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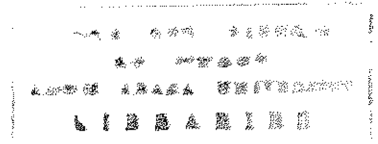

INVESTIGATION OF THE MECHANISMS OF RESISTANCE TO STREPTOMYCIN IN *Mycobacterium tuberculosis*

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INVESTIGATION OF THE MECHANISMS OF RESISTANCE TO STREPTOMYCIN IN *Mycobacterium tuberculosis*

A thesis submitted to the School of Graduate Studies, Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Biology

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by

Yishak Gebrekidan

Dedication

To My mother Zufan Woldemichael

&

My father Gebrekidan Woldemichael

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TABLE OF CONTENTS

	PAGE
ACKNOWLEDGMENTS	i
LIST OF TABLES	ii
LIST OF FIGURES	iii
ABBREVIATIONS	iv
ABSTRACT	vi
1. INTRODUCTION	1
1.1. Tuberculosis	1
1.1.1. Global Situation	1
1.1.2. The Disease	3
1.2. The causative agent of tuberculosis	6
1.3. Mechanisms of resistance to anti-tuberculosis drugs8	
1.4. Molecular mechanisms of resistance to antituberculosis drugs in *M.tuberculosis*	12
1.5. Streptomycin	15
1.6. Genes responsible for streptomycin resistance in *M.tuberculosis*	16
1.7. objectives of the study	19
2. MATERIAL AND METHODS	21
2.1. Determination of MIC value	21
2.1.1. *M. tuberculosis* strains	21
2.1.2. Media preparation	21
2.1.3. Incorporation of streptomycin	22
2.1.4. Inoculation of 7H10 plates with the *M. tuberculosis*	23
2.2. DNA Extraction	23
2.3. Amplification of *rpsL* and *rrs* genes	25
2.4. Sequencing of rpsL and *rrs* genes	29
2.4.1. Sequencing strategy	29
2.4.2. Radioactive sequencing	30
2.4.2.1. Cycle sequencing reaction	30

2.4.2.2. Preparation and casting of sequencing gel 32
2.4.2.3. Electrophoresis of sequencing reaction products 33
2.4.3. Non-Radioactive (Fluorescence) Sequencing34
2.4.3.1. PCR purification 34
2.4.3.2. Sequencing reaction 34
2.4.3.3. Sequencing gel preparation 35
2.5. Functional analysis of the rRNA of mycobacteria 36
2.5.1. Digestion of plasmid DNA 36
2.5.2. Gene cleaning 39
2.5.3. Ligation reaction 39
2.5.4. Transformation of *E. coli* 40
2.5.5. Isolation of plasmid DNA 42
2.5.6. Transformation of *M. smegmatis* mc^2 155 43
2.5.7. Southern blot analysis 45
2.6. Generation of streptomycin resistant mutants of *M. smegmatis* with one functional rRNA operon 48

3. RESULTS 50
3.1. MIC value of *M. tuberculosis* strains 50
3.2. PCR amplification 53
3.3. Sequence of *rpsL* and *rrs* genes 56
3.4. Inactivation of one rRNA operon of *M. smegmatis* mc^2 155 61
3.5. streptomycin resistant mutants of *M. smegmatis* with one functional rRNA operon 67

4. DISCUSSION 70
4.1. MIC value determination 70
4.2. Mutational status of the streptomycin resistant *M. tuberculosis* strains 71
4.3. Generation of *M. smegmatis* strains with

one functional rRNA operon 72
4.4. Streptomycin resistant mutants of *M. smegmatis* strains with one functional rRNA operon 74

5. CONCLUSION AND RECOMMENDATIONS 75

6. REFERENCES 77

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LIST OF TABLES

	Page
1. Location of the annealing and ending position on the genes, length and base sequence of each of the primers.	26
2. Culture status and MIC values of each strain.	51
3. Results of the sequencing of the *rpsL* gene giving special attention to codon 43 and 88 of the high level and low level streptomycin resistant and the susceptible strain of *M. tuberculosis*.	57
4. Comparison of the MIC value and observed mutation of the high level streptomycin resistant *M. tuberculosis*.	60
5. Number of colonies obtained on different plates after transformation of *M. smegmatis*.	64

LIST OF FIGURES

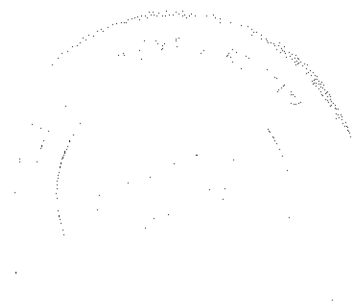
Page

1. Diagrammatic representation of the alignment of each of the primers along the genes. 27
2. PCR amplification of the entire *rpsL* of the high level and low level streptomycin resistant strains and the susceptible control strain of *M. tuberculosis* using S12-3 + S12-4 primers. 54
3. PCR amplification products of the 650 bp fragment of *rrs* gene from the high level and low level streptomycin resistant and the susceptible control strains of *M. tuberculosis* after amplification using 16S-1+16S-4 primers. 55
4. An example of *rpsL* gene radioactive sequence result of the *M. tuberculosis*. 58
5. An example of *rpsL* non-radioactive sequence result of the *M. tuberculosis*. 59
6. Plasmid pRRNA4-4K1. 62
7. Plasmid pLO2. 62
8. Plasmid pRRNA4-4K1-*SacB*. 63
9. Southern blot analysis of the *M. smegmatis* transformants. 66
10. Plasmid ptrPA-1-rpsL. 68
11. Plasmid pHINT 1. 68
12. Plasmid pHINT-rpsL. 69

LIST OF ABBREVIATIONS

AHRI = Armauer Hansen Research Institute
AIDS = Acquired Immune Deficiency Syndrome
Arg = Arginine
BHI =Brain Heart Infusion
bp = base pair
Ca = Calcium
DNA = Deoxyribonucleic acid
EDTA = Ethylenediaminetetra acetate
e.g. = Example
Gln = Glutamine
HEPES= N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid
HIV = Human Immunodeficiency Virus
hr = Hour
kb = Kilo base
km = kilo meter
LB = Luria-Bertani
Lys = Lysine
MDRTB=Multi-drug Resistant Tuberculosis
Mg = Magnesium
mg = milligram
MHH = Medizinische Hochschule Hannover
MIC = Minimum Inhibitory Concentrations
ml = milliliter
mm = millimeter
MOH = Ministry of Health
mRNA= Messenger ribonucleic acid
NaCl = Sodium chloride
NAD = Nicotinamide adenine dinucleotide
NaI = Sodium iodide

NaOH = Sodium hydroxide
OD = Optical density
PBS = Phosphate Buffer Saline
PCR = Polymerase Chain Reaction
RNA = Ribonucleic acid
rRNA = Ribosomal ribonucleic acid
SDS = Sodium dodecylsulfate
SIID = Swedish Institute for Infectious Diseases Control
TAE = Tris- acetic acid EDTA
Taq = *Thermophillus aquaticus*
TB = Tuberculosis
TBE = Tris-boric acid EDTA
TE =Tris-EDTA
TEMED= Tetramethylethylenediamine
Thr = Threonine
tRNA = Transferase ribonucleic acid
UV = Ultraviolet
WHO = World health organization
μg = microgram
μl = microliter



ABSTRACT

MIC value of streptomycin resistant *M. tuberculosis* strains was determined using the proportional method. Sequence analysis of *rpsL* and *rrs* genes was performed using both the radioactive and non-radioactive cycle sequencing protocols. For functional analysis of rRNA of *M. tuberculosis*, *M. smegmatis strain* mc^2 155 was used as a model system.There were 34 streptomycin resistant *Mycobacterium tuberculosis* strains subjected for MIC value determination. This included 18 strains from Sweden and 16 strains from Ethiopia. Their streptomycin resistance character had been previously confirmed by culturing in BACTEC media containing 4μg/ml streptomycin. Eleven strains growing on 7H10 plates containing ≥ 160 μg/ml streptomycin were classed as high level resistant, 13 strains growing on 5-10 μg/ml streptomycin classed low level resistant and 10 strains were found to be susceptible to streptomycin. DNA was extracted from the 11 high level and from 10 of the 13 low level streptomycin resistant strains. The extracted DNA was PCR amplified by targeting the *rpsL* and *rrs* genes. PCR products of all the high level and 6 of the low level resistant strains were sequence analyzed. For the *rpsL* gene, 9 of the high level resistant strains had the previously documented mutation at codon 43, and one strain has mutation at codon 88. One of the high level streptomycin resistant strains was negative for PCR amplification of the *rpsL* gene. All sequence analyzed low level streptomycin resistant strains were wild type for the *rpsL* and *rrs* genes. Since the low level resistant strains did not show any of the documented mutations, it is likely that they might have mutations in other ribosomal genes. This possibility was explored by manipulating the mycobacterial relative *M. smegmatis* for functional analysis of the rRNA. *M. smegmatis* with only one functional rRNA operon was generated by replacing the second rRNA operon with plasmid derived inactivated rRNA operon. For replacing one of the rRNA operons, a replacement plasmid was constructed. The replacement plasmid contained one rRNA operon inactivated by kanamycin resistant marker. As a counter selectable marker, the plasmid is equipped with the *SacB* gene which is lethal to mycobacteria in the presence of 15% sucrose. After transformation, colonies were selected on LB plates containing kanamycin 25μg/ml and different sucrose concentrations. *M. smegmatis* transformants resulted from double crossover and homologous recombinations were identified by their kanamycin and sucrose resistant phenotypes.To check for replacements of one of the rRNA operon, transformants were analyzed by Southern blotting. Competent cells of *M. smegmatis* with one functional rRNA operon were prepared and transformed with plasmid construct that contained the wild type *rpsL* gene. Transformants were selected on LB plates containing 25μg/ml streptomycin. Determination of MIC value of the streptomycin resistant *M. smegmatis* transformants and sequencing of *rrs*, *rpsL* and the entire genes of the rRNA operon will need to be determined. This will be necessary to establish the mutation induced. If established the hitherto undocumented mutation will be useful to trace the mechanisms of resistance in low level streptomycin resistant *M. tuberculosis* strains.

1. INTRODUCTION

1.1. TUBERCULOSIS

1.1.1. Global Situation

Tuberculosis is the leading cause of death from an infectious agent in man world-wide. It is currently of such concern that the WHO has declared it to be a global emergency, the first disease to be so called (Marsh *et al.*, 1996). The disease, almost from a single infectious agent, is responsible for 60 million people suffering from active tuberculosis with annual risk of 10 million new cases (Kaufman *et al.*, 1993; Britton *et al.*, 1994). Generally the disease is responsible for more than three million annual deaths, accounting for one fourth of the world's annual deaths due to preventable diseases (Daniel *et al.*, 1994). It is a chronic granulomatous disease affecting man, many other mammals, marsupials, birds, fish, amphibians and reptiles (Grange, 1992). There are four related species of the genus *Mycobacterium* which are known to be the causative agents of mammalian tuberculosis. *Mycobacterium tuberculosis* (the human tubercle bacillus), *Mycobacterium bovis* (the bovine tubercle bacillus), *Mycobacterium microti* (the vole tubercle bacillus) and *Mycobacterium africanum* (human tubercle bacilli isolated from east and west africa) (O'Reilly *et al.*, 1995).

Although most human tuberculosis is caused by *Mycobacterium tuberculosis* (*M.tuberculosis*) a small percentage of human cases are also reported due to infection with *Mycobacterium bovis* (*M. bovis*) (O'Reilly *et al.*, 1995; Pollock *et al.*, 1996). As compared to the classic epidemics of acute infectious communicable diseases,

tuberculosis is disseminated slowly. This is one feature of the disease which makes it difficult to observe and to follow up its epidemic cycle. In many countries the disease has taken several generations to evolve and complete its continuing cycle (Lowell, 1984).

In the previous few decades, a striking decline in incidence of the disease had been observed in industrialized countries. This could have been the result of wide spread application of anti-tuberculosis chemotherapy in conjunction with improved living conditions. Unfortunately this trend was reversed as a consequence of the Acquired Immunodeficiency Syndrome (AIDS) epidemic, together with increased poverty, homelessness, and inadequate health care in certain cities particularly in developing countries. At no time in recent history has tuberculosis been as great a concern as it is today. An additional serious aspect of the problem is the development of multi-drug resistant tuberculosis (MDRTB) (Wieles *et al.*, 1995; Sorensen *et al.*,1995).

The risk of tuberculosis infection is not the same for the whole population. Certain groups of the population may show higher prevalence due to their higher likelihood of exposure, which will lead to infection or progression of the disease to an active form. Medically under-served ethnic minorities, homeless persons, prison inmates, alcoholics, injecting drug users, and persons in contact with active tuberculosis are expected to have higher prevalence than the general population.

As in much of the developing countries, tuberculosis is the major cause of morbidity and mortality, both in and out of hospital in Ethiopia. Based on some direct and indirect evidences there is an indication of high magnitude of tuberculosis in

Ethiopia. In the year 1983 to 1989, tuberculosis was the third leading cause of hospitalization and the first leading cause of hospital deaths (MOH, 1983/84; MOH, 1986/87; MOH, 1988/89). The tuberculin test survey which was conducted nation wide during 1988-1990, showed 1.4% average annual risk of tuberculosis infection in rural children, while it is over 50% higher in Addis Ababa (Hodes *et al.*, 1993). Inadequate health service and lower living standard in the country together with the present HIV epidemic, the problem is expected to worsen.

1.1.2. The Disease

Tuberculosis is transmitted aerogenically and the causative agent may gain entry into the body by inhalation, ingestion or through the skin. The causative agent is spread from coughing active pulmonary tuberculosis patients, and is carried in the form of droplet nuclei. The droplet nuclei about 1-5 μm in diameter contain one or two viable bacilli which may be inhaled and lodged in the terminal airway of the lung, and the bacilli are then engulfed by alveolar macrophages (Nolte *et al.*, 1995). The alveolar macrophages vary in their capacity to destroy the tubercle bacillus, being more or less rich in enzymes and microbicidins (Dannenberg, 1984). If infection takes place, it will lead to one of the following three outcomes, based on the host immune status and the host factors.

1. Acute disease shortly after the infection (Primary tuberculosis -TB).
2. Establishment of a chronic latent infection.
3. Active disease many years of inactive infection (Reactivation TB).

If the engulfed bacilli are not destroyed or inhibited they start to multiply intracellularly, the alveolar macrophage dies and other alveolar macrophages or macrophages derived from circulating monocytes ingest the bacillary loads. The bacilli within the macrophage continue to divide and result in massive tissue damage in any organ where they successfully reside. Eventually in a susceptible individual the total number of organisms harbored by the host may reach hundreds of millions, leading to tubercles in the affected organ. However, under normal conditions further multiplication and spread of the causative agent within the body is checked by the host cell-mediated immune response.

There are many factors which confer virulence on the bacilli. Glycolipids, including " cord-factor", inhibit the normal respiratory metabolism of the host cell by affecting the host mitochondrial membrane function (Dannenberg, 1984). Inhibition of lysosome-phagosome fusion within the macrophage by sulfolipids together with inhibition of phagosome acidification by selectively excluding the proton ATPase are some of the mechanisms by which the bacilli manage to survive intracellularly (Riley, 1995). This is important because if lysosome-phagosome is allowed to occur the hydrolytic enzymes in the phagolysosome remove the outer lipid coats of the bacilli and expose the hydrolyzable peptidoglycan backbone.

The most dramatic characteristic of the disease is its ability to reactivate and cause severe damage after many years of dormancy. This is achieved as a result of persistence of the bacilli within the host, lying quiescent by adapting to the microaerophilic and anaerobic conditions within the micro-environment. Bacilli in an

in vitro model of the dormant state, have been found to be resistant to the bactericidal action of antimicrobial agents that would be lethal to actively replicating bacilli. Moreover, the *in vitro* dormant bacilli exhibit a modified enzyme composition, including shift into the glyoxylate cycle (Wayne *et al.*, 1996). After transfer to oxygen-rich media the bacteria undergo a synchronous replication (Wayne *et al.*, 1996). People harboring dormant viable bacilli have a 10% risk of developing active tuberculosis at some stage of their life, but HIV co-infected individuals have an annual risk of 10% - 15% (Nolte *et al.*, 1995).

