An investigation into the molecular genetics of cell wall biosynthesis in *Mycobacterium leprae*

Lucy Anna Brooks

A thesis submitted for the degree of Doctor of Philosophy in the University of London
February 1996

Department of Clinical Sciences
London School of Hygiene & Tropical Medicine
Keppel Street
London
WC1E 7HT

NUMEROUS ORIGINALS
IN COLOUR

ABSTRACT

The mycobacterial cell envelope is a complex structure the components of which have been implicated in the survival, virulence, permeability and resistance to antibiotics of *Mycobacterium*. The elucidation of the synthesis of the cell envelope would improve the understanding of the structure and its influential properties and could lead to the identification of drug target sites. The isolation of genes encoding proteins involved in the biosynthesis of the cell envelope structures and their mutants would enable the biosynthetic pathways and functions of individual structures to be determined.

The objective of this project was to isolate genes encoding cell envelope structures in *Mycobacterium leprae*, using *Mycobacterium smegmatis* as model organism. The aim was to isolate an *M. smegmatis* strain with a mutant cell envelope and to complement the mutation using a genomic library of *M. leprae*.

M. smegmatis mc^2155 was successfully mutagenised using *N*-methyl-*N*-Nitro-*N*-nitrosoguanidine (NTG), generating 0.1-0.2% auxotrophic mutants. A bank of 2,000 NTG-treated *M. smegmatis* strains were screened for alterations in phenotypes that may have reflected a change in the cell envelope i.e. mycobacteriophage resistance, temperature sensitivity and increased resistance and sensitivity to antibiotics. *M. smegmatis* strains with increased resistance to ofloxacin and ciprofloxacin (15) were isolated along with strains more sensitive to penicillin G(5) and pyrazinamide (1).

The pyrazinamide sensitive mutant, PyramidII was further characterised and found to be 20% more sensitive to pyrazinamide than the *M. smegmatis* mc^2155 wild type strain. PyramidII was found to be less hydrophobic than the wild type strain, variably more sensitive to penicillin G and to exhibit a smooth colony morphology. The cell wall components of Pyramid II were analysed but no gross differences were observed in comparison to the wild type *M. smegmatis* strain. PyramidII is believed to contain a mutation effecting its permeability to pyrazinamide.

PyramidII was transformed with an *M. leprae* genomic pYUB18 shuttle vector library and complementing clones isolated (6%). A complementing cosmid, 57, found to map to cosmid B1308 in the ordered *M. leprae* library, was used to create a sub-library in pMV206 from which a 3.5kb fragment of complementing *M. leprae* DNA was isolated and found to contain three complete putative coding regions, possibly involved in osmoregulation.

CONTENTS

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

aa-	Amino acids
APS-	Ammonium persulphate
ATP-	Adenosine 5'-triphosphate
bp-	base pairs
b.p.-	boiling point
CTP-	Cytidine 5'-triphosphate
Da-	Dalton
dATP-	Deoxyadenosine 5'-triphosphate
DMF-	Dimethylformamide
DMSO-	Dimethylsulphoxide
DNA-	Deoxyribonucleic acid
DNAase-	Deoxyribonuclease
dNTPs-	Doexynucleoside 5'-triphosphates
DTT-	Dithiothreitol
EDTA-	Ethylenediaminetetraacetic acid
GTP-	Guanosine 5'-triphosphate
HPLC-	High performance liquid chromatography
INH-	Isonicotinic acid hydrazide (Isoniazid)
IPTG-	*iso*-propyl-β–D-thiogalactopyranoside
Kan-	Kanamycin
kb-	Kilobase
kDa-	Kilodalton
mAb-	Monoclonal antibody
Mb-	Megabase
MIC-	Minimum inhibitory concentration
MOPS-	4-morpholinepropanesulphonic acid
mRNA-	Messenger ribonucleic acid
NADH-	Nicotinamide adenine dinucleotide
NADP-	Nicotinamide adenine dinucleotide phosphate
nt-	Nucleotide
NTG-	N-methyl-N'-nitro-N-nitrosoguanidine
ORF-	Open reading frame
PCR-	Polymerase chain reaction
PCR-SSCP	Polymerase chain reaction-single stranded conformation polymorphism
PTFE-	Polytetrafluoroethylene
rBCG-	Recombinant *M.bovis* BCG
RNA-	Ribonucleic acid
RNAase-	Ribonuclease
rRNA-	Ribosomal ribonucleic acid
SDS-	Sodium dodecyl sulphate
TEMED-	N,N,N',N'-tetramethylethylenediamine
TLC-	Thin layer chromatography
Tris-	2-amino-2-hydroxymethylpropane-1,3-diol
tRNA-	Transfer ribonucleic acid
ts-	Temperature sensitive
Xgal-	4-bromo-3-chloro-2-indolyl-β-galactoside
UTP-	Uridine 5'-triphosphate
UV-	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 THE MYCOBACTERIA

The mycobacteria are actinomycetes and are closely related to *Nocardia*, *Corynebacterium* and *Rhodococcus*. They are Gram-positive, aerobic, rod-shaped organisms, identified by their acid-fastness, whose genomes are 2-5x10^9Da in size with a G+C content of 58-69% (Clark-Curtiss, 1990). The majority of mycobacteria are saprophytic soil dwelling bacteria i.e. *Mycobacterium smegmatis* and *Mycobacterium avium*, but four members of the genera *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis* and *Mycobacterium leprae* are obligate pathogens. The mycobacteria can be grouped into two classes, the fast-growers and the slow-growers. The fast-growers which grow in less than seven days and are generally environmental bacteria e.g. *Mycobacterium chelonae*. The slow-growers require 2-4 weeks for growth and include the pathogens. These grouping were reinforced by the conserved 16SRNA sequence data obtained for slow and fast-growing mycobacteria (Clark-Curtiss, 1990).

The slow-growers are further divided into nonphotochromogenic species i.e. the *M. tuberculosis* complex and *M. avium* complex and the photochromogenic species. The members of the *M. tuberculosis* complex are the causative agents of tuberculosis, *M. tuberculosis*, *Mycobacterium bovis* and *Mycobacterium africanum*, members of this complex are slow-growing, acid-fast bacilli that display 95% DNA/DNA homology with each other. *M. tuberculosis* can be distinguished as it is niacin and nitrate reductase positive. The *M. avium* complex cause pulmonary and nonpulmonary infections, primarily in the immunocompromised and is composed of *M. avium* and *Mycobacterium intracellulare* although their DNA/DNA homology clearly indicated they are different species. The members of this complex are niacin and urease-negative and hybridise to MAC specific insertion element DNA probes (McFadden, 1990).

The slow-growing photochromogenic species are further subdivided into Runyon group I, which produce pigment on exposure to light e.g. *Mycobacterium kansasii* and *Mycobacterium*

marinum and Runyon group II species which produce pigment in the dark e.g. *Mycobacterium gordonae*.

Fast-growing mycobacteria include the *Mycobacterium fortuitum* complex and *M. smegmatis*. The members of the *M. fortuitum* complex cause the most human disease out of the fast growers. The complex consists of *M. chelonae*, *Mycobacterium peregrinum*, and *Mycobacterium abscessus*.

Mycobacterium leprae is a straight rod bacillus of 1-8μm long by 0.3μm in diameter, the causative agent of leprosy and is unculturable *in vitro*, it has therefore been classed in a group alone. *M. leprae* is detected using specific antibodies, DNA probes and the identification of acid-fast bacilli (reviewed by Shinnick, 1994).

1.2 THE MYCOBACTERIAL CELL ENVELOPE

A simplified model of the mycobacterial cell envelope is shown in Fig 1., this model was initially based on the chemical properties of individual cell wall components and has subsequently been borne out by other experimentation i.e. x-ray crystallography and electron microscopy (EM) (Minnikin, 1982; Nikaido *et al.*, 1993; Brennan and Nikaido, 1995). The mycobacterial plasma membrane is surrounded by a cell wall, thought to be an asymmetrical bilayer, the major components of which are lipid and polysaccharide.

The major lipid components of the cell wall are the characteristic mycolic acids, 70-90 carbons long, these are only found in other actinomycetes e.g. *Corynebacterium* and *Nocardia* where they are 40-60 carbons long. The major mycobacterial polysaccharides are peptidoglycan, which is of chemotype IV, and the unique arabinogalactan.

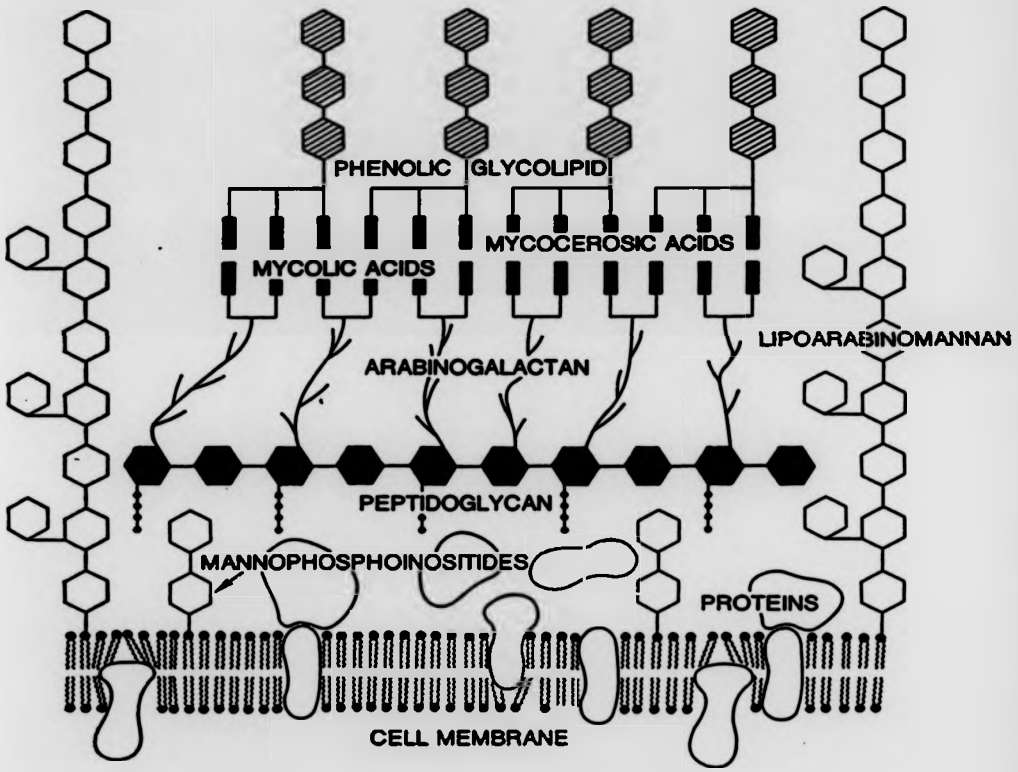


Fig.1. **A cartoon depiction of the cell envelope of *Mycobacterium leprae*** (This picture was taken from Brennan, 1989).

The cell wall consists of peptidoglycan covalently linked to arabinoglactan to which mycolic acids are covalently bound to form a surrounding monolayer and creating the mycolylarabinogalactan-peptidoglycan complex, mAGP. The mAGP complex, or covalent skeleton of the cell wall, is believed to make up the inner layer of the cell wall bilayer. The mycolates are arranged with their main and branched chains parallel to each other and perpendicular to the plane of the cell surface and the peptidoglycan (Brennan and Nikaido, 1995). The mycolates therefore produce a close packed inner structural permeability barrier (Nikaido *et al.*, 1993; Besra *et al.*, 1995). The inner leaflet of mycolic acids is in turn believed to be covered by an outer leaflet of variable intercalating extractable lipids. The whole structure thus producing an asymmetrical lipid bilayer (Fig.1). The extractable lipids are species-specific glycolipids and peptidolipids, the main variables in an otherwise fairly generic cell wall. The other dominant cell wall component is lipoarabinomannan, LAM, which is believed to be anchored in the plasma membrane and to span the cell wall; it is analogous to the lipopolysaccharide (LPS) of Gram-negative bacteria. The cell wall also contains intercalating proteins and porins which span the cell wall. The cell wall is in fact more analogous to the outer membranes found beyond the peptidoglycan in the cell walls of Gram-negative bacteria (Brennan and Nikaido, 1995).

1.2.1 CELL ENVELOPE COMPONENTS

The mycobacterial cell envelope is composed of numerous individual components of varying complexity, many of which are lipid based. As a result the cell envelope is 30 to 60% lipid, depending on the growth environment (Ratledge, 1982). The lipids range from non polar waxes to sugar and amine-containing polar lipids. Mycobacteria contain a number of novel lipid components unobserved in most bacteria other than actinomycetes (Minnikin and Goodfellow, 1980; Minnikin, 1982) e.g. phosphatidylinositol mannosides (PIMs), tuberculostearic acid and mycolic acids. Other conventional lipids e.g. isoprenoids, quinones, carotenoids and membrane polar lipids are also found in mycobacteria. Polysaccharides and

proteins are the other main components of the cell wall.

The major and unique lipid, polysaccharide and protein components of the mycobacterial cell wall and their location within the cell envelope are discussed below.

1.2.1.1 The Plasma membrane

The plasma membrane of the mycobacterial cell envelope is a typical phospholipid bilayer, which appears asymmetrical when observed by EM (Paul and Beveridge, 1992). Other components such as proteins are integrated primarily into the outer layer of the membrane, hence its increased diameter. Some components of the plasma membrane are discussed below.

1.2.1.1.1 Phospholipids

These are the most common polar lipid types in mycobacteria and are generally phosphodiacylglycerol derivatives of phosphatidic acid. Phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and PIMs are the most common. They are all amphipathic, being composed of polar phosphatidic heads with long chain fatty acids of less than 20 carbons esterified to C-1 and C-2 (Fig.2a). The glycerol, ethanolamine, inositol or phosphatidylglycerol groups are attached to the oxygen of the phosphate group. The fatty acids are a mixture of straight chain monounsaturated palmitic (C_{16}:1) oleic (C_{18}:1) and 10-methyl branched tuberculostearic acids (Minnikin, 1982).

PE is almost universally found, DPG and PI are widespread and PG is less common (Minnikin, 1982). PIMs (Fig.2b) are the most unusual and highly characteristic phospholipids found in mycobacteria and other actinomycetes (Minnikin and Goodfellow, 1980; Minnikin, 1982). Phosphatidylinositol dimannoside (PIM_2) from *M. tuberculosis* and *Mycobacterium phlei* was found to consist of a glycerol phosphate moiety attached to the L-1 position of the *myo*-inositol ring with mannose residues glycosidically-linked to the 2 and 6 positions of the

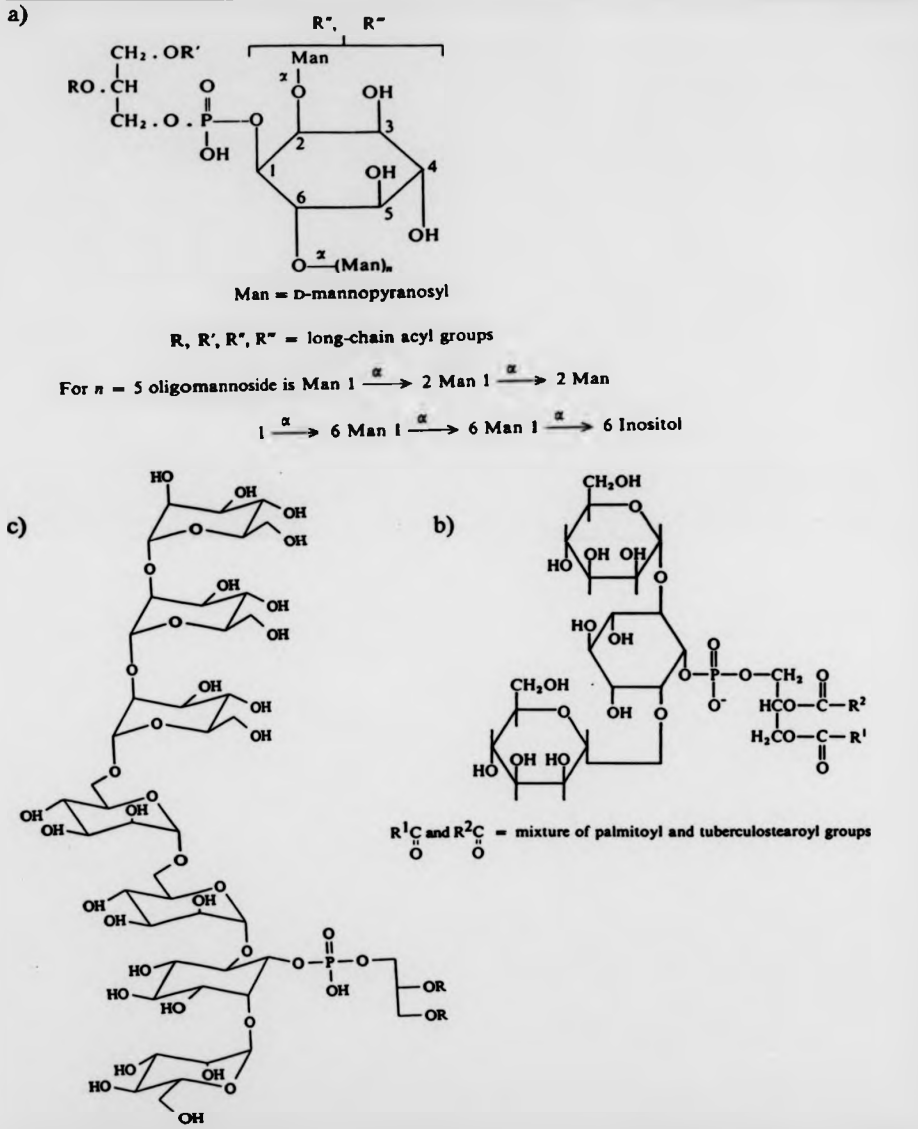


Fig.2. The general structure of phospholipids and phosphatidylinositolmannosides, PIM_2 and PIM_6

a) generalised phospholipid structure (taken from Minnikin, 1982)

b) phosphatidylinositol dimannoside PIM_2 (taken from Besra and Chatterjee, 1994)

c) phosphatidylinositol hexamannoside PIM_6 (taken from Besra and Chatterjee, 1994)

inositol ring (Lee & Ballou, 1964) (Fig.2b). PIM_2 (n=1) is the most commonly isolated PIM in mycobacteria (Minnikin 1982). Phosphatidylinositol mannoside homologs PIM_3 (n=2), PIM_4 (n=3), PIM_5 (n=4) and PIM_6 (n=5), (Fig.2c), have also been isolated (Lee and Ballou, 1965; Chatterjee *et al.*, 1992a). They also have a single α-D-mannopyranosyl group at the 2 position of the mannose oligosaccarides e.g. [α-D-Man*p*-(1→2)-]$_2$-[α-D-Man*p*-(1→6)-]$_2$-α-D-Man*p* in PIM_6 are found at the 6 position on the inositol ring.

PIM_2 has been shown to have an additional acyl group attached at the 2 or 6 position of the dimannoside (Brennan and Ballou, 1967; Khoo *et al.*, 1995a), this structure is known as a tri or monophosphatidylinositol dimannoside. PIMs are also believed to contain a second acyl group which is thought to be on the C-3 position of the inositol ring (Ballou, 1972). The diacylated PIMs are not found in *M. leprae*, where they seem only to exist in a mono-acylated form (Minnikin *et al*, 1985).

1.2.1.1.2 Isoprenoid and related compounds

These are lipids synthesised by condensation of isoprene units to form polyterpene-based products e.g. menaquinones and carotenoids.

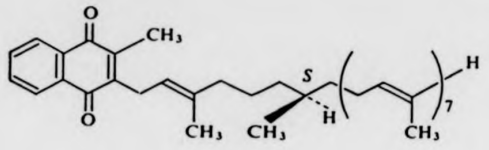
1.2.1.1.2.1 Menaquinones

Menaquinones (2-methyl-3-polyprenyl-1,4,-naphthoquinones) are the predominant isoprenoid lipoquinones found in mycobacteria and are involved in electron transport. They generally have nine isoprene units, one of which is hydrogenated; the precise structure of a menaquinone from *M. phlei* is shown in Fig.3a. This has all *trans* double-bond configurations. Non-isoprenoid lipoquinones have been isolated in *M. avium* (Minnikin, 1982).

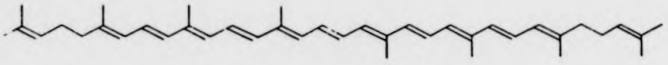
1.2.1.1.2.2 Carotenoids

These are linear polyterpenes containing around 40 carbons (Fig.3b) and are responsible for

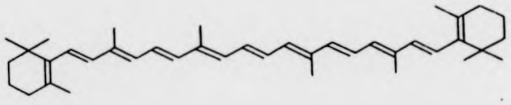
a)



b) 1)



2)



c)

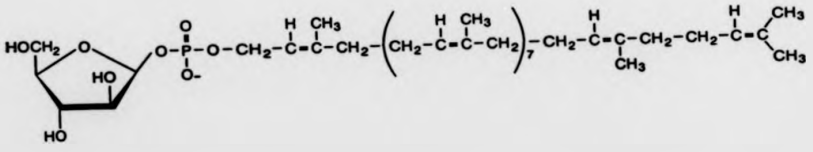


Fig.3. **The general structure of mycobacterial menaquinones, carotenoids and polyprenols**
a) The most abundant menaquinone structure found in *M. phlei* (taken from Minnikin, 1982)
b) Carotenoid structures 1) lycopene 2)β-carotene (taken from Minnikin, 1982)
c)Phosphodecaprenol with a β-D-linked-arabinofuranosyl unit attached, β-D-Arabinofuranosyl-1-phosphodecaprenol (Wolucka et *al.*, 1994) (taken from Besra and Chatterjee, 1994).

the characteristic yellow-orange colour of scotochromogenic and photochromogenic mycobacteria such as *M. gordonae* and *M. kansasii*.

1.2.1.1.3 Glycosylphosphopolyprenols

Glycosylphosphopolyprenols of mycobacteria contain the polyprenols decaprenol and octahydroheptaprenol, rather than the undecaprenol found in common bacteria, and are believed to be carrier molecules associated with the plasma membrane (Brennan and Nikaido, 1995). β-D-mannopyranosyl phosphodecaprenol and β-D-mannopyranosyl-phosphooctaheptaprenol were discovered in *M. smegmatis* and *M. tuberculosis* (Takayama *et al.*, 1973; Takayama and Goldman, 1970) Fig.3c. Recently, β-D-arabinofuranosyl phosphodecaprenol, β-D-ribosyl-1-monophosphodecaprenol and 6-*O*-mycolyl-β-D-mannopyranosyl phosphooctahydroheptaprenol have been found in *M. smegmatis* (Wolucka *et al.*, 1994; Wolucka *et al.*, 1995; Besra *et al.*, 1994a). The polyprenols are thought to be involved in cell wall biosynthesis as carrier molecules for arabinose, ribose and mycolates.

1.2.1.2 The cell wall skeleton

The covalently-linked skeleton of the cell wall consists of the peptidoglycan linked to the arabinogalactan, the side chains of which are esterified at their distal ends with mycolic acids to form mAGP.

1.2.1.2.1 The peptidoglycan

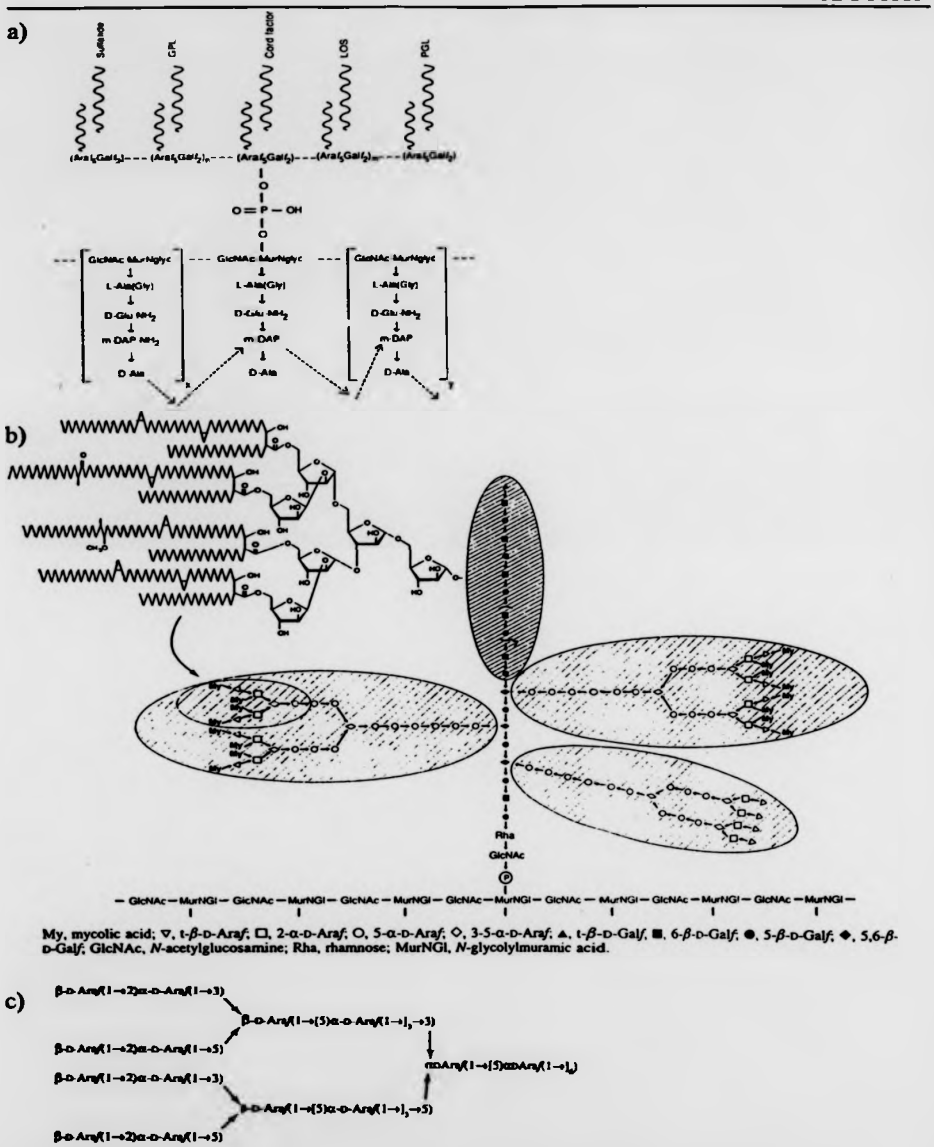
Peptidoglycan makes up 18-25% of the cell wall and lends it rigidity and shape. It consists of long polysaccharide chains cross-linked by short polypeptide chains to form a net-like macromolecule. The polysaccharide chains of the mycobacteria and the Nocardia are composed of repeating *N*-acetyl-β-D-glucosaminyl-(β1−4)-*N*-glycosylmuramic acid units; in other bacterial species the muramic acid residues are *N*-acetylated. The cross-linking peptide chains consist of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine, the

diaminopimelic acids (mDAP) being amidated. *M. leprae* is an exception in that it contains glycine in the place of L-alanine (Draper *et al.*, 1986). The peptide chains are linked to each other by interpeptide linkages between alanine and mDAP residues and between mDAP residues. The peptide chains are in turn linked to the carboxyl groups of the *N*-glycolyl muramic acid residues of the polysaccharide chains (Fig.4a)

1.2.1.2.2 Mycolyl-AG

Arabinogalactan constitutes about 35% of the cell wall mass of mycobacteria. Arabinogalactan is linked to the peptidoglycan via an unusual diglycosylphosphoryl bridge, 5-Gal*f*(1→4)-L-Rhap-(1→3)-D-GlcNAc(1→P) to the C-6 of the muramic acid residues (McNeil *et al.*, 1990).

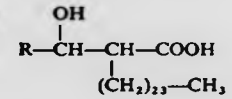
Arabinogalactan consists of arabinosyl (Ara) and galactosyl (Gal) residues, all in the furanose form which is rarely found in nature. The galactan segment is thought to exist as a homopolysaccharide linear strand of alternating 5- and 6-linked β-D-Gal*f* residues, [5)-β-D-Gal*f*-(1→6)-β-D-Gal*f*-(1→]$_n$, up to 30 units long (Daffe *et al.*, 1990; Besra *et al.*, 1995). The arabinan branched chains are thought to be attached to the C-5 of C-6-linked Gal*f* residues 24-27 at the reducing end of the galactan backbone, near the peptidoglycan (Besra *et al.*, 1995) (Fig.4b). Arabinan exists as branched chains (Fig.4c), the majority of the homoarabinan chains are composed of linear 5-linked α-D-Ara*f* residues with branching introduced by 3,5-α-D-Ara*f* residues, substituted at both branch positions with 5-α-D-Ara*f* (Fig.4c). Arabinan chains were found to be terminated by a unique branched hexaarabinofuranosyl structure [β-D-Ara*f*-(1 → 2)-α-D-Ara*f*]$_2$-3,5,-α-D-Ara*f*-(1→5)-α-D-Ara*f* (Daffe *et al.*, 1990; McNeil *et al.*, 1994). Recent work has shown the arabinan branches are now believed to consist of two hexaarabinofuranosyl residues linked to two 1→5α-D-Ara*f* residues joining at a 3→5 α-D-Ara*f* making a 23-residue arabinan unit (Besra *et al.*, 1995).



My, mycolic acid; ▽, t-β-D-Araf; □, 2-α-D-Araf; ○, 5-α-D-Araf; ◇, 3-5-α-D-Araf; ▲, t-β-D-Galf; ■, 6-β-D-Galf; ●, 5-β-D-Galf; ◆, 5,6-β-D-Galf; GlcNAc, *N*-acetylglucosamine; Rha, rhamnose; MurNGl, *N*-glycolylmuramic acid.

Fig.4. **The structure of mycobacterial peptidoglycan and arabinogalactan**
a) the general structure of chemotype IV peptidoglycan, where f=furanosyl;n+m =8-10 units and x+y=8-10 units. (taken from Brennan, 1989)
b) the proposed structure of arabinogalactan (taken from Besra et al., 1995)
c) the proposed structure of arabinogalactan branching (taken from Brennan and Nikaido, 1995)

a)



b) Table 1. **The structures of some mycobacterial mycolic acids**

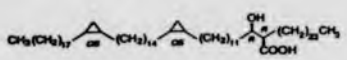
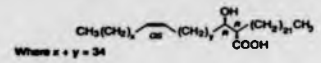
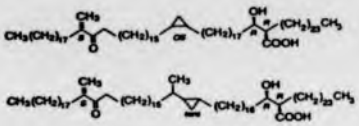
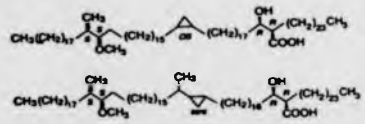
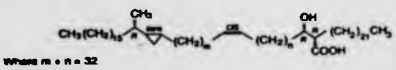
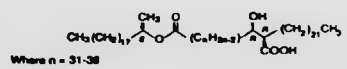
 α - Mycolates	76-82 carbons, do not contain oxygen functions, apart from the inherent hydroxy group. Can be unsaturated, methyl-branched or contain cyclopropane rings, as in the example shown. from *M. tuberculosis*. Found in all mycobacteria.
 α'-Mycolates	46-68 carbons, shorter versions of the mycolates, which are diunsaturated or monounsaturated and slightly more polar than mycolates. Found in *M. smegmatis* and *M. chelonae*.
 Ketomycolates	Contain ketogroups in their main carbon backbone. *M. tuberculosis cis* and *trans* ketomycolates are shown, the *trans* form is the most abundant. Found in many mycobacteria including *M. kansassi*, *M. leprae*, *M. bovis* and *M. avium* (Minnikin and Goodfellow, 1980)
 Methoxymycolates	Contain a methoxy group in their main carbon backbone again the methoxymycolates of *M. tuberculosis* shown are *cis* and *trans* forms, the *cis* being the most common. Also found in *M. bovis*, *M. kansassi*, *M. marinum* and *M. gordonae* (Reviewed by G. Dobson *et al.*, 1985)
 Epoxymycolates	Contain an epoxy group in their main carbon backbone and have only been found in *M. smegmatis* and *M. fortuitum* (reviewed by Dobson *et al.*, 1985)
 Wax ester mycolic acids	Wax ester mycolic acids have been observed in *M. avium*, *M. intracellulare*, *M.scrofulaceum*, *M. vaccae*, *M. aurum* and *M. paratuberculosis* among (reviewed by Dobson *et al.*, 1985). Wax ester mycolates are difficult to isolate intact as they are degraded to ω-carboxymycolates and 2-eicosanol or alkali hydrolysis

Fig.5. **The general structure of mycolates and a table of the various types**
a) general mycolate structure (taken from Minnikin, 1982)
b) Table.1. The structure of the mycolates and the species in which they are found

Two thirds of the hexaarabinofuranosyl units are then mycolated. Long chain α-alkyl-β-hydroxy mycolic acids are selectively and equally distributed on the 5-hydroxyl functions of terminal β-D-Ara*f* and internal 2 α-D-Ara*f* residues (Fig.4b). Mycolyl units are clustered in groups of four in each hexaarabinofuranosyl non-reducing terminus; they are packed parallel to each other and extend upwards from the arabinogalactan, perpendicular to the cell surface (McNeil *et al.*, 1991; Besra *et al.*, 1995).

1.2.1.1.3 Mycolic acids

Mycolic acids are high molecular weight long chain β(C-3)-hydroxy fatty acids of 70 to 90 carbons in length. The acid group in the C-1 position is at the terminus of the main backbone of 50-60 carbons with α(C-2) side chains of 20-24 carbons and a hydroxy group at the C-3 position (Fig.5a). The main carbon backbone, or meromycolate, may also contain up to two unsaturations, double bonds (*cis* or *trans*), cyclopropane rings, oxygen functions (additional to the β-hydroxy group e.g. keto groups) or methyl branches. These additional features of the carbon backbone distinguish mycobacterial mycolic acids from those of *Corynebacterium*, *Nocardia* and *Rhodococcus*.

The structure of different types of mycolates has been reviewed by Minnikin (1982) and Besra and Chatterjee (1994). Six types of mycolates have been defined: α, α', keto, methoxy, epoxy and wax ester mycolates. The structures of the various mycolates and the mycobacterial species in which they are found are described in Table 1 (Fig.5b).

1.2.1.3 Extractable cell wall lipids

A wide variety of cell wall lipids are not covalently bound and can be extracted by treatment with appropriate solvents. Extractable lipids are believed to make up the outer lipid leaflet which intercalates with the bound mycolates and those present in each species of mycobacteria vary. The most commonly found extractable lipids are described below in order of increasing

polarity, which generally coincides with a larger sugar component.

1.2.1.3.1 Waxes

True waxes consist of long chain fatty acids esterified to long chain alcohols. An array of waxes are found in mycobacteria, particularly slow growers i.e. *M. leprae*. They are generally long chain diols, phthiocerols A and B, phthiodiolone A and phthiotriol A, in which mycocerosic acids are esterified to both hydroxyl groups (Fig.6: Table.2). The phenolphthiocerols are a related family, which form the basic lipid core of phenolic glycolipids (Fig.6: Table 2) (Minnikin, 1982; Besra and Chatterjee, 1994).

1.2.1.3.1.1 Mycocerosates

The mycocerosic acids isolated from waxes are a complex mixture of multi-methyl branched acids, the major component of which is C_{32} 2,4,6,8,-tetramethyloctacosanoate (Fig.6: Table 2) (Minnikin, 1982; Besra and Chatterjee, 1994).

1.2.1.3.2 Phenolic glycolipids

Phenolic glycolipids (phenol phthiocerol glycosides, PGLs) have been found in five mycobacterial species to date: *M. kansasii* (Mycoside B), *M. bovis* (Mycoside B), *M. marinum* (Mycoside G), *M. leprae* (PGL-1) and some strains of *M. tuberculosis* (attenuator lipid) (reviewed in Brennan, 1989). PGLs consist of a C_{36} phenolic phthiocerol group with two C_{34} mycocerosate fatty acids attached (Fig.7a. where R=mycoserosate). There is also an oligosaccharide attached to the phenol group which consists of 1-4 sugar residues depending on the species. The oligosaccharide of *M. bovis* is a monoglycosyl unit, *M. leprae* and *M. tuberculosis* (Canetti) have triglycosyl units and *M. kansasii* has a tetraglycosyl unit (Fig.7b, Table. 3). The sugar residues are not very hydrophilic, consisting of deoxy sugars that are multiply *O*-methylated. PGLs are the major extractable lipid produced by *M. leprae in vivo*.

Table.2

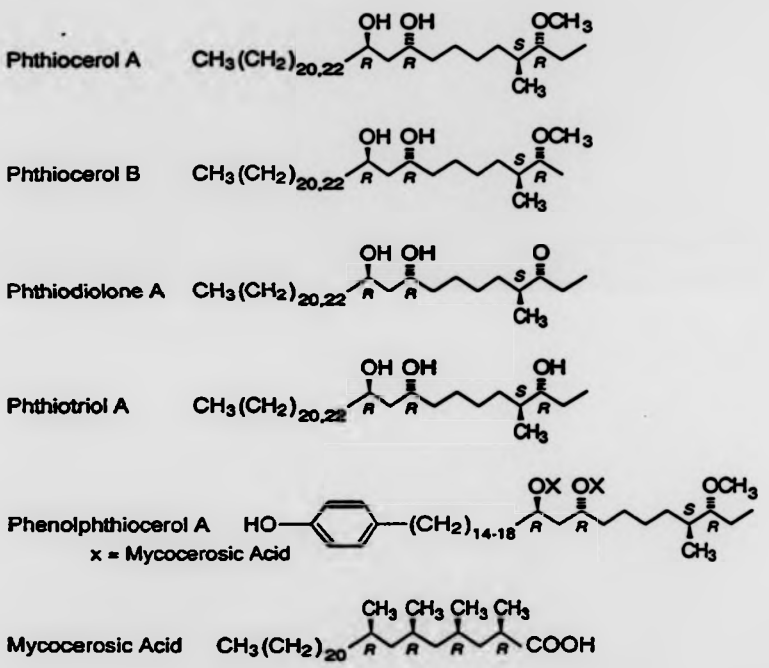
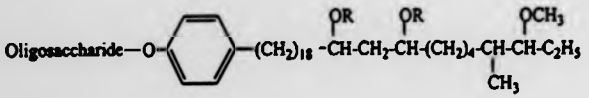


Fig. 6.**Waxes and mycocerosates found in the mycobacteria**
Table 2. **The structure of waxes and mycocerosates found in *M. tuberculosis*** (taken from Besra and Chatterjee, 1994)

a)



R = Long Chain Fatty Acyl

b) Table. 3. Summary of structures of major phenolic glycolipids from *Mycobacterium* species.

Mycobacterial species	Structure of carbohydrate chain
Mycobacterium bovis	2-*O*-Me-α-L-Rha*p*-(1→phenol-dimycocerosyl phthiocerol)
Mycobacterium lepree	3,6-di-*O*-Me-β-D-Glc*p*(1→4)-2,3-di-*O*-Me-α-L-Rha*p*-(1→4)-3-*O*-Me-α-L-Rha*p*-(1→phenol-dimycocerosyl phthiocerol)
Mycobacterium kansasii	2,6-dideoxy-4-*O*-Me-α-*arabino*-hex*p*-(1→3)-4-*O*-Ac-2-*O*-Me-α-Fuc*p*-2-*O*-Me-α-Rha*p*-(1→3)-2,4-di-*O*-Me-α-Rha*p*-(1→ phenol-dimycocerosyl phthiocerol)
Mycobacterium tuberculosis	2,3,4-tri-*O*-Me-α-L-Fuc*p*-(1→3)-α-L-Rha*p*-(1→3)-2-*O*-Me-α-L-Rha*p*-(1→phenol-dimycocerosyl phthiocerol)

Fig.7.The structure of mycobacterial phenolic glycolipids and their specific oligosaccharide haptens.
a) General structure of phenolic glycolipids (taken from Besra and Chatterjee, 1994)
b) Table.2. **The specific oligosaccharide haptens of PGLs from the PGL containing mycobacterial species** (taken from Brennan, 1989)

1.2.1.3.3 Glycopeptidolipids

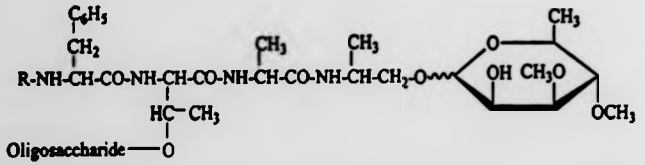
Glycopeptidolipids (GPLs) are peptide-containing surface glycolipids and can be divided into two main types, simple apolar and complex polar hydrophilic molecules. Generally, GPLs consist of a core head group, the tripeptide, D-Phe-D-*allo*Thr-D-Me-L-alaninol, where the alaninol is substituted with a 3,4 di-*O*-methyl-X-rhamnose and the aminogroup of the D-phenylalanine residue is substituted with a fatty acid residue. The D-*allo*-threonine residue is substituted with oligosaccharides of varying length, the proximal portion of which are generally α-L-rhamnopyranosyl (1→2)-6-deoxy-L-talopyranose in complex GPLs (Fig.8a). The simple polar GPLs differ from the complex GPLs in that they are singly glycosylated at the threonine substituent and are found in species which contain complex GPLs and others e.g. *M. smegmatis* which do not.

Complex GPLs are the major cell surface antigens of *M. avium*, *M. intracellulare* and *M. scrofulaceum* (MAIS complex) and *M. peregrinum*, *M. chelonae* and *M. absessus*, of the *M. fortuitum* complex. The variable oligosaccharide region of the MAIS complex GPLs is serospecific allowing the MAIS complex to be subdivided into 31 distinct serotypes, 12 of which have been completely defined (Fig.8b: Table.4) (reviewed by Brennan, 1989; Belisle and Brennan, 1994). The GPLs of the *M. fortuitum* complex vary slightly in that these oligosaccharides are attached to the alaninol (López Marin *et al.*, 1991, 1992 and 1994). Other variations include sulphated GPLs in *M. peregrimum*, *Mycobacterium senegalense* and *Mycobacterium porcinum* (López-Marin *et al.*, 1992 and 1993) and a novel GPL with a serine-containing lipoprotein core structure in *M. xenopi* (Rivière and Puzo, 1992; Rivière *et al.*, 1993).

1.2.1.3.4 Acylated trehaloses

There are three families of trehalose based lipids, trehalose 6,6'-dimycolate (cord factor) (Fig.9a), the simple acylated trehaloses, which contain a combination of saturated straight

a)



b) Table. 4. Structure of oligosaccharide haptens from the glycopeptidolipids of some serovariants of *Mycobacterium avium*.

Serovariant no.	Structure
2	2,3-Di-*O*-Me-L-Fuc*p*(α1→3)-L-Rha*p*(α1→2)6-d-L-Tal
4	4-*O*-Me-L-Rha*p*(α1→4)2-*O*-Me-L-Fuc*p*)(α1→3)n-Rha*p*(α1→2)6d-L-Tal
8	4,6-(1'-carboxyethylidene)3-*O*-Me-D-Glc*p*(β1→3)-L-Rha*p*(α1→2)6d-L-Tal
9	2,3-Di-*O*-Me-L-Fuc*p*(α1→4)-D-GlcA*p*(β1→4)2,3-Di-*O*-Me-L-Fuc*p*(α1→3)-L-Rha*p*(α1→2)6d-L-Tal
12	4-*N*-lactyl-3-*O*-Me-4,6-dideoxyglυco(1→3)-4-*O*-Me-L-Rha*p*(α1→3)-L-Rha*p*(α1→3)-L-Rha*p*(α1→2)-6-d-L-Tal
14	4-Formamido-3,4,6-trideoxy-2-*O*-Me-3-*C*-Me-Man(1→3)2-*O*-Me-D-Rha*p*(α1→3)2-*O*-Me-L-Fuc*p*(α1→3)n-Rha*p*(α1→2)6-d-L-Tal
17	4-(2'-methyl-3'-hydroxy-butyr)amido-4,6-dideoxy-Hex(1→3)4-*O*-Me-L-Rha*p*(α1→3)n-Rha*p*(α1→3)n-Rha*p*(α1→2)6-d-L-Tal
19	3,4-Di-*O*-Me-D-GlcA*p*(β1→3)2,4-Di-*O*-Me-3-*C*-Me-6-dHex(α1→3)n-Rha*p*(α1→3)n-Rha*p*(α1→2)6-d-L-Tal
20	2-*O*-Me-D-Rha*p*(α1→3)2-*O*-Me-L-Fuc*p*(α1→3)n-Rha*p*-(α1→2)6-d-L-Tal
21	4,6-(1'-carobyxyethylidene)-D-Glc*p*(β1→3)-L-Rha*p*-(α1→2)-6-d-L-Tal
25	4-acetamido 4,6-dideoxy 2-*O*-Me-Hex(α1→4)D-GlcA*p*(β1→4)2-*O*-Me-L-Fuc*p*(α1→3)n-Rha*p*(α1→2)6-d-L-Tal
26	2,4-Di-*O*-Me-Fuc*p*(α1→4)GlcA*p*(β1→4)2-*O*-Me-L-Fuc*p*-(α1→3)n-Rha*p*-(α1→2)6-d-L-Tal

Fig.8. The general structure of the glycopeptidolipids found in mycobacteria and the serovar specific oligosaccharide haptens of the *M. avium* complex

a) General GPL structure (taken from Besra and Chatterjee, 1994)

b) Table. 4. **The serovar specific oligosaccharide haptens from 12 *M. avium* serovars** (taken from Brennan, 1989)

chain mycocerosate (C_{16}-C_{19}, C_{21}-C_{25}), mycolipanolic (C_{24}-C_{28}) and mycolipenic fatty acids (C_{25}-C_{27}) and the sulpholipids (Fig.9b). The sulpholipids consist of trehalose 2'-sulphate acylated with hydroxyphthioceranic, phthioceranic and saturated straight-chain fatty acids (Besra and Chatterjee, 1994).

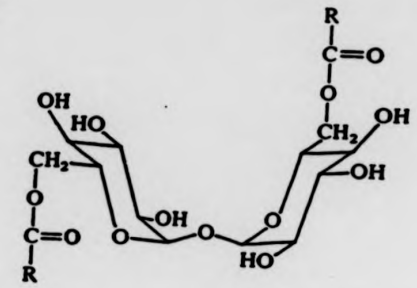
1.2.1.3.5 Lipooligosaccharides

Lipooligosaccharides (LOSs) consist of variable oligosaccharides with an acylated α, α' trehalose unit at one end (Fig.9c). LOSs vary from multi-glycosylated forms found in *M. kansasii* (Hunter *et al.*, 1985) to the simpler singly glycosylated compounds found in *M. smegmatis* and *M. fortuitum* (Kamisango *et al.*, 1985; Besra *et al.*, 1992).

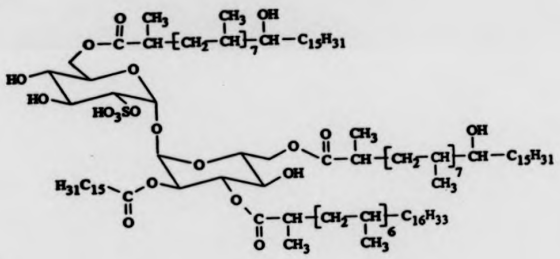
The multi-glycosylated acyltrehaloses of *M. kansasii* were isolated from eight smooth colony variants (Hunter *et al.*, 1985). Their oligosaccharides are composed of variable residues of xylose, 3-*O*-methylrhamnose, fucose and N-acylosamine, a novel N-acylamino sugar linked to a common tetraglucose core, which contains an α α'-trehalose moiety. The terminal glucose residue of the trehalose is usually acylated at positions 3, 4 and 6 by 2,4-dimethyltetradecanoic acid residues. Multiglycosylated LOSs with similar structures to those found in *M. kansasii* have been found in *M. gordonae* and *M. tuberculosis* Canetti (Besra *et al.*, 1993; Daffe *et al.*, 1991).

The simpler LOS of *M. smegmatis* consists of a pentaoligosaccharide unit of D-glucose residues. Two of the glucose residues are pyruvylated and one is 3-*O*-methylated. Acyl groups, 2,4-dimethyl-2-eicosenoic acid and hexadecanoic acid are found on the 4 and 6 positions respectively, on both glucose molecules of the trehalose unit (Kamisango *et al.*, 1985). The two pyruvate methyl groups and the 3-*O*-methyl ether group lie together on one side of the sugar and with the fatty acid chains are thought to result in a molecule with a non-polar surface.

a)



b)



c)

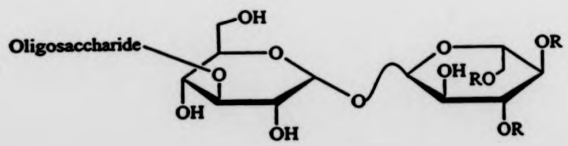


Fig.9. The structure of trehalose containing mycobacterial lipids
a) Trehalose 6,6,'dimycolate (taken from Besra and Chatterjee, 1994)
b) Sulpholipids of *M. tuberculosis* (taken from Besra and Chatterjee, 1994)
c) General structure of LOSs (taken from Besra and Chatterjee, 1994)

1.2.1.3.5 Glycerophospholipids

Recent work by Rosenburg and Nikaido has revealed that the cell wall of *M. chelonae* contains at least two short chain (C_{16}-C_{18}) fatty acid residues per bound molecule of mycolic acid residue (Nikaido *et al.*, 1993), some of the short chain acids found in PIMs (Brennan and Nikaido, 1995), implying PIMs are present in the cell wall. The cell wall extracts of *M. chelonae* contained less than 1% plasma membrane so it is unlikely that the glycerophospholipids found were contaminants from the plasma membrane.

1.2.1.4 Lipoarabinomannan

Lipoarabinomannan (LAM) is a major constituent of mycobacterial cell envelopes and is believed to be present in all mycobacterial species. Detailed structural analysis of LAM from *M. leprae* and *M. tuberculosis* Erdman has revealed that LAM is a multiglycosylated extension of PIM_2, consisting of a mannose backbone with branched arabinogalactan extending perpendicularly (Fig.10) (Hunter *et al.*, 1986; Chatterjee *et al.*, 1992a). The C_6 of the inositol is the point of attachment of a mannan backbone (Chatterjee *et al.*, 1992a) which consists of α(1→6)-linked D-mannose (Man*p*) residues, with a variable degree of α(1→2)-Man*p* linked side branches (Misaki *et al.*, 1977; Chatterjee *et al.*, 1992a; Venisse *et al.*, 1995). The main arabinan backbone consists of α(1→5)-linked α-D-arabinofuranosyl (Ara*f*) residues, with branches introduced by α-3,5-α-D-Ara*f* linkages. There are two types of arabinan branches shown in Fig.10. One type [β-D-Ara*f*-(1→2)-α-D-Ara*f*]$_2$-3,5-α-D-Ara*f*-(1→5)-α-D-Ara*f* is similar to arabinogalactan and the other, β-D-Ara*f*-(1→2)-α-D-Ara*f*(1→5)-α-D-Ara*f*, is linear (Chatterjee *et al.*, 1991).

Recently, the arabinose terminus of LAM from *M. tuberculosis* Erdman, H37RV and H37Ra (Chatterjee *et al.*, 1992b; Khoo *et al.*, 1995b), *M. bovis* BCG (Prinzis *et al.*, 1993) and *M. leprae* (Khoo *et al.*, 1995b) have been shown to be mannose-capped (ManLAM). The branched or linear termini are extensively capped with mono, di or trimannosides (1→5)

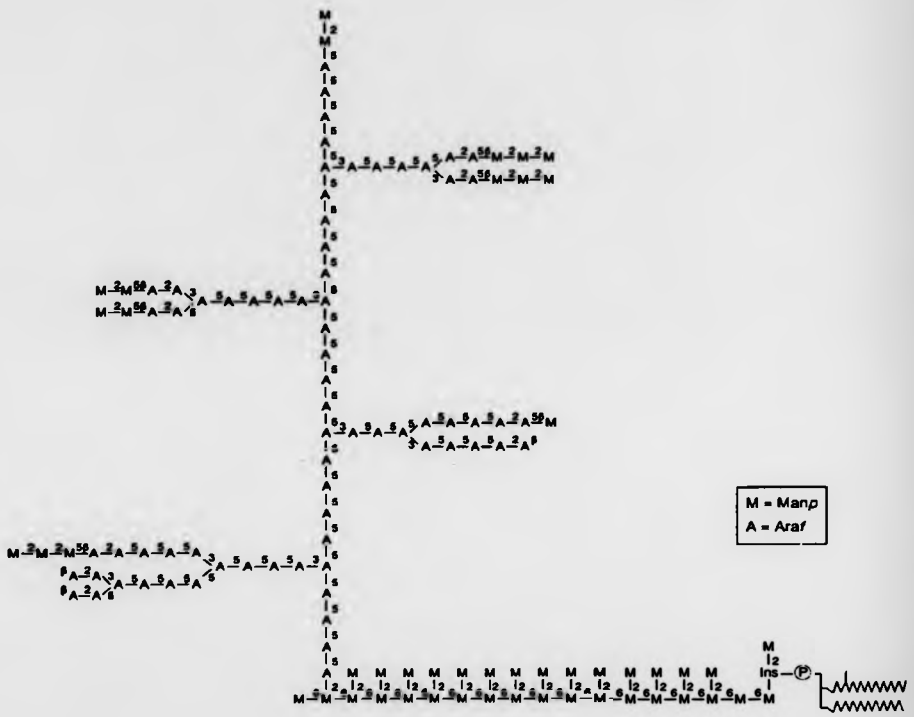


Fig. 10. **The structure of lipoarabinomannan, LAM**
(taken from Besra and Chatterjee, 1994)

linked to an α-Ara*f* residue (Fig.10) and the terminal Man*p* residues are α-D-Man*p*-(1-2)-α-D-Man*p*-linked (Fig.10), an arrangement that is uncommon in mycobacteria but is common on the glycoproteins of mammalian cell surfaces. The extent of the mannose-capping varies between species from 70% in *M. tuberculosis* Erdman to 30% in *M. leprae* (Khoo *et al.*, 1995b).

The non-reducing terminus of LAM in fast-growing mycobacteria e.g. *M. smegmatis*, is devoid of mannose-capping and has been shown to exhibit a degree of inositol phosphate capping (Khoo *et al.*, 1995b). The inositol capping is believed to be restricted to the linear arabinan termini and attached at position 5 of the terminal β-Ara*f* residue.

The general structure of LAM does vary slightly from species to species e.g. *M. bovis* BCG ManLAM has a shorter mannose backbone of only 5 units of 2,6 linked mannose, a highly branched arabinofuranosyl side chain and up to three 2-linked mannopyranosyl segments at the non-reducing ends of the molecule (Prinzis *et al.*, 1993).

LAM was believed to be anchored in the plasma membrane by a PIM anchor and to span the cell wall, however the recent discovery of PIMs in the cell wall imply that LAM could be extractable and embedded in the cell wall (Brennan and Nikaido, 1995) .

1.2.1.5 Cell wall associated proteins

The lipoproteins found in *M. tuberculosis* are believed to be analogous to the periplasmic proteins of Gram-negative bacteria and to be cell wall associated (Young and Garbe, 1991). The 38kDa protein of *M. tuberculosis* has 30% homology to the periplasmic *Pst*S *(PhoS)* from *E. coli* which captures phosphate transported through the porin and releases it via a phosphate specific phosphate channel which spans the cytoplasmic membrane. The synthesis of the 38kDa protein is upregulated during phosphate starvation, implying the protein could

be involved in phosphate biosynthesis (Andersen *et al.*, 1990).

The 19kDa *M. tuberculosis* lipoprotein is anchored in the cell wall (Young and Garbe, 1991) and is also believed to be involved in phosphate uptake (Andersen and Brennan, 1994) but its exact functions are unknown.

Excreted proteins e.g. superoxide dismutase (Zhang *et al.*, 1991; Abou-Zeid *et al.*, 1988; Thole *et al.*, 1992) must transverse the cell wall to reach the exterior. The 30/31kDa antigen 85 complex contains signal sequences required to transverse the cell wall and has been found to be at least temporarily associated with the cell wall surface before release into the exterior (Rambukkana *et al.*, 1991).

The high affinity iron binding compounds, exochelins and mycobactins are also cell wall associated. Exochelins are excreted into the extracellular environment, while mycobactins are thought to facilitate the transport of iron across the cell wall to the interior of the bacterium (Ratledge, 1982). The mycobactins are highly lipophilic and the exochelins have recently been shown to share a common core structure with the mycobactins (Sharman *et al.*, 1995).

Cell wall associated proteins with unknown functions have been found in a number of mycobacterial species e.g. the major 35kDa *M. leprae* protein (Hunter *et al.*, 1990) and the 30kDa protein of *M. chelonae* (Nikaido *et al.*, 1993). Porins have also been found in the cell walls of *M. chelonae* and *M. smegmatis* (Trias *et al.*, 1992; Trias and Benz, 1994).

1.2.1.6 Physical organisation of the cell envelope

The proposed model for the physical organisation of the cell envelope of a plasma membrane surrounded by a bilipid cell wall was described in section 1.2. The model, based on the chemical properties of the cell envelope components (Minnikin, 1982) has largely been borne

out by subsequent experimental evidence (Nikaido *et al.*, 1993; Brennan and Nikaido, 1995).

1.2.1.6.1 Ultrastructure evidence

The ultrastructure of mycobacteria has been extensively studied using a variety of techniques including freeze-fracture, freeze-etching, negative staining, metal shadowing, thin sectioning and freeze-substitution (Reviewed by Draper, 1991; Brennan and Draper, 1994). These experiments have attempted to relate the visible structure to the physical organisation of the chemical components in the cell envelope.

The mycobacterial plasma membrane appears as two electron dense layers separated by a transparent layer, with integral membrane proteins and a freeze-fracture plane across the transparent layer (Nguyen *et al.*, 1979; Silva and Macedo, 1983). The plasma membrane is asymmetrical, the outer electron dense layer appears thicker than the inner layer, due perhaps to the presence of carbohydrate in molecules such as PIMs or LAM (Silva and Macedo, 1983; Paul and Beveridge, 1992). A pseudo-periplasmic space between the plasma membrane and the cell wall has not been categorically established through electron microscopy.

Observation of the cell wall of mycobacteria by electron microscopy has given variable and contradictory data depending on the methods used. The wall appears to consist of three layers; an inner layer (IL) of moderate electron density, a wider layer electron-transparent layer (ETL) and an outer electron-opaque layer (OL) of variable thickness and appearance.

The (IL) is 5.6-6.2nm wide (Paul and Beveridge, 1992) and is believed to contain the peptidoglycan because of the consistency of its moderate electron density across the species and the ability of the carboxyl groups of diaminopimelic acid in this layer to bind metal ions during staining (Beveridge and Murray, 1980).

The ETL represents the arabinogalactan-mycolate region of the cell wall. It is the most hydrophobic region and the most lacking in reactive chemical groups to bind the electron microscopy stains. The mycolic acids are thought to prevent access of the stain to the arabinogalactan making it part of the electron-transparent layer (Rastogi *et al.*, 1984). The ETL disappeared following extraction of lipids with acetone in *M. kansasii*, and the lipid extracted was also shown by TLC analysis to contain large quantities of phenolic glycolipid. (Paul and Beveridge, 1994). This experiment implies that the ETL contains shorter-chain lipids predicted to intercalate with the mycolates in the models of the cell wall (Minnikin 1982; Nikaido *et al.*, 1993; Brennan and Nikaido, 1995). The ETL was found to be 5-10nm in width by freeze-substitution (Paul and Beveridge, 1992).

The OL, approximately 5nm wide (Paul and Beveridge, 1992), is the most variable from species to species and has been identified in 18 mycobacterial species (Brennan and Draper, 1994). The OL appears fibrillar, granular, or homogenous and varies in thickness and electron density; this is thought to be due to the various staining methods and growth conditions employed, as well as genuine differences between species. The OL is believed to consist of delicate surface structures and has been shown to contain polysaccharides, glycoproteins, proteins, phospholipids and complex glycolipids in *M. avium* (David *et al.*, 1988).

Ruthenium red staining was the first to allow clear and consistent observation of the OL layer in different species. The dye binds to charged groups and does not penetrate the membrane; it is not clear which specific components of the mycobacterial cell wall bind to the dye (Rastogi *et al.*, 1984). Recent freeze-substitution experiments of *M. kansasii* showed a very faint OL towards the edge of the ETL but contained within it, suggesting that the components of the OL may be found in structures that also make up part of the ETL (Paul and Beveridge, 1994). This indicates that the OL contains the sugar and protein components of the intercalating lipid of the cell wall leaflet.

A freeze-fracture plane has also been observed in the mycobacterial cell wall and supports the concept that the cell wall exists as a lipid bilayer (Draper and Brennan, 1994; Brennan and Nikaido, 1995).

1.2.1.6.1.1 Intracellular ultrastructure

The ultrastructure of some mycobacteria inside macrophages has been studied. The mycobacteria were generally found within a vacuole but separated from its contents by a distinct "electron transparent zone" (ETZ). This ETZ is different from the ETL in mycobacteria grown *in vitro* and the boundary between the two is generally visible inside vacuoles. The ETZ corresponds with the position of the outer layer of cells grown *in vitro*.

In *M. leprae* an ETZ was observed surrounding the bacilli *in vivo* (Nishiura *et al.*, 1977). The use of a specific PGL-1 monoclonal antibody and indirect immunofluorescence resulted in a fluorescent zone surrounding the bacilli (Gaylord and Brennan, 1987) demonstrating the surface location of the glycolipid antigen and implying the presence of PGL-1 in the ETZ of *M. leprae*. In *M. avium* the ETZ consisted of a multilamella coat of parallel straight fibrils and is thought to contain GPLs (Rulong *et al.*, 1991).

1.2.1.6.2 Biochemical evidence

The proposed arrangement of the arabinan branching in the arabinogalactan, at the base of the galactan chain (Besra *et al.*, 1995) would allow the mycolates to extend upwards perpendicular to the cell surface, as proposed in the model.

The amounts of mycolic acid present in whole cell hydrolysates are comparable with the nonhydroxylated fatty acids of the plasma membrane suggesting there are sufficient mycolates to form the proposed lipid domain (Minnikin, 1982).

The lack of acylation of the β-hydroxyl group of the mycolates implies that intramolecular and intermolecular hydrogen bonds could form between the hydroxy group and the carbonyl group of each mycolate (Besra *et al.*, 1995). It is thought that the hydrogen bond would result in the α branch and the meromycolate backbone of each mycolate being parallel to each other forming the predicted parallel packing of the mycolates. Recent x-ray diffraction studies of the cell wall of *M. chelonae* showed that a large part of the hydrocarbons are tightly packed in a parallel array, perpendicular to the surface (Nikaido *et al.*, 1993).

If this arrangement is correct the mycolic acid structure would be predicted to produce domains of different fluidity, an inner region of low fluidity and a distal region of increased fluidity (Brennan and Nikaido, 1995). The α branch is without double bonds or cyclopropane rings and the proximal portion of the meromycolate chain generally contains a *trans* double bond that does not introduce kinks, which would result in low fluidity in the region close to the arabinogalactan. In contrast, the distal end of the mycolates contains the second double bond or cyclopropane group with a *cis* configuration; oxygen-containing substituents may also be present at this position. These groups would strongly disrupt the lateral packing at the distal end of the mycolates leading to increased fluidity towards the outer edge of the mycolate region (Brennan and Nikaido, 1995). The x-ray data showed that a large fraction of the cell wall lipid was in an almost crystalline arrangement of low fluidity in *M. chelonae* (Nikaido *et al.*, 1993). These two regions of fluidity would assist in the formation of the predicted outer bilayer (Brennan and Nikaido, 1995).

The model also requires sufficient amounts of extractable lipids as components of the outer leaflet. As the meromycolates and α branches differ by about 26 carbons, lipids and fatty acids with between 14-18 and 30-40 residues are required to intercalate with the mycolates to form the outer leaflet of the lipid bilayer. *M. chelonae* was found to contain glycerophospholipids, GPLs and unknown lipids with intermediate chain length in ample

quantities to form an outer leaflet (Nikaido *et al.*, 1993; Brennan and Nikaido, 1995). Other species also have extensive quantities of extractable lipids which are thought to be located in the outer leaflet of the bilayer, e.g. PGLs of *M. leprae* (Gaylord and Brennan, 1987) and GPLs of *M. avium* (Rulong *et al*, 1991).

Some lipids may form independent aggregates outside the bilayer, particularly when over produced. The form in which these lipids exist outside the cell will vary e.g. PGL-I of *M. leprae* is apolar and exists as oil droplets (Gaylord and Brennan, 1987) whereas GPLs of *M. avium* are amphiphilic and form micelles or fibrils (Barrow *et al.*, 1980). Other extractable lipids, i.e. trehalose dimycolate, LOSs and sulpholipids, are more ideally shaped to form the lipid bilayer and are unlikely to exist outside the cell wall bilayer (Brennan and Nikaido, 1995).

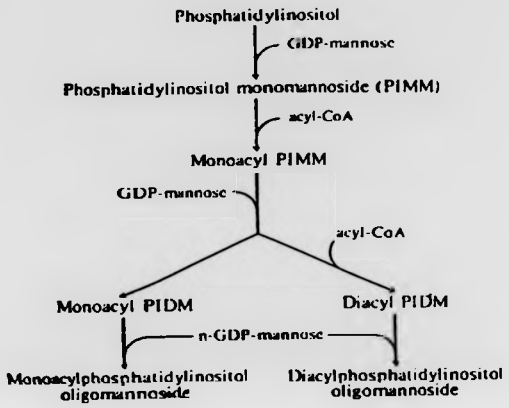
1.2.2 BIOSYNTHESIS OF SOME CELL WALL COMPONENTS

The elucidation of biosynthetic pathways is important in the search for drug target sites for mycobacteria. Mutational analysis of operons encoding the cell wall components would help elucidate the pathways as shown by Mills *et al.*, (1994) with the GPLs of *M. avium* serovar2. Generally a larger number of individual enzymes are required to form oligosaccharides, while lipid synthesis appears to involve multifunctional enzyme complexes. This section describes what is known about the some of the more unique mycobacterial cell wall components.

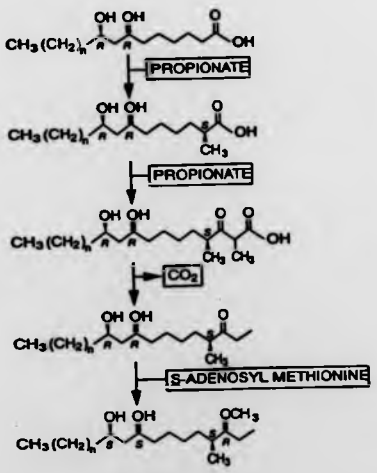
1.2.2.1 PIM and LAM biosynthesis

A proposed biosynthetic pathway for PIMs is shown in Fig.11a. The recent discovery that an additional acyl group is attached either to the C-6 or C-2 position of the dimannoside moiety (Khoo *et al.*, 1995a) has implications for LAM biosynthesis, as the addition of an acyl group to the C-6 position would prevent the formation of the mannose backbone.

a)



b)



n = 20 or 22.

Fig.11. **The proposed pathways of PIM and phthicerol biosynthesis**
a) The proposed pathway of PIM biosynthesis (taken from Minnikin, 1982)
b) The later stages of phthiocerol A biosyntheis (taken from Besra and Chatterjee, 1994)

1.2.2.2 Arabinogalactan

Little is known of the biosynthesis of this compound. However, recently, a family of monoglycosyl polyprenylphosphates have been isolated from *M. smegmatis* which contain arabinose and ribose, β-D-arabinofuranosyl phosphodecaprenol and β-D-ribosyl-1-monophosphodecaprenol (Wolucka *et al.*, 1994 and 1995). The polyprenols are thought to be involved in cell wall biosynthesis as carrier molecules for arabinose and ribose. The destiny of the ribose is as yet unknown; it could be a component of an unknown polysaccharide or a precursor for arabinose (Wolucka *et al.*, 1995). Pulse-chase metabolic labelling of cells with [^{14}C]-glucose indicated that decaprenyl-P-arabinose is an active intermediate in the biosynthesis of arabinogalactan and arabinomannan (Wolucka *et al.*, 1994).

1.2.2.3 Mycolic acids

The complete biosynthetic pathways of mycolate formation have not yet been determined, although a number of individual steps have been established.

Initial studies on *Corynebacterium diphtheriae* (Gastambide-Odier and Lederer, 1960) showed that C_{32} mycolates were formed by Claisen condensation and reduction of two C_{16} acids. Similar condensations were subsequently revealed in the formation of mycolates of *M. smegmatis* (Etémadi, 1967a, b and c) as reviewed by Minnikin (1982).

A general pathway for the synthesis of the α dicyclopropyl mycolates of *M. tuberculosis* H37Ra was initially proposed by Takayama and coworkers following detailed investigations (reviewed by Minnikin (1982)). An updated version of this pathway has been proposed by G. Besra (Brennan and Nikaido, 1995) (Fig.12)

A C_{24} fatty acid, formed in the cytosol by fatty acid synthesis FASI/FASII, is believed to enter one of two branches of the pathway. In one branch of the pathway the C_{24} acid is thought to

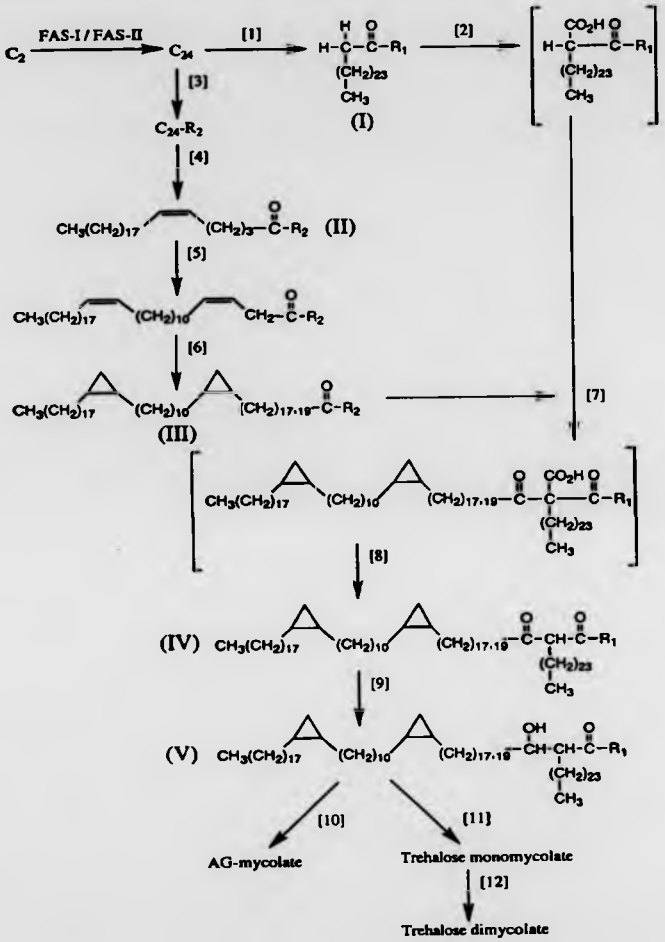


Fig. 12. **The proposed pathway of α mycolate biosynthesis in *M. tuberculosis* H37Ra** The proposed anabolic pathway of mycolic acid biosynthesis in *M. tuberculosis* H37Ra was taken from Brennan and Nikaido,1995. The key products are (I) hexacosanoate-R1 (II) Δ-5-tetracosanoate-R2, (III) $C_{52,54}$ meromycolate-R_2, and (IV)oxomycolate-R_1, and (V) mycolate-R_2. Carrier group$_1$ R is believed to be the β-D-mannopranosyl-monophosphoryl-polyisoprenol, and R_2 may be either CoA or ACP. The reactions are 1) elongation and introduction of carrier group R_1 2) carboxylation 3) introduction of carrier group R_2 4)Δ-5-desaturation 5) elongation and Δ-3-desaturase 6) introduction of cyclopropane rings and elongation 7) Claisen type condensation 8) decarboxylation 9) reduction 10) mycolate transfer to AG 11) mycolate transfer to trehalose and 12) trehalose mycoyltransferase.

be elongated to a C_{26} molecule by the addition of C_2 attached to a carrier molecule R (Fig.12). The carrier molecule R_1 is possibly the recently discovered mycolyl phospholipid (Myc-PL), 6-0 mycolyl-β-D-mannopyranosyl-phosphaoctahydroheptaprenol, which contains an appropriate acyl carrier group (Besra *et al.*, 1994a). The C_{26} molecule is then thought to be carboxylated to form an acid, tetracosanyl malonate, $(CH_3(CH_2)_{23}CH(COOH)_2)$ (Fig.12).

