence can be cloned.

2.5.3 ii. Method employed

The methylation reactions were set up, for the M.tuberculosis and the control DNA, in 1.5 ml microcentrifuge tubes on ice. The components were added in the following order:

Tube A	volume
Sheared M.tuberculosis DNA, 1 ug	4 ul
Methylase buffer (appendix 2)	4 ul
100 uM s-adenosyl methionine (New England Biolabs, Beverly, MA, USA)	2 ul
Sterile distilled water to bring the final reaction volume to 20 ul including the methylase enzyme.	

Tube B	volume
Control DNA, 1 ug	5 ul
Methylase buffer (appendix 2)	4 ul
100 uM s-adenosyl methionine (New England Biolabs, Beverly, MA,USA)	2 ul
Sterile distilled water to bring the final reaction volume to 20 ul including the methylase enzyme.	

The components of each tube were mixed gently and spun for a few seconds in a microcentrifuge tube to collect the contents to the bottom of the tube. Twenty units of Eco R1 methylase (New England Biolabs, Beverly, MA, USA) were added to each reaction tube. The reaction components were mixed gently, and incubated at 37°C for 60 minutes, followed by a further incubation at 70°C for 10 minutes to heat-inactivate the enzyme.

2.5.4 Addition of Eco R1 linkers to the DNA

2.5.4 i. Introduction

In order to enable the covalent attachment of the insert DNA to the vector DNA at the Eco R1 insertion site, synthetic Eco R1 linkers must be attached to the DNA (Wu, Wu, and Ray, 1987). Because efficient blunt-end ligation requires a vast molar excess of linker DNA molecules to drive the reaction (Wu, Wu, and Ray, 1987), this results in the covalent attachment of oligomers of the linkers to the ends of the insert DNA. It is therefore necessary to subsequently cleave the reaction product with Eco R1 to remove any excess linkers (Huynh, Young, and Davis, 1985). A single Eco R1 "sticky end" on each terminus of the insert DNA is produced by digesting and releasing the excess linker molecules.

2.5.4 ii. Method

Reactions were set up in microcentrifuge tubes on ice. To 20 ul of methylated M.tuberculosis and control DNA, 3 ul of T4 DNA ligase buffer (appendix 2) were added, followed by 1.5 ug of phosphorylated Eco R1 linkers (d(pGGAATTCC; New England Biolabs, Beverly, MA, USA). Sterile distilled water was added to bring the final reaction volume, after the addition of the ligase enzyme, to 30 ul. The components were

mixed gently and spun in a microcentrifuge for a few seconds. Seven point five units of T4 DNA ligase (New England Biolabs, Beverly, MA, USA) were added, the reactions mixed gently, and incubated in a 15°C waterbath for 20 hours. The reactions were then stopped by incubating the tubes at 70°C to heat-inactivate the enzyme.

Following ligation, Eco R1 digestions of the linkered DNA were set up on ice. Ten microlitres of Eco R1 buffer (appendix 2) were added to each reaction tube, followed by sufficient sterile distilled water to bring the final reaction volume, after the addition of the Eco R1 enzyme, to 100 ul. The components were mixed gently, and 250 units of Eco R1 (New England Biolabs, Beverly, MA, USA) were added to each reaction. The tube contents were gently mixed, centrifuged for a few seconds, and incubated at 37°C for 7 hours. The reactions were then incubated at 70°C for 10 minutes to heat-inactivate the enzyme.

2.5.5 Removal of excess Eco R1 linkers and size fractionation of the DNA

2.5.5 i. Introduction

Before the DNA can be inserted into λgt 11, excess linker molecules must be removed, since ligation of the linkers into the vector DNA results in the appearance of false

recombinants in the library (Huynh, Young, and Davis, 1985). The DNA is then fractionated by size, and excess linkers are removed in a single step by running the linker reactions through a gel filtration column (Huynh, Young, and Davis, 1985).

2.5.5 ii. Method

Separate gel filtration columns (supplied as part of the λ gt 10 cloning kit, Amersham International, Little Chalfont, Buckinghamshire, England) were used for the fractionation of the M.tuberculosis and the control DNA.

Fractionation was carried out at room temperature. The columns were washed twice with 3 ml of STE buffer (appendix 3), and then equilibrated by passing a further 2-3 ml volumes of STE buffer through each column.

The linker reaction solutions (100 ul) were loaded on the top of the column beds, and when these had entered the matrix, 100 ul of STE buffer were added to the top of each column. The column eluates (100 ul) were collected into 2 microcentrifuge tubes, and a further 200 ul of STE buffer were loaded onto each column. The eluates (200 ul) were collected into 2 tubes, and the procedure repeated until 10 eluate fractions were collected for each column. The eluate fractions were analysed for the presence of DNA by

electrophoresing a 30 ul aliquot of each fraction through a 0.7% agarose gel (Appendix 1). The fractions containing the major peaks of DNA were pooled, and all remaining fractions, containing buffer or linkers, were discarded.

The linkered DNA was ethanol-precipitated by adding 1/10 volume of 3 M sodium acetate, followed by 2 volumes of ice-cold absolute ethanol. The solutions were gently mixed, and the DNA precipitated at -20°C for 16 hours. Precipitated DNA was collected by centrifuging at 8 500 g in a microcentrifuge for 30 minutes at 4°C. The supernatants were carefully removed, the DNA dried at room temperature, and finally resuspended in STE buffer (Appendix 8) to give a final concentration of approximately 100 ug/ml.

2.5.6 Ligation of the DNA to λ gt 11 arms

2.5.6 i. Introduction

At this point the DNA had Eco R1 cohesive ends and was ready for ligation into the λ gt 11 vector DNA. There are 2 important parameters to consider when setting up a ligation reaction (Maniatis, Fritsch, and Sambrook, 1982): the ratio of arms to potential inserts, and the concentration of each of the DNA species. The best substrate for *in vitro* packaging is a concatemer of the form [n X (left arm - insert - right arm)]. Each arm of the λ DNA carries 2

different cohesive termini: one cos that is compatible only with the complementary cos sequence at the end of the other arm, and one, ct, that is compatible with both termini of the insert and with the remaining terminus of the other arm (Maniatis, Fritsch, and Sambrook, 1982). To obtain the maximum yield of the desired concatemer, the ligation reaction should contain equimolar concentrations of the ct ends of each of the 3 species of DNA. Because each bacteriophage λ arm contains only a single ct end, whereas each potential insert contains 2 ct ends, the ratio of molecules in the reaction should be 2:1:2 (left arm: insert: right arm). This results in a 2:1 molar ratio of annealed arms to potential inserts (Maniatis, Fritsch, and Sambrook, 1982). The absolute concentration of DNA in the reaction mixture is also important. The concentration must be high enough to ensure that the intermolecular ligation, which leads to the formation of concatemers, is favoured over self-ligation, which leads to the cyclization of the DNA molecules (Maniatis, Fritsch, and Sambrook, 1982).

It is important at this stage of the cloning procedure that a number of control reactions be included. These serve the dual functions of testing various stages in the whole cloning process, and of providing the background data necessary to analyse the results.

The major control reactions for the ligation and packaging steps are (Maniatis, Fritsch, and Sambrook, 1982):

i. λ gt 11 Eco RI arms without any insert DNA.
   This monitors the efficiency of the ligation reactions, and allows the estimation of false recombinants which appear independent of added insert DNA.

ii. λgt 11 arms and linkered control fragments.
   This control DNA is processed throughout the procedure, and checks the performance of the whole cloning system.

iii. packaging of whole λ DNA.
   This monitors the overall efficiency of the *in vitro* packaging reactions.

iv. a mock packaging reaction.
   This contains no DNA whatsoever, and gives a measure of any background that might be contributed by the packaging extracts.

2.5.6 ii. Method

Ligation reactions were set up on ice as described in Table 4. The tube contents were mixed gently, and a 1 ul aliquot was removed from each reaction and added to 10 ul TE buffer for subsequent electrophoresis. T4 DNA ligase (New England Biolabs, Beverly, MA, USA) was then added as specified in Table 4, and the reactions incubated at 16^{0}C for 20 hours. After ligation, another aliquot (1 ul) was removed from each

TABLE 4 LIGATION REACTIONS

Tube number	Insert DNA	λgt 11 arms	10 X ligase buffer*	distilled water	T4 DNA ligase
1	-	1.0 ug (2 ul)	1 ul	1.0 ul	5 units
2	positive control 0.2 ug (1 ul)	0.5 ug (1 ul)	1 ul	4.5 ul	2.5 units
3	M.tuberculosis 100 ng (1 ul)	1.0 ug (1 ul)	1 ul	1.0 ul	5.0 units
4	M.tuberculosis 200 ng (2 ul)	1.0 ug (1 ul)	1 ul	-	5.0 units

* : T4 DNA ligase buffer (appendix 2)

reaction tube and added to 10 ul TE buffer (Appendix 8). These aliquots, together with those set aside before the addition of the ligase, were heated to 70°C to for 5 minutes to denature any unligated λ cohesive ends. These samples were analysed for successful ligation by electrophoresis through a 0.7% agarose gel (Appendix 1).

