A RIBOSOMAL GENE MUTATION IN STREPTOMYCIN RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES

JOHN WINGFIELD DOUGLASS

A thesis submitted in fulfilment of the requirements for part III of the degree M.Med (Clinical Pathology)

April 1994

ACKNOWLEDGEMENTS

I extend my thanks to my supervisor, Professor Lafras Steyn, who willingly took me into the molecular medical microbiology laboratory, taught me, and guided me through this project. To Professor Forder, for the encouragement he has extended, both during my time in the Medical Microbiology department and after. Thanks also to the researchers with whom I worked, who taught me the techniques and helped to clarify problematic issues in discussion (Karen, Patrick, Niki, Bruce, Gay and Fernanda).

LIST OF ABBREVIATIONS

α	Alpha
ARMS	Amplification refractory mutation system
bp	Base pairs
β	Beta
°C	Degrees Celsius
dATP (A)	Doxyadenosine triphosphate
dCTP (C)	Deoxycytidine triphosphate
dGTP (G)	Deoxyguanosine triphosphate
dTTP (T)	Doxythymidine triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
g	Gram
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-thio-galactoside
kb	Kilobase pair
M	Molar
m	Milli-
n	Nano-
PAS	para-aminosalicylate
PCR	Polymerase chain reaction
p-	Pico-
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
^{35}S	dATP radioactively labelled with Sulphur-35
SDS	Sodium dodecyl sulphate
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	Tris-EDTA-sodium chloride
Tris	Tris (hydroxymethyl)aminomethane
μ	Micro
UV	Ultraviolet

V	Volt
V/V	Volume in volume
W	Watt
X-GAL	5-bromo-4-chloro-3-indolyl-β-galactoside

ABSTRACT

Tuberculosis is a disease which continues to be a major cause of morbidity and mortality in the third world. It has also resurged in first world countries linked to the Human Immunodeficiency Virus epidemic. Added to this is the increasing prevalence of multidrug-resistant tuberculosis, related primarily to erratic therapy. When multidrug-resistant isolates are found in association with the acquired immunodeficiency syndrome, a high case-fatality rate is seen. This formidable combination of diseases has caused great concern, and has resulted in renewed interest in the mechanisms of mycobacterial drug resistance.

Streptomycin is one of the oldest and best studied antimicrobial agents; the ribosome being an important target of streptomycin action, if not the most important target. Streptomycin exerts it's therapeutic action via binding to the smaller subunit of the ribosome (30S). The molecules involved in the site of streptomycin binding and action are the 16S rRNA and a number of ribosomal proteins, particularly protein S12. The 16S RNA forms the backbone of the structure of the 30S subunit, with a number of regions, separated by several hundreds of bases, all implicated in binding to streptomycin. One region, in particular, has been shown to be involved in this interaction. This is the 915 region which we investigated in streptomycin resistant *Mycobacterium tuberculosis* isolates.

A 913 A→G mutation was identified in 3 of the 41 multidrug-resistant *M. tuberculosis* isolates investigated using the PCR based amplification refractory mutation system (ARMS) to screen the isolates for the presence of either A or G at the 913 position.

Twenty-five of the isolates were sequenced, either after cloning the region into a vector, or directly from PCR generated DNA fragments. This was done to determine whether any other mutations, which might affect the streptomycin binding, were present in 915 region, as well as to confirm the ARMS data.

The ARMS method of point mutation detection offers the possibility of revolutionising drug sensitivity testing where chromosomal mutations occur in genes encoding proteins or rRNA that bind antimicrobial drugs.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF ABBREVIATIONS iii

ABSTRACT v

TABLE OF CONTENTS vi

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction 1

1.2 Tuberculosis 1

1.2.1 History 1

1.2.2 Microbiology 2

1.2.2.1 Classification 2

1.2.3 Laboratory diagnosis 4

1.2.3.1 Microscopy 4

1.2.3.2 Culture 4

1.2.3.3 Molecular techniques 4

1.2.4 Therapy 5

1.2.5 Epidemiology 5

1.3 Streptomycin 6

1.3.1 History 6

1.3.2 Structure 6

1.3.3 Mechanism of action and resistance 7

1.3.3.1 Introduction 7

1.3.3.2 Transport across the cell wall and membrane 7

1.3.3.3 Enzymatic modification of aminoglycosides 8

1.3.3.4 The streptomycin-ribosome interaction 8

1.3.3.4.1 The 30S proteins 8

1.3.3.4.2 The 16S rRNA 11

1.3.3.4.2.1 Cross-linking experiments 11

1.3.3.4.2.2 RNA footprinting 12

1.3.3.4.3 Ribosomal RNA mutations 13

1.3.3.4.3.1 Position 912 13

1.3.3.4.3.2 Position 913 and 915 14

1.3.3.4.3.3 Position 13 and 13+914 15

1.3.3.4.3.4 The 530 loop 15

1.3.3.4.4 Involvement of the 50S subunit 16
1.4 Conclusions and aims of this study 16

CHAPTER 2: MATERIALS
2.1 Plasmid and host cells 17
2.2 *M. tuberculosis* isolates 17
2.3 Enzymes, chemicals, solutions and media 17
2.3.1 Enzymes and kits 17
2.3.2 Chemicals and reagents 18
2.3.3 Media and solutions 19
2.3.4 Suppliers 20

CHAPTER 3: CLONING OF THE 915 REGION OF THE 16S rRNA GENE OF A STREPTOMYCIN RESISTANT ISOLATE OF *M. TUBERCULOSIS*
3.1 Introduction 21
3.2 Methods 21
3.2.1 Preparation of mycobacterial DNA 21
3.2.2 PCR amplification of a 336bp fragment of the 16S rRNA gene 22
3.2.3 Large scale plasmid preparation ('maxiprep') 23
3.2.3.1 Isopycnic gradient centrifugation 23
3.2.3.2 Removal of ethidium bromide and CsCl 24
3.2.4 Restriction enzyme digestion 25
3.2.5 Agarose gel electrophoresis 25
3.2.6 Preparation of vector for ligation 26
3.2.6.1 Construction of a T-tailed vector 26
3.2.7 Ligation reaction 27
3.2.8 Preparation of competent cells 27
3.2.9 Transfomation of E. coli LK111 cells 28
3.2.10 Small scale plasmid preperation ('miniprep') 28
3.2.11 Screening transformants for recombinant plasmids 29
3.2.11.1 *Eco*RI linearisation 29
3.2.11.2 *Hin*dIII/*Eco*RI digestion 30
3.3 Results 30

CHAPTER 4: DNA SEQUENCING OF THE 915 REGION OF THE 16S rRNA GENE OF A STREPTOMYCIN RESISTANT ISOLATE OF *M. TUBERCULOSIS*

4.1 Summary 34
4.2 Methods 34
4.2.1 Plasmid extraction 34
4.2.2 Sequencing reaction 35
4.2.3 Polyacrylamide gel electrophoresis 37
4.3 Results 38

CHAPTER 5: SCREENING STREPTOMYCIN RESISTANT *M. TUBERCULOSIS* ISOLATES BY THE AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)

5.1 Introdution 41
5.2 Methods 41
5.2.1 The amplification refractory mutation system 41
5.2.1.1 Optimisation 42
5.2.1.1.1 Comparison of two annealing temperatures on specificity 42
5.2.1.1.2 Comparison of two denaturation temperatures on sensitivity 43
5.2.1.1.3 Comparison of various concentrations of template and two concentrations of primers 43
5.2.1.1.4 Comparison of DNA extraction methods 43
5.2.1.2 ARMS 44
5.3 Results 44

CHAPTER 6: DIRECT SEQUENCING OF 16S rRNA PCR FRAGMENTS FROM STREPTOMYCIN RESISTANT *M. TUBERCULOSIS* ISOLATES

6.1 Introduction 51
6.2 Methods 51
6.2.1 PCR of a 400bp fragment 51
6.2.2 Purification of the PCR fragment 52
6.2.2.1 Well to well elution 52
6.2.3 Sequencing 53
6.2.4 Polyacrylamide gel electrophoresis 54
6.3 Results 54

CHAPTER 7: CONCLUSIONS AND DISCUSSION 57
REFERENCES 58
APPENDIX A 68
APPENDIX B 69

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The history of tuberculosis and streptomycin is presented briefly as a background to this dissertation. Streptomycin resistance of microorganisms, and the mechanisms of resistance, with particular reference to the streptomycin-ribosome interaction are covered, and the data concerning the recent strides made in uncovering these mechanisms in *Mycobacterium tuberculosis*, are presented.

1.2 TUBERCULOSIS

1.2.1 HISTORY

Tuberculosis is a disease of great antiquity. It has been a scourge of immense proportions which continues until the present to be a major cause of morbidity and mortality, particularly in the third world. In South Africa the reported incidence of tuberculosis peaked in 1963 with 372 cases per 100 000 of the total population. In 1990 this incidence was 237 cases per 100 000. The estimated total number of persons infected with *M. tuberculosis* was 6 to 10 million in South Africa in 1990 (Weyer & Kleeberg, 1992). One-third of the world's population is infected with *M. tuberculosis* (Ehlers, 1993).

Ancient descriptions of the disease are recordered as far back as 5000 BC. An accurate clinical description was made by Hippocrates in approximately 460-370 BC (Burke, 1955). Knowledge of the disease was furthered by the description of tubercles by Sylvius in 1679, as well as by the post mortem studies of Laennec in 1819, in which he recognized the unitary nature of the disparate symptoms and signs of the disease. In 1865 Villemin demonstrated the infectious nature of the disease by inoculating rabbits with material taken from human patients. This was followed by the discovery of the aetiology of tuberculosis, the tubercle bacillus, by Dr Robert Koch who announced his discovery in a paper read in Berlin on 24 March, 1882 (Koch, 1932). It

was Koch too, who prepared tuberculin, and demonstrated the pathogenicity of the bacillus experimentally.

It is estimated that a quarter of all adult deaths in Europe was caused by tuberculosis during the seventeenth and eighteenth centuries (Des Prez & Heim, 1990). Yet the treatment developed then, consisting of rest and fresh air, remained in practice until the middle of this century. This was to change with the discovery of antitubercular drugs. In 1942 Feldman *et al* showed that sulphones exerted a therapeutic effect in experimentally produced tuberculosis, followed in 1943 by Waksman's discovery of streptomycin (Schatz *et al*, 1944) which heralded the 'streptomycin era' (1947-1952). Thereafter the discovery of para-aminosalicylic acid (PAS) and thiosemicarbazones in 1946, isoniazid in 1951, rifampicin in 1970, and pyrazinamide transformed therapy. This changed the prognosis of the disease from a very poor one (approximately 50% of patients died within two years, while only 25% recovered), to one one which was eminently curable (98-99% cure in previously untreated pulmonary tuberculosis).

1.2.2 MICROBIOLOGY

1.2.2.1 CLASSIFICATION

M. tuberculosis is a species of the family Mycobacteriaceae, order Actinomycetales. *M. tuberculosis* and *M. bovis* are both included by the description 'tubercle bacillus', but *M. bovis* as a cause of disease in humans is now rare.

M. tuberculosis is an aerobic, non-motile, non-spore forming bacteria which has a high content of waxy materials in the cell wall. This waxy cell wall results in the characteristic acid fastness seen on staining with carbol fuchsin in the Ziehl-Neelsen stain.

Mycobacteria are widespread in nature ranging from soil-dwelling saprophytes to pathogens of humans and animals. *M. tuberculosis* falls into this latter group, being an obligate pathogen of humans, and mammals found in close proximity to humans, e.g. dogs.

M. tuberculosis has a slow growth rate with a generation time of between 15 and 20 hours, with visible colonial growth taking at least 3 weeks and usually 4 to 6 weeks on solid media.

Using differences in growth rate and pigmentation, the genus *Mycobacterium* has been divided into four groups: group I, the photochromogens (slow growing); group II, the scotochromogens (slow growing); group III, the non-photochromogens (slow growing); and group IV, the rapid growers (Sommers & Good, 1985). This classification was first developed by Runyon in 1959 as a classification of 'anonymous' or non-tuberculous mycobacteria; mycobacterial isolates which were recognised as displaying unusual characteristics that differed from the tubercle bacillus.

The identification of mycobacteria by species is now preferred. This requires a variety of specialised and complex tests to be done; enzyme assays, growth charateristics, morphology and drug susceptibility. In an effort to improve on these methods other approaches have been suggested, including immunological methods and DNA composition (Bradley, 1973). Recently the nucleic acid sequence of 16S and 23S rRNA has received much attention as a useful way of establishing phylogenetic relationships within the genus *Mycobacterium* (Rogall *et al*, 1990; Edwards *et al*, 1989; Liesack *et al*, 1990; Liesack *et al*, 1991; Suzuki *et al*, 1988; Estrada-G *et al*, 1989; reviewed by Olsen, 1993). A phylogenetic tree was constructed using the 16S rRNA (Rogall *et al*, 1990) and is presented in figure 1.1. Of note, the sequences for *M. tuberculosis*, *M. tuberculosis* H37, *M. bovis* and *M. bovis* BCG were identical, and are therefore represented in the tree by a single branch.

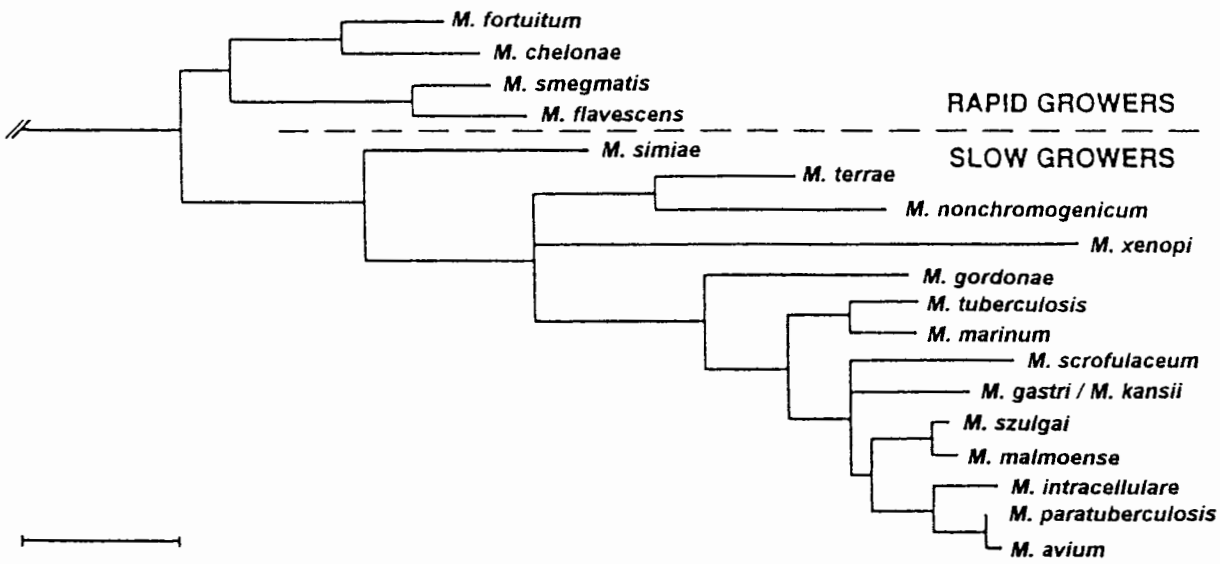


FIGURE 1.1: Phylogenetic tree showing relationships of species belonging to the genus *Mycobacterium*. After Rogall *et al* (1990). Bar = 10 nucleotide positions.

1.2.3 LABORATORY DIAGNOSIS

1.2.3.1 MICROSCOPY

The initial mode of mycobacterial identification is microscopic examination of a specimen after staining. Gram staining is not useful as the organisms demonstrate weak Gram-positive staining or remain colourless. Mycobacteria have the unusual staining characteristic known as acid-fastness. This has been attributed to the high mycolic acid content of the cell wall (Minniken, 1982; Ehlers, 1993). The organisms, when stained by carbol fuchsin, are resistant to decolourisation by acid-alcohol. This property is seen when the Ziehl-Neelsen and Kinyoun staining method is used. Fluorochrome staining with phenolic auramine or auramine-rhodamine also rely on the acid-fastness for detection of mycobacteria. Microscopy is not sensitive, requiring approximately 5 000 to 10 000 organisms per ml of sputum for detection (Hobby *et al*, 1973).

1.2.3.2 CULTURE

Laboratory culture is a far more sensitive method of detection, being able to detect as few as 10 organisms per inoculum (Hobby *et al*, 1973). Culture of sputum requires a decontamination procedure, while specimens taken from "sterile" sites under aseptic conditions can be inoculated directly onto/into culture medium, Lowenstein and Jensen, a solid egg based medium, and Kirschner, a liquid medium. The major problem is that these culture methods may take up to six weeks to become positive, with sensitivity testing requiring a further 3-6 weeks.
Newer culture methods e.g. the Bactec system, are based on the measurement of a growth index which measures microbial growth by detecting the conversion of a radiolabelled carbon source to $^{14}CO_2$. This system is capable of detecting organisms very much quicker (2-6 days).

1.2.3.3 MOLECULAR TECHNIQUES

Molecular methods offer the possibility of even quicker detection and identification. DNA probes use labelled DNA to hybridise to specific DNA or RNA sequences of extracted nucleic acids from cultured mycobacteria. A limited number of species can be identified in this way. However, direct detection of mycobacteria in clinical specimens is approximately 100 times poorer than culture, and false positives occur (McFadden *et al*, 1990). DNA fingerprinting, in which the extracted

DNA is restriction enzyme digested and probed with labelled DNA according to the Southern blot procedure, is useful for identifying strains. High performance liquid chromatography (HPLC) of the mycolic acids from a culture of the organisms is able to identify a wide range of species based on the chromatographic pattern produced (Desmond, 1992). The polymerase chain reaction (Saiki *et al*, 1985) offers the possibility of rapid detection (De Wit *et al*, 1990; Lombard *et al*, 1994) and identification. It may even be used in drug susceptibility testing (Douglass & Steyn, 1993; Honoré & Cole, 1994).

1.2.4 THERAPY

A variety of treatment regimens have been used since the 1950s. They all have in common the use of multiple drugs in combination, in an effort to avoid the appearance of drug resistance and to kill the mycobacterial population quickly. In addition variations in the treatment period, supervised drug administration, intermittent treatment and number and type of drug have been assessed.

A standard regimen in South Africa would be to use isoniazid, rifampicin and pyrazinamide for 6 months, with ethambutol added in areas known to have a high incidence of isoniazid resistance (Weyer & Kleeberg, 1992). Streptomycin, which was used until recently, has been substituted by ethambutol without a loss of treatment potency, thereby converting the regimen to oral drugs only (Snider *et al*, 1986).