The C_{24} acid entering the other branch of the pathway is thought to be attached to an acyl carrier molecule R_2, which could be CoA, an acyl carrier protein (ACP) or a membrane-bound protein (Brennan and Nikaido, 1995). The C_{24}-R_2 molecule is thought to be desaturated by Δ-5-desaturase to form $C_{24:1}$, Δ-5-tetracosanoate-R_2. The Δ-5-tetracosanoate (Fig.12 II) is then thought to be elongated to a C_{32} molecule by the sequential addition of C_2 molecules before undergoing a second desaturation. The cyclopropane rings are then thought to be formed at the positions of the double bonds before further elongation of the C_{32} molecule to a meromycolate (C_{52}-C_{54}) (Fig.12 III). The meromycolates and the C_{26} acid, tetracosanyl malonate-R_1, are then thought to undergo a Claisen condensation producing a ketomycolyl-R_1 intermediate. The intermediate is thought to be decarboxylated yielding an oxomycolate-R_1 which is reduced to give mycolate-R_1. The mycolate-R_1 (R_1 = Myc-PL) may then be directly transferred to the non-reducing terminus of the D-arabinose residue of arabinogalactan matrix. Myc-PL could transfer the mycolate residues to trehalose to form trehalose monomycolates which are also thought to be the carriers of mycolic acid to the D-arabinose residues (Takayama and Armstrong, 1976).

Evidence has been gathered for some steps in the proposed pathway from a wide range of studies, including investigations into the effect of antituberculous drugs.

That Δ5-unsaturated C_{24} fatty acids are precursors of mycolates has been implicated by a number of groups. Asselineau *et al.* (1970) showed that a series of Δ5-unsaturated C_{22}, C_{24}

and C_{26} non-hydroxylated fatty acids (FA), from *M. phlei*, were structurally similar to the hydrocarbon terminal portion of its di-unsaturated mycolates. Oxidative cleavage of the double bonds in both the mycolates and FA gave identical degradation products. It was therefore suggested that Δ5-acids may be formed by aerobic desaturation of C_{24} tetracosanoic acids. Investigation of the monounsaturated FA of H37Ra (Takayama *et al.*, 1978) revealed the position of the double bonds in the FA corresponded exactly with positions of the cyclopropane rings in the alkyl terminal portion of the α mycolates (Qureshi *et al.*, 1978). It was concluded that these unsaturated FA esters may be precursors of mycolic acids.

A system was developed to follow mycolic acid synthesis in intact *M. tuberculosis* H37Ra by measuring incorporation of a radioactive [^{14}C]-acetate precursor into mycolates (Takayama *et al.*, 1972). A cell-free system able to synthesize whole mycolic acids was subsequently developed for *M. aurum*, again using [^{14}C]-acetate as a precursor (Lacave *et al.*, 1990). The synthetic activity was found in an insoluble fraction extracted from disrupted *M.aurum* cells. The addition of isoniazid (INH) to both systems inhibited mycolic acid synthesis and led to the accumulation of non-hydroxylated fatty acids, implying that non-hydroxylated fatty acids are the precursors of mycolic acid.

Analysis of the [^{14}C]-acetate distribution in the newly synthesized mycolic acids of the cell-free system revealed that the [^{14}C]-acetate was not uniformly incorporated into the unsaturated, oxy, dicarboxy and meromycolates formed by the system (Lacave *et al.*, 1990b). Extraction and oxidation of the resulting mycolates fragmented the meromycolate chains at their double bonds and demonstrated that the methyl terminus and the next 18 carbon atoms were virtually unlabelled, indicating an endogenous C_{24}:1 *cis*5 precursor.

The C_{24} - C_{26} fatty acids are probably synthesized by fatty acid synthases (FASs) FASI and FASII. In *M. tuberculosis* this is a multifunctional enzyme system, a *de novo* synthase forms

C_{16} acids which are elongated to the C_{24} or C_{26} acids required as mycolate precursors.

Recently, *cis* tetracos-5-enoic (Δ^5 tetracosenoic acid) acid was positively identified as a putative precursor in mycolic acid biosynthesis (Wheeler *et al.*, 1993a). Following extraction with hexane to remove endogenous fatty acids, the incorporation of [^{14}C]- acetate into the α, α' and epoxymycolates of *M. smegmatis* was observed, following addition of the *cis* tetracosenoic as a precursor; other lengths of fatty acids had no effect. The use of a structural analogue in this system, methyl-4-(2-octadecylcyclopropene-1-yl) butanoate, inhibited mycolic acid biosynthesis (Wheeler *et al.*, 1993b).

The subsequent elongation of the C_{24} fatty acid or C_{24}:1 *cis*-5-precursor in mycolate synthesis could be initiated by FAS enzymes or specific elongases. Analysis of the cell free system of mycolate formation (Lacave *et al.*, 1990a) revealed that [^{14}C]-acetate incorporation into mycolates was up regulated by CoA, biotin, $KHCO_3$ and $MgCl_2$. As mycolate biosynthesis requires elongation, condensation, dehydrations and desaturation, Lacave *et al.*, (1990a) concluded that a multi-enzyme complex is likely to exist. The recently discovered *inhA* gene, when mutated or over-expressed, confers resistance to INH by preventing the inhibition of mycolic acid biosynthesis (Banerjee *et al.*, 1994). Initial characterisation of the gene and its product revealed homology to *env*M of *E. coli.* NAD/NADH binding and a β-ketoacyl-ACP-reductase gene downstream of the *inh*A gene suggested it was an enoyl-ACP-reductase and therefore, involved in elongation events. The 32kDa InhA protein has been expressed revealing its crystalline structure which contains an NADH binding site (Dessen *et al.*, 1995). The protein has recently been shown to catalyse the NADH-specific reduction of 2-trans-enoyl-ACP (Quemard *et al.*, 1995) and possibly exists as a multienzyme-complex involved in mycolic acid biosynthesis.

The incorporation of (S)-methyl-[^{14}C]-adenosyl-methionine provides the methyl groups for cyclopropane rings. This is thought to occur late in the pathway as incorporation of (S)-methyl-[^{14}C] adenosyl methane and unlabelled malonyl CoA gave C_{48}-C_{56} acids in a cell-free extract of *M. tuberculosis* H37Ra (Qureshi *et al.*, 1984). The correct double bond position was also essential for effective elongation. Recently, an *M. tuberculosis* gene *cma*1 has been isolated which confers the ability to form cyclopropane rings at the double bond positions of α mycolates in *M. smegmatis*, which is normally unable to produce cyclopropane rings (Yuan *et al.*, 1995). Regions of the *M. tuberculosis* DNA adjacent to the *cma1* gene display homology to other fatty acid synthases and may represent part of an operon involved in mycolic acid biosynthesis (Yuan *et al.*, 1995).

The mycolic acids of a number of mycobacteria have been found in the trehalose monomycolate (TM) form. These esters were thought to be carriers of the mycolic acids to the cell wall. The recently discovered Myc-PL may be a precursor of TM or may transfer the mycolate residue to trehalose (Besra *et al.*, 1994; Brennan and Nikaido, 1995) .

The proposed biosynthetic pathway described was for α mycolates of *M. tuberculosis*. Other studies indicated that a parallel pathway exists for the formation of oxygen containing mycolates (Quemard *et al.*, 1992). The absolute configuration of the methyl group in epoxymycolates when compared to the (S) methyl group adjacent to keto, methoxy and wax-ester function has ruled out epoxides as common intermediates (Minnikin, 1982). Metabolic studies with different chain lengths indicate that α mycolates are not precursors of oxygenated mycolates. Pathways for α mycolates and oxygenated mycolates appear to diverge from a common intermediate.

A strain of *M. smegmatis* with defective mycolate synthesis has been isolated (Kundu *et al.*, 1991) which is believed to be unable to undergo the Claisen type condensation as it only

produces short chain fatty acids. The isolation of other such mutants or those resistant to drugs which inhibit mycolate biosynthesis should help elucidate this pathway.

1.2.2.4 Phenolic glycolipids

The carbon atoms in the methyl branched structures of the mycocerosates and in phthiocerol had previously been shown to be derived from propionate (Gastambide-Odier *et al.*, 1963) (Fig.11b). The mycocerosates themselves have recently been shown to be synthesised by a mycocerosic acid synthase (MAS), a novel fatty acid elongating multifunctional enzyme from the *M. tuberculosis* complex. The operon encoding the MAS enzyme contains a ketoacyl synthase, acyl transferase, dehydratase, enoyl reductase, β-ketoreductase, and acyl carrier protein in that order (Mathur and Kolattukudy, 1992).

The phenol-phthiocerols come from *p*-hydroxybenzoate, which is then elongated to yield long-chain diols (Gastambide-Odier, 1970). The aromatic ring of the phenol can be derived from tyrosine but not from phenylalanine (Minnikin, 1982).

Recent experiments in which *M. microti* was grown in the presence of 2-[^{14}C]-propionate led to the isolation of two lipids which were identified as phthiocerol dimycoserosate and phenolphthiodiolone dimycocerosate, the aglycosyl derivatives of mycoside B (the PGL of *M. microti* and *M. bovis)*. Cell-free extracts of the organism were able to glycosylate the two lipids to form the PGL *in vitro*. The lipids are therefore believed to be intermediates in the biosynthesis of PGLs in mycobacteria (Thurman *et al.*, 1993).

1.2.2.5 Glycopeptidolipids

Recent experiments have begun to elucidate the biosynthetic pathway of GPLs using a genetic approach (Belisle *et al.*, 1991; Mills *et al.*, 1994). Initially, the gene cluster *ser2* encoding the oligosaccharide hapten responsible for the serovar specificity of *M. avium* serovar 2 was cloned and expressed in *M. smegmatis*. Expression of the *ser2* genes in *M. smegmatis* produced a recombinant serovar 2-specific GPL, with a non-specific GPL (nsGPL) *M. smegmatis* base and the serovar 2-specific disaccharide 2,3,di-0-methyl-α-L-fucopyranosyl-(1→3)-α-L-rhamnopyranose attached to the 6-deoxytalose of the nsGPL base (Belisle *et al.*, 1991). Subsequent transposon mutagenesis of the *ser2* gene cluster and screening of the recombinants for *ser2* expression revealed four essential loci spanning 14kb of *M. avium* DNA. These loci are believed to encode at least a rhamnosyltransferase, a fucosyltransferase and the methyltranferases required to methylate the fucose. It is possible the enzymes responsible for the *de novo* production of fucose are also encoded within this region (Mills *et al.*, 1994). Isolation of truncated versions of the recombinant GPL following transposon mutagenesis of *ser2* implied a chain of events in the biosynthetic pathway of the GPLs of serovar 2. Synthesis is thought to be initiated by the transfer of a rhamnose unit to the 6-deoxytalose of the peptide core followed by the addition of the fucose and subsequent methylation of the fucosyl unit.

1.2.3 FUNCTIONS ASSOCIATED WITH THE COMPONENTS OF THE CELL WALL

1.2.3.1 Antigenicity

The glycolipids present in the cell wall outer leaflet of a number mycobacterial species are antigenic as a result of their specific oligosaccharide components e.g. PGLs, LOSs, LAM and GPLs. The glycolipids can be sufficiently antigenic to evoke specific antibodies and thereby

allow serodiagnosis or serotyping of the mycobacterial species in question. The glycolipids, although antigenic, are not always immunogens; for example, *M. fortuitum* LOS reacts with antiserum raised against the whole bacteria strongly but only weakly against antibodies raised specifically against the glycolipid (Besra *et al.*, 1992).

Multiglycosylated species-specific glycopeptidolipids (ssGPLs) are the major surface glycolipids of *M. avium*, *M.intracellulare* and *M. scrofulaceum* species and their oligosaccharide components are the determinants of the 31 serovars within the MAIS complex. Glycopeptidolipids have also been isolated from *M. chelonae*, *M. peregrinum* and *M. absessus* (of the *M. fortuitum* complex) and *M. porcinum*. Non-specific singly glycosylated glycolipids are also found in these species.

M. gordonae is a serocomplex like the *M. avium* complex, but based on the trehalose containing LOSs rather than glycopeptidolipids as demonstrated by the dominant LOS of *M. gordonae* 990 reacting strongly with antiserum raised against it, but only very weakly with antiserum raised against *M. gordonae* 989 and *vice versa* (Besra *et al.*, 1993). The multiglycosylated LOSs of *M. kansasii*, *M. szulgai*, *M. gordonae* and *M. tuberculosis* Canetti are also highly antigenic (Brennan and Nikaido, 1995; Daffe *et al.*, 1991). The serological dominance is resident in the terminal non-reducing arrangements of the most polar LOSs from *M. kansasii*, *M. szulgai* and *M. gordonae* (Besra *et al.*, 1993).

PGLs are particularly antigenic evoking specific antibodies, e.g. in *M. leprae* recognition of PGL-1 with a monoclonal antibody (mAb) can be used to diagnose the disease. LAM found in all mycobacteria is also highly antigenic (Hunter *et al.*, 1986; Gaylord and Brennan, 1987).

1.2.3.2 Interaction of cell wall components with the host

1.2.3.1.1 PGLs, LOSs and GPLs

PGL-1 has also been implicated in the pathogenicity of *M. leprae*. It is believed to contribute to the intracellular survival of *M. leprae* within macrophages through its ability to scavenge oxygen radicals (Gaylord and Brennan, 1987). PGL-1 has also been implicated as an acceptor molecule on the bacilli for the C3 ligand which combines with the complement receptor to form a complex which initiates phagocytosis of the bacteria by the macrophage (Schlesinger and Horwitz, 1991). Ligation of complement receptors by C3 does not trigger an appreciable oxidative burst and ingestion of *M. leprae* is not accompanied by a significant oxygen burst. The C3 fixation to *M. leprae* has been shown to be mediated by natural antibody in the serum of nonimmune hosts (Schlesinger and Horwitz, 1994).

The LOSs are not thought to interact directly with the host but are thought to mask underlying virulence determinants such as LAM, preventing their pathogenic effects (Daffe *et al.*, 1991).

There is mounting evidence from *in vitro* studies that GPLs may play a role in host-parasite interactions. GPLs have been shown to be immunosuppresive, decreasing the lymphoproliferation of murine splenic lymphocytes (Brownback and Barrow, 1988). Subsequent experiments with β-lipid, essentially the peptide core of the GPLs, show it also had the ability to suppress lymphoproliferation of splenic lymphocytes (Tassell *et al.*, 1991). Purified GPL and β-lipid also induced secretion of tumour necrosis factor α (TNFα) (Pourshafie *et al.*, 1993). The peritoneal macrophage membranes were disturbed by both GPL and β-lipid which may have caused their altered function. Barrow *et al.*, (1993) gained similar

results with human peripheral blood mononuclear cells (HPBMs) and serovar 4 β-lipid.

The oligosaccharide moiety of serovar 4 or 2 was not immunosuppressive nor did it induce TNFα or prostaglandin 2 (PGE_2). However, *M. avium* serovar 8 GPL has been shown to induce secretion of PGE_2, which GPLs of serovars 4 and 2 were unable to do, implying induction was due to the variable oligosaccarides of the GPLs (Barrow *et al.*, 1995).

1.2.3.1.2 Trehalose 6,6'-dimycolate

Trehalose 6,6'-dimycolate (cord factor) has been shown to induce cytokine-mediated events such as systemic toxicity (Kato, 1973), anti-tumour activity (Lepoivre *et al*, 1982), granulomagenic activity and macrophage release of chemotactic factors (Matsunaga *et al* 1990). Cord factor also inhibits Ca^{2+} induced fusion between phospholipid vesicles (Spargo *et al.*, 1991) and migration of leukocytes (Goren and Brennan, 1979). Recently, lung inflammation produced by cord factor was shown to induce a coagulative response in murine lungs rather than the fibrinolytic response associated with lungs resistant to cord factor (Perez *et al.*, 1994).

1.2.3.1.3 Sulpholipids

Sulpholipids are thought to inhibit phagosome activation thus promoting intracellular survival of the pathogen and have been shown to have multiple affects on the signal transduction pathways leading to phagosome activation and priming (Zhang *et al.*, 1991b). They have been shown to prevent the release of superoxide (O^{2-}) by the macrophage-activating factors LPS gamma interferon (IFN-γ) interleukin-1β (IL-1β), TNFα and muramyl dipeptide. The inhibition of macrophage priming was accompanied by an increase in the secretion of IL-1β

and TNFα (Brozna *et al.*, 1991).

1.2.3.1.4 LAM

In earlier investigations, LAM of *M. leprae* and *M. tuberculosis* (now known to be ManLAM) was shown to inhibit T-cell proliferation (Kaplan *et al.*, 1987; Molloy *et al.*, 1990). The IFN-γ mediated activation of macrophages via T-cell lymphokines was also inhibited by the presence of *M. leprae* LAM in murine macrophages (Sibley *et al.*, 1988). Both inhibitory effects were prevented by the deacylation of LAM (Molloy *et al.*, 1990; Sibley *et al.*, 1988). LAM of *M. leprae* and *M. tuberculosis* was also shown to scavenge potentially cytotoxic oxygen free radicals and inhibit protein kinase C activity (Chan *et al.*, 1991). The scavenging was upregulated by deacylation of LAM (Chan *et al.*, 1991).

LAM has also been shown to induce an array of cytokines. AraLAM of the attenuated *Mycobacterium* strain initially thought to be *M. tuberculosis* H37Ra (Chatterjee *et al.*, 1992c) was shown to induce TNFα at levels 100-fold greater than those induced by ManLAM (Chatterjee *et al.*, 1992c). AraLAM was subsequently shown to increase production of TNFα, granulocyte macrophage-colony-stimulating factor (GM-CSF), IL-1α, IL-1β, IL-6, IL-8 and IL-10, generally produced by macrophages and resulting in increased antigen-induced T-cell proliferation (Adams *et al.*, 1993). ManLAM, lipomannan (LM) and PIMs were also found to induce the same cytokines to a lesser extent in both experiments. Deacylation of both types of LAM prevented the molecule inducing cytokines. (Chatterjee *et al.*, 1992c; Barnes *et al.*, 1992).

AraLAM has also been shown to induce macrophage early gene expression (c-*fos*, KC and JE) and TNFα in murine macrophages which ManLAM was unable to do (Roach *et al.*, 1994). AraLAM has recently been shown to be a potential stimulator of intracellular nitric oxide synthase (iNOS) expression whereas ManLAM was not; the inability of ManLAM to induce iNOS was not due to the induction of inactivating IL-10, as AraLAM also induced IL-10 production. IFN-γ was however found to completely inhibit the IL-10 production induced by AraLAM, implying an immunoregulatory role in determining the macrophage response (Roach *et al.*, 1995). The TNFα induction by AraLAM has been shown to be modulated through the binding activities of transcriptional factors, NF-kb and NBF1, as with LPS. ManLAM was found to be considerably less potent at stimulating NF-kb (Brown *et al.*, 1995). These experiments indicate that LAM is a potential regulator of macrophage functions.

Recently ManLAM has been shown to selectively enhance adherence of polystyrene microspheres to human monocyte derived macrophages whereas AraLAM did not. Adherence was decreased by down-modulating the mannose receptor on the macrophages or by removing the terminal mannose units of ManLAM, implying that ManLAM enhances adherence by interacting with the macrophage mannose receptors, providing a novel receptor-ligand pathway in phagocytosis of *M. tuberculosis* (Schlesinger *et al.*, 1994).

ManLAM from *M. leprae* has been shown to be presented to T-cells by human CD1b; this presentation required internalisation and endosomal acidification as well as the presence of mannosides with (1-2) linkages and a phosphatidylinositol unit. LAM, a lipoglycan, can act as an antigen and be recognised by human T-cells (Sieling *et al.*, 1995)

1.3 PERMEABILITY OF THE MYCOBACTERIAL CELL ENVELOPE

1.3.1 PERMEABILITY OF THE MYCOBACTERIAL CELL WALL

The mycobacterial cell wall acts as an effective permeability barrier to both hydrophilic and hydrophobic solutes. Hydrophilic molecules are expected to transverse the cell wall through porins whereas lipophilic solutes are more likely to diffuse through the lipid bilayer.

1.3.1.1 Permeability to hydrophilic solutes

The permeability to hydrophilic solutes has been tested in two non-pathogenic mycobacterial species, *M. chelonae* and *M. smegmatis* mc^2155 (Jarlier and Nikaido, 1990; Trias and Benz, 1994). Precise measurement of the permeability of mycobacteria has not been straightforward as attempts to measure uptake of radioactively-labelled drugs were hampered by drugs binding to their targets, an inability to distinguish between partition into the lipid interior of the walls from true entry across the barriers, unequal distribution caused by the proton motive force across the membrane and the modification of some drugs e.g. penicillins.

The Zimmermann-Rosselet method was used to measure the permeability of intact mycobacteria to hydrophilic cephalosporins by measuring their rate of hydrolysis. The cell wall permeability was calculated by assuming that the drug molecules diffused through the cell wall following Flick's first law of diffusion and that they were then hydrolysed by periplasmic β-lactamase, following Michaelis-Menten kinetics. The rates of hydrolysis were then used to determine the permeability coefficients. The cell wall permeability coefficient of *M. chelonae* ranged between $0.9x10^{-5}$-$10x10^{-5}$, three orders of magnitude lower than *E. coli* and one order of magnitude lower than the notoriously impermeable *Pseudomonas aeruginosa*. Penetration of the cephalosporins was not dependent on temperature or the hydrophobicity of the

cephalosporin and the rate of diffusion was directly proportional to the hydrated size of the cephalosporin. These results indicated that permeation occurred mainly through aqueous channels. The permeability to hydrophilic nutrient molecules such as glucose and glycerol was also low. It was also noted that cephalosporins with no net charge diffused more rapidly than monoanionic cephalosporins. The cell wall permeability of *M. smegmatis* mc^2155 and *M. tuberculosis* to hydrophilic solutes, e.g. hydroxalamine, has recently been investigated and found to be an order of magnitude higher than in *M. chelonae*, although this is still low in comparison to other bacteria (Trias and Benz, 1994; Brennan and Nikaido, 1995).

Porins (aqueous diffusion channels) have been identified in *M. chelonae* and *M. smegmatis* (Trias *et al.*, 1992; Trias and Benz, 1994). *M. chelonae* cell wall proteins were extracted, added to reconstituted liposomes and the permeability of the liposome investigated by studying the diffusion of hydrophilic compounds, e.g glycine, glucose and maltose (Trias *et al.*, 1992). The porin was formed by a 59kDa protein, which was found in small amounts in the cell wall. The porin formed was a water-filled channel with a large pore size, 2.2nm, and a low ion specificity (Trias *et al.*, 1992). The channels were voltage-gated; at 40mV of applied voltage the porin switched to a closed configuration; they were observed in an open, closed or flickering state in the reconstituted membranes (Trias *et al.*, 1992; Trias *et al.*, 1993).

The porins are cation-selective due to the presence of 2.5 negative point charges at the mouth of the pore (Trias *et al.*, 1993). The 59kDa protein appeared to be the only porin-like protein present in the cell wall of *M. chelonae*; the major 30kDa cell wall protein of *M. chelonae* showed no porin-like activity (Trias *et al.*, 1992).

Similar porins were also found in *M. smegmatis* mc^2155; they are non specific water filled cation selective channels with a 3nm diameter and four negative point charges (Trias and Benz, 1994).

1.3.1.2 Permeability to hydrophobic solutes

The lipid bilayer is generally permeable to hydrophobic solutes but the permeability is inversely proportional to the fluidity of the lipid membrane. The rigid organisation of the mycolates within the mycobacterial cell wall implies an inner region of very low fluidity. However, due to the relatively low permeability of the hydrophilic pathway, the hydrophobic pathway is of greater importance in mycobacteria. This is borne out by the large number of relatively lipophilic antimycobacterial agents e.g. tetracyclines, rifamycins, macrolides and fluoroquinolones. It has also emerged that the lipophilic derivatives of a number of these classes of antimycobacterials are more active against mycobacteria, presumably as a result of their improved penetration rate. The efficacy of the fluoroquinolones increases with increased lipophilicity, e.g. more hydrophobic derivatives such as WIN57273 and sparfloxacin, are more effective than ciprofloxacin against *M. leprae* (Franzblau and White 1990; Gelber *et al.*, 1992). The more hydrophobic tetracyclines such as minocycline and doxycycline are also more effective against mycobacteria than hydrophilic tetracyclines. There is no correlation between lipophilicity and efficacy in other bacteria such as *Staphylococcus aureus* because of the already rapid diffusion of the compounds across the lipid membrane (Nikaido and Thanassi, 1993).

The addition of alkyl groups to known antimycobacterials has also been known to increase their efficacy, for example the addition of palmitoyl to the hydrophilic agent INH, believed to

utilise the porin route, enhanced the efficacy of INH against *M. avium*, which appeared to have a low hydrophilic permeability (Rastogi and Goh, 1990).

The penetration rate of certain antimycobacterial agents is improved with an increase in temperature indicating the importance of the lipid pathway for diffusion in mycobacteria. For example the penetration rates of norfloxacin into *M. tuberculosis* increased six-fold with a 10°C increase in temperature (Brennan and Nikaido, 1995).

The permeability of different species of mycobacteria is variable in saprophytic fast-growing and soil-dwelling mycobacteria, e.g. *M. chelonae*, *M. fortuitum* and *M. smegmatis*, have less permeable outer membranes and are intrinsically more resistant to lipophilic antibiotics. This is presumably as they must defend themselves against antibiotics and other toxic molecules in their natural habitat. Members of the *M. avium* complex, although slow growing, are thought to exist in the soil and are also resistant to many lipophilic and hydrophilic agents and presumably have poor porin permeability as well. The mycobacteria pathogenic for mammalian hosts, e.g. *M. tuberculosis* and *M. leprae*, are more susceptible to lipophilic agents, dyes and detergents. This is illustrated by the resistance of all the soil-species mentioned to rifampicin which is active against *M. tuberculosis* and *M. leprae*.

It is thought that the resistance of the soil organisms stems from the type of mycolates found in these species. 41-75% of the α mycolates of *M. chelonae*, *M. fortuitum*, *M. smegmatis* and *M. phlei* contain *trans* double bonds at the proximal (inner) ends of the mycolates. The *trans* bonds decrease the fluidity and therefore the permeability of the innermost part of the bilayer (Brennan and Nikaido, 1995). The more susceptible species however have *cis* double bonds

or cyclopropane rings at this position, which would increase the fluidity and therefore the permeability of the innermost part of the cell wall (Brennan and Nikaido, 1995).

Some organisms exhibit intermediate levels of permeability with, for example, a low permeability to hydrophilic agents such as INH but susceptible to more hydrophobic compounds such as rifamycins e.g. *M. marinum*. In this case it is assumed that the porin pathway has an exceptionally low permeability.

Despite the low permeability of the mycobacterial cell wall the half-equilibration times (the time needed for intracellular concentration to reach one-half of the external concentration) across the cell wall are only a few minutes, much less than the generation time of the organisms.

1.3.1.3 Efflux systems

For an organism to display resistance the low permeability must act in synergy with another mechanism such as hydrolysis or efflux of the compound. Efflux systems play an important role in the resistance of other bacteria to antibacterials. There are currently four major efflux systems; the major facilitator family, the resistance-nodulation division family, staphylococcal multidrug resistance and the ABC adenosine triphosphate binding cassette. As yet no efflux systems have been identified in mycobacteria, although six ABC-type transporters genes have been identified in the *M. leprae* genome. The intrinsic resistance of *P. aeruginosa* was shown to be due as much to an efflux system as to the low permeability of the outer membrane. An efflux operon, *mexαmex*B-*opr*K, believed to function in the export of siderophores was recently discovered in *P. aeruginosa*; when inactivated by insertional mutagenesis the strain

became nearly as susceptible as *E. coli* to tetracycline and chloramphenicol (Poole *et al.*, 1993). The efflux system is thought to be the main reason the species displays the general drug-resistance phenotype and a similar system could exist in mycobacteria (Nikaido, 1994).

1.3.2 PERMEABILITY OF THE CELL MEMBRANE

Information available about the permeability of the cell membrane in mycobacteria is limited and has been gleaned through studies of nutrient uptake. Evidence for both active and facilitated diffusion of nutrients has been obtained. Glycerol is universally transported by facilitated diffusion and kinetic evidence for uptake of glycerol in mycobacteria suggests they are no exceptions (Connell and Nikaido, 1994). Studies in *M. smegmatis* showed that D-glutamic acid and D-aspartic acid are also taken up by passive diffusion, whereas D-alanine is taken up by active transport which would require a permease (Connell and Nikaido, 1994). Proline uptake in *M. phlei* has been the most extensively studied in membrane vesicles. It was found to be an active transport which was dependent on the oxidation of the substrate and had a requirement for Na^+ or Li^+ (Lee and Brodie, 1979). The Na^+ ions increased the activity of the active transport but not the specificity (Lee and Brodie, 1978). The transport was driven by an electrochemical gradient as it was found to be sensitive to uncouplers, which change the pH gradient, but not to ionophores which change the permeability of the membrane to ions (Lee *et al.*, 1979). These results suggested a symport system where the substrate and H^+ move across the membrane simultaneously driven by a proton-motive force created by a chemical gradient. The transport was also insensitive to respiratory inhibitors and is thought to bypass the respiratory chain using molecular oxygen as a terminal acceptor. A 20kDa monomeric protein was isolated from the membrane vesicles which when reconstituted into liposomes restored specific proline uptake (Lee *et al.*, 1979) and is likely to be a permease. The

membrane vesicles of *M. phlei* are also capable of active transport of glutamine and glutamic acid.

The *M. leprae* 46kDa protein is thought to be membrane associated; its exact function is unknown (Oskam *et al.*, 1995). The *M. leprae* genome sequencing project has also revealed 67 genes showing homology to known membrane transport proteins in other species.

1.4 COLONY MORPHOLOGY

The colony morphologies of strains of mycobacteria within a given species differ. This has previously been attributed to alterations in the components of the cell wall; recently cytosolic proteins have also been implicated (Prinzis *et al.*, 1994). There appears to be a complex link between morphology and virulence.

A number of cell wall components have been shown to help determine colony morphology. Strains of *M. kansasii* containing LOSs were found to be smooth variants, while those without LOSs had a rough colony morphology (Hunter *et al.*, 1985). Smooth *M. kansasii* were avirulent whereas rough colony variants of *M. kansasii* contained no LOS and were found to be virulent. The LOS-containing avirulent strain Canetti of *M. tuberculosis* has a smooth colony morphology, whereas virulent *M. tuberculosis* strains, e.g. H37Rv, have a rough colony morphology and are devoid of LOSs (Daffe *et al.*, 1991). However, subsequent work showed that a spontaneous rough mutant of the *M. tuberculosis* Canetti strain still contained LOS and that other smooth variants of *M. bovis* and *M. tuberculosis* H37Rv did contain LOS (Lemassu *et al.*, 1992).

LOS-containing strains of *M. fortuitum biovar fortuitum* also exhibit rough colony morphology. The LOSs of these strains are however elemental acyl trehaloses and the lack of a large oligosaccharide could account for their rough colony morphology (Besra *et al.*, 1992).

Three distinct colony forms are described for the *M. avium* complex; smooth transparent (SmT), smooth domed (SmD) and rough (Rg) (Vestal & Kubica, 1966; Belisle and Brennan, 1994).

The genetic basis of colony morphology has been investigated in *M. avium* serovar 2 (Belisle *et al.*, 1993b). The studies revealed that two types of Rg variant occur which arise from separate genomic deletions. Those Rg variants still able to produce the lipopeptide core had a deletion in the *ser2* cluster in the region responsible for the synthesis of the haptenic oligosaccharide. Those Rg variants that have lost total GPL had a large deletion to the right of the *ser2* gene cluster; this implied the region was encoding the lipopeptide or controlled the regulation of its production (Belisle *et al.*, 1993b).

The difference in morphology between the SmT and the SmD has recently been investigated. The smooth transparent variants have been shown to produce significantly larger quantities of nsGPL and generally more total GPL than the SmD variants (Belisle *et al.*, 1989). Subcellular fractions of the different morphological forms of *M. avium* serovar 2 and serovar 4 were extracted and analysed. Cell wall proteins were remarkably conserved but a novel cytosolic protein of 66 kDa was observed in large amounts in all batches of SmT cells and was barely visible in other variants (Prinzis *et al.*, 1994). Three proteins of 33.41 and 66 kDa have

been observed previously in smooth but not rough variants of *M. avium* serovar 20 (Ramasesh *et al.*, 1992).

1.5 ANTIMYCOBACTERIAL DRUG TARGETS

1.5.1 CELL WALL BIOSYNTHESIS

Cell wall biosynthesis is believed to be a target of a number of antimycobacterial drugs including ethambutol, ethionamide, INH and cycloserine.

1.5.1.2 Cycloserine

Cycloserine inhibits incorporation of D-alanine into the peptidoglycan precursor, by competitive inhibition of the alanine racemase and D-alanine:D-alanine synthase by binding to the D-alanine binding sites in the enzymes. Alanine racemase converts L-alanine to D-alanine (Winder, 1982).

1.5.1.3 Ethambutol

Ethambutol, a polyamine, inhibits the biosynthesis of the arabinan and galactan components of arabinogalactan (Takayama *et al.*, 1989; Deng *et al.*, 1995) and LAM to a lesser extent (Deng *et al.*, 1995). Treatment with ethambutol also results in the cleavage of arabinosyl residues; over 50% were removed from the cell wall following a 1hr exposure to the drug resulting in severe disruption to the cell wall (Deng *et al.*, 1995). Ethambutol also results in accumulation of the decaprenyl-P-arabinose implying that the drug interferes with the transfer of arabinose to the acceptor molecule from the donor, decaprenol carrier (Wolucka *et al.*, 1994). If ethambutol interferes with an arabinose transfer enzyme then an explanation is still required for the arabinosyl cleavage activity; this may be an inherent function noticed only

when arabinose synthesis is interrupted or the ethambutol may upregulate the function in some way (Deng *et al.*, 1995). Ethambutol may also inhibit early glucose biosynthesis, as its effects can be overcome by the addition of specific monosaccharides (Silve *et al.*, 1993). The exact mode of action remains unclear although the ability to transfer resistance between strains using cloned DNA fragments may elucidate the situation (Young, 1994).

1.5.1.3 INH and Ethionamide

INH and ethionamide are both believed to inhibit the biosynthesis of mycolic acids. Mycolic acid synthesis in whole cells of *M. tuberculosis* were shown to be inhibited by INH by following the incorporation of radioactively labelled [^{14}C]-acetate (Takayama *et al.*,1972). INH and ethionamide have both been shown to inhibit mycolic acid synthesis in cell-free systems of wall extracts from *M. aurum*, again inhibiting the incorporation of radioactively [^{14}C]-acetate into mycolates (Lacave *et al.*, 1990b; Quemard *et al.*, 1991 and 1992). The inhibition of mycolic acid biosynthesis by INH appears to be non-reversible in cell-free systems implying that INH is covalently bound to a component of mycolate biosynthesis (Quemard *et al.*, 1992).

In recent experiments an INH and ethionamide resistant strain of *M. smegmatis* (mc^{2}651), was isolated (Banerjee *et al.*, 1994). A genomic library was made from this strain and used to transform wild type *M. smegmatis*; recombinant clones were screened for their ability to confer drug resistance. A single gene, *inhA*, thought to encode a 32kDa enoyl-ACP-reductase protein (section 1.2.2.2), was found to confer INH resistance when present on a multicopy plasmid in *M. smegmatis* and *M. bovis* BCG(Banerjee *et al.*, 1994). A single point mutation was revealed in the *inhA* genes of resistant strains of *M. smegmatis* and *M. bovis* which

converted a serine to an alanine (Banerjee *et al.*, 1994) in the NADH binding site of the protein, suggesting that INH binds to this region of the protein preventing the NADH-specific reduction of 2-trans-enoyl-ACP (Dessen *et al.*, 1995; Quemard *et al.*, 1995). The InhA protein may represent a common target for both drugs and appears to explain the ability of INH to inhibit the mycolic acid synthesis.

The development of even higher levels of INH resistance (up to 0.5mg/ml) has been associated with the loss of catalase and peroxidase activities, which has been confirmed by genetic evidence. The gene encoding catalase-peroxidase *(katG)* was found to be absent in a number of resistant *M. tuberculosis* and *M. smegmatis* strains and transformation of these strains with the functional *katG* gene restored their INH sensitivity (Zhang *et al.*, 1992 and 1993). The sequencing of the entire *katG* gene (Heym *et al.*,1993) has allowed the use of PCR-SSCP on resistant clinical isolates of *M. tuberculosis* and *M. bovis* (Heym *et al.*, 1993). Sequencing of the PCR products has revealed missense mutations in the N-terminal peroxidase domain of the protein which reduces the enzyme activity and in the C-terminal domain of the protein (Heym *et al.*, 1995; Wilson *et al.*, 1995). INH is thought to act as a prodrug, being converted to an active form inside the bacteria, possibly by the catalase enzyme and it is this active form which appears to inhibit mycolic acid biosynthesis.

1.5.2 PROTEIN SYNTHESIS AND NUCLEIC ACIDS

Protein synthesis is the target of the aminoglycosides streptomycin, kanamycin and amikacin. The drugs are believed to bind to the 16S rRNA resulting in misreading of the genetic code and the inhibition of translational initiation. The ribosomal S12 protein is believed to stabilise a pseudoknot in the 530 loop of the 16S rRNA involved in the selection of cognate tRNAs

at the ribosomal A site. Streptomycin resistant *M. tuberculosis* strains have been found to have point mutations in the 530 loop of 16S rRNA and in their S12 protein *(rpsL)* genes (Finken *et al.*, 1993; Meier *et al.*, 1994; Honore and Cole., 1994).

Fluoroquinolones disrupt the bacterial chromosome by inhibiting the supercoiling activity of DNA gyrase, DNA topisomerase II. The DNA gyrase genes, *gyrA* and *gyrB*, of *M. tuberculosis* have been sequenced and resistant *M. tuberculosis* strains were found to have point mutations in the *gyrA* gene, causing mutations in the N-terminal sequence of the gyrase protein and presumably preventing binding of the fluroquinones (Takiff *et al.*, 1994).

Rifampicin inhibits transcription by interacting with the β-subunit of the RNA polymerase. The *M. leprae* and *M. tuberculosis rpoB* genes encoding the β-subunit have been cloned and analysed (Honore and Cole, 1993; Telenti *et al.*, 1993) Point mutations within the *rpoB* genes of *M. leprae*, *M. tuberculosis* and *M. avium* have been shown to confer resistance to rifampicin and have been found in two main regions of the gene, at a 23 aa cluster and at the serine-531, areas which correspond to the mutated regions in *E. coli* mutants.

1.5.3 PYRAZINAMIDE

Pyrazinamide is a bacteriostatic, prodrug only active against *M. tuberculosis* (Konno *et al.*, 1967; Stottmeier *et al.*, 1967). Pyrazinoic acid is the active form and has been shown to be active against resistant strains of *M. tuberculosis* (Konno *et al.*, 1967; Speirs *et al.*, 1995). *M. tuberculosis* sensitive and resistant strains are also susceptible to an ester of pyrazinoic acid, n-propyl-pyrazinoate (Speirs *et al.*, 1995). Pyrazinamide is converted to pyrazinoic acid by a pyrazinamidase enzyme, thought to be a pyrazinamidase/nicotinamidase enzyme, as cross

resistance to both nicotinamide and pyrazinamide has been observed in *M. tuberculosis* (Konno *et al.*, 1967). The activity of pyrazinamide is reliant on low pH (pH5.2) *in vitro*, not believed to reflect the intracellular conditions within phagocytic vesicles (Crowle *et al.*, 1991). The low pH is not a requirement for the action of the pyrazinamidase which has an optimal pH of 7.0 (Konno *et al.*, 1967) but it may be a requirement for the action of pyrazinoic acid on its target site. The specific site of action of pyrazinamide is unknown.

The natural resistance of *M. bovis* to pyrazinamide is believed to be due to a lack of active pyrazinamidase enzyme, as pyrazinoic acid is active against *M. bovis* (Konno *et al.*, 1967). The mechanisms of resistance of other mycobacterial species which possess pyrazinamidase, e.g. *M. smegmatis*, are unknown. Pyrazinoic acid has been shown to be inactive against *M. smegmatis* indicating poor uptake or an internal mechanism to prevent the action of the drug (Konno *et al.*, 1967). The resistance may result from a lower level of permeability to the drug, increased efflux of pyrazinamide or pyrazinoic acid, a lack of target site or a combination of a number of these factors.

1.6 MYCOBACTERIOPHAGES

Over 250 types of mycobacteriophage have been identified which have been reviewed by Grange and Redmond (1978). Mycobacteriophages have been widely employed for the phage typing of strains due to their variable host range and more recently for genetic studies.

1.6.1 STRUCTURE OF THE MYCOBACTERIOPHAGES

Mycobacteriophages generally have hexagonal or oval heads and long non-contractile tails (Grange and Redmond, 1978), although occasional variants have been found, e.g. the

contractile tail of I3. The head has been shown to consist of distinct circular capsonars, containing double stranded DNA (Grange and Redmond, 1978). Mycobacteriophages also show differences in the terminal structure of their tail and in the length and number of tail fibres. Mycobacteriophages also contain lipids which allow their inactivation by organic solvents i.e. chloroform and acetone (Castelnuovo *et al.*, 1970).

Mycobacteriophages infect by initial adsorption to a receptor site on the cell wall through a terminal structure on their tails. Tail termini vary between mycobacteriophages indicating that their receptor sites on the cell wall are also variable (Imadeda and San Blas, 1969). The simple apolar nsGPLs have been shown to be receptor sites for mycobacteriophage D4 in *M. smegmatis* (Goren *et al*, 1972) and other mycobacterial species. Other strains of *M. scrofulaceum*, *M. avium* and *Mycobacterium butyricum* were however unsusceptible to infection by D4. This has been shown to be due to the haptenic oligosaccarides of the ssGPLs which are thought to mask the terminal methylated rhamnose phage receptor site, thereby preventing phage adsorption and subsequent infection (Dhariwal *et al.*, 1986).

The pyruvulated simple LOSs of *M. smegmatis* have recently been shown to be the receptor sites for mycobacteriophage D29 (Besra *et al.*, 1994b). The LOSs in *M. smegmatis* strains pseudolysogenic for D29 were found to exhibit extensive *0*-methylation and *0*-acylation, increasing the hydrophobicity of the molecule and rendering the bacteria resistant to infection by further D29 mycobacteriophages (Besra *et al.*, 1994b). D29 is a lytic phage of *M.smegmatis*, that has also been shown to absorb to the surface of *M.leprae* (David *et al.*, 1978). Other phage receptor substances, although not fully characterized, appear to contain lipids and sugars and are probably lipopolysaccharides (Imaeda and San Blas, 1969; Grange

and Redmond, 1978).

1.6.2 MYCOBACTERIOPHAGE INFECTION

Infecting mycobacteriophages establish a lytic, lysogenic or pseudolysogenic relationship with the mycobacterial host.

1.6.2.1 Lytic and lysogenic infection

The mechanisms by which a lytic or a lysogenic infection are established have been greatly elucidated by the work of Hatfull, on the temperate mycobacteriophage L5. The whole linear genome (52kb) of L5 has been sequenced and found to contain 85 putative coding regions (Fig.13) including a central *att*P site with an adjacent integrase, *int* gene (Hatfull and Sarkis, 1993; Lee and Hatfull, 1991). The genes on the right arm are transcribed in the leftwards direction and control the lysogenic state while the genes on the left arm encode the assembly proteins and are transcribed in the rightwards direction (Hatfull and Sarkis, 1993; Donnelly-Wu *et al.*, 1993; Nesbit *et al.*, 1995). The lysogenic state is thought to be induced by the production of the gp71 protein shown to confer superinfection immunity (Donelley-Wu *et al.*, 1993). The protein is initially expressed from the P_{left} promoter and thought to be established by a phage encoded protein in the 72-82 region. This establishment protein prevents degradation of gp71 by the host enzymes; the P_{left} promoter is then repressed by gp71 and gp71 can initiate its own production from three promoters just upstream of it (P1, 2 and 3). The high expression is enough to maintain lysogeny despite still being degraded to some extent by the host proteins as the establishment protein is now switched off.

An imbalance in the gp71 and degradative host proteins is thought to lead to the induction of

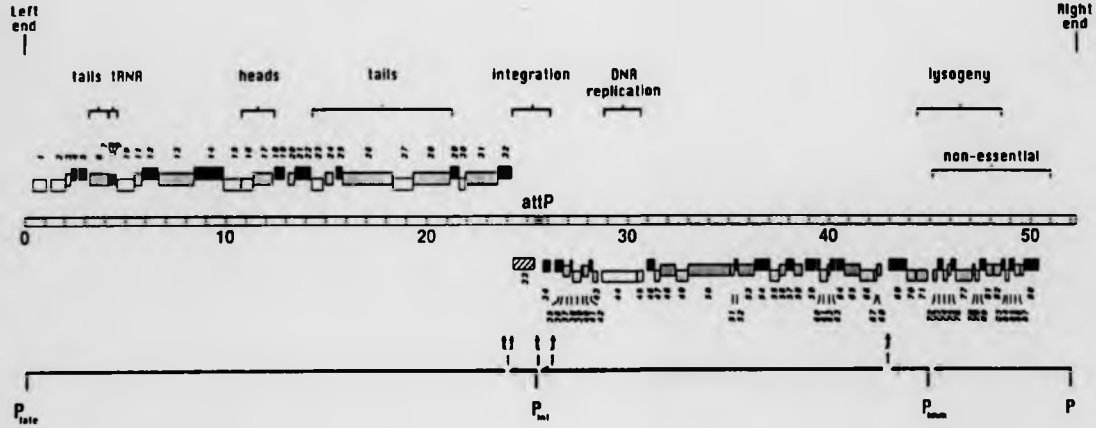


Fig. 13. **Map of the phage L5 genome**. The 52,927-bp genome of L5 is shown as the horizontal bar, with vertical bars spaced 1kb apart; the attachment site (attP) is located near the center of the genome. The shaded boxes represent the putative L5 genes with those above the genome being transcribed rightward (assembly proteins) and those below the genome being transcribed leftward (genes controlling the lysogenic state); the different shadings and vertical heights depict the reading frame for each gene. The functions of some of the genes are indicated. P represents the P_{left} promoter while P_{imm} represents the region containing the three promoters p1, p2 and p3 which regulate the gp71 repressor protein gene (Nesbit *et al.*, 1995) (taken from Hatfull and Jacobs, 1994).

the lytic cycle. Transcription from P_{left} is initiated due to reduced gp71 which will initiate production of the early lytic genes, resulting in the inhibition of host RNA polymerase and total shutdown of host protein production. Late lytic expression of left arm genes may overcome the host shutdown by modification of the host RNA polymerase (Nesbit *et al.*, 1995).

1.6.2.2 Pseudolysogeny

Pseudolysogeny is a state where the interaction of the phage with their host superficially stimulates the conditions of lysogeny, not replicating or integrating, but preventing further infection by other mycobacteriophage (Hayes, 1968). There are two mechanisms by which this is achieved; the first in which the host is infected with phage and strains are produced which appear to be resistant to phage infection, however the subsequent generations of the resistant strains are susceptible to infection. This is caused by lysed bacteria releasing an enzyme which removes all the phage receptors from the surrounding cells preventing their infection. The second is carrier state pseudolysogeny in which the phage exist as a non-integrating prophage within the cell which prevents infection. The carrier cells will only allow the transfer of the prophage to one daughter cell following replication and so a proportion of second generation cells become infectable. However, a proportion of unstable prophages will also enter the lytic cycle, reinfecting surrounding daughter cells no longer harbouring phage and so maintaining the pseudolysogenic state in subsequent generations.

Pseudolysogeny has been reported in a number of mycobacteria including *M. kansasii*, *M. chelonae*, *M. marinum* and *M. smegmatis* (Grange and Redmond, 1978).

1.7 MOLECULAR GENETICS OF MYCOBACTERIA

1.7.1 EXPRESSION SYSTEMS FOR RECOMBINANT DNA IN MYCOBACTERIA

1.7.1.2 Expression systems in E. coli

Early work in the isolation of mycobacterial genes was carried out using *E. coli*-based vectors and was mainly directed towards the cloning and expression of protein antigens for possible use in recombinant vaccines.

E. coli cosmid vectors such as pHC79 and Lorist 6 have been used to construct genomic libraries of mycobacteria such as *M. leprae* (Clark-Curtiss *et al.*, 1985; Eiglmeier *et al.*, 1993). However, it was found that mycobacterial genes were not always expressed in these *E. coli* vectors e.g. a citrate synthase mutant of *E.coli* could not be complemented by an *M. leprae* homolog present in pHC79; only when the *M. leprae* homolog was expressed from a *Streptococcus* promoter in the *E. coli* pYA626 was complementation observed (Clark-Curtiss *et al.*, 1985). Defective expression of mycobacterial genes in *E. coli* was widespread, but not complete e.g. BCG libraries in pBR322 (Moss *et al.*, 1987) and pEMBL3 (Thole *et al.*, 1985), without additional promoters were found to express the 65kDa antigen.

Recombinant DNA expression libraries of *M. leprae* and *M. tuberculosis* were constructed in the *E. coli* expression vector λgt11 to improve mycobacterial gene expression (Young *et al.*, 1985a and b). The DNA (1-7kb) was inserted into the β-galactosidase gene (*lacZ*) and genes were expressed as fusion proteins from *E. coli* transcription signals. Plaques formed were immunoblotted with monoclonal antibodies and polyclonal antisera (Young *et al.*, 1985a and b; Young *et al.*, 1987; Vega-Lopez *et al.*, 1988) and a number of *M. leprae* and *M. tuberculosis* antigen genes isolated, e.g. members of the heat shock protein family, such as the

65kDa (GroEL) of *M. tuberculosis* and *M. leprae* (Young *et al.*, 1985a and b)

The first mycobacterial genes, encoding protein antigens of *M. leprae* (Young *et al.*, 1985a; Clark-Curtiss *et al.*, 1985) *M. tuberculosis* (Young *et al.*, 1985b) and *M. bovis* BCG (Thole *et al.*, 1985) were therefore cloned and expressed in *E. coli* based vectors. Again, many mycobacterial genes could be not be expressed in these systems which was attributed to the inability of the *E. coli* transcriptional apparatus to recognise mycobacterial transcriptional signals and emphasised the need for systems to express recombinant genes encoding mycobacterial antigens and enzymes in surrogate mycobacterial hosts e.g. *M. smegmatis*, *M. bovis*, *M. vaccae* and *Mycobacterium* w (Snapper *et al.*, 1988; Jacobs *et al.*, 1991; Winter *et al.*, 1991; Garbe *et al.*, 1994).

1.7.1.2 Expression systems in Mycobacteria

The nature of the mycobacterial cell wall has made it difficult to extract the large amounts of recombinant DNA required for manipulation in expression systems, from mycobacteria. As a result the majority of vectors developed for use within mycobacteria are shuttle vectors, also able to replicate in *E. coli*.

1.7.1.2.1 Shuttle phasmids

Shuttle phasmids were among the first mycobacterial vectors to be developed and consisted of the *E. coli* cosmid pHC7 containing the regions of mycobacteriophage TM4 or L1 (phAE19) genome required for replication in mycobacteria (Jacobs *et al.*, 1987; Snapper *et al.*, 1988). The shuttle phasmids replicate as phages in mycobacteria, which can go into lytic or lysogenic cycle, and as cosmids in *E. coli*. In a lysogenic state these vectors provide the

means to introduce and express foreign genes in mycobacteria. The TM4 vectors did not form stable lysogens, unlike the L1 shuttle phasmid (phAE19) which proved capable of expressing the kanamycin resistance *aph* gene (Tn*903*), in *M. smegmatis* and *M. bovis* BCG and also prevented L1 superinfection (Snapper *et al.*, 1988). The size of DNA (2-4kb) which can be cloned into these vectors is limited by the requirements of mycobacteriophage packaging.

1.7.1.2.2 Shuttle plasmid-based expression systems

Plasmid shuttle vectors with an increased cloning capacity, a higher copy number (5 copies/ genome (Stover *et al.*, 1991)) and an improved ease of manipulation, compared to the shuttle phasmids, were developed for mycobacteria. When the first plasmid vector was produced no native plasmids had been identified in slow-growing mycobacteria, so a derivative of the *M. fortuitum* plasmid, pAL5000 (Labidi *et al.*, 1985) was created. The plasmid developed, pYUB12, was constructed from the *E. coli* pIJ666 plasmid (6.2kb), which contains the *Tn5* neomycin gene, the p15A origin of replication and the chloramphenicol acetyl transferase (CAT) gene and pAL5000. pIJ666 was randomly inserted into pAL5000 (5kb) and kanamycin selected transformants able to grow in *M. smegmatis* were isolated; the plasmid DNA from one of these transformants was used as the vector, pYUB12. (Snapper *et al.*, 1988 and 1990).

The pIJ666 plasmid was consistently inserted into one side of the plasmid implying that the other side of pAL5000 contained the mycobacterial origin of replication. pYUB72 contains a 2.6kb *Eco*RV-*Hpa*I fragment from the side of pAL5000 apparently essential for replication and was found to stably transform *M. smegmatis* and *M. bovis* BCG when ligated to the Tn*903 aph* gene (Snapper *et al.*, 1990). The number of open reading frames (ORFs) believed to be present in pAL5000 varies from two (Labidi *et al.*, 1992) to five (Rauzier *et al.*, 1988).

The *EcoRI-HpaI* region of the genome contains ORF1 (Labidi *et al.*, 1992) or ORF1, 2 and 5 (Rauzier *et al.*, 1988). Construction of second generation shuttle plasmids further localised the mycobacterial replicon to 1.8kb (Ranes *et al.*, 1990; Stover *et al.*, 1991).

Various derivatives of the pAL5000 plasmid replicon have been combined with *E. coli* plasmid replicons, e.g. ColE1 and p15A, to form shuttle vectors. The vector pMV261, an example of a second generation plasmid, also contains a multiple cloning site, the *M. leprae* hsp60 promoter and a transcriptional terminator (Stover *et al.*, 1991). Selection markers other than kanamycin cassettes have also been used, section 1.7.1.2.4.

1.7.1.2.2.1 Integrative vectors

Integrating plasmid vectors have been developed to express single copies of genes in mycobacteria that can be maintained for many generations without selection which may be useful for vaccine formation. These vectors can be formed by insertion of the integration region of a temperate phage or integrating plasmid.

The non-replicative integration-proficient vectors pMH94 and pMV361 were constructed by inserting the *attP-int* region of the mycobacteriophage L5 (Lee *et al.*, 1991) into pUC119 and pMV261 (in place of *oriM*) respectively (Lee *et al.*, 1991; Stover *et al.*, 1991). pMH94 was found to form stable recombinants in *M. smegmatis, M. tuberculosis* and *M. bovis* BCG (Lee *et al.*, 1991) while pMV361 has been found to stably express foreign genes i.e. HIV-1 gp120 as fusion proteins in *M. bovis* BCG (Stover *et al.*, 1991).

The integrating region of plasmid pSAM2 from *Streptomyces ambofaciens* was inserted into

a vector containing the *E.coli* pBR322 replicon and Tn*903* to create pTSN39, which stably integrated into *M. smegmatis* (Martin *et al.*, 1991). There is also an *attB* attachment site in the *M. leprae* chromosome (Eiglmeier *et al.*, 1991) so it may therefore be possible to follow the growth, viability and drug susceptibility using pTSN39 with a reporter gene e.g. luciferase (Jacobs *et al.*, 1993).

Suicide and conditional plasmids based on transposons have recently been created for insertional mutagenesis (section 1.7.3.2) (McAdam *et al.*, 1995; Guilhot *et al.*, 1994)

1.7.1.2.3 Shuttle cosmid-based expression systems

A number of *E. coli-Mycobacterium* shuttle cosmid vectors have been constructed appropriate for the formation of mycobacterial genomic libraries. The large insert size (30-40kb) allows the mycobacterial genome (3-5Mb) to be represented in approximately 100 clones and to provide the possibility for operons of genes encoding complex polysaccharides or lipids be cloned together, thereby increasing the likelihood of their expression; e.g. the glycolipids of *M. avium* cloned were expressed in *M. smegmatis* (Belisle *et al.*, 1991).

Shuttle cosmids have been constructed by the insertion of bacteriophage λ *cos* sequence into plasmid shuttle vectors e.g. λ*cos* was inserted into pYUB12 to form pYUB18 (Jacobs *et al.*,1991). Other shuttle cosmids include pMSC1 (Hinshelwood and Stoker, 1992a) and Tropist4 based on the λ origin double cos-site vector *E. coli* vector Lawrist4, the *Eco*Rv-*Hpa*I fragment of pAL5000 and a number of unique cloning sites (De Smet *et al.*, 1993).

1.7.1.2.3.1 Integrating cosmid vectors

An integrative cosmid vector pYU178 (5kb) has been developed which consists of the *oriE*, from pUC19, the L5 *attP-int* region, the *aph* kanamycin gene (Tn*903*) and the λ *cos* sequence (Pascopella *et al.*, 1994). This vector has been used to form integrating genomic libraries of *M. tuberculosis* H37Rv and *M. bovis* BCG in surrogate mycobacterial host genomes *M. tuberculosis* H37Ra (Pascopella *et al.*, 1994) and *M. smegmatis*, (Falcone *et al.*, 1995), respectively.

1.7.1.2.4 Selectable markers

The choice of antibiotic markers available for use in mycobacteria is limited by their natural resistance to a number of antibiotics. The *E. coli* kanamycin resistance genes from Tn*5* and Tn*903*, encoding aminoglycoside phosphotranferase are the most commonly used selection markers in mycobacteria (Snapper *et al.*, 1988; Ranes *et al.*, 1990; Stover *et al.*, 1991). Hygromycin was first used as a selectable marker in *M. smegmatis* and *M. bovis* BCG (Radford and Hodgson, 1991); since then problems encountered in recovering transformants from *M. tuberculosis* (Zhang *et al.*, 1992) *Mycobacterium vaccae* and *Mycobacterium* w (Garbe *et al.*, 1994), when using kanamycin resistance, have been overcome by the use of hygromycin as a selectable marker (Zhang *et al.*, 1993; Garbe *et al.*, 1994). Hygromycin is possibly more efficiently expressed than kanamycin in some *Mycobacterium* species as the gene is from *Streptomyces hygroscopicus*. It also provides a second selectable marker for genetic experiments in mycobacteria. Other selectable markers include the chloramphenicol acetyl transferase gene (*cat*) functional in *M. smegmatis* (Snapper *et al.*, 1988) and the mercury resistant gene (*mer*) from *Pseudomonas aeruginosa*, functional in *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis*. The mycobacteriophage L5 repressor gene 71 (Donnelly-Wu *et al.*, 1993), conferring superinfection immunity to phage L5, could be used as a selectable

marker in recombinant vaccines thereby avoiding the use of antibiotic selection which may be transferred to a pathogenic bacteria in the host. A green fluorescence protein, (GFP) has recently been used as a selectable marker in *M. smegmatis* and *M. bovis* BCG on agar and in liquid culture. GFP has the advantage of not requiring a substrate as it is detectable by exposure to ultraviolet (UV) light (Kremer *et al.*, 1995a).

1.7.1.2.5 Reporter genes

A number of reporter genes have been successfully used in mycobacteria. The *E. coli lacZ* gene was first used to form fusion proteins with mycobacterial genes in *E. coli* expression systems (Young *et al.*, 1985 a and b) and since has been successfully expressed in *M. smegmatis* and *M. bovis* BCG as a fusion protein (Timm *et al.*, 1994). Transcriptional fusions have also been created with the *lacZ* gene to isolate and analyse mycobacterial promoters from *M. smegmatis* and *M. bovis* BCG (Barletta *et al.*, 1992; Dellagostin *et al.*, 1995). The *lacZ* gene also proved a successful reporter gene for mycobacterial gene expression in *M. bovis* BCG within macrophages (Dellagostin *et al.*, 1995). *E. coli phoA* has proved a reliable reporter gene for the isolation of *M. tuberculosis* and *M. fortuitum* genes encoding exported proteins in an *M. smegmatis* host (Lim *et al.*, 1995; Kremer *et al.*,1995b; Timm *et al.*,1994). The *E. coli cat* gene (Das Gupta *et al.*, 1993) and the *xylE* (Curcic *et al.*, 1994) have also been used to analyse and isolate *M. tuberculosis* promoters. The green fluoresence protein *gfp* gene (Kremer *et al.*, 1995a) and the firefly luciferase *lux* gene (Jacobs *et al.*, 1993) are also important mycobacterial reporter genes.

1.7.1.2.6 Introduction of DNA into mycobacteria

The cell wall of mycobacteria make them difficult to transform with DNA. Protoplasts were

initially used to transform mycobacteria with plasmid and phage DNA at efficiencies of 10^3-10^4 pfu or cfu/μg of DNA (Jacobs *et al.*, 1987). This was superseded by electroporation with efficiencies of 10^4-10^6 cfu/μg in *M. smegmatis* mc^2155 (Snapper *et al.*, 1990) and *M. bovis* BCG (Snapper *et al.*, 1988; Jacobs *et al.*, 1991). *M. smegmatis* mc^2155 is an electroporation-efficient strain believed to have a mutation that affects plasmid replication or maintenance, as the efficiency of phage transfection and transformation of integrating plasmids is similar in mc^2155 and the parent *M. smegmatis* mc^26 (Snapper *et al.*, 1990). The introduction of DNA into *M. smegmatis*, *M. tuberculosis* and *M. bovis* BCG by means of recombinant mycobacteriophages e.g. phAE40 is even more efficient (Jacobs *et al.*, 1993). Conjugative transfer of plasmid DNA between *E. coli* and *M. smegmatis* has also been reported (Gormley and Davies., 1991).

1.7.1.2.7 Mycobacterial cloning host

M. bovis BCG has proved a useful host for the expression of foreign and mycobacterial genes (section 1.7.2.4) (Stover *et al.*, 1991 and 1993; Haeseleer *et al.*, 1993).

M. smegmatis is a useful model host for the expression of mycobacterial genes as it is a non-pathogenic fast-growing species. *M. smegmatis* has proved a successful surrogate host for the faithful expression of mycobacterial genes of *M. tuberculosis* (Zhang *et al.*, 1992; Garbe *et al.*, 1993; Yuan *et al.*, 1995), *M. avium* (Belisle *et al.*, 1991; Mills *et al.*, 1994) and *M. bovis* BCG (Falcone *et al.*, 1995). *M. smegmatis* is however not always the most appropriate host; for example hybrid superoxide dismutase enzyme is produced when the *M. tuberculosis sodA* gene is expressed in *M. smegmatis* (Zhang *et al.*, 1991; Garbe *et al.*, 1994). However, the recombinant *M. tuberculosis sodA* gene is expressed in the native form in *M. vaccae* (Garbe

et al., 1994) which may prove to be a more appropriate cloning host for some mycobacterial genes.

1.7.2 APPLICATIONS OF RECOMBINANT DNA SYSTEMS IN MYCOBACTERIA

Gene transfer systems for mycobacteria have provided an important new tool for the study of mechanisms of drug resistance (section 1.5), the generation of novel recombinant vaccines, the isolation and regulation of virulence factors and for genome mapping and sequencing.

1.7.2.1 Genome organisation and sequence

The formation of ordered genomic cosmid libraries of the two major mycobacterial pathogens *M. leprae* (Eiglmeier *et al.*, 1993) and *M. tuberculosis* (Cole and Smith, 1994) has unified the known genetic information and facilitated the project to sequence the genomes. The revealed sequence of both the *M. leprae* and the *M. tuberculosis* genome will provide an invaluable source of information (D. Smith, Collaborative Research Inc.) in the biochemical and immunological characterization of mycobacteria by identifying genes encoding relevant antigens and enzymes.

1.7.2.1.1 The M. leprae genome

The ordered cosmid library of *M. leprae* was made in Lorist 6 (Eiglmeier *et al.*, 1993) by blunt end ligation of 35-50kb fragments of *M. leprae* DNA into the vector, yielding 1000 clones. The clones were subjected to fingerprint analysis and chromosome walking experiments from which the inserts were grouped into four contigs. Attempts made to close the gaps in the chromosomal map failed as adjacent clones are often highly unstable and readily delete on subculturing, implying that the regions could be toxic sequences (Eiglmeier

et al., 1993). With average insert sizes of 40kb the genome size was estimated at 2.8Mb which was in keeping with the predicted 3.3 ± 0.5Mb (Clark-Curtiss *et al.*, 1985; Eiglmeier *et al.*, 1993).

Thirty known *M. leprae* genes were mapped to positions on the contig map (Fig.14) along with 13 housekeeping genes from *M. tuberculosis*, *E. coli*, *Bacillus subtilis* and various *Streptomyces* species, which, among others, enabled the *rpoB*, *rpoC*, *tuf*, *rpoD*, *aroA*, *asd* and *recA* genes of *M. leprae* to be identified. Most antigen coding sequences were found to be randomly distributed, whilst multiple copies of known *M. leprae* repetitive elements RLEPs, were found in some regions and not in others. A total of 29 RLEPs were identified which were grouped into four classes. Clustered RLEPs may represent a site recognised by proteins maintaining the structural integrity of the chromosome.

The ordered set of *M. leprae* clones provided the starting material for the systematic automated sequencing of the *M. leprae* genome by Collaborative Research Inc. The sequencing is carried out using the multiplex sequencing method (Church and Kiefer-Higgins, 1988) which is based on sample mixing and molecular decoding by oligonucleotide hybridisation (Fig. 15). Complete sequences are submitted to GenBank and MycDB (Bergh and Cole, 1994), which also contains all the physical mapping data from the *M. leprae* and *M. tuberculosis* ordered libraries. 2,238,856nt of *M. leprae* and *M. tuberculosis* DNA has been sequenced to date and 3,234 sequence objects identified (GenBank update 1995; World Wide Web site). Of the *M. leprae* ORFs identified just over half have been assigned functions based on their homologies with other known bacterial genes. The identified genes include those involved in DNA replication, recombination and repair, respiration, catabolism of amino

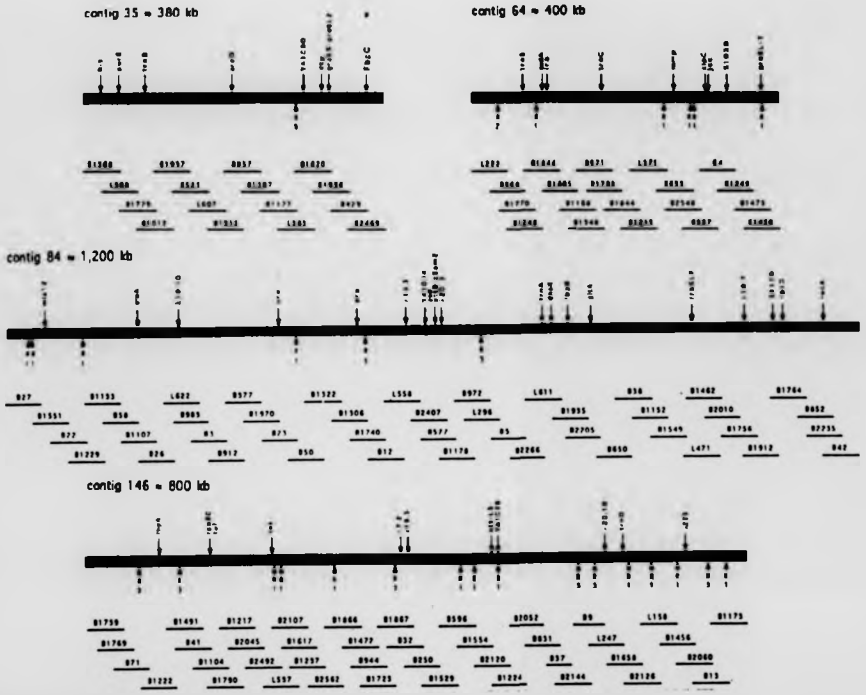


Fig. 14. **The contig map of the *M. leprae* genome formed using an ordered cosmid library**
The contig map shows the position of selected sets of cosmids, their overlapping regions are shown below the the contigs. The estimated size of each cosmid is also given. The positions of all known *M. leprae* genes (at the time of writing ,1993) and the RLEP sequences and their respective classes (1,2,3,5) (taken from Eiglmeier *et al.*, 1993).Contig 35 contains cosmid BI308 at its left hand end (see section 3.3.1).

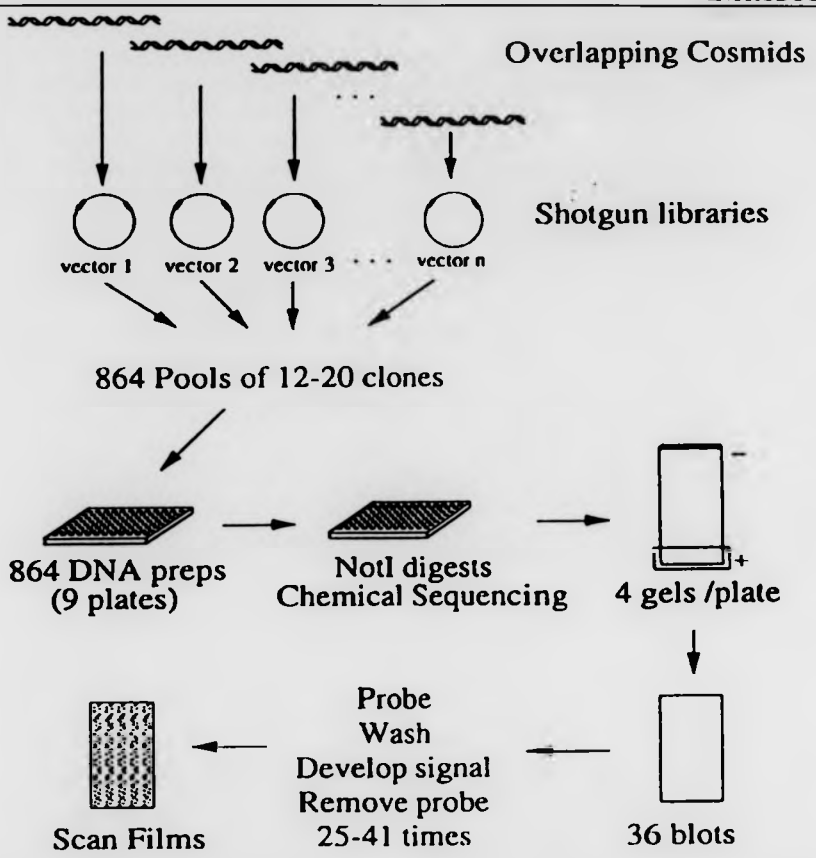


Fig .15 The sequencing strategy for the Genome Sequencing Projects of *M. leprae* and *M. tuberculosis*

High purity DNA preparations are made for each cosmid to be sequenced and a shotgun subclone library is then constructed in one of a set of 20 uniquely tagged "plex" vectors . Individual clones from 15-20 different subclone libraries are pooled and the DNA is purified from enough of the pools to guarantee a final eightfold random coverage for each cosmid. These samples are chemically sequenced, separated on polyacrylamide gels and transferred onto nylon membranes by using the direct transfer electrophoresis technique. The membranes are probed with labeled oligonucleotides to visualise sequence ladders from each subclone library individually. The membranes are stripped and reprobed upto 40 times providing a large number of films from each gel. The films are scanned using a CCD-based film or a lazer-scanning device, entered into the computer and assembled using the REPLICA and GTAC programmes (taken from Cole and Smith, 1994). Finished sequences are submitted electronically to GenBank and a mycobacterial mapping and sequence database MycDB.

acids, organic acids, and aromatic compounds, regulatory functions, nucleotide biosynthesis, fatty acid and lipid biosynthesis, protein and carbohydrate synthesis, transport across the cell wall and cell wall synthesis. Operons of genes have also been observed e.g. the polyketide synthesis gene complex, in cosmid B518.