2.5.7 In vitro packaging of ligated DNA

2.5.7 i. Introduction

The establishment of *in vitro* packaging systems has been the decisive step in making λ phage such a highly efficient and favoured vector for DNA cloning (Perbal 1984). The *in vitro* packaging of recombinant λ DNA molecules into mature phage particles results in 3-5 logs greater plating efficiencies than do calcium chloride transfection procedures (Perbal, 1984). In addition, the packaged phage mixture can be stored at 4°C as a stable phage preparation for several months. Phage packaged by *in vitro* methods may be plated at a 100-fold greater density than a similar transfection mixture. They can be easily amplified, and contain a high percentage of recombinant molecules (Perbal, 1984). Successful packaging of recombinant molecules is dependent on the physical nature of the substrate DNA. There is a strict requirement for the presence of the λ cohesive termini (*cos* sites), which, for efficient packaging, must be

separated by 35-52 kb of DNA (75%-105% length of wild type DNA) (Perbal, 1984). Although linear, multimeric linear (concatemers), and multimeric circular DNA's are all packaged, monomeric circular molecules are not packaged *in vivo* or *in vitro* (Perbal, 1984). It is thus necessary to perform ligations for packaging under conditions which limit monomeric circle formation.

Packaging of bacteriophage λ DNA *in vitro* was initially developed by Becker and Gold (1975) using mixtures of extracts prepared from bacteria infected with mutants of bacteriophage λ that map in genes required for assembly of bacteriophage particles (Maniatis, Fritsch, and Sambrook, 1982). A diagrammatic representation of the various stages of bacteriophage λ packaging, the gene products required, and the stages at which various mutations affect the process is shown in Figure 3.

The E protein is the major component of the bacteriophage head, and is required for the assembly of the earliest identifiable precursor (Maniatis, Fritsch, and Sambrook, 1982). Mutants in E protein accumulate all the components of the viral capsid. The D protein is localised on the outside of the phage head, and is involved in the coupled process of insertion of λ DNA into the head precursor and subsequent maturation of the head (Maniatis, Fritsch, and Sambrook, 1982). Mutants in the D gene accumulate the immature

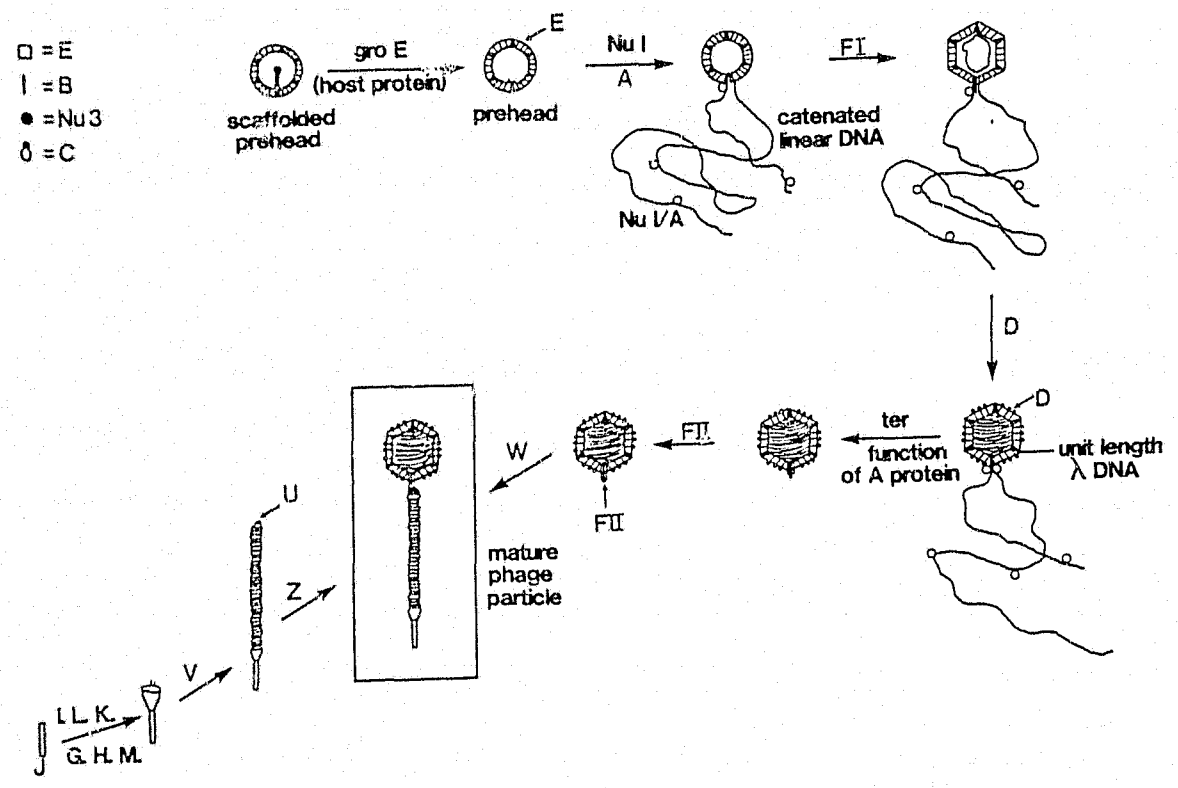


FIGURE 3 A schematic diagram of the assembly of bacteriophage (Maniatis, Fritsch, and Sambrook, 1982). The gene products involved in each step are indicated.

"prehead" but do not allow insertion of λ DNA into the head. The A protein is involved in the insertion of the λ DNA into the bacteriophage head and cleavage of the conoatenated, precursor DNA at the cohesive end sites (Maniatis, Fritsch, and Sambrook, 1982). Mutants in the A gene also accumulate empty preheads.

Complementary extracts have been prepared from cells infected with A^- and E^- , or D^- and E^- strains (Maniatis, Fritsch, and Sambrook, 1982). Extracts are usually prepared from cells containing bacteriophage λ lysogens of the appropriate genotype (amber mutations in the A, E or D genes) (Maniatis, Fritsch, and Sambrook, 1982). The lysogens also carry the following mutations (Maniatis, Fritsch, and Sambrook, 1982):

cIts 857: specifies a temperature-sensitive bacteriophage λ repressor molecule, which causes the λ DNA to be maintained in the lysogenic state when the bacteria are grown at 32°C, and to revert to lytic growth when grown at 42°C.

Sam 7: an amber mutation in the bacteriophage S gene that is required for cell lysis and which causes capsid components to accumulate for several hours following induction of the cIts 857 lysogen.

b region deletion: a deletion in the bacteriophage genome

that effectively removes the λ DNA attachment site (att). This mutation reduces the packaging of endogenous DNA molecules in extracts made from the induced cells.

red_3 (in λ) and
rec_A (in E.coli): mutations that inactivate the generalised recombination systems of bacteriophage λ and the host, thereby minimising recombination between the endogenous λ DNA in the extract and the exogenously added recombinant genomes.

The Packagene Lambda DNA packaging system (Promega Biotech, Madison, WI, USA) was used for packaging the recombinant phage. The system has a high capacity, packaging up to 5 ug of DNA per extract. It is derived from a lysogen carrying the cos_2 mutation which prevents packaging of prophage DNA (Kobayashi, Murialdo, Craseman, et al., 1987). The extracts contain all the components and buffers needed to perform the packaging reaction, and require only the addition of substrate DNA.

2.5.7 ii. Method

Packaging was performed as suggested by the manufacturers of the in vitro packaging extracts. The packaging extracts

(Packagene in vitro packaging systems, Promega Biotech, Madison, WI, USA) were thawed on ice, briefly centrifuged to collect the contents to the bottom of the tubes, and immediately transferred to the tubes containing the ligated DNA, and to a tube containing DNA to be used as a packaging control. The control DNA (1 ug) used was concatemeric lambda C 1857 Sam 7 DNA, ligated with an excess of T4 DNA ligase (Promega Biotech, Madison, WI, USA). The tubes were gently mixed, and the packaging reactions incubated at 22°C for 3 hours. After packaging, 500 ul of SM buffer (appendix 8) were added to each tube, followed immediately by 25 ul of chloroform. Tubes were mixed gently, and packaged phage libraries were stored at 4°C until required.

2.5.8 Titration of λgt 11 recombinants

2.5.8 i. Introduction

In order to estimate the number of recombinant phage which may be screened for isolation of the recombinants of interest, the numbers of recombinants in the library must first be determined. The lon protease-deficient host, E.coli Y 1090, used to plate out libraries for screening with antibody probes, is not defective in host-controlled restriction and modification enzyme activities (Huynh, Young, and Davis, 1985). Therefore, the initial packaging mix of libraries cloned in λ gt 11 should not be plated

directly on Y 1090 for screening, but should first be amplified through E.coli Y 1088, which is deficient in the abovementioned enzyme activities. In addition, to avoid creating a bias against relatively poor-growing recombinants during amplification of the library, it is important to repress production of toxic polypeptides (in the form of β-galactosidase fusion proteins) encoded by the recombinant phage. Therefore, E.coli Y 1088, which produces lac repressor, is used as the host to amplify the library (Huynh, Young, and Davis, 1985), and higher phage titres are further obtained by growing the λ gt 11 libraries at 42°C instead of 37°C (Young and Davis, 1985).