Spontaneous resistance occurs at a rate between 1×10^{-6} and 1×10^{-8} per generation. This is important if only one drug is used, and a large enough number of organisms are present. This situation is found in pulmonary cavitatory disease when there may be $>10^{9}$ organisms present. Erratic treatment is known to select drug resistant organisms (Mitchison, 1984).

1.2.5 EPIDEMIOLOGY

The infection is usually spread by airborne droplets, and the incidence of infection is related to overcrowded conditions and poor inherent resistance of individuals. First world countries such as the USA and Britain, have seen a steady decline in the incidence of tuberculosis since the 1950's. In contrast in third world countries the incidence of tuberculosis has remained unacceptably high (Comstock, 1982). This includes South Africa, and in particular the Western Cape, which has a disproportionately high incidence of tuberculosis; the 1990 reported incidence for "Coloureds" was 626 per 100 000 (Weyer & Kleeberg, 1992). With the spread of human

immunodeficiency virus (HIV) infections worldwide, however, there has been an increasing incidence of tuberculosis in first world countries (Sumartojo, 1993), and an increased incidence of drug resistant cases (Hurley and Andrew, 1993; Grandes *et al*, 1993; Idigbe *et al*, 1992). This has resulted in a renewed interest in tuberculosis (Nardell, 1993; Weiss, 1992), and the molecular mechanisms of resistance (Ahern, 1993; Beardsley, 1992).

1.3 STREPTOMYCIN

1.3.1 HISTORY

In 1944 Waksman announced the discovery of streptomycin (Schatz *et al*, 1944). It's efficacy in experimentally produced tuberculosis was demonstrated (Feldman *et al*, 1945; Youmans & McCarter, 1945), and it was used in a clinical trial of therapy in 24 patients with pulmonary tuberculosis (Hinshaw *et al*, 1945). This was soon followed by a larger controlled trial (the first controlled randomised clinical trial of a drug!) in which the beneficial effects of streptomycin were clearly shown (British Medical Research Council, 1948). These trials also revealed the presence of bacterial drug resistance and drug toxicity. This resistance to therapy using streptomycin was seen as the major drawback to it's clinical usefulness. However, experimentation with para-aminosalicylate (PAS) had suggested it's efficacy, and subsequently the combination of the two drugs was compared to using streptomycin only (British Medical Research Council, 1949; British Medical Research Council, 1950). The most remarkable finding was that the incidence of streptomycin-resistant organisms was 70% in the streptomycin only group, as compared with 9% in the group receiving both drugs. These trials established a place for the use of combination therapy in the treatment of tuberculosis.
Streptomycin remained one of the mainstays of the treatment of tuberculosis until recent times when alternative less toxic, and easily administered drugs, have become preferred.

1.3.2 STRUCTURE

Streptomycin, derived from *Streptomyces griseus*, is an aminoglycoside i.e. it contains two aminosugars linked by glycosidic bonds to an aminocyclitol ring (streptidine). The structure can be seen in Figure 1.2.

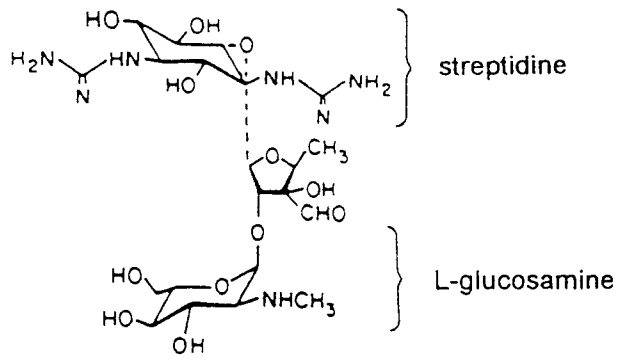


FIGURE 1.2 The atomic structure of streptomycin, from Lietman, 1990.

1.3.3 MECHANISM OF ACTION AND RESISTANCE

1.3.3.1 INTRODUCTION

The mechanism of action of streptomycin and resistance are discussed together because of the intimate way research into the one subject is related to the other.

Organisms can be resistant to aminoglycosides by one, or combinations, of three possible mechanisms:

1.) by ineffective uptake of the drug,

2.) by enzymatic modification the drug, or;

3.) by reduced affinity between drug and ribosome.

1.3.3.2 TRANSPORT ACROSS THE CELL WALL AND MEMBRANE

The precise nature of the transport of streptomycin (and other aminoglycosides) across the cell wall and membrane remains a subject for debate (Hancock, 1981). There is evidence for an energy dependant mechanism of transport involving a carrier molecule (Bryan & van den Elzen, 1976; Nichols, 1987), but not stringent proof for the existence of a carrier (Hancock, 1981). Resistance in bacteria, caused by ineffective transport, is thought to be due to the inability of the cell to maintain the required electromotive potential (Hancock, 1981). Mutations involving many different sites, including ribosomal mutations (e.g. the S12 mutation), have been shown to affect

aminoglycoside transport (Hancock, 1981). There is speculation that this mechanism of resistance may be present in mycobacteria (Honoré & Cole, 1994), but, as yet there is no proof.

1.3.3.3 ENZYMATIC MODIFICATION OF AMINOGLYCOSIDES

In many bacteria, aminoglycoside resistance is due to the production of aminoglycoside modifying enzymes (Edson & Terrell, 1991). These are usually coded for by genes found on plasmids or transposable elements. Although certain of the Mycobacteria have been found to harbour such elements and aminoglycoside modifying activity has been detected in fast growing species (Martin *et al*, 1990), they have not been found in *M. tuberculosis* (Martin *et al*, 1990). This mechanism will therefore not be discussed further.

1.3.3.4 THE STREPTOMYCIN-RIBOSOME INTERACTION

Streptomycin inhibits protein synthesis by interaction with the ribosome (Spotts & Stanier, 1961), specifically the 30s subunit (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973). The effect of streptomycin at the ribosome is to cause an increased error of translation, and stimulating the breakdown of polysomes to monosomes (Modolell & Davis, 1970). This prevents chain elongation and prevents effective initiation of translation.

Hancock (1981) argued that inhibition of protein synthesis may not be the lethal event. According to his argument the action of streptomycin is not confined to only one critical target, but may act at two targets. He suggests that one of five other possibilities occurs as the lethal event; cell wall destruction, cytoplasmic membrane puncture, a lethal hit on an essential gene, cross-linking or double strand breaks in the DNA or disruption of the DNA-membrane attachment site.

Davis *et al* (1986) proposed that the membrane is damaged by the incorporation of misread proteins which create abnormal channels through which streptomycin enters irreversibly at an increased rate.

1.3.3.4.1 THE 30S PROTEINS

Streptomycin binds the 30S subunit of the ribosome of *E. coli* at a single site (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973; Grisé-Miron & Brakier-Gingras, 1982). Early work pointed

toward the ribosomal proteins (see figure 1.2) as the important mediators of the streptomycin-ribosomal interaction (Ozaki *et al*, 1969; Birge & Kurland, 1969; Schreiner & Nierhaus, 1973). Protein S12 was shown to be necessary for the action of streptomycin on the ribosome; the absence of S12 or the presence of a mutation in this protein caused the 30S subunit to bind streptomycin weakly when compared to subunits containing wild-type S12 (Ozaki *et al*, 1969). Birge and Kurland (1969) used ribosomes from streptomycin dependent *E. coli* (these require streptomycin for maximal polypheylalanine synthesis when primed by poly(U) mRNA). Using reconstitution experiments they showed that a single 30S subunit protein was altered in the streptomycin dependent ribosomes; the same protein which Ozaki *et al* (1969) had identified in streptomycin resistant ribosomes, namely S12.

Proteins S4 and S5 were postulated to be part of a single binding site of dihydrostreptomycin on the 30S subunit (Schreiner & Nierhaus, 1973). They discovered two types of binding; one to the 30S subunit which predominated, was protein dependent, specifically involving proteins S4 and S5 and is at one site only. The second type of binding is mentioned only briefly; that to the 16S and 23S RNA and the 50S subunit, which was a far weaker binding. It is interesting to note that they did not demonstrate binding to S12, although it was specifically tested for.

Further S12 mutations were discovered in streptomycin resistant ribosomes of many species (Galili *et al*, 1989; Salles *et al*, 1992). Two regions may be altered; position 42(Lys) and the region from positions 85 to 91 (Funatsu & Wittmann, 1972; Galili *et al*, 1989; Salles *et al*, 1992).

Affinity labeling experiments using a streptomycin analogue and *E. coli* ribosomes (Pongs & Erdmann, 1973; Pongs *et al*, 1974), showed the analogue to react irreversibly and specifically to the 70S ribosome. They were furthermore able to show that the analogue was attached to the 30S subunit, probably S3 and S4. They did not detect any reaction to the rRNA.

S4 and S5 *ram* (ribosomal ambiguity) mutations were shown to enhance streptomycin binding and therefore increase the sensitivity of ribosomes to streptomycin (Böck *et al*, 1979). The introduction of *ram* mutations into ribosomes containing a S12 mutation, results in the reversal of the restriction of translational ambiguity caused by the S12 mutation. i.e. they have opposite effects (Biswas & Gorini, 1972).

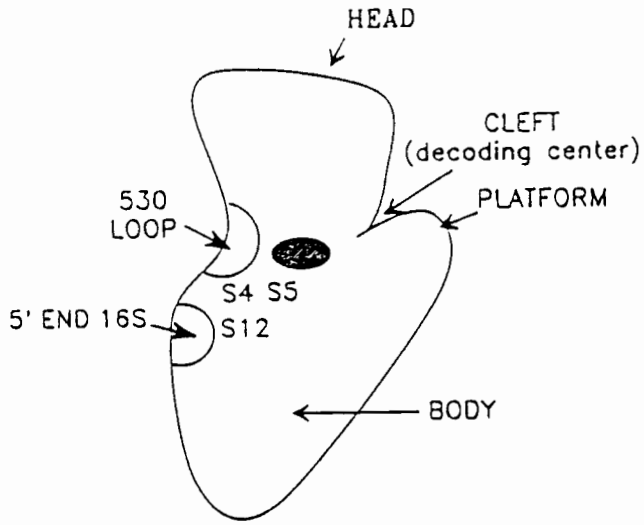


FIGURE 1.2: The 30S subunit of the ribosome as viewed from the interface betwen the two subunits, showing the relative positions of proteins and RNA loops involved in streptomycin binding. The central shaded area indicates the position of the 5' terminal, beneath which lies the 900 helix. (from Leclerc *et al* (1991)

M. tuberculosis ribosomal proteins from streptomycin sensitive and resistant isolates were compared by Medow *et al* (1987). They detected two additional proteins in the 30S subunit; 28 proteins as compared to 26 in sensitive ribosomes, and an altered mobility of the protein L34 on two dimensional electrophoretic gels. It is interesting to note that the total number and molecular weights of ribosomal proteins was found to be different in different species, e.g. *E. coli* and *M. tuberculosis*.

Very recently, a mutation in protein S12 from streptomycin resistant *M. tuberculosis* isolates has been discovered (Finken *et al*, 1993). This is present at codon 88 of the *rpsL* gene. A mutation was also found in the *rpsL* gene at codon 88(Lys to Arg) in a streptomycin resistant *M. tuberculosis* isolate which had a simultaneous 913 A→G mutation in the 16S rRNA gene (Meier *et al*, 1994).

Honoré and Cole (1994) reported S12 mutations in two out of six streptomycin resistant *M. tuberculosis* isolates. They showed that two of the six isolates investigated contained mutations at position 43(Lys to Arg) in the S12 (*rpsL)* gene. Two of the isolates contained 912 mutations of the 16S rRNA (*rrs*) gene, and in two no mutations were found.

A paper has also been presented on this topic (Morris *et al*, 1993), the abstract of which I have read. Five streptomycin resistant *M. tuberculosis* isolates were reported to contain an identical mutation (A→G), at codon 43 of the gene encoding protein S12, while the parental strains and

two further streptomycin resistant *M. tuberculosis* isolates were not mutated. This work has not yet been published in full.

1.3.3.4.2 THE 16S rRNA

Evidence which supported direct binding of streptomycin to naked 16S rRNA appeared after it had been established that protein-streptomycin binding was important. (Biswas & Gorini, 1972; Garvin *et al*, 1974; Melançon *et al*, 1984). Gorini's group used radiolabeled streptomycin and dihydrostreptomycin in equilibrium dialysis experiments to show that the drug binds stably to the 16S rRNA, and not to the 23S rRNA (Biswas & Gorini, 1972; Garvin *et al*, 1974).

1.3.3.4.2.1 CROSS-LINKING EXPERIMENTS

Melançon *et al* (1984) used the bifunctional cross-linking agent phenyldiglyoxal to determine precisely the site at which streptomycin binds. The streptomycin-30S complex was allowed to form after which phenyldiglyoxal was added. This caused covalent cross-linking to occur by reacting with the guanosine of ssRNA and guanidine groups of arginine residues in the proteins. The potential for cross-linking streptomycin through it's guanidine residues to the guanosine of ssRNA, was recognised and used for these experiments. They showed that streptomycin bound reversibly to the 30S subunit, and that the binding was tighter to the 30S subunit of a streptomycin-sensitive *E. coli* than to the 30S subunit of a streptomycin-resistant *E. coli*. They showed that streptomycin binds a specific site by succesfully abolishing cross-linking in the presence of bluensomycin, an agent which competes for the streptomycin binding site (Chang & Flaks, 1972). This excluded the possibility that phenyldiglyoxal might have caused cross-linking at indiscriminate sites on the 30S subunit. The cross linked 30S subunit was then incubated either with proteinase K or RNAse before precipitation and counting the radioactivity of the precipitate. This showed that the streptomycin was binding mainly to the RNA (75%), and to a lesser extent to ribosomal proteins. Protein electrophoresis of extracted 30S ribosomal proteins on 1 dimensional and 2 dimensional polyacrylamide gels, identified the bound proteins to be S1, S5, S11 and S13. Interestingly S12 did not show cross-linking to streptomycin suggesting that it is not located in the streptomycin binding site. Yet it is known to be involved in the ribosomal response to streptomycin (Ozaki *et al*, 1969; Birge & Kurland, 1969). This, together with results of experiments reported by the same group (Brakier-Gingras *et al*, 1974), in which it was shown that streptomycin induces conformational changes in streptomycin sensitive but not in

streptomycin-resistant ribosomes, led the authors to postulate that the binding of streptomycin occurs in a two-step reaction. An initial interaction would be followed by a conformational change in which the association with antibiotic is tightened and protein synthesis is interrupted. Thus protein S12 would occupy a site close to the streptomycin binding site which controls the ability of the ribosome to undergo the conformational change.

Gravel *et al* (1987) then went on to localise the site of cross-linking to the 16S RNA. Tritiated streptomycin was cross-linked to the 30S subunit or to the 70S ribosome of *E. coli* using nitrogen mustard. This was then hybridized to restriction fragments of the 16S RNA gene. By using a variety of restriction enzymes the region of the gene to which the labeled RNA hybridized was localized. Two regions were identified, one corresponding to positions 892-917, and the other to positions 1394-1415.

1.4.4.3.2 RNA FOOTPRINTING

RNA footprinting done by Moazed and Noller (1987), indicated direct binding of the antibiotic to the 16S rRNA. In these experiments ribosomes from *E. coli* were incubated with streptomycin prior to chemical attack of covalent bonds within bases by dimethylsulphate (DMS). The RNA was then subject to primer extension using reverse transcriptase. Where the bases are damaged, a termination occurs; where the bases are protected readthrough occurs. In this way bases protected by the binding of the antibiotic can be identified. They showed that three adenosines at positions 913, 914 and 915 are strongly protected by streptomycin and that there is weaker protection of bases U911 and C912 (see figures 1.3 and 1.4). The experiments were repeated with the 30S subunit alone. This showed additional weak protection of bases A909, A1413, G1487 and G1494. Some of these sites (909, 1413 and 1487) are also protected by tRNA and by 50S RNA in a non-competitive manner (known as class III sites). Therefore, they suggested that these sites are not protected by directly binding streptomycin, but possibly because a conformational change is induced by the streptomycin binding, or due to the formation of a pocket when both ribosomal subunits are present, which can be blocked by the drug.

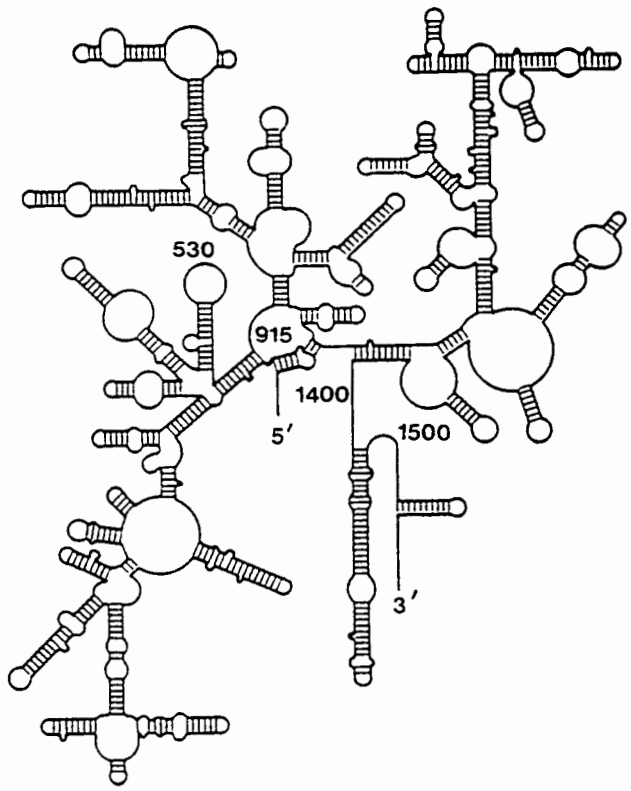


FIGURE 1.3: Secondary structure of the 16S rRNA highlighting the 530, 915 and 1440 loops. The 915 loop is central to the structural of the 16S rRNA, and it is at this point that movement is thought to occur during conformational changes (from Moazed and Noller, 1987).

1.3.3.4.3 RIBOSOMAL RNA MUTATIONS

1.3.3.4.3.1 POSITION 912

Montandon *et al* (1985) reported the finding of a 912 C→T base change in the chloroplast 16S rRNA gene of two streptomycin resistant *Euglena gracilis* isolates. To support the postulate of linkage between the mutation and streptomycin resistance, they argued that this is a highly conserved position across phylogenies, and an invariant position in eubacteria and chloroplast DNA. Furthermore, the S12 gene in these resistant chloroplasts was unchanged, and that archaebacteria, which are resistant to streptomycin, contain the same base at the analogous position. Later the same position was found to be mutated (912 C→A) in the 16S rRNA gene of streptomycin resistant *Nicotiana tabacum* chloroplasts (Etzold *et al*, 1987).