Two sequenced *M. leprae* cosmids have been analysed in detail, cosmid B1790 (Honore *et al.*, 1993) and B961 (Fsihi and Cole, 1995). B1790 was found to contain 12 ORFs in 36,716bp, 40% of the coding capacity, which encoded 5 ribosomal proteins, two elongation factors, the β and β' subunits of ribosomal RNA and two unknown proteins. One of the unknown proteins, *end* gene is homologous to the *nfo* gene of *E. coli* and the apurinic endonuclease of yeast both of which are involved in the repairing the base-free lesions in DNA caused by free radicals which may be important for the survival of *M. leprae* in the macrophage, a prolific source of free-radicals (Honore *et al.*, 1993). B961 revealed 10 ORFs, 7 isocitrate catabolite genes, 2 ATP-dependent transport systems and a novel *polA* locus flanked by RLEPs, which may contain a promoter site (Fsihi and Cole, 1995).

Seventy-five percent of the codons end in G or C, initiation codons GTG and ATG were found and TAG was the most common termination codon in both cosmids (Honore *et al.*, 1993; Fsihi and Cole, 1995).

1.7.2.1.2 M. tuberculosis genome and sequencing

Two cosmid libraries of *M. tuberculosis*, in shuttle vector pYUB18 (Jacobs *et al.*,1991) and *E. coli* vector pYUB328 (Cole and Smith, 1994) are being ordered to form a map of the chromosome which currently contains 15 contigs. The contigs account for most of the

chromosome (4Mb) predicted by the *Dra*I physical map formed using pulse field gel electrophoresis (PFGE), with which the contigs can be correlated (Philip *et al.*, 1993).

Twenty five known *M. tuberculosis* genes have been mapped to cosmids, these include *katG*, *inhA*, *regX* and the fibronectin binding protein genes (Cole and Smith 1994). The one *M. tuberculosis* cosmid (TBC2, 32kb) sequenced so far was found to contain 23 ORFs which include the polyketide synthase operon and a homolog of the *M. bovis* BCG mycocerosic acid synthase gene (*mas*) (Cole and Smith, 1994); other cosmids are currently being sequenced.

1.7.2.2 Cloning of genes involved in cell wall biosynthesis

The products of genes involved in cell wall biosynthesis may be potential drug target sites and possible virulence determinants; a few of these genes have been cloned.

The *mas* gene of *M. bovis* BCG was cloned by N-terminal sequencing of the purified protein and screening of a λgt11 genomic library expression library (Mathur and Kolattukudy, 1992). The ORF was 6.3kb long, encoding a 22.5kDa elongating multifunctional enzyme with 5 domains: β-ketoacyl synthase, acyl transferase, dehydratase-enoyl reductase, β-ketoreductase and an acyl carrier protein (Mathur and Kolattukudy, 1992). The *M. leprae* and *M. tuberculosis accBC* genes, for the biotin carboxylase-biotin carrier protein have been cloned; the protein is believed to be a subunit of acyl-CoA carboxylase which carries out the first committed step fatty acid biosynthesis (Norman *et al.*, 1994). This was achieved by the selection of a false-positive, biotin containing protein when screening an *M. leprae* λgt11 due to the streptavidin detection system (Norman *et al.*, 1994). The *M. bovis* BCG NADP-dependent alcohol dehydrogenase gene, *adh* has been cloned, the protein is required for the

esterification of long chain alcohols to form cell wall waxes (Stelandre *et al.*, 1992).

The genes, *ser*2, involved in the biosynthesis of the oligosaccharide segment of the *M. avium* *ser*ovar 2-specific glycopeptidolipid were cloned and expressed in *M. smegmatis* from a pYUB18:*M. avium* genomic library (Belisle *et al.*, 1991). The region of the *M. avium* genome responsible for the expression has been narrowed down to 5.7kb, with 4 essential loci, *serA-D*; *serB* and *D* encode methyltransferases, *serA* rhamnosyltransferases and *serC* or *serD* the fucosyltransferases (Mills *et al.*, 1994) (section 1.2.2.5). *M. smegmatis* was also used as a surrogate host to clone and express the *M. tuberculosis cma1* gene responsible for the formation of cyclopropane rings at the double bond positions of *M. smegmatis* α mycolates (Yuan *et al.*, 1995). The *inhA* gene encodes an enoyl-ACP-reductase thought to be involved in mycolic acid biosynthesis (sections 1.2.2.2 and 1.5.1.3) (Banerjee *et al.*, 1994; Dessen *et al.*, 1995; Quemard *et al.*, 1995). The trehalose-6-phosphate synthases of *M. leprae* and *M. smegmatis* have been cloned and expressed (De Smet *et al.*, unpublished, 1995). The *M. bovis* BCG *meso*-diaminopimelate genes (*m-dap*) involved in peptidoglycan biosynthesis were cloned by complementation of *E. coli* mutants (Cirillo *et al.*, 1994). The *M. tuberculosis* UDP-N-acetyl-glucosamine-enolpyruval transferase (*murA*) gene which catalyses the first step in peptidoglycan biosynthesis has also been cloned (Kempsell *et al.*, 1995, unpublished).

1.7.2.3 Virulence determinants

The construction of integrative cosmid vectors has allowed the non-virulent H37Ra strain of *M. tuberculosis* to be transformed with the entire genome of the virulent strain H37Rv in an attempt to restore virulence. A 25kb genomic fragment, *ivg*, was isolated from the pYUB178::H37Rv library that conferred a more rapid growth rate of *M. tuberculosis* H37Ra

on passage through mice (Pascopella *et al.*, 1994). The same *in vivo* complementation technique has been used to restore virulence to attenuated strains of *M. bovis* as assessed by a guinea pig model and lead to the isolation of a 2.7kb fragment of DNA which restored virulence (Collins *et al.*, 1995). The complementing DNA encoded a principal sigma factor, *rpoV*, which was shown to contain a point mutation, that caused an arginine to histidine change in a region of the protein known to interact with promoters. The RpoV is homologous to the sigma factors of *M. smegmatis* (Predich *et al.*, 1995) and is likely to be involved in the regulation of virulence genes (Collins *et al.*, 1995). The formation of promoter probe and gene fusion libraries with reporter genes such as *lacZ* (Dellagostin *et al.*, 1995), *gfp* (Kremer *et al.*, 1995) and *phoP* (Timm *et al.*, 1994) are being used to investigate the regulation of genes expressed inside macrophages (Dellagostin *et al.*, 1995; Dhandayuthapani *et al.*, 1995;). The *phoA* reporter gene is also being used to isolate genes encoding secreted proteins (Lim *et al.*, 1995) which may also be virulence factors. The differential production of *M. bovis* BCG (Monahan *et al.*, 1994) and *M. tuberculosis* (Lee and Horwitz, 1995) proteins inside macrophages is also being studied and a number of potential virulence proteins have been observed. The differential intracellular gene expression of *M. avium* in human macrophages is also being analysed by subtractive RNA analysis (Plum and Clark-Curtiss, 1994).

1.7.2.4 Recombinant vaccines

The one of the goals for mycobacterial molecular genetics is the development of a recombinant BCG (rBCG) vaccine system delivering heterologous antigens. Foreign genes e.g. HIV-1p17 *gag* have been successfully expressed in *M. bovis* BCG (Stover *et al.*, 1991) using pMV261 and pMV361 and were shown to elicit an immune response. Foreign proteins expressed in rBCG have also been shown to be protective in animal models of disease e.g.

BCG expressing the OpsA protein of *Borellia burgdorferi* has been shown to elicit sterilising immunity in mice (Stover *et al.*, 1993).

The simultaneous expression and delivery of multiple foreign antigens is also a priority in the development of rBCG vaccine vectors. The simultaneous expression of two antigens from *P. falciparium* in rBCG (Haeseleer *et al.*, 1993) is an encouraging result for the potential production of multivalent rBCG vaccines, which would elicit a protective immune response to each foreign antigen.

Early experiments in *E. coli* expression vectors led to the isolation of a number of mycobacterial protein antigens in the search for protective antigens. It remains unclear which additional *M. tuberculosis* protein antigens would provide effective protection against infection when expressed from rBCG. *M. smegmatis* is also a potential vaccine delivery vehicle and has been shown to express immunogenic recombinant *M. bovis* BCG genes (Falcone *et al.*, 1995).

1.7.3 MUTAGENESIS IN MYCOBACTERIA

The isolation and characterisation of mutations in the genomes of mycobacterial species is crucial for the analysis of gene function and regulation. A variety of strategies for the creation of mutant strains of the fast and slow-growing mycobacterial species have been developed.

1.7.3.1 Chemical mutagenesis

A number of fast growing mycobacterial species i.e. *M. smegmatis* (Holland and Ratledge, 1971; Subramanyam *et al.*, 1989; Hinshelwood and Stoker, 1992b) *M. fortuitum*, *M. vaccae*

(Subramanyam *et al.*, 1989) and *M. phlei* (Konickova-Radochova *et al.*, 1970) have been successfully mutagenised using the chemical mutagen N-methyl-N'-nitro-N-nitrosguanidine (NTG). The percentage of stable auxotrophic mutants produced by the treatment varied from 0.02% in *M. vaccae* to 0.46% in *M. smegmatis* (Subramanyam *et al.*, 1989); the highest values were found in *M. smegmatis* and *M. phlei*. INH-enrichment of mutagenised cultures increased the efficacy of mutagenesis up to 0.9% in *M. smegmatis* (Holland and Ratledge, 1971). As INH is only effective against actively growing bacteria it enriches for slow growing auxotrophs. Other mutagens tested on *M. phlei* included UV irradiation, hydroxylamine and ethyl methanesulfonate, only the latter produced levels of stable mutants comparable with NTG (Konickova-Radochova., 1970).

1.7.3.2 Transposon mutagenesis

Transposon mutagenesis is an efficient means of generating libraries of insertion mutants. The mutant mycobacterial strains can be easily selected by antibiotic resistance marker, e.g. kanamycin, and the problems of clumping are avoided. Two transposon mutagenesis libraries have been formed in mycobacteria, one in the slow-growing *M.bovis* BCG (McAdam *et al.*, 1995) and the other in the fast-growing *M. smegmatis* (Guilhot *et al.*, 1994).

The *M. bovis* BCG mutagenesis procedure has employed the use of a suicide vector which is unable to replicate in *M. bovis* BCG, transformants can therefore be isolated by selection on the selectable marker encoded for by the transposon. The transposon was constructed by inserting the *aph* kanamycin resistance gene into the *M. smegmatis* IS*1096* insertion element. The transposon was cloned into a derivative of pMV261 in which the *oriM* had been disabled and which contained a tetracycline resistance gene *(tetr)*. The vector was transformed into *M.*

bovis BCG by electroporation and kanamycin resistant transformants (10^2-10^3 cfu/μg) selected. Of 923 transformants screened 3 auxotrophs (two leucine and one methionine) were isolated, an efficiency of 0.325%. The auxotrophs were all stable with reversion frequencies of $1x10^{-7}$-$2x10^{-7}$ (McAdam *et al.*, 1995).

The *M. smegmatis* transposon library was formed using a conditionally- replicating plasmid (Guilhot *et al.*, 1992). The plasmid, pCG79, is a thermosensitive shuttle vector (pCG63) that replicates at 30°C but not at 39°C, (due to the thermosensitive mutations for replication in the pAL5000 replicon) and that contains the Tn*611* transposon (Guilhot *et al.*, 1994). Stable auxotrophic mutants were isolated at frequencies of 0.1-0.4% with reversion rates of $2x10^{-6}$/cell.

Random shuttle insertional mutagenesis has also been successfully performed on an *M. smegmatis* plasmid DNA library in an *E. coli* host using an *E. coli* transposon Tn*5 seql*. The transposon containing recombinant plasmids were reintroduced into the *M. smegmatis* chromosome by homologous recombination and three auxotrophic mutants were isolated (Kalpana *et al.*, 1991). This approach has been used subsequently for mutational analysis of the *M. avium ser2* loci (Mills *et al.*, 1994).

1.7.3.3 Homologous recombination

Homologous recombination has been successfully employed in other bacterial systems to create specific chromosomal mutations to investigate gene functions. Although homologous recombination has been achieved in *M. smegmatis* (Husson *et al.*, 1990; Kalpana *et al.*, 1991) attempts to use this approach in the clinically important slow-growing *M. tuberculosis* and

M. bovis BCG have been unsuccessful (Kalpana *et al.*, 1991). *M. tuberculosis* and *M. bovis* BCG exhibit high levels of illegitimate recombination (Kalpana *et al.*, 1991; Colston and Davies, 1994) and single rather than double cross-over events obtained (Aldovini *et al.*, 1993). Recently, gene replacement has been achieved in *M. bovis* BCG, with the *accBC* gene (Norman *et al.*,1995) and the *M. tuberculosis* urease gene, *ureBC*, (Reyrat *et al.*, 1995), in *M. intracellulare* (Marklund *et al.*, 1995) and in *M. tuberculosis* with 40-50kb lengths of DNA (Balasubramanian *et al.*, 1996).

Homologous recombination may be limited by the unusual nature of the *M. tuberculosis* RecA protein which contains an additional internal sequence (intein), that has to be removed by a protein splicing mechanism (Davis *et al.*, 1991; Davis *et al.*, 1992; Davis *et al.*, 1994). A similar structure has been observed for *M. leprae* RecA but not other mycobacteria e.g. *M. smegmatis* (Davis *et al.*, 1994; Colston and Davis, 1994).

1.8 PROJECT AIMS AND OBJECTIVES

The main objective of this project was to isolate *M. leprae* genes encoding cell envelope biosynthetic enzymes using an *M. smegmatis* model system.

The cell envelope of mycobacteria is a complex structure which is believed to contain many of the elements responsible for the pathogenicity, toxicity and virulence associated with *M. leprae*, *M. tuberculosis* and *M. avium*. The cell envelope also confers a permeability barrier that underlies the resistance of mycobacteria to many existing antibacterial agents. The isolation and subsequent mutational analysis of cell envelope genes should elucidate the function and biosynthetic pathways of encoded cell envelope components. Ultimately, this

may lead to the design of target specific antimycobacterial drugs.

The inability to culture *M. leprae* has made it extremely difficult to determine the functions of *M. leprae* components. The proven ability to express *M. leprae* genes in a surrogate mycobacterial host e.g. *M. smegmatis* would provide a useful system for the further study of *M. leprae* and so the decision was made to try and isolate *M. leprae* genes functional in an *M. smegmatis* host.

The aim of the project was to isolate a cell envelope mutant of *M. smegmatis* and to complement the mutation with a functional, possibly homologous *M. leprae* gene from an *M. leprae* genomic shuttle cosmid library.

The isolation of a cell envelope mutant of mycobacteria is difficult as there are no clear cut phenotypes to select such mutants. Phenotypic changes which may define cell envelope mutations include alterations in antibiotic resistance and sensitivity, phage resistance, the binding of mAb specific to cell wall components, e.g. PGL-1, and binding of lectins to carbohydrate components of the cell wall. A single cell wall mutant of *M. smegmatis*, defective in its mycolate formation (Kundu *et al.*, 1991), had been isolated at the initiation of this project. This mutant was isolated by the screening of *M. smegmatis* mutants for increased sensitivity to penicillin G.

1.8.1 THE ISOLATION OF AN *M. SMEGMATIS* MUTANT

The initial aim was to screen *M. smegmatis* mc^2155 for spontaneous resistance to mycobacteriophage which may be caused by the alteration of the phage receptor in the

mycobacterial cell wall.

The screening for spontaneous phage resistance proved unsuccessful and it was decided to form an *M. smegmatis* mutant bank which could be screened for a number phenotypic changes. It was decided to create mutant *M. smegmatis* mutants using the chemical mutagen NTG shown to generate 0.1-0.9% auxotrophic mutants (Holland and Ratledge, 1971) as the methodology was available in the laboratory (Hinshelwood and Stoker, 1992b). Transposon mutagenesis was not available and as NTG produces point mutations rather than truncated proteins it would possibly result in fewer lethal mutations of cell wall components.

1.8.1.1 Screening methods for potential cell envelope mutants of M. smegmatis

A bank of these treated mutants were screened for resistance to lytic mycobacteriophage, e.g. D29, D4, D33, and changes in antibiotic sensitivity and resistance.

Lytic mycobacteriophage were used, some which have known receptor sites on the cell wall e.g. D29, the LOSs (Besra *et al.*, 1994b) and D4 the GPLs (Goren *et al.*, 1972).

Resistance to antibiotics could be caused either by a change in the permeability of the cell envelope or by an alteration in the drug target sites. NTG-treated *M. smegmatis* strains were therefore mainly screened for resistance to drugs whose target site is thought to be associated with cell wall biosynthesis i.e. ethambutol, ethionamide and cycloserine; as well as for resistance to other antibiotics in which resistance could be caused by a decreased permeability.

Increased antibiotic sensitivity could be due to increased cell envelope permeability, the NTG-treated strains were therefore screened for increased sensitivity to penicillin G and pyrazinamide, to which *M. smegmatis* is naturally resistant; this was a similar method to that employed by Kundu *et al.*(1991) to isolate the mycolate mutant of *M. smegmatis*.

Any mutants isolated would be fully characterised in an attempt to determine the target of the mutation, the analysis would include biochemical analysis of the lipid components of the cell wall to look for any alterations in the components of the cell wall (Dobson *et al.*, 1985).

1.8.1 COMPLEMENTATION WITH *M. LEPRAE* GENOMIC LIBRARY

Having isolated an *M. smegmatis* mutant the aim was to complement the mutation using an *M. leprae* cosmid library. The mutant strain would be transformed with the *M. leprae*::pYUB18 cosmid library. It was elected to use a cosmid library because if the complementing *M. leprae* gene was part of an operon it was more likely to be expressed. Operons of mycobacterial genes have been shown to be expressed in surrogate hosts i.e. the *ser2* genes of *M. avium* have been expressed in *M. smegmatis* (Belisle *et al.*, 1991). The complementing region of the cosmid DNA would then be isolated by the formation of a sublibrary of the cosmid DNA in the plasmid pMV206. The complementing DNA could also be sent for mapping to the ordered *M. leprae* cosmid library (Eiglmeier *et al.*, 1993), to determine whether it had been sequenced in the *M. leprae* genome sequencing project.

This thesis describes the formation of a bank of *M. smegmatis* mc^2155 NTG treated cells with 0.1-0.2% levels of auxotrophy and the isolation of a number of *M. smegmatis* mutants including a pyrazinamide sensitive *M. smegmatis* mutant, with decreased hydrophobicity

which has been complemented using the *M. leprae* pYUB18 genomic cosmid library. The complementing region of *M. leprae* DNA, which restores pyrazinamide resistance to the *M. smegmatis* mutant, is 3.5kb region of the *M. leprae* B1308 cosmid and has been shown to contain three complete putative *M. leprae* open reading frames (MycDB and GenBank, 1995; D. Smith, Collaborative Research, Inc).

CHAPTER 2

MATERIALS AND METHODS

2.1 DETERMINATION OF CONCENTRATION AND PURITY OF DNA SOLUTIONS

100-fold dilutions of sample DNA were prepared with sterile distilled water (SDW) in 1.5ml microfuge tubes. A quartz cuvette containing SDW was used to blank a UV spectrophotometer set at a wavelength of 260nm. 1ml of the dilute DNA solution was placed in the quartz cuvette and placed in the spectrophotometer and an absorbance (A_{260}) reading taken. This was repeated with the spectrophotometer set at a 280nm wavelength. The concentration and purity of the DNA was determined whereby $1.0A_{260}$=50µg/ml of double stranded DNA and the sample is pure when $A_{260}/_{280}$=1.8or greater, as stated in Sambrook *et al.*, (1989).

2.2 GEL ELECTROPHORESIS

Electrophoresis in agarose gels was used according to the methods described in Sambrook *et al.*, (1989). Agarose gel electrophoresis was carried out using 0.8% agarose (Electrophoresis grade agarose, Gibco, BRL) containing 0.2μg/ml ethidium bromide (Sigma), in TAE buffer (Appendix B), unless otherwise stated. Medium gels 16.5x12cm (Flowgen) or 15x14cm (NBL) were run at 80V for 3-4hr or at 15V for 16-20hr. Minigels 7x7.5 cm (NBL) were run at 100mA for 20-40min. 2μl of bromophenol blue loading dye (0.25% w/v bromophenol blue [Sigma], 0.25% w/v xylene cyanol FF [Sigma], 30%v/v glycerol [BDH]) were added for every 10μl of DNA sample prior to loading on the gel for samples >1.5kb. Orange G dye (0.25%w/v Orange G [Sigma], 30%v/v glycerol [BDH]) was used with DNA samples <1.5kb in size, often PCR products, as the dye runs ahead of small products ensuring the DNA remains visible under UV light. 3μl of Orange G were used for every 10μl of DNA sample to be loaded. DNA size markers λ*Hin*dIII (λH3 [Promega]) and λ*Hin*dIII/*Eco*RI (λH3E1 [Promega]) were diluted with bromophenol blue loading buffer and SDW to give a

concentration of 0.05μg/μl and 10μl run on gels to determine the size of the sample DNA loaded. For DNA samples <1.5kb such as PCR products a 123bp ladder (NBL) was diluted with SDW and Orange G loading buffer to a concentration of 0.05μg/μl and 10μl were loaded. The DNA was visualised on a UV transilluminator and recorded photographically with a Gel Documentation System (UVP)

2.3 PHENOL EXTRACTION OF DNA

Phenol extractions were performed as stated in Sambrook *et al.*, (1989). The aqueous DNA solution was made up to at least 200μl with SDW when extracting impurities. An equal volume of TE-equilibrated phenol pH 7.5 (Sigma) was added to the DNA , in a 1.5ml microfuge tube (unless otherwise stated). The solution was mixed by inversion, and the two phases separated by centrifugation at 10,000xg (13,000rpm) in a microfuge tube, (unless otherwise stated) for 1min. The upper aqueous phase was transferred to a new 1.5ml microfuge tube taking care not to carry over any debris from the interface. An equal volume of phenol: chloroform: isoamylalcohol (25:24:1) (Sigma) was added, the phases mixed and then separated by centrifugation. Again the aqueous phase was removed and re-extracted with an equal volume of chloroform:isoamylalcohol (24:1) (Sigma), as above. The DNA was then recovered from the final aqueous solution by ethanol precipitation (section 2.4).

2.4 ETHANOL PRECIPITATION OF DNA.

Ethanol precipitations were performed as stated in Sambrook *et al.*, (1989). DNA was precipitated from aqueous solution by the addition of 0.1 volume 3M Na acetate pH4.8, and 2 volumes 100% ethanol and followed by incubation at -20°C for 30min. The solution was then centrifuged at at 10,000xg (13,000rpm) for 15min to pellet the DNA. The excess ethanol was removed with a 200μl Gilson pipette, taking care not to dislodge the DNA pellet. The DNA pellet was washed, to remove excess salt by resuspension in a 0.1 volume of 70% ethanol followed by centrifugation as above. The 70% ethanol was removed and the DNA

pellet was air-dried and resuspended in TE buffer (Appendix B).

2.5 DNA MODIFICATION PROCEDURES

The majority of these procedures were carried out as stated in Sambrook *et al.*, (1989), unless otherwise stated.

2.5.1 RESTRICTION ENZYME DIGESTS

Restriction enzyme digestions were carried out according to the manufacturers instructions i.e. with one unit of enzyme/μg of DNA present in the reaction volume. Reaction buffers were added in 0.1 or 0.2 volumes depending on the concentration of the buffer provided by the manufacturer. The reactions were usually carried out in 20μl for small digests (1μg) and 100μl for large scale digests (10-20μg). Care was taken to ensure that the volume of enzyme added did not exceed 1/10th of the entire reaction volume, above which the glycerol in the enzyme storage buffer could become inhibitory. The reactions were left to proceed at 37°C, unless otherwise stated, for 1hr or overnight. Restriction enzymes were supplied by Promega, Gibco BRL and Boehringer Mannheim.

2.5.2 ALKALINE PHOSPHATASE TREATMENT

Alkaline phosphatase treatment was used to prevent the re-ligation of compatible ends of linear DNA, when required. The linear DNA was treated with 0.1 unit/μg of calf intestinal alkaline phosphatase (CIAP, Boehringer Mannheim) in a reaction volume containing 0.1 volume of 10x CIAP buffer, at 37°C for 15min. The solution was then heated at 72°C for 10min to halt the reaction and the DNA cleaned using the Wizard DNA Clean Up Kit (Promega; section 2.6.1).

2.5.3 DNA END-REPAIR

End repair of sonicated insert was performed to create blunt ended DNA. 2.5mM dNTPs and

2M $MgCl_2$ were added in 1/10th volumes to a given volume of DNA solution resulting in a final concentration of 0.5mM dNTPs and 50mM $MgCl_2$. 1.5μl (5-10u/μl) of T4 DNA polymerase (Promega) were added to the solution which was left at room temperature for 15 min. This was followed by the addition of 1μl (5-10 units/μl) Klenow DNA polymerase (Promega) and a further 15min incubation at room temperature. The reaction was inhibited by heating at 68°C for 15min.

2.5.4 DNA LIGATION

Ligation reactions were usually performed in a 10μl reaction volume containing 750ng of insert DNA, 250ng of vector DNA, 1μl of 10x ligase buffer and 3 units of T4 DNA ligase enzyme (3,000u/ml, Boehringer Mannheim). The reactions were left to proceed overnight at 4°C.

2.6 DNA PURIFICATION

Following restriction enzyme digestion, alkaline phosphatase treatment or DNA size selection from agarose gels, DNA samples required purification. The removal of enzymes, buffer and agarose allowed the DNA to be successfully manipulated in further reactions.

2.6.1. WIZARD CLEAN UP SYSTEM (PROMEGA)

DNA purification was achieved with the Wizard DNA Clean Up Kit produced by Promega. The kit was used according to the manufacturers instructions. 1ml of DNA purification resin was added to the designated DNA solution in a 1.5ml microfuge tube and mixed by gentle inversion. On purification of DNA from agarose, the DNA band was excised using a sterile scalpel blade in approximately 300mg of agarose and transferred to a 1.5ml microfuge tube. The agarose was then incubated at 70°C, with 3x w/v 6M NaI until it had completely dissolved, before adding the resin. A 2ml disposable syringe was taken, the plunger removed and the syringe attached to the barrel of a Wizard minicolumn. The resin/DNA mix was

pipetted into the syringe and the slurry gently pushed through the minicolumn with the syringe plunger. The syringe was removed from the minicolumn, its plunger was removed and the syringe reattached to the minicolumn. 2ml of 80% isopropanol were then pipetted into the syringe and gently pushed through the minicolumn with the plunger. The minicolumn was then transferred to a 1.5ml microfuge tube and centrifuged for 1min at 10,000xg (13,000 rpm) to dry the resin. The minicolumn was transferred to a new 1.5ml microfuge tube and 30-50μl of TE buffer pre-heated to 70°C was added to the minicolumn and left for 1min. The minicolumn was then centrifuged at 10,000xg (13,000rpm) for 1min to elute the DNA. The 30-50μl of DNA sample were stored at -20°C.

2.7 CULTURE OF *E. COLI*

Cultures were grown in Luria Broth (LB; Appendix A) and incubated at 37°C with shaking (200rpm), for 16-20hr, or on LB plates and incubated at 37°C for 16-20hr. Antibiotic selection for plasmid or cosmid maintenance was achieved by the addition of kanamycin (Sigma) or ampicillin (Sigma) to give a final concentration of 50μg/ml.

2.8 PRODUCTION OF COMPETENT *E. COLI* CELLS.

The following method was described by D. Hanahan and developed by V. Simanis (Imperial College London). 100ml of SOB (Appendix A) in a 1l flask was inoculated with several, 2-3 mm, *E. coli* colonies grown overnight on minimal media, at 37°C. The culture was incubated for 4 hours at 37°C until the cell density was 4-7 x10^7 viable cells/ml (A_{600} 0.6-0.8). The culture was transferred to two chilled 50ml polypropylene tubes and incubated on ice for 15min. The cells were pelleted by centrifugation at 3500xg (4,000rpm) for 15min at 4°C. The supernatant was discarded and the pellet gently resuspended in 1/3 volume of RF1 (100mM $RbCl_2$, 50mM $MnCl_2.4H_2O$, 30mM K acetate, 10mM $CaCl_2.2H_2O$, glycerol 15%w/v, pH5.8) and the cell suspension incubated on ice for 15min. The cells were pelleted, as before, resuspended in 1/25 volume RF2 (10mM MOPS, [BDH] 10mM $RbCl_2$, 75mM $CaCl_2$, 15%

glycerol, pH6.8) and incubated on ice for a further 15min. 1ml aliquots of the cell suspension were then distributed into chilled screwcapped tubes, snap frozen in an ethanol/dry ice bath and stored at -70°C.

2.9 TRANSFORMATION OF *E. COLI*

Transformation of competent *E. coli* was carried out using the method described in Sambrook *et al.*, (1989). A 200μl aliquot of competent *E. coli* cells (section 2.8), thawed on ice, was added to a pre-chilled 1.5ml microfuge tube containing 1μg of the appropriate DNA solution. The cells were incubated on ice for 30min, heat shocked at 42°C for 90s and returned to ice for 2min. The cells were then inoculated into 1ml of LB and incubated at 37°C with shaking for 1 hr. When small (ng) or unknown amounts of DNA were used the cells were pelleted by centrifugation at 3500xg (4,000rpm), resuspended in 200μl of LB and spread on LB agar plates (6cm), containing the appropriate antibiotic. When larger amounts of DNA (μg) were used 200μl (1/5 volume) of cell culture was spread onto the plate, without pelleting the cells first. All plates were then incubated at 37°C for 16-20hr to allow colony formation.

2.10 TRANSFORMATION OF *E. COLI* WITH *M.LEPRAE* COSMID LIBRARY PHAGE LYSATE

This method was carried out as stated by Eiglmeier *et al.*, (1993). 100ml of LB supplemented with 0.02% maltose and 10mM $MgSO_4$ were inoculated with 2-3 colonies of *E. coli* NM554 and the culture was incubated at 37°C for 3-4hr with shaking (200rpm). When the culture reached late log-phase growth (0.6-0.8A_{600}), the cells were pelleted by centrifugation at 3500xg (4,000rpm) in 50ml polypropylene tubes. The cells were washed twice in 10ml of 10mM $MgSO_4$ and finally resuspended in 8ml LB. 100μl of cell suspension was placed in a 1.5ml microfuge tube and 5μl of phage lysate added. The cells were left at room temperature for 30min whereupon 800μl of LB were added and the solution was incubated at 37°C

(without shaking) for 1hr. The cells were then pelleted by centrifugation at 10,000xg (13,000rpm) for 2min and resuspended in 200μl LB. The cells were then spread on selective LB agar plates containing kanamycin at a concentration of 50μg/ml.

2.11 SMALL SCALE PLASMID PREPARATION FROM *E. COLI*

All plasmid DNA isolation from *E. coli* was based on the alkaline lysis method of Birnboim and Doly (1979).

Cells from a 5ml overnight culture, incubated at 37°C in Luria broth (LB) containing 50μg/ml of the selective antibiotic, were pelleted by centrifugation at 3500xg (4,000rpm) for 7min. The pellet was resuspended in 200μl of GET (50mM glucose, 70mM EDTA, 50mM Tris-Cl pH8) containing 2mg/ml lysozyme (Sigma), transfered to a 1.5ml microfuge tube and placed on ice for 5min. 400μl of alkaline SDS solution (0.2M NaOH, 1%SDS) were added, the solution mixed thoroughly and left on ice for 5min. This was followed by the addition of 300μl of 3M sodium acetate pH4.8 and a 5min incubation on ice. The precipitate was pelleted by centrifugation at 10,000xg for 10min and the supernatant was decanted into a clean 1.5ml microfuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added to the supernatant, the solutions mixed by inversion and centrifuged at 10,000xg for 1min. The upper aqueous layer was retained, transferred to another 1.5ml microfuge and the extraction procedure repeated. A 0.5 volume of isopropanol (BDH) was added to the retained aqueous upper layer, the solution was left at room temperature for 15min before being centrifuged at 10,000xg for 15 min to pellet the DNA. The supernatant was discarded and the DNA pellet air dried before being washed in 200μl of 70% ethanol. The solution was recentrifuged at 10,000xg for 10min to repellet the DNA and the 70% ethanol discarded. The DNA pellet was air dried and resuspended in 50μl of TE buffer. 1μl (19 units) of RNAase T1 (1930 units/μl; Boehringer Mannheim) was added to the DNA solution which was incubated at 37°C for 1hr.

2.12 MIDI PLASMID PREPARATION FROM *E. COLI*.

The strain of *E. coli* carrying the plasmid DNA was streaked out onto LB agar containing 50μg/ml of the selective antibiotic required by the plasmid and grown for 16-20hr at 37°C. 50ml of LB, containing 50μg/ml of the appropriate antibiotic, was inoculated with a single colony from the overnight plate. The culture was then incubated at 37°C, with shaking, for 16-20hr. The cells were then pelleted by centrifugation at 3,500xg (4,000rpm) in 50ml polypropylene tubes for 15min. All traces of excess medium were removed from the *E. coli* cells, which were the resuspended in 5ml GET solution (section 2.11) containing lysozyme (Sigma) at 2mg/ml and incubated on ice for 30min. The cells were then lysed by the addition of 10ml alkaline SDS (section 2.11) followed by incubation on ice for 10min. 7.5ml of 3M Na acetate was then added to the solution and incubated for a further 60min before pelleting the cell debris by centrifugation of the solution at 3,500xg for 20min. The supernatant was retained and placed in a clean 50ml polypropylene tube with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1 [Sigma]) and mixed by inversion. The two phases were separated by centrifugation and the upper aqueous layer was removed without disturbing the debris at the interface of the two phases. An equal volume of chloroform was added to the aqueous layer and the solution mixed and centrifuged, as stated above. Again the aqueous upper layer was retained and 0.5 volumes of isopropanol were added. The solution was mixed by inversion and left to stand at room temperature for 15min. The DNA was then pelleted by centrifugation of the solution at 3,500xg for 20min. The supernatant was discarded and any excess removed using a Gilson pipette 200μl tip. The DNA pellet was washed in 1ml 70% ethanol, to remove any excess salt and recovered by centrifugation of the solution at 3500xg for 10min. Excess ethanol was removed and the DNA pellet left to air dry before being resuspended in 500μl TE buffer. 200U of RNAase T1 (Gibco BRL) were added to the DNA solution and incubated at 37°C for 16-20hr to digest any RNA present. The RNAase and excess nucleotides were removed by further phenol:chloroform and chloroform extractions (section 2.3), the DNA was then recovered by ethanol precipitation (section 2.4). The DNA

was resuspended in 300μl of TE and stored at -20°C until use.

2.13 LARGE SCALE PLASMID PREPARATION FROM *E. COLI* USING THE QIAGEN METHOD

The Qiagen maxi prep method has a maximum yield of 500mg DNA from 150-500ml. The cells of a 400ml culture, grown at 37°C overnight, were pelleted by centrifugation at 3,500xg (4,000rpm) for 15min. The cells were gently resuspended in 10ml of solution P1 (RNAase A [100mg/ml] in 50mM Tris-Cl, 10mM EDTA pH8.0) and transferred to a 50ml Beckman centrifuge tube. 10ml of solution P2 (0.2M NaOH, 1% SDS) was then added and the solution incubated at room temperature for 5min. 10ml of solution P3 (2.55M K acetate pH4.8) were added immediately, the solution was mixed gently forming a precipitate and then centrifuged at 20,000xg (17,000rpm) for 30 min, at 4°C. The supernatant was retained and recentrifuged under the same conditions for 10 minutes, to give a particle-free clear lysate. A Qiagen ion exchange 500 column was equilibrated by the addition of 10ml of QBT buffer (750mM NaCl, 50mM MOPS, 15% ethanol pH7.0). The lysate was then poured into the Qiagen column, invading the resin by gravity flow. The Qiagen column was washed three times with 10ml of QC buffer (100mM NaCl, 50mM MOPS, 15% ethanol pH 7.0) and the DNA eluted with 15ml of QF buffer (120mM NaCl, 50mM MOPS, 15% ethanol) into a 50ml polypropylene tube. 0.7 volumes of isopropanol were added to the eluate and the solution was left at room temperature for 30min before being centrifuged for 15min at 3,500xg (4,000rpm). The DNA pellet was then recovered by centrifugation, washed with 1ml 70% ethanol, repelleted under the same centrifugation conditions, air dried and dissolved in 1ml TE.

2.14 COSMID EXTRACTION FROM *E. COLI*

2ml of LB containing 50μg/ml of selective antibiotic was inoculated with a single colony of a cosmid-containing *E. coli* strain, maintained on LB agar (plus antibiotic) at 37°C. The culture was then incubated at 37°C to grow, for 16-20hr. The cells were pelleted by

centrifugation at 3,500xg (4,000rpm) for 5min, the supernatant discarded and the cells resuspended in 200μl of GET buffer (section 2.11) containing 2mg/ml lysozyme and transferred to 1.5ml microfuge tube. The cells were left on ice for 30min before the addition of 400μl of alkaline/SDS (section 2.11) and a further 10min incubation on ice. 300μl of 3MNa acetate were then added to the solution which was left on ice for a further 60min. The cell debris was pelleted by centrifugation at 10,000xg (13,000rpm) in a microfuge for 15min. The supernatant was retained and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and the solution mixed by inversion. The two phases were separated by centrifugation at 10,000xg (13,000rpm) for 1min and the upper aqueous layer removed, taking care not to disturb any debris at the interface. This step was repeated, again retaining the upper aqueous layer, to which 0.5 volumes of isopropanol were added. The solution was mixed, by inversion and left to stand at room temperature for 15min. The solution was centrifuged at 10,000xg (13,000 rpm) for 15min to pellet the DNA, which was then washed in 70% ethanol, repelleted and left to air dry. The DNA pellet was dissolved in 25μl TE, 19units (1μl) of RNAase T1 (Gibco BRL) was added and the solution incubated at 37°C for 1hr before storing the DNA sample at -20°C until used.

2.15 PLASMID PREPARATION FROM *M. SMEGMATIS* MC^{2}155

This method is based on the alkaline lysis method of Birnboim and Doly, (1979) but has been adapted for mycobacteria by the addition of lipase IV to the GET buffer. 5ml-10ml Middlebrook 7H9 media, containing kanamycin at 25μg/ml, were inoculated with a single colony taken from an *M. smegmatis* plasmid-containing strain maintained on Middlebrook agar, containing kanamycin (25μg/ml), at 37°C. The culture was incubated at 37°C, with shaking, for 48-72hr (until the culture reached late log-phase) and the cells were pelleted by centrifugation at 3,500xg for 10min. The supernatant was discarded and any recalcitrant liquid removed by pipetting. The cells were resuspended in 200μl GET buffer containing 30mg/ml lipase IV (Sigma) and 10mg/ml lysozyme (Sigma). The suspension was incubated at 37°C

with shaking for 16-20hr before being transferred to an 1.5ml microfuge tube and placed on ice for 30min. 400μl of alkaline/SDS solution was added to the suspension which was kept on ice for 60min before the addition of 300μl 3M Na acetate solution and a further 60min incubation on ice. The cell debris was pelleted by centrifugation at 10,000xg for 20min; the supernatant was retained and phenol:chloroform extracted (section 2.3). The DNA was then recovered by ethanol precipitation (section 2.4), air dried and resuspended in 25μl of TE. The plasmid recovery from *M. smegmatis* is generally less efficient (0.05μg/ml) than from *E. coli*; in order to prevent further loss of the plasmid DNA it was not RNAase treated and the whole sample was transformed directly into *E. coli* DH5α.

2.16 COSMID PREPARATION FROM *M. SMEGMATIS* MC^{2}155

Cosmid preparation from *M. smegmatis* mc^{2}155 was identical to that stated for plasmid preparation (section 2.15) except that smaller 5ml cultures were always used due to the instability of the cosmid DNA. Cosmid DNA recovery was poorer than plasmid recovery (<0.05μg), so to prevent further loss of the cosmid DNA it was not RNAase T1 treated and the whole sample was then transformed into *E. coli* DH5α.

2.17 SEQUENCING

2.17.1 PREPARATION OF DOUBLE STRANDED RECOMBINANT DNA FOR SEQUENCING

50μl (3μg) of dsDNA were added to 50μl of denaturation solution (0.4M NaOH, 0.2mM EDTA) and incubated at 37°C for 30min, denaturing the DNA. The single stranded ssDNA was precipitated by the addition of 10μl of 3M Na acetate pH5.2 and 200μl of 100% ethanol, followed by a 30min incubation at -20°C. The solution was centrifuged in a microfuge at 10,000xg (13,000rpm) for 15min to pellet the DNA. The DNA pellet was washed in 200μl of 70% ethanol, dried and dissolved in 7μl of TE. This ss DNA was sequenced as stated in section 2.17.2.

2.17.2 SINGLE STRANDED SEQUENCING OF RECOMBINANT DNA

Single stranded recombinant plasmid DNA was prepared as described above (section 2.17.1) and sequenced using the United States Biochemical (USB) Sequenase Kit Version 2.0, according to the instructions provided by the manufacturers. 3µg (7µl) of ss recombinant plasmid DNA was mixed with 3ng (1μl) of primer (-40) and 2μl of 5x Sequenase Buffer (200mM Tris-Cl pH7.5, 100mM $MgCl_2$, 250mM NaCl) in a 1.5ml microfuge tube and placed in a water bath maintained at 65°C for 2min. The water bath was then left at room temperature until it reached 30°C. A labelling master mix had been prepared which contained 4.4μl 0.1M Dithiothreitol (DTT), 8.8μl 5X Labelling Mix (7.5μM dGTP, 7.5μM dCTP, 7.5μM dTTP), 2.2μl [α-^{35}S]dATP (10MCi/ml, Amersham) and 8.0μl of diluted Sequenase v2.0 enzyme (1μl enzyme and 7μl Enzyme Dilution Buffer (10mM Tris-Cl pH7.5, 5mM DTT, 0.5mg/ml BSA), enough for four reactions. 5.5μl of the labelling "cocktail" was immediately added to the primer/template DNA and left at room temperature for 3min. Four 0.5ml microfuge tubes were taken and 2.5μl of the dideoxynucleotide termination mixes were placed in each and preheated to 37°C. 3.5μl of the labelled template/primer was added to each termination mix and incubated at 37°C for 5min. The reaction was stopped by the addition of 4μl of Stop solution buffer. Samples were then denatured by heating to 80°C, in a heating block, for 7min prior to loading on the sequencing gel.

2.17.3 PREPARATION AND ELECTROPHORESIS OF SEQUENCING GEL

Two sequencing plates, 33x39.5cm and 33x42cm, were cleaned thoroughly with detergent (Decon) and wiped down with absolute ethanol. The smaller plate was wiped down with Sigmacote (Sigma) then placed on top of the larger plate with 0.3mm spacers in between the plates at their outer edges. The plates were sealed around three sides with electrical tape. 75ml of a 6% polyacrylamide gel mix was prepared from a 40% acrylamide:*bis* acrylamide (19:1) stock solution (CAMLAB) in 1x TBE and 8M urea (Sigma). 240µl 20%(w/v) ammonium persulphate (APS, Sigma) and 100µl N,N,N',N'-tetramethyl-ethylenediamine (TEMED,

Sigma) were added to 70ml of gel solution and mixed gently. The gel solution was taken up in a 50ml syringe and injected between the plates from one corner, allowing the plates to fill from one side, reducing the risk of air bubbles. Any air bubbles formed were removed with a Bubble Remover (Promega). A shark's tooth comb with 23 teeth was immediately inserted inverted into the top of the gel, to later form twenty arch shaped wells. Generally polymerization occurred within 5min, but gels were left 16-20hr before electrophoresis. The lower strip of tape was removed from the plates, which were placed in the sequencing apparatus (Life Technologies) and secured. The gels were prewarmed by running in 1xTBE buffer for 30min at 60V until they reached 55°C. Urea which had accumulated in the wells was flushed out using a 20ml syringe containing 1xTBE buffer from the upper chamber of the gel tank. 2μl of the four termination reactions, for every sequenced clone, was loaded into four adjacent wells (TCGA). The reactions were loaded using a flat-ended sequencing tip (Gilson). The gels were run at 60W for 1-3hr, depending on the length of sequence to be obtained; gels were run for 1hr to read approximately 200bp of sequence (blue dye migrated 3/4 length of the gel) and 3hr (blue dye run off the gel) to read 200-400bp of a given clone. Following electrophoresis the shorter plate was removed, the gel adhering to the larger plate. The larger plate and gel were placed in a tray and submerged in 1l of fixer (5% acetic acid [BDH], 15% methanol [BDH]) for 15min. The plate was removed from the tank, draining excess fixer and the gel carefully transferred to Whatman 3MM paper. The gel was covered in Saran wrap and dried for 90min at 80°C, on a heated vacuum gel dryer. The dried gel was placed in an autoradiography cassette and a photographic X-film (Kodak) placed on the gel. The film was exposed to the gel for 16-72hr, depending on the strength of the signal and then developed in an automatic film processor. Sequence data were read manually, entered into the computer and assembled using the Staden software. The software was accessed through the MRC Human Genome Mapping Project.

2.18 CULTURE OF *MYCOBACTERIUM SMEGMATIS*

M. smegmatis mc^2155 (Snapper *et al.*, 1990) was obtained from W.R.Jacobs and mutant strains were created by NTG mutagenesis (section 2.27). Both wild type and mutant strains of *M. smegmatis* mc^2155 were stored as frozen cultures in 25% glycerol at -70°C. The strains were maintained at room temperature on Lowenstein-Jensen (LJ) slopes (Difco) following initial growth at 37°C for 16-20hr.

M. smegmatis strains were streaked to single colonies on Lemco (Appendix A) or Middlebrook 7H10 agar (Difco) and incubated at 37°C, for 2-6 days or at 28°C for 4-10 days. Small scale liquid cultures (5-10ml) were generally grown in Lemco broth containing 0.1% Tween, but in certain circumstances in Middlebrook 7H9 media containing 0.1% Tween 80 was used. Large scale liquid cultures (100ml-1l) of *M. smegmatis* were grown in Middlebrook 7H9 medium containing 0.1% Tween 80 for 1-3 days at 37°C or 2-6 days at 28°C with shaking at 150rpm (incubation times were dependent on the size of the inoculum and the volume of the culture). 0.1% Tween 80 was used in all liquid cultures unless otherwise stated, to reduce clumping.

Kanamycin selection for the mycobacterial plasmid and cosmid DNA used in *M. smegmatis* strains was achieved by using the antibiotic at 15μg/ml and 20μg/ml in Lemco and Middlebrook media, respectively. *M. smegmatis* cosmid-containing strains were grown in 5ml Middlebrook 7H9 media at 37°C for DNA extraction and in 20ml volumes at 28°C for lipid extraction (section 2.29).

2.19 ACID-FAST STAINING OF *M. SMEGMATIS*

Cultures of *M. smegmatis* were acid-fast stained to ensure their identity and their purity, using the TB Staining Kit (Difco). 1ml of a culture was transferred to an microfuge tube and the cells pelleted by centrifugation in a microfuge for 2min at 10,000xg (13,000rpm). The excess

medium was discarded and the cells resuspended in 200μl of medium. The cell suspension was spread on a glass slide, air dried and flamed to heat fix the cells. The slide was left to cool before being covered in carbolfuschin for 5min. The carbolfuschin was gently poured off and the slide covered in acid-alcohol (15% ethanol 5% H_2SO_4) for 30s to decolourise the cells. The slide was gently washed with water and then counterstained with malachite green for 5min. Again the slide was rinsed in water and then left to air dry. The cells were viewed under a microscope at 1000x magnification using immersion oil to ensure they were stained the characteristic magenta of acid fast bacilli and that there were no green staining contaminants present.

2.20 LARGE SCALE MYCOBACTERIOPHAGE PREPARATION

Four plaques were picked with a sterile wire loop and placed in 10ml of Lemco broth (without Tween) and incubated at 37°C, with shaking (200rpm) for 16-20hr. The culture was taken up in a 10ml syringe and pushed through a 0.2μm sterilising filter (Acrodisc). The retained phage suspension was stored at 4°C. To determine the phage concentration 1-1x10^{-9} dilutions of the solution were made and 10μl of each used to infect 200μl of *M. smegmatis* as stated (section 2.21). The number of plaques on each plate was counted and the number of plaque forming units/ml (pfu/ml) calculated for a given phage type.

2.21 INFECTION OF *M. SMEGMATIS* WITH MYCOBACTERIOPHAGES

Mycobacteriophage were provided by Dr.J.Grange, The Middlesex Hospital, London. The mycobacteriophages were either stored in Lemco broth at 4°C (short term) or freeze-dried in glass ampoules (long term). The mycobacteriophages were released from long term storage by adding the freeze-dried material to 1ml Lemco broth, in a 1.5ml microfuge tube, mixing them by inversion and incubating at 37°C for 4hr. 10ml of Luria broth (without Tween) was inoculated with a single colony of *M. smegmatis*, maintained on Middlebrook agar at 37°C and incubated at 37°C for 16-20hr. 10μl of a given mycobacteriophage solution (1x10^5

pfu/ml) was added to 200μl of the overnight *M. smegmatis* late log-phase (A_{600} 0.8) culture and left for 30min for the phage to adhere to the bacteria. The mixture was then added to 3ml of Lemco soft top agar and poured on to a Lemco agar plate, allowed to set and incubated at 37°C for 24 -36hr until a lawn of bacteria formed and any plaques were visible.

2.22 DROP METHOD FOR TESTING PHAGE SENSITIVITY

200μl of an overnight late log-phase *M. smegmatis* mc^2155 culture were added to 3ml of Lemco soft top agar, mixed by inversion, poured onto a Lemco agar plate and left to set. 10μl of mycobacteriophage solution were then pipetted ("dropped") onto the surface of the Lemco soft top agar containing the *M. smegmatis* cells. The plates were then incubated at 37°C for 12-24hr until a bacterial lawn was formed and any clearing caused by phage infection could be observed. On testing of a given *M. smegmatis* strain for infectability with a number of mycobacteriophage, the phage solutions were placed in the wells of a microtitre plate (in a known order). A multichannel pipette was used to take up 5μl of each phage solution. The solutions were then released from the pipette onto the soft agar containing the *M. smegmatis* cells, and the test plate was then incubated at 37°C as above. The maximum number of phages which could be used to test a given *M. smegmatis* strain for infectability on a single plate was six.

2.23 OVERINFECTION METHOD FOR THE ISOLATION OF PHAGE RESISTANT COLONIES

$1x10^9$ pfu (100μl of $1x10^{10}$ pfu/ml phage stock) were added to 200μl of late log-phase *M. smegmatis* mc^2155 in a 1.5ml microfuge tube and left for 20min at room temperature. The infected culture was then plated out in 3ml Lemco soft top agar onto Lemco agar before being incubated at 37°C. The infection plates were observed after 24hr to ensure complete lysis had occurred and no bacterial lawn had been formed. The plates were then incubated at 37°C for a further 4 days to allow for the formation of colonies by any phage resistant *M. smegmatis*

cells.

2.24 TESTING FOR PSEUDOLYSOGENY IN *M. SMEGMATIS*

Mycobacteria often exhibit pseudolysogeny i.e. they are able to harbour individual phage which inhibit infection by other phage, but are not integrated into the mycobacterial genome. The following method was developed to determine if a given strain of *M. smegmatis* was pseudolysogenic. The test strain and the wild type *M. smegmatis* strains were streaked out from frozen culture to single colonies on Lemco agar, and grown at 37°C for 20-40hr. Single colonies of each strain were inoculated into 10ml of Lemco broth (no Tween) and incubated at 37°C, shaking, for 16-20hr. 200μl of *M. smegmatis* culture were added to 3ml of Lemco soft top agar and plated out onto a Lemco agar plate. 10μl of the test strain were pipetted on to the centre of one half of the soft top agar and 10μl of the wt on to the other half. This procedure was carried out in duplicate. The plate was incubated at 37°C for 24-36hr until a bacterial lawn had formed. Any clearing in the lawn around the test strain indicated the presence of phage in the strain infecting the susceptible wild type *M. smegmatis*.

2.25 PRODUCTION OF COMPETENT *M. SMEGMATIS* MC^{2}155

Competent *M. smegmatis* were produced using a method developed by Snapper *et al.*, (1988 and 1990). A 5ml volume of Middlebrook 7H9 media was inoculated with a single colony of *M. smegmatis* and grown for 16-20hr at 37°C, with shaking. A 500ml volume of Middlebrook 7H9 was inoculated with 1ml of the overnight *M. smegmatis* culture, incubated at 37°C with shaking (200rpm) and grown for 24-36hr, to late-log phase (A_{600}=0.8). The flask was placed on ice and the culture incubated for 60min. The culture was transferred to a 1l Beckman container and centrifuged at 3500xg (4,000rpm) to pellet the cells. The cells were resuspended in 100ml (1/5 volume) of ice cold 10% (v/v) glycerol (BDH) and repelleted by centrifugation at 3500xg (4,000rpm) for 15min. The cells were then resuspended in 20ml (1/25 original volume) of ice cold 10% (v/v) glycerol and pelleted by centrifugation as before. The cells

were finally resuspended in 5ml (1ml for library efficiency) of ice cold 10% (v/v) glycerol, transferred to 1.5ml microfuge tubes and stored frozen at -70°C. This gives a final concentration of between 2-5x10^9 cfu/ml and a transformation efficiency of 10^4-10^6 cfu/μg DNA .

2.26 TRANSFORMATION OF *M. SMEGMATIS* MC^{2}155 BY ELECTROPORATION

M. smegmatis cells were transformed using the method developed by Snapper *et al.*, (1988 and 1990). A frozen stock of competent *M. smegmatis* cells (section 2.25) were thawed on ice, aliquotted and placed into a pre-chilled 1.5ml microfuge tube containing the DNA solution to be transformed. Care was taken to ensure the DNA and cells were evenly suspended and the DNA/cell suspension was left on ice for 30min. The cell suspension was mixed gently by pipetting, avoiding the introduction of air bubbles and was transferred to a pre-chilled 200μl electroporation cuvette. A Bio-Rad Gene Pulse and Pulse Controller were set at 2.5kV, 25μF and 1000Ω. The cuvette was placed in the chamber of the pulser and the cells electroporated. The cuvette was placed on ice for 10min before the cell suspension was transferred using 2ml of Lemco broth into a universal and incubated, with shaking (200rpm), at 37°C for 2hr. The cells were then centrifuged at 3,500xg (4,000rpm) for 10min and 1ml of the Lemco broth removed. The cells were resuspended in the remaining 1ml of Lemco broth and spread on Lemco or Middlebrook 7H9 agar (12cm plates), containing the appropriate selective antibiotic. The cells were then incubated at 37°C for 4 days or at 28°C for 8 days.

2.27 MUTAGENESIS OF *M. SMEGMATIS* WITH N-METHYL-N-NITRO-N-NITROSOGUANIDINE (NTG)

The mutagenesis procedure was carried out according to a method established by Holland and Ratledge (1971). 100ml supplemented minimal medium (MM+AA; Appendix A) containing 0.1% Tween 80 were inoculated with 1ml of an overnight *M. smegmatis* mc^{2}155 culture and

incubated at 37°C, with shaking (150rpm) until the culture reached late log-phase (A_{600}0.8), approximately 40hr. 1ml of the culture was removed at this point and plated onto Lemco plates in serial dilutions (1-1x10^{-9}) to determine the percentage killing of cells following the NTG treatment. The remaining cells were harvested by centrifugation in J6B rotor (Beckman) at 3,500xg (4,000rpm) for 7min and resuspended in 10ml of unsupplemented minimal media, pH6.3. 10ml of minimal medium (pH6.3) had previously been injected into a sealed bottle containing 10mg of NTG (Sigma) and the 10ml of *M. smegmatis* mc^2155 cell suspension was injected into the sealed bottle giving a final concentration of 0.5mg/ml NTG. The *M. smegmatis*/NTG suspension was incubated at 37°C, with shaking (150rpm) for 45min, in the sealed container. The suspension was removed from the bottle with a syringe and the NTG-treated cells were pelleted, washed four times with 0.2M phosphate buffer (KH_2PO_4/KOH pH6.3) containing 0.5% Tween 80 and finally resuspended in 2ml MM. The wash solutions containing NTG were pooled and treated with 0.1M HCl to destablise the remaining NTG before disposal. This procedure was carried out on two individual *M. smegmatis* mc^2155 cultures and known as the first and second mutagenesis procedures. The NTG-treated cells of these two procedures were treated differently following exposure to NTG.

2.27.1 FIRST MUTAGENESIS - INH TREATMENT

The use of INH in this method was previously described by Holland and Ratledge (1971). The 2ml of NTG-treated *M. smegmatis* cells were divided into two. 1ml of NTG-treated cells were resuspended evenly in 100ml MM+AA (0.5% Tween 80). 1ml of this cell suspension was removed, serially diluted 1-1x10^{-9} and plated in 200μl volumes onto Lemco agar (12cm plates) and incubated at 37°C for 24hr. The cells were then incubated at 28°C for 48hr to allow the cells to recover. These NTG-treated cells left to recover at 28°C were known collectively as SetA.

The remaining NTG-treated cells from the first mutagenesis were evenly resuspended in

100ml MM+AA (0.5% Tween 80) containing 50μg/ml INH and then incubated at 37°C for 24hr, with shaking (150rpm). The INH-treated cell culture was transferred to 50ml Falcon tubes, the cells precipitated by centrifugation at 3500xg (4,000rpm) and the cells were then resuspended in 30ml MM. The cells were washed 3x in MM and finally resuspended in 100ml MM+AA (0.5% Tween 80) and incubated at 28°C, with shaking (150rpm) for 48hr. The NTG and INH-treated *M. smegmatis* cells were known collectively as SetB(INH).

2.27.1.1. Dilution procedure of NTG-treated M. smegmatis from the first mutagenesis

Following recovery at 28°C the NTG-treated *M. smegmatis* cells of SetA and SetB(INH) were transfered to 50ml Falcon tubes (50ml stored at 4°C and 50ml stored at -20°C, from each of SetA and SetB(INH)). 10ml of culture from SetA and SetB(INH) were taken and dilutions of $1\text{-}10^{-9}$ in final volumes of 5ml were made up in 10ml universals with SDW. 1ml volumes of each dilution from SetA and SetB(INH) were plated onto MM+AA and MM agar plates (14cm), in duplicate. The 1ml volumes of each dilution were passed through 25 gauge needles, to reduce clumping. One set of MM+AA and MM serial dilution plates from both SetA and SetB(INH) were incubated at 37°C for 48 hr; the duplicate sets of MM+AA and MM agar serial dilution plates, from SetA and SetB(INH), were incubated at 28°C for four days. The number of colonies formed on MM+AA and MM, at comparable dilutions were compared within SetA and SetB(INH); as well as the colonies formed at 28°C and 37°C on MM+AA and MM, within SetA and SetB(INH).

1000 colonies from MM+AA dilution plates of SetA and SetB, incubated at 28°C, were randomly selected and transferred to Middlebrook 7H10 agar. The 2000 colonies were arrayed in the dimensions of a 96 well microtitre plate, on the Middlebrook 7H10 agar plates (14cm), and incubated at 28°C for 6 days. The set of 2000 colonies was known collectively as Bank 1.

2.27.2 SECOND MUTAGENESIS

Following treatment with NTG the *M. smegmatis* cells of the second mutagenesis procedure were left to recover at 28°C in 100ml MM+AA (0.5% Tween 80) for 48hr. The culture was then transferred to two 50ml polypropylene tubes and all but 10ml was stored at -20°C. The 10ml of NTG-treated cells were used to make serial dilutions $1\text{-}1x10^{-9}$ which were then plated in 1ml volumes onto Middlebrook 7H10 agar and incubated at 28°C for 6 days to allow colony formation. 1,000 of the colonies formed were randomly selected and transferred to Middlebrook 7H10 plates in arrays of 96 as above, the arrays were incubated at 28°C to recover. The 1,000 colonies of NTG-treated *M. smegmatis* from the second mutagenesis were known collectively as Bank 2.

2.28 SCREENING OF NTG-TREATED *M. SMEGMATIS* COLONIES FOR RESISTANCE OR SENSITIVITY TO ANTIBIOTICS

2.28.1. REPLICA PLATE SCREENING OF NTG-TREATED *M. SMEGMATIS*

Initially arrayed NTG-treated *M. smegmatis* colonies from Bank 1 or 2 were replica plated onto Lemco agar containing the test antibiotic at the MIC, when looking for resistance and just below the MIC, when looking for sensitivity to the test antibiotic. The colonies were replica plated using a replicating device with 96 pins arranged in the dimensions of a microtitre plate. The 96 pin device was sterilised, placed in the arrayed colonies, removed and plunged into the Lemco agar containing the test antibiotic. This was repeated for all the colonies in the Bank and the inoculated Lemco agar plates containing the test antibiotic were incubated at 28°C for 8 days.

2.28.2 LARGE SCALE SCREENING FOR MYCOBACTERIOPHAGE AND ANTIBIOTIC RESISTANCE

100ml of Middlebrook 7H9 media was seeded with 1ml of NTG-treated cells from a frozen stock. The cell suspension was incubated at 28°C with shaking for 48hr until the culture

reached a 0.8A_{600}. 10μl, 100μl and 1ml volumes of the NTG-treated *M. smegmatis* mc^2155 cells were plated onto Lemco agar plates (14cm) containing the test antibiotic (MIC) or spread with 1ml phage solution (x10^6pfu/ml). The test plates were then incubated at 28°C for 8 days before being read for growth. Any resistant strains were analysed further.

2.28.3 CONFIRMATION OF ANTIBIOTIC RESISTANCE

Any *M. smegmatis* strains which appeared resistant to a given antibiotic were subcultured from the master plates and subsquently retested against the same concentration of the given antibiotic. Initially a single colony of the subcultured strain was streaked out onto a Lemco plate containing the test antibiotic at the same concentration. If the *M. smegmatis* strain remained sensitive or resistant to the test antibiotic the strain was subcultured and a single colony was retested on solid agar (plus antibiotic) or used to seed 5ml Lemco broth (0.5% Tween) which was incubated at 28°C until the culture reached late log-phase (A_{600} 0.8); the liquid culture was then streaked on Lemco agar containing the same concentration of the test antibiotic. Any strains remaining resistant or sensitive to the test antibiotic at this stage were subcultured again, a Lemco liquid culture created as before and again cells streaked onto two Lemco agar plates containing the test antibiotic, one of which was incubated at 37°C (48-72hr) and the other at 28°C (6-8 days). In some cases equal numbers of the test strain cells (2.5x10^7) and *M. smegmatis* mc^2155, cells were added to 5ml liquid Lemco containing the test antibiotic in the test concentrations. Control cultures of wild type and test strains without antibiotic were also set up. The liquid cultures were incubated at 37°C, with shaking (150rpm), for 48hr. The A_{600} of the test and wild type cultures with and without the test antibiotic were read and compared.

2.28.4 ANTIBIOTIC SENSITIVITY TESTING IN LIQUID CULTURE

An overnight culture, seeded with a single colony of an antibiotic sensitive strain of *M. smegmatis*, was grown to late log-phase culture (0.8 A_{600}). 100μl of the cell culture (2.5x10^7

cfu, unless otherwise stated) was then added to a series of 9x5ml volumes of Lemco, eight of which contained increasing concentrations of the test antibiotic (200-900μg/ml); the ninth, control culture, contained no antibiotic. An identical series was set up seeded with $2.5x10^7$ *M. smegmatis* mc^2155, wild type cells. The two test series were incubated at 37°C or 28°C until the control cultures reached 0.8 A_{600} or 1.5 A_{600} and then the A_{600} of all the cultures in the two series were read. The growth of the test strain at each increasing concentration of the antibiotic was then compared with that of the wild type at the same concentration of antibiotic.

2.29 EXTRACTION OF FREE MYCOBACTERIAL LIPIDS

These methods were carried out as described by G.Dobson *et al.*, (1985).

2.29.1 NON POLAR LIPIDS

A 50mg dry biomass sample was placed in an 8.5ml PTFE-capped Corning tube. 2ml of aqueous methanol (10ml 0.3% NaCl:100ml methanol [BDH, HPLC grade]) and 1ml of petroleum ether (BDH, HPLC grade) boiling point (b.p.) 60-80°C were added to the sample and mixed vigorously on a shaking platform for 15min. The solution was then centrifuged at 3,500xg (4,000rpm) for 7min to separate the two phases. The upper phase was transferred with a glass pasteur pipette to 5ml glass vial and another 1ml of petroleum ether (b.p. 60-80°C) added to the remaining lower layer. The mixture was mixed vigorously for a further 15min, centrifuged again for 7min and the upper layer retained with the previous one. The upper layers were dried with N_2 at <37°C, redissolved in 70μl of chloroform:methanol (2:1) and stored at -20°C until use. The lower layer was retained for the extraction of polar lipids.

2.29.2 POLAR LIPIDS

The retained lower layer residue was heated at 100°C in a heating block for 5min and allowed to cool to 37°C before the addition of 2.3ml of solution 1 (chloroform: methanol:0.3% NaCl,

90:100:30). The mixture was shaken vigorously for 60min and centrifuged for 5min, at 3,500xg (4,000rpm) to pellet the cell debris. The upper solution was retained and the cell debris resuspended in 0.75ml of solution 2 (chloroform: methanol: 0.3% NaCl, 50:100:40). The suspension was mixed vigorously for 30min and centrifuged at 3,500xg (4,000 rpm) for 7min to pellet cell debris. The upper aqueous layer was retained and added to the 8.5ml tube containing the previous extraction. This step was then repeated. The supernatants were combined by the addition of 1.3ml chloroform and 1.3ml 0.3% NaCl, followed by vigorous mixing for 30min. The two phases were separated by centrifugation at 3,500xg (4,000rpm) for 7min and the upper layer was discarded, carefully removing any debris at the interface. The lower layer was retained and evaporated with N_2 The residue was then dissolved in 70μl of chloroform:methanol, 2:1 and stored at -20°C.

2.30 TWO DIMENSIONAL THIN LAYER CHROMATOGRAPHIC (TLC) SYSTEMS FOR THE ANALYSIS OF POLAR AND NON POLAR LIPIDS OF MYCOBACTERIA.

A 7μl sample of a nonpolar or polar lipid fraction was applied to a 6.6x6.6cm aluminium backed TLC plate (Merck 5554) using a glass capillary tube in one corner of the plate, 1cm away from each side and left to air dry. One of the following solvent systems was then applied.

2.30.1 SYSTEM A

This system is used to detect menaquinones, triacylglycerols and mycocerosates of the phthiocerol family as it separates the least polar of the non polar lipids. A TLC plate loaded with non polar lipid extract, was developed with petroleum ether (bp 60-80°C): ethyl acetate (98:2 v/v) the first direction solvent, until the solvent reached the top of the plate. The TLC plate was removed and left to air dry; this procedure was repeated twice. The TLC plate, was turned 90° and developed with petroleum ether:acetone (98:2), the second direction solvent, once. The TLC plate was left to air dry, before observation under ultraviolet light for the

presence of menaquinones. The separated lipid components could then be revealed by spraying the plate with 5% (w/v) ethanolic molybdophosphoric acid (MPA) solution and subsequent heating at 180°C for 10min.

2.30.2 SYSTEM D

This system separated the most polar of the non polar lipids and the least polar of the polar lipids. A TLC plate loaded with non polar or polar lipid extract was developed with chloroform:methanol water (100:14:0.8), the first direction solution, until the solvent reached the top of the plate when it was removed and left to air dry. The plate was then turned 90° and developed with the second direction solvent, chloroform:acetone:methanol (50:60:2.5), until the solvent reached the top of the plate. The plate was left to air dry before being sprayed with MPA and placed at 180°C for 10min, to detect lipids. In order to detect any sugars present on the lipids, the system was repeated and the plate ultimately sprayed with α-naphthol-sulphuric acid (NAS) and incubated at 120°C for 2min. This system detected trehalose dimycolates and sulpholipids from non polar extracts and glycopeptidolipids from polar lipid fractions.

2.30.3 SYSTEM E

This system detected the most polar lipids in the polar lipid extract. A TLC plate loaded with polar lipid extract was placed in a TLC tank containing chloroform:methanol:water (10:5:1) until the solvent reached the top of the plate. The plate was air dried before being placed, at a 90° turn, in the second direction solvent, chloroform:acetic acid:methanol:water (40: 25:3:6), until the solvent reached the top of the plate. The plate was sprayed with MPA and heated at 180°C to detect the lipids. The process was repeated and the plate sprayed with NAS and heated at 120°C to detect sugars. This system detects phospholipids and glycolipids including forms of phosphatidylinositol.

2.30.4 SYSTEM F

This system also detected the most polar lipids in the polar lipid extract and was an adaptation of system E. A TLC plate loaded with polar lipid extract was developed with a first direction solvent chloroform: methanol: water (65:25:4) until the solvent reached the top of the plate. The plate was left to air dry before being placed, at a 90°turn in the second direction solvent chloroform: acetic acid: methanol: water (80:15:12:4) until the solvent reached the top of the plate. The plate was sprayed with MPA and heated at 180°C for 10min to detect the lipids. The process was repeated and the plate sprayed with NAS and heated at 120°C, to detect sugars. This system detects phospholipids and glycolipids including forms of phosphatidylinositol.

2.31 EXTRACTION AND DETECTION OF MYCOLIC ACIDS

50mg samples of dried biomass were placed in an 8.5ml Corning tube, 2ml of 5%v/v aqueous tetrabutylammoniumhydroxide was added and the mixture placed at 100°C for 16-12hr. The mixture was cooled, centrifuged and the supernatant transferred to a Corning tube containing 2ml dichloromethane (Sigma) and 25μl iodomethane (Sigma). The preparation was shaken for 30min, the upper layer discarded, and the lower layer washed with 1ml of 10% aqueous HCl followed by 1ml distilled water and evaporation to dryness under N_2.

