INVESTIGATIONS ON THE BIOLOGICAL COSTS OF RIFAMPICIN RESISTANCE AND ON THE DEVELOPMENT OF MULTIDRUG-RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

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LIST OF ABBREVIATIONS

ACP – Acyl carrier protein

ADC – Albumin dextrose catalase

AIDS – Acquired Immunedeficiency Syndrome

ATP – Adenosine triphosphate

BCG - bacille Calmette-Guerin

bp –base-pair

CFU – Colony forming units

DNA – Deoxyribonucleic acid

DR – Direct repeat

ETH - Ethionamide

HIV – Human immunodeficiency virus

HPLC – High performance liquid chromatography

hr – hour

INH – Isonicotinic acid hydrazide

L - liter

LJ – Lowenstein Jensen

LRP - Luciferase reporter mycobacteriophage

MDR-TB – Multidrug-resistant tuberculosis

mg - milligram

MIC – Minimal inhibitory concentration

min – minutes

ml – milliliter

mM - millimolar

NAD – Nicotinamide Adenine Dinucleotide

NADP- Nicotinamide Adenine Dinucleotide Phosphate

NAP - p-nitro-α-acetylamino-β-hydroxy propiophenone

OADC – Oleic-acid albumin dextrose complex

PCR – Polymerase chain reaction

PCR-SSCP - Polymerase chain reaction-single-stranded conformation polymorphism

PGRS – Polymorphic GC-rich sequences

PPD – Purified protein derivative

PZA - Pyrazinamide

RFLP – Restriction fragment length polymorphism

RNA – Ribonucleic acid

rRNA – ribosomal RNA

SDS – Sodium dodecyl sulfate

TB - Tuberculosis

TE – Tris EDTA

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ABSTRACT

The biological cost of rifampicin resistance mutations in isogenic isolates of a *M. tuberculosis* strain and the development of multidrug resistance in serial clinical Beijing isolates were studied. Rifampicin is a major drug used in the treatment of tuberculosis. Increasing rifampicin resistance represents a global clinical problem. Most (about 96%) of the resistance to rifampicin is caused by mutations in a small segment of the *rpoB* gene, which encodes the β-subunit of RNA polymerase. The effect of three different *rpoB* mutations on the fitness of *M. tuberculosis* was examined. Rifampicin-resistant mutatnts were initially isolated from a virulent clinical isolate of *M. tuberculosis* (strain Harlingen) at a mutation frequency of 2.3 x 10^{-8}. Mutations in the *rpoB* gene were identified by genotypic sequencing and the growth rates of three defined mutants were measured by competition with the susceptible parent strain in laboratory medium and by single cultures in a macrophage cell line and in laboratory medium. All mutants exhibited a decrease in growth rate compared with the susceptible parent in all three assays. The relative fitness of the mutants varied between 0.29 and 0.96 depending on the mutant and assay system used. The results revealed that rifampicin resistance is associated with a cost that is conditional. The Beijing isolates analyzed included eight serial isolates from a single patient in a space of nine years. The RFLP and spoligotype patterns of all these isolates were identical. The results from the Beijing isolates showed that Beijing genotype is associated an increased tendency toward the development of multidrug resistance once resistance to a single first-line drug develops.

Chapter 1 Introduction

1.1 A Brief History of Tuberculosis and BCG

Tuberculosis is a disease caused by the bacterium *Mycobacterium tuberculosis* that mainly afflicts the lungs. It now affects every country in every continent with the rate of incidence being highest in certain regions of Asia and Africa. However, until about a century ago, tuberculosis (TB) in these regions was almost unknown or uncommon whereas prior to that the incidence of TB in western Europe and North America was high (Daniel et al, 1994). Moreover, even the causative agent of TB was unknown and was thought to be a bad omen or a form of tumor or abnormal gland.

In 1882, Robert Koch described the tubercle bacillus, *M. tuberculosis* and demonstrated it to be the cause of TB. This changed the widespread misconceptions about TB that had been upheld until then. He also pioneered staining and culture techniques.

The development of the first TB vaccine was rather accidental. Calmette and Guerin developed the first TB vaccine (that is still in use today) by first growing a virulent strain of the bovine tubercle bacillus with ox bile in potato-glycerin culture medium. After 39 passages, they obtained a variant strain with altered colony morphology. Infection of various animals with this strain not only produced no evidence of reversion to virulence but also conferred protection from challenge with virulent strains of bovine and tubercle bacilli (Bloom and Fine, 1994). Subsequent administration of this strain to children proved to confer a significant level of protection. This strain has since been used as a vaccine bearing their name (bacille

Calmette-Guerin, BCG). Since then, a number of BCG vaccine strains grown and produced under different conditions have been in use (e.g., BCG Glaxo, Danish, BCG Pasteur). These vaccine strains have variable degree of protective efficacy (BlooAm and Fine, 1994).

A refined culture filtrate of *M. tuberculosis* called purified protein derivative (PPD) has been developed to detect past infection by skin testing. However, because PPD is unspecific to *M. tuberculosis*, this complicates interpretation of skin test results.

1.2. Antituberculosis Chemotherapy, Development of Resistance and Elucidation of Resistance Mechanisms

The first TB drug to be introduced was streptomycin in the 1940's. Another first-line drug, isonicotinic acid hydrazide (INH) was introduced in the early 1950's. More first- and second-line drugs were subsequently added, with the last first-line drug (rifampicin) being introduced in the late 1960's. Since then, no new drugs have been introduced. Due to the use of those drugs, tuberculosis appeared to have been brought under control. However, tuberculosis is once again a major health problem and variants of *M. tuberculosis* that are resistant to one or more of the major drugs are now rampant. As much as drug resistance has hampered tuberculosis control efforts, it has also given the impetus for a renewed interest in the understanding of the mechanisms of both drug action and resistance. Thus, within the last decade, several mutations occurring in drug targets and responsible for resistance to the major drugs have been elucidated (Table 1 gives a summary of drug action and resistance mechanisms for the major anti-TB drugs). In tubercle bacilli, there is no known

mechanism of drug resistance other than individual chromosomal mutations of genes for drug targets or modifying enzymes (Heym et al, 1994, Heym et al, 1995, Rastogi and David, 1993).

Drug-resistant tuberculosis can be either primary or secondary (acquired) resistance. Primary resistance occurs because of infection by a resistant *M. tuberculosis* strain and is meant to apply to patients who have never taken antituberculosis drugs. Secondary resistance is that which develops during treatment, especially when treatment is inadequate. However, WHO recommends the use the terms ''drug resistance among new cases'' and ''drug resistance among previously treated cases'' for primary and secondary resistance respectively (Antituberculosis Drug Resistance, Report No. 2, WHO, 2000).

The extensive worldwide spread of TB again, the increase in the percentage world population at risk for TB (because of increased population density, fast travel, etc) and the deadly synergy between TB and AIDS complicate the control of TB.

Table 1. Antituberculosis drugs, their targets and mechanisms of resistance

Drug (target or effect)	Gene (function)	Resistance mechanism(s)	Reference(s)
Isoniazid (inhibits mycolic acid synthesis)	*KatG* (encodes catalase peroxidase)	Missense mutations in *katG* or deletion of *katG*	Zhang et al, 1992 Heym et al, 1993
	inhA (mycolic acid synthesis)	(i) mutations in *inhA* structural gene (ii) overexpression of inhA	Banerjee et al, 1994 Basso et al, 1998 Larsen et al, 2002 Kremer et al, 2003
	ndh (NADH dehydrogenase)	(i) mutations in *ndh* (ii) defect in NADH dehydrogenase activity	Lee et al, 2001 Miesel et al, 1998
Rifampicin (inhibits RNA chain initiation)	*rpoB* (encodes a subunit of RNA polymerase)	mutations in codons 507-531 of *rpoB*	Telenti et al, 1993
Streptomycin (inhibits initiation of translation)	*rpsL* (encodes S12 protein of 30S)	mutations in codons 43 and 87 of *rpsL*	Nair et al, 1993 Finken et al, 1993
	rrs (encodes 16SrRNA)	mutation at position 513 in *rrs*	Meier et al, 1996
Ethambutol (targets the mycobacterial cell wall)	*embB* (encodes arabinosyl transferases)	point mutations in *embB*	Telenti et al, 1997 Sreevastan et al, 1997, Lety et al, 1997
Pyrazinamide (active against semi-dormant bacilli)	*pncA* (encodes pyrazinamidase)	point mutations in *pncA*	Scorpio & Zhang, 1996, Zhang et al, 1999
Amikacin ()	*rrs*	mutation in nucleotide 1400	Prammananan et al, 1998

1.3. The Extensive use of Antibiotics and the Biological Cost of Resistance

It is believed that restricted use of antibiotics will lead to reduction in the proportion of resistant bacteria in the bacterial population. The underlying assumption to this idea is that drug-resistant bacteria are less fit, and in the absence of selective pressure, drug-resistant bacteria will be outcompeted by drug-sensitive bacteria. While the importance of restricted and prudent use of antibiotics should never be discounted, there are indications that these measures alone will not lead to the disappearance of drug resistance from the bacterial population. These indications include the ability of bacteria to adapt to fitness losses by compensatory mutations, the occurrence of low- or no-cost mutations, and the maintenance of virulence along with resistance. Moreover, as a recent study (Austin et al, 1999) indicated, the time scale for emergence of resistance under a constant selective pressure is much shorter than the time it takes for significant decline in resistance in the absence of antibiotic use.

Other recent studies also indicated that resistance may not disappear following reduction or sessation of antibiotic use. For example, the frequency of sulphonamide-resistant *E. coli* increased from 40% in 1991 to 46% in 1999 despite a huge decrease in sulphonamide prescriptions in the UK between 1991 and 1999 ((Enne et al, 2001). In another study with clarithromycin-resistant *Helicobacter pylori*, Sjolund et al demonstrated that treatment selects for highly resistant enterococci that can persist for at least 3 years without further selection (Sjolund et al, 2003).

In many bacterial species, resistance mutations occur in genes that are essential for the metabolism of bacteria. This suggests that resistant bacteria pay a biological cost for acquiring those mutations.

The major factor driving changes in the frequency of antibiotic resistance in both community- and hospital-acquired infections is the volume of drug use (Andersson, 2003, Gillespie et al, 2002). Other factors include the biological cost of resistance and the ability of bacteria to compensate for such costs (Austin et al, 1999, Björkman et al, 2000).

The extensive use of antibiotics has created an environment that not only favors resistant mutants, but also one that favors mutations that compensate for the fitness losses rather than reversion to wild-type (Levin et al, 2000). Compensatory evolution can occur under continuous selective pressure (or even in the absence of) in vitro and/or in vivo as was shown in various bacterial species including *Escherichia coli* (Schrag and Perrot, 1996, Reynolds, 2000), *Salmonella typhimurium* (Björkman et al, 1998, 2000) and *Staphylococcus aureus* (Nagaev et al, 2001). Additionally, the occurrence of low- or no-cost mutations has been reported for various bacterial species including *S. aureus* (Wichelhaus et al, 2002), *Salmonella typhimurium* (Björkman et al, 1998) and *M. tuberculosis* (Böttger et al, 1998). Moreover, some resistant variants of *M. tuberculosis* have been shown to have the same degree of virulence and transmissibility as their wild-type counterparts (Martilla et al, 1998, Pym et al, 2002, van Soolingen et al, 2000).

1.4. Genetic Systems for Mycobacteria and Elucidation of Genome Sequences

The development of genetic systems for mycobacteria had lagged behind those of other bacteria for several reasons. One of these was the withdrawal of the attention given to TB following its successful control after the introduction of effective chemotherapy in the 1950's. Other reasons included the lack of molecular genetic systems for the study of mycobacterial pathogenesis, the design of vaccines, the identification of virulence factors and specific metabolic and resistance genes.