Frattali *et al* (1990) investigated the effect of introducing four different mutations into position 912 of *E. coli* ribosomes. They introduced the mutations (912 C→U, 912 C→G, 912 C→A and a

deletion at 912) into the *rrn*B operon of the multicopy plasmid, pKK3535, which was used to transform *E. coli*. They found that two of the mutations (912 C→U and 912 C→G) conferred a low level of resistance to streptomycin, and that after selection in progressively higher concentrations of streptomycin, a high level of resistance was obtained in cells containing the 912 C→U mutation. However after removal of the plasmid the cells retained a higher level of resistance than the original wild type strain, even though the 912 C→U mutation was not present due to a homologous recombination event in the chromosomal DNA. Retransformation of these cells to introduce the 912 C→U mutation again caused an enhanced resistance to streptomycin. They also introduced the 912 C→U mutation into ribosomes from three strains of *E. coli* which were streptomycin dependent by virtue of S12 protein mutations. These *E. coli* strains require the binding of streptomycin. The plasmid was lethal in one strain and caused ribosomes from two other strains to be non-functional; the explanation being that the mutation prevented the proper binding of streptomycin.

Honoré and Cole (1994) have reported 912 mutations in two streptomycin resistant *M. tuberculosis* isolates. They showed that two of the six isolates investigated contained 912 C→G or 912 C→A substitutions respectively. Two of the isolates contained S12 mutations at position 43(Lys to Arg) and in two, no mutations were found in the S12 (*rpsL)* gene or in selected regions of the 16S rRNA (*rrs*) gene.

1.3.3.4.3.2 POSITION 913 AND 915

Leclerc *et al* (1991) succesfully introduced two mutations, 913 A→G and 915 A→G, into 16S rRNA of ribosomes in *E. coli* (see figure 1.4). Using these ribosomes, as well as ribosomes containing a 912 U→C mutation, they investigated the streptomycin-ribosome interaction. Approximately 67% of ribosomes extracted from each *E. coli* clone contained the mutation, which had been introduced into the *rrn*B operon of pKK3535. Ribosomes were assayed for misreading of a poly(U) messenger in the presence of streptomycin. They showed that the mutations restricted the misreading caused by streptomycin. The 913 A→G and 915 A→G mutations did not cause a decreased protein synthesis in the absence of streptomycin, but did restrict the inhibition of protein synthesis in the presence of streptomycin. Lastly, they showed that the mutant ribosomes had a decreased binding affinity for streptomycin.

In this report we describe an A→G point mutation at position 913 in streptomycin resistant *M. tuberculosis* isolates (Douglass & Steyn, 1993). These results have recently been confirmed by Meier *et al*, (1994).

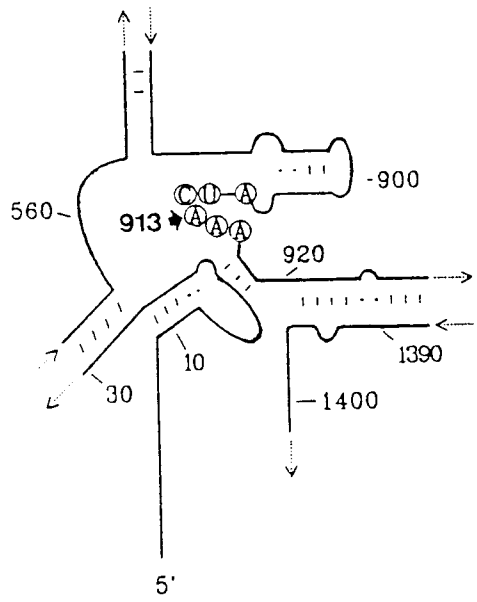


FIGURE 1.4: The 915 region of the 16S rRNA (from Leclerc *et al*, 1991a).

1.3.3.4.3.3 POSITION 13 AND 13+914

Pinard *et al*, (1993) investigated the idea that the 5' proximal helix of the ribosome is involved in streptomycin binding by a conformational change to stabilize the binding of the drug (Leclerc & Gingras, 1991). Four mutations (13 U→A, 13U→C, 914 A→U and 914 A→G) were introduced, either individually or as double mutations (13 U→A and 914 A→U or 13U→C and 914 A→G) into *E. coli*. None of the mutations affected the growth rate, but they all impaired the binding of streptomycin to the ribosome, and consequently protected the ribosomes against streptomycin induced misreading. However, the double mutations, which restore the capacity of position 13 to base pair with position 914, did not restore the binding of streptomycin. Therefore, the idea that a conformational change produces a novel helix which is essential for streptomycin binding, was not supported by their data.

1.3.3.4.3.4 THE 530 LOOP

An A→C mutation was introduced at position 523 of the 530 loop (see figure 1.3) of *E.coli* ribosomes (Melançon *et al*,1988; Leclerc *et al*, 1991b) in the same way as described above. The effect was to decrease streptomycin binding to the 30S subunit and restrict the misreading caused by streptomycin.

Meier *et al* (1994) found point mutations, 501 C→U and 522 C→U, in two streptomycin resistant *M. tuberculosis* isolates, while none of the 35 control isolates contained a mutation.

1.3.3.4.4 INVOLVEMENT OF THE 50S SUBUNIT

Melançon *et al* (1992) investigated the effect of two mutatons (2661 G→C and 2661 G→U) on the binding of streptomycin, neomycin and gentamycin to ribosomes, and misreading of the ribosomes in the presence of the aminoglycosides. This was done using pKK3535 and *E.coli*. They found that although the mutations did protect the ribosomes against misreading, although neither affected the drug-ribosome binding. The effects of the 2661 mutations were similar to those obtained for L6 mutations (Kühberger *et al*, 1979; Hummel *et al*, 1980), a 50S protein found in close proximity to the 2660 loop. They proposed that the 2660 loop is also part of the ribosomal site which controls the proofreading step.

1.4 CONCLUSION AND AIMS OF THIS STUDY

The ongoing prevalence of tuberculosis in our country and the world with the incumbent problem of resistance of the organism to effective drug therapy makes the study of the mechanisms of drug resistance in *M. tuberculosis* most important.

Streptomycin is one of the oldest and best studied antimicrobial agents. The interaction of streptomycin with the cell is complex. I have sketched the major points of it's interaction with the ribosome, known to be one of the most important targets of it's action, if not the most important target.

The level of understanding of the streptomycin-ribosomal interaction is well developed, but still not complete. The small subunit of the ribosome, particularly the 16S rRNA and protein S12 have received much attention in *E. coli*. The importance of the 16S rRNA for streptomycin binding and action are well demonstrated at ribosomal level, with the 915 region structurally pivotal to all of the sites implicated in the streptomycin-ribosomal interaction.

We investigated the possibility that a 16S rRNA mutation in the 915 region may be responsible for the resistant phenotype displayed by *M. tuberculosis* isolates. The *M. tuberculosis* chromomsome is known to contain only one set of of RNA genes as opposed to the seven sets carried by *E. coli*. A single point mutation which decreases streptomycin binding should, therefore, be immediately apparent at the phenotypic level.

CHAPTER 2

MATERIALS

2.1 PLASMID AND HOST CELLS

pUC18, a *lacZ'* vector with ampicillin as the antibiotic marker (Yanisch-Perron *et al*, 1985), and *E. coli* LK111 cells, a derivative of K514, *lacI*$^-$, *lacZ*M15, *lacY*$^+$ (Zabeau & Stanley, 1982), were provided by Professor Lafras Steyn, Department of Medical Microbiology, UCT Medical School, Observatory. A map of pUC18 is shown in appendix A.

2.2 *M. TUBERCULOSIS* ISOLATES

Streptomycin resistant *M. tuberculosis* isolates were obtained from the mycobacterial laboratory, Groote Schuur Hospital, Observatory and the South African Institute of Medical Research, Orange street, Cape Town. DNA from two isolates was received from Ian Wiid, Department of Medical Biochemistry, Tygerberg Hospital, Tygerberg.

2.3 ENZYMES, CHEMICALS, SOLUTIONS AND MEDIA

All reagents and suppliers are listed below.

2.3.1 ENZYMES AND KITS

*Eco*RI	Boehringer Mannheim
*Hin*dIII	Boehringer Mannheim
Proteinase K	Boehringer Mannheim
Sequenase	United States Biochemicals
*Sma*I	Boehringer Mannheim
Taq polymerase	Boehringer Mannheim

T4 DNA ligase	Boehringer Mannheim
^{T7}Sequencing kit	Pharmacia
RNAse	Sigma
Uracil DNA Glycosylase	BRL

2.3.2 CHEMICALS AND REAGENTS

Acetic acid	BDH
Acetone	BDH
Acrylamide	BDH
Agarose	Promega Corp
Ammonium Acetate	BDH
Ampicillin	Beechams
Bis (N,N'-methylene bis acrylamide)	Sigma
Boric Acid	Merck
Bromophenol blue	Sigma
Calcium chloride	Merck
Caesium chloride	Boehringer Mannheim
Chloroform	Merck
Chromatography paper 3mm	Whatman
Deoxynucleotide triphosphates	Boehringer Mannheim
Dimethyl formamide	BDH
Dimethyl sulphoxide	Merck
Ethylenediaminetetraacetic acid (EDTA)	Boehringer Mannheim
Ethidium bromide	Merck
Ethanol	BDH
Fixer (AMFIX)	Maybaker
Formamide	BDH
Glycerol	BDH
Glycogen	Boehringer Mannheim
Hydrochloric acid	BDH
8-Hydroxyquinolone	Merck
IPTG (isopropyl-β-D-thiogalacto-pyranoside)	Boehringer Mannheim

Isoamyl alcohol	Merck
Isopropanol	BDH
Lysozyme	Boehringer Mannheim
Lithium Chloride	Sigma
2-Mercaptoethanol	Merck
N-cetyl-N,N,N-trimethyl ammonium bromide (cetramide)	Sigma
Nonidet P40	Sigma
Phenol	BDH
Potassium acetate	Fluka
^{35}S-dCTP	Amersham & Du Pont
Sodium chloride	Merck
Sodium citrate	BDH
Sodium deoxycholate	BDH
Sodium dodecyl sulphate (SDS)	Sigma
Sodium hydroxide	Saarchem
Sterile distilled water	BDH
Sucrose	BDH
TEMED(N,N,N',N',-tetramethyl-ethylenediamine)	Sigma
Tris(hydroxymethyl)amino-methane (Tris)	Boehringer Mannheim
Triton X-100	BDH
Tween 20	Sigma
X-Gal (5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside)	Boehringer Mannheim
X-ray film (curix)	Agfa
X-ray film developer (Ilford Phenisol)	Isophoto

2.3.3 MEDIA AND SOLUTIONS

Luria agar and Luria broth were prepared by the media division of the Department of Medical Microbiology, University of Cape Town. These were stored at room temperature. The agar was melted in a microwave oven before being poured into petri dishes for use.

Lowenstein Jensen slopes were prepared by the media division of the Department of Medical Microbiology, University of Cape Town.

Saturated phenol was prepared as described by Sambrook *et al* (1989) and stored frozen (-20°C) until use.

Ampicillin (25 mg/ml) was stored at -20°C in aliquots and used at a final concentration of 50ug/ml.

10 × NNB buffer:	162g Tris base
	27.5g boric acid
	9.3g $Na_2EDTA.2H_2O$
	made up to 1l with sterile distilled water.
50% acrylamide stock:	125g acrylamide
	12.5g bis
	made up to 250ml with sterile, distilled water, filtered, and stored in the dark at 4°C.

2.3.4 SUPPLIERS

Amersham	Little Chalfont. Buckinghamshire, England.
BDH	Poole, BH15 ITD, England.
Boehringer Mannheim GmbH	Biochemica, P O Box 310, 120, D-6800, Mannheim 31, West Germany.
BRL	Cowley Mill Trading Estate. Longbride Way, Uxbridge, Middlesex, England.
Du Pont de Nemours & Co	NEN Product, 549 Albany street, Boston, MA 02118, USA.
Isophoto	P.O. Box 2435, Rivonia, 2128, RSA.
Maybaker (SA)	21 McHardy Avenue, Port Elizabeth, RSA.
Merck	Frankfurter Strasse 250, D-6100, Darmstadt, Germany.
Pharmacia	LKB Biotechnology AB, Uppsala, Sweden.
Promega Corp	Madison, WI, 53711-5399, USA.
Saarchem	P.O. Box 144, Muldersdrift, 1747, RSA.
Sigma	P O Box 14508, St Louis MO 63178, USA.
United States Biochemicals:	P.O. Box 22400, Cleveland, Ohio, 44122, USA.

CHAPTER 3

CLONING OF THE 915 REGION OF THE 16S rRNA GENE OF A STREPTOMYCIN RESISTANT *M. TUBERCULOSIS* ISOLATE

3.1 INTRODUCTION

The basic molecular biology techniques/methods used in this investigation are described in this chapter. The preparation of a recombinant plasmid for the sequencing of the streptomycin binding region of the 16S rRNA gene of a clinical isolate of *M. tuberculosis* resistant to streptomycin is described. The streptomycin binding region of the gene was amplified by the polymerase chain reaction and ligated into a T-tailed *Sma*I site of pUC18. *E. coli* LK111 cells were transformed with this construct and selection was performed using the blue-white selection system on agar containing ampicillin (described in 3.2.6).

3.2 METHODS

3.2.1 PREPARATION OF MYCOBACTERIAL DNA

A clinical isolate of *M. tuberculosis* (designated M.tbA) resistant to streptomycin, rifampicin, isoniazid, ethionamide, thiacetazone and cycloserine was obtained from the South African Institute of Medical Research, Cape Town. This was cultured on streptomycin-free Lowenstein and Jensen medium for approximately 4 months at 37°C in air prior to DNA extraction. DNA was extracted by a modification of the method described by van Soolingen *et al* (1991). A suspension of cells was prepared in 1.5 ml of TES buffer (10mM tris-HCl ; 1mM EDTA ; 150mM NaCl [pH 8.5]) in 5ml Greiner tubes and heated to 75°C for 2 hours to ensure complete killing of all cells. (Experimentation done in this laboratory by Mr Bruce Allan showed that viable mycobacteria were still present if only 30 minutes were used for the heat killing. It was therefore decided to err on the side of caution because of the potential health hazard posed by the organism under investigation.) The killed cells were pelleted by centrifugation for 5 minutes at 3 000rpm in a Beckman GS-6 centrifuge, and resuspended in 500μl of TE buffer (10mM Tris-HCl, 1mM EDTA

[pH 8.0]). The cell suspension was transferred to a 1.5ml Eppendorf tube. A spatula-tip of lysozyme powder was added, mixed by vortexing, and the solution incubated at 37°C for 1 hour. Following this, 70μl of 10% SDS (w/v) and 6μl of proteinase K (10mg/ml) were added, mixed and incubated at 65°C for 10 minutes. Thereafter, 80μl of *N*-cetyl-*N,N,N*-trimethyl ammonium bromide (cetramide) was added, mixed and incubated at 65°C for 10 minutes. Proteins and lipids were extracted by adding an equal volume of chloroform-isoamylalcohol (24:1 V/V), the solution mixed, and the phases separated by centrifugation for 5 minutes at 14 000rpm in a microfuge (Eppendorf 5415). The DNA was precipitated by addition of a 0.6 volume of isopropanol to the supernatant fluid, the solution was mixed by inverting, and incubated at room temperature for 15 minutes. The DNA was pelleted by centrifugation for 15 minutes at 14 000rpm in a microfuge. The supernatant fluid was discarded and excess salt removed by centrifugation of the pellet in 1ml 70% ethanol with the tube orientated in the opposite direction to that used to precipitate the DNA, such that the pellet migrated through the ethanol. The supernatant fluid was discarded and the pellet was dried under vacuum in a Servant speedivac concentrator, and finally dissolved in sterile, distilled water.

3.2.2 PCR AMPLIFICATION OF A 336bp FRAGMENT OF THE 16S rRNA GENE

Published mycobacterial 16S rRNA sequences (Rogall *et al*, 1990) were used to design 19-mer oligonucleotide primers flanking the 915 region. These were synthesized by Professor D. Botes of the Department of Biochemistry, UCT.

The DNA sequences of the primers used were 5'-AACGCATTAAGTACCCCGC-3' (forward, designated F2), and 5'-CTTGACGTCATCCCCACCT-3' (reverse, designated R1). These correspond to positions 863-882 and 1179-1198 of the 16SrRNA gene (*E. coli* numbering).

A PCR reaction mix was prepared using buffer, nucleotides and *Taq* polymerase purchased from Boehringer Mannheim. The final 50μl mixture was composed as follows; 5μl of 10×buffer, 1μl of 10mM dATP, 1μl of 10mM dGTP, 1μl of 10mM dCTP, 1μl of 10mM dTTP, 0.25μl of *Taq* polymerase (1.25 units), 1μl of forward primer (11.86pmol/μl to give a final concentration of 0.24μM), 1μl of reverse primer (9.99pmol/μl to give a final concentration of 0.2μM), 10μl of M.tbA mycobacterial DNA (approximately 400-600ng) and 29μl of water.

PCR was performed on a JDI 8012 thermocycler using the following parameters: an initial temperature of 95°C for 7 minutes, followed by 30 cycles of; 95°C for 10 seconds, 54°C for 30 seconds and 70°C for 30 seconds.

A 10μl aliquot of the solution was then loaded onto a 3% agarose gel and subjected to electrophoresis (as described in 3.2.5), to confirm the amplification of a 336bp product and to estimate the concentration of DNA.

3.2.3 LARGE SCALE PLASMID PREPARATION ('Maxiprep')

The maxiprep method used is based on that of Greenaway and Dale (1983).

Plasmids were prepared from and maintained in *E. coli* cells (DK1 or LK111). A single colony was picked from a Luria agar plate containing the appropriate antimicrobial agent (ampicillin or kanamycin) and inoculated into 5ml of Luria broth containing antibiotic. This was incubated at 37°C, with shaking, for approximately 4 hours.

A 4ml aliquot of the culture was added to 150ml prewarmed Luria broth containing antibiotic and incubated at 37°C overnight (approximately 18 hours) with shaking. Erlenmyer flasks (500ml) stoppered with cotton wool were used to ensure maximal aeration during the incubation.

The cells were harvested by centrifugation at 5 000rpm at 4°C for 20 minutes in a JA-10 rotor. The supernatant fluid was discarded. Cells were resuspended in 5ml of 25% sucrose in 0,05M Tris-HCl [pH 8.0] and transferred to a polypropelene centrifuge tube.

To lyse the cells, a spatula-tip of dried lysozyme was added to the suspension, and mixed by intermittent swirling for 5 minutes. The suspension was kept on ice between times. Following this, 5 ml of 0.25M EDTA [pH 8.0] was added, the solution was swirled intermittently, and replaced on ice.

After 5 minutes, 8 ml of Triton/Doc solution (1% Triton, 0.4% Sodium deoxycholate in 10mM Tris, 1mM Na_2 EDTA [pH8.0]) was added rapidly from a 10ml pipette, and mixed by drawing up and expelling the solution three times. This was then left to stand on ice for approximately 20 to 30 minutes.