The transcription of the lacZ gene product, and the production of fusion proteins by recombinant λ gt 11 phages can be induced by the addition of IPTG, a molecule which has a β-galactoside linkage that is found in lactose, and which releases the lacZ gene repressor, presumably by interaction with the repressor molecule (Birge, 1931). Insertion of foreign DNA into λ gt 11 inactivates the lacZ gene product, and as such, plaques formed by lacZ⁻ λ gt 11 phages on a lacZ⁻ host cannot metabolise the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal), and therefore form clear plaques. LacZ- non-recombinants (λ gt 11 phages containing no foreign inserts) are gal⁺ and can metabolize Xgal, producing blue plaques. This allows for colorimetric selection of recombinants by distinguishing

between insert-bearing (clear) and non insert-bearing (blue) λ gt 11 plaques (Davis, Dibner, and Battey, 1986).

2.5.8 ii. Method

Phage plating cells were prepared by streaking a loopful of indicator bacteria (E.coli Y 1088 for λ gt 11 recombinants; E.coli LE 392 for the packaging control DNA) onto separate dry Luria-Bertani (LB) plates (appendix 4). The plates were incubated at 37°C overnight. An individual colony from each plate was inoculated into 50 ml LB broth containing 0.4% maltose (appendix 4), and the flasks incubated at 37°C with good aeration until the $O.D._{600}$ of the cultures was approximately 0.6. The cultures were cooled on ice, spun at 1 500 g for 10 minutes at 4°C, and the cells resuspended in 15 ml of ice-cold 10 mM $MgSO_4$. The cells were now considered ready for infection with phage, and were stored at 4°C until required. Fresh (up to 36 hours old) plating cells were used for all phage titrations, since storage of the cells at 4°C for long periods of time leads to a significant loss of packaging efficiency (Maniatis, Fritsch, and Sambrook, 1982).

The packaged phage was then titrated essentially as described by Huynh, Young, and Davis (1985). Serial tenfold dilutions (from 10^{-1} to 10^{-6}) of the packaged phage were made in SM buffer. One hundred microlitres of each phage dilution

were mixed with 100 ul of of the appropriate plating cells, and phage was allowed to absorb for 20 minutes at 37°C. Five millilitres of molten (45°C) LB agar (appendix 4) were added to each tube, the tubes quickly inverted several times, and the phage plated onto dry (at least 2 days old) LB plates (Appendix 4). Plates were incubated at 42°C overnight. The phage titres were tabulated and the optimal phage dilutions determined for each recombinant phage library.

In order to determine the percentage of the library that was recombinant, and to have an estimate of the complexity of the library, 100 ul of the appropriate dilutions of the packaged phage were mixed with 100 ul of fresh Y 1088 plating cells, and incubated at 37°C for 20 minutes. Five millilitres of molten LB agar containing 20 ul Xgal (50 mg/ml in dimethylformamide; Sigma Chemical Co., St. Louis, MO, USA) and 20 ul IPTG (40 mg/ml in distilled water; Sigma Chemical Co., St. Louis, MO, USA) were added to each tube, and the phage plated onto dry LB plates. The number of clear and blue plaques on the plates were counted, the cloning efficiency of the mycobacterial DNA calculated, and the percentage of recombinant (clear) plaques determined.

2.5.9 Amplification of the library and preparation of recombinant phage stocks

For amplification, the library constructed with 200 ng

M.tuberculosis DNA was plated out at a density of 10^2 plaque forming units (pfu's) per 90 mm petri dish, using 200 ul of Y 1088 cells and fresh LB plates. Five millilitres of molten agar agar containing 20 ul Xgal and 20 ul IPTG stock solutions were used per plate. Plates were incubated overnight at 42°C.

Recombinant phage stocks were prepared using a modification of the technique described by Davis, Botstein, and Roth, (1980). One hundred clear (recombinant) plaques were cored from the plates containing the amplified library using a sterile Pasteur pipette, and resuspended in a sterile Bijou bottle containing 5 ml SM buffer. A 300 ul aliquot of the phage suspension from the bottle was then added to 5 ml of fresh Y 1090 plating cells. The phage were allowed to absorb to the cells for 30 minutes at 37°C. The cells and phage were then transferred to 50 ml of warm (37°C) LB broth containing 5 mM $CaCl_2$ ($CaCl_2$ was made up as a 500 mM stock solution, filter sterilised, and the required volume added to cool LB broth to provide the final concentration required). Flasks were incubated at 37°C with good aeration for 5 hours, after which 500 ul of chloroform were added to each flask, and the cultures incubated at 37°C for a further 10 minutes. Bacteria and debris were removed by centrifugation at 2 500 g for 15 minutes. The supernatant was centrifuged a second time at 2 500 g to remove any remaining debris. The resultant phage stock was titrated,

and stored at 4°C until required. For long term storage, dimethylsulfoxide (DMSO; Merck, Darmstadt, W.Germany) was added to the stocks at a final concentration of 7%, and the amplified recombinant phage stocks stored in liquid nitrogen

2.5.10 Titration of the recombinant phage stock

Serial tenfold dilutions (10^{-7} - 10^{-12}) of the recombinant phage stock were made with SM buffer. One hundred microlitres of each phage dilution were mixed with 100 ul of Y 1090 plating cells, and the mixture incubated at 37°C for 20 minutes. Five millilitres of molten agar containing 20 ul IPTG and 20 ul Xgal stock solutions were added to each tube, and the phage plated onto dry LB plates. IPTG and Xgal were added to the agar to confirm that the stock was recombinant. Plates were incubated at 37°C overnight, and the numbers of blue and clear plaques counted.

2.6 SCREENING OF THE RECOMBINANT LIBRARY WITH ANTIBODY PROBES

2.6.1. Introduction

λ gt 11 libraries can be screened with antibody probes as plaques on a lawn of E.coli Y1090. Y 1090 contains the following features which make it an ideal host for screening libraries (Huynh, Young, and Davis, 1985):

i. lac repressor, which prevents lacZ-directed gene expression until it is induced by the addition of IPTG to the medium.

ii. a deficiency in the lon protease, which increases the stability of recombinant fusion proteins.

iii. sup F to suppress the phage mutation causing defective lysis (S 100).

To ensure that recombinant proteins toxic to the host will not inhibit the growth of particular members of the library, plaque formation is initiated without expression from the lacZ promoter. After the number of infected cells surrounding the plaques is sufficiently high, lacZ-directed gene expression is induced by the addition of IPTG (Huynh, Young, and Davis, 1985). The IPTG is added by overlaying the bacterial lawn with an IPTG-impregnated nitrocellulose filter. After 3 - 8 more hours of incubation, the filter is removed from the lawn, and proteins from the lysed cells will be bound to the filter. The filter is washed, and incubated with the primary antibody solution. The filter is then incubated with an enzyme-conjugated secondary antibody solution, followed by the enzyme substrate to visualize antibody binding.

Successful immunoscreening depends on the quality of the antibody. High titre antibodies produce better signals than do low titre antibodies (Huynh, Young, and Davis, 1985), and

antibodies that produce strong signals on Western blots (immunoblots) usually also produce good signals in the λ gt 11 screening procedure (Huynh, Young, and Davis, 1985; Snyder, Elledge, Sweetser, et al., 1987). Both monoclonal and polyclonal antisera have been used successfully, but polyclonal antibodies have an advantage over monoclonal antibodies when used as probes, as they are usually not restricted to the recognition of a single epitope on any given protein (Huynh, Young, and Davis, 1985). The ability to detect a variety of epitopes on a protein is important, since genomic inserts will often not be full length and thus only a portion of the polypeptide will be expressed (Snyder, Elledge, Sweetser, et al., 1987). However, polyvalent antibodies often contain components which bind to antigens normally produced by E.coli. In order to avoid this high background, steps must be taken to reduce such non-specific binding. This can be accomplished by immobilising an E.coli lysate on a Sepharose resin or on nitrocellulose filters, and incubating the antibody with the bound bacterial lysate (Huynh, Young, and Davis, 1985). In addition, because the antibody solution can be re-used many times, anti-E.coli components are removed by adsorption every time the serum is re-used, and background binding often progressively decrease (Huynh, Young, and Davis, 1985; Snyder, Elledge, Sweetser, et al., 1987).

2.6.2 Method: Adsorption of anti-E.coli components from anti-M.tuberculosis serum.

The method described by Huynh, Young, and Davis, (1985) was employed to adsorb anti-E.coli components from the serum. The E.coli lysate was prepared by growing E.coli Y 1090 to saturation in 50 ml LB medium at 37^{0}C with good aeration (approximately 4 - 5 hours incubation). The cells were harvested by centrifugation at 1 500 g for 10 minutes at room temperature, and then resuspended in 20 ml 100 mM Tris-HCl, pH 7.5. The resuspended cells were immediately frozen in liquid nitrogen, and then thawed at room temperature. This resulted in lysis of essentially all the cells. The lysate was then sonicated (18 um peak to peak) at 4^{0}C in a MSE Soniprep 150 sonicator to reduce its viscosity. To bind the lysate to the solid phase, 82 mm nitrocellulose filters (Hybond C, 45 um; Amersham International, Little Chalfont, Buckinghamshire, England) were dipped in the lysate, and incubated for 30 minutes at room temperature. The filters were then washed twice, 5 minutes each wash, in TBS (appendix 8), and then blocked by incubating them in 1% BSA in TBS for 15 minutes at room temperature. Filters were then washed as before. The anti-M.tuberculosis and control sera were diluted 1/10 with TBS, and incubated with the filters for 30 minutes at room temperature. The antisera were removed, and incubated with a second batch of filters as before. The antisera were then carefully removed and

stored at 4^0C until required or at -20^0C for long term storage.

