

Regulatory responses to rifampicin exposure in *Mycobacterium tuberculosis*

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Declaration

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Summary

There is increasing evidence that the mechanisms of drug resistance in *Mycobacterium tuberculosis* are not limited to mutations in the known drug resistance causing genes. Classically a mutation in one gene confers resistance to a drug, for example a mutation in the *rpoB* gene confers rifampicin resistance. However varying levels of resistance and fitness observed, as well as the lack of mutations in some clinically resistant isolates has guided researchers to focus on alternate genetic and physiological factors influencing drug resistance in *M. tuberculosis*. Whole genome sequencing (WGS) has previously identified polymorphisms in the *rpoC* gene of *M. tuberculosis* which were later confirmed to compensate for the loss of fitness occurring with the acquisition of *rpoB* mutations. Similarly, numerous mutations have been identified to be ancillary to drug resistance. Additionally, WGS has been used to investigate the evolution of drug resistance in *M. tuberculosis*. In addition to genetic factors, the activity of energy dependent efflux pumps has been associated with drug resistance and has been demonstrated to provide low levels of drug resistance to anti-TB drugs to allow for the development of drug resistance causing mutations. This study aimed to further investigate both genetic and efflux based mechanisms of resistance in *M. tuberculosis*, as well the response of *M. tuberculosis* to rifampicin exposure. It was therefore hypothesised that *M. tuberculosis* acquires additional genomic mutations during the evolution of rifampicin mono-resistance to multidrug resistant (MDR). In addition this study hypothesised that efflux pump activity contributes to the level of rifampicin resistance in *M. tuberculosis*, and this activity is dependent on the presence of specific *rpoB* mutations. Lastly, this study hypothesised that rifampicin exposure induces the expression of energy metabolism genes and efflux pumps in *M. tuberculosis*.

The Department of Biomedical Sciences strain bank was interrogated and a total of 6 clinical isolates originating from 3 patients representing evolution from rifampicin mono-resistance to MDR were selected for WGS analysis. WGS identified novel genetic variants occurring during the evolution of drug resistance. Numerous variants were only present in a proportion of the population and were observed to change (emerge or disappear) during the course of treatment. This heterogeneity suggests that *M. tuberculosis* populations are dynamic during the evolution of drug resistance, accumulating numerous genetic changes.

Experiments investigating the role of efflux pump activity in rifampicin resistance demonstrated that efflux pump activity differs in *M. tuberculosis* isolates with an *rpoB* Ser531Leu mutation compared to those with an *rpoB* His526Leu mutation. In addition, the efficacy of verapamil as an efflux pump inhibitor differed between these isolates. This finding may have implications for the consideration of the inclusion of efflux pump inhibitors in treatment regimens.

Lastly, this study is the first to identify *mmpL5* expression in response to rifampicin exposure; however the consequence of this association remains unknown. An additional finding of the investigation into the response of *M. tuberculosis* to rifampicin exposure is the identification of a proposed transcriptional regulator responding to rifampicin exposure. Together these findings demonstrate the numerous genetic and physiological mechanisms contributing to drug resistance in *M. tuberculosis*.

Opsomming

Toenemende bewyse stel voor dat meganismes van weerstandigheid in *Mycobacterium tuberculosis* is nie beperk tot slegs mutasies in gene wat bekend is vir die oorsaak van middelweerstandigheid nie. Dit word algemeen aanvaar dat 'n mutasie in een geen weerstandigheid teen 'n middel veroorsaak, byvoorbeeld 'n mutasie in die *rpoB* geen veroorsaak rifampisien weerstandigheid. Daar is egter al waargeneem dat verskillende vlakke van weerstandigheid en fiksheid voorkom, asook die afwesigheid van mutasies in sommige kliniese middelweerstandige isolate. Dit het navorsers gelei om te fokus op alternatiewe genetiese en fisiologiese faktore wat weerstandigheid in *M. tuberculosis* kan beïnvloed. Mutasies in *rpoC* is voorheen geïdentifiseer deur heelgenoom volgorde bepaling en is later bevestig om vergoedend te wees vir die afname in fiksheid as gevolg van die verwerwing van *rpoB* mutasies. Soortgelyk is dit ook gevind dat verskeie mutasies aanvullend is tot middelweerstandigheid. Verder is heelgenoom volgorde bepaling ook gebruik om die evolusie van middelweerstandigheid in *M. tuberculosis* te bestudeer. Ten spyte van genetiese faktore, is daar ook gevind dat die aktiwiteit van energie afhanklike uitvloeipompe geassosieer is met middelweerstandigheid en daar is gewys dat dit 'n verlaagde vlak van weerstandigheid voorsien sodat middelweerstandige mutasies kan ontwikkel. Hierdie studie stel ten doel om beide die genetiese en uitvloeipompe meganismes van weerstandigheid in *M. tuberculosis* verder te ondersoek, asook die reaksie van *M. tuberculosis* op rifampisien blootstelling. Hierdie studie het uit drie aparte navorsing vrae bestaan om die volgende hipoteses aantespreek: *M. tuberculosis* verkry addisionele genomiese mutasies gedurende die evolusie van rifampisien enkelweerstandigheid na meervoudige middelweerstandigheid (MDR); dat uitvloeipompe bydra tot die vlakke van rifampisien weerstandigheid in *M. tuberculosis* en dat hierdie aktiwiteit afhanklik is van spesifieke *rpoB* mutasies; dat die blootstelling van *M. tuberculosis* aan rifampisien lei tot die verhoging in geen uitdrukking van energie metabolisme en uitvloeipompe.