Tuberculosis occurs at any site of the body producing symptoms which are both local and systemic in nature (Hopewell, 1994). Pulmonary tuberculosis, is characterized by coughing at the early stages of the illness and with progression of the disease it will be accompanied by sputum production. In pulmonary tuberculosis the middle or lower zones of the host lung are the initial focus, because the majority of the inhaled air is directed to these parts of the lung (Bates, 1984). With continuous replication of the bacilli within the macrophage tubercles will form. The site of infection will be surrounded by a zone of lymphocytes and mononuclear cells, and often there is a proliferation of connective tissue at the periphery that appears as a capsule. As the lesion increases in size, there may be mineralization and/or caseous necrosis (Thoen *et al.*, 1984). If the tubercle bursts, the bacilli are released for further spread via the bronchi and eventual lung destruction (Bates, 1984). If the tubercle bursts into a blood vessel, there will be spread of *M.tuberculosis* throughout the body via lymphatic and blood streams which can result in occurrence of extrapulmonary tuberculosis. This type

of tuberculosis has been increasing in prevalence with the onset of the HIV epidemic, and often leads to the disseminated or miliary form of tuberculosis (considered as one of the extrapulmonary forms of tuberculosis because of the multiplicity of organs affected) (Hopewell, 1994).

1.2. THE CAUSATIVE AGENT OF TUBERCULOSIS

M. tuberculosis is a member of the family *Mycobacteriaceae* in the only genus, *Mycobacterium*. It belongs to a closely related slow growing group of mycobacteria, called the *M. tuberculosis* complex, which comprises the species *M. tuberculosis, M. bovis*,(including *M. bovis* BCG), *M. africanum* and *M. microti* (Goodfellow *et al.*, 1982). As a result of their staining properties, members of the genus *Mycobacterium* are often referred to as " acid fast" bacilli. They are difficult to stain, but once stained with carbolfuchsin or auramine o fluorochrome stains they retain the stain during treatment with acid alcohol for decolorization. The intact mycobacterial cell wall incorporates the stain into its interior or binds to the mycolic acid residues of the outer cell wall, to form a stable complex. The large amount of lipids present in their cell wall render them impermeable to the dyes used in the gram stain.

The mycobacterial cell wall is rich in lipids, free lipid accounts for about 25% of the bacterial weight. In addition the cell wall has a frame work structure called a "covalent skeleton", which consists of peptidoglycan plus a small amount of neutral sugars, lipids, and non-peptidoglycan amino acids, together with arabinogalactan which

is covalently linked to the peptidoglycan and esterified by mycolic acids (Jean-Francois *et al.*, 1984). Mycolic acids are major constituents of the cell wall and the α-alkyl, β-hydroxy fatty acids range in size from C_{22} to C_{90} (Kuni *et al.*, 1984).

The relationship between the disease tuberculosis and the tubercle bacilli was established for the first time by Robert Koch. The organism was named *Mycobacterium tuberculosis* in 1886 (Nolte *et al.*, 1995). *M. tuberculosis* is one of the most successful bacterial parasites of human. Tuberculosis produced in humans by the highly related *M.bovis,* is indistinguishable from that caused by *M. tuberculosis* and it is treated similarly. *M. bovis* which is less aero-tolerant than *M. tuberculosis*, can be spread from infected cattle to man by way of milk. *M. africanum* (it shows characteristics intermediate between *M. tuberculosis* and *M. bovis)* is also a cause of human tuberculosis in tropical Africa (Nolte *et al.*, 1995; O'Reilly *et al.*, 1995).

M. tuberculosis, the typical tubercle bacillus, is a slender, straight or slightly curved, rod in shape. It may exist singly or form thread like structures. The size of the bacillus ranges in size from 0.3 μm to o.6 μm in width and from o.5 μm to 4.0 μm in length. *M. tuberculosis* is slow growing, non-encapsulated, non-spore forming, non-motile, lipid rich, and hydrophobic (Laidlaw, 1989; Barkley *et al.*, 1994).

A classic description of tubercle bacilli, which would be only partially accurate, is that they are fastidious and strictly aerobic. The fastidiousness of some isolates may be a consequence of their injury by conditions in host tissue or by the processing of the clinical specimen (Wayne, 1994). Once isolated, *M. tuberculosis* is capable of adapting

to growth on extremely simple media containing a simple source of carbon and nitrogen with some buffer salts and trace elements, and can grow under a wide range of partial oxygen pressures. In an *in vitro* model experiment, *M. tuberculosis* remain viable for a considerable period of time under microaerophilic and anaerobic conditions without replication (Wayne *et al.*, 1982). *M. tuberculosis* grows in the temperature ranges between 30°C - 41°C. Optimally they grow well at 35°C - 37°C, they fail to grow at 25°C or 42°C, and they die at 60°C with in 15 minutes of incubation (Grange, 1992; Heifets *et al.*, 1994). Although tubercle bacilli are obligate pathogens, they survive in milk and other organic materials, in dust, and in dried sputum for many days (Grange, 1992; Barkley *et al.*, 1994). They are highly sensitive to sun light and ultraviolet exposure, but they are relatively resistant to chemicals and drying.

M. tuberculosis grows well both on solid and in liquid media. The buff coloured colonies of *M. tuberculosis* growing on 7H10 or 7H11 agar plates have a rough, dry surface with irregular edges. Under optimal conditions one cycle of replication of *M. tuberculosis* requires 16-18 hours. So a single bacillus, to yield a visible colony on solid media takes at least two weeks.

1.3. MECHANISMS OF RESISTANCE TO ANTI-TUBERCULOSIS DRUGS

The resurgence of tuberculosis, compounded by the emergence of multi-drug resistance in *M. tuberculosis* demands continuing improvement of our understanding of the mechanisms of action of antimycobacterial agents and molecular mechanisms of

resistance to the drugs (Garbe *et al.*, 1996). Tuberculosis caused by drug resistant strains of *M. tuberculosis* is a growing problem in several countries including Ethiopia. Significant progress has been made toward understanding the molecular basis of resistance to streptomycin and other antituberculosis drugs in *M. tuberculosis* strains (Honore *et al.*, 1995). The knowledge that will be obtained on how the bacilli develop resistance against the drugs, will help in designing effective control measures, methods of detection of drug resistant tuberculosis and improve our understanding of the mechanisms of action of the drugs so that rational drug design can be employed to develop more effective tuberculosis drugs.

Drugs which are recommended for the treatment of tuberculosis attack the bacilli by interrupting a variety of metabolic pathways. Some inhibit mycolic acid synthesis, some inhibit peptidoglycan synthesis, and others the transcription or translational process. For effective treatment of the disease the powerful weapon available is short-course chemotherapy. At the initial intensive phase of the treatment, patients are treated with four drugs (rifampicin, isoniazid, pyrazinamide and ethambutol or streptomycin) which will last for 2 months. During the continuation phase, which will last for 4 months only rifampicin and isoniazid are administered to kill any persisting organism (Cole, 1994). In Ethiopia, streptomycin, para-aminosalicylic acid and isoniazid were described as principal antituberculosis drugs 40 years ago. At that time although treatment of tuberculosis with isoniazid alone was also practiced, a combination of two and preferably three drugs was recommended (Gorden, 1968). The short-course therapy of tuberculosis has been used successfully in approximately 20,000 Ethiopian Jews who

migrated from Gonder to Addis Ababa in 1990 (Hodes *et al.*, 1993). A 6-month program using the four bacteriocidal drugs daily for 2 weeks and then twice weekly was conducted. There are different tuberculosis control programs and studies are being initiated in the National tuberculosis Centre and in St. Peter's tuberculosis hospital in Addis Ababa. For implementing tuberculosis control program in the country, the challenges are enormous, as 90% of the population lives in rural areas of which 85% lives 30 km or more away from a paved road, and at least 50% without access to any form of medical services. Thus, delivery of health care is tremendously difficult (Hodes *et al.*, 1993). As in all the developing countries rifampicin which is vital for the success of short-course therapy, can be limited to the initial 2 months for financial reasons. The logic behind the use of multidrug treatment is to ensure that mutants that are resistant to a single drug cannot emerge. Although plasmid mediated resistance has not been confirmed, drug resistance in *M. tuberculosis* occurs by random, single-step, spontaneous chromosomal mutations at a low but predictable frequency which are not linked. The probability of occurrence of a drug resistant mutant is directly proportional to the size of the bacterial population. Spontaneous resistance to isoniazid is estimated to occur one in every 10^6 organisms, for streptomycin one in every 10^5 organisms, for ethambutol one in every 10^6 organisms and one in every 10^8 organisms in case of rifampin (Chapman *et al*, 1994). The probability of spontaneous mutants being simultaneously resistant to two or more drugs is the product of the individual mutant frequency.

Application of the use of multiple drugs for the treatment of tuberculosis is based

on the premise that resistance mutations are infrequent and are not linked. Generally anti-tuberculosis drugs can be grouped in to three major groups (Grange, 1992). Sterilizing drugs are those drugs that can effectively sterilize the tuberculous lesion, (e.g. rifampicin and pyrazinamide). Bactericidal drugs, like isoniazid, streptomycin, ethambutol, kill the tubercle bacilli but often only in certain situations. Isoniazid can kill the bacilli only during replication and streptomycin is ineffective against bacilli harbored within macrophages and acidic inflammatory tissue. Bacteriostatic drugs inhibit further replication and multiplication of the bacillus but do not kill it. Ethionamide, prothionamide, thiacetazone, p-aminosalicylic acid, and cycloserine are the most commonly used bacteriostatic drugs (Winder, 1982; Grange, 1992).

The resurgence of tuberculosis this decade has been accompanied by emergence of multi-drug resistant *M. tuberculosis* isolates. Particularly in the developing world, AIDS-related tuberculosis has been associated with emergence of new *M. tuberculosis* strains that are resistant to some, or all currently used anti-tuberculosis agents (Levy, 1993; Heym *et al.*, 1994). The situation of this drug resistance complicates treatment and prevention programmes and could potentially hinder all efforts employed to control the upsurge of tuberculosis (Chapman *et al.*, 1994).

Patients develop drug-resistant tuberculosis in one of the following two ways. Initial, or primary drug resistance occurs when a patient is infected with resistant bacilli before any treatment with the anti-tuberculosis agent, and is caused by infection with drug resistant strains shed from existing patients. Acquired, or secondary resistance occurs when drug-resistant mutants are selected in a patient who has had ineffective

therapy. The wide-spread use and misuse of anti-tuberculosis drugs, such as patient default, prescription of anti-tuberculosis drugs for other diseases, adding a single agent to a failing regimen, using a sub-optimal dosage, interrupted or irregular treatment schedules, and poor drug absorption (e.g. due to gastrointestinal disease) produces selective pressure for the emergence of anti-tuberculosis resistant *M. tuberculosis* (Chapman *et al.*, 1994).

1.4. MOLECULAR MECHANISMS OF RESISTANCE TO ANTITUBERCULOSIS DRUGS IN M. TUBERCULOSIS

On a molecular level, drug resistance can be caused by many mechanisms. Common mechanisms include the acquisition of genes encoding enzymes that inactivate the drugs, modification or alteration of the drug target, active efflux of the drugs, increased production of the target, and permeability barriers (Banerjee *et al.*, 1994; Chapman *et al.*, 1994; Spratt, 1994).

M. tuberculosis has no known naturally occurring plasmid, and so resistance to drugs is encoded in the genome. This means there is no horizontal transfer of resistant elements in the species (Chapman *et al.*, 1994). Recently some advances have been made in our knowledge about the mechanisms of resistance to some of the anti-tuberculosis drugs, and about the molecular mechanisms by which certain anti-tuberculosis drugs act on non resistant tuberculosis.

Isonicotinic acid hydrazide (isoniazid) is thought to block the synthesis of

mycolic acid, which is confined essentially to mycobacteria and is thus a selective target for drugs. Catalase-peroxidase, the enzyme encoded by the *KatG* gene, is responsible for the toxicity of isoniazid by changing it into an active form. Resistance to isoniazid in some strains of *M. tuberculosis* is associated with deletion or inactivating mutations in the *KatG* gene (Cole, 1994; Marttila *et al.*, 1996). Only 10% of isoniazid resistant *M. tuberculosis* strains are found to be catalase negative, so that means in addition to mutations in the *KatG* gene there should be other mechanisms for resistance to isoniazid in 90% of the strains (Jacob,1993). The recent discovery of a novel gene, *inhA*, may provide a link between resistance to isoniazid , mycolic acid synthesis and the NAD pool (Cole, 1994). In the experiment conducted (Wilson *et al.*, 1996), more than a quarter of isoniazid resistant clinical strains of *M. tuberculosis*, were with no mutations in either *KatG* or *inhA*. Some workers were able to identify a third gene, *ahpC*, that appears to play a role in development of isoniazid resistant strains of the M. tuberculosis complex (Wilson *et al.*, 1996).

Rifampicin, which is a lipophilic ancamycin, is a drug which is very active against mycobacteria in part because of its ability to rapidly diffuse across the hydrophobic cell envelope. Rifampicin blocks transcription by binding to the β-subunit of RNA polymerase (Cole, 1994). The β-subunit of RNA polymerase is encoded by the *rpoB* gene. Resistance to rifampicin in *M. tuberculosis* results from missense mutations in the *rpoB* gene, which lead to substitution of key amino acids resulting in conformational changes and defective binding of the drug (Telenti *et al.*, 1993).

The molecular basis of the mechanisms of resistance and modes of action of

some other front-line drugs, like pyrazinamide and ethambutol, is not well known. With weak evidence, ethambutol is believed to act on the biosynthesis of mycobacterial cell wall. Although the mechanism of action or resistance has not been elucidated, pyrazinamide, which works optimally at acidic pH, is thought to act against intracellular organisms (Cole, 1994). It exerts its action after conversion into pyrazinoic acid by the enzyme pyrazinamidase.

Second-line drugs are those drugs used for the treatment of tuberculosis in rare cases of drug resistant *M. tuberculosis*, although in developing countries they may be included in the initial regimen because of their lower cost. Most of these compounds are only weakly active and have high levels of secondary effects (Cole, 1994). They included amikacin, capreomycin, ethionamide, fluoroquinolones, kanamycin, p-aminosalicylic acid, thiacetazone, and viomycin. Concerning the knowledge of the mechanisms of resistance to the second-line drugs a very little is known. The site of action of amikacin and kanamycin is the 30S ribosomal sub-unit, they disrupt the process of decoding of the aminoacyl-tRNA. Capreomycin and viomycin acts on the 50S ribosomal sub-unit and affect the translocation reaction in peptide formation (Bottger, 1994). Ethionamide seems to share a target, *inhA*, gene with isoniazid (Banerjee *et al.*, 1994). The principal target of fluoroquinolones is the DNA gyrase, which is composed of two sub-units, A and B, encoded by *gyrA* and *gyrB* genes, respectively (Takiff *et al.*, 1994; Kocagoz *et al.*, 1996). Mutation occurring in any or both of the DNA gyrase sub-units is found to confer resistance to fluoroquinolones (Kocagoz *et al.*, 1996).

1.5. STREPTOMYCIN

Streptomycin is one of the front-line drugs for the treatment of tuberculosis. It is in the group of aminoglycoside antibiotics mainly used for the treatment of tuberculosis, but it is also active against a wide range of bacteria. Streptomycin sulphate, the basis of the drug is produced by the growth of certain strains of *Streptomyces griseus* (Winder, 1982; Dollery, 1994). Like other aminoglycosides, streptomycin is actively transported across the bacterial cell membrane by an oxygen-dependent system. The drug penetrates the bacterial cell wall and cell membrane to bind with ribosomes, thus altering protein synthesis and causing active death of the bacteria. Factors such as the divalent cation concentration (mg^{2+} and ca^{2+}), environmental pH and oxygen tension are determinant for the rate of intracellular accumulation of the drug.

Streptomycin is a potent bactericidal drug against bacilli in a neutral or alkaline environment, greatest activity of which is at a pH of 7.8, but it becomes inactive under anaerobic and under acidic conditions (Raleigh, 1984). Most strains of *M. tuberculosis* are sensitive to less than 10 mg/L concentration of streptomycin, so a 10 mg/L or greater of streptomycin in a patient is generally accepted as being therapeutically adequate (Dollery, 1994).

Streptomycin has a selectively toxic action on the eighth cranial nerve. Vestibular damage is much commoner than auditory damage, which may include deafness, but both can occur together. When the drug is administered by intrathecal injection, it can cause root pain, pleocytosis in cerebrospinal fluid, and rarely evidence of brain stem irritation, such as neck pain, temporary retention of urine (Dollery, 1994).

