Investigation of the Genetic Basis of Antibiotic Resistance in *Mycobacterium tuberculosis*

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For Mum

"*The greatest disaster that can happen to a patient with tuberculosis is that his organisms become resistant to two or more of the standard drugs. Fortunately we can prevent the emergence of drug resistance in virtually all cases if we take enough trouble to ensure that the best drug combinations are prescribed and that the patient takes them as directed. It is often not realized how venial a sin can result in ultimate disaster. It might be suggested that giving a risky combination of drugs, or even giving a drug alone, will not matter if it is only for a short time. It is true that it may not matter in a number of patients, but in some it can matter very much and may make all the difference between survival and death.*

The development of drug resistance may be a tragedy not only for the patient himself but for others. For he can infect other people with his drug-resistant organisms. In such patients the disease would not be sensitive to the drug in question. A recent survey by the Medical Research Council (1957) in various clinics all over the country has shown that no less than 5% of newly diagnosed patients were infected with organisms resistant to at least one of the three main drugs. If physicians come to apply thoroughly the present knowledge about preventing drug resistance, this percentage should steadily diminish".

- Sir John Crofton (1912 – 2009)

CONTENTS

DECLARATION

I, Joanna Evans, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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SINGLE LETTER NUCLEOTIDE CODE

A	Adenine
T	Thymine
C	Cytosine
G	Guanine
N	Any nucleotide

SINGLE LETTER AMINO ACID CODE

G	Glycine
P	Proline
A	Alanine
V	Valine
L	Leucine
I	Isoleucine
M	Methionine
C	Cysteine
F	Phenylalanine
Y	Tyrosine
W	Tryptophan
H	Histidine
K	Lysine
R	Arginine
Q	Glutamine
N	Asparagine
E	Glutamic Acid
D	Aspartic Acid
S	Serine
T	Threonine

ABBREVIATIONS

%	percent
AAC	aminoglycoside acetyltransferase
ABC	ATP-binding cassette
ACP	acyl carrier protein
ADC	albumin dextrose catalase
AGE	agarose gel electrophoresis
AIDS	Acquired Immune Deficiency Syndrome
AME	aminoglycoside modifying enzyme
ASF	alternative sigma factor
ATP	adenosine tri-phosphate
BCG	bacillus Calmette-Guerin
bp	base pair(s)
β	beta
°C	degrees Celsius
CAS	Central Asian
CCCP	*m*-chlorophenylhydrazone
cDNA	complementary DNA
CoA	coenzyme A
CO_2	carbon dioxide
C_t	cycle threshold
CTAB	Hexadecyltrimethylammonium bromide
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DR	direct repeat
DST	drug susceptibility testing

EAI	East African Indian
ECF	extracytoplasmic function
EDTA	ethylenediamine tetraacetic acid
FAM	5-carboxyfluorescein
FASI	fatty acid synthase I
FASII	fatty acid synthase II
g	gram(s)
GSH	Groote Schuur Hospital
HIV	Human Immunodeficiency Virus
H_20_2	hydrogen peroxide
IS	insertion sequence
Isoniazid	isonicotinic acid hydrazide
kb	kilobase(s)
kDa	kiloDalton(s)
l	litre(s)
LAM	Latin American-Mediterranean
LSP	large sequence polymorphism
M	molar
MAS-PCR	multiplex allele-specific-PCR
MDR	multidrug resistant
MFS	major facilitator family
mg	milligram(s)
MGB	minor groove binder
$MgCl_2$	magnesium chloride
MGIT	mycobacterial growth indicator tube
MIC	minimum inhibitory concentration
MIRU	mycobacterial interspersed repetitive unit
ml	millilitre(s)
mM	millimolar

M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger RNA
MST	multispacer sequence typing
µg	microgram(s)
µl	microlitre(s)
NaCl	sodium chloride
NAD	Nicotinamide Adenine Dinucleotide
NADH	reduced NAD
NADPH	reduced NAD phosphate
NCCLS	National Committee for Clinical Laboratory Standards
ng	nanogram(s)
OADC	oleic albumin dextrose catalase
OD	optical density
PCR	polymerase chain reaction
pmoles	picomoles
POA	pyrazinoic acid
PZase	pyazinamidase
QRDR	quinolone resistance determining region
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RRDR	rifampicin resistance determining region
rRNA	ribosomal RNA
RT-qPCR	real time quantitative PCR
SDS	sodium dodecyl sulphate
SLDs	second-line drugs
SNP	single nucleotide polymorphism
σ	sigma
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*

TB	tuberculosis
U	unit(s)
UV	ultraviolet
VNTR	variable number tandem repeat
v/v	volume per volume
WHO	World Health Organisation
w/v	weight per volume
XDR	extensively drug resistant

LIST OF FIGURES

LIST OF TABLES

Abstract

The emergence of antibiotic resistant strains of *Mycobacterium tuberculosis*, coupled with the time it takes to perform phenotypic drug susceptibility testing of this organism, makes the treatment of tuberculosis increasingly difficult. Several genotypic assays for the rapid detection of drug resistance in *M. tuberculosis* have been developed, but the sensitivity with which these assays identify resistance differs geographically. Additionally, the identification of phenotypically resistant isolates with no identifiable genotypic marker suggests that other factors, such as differential gene expression, may play a role in the development of drug resistance in *M. tuberculosis*. This investigation aims to both develop and evaluate rapid genotypic assays for the detection of resistance to both first- and second-line drugs in *M. tuberculosis*, and to investigate the role of alternative sigma factors in the progression to multidrug resistant *M. tuberculosis*.

The sensitivity of the GenoType® MTBDR*plus* [HAIN Lifescience] assay for the detection of rifampicin and isoniazid resistance was evaluated in clinical *M. tuberculosis* isolates with varying phenotypic susceptibility profiles from Cape Town, South Africa. Additionally, the use of multiplex allele-specific-PCR assays for the detection of two commonly identified isoniazid resistance determinants was evaluated in parallel. The GenoType® MTBDR*plus* [HAIN Lifescience] assay identified rifampicin and isoniazid resistance in clinical *M. tuberculosis* isolates with sensitivities of 93.5% and 82.1%, respectively, and similar results were obtained using multiplex allele-specific-PCR assays for the detection of isoniazid resistance.

Novel multiplex allele-specific-PCR assays for the detection of resistance to ofloxacin and kanamycin/amikacin in *M. tuberculosis* were designed, and their ability to correctly identify resistance to these second-line drugs was evaluated. Multiplex allele-specific-PCR assays for the detection of GyrA D94G and *rrs* A1401G correctly identified ofloxacin and kanamycin/amikacin resistance in *M. tuberculosis* in 64.3% and 80.0% of phenotypically resistant isolates, respectively. Whilst the development of genotypic assays for the rapid detection of drug resistance in *M. tuberculosis* show promise, variation in the geographical distribution of specific resistance determinants necessitates that phenotypic drug susceptibility testing be performed in parallel. However, screening for GyrA D94G and A90V together with *rrs* A1401G would

identify up to 88.0% of XDR-TB in this region prior to obtaining phenotypic drug susceptibility results, making these assays extremely useful as a rapid genotypic tool for the detection of second-line drug resistance in this setting.

The role of alternative sigma factor expression in the progression to drug resistance in *M. tuberculosis* was investigated in rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants using real-time quantitative PCR assays. Investigation of rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants indicated an association between specific RpoB mutations and an enhanced ability to grow in the presence of isoniazid. Furthermore, mutants that were able to grow in the presence of isoniazid displayed upregulation of *sigE*, which encodes a sigma factor involved in the maintenance of cell wall structure. A role for differential gene expression induced by the use of alternative sigma factors in the development of drug resistance in *M. tuberculosis* was demonstrated in rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants, and further confirmed in clinical isolates of *M. tuberculosis*.

CHAPTER 1:

General Introduction

1.1 Introduction

1.2 Phenotypic drug susceptibility testing of *M. tuberculosis*

1.3 Molecular mechanisms of antibiotic resistance in *M. tuberculosis*

1.3.1 Rifampicin resistance

1.3.2 Isoniazid resistance

1.3.2.1 Catalase-peroxidase-peroxynitrase T (KatG)
1.3.2.2 NADH-dependent enoyl-acyl carrier protein reductase (InhA)
1.3.2.3Oxidative-stress regulator (OxyR) and alkyl hydroperoxide reductase C (AhpC)
1.3.2.4 NADH dehydrogenase (*ndh*)
1.3.2.5 3-Oxoacyl-[acyl-carrier protein] synthase 1(KasA)

1.3.3 Ethambutol resistance

1.3.4 Pyrazinamide resistance

1.3.5 Fluoroquinolone resistance

1.3.6 Aminoglycoside resistance

1.4 Genotyping of *M. tuberculosis*

1.4.1 IS*6110* restriction fragment length polymorphism

1.4.2 Spacer oligonucleotide typing

1.4.3 Mycobacterial Interspersed Repeat Unit-Variable Number Tandem Repeat Typing

1.5 Gene expression in *M. tuberculosis*

1.5.1 Sigma factors in *M. tuberculosis*

1.5.1.1 SigA
1.5.1.2 SigB
1.5.1.3 SigF
1.5.1.4 Extracellular cytoplasmic function sigma factors

1.5.2 Anti-sigma factors

1.6 Aims of the study

1.1 Introduction

The isolation of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), by Robert Koch in 1882 (Kaufmann, 2005) has prompted extensive investigation into both the biology of the organism and the host immune response to infection with the organism. The use of molecular techniques indicates that *M. tuberculosis* has been plaguing mankind for up to 1000 years (Salo *et al*, 1994) and yet the TB epidemic has never been more of a global threat than it is today.

TB remains the leading cause of bacterial death in low-income countries, and it was declared a global emergency by the World Health Organisation (WHO) in 1993. It is currently estimated that one third of the world's population is latently infected with *M. tuberculosis*, and nearly 363 cases of TB per 100 000 population are seen in Africa alone (WHO, 2008a). South Africa is classified as a high-burden TB country and has the seventh highest global TB incidence, with 692 cases of TB per 100 000 population recorded in 2007 (WHO, 2009). In particular, the Western Cape region, together with the Northern Cape and KwaZulu-Natal provinces, has the highest average TB notification rate in the country at 47% (Fig. 1.1) (WHO, 2009).

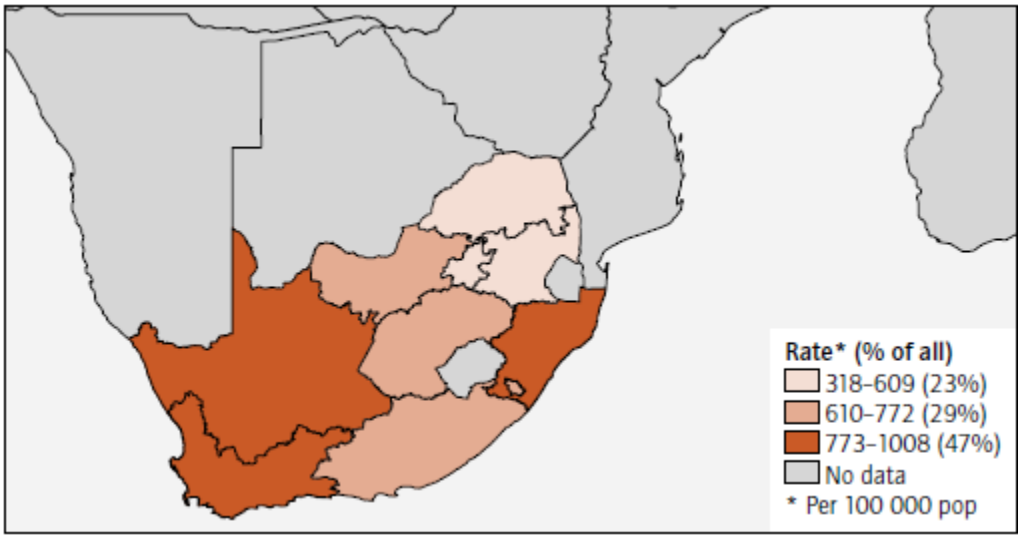

Figure 1.1: TB case notification rates of both new and relapse cases recorded in the nine provinces of South Africa in 2007. (Adapted from WHO, 2009)**.**

The association of TB incidence with HIV prevalence is clearly demonstrated in South Africa, with up to 70% of TB patients co-infected with HIV (Abdool Karim *et al*, 2009). The ability of *M. tuberculosis* to remain in a dormant state, in which the immune response contains the infection and no symptoms of active disease are observed, remains poorly understood, but is key to the success of this organism as a human pathogen.

1.2 Phenotypic drug susceptibility testing of *M. tuberculosis*

Efforts to control the spread of TB include the use of antibiotic therapy. However, the slow generation time of *M. tuberculosis* of approximately 24-36 hours necessitates the implementation of lengthy treatment regimes. Currently, standard chemotherapy for TB patients in South Africa consists of a regimen of rifampicin, isoniazid, ethambutol and pyrazinamide for a period of 2 months, followed by rifampicin and isoniazid only for a further 4 months (Jindani *et al*, 2004). Streptomycin is added to the initial 2 month regimen if the patient has previously been treated for TB (WHO, 2006). A regimen such as this is usually effective at eradicating drug-susceptible *M. tuberculosis*, but in the case of drug-resistant isolates, more costly, less effective and more toxic second-line drugs must be used.

The inappropriate use of antibiotics for the treatment of TB has, to a large extent, contributed to the emergence of drug-resistant strains of *M. tuberculosis*, and isolates that are simultaneously resistant to both rifampicin and isoniazid are defined as multidrug resistant (MDR) (WHO, 1997). The most recent global survey on the incidence of drug resistance between 2002 and 2007 revealed that 2.9% of new cases and 15.3% of previously treated cases of TB are MDR, respectively, which equates to more than 400 000 cases of MDR-TB emerging each year (WHO 2010; WHO, 2008b). It is notable, however, that more than 50% of the global MDR-TB burden in previously treated cases is in China, India and the countries of the former Soviet Union, with much lower rates observed in the rest of the world (WHO, 2008b).

In South Africa in 2007 an estimated 1.8% of new cases and 6.7% of previously treated cases were MDR (WHO, 2009). The treatment of these patients requires a 4-month course of antibiotic therapy consisting of 5 drugs, including kanamycin,

ethionamide, pyrazinamide, ofloxacin and cycloserine or ethambutol, followed by 12-18 months of ethionamide, ofloxacin and cycloserine or ethambutol (Department of Health, RSA, 1999). The increasing incidence of drug-resistant strains of *M. tuberculosis* highlights the urgent need for rapid and reliable methods of drug susceptibility testing (DST) in order to facilitate the implementation of appropriate and individualised treatment regimens.

MDR isolates with additional resistance to a fluoroquinolone and at least one of the injectable aminoglycosides, kanamycin and amikacin, or capreomycin are defined as extensively drug resistant (XDR) (WHO, 2008b). By March 2006 XDR-TB had been reported in all parts of the world, and it is especially prevalent in countries with a high HIV burden, such as South Africa. As there are limited antituberculous agents available for the treatment of XDR-TB, infection with these strains is associated with a high mortality rate (Gandhi *et al*, 2006). The most recent survey of drug-resistance in *M. tuberculosis* in South Africa indicated that in 2008 10.5% of MDR-TB cases were XDR, as compared to the estimated global prevalence of 5.4% (WHO, 2010).

In South Africa, DST is only performed on request by the physician due to the substantial cost associated with it. It was estimated that the cost of managing MDR-TB during 2007 would amount to almost 2 billion South African Rand (Department of Health, RSA, 2006), and this figure will continue to increase each year. Current protocol dictates that DST should be performed if the patient has had TB previously, if the patient remains smear positive after 2 months of treatment and/or if the patient has been in close contact with confirmed MDR-TB patients (Singh *et al*, 2007; Department of Health, RSA, 1999 & 2000). Standard DST is traditionally performed using the indirect proportions method, which involves growing the organism in drug-free media and in media containing the antibiotic at critical concentrations. The critical concentration of an antibiotic is defined as the concentration at which the growth of most of the susceptible bacterial cells is inhibited (WHO, 2008c). If more than 1% of the bacillary population is resistant to the critical concentration of the drug, the isolate is considered to be resistant to that drug (WHO, 2008c). The critical concentration of an antibiotic also varies depending on the type of media used (Table 1.1), and this must be taken into account when determining drug sensitivities.

Table 1.1: Critical concentrations of antibiotics recommended by the World Health Organisation in 2008 for the phenotypic susceptibility testing of first- and second-line drugs used in the treatment of tuberculosis.

Drug Group	Drug	[a]DST Category	DST Method Available	DST Critical Concentrations (µg/ml)				
				Lowenstein-Jensen	Middlebrook 7H10	Middlebrook 7H11	BACTEC460	MGIT960
Group 1 First-line oral anti-TB agents	Isoniazid Rifampicin Ethambutol Pyrazinamide	I I II II	Solid, liquid Solid, liquid Solid, liquid Liquid	0.2 40.0 2.0 -	0.2 1.0 5.0 -	0.2 1.0 7.5 -	0.1 2.0 2.5 100.0	0.1 1.0 5.0 100.0
Group 2 Injectable anti-TB agents	Streptomycin Kanamycin Amikacin Capreomycin Viomycin	II II II II V	Solid, liquid Solid, liquid Liquid Solid, liquid None	4.0 30.0 - 40.0 -	2.0 5.0 - 10.0 -	2.0 6.0 - 10.0 -	2.0 4.0 1.0 1.25 -	1.0 - 1.0 2.5 -
Group 3 Fluoroquinolones	Ciprofloxacin Ofloxacin Levofloxacin Moxifloxacin Gatifloxacin	III III IV IV IV	Solid, liquid Solid, liquid Solid, liquid Liquid Solid	2.0 2.0 - - -	2.0 2.0 2.0 - 1.0	2.0 2.0 - - -	2.0 2.0 - 0.5 -	1.0 2.0 2.0 0.25 -
Group 4 Oral bacteriostatic second-line anti-TB agents	Ethionamide Prothionamide Cycloserine Terizidone *P*-aminosalicylic acid Thioacetazone	IV IV IV IV IV V	Solid, liquid Solid, liquid Solid None Solid, liquid None	40.0 40.0 40.0 - 1.0 -	5.0 - - - 2.0 -	10.0 - - - 8.0 -	2.5 1.25 - - 2.0 -	5.0 2.5 - - - -
Group 5 Anti-TB agents with unclear efficacy (not recommended by WHO for routine use in MDR-TB patients)	Clofazimine Amoxicillin/clavulanate Clarithromycin Linezolid	V V V V	Liquid None None Liquid	- - - -	- - - -	- - - -	4.0 - - 1.0	- - - 1.0

[a]Categories determined by results of published studies of clinical outcome data, laboratory studies and expert opinion. Range from category I antibiotics, which have been extensively studied and for which extensive clinical data is available, to category V for which no clinical outcomes data is available. (Adapted from WHO, 2008d).

It is recommended that second-line DST be performed on all confirmed cases of MDR-TB (Department of Health, RSA, 2006). There is, however, no current international consensus for second-line DST, and critical concentrations allowing accurate DST and reproducible results have not yet been determined for all SLDs (Table 1.1) (Jureen *et al*, 2010; WHO, 2008d). In South Africa, there are also only a limited number of accredited laboratories that are equipped to facilitate second-line DST (Department of Health, RSA, 2006). The increasing incidence of XDR-TB together with the delay associated with phenotypic DST highlight the need for the development of more rapid and more accurate genotypic assays for the detection of resistance to both first- and second-line drugs. In order to facilitate this, it is imperative that further research into the molecular mechanisms of drug resistance in *M. tuberculosis* be carried out.

1.3 Molecular mechanisms of antibiotic resistance in *M. tuberculosis*

Antimicrobial chemotherapy for the treatment of TB is severely compromised by the emergence of *M. tuberculosis* isolates resistant to antituberculous agents. Contributing factors to the development of resistance include incomplete adherence and the prescription of inappropriate treatment regimens, both of which are influenced by the time delay associated with standard phenotypic DST.

The genetic basis of resistance of *M. tuberculosis* to antimicrobial agents has been extensively investigated and chromosomal mutations associated with resistance to several drugs have been described. The characterisation of such resistance determinants has led to the design of a variety of rapid molecular assays for the detection of drug resistance in *M. tuberculosis*, including single-strand conformation polymorphism and dideoxy DNA sequencing (Felmlee *et al*, 1995), line-probe assays such as INNO-Lipa Rif.TB [Innogenetics, Belgium] (Traore *et al*, 2000; Cooksey *et al*, 1997), GenoType MTBDR*plus* [HAIN Lifescience] (Evans *et al*, 2009; Barnard *et al*, 2008; Hillemann *et al*, 2007) and GenoType MTBDR*sl* [HAIN Lifescience] (Hillemann *et al*, 2009), PCR-single strand conformation polymorphism (SSCP) analysis (Telenti *et al*, 1993), molecular beacon assays (Varma-Basil *et al*, 2004), multiplex allele-specific (MAS)-PCR assays (Evans & Segal, 2010; Prammananan *et al*, 2008; Leung *et al*, 2006; Mokrousov *et al*, 2003; Mokrousov *et al*, 2002a,b) and DNA microarray analysis (Aragón *et al*, 2006), among others.

The implementation of assays such as these in the clinical setting would significantly reduce the time it takes to perform phenotypic DST, leading to the more rapid administration of appropriate therapies. A limitation of molecular assays however is that they only target selected resistance determinants, meaning that drug-resistant isolates carrying other resistance determinants would be identified as susceptible. To this end, results obtained using molecular assays alone should not always be taken as conclusive in the case of isolates that appear susceptible. In addition, many molecular assays are unable to provide conclusive results in the case of mixed infections when a susceptible strain and a resistant strain are present (Telenti *et al*, 1997a) or in the case of heteroresistance, when a small subpopulation of bacteria are resistant whilst the majority are susceptible (Falagas *et al*, 2008). Nonetheless, the significance of molecular assays in identifying resistance in those strains in which mutations can be detected should not be overlooked.

In addition to chromosomally encoded resistance determinants due to point mutations, *M. tuberculosis* also has intrinsic resistance to a large number of antibiotics, including β-lactams (Chambers *et al*, 1995), clarithromycin (Buriánková *et al*, 2004; Truffot-Pernot *et al*, 1995), azithromycin (Hoffner *et al*, 1997) and trovafloxacin (Hoffner *et al*, 1997). This both limits the use of these drugs and also reduces options for the discovery of novel antibiotics with activity against this organism. The complexity of the mycobacterial cell wall is thought to contribute to the decreased susceptibility of *M. tuberculosis* to some antibiotics by inhibiting their passage into the bacterial cell (Nguyen & Thompson, 2006; Jarlier & Nikaido, 1994). However, this cell wall impermeability is not sufficient to cause high-level resistance in the absence of additional resistance determinants, including active efflux (Nguyen & Thompson, 2006; Jarlier & Nikaido, 1994).

Several efflux pumps belonging to both the major facilitator family (MFS) and the ATP-binding cassette (ABC) transporters were identified in the *M. tuberculosis* genome (Cole *et al*, 1998). Though only partially characterised, a number have been implicated in contributing to low level resistance in *M. tuberculosis* to fluoroquinolones (Pasca *et al*, 2004), streptomycin (Spies *et al*, 2008) tetracycline (Silva *et* al, 2001; Aínsa *et al*, 1998), ampicillin (Danilchanka *et al*, 2008) and aminoglycosides (Danilchanka *et al*, 2008; Silva *et al*, 2001) through expression analysis in *Mycobacterium smegmatis*. However, given the vast differences in growth

and metabolism between the fast-growing *M. smegmatis* and the slow-growing *M. tuberculosis*, the same phenotypes might not be observed in *M. tuberculosis*.

Whilst a number of bacteria readily acquire traits such as virulence and antimicrobial resistance through the exchange of genetic material by horizontal gene transfer (Ochman *et al*, 2000), there is little evidence that this occurs in *M. tuberculosis* to contribute to its establishment as a successful human pathogen (Ochman *et* al, 2000; Cole *et al*, 1998). Rather, the predominant mechanism of acquired drug resistance in this organism is through nucleotide point mutations in chromosomally-encoded genes (Johnson *et al*, 2006; Gillespie, 2002; Billington *et al*, 1999; Ramaswamy & Musser, 1998). The rate of acquisition of mutations resulting in drug resistance in *M. tuberculosis* is relatively low, with 10^{-10} mutations occurring per cell division in the presence of rifampicin (David, 1970) as compared to 10^{-6} to 10^{-8} in *Escherichia coli* (Matic *et al*, 1997). These mutations that result in drug resistance in *M. tuberculosis* are frequently associated with alteration of the drug target or with drug titration through overproduction of the drug target (Hazbón *et al*, 2006).

The identification of common resistance determinants resulting in resistance to first-line drugs has enabled the development of rapid molecular assays for use worldwide. The high mortality rates associated with XDR-TB highlight the need for a better understanding of the molecular mechanisms by which *M. tuberculosis* acquires resistance to second-line drugs as well. In addition, as *M. tuberculosis* populations and their associated resistance determinants vary geographically, so too does the sensitivity with which these assays can accurately detect resistance determinants. It is therefore imperative that investigations into the genetic basis of resistance of *M. tuberculosis* to all drugs used in the treatment of TB continues.

1.3.1 Rifampicin resistance

Rifampicin, a lipophilic antibiotic belonging to the rifamycin group, is the primary first-line drug used in the treatment of TB. Rifampicin exerts its bactericidal effect on *M. tuberculosis* by specifically binding to the DNA-dependant RNA polymerase, thereby inhibiting transcription (Zenkin *et al*, 2005; Harshey & Ramakrishnan, 1976; Hartmann *et al*, 1967). It therefore follows that 90-98% of rifampicin resistance occurs as a result of mutations in *rpoB* (Riska *et al*, 2000), which encodes the

β-subunit of the bacterial DNA-dependant RNA polymerase. Up to 95% of *rpoB* mutations that result in resistance to rifampicin occur within an 81bp rifampicin resistance determining region (RRDR) (Fig. 1.2) (Sandgren *et* al, 2009; Ramaswamy & Musser, 1998), making this region an ideal target for use in molecular assays for the rapid detection of rifampicin resistance. It must be taken into consideration, however, that rifampicin resistance in up to 5% of isolates with mutations outside the RRDR (Sandgren *et* al, 2009; Rigouts *et al*, 2007; Yue *et al*, 2003; Heep *et al*, 2001) will go undetected using assays directed only at the RRDR of *rpoB*. As the identification of rifampicin mono-resistance is rare and the association of rifampicin resistance with resistance to isoniazid is common, rifampicin resistance is currently considered a marker for the identification of MDR-TB (Watterson *et al*, 1998). The high sensitivity with which rifampicin resistance can be detected by screening the RRDR therefore makes this region a very useful target for the rapid diagnosis of MDR-TB.

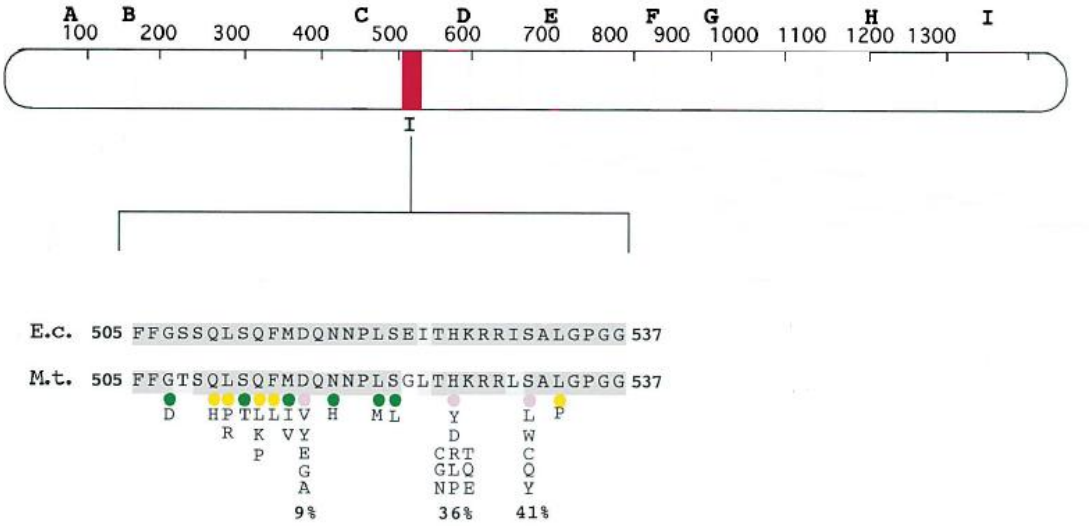

Figure 1.2: Mutations identified within the 81bp rifampicin resistance determining region (codons 507 to 533) of rpoB that are associated with rifampicin resistance in *M. tuberculosis*. The bar at the top schematically represents the structural *rpoB* gene with amino acids numbered above. The red rectangle represents the rifampicin resistance determining region; mutations that confer rifampicin resistance in *M. tuberculosis* are indicated below the sequence alignment of the *E. coli* (E.c.) and *M. tuberculosis* (M.t.) RpoB. Percentages indicate the overall global frequency with which the three most commonly mutated codons, 531, 526 and 516, are identified. (Adapted from Campbell *et al*, 2001).

Although up to 35 mutations resulting in resistance to rifampicin have been described within the RRDR of RpoB (Fig. 1.2) (Sandgren *et al*, 2009; Ramaswamy & Musser, 1998), mutations at codons 516, 526 and 531 are the most frequently observed (Cavusoglu *et al*, 2002; Valim *et al*, 2000; Ramaswamy & Musser, 1998). A serine to leucine amino acid substitution at codon 531 accounts for up to 40% of global rifampicin resistance in clinical isolates (Ramaswamy & Musser, 1998), but the distribution of mutations does vary geographically (Caws *et al*, 2006; Mokrousov *et al*, 2004; Yue *et al*, 2003; Valim *et al*, 2000; Kapur *et al*, 1994). Mutations at codons 531 and 526 appear to be associated with high-level rifampicin resistance, resulting in minimum inhibitory concentrations (MIC) of ≥ 50μg/ml (Moghazeh *et al*, 1996; Taniguchi *et al*, 1996), whilst mutations at codon 516 result in lower levels of resistance (2 – 32μg/ml) (Ohno *et al*, 1996).

The high incidence of RpoB S531L mutations associated with resistance to rifampicin is likely due to the minimal fitness cost it conveys upon the bacterium *in vitro* (Gagneux *et al*, 2006; Toungoussova *et al*, 2004; Billington *et al*, 1999). Because antibiotics exert their effect by targeting metabolic processes that are essential for the growth of the bacterium, it follows that mutations within genes associated with drug resistance may confer a fitness cost upon the bacterium (Toungoussova *et al*, 2004; Davies *et al*, 2000; Billington *et al*, 1999). Other RpoB mutations, such as the S522L amino acid substitution, confer a significant fitness cost (Gagneux *et al*, 2006; Huitric *et al*, 2006; Mariam *et al*, 2004) and, together with the low-level of rifampicin resistance associated with such mutations, may account for the minimal frequency with which these mutations are observed in clinical *M. tuberculosis* isolates (Huitric *et al*, 2006). Similarly, the RpoB S531W mutation, which is associated with high-level resistance to rifampicin, is rarely seen in clinical *M. tuberculosis* isolates, likely due to the significantly decreased fitness of strains harbouring this mutation (Mariam *et al*, 2004).

Whilst the RRDR of RpoB presents a useful target for the molecular detection of rifampicin resistance in *M. tuberculosis*, continued investigation into the effects of specific mutations within this region is still required, as is illustrated in the case of RpoB codon 533. Until recently mutation of this codon was considered an indicator of rifampicin resistance, but its subsequent identification in rifampicin susceptible *M. tuberculosis* isolates suggests that it does not play a role in resistance to this drug

(Ma *et al*, 2006; Ohno *et al*, 1996). In order to reduce the risk of unnecessary removal of rifampicin, one of the most effective drugs available for the treatment of TB, from treatment regimens, it is important that mutations that do not confer rifampicin resistance should not be included in rapid molecular assays for the detection of resistance to this drug.

1.3.2 Isoniazid resistance

In the 1940s, and up until the early 1950s, standard chemotherapy for the treatment of TB consisted of streptomycin, which, when used in conjunction with para-aminosalicylic acid, was seen to reduce the emergence of resistant bacilli (Lapp *et al*, 1952). In 1952, however, the identification of a nicotinamide derivative, isoniazid (isonicotinic acid hydrazide) (Robitzek and Selikoff, 1952), that displayed strong activity against MTB complex species (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*), was received with great anticipation within the medical fraternity. In fact, to this day, more than 50 years after the discovery of isoniazid, this antibiotic remains one of the most potent antibacterial agents available for the treatment of TB.

Though the bactericidal activity of isoniazid was evident, the precise mechanism of action of this antibiotic was only elucidated many years after its inclusion in TB treatment regimens (Takayama *et al*, 1972). It is now known that the ultimate mode of action of isoniazid in mycobacteria is inhibition of mycolic acid synthesis, with almost complete inhibition occurring after 60 minutes at 0.5µg/ml of isoniazid (Takayama *et al*, 1972). The mycobacterial cell wall is unique in that it has a lipid-rich layer surrounding the peptidoglycan layer that is found in all Gram-positive organisms. This lipid-rich layer is composed of unusual lipids, glycolipids and polysaccharides, including mycolic acids, the latter of which are unique to mycobacteria. Mycolic acids are branched β-hydroxy fatty acids that consist of a long meromycolate chain (C54-C63) with shorter (C24-C26), saturated α side chains attached (Wilson *et al*, 1999; Asselineau & Lederer, 1950). By inhibiting the synthesis of mycolic acids, the protective barrier surrounding the bacterium becomes destabilised, rendering it susceptible to external environmental stimuli. Following removal of isoniazid from cells in which the mycolic acid pathway had been inhibited by this drug *in vitro*, these cells recovered their ability to synthesise mycolic acids, indicating that isoniazid is initially bacteriostatic before bactericidal (Takayama *et al*,

1972). Importantly, this recovery of mycolic acid synthesis following the removal of isoniazid clearly emphasises the need for prolonged combination therapy with isoniazid for the treatment of TB.

Despite the potent activity of isoniazid against *M. tuberculosis*, resistance to this drug was soon observed in clinical isolates, prompting investigation into the precise mechanism by which isoniazid inhibits mycolic acid synthesis (Zhang *et al*, 1992; Middlebrook *et al*, 1954). The complex mycolic acid synthesis pathway involves a large number of genes, making the exact mode of action of isoniazid difficult to elucidate. However, several loci, particularly *katG* and *inhA*, have been well characterised as isoniazid resistance determinants, and others such as *oxyR-ahpC*, *ndh* and *kasA* have been implicated in playing a role in resistance to isoniazid.

1.3.2.1 Catalase-peroxidase-peroxynitritase T (KatG)

The first mutation associated with resistance to isoniazid in *M. tuberculosis* was identified in *katG* (Zhang *et al*, 1992). This sole catalase-peroxidase in *M. tuberculosis* plays a crucial role in the detoxification of harmful reactive oxygen species encountered within the macrophage, wherein the organism resides (Sherman *et al*, 1996). Given the apparent indispensability of a functional KatG, the association of mutations in the gene encoding this protein and resistance to isoniazid was unexpected. Further investigation revealed that KatG is required for the oxidation of the isoniazid prodrug, thereby converting isoniazid into its active form (Johnsson & Schultz, 1994; Zhang *et al*, 1992). Consequently, mutations in *katG* that prevent activation of the prodrug result in resistance to isoniazid (Quemard *et al*, 1995; Zhang *et al*, 1992). In clinical isolates of *M. tuberculosis*, *katG* mutations associated with resistance to isoniazid do not completely abolish the catalase-peroxidise activity of KatG, and therefore do not compromise the survival or virulence of the organism to a great extent (Yu *et al*, 2003). It is noteworthy that a large proportion of *in vitro*-generated isoniazid resistant mutants lose *katG* entirely, which is rarely observed in clinical isolates (Rouse & Morris, 1995) and confirms the crucial role of KatG in the intracellular survival of *M. tuberculosis* (Slayden & Barry, 2000).

A recent review of isoniazid resistance mechanisms in *M. tuberculosis* reports the identification of at least 130 mutations within *katG* that can result in a broad spectrum of isoniazid MICs, ranging from 0.2 to 256µg/ml (Vilchèze & Jacobs, 2007). Of these, a serine to threonine amino acid substitution at codon 315 of KatG is the most frequently identified (Sandgren *et al*, 2009; Ramaswamy & Musser, 1998), accounting for almost 50% of isoniazid resistance worldwide (Hazbón *et al*, 2006) and making it an ideal target for the design of rapid assays to detect isoniazid resistance in *M. tuberculosis*. Despite its prevalence, KatG S315T does not appear to confer high-level resistance to isoniazid, with intermediate MICs of 1-2µg/ml observed (Rouse & Morris, 1995). Isothermal titration calorimetry experiments have demonstrated that this S315T mutation reduces the affinity of KatG for isoniazid (Yu *et al*, 2003), thereby leading to decreased susceptibility in strains carrying this mutation due to decreased activation of the isoniazid prodrug. The KatG S315T mutation also has a minimal effect on the catalase-peroxidase activity of the enzyme, with catalase and peroxidise activities decreased by only 2-fold and 4-fold, respectively (Hazbón *et al*, 2006; Yu *et al*, 2003). The presence of this mutation therefore imposes less of a fitness cost upon the bacterium, which may account for the frequency with which it is observed in association with isoniazid resistance, despite conferring only a low level of resistance.

1.3.2.2 NADH-dependent enoyl-acyl carrier protein reductase (*inhA*)

The identification of isoniazid resistant *M. tuberculosis* isolates with no *katG* mutations prompted further investigation into the actual target of activated isoniazid. The identification of an S94A amino acid substitution in InhA in *M. smegmatis* and *M. bovis* that conferred resistance to both isoniazid and the structurally related ethionamide led to the speculation that this protein may be a target of isoniazid in *M. tuberculosis* (Dessen *et al*, 1995; Banerjee *et al*, 1994). InhA is an enoyl-acyl carrier protein reductase that, in the presence of NADH, catalyses the reduction of 2-*trans*-enoyl ACP (acyl carrier reductase), an enzyme involved in fatty acid synthesis (Quemard *et al*, 1995). The S94A mutation is situated within the binding pocket of NADH and prevents the binding of cofactor NADH to InhA, thereby inhibiting ACP reduction and disrupting the fatty acid synthesis pathway (Quemard *et al*, 1995).

That purified InhA cannot directly bind isoniazid *in vitro* confirms the necessity of KatG-mediated activation of the isoniazid prodrug (Dessen *et al*, 1995). Activated isoniazid forms a covalent bond with the nicotinamide group of NAD, and this adduct is then able to bind to and directly inhibit InhA, ultimately resulting in the disruption of mycolic acid synthesis (Rawat *et al*, 2003; Quemard *et al*, 1996). In the presence of a KatG S315T mutation, the isoniazid-NAD adduct is not formed and therefore InhA is not inhibited (Ghiladi *et al*, 2004).

A number of other mutations in InhA that confer resistance to isoniazid in *M. tuberculosis* have been identified, including I16T, I21V, I47T and I95P (Sandgren *et al*, 2009; Basso *et al*, 1998). These mutations are also located in the NADH binding pocket and therefore result in resistance to isoniazid due to the reduced affinity of InhA for NADH (Basso *et al*, 1998; Quemard *et al*, 1996; Dessen *et al*, 1995). It should be noted that in a more recent study by Rawat *et al* (2003) it was demonstrated that although the isoniazid-NAD adduct is a potent inhibitor of InhA, the I21V, I47T and S94A mutations did not result in a decreased affinity of the isoniazid-NAD adduct for the enzyme. This suggests that these mutations alone are not sufficient to prevent the inhibition of InhA by isoniazid.

In clinical isolates, however, mutations are rarely identified within the *inhA* structural gene (Hazbón *et al*, 2006), with a C-15T nucleotide substitution within the promoter region of *inhA* accounting for up to 27.2% of isoniazid resistance (Herrera *et al*, 2004). This promoter mutation leads to overexpression of *inhA*, thereby resulting in resistance to isoniazid due to drug titration (Larsen *et al*, 2002; Banerjee *et al*, 1994).

1.3.2.3 Oxidative-stress regulator (OxyR) and alkyl hydroperoxide reductase C (AhpC)

Although up to 90% of isoniazid resistance can be attributed to mutations in *katG* or *inhA*, the identification of resistant clinical isolates of *M. tuberculosis* lacking mutations in either of these genes suggests that there must be another isoniazid target(s) in this organism. In enteric bacteria, the gene encoding the OxyR transcriptional factor is located upstream of, and regulates the expression of, genes involved in the oxidative stress response, including *ahpC*, which encodes the small subunit of an alkyl hydroperoxide reductase (Sherman *et al*, 1996). The

M. tuberculosis oxyR, however, is non-functional due to the presence of multiple mutations and deletions (Deretic *et al*, 1995; Sherman *et al*, 1995). The transformation of *M. tuberculosis* H37Rv with a functional *oxyR* from the inherently isoniazid-resistant *M. leprae* results in an increase in the isoniazid MIC from 0.05µg/ml to 5µg/ml (Deretic *et al*, 1995). This, taken together with the fact that mutations in *oxyR* from *E. coli*, which is also naturally resistant to isoniazid, result in susceptibility of the bacterium to this drug (Rosner, 1993), suggests that the defective *M. tuberculosis oxyR* plays a major role in the sensitivity of this organism to isoniazid. This may be due to lack of induction of *ahpC* by OxyR (Dhandayuthapani *et al*, 1996; Zhang *et al*, 1996), as insertional inactivation of *ahpC* in *M. smegmatis* results in increased susceptibility to isoniazid (Zhang *et al*, 1996).

Though expression of *ahpC* in wildtype *M. tuberculosis* is too low to be detected (Dhandayuthapani *et al*, 1996), mutations within the promoter region of this gene lead to detectable levels of expression (Zhang *et al*, 1996). In addition, mutations within the *oxyR-ahpC* intergenic region have been identified in clinical isolates of *M. tuberculosis* displaying high level isoniazid resistance. These isolates have additional mutations in *katG*, and therefore the role of *oxyR-ahpC* in conferring resistance to isoniazid cannot be confirmed (Dhandayuthapani *et al*, 1996). As overexpression of *ahpC* in *M. tuberculosis* results in protection against organic peroxides but does not appear to increase isoniazid resistance levels (Sherman *et al*, 1996), it may be that the resultant increase in expression of AhpC compensates for the loss of KatG activity rather than playing a direct role in contributing to low-level isoniazid resistance (Dhandayuthapani *et al*, 1996). The identification of an isolate with low-level isoniazid resistance along with detectable levels of AhpC, but no promoter mutations, suggests that there may be a transcriptional regulator of *ahpC* that plays a role in isoniazid resistance in *M. tuberculosis* (Zhang *et al*, 1996). Identification of such a regulator may facilitate the identification of isoniazid-resistant *M. tuberculosis* isolates that do not carry *katG* or *inhA* mutations.

1.3.2.4 NADH dehydrogenase (*ndh*)

Other than the major determinants discussed above, additional isoniazid resistance markers have been suggested. The identification of mutations in *ndh* in isoniazid resistant *M. smegmatis* isolates suggested that this gene may play a role in resistance to isoniazid in *M. tuberculosis* (Miesel *et al*, 1998). Since mutations in *ndh*, which encodes an NADH dehydrogenase, result in a markedly increased NADH to NAD^+ ratio, NADH may compete with isoniazid for binding to InhA (Vilchèze *et al*, 2005; Miesel *et al*, 1998). Though Lee *et al* (2001) identified *ndh* mutations in clinical isoniazid-resistant isolates, others report no mutations in *ndh* (Guo *et al*, 2006), or *ndh* mutations in conjunction with *katG* mutations (Cardoso *et al*, 2007). The role of *ndh* in the development of resistance to isoniazid is therefore inconclusive.

1.3.2.5 3-Oxoacyl-[acyl-carrier protein] synthase 1 (KasA)

KasA, a β-ketoacyl-ACP-synthase that is part of the fatty acid synthase II (FASII) pathway, has been suggested as an additional marker of isoniazid resistance. Isoniazid inhibits mycolic acid synthesis by the FASII pathway, resulting in the intracellular accumulation of saturated fatty acids (C24-C26) (Mdluli *et al*, 1996). The accumulation of these short-chain fatty acids induces the expression of *acpM* and *kasA* (Fu, 2006; Wilson *et al*, 1999; Mdluli *et al*, 1998) of the *kas* operon (Fig. 1.3) (Cole *et al*, 1998). Isoniazid also induces increased expression of *acpM* and *kasA*, as well as *kasB* and *accd6* in the *kas* operon (Fig. 1.3) (Hughes *et al*, 2006). Increased expression of *fabD* in the *kas* operon following isoniazid exposure is inconclusive (Hughes *et al*, 2006; Wilson *et al*, 1999).

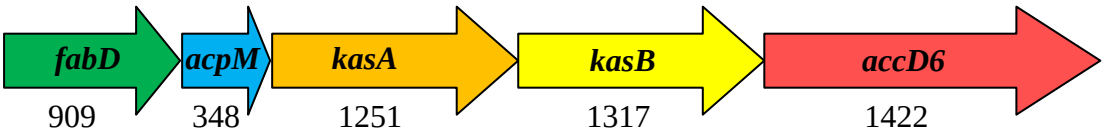

Figure 1.3: A Schematic representation of the *kas* operon of *M. tubuerculosis* H37Rv. Numbers indicate the nucleotide length (bp) of each gene.

The increased expression of *acpM* and *kasA* following exposure to isoniazid occurs within 20 minutes, suggesting that interruption of the FASII cycle occurs very rapidly in *M. tuberculosis* (Wilson *et al*, 1999). The specific role of KasA in resistance to isoniazid, however, is unclear, as isoniazid resistance is not always associated with upregulation of *kasA* (Larsen *et al*, 2002). As the reaction catalysed by InhA precedes that catalysed by KasA in the FASII pathway, mutations in *kasA* may prevent InhA binding to KasA, thereby arresting the FASII pathway (Kruh *et al*, 2007). However, although mutations within the NADH binding domain of InhA reduce the affinity of the enzyme for NADH, they have no effect on the binding of KasA, and therefore do not actually arrest mycolic acid synthesis (Kruh *et al*, 2007). This suggests, therefore, that in isolates with structural InhA mutations, another mechanism(s) of resistance likely accounts for the isoniazid resistant phenotype, further illustrating the complexity of the acquisition and maintenance of isoniazid resistance in *M. tuberculosis*.

1.3.3 Ethambutol resistance

Ethambutol [dextro-2,2`-(ethylenediimino)di-1-butanol] is one of the four drugs included in the first-line regimen for the treatment of TB in South Africa (Department of Health, RSA, 2006). Though ethambutol mono-resistance is rarely observed (Mokrousov *et al*, 2002a; Telenti *et al*, 1997b), up to 87.2% of ethambutol resistance in *M. tuberculosis* isolates from the Western Cape is associated with MDR (Johnson *et al*, 2006b).

Ethambutol inhibits arabinosyltransferases, which convert cell wall arabinan into arabinogalactan and lipoarabinogalactan (Deng *et al*, 1995; Takayama & Kilburn, 1989). The *emb* operon, comprising *embC*, *embA* and *embB*, is thought to encode integral membrane proteins that may play a role in resistance to ethambutol (Alcaide *et al*, 1997; Telenti *et al*, 1997b). Mutations located at the 5` end of *embB*, particularly at codon M306, appear to be associated with ethambutol resistance (Alcaide *et al*, 1997; Telenti *et al*, 1997b; Sreevatsan *et al*, 1997a). This region of EmbB is located extracellularly (Ramaswamy *et al*, 2000) and may favour drug-protein interactions that lead to diminished drug binding (Srivastava *et al*, 2006). An M306I substitution results in MICs of 20µg/ml, whilst M306L and M306V result in higher MICs of 40µg/ml (Ramaswamy *et al*, 2000; Sreevatsan *et al*, 1997a). Mutations at codons 406

and 497 result in ethambutol MICs of ≥30μg/ml (Ramaswamy *et al*, 2000). Though mutations within the *embC-embA* intergenic region have been described (Ramaswamy *et al*, 2000), all isolates harbouring these mutations had additional mutations in other ethambutol resistance-associated genes, making the association of these mutations with ethambutol resistance inconclusive. However, these mutations introduce transcriptional start sites into a region that appears to lack any (Telenti *et al*, 1997b), and may thereby result in altered expression of the *emb* operon (Ramaswamy *et al*, 2000).

Rapid molecular assays such as line-probe assays MAS-PCR, PCR-restriction fragment length polymorphism (RFLP) for the detection of ethambutol resistance in *M. tuberculosis* are based solely on the identification of mutations at codon 306 of EmbB (Brossier *et al*, 2010; Hillemann *et al*, 2009; Mokrousov *et al*, 2002c; Rinder *et al*, 2001). However, mutations at codon 306 of EmbB have been described in a large proportion of MDR isolates that are phenotypically susceptible to ethambutol (Tracevska *et al*, 2004; Mokrousov *et al*, 2002c; van Rie *et al*, 2001). In addition, *M. tuberculosis* isolates of the W-Beijing lineage, a particularly virulent lineage, are more likely to harbour EmbB codon 306 substitutions than non-W-Beijing isolates, regardless of ethambutol susceptibility (Mokrousov *et al*, 2002c; van Rie *et al*, 2001). EmbB codon 306 alone is therefore not sufficient to confer resistance to ethambutol, and should not form the basis of molecular assays for the detection of ethambutol resistance in *M. tuberculosis*. Rather, as overexpression of *embB* is associated with resistance to ethambutol in *M. smegmatis* (Telenti *et al*, 1997b), there may be a role for transcriptional regulators in the development of ethambutol resistance in *M. tuberculosis*, which would provide an additional marker for the molecular detection of ethambutol resistance.