Die departement van Biomediese Wetenskappe se isolaat versameling is ondersoek en 'n totaal van ses kliniese isolate afkomstig van drie pasiënte wat die evolusie van rifampisien enkelweerstandigheid na MDR voorstel is met heelgenoom volgorde bepaling ondersoek. Heelgenoom volgorde bepaling het nuwe genetiese variante geïdentifiseer tydens die evolusie van weerstandigheid. Verskeie variante was slegs teenwoordig in 'n gedeelte van die populasie

wat verander het (verskyn of verdwyn) tydens die behandelingskursus. Hierdie heterogenetiese eienskappe stel voor dat *M. tuberculosis* populasies dinamies is gedurende die evolusie van middel weerstandigheid deur die akkumulering van verskeie genetiese verandering.

Eksperimente wat die rol van uitvloei pomp aktiwiteit in rifampisien weerstandigheid ondersoek het gewys dat uitvloei pomp aktiwiteit verskil tussen *M. tuberculosis* isolate met *rpoB* Ser531Leu en *rpoB* His526Leu mutasies. Verder was die effektiwiteit van verapamil as uitvloei pomp inhibeerder verskillend tussen hierdie isolate. Hierdie bevinding mag dalk implikasies inhou vir die oorweging om uitvloei pomp inhibeerders intesluit by behandelingskursusse.

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List of abbreviations

Δ pH	Transmembrane Proton Gradient
$\Delta\phi$	Membrane Potential
$^{\circ}$ C	Degree Celsius
ABC	ATP Binding Cassette
ADC	Albumin Dextrose Catalase
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
BSA	Bovine Serum Albumin
BWA	Burrows Wheeler Alignment
CCCP	Carbonyl Cyanide m-chlorophenyl Hydrazone
Cfu	Colony Forming Units
CIP	Ciprofloxacin
CPGR	Centre for Proteomic and Genomic Research
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSA	n-Decanosulfonylacetamide
DST	Drug Susceptibility Testing
dATP	Deoxyadenosine Triphosphate
dNTP	Deoxyribonucleotide Triphosphate
EAI	East Africa Indian
EBA	Early Bactericidal Assay
EDTA	Ethylene Diamine Tetra Acetic Acid
ETC	Electron Transport Chain
FQ	Fluoroquinolone
g	Grams
GatK	Genome Analysis Toolkit
GITC	Guanidium Thiocyanate
GU	Growth unit
INH	Isoniazid
InDel	Insertion Deletion

KAN	Kanamycin
kb	Kilo bases
LAM	Latin-American and Mediterranean
LCC	Low Copy Clade
LJ	Lowenstein Jensen
M	Molar
MATE	Multidrug and Toxic Compound Extrusion
MCL	Maximum Composite Likelihood
MDR	Multi-drug Resistant
MFS	Multi facilitator Superfamily
MGIT	Mycobacterial Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MK	Menaquinone
MKH ₂	Menaquinol
ml	Millilitres
µl	Microlitres
µM	Micro Molar
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MXF	Moxifloxacin
NDH	NADH Dehydrogenase
NOX-2	NADPH Oxidase
ng	Nanograms
NGSF	Next Generation Sequencing Facility
NRP	Non-replicating Persistence
nsSNP	Non Synonymous Single Nucleotide Polymorphism
OADC	Oleic Acid Albumin Dextrose Catalase
OD	Optical Density
OFL	Ofloxacin
PCR	Polymerase Chain Reaction
REST	Relative Expression Software Tool
RFLP	Restriction Fragment Length Polymorphism