Plasmid DNA was separated from chromosomal DNA by pelleting the cell debris, and with it the attached chromosomal DNA, by centrifugation at 15 000rpm at 4°C for 45 minutes in a JA-20 rotor.

3.2.3.1 ISOPYCNIC GRADIENT CENTRIFUGATION

Plasmid DNA was purified by isopycnic gradient centrifugation. Macromolecules, in this case DNA, are separated according to density in a solution of caesium chloride (CsCl) and ethidium bromide.

While pelleting the cell debris by centrifugation, the CsCl solution was prepared by weighing out 9.5g of CsCl into a conical tube. On completion of the centrifugation, 10ml of the watery supernatant fluid (containing plasmid DNA), was added to the CsCl, and dissolved by gentle swirling.

A 0.2ml aliquot of 10mg/ml ethidium bromide was added.

The solution was transferred to polyallomer centrifugation tubes using a syringe to fill the tubes. The necks of the tubes were dried, and heat sealed.

These were then loaded into a VTi65 ultracentrifuge and centrifugation at 50 000 rpm at 20°C was continued for 20 hours.

Following centrifugation the separated DNA was visualised in a darkroom under ultraviolet light by virtue of the orange fluorescence produced by intercalated ethidium bromide. Two bands were visible; a distinct lower band corresponding to the covalently closed coiled plasmid DNA, and a less distinct higher band corresponding to chromosomal and/or nicked plasmid DNA.

Plasmid DNA was eluted into plastic Greiner tubes by puncturing the polyallomer tube at the top and bottom and allowing drainage downward. The speed of flow was controlled by finger pressure on the upper puncture which allowed accurate collection of plasmid DNA while excluding the upper chromosomal DNA.

3.2.3.2 REMOVAL OF ETHIDIUM BROMIDE AND CsCl

Ethidium bromide was removed from the eluted plasmid DNA by adding an equal volume of isoamylalcohol, mixed by vortexing, and separation by centrifugation. The upper organic layer was discarded and the procedure repeated (usually twice more) until no pink colour was visible.

To remove the CsCl, the plasmid preparation was dialysed overnight at 4°C against 500ml TE buffer (10mM Tris, 1mM EDTA, [pH 8.0]). Three changes of buffer were made.

The solution was removed from the dialysis bag, aliquotted into Eppendorf tubes, and the DNA precipitated by adding one-tenth volume of 4M LiCl and 2.5 volumes of absolute ethanol. This was left at -20°C overnight, followed by centrifugation at 14 000rpm for 15 minutes in a microfuge. The supernatant fluid was discarded, 70% ethanol was added and the tube centrifuged at 14 000rpm for 5 minutes in a microfuge. The supernatant fluid was discarded and the pellet dried under vacuum. The pellet was then resuspended in sterile, distilled water. The concentration and purity of the DNA was assessed by measuring absorbance of an aliquot of the solution at 260nm and 280nm. The absorbance at 260nm is directly proportional to the DNA concentration, and the ratio of absorbance 260nm:280nm allows an assessment of purity (the ratio 1.8 being uncontaminated DNA).

3.2.4 RESTRICTION ENZYME DIGESTION

The pUC18 plasmid is designed to include a number of unique restriction sites in a multiple cloning site, as shown in Appendix A. Digestion of the plasmid using a restriction enzyme causes linearisation of the plasmid. This allows one to introduce compatible DNA fragments into this site by a ligation reaction. Restriction enzyme digestion can also be used to identify recombinant plasmids which on linearisation can be seen to be larger than the parent plasmid. Digestion can be performed to remove the insert from the parent plasmid which is recognised as a second band of DNA when visualised on agarose gel following electrophoresis. Restriction enzymes were used for each of these applications.

Restriction enzyme digestion was performed according to the manufacturers recommendations. The reactions were terminated by adding one-tenth volume of a 10× stop buffer (50% glycerol, 100mM Na_2EDTA [pH8.0], 1% SDS, 0.1% bromophenol blue, 0.1% cyanol).

3.2.5 AGAROSE GEL ELECTROPHORESIS

DNA fragments were separated by electrophoresis in agarose gels of varying concentrations, chosen according to the size of the fragments to be separated. The concentration used varied from 0.8% to 3% agarose, with the lower concentration used for detecting the recombinant plasmids, and the higher concentration used for detecting the PCR fragments.

Gels were prepared by dissolving appropriate amounts of agarose in TAE buffer (40mM Tris-acetate, 1mM EDTA) aided by heating the solution in a microwave oven. A 100µl aliquot of 100µg/ml ethidium bromide was added to 100ml of the agarose solution. The solution was allowed to cool to 56°C prior to being poured into perspex or plastic moulds, with a comb lowered into the molten agarose to form wells. Once the agarose had gelled it was placed in an electrophoresis tank with the gel covered by buffer. Aliquots of DNA were introduced into the preformed wells and electrophoresis performed at a constant voltage.

After seperation the DNA was visualised in ultraviolet light on a UV illuminator.

3.2.6 PREPARATION OF VECTOR FOR LIGATION

pUC18 contains a β-lactamase gene which confers ampicillin resistance to cells which contain the plasmid. pUC18 also contains a portion of a β-galactosidase gene into which a multiple cloning site has been engineered. The complementary portion of the beta-galactosidase gene is found in the genome of LK111. Isopropyl-β-D-thiogalactopyranoside (IPTG) induces the *lac* operon causing the production of β-galactosidase. This enzyme hydrolyses 5-bromo-4-chloro-3-indolyl-β-galactosidase (X-gal), resulting in the production of bromochloroindole, a blue dye.
This allows for the selection of transformed cells, as well as cells harbouring recombinant plasmids on agar containing ampicllin, IPTG and X-gal. Transformed cells will grow in the presence of ampicillin and produce the blue dye; therefore they are seen as blue colonies. However the β-galactosidase gene is interrupted if extraneous DNA is introduced into the multiple cloning site, preventing the production of β-galactosidase, and therefore colonies containing a recombinant plasmid will be white.

One microgram of pUC18 was digested with *Sma*I to produce a blunt-ended plasmid in a reaction mixture composed as follows; 5μl of pUC18 DNA, 1μl of *Sma*I, 1μl of buffer A (Boehringer) and 3μl of water. This was incubated at 37°C for 3 hours.

3.2.6.1 CONSTRUCTION OF A T-TAILED VECTOR

PCR amplification by *Taq*-polymerase causes the addition of a nucleotide (almost exclusively adenine) to the 3' end of the newly synthesized DNA (Clark, 1988). This causes inefficient ligation of the insert to blunt-ended plasmid DNA (Mole *et al*, 1989). Therefore a method which improves the efficiency of ligation, the 'T-tailing' of the vector, was used (Marchuk *et al*, 1990).

The entire 10μl of *Sma*I digested pUC18 was used. To this 1μl of 10mM dTTP (final concentration of 0.5mM), 5μl of 10× buffer (PCR buffer of Boehringer-Mannheim), 0.2μl *Taq* polymerase and 33.8μl of water were added. This 50μl reaction mix was incubated at 70°C for 2 hours. The high temperature inactivates the *Sma*I, while *Taq* polymerase catalyses the addition of a 3' thymidine. This produces a vector with a sticky-end complementary to PCR amplified DNA.
The solution was extracted with phenol and chloroform-isoamylalcohol. A half volume of saturated phenol and a half volume of chloroform-isoamylalcohol (24:1 V/V) was added to the

supernatant fluid. The solution was mixed by vortexing, and the phases separated by centrifugation at 14 000rpm for 2 minutes in a microfuge. This step was repeated, followed by two extractions with a half volume of chloroform-isoamylalcohol (24:1 V/V). The DNA was precipitated by adding one-tenth volume of LiCl and 2½volumes of ice-cold absolute ethanol, mixed by inverting, and incubated at -20°C overnight. This was followed by centrifugation at 14 000rpm for 15 minutes in a microfuge to pellet the DNA. The supernatant fluid was discarded and the pellet washed by the adding 1ml of 70% ethanol and centrifugation at 14 000rpm for 1 minute in a microfuge.

The pellet was redissolved in 15μl of water. The DNA concentration was estimated roughly by agarose gel electrophoresis of 1μl. The remaining 14μl was made up to 20μl with sterile, distilled water.

3.2.7 LIGATION REACTION

Ligation of the 336bp fragment amplified from M.tbA into the T-tailed *Sma*I site of pUC18 was done using plasmid and mycobacterial DNA in approximately 1:1 molar ratios. A 20μl ligation reaction mixture was made up as follows; 1μl of the pUC18 solution (approximately 50ng DNA), 2μl of PCR products containing the 336bp fragment to be inserted (approximately 5-10ng DNA), 1μl of T4 DNA-ligase, 1μl of *Sma*I, 2μl of 10× ligase buffer and 13μl of water.

The *Sma*I was included to prevent recircularisation of pUC18.

Ligation in the presence of *Sma*I was compared to ligation not including *Sma*I, which was run in tandem with the above experiment. The final 10μl solution contained; 1μl of pUC18, 2μl of the 336bp fragment, 1μl of T4 DNA ligase, 1μl of 10× ligase buffer and 5μl of water.

These were incubated at 15°C overnight, following which the solutions were stored at 4°C.

3.2.8 PREPARATION OF COMPETENT CELLS

Competent DK1 cells were prepared by the following method for use in cloning experiments which did not require blue/white selection (not described).

Luria broth (10ml) was inoculated with a colony of DK1 cells. This was incubated overnight (approximately 18 hours) at 37°C with aeration. A 0.5ml aliquot was removed and added to a 500ml flask and incubated for approximately 4 hours (to reach an optical density of 0.5-0.6 at 600nm). The culture was then cooled on ice, transferred to corex tubes and subjected to centrifugation at 4 000rpm at 4°C for 5 minutes in a YA-20 rotor.

The supernatant fluid was discarded and the cells in each corex tube resuspended in 3.25ml of 60mM CaCl, 10mM Hepes [pH 7,2]. The cell suspensions were pooled into 2 tubes and incubated on ice for 20 minutes, prior to centrifugation at 4 000rpm at 4°C for 5 minutes.
The supernatant fluid was discarded and the cells in each corex tube resuspended in 1.5ml of 60mM CaCl, 10mM Hepes, 15% glycerol.
The cells were divided into 100μl aliquots and snap frozen for storage at -70°C.
The competence of the cells was checked by transforming with circular closed plasmid and selection on Luria agar containing antibiotic.

3.2.9 TRANSFORMATION OF *E. coli* LK111 CELLS.

Previously prepared LK111 competent cells (efficiency estimated at $5\times10^6/\mu$g of DNA) stored in 100μl aliquots at -70°C were used. The cells were thawed on ice prior to the addition of the ligation mix. A 10μl aliquot of the ligation mix including *Sma*I was used, while 5μl of the ligation mix without *Sma*I was used. These volumes corresponded to an estimated 25ng of pUC18 DNA per transfomation reaction. The cells were mixed gently by swirling intermittently and kept on ice for 30 minutes.
The cells were heat shocked at 42°C for precisely 2 minutes, then placed onto ice for 2 minutes. One milliliter of prewarmed Luria broth was added to the cells, and incubated at 37°C for 1 hour to allow expression of the β-lactamase gene.
Aliquots of 50μl of the cell suspension were then spread onto selective media; Luria agar plates containing ampicillin (50ng/μl), 2% X-gal in dimethyl formamide (250μl/100ml), and IPTG (50μl/100ml). After allowing for absorption of the liquid, the plates were inverted and incubated at 37°C overnight.
Transformed cells containing the M.tb fragment inserted into pUC18 were recognized as white colonies, because the β-galactosidase gene was interrupted, amongst blue colonies. The blue colonies are those cells which contain pUC18, and are therefore resistant to ampicillin, but do not contain an insert to interrupt the β-galactosidase gene.

3.2.10 SMALL SCALE PLASMID PREPARATION ('Miniprep')

To screen selected colonies for the presence of recombinant plasmid an alkaline lysis method of plasmid extraction based on that of Birnboim and Doly (1979) was used.

A single colony of transformed *E. coli* was inoculated into 10ml of Luria broth and incubated at 37°C overnight (approximately 18 hours), while shaking.

A 1.5ml Eppendorf tube was filled with culture broth followed by centrifugation at 14 000rpm for 30s to 1 minute in a microfuge. The supernatant fluid was discarded and the cells resuspended in 90µl of Tris/EDTA/glucose (25mM Tris-HCl, 10mM EDTA, 50mM glucose [pH 8.0]).

Cells were lysed by adding a spatula-tip of powdered lysozyme, and the mixture incubated at room temperature for 5 minutes. Thereafter, 200µl of freshly prepared NaOH/SDS solution (0.2N NaOH, 1% SDS) was added, mixed by inverting, and incubated on ice for 5 minutes.

To precipitate chromosomal DNA and protein, 150µl of ice cold 'high salt' (3M K, 5M acetate) was added, mixed by vortexing, and incubated on ice for 15 minutes. The cellular debris and chromosomal DNA was pelleted by centrifugation at 14 000rpm for 5 minutes in a microfuge.

The supernatant fluid was carefully removed and extracted with phenol and chloroform/isoamylalcohol. One-half volume of saturated phenol and 0.5 volume of chloroform/isoamylalcohol (24:1 V/V) were added to the supernatant fluid, the solution was mixed thoroughly by vortexing, and the phases seperated by centrifugation at 14 000rpm for 2 minutes in a microfuge. This step was repeated twice, but with 0.25 volume of phenol and 0.75 volume of chloroform/isoamylalcohol.

Plasmid DNA was precipitated by the addition of 0.6 volume of isopropanol. The solution was mixed by inversion, incubated at room temperature for 5 minutes, and the DNA pelleted by centrifugation at 14 000rpm for 15 minutes in a microfuge. (Alternatively the DNA was precipitated by adding 0.1 volume of 4M LiCl and 2.5 volumes of ice-cold 100% ethanol. This was incubated at -20°C overnight or at -70°C for 15 minutes prior to centrifugation.)

The supernatant fluid was discarded and excess salt removed by centrifugation of the pellet in 70% ethanol.

The supernatant fluid was discarded and the pellet dried under vacuum. Finally the DNA pellet was dissolved in an appropriate volume of sterile, distilled water (usually 10-20µl).

3.2.11 SCREENING TRANSFORMANTS FOR RECOMBINANT PLASMIDS

3.2.11.1 *Eco*RI LINEARISATION

Ten white colonies were picked off of the selection medium and used to prepare plasmid DNA for digestion. *Eco*RI digested linear fragments were produced to screen for larger plasmids corresponding to the 336bp fragment ligated into pUC18.

The digestion mix consisted of 5µl of miniprep DNA, 1µl of H buffer (Boehringer), 1µl of *Eco*RI, 1µl of RNAse and 2µl of water. This was incubated at 37°C for 2½ hours and then electrophoresed in a 0.8% agarose gel containing ethidium bromide to enable visualisation of the DNA under ultraviolet light.

3.2.11.2 *Hin*dIII/*Eco*RI DIGESTION

Because the vector, pUC18, was T-tailed at the *Sma*I site, the inserted fragment of DNA could not simply be cut out of the plasmid using *Sma*I. Instead, two enzymes, which are known to have restriction sites on either side of the *Sma*I site in the multiple-cloning site (see appendix A), were used to digest the clones. This produced two fragments, one approximately 2.8kb, and the other slightly larger than the original 336bp insert.
The 20µl digestion mix was constituted as follows; 2µl of plasmid DNA (produced by miniprep), 2µl of B buffer (Boehringer), 1µl of *Eco*RI, 1µl of *Hin*dIII, 1µl of RNAse and 13µl of water. This was incubated at 37°C overnight before electrophoresis in a 0.8% agarose gel.

3.3 RESULTS

The mycobacterial DNA, produced by the method described, was seen as a compact band on 0.8% agarose gel electrophoresis. No smearing of DNA, indicative of smaller sheared fragments, was seen. The DNA could be used for all the manipulations described in this chapter. The concentration of mycobacterial DNA produced by this method varied between preparations from 24-990ng/µl.

The polymerase chain reaction of M.tbA DNA successfully amplified a fragment of DNA of approximately 336bp. This was the size expected for the primers used. The fragment can be seen compared to a molecular weight marker in Figure 1. The solution was estimated to contain approximately 5-10ng/µl of the DNA fragment.

The large scale plasmid DNA preparation gave a wide ranging yield; from 20µg to 350µg per maxiprep, with the average yield approximately 100µg. The DNA prepared was of a high quality with the 260nm/280nm ratios varying from 1.75 to 1.80.

The pUC18 plasmid, linearised by *Sma*I and T-tailed as described in 3.2.6, was seen as a ±2.7kb fragment on a 0.8% agarose gel and the concentration estimated at approximately 50ng/μl (result not shown).

The DK1 cells, prepared as described in 3.2.8, had an efficiecy of 1×10^{8}/μg DNA. However these cells were not used for the selection of the cloned 915 region of M.tbA, as DK1 cells do not exhibit the blue/white selection which was required.

The transformation of *E. coli* LK111 cells by the ligation mix which did not include *Sma*I, resulted in the growth of 8 white colonies in a total of 233. Transformation of *E. coli* LK111 cells by the ligation mix which included *Sma*I, gave 53 white colonies in a total of 164. This represents a 10 times improvement in efficiency when *Sma*I is included during the ligation reaction. (3% versus 32% white of total colonies.) The presence of so many blue colonies despite the use of *Sma* I may be caused by conditions for ligation (the use of ligation buffer and low temperature) not being optimal for the activity of *Sma* I.

Two of the ten colonies screened contained plasmids, which on linearisation by *Eco*RI digestion, were larger than control linearised pUC 18 (Figure 3.2).
Each *Eco*RI/*Hin*dIII digestion of plasmid DNA from the two selected colonies produced two fragments; one of approximately 2.7kb and one of approximately 336bp. These are shown in figure 3.2.

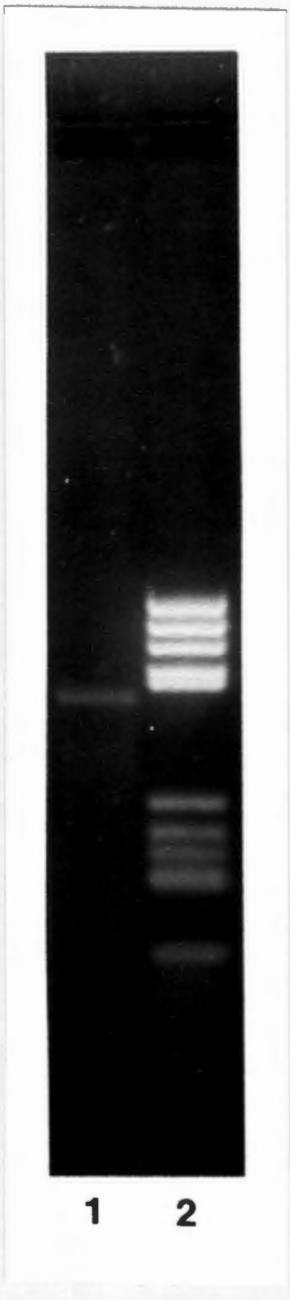


FIGURE 3.1: PCR amplified fragment of 16S rRNA gene from the streptomycin resistant *M. tuberculosis* isolate, M.tbA, seen on 3% agarose gel after electrophoresis. Lane 1: PCR amplified fragment. Lane 2: Boehringer Mannheim marker V (see appendix B).