1.3.4 Pyrazinamide resistance

Pyrazinamide is an important component of the current first-line regimen for the treatment of TB as it is *M. tuberculosis*-specific, displaying no activity against other mycobacteria, such as *M. smegmatis* (Zhang *et al*, 1999a) and is therefore used solely for the treatment of TB. Initially, pyrazinamide showed no *in vitro* activity against *M. tuberculosis* (Tarshis & Weed, 1953), but the efficacy of pyrazinamide as an antituberculous drug was enhanced by activation in an acidic environment

(McDermott & Tompsett, 1954). The low pH environment (pH 5.5) enhances the accumulation of pyrazinoic acid (POA), the active moiety of pyrazinamide, due to a pH gradient (Zhang *et al*, 1999a). For this reason, pyrazinamide targets slow-growing, semi-dormant bacilli found in acidic environments in the lysosomes and phagolysosomes of macrophages and at active inflammation sites (Mitchison, 1985).

Pyrazinamide, a prodrug, is hydrolysed by pyrazinamidase (PZase) (Zhang *et al*, 1999a; Scorpio & Zhang, 1996; McClatchy *et al*, 1981), encoded by *pncA*, into its active form, POA (Scorpio & Zhang, 1996). Mutations within the active site of *pncA* correlate with resistance to pyrazinamide (Sandgren *et al*, 2009; Unissa *et al*, 2009; Barco *et* al, 2006; Cheng *et* al, 2000; Sreevatsan *et al*, 1997b; Scorpio *et al*, 1997) and presented as a good indicator of pyrazinamide resistance (Trivedi & Desai, 1987; McClatchy *et al*, 1981). However, the susbequent identification of strains with high-level resistance to pyrazinamide that are PZase-positive and have no mutations in the structural *pncA* gene nor in the promoter region suggest that there is another mechanism(s) of resistance to pyrazinamide in *M. tuberculosis* (Barco *et* al, 2006; Cheng *et al*, 2000; Butler & Kilburn, 1983). The accumulation of POA is thought to reduce the environmental pH, leading to non-specific inhibition of cellular metabolism (Boshoff *et al*, 2002; Boshoff & Mizrahi, 2000). Though POA was thought to act upon fatty acid synthase I (FASI) during the first stage of mycolic acid synthesis (Zimhony *et al*, 2007), no significant inhibition of mycobacterial FASI could be detected, suggesting that this enzyme is not the ultimate target of POA (Boshoff *et al*, 2002).

Because pyrazinamide is only active *in vitro* under acidic conditions and many clinical *M. tuberculosis* isolates grow poorly under such conditions, the standardisation of phenotypic DST of pyrazinamide is very difficult (McClatchy *et al*, 1981). The development of a rapid molecular assay for the detection of mutations associated with resistance to pyrazinamide would therefore be very useful for identifying *M. tuberculosis* isolates resistant to this drug. That more than 120 mutations have been implicated in resistance to pyrazinamide (Cheng *et al*, 2000) and that these mutations occur throughout the entire gene (Hirano *et al*, 1997; Sreevatsan *et al*, 1997b), however, makes the development of such an assay with high sensitivity a challenge. The identification of pyrazinamide-resistant *M. tuberculosis* isolates that are PZase-negative and yet have no identifiable mutations in *pncA* or the promoter

region, suggests the role of a regulator of *pncA* expression, and the identification of the gene encoding such a protein may be useful in the design of molecular assays for the detection of pyrazinamide resistance in *M. tuberculosis* (Cheng *et al*, 2000; Hirano *et al*, 1997).

1.3.5 Fluoroquinolone resistance

The fluoroquinlones ofloxacin and ciprofloxacin are included in the treatment regimen for MDR-TB cases in South Africa (Department of Health, RSA, 1999). This group of antimicrobials target bacterial DNA gyrase and topoisomerase IV enzymes, both of which are involved in DNA replication (Ruiz, 2003). Quinolones such as ofloxacin, ciprofloxacin and levofloxacin are considered older quinolones, with moxifloxacin, gatifloxacin and sparfloxacin, which generally have lower MICs, considered as newer quinolones (Ginsburg *et al*, 2003a). Across bacterial species, different fluoroquinolones favour different enzyme targets; in Gram-negative bacteria, *gyrA* and *gyrB*, which encode the DNA gyrase subunits, are generally the primary targets, whereas in Gram-positives, *parA* and *parC*, which encode the subunits of topoisomerase IV, are favoured (Hooper, 2001; Hooper, 2000). *M. tuberculosis*, however, lacks a topoisomerase IV, and only has a DNA gyrase consisting of two A subunits, encoded by *gyrA*, and two B subunits, encoded by *gyrB* (Cole *et al*, 1998). DNA gyrase binds to DNA and introduces the negative superhelical twists that are required for the initiation of DNA replication (Drlica & Zhao, 1997). Fluoroquinolones act by stabilising the DNA-DNA gyrase complex, thereby blocking the movement of the replication fork and inhibiting DNA replication, resulting ultimately in cell death (Hooper 2001; Drlica, 1999).

Mutations resulting in fluoroquinolone resistance in *M. tuberculosis* occur predominantly within the quinolone resistance determining region (QRDR) of *gyrA*, with amino acid substitutions at codon 94 of GyrA most frequently observed (Wang *et al*, 2007; Pitaksajjakul *et al*, 2005; Takiff *et al*, 1994). However, different mutations result in different levels of fluoroquinolone resistance (Alangaden *et al*, 1995), and high-level resistance appears to arise due to the step-wise accumulation of acquired mutations (Zhou *et al*, 2000; Kocagöz *et al*, 1996; Takiff *et al*, 1994). That there have only been five reports of mutations within *gyrB* in clinical *M. tuberculosis* isolates (Von Groll *et al*, 2009; Wang *et al*, 2007; Aubry *et al*, 2006; Pitakksajjakul *et al*,

2005; Lee *et al*, 2002) suggests that this subunit does not contribute significantly to fluoroquinolone resistance. Furthermore, the complexity of fluoroquinolone resistance is highlighted by the identification of strains carrying both a T80A and a A90G mutation in GyrA that have reduced MICs compared to the *M. tuberculosis* H37Rv parent strain (Aubrey *et al*, 2006). It may be that the presence of both mutations enhances quinolone binding, thus stabilising the DNA-DNA gyrase complex even more (Aubrey *et al*, 2006). A large proportion of isolates carry a S95T amino acid substitution in GyrA, but rather than be associated with drug resistance (Takiff *et al*, 1994) this mutation is thought to be an evolutionary marker (Sreevatsan *et al*, 1997c).

Though cross-resistance within the fluoroquinolone group of antibiotics is common (Alangaden *et al*, 1995), fluoroquinolone resistance is seemingly not associated with resistance to rifampicin or isoniazid (Alanganden *et al*, 1995; Collins & Uttley, 1985). Instead, fluoroquinolone resistance is primarily seen in patients with MDR-TB (Wang *et al*, 2007; Bozeman *et al*, 2005), due to inclusion of this drug in failing multidrug regimens (Huang *et al*, 2005; Ginsburg *et al*, 2003a). In addition, the empiric use of fluoroquinolones for the treatment of other bacterial infections such as pneumococcal infections may lead to an increase in the prevalence of fluoroquinolone-resistant *M. tuberculosis* (Von Groll *et al*, 2009; von Gottberg *et al*, 2008; Ginsburg *et al*, 2003b), although this is not confirmed (Huang *et al*, 2005).

It is notable that although mutations within the QRDR are the most frequently observed mechanism of resistance to fluoroquinolones in clinical *M. tuberculosis* isolates, they are reported in 42-85% of cases, suggesting that mutations elsewhere in *gyrA*, or other mechanisms of resistance, such as active efflux, may play a role in fluoroquinolone resistance in this organism (Pasca *et al*, 2004; Ginsburg *et al*, 2003a). Thus, the use of the QRDR alone for rapid molecular assays that detect resistance to fluoroquinolones is not sufficient, and the identification and inclusion of other resistance markers in such assays is needed for increased sensitivity.

1.3.6 Aminoglycoside resistance

The aminoglycoside group of antibiotics inhibit protein synthesis by binding to the 30S subunit of the bacterial ribosome, which is required for high-fidelity translation (Vakulenko & Mobashery, 2003). In 1944, streptomycin was the first of the

aminoglycoside antibiotics to be discovered because of its activity against *M. tuberculosis* (Vakulenko & Mobashery, 2003; Blanchard, 1996). Resistance to streptomycin, however, was soon observed in *M. tuberculosis* (Crofton & Mitchison, 1948), and the molecular mechanisms of resistance to this drug have since been extensively investigated.

Streptomycin resistance occurs due to point mutations in *rpsL* and *rrs*, which encode the ribosomal protein S12 and the 16S rRNA, respectively (Sandgren *et al*, 2009; Douglass & Steyn, 1993; Finken *et al*, 1993). RpsL interacts with 16S rRNA and maintains the higher-order structure of 16S rRNA, and therefore mutations in RpsL alter the RpsL-16S rRNA interaction, leading to streptomycin resistance (Sreevatsan *et al*, 1996). Although mutations in both genes can result in streptomycin resistance, *rpsL* is implicated in resistance to this antibiotic in *M. tuberculosis* more often than *rrs* (Tracevska *et al*, 2004; Blanchard, 1996; Sreevatsan *et al*, 1996; Morris *et al*, 1995; Heym *et al*, 1994). Mutations at codons 43 and 88 of RpsL are most frequently observed in association with streptomycin resistance worldwide, with organisms harbouring codon Lys43Arg mutations displaying MICs of >200µg/ml (Via *et al*, 2010; Riska *et al*, 2000; Sreevatsan *et al*, 1996). Mutations in *rpsL* are associated with high-level resistance (500-1000µg/ml) to streptomycin, whilst mutations in *rrs* more frequently result in intermediate-level resistance (50-500µg/ml) to this drug (Cooksey *et al*, 1996; Meier *et al*, 1996). Though rare (Sreevatsan *et al*, 1996), isolates with mutations in both genes display elevated levels of resistance (Meier *et al*, 1996). With approximately 25-35% of streptomycin resistant isolates having wildtype *rpsL* and *rrs* genes, the existence of other mechanism(s) of resistance to streptomycin in *M. tuberculosis* is likely (Sreevatsan *et al*, 1996).

In 1993, amikacin was found to have activity against MDR-TB and, together with kanamycin, has since been a critical component of the second-line regimen used to treat infection with drug-resistant *M. tuberculosis*. In contrast to streptomycin, the majority of amikacin and kanamycin resistance determinants are located in *rrs* rather than *rpsL* (Via *et al*, 2010; Suzuki *et al*, 1998), and the most frequently observed mutation in *rrs* is an A1401G nucleotide substitution (Brossier *et al*, 2010; Maus *et al*, 2005a; Camus *et al*, 2002; Alangaden *et al*, 1998; Suzuki *et al*, 1998). This mutation is associated with high-level resistance to both amikacin and kanamycin at >256µg/ml (Maus *et al*, 2005b; Alangaden *et al*, 1998), whilst isolates with MICs ≤64µg/ml do

not carry this mutation (Alangaden *et al*, 1998). Amikacin and kanamycin have almost complete cross-resistance (WHO, 2006; Alangaden *et al*, 1998), with resistance to one of these drugs automatically excluding the use of the other in treatment regimens (Department of Health, RSA, 1999). However, the identification of an aminoglycoside acetyltransferase, Eis, that results in low-level resistance to kanamycin but not amikacin suggests that this is not always the case (Zaunbrecher *et al*, 2009).

Capreomycin is used to treat drug-resistant TB, and is one of the only antituberculous drugs available that is bactericidal for non-replicating *M. tuberculosis* (Heifets *et al*, 2005). Though little is known about the mechanism of action, capreomycin appears to interfere with translation in mycobacteria (Maus *et al*, 2005a). TlyA is an rRNA methyltransferase, and mutation of *tlyA* appears to confer resistance to both capreomycin and viomycin in *M. tuberculosis* (Maus *et al*, 2005a). TlyA-mediated methylation of rRNA is required for capreomycin susceptibility, and therefore unmethylated ribosomes result in capreomycin resistance (Johansen *et al*, 2006; Maus *et al*, 2005a). Sequence analysis of *rrs* from 30 *M. tuberculosis* H37Rv *in vitro* generated capreomycin resistant mutants revealed that none had mutations in this gene (Maus *et al*, 2005a), indicating that *rrs* is not an appropriate marker for detecting capreomycin resistance. However, investigation of clinical *M. tuberculosis* isolates indicated complete cross-resistance between kanamycin, amikacin and capreomycin in 100% of isolates carrying *rrs* A1401G (Via *et al*, 2010). Mutations in *tlyA* alone appear to confer resistance to capreomycin and viomycin, but not amikacin and kanamycin (Maus *et al*, 2005a,b). However, *tlyA* mutants grown *in vitro* in the presence of high concentrations of amikacin or kanamycin acquire resistance to these antibiotics due to *rrs* A1401G, simultaneously resulting in approximately 4-fold increases in capreomycin and viomycin MICs (Maus *et al*, 2005b).

The identification of multiple resistance determinants for each antituberculous drug, together with the variable geographical distribution of mutations, makes the design of molecular assays that are sufficiently sensitive to detect drug-resistance in *M. tuberculosis* worldwide a challenge. Genotypic analysis of *M. tuberculosis* has enabled the classification of isolates into different lineages, and has led to the suggestion that particular lineages may be more likely to acquire resistance determinants than others (Brimacombe *et al*, 2007). Due to the slow mutation rate of

M. tuberculosis, dissemination of drug-resistant *M. tuberculosis* strains within localised areas would presumably result in an overrepresentation of particular mutations in those regions (Johnson *et al*, 2006c; Marais *et al*, 2006). To this end, genotypic analysis of the predominant local *M. tuberculosis* strain population(s) present in specific geographic locations may provide insight into the design of molecular assays with higher sensitivities of resistance detection in *M. tuberculosis* isolates from those regions.

1.4 Genotyping of *M. tuberculosis*

Genotyping techniques are not only valuable epidemiological tools, but they also provide a wealth of information with regard to the phylogeny of the *M. tuberculosis* complex (Sola *et al*, 2003). Indeed, genotypic analyses have provided evidence that *M. tuberculosis* may have emerged as recently as 15 000 to 20 000 years ago (Ramaswamy *et al*, 2000). A variety of genotyping tools are commonly used for investigating outbreaks of TB and for determining the epidemiology of disseminated clones within communities (Telenti *et al*, 1997a).

The most important consideration when devising any epidemiological assay is its ability to discriminate between closely related lineages. This is of particular importance when considering *M. tuberculosis*, due to the extremely slow rate of mutation associated with this organism. Currently, genotypic analysis of *M. tuberculosis* requires the use of a combination of genotyping methods for differentiation of strain lineages and to track epidemiological links (Sola *et al*, 2003; Cowan *et al*, 2002). IS*6110* RFLP, spacer oligotyping (spoligotyping) and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) are frequently used for genotyping *M. tuberculosis*. Other methods include single nucleotide polymorphism (SNP) allele typing (Gutacker *et al*, 2002), large sequence polymorphism (LSP) or deletion analysis (Parsons *et al*, 2002) and multispacer sequence typing (MST) (Djelouadji *et al*, 2008). However, these methods can be time-consuming and expensive, and their reproducibility has yet to be demonstrated globally.

1.4.1 IS*6110* restriction fragment length polymorphism

The ability of insertion sequence (IS) elements to readily transpose within the genome would seemingly make them an unlikely tool for the epidemiological typing of any organism. In 1990, however, an IS element unique to *M. tuberculosis* complex bacteria, designated IS*6110*, was identified, and now forms the basis of a commonly used genotyping tool for *M. tuberculosis* (McAdam *et al*, 1990; Thierry *et al*, 1990). Though IS*6110* is present in variable copy number and integrates at multiple loci in *M. tuberculosis* (McAdam *et al*, 1990), it is relatively stable within the genome despite serial passage of up to 24 generations (van Soolingen *et al*, 1991). This led to the development of IS*6110* RFLP as a highly discriminatory, standardised genotyping tool (van Embden *et al*, 1993; Hermans *et al*, 1990).

IS*6110* RFLP involves identifying the number of copies of IS*6110* in each isolate, as well as the relative insertion sites of each copy through restriction of the genome with endonucleases (van Embden *et al*, 1993). It is a particularly useful technique for investigating the evolution of *M. tuberculosis* isolates as the infrequent transposition of IS*6110* over time can serve as an indicator of strain divergence, and this may aid in determining the origins of particular strain lineages (van Soolingen *et al*, 1995). IS*6110* RFLP typing is, however, a labour-intensive technique (Supply *et al*, 2001), with a major drawback being its poor discriminatory power when used on isolates with low IS*6110* copy number (Barlow *et al*, 2001; van Soolingen *et al*, 1993). This then necessitates further strain differentiation using alternative genotyping techniques (Barlow *et al*, 2001; Warren *et al*, 1996). In addition, it is possible, particularly with low-copy number strains, that IS*6110* translocation may have occurred independantly at the same position(s) in two distinct isolates, causing these isolates to appear to be related (Warren *et al*, 1996). Despite the development of other techniques with better reproducibility, IS*6110* RFLP typing remains the gold-standard for genotyping *M. tuberculosis* as it is both reproducible and sufficiently discriminatory to facilitate the comparison of global genotype distribution (van Embden *et al*, 1993).

1.4.2 Spacer oligotyping

Unlike IS*6110*, IS*987* in *M. bovis* BCG is present as a single copy in one chromosomal location (van Soolingen *et al*, 1991; Hermans *et al*, 1991; Hermans *et al*, 1990). Cloning of IS*987* from *M. bovis* BCG revealed that this element is located within a region of the genome that contains several direct repeats (DR) of 36bp each, interspersed with variable segments of spacer DNA (Hermans *et al*, 1991). Investigation of this region in *M. tuberculosis* complex bacteria revealed various combinations of DRs in different species of the complex (Fig. 1.4), thought to have arisen due to homologous recombination between different members of the complex (Fang *et al*, 1998; Groenen *et al*, 1993). In *M. tuberculosis*, the number of DRs present varies between isolates and the spacer sequences located between these DRs are unique (Hermans *et al*, 1991). Interestingly, multiple copies of IS*6110* are also located in the DR region of the genome, suggesting that this may be a hot-spot particularly susceptible to IS integration in *M. tuberculosis* (Hermans *et al*, 1991).

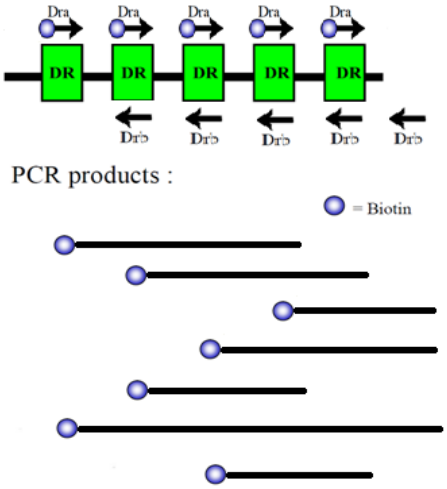

Figure 1.4: Schematic representation of the direct repeat regions in the *M. tuberculosis* genome and the primers used in the amplification of the variable spacer regions in spoligotyping. The production of biotinylated amplicons of various lengths facilitates visualisation following hybridisation to covalently-bound spacer regions. (Adapted from the Spoligotyping Kit Manual, Isogen Biosolutions).

The variation in the sequence and the presence of spacer regions in different *M. tuberculosis* isolates forms the basis of a genotyping tool termed spacer oligotyping, or 'spoligotyping' (Kamerbeek *et al*, 1997). Primers that anneal to the conserved DR regions within the chromosome are used to amplify the variable spacer regions (Fig. 1.4). Following hybridisation of the amplicons to spacer regions

covalently linked to a nitrocellulose membrane, a pattern representative of the spacer sequences present in a particular isolate is obtained. Although less discriminatory than IS*6110* RFLP, the major advantage of spoligotyping is that it is relatively simple to perform and is highly reproducible, which has enabled the formation of large databases to monitor global strain distributions (Filliol *et al*, 2002). In addition, reliable results can be obtained using poor quality DNA or low concentrations of DNA that would preclude typing by IS*6110* RFLP (Warren *et al*, 2002).

1.4.3 Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat Typing (MIRU-VNTR)

Mycobacterial interspersed repetitive units (MIRUs) (Supply *et al*, 2001; Supply *et al*, 2000) are regions of the *M. tuberculosis* genome containing variable number tandem repeats that can be used for distinguishing between genotypically distinct isolates (Frothingham & Meeker O'Connell, 1998) (Fig. 1.5). These repeat regions can be polymorphic or exact repeats, and although copy numbers of several repeat regions remain fairly constant in most strains of *M. tuberculosis*, there are several that are sufficiently variable to make them valuable as epidemiological tools (Frothingham & Meeker O'Connell, 1998) (Fig. 1.5).

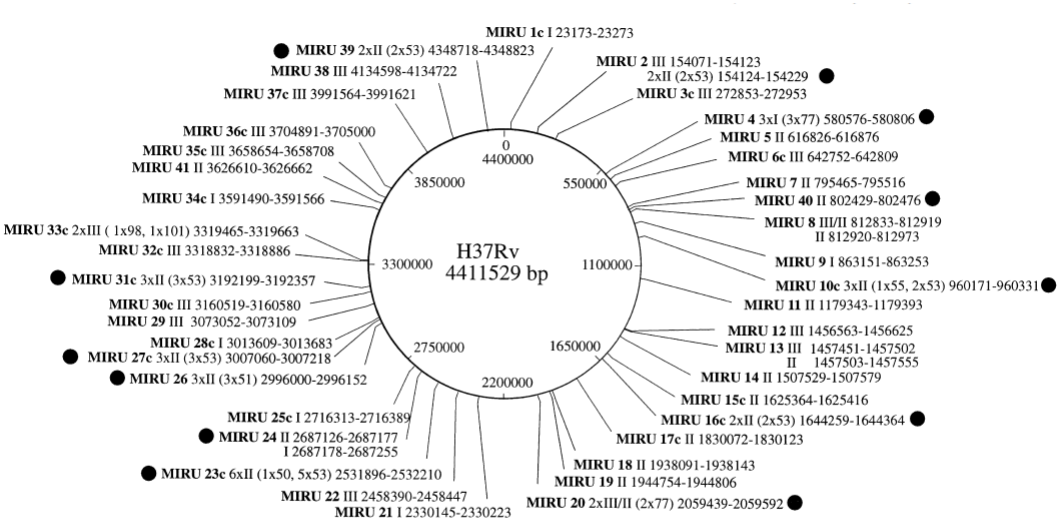

Figure 1.5: A schematic representation of the positions of the MIRU loci on the *M. tuberculosis* H37Rv chromosome. Black dots indicate the 12 variable loci used in MIRU-VNTR typing. (Adapted from Supply *et al*, 2000).

MIRU- VNTR was originally devised using 12 loci to distinguish between isolates of varying lineages (Supply *et al*, 2001), but has since become even more discriminatory by including 24 loci (Maes *et al*, 2008). Although it has been modified for use on an automated DNA sequencer, this PCR-based technique can be carried out with relative ease by agarose gel electrophoresis in rural laboratories that lack access to automated analysis, making this a valuable genotyping tool (Sola *et al*, 2003).

MIRU-VNTR is comparatively as discriminatory as IS*6110* RFLP (Supply *et al*, 2001), and it is particularly useful for characterising isolates with low IS*6110* copy number (Cowan *et al*, 2002). It is also a much more discriminatory secondary genotyping tool to IS*6110* RFLP than spoligotyping (Kwara *et al*, 2003; Cronin *et al*, 2001). This, taken together with the fact that it is significantly less labour intensive and can be performed much more rapidly, makes it a potential candidate to replace IS*6110* RFLP as gold standard method of genotyping *M. tuberculosis* isolates (Maes *et al*, 2008).

1.5 Gene expression in *M. tuberculosis*

M. tuberculosis isolates belonging to distinct lineages are defined by unique characteristics, including virulence (Valway *et al*, 1998), ability to evade host immune responses (Wu *et al*, 2009) and acquisition of drug resistance (Marais *et al*, 2006; Bifani *et al*, 2002), which manifest as a result of transcriptional regulation in response to external stimuli. Additionally, the ability of *M. tuberculosis* to proliferate within the acidic phagosome (Schaible *et al*, 1998; Via *et al*, 1998) and to withstand the reactive oxygen/nitrogen intermediates produced by the macrophages (Chan *et al*, 1992; Ding *et al*, 1988) leads to the selection of strains expressing genes that enable survival in these conditions. The transcriptional regulation of these genes is therefore critical to the survival of the invading *M. tuberculosis* isolates.

Gene expression is largely regulated by the selective transcription of bacterial genes in a complex regulatory network in response to both internal and external stimuli. Prokaryotic transcription is carried out by the DNA-dependant RNA polymerase, the core enzyme of which consists of 5 subunits, $\beta\beta`\alpha_2\omega$ (Burgess, 1969). The β and β` subunits form the active site of the core enzyme, facilitating both template DNA and RNA product binding (Korzheva *et al*, 2000). In the assembly of the core enzyme,

the two α subunits associate first, and the amino-terminal domains of the α subunits then facilitate the assembly of the β and β` subunits (Hayward *et al,* 1991). Despite the presence of a ω subunit in all bacterial species, the ability to assemble a functional RNA polymerase in its absence makes its role in transcription harder to define (Hampsey, 2001; Burgess, 1969). It has been proposed, however, that it may assist in the folding of the β` subunit (Hampsey, 2001).

The primary step in transcription involves the recognition of and binding to specific promoter sequences located upstream of the gene to be expressed (McClure, 1985). Whilst the core RNA polymerase directs transcription elongation (Polyakov *et al*, 1995), it is the binding of the σ factor to the core enzyme to form the holoenzyme that facilitates promoter recognition, template binding and initiation of transcription (Lesley & Burgess, 1989; Burgess, 1969). Following binding of the holoenzyme to the promoter, the RNA polymerase forms the 'transcription bubble', a region of unwound double-stranded template DNA in the vicinity of the transcriptional start point and elongation of the transcript can then proceed (Murakami *et al*, 2002; Vassylyev *et al*, 2002).

It was traditionally thought that the sigma factor dissociated from the holoenzyme following initiation of transcription, to be reused in subsequent transcriptional processes (Krummel & Chamberlin, 1989; Hansen & McClure, 1980; Travers & Burgess, 1969). Recent evidence suggests, however, that under certain circumstances the sigma factor may actually remain associated with the RNA polymerase throughout elongation (Bar-Nahum & Nudler, 2001; Mukhopadhyay *et al*, 2001). In addition, the sigma factor is more likely to remain associated with the core enzyme during elongation in stationary phase than in exponential phase (Bar-Nahum & Nudler, 2001). This may reflect the tighter regulation of specific subsets of genes required for maintenance of stationary phase growth as compared to vegetative growth in which a much larger range of metabolic processes are mediated.

The specificity of transcriptional regulation mediated by sigma factors arises from their recognition of specific promoter sequences upstream of the genes within their regulons (Beaucher *et al*, 2002). The identification of promoter motifs specific to each individual sigma factor therefore facilitates the identification of potential gene candidates regulated by that sigma factor. *M. tuberculosis* promoters have a high

GC content (57%) as compared to those of *M. smegmatis* (43%), and transcription from *M. tuberculosis* promoters usually initiates at a purine nucleotide (Bashyam *et al*, 1996). Mycobacterial promoters are characterised by the presence of a Pribnow box sequence that is located near to nucleotide position -10 (Bashyam *et al*, 1996). That a larger proportion of *E. coli* promoters are recognised in *M. smegmatis* (Bashyam *et al*, 1996) than vice versa (Das Gupta *et al*, 1993) suggests that mycobacterial sigma factors recognise a more diverse selection of sequences than *E. coli* sigma factors. Additionally, that *M. tuberculosis* has almost twice as many sigma factors (Cole *et al*, 1998) as the 7 identified in *E. coli* (Lacour *et al*, 2004) likely accounts for the greater variation of promoter sequences recognised in mycobacteria. Several mycobacterial genes have more than one promoter (Unniraman *et al*, 2002; Mulder *et al*, 1999; Dhandayuthapani *et al*, 1997) and it may be that these are each recognised by different sigma factors, thereby enabling differential regulation of expression under varying conditions (Mulder *et al*, 1999). This tighter regulation of various subsets of genes in *M. tuberculosis* may enable survival in very diverse environments and metabolic states, and thereby contribute to its success as a human pathogen.

As different sigma factors recognise different promoter sequences (Beaucher *et al*, 2002), the presence of alternative sigma factors is a very elegant way of controlling gene expression (Rodrigue *et al*, 2006). Transcriptional regulation and alternative sigma factor usage play an essential role in virulence by facilitating the expression of different subsets of genes depending upon the stage of infection (Talaat *et al*, 2004). Analysis of the *M. tuberculosis* H37Rv genome identified a total of 13 putative sigma factors (Cole *et al*, 1998). The ability of *M. tuberculosis* to remain dormant for extended periods during latency and then reactivate to cause active disease indicates that gene expression in *M. tuberculosis* is tightly regulated. Further investigation into the selective use of alternative sigma factors and their promoter recognition sequences in this organism may provide more insight into the mechanisms by which this pathogen is able to exist in such diverse environments and metabolic states, and to survive different selective pressures.

1.5.1 Sigma factors in *M. tuberculosis*

Prokaryotic sigma factors can be divided into two major families, namely σ^{70} and the structurally unrelated σ^{54} families (Merrick, 1993; Lonetto *et al*, 1992). Although sigma factors belonging to both of these families bind to the same core polymerase (Buck *et al*, 2000), the σ^{54} family are rare (Kill *et al*, 2005) and are not found in *M. tuberculosis* (Cole *et al*, 1998). This family of sigma factors differ from the σ^{70} family in that they recognise regions -12 and -24 relative to the transcriptional start site, rather than the consensus -10 and -35 regions recognised by σ^{70} factors (Ninfa *et al*, 1989). The σ^{54} family are found in a variety of organisms including *E. coli*, *Salmonella typhimurium*, *Pseudomonas* spp. and *Klebsiella* spp., where they mediate a wide range of processes, including nitrogen fixation, pilin and flagellin synthesis and amino acid utilisation (Merrick, 1993).

The σ^{70} family are ubiquitous and consist of both the principal sigma factors responsible for the transcription of the major housekeeping genes, and additional related alternative sigma factors that regulate specific subsets of genes when metabolic changes are conferred upon the organism, such as heat-shock, sporulation and stationary phase growth (Browning & Busby, 2004; Gruber & Gross, 2003; Paget & Helmann, 2003). Sigma factors belonging to the σ^{70} family range in number from zero in *Chlorobium tepidum*, which lacks a housekeeping sigma factor altogether (Kill *et al*, 2005), to 65 in *Streptomyces coelicolor*, 45 of which are extracellular cytoplasmic function (ECF) sigma factors (Bentley *et al*, 2002). The natural habitat of *S. coelicolor* is in soil, and the ability of this organism to survive for long periods in a dormant spore-like state likely requires the interaction of several regulatory networks involving a large number of sigma factors (Lonetto *et al*, 1994). In addition, the presence of multiple internal promoters within *S. coelicolor* operons suggests that a large number of sigma factors are required for the recognition of a wide variety of specific promoter sequences (Laing *et al*, 2006). In general, however, the number of sigma factors present in an organism appears to correlate with both the size of the genome and the diversity of environmental stimuli encountered by the organism (Kill *et al*, 2005). It is therefore not surprising that the *M. tuberculosis* genome contains 13 putative sigma factors, which is a larger number than found in most bacteria (Kill *et al*, 2005). The presence of 13 sigma factors suggests that the regulation of

transcription in *M. tuberculosis* is complex, and likely plays a major role in virulence and antibiotic resistance in this organism (Rodrigue *et al*, 2006; Cole *et al*, 1998).

The function of sigma factors relates directly to their structure, and based on this they can be further subdivided into phylogenetic groups (Paget & Helman, 2003; Lonetto *et al*, 1994) (Fig. 1.6). The first includes the essential primary sigma factors, which are those most closely related to σ^{70} of *E. coli*; the second comprises the non-essential secondary sigma factors that are structurally related to the primary σ^{70}; the third includes the more distantly related sigma factors; and the fourth consists of the ECF subfamily of sigma factors that respond to external environmental stimuli.

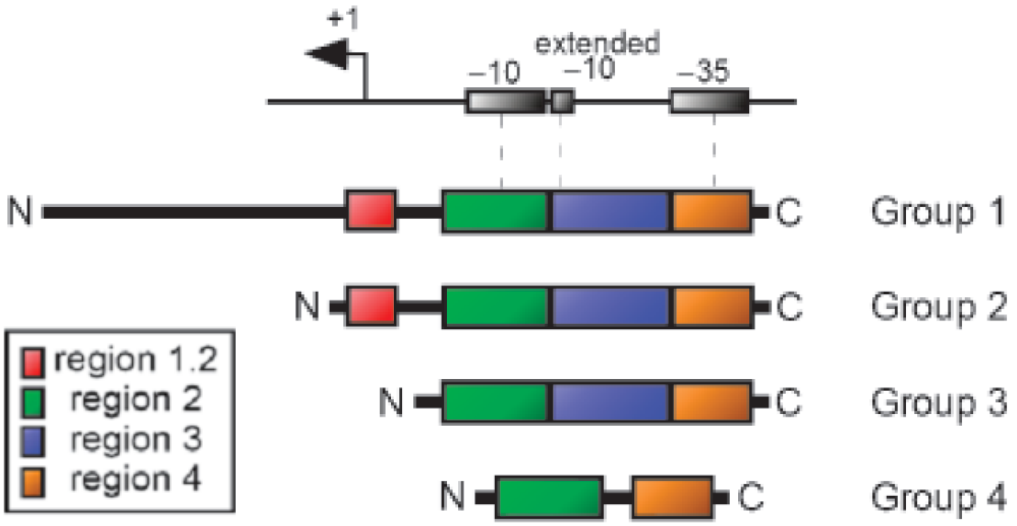

Figure 1.6: Schematic representation of the four phylogenetic groups of the σ^{70} family. The dashed lines indicate the region of the sigma factor that recognises the corresponding promoter region. The transcriptional start site and the direction in which transcription proceeds is indicated by an arrow. (Adapted from Rodrigue *et al*, 2006).

All σ^{70}-related sigma factors have four conserved regions, 1 to 4, and these regions are further classified into subregions (Fig. 1.7) (Lonetto *et al*, 1992). It is interesting to note that region 1 is present in all primary sigma factors but is absent in sigma factors from the other phylogenetic groups, whilst the remainder of the protein is fairly conserved throughout all groups (Lonetto *et al*, 1992). This suggests a central role for region 1 of primary sigma factors in regulating housekeeping genes, and it may be that region 1 is required for the recognition of promoter sequences upstream of these essential genes. Region 2.1 is the core binding domain of the sigma factor,

which interacts with the RNA polymerase core enzyme in the formation of the holoenzyme (Fig. 1.7) (Lesley & Burgess, 1989). Recognition of the -10 promoter sequence occurs via region 2.4 of the sigma factor (Fig. 1.7) (Paget & Helman, 2003; Siegele *et al*, 1989), and it follows that this region is less conserved amongst the alternative factors than the primary sigma factors, as it facilitates the recognition of distinct promoter sequences (Lonetto *et al*, 1992). Following recognition of the -35 element by region 4.2 (Fig. 1.7) (Paget & Helman, 2003), region 2.3 facilitates the unwinding of the DNA template from positions -11 to +4 (Paget & Helman, 2003), allowing transcription to proceed.

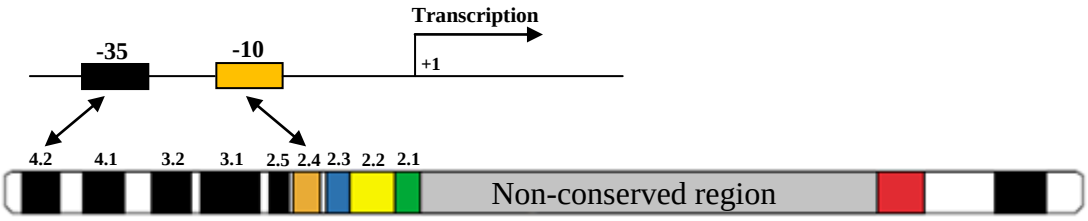

Figure 1.7: Schematic representation of sigma factors belonging to the σ^{70} family. Sigma factors can be divided into 4 regions, each of which is further divided into subregions. In sigma factor-mediated promoter recognition, regions 2.4 and 4.2 interact with the -10 and -35 sequences, respectively. (Adapted from Burgess & Anthony, 2001).

In *M. tuberculosis*, SigA is the principal group 1 sigma factor, SigB (Gomez *et al*, 1997) is a non-essential secondary sigma factor (Gomez *et al*, 1997) and SigF is a stress response sigma factor (Rodrigue *et al*, 2006; Hu & Coates, 1999; De Maio *et al*, 1996) The remaining 10 *M. tuberculosis* sigma factors belong to the ECF subgroup, which transcribe specific subsets of genes in response to external stimuli, such as misfolded proteins or environmental stresses (Rodrigue *et al*, 2006). The relatively large number of ECF sigma factors identified in this organism highlights the tight regulation of gene subsets involved in the response to the various stresses that this organism encounters throughout infection of the human host.

1.5.1.1 SigA

That SigA is constitutively expressed in *M. tuberculosis* and that attempts to inactivate *sigA* have been unsuccessful suggests that this is the primary sigma factor

that controls vegetative growth (Hu & Coates, 1999; Gomez *et al*, 1997). In addition, *sigA* mRNA is stable, with a half-life of 40 minutes, which suggests that this may be the mechanism by which its expression is maintained during growth (Hu & Coates, 1999). Although the -10 region of *sigA* in mycobacteria, TACAAT, is almost identical to the TATAAT consensus of most other prokaryotes, the -35 region, TGTACT, shows poor identity to the *E.coli* TTGACA consensus and to other known mycobacterial -35 regions (Hu & Coates, 1999; Bashyam *et al*, 1996). This suggests that during the evolutionary divergence of *M. tuberculosis* from other mycobacterial species, this organism acquired unique promoter regions (Bashyam *et al*, 1996). Furthermore, the lack of a -35 consensus in *M. tuberculosis* suggests that sigma factor usage in this organism is very specific and not readily interchangeable. Mycobacterial SigA proteins have a conserved region between domains 1.2 and 2.1 that is usually variable in other bacteria (Doukhan *et al*, 1995), and regions 1.2, 2, 3 and 4 are also highly conserved, whilst region 1.1 of SigA is poorly conserved (Doukhan *et al*, 1995). Since region 1.1 enhances promoter recognition by preventing sigma factors that are not associated with the core enzyme from binding to the DNA (Bowers & Dombroski, 1999; Dombroski *et al*, 1992), this may reflect an additional regulatory control of transcriptional regulation of housekeeping genes in *M. tuberculosis*. The ability of *M. tuberculosis* to proliferate within macrophages during the first stages of infection is associated with increased expression of *sigA*, suggesting that this sigma factor controls the regulation of genes that are central to the pathogensis of this organism (Volpe *et al*, 2006; Wu *et al*, 2004).

Enhanced survival of *M. smegmatis* in a human macrophage cell line follows introduction of the gene encoding the *M. tuberculosis* enhanced intracellular survival protein, Eis (Wei *et al*, 2000). This gene has since been identified as a member of the SigA regulon (Wu *et al*, 2009; Roberts *et al*, 2004), and is present in only the pathogenic *M. tuberculosis* and *M. bovis* BCG but not in any of the non-pathogenic mycobacteria such as *M. smegmatis* (Wei *et al*, 2000). This suggests that the role of SigA as the primary sigma factor in *M. tuberculosis* is not only in the regulation of housekeeping genes, but that it also plays a role in maintaining the pathogenicity of the organism (Wu *et al*, 2009). Interestingly, Eis has recently been identified as an aminoglycoside acetyltransferase, overexpression of which has been implicated in conferring low-level resistance to kanamycin following acetylation and inactivation of the antibiotic in *M. tuberculosis* (Zaunbrecher *et al*, 2009). That Eis has been

identified in the cytoplasm, cell wall, cell membrane and in culture supernatant (Dahl *et al*, 2001) suggests that this protein is integral to the survival of *M. tuberculosis*, and likely mediates many other processes in addition to virulence and antibiotic resistance in this organism. Though *sigA* expression is induced by SDS stress, the increase in transcript levels under these conditions does not lead to a corresponding increase in SigA protein levels (Pang & Howard, 2007). It is suggested that under these conditions the transcript accumulates in order to be rapidly translated upon removal of the stress (Pang & Howard, 2007).

1.5.1.2 SigB

The gene encoding SigB is adjacent to *sigA* in the *M. tuberculosis* genome, separated by a 3.0kb intergenic sequence (Doukhan *et al*, 1995), but it is independently transcribed (Gomez *et al*, 1997) and autoregulated (Lee *et al*, 2008a). Indeed, the putative -10 region of *sigB* is TTAAAC, which bears little resemblance to the *sigA* -10 or to the consensus sequences of other bacterial promoters, confirming that expression of *sigA* and *sigB* themselves likely arises due to induction by different sigma factors (Hu & Coates, 1999). As the principal sigma factors from most bacterial species are relatively structurally conserved, the similarity observed between SigA and SigB in *M. tuberculosis* suggests that SigB is the non-essential, secondary principal sigma factor (Fontàn *et al*, 2009; Gomez *et al*, 1997). The mycobacterial SigB proteins are less conserved, especially in regions 3 and 4, but regions 2.4 and subregion 4.2 are highly conserved (Doukhan *et al*, 1995). Since regions 2.4 and 4.2 recognise the -10 and -35 promoter regions, respectively, their conserved sequences within SigB proteins likely reflects their specificity for particular promoter motifs. As with SigA, the region between 1.2 and 2.1 is highly conserved, but unlike SigA, the 1.1 region is highly conserved in mycobacterial SigB (Doukhan *et al*, 1995). This may be a distinguishing feature of the non-essential secondary sigma factor as compared to the primary sigma factor, with the more variable 1.1 region of SigA facilitating recognition of a wider range of promoter sequences and independent expression of various subsets of housekeeping genes.

The expression of *sigB* is both autoregulated and regulated by SigC (Sun *et al*, 2004; Hu & Coates, 1999) and SigE (Rodrigue *et al*, 2006), whereas SigB does not appear to regulate the expression of any other sigma factors (Lee *et al*, 2008a). Whilst

M. tuberculosis sigA is constitutively expressed under stresses such as hydrogen peroxide exposure and heat-shock in both log- and stationary-phases, *sigB* expression is growth phase dependant, and it is expressed at a higher level during stationary phase than exponential phase when exposed to these external stresses (Hu & Coates, 1999). The SigB of *B. subtilis* is a non-essential stress-response sigma factor that is induced under heat or alcohol stress (Boylan *et al*, 1993), and, as with *M. tuberculosis*, its expression is induced in stationary phase (Benson and Haldenwang, 1993). Insertional inactivation of *sigB* in *Staphylococcus aureus* significantly reduces methicillin resistance levels (Wu *et al*, 1996) and virulence (Deora *et al*, 1997). In *M. tuberculosis*, the SigB regulon includes genes that play a role in mediating cell membrane stress, by regulating genes such as *kasA* (Lee *et al*, 2008a), and the oxidative stress response (Fontàn *et al*, 2009).

1.5.1.3 SigF

The mycobacterial SigF recognises a promoter that is similar to the consensus of the *B. subtilis* SigB promoter (Beaucher *et al*, 2002), and *sigF* expression in *M. tuberculosis* closely mimics that of *sigB* in *B. subtilis* (Hu & Coates, 1999; DeMaio *et al*, 1996), which suggests that this sigma factor may be an additional secondary sigma factor in *M. tuberculosis*. However, SigF expression is minimal during exponential growth and increases approximately 160-fold during stationary phase (Michele *et al*, 1999; DeMaio *et al*, 1996), suggesting that its major role, unlike SigB, involves the regulation of stress response genes rather than housekeeping genes during vegetative growth. SigF is therefore classified as a group 3 sigma factor rather than a primary one.

In addition to SigF playing a role in the regulation of genes involved in stationary phase, *M. tuberculosis* isolates deficient in *sigF* display attenuated virulence in mice (Geiman *et al*, 2004), indicating a potential role in pathogenesis in this organism. That the sole difference between the sigma factors of *M. tuberculosis* and the less pathogenic *M. microti* is a nonsense mutation in *sigF* further implicates SigF in virulence (Rodrigue *et al*, 2006). The expression of *sigF* is also induced following heat shock, mild cold shock and nutrient depletion (Manganelli *et al*, 2004; Manganelli *et al*, 1999). Interestingly, *sigF* expression is increased following exposure to the antimicrobials rifampicin, ethambutol, streptomycin and cycloserine,

suggesting that this sigma factor may play a role in modulating antibiotic resistance in *M. tuberculosis* (Michele *et al*, 1999). This effect is not seen, however, following exposure to isoniazid (Michele *et al*, 1999), but *sigF* mutants do show a significant increase in *ahpC* expression levels (Geiman *et al*, 2004), suggesting that SigF may regulate genes involved in the oxidative stress response.

1.5.1.4 Extracellular cytoplasmic function sigma factors

Extracellular cytoplasmic function (ECF) sigma factors were first identified in *S. coelicolor* and, as their name suggests, they regulate genes encoding proteins that are induced in response to stimuli both from within the bacterial cell and from the external environment (Lonetto *et al*, 1994). Structurally, ECF sigma factors differ from sigma factors in other phylogenetic groups in that they completely lack region 3 and that they have a significantly different region 2.4 than other sigma factors of the σ^{70} family (Paget & Helman, 2003; Lonetto *et al*, 1994). Since region 2.4 is required for recognition of the -10 promoter region, the variability observed in ECF sigma factors in this region reflects the diversity of promoter sequences recognised and, thereby, the differential regulation of specific subsets of genes. Interestingly, all *M. tuberculosis* ECF sigma factors, as well as SigF, lack region 1.2 (Rodrigue *et al*, 2006), which, when present, allosterically increases the affinity of region 2.4 for the -10 element, facilitating optimal binding (Zenkin *et al*, 2007; Baldwin and Dombroski, 2001). It may be that because *M. tuberculosis* ECF sigma factors recognise such different promoter sequences (Rodrigue *et al*, 2006) the additional specificity afforded by region 1.2 is not required and this region has been deleted during evolution.

The number of ECF sigma factors found in different bacterial species varies, with up to as many as 48 in *Bacteroides thetaiotamicron* (Fig. 1.8) (Kill *et al*, 2005). In *M. tuberculosis*, the identification of 10 putative ECF sigma factors highlights the complexity of the transcriptional network regulating responses to environmental stimuli in this organism. Cross-regulation of certain ECF sigma factors by others (Kazmierczak *et al*, 2005) further highlights the complex nature of transcriptional regulation in response to external stimuli.