2.6.3 Method: Screening of the recombinant library with antibody probes

The method described by Huynh, Young, and Davis, (1985), Davis, Dibner, and Battey (1986), and Snyder, Elledge, Sweetser, et al., (1987) was used to screen the recombinant library. The recombinant library stock was plated out on a lawn of Y 1090 cells: 200 ul of Y1090 plating cells and approximately 1 X 10^3 pfu's of library were mixed, incubated at 37^0C for 20 minutes, and plated with 5 ml LB molten agar onto dry 90 mm LB plates. The plates were incubated at 42^0C for 3-4 hours until the bacterial lawn just became visible. Plates were then overlaid with IPTG-saturated nitrocellulose filters, (Hybond C, 45 um; Amersham International, Little Chalfont, Buckinghamshire, England), pretreated by incubating the filters in a solution of 10 mM IPTG in distilled water for 1 hour, and allowed to air-dry on plastic wrap film. The plates were further incubated at 42^0C for 8-10 hours. At the end of the incubation period, the lids were removed from the plates and the plates incubated at 37^0C for an additional 10 minutes to prevent the agar from sticking to the filters.

The position of the filters on the plates was marked with a

needle, and the filters carefully lifted off the plates. The plates were stored at 4^0C for subsequent reference.

The nitrocellulose filters were briefly rinsed twice in TBS plus 0.01% Tween (appendix 8), and blocked by incubating them in 1 % BSA in TBS (15 ml per filter) for 1 hour at room temperature. Filters were washed 4 times, 5 minutes each wash, with TBS-Tween (15 ml per filter). The filters were then probed with pre-adsorbed anti-M.tuberculosis or control rabbit serum, diluted 1/30 in TBS-Tween, or with anti-β-galactosidase monoclonal antibodies (10 ml per filter) diluted 1/1 000 with TBS-Tween, by incubating at 4^0C overnight. After washing 4 times with TBS-Tween as before, filters were treated with peroxidase-conjugated swine anti-rabbit IgG (Dako Immunoglobulins, Denmark) (10 ml per filter) diluted 1/400 with TBS-Tween, or with peroxidase conjugated rabbit-anti-mouse immunoglobulins (Dako Immunoglobulins, Denmark) (10 ml per filter) diluted 1/200 for 1 hour at room temperature. Filters were washed 4 times with TBS (no Tween), and incubated at room temperature with the enzyme substrate (appendix 5) until positive signals appeared. The reaction was stopped by rinsing the filters with several changes of distilled water. Filters were air-dried, and positive signals aligned with the corresponding plaques on the plates.

Twenty five putative positive plaques were cored from the plates, and each plaque was amplified. Each agar plug containing a plaque was transferred to 0.5 ml of Y 1090 plating cells in a sterile 50-ml centrifuge tube, and mixed vigorously for 30 seconds. The phage was allowed to adsorb at 37^0C for 20 minutes. A further 10 ml of LB broth containing 5 mM $CaCl_2$ were added to each tube, and tubes were incubated at 37^0C with good aeration for 4 hours. Two drops of chloroform were then added to each tube, the tubes shaken at 37^0C for a further 5 minutes, and the bacteria and debris removed by 2 centrifugation steps at 1 500 g for 15 minutes. The phage stocks were titrated on Y 1090 as described in section 2.5.10 and screened again. Amplification and screening was repeated until all the plaques on the plates produced a positive signal.

2.7 PREPARATION OF RAPID LYSATE DNA FROM PHAGE CLONES SHOWING POSITIVE SIGNALS

2.7.1 Introduction

In order to ascertain that the positive phage clones contained insert DNA fragments, and to determine the size of the inserts, the DNA from such clones was isolated, and its Eco R1 restriction patterns analysed.

2.7.2 Phage purification

Phage was purified and the DNA extracted using a modification of the method described by Davis, Botstein, and Roth (1980). Five milliliters of each of the positive recombinant phage stocks were centrifuged at 1 200 g for 190 minutes at room temperature. Centrifuge tubes were previously prepared by soaking them first in ethanol for 30 minutes, and then rinsing them in distilled water for 60 minutes. After centrifugation, the supernatants were immediately discarded, and the translucent phage pellets resuspended in 1 ml TE buffer (appendix 8). Aliquots (0.5 ml) of each of the purified phage preparations were used for the subsequent DNA extractions.

To each of the phage preparations, SDS (10% stock solution, pH 7.2) and EDTA (0.5 M stock solution, pH 8.0) were added to give final concentrations of 0.25% and 10 mM respectively. Proteinase K (200 ug/ml; Boehringer Mannheim, West Germany) was added to each tube, the tubes gently mixed and centrifuged for a few seconds to collect the contents to the bottom. The tubes were incubated at 37^0C for 60 minutes, after which 100 ug/ml RNase (10 mg/ml stock solution in distilled water; Sigma Chemical Co., St.Louis, MO, USA) was added to each tube. The tubes were incubated at 37^0C for a further 40 minutes. An equal volume of phenol/chloroform (1:1) was added to each tube, the

solutions mixed gently for 1 minute, and then centrifuged in a microcentrifuge tube to separate the phases.

The aqueous phases were carefully removed and placed in clean tubes, An equal volume of chloroform/isoamylalcohol (24:1) was added to each tube, the solutions mixed for 1 minute, and then microcentrifuged for 30 seconds. The aqueous phase was removed, placed in small (5 ml volume) glass tubes, and adjusted to 0.1 M sodium acetate, pH 5.5. Three volumes of ice-cold absolute ethanol were slowly added, and the tubes gently inverted several times. The precipitated DNA was spooled out and transferred to 200 ul of sterile distilled water. When the DNA had resuspended, it was quantified spectrophotometrically (appendix 3) and analysed by electrophoresis through a 1% agarose gel (Appendix 1).

2.7.3 Restriction digests of phage DNA

The following reactions were set up on ice for each phage DNA sample:

10 X Eco R1 buffer (appendix 2)	1.5 ul
DNA	0.25 ug
Sterile distilled water	sufficient to bring the final reaction

volume to 12 ul
including the enzyme.

The tubes were gently mixed, and 40 U of Eco R1 (New England Biolabs, Beverly, MA, USA) were added for each reaction. Tubes were briefly centrifuged to collect the contents to the bottom, and the reactions were incubated at 37^0C for 2.5 hours. The Eco R1 was then inactivated by heating the tubes to 70^0C for 10 minutes. Restriction patterns were analysed on 1% agarose gels (Appendix 1).

2.8 PREPARATION OF RECOMBINANT ANTIGENS FROM λgt 11 RECOMBINANT LYSOGENS

2.8.1 Introduction

Lysates containing a particular recombinant antigen can be prepared easily by expressing a λ gt 11 recombinant as a lysogen in E.coli Y 1089 (Huynh, Young, and Davis, 1985). Y 1089 contains :

i. the lac repressor (lacI gene product), which prevents lacZ-directed gene expression until derepressed by the addition of IPTG to the medium;

ii. a deficiency in the lon protease, which increases the stability of the recombinant fusion protein;

iii. a mutation which enhances the frequency of phage lysogeny (hfl A 150).

To produce the recombinant fusion protein, Y 1089 is lysogenised with the recombinant clone of interest. Lysogens usually arise at a frequency between 10% and 70% (Perbal, 1984; Snyder, Elledge, Sweetser, et al., 1987). The lysogen is grown to high cell density, lacZ-directed fusion protein production is induced by the addition of IPTG to the medium, and the cells are harvested and lysed. If crude antigen is required, the crude lysate can be used direct'y. If pure antigen is needed, the β-galactosidase fusion protein can be purified in several different ways (Huynh, Young, and Davis, 1985). The size of the β-galactosidase fusion protein (the β-galactosidase portion of the fusion protein accounts for 114 kDa) enanbles its resolution from other proteins on SDS-polyacrylamide gels. Preparative gels can be used to isolate large quantities of denatured protein. If pure antigen in native form is required, the protein may be purified by classical column chromatography. The availability of monoclonal antibodies to the required antigen makes it possible to purify the antigen using monoclonal antibody affinity columns (Huynh, Young, and Davis, 1985).

2.8.2 Method

2.8.2 i. Generation of recombinant lysogens in Y 1089

Lysogens were generated using the method described by Huynh, Young, and Davis, (1985). Y 1089 cells were grown to saturation at 37^0C overnight in LB medium containing 0.2% maltose. The cells were supplemented with 10 mM $MgCl_2$, and were infected with each of the recombinant phage stocks at a multiplicity of approximately 10: the volume of the cell suspension was kept constant at 100 ul, and the phage stock solution volumes adjusted to obtain the required multiplicity. The phage was adsorbed for 20 minutes at 37^0C, and the cells were then plated onto fresh LB plates at a density of approximately 200 cells per plate. Plates were incubated overnight at 32^0C. At this temperature, the temperature-sensitive phage repressor is functional. Individual colonies from the plates were tested for temperature sensitivity at 42^0C: cells from individual colonies were spotted in duplicate onto each of 2 LB plates; one set of plates was incubated at 32^0C, and the other at 42^0C. Colonies which grew at 32^0C, but not at 42^0C, were assumed to be lysogens.