Introduction of recombinant DNA into mycobacteria had not been possible until its first demonstration in 1987 (Jacobs et al, 1987). The first attempts of gene inactivation and foreign gene expression in mycobacteria were reported in 1988 (Snapper et al, 1988) using *M. smegmatis* or BCG as model organisms. Subsequently, a high efficiency transformation mutant of *M. smegmatis*, mc^2155, was isolated and characterized (Snapper et al, 1990). But difficulties in the genetic analysis of mycobacteria remained, among others, due to the high rates of illegitimate recombination and low rates of homologous recombination in *M. tuberculosis* (Kalpana et al, 1991). However, subsequent experiments showed that homologous recombination was achievable in the slow-growing mycobacteria (Balasubramanian et al, 1996) and later experiments confirmed that *M. tuberculosis* has similar rates of homologous recombination as the faster-growing *M. smegmatis* (Pelicic et al, 1997).

Attempts to develop methods to generate allelic exchange mutants in mycobacteria were complemented by the development of suitable counterselectable markers for mycobacteria. Commonly used counterselectable markers in

mycobacteria include *sacB* and *rpsL*$^+$. *sacB* codes for levansucrase, which converts sucrose to levans that accumulate in the periplasm of Gram-negative bacteria and becomes toxic. This lethal effect of the expression of the *sacB* gene has been demonstrated in Gram-negative bacteria (*Legionella pneumophila, Klebsiella pneumoniae, Helicobacter pylori, Yersinia* sp, *Rhizobium* sp, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Corynebacterium glutamicum* (Jäger et al, 1992) and *Mycobacterium* sp (Pelicic et al, 1996).

rpsL codes for the small ribosomal subunit protein S12, which is the target of streptomycin. *rpsL*$^+$, the wildtype allele, confers sensitivity to streptomycin. Streptomycin sensitivity is dominant over resistance. Thus, *rpsL*$^+$ provides for positive selection of streptomycin sensitive bacteria. This selection scheme has been demonstrated in *E. coli* (Dean, 1981), *B. pertusis* (Stibitz, et al, 1986), *M. smegmatis* (Sander et al, 1995). Thus difficulties in the isolation of allelic exchange mutants due to the low frequency of double crossover events and high frequency of illegitimate recombination could be circumvented by positive selection of such rare allelic exchange mutants using counterselectable markers (Reyrat et al, 1995, Smith, 2003, Clark-Curtis and Haydel, 2003).

Aside from development of systems for homologous recombination, a phage integration system has also been developed for mycobacteria. This makes use of phage integration vectors that can be used to site-specifically insert foreign DNA into mycobacteria for which temperate phages are available. Notable among these is mycobacteriophage L5, which has been studied in detail. It infects both fast- and slow-growing mycabacteria. However, since then several other mycobacteriophages have been isolated, sequenced and characterized (Pedulla, et al, 2003) and more are

expected to be discovered. It is hoped these phages will be useful in studies of mycobacterial genetics.

The complete genome sequence of *M. tuberculosis* strain H37Rv has been published (Cole, et al, 1998). Among other things, the completion of this project has shown that the genome comprises 4,411,529 base pairs with about 4000 genes. The genome has ~ 60% G+C content. The genome sequences of *M. tuberculosis* CDC 1551 and *M. bovis* have also been published (Fleishman et al, 2002, Garnier et al, 2003).

The completion of the genome sequence enables identification and description of more virulence-associated genes and gene products which would also mean better understanding of the molecular basis of mycobacterial virulence. Before the completion of the mycobacterial genome sequence, only three virulence factors (catalase-peroxidase, *mce* and *sigA*) had been described (Arruda et al, 1993, Collins, 1996). In addition, availability of whole genome sequences makes it possible, among others, to understand the biology of microorganisms at the gene level; to design new antimycobacterial agents based on identified drug targets; to design new vaccines; to define molecular mechanisms of pathogenicity; to identify homologous sequences and assign putative functions to genes based on homology to other sequences of known function, especially for genes involved in transcription and translation as the number of genes involved in these functions is quite similar in several bacterial species (Fraser, 2000).

Chapter 2 The *M. tuberculosis* Complex and other Mycobacteria

The tubercle bacilli are rod-shaped obligate aerobes and may exist singly or form threads. The size ranges from 0.3 to 0.6 μm by 0.5 to 4.0 μm. The bacilli are non-encapsulated, non-spore-forming and nonmotile. The bacilli gain entry into the body by inhalation, ingestion or through the skin. The mycobacterial cell wall contains mycolic acid, a highly saturated fatty acid, which is a strong barrier against the influx of antibiotics (Nikaido, 1994). The *M. tuberculosis* complex consists of *M. tuberculosis* , *M. bovis, M. bovis* BCG, *M. africanum, M. microti* and *M. canetti.* These organisms are closely related and share more than 99% similarity at the nucleotide level (for some loci, however, they differ in morphology, biochemistry, host range and disease patterns in experimental animals.

M. microti is capable of causing human tuberculosis and infection with this bacterium has been documented in Europe in both immunocompetent and immunocompromised individuals (van Soolingen, 2001). *M. africanum* causes the same syndromes in humans as does *M. tuberculosis*. *M. bovis* also causes human tuberculosis but, unlike *M. tuberculosis*, it has a broad host range. *M. canetti* has been isolated from human patients (van Soolingen, 2001). The most common cause of tuberculosis in humans, however, is *M. tuberculosis*. All other mycobacteria (about 150 species) are refered to as mycobacteria other than tuberculosis (MOTT) or atypical mycobacteria. These include, among others, *M. smegmatis, M avium, M. intracellulare, M. fortuitum, M. chelonae*, and *M. kansassi, M. malmoense, M, simiae, M. marinum, M. haemophilium, M. ulcerans, M* and *scrofulaceum.* Prior infection by atypical mycobacteria was shown to provide some degree of protection against challenge with *M.*

tuberculosis (Bloom and Fine, 1994). However, some atypical mycobacteria can be pathogenic to humans; for example *M avium* is known to cause generalized disease in HIV-infected individuals (Heifets and Good, 1994).

2.1. Microscopic Detection of Mycobacteria

Mycobacteria are able to form stable complexes when stained with certain dyes such as fuchsin and auramine O. The mycolic acid residues of the cell wall retain the primary stain even after exposure to acid alcohol or strong mineral acids and extensive washing. Because of this property, they are called acid-fast bacilli. A counterstain is used for microscopic recognition. In the carbol-fuchsin staining procedure such as Ziehl-Nielsen and Kinyoun staining, acid-fast bacilli appear red against a blue or green background when examined with a 100X oil immersion lens using a light microscope. With the auramine O or auramine-rhodamine staining procedure, acid-fast bacilli fluoresce yellow to orange when examined with a fluorescence microscope.

2.2. Cultivation of *M. tuberculosis*

Either solid and/or liquid media may be used for the growth of *M. tuberculosis*. Solid media may be agar-based or egg-based. Agar-based media are transparent and thus suitable for susceptibility testing and morphologic studies. Agar-based media include Middlebrook 7H10 and 7H11 media. The 7H11 formulation differs from 7H10 in containing 0.1% enzymatic hydrolysate of casein to improve recovery of INH-resistant strains. Egg-based media have long shelf lives and support good growth of most mycobacteria. However, it is difficult to distinguish mycobacterial colonies from debris and are not suitable for drug susceptibility testing because it is not possible to achieve uniform drug concentrations and the heat

required to solidify the media inactivates drugs. Still, egg-based media (Lowenstein-Jensen) are widely used in many countries and are especially suited for primary isolation of mycobacteria.

The liquid medium most commonly used is Middlebrook 7H9 broth. It is used for subculturing stock strains and preparing inocula for drug susceptibility tests and other in vitro tests.

2.3. Standard Tuberculosis Regimen

In the treatment of tuberculosis, multi-drug therapy can reduce or avoid the risk of emergence of drug-resistance because there is less likelihood of mycobacteria surviving multi-drug therapy than single-drug therapy. This is because the simultaneous development of resistance mutations to two or more drugs is highly unlikely. The standard antituberculosis regimen of first line drugs includes isoniazid, rifampicin, pyrazinamide and either ethambutol or streptomycin (Dooley et al, 1992, Cole, 1994). Infection with multidrug-resistant tuberculosis (MDR-TB) necessitates the use of second-line drugs. The second line drugs include ethionamide, quinolones, p-aminosalicylic acid, kanamycin, amikacin and capreomycin (Heym et al, 1994).

2.4. Drug-Resistant Tuberculosis: The Magnitude of the Problem

The ease and speed with which transmission of infection with *Mycobacterium tuberculosis* occurs and the deadly synergy between tuberculosis and human immunodeficiency virus (HIV) infection has made the control of TB challenging. The magnitude of the problem can be even more appreciated when the emergence of more and more drug-resistant variants of the tubercle bacillus is taken into consideration. Several factors contribute to outbreaks of multidrug-resistant tuberculosis (resistance to

two or more drugs, especially to isoniazid and rifampicin) (Dooley et al, 1992). These include: poor management of tuberculosis patients, poor compliance with drug therapy, inadequate infection control programs, delays in laboratory identification and screening and delayed initiation of treatment of patients by which time many others could have been infected. The increasing frequency of emergence of strains resistant to TB drugs creates a dilemma as a delay in the diagnosis and effective treatment of tuberculosis is bound to cause further transmission of infection in the general public but, on the other hand, treatment of tuberculosis with available drugs before resistance patterns are known is likely to favor selection and proliferation of drug-resistant strains.

Tuberculosis causes about eight million new infections and three million deaths annually. A significant fraction of these cases is attributable to multidrug-resistant tuberculosis. A recent four-year survey in 50 countries in six continents to assess trends in resistance to antituberculosis drugs showed that the prevalence of resistance to any drug among new cases ranged from as low as 1.7% to as high as 37% in countries of the former Soviet Republics. The prevalence of resistance to any drug among previously-treated cases ranged from 0% to as high as 94% with a median prevalence of 23% (Espinal et al, 2001). This survey also revealed that the multidrug-resistant tuberculosis continues to be a serious problem, particularly among some countries of Eastern Europe and identified areas with a high prevalence of multidrug-resistant tuberculosis in such countries as China and Iran (Espinal et al, 2001).

Chapter 3 Identification and Typing Methods and Tests

3.1. Phenotypic Methods

3.1.1. Microscopy

Microscopy is a cornerstone of tuberculosis identification and control. It identifies smear-positive cases from sputum. It is also rapid and cheap, but it has a limited specificity for *M. tuberculosis*. For detection by smear positivity, a minimum of $5x10^3$ to $5x10^4$ bacilli per ml is required.

3.1.2. Culture

Culture is used for detection of mycobacteria in clinical specimen. Culture detects as few as 10 to 100 viable organisms in a specimen. Thus, culture has the capacity to detect mycobacteria in smear-negative specimen. The disadvantage of culture, however, is that it usually takes between 4 and 6 weeks to detect positive results.

3.1.3. Biochemical Tests

3.1.3.1. Catalase Test

Catalase is an intracellular enzyme that is capable of degrading hydrogen peroxide(H_2O_2) into water (H_2O) and oxygen (O_2). The presence of the enzyme is detected by adding H_2O_2 to a culture of the test organism and observing for the formation of O_2 bubbles. Species of catalase-producing mycobacteria can be distinguished by quantitative differences in catalase activity demonstrated by intact cells in the semiquantitative catalse test and by differences in heat stability detected by the 68°C catalase test.

3.1.3.2. Semiquantitative Catalse Test

The semiquantitative catalse test divides the mycobacteria into two groups: those producing less than 45 mm of bubbles (low catalase) and those producing more than 45 mm of bubbles (high catalase). Among those that produce less than 45 mm of bubbles are *M. tuberculosis, M avium* complex, *M. bovis,,M. malmoense, M, xenopi, M. marinum, M. haemophilium,* and *M. gastrii. M. kansasi* has two subgroups: one produces less than 45 mm of bubbles whereas strains more commonly associated with disease produce more than 45 mm of bubbles.

3.1.3.3. Heat Stable Catalase Test

Some mycobacteria lose catalase activity when suspended in pH 7.0 buffer and heated to 68°C for 20 min. Included in this group are *M. tuberculosis, M. bovis, M. gastri* and *M. haemophilium.*

3.1.3.4. Niacin Accumulation Test

Niacin is a precursor in the biosynthesis of NAD and NADP. *M. tuberculosis* accumulates niacin and excretes it into the culture medium. A pure, 3-4 week old culture of *M. tuberculosis* along with reagent-impregnated paper test strips are needed for the test. The niacin is extracted from the medium and then detected by its reaction with a cyanogen halide in the presence of a primary amine. The test is based on the formation of cyanogen chloride by the reaction of chloramines T and potassium thiocyanate in the presence of citric acid. Niacin then reacts with cyanogen chloride and couples with a primary aromatic amine to produce a yellow color.