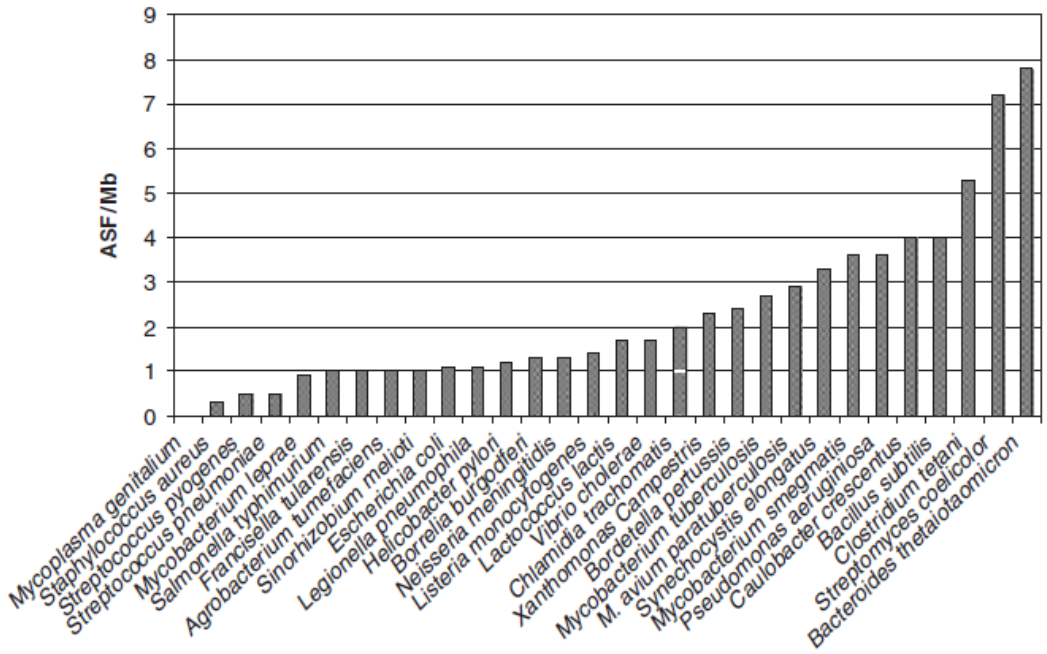

Figure 1.8: A representation of the ratio of alternative sigma factors to mega-base pairs (ASF/Mb) in a variety of different bacteria. (Adapted from Rodrigue *et al*, 2006)**.**

The complexity of the sigma factor regulatory network has made elucidation of transcriptional regulation of and by ECF sigma factors in *M. tuberculosis* difficult to characterise. A number of ECF sigma factors have been implicated in mediating virulence in this organism, including *sigC*, which is expressed during exponential growth to regulate metabolic processes as well as during stationary phase to regulate stress-related genes (Sun *et al*, 2004; Manganelli *et al*, 1999). SigC regulates expression of virulence factors *hspX*, *mtrA* and *senX3*, all of which are involved in survival within the macrophage (Sun *et al*, 2004). Furthermore, *M. tuberculosis* strains deficient in *sigC* cause limited pathogenesis in both the murine model (Sun *et al*, 2004) and the guinea pig model through production of non-necrotic granulomas (Karls *et al*, 2006). Not surprisingly, SigF, which is strongly implicated in virulence regulation in *M. tuberculosis* (Geiman *et al*, 2005), also regulates expression of *sigC* (Lee *et al*, 2008a; Karls *et al*, 2006), thereby highlighting a further level of control of this important survival characteristic of *M. tuberculosis*. SigD, which is autoregulatory and is not essential for growth *in vitro* (Calamita *et al*, 2005; Raman *et al*, 2004), is also implicated in virulence as mutants lacking this sigma factor display reduced lethality in the mouse model (Calamita *et al*, 2005). The expression of *sigG*, *sigA* and *sigE* is upregulated in macrophages, suggesting that these sigma factors

contribute to survival within the macrophage (Cappelli *et al*, 2006; Volpe *et al*, 2006) through interaction with the regulatory network.

The environment within the macrophage is unfavourable, due to the acidic pH and the production of several reactive oxygen and nitrogen species. Through an oxidative stress response regulated by sigma factors SigE (Wu *et al*, 1997), SigH (Manganelli *et al*, 2002; Raman *et al*, 2001; Fernandes *et al*, 1999), SigG (Lee *et al*, 2008b) and SigJ (Hu *et al*, 2004; Hu & Coates, 2001), *M. tuberculosis* is able to survive these conditions. SigE mediates regulation of the oxidative stress response by regulation of *sodA* (Kazmierczak *et al*, 2005), which encodes a superoxide dismutase that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide (Harth & Horwitz, 1999). In addition, a SigE mutant is more susceptible to acid shock, heat shock and detergent stress (Wu *et al*, 1997). Although a *sigJ* mutant displays increased sensitivity to H_20_2 (Hu & Coates, 2001), *katG* expression in this mutant remains unchanged, suggesting that SigJ may mediate the oxidative stress response via an alternative mechanism (Hu *et al*, 2004).

Whilst detergent and oxidative stresses have no effect on the expression of *sigL* (Hahn *et al*, 2005) or *sigM*, *sigM* transcript levels increase following heat shock and during late stationary phase, indicating that SigM is involved in long-term survival rather than virulence (Agarwal *et al*, 2007). SigM may therefore play a role in mediating latency (Agarwal *et al*, 2007; Raman *et al*, 2006), a key feature of *M. tuberculosis* that ensures its long-term survival and widespread dissemination. Additionally, SigL, which is involved in the synthesis of cell membrane lipids (Dainese *et al*, 2006), is expressed at very low levels during vegetative growth *in vitro* (Hahn *et al*, 2005). In the absence of an effective model of latency in which to investigate gene expression under these conditions, it may be that increased expression of SigL serves to maintain the cell wall during this period of dormancy. Similarly, SigH may be involved in maintaining latency as mutants deficient in *sigH* do not display any variation in global gene expression during exponential phase when compared to the wildtype parent strains (Manganelli *et al*, 2002).

The remaining 2 sigma factors, SigK and SigI, are not well characterised, but the SigK regulon appears to consist of only a small number of genes (Veyrier *et al*, 2008), suggesting that its role in *M. tuberculosis* may be highly specific. Whilst the complex network of regulatory pathways in *M. tuberculosis* is as yet poorly understood, it is clear that individual sigma factors do not target unique subsets of genes, but rather

regulate a wide range of responses to external stimuli. Furthermore, not only do sigma factors regulate expression of metabolic genes, they also regulate the expression of other sigma factors, further complicating the cascade. The gene encoding SigI is located downstream of *sigJ* and is also regulated by SigJ (Homerova *et al*, 2008). Additionally, SigG regulates the expression of both *sigD* and *sigH* (Lee *et al*, 2008b), and SigH, in turn, induces the expression of *sigE* and *sigB* (Raman *et al*, 2001). This complex sigma factor-mediated transcription cascade highlights the elegance of the regulatory network contributing to the success of *M. tuberculosis* as a pathogen.

1.5.2 Anti-sigma factors

Clearly, transcriptional regulation in *M. tuberculosis* is critical, as is evidenced by the many levels of control and the complex cascades of cross-regulation discussed above. Other than transcriptional regulation of sigma factor-mediated gene expression via recognition of specific promoter sequences, the direct association of sigma factors with anti-sigma factors further compounds these regulatory cascades. Inhibition of sigma factor binding to the RNA polymerase by anti-sigma factors provides another means of regulating the expression of specific regulons (Beaucher *et al*, 2002). Anti-sigma factors are often cell membrane-associated and remain bound to the sigma factor until an external stress triggers dissociation of the anti-sigma factor, thereby freeing the sigma factor for the regulation of genes involved in the response to the external stress (Helmann, 1999). Anti-sigma factors were first identified in *Pseudomonas aeruginosa* through investigation of the mucoid phenotype of cystic fibrosis isolates, which was found to be under the control of AlgU (Schurr *et al*, 1996; Xie *et al*, 1996). In *M. tuberculosis*, SigF is negatively regulated by anti-sigma factor UsfX, encoded by the gene located upstream of *sigF* (Beaucher *et al*, 2002). As UsfX is SigF-specific and cannot interact with SigA, it is likely that anti-sigma factors are specific for certain sigma factors (Beaucher *et al*, 2002). SigH has been shown to interact with RsrA, an anti-sigma factor encoded by a co-transcribed gene within the *sigH* operon (Song *et al*, 2003). Interestingly, RsrA inhibits SigH in a redox-dependant manner (Song *et al*, 2003). This confirms the role of SigH in response to oxidative stress, as under oxidative stress, RrsA releases SigH and transcription of the oxidative stress response genes can proceed (Song *et al*, 2003). Similarly, SigE from *M. tuberculosis* is negatively regulated by the direct binding of anti-sigma factor RseA (Donà *et al*, 2008). It was recently demonstrated that the interaction between

SigE and RseA does not occur following oxidative stress, and that exposure to vancomycin or SDS results in degradation of RseA (Barik *et al*, 2010). This neatly illustrates the mechanism by which external stimuli can trigger the expression of genes involved in the stress response via the alternative sigma factor pathway.

The elucidation of the genome sequence of *M. tuberculosis* H37Rv (Cole *et al*, 1998) has provided invaluable information with regard to the biology of this organism, enabling the identification of several drug resistance determinants. However, the nucleotide sequence alone is not sufficient to facilitate an understanding of the role of gene expression in the development of drug resistance. Since ECF sigma factors respond to both internal and external stimuli, it follows that in the presence of an antibiotic a transcriptional response may be induced. Ethambutol, rifampicin, streptomycin and cycloserine all induce expression of *sigF* in *M. tuberculosis* whilst isoniazid has no effect on *sigF* expression (Michele *et al*, 1999). It may be that upon exposure to ethambutol, rifampicin, streptomycin and/or cycloserine, differential expression of the SigF regulon results in a protective response in *M. tuberculosis*. In contrast to *sigF*, rifampicin treatment has no effect on the expression of *sigJ* (Hu & Coates, 2001), further confirming that unique stress responses are induced by individual sigma factors.

It is interesting to speculate that in drug resistant *M. tuberculosis* isolates with no identifiable resistant determinants, transcriptional regulation may play a role in the development of antibiotic resistance. It is clear, however, that our current understanding of transcriptional regulation in *M. tuberculosis* is limited and that further investigation is necessary so as to gain more insight into the extent to which this regulatory network facilitates the survival of *M. tuberculosis*.

1.6 Aims of the study

The global threat of the TB epidemic is becoming increasingly evident, especially in the context of increasing HIV infection rates in developing countries. In addition, the emergence of *M. tuberculosis* strains with resistance to multiple antituberculous drugs makes the treatment and management of TB increasingly difficult. A greater understanding of the biology of this organism and the mechanisms by which it is able to both adapt to survival within the host and to acquire resistance to antibiotics is necessary in order to improve treatment options through the use of effective rapid assays for the detection of drug resistance. To this end, this study was carried out in order to investigate and further characterise the molecular mechanisms by which *M. tuberculosis* responds to antibiotic pressures and to gain further insight into the role of transcriptional regulation in this process.

The aims of this study are four-fold:

1) to characterise the genetic basis of resistance, the prevalence and the epidemiology of MDR- and XDR-TB in Cape Town, South Africa.

2) to evaluate the efficacy of rapid molecular assays for the detection of resistance to first-line drugs in *M. tuberculosis*.

3) to develop and evaluate a rapid genotypic assay for the detection of resistance to second-line drugs in *M. tuberculosis*.

4) to investigate the role of transcriptional regulation in the progression to antibiotic resistance in *M. tuberculosis*.

CHAPTER 2:

Detection of Drug Resistance Markers in *Mycobacterium tuberculosis* and the Association with Strain Lineage

2.1 Introduction

2.2 Materials and Methods

- **2.2.1 Bacterial isolates and susceptibility testing**
- **2.2.2 Genomic DNA preparation and agarose gel electrophoresis**
- **2.2.3 GenoType® MTBDR*plus* assays**
- **2.2.4 Multiplex allele-specific –PCR assays**
- **2.2.5 DNA sequencing and analysis**
- **2.2.6 Spacer oligonucleotide typing**
- **2.2.7 Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat Typing**

2.3 Results

- **2.3.1 Phenotypic drug susceptibilities of *M. tuberculosis* isolates**
- **2.3.2 Detection of resistance to rifampicin using the GenoType® MTBDR*plus* assay**
- **2.3.3 Detection of resistance to isoniazid using the GenoType® MTBDR*plus* assay**
- **2.3.4 Correlation between standard phenotypic susceptibility testing and the GenoType® MTBDR*plus* assay for the detection of rifampicin and isoniazid resistance**
- **2.3.5 Detection of isoniazid resistance using MAS-PCR assays**
- **2.3.6 Correlation of GenoType® MTBDR*plus* and MAS-PCR assays for the detection of isoniazid resistance in *M. tuberculosis***
- **2.3.7 Relationship between genotype and resistance phenotype using spacer oligonucleotide typing and MIRU-VNTR typing**

2.4 Discussion

2.1 Introduction

The use of rapid molecular assays to detect drug resistance in *M. tuberculosis* would significantly reduce the time delay associated with standard phenotypic DST. This has prompted advances in molecular genetics that have facilitated a greater understanding of the genetic basis of resistance of *M. tuberculosis* to many antibiotics. As a result, a wide variety of rapid molecular assays for detecting drug resistance in *M. tuberculosis* have been developed and evaluated. These include, among others, direct DNA sequencing (Yam *et al*, 2004; Sintchenko *et al*, 1999), molecular beacon analysis (Piatek *et al*, 2000; Piatek *et al*, 1998), biprobe analysis (Edwards *et al*, 2001), MAS-PCR assays (Leung *et al*, 2006; Mokrousov *et al*, 2003; Mokrousov *et al*, 2002a,b) and line probe assays (Hillemann *et al*, 2009; Hillemann *et al*, 2007; Juréen *et al*, 2004; Marttila *et al*, 1999). However, many of these methods are often expensive and require the training of specialized personnel.

Following the WHO recommendation of the use of line probe assays for the rapid screening of patients at risk of MDR-TB under certain guiding principles (WHO, 2008e), the South African National Department of Health began the roll out of the GenoType® MTBDR*plus* [HAIN Lifescience] assay nationwide (NTRF, RSA, 2008). This assay uses a reverse hybridization-based technique to rapidly detect the mutations most frequently associated with MDR-TB (Barnard *et al*, 2008; Hillemann *et al*, 2007). The assay utilizes eight *rpoB* wild-type probes that span the entire RRDR, with the absence of a hybridization signal from any of these probes indicating the presence of a mutation within that specific region (Fig. 2.1). Additionally, the presence of a hybridisation signal from one or more of the four mutant probes indicates the presence of specific mutations S531L, H526Y, H526D and D516V in RpoB, which are frequently identified in rifampicin resistant *M. tuberculosis* isolates (Fig. 2.1). Similarly, high-level isoniazid resistance due to the presence of a KatG S315T (Lacoma *et al*, 2008; Silva *et al*, 2003) amino acid substitution or lower level isoniazid resistance as a result of an *inhA* C-15T (Lacoma *et al*, 2008) promoter mutation can also be identified using this assay.

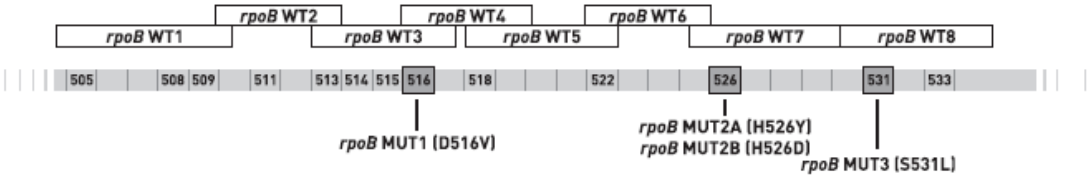

Figure 2.1: A schematic representation of the RRDR of RpoB and the rifampicin resistance determinants that can be identified using the GenoType® MTBDR*plus* assay. Numbers represent the RpoB codons within the RRDR and the codons most frequently associated with rifampicin resistance worldwide are highlighted. (Adapted from HAIN Lifescience GenoType® MTBDR*plus* handbook).

Several independent evaluations of the GenoType® MTBDR*plus* assay (Huang *et al*, 2009; Barnard *et al*, 2008; Lacoma *et al*, 2008; Miotto *et al*, 2008; Hilleman *et al*, 2007) have shown that the assay is highly sensitive for the detection of rifampicin resistance (99-100%) and less sensitive at detecting isoniazid resistance (73-92%), whilst specificities for the detection of both rifampicin and isoniazid resistance range from 93-100% (Ling *et al*, 2008). Interestingly, this assay is more sensitive at detecting high-level isoniazid resistance than lower level isoniazid resistance (Lacoma *et al*, 2008), suggesting that low-level isoniazid resistance may frequently arise as a result of mutations other than *inhA* C-15T. Whilst the GenoType® MTBDR*plus* assay has proven relatively effective at identifying mutations associated with MDR, the cost associated with the routine implementation of this assay in developing countries that have a high burden of TB, such as South Africa, must be considered.

MAS-PCR assays designed to detect the isoniazid resistance determinants KatG S315T (Mokrousov *et al*, 2002) and *inhA* C-15T (Leung *et al*, 2006) are relatively simple and inexpensive to perform. Two external primers amplify an internal control that confirms that PCR amplification has proceeded successfully. The internal primer is designed so that its 3` end is complementary to the wild-type sequence, and it therefore cannot be extended in the presence of a mutation at this locus (Fig 2.2). The amplicons obtained can easily be visualised by agarose gel electrophoresis, and the presence or absence of the expected mutation is deduced by the size of the amplicons that are produced. That up to 80% of global isoniazid resistance can be attributed to the acquisition of one or both of these mutations (Hazbón *et al*, 2006) makes rapid, simple assays such as these extremely valuable for the preliminary determination of susceptibility to isoniazid.

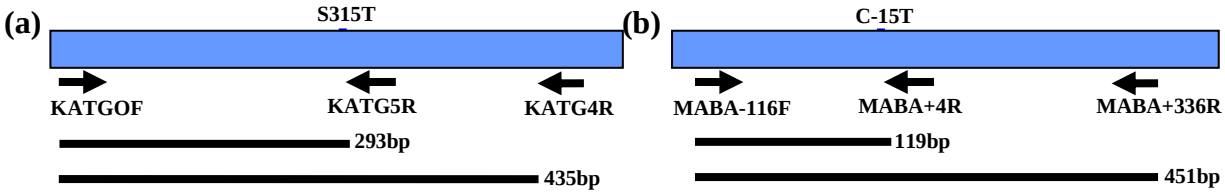

Figure 2.2: Schematic representation of MAS-PCR assays used for the detection of (a) KatG S315T and (b) *inhA* C-15T isoniazid resistance determinants. Elongation of internal primers KATG5R and MABA+4R, which produce the smaller amplicon in each assay, can only proceed in the absence of a mutation. Horizontal lines represent the sizes of the expected amplicons.

The infrequency of horizontal gene transfer in *M. tuberculosis* together with the slow mutation rate of this organism suggests that related isolates would carry the same chromosomal mutations. Genotypic analysis of *M. tuberculosis* has enabled the classification of isolates into different lineages, and has led to the suggestion that particular lineages may be more likely to acquire particular resistance determinants than others (Brimacombe *et al*, 2007). In particular, isolates of the W-Beijing lineage have been extensively studied as they appear to be associated with increased virulence, epidemiological outbreaks of TB and drug resistance (Marais *et al*, 2006; Bifani *et al*, 2002; Toungoussova *et al*, 2002), all of which may be attributed to their enhanced ability to evade host defenses (Zhang *et al*, 1999b). Although the association of W-Beijing isolates with drug resistance is well documented (Marais *et al*, 2006; Bifani *et al*, 2002), it is not known whether isolates of particular lineages are more likely to carry specific resistance determinants than others.

The frequency with which specific resistance determinants are identified in a collection of isolates may vary somewhat geographically, as is illustrated by the high incidence of *inhA* C-15T promoter mutations in *M. tuberculosis* isolates from Madrid as compared to those from New York (Table 2.1) (Piatek *et al*, 2000). It is therefore important to ascertain the incidence of resistance determinants in a region and what proportion of resistant isolates carry less frequently observed mutations as these would be missed following the implementation of rapid molecular assays for the detection of antibiotic resistance.

Table 2.1: Variation in the geographical distribution of isoniazid resistance determinants in Madrid and New York. (Adapted from Piatek *et al*, 2000).

Mutation	No. (%) of isolates				*P*
	Madrid isolates		New York isolates		
	Susceptible (n = 7)	Resistant (n = 49)	Susceptible (n = 48)	Resistant (n = 45)	
katG codon 315 alone		15 (30.6)		26 (57.8)	<0.012
inhA ribosomal binding site alone		21 (42.9)		4 (8.8)	<0.001
ahpC-oxyR intragenic region alone		4 (8.2)		2 (4.4)	NS[a]
katG plus *inhA*		1 (2)		1 (2.2)	NS
katG plus *ahpC-oxyR*		1 (2)		1 (2.2)	NS
inhA plus *ahpC-oxyR*		5 (10.2)			NS
kasA codon 66					NS
kasA codon 269			5 (10.4)	5 (11.1)	NS
kasA codon 312					NS
kasA codon 413					NS
No mutation detected	NA[b]	3 (6.1)	NA	11 (24.4)	0.02

[a] NS, not significant.
[b] NA, not applicable.

The GenoType® MTBDR*plus* assay was therefore evaluated for the detection of resistance to rifampicin and isoniazid in *M. tuberculosis* isolates from patients at a tertiary care hospital in the Western Cape, and compared to MAS-PCR assays designed for the detection of isoniazid resistance. In addition, the association between resistance determinants and strain lineage was investigated in this setting.

2.2 Materials and Methods

2.2.1 Bacterial isolates and susceptibility testing

All isolates included in this evaluation were obtained from patients at Groote Schuur Hospital (GSH), Cape Town, in 2006 [Appendix A] and were identified as *M. tuberculosis* by Ziehl-Neelsen staining and *M. tuberculosis*-specific PCR (De Wit *et al*, 1990). Drug susceptibility testing (DST) for rifampicin and isoniazid was carried out on 652 *M. tuberculosis* isolates in the diagnostic laboratory at GSH using the BACTEC MGIT 960 system (MGIT 960; Becton Dickinson Diagnostic Systems, Sparks, MD) (NCCLS, 2003). Standard critical concentrations of 1µg/ml for rifampicin and 0.1µg/ml for isoniazid were used.

2.2.2 Genomic DNA preparation and agarose gel electrophoresis

M. tuberculosis isolates were grown on Lowenstein-Jensen (LJ) slanted agar, following which colonies were resuspended in 500µl distilled H_2O (dH_2O) and heat inactivated at 80˚C for 1 hour. Genomic DNA was extracted as previously described (van Soolingen *et al*, 1991). Briefly, bacterial cells were lysed using SDS and

proteinase K, after which hexadecyl trimethylammonium bromide (CTAB)/NaCl solution was used to aggregate and precipitate cell debris and denatured proteins. Genomic DNA was obtained by chloroform:isoamyl alcohol (24:1) extraction and precipitation using isopropanol, prior to resuspension in 55µl dH_20. Alternatively, genomic DNA was obtained from cells in 2ml MGIT culture by centrifugation of samples and resuspension in 500µl dH_2O following heat inactivation at 80˚C for 1 hour.

Separation and visualisation of nucleic acid products was carried out by agarose gel electrophoresis using a 1X Tris-acetate EDTA (TAE) running buffer. Agarose concentrations of 1% (w/v) were used and ethidium bromide was added to the agarose at a final concentration of 10ng/µl. Visualisation of nucleic acid products was carried out using a Fotodyne Inc. UV light box (302nm) and the results were captured using a Kodak EDAS 290 camera.

2.2.3 GenoType® MTBDR*plus* assays

The efficacy of the GenoType® MTBDR*plus* assay [HAIN Lifescience] for the identification of resistance determinants associated with rifampicin and isoniazid resistance in isolates with known phenotypic DST results was investigated. A multiplex PCR consisting of 35µl primer nucleotide mix [GenoType® MTBDR*plus*], 3mM $MgCl_2$, 1U HotStart*plus* *Taq* DNA polymerase [QIAGEN] and 5-25ng genomic DNA [2.2.2] was carried out according to the manufacturer's instructions. Reverse hybridization of the amplicons obtained to membrane-associated probes enables detection of the mutations most frequently associated with MDR worldwide. These include mutations at codons 531, 526 and 516 of RpoB that result in resistance to rifampicin, and KatG S315T and *inhA* C-15T mutations, which are associated with resistance to isoniazid. Included on each strip are a conjugate control (CC), an amplification control (AC) and a TUB control, the latter of which confirms that the isolate belongs to the *M. tuberculosis* complex. Hybridisation and stringency washes were carried out according to the manufacturer's instructions and were performed using a TwinCubator® [HAIN Lifescience]. All hybridization patterns obtained were interpreted in a blinded manner.

2.2.4 Multiplex allele-specific -PCR assays

MAS-PCR assays for the detection of KatG S315T and *inhA* C-15T were carried out as previously described (Mokrousov *et al*, 2002; Leung *et al*, 2006) using a GeneAmp PCR System 2400 [Perkin Elmer] thermocycler. A 3` mismatch between a primer (Table 2.2; Fig. 2.3 & 2.4) and the targeted mutation enables detection of the mutation using these assays. In the absence of a KatG S315T mutation, primers KATG5R and KATG0F (Table 2.2; Fig. 2.3) amplify a product of 293bp (Fig. 2.2a & 2.3). The presence of a KatG S315T mutation, however, prevents elongation of KATG5R and instead amplification from KATG4R and KATG0F results in a 435bp product (Fig. 2.2a & 2.3). In absence of an *inhA* C-15T mutation, amplification from both primers MABA+4R and MABA+336R with MABA-116F (Table 2.2; Fig. 2.4) can proceed, resulting in the production of two amplicons of 451bp and 119bp (Fig. 2.2b & 2.4). The presence of *inhA* C-15T inhibits elongation from internal primer MABA+4R, resulting in the production of only the larger 451 bp amplicon (Fig. 2.2b & 2.4).

Table 2.2: Primers used in MAS-PCR assays for the identification of isoniazid resistance determinants KatG S315T and *inhA* C-15T.

Primer Name	Primer Sequence (5` → 3`)
KATG0F	GCAGATGGGGCTGATCTACG
KATG4R	AACGGGTCCGGGATGGTG
KATG5R	TACGACCTCGATGCCGC
MABA-115F	ACAAACGTCACGAGCGTAACC
MABA+4R	TCACCCGACAACCTATCG
MABA+336R	GTTGGCGTTGATGACCTTCTC

Amplification reactions were to a final volume of 50µl and consisted of 0.2mM of each dNTP [Fermentas], 3mM $MgCl_2$ [Promega], 1 X GoTaq PCR buffer [Promega], 20pmoles of each primer (Table 2.2), 1.25U GoTaq® DNA polymerase [Promega] and 50-100ng template genomic DNA [2.2.2]. All primers used in this study were synthesised at the Synthetic DNA Laboratory of the University of Cape Town, using a Beckman 1000M DNA synthesiser on the high purity program. The MAS-PCR cycling conditions consisted of an initial denaturation step at 95°C for 3 minutes, followed by 5 cycles of denaturation at 95°C for 1 minute, primer annealing at 64°C for 1 minute and primer extension at 72°C for 30 seconds, 5 cycles of

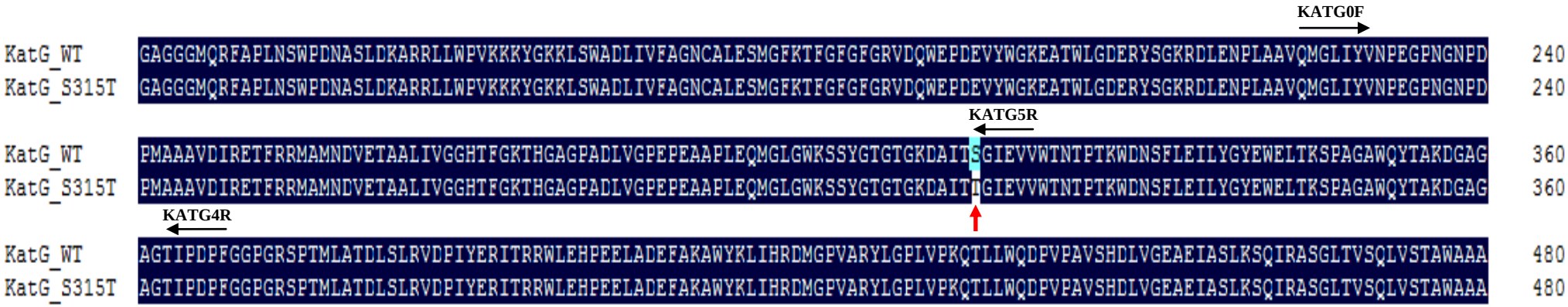

Figure 2.3: Amino acid sequence alignment of KatG from *M. tuberculosis* isolates included in all *katG* MAS-PCR assays. Primer binding sites are indicated by black arrows. The position of the KatG S315T amino acid substitution is indicated by the red arrow. Regions shaded in black represent regions of 100% sequence identity. The numbering on the right indicates the amino acid position relative to the start codon of KatG.

Figure 2.4: Nucleotide sequence alignment of the promoter region of *inhA* from *M. tuberculosis* control isolates included in all *inhA* MAS-PCR assays. Primer binding sites are indicated by black arrows. The position *inhA* C-15T nucleotide substitution is indicated by the red arrow. Regions shaded in black represent regions of 100% sequence identity. The GTG start codon of *inhA* is boxed in red.

denaturation at 95°C for 1 minute, primer annealing at 62°C for 40 seconds and primer extension at 72°C for 30 seconds, and 20 cycles of denaturation at 94°C for 1 minute, primer annealing at 60°C for 40 seconds and primer extension at 72°C for 30 seconds. A final elongation step at 72°C for 3 minutes was performed to complete elongation of all unfinished or incomplete products, and PCR products were separated and visualised by agarose gel electrophoresis using 1.5% (w/v) agarose [2.2.2].

2.2.5 DNA sequencing and analysis

Following agarose gel electrophoresis [2.2.2], PCR products of interest were excised and purified from gels using the MinElute Gel Extraction Kit [QIAGEN] according to manufacturer's instructions. Purified amplicons were quantified by agarose gel electrophoresis alongside a quantitative molecular weight marker, Hyperladder I or Hyperladder IV [Bioline] [Appendix C]. Automated DNA sequencing of purified products was carried out at the Stellenbosch University DNA Sequencing Facility, Cape Town, using an ABI 3130 Genetic Analyser. DNA sequences were analysed using DNAMAN [version 4.0, Lynnon Biosoft], and nucleotide and amino acid sequences obtained were compared with existing sequences in the database using BLAST (Altschul *et al*, 1997).

2.2.6 Spacer oligonucleotide typing

Spacer oligonucleotide typing, or 'spoligotyping', (Kamerbeek *et al*, 1997) was carried out on all *M. tuberculosis* isolates included in this study. PCR amplification of the spacer regions present in each isolate was carried out using primers DRa (5`-GGT TTTGGGTCTGACGAC-3`, 5` biotinylated) and DRb (5`-CCGAGAGGGGACGG AAAC-3`), which are directed at the DR region. Amplification reactions were to a final volume of 25µl and consisted of 0.2mM of each dNTP [Fermentas], 3mM $MgCl_2$ [QIAGEN], 1 X HotStart*plus* PCR buffer [QIAGEN], 20pmoles of each primer, 1U HotStart*plus* *Taq* DNA polymerase [QIAGEN] and 50-100ng template genomic DNA. Following an initial denaturation at 96°C for 15 minutes, amplification reactions consisted of 25 cycles of 96°C for 1 minute, primer annealing at 55°C for 1 minute and elongation at 72°C for 1 minute. This was followed by a final elongation at 72°C for 5 minutes. The amplicons obtained were hybridized to corresponding membrane-associated spacer oligonucleotides and conjugated to streptavidin

peroxidase. Signal detection was carried out using the ECL Direct Nucleic Acid Labelling and Detection System [Amersham Biosciences]. Hybridization patterns obtained were analysed using SPOTCLUST SpolDB3-based model (http://cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html) in order to assign isolates to specific genotype families (Filliol *et al*, 2002).

2.2.7 Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat Typing

All MDR W-Beijing, susceptible W-Beijing, rifampicin mono-resistant LAM3 and isoniazid mono-resistant X3 isolates identified by spoligotyping were further differentiated using 12-locus mycobacterial interspersed repetitive unit (MIRU)-variable number tandem repeat (VNTR) analysis (Supply *et al*, 2001). Four multiplex PCR assays consisting of three primer pairs each were carried out (Table 2.3), all of which were to a final volume of 25µl and consisted of 1 X HotStart*plus* PCR buffer [QIAGEN], 0.2mM of each dNTP [Fermentas] and 1U HotStart*plus* *Taq* DNA polymerase [QIAGEN]. All primers were used at a final concentration of 0.4µM, with the exception of primer pairs 4 and 24 which were at 0.25µM, and $MgCl_2$ [QIAGEN] was added at 1.5mM to mixture A and at 0.5mM to mixtures B and C (Table 2.3). Amplification reactions for all assays consisted of an initial denaturation at 96°C for 15 minutes follwed by 40 cycles of denaturation at 94°C for 90 seconds, primer annealing at 58°C for 90 seconds and elongation at 72°C for 2 minutes and a final elongation at 72°C for 10 minutes. One primer from each pair was 5`-labelled with a fluorescent dye (FAM, HEX or NED) (Table 2.3), facilitating the use of an ABI 3100 analyzer [Applied Biosystems] for the determination of the number of tandem repeats observed at each locus. The relationship between the MIRU-VNTR genotype of each isolate was determined using MIRU-VNTR*plus* (Allix-Beguec *et al*, 2008; http://www.miru-vntrplus.org/) and the BURST algorithm (http://eburst.mlst.net/).

Table 2.3: Primers used in four multiplex PCR assays for genotyping *M. tuberculosis* isolates by 12-locus MIRU-VNTR analysis.

PCR Assay	MIRU locus	[fluorescent label] Primer Sequence (5`→3`)
A	4	[a][FAM]GCGCGAGAGCCCGAACTGC
		GCGCAGCAGAAACGTCAGC
	26	TAGGTCTACCGTCGAAATCTGTGAC
		[b][HEX]CATAGGCGACCAGGCGAATAG
	40	[c][NED]GGGTTGCTGGATGACAACGTGT
		GGGTGATCTCGGCGAAATCAGATA
B	10	GTTCTTGACCAACTGCAGTCGTCC
		[FAM]GCCACCTTGGTGATCAGCTACCT
	16	TCGGTGATCGGGTCCAGTCCAAGTA
		[HEX]CCCGTCGTGCAGCCCTGGTAC
	31	ACTGATTGGCTTCATACGGCTTTA
		[NED]GTGCCGACGTGGTCTTGAT
C	2	TGGACTTGCAGCAATGGACCAACT
		TACTCGGACGCCGGCTCAAAAT [FAM]
	23	CTGTCGATGGCCGCAACAAAACG [HEX]
		AGCTCAACGGGTTCGCCCTTTTGTC
	39	CGCATCGACAAACTGGAGCCAAAC
		CGGAAACGTCTACGCCCCACACAT [NED]
D	20	TCGGAGAGATGCCCTTCGAGTTAG [FAM]
		GGAGACCGCGACCAGGTACTTGTA
	24	CGACCAAGATGTGCAGGAATACAT
		GGGCGAGTTGAGCTCACAGAA [HEX]
	27	TCGAAAGCCTCTGCGTGCCAGTAA
		GCGATGTGAGCGTGCCACTCAA [NED]

[a]FAM, 5-carboxyfluorescein; [b]HEX, 6-carboxy-4,7,2`,4`,5`,7`-hexachlorofluorescein; [c]NED, 2,7`,8`-benzo-5`-fluoro-2`,4,7-trichloro-5-carboxyfluorescein.

2. 3 Results

2.3.1 Phenotypic drug susceptibilities of *M. tuberculosis* isolates

Phenotypic DST [2.2.1] was carried out on a total of 652 *M. tuberculosis* isolates obtained from patients at GSH in 2006 [Appendix A]. Of these, 95 isolates were identified as MDR (14.6%), 67 isolates as isoniazid mono-resistant (10.3%) and 11 as rifampicin mono-resistant (1.5%). Multiple isolates from the same patient were excluded in order to eliminate the bias associated with multiple strains harbouring the same resistance determinant. A total of 82 MDR, 41 isoniazid mono-resistant and 11 rifampicin mono-resistant isolates were included in this study (Table 2.4) as the remaining isolates were non-viable when the study commenced. A total of 90 randomly selected isolates susceptible to both rifampicin and isoniazid were included

as controls in both genotypic assays and spoligotyping. The genotypic assays were evaluated against the gold standard DST results and sequence analysis was carried out on isolates with discrepant results.

Table 2.4: Rifampicin and isoniazid susceptibilities of *M. tuberculosis* strains from Groote Schuur Hospital in 2006.

M. tuberculosis isolates (n)	RIF	INH
82	R	R
41	S	R
11	R	S
90	S	S

RIF, rifampicin; INH, isoniazid; R, resistant; S, susceptible

2.3.2 Detection of resistance to rifampicin using the GenoType® MTBDR*plus* assay

The GenoType® MTBDR*plus* [HAIN Lifescience] assay was evaluated for determination of the rifampicin susceptibility profiles of all 224 *M. tuberculosis* isolates with known phenotypic DST results that were included in this study. Resistance to rifampicin was correctly indicated using this assay in 80 of 82 (97.6%) MDR isolates. Of these, resistance was attributed to a serine to leucine amino acid substitution at codon 531 (S531L) in RpoB in 71 (86.6%) isolates, determined by the absence of a hybridization signal from wild-type probe 8 and the presence of a signal from the S531L mutant probe (Fig. 2.5, strips 1 & 2). Phenotypic rifampicin resistance could be attributed to the presence of a H526D substitution in RpoB in one isolate due to absence of a hybridization signal from wild-type probe 7 and the presence of a signal from the H526D mutant probe, and a further two isolates were identified as carrying a D516V substitution in RpoB due to the absence of a hybridization signal from wild-type probes 3 and 4, and the presence of a hybridization signal from the D516V mutant probe. Of the remaining six isolates, five were scored as rifampicin resistant due to the absence of wild-type signals that correspond to mutations at codons 516 in one isolate (Fig. 2.5, strip 3), 526 in 2 isolates and either 531 or 533 in 2 isolates. Though the specific amino acid alterations cannot be identified as there was no hybridization signal from a corresponding mutant probe, these isolates were considered rifampicin resistant. One isolate had all 8 *rpoB* wild-type probes and the MUT3 probe binding, which suggests either a mixed

infection with at least one rifampicin susceptible and one rifampicin resistant strain, or the presence of a heterogeneous isolate that is only partially resistant to rifampicin (Miotto *et al*, 2006). It is recommended by the manufacturers of the assay that in cases such as this the isolate be interpreted as rifampicin resistant. The remaining two MDR isolates produced signals representative of rifampicin susceptible isolates using the GenoType® MTBDR*plus* assay.

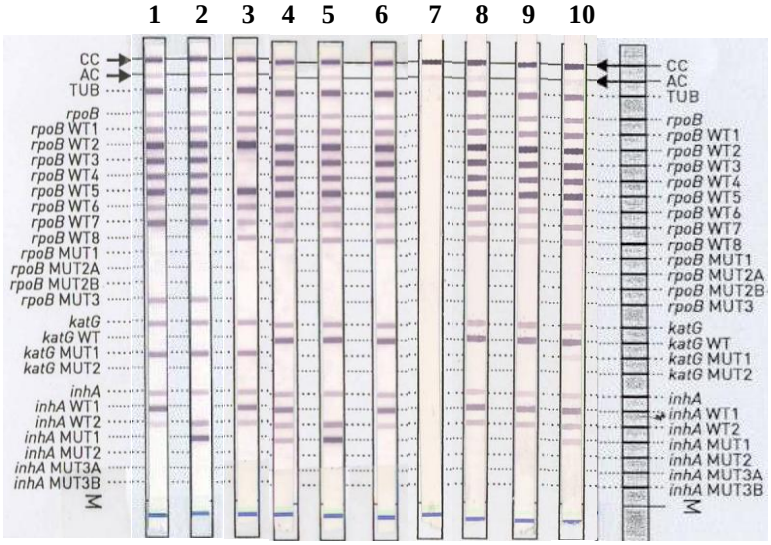

Figure 2.5: GenoType® MTBDR*plus* strips following amplification and hybridization of amplicons from *M. tuberculosis* isolates with varying resistance profiles. Strip 1, rifampicn resistant due to RpoB S531L and isoniazid resistant due to KatG S315T; strip 2, rifampicin resistant due to RpoB S531L and isoniazid resistant due to both KatG S351T and *inhA* C-15T; strip 3, rifampicin resistant due to an unidentifiable mutation at RpoB codon 516 and isoniazid resistant due to KatG S315T; strip 4, isoniazid mono-resistant isolate with both *inhA* wild-type and mutant probes binding, indicative of a mixed infection; strip 5, isoniazid mono-resistant due to KatG S315T; strip 6, isoniazid mono-resistant isolate with a hybridization profile representative of a susceptible isolate; strip 7, isoniazid mono-resistant isolate lacking a TUB control, indicative of a non-*M. tuberculosis* complex isolate; strips 8 & 9, isolates susceptible to both rifampicin and isoniazid; strip 10, susceptible isolate with both *inhA* wild-type and mutant probes binding, indicative of a mixed infection.

Resistance to rifampicin was correctly indicated by the GenoType® MTBDR*plus* assay in 7 of 11 (63.6%) rifampicin mono-resistant isolates, of which 3 (27.3%) isolates carried an S531L substitution in RpoB. The absence of a signal from wild-type probes 4 and 5 in one isolate suggests resistance due to the presence of a mutation at codon 518 of RpoB. One isolate was found to harbour a mutation at codon 526, as indicated by the absence of a hybridization signal from wild-type probe 7, and another isolate a mutation at either codon 531 or 533 due to the lack of wild-type

probe 8 binding. A mutation at either codon 514 or 515 of RpoB was identified in the remaining isolate, which was indicated by the absence of a hybridization signal from wild-type probe 3. A total of 4 phenotypically rifampicin mono-resistant isolates produced profiles representative of rifampicin susceptibility using this assay.

Of the 41 isolates indicated as isoniazid mono-resistant by phenotypic DST, the GenoType® MTBDR*plus* assay indicated additional resistance to rifampicin in 3 isolates. In two of these the absence of signal from wild-type probes 3 and 4 is suggestive of a mutation at codon 516 of RpoB (Fig. 2.5, strip 3). The remaining isolate lacked a hybridization signal from wild-type probe 7, indicating the presence of a mutation at codon 526.

All 90 phenotypically susceptible *M. tuberculosis* isolates included in this study (100%) were indicated as susceptible to rifampicin using the GenoType® MTBDR*plus* assay (Table 2.5; Fig. 2.5, strips 8-10).

2.3.3 Detection of resistance to isoniazid using the GenoType® MTBDR*plus* assay

Resistance to isoniazid due to the presence of a KatG S315T amino acid substitution and/or an *inhA* C-15T nucleotide substitution was determined for all isolates using the GenoType® MTBDR*plus* assay [Appendix A]. This assay correctly indicated isoniazid resistance in 75 of 82 (91.6%) MDR isolates. Of these, 44 (53.7%) carried a KatG S315T mutation, 21 (25.6%) carried an *inhA* C-15T mutations and 10 of 82 (12.2%) carried both KatG S315T and *inhA* C-15T mutations (Table 2.5; Fig, 2.5). The remaining 7 isolates produced hybridization patterns representative of isoniazid susceptible isolates. Additionally, the GenoType® MTBDR*plus* assay identified a KatG S315T mutation in a rifampicin mono-resistant isolate that produced hybridization signals characteristic of a rifampicin susceptible isolate using this assay.

A total of 26 of 41 (63.4%) isoniazid mono-resistant isolates were correctly identified as resistant to isoniazid using this assay (Table 2.5). Of these, 13 (31.7%) isolates carried KatG S315T and 11 (26.8%) carried *inhA* C-15T (Table 2.5). A further 2 isoniazid mono-resistant isolates produced both wild-type and mutant *inhA*

Table 2.5: Evaluation of the GenoType® MTBDR*plus* assay for detection of isoniazid and rifampicin resistance in *M. tuberculosis*.

Phenotypic susceptibility profile of *M. tuberculosis* Isolates (n)	RIF								INH						
	RpoB				No R detected	HR	ND	R detected (%)	KatG S315T	*inhA* C-15T	[b]Both	[c]No R detected	HR	ND	R detected (%)
	S531L	H526D	D516V	Other											
MDR (82)	71	1	2	5	2	1	0	97.6	44	21	10	7	0	0	91.6
INH^R RIF^S (41)	0	0	0	[a]3	38	0	0	7.3	13	11	0	13	2	2	63.4
RIF^R INH^S (11)	3	0	0	4	3	0	0	70	1	0	0	10	0	0	9.1
RIF^S INH^S (90)	0	0	0	0	90	0	0	0	0	0	0	89	1	0	1.1

RIF, rifampicin; INH, isoniazid; R, resistance; HR, heteroresistance; ND, not determined; MDR, multi-drug resistant; [R], resistant; [S], susceptible.
[a]RpoB mutations at codon 516 (n = 2) and 526 (n = 1) were confirmed by sequence analysis.
[b]Both KatG S315T and *inhA* C-15T mutations detected in the same isolate.
[c]Neither KatG S315T nor *inhA* C-15T detected in an isolate.

hybridisation signals (Fig. 2.5), which may indicate a mixed infection in which one isolate is susceptible to isoniazid and the other is resistant (Miotto *et al*, 2006). Alternatively this may indicate heteroresistance, in which a small subpopulation of bacteria are resistant while the majority of the organisms are susceptible (Falagas *et al*, 2008). In this case it is recommended that the isolate should, however, be considered resistant to isoniazid. No isoniazid mono-resistant isolates harboured both KatG S315T and *inhA* C-15T mutations simultaneously. It is notable that the GenoType® MTBDR*plus* assay did not identify isoniazid resistance in 13 of 41 (31.7%) isolates indicated as isoniazid mono-resistant by phenotypic DST.

Of the 90 phenotypically rifampicin and isoniazid susceptible *M. tuberculosis* isolates, 89 (98.9%) were indicated as susceptible to isoniazid using the GenoType® MTBDR*plus* assay (Table 2.5; Fig. 2.5, strips 8 & 9). In one susceptible isolate, a signal was obtained from both the *inhA* wild-type probes and the MUT1 probe (Fig. 2.5, lane 10), suggesting isolate heterogeneity or a mixed infection (Miotto *et al*, 2006) and this isolate was therefore considered to be resistant to isoniazid.

2.3.4 Correlation between standard phenotypic susceptibility testing and the GenoType® MTBDR*plus* assay for the detection of rifampicin and isoniazid resistance

The GenoType® MTBDR*plus* assay confirmed standard DST results for rifampicin and isoniazid in 75 of 82 (91.6%) MDR isolates, 23 of 41 (56.1%) isoniazid mono-resistant isolates, and 7 of 11 (63.6%) rifampicin mono-resistant isolates (Table 2.5). Seven MDR isolates were, however, indicated as isoniazid susceptible using this assay.

Three phenotypically isoniazid mono-resistant isolates were indicated as MDR due to the absence of hybridization signals from *rpoB* wild-type probes. Of these, two were shown to have a mutation at codon 516 due to the absence of a signal from wild-type probes 3 and 4. The remaining isolate did not produce a hybridization signal from wild-type probe 7, indicating the presence of a mutation at codon 526 of RpoB in this isolate. Four rifampicin mono-resistant isolates were indicated as rifampicin susceptible by the GenoType® MTBDR*plus* assay, one of which was also indicated as isoniazid resistant due to the presence of KatG S315T.

A total of 13 isoniazid mono-resistant isolates produced hybridization signals characteristic of isoniazid susceptible isolates following interpretation of this assay (Table 2.5). Two isolates produced both wild-type and mutant hybridization signals, suggesting a possible mixed infection, but in accordance with the recommended interpretation of this type of result they were nonetheless correctly identified as isoniazid resistant. One isoniazid mono-resistant isolate produced no *M. tuberculosis* complex (TUB) hybridization signal (Fig. 2.5, lane 7), which suggests that this isolate was not *M. tuberculosis*. However, the use of the GenoType® Mycobacterium CM assay [HAIN Lifescience] confirmed this isolate as *M. tuberculosis*. In another isolate, complete absence of hybridization to any *katG* probes was noted, suggesting that *katG* may be deleted in this isolate.

2.3.5 Detection of isoniazid resistance using MAS-PCR assays

MAS-PCR assays designed to detect the S315T KatG amino acid substitution and *inhA* C-15T promoter mutation were carried out on all isolates [Appendix A]. Products corresponding to wild-type for both *katG* and *inhA* assays obtained from one isolate were purified and sequenced to confirm the absence of the mutations, and this isolate was included as a wild-type control in all subsequent assays. Similarly, products suggestive of the presence of a KatG S315T substitution or an *inhA* C-15T mutation were obtained from two mutant control isolates and, following sequence analysis to confirm the presence of each of the mutations (Fig. 2.3 & 2.4), these isolates were also included in all subsequent MAS-PCR assays (Fig. 2.6 & 2.7).

MAS-PCR analysis of 82 MDR isolates identified a KatG S315T mutation (Fig. 2.6; Table 2.6) in 44 (53.7%) due to the amplification of a 435 bp product, and an *inhA* C-15T mutation in 21 (25.6%) due to the amplification of a 451 bp product only (Fig. 2.7; Table 2.6). A total of 10 (12.2%) MDR isolates were shown to carry both KatG S315T and *inhA* C-15T mutations (Table 2.6). Amplicons representative of wild-type isolates were obtained using both assays in the remaining 7 MDR isolates, which is suggestive of isoniazid susceptibility (Table 2.6). MAS-PCR assays also detected a KatG S315T mutation in 1 of 11 rifampicin mono-resistant isolates.