1.6. GENES RESPONSIBLE FOR STREPTOMYCIN RESISTANCE IN M. TUBERCULOSIS

Resistance to streptomycin in *M. tuberculosis* has been found to be associated with missense mutations in the genes encoding two components of the ribosome, the 16S rRNA (encoded by *rrs*) and the S12 protein(encoded by *rpsL*). In the ribosomal structure, the 16S rRNA forms a conserved pseudoknot structure. This pseudoknot, with two highly conserved loops that are adjacent in the 16S rRNA, is stabilized by the S12 ribosomal protein (Sreevatsan *et al.*, 1996). Mutations associated with resistance to streptomycin in *M. tuberculosis,* are the mutations clustered in two regions around nucleotide 530 and 915 of the 16S rRNA. So far, mutations that occur at the nucleotide *530 and 915* loop of the 16S rRNA have been shown to be responsible. Substitution of cytosine at *512*, adenine at 513, cytosine at 516 by other bases distort the structure of the loop, which will inhibit binding of the drug and would result in higher level resistance (Bottger, 1994). Alternatively insertion of an extra base after position 512 in the 16S rRNA may result in a bulge in the 530 loop which inhibits binding of the drug (Honore *et al.*, 1995). The complex cellular biological process of protein synthesis requires the alignment of the ribosome to tRNA with mRNA. There must be correct codon-anticodon pairing at the decoding site and to catalyze formation of the peptide bond at the other end of the tRNA at the peptidyl-transferase site (Bottger, 1994). Other mutations observed so far in association with streptomycin resistance is substitution of amino acid *Lys 43 or Lys 88* by arginine in the S12 protein which affect the higher-order structure of 16S rRNA (Bottger, 1994; Cooksey *et al.*, 1996). Since streptomycin

usually binds to the 16S rRNA sub-unit of the ribosome, the disrupted structure alters the binding nature of streptomycin.

The most frequently occurring mutations that result in high level resistance to streptomycin in *M. tuberculosis* are the mutations in the *rpsL* gene (Nair *et al.*, 1993). The most commonly occurring mutation results in replacement of Lys 43 or Lys 88 by Arg (Bottger, 1994; Cooksey *et al.*, 1996). This is caused by a point mutations in the codon AAG for Lysine to AGG for Arginine. Other amino acid substitutions associated with streptomycin resistance are also observed at these two sites, for example the substitution of *Lys 43* by *Thr* and *Lys 88 by Gln*, but the significance of *Lys 88* replacement by *Gln* is less clear (Honore *et al.*, 1995; Sreevatsan *et al.*, 1996).

However, despite the detail of our knowledge on the effects of the above mutations, recent studies show that about 20% of strains streptomycin resistant *M. tuberculosis* have neither *rpsL* nor *rrs* gene alterations (Finken *et al.*, 1993) Out of the 15 streptomycin resistant isolates examined by Bottger's laboratory for the occurrence of mutations in the *rpsL* and *rrs* genes, 4 of the isolates (which had low level resistance) were found to have no mutations in either *rpsL* or *rrs* genes (Finken *et al.*, 1993). Also, Morris *et al.* (1995) showed that 12 strains of 44 total isolates did not have mutations in *rpsL* or *rrs*, and that some of these strains had high level of resistance to streptomycin. These isolates with wild type *rpsL and rrs* genes must have developed other mechanisms of resistance to streptomycin. According to Shaila *et al.* (1973) low level resistance to streptomycin might be attributed to changes in cell wall permeability, but this has not been demonstrated. Altered permeability of the mycobacteria cell wall,

which constitutes an effective barrier to all kinds of antibacterial agents, could be responsible for blocking the uptake of streptomycin (Heym *et al.*, 1996). Other possible mechanisms of resistance suggested by Honore *et al.* (1994) are modification of the components of the ribosome and the acquisition of genes encoding aminoglycoside modifying enzymes which requires additional studies. Cooksey *et al.* (1996) and Meier *et al.* (1996) generalized the mechanisms of resistance to streptomycin in *M.tuberculosis* as a ribosomal and a permeability barrier. This concept with the knowledge of the ribosomal RNA playing active role in translation rather than being a mere " scaffold" for ribosomal proteins, invites further functional investigation of the ribosomal RNA (Sander *et al.*, 1996). Although the slow growth and pathogenicity of *M. tuberculosis* presents a major challenge, many important questions can be addressed by using the fast growing mycobacterial relative, *Mycobacterium smegmatis*. The presence of only two functional rRNA operons in the *M. smegmatis* chromosome is also an advantage to use the organism as a model to study the function of rRNA in relation to streptomycin resistance. The generation of *M. smegmatis* with one functionally inactivated chromosomal rRNA operon can be further manipulated to allow the introduction of site directed mutations within the remaining chromosomal rRNA operon (Sander *et al.*, 1996). Exploiting the homologous recombination properties of the cell one of the functional chromosomal rRNA operons of *M. smegmatis* can be replaced with an inactivated copy of vector-derived rRNA operon. Homologous recombination, which could be achieved from double-cross over, results in generating transformed *M. smegmatis* having a single functional rRNA operon. *M. smegmatis* mc^2 155 strain with

one functional chromosomal rRNA operon and site directed mutations would greatly solve the problem of generating mutants resistant to streptomycin. In parental *M. smegmatis* strains, due to the presence of two functional rRNA operons and the dominance of aminoglycoside sensitivity over resistance prohibited the isolation of aminoglycoside resistant mutants at the expected frequency (Sander *et el.*, 1996).

Mutation occurring in one operon of the parental *M. smegmatis* mc^2 155 strain will generally be suppressed phenotypically by wild-type rRNA transcribed from the unmutated gene.

1.7. OBJECTIVES OF THE STUDY

General objective:

A better understanding of the mechanisms of action of streptomycin and of the mechanisms of development of resistance to streptomycin will lead to the development of better therapeutic and prophylactic strategies and diagnostic tests for drug resistant *M. tuberculosis*.

Specific objectives:

1. Primary objective. To examine the molecular mechanisms of resistance to streptomycin in *M. tuberculosis* clinical isolates from Ethiopia.
2. Secondary objective. To investigate the possible mechanisms responsible for

resistance to streptomycin in resistant strains of *M. tuberculosis*, with out mutations in *rpsL* and *rrs* genes. Comparative study will be conducted on ribosomal RNA of the mycobacterial relative, *Mycobacterium smegmatis* (*M. smegmatis* mc^2 155).

2. MATERIAL AND METHODS

2.1. DETERMINATION OF MIC VALUE.

2.1.1. *M. tuberculosis* Strains

Twenty two streptomycin resistant isolates of *M.tuberculosis* which had been collected from Addis Ababa Tuberculosis Demonstration and Training Center, Ethiopia, were stored in the Armauer Hansen Research Institute (AHRI) laboratory and were used for this study. For all the isolates, resistance to streptomycin had been previously confirmed in Sweden (Swedish institute for infectious disease control, SIID) by culturing in BACTEC media containing 4 μg/ml streptomycin concentration (Abate *et al.*, 1994). In addition to these strains 5 streptomycin resistant strains of *M. tuberculosis* (which had been tasted by BACTEC and brought from Sweden (SIID) and stored in the AHRI laboratory were included in the study.

Seventeen streptomycin resistant strains of *M. tuberculosis* were brought from Sweden (SIID). In addition, one *M. tuberculosis* strain (S:29) which is susceptible to the front-line drugs (Abate *et al.*, 1994) was included in experiments as control.

2.1.2. Media Preparation

The Minimum Inhibitory Concentration (MIC) value of the strains was determined by culturing each of the strains on a Middlebrook-cohn 7H10 agar media. As described by Jacobs *et al.*, (1991) 9.5 g of Middlebrook 7H10 agar (Difco laboratories, Detroit, Michigan, USA) was dissolved in 450 ml distilled water in a bottle

with a 600 volume of fluid capacity. Six ml of 40% sterile Glycerol (BDH laboratory supplies, Poole, England) was added for better growth of the *M. tuberculosis* bacilli. The mixture was subjected to sterilization by autoclaving for 20 minutes at 120°C under 15 atmospheric pressure. Then the bottle was equilibrated in a water bath to 55°C before the enrichment media Middlebrook OADC (Difco laboratories) was added. The media (total volume of 500 ml) was then immediately poured into 60 mm diameter petriplates, approximate volume of 9 ml. All procedures were conducted in a laminar flow hood. Finally, the petriplates were wrapped with parafilm, and stored at 4°C.

2.1.3. Incorporation of streptomycin

The antibiotic streptomycin sulphate (Sigma Chemical Company., St. Louis, USA) was purchased in the powder form. Since it's activity was 75.7%, to get a 100 mg/ml stock with 100% activity, 132 mg of streptomycin sulphate was suspended in 1 ml of distilled sterile water. 6 different concentrations (0, 5μg/ml, 10μg/ml, 40μg/ml, 160μg/ml, 640μg/ml) in duplicate were used for the determination of the MIC of the strains, following (Heym *et al.*, 1994; Morris *et al.*, 1995). The appropriate amounts of streptomycin were incorporated by spreading the liquid evenly over the surface with a bent glass rod into each of the petriplate.

Streptomycin-containing media were wrapped with parafilm and stored at 4°C for 24 hrs to allow the streptomycin to diffuse into the media.

2.1.4. Inoculation of 7H10 plates with the *M. tuberculosis*

Bacterial suspensions were prepared by taking 3 loopfuls (1μl volume loop) of the bacterial colony from the sub-culture, and adding to small sterile bottle containing 3 ml of Phosphate Buffer Saline (PBS) and 5 glass beads (3 mm diameter). The glass beads disrupt the bacterial colonies to form a good suspension when the bottles were subjected for shaking at 250 rpm for 1 hr. From the suspension, dilutions were prepared to inoculate the first set of the petriplates with approximately 10^4 bacteria and each of the second set of the petriplates with approximately 10^3 bacteria. 6 loopfuls of the bacterial suspension was added to 54 μl of PBS (ie. a 1 in 10 dilution of the stock), and 6 loopfuls of this was added to another 54 μl of PBS (ie. a 1 in 100 dilution of the stock suspension). Inoculation of the plates was carried out by taking 3 loopfuls from either of the two dilutions and spreading on the surface of the correspondingly labelled medium. All procedures of the bacterial inoculation were done in the AHRI TB laboratory (level 3 containment facility). Following inoculation, plates were kept at 37°C for about 4 weeks.

2.2. DNA EXTRACTION

DNA of the high level and low level streptomycin resistant strains of *M. tuberculosis* was extracted using DNAstat-60 (Tel-Test "B", Inc. Friends wood, Texas) following the manufacturer's instructions. In the tuberculosis laboratory safety cabinet, 5-7 loopfuls (1μl capacity loop) of bacteria was taken from the Middlebrook 7H10

medium culture, and added into eppendorf tube (1.5 ml capacity) containing 1 ml of DNAstat-60. The mixture was homogenized by shaking for 2 minutes to facilitate lysis. The homogenized mixture heated to 80°C, for 10 minutes to kill any remaining viable bacilli before the mixture was taken out from the TB laboratory for further DNA extraction processing. The homogenate was passed up and down a 21 gauge needle about 20 times to mechanically disrupt the bacteria. 200 μl of chloroform per 1 ml of DNAstat-60 used was then added , shaken vigorously for 15 seconds, stood for 2-3 minutes then centrifuged for 15 minutes at 12000 rpm, 4°C. After centrifugation the homogenate separates into two phases, a lower organic phase and upper aqueous phase where the DNA is found.

The aqueous phase was transferred into a fresh eppendorf tube and mixed with 500 μl of isopropanol per 1 ml of the DNAstat-60 used to precipitate the DNA. The mix was incubated for 5-10 minutes under room temperature, then centrifuged for 10 minutes under 12000 rpm at 4°C. The supernatant was discarded from the tube, leaving a small white DNA pellet, which was washed with 75% ethanol, by vortexing and subsequent centrifugation under 7500 rpm for 5 minutes at 4°C. Finally the DNA pellet was dried in speed-vac Concentrator (Savant Instruments, Inc. Farmingdale, NY. USA) attached to a vacuum pump for 5-10 minutes and 20 μl of sterile distilled water was added to dissolve the pellet. DNA extracts were stored at -20°C until needed for further PCR amplification.

2.3. AMPLIFICATION OF *rpsL* AND *rrs* GENES

To amplify the *rpsL* gene (encoding the ribosomal protein S12) from strains of *M. tuberculosis*, PCR amplification was conducted using S12-3 + S12-4 primers, which amplify the entire *rpsL* gene (375 bp).

Amplification of the *rrs* gene (encoding the 16S rRNA) was conducted using primers 16S-1 + 16S-4. To amplify a 650 base pair fragment. Already documented mutations of the *rrs* gene are confined within this segment of the gene.

Table: 1. Location of the annealing and ending position on the genes, length and base sequence of each of the primer.

Ser.No	Primers	The gene	Position in the gene		Length of primers (base sequence)
			Start	Ends	
1	S12-1	rpsL	109	128	5'GTG TAC ACC ACC ACT CCG AA 3'
2	S12-2	rpsL	282	263	5'GCG CAC ACC AGG CAG GTC CT 3'
3	S12-3	rpsL	1	19	5'ATG CCA ACC ATC CAG CAG C 3'
4	S12-4	rpsL	375	355	5'TCA GCC CTT CTC CTT CTT AGC 3'
5	16S-1	rrs	349	365	5'TGC AGC GAC GCC GCG TG 3'
6	16S-2	rrs	591	574	5'TCG CCC GCA CGC TCA CAG 3'
7	16S-4	rrs	1005	987	5'GCA CCA CCT GCA CAC AGG C 3'

A. PARTIAL SEQUENCE OF *rpsL* GENE

```
 S12-3→
1   5'ATGCCAACCA TCCAGCAGCT GGTCCGCAA GGGTCGTCGGG ACAAGATCAG TAAGGTCAAG
                                 S12-1→
61  ACCGCGGCTC TGAAGGGCAG CCCGCAGCGT CGTGGTGTAT GCACCCGCGT GTACACCACC
121 ACTCCGAAGA AGCCGAACTC GGCGCTTCGG AAGGTTGCCC GCGTGAAGTT GACGAGTCAG
181 GTCGAGGTCA CGGCGTACAT TCCCGGCGAG GGCCACAACC TGCAGGAGCA CTCGATGGTG
241 CTGGTGCGCG GCGGCCGGGT GAAGGACCTG CCTGGTGTGC GCTACAAGAT CATCCGCGGT
                              ←S12-2
301 TCGCTGGATA CGCAGGGTGT CAAGAACCGC AAACAGGCAC GCAGCCGTTA CGGCGCTAAG
361 AAGGAGAAGG GCTGA 3'
        ←S12-4
```

B. PARTIAL SEQUENCE OF *rrs* GENE

```
                    16S-1  →
316 GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGT TGTAAACCTC
396 TTTCACCATCGACGAAGGTCCGGGTTCTCT CGGATTGACG GTAGGTGGAG AAGAAGCACCGGCCAACTAC GTGCCAGCAG
476 CCGCGGTAAT ACGTAGGGTGCGAGCGTTGT CCGGAATTAC TGGGCGTAAA GAGCTCGTAGGTGGTTTGTC GCGTTGTTCG
556 TGAAATCTCACGGCTTAACTGTGAGCGTGCGGGCGATACGGGCAGACTAGAGTACTGCAGGGGAGACTGG AATTCCTGGT
                    ← 16S-2
636 GTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGC
716 GAAAGCGTGGGGAGCGAACAGGATTAGATA CCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTG GGTTTCCTTC
796 CTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC AAAGGAATTG
876 ACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTT GACATGCACA
956 GGACGCGTCT AGAGATAGGC GTTCCCTTGT GGCCTGTGTG CAGGTGGTGC ATGGCTGTCG TCAGCTCGTG TCGTGAGATG
                              ← 16S-4
```

Fig.1. Diagrammatic representation of the alignment of each of the primer along the genes.

The PCR mix for one template was prepared in a total volume of 50 μl. The reaction mixture consists of 10X Buffer (Boehringer Mannheim, GmbH. Germany) contained 10 mM Tris HCl (pH 8.3); 1.5 mM $Mgcl_2$; 50 mM Kcl, 10 mM deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP), 10 pmol each of the forward primer and reverse primer (Johns Hopkins University, School of Medicine, USA) and 5 unit of heat stable Taq DNA polymerase (Boehringer Mannheim) derived from *Thermophillus aquaticus* in 0.5 ml capacity micro-centrifuge tube. After adding 1 μl of the template (Genomic DNA), the mixture was overlaid with one drop of mineral oil (Sigma Chemical Comp.).

The PCR machine (Hybaid, OmniGene, UK) was programmed as follows; the second stage being repeated for 35 cycles :-

First stage	95°C	For 5 minutes (Denaturation)
Second stage	95°C	For 1 minute (Denaturation)
	62°C	For 1 minute (Annealing)
	72°C	For 1 minute (Extension)
Third stage	72°C	For 10 minutes (Finalization)

In all cases 5μl PCR products were mixed with 2μl of 6X loading buffer (0.05% w/v bromophenol blue, 40% w/v sucrose, 0.1 1M EDTA) (Sigma chemical Comp.) and loaded on 2% agarose gel and electrophoresed together with molecular weight marker

(1kb DNA marker) (Gibco BRL. Life Technologies, Paisely, Scotland). 2% agarose gel was prepared by dissolving 1 g of agarose (Pharmacia Biotech. AB Uppsala, Sweden) in 50 ml of Tris acetate EDTA(TAE) buffer(1X TAE= 4.84 g Tris-base, 1.14 ml glacial acetic acid and 0.744 g Na_2 EDTA.$2H_2O$ dissolved in a liter of distilled water) (Sambrook *et al.*, 1989). After the agarose completely dissolved, 1.5 μl of ethidium bromide (EtBr) (10 μg/ml) was added, and shaken to mix it with the gel solution. Ethidium bromide containing gel solution was poured into the well set gel plate (150 ml capacity), and let it solidify for about 30 minutes. When the gel was solidified , it transferred into the electrophoresis tank containing 250 ml of Tris acetate EDTA (TAE) buffer into which 6.5 μl ethidium bromide(EtBr) (10μg/ml) was added.