2.8.2 ii. Preparation of crude lysates from λ gt 11 recombinant lysogens

Aliquots (100 ml each) of LB medium were inoculated with single colonies of the Y 1098 lysogens. Control cultures contained (1) no lysogen (i.e. a colony of non-lysogenised Y1039); (2) no IPTG; (3) no lysogen plus no IPTG; and (4) lysogens of non-recombinant λ gt 11. Cultures were incubated at 32^{0}C with good aeration. When the O.D.$_{600}$ of the cultures reached approximately 0.5, the temperature of the cultures was rapidly raised to 42^{0}C, and the cultures incubated for a further 20 minutes at the elevated temperature. IPTG was added to the cultures to a final concentration of 10 mM, and the cultures incubated at 37^{0}C for a further 60 minutes. The cells were rapidly harvested by centrifugation at 2 500 g for 10 minutes at room temperature, and immediately resuspended, in 1/10 the original culture volume, in 100 mM Tris-HCl, pH 7.5. The resuspended cells were immediately frozen in liquid nitrogen, and lysed by thawing the cells at room temperature. The protein concentration of the lysates was estimated using the Biorad protein estimation kit (Biorad Laboratories, Richmond, CA). Lysates were aliquoted and stored at -20^{0}C until required.

2.9 ANALYSIS OF RECOMBINANT PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS.

Proteins in the lysates were resolved by SDS-PAGE on polyacrylamide slab gels according to the discontinuous buffer system of Laemmli (1970). Lysates were diluted 1:1 in 100 mm Tris-HCl, pH 7.5, and the diluted lysates were prepared for electrophoresis by adding 1/3 the lysate volume splitting solution (2% SDS; 5% β-mercaptoethanol (Sigma Chemical Co. St.Louis, MO, USA); 8 M urea (BDH, Poole, England); 10% sucrose (BDH, Poole, England); 0.1% bromophenol blue (Biorad Laboratories, Richmond, CA) and boiling for 5 minutes. The denatured samples were then applied to 7.5%, 10%, and 15% polyacrylamide gels (appendix 7) at a concentration of approximately 50 ug per lane. Samples were electrophoresed in a Tris-glycine-SDS buffer system (appendix 7), using the Biorad Protean II slab gel apparatus (Biorad Laboratories, Richmond, CA) under conditions of constant current (25 mA/gel). High and/or low molecular weight standards (Pharmacia Fine Chemicals, Uppsala, Sweden) were electrophoresed on each gel for the determination of molecular weight. After completion of electrophoresis, gels were fixed in 15% trichloroacetic acid for 20 minutes, stained overnight in 0.25% Coomassie brilliant blue R 250 (Biorad Laboratories, Richmond, CA) in 45% methanol/7% glacial acetic acid, and destained in 45% methanol/7% glacial acetic acid.

For molecular weight determination, a standard curve of Rf values versus log molecular weight was constructed for the molecular weight protein standards. Rf values were calculated for the proteins of interest, and their molecular weights determined from the standard curve.

2.10 IMMUNOBLOTTING OF RECOMBINANT LYSATES

2.10.1 Introduction

Polyacrylamide gel electrophoresis has become a standard tool for the analysis and purification of proteins. The transfer of proteins onto nitrocellulose membranes is a useful technique for the screening of serum or monoclonal antibodies for specific antibody activity (Lin and Kasamatsu, 1983). The entire process involves the elution of polypeptides onto nitrocellulose, and treatment of the nitrocellulose replica for immunological detection of specific antigens (Towbin, Staehelin, and Gordon, 1979).

2.10.2 Method

Electroblotting was performed essentially as described by Towbin, Staehelin, and Gordon, (1979). Briefly, lysate samples were diluted, split, and electrophoresed on 10% polyacrylamide gels as described in Section 2.9. Once electrophoresis was complete, the gels were equilibrated in

cold (4^{0}C) electroblotting transfer buffer (25 mM Tris; 192 mM glycine; 20% methanol) for 15 minutes, and then blotted onto nitrocellulose sheets (Hybond C, 45 um; Amersham International, Little Chalfont, Buckinghamshire, England). Electroblotting was carried out in Tris-glycine-methanol (25 mM Tris; 192 mM glycine; 20% methanol) transfer buffer, under conditions of constant current and voltage (0.3 A; 70 V) for 3 hours. The Biorad TM Transblot Cell apparatus (Biorad Laboratories, Richmond, CA) was used for electroblotting.

Upon completion of electroblotting, the nitrocellulose sheets were rinsed twice in TBS (appendix 8), 5 minutes each wash, and non-bound sites on the filters were blocked with 1% BSA for 1 hour at room temperature, or overnight at 4^{0}C. The blots were washed 4 times with TBS, 5 minutes each wash, and probed with pre-adsorbed rabbit anti-M.tuberculosis serum (diluted 1/30 with TBS) or anti-β-galactosidase monoclonal antibodies (diluted 1/1 000 with TBS) for 2 hours at room temperature with gentle agitation. After washing with TBS, horseradish peroxidase-labelled swine anti-rabbit IgG (Dako Immunoglobulins, Denmark), diluted 1/400 with TBS, or peroxidase-labelled rabbit anti-mouse immunoglobulins (Dako Immunoglobulins, Denmark), diluted 1/200 with TBS, were added for 1 hour at room temperature. Blots were gently agitated during incubations. The blots were washed a final 4 times, and then incubated in the enzyme substrate solution

(appendix 5) at room temperature. When colour had developed (after approximately 30 minutes incubation), the blots were rinsed several times with distilled water, and dried on blotting paper at room temperature. The blots were then compared to their corresponding stained gels to determine the size of the bands to which the antisera bound.

CHAPTER 3 RESULTS

3.1 DETECTION OF ANTIBODIES TO M.tuberculosis

Antibodies to sonicated M.tuberculosis antigens were prepared in 3 rabbits, and tested for recognition of M.tuberculosis antigens using a standard ELISA assay. Serum from each of the 3 rabbits was found to recognise mycobacterial antigens in the ELISA. Pooled serum from normal saline-immunised rabbits did not react to these mycobacterial antigens (Table 5).

In order to establish that antibodies from immunised rabbits could detect specific mycobacterial antigens, the M.tuberculosis sonicate was fractionated by SDS-PAGE, the proteins transferred to nitrocellulose by Western blotting, and reacted with the anti-M.tuberculosis antibodies. Figure 4 demonstrates the results from such experiments. The M.tuberculosis sonicate revealed several protein bands, with 2 major protein bands of molecular weights 65 kDa and 43 kDa - 45 kDa. The sera raised against the mycobacterial sonicate showed a strong positive reaction to both the 65 kDa and 43 kDa - 45 kDa antigens. Control serum from saline-immunised rabbits did not react with any of these antigens.

Since the anti-M.tuberculosis serum effectively recognised several mycobacterial antigens immobilised on nitrocellulose, it was considered suitable for use in screening the recombinant λ gt 11 library for phage clones which produced specific mycobacterial proteins.

TABLE 5 ELISA to detect antibodies to antigens derived from sonicated M.tuberculosis

Specimen	$O.D._{492}$ value	Significantly different from control
anti-M.tuberculosis serum		
rabbit 1	0.318 + 0.097	yes ($p < 0.01$)
rabbit 2	0.349 + 0.056	yes ($p < 0.001$)
rabbit 3	0.532 + 0.056	yes ($p < 0.001$)
control rabbits (pooled sera from 3 rabbits)	0.096 + 0.061	

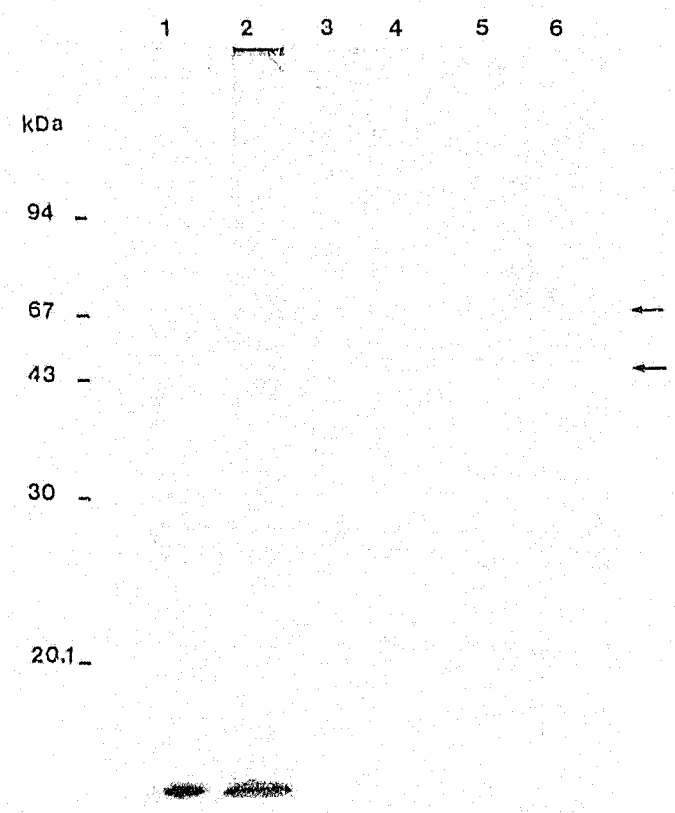


FIGURE 4 SDS-PAGE electrophoresis and Western blotting of whole M.tuberculosis sonicate.