3.1.3.5. Catalase Drop Test

The catalase drop test can be used for quick determination of significant INH resistance of *M. tuberculosis*. If a colony fails to produce bubbles after a drop of reagent is added, then the isolate is probably resistant to INH. However, some catalase-producing colonies can be INH-resistant.

3.1.3.6. Pyrazinamidase Test

Pyrazinamidase hydrolyzes pyrazinamide to pyrazinoic acid. This acid is detected by the addition of ferrous ammonium sulfate to the culture medium. The formation of a pink ferrous-pyrazinoic acid complex indicates a positive test. This test is most useful in separating *M. marinum* from *M. kansasii* and *M. bovis* from *M. tuberculosis*. *M. bovis* is negative even at 7 days, whereas *M. tuberculosis* is positive within 4 days.

3.1.3.7. Nitrate Reduction Test

The nitrate reduction test is valuable for the identification of some mycobacteria that possess similar characteristics of colony morphology, growth rate and pigmentation. The test can be performed by using chemical reagents or reagent-impregnated chemical strips or by combining the nitrate reduction test with the niacin test. *M. tuberculosis* reduces nitrate into nitrite producing a color change to pink or red. In the presence of drugs, this test is also used as a drug susceptibility test because only resistant bacteria reduce the nitrate.

3.2. Molecular Methods

3.2.1. Polymerase Chain Reaction (PCR)

Various PCR assays used for identifying mycobacterial DNA include (i) amplification of the genes encoding mycobacterial antigens such as the 65 kDa protein (Brisson-Noel et al, 1989, Pao et al, 1990), (ii) amplification of rRNA (Boddinghaus et al, 1990, Kirschner et al, 1996), (iii) amplification of repetitive sequences, (iv) DR (Groenen et al, 1991) and MPB64 protein (Manjunath et al, 1991, Sjobring et al, 1990).

3.2.2. The Gen-Probe Rapid Diagnostic Test

The first DNA probes to be commercially available for mycobacterial detection were those marketed by Gen-Probe. The diagnostic systems use ^{125}I-labaled single strand synthetic oligonucleotide complementary to specific ribosomal RNA sequences as probes. Since rRNA is present in ~ 10,000 copies per cell, the use of rRNA as target should increase sensitivity of detection. The sequence of rRNA is highly conserved, relative to most other genes, and therefore may be considered an unlikely choice for developing species-specific probes.

3.2.3. Genetic Markers

Almost all *M. tuberculosis* strains carry the repetitive DNA element, insertion sequence IS*6110*. Detection of IS*6110* sequences by DNA hybridization provides a means of demonstrating that *M. tuberculosis* is present in clinical specimens. The insertion sequence IS*6110* was shown to be specific to members of the *M. tuberculosis* complex (Thierry et al, 1990) and strain identification based on this repetitive element is the recommended typing method (van Embden et al, 1993).

The application of IS*6110*-based restiction fragment length polymorphism (RFLP) typing and other molecular typing methods has contributed significantly to understanding transmission of mycobacteria (van Soolingen, 2001). Strains with the same IS*6110* fingerprint pattern are epidemiologically related. *M. tuberculosis* strains carry multiple copies of IS*6110* with the insertion sites being variable among strains. This variability in both the number and location of IS*6110* provides the polymorphism for identification. IS*6110* band patterns are quite stable and IS*6110* transposition is very low.

Another transposable element known in *M. tuberculosis* is IS*1081*. In contrast to IS*6110*, IS*1081* is distributed rather homogeneously in different *M. tuberculosis* strains. Fingerprints obtained with IS*1081* are much less polymorphic. Therefore, IS*1081* is not a useful marker for distinguishing between strains of *M. tuberculosis*. However, because IS*1081* is present on a characteristic *Pvu*II fragment in *M. tuberculosis*, it may be used to differentiate BCG from other strains of *M. tuberculosis*.

The RFLP analysis has been standardized by the introduction of internal and external molecular weight standards and computer-assisted analysis of DNA fingerprints (van Soolingen, 2001). These have made it possible to establish international databases of RFLP patterns, which in turn enable tracing source(s) of infection and also compare results obtained in different laboratories. S*6110* is most useful in identifying isolates with unique fingerprint patterns and those in clusters in which the isolates contain six or more copies of IS*6110* (Chaves et al, 1996).

Other genetic markers have been shown to be useful in discriminating between strains that can not be differentiated with IS*6110* because of the presence of

five or fewer copies of IS*6110* (van Soolingen et al, 1993, Chaves et al, 1996). These include the polymorphic GC-rich repetitive sequence (PGRS) and the 36-bp direct repeat (DR) sequence. The PGRS is contained in pTBN12, a recombinant plasmid.

The DR sequence is clustered at a single chromosomal locus and is interspersed by nonrepetitive DNA of 36 to 41 bp in length. The majority of *M. tuberculosis* strains contain one or more IS*6110* elements in the DR region. The DR region of most *M. tuberculosis* strains also contains IS elements. The integration sites of these IS elements and the sizes of the DR regions revealed that the DR-containing region of these *M. tuberculosis* strains is polymorphic in size and composition.

The DR locus contains multiple, conserved 36 bp direct repeats that are interspersed by nonrepetitive spacers that are 35 to 41 bp long. The DR region is clustered in a single chromosomal region but, because strains vary in both the number of DRs and spacers, this creates the polymorphism that is the basis for the typing method called spoligotyping. The DR locus is amplified by PCR and the amplified DNA is hybridized with multiple synthetic spacer oligonucleotides immobilized on a membrane (Kamerbeek et al, 1997). Spoligotyping may be useful for the differentiation of strains with few IS*6110* copy number and for the differentiation of *M. bovis,* which often contains a single IS*6110* element, from *M. tuberculosis* (Kamerbeek et al, 1997). Because most *M. bovis* isolates contain only one copy of the IS*6110* element at a fixed genomic position, IS*6110* typing is not very useful for investigating transmission of *M. bovis* (van Soolingen, 2001).

Another typing method, based on the pTBN12 probe, has been shown to be useful in distinguishing between isolates with fewer than six copies of IS*6110* as well as in distinguishing *M. bovis* isolates (Ross et al, 1992). pTBN12 is a recombinant

plasmid that contains an insert referred to as polymorphic GC-rich repetitive sequence (PGRS), a sequence found multiple times in the genomes of *M. tuberculosis* and other mycobacteria. A more recent study showed that the pTBN12 method has even higher discriminating power than does spoligotyping (Yang et al, 2000).

3.3. Chromatographic Method

High performance liquid chromatography (HPLC) is the chromatographic method most commonly used for the identification of mycobacteria. HPLC uses a liquid mobile phase at high pressure to carry a sample through a column packed with particulate material or stationary phase. Mycolic acids extracted from saponified mycobacterial cells are converted to the p-bromophenacyl esters (UV-absorbing derivatives) and the unique mycolic acid pattern associated with each species is detected by separation of the esters. The analysis is based on a comparison of retention time of the peaks and of their height ratios.

The mycolic acid patterns are similar among the members of the *M. tuberculosis* complex but the pattern of BCG is quite different. This distinction cannot be made by the AccuProbe technique. Regarding species other than *M. tuberculosis*, HPLC techniques cannot distinguish between *M. avium* and *M. intracellulare*, but the AccuProbe method can do so if separate *M. avium* and *M. intracellulare* probes are used.

As with the probe, HPLC could not separate *M. tuberculosis* from *M. bovis* but did allow for the differentiation of the BCG strains. Other methods used to identify *M. tuberculosis* based on the chromatographic analysis of cell wall lipids

include thin-layer chromatography and gas-liquid chromatography (Heifets and Good, 1994).

Chapter 4 Methods of Susceptibility Testing for Slowly-Growing Mycobacteria

4.1. Phenotypic Methods

Drug-susceptibility testing of *M. tuberculosis* can be performed using either a direct or an indirect procedure. In the direct procedure, a digested, decontaminated and concentrated smear-positive specimen is inoculated directly into the drug-containing and control media. In the indirect procedure, a pure culture grown on primary isolation medium is inoculated into the drug-containing and control media. Careful attention should be given to the selection of colony types so that the final inoculum is representative of all types present in order to ensure a balance of potentially resistant and susceptible bacilli ((Siddiqi, 1992).

4.1.1. Proportion Method

In the indirect Proportion method, a standardized suspension of the test organism is inoculated onto both agar containing an antimicrobial agent and a control agar plate.The recommended medium is Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase. Plates are incubated for 3 weeks at 37°C and 5% CO_2. Growth on the drug-containing plate is compared with growth on the drug-free plate. Resistance is defined as > 1% growth on the drug-containing plate when compared with growth on the drug-free plate.

4.1.2. BACTEC Susceptibility Test

Mycobacteria are inoculated into vials containing BACTEC 12B medium with and without antimycobacterial agents (control). The BACTEC vials contain Middlebrook 7H12 medium with ^{14}C-labeled palmitate. The number of bacilli added

to the control vial is diluted to one-hundredth of that in the drug-containing vial. An undiluted inoculum is also added into a drug-free BACTEC vial as additional growth control. The vials are incubated at 37°C and 5% CO_2 and read everyday in the BACTEC 460 system. Mycobacteria resistant to the drug utilize the ^{14}C-labeled medium and produce $^{14}CO_2$, which is quantitatively measured by the BACTEC 460 system. The results are interpreted by evaluating the rate of increase in the readings, described as growth index (GI). If the increase in GI in the drug-containing vial is less than the GI in drug-free vial containing the one-hundredth bacterial inoculum, the isolate is considered susceptible to the tested drug concentration; if it is greater, the isolate is considered resistant ((Siddiqi, 1992).

4.1.3. Mycobacteriophage-based Assay

A mycobacteriophage-based assay that can be used for detection and susceptibility testing of *M. tuberculosis* has been developed (Jacobs et al, 1993). This exploits the use of the recombinant vector phAE40, constructed from mycobacteriophage TM4 carrying the firefly luciferase gene (*fflux*) under the control of the mycobacterial promoter BCG hsp60, as a reporter gene. Upon infection of mycobacteria with phAE40 in the presence of substrates (exogenously-added luciferin and endogenous ATP), mycobacteria generate photons that can be detected by luminometer. In the presence of antitubercular drugs, no photons will be generated after infection with phAE40, confirming the susceptibility of *M. tuberculosis*. The *fflux* gene is expressed only if mycobacteria are viable.

Because the host range of phAE40 includes those mycobacteria outside the *M. tuberculosis* complex, the specificity of the luciferase reporter mycobacteriophage (LRP) assay was enhanced by use of p-nitro-α-acetylamino-β-hydroxy

propiophenone (NAP), which has previously been shown to have selective inhibitory activity against members of the *M. tuberculosis* complex (Riska et al, 1997).

Furthermore, the lytic nature of TM4 from which phAE40 was constructed caused rapid lysis of mycobacteria, resulting in loss of detectable light output. This led to the development of newer generations of phage vectors that continue to be improved. The latest one of them is phAE142, which uses a potent promoter (P_{left} of mycobacteriophage L5) to drive luciferase expression, produce more light from a given concentration of *M. tuberculosis* and thus enhances the sensitivity of detection (Bardarov et al, 2003). This phage has been used for detection and drug susceptibility testing of *M. tuberculosis* from digested and decontaminated sputum (Bardarov et al, 2003). This assay is not yet available for wide-scale application.

4.2. Molecular Methods

4.2.1. PCR-SSCP

PCR-SSCP (polymerase chain reaction single-stranded conformation polymorphism) is based on PCR amplification of the region of interest, denaturation of the amplified fragment and running in polyacrylamide gel (Telenti et al, 1993, 1998). Point mutations cause conformational changes that result in altered electrophoretic mobility when compared with reference control. This method has most often been used to detect drug resistance to rifampicin but less so to isoniazid. It does not work well for detecting drug resistance to other drugs (Viedma, 2003).