Electrophoresis was performed for about 1 hr at 120 volts. The DNA was visualized by exposure to uv transilluminator(UVP, Inc, San Gabriel, USA) and the gel was photographed using Polaroid black and white print film (type 667, Sigma chemical Comp.).

2.4. SEQUENCING OF *rpsL* AND *rrs* GENES

2.4.1. Sequencing Strategy

For identifying key mutations in the *rpsL* gene PCR products amplified using S12-3 + S12-4 primers, was used as template DNA for the sequencing reaction. The reverse primer S12-2 was used as the sequencing primer inorder to identify mutations at codon 43 . The sequence was confirmed by sequencing in the opposite direction using

the forward primer S12-3. To identify mutations at codon 88 of the *rpsL* gene, the forward primer S12-1 was used (Table. 1).

For identifying key mutations in the *rrs* gene, PCR product of primers 16S-1 + 16S-4 was used as a template. The forward primer 16S-1 was used as primer in the cycle sequencing reaction to focus on mutational site of 915 loop on the *rrs* gene. For detection of the base sequence of the 530 loop of the *rrs* gene, the reverse primer 16S-2 was used as primer (Table. 1).

2.4.2. Radioactive Sequencing

2.4.2.1. Cycle sequencing reaction

Sequencing of the *rpsL* and *rrs* genes was conducted using a cycle sequencing kit (Pharmacia Biotech) following the manufacturer's instructions.

Four 0.5 ml capacity micro-centrifuge tubes were labelled as "A", "C", "G", and "T" for the respective termination reactions. Into each of these four tubes 2 μl of the corresponding A,C,G or T termination mix was added, and the tubes were placed on ice until needed.

Cycle sequencing reaction mix for one template was prepared in a total volume of 25 μl in a 1.5 ml capacity micro-centrifuge tube. The mixture consisted of 5X sequencing buffer (Pharmacia Biotech) contained in 350 mM Tris HCl (pH 9.0); 7.5 mM $Mgcl_2$; 52% DMSO, 10 pmol of sense primer (forward primer) or anti-sense

primer (reverse primer), deoxynucleoside triphosphate (80μM each dATP, dCTP, dGTP and dTTP), 1 unit Taq DNA polymerase, 10 mCi/ml of dATP labelled with [α-S^{35}] (Amersham International. UK) and 3 μl of DNA template. S^{35} dATP is a radioactive chemical and stored at -20°C in a Radioisotope laboratory. It was taken with great precautions under a cabinet protected with glass frame, to avoid eye and skin contact. The components mixed gently, then centrifuged briefly, and placed on ice. From this master mix 5 μl was aliquoted into each of the labelled tubes containing the termination mix, and gently pipette to mix. A drop of mineral oil was added into each of the labelled tubes, then the tubes were subjected to centrifugation for 20 seconds to remove any bubbles. The tubes were then placed into a pre-heated (95°C) thermal cycler(Hybaid, OmniGene, UK) for 2 minutes.

The thermal cycler was programmed as follows for 25 cycles.

95°C For 30 seconds (Denaturation)

55°C For 36 seconds (Annealing)

72°C For 84 seconds (Extension)

Finally, the reaction was stopped by adding 3 μl of the stop solution (97.5% deionized formamide, 10 mM EDTA, 0.3% xylene cyanol, 0.3% bromophenol blue) into each of the labelled tubes; centrifuged briefly and frozen at -20°C, until ready for electrophoresis.

2.4.2.2. Preparation and Casting of Sequencing Gel

64 and 61 cm long glass sequencing plates(long and short plate, respectively) (Bio-Rad laboratories, Inc.) were washed with tap water then with 100% acetone using tissue paper. The shorter plate was coated with bind coating (containing 80% ethanol (95%)(BDH laboratory supplies), 20% acetic acid (10%) (MERCK, Darmstadt, Germany), 0.3% bind silane (Pharmacia Biotech). The longer plate was coated with 100% repel silane (2% dimethyldichlorosilane in 1,1,1, trichloroethane) (Pharmacia Biotech). Plates were then cleaned with 95% ethanol using tissue paper. The 0.04 mm thicker spacers and shark tooth comb were also cleaned with ethanol and distilled water. The two plates and spacers were assembled and held together with spring clips on both sides and laid flat for pouring the gel.

6% acrylamide gel solution was prepared by dissolving 31.5 g of urea in 35 ml of distilled water in 37°C adjusted water bath. With complete dissolving of the urea, 40% acrylamide solution(38% acrylamide - 2% bis- acrylamide), 10X Tris borate EDTA (TBE) buffer (10x TBE= 109 g Tris base, 55.6 g of Boric acid (powder) and 7.44 g Na_2 EDTA.$2H_2O$ dissolved in a liter of distilled water) was added (Sambrook *et al.*, 1989). The gel solution was filtered through a 0.2 μm filter using vacuum suction and degassed for 5 minutes to remove air bubbles and reduce polymerization time. 450 μl of 10% Ammonium Per Sulphate (APS) and 65 μl of TEMED (Tetramethyl ethylenediamine) (Serva, FeinBiochemica. Heidelberg) was added and mixed gently, and then the solution was poured immediately between the two glass plates in a small stream. The comb was placed in the top of the plate blunt side down to create a flat

edge to the top of the gel. The gel polymerized within 45 minutes.

2.4.2.3. Electrophoresis of Sequencing Reaction Products

After polymerization of the gel the spring clips were removed, and the plates assembled on the electrophoresis apparatus (Pharmacia LKB) attached with SA Model Stabilizer Base Plate (Life Technologies, Inc. USA). The comb was taken out carefully, and the space cleaned with running buffer (10% Tris borate EDTA (TBE)). Urea that had leached out from the gel was removed by flushing with running buffer. The gel was pre-run at 23 mA, 2000 v, 46 W for 45 minutes to warm the gel to approximately 50°C with stop solution loaded into alternate wells to check for well leakage.

The cycle sequencing reaction products were denatured at 95°C for 2 minutes, then stored on ice. 2-3 μl of each sample was loaded per well in the order G, A, T, C, and electrophoresed for about 4 hrs. The gel was then dismantled and the two glass plates pried apart. The gel on the shorter glass plate was incubated in fixer solution contained 10% glacial acetic acid (100%) (MERCK) and 10% methanol (99.7-100% (v/v)) (BDH laboratory supplies) for 1 hr, then dried at 65°C for 90 minutes. The gel was exposed to Hyper film β max (Amersham international)in the dark at room temprature for about 14 days at AHRI (because of "old film") and for one day at Medizinische Hochschule Hannover (MHH), then developed with GBX developer (Sigma Chemical Comp.) and fixed with GBX fixative chemical (Sigma Chemical Comp.). Sequences were read manually. Sequence analysis was carried out using the

computer programme PC gene.

2.4.3. Non-Radioactive (Fluorescence) sequencing

2.4.3.1. PCR purification

PCR products were purified as per instructions in QIA quick PCR purification Kit (250), QIA quick hand book, Germany. 5 volume of buffer PB (QIA GEN^R) was added to the PCR product. After the QIA quick spin column placed in a 2ml volume collection tube the sample was applied and centrifuged at 13000 rpm for 60 seconds. The flow-through solution was discarded and QIA quick column placed back into the same tube. The column was washed with 750μl of buffer PE(QIA GEN^R) and centrifuged at 13000 rpm for 60 seconds. The flow-through solution was again discarded and QIA quick column centrifuged for an additional 1 minute at 13000 rpm. QIA quick column was placed in a clean 1.5 ml eppendorf tube. To elute the DNA, 50μl of sterile distilled water was added to the center of the QIA quick column and centrifuged at 13000 rpm for 1 minute. DNA was stored at -20°C.

2.4.3.2. Sequencing Reaction

Following the instructions in Perkin-Elmer cycle sequencing protocol (Perkin-Elmer corporation. Norwalk, USA), the sequencing reaction mix was prepared in a volume of 20 μl using 0.2ml capacity Gene Amp tube. The reaction mixture (one tube

for one sample) consists of 1/5 volume of terminator ready mix (containing different fluorescent labelled deoxynucleoside triphosphate, 1 unit of Taq DNA polymerase), 100 ng of purified PCR product, and 10 pmole primer. Sequencing reaction containing tubes were placed in the thermal cycler (Gene Amp PCR system, 9600, Perkin-Elmer corporation). The thermal cycler was programmed for 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Cycle sequencing products were purified by treating with 1/10 volume of 3M sodium acetate (pH 4.6) and 2.5 volume of 95% ethanol in a 1.5 ml eppendorf tube. The mixture was vortexed and placed on ice for 10 minutes. After centrifugation ethanol was aspirated, pellet rinsed with 70% ethanol and the ethanol aspirated again. Pellet was dried in a speed vac (Savant Instruments) for 15 minutes and stored at 4°C until needed.

2.4.3.3. Sequencing Gel Preparation

34 cm long 5% acrylamide gel was prepared by dissolving 31.5 g of urea in 23.5 ml of distilled water and 40% acrylamide solution. Mixture was placed in 37°C adjusted water bath and stirred using magnetic stirrer. Into the mixture 6 ml of 10X Tris borate EDTA (TBE) buffer was added and filtered through 0.2 mm filter using water pressure suction filter. 27μl of APS and the filtered gel solution was poured into a new Erlenmeyer flask and 15 μl of TEMED was added. After mixing gently, the solution was poured immediately between the two well set glass plates in a small stream. Glass plates were washed with water, detergent solution and sprayed with isopropanol for

better drying and removal of water droplet. Before pouring the gel solution both the spacers and the shark tooth comb were placed properly.

Unlike the radioactive sequencing, in non-radioactive sequencing for each sample only one well was required and electrophoresed for over night. The automatic sequencer (Applied Biosystem 373 DNA sequencer) is equipped with laser reader. The laser reader detects each of the bases based on their fluorescence as they pass through the gel and convert into densitometer in different colours. The out put was available in the form of peaks as well as base designation (G,A,T,C) which could either be stored in the computer or hard copy print out (Fig. 5).

2.5. FUNCTIONAL ANALYSIS OF THE rRNA OF MYCOBACTERIA

2.5.1. Digestion Of Plasmid DNA

To construct the replacement vector, for the inactivation of one rRNA operon of *M. smegmatis* mc^2 155, plasmid pRRNA4-4-K1, constructed at MHH, Germany, was used (Fig. 6). The plasmid is consists of one rRNA operon, part of plasmid pBluescript II ks+ (Stratagene) and the kanamycin resistance marker (aph) from plasmid pUC4K (Pharmacia Biotech). The *M. smegmatis* origin 6.4 kb rRNA operon was obtained by digesting the phagemid pBK-CMV (into which the rRNA operon was packaged) using Xba I and Sal I restriction enzymes. The rRNA operon was cloned to the XbaI + SaI I digested pBluescript II ks+. The 1.3kb, *PstI* restriction enzyme fragment, kanamycin resistant marker interrupts the rRNA operon.

At the sal I cloning site of the plasmid pRRNA4-4-K1, the *sacB* suicide fragment of plasmid pLO2 was cloned. Plasmid pLO2 (Fig. 7) contains the *Bacillus subtilis* gene, *sacB*, that encodes the secreted enzyme Levansucrase. This enzyme has been found to be lethal to mycobacteria in the presence of 15% sucrose (Pelicic *et al.*, 1996). The enzyme, at the mycobacterial periplasm catalyzes hydrolysis of sucrose and synthesis of levans which are high-molecular weight fructose polymers. The toxicity of the levans could be due to an accumulation which might encumber the periplasm, or transfer of fructose residues to inappropriate acceptor molecules which could result a toxic effect on the bacterial cells (Pelicic *et al.*, 1996). A sucrose counter-selectable suicide plasmid was used to deliver an inactivated copy of the rRNA operon. The *sacB* gene used as a marker for positive selection of gene-replacement events into *M. smegmatis*. First, linearization of the pRRNA4-4-K1 was done by digestion with the restriction enzyme sal I. The digestion mix was prepared in a total volume of 30μl, containing 10x reaction buffer (33 mM Tris-acetate; 10 mM Mg-acetate; 66 mM K-acetate; 0.5 mM Dithiothreitol), 30 units of sal I (Boehringer Mannheim) and 10μl (9μg) of plasmid DNA. After 30 seconds of centrifugation at 13000 rpm the digestion mix was incubated at 37°C over night. The 2Kb fragment *sacB* gene of plasmid pLO2 was obtained by digestion with BamH I and EcoR V restriction enzymes. The 30 μl digestion mix consisted of 10X buffer B and 10X buffer H (contained 10 mM Tris-HCl; 100 mM NaCl; 5 mM $MgCl_2$; 1 mM 2-Mercaptoethanol and 50 mM Tris-HCl; 10 mM $MgCl_2$; 100 mM NaCl; 1 mM Dithioerythritol respectively), 40 units of BamHI and 20 units of EcoR V, and 10 μl (15μg) of the plasmid DNA. It was incubated at 37°C overnight.

Digestion products were precipitated by adding 1 volume of Phenol-Chloroform-Isoamyl alcohol (25:24:1), 1/10 volume of 3M NaAc and 3 volumes of absolute ethanol (Mallinckrodt Backer B.V. Deventer, Holland), then centrifuged at 13000 rpm for 10 minutes and the pellet resuspended in 20 μl of Tris-EDTA (TE) buffer (contained 10 mM Tris and 1 mM EDTA) buffer. To get blunt ended plasmid DNA, phenolized products were treated with T4- DNA -polymerase in a total volume of 21μl, containing 2μl of 10X T4-DNA-polymerase buffer (New England Biolabs) consists of 33 mM Tris-acetate, pH 7.9, 66 mM Potassium acetate, 10 mM Magnesium acetate, 0.5 mM DTT, and 0.5 mg/ml BSA); 20 mM deoxynucleoside triphosphate, and 10 units of T4-DNA-polymerase (Promega, Medison. Wi, USA) were added to 16μl of the resuspended DNA. The mixture was incubated at 11°C for 20 minutes, then the reaction was stopped by adding 0.5 M EDTA. The phenolization step was repeated to the vector (plasmid pRRNA4-4-K1) before the process of dephosphorylation to prevent religation of the empty vector during the subsequent ligation reaction. The blunt-ended vector was dephosphorylated in 1 unit of Calf intestine alkaline phosphatase (Boehringer Mannheim) added to 18μl of the DNA, and incubated at 37°C for 1 hr. An additional 1 unit of phosphatase was added, and the reaction incubated for one more hour, then the reaction product was stored at -20 °C until it was needed.

Treated digested products were loaded on 0.7% agarose gel with 10μl of loading buffer and electrophoresed. The gel was visualized by exposure to a UV transilluminator (Herolab, Molekulare Trenntechnik, Germany). The band of interest (approximately 8.6 Kb of the linearized pRRNA4-4-K1 and 2Kb of the *sacB* fragment) were excised from

the gel for gene cleaning.

2.5.2. Gene Cleaning

Gene cleaning was performed following the manufacture's instructions (Gene clean II Kit, Bio 101. Inc. USA). Into the excised vector and insert plasmid DNA, in separate 1.5 ml eppendorf tubes, 3 volumes of stock NaI solution was added to dissolve the agarose gel. After 10 minutes of incubation at 55°C, 10μl of glass milk (insoluble silica matrix) was added to bind the DNA, then the mixture vortexed for 5 minutes and centrifuged at 13000 rpm for 30 seconds. After discarding the supernatant the pellet was washed 3 times with New Wash solution (made with 280 ml of autoclaved water, and 310 ml of absolute ethanol plus the 14 ml of the kit's "New concentrate"). The pellet was dried in a speed vac concentrator (Savant Instruments) for 15 minutes. To elute the plasmid DNA from the glass milk, the pellet was resuspended in 40μl of TE buffer, and incubated for 10 minutes at 55°C. Following centrifugation at 13000 rpm for 30 seconds, the supernatant was transferred into a fresh 1.5 ml eppendorf tube.