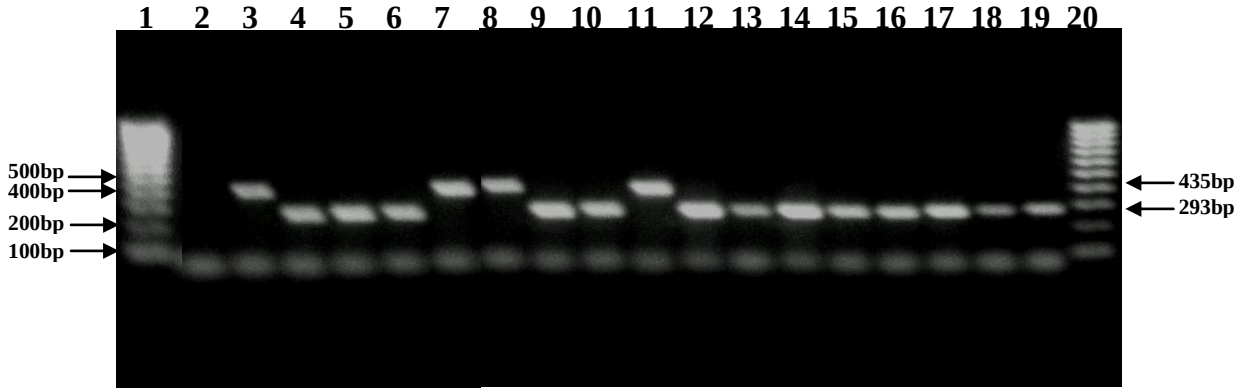

Figure 2.6: Agarose gel electrophoresis of *katG* MAS-PCR amplicons obtained from representative MDR-TB isolates. Lane 1, Hyperladder IV [Bioline]; lane 2, no DNA control; lane 3, KatG S315T mutant control; lane 4, wild-type control; lanes 5-6, 9-10 and 12-19, wild-type isolates; lanes 7-8 and 11, KatG S315T mutants.

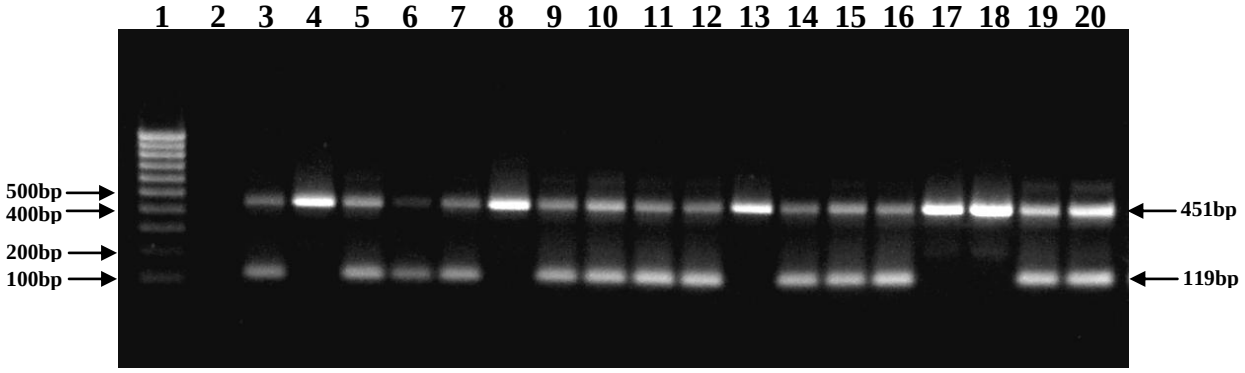

Figure 2.7: Agarose gel electrophoresis of *inhA* MAS-PCR amplicons obtained from representative MDR-TB isolates. Lane 1, Hyperladder IV [Bioline]; lane 2, no DNA control; lane 3, wild-type control; lane 4, *inhA* C-15T mutant control; lanes 5-7, 9-12, 14-16 and 19-20, wild-type isolates; lanes 8, 13 and 17-18, *inhA* C-15T muants.

Of 41 isoniazid mono-resistant isolates included in this study, MAS-PCR identified KatG S315T in 12 (29.3%), *inhA* C-15T in 10 (24.4%) and no isolates were identified as having both mutations (Table 2.6). Notably, neither KatG S315T nor *inhA* C-15T mutations were detected in 16 of 41 (39.0%) isoniazid mono-resistant isolates (Table 2.6). No products could be amplified by either assay for two isolates. In the remaining isolate amplicons representative of a wild-type isolate were obtained for the *inhA* assay, whilst no products were obtained for the *katG* assay.

Table 2.6: Detection of isoniazid resistance in *M. tuberculosis* using MAS-PCR assays.

M. tuberculosis Isolates (n)	INH					
	KatG S315T	*inhA* C-15T	Both	Neither	ND	R detected (%)
MDR (82)	45	21	10	7	0	91.5
INH^R (41)	12	10	0	[a]16	3	53.7
RIF^R (11)	1	0	0	0	0	9.1
RIF^S INH^S (25)	0	0	0	25	0	0

INH, isoniazid; ND, not determined; R, resistance; MDR, multi-drug resistant; ^{R}, resistant; RIF, rifampicin; ^{S}, susceptible.
[a]The presence of a KatG S315T (n=1) and an *inhA* C-15T (n=1) mutation in two isolates that were detected by GenoType MTBDR*plus* assay but not by MAS-PCR was confirmed by sequence analysis.

MAS-PCR assays for the detection of KatG S315T in 25 isoniazid susceptible clinical *M. tuberculosis* isolates produced amplicons of 293 bp, representative of isolates that are wild-type at this locus (Fig. 2.8; Table 2.6). Similarly, the production of both 451 bp and 119 bp amplicons following MAS-PCR for the detection of *inhA* C-15T (Fig. 2.9; Table 2.6) indicated that none of the susceptible isolates carry this mutation.

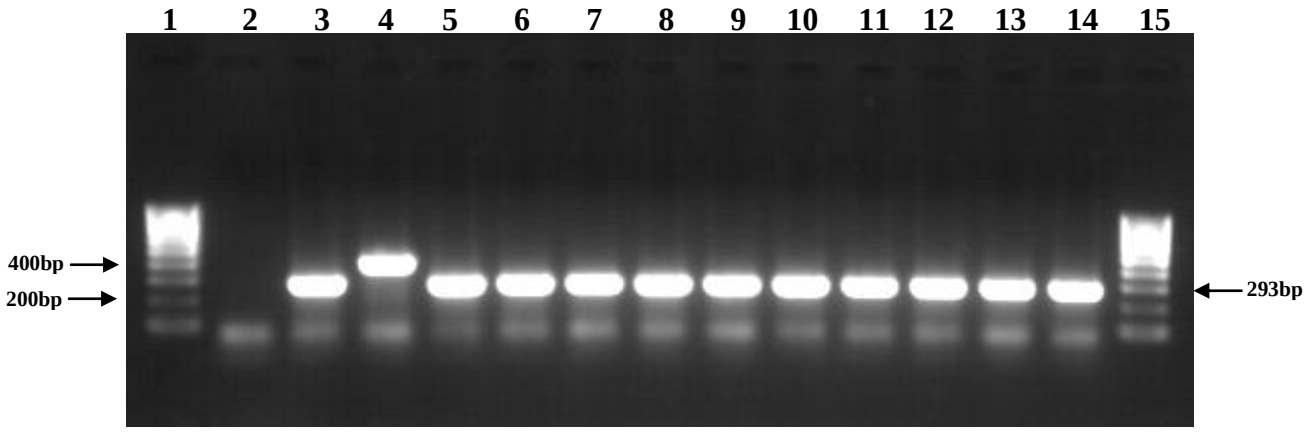

Figure 2.8: MAS-PCR amplification for the detection of KatG S315T in isoniazid susceptible clinical isolates of *M. tuberculosis*. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, wild-type control; lane 4, KatG S315T mutant control; lanes 5 to 14, isoniazid susceptible *M. tuberculosis* clinical isolates; lane 15, molecular weight marker Hyperladder I [Bioline].

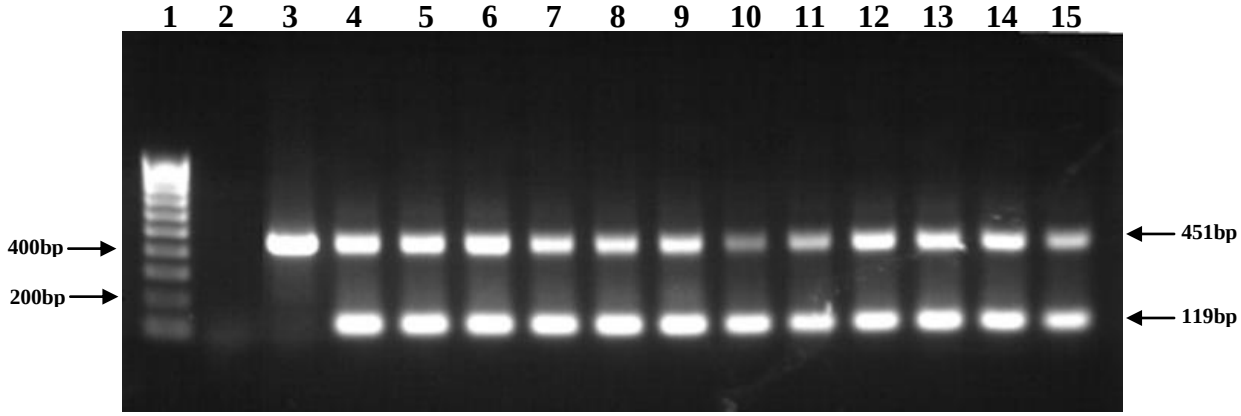

Figure 2.9: MAS-PCR amplification for the detection of *inhA* C-15T in isoniazid susceptible clinical isolates of *M. tuberculosis*. Lane 1, molecular weight marker Hyperladder I [Bioline]; lane 2, no DNA control; lane 3, *inhA* C-15T mutant control; lane 4, wild-type control; lanes 5 to 15, isoniazid susceptible *M. tuberculosis* clinical isolates.

2.3.6 Correlation of GenoType® MTBDR*plus* and MAS-PCR assays for the detection of isoniazid resistance in *M. tuberculosis*

In all 82 MDR isolates investigated, interpretable results were obtained using both the GenoType® MTBDR*plus* assay and MAS-PCR analysis. Both assays indicated the presence of a KatG S315T amino acid substitution in the same 44 isolates, an *inhA* C-15T mutation in the same 21 isolates and both KatG S315T and *inhA* C-15T in the same 10 isolates. It is notable that both assays detected a KatG S315T amino acid substitution in the same rifampicin mono-resistant strain. Repeat phenotypic DST for isoniazid on this isolate could not be carried out, however, due to lack of viability of the organism at the time of testing.

Results within the isoniazid mono-resistant subgroup of strains were more variable, with both assays producing the same results in 34 of 41 (82.9%) strains. The GenoType® MTBDR*plus* assay detected a KatG S315T mutation in two strains that were not identified by MAS-PCR; in one strain amplicons representative of wild-type strains were obtained and in the other strain no MAS-PCR products could be amplified using either the KatG or *inhA* MAS-PCR assays. Sequence analysis of *katG* in both of these isolates identified an AGC (Ser) to ACC (Leu) nucleotide substitution at codon 315, confirming the results obtained by the GenoType® MTBDR*plus* assay (Fig. 2.10a,b). The GenoType® MTBDR*plus* assay also identified a C-15T substitution in one strain that was not detected by MAS-PCR, and sequence analysis

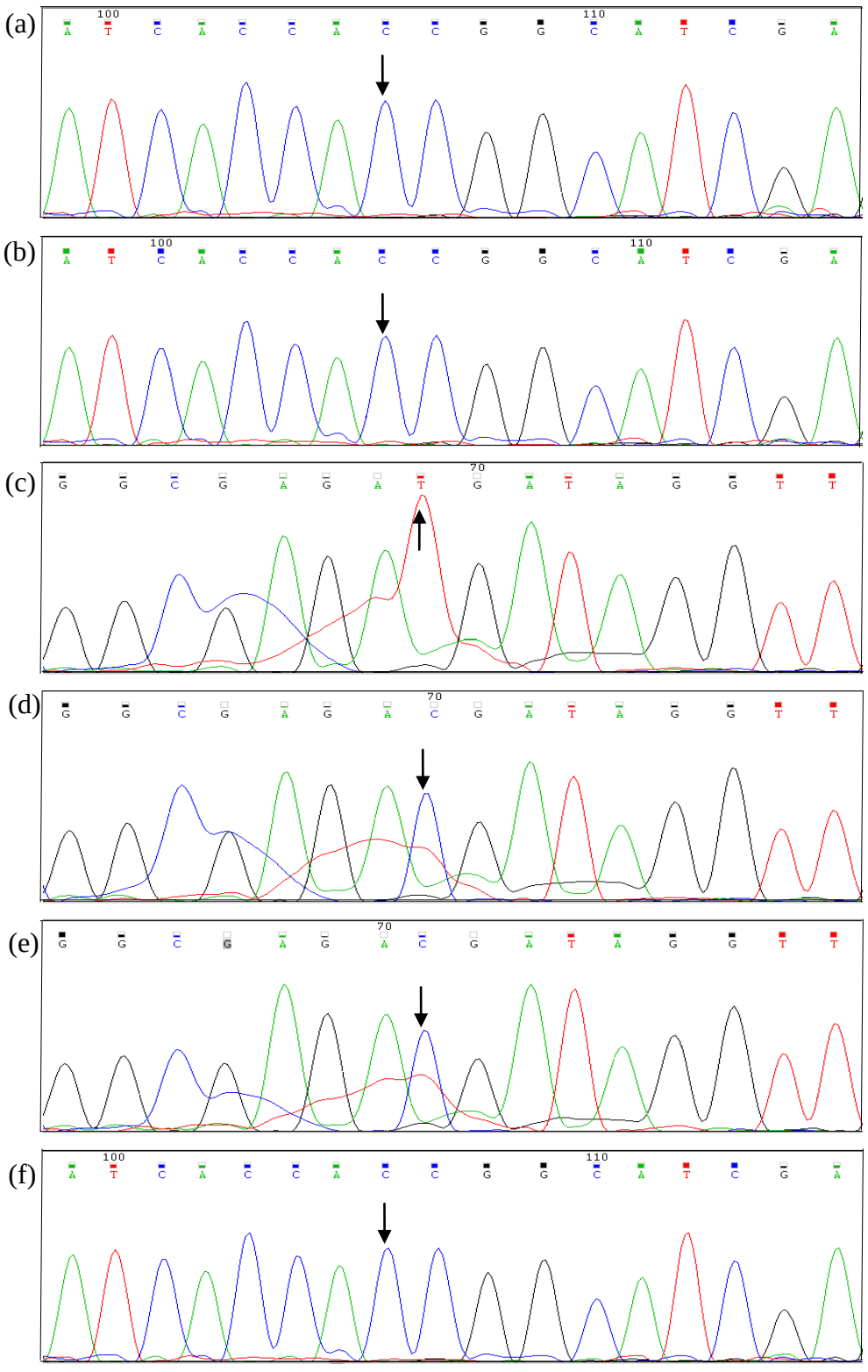

Figure 2.10: Electropherograms representing regions of *katG* and *inhA* nucleotide sequence in *M. tuberculosis* isolates from which discrepant results were obtained by the GenoType® MTBDR*plus* assay and MAS-PCR. Arrows indicate nucleotides of interest. (a), (b) & (f) confirmation of an AGC (Ser) to ACC (Tyr) nucleotide mutation in *katG* at codon 315; (c) a C-15T nucleotide mutation in the promoter region of *inhA*; (d) & (e), isolates wild-type at the *inhA* C-15T locus.

confirmed the presence of this mutation in *inhA* (Fig. 2.10c). In two strains, both wild-type and mutant *inhA* hybridization signals were obtained using the GenoType® MTBDR*plus* assay, whilst amplification products characteristic of isoniazid susceptible isolates were obtained in the MAS-PCR assay and confirmed by sequence analysis (Fig. 2.10d,e). In another isolate, MAS-PCR detected a KatG S315T mutation, but no TUB hybridization was obtained using the GenoType® MTBDR*plus* assay. This isolate was, however, confirmed as *M. tuberculosis* complex using the GenoType® Mycobacterium CM assay [HAIN Lifescience], and the presence of the KatG S315T mutation was confirmed by sequence analysis (Fig. 2.10f).

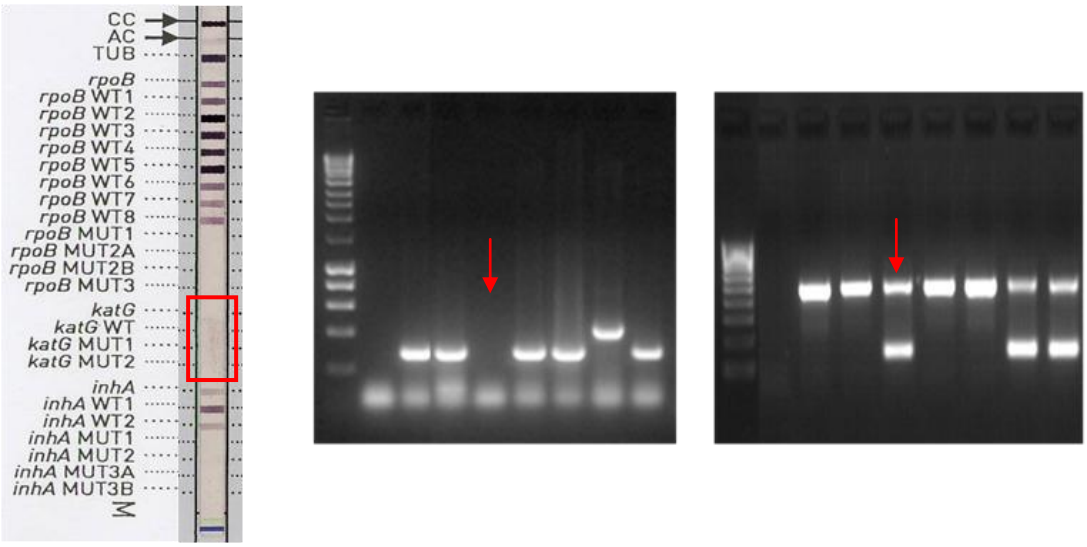

Figure 2.11: GenoType® MTBDR*plus* strip of an isolate showing no hybridization from any *katG* probes (a) and agarose gel electrophoresis of *katG* (b) and *inhA* (c) MAS-PCR amplicons obtained from the same isolate. The absence of the wild-type *katG* internal hybridisation control (indicated within the red box) and *katG* amplicons (indicated by red arrows) despite successful amplification of *inhA* suggests that *katG* may be deleted in this isolate.

Two isolates produced no amplicons in either MAS-PCR assay, suggesting that the starting DNA template may have been insufficient in concentration and/or integrity to result in the production of amplicons. Interpretable results were however obtained for both isolates using the GenoType® MTBDR*plus* assay. This indicates that the quality of the template DNA to be used must be taken into account prior to using MAS-PCR assays for the detection of resistance determinants. In one isolate both the GenoType® MTBDR*plus* assay and MAS-PCR produced hybridization signals (Fig. 2.11a) and amplicons (Fig. 2.11b,c), respectively, representative of wild-type

inhA, but neither assay produced any products representative of *katG*, suggesting that *katG* may be deleted in this isolate (Fig 2.11a,b,c).

2.3.7 Relationship between genotype and resistance phenotype using spacer oligonucleotide typing and MIRU-VNTR typing

Spoligotyping of all 224 isolates included in this study [Appendix A] identified more than half (56.9%) as either W-Beijing (39.9%) or LAM3/F11 (17.0%) (Fig. 2.12; Table 2.7), which is in accordance with previous reports from this region (Marais *et al*, 2006; Nicol *et al*, 2005). W-Beijing isolates are characterized by the presence of at least three spacers between 35-43 and an absence of spacers 1-34 (Kremer *et al*, 2004; van Soolingen *et al*, 1995), whilst LAM3/F11 isolates are identified by the absence of spacers 9-11, 21-24 and 33-36 (Victor *et al*, 2004) (Fig 2.12).

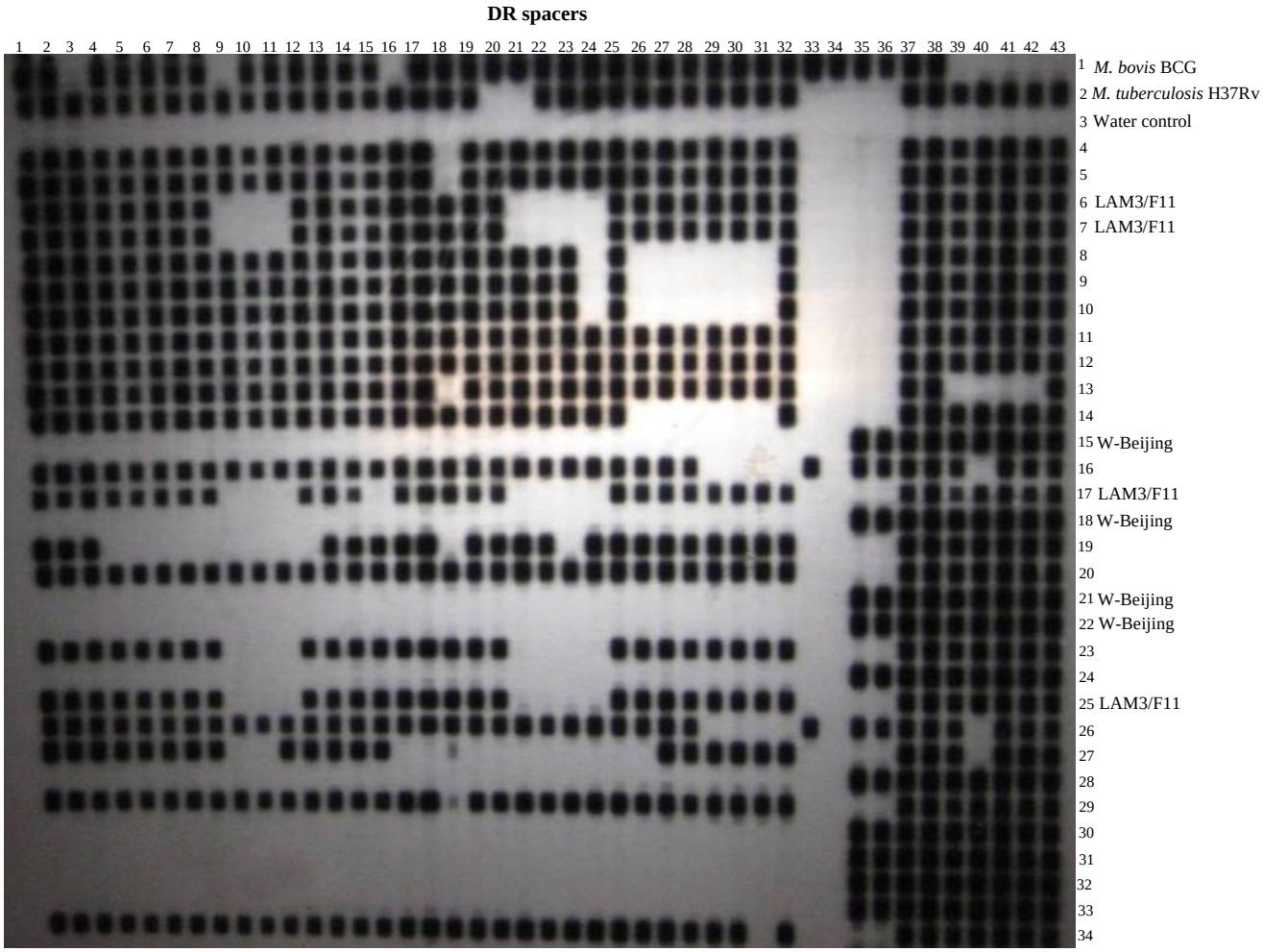

Figure 2.12: Autoradiograph of the spoligotyping profiles of representative MDR-TB isolates. DR spacers 1 – 43 are indicated above the autoradiograph; row 1, *M. bovis* BCG; row 2, *M. tuberculosis* H37Rv; row 3, water negative control; rows 4 – 34, MDR clinical *M. tuberculosis* isolates; representative W-Beijing and LAM3/F11 isolates are indicated.

Notably, 49 (59.8%) of 82 MDR-TB isolates included in this study were of the W-Beijing lineage (Table 2.7), confirming previous reports of an association between W-Beijing isolates and the MDR phenotype (odds ratio, 3.29; 95% confidence interval, 1.76 to 6.16) (Marais *et al*, 2006). By contrast, 9.1% (1 of 11) rifampicin mono-resistant, 26.8% (11 of 41) of isoniazid mono-resistant and 31.1% (28 of 90) of isolates susceptible to both rifampicin and isoniazid were W-Beijing lineage isolates (Table 2.7). A total of 9 (11%) MDR isolates belong to the LAM3/F11 family, compared with 9.8% (4 of 41 of isoniazid mono-resistant isolates, 18.9% (17 of 90) of susceptible isolates and, interestingly, 72.7% (8 of 11) of rifampicin mono-resistant isolates (Table 2.7). Within the isoniazid mono-resistant group of isolates, the W-Beijing lineage (26.8%) and X3 lineage (24.4%) were observed with the highest frequency. The majority of the rifampicin and isoniazid susceptible isolates were W-Beijing (31.1%), LAM3/F11 (18.9%), T1 (16.7%) and isolates with unique spoligotyping profiles (17.8%). The remaining isolates were either T1, T3, T4, T5, LAM4, LAM9, EAI1, H1, H3, S, X1, X2, X3, CAS or Family 36 and these were all observed with low frequency (Table 2.7). Whilst a relatively large proportion of susceptible isolates had unique profiles (17.8%), these were seen in only 2 (2.4%) MDR isolates and 3 (7.3%) isoniazid mono-resistant isolates (Table 2.7). No rifampicin mono-resistant isolates had unique spoligotyping profiles (Table 2.7).

Table 2.7: Relationship between resistance phenotype and *M. tuberculosis* lineage as determined by spacer oligonucleotide typing.

M. tuberculosis Isolates (n)	Spoligotyping Profile																		
	W-B	LAM3/ F11	X3	T1	T3	T4	T5	LAM4	LAM9	EAI1	H1	H3	S	X1	X2	CAS	Family 36	Unique	Mixed
MDR (82)	49	9	4	2	1	0	2	2	2	2	1	0	1	4	1	0	0	2	0
INH^R (41)	11	4	10	5	1	1	0	0	0	1	0	2	2	0	0	0	0	3	1
RIF^R (11)	1	8	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
INH^S RIF^S (90)	28	17	3	15	0	3	0	0	1	0	1	0	0	0	2	1	2	16	1

MDR, multi-drug resistant; INH, isoniazid; ^{R}, resistant; RIF, rifampicin; ^{S}, susceptible.

Of 49 MDR W-Beijing isolates, 42 (85.7%) carried an S531L mutation in RpoB that accounted for resistance to rifampicin. One MDR W-Beijing isolate carried a H526D mutation in RpoB, two carried a D516V mutation and one was identified by the GenoType MTBDR*plus* assay as having a mutation at either codon 531 or 533. Three MDR W-Beijing isolates produced hybridisation patterns representative of rifampicin susceptible isolates using this assay. Isoniazid resistance in 40.8% of MDR W-Beijing isolates could be attributed to an *inhA* C-15T mutation, while 30.6% carried a KatG S315T mutation and 16.3% carried both *inhA* C-15T and KatG S315T mutations (Fig. 2.13). A total of 8.2% of MDR W-Beijing isolates produced profiles representative of isoniazid susceptible isolates using both the GenoType MTBDR*plus* assay and MAS-PCR assays (Fig. 2.13), suggesting that isoniazid resistance in these isolates is due to resistance determinants other than *inhA* C-15T and KatG S315T.

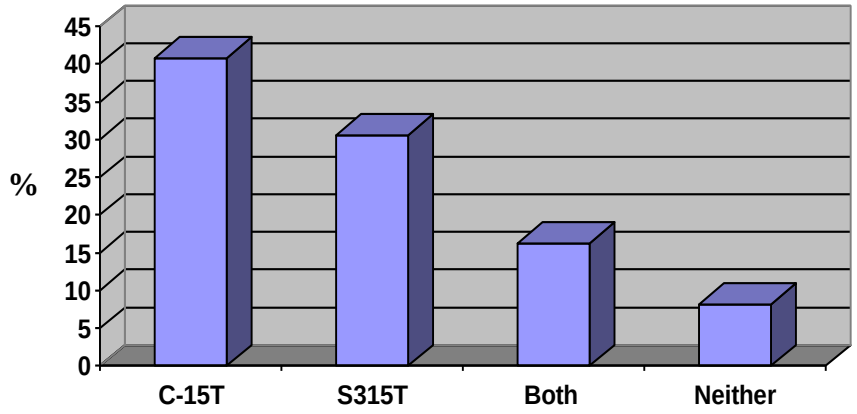

Figure 2.13: Distribution of isoniazid resistance determinants amongst MDR isolates of the W-Beijing lineage.

The eight rifampicin mono-resistant LAM3/F11 isolates carried various rifampicin resistance determinants, including RpoB S531L (n = 3), Δ518 (n = 1) and Δ531/533 (n = 1). Three rifampicin mono-resistant LAM3/F11 isolates produced GenoType MTBDR*plus* hybridisation profiles representative of rifampicin susceptible isolates.

It is noteworthy that 7 of the 13 (53.8%) isoniazid mono-resistant isolates that were identified as isoniazid susceptible by both the GenoType MTBDR*plus* assay and MAS-PCR were of the X3 lineage. Also, 9 of 11 (81.8%) isoniazid mono-resistant W-Beijing isolates carry *inhA* C-15T, whilst only 2 (18.2%) carry KatG S315T.

Further discrimination of 49 MDR W-Beijing isolates, 24 susceptible W-Beijing isolates, 8 rifampicin mono-resistant LAM3/F11 isolates and 8 isoniazid mono-resistant X3 isolates was carried out by MIRU-VNTR analysis. The relatedness of all isolates was determined using the MIRU-VNTR*plus* online application and isolates were defined as belonging to same cluster complex if they differed from each other at two alleles or less. All of the W-Beijing isolates clustered together in one complex, while the rifampicin mono-resistant LAM3/F11 isolates and the isoniazid mono-resistant X3 isolates formed another cluster complex distinct from the W-Beijing isolates (Fig 2.14). The MIRU-VNTR digital profiles of all W-Beijing and LAM3/F11 isolates can be found in Appendix D.

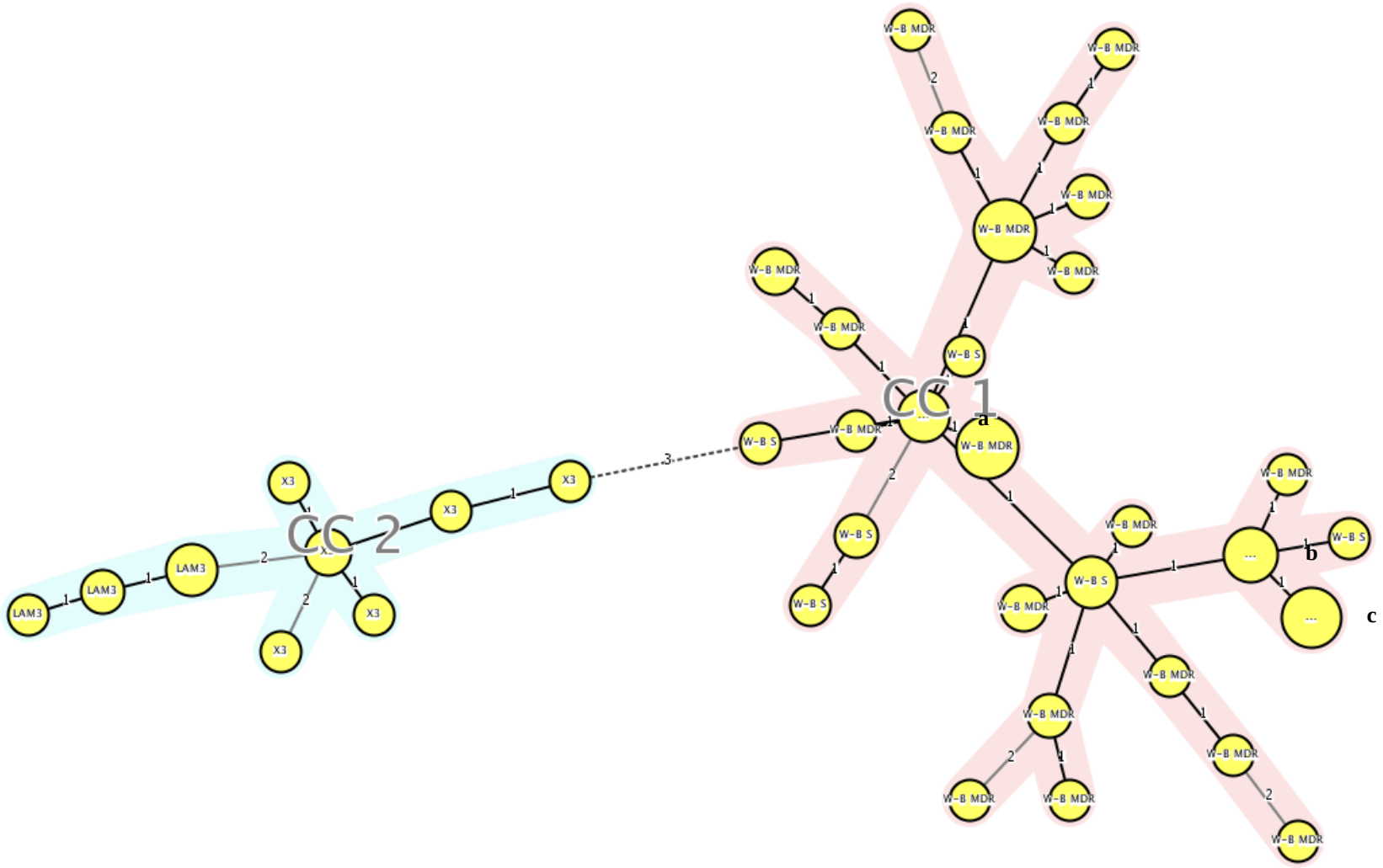

Figure 2.14: Minimum spanning tree showing the genetic relatedness of *M. tuberculosis* isolates as determined by 12-locus MIRU-VNTR analysis. Cluster complex 1 (CC 1), consisting of W-Beijing isolates, is indicated in pink. Cluster complex 2 (CC 2), consisting of the rifampicin mono-resistant LAM3/F11 and isoniazid mono-resistant X3 isolates, is indicated in blue. The size of the circles is proportionate to the number of identical isolates within each group. Numbers indicate the allele differences between the connected strains. a, group contains four MDR W-Beijing isolates and one susceptible W-Beijing isolate; b, group contains one MDR W-Beijing isolate and five susceptible W-Beijing isolates; c, group contains one MDR W-Beijing isolate and 7 susceptible W-Beijing isolates.

Interestingly, the majority of the MDR W-Beijing isolates harbouring the *inhA* C-15T isoniazid resistant determinant were found to cluster together (Fig. 2.15). These isolates were also all resistant to rifampicin due to an S531L mutation in RpoB, suggesting dissemination of a W-Beijing MDR clone in this setting.

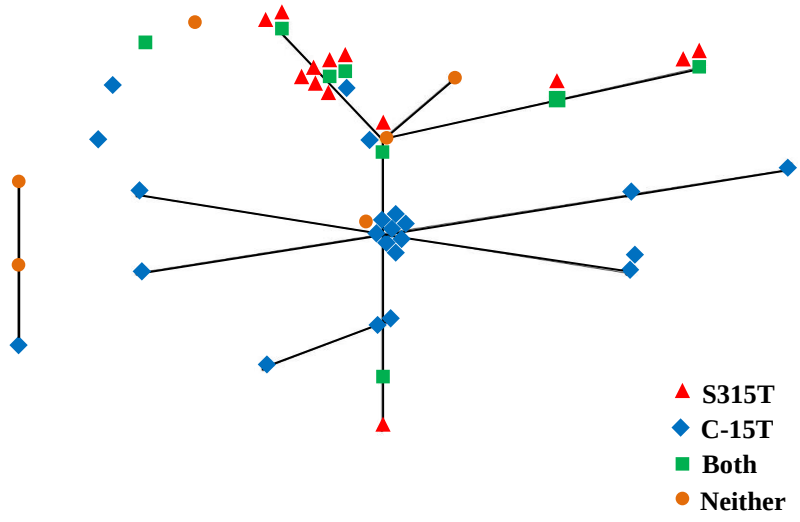

Figure 2.15: Genetic clustering of W-Beijing MDR isolates, as determined by MIRU-VNTR analysis, according to their isoniazid resistance determinants. Each isolate is represented by a shape. Isolates separated by a straight line differ from one another at a single allele. Outliers differ from all other isolates at two or more alleles.

2.4 Discussion

The increasing emergence of *M. tuberculosis* isolates that are resistant to one or more antituberculous drugs poses a serious threat to global tuberculosis control. In 2006, DST was performed on 652 *M. tuberculosis* isolates at GSH, and of these 14.6% were indicated as MDR, 10.3% as isoniazid mono-resistant and 1.7% as rifampicin mono-resistant. This high incidence of drug resistant *M. tuberculosis* in the Western Cape is inflated, however, since in South Africa only isolates suspected resistant due to treatment failure undergo DST. Conversely, it should also be taken into account that phenotypic DST can sometimes underestimate the proportion of drug resistant isolates due to the difficulties associated with growing *M. tuberculosis in vitro*.

Another short-coming of phenotypic DST is the length of time to detection of growth, which can take up to 6 weeks. The advent of molecular assays for the detection of drug resistance in *M. tuberculosis* has enabled earlier detection of drug resistance and

tailoring of treatment regimens. The most recently developed assay for the detection of resistance to both rifampicin and isoniazid, GenoType® MTBDR*plus* assay [HAIN Lifescience], was evaluated in this study. Overall MDR, isoniazid mono-resistance and rifampicin mono-resistance were detected by the GenoType® MTBDR*plus* assay with sensitivities of 91.5%, 56.1% and 63.6%, respectively. These results are in contrast to a large-scale evaluation of the GenoType® MTBDR*plus* assay recently undertaken in the Western Cape, in which sensitivities of 97.7%, 96.5% and 72.7% for MDR, isoniazid mono-resistance and rifampicin mono-resistance, respectively, were reported (Barnard *et al*, 2008). However, overall specificities obtained in this study of 98.9%, 98.9% and 100.0% for MDR, isoniazid resistance and rifampicin resistance detection, respectively, are similar to those described by Barnard and colleagues (2008). Both of these evaluations were conducted in the Western Cape region, but the *M. tuberculosis* isolates included in this investigation were likely from a smaller area in and around Cape Town, whilst Barnard *et al* (2008) obtained isolates from the entire Western Cape region. It may be that *M. tuberculosis* isolates with unusual resistance determinants, particularly in the case of isoniazid, are being disseminated in and around the city centre as compared to the rest of the province.

The GenoType® MTBDR*plus* assay was effective at identifying rifampicin resistance in the MDR isolates, with a sensitivity of 97.6%. Resistance to rifampicin in two isolates indicated as rifampicin susceptible using this assay is likely due to mutations outside the RRDR of *rpoB* (Yue *et al*, 2003; Heep *et al*, 2001), or possibly in an as yet unidentified locus. The RpoB S531L amino acid substitution accounted for rifampicin resistance in 86.6% of MDR and 27.3% rifampicin mono-resistant isolates. The overall prevalence of RpoB S531L is 80.4%, which is somewhat higher than reported worldwide, but comparable to the 70.5% reported in this geographical area (Barnard *et al*, 2008). In contrast, the proportion of mutations at codons 526 (4.3%) and 516 (3.3%) of RpoB is lower than that observed elsewhere (Ramaswamy & Musser, 1998). The low fitness cost associated with RpoB S531L may account for the high frequency with which it is observed (Gagneux *et al*, 2006), especially amongst MDR isolates. Additionally, the presumably higher fitness cost associated with the other less commonly observed mutations in the rifampicin mono-resistant isolates may account, in part, for the low frequency with which rifampicin mono-resistant isolates are

observed in clinical practice. The HIV status of these patients is unknown, but it may be that the high rate of HIV/AIDS in patients in this region facilitates the proliferation of these less fit rifampicin mono-resistant isolates that would usually be contained in immunocompetent people.

A caveat in the interpretation of the GenoType® MTBDR*plus* assay with respect to the detection of rifampicin resistance is that resistance may be indicated by the absence of a wild-type hybridization signal alone, without confirmation by a mutant probe signal, as was seen in 6.1% MDR and 36.4% of rifampicin mono-resistant isolates in this study. Since a wild-type probe may not hybridize due to a mutation in the RRDR that is not associated with a resistance phenotype (Ma *et al*, 2006), such rifampicin susceptible isolates would be called resistant and may lead to the unnecessary removal of rifampicin from treatment regimens. In addition, the recent identification of mutations at codon 533 of rifampicin susceptible isolates suggests that isolates indicated as resistant by GenoType® MTBDR*plus* due to a mutation at codon 533 may in fact be rifampicin susceptible (Ma *et al*, 2006) and similar caution should be observed. Of more concern is that the GenoType® MTBDR*plus* assay failed to detect rifampicin mono-resistance in 30% of cases. Treatment of these patients with standard TB regimens may therefore hasten the emergence of MDR isolates. Furthermore, 8 of 11 rifampicin mono-resistant LAM3/F11 isolates fall within the same MIRU-VNTR cluster, which may suggest expansion of a rifampicin mono-resistant LAM3/F11 isolate. The identification of different resistance determinants in this group of isolates, however, suggests that they may not be descendant of a common clone. Rather, LAM3/F11 isolates may have a predisposition to develop rifampicin mono-resistance, which is rarely seen amongst clinical *M. tuberculosis* isolates.

MAS-PCR assays are rapid and simple to perform, and are less costly than commercial assays, which is of considerable importance in developing countries with limited resources and a high TB disease burden, such as South Africa. MAS-PCR assays were evaluated for the detection of KatG S315T and *inhA* C-15T isoniazid resistance determinants and compared with detection of resistance using the GenoType® MTBDR*plus* assay. Comparable results for the detection of isoniazid resistance were obtained using both assays. As MAS-PCR assays are much cheaper

and easier to perform than the GenoType® MTBDR*plus* assay, this has significant implications for the molecular detection of isoniazid resistance in low-income settings where the standardized use of the GenoType® MTBDR*plus* assay is not feasible. The same genetic basis of isoniazid resistance was identified in 75 of 82 (91.6%) MDR isolates and 34 of 41 (82.9%) isoniazid mono-resistant isolates. Neither assay detected a mutation in 7 MDR isolates and 13 isoniazid mono-resistant isolates. These isolates would therefore have been interpreted as isoniazid susceptible by both genotypic assays, and it is likely that they carry an isoniazid resistance determinant other than KatG S315T or *inhA* C-15T. The GenoType® MTBDR*plus* assay identified a KatG S315T mutation in two isoniazid mono-resistant isolates and an *inhA* C-15T mutation in one isoniazid mono-resistant isolate that were not detected using MAS-PCR, and the presence of all three muations was confirmed by sequence analysis. That no products could be amplified by either MAS-PCR assay in two of these isolates suggests that the GenoType® MTBDR*plus* assay is more sensitive than MAS-PCR at detecting resistance determinants when the starting DNA template is low in concentration or of poor integrity. Both assays suggested deletion of *katG*, and hence isoniazid resistance (Ramaswamy *et al*, 2003; Ramaswamy & Musser, 1998; Marttila *et al*, 1996), in one isolate as no PCR amplicons could be obtained using *katG*-specific primer sets and no *katG*-specific hybridization signals were obtained from the GenoType MTBDR*plus* assay. Neither assay could confirm the mechanism of resistance to isoniazid in this isolate, however, as a specific resistance determinant could not be identified. In addition, sequencing of *inhA, ndh* and the *oxyR-ahpC* region of this isolate (personal communication, L. Ah Tow) did not identify any mutations that could account for isoniazid resistance or compensate for the loss of KatG activity.

Both the GenoType® MTBDR*plus* assay and MAS-PCR identified a KatG S315T mutation in 53.7% of MDR isolates and in 29.3% of isoniazid mono-resistant isolates, providing further evidence for an association between KatG S315T and MDR *M. tuberculosis* (Hazbón *et al*, 2006; Aktas *et al*, 2005). A C-15T mutation in the promoter region of *inhA* was observed in 25.6% of MDR isolates and 24.4% of isoniazid mono-resistant isolates. It is interesting to note that despite the association between KatG S315T and MDR, the majority of W-Beijing MDR isolates carried an *inhA* C-15T mutation that accounted for resistance to isoniazid in these isolates. In

addition, all of these isolates were resistant to rifampicin due to an RpoB S531L mutation. The drug-resistant W-Beijing cluster 220 subgroup is well described in the Western Cape region (Johnson *et al*, 2006c; Marais *et al*, 2006), and all isolates within this subgroup carry an *inhA* C-15T mutation and the predominant rifampicin resistance marker in this strain is RpoB S531L (Johnson *et al*, 2006). It is therefore likely that the data reported here reflects the identification of this subgroup, which was first defined by IS*6110* RFLP typing. This is further supported by the observation that these isolates cluster together following MIRU-VNTR analysis, confirming that they are descendant of the same clonal line. The extensive transmission of this virulent MDR *M. tuberculosis* strain in the Western Cape region suggests that these W-Beijing cluster 220 isolates may be more fit than other W-Beijing strains (Johnson *et al*, 2006). This further highlights the need for the timely administration of appropriate therapies in order to reduce dissemination of drug-resistant strains such as this. The GenoType® MTBDR*plus* assay targets both the rifampicin and the isoniazid resistance determinants most frequently identified in W-Beijing cluster 220 isolates, making it an effective tool for identifying MDR-TB in regions where this strain is prominent.

However, that 36.6% and 46.3% of isoniazid resistance in isoniazid mono-resistant isolates was not detected by either GenoType® MTBDR*plus* assay or MAS-PCR, respectively, is of concern as these isolates present a potential reservoir for future MDR-TB. This high prevalence of isoniazid resistant isolates harbouring neither KatG S315T nor *inhA* C-15T contrasts with reports attributing isoniazid resistance in the majority of isolates to either or both of these mutations (Barnard *et al*, 2008; Hazbón *et al*, 2006). It may be that less prevalent mutations in *katG*, *inhA*, *ndh*, and *oxyR-ahpC*, or in a novel marker, play a role in isoniazid resistance in these isolates. Since 7 of the 13 isoniazid mono-resistant isolates that were indicated as susceptible in these assays are of the X3 lineage, this may indicate dissemination of a dominant isoniazid mono-resistant clone with a unique isoniazid resistance determinant in the Western Cape. The identification of an isoniazid resistance marker in these isolates for inclusion in molecular assays would increase the sensitivity with which isoniazid resistance is identified in this region, particularly isoniazid mono-resistance. The ability to rapidly identify isoniazid resistance in these isolates would contribute both to limiting their dissemination and their progression to MDR.

This evaluation illustrates that the geographical distribution of mutations resulting in drug resistance in *M. tuberculosis* in this region is different from that reported elsewhere (Huang *et al*, 2009; Hillemann *et al*, 2005; Herrera *et al*, 2004). The high sensitivity (97.6%) with which rifampicin resistance could be detected by GenoType® MTBDR*plus* in MDR-TB isolates in this setting is encouraging and highlights the benefits of assays such as this. More specifically, rifampicin resistance in 86.6% of MDR-TB isolates could be attributed to an RpoB S531L amino acid substitution. The development of a more cost-effective assay, such as MAS-PCR, for the detection of this single mutation would therefore be able to detect a large proportion of rifampicin resistance in rural areas that are not equipped to perform routine GenoType® MTBDR*plus* assays. This may have important implications for the standardised use of rapid genotypic tests for the identification of drug-resistant *M. tuberculosis*, and may, in turn, impact the progression of *M. tuberculosis* to MDR- and, ultimately, XDR-TB. In particular, the identification of a high proportion of potentially clonally related rifampicin and clonally related isoniazid mono-resistant strains that are not identified as resistant by the GenoType® MTBDR*plus* assay is of concern. If rapid genotypic assays for the detection of drug resistance are to be widely used, there is a need to continually monitor local patterns of drug-resistance mutations to ensure that if clonal groups of *M. tuberculosis* do emerge, they do not go undiagnosed as drug resistant.

Although neither the GenoType® MTBDR*plus* assay nor MAS-PCR detected isoniazid resistance in a large proportion of isolates in this setting, the majority of rifampicin resistance in this setting can be detected using the GenoType® MTBDR*plus* assay. Since isolates that are rifampicin resistant are treated as MDR regardless of isoniazid susceptibility, this evaluation indicates that the GenoType® MTBDR*plus* effectively identifies MDR-TB with high sensitivities in this setting. This is encouraging, as rapid diagnosis of drug resistant *M. tuberculosis* can inform individualized treatment regimens at the outset of therapy. Similarly, the characterization of predominant SLD resistance determinants in this region would facilitate the development of molecular assays for the rapid detection of MDR-TB isolates with additional resistance to SLDs, thereby contributing to minimizing the emergence and dissemination of XDR-TB.