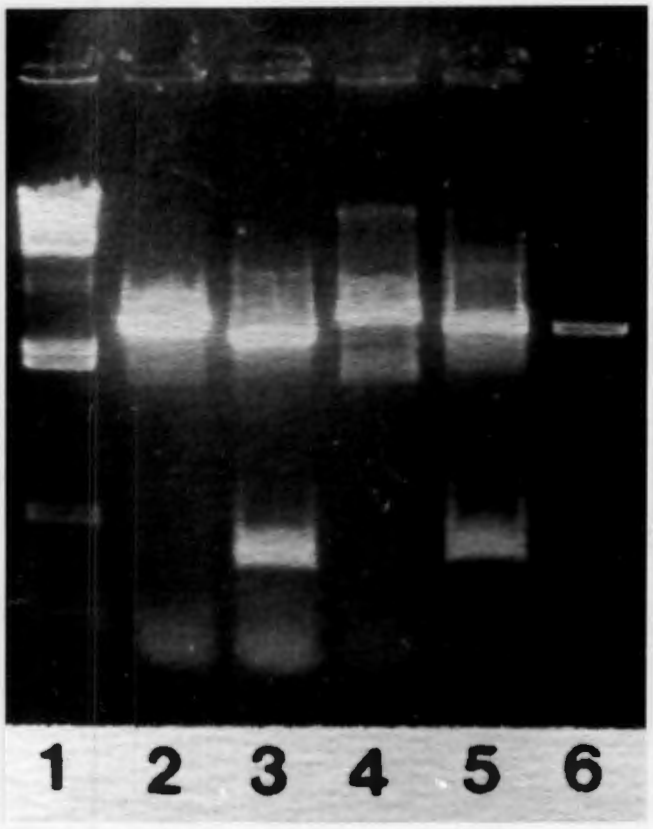


FIGURE 3.2: Two recombinant plasmids (designated pRIB1 and pRIB2), digested by *Eco*RI or double digested by *Eco*RI and *Hin*dIII, seen on 0.8% agarose gel after electrophoretic seperation. The linearised recombinant plasmids in lanes 2 and 4 can be seen to be larger than the parent plasmid, pUC18, in lane 6. Lanes 3 and 5 show a second band of DNA of ±336bp excised from the recombinants by the double digestion.
Lane 1: Lambda *Hin*dIII digested DNA. Lane 2: pRIB1 *Hin*dIII digested DNA. Lane 3: pRIB1 *Eco*RI and *Hin*dIII digested DNA. Lane 4: pRIB2 *Hin*dIII digested DNA. Lane 5: pRIB2 *Eco*RI and *Hin*dIII digested DNA. Lane 6: pUC18 *Sma*I linearised DNA.

CHAPTER 4

DNA SEQUENCING OF THE 915 REGION OF THE 16S rRNA GENE OF A STREPTOMYCIN RESISTANT *M. TUBERCULOSIS* ISOLATE

4.1 SUMMARY

The DNA sequence was determined for the 915 region of the 16S rRNA gene of a *M. tuberculosis* isolate, M.tbA. This organism is resistant to multiple antitubercular drugs, including streptomycin. Two recombinant plasmids, pRIB1 and pRIB2, were constructed as described in chapter 3. These contain a 336bp fragment of the 915 region of M.tbA inserted into the T-tailed *Sma*I site of pUC18. The plasmids were extracted from transformed LK111 cells by a modification of the method of Birnboim and Doly (1979), and sequenced by the dideoxy-Sanger method (Sanger *et al*, 1975; Sanger *et al*, 1977). The DNA sequence was then compared to the equivalent published sequences of *M. tubeculosis* (Rogall *et al*, 1990) and *E. coli* and found to contain an A→G transition at position 913 (*E. coli* numbering).

4.2 METHODS

4.2.1 PLASMID EXTRACTION

The method used to extract plasmid DNA for sequencing was modified from that described previously (chapter 3.2.10). The changes were made to improve the quantity and quality of DNA, as sequencing done on DNA prepared by the method described in 3.2.10 did not give good quality results.

The changes made were:

1.) the entire 10ml culture was extracted to give as much template DNA as possible for the sequencing reaction;

2.) larger volumes of solutions were used, i.e. 400µl of the NaOH/SDS solution, 300µl of the 'high salt' solution;

3.) the purification steps differed in that the plasmid DNA in the supernatant fluid was precipitated and resuspended in TES (10mM Tris-HCl; 1mM EDTA; 150mM NaCl [pH 8.5]), prior to extraction with phenol and chloroform-isoamylalcohol; and finally,

4.) the DNA pellet was dissolved in 30µl sterile, distilled water.

A 2µl aliquot of the final solution was diluted in water and scanned in a Beckman DU-40 spectrophotometer from 220-310nm to estimate the concentration and purity of the DNA.

4.2.2 SEQUENCING REACTION

Sequencing was performed using Sequenase Version 2.0 from United States Biochemicals. The method is based on the dideoxy-Sanger method (Sanger *et al*, 1975; Sanger *et al*, 1977).

In this method a single strand of DNA (either M13 or denatured double-stranded plasmid DNA) is used as a template. An oligonucleotide primer is chosen which anneals to a known sequence of the template towards the 3' end of the area to be sequenced, and extension of the complementary strand of DNA is commenced. However the extension is terminated by the incorporation of nucleotide analogues (2', 3'-dideoxynuleoside 5'-triphosphates), which do not have the 3'-OH group needed for further chain elongation. Four separate reactions are performed; each reaction vessel contains all the constituents needed for DNA polymerisation, but also includes a terminating nucleotide analogue of one base type. Therefore, in each reaction the DNA strand is terminated at one of the four bases, resulting in multiple lengths of DNA which all terminate with the same base. The DNA is labelled by the inclusion of a radiolabelled nucleotide. The four completed reactions are then loaded onto a denaturing polyacrylamide gel, and the DNA strands are separated electrophoretically. DNA fragments differing in length by a single base or more will be separated. Autoradiography of the gel then allows reading of the sequence. The shorter chains migrate faster, therefore the sequence is read from bottom to top in the direction of elongation, i.e. 5' to 3'.

Half of the plasmid DNA was used for the forward primer sequencing reaction and half for the reverse reaction. Using the modified method of plasmid extraction described above (4.2.1), up to 50µg of DNA per reaction was used. The primer used for forward sequencing, 5'-AACGCATTAAGTACCCCGC-3' (positions 863-882), was the same primer used for amplification of the insert (F2). The primer used for reverse sequencing, 5'-CGCGTTGCATCGAATTAAT-3', corresponds to a sequence 50bp downstream of the 915 region (positions 955-974, designated R2).

Denaturation.

The double stranded plasmid DNA was denatured by the addition of 2µl of 2M NaOH to 8µl of the plasmid DNA (an estimated 28pmols; in sterile distilled water). This was incubated at 37°C for 30 minutes.

Annealing and neutralisation.

Two microliters of either primer (949pmols of F2 for the forward reaction, or 470pmols of R2 for the reverse reaction), 2µl of 1M HCl, 2µl of reaction buffer and 4µl of water were added to give a final volume of 20µl. This solution was incubated at 37°C for 30 minutes.
The DNA was precipitated by the addition of 50µl of absolute ethanol, incubated on dry ice for 30 minutes, and followed by centrifugation at 14 000rpm for 15 minutes in a microfuge. The pellet was washed in 70% ethanol and dried under vacuum.
During this time the termination mixes were prepared by adding 2.5µl of each of the solutions containing ddCTP, ddATP, ddTTP, and ddGTP to individual Eppendorf tubes. These were incubated at 37°C until needed.

Extension and Labelling.

The DNA was redissolved in 8µl water, and 2µl of the reaction buffer was added, followed by the addition of 1.7µl dimethylsulphoxide (DMSO), 1µl of 0.1M dithiothreitol (DTT), 2µl of labelling mix (from a 1:15 dilution in water of the dGTP 5× concentrate to give a final concentration of 55nM of each of dGTP, dCTP and dTTP), 1µl of ^{35}S ([α-^{35}S]-dATP), and 2µl of sequenase (DNA polymerase diluted 1:8 with the enzyme dilution buffer).

Termination.

The solution was vortexed briefly and a 3.5µl aliquot immediately added to each of the prewarmed 2.5µl termination mixes. These were incubated at 37°C for 4 minutes prior to the addition of 4µl of stop buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol).
These were stored at -20°C until polyacrylamide gel electrophoresis.

4.2.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

High resolution separation of DNA fragments was performed using 8% polyacrylamide gel electrophoresis. Denaturing conditions (8M urea), and the high running temperature of the gel prevented the formation of secondary structures within the DNA strands with resultant poor resolution. A buffer gradient was also used both to retard the movement of the faster migrating DNA strands and to improve resolution.

Glass plates were prepared immediately before the pouring of the gel by a simple washing step using soap and water, followed by 70% ethanol, and finally acetone. The plates were not siliconised as no difficulty was encountered removing gels.

The plates were apposed, separated by two 2mm spacers and 3mm Whatman number 1 filter paper. The plates used were of unequal length, resulting in an overhang by the longer plate. This is used to form the fourth side of the upper buffer tank during electrophoresis while allowing the gel to be bathed in buffer.

Approximately 40 ml of 8% polyacrylamide in 0.5× NNB buffer (see reagents) was used per gel. A 140µl aliquot of freshly prepared 25% ammonium persulphate (AMPS) and 35µl of NNN'N'-tetramethylethylenediamine (TEMED) was added and mixed briefly by swirling, to initiate polymerisation.

The polyacrylamide solution was poured immediately, taking care to prevent bubbles from becoming trapped in the gel, as these distort the final DNA pattern. When filled to the top edge of the shorter plate a comb was inserted, blunt edge leading, to form a smooth surface.

After polymerisation, the gel was used immediately or kept overnight sealed with Saran wrap.

The comb was removed, and the gel clamped vertically against the electrophoretic apparatus with the shorter plate toward the upper buffer tank. Enough buffer was added to the upper tank to cover the upper surface of the gel, and 400ml added to the bottom tank.

The gel was preheated by applying an electric field at constant power (30W) for 30 minutes.

Samples were denatured at 100°C for at least 3 minutes before loading. The comb was then inserted between the plates at the top of the gel. This formed the wells. The wells were rinsed thoroughly with buffer to remove the highly concentrated urea which leaches out of the gel during the preheating step. If not well rinsed, the urea prevents the sample loaded from forming a sharp band, and results in poor resolution.

The samples were loaded into the wells, 3µl per well, in the order CATGC. This order was always used when running sequencing gels.

Electrophoresis was then performed at 40-45W for 1-1½ hours. When the leading blue marker was half way down the gel, 200ml of 3M sodium acetate was added to the bottom tank to create

a buffer gradient. The electrophoresis was continued until the leading blue marker reached the blotting paper at the bottom edge of the gel.

The plates and gel were removed from the apparatus and the plates prised apart leaving the gel on the lower plate. The upper glass plate was removed, a sheet of Whatman 3MM paper was laid onto the gel and smoothed onto it carefully until the gel adhered to the paper. The paper and attached gel were then carefully peeled off of the bottom plate. This was laid onto a slab gel drier (with the paper below the gel), a film of saran wrap laid over the gel, and dried at 60°C under vacuum for 2-3 hours.

Once dry, the gel was autoradiographed for 18 hours to 1 week, depending on the intensity of the bands.

4.3 RESULTS

The sequences of both clones (see Figures 4.1 and 4.2) were identical to each other but differed from published mycobacterial 16S rRNA sequences at a single position. A 913A→G transition was identified in both clones.

FIGURE 4.1: Polyacrylamide sequencing gel of pRIB1 and pRIB2. This shows the 915 region of the 16S rRNA gene of M.tbA sequenced in both the forward (sequences 1 & 4) and reverse (sequences 2 & 3) directions. The 913A→G transition is marked by an open arrow (⇨ or ⇦) in the forward sequences, and a closed arrow (➔ or ➔) in the reverse sequences.

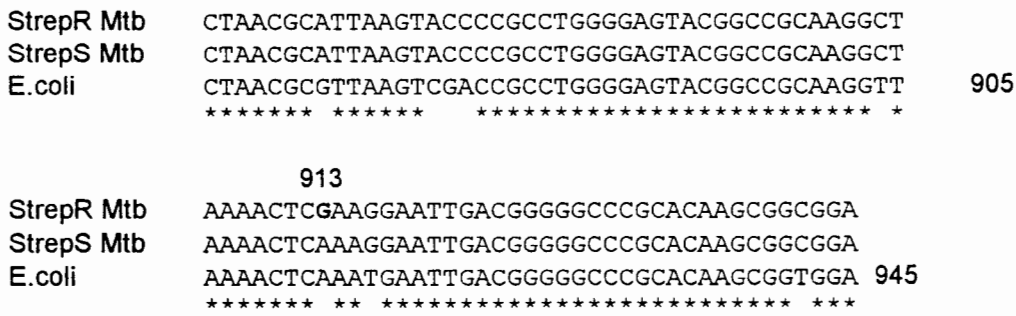


FIGURE 4.2: Sequence alignment of the streptomycin binding region of the 16S rRNA gene of the streptomycin resistant *M. tuberculosis* (StrepR Mtb) with the equivalent region of published *M. tuberculosis* (StrepS Mtb) and *E. coli*.

The numbers to the right of the sequences correspond to the position in the 16S rRNA gene of the nucleotide shown at the end of each row; the mycobacterial 16S rRNA gene is shorter than the *E. coli* gene. The A→G transition at position 913 is labelled and shown in bold. The sequences were aligned using the 'Clustal V' programme (Higgins *et al*, 1992); stars indicate base identity.

CHAPTER 5

SCREENING STREPTOMYCIN RESISTANT *M. TUBERCULOSIS* ISOLATES BY THE AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)

5.1 INTRODUCTION

With the discovery of the 913A→G transition in an isolate of streptomycin resistant *M. tuberculosis*, we were interested in investigating the possibility that this mutation was present in other streptomycin resistant *M. tuberculosis* isolates. Forty isolates of streptomycin resistant *M. tuberculosis* were screened using the PCR based amplification refractory mutation system (ARMS) described by Newton *et al*, (1989).

5.2 METHODS

Isolates of *M. tuberculosis* were collected from the mycobacteria laboratory of Groote Schuur Hospital, and the Orange street laboratory of the South African Institute of Medical Research (SAIMR). All isolates were found to be resistant to streptomycin by the SAIMR. Aliquots of DNA from two streptomycin resistant *M. tuberculosis* isolates were kindly supplied by Ian Wiid of the Medical Biochemistry Department, Tygerberg.

5.2.1 THE AMPLIFICATION REFRACTORY MUTATION SYSTEM

To clone and sequence a large number of isolates is not a pratical means of detecting a single base mutation in a large number of isolates. The amplification refractory mutation system (ARMS) is a PCR based method of detecting single base changes. Two PCR reactions are performed simultaneously for each isolate screened. The 3' end of one of the primers corresponds to the base under investigation. It is this 3' end which differs in the two reactions; in one reaction the base at the 3' end of the primer is complementary to one of the possible bases

at the mutation position, while the other tube contains a primer whose 3' end is complementary to the alternate base (assuming there are only two possibilities). The method relies on the finding that a 3' mismatch in one of a set of primers effectively prevents amplification by PCR, while the simultaneous PCR reaction with the matched primer will amplify a fragment. Both of these results must be obtained for the test to be reliable, i.e. one positive and one negative.

The two 18-mer forward primers were 5'-CCGCAAGGCTAAAACTCA-3' and 5'-CCGCAAGGCTAAAACTCG-3'. The forward primers were labelled strepS and strepR respectively. Each was used in combination with the same reverse primer, 5'-TGACGTCATCCCCACCT-3' (R1). This was one of the primers used to PCR amplify the 336bp fragment used for cloning as described in chapter 3.2.2.

5.2.1.1 OPTIMISATION

Mycobacterial DNA was prepared by the method described in chapter 3.2.1. DNA from the streptomycin resistant isolate, M.tbA, and from a streptomycin sensitive isolate was used.

The PCR mix incorporated dUTP and Uracil DNA glycosylase (UDG) in an effort to control contamination by previously generated fragments. This practice was later discontinued because the UDG was found to be incompletely inactivated at 95°C by Mr Bruce Allan (personal communication).

PCR was performed using a JDI 8012 thermocycler using the following parameters; 37°C for 5 minutes, 95°C for 5 minutes, followed by 40 cycles of; 95°C for 20 seconds, 56.3°C for 30 seconds and 70°C for 30 seconds.

A 10µl aliquot from each reaction was then loaded onto a 3% agarose gel for electrophoresis.

5.2.1.1.1 Comparison of two annealing temperatures on specificity.

PCR was performed on M.tbA using both strepR and strepS forward primers in combination with a reverse primer (R1) in the format of an ARMS test. The template DNA was added at 120ng per reaction. Primer concentration was approximately 2.5µM of each. Two sets of identical reaction mixes were made. Thermal cycling was performed using the programme given in 5.2.1.1 for the first set of tubes, i.e. the annealing temperature was 56.3°C. During the thermal cycling of the second set, however, the annealing temperature was 50°C.

5.2.1.1.2 Comparison of two denaturation temperatures on sensitivity.

ARMS was performed on M.tbA using strepR and strepS forward primers in combination with a reverse primer (R1). The template DNA was added at 120ng per reaction. Primer concentration was 2.5µM of each. Two sets of identical reaction mixes were made. Thermal cycling was performed using the programme given in 5.2.1.1 for the first set of tubes. i.e. the denaturation temperature was 95°C. During the thermal cycling of the second set, however, the denaturation temperature was 96°C.

5.2.1.1.3 Comparison of various concentrations of template DNA and two concentrations of primers.

A dilution series of M.tbA (streptomycin resistant) DNA was made, and 10µl added to a PCR tube to give a final total of approximately 12pg, 120pg, 1.2ng, 12ng and 120ng of DNA per tube. Each of these was subject to PCR at two concentrations of primer. One reaction contained 150ng (0.625µM) of each primer (strepR forward primer and R1 reverse primer), and the other 600ng (2.5µM) of each primer.
Simultaneously an experiment using a streptomycin sensitive *M.tuberculosis* isolate and the strepS forward primer was done. The concentration of primer was the same and template DNA concentration was approximately the same. viz. 10pg, 100pg, 1ng, and 10ng.