2.5.3. Ligation Reaction

The insert DNA (blunt ended DNA of the *sacB*) was ligated to the linearized dephosphorylated-blunt ended vector DNA (plasmid pRRNA4-4-K1). Based on the length ratio of vector to insert DNA, a one to one molar ratio and a one to three molar ratio of vector to insert was calculated for the ligation reaction. The 20μl ligation

reaction, contained 250 mM Tris-HCl,pH 7.6; 50 mM $MgCl_2$; 5mM ATP; 5mM DTT; 25% (w/v) polyethylene glycol-8000; 1 unit of T4-DNA ligase (GibcoBRL. Life Technologies) and 20μM Hexamine Cobalt Chloride (HCC) (Sigma Chemical Comp.) and vector and insert DNA. Ligation reactions were carried out at 16°C overnight. Reaction products were purified by adding 80μl autoclaved water, 100μl of phenol-chloroform-isoamyl alcohol, and subsequent centrifugation at 13000 rpm for 10 minutes. The supernatant was transferred into a fresh eppendorf tube, and 100μl of diethylether (Riedel-de-Haen, Germany) was added. After brief vortexing and centrifugation for 15 minutes, the most upper phase of the supernatant was discarded. The DNA was precipitated by adding 0.06 μl by volume of 5M NaCl, 200 ng/μl of acrylamide and 1.8 μl by volume of absolute ethanol were added; then the mixture vortexed and incubated for 30 minutes in -70 °C. Finally, after 20 minutes centrifugation and twice washing with 1 volume of 70% ethanol, the pellet was dried and resuspended in 20μl of autoclaved water.

2.5.4. Transformation of *E. coli*

Competent cells (cells ready for transformation) of the *Escherichia coli* strain, XL1-Blue-MRF, were prepared by culturing a single colony of the strain in 5 ml Luria-Bertani (LB) medium (GibcoBRL. Life Technologies). After overnight incubation at 37°C on 250 rpm shaker, 1ml of the shaken culture was inoculated into 500 ml of LB medium, and the culture was incubated overnight at 37°C, 250 rpm. When the optical

density reached between 0.5-0.7 OD, the culture was incubated on ice for 15 minutes, and cells were harvested by centrifuging the culture at 5000 rpm, at 4°C for 20 minutes by using a Beckman centrifuge, JA-10 rotor (Beckman Instruments, Inc. Palo Alto, California). The harvested cell pellet was resuspended in 50 ml ice cold N-2-hydroxyethyl piperazine-N-2- ethane sulfonic acid (HEPES) (Sigma Chemical Comp.) and centrifuged using MiniFuge GL centrifuge (Heraeus-christ, GmbH. Germany) at 5000 rpm, 4°C for 20 minutes. This step was repeated 5 times. Finally, the cell pellet was resuspended in 2-5 ml of ice-chilled 10% glycerol (MERCK). 300 -400 μl of the resuspended cells were aliquoted into 1.5 ml eppendorf tubes, and stored in -70°C.

For electroporation(the process by which plasmid DNA can be transferred to the bacteria, electroporation makes the bacterial cell cavitation to take the plasmid DNA) 35 μl of the competent cells were mixed with 2 μl of the purified ligation product in an sterile electroporation cuvette and chilled in ice. The electroporator (BioRad Gene pulser) was adjusted to a resistance of 400 Ohms, voltage of 2.5 KV and 25 μF capacitance. After electroporation, transformed competent cells were mixed with 1 ml of SOC broth medium (made with SOB broth medium, 10 mM $MgCl_2$, 10 mM $MgSo_4$, and 20 mM glucose) and incubated at 37°C for one hour. 200 μl of the culture was plated onto a LB agar plate (GibcoBRL. Life Technologies) containing 50 μg/ml kanamycin. Plates were incubated at 37°C for over night. Random colonies were selected from the plates and inoculated into 5 ml LB broth medium containing 50 μg/ml kanamycin. After over night incubation at 250 rpm, 37°C, sensitivity tests of the selected colonies was done by spreading 20 μl of the culture on the surface of both LB-

kanamycin 50 μg/ml duplicate plates and on LB-kanamycin 50 μg/ml+7.5% sucrose duplicate plates. The plates were incubated at 37°C for overnight.

2.5.5. Isolation Of Plasmid DNA

2 ml culture which were grown on LB-kanamycin 50 μg/ml plates but not on the plates containing sucrose, were inoculated into 150 ml LB broth containing 50 μg/ml kanamycin, and incubated at 37°C over night. Plasmid DNA was extracted using QIAGEN-tip 100 (QIAGEN. Inc, Germany), following the manufacturer's instructions. The cell pellets were harvested by centrifuging the culture at 5000 rpm, at 4°C for 15 minutes using the MiniFuge GL centrifuge (Heraeus-christ). The pellet was resuspended with 4 ml resuspension buffer (containing 100 μg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and subsequently 4 ml of the lysing buffer (containing 200 mM NaoH, 1% SDS) was added, then incubated at room temperature for 5 minutes, for the cells to lyse. After adding 4 ml of ice chilled neutralization buffer (3M KAc, pH 5.5) the mixture was incubated on ice for 15 minutes. The mixture was centrifuged at 16000 rpm at 4°C using the Beckman centrifuge(JA-20 rotor). The supernatant was filtered through the equilibrated QIAGEN tip-100, then the filter tube was washed twice with 10 ml of the washing buffer (containing 1 M NaCl, 50 mM Mops, 15% ethanol, pH 7.0). Plasmid DNA was eluted from the QIAGEN tip-100 by adding 5 ml of elution buffer (containing 1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol,pH 8.5). DNA was precipitated with 0.7 volume of isopropanol, and centrifuged at 16000 rpm at 4°C for

30 minutes in Beckman centrifuge. Finally, the DNA was washed with cold 70% ethanol, air dried and redissolved in 150 μl of autoclaved water.

2.5.6. Transformation of *Mycobacterium smegmatis*

Due to its fast growth rate and ease of electroporation mc^2 155 strain of *M. smegmatis* was selected for the electroporation procedure. Competent cells were prepared by inoculating a single colony of the strain into 5 ml of Brain Heart Infusion (BHI) (Unipath. LTD, Hampshire, England) medium containing 0.05 % Tween 80. The culture was incubated for 1-2 days at 37°C. 2 ml of the culture was then inoculated into 500 ml BHI-Tween 80 medium, and incubated over night at 37°C. When the culture had attained an optical density of 0.5-1, it was incubated on ice for one hour. Cells were harvested by centrifuging at 7000 rpm, 4°C for 15 minutes using a Beckman centrifuge (JA-10 rotor). The cell pellet was resuspended in 10 % ice-chilled glycerol and centrifuged in the same manner. Washing of the cell pellet and centrifugation at 5000 rpm, 4°C, for 20 minutes using a MiniFuge GL centrifuge (Heraeus-christ) was repeated 4 times. Finally, the cell pellet was resuspended in 2-3 ml ice-chilled 10 % glycerol.

For electroporation, 100μl of the competent cells were mixed with 1μg of the plasmid DNA. The electroporator was adjusted to a resistance of 1000 Ohms, voltage of 2.5 KV and 25 μF capacitance. After electroporation, cells were mixed with 1 ml

BHI-Tween 80 broth and incubated at 37°C for 2 hours. 200 μl of the culture was plated on LB plates containing 25 μg/ml kanamycin and LB plates containing 25 μg/ml kanamycin and different concentrations of sucrose (10 %, 15 %, 25 %) and incubated for 4-5 days.

Random colonies were selected from each of the plates and amplified on kanamycin 25 μg/ml containing LB plates. From the amplified colonies genomic DNA was extracted following the SDS/ Proteinase K procedure. Cells were harvested from the plates and resuspended in 0.1 M NaCl, and inactivated by heating at 80°C for 10 minutes, then pelleted. 360 μl of lysozyme buffer containing 10 mM Tris, 10 mM EDTA, 0.1 % Tween 80, and 2 mg/ml lysozyme (Boehringer Mannheim) was added and cells incubated at 37°C for 2 hours. After adding 40 μg of proteinase K (Boehringer Mannheim) and 20% SDS (Sodium dodecylsulfate), the cell mixture was incubated for one hour at 50°C. To remove protein and other contaminants from the DNA 400 μl phenol-chloroform isoamyl alcohol was added, the mixture was centrifuged at 13000 rpm for 5 minutes. The supernatant was transferred into a fresh 1.5 ml eppendorf tube, then the phenol chloroform treatment and centrifugation repeated . After transferring the supernatant into a fresh eppendorf tube 1/40 volume of 5M NaCl was added, the mixture was incubated on ice for 10 minutes, and subsequently centrifuged. The supernatant was transferred into a fresh 1.5 ml eppendorf tube. To precipitate the DNA, 1/50 volume of 5 M NaCl plus 2.5 volumes of absolute ethanol was added, and the mixture incubated at -70°C for one hour. After centrifugation for 20 minutes, DNA pellet was washed twice with 70% ethanol, air dried and redissolved

in 50 μl of TE buffer.

2.5.7. Southern Blot Analysis

To detect the inactivated rRNA operon of *M.smegmatis* transformants, extracted genomic DNA of the sample colonies and positive controls (ko10 and ko14 *M. smegmatis* knock out strains) were digested with 10 units of Sma I (Pharmacia Biotech), which cuts the genomic DNA at several sites. Digestion products were loaded on 1.5 % agarose gel in parallel with the 1Kb DNA marker. After electrophoresis the gel was immersed in 0.25 M HCl for 20 minutes, then, twice for 20 minutes in a denaturation solution that contained 1.5 M NaCl and 0.5 M NaoH and neutralized by immersing twice for 20 minutes in a solution that consists of 0.3 M NaCl, 0.03 M Na_3-citrate and 0.5 M Tris-HCl (pH 7.5). The southern blot was set up according to Sambrook *et al*, (1982), and digested DNA fragments from the gel were transferred to the nitrocellulose filter paper. Stack of glass plate was wrapped with Whatman 3mm paper (Whatman International Ltd. Maidstone, England), the wrapped support was placed inside a large backing dish filled with 20x SSC (0.3 M NaCl and 0.03 M Na_3-citrate, pH 7.0). The gel was placed on the top of the Whatman paper in an inverted position (under side became upper most), then air bubbles removed in between the Whatman and the gel. Nitrocellulose filter paper (Schleicher and Schuell, Filtration life Science, Germany) about 1-2 mm larger than the gel in both dimensions was cut and wetted completely by immersing on the surface of the 20x SSC solution. The wet nitrocellulose filter paper

was placed on the top of the gel, with out creating any air bubbles. Four pieces of Whatman papers, with the same size as the gel was immersed in the 20x SSC solution and placed on the top of the nitrocellulose filter paper. Stack of paper towels having 5-8 cm high was placed on the top of 3 mm paper, then glass plate and a considerable weight were added on the top of the paper towels respectively. The setup allows a flow of liquid from the reservoir through the gel and the nitrocellulose filter paper so that DNA fragments are eluted from the gel and are deposited on to the nitrocellulose filter paper. The relative positions of the DNA fragments in the gel are preserved during their transfer to the filter paper. The filter paper was dried at room temprature on a sheet of 3MM whatman paper, and then placed between two sheets of the whatman papers and baked for 2 hrs at 80°C under vacuum. The DNA attached to the filter is then hybridized to p^{32}-labelled DNA probe, and autoradiography was used to locate the position of any bands complementary to the radioactive labelled probe. For the hybridization process of the southern blot analysis, rRNA operon probes were generated by PCR amplification of the genomic DNA of the wild type *M. smegmatis* mc^2 155. Primers were selected to amplify the 5' end of the rRNA operon, approximately 1.2 Kb (primer 283, 5'GAG TTT GAT CCT GGC TCA GGA 3', and primer 264, 5' TGC ACA CAG GCC ACA AGG GA 3'). PCR products were precipitated by adding NaCl to 0.1 M and 0.7 volume of isopropanol and incubating at room temperature for 30 minutes. After 20 minutes centrifugation at 13000 rpm the supernatant was discarded , the DNA pellet washed with 300μl of 70 % ethanol, centrifuged, air dried and resuspended with 50 μl autoclaved water. To obtain the 800 bp fragment of the rRNA

operon, 24 μl of the PCR product was digested with 60 units of BamH I restriction enzyme in a total volume of 30 μl digestion reaction. The digestion product was loaded on 0.7% agarose gel for electrophoresis, and the 800bp band was excised and gene cleaned.

Hybridization of southern filter was conducted following all the procedures indicated in Sambrook, (1982). The baked filter paper was immersed in the 6x SSC solution for 2 minutes and slipped into a heat sealable plastic bag. Prehybridization fluid (containing 6X SSC; 0.5% SDS; 5X Denhardt's solution; and 100μg/ml denatured, Salmon sperm DNA) warmed to 68°C was added about 0.2 ml for each square centimeter of the nitrocellulose filter paper. After squeezing as much air as possible, the open bag was sealed with heat sealer and incubated in a water bath at 68°C for 3 hrs. The bag was removed from the water bath and opened by cutting off one corner with scissors, prehybridization solution was discarded. Using a pasteur pipette, the hybridization solution(containing 6X SSC; 0.01 M EDTA; P^{32} labelled denatured probe DNA; 5X Denhardt's solution; 0.5 %SDS; and 100 μg/ml denatured, Salmon sperm DNA) was added into the bag. By squeezing the bag much of the air was removed and the cut edge of the bag was sealed with heat sealer leaving as few trapped air bubbles as possible. The bag was incubated in a water bath at 68°C for overnight. The bag was removed from the water bath and cut quickly along the length of three sides. Using gloves, the filter paper was removed and immediately submerged in a tray containing a solution of 2X SSC and 0.5 % SDS at room temprature. After 5 minutes the filter paper was transferred into a fresh tray containing a solution of 2X SSC and 0.1 % SDS,

and incubated for 15 minutes at room temprature with occasional gentle agitation. The filter paper was transferred into a flat-bottomed plastic box containing a solution of 0.1X SSC and 0.5 % SDS, and then incubated at 68°C for 2 hrs with gentle agitation. The washing process was carried out under less stringent conditions. Finally the filter paper was dried at room temprature on a sheet of whatman 3MM paper and wrapped in saran wrap, and apply to X-ray film to obtain an autoradiographic image.

2.6. GENERATION OF STREPTOMYCIN RESISTANT MUTANTS IN M. SMEGMATIS MC^2 155 STRAINS WITH ONE FUNCTIONAL rRNA OPERON

In streptomycin resistant *M. tuberculosis* mutants, approximately 30% of the strains showed mutations in the rrs gene, whereas, the other strains had mutations in the rpsL gene. The proportion of streptomycin resistant *rrs* mutants could be dramatically increased when a single rRNA allelic *M. smegmatis* are transformed with an additional wild type *rpsL* gene.

For the generation of streptomycin resistant mutants from the *M. smegmatis* strains with one inactivated rRNA operon plasmid ptrpA-1-rpsL was used as a source of the wild type *rpsL* gene (Fig. 10). Plasmid ptrpA-1-rpsL, which was constructed at MHH, contains *M.bovis rpsL* gene. To get the 1kb *rpsL* containing fragment the plasmid was digested with EcoR V and Xba I restriction enzymes. The fragment was treated as insert to ligate into the pHINT 1 plasmid (Fig. 11). The 6,23 kb pHINT 1

plasmid was linearized with Xho I restriction enzyme and treated as a vector to construct plasmid pHINT-rpsL. After ligation, *E. coli* strains (XL1-Blue-MRF) competent cells were transformed with the constructed plasmid. Colonies were selected on LB-plates containing 80 μg/ml ampicillin. Random colonies were selected and cultured in 5 ml LB-80 μg/ml ampicillin, 5ml LB-100 μg/ml hygromycin and 5 ml LB-80 μg/ml ampicillin + 100 μg/ml hygromycin broth medium. After 24 hrs of incubation at 37°C, 1 ml of the culture was added into 150 ml of the respective medium. Plasmid DNA was extracted using QIAGEN Tip 100 (QIA GEN 100).

Competent cells were prepared both from the one rRNA operon inactivated and the wild type *M. smegmatis* mc^2 155. The competent cells transformed with the extracted plasmid DNA. Transformants were selected on LB-plates containing 50 μg/ml hygromycin. Random colonies were picked from the plate and amplified in 20 ml BHI-Tween medium by incubating for 2-3 days at 37°C and 250 rpm. Streptomycin resistant transformants were screened by spreading 300μl of the culture on LB-plates containing 20μg/ml streptomycin. Random colonies were picked and amplified on LB-plates containing 20μg/ml streptomycin.

From the amplified colonies genomic DNA will be extracted following the SDS/Proteinase K procedure. Genomic DNA extract will be sequenced for *rrs*, *rpsL* and other genes in the rRNA operon.