CHAPTER 3:

Characterisation of Second-Line Drug Resistance in *Mycobacterium tuberculosis* Clinical Isolates

3.1 Introduction

3.2 Materials and Methods

3.2.1 Bacterial isolates and phenotypic drug susceptibility testing

3.2.2 Multiplex Allele-Specific PCR assays for the detection of resistance to second-line drugs

3.2.3 Efflux inhibitor assays

3.3 Results

3.3.1 Phenotypic drug susceptibilities of *M. tuberculosis* isolates

3.3.2 Detection of ofloxacin resistance and kanamycin resistance using MAS-PCR assays

3.3.3 Correlation of ofloxacin and kanamycin MAS-PCR assays with phenotypic drug susceptibility testing

3.3.4 The role of active efflux in ofloxacin resistance in *M. tuberculosis*

3.3.5 Genotypic characterisation of MDR- and XDR-TB isolates using spacer oligonucleotide typing and MIRU-VNTR typing

3.4 Discussion

3.1 Introduction

Whilst therapies available for the treatment of drug-susceptible TB are effective when adhered to, the treatment of patients with drug-resistant *M. tuberculosis* is more difficult due to the lack of availability of effective antibiotics. The global incidence of MDR-TB is increasing and is currently estimated to be 2.9% in new cases and 15.3% in previously treated cases of TB, with more than 400 000 cases of MDR-TB emerging per annum (WHO, 2010; WHO, 2008c). The increase in MDR-TB prevalence has led to a proportional increase in the use of SLDs, which, in turn, has likely contributed to the emergence of extensively drug resistant (XDR)-TB (WHO, 2008). The most recent global survey revealed that approximately 5.4% of MDR-TB cases from a total of 46 countries are XDR (WHO, 2010), and a total of 58 countries had reported at least one case of XDR-TB by January 2010 (Fig. 3.1) (WHO, 2010). It is probable, however, that this number is underestimated due to the lack of data from some countries, particularly those in Africa where the incidence of XDR-TB may be high.

Figure 3.1: A global map indicating a total of 58 countries (indicated in red) with at least one reported case of XDR-TB by January 2010. (Adapted from WHO, 2010).

The impact of the drug-resistant TB epidemic was highlighted in 2006 when an outbreak of XDR-TB in Tugela Ferry, KwaZulu Natal, killed 52 of 53 people infected with a median time to death of only 16 days (Gandhi *et al*, 2006). By March 2006 XDR-TB had been reported in all parts of the world, with a particular prevalence in countries with a high HIV burden, such as South Africa (WHO, 2010). However, despite the strong association between HIV and TB, HIV status alone is not sufficient to imply treatment failure in patients infected with MDR-TB strains, and a poor prognosis should be anticipated in HIV negative patients as well (Kim *et al*, 2007). The limited availability of antibiotics with activity against XDR-TB means that infection with these strains is associated with a high mortality rate. XDR-TB has now been identified in all 9 provinces of South Africa, and recent data indicates that 10.5% of MDR-TB cases in this country are XDR, making South Africa one of only 8 countries in the world with proportions of greater than 10% (WHO, 2010).

The current SLD regimen used for the treatment of MDR-TB in South Africa includes ofloxacin and kanamycin as the fluoroquinolone and aminoglycoside components, respectively (Department of Health, RSA, 1999). The availability of the *M. tuberculosis* H37Rv genome sequence (Cole *et al*, 1998) has facilitated extensive investigation into the genetic basis of resistance of *M. tuberculosis* to both of these antibiotics. Through the use of molecular biology specific resistance determinants frequently associated with resistance to ofloxacin and kanamycin have been identified that could facilitate the development of rapid assays for the detection of resistance to these drugs.

The fluoroquinolone group of antibiotics, which are synthetically derived from nalidixic acid, have potent *in vitro* activity against *M. tuberculosis*. Following cleavage of the phosphodiester bond on one strand of the double-stranded DNA, fluoroquinolones form a complex with the DNA, thereby blocking the movement of the replication fork (Hooper 2001; Drlica, 1999). Though fluoroquinolones have potent activity against MDR-TB, these drugs are not reserved for the treatment of MDR-TB. Instead, fluoroquinolones are widely prescribed for the treatment of other Gram-negative infections, such as pneumonia (Mandell *et al*, 2007; Dooley *et al*, 2002). The incidence of pre-existing fluoroquinolone-resistant isolates of *M. tuberculosis* as a result of exposure to these antibiotics is therefore of concern

(Von Groll *et al*, 2009; von Gottberg *et al*, 2008; Ginsburg *et al*, 2003b). The development of new drugs that can be reserved for the treatment of TB may contribute to reducing the emergence of fluoroquinolone-resistant *M. tuberculosis*. A new generation fluoroquinolone, moxifloxacin, is currently undergoing phase III clinical trials with a view to it replacing isoniazid or ethambutol in the first-line TB regimen (TB Alliance, 2010). Moxifloxacin reduces the time to eradication of infection from 6 months to 4 months when used as a replacement for isoniazid in the mouse model (Nuermberger *et al*, 2004), making it a potentially useful antituberculous drug. However, moxifloxacin has almost complete cross-resistance with ofloxacin and ciprofloxacin (Devasia *et al*, 2009; Von Groll *et al*, 2009), and therefore cannot be used to treat *M. tuberculosis* already resistant to these older fluoroquinolones.

Resistance to the fluoroquinolone group of antibiotics was first observed in *M. tuberculosis* in 1995 (Chan *et al*, 2004), with 42-85% of fluoroquinolone resistance attributed to mutations within the quinolone resistance determining region (QRDR) of *gyrA* (Brossier *et al*, 2010; Duong *et al*, 2009; Xu *et al*, 2009). Resistance to ofloxacin, specifically, arises due to mutations within *gyrA* in up to 81.5% isolates, making this gene a good candidate for the molecular screening of ofloxacin resistance (Xu *et al*, 2009). The most frequently observed mutations in GyrA in clinical isolates of *M. tuberculosis* are at codons D94 and/or A90 (Feuerriegel *et al*, 2009; Hillemann *et al*, 2009; Wang *et al*, 2007; Giannoni *et al*, 2005; Pitakksajjakul *et al*, 2005; Takiff *et al*, 1994). A GyrA A90V mutation results in a 16-fold decrease in susceptibility to ciprofloxacin, whilst a D94H/N/Y amino acid substitution is associated with a 30-fold reduction in susceptibility to this drug (Xu *et al*, 1996). This reduction is decreased a further 2-fold if the aspartate at codon 94 is substituted for a glycine (Xu *et al*, 1996). A GyrA G88A amino acid substitution results in decreased inhibition of gyrase activity, and the role of this mutation in conferring fluoroquinolone resistance has been confirmed by site-directed mutagenesis (Matrat *et al*, 2006). The role of *gyrB* in fluoroquinolone resistance is not clear. There have been reports of mutations within *gyrB* in resistant clinical isolates of *M. tuberculosis*, but their role in conferring resistance has not been confirmed (Brossier *et al*, 2010; Duong *et al*, 2009; Veziris *et al*, 2007). It may be that mutations in *gyrB* are more likely to occur in isolates resistant to moxifloxacin and gatifloxacin than to ofloxacin (Von Groll *et al*, 2009)

and mutations in *gyrB* may therefore be more frequently reported as the use of these drugs becomes more widespread.

In the absence of mutations in *gyrA* and *gyrB*, active efflux may play a role in conferring low-level resistance to fluoroquinolones in *M. tuberculosis* (Duong *et al*, 2009; Pasca *et al*, 2004; Ginsburg *et al*, 2003a). There have been several reports of decreased susceptibility to fluoroquinolones due to active efflux in *M. smegmatis* (Li *et al*, 2004; Liu *et al*, 1996), and several *M. tuberculosis* efflux pumps have been implicated in fluoroquinolone resistance following expression in *M. smegmatis* (Pasca *et al*, 2004; De Rossi *et al*, 2002) and *M. bovis* BCG (Ramon-Garcia *et al*, 2009). In an *M. tuberculosis* clinical isolate with high-level resistance to both ofloxacin and rifampicin, expression levels of Rv1258c, which encodes an efflux pump, were increased 6-fold and 10-fold in the presence of ofloxacin and rifampicin, respectively (Siddiqi *et al*, 2004). Although this isolate also carried commonly identified resistance determinants in both *gyrA* and *rpoB*, the combination of these mutations with the effect of efflux may have resulted in the increased MICs observed (Siddiqi *et al*, 2004).

A large number of putative efflux pumps have been identified in *M. tuberculosis* (Cole *et al*, 1998). The ATP-binding cassette (ABC) superfamily of transporters account for 2.5% of the *M. tuberculosis* genome (Braibant *et al*, 2000), and at least 14 putative members of the major facilitator superfamily (MFS) have been identified in this organism (Cole *et al*, 1998). The ABC transporters are driven by ATP (De Rossi *et al*, 2002; Saier, 2000) and can therefore be inhibited by reserpine, which blocks ATP-dependant pumps (Choudhuri *et al*, 1999), and the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) disrupts the proton motive force (Williams & Piddock, 1998) that drives the MFS transporters (De Rossi *et al*, 2002; Saier, 2000). The use of these efflux inhibitors provides a means of investigating the collective role of active efflux in conferring resistance in *M. tuberculosis* without targeting each efflux pump individually (Danilchanka *et al*, 2008; Escribano *et al*, 2007).

Fluoroquinolone resistance in *M. tuberculosis* is often associated with resistance to other antituberculous drugs, particularly MDR, and is rarely identified in pan-susceptible isolates (Xu *et al*, 2009). In some cases this may necessitate the exclusion of fluoroquinolones from regimens for the treatment of drug-resistant *M. tuberculosis*. This requires the use of alternative second-line drugs, with aminoglycosides such as kanamycin, amikacin and capreomycin forming an important part of the MDR-TB treatment regimen. Following the emergence of streptomycin resistance in *M. tuberculosis*, kanamycin and its synthetic derivative, amikacin, were introduced into the TB treatment regimen. Amikacin and kanamycin are almost completely cross-resistant, but they display little cross-resistance to streptomycin (Via *et* al, 2010; Alandagen *et al*, 1998), making them useful for the treatment of streptomycin-resistant *M. tuberculosis* isolates. Additionally, the high efficiency with which amikacin reduces bacterial load in the mouse model (Lounis *et al*, 1997) illustrates the importance of these drugs in the treatment of TB.

Resistance to amikacin and kanamycin arises predominantly due to mutations in *rrs*, the gene encoding *M. tuberculosis* 16S rRNA (Suzuki *et al*, 1998). Specifically, an A1401G mutation in *rrs*, which prevents ribosomal binding of kanamycin (Moazed & Noller, 1987), accounts for 60.5% to 85% of global amikacin and kanamycin resistance (Via *et al*, 2010; Feuerriegel *et al*, 2009; Alangaden *et al*, 1998; Suzuki *et al*, 1998). This mutation is associated with high-level resistance (MICs >256μg/ml) to amikacin and kanamycin, and is rarely identified in isolates with MICs of ≤64μg/ml (Alangaden *et al*, 1998; Taniguchi *et al*, 1997). The molecular basis of resistance of *M. tuberculosis* to capreomycin is associated with mutations in the TlyA rRNA methyltransferase (Johansen *et al*, 2006; Maus *et al*, 2005a). The *rrs* A1401G mutation can also be an indicator of low-level capreomycin resistance (Brossier *et al*, 2010; Maus *et al*, 2005b), making this mutation a particularly useful marker as it can simultaneously detect resistance to kanamycin/amikacin and capreomycin.

Despite the frequency with which *rrs* A1401G is associated with resistance to amikacin and kanamycin, the identification of phenotypically resistant strains of *M. tuberculosis* that lack this marker suggests the existence of other mechanisms of resistance to these drugs. Aminoglycoside modifying enzymes (AMEs) can modify aminoglycosides by phosphorylation, nucleotidylation and acetylation (Hegde *et al*,

2001), thereby preventing binding of the modified aminoglycoside to the ribosome and resulting in resistance. AMEs play a significant role in the development of resistance to aminoglycosides in Gram-negative bacteria (Vakulenko & Mobashery, 2003) but, despite the fact that aminoglycoside acetyltranferases (AAC(2`)) are ubiquitous in mycobacteria (Ainsa *et al*, 1997), they have not been implicated in aminoglycoside resistance in *M. tuberculosis* (Hegde *et al*, 2001; Ho *et al*, 2000) until recently (Zaunbrecher *et al*, 2009). The identification of an aminoglycoside acetyltransferase, Eis, in *M. tuberculosis* that has activity against kanamycin may serve as an additional molecular marker for the detection of kanamycin resistance in isolates with no *rrs* mutations. Although it is widely accepted that there is complete cross-resistance between amikacin and kanamycin, there have been reports of kanamycin-resistant and amikacin-susceptible *M. tuberculosis* isolates (Brossier *et al*, 2010; Krunner *et al*, 2003). As overexpression of Eis due to a C-14T mutation in the promoter region confers resistance to kanamycin but not to amikacin (Zaunbrecher *et al*, 2009) it may be that Eis activity is increased in these isolates.

Currently, ofloxacin and kanamycin are used in the second-line treatment regimen for MDR-TB in South Africa (Department of Health, RSA, 1999). The development of resistance to one or both of these drugs due to initiation of inappropriate therapy is of concern as alternative treatment options are limited. The availability of molecular assays to rapidly detect resistance to these antibiotics at the outset of treatment may contribute to reducing the emergence of *M. tuberculosis* strains resistant to these drugs, ultimately reducing the emergence of XDR-TB. Given that 20-35% of ofloxacin resistance (Hillemann *et al*, 2009; Wang *et al*, 2007; Huang *et al*, 2005; Pitakksajjakul *et al*, 2005; Cheng *et al*, 2004; Takiff *et al*, 1994) and 60-87% of kanamycin/amikacin resistance (Maus *et al*, 2005a; Alangaden *et al*, 1998; Suzuki *et al*, 1998) worldwide are associated with GyrA D94G and *rrs* A1401G mutations, respectively, novel MAS-PCR assays targeting these mutations were designed and their efficacy at identifying resistance to ofloxacin and kanamycin in *M. tuberculosis* isolates from Cape Town was evaluated.

3.2 Materials and Methods

3.2.1 Bacterial isolates and phenotypic drug susceptibility testing

In addition to 224 *M. tuberculosis* isolates obtained from patients at Groote Schuur Hospital (GSH) in 2006 [2.2.1; 2.3.1], a further 84 MDR *M. tuberculosis* isolates identified [2.2.1] during 2009 were investigated. In addition, second-line drug susceptibility testing of ofloxacin and kanamycin was carried out by the indirect proportions method. Isolates were grown in Middlebrook 7H9 broth [Appendix B] supplemented with ADC and Tween 80 (0.05% v/v) in the absence of selection to OD_{600} = 0.25 (approximately equivalent to a McFarland 1.0). A volume of 100µl of this suspension was inoculated onto Middlebrook 7H11 agar supplemented with OADC [Appendix B] containing no selection, kanamycin [Sigma-Aldrich] at 6µg/ml and 8µg/ml, and ofloxacin [Sigma-Aldrich] at 2µg/ml and 4µg/ml. An *M. tuberculosis* H37Rv susceptible control was inoculated in parallel with each batch of isolates tested. Agar plates were incubated at 37°C in 8.5% CO_2 and results were interpreted following 10 days of incubation.

3.2.2 Multiplex Allele-Specific PCR assays for the detection of resistance to second-line drugs

MAS-PCR assays were designed to indicate the presence of GyrA D94G and *rrs* A1401G mutations, which account for ofloxacin and kanamycin resistance, respectively. Both PCR assays were to a final volume of 50µl and contained 1 X GoTaq PCR Buffer [Promega], 1.25U GoTaq® DNA polymerase [Promega] and 50-200ng genomic DNA [2.2.2] as template. When amplifying *gyrA*, 0.15mM of each dNTP [Fermentas], 10pmoles of primer GYRAF (Table 3.1), 30pmoles of GYRAR1, 20pmoles of GYRAR2 and 3mM Mg^{2+} [Promega] were used. The reaction conditions consisted of an initial denaturation step at 95°C for 5 minutes followed by 5 cycles of 95°C for 15 seconds, 68°C for 5 seconds and 72°C for 20 seconds, 5 cycles of 95°C for 15 seconds, 64°C for 5 seconds and 72°C for 20 seconds and 25 cycles of 94°C for 15 seconds, 62°C for 5 seconds and 72°C for 20 seconds, followed by an final elongation step at 72°C for 5 minutes. For amplification of *rrs*, 0.2mM of each dNTP [Fermentas], 20pmoles of primer RRSF (Table 3.1), 5pmoles of RRSR1, 40pmoles of

RRSR and 1.5mM Mg^{2+} [Promega] were used. The cycling conditions for the *rrs* MAS-PCR were as for *gyrA* except that primer annealing was carried out at 66°C for the first 5 cycles, then at 64°C for the next 5 cycles and at 62°C for the remaining 25 cycles. A mismatch between a primer and the targeted mutation enables the detection of the mutation using these assays (Fig. 3.2a,b). Amplification of 427bp and 260bp products following *gyrA* MAS-PCR and 481bp and 353bp following *rrs* MAS-PCR (Fig. 3.2a,b) indicates that the corresponding wild-type sequences are present. The absence of the smaller product in both assays indicates detection of the GyrA D94G and *rrs* A1401G mutations. Sequencing on both strands of the products from wild-type and mutant isolates confirmed the absence or presence of the mutations, and these isolates were used as controls in all subsequent MAS-PCR assays.

Table 3.1: Primers designed for use in MAS-PCR assays for the identification of GyrA D94G and *rrs* A1401G resistance determinants.

Primer Name	Primer Sequence (5` → 3`)
GYRAF	CCGGATCGAACCGGTTGAC
GYRAR1	CCATGCGCACCAGGCTGT
GYRAR2	GTTAGGGATGAAATCGACTG
RRSF	GTGAGATGTTGGGTTAAGTCC
RRSR1	GTTACCGACTTTCATGACGT
RRSR	TGGTGCTCCTTAGAAAGGAG

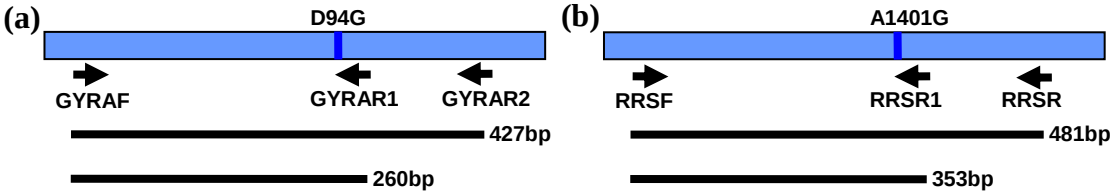

Figure 3.2: Schematic representation of MAS-PCR assays designed for the identification of (a) GyrA D94G and (b) *rrs* A1401G resistance determinants. The position of each mutation is represented by a vertical line; arrows represent the primers used for PCR amplification; horizontal lines represent the sizes of the expected amplicons.

3.2.3 Efflux inhibitor assays

Efflux inhibitor assays were carried out, using efflux inhibitors reserpine and CCCP, to investigate the role of active efflux in fluoroquinolone resistant *M. tuberculosis* isolates. Selected isolates were grown in Middlebrook 7H9 broth supplemented with ADC and Tween 80 (0.05% v/v) to an OD_{600} = 0.25 (approximately equivalent to a McFarland 1.0). A volume of 100µl of this suspension was inoculated onto Middlebrook 7H11 agar supplemented with OADC containing no selection, ofloxacin [Sigma-Aldrich] at 2µg/ml, reserpine [Sigma-Aldrich] at 12µg/ml, CCCP [Sigma-Aldrich] at 1.25µg/ml, reserpine at 12µg/ml plus ofloxacin at 2µg/ml, and CCCP at 1.25µg/ml plus ofloxacin at 2µg/ml. Agar plates were incubated at 37°C in 8.5% CO_2 and results were interpreted following 10 days of incubation.

3.3 Results

3.3.1 Phenotypic drug susceptibilities of *M. tuberculosis* isolates

Phenotypic DST carried out on *M. tuberculosis* isolates obtained from patients at GSH in 2006 and 2009 identified 166 of 308 isolates as MDR. Additional second-line phenotypic DST of 84 viable MDR-TB isolates from 2009 by the indirect proportions method [3.2.1] indicated 5 (6.0%) as resistant to ofloxacin alone and 6 (7.1%) as resistant to kanamycin alone (Table 3.2), and these isolates are defined as pre-XDR (Mlambo *et al*, 2008). A further 9 (10.7%) isolates were indicated as XDR since they were resistant to both ofloxacin at 2µg/ml and kanamycin at 6µg/ml (Fig 3.3 & 3.4; Table 3.2). *M. tuberculosis* H37Rv inoculated in parallel with all clinical isolates was consistently susceptible to both ofloxacin and kanamycin. Kanamycin and ofloxacin phenotypic DST of the 82 MDR, 41 isoniazid mono-resistant, 10 rifampicin mono-resistant isolates and 90 isolates susceptible to both rifampicin and isoniazid from 2006 was not carried out as these isolates were no longer viable. Current WHO guidelines (2008) for SLD susceptibility testing recommend the use of ofloxacin at 2µg/ml and kanamycin at 6µg/ml when using the indirect proportions method on Middlebrook 7H11 agar (Table 1.1). In this study, all isolates were additionally inoculated onto agar containing ofloxacin at 4µg/ml and kanamycin at 8µg/ml. All isolates tested were either resistant or susceptible to

kanamycin at both 6µg/ml and 8µg/ml (Table 3.2). Of 14 MDR-TB isolates that were identified as resistant to ofloxacin, 2 XDR isolates were resistant to ofloxacin at 2µg/ml but susceptible at 4µg/ml (Table 3.2).

Table 3.2: Number of MDR *M. tuberculosis* isolates from Groote Schuur Hospital in 2009 identified as phenotypically ofloxacin and kanamycin resistant.

MDR *M. tuberculosis*	OFX		KAN	
Isolates (n=84)	2µg/ml	4µg/ml	6µg/ml	8µg/ml
OFX^R (n=5)	5	5	0	0
KAN^R (n=6)	0	0	6	6
OFX^R KAN^R (n=9)	9	7	9	9
OFX^S KAN^S (n=64)	0	0	0	0

MDR, multidrug resistant; OFX, ofloxacin; KAN, kanamycin; ^{R}, resistant; ^{S}, susceptible.

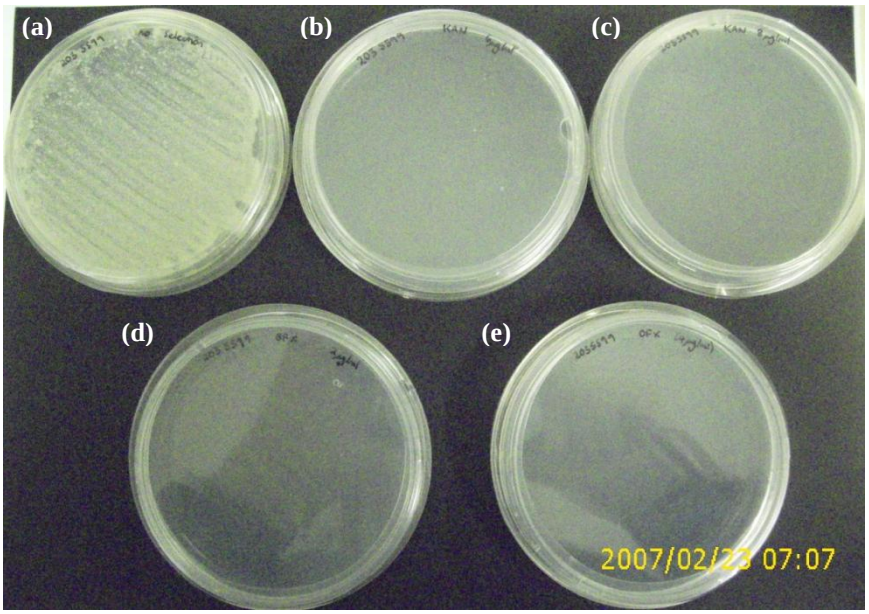

Figure 3.3: Phenotypic drug susceptibility testing of an MDR-TB isolate susceptible to both ofloxacin and kanamycin. The MDR-TB isolate was inoculated onto Middlebrook 7H11 agar containing (a) no selection; (b) kanamycin at 6µg/ml; (c) kanamycin at 8µg/ml; (d) ofloxacin at 2µg/ml; (e) ofloxacin at 4µg/ml.

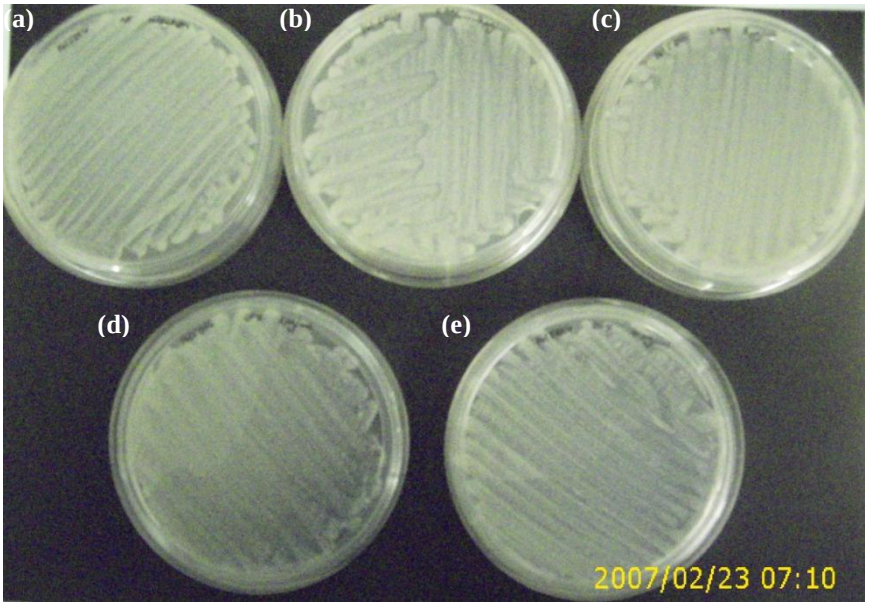

Figure 3.4: Phenotypic drug susceptibility testing of an MDR-TB isolate resistant to both ofloxacin and kanamycin. The MDR-TB isolate was inoculated onto Middlebrook 7H11 agar containing (a) no selection; (b) kanamycin at 6µg/ml; (c) kanamycin at 8µg/ml; (d) ofloxacin at 2µg/ml; (e) ofloxacin at 4µg/ml.

3.3.2 Detection of ofloxacin resistance and kanamycin resistance using MAS-PCR assays

MAS-PCR assays designed to detect GyrA D94G (Fig. 3.5) and *rrs* A1401G (Fig. 3.6) were carried out on all 308 *M. tuberculosis* isolates. Amplicons corresponding to wild-type genotype were obtained from the *gyrA* and *rrs* wild-type controls, and products indicating a GyrA D94G substitution or an *rrs* A1401G mutation were obtained from the corresponding mutant control isolates (Fig. 3.5, lanes 2 and 3 & Fig. 3.6, lanes 2 and 3).

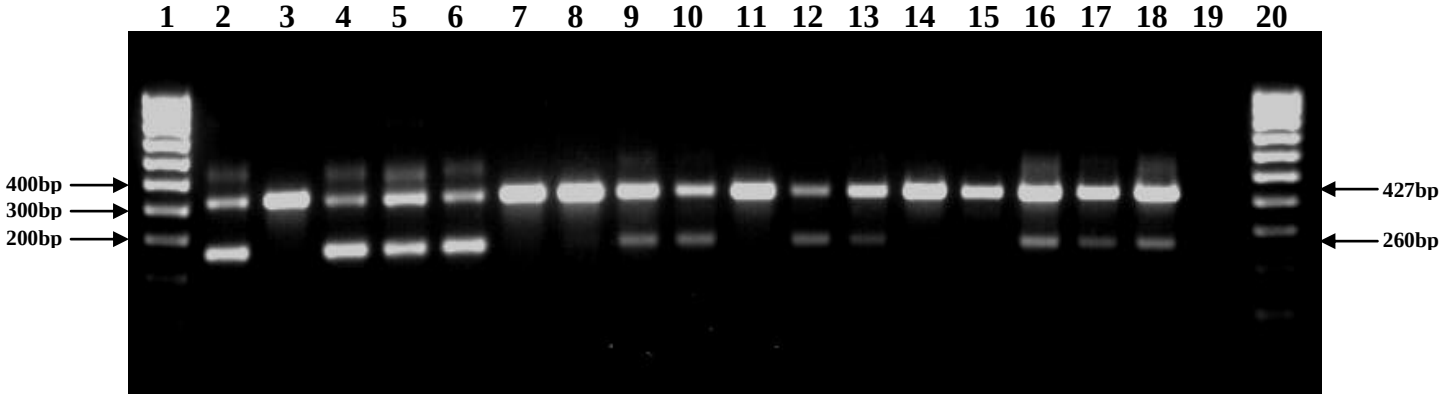

Figure 3.5: Agarose gel electrophoresis of amplicons obtained using *gyrA* MAS-PCR assay. Lane 1, Hyperladder IV [Bioline]; lane 2, wildtype *gyrA* control; lane 3, D94G GyrA control; lanes 4 – 18, multidrug resistant *M. tuberculosis* isolates; lane 19, water control; lane 20, Hyperladder IV [Bioline].

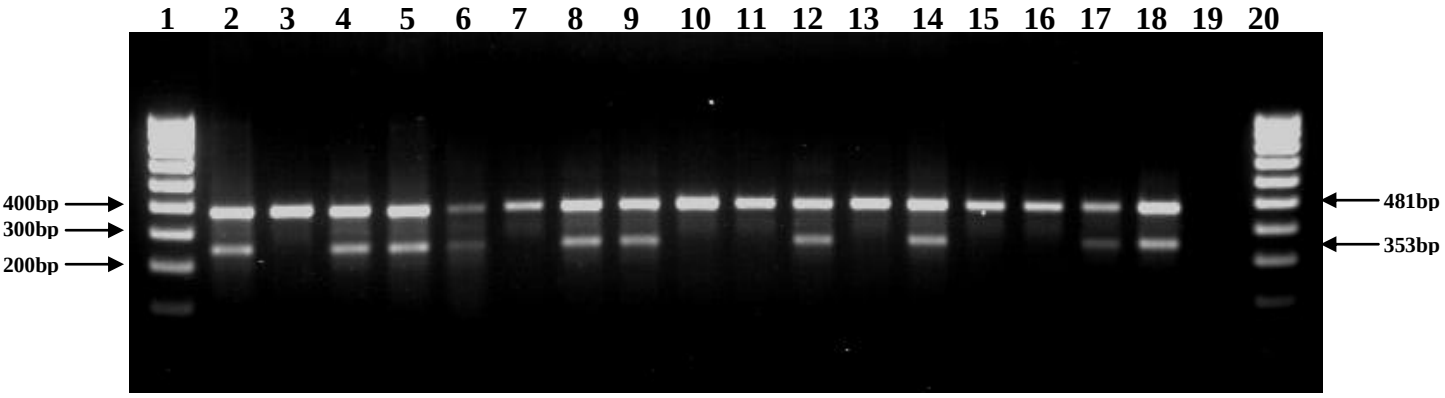

Figure 3.6: Agarose gel electrophoresis of amplicons obtained using *rrs* MAS-PCR assay. Lane 1, Hyperladder IV [Bioline]; lane 2, wildtype *rrs* control; lane 3, A1401G *rrs* control; lanes 4 – 18, multidrug resistant *M. tuberculosis* isolates; lane 19, water control; lane 20, Hyperladder IV [Bioline].

Following MAS-PCR analysis, the GyrA D94G mutation was indicated in 18 of 166 (10.8%) MDR-TB isolates (Table 3.3), with the remaining 290 isolates indicated as containing a wild-type *gyrA* at this locus. The *rrs* A1401G nucleotide substitution was indicated in 34 of 166 (20.5%) MDR-TB isolates, 2 of 41 (4.9%) isoniazid mono-resistant isolates and 17 of 90 (18.9%) isolates susceptible to rifampicin and isoniazid (Table 3.3). The remaining 255 isolates produced amplicons indicative of wild-type *rrs* at this locus. Of the isolates carrying GyrA D94G (18) and *rrs* A1401G (34), 12 (23.1%) carried both mutations (Table 3.3), indicating them as XDR.

Table 3.3: Identification of *M. tuberculosis* isolates carrying GyrA D94G and *rrs* A1401G mutations using MAS-PCR assays.

M. tuberculosis Isolates (n)	GyrA D94G	*rrs* A1401G	GyrA D94G & *rrs* A1401G
MDR (166)	6	22	12
INH^R (41)	0	2	0
RIF^R (11)	0	0	0
RIF^S INH^S (90)	0	17	0

MDR, multi-drug resistant; INH, isoniazid; RIF, rifampicin; ^{R}, resistant; ^{S}, susceptible.

The proportion of MDR-TB isolates from GSH that were identified as XDR using MAS-PCR was 8.5% in 2006 and 4.8% in 2009 (Fig. 3.7), which suggests that the incidence of XDR-TB amongst MDR isolates in this region has decreased over this time period. Phenotypic DST of the 2009 MDR isolates, however, indicates 10.7% as XDR (Table 3.2). Although rapid molecular assays are invaluable for the rapid determination of susceptibility, this highlights the importance of performing phenotypic drug susceptibility testing in parallel with molecular assays for the detection of drug resistance. The proportion of pre-XDR isolates, which are MDR isolates with additional resistance to either ofloxacin or kanamycin (Mlambo *et al*, 2008), identified using MAS-PCR decreased from 23.2% in 2006 to 15.5% in 2009. This may indicate a progression from pre-XDR to XDR-TB due to the sequential acquisition of additional resistance determinants over this time. Alternatively, it may be that increased awareness of the XDR-TB epidemic has necessitated improved detection and screening methods during this period, resulting in more rapid implementation of appropriate treatment regimens and, thereby, a decrease in the rate of emergence of pre-XDR isolates.

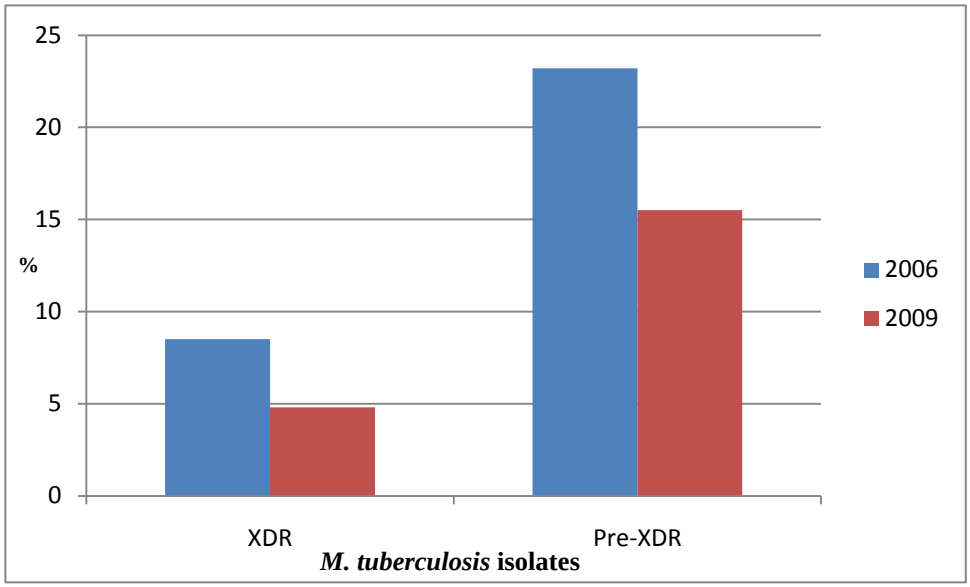

Figure 3.7: The proportion of MDR *M. tuberculosis* isolates from 2006 (n=82) and 2009 (n=84) identified as pre-XDR and XDR using MAS-PCR. Values are reported as the percentage of MDR-TB isolates from each year that had additional resistance to second-line drugs.

3.3.3 Correlation of ofloxacin and kanamycin MAS-PCR assays with phenotypic drug susceptibility testing

The sensitivity with which MAS-PCR assays identify ofloxacin and kanamycin resistance was determined by comparison with the gold-standard phenotypic DST results. Ofloxacin resistance was detected by phenotypic DST in 14 of 84 (16.7%) MDR-TB isolates tested. MAS-PCR assays indicated a GyrA D94G mutation in 9 of the 14 (64.3%) ofloxacin resistant isolates (Table 3.4). Of the 15 MDR isolates phenotypically resistant to kanamycin, 12 (80.0%) had an *rrs* A1401G nucleotide substitution indicated by MAS-PCR (Table 3.4). Two pre-XDR isolates, one of which was resistant to ofloxacin and one to kanamycin, were indicated as susceptible to these antibiotics using MAS-PCR assays. A total of 9 isolates were identified as XDR following phenotypic DST, of which 4 (44.4%) carried both a GyrA D94G substitution and an *rrs* A1401G mutation. Of the remaining 5 isolates, one had a GyrA D94G substitution and no *rrs* A1401G mutation, and 3 had *rrs* A1401G mutations and no GyrA D94G mutation. One phenotypically XDR isolate had neither a GyrA D94G nor an *rrs* A401G mutation.

Table 3.4: Correlation between MAS-PCR assays for detection of ofloxacin and kanamycin resistance in MDR *M. tuberculosis* isolates and phenotypic drug susceptibility testing.

MDR *M. tuberculosis* Isolates (n)	Phenotypic DST			MAS-PCR Assay		
	OFX^R	KAN^R	OFX^R KAN^R	GyrA D94G	*rrs* A1401G	GyrA D94G + *rrs* A1401G
84	5	6	9	5	8	4

OFX, ofloxacin; KAN, kanamycin; ^{R}, resistant.

PCR amplification [3.2.2] and sequence analysis [2.2.5] of the GyrA QRDR of the 5 isolates identified as phenotypically resistant to ofloxacin in the absence of a GyrA D94G mutation was carried out using primers GYRAF and GYRAR2 (Table 3.1). A GyrA A90V mutation accounting for ofloxacin resistance was identified in 4 isolates, whilst the remaining isolate had no identifiable mutation within this region (Fig. 3.8). Of the 4 isolates with A90V mutations, 3 carried an additional *rrs* A1401G nucleotide substitution, whilst the remaining isolate had no identifiable kanamycin resistance determinant. All 5 isolates were found to carry an additional nonsynonymous GyrA S95T amino acid substitution (Fig. 3.8). This mutation is not associated with fluoroquinolone resistance, but occurs at a high frequency in *M. tuberculosis* and is used as a marker of evolutionary divergence (Sreevatsan *et al*, 1997c).

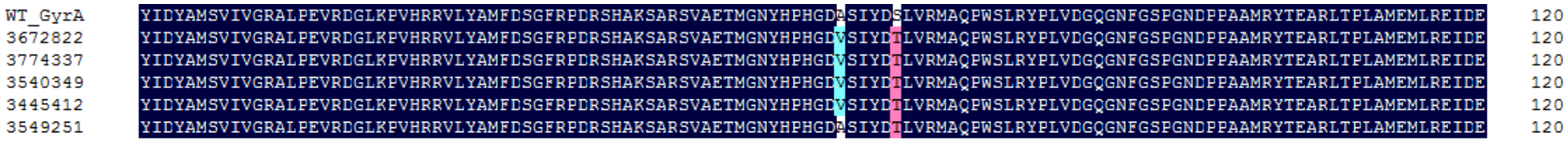

Figure 3.8: Amino acid sequence alignment of GyrA from phenotypically ofloxacin resistant *M. tuberculosis* isolates with no GyrA D94G resistance determinant. Regions shaded in black represent regions of 100% sequence identity. An A90V amino acid substitution in 4 isolates is highlighted in blue. The nonsynonomous GyrA S95T mutation is highlighted in pink.

3.3.4 The role of active efflux in ofloxacin resistance in *M. tuberculosis*

Of the 4 isolates identified as carrying GyrA A90V mutations, 2 were phenotypically resistant to ofloxacin at 2µg/ml but susceptible at 4µg/ml. In order to investigate whether an additional resistance mechanism such as active efflux was contributing to the higher level of resistance in the 2 isolates resistant to ofloxacin at 4µg/ml, efflux inhibitor assays [3.2.3] were carried out. The isolate with no identifiable ofloxacin resistance determinant was also included in the assay. Reserpine and CCCP inhibit ABC and MFS transporters, respectively, and an increased susceptibility to a specific antibiotic in the presence of these inhibitors therefore indicates active efflux of the antibiotic from within the bacterial cell. Growth of the *M. tuberculosis* H37Rv control was comparable without selection and in the presence of reserpine or CCCP alone, indicating that the inhibitors do not affect growth of the organism in the absence of selection. As expected, no growth of *M. tuberculosis* H37Rv was observed in the presence of ofloxacin.

The growth of the four GyrA A90V mutants, the ofloxacin resistant mutant with no *gyrA* QRDR mutation and a GyrA D94G control isolate was not affected by the presence of either inhibitor in combination with ofloxacin. This suggests that active efflux does not contribute to ofloxacin resistance in any of these isolates. However, it may be that an efflux pump transporter that is not inhibited by reserpine or CCCP could contribute to the higher level of resistance observed in 2 of the GyrA A90V mutants. Alternatively, these mutants may have mutations in *gyrB* or at other loci that contribute to increased resistance to ofloxacin. Given that GyrA A90V mutations are usually associated with ofloxacin MICs of 8µg/ml (Giannoni *et al*, 2005) to 160µg/ml (Kocagöz *et al*, 1996), it may be that the presence of a compensatory mutation results in increased susceptibility in the two isolates resistant to ofloxacin at 2µg/ml but not at 4µg/ml.

3.3.5 Genotypic characterisation of MDR- and XDR-TB isolates using spacer oligonucleotide typing and MIRU-VNTR Typing

Spoligotyping [2.2.6] identified 65 of 120 (54.2%) MDR isolates, 24 of 30 (80.0%) pre-XDR and 15 of 16 (93.8%) XDR-TB isolates as W-Beijing (Table 3.5). That a total of 104 of 166 (62.7%) drug-resistant isolates were of the W-Beijing lineage further confirms the association between W-Beijing and drug resistance (odds ratio, 3.71; 95% confidence interval, 2.15 to 6.41) (Evans *et al*, 2009; Marais *et al*, 2006). Interestingly, however, the association between MDR isolates with no additional resistance to ofloxacin and/or kanamycin and W-Beijing (odds ratio, 2.62; 95% confidence interval, 1.48 to 4.64) was relatively weak as compared to the pre-XDR and XDR-TB isolates, which were more strongly associated with this lineage (odds ratio, 12.34; 95% confidence interval, 4.92 to 30.96). The remaining 6 pre-XDR isolates were either LAM3/F11 (n=2), LAM9 (n=2) or X3 (n=2), with one XDR isolate belonging to the LAM3/F11 family (Table 3.5).

Table 3.5: Relationship between resistance phenotype and *M. tuberculosis* lineage as determined by spacer oligonucleotide typing.

M. tuberculosis	*M. tuberculosis* Lineage				
Isolates (n=166)	W-Beijing	LAM3/ F11	LAM9	X3	[a]Other
MDR (120)	65	9	4	5	33
MDR + OFX^R (7)	4	1	2	0	0
MDR + KAN^R (23)	20	1	0	2	0
XDR (16)	15	1	0	0	0

MDR, multidrug resistant; OFX, ofloxacin; ^{R}, resistant; KAN, kanamycin; ^{S}, susceptible; XDR, extensively drug resistant.
[a]Other isolate lineages include LAM4, X1, X2, H1, T1, T3, T4, T5, S, EAI1, EAI5, Family 33 and unique spoligotype profiles.

Further discrimination of all pre-XDR and XDR *M. tuberculosis* isolates was carried out using MIRU-VNTR analysis [2.2.7]. The relatedness of all isolates was determined using the MIRU-VNTR*plus* application (Allix-Béguec *et al*, 2008) and isolates were inferred as belonging to same cluster complex if they differed from each other at two alleles or less. All isolates clustered together in one complex, with the exception of one kanamycin-resistant W-Beijing pre-XDR isolate that differed from

all other isolates at three alleles (Fig. 3.9a,b). Although the W-Beijing isolates cluster together within cluster complex 1 (Fig. 3.9a), they do not cluster according to resistance profile (Fig. 3.9b). This suggests that there is not dissemination of one dominant clone, but that these W-Beijing isolates acquired their resistance determinants independently. The MIRU-VNTR digital profiles of all W-Beijing and LAM3/F11 isolates can be found in Appendix D.

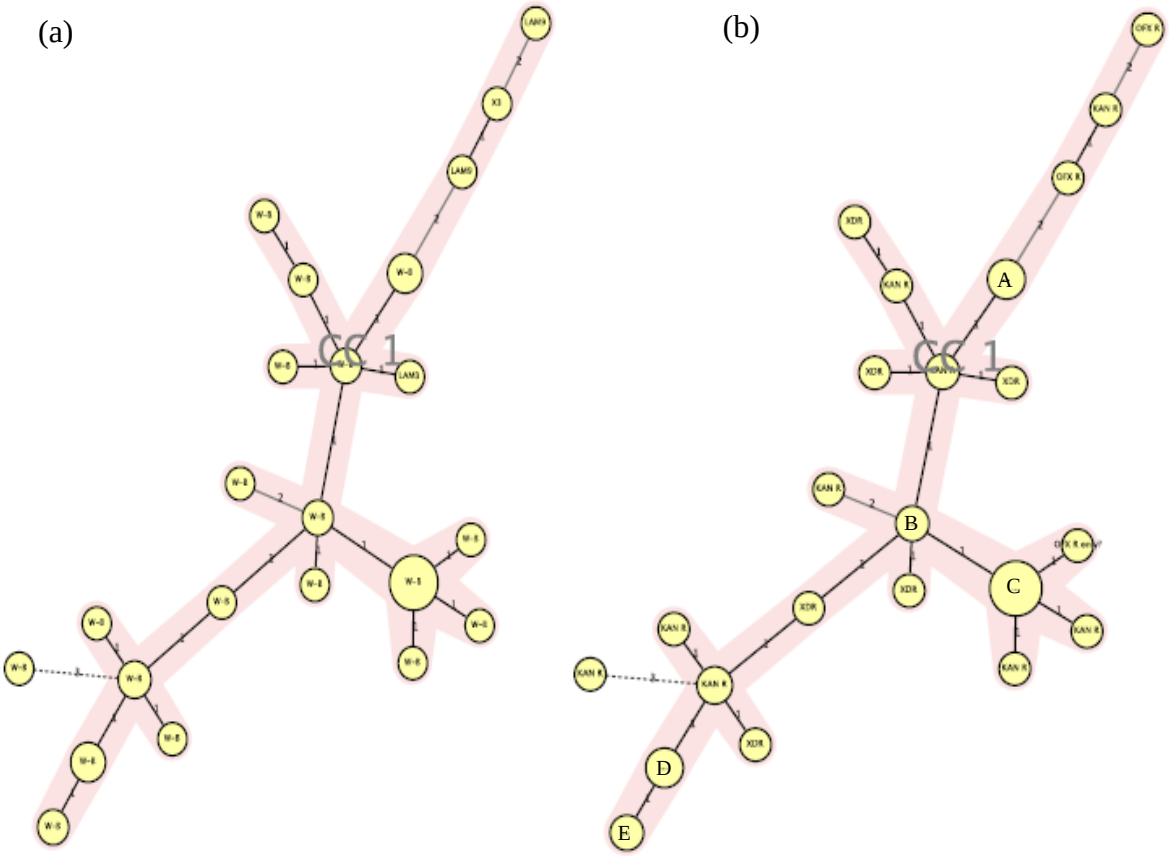

Figure 3.9: Minimum spanning tree showing the genetic relatedness of *M. tuberculosis* isolates by (a) spoligotype and (b) phenotypic resistance profile as determined by 12-locus MIRU-VNTR analysis. The size of the circles is proportionate to the number of identical isolates within each group. Numbers indicate the allele differences between the connected strains. A, group contains one isolate resistant to kanamycin only and three isolates resistant to ofloxacin only; B, group contains one kanamycin resistant isolate and one XDR isolate; C, group contains four kanamycin-resistant isolates, two ofloxacin resistant isolates and 5 XDR isolates; D, group contains one kanamycin resistant isolate and three XDR isolates; E, group contains one kanamycin resistant isolate and one ofloxacin resistant isolate.

3.4 Discussion

The emergence of *M. tuberculosis* isolates resistant to one or more first- and second-line drugs severely compromises effective treatment regimens, leading to the inappropriate use of antituberculous agents and potentially contributing to the emergence of strains with additional resistance. The high incidence of HIV in South Africa makes the emergence of multidrug-resistant *M. tuberculosis* isolates of particular concern, as the combination of *M. tuberculosis* isolates resistant to conventional therapies together with an immunocompromised host results in high mortality rates (Gandhi *et al*, 2006). The rapid implementation of effective drugs at the outset of treatment could contribute to significantly decreasing this trend, but the time delay associated with conventional phenotypic DST makes this difficult. The BACTEC MIGIT 960 produces results comparable to the proportions method in a shorter time (Devasia *et al*, 2009), but the delay associated with this method is still significant and the equipment required is costly. Molecular methods to detect drug resistance more rapidly could therefore be of great value in aiding the timely administration of appropriate antibiotics to TB patients infected with drug-resistant strains.

MAS-PCR assays for the detection of isoniazid resistance due to a KatG S315T or an *inhA* C-15T mutation were successful at identifying these resistance determinants in a large proportion of isoniazid-resistant *M. tuberculosis* isolates investigated (Evans *et al*, 2009). These assays are simple to perform and are less costly than line-probe based assays, making them useful for the detection of drug resistance in *M. tuberculosis*, particularly at point-of-care facilities in rural areas. The availability of similar assays for the detection of second-line drug resistance would therefore be beneficial in such settings, where the burden of XDR-TB is usually very high.