4.2.2. DNA Sequencing

The most direct way of detecting resistance mutations is by PCR amplification of the target region followed by direct automated sequencing. Automated sequencing

machines are available but their cost is so high that they are not in ordinary use in most routine microbiology laboratories.

4.2.3. Heteroduplex Analysis

Heteroduplex analysis involves mixing amplified DNA from the sample with that of reference drug-sensitive strains. The DNA is then denatured and cooled again to allow the DNA to hybridize into hybrid double-stranded DNA. The DNA is then analyzed on a denaturing electrophoresis gel. Where the sample DNA carries a drug-resistant mutation, there will be a mismatch in the complementary base-pairing, and the heteroduplex will have a different mobility than the homoduplex (Caws and Drobniewski, 2001).

4.2.4. Hybridization on Strips

Solid-phase hybridization analysis involves the immobilization of DNA probes complementary to the most common mutations and to drug-sensitive patterns. Amplified DNA can then be hybridized to the probes and detected through fluorescence, radiolabel, or colorimetric reaction in the normal way. One such assay that is commercially available is the line-probe assay (LiPA, Innogenetics, Zwijndrecht, Belgium) for the detection of rifampicin resistance (Caws and Drobniewski, 2001).

Chapter 5 Antituberculosis Drugs: Mechanisms of Action and Resistance

5.1. RIFAMPICIN

The antibiotic rifampicin is a transcription inhibitor that binds to the ß- subunit of RNA polymerase. Rifampicin inhibits the initiation of RNA chain growth. It is without effect on the elongation and termination processes. Resistance to rifampicin has been reported to be due to mutations in the *rpoB* gene, the gene that codes for the ß-subunit of RNA polymerase (Telenti et al, 1993). Emergence of resistance to rifampicin appears to be facilitated by the fact that the drug acts only on a single target which may exhibit significant reductions in its affinity for the antibiotic by mutations (Spratt, 1994). Nearly 95% of the mutations in *M. tuberculosis* that confer resistance to rifampicin occur within a sequence of 81 nucleotide residues (codons 507-533) near the center of the *rpoB* gene (Cole, 1994). Within this region, 78% of the mutations occur between codons 527 and 533. Analysis of mutations between codons 511 and 533 of the *rpoB* gene (which encodes the rifampicin-binding domain) in 122 clinical isolates of which 56 were sensitive and 66 were resistant to rifampicin revealed mutations involving 8 conserved amino acids in all but 2 of the 66 resistant isolates (Telenti et al, 1993). The absence of mutations in this region in the 2 resistant isolates suggests there may be mutations at other sites of the *rpoB* gene which can confer resistance. This has been corroborated by a study (Williams et al, 1994) which examined a 305 base pair fragment of the *rpoB* gene from 110 resistant isolates, 8 of which contained no mutations and the remaining had 16 different mutations affecting 13 amino acids in this region of the *rpoB* gene.

5.2. ISONIAZID

The antibiotic isoniazid (INH) was introduced as an antitubercular drug in the early 1950's. Though it continued to be used, its bactericidal activity remained unclear. In the early 1970's, studies of the effect of INH demonstrated that INH inhibited the biosynthesis of mycolic acids (Winder and Collins, 1970, Takayama et al, 1972).

INH-resistant isolates of *M. tuberculosis* were reported shortly after the introduction of INH (Middlebrook, 1954, Middlebrook and Cohen, 1953). The first *M. tuberculosis* gene identified as conferring INH resistance was *katG* (Zhang et al, 1992). Transformation of INH-resistant *M. smegmatis* with the wild type *M. tuberculosis katG* restored sensitivity to INH (Zhang et al, 1992). Later, transformation of INH-resistant *M. tuberculosis* with the wild type *M. tuberculosis katG* was shown to restore sensitivity to INH (Zhang et al, 1993). These results suggested that the *katG* gene product, catalase-peroxidase, encoded an activator of INH. INH is a prodrug that requires conversion into its active form. The mutant KatG has a reduced catalase-peroxidase activity, leading to reduced conversion of INH to its active form (Heym et al, 1995, Wengenack et al, 1997).

The second *M. tuberculosis* gene associated with INH resistance was found to be *inhA* (a gene whose protein product is required for mycolic acid biosynthesis) and was shown to confer INH resistance through both mutations in the structural gene and overexpression (Banerjee et al, 1994). Thus, allelic exchange experiments showed that a point mutation (Ser94Ala) within *inhA* of an INH-resistant *M. smegmatis* was sufficient to transfer INH and ethionamide (ETH, a structural analog of INH) resistance to *M. smegmatis*. Allelic exchange experiments were also performed by transforming the spontaneous INH-resistant mutant, mc^2651, with a 45kb *M. smegmatis* DNA containing

the wild type *inhA* linked to a kanamycin resistance gene. This resulted in cotransformation of kanamycin resistance and INH-sensitivity in 72% of the transformants, indicating that the single Ser→Ala substitution caused INH resistance (Banerjee et al, 1994). Resistance to INH and ETH were also conferred on sensitive strains of *M. smegmatis* and *M. bovis* BCG by overexpression (transfer on a multicopy plasmid) of the wildtype *inhA* gene from *M. tuberculosis* or *M. smegmatis* (Banerjee et al, 1994). Shortly thereafter, InhA was purified, its crystal structure determined and shown to be an NADH-dependent enoyl acyl carrier protein (ACP) reductase (Dessen et al, 1995, Quemard et al, 1995). The mechanism of action of INH was shown to be covalent attachment of the activated INH to the nicotinamide ring of NAD, generating an INH-NAD adduct that binds to InhA (Rozwarski et al, 1998). Mutations in both the *inhA* structural gene and the *inhA* promoter region have been identified in INH-resistant clinical isolates of *M. tuberculosis* and InhA enzymes prepared from INH-resistant strains of *M. tuberculosis* were resistant to inhibition by KatG-activated INH (Quemard et al, 1995, Basso et al, 1998).

Another study (Miesel et al, 1998) described a new mechanism for INH resistance. This study found that many INH-resistant mutants are defective in NADH dehydrogenase activity (Ndh, type II) which oxidizes NADH; this results in increased NADH concentration which might lead to INH resistance by one or both of two mechanisms: interference with KatG-mediated peroxidation of INH and/or displacement of the INH-NAD adduct from the active site of InhA. In addition to INH resistance, such mutants also exhibit phenotypes including resistance to ethionamide, thermosensitivity and auxotrophy (Miesel et al, 1998).

However, mutations in *katG* and *inhA* do not account for all observed INH resistance. Some 20% of strains resistant to INH are wild type in both *katG* and *inhA*. A new putative target of INH, termed β-ketoacyl ACP syntase (*kas*) was reported (Mdluli et al, 1998). In INH-treated *M. tuberculosis*, *kasA* was identified in a covalent complex along with INH and AcpM (a 12 kDa acyl carrier protein on which a saturated hexacosanoic acid, C26:0, accumulated during INH treatment) (Mdluli et al, 1998). The report indicated that 5-20% of INH-resistant isolates have *kasA* mutations.

However, other studies provide evidence that contradict the above report and maintain that *inhA* is the primary target of INH. One such study (Larsen et al, 2002) demonstrated that overexpression of *inhA* conferred resistance to INH and ETH whereas overexpression of *kasA* conferred resistance to neither drug. Another study (Kremer et al, 2003) showed that the formation of the KasA-containing complex was induced by the inhibition of InhA activity but not by inhibition of KasA activity, that InhA was not part of the complex, and that InhA did not target kasA.

5.3. STREPTOMYCIN

In *E. coli,* streptomycin causes misreading of the genetic code and inhibits initiation of translation by binding to the S12 protein and 16S rRNA of the 30S ribosomal subunit encoded by the *rpsL* and *rrs* genes respectively (Noller, 1984, Moazed and Noller, 1987). Mutational changes in these targets lead to resistance by decreased affinity for the antibiotic. Such mutations have been found in resistant strains of *M. tuberculosis* (Davies, 1994, Finken et al, 1993). The majority of mutations which confer streptomycin resistance in *M. tuberculosis* involve substitution of Lys by Arg at positions 43 and 88 of protein S12 (Finken et al, 1993, Nair et al, 1993, Sreevastan et al,

1996). Less frequently, Lys 43 is also replaced by Thr and Asn in resistant isolates . Mutations at position 43 also confer streptomycin resistance in *E. coli* and *M. luteus* (Nair et al, 1993, Bottger, 1994) indicating that this region is highly conserved and is the binding site for streptomycin.

Mutations in *rrs* that confer resistance to streptomycin in *M. tuberculosis* were found in the 520 region (corresponding to the 530 loop in *E. coli*) in the secondary structure of 16S rRNA at positions 491, 512 and 516 (Finken et al, 1993, Bottger, 1994, Ramaswamy and Musser, 1998). These are C to T transitions corresponding to *E. coli* positions 501, 522, and 526 respectively and A to C transversion at position 513 (position 523 in *E. coli*). The corresponding region (530 loop) in *E. coli* is known to interact with S12 protein in the selection of tRNAs at the A site. The 530 region is protected from chemical modification by the S12 protein, providing evidence for the interaction between the 530 region and S12 protein (Finken et al, 1993, Bottger, 1994).

Previously undescribed mutations in the 915 region at positions 798 (C→T), 877 (G→A) and 906 (A→C) of *rrs* that conferred streptomycin resistance have also been identified (Sreevastan et al, 1996).

Nucleotide residues 524-526 in the 530 region (residues 514-516 in *M. tuberculosis*) are also involved in base-pairing with residues 505-507 in the 510 region bulge loop (residues 495-497 in *M. tuberculosis*) of the secondary structure of 16S rRNA known as a pseudoknot (Finken et al, 1993, Bottger, 1994). The pseudoknot is needed for ribosome function. This was shown by introducing G-U wobble base pairs between residues 524-526 and 505-507, which resulted in streptomycin resistance (Dam et al, 1992).

Resistance to streptomycin is not solely due to mutations in the *rrs* or *rpsL* genes. Bottger (Bottger, 1994) examined mutations in 16S rRNA and S12 of 26 multidrug-resistant, 15 streptomycin-resistant and 35 susceptible isolates of *M. tuberculosis*. All 35 susceptible isolates were free of mutations of any sort, but in 5 of the 26 multidrug-resistant isolates and in 4 of the 15 streptomycin-resistant isolates, no mutations were found in either *rrs* or *rpsL*. Other reports also showed that 26% (Meier et al, 1996), and about 30% (Sreevatsan et al, 1996) of streptomycin-resistant isolates were wild-type in both *rpsL* and *rrs* genes, indicating that genes other than *rpsL* and *rrs* might be involved in streptomycin resistance. Thus resistance to streptomycin due to mutations in *rrs* or *rpsL* genes accounted for about 80% of the streptomycin resistance in *M. tuberculosis* strains.

5.4. PYRAZINAMIDE

Pyrazinamide is an analog of nicotinamide that is active against semi-dormant tubercle bacilli residing in acidic environment (Heifets and Lindholm-Levy, 1992). Pyrazinamide has no activity against *M. tuberculosis* at neutral or alkaline pH. Pyrazinamide is a prodrug whose activation requires conversion into pyrazinoic acid by pyrazinamidase. *M. tuberculosis* contains pyrazinamidase, while the naturally pyrazinamide-resistant *M. bovis* lacks this enzyme and this biochemical difference is used to distinguish *M. tuberculosis* from *M. bovis*.

Pyrazinamide-resistant *M. tuberculosis* are not uncommon though, and this has been shown to be mainly due to mutations in *pncA*, a gene that encodes for pyrazinamidase (Scorpio & Zhang, 1996). In 4 different isolates examined, amino acid substitutions in 3 isolates at codons 63 (Asp→His), 138 (Cys→Ser), and 141 (Gln→Pro)

and deletion of nucleotide G at position 162 were reported. Transformation of both the naturally pyrazinamide-resistant BCG and pyrazinamide-resistant derivative of H37Rv with cosmid DNA containing the wild-type *pncA* gene restored pyrazinamidase activity and PZA susceptibility (Scorpio & Zhang, 1996).

pncA mutations are not responsible for all of the observed pyrazinamide resistance. For example, one study reported *pncA* mutations in only 72% of pyrazinamide-resistant isolates examined and a total of 17 previously undescribed mutations in *pncA* including upstream mutations, missense changes, insertions, deletions and terminations (Sreevastan et al, 1997). In other reports of pyrazinamide resistance, 33 of 34 (Scorpio et al, 1997) and 32 of 33 (Hirano et al, 1998) pyrazinamide-resistant isolates were found to have mutations at various positions in the *pncA* gene, implicating *pncA* mutations as a major mechanism of pyrazinamide resistance.