5.2.1.1.4 Comparison of DNA extraction methods.

A simple method of extraction was sought for use during the screening of isolates in order to avoid the longer, more formal, 'cetramide' extraction method.
Three simple methods were compared: 1) boiling in water for 5 minutes; 2) sonication in water for 10 minutes in a Branson 2000 sonicator; and 3) boiling and sonication.
A loopful of M.tbA culture was scraped from a L&J slope, swirled in 200µl water in 3 Eppendorf tubes and sealed. Two of these were placed into boiling water for 5 minutes. On removal, one of the tubes was kept on ice, while the second tube along with the third tube was placed in the sonicator for 10 minutes. The three tubes were then spun in a microfuge at 14 000rpm for 30 seconds to pellet the debris.

A 10µl aliquot of the supernatant fluid was used from each for PCR using the strepR-R1 primer combination. The PCR parameters were as before; 37°C for 5 minutes, 95°C for 5 minutes, followed by 40 cycles of; 95°C for 20 seconds, 56.3°C for 30 seconds and 70°C for 30 seconds. DNA extracted by the cetramide method was subject to the same PCR reaction, as was an aliquot of water, to serve as positive and negative controls respectively.
Following PCR amplification, a 20µl aliquot was electrophoresed on a 3% agarose gel.

5.2.1.2 ARMS SCREENING

The routine used for ARMS screening was:
1) extraction of DNA by boiling the culture in water,
2) ARMS PCR using 10µl of supernatant fluid,
3) electrophoresis of a 10µl aliquot on a 3% agarose gel with positive and negative controls.

As a negative control 10µl of water was substituted for the 10µl of DNA. M.tbA was used as the positive control for the strepR-R1 combination. A positive control for the strepS-R1 primer combination was not always included on each run because the majority of test results were positive using these primers. However on occasions that a run did not include a positive result with these primers, the screen was repeated using DNA from one of the previously screened isolates known to give a positive result with these primers.

5.3 RESULTS

When comparing the two annealing temperatures, 50°C and 56.3°C, both sets of ARMS reactions produced identical results at both temperatures (figure 5.1). Both gave a positive result with the strepR-R1 primer combination, and a negative result with the strepS-R1 combination, as was expected for this 913 A→G mutant isolate. Because no false positive amplification occurred by the strepS-R1 primer combination at the lower annealing temperature the technique is highly specific. Thus lending a degree of confidence to the results.

The fragment of DNA amplified by PCR using a higher denaturation temperature was seen on an agarose gel as a faint band (figure 5.1). It was not as distinct as that produced when using the lower denaturation temperature. The increase in denaturation temperature did not appear to improve sensitivity, but rather to decrease the sensitivity .

The lowest concentration of template DNA giving a visible product was found to be approximately 1ng. This was seen in tubes in which 150ng of primer was used per reaction. The higher concentration of primer did not improve the sensitivity, on the contrary it caused the lower limit of detection to be approximately ten times higher. See figure 5.2.

A PCR product was clearly visualised when using the boiling, as well as the boiling plus sonication methods (see figure 5.3). Sonication, *per se*, did not allow production of a distinct band, if any.
The simple boiling of culture is adequate to release DNA into the water, and amplification by PCR of this DNA is possible. The addition of sonication does not improve the sensitivity appreciably.

An example of a result obtained during the screening is shown in figure 5.4. The results of the entire screening are shown in table 5.1.
Three isolates contained the 913A→G transition as screened for by ARMS. This represents <10% of the isolates screened; less than we had expected. In retrospect, the finding of the mutation in the first isolate sequenced (M.tbA), was perhaps very fortunate.

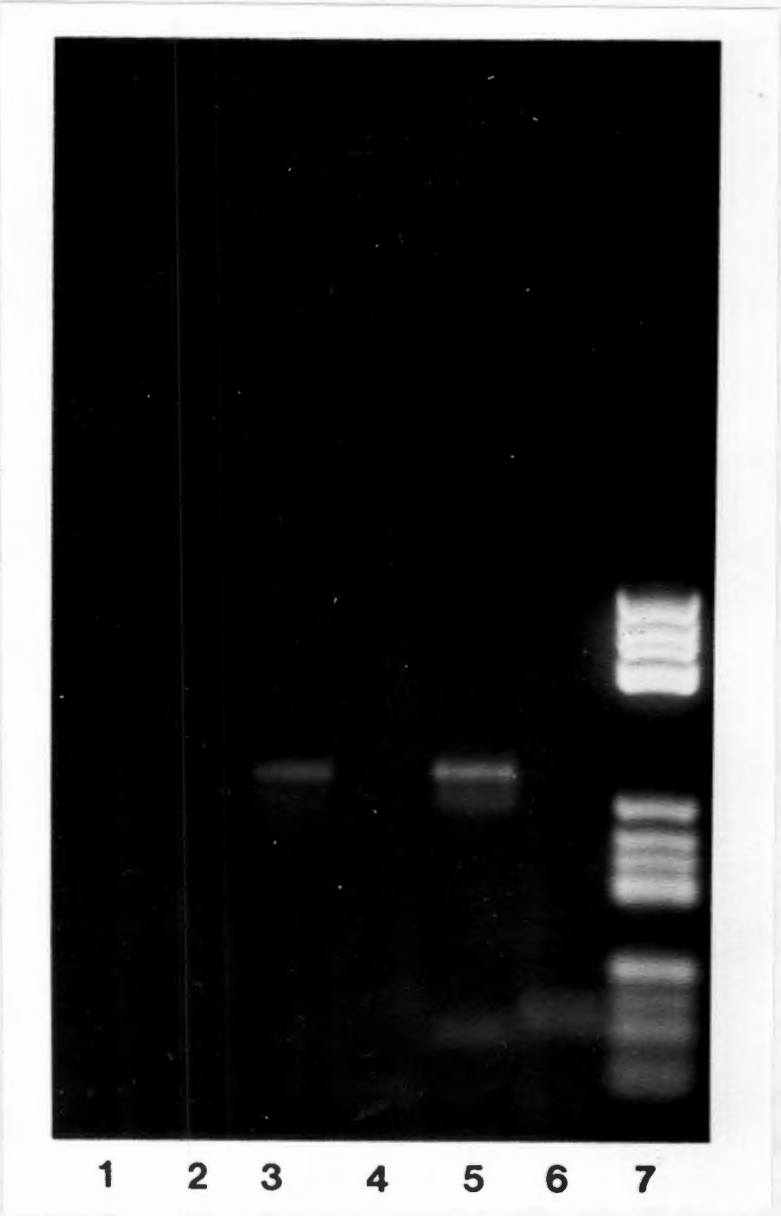


FIGURE 5.1: Agarose gel electrophoresis of ARMS done on M.tbA at two annealing temperatures and two denaturation temperatures. Lanes 1 & 2 denatured at 96°C, annealed at 56.3°C. Lane 1: strepR-R1 of M.tbA. Lane 2: strepS-R1 of M.tbA. Lanes 3 & 4 annealed at 50°C, denatured at 95°C. Lane 3: strepR-R1 of M.tbA. Lane 4: strepS-R1 of M.tbA. Lanes 5 & 6 annealed at 56.3°C, denatured at 95°C. Lane 5: strepR-R1 of M.tbA. Lane 6: strepS-R1 of M.tbA. Lane 7: marker V.

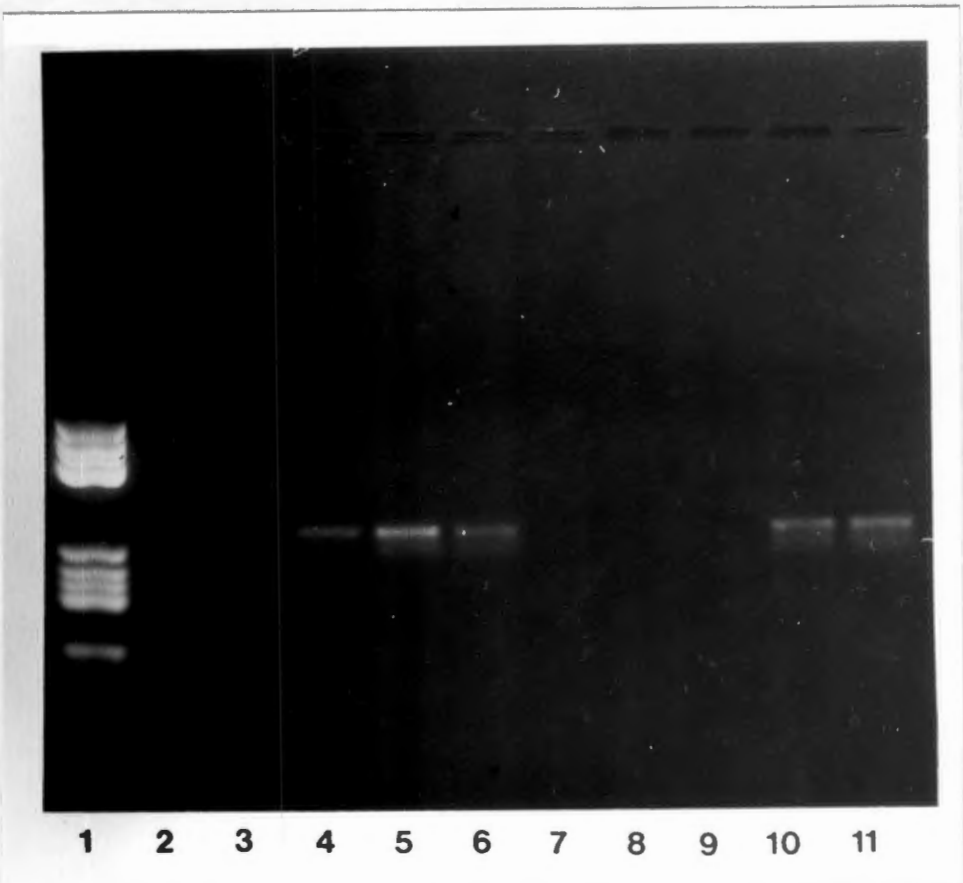


FIGURE 5.2: Agarose gel electrophoresis of PCR fragments generated by the primer pair ,strepR-R1, at various concentrations of template DNA (M.tbA), and two concentrations of primer (strepR-R1). Lane 1: marker V. Lanes 2-6: primer concentration 0.625μM. The amount of template is increased in each lane from left to right by a factor of 10. Lane 2: ±12 pg of M.tbA. Lane 3: ±120 pg of M.tbA. Lane 4: ±1.2 ng of M.tbA. Lane 5: ±12 ng of M.tbA. Lane 6: ±120 ng of M.tbA. Lanes 7-11: primer concentration 2.5μM, with the amount of template increased in each lane from left to right by a factor of 10. Lane 7: ±12 pg of M.tbA. Lane 8: ±120 pg of M.tbA. Lane 9: ±1.2 ng of M.tbA. Lane 10: ±12 ng of M.tbA. Lane 11: ±120 ng of M.tbA. The lower primer concentration is seen to be ten times more sensitive.

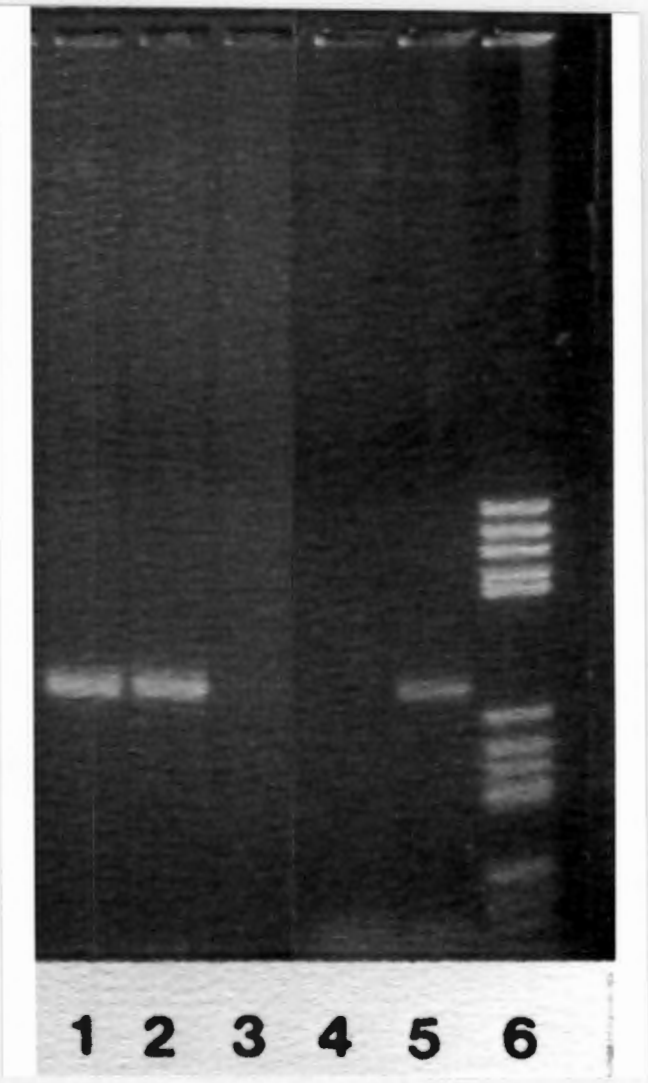


FIGURE 5.3: Agarose gel electrophoresis of PCR fragments generated by the primer pair strepR-R1 on DNA extracted from M.tbA by different methods. Lane 1: boiled M.tbA. Lane 2: boiled and sonicated M.tbA. Lane 3: sonicated M.tbA. Lane 4: negative control. Lane 5: positive control. Lane 6: marker V.

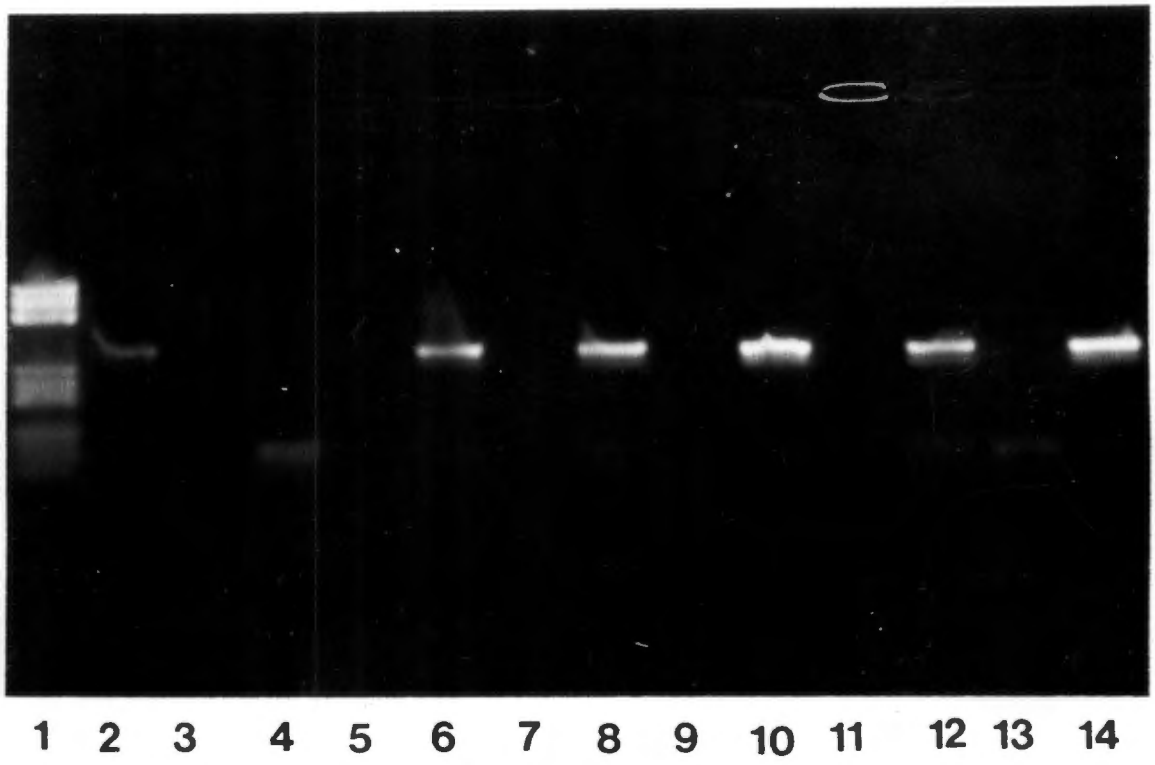


FIGURE 5.4: Agarose gel electrophoresis of ARMS screening of five isolates all giving a result indicative of the presence of the base adenine at position 913. Lane 1: marker V. Lane 2: positive control (M.tbA template, strepR-R1 primer pair). In lanes 3-14 the primer pairs alternate. i.e.strepR-R1 followed by strepS-R1. Lane 3 & 4: negative control. Lane 5 & 6: isolate number 10. Lane 7 & 8: isolate number 15. Lane 9 & 10: isolate number 12. Lane 11 & 12: isolate number 6. Lane 13 & 14: isolate number 5.

TABLE 5.1: Results of the ARMS screening done on M.tbA and a further 40 streptomycin resistant *M. tuberculosis* isolates.

ISOLATE	ARMS RESULT
1 (M.tbA)	913 A→G
2	913 A→G
3	913 A
4	913 A
5	913 A
6	913 A
7	913 A
8	913 A
9	913 A
10	913 A
11	913 A
12	913 A
13	913 A
14	913 A
15	913 A
16	913 A
17	913 A
18	913 A
19	913 A
20	913 A
21	913 A
22	913 A
23	913 A
24	913 A
25	913 A
26	913 A
27	913 A
28	913 A
29	913 A
30	913 A
31	913 A
32	913 A
33	913 A
34	913 A
35	913 A
36	913 A
37	913 A
38	913 A
39	913 A→G
40	913 A
41	913 A

CHAPTER 6

DIRECT SEQUENCING OF 16S rRNA PCR AMPLIFIED FRAGMENTS FROM STREPTOMYCIN RESISTANT *M. TUBERCULOSIS* ISOLATES

6.1 INTRODUCTION

Twenty-four of the isolates screened for the 913A→G transition were sequenced in the 915 region. This was done, firstly; to confirm the results of the ARMS, and secondly; to determine whether any other mutations exist in this region of *M. tuberculosis* isolates resistant to streptomycin. The sequencing was done after amplifying the 915 region, using the polymerase chain reaction, and purifying the resulting linear double stranded DNA fragment, which was sequenced directly. This avoided the time consuming process of cloning as well as sequencing errors caused by *Taq* polymerase infidelities.

6.2 METHODS

6.2.1 PCR OF A 400bp FRAGMENT

The upstream primer, 5'-GTAGTCCACGCCGTAAACG-3', (designated F1), was used in combination with the downstream primer, R1, described previously.