Novel MAS-PCR assays were designed to detect GyrA D94G and *rrs* A1401G mutations, which are associated with ofloxacin and kanamycin resistance, respectively. Validation of both MAS-PCR assays was carried out by phenotypic DST using the indirect proportions method on 84 viable MDR-TB isolates from 2009. The MAS-PCR assay for the detection of resistance to kanamycin identified an *rrs* A1401G mutation in 80.0% of isolates phenotypically resistant to kanamycin, with a

specificity of 100%. Kanamycin resistance in three isolates that did not carry an *rrs* A1401G mutation is likely due to an alternative kanamycin resistance mechanism, such as an alternative mutation in *rrs* (Perdigão *et al*, 2010; Maus *et al*, 2005a) or increased expression of the gene encoding the aminoglycoside acetyltransferase Eis (Zaunbrecher *et al*, 2009).

MAS-PCR analysis of the non-MDR isolates from 2006 identified no isolates that carried both GyrA D94G and *rrs* A1401G mutations. That 4.9% of isoniazid mono-resistant and 16.8% of susceptible isolates carried an *rrs* A1401G mutation alone, and that 13.3% of the MDR isolates carried *rrs* A1401G alone and only 3.6% harboured GyrA D94G alone, suggests that resistance to kanamycin may be more readily acquired than resistance to ofloxacin. Whilst prior exposure to streptomycin may impact the increased incidence of kanamycin resistance, that streptomycin resistance occurs primarily due to mutations in *rpsL* suggests that the *rrs* A1401G mutations identified are likely not due to streptomycin exposure. Additionally, that 66.7% of ofloxacin resistant MDR isolates also carry an *rrs* A1401G mutation suggests that ofloxacin resistance in the absence of kanamycin resistance is rare. Identification of kanamycin resistance may therefore serve as a marker for the detection of pre-XDR isolates with increased potential to progress to XDR-TB. That MAS-PCR detects kanamycin resistance with a sensitivity of 80.0% makes this a useful tool for identifying kanamycin-resistant isolates, facilitating rapid implementation of appropriate treatment and thereby reducing the progression to XDR in these strains.

The MAS-PCR assay for the detection of GyrA D94G identified 64.3% of MDR-TB isolates as ofloxacin resistant, with a specificity of 100%. Sequence analysis of *gyrA* identified GyrA A90V in 4 of 5 (80.0%) ofloxacin resistant isolates that did not carry the D94G substitution. This is equivalent to previously reported frequencies for the detection of GyrA A90V (An *et al*, 2009). In the remaining isolate, a mutation outside the QRDR of GyrA or within the QRDR of GyrB (Mokrousov *et al*, 2008) or active efflux (Escribano *et al*, 2007; Siddiqi *et al*, 2004) may account for ofloxacin resistance. Of the 4 isolates carrying GyrA A90V, 2 were resistant to ofloxacin at 2µg/ml but susceptible at 4µg/ml. That the remaining two isolates carrying a GyrA A90V mutation, however, were resistant to ofloxacin at both 2µg/ml and 4µg/ml, suggests the contribution of an additional mechanism that accounts for higher-level

resistance to ofloxacin in these two isolates. Though active efflux by ABC and MFS transporters was ruled out in both A90V mutants resistant to ofloxacin at 4µg/ml and in the isolate with no identifiable ofloxacin resistance determinant, transporters not inhibited by reserpine and CCCP may contribute to ofloxacin resistance in these isolates. Alternatively, an additional mutation outside the QRDR of *gyrA* or in another gene, such as *gyrB*, may account for the higher levels of resistance observed in these isolates.

Previous investigations have identified GyrA D94G in association with only up to 35% of global ofloxacin resistance (Wang *et al*, 2007; Huang *et al*, 2005; Cheng *et al*, 2004; Takiff *et al*, 1994). That up to 64% of MDR isolates with additional phenotypic resistance to ofloxacin in this study carry GyrA D94G suggests that this mutation is over-represented in ofloxacin-resistant *M. tuberculosis* isolates in this local setting. MAS-PCR assays for the detection of GyrA D94G and A90V would therefore identify ofloxacin resistance in *M. tuberculosis* isolates in this region with a sensitivity of up to 92.9%, making such MAS-PCR assays very useful rapid molecular tools for detecting ofloxacin resistance.

Together, phenotypic DST and MAS-PCR assays for the identification of ofloxacin and/or kanamycin resistance in MDR isolates from the Western Cape indicated a total of 18.1% as resistant to either ofloxacin or kanamycin, or pre-XDR (Mlambo *et al*, 2008). A further 9.6% of isolates were additionally resistant to both antibiotics, which is in accordance with the WHO's most recent estimated incidence of 10.5% XDR-TB amongst MDR isolates in South Africa (WHO, 2010). The proportion of pre-XDR (23.2%) isolates within the MDR group was higher than XDR (8.5%) in 2006, whilst the proportion of pre-XDR (13.1%) and XDR (10.7%) isolates amongst the 2009 MDR group were equivalent. The decrease in prevalence of pre-XDR isolates over this period may reflect an increase in the progression of pre-XDR isolates to XDR in the Western Cape. However, since the XDR-TB prevalence has only increased slightly during this time, it may be that improved screening procedures have led to the implementation of more effective treatment regimens, thereby resulting in decreased emergence of pre-XDR isolates.

Interestingly, 65.5% of the MDR-TB isolates with and without additional resistance to ofloxacin and/or kanamycin from 2009 are W-Beijing and 59.8% of MDR-TB isolates from 2006 are W-Beijing (Evans *et al*, 2009), suggesting a slight increase in the prevalence of MDR W-Beijing isolates in this region. Not surprisingly, 84.4% of all pre-XDR and XDR-TB isolates were W-Beijing. However, the proportion of pre-XDR and XDR W-Beijing isolates increased from 76.9% in 2006 to 94.7% in 2009. This may indicate increased dissemination of the virulent, multidrug resistant W-Beijing *M. tuberculosis* lineage in the Cape Town region. The remaining XDR isolate and 33.3% of the remaining pre-XDR isolates were LAM3/F11, with another 33.3% of pre-XDR isolates identified as LAM9. This may indicate a predisposition of LAM family isolates to acquire additional second-line resistance. However, together with W-Beijing family isolates, LAM3/F11 strains account for up to 55% of *M. tuberculosis* infections in the Western Cape (Nicol *et al*, 2005; Streicher *et al*, 2004), which may account for the overrepresentation of this strain in the pre-XDR and XDR isolates. One isoniazid mono-resistant isolate carrying *rrs* A1401G was identified as W-Beijing and one was X3. Of 17 rifampicin and isoniazid susceptible isolates carrying *rrs* A1401G mutations, 64.7% were non-W-Beijing, including LAM3/F11 (n=3), X3 (n=2), H37Rv (n=1) and unique hybridisation profiles (n=5). Only 35.3% of susceptible isolates with additional resistance to kanamycin were W-Beijing, further confirming the association between W-Beijing isolates and the MDR phenotype. MIRU-VNTR analysis of all pre-XDR and XDR isolates indicated that resistance in these isolates was likely due to independent acquisition of mutations rather than dissemination of resistant strains. Whilst this does indicate that XDR-TB has arisen on multiple occasions, it is encouraging as it suggests that more rapid implementation of appropriate therapies and better adherence may curb the emergence of XDR-TB in this region.

A line-probe assay for the detection of resistance to SLDs has recently been developed that facilitates the identification of GyrA D94G and *rrs* A1401G resistance determinants in association with ofloxacin and kanamycin resistance, respectively. The GenoType® MTBDR*sl* [HAIN Lifescience] assay detects fluoroquinolone resistance using probes that span the QRDR of *gyrA* and specifically detect GyrA A90V, S91P and D94A/N/Y/G mutations. In addition, kanamycin/amikacin resistance due to *rrs* A1401G, C1402T and G1484T mutations, and ethambutol resistance due to

mutations of codon 306 of EmbB can be identified using this assay. Though this assay was not commercially available in South Africa at the time of this investigation, a preliminary evaluation of the GenoType® MTBDR*sl* assay on clinical *M. tuberculosis* isolates indicated sensitivities of 90.6% and 84.8% for the detection of ofloxacin and amikacin/kanamycin resistance, respectively, and 100% specificity for both (Hillemann *et al*, 2009). The use of MAS-PCR for the detection of GyrA D94G and *rrs* A1401G indicated that up to 64.3% and 80.0% of ofloxacin and kanamycin resistance, respectively, can be detected. Additionally, the development of a similar MAS-PCR assay for the detection of GyrA A90V would increase the sensitivity of ofloxacin resistance detection to 92.9%, producing similar sensitivities for the detection of resistance to ofloxacin and kanamycin as the GenoType® MTBDR*sl* [HAIN Lifescience] assay. As MAS-PCR assays are significantly less costly and technically less difficult to perform than the GenoType® MTBDR*sl* [HAIN Lifescience] assay, this makes them very useful for rapidly detecting drug resistance in *M. tuberculosis* in resource-poor settings such as South Africa.

CHAPTER 4:

Investigation of the Association of Transcriptional Regulation with Progression to Antibiotic Resistance in *M. tuberculosis*

4.1 Introduction

4.2 Materials and Methods

4.2.1 Generation and characterisation of *M. tuberculosis* H37Rv rifampicin-resistant mutants

4.2.2 Exposure of rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants to isoniazid

4.2.3 Real-time quantitative reverse transcriptase PCR assays

4.2.3.1 RNA extraction and DNase treatment

4.2.3.2 Validation of PCR efficiency

4.2.3.3 cDNA synthesis and real time qRT-PCR

4.2.4 Molecular characterisation of *rseA* and *mprA*

4.3 Results

4.3.1 Genetic characterisation of rifampicin resistant *M. tuberculosis* H37Rv isogenic mutants

4.3.2 Investigation of the growth of rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants in isoniazid

4.3.3 Analysis of sigma factor expression in rifampicin resistant *M. tuberculosis* H37Rv mutants using real-time quantitative PCR

4.3.4 Molecular characterisation of sigma factor regulators *rseA* and *mprA*

4.3.5 Analysis of *sigE* expression in clinical *M. tuberculosis* isolates

4.3.6 Analysis of FASII cycle gene expression prior to and following exposure of rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants to isoniazid

4.3.7 Analysis of FASII cycle gene expression in *M. tuberculosis* clinical isolates

4.4 Discussion

4.1 Introduction

In any given population of bacteria there exist a small number of spontaneous mutants (Zhao & Drlica, 2002), which can arise as a result of errors during DNA replication and recombination, or oxidative damage of DNA (Foster, 2006; Miller, 1996). The acquisition of point mutations within genes involved in essential metabolic processes may confer a survival advantage, facilitating the proliferation of such mutants to become the dominant bacterial population. Similarly, acquisition of spontaneous point mutations in genes involved in antibiotic resistance can lead to the selection of these resistant mutants during antibiotic therapy. The use of combination therapy increases the likelihood of eradicating all organisms as the probability of a spontaneous mutant resistant to all antibiotics occurring is unlikely.

The occurrence of spontaneous *M. tuberculosis* mutants highlights the importance of rapidly identifying drug resistance in this organism. Primary drug resistance is defined as infection with an *M. tuberculosis* strain that is already resistant to a given antibiotic(s). However, by the time the susceptibility profile of the strain has been determined, treatment with the standard TB regimen has already been administered. In the case of infection with an MDR-TB strain, the rifampicin and isoniazid components of the treatment regimen will be ineffective, increasing the chance that proliferation of spontaneous mutants with additional resistance to ethambutol and/or pyrazinamide may occur. In this manner, the sequential acquisition of antibiotic resistance determinants by the bacterial population results in infection with strains against which no available drugs are effective (Fig. 4.1). The use of molecular assays for the detection of drug resistance and tailoring of treatment early during infection would contribute to reducing the selection and proliferation of drug-resistant spontaneous *M. tuberculosis* mutants.

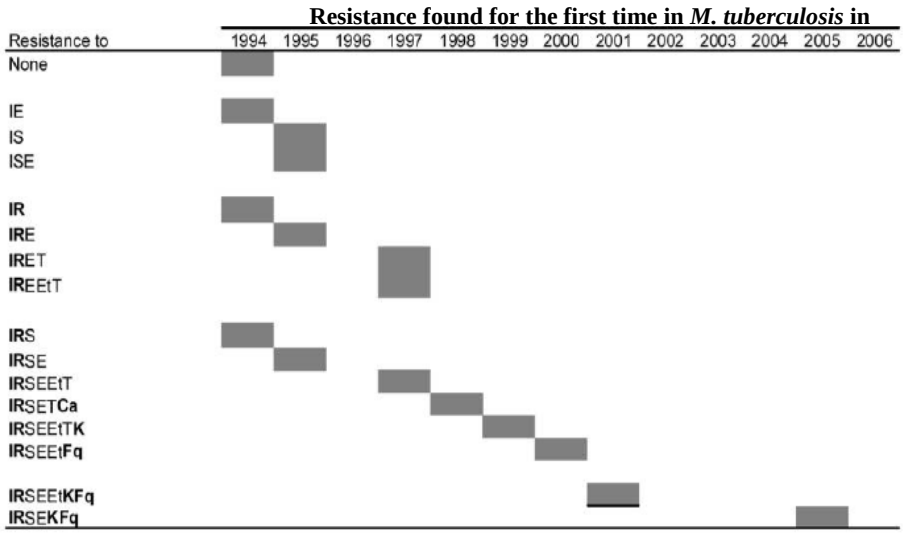

Figure 4.1: The sequential acquisition of resistance in *M. tuberculosis* to individual antibiotics from 1994 to 2006. I, isoniazid; E, ethambutol; S, streptomycin; R, rifampicin; T, thiacetazone; Et, ethionamide; Ca, capreomycin; K, Kanamycin/amikacin; Fq, fluoroquinolones. (Adapted from Pillay and Sturm, 2007).

Furthermore, antibiotics exert their effect by targeting essential metabolic processes and it therefore follows that mutations resulting in antibiotic resistance are associated with a fitness cost to the bacterium. Different mutations are associated with varying degrees of fitness cost (Gagneux *et al*, 2006), and spontaneous mutants carrying mutations with minimal effect on normal metabolic growth are more likely to proliferate and predominate than their counterparts harbouring mutations associated with higher fitness cost (Sander *et al*, 2002; Böttger *et al*, 1998). This is clearly demonstrated in the case of rifampicin resistance due to mutations in RpoB, with *in vitro*-generated RpoB S531L mutants having minimal fitness cost relative to their susceptible parent strains, whilst RpoB R529Q mutants have a high relative fitness cost (Gagneux *et al*, 2006). The minimal fitness cost associated with RpoB S531L mutations likely accounts for the frequency with which it is observed in association with rifampicin resistance worldwide, whereas RpoB R529Q is rarely identified in clinical *M. tuberculosis* isolates (Rossau *et al*, 1997).

Since cell metabolism and functionality is governed predominantly by transcriptional regulation of gene expression, it follows that spontaneous mutations that affect transcription may occur. Such mutations may lead to increased or decreased expression of genes, thereby conferring a selective advantage upon the bacterium.

This is the case with the *inhA* C-15T promoter mutation in *M. tuberculosis* that results in isoniazid resistance (Basso *et al*, 1998; Quemard *et al*, 1996; Dessen *et al*, 1995). The point mutation leads to increased expression of InhA and thereby isoniazid resistance occurs due to drug titration (Larsen *et al*, 2002; Banerjee *et al*, 1994). Together with mutations in KatG, this *inhA* promoter mutation accounts for up to 80% of global isoniazid resistance in *M. tuberculosis* (Hazbón *et al*, 2006). However, the isolation of isoniazid resistant isolates with no identifiable resistance determinants suggests the occurrence of alternative mechanisms of resistance to this drug.

The mechanism of action of isoniazid is inhibition of the synthesis of mycolic acids (Takayama *et al*, 1972), which are key components of the complex layer of lipids surrounding the mycobacterial cell wall. Mycolic acids are unique to mycobacteria and are located on the extracellular side of the arabinogalactan layer of the cell wall, where they are covalently attached to the terminal arabinose residue (Fig. 4.2) (Veyron-Churlet et al, 2010; Bhatt *et al*, 2007). These long chain fatty acids are important in maintaining cell wall impermeability and they also account for the acid-fastness of mycobacteria (Veyron-Churlet et al, 2010).

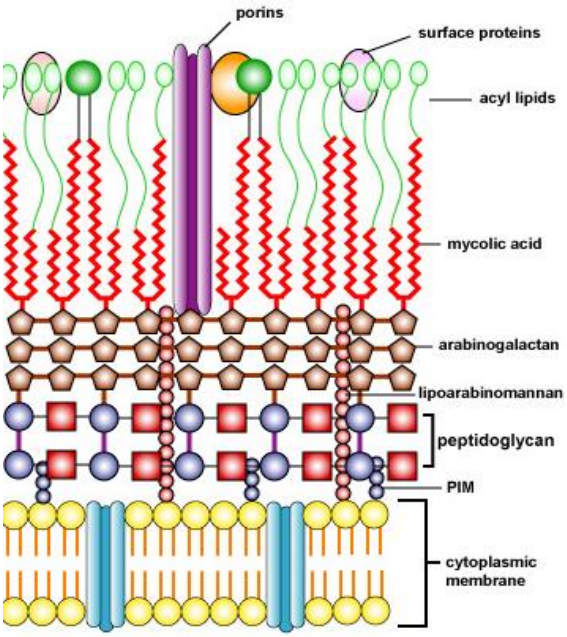

Figure 4.2: Schematic representation of the structure of the mycobacterial cell wall. (http://student.ccbcmd.edu/courses/bio141/lecguide/unit2/bacpath/images/afcw_illus.jpg).

The synthesis of mycolic acids occurs in two steps, the first of which involves the production of short-chain fatty acids by the eukaryotic-like FASI (Schaeffer *et al*, 2001). The prokaryotic-like FASII system (Fig. 4.3) extends these short-chain fatty acids to generate longer meromycolic acids, which are the direct precursors of mycolic acids (Bhatt *et al*, 2007). The acyl-CoA molecules generated during FASI are introduced into the FASII stage of mycolic acid synthesis following their conversion into β-ketoacyl-ACP by FabH, which condenses malonyl-ACP generated by FabD, a malonyl-CoA-ACP transacylase, to the acyl-CoAs generated from FASI (Fig. 4.3) (Kremer *et al*, 2001; Schaeffer *et al*, 2001). Unlike other bacterial FASII systems, the mycobacterial FASII is incapable of generating fatty acids without the precursors generated during FASI, making FabH an essential component of mycolic acid synthesis in *M. tuberculosis* by providing the preferred precursor of FASII (Kremer *et al*, 2001).

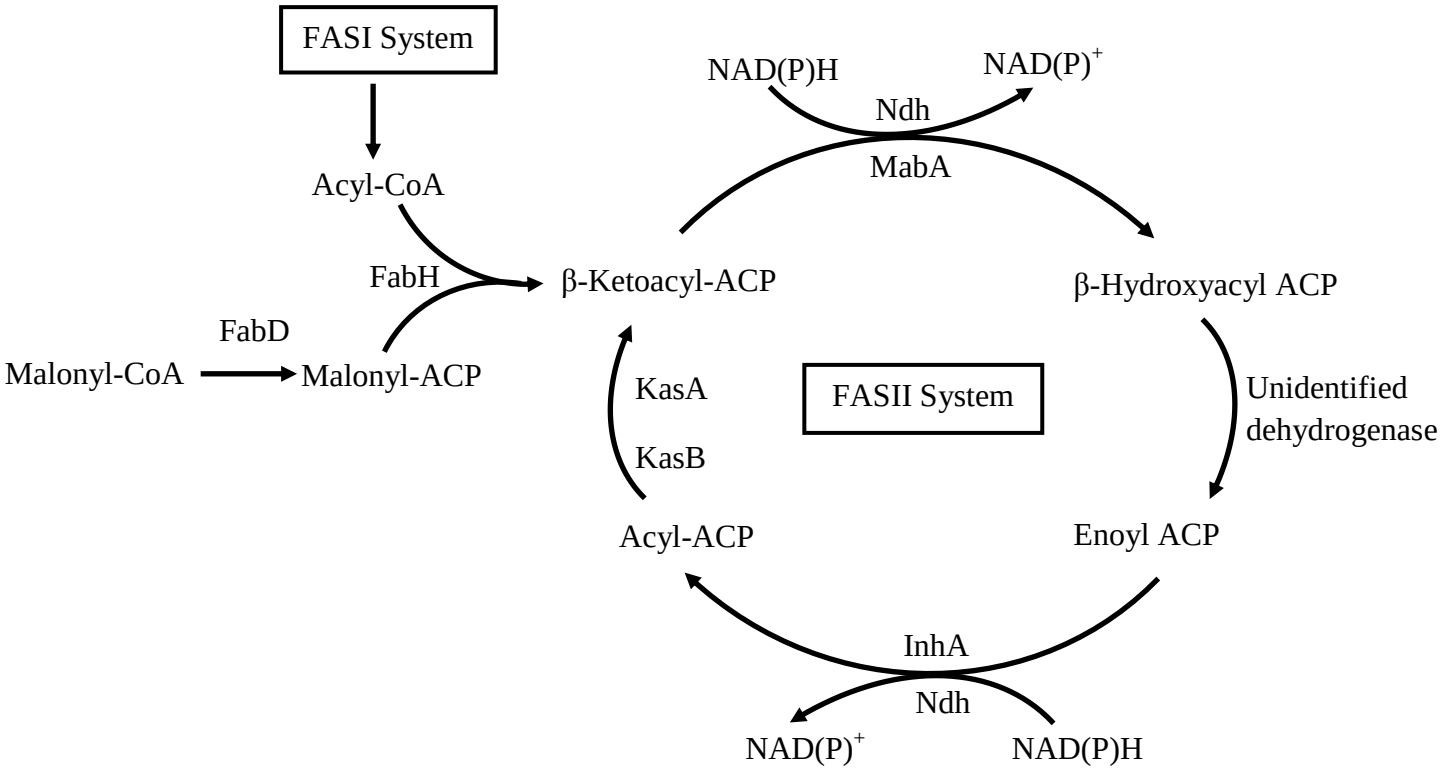

Figure 4.3: A schematic representation of the FASII stage of mycolic acid synthesis that occurs following the generation of short-chain fatty acids during the FASI cycle in *M. tuberculosis*.

On entry into the FASII cycle, two β-ketoacyl-ACP-synthases, KasA and KasB (Fig. 4.3), catalyse elongation of the meromycolate chain by condensation of acyl-ACP and malonyl-ACP (Schaeffer *et al*, 2001). Both enzymes have higher specific activities when acting upon substrates that are bound to the mycobacterial acyl carrier protein (ACP), AcpM (Fig. 4.3) (Schaeffer *et al*, 2001; Kremer *et al*, 2001; Mdluli *et al*, 1998). AcpM is one of three proteins in *M. tuberculosis* that contain the ACP signature sequence (Cole *et al*, 1998), and this protein cross-links to KasA (Mdluli *et al*, 1998). Hydrophobic interactions between the four helices of AcpM maintain its structure, which is highly homologous to the structure of ACPs from *E. coli* and *B. subtilis* (Wong *et al*, 2002). AcpM from *M. tuberculosis*, however, has an additional unique carboxyl terminal region not seen in other bacterial ACPs, whose function is unknown (Wong *et al*, 2002).

Although the exact difference between the roles of KasA and KasB is not precisely known, KasA is thought to initiate the first stage of FASII by condensing malonyl-AcpM to an existing carbon chain esterified to AcpM, C_{16}-AcpM (Kremer *et al*, 2002), thereby initiating the elongation process, whilst KasB is thought to be involved in the continuation of elongation of C_{16}-AcpM at a later stage in the cycle (Molle *et al*, 2006; Schaeffer *et al*, 2001). That KasB mutants can be generated whilst KasA mutants cannot, however, suggests that KasA is essential and KasB is non-essential (Bhatt *et al*, 2005). Mutation of KasA Cys171, His311, Lys340 and His345 abolish its condensation-elongation activity *in vitro* (Kremer *et al*, 2002), indicating that these amino acid residues are located within the active site of KasA and are required for mycolic acid synthesis. The identification of the same mutations within *kasA* in both isoniazid resistant and susceptible *M. tuberculosis* isolates, however, suggests that KasA is not directly involved in the development of resistance to isoniazid (Hazbón *et al*, 2006).

The second stage of FASII elongation involves the reduction of the β-ketoacyl product generated by KasA/KasB to form β-hydroxyacyl, and this is carried out by MabA, an NADPH dependant β-ketoacyl reductase (Banerjee *et al*, 1998). Following the conversion of β-hydroxyacyl to enoyl ACP by an as yet unidentified dehydrogenase, InhA generates acyl-ACP, the substrate for KasA/KasB (Fig. 4.3).

Throughout the entire elongation cycle, the growing meromycolate chain remains attached to AcpM through thioester linkage (Yuan *et al*, 1998).

The FASII stage of the mycolic acid synthesis pathway is well characterised as the target of isoniazid in *M. tuberculosis*, as is demonstrated by the accumulation of C_{26} fatty acids following isoniazid treatment (Takayama *et al*, 1972). The primary target of isoniazid, however, remains unknown. Although InhA has a clearly defined role in isoniazid resistance through mutations within its promoter region (Hazbón *et al*, 2006), evidence suggests that it is not the primary target of isoniazid in *M. tuberculosis*. Inactivation of InhA does not result in C_{26} fatty acid accumulation and direct binding of isoniazid to InhA has never been observed (Mdluli *et al*, 1996), suggesting an alternative target. Though *mabA* and *inhA* are cotranscribed in *M. tuberculosis* (Banerjee *et al*, 1994), *mabA* does not appear to be involved in the development of resistance to isoniazid in *M. tuberculosis* or in *M. smegmatis* (Musser *et al*, 1996). Investigation into whether KasA was the primary target of isoniazid in *M. tuberculosis* led to the elucidation of an 80-kDa complex consisting of isoniazid, KasA and AcpM (Mdluli *et al*, 1996). However, the lack of significant depletion of unbound KasA in isoniazid treated versus untreated cells in *M. kansasii*, *M. bovis* BCG, *M. smegmatis* and *M. tuberculosis* suggests that it is not the primary target of isoniazid (Kremer *et al*, 2003). In addition, that KatG-activated isoniazid significantly inhibits InhA activity *in vitro* but does not affect KasA activity, suggests that isoniazid does not directly target KasA (Kremer *et al*, 2003).

In the absence of a defined primary target for isoniazid despite investigation of all gene products of the FASII cycle, it is interesting to speculate that regulation of these or other genes involved in the FASII cycle could play a role in the progression of *M. tuberculosis* to isoniazid resistance. This may be effected through differential gene expression due to point mutations within the regulatory region or through alternative sigma factors that alter transcriptional regulation within the cell. Specifically, ECF sigma factors provide an elegant means of regulating gene expression in response to external stimuli, such as antibiotic exposure. Following exposure to isoniazid, the expression of all five genes of the kas/FASII operon is induced (Wilson *et al*, 1999). Additionally, SigE, an *M. tuberculosis* ECF sigma factor, is involved in the regulation of expression of *acpM* and *fabD* of the kas/FASII operon (Manganelli *et al*, 1999). It

may be, therefore, that exposure to isoniazid induces alternative sigma factor-mediated transcriptional regulation that assists the bacterium in overcoming the selective pressure.

Unlike isoniazid mono-resistance, rifampicin mono-resistance is rarely seen, as rifampicin resistance is usually identified in association with resistance to isoniazid. Rifampicin binds to the β-subunit of RNA polymerase (Fig. 4.4), RpoB, inhibiting transcription by preventing elongation of the RNA transcript from the 5` end (Campbell *et al*, 2001). It therefore follows that mutations within the rifampicin binding pocket of RpoB that affect rifampicin binding prevent inhibition of transcription by rifampicin, thereby resulting in resistance to this antibiotic (Campbell *et al*, 2001). Twelve of the well described rifampicin resistance determinants in RpoB, including S531, H526 and D516, are located within the rifampicin binding pocket where they interact directly with rifampicin (Campbell *et al*, 2001).

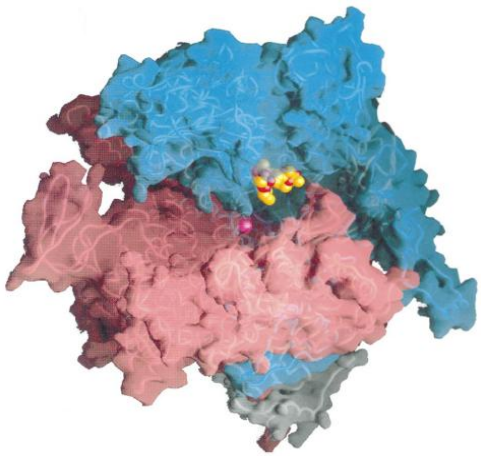

Figure 4.4: A three-dimensional representation of a rifampicin molecule within the rifampicin binding pocket of the RNA polymerase core enzyme. The β subunit is indicated in blue, the β` subunit is indicated in pink, the ω subunit is indicated in grey and the α subunits, located behind the RNA polymerase, are not visible. (Adapted from Cambell *et al*, 2001).

The β` subunit of RNA polymerase contains the primary binding site of σ^{70} (Arthur *et al*, 2000; Arthur & Burgess, 1998), which is located between amino acid residues 260 and 309. However, the β flap of the β subunit is also required for sigma factor binding, and interacts directly with region 4 of the sigma factor (Nickels *et al*, 2005;

Kuznedelov *et al*, 2002). In the absence of the β flap, the interaction between region 4.2 of the sigma factor and the -35 promoter region is abolished (Kuznedelov *et al*, 2002). Upon binding of the sigma factor to the core RNA polymerase, regions 2.1, 2.3, 3.1, 3.2 and 4.2 of the sigma factor are all proximal to the conserved β region located between amino acids 383 and 554, which includes the rifampicin resistance determining region (amino acids 507 to 534) (Owens *et al*, 1998). As sigma factors are responsible for recognition and transcription of specific subsets of genes, mutations in RpoB that result in rifampicin resistance may change the affinity of the core polymerase for certain sigma factors. It follows that with altered sigma factor binding comes expression of different subsets of genes. That some of these genes may be implicated in reduced susceptibility to isoniazid could account for a relatively rapid progression of rifampicin mono-resistant mutants to MDR. This provides an interesting hypothesis for the rarity of observed rifampicin mono-resistance. To test this hypothesis, rifampicin-resistant isogenic *M. tuberculosis* H37Rv mutants were generated and characterised and progression to MDR was investigated. This included investigation of the role of sigma factors in altered gene expression in these *M. tuberculosis* H37Rv isogenic mutants through measuring sigma factor expression using real-time quantitative PCR assays.

4.2 Materials and Methods

4.2.1 Generation and characterisation of *M. tuberculosis* H37Rv rifampicin-resistant mutants

M. tuberculosis H37Rv with wild-type *rpoB* was grown to mid-log phase (OD_{600} = 0.6) in 10ml Middlebrook 7H9 broth supplemented with ADC and Tween 80 (0.05% v/v) at 37°C with shaking. This starter culture was then diluted 1:100 to a final volume of 10ml in Middlebrook 7H9 broth supplemented with ADC and Tween 80 (0.05% v/v) containing rifampicin at 1μg/ml and grown to early log phase (OD_{600} = 0.2-0.4). A volume of 100μl of culture was inoculated onto 25 Middlebrook 7H11 agar plates supplemented with OADC and containing rifampicin at 2μg/ml. Agar plates were incubated at 37°C in the presence of 8% CO_2 for 20 - 25 days. A total of 50 individual colonies were inoculated into 10ml Middlebrook 7H9 broth containing rifampicin at 2μg/ml and grown to mid-log phase (OD_{600} = 0.6), following

which genomic DNA [2.2.2] was extracted and glycerol stocks were made and stored at -80°C.

PCR amplification of the RRDR of *rpoB* from 50 randomly selected mutants was carried out using primers RPOBF (5`-GAGATCAAGAGTCAGAC-3`) and RIR (Mokrousov *et al*, 2003) to characterise the rifampicin resistance determinants. PCR assays were to a final volume of 50µl and contained 1X GoTaq PCR buffer [Promega], 0.2mM of each dNTP [Fermentas], 3mM $MgCl_2$ [Promega], 20 pmoles of each primer, 1.25U GoTaq® DNA polymerase [Promega] and 50-100ng template genomic DNA. Following an initial denaturation step at 95°C for 5 minutes, both PCR reactions consisted of 35 cycles of denaturation at 95°C for 60 seconds, primer annealing at 53°C for 60 seconds and primer extension at 72°C for 90 seconds. A final elongation step at 72°C for 5 minutes was performed to complete elongation of all incomplete products. PCR assays were carried out using a GeneAmp PCR System 2400 [Perkin Elmer] thermocycler and all PCR products were visualised by agarose gel electrophoresis [2.2.2] and sequenced [2.2.5].

4.2.2 Exposure of rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants to isoniazid

M. tuberculosis H37Rv and isogenic mutants carrying RpoB S531L, S531W, S522L, H526D and H526Y rifampicin resistance determinants were grown to early-log phase (OD_{600} = 0.2) in 10ml Middlebrook 7H9 broth supplemented with ADC and Tween 80 (0.05% v/v) at 37°C with shaking. A total of 10^4 colony forming units of each isogenic mutant was inoculated in triplicate into 100ml Middlebrook 7H9 broth supplemented with ADC and Tween 80 (0.05% v/v) without selection and in the presence of isoniazid (0.2µg/ml). Cultures were incubated at 37°C with continuous rolling for 24 days and OD_{600} readings were measured every 48 hours.

4.2.3 Real-time quantitative reverse transcriptase PCR assays

4.2.3.1 RNA extraction and DNase treatment

Total cellular RNA was extracted from 3 cultures of *M. tuberculosis* H37Rv and from 3 cultures of each of the isogenic mutants carrying RpoB S531L, S531W, S522L, H526D and H526Y rifampicin resistance determinants using the FastRNA® Pro Blue Kit [MP Biomedicals] according to the manufacturer's instructions. Briefly, cells were pelleted from 10ml cultures grown to mid-log phase (OD_{600} = 0.6) in Middlebrook 7H9 broth supplemented with ADC, and pellets were resuspended in 1ml RNApro™ solution [MP Biomedicals] in order to initiate RNase inhibition. The cells were lysed by processing at a setting of 6.0 for 40 seconds using a FastPrep-24 system [MP Biomedicals], and cellular debris was pelleted by centrifugation. Total RNA was extracted from the supernatant by chloroform extraction and ethanol precipitation (Ausubel *et al*, 1987) and resuspended in 60µl DEPC-dH_20 prior to storage at -80°C.

Following quantification, 1µg of total RNA was incubated at 37°C for 30 minutes in the presence of 4U TURBO™ DNase [Ambion]. The DNase-treated RNA was purified by acid phenol:choloroform:isoamyl alcohol (125:24:1) [Ambion] extraction and ethanol precipitation and the pellet was resuspened in 12µl DEPC-dH_20. The DNase treatment and purification steps were repeated to increase the purity of the RNA samples.

4.2.3.2 Validation of PCR efficiency of all primer and probe sets

Gene expression assays were carried out using a 5` nuclease-based assay to cleave a sequence-specific probe during PCR. TaqMan minor groove binder (MGB) probes and primers (Table 4.1) were designed using Primer Express® Software v2.0 [Applied Biosystems] and probes were 5`-FAM-labelled. Standard curves were generated using 10-fold dilutions of *M. tuberculosis* H37Rv genomic DNA [2.2.2] in order to ensure that PCR efficiency was equivalent using all primer/probe sets. From the slope of the standard curve, PCR efficiency was calculated using the formula $E = 10^{[-1/slope]}$, where

a slope of -3.3 corresponds to 100% efficiency and indicates that the amount of PCR product is doubled in after each cycle (Fig. 4.5). The coefficient of determination, R^2, of the slopes produced by all primer/probe sets was ≥0.98.

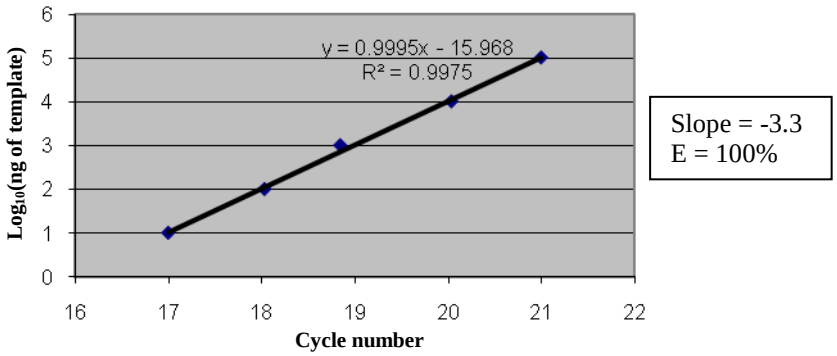

Figure 4.5: Standard curve generated for validation of the 16S rRNA primer/probe set used for quantification normalisation in all qPCR assays. The coefficient of determination (R^2) approximates 1 and the slope of the curve is -3.3, which corresponds to 100% PCR efficiency.

Amplification using all other primer/probe sets was optimised to produce amplification efficiencies of 90-110%, with no more than a 10% difference in amplification efficiency from that of 16S rRNA. Where PCR efficiency was greater than 110%, 10µM EDTA was added to reduce it to 90-110% (Fig. 4.6a,b), and 1mM Mg^{2+} was used to increase PCR efficiency (Fig. 4.7a,b) in the case of primer/probe sets with efficiencies of less than 90%. Despite optimisation, acceptable amplification efficiencies could not be obtained for the *sigD* primer/probe set, and this set was therefore excluded from further analysis.

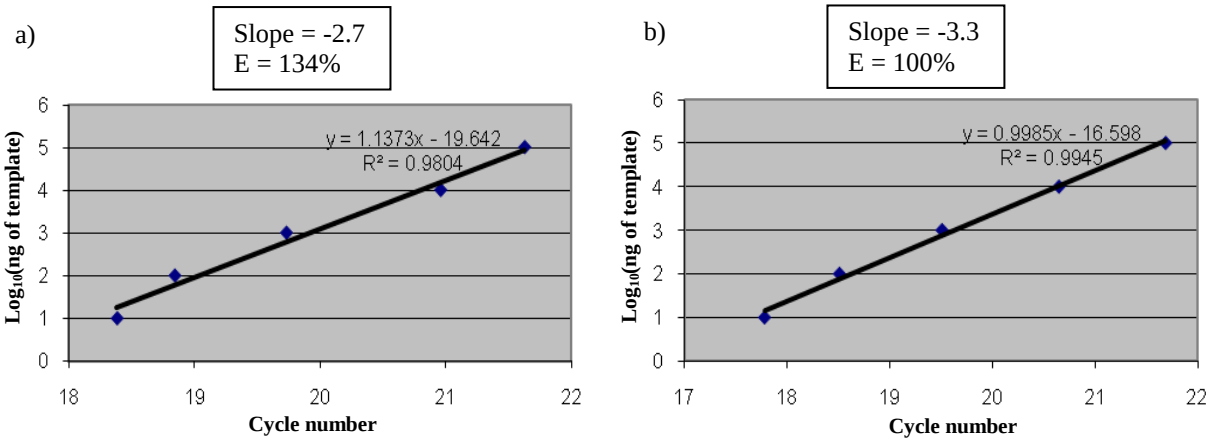

Figure 4.6: Standard curve generated for validation of the *sigB* primer/probe set (a) prior to and (b) following addition of EDTA (b). The coefficient of determination (R^2) approximates 1 and the addition of 10µM EDTA reduces the PCR efficiency from 134% to 100%.

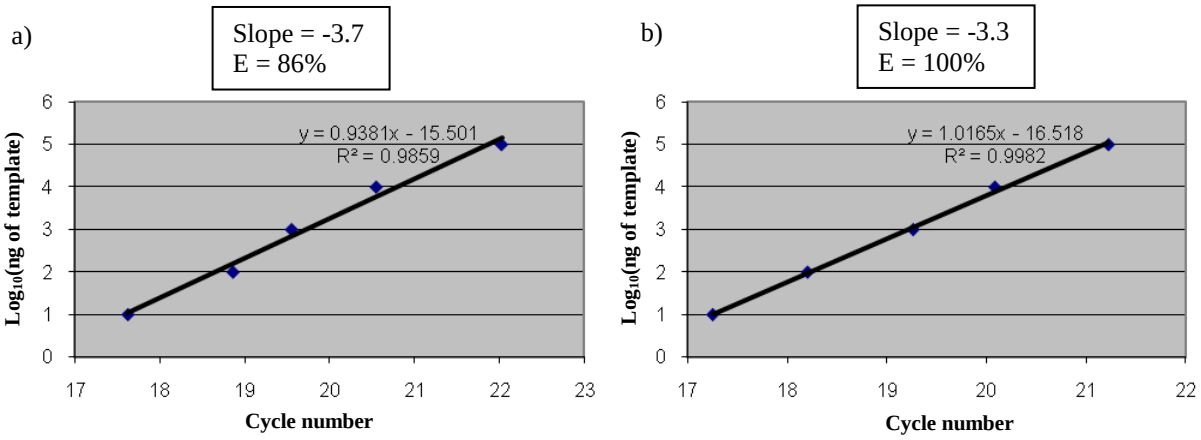

Figure 4.7: Standard curve generated for validation of the *sigG* primer/probe set (a) prior to and (b) following addition of Mg^{2+}. The coefficient of determination (R^2) approximates 1 and the addition of 1mM Mg^{2+} increases the PCR efficiency from 86% to 100%.

4.2.3.3 cDNA synthesis and real-time qRT-PCR

A total of 40ng DNase-treated RNA and 0.5µg random hexamers were denatured at 70°C for 5 minutes using a GeneAmp PCR System 2400 [Perkin Elmer] thermocycler. Following immediate incubation on ice for 5 minutes, RNA was reverse

transcribed into cDNA using 200U M-MLV reverse transcriptase [Promega] in the presence of 20U Protector RNase Inhibitor [Roche] and 0.2mM of each dNTP for 60 minutes at 37°C. A reverse transcription negative control was included for each sample to confirm the absence of contaminating genomic DNA in the RNA preparations. This control consisted of all components of the reverse transcription reaction except the M-MLV reverse transcriptase. In addition, a water control containing no RNA template was included for each cDNA synthesis preparation in order to confirm the absence of contaminating genomic DNA in any of the reagents. Experimental data was only evaluated if no amplification was observed in any of these controls.

A volume of 2µl of cDNA was used as the template in real-time PCR assays consisting of 1 X TaqMan® Universal PCR Master Mix containing AmpErase uracil N-glycosylase (UNG) [Applied Biosystems], 900nM of each primer (Table 4.2.1) and 250nM of TaqMan® MGB probe (Table 4.2.1) to a final volume of 25µl with dH_20. Cycling conditions consisted of 10 minutes at 50°C for 2 minutes to activate the UNG, which minimises cross-contamination with PCR products by incorporating dUTP, 95°C for 10 minutes to activate AmpliTaq Gold, followed by 35 cycles of 95°C for 15 seconds and annealing at 60°C for 30 seconds. All qRT-PCR assays were performed in duplicate on each biological triplicate using an ABI Prism® 7000 [Applied Biosystems].

The $2^{-\Delta\Delta Ct}$ method was used to calculate fold differences in expression levels between the rifampicin resistant mutant test samples and the *M. tuberculosis* H37Rv calibrator (Livak & Schmittgen, 2001). Amplification of *M. tuberculosis* H37Rv 16S rRNA was used as the reference gene for quantification normalisation. Cycle threshold (C_t) values of above 32 indicate a very low level of expression and therefore result in poorly reproducible data. For the purposes of this study, since all C_t values obtained were from three independant RNA extractions, each evaluated in duplicate, genes producing C_t values of 32 or above were considered to be not significantly expressed.

Table 4.1: Primers and probes used for real-time PCR amplification of *M. tuberculosis* sigma factors.

Primer Name	Primer Sequence (5` → 3`)	[a]TaqMan® MGB Probe Sequence (5` → 3`)
16SF	GCCCTGCACTTCGGGATAA	CCTGGGAAACTGGGTC
16SR	CCCGTGGTCCTATCCGGTAT	
SIGAF	CCGATGACGACGAGGAGATC	AAGGACAAGGCCTCC
SIGAR	GTCTTCATCCCAGACGAAATCAC	
SIGBF	GCCGTTGCTGGACCTCAT	AGGAGGGCAACCTG
SIGBR	TTCTCCATCGCTCGGATCA	
SIGCF	GCGTCCCGAACATCTCATAGA	CGCGGATTCGAAGAC
SIGCR	GTTAGGTCGGCGATCATCGT	
SIGEF	TCCGGTCGGTCCAGAATTAC	CGGGCACCTTCGAA
SIGER	TTGGTGGTGATGCGGTGTAG	
SIGFF	AACAGCTGGTCGGTCAAGGT	CCCGGCGTCTCAA
SIGFR	CCTAGCCGCAGATGCAGTTC	
SIGGF	AGCCCTACCGGCGTGAAC	TCGCACACTGCTATC
SIGGR	TGCAGCGAGCCAGTCATG	
SIGHF	GGCCTGGCTCTACCGGATAC	CCAACACCTACATCAAC
SIGHR	CCGCTGTTTCTTGCGATAGC	
SIGIF	GACCTCGCGTTTCGTATGGTA	ATCGGCGTGGCCGA
SIGIR	AAATGCCTCTTGCACCATGTC	
SIGJF	TGCGCGACGGAAAGGT	CGCTGTGGGATATC
SIGJR	GGTGAACTTGTCGGGATTGG	
SIGKF	GATACCGGCTACAGCGAAGAA	CCACCCAGGAGATC
SIGKR	GTTCCGCCACACCTCAAGAT	
SIGLF	CGTGATCCAGCGGTCCTACT	CCGCGGATGGTC
SIGLR	ACCGTTCCTTCGGCAATTC	
SIGMF	GCTGCTGGCCGCCCATGTC	GGCGACCGGTACGC
SIGMR	TGACGGCGGAACAACTGATC	
INHAF	CCTACGCGGATGTGTCCAA	CATCCACATCTCGGC
INHAR	TTGGCCATCGAAGCATACG	
KATGF	GGCACCGGAACCGGTAAG	ATGCCGCTGGTGAT
KATGR	AGGAAACTGTTGTCCCATTTCGT	
FABDF	CGGCAAGGACGTCATCGT	CCACTCCGTCGGCG
FABDR	GCTATCACACCGGCGATTG	
ACPMF	TCGACTCGCTGTCGATGGT	AGATCGCCGTGCAGAC
ACPMR	GGATCTTGACGCCGTACTTGTC	

[a]5`-FAM (5-carboxyfluorescein)-labelled.

4.2.4 Molecular characterisation of sigma factor regulators *rseA* and *mprA*

The functional *rseA* and *mprA* genes were PCR amplified using primers RSEAF (5`-TCGGTCCCGAATCCTGG-3`), RSEAR (5`-GTTGTCCGTGTCCACCC-3`), MPRAF (5`-CCAATGTGGCTGATGTGG-3`) and MPRAR (5`-TCGCCAGCAG CATGACC-3`) (Fig. 4.8a,b) and amplicons were sequenced. Both PCR assays were to a final volume of 50µl and contained 1X GoTaq PCR buffer [Promega], 0.2mM of each dNTP [Fermentas], 3mM $MgCl_2$ [Promega], 20 pmoles of each primer, 1.25U GoTaq® DNA polymerase [Promega] and 50-100ng template genomic DNA [2.2.2]. Following an initial denaturation step at 95°C for 5 minutes, both PCR assays consisted of 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds and primer extension at 72°C for 30 seconds. A final elongation step at 72°C for 5 minutes was performed to complete elongation of all incomplete products. PCR assays were carried out using a GeneAmp PCR System 2400 [Perkin Elmer] thermocycler and all PCR products were visualised by agarose gel electrophoresis [2.2.2] and sequenced [2.2.5].

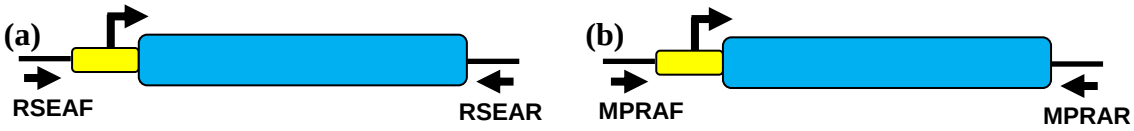

Figure 4.8: Schematic representation of the location of primers used for the amplification of sigma factor regulators *resA* and *mprA*. The structural gene is indicated by the blue rectangle; the promoter region is indicated in yellow; the bent arrow indicates the direction of transcription; primers are shown by arrows flanking the functional gene.