3. RESULTS

3.1. MIC VALUE OF *M. tuberculosis* STRAINS

There were 44 strains subjected to the determination of the MIC value. Based on classification of Cole, (1994) and Morris *et al.* (1995) susceptible strains were grouped as those only growing on 7H1O plates at a streptomycin concentration of < 5 μg/ml. Low level resistant strains those growing on 5-10 μg/ml streptomycin concentration, as medium level resistant strains those growing at 40 μg/ml streptomycin concentration. High level resistant strains those growing on $\geq$ 160 μg/ml streptomycin concentration. Plates with $< 1\%$ of the colonies growing on the control plate were considered as susceptible to that concentration of drug. Plates with $> 1\%$ of the colonies growing on the control plate were considered as resistant to that concentration of drug. The data collected on the growth condition, susceptibility status, and MIC value of the strains is shown in table 2.

In this study no *M. tuberculosis* strain was found to be within the range of the medium level streptomycin resistant.

Table: 2. Culture status and MIC values of each strain. (Source of *M. tuberculosis* strains: from serial No. 1-22, Ethiopia (Addis Ababa Tuberculosis Demonstration and Training Center): from serial No. 23-44, Sweden (SIID)).

Ser.No	Strain No	Higher strepto. conc.(μg/ml) where growth observed	MIC value of strains	Remarks
1	S:01	5	10	Low level resistant
2	S:03	5	10	Low level resistant
3	S:04	0	< 5	Susceptible
4	S:05	5	10	Low level resistant
5	S:06	-	-	Highly contaminated
6	S:07	0	< 5	Susceptible
7	S:08	640	> 640	High level resistant
8	S:15	10	40	Low level resistant
9	S:36	5	10	Low level resistant
10	S:37	5	10	Low level resistant
11	S:41	0	< 5	Susceptible
12	S:63	10	40	Low level resistant
13	S:66	-	-	No growth on the sub-culture
14	S:74	-	-	No growth on the sub-culture
15	S:83	640	> 640	High level resistant
16	S:85	0	< 5	Susceptible
17	S:106	5	10	Low level resistant
18	S:110	-	-	No growth on the sub-culture
19	S:116	-	-	No growth on the sub-culture
20	S:129	0	< 5	Susceptible
21	S:137	160	640	High level resistant
22	S:141	-	-	No growth on the sub-culture
23	A:41295	0	< 5	Susceptible
24	A:45841	640	> 640	High level resistant
25	A:46949	5	10	Low level resistant
26	S:90/95	-	-	No growth on the sub-culture
27	S:93/95	-	-	No growth on the sub-culture
28	S:94/95	-	-	No growth on the sub-culture

29	H:87/94	0	< 5	Susceptible
30	H:987/94	0	< 5	Susceptible
31	H:1005/94	0	< 5	Susceptible
32	H:1081/94	-	-	No growth on the sub-culture
33	H:1173/94	10	40	Low level resistant
34	H:1261/94	10	40	Low level resistant
35	E:84/94	640	> 640	High level resistant
36	E:134/94	160	640	High level resistant
37	E:187/94	640	> 640	High level resistant
38	E:200/94	0	< 5	Susceptible
39	P31/94	640	> 640	High level resistant
40	P32/94	640	> 640	High level resistant
41	P2	5	10	Low level resistant
42	P24	640	> 640	High level resistant
43	S:2351	640	> 640	High level resistant
44	9451/83	5	10	Low level resistant

The culture status and MIC values of the *M. tuberculosis* strains can be summarized as follows, based on the origin and number of isolates:-

Ethiopian isolates 3/25 High level resistant

8/25 Low level resistant

5/25 Susceptible to streptomycin

6/25 no growth/ contaminated

Swedish isolates 8/28 High level resistant

5/28 Low level resistant

5/28 Susceptible to streptomycin

4/28 no growth, either in the original sample or after sub-culturing

3.2. PCR AMPLIFICATION

DNA was extracted from each of the high level streptomycin resistant strain, 10 of the 13 low level streptomycin resistant strains, and from the susceptible control strain. The extracted DNA was subjected to PCR amplification to amplify the *rrs* and *rpsL* genes for sequence analysis. In the PCR amplification of the entire *rpsL* gene (375 bp) using S12-3 and S12-4 primers, 9 of the 11 high level resistant strains gave a PCR product (Fig. 2). All the tested 10 low level resistance strains gave an amplification product for the *rpsL* gene (Fig. 2).

For the PCR amplification of about 650 bp of the *rrs* gene(which includes the known areas of mutation) using 16S-1 and 16S-4 primers, all the high level streptomycin resistant, 10 of the amplified 10 low level resistant and the susceptible control *M. tuberculosis* strains gave a PCR product of the correct size which could then be sequenced (Fig. 3).

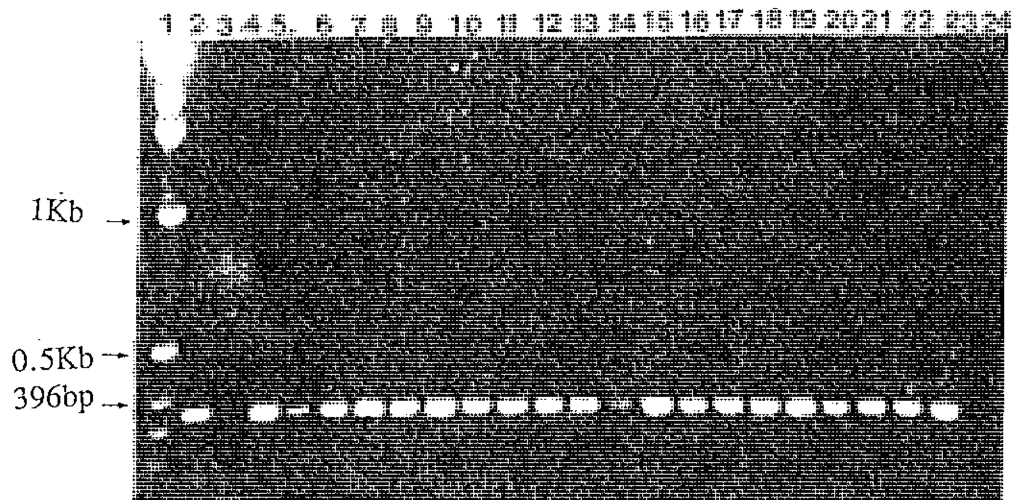


Fig. 2. PCR amplification of the entire *rpsL* of the high level and low level streptomycin resistant strains and the susceptible control strain of *M. tuberculosis* using S12-3 + S12-4 primers. **High level resistant strains**, Lane 1: 1kb DNA Marker; Lane 2. S:08; Lane 3. S:0.83; Lane 4. S:137; Lane 5. A:45841; Lane 6. E:84/94; Lane 7. E:134/94; Lane 8. P31/94; Lane 9. P32/94; Lane 10. P24; Lane 11. S:2351; Lane 12. E:187; **Low level resistant strains**, Lane 13. S:01; Lane 14. S:05; Lane 15. S:15; Lane 16. S:36; Lane 17. S:37; Lane 18. S:63; Lane 19. A:46949; Lane 20. H:1173/94; Lane 21. H:1261/94; Lane 22. P2; Lane 23. S:29 (positive control); Lane 24. Negative control.

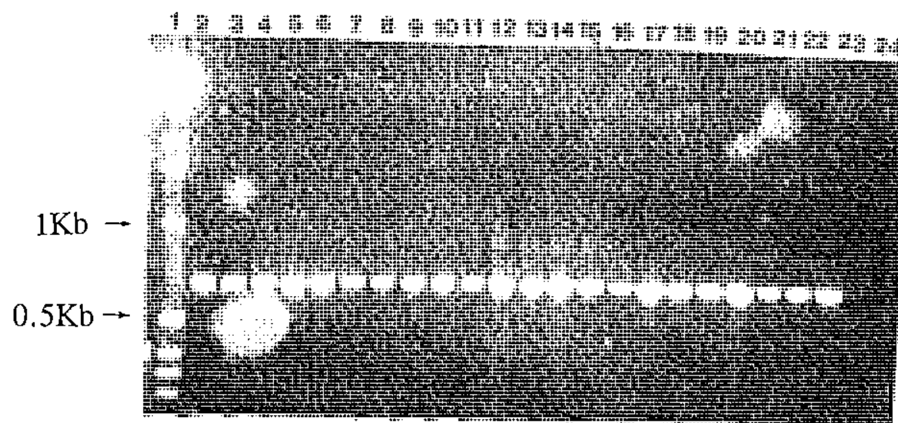


Fig. 3. PCR amplified products of the 650 bp fragment of *rrs* gene from the high level , low level streptomycin resistant and the susceptible control strains of *M. tuberculosis* after amplification using 16S-1 + 16S-4 primers. **High level resistant strains,** Lane 1. 1kb DNA Marker; Lane 2. S:08; Lane 3. S:0.83; Lane 4. S:137; Lane 5. A:45841; Lane 6. E:84/94; Lane 7. E:134/94; Lane 8. P31/94; Lane 9. P32/94; Lane 10. P24; Lane 11. S:2351; Lane 12. E:187. **Low level resistant strains,** Lane 13. S:01; Lane 14. S:05; Lane 15. S:15; Lane 16. S:36; Lane 17. S:37; Lane 18. S:63; Lane 19. A:46949; Lane 20. H:1173/94; Lane 21. H:1261/94; Lane 22. P2; Lane 23. S:29 (Positive control); Lane 24. Negative control.

3.3. SEQUENCE OF *rpsL* AND *rrs* GENES

In wild type strains the nucleotide sequence of the *rpsL* gene at the site of codon 43 is TTC, whereas in 9 of the 10 high level resistant strains it was found to be TCC (Fig. 4). This condition results in a change of amino acid from Lysine to Arginine. Next to the site of codon 43 of the *rpsL* gene there was a compressed area of sequence. However, sequencing of the gene in the opposite direction using primer S12-3 was done both manually at AHRI and at MHH, Germany using an Applied. Biosystem 373 DNA automatic sequencer, randomly on 3 of the 9 mutant strains, demonstrated that the sequence of this compressed area was the wild type sequence (a T rich). Sequencing of the *rpsL* gene for observation of codon 88 was conducted for strains S:137, E:187/94 S:2351, E:134/94 and the susceptible control S:29 at MHH, Germany. For this sequencing reaction the forward primer S12-1 was used. Strain S:137 appeared to be mutant for the nucleotide sequence of the *rpsL* gene at the site of codon 88 (Fig. 5) In some of the high level resistant strains there was a substitution of one nucleotide by another nucleotide, Guanine by Thymine at codon 56, Guanine by Adenine at codon 58 and codon 96 of the *rpsL* gene. These mutations are merely a silent mutations which do not result in a change of amino acids.

All sequence analyzed low level streptomycin resistant strains of *M. tuberculosis* were found to be wild type for their *rrs* gene.

Table: 3. Results of the sequencing of the *rpsL* gene giving special attention to codons 43 and 88 of the high level and low level streptomycin resistant and the susceptible strains of *M.tuberculosis*.

Ser. No.	Strain No.	Sequencing Result	codon changes
1	S:08	Mutant at codon 43	TTC → TCC
2	S:83	Not sequenced- no PCR product obtained	
3	S:137	Mutant at codon 88, wild type codon 43	TTC → TCC
4	A:45841	Mutant at codon 43	TTC → TCC
5	E:84/94	Mutant at codon 43	TTC → TCC
6	E:134/94	Mutant at codon 43, wild type codon 88	TTC → TCC
7	P31/94	Mutant at codon 43	TTC → TCC
8	P32/94	Mutant at codon 43	TTC → TCC
9	P24	Mutant at codon 43	TTC → TCC
10	S:2351	Mutant at codon 43, wild type codon 88	TTC → TCC
11	E:187/94	Mutant at codon 43, wild type codon 88	TTC → TCC
12	S:29	Wild type codon 43 and codon 88	No change, TTC
13	S:01	Wild type codon 43 and codon 88	No change, TTC
14	S:15	Wild type codon 43 and codon 88	No change, TTC
15	S:37	Wild type codon 43 and codon 88	No change, TTC
16	A:46949	Wild type codon 43 and codon 88	No change, TTC
17	H:1173/94	Wild type codon 43 and codon 88	No change, TTC
18	H:1261/94	Wild type codon 43 and codon 88	No change, TTC

NB From serial number 1-11 are high level streptomycin resistant.

12 is the streptomycin susceptible strain

13-18 are the Low level streptomycin resistant strains

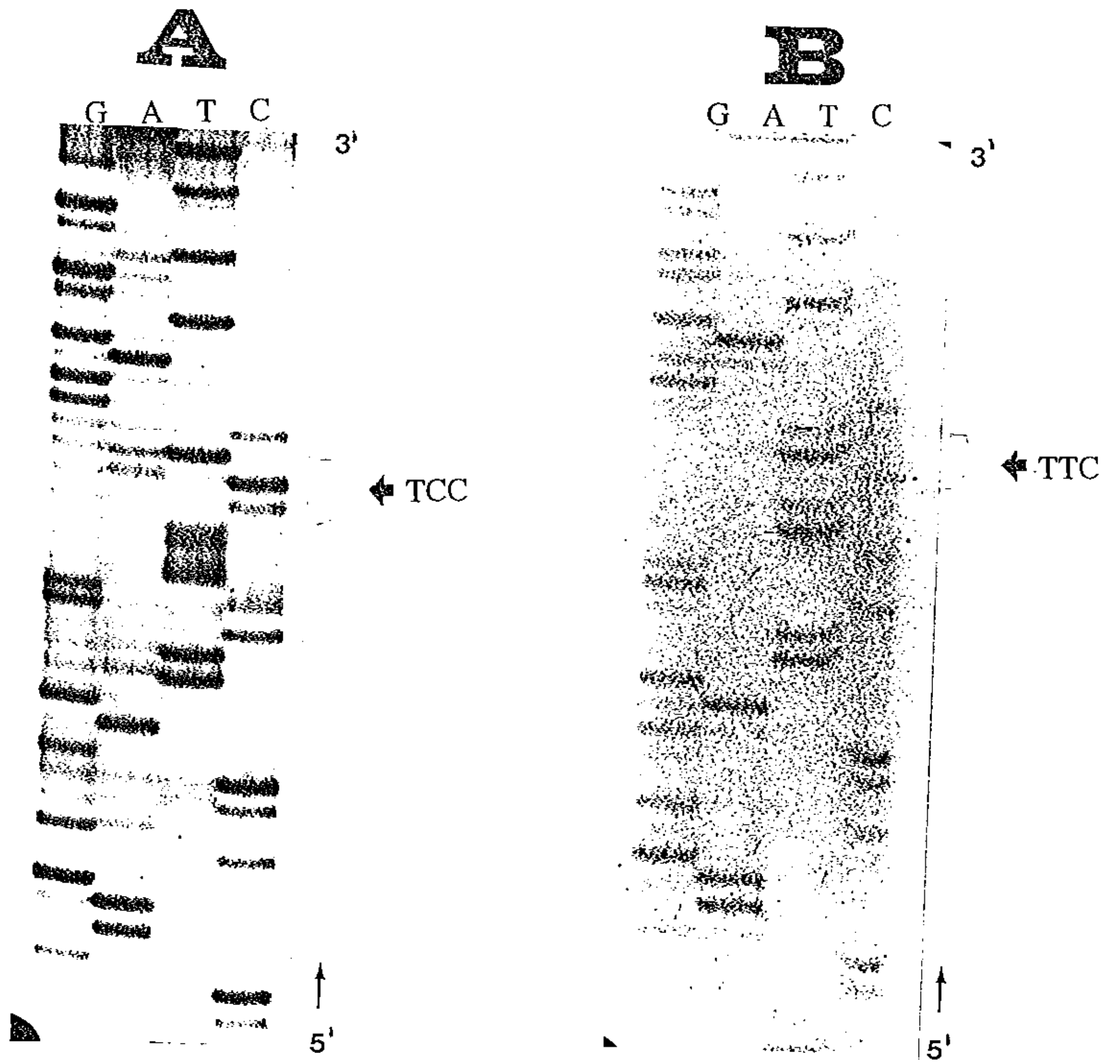


Fig. 4. An example of *rpsL* gene Radioactive sequence result of the *M. tuberculosis* strains. A. high level streptomycin resistant mutated at codon 43. B.Wild type codon 43.