Another report (Zhang et al, 1999) showed that under conditions of low pH, accumulation of pyrazinoic acid in pyrazinamide susceptible *M. tuberculosis* cells was enhanced when [^{14}C] pyrazinamide was added in the bacterial suspension. This intracellular accumulation of pyrazinoic acid was not seen in pyrazinamide-resistant *M. tuberculosis*, but accumulated [^{14}C] pyrazinoic acid when this was supplied in the medium; however, naturally pyrazinamide-resistant *M. smegmatis* converted pyrazinamide into pyrazinoic acid, but didn't accumulate it even at acidic pH. So it has been proposed (Zhang et al, 1999) that the pyrazinamide resistance phenotype in *M. smegmatis* is not due to a defective pyrazinamidase but rather due to an efflux system that prevents accumulation of pyrazinoic acid. Conversely, the intracellular accumulation of pyrazinoic acid and pyrazinamide susceptibility of *M. tuberculosis* is due to a much weaker efflux mechanism. The recent report (Boshoff and Mizrahi, 1998)

of targeted knockout of the *M. smegmatis pncA* which resulted in more than 300% reduction in the enzyme's activity, and overexpression of this gene in *M. smegmatis* which conferred pyrazinamide susceptibility are consistent with this proposition that the natural pyrazinamide resistance of *M. smegmatis* is not due to a defective pyrazinamidase.

5.5. ETHAMBUTOL

Studies have shown that the target of ethambutol (EMB) is the mycobacterial cell wall. EMB has been shown to inhibit the transfer of arabinogalactan into the cell wall of *M. smegmatis*, as evidenced by the accumulation upon treatment with EMB of trehalose mono- and di-mycolates for lack of mycolate attachment (Takayama and Kilburn, 1989). Ethambutol was also shown to inhibit the addition of glucose into arabinose residue of arabinogalactan (Silve et al, 1993).

EMB treatment of mycobacteria resulted in accumulation of decaprenol P-arabinose, indicating both that decaprenol P-arabinose serves as the arabinan donor and that the drug interefers with the transfer of arabinose to the arabinogalactan chain (Takayama and Kilburn, 1989, Wolucka et al, 1994, Brennan and Nikaido, 1995). Addition of EMB into growing EMB-sensitive *M. smegmatis* culture inhibited polymerization of arabinan of arabinogalactan and consequently resulted in accumulation of free lipids because of lack of mycolate attachment sites (Mikusova et al, 1995).

Recently, genetic evidence for EMB interference in cell wall biosynthesis was presented (Belanger et al, 1996). The targets of EMB in *M. avium* were shown to be two genes, *embA* and *embB*, that encode for arabinosyl transferases responsible for the polymerization of arabinose into the arabinan component of arabinogalactan. The

overexpression of these otherwise EMB-sensitive target genes by a plasmid vector in *M. smegmatis* led to resistance to EMB.

More recently, Telenti et al (Telenti et al, 1997) identified, cloned, sequenced and characterized a 3-gene contiguous region supposedly encoding the EMB traget (arabinosyl transferases) and designated *embCAB* in *M. smegmatis*. Resistance to EMB was shown to result from overexpression of the EMB protein(s), structural mutation in EmbB or both. The genes were identified by introduction into wild type *M. smegmatis* of a genomic library from an EMB-resistant mutant of *M. smegmatis*. This region is also conserved in *M. tuberculosis* and *M. leprae* as identified from an ordered cosmid library and two of the three genes (*embAB*) are homologous to the *embAB* genes in *M. avium* (Belanger et al, 1996, Telenti et al, 1997). Subsequent reports (Sreevastan et al, 1997, Lety et al, 1997) also showed that point mutation in *embB* confers resistance to EMB. This mutation was found in most (89%) of epidemiologically unrelated isolates involving substitutions at amino acid 306 of EmbB (Met306Leu, Met306Val, Met306Ile) in *M. tuberculosis* (Sreevastan et al, 1997) or in three different regions of EmbB in *M. smegmatis* (Lety et al, 1997).

Objectives of the Study

- To obtain spontaneous, rifampicin-resistant mutants of clinical isolates of *M. tuberculosis*, identify mutations and study the effect(s) of rifampicin-resistance mutations on bacterial fitness in in vitro and macrophage culture models.
- To study the development of multidrug resistance in serial clinical isolates of *M. tuberculosis* and identify the relevant mutations.
- To study the bacterial population dynamics and stability of genetic markers in a collection of serial, MDR clinical isolates of *M. tuberculosis*.

Chapter 6 **Materials and Methods**

Growth and Storage of rifampicin-resistant strains.

Strains were grown in Middlebrook 7H9 broth with 10% ADC enrichment and 0.05% Tween 80 (hereafter referred to as 7H9 medium) for 3 weeks. Bacterial cells were stored in glycerol-containing medium in –70°C freezer. Strain Harlingen is a clinical isolate of *M. tuberculosis* that was isolated as a particularly transmissible and virulent strain in Holland in 1993 (Kiers et al, 1997).

Isolation of Spontaneous Rifampicin-Resistant Mutants.

The parent strain was grown in 7H9 medium to mid-log phase and then used to inoculate 60 independent cultures which were grown to OD600 ≈ 1.00. Approximately 10^8 cells from each of these 60 independent cultures were spread on Middlebrook 7H10 plates containing 1 μg/ml rifampicin. The plates were incubated at 37°C in a 5% CO_2 incubator. The appearance of single colonies was periodically examined using a dissecting microscope. One single colony was picked from each independent plate and inoculated into 7H9 medium. After growth these bacteria were pelleted by centrifugation and the pellets resuspended in glycerol-containing storage medium, aliquoted and stored in a –70°C freezer.

Characterization of the Rifampicin-Resistant Isolates.

Determination of minimum inhibitory concentration (MIC): The MIC values for the different isolates were determined using the Bactec 460 system.

Extraction of DNA: Mycobacteria were grown on LJ medium. A 10 μl loop was used to transfer cells into a microcentrifuge tube containing 250 μl of 1X TE

buffer. The cells were killed by heating at 80°C for 1 hr. Cells were then centrifuged at 13000 rpm for 2 min. The supernatant was discarded and the pellet resuspended in 500 µl of 150 mM NaCl, followed by centrifugation. This was repeated once. Finally, the DNA pellet was resuspended in 25 µl of TE.

PCR Amplification and Sequencing of rpoB Gene: A 258 base-pair region of the *rpoB* gene which encompasses all the codons in which mutations that lead to rifampicinampicin resistance was amplified by PCR. The forward primer was 5'ATCAACATCCGGCCGGTGGT 3' and the reverse primer was 5' TACACCGACAGCGAGCCGAT 3' (Morlock et al, 2000). PCR was run in a 50 µl reaction containing 0.5 µM of each primer, 200 µM of each dNTP, 2 mM $MgCl_2$, 5 µl of 10X PCR buffer, 0.25 µl of AmpliTaq DNA polymerase, and 1 µl of DNA. The PCR conditions were an initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. PCR products were purified using GFX™ PCR and Gel Band Purification Kit (Amersham Pharmacia Biotech). Cycle sequencing was performed according to Applied Biosystems ABI PRISM Big Dye Terminator Cycle Sequencing Kit using ABI PRISM 310 or 3100 Genetic Analyzer.

Competition Experiments.

The wild-type and the different mutants were grown separately in 7H9 broth containing 0.1% Tween 80. The Ser522→Leu (TCG→TTG), His526→Tyr (CAC→TAC), and Ser531→Trp (TCG→TGG) mutants were used in this experiment. To start mixed competition cultures, the optical densities of the wild-type

and mutant isolates were first adjusted to the same value. Then 30 μl of the mutant and 10 μl of the wild-type bacterial suspensions were mixed in 20 ml 7H9 medium. Immediately after mixing (Day 0), 10-fold serial dilutions were prepared from each mixed culture and plated on both drug-free and drug-containing Middlebrook 7H10 plates with 10% OADC enrichment. This procedure was repeated on the 3rd, 7th and 14th day. The CFU were counted on the 25th day. The CFU of the wild-type on days 0, 3, 7 and 14 was obtained by subtracting the CFU on drug-containing plates from the total CFU on drug-free plates.

Macrophage Infection Experiments.

Growth of Bacteria and Cell Line: The same mutants as above were used for the macrophage experiments. Bacteria were pre-grown in 7H9 medium. To prepare the inoculum for infection, the bacterial cultures were centrifuged and washed once with fresh 7H9 medium. The pellets were resuspended in 3 ml 7H9 medium and then successively agitated with sterile glass beads for 30 minutes, sonicated twice (30 seconds each) and then filtered through a 5 μm pore-size filter unit (Millipore) to disrupt clumps in the bacterial suspensions. Finally, the optical densities of the suspensions were adjusted to give a 1:1 infection ratio.

A frozen ampoule of the cell line (U937) was thawed at 37°C, washed in warm complete RPMI medium containing 10% fetal bovine serum (GIBCO™, Invitrogen Corporation) and then viability of the cells determined. These were then cultured in complete RPMI medium for 3 days and then fresh medium added and cultured for a further 4 days. Then viability and the number of viable cells per ml were determined, PMA added, approximately 10^5 cells dispensed into each well of 8-

well slide flasks (Lab-Tek II Chamber Slide™ System) and incubated to mature for 3 days.

Infection of Macrophages: For a 1:1 multiplicity of infection (MoI), 200 μl of each bacterial suspension was mixed with RPMI. Prior to infection, the medium from each well was removed and the wells washed with warm RPMI. Then 0.5 ml of the mixture of bacteria and medium was pipetted into each well. For Day 0, two wells each for microscopy and CFU were infected. For Days 3, 5 and 7, three wells each for microscopy and CFU were infected. After a 4 hr infection period, infecting medium from each well was removed and each well vigorously washed twice with 0.5 ml RPMI. Day 0 slide flasks were processed immediately. Day 3, 5 and 7 slide flasks were filled with 0.5 ml fresh complete medium and returned into the incubator.

Quantitation of CFU and Microscopy: Previous experiments have shown that the number of bacteria removed with the medium on Day 3 is less than 2% of the total while the number of bacteria removed with the medium on Day 7 can be 20% or more. Thus, to quantitate the CFU within lysates from wells on days 0 and 3, the medium was discarded whereas on days 5 and 7, the medium was lysed and combined with the lysate from the well to quantitate the CFU. Lysis was performed with a 10 minute incubation in sterile distilled water containing 0.036% SDS. After the lysis period, 50 μl of 20% BSA was added to the lysate (Paul et al, 1996, Zhang, et al, 1998). Then 10-fold dilutions were prepared and plated on Middlebrook 7H10 medium with 10% OADC. Plates were incubated in 37°C incubator with 5% CO_2. The CFU were counted after 3 weeks. For microscopy, about 2500 macrophages were counted for each time point and strain. Twenty different fields from each well were

counted and the number of macrophages infected with variable number of bacilli scored.

Characterization of Multidrug-Resistant Isolates.

The isolates that were characterized were clinical isolates obtained from the same patient over the course of a decade (1991-1999). These isolates were kept frozen at –70°C in Middlebrook 7H9 broth containing 10% glycerol. A fresh aliquot was taken for analysis. The isolates and the time of isolation together with their drug resistance patterns are listed in Table 2.

DNA Extraction. For DNA extraction, the isolates were first grown on Lowenstein-Jensen medium. Bacterial cells were then transferred into a microcentrifuge tube containing 250 μl of 1X TE buffer using a sterile loop followed by killing by heating at 80°C for 1 hr. Cells were then centrifuged at 13000 rpm for 2 min. The supernatant was discarded and the pellet resuspended in 500 μl of 150 mM NaCl, followed by centrifugation. This was repeated once. Finally, the DNA pellet was resuspended in 25 μl of TE. To check the identity of mutations for streptomycin and amikacin resistance, all 8 isolates were first grown on Middlebrook 7H10 medium containing 10% OADC enrichment to obtain single colonies. Then, 10 single colonies were randomly picked from two different plates between days 14 and 16 of culture and grown in Middlebrook 7H9 broth containing 10% ADC enrichment for 3 weeks followed by inoculation onto LJ medium. DNA from these was extracted as described above.