Lane 1: molecular weight markers (kDa); lane 2: M.tuberculosis sonicate (100 ug/lane); lane 3: immunoblot of M.tuberculosis sonicate incubated with pooled negative control rabbit serum; lanes 4, 5, and 6: immunoblots of M.tuberculosis sonicate, incubated with anti-M.tuberculosis serum from rabbit 1 (lane 4), rabbit 2 (lane 5), and rabbit 3 (lane 6). The major mycobacterial protein bands recognised by the anti-M.tuberculosis antibodies are marked with an arrow (←).

3.2 EXTRACTION OF M.tuberculosis DNA

DNA was extracted from M.tuberculosis organisms obtained from sputum cultures. After phenol/chloroform extraction, the DNA was precipitated with absolute ethanol. Any remaining protein or RNA was removed enzymatically, and the DNA was re-extracted, precipitated, and resuspended in TE buffer, pH 8.0. The concentration and purity of the preparation was assessed spectrophotometrically and by agarose gel electrophoresis. The concentration of the DNA was found to be approximately 0.35 mg/ml. An $O.D._{260}$ /$O.D._{280}$ value of 1.75 was obtained for the preparation, indicating that it was pure and free from contaminants.

The electrophoretic pattern of the DNA through a 0.7% agarose gel is shown in Figure 5. Although the gel is somewhat overloaded, clean high molecular weight M.tuberculosis DNA, containing no detectable degraded DNA or RNA (which migrate faster than high molecular weight DNA) can be seen (lane 2). The sheared M.tuberculosis DNA ranged in size from approximately 10 kb to 0.5 kb, with the majority of the DNA concentrated in the 3 kb - 6 kb range (lane 3). λ gt 11 left and right arms, the sizes of which are 19.6 kb and 24.1 kb respectively, were also run on the gel (lane 4), and ran as a single band of approximately 20 kb in size.

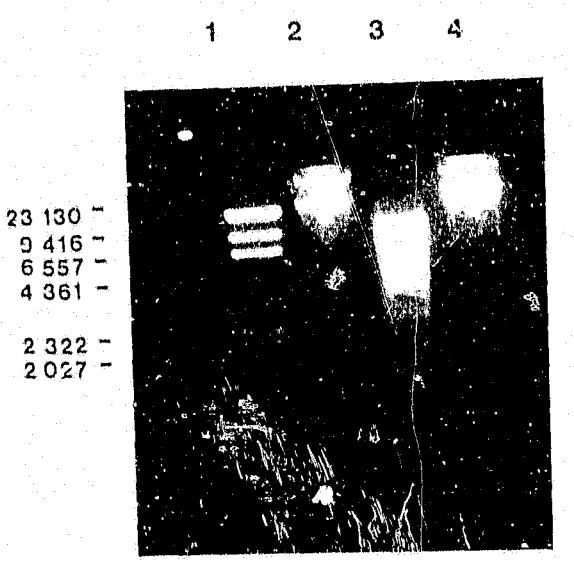


FIGURE 5 Agarose gel electrophoresis of M.tuberculosis and λ gt 11 DNA.

The DNA was isolated as described in the text, and electrophoresed through a 0.7% agarose gel. The sizes (base pairs) of the DNA molecular weight markers (lane 1) are indicated on the left. Lane 2 - M.tuberculosis DNA, 2.5 ug; lane 3 - mechanically sheared M.tuberculosis DNA, 2.5 ug; lane 4 - λgt 11 arms, 100 ng.

3.3 CONSTRUCTION OF THE RECOMBINANT LIBRARY

3.3.1 Methylation and size fractionation of DNA

Internal Eco R1 sites in the M.tuberculosis DNA and the control DNA (a 3 kb plasmid fragment, obtained commercially) were methylated as described in Section 2.5.3 ii. Synthetic Eco R1 linkers were covalently attached to the DNA, and excess linkers were then removed by size fractionation of the DNA through gel filtration columns. The results of the fractionation are shown in Figure 6. M.tuberculosis DNA eluted from the column in fractions 4 and 5. These 2 fractions were pooled and set aside for further studies, and other remaining fractions collected from that column were discarded. Since the size of the control DNA was slightly smaller than the average size of the M.tuberculosis DNA, it was expected to elute from the column slightly later than the M.tuberculosis DNA. The control DNA was fractionated on a separate column and eluted in fractions 5 and 6. These 2 fractions were pooled and kept for further investigations, and all other fractions discarded. Excess Eco R1 linkers, which, due to their very small size, were expected to elute in the final fractions from both columns (fractions 7 to 10), are not visible in the agarose gel, since their small size precludes their being detected under ultraviolet light.

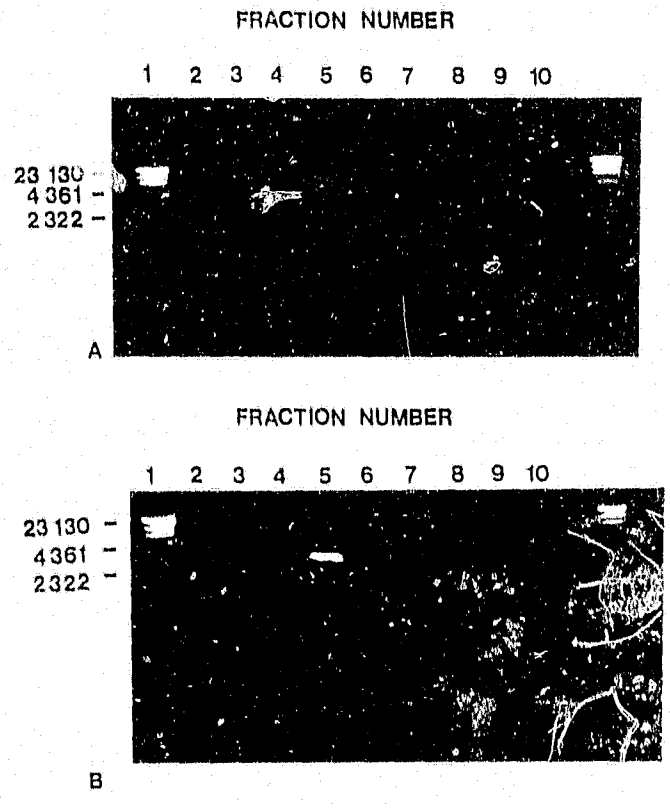


FIGURE 6 Agarose gel electrophoresis of fractionated DNA eluted from gel filtration columns.

Plate A - fractionated, double-stranded Eco RI-ligated *M.tuberculosis* DNA. The major DNA peaks can be seen in fractions 4 and 5.

Plate B - control DNA. The DNA peaks can be seen in fractions 5 and 6. The position and sizes of the molecular weight markers (base pairs) are indicated on the left.

3.3.2 Ligation of the DNA to λ gt 11 arms

Ligation reactions were set up as described in Table 4. A 1 ul aliquot was removed from each ligation reaction prior to the addition of the ligase enzyme, and another following completion of the reaction. Both sets of aliquots were electrophoresed through a 0.7% agarose gel to determine whether successful ligation had taken place. Successful ligations are indicated by the presence of DNA of at least the size of intact λ DNA (approximately 40 kb). The presence of DNA of molecular weight lower than that of the λ arms indicates that ligation between the insert DNA and the λ arms has not occurred. Electrophoretic analysis of the ligation reactions can be seen in Figure 7. M.tuberculosis and control DNA can be seen before the addition of the ligase enzyme (Plate A; not clear on photographing); after the addition of the enzyme, only DNA of high molecular weight is visible (Plate B). The results indicate that most of the insert DNA had ligated to the λ gt 11 arms to produce recombinant DNA of higher molecular weight.

The ligated reactions were therefore packaged as described in Section 2.5.7 ii.

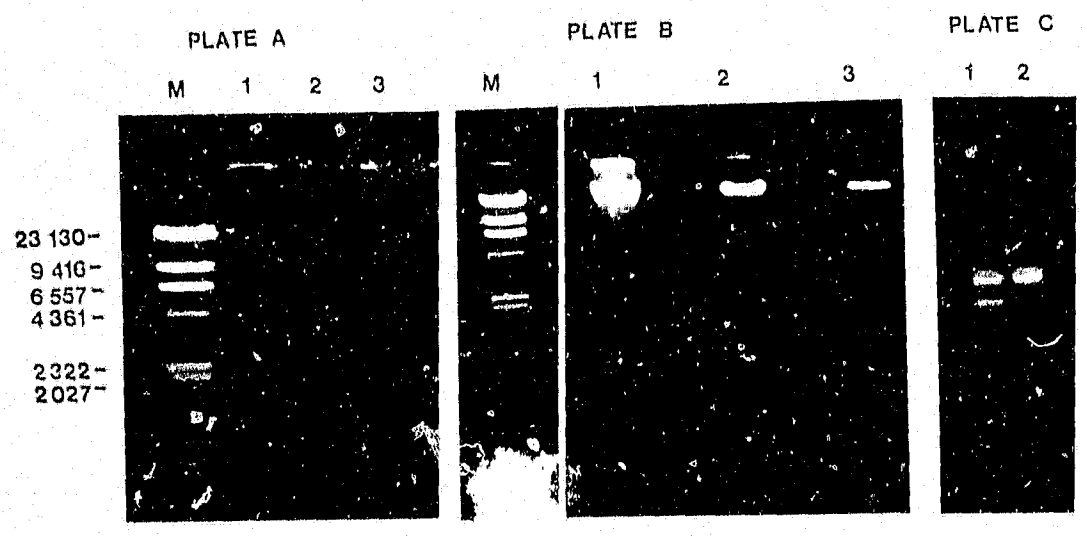


FIGURE 7 Electrophoretic analysis of the λ gt 11 arms/ insert DNA ligations.