2.31.1 TLC OF MYCOLIC METHYL ESTERS

The alkaline methanolysates were dissolved in 0.1ml petroleum ether (b.p. 60-80°C) and 2μl applied to 6.6 x 6.6cm aluminium backed TLC plates. The plates were developed three times with petroleum ether (b.p. 60-80°C):acetone (95:5 v/v) in the first direction and once with toluene:acetone (97:3) in the second direction. The positions of the separated components were revealed by spraying with 5%, w/v ethanolic molybdophosphoric acid followed by heating at 180°C for 15min.

2.32 ANALYSIS OF BACTERIAL HYDROPHOBICITY

The hydrophobicity of *M. smegmatis* cultures was measured using an adaptation of the method of Rosenberg *et al.*, (1980). Two 200ml cultures of the test strain and of the wild type strain of *M. smegmatis* were grown to late-log phase (0.8 A_{600}) and early stationary phase growth (1.6 A_{600}). The cells from each culture were pelleted following centrifugation at 3500xg (4,000rpm) and washed twice and resuspended in 20ml of PUM buffer (22.2g $K_2HP0_4.3H_20$, 7.26g KH_2P0_4, 1.8g urea, 0.2g $MgSO_4.7H_20$ in 1l, pH7.1). 1.5ml of each of the cell suspensions were added to individual round bottomed Corning tubes followed by addition of 15μl of the hydrocarbon n-hexadecane (Sigma) and incubation at 30°C. Each suspension was vortexed for 120s before being left to stand at room temperature for 15min. The lower aqueous phase was removed carefully with a glass Pasteur pipette and transferred to a 1ml cuvette. The optical density of the aqueous phase was measured at 400nm, using a spectrophotometer. The optical densities of the test strain and the wild type strain from the same stage of growth were compared, as were the A_{400} from different stages of growth of the same strain.

2.33 API-ZYM TEST

The Api-Zym test was carried out according to the manufactures instructions. An overnight *M. smegmatis* culture was diluted with SDW to 0.5-0.6 A_{600} (10^5-10^7 cfu/ml). An incubation tray was taken, 5ml of water placed in the bottom, and an Api-Zym strip was then placed in the incubation tray. Using a Pasteur pipette, two drops of culture were added into each cupule of the strip. Following inoculation, the plastic lid was placed on the tray and the tray incubated at 37°C for 4hr. After incubation one drop of ZYM A (25% w/v Tri-hydroxmethyl-amino-methane,3.7% HCl, 10% w/v Laryl sulphate) and one drop of ZYM B (0.35% w/v Fast blue BB [2-methoxyethanol diluent]) were added to each cupule. The strips were left in daylight for five minutes for the colour to develop. A value ranging from 0-5 was assigned to the colours produced when compared with the colour chart where 0 is a negative result, 1- 4 are

intermediate reactions (1=5 nmoles, 2=10 nmoles, 3=20 nmoles, 4=30 nmoles) and 5 is a reaction of maximum intensity (5=40nmoles and above).

CHAPTER 3

RESULTS

3.1 THE ATTEMPT TO ISOLATE A CELL ENVELOPE MUTANT *M. SMEGMATIS* MC²55 MUTANT

The initial approach to isolating a cell wall mutant of *M. smegmatis* mc²155 was to find or create a mycobacteriophage-resistant strain of *M. smegmatis* mc²155. This strategy was based on the theory that as mycobacteriophages adhere to receptor sites on the cell wall (Grange and Redmond, 1978; Goren *et al.*, 1972; David *et al.*, 1978) of the bacteria then resistance to phage infection may be as a result of an altered cell wall, which lacked the appropriate phage receptor site.

3.1.1 ISOLATING SPONTANEOUS PHAGE RESISTANT MUTANTS OF *M. SMEGMATIS* MC²155

The original aim was to isolate a spontaneous phage resistant mutant of *M. smegmatis* mc²155.

3.1.1.1 Overinfection method

The strategy adopted was to overinfect an *M. smegmatis* mc²155 population with sufficient numbers of a lytic mycobacteriophage so as to cause confluent lysis of the infectable portion of the population. Any spontaneously resistant *M. smegmatis* cells within the population would be able to replicate in the surrounding "sea" of phages and form visible colonies. The overinfection method was used in an attempt to isolate strains of *M. smegmatis* mc²155 resistant to three mycobacteriophage D29, D4, and D33. D4 and D29 were chosen as their receptors had been shown to be a nsGPL in *M. avium* (Goren *et al.*, 1972: Dhariwal *et al.*, 1986) and a simple LOSs in *M. smegmatis* (Besra *et al.*, 1994b), respectively. D29 has also been shown to adsorb to the cell wall of *M. leprae* (David *et al.*, 1978)

Initially straightforward infections were performed with $1x10^3$ pfu/ml of each phage (section

2.21), to ensure infection of *M. smegmatis* mc^2155 was possible. The three phages D29, D4 and D33 all successfully infected *M. smegmatis* mc^2155. D29 produced 2mm clear circular plaques, D4 produced large, 3-4mm, clear circular plaques and D33 produced small 0.5-1mm clear circular plaques.

Having established that the three phages infected wild type *M. smegmatis* mc^2155, the overinfection method (section 2.23) was performed with each phage type. The overinfection plates were observed after 24hr, to ensure that total lysis of the *M. smegmatis* lawn had occurred and then incubated at 37°C for a further 4 days to allow for the formation of colonies by any phage resistant *M. smegmatis* cells. This method was carried out three times for each phage with three independent *M. smegmatis* mc^2155 cultures, seeded with single colonies, to increase the chances of isolating independent mutants.

The number of colonies formed on the overinfection plates varied slightly between the three phages but remained consistent for each of the three infections for individual phages. On average $1x10^2$ colonies were formed on D29 infection plates; they were mucoid and 1-3mm in diameter. $2x10^2$ colonies of 0.5-1mm in diameter were formed on D4 infection plates; the colony morphology was of two types, mucoid and rough, in equal proportions. $1x10^2$ colonies formed on average on the D33 infection plates and were small (1mm) in diameter and mucoid.

Twelve colonies which were "resistant" to each phage were selected, four from each of the three overinfection plates for each mycobacteriophage, and subcultured. The 36 "resistant" strains were then retested for infectability by the phage to which they were "resistant" (section 2.21). Control infections, to which no phages were added, were set up for each "resistant" strain.

Initially four D4, eight D29 and twelve D33 "resistant" strains remained resistant to infection.

These strains were subcultured again and retested. On retesting, more strains became sensitive to phage infection and only two D4, six D29 and five D33 strains remained resistant to infection. However, the control plates of these thirteen strains produced mottled bacterial lawns, which appeared to contain a background of plaques, or in some cases clear plaques. Following four subsequent subcultures and retesting, all of the strains became infectable.

These results implied that the strains were losing their "resistance" on serial transfer, which would be expected from unstable mutant strains; however the presence of clear and background plaques in the control infections of the strains indicated that the strains were in fact harbouring phages.

This suggested that the "resistant" strains were in fact pseudolysogenic, containing a non integrated phage which prevented induction of the lytic cycle on attempted reinfection, while forming clear plaques on subculture without infection due to the instability of the lysogenic state.

3.1.1.2 Pseudolysogeny

A rapid method was developed to test large numbers of resistant strains for pseudolysogeny. The test was simply to "drop" liquid culture (10μl of late log-phase culture; 0.8 A_{600}) of the test strain onto a lawn of wild type *M. smegmatis* cells and to incubate the plate overnight at 37°C (section 2.24). The rationale was that some cells in the liquid culture of a pseudolysogenic strain would revert to the lytic cycle releasing phage which would infect the wild type *M. smegmatis* cells, forming a visible "halo" of clearing. Non-pseudolysogenic strains should not create a clearing "halo" and would form a confluent bacterial lawn.

The original 36 "resistant" strains obtained from the D4, D33 and D29 overinfection plates were tested for pseudolysogeny using this method. Three visible outcomes were obtained

from the pseudolysogeny test of the 36 phage "resistant" strains tested. The predicted "halo" of clearing was observed around a few pseudolysogenic strains, caused by lysis of the surrounding *M. smegmatis* mc^2155 lawn cells (Fig. 16a). The region in which the culture was "dropped" of the majority of these strains was mucoid and translucent, apparently caused by lysis of the underlying lawn cells. A larger number of test strains did not produce distinct clearing and formed a uniform lawn were the culture had been "dropped", (Fig. 16b).

Of the 36 strains retested for pseudolysogeny 34 were found to be pseudolysogenic. One D4 and one D29 strain did not appear to be pseudolysogenic. Both the D4 and D29 non-pseudolysogenic and "resistant" strains were retested for infectability, using fresh cultures seeded with single colonies. The non-pseudolysogenic D4 strain was infectable, as it had been when previously tested, in the overinfection experiment. The non-pseudolysogenic D29 strain was not infectable, but had previously only been infectable following two subcultures of the frozen stock whereas the colonies used for this D29 infection and the pseudolysogeny test were formed directly from frozen stocks. The non-pseudolysogenic D29 resistant strain was therefore subcultured and six cultures, seeded from single colonies, were tested for pseudolysogeny and infectability. One culture was pseudolysogenic but uninfectable while the remaining five were infectable but not pseudolysogenic.

The results suggested that this D29 "resistant" strain contained a mixed population of pseudolysogenic and non-pseudolysogenic cells and therefore colonies which gave varying results in culture i.e. both pseudolysogenic uninfectable, and non-pseudolysogenic infectable cultures. The "nonpseudolysogenic" D4 strain could well have been a mixed population of pseudolysogenic and non-pseudolysogenic strains but the two cultures tested were non-pseudolysogenic and infectable concurring with a negative test.

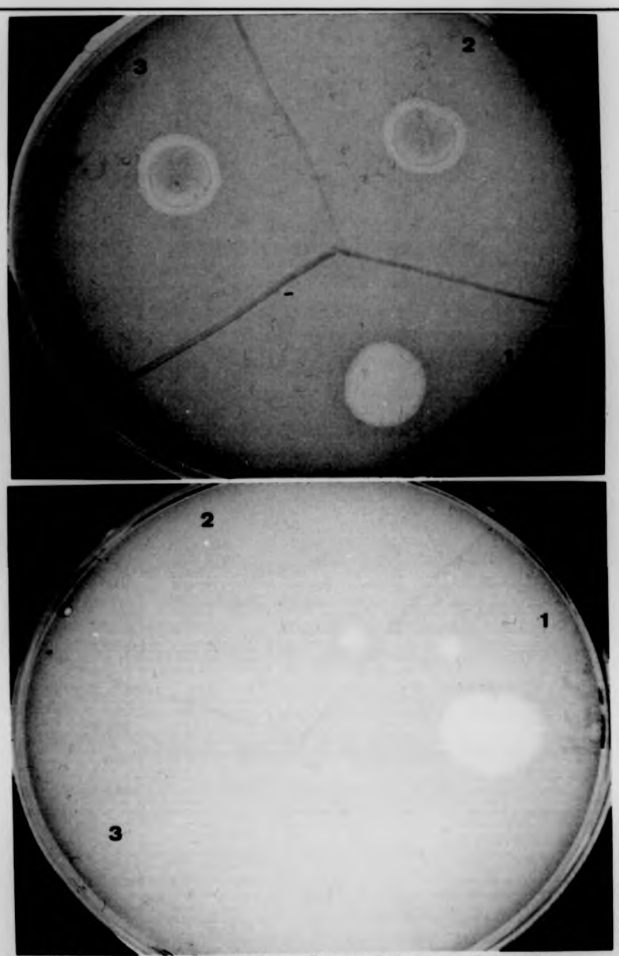


Fig.16. **The pseudolysogeny test results of pseudolysogenic and non pseudolyogenic *M. smegmatis* mc²155 strains**.

a) shows the test plate for a strain pseudolysogenic for mycobacteriophage D4.
b) shows the test plate for a strain non pseudolysogenic for mycobacteriophage D29.
a+b show *M. smegmatis* mc²155 bacterial lawns formed in Lemco soft top agar following incubation at 37°C for 24-48hr divided into sections 1, 2 and 3. 5μl of mycobacteriophage solution 1x10⁵pfu/ml), was "dropped" onto section 1 of each plate, D4 on 1a and D29 on 1b. The test cultures (10μl of late log phase culture) were "dropped" onto sections 2 and 3. 2a and 3a clearly show the "halo" of clearing created by the lysis of lawn cells with D4 phage released from the pseudolysogenic strain. In sections 2b and 3b a complete bacterial lawn was formed following the addition of a non pseudolysogenic from an apparently D29 resistant strain.

3.1.1.3 UV induction of the lytic cycle in pseudolysogenic strains

In order to improve the clarity of the pseudolysogeny test, by creating unequivocal "halos" of clearing, the overinfection plates were exposed to UV following complete lysis in an attempt to induce the lytic cycle in any pseudolysogenic strains.

Two overinfection plates were set up for D29, D33 and D4, respectively using the same *M. smegmatis* culture for lawn cells. One overinfection plate for each phage type was exposed to 40x10-3J of UV, following total lysis of the lawn cells, before reincubation of the plates (4 days at 37°C) to allow for colony formation. Similar numbers of colonies were produced on the overinfection plates following exposure to UV as were formed on the unexposed plates and had previously formed on the overinfection plates. A total of 50 strains "resistant" to each of the three phage types were selected, 25 from the UV exposed plates and 25 from the unexposed overinfection plates. All the strains were subcultured and tested for pseudolysogeny. 144 strains appeared to be pseudolysogenic. The pseudolysogenic strains of D4 and D33 exposed to UV light exhibited more distinguishable clearing zones than those that had not been exposed to UV light; 80% in D4 strains and 20% in D33 strains. The UV exposed D29 strains did not exhibit any increase in those producing a distinct clearing zone.

The six apparently non-pseudolysogenic strains were all isolated from UV exposed overinfection plates, two D33, three D4 and one D29 strains. All six of these non-pseudolysogenic possibly resistant strains were found to be infectable with their respective phage. No truly phage resistant strains were obtained from this screen of 150 "resistant" colonies produced on overinfection plates.

The results of the pseudolyogeny testing suggested that within a given strain, varying proportions of cells were pseudolysogenic and that this proportion decreased with serial subculture, which would be expected from an unstable lysogen. The pseudolysogeny test

whilst screening out the vast majority of positively pseudolysogenic strains could also give false negatives in strains with higher proportions of non-pseudolysogenic cells. These false negative pseudolysogenic strains could give a negative infectability test if the culture used was generated from a pseudolysogenic single colony of the strain; this could give the impression that the strain was phage resistant. The false negative pseudolysogeny results could have been reduced by carrying out the initial pseudolysogeny tests on the same culture of each strain and by performing both tests on more than one culture from each strain at a time, however this would have defeated the object of providing a quick screen for pseudolysogeny. The exposure to UV increased the clarity of the test, although this may have been improved upon by increasing the amount of UV to which the overinfection plates were exposed. Overall the pseudolysogeny test proved a quick method of screening large numbers of "resistant" strains produced by the overinfection method for pseudolysogeny.

No mycobacteriophage resistant mutants were isolated by the overinfection method which was possibly because an insufficient number of "resistant" strains had been screened. Due to the difficulties associated with this method, caused by pseudolysogeny, the search for a spontaneous *M. smegmatis* mutant was abandoned.

An alternative approach was to increase the probability of finding a resistant strain by mutating a population of *M. smegmatis* mc^2155 cells and screening for phage resistant strains. The strategy had the advantage that the *M. smegmatis* strains would not have been previously exposed to phage and would not therefore be pseudolysogenic.

3.1.2 MUTAGENESIS OF *M. SMEGMATIS* MC^2155 WITH N-METHYL-N'-NITRO-N-NITROSOGUANIDINE (NTG)

The proposal was to create and isolate a mutant strain of *M. smegmatis* mc^2155 with a defective cell wall, which would manifest itself as a screenable altered phenotype i.e. phage

resistance or antibiotic resistance. NTG was used to generate mutated strains of *M. smegmatis*, acting by intercalation into the DNA double helix during replication and causing point mutations by preventing correct incorporation of nucleotides. The formation of point mutations can be an advantage as mutations in essential genes could possibly be isolated. A bank of NTG-treated strains would then be screened for phenotypic alterations which may indicate an altered cell wall structure.

M. smegmatis mc^2155 cultures were treated with NTG as described in section 2.27. A late log-phase *M. smegmatis* mc^2155 culture was treated with 0.5μg/ml NTG at 37°C for 45min, before being washed with buffer, resuspended in minimal medium supplemented with Casamino acids (MM+AA) and incubated at 28°C; this temperature was used because the mycolic acid mutant (Kundu *et al.*, 1991) was isolated at 28°C and found to be thermosensitive, presumably due to the altered properties of the lipids in the cell envelope (Dr. P. Chakrabati, personal communication). This mutagenesis procedure was performed twice, on individual late log-phase cultures of *M. smegmatis* mc^2155, and described below.

Following recovery of the cells at 28°C the NTG-treated cells of the first mutagenesis procedure were serially diluted ($1\text{-}1x10^{-9}$) and plated onto duplicate sets of minimal media (MM) agar and MM+AA agar plates and incubated at 28°C and 37°C (section 2.27.1.1; Fig.17). This was done in order to be able to determine the levels of auxotrophy and temperature sensitivity within the NTG-treated population, directly after the mutagenesis event, before subculture.

On the basis of the results obtained from phenotypic screening of the first mutagenesis a second mutagenesis procedure was carried out. NTG-treated cells were diluted ($1\text{-}1x10^{-9}$), plated onto Middlebrook 7H10 agar and incubated at 28°C (section 2.27.2)

The colonies initially formed by the NTG-treated cells, from both mutagenesis procedures, were mucoid after incubation at 28°C and 37°C. Some white and orange colonies, with altered chromogenesis, were observed on plates of colonies from each mutagenesis, at both temperatures.

3.1.2.1 Percentage survival following the NTG treatment of M. smegmatis mc²155 cultures

It was important to ensure a balance was kept between the loss of viability and effective mutagenesis caused by the NTG treatment of *M. smegmatis* cultures. The percentage survival was therefore measured following each NTG treatment as a measure of this balance and therefore as an indication of the success of the mutagenesis procedure. The results for both the mutagenesis procedures are shown in Table 5.

Table 5. **Effect on survival of *M. smegmatis* mc²155 following exposure to NTG**

Mutagenesis procedure	Untreated cells cfu/ml	Treated cells (NTG) cfu/ml	% Killing	% Survival
1	$2x10^9$	$1.6x10^8$	92	8
2	$1.36x10^9$	$2.4x10^8$	82.3	17.6

The percentage survival was 8% and 17.6% following NTG treatment in the first and second mutagenesis procedures respectively. These results were within the levels described by Holland and Ratledge (1971) and Hinshelwood and Stoker (1992b) as resulting in successful mutagenesis without a damaging loss of viability.

3.1.2.2 INH enrichment

INH kills only actively growing cells and has been found to enrich for mutant strains with poor growth at 37°C in mycobacteria (Holland and Ratledge, 1971). In an attempt to enrich for mutated strains, half of the NTG-treated *M. smegmatis* cells from the first mutagenesis

experiment were exposed to INH (50μg/ml) at 37°C (24hr) before being left to recover at 28°C (2.27.1.). The first mutagenesis therefore resulted in two groups of NTG-treated cells SetA, not exposed to INH and SetB(INH) which were INH treated (Fig.17). Following recovery at 28°C the NTG-treated cells of the first procedure, SetA and SetB (INH), were serially diluted, each dilution was plated onto MM and MM+AA in duplicate and incubated at 37°C and at 28°C (section 2.27.1.1; Fig.17). The numbers of colonies formed were compared at corresponding dilutions (Table.6).

SetB(INH) from the first mutagenesis produced a greatly reduced number of colonies on both MM and MM+AA than at comparable dilutions for SetA at both incubation temperatures. The percentage survival of SetB(INH) following INH treatment varied from 5% at 37°C to 13.6% at 28°C on MM+AA. On unsupplemented MM the percentage survival following INH treatment was slightly lower at both temperatures, 11.6% at 28°C and 4% at 37°C. Strains which were incubated at 28°C following INH treatment showed higher survival levels than those incubated at 37°C, (Table.6).

Table 6. **Effects of INH treatment and subsequent incubation temperature on survival of NTG-treated strains**

Incubation temperature	Percentage survival on MM+AA SetB/SetA	Percentage	Percentage survival on MM SetB/SetA	Percentage
28°C	676/5325	12.7	515.5/5087	10
37°C	68/1314	5.1	55/1318	4

These initial results suggested that the INH treatment had significantly reduced the percentage survival under all conditions and appeared to be enriching for NTG-treated strains better able to survive at 28°C.

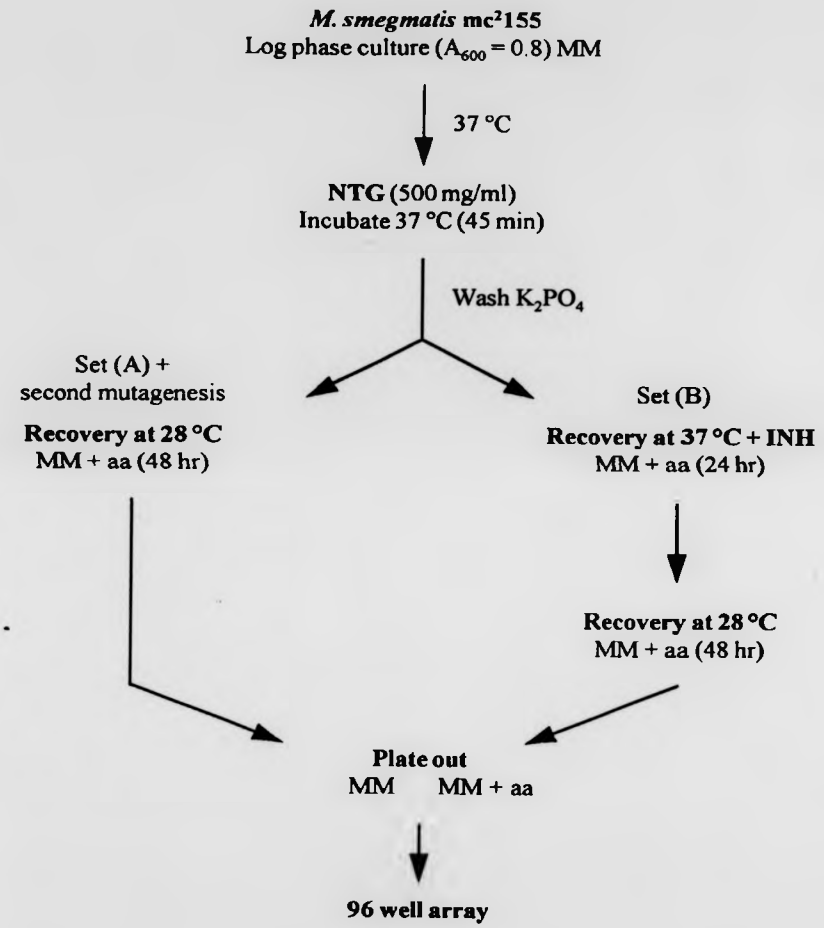


Fig.17. A schematic diagram of the first mutagenesis procedure.

3.1.2.3 Auxotrophy

3.1.2.3.1 First mutagenesis

The NTG-treated *M. smegmatis* mc^2155 strains from both procedures were screened for auxotrophs, as a marker of the efficiency of the mutagenesis.

An initial estimate of the level of auxotrophy in SetA and SetB(INH) was made by comparing the number of colonies formed on the original MM and MM+AA dilution series (Fig.17 and 2.27.1.1), at 37°C and 28°, (Table.7).

Table 7. **The initial levels of auxotrophy observed following the first NTG mutagenesis procedure**

NTG-treated *M. smegmatis* (1st procedure)	Level of auxotrophy at 28°C No. of colonies MM/MM+AA	Percentage	Level of auxotrophy at 37°C No. of colonies MM/MM+AA	Percentage
Set A	5175/5450	5.3	1318/1314	-0.3
Set B (INH)	600/744	24.0	575/696	21.0

The estimated percentage of auxotrophs within the NTG-treated *M. smegmatis* strains of SetA was 5.3% at 28°C and -0.3% at 37°C. The estimated percentage of auxotrophs within SetB(INH) was 24% at 28°C and 21% at 37°C, higher than in the cells of SetA, which were not INH treated. The estimated levels of auxotrophy were higher at 28°C than at 37°C for both sets of NTG-treated cells.

The actual percentage of stable auxotrophs within the NTG-treated cell populations of SetA and SetB(INH) was then established. 2,000 colonies were randomly selected, 1,000 from SetA and 1,000 from SetB(INH) from the MM+AA plates incubated at 28°C. The colonies were maintained on Middlebrook 7H10 plates arranged in arrays of 96 and were known

collectively as Bank 1.

The 2,000 colonies of Bank 1 were replica plated onto MM and MM+AA plates, at 28°C. 36 of the NTG-treated strains of Bank 1 showed no growth on MM while exhibiting growth on MM+AA. 14 (0.67%) of the 36 strains were from SetA (1.4%/of SetA) and 22 (1.11%) from SetB(INH) (2.2% of SetB). These strains were picked from the MM+AA plates, grown to late log-phase in liquid culture at 28°C, before being individually retested on MM and MM+AA agar at both 28°C and 37°C. Five strains (0.25%), one from SetA and four from SetB remained auxotrophic only growing on MM+AA, at both temperatures; AM2 and AM1,3,4,5 although three of these appeared leaky showing a little growth on MM agar, at both temperatures.

3.1.2.3.2 Nutritional requirements of the auxotrophs

Determination of the nutritional requirements of the auxotrophs was then carried out. To determine the amino acid required for survival, each of the five auxotrophic strains (AM1-5) were tested on "dropout" plates containing 19 of the 20 Casamino acids. Strains AM2 and AM4 exhibited weak growth on drop out plates without tryptophan, proline, asparagine, glycine or methionine. AM1 did not grow on agar without proline. AM3 and AM5 grew on all 20 dropout plates.

The five strains were then tested on MM agar containing single amino acids. AM1 only grew on proline-containing MM agar. AM2 grew on MM agar containing only arginine, aspartic acid, phenylalanine, serine or valine. AM3, 4 and 5 grew on all 20 single amino acid plates.

Thus two stable auxotrophs were isolated: AM1 a proline mutant from SetB(INH) and AM2 (SetA), a mutant requiring aspartate, phenylalanine, arginine, serine and valine. The overall percentage of stable auxotrophs was found to be 0.1% within this population of NTG-treated

M. smegmatis mc²155. The percentage of stable auxotrophs was the same within both the INH treated, SetB(INH), and untreated sets of cells (SetA), although three additional leaky auxotrophs (AM3, AM4, AM5) were isolated from SetB(INH). The reduction in the estimated levels of auxotrophy to the actual level of stable auxotrophs from initial estimates of 5.3% in SetA and 24% SetB(INH) to 0.1% in both was also observed by other groups (Konickova-Radochova *et al.*, 1970; Subramanyam *et al.*, 1989).

3.1.2.3.3.Second mutagenesis

One thousand NTG-treated *M. smegmatis* strains from the second mutagenesis were randomly selected and maintained on Middlebrook 7H10 agar arranged in arrays of 96 following incubation at 28°C. The 1,000 strains, known collectively as Bank 2, were then replica plated on to MM and MM+AA agar and incubated at 28°C. Fifty eight strains grew poorly or not at all on MM agar and these strains were subcultured from the master plates before being retested individually. Two stable strains 570 and 657 were consistently unable to grow on MM following subculture. Although the nutritional requirements of these auxotrophs has not yet been determined, they were taken to be auxotrophs. The cell population of the second mutagenesis therefore had a 0.2% level of auxotrophy.

The actual percentage of stable auxotrophs derived from both mutagenesis procedures 0.1% and 0.2% were comparable to those found by other groups (Holland and Ratledge, 1971; Hinshelwood and Stoker, 1992b).

3.1.2.4 Altered chromogenesis

A number of NTG-treated strains with altered chromogenesis were observed in SetA but not in SetB(INH) following the first mutagenesis procedure. Three strains (17, 143 and 186) appeared to have no colouring, remaining white even after prolonged growth and exposure to light. Two strains 96 and 356 were observed to be a bright orange colour from an early

stage of growth. One white strain, 17, one orange strain, 96, and the parent *M. smegmatis* mc^2155 were cultured at 28°C and 37°C to late stationary phase (0.8 A_{600}). The cells were freeze dried, their pigmentS extracted and their absorption spectra analysed. The pigment analysis was carried out by Dr.G.Britton, Department of Biochemistry, University of Liverpool.

The analysis revealed small amounts of a β-carotene like-pigment in wild type mc2155 *M. smegmatis* strain but the main pigment was theophytin, a chlorophyll degradation product which absorbed at 409nm. No pigment was found in the white NTG-treated *M. smegmatis* strain, 17, while the orange strain 96, produced higher levels of both pigments. The amounts of pigment produced by the strain 96 at 37°C were slightly higher than the amounts produced at 28°C.

3.1.2.5 Melanin and Mitomycin C mutants

One thousand of the NTG-treated strains of Bank 1 were also screened for a number of other altered phenotypes, including increased sensitivity to Mitomycin C and lack of melanin production. This work was carried out by R.Rees, LSHTM. The 1,000 NTG-treated strains were screened for Mitomycin C sensitivity, in the search for a *recA*⁻ strain. Initially the strains were screened by replica plating onto Lemco plates containing 0.5μg/ml Mitomycin C and 72 strains with poor or no growth were isolated. The 72 strain were subcultured, retested and ultimately 12 stable Mitomycin C sensitive strains were isolated, strains 42, 43, 147, 149, 218, 316, 386, 426, 427, 460, 681, and 734 all from SetB(INH).

The screen for melanin mutants initially produced 10 possible mutants, with pale colour when replica plated onto tyrosine Lemco agar containing $CuSO_4$. These strains were subcultured and retested, revealing 2 strains which were consistently white when grown on tyrosine medium, 387(INH) and 418(INH). The Mitomycin C sensitive strains and the melanin mutants are

currently being investigated.

The isolation of auxotrophic mutants at 0.1 and 0.2% indicated that both mutagenesis experiments had been successful. The additional chromogenesis, Mitomycin C and melanin mutants retrieved from Bank 1 were further evidence that the first mutagenesis was successful and had produced diverse mutations. Having established the success of the mutagenesis procedures the NTG-treated strains could then be screened for any alteration in phenotypes that may indicate a change in the cell envelope of the strain.

3.1.3 SCREENING AND SELECTION FOR *M. SMEGMATIS* MUTANTS WITH ALTERED CELL ENVELOPES

In order to isolate cell envelope mutant strains of *M. smegmatis* from the NTG-treated population the strains were screened for altered phenotypes which may indicate a defective cell envelope. The NTG-treated *M. smegmatis* strains were initially screened for temperature sensitivity and mycobacteriophage resistance and then for increased resistance and sensitivity to a variety of antibacterials.

3.1.3.1 Mycobacteriophage resistance

Following unsuccessful attempts to isolate a spontaneous phage resistant mutant of *M. smegmatis* mc^2155 NTG-treated *M. smegmatis* strains were screened for resistance to a number of mycobacteriophages.

3.1.3.1.1 First screening of Bank 1 strains

1170 colonies from Bank 1 of NTG-treated *M. smegmatis* strains, 584 from SetA and 586 from SetB(INH), were screened for resistance to four mycobacteriophages: D29, D4, D33 and DNAIII8. The strains were initially screened using the "drop" method (section 2.22). The basis of this method was to pipette mycobacteriophage solution (10^5 pfu/ml) onto a lawn of

the NTG-treated test culture and to incubate at 37°C. Resistant strains would form a complete lawn where as infectable strains should form a clearing zone, caused by mycobacteriophage lysis of the surrounding cells. The NTG-treated *M. smegmatis* mc^2155 strains were replica plated into 96 well micronic tubes containing 800μl of Middlebrook 7H9 media and incubated at 37°C with shaking for 48hr before 200μl of each culture was plated out in Lemco soft top agar. 5μl of D29, D4 D33 and DNAIII8 solutions were then dropped onto the soft top and the plates incubated at 37°C for 48hr.

A total of 28 NTG-treated *M. smegmatis* strains, 14 each from SetA and SetB(INH), appeared resistant to one or more of the mycobacteriophage tested (Table.8). Seven strains initially appeared resistant to all four mycobacteriophages and another seven were apparently resistant to D29, D33 and DNAIII8. Two strains appeared resistant to both D33 and DNAIII8 and three to D29 and DNAIII8. Six strains were resistant to D29, the most frequent resistance to a single mycobacteriophage, with single strains showing resistance to D33, DNAII8 and D4.

Table 8. **The results of the initial screening of Bank 1 strains for mycobacteriophage resistance**

Type of phage resistance	No. of NTG-treated resistant strains
D29, D4, D33 and DNAIII8	7
D29, D33 and DNAIII8	7
D33 and DNAIII8	2
D29 and DNAIII8	3
D29	6
D33	1
D4	1
D29	1

The 28 "resistant" strains were subcultured onto Middlebrook 7H10 plates before being retested for phage resistance by the infection method (section 2.21). On retesting all 28 strains were found to be sensitive to infection by all four mycobacteriophage types.

3.1.3.1.2 Second screening of an additional 1,000 SetA mutants

Having been unable to isolate any stable mycobacteriophage resistant strains from Bank 1 of NTG-treated strains, a further batch of 1,000 NTG strains from SetA were screened. The 1,000 NTG-treated *M. smegmatis* strains were grown to late log-phase in 5ml Lemco at 37°C, with shaking. Each culture was then tested for infectability with D29, D4, DNAIII8, D33 and Lg ($1x10^5$ pfu/ml) using the "drop" method (section 2.22). Those strains which appeared to be resistant following this initial screen were retested following subculture using the infection method (section 2.21).

Table 9. **The results of serial screening of 1,000 NTG-treated *M. smegmatis* for mycobacteriophage resistance**

Phage	Screen 1 "Drop" Method	Screen 2 Infection	Screen 3 Infection	Screen 4 Infection
D4	4	0	-	-
D29	94	24	4	0
D33	0	-	-	0
DNAIII8	2	0	-	-
Lg	0	-	-	-

Following the initial screen using the drop method 100 strains appeared resistant; 94 of these (94%) to D29, 4 to D4 and 2 to DNAIII8 (Table.9). The high numbers of D29 "resistant" strains indicated that the titre of the phage solution had fallen and a new preparation (section 2.20) was used in subsequent experiments. On retesting of the 100 strains using the infection method (section 2.21) only 24 strains resistant to D29 remained, a five fold reduction. After

subculture the 24 strains were retested twice following subculture, using the infection method and all the strains were found to be infectable with D29 implying the strains were unstable mutants which would have been unsuitable for complementation.

3.1.3.1.3 Large scale selection for mycobacteriophage resistance

To increase the chances of isolating a mycobacteriophage resistant strain of *M. smegmatis* larger numbers of NTG-treated *M. smegmatis* mc^2155 strains, derived from frozen stocks and grown to late log-phase at 28°C, were screened for mycobacteriophage resistance (section 2.28.2). The selection involved plating large numbers of NTG-treated *M. smegmatis* strains onto plates soaked in mycobacteriophage and analysing any *M. smegmatis* strains able to survive this "sea" of phage. The mycobacteriophage solutions used were of lower concentrations ($1x10^4$-$1x10^5$ pfu/ml) than those which had previously resulted in pseudolysogenic strains ($1x10^8$-$1x10^9$pfu/ml)(section 3.1.1). 1ml ($3x10^8$ cfu) 100μl ($3x10^7$cfu) and 10μl ($3x10^6$ cfu) of a late log-phase culture of NTG-treated *M. smegmatis* cells from SetB(INH) was spread onto Lemco plates soaked with 1ml of individual mycobacteriophage solution ($1x10^4$-$1x10^5$pfu/ml). Colonies were only formed on plates seeded with $3x10^8$ cfu, no colonies were observed when lower numbers of NTG-treated *M. smegmatis* strains were used. No colonies were formed on any plates spread with equal numbers of wild type *M. smegmatis* mc^2155 cells. A similar number of resistant colonies were formed for each phage type used, 35 with D29, 30 with DNAIII8, 25 with D33 and 24 with D4.

The "resistant" strains were all subcultured, tested for pseudolysogeny (section 2.24) and then infectabilty with D29, D33, DNAIII8 and D4, using the drop method (section 2.22). All 24 D4 strains were found to be non-pseudolysogenic but infectable with D4 and the three other phage on retesting. All 30 DNAIII8 and D29 strains were found to be pseudolysogenic and were not therefore tested for infectability. Of the 25 D33 resistant strains only 13 were successfully subcultured and tested for pseudolysogeny and infectability. Seven D33 strains

were non-pseudolysogenic and infectable with all four phages.

Six D33 "resistant" strains remained uninfectable with both D33 and also DNAIII8 but four of these were pseudolysogenic on the initial screen. Two uninfectable strains 2 and 21 were retested for pseudolysogeny and infectability, using the infection method. On rescreening two strains were found to be pseudolysogenic and remained resistant to infection by both D33 and DNAIII8.

This large scale screen for phage resistant mutants was unsuccessful in isolating any stable mycobacteriophage resistant strains of *M. smegmatis*, although initially colonies were formed on phage-soaked plates spread with NTG-treated *M. smegmatis* which were not found on the wild type plates, suggesting these were resistant strains. The resistant strains were all pseudolysogenic apart from the D4 derived colonies. The experiment implied that strains pseudolysogenic for D33 were resistant to infection with DNAIII8 as well as D33. Unfortunately as the DNAIII8 strains were all pseudolysogenic they were not tested for infectability with any phage including D33. The levels of pseudolysogeny observed for DNAIII8, D29 and D33 indicated that the $1x10^4$-$1x10^5$ pfu/ml required to cause confluent lysis, was still capable of producing pseudolysogenic strains.

The individual (2170) colonies and large scale screening of NTG-treated *M. smegmatis* strains for resistance to a range of mycobacteriophage failed to isolate a stable phage resistant mutant strain of *M. smegmatis*. The decision was made to discontinue this approach to the isolation of *M. smegmatis* cell wall mutant strains and to commence the search for temperature sensitive strains or strains with altered antibiotic resistance or sensitivity as a phenotypic indication of an altered cell wall.

3.1.3.2 Temperature sensitivity

All NTG-treated *M. smegmatis* cells were left at 28°C to recover in an attempt to retain any temperature sensitive mutants, as there is some evidence that cell wall mutants may survive better at lower temperatures. This approach was also adopted by P. Chakrabarti (personal communication) when isolating the *M. smegmatis* mycolic acid mutant strain (Kundu *et al.*, 1991).

An initial estimate of the percentage of temperature sensitive mutants, from the first mutagenesis was determined by comparing the number of colonies formed on the initial MM+AA dilution plates (2.27.1.1 and Fig.17) at 28°C and at 37°C, for SetA and SetB(INH)(Table.10).

Table 10. **Initial levels of temperature sensitivity observed following the first mutagenesis procedure**

NTG-treated *M. smegmatis*	Levels of temperature sensitivity No. of colonies on MM+AA 28°C/37°C	Percentage	Levels of temperature sensitivity No.of colonies on MM 28°C/37°C	Percentage
Set A	1325/1314	0.83	1087/1318	-21.0
Set B(INH)	744/696	6.9	600/575	4.3

A greater number of colonies were formed on MM+AA plates incubated at 28°C than those incubated at 37°C, for both SetA and SetB(INH). The estimated percentage of temperature sensitive strains was 0.83% for SetA and 6.9% for SetB(INH). These results implied that both recovery at 28°C and particularly INH treatment of the NTG-treated cells had initially enriched for strains only able to survive at 28°C on MM+AA.

The initial number of colonies formed from SetA and SetB(INH) on MM at 28°C and 37°C was also compared (Table.10). Within SetA the number of colonies able to survive at 37°C

on MM was 21% higher than those able to survive at 28°C. The colony counts for SetB(INH) were 4.3% higher for those able to survive at 28°C than at 37°C on MM. These results suggested that the INH treatment was enriching for temperature sensitive mutants, although the numbers able to survive on MM were lower than those able to survive on MM+AA. Recovery at 28°C was insufficient to select for mutants which were stable at 28°C on MM.

These initial results on MM indicated that while INH treatment was probably enriching for strains truly stable at 28°C and sensitive to growth at 37°C. Recovery at 28°C was possibly allowing metabolic mutants to survive at the slower growth rate incurred at 28°C on MM+AA; while on MM with the added disadvantage of poor nutrition these strains were unable to survive.

In an attempt to isolate stable temperature sensitive strains, Bank 1 was replica plated onto duplicate sets Middlebrook 7H10 agar plates. One set of duplicates was incubated at 28°C and the other at 37°C. The colonies were then screened for those with poor growth at 37°C and strong growth at 28°C. Twenty one strains were found which fulfilled this criteria, five of these strains were from SetB(INH) and 16 from SetA. The 21 strains were subcultured onto duplicate 7H10 agar plates and their growth at 28°C and 37°C retested. Three strains continued to show poor growth at 37°C while retaining proliferative growth at 28°C; one strain, 228, from SetA and strains 166 and 621 from SetB(INH). A subsequent subculture of these three strains and a repeat testing of their growth at 37°C and 28°C showed an apparent reversion to growth at 37°C. No stable temperature sensitive mutants were isolated from Bank 1.

3.1.3.3 Screening for altered antibiotic resistance and sensitivity

There are a variety of antibiotics whose mode of action is thought to involve the mycobacterial cell wall, as well as those whose uptake would be affected by a change in the

permeability of the cell envelope (section 1.5). An increased antibiotic resistance or sensitivity could therefore be due to an altered cell envelope. The NTG-treated *M. smegmatis* strains were screened for altered antibiotic resistance and sensitivity in an attempt to isolate a strain with a defective cell wall, while remaining aware that other non-cell wall target site mutations could be involved.

3.1.3.3.1 Minimum Inhibitory Concentrations, MICs

Initially MICs of the selected antibiotics were determined on solid Lemco agar at 28°C . A late log-phase (0.8-1 A_{600}) liquid culture of wild type *M. smegmatis* mc^2155 was streaked onto Lemco agar containing increasing concentrations of the given antibiotic, from 0 - 1mg/ml, in doubling increments i.e. 2, 4, 8, 16, 32, etc. The plates were incubated at 28°C for 6 days before being read. The results are shown in Table 11.

The MICs of each antibiotic for *M. smegmatis* mc^2155 on Lemco agar at 28°C were on the whole similar to those found by other groups (Table.11); variations were put down to the differing conditions of media, temperature and growth stage and size of inoculum under which these values were established. *M. smegmatis* mc^2155 was, as expected, sensitive to antimycobacterials 1-7 (Table.11) and resistant to penicillin G and pyrazinamide. The most suitable concentrations for the initial screening of the banks of NTG-treated *M. smegmatis* strains for increased resistance to antimycobacterials 1-7, or sensitivity to penicillin G and pyrazinamide, were determined (Table.11).

Table 11. The Minimum Inhibitory Concentrations (MICs) of nine antibiotics against *M. smegmatis* mc²155 at 28°C

Antibiotic	*M. smegmatis* mc²155 MIC (μg/ml) at 28°C (on Lemco media)	Published *M. smegmatis* MIC's (μg/ml), at 37°C (in various media)	Screening concentration (μg/ml)
1) Minocycline	32	2-5 (Yamada *et al.*, 1991)	50
2) Ethambutol	2-4	3-5 (Silve *et al.*, 1993)	5
3) Ethionamide	32	10 (Banerjee *et al.*, 1994)	50
4) Amikacin	32-64	Unknown (5-20 for *M. tuberculosis* Dulwich, PHL)	75
5) Cycloserine	128	Unknown (< 50 for *M. tuberculosis* , Dulwich PHL)	200
6) Ofloxacin	2-4	< 2 in *M. tuberculosis*	5
7) Ciprofloxacin	2-4		5
8) Penicillin G	1024		200 + 500
9) Pyrazinamide	600	at least 500 (Konno et *al.*, 1967)	500

3.1.3.3.2 Antibiotic resistance

NTG-treated *M. smegmatis* were screened for resistance to ethambutol, ethionamide and cycloserine as their target site is believed to be in the cell wall (section 1.5.1.); the tetracycline, minocycline, was used as its efficacy against mycobacteria is attributed to its ability to traverse the lipid permeability barrier, an alteration in the cell wall could result in increased resistance to this antibiotic. Amikacin is a polycationic aminoglycoside whose uptake is self promoted and believed to be due to the negative surface charges on the mycobacterial cell wall; the drug was used as an alteration in the cell wall could again lead to reduced uptake of the drug. The fluoroquinones, despite the fact that their main target site is DNA gyrase, were used as a

permeability alteration could also result in increased resistance.

Bank 1 of 2,000 NTG-treated *M. smegmatis* strains were screened for resistance to seven antibiotics. The 2,000 strains were replica plated onto Lemco agar containing an inhibitory concentration of the given antibiotic and subsequently incubated at 28°C for 5 days. Any strains able to grow were subcultured onto Middlebrook 7H10 agar before being retested by streaking single colonies onto Lemco agar containing an inhibitory concentration of antibiotic. The strains were subsequently incubated at 28°C for 5 days. Any resistant strains isolated were retested in liquid Lemco media containing increasing concentrations of the antibiotics.

If no resistant strains were isolated from Bank 1 then a large scale screen of NTG-treated *M. smegmatis* strains was undertaken. 1ml ($3x10^8$ cfu) of a late log-phase culture of NTG-treated *M. smegmatis* strains, seeded from frozen stocks of SetB(INH), was spread onto Lemco agar containing the inhibitory concentration of the antibiotic. Again any resistant strains were subcultured and retested individually.

3.1.3.3.2.1 Minocycline

Bank 1 was replica plated onto Lemco agar containing 50μg/ml of minocycline and 12 apparently resistant strains were observed. These 12 strains were found to be sensitive to the same concentration when subcultured and retested. No stable minocyline resistant strains were isolated from Bank 1.

A large scale screen (section 2.28.2) for minocycline resistant strains at 50μg/ml concentration was then carried out which was also fruitless .

3.1.3.3.2.2 Ethambutol

Bank 1 was replica plated onto Lemco agar containing 2μg/ml ethambutol and incubated at 28°C. The majority of the Bank 1 strains appeared sensitive to the 2μg/ml MIC, which had been difficult to determine exactly. Twenty-nine strains, 18 from SetA and 11 SetB(INH), grew on the ethambutol containing agar. These strains were subcultured and restreaked onto agar containing ethambutol at 2μg/ml and 5μg/ml. One strain showed growth on 2μg/ml ethambutol, five strains showed some growth on 2 and 5μg/ml of ethambutol. The other 23 strains showed no growth on either concentration of ethambutol. Concentrations of 2μg/ml and 5μg/ml ethambutol were used to determine the degree of resistance exhibited by resistant strains. The six apparently resistant strains were retested on 2μg/ml and 5μg/ml of ethambutol on solid and liquid Lemco agar and were found to be sensitive to both concentrations on solid and in liquid media.

Large scale screening (section 2.28.2) yielded two apparently resistant strains which were found to be sensitive on subculture and retesting, again no strain was found.

3.1.3.3.2.3 Ethionamide

Bank 1 was replica plated onto Lemco agar containing 50μg/ml of ethionamide. Thirty nine strains showed growth on ethionamide, 24 from SetA and 15 from SetB(INH). On subculture and retesting, nine strains, all from SetA were found to be weakly resistant, whereas the other 30 were all sensitive. On subsequent subculture and retesting of these 9 strains on solid and in liquid media the resistance was not maintained.

Large scale screening (section 2.28.2) for ethionamide resistance involved the plating out of NTG-treated *M. smegmatis* strains from SetA and SetB(INH). The screen yielded 45 resistant colonies, 21 from SetA and 24 from SetB(INH). The 45 strains were subcultured and restreaked onto plates containing ethionamide; 19 SetA strains and 20 SetB(INH) strains

remained resistant. The test was repeated by streaking from liquid cultures of each strain on to ethionamide plates. This repeat test resulted in 4 strains from SetA and 3 from SetB(INH) retaining very weak resistance, only 3-4 resistant colonies per plate. The seven weakly resistant strains were deemed unsuitable for complementation experiments and therefore further investigation.

3.1.3.3.2.4 Amikacin

Bank 1 was replica plated onto Lemco agar containing 75μg/ml amikacin. Four strains were found to grow strongly on the Lemco agar containing amikacin. These strains were subcultured and retested on Lemco agar with 75μg/ml amikacin. The four strains no longer showed resistance to this concentration of amikacin.

A large scale amikacin screen (section 2.28.2) did not produce any resistant strains. No stably amikacin resistant strains were isolated.

3.1.3.3.2.5 Cycloserine

Bank 1 was replica plated onto Lemco agar containing 200μg/ml cycloserine. 150 strains were found to grow on cycloserine following this initial screen, 65 from SetA and 85 from SetB(INH). These strains were subcultured and retested on Lemco agar containing 200μg/ml cycloserine. Forty nine strains retained resistance, 22 from SetA and 27 from SetB(INH). These strains were again subcultured and retested and all forty nine strains regained their sensitivity.

3.1.3.3.2.6 Ofloxacin

Bank 1 was replica plated onto Lemco agar containing 5μg/ml ofloxacin. Forty five strains showed growth on 5μg/ml ofloxacin, 25 from SetA and 20 from SetB(INH). Of these only 12, eight from SetA and four from SetB(INH), were found to remain resistant following

subculture and subsequent retesting. The strains from SetA were 4, 134, 290, 291, 486, 934, 944 and 1008, those from SetB(INH) were 259, 601, 712 and 720. The strains retested on Lemco agar containing 5μg/ml and 10μg/ml ofloxacin and found to remain strongly resistant to 5μg/ml and weakly resistant to 10μg/ml.

The resistance of the 12 strains was also tested in 5ml Lemco media with a 5μg/ml concentration. Seven of the strains were found to remain resistant to 5μg/ml in liquid culture, while five (134, 486, 1008, 259(INH) and 712(INH)) were inhibited at this concentration in liquid culture.

The 12 strains were also tested for cross reactivity to ciprofloxacin by streaking them onto 5 and 10μg/ml of ciprofloxacin.

Two strains 486 and 712(INH) were found to be sensitive to 5μg/ml ciprofloxacin, the other ten strains were resistant. It was more likely that those strains resistant to both ofloxacin and ciprofloxacin had all sustained mutations in their *gyrA* or *gyrB* genes, the primary site of action of both drugs. It was also conceivable that those strains resistant to ofloxacin only had acquired mutations in other genes which resulted in resistance through decreased permeability possibly due to an alteration in the cell envelope.

3.1.3.3.2.7 Ciprofloxacin

Bank 1 of NTG-treated *M. smegmatis* cells were replica plated onto Lemco agar containing 5μg/ml ciprofloxacin. Eighteen strains were found to grow on this concentration of ciprofloxacin, 12 strains from SetA and 6 from SetB(INH). The strains were subcultured and retested and three of the strains from SetA remained resistant (273, 487 and 719). The other 15 strains were sensitive to 5μg/ml ciprofloxacin. None of the cross resistant ofloxacin resistant strains were picked up from this screen for ciprofloxacin resistance.

In summary, NTG-treated strains were screened for resistance to seven antimycobacterial agents at concentrations to which they were normally susceptible. Although initial screens appeared to pick up some strains resistant to each antibiotic the majority of these strains became sensitive following serial subculture and retesting. Finally, the only stable NTG-treated *M. smegmatis* mutant strains isolated were resistant to ofloaxcin and ciprofloxacin (Table.12)

3.1.3.3.3 Antibiotic Sensitivity Testing

NTG-treated *M. smegmatis* mc^2155 strains were screened for increased sensitivity to compounds to which *M. smegmatis* mc^2155 possessed a naturally high level of resistance i.e. pyrazinamide and penicillin G (Table.11). An increased level of sensitivity may be have been due to a change in permeability, caused by an alteration in the cell envelope structure, as with the penicillin G sensitive mycolic acid mutant of *M. smegmatis* (Kundu *et al.*, 1991).

3.1.3.3.3.1 Penicillin G

Bank 1 was screened for sensitivity to penicillin G by replica plating onto two sets of Lemco plates containing 500μg/ml and 200μg/ml penicillin G respectively, followed by incubation at 28°C. As the MIC for penicillin G was >1mg/ml at 28°C, any strains unable to survive these concentrations of penicillin G would be exhibiting increased sensitivity to the antibiotic. From the initial screening nine strains were isolated from SetA and five of these showed weak growth at 200μg/ml penicillin G as well as no growth at 500μg/ml penicillin G. Eight strains showing no growth on 500μg/ml penicillin G were isolated from SetB(INH). These seventeen strains were retested for sensitivity to 200μg/ml and 500μg/ml. Seven SetA strains and one SetB(INH) strain continued to show increased sensitivity to penicillin G at 500μg/ml. These strains 251, 255, 357, 396, 730, 758 and 826 from SetA and 722 from SetB(INH), were subcultured and further analysed. Two single colonies from each strain were streaked out onto Lemco plates containing penicillin at 200μg/ml and 500μg/ml. All showed increased

sensitivity in that they were sensitive to 500μg/ml penicillin G, although resistant to 200μg/ml on solid medium.

The penicillin G sensitivities of liquid cultures of these strains were then investigated at 28°C and 37°C. Eight single colonies from each of the penicillin sensitive strains were picked and used to inoculate 5ml of Lemco broth. Four cultures from each strain were incubated at 28°C and four from each strain were incubated at 37°C. The cultures were then streaked onto duplicate 200μg/ml penicillin G and 500μg/ml penicillin Lemco plates, one set of duplicates were incubated at 37°C and the other set at 28°C. The eight cultures derived from single colonies of the eight initially sensitive strains were all resistant to 200μg/ml, at 28°C and 37°C. All cultures of the three strains 251, 357 and 826 also showed complete resistance to 500μg/ml penicillin G at both temperatures. In contrast five strains 255, 396, 758, 730 and 722 (INH) showed variable results when their eight liquid cultures were plated onto solid Lemco containing 500μg/ml penicillin G; although the results were consistent at both temperatures.

These five cultures showing variable results were analysed further. The cultures of each strain unable to survive 500μg/ml penicillin were subcultured onto Lemco plates at 28°C, five single colonies from each of these strains were cultured and retested on 500 μg/ml penicillin G at 28°C and 37°C. Three strains (255, 396 and 758) were found to be resistant to 500μg/ml penicillin G at both temperatures. The cultures of strains 730 and 722 showed variable sensitivities to penicillin G. Four cultures of 722 were sensitive to 500μg/ml penicillin G and one was resistant, at both temperatures. Two cultures of 730 were sensitive to 500μg/ml at 37°C and 28°C. One 730 culture was sensitive to 500μg/ml penicillin G at 28°C one at 37°C and one was contaminated. The inconsistency of the results obtained for both strains 730 and 722 indicated that the strains were mixed or unstable and had reverted to the wild type in some of the cultures.

In conclusion, two strains, 730 and 722, showing some penicillin sensitivity were isolated; since they did not appear stable it was concluded that neither were suitable for further use.

3.1.3.3.3.2. Screening of NTG-treated strains from the second mutagenesis for penicillin sensitivity

The screen for penicillin sensitivity had unearthed some sensitive strains, albeit unstable and the decision was taken to continue the search. The majority of the sensitive strains isolated from the previous screen were from SetA, strains which had not been INH treated; the repeat screen was therefore carried out on the INH untreated strains of Bank 2 from the second mutagenesis procedure (section 2.27). The NTG-treated strains were again screened by replica plating for sensitivity to penicillin G at 28°C.

57 strains initially appeared sensitive to penicillin G, these were subcultured and retested on Lemco agar containing 500μg/ml penicillin G from single colonies on Lemco plates. Seventeen strains appeared sensitive, showing no growth on 500μg/ml of penicillin G. The strains were retested by streaking liquid culture, derived from single colonies, onto Lemco agar containing 500μg/ml penicillin. 15 strains were found to remain sensitive to a 500μg/ml concentration of penicillin G at both 28°C and 37°C.

The antibiotic sensitivity of these fifteen strains was then compared to the wild type in liquid culture. Equal numbers ($2x10^7$ cfu) of test strain cells were added to a series of 5ml volumes of Lemco media containing increasing concentrations of penicillin G (100μg/ml-1mg/ml). A series was set up for each test strain and wild type *M. smegmatis* mc^2155, all the series were incubated at 37°C for 48hr (section 2.28.4). The A_{600} of the individual cultures of each series was taken as a measure of growth at each concentration of penicillin. The antibiotic sensitivity of the fifteen test strains were then compared to the wild type. The results represented graphically in Fig.18.

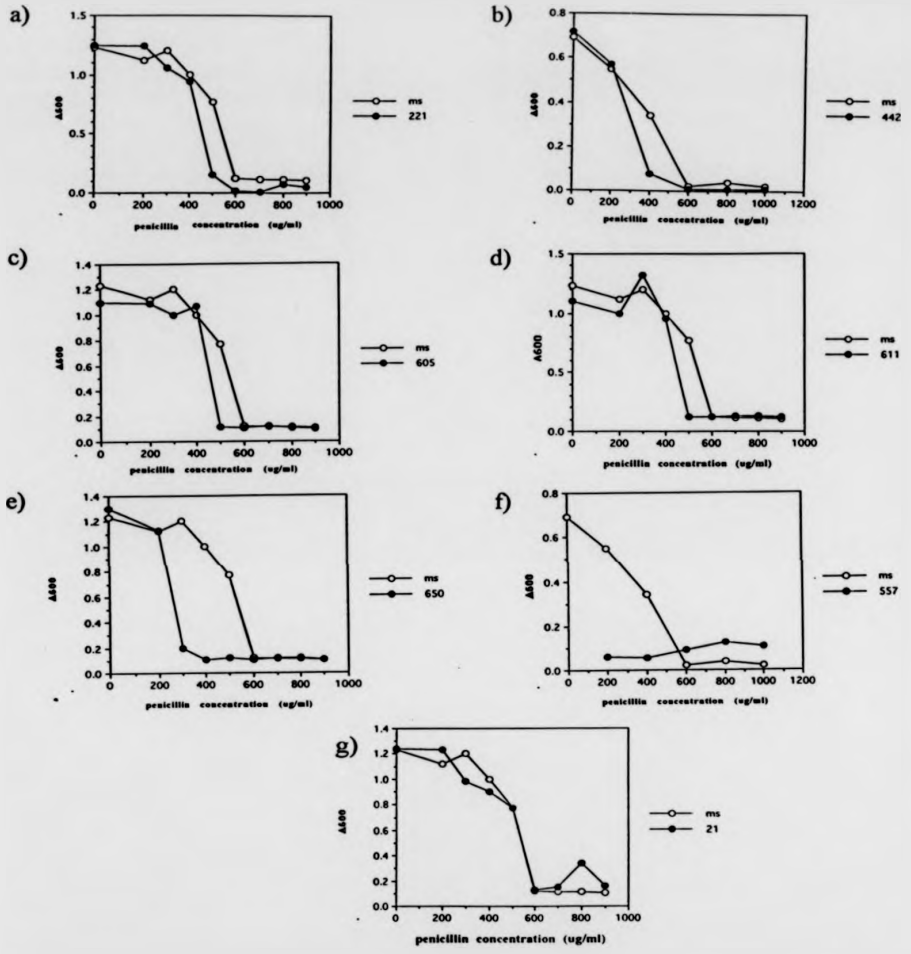


Fig. 18. **Graphs depicting the penicillin G sensitivity of NTG treated *M. smegmatis* strains in liquid culture**

An approximately equal number of cells ($2x10^7$) of each strain were added to 5ml Lemco containing increasing concentrations (100μg/ml) of penicillin G and incubated at 37°C. The A_{600} of each of the individual cultures, at increasing penicillin G concentrations, of each strain were then taken. Strains 221, 442, 605, 611 and 650 (a-e) exhibited increased sensitivity to penicillin G. Strain 557 (f) showed such poor growth that a proper comparision with wild type could not be made. Strain 21 (g) is an example of a strain exhibiting no increased sensitivity to penicillin G.

Five of the strains clearly showed increased sensitivity to penicillin G: 221, 442, 605, 611 and 650 (Fig.18). Eight of the strains (140, 190, 350, 463, 570, 557, 879, 889) showed poor growth in comparison to the wild type in Lemco alone (only 557 is shown in Fig.18) and as well as at the lower concentrations of penicillin G. One of these strains, 570, was also pulled out on the screen for auxotrophs from the second mutagenesis procedure. Four strains, 21, 922, 939 and 806 showed no increased sensitivity to penicillin G (only 21 is shown Fig.18). This experiment also revealed a reduced MIC of 500μg/ml for penicillin G against the *M. smegmatis* mc^{2}155 strain in liquid culture at 37°C following a 48hr incubation when compared with the 1mg/ml MIC on solid media. Similar results were obtained on repetition of this experiment.

Penicillin G sensitive strains 221, 442, 605 and 611 showed increased sensitivity to penicillin G with an MIC of 400μg/ml, 100μg/ml less than the wild type (500μg/ml) in liquid culture. Strain 650 showed the greatest increase in sensitivity, with an MIC of 200μg/ml, 300μg/ml lower than the wild type in liquid culture (Fig.18). The five penicillin G sensitive strains were therefore candidates for further analysis and possible complementation.

3.1.3.3.3.3 Pyrazinamide

The bank of 2,000 NTG-treated colonies was screened for increased sensitivity to pyrazinamide by replica plating onto Lemco agar pH5.2 containing 500μg/ml pyrazinamide, at a concentration of 100μg/ml lower than the 600μg/ml MIC. Fourteen strains from SetA and from SetB(INH) were isolated which appeared to show increased sensitivity to pyrazinamide. These were subcultured and retested on solid medium at pH5.2, at a concentration of 500μg/ml pyrazinamide at 28°C and 37°C. Two strains, 826 and 415, were found to remain sensitive to 500μg/ml pyrazinamide at 28°C, showing no growth on solid medium. The 415(INH) strain showed weak growth at 37°C and with strain 826 only the inoculum was visible. The two strains were subcultured, the test repeated and the same results

obtained.

Both strains 415(INH) and 826 had been selected from other screens. Strain 415(INH)(AM2) had previously been isolated from the auxotroph screen and was found to require aspartate, phenylalanine, arginine, serine or valine for growth. Strain 826 had been selected on the initial screen for penicillin G sensitive strains although it appeared not to be consistently sensitive to penicillin G on retesting.

The sensitivity of 415(INH) and 826 to pyrazinamide was then compared to the wild type strain in liquid culture (section 2.28.3). Equal numbers of cells ($5x10^6$ cfu) from strains 415, 826 and the parent *M. smegmatis* mc^2155 were added to a series of ten 5ml volumes of Lemco broth pH5.2 containing concentrations of pyrazinamide, from 0-1054μg/ml in doubling quantities. The series were set up in duplicate, one series for each strain was incubated at 37°C and the duplicate at 28°C for 48hr and 72hr, respectively.

The MIC of pyrazinamide for the wild type strain was reduced in liquid culture (by 100μg/ml to 500μg/ml) as had been observed for penicillin G. Strain 415(INH) did not show significant sensitivity to pyrazinamide in liquid culture at either temperature when compared with the wild type, (Fig.19a+b). Strain 826 was considerably more sensitive than the wild type to pyrazinamide in liquid culture, particularly at 28°C (Fig.19a+b). As was observed for penicillin G, the MIC of pyrazinamide for the wild type strain was reduced in liquid culture, by 100μg/ml to 500μg/ml.

The fact that 415 was not sensitive to pyrazinamide in liquid culture implied that 415(INH) was not specifically sensitive to pyrazinamide but showed poor growth on solid media containing pyrazinamide due to another mutation which resulted in a poor growth rate; alternatively the strain could have been specifically pyrazinamide sensitive but unstable in

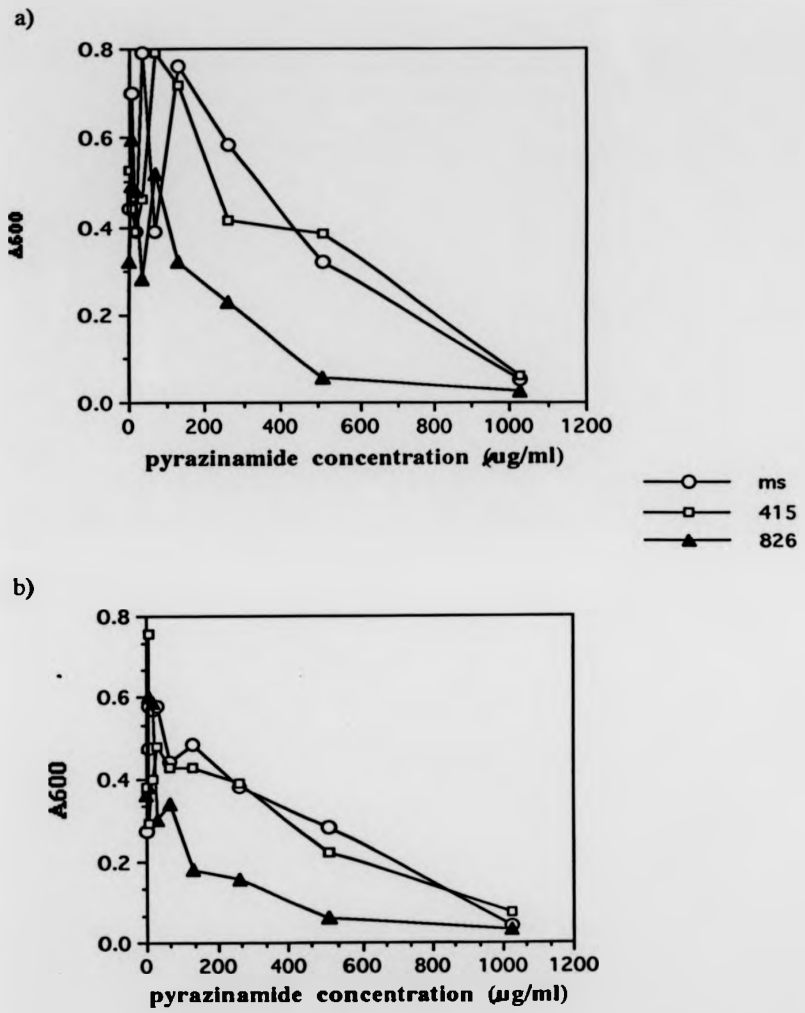


Fig 19. **A graph depicting the pyrazinamide sensitivity of two NTG treated strains of *M. smegmatis*.**
An approximately equal number of cells ($5x10^6$) were added to 5ml Lemco (pH 5.2) with increasing concentrations of pyrazinamide (2μgml-1054μg/ml in doubling quantities) which were incubated at a) 37°C and b) 28°C Strain 826 (Pyramid II) exhibited increased sensitivity to pyrazinamide in liquid culture, (particularly at 28°C) while strain 415 did not, when compared with the *M. smegmatis* mc^2155 wild type resistance (500µg/ml).

liquid culture.

Strain 826 appeared to be a stable specific pyrazinamide sensitive mutant and as such was considered a candidate for further investigation and possible complementation experiments.

In conclusion the screen of NTG-treated *M. smegmatis* strains for sensitivity to penicillin G and pyrazinamide yielded five stable penicillin G-sensitive strains and one stable pyrazinamide-sensitive strain. The two sensitivity screens also selected seven other slow growing, possibly mutant strains, two known auxotrophic strains 415(INH)(AM2) and 570 and the pyrazinamide mutant 826 on the penicillin G screen.

This suggested that the initial sensitivity screening was less specific than the resistance screening, picking up other mutant strains which were not specifically sensitive to the test drug. These nonspecific mutant strains were probably picked up because of their poor growth rates. The sensitivity screening ultimately proved successful in isolating a number of mutant strains.

3.1.3.3.4 Summary of antibiotic screening

The screen for antibiotic resistant and sensitive strains of NTG-treated *M. smegmatis* successfully isolated 12 ofloxacin and 3 ciprofloxacin resistant strains as well as 5 penicillin G and 1 pyrazinamide sensitive mutant (Table.12). The isolation of strains resistant to antibiotics whose mode of action was thought to be involved directly with the cell wall i.e. ethambutol, ethionamide proved elusive.

The initial screening procedure by replica plating pulled out apparently resistant strains or sensitive strains for every antibiotic tested. However, on subsequent subculture and retesting the majority of these strains appeared to lose their resistance or sensitivity (Table.12).

	Number of serial transfers			
(a) Antibiotic resistance	**1**	**2**	**3**	**Actual mutants**
Minocycline	12	0		0
Ethambutol	30	6	0	0
Ethionamide	39	9	0	0
Amikacin	4	0		0
Cycloserine	150	49	0	0
Ofloxacin	45	12	12	12
Ciprofloxacin	18	3	3	3
(b) Antibiotic sensitivity				
Penicillin G - Bank 1	17	8	5	2 variable
Penicillin G - Bank 2	17	15	5	5
Pyrazinamide	14	2	1	1

Table. 12. **Antibiotic resistant and sensitive mutant strains of isolated from Bank1 and Bank2 of NTG-treated *M. smegmatis* strains**
a) shows the results of screening for antibiotic resistance
b) shows the results of screening for antibiotic sensitivity
The numbers of potential mutants following each serial transfer are shown as well as the final number of mutants isolated.

This phenomenon has been observed by other groups screening NTG-treated mycobacteria and was put down to the instability of the mutations.

At this point a decision had to be made about which strains to investigate further and it was considered appropriate to pursue the penicillin G and pyrazinamide sensitive mutants. The basis for the selection of the penicillin G mutants was the previous isolation of a penicillin G sensitive mutant with defective mycolic acid biosynthesis (Kundu *et al.*, 1991); while remaining aware that the penicillin G sensitive strains may have defective β-lactamases resulting in increased sensitivity to penicillin. Of the penicillin G sensitive strains, 650 (PenG 650) was selected for further investigation as it exhibited the greatest degree of sensitivity. The pyrazinamide sensitive mutant 826 (PyramidII) was chosen as it could be a permeability mutant with some form of cell envelope alteration; as little was known of the mode of action of pyrazinamide and the levels of resistance to the drug in *M. smegmatis* it was considered preferable to follow this mutant further.

3.1.4 BIOCHEMICAL ANALYSIS OF CANDIDATE STRAINS FOR COMPLEMENTATION

As described above, two strains, a pyrazinamide sensitive strain (PyramidII) and a penicillin G sensitive strain (PenG 650) were considered candidate strains for further analysis. The initial decision taken was to investigate the lipid content of the cell envelope of the strains to look for any gross alterations.

The mycobacterial lipids of strains PyramidII and PenG 650 were systematically analyzed using the strategy created by G. Dobson *et al.* (1985), in order to locate any gross alterations in the components of the cell envelope. The lipids of wild type *M. smegmatis* and the uncharacterised auxotroph (570) from the second mutagenesis were analysed as controls. This work was carried out under the supervision of Dr.D.Minnikin and Mr R.Bolton, Department of Biochemistry, University of Newcastle.

3.1.4.1 Non Polar and Polar "free" lipid analysis

The free lipids were extracted from 50mg of the freeze-dried cells from a late log-phase culture (0.8 A_{600}) of each strain (section 2.29). There are a total of six specifically developed two-dimensional thin layer chromatographic (2D-TLC) systems (A-F) which are used to analyze the range of polar and non polar lipids. Three of these systems, A, D and F (section 2.30.1-3) were used to analyze the "free" lipids of the strains, PyramidII (826), Aux 570, PenG 650 and *M. smegmatis* mc^2155.