4.3 Results

4.3.1 Genetic characterisation of rifampicin resistant *M. tuberculosis* H37Rv isogenic mutants

Following growth of *M. tuberculosis* H37Rv in the presence of rifampicin more than 1000 rifampicin-resistant mutants were obtained. Sequence analysis of *rpoB* from 50 randomly selected mutants identified an RpoB S531L mutation in 38 (76.0%) and an

RpoB S531W mutation in 4 (8.0%) mutants (Figure 4.9). A total of eight mutants had mutations at codon 526, with 4 (8.0%) carrying H526D and 4 (8.0%) carrying H526Y substitutions (Figure 4.9).

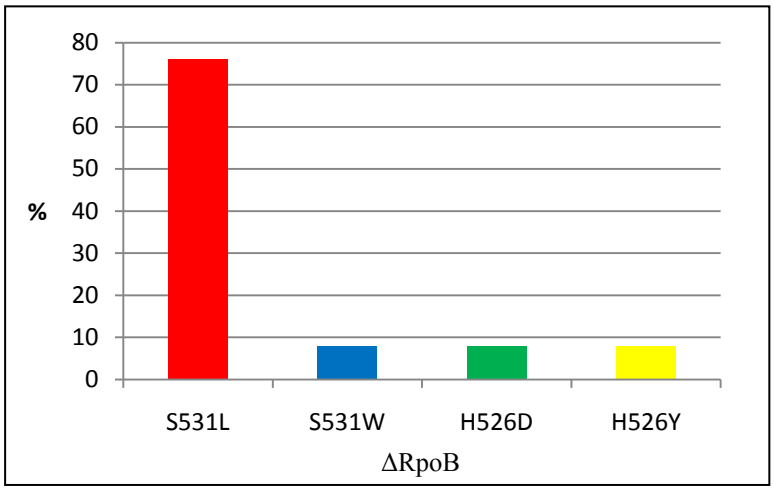

Figure 4.9: Distribution of RpoB mutations identified within the rifampicin resistance determining region of 50 isogenic *M. tuberculosis* H37Rv mutants.

Though RpoB 531 mutations are most frequently associated with rifampicin resistance in clinical *M. tuberculosis* isolates (Ramaswamy *et al*, 1998), codon 526 mutations are usually seen at a higher frequency in *in vitro* generated mutants (Huitric *et al*, 2006; Anthony *et al*, 2005). Since only 50 of over 1000 rifampicin mono-resistant mutants obtained were characterised, it is possible that these results reflect some selection bias. However, as all cultures were inoculated from the same starter culture, it may be that the high proportion of RpoB S531L mutants obtained represents proliferation of existing spontaneous RpoB S531L mutants in the population that were selected in the presence of rifampicin.

MAS-PCR assays for the detection of KatG S315T and *inhA* C-15T (2.2.4) were carried out on one representative mutant carrying each RpoB mutation. Neither mutation was identified in any of these isogenic mutants, confirming no spontaneous acquisition of isoniazid resistance due to either of these resistance determinants.

4.3.2 Investigation of the growth of rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants in isoniazid

The association of specific RpoB mutations with ability to grow in the presence of isoniazid was investigated by growth, in triplicate, of isogenic rifampicin-resistant *M. tuberculosis* H37Rv mutants in the presence and absence of 0.2µg/ml isoniazid. In the absence of isoniazid, all 4 mutants (RpoB S531L, S531W, H526D and H526Y) reached early to mid-log phase growth (OD_{600} = 0.2 - 0.4) by day 22, following a lag phase of 8-10 days (Fig. 4.10). In the absence of isoniazid, the RpoB S531L, S531W and H526D mutants took 2 days longer to reach exponential growth phase than the parent *M. tuberculosis* H37Rv strain (Fig. 4.10), suggesting that the *rpoB* mutations conferred little fitness cost. The isogenic mutant carrying RpoB H526Y grew equivalently up to day 12 and then the growth rate of this mutant slowed relative to the parent *M. tuberculosis* H37Rv strain (Fig. 4.10). This suggests that an H526Y mutation in RpoB is associated with a higher degree of fitness cost than RpoB S531L, S531W or H526D mutations.

Both the RpoB S531L and S531W mutants grew equivalently in the presence and absence of isoniazid (Fig. 4.10). Since no growth of the parent *M. tuberculosis* H37Rv strain was observed in the presence of isoniazid, this suggests that mutations at codon 531 of RpoB enhance the ability of *M. tuberculosis* to grow in the presence of isoniazid. A longer lag phase of 2 days was observed in the case of the H526D mutant in the presence of isoniazid as compared to without (Fig. 4.10), indicating that resistance to isoniazid in the presence of RpoB H526D is not acquired as rapidly as with S531L/W mutations. Interestingly, no growth was observed up to day 24 in the presence of isoniazid of the RpoB H526Y mutant (Fig. 4.10). This suggests that the presence of an RpoB H526Y mutation does not contribute towards facilitating growth in the presence of isoniazid.

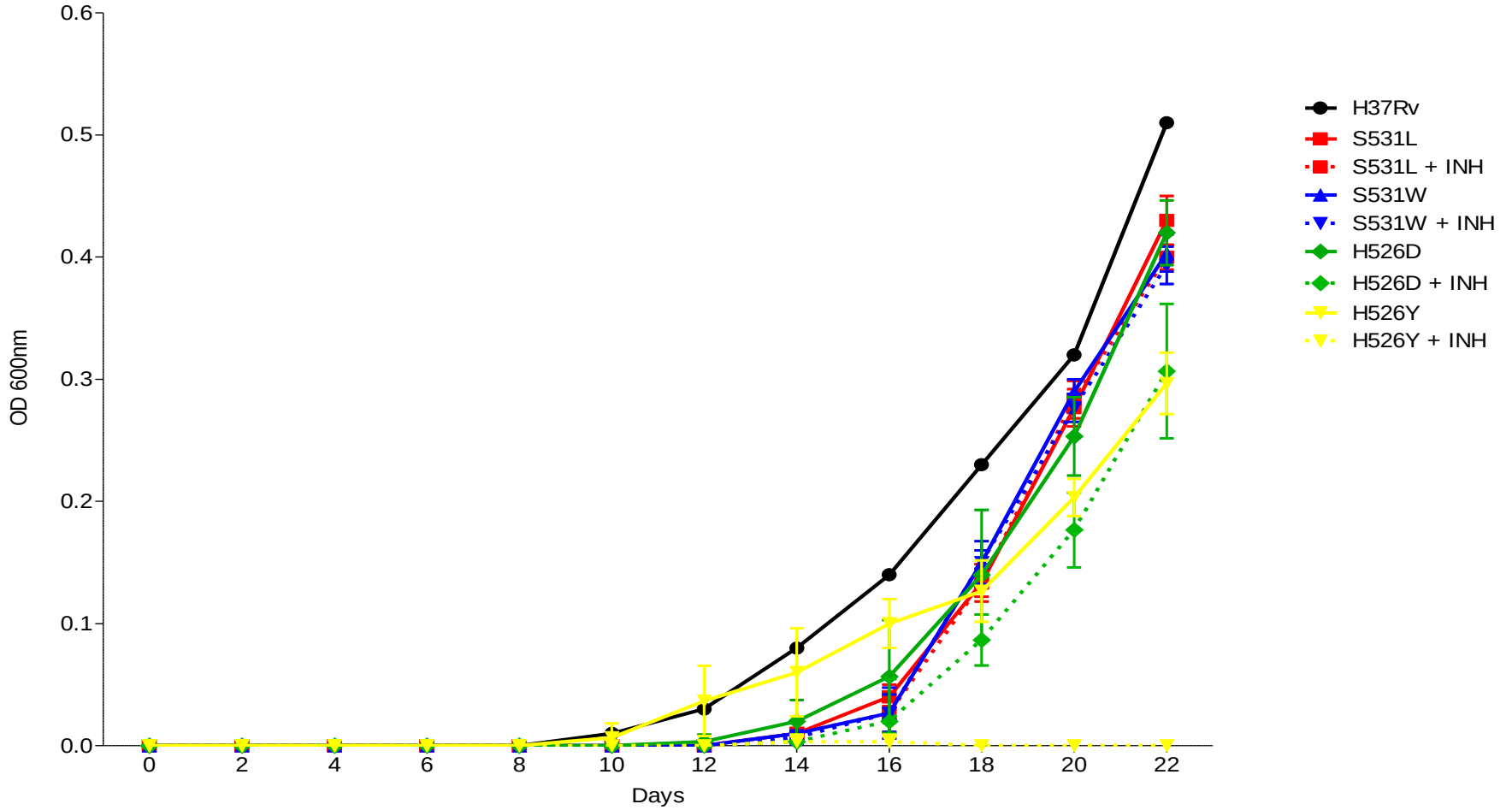

Figure 4.10: Growth curves of rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants in the presence and absence of isoniazid at 0.2µg/ml. Each data point represents the average of three OD_{600} readings, each from independent cultures; error bars represent the standard deviation of the mean.

Following growth of the RpoB S531L, S531W and H526D mutants in the presence of isoniazid, MAS-PCR assays for the detection of KatG S315T and *inhA* C-15T identified neither mutation in any of the isogenic mutants. However, KatG S315T and *inhA* C-15T mutations are rarely identified *in vitro*, and it is therefore likely that alternative isoniazid resistance determinants, such as mutations elsewhere in *katG*, were acquired in these isolates (Bergval *et al*, 2009).

4.3.3 Analysis of sigma factor expression in rifampicin resistant *M. tuberculosis* H37Rv mutants using real-time quantitative PCR

Following validation of all primer and probe sets to ensure equivalent PCR efficiencies [4.2.3.2], sigma factor expression in rifampicin-resistant *M. tuberculosis* H37Rv isogenic mutants was investigated. RT-qPCR assays [4.2.3] were carried out on genes encoding sigma factors in isogenic mutants carrying RpoB S531L, S531W, H526D and H526Y resistance determinants. The threshold (C_t) values obtained for *sigC*, *sigF*, *sigI*, *sigL* and *sigM* from the parent *M. tuberculosis* H37Rv strain and for each of the isogenic mutants were above 32 cycles, indicating that *sigC*, *sigF*, *sigI*, *sigL* and *sigM* are not expressed at detectable levels.

Expression of *sigA*, the primary sigma factor of *M. tuberculosis*, was equivalent to that of the parent *M. tuberculosis* H37Rv strain in isogenic mutants carrying RpoB S531W, H526D and H526Y (Table 4.2; Fig. 4.11). Interestingly, in the RpoB S531L mutant, *sigA* was upregulated 2.92-fold (Table 4.2; Fig. 4.11), and this may account in part for the reduced fitness cost observed in association with RpoB S531L. Expression levels of *sigB*, encoding the *M. tuberculosis* secondary sigma factor, were equivalent in all isogenic mutants to that of the *M. tuberculosis* H37Rv parent strain (Table 4.2; Fig. 4.11), as was expression of the ECF sigma factors *sigG*, *sigH*, *sigJ* and *sigK* (Table 4.2; Fig. 4.11).

Table 4.2: Fold increase in expression of genes encoding *M. tuberculosis* sigma factors in rifampicin-resistant isogenic mutants as compared to the parent *M. tuberculosis* H37Rv strain.

	ΔRpoB			
[a]Gene	S531L	S531W	H526D	H526Y
sigA	[b]2.92	1.24	1.11	1.23
sigB	1.74	1.70	0.91	1.01
sigE	9.03	7.30	6.84	1.87
sigG	1.78	2.30	0.79	1.03
sigH	1.90	0.54	0.65	1.60
sigJ	0.55	0.97	0.85	1.02
sigK	0.99	0.67	0.46	0.77

[a]Genes encoding *M. tuberculosis* sigma factors *sigC*, *sigD*, *sigF*, *sigI*, *sigL* and *sigM* are not indicated since they were not expressed at detectable levels.
[b]Values represent an average of two technical repeats performed on each of three independant RNA extractions from each rifampicin-resistant isogenic mutant, calculated relative to *M. tuberculosis* H37Rv and normalised to 16S rRNA expression.

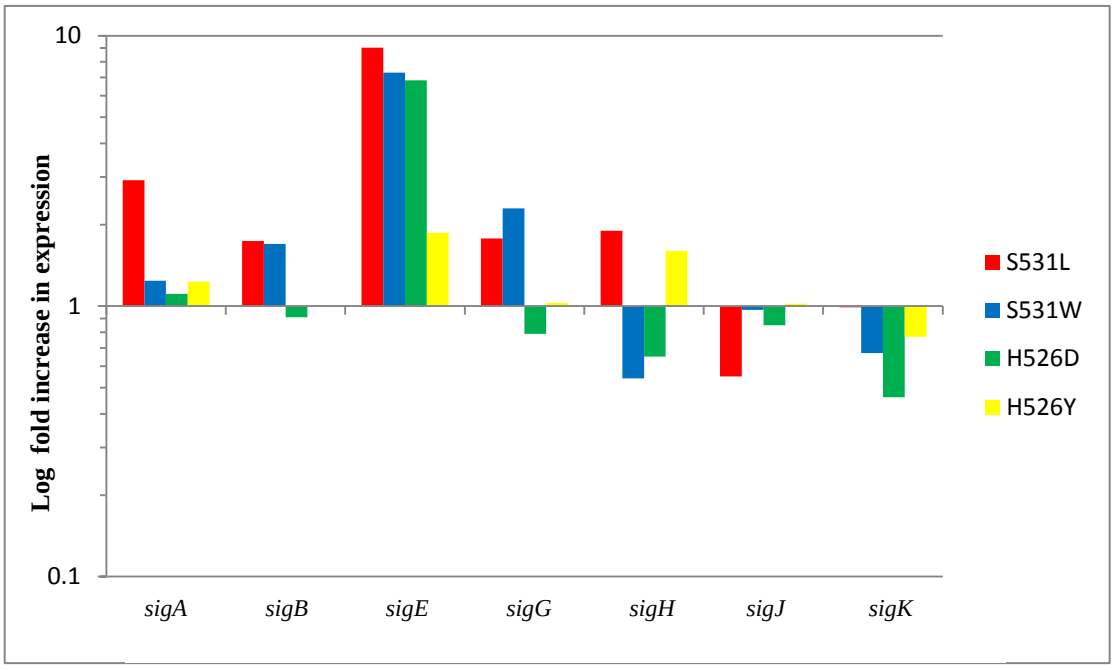

Figure 4.11: Expression levels of sigma factor transcripts in rifampicin resistant *M. tuberculosis* H37Rv mutants compared to the parental *M. tuberculosis* H37Rv. Values are expressed as ratios of cDNA copies detected from exponentially growing cultures, calculated relative to *M. tuberculosis* H37Rv and normalised to 16S rRNA expression. The reported values indicate the average of two technical repeats of each of three independant RNA preparations from each isogenic mutant. Genes encoding *M. tuberculosis* sigma factors *sigC*, *sigD*, *sigF*, *sigI*, *sigL* and *sigM* are not indicated since they were not expressed at detectable levels.

RT-qPCR analysis identified a 9.03-fold, 7.30-fold and 6.84-fold increase in *sigE* expression in the RpoB S531L, S531W and H526D mutants, respectively (Table 4.2; Fig. 4.11), whilst *sigE* expression in the RpoB H526Y mutant was equivalent to that of the *M. tuberculosis* H37Rv parent strain (Table 4.2; Fig. 4.11). Interestingly, the magnitude of *sigE* upregulation appears to directly correlate with the ability of these mutants to grow in the presence of isoniazid. Isogenic mutants carrying RpoB S531L, S531W and H526D mutations, in which *sigE* expression was highly upregulated, grew rapidly in the presence of isoniazid (Table 4.2; Fig. 4.10; Fig. 4.11). The expression of *sigE* in the RpoB H526Y mutant was equivalent to that of the parent strain, and no growth of the parent strain or of the RpoB H526Y mutant in the presence of isonaizid was observed at up to 24 days (Table 4.2; Fig. 4.10; Fig. 4.11). These results indicate that increased *sigE* expression in *M. tuberculosis* is directly proportional to an enhanced ability to grow in the presence of isoniazid. This suggests that SigE may play an integral role in the progression of *M. tuberculosis* from rifampicin mono-resistance to MDR and that this regulation may be effected through differential gene expression.

4.3.4 Molecular characterisation of sigma factor regulators *rseA* and *mprA*

Though the transcriptional regulatory network in *M. tuberculosis* is complex, it is interesting to consider the role of regulators of *sigE* in the observed altered expression of this sigma factor in the isogenic RpoB mutants. SigE expression is regulated by anti-sigma factor RseA, which post-transcriptionally binds to free SigE, sterically inhibiting interaction with the core RNA polymerase (Donà *et al*, 2008). In addition to this anti-sigma factor regulation, *sigE* expression is regulated at the transcriptional level by MprA, the response regulator of the two-component system, *mprAB* (Pang *et al*, 2007; He *et al*, 2006). To determine whether either *rseA* or *mprA* carried nucleotide changes that could account for increased expression of *sigE*, both functional genes were PCR amplified from isogenic mutants carrying RpoB S531L, S531W, H526D and H526Y mutations. Following analysis of the sequencing data, no mutations were identified (Fig. 4.12 & Fig. 4.13). The absence of nucleotide changes in *rseA* and *mprA* suggests that increased expression of *sigE* in RpoB S531L, S531W and H526D mutants is not due to altered regulation of this sigma factor by its anti-sigma factor or by the transcriptional regulator MprA.

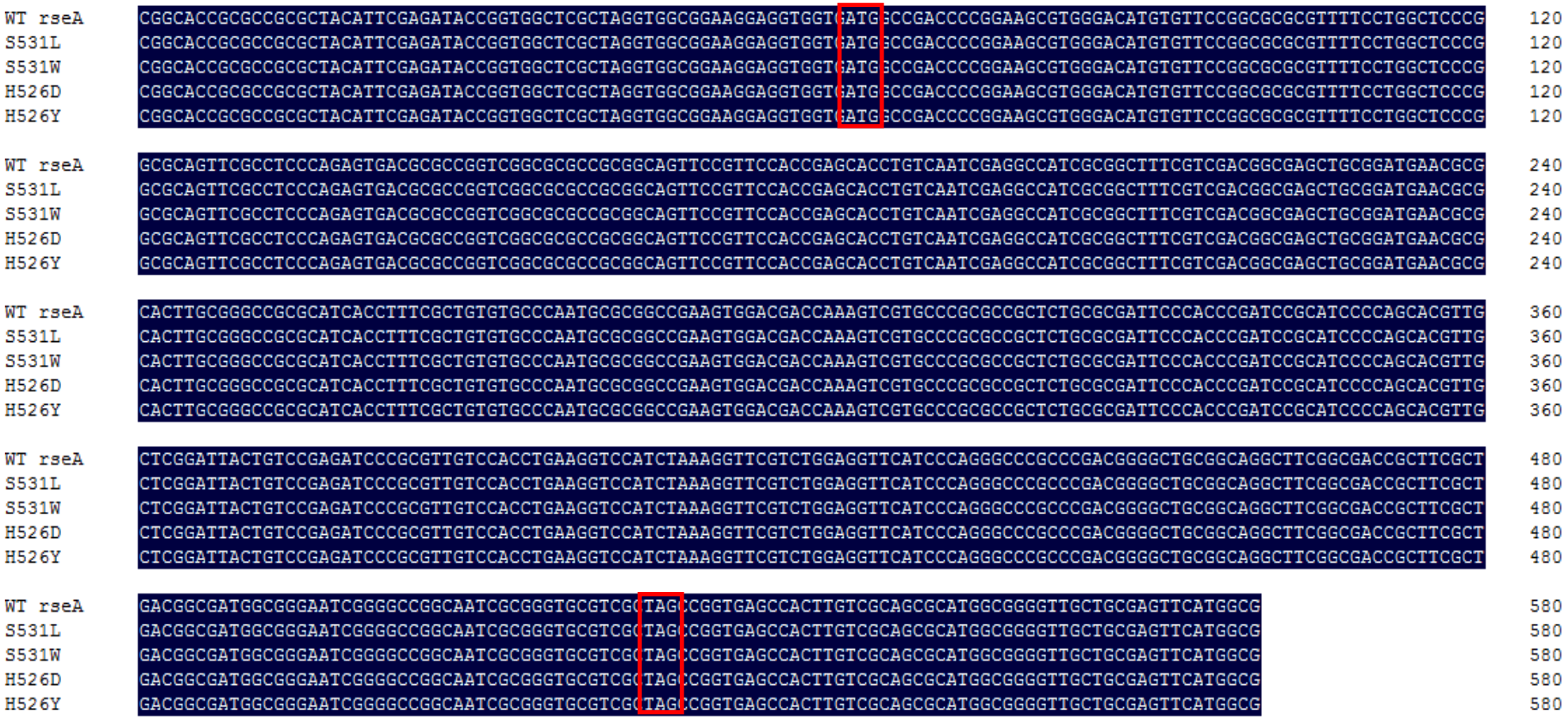

Figure 4.12: Nucleotide sequence alignment of the functional *rseA* from rifampicin-resistant *M. tuberculosis* H37Rv isogenic mutants. Regions shaded in black represent regions of 100% sequence identity. The ATG start codon and TAG stop codons of *rseA* are boxed in red.

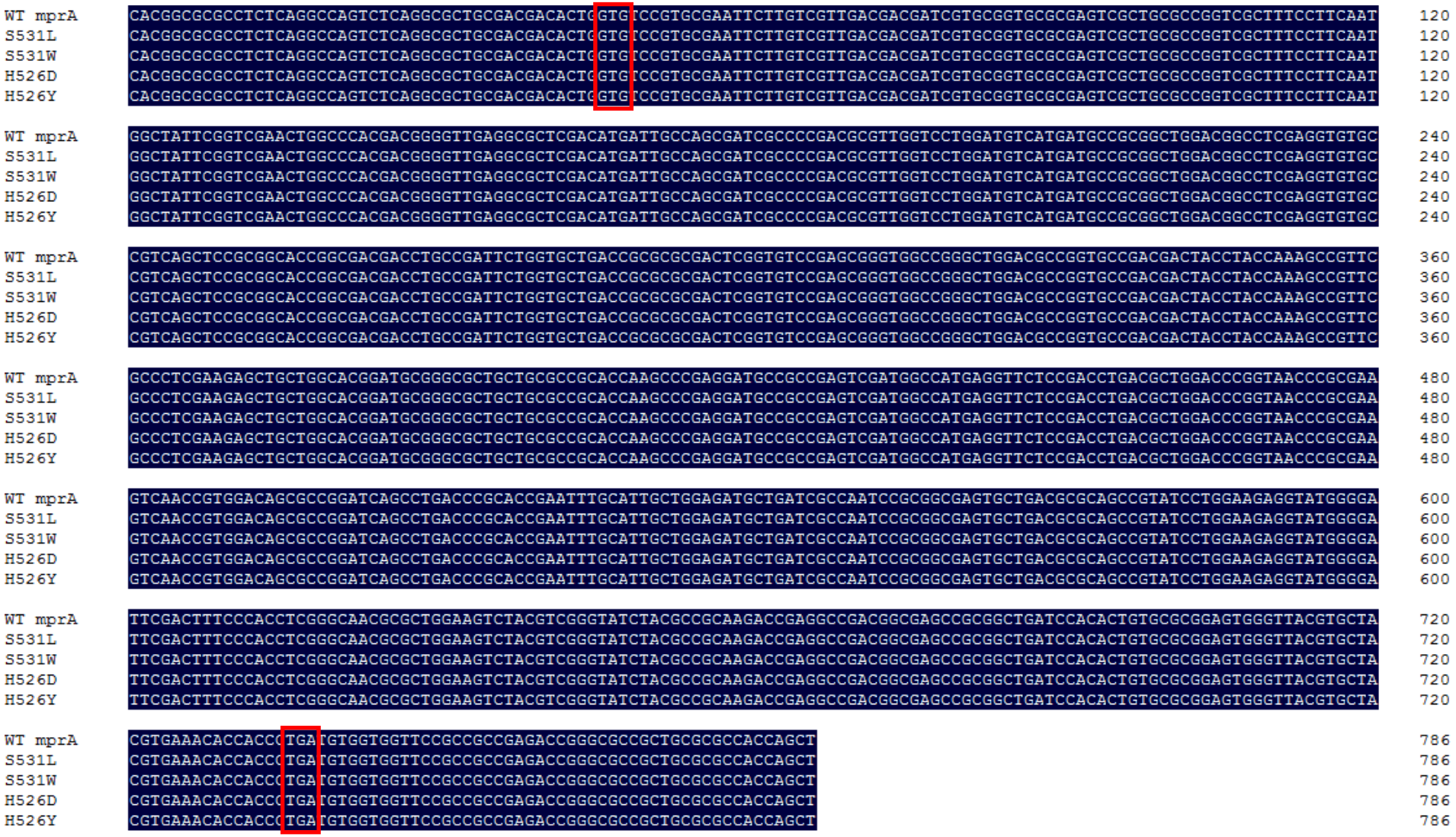

Figure 4.13: Nucleotide sequence alignment of the functional *mprA* from rifampicin-resistant *M. tuberculosis* H37Rv isogenic mutants. Regions shaded in black represent regions of 100% sequence identity. The GTG start codon and TGA stop codons of *mprA* are boxed in red.

4.3.5 Analysis of *sigE* expression in clinical *M. tuberculosis* isolates

To determine whether increased *sigE* expression was associated with the progression of rifampicin mono-resistant clinical *M. tuberculosis* isolates to MDR-TB, the expression of *sigE* in five clinical *M. tuberculosis* isolates with different rifampicin and isoniazid resistance determinants (Table 4.3) was investigated using RT-qPCR (4.2.3). One rifampicin mono-resistant isolate carrying an RpoB S531L mutation was included (Table 4.3). Of the four phenotypically MDR isolates investigated, one carried an RpoB S531L mutation and both KatG S315T and *inhA* C-15T, one carried RpoB S531L and KatG S315T and one carried RpoB H526D and KatG S315T (Table 4.3). The remaining MDR isolate carried RpoB S531L and no known isoniazid resistance marker.

Table 4.3: *M. tuberculosis* clinical isolates used in RT-qPCR assays for the investigation of *sigE* expression.

M. tuberculosis Isolate	Phenotypic Susceptibility	Rifampicin Resistance	Isoniazid Resistance		[a]Fold Increase in *sigE* Expression
		RpoB	KatG	*inhA*	
2148275	RIF^R	S531L	-	-	3.54
2035598	MDR	S531L	S315T	C-15T	4.82
2077418	MDR	S531L	S315T	-	4.42
2187813	MDR	H526D	S315T	-	14.04
1953350	MDR	S531L	-	-	3.83

RIF^R, rifampicin mono-resistant; MDR, multidrug resistant.
[a]Values represent an average of two technical repeats performed on each of three independant RNA extractions from each rifampicin-resistant isogenic mutant, calculated relative to *M. tuberculosis* H37Rv and normalised to 16S rRNA expression.

As with the rifampicin-resistant isogenic *M. tuberculosis* H37Rv mutants, *sigE* expression was increased in the clinical *M. tuberculosis* isolates carrying RpoB S531L, although to a lesser extent (Table 4.3; Table 4.2). Both the MDR isolate with no identified isoniazid resistance determinant and the rifampicin mono-resistant isolate displayed upregulaton of *sigE* with an equivalent increase of 3.83-fold and 3.54-fold, respectively (Table 4.3), which is lower than the 9.03-fold increase in *sigE*

expression observed in the RpoB S531L isogenic mutant (Table 4.2; Fig. 4.11). Similarly, the fold increase in *sigE* expression in the RpoB S531L mutant carrying both KatG S315T and *inhA* C-15T (4.82-fold) and in the RpoB S531L mutant carrying only KatG S315T (4.42-fold) were equivalent. Interestingly, *sigE* expression was increased 14.04-fold in the MDR clinical isolate carrying RpoB H526D (Table 4.3), whilst only a 6.84-fold increase in *sigE* expression was observed in the isogenic mutant carrying this mutation (Table 4.2; Fig. 4.11).

4.3.6 Analysis of the FASII cycle gene expression prior to and following exposure of rifampicin mono-resistant M. tuberculosis H37Rv isogenic mutants to isoniazid

Isoniazid targets the FASII cycle of mycolic acid synthesis and two genes involved in this cycle, *acpM* and *fabD*, are regulated by SigE (Manganelli *et al*, 1999). To determine whether differential expression of either of these genes in association with upregulation of *sigE* was involved in adaption to growth in the presence of isoniazid, RT-qPCR analysis of these genes in rifampicin mono-resistant *M. tuberculosis* isogenic mutants prior to and following isoniazid exposure was carried out. In addition, expression of the two genes most frequently associated with resistance to isoniazid, *katG* and *inhA*, was investigated.

The C_t values obtained for *fabD* from both the parent *M. tuberculosis* H37Rv strain and for each of the isogenic mutants were above 32 cycles, suggesting that *fabD* is not expressed at significant levels in these isolates. Expression of *inhA* and *katG* was equivalent to that of the parent *M. tuberculosis* H37Rv strain in all mutants prior to isoniazid exposure (Table 4.4), suggesting that neither of these genes is regulated by SigE. Interestingly, a 3.94-fold to 5.76-fold increase in *acpM* expression was observed in the RpoB S531L, S531W and H526D mutants (Table 4.4), which corresponds to the mutants that had displayed increased *sigE* expression (Table 4.2), whilst no increase in *acpM* expression was observed in the RpoB H526Y mutant (Table 4.4), which did not display increased *sigE* expression (Table 4.4; Table 4.2). This confirms a role for SigE in the regulation of expression of *acpM*.

Table 4.4: Fold increase in expression of genes involved in the FASII cycle in rifampicin-resistant *M. tuberculosis* isogenic mutants as compared to the parent *M. tuberculosis* H37Rv strain.

	ΔRpoB						
	S531L		S531W		H526D		H526Y
Gene	- INH	+ INH	- INH	+ INH	- INH	+ INH	-INH
sigE	[a]9.03	7.42	7.30	10.35	6.84	19.32	1.87
inhA	1.21	0.90	1.88	1.39	0.99	1.42	0.74
katG	2.15	0.42	2.08	1.76	0.58	3.11	1.86
acpM	3.94	5.62	4.35	8.18	5.76	8.70	1.75

- INH, growth in the absence of isoniazid; +INH, growth in the presence of isoniazid
Expression of *fabD* is not indicated since this gene was not expressed at detectable levels.
[a]Values represent an average of two technical repeats performed on each of three independant RNA extractions from each rifampicin-resistant isogenic mutant, calculated relative to *M. tuberculosis* H37Rv and normalised to 16S rRNA expression.

The expression of *acpM*, *fabD*, *inhA* and *katG*, as well as that of *sigE*, was also investigated in the RpoB S531L, S531W and H526D isogenic mutants following exposure to isoniazid. The lack of expression of *fabD* in the parent *M. tuberculosis* H37Rv strain prevented accurate quantification of the fold increase using the $2^{-\Delta\Delta Ct}$ method, but the ΔC_t values prior to and following exposure to isoniazid indicated an approximate 2 log-fold difference in expression, confirming induced expression of *fabD* following exposure to isoniazid. The presence of isoniazid had no significant effect on the expression of either *inhA* or *katG* in any of the mutants (Table 4.4), indicating that the enhanced ability of rifampicin mono-resistant mutants carrying RpoB S531L, S531W and H526D to grow in the presence of isoniazid was not due to differential expression of either of these genes. This was not unexpected, since MAS-PCR assays for the detection of KatG S315T and *inhA* C-15T mutations in these isolates prior to and following isoniazid exposure identified neither mutation.

Interestingly, *sigE* expression in the RpoB S531L mutant decreased from 9.03-fold to 7.42-fold following exposure to isoniazid (Table 4.4), whilst expression of *sigE* in the RpoB S531W and H526D mutants was further increased to 10.35-fold and 19.32-fold, respectively (Table 4.4). This suggests that whilst *sigE* expression is further induced by isoniazid in the presence of RpoB S531W and H526D mutations, the presence of

an RpoB S531L mutation does not lead to a further increase in *sigE* expression following isoniazid exposure.

Expression of *acpM* was also further increased following exposure to isoniazid, but this effect was seen in the RpoB S531L mutant as well as in the RpoB S531W and H526D mutants (Table 4.4). Since *acpM* expression is regulated by SigE, it follows that increased expression of *sigE* is associated with an increase in *acpM* expression in the RpoB S531W and H526D mutants. It may be that increased *sigE* expression as a result of specific RpoB mutations enhances the progression of rifampicin mono-resistance to MDR through overexpression of *acpM*, the acyl carrier protein that forms the scaffold for the synthesis of mycolic acids.

4.3.7 Analysis of FASII cycle gene expression in *M. tuberculosis* clinical isolates

To investigate the role of differential expression of genes in the FASII cycle in isoniazid resistance in clinical *M. tuberculosis* isolates, RT-qPCR assays were carried out. No significant change in *inhA* expression was observed in the rifampicin mono-resistant isolate or in the two MDR isolates carrying KatG S315T mutations (Table 4.5). The MDR isolate carrying an *inhA* C-15T promoter mutation, however, displayed a 31.34-fold increase in *inhA* expression (Table 4.5), confirming that this mutation does result in upregulation of *inhA*. The MDR isolate with no identified isoniazid resistance determinant displayed a 6.61-fold increase in *inhA* expression (Table 4.5). Sequence analysis of the promoter region of *inhA* in this isolate did not identify mutations such as G-17T or T-8A/C, which have previously been shown to contribute to isoniazid resistance (Hazbon *et al*, 2006), suggesting that upregulation of *inhA* in this isolate may be due to altered expression of a transcriptional regulator.

Expression of *katG* in all clinical isolates carrying RpoB S531L mutations was equivalent to that in *M. tuberculosis* H37Rv. No *katG* expression was observed in the MDR isolate carrying RpoB H526D (Table 4.5) despite the presence of a KatG S315T mutation (Table 4.3). Sequence analysis of the functional *ahpC* identified no mutations that could compensate for the absence of KatG catalase/peroxidise activity, but it may be that *ahpC* expression is upregulated via an alternative mechanism, such as upregulation of a transcriptional activator, in this isolate.

Table 4.5: Analysis of FASII cycle gene expression in clinical *M. tuberculosis* isolates.

M. tuberculosis Isolate	Phenotypic Susceptibility	Rifampicin Resistance	Isoniazid Resistance		[a]Fold Increase in Gene Expression			
		RpoB	KatG	*inhA*	*sigE*	*inhA*	*katG*	*acpM*
2148275	RIF^R	S531L	-	-	3.54	1.71	0.66	8.52
2035598	MDR	S531L	S315T	C-15T	4.82	31.34	0.53	5.25
2077418	MDR	S531L	S315T	-	4.42	0.55	1.48	32.52
2187813	MDR	H526D	S315T	-	14.04	1.64	Not detectable	9.55
1953350	MDR	S531L	-	-	3.83	6.61	1.91	3.74

RIF^R, rifampicin mono-resistant; MDR, multidrug resistant.

[a]Values represent an average of two technical repeats performed on each of three independant RNA extractions from each rifampicin-resistant isogenic mutant, calculated relative to *M. tuberculosis* H37Rv and normalised to 16S rRNA expression.

The expression of *acpM* was increased 3.74-fold to 32.52-fold in the clinical *M. tuberculosis* isolates investigated (Table 4.5), corresponding with increased *sigE* expression in these isolates. An 8.52-fold increase in *acpM* expression was observed in the rifampicin mono-resistant isolate (Table 4.5) carrying RpoB S531L. Since a 3.54-fold increase in *sigE* expression was also observed in this isolate, it may be that this isolate is primed to progress to MDR upon exposure to isoniazid. A 32.52-fold increase in *acpM* expression was observed in the MDR isolate carrying RpoB S531L and KatG S315T resistance determinants (Table 4.5). The duration of isoniazid therapy that the patient infected with this strain would have undergone is unknown, but since isoniazid induces further upregulation of *acpM*, it may be that prolonged exposure of this isolate to isoniazid accounts for the large degree of increased *acpM* expression observed. Interestingly, the RpoB H526D mutant, in which no *katG* expression was observed, displayed a 14.04-fold increase in *sigE* in combination with a 9.55-fold increase in *acpM* expression, both of which are higher than observed in the isogenic mutant carrying RpoB H526D. It may be that the higher levels of expression of *sigE* and *acpM* observed in the clinical isolate are associated with the isoniazid resistant phenotype of this isolate in the absence of *katG* expression.

4.4 Discussion

The regulation of global gene expression in prokaryotes is controlled largely by recognition of specific promoter sequences by different sigma factors (Beaucher *et al*, 2002). The use of alternative sigma factors therefore provides an elegant means of regulating the expression of various subsets of genes in response to environmental stimuli, such as antibiotic pressure. Rifampicin mono-resistance is rarely identified in clinical isolates of *M. tuberculosis*, suggesting that acquisition of resistance to rifampicin is associated with a rapid progression to MDR-TB. Since rifampicin resistance occurs due to mutations in RpoB, and this subunit of RNA polymerase interacts with the sigma factor upon formation of the holoenzyme (Kuznedelov *et al*, 2002; Owens *et al*, 1998), it was hypothesised that RpoB mutations resulting in rifampicin resistance may alter sigma factor binding, thereby regulating expression of specific subsets of genes. That some of these genes may play a role in the acquisition of resistance to isoniazid provides an interesting explanation for the rarity with which rifampicin mono-resistance in observed.

Should an RpoB mutation be associated with progression to isoniazid resistance, it follows that these mutations would confer a growth advantage on the isolates in the presence of isoniazid. The association of different RpoB mutations with ability to grow in the presence of isoniazid was investigated using rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants. As anticipated, the different RpoB mutations were associated with a varying ability to grow in the presence of isoniazid. That the parent *M. tuberculosis* H37Rv strain and the RpoB H526Y mutant were unable to adapt to growth in the presence of isoniazid, whilst isogenic mutants carrying RpoB S531L, S531W and H526D mutations were, suggests that the presence of these latter mutations may facilitate the acquisition of resistance to isoniazid upon exposure to this drug.

Of particular significance is that the association of RpoB S531L with ability to adapt to growth in isoniazid, together with the overrepresentation of this mutation in rifampicin resistant clinical isolates from this region, may account for the low frequency of rifampicin mono-resistance observed in the Cape Town area (Evans *et al*, 2009). Furthermore, that 72.7% of rifampicin mono-resistant clinical isolates investigated carry rifampicin resistance determinants other than RpoB S531L (Evans *et al*, 2009), may indicate that these less frequently identified RpoB mutations are not associated with rapid progression to MDR, resulting in these isolates remaining rifampicin mono-resistant.

This lack of inhibition of growth by isoniazid of the RpoB S531L and S531W mutants may also reflect the minimal fitness cost associated with mutations at this codon (Gagneux *et al*, 2006). However, although mutations at codon 526 of RpoB are associated with a higher fitness cost than those at codon 531, it is expected that the presence of H526D and H526Y mutations would confer an equivalent fitness cost (Gagneux *et al*, 2006). That the RpoB H526D isogenic mutant adapted to growth in isoniazid following an extended lag phase of 2 days, while the H526Y mutant was unable to grow in the presence of isoniazid at up to 24 days, suggests, therefore, that adaption to growth in isoniazid may not be due solely to the acquisition of particular mutations in RpoB, but that these mutations may induce additional adaptive mechanisms.

Differential regulation of expression of sigma factors may contribute to adaption of isolates in the presence of selection, and this may account for the observed fitness cost associated with certain mutations. To determine whether alternative sigma factor usage due to the

acquisition of RpoB S531L, S531W and H526D mutations accounted for the enhanced ability of these mutants to grow in the presence of isoniazid, expression of genes encoding sigma factors was investigated. In the generation of rifampicin-resistant *M. tuberculosis* H37Rv isogenic mutants, it is assumed that only the RpoB mutation resulting in the observed phenotypic resistance to rifampicin is acquired. It cannot, however, be overlooked that compensatory mutations may be acquired in parallel, and that these could have an additional effect on gene expression. It may be that in addition to acquisition of the RpoB S531L mutation, a further mutation was simultaneously acquired that resulted in the 2.92-fold increase in *sigA* expression observed for this mutant. Further investigation of multiple mutants with the same resistance determinants would serve to confirm this. Alternatively, the presence of an RpoB S531L mutation may increase the affinity of the RNA polymerase for SigA, thereby resulting in its upregulation. Since SigA is the primary sigma factor that controls expression of essential housekeeping genes in *M. tuberculosis*, increased expression of *sigA* in the RpoB S531L isogenic mutant may compensate for any adverse metabolic effects imparted by the RpoB S531L mutation. This would account for the minimal fitness cost associated with RpoB S531L (Gagneux *et al*, 2006), and therefore also for the frequency with which this mutation is identified in rifampicin resistant clinical *M. tuberculosis* isolates.

Since *sigC*, *sigF* and *sigL* are involved in virulence (Dainese *et al*, 2006; Karls *et al*, 2006; Geiman *et al*, 2005), their expression may not be required *in vitro*, which would account for the lack of expression of these genes observed in both the parent *M. tuberculosis* H37Rv strain and in the rifampicin resistant isogenic mutants. Similarly, the lack of expression of *sigI in vitro* suggests that this sigma factor may also play a role in virulence in *M. tuberculosis*. Both *sigF* and *sigM* are expressed during stationary phase (Agarwal *et al*, 2007; Michele *et al*, 1999; DeMaio *et al*, 1996), and would therefore not be detected during mid-log phase.

Interestingly, increased *sigE* expression was observed in mutants carrying RpoB S531L, S531W and H526D mutations, which corresponds to the mutants that grew in the presence of isoniazid, whilst expression of *sigE* in the RpoB H526Y mutant that did not grow in the presence of isoniazid was equivalent to that of the parent *M. tuberculosis* H37Rv strain. Furthermore, the RpoB S531L and S531W mutants displayed comparatively higher fold increases in *sigE* expression of 9.03 and 7.30, respectively, compared to the 6.84-fold increase in *sigE* expression seen in the the RpoB H526D mutant. Since the RpoB H526D

mutant took longer to adapt to growth in isoniazid than the RpoB S531L and S531W mutants, this suggests that upregulation of *sigE* is directly proportional to an increased ability to adapt to growth in the presence of isoniazid, suggesting a crucial role for SigE in the progression from rifampicin mono-resistance to MDR-TB in the presence of isoniazid selection.

In *M. tuberculosis* (Manganelli *et al*, 2001) and in *Salmonella* (Muller *et al*, 2009), SigE is posttranscriptionally regulated by anti-sigma factor, RseA, the gene encoding which is located downstream of *sigE* in *M. tuberculosis* (Cole *et al*, 1998). In *Salmonella*, proteolysis of RseA occurs in the presence of unfolded outer membrane proteins, which signifies envelope stress, releasing SigE for association with the core RNA polymerase (Muller *et al*, 2009). Since isoniazid interferes with cell wall synthesis, it may be that exposure of rifampicin mono-resistant *M. tuberculosis* isolates to isoniazid induces a similar mechanism of release of SigE from RseA as in *Salmonella*. That a large number of *M. tuberculosis* sigma factors are autoregulated (Lee *et al*, 2008; Calamita *et al*, 2005; Raman *et al*, 2004), suggests that, following release from RseA, SigE may induce its own expression, accounting for the upregulation of *sigE* in the isogenic mutants that were able to grow in the presence of isoniazid.

In addition to anti-sigma factor mediated SigE regulation, the expression of *sigE* in *M. tuberculosis* is directly regulated by a two-component system, MprAB (Pang *et al*, 2007; He *et al*, 2006), in which MprA is the response regulator and MprB is the histidine kinase (Pang & Howard, 2007). MprA is autoregulated (Haydel & Clark-Curtiss, 2006) and also regulates *sigE* expression by binding directly to the *sigE* promoter (Pang & Howard, 2007). In addition, the *mprAB* operon is itself regulated by SigE in response to SDS stress (Manganelli *et al*, 2001), and several genes involved in fatty acid metabolism are upregulated in the *mprAB* deletion mutant (Pang *et al*, 2007). Sequence analysis of the functional *rseA* and *mprA* genes in the RpoB S531L, S531W and H526D isogenic mutants, however, identified no mutations. This suggests that differential expression of one or both of these genes by a mechanism other than a promoter mutation(s) may account for the observed increase in *sigE* expression. Alternatively, the upregulation of *sigE* observed in these mutants may be mediated by yet another regulatory mechanism, further highlighting the complexity of transcriptional regulation in *M. tuberculosis*.

Since isoniazid targets cell wall synthesis, it is interesting that SigE regulates genes involved in the maintenance of cell wall integrity in *S. coelicolor* (Paget *et al*, 1999), *Salmonella* (Muller *et al*, 2009) and *M. tuberculosis* (Dutta *et al*, 2010; Schnappinger *et al*, 2003; Manganelli *et al*, 2001). SigE regulates expression of *acpM* and *fabD*, two genes involved in the FASII stage of mycolic acid synthesis in *M. tuberculosis* (Manganelli *et al*, 1999), the expression of both of which is induced following exposure to isoniazid (Wilson *et al*, 1999). Since increased *sigE* expression is associated with growth in the presence of isoniazid, and isoniazid targets the FASII cycle, *acpM* and *fabD* expression was investigated in the rifampicin mono-resistant *M. tuberculosis* H37Rv isogenic mutants to determine whether these gene products enable enhanced growth in the presence of isoniazid. Additionally the effect of isoniazid exposure on *sigE* expression was investigated. Expression of *sigE* was further upregulated in the RpoB S531W and H526D mutants following exposure to isoniazid, whilst *sigE* expression in the RpoB S531L mutant decreased from 9.03- to 7.42-fold. It may be that upon exposure to isoniazid, *sigE* expression in the RpoB S531W and H526D mutants is further upregulated in order to overcome the selective pressure imposed by the drug. In the case of the RpoB S531L mutant, however, a further increase in *sigE* expression is not required as the upregulation of *sigA* in this mutant already confers a survival advantage. Whilst no detectable expression of *fabD* was observed in any of the isogenic mutants or the parent *M. tuberculosis* H37Rv strain prior to isoniazid exposure, detectable levels of expression of this gene were observed in the RpoB S531L, S531W and H526D mutants following exposure to isoniazid. Upregulation of *acpM* was observed in the RpoB S531L, S531W and H526D mutants that displayed increased *sigE* expression, whilst no upregulation of *acpM* was seen in the RpoB H526Y mutant, further confirming the SigE-mediated regulation of *acpM*. As with *fabD*, expression of *acpM* was also further increased following exposure to isoniazid in these mutants.

Investigation of the association of different RpoB mutations with increased expression of *sigE* and *acpM* in clinical *M. tuberculosis* isolates produced similar results to those obtained with the isogenic mutants. Whilst increased *sigE* expression was observed in all clinical isolates carrying RpoB S531L, the fold-increase in expression of 3.54- to 4.82-fold was lower than the 9.03-fold increase seen in the RpoB S531L isogenic mutant. Similarly, increased expression of *acpM* was observed in the 5 clinical isolates investigated, but as opposed to *sigE*, the increases observed were higher than those seen in the isogenic mutants. Since the genetic backgrounds of clinical *M. tuberculosis* isolates are likely to be much more diverse

than that of the isogenic mutants, a number of other mutations and regulatory pathways may be altered in clinical isolates, accounting for this difference in expression. Nonetheless, an association between the presence of RpoB S531L and H526D mutations and upregulation of *acpM* as a result of increased *sigE* expression in clinical *M. tuberculosis* isolates was confirmed.

Expression of *sigE* in the clinical MDR *M. tuberculosis* isolates was not associated with the isoniazid resistance determinant present, as no significant differences in *sigE* expression were observed in the 3 MDR isolates carrying RpoB S531L and either KatG S315T, *inhA* C-15T or both mutations. Similarly, *sigE* expression in the rifampicin mono-resistant clinical isolate was equivalent to that of the MDR isolates. This, taken together, suggests that following SigE-mediated acquisition of isoniazid resistance in RpoB S531L mutants, the expression of *sigE* is not further influenced. This is supported by the fact that exposure of the RpoB S531L isogenic mutant to isoniazid resulted in a slight decrease in expression of *sigE*, from 9.03- to 7.42-fold, whilst *sigE* expression in the RpoB S531W and H526D mutants increased significantly following exposure to isoniazid. Since phenotypic DST is carried out in South Africa only upon request of the physician, and this is usually as a result of treatment failure, it is likely that all 5 clinical isolates investigated had been exposed to isoniazid during standardised first-line TB therapy prior to inclusion in this study. This accounts for the fact that *sigE* expression in the clinical *M. tuberculosis* iolates was more comparable to *sigE* expression in the isogenic mutants following exposure to isoniazid than prior to exposure. A much higher 14.04-fold increase in *sigE* expression was observed in the RpoB H526D clinical isolate as compared to the 3.54- to 4.82-fold increases in *sigE* expression in the RpoB S531L clinical isolates. These levels of expression are more comparable to the 19.02-fold and 7.42-fold increase in *sigE* expression observed in the RpoB H526D and S531L isogenic mutants, respectively, following exposure to isoniazid, as compared to 9.03- and 6.84-fold increases observed prior to isoniazid exposure. Whether an *M. tuberculosis* clinical isolate carrying RpoB S531W has increased *sigE* expression as indicated in the RpoB S531W isogenic mutant following exposure to isoniazid remains to be determined. That the fold increase in expression of *sigE* was much lower in the clinical *M. tuberculosis* isolates than in the *M. tuberculosis* H37Rv isogenic mutants following exposure to isoniazid is likely due to the significantly lower concentrations of isoniazid encountered by the clinical isolates within the host.