Plate A: DNA before the addition of the ligase enzyme - lane 1, λ gt 11 arms only; lane 2, λ gt 11 arms and 100 ng *M.tuberculosis* DNA; lane 3, λ gt 11 arms and 200 ng *M.tuberculosis* DNA (faint smear).

Plate B: the same reactions after the addition of the ligase enzyme - high molecular weight DNA only can be seen in lanes 1. 2 and 3.

Plate C: λ gt 11 and positive control insert before the addition of the ligase (lane 1) and after completion of ligation (lane 2).

The positions and sizes of the molecular weight markers (M; base pairs) are indicated on the left.

3.4 TITRATION OF RECOMBINANT LIBRARIES

The packaged libraries were titrated on E.coli Y 1088 (λ gt 11 libraries) or E.coli LE 392 (packaging control), using LB agar plates containing IPTG and Xgal (Figures 3, 9, and 10). Both the total number of plaques per plate (blue and clear plaques) and the number of recombinant plaques (clear plaques only) were stored. Titres were expressed as pfu/ml and pfu/ug DNA (Table 6). The percentage of plaques which were recombinant was calculated by dividing the recombinant titre (clear plaques only) by the total library titre (clear and blue plaques), and multiplying the result by 100.

The titres of all the reactions were analysed to determine whether the generation of a recombinant library had been successful. The M.tuberculosis and positive control DNA library titres were compared to the titres for the control reactions. Titres from the latter reactions reflect the efficiency of the cloning and packaging as a whole. As can be seen in Table 6, no phage were produced by the packaging extracts alone, whereas the packaging control reaction produced a high titre (10×10^8 pfu/ml) of phage. When λ gt 11 arms alone were packaged, a titre of 2×10^4 pfu/ ml was obtained, in contrast to the 100-fold increase in the phage titre when the positive control DNA was used (Table 6, reactions 3 and 4). A recombinant titre of 2.6×10^4 pfu/ml was obtained when 0.1 ug of M.tuberculosis DNA was ligated to

TABLE 6 Titration of λ gt 11 recombinant libraries

Reaction number	λ DNA	Insert	Library titre * pfu/ml	Library titre * pfu/ug DNA	recombinant titre * pfu/ml	recombinant titre * pfu/ug DNA	percentage recombinants
1 packaging extract only	-	-	0	0	0	0	0
2	-	packaging control DNA 2.5 ug	10×10^8	2×10^8	10×10^8	2×10^8	100
3	λ gt 11 arms 1 ug	-	2×10^4	1×10^4	1.6×10^2	80	0.8
4	λ gt 11 arms 0.5 ug	positive control DNA 0.2 ug	3×10^6	7.5×10^6	2.8×10^5	7.1×10^6	94
5	λ gt 11 arms 1 ug	M.tuberculosis DNA 0.1 ug	2×10^5	1×10^6	2.6×10^4	1.28×10^5	13
6	λ gt 11 arms 1 ug	M.tuberculosis DNA 0.2 ug	2×10^5	5×10^5	1.27×10^5	3.2×10^5	63.5

* - Results shown are means of three separate titration experiments

λ gt 11 arms. Increasing the concentration of mycobacterial DNA for ligation from 0.1 ug to 0.2 ug did not increase the library titre, but significantly increased the percentage of recombinant phage in the library (Table 6, reactions 5 and 6) from 13% to 63 %.

Comment

[illegible] plaques were expected to be produced by the packaging extracts alone (Table 6, reaction 1), since no packageable DNA was present. This absence of plaques also indicates that no backgr[illegible] plaques were produced by the extracts themselves.

The high titre for the packaging control reaction (10 X 10^8 pfu/ml) indicates that the packaging extracts were functioning well and were able to package a high concentration of DNA and to maximise phage yields.

A low library titre of 2 X 10^4 pfu/ml with λ gt 11 arms alone indicates that the arms were able to ligate successfully, and that a small number of ligated λ gt 11 arms were packaged. This low background titre was expected.

The positive control insert titre was high - 3 X 10^6 pfu/ml -, and approximately 100-fold greater than the titre for the λ gt 11 arms alone, indicating that the cloning system as a whole was functioning efficiently.

The titre for the library constructed using 0.1 ug of M.tuberculosis DNA was high - 2 X 10^5 pfu/ml. This titre was only 10- fold lower than the titre obtained using the positive control insert DNA under optimal conditions of size, concentration and intact DNA; this is indicative of efficient cloning of the M.tuberculosis DNA. A dramatic increase in the percentage of recombinant phage titres when the concentration of ligated M.tuberculosis DNA was increased from 0.1 ug to 0.2 ug indicates that although maximum ligation capacity was obtained using a ratio of 0.1 ug of DNA to 1 ug of λ gt 11 arms, the selection of DNA inserts for ligation was increased, thus resulting in an increased recombinant library titre.

For a more accurate comparison of the library titres with known or optimal titres of λ gt 11 libraries, the titres were calculated as pfu/ug DNA (the weight of the DNA being that of the insert DNA, or of the λ gt 11 arms alone where no insert was added) (Table 6). When calculating the titres as pfu/ug DNA, the total packaged library volume of 500 ul per library was taken into account.

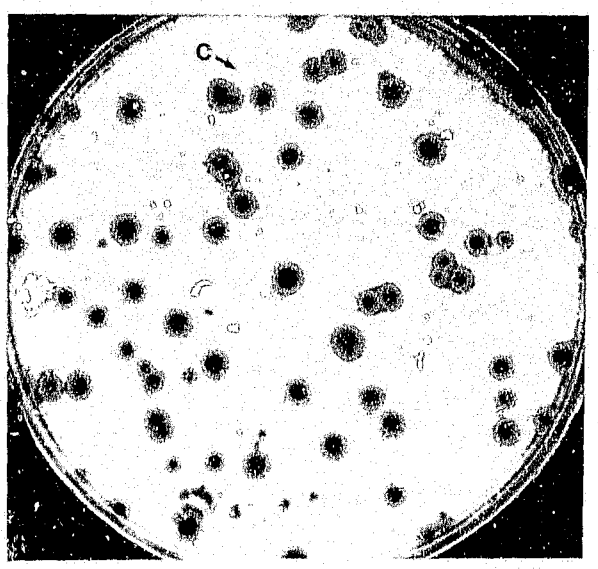


FIGURE 8 Ligated and packaged λ gt 11 arms, containing no inserts, plated on E.coli Y 1088.

, oximately 99% of the phage appear not to contain inserts (blue plaques) when plated in the presence of IPTG and Xgal. One clear (C) plaque can be seen.

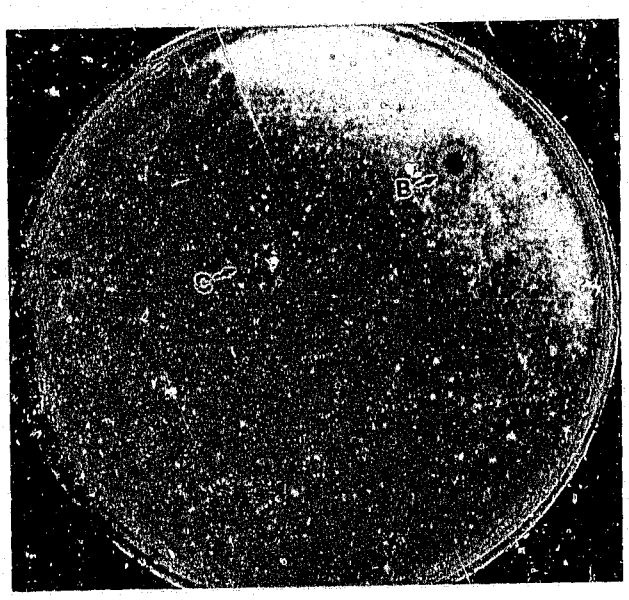


FIGURE 9 Ligated and packaged λ gt 11 arms and a positive control insert (3 kb fragment of plasmid DNA).

Phage were plated on Y 1088 in the presence of IPTG and Xgal. The majority of the phage contain inserts (clear plaques - C). One non insert-bearing phage plaque (B) can be seen



A

B

FIGURE 10 λ gt 11 library constructed with 100 ng, (plate A), and 200 ng (plate B) M.tuberculosis DNA.

Phages were plated on a lawn of E.coli Y 1088 in the presence of IPTG and Xgal. Clear plaques (C) were produced by insert-bearing recombinant phage. Blue plaques were produced by non insert-bearing phage.



FIGURE 11 Amplified stock of the λ gt 11 library constructed with 200 ng M.tuberculosis DNA.

Phage were plated on a lawn of E.coli Y 1090 in the presence of IPTG and Xgal. The majority of the plaques (> 90%) are clear, indicating that they are produced by insert-bearing recombinant phage.

3.5 AMPLIFICATION OF THE RECOMBINANT LIBRARY

The 0.2 ug *M.tuberculosis* library, which provided the greatest percentage of recombinant phage, was then amplified through *E.coli* Y 1088, and recombinant phage stocks were prepared using small-scale liquid culture. The titre of the resultant phage stock, determined as described in Section 2.5.10, was 10^{11} pfu/ml, indicating good library amplification and a resultant library sufficiently amplified for plaque screening with antibody probes. This amplified library consisted of essentially greater than 90% recombinants when plated on plates containing IPTG and Xgal (Figure 11).