The template DNA was extracted by simply boiling *M. tuberculosis* culture in 200μl of water, and using 10μl of the supernatant fluid for PCR.

A 10μl aliquot of water was added to a PCR tube as a negative control.

The PCR mix contained 150ng (±0.625μM) of each primer, the nucleotides dATP, dCTP, dGTP and dTTP, *Taq* polymerase and the appropriate buffer.

Thermal cycling was done using the following progamme: 95°C for 5 minutes, followed by 40 cycles of; 95°C for 10 seconds, 54°C for 30 seconds, 70°C for 30 seconds, and finally ending with a 5 minute chase at 70°C. An aliquot was loaded onto an agarose gel for electrophoresis to

check for the production of the correct sized fragment (400bp) and to estimate the concentration of DNA.

6.2.2 PURIFICATION OF THE PCR PRODUCT

Purification was performed using well to well elution, as described by Hansen *et al* (1992). This is a simple, quick method, with a good rate of recovery.

6.2.2.1 WELL TO WELL ELUTION

An agarose gel (3%) was poured placing two combs into the gel parallel to each other. Once the agarose had gelled, it was placed into an electrophoretic apparatus with the buffer not covering the gel as can be seen in figure 6.1. The PCR-amplified DNA solution was placed into the cathodal well and electrophoresis commenced. The progress of the band of DNA was monitored using a hand-held UV source. When the band was within 1-2mm of the anodal (retrieval) well, the electrophoresis was stopped. The buffer in the well was removed and replaced with a running buffer containing 10% 3M sodium acetate [pH6.0] and 10% sucrose in TAE. The electrophoresis was continued. The buffer caused the DNA band to become very sharp as it entered the well. This allowed most of the DNA to be removed in a small volume of buffer, and the fragment is separated from all other nucleic acids present in the PCR mix. The DNA was precipitated by adding 1µl of glycogen and an equal volume of isopropanol. This was incubated at room temperature for 5 minutes and pelleted by cetrifugation at 14 000rpm in a microfuge. The DNA was then washed in 70% ethanol, dried, and redissolved in sterile, distilled water.
The method was easy and quick, and produced DNA which could be sequenced nearly every time a sequencing reaction was performed.

Sequencing after well to well elution was compared to sequencing after ammonium acetate precipitation. Two identical PCR reactions were performed. The DNA in one was precipitated using ammonium acetate. The DNA in the other was purified by well to well elution. These were then sequenced as described below, followed by denaturing PAGE and autoradiography.

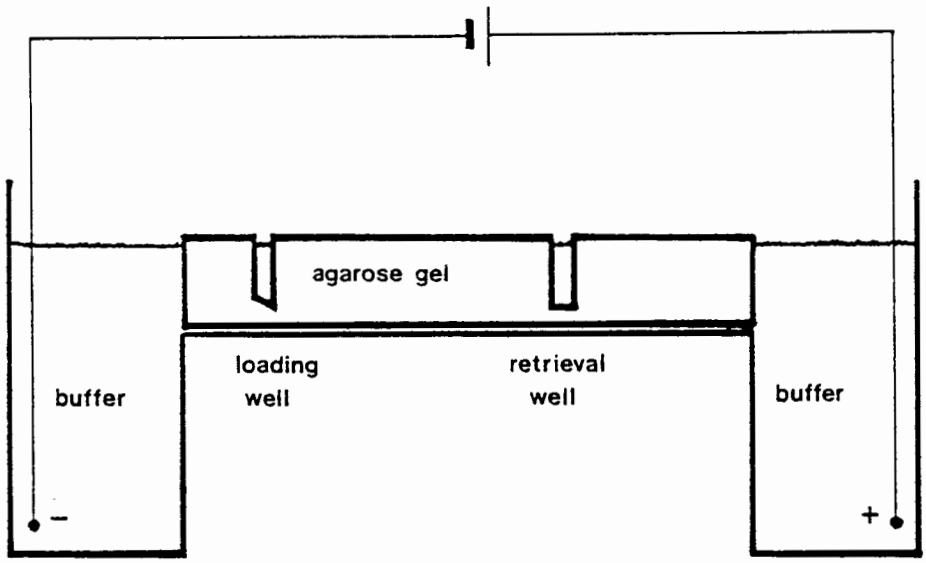


FIGURE 6.1: Schematic diagram of well to well electrophoretic apparatus used for purifying PCR generated fragments.

6.2.3 SEQUENCING

The method used for sequencing the fragments produced by PCR differed somewhat from that used for sequencing of plasmid DNA. The template was heat denatured (Casanova *et al*, 1990). Detergents were used to minimize reannealing of the template (Bachman *et al*, 1990). The annealing buffer was added to a frozen mixture of template, primer and detergent, to improve clarity of the sequencing gel (Andersen *et al*, 1992). A manganese buffer was used to enable reading of the sequence close to the primer. The ratio of primer to template was ±100:1, which has been shown to be a good ratio for direct sequencing (Casanova *et al*, 1990).

Denaturing and annealing.

The DNA template was dissolved in 9µl of water. A 1µl aliquot of primer F2 (±474pmoles), and 2µl of the detergent mix (0.5% Tween 20 and 0.5% NP40 in a 1:1 ratio) were added. This was heated at 100°C for 3 minutes before cooling at -70°C. Once the solution had solidified a 2µl aliquot of annealing buffer was added.

Labelling reaction.

Three microlitres of labelling buffer was added, 1μl of ^{35}S-dATP, 2μl of T7 DNA polymerase (Pharmacia), 1.75μl of the detergent mix and 1μl of Mn^{2+} buffer (from Sequenase kit). This was mixed in a picofuge and incubated at room temperature for no longer than 2 minutes.

Termination.

A 2μl aliquot of each of the four dideoxynucleotide termination mixes was added to separate Eppendorf tubes, together with 0.5μl of the detergent mix per tube.
The annealed and labelled DNA was then divided between the four tubes; 4.5μl per reaction tube. These were incubated at 40°C for 3 minutes, after which the reaction was stopped by the addition of 5μl of stop buffer per tube.
The DNA was ready for PAGE immediately, or after storage at -20°C.

6.2.4 POLYACRYLAMIDE GEL ELECTROPHORESIS

The sequencing reaction was heated at 100°C for 3-5 minutes before loading onto an 8% polyacrylamide gel for electrophoresis and autoradiography in exactly the same way as described in chapter 4.2.3.

6.3 RESULTS

PCR amplification produced an estimated 500ng to 1.5μg (1.9-5.7 pmoles) of DNA per reaction. Initially 5 tubes were combined for each sequencing reaction. However this was found to be unnecessary, as the DNA from a single PCR reaction produced sequencing gels of good quality.
The well to well method of elution was found to be quick and easily performed. When compared to electroelution it was found that the amount of DNA recovered was very similar. (Result not shown.)
Sequencing after DNA precipitation by ammonium acetate, without purification of the DNA template by well to well elution, gave a poor result when compared to well to well purification, as can be seen in figure 6.2.

The sequencing confirmed the ARMS results for the two isolates containing the 913 A→G transition, and for 22 isolates without the transition (Table 6.1). No other mutations were identified in this region.

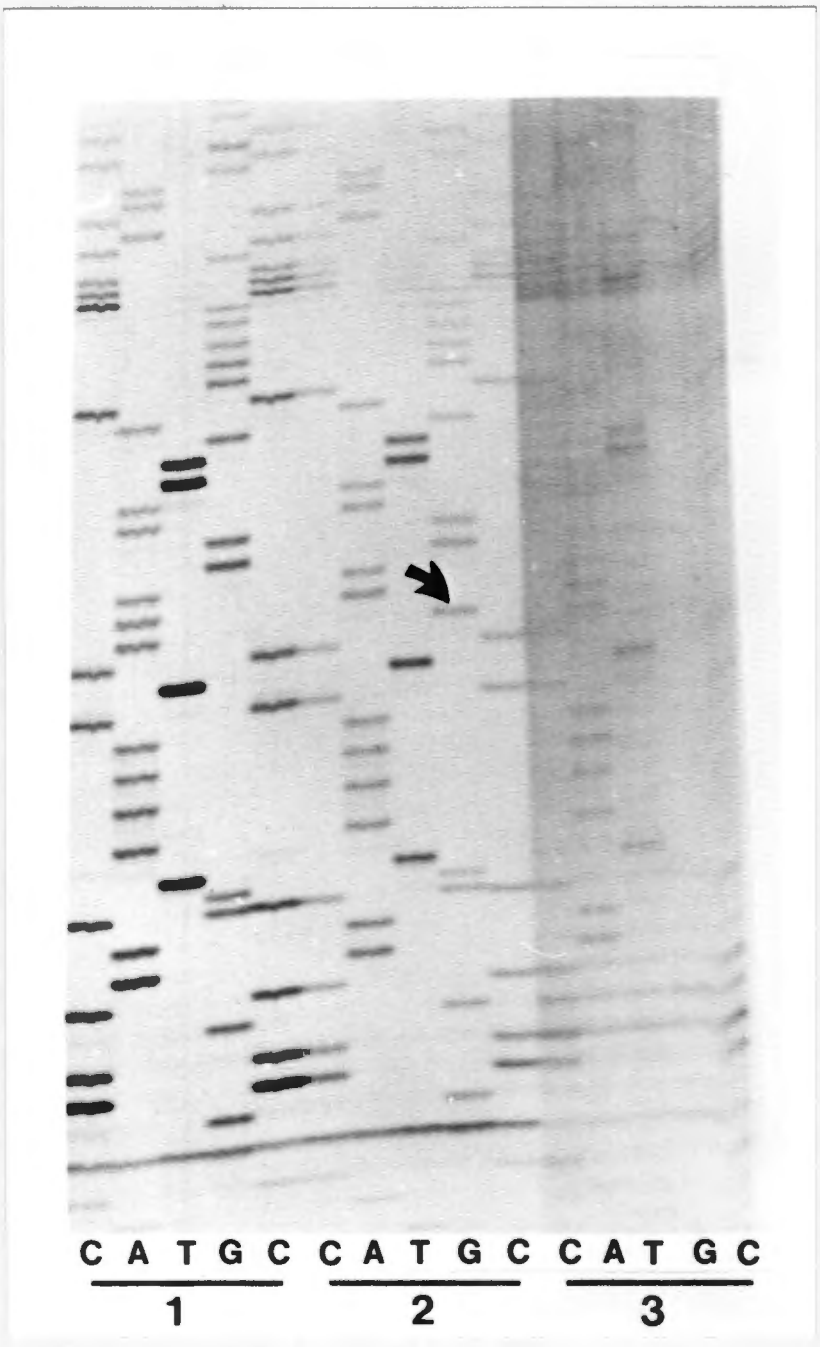


FIGURE 6.2: Autoradiograph of three sequencing reactions showing an example of the 913 A→ G transition (➔). Sequence 1 and 3 are the sequence of isolate number 3, comparing well to well elution (1) to simple precipitation (3). Sequence 2 is the sequence of isolate number 39 which contains the 913 A→G transition (➔).

TABLE 6.1: Sequencing results for the 915 region of M.tbA and 24 further streptomycin resistant *M. tuberculosis* isolates.

ISOLATE	ARMS RESULT	SEQUENCING
1 (M.tbA)	913 A→G	913 A→G
2	913 A→G	913 A→G
3	913 A	913 A
4	913 A	913 A
5	913 A	913 A
6	913 A	913 A
7	913 A	913 A
8	913 A	913 A
9	913 A	913 A
10	913 A	913 A
11	913 A	913 A
12	913 A	913 A
13	913 A	913 A
14	913 A	913 A
15	913 A	913 A
16	913 A	913 A
18	913 A	913 A
19	913 A	913 A
20	913 A	913 A
21	913 A	913 A
22	913 A	913 A
23	913 A	913 A
24	913 A	913 A
25	913 A	913 A
39	913 A→G	913 A→G

CHAPTER 7

CONCLUSIONS AND DISCUSSION

Tuberculosis is a resurgent disease worldwide, with increasing levels of resistance to anti-mycobacterial agents.

We investigated one of the most important ribosomal sites of the streptomycin-ribosomal interaction in streptomycin resistant *M. tuberculosis* isolates using established methods of cloning and sequencing as well as the newer amplification refractory mutation system (ARMS) and direct sequencing of PCR fragments.

We have shown; firstly, that a small proportion (3 of the 41 isolates screened) of the streptomycin resistant isolates contain a 913 A→G mutation in the 16S rRNA gene. Mutations in this region of *M. tuberculosis* had not been demonstrated previously. Secondly, the ARMS method is a sensitive, specific, quick and easy method for the detection of this mutation.

Meier *et al* (1994) confirmed our findings in one of the three streptomycin resistant isolates which they investigated. Interestingly, the same isolate also contained an S12 mutation at codon 88(Lys to Arg), emphasising the importance of excluding S12 mutations in these organisms.

We have not proven that this mutation is a cause of streptomycin resistance in *M. tuberculosis*. This could be tested by introducing a gene mutated in the 913 position into *M. smegmatis* and measuring the streptomycin MIC (minimal inhibitory concentration). A significant increase in MIC would indicate that the single base mutation causes streptomycin resistance.

The 913 A→G mutation occurs infrequently in streptomycin resistant *M. tuberculosis* isolates, while S12 mutations may occur more frequently, especially in multidrug-resitant isolates. It may be speculated, therefore, that the 913 A→G mutation and other rRNA mutations in highly conserved regions are found in organisms demonstrating primary resistance, while S12 mutations are found in organisms with secondary resistance.

If a limited number of mutations are responsible for all streptomycin resistance in *M. tuberculosis*, and if these mutations are identified, the amplification refractory mutation system could be used as a revolutionary method of sensitivity testing.

REFERENCES

AHERN, H. (1993). Insights into Molecular Mechanisms of Drug-Resistant TB. ASM News **59**: 593-594.

ANDERSEN, A.S.; PETTERSEN, A.F.; KJELDSEN. (1992). A Fast and Simple Technique for Sequencing Plasmid DNA with Sequenase®Using Heat Deaturation. Biotechniques **13**: 878-879.

BACHMANN, B.; LUKE, W.; HUNSMANN, G. (1990). Improvement of PCR amplified DNA sequencing with the aid of detergents. Nucl Acids Res **18**: 1309.

BEARDSLEY, T. (1992). Paradise Lost? Sci Am November: 12-13.

BISWAS, D.K. & GORINI, L. (1972). Restriction, De-restriction and Mistranslation in Missense Suppression. Ribosomal Discrimination of Transfer RNA's. J Mol Biol **64**: 119-134.

BISWAS, D.K. & GORINI, L. (1972). The Attachment site of Streptomycin to the 30S Ribosomal Subunit. Proc Natl Acad Sci USA **69**: 2141-2144.

BIRGE, E.A. & KURLAND, C.G. (1969). Altered Ribosomal Protein in Streptomycin-Dependent *Escherichia coli*. Science (Washington D.C.) **166**: 1282-1284.

BIRNBOIM, H.C.; DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acids Res **7**: 1513-1523.

BÖCK, A.; PETZET, A.; PIEPERSBERG, W. (1979). Ribosomal ambiguity mutations (*ram*) facilitate dihydrostreptomycin binding to ribosomes. FEBS Lett **104**: 317-321.

BRAKIER-GINGRAS, L.; PROVOST, L.; DUGAS, H. (1976). Conformational changes induced in *Escherichia coli* ribosomes by streptomycin. A spin label study. Biochem and Biophys Res Comm **60**: 1238-1244.

BRADLEY, S.G. (1973). Relationships Among Mycobacteria and Nocardiae Based upon Deoxyribonucleic Acid Reassociation. J Bact **113**: 645-651.

BRITISH MEDICAL RESEARCH COUNCIL. (1948). Streptomycin treatment of pulmonary tuberculosis. A Medical Research Council investigation. Br Med J **2**: 769-782.

BRITISH MEDICAL RESEARCH COUNCIL.(1949). Treatment of pulmonary tuberculosis with para-aminosalicylic acid and streptomycin. Preliminary report. Br Med **J** 2: 1521.

BRITISH MEDICAL RESEARCH COUNCIL.(1950). Treatment of pulmonary tuberculosis with streptomycin and para-aminosalicylic acid. A Medical Research Council investigation. Br Med J **2**: 1073-1085.

BRYAN, L.E. & VAN DEN ELZEN. (1976). Streptomycin accumulation in susceptible and resistant strains of Escherichia coli and Pseudomonas aeruginosa. Antimicrob Agents Chemother **9**: 928-938.

BURKE, R.M. (1955). An Historical Chronology of Tuberculosis, second edition. Published by Charles C. Thomas, Springfield Illinois, USA.

CASANOVA, J. L.; PANNETIER, C.; JAULIN, C.; KOURILSKY, P. (1990). Optimal conditions for sequencing double-stranded PCR products with sequenase. Nucl Acids Res **18**: 4028.

CHANG, F.N.; FLAKS, J.G. (1972). Binding of Dihydrostreptomycin to Escherichia coli Ribosomes: Characteristics and Equilibrium of the Reaction. Antimicrob Agents Chemother **2**: 294-307.

CLARK, J.M. (1988). Novel non-templated nucleotide addition reactions catalyzed by prcaryocytic and eucaryocytic DNA polymerases. Nucl Acids Res **16**: 9677-9686.

COMSTOCK, G.W. (1982). Epidemiology of Tuberculosis. Am Rev of Resp Dis **125**: 8-15.

DAVIS, B.D.; CHEN, L.; TAI, P.C. (1986). Misread protein creates membrane channels: An essential step in the bactericidal action of aminoglycosides. Proc Natl Acad Sci USA. **83**: 6164-6168.

DESMOND, E.P. (1992). Molecular Approaches to the Idetification of Mycobacteria. Clin Micro Newslet **14**: 145-149.

DES PREZ, R.M. & HEIM, C.R. (1990). Mycobacterium tuberculosis, p. 1877-1905. *In* G.L. Mandell; R.G. Douglas; J.E. Bennett (eds.), Principles and Practice of Infectious Diseases. Churchill Livingstone, New York, USA.

DE WIT, D.; STEYN, L.M.; SHOEMAKER, S.; SOGIN, M. (1990). Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. J Clin Microbiol **28**:

DOUGLASS, J.W. & STEYN, L.M. (1993). A Ribosomal Gene Mutation in Streptomycin-Resistant *Mycobacterium tuberculosis* Isolates. J Inf Dis **167**: 1505-1506.

EDSON, R.S. & TERRELL, C.L. (1991). The Aminoglycosides. Mayo Clin Proc **66**: 1158-1170.

EDWARDS, U.; ROGALL, T.; BLÖCKER, H.; BÖTTGER, E.C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nuc Acids Res **17**: 7843-7853.

EHLERS, M.R.W. (1993). The wolf at the door. Some thoughts on the biochemistry of the tubercle bacillus. South Afr Med J **83**: 900-903.