3.1.4.1.1 System A

System A is the least polar system and this was used to search for menaquinones, characteristically found in *M. smegmatis*, in the four strains. Menaquinones were observed under UV light in all four strains. This system can also be used to detect mycoserosates and phthiocerols.

3.1.4.1.2. System D_1

The most polar of the non polar lipid extracts were analyzed using system D_1, which detects trehalose dimycolates (cord factor) and the sulpholipids found in *M.tuberculosis*. Trehalose dimycolate (cord factor) was observed in PyramidII (826), *M. smegmatis*, Aux 570 and PenG 650 (Fig.20).

Sulpholipids were not present as would be expected. Other identical but unknown glycolipids were also observed in all four strains under this system, all of which reacted positively with the α-naphthol sulphuric acid spray, NAS which detects sugar moieties (Fig.20).

3.1.4.1.3 System D_2

The least polar of the polar free lipids were also analyzed using system D which revealed the glycopeptidolipids or C-mycosides. All four strains appeared to have the same simple

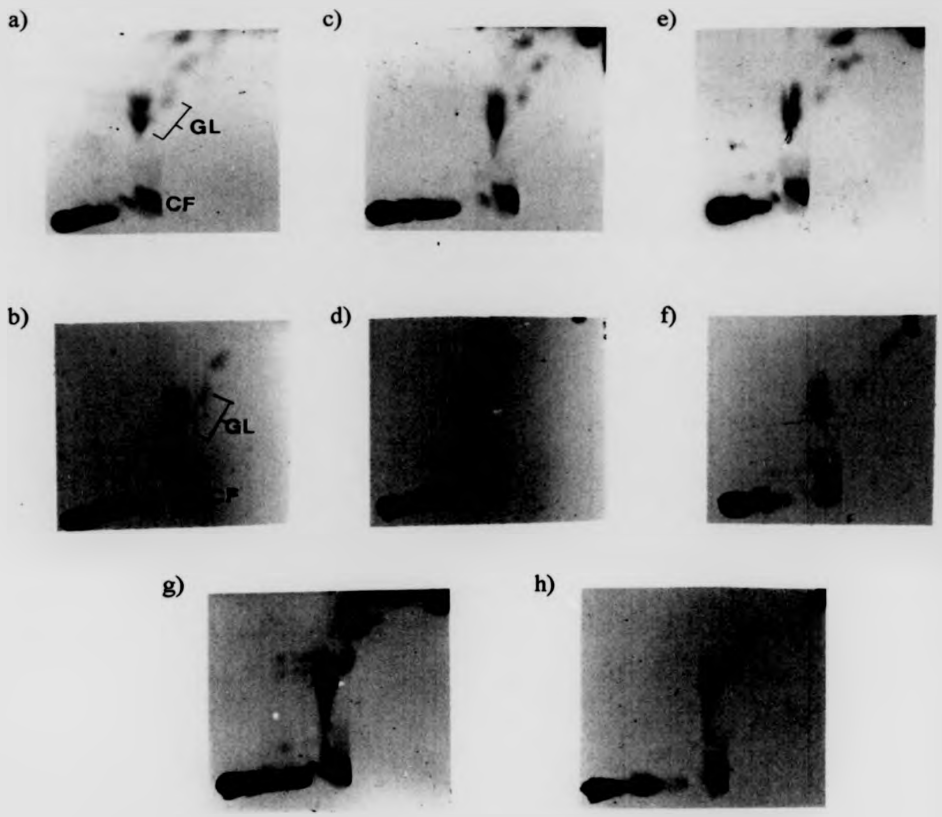


Fig.20. **Two dimensional TLCs of the most polar class of lipids in the non-polar lipid fraction of mycobacterial "free" lipids (System D_1)**
1st direction, chloroform:methanol:water (100:14:0.8 v/v/); 2nd direction, chloroform : acetone : methanol (50:60:2.5 v/v). Organisms: a+b) *M. smegmatis* mc^2155, c+d) PyramidII *M. smegmatis*, e+f) Aux 570 *M. smegmatis*, g+h) PenG 650 *M. smegmatis*.
Detection: a,c,e + g - 5% ethanolic molybdophosphoric acid MPA, all lipids; b,d,f + h - α-naphthol sulphuric acid for sugars components i.e.CF and GL.
CF - cord factor (trehalose dimycolate)
GL - glycolipids

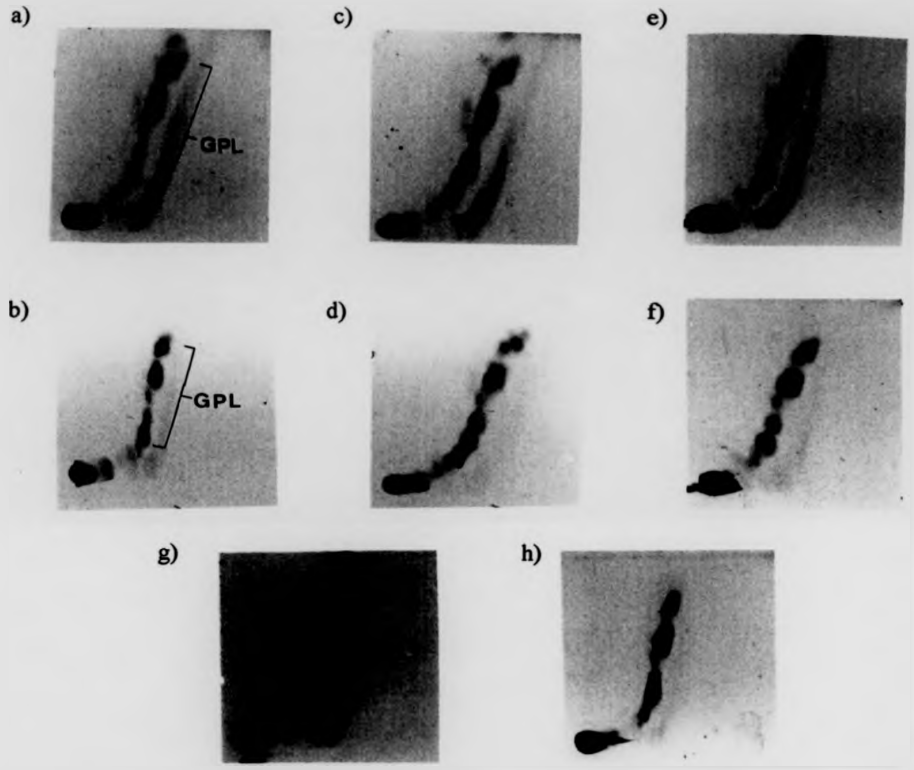


Fig.21. **Two dimensional TLCs of the least polar class of lipids in the polar lipid fraction of mycobacterial "free" lipids (System D_2).**
1st direction, chloroform:methanol:water (100:14:0.8 v/v/); 2nd direction, chloroform : acetone : methanol (50:60:2.5 v/v). Organisms: a+b) *M. smegmatis* mc^2155, c+d) PyramidII *M. smegmatis*, e+f) Aux 570 *M. smegmatis*, g+h) PenG 650 *M. smegmatis*.
GPL - glycopeptidolipid
Detection: a,c,e + g - 5% ethanolic molybdophosphoric acid MPA, all lipids; b,d,f + h - α-naphthol sulphuric acid for sugars components i.e. GPL

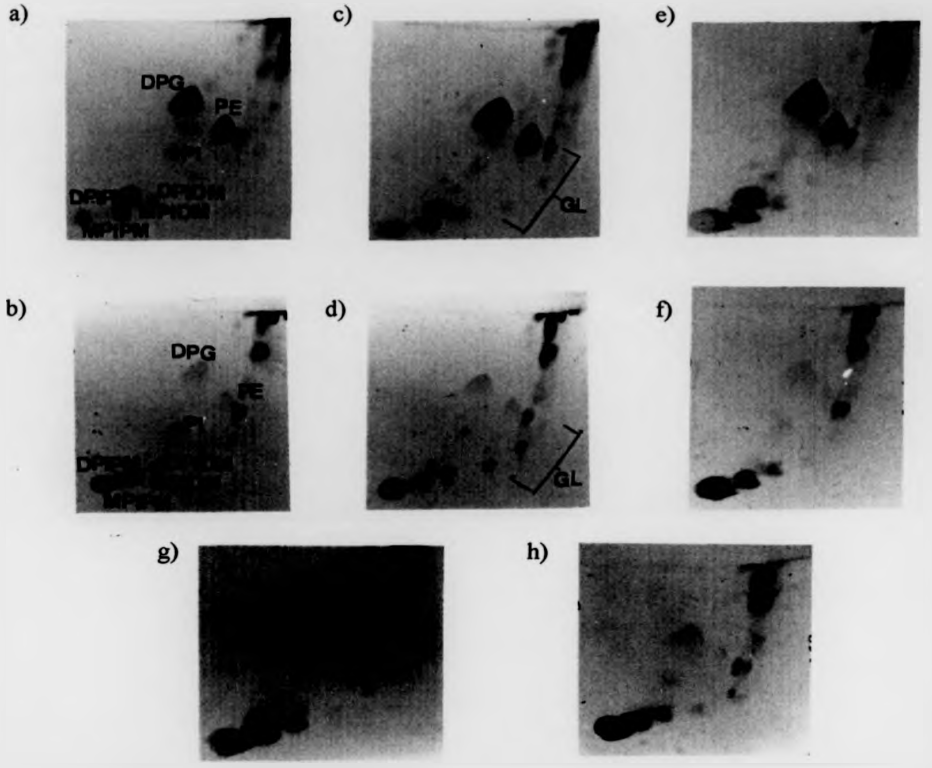


Fig.22. **Two dimensional TLCs of the most polar class of lipids found in the mycobacterial polar "free" lipid fraction (System F).**
1st direction, chloroform:methanol:water (65:25:4 v/v); 2nd direction chloroform:acetic acid: methanol water (80:15:12:4 v/v).
Organisms: a+b) *M. smegmatis* mc^2155, e+f) Aux 570 *M. smegmatis* strain, c+d) PyramidII *M. smegmatis* strain, g+h) PenG 650 *M. smegmatis* strain.
DPG - diphosphatidylglycerol
PE - phosphatidylethanolamine acid
PI - phosphatitylinsositol
DPIDM + MPIDM - di and mono acylphosphatidylinsositol dimannosides
DPIPM + MPIPM - di and mono acylphosphatidylinsositol pentamannosides
GL - glycolipid
Detection system: a,c,e + g - 5% ethanolic molybdophosphoric acid spray (MPA) for all lipids. b,d,f + h α-naphthol sulphuric acid (NAS), for sugar components, i.e. GL, DPIDM, MPIDM, DPIPM and MPIPM.

glycopeptidolipid components, revealed with both 5% molybodophosphoric acid (MPA) and the α-naphthol sulphuric acid sprays (Fig.21).

3.1.4.1.4 System F

This system was used to analyze the most polar lipids of the polar lipid fraction, phospholipids found in mycobacteria and the polar glycolipids. The polar lipids were detected with MPA (Fig.22). The largest proportion of phospholipids observed were diphosphatidylglycerol and phosphatidylethanolamine (Fig.22). Phosphatidylinositol-mannosides (PIMs), were also observed in smaller amounts. No gross differences in the proportions of any of these components were noted between the wild type mc^2155 and the mutant strains Aux 570, PenG 650 and PyramidII.

Fig.22 also shows System F TLC plates of the four strains sprayed with α-naphthol sulphuric acid spray, in which the polar glycolipids of each strain could be distinguished better. Three glycolipids, GL 1, 2 and 3, were clearly visible, particularly strongly in the Pyramid II strain. In strains Aux 570 and PenG 650 another glycolipid appeared to be running to the right of glycolipid 3; a fourth glycolipid was also weakly visible in the wild type strain. This glycolipid did not appear to be present in the PyramidII strain.

3.1.4.2 Mycolate analysis

The mycolates of the *M. smegmatis* mc^2155, Aux 570, PyramidII and PenG 650 were also analyzed by a 2D-TLC system following an alkali extraction which hydrolysed and the esterified the lipids to obtain fatty methyl esters and methyl mycolates (section 2.31). Fig.23 shows the mycolates of the four strains, the characteristic α, α' and epoxymycolates of *M. smegmatis* were observed in all four strains, although there may be differences in the actual amounts of each mycolate component in each strain.

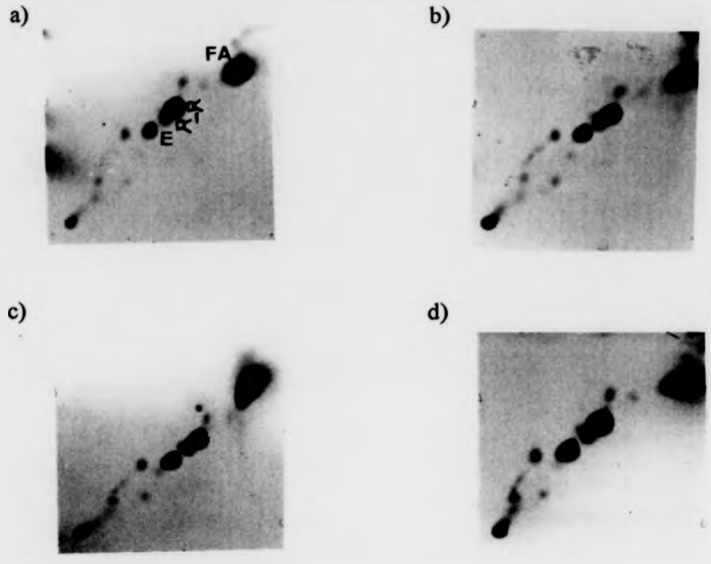


Fig.23. **Two dimensional TLC of the mycolic acid methylester obtained from the extraction of mycolic acids**
1st direction, petroleum ether (b.p. 60-80°C), acetone (95:5v/v), three times; 2nd direction toluene, acetone (97:3, v/v).
Organisms: a) *M. smegmatis* mc^2155, b) Aux 570 *M. smegmatis* c) PyramidII *M. smegmatis* d) PenG 650 *M. smegmatis*. Detection: MPA all lipids
α - α mycolates
α'- α' mycolates
E - epoxymycolates
FA - Fatty acids
All strains exhibited the expected α, α' and epoxymycolates expected from *M. smegmatis* strains in similar proportions.

3.1.4.3 Fatty acid analysis using gas chromatography

The fatty acid methyl esters of the fatty acids produced by the mycolate extraction were extracted and analysed by gas chromatography. The gas chromatography was carried out by Mr.R.Bolton, University of Newcastle.

Similar amounts of the various fatty acids were found in all four strains (Table.13). The majority of the fatty acids in the strains were oleic acids (40%), closely followed by palmitic acid at 30%. Palmitoleic acid and tuberculostearic acid were found in similar amounts at about 12%. The only slight variation was in the strain PenG 650 which contained slightly less oleic acid (32.6%).

Table.13. **The fatty acid composition of three mutant *M. smegmatis* strains and the wild type *M. smegmatis* mc^{2}155 strain**

Fatty Acid	*M. smegmatis* mc^{2}155	PyramidII	Aux 570	PenG 650
Palmitoleic acid (C16:1) %	11.90	12.21	12.54	10.98
Palmitic acid (C:16) %	28.89	31.58	27.2	32.42
Oleic acid (C18:1) %	39.83	37.68	40.00	32.60
Tuberculostearic acid (C19Br) %	10.72	12.79	13.23	18.66

In conclusion, no gross alterations were observed in the lipid content of the two mutant strains, PyramidII and PenG 650, or in the control mutant strain Aux 570, following this systematic analysis.

3.1.5 FURTHER ANALYSIS OF PYRAMIDII

Given that no gross alterations were observed in the lipid content of PyramidII and PenG 650, it was difficult to make the decision on which one to investigate further. On balance the decision was made to pursue PyramidII, as even if it turned out not to be the permeability mutant of interest, little is known of the mechanisms employed by mycobacteria to cope with pyrazinamide. It was also conceivable that PenG 650 had a defective β-lactamase as opposed to a more generalised permeability mutant.

3.1.5.1 Detailed analysis of the pyrazinamide sensitivity of PyramidII in liquid culture.

The pyrazinamide sensitivity experiment in liquid culture was repeated with strain PyramidII and wild type *M. smegmatis* mc²155 at 37°C in duplicate (section 2.28.4). The control cultures for each strain were grown to two stages of growth, mid log-phase ($0.6A_{600}$) and very early stationary phase growth ($1.0\ A_{600}$). The results of these experiments are shown in Fig.24. It was observed that strain PyramidII was more sensitive to pyrazinamide than the wild type *M. smegmatis* at both stages of growth. However, the resistance of both strains to pyrazinamide increased with increasing age of the cells in each culture and extended exposure of the cells to a given concentration of the drug. In mid log-phase the greatest difference in the sensitivity between the two strains was observed at a 500μg/ml pyrazinamide where the PyramidII culture had an $0.255A_{600}$ ($2x10^7$ cfu/ml) while the *M. smegmatis* mc²155 value was $0.55\ A_{600}$ (1.4x108 cfu/ml), a difference of 0.325_{600}A , which represented a seven fold difference in the cfu/ml of each of the cultures of the two strains (Fig.24a). At early stationary-phase growth the most substantial difference in sensitivity between the two cultures was observed at 700μg/ml of pyrazinamide, when the two cultures had a $0.425\ A_{600}$ differential, representing a 10 fold difference in cfu/ml (Fig.24b). Similar observations were made on repetition of this experiment and at other phases of growth. Strain PyramidII was, at all stages of growth, significantly more sensitive to pyrazinamide than the wild type *M. smegmatis* mc²155, however the resistance of both *M. smegmatis* strains to

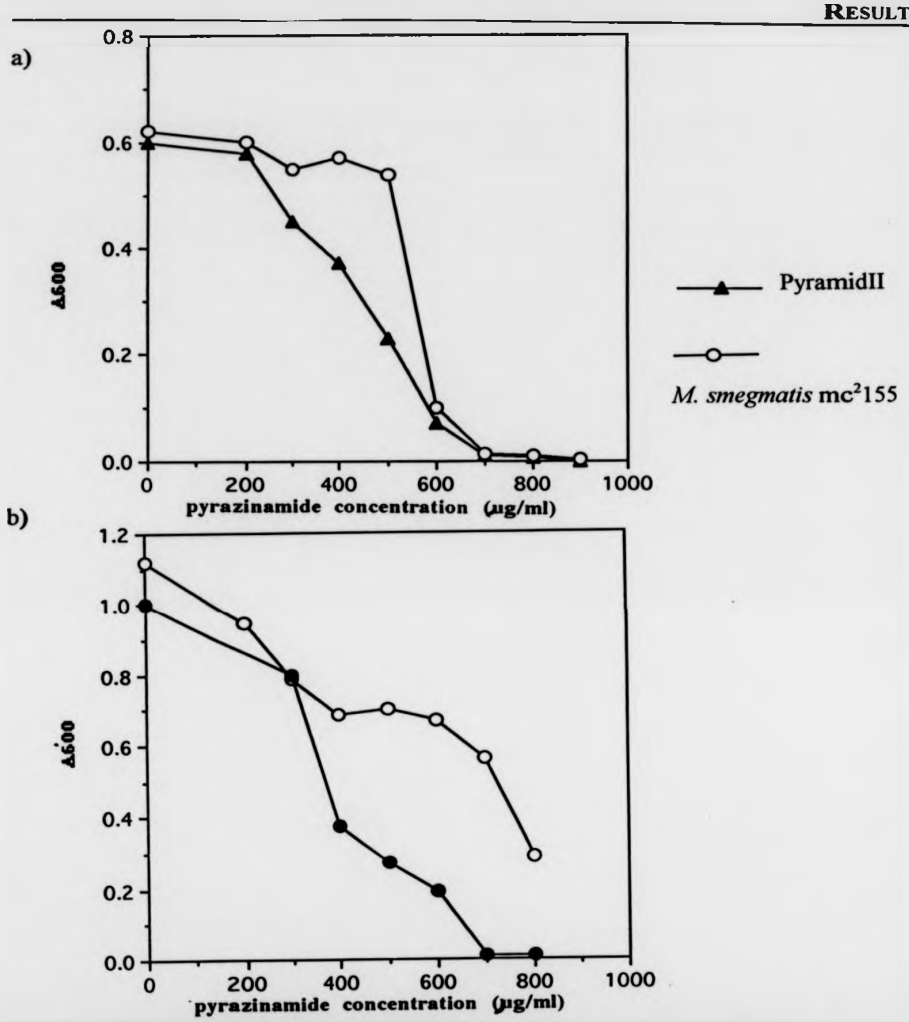


Fig. 24. **A graph depicting the pyrazinamide sensitivity of the PyramidII *M. smegmatis* strain in liquid culture, at two growth stages.**

An equal number of cells ($3x10^7$) and were added to two sets of 5ml Lemco (pH5.2) containing increasing concentrations (200μg/ml-900μg/ml) of pyrazinamide, for PyramidII and *M. smegmatis* mc²155 strains. Control cultures without pyrazinamide were also set up. The A_{600} of each culture for set 1 was read when the control reached mid log-phase growth A_{600} 0.6 and for set 2 when the control reached late log/early stationary phase growth 1.0 A_{600}. Graph a) represents mid-log phase growth and graph b) represents early stationary phase growth. At both stages of growth PyramidII remained more sensitive to pyrazinamide than the wild type *M. smegmatis* mc²155, although the resistance of both strains increased with stage of growth and also time.

pyrazinamide increased with the age of the cells and continuing exposure to the drug. This increase in resistance with age was more pronounced in the wild type *M. smegmatis* mc^2155 strain than the PyramidII strain. The increase in resistance with age was probably due to a number of factors such as the decrease in permeability of elderly cell walls, an increase in the pH of the culture with time reducing the efficacy of the drug and the bacteriostatic nature of the drug. It was concluded that strain PyramidII was a stable pyrazinamide sensitive mutant that would be suitable for further analysis.

3.1.5.2 Penicillin sensitivity of PyramidII

In order to determine whether the increased sensitivity of PyramidII was specific to pyrazinamide, the penicillin sensitivity of PyramidII was retested in liquid culture (section 2.28.4.) The experiment was carried out five times with concentrations of penicillin G increasing from 1-1,000μg/ml in 100μg/ml increments. Two out of the five times PyramidII showed similarly increased levels of sensitivity to penicillin G as to pyrazinamide; on the other three occasions PyramidII showed resistance levels similar to the wild type *M. smegmatis* mc^2155 (data not shown).

3.1.5.3 Morphology and Acid-Fast Staining

The colonies of PyramidII had a smoother morphology than the rougher wild type *M. smegmatis* mc^2155 strain on both Lemco and Middlebrook 7H10 agar at 28°C or 37°C, as illustrated in Fig.25. Both strains were acid fast; although the PyramidII strain did appear very slightly less acid fast than the wild type cells, this may have been due to a slight variation in the stage of growth of the cells.

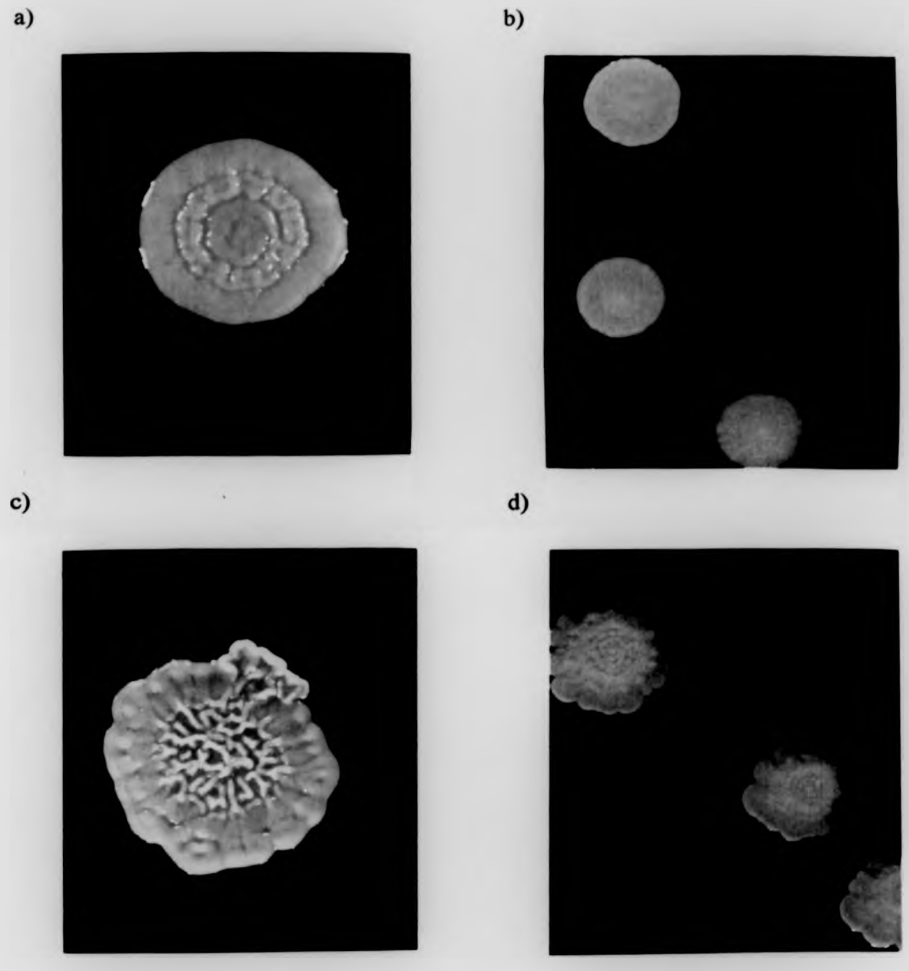


Fig.25 **Colony morphology of strain Pyramid II in comparison to wild type *M. smegmatis* mc²155**
a+b) PyramidII colonies on Middlebrook 7H10 agar
c+d) *M. smegmatis* mc²155 on Middlebrook 7H10 agar
All colonies were produced following 10 days growth at 37°C but from different dilutions of an early stationary phase culture 1.5 A_{600} a+c) 1x10-^{9} dilution and b+d) 1x10^{-8} dilution.

3.1.5.4 Api-Zym Test

To ensure both strains were *M. smegmatis*, an Api-Zym test was carried out according to the manufacturer's instructions (section 2.33) on cultures of the wild type *M. smegmatis* mc^2155 and PyramidII strains. The test results for both strains were concurrent with those for *M. smegmatis* mc^2155 as shown in Table.14 and Fig.26. The only variable result between the two strains was test 12 for naphthol-β1-phosphohydrolase activity which appeared to be lower for the PyramidII mutant strain as indicated by a weak positive blue colour (Fig.26). The results for both strains were consistent with their being *M. smegmatis*.

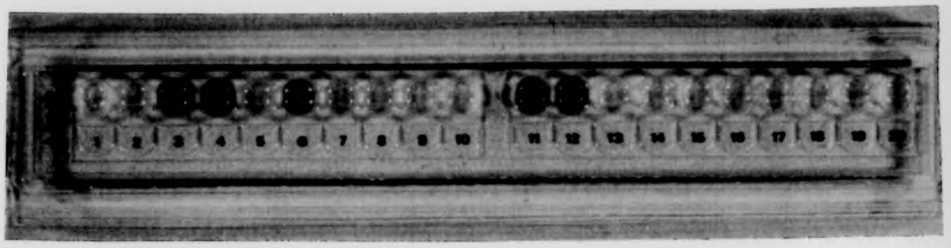
3.1.5.5 Growth curves

The growth of PyramidII was compared to the wild type *M. smegmatis* mc^2155 strain by following the A_{600} of both strains over time. As shown in Fig.27 the PyramidII strain has a lag-phase at least eight hours longer than the wild type *M. smegmatis* mc^2155 strain, having initially been seeded with a similar number of cells, $1x10^8$. The growth rate of both strains during log-phase growth appeared identical in both strains. The cfu/ml at each stage of growth were very slightly higher in the PyramidII strain (Fig.27b). The cells of PyramidII in culture were observed to form more of an even suspension in culture than the *M. smegmatis* mc^2155 strain and this reduction in clumping may have accounted for the slightly higher numbers of colonies formed by the PyramidII strain in the growth experiment.

3.1.5.6. Further biochemical analysis

PyramidII strain was further analyzed by Dr.G.Besra, Colorado State University, Colorado for alterations in the glycosyl composition by HPLC. No alterations were found in the arabinogalactan or in the lipoglycan fraction composed of LAM, LM and PIMs. The glycolipids, PIMs and trehalose mycolate profiles were re-analyzed by 2D-TLC and again no differences were found in PyramidII lipids when compared to the wild type, *M. smegmatis* mc^2155 lipids.

a)



b)

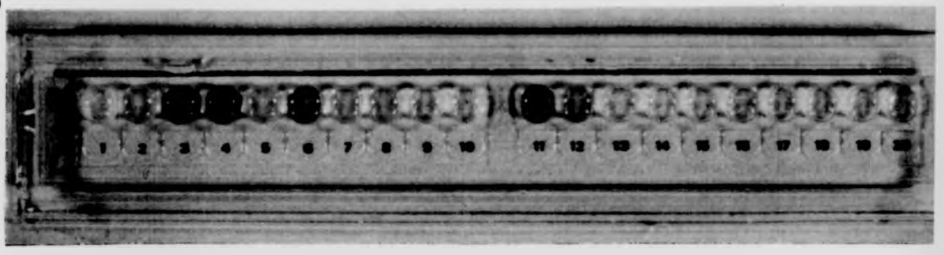


Fig. 26. **The results of the Api-Zym test of PyramidII and wild type mc²155, *M. smegmatis* strains**
a) *M. smegmatis* mc²155 wild type and b) PyramidII *M. smegmatis*
The results indicated that both both strains were *M. smegmatis* (Table 14). The results obtained from both strains were the same apart from test no.12 the assay for Naphol-AS-BI-phosphate phosphohydrolase which was distinctly lighter in the PyramidII strain.

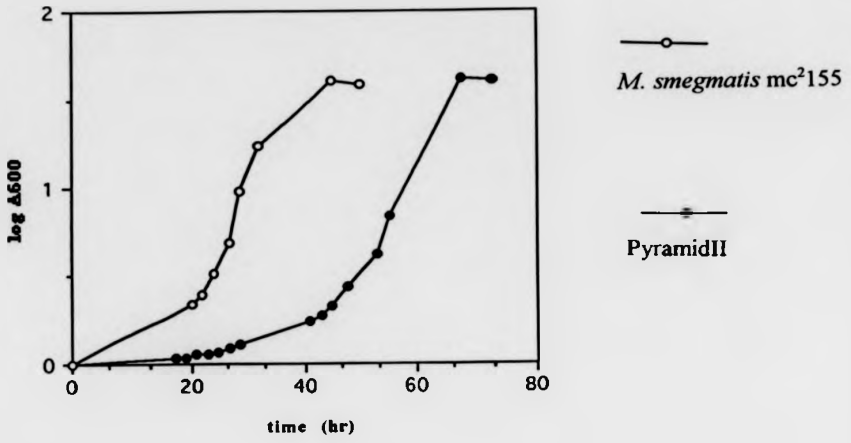
Enzymes assayed for:-

1) Control	8) Cystine arylamidase	15) β glucuronidase
2) Phosphatase alcaline	9) Trypsin	16) α glucosidase
3) Esterase (C4)	10) Chemotrypsin	17) β glucosidase
4) Esterase Lipase (C8)	11) Phosphatase acid	18) N-acetyl-β glucosaminidase
5) Lipase (C14)	12) Napthol-AS-BI-phosphohydrolase	
6) Leucine arylamidase	13) α galactosidase	19) α mannosidase
7) Valine arylamidase	14) β galactosidase	20) α fucosidase

Enzyme assayed for	Substrate	Wild-type *M.smegmatis*	Pyramid II
Phosphatase alcaline	2-naphthyl phosphate	+	+
Esterase (C4)	2-naphthyl butyrate	+	+
Esterase Lipase (C8)	2-naphthyl caprylate	+	+
Lipase (C14)	2-naphthyl myristate	-	-
Leucine arylamidase	L-leucyl-2-naphthylamide	++	++
Valine arylamidase	L-valyl-2-naphthylamide	+	+
Cystine arylamidase	L-cystyl-naphthylamide	-	-
Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	+	+
Chymotrypsin	N-glutanyl-phenylalanine-2-naphthylamide	-	-
Phosphatase acid	2-naphthyl phosphate	+	+
Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	+	±
α galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	-	-
β galactosidase	2-naphthyl-βD-galactopyranoside	-	-
β glucuronidase	Naphthol-AS-BI-βD-glucuronide	-	-
α glucosidase	2-naphthyl-αD-glucopyranoside	+	+
β glucosidase	6-Br-2-naphthyl-βD-glucopyranoside	++	++
N-acetyl-β-glucosaminidase	1-naphthyl-n-acetyl-βD-glucosaminide	-	-
α mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	-	-
α fucosidase	2-naphthyl-αL-fucopyranoside	-	-

Table.14. **The API-ZYM test results for *M. smegmatis* mc²155 and PyramidII**

a)



b)

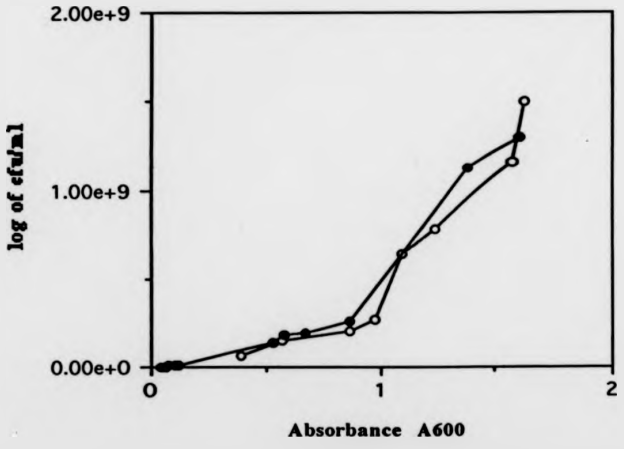


Fig.27 . **Graphs comparing the growth of PyramidII and wild type *M. smegmatis* mc²155**
a) The growth measured in optical density, A_{600} against time of a 200ml culture of each strain in Middlebrook 7H9 medium 0.1% Tween 80, of PyramidII and *M. smegmatis* mc²155.
b) The growth measured by colony forming units (cfu)/ml against optical density A_{600}
Graph a) shows a lag-phase of 8-10 hr for the PyramidII strain followed by a log-phase growth rate not significantly different from the wild type *M. smegmatis* mc²155.
Graph b) shows a slight increase in the cfu's produced by PyramidII when compared with *M. smegmatis* mc²155 at stages of growth defined by A_{600}.

3.1.5.7 Hydrophobicity

A change in the hydrophobicity of a bacterial strain can indicate an alteration in the structure of its outer surface (Rosenberg *et al.*, 1980). The hydrophobicity of PyramidII was measured in comparison to the wild type *M. smegmatis* mc^2155 using the method of Rosenberg *et al.* (1980)(section 2.32). The basis of this method was to determine the solubility of cells in an organic solvent as a measure of their hydrophobicity. The solubility of a culture in the hydrocarbon was assessed by measuring the change in optical density of the aqueous layer on the addition of the hydrocarbon.

A standard volume of hexadecane was added to 1.5ml of the test strain cultures, which were at the same stage of growth and incubated at 28°C for 10min. The solutions were then vortexed, left to settle and the A_{400} of the aqueous layer was then measured. The original system was adapted slightly to use a non limiting number of cells i.e. 200ml of culture in 7H9 broth were concentrated ten-fold. The hydrophobicity of the test strains, PyramidII and mc^2155, was measured at two stages of growth, late log-phase ($0.8A_{600}$) and early stationary stage ($1.6A_{600}$), following growth at 37°C. The optical density of the aqueous layer of PyramidII was measured at 400nm and was significantly higher than the wild type culture at both stages of growth, by at least $0.5A_{400}$, (Table 15).

Table 15. **The hydrophobicity of PyramidII and *M. smegmatis* mc^2155**

Growth stage of the culture	Pyramid II aqueous layer A_{400}	*M. smegmatis* mc^2155 aqueous layer A_{400}
Late log-phase ($0.8A_{600}$)	0.59 ± 0.1	0.167 ± 0.014
Early stationary-phase ($1.6A_{600}$)	1.53 ± 0.05	0.92 ± 0.082

The results shown were the average of two readings for each strain. The results indicated that PyramidII was more hydrophilic than the wild type *M. smegmatis*. The hydrophilic nature of PyramidII may have been due to the lack of a hydrophobic molecule on the cell surface or the increase in a hydrophilic molecule which could have resulted in the strain being more permeable to hydrophilic molecules such as pyrazinamide.

In summary strain PyramidII was proven to be more susceptible to pyrazinamide than the wild type *M. smegmatis* mc^2155 strain and variably sensitive to penicillin G. PyramidII was shown to be *M. smegmatis* through Acid-Fast staining and Api-Zym testing. PyramidII also exhibited a smooth colony morphology and a lag-phase 8-10hr longer than the wild type strain. Although no gross differences were detected in the lipid content of the PyramidII strain a distinct decrease in the hydrophobicity of the strain was discovered which may be caused by some cell envelope defect and may also have caused the increased permeability of the strain to hydrophilic molecules such as pyrazinamide.

3.2 COMPLEMENTATION OF PYRAMIDII WITH A COSMID PYUB18::*M. LEPRAE* GENOMIC LIBRARY.

The next objective was to isolate a region of *M. leprae* DNA which would complement the pyrazinamide sensitive phenotype of the *M. smegmatis* PyramidII strain.

The proposed strategy was to transform competent PyramidII *M. smegmatis* cells with the cosmid shuttle vector pYUB18::*M. leprae* genomic library by electroporation. The PyramidII::*M. leprae* transformants would then be screened for increased resistance to pyrazinamide. Apparent revertants and their cosmids would then be analysed in more detail.

3.2.1 TRANSFORMATION OF PYRAMIDII WITH COSMID PYUB18::*M. LEPRAE* GENOMIC LIBRARY.

The *M. leprae* pYUB18 shuttle vector cosmid library was provided by Dr. W.R. Jacobs as a phage lysate with a titre of $1x10^9$ pfu/ml. *E. coli* NM554 cells were transformed with 5μl of phage lysate (section 2.10) and $2x10^4$ colonies were produced. The transformation efficiency of $4x10^6$ cfu/ml was lower than the $1x10^9$ (or $5x10^6$ cfu) expected, however $2x10^4$ colonies were considered sufficient to be a representative sample of the entire library.

As cosmid DNA is particularly unstable in mycobacteria, small culture volumes were used to reduce the loss of unstable cosmids, on the basis that the cells would go through a reduced number of cell divisions. The colonies were therefore resuspended in 10ml LB (Kan 50μg/ml) and 1ml volumes of the resuspension were added to ten 2ml volumes of LB (Kan 50μg/ml) to form overnight cultures for small scale cosmid preparation (section 2.16). The cosmid DNA from each of the extractions was pooled to give a final concentration of 0.5μg/μl.

Competent cells of the *M. smegmatis* PyramidII strain were produced (section 2.25) and transformed (section 2.26) with 1μg pYUB12 (11.2kb) forming 1028 colonies on Lemco

(Kan15μg/ml) after 4 days at 37°C, a transformation efficiency of $1x10^3/\mu g$. 400μl of PyramidII competent cells were transformed with 2.5μg pYUB18:*M. leprae* cosmid library DNA. Three hundred and twelve transformants were formed, a transformation efficiency of $1.2x10^2/\mu g$, eight fold lower than pYUB12, which would be expected due the large size (30kb) of the *M. leprae* insert DNA.

The PyramidII transformants were of two colony types, smooth and crinkly. The smooth colonies were akin to the parental strain and the crinkly ones were crenellated and invaginated, generally to an even greater extent than observed in wild type *M. smegmatis* mc^2155. Of the 312 transformants, 48% (150) were smooth and 52% (162) were crinkly. The size of the transformed colonies also varied, from 0.5-5mm; the smooth colonies were generally the larger.

The transformants were transferred onto Lemco (Kan 15μg/ml) agar and arranged in arrays of 96, divided on the basis of their apparent morphology. The transferred colonies were then incubated at 37°C for four days to grow. 130 (41%) of the original transformants did not survive the transfer onto Lemco (Kan 15μg/ml) and 90% of those lost were crinkly colonies, with a diameter of 0.5-1mm. This loss of transformants was thought to be due to the small colony size of the majority of lost transformants, which made transfer difficult; it may also have been due to the instability of the cosmid DNA.

182 (59%) transformants were successfully transferred onto Lemco (Kan15μg/ml) and assuming, conservatively, an average insert size of 30kb this was considered a sufficient number of transformants to represent the entire estimated genome size of 2.8Mb for *M. leprae* (Clark-Curtiss *et al.*, 1985; Eiglmeier *et al.*, 1993).

3.2.2 SCREENING OF PYRAMIDII::M.LEPRAE TRANSFORMANTS FOR INCREASED RESISTANCE TO PYRAZINAMIDE

The 182 arrayed PyramidII::*M. leprae* transformants were then screened for increased resistance to pyrazinamide at 28°C, to obtain the clearest possible phenotype. The 182 colonies were replica plated onto Lemco pH5.2 (Kan 15μg/ml) containing pyrazinamide (500μg/ml) agar and Lemco (Kan 15μg/ml) agar. The replicated transformants were then incubated at 28°C to grow for six days.

132 (75%) transformants grew strongly at 28°C on Lemco (Kan 15μg/ml) and fourteen (9.8%) of these colonies grew on Lemco pH5.2 (Kan 15μg/ml) agar containing 500μg/ml pyrazinamide. Hence 75% of the 182 PyramidII::*M. leprae* transformants were stable at 28°C and 37°C, while 25% were only stable at 37°C. 9.8% of the 182 transformants initially appeared resistant to 500μg/ml pyrazinamide. This which was a fairly high level of complementation, 1-2% being the expected result. The complementing strains were then analysed in more detail.

3.2.2.1 Analysis of the fourteen PyramidII:M. leprae transformants selected on pyrazinamide (500μg/ml)

3.2.2.1.1 Resistance on solid media at 28 °C

The resistance of the 14 transformants was first assessed on solid media at 28°C. The fourteen PyramidII::*M. leprae* transformants which showed increased resistance to 500μg/ml of pyrazinamide were placed in 5ml Middlebrook 7H9 broth (Kan 20μg/ml) and incubated at 28°C until each culture reached late log-phase culture (0.8 A_{600}). The pyrazinamide sensitivity of the 14 transformants was retested by streaking the late log-phase cultures onto Lemco pH5.2 pyrazinamide 500μg/ml (+Kan 20μg/ml for the transformants) agar. The growth of the 14 PyramidII:: *M. leprae* clones was then compared to the PyramidII and wild type mc^2155

strains of *M. smegmatis* following incubation at 28°C for five days.

The sensitive *M. smegmatis* PyramidII strain showed no growth, while PyramidII::*M. leprae* transformants 42, 59 and 78 showed very weak growth on the pyrazinamide (500μg/ml) containing media. Eleven of the PyramidII::*M. leprae* strains showed stronger growth on Lemco pH5.2 pyrazinamide (500μg/ml) agar than the parental PyramidII strain. The growth of the eleven transformants was, however, not as strong as the wild type mc^{2}155 *M. smegmatis*; this may have been due to a reduction in growth rate caused by the presence of cosmid DNA (20-30kb) in the transformants.

An accurate assessment of the individual levels of pyrazinamide resistance exhibited by the 14 transformants was difficult on solid media as the transformants growth rate became the only measure of individual levels of pyrazinamide resistance. However, the presence of 30-40kb of cosmid DNA within these strains slowed down their growth rate and made individual levels of resistance impossible to ascertain on solid media. In order to gain a true comparison of the individual levels of pyrazinamide resistance found in the transformants their sensitivity had to be measured at the same stage of growth. This could be done in liquid culture.

3.2.2.1.2 Resistance in liquid culture at 37 °C

In order to observe the degree of increased pyrazinamide resistance in the 14 PyramidII::*M. leprae* clones at the same stage of growth as the PyramidII and mc^{2}155 strains further analysis was carried out in liquid culture (section 2.28.4).

A set of eight tubes containing 5ml of Lemco pH5.2 (Kan 15μg/ml) with pyrazinamide concentrations increasing in 100μg/ml quantities, from 200μg/ml - 900μg/ml were set up for each strain. The range of pyrazinamide concentrations selected was based on the results obtained for the growth of the wild type and the mutant PyramidII strain (section 3.1.3.3.3).

A control was set up for each strain containing 5ml Lemco pH5.2 (Kan 20μg/ml) only. The 14 PyramidII::*M. leprae* clones were grown to early stationary stage (1.5 A_{600}) and an approximately equal number of cells ($1x10^8$) from each strain were then added to a series of nine tubes. The each series of tubes were incubated at 37°C, with shaking, until the control cultures i.e. those without pyrazinamide, reached mid log-phase growth (0.6 A_{600}). The A_{600} of the cultures within each series then read.

The resistance of each PyramidII::*M. leprae* clone to increasing concentrations of pyrazinamide represented and compared to the parental and wild type *M. smegmatis* strains. The growth of the 14 PyramidII::*M. leprae* clones was also compared to that of PyramidII (His), the PyramidII strain harbouring a cosmid containing the histidinol D gene, ***hisD*** (Hinshelwood and Stoker, 1992b). This was a control to determine wether the presence of random genes in a cosmid would confer a growth advantage that could be misinterpreted as increased resistance to pyrazinamide.

Variable levels of resistance to pyrazinamide were observed for each PyramidII::*M. leprae* clone in liquid culture, as shown in Figs. 28, 29 and 30. Six clones 10, 33^1, 45, 57, 83 and 116 consistently showed levels of growth over and above the parental PyramidII, PyramidII(His) and the wild type mc^2155 *M. smegmatis* strains in increasing concentrations of pyrazinamide (Fig.28). Clones 32, 33^2, 34 and 38 showed an intermediate increase in resistance to pyrazinamide (Fig.29). Clones 42, 59 and 78 showed no increased resistance to pyrazinamide in liquid culture (Fig.30) The results for clone 54 are not shown as the strain was tested in liquid culture only once before the strain became unculturable from frozen stocks.

As is evident from the growth of the PyramidII (His) strain in liquid culture and on solid media the presence of these random genes in a given cosmid did not confer any significant growth advantage on the parental PyramidII strain in the presence of pyrazinamide.

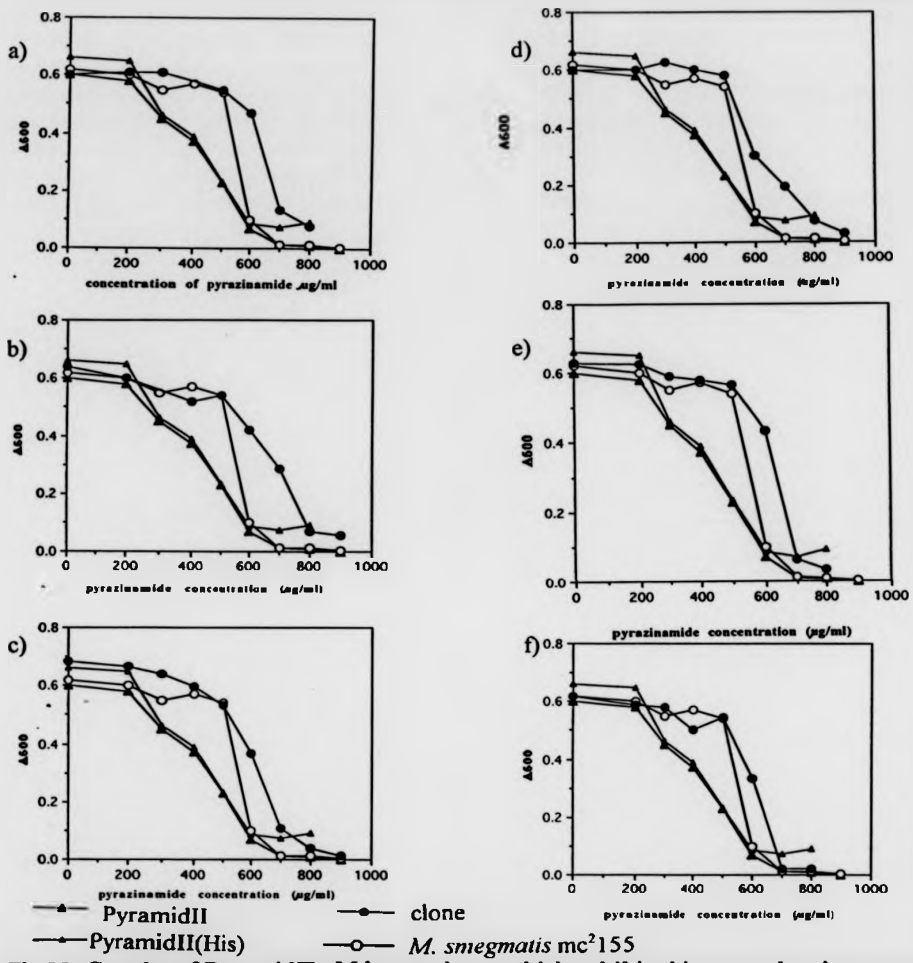


Fig.28. Graphs of PyramidII::*M.leprae* clones which exhibited increased resistance to pyrazinamide in liquid culture

An equal number of cells ($3x10^7$) and were added to a set of 5ml Lemco (pH5.2) containing increasing concentrations (200μg/ml-900μg/ml) of pyrazinamide, for six PyramidII:*M.leprae* clones 10, 331, 45, 57, 83, 116. Control cultures without pyrazinamide were also set up. The A_{600} of each culture, in the six sets, was read when the control reached mid log-phase growth $0.6A_{600}$. The sensitivity of each clone to pyrazinamide was compared to the parental strain, the parental strain harbouring an unselected cosmid (PyramidII[His]) and the wild type mc^2155. The graphs represent the following PyramidII::*M.leprae* clones a) 10 b) 33^1 c) 45 d) 57 e) 83 and f) 116. All six of these PyramidII::*M.leprae* clones exhibited an increase in resistance to pyrazinamide over and above the parental PyramidII, PyramidII and the wild type *M. smegmatis* mc^2155 strains.

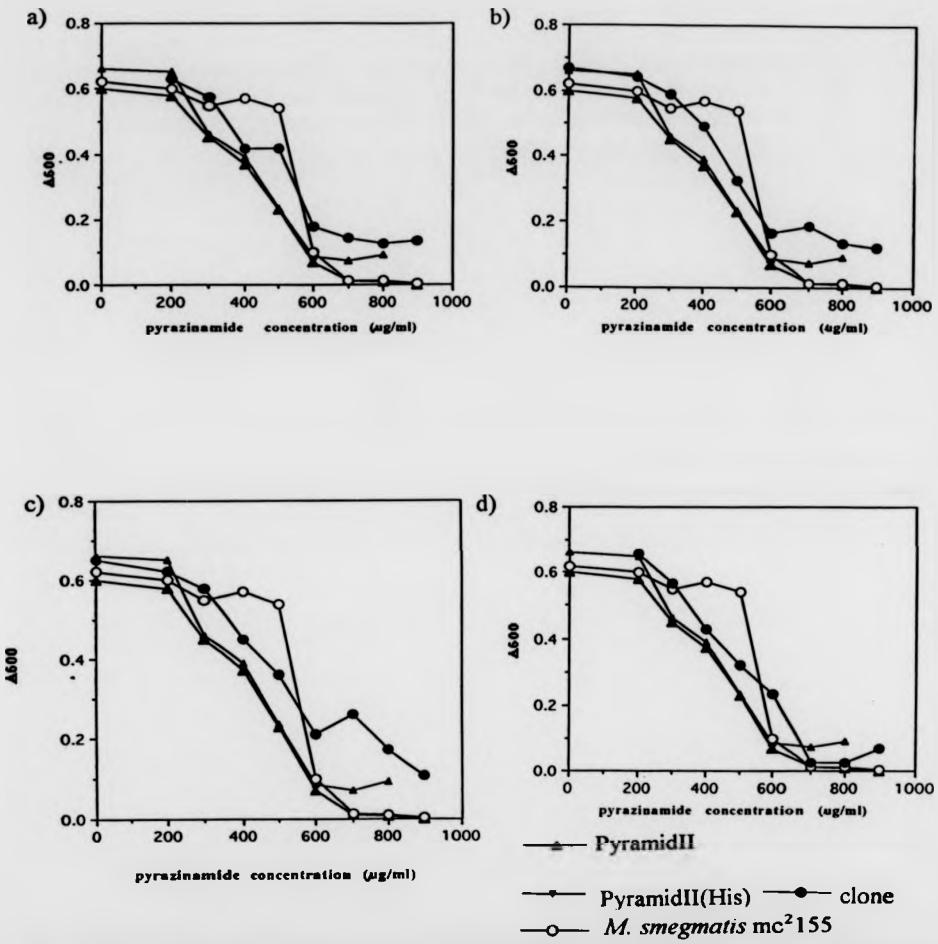


Fig. 29. **Graphs of PyramidII::*M.leprae* clones which exhibited an intermediate increase in resistance to pyrazinamide in liquid culture, in comparison to wild type levels.**
An equal number of cells ($3x10^7$) and were added to a set of 5ml Lemco (pH5.2) containing increasing concentrations (200μg/ml-900μg/ml) of pyrazinamide, for four PyramidII::*M.leprae* clones 32, 33^2, 34 and 38. Control cultures without pyrazinamide were also set up. The A_{600} of each culture, in the four sets, was read when the control reached mid log-phase growth 0.6 A_{600}. The sensitivity of each clone to pyrazinamide was compared to the parental strain, the parental strain harbouring an unselected cosmid (PyramidII[His]) and the wild type mc^2155. The graphs represent the following PyramidII::*M.leprae* clones a)32 b)33^2 c)34 and d)38. The four PyramidII::*M.leprae* clones exhibited an intermediate increase in pyrazinamide resistance in comparison to the wild type and parental strains, in liquid culture.

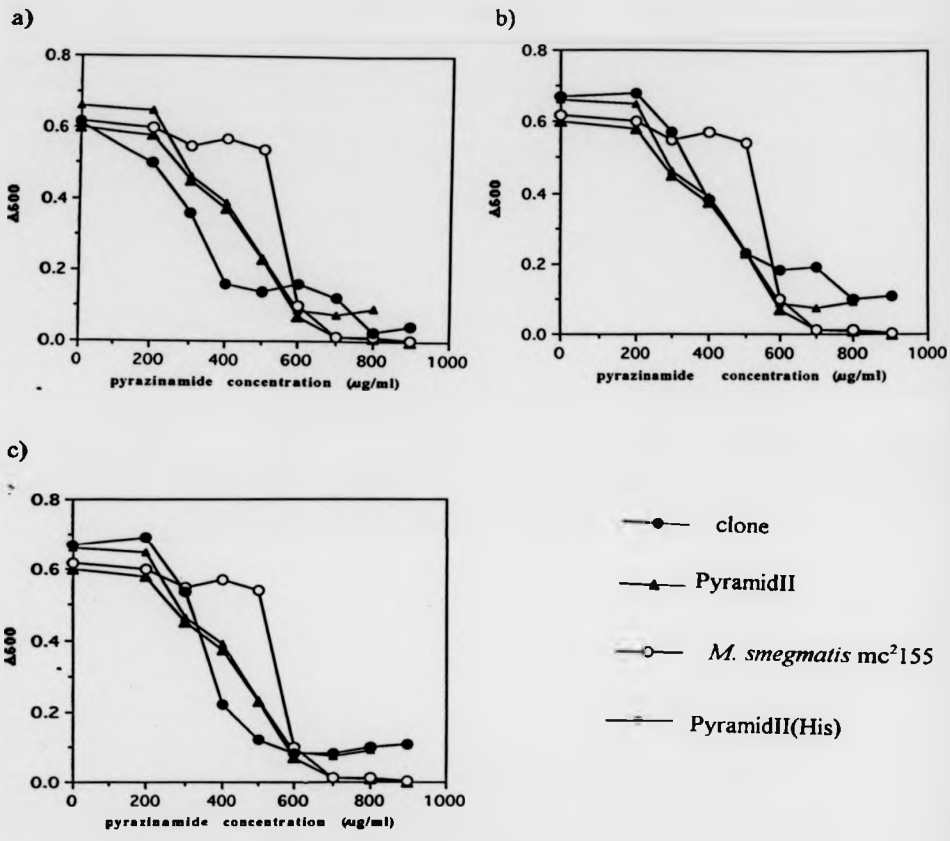


Fig.30. **Graphs of PyramidII::*M.leprae* clones which did not exhibit any increase in resistance to pyrazinamide, in liquid culture.**
An equal number of cells ($3x10^7$) and were added to a set of 5ml Lemco (pH5.2) containing increasing concentrations (200μg/ml-900μg/ml) of pyrazinamide, for three PyramidII::*M.leprae* clones 42, 59, and 78. Control cultures without pyrazinamide were also set up. The A_{600} of each culture, in the three sets, was read when the control reached mid log-phase growth 0.6 A_{600}. The sensitivity of each clone to pyrazinamide was compared to the parental strain, the parental strain harbouring an unselected cosmid (PyramidII[His]) and the wild type mc^2155. The graphs represent the following PyramidII::*M.leprae* clones a)42 b)59 c)78. These three PyramidII::*M.leprae* clones, initially isolated as exhibiting some increased resistance to pyrazinamide showed no increased resistance in liquid culture when compared to the wild type and parental strains, in liquid culture.

In summary, of the initial 14 PyramidII::*M. leprae* clones isolated due to their survival following replica plating of 182 PyramidII::*M. leprae* transformants onto pyrazinamide (500μg/ml), 11 showed an increased level of resistance when compared with the parental PyramidII strain. Six of the clones exhibited levels of resistance to pyrazinamide in excess of the wild type *M. smegmatis* mc^2155. Four PyramidII::*M. leprae* clones showed levels of resistance between the parental and the wild type strains. The eleventh strain (54) showed resistance to pyrazinamide 500μg/ml on solid media at 28°C.

The final level of complementing PyramidII::*M. leprae* transformants was 6% of the 182 transformants which survived the initial transfer onto Lemco (Kan 15μg/ml) agar. Values are higher than would be estimated for the percentage of complementing cosmids; this and the varying levels of pyrazinamide resistance exhibited by the 11 complementing PyramidII::*M. leprae* clones suggested that there may be more than one region of *M. leprae* DNA able to complement the pyrazinamide sensitive phenotype.

3.2.3 ISOLATION OF COSMID DNA FROM PYRAMIDII::*M. LEPRAE* TRANSFORMANTS

Cosmid DNA was extracted from all of the initially isolated fourteen PyramidII::*M. leprae* transformants, in order to compare the DNA of the complementing and non complementing clones.

The cosmid DNA was extracted using an adaptation of the method described for the extraction of *E. coli* DNA (section 2.16). Cell pellets from 48hr 5ml Lemco (Kan 15μg/ml) cultures of each transformant strain were resuspended in GET containing lipase (30mg/ml) and lysozyme (10mg/ml) and incubated overnight at 37°C, with shaking. The DNA was then extracted by the alkaline lysis method described for small scale cosmid preparation (section 2.16).

The DNA extracted from each strain was transformed directly into competent *E. coli* DH5α (transformation efficiency $1x10^6$ cfu/μg). The number of *E. coli* transformants obtained from the extracted cosmid DNA of each PyramidII::*M. leprae* transformant varied between PyramidII::*M. leprae* strains, but was consistent for individual strains. Generally the number of transformants obtained from PyramidII transformants 10, 32, 33^1, 33^2, 34, 38, 59 and 42 was $1x10^3$cfus which was 100 fold higher than from strains 45, 57, 83 and 116. *E. coli* DH5α transformants from each PyramidII transformant strain were inoculated into 2ml Lemco (Kan 15μg/ml) to reduce rearrangements, incubated at 37°C overnight before the cosmid DNA was extracted using the normal method (section 2.16).

Initially the mycobacterial cosmid DNA was transformed into the *E. coli* strain TG2 but the majority of the cosmids appeared unstable. The cosmid DNA extracted from repeated transformations of TG2 with mycobacterial cosmid DNA from a given PyramidII::*M. leprae* strain varied in size and exhibited varying restriction patterns. A recombination stable *E. coli* strain, DL795, was also tried and while those cosmids recovered appeared stable, the transformation efficiency obtainable with DL795 was too poor ($1x10^4$ cfu/μg) to produce transformants from all fourteen mycobacterial cosmids.

Cosmid DNA was obtained from thirteen PyramidII::*M. leprae* clones, all except 54, following transformation of the mycobacterial cosmid DNA into the *E. coli* DH5α strain. On the whole, the cosmid DNA extracted from DH5α was consistent for each PyramidII:*M. leprae* clone, although occasional rearrangements and deletions of the entire insert DNA were observed.

3.2.3.1. Analysis of the cosmid DNA in the PyramidII transformants

Cosmid DNA from each of the thirteen PyramidII::*M. leprae* clones (10 complementing and 3 non complementing) was individually digested with *Bam*HI, *Pst*I, *Eco*RI and *Hin*dIII (Fig.31 and Fig.32). Common band sizes were observed in a number of the cosmids suggesting some were overlapping (Table.16). The *Pst*I and *Eco*RI digests both resulted in the formation of five bands common to a minimum of two cosmids. The *Eco*RI digests created fragment sizes of 2.55kb from cos83 and cos116, 4.2kb from cos33^2 and cos45, 6.4kb from cos57 and cos116, 7.6kb from cos33^1 and cos45 and 9.6kb from cos45 and cos57 . The sixth *Eco*RI fragment size 4.64kb common to all thirteen cosmids was a fragment from the pYUB18 cosmid shuttle vector itself (Fig.32). The *Pst*I digests created fragment sizes of 0.2kb in cos34 and cos57, 1.1kb from cos34 and cos57, 2.45kb from cos34, cos45 and cos83, 2.75kb from cos33^1 and cos116 and 5.5kb from cos45 and cos57. A 0.85kb fragment common to all the cosmids is produced from the pYUB18 vector (Fig.31). *Hin*dIII digests created a 2.4kb fragment found in cos10, cos32, cos116 and cos57. A 7.2kb fragment was found in cos33^1 and cos57 and a 6.9kb fragment was found in cos42 and cos 33^2 (Fig.32). A 5.8kb band common to all the cosmids was from the pYUB18 vector (Fig.32). The *Bam*HI digests created fragments of 1.75kb in cos45 and 33^1, 2.6kb in cos45 and cos33^2 and a 3.4kb fragment in cos57 and cos78 (Fig.31). pYUB18 vector only DNA was not available for analysis.

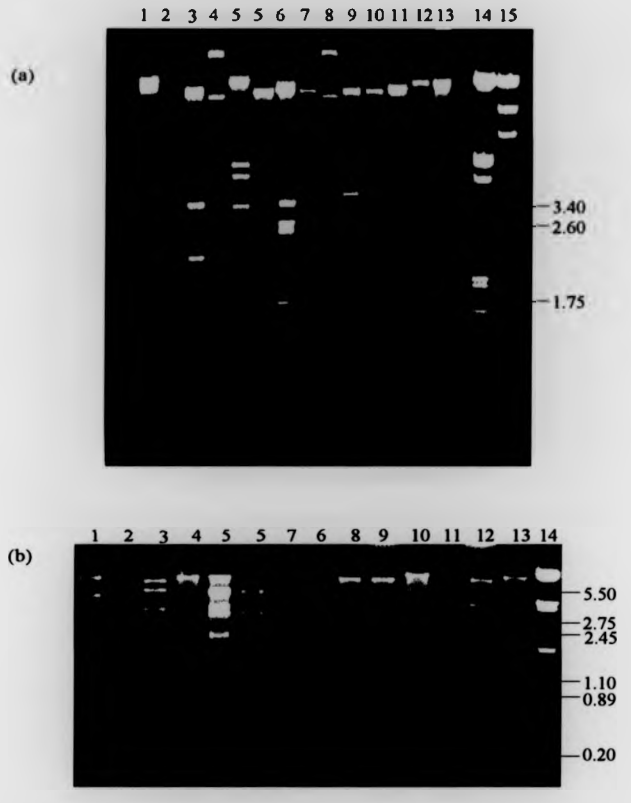


Fig. 31. **Restriction analysis of complementing and non-complementing cosmids using a) *BamH*1 and b) *Pst*I showing shared band sizes (kb).**

Complementing cosmids:		Non-complementing cosmids	Markers:
lane 1) cos116	lane 9) cos34	lane 3) cos78	lane 14) λ*Hin*dIII
lane 2) cos83	lane 10) cos33[2]	lane 4) cos59	lane 15) λ*Hin*dIII/*Eco*RI
lane 5) cos57	lane 11) cos33[1]	lane 6) cos 42	
lane 7) cos45	lane 12) cos32		
lane 8) cos38	lane 13) cos10		

a) Shared bands in *Bam*HI digest: 1.75kb in cos45 and cos331, 2.6kb in cos45 and cos331 and 3.4kb in cos57 and cos78.

b) Shared bands in *Pst*I digest: 0.2kb in cos34 and cos57, 1.1kb in cos34 and cos57, 2.45kb in cos34, cos45 and cos83, 2.75kb in cos33[1] and cos116 and 5.5kb in cos45 and cos57. The 0.89kb fragment is from the vector pYUB18 common to all the cosmids.

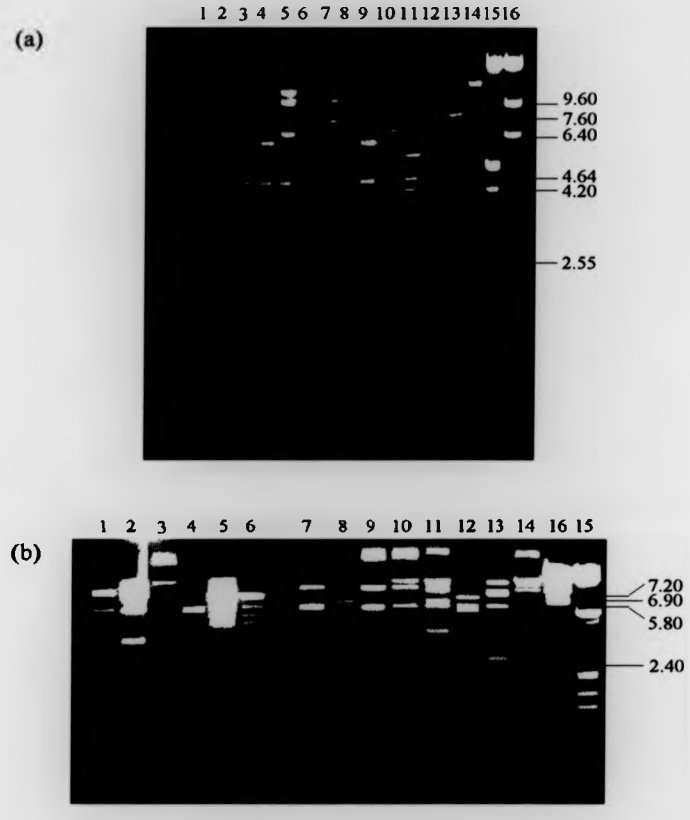


Fig. 32. **Restriction analysis of complementing and non-complementing cosmids using a) *Eco*RI and b) *Hin*dIII showing shared band sizes (kb).**

Complementing cosmids:		Non-complementing cosmids	Markers:
lane 1) cos116	lane 9) cos34	lane 3) cos78	lane 14) λ*Hin*dIII
lane 2) cos83	lane 10) cos33[2]	lane 4) cos59	lane 15) λ*Hin*dIII/*Eco*RI
lane 5) cos57	lane 11) cos33[1]	lane 6) cos 42	
lane 7) cos45	lane 12) cos32		
lane 8) cos38	lane 13) cos10		

a) Shared bands in *Eco*RI digests: 2.55kb in cos83 and cos116, 4.2kb in cos33[2] and cos45, 6.4kb in cos57 and cos116, 7.6kb in cos33[1] and cos45 and 9.6kb in cos45 and cos57. The 4.6kb fragment common to all cosmids is from the vector pYUB18.

b) Shared bands in *Hin*dIII digests: 2.4kb in cos10, cos32, cos116 and cos57, 7.2kb in cos33[1] and cos57 and 6.9kb in cos42 and cos33[2]. The 5.8kb band common to all is from the vector pYUB18.

Table.16 **Showing the restriction fragment sizes of various cosmid digests of PyramidII:*M. leprae* clones**

Restriction enzyme	Fragment sizes (kb)	PyramidII:*M. leprae* cosmid DNA (kb)
*Bam*HI	1.75 2.60 3.40	45, 33^1 45, 33^2 57, 78
*Eco*RI	2.55 4.20 6.40 7.60 9.60	83, 116 33^2, 45 57, 116 33^1, 45 45, 57
*Pst*I	0.20 1.10 2.45 2.75 5.50	34, 57 34, 57 34, 45, 83 33^1, 116 45, 57,
*Hind*III	2.40 7.20 6.90	10, 32, 116 33^1, 57 42, 33^2

3.2.3.2 Insert sizes

The average sizes of the *M. leprae* insert DNA contained in the cosmids were calculated from an average of the sum of the fragments from each of the four digests (Table.17). The largest inserts were found in cos116, cos57 cos83 and cos45 which were 31.6kb, 26kb, 22.1kb and 18.6kb respectively. The insert sizes of cos10, cos32, cos33^1, cos33^2, cos34 and cos78 varied between 6-9kb, small for cosmid inserts. Due to the very small predicted insert size and the error margin on its calculation it was concluded that cos42 was possibly pYUB18 vector alone. Cosmid 59 and cos38 gave an average size of 13.8kb $\pm$ 4.46 implying that the DNA could have been smaller than the vector.

Table 17. **Showing the total cosmid sizes and *M. leprae* insert sizes**

Cosmid	Cosmid size (kb)	Insert size (kb)
10	20.50 ± 2.56	8.65 ± 3.0
32	19.84 ± 3.78	7.35 ± 3.64
33[1]	19.00 ± 1.02	7.65 ± 1.38
33[2]	17.95 ± 5.07	5.65 ± 5.00
34	18.66 ± 2.38	6.18 ± 2.10
38	13.80 ± 4.30	N/A
42	15.30 ± 3.47	3.32 ± 3.28
45	30.95 ± 2.30	38.60 ± 2.46
57	38.60 ± 2.46	26.90 ± 2.47
59	13.70 ± 4.30	N/A
78	21.04 ± 0.83	8.37 ± 1.04
83	34.30 ± 4.75	22.13 ± 4.70
116	43.80 ± 4.46	31.60 ± 4.46

From the digests and the cosmid sizes estimated for cos42 it was concluded that the DNA consistently extracted from *E. coli* was probably vector pYUB18 DNA. Both cos59 and cos38 were undigestable with *Bam*HI and gave band sizes smaller than predicted for the pYUB18 vector alone with *Hin*dIII, *Eco*RI and *Pst*I i.e. bands of 5.6kb and 5.8kb were produced from a *Hin*dIII digest instead of the 6.3 and 5.8kb which would be predicted. This implied a deletion of at least 1kb had occurred, which included the *Bam*HI cloning site, and possibly unstable *M. leprae* insert DNA. An early cosmid extraction of transformant 38 from TG2 yielded cosmid DNA which was found to contain an insert on *Bam*HI digestion.

Following single and double digests (not shown) of the other ten cosmids, nine from complementing PyramidII::*M. leprae* clones and one from a non complementing clone (cos78) similar band sizes were observed although a restriction map overlapping all the complementing cosmids could not be formed. It may have been that a number of different regions of *M.*

leprae DNA were capable of phenotypically complementing the mutant *M. smegmatis* mc^2155 strain.