Amplification of DNA by PCR. The PCR reactions were carried out in a 50 μl volume containing 0.5 μM of each primer, 200 μM of each dNTP, 5 μl of 10X PCR

buffer, 0.25 μl of AmpliTaq DNA polymerase, 1 μl of DNA and 2 mM $MgCl_2$ except for primer pair RF3/RR3 for which the $MgCl_2$ concentration was 3 mM. The primers and cycling conditions are listed in Table 3. PCR products were purified using GFX™ PCR and Gel Band Purification Kit (Amersham Pharmacia Biotech). Sequences were obtained using ABI PRISM 3100 Genetic Analyzer.

Table 2. Dates of isolation and MIC of MDR isolates.

Isolate (date isolated, D/M/Y)	MIC (μg/ml)					
	INH	Rifampin	Streptomycin	Ethambutol	Amikacin	Ofloxacin
1 (2/4/91)	2	≤2	≤2	5	1	1
2 (12/7/91)	>2	>32	≤2	5	1	1
3 (11/9/91)	4	>32	>16	5	1	
4 (13/12/91)	4	>8	>16	5	1	1
5 (5/2/92)	2	>32	>16	5	1	1
6 (14/12/92)	4	>32	>16	5	1	2
7 (5/2/93)	2	>32	>16	5	2	
8(_/3/99)	4	>32	>16	5	>8	1

Table 3. Primers and PCR conditions for MDR isolates

Gene name	Primer pairs	Fragment length(bp)	Cycling conditions (30 cycles)
KatG	5'ATGGGGCTGATCTACGTGAA3' 5'TCCTTGGCGGTGTATTGC3'	410	95°C 5min; 94°C 30s, 55°C 30s, 72°C 30s, 72°C 7min.
	5'TACGAGTGGGAGCTGACGAA3' 5'ATGCGGTCGAAACTAGCTGT3'	429	
rpoB	5'ATCAACATCCGGCCGGTGGT3' 5' TACACCGACAGCGAGCCGAT3'	258	95°C 5min; 94°C 20s, 55°C 20s, 72°C 30s, 72°C 7min.
rpsL	5'ATGCCAACCATCCAGCAG3' 5'TTCTCTTTCTTAGCGCCGTA3'	368	95°C 5min; 94°C 30s, 55°C 30s, 72°C 30s, 72°C 7min.
embB	5'TGATATTCGGCTTCCTGCTCT3' 5'TTGTTGAACGGCATCCAC 3'	359	95°C 5min; 94°C 30s, 55°C 30s, 72°C 30s, 72°C 7min.
	5'TGGATGCCGTTCAACAAC 3' 5'TTCTCGGTATACCACGCCTG3'	385	
rrs	5'GAAACTGGGTCTAATACCGGA3' 5'AAGGAAGGAAACCCACACCTA3'	688	95°C 5min; 94°C 30s, 55°C 30s, 72°C 50s, 72°C 7min.
	5'TAGGTGTGGGTTTCCTTCCTT3' 5'ATCCCACCTTCGACAGCTC3'	660	

Chapter 7 **Results**

The rifampicin resistant isolates studied in this work were the TCG531→TGG, CAC526→TAC and TCG522→TTG, mutants (Fig.1 and Table 4). These mutants were tested in all three culture systems (i.e., single, competition and macrophage cultures) for their growth rates.

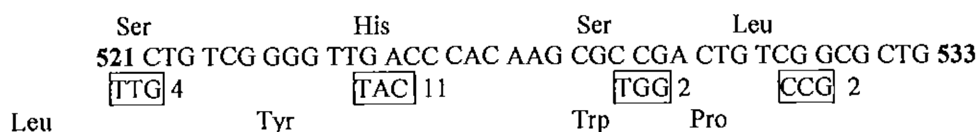


Figure 1. Nucleotide and amino acid substitutions in *rpoB* mutants. Below the sequence row are shown the mutated codons, the number of independent isolates with those mutations and the amino acid changes resulting from those mutations.

Table 4. Characteristics of rifampicin-resistant mutants.

Number of independent isolates	MIC (μg/ml)	Mutation in *rpoB*
2	> 32	Ser531→Pro (TCG→TGG
11	> 32	His526→Tyr (CAC→TAC)
4	≥16	Ser522→Leu (TCG→TTG)
10	> 32	None

Growth rates of rifampicin-resistant isolates in single (broth) cultures. The TCG531→TGG mutants exhibited significantly reduced growth rates in broth culture. Of the three mutants, the TCG531→TGG mutants had the lowest fitness with the longest doubling time and the lowest number of divisions in the assay period. The CAC526→TAC and TCG522→TTG mutants exhibited only slightly reduced growth rates with calculated fitness values of 0.87 ± 0.03 and 0.96 ± 0.02 relative to the wild-type parental strain with fitness value of 1.00 (Table 5).

TABLE 5. Growth rates of susceptible and resistant *M. tuberculosis* during growth *in vitro* in single cultures and competitions. Data shown represent the average of 2-3 independent replicate experiments for each of 2 independent isolates of the same mutant type.

Strain	Mutation	MIC (µg/ml)	Single culture			Competition culture				
			Doubling time	Number of divisions	Relative fitness	Doubling time (hrs)		Number of divisions		Relative fitness
						Mutant	Wild type	Mutant	Wildtype	
Harlingen	Wild type		22.4	15.0	1.00					
Harlingen	Ser531→Pro (TCG→TGG)	> 32	31.6	10.6	0.63±0.09	36.3	24.3	9.4	13.9	0.67±0.06
Harlingen	His526→Tyr (CAC→TAC)	> 32	26.2	12.8	0.87±0.03	30.6	27.2	11.1	12.5	0.88±0.05
Harlingen	Ser522→Leu (TCG→TTG)	≥16	23.6	14.2	0.96±0.02	44.4	23.8	7.7	13.7	0.54±0.03
H37Ra	Wild type	0.25	24.5	13.7	0.94±0.05					
H37Rv	Wild type	0.25	24.8	13.5	0.94±0.05					

Growth rates of rifampicin-resistant isolates in competition cultures. The ratios of CFU of resistant (R) to wildtype (S) on days 0, 3, 7 and 14 were calculated. In all cases, these ratios decreased from day 0 to day 14. This decrease was evident even when the initial R:S ratio on day 0 was much higher than the intended 3:1 ratio as described in the Materials and Methods. An example is shown in Fig. 2. Thus, the susceptible parent strain had the shorter doubling time and the higher number of divisions in the competition cultures. (Table 5).

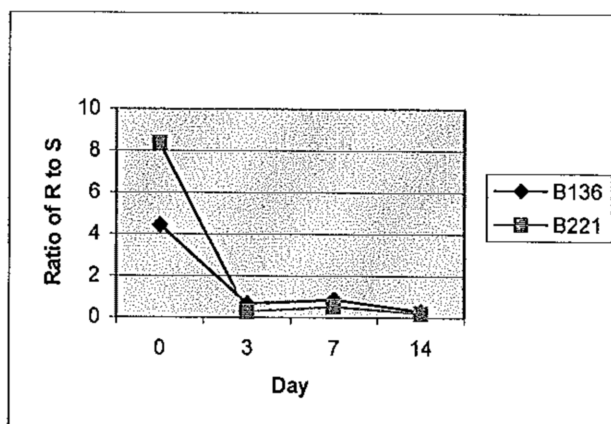
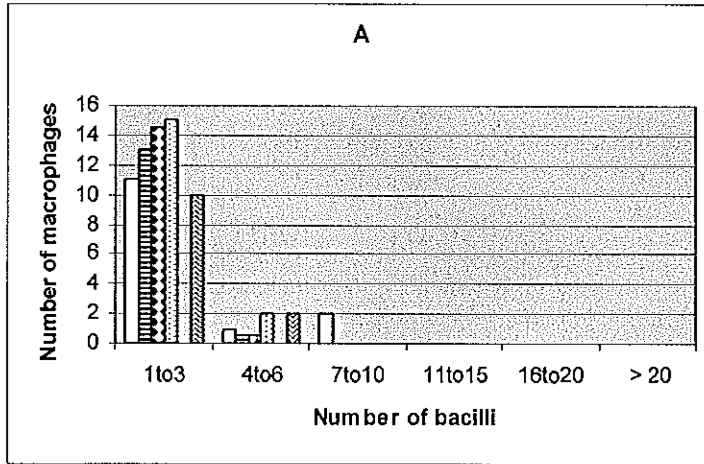


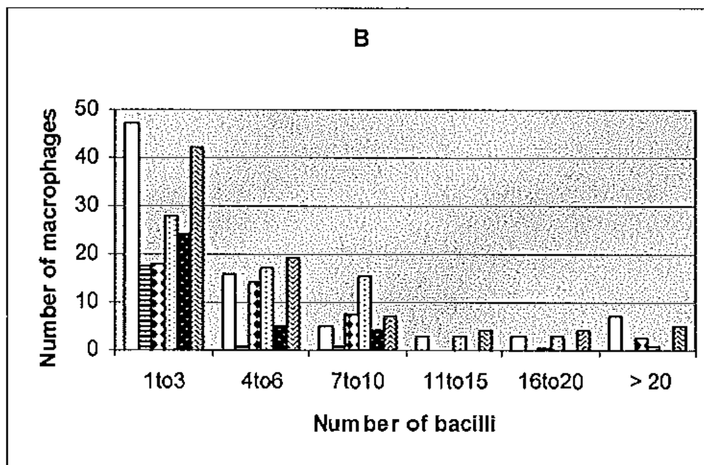
Figure 2. Ratios of resistant to sensitive bacteria as determined by plating on days 0,3,7 and 14 for TCG531→TGG mutants B136 and B221.

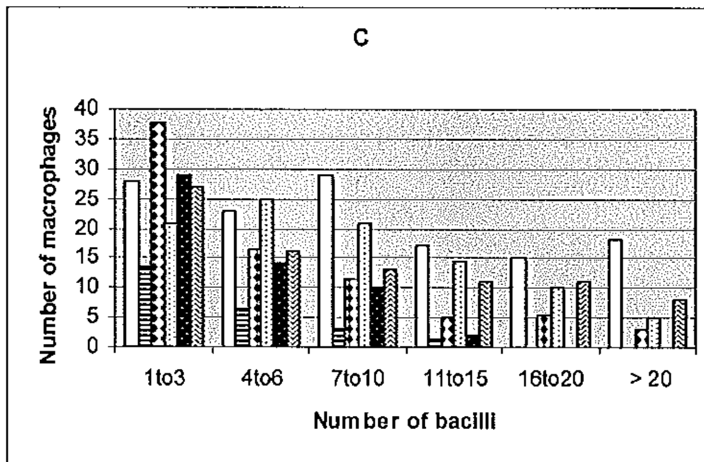
Growth rates of rifampicin-resistant isolates in macrophage cultures. To further assess the effects of these mutations on fitness, the growth rates of the mutants were measured in a macrophage cell line. The number of macrophages containing variable number of bacilli on days 0 (4 hours after infection), 3, 5 and 7 are shown in Fig. 3A, B, C and D respectively. On day 0, most of the infected macrophages were infected with 1 to 3 bacilli. A

much smaller number of macrophages were each infected with 4-6 bacilli. The number of macrophages infected with more and more bacilli increased as the culture period increased from day 0 to day 7.

Figure 3E shows the CFU obtained from the macrophage cultures of the different mutants and the control strains. Since the live bacterial densities in the original suspensions were not equivalent, data are presented as fold increases over day 0 for each isolate. The CAC526→TAC mutants grew from day 0 to day 5 and then their growth declined by day 7. A similar trend was observed for the TCG522→TTG mutants. However, the TCG531→TGG mutants showed very minimal growth and eventually decreased by day 7. The avirulent strain, H37Ra, showed a slight increase by day 3 and then declined with no change in CFU between days 5 and 7. The CFUs for both H37Rv and Harlingen continued to increase into day 7. Table 6 gives the fitness ratings.