RT	Reverse Transcriptase
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
RIF	Rifampicin
PMF	Proton Motive Force
PZA	Pyrazinamide
rpm	Revolutions per Minute
RNA	Ribonucleic Acid
RND	Resistant/Nodulation/Division
RRDR	Rifampicin Resistance Determining Region
rRNA	Ribosomal Ribonucleic Acid
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SAM	Sequence Alignment/Mapper
SDS	Sodium Dodecyl Sulphate
SDH	Succinate Dehydrogenase
SMR	Small Multidrug Resistance
SNP	Single Nucleotide Polymorphism
TB	Tuberculosis
TE	Tris/EDTA
TET	Tetracycline
TDR	Totally Drug Resistant
THZ	Thioridazine
T _m	Melting Temperature
Tris	Trishydroxymethylaminomethane
U	Units
V	Volt
WHO	World Health Organisation
WGS	Whole Genome Sequencing
XDR	Extensively Drug Resistant
ZN	Ziehl-Neelsen

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CHAPTER 1

General introduction

South Africa is ranked by the World Health Organisation (WHO) as having one of the highest incidences of Tuberculosis (TB) globally, with a reported incidence of 1003 cases per 100 000 population in South Africa in 2012 (1). The emergence of drug resistance is an increasing problem which negatively impacts on the TB control program. The WHO 2013 Global Tuberculosis Control Report estimates that in South Africa there were approximately 4600 new cases of multi-drug resistant (MDR) TB (resistant to at least isoniazid and rifampicin) and 3500 retreatment cases of MDR (2), indicating a high rate of treatment failure. Treatment success rates are below 50% for MDR-TB with poorer outcomes for extensively drug resistant (XDR)-TB (75% mortality over a 5 year period) (2). Conventionally, drug resistance in *Mycobacterium tuberculosis*, the causative agent of TB, has been associated with mutations in specific target genes followed by antibiotic selection during periods of poor adherence. For example, mutations in the Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene have been associated with rifampicin resistance (3, 4). However, mutations in known drug resistance causing genes are not the sole mechanisms of drug resistance. For example, only 95% of clinical *M. tuberculosis* isolates resistant to rifampicin harbour an *rpoB* mutation (5). Therefore the possibility of alternate mechanisms contributing to drug resistance in *M. tuberculosis* needs to be explored.

Numerous approaches have been followed to identify novel mechanisms of drug resistance, or mechanisms which may be contributing to the level of drug resistance in *M. tuberculosis*. Efflux has largely been investigated for its role in defining the level of drug resistance, as well as its role in mechanisms of drug tolerance (6–13). In addition, it has been shown that the activation of efflux pumps may act as an initial gateway mechanism enabling acquisition of resistance causing mutations (14). Although efflux has been widely recognised to contribute to the problem of drug resistance, it has recently been highlighted that there is no evidence that efflux pump activity may be the sole cause of clinical drug resistance (14, 20). Numerous studies have shown the success of including efflux inhibitors in treatment regimens to improve the outcome of anti-TB therapy (15–18).

Despite the role of efflux and the success of efflux pump inhibitors as a complementary treatment to current and new regimens, other mechanisms contributing to resistance have also been investigated. Whole genome sequencing (WGS) has successfully been used to investigate compensatory mutations, as well as novel mutations conferring drug resistance (19–23). In addition, mutations thought to be ancillary to drug resistance have also been identified in a

number of studies (14). WGS techniques have also allowed for investigation into genetic diversity in *M. tuberculosis*, during the course of infection in single isolates as well as between numerous samples within an outbreak (14, 24–26). Investigation into strain diversity has highlighted the implications of genetic diversity in the development on new rapid diagnostic techniques (27, 28). These findings demonstrate the importance of identifying and studying sub-populations within patients at different time points, as well as investigating the genomic occurrences during the evolution of drug resistance.

While WGS is increasingly being used to investigate drug resistance and evolution in *M. tuberculosis*, there is still little known about genomic changes that occur during the evolution of drug resistance, beyond the known drug resistance causing mutations. In addition, little research has focused on the physiological consequences of specific mutations as well as the transcriptional response of *M. tuberculosis* to drug exposure. This study aims to utilise WGS to investigate novel mechanisms contributing to the evolution of drug resistance, as well as use efflux pump activity assays and gene expression analysis to investigate metabolic changes which may be involved in drug resistance in *M. tuberculosis*.