There was therefore no single *M. leprae* DNA fragment from one of the cosmids deemed suitable for use as a probe in Southern hybridisation, to determine whether any of the cosmids were related, at this point.

3.2.4 MORPHOLOGY OF PYRAMIDII::M.LEPRAE TRANSFORMANT STRAINS

The morphology of the fourteen PyramidII:*M. leprae* strains was of two types, smooth and crinkly, on both Lemco and Middlebrook agar. Complementing strains 10, 32, 33^2, 34 and non complementing strains 42 and 59 showed similar morphology to the PyramidII strain, (Fig.33) The five other complementing strains 33^1, 45, 57, 83, and 116 exhibited a crinkly morphology stronger than the wild type (Fig.33). Complementing strain 38 exhibited a mixture of smooth and crinkly morphology colonies, it is possible that the insert DNA had deleted out of the cosmids in the smooth colonies. There was no clear cut correlation observed between crinkly morphology and complementation. Four transformants 116, 57, 83 and 45 exhibiting crinkly morphology had larger *M. leprae* insert DNA in their cosmids i.e. 116, 57 and 45 see Table.17.

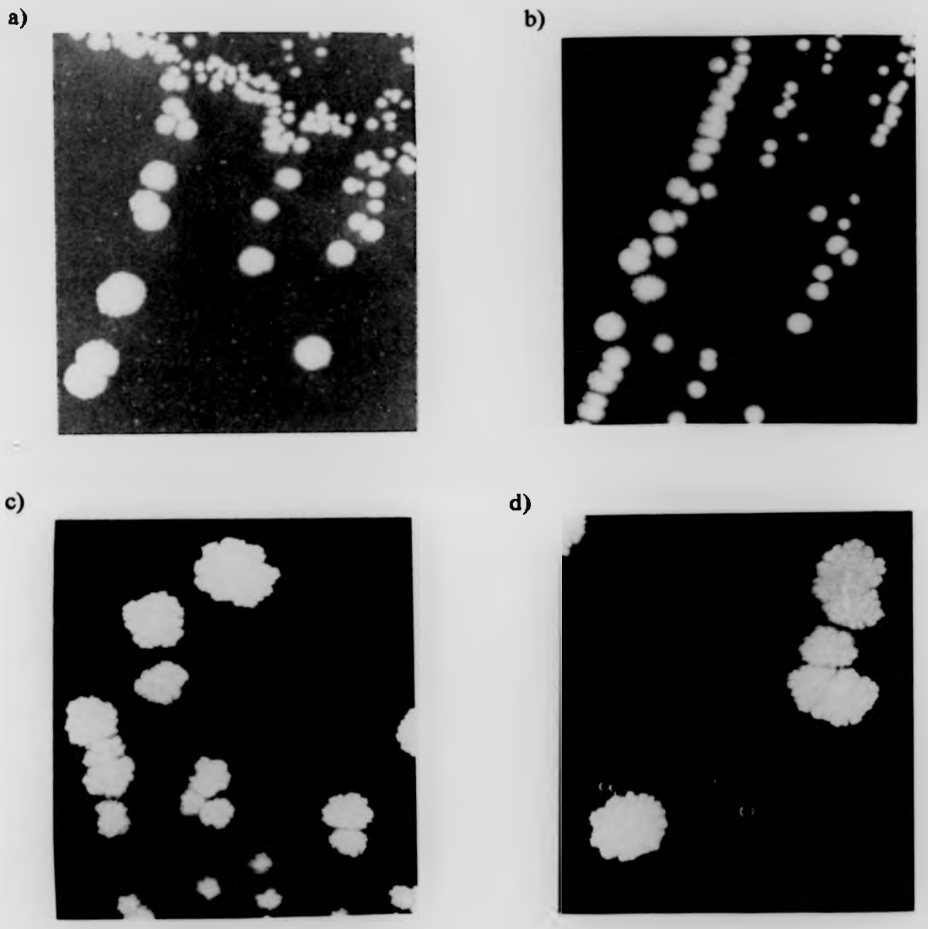


Fig.33. **The colony morphology of the PyramidII::*M.leprae* clones.**
The PyramidII::*M.leprae* clones showed two morphological types, shown above on Middlebrook 7H10 agar. Smooth colony morphology similar to the parental strain was exhibited by complementing and non complementing PyramidII::*M.leprae* clones. a) complementing clone 32 b)non complementing clone 42. Only complementing clones exhibited crinkly colony morphology c) complementing clone 57 d) complementing clone 45.

3.2.5 BIOCHEMICAL ANALYSIS OF PYRAMIDII::*M. LEPRAE* CLONES

3.2.5.1 Analysis of polar and non polar "free" lipids

The lipids of a number of the initial 14 PyramidII::*M. leprae* clones isolated were analysed to establish whether the presence of the *M. leprae* containing cosmid DNA had altered the lipid profile of PyramidII.

Initially, four complementing PyramidII:*M. leprae* clones 54, 57, 116 and one non complementing clone 78, were investigated. The three complementing clones also had a crinkly morphology whereas the non complementing clone had a smoother morphology. Clone 54 was still culturable from frozen culture at the time of this experiment.

The free lipids, polar and non polar, and the covalently bound lipids were extracted as before (section 2.29). The free lipids were analysed by 2D-TLC systems A, D and F. The lipid profiles were compared with those of the mc^2155 wild type and the parental PyramidII strains of *M. smegmatis*.

3.2.5.1.1 Systems A and D

Two dimensional TLC system A revealed menaquinones in all four PyramidII::*M. leprae* clones 54, 57, 116 and 78, in the same proportions as wild type.

The most polar lipids of the non polar lipid fraction of each strain were analysed using 2D-TLC system D_1 to disclose trehalose dimycolates, again in similar proportions to those found in the wild type and PyramidII *M. smegmatis* strains (Fig.34 and Fig.35). None of the three unidentified glycolipids found in *M. leprae* (Minninkin *et al.*, 1985) using this system, were observed.

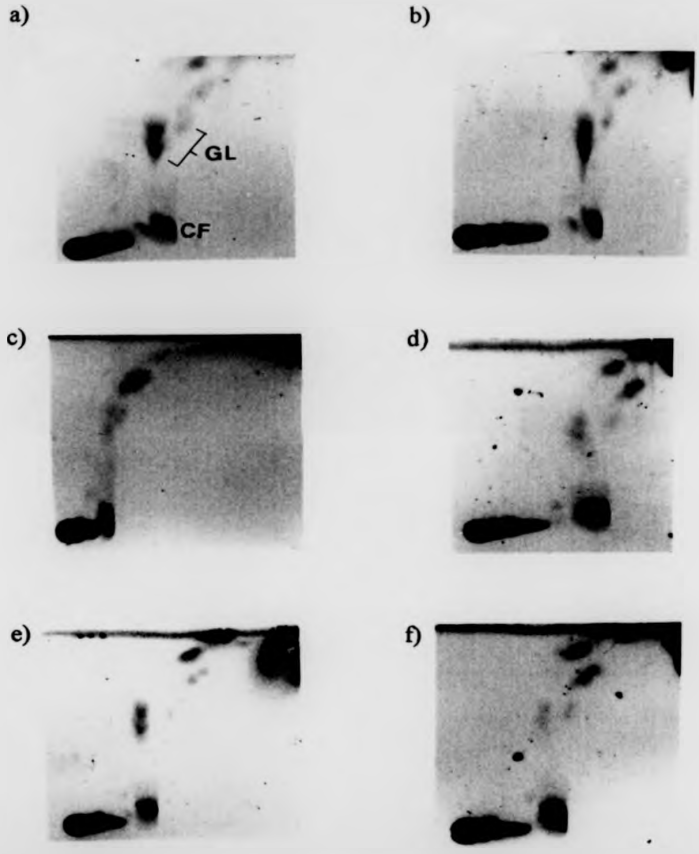


Fig.34. **Two dimensional TLCs of the most polar class of lipids in the non-polar lipid fraction of mycobacterial "free" lipids of PyramidII::*M.leprae* clones, (System D_1, with MPA)**

1st direction, chloroform:methanol:water (100:14:0.8 v/v/), 2nd direction, chloroform : acetone : methanol (50:60:2.5 v/v). Organisms: a) *M. smegmatis* mc^2155, b) PyramidII *M. smegmatis*, Pyramid::*M.leprae* clones c) 78 (non complementing) d) 54 e) 57 f) 116 (complementing).

Detection: a-f 5% ethanolic molybdophosphoric acid, MPA, all lipids.

CF - cord factor (trehalose dimycolate)

GL - glycolipids

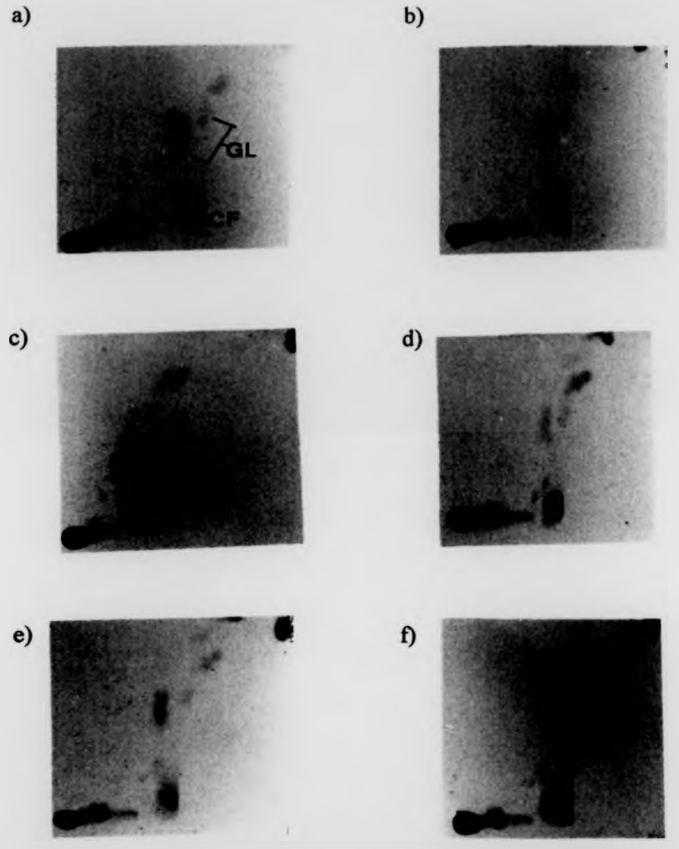


Fig.35. **Two dimensional TLCs of the most polar class of lipids in the non-polar lipid fraction of mycobacterial "free" lipids of PyramidII::*M.leprae* clones, (System D_1, with NAS).**

1st direction, chloroform:methanol:water (100:14:0.8 v/v/); 2nd direction, chloroform : acetone : methanol (50:60:2.5 v/v). Organisms: a) *M. smegmatis* mc^2155, b) PyramidII *M. smegmatis*, Pyramid::*M.leprae* clones c) 78 (non complementing) d) 54 e) 57 f) 116 (complementing).

Detection: a-f 5% α-naphthol sulphuric acid, NAS, specifically detects sugar components i.e. CF and GL.

CF - cord factor (trehalose dimycolate)

GL - glycolipids

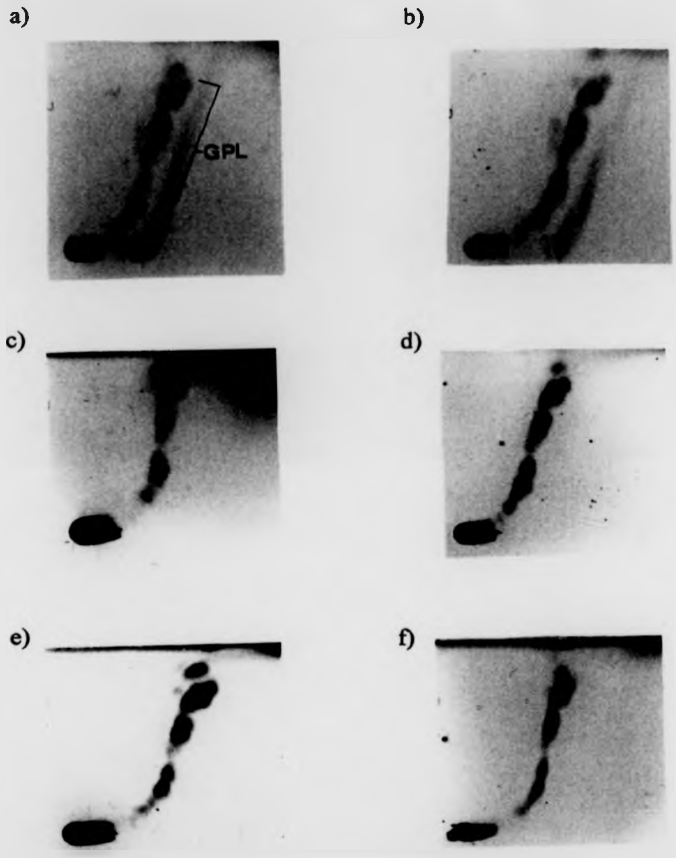


Fig.36. **Two dimensional TLCs of the least polar class of lipids in the polar lipid fraction of mycobacterial "free" lipids of complementing and non complementing PyramidII::*M.leprae* clones (System D_2, with MPA).**
1st direction, chloroform:methanol:water (100:14:0.8 v/v/); 2nd direction, chloroform : acetone : methanol (50:60:2.5 v/v). Organisms: a) *M. smegmatis* mc^2155, b) PyramidII *M. smegmatis*, Pyramid::*M.leprae* clones c) 78 (non complementing) d) 54 e) 57 f) 116 (complementing).
GPL - glycopeptidolipid

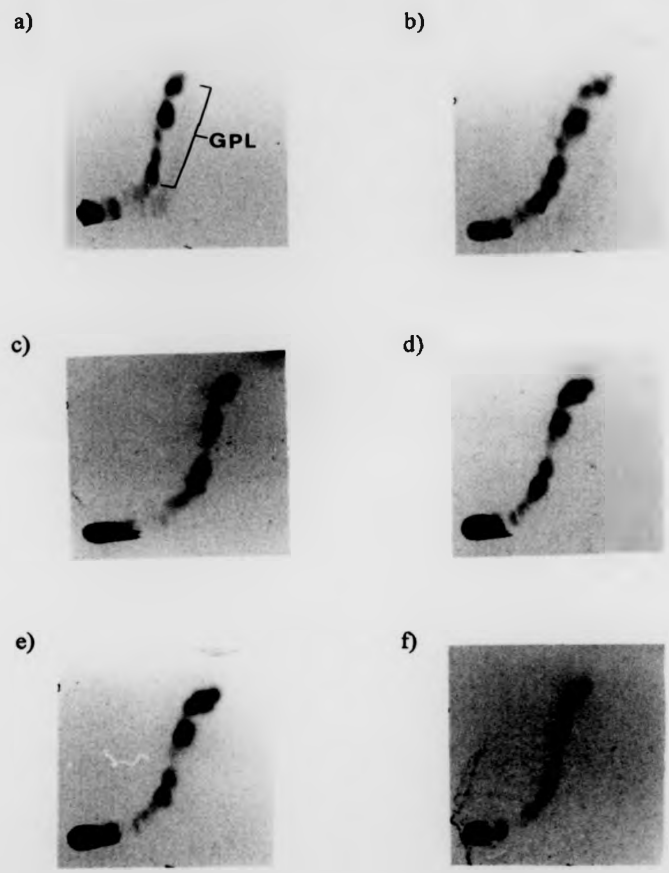


Fig.37. **Two dimensional TLCs of the least polar class of lipids in the polar lipid fraction of mycobacterial "free" lipids of complementing and non complementing PyramidII::*M.leprae* clones (System D_2, with NAS).**
1st direction, chloroform:methanol:water (100:14:0.8 v/v/); 2nd direction, chloroform : acetone : methanol (50:60:2.5 v/v). Organisms: a) *M. smegmatis* mc^2155, b) PyramidII *M. smegmatis*, Pyramid::*M.leprae* clones c) 78 (non complementing) d) 54 e) 57 f) 116 (complementing).
GPL - glycopeptidolipid
Detection: a-f α-naphthol sulphuric acid, NAS, for all sugars components i.e. GPL

The least polar lipids were also analysed using system D_2. The four PyramidII::*M. leprae* clones displayed the same pattern of glycopeptidolipids that were present in the wild type and parental *M. smegmatis* strains and in similar proportions (Fig.36 and Fig.37).

3.2.5.1.2 System F

This was used to investigate the most polar of the polar lipid extracts from each of the four clones i.e. phospholipids and phosphatidylinositolmannosides, PIMs. The phospholipids, PIMs and glycolipids present in the parental PyramidII and wild type mc^2155 *M. smegmatis* strains were also present in the four PyramidII::*M. leprae* clones in similar proportions with one exception, (Fig.38 and Fig.39). A large quantity of an unknown lipid, P, was detected in the complementing clones 54, 57 and 116 which was barely visible in the non complementing clone 78, the parental PyramidII and the wild type *M. smegmatis* mc^2155, (Fig.38). The unknown lipid was not visible under α-naphthol spray (Fig.39) and therefore contained no sugar moieties such as mannose.

The free lipid extraction and analysis by system F was repeated in duplicate, with clones 57 and 116 wild type mc^2155 and parental strain PyramidII. The same unknown lipid, P, was detected when the plates were sprayed with MPA to detect the lipid. The unknown lipid P spot was also detected when plates were sprayed with Molybdenum blue, used to detect phospholipids, implying that it was a phospholipid. No phospholipids were detected with this spray at the same position in the parental PyramidII or *M. smegmatis* mc^2155 wild type strain, although small amounts of a lipid were detected in a similar position (Fig.40).

The position of the unknown lipid P, in system E, from which system F was derived, seems to correspond to an unknown lipid observed in *M. leprae* using the same system (Minnikin *et al.*, 1985) (Fig.41).

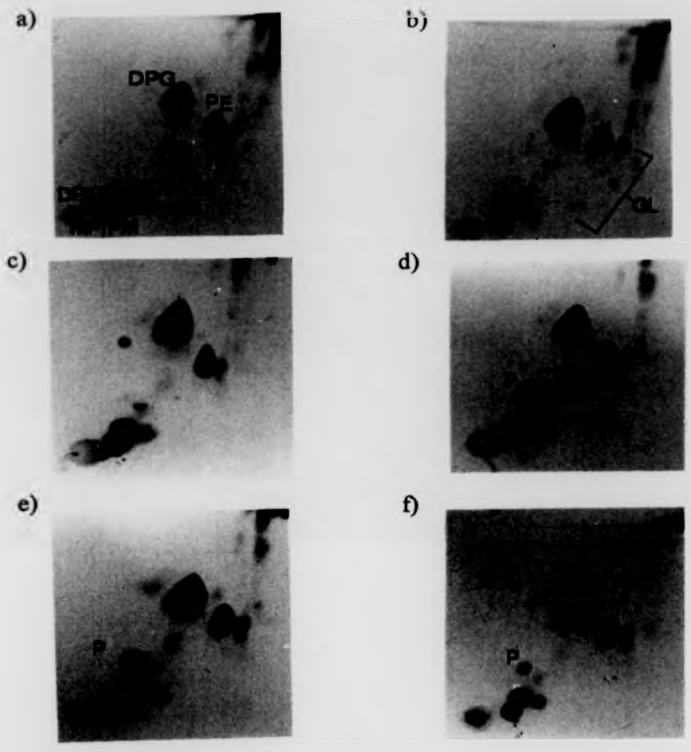


Fig.38.**Two dimensional TLCs of the most polar class of lipids found in the mycobacterial polar "free" lipid fraction of PyramidII::*M.leprae* complementing and non complementing strains (System F, with MPA).**
1st direction, chloroform:methanol:water (65:25:4 v/v); 2nd direction chloroform:acetic acid: methanol water (80:15:12:4 v/v).
Organisms: a) *M. smegmatis* mc^2155, b) PyramidII *M. smegmatis*, Pyramid::*M.leprae* clones c) 78 (non complementing) d) 54 e) 57 f) 116 (complementing).
DPG - diphosphatidylglycerol
PE - phosphatidylethanolamine acid
PI - phosphatitylinsositol
DPIDM + MPIDM - di and mono acylphosphatidylinsositol dimannosides
DPIPM + MPIPM - di and mono acylphosphatidylinsositol pentamannosides
GL - glycolipid
P- unknown lipid
Detection system: a-f 5% ethanolic molybdophosphoric acid spray (MPA) for all lipids.
An unknown lipid was found in the complementing PyramidII::*M.leprae* clones (54, 57,116) which was not found in the non complementing clone (78), the parental or wild type strains in the same quantities.

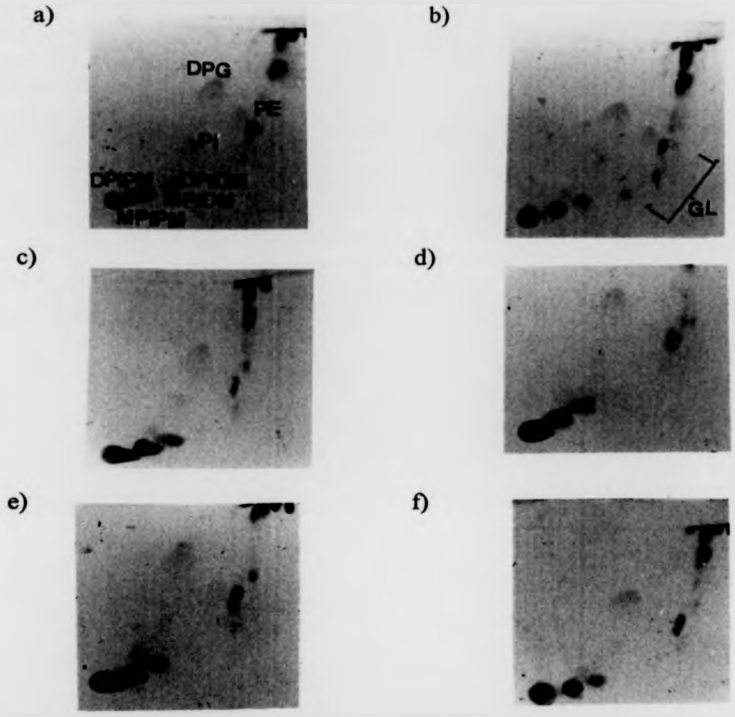


Fig.39.**Two dimensional TLCs of the most polar class of lipids found in the mycobacterial polar "free" lipid fraction of PyramidII::*M.leprae* complementing and non complementing strains (System F, with NAS).**
1st direction, chloroform:methanol:water (65:25:4 v/v); 2nd direction chloroform:acetic acid: methanol water (80:15:12:4 v/v).
Organisms: a) *M. smegmatis* mc^2155, b) PyramidII *M. smegmatis*, Pyramid::*M.leprae* clones c) 78 (non complementing) d) 54 e) 57 f) 116 (complementing).
DPG - diphosphatidylglycerol
PE - phosphatidylethanolamine acid
PI - phosphatitylinsositol
DPIDM + MPIDM - di and mono acylphosphatidylinsositol dimannosides
DPIPM + MPIPM - di and mono acylphosphatidylinsositol pentamannosides
GL - glycolipid
Detection system: a-f α-naphthol sulphuric acid (NAS), for sugar components, i.e. GL, DPIDM, MPIDM, DPIPM and MPIPM.
P, the unknown lipid found in the complementing PyramidII::*M.leprae* clones (54, 57,116) but not in the non complementing clone (78), the parental or the wild type strains in the same quantities was not dectable with NAS and therefore did not contain a sugar component.

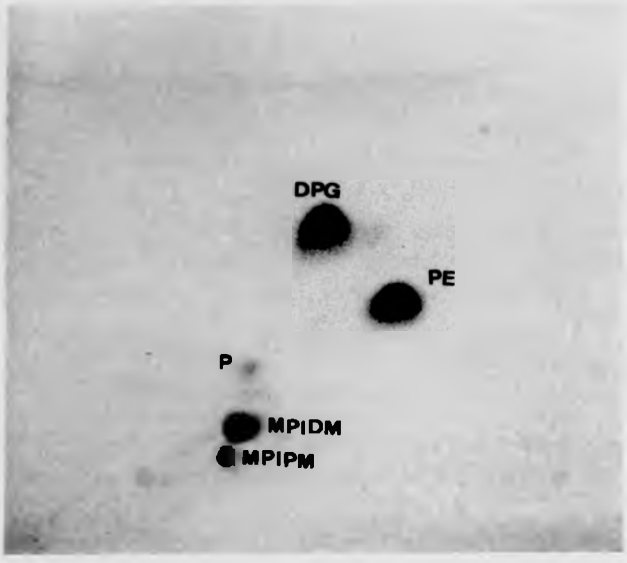


Fig 40. **Two dimensional TLC of the most polar class of lipids found in the mycobacterial polar "free" lipid fraction of PyramidII::M. leprae complementing clone 57, showing the unknown phospholipid**
1st direction, chloroform: methanol: water (65:25:4 v/v); 2nd direction chloroform:acetic acid:methanol:water (80:15:12:4 v/v);
Organism: Pyramid::*M. leprae* complementing clone 57.
Spray: Molybdenum blue which detects phospholipids
DPG- diphosphatidylglycerol
PE- phosphatidylethanolamine
PI- phosphatidylinositol
DPIDM + MPIDM - di and mono acylphosphatidylinositol dimannosides
DPIPM + MPIPM - di and mono acylphosphatidylinositol pentamannosides
P confirmed as a phospholipid, found in the complementing clones 57, 116 and 54.

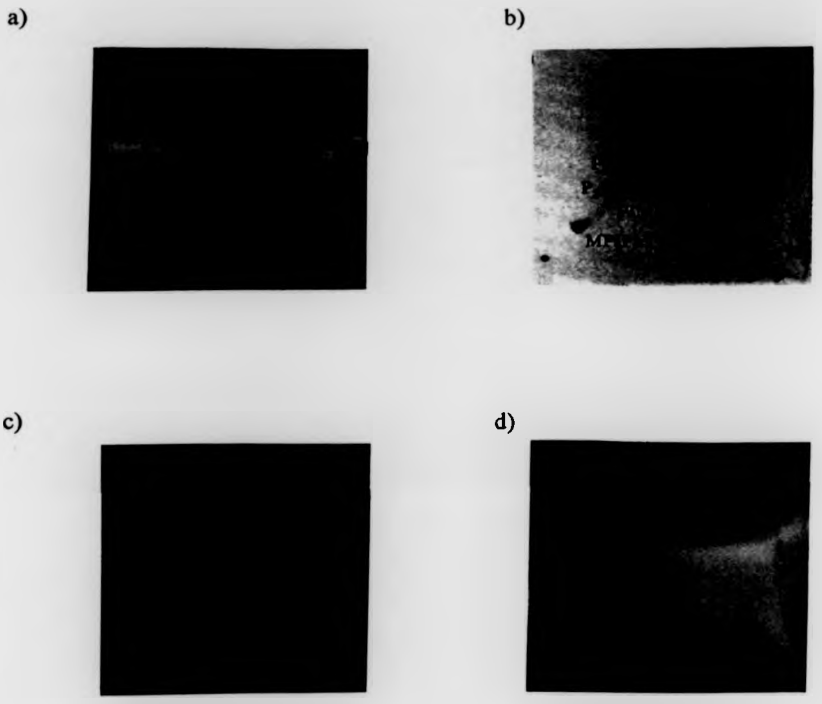


Fig.41.**Two dimensional TLCs of the most polar class of lipids found in the mycobacterial polar "free" lipid fraction of PyramidII, PyramidII::*M. leprae* complementing clone 57 and 116 compared with *M. leprae* using system E (with MPA)** 1st direction, chloroform: methanol: water (10:5:1 v/v); 2nd direction chloroform:acetic acid:methanol:water (40:25:3:6 v/v);
Organisms a) PyramidII *M. smegmatis*, b) *M. leprae* taken from Minnikin *et al.*, 1985) c and d) Pyramid:*M. leprae* complementing clones 116 and 57 respectively.
DPG- diphosphatidylglycerol
PE-phosphatidylethanolamine
PI- phosphatidylinositol
DPIDM + MPIDM - di and mono acylphosphatidylinositol dimannosides
DPIPM + MPIPM - di and mono acylphosphatidylinositol pentamannosides
P unknown phospholipid found in the complementing clones 57 and 116 and in *M. leprae* (Minnikin *et al.*, 1985) but not in the parental PyramidII *M. smegmatis*.

In an attempt to identify the phospholipid produced by the PyramidII::*M. leprae* clones, ngs of the phospholipid were isolated and are currently being analysed by gas chromatography mass spectrometry. This analysis is being carried out by Dr.P.Wheeler, LSHTM and Dr.W.Blackstock, Glaxo plc.

3.2.5.2 Covalently bound lipids

The mycolates of the four PyramidII:*M. leprae* clones were analysed (Fig.42). The characteristic α, α' and epoxymycolates of *M. smegmatis* were present in all four clones in similar proportions to those found in the wild type and PyramidII *M. smegmatis* strains.

3.2.5.3 Fatty acid analysis using gas chromatography

The fatty acid composition of the four clones was also analysed using gas chromatography. The proportions of the dominant types of non hydroxylated fatty acids were altered in all four of the PyramidII:*M. leprae* clones in comparison with *M. smegmatis* mc^2155 and the parental PyramidII strains (Table.18).

Table 18. **The fatty acid composition of the PyramidII:M.leprae clones in comparison with parental and wild type composition**

% Fatty acid	mc^2155	PyramidII	78	57	54	116
Palmitoleic acid (C16:1)	11.90	12.21	10.33	10.67	8.52	11.74
Palmitic acid (C16)	28.89	31.58	41.46	38.53	35.34	36.35
Oleic acid (C18:1)	39.83	37.68	15.67	14.65	13.59	16.06
Stearic acid (C18)	2.10	1.40	3.16	4.28	3.40	2.50
Tuberculostearic acid (C19:Br)	10.72	12.79	23.82	22.35	35.14	27.6

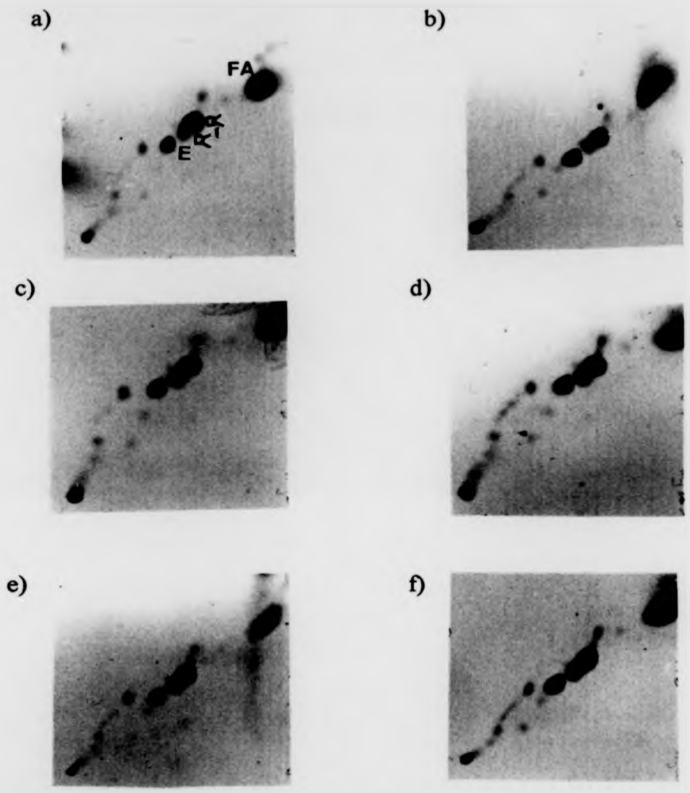


Fig.42. **Two dimensional TLC of the mycolic acid methylester obtained from the extraction of mycolic acids**
1st direction, petroleum ether (b.p. 60-80°C), acetone (95:5v/v), three times; 2nd direction toluene, acetone (97:3, v/v).
Organisms: a) *M. smegmatis* mc^2155, b) PyramidII *M. smegmatis*, Pyramid::*M.leprae* clones c) 78 (non complementing) d) 54 e) 57 f) 116 (complementing).
Detection: MPA all lipids
α - α mycolates
α'- α' mycolates
E - epoxymycolates
FA - Fatty acids
All PyramidII::*M.leprae* clones exhibited the expected α, α' and epoxymycolates expected from *M. smegmatis* strains in similar proportions.

The percentage of oleic acid in the clones decreased from 38% in the wild type to 15% in the clones. The levels of palmitic acid increased by 5-10% in the clones as compared to the wild type or parental strains. The amounts of tuberculostearic acid were up by 10-17% in three clones 57, 78 and 116 and by 25% in clone 54, when compared to wild type levels.

In conclusion, the systematic biochemical analysis of the four clones resulted in the detection of an unknown phospholipid in the three complementing strains 54, 57 and 116, which was not present in the non complementing clone, 78. The proportions of the individual fatty acids in the PyramidII::*M. leprae* clones also differed significantly from those found in the wild type mc^2155 and the parental PyramidII *M. smegmatis*.

3.3 FURTHER ANALYSIS OF PYRAMIDII COMPLEMENTING COSMID 57

PyramidII::*M. leprae* transformant containing cosmid 57 showed increased pyrazinamide resistance on solid and in liquid medium, exhibited a crinkly morphology and produced an unknown phospholipid in larger quantities than were visible in the parental PyramidII or *M. smegmatis* mc^2155. Strain 116 also displayed these properties, the only difference being that it produced slightly smaller amounts of the unknown phospholipid. Cos57 was therefore chosen as the cosmid from which to isolate a specific region of *M. leprae* DNA which complemented the pyrazinamide sensitive phenotype of the PyramidII strain. The relationship, if any, between the *M. leprae* DNA complementing the pyrazinamide sensitivity, the crinkly morphology and the phospholipid production, of strain 57 could also be investigated.

3.3.1 THE MAPPING OF COSMID 57 TO THE ORDERED *M. LEPRAE* COSMID LIBRARY

An ordered *M. leprae* cosmid library exists (Eiglmeier *et al.*, 1993) and the position of cosmid 57 *M. leprae* insert DNA in the *M. leprae* genome could be determined by Southern hybridisation of a fragment of cos57 DNA to the ordered library. A random fragment of cosmid 57 was therefore cloned.

A 1.2kb fragment created by a *Bam*HI digest of cosmid 57 (Fig.43) was cloned into the *Bam*HI site of pMV206 producing pC57B2. 2μg of the construct was digested with *Bam*HI and purified as described in section 2.6.1.

The 1.2kb insert DNA was labelled and hybridised to filters of the ordered *M. leprae* cosmid library by Dr.K.Eiglmeier, Institute Pasteur, Paris. The insert DNA was found to hybridise to a 1.17kb fragment of cosmid B1308 in the ordered cosmid library. This cosmid had been sequenced and was adjacent to a second sequenced cosmid, L308, at the end of contig 35 (Fig.14) (Eiglmeier *et al.*, 1993).

3.3.1.1 Mapping of cosmid 57 to cosmid B103

The results of the hybridisation indicated that the length of *M. leprae* insert DNA contained in cosmid 57 mapped within cosmid B1308 or overlapped one of the adjacent cosmids, L308 or Y236. To ascertain the position of cosmid 57 within the ordered cosmid library and how much of the cosmid was sequenced, the restriction patterns of cosmid 57 were compared with those of cosmid B1308, available from the Mycobacterial Genome Database, MycDB (Bergh and Cole, 1994). 0.5μg of cosmid 57 was digested with each of the restriction endonucleases *Bam*HI, *Pst*I, *Hin*dIII, *Eco*RI, *Not*I and *Eco*RV. The restriction fragments of cosmid 57 produced by *Pst*I and *Bam*HI digests and the loci of these individual fragments in cosmid B1308 are shown in Fig.43.

The average size of the *M. leprae* DNA insert in cosmid 57, calculated from the band sizes produced by the six digests, was 25.7kb $\pm$ 1.9kb. Cosmid B1308 is 33,312bp long and and the largest region of B1308 initially shown to be present in cosmid 57 was the 24.059kb between 5,992-30,051 defined by the *BamH*I and *Pst*I digest of cosmid 57 (Fig.43). The *PstI* site at 30,051 was confirmed by sequencing in subsequent experiments (3.5.2.4). The *Eco*RV digest also produced a 1.443kb fragment which requires a site at 30,603 also found in later

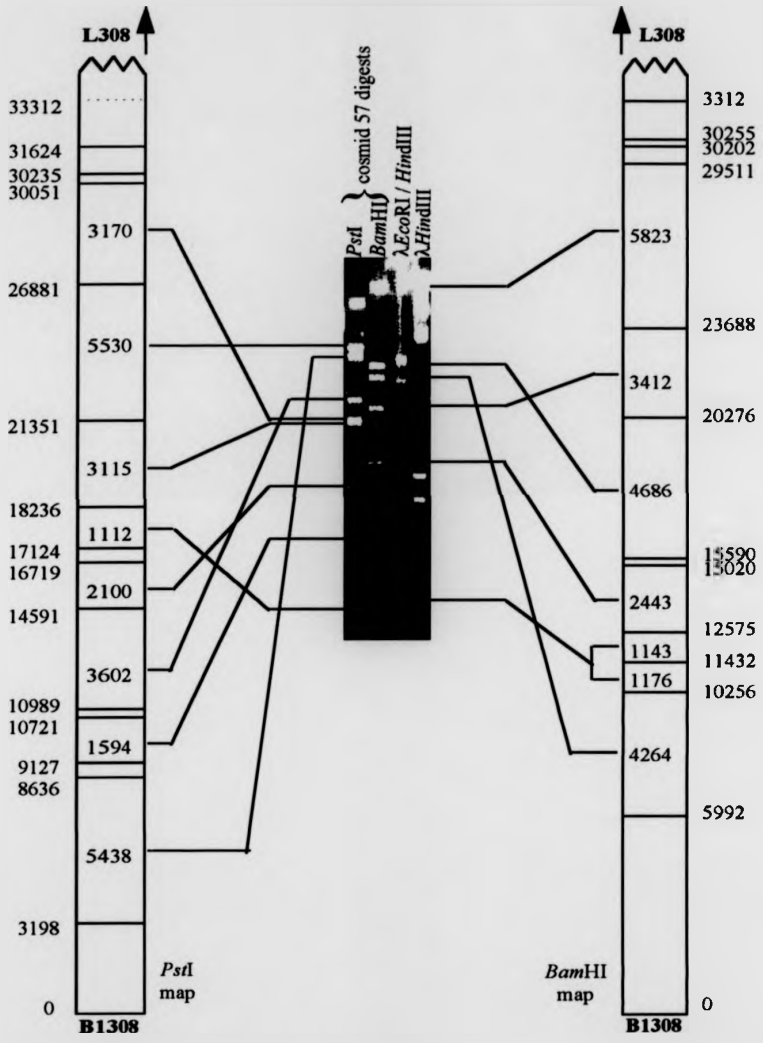


Fig 43. **The alignment of the *Pst*I and *Bam*HI fragments of cosmid57 with restriction maps of B1308 as quoted in MycDB**.

experiments (Fig.44 and 45) which would extend the insert size to at least 24.611kb. This is not in agreement with the *Bam*HI digest shown, which would be expected to produce a 5.823kb and 0.69kb fragment due to sites at 29,511, 30,255 and 30,255; it is thought that the digest shown is a partial and that the sites closest to the vector have not been cut, due to the 19kb fragment present which would appear to be pYUB18 (12kb) and at least another 7kb of DNA. It is also possible that one of the *Bam*HI sites has been lost during cloning. The distal end of cosmid 57 was known not to extend beyond 31,624, as there was a *Pst*I site at this locus which would have produced a 1.385kb band not visible following the *Pst*I digest of cosmid 57, (Fig.43). Later experiments implied that another 1.3kb of *M. leprae* DNA was present in cosmid 57 at the distal locus, extending to position 31,540 of B1308. There is also a band of 5.4kb in the *Pst*I digest which would extend the proximal region of the insert to 3,198, however it is possible that this fragment consists of 2kb of pYUB18 vector DNA which would be created by a *Pst*I digest and 3.4kb of proximal insert DNA; this would extend the proximal end of the insert to at least 5, 236, giving an insert size of at least 26.548kb.

In summary the insert DNA of cosmid 57 was contained within cosmid B1308 and was thought to extend from at least bases 5,230 to 31,540 giving a 26.3kb insert. The mapping of cosmid 57 within cosmid B1308 confirmed that the entire sequence of cosmid 57 was known. Having determined the approximate coordinates of cosmid 57 within B1308, the business of narrowing down the complementing region of cosmid 57 was begun.

3.3.2 THE ISOLATION OF THE SPECIFIC LENGTH OF COMPLEMENTING DNA IN COSMID 57

3.3.2.1 Cloning of the BamHI fragments of cosmid 57

The initial strategy for narrowing down the area of *M. leprae* DNA within cosmid 57 complementing the mutant phenotype, was to clone the five *Bam*HI fragments of cosmid 57 into the pMV206 shuttle vector plasmid. The five constructs would be individually transformed into PyramidII and the five transformed PyramidII strains would then be tested

for their sensitivity to pyrazinamide.

*Bam*HI fragments were chosen since the sites interrupted only one gene, lysine-6-amino transferase, in the cosmid, whereas other restriction enzymes cut through a greater number of open reading frames. This information was gleaned from the restriction maps and positions of the genes within cosmid B1308 available from MycDB. The terminal lengths of *M. leprae* DNA would remain attached to the pYUB18 vector following a *Bam*HI digest of cosmid 57 and was not therefore a complete screen of all the insert DNA for complementation.

5μg of cosmid 57 DNA was digested with *Bam*HI restriction enzyme. The digested DNA was loaded into the extended well of a 0.8% agarose gel and run at 20V overnight. Fig.43 shows the bands the bands of fragment sizes 1.1kb, 2.4kb, 3.4kb, 4.2kb and 4.6kb which were excised and the DNA recovered using a Wizard DNA Clean Up Kit (Promega)(section 2.6.1). 5μg of plasmid vector pMV206 was cut, treated with alkaline phosphatase and run on a 0.8% LMP agarose gel from which only cut vector was excised and recovered. Ligation reactions were set up for each of the five fragments with 750ng insert DNA and 250ng vector DNA and these were subsequently transformed into *E.coli* DH5α.

The ligation reactions of fragments 1.1kb and 2.4kb produced on average $1x10^3$ transformants/μg and those of fragments 3.4kb, 4.2kb and 4.6kb produced $2x10^2$ transformants/μg. Recombinant plasmids containing inserts of 1.1kb and 2.4kb were successfully recovered from the transformants. Recombinant plasmids with the correct insert size were not obtainable for 3.4kb, 4.2kb and 4.6kb fragments, after repeated attempts. The subcloning of the *Bam*HI fragments was eventually discontinued.

3.3.3 CREATION OF A SUB-LIBRARY OF COSMID 57 IN PMV206

The next approach was to make a *Sau*3AI sublibrary of cosmid 57. 5μg of cosmid 57 DNA was partially digested with *Sau*3AI, run on a 0.8% LMP agarose gel and DNA fragments of 3kb and 6kb were size selected by excision. The size selected DNA fragments were purified from the agarose using Wizard DNA Clean Up Kit (Promega). 5μg of pMV206 was linearised by digestion with *Bam*HI and alkaline phosphatase treated at 37°C for 15min. The cut, phosphatased pMV206 DNA was run on an LMP agarose gel at 80V for 3hr and the linear vector band excised and recovered using the Wizard DNA Clean Up Kit (2.6.1).

Individual ligation reactions were set up for both sizes of insert DNA, 3kb and 6kb. 1μg of insert was added to 350ng of cut and phosphatased pMV206 vector. A control vector only ligation was also set up. The three ligations were transformed into competent *E.coli* DH5α with a transformation efficiency of $1x10^6$cfu/μg. $1x10^3$ and $1.5x10^4$ transformants were produced from the 3kb and the 6kb ligation reactions respectively. The plasmid DNA was extracted from five colonies of the 3kb cos57 sub-library and eight colonies from the 6kb cos57 sub-library using the small scale plasmid extraction method (section 2.11). The remaining transformants of each sub-library were pooled and used to seed two 100ml Lemco which were incubated at 37°C overnight. The cells from each of two "sub-library" cultures were pelleted and the plasmid DNA extracted using the midi plasmid preparation (section 2.12). 0.5μg of plasmid DNA extracted from the two pooled libraries and from the thirteen individual plasmid DNAs was digested with *Pst*I. As there was only one *Pst*I site in pMV206, digestion with the enzyme linearised the pMV206 plasmid DNA; those plasmids containing inserts could be distinguished by increased size of the plasmid and by "drop out" fragments caused by the presence of *Pst*I sites in the cosmid 57 insert DNA.

Of the five plasmid DNAs of the 3kb sub-library, four contained small inserts, up to 1kb, one strain appeared to contain no insert DNA. Of the eight plasmid DNAs of the 6kb sub-library,

six appeared to contain inserts ranging in size from 0.2kb-5kb. The *Pst*I digests of the pooled plasmid DNA of both the 3kb and the 6kb sub-libraries produced the expected DNA "smears". The fragment sizes produced from the 6kb sub-library ranged from 9kb to 1kb, while the fragment sizes of the 3kb sub-library ranged from 6kb to 1kb. Strong bands were observed at 4kb in the digests of pooled plasmid DNA from both libraries, probably consisting mainly of linearised pMV206 without inserts. The 6kb sub-library appeared to be the superior library, with a greater number of plasmids which contained insert DNA than the 3kb sub-library; this was deduced from the range and brightness of the DNA smear produced from the *Pst*I digestion of the 6kb sub-library DNA in comparison with that of the 3kb sub-library DNA.

3.3.3.1 Transformation of PyramidII with the 6kb sub-library of cosmid 57

Competent PyramidII *M. smegmatis* cells were transformed with 2μg of the 6kb sub-library DNA, the cells were then plated onto Lemco (Kan 15μg/ml) and incubated at 37°C for four days. $2x10^3$ transformants were produced, the colonies all had a smooth colony morphology, except for one which had a crinkly morphology.

3.3.3.2 Screening for increased pyrazinamide resistance

Initially 96 transformed strains, all with a smooth colony morphology, were selected and transferred onto a Lemco (Kan 15μg/ml) plate and incubated at 37°C for four days to recover. The strains were then replica plated on to Lemco pH5.2 agar containing 500μg/ml pyrazinamide and a control Lemco agar plate. Both arrays of the strains were incubated at 28°C for six days. Fourteen strains grew on 500μg/ml pyrazinamide, while all strains grew on Lemco (Kan 15μg/ml) at 28°C. The fourteen "resistant" strains were sub-cultured onto Lemco (Kan 15μg/ml) and incubated at 37°C to recover.

The fourteen PyramidII::cos57 transformant strains were named Pyr1-14. Single colonies from Pyr1-14 were used to inoculate 5ml Lemco (Kan 15μg/ml) cultures which were incubated at 37°C until the cultures reached approximately late log-phase of growth 0.8A_{600}. Control cultures of PyramidII and wild type *M. smegmatis* mc^2155 strains were also grown to late-log phase (0.8A_{600}). The PyramidII::cos57 cultures, Pyr1-14, were then streaked onto Lemco pH5.2 (Kan 15μg/ml) containing 500μg/ml pyrazinamide and incubated at 28°C for five days. The control cultures were streaked onto Lemco pH5.2 agar containing pyrazinamide 500μg/ml and also incubated at 28°C for five days. The mutant PyramidII *M. smegmatis* strain did not grow on 500μg/ml pyrazinamide. Ten of the PyramidII::cos57 strains, Pyr1,3-6 and 10-14, exhibited stronger growth on 500μg/ml pyrazinamide than the wild type *M. smegmatis* mc^2155 while four of the strains Pyr 2,7,8, and 9 showed no growth on 500μg/ml pyrazinamide. This experiment was repeated and the same results obtained.

3.3.3.3 Analysis of the plasmid DNA in the PyramidII transformants

The recombinant plasmid DNA contained in the fourteen Pyr1-14 strains was extracted using the small scale plasmid prep method for mycobacteria from overnight cultures of each strain grown in 5ml Lemco (Kan 15μg/ml) media at 37°C with shaking. The recombinant plasmid DNA recovered from each strain was investigated by restriction digest analysis. Initially, the plasmids were double digested with *Pst*I and *Eco*RV and by *Pst*I and *Kpn*I restriction enzymes. These pairs of sites were situated either side of the *Bam*HI site in the multiple cloning region of pMV206 into which the cos57 insert DNA was cloned. Double digests of the plasmid DNAs with these enzyme pairs would therefore "dropout" the insert DNA of each recombinant plasmid. Single digests with *Pst*I, *Kpn*I and *Eco*RV were also carried out. The results of the double digests are shown in Fig.44 and illustrate the similarity of the recombinant plasmid DNA in each of complementing strains Pyr1, 3-6 and 10-14, whereas the non complementing strains Pyr 2, 7, 8 and 9 were visibly different. The ten complementing strains produced 0.8-0.9kb and 1.8kb fragments following a *Pst*I, *Eco*Rv double digest, not

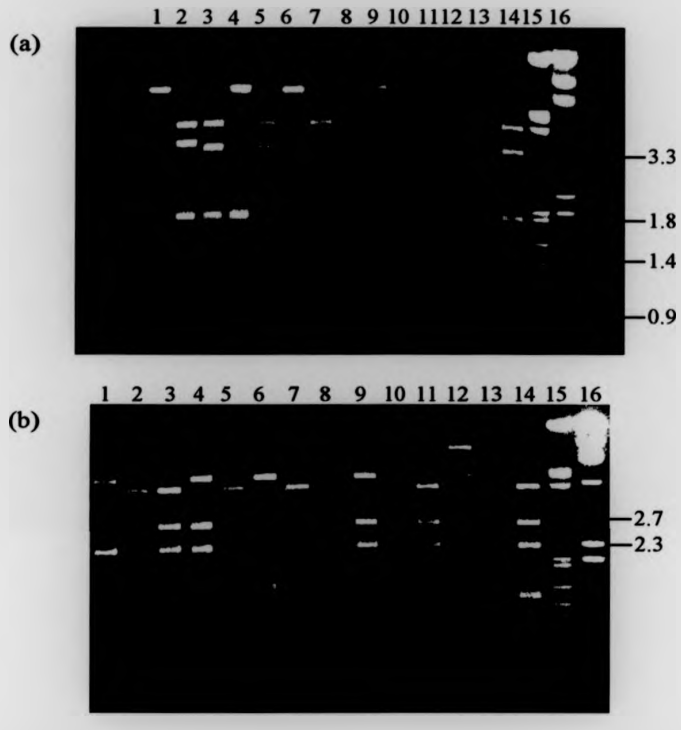


Fig. 44. *Pst*I / *Eco*RV and b) *Pst*I / *Kpn*I double digests of ten complementing and four non-complementing plasmids.

Complementing recombinant plasmids		Non-complementing plasmids		Markers
lane 1) pYR14	lane 9) pYR6	lane 6) pYR9		lane 15) λ*Hin*dIII / *Eco*RI
lane 2) pYR13	lane 10) pYR5	lane 7) pYR8		lane 16) λ*Hin*dIII
lane 3) pYR12	lane 11) pYR4	lane 8) pYR7		
lane 4) pYR11	lane 12) pYR3	lane 13) pYR2		
lane 5) pYR10	lane 14) pYR2			

pYR 3, 5, 7, 10 and 14 all appear to have lost the *Eco*RV and *Kpn*I sites during cloning.

a) Shows the 0.9kb fragment found in all complementing plasmids. The approximate 1.8kb fragment was found in pYR1, 3, 4, 6, 10, 11, 12 and 13 and is contained within the region between the *Eco*RI site at 29160 and pMV206 *Pst*I cloning site (Fig.45). The size of this band varies in the different plasmids, only 1.4kb of the region is apparent in pYR5 and 14. The 3.3kb region is part of pYUB18 which contains part of the mycbacterial origin of replication pAL500 (Fig. 45).

b) The 2.7kb represents the region from the *Pst*I site at 30051 to the *Pst*I site in pMV206 and is present in all the clones containing the 1.8kb *Eco*RV/*Pst*I fragment (Fig.45). The 2.3kb fragment represents the region between the *Kpn*I site present in pYUB18 and the *Pst*I site at 30051 (Fig.45).

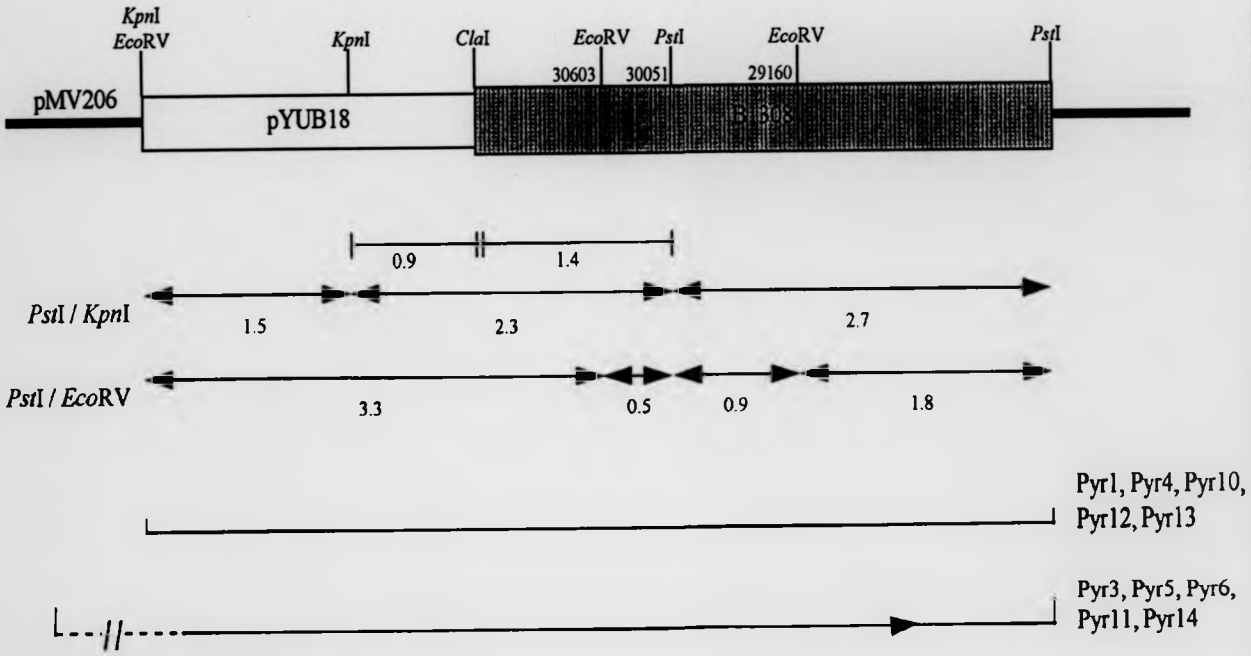


Fig.45. Restriction map of the complementing DNA found in PyramidII strains complemented with a sublibrary of complementing cosmid 57.

The complemented clones all contained at least 3.5kb from the distal end of cosmid, cosmid57 which correponds to the distal region of B1308. All the complementing recombinant DNA also contains variable lengths of the pAL5000 region of pYUB18 cloned between the *Eco*RV/*Kpn*I and *Pst*I sites of pMV206. The coordinates shown in the region of B1308 are taken from MycBD. The map shows the coordinates for the plasmid DNA of Pyr1,4,10,12 and 13. Pyr14 and Pyr5 contain approximately 0.5-0.6kb less *M. leprae* DNA proximal to the *Pst*I site and so the complementing region as defined so far is approximately 3.5kb. The plasmid DNA of Pyr3,5,6,11 and 14 appears to have lost its *Eco*RV and *Pst*I sites.

present in the *Pst*I only digest. A 2.75kb fragment produced by a *Pst*I only digest was not present following digestion with *Pst*I, *Eco*RV.

With the aid of the restriction patterns produced for cosmid B1308, available from the MycDB database the complementing length of cosmid 57 DNA was mapped to a 3.5kb region of cosmid B1308 (Fig.45), located at the distal end of both cosmid57 and B1308, between 28,000 and 31,500 of B1308 (The coordinates are those found in MycDB which are in the opposite orientation to those in the EMBL database).

3.3.3.4 Sequencing Analysis

In order to confirm the mapping of the complementing DNA to the distal end of both cosmid 57 and B1308 the ends of a fragment of the complementing plasmid were sequenced.

A 0.9kb *PstI/Eco*Rv fragment ([E] 29,160-30,0051 [P]) found in all the complementing plasmids was subcloned into the *PstI/Eco*Rv sites of pMV206. A large scale *PstI/Eco*Rv digest of 5μg of plasmid DNA extracted from Pyr1 was run on an 0.8% agarose gel and the 0.9kb band excised and recovered with Wizard DNA clean up kit. 750ng of 0.9kb insert DNA was placed in a ligation reaction with 250ng of *PstI/Eco*RV digested pMV206. The ligation reaction was transformed into *E.coli* DH5α, small scale plasmid preparations were carried out on overnight cultures of the transformants and the plasmid DNA recovered was digested with *PstI/Eco*Rv to confirm that it contained the 0.9kb insert DNA. The procedure was then repeated with a *Hin*dIII/*Xba*I digest of the pMV206 plasmid, dropping out the 0.9kb fragment, and the subsequent cloning of the 0.9kb insert into *Hin*dIII/*Xba*I digested pUC19.

Having successfully cloned the 0.9kb fragment into pUC19, universal forward and reverse primers could be used to sequence across the ends of the insert DNA. The double stranded sequencing was carried out (section 2.17.1, 2 and 3) and the sequence generated aligned to

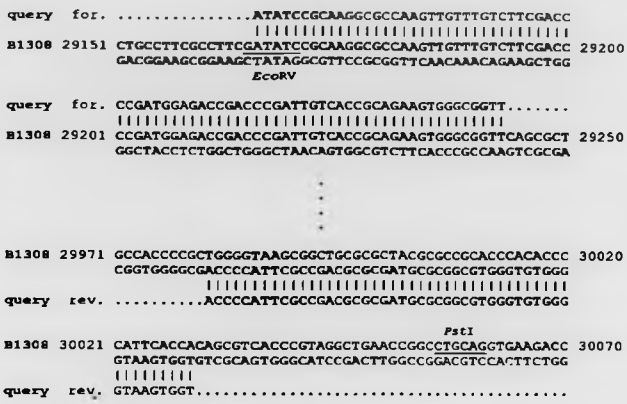


Fig.46.a) **An alignment of a sequenced region of cosmid 57 with B1308**
An alignment of the sequence obtained from the ends of the0.9kb *PstI*/*Eco*RV fragment isolated from complementing recombinant plasmid pYR1 with B1038. One end of the 0.9kb fragment aligned with the 30,0051 *PstI* site and the other end with the *Eco*RV 29,160 site so confirming the restriction mapping analysis of the region of B1308/57 contained within the complementing clones.

Fig 45.b) Table.19. **Homologies of six putative coding regions found at the distal end of cosmid B1308**

Putative coding regions	Location on B1308 (MycDB)	Best match	Probability of match	Database
p	31084-32187	propionyl CoA carboxylase	$5x10^{-139}e$	EMBL/ Swissprot
i	29489-29983	no significant match	-	-
a / c	28118-28486	no significant match	-	-
b	28715-28965	no significant match	-	-
A	25571-26125	*P.satirum* turgor response protein / aldehyde dehydrogenase	$6x10^{-21}e$	EMBL/ Swissprot
C	25571-26125	DNA binding repressor protein of *E.coli* osmoregulatory system	$2x10^{-4}e$	EMBL/ Swissprot

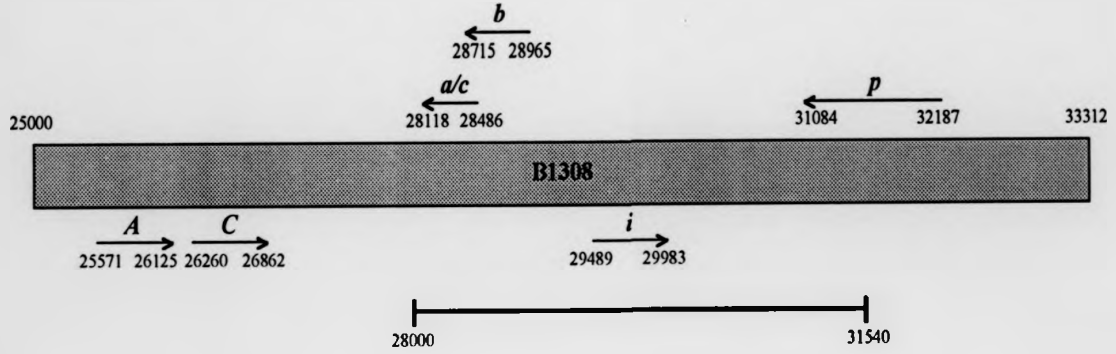


Fig.47. **A map of the region the distal region of cosmid B1308 showing putative coding regions**
The map show the positions of the putative coding regions *p*, *i*, *a*,and *b* found in the region of B1308 which complements PyramidII and of two coding regions upstream of *i* and the complementing region of *M. leprae* DNA, which are believed to be involved in osmoregulation (Table.19. Fig.46). The complementing region of DNA is represented by the black bar.

Forward strand	Reverse strand
	4406-4672 transport protein
5087-6100 *pab*B antigen B	
7549-7812 *M.leprae* gene sequence	
7761-8942 *acd* acyl coA dehydrogenase	
9197-9516 *pur*6 phosphoribosylaminoimidazole carboxylase	
9540-10907 *pur*K phosphoribosylaminodazole carboxylase (ATPase)	
10941-11633 *M.leprae* gene sequence	
11589-12238 *M.leprae* gene sequence	
12802-13134 *M.leprae* gene sequence	13089-14774 *pcc* propionyl coA carboxylase
	14796-15662 *M.leprae* specific
	15860-16753 *thi*R Thiosulfate sulfotransferase
19208-19621 *rps*B sigma factor B	17231-19084 biotin carboxyl carrier protein- met
19950-20228 *M.leprae* gene sequence	
21497-22045 *lat*A Lysine-6-amino transferase	23999-24283 *M.leprae* gene sequence
	25191-25403 aldehyde dehydrogenase
	25571-26125 probable dehydrogenase *A*
	26115-26858 *M.leprae* gene sequence *C*
28118-28486 *M.leprae* gene sequence *a/c*	
28715-28965 *M.leprae* gene sequence *b*	
31084-32187 *M.leprae* gene sequence *p*	29489-29983 *M. leprae* gene sequence *i*

Fig. 48. **A list of open reading frames found in cosmid B1308 and their coordinates on the cosmid as defined by MycBD** (opposite orientation to GenBank)
The numbers given show the position of each ORF identified in relation to the length of the cosmid. ORFs on the left are on the forward strand; ORFs on the right are on the reverse strand. ORFs putatively identified by homolgy with known genes are named; genes without homologies to sequences in the database are shown as *M. leprae* gene sequences.

that of B1308 in the region of the 0.9kb *PstI*/*Eco*RV fragment (Fig.46a). This confirmed the position of this fragment and the other mapping of the complementing plasmids generated from cosmid 57.

The 3.5kb complementing region has been shown to contain 3 entire open reading frames a/c (70aa) b(111aa) and i(142aa) and part of a fourth at the most distal end, p (303) in MycDB; while in Genbank (1995) the region was predicted to contain the complete coding region i and part of p at the distal end (Fig.47). The region was reanalysed for possible coding regions using PC gene and shown to contain the same ORFs as predicted on MycDB and in GenBank.

Blast searches were repeated on the four MycDB predicted ORFs contained within the region, the genes all appeared *M. leprae* specific showing no significant homologies to genes of bacterial or eukaryotic species listed in the EMBL and Swissprot databases (Fig.46b). The results confirmed those of MycDB and GenBank. The complete list of all known and putative coding regions on cosmid B1308 are listed in Fig.48.

3.3.4 PYRAZINAMIDE SENSITIVITY AND HYDROPHOBICITY OF PYRAMIDII:COS57 RECOMBINANT STRAIN PYR1

The pyrazinamide sensitivity of recombinant strain Pyr1 was measured in liquid culture (section 2.28.4). Strain Pyr1 exhibits significantly increased resistance to pyrazinamide when compared with the parental mutant strain PyramidI and with wild type *M. smegmatis* mc^2155 in liquid culture (Fig.49).

The hydrophobicity test was repeated for 200ml of early-stationary phase Pyr1 culture. The strain was found to have a greater hydrophobicity than both the parental PyramidII strain and the wild type *M. smegmatis* strain, with an aqueous layer A_{400} of 0.314 compared to 0.9 and 0.5 for PyramidII and *M. smegmatis* mc^2155 respectively.

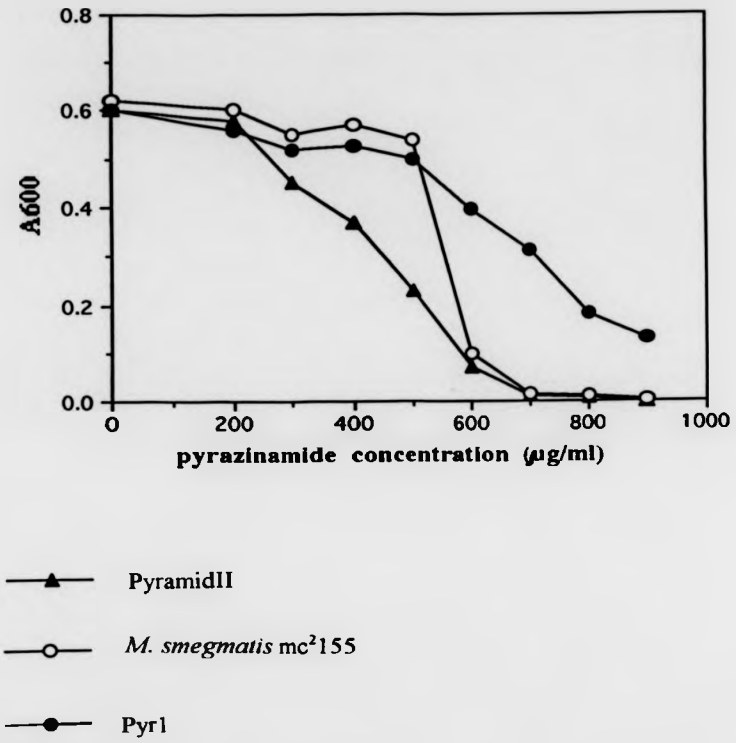


Fig.49. A graph of strain Pyr1 which exhibits increased resistance to pyrazinamide in liqiud culture, when compared with the parental strain.

An equal number of cells were added to a set of 5ml Lemco(pH5.2) containing increasing concentrations (200 µg/ml-900µg/ml) of pyrazinamide. Control cultures without pyrazianmide were also set up. The A_{600} was taken when the control cultures reached, mid log-phase growth $0.6A_{600}$ Pyr1 was found to exhibit an increased resistance to pyrazinamide over and above the levels attained by *M. smegmatis* mc²155 wild type cells.

The levels of resistance to pyrazinamide and increased hydrophobicity exhibited over and above those found in the wild type may be due to increased copy number or to upregulation of the gene or genes involved by a mycobacterial promoter found in the length of pYUB18 vector cloned into pMV206 beside the *M. leprae* genes, or both.

3.3.5 BIOCHEMISTRY

The free lipids of strains Pyr1 and another recombinant strain of PyramidII containing the 2.2kb *Bam*HI fragment of cosmid57, in pMV206 were analysed. The phospholipids of the two strains were analysed with the 2D-TLC system F (data not shown) . The Pyr1 strain does not produce the unknown phospholipid in the same quantities as were found in the PyramidII strain containing cosmid 57. This implies that the phospholipid is not encoded by the *M. leprae* genes contained in the recombinant plasmid of the Pyr1 strain. The genes encoding or controlling production of the phospholipid may be located elsewhere on cosmid 57.

3.3.6 SUMMARY

In summary a 3.5kb region of *M. leprae* DNA was mapped to cosmid B1308 and shown to contain four *M. leprae* putative coding regions (MycDB), confers increased pyrazinamide resistance and hydrophobicity in the pyrazinamid sensitive, PyramidII strain.

CHAPTER 4

DISCUSSION

4.1 THE ISOLATION OF *M. SMEGMATIS* MC²155 WITH MUTANT PHENOTYPES

4.1.1 PSEUDOLYSOGENY AND THE ISOLATION OF *M. SMEGMATIS* MC²155 PHAGE RESISTANT MUTANTS

The attempt to isolate spontaneous mycobacteriophage resistant mutants failed due to the prevalence of pseudolysogeny in *M. smegmatis* mc²155; what appeared to be phage resistant colonies on the overinfection plates were in fact pseudolysogenic strains (section 3.1). A number of facts were indicative that the "resistant" strains were pseudolysogenic: the strains released mycobacteriophages on spotting of cultures onto a wild type lawn and formed spontaneous clear plaques and background plaques on their own uninfected lawns. Approximately, $1\text{-}2\text{x}10^2$ phage "resistant" colonies were formed on the overinfection plates for each phage type plated with $1\text{-}3\text{x}10^8$ *M. smegmatis* mc² 155 cells, a frequency of pseudolysogeny of $0.5\text{-}1\text{x}10^{-6}$ (section 3.1).

The pseudolysogeny observed in *M. smegmatis* mc²155 is believed to be of the carrier type found in other mycobacteria (Grange and Redmond, 1978) in which the prophage does not replicate or integrate into the genome and is passed into one daughter cell of the next generation. The prophage can be induced into lytic growth thereby reinfecting daughter cells which do not retain the prophage and maintaining the pseudolysogenic state. A number of factors lead to this conclusion; some colonies (derived from single cells) within a strain were positive and some negative when tested for pseudolysogeny, the level of "resistant" colonies within a given strain was reduced by serial subculture and the appearance of background plaques within the bacterial lawn (mottled effect). The mottled lawns containing background plaques suggested that some phage-infected cells within the lawn were undergoing a lytic cycle, infecting others and preventing their reinfection to allow a masking layer of

pseudolysogenic cells to overgrow plaques within the lawn. The mottled backgrounds often occurred in strains that had been serially subcultured.

Pseudolysogenic colonies were smooth and mucoid, a phenomenon noted in other pseudolysogenic species, for example *M. kansasii* mucoid strains all released phage without prior infection (Grange and Bird, 1977). This change in morphology may be mediated by the chemical alteration of the phage receptor site, presumably undertaken to prevent further phage infection e.g. the simple LOSs of a D29 resistant strain of *M. smegmatis* mc^2155 were found to be *O*-methylated and *O*-acylated (Besra *et al.*, 1994b).

The lytic cycle was induced by exposure to UV in most pseudolysogenic strains, improving the accuracy of the pseudolysogenic test in most cases and reducing the mucoid nature of the "resistant" strains. Only strains pseudolysogenic for D29 appeared unaffected by exposure to UV; that is the lytic cycle was not readily induced and the strains retained a mucoid appearance. It appears that the D29 prophage is more stable within *M. smegmatis* than the other phages tested.

DNAIII8 and 33D appeared to confer superinfection immunity to each other, a phenomenon noted with other mycobacteriophage e.g L5 and D29 (Donnelly-Wu *et al.*, 1993); this effect was not exhibited by any of the other mycobacteriophage used in these experiments.

The number of pfus ($1x10^9$/plate) used to cause confluent lysis was comparable with numbers ($1x10^8$/plate) used to select L5 gp71 transformants (Donnelly-Wu *et al.*, 1993). The numbers of pfus used to produce confluent lysis were reduced (10^6) in an attempt to lower the levels of pseudolysogeny in later experiments when screening NTG-treated *M. smegmatis* mc^2155 ($1\text{-}3x10^8$) cells; however, this approach was unsuccessful and it may have been preferable to have reduced the numbers of *M. smegmatis* mc^2155 cells plated rather than the number of

pfus.