A
Number of macrophages
16
14
12
10
8
6
4
2
0
1to3
4to6
7to10
11to15
16to20
> 20
Number of bacilli


B
Number of macrophages
50
40
30
20
10
0
1to3
4to6
7to10
11to15
16to20
> 20
Number of bacilli


C
Number of macrophages
40
35
30
25
20
15
10
5
0
1to3
4to6
7to10
11to15
16to20
> 20
Number of bacilli

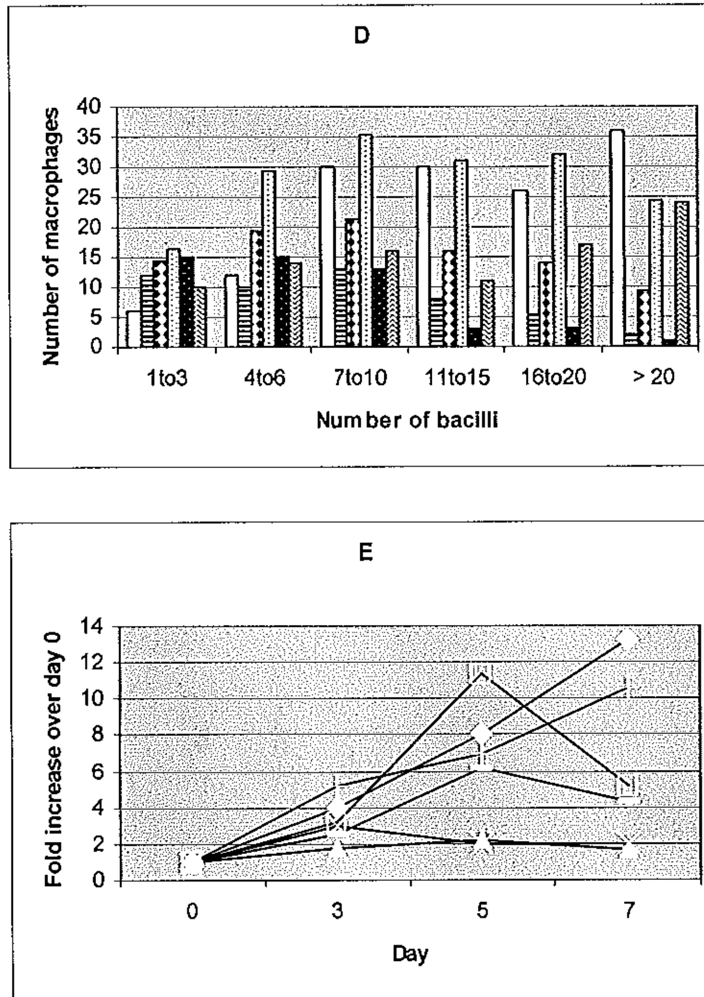


Figure 3. Number of macrophages (y-axis) containing the indicated number of bacilli (x-axis) on days 0 (A), 3 (B), 5 (C), and 7 (D) and fold increases in CFU over day 0 (E) of the macrophage cultures for the various rifampicin-resistant mutants and the susceptible control strains. A – D: white, Harlingen; ladder, Ser531→Pro (TCG→TGG); diamond, His526→Tyr (CAC→TAC); dots, white bar, Ser522→Leu (TCG→TTG); dots, black bar, H37Ra; and zigzag, H37Rv. E: diamond, Harlingen; triangle, Ser531→Pro (TCG→TGG); square, His526→Tyr (CAC→TAC); hyphen, Ser522→Leu (TCG→TTG); ×, H37Ra; and +, H37Rv.

TABLE 6. Growth rates of resistant mutants and susceptible *M. tuberculosis* in the macrophage cell line U937. Data shown represent the average of 2-3 independent replicate experiments for each of 2 independent isolates of the same mutant type.

Strain	Mutation	MIC (μg/ml)	Doubling time (hrs)	Number of divisions	Relative fitness
Harlingen	Wild type		46.0	3.6	1.00
Harlingen	Ser531→Pro (TCG→TGG)	> 32	166.0	1.0	0.29±0.06
	His526→Tyr (CAC→TAC)	> 32	72.9	2.4	0.64±0.02
	Ser522→Leu (TCG→TTG)	≥16	91.3	1.9	0.53±0.16
H37Ra	Wild type		111.2	1.5	0.33±0.12
H37Rv	Wild type	0.25	50.6	3.3	0.91±0.06

Drug susceptibility of multidrug-resistant isolates. Drug susceptibility testing of the serial isolates showed that the isolates exhibited resistance to an increasing number of drugs. The first isolate was resistant to isoniazid only. The second isolate added rifampicin resistance. The third through seventh isolates were resistant to isoniazid, rifampicin and streptomycin. The eigth isolate developed resistance to all these drugs and to the second-line drug amikacin. The MIC values are shown in Tables 2.

Sequencing of multidrug-resistant isolates for mutation detection. These isolates were subjected to PCR-DNA sequencing to detect the presence of mutations in *katG*, *rpoB*, *rpsL*, *embB* and *rrs*. Initial sequencing revealed that all eight isolates carried the AGC315→ACC mutation and an additional CGG463→CTG mutation in *katG*. The bacterial

population in some of these isolates was composed of a mixture of CGG→CTG mutant and CGG→CGG wild-type at codon 463. All but the first isolate harboured the GAC516→GTC mutation in the *rpoB* gene. These isolates did not carry *embB*306 mutation and the MIC for ethambutol was found to be 5 μg/ml. All isolates were susceptible to amikacin except isolate 8, which carried the A1400→G mutation in *rrs*, which has been shown to be responsible for resistance to amikacin in both clinical and in vitro isolates of *M. chelonae* and *M. abscessus* (Prammananan et al, 1998).

This initial sequencing of all isolates showed the presence of one of three different mutations in *rpsL* or *rrs*. These were the AAG87→AGG and AAG42→AGG in *rpsL* and A513→C in *rrs* in isolates 3-8. The A513→C mutation alone in *rrs* has been shown to confer streptomycin resistance in *E. coli* (Melancon et al, 1988) and clinical isolates of *M. tuberculosis* (Finken et al, 1993). The absence of cross-resistance between streptomycin and amikacin or other aminoglycoside antibiotics in *M. tuberculosis* has also been demonstrated (Tsukamura and Mizuno, 1975, Meier et al, 1996). Thus, to assess the genotypic homogeneity of the bacterial population in each isolate with respect to streptomycin (and also amikacin) resistance, 10 randomly-selected single colonies (subisolates) were isolated from plate cultures of the 8 isolates. As shown in Table 7, of the 10 subisolates of isolate 3, 8 had the AAG87→AGG mutation, 1 had the AAG42→AGG mutation and 1 was wild-type in *rpsL*. This subisolate with wild-type *rpsL* had the A513→C mutation in *rrs*. Of the 10 subisolates of isolate 4, 9 had the AAG87→AGG mutation while 1 had the AAG42→AGG mutation. Isolate 5 was a mixed population as there were both AAG87→AGG (8 subisolates) and AAG87→ANG (2 subisolates). All of the 10 subisolates from isolate 6 contained the AAG42→AGG mutation in *rpsL*. None of the subisolates of isolates 4, 5 and 6 had the

A513→C mutation. Two of the 10 subisolates of isolate 7 had the AAG42→AGG mutation while 8 were wild-type in *rpsL*; but these 8 subisolates had the A513→C mutation in *rrs*. Nine out of 10 subisolates of isolate 8 had the AAG42→AGG mutation and one was wild-type in *rpsL*. However, this isolate was tested and found to be resistant to streptomycin. All 10 subisolates of isolate 8 had the A1400→G mutation in *rrs*.

RFLP and spoligotyping pattern of multidrug-resistant isolates. The RFLP pattern of the isolates were identical as shown in Fig. 4, indicating no change in the number and positions of IS*6110* banding pattern. The spoligotyping pattern also showed that all eight isolates have the 9-band hybridization to spacers 35-43, a banding pattern characteristic of Beijing family isolates (Fig. 4).

Table 7. Mutations identified in various genes of the MDR isolates.

Isolate (date isolated, D/M/Y)	*katG*	*rpoB*	*rpsL*	*rrs*	
				A513→C	A1400→G
1 (2/4/91)	AGC315→ACC CGG463→CTG	wt	wt	wt	
2 (12/7/91)	AGC315→ACC CGG463→CTG	GAC516→GTC	wt	wt	
3 (11/9/91)	AGC315→ACC CGG463→CTG	GAC516→GTC	AAG87→AGG (8) AAG42→AGG (1) wt (1)	Wt (9) A513→C (1)	wt
4 (13/12/91)	AGC315→ACC CGG463→CTG	GAC516→GTC	AAG87→AGG (9) AAG42→AGG (1)	wt	wt
5 (5/2/92)	AGC315→ACC CGG463→CTG	GAC516→GTC	AAG87→AGG (8) AAG87→ANG (2)	wt	wt
6 (14/12/92)	AGC315→ACC CGG463→CTG	GAC516→GTC	AAG42→AGG (10)	wt	wt
7 (5/2/93)	AGC315→ACC CGG463→CTG	GAC516→GTC	AAG42→AGG (2) wt (8)	A513→C (8) wt (2)	wt
8(_/3/99)	AGC315→ACC CGG463→CTG	GAC516→GTC	AAG42→AGG (9), wt (1)	wt (10)	A1400→G

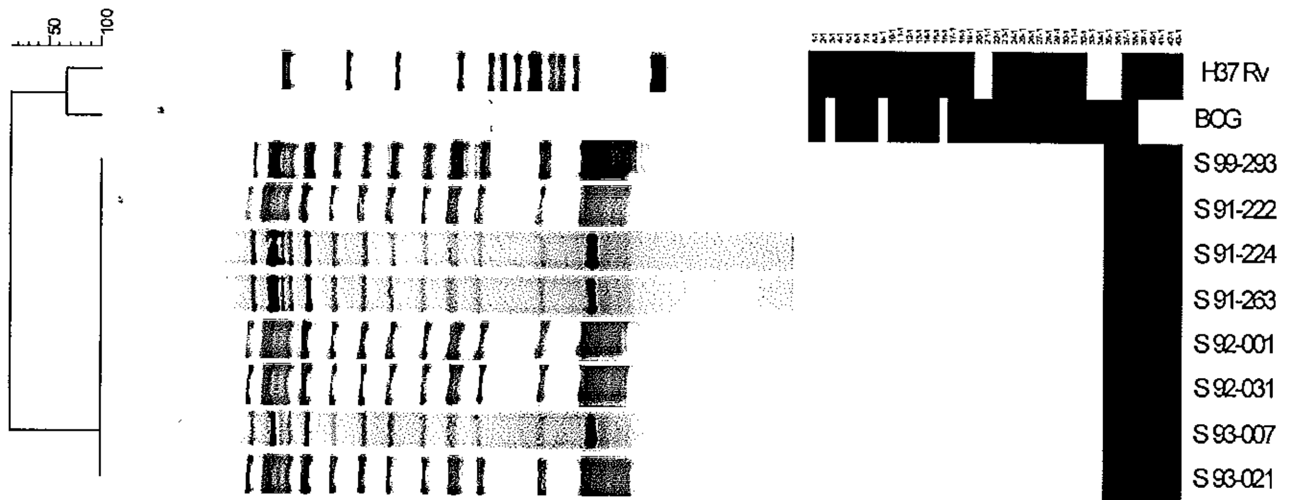


Figure 4. Spoligotyping and RFLP pattern of Serial clinical isolates of *M. tuberculosis*. S99-293 = isolate 8, S91-222 = isolate 1, S91-224 = isolate 2, S91-263 = isolate 3, S92-001 = isolate 4, S92-031 = isolate 5, S93-007 = isolate 6, S93-021 = isolate 7.