ESTRADA-G, I.C.E.; COLSTON, M.J.; COX, R.A. (1989). Dtermination and evolutionary significance of nucleotide sequences near to the 3' -end of 16S ribosomal RNA of mycobacteria. FEMS Micro Lett **61**: 285-290.

ETZOLD, T.; FRITZ, C.C.; SCHELL, J.; SCHREIR, P.H. (1987). A point mutation in the chloroplast 16S rRNA gene of a streptomycin resistant *Nicotiana tabacum*. FEBS Lett **219**: 343-346.

FELDMAN, W.H.; HINSHAW, H.C.; MANN, F.C. (1945). Streptomycin in experimental tuberculosis. Am Rev Tuberc **52**: 269-298.

FINKEN, M.; KIRSCHNER, P.; MEIER, A.; BÖTTGER, E.C. (1993). Molecular basis of of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal S12 gene and point mutations with a functional 16S ribosomal RNA pseudoknot. Mol Microbiol **9**: 1239-1246.

FRATTALI, A.L.; FLYNN, M.K.; DE STASIO, E.A.; DAHLBERG, A.E. (1990). Effects of mutagenesis of C912 of *Escherichia coli* 16S ribosomal RNA. Biochim et Biophys Acta **1050**: 27-33.

FUNATSU, G. & WITTMANN, H.G. (1972). Location of Amino-acid Replacements in Protein S12 isolated from Escherichia Mutants Resistant to Streptomycin. J Mol Biol **68**: 547-550.

GALILI, S.; FROMM, H.; AVIV, D.; EDELMAN, M.; GALUN, E. (1980). Ribosomal protein S12 as a site for streptomycin resistance in *Nicotina* chloroplasts. Mol Gen Genet **218**: 289-292.

GARVIN, R.T.; BISWAS, D.K.; GORINI, L. (1974). The Effects of Streptomycin or Dihydrosreptomycin Binding to 16S RNA or to 30S Ribosomal subunits. Proc Nat Acad Sci U.S.A. **71**: 3814-3818.

GRANDES, G.; LOPEZ-DE-MUNAIN, J.; DIAZ, T.; RULLAN, J.V. (1993). Drug-resistant Tuberculosis in Puerto Rico, 1987-1990. Am Rev Respir Dis **148**: 6-9.

GRAVEL, M.; MELANÇON, P.; BRAKIER-GINGRAS, L. (1987). Cross-Linking of Streptomycin to the 16S Ribosomal RNA of Escherichia coli. Biochemistry **26**: 6227-6232.

GREENAWAY, P.J.; DALE, J. (1983). Genetic Manipulation. A practical introduction to basic techniques.

GRISÉ-MIRON, L.; & BRAKIER-GINGRAS, L. (1982). Effect of Neomycin and Protein S1 on the Binding of Streptomycin to the Ribosome Eur J Biochem **123**: 643-646.

HANCOCK, R.E.W. (1981). Aminoglycoside uptake and mode of action - with special reference to streptomycin and gentamycin. J Antimicr Chem **8**: 248-276 & 429-445.

HANSEN, H.; LEMKE, H.; BODNER, U. (1993). Rapid and simple purification of PCR products by direct band elution during agarose gel electrophoresis. Biotechniques **14**: 28-30.

HIGGINS, D.G.; BLENSBY, A.J.; FUCHS, R. (1992). Clustal V: improved software for multiple sequence alignment. Comp Applic Biosci **8**: 189-191.

HINSHAW, H.C.; FELDMAN, W.H. (1945). Streptomycin in treatment of clinical tuberculosis: a preliminary report. Proc Staff Meet Mayo Clin **20**: 313-318.

HOBBY, G.L.; HOLMAN, A.P.; ISEMAN, M.D.; JONES, J.M. (1973). Enumeration of Tubercle Bacilli in Sputum of Patients with Pulmonary Tuberculosis. Antimicrob Ag Chemother **4**: 94-104.

HONORÉ, N. & COLE, S.T. (1994). Streptomycin resistance in Mycobacteria. Antimicrob Ag Chemother **38**: 238-242.

HUMMEL, H.; PIEPERSBERG, W.; BÖCK, A. (1980). 30S Subunit Mutations Relieving Restriction of Ribosomal Misreading Caused by L6 Mutations. Molec Gen Genet **179**: 147-153.

HURLEY, J.C. & ANDREW, J.H. (1993). Bacteriology and drug susceptibility of tuberculosis at St Vincent's Hospital, Melbourne 1962-1991. Tubercl Lung Dis **74**: 163-166.
IDIGBE, E.O.; DUQUE, J.P.; JOHN, E.K.O.;ANNAM. O. (1992). Resistance to antituberculosis drugs in treated patients in Lagos, Nigeria. J Trop Med Hyg **95**: 186-191.

KIRSEBOM, L.A.; AMONS, R.; ISAKSSON, L.A. (1986). Primary structure of mutationally altered ribosomal protein L7/L12 and their effects on cellular growth and translational accuracy. Eur J Biochem **156**: 669-675.

KOCH, R. (1932). The aetiology of Tuberculosis. A Translation by Berna Pinner and Max Pinner. Am Rev Tuberc **25**: 285-323.

KÜHBERGER, R.; PIEPERSBERG, W.; PETZET, A.; BUCKEL, P.; BÖCK, A. (1979). Alteration of Ribosomal Protein L6 in Gentamicin-Resistant Strains of *Escherichia coli.* Effects on fidelity of Protein Synthesis. Biochemistry **18**: 187-193.

LECLERC, D.; MELANÇON, P.; BRAKIER-GINGRAS, L. (1991a). Mutations in the 915 region of *Escherichia coli* 16S ribosomal RNA reduce the binding of streptomycin to the ribosome. Nucl Acids Res **19**: 3973-3977.

LECLERC, D.; MELANÇON, P.; BRAKIER-GINGRAS, L. (1991b). The interaction between streptomycin and ribosomal RNA. Biochimie **73**: 1431-1438.

LECLERC, D. & BRAKIER-GINGRAS, L. (1991). A conformational switch involving the 915 region of *Escherichia coli* 16S ribosomal RNA. FEBS Lett **279**: 171-174.

LIESACK, W.; PITULLE, C.; SELA, S.; STACKEBRANDT, E. (1990). Nucleotide sequence of the 16S rRNA from *Mycobacterium leprae*. Nucl Acids Res **18**: 5558.

LIESACK, W.; SELA, S.; BERCOVIER, H.; PITULLE, C.; STACKEBRANDT, E. (1991). Complete nucleotide sequence of the *Mycobacterium leprae* 23S and 5S rRNA genes plus flanking regions and their potentialin designing diagnostic probes. FEBS Lett **281**: 114-118.

LIETMAN, P.S. (1990). Aminoglycosides and spectinomycin: aminocyclitols, p. 269-283. *In* G.L. Mandell; R.G. Douglas; J.E. Bennett (eds.), Principles and Practice of Infectious Diseases. Churchill Livingstone, New York, USA.

LOMBARD, E.H.; VICTOR, A.J.; VAN HELDEN, P.D. (1994). The detection of *Mycobacterium tuberculosis* in bone marrow aspirate using the polymerase chain reaction. Tuberc Lung Dis **75**: 65-69.

MARCHUCK, D.; DRUMM, M.; SAULINO, A.; COLLINS, F.S. (1990). Construction of T-vectors, a rapid and general system for direct cloing of unmodified PCR products. Nucl Acids Res **19**: 1154.

MARTIN, C.; TIMM, J.; RAUZIER, J.; GOMEZ-LUS, R.; DAVIES, J.; GICQUEL, B. (1990). Transposition of an antibiotic resistance element in mycobacteria. Nature **345**: 739-743.

MARTIN, C.; RANES, M.; GICQUEL, B. (1990). Plasmids, antibiotic resistance, and mobile genetic elements in mycobacteria, p. 121-138. *In* J. McFadden (ed.), Molecular biology of the mycobacteria. Surrey University Press, Guildford, United Kingdom.

McFADDEN, J.; KUNZE, Z.; SEECHURN, P. (1990). DNA probes for detection and identification, p. 139-172. *In* J. McFadden (ed.), Molecular biology of the mycobacteria. Surrey University Press, Guildford, United Kingdom.

MEDOW, I.; RÜSCH-GERDES, S.; SCHRÖDER, K-H. (1987). Comparison of Ribosomes and Ribosomal Proteins of Sensitive and Resistant Mycobacteria. Zbl Bakt Hyg **266**: 359-369.

MEIER, A.; KIRSCHNER, P.; BANGE, F-C.; VOGEL, U.; BÖTTGER, E.C. (1994). Genetic alterations in Streptomycin-Resistant Mycobacteria tuberculosis: Mapping of Mutations Conferring Resistance. Antimicrob Ag Chemother **38**: 228-233.

MELANÇON, P.; BOILEAU, G.; BRAKIER-GINGRAS, L. (1984). Cross-Linking of Streptomycin to the 30S Subunit of *Escherichia coli* with Phenyldiglyoxal. Biochemistry **23**: 6697-6703.

MELANÇON, P.; LEMIEUX, C.; BRAKIER-GINGRAS, L. (1988). A mutation in the 530 loop of *Escherichia coli* 16S ribosomal RNA causes resistance to streptomycin. Nucl Acids Res **16**: 9631-9639.

MELANÇON, P.; TAPPRICH, W.E.; BRAKIER-GINGRAS, L. (1992). Single-Base Mutations at Position 2661 of *Escherichia coli* 23S rRNA Increase Efficiency of Translational Proofreading. J Bact **174**: 7896-7901.

MINNEKIN, D.E. (1982). Lipids: complex lipids, their chemistry, biosynthesis and roles, p. 95-184. *In* C. Ratledge & J.L. Stanford (ed.), The Biology of the Mycobacteria, Vol. 1. Academic Press, London.

MITCHISON, D.A. (1984). Drug resistance in mycobacteria. Br Med Bull **40**: 84-90.

MOAZED, D.; NOLLER, H.F. (1986). Transfer RNA shields specific nucleotides in 16S ribosomal RNA from attack by chemical probes. Cell **47**: 985-994.

MOAZED, D.; NOLLER, H.F. (1987). Interactions of antibiotics with functional sites in 16S ribosomal RNA. Nature **327**: 389-394.

MODOLELL, J.; DAVIS, B. D. (1970). Breakdown by streptomycin of initiation complexes found on ribosomes of *Escherichia coli*. Proc Natl Acad Sci **67**: 1148-1155.

MONTANDON, P.; NICOLAS, P.; SCHÜRMANN, P.; STUTZ, E. (1985). Streptomycin-resistance of *Euglena gracilis* chloroplasts: identification of a point mutation in the 16S rRNA gene in an invariant position. Nucl Acids Res **13**: 4299-4310.

MOLE, S.E.; IGGO, R.D.; LANE, D.P. (1989). Using the polymerase chain reaction to modify expression plasmids for epitope mapping. Nucl Acids Res **17**: 3319.

MORRIS, S.; ROUSE, D.; NAIR, J. (1993). The ribosomal S12 protein and streptomycin resistance in single and multiple drug resistant strains of *Mycobacterium tuberculosis*. *In* Abstracts of the Second International Conference on the Pathogenesis of Mycobacterial Infections. Abstract, p. 18. Stockholm, Sweden.

NARDELL, E.A. (1993). Beyond Four Drugs. Am Rev Respir Dis **148**: 2-5

NEWTON, C.R.; GRAHAM, A.; HEPTINSTALL, L.E.; POWELL, S.J.; SUMMERS, C.; KALSHEKER, N.; SMITH, J.C.; MAKHAM, A.F. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucl Acids Res **17**: 2503-2516.

NICHOLS, W.W. (1987). On the mechanism of translocation of dihydrostreptomycin across the bacterial cytoplasmic membrane. Biochim Biophys Acta **895**: 11-23.

OLSEN, G.J. & WOESE, C.R. (1993). Ribosomal RNA: a key to phylogeny. FASEB J **7**: 113-123.

OZAKI, M.; MIZUSHIMA, S.; NOMURA, M. (1969). Identification and Functional Characterisation of the Protein controlled by the Streptomycin-resitant Locus in *E. coli*. Nature **222**: 333-339.

PINARD, R.; PAYANT, C.; MELANÇON, P.; BRAKIER-GINGRAS, L. (1993). The 5' proximal helix of 16S rRNA is involved in the binding of streptomycin to the ribosome. FASEB J **7**: 173-176.

PONGS, O. & ERDMANN,V.A. (1973). Affinity labeling of *E. coli* Ribosomes with a Streptomycin-Analogue. FEBS Lett **37**:47-50.

PONGS, O.; NIERHAUS, K.H.; ERDMANN, V.A. & WITTMANN, H.G. (1974). Active sites in *Escherichia coli* Ribosomes. FEBS Lett **40**: S28-S37.

ROGALL, T.; WOLTERS, J.; FLOHR, T.; BÖTTGER, E.C. (1990). Towards a Phylogeny and Definition of Species at the Molecular Level within the Genus *Mycobacterium*. Int J Syst Bacteriol **40**: 323-330.

SAIKI, R.K.; SCHARF, S.; FALOONA, F.; MULLIS, K.B.;HORN, G.T.; ERLICH, H.A.; ARNHEIM, N. (1985). Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anaemia. Science **230**: 1350-1354.

SALLES, C.; CRÉANCIER, L.; CLAVERYS, J-P.; MÉJEAN, V. (1992). The high level streptomycin resitance gene from *Streptococcus pneumoniae* is a homologue of the ribosomal protein S12 gene from *Escherichia coli*. Nucl Acids Res **20**: 6103.

SAMBROOK, J.; FRITSCH, E.F.; MANIATIS, T. (1989). Molecular cloning: A laboratory manual: second edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

SANGER, F.; COULSON, A.R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol **94**: 441-448.

SANGER, F.; NICKLEN, S.; COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74**: 5463-5467.

SCHATZ, A.; BUGIE, E.; WAKSMAN, S.A. (1944). Streptomycin, a substance exhibiting antibiotic activity against a gram-positive and gram-negative bacteria. Proc Soc Exper Biol Med **55**: 66-69.

SCHREINER, G.; NIERHAUS, K.H. (1973). Protein Involved in the Binding of Dihydrostreptomycin to Ribosomes of *Escherichia coli*. J Mol Biol **81**: 71-82.

SOMMERS, H.M. & GOOD, R.C. (1985). *Mycobacterium*, p. 216-248. *In* E.H. Lennette; A. Balows; W.J. Hausler, jnr; H.J. Shadowmy (eds.), Manual of clinical microbiology, 4th ed. American Society of Microbiology, Washington, D.C.

SPOTTS, C.R. & STANIER, R.Y. (1961). Mechanism of Streptomycin Action on Bacteria: A Unitary Hypothesis. Nature**192**: 633-637.

SUMARTOJO, E. (1993). When Tuberculosis treatment fails. Am Rev of Resp Dis **147**:1311-1320.

SUZUKI, Y.; NAGATA, A.; ONO, Y.; YAMADA, T. (1988). Complete Nucleotide sequence of the 16S rRNA Gene of Mycobacterium bovis BCG. J Bact **170**: 2886-2889.

VAN SOOLINGEN, D.; HERMANS, P.W.M.; DE HAAS, P.E.W.; SOLL, D.R.; VAN EMDEN, J.D.A. (1991). Occurrence and Stability of Insertion Sequences in *Mycobacterium tuberculosis* Complex strains: Evaluation of an Insertion Sequence-Dependent DNA Polymorphism as a tool in the Epidemiology of Tuberculosis. J Clin Microbiol **29**: 2578-2586.

WEYER, K.; KLEEBERG, H.H. (1992). Primary and acquired drug resistance in adult black patients with tuberculosis in South Africa: results of a continuous national drug resistance surveillance programme involvement. Tuberc Lung Dis **73**: 106-112.

WEISS, R. (1992). On the Track of "Killer" TB. Science **255**: 148150.

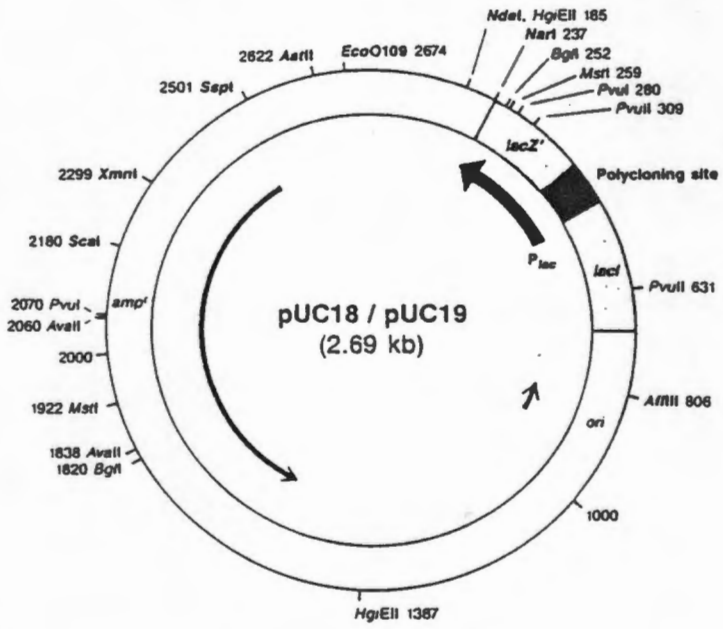
YANISCH-PERRON, C.; VIEIRA, J.; MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**: 103-119.

YOUMANS, G.P.; McCARTER, J.C. (1945). Streptomycin in experimental tuberculosis. It's effect on tuberculous infection in mice produced by M. tuberculosis var. hominis. Am Rev Tuberc **52**: 432-439.

ZABEAU, M. & STANLEY, K.K. (1982). Enhanced expression of cro-β-galactosidefusion proteins under the control of the P_R promoter of bacteriophage lambda. EMBO J **1**: 1217-1224.

APPENDIX A

pUC18



Polycloning Sites

pUC18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser		Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC		TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC
					EcoRI			*SacI*		*KpnI*		*SmaI* *XmaI*		*BamHI*		*XbaI*		*SalI* *AccI* *HincII*		*PstI*		*SphI*		*HindIII*			

pUC19

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC
					HindIII		*SphI*		*PstI*		*SalI* *AccI* *HincII*		*XbaI*		*BamHI*		*SmaI* *XmaI*		*KpnI*		*SacI*		*EcoRI*			

In pUC18, the *EcoRI* site lies immediately downstream from P_{lac}.
In pUC19, the *HindIII* site lies immediately downstream from P_{lac}.

APPENDIX B

MOLECULAR WEIGHT MARKERS USED

B.1 Lambda DNA digested with *Hin*dIII.

Fragment sizes (bp):

23 130
9 416
6 682
4 361
2 322
2 027
564
125

B.2 Marker V (Boehringer Mannheim).

Fragment sizes (bp):

587
540
504
458
434
267
234
213
192
184
124
123
104
89
80
64
57
51
21
18
11
8