3.6 SCREENING OF THE RECOMBINANT M.tuberculosis LIBRARY WITH ANTIBODY PROBES

The amplified recombinant M.tuberculosis library was screened for the presence of phage which produced M.tuberculosis antigens. Preadsorbed anti-M.tuberculosis serum and anti-β-galactosidase antibodies were used for this purpose. Seventy five filters (82 mm diameter), each containing antigen from approximately 1 X 10^3 individual plaques, were screened. Of these, 60 filters were incubated with immune rabbit serum, and 15 with anti-β-galactosidase antibodies. Nine putative positive signals were observed when filters were screened with the immune rabbit serum, and 13 putative positive signals were obtained with the anti-β-galactosidase monoclonal antibodies (Figure 12).

The signal-producing plaques on each plate were then located by comparing the background pattern produced by neighbouring plaques on each nitrocellulose filter with the pattern of plaques on the corresponding agar plates. Each signal-producing plaque was then amplified and screened again to ensure its clonality. The results of a representative experiment with clone TB 12 are shown in Figure 13 - positive signals are more numerous than those produced after the primary screening. The amplified stocks were then re-amplified until all the plaques produced a positive signal (data not shown). Of the 22 clones producing putative positive

signals, 7 again produced positive signals after amplification and screening with immune rabbit serum, and 3 produced positive signals when re-screened with anti-β-galactosidase antibodies.

The phage stocks that produced positive signals when screened with immune rabbit serum were then re-screened with anti-β-galactosidase antibodies. Similarly those plaques producing positive signals when screened with anti-β-galactosidase antibodies were re-screened using immune serum. Of these phage stocks, only 1 reproduced a positive reaction when screened with both antibodies.

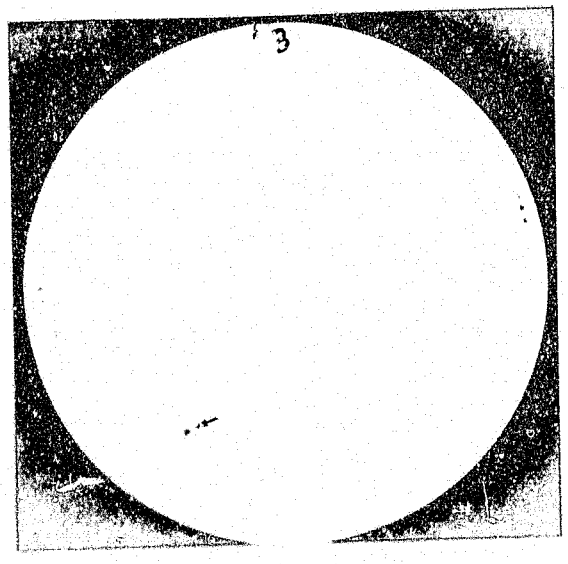
The combined results of the screening procedures are tabulated in Table 7: clones TB 6, TB 13, TB 14, TB 15, TB 16, TB 18, TB 19, and TB 23 produced positive signals when screened with immune rabbit serum. Of these, all but clones TB 13 and TB 19 again produced positive signals when amplified and re-screened. Clones TB 2, TB 3, TB 7, TB 8, TB 9, TB 10, TB 11, TB 12, TB 18, TB 20, TB 21, TB 22, TB 24, and TB 25 produced positive signals when screened with anti-β-galactosidase antibodies. Of these, only clones TB 3, TB 12, and TB 18 again produced positive signals after amplification and re-screening. Clone TB 18 produced positive signals when screened with both the anti-M.tuberculosis serum and the anti-β-galactosidase antibodies. All the clones which produced positive signals when re-screened, viz. clones TB 3, TB 6,

TB 12, TB 14, TB 15, TB 16, TB 17, TB 18, and TB 23 were used for further investigation.

It was presumed that the phage clones which did not produce positive signals after re-isolation and amplification subsequent to the initial screening were producing false-positive signals initially, and these phage stocks were thus discarded. In order to eliminate the possibility of false positive signals arising due to non-specific binding of the rabbit or mouse antibodies to phage or E.coli proteins, the 10 positive phage clones were also screened with normal rabbit or mouse serum (negative control mouse ascites fluid was not available). No positive signals were obtained for any of these clones (results not shown).

TABLE 7 Immunoblotting analysis of recombinant phages

Phage clone number	Recognition by anti-*M.tuberculosis* immune serum		Recognition by anti-β-galactosidase antibodies	
	primary screen	amplified library	primary screen	amplified library
TB 2			+	
TB 3			+	+
TB 6	+	+		
TB 7			+	
TB 8			+	
TB 9			+	
TB 10			+	
TB 11			+	
TB 12			+	+
TB 13	+			
TB 14	+	+		
TB 15	+	+		
TB 16	+	+		
TB 17	+	+		
TB 18	+	+	+	+
TB 19	+			
TB 20			+	
TB 21			+	
TB 22			+	
TB 23	+	+		
TB 24			+	
TB 25			+	



A

B

FIGURE 12 Identification of recombinant phages from the M.tuberculosis recombinant library.

The library was probed with anti-M.tuberculosis antibodies (plate A) and anti-β-galactosidase antibodies (plate B). Positive signals are seen as dark spots (←) over the background pattern of plaques.

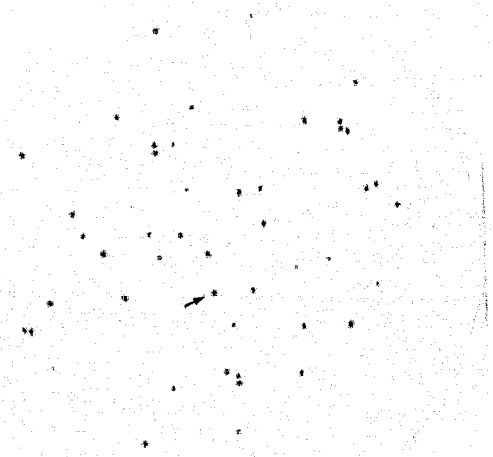


FIGURE 13 First re-isolation and amplification of recombinant phage stock TB 12 probed with anti-β-galactosidase antibodies.

Positive signals (←) can be seen over the background pattern of plaques.

3.7 ANALYSIS OF THE DNA OF RECOMBINANT PHAGE CLONES

DNA from positive phage clones (when screened with both anti-*M.tuberculosis* and anti-β-galactosidase antibodies) was extracted as described in Section 2.7.1, and analysed for the presence of inserts by Eco R1 digestion and agarose gel electrophoresis. DNA inserts were identified in each of the 10 positive recombinant phage stocks analysed (Figure 14). The results of this analysis are tabulated in Table 8. Of the clones assessed, clones TB 3, TB 6, TB 9, TB 12, TB 14, and TB 16 each contained inserts of 4 000 bp, 1 900 bp, 2 000 bp, 4 200 bp, 3 800 bp, and 2 400 bp respectively. Clones TB 15, TB 17, TB 18, and TB 23 were found to contain more than one insert (Table 8).

Comment

The results indicating the presence of more than one insert following digestion could be explained by the fact that the mycobacterial DNA inserted in these clones may have contained internal Eco R1 sites; these sites were protected by the methylation reaction described in Section 2.5.3. Restriction of the DNA at these sites may have resulted in the generation of several small fragments of DNA. The number of small fragments produced depends on the number of Eco R1 restriction sites contained in the original inserted DNA.

In addition to the small DNA fragments, clones TB 15, TB 17, TB 18, and TB 23 also contained maximum length inserts approximately 8 000 bp in size (λ gt 11 can accommodate up to 8.3 kb of insert DNA, assuming a maximum packageable phage DNA length of 52 kb; Young and Davis, 1983). The presence of the entire maximum length insert DNA, in addition to several smaller DNA fragments after restriction, indicates that although some of the insert DNA was restricted, some of the insert DNA may have been unaffected by the restriction endonuclease. This would then result in the presence of maximum length inserts, in addition to several smaller insert DNA fragments.

The 20 kb band present in each recombinant clone (Figure 14) represents the λ gt 11 arms, which are 19.6 kb (left arm) and 24.1 kb (right arm) in size. In addition to the inserts of 8 000 bp or less, clones TB 15, TB 17, and TB 18 also contained inserts ranging in size from 10 000 to 18 000 kb: the presence of such large fragments may be explained by the fact that the λ arms of these clones may have sustained some damage during the extraction or restriction procedures: these bands would therefore represent damaged λ gt 11 arms. Alternatively, the presence of phage clones lacking one of the Eco R1 sites flanking the inserted DNA could also explain the presence of these large fragments.

TABLE 8 Analysis of the sizes of the DNA inserts in the recombinant clones*

Recombinant phage clone number	Size of insert DNA (base pairs)
TB 3	4 000
TB 6	1 900
TB 9	2 000
TB 12	4 000
TB 14	3 800
TB 15	8 000 4 000
TB 16	2 400
TB 17	12 600 10 000 6 300 5 000 1 900
TB 18	12 600 8 000 4 500
TB 23	8 000 5 300 2 600

* - DNA from the clones which produced positive signals when treated with anti-M.tuberculosis and anti-β-galactosidase antibodies was analysed.

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