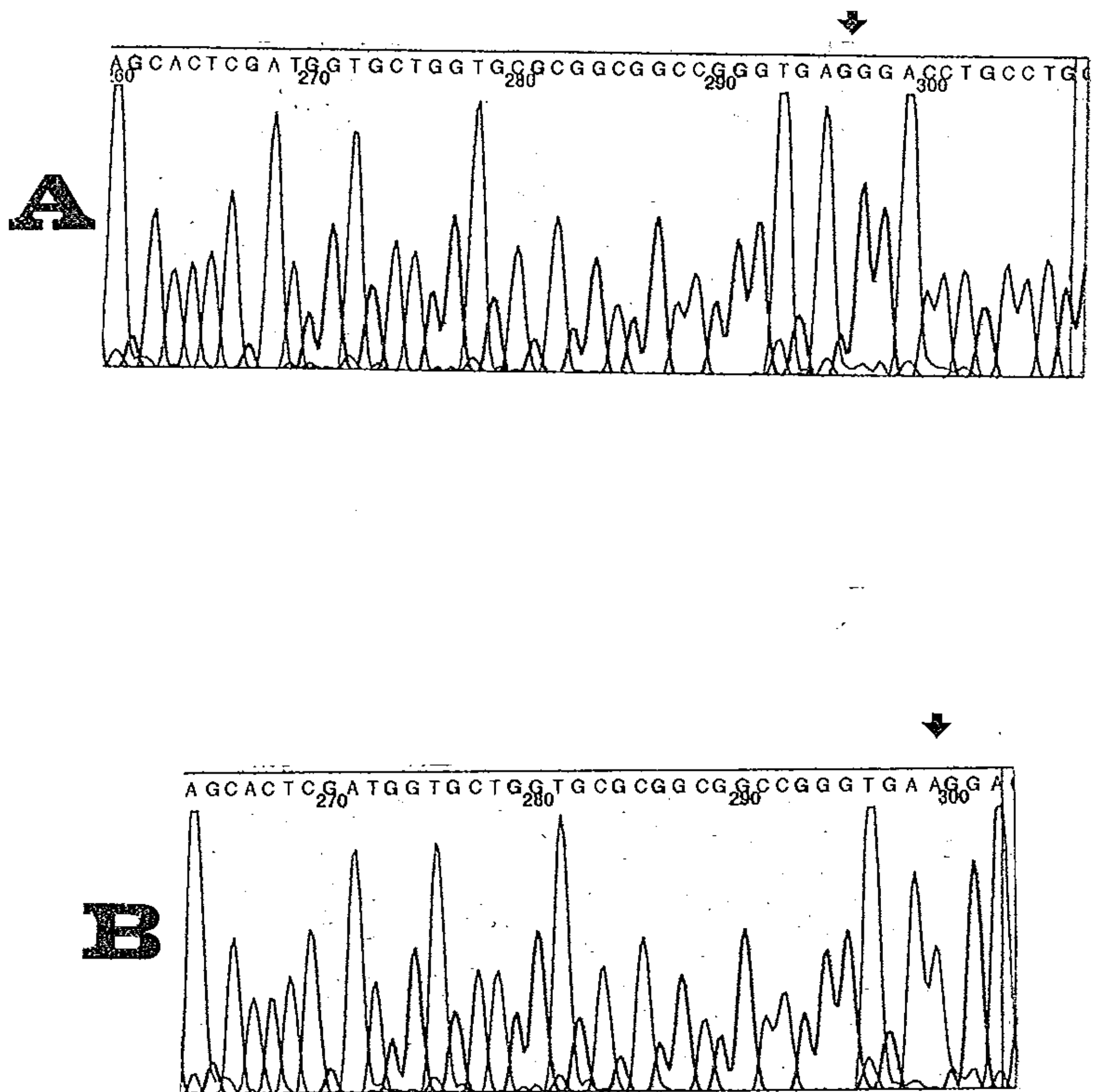


Fig. 5. An example of *rpsL* gene Non-Radioactive sequence result of the *M. tuberculosis*, using primer S12-1. A. High level streptomycin resistant strain mutated at codon 88. B. Wild type codon 88.

Table: 4. Comparison of the MIC value and observed mutation of the high level streptomycin resistant *M. tuberculosis* strains.

Ser. No	Strain No.	MIC value	Mutational site	Codon Change
1	S:08	>640 μg/ml	Codon 43	TTC---→TCC
2	S:137	640 μg/ml	Codon 88	TTC---→TCC
3	A:45841	>640 μg/ml	Codon 43	TTC---→TCC
4	E:84/94	>640 μg/ml	Codon 43	TTC---→TCC
5	E:134/94	640 μg/ml	Codon 43	TTC---→TCC
6	P31/94	>640 μg/ml	Codon 43	TTC---→TCC
7	P32/94	>640 μg/ml	Codon 43	TTC---→TCC
8	P24	>640 μg/ml	Codon 43	TTC---→TCC
9	S:2351	>640 μg/ml	Codon 43	TTC---→TCC
10	E:187/94	>640 μg/ml	Codon 43	TTC---→TCC

Out of the 11 high level streptomycin resistant strains 10 strains were analyzed by sequencing the *rpsL* gene and 10 of them had the documented mutations at the site of amino acid 43 encoding gene or at the site of amino acid 88 encoding gene (Table. 3). Six strains of the low level streptomycin resistant and the susceptible control strain were also sequence analyzed for *rpsL* and the *rrs* genes and they appeared to be wild type.

3.4. INACTIVATION OF ONE rRNA OPERON OF M. SMEGMATIS MC^2 155

Screening for the correct construct, pRRNA4-4-K1 containing the *sacB* insert was done by transforming *E.coli* strain (XL1-Blue-MRF) with the ligation reaction products. There were a total of 28 colonies on the kanamycin 50 μg/ml containing LB plates. 16 colonies, including 4 colonies from the control plates (transformed with pRRNA4-4-K1), were selected randomly and cultured in 5ml LB broth containing 50 μg/ml kanamycin. All colonies were tested for their sucrose sensitivity by streaking on sucrose (7.5%)-containing LB-kanamycin 50 μg/ml plates. Colonies number 3 and 7 out of the 28 sample colonies were found to be sensitive to a sucrose concentration of 7.5 %. *M. smegmatis* mc^2 155 were transformed with plasmid DNA extracts, pRRNA4-4K1-*SacB* (Fig. 8) from these sucrose sensitive colonies of *E. coli* transformants.

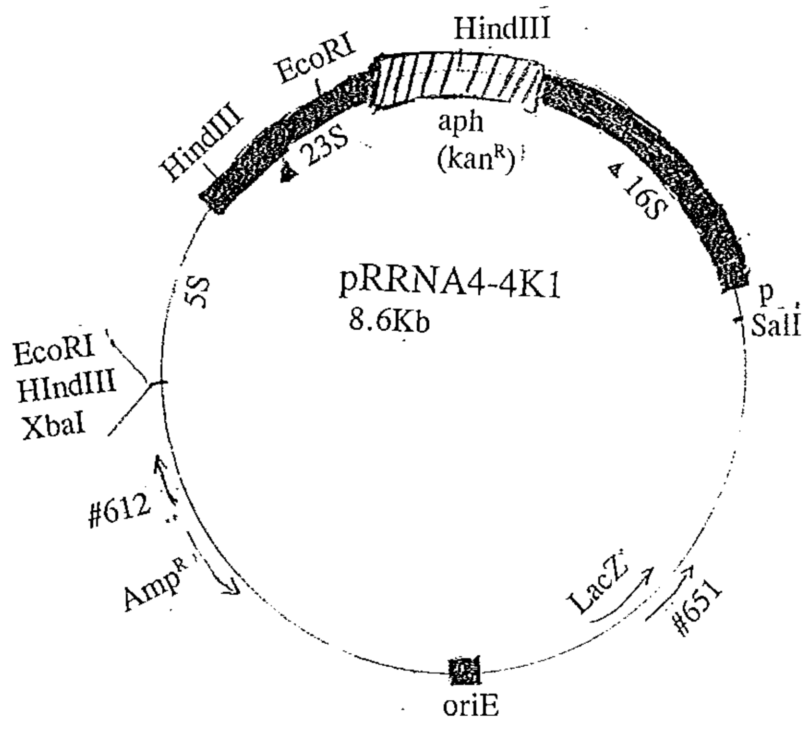


Fig. 6. Plasmid pRRNA4-4K1

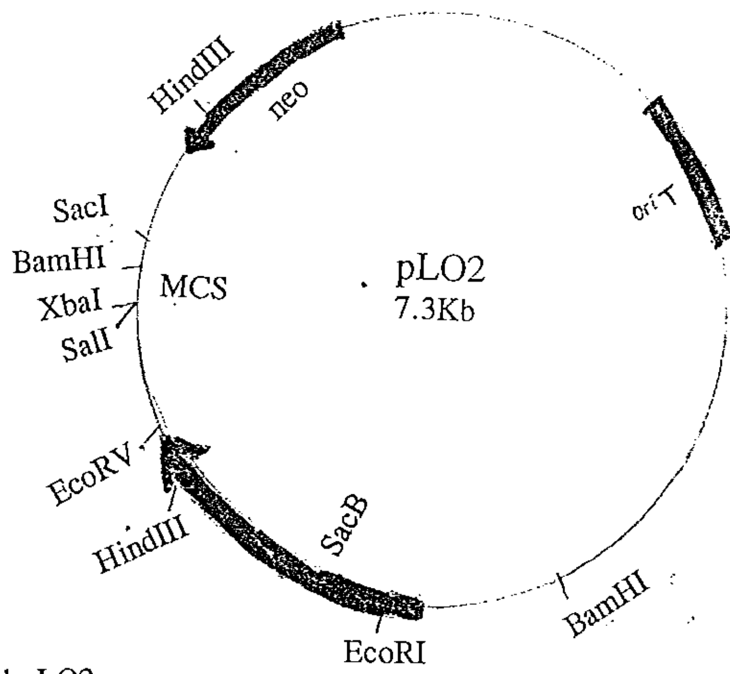
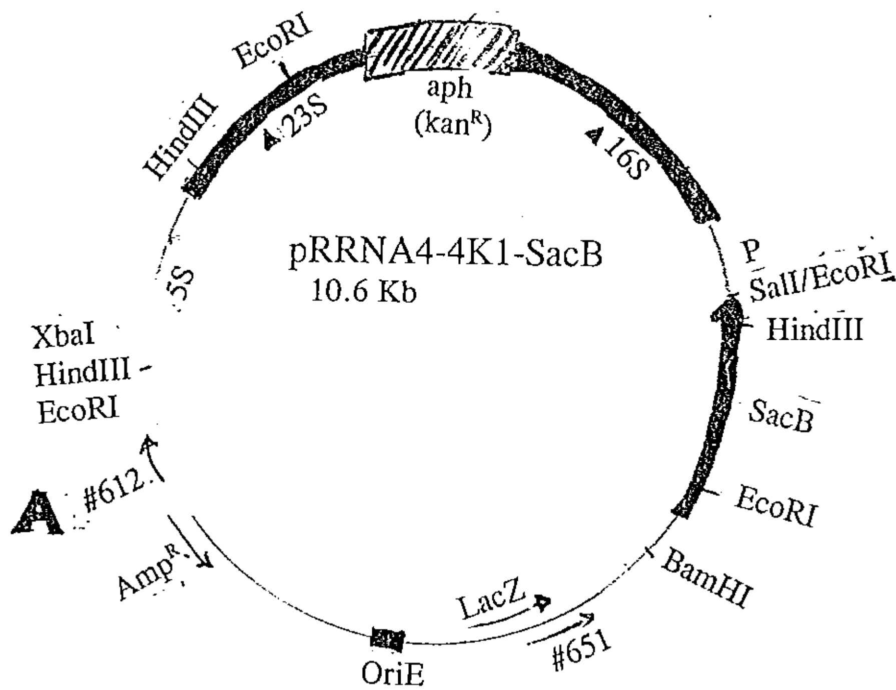


Fig. 7. Plasmid pLO2



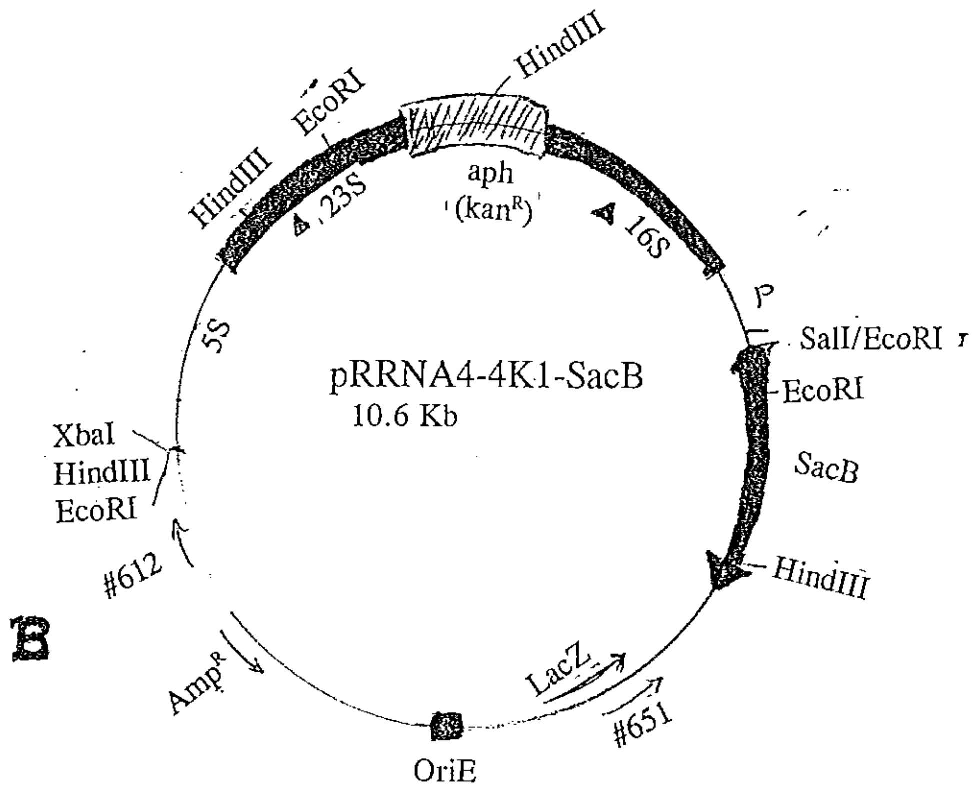


Fig. 8. Plasmid pRRNA4-4K1-SacB

A. Extracted from *E. coli* transformants, colony 3: pRRNA4-4K1-SacB C-3

B. extracted from *E. coli* transformants, colony 7: pRRNA4-4K1-SacB C-7

Table: 5. Number of colonies obtained on different plates after transformation of *M. smegmatis* mc[2] 155 with plasmid DNA extracted from the *E. coli* colonies containing the *sacB* gene.

Plasmid DNA	Number of colonies on LB plates containing			
	kan 25 μg/ml	kan 25 μg/ml + different sucrose conc.		
		10 %	15%	25%
pRRNA4-4K1-SacB C-3	61	45	45	27
pRRNA4-4K1-SacB C-7	58	2	1	1

NB. kan=kanamycin

From the *M. smegmatis* competent cells transformed with colony 3 and colony 7 derived plasmid DNA, colonies were obtained on the respective plates (Table. 5).

To select colonies of *M. smegmatis* in which the interrupted rRNA operon of the plasmid had replaced one of the rRNA operon, southern blot analysis was done. After southern blot analysis 3 of the strains showed a shift in bands as compared to the wild type negative control *M. smegmatis* mc^2 155 and the positive controls used (Fig. 9). A shift of one band in the single allelic rRNA mutants, compared to that of the wild type *M. smegmatis* (wt) and with single allelic mutant (KO10 and KO14) positive controls, had replaced one of the genomic rRNA operons.

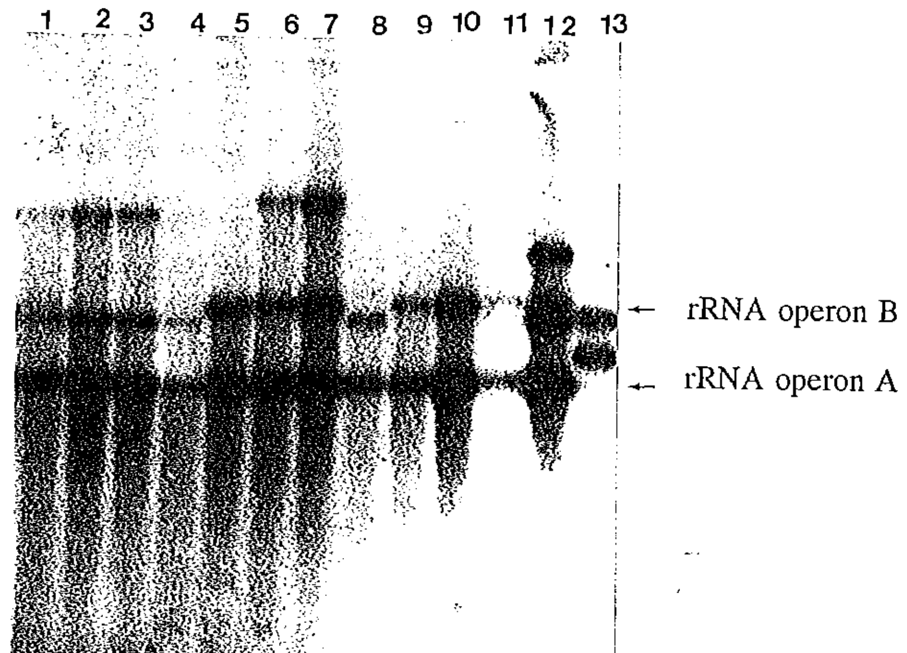


Fig. 9. Southern blot analysis of the *M.smegmatis* transformants. The two bands correspond to the two rRNA operons. **from colonies transformed with Plasmid (pRRNA4-4k1-SacB) extracted from *E. coli* colony 3**. Lane 1. from kan 25μg/ml plate; Lane 2-4. from kan 25 μg/ml+ Sucrose 15% plate; **Control**, Lane 5. from *M. semegmatis* K010; **From colonies transformed with plasmid (pRRNA4-4k1-SacB)extracted from *E. coli* colony 7**. Lane 6-7. from kan 25μg/ml plate, Lane 9-10. from kan 25 μg/ml + Sucrose 10% plate, Lane 11. from kan 25 μg/ml + Sucrose 15% plate, Lane 12. from kan 25 μg/ml + Sucrose 25% plate; **controls**. Lane 8. from *M. smegmatis* (wt); Lane 13. from *M. smegmatis* K014.