M. smegmatis infected with L1 has been described as having an intermediate host/phage relationship between pseudolysogeny and true lysogeny; although phage L1 establishes a lysogenic relationship with the host, this broke down at high frequencies and led to lysis or curing of the host bacterium. Cured bacteria were then susceptible to reinfection (Tokunaga and Sellers, 1970). *M. smegmatis* mc^2155 has been shown from these experiments to establish a similar relationship with D29, 33D, D4 and DNAIII8. How this relationship is maintained is a matter of speculation and some light may be shed on the situation by comparison to the biology of L5 where lysogeny is host induced. For example, if the phage produced residual amounts of a repressor protein which switches off the lytic cycle and the levels of this protein are controlled by a host protein as predicted for L5 (Nesbit *et al.*, 1995), an altered host environment could lead to a reduction in the host degradative enzyme and an increase in the levels of the phage inhibitory protein, inhibiting the lytic cycle; if the phages have no integrative apparatus i.e. *int* or *att*P then they would be unable to form the more stably inherited lysogens (Hatfull and Sarkis, 1993).

A total of sixty two strains produced on the overinfection plates were screened for resistance to each phage. On the basis that spontaneous L5 resistant strains were observed at a frequency of $1x10^{-8}$ on confluent lysis plates when plated with 10^8 *M. smegmatis* mc^2155 cfus (Donnelly-Wu *et al.*, 1993), 1-3 in 100 would have been expected to be spontaneously resistant. It was therefore conceivable that a spontaneously resistant mutant would have been identified by screening 62 strains, although these numbers were low. Pseudolysogeny would mask a strain resistant to phage infection as the result of a mutation which prevented replication. It is unlikely, however that it could mask a mutant resistant through an altered receptor as the phage would have been unable to infect the strain to cause pseudolysogeny. However, it is possible that a phage resistant strain caused by a receptor mutation would not have been

detected if it was a mixed culture of pseudolysogenic and phage resistance as resistance to infection may have been attributed to a positive pseudolysogeny test.

Phage D4 overinfection produced four colonies which were rough not mucoid and non pseudolysogenic, but were infectable on retesting; it is possible that these strains were genuine phage resistant mutants that were unstable. This also applies to the two 33D and one D29 non pseudolysogenic strains isolated, which were infectable on retesting.

4.1.2 MUTAGENESIS

The NTG mutagenesis of *M. smegmatis* mc^2155 resulted in the creation of 0.1-0.2% of stable auxotrophic mutants, without INH selection. These levels are comparable with those found by other groups using NTG to mutagenise *M. smegmatis* and *M. phlei*, (0.46% and 0.11%, respectively) (Subramanyan *et al.*, 1989; Konickova-Radochova *et al.*, 1970). The use of INH enrichment increased the levels to 0.4%, again comparable to the levels found by others using this selection on *M. smegmatis*, 0.37% (Hinshelwood and Stoker, 1992b) and 0.1-0.9% (Holland and Ratledge, 1971). The level of auxotrophy achieved by INH enrichment is variable as shown by the results of Holland and Ratledge (1971). In *M. phlei* and in *M. smegmatis*, this was shown to be due to the percentage survival following the NTG treatment, a significant increase in INH enrichment was observed at survival levels below 5%, the maximum effect was seen at less than 1% survival auxotrophy (Konickova *et al.*, 1978; Holland and Ratledge, 1971). The percentage survival was 8% in the INH-enriched NTG-treated *M. smegmatis* cells in these experiments. This may account for the fact that 3 of the 4 auxotrophs isolated following INH enrichment were leaky. The colonies formed following mutagenesis were initially mucoid, a phenomenon also noted by Holland and Ratledge (1971).

Both the INH and non-INH NTG-treated sets of *M .smegmatis* mc^2155 cells produced mutants, with a variety of phenotypes (Mitomycin C resistance, melanin mutants, quinolone

resistance, penicillin G and pyrazinamide sensitivity) implying that NTG is an appropriate mutagen for the creation of mutant strains of *Mycobacterium*, corresponding with the findings of other groups (Konickova-Radochova *et al.*, 1970).

The levels of stable auxotrophs formed were also comparable with the levels formed in transposon mutagenesis libraries of *M. smegmatis* mc^2155 (Guilhot *et al.*, 1994) and *M. bovis* BCG, (McAdam *et al.*, 1995). Transposon mutagenesis has the advantages of being able to directly select mutants with antibiotic selection markers e.g. kanamycin, before screening for altered phenotypes, and of stably maintaining the mutations by selection on kanamycin. Transposon mutagenesis is also less likely to cause multiple mutations and may have been used if the technology were available at the initiation of this project. However NTG has the advantage of creating point mutations less likely to form lethal mutations which may have involved cell wall biosynthesis.

One of the main observations of the NTG procedure was the apparently high levels of reversion, within the auxotrophic population. Levels of auxotrophy within the INH enriched group fell from 24% on the initial plating to 2.2% on the first serial transfer to a final value of 0.4% following individual testing. The first set of values for auxotrophs from Bank1 may be slightly increased by the decision to test all strains with weak looking growth, but this may also have been counter balanced by any accidental elimination of auxotrophic strains due to carry over of media to the MM test plates, allowing their growth. Again this phenomenon has been observed by others using NTG as a mutagen and the reasons for it are unknown. One proposal is that the strain possesses a mutational hotspot which is particularly unstable, resulting in high levels of reversion as was observed with glycine/serine mutations of *M. phlei* with a number of mutagens (Konickova *et al.*, 1970). Alternatively, point mutations may be more readily resolved in *Mycobacterium* in comparison with other genera, for example *E. coli* which formed 40% stable auxotrophs following NTG treatment (Adelberg *et al.*, 1965). The

high levels of reversion were also noted when screening for other phenotypes such as antibiotic resistance and phage resistance.

4.1.2.1 Conditions for the survival of cell wall mutants

The penicillin sensitive *M. smegmatis* mycolic acid mutant described by Kundu *et al.*, (1991) was isolated at 28°C and is temperature sensitive (Dr. P. Chakrabaty personal communication). This may be because the production of cell wall components such as mycolic acids are reduced at 27°C (Takayama *et al.*,1978) and the levels of phospholipids increase (Taneja *et al.*, 1978) forming a barrier to the external environment. This natural adaptation to low temperatures may therefore increase the chances of survival of a cell wall mutant by increasing its levels of phospholipid to create a barrier to the environment that the mutant would not possess at 37°C. For these reasons the NTG-treated *M. smegmatis* cells were left to recover at 28°C and at 37°C in an attempt to select for temperature sensitive mutants and to enhance the survival of cell wall mutants.

The initial plating of NTG-treated cells which had been INH-enriched appeared to successfully select for temperature sensitive mutants. Twenty four percent of INH-enriched cells were only able to grow at 28°C on the initial plating of cells onto MM+AA, indicating that the INH treatment enriched for temperature sensitive strains better able to survive at 28°C, even though no stable temperature sensitive mutants were isolated. As the NTG-treated *M. smegmatis* strains were grown at 37°C when treated with INH and INH is only bactericidal to growing strains this was as predicited.

The INH-enriched NTG-treated *M. smegmatis* strains did not display any chromatogenic mutants, as opposed to the 0.3% found in SetA, the NTG-treated *M. smegmatis* cells allowed to recover at 28°C; chromatogenic mutants may have grown rapidly enough at 37°C to have been selected out by the INH treatment. There did however, seem to be a slight variation in

the types of mutant phenotypes selected for by recovery at 28°C alone and subsequent INH treatment at 37°C. The initial antibiotic sensitive mutants were selected from SetA which had not been INH-enriched; on the basis of these results a second mutagenesis was carried out which was not INH-enriched in order to select for antibiotic sensitivity. Although selecting for mutants which may have been temperature sensitive as result of an altered cell wall by recovery at 28°C, using the INH selection may have selected against an altered cell wall phenotype merely by the required incubation at 37°C. The INH selection may also have selected out cell membrane transport protein mutations which if involved in active transport are more likely to be able to overcome a mutation at 37°C than at 28°C, when their uptake is reduced anyway. If this is the case then they would have been selected out by INH at 37°C due to their faster growth. It is possible that simple selection by recovery at 28°C may have been the most appropriate way to isolate cell envelope mutants of *M. smegmatis* mc^2155.

4.1.3 SCREENING FOR MUTANT PHENOTYPES

4.1.3.1.Mycobacteriophage resistance

Bank1 and an additional 1,000 strains from SetA were screened for resistance to a number of mycobacteriophage, but no phage resistant colonies were isolated. On initial screening for phage resistance 2.8% and 2.4% respectively appeared resistant, but on subculture and reinfection these were all found to be sensitive. These levels of initial resistance were comparable to those initially found for auxotrophy in the Bank1 strains and imply that the strains could have been genuinely phage resistant but unstable. It is also possible that the strains appeared resistant through an artifact such as low phage titre in the sample added to the lawn of the test strain. If these initial strains were genuinely resistant then the seven strains from Bank1 which were initially resistant to all phage types tested were likely to have had mutations effecting the internal mechanisms of phage production, as phage D4 and D29 are known to have different phage receptors. An example of such an internal mutation could be an alteration in the host RNA polymerase which allowed host protein production but not

adaptation by the mycobacteriophage for their use.

Screening of the NTG-treated *M. smegmatis* strains of Bank1 and the additional 1,000 strains from SetA had the advantage that the strains had not previously been exposed to mycobacteriophage when infected and were extremely unlikely to be pseudolysogenic.

The large scale screening of NTG-treated *M. smegmatis* strains for resistance to mycobacteriophage was carried out by plating out large numbers of cells ($1x10^8$) onto agar spread with 10^6 pfu/ml, which resulted in confluent lysis and the formation of 25-35 "resistant" strains, a frequency of $1x10^{-7}$. These strains were all pseudolysogenic except for those formed on plates spread with D4. The D4 colonies could have been unstably resistant or unstably pseudolysogenic strains. These experiments implied that pseudolysogeny is dependent on a host mutation as lowering the number of pfus used did not prevent its occurrence.

The inability to isolate stable mycobacteriophage resistant strains may have been dictated by a number of factors. The specificity of the mycobacteriophage receptors to oligosaccharide units of cell wall components e.g. D4 and GPLs (Dhariwal *et al.*,1986) and the large number of genes encoding enzymes involved in the formation of those structures may make it more difficult to mutate the specific enzyme involved in the addition of the specific receptor sugar. Mutations further back in the pathway of these cell wall structures are also more likely to prove lethal to the host. The mutations required for resistance to mycobacteriophage through inhibition of the lytic cycle may also have proved lethal to the host e.g. an RNA polymerase mutation.

4.1.3.2 Temperature sensitive mutations

Again a number of temperature sensitive (ts) mutations were initially isolated from Bank1 at 1%, levels slightly lower than those of the initial auxotrophic mutants found. On subculture

0.15% remained temperature sensitive; however the temperature sensitivity was lost on further subculture from frozen stocks. Temperature sensitive mutations were noted by Holland and Ratledge, 1971 using NTG mutagenesis but were apparently not subcultured at all and so their degree of stability is unknown. The three ts mutants lost from frozen culture may have had cell wall mutations which could not survive being frozen and recultured.

4.1.3.3 Antibiotic resistance and sensitivity mutations

The MICs of the antibiotics to be determined were tested at 28°C and the MICs for minocycline, and ethionamide were found to be ten fold higher than those reported by other groups, at 37°C (Yamada *et al.*, 1992; Banerjee *et al.*, 1994).

Minocycline is the most effective tetracycline against mycobacteria and this is thought to be due to its lipophilicity and consequent ability to penetrate the lipid permeability barrier of the mycobacterial cell wall. It is thought that the decrease in temperature, causing a decrease in the fluidity of the cell wall, has reduced the ability of minocycline to transverse the cell wall. Mycolic acid biosynthesis is believed to be the site of action of ethionamide and the reduced production of mycolates at low temperatures may have rendered ethionamide less effective, as the cell's ability to survive without mycolates is improved at these low temperatures.

The MICs of the other antibiotics used appeared unaffected by incubation at 28°C. In some cases MICs were not available for *M. smegmatis* and so were compared to MICs for *M. bovis* BCG and *M. tuberculosis* which are generally ten fold lower (Public Health Laboratories, Dulwich, personal communication).

The concentrations used in order to isolate antibiotic resistant and sensitive mutants were generally close to the MICs in an attempt to isolate mutants with lower levels of resistance and sensitivity, where mutants are perhaps less likely to be in the primary target site of the

drug and perhaps more likely to be cell envelope associated e.g. low level INH resistance mutants lead to the isolation of the *inh*A gene (Banerjee *et al.*, 1994) rather than the higher level resistance observed with mutations of the *kat*G gene (Zhang *et al.*, 1993).

4.1.3.3.1 Antibiotic resistance

Of the seven antibiotics tested only NTG-treated *M. smegmatis* strains resistant to the quinolones ofloxacin (12) and ciprofloxacin (4) were isolated. The initial levels of possible antibiotic mutants isolated from Bank1 for amikacin (0.2%), minocycline, (0.6%), ethambutol (1.45%) and ethionamide (1.95%) were lower than those seen for auxotrophic mutants (2.2%) and were all found to be unstable on further subculture. The initial levels of cycloserine resistance were very high at 7.8% but again resistance was lost on subculture and retesting.

Large scale screening for resistance to minocycline, ethionamide, ethambutol and amikacin only yielded colonies on the ethionamide and ethambutol plates at frequencies of $1.5x10^{-7}$ and $0.6x10^{-9}$ respectively, which are lower than the spontaneous levels of INH resistance for *M. smegmatis* mc^2155 (Banerjee *et al.*, 1994). These results implied that the NTG-treated *M. smegmatis* cells, grown from frozen culture, had not retained their mutations at a level much above wild type on reculture at 28°C.

The higher numbers of quinolone resistant mutants (0.75%) isolated also indicate that it is easier to form mutations in some loci than others; the mutations in the quinolone resistant strains are more likely to be found in the *gyrA* and *gyrB* genes.

The isolation of stable antibiotic resistant mutants appeared more difficult even from initial levels of possible mutants, this may have been caused to some extent by the selection concentration being too high, particularly in the case of minocycline and amikacin. Resistance to all of these drugs particularly ethambutol and ethionamide could have been caused by a cell

wall mutation; it is possible that the essential nature of some of the cell wall components may lead to a selection pressure for revertants, resulting in the isolation of unstable mutants. A mutation in less essential genes, possibly the *gyr*A gene, may not adversely affect the strain resulting in the maintenance of the mutation.

4.1.3.3.2 Antibiotic sensitivity screening

The screening for increased antibiotic sensitivity was generally more successful than the screening for antibiotic resistance. A pyrazinamide sensitive strain (0.05%), five penicillin G sensitive strains (0.5%) and eight other strains with poor growth were isolated, the majority from the second mutagenesis procedure. Screening for sensitivity tended to throw up other types of mutants with slow growth as well as strains genuinely sensitive to the test antibiotic e.g. auxotrophic mutants AM2 and 570 were isolated from screens for pyrazinamide and penicillin G respectively. The antibiotic sensitive strains seemed to have survived better without INH enrichment as discussed above. Again the MICs of *M. smegmatis* mc^2155 wild type differed at 37°C and 28°C by decreasing at 37°C, presumably as the result of an increase in the permeability of the strain at this higher temperature.

4.2 CHARACTERISATION OF MUTANT STRAINS OF *M. SMEGMATIS*

4.2.1 AUXOTROPHIC MUTANTS

One stable proline mutant (AM1) and one mutant auxotrophic for aspartate, phenylalanine, serine and valine were isolated from Bank1.

Proline biosynthesis involves the conversion of glutamic acid to glutamate semialdehyde dehydrogenase which spontaneously converts to Δ-1-pyrroline-5-carboxylate, which is converted to proline by pyrroline-5-carboxylase. The proline mutant could have altered glutamate dehydrogenase or pyrroline-5-carboxylase enzymes. Which enzyme is mutated could be determined by adding only glutamic acid semialdehyde to MM. If the strain did not

grow on glutamic acid semialdehyde it would indicate a pyrroline-5-carboxylase reductase mutation, whereas if the strain grew on the glutamic acid semialdehyde this would indicate that the mutation was in the glutamate kinase or hydrogenase enzyme.

Proline mutants of mycobacteria had been previously isolated (Subramanyan *et al.*, 1989) and the *M. leprae* genes encoding glutamate kinase (*arg*B) and dehydrogenase (*gad*C/D) and pyrroline-5-carboxylate reductase (*pro*C) have been identified (GenBank., 1995).

The second auxotrophic mutant investigated, AM2, was able to grow on single amino acid plates containing either aspartate, arginine, phenylalanine, serine or valine. The exact nature of this mutation is not obvious; there may be an alteration in the transaminase enzyme glutamate dehydrogenase, or in the glutamine synthetase and glutamate synthase. However, the synthesis of other amino acids, tyrosine and L-alanine also require transamination. *E. coli* posseses several glutamate dehydrogenase enzymes that are specific for linear, branched and aromatic amino acid biosynthesis. *M. smegmatis* may also possess several glutamate dehydrogenase enzymes , some of which may be specific to the formation of aspartate, phenylalanine, serine and valine. Although it is not specifically transaminated, arginine biosynthesis requires α-oxoglutarate, the by product of transamination from glutamate; reduced transamination may therefore result in low levels of α-oxoglutarate and hence arginine.

The mutant would not grow on "drop out" plates without methionine, tryptophan, proline, asparagine and glycine. The biosynthesis of methionine and asparagine, glycine and tryptophan requires aspartate and serine respectively. It therefore also possible that AM2 is a double mutant in serine and aspartate production.

4.2.2 PIGMENTATION MUTANTS

Two mutants with altered pigmentation, one orange and one white were isolated from Bank1 and their pigmentation analysed, along with wild type *M. smegmatis* mc^2155.

The adsorption spectra analysis of the pigments of *M. smegmatis* mc^2155 wild type revealed the presence of two pigments; small amounts of a β-carotene like xanthophyll 4-ketorulene, and the main pigment theophytin, a chlorophyll degradation product which absorbs at 409nm. The white mutant (17) contained no pigments while the orange mutant (96) contained more 4-ketorulene. *M. smegmatis* is known to contain 4-ketorulene, but not the leprotene or α-carotene found in *M. leprae* and *M. aurum* or the β-carotene found in *M. phlei* (David, 1984). The formation of the 4-ketorulene is believed to occur via the route phytoene→phytofluene→carotene→neurosporene→4-ketorulene (David, 1984). In most other species neurosporene is converted to lycopene which undergoes cyclisation to form first the mono and then the dicyclic leprotene, α and β carotenes. The xanthophylls in other species are also generally formed using lycopene as a precursor, however *M. smegmatis* does not contain lycopene.

Carotenoid mutants, one white and one red, have been used in *M. aurum* to isolate the leprotene and α-carotene coding region (Houssaini-Iraqui *et al.*,1992 and 1993a). The genes encoding the biosynthesis of carotenes in *M. aurum* are clustered in a 10.83kb operon, while the genes to convert lycopene into xanthophylls appeared to be located elsewhere in the *M. aurum* genome, as when the gene cluster was placed in *M. smegmatis* only small amounts of xanthophyll accumulated (Houssaini-Iraqui *et al.*, 1993b). The white (17) and orange (96) mutants could be used to investigate the unusual production of the xanthophyll 4-ketorulene and the theophytin pigment. The pigment encoding genes are probably regulated from a similar area or found in an operon, as the white mutant contains neither pigment and the levels of both pigment are increased in the orange mutant.

Two *M. smegmatis* strains were isolated with an inability to synthesis melanin on tyrosine containing media, one of which was leaky. The melanin mutant has been complemented with a *M. smegmatis* cosmid library and the complementing genes cloned; it is hoped that melanin production could be used as a selectable marker in mycobacteria that do not produce melanin and in mutant strains of those that do.

4.2.3 MITOMYCIN C MUTANTS

Relatively large numbers of mutants (2.4%) were found on screening for sensitivity to mitomycin C which interferes with DNA recombination. These strains were deficient in their recombinational and repair mechanisms; the fact that there are large numbers of enzymes involved in these processes would explain the higher frequencies of mutants isolated from the mitomycin C screen. This screen was carried out in the search for a *rec*A mutant. The twelve stable strains were sent to Dr. E. Davis, NIMR for further analysis.

4.2.4 QUINOLONE RESISTANCE

The 12 ofloxacin and 3 ciprofloxacin resistance mutants isolated could contain mutations in their *gyrA* and/or *gyrB* genes, which code for the A and B subunits of topoisomerase DNA II. Point mutations have been identified in the *gyrA* genes of ciprofloxacin resistant mutants of *M. tuberculosis* and *M. bovis* (Takiff *et al.*, 1994). None were observed in the *gyrB* gene which has been shown to result in low level resistance in other species. Mutations in the *gyrA* gene are thought to cause high level resistance as the A subunit of the DNA topoisomerase II is the binding site of the quinolones.

The ten ofloxacin resistant strains were cross-resistant to ciprofloxacin and must have mutations in a site of action of both drugs i.e. *gyrA* or *gyrB* genes, or a transport protein. The *M. tuberculosis gyrA* and *gyrB* sequences could be used to design PCR primers to the *gyrA* and *gyrB* genes of the resistant *M. smegmatis* strains; PCR products could be screened for

single strand conformational polymorphisms which would indicate defective *gyrA* or *gyrB* genes. Any strains not defective in these genes could have altered permeability caused by a change in the cell wall structure or transport proteins across the cell wall or the cell membrane or other novel sites of action of the drugs, and could be investigated further. Three ciprofloxacin mutants and 2 ofloxacin mutants were not cross-resistant, which could imply that the mode of transport of the two drugs varies, possibly differing uptake proteins in the cell membrane

4.2.5 PENICILLIN G

Five stable penicillin G sensitive strains were isolated, four of which showed low level sensitivity (100μg/ml lower than wild-type), and one which showed high level sensitivity, PenG 650, (300μg/ml less) in comparison with the wild type (500μg/ml at 37°). It was not possible to compare the levels of penicillin G sensitivity of these strains with the mycolic acid mutant (Kundu *et al.*, 1991) as the degree of penicillin sensitivity of the strain was not published.

High levels of resistance to penicillin and other β-lactams may result from factors including low permeability to the drugs, β-lactamase activity and the affinity of penicillin binding proteins (PBPs) for the drug (Jarlier *et al.*, 1991; Fattorini *et al.*, 1992). The degree of the effect of each factor on penicillin resistance is variable, although altered permeability and lower affinity of PBPs have been shown to produce up to 512-fold greater resistance to amoxicillin in a *M. fortuitum* mutant with normal β-lactamase activity (Fattorini *et al.*, 1992). Increased sensitivity could have resulted from a mutation which caused increased permeability to the drug, reduced β-lactamase activity or increased affinity of the PBPs for the drug.

Mutant PenG650 was chosen for further analysis as it was the most sensitive to penicillin G, and since high levels of resistance were noted with permeability changes in *M. fortuitum* it was

possible that they could be the cause of high level sensitivity as well. PenG650 was analysed for any gross changes in the cell envelope lipids on the basis that the *M. smegmatis* mycolic acid mutant was penicillin sensitive and it may therefore contain cell wall alterations; whilst remaining aware it was equally possible that the increased sensitivity could be caused by decreased β-lactamase activity or an increased affinity of PBPs for penicillin G.

A change in permeability could have been caused by an alteration in the cell wall structure, but no gross changes were noted in any of the cell wall components studied; α α' and epoxy mycolates were all present, as were the glycolipids, GPLs, LOSs, cord factor and phospholipids. The increased permeability could still have been caused by a more subtle change in a cell wall component; for example the amounts of each mycolate type present may vary - this could be determined by HPLC analysis of the mycolates which is more quantitative. Increased permeability could also be caused by an upregulation of a transport protein or by an increase in porin production.

The five penicillin G mutants require further analysis to determine the exact nature of their increased sensitivity which could be identified by assessing their β-lactamase activity and PBPs affinities for penicillin G, as well as by assaying for a change in permeability.

4.2.6 PYRAZINAMIDE SENSITIVE MUTANTS

Two pyrazinamide-sensitive mutants were isolated from BankI; PyramidII (discussed below) and AM2 (415). As discussed above, the screening for antibiotic sensitive mutants resulted in the isolation of other slow growing mutants e.g. auxotrophs. The possible transaminase mutant AM2 was isolated on the screen for pyrazinamide sensitive mutants and appeared genuinely sensitive to pyrazinamide at 28°C but not at 37°C. It is possible that the increased sensitivity to pyrazinamide is a secondary effect of a lack of transaminase enzyme, which may result in the reduction of substrate for the pyrazinamidase/nicotinamidase enzyme; this would

reduce any competition for the enzyme leaving it free to hydrolyse pyrazinamide, producing more pyrazinoic acid and apparently increasing the sensitivity of the mutant to pyrazinamide. This effect is probably recognised to a greater degree at 28°C where the active uptake of the required amino acids will be lower resulting in an internal lack of substrate for the pyrazinamidase.

4.3 CHARACTERISTICS OF PYRAMID II

4.3.1 PYRAZINAMIDE SENSITIVITY ON SOLID AND IN LIQUID CULTURE

PyramidII exhibits increased sensitivity to pyrazinamide on solid and in liquid media, when compared with the wild type *M. smegmatis* strain at 28°C and at 37°C. The actual MIC for PyramidII varied under different conditions but was consistently at least 100μg/ml lower than the wild type MIC, a 20% increase in sensitivity. For example on solid media at 28° PyramidII was unable to grow on a 500μg/ml concentration, 100μg/ml lower than the wild type MIC 600μg/ml, whereas at 37°C PyramidII was unable to grow on 400μg/ml while the MIC for wild type was 500μg/ml. The MIC for liquid culture increased with the growth stage of the cultures as pyrazinamide is bacteriostatic not bactericidal.

The level of sensitivity of PyramidII and the wild type to pyrazinamide appeared to be higher in mid log-phase growth as opposed to early stationary-phase growth and the differential between them was higher at early stationary-phase growth. Both strains would be expected to exhibit higher levels of sensitivity during mid log-phase compared to stationary phase growth as the permeability of the cell envelope would be predicted to decrease towards the end of stationary growth; However, the fact that the difference between the two strains increases from a 7-10 fold differential in the numbers of cfu/ml indicates that pyrazinamide is able to maintain a greater effect on early stationary phase PyramidII cells than on early stationary phase wild type cells. This may indicate that the mutant does not present as strong a permeability barrier as the wild type cells at stationary phase.

The exact mode of action of pyrazinamide is unknown, pyrazinamide is known to be a pro-drug converted by pyrazinamidase to pyrazinoic acid, which is believed to be the active form. However, as *M. smegmatis* is ostensibly resistant to pyrazinamide despite the fact that it produces pyrazinamidase, unlike the resistant *M. bovis* which does not produce pyrazinamidase (Konno *et al.*, 1967) it must employ other mechanisms of resistance to pyrazinamide. Possible resistance mechanisms include reduced permeability caused by the cell wall structure of *M. smegmatis*, increased efflux of pyrazinamide or pyrazinoic acid, or a mechanism capable of degrading pyrazinoic acid. PyramidII may have a mutation which causes increased permeability, this may be an alteration in a cell wall structure, an upregulated transport protein or an increase in porin production. The increased sensitivity of PyramidII to pyrazinamide could also be a result of down regulated efflux or pyrazinoic acid control system or upregulation of the pyrazinamidase enzyme.

4.3.2 IDENTITY, COLONY MORPHOLOGY AND GROWTH

The identity of PyramidII as a mycobacterium was confirmed by the results of the acid-fast test and as *M. smegmatis* mc^2155 by the results of the API-ZYM test.

PyramidII colonies were smooth and filamentous, as opposed to the wild type which are uneven and invaginated, which was also reported for the mycolic acid mutant of *M. smegmatis* (Kundu *et al.*,1991). Changes in morphology have been associated with alterations in cell wall components e.g. rough morphology mutants of *M. kansasii* and *M. avium* have lost the oligosaccharide components of their LOSs and GPLs respectively, resulting in a loss of smooth morphology (Hunter *et al.*, 1985; Belisle and Brennan, 1989). These results would imply that smooth morphology is associated with the presence of oligosaccharides on the cell surface, although the *M. avium* SmT smooth variant has recently been shown to contain a 66kDa cytosolic protein not present in the SmD or the rough variants (Prinzis *et al.*, 1994). It is possible that the PyramidII strain is upregulating a cytosolic protein that is causing a

morphological change or that an additional oligosaccharide has been exposed or expressed on the cell surface which was too small to have been detected by the biochemical analysis.

PyramidII formed an even suspension in liquid culture; the reduction in clumping may have been due to a slight alteration in the cell wall surface, preventing adherence to neighbouring cells. The slightly variable acid-fast stain may also have been the result of cell wall alteration, although an internal mutation may have had a similar effect. The slight variation in growth phase of the cells in comparison with a wild type culture may also have created this effect.

PyramidII was able to grow at 28°C and 37°C and exhibited an additional 8hr lag-phase in comparison to wild type growth, when grown at 37°C. This implied that the mutation was in a function that is required for the early growth of the strain to the point at which log-phase growth can be initiated. The log-phase growth rate appears identical to the wild type *M. smegmatis* mc^2155 strain implying that the mutation is no longer inhibitory to the growth of the strain. The viable cell counts for PyramidII are slightly higher than for the mutant, but this is believed to be caused by the reduced clumping of the strain in comparison with *M. smegmatis* mc^2155.

4.3.3 BIOCHEMISTRY AND HYDROPHOBICITY

The systematic analysis of the cell envelope lipid components of PyramidII by 2D-TLC analysis did not reveal any gross alterations in the mycolates, glycolipid, glycopeptidolipid or phospholipids. HPLC analysis of the arabinogalactan, LAM and LM (Dr.G. Besra, Colorado State University, Colorado) did not reveal any alterations either. These results indicate that there is no gross alteration of a cell envelope component, and suggest that the strain is not harbouring a major cell wall mutation. It is however possible that subtle changes are present, only detectable by techniques such as GCMS, NMR as with the *O*-methylation and *O*-acytylation of the simple LOS in *M. smegmatis* resistant to D29 infection (Besra *et al.*, 1994).

Alterations in the quantities of mycolates formed would not have been detected using these methods, but should be found using HPLC analysis. The alterations in waxes and mycocerosate components were not screened for using the 2D-TLC system and so alterations in these would not be noted.

PyramidII was found to be less hydrophobic than the wild type *M. smegmatis* in late log-phase and early stationary phase, which indicates an alteration in the cell surface. In other species *E. coli* and *Salmonella typhimurium*, rough mutants lacking oligosaccharides have been shown to be more hydrophobic (Rosenburg *et al.*, 1980) using a similar methodology. The reduced hydrophobicity of PyramidII may be caused by the upregulated production of a sugar moiety at the cell surface or by the reduced production of fatty acid components such as mycolates.

4.3.4 SUMMARY OF PYRAMIDII

The exact mutation of PyramidII is unknown. An alteration in its cell surface is suggested by its decreased hydrophobicity and its smooth colony morphology; however, no alterations in the cell wall components which may be responsible have been detected. Other possibilities include a mutation resulting in an increased pyrazinamidase activity, a decreased efflux or a change in an other internal mechanism controlling the composition of the pyrazinoic acid. The PyramidII mutant was chosen for complementation without being categorically defined as a cell envelope mutation as little is known of the resistance mechanisms of *M. smegmatis* or other mycobacteria e.g. *M. leprae* and *M. avium* to pyrazinamide.

4.4 COMPLEMENTATION OF PYRAMID II

4.4.1 TRANSFORMATION WITH PYUB18::*M. LEPRAE* GENOMIC COSMID LIBRARY

PyramidII was successfully transformed with a representative coverage of the *M. leprae* genome in pYUB18 shuttle vector cosmid; the transformants exhibited variable colony

morphology which was attributed to the expression of the *M. leprae* genes contained in the transformants.

Complementing cosmid clones were isolated at a frequency of 6%. This is a relatively high frequency of complementation, which should theroretically be 1-2% (Hinshelwood and Stoker, 1992b). The high frequency of complementation may have been a result of the ability of more than one gene to complement the mutation or due to a higher representation of the complementing cosmid in the pYUB18::*M. leprae* DNA used to transform the strain.

The 11 complementing clones all grew well on pyrazinamide in solid media and exhibited intermediate (4) and higher than wild type (7) resistance in liquid culture. The intermediate degree of complementation in liquid culture could have been caused by a number of factors: a number of different cosmids may be complementing the mutation, conferring different levels of complementation, or if all of the required complementing region is not present the DNA may only be partially complementing the mutation. It is also possible that the recombinant strains with intermediate growth contain cosmids with unstable insert DNA which has been deleted in a proportion of the cells resulting in a mixed culture of complemented and non-complemented cells, e.g. cos38 is unstable in *E. coli*; the recombinant *M.* smegmatis strain exhibits intermediate resistance to pyrazinamide in liquid culture and on solid media displays colonies with both smooth and crinkly morphology types.

When using the liquid culture method, the results were taken when the control was as close to $0.6A_{600}$ as possible, and the slower growth of cosmid containing clones meant that the incubation times required to reach this A_{600} were generally longer.

Three clones which appeared resistant to pyrazinamide on the initial replica screening of the transformants onto pyrazinamide were found to grow very weakly on pyrazimamide in solid

agar while apparently not exhibiting any increased resistance in liquid culture. The bacteriostatic nature of pyrazinamide may have allowed the initial growth of these strains.

4.4.2 CHARACTERISATION OF THE COMPLEMENTING *M. LEPRAE* DNA

The extraction of complementing cosmid DNA from *M. smegmatis* strains proved difficult, despite adapting the cosmid DNA extraction method for mycobacteria by the addition of lipase type IV to the overnight cultures to improve the lysis step. Cosmid DNA was found to be unstable in *E. coli* despite the use of recombination deficient strains and small culture volumes, the cosmid DNA of all the complementing recombinant strains was unstable to some extent. The instability of mycobacterial insert DNA from both plasmid and cosmid vectors in *E. coli* has been noted by other groups and generally occurs in the form of deletions. Instability of recombinant DNA within the homologous *M. smegmatis* and *M. bovis* BCG strains has also been noted (Haeseleer, 1994)).

Restriction analysis of the complementing clones revealed a number of similar bands between cosmids but no clear map could be formed. The insert sizes of five of the cosmids were particularly small (6-9kb) and the insert appeared to have been completely deleted from other cosmids e.g.cos38.

The complementing cosmid cos57 was mapped to cosmid B1308 of the overlapping *M. leprae* cosmid library (Eiglmeier *et al.*, 1993) .This cosmid is found at one end of the four contigs and any of the complementing cosmids containing *M. leprae* DNA which extends towards the end of the contig is likely to be unstable as this region of DNA has so far proved unclonable (Eiglmeier *et al.*, 1993). This could also account for the small predicted insert sizes (6-9kb) of some clones and the complete deletion of others. The inability to form a map may be due to deletions of regions of the *M. leprae* insert within certain cosmids, or it may be due to the fact that different genes are complementing the mutation, or both.

Successful complementation of the pyrazinamide sensitivity of PyramidII with the *M. leprae* DNA indicates that *M. leprae* genes can be expressed in *M. smegmatis* mc^2155.

4.4.3 COMPLEMENTATION WITH COS57

Complemented recombinant strain 57, exhibited increased pyrazinamide resistance, a crinkly morphology and the production of the unknown phospholipid, and was used to narrow down the region of complementing DNA to investigate these phenomena.

The cosmid was shown to map to B1308; open reading frames and their possible functions as predicted in MycDB and in GenBank (GB) 1995, are listed in Fig 48. Using the available information on MycBD an attempt to narrow down the complementing region was made by subcloning *Bam*HI fragments known not to disrupt ORFs, however the larger fragments proved unclonable.

Transformation of PyramidII with a plasmid sublibrary of cos57 DNA in pMV206 resulted in the isolation of 1% of transformants which restored resistance to pyrazinamide at a level over and above that of wild type, on solid and in liquid media. The 3.5kb complementing fragment of *M. leprae* DNA contained within the transformants was mapped to the distal end of B1308 between 28,000-31,500 (1,812-5,312 GB). This region of B1308 maps next to L308 on the contig, away from the unknown end of the contig (Fig.47). This region is predicted to contain three complete ORFs of unknown function; ORF *i* (142aa), ORF *a/c* (70aa) and ORF *b*(111aa) and the terminal end of ORF *p* (303aa) predicted in the MycDB database while in the GenBank database the region is predicted to contain only the terminal end of ORF *p* and the complete ORF*i*.

PC Gene was used to predict potential ORFs in the 3.5kb region; these were found to be in keeping with the predictions put forward by both groups and suggested that the GenBank

predictions had eliminated any ORFs under 300bp. Repeat searches of the EMBL and SWISSPROT databases were carried out using the predicted amino acid sequences of ORFs *i*, *b*, and *a/c*; no significant homologies were found for *b* or *a/c* in either database (Table19).

As ORF*i* is found in all complementing clones it is possible that this is the complementing region. ORF *i* is contained within the 0.8-0.9kb *Eco*RV-*Pst*I fragment subcloned for sequence analysis. This region was subcloned into pMV206 and used to transform PyramidII. The recombinant clone was then tested for pyrazinamide sensitivity, preliminary data suggest that the fragment does not complement PyramidII, however the fragment used only contained a 300bp region upstream of ORF*i* and may not contain the promoter region of the gene. No putative promoters were found in the upstream region of ORF*i* but the promoter may be an unrecognisable mycobacterial type of promoter; alternatively it is possible that the ORF*i* is expressed as part of an operon from a promoter upstream of ORFs *a* and *c* (Fig.47).

The region upstream from the complementing region contains a putative turgor response aldehyde dehyrogenase protein, *A* (179aa) and an osmotic repressor protein, *C* (201aa), it is therefore conceivable that protein *I* is encoded within the complementing region and is part of an osmoregulatory operon.

The homologies found for the ORFs *a/c* and *b* were generally proteins from other GC rich organisms and did not appear significant; it is therefore possible that the ORFs do not represent genuine coding regions (Table 19). Alternatively it is possible that ORFs *a/c* and *b* are genuine and expressing the complementing gene products.

The region of complementing DNA requires further analysis to determine the exact source of the complementation. This can be achieved by further subcloning regions e.g. the 2.75kb *Pst*1 fragment which covers the region containing ORF*i* and approximately 1.9kb of upstream

sequence. Alternatively, transposon mutagenesis of the region could be carried out to determine the exact complementing region (Jacobs *et al.*, 1991; Mills *et al.*, 1994))

4.5 BIOCHEMICAL ANALYSIS

The expected *M. smegmatis* lipid components were found in both *M. smegmatis* and PyramidII, although the actual amounts of each component present are unknown, and no alterations were found in the lipoglycan fraction analysed by HPLC (Dr. G Besra, Colorado State University, Colorado). The specific amounts and proportions of the mycolates in all strains could be measured by HPLC which may uncover differences not apparent in the 2D-TLC system. Equally the PyramidII strain could be analysed further with techniques (NMR) which may pick up subtle changes e.g. O-methylation or O-acylation of sugars which may not have been detected by the systems used.

The lipid components of the cosmid-containing complemented clones 57, 54 and 116 analysed contained all the components observed in wild type *M. smegmatis* mc^2155 strain as well as an unknown phospholipid. This phospholipid appears to be present in *M. leprae* (Minnikin *et al.*, 1985) and may be a direct product of an *M. leprae* gene or a secondary product. The second explanation seems most likely, since acetyl CoA carboxylase (*accC)*, the nucleotide biosynthesis genes *pur6* and *purk* and the phosphate-transport protein genes (*pstB)* which are all found on B1308 (Fig.48) all have the potential to indirectly alter phospholipid biosynthesis. Phospholipid accumulation may have been enhanced by the apparently reduced activity of phosphohydrolase enzyme found in Pyramid II.

Changes in the regulation of phospholipid biosynthesis could also lead to the accumulation of a secondary product which is not generally visible. The phospholipid is less polar than PI and PIMs and more polar than DPG and PE; the decrease in polarity in comparison with PI may be due to methylation or acylation which may then block alternative pathways. This

methylation or acylation may be encoded by a gene found on the cosmids. It is also possible that a specific *M. leprae* gene on the cosmids encodes an enzyme responsible for a methylation or acylation event which causes a block in the pathway, again leading to a visible intermediate.

The unknown phospholipid produced in complementing strains 57, 54 and 116 has not yet been specifically identified by GCMS.

The fatty acid content of the cosmid-containing strains appeared to be significantly altered in the levels of palmitic acid and tuberculostearic acid, these increased by approximately 10% in all cosmid-containing strains. The increase in palmitic acid and the larger tuberculostearic acid, both of which contain no saturated bonds, would result in a decreased fluidity of the cell membrane and may be connected to the mechanism of kanamycin resistance since it occurred in all cosmid containing strains.

4.6 FUTURE WORK

The nature of the PyramidII mutant should be investigated further; the permeability of the cell envelope could be measured (Nikaido and Jarlier, 1991; Connell and Nikaido, 1994) and sensitivity to other antibiotics investigated. An amide test could be carried out to ensure the mutation was not caused by upregulation of the pyrazinamidase enzyme.

The complementing region of *M. leprae* DNA needs to be narrowed down to a gene function as stated and could be achieved by subcloning or transposon mutagenesis (Jacobs *et al.*, 1991; Mills *et al.*, 1994). The 0.9kb fragment containing ORF*i* could be cloned into an expression vector to determine whether it was able to complement, when expressed from a mycobacterial promoter. The 0.9kb fragment could also be used as a probe to analyse the other complementing cosmids for homologous regions to categorically determine whether they all

contain the same complementing cosmids or whether they encode varying complementing regions. The complementing gene could be used to probe a *M. smegmatis* genomic library to determine whether the gene is present in *M. smegmatis*, if so it could be cloned and PCR-SSCP analysis carried out to determine wether the *M. smegmatis* homolog was in fact mutated. PyramidII could be transformed with an *M. smegmatis* cosmid or plasmid genomic library and the complementing region of DNA determined directly; this could then be used to probe the *M. leprae* complementing region to determine whether they were homologs. The complementing DNA, when isolated may also be used to carry out a zoo blot on a number of mycobacterial strains, including *M. smegmatis* to determine whether the gene is *M. leprae*-specific and therefore not the true homolog of the mutation or if it is present in other strains.

Cell free extracts of complemented PyramidII (containing pYRI) could be extracted and western blots run to try and identify any protein being overproduced which could be purified and further analysed.

Other mutants generated by this project e.g. penicillin G sensitive mutants, auxotrophic mutants and fluoroquinone mutants all require further analysis and transformation with an *M. smegmatis* genomic library to isolate the complementing genes.

4.7 SUMMARY

This project has resulted in the formation of two banks of NTG-treated *M. smegmatis* mc^2155 and has led to the isolation of a number of *M. smegmatis* mutants with alterations in a variety of phenotypes e.g. penicillin sensitivity, fluoroquinone resistance and melanin production.

A pyrazinamide sensitive mutant with decreased hydrophobicity, smooth colony morphology and variable increased sensitivity to penicillin was isolated. PyramidII may have altered cell envelope permeability which could be due to a variety of factors i.e. upregulation of porin

production or a subtle alteration in the cell wall structure, implied by the decrease in hydrophobicity and the altered colony morphology. Increased sensitivity to pyrazinamide could be caused by an upregulated efflux system, upregulated pyrazinamidase enzyme or by another mutation in an unknown internal mechanism for resistance to pyrazinamide in *M. smegmatis*.

A 3.5kb region of *M. leprae* DNA was found to complement this mutation and contains three putative ORFs. The region is found down stream of a putative osmoregulatory site and it is possible that one or all three of these coding regions are involved in osmoregulation. If PyramidII has altered permeability it would follow that it would require a change in its osmoregulation, to overcome the change in permeability. It is therefore possible that the complementing region of *M. leprae* DNA is involved in osmoregulation.

ACKNOWLEDGEMENTS

I would like to thank Dr Neil Stoker for giving me the opportunity to undertake a PhD as well as for his supervision throughout. Dr P Wheeler for patiently sharing his biochemical know how and for his continued interest and support. I would also like to thank Dr. D.Minikin and Mr.R.Bolton for teaching me how to analyse mycobacterial lipids and for putting up with me in their laboratory. Thanks also to Dr. G.Besra for his further biochemical analysis of PyramidII, Dr.K.Eighlmeier for the hybridisation to the cosmid library and to Dr.G.Britton for pigment analysis. Dr. G.Bancroft for the loan of his computer for an inordinately long time and for his encouragement.

I would also like to acknowledge the support of the trustees of The Hospices of St.Giles who have been interested and generous .

I would like to thank for getting me started in the laboratory Steve Hinshelwood and Koen de Smet and for keeping me going I would particularly like to thank Dr.J.Keer for her unending help and friendship over the last four years. Dr.T.Parish, for her help and clarity and to Jason, Nicki, Caroline and Rebecca for helping redress the insanity! I would specifically like to thank Jason, Nicki, Tanya and Jacquie for their help unstinting help in the last few weeks.

To my family thanks for all their love and support over many years and to my other friends thanks for still asking me out!

APPENDIX A: MEDIA AND ANTIBIOTICS

M9 MINIMAL MEDIUM (MM)

5x M9 salts *	200ml
Distilled water	800ml

sterilize by autoclaving
add 20ml 20% glucose (autoclaved) and 1ml 1% thiamine (filter sterilized)
for agar plates Bacto agar was added to 1.5%
cas-amino acids were added at 1g/l for MM+AA

***5x M9 salts**

$Na_2HPO_4.7H_2O$	64g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5g
Distilled water	to 1000ml

sterilize by autoclaving

LURIA BROTH (LB)

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
Distilled water	to 1000ml

sterilize by autoclaving
for agar plates Bacto agar was added to 1.5%

SOB

Bacto-tryptone	20g
Yeast extract	5g
NaCl	0.5g
Distilled water	to 1000ml

sterilize by autoclaving

LEMCO BROTH

Bacto-peptone	10g
Bacto Lab Lemco powder	5g
NaCl	5g
Distilled water	to 1000ml

pH to 7.2 with 2M NaOH, sterilize by autoclaving
for Lemco soft top, Bacto agar was added to 0.75%
for agar plates Bacto agar was added to 1.5%

MIDDLEBROOK 7H9 MEDIUM

Ammonium sulphate	0.5g
Monopotaasium phosphate	1.0g
Disodium phosphate	2.5g
Sodium citrate	0.1g
Magnesium sulphate	0.05g
Calcium chloride	0.0005g
Zinc sulphate	0.001g
Copper sulphate	0.001g
L-glutamic acid	0.5g
Ferric ammonium citrate	0.04g
Pyridoxine	0.001g
Biotin	0.0005g
Distilled water	to 900ml

sterilize by autoclaving, cool to 50-55°C and add 100ml ADC$^+$ enrichment (filter sterilized)

MIDDLEBROOK 7H10 AGAR

Ammonium sulphate	0.5g
Monopotaasium phosphate	1.5g
Disodium phosphate	1.5g
Sodium citrate	0.4g
Magnesium sulphate	0.025g
Calcium chloride	0.0005g
Zinc sulphate	0.001g
Copper sulphate	0.001g
L-glutamic acid	0.5g
Ferric ammonium citrate	0.04g
Pyridoxine	0.001g
Biotin	0.0005g
Malachite green	0.00025g
Bacto agar	15g
Distilled water	to 900ml

sterilize by autoclaving, cool to 50-55°C and add 100ml ADC$^+$ enrichment (filter sterilized)

MIDDLEBROOK 7H11 AGAR

Pancreatic digest of casein	1g
Ammonium sulphate	0.5g
Monopotaasium phosphate	1.5g
Disodium phosphate	1.5g
Sodium citrate	0.4g
Magnesium sulphate	0.05g
L-glutamic acid	0.5g
Ferric ammonium citrate	0.04g
Pyridoxine	0.001g
Biotin	0.0005g
Malachite green	0.001g
Bacto agar	15g
Distilled water	to 900ml

sterilize by autoclaving, cool to 50-55°C and add 100ml ADC* enrichment (filter sterilized)

ADC* ENRICHMENT

Albumin Fraction V, Bovine	5g
Dextrose	2g
Catalase (beef)	0.003g
distilled water	100ml

sterilize by filtration

ANTIBIOTIC STOCK SOLUTIONS

Penicillin G	10 mg/ml in H_2O (buffered with NaOH)
Pyrazinamide	10 mg/ml in H_2O (pH to 5.2 with HCl)
Ethionamide	
Ethambutol	
Kanamycin	50 mg/ml in H_2O
Ampicillin	50 mg/ml in H_2O
Ciprofloxacin	50 mg/ml in H_2O
Oxofloxacin	50 mg/ml in H_2O
Minocycline	50 mg/ml in DMF
Amikacin	50 mg/ml in H_2O

APPENDIX B: BUFFERS

TAE

50x stock	
Tris base	242g
glacial acetic acid	57.1ml
0.5M EDTA pH8.0	100ml
Distilled water	to 1000ml

TBE

5x stock	
Tris base	54g
boric acid	27.5g
0.5M EDTA pH8.0	20ml
Distilled water	to 1000ml

TE

2M Tris-Cl pH8.0	0.5ml
0.5M EDTA pH8.0	0.2ml
Distilled water	to 100ml

sterilize by autoclaving

APPENDIX C: BACTERIAL STRAINS AND PLASMIDS

E.coli

DH5α	F-, ϕ80D*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A46, *thi*-1, *hsd*R17 (r_K-,m_K+), *sup*E44, *rel*A1,*deo*R, Δ(*lac*ZYA-*arg*F)U169
TG2	*sup*E, *hsd*Δ5, *thi*, Δ(*lac-pro*AB), Δ (*srl-rec*A) 306::Tn10 (*tet*^{r}), F'{*traD* 36 *pro*AB+ *lac*I^q *lac*ZΔM15}
NM554	*rec*A13 *ara*D139 Δ(*ara-leu*)7696 Δ(*lac*)*l*7A *gal*U *gal*K *hsd*R *rps*L (Str^r) *mcr*A *mcr*B
DL795	Δ*hsd*RMS, *mcr*BC, *mrr*, e14 *mcr*A°; *sbc*C201; *rec*A::Cm^r

M.smegmatis

mc^2155	high efficiency of electroporation strain of *M.smegmatis* 607

PLASMIDS

pYUB18::	
M.leprae	library of genomic *M.leprae* DNA in shuttle cosmid pYUB18 (kan^r)
pYUB12	*E.coli*-mycobacterium shuttle plasmid (kan^r)
pMV206	*E.coli*-mycobacterium shuttle plasmid (kan^r)
pUC19	*E.coli* cloning vector (amp^r)

BIBLIOGRAPHY

AbouZeid C., Ratliff T.L., Wiker H.G., Harboe M., Bennedsen J., Rook G.A.W. 1988. Characterisation of fibronectin-binding antigens released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. *Infection and Immunity,* **56:** 3046-3051.

Adams L.B., Fukutomi Y., Krahenbuhl J.L. 1993. Regulation of murine macrophage effector functions by lipoarabinomannan from mycobacterial strains with different degrees of virulence. *Infection and Immunity,* **61:** 4173-4181.

Adelberg E.A., Mandel M., Grace Chein Ching Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications,* **18:** 788

Aldovini A., Husson R.N., Young R.A. 1993. The *ura*A locus and homologous recombination in *Mycobacterium bovis* BCG. *Journal of Bacteriology,* **175:** 7282-7289.

Andersen A.B., Brennan P.J. 1994. *M. tuberculosis* proteins and antigens. In *Bloom B.R.* (ed.), *Tuberculosis: pathogenesis, protection and control.* American Society for Microbiology, Washington DC. pp. 307-332.

Andersen A.B., Ljungqvist L., Olsen M. 1990. Evidence that protein antigen b of *Mycobacterium tuberculosis* is involved in phosphate metabolism. *Journal of General Microbiology,* **136:** 477-480.

Asselineau C., Lacave C.S., Montrozier H.L., Prome J.C. 1970. Relations structurales entre les acides mycoliques insatures et les acides inferieurs insatures synthetises par *Mycobacterium phlei. European Journal of Biochemistry,* **14:** 406-410.

Balasubramanian V., Pavelka Jr M.S., Bardarov S.S., Martin J., Weisbrod T.R., McAdam R.A., Bloom B.R., Jacobs Jr W.R. 1996. Allelic exchange in *Mycobacterium tuberculosis* with long linear recombination substrates. *Journal of Bacteriology,* **178:** 273-279.

Ballou C.E. 1972. Biosynthesis of mannophosphoinositides in *Mycobacterium phlei. Methods in Enzymology,* **28:** 493-500.

Banerjee A., Dubnau E., Quemard A., Balasubramanian V., Um K.S., Wilson T., Collins D., De Lisle G., Jacobs W.R., Jr. 1994. *inh*A, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis. Science,* **263:** 227-230.

Barletta R.G., Kim D.D., Snapper S.B., Bloom B.R., Jacobs W.R., Jr. 1992. Identification of expression signals of the mycobacteriophages Bxb1, L1 and TM4 using the *Escherichia-Mycobacterium* shuttle plasmids pYUB75 and pYUB76 designed to create translational fusions to the *lac*Z gene. *Journal of General Microbiology,* **138:** 23-30.

Barnes P.F., Chatterjee D., Abrams J.S., Lu S., Wang E., Yamamura M., Brennan P.J., Modlin R.L. 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan: relationship to chemical structure. *Journal of Immunology,* **149:** 541-547.

Barrow W.W., Davis T.L., Wright E.L., Labrousse V., Bachelet M., Rastogi N. 1995. Immunomodulatory spectrum of lipids associated with *Mycobacterium avium* serovar 8. *Infection and Immunity,* **63:** 126-133.

Barrow W.W., De Sousa J.P.C., Davis T.L., Wright E.L., Bachelet M., Rastogi N. 1993. Immunomodulation of human peripheral blood mononuclear cell functions by defined lipid fractions of *Mycobacterium avium. Infection and Immunity,* **61:** 5286-5293.

Barrow W.W., Ullom B.P., Brennan P.J. 1980. Peptidoglycolipid nature of the superficial cell wall sheath of smooth-colony-forming mycobacteria. *Journal of Bacteriology,* **144:** 814-822.

Belisle J.T., Brennan P.J. 1989. Chemical basis of rough and smooth variation in mycobacteria. *Journal of Bacteriology,* **171:** 3465-3470.

Belisle J.T., Brennan P.J. 1994. Molecular basis of colony morphology in *Mycobacterium avium. Research in Microbiology,* **145:** 237-242.

Belisle J.T., Klaczkiewicz K., Brennan P.J., Jacobs W.R., Jr., Inamine J.M. 1993b. Rough morphological variants of Mycobacterium avium. Characterization of genomic deletions resulting in the loss of glycopeptidolipid expression. *Journal of Biological Chemistry,* **268:** 10517-10523.

Belisle J.T., McNeil M.R., Chatterjee D., Inamine J.M., Brennan P.J. 1993a. Expression of the core lipopeptide of the glycopeptidolipid surface antigens in rough mutants of *Mycobacterium avium. Journal of Biological Chemistry,* **268:** 10510-10516.

Belisle J.T., Pascopella L., Inamine J.M., Brennan P.J., Jacobs W.R., Jr. 1991. Isolation and expression of a gene cluster responsible for biosynthesis of the glycopeptidolipid antigens of *Mycobacterium avium. Journal of Bacteriology,* **173:** 6991-6997.

Bergh S., Cole S.T. 1994. MycDB: An integrated mycobacterial database. *Molecular Microbiology,* **12:** 517-534.

Besra G.S., Chatterjee D. 1994. Lipids and carbohydrates of *Mycobacterium tuberculosis.*. In *Bloom B.R.* (ed.), *Tuberculosis: Pathogenesis, protection and control.* American Society for Microbiology, Washington DC. pp. 285-306.

Besra G.S., Khoo K.H., Belisle J.T., McNeil M.R., Morris H.R., Dell A., Brennan P.J. 1994b. New pyruvylated, glycosylated acyltrehaloses from *Mycobacterium smegmatis* strains, and their implications for phage resistance in mycobacteria. *Carbohydrate Research,* **251:** 99-114.

Besra G.S., Khoo K.H., McNeil M.R., Dell A., Morris H.R., Brennan P.J. 1995. A new interpretation of the structure of the mycolyl-arabinogalactan complex of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycosylalditol fragments by fast-atom bombardment mass spectrometry and 1H nuclear magnetic resonance spectroscopy. *Biochemistry,* **34:** 4257-4266.

Besra G.S., McNeil M.R., Brennan P.J. 1992. Characterization of the specific antigenicity of *Mycobacterium fortuitum. Biochemistry (USA),* **31:** 6504-6509.

Besra G.S., McNeil M.R., Khoo K.H., Dell A., Morris H.R., Brennan P.J. 1993. Trehalose-containing lipooligosaccharides of *Mycobacterium gordonae*: Presence of a mono-O-methyltetra-O-acyltrehalose 'core' and branching in the oligosaccharide backbone. *Biochemistry (USA),* **32:** 12705-12714.

Besra G.S., Sievert T., Lee R.E., Slayden R.A., Brennan P.J., Takayama K. 1994a. Identification of the apparent carrier in mycolic acid synthesis. *Proceedings of the National Academy of Sciences of the United States of America,* **91:** 12735-12739.

Beveridge T.J., Murray R.G.E. 1980. Sites of metal deposition in the cell wall of *Bacillus subtilis. Journal of Bacteriology,* **141:** 876-887.

Birnboim H.C., Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research,* **7:** 1513

Brennan P.J. 1989. Structure of mycobacteria: Recent developments in defining cell wall carbohydrates and proteins. *Reviews of Infectious Diseases,* **11:** S420-S429.

Brennan P.J., Ballou C.E. 1967. Biosynthesis of mannophosphoinositides by *Mycobacterium phlei. Journal of Biological Chemistry,* **242:** 3046-3056.

Brennan P.J., Draper P. 1994. Ultrastructure of *M. tuberculosis.*. In *Bloom B.R.* (ed.), *Tuberculosis: Protection, pathogenesis and control.* American Society for Microbiology, Washington DC. pp. 271-284.

Brennan P.J., Nikaido H. 1995. The envelope of mycobacteria. *Annual Review of Biochemistry,* **64:** 29-63.

Brown M.C., Taffet S.M. 1995. Lipoarabinomannans derived from different strains of

Mycobacterium tuberculosis differentially stimulate the activation of NF-kappa B and KBF1 in murine macrophages. *Infection and Immunity,* **63:** 1960-1968.

Brownback P.E., Barrow W.W. 1988. Modified lymphocyte response to mitogens after intraperitoneal injection of glycopeptidolipid antigens from *Mycobacterium avium* complex. *Infection and Immunity,* **56:** 1044-1050.

Brozna J.P., Horan M., Rademacher J.M., Pabst K.M., Pabst M.J. 1991. Monocyte responses to sulfatide from *Mycobacterium tuberculosis*: Inhibition of priming for enhanced release of superoxide, associated with increased secretion of interleukin-1 and tumor necrosis factor alpha, and altered protein phosphorylation. *Infection and Immunity,* **59:** 2542-2548.

Castelnuovo G., Bellezza G., Yamanaka S. 1970. Phage inhibiting substances of mycobacteria. 1. -The nature of phage inhibiting substances of *Mycobacterium phlei. Ann Inst Pasteur,* **119:** 302-311.

Chan J., Fan X., Hunter S.W., Brennan P.J., Bloom B.R. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. *Infection and Immunity,* **59:** 1755-1761.

Chatterjee D., Bozic C.M., McNeil M., Brennan P.J. 1991. Structural features of the arabinan component of the lipoarabinomannan of *Mycobacterium tuberculosis. Journal of Biological Chemistry,* **266:** 9652-9660.

Chatterjee D., Hunter S.W., McNeil M., Brennan P.J. 1992a. Lipoarabinomannan: multiglycosylated form of the mycobacterial mannosylphosphatidylinositols. *Journal of Biological Chemistry,* **267:** 6228-6233.

Chatterjee D., Lowell K., Rivoire B., McNeil M.R., Brennan P.J. 1992b. Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannosyl residues in some strains. *Journal of Biological Chemistry,* **267:** 6234-6239.

Chatterjee D., Roberts A.D., Lowell K., Brennan P.J., Orme I.M. 1992c. Structural basis of capacity of lipoarabinomannan to induce secretion of tumor necrosis factor. *Infection and Immunity,* **60:** 1249-1253.

Church G., Kieffer-Higgins S. 1988. Multiplex DNA sequencing. *Science,* **240:** 185-188.

Cirillo J.D., Weisbrod T.R., Banerjee A., Bloom B.R., Jacobs W.R., Jr. 1994. Genetic determination of the meso-diaminopimelate biosynthetic pathway of mycobacteria. *Journal of Bacteriology,* **176:** 4424-4429.

Clark-Curtiss J.E. 1990. Genome structure of mycobacteria. In *McFadden J.* (ed.),

Molecular biology of the mycobacteria. Academic Press, London. pp. 77-96.

Clark-Curtiss J.E., Jacobs W.R.J., Docherty M.A., Ritchie L.R., Curtiss R.I. 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae.*. *Journal of Bacteriology,* **161:** 1093-1102.

Cole S.T., Smith D.R. 1994. Towards mapping and sequencing the genome of *Mycobacterium tuberculosis.*. In *Bloom B.R.* (ed.), *Tuberculosis: Pathogenesis, protection and control.* American Society for Microbiology, Washington DC. pp. 227-238.

Collins D.M., Kawakami R.P., De Lisle G.W., Pascopella L., Bloom B.R., Jacobs WR Jr. 1995. Mutation of the principal sigma factor causes loss of virulence in a strain of the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Sciences of the United States of America,* **92:** 8036-8040.

Colston M.J., Davis E.O. 1994. Homologous recombination, DNA repair, and mycobacterial *recA* genes. In *Bloom B.R.* (ed.), *Tuberculosis: Pathogenesis, protection and control.* American Society for Microbiology, Washington, DC 20005. pp. 217-226.

Connell N.D., Nikaido H. 1994. Membrane permeability and transport in *Mycobacterium tuberculosis.* In *Bloom B.R.* (ed.), *Tuberculosis: Pathogenesis, protection and control.* American Society for Microbiology, Washington DC. pp. 333-352.

Crowle A.J., Dahl R., Ross E., May M.H. 1991. Evidence that vesicles containing living virulent *Mycobacterium tuberculosis or Mycobacterium avium* in cultured human macrophages are not acidic. *Infection and Immunity,* **59:** 1823-1831.

Curcic R., Dhandayuthapani S., Deretic V. 1994. Gene expression in mycobacteria: Transcriptional fusions based on *xyl*E and analysis of the promoter region of the response regulator *mtr*A from *Mycobacterium tuberculosis. Molecular Microbiology,* **13:** 1057-1064.

Daffe M., Brennan P.J., McNeil M. 1990. Predominant structural features of the cell wall arabinogalactan of *Mycobacterium tuberculosis* as revealed through characterisation of oligoglycosyl alditol fragments by gas chromatography/mass spectrometry and by ^{1}H and ^{13}C-NMR analyses. *Journal of Biological Chemistry,* **265:** 6734-6743.

Daffe M., McNeil M., Brennan P.J. 1991. Novel type-specific lipooligosaccharides from *Mycobacterium tuberculosis. Biochemistry (USA),* **30:** 378-388.

Das Gupta S.K., Bashyam M.D., Tyagi A.K. 1993. Cloning and assessment of mycobacterial promoters by using a plasmid shuttle vector. *Journal of Bacteriology,* **175:** 5186-5192.

David,H.L. 1984. Carotenoid pigments of the mycobacteria. In *Kubica,G.P., Wayne,L.G.* (eds), *The Mycobacteria: a sourcebook, Part A.* New York: Marcel Dekker, Inc. pp696-699.

David H.L., Clement F., Meyer L. 1978. Adsorption of mycobacteriophage D_{29} on *Mycobacterium leprae. Annales de Microbiologie,* **129:** 563-566.

David H.L., Levy-Frebault V., Thorel M.F. 1988. Characterisation of distinct layers of the *Mycobacterium avium* envelope in respect of their composition by fatty acids, proteins, oligosaccharides and antigens. *Zentralblatt fur Bakteriologie Mikrobiologie und Hygiene - Abt 1 Orig A,* **A268:** 193-208.

Davis E.O., Jenner P.J., Brooks P.C., Colston M.J., Sedgwick S.G. 1992. Protein splicing in the maturation of M. tuberculosis RecA protein: A mechanism for tolerating a novel class of intervening sequence. *Cell,* **71:** 201-210.

Davis E.O., Sedgwick S.G., Colston M.J. 1991. Novel structure of the *rec*A locus of *Mycobacterium tuberculosis* implies processing of the gene product. *Journal of Bacteriology,* **173:** 5653-5662.

Davis E.O., Thangaraj H.S., Brooks P.C., Colston M.J. 1994. Evidence of selection for protein introns in the RecAs of pathogenic mycobacteria. *EMBO Journal,* **13:** 699-703.

De Smet K.A.L., Jamil S., Stoker N.G. 1993. Tropist3: A cosmid vector for simplified mapping of both G + C-rich and A + T-rich genomic DNA. *Gene,* **136:** 215-219.

De Smet K.A.L., Weston T., Young D.B. 1995. Trehalose synthesis in mycobacteria. Abstract for the *Glaxo "Action TB: Scientific conference and workshops",* 18 August 1995.

Dellagostin O.A., Esposito G., Eales L.J., Dale J.W., McFadden J. 1995. Activity of mycobacterial promoters during intracellular and extracellular growth. *Microbiology,* **141:** 1785-1792.

Deng L., Mikusova K., Robuck K.G., Scherman M., Brennan P.J., McNeil M.R. 1995. Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. *Antimicrobial Agents and Chemotherapy,* **39:** 694-701.

Dessen A., Quemard A., Blanchard J.S., Jacobs W.R., Jr., Sacchettin J.C. 1995. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis. Science,* **267:** 1638-1641.

Dhandayuthapani S., Via L.E., Thomas C.A., Horowitz P.M., Deretic D., Deretic V. 1995. Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages. *Molecular Microbiology,* **17:** 901-912.

Dhariwal K.R., Liav A., Vatter A.E., et al. 1986. Haptenic oligosaccharides in antigenic variants of mycobacterial C- mycosides antagonize lipid receptor activity for mycobacteriophage D4 by masking a methylated rhamnose. *Journal of Bacteriology,* **168:** 283-293.

Dobson G., Minnikin D.E., Minnikin S.M., Parlett J.H., Goodfellow M., Ridell M., Magnusson M. 1985. Systematic analysis of complex mycobacterial lipids. In *Goodfellow M., Minnikin D.E.* (ed.), *Chemical methods in bacterial systematics.* Academic Press, London. pp. 237-265.

DonnellyWu M.K., Jacobs W.R., Jr., Hatfull G.F. 1993. Superinfection immunity of mycobacteriophage L5: Applications for genetic transformation of mycobacteria. *Molecular Microbiology,* **7:** 407-417.

Draper P. 1986. Structure of *Mycobacterium leprae. Leprosy Review,* **57:** 15-20.

Draper P. 1991. The structure of the mycobacterial cell envelope is not yet understood. *Research in Microbiology,* **142:** 420-422.

Eiglmeier K., Honore N., Cole S.T. 1991. Towards the integration of foreign DNA into the chromosome of *Mycobacterium leprae.. Research in Microbiology,* **142:** 617-622.

Eiglmeier K., Honore N., Woods S.A., Caudron B., Cole S.T. 1993. Use of an ordered cosmid library to deduce the genomic organization of Mycobacterium leprae. *Molecular Microbiology,* **7:** 197-206.

Etemadi A.-H. 1967a. Correlations structurales et biogenetiques des acides mycoliques en rapport avec la phylogenese de quelques genres d'actinomycetales. *Bulletin de la Societe de chimie biologique,* **49:** 695-706.

Etemadi A.-H. 1967b. Les acides mycoliques structure, biogenese et interet phylogenetique. *Exposes Annuels de Biochimie Medicale,* **28:** 77-109.

Etemadi A.-H. 1967c. The use of pyrolysis gas chromatography and mass spectroscopy in the study of the structure of mycolic acids. *Journal of Gas Chromatography,* **5:** 447-456.

Falcone V., Bassey E., Jacobs W Jr, Collins F. 1995. The immunogenicity of recombinant *Mycobacterium smegmatis* bearing BCG genes. *Microbiology,* **141:** 1239-1245.

Fattorini L., Orefici G., Shao Hong Jin;, Scardaci G., Amicosante G., Franceschini N., Chopra I. 1992. Resistance to beta -lactams in *Mycobacterium fortuitum. Antimicrobial Agents and Chemotherapy,* **36:** 1068-1072.

Finken M., Kirschner P., Meier A., Wrede A., Bottger E.C. 1993. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: Alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Molecular Microbiology,* **9:** 1239-1246.

Franzblau S.G., White K.E. 1990. Comparative in vitro activities of 20 fluoroquinolones against *Mycobacterium leprae. Antimicrobial Agents and Chemotherapy,* **34:** 229-231.

Fsihi H., Cole S.T. 1995. The *Mycobacterium leprae* genome: Systematic sequence analysis identifies key catabolic enzymes, ATP-dependent transport systems and a novel *pol*A locus associated with genomic variability. *Molecular Microbiology,* **16:** 909-919.

Garbe T., Harris D., Vordermeier M., Lathigra R., Ivanyi J., Young D. 1993. Expression of the *Mycobacterium tuberculosis* 19-kilodalton antigen in *Mycobacterium smegmatis*: Immunological analysis and evidence of glycosylation. *Infection and Immunity,* **61:** 260-267.

Garbe T.R., Barathi J., Barnini S., Zhang Y., AbouZeid C., Tang D., Mukherjee R., Young D.B. 1994. Transformation of mycobacterial species using hygromycin resistance as selectable marker. *Microbiology,* **140:** 133-138.

Gastambide-Odier M., Delaumeny J.M., Lederer E. 1963. Biosynthesis of phthiocerol: incorporation of methionine and propionic acid. *Chem Ind,* **1963:** 1285-1286.

Gastambide-Odier M., Lederer E. 1960. Biosynthese de l'acide corynomycolique a partir de deux molecules d'acide palmitique. *Biochem Z,* **333:** 285-295.

Gastambide-Odier M., Sarda P. 1970. Contribution a etude de la structure et de la biosytnese de glycolipides specifiques isoles de mycobacterias: les mycosides A et B. *Pneumologie,* **42:** 241-255.

Gaylord H., Brennan P.J. 1987. Leprosy and the leprosy bacillus: recent developments in characterization of antigens and immunology of the disease. *Annu Rev Microbiol,* **41:** 645-675.

Gelber R.H., Iranmanesh A., Murray L., Siu P., Tsang M. 1992. Activities of various quinolone antibiotics against *Mycobacterium leprae* in infected mice. *Antimicrobial Agents and Chemotherapy,* **36:** 2544-2547.

Goren M.B. 1972. Mycobacterial lipids: Selected topics. *Bacteriological Reviews,* **36:** 33-38.

Goren M.B., Brennan P.J. 1979. Mycobacterial lipids: chemistry and biologic activities. In *Youmans G.P.* (ed.), *Tuberculosis.*. The W.B.Saunders Co. Philadelphia. pp. 69-193.

Gormley E.P., Davies J. 1991. Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. *Journal of Bacteriology*, **173:** 6705-6708.