Whilst the transcriptional regulatory network in *M. tuberculosis* is incredibly complex, the results of this investigation suggest that the presence of different mutations within RpoB of rifampicin resistant *M. tuberculosis* isolates may influence the progression to MDR-TB through altered gene regulation. The mechanism by which specific RpoB mutations lead to increased expression of *sigE* is not known, but it may be that alteration of the structure of the β subunit of RNA polymerase by certain RpoB mutations increases the affinity of the RNA polymerase for SigE, thereby resulting in a global change in gene expression. In particular, RpoB S531L, S531W and H526D mutations appear to directly regulate aspects of the mycolic acid synthesis pathway via upregulation of *sigE* and, thereby, *acpM*. Since upregulation of *inhA*, another component of the FASII cycle, results in resistance to isoniazid through a drug-titration effect, it may be that upregulation of *acpM*, which forms the backbone of the growing meromycolate chain, facilitates continued mycolic acid synthesis even in the presence of isoniazid, thereby contributing to the enhanced ability of these mutants to grow in the presence of this drug.

CHAPTER 5:

General Conclusions

With the increasing emergence of *M. tuberculosis* strains resistant to available antibiotics, the global TB epidemic is of greater concern than ever. South Africa has one of the highest incidences of TB in the world (WHO, 2010), and the limited access to adequate treatment and poor living conditions affecting the majority of the infected population exacerbate the problem. The extremely high rate of HIV infection is an additional confounder, with a current estimated 70% of HIV infected persons in this country co-infected with *M. tuberculosis* (Abdool Karim *et al*, 2009). The time to diagnosis of drug resistant *M. tuberculosis* can be up to 6 weeks, during which time patients return to densely populated communities where the risk of transmission is high. Additionally, since phenotypic DST is usually only performed following treatment failure in South Africa, the time to diagnosis of drug resistance is even longer, further increasing the likelihood of dissemination of drug resistant strains. Rapid molecular assays for the detection of drug resistance in *M. tuberculosis* significantly reduce the time to diagnosis, facilitating more rapid implementation of effective treatment regimens and thereby contributing to reducing the spread of disease. Furthermore, the implementation of effective therapy at the outset of treatment reduces the risk of selecting existing spontaneous resistant mutants within the bacterial population, thereby curbing the emergence of MDR *M. tuberculosis*.

Global evaluations of rapid molecular assays for the detection of antibiotic resistance in *M. tuberculosis* produce varying results. Since up to 95% of rifampicin resistance worldwide can be attributed to mutations at 3 codons of RpoB (Ramaswamy & Musser, 1998), molecular screening for rifampicin resistance is usually highly sensitive (Barnard *et al*, 2008; Hillemann *et al*, 2007). Resistance to other antituberculous drugs, however, is attributed to a larger variety of resistance determinants, making molecular screening for these mutations more complex and less sensitive. In addition, the variation in geographical distribution of specific resistance determinants (Piatek *et al*, 2000) necessitates the characterisation of the proportions of common resistance determinants in a region prior to implementing molecular assays, in order to ensure that adequate sensitivities will be obtained.

The GenoType MTBDR*plus* assay identified rifampicin and isoniazid resistance in this setting with sensitivities of 93.5% and 82.1%, respectively. However, whilst isoniazid resistance was detected in 91.6% of MDR-TB isolates, low sensitivities of 63.4% were obtained for isoniazid mono-resistant isolates. Molecular screening for KatG S315T and *inhA* C-15T isoniazid resistance determinants alone would therefore result in up to 40% of isoniazid mono-resistance going undetected. In such cases, the isoniazid component of treatment regimens would be ineffective, increasing the chance of selecting subpopulations of spontaneous mutants resistant to other drugs in the regimen. The identification of a predominant isoniazid resistance marker in these isolates would facilitate the development of a rapid molecular assay that would be useful in this setting. However, that no known isoniazid resistance markers could be detected in these isolates highlights the need for continuation of phenotypic DST in parallel with molecular assays.

The predominant mechanism of resistance to isoniazid in *M. tuberculosis* is an S315T mutation in KatG (Vilchèze & Jacobs, 2007; Hazbón *et al*, 2006; Ramaswamy & Musser, 1998), and the presence of this mutation is frequently associated with resistance to other antituberculous drugs (Hazbón *et al*, 2006; Ahmad *et al*, 2002; van Soolingen *et al*, 2000). It is likely that the predominant mutations observed in association with resistance to various antibiotics in *M. tuberculosis* are those that confer the least fitness cost, as is the case with rifampicin resistance determinant RpoB S531L (Gagneux *et al*, 2006; Toungoussova *et al*, 2004; Billington *et al*, 1999). Since KatG S315T has minimal effect on the catalase-peroxidase activity of the enzyme (Hazbón *et al*, 2006; Yu *et al*, 2003), it likely confers little fitness cost. In this study, 80.4% of pre-XDR and XDR-TB isolates carry a KatG S315T mutation, further confirming this association. The ability of *M. tuberculosis* isolates to proliferate likely decreases as the stepwise accumulation of resistance determinants confer increased fitness cost. It may therefore be that due to the minimal fitness cost imposed by KatG S315T, isolates carrying this mutation are more readily able to acquire and maintain additional resistance determinants, whilst still retaining their virulence and transmissibility.

Identification of resistance to SLDs in *M. tuberculosis* by phenotypic DST is less reliable and less well characterised than for first-line antibiotics. In addition, laboratories with facilities to perform these tests in South Africa are limited, and are lacking in rural areas where this service is most needed. MAS-PCR assays designed for the detection of GyrA D94G and

A90V and *rrs* A1401G could detect up to 88.9% of XDR-TB cases in the Western Cape. As MAS-PCR assays are rapid, inexpensive to perform and do not require the training of specialised personnel, they are ideal for use in the rural setting. While these MAS-PCR assays may not completely replace phenotypic DST because of other mechanisms of resistance such as efflux (Danilchanka *et al*, 2008; Spies *et al*, 2008; Pasca *et al*, 2004), they can provide an essential initial diagnosis that can inform individualised treatment regimens in patients harbouring lethal, drug-resistant strains of *M. tuberculosis*.

The association of drug resistance with *M. tuberculosis* W-Beijing isolates is well documented (Marais *et al*, 2006; Bifani *et al*, 2002) and it is therefore not surprising that 54.2% of MDR isolates included in this study belong to this lineage. It is noteworthy, however, that 37.5% of these MDR W-Beijing isolates are already either pre-XDR or XDR, and they account for 80.4% of all pre-XDR and XDR isolates identified in this investigation. This suggests that the remaining 62.5% of MDR W-Beijing isolates may be more likely to progress to pre-XDR and, ultimately, XDR-TB, than isolates of other lineages. That the proportion of W-Beijing pre-XDR and XDR isolates increased from 76.9% in 2006 to 95.0% in 2009 supports this observation. Additionally, that the MIRU-VNTR profiles of the pre-XDR and XDR *M. tuberculosis* isolates are not identical, suggests that these isolates acquired their resistance independently and do not represent dissemination of a common pre-XDR or XDR clone. Given the increased virulence and transmissibility associated with W-Beijing isolates (Manca *et* al, 2005; Bifani *et al*, 1996), there may be value in genotyping isolates confirmed as MDR and closely monitoring those patients infected with W-Beijing strains in order to prevent the emergence of additional resistance to SLDs in these isolates.

A large proportion of drug resistance in *M. tuberculosis* isolates can be attributed to the acquisition of specific chromosomal point mutations (Johnson *et al*, 2006, Gillespie, 2002; Billington *et al*, 1999; Ramaswamy & Musser, 1998). However, the emergence of drug resistant strains of *M. tuberculosis* with no identifiable resistance determinants suggests the role of additional mechanisms in the development of resistance. Global gene expression is regulated by sigma factors, which direct transcription of specific subsets of genes following association with the core RNA polymerase through interaction with the β` and β subunits (Beaucher *et al*, 2002; Lesley & Burgess, 1989). Since rifampicin resistance occurs due to mutations in *rpoB* (Riska *et al*, 2000; Ramaswamy & Musser, 1998), which encodes the β subunit, it was hypothesised that mutations resulting in resistance to rifampicin may affect

sigma factor binding, thereby influencing global gene regulation in *M. tuberculosis* and contributing to the development of resistance to other drugs.

Interestingly, investigation of the progression from rifampicin mono-resistance to MDR-TB using isogenic *M. tuberculosis* H37Rv mutants indicated that RpoB S531L, S531W and H526D mutations enhance the ability of *M. tuberculosis* to adapt to growth in the presence of isoniazid, whilst RpoB H526Y does not facilitate enhanced growth in the presence of this drug. Furthermore, RpoB S531L, S531W and H526D mutants displayed upregulation of *sigE*, encoding a sigma factor involved in the maintenance of cell wall integrity in *M. tuberculosis* (Dutta *et al*, 2010; Schnappinger *et al*, 2003; Manganelli *et al*, 2001), whilst the RpoB H526Y mutant did not. The target of isoniazid is the FASII cycle of mycolic acid synthesis (Zhang *et al*, 1992; Takayama *et al*, 1972) and one of the components of this pathway, *acpM*, is regulated by SigE (Manganelli *et al*, 1999). It was therefore hypothesised that SigE-mediated upregualtion of *acpM* may account for the ability of RpoB S531L, S531W and H526D mutants to grow in the presence of isoniazid. The simultaneous upregulation of *acpM* in association with increased *sigE* expression in the rifampicin mono-resistant RpoB S531L, S531W and H526D isogenic mutants, but not in the RpoB H526Y mutant, further supports this hypothesis. Additionally, that exposure of the RpoB S531L, S531W and H526D isogenic mutants to isoniazid induces a further increase in *acpM* expression provides additional evidence that *acpM* may be involved in the progression of rifampicin mono-resistance to MDR-TB. However, whilst an enhanced ability to grow in the presence of isoniazid appears to be associated with upregulation of *sigE*, this effect could be conferred by any of the other genes in the SigE regulon as well as by *acpM*. Whole-genome microarray analysis of these isogenic mutants would identify all genes that are differentially expressed as a result of the upregulation of *sigE*, providing further insight into the role of SigE in the progression from rifampicin mono-resistance to MDR-TB.

The mechanism by which mutation of RpoB results in upregulation of *sigE* is unknown, but it may be that specific mutations, namely S531L, S531W and H526D, result in a higher affinity of the core polymerase for SigE than other mutations, such as H526Y. This would lead to differential regulation of a large number of genes, some of which may be implicated in the development of resistance to isoniazid. The infrequency with which rifampicin mono-resistance is identified, together with the overrepresentation of RpoB S531L mutations in 86.6% of MDR clinical isolates in this study suggests that increased expression of *sigE*

following acquisition of RpoB S531L may have contributed to rapid progression to isoniazid resistance in these isolates. Furthermore, that 54.5% of rifampicin mono-resistant *M. tuberculosis* clinical isolates carried either no identifiable mutations in *rpoB* or mutations that are rarely observed in clinical *M. tuberculosis* isolates, may account for the lack of progression of these isolates to MDR-TB. The 3.54-4.82-fold increase in *sigE* expression oberved in clinical *M. tuberculosis* isolates carrying RpoB S531L mutations supports this hypothesis. Whilst the same holds true for RpoB S531W and H526D mutations, the simultaneous upregulation of *sigA* in association with RpoB S531L may account for the higher incidence of RpoB S531L mutations. Identification of increased *sigA* expression in clinical *M. tuberculosis* isolates would serve to further confirm this.

In conclusion, the results of this study demonstrate that whilst phenotypic DST should not be completely replaced by rapid molecular assays for the detection of drug resistance in *M. tuberculosis*, these assays are invaluable for rapid identification of drug resistance in isolates carrying commonly identified resistance determinants. Characterisation of the predominant mutations associated with antibiotic resistance in *M. tuberculosis* isolates from Groote Schuur Hospital indicates that molecular assays can detect resistance to both first- and second-line drugs with relatively high sensitivities in the case of MDR isolates. Furthermore, the ability of MAS-PCR assays to detect a large proportion of isoniazid, ofloxacin and kanamycin resistance in this setting is of significant importance due to the ease with which these assays can be performed and the reduced cost associated with them. In addition, this study has provided an increased understanding of the role of transcriptional regulation in the progression to antibiotic resistance in *M. tuberculosis*. In particular, the association of increased *sigE*, *acpM* and *sigA* expression with the ability of rifampicin mono-resistant RpoB S531L isogenic mutants to grow in the presence of isoniazid may account for the association of rifampicin resistance with resistance to isoniazid, and hence the rarity of rifampicin mono-resistance.

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Appendix A: *Mycobacterium tuberculosis* isolates from patients at Groote Schuur Hospital, Cape Town, in 2006 and 2009.

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
1574027	2006	RZ	ND	W-B	S531L	S315T	-	-	A1401G
1575943	2006	RZ	ND	W-B	S531L	S315T	-	-	-
1581019	2006	RZ	ND	W-B	S531L	S315T	C-15T	-	A1401G
1591317	2006	RZ	ND	W-B	S531L	-	-	-	-
1604226	2006	RZ	ND	LAM9	S531L	S315T	-	-	-
1615016	2006	RZ	ND	W-B	D516V	S315T	-	D94G	A1401G
1615262	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
1641739	2006	RZ	ND	X3	S531L	S315T	-	-	-
1649314	2006	RZ	ND	LAM4	S531L	S315T	-	-	-
1666020	2006	RZ	ND	T5	S531L	S315T	-	-	-
1667182	2006	RZ	ND	T3	S531L	S315T	-	-	-
1668671	2006	RZ	ND	LAM3	S531L	S315T	-	-	-
1675823	2006	RZ	ND	T1	S531L	-	-	-	-
1675839	2006	RZ	ND	W-B	S531L	S315T	-	-	-
1687190	2006	RZ	ND	W-B	S531L	S315T	C-15T	D94G	A1401G
1704487	2006	RZ	ND	W-B	D516V	S315T	-	-	A1401G
1717310	2006	RZ	ND	X3	S531L	S315T	C-15T	-	A1401G
1723396	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
1730165	2006	RZ	ND	W-B	S531L	S315T	-	-	-
1737685	2006	RZ	ND	W-B	S531L	S315T	-	D94G	A1401G
1745531	2006	RZ	ND	W-B	S531L	S315T	C-15T	-	-
1745818	2006	RZ	ND	EAI1	Δ526	S315T	-	-	-
1752121	2006	RZ	ND	LAM9	Δ531/533	S315T	-	D94G	-
1757523	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
1761096	2006	RZ	ND	W-B	Δ531/533	-	-	-	A1401G
1766109	2006	RZ	ND	LAM3	S531L	-	C-15T	D94G	A1401G

R, rifampicin; Z, isoniazid; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
1766512	2006	RZ	ND	W-B	S531L	S315T	-	-	A1401G
1772738	2006	RZ	ND	LAM3	S531L	S315T	-	-	A1401G
1774234	2006	RZ	ND	X1	S531L	S315T	-	-	-
1778309	2006	RZ	ND	X1	S531L	S315T	-	-	-
1778311	2006	RZ	ND	*T1(P=0.35) X1(P=0.65)	S531L	S315T	-	-	-
1778314	2006	RZ	ND	X1	S531L	S315T	-	-	-
1786691	2006	RZ	ND	W-B	S531L	S315T	-	-	-
1796097	2006	RZ	ND	W-B	S531L	S315T	C-15T	-	A1401G
1841037	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
1842109	2006	RZ	ND	X2	S531L	S315T	-	-	-
1852344	2006	RZ	ND	W-B	S531L	-	C-15T	-	A1401G
1857336	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
1918170	2006	RZ	ND	W-B	S531L	S315T	C-15T	D94G	A1401G
1932914	2006	RZ	ND	W-B	S531L	S315T	C-15T	-	-
1933781	2006	RZ	ND	EAI1	Δ526	S315T	-	-	-
1938151	2006	RZ	ND	LAM3	S531L	S315T	-	D94G	-
1949228	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
1953350	2006	RZ	ND	LAM3	S531L	-	-	-	-
1953765	2006	RZ	ND	W-B	S531L	S315T	-	-	-
1955012	2006	RZ	ND	*T1(P=0.35) X1(P=0.65)	S531L	S315T	-	-	-
1957267	2006	RZ	ND	LAM3	S531L	-	-	-	-
1981629	2006	RZ	ND	W-B	S531L	-	C-15T	-	A1401G
1982956	2006	RZ	ND	X3	S531L	S315T	C-15T	-	A1401G
1986517	2006	RZ	ND	W-B	S531L	S315T	-	-	A1401G
1986724	2006	RZ	ND	LAM3	S531L	S315T	-	-	-
1987257	2006	RZ	ND	W-B	S531L	-	C-15T	-	-

R, rifampicin; Z, isoniazid; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
1998862	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2015741	2006	RZ	ND	W-B	S531L	S315T	-	-	A1401G
2022563	2006	RZ	ND	W-B	S531L	S315T	-	D94G	A1401G
2025037	2006	RZ	ND	T1	Δ516	S315T	-	-	-
2031340	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2035598	2006	RZ	ND	W-B	S531L	S315T	C-15T	-	-
2035599	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2036204	2006	RZ	ND	W-B	S531L	S315T	C-15T	-	A1401G
2054728	2006	RZ	ND	W-B	S531L	S315T	-	-	-
2058791	2006	RZ	ND	X3	S531L	S315T	-	-	-
2058806	2006	RZ	ND	LAM4	S531L	S315T	-	-	-
2058812	2006	RZ	ND	W-B	Hetero	S315T	-	-	-
2059587	2006	RZ	ND	X1	S531L	S315T	-	-	-
2070379	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2070402	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2073933	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2076515	2006	RZ	ND	W-B	-	-	-	-	-
2077418	2006	RZ	ND	H1	S531L	S315T	-	-	-
2078536	2006	RZ	ND	W-B	S531L	-	C-15T	D94G	A1401G
2109261	2006	RZ	ND	W-B	-	-	-	-	A1401G
2121604	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2128144	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2129235	2006	RZ	ND	LAM3	S531L	S315T	-	-	-
2140061	2006	RZ	ND	W-B	S531L	-	C-15T	-	-
2161465	2006	RZ	ND	LAM3	S531L	S315T	-	-	-
2161469	2006	RZ	ND	W-B	S531L	S315T	-	-	A1401G
2175589	2006	RZ	ND	T5	S531L	S315T	-	-	-
2175594	2006	RZ	ND	S	S531L	S315T	-	-	-

R, rifampicin; Z, isoniazid; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
2181974	2006	RZ	ND	W-B	S531L	-	C-15T	-	A1401G
2187813	2006	RZ	ND	W-B	H526D	S315T	-	-	-
1576613	2006	R	ND	LAM3	-	-	-	-	-
1699924	2006	R	ND	LAM3	Δ518	-	-	-	-
1713827	2006	R	ND	H3	-	-	-	D94G	-
1732114	2006	R	ND	LAM3	Δ531/533	-	-	-	-
1772394	2006	R	ND	LAM3	-	S315T	-	-	-
2070407	2006	R	ND	T3	Δ514/515	-	-	-	-
2096822	2006	R	ND	W-B	Δ526	-	-	-	-
2140030	2006	R	ND	LAM3	S531L	-	-	-	-
2140034	2006	R	ND	LAM3	S531L	-	-	-	-
2148275	2006	R	ND	LAM3	S531L	-	-	-	-
2189529	2006	R	ND	LAM3	-	-	-	-	-
1574845	2006	Z	ND	EAI1	Δ526	S315T	-	-	-
1675804	2006	Z	ND	S	-	-	-	-	-
1686065	2006	Z	ND	X3	-	-	-	-	-
1687323	2006	Z	ND	H3	-	S315T	-	-	-
1688883	2006	Z	ND	T4	-	S315T	-	-	-
1693171	2006	Z	ND	X3	-	S315T	-	-	-
1722010	2006	Z	ND	W-B	-	S315T	-	-	-
1724629	2006	Z	ND	W-B	-	-	C-15T	-	-
1737678	2006	Z	ND	X3	-	-	-	-	-
1761073	2006	Z	ND	* T1 (P=0.35) X1 (P=0.65)	-	-	-	-	-
1773315	2006	Z	ND	T3	Δ515/516	S315T	-	-	-
1776699	2006	Z	ND	X3	-	-	-	-	-
1777449	2006	Z	ND	H3	-	S315T	-	-	-
1781603	2006	Z	ND	T1	-	S315T	-	-	-

R, rifampicin; Z, isoniazid; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
1782003	2006	Z	ND	T1	-	-	C-15T	-	-
1808291	2006	Z	ND	S	-	-	-	-	-
1818113	2006	Z	ND	* LAM9 (P=0.34) LAM1 (P=0.66)	-	S315T	-	-	-
1828958	2006	Z	ND	T1	-	-	C-15T	-	-
1829203	2006	Z	ND	* S (P=0.78) T1 (P=0.22)	-	-	-	-	-
1838765	2006	Z	ND	X3	-	-	-	-	-
1849334	2006	Z	ND	T1	-	-	-	-	-
1874465	2006	Z	ND	X3	-	-	-	-	
1908146	2006	Z	ND	W-B	-	-	C-15T	-	A1401G
1912853	2006	Z	ND	LAM3	-	*katG* del	-	-	-
1925664	2006	Z	ND	W-B	-	-	C-15T	-	-
1932912	2006	Z	ND	X3	-	-	-	-	-
1995193	2006	Z	ND	W-B	No TUB	S315T	-	-	-
1999950	2006	Z	ND	W-B	-	-	C-15T (seq)	-	-
2007945	2006	Z	ND	mixed	-	-	hetero	-	-
2016107	2006	Z	ND	T1	-	-	-	-	-
2018953	2006	Z	ND	LAM3	-	S315T	-	-	-
2030137	2006	Z	ND	X3	-	S315T	-	-	-
2088135	2006	Z	ND	X3	-	-	-	-	-
2096166	2006	Z	ND	X3	-	-	-	-	A1401G
2096172	2006	Z	ND	LAM3	Δ515/516	S315T	-	-	-
2100729	2006	Z	ND	W-B	-	-	C-15T	-	-
2140044	2006	Z	ND	W-B	-	-	C-15T	-	-

R, rifampicin; Z, isoniazid; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
2141088	2006	Z	ND	W-B	-	-	C-15T	-	-
2146324	2006	Z	ND	LAM3	-	S315T	-	-	-
2161450	2006	Z	ND	W-B	-	-	C-15T	-	-
2161462	2006	Z	ND	W-B	-	-	C-15T	-	-
1579209	2006	No R	ND	W-B	-	-	-	-	-
1579315	2006	No R	ND	LAM3	-	-	-	-	-
1582801	2006	No R	ND	W-B	-	-	-	-	-
1589906	2006	No R	ND	X3	-	-	-	-	A1401G
1595780	2006	No R	ND	T1	-	-	-	-	-
1603093	2006	No R	ND	T1	-	-	-	-	-
1603297	2006	No R	ND	LAM3	-	-	-	-	A1401G
1607737	2006	No R	ND	LAM3	-	-	-	-	-
1610602	2006	No R	ND	* S (P=0.78) T1 (P=0.22)	-	-	-	-	-
1613293	2006	No R	ND	Family 36	-	-	-	-	-
1623004	2006	No R	ND	Haarlem 1	-	-	-	-	-
1626535	2006	No R	ND	W-B	-	-	-	-	-
1634143	2006	No R	ND	S (P=0.92)	-	-	-	-	-
1659358	2006	No R	ND	T4	-	-	-	-	-
1660666	2006	No R	ND	LAM3	-	-	-	-	-
1669371	2006	No R	ND	W-B	-	-	-	-	-
1683439	2006	No R	ND	LAM9	-	-	-	-	-
1684292	2006	No R	ND	* S (P=0.78) T1 (P=0.22)	-	-	-	-	-
1685738	2006	No R	ND	W-B	-	-	-	-	A1401G

No R, No resistance detected; Z, isoniazid; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
1701951	2006	No R	ND	T1	-	-	-	-	-
1724126	2006	No R	ND	T1	-	-	-	-	-
1736195	2006	No R	ND	W-B	-	-	-	-	-
1740673	2006	No R	ND	S (P=0.93)	-	-	-	-	A1401G
1744429	2006	No R	ND	H37Rv (P=0.98)	-	-	-	-	A1401G
1748870	2006	No R	ND	* S (P=0.78) T1 (P=0.22)	-	-	-	-	A1401G
1749233	2006	No R	ND	LAM3	-	-	-	-	-
1754130	2006	No R	ND	LAM3	-	-	-	-	-
1766503	2006	No R	ND	Mixed	-	-	-	-	-
1770076	2006	No R	ND	* Haarlem3 (P=0.77) T1 (P=0.23)	-	-	-	-	-
1772552	2006	No R	ND	T1	-	-	-	-	-
1790182	2006	No R	ND	T1	-	-	-	-	-
1795904	2006	No R	ND	* Haarlem3 (P=0.77) T1 (P=0.23)	-	-	-	-	-
1798217	2006	No R	ND	Family 36	-	-	-	-	-
1798322	2006	No R	ND	W-B	-	-	-	-	A1401G
1804576	2006	No R	ND	W-B	-	-	-	-	-
1815424	2006	No R	ND	T1	-	-	-	-	-
1829870	2006	No R	ND	T1	-	-	-	-	-
1830879	2006	No R	ND	LAM3	-	-	-	-	-
1845470	2006	No R	ND	* T2 (P=0.82) T1 (P=0.18)	-	-	-	-	A1401G
1873134	2006	No R	ND	W-B	-	-	-	-	A1401G

No R, No resistance detected; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
1887684	2006	No R	ND	W-B	-	-	-	-	A1401G
1890070	2006	No R	ND	W-B	-	-	-	-	A1401G
1895689	2006	No R	ND	T1	-	-	-	-	-
1905052	2006	No R	ND	* Haarlem3 (P=0.77) T1 (P=0.23)	-	-	-	-	A1401G
1923217	2006	No R	ND	W-B	-	-	-	-	-
1923470	2006	No R	ND	LAM3	-	-	-	-	-
1930586	2006	No R	ND	LAM3	-	-	-	-	-
1931379	2006	No R	ND	* S (P=0.78) T1 (P=0.22)	-	-	-	-	A1401G
1932700	2006	No R	ND	LAM3	-	-	-	-	-
1932991	2006	No R	ND	T1	-	-	-	-	-
1932993	2006	No R	ND	LAM3	-	-	-	-	-
1933142	2006	No R	ND	W-B	-	-	-	-	-
1938261	2006	No R	ND	CAS	-	-	-	-	-
1941484	2006	No R	ND	W-B	-	-	-	-	A1401G
1952795	2006	No R	ND	W-B	-	-	-	-	-
1955296	2006	No R	ND	W-B	-	-	-	-	-
1956582	2006	No R	ND	W-B	-	-	-	-	-
1958984	2006	No R	ND	LAM3	-	-	-	-	-
1973015	2006	No R	ND	W-B	-	-	-	-	-
1977754	2006	No R	ND	LAM3	-	-	-	-	A1401G
1993959	2006	No R	ND	W-B	-	-	-	-	-
1999948	2006	No R	ND	T1	-	-	-	-	-
1999953	2006	No R	ND	W-B	-	-	-	-	-
2003165	2006	No R	ND	LAM3	-	-	-	-	A1401G
2010607	2006	No R	ND	T4	-	-	-	-	-

No R, No resistance detected; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
2010674	2006	No R	ND	W-B	-	-	-	-	-
2011466	2006	No R	ND	T1	-	-	-	-	-
2016121	2006	No R	ND	T1	-	-	-	-	-
2022425	2006	No R	ND	W-B	-	-	-	-	-
2022434	2006	No R	ND	LAM8 (P=0.94)	-	-	-	-	-
2032739	2006	No R	ND	* Haarlem3 (P=0.77) T1 (P=0.23)	-	-	-	-	-
2036200	2006	No R	ND	LAM3	-	-	-	-	-
2039748	2006	No R	ND	T1	-	-	-	-	-
2049494	2006	No R	ND	W-B	-	-	-	-	-
2083339	2006	No R	ND	X2	-	-	-	-	-
2116919	2006	No R	ND	LAM3	-	-	-	-	-
2116979	2006	No R	ND	X3	-	-	-	-	A1401G
2121553	2006	No R	ND	* X1 (P=0.65) T1 (P=0.35)	-	-	-	-	-
2123009	2006	No R	ND	W-B	-	-	-	-	-
2123524	2006	No R	ND	T1	-	-	-	-	-
2124429	2006	No R	ND	T4	-	-	-	-	-
2127096	2006	No R	ND	X3	-	-	-	-	-
2132434	2006	No R	ND	X2	-	-	-	-	-
2134232	2006	No R	ND	LAM3	-	-	-	-	-
2134238	2006	No R	ND	W-B	-	-	-	-	-
2141481	2006	No R	ND	* S (P=0.81) T1 (P=0.19)	-	-	-	-	-

No R, No resistance detected; ND, not determined; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
2151646	2006	No R	ND	* Haarlem3 (P=0.77) T1 (P=0.23)	-	-	-	-	-
2161993	2006	No R	ND	W-B	-	-	-	-	-
3274790	2009	RZ	-	X1 (P=65.3) T1 (P=34.6)	ND	-	C-15T		
3396641	2009	RZ	-	W-B	ND	-	C-15T		
3403831	2009	RZ	-	W-B	ND	-	C-15T		
3431130	2009	RZ	-	LAM3	ND	-	-		
3433645	2009	RZ	-	W-B	ND	-	C-15T		
3438835	2009	RZ	-	W-B	ND	S315T	-		
3445412	2009	RZ	OK	W-B	ND	S315T	C-15T		
3457329	2009	RZ	-	LAM9	ND	-	-		
3463728	2009	RZ	-	W-B	ND	S315T	-		
3466368	2009	RZ	-	W-B	ND	-	C-15T		
3468913	2009	RZ	-	W-B	ND	S315T	-		
3470221	2009	RZ	-	W-B	ND	S315T	-		
3472096	2009	RZ	OK	W-B	ND	S315T	-		
3479710	2009	RZ	-	W-B	ND	S315T	-		
3486758	2009	RZ	-	W-B	ND	-	C-15T		
3492810	2009	RZ	OK	W-B	ND	S315T	-		
3495032	2009	RZ	OK	W-B	ND	S315T	C-15T		
3499251	2009	RZ	-	LAM9	ND	S315T	-		
3501319	2009	RZ	-	W-B	ND	-	C-15T		
3510648	2009	RZ	OK	W-B	ND	S315T	C-15T		
3527781	2009	RZ	-	W-B	ND	-	C-15T		
3530096	2009	RZ	-	W-B	ND	-	C-15T		
3534245	2009	RZ	-	W-B	ND	-	C-15T		

No R, No resistance detected; ND, not determined; R, rifampicin; Z, isoniazid; O, ofloxacin; K, kanamycin; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
3540349	2009	RZ	OK	W-B	ND	S315T	-		
3541254	2009	RZ	O	W-B	ND	S315T	C-15T		
3543001	2009	RZ	-	W-B	ND	-	C-15T		
3548886	2009	RZ	-	W-B	ND	-	C-15T		
3549251	2009	RZ	O	LAM9	ND	S315T	-		
3568287	2009	RZ	-	W-B	ND	-	-		
3600852	2009	RZ	-	LAM3	ND	-	C-15T		
3603757	2009	RZ	K	W-B	ND	S315T	-		
3609388	2009	RZ	-	W-B	ND	S315T	-		
3627071	2009	RZ	-	W-B	ND	-	C-15T		
3627078	2009	RZ	K	W-B	ND	S315T	C-15T		
3627458	2009	RZ	-	W-B	ND	-	C-15T		
3628062	2009	RZ	-	X1 (0.68) T1 (0.32)	ND	S315T	-		
3636630	2009	RZ	O	W-B	ND	S315T	-		
3643609	2009	RZ	-	W-B	ND	-	C-15T		
3672812	2009	RZ	-	LAM1 (0.66) LAM9 (0.33)	ND	-	-		
3672816	2009	RZ	-	X3	ND	-	-		
3672818	2009	RZ	-	X3	ND	S315T	-		
3672822	2009	RZ	OK	W-B	ND	S315T	-		
3673034	2009	RZ	-	W-B	ND	S315T	-		
3675540	2009	RZ	-	LAM3	ND	S315T	-		
3693592	2009	RZ	-	W-B	ND	-	C-15T		
3707661	2009	RZ	-	S (0.78) T1 (0.22)	ND	-	-		
3727846	2009	RZ	-	W-B	ND	-	C-15T		
3728591	2009	RZ	-	W-B	ND	-	C-15T		

ND, not determined; R, rifampicin; Z, isoniazid; O, ofloxacin; K, kanamycin; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
3742833	2009	RZ	-	X1 (0.65) T1 (0.35)	ND	-	C-15T		
3774337	2009	RZ	OK	W-B	ND	S315T	-		
3786720	2009	RZ	-	W-B	ND	-	C-15T		
3799787	2009	RZ	-	LAM9	ND	-	C-15T		
3812272	2009	RZ	-	T4	ND	-	-		
3815930	2009	RZ	-	W-B	ND	-	C-15T		
3830756	2009	RZ	-	W-B	ND	-	C-15T		
3850483	2009	RZ	-	LAM3	ND	S315T	-		
3850495	2009	RZ	-	X3	ND	S315T	-		
3850531	2009	RZ	-	X3	ND	S315T	-		
3853072	2009	RZ	-	W-B	ND	-	C-15T		
3876284	2009	RZ	K	W-B	ND	S315T	C-15T		
3883261	2009	RZ	-	W-B	ND	-	C-15T		
3892439	2009	RZ	-	T1	ND	-	-		
3901555	2009	RZ	-	T4	ND	S315T	-		
3906872	2009	RZ	-	X1 (0.65) T1 (0.35)	ND	S315T	-		
3922645	2009	RZ	-	W-B	ND	-	C-15T		
3922822	2009	RZ	-	W-B	ND	S315T	C-15T		
3936659	2009	RZ	-	W-B	ND	-	C-15T		
3961019	2009	RZ	-	W-B	ND	-	C-15T		
3981479	2009	RZ	-	W-B	ND	-	C-15T		
4009081	2009	RZ	-	W-B	ND	-	C-15T		
4021867	2009	RZ	-	X3	ND	-	-		
4050595	2009	RZ	-	W-B	ND	S315T	-		
4079310	2009	RZ	-	CAS	ND	-	-		
4093471	2009	RZ	-	X3	ND	-	-		

ND, not determined; R, rifampicin; Z, isoniazid; O, ofloxacin; K, kanamycin; W-B, W-Beijing

M. tuberculosis isolate	Year of Isolation	First-line Phenotypic Resistance Profile	Second-line Phenotypic Resistance Profile	Spoligotype	RpoB	KatG	*inhA*	GyrA	*rrs*
4102922	2009	RZ	-	EAI5 (0.95)	ND	-	-		
4104285	2009	RZ	OK	W-B	ND	S315T	-		
4105247	2009	RZ	-	W-B	ND	S315T	C-15T		
4109544	2009	RZ	K	W-B	ND	S315T	C-15T		
4119646	2009	RZ	-	LAM3	ND	-	-		
4129268	2009	RZ	-	W-B	ND	-	C-15T		
4151468	2009	RZ	-	W-B	ND	-	C-15T		
4156958	2009	RZ	-	W-B	ND	S315T	C-15T		
4175521	2009	RZ	-	W-B	ND	-	-		
4175540	2009	RZ	-	T1	ND	S315T	-		
4181315	2009	RZ	-	T1	ND	S315T	-		
4198711	2009	RZ	O	W-B	ND	S315T	-		
4209765	2009	RZ	-	W-B	ND	-	C-15T		
4210549	2009	RZ	-	W-B	ND	-	C-15T		
4218235	2009	RZ	-	Family 33	ND	-	C-15T		

ND, not determined; R, rifampicin; Z, isoniazid; O, ofloxacin; K, kanamycin; W-B, W-Beijing

Appendix B: Media

Middlebrook 7H9 broth:

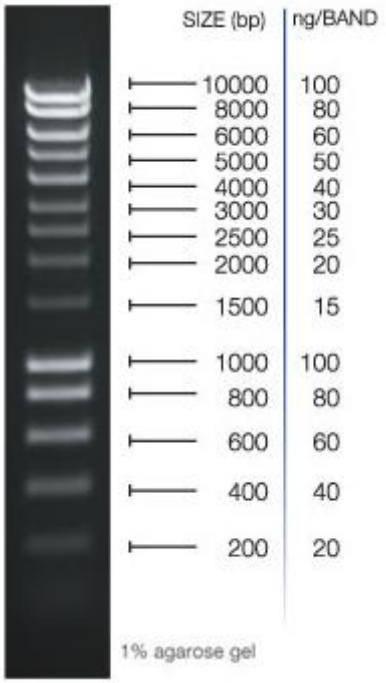
Middlebrook broth powder	4.7g
Tween 80 (10%)	5ml
ADC	100ml
dH_2O to 1 litre.	

Middlebrook 7H11 agar:

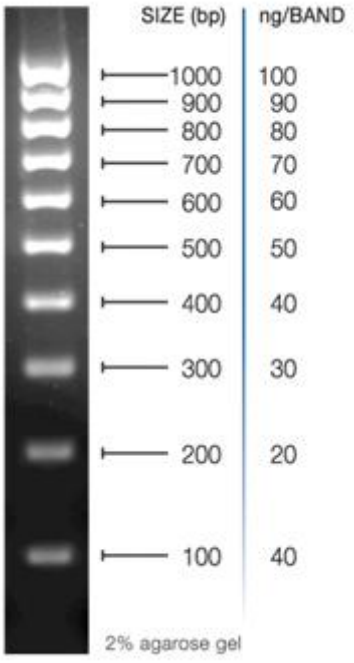
Middlebrook agar powder	19g
Glycerol	5ml
OADC	100ml
dH_2O to 1 litre.	

Appendix C: Molecular Weight Markers

C1: Hyperladder I [Bioline]



C2: Hyperladder IV [Bioline]



Appendix D: MIRU-VNTR allele profiles of *M. tuberculosis* isolates from patients at Groote Schuur Hospital.

M. tuberculosis Isolate	Year	Phenotypic DST	Spoligo Profile	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
2189529	2006	R	LAM3	2	2	4	3	2	6	1	5	3	3	2	3
1732114	2006	R	LAM3	2	2	4	3	2	6	1	5	3	3	2	3
2140030	2006	R	LAM3	2	2	3	3	2	4	1	5	3	3	2	3
1699924	2006	R	LAM3	2	2	4	3	2	6	1	5	3	3	2	3
1772394	2006	R	LAM3	2	2	4	3	2	6	1	5	3	3	2	3
1576613	2006	R	LAM3	2	2	4	3	2	6	1	5	3	3	2	3
2140034	2006	R	LAM3	2	2	3	3	2	6	1	5	3	3	2	3
2148275	2006	R	LAM3	2	2	3	3	2	6	1	5	3	3	2	3
1686065	2006	Z	X3	2	2	4	3	2	5	1	5	3	3	2	4
1737678	2006	Z	X3	2	2	4	2	2	5	1	5	3	3	2	5
1776699	2006	Z	X3	2	2	4	3	2	5	1	5	1	3	2	4
1838765	2006	Z	X3	2	2	4	3	2	5	1	5	3	3	2	4
1874465	2006	Z	X3	2	2	4	3	2	5	1	5	3	3	2	4
1932912	2006	Z	X3	2	2	4	3	2	5	1	5	3	2	2	4
2096166	2006	Z	X3	2	2	4	3	2	5	1	4	3	3	2	4
2088135	2006	Z	X3	2	2	3	3	2	5	1	4	3	3	2	4
1575943	2006	RZ	W-B	2	0	3	3	2	5	1	7	3	5	3	3
1591317	2006	RZ	W-B	2	2	3	3	3	5	1	7	3	5	3	3
1615262	2006	RZ	W-B	2	2	3	3	2	5	1	8	3	5	3	3
1675839	2006	RZ	W-B	2	0	3	3	2	5	1	7	3	5	3	3
1723396	2006	RZ	W-B	2	2	3	3	2	5	1	8	3	5	3	3
1730165	2006	RZ	W-B	2	2	3	3	2	5	1	6	3	5	3	1
1745531	2006	RZ	W-B	2	0	3	3	2	5	1	7	3	5	3	3
1757523	2006	RZ	W-B	2	2	3	3	2	5	1	8	3	5	4	3

R, rifampicin; Z, isoniazid; W-B, W-Beijing

M. tuberculosis Isolate	Year	Phenotypic DST	Spoligo Profile	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
1786691	2006	RZ	W-B	2	0	3	3	2	5	1	7	3	5	4	3
1841037	2006	RZ	W-B	2	2	3	3	2	5	3	8	3	6	4	3
1857336	2006	RZ	W-B	2	2	3	3	3	5	1	8	3	5	3	3
1932914	2006	RZ	W-B	2	0	3	3	2	5	1	7	3	5	4	3
1949228	2006	RZ	W-B	2	2	3	3	3	5	1	8	3	5	3	3
1953765	2006	RZ	W-B	2	2	3	3	2	5	1	6	3	5	4	1
1987257	2006	RZ	W-B	2	2	4	4	2	5	1	8	3	6	3	3
1998862	2006	RZ	W-B	2	3	3	3	3	5	1	8	3	5	3	3
2031340	2006	RZ	W-B	2	2	3	3	2	5	1	7	3	5	3	3
2035598	2006	RZ	W-B	2	2	3	3	2	5	1	7	3	5	3	3
2035599	2006	RZ	W-B	2	1	3	3	2	5	1	8	3	5	3	3
2054728	2006	RZ	W-B	2	2	3	3	2	5	1	6	3	5	3	1
2058812	2006	RZ	W-B	2	2	3	3	2	5	1	7	3	5	3	3
2070379	2006	RZ	W-B	2	2	2	3	2	5	1	7	4	5	4	3
2070402	2006	RZ	W-B	2	2	4	3	2	5	1	8	3	5	4	3
2073933	2006	RZ	W-B	2	2	3	3	2	5	1	8	3	5	3	3
2076515	2006	RZ	W-B	2	2	2	3	3	5	1	7	3	5	4	3
2121604	2006	RZ	W-B	2	2	3	3	2	5	1	8	3	5	3	3
2128144	2006	RZ	W-B	2	2	3	3	2	5	1	8	3	5	3	3
2140061	2006	RZ	W-B	2	2	4	3	2	5	1	8	3	5	3	3
2187813	2006	RZ	W-B	2	2	3	3	2	5	3	7	3	5	3	3
1752121	2006	RZO	LAM9	2	2	3	3	2	5	1	5	3	3	2	3
1938151	2006	RZO	LAM3	2	2	3	3	2	6	1	5	3	3	2	3
3541254	2009	RZO	W-B	2	0	3	3	2	5	1	7	3	5	3	3
3549251	2009	RZO	LAM9	1	2	4	3	2	6	1	5	3	5	2	4
3636630	2009	RZO	W-B	2	0	3	3	2	5	1	7	3	5	3	3
4151468	2009	RZO	W-B	2	2	3	3	2	5	1	8	3	5	2	3
4198711	2009	RZO	W-B	2	0	3	3	2	5	1	7	3	5	0	3

R, rifampicin; Z, isoniazid; O, ofloxacin; W-B, W-Beijing

M. tuberculosis Isolate	Year	Phenotypic DST	Spoligo Profile	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
1574027	2006	RZK	W-B	2	0	3	3	2	5	1	7	3	5	4	3
1581019	2006	RZK	W-B	2	2	3	3	2	5	1	6	3	5	3	1
1704487	2006	RZK	W-B	2	2	3	3	2	5	1	6	3	5	3	1
1717130	2006	RZK	X3	2	2	3	3	2	5	1	5	3	5	2	4
1761096	2006	RZK	W-B	2	2	2	3	2	5	1	7	3	5	4	3
1766512	2006	RZK	W-B	2	0	3	3	2	5	1	6	3	5	3	3
1772738	2006	RZK	LAM3	2	2	3	3	2	6	1	5	3	3	2	3
1796097	2006	RZK	W-B	2	2	3	3	2	5	1	6	3	4	3	1
1852344	2006	RZK	W-B	2	2	3	3	2	5	1	8	3	5	3	3
1981629	2006	RZK	W-B	2	2	3	3	2	5	1	8	3	5	4	3
1982956	2006	RZK	X3	2	2	4	3	2	5	1	5	3	5	2	4
1986517	2006	RZK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
2015741	2006	RZK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
2036204	2006	RZK	W-B	2	3	4	3	2	5	1	6	3	5	4	1
2109261	2006	RZK	W-B	2	2	3	3	2	5	1	7	3	5	3	3
2161469	2006	RZK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
2181974	2006	RZK	W-B	2	2	3	3	2	5	1	8	3	5	3	3
3603757	2009	RZK	W-B	2	2	3	3	2	5	1	6	3	5	3	1
3627078	2009	RZK	W-B	2	2	3	3	2	5	1	6	3	5	2	1
3876284	2009	RZK	W-B	2	2	3	3	2	5	1	7	3	5	3	3
3922822	2009	RZK	W-B	2	2	3	3	2	5	1	8	3	5	2	3
4109544	2009	RZK	W-B	2	2	3	3	2	5	1	6	2	5	2	1
4210549	2009	RZK	W-B	2	2	3	3	2	5	1	8	3	5	2	3
1615016	2006	RZOK	W-B	2	2	3	3	2	5	1	7	3	5	3	1
1687190	2006	RZOK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
1737685	2006	RZOK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
1766109	2006	RZOK	LAM3	2	2	3	3	2	5	1	8	3	6	3	3
1918170	2006	RZOK	W-B	2	2	3	3	2	5	1	6	3	5	4	3

R, rifampicin; Z, isoniazid; O, ofloxacin; K, kanamycin; W-B, W-Beijing

M. tuberculosis Isolate	Year	Phenotypic DST	Spoligo Profile	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
2022563	2006	RZOK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
2078536	2006	RZOK	W-B	2	3	3	3	2	5	1	8	3	5	3	3
3445412	2009	RZOK	W-B	2	2	3	3	2	5	1	6	3	5	2	1
3472096	2009	RZOK	W-B	2	2	3	3	2	5	1	7	3	5	3	3
3492810	2009	RZOK	W-B	2	2	3	3	2	5	1	7	3	6	3	3
3495032	2009	RZOK	W-B	2	2	3	3	2	5	1	6	3	5	2	1
3510648	2009	RZOK	W-B	2	2	3	3	2	5	1	6	3	6	3	1
3540349	2009	RZOK	W-B	2	2	3	3	2	5	1	6	3	5	2	1
3672822	2009	RZOK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
3774337	2009	RZOK	W-B	2	0	3	3	2	5	1	7	3	5	3	3
4104285	2009	RZOK	W-B	2	2	3	3	2	5	1	6	2	5	2	1
1890070	2006	K	W-B	2	2	2	3	2	5	1	7	3	5	4	3
1685738	2006	K	W-B	2	2	2	3	2	5	1	7	4	5	4	3
1798322	2006	K	W-B	2	2	2	3	2	5	1	7	3	5	4	3
1873134	2006	K	W-B	2	2	2	3	2	5	1	7	2	5	2	3
1887684	2006	K	W-B	2	2	2	3	2	5	1	7	4	5	4	3
1941484	2006	K	W-B	2	2	2	3	2	5	1	7	3	5	4	3
1955296	2006	No R	W-B	2	2	2	3	2	5	1	7	4	5	4	3
1956582	2006	No R	W-B	2	2	3	3	2	5	1	7	3	5	4	3
2022425	2006	No R	W-B	2	2	2	3	2	5	1	7	4	5	4	3
1993959	2006	No R	W-B	2	2	2	3	2	5	1	7	3	5	4	3
1999959	2006	No R	W-B	2	2	2	3	2	5	1	7	3	5	4	3
2010674	2006	No R	W-B	2	2	3	3	2	5	1	7	3	5	3	4
2049494	2006	No R	W-B	2	2	3	3	2	5	1	7	3	5	4	3
2134238	2006	No R	W-B	2	3	2	3	2	5	1	7	3	5	4	3
2123009	2006	No R	W-B	2	2	2	3	2	5	1	7	4	5	4	3
2161993	2006	No R	W-B	2	2	2	3	2	5	1	7	4	5	4	3
1933142	2006	No R	W-B	2	2	3	3	2	5	1	7	3	5	4	3

R, rifampicin; Z, isoniazid; O, ofloxacin; K, kanamycin; No R, no resistance detected; W-B, W-Beijing

M. tuberculosis Isolate	Year	Phenotypic DST	Spoligo Profile	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
1579209	2006	No R	W-B	2	2	3	3	2	5	1	7	2	5	2	3
1582801	2006	No R	W-B	2	2	3	3	2	5	1	7	3	5	4	3
1669371	2006	No R	W-B	2	2	3	3	2	5	1	7	3	5	4	3
1736195	2006	No R	W-B	2	2	2	3	2	5	1	7	4	5	4	3
1804576	2006	No R	W-B	2	2	3	3	2	5	1	7	2	5	2	3
1952795	2006	No R	W-B	2	2	3	3	2	5	1	7	3	5	3	3
1973015	2006	No R	W-B	2	2	3	2	2	5	1	7	3	5	3	3

No R, no resistance detected; W-B, W-Beijing