3.5. STREPTOMYCIN RESISTANT MUTANTS OF M. SMEGMATIS WITH ONE FUNCTIONAL rRNA OPERON

A total of 36 colonies of *E. coli*, transformed with ligation products, were picked from 80 μg/ml ampicillin containing LB plates and 12 colonies each were cultured in LB broth media containing ampicillin, hygromycin and ampicillin + hygromycin respectively. 8 colonies which were cultured in 80 μg/ml ampicillin containing LB-medium and 3 colonies from 100 μg/ml hygromycin containing LB-medium showed good growth. Two out of the 8 colonies and one from the three colonies were randomly selected for plasmid DNA extraction. The constructed plasmid, pHINT-rpsL (Fig. 12) has *E. coli* origin of replication. It has also the *int* gene which would allow the plasmid to integrate in the *M. smegmatis* genome. Eighteen colonies from the transformed *M. smegmatis* strain with one rRNA operon inactivated and 9 colonies form the transformed wild type were selected randomly and cultured in 3 ml of BHI-Tween medium. For amplification in 25 ml BHI medium 6 out of the 18 cultures and three of the 9 cultures were randomly selected. For screening streptomycin resistant mutants, more than thousand colonies per plate were obtained.

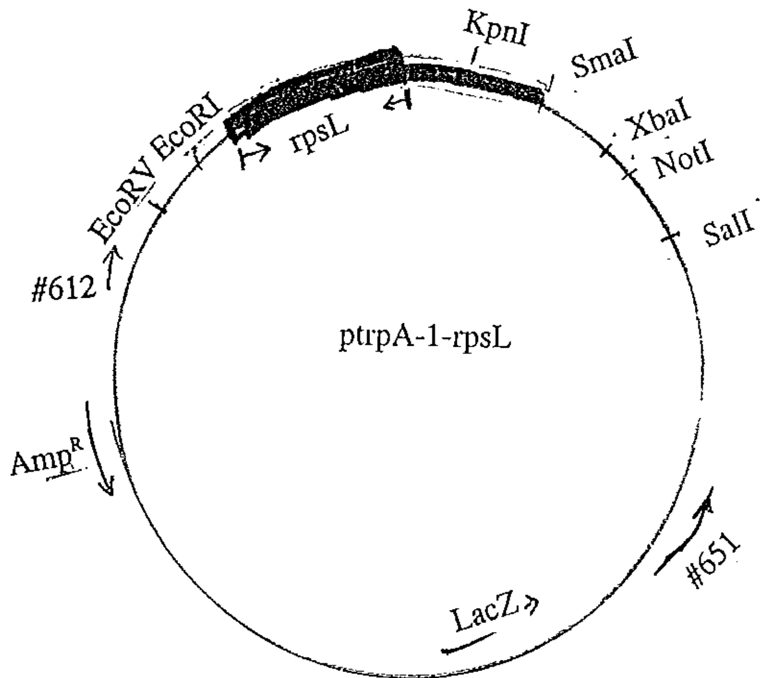


Fig. 10. Plasmid ptrpA-1-rpsL

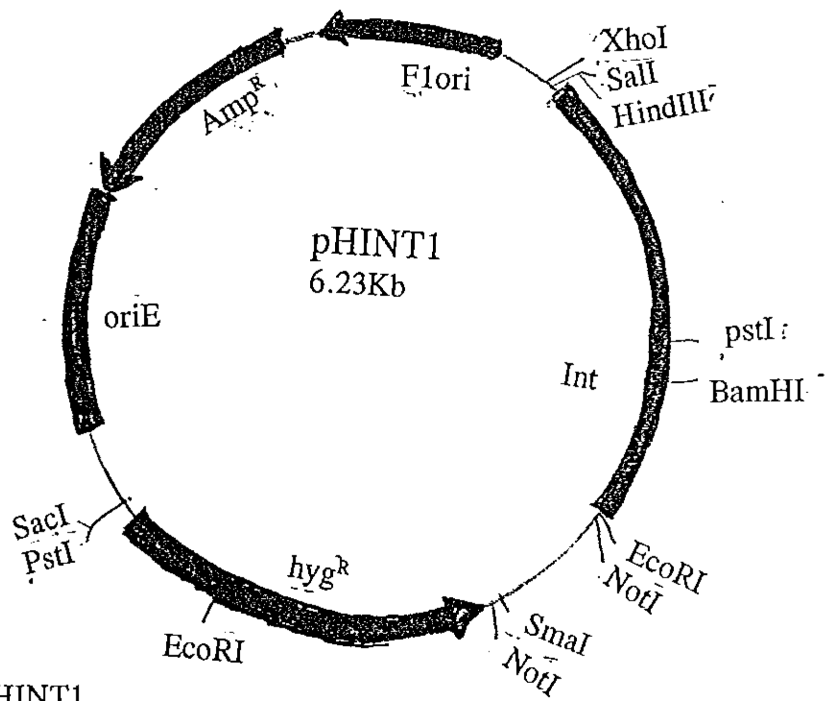


Fig. 11. Plasmid pHINT1

hyg^R= Hygromycin resistance marker

Amp^R= ampicillin resistance marker

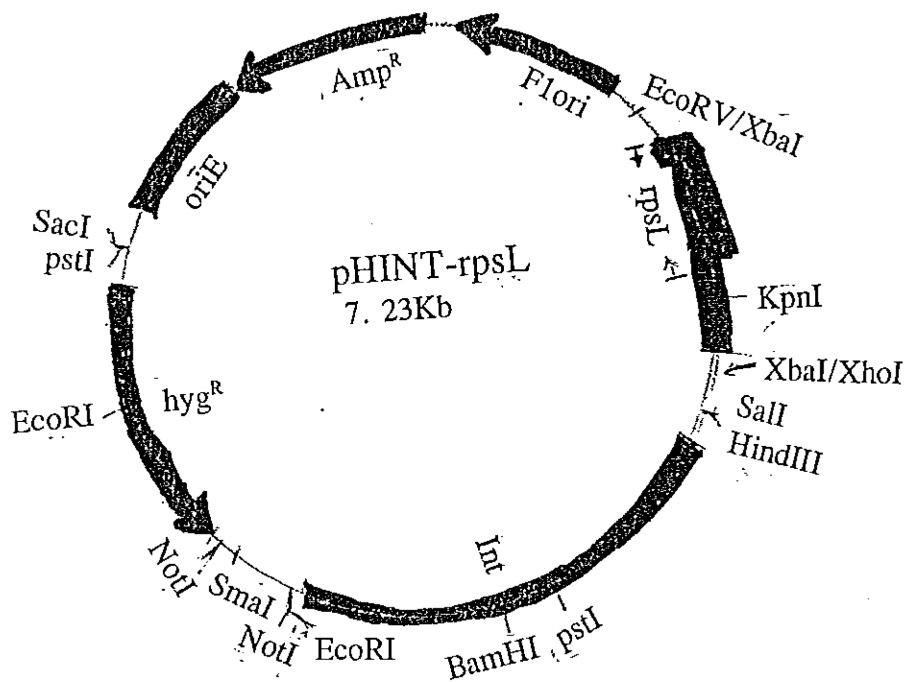


Fig. 12. Plasmid pHINT-rpsL

4. DISCUSSION

4.1. MIC VALUE DETERMINATION

In this study, for 34 viable *M. tuberculosis* strains, which had previously been shown to exhibit at least a low level of resistance to streptomycin, the MIC value was determined. Compared with previous studies, the present results showed a high proportion of low level streptomycin resistant strains than expected. In previous studies, out of the total 19 streptomycin resistant *M. tuberculosis* strains examined by Meier *et al.* (1996), 5 of the strains were low level resistant; Finken *et al.* (1993), had reported 4 low level resistance out of the total 15 streptomycin resistant strains. Sreevatsan *et al.* (1996) in their characterization of *rpsL* and *rrs* mutations in streptomycin resistant *M. tuberculosis* isolates, reported 25 low level resistant strains out of the total 78 streptomycin resistant strains. This high proportion of low level resistant could be a real phenomenon or it could be the result of repeated sub-culturing in the absence of antibiotics. Strains may lose their resistance as a result of frequent sub-culturing, since there is no longer any selection pressure to maintain the mutation responsible for streptomycin resistance within the bacterial population. Without the selection pressure which could be induced by the antibiotics the mutation may leave the bacteria at a competitive disadvantage so that they will be outgrown by the wild type.

In this study some of the *M. tuberculosis* strains were found to be susceptible to streptomycin although they were characterized for resistance to streptomycin. This

phenomenon could happened as a result of the lower streptomycin concentration (5μg/ml) used in this experiment, whereas resistant character of the strains to streptomycin was previously determined in a concentration of 4μg/ml. Also, due to lack of a deep freezer, the Ethiopian *M. tuberculosis* isolates which had been repeatedly sub-cultured, were not in good condition and may have lost some of their original resistance characteristics.

4.2. MUTATIONAL STATUS OF THE STREPTOMYCIN RESISTANT M. TUBERCULOSIS STRAINS

The proportion of high level resistant strains with mutations of codon 43 and codon 88 of the *rpsL* gene, is comparable to the result of other workers. The result obtained by Sreevatsan *et al.* (1996) out of the total 78 streptomycin resistant *M. tuberculosis* strains, 42 strains had mutations at the *rpsL* gene. Out of the 19 streptomycin resistant strains 8 strains were with altered *rpsL* (4 strains each of codon 43 and codon 88) (Meier *et al.*, 1996). According to Heym *et al.* (1996); Meier *et al.* (1996), strains with high level of streptomycin resistance phenotype were those strains with an altered *rpsL* gene. In this study mutations at codon 43 of the *rpsL* gene was found on those *M. tuberculosis* strains which were resistant to streptomycin at higher concentration (>640 μg/ml). One high level resistant strain (S:0.83) was found not to give a PCR product for the *rpsL* gene although the same DNA could be amplified for

the *rrs* gene. This difference in PCR results could possibly have been due to some contaminants within the extracted DNA. If the DNA extract contain contaminants, it could interfere with annealing of certain primers but not others. The presence of mutation close to the 3' end of the primers could also affect annealing of the primer. These are the most likely explanation, since it is highly improbable that the *rpsL* gene would have been deleted, because it is considered crucial for survival. Sequence analysis of *rrs* gene of strain S:0.83 was attempted, but it was not clear enough to decide.

Six of the sequence analyzed low level streptomycin resistant strains were wild type for the *rpsL* and *rrs* genes. This observation suggests that there may be additional mechanisms mediating streptomycin resistance. Since the low level resistant strains did not show any of the documented mutations, it is likely that they might have mutations in other ribosomal genes. The best way to explore this possibility is by manipulating the mycobacterial relative *M. smegmatis*.

4.3. GENERATION OF M. SMEGMATIS STRAINS WITH ONE FUNCTIONAL rRNA OPERON

After transformation, the *sacB* containing pRRNA4-4-K1 plasmid was expected to replicate in *E. coli*, since the plasmid contained an *E. coli* origin of replication. This replicative possibility allows to harvest perfectly ligated construct containing both the insert and vector DNA. The expression of the plasmid construct can easily be detected by the sucrose sensitivity and kanamycin resistance of the transformed *E. coli* strain.

The plasmid will not replicate in mycobacteria, because it lacks a mycobacterial origin of replication, but it will integrate into the genomic DNA of the *M. smegmatis* transformants. In the process of homologous recombination within the *M. smegmatis* cell, there could be single or double crossover at the rRNA operon. If homologous recombination had resulted from double crossover, the replacement of one of the rRNA operons of the *M. smegmatis* by the homologous plasmid derived inactivated rRNA operon would be likely to happen. *M. smegmatis* transformants which undergo double crossover can be selected by kanamycin and sucrose resistance. Phenotypically, *M. smegmatis* transformants resulted from single crossover were identified by their kanamycin resistance but sensitive to sucrose. In this study, the tendency of decreasing colony number with increasing sucrose concentration was clearly seen. This could happen as a result of the presence of double and single crossover *M. smegmatis* transformants within the mixed population.

Replacement of one rRNA operon of *M. smegmatis* by the inactivated plasmid derived rRNA operon can be revealed by southern blot analysis. Since the plasmid derived operon contains the *aph* cassette for inactivation, this cassette would result in increasing the molecular weight as compared to the wild type operon. Change of molecular weight within the rRNA operon can be detected by a shift of bands in the southern blot. The phenomenon of single crossover was also clearly seen on the southern blot, characterized by the appearance of 3 bands. According to Sander *et al.* (1996), two of the rRNA operons were identified as lower band for operon A and the upper band for rRNA operon B.

4.4. STREPTOMYCIN RESISTANT MUTANTS OF M. SMEGMATIS WITH ONE FUNCTIONAL rRNA OPERON

Wild type *M. smegmatis* strains have one *rpsL* gene and 2 rRNA operons. Mostly, the mutation that confer streptomycin resistance occurs in the *rpsL* gene. One rRNA operon inactivated *M. smegmatis* strains has one *rpsL* and one functional rRNA operon. In *M. smegmatis* strains with only one functional rRNA operon, the mutations that lead to streptomycin resistance are expected to take place approximately equally in the *rrs* and *rpsL* genes.

When the wild type *rpsL* gene containing plasmid, pHINT-rpsL is introduced into the *M. smegmatis* strains with only one functional rRNA operon, high proportion of streptomycin resistant mutants with mutation in the *rrs* or other genes of the rRNA operon would be expected. Sequence results of the *rrs*, *rpsL* and the entire rRNA operon could help to trace the mechanisms of resistance to streptomycin in low level resistant *M. tuberculosis*.

5. CONCLUSION AND RECOMMENDATIONS

Identification of the molecular mechanisms underlying drug resistance may offer the possibility for rapid recognition of drug-resistant *M. tuberculosis* strains and may allow prompt initiation of effective chemotherapy. Unrecognized drug resistance may increase the risk of transmission both in the community and in the hospital if patients have tuberculosis disease for weeks or months while they receive ineffective therapy. In this study, the mechanism of resistance to streptomycin in the high level resistant *M. tuberculosis* strains was investigated. All the tested high level streptomycin resistant strains showed the previously documented mutations at codon 43 or codon 88 of the *rpsL* gene. The mechanisms of resistance to streptomycin in the low level resistant strains of *M. tuberculosis* remains obscure, because all the tested strains appeared to possess wild type *rpsL* and *rrs* genes. As a first step towards the goal of identification of the mechanisms of resistance to streptomycin in the low level resistant strains of *M. tuberculosis*, a comparative study was demonstrated on *M. smegmatis*. Functional analysis of the rRNA of mc^2 155 *M. smegmatis* strain will give a strong evidence as to how the low level streptomycin resistant *M. tuberculosis* strains developed resistance. *M. smegmatis* strain with only one functional rRNA operon was generated. Introduction of an additional wild type *rpsL* into this *M. smegmatis* (with one rRNA operon knocked out) make the number of *rpsL* genes two. This strategy will help to get streptomycin resistant mutants of *M. smegmatis* with one rRNA inactivated and one additional wild type *rpsL* gene having mutation (mutations) at any point of the rRNA genes.

Streptomycin resistant colonies of these modified *M. smegmatis*, with only one rRNA operon and an additional *rpsL* gene were detected in the streptomycin containing LB plate. However, determination of the MIC value and sequencing of the *rrs, rpsL* and the entire genes of the rRNA operon will need to be determined. This will be necessary to establish the mutation induced. If established the hitherto undocumented mutation will be useful to trace the mechanisms of resistance in low level streptomycin resistance *M. tuberculosis* strains.

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