Chapter 8 Discussion

The point mutation at codon 531 of *rpoB* gene reported in this work involves the change of TCG(Ser) → TGG(Trp). Although it is associated with high-level resistance, this mutation is rare. The other point mutation at codon 531 involves the change of TCG(Ser) → TTG(Leu) and occurs at a much higher frequency irrespective of geographical location. Rifampicin-resistant *M. tuberculosis* isolates from different regions exhibit these two mutations at frequencies ranging from 0 - 14% and 86 –100% for the TCG→TGG and TCG→TTG mutations respectively (Ohno et al, 1996, Qian et al, 2002, Ramaswamy and Musser et al, 1998, Yang et al, 1998, Yuen et al, 1999). The rarity of the Ser→Trp mutation might be associated with a higher fitness cost that this mutation imposes. The TCG→TTG mutation at codon 531 followed by the CAC→TAC mutation at codon 526 of the *rpoB* gene were reported to be the most frequent mutations responsible for rifampicin resistance in *M. tuberculosis* strains from various regions (Ramaswamy and Musser, 1998).

While there seems to be a correlation between frequency of mutation and relative fitness, there is no correlation between frequency of mutation and level of drug resistance. Nor there seems to be a correlation between level of fitness and level of drug resistance. This lack of correlation has also been seen in rifampicin-resistant *Escherichia coli* (Reynolds, 2000), where clones isolated after serial passage for 200 generations in the absence of rifampicin were more fit than their ancestor whereas clones isolated in the presence of rifampicin showed increases in both levels of resistance and fitness. Sander et al (Sander et al, 2002) also showed that there is a positive correlation between the frequency of isolates

with a given mutation and the relative fitness that mutation confers in clinical isolates of *M. tuberculosis* resistant to streptomycin.

The increase in the number of macrophages containing increasing numbers of bacilli from day 0 to day 7 together with the fact that RPMI medium does not support the extracellular growth of bacilli (Paul et al, 1996, Zhang et al, 1998) and the fewer number of bacilli/macrophage on day 0, strongly indicate that fewer macrophages were infected with one or two bacilli on day 0, but as the infection progressed, the bacilli reproduced within the macrophages, lysed macrophages and infected previously uninfected neighboring macrophages.

The results from plate cultures of macrophage lysates showed that the CFU of all resistant isolates decreased by day 7 (or even by day 5 for some of them) while those of only H37Rv and the wild type Harlingen continued to increase. The microscopy data revealed that the number of macrophages containing variable numbers of bacilli did increase for the resistant isolates as well as for H37Rv and Harlingen. However, microscopy does not discriminate between live and dead bacilli. Considering the resistant isolates, the increase in the number of bacilli/macrophage and the decrease in the CFU strongly indicates killing of the resistant isolates in macrophages, probably between days 5 and 7.

The persistence and proliferation of antibiotic-resistance can be exacerbated by the presence of no-cost, low-cost, or compensatory mutations. Some examples that make these the likely scenario in various microorganisms can be cited as follows (Table 8 also gives rifampicin resistance mutations and their costs in various bacterial species).

<u>INH-resistant *M. tuberculosis* strains</u>: More than 50% of INH resistance is caused by mutations in *katG* gene. The most-commonly-occurring *katG* mutation is Ser315→Thr and is

associated with both high level resistance to INH, transmission rate as often as the INH-susceptible strains, and without significant loss of fitness (Martilla et al, 1998, Pym et al, 2002, van Soolingen et al, 2000).

Streptomycin- resistant *M. tuberculosis* strains: As described earlier, mutations at codon 42 of *rpsL* in *M. tuberculosis* (Lys→Arg, Lys→Asn and Lys→Thr) cause streptomycin resistance. Of these, Lys→Arg is the most frequent and is associated with less than 1% fitness cost per generation (Sander et al, 2002). The Lys→Arg mutation at codon 42 of the *rpsL* gene confers no significant cost in both clinical and in vitro isolates whereas the mutations Lys→Thr and Lys→Asn at the same codon both confer a fitness cost of 15% (Sander et al, 2002). Similar observations have been reported for the bacterium *Salmonella typhimurium* (Björkman et al, 1998) and the mutations have been classified as nonrestrictive.

Fluoroquinolone-resistant *Streptococcus pneumoniae*: The relative growth rate of a mutant strain of *S. pneumoniae*, compared with its susceptible isogenic parent in comparative growth experiments showed no significant deficit (Gillespie et al, 2002).

Streptomycin-resistant mutants of *E. coli*: Maintenance of spontaneous, streptomycin-resistant mutants of *E. coli* in culture for 180 generations in antibiotic-free medium resulted in evolved strains with reduced cost of resistance and higher peptide chain elongation rates (expressed as amino acids incorporated per second) relative to the unevolved, parental streptomycin-resistant strains (Schrag and Perrot, 1996).

Rifampicin-resistant strains of *E. coli*:. A study showed that adaptation to fitness costs associated with resistance in rifampicin-resistant strains of *E. coli* occurs by compensatory evolution rather than reversion. This compensatory evolution occurred in the absence as well

as in the presence of rifampicin but for nearly all strains, overall levels of resistance increased as did relative fitness in cultures that evolved in the presence of rifampicin.

Streptomycin- and fusidic acid-resistant mutants of *S. typhimurium*: Streptomycin- and fusidic acid-resistant mutants of *S. typhimurium* were examined to determine how amelioration of costs occurs under in vitro (LB) and in vivo (mice) conditions. For both LB- and mouse-selected streptomycin-resistant mutants, amelioration of costs occurred exclusively by compensatory mutations with none of the mutants reverting to wild type. However, the fusidic acid-resistant mutants differed from the streptomycin-resistant mutants in the manner of amelioration of costs. That is, almost all LB-selected mutants acquired compensatory mutations whereas slightly more (56%) of the mouse-selected mutants reverted to wild type than those that acquired compensatory mutations (44%) (Björkman et al, 2000).

Protease inhibitor-resistant variants of HIV: Variants of the virus that progressively accumulated mutations at different sites in the protease emerged upon exposure to a protease inhibitor. The initially selected variants had a low level of resistance as well as markedly reduced replication. Further accumulation of mutations at secondary sites occurred leading to both increased drug resistance and replication. These secondary mutations continued to accumulate at different sites in the protease, even in the absence of drug and did not revert to wild-type during passage in drug-free conditions (Borman et al, 1996). Another paper (Nijhuis et al, 1999) described the emergence of variants of drug-resistant HIV with increased protease activity and enhanced replicative capacity after an initial selection of mutants with reduced protease activity and replicative capacity.

Table 8. Rifampicin resistance mutations and fitness for various bacterial species. These were assayed in competition experiments in *in vitro* cultures. Only the subset of mutations that have been identified in all three species are shown.

Bacterial species	*rpoB mutation*	Relative fitness	Reference
E. coli	His526→Tyr His526→Leu	0.91 0.94	Reynolds, 2000
S. aureus	His481→Tyr (corresponds to *E. coli* codon 526) His481→Asn (" " " " 526) Ser486→Leu (" " " " 531)	0.93 1.00 0.86	Wichelhaus et al, 2002
M. tuberculosis	His526→Tyr Ser531→Leu	0.79 0.84	Billington et al, 1999
M. tuberculosis	His526→Tyr Ser531→Pro	0.89 0.67	This work

Drug susceptibility testing of the serial isolates showed that the isolates exhibited resistance to an increasing number of first- and second-line drugs. The first isolate was resistant to isoniazid only and had no mutations in *rpoB*, *rpsL* or *rrs*. The second isolate was resistant to both isoniazid and rifampicin. The third isolate was resistant to isoniazid, rifampicin and streptomycin. The eigth isolate developed resistance to all the major drugs and to the second-line drug amikacin. The MIC values and the mutations responsible for the resistance are shown in Tables 2 and 7 respectively.

From both the MIC data and genotypic sequencing, it is clear that the original bacterial population was monodrug-resistant (resistant to isoniazid but susceptible to all other drugs). However, the isolates exhibited resistance to an increasing number of the major antituberculosis drugs including, in the case of the last isolate, to the second line drug

amikacin. The sequential accumulation of mutations responsible for resistance to the various drugs indicates an increased tendency towards chronic resistance development.

The spoligotype patterns of the 8 isolates were determined to see if they belong to the Beijing family. As shown in Figure 4, the isolates have only 9 of the 43 spacer sequences in the spoligotypes, characteristic of Beijing family strains (van Soolingen et al, 1995). The 9 band spoligotype pattern has been shown to be superior to the IS*6110* RFLP pattern in identifying strains of the Beijing genotype (Kremer et al, 2004). In addition to an identical spoligotype, the eight isolates also exhibited identical RFLP pattern.

Interpretation of the presence of chain of transmission based on similar or identical molecular fingerprinting patterns may be complicated when transmission is due to (isolates) (strains) that are genetically but not epidemiologically related. If, for example, rate of change of RFLP or spoligo patterns is so low, then it might lead to an overestimation of transmission. Conversely, if the rate of change of fingerprinting patterns is fast, it will inevitably result in underestimation of ongoing transmission.

The widespread distribution of the W-Beijing strains is an indication of their ability to both sequentially acquire drug-resistance mutations and enhanced adaptability. This may be explainable, at least in part, by the recent report by Rad et al (Rad et al, 2003), who described the sequential acquisition of missense mutations in two *mutT* genes involved in DNA repair in the vast majority of a collection of W-Beijing genotype strains originating from 35 countries. These mutations were not seen in the non-Beijing isolates included in the study. An increased mutation rate in combination with continuous selective pressure might correspondingly increase the proportion of mutant bacteria in the bacterial population as for example, shown in *Escherichia coli* (Mao et al, 1997). A link between hypermutable strains of *Pseudomonas aeruginosa* and persistent lung colonization was found in a significant

proportion of cystic fibrosis patients (Oliver et al, 2000). *M. tuberculosis*, like *E. coli* and other microorganisms, possesses several mutator genes involved in DNA repair and would act such that mispairing of bases is either prevented or a mispaired base is removed (Mizrahi and Andersen, 1998, Horst et al, 1999). A recent report has shown that drug susceptible Beijing genotype does not have an enhanced mutation rate and tendency for drug resistance development (Werngren and Hoffner, 2003).

The IS*6110* RFLP patterns of the eight isolates in this study are identical, indicating no change has occurred in the eight years time interval between the first and last isolates. Most studies dealing with stability of IS*6110* RFLP patterns of serial isolates involve time intervals from a few months to a few years. In one such study, de Boer et al (de Boer et al, 1999) used isolates with time interval less than 3 months and calculated a half-life of 3.2 years with only 4.6% of the isolates changing patterns. In another study (Niemann et al, 1999), 10 serial isolates from a single patient showed no change in their IS*6110* patterns in 2.1 years. This study also indicated an increased tendency for changes in IS*6110* patterns with longer time intervals whereas another study (Yeh et al, 1998) suggested no association between changes in IS*6110* patterns and time interval upto 3 years but both this and another study (Warren et al, 2002a) agreed that there was no association between changes in IS*6110* patterns and drug resistance. The latter appears to be the case in our isolates also as the RFLP patterns of the first and subsequent isolates are the same but with marked differences in the drug susceptibility of the isolates. The number of IS*6110* bands of most of the isolates in the two studies were also similar [3-17 in (Niemann et al, 1999) and 1-21 in (Yeh et al, 1998)]. However, interpretations of stability with respect to time interval need to take into consideration that the bacterial population remains the same throughout.

Warren et al (Warren et al, 2002b) also calculated a half-life of 8.74 years and 10.69 years corresponding to early and late disease phases respectively. A more recent report (Eilers et al, 2004) argues that most changes in RFLP pattern occur before diagnosis with extremely low changes thereafter and that the proportion of isolates with changed bands, not half-life or rate of change, is the determinant factor for changes in RFLP pattern.

Conclusions

- Rifampicin-resistance mutations are associated with biological costs. The severity of these costs is dependent on the assay or culture system. The wild-type parental strain has the highest fitness in both in vitro and in macrophage cultures.

- There is a strong evidence for an inverse correlation between frequency of mutation and the cost of that mutation. The most frequent mutations carry the least biological costs.

- In vitro and in vivo assays of the biological costs of resistance mutations are useful indicators for identification of potential drug targets.

- *M. tuberculosis* Beijing genotype strains are associated with an increased tendency to develop multi-drug resistance once resistance to a single first-line develops.

- The stability of the genetic marker IS*6110* in clinical Beijing isolates can extend over several years. This complicates the identification of mixed infection with multiple strains of the Beijing genotype.

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