Grange J.M., Bird R.G. 1977. Lysogeny associated with mucoid variation in *Mycobacterium kansasii*.. *Journal of Medical Microbiology*, **11:** 1-6.

Grange J.M., Redmond W.B. 1978. Host-phage relationships in the genus *Mycobacterium* and their clinical significance. *Tubercle*, **59:** 203-225.

Guilhot,C.B., Gicquel,B., Martin,C. 1992. Temperature-sensitive mutants of the *Mycobacterium* plasmid pAL5000. Fems Microbiology Letters. **98**: 181-186.

Guilhot C., Otal I., Van Rompaey I., Martin C., Gicquel B. 1994. Efficient transposition in mycobacteria: Construction of *Mycobacterium smegmatis* insertional mutant libraries. *Journal of Bacteriology*, **176:** 535-539.

Haeseleer F. 1994. Structural instability of recombinant plasmids in mycobacteria. *Research in Microbiology*, **145:** 683-687.

Haeseleer F., Pollet J.F., Haumont M., Bollen A., Jacobs P. 1993. Stable integration and expression of the *Plasmodium falciparum* circumsporozoite protein coding sequence in mycobacteria. *Molecular and Biochemical Parasitology*, **57:** 117-126.

Hatfull G.F., Sarkis G.J. 1993. DNA sequence, structure and gene expression of mycobacteriophage L5: A phage system for mycobacterial genetics. *Molecular Microbiology*, **7:** 395-405.

Hatfull,G.F., Jacobs, W.R.Jr. 1994. Mycobacteriophages: Cornerstones of Mycobacterial research. In *Bloom B.R.* (ed.), *Tuberculosis: Pathogenesis, protection and control.* American Society for Microbiology, Washington DC. pp. 165-184.

Hayes W. *The genetics of bacteria and their viruses*. 2nd ed. London and Edinburgh: Blackwell Scientific Publications; 1968;

Heym B., Alzari P.M., Honore N., Cole S.T. 1995. Missense mutations in the catalase-peroxidase gene, katG, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Molecular Microbiology*, **15:** 235-245.

Heym B., Honore N., TruffotPernot C., Banerjee A., Schurra C., Jacobs W.R., Jr., Van Embden J.D.A., Grosset J.H., Cole S.T. 1994. Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: A molecular study. *Lancet*, **344:** 293-298.

Heym B., Zhang Y., Poulet S., Young D., Cole S.T. 1993. Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. *Journal of Bacteriology*, **175:** 4255-4259.

Hinshelwood S., Stoker N.G. 1992a. An *Escherichia coli-Mycobacterium* shuttle cosmid vector, pMSC1. *Gene*, **110:** 115-118.

Hinshelwood S., Stoker N.G. 1992b. Cloning of mycobacterial histidine synthesis genes by complementation of a *Mycobacterium smegmatis* auxotroph. *Molecular Microbiology*, **6:** 2887-2895.

Holland K.T., Ratledge C. 1971. A procedure for selecting and isolating specific auxotrophic mutants of *Mycobacterium smegmatis.*. *Journal of General Microbiology*, **66:** 115-118.

Honore N., Bergh S., Chanteau S., DoucetPopulaire F., Eiglmeier K., Garnier T., Georges C., Launois P., Limpaiboon T., Newton S., Niang K., Del Portillo P., Ramesh G.R., Reddi P., Ridel P.R., Sittisombut N., WuHunter S., Cole S.T. 1993. Nucleotide sequence of the first cosmid from the *Mycobacterium leprae* genome project: Structure and function of the *Rif-Str* regions. *Molecular Microbiology*, **7:** 207-214.

Honore N., Cole S.T. 1993. Molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrobial Agents and Chemotherapy*, **37:** 414-418.

Honore N., Cole S.T. 1994. Streptomycin resistance in mycobacteria. *Antimicrobial Agents and Chemotherapy*, **38:** 238-242.

HoussainiIraqui M., Clavel-Seres S., Rastogi N., David H.L. 1993b. Partial physical and functional map of a *Mycobacterium aurum* carotenogenesis operon. *Current Microbiology*, **26**: 65-74.

HoussainiIraqui M., David H.L., Calvel-Seres S., Hilali F., Rastogi N. 1993a. Characterisation of *car* α, *car Lep*, and *Crt I* genes controlling the biosynthesis of carotenes in *Mycobacterium aurum*. *Current Microbiology*, **27**: 317-322.

HoussainiIraqui M., Lazraq R., ClavelSeres S., Rastogi N., David H.L. 1992. Cloning and expression of *Mycobacterium aurum* carotenogenesis genes in *Mycobacterium smegmatis*. *FEMS Microbiology Letters*, **90:** 239-244.

Hunter S.W., Gaylord H., Brennan P.J. 1986. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *Journal of Biological Chemistry*, **261:** 12345-12351.

Hunter S.W., Jardine I., Yanagihara D.L., Brennan P.J. 1985. Trehalose-containing lipooligosaccharides from mycobacteria: structures of the oligosaccharide segments and recognition of a uniqueN-acylkanosamine-containing epitope. *Biochemistry,* **24:** 2798-2805.

Hunter S.W., Rivoire B., Mehra V., Bloom B.R., Brennan P.J. 1990. The major native proteins of the leprosy bacillus. *Journal of Biological Chemistry,* **265:** 14065-14068.

Husson R.N., James B.E., Young R.A. 1990. Gene replacement and expression of foreign DNA in mycobacteria. *Journal of Bacteriology,* **172:** 519-524.

Imaeda T., San Blas F. 1969. Adsorption of mycobacteriophage on cell-wall components. *Journal of General Virology,* **5:** 493-498.

Jacobs W.R., Tuckman M., Bloom B.R. 1987. Introduction of foreign DNA into mycobacteria using a shuttle plasmid. *Nature,* **327:** 532-535.

Jacobs W.R., Jr., Barletta R.G., Udani R., Chan J., Kalkut G., Sosne G., Kieser T., Sarkis G.J., Hatfull G.F., Bloom B.R. 1993. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science,* **260:** 819-822.

Jacobs W.R., Jr., Kalpana G.V., Cirillo J.D., Pascopella L., Snapper S.B., Udani R.A., Jones W., Barletta R.G., Bloom B.R. 1991. (25) Genetic systems for mycobacteria. *Methods in Enzymology,* **204:** 537-555.

Jarlier V., Gutmann L., Nikaido H. 1991. Interplay of cell wall barrier and beta-lactamase activity determines high resistance to beta-lactam antibiotics in *Mycobacterium chelonae*. *Antimicrobial Agents and Chemotherapy,* **35:** 1937-1939.

Jarlier V., Nikaido H. 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. *Journal of Bacteriology,* **172:** 1418-1423.

Jarlier V., Nikaido H. 1994. Mycobacterial cell wall: Structure and role in natural resistance to antibiotics. *FEMS Microbiology Letters,* **123:** 11-18.

Kalpana G.V., Bloom B.R., Jacobs W.R., Jr. 1991. Insertional mutagenesis and illegitimate recombination in mycobacteria. *Proceedings of the National Academy of Sciences of the United States of America,* **88:** 5433-5437.

Kamisango K., Saadat S., Dell A., Ballou C.E. 1985. Pyruvylated glycolipids from *Mycobacterium smegmatis*. Nature and location of the lipid components. *Journal of Biological Chemistry,* **260:** 4117-4121.

Kaplan G., Gandhi R.R., Weinstein D.E., Levis W.R., Patarroyo M.E., Brennan P.J.,

Cohn Z.A. 1987. *Mycobacterium leprae* antigen-induced supression of T cell proliferation *in vitro*. *Journal of Immunology*, **138:** 3028-3034.

Kato M. 1973. Effect of anti-cord factor antibody on experimental tuberculosis in mice. *Infection and Immunity*, **7:** 14-21.

Kempsell K.E., Kelly V.A., Hutchinson S.E., Duncan K. 1995. Cloning and expression of the *Mycobacterium tuberculosis* homologue for UDP-N-acetylglucosamine enolpyruvyl transferase. Abstract for the *Glaxo "Action TB: Scientific conference and workshops"*, 18 August 1995.

Khoo K.H., Dell A., Morris H.R., Brennan P.J., Chatterjee D. 1995b. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. *Journal of Biological Chemistry*, **270:** 12380-12389.

Khoo K.H., Dell A., Morris H.R., Brennan P.J., Chatterjee D. 1995a. Structural definition of acylated phosphatidylinositol mannosides from *Mycobacterium tuberculosis*: Definition of a common anchor for lipomannan and lipoarabinomannan. *Glycobiology*, **5:** 117-127.

Konickova-Radochova M., Konicek J., Malek I. 1970. The study of mutagenesis in *Mycobacterium phlei*. *Folia Microbiologica*, **15:** 88-102.

Konno K., Feldmann F.M., McDermott W. 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *American Review of Respiratory Disease*, **95:** 461-469.

Kremer L., Baulard A., Estaquier J., Content J., Capron A., Locht C. 1995b. Analysis of the *Mycobacterium tuberculosis* 85A antigen promoter region. *Journal of Bacteriology*, **177:** 642-653.

Kremer L., Baulard A., Estaquier J., PoulainGodefroy O., Locht C. 1995a. Green fluorescent protein as a new expression marker in mycobacteria. *Molecular Microbiology*, **17:** 913-922.

Kundu M., Basu J., Chakrabarti P. 1991. Defective mycolic acid biosynthesis in a mutant of *Mycobacterium smegmatis*. *Journal of General Microbiology*, **137:** 2197-2200.

Labidi A., David H.L., RoullandDussoix D. 1985. Cloning and expression of mycobacterial plasmid DNA in *Escherichia coli*.. *FEMS Microbiology Letters*, **30:** 221-225.

Labidi A., Mardis E., Roe B.A., Wallace R.J.J. 1992. Cloning and DNA sequence of the *Mycobacterium fortuitum var fortuitum* plasmid pAL5000. *Plasmid*, **27:** 130-140.

Lacave C., Laneelle M.A., Laneelle G. 1990a. Mycolic acid synthesis by *Mycobacterium aurum* cell-free extracts. *Biochimica et Biophysica Acta*, **1042:** 315-323.

Lacave C., Quemard A., Laneelle G. 1990b. Cell-free synthesis of mycolic acids in *Mycobacterium aurum*: radioactivity distribution in newly synthesised acids and presence of cell wall in the system. *Biochimica et Biophysica Acta*, **1045:** 58-65.

Lee B.Y., Horwitz M.A. 1995. Identification of macrophage and stress-induced proteins of *Mycobacterium tuberculosis*. *Journal of Clinical Investigation*, **96:** 245-249.

Lee M.H., Pascopella L., Jacobs W.R.J., Hatfull G.F. 1991. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for *Mycobacterium smegmatis, Mycobacterium tuberculosis*, and *bacille Calmette-Guerin*. *Proceedings of the National Academy of Sciences of the United States of America*, **88:** 3111-3115.

Lee S.-H., Brodie A.F. 1978. A model proteoliposomal system for proline transport using a purified proline carrier protein from *Mycobacterium phlei*. *Biochemical and Biophysical Research Communications*, **85:** 788-794.

Lee S.-H., Cohen N.S., Jacobs A.J., Brodie A.F. 1979. Isolation, purification and reconstitution of a proline carrier protein from *Mycobacterium phlei.*. *Biochemistry*, **18:** 2232-2238.

Lee S.-H., Brodie A.F. 1979. Resolution and reconstitution of active transport of calcium by a protein(s) from *Mycobacterium phlei*. *Journal of Biological Chemistry*, **254:** 6861-6864.

Lee Y.C., Ballou C.E. 1964. Structural studies on the myo-inositol mannosides from the glycolipids of *Mycobacterium tuberculosis* and *Mycobacterium phlei*. *Journal of Biological Chemistry*, **239:** 1316-1327.

Lee Y.C., Ballou C.E. 1965. Complete structures of the glycophospholipids of mycobacteria. *Biochemistry*, **4:** 1395-1404.

Lemassu A., LevyFrebault V.V., Laneelle M.A., Daffe M. 1992. Lack of correlation between colony morphology and lipooligosaccharide content in the *Mycobacterium tuberculosis* complex. *Journal of General Microbiology*, **138**: 131-137

Lepoivre M., Tenu J.P., Lemaire G., Petit J.F. 1982. Anti-tumour activity and hydrogen peroxide release by macrophages elicited by trehalose clusters. *Journal of Immunology*, **129:** 860-866.

Lim,E.M., Rauzier,J., Timm,J., Torrea,G., Murray,A., Gicquel,B., Portnoi,D. 1995. Identification of *Mycobacterium tuberculosis* DNA sequences encoding exported proteins by

using *phoA* gene fusions. *Journal of Bacteriology*, **177**: 59-65.

Lopez Marin L.M., Laneelle M.A., Prome D., Daffe M. 1993. Structures of the glycopeptidolipid antigens of two animal pathogens: *Mycobacterium senegalense* and *Mycobacterium porcinum*. *European Journal of Biochemistry*, **215:** 859-866.

Lopez Marin L.M., Laneelle M.A., Prome D., Daffe M., Laneelle G., Prome J.C. 1991. Glycopeptidolipids from *Mycobacterium fortuitum*: A variant in the structure of C-mycoside. *Biochemistry*, **30:** 10536-10542.

Lopez Marin L.M., Laneelle M.A., Prome D., Laneelle G., Prome M. 1992. Structure of a novel sulfate-containing mycobacterial glycolipid. *Biochemistry*, **31:** 11106-11111.

Lopez Marin L.M., Quesada D., Lakhdar-Ghazal F., Tocanne J.F., Laneelle G. 1994. Interactions of mycobacterial glycolipids with membranes: Influence of carbohydrate on induced alterations. *Biochemistry*, **33:** 7056-7061.

Marklund B.I., Speert D.P., Stokes R.W. 1995. Gene replacement through homologous recombination in *Mycobacterium intracellulare*. *Journal of Bacteriology*, **177:** 6100-6105.

Martin C., Mazodier P., Mediola M.V., Gicquel B., Smokvina T., Thompson C.J., Davies J. 1991. Site-specific integration of the *Streptomyces* plasmid pSAM2 in *Mycobacterium smegmatis*. *Molecular Microbiology*, **5:** 2499-2502.

Mathur M., Kolattukudy P.E. 1992. Molecular cloning and sequencing of the gene for mycocerosic acid synthase, a novel fatty acid elongating multifunctional enzyme, from *Mycobacterium tuberculosis var. bovis Bacillus Calmette-Guerin*. *Journal of Biological Chemistry*, **267:** 19388-19395.

Matsunaga I., Oka S., Inove T., Yano I. 1990. Mycolyl glycolipids stimulate macrophages to release chemotactic factors. *FEMS Microbiology Letters*, **67:** 49-54.

McAdam R.A., Weisbrod T.R., Martin J., Scuderi J.D., Brown A.M., Cirillo J.D., Bloom B.R., Jacobs W.R.J. 1995. *In vivo* growth characteristics of leucine and methionine auxotrophic mutants of *Mycobacterium bovis* BCG generated by transposon mutagenesis. *Infection and Immunity*, **63:** 1004-1012.

McFadden J., Kunze Z., Seechurn P. 1990. DNA probes for detection and identification. In *McFadden J.* (ed.), *Molecular biology of the mycobacteria*. Academic Press, London. pp. 139-172.

McNeil M., Brennan P.J. 1991. Structure, function, and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis, and drug resistance; some

thoughts and possibilities arising from recent structural information. *Research in Microbiology*, **142:** 451-463.

McNeil M., Daffe M., Brennan P.J. 1990. Evidence for the nature of the link between the arabinogalactan and peptidoglycan of mycobacterial cell walls. *Journal of Biological Chemistry*, **265:** 18200-18206.

McNeil M.R., Robuck K.G., Harter M., Brennan P.J. 1994. Enzymatic evidence for the presence of a critical terminal hexa-arabinoside in the cell walls of *Mycobacterium tuberculosis*. *Glycobiology*, **4:** 165-173.

Meier A., Kirschner P., Bange F.C., Vogel U., Bottger E.C. 1994. Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis*: Mapping of mutations conferring resistance. *Antimicrobial Agents and Chemotherapy*, **38:** 228-233.

Mills J.A., McNeil M.R., Belisle J.T., Jacobs W.R., Jr., Brennan P.J. 1994. Loci of *Mycobacterium avium ser2* gene cluster and their functions. *Journal of Bacteriology*, **176:** 4803-4808.

Minnikin D.E. 1982. Lipids: complex lipids, their chemistry, biosynthesis and roles. In *Ratledge C., Stanford J.L.* (ed.), *The Biology of the Mycobacteria.* Academic Press, London. pp. 95-184.

Minnikin D.E., Dobson G., Draper P. 1985. The free lipids of *Mycobacterium leprae* harvested from experimentally infected nine-banded armadillos. *Journal of General Microbiology*, **131:** 2007-2011.

Minnikin D.E., Goodfellow M. 1980. Lipid composition in the classification and identification of acid-fast bacteria. In *Goodfellow M., Board R.G.* (ed.), *Microbiological Classification and Identification.* Academic Press, London. pp. 189-256.

Misaki A., Azuma I., Yamamura Y. 1977. Structural and immunochemical studies on D-arabino-D-mannans and D-mannans of *Mycobacterium tuberculosis* and other *Mycobacterium* species. *Journal of Biological Chemistry*, **82:** 1759-1770.

Molloy A., Gaudernack G., Levis W.R., Cohn Z.A., Kaplan G. 1990. Suppression of T-cell proliferation by *Mycobacterium leprae* and its products: The role of lipolysaccharide. *Proceedings of the National Academy of Sciences of the United States of America*, **87:** 973-977.

Monahan I.M., Banerjee D.K., Butcher P.D. 1994. Gene expression of *Mycobacterium bovis* BCG induced in vitro by stress stimuli associated with infection. *Biochemical Society Transactions*, **22:** 89Sl.

Lee M.H., Hatfull G.F. 1993. Mycobacteriophage L5 integrase-mediated site-specific integration *in vitro*. *Journal of Bacteriology*, **175:** 6836-6841.

Moss, M.T. Cloning and expression of mycobacterial genes in *Escherichia coli*. University of Surrey; 1987. PhD thesis.

Nesbit C.E., Levin M.E., DonnellyWu M.K., Hatfull G.F. 1995. Transcriptional regulation of repressor synthesis in mycobacteriophage L5. *Molecular Microbiology*, **17:** 1045-1056.

Nguyen H.T., Trach D.D., Man N.V., Ngoan T.H., Dunia I., Ludosky-Diawara M.A., Benedetti E.L. 1979. Comparative ultrastructure of *Mycobacterium leprae* and *Mycobacterium lepraemurium* cell envelopes. *Journal of Bacteriology*, **138:** 552-558.

Nikaido H. 1994. Prevention of drug access to bacterial targets: Permeability barries and active efflux. *Science*, **264:** 382-388.

Nikaido H., Jarlier V. 1991. Permeability of the mycobacterial cell wall. *Research in Microbiology*, **142:** 437-443.

Nikaido H., Kim S.H., Rosenberg E.Y. 1993. Physical organization of lipids in the cell wall of *Mycobacterium chelonae*. *Molecular Microbiology*, **8:** 1025-1030.

Nikaido H., Thanassi D.G. 1993. Penetration of lipophilic agents of multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. *Antimicrobial Agents and Chemotherapy*, **37:** 1393-1399.

Nishiura M., Izumi S., Mori T., Takeo K., Nonaka T. 1977. Freeze-etching study of human and murine leprosy bacilli. *International Journal of Leprosy*, **45:** 248-254.

Norman E., De Smet K.A.L., Stoker N.G., Ratledge C., Wheeler P.R., Dale J.W. 1994. Lipid synthesis in mycobacteria: Characterization of the biotin carboxyl carrier protein genes from *Mycobacterium leprae* and *M. tuberculosis*. *Journal of Bacteriology*, **176:** 2525-2531.

Norman E., Dellagostin O.A., McFadden J., Dale J.W. 1995. Gene replacement by homologous recombination in *Mycobacterium bovis* BCG. *Molecular Microbiology*, **16:** 755-760.

Oskam L., Hartskeerl R.A., Hermans C.J., De Wit M.Y.L., Jarings G.H., Nicholls R.D., Klatser P.R. 1995. A 46 kDa integral membrane protein from *Mycobacterium leprae* resembles a number of bacterial and mammalian membrane transport proteins. *Microbiology*, **141:** 1963-1968.

Pascopella L., Collins F.M., Martin J.M., Mong Hong Lee;, Hatfull G.F., Stover C.K.,

Bloom B.R., Jacobs W.R., Jr. 1994. Use of in vivo complementation in *Mycobacterium tuberculosis* to identify a genomic fragment associated with virulence. *Infection and Immunity,* **62:** 1313-1319.

Paul T.R., Beveridge T.J. 1992. Reevaluation of envelope profiles and cytoplasmic ultrastructure of mycobacteria processed by conventional embedding and freeze-substitution protocols. *Journal of Bacteriology,* **174:** 6508-6517.

Paul T.R., Beveridge T.J. 1994. Preservation of surface lipids and determination of ultrastructure of *Mycobacterium kansasii* by freeze-substitution. *Infection and Immunity,* **62:** 1542-1550.

Perez R.L., Roman J., Staton G.W., Jr., Hunter R.L. 1994. Extravascular coagulation and fibrinolysis in murine lung inflammation induced by the mycobacterial cord factor trehalose-6, 6'-dimycolate. *American Journal of Respiratory And Critical Care Medicine,* **149:** 510-518.

Philipp W, Poulet S, Pascopella L *et al.* Genome organisation of *Mycobacterium tuberculosis*.[Unpublished] 1993; Quoted in Cole and Smith, 1994.

Plum G., ClarkCurtiss J.E. 1994. Induction of *Mycobacterium avium* gene expression following phagocytosis by human macrophages. *Infection and Immunity,* **62:** 476-483.

Poole K., Krebes K., McNally C., Meshat S. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *Journal of Bacteriology,* **175:** 7363-7372.

Pourshafie M., Ayub Q., Barrow W.W. 1993. Comparative effects of *Mycobacterium avium* glycopeptidolipid and lipopeptide fragment on the function and ultrastructure of mononuclear cells. *Clinical and Experimental Immunology,* **93:** 72-79.

Predich M., Doukhan L., Nair G., Smith I. 1995. Characterization of RNA polymerase and two sigma-factor genes from *Mycobacterium smegmatis*. *Molecular Microbiology,* **15:** 355-366.

Prinzis S., Chatterjee D., Brennan P.J. 1993. Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG. *Journal of General Microbiology,* **139:** 2649-2658.

Prinzis S., Rivoire B., Brennan P.J. 1994. Search for the molecular basis of morphological variation in *Mycobacterium avium*. *Infection and Immunity,* **62:** 1946-1951.

Quemard A., Lacave C., Laneelle G. 1991. Isoniazid inhibition of mycolic acid synthesis by cell extracts of sensitive and resistant strains of *Mycobacterium aurum*. *Antimicrobial Agents*

and Chemotherapy, **35:** 1035-1039.

Quemard A., Laneelle G., Lacave C. 1992. Mycolic acid synthesis: A target for ethionamide in mycobacteria? *Antimicrobial Agents and Chemotherapy,* **36:** 1316-1321.

Quemard A., Sacchettini J.C., Dessen A., Vilcheze C., Bittman R., Jacobs WR Jr, Blanchard J.S. 1995. Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis. Biochemistry,* **34:** 8235-8241.

Qureshi N., Sathyamoorthy N., Takayama K. 1984. Biosynthesis of C_{30} to C_{56} fatty acids by an extract of *Mycobacterium tuberculosis* H37Ra. *Journal of Bacteriology,* **157:** 46-52.

Qureshi N., Takayama K., Jordi H.C., Schnoes H.K. 1978. Characterisation of the purified components of a new homologous series of α -mycolic acids from *Mycobacterium tuberculosis* H37Ra. *Journal of Biological Chemistry,* **253:** 5411-5417.

Radford A.J., Hodgson A.L.M. 1991. Construction and characterisation of a *Mycobacterium - Escherichia coli* shuttle vector. *Plasmid,* **25:** 149-153.

Ramasesh N., Wright E.L., Barrow W.W. 1992. Cell-free system responsible for internal radiolabeling of glycopeptidolipids of the Mycobacterium avium complex. *Infection and Immunity,* **60:** 308-311.

Rambukkana A., Das P.K., Chand A., Baas J.G., Groothuis D.G., Kolk A.H.J. 1991. Subcellular distribution of monoclonal antibody defined epitopes on immunodominant *Mycobacterium tuberculosis* proteins in the 30-kDa region: Identification and localisation of 29/33-kDa doublet proteins on mycobacterial cell wall. *Scandinavian Journal of Immunology,* **33:** 763-775.

Ranes M.G., Rauzier J., Lagranderie M., Gheorghiu M., Gicquel-Sanzey B. 1990. Functional analysis of pAL5000, a plasmid from *Mycobacterium fortuitum*: construction of a "mini" mycobacterium-*Escherichia coli* shuttle vector. *Journal of Bacteriology,* **172:** 2793-2797.

Rastogi N., Frehel C., David H.L. 1984. Evidence for taxonomic utility of periodic acid-thiocarbohydrazide-silver proteinate cytochemical staining for electron microscopy. *Int J Syst Bacteriol,* **34:** 293-299.

Rastogi N., Goh K.S. 1990. Action of 1-isonicotinyl-2-palmitoyl hydrazine against the *Mycobacterium avium* complex and enhancement of its activity by m-fluorophenylalanine. *Antimicrobial Agents and Chemotherapy,* **34:** 2061-2064.

Ratledge C. 1982. Nutrition, Growth and Metabolism. In *Ratledge C., Stanford J.L.* (ed.),

The Biology of the Mycobacteria. Academic Press, London. pp. 185-271.

Rauzier J., Moniz-Pereira J., Gicquel-Sanzey B. 1988. Complete nucleotide sequence of pAL5000, a plasmid from *Mycobacterium fortuitum. Gene,* **71:** 315-321.

Reyrat J.M., Berthet F.X., Gicquel B. 1995. The urease locus of *Mycobacterium tuberculosis* and its utilization for the demonstration of allelic exchange in *Mycobacterium bovis bacillus Calmette- Guerin. Proceedings of the National Academy of Sciences of the United States of America,* **92:** 8768-8772.

Riviere M., Auge S., Vercauteren J., Wisingerova E., Puzo G. 1993. Structure of a novel glycopeptidolipid antigen containing a O-methylated serine isolated from *Mycobacterium xenopi*. Complete 1H-NMR and 13C-NMR assignment. *European Journal of Biochemistry,* **214:** 395-403.

Riviere M., Puzo G. 1992. Use of 1H NMR ROESY for structural determination of O-glycosylated amino acids from a serine-containing glycopeptidolipid antigen. *Biochemistry (USA),* **31:** 3575-3580.

Roach T.I.A., Barton C.H., Chatterjee D., Liew F.Y., Blackwell J.M. 1995. Opposing effects of interferon-gamma on iNOS and interleukin-10 expression in lipopolysaccharide- and mycobacterial lipoarabinomannan-stimulated macrophages. *Immunology,* **85:** 106-113.

Roach T.I.A., Chatterjee D., Blackwell J.M. 1994. Induction of early-response genes KC and JE by mycobacterial lipoarabinomannans: Regulation of KC expression in murine macrophages by *Lsh/Ity/Bcg* (candidate Nramp). *Infection and Immunity,* **62:** 1176-1184.

Rosenberg M.D., Gutnik D., Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters,* **9:** 29-33.

Rulong S., Aguas A.P., Da Silva P.P., Silva M.T. 1991. Intramacrophagic *Mycobacterium avium* bacilli are coated by a multiple lamellar structure: Freeze fracture analysis of infected mouse liver. *Infection and Immunity,* **59:** 3895-3902.

Sambrook J., Fritsch E.F., Maniatis T. 1989. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press,

Schlesinger L.S., Horwitz M.A. 1991. Phenolic glycolipid-1 of *Mycobacterium leprae* binds complement component C3 in serum and mediates phagocytosis by human monocytes. *Journal of Experimental Medicine,* **174:** 1031-1038.

Schlesinger L.S., Horwitz M.A. 1994a. A role for natural antibody in the pathogenesis of

leprosy: Antibody in nonimmune serum mediates C3 fixation to the *Mycobacterium leprae* surface and hence phagocytosis by human mononuclear phagocytes. *Infection and Immunity,* **62:** 280-289.

Schlesinger L.S., Hull S.R., Kaufman T.M. 1994b. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. *Journal of Immunology,* **152:** 4070-4079.

Sharman G.J., Williams D.H., Ewing D.F., Ratledge C. 1995. Isolation, purification and structure of exochelin MS, the extracellular siderophore from *Mycobacterium smegmatis*. *Biochemical Journal,* **305:** 187-196.

Shinnick T.M., Good R.C. 1994. Mycobacterial taxonomy. *European Journal of Clinical Microbiology and Infectious Diseases,* **13:** 884-901.

Sibley L.D., Hunter S.W., Brennan P.J., Krahenbuhl J.L. 1988. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. *Infection and Immunity,* **56:** 1232-1236.

Sieling P.A., Chatterjee D., Porcelli S.A., Prigozy T.I., Mazzaccaro R.J., Soriano T., Bloom B.R., Brenner M.B., Kronenberg M., Brennan P.J., Modlin R.L. 1995. CD1-Restricted T cell recognition of microbial lipoglycan antigens. *Science,* **269:** 227-230.

Silva M.T., Macedo P.M. 1983. The interpretation of the ultrastructure of mycobacterial cells in transmission electron microscopy of ultrathin sections. *International Journal of Leprosy,* **51:** 225-234.

Silve G., ValeroGuillen P., Quemard A., DuPont M.A., Daffe M., Laneelle G. 1993. Ethambutol inhibition of glucose metabolism in mycobacteria: A possible target of the drug. *Antimicrobial Agents and Chemotherapy,* **37:** 1536-1538.

Snapper S.B., Lugosi L., Jekkel A., Melton R.E., Kieser T., Bloom B.R., Jacobs WR Jr. 1988. Lysogeny and transformation in mycobacteria: Stable expression of foreign genes. *Proceedings of the National Academy of Sciences of the United States of America,* **85:** 6987-6991.

Snapper S.B., Melton R.E., Mustafa S., Kieser T., Jacobs WR Jr. 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Molecular Microbiology,* **4:** 1911-1919.

Spargo B.J., Crowe L.M., Ioneda T., Beaman B.L., Crowe J.H. 1991. Cord-factor (α -α' -trehalose 6,6' dimycolate) inhibits fusion between phospholipid vesicles. *Proceedings of the National Academy of Sciences of the United States of America,* **88:** 737-740.

Speirs R.J., Welch J.T., Cynamon M.H. 1995. Activity of n-propyl pyrazinoate against pyrazinamide-resistant *Mycobacterium tuberculosis*: Investigations into mechanism of action of and mechanism of resistance to pyrazinamide. *Antimicrobial Agents and Chemotherapy,* **39:** 1269-1271.

Stelandre M., Bosseloir Y., De Bruyn J., Maes P., Content J. 1992. Cloning and sequence analysis of the gene encoding an NADP-dependent alcohol dehydrogenase in *Mycobacterium bovis* BCG. *Gene,* **121:** 79-86.

Stottmeier K.D., Beam R.E., Kubica G.P. 1967. Determination of drug susceptibility of mycobacteria to pyrazinamide in 7H10 agar. *American Review of Respiratory Disease,* **95:** 1072-1075.

Stover C.K., Bansal G.P., Hanson M.S., Burlein J.E., Palaszynski S.R., Young J.F., Koenig S., Young D.B., Sadziene A., Barbour A.G. 1993. Protective immunity elicited by recombinant *bacille Calmette-Guerin* (BCG) expressing outer surface protein A (OspA) lipoprotein: A candidate Lyme disease vaccine. *Journal of Experimental Medicine,* **178:** 197-209.

Stover C.K., De la Cruz V.F., Fuerst T.R., Burlein J.E., Benson L.A., Bennett L.T., Bansal G.P., Young J.F., Lee M.H., Hatfull G.F., Snapper S.B., Barletta R.G., Jacobs W.R.J., Bloom B.R. 1991. New use of BCG for recombinant vaccines. *Nature,* **351:** 456-460.

Subramanyam V.R., Pal B.B., Mohanty K.K. 1989. Inducibility and stability of auxotrophic mutations in *Mycobacterium fortuitum, M. smegmatis* and *M. vaccae. Letters in Applied Microbiology,* **8:** 161-164.

Takayama K., Armstrong E.L. 1976. Isolation, characterisation and function of 6-mycolyl-6'-acetyltrehalose in the H37Ra strain of *Mycobacterium tuberculosis. Biochemistry,* **15:** 441-447.

Takayama K., Armstrong E.L., Davidson L.A., Kunugi K.A., Kilburn J.O. 1978. Effect of low temperature on growth, viability and synthesis of mycolic acids of *Mycobacterium tuberculosis* strain H37Ra. *American Review of Respiratory Disease,* **118**: 113-117.

Takayama K., Goldman D.S. 1970. Enzymatic synthesis of mannosyl-1-phosphoryldecaprenol by a cell-free system of *Mycobacterium tuberculosis. Journal of Biological Chemistry,* **245:** 6251-6257.

Takayama K., Kilburn J.O. 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis. Antimicrobial Agents and Chemotherapy,* **33:** 1493-1499.

Takayama K., Qureshi N., Schnoes H.K. 1978. Isolation and characterisation of the monounsaturated long chain fatty acids of *Mycobacterium tuberculosis*. *Lipids*, **13:** 575-579.

Takayama K., Schnoes H.K., Semmlier E.J. 1973. Characterization of the alkali-stable mannophospholipids of *Mycobacterium smegmatis*. *Biochimica et Biophysica Acta*, **136:** 212-221.

Takayama K., Wang L., David H.L. 1972. Effect of isoniazid on the *in vivo* mycolic acid synthesis, cell growth and viability of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, **2:** 29-35.

Takiff H.E., Salazar L., Guerrero C., Philipp W., Wai Mun Huang;, Kreiswirth B., Cole S.T., Jacobs W.R., Jr., Telenti A. 1994. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyr*A and *gyr*B genes and detection of quinolone resistance mutations. *Antimicrobial Agents and Chemotherapy*, **38:** 773-780.

Taneja R., Malik U., Khuller G.K. 1979. Effect of growth temperature on the lipid composition of *Mycobacterium smegmatis* ATCC 607. *Journal of General Microbiology*, **113:** 413-416.

Tassell S.K., Pourshafie M., Wright E.L., Richmond M.G., Barrow W.W. 1991. Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids. *Infection and Immunity*, **60:** 706-711.

Telenti A., Lowrie D., Matter L., Imboden P., Cole S., Schopfer K., Marchesi F., Colston M.J., Bodmer T. 1993. Detection of rifampicin-resistance mutation in *Mycobacterium tuberculosis*. *Lancet*, **341:** 647-650.

Thole J.E.R., Dauwerse H.G., Das P.K., Groothuis D.G., Berwald L.G., Tiesjema R.H., Van Embden J.D.A. 1985. Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli.*. *Infection and Immunity*, **50:** 800-806.

Thole J.E.R., Schoningh R., Janson A.A.M., Garbe T., Cornelisse Y.E., Clark-Curtiss J.E., Kolk A.H.J., Ottenhoff T.H.M., DeVries R.R.P., Abou-Zeid C. 1992. Molecular and immunological analysis of fibronectin-binding protein antigen secreted by *Mycobacterium leprae*. *Molecular Microbiology*, **6:** 153-163.

Thurman P.F., Chai W., Rosankiewicz J.R., Rogers H.J., Lawson A.M., Draper P. 1993. Possible intermediates in the biosynthesis of mycoside B by *Mycobacterium microti*. *European Journal of Biochemistry*, **212:** 705-711.

Timm J., Perilli M.G., Duez C., Trias J., Orefici G., Fattorini L., Amicosante G.,

Oratore A., Joris B., Frere J.M., Pugsley A.P., Gicquel B. 1994. Transcription and expression analysis, using *lac*Z and *pho*A gene fusions, of *Mycobacterium fortuitum* beta -lactamase genes cloned from a natural isolate and a high-level beta -lactamase producer. *Molecular Microbiology,* **12:** 491-504.

Trias J., Benz J. 1994. Permeability of the cell wall of *Mycobacterium smegmatis*. *Molecular Microbiology,* **14:** 283-290.

Trias J., Benz R. 1993. Characterization of the channel formed by the mycobacterial porin in lipid bilayer membranes. Demonstration of voltage gating and of negative point charges at the channel mouth. *Journal of Biological Chemistry,* **268:** 6234-6240.

Trias J., Jarlier V., Benz R. 1992. Porins in the cell wall of mycobacteria. *Science,* **258:** 1479-1481.

Vega-Lopez F., Stoker N.G., Locniskar M.F., Dockrell H.M., Grant K.A., McAdam K.P.W.J. 1988. Recognition of mycobacterial antigens by sera from patients with leprosy. *Journal of Clinical Microbiology,* **26:** 2472-2479.

Venisse A., Riviere M., Vercauteren J., Puzo G. 1995. Structural analysis of the mannan region of lipoarabinomannan from *Mycobacterium bovis* BCG. Heterogeneity in phosphorylation state. *Journal of Biological Chemistry,* **270:** 15012-15021.

Vestal A.L., Kubica G.P. 1966. Differential colony characteristics of mycobacteria on Middlebrook and Cohn 7H10 agar-based medium. *American Review of Respiratory Disease,* **94**: 247-252.

Wheeler P.R., Besra G.S., Minnikin D.E., Ratledge C. 1993a. Stimulation of mycolic acid biosynthesis by incorporation of *cis*-tetracos-5-enoic acid in a cell wall preparation from *Mycobacterium smegmatis*. *Biochimica et Biophysica Acta,* **1167:** 182-188.

Wheeler P.R., Besra G.S., Minnikin D.E., Ratledge C. 1993b. Inhibition of mycolic acid biosynthesis in a cell wall preparation from *Mycobacterium smegmatis* by methyl 4-(2-octadecylcyclpropen-1-yl) butanoate, a structural analogue of a key precursor. *Letters in Applied Microbiology,* **17:** 33-36.

Wilson T.M., De Lisle G.W., Collins D.M. 1995. Effect of *inh*A and *kat*G on isoniazid resistance and virulence of *Mycobacterium bovis*. *Molecular Microbiology,* **15:** 1009-1015.

Winder F.G. 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of the mycobacteria. In *Ratledge C., Stanford J.* (ed.), *The Biology of the Mycobacteria.* Academic Press, London. pp. 354-440.

Winter N., Lagranderie M., Rauzier J., Timm J., Leclerc C., Guy B., Kieny M.P., Gheorgiu M., Gicquel B. 1991. Expression of heterologous genes in *Mycobacterium bovis* BCG: Induction of a cellular response against HIV-1 Nef protein. *Gene,* **109:** 47-54.

Wolucka B.A., De Hoffmann E. 1995. The presence of beta-D-ribosyl-1-monophosphodecaprenol in mycobacteria. *Journal of Biological Chemistry,* **270:** 20151-20155.

Wolucka B.A., McNeil M.R., De Hoffmann E., Chojnacki T., Brennan P.J. 1994. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. *Journal of Biological Chemistry,* **269:** 23328-23335.

Yamada T., Mizuguchi Y., Isono S., Isono K. 1992. Genetic and biochemical analysis of ribosomal proteins of minocycline-susceptible and -resistant *Mycobacterium smegmatis*. *Microbiology and Immunology,* **36:** 139-148.

Young D.B. 1994. Strategies for new drug development. In *Bloom B.R.* (ed.), *Tuberculosis: Pathogenesis, protection and control.* American Society for Microbiology, Washington DC. pp. 559-568.

Young D.B., Garbe T.R. 1991. Lipoprotein antigens of *Mycobacterium tuberculosis*. *Infection and Immunity,* **142:** 55-65.

Young D.B., Kent L., Young R.A. 1987. Screening of a recombinant mycobacterial DNA library with polyclonal antiserum and molecular weight analysis of expressed antigens. *Infection and Immunity,* **55:** 1421-1425.

Young L.S., Wormser G.P. 1994. The resurgence of tuberculosis. *Scandinavian Journal of Infectious Diseases,Supplement,* 9-19.

Young R.A., Bloom B.R., Grossinsky C.M., Ivany J., Thomas D., Davis R.W. 1985a. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. *Proceedings of the National Academy of Sciences of the United States of America,* **82:** 2583-2587.

Young R.A., Mehra V., Sweetser D., Buchanan T., Clark-Curtiss J.E., Davis R.W., Bloom B.R. 1985b. Genes for the major protein antigens of the leprosy parasite *Mycobacterium leprae.. Nature,* **316:** 450-452.

Yuan Y., Lee R.E., Besra G.S., Belisle J.T., Barry CE I.I.I. 1995. Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America,* **92:** 6630-6634.

Zhang L., English D., Andersen B.R. 1991b. Activation of human neutrophils by *Mycobacterium tuberculosis-derived* sulfolipid-1. *Journal of Immunology,* **146:** 2730-2736.

Zhang Y., Garbe T., Young D. 1993. Transformation with katG restores isoniazid-sensitivity in *Mycobacterium tuberculosis* isolates resistant to a range of drug concentrations. *Molecular Microbiology,* **8:** 521-524.

Zhang Y., Garcia M.J., Lathigra R., Allen B., Moreno C., Van Embden J.D.A., Young D. 1992. Alterations in the superoxide dismutase gene of an isoniazid-resistant strain of *Mycobacterium tuberculosis. Infection and Immunity,* **60:** 2160-2165.

Zhang Y., Heym B., Allen B., Young D., Cole S. 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis. Nature,* **358:** 591-593.

Zhang Y., Lathigra R., Garbe T., Catty D., Young D. 1991a. Genetic analysis of superoxide dismutase, the 23 kilodalton antigen of *Mycobacterium tuberculosis. Molecular Microbiology,* **5:** 381-391.

APPENDUM

5.1 *M. LEPRAE* GENES IDENTIFIED THAT MAY BE RELEVANT TO PERMEABILITY

The permeability of bacterial cell envelopes are defined by the structure of their cytoplasmic and outer membranes and the transport systems found within these membranes. The relatively low permeability of the mycobacterial cell envelope is believed to be effected by the complex cell wall structure but may also be effected by low activity of transport systems e.g. specific uptake proteins and porins and the presence of efflux systems in the cytoplasmic cell membrane. The effects on permeability made by selected *M. leprae* gene products may be investigated by cloning the genes by PCR, overexpressing them in a model organism such as *M.smegmatis* or *M. bovis* BCG and measuring their permeability (5.4). Alternatively the homologous genes could be cloned from the model organisms by degenerative PCR, knockouts made using homologous recombination and the permeability of the mutated strains could then be investigated.

General alteration of permeability is most likely to be caused by the mutation of genes encoding products involved in the formation of the cell wall. *M.leprae* homologues of the peptidoglycan *mur* genes (*B,C,D,G* and *Z*) would be possible candidates for investigation as would genes involved in glucose metabolism, such as *glgC*, as glucose is thought to be a precursor of arabinogalactan (Silve *et al.*, 1993). The gene products involved in the formation of mycolic acids may effect the permeability e.g. the *M. leprae* homologue of *cma2* which forms the proximal cyclopropane ring in mycolates.. Other *M. leprae* homologues which may be involved in mycolate biosynthesis include the *inhA* gene encoding an enoyl-ACP-reductase, the fatty acid biosynthesis and synthase genes *fab B, D, G* and *J* and *u13s, u13t* and *u13u* respectively (as the short chain fatty acids are thought to be precursors of mycolates) and the *xclC* long chain fatty acid ligase gene. The *M. leprae* mycocerosate synthase genes *masA, B* and *C* may also be worth investigating as an altered expression could lead to a change in permeabilty.

The *M. leprae* genome sequencing project has identified sixty seven ORFs which show significant homology to genes in other species to encoding transport proteins, found in the cytoplasmic membrane or in the 'periplasmic' space. Permeability to specific substrates such as cystidine, maltose and phosphate could be investigated by the altered expression of the *M. leprae* homologue encoding their transport

systems *cysA*, *malf* and *g* and *phoS*, *T*, *U*, *W*, *X* and *Y* respectively. The investigation of possible efflux proteins would be of particular interest as they would affect the apparent permeability to certain antibiotics; the *M. leprae* homologues of efflux proteins *drrA* and *ddrB* as well the *arsA* gene which extrude danorubicin and arsenicals respectively, are possible candidates. No *M. leprae* porin homologues have been identified as yet and it is possible that they do not contain any.

5.2 ALTERNATIVE SECRETORY PATHWAYS IN BACTERIA

Bacterial proteins destined for secretion usually contain a cleavable hydrophobic signal peptide at the N-terminus of the polypeptide that target them to the cytoplasmic membrane via the secretion apparatus encoded by the *sec* genes, the classical secretory pathway. Signal peptide independant pathways also exist in bacteria, particularly for toxins e.g. the related *E.coli* haemolysin and *Pasturella hemolytica* leucotoxin and unrelatd *E.coli* colicin V. The targeting signals for all these toxins have been located in the last 100aa of the carboxy terminus which are predicted to form amphipathic α-helices. The related toxins also contain conserved repeat domains of a glycine rich sequence GGXGXD located close to the carboxy-terminal sequence, thought to be involved in correct folding for passage through the membranes. The secretion and translocation of these Gram-negative toxins occurs as a single event across both membranes without stopping in the periplasmic space at the junction of the two membrane (Wandersman, 1992).

The superoxide dismutase and ESAT6 proteins of *M. tuberculosis* are apparently secreted into the culture supernatant of early logarithmic cultures but contain no signal peptides. It is possible they are released by damaged cells within the culture or they contain as yet undefined signal sequences such as the carboxyterminal or internal sequences of toxins such as haemolysin which allow their secretion.

5.3 THE STRUCTURE AND FUNCTION OF PORINS AND EFFLUX PROTEINS

5.3.1 PORINS

Porins are transport proteins (Nikaido, 1992) found in the outer membranes of Gram-negative bacteria which have recently been detected in the cell walls of the Gram-positive *Mycobacterium* (Trias *et al.*, 1992; Trias and Benz, 1993 and 1994). 'Classical' non-specific porins of the enteric bacteria studied

include the OmpF, OmpC and PhoE porins of *E.coli*. These porins are 34-50kDa in size and exist as trimeric complexes, each monomer producing a water filled channel. The porin proteins are not hydrophobic, instead the monomeric polypeptide chain traverses the membrane as antiparallel β-strands which form a β-barrel thus creating a channel. Loops extend from the β-barrel of the polypeptides into the periplasmic and external side of the outer membrane and one of the external loops appears to fold back into the channel producing a narrowing of the channel opening to a diameter of approximately 1nm (Nikaido and Saier, 1992). Specific porin-like channels have similar structures but with specific ligand binding sites. Monomeric porins found have been shown to have similar β-sheet structures e.g. the non-specific OprF of *P. aeruginosa* (Nikaido, 1992).

Porins have been shown to exist in both *M. chelonae* and *M. smegmatis* and are thought to exist in *M. tuberculosis* (Trias *et al.*, 1992; Trias and Benz, 1994; Brennan and Nikaido, 1995). The *M. cheolonae* protein is known to be 59kDa, present in relatively small amounts and the only porin present. *M. smegmatis* and *M. chelonae* porins have a relatively large pore size, 3nm and 2nm repectively, with negative point charges at their openings of 3.5 and 2.5 respectively. The exact structure and the presence of β-sheets in mycobacterial porins has not yet been established but the *M. chelonae* porin is believed to be monomeric.

The function of porins is to transport specific or non-specific substrates, nutrients and waste products across the outer membranes. The majority of Gram-negative bacteria contain more than one type of porin which may be specific or general diffusion porins, trimeric or monomeric and are often the most abundant outer membrane proteins present. The penetration rates of solutes through general diffusion channels is inversely proportional to the size of the molecules and reduced for hydrophobic molecules. General porins may also display a preference for cations or anions as a result of charges present on the diameter constricting loops at the channel openings e.g. *M. smegmatis*, *M. chelonae* OmpC and OmpF channels prefer cations. Mycobacterial porins are apparently less active than the porins of other bacteria e.g. *E.coli* this may be because they are monomeric and present in small numbers (Trias and Benz, 1994).

5.3.2 EFFLUX PROTEINS

There are four families of efflux systems in bacteria; the major facilitator (MF) family, the resistance-nodulation-division-family (RND), the staphylococcal multidrug resistance family (Smr) and the ABC type systems. The MF, RND and Smr systems consist of a single cytoplasmic membrane protein that extrudes drugs using a proton motive force i.e. as an H+ antiporter. The ABC system consists of two transmembrane transporter proteins and two ATP-binding subunits which function as ATPases. The cytoplasmic membrane proteins contain an even number of hydrophobic transmembrane α helices, only 4 in Smr transporters. MF efflux proteins differ from nutrient transporters in that they contain the consensus sequence G-X-X-X-G-P-X-X-G-G. The function of efflux proteins is to extrude toxic substances, antibiotics and proteins produced by the bacteria.

An efflux protein sequence, *lfrA*, has recently been identified in *M. smegmatis* by selection for increased resistance to fluoroquinones (Takiff *et al.*, 1996). The protein is predicted to be 504 amino acids long and to contain 14 transmembrane helices, as predicted for the encoded streptomyces MF efflux proteins of *quaC*, *actII*, *tcmA* and *mmr* (35-27% identity and 60-52% similarity). The LfrA protein of *M. smegmatis* extrudes ciprofloxacin, ofloxacin, levofloxacin, EtBr, acriflavine and CTAB but not the hydrophobic fluoroquinolones such as sparfloxacin, similar substrates as the efflux proteins to which it is homologous. However, *lfrA* was not mutated and conferred resistance by increased expression from a vector promoter. It is possible that the resistant *M. smegmatis* strain has a mutation in another pump or in a regulatory locus e.g. *mar* which would increase the expression of LfrA or the relevant efflux pump.

5.4 METHODS FOR MEASURING THE PERMEABILITY OF *M. SMEGMATIS* MUTANTS

5.4.1 MEASUREMENT OF THE PERMEABILITY TO B-LACTAM CEPHALOSPORINS

The permeability coefficient, P, a measure of permeability, is determined by measuring the comparative rates of hydrolysis of β-lactam cephalosporins by intact *M. smegmatis* cells and cell extracts (Trias and Benz, 1994), using a similar method to that developed for *M. chelonae* (Jarlier and Nikaido, 1990). The method is based on the principles that 1) the net rate of diffusion across the cell wall (V_1) should occur according to Fick first law of passive diffusion 2) that the rate of hydrolysis of the cephalosporin in the space between the cell wall and the cell membrane (V_2) occurs according to Michaelis-Menten kinetics

and 3) at steady state the net rate of drug influx should be equal to the rate of drug degradation i.e. $V_1=V_2$.

The hydrolysis of the cephalosporins leads to the disruption of the β-lactam ring and the loss of u.v. absorption in the 260nm range allowing the rate of hydrolysis to be measured spectrophotometrically. To measure rates of hydrolysis of cephalosporins (cephaloride, cephalothin, cefamandole) intact cells were resuspended in PIPES buffer pH6-6.5 to a turbidity of 100Kletts units and a final 0.5mM concentration of cephalosporin, the optical density of the solution was measured over 40min at 25°C. The β-lactamase activity of supernatants and cell extracts obtained from a portion of the original cell suspension were assessed in the same way. The Michaelis-Menten constants, K_m for each cephalosporin used were determined by measuring their rates of hydrolysis with increasing concentrations. The internal rate of hydrolysis of cephalosporins, V_2 ,was calculated in μmol/min/mg from the rate of change of A_{260} with the sonicated cell extract and the V_{max} was determined. As $V_2=V_{max}$ Ci/(K_m+Ci) Ci the internal concentration of the drug could then be determined. From Fick's law of diffusion V_1=PA (Co-Ci) where A is the cell surface/unit weight of cells and Co is the external concentration of the drug, V_1 was calculated in μmol/min/mg from the rate of change of A_{260} with the intact cells, so P, could be determined.

5.4.2 MEASUREMENT OF PERMEABILITY TO SMALL HYDROPHILIC MOLECULES

This method was employed to measure the permeability of *M. chelonae* (Jarlier and Nikaido, 1990) but may also be feasible for *M. smegmatis* strains. Permeability to small hydrophobic molecules can be estimated from their uptake kinetics. Cell suspensions are diluted in 2mM PIPES-NaOH (pH6.5) and 0.5mM $MgCl_2$ to give a final turbidity of 1,000 Klett units. Increasing final concentrations of [^{14}C] glucose, [^{3}H] glycerol, [^{14}C] glycine and [H] leucine were added to samples of the cell suspension. Samples were removed at regular time intervals, washed, dried and scintillation counted. The initial cell suspension was dried and weighed to measure the exact concentration of cells in the assay. Rates of uptakes were determined in μmoles/mg/min, the V_{max} and K_m of overall transport were calculated graphically from rates of uptake and at different concentrations of substrates. The permeability coefficients were calculated assuming that the overall transport system was functioning at half maximal capacity i.e. $V_{max}/2$ and Co=K_m and that Ci is negligible due active transport therefore P = ($V_{max}/2/A \times K_m$).

a)

i

```
16 LAGQLLDHVELRELCNPEVITATERQLQNLLDNRVGHDTEAIADLLRLLGPLTAADAAAWSNGCSD 81
   L   LL  V+  EL +P+VI   E +LQ L   R     E + DLLR LGP+T  D A    G S+
3630 LLRNLLGQVDPGELLDPQVIRQVEEELQRLAPGRRAKGEEGLFDLLRELGPMTVEDLAQRHTGSSE 3827
```

rnt

b)

a/c

```
16 WRSRNSRNSSQRNACRMCRGPGGS 39          30 CRMCRGPGGSVL 41
   WR+R  R   ++R A   C G   S              -CR+CRG G  VL
13147 WRARARRARARRRARCACSGARSS 13218   1640 CRVCRGRGS*VL 1675
```

ryanodine receptor gene

a/c

```
34 RGPGGSVLRSRRRVGLGGR 52              9 RPATPTGWRSRNSR 22
   RGPGG  +R R R+GLGGR                   RPATP G R   SR
13922 RGPGGCRVRGRLRLGLGGR 13978     4070 RPATPGGGRGPGSR 4111
```

ryanodine receptor gene

c)

b

```
12 ARRSCAAAPRMGCPGP 27          10 VWARRSCAAAP 20
   ARRS A+ PR G   P                VW  R C AAP
16783 ARRSVASRPRAGRSAP 16830    880 VWTARRCTAAP 912
```

S. lincolnensis lincomycin gene

b

```
11 WARRSCAAAPRMGCPG 26          26 GPDGVAPLAG 35
   WARR+   APR G  G                GPDG+AP+ G
15473 WARRTGRGAPRCGSTG 15520   16981 GPDGLAPVGG 17010
```

S. lincolnensis lincomycin gene

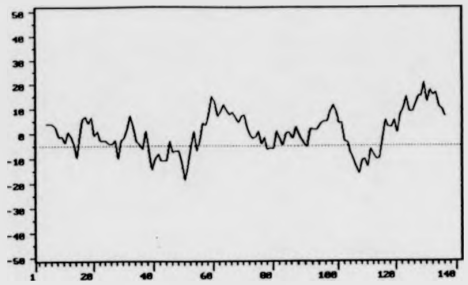
Fig. 50 **Alignments of the three complete ORFs *i*, *a/c* and *b* in the complementing region with possible protein homologues**

a) Shows the alignment of 65aa of a putative protein of 141aa from ORF *i* with the long helicase-related region of the *E.coli* RnaseT (rnt) gene, with a 40% identitiy and a 51% similarity in this region. It is possible that ORF *i* encodes an area of helicase activity rather than encoding a protein, although the region of homology is small and may not be significant.

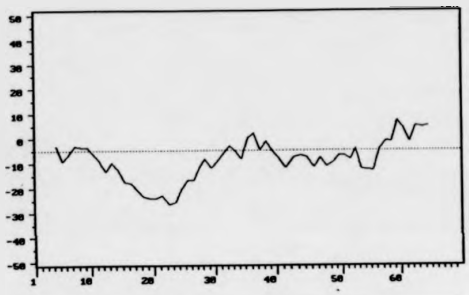
b) Shows the alignment of several short regions of 70aa the putative polypeptide encoded by *a/c* with the rabbit skeletal muscle mRNA for ryanodine receptor

c) Shows the alignment of the 111aa putative protein encoded by ORF *b* with several short regions of of the protein with the ***Streptococcus lincolnensis*** lincomycin production genes, presumably pulled out due to the GC rich DNA of streptomyces.

a)



b)



c)

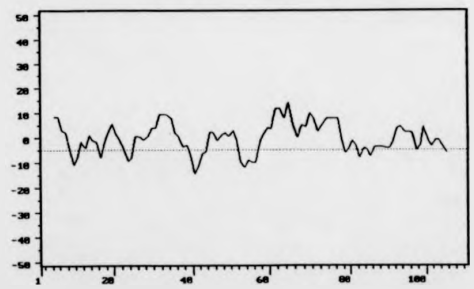


Fig 51. **Hydrophobicity analysis of the three complete OFRs *i*, *a/c* and *b* in the complementing region of *M. leprae* DNA.**

a) Shows the hydrophobicity plot of putative protein i , with one hydrophobic region itis predicted to be an integral membrane protein, by the PC gene program.

b) Shows the hydrophobicty plot of putative protein a/c predicted to be a peripheral protein associated with cell membrane

c) Shows the hydrophobicity plot of putative protein b predicted to be a peripheral protein, associated with the cell membrane.

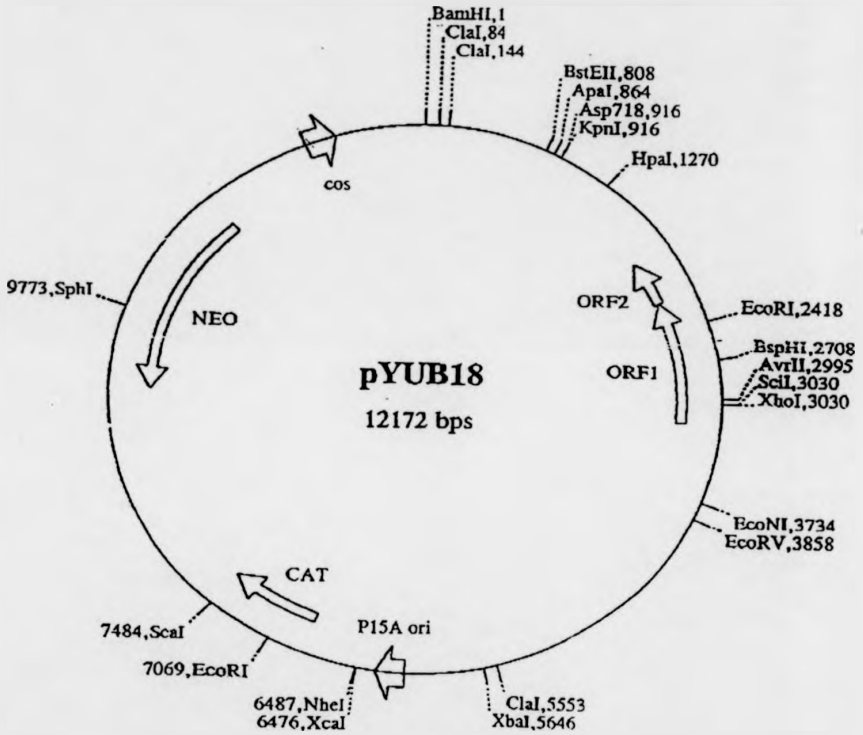
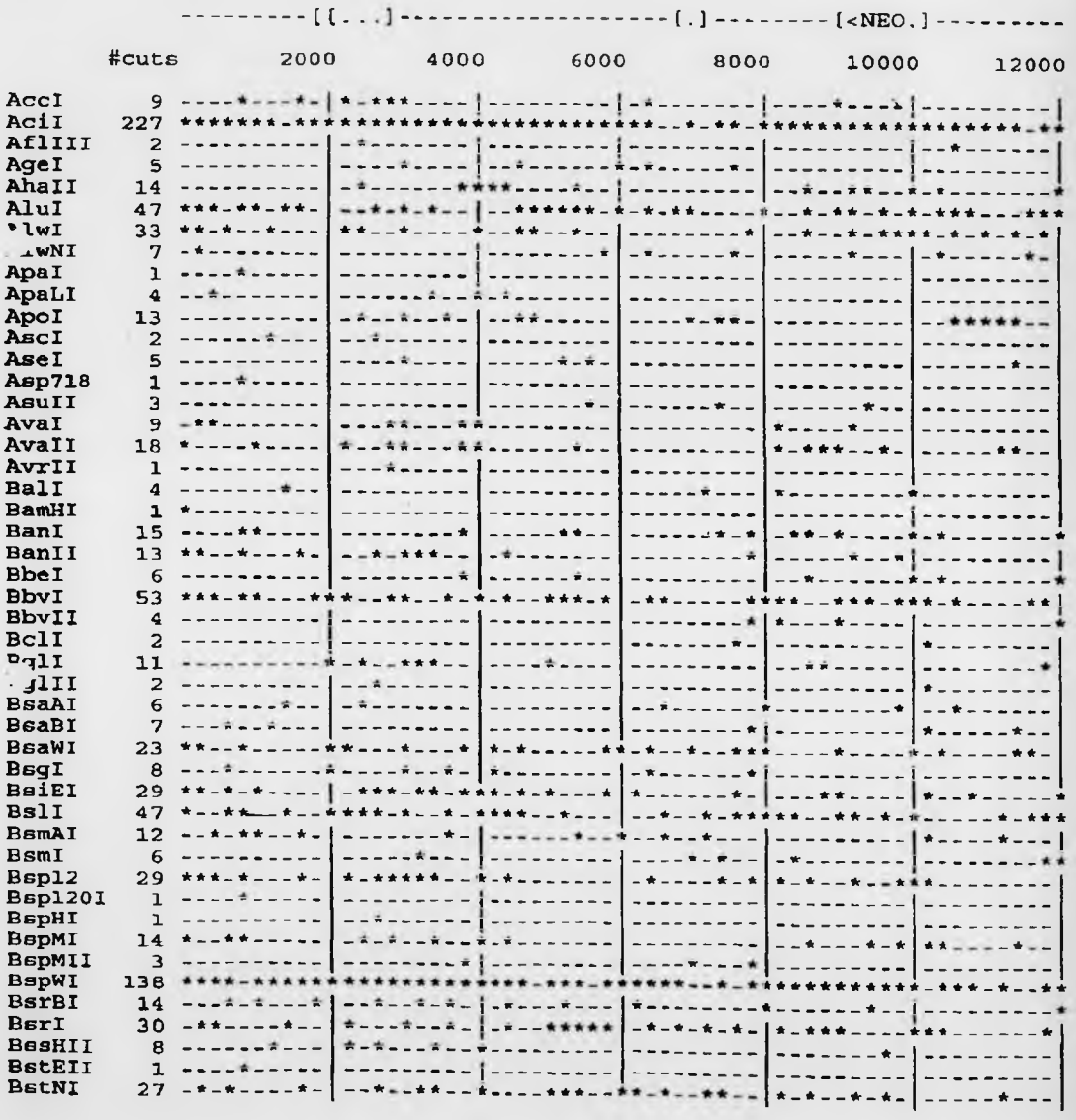
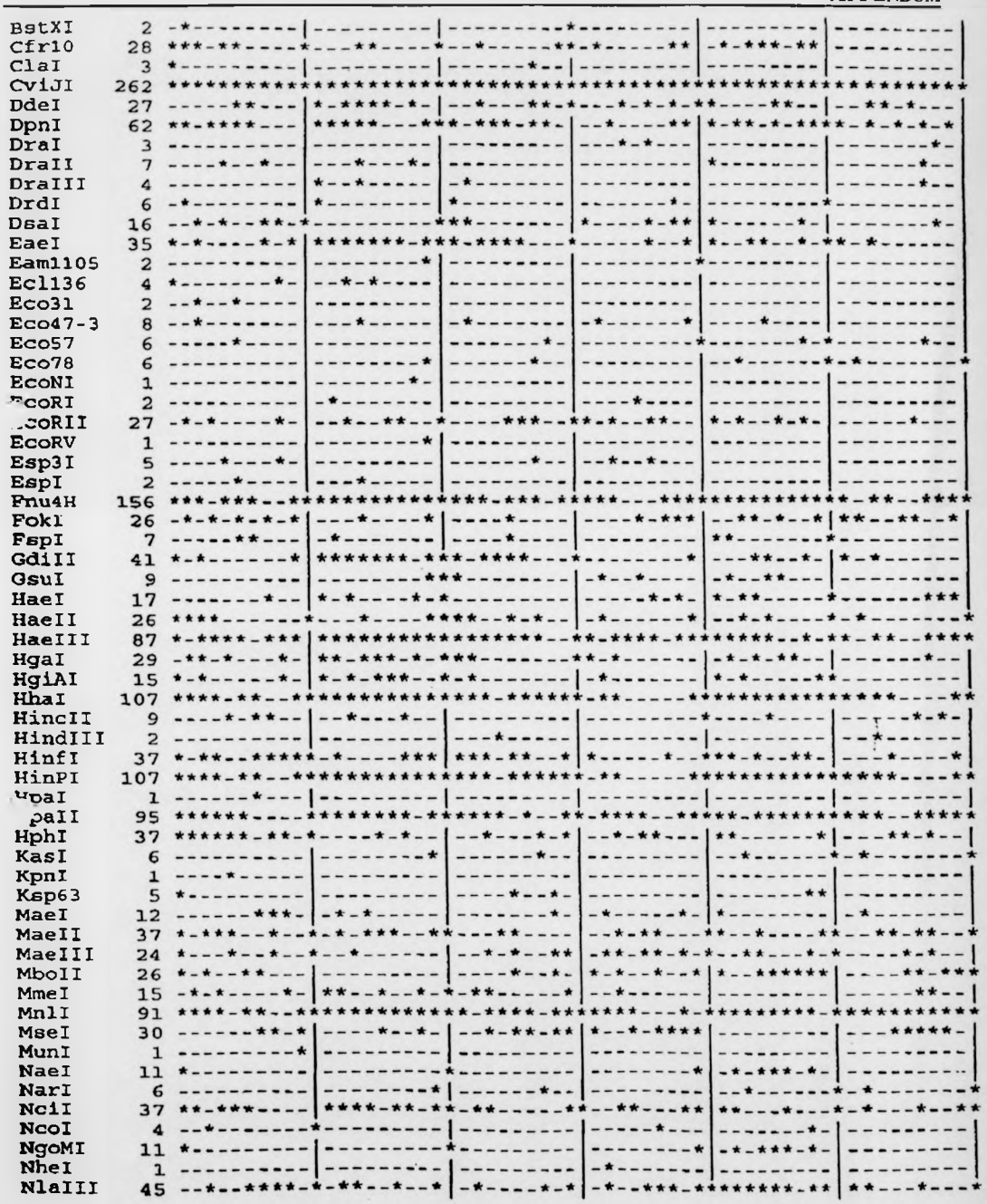
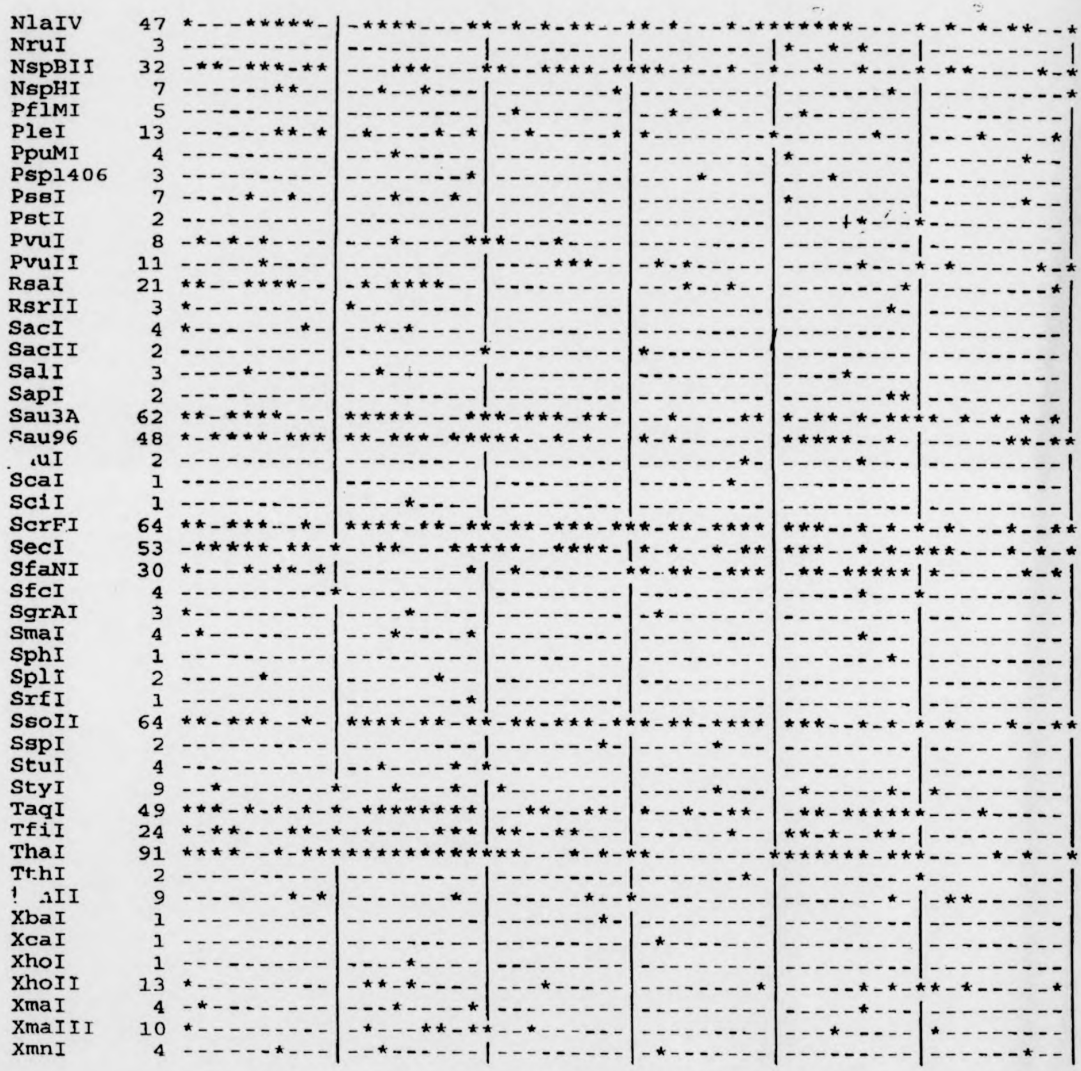


Fig.52 a) **A restriction map of pYUB18**

Fig. 52 b) **Other restriction sites of pYUB18**






NlaIV 47
NruI 3
NspBII 32
NspHI 7
PflMI 5
PleI 13
PpuMI 4
Psp1406 3
PssI 7
PstI 2
PvuI 8
PvuII 11
RsaI 21
RsrII 3
SacI 4
SacII 2
SalI 3
SapI 2
Sau3A 62
Sau96 48
ScaI 2
SciI 1
ScrFI 1
SecI 64
SfaNI 53
SfcI 30
SgrAI 4
SmaI 3
SphI 4
SplI 1
SrfI 2
SspI 1
StuI 2
StyI 4
TaqI 9
TfiI 49
ThaI 24
TthI 91
XbaI 2
XcaI 1
XhoI 1
XhoII 1
XmaI 13
XmaIII 4
XmnI 10
4