The overall aim of this study is therefore to further investigate mechanisms contributing to drug resistance, with a specific focus on rifampicin resistance. Investigation into mechanisms contributing to drug resistance was carried out in three independent chapters. The study is structured as follows:

- Chapter 2 reviews literature and discusses the role of energy metabolism and efflux pumps in drug resistance in *M. tuberculosis*.
- Chapter 3 describes the methods used in Chapters 4, 5 and 6.
- In Chapter 4, WGS of serial *M. tuberculosis* isolates was used to investigate the *in vivo* evolution of drug resistance from rifampicin mono-resistance to MDR.
- Chapter 5 focuses on the role of efflux pumps in drug resistance in *M. tuberculosis*, with specific focus on the role of different *rpoB* mutations on the response of *M. tuberculosis* to verapamil treatment.
- Chapter 6 investigates the response of *M. tuberculosis* to rifampicin exposure, focusing on the transcriptional response of efflux pumps and components involved in energy metabolism in *M. tuberculosis*.

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CHAPTER 2

Energy metabolism and drug efflux in *Mycobacterium tuberculosis*

The following chapter was published in Antimicrobial Agents and Chemotherapy, and is presented here as the final published unit:

Energy Metabolism and Drug Efflux in *Mycobacterium tuberculosis*

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Abstract

The inherent drug susceptibility of microorganisms is determined by multiple factors, including growth state, the rate of drug diffusion into and out of the cell, and the intrinsic vulnerability of drug targets with regard to the corresponding antimicrobial agent. *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), remains a significant source of global morbidity and mortality, further exacerbated by its ability to readily evolve drug resistance. It is well accepted that drug resistance in *M. tuberculosis* is driven by the acquisition of chromosomal mutations in genes encoding drug targets/promoter regions; however, a comprehensive description of the molecular mechanisms that fuel drug resistance in the clinical setting is currently lacking. In this context, there is a growing body of evidence suggesting that active extrusion of drugs from the cell is critical for drug tolerance. *M. tuberculosis* encodes representatives of a diverse range of multidrug transporters, many of which are dependent on the proton motive force (PMF) or the availability of ATP. This suggests that energy metabolism and ATP production through the PMF, which is established by the electron transport chain (ETC), are critical in determining the drug susceptibility of *M. tuberculosis*. In this review, we detail advances in the study of the mycobacterial ETC and highlight drugs that target various components of the ETC. We provide an overview of some of the efflux pumps present in *M.*

tuberculosis and their association, if any, with drug transport and concomitant effects on drug resistance. The implications of inhibiting drug extrusion, through the use of efflux pump inhibitors, are also discussed.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a global health problem, causing 8.8 million incident cases and 1.1 million deaths in 2012 (1, 2). In many countries, the TB epidemic continues unabated in the face of combination chemotherapy which involves the administration of at least four drugs. The most significant barrier to the eradication of TB is the rapid emergence of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) TB, which has rendered current treatments ineffective and placed an enormous patient management burden on TB control programs. Treatment of drug-resistant TB is costly and requires the use of highly toxic drugs, leading to numerous side effects. Moreover, the spread of MDR strains makes for an alarming situation which provides an ideal breeding ground for further, more advanced forms of drug resistance (3). Given the limited number of anti-TB drugs currently available and the duration of treatment required to achieve cure, there is a global need for the discovery of novel drugs with rapid sterilizing activity against active and dormant bacteria. These drugs should ideally shorten treatment duration and reduce the pill burden (2, 4). Moreover, maintaining the fidelity of the current antibiotics and further understanding the mechanism of emergence of drug resistance require immediate attention to address this growing problem.

Suboptimal intracellular concentrations of drugs often lead to transient drug tolerance, which may be a precursor to chromosomally encoded, stable drug resistance. There is a growing body of evidence that suggests that *M. tuberculosis* retains the capacity to extrude drugs from the cell, resulting in drug tolerance effects (reviewed in reference 5). In many cases, these processes are postulated to be dependent on the proton motive force (PMF) and the presence of sufficient ATP concentrations within the cell. This suggests that the activity of the mycobacterial electron transport chain (ETC) under different conditions plays a key role in determining the inherent susceptibility of *M. tuberculosis* to various drugs. In this review, we provide an overview of the mycobacterial ETC and discuss this component of *M. tuberculosis* metabolism as a target for novel antitubercular agents. We also highlight the importance of energy metabolism in mediating drug tolerance through efflux.

The mycobacterial ETC

In bacteria, the ETC is integrally involved in the generation of energy via oxidative phosphorylation. The mycobacterial components involved in oxidative phosphorylation and energy production have been extensively reviewed, and the reader is referred to these for more-detailed information (6–12). Electrons enter and are shunted through the ETC in a variety of ways, depending on the source of growth substrates and the availability of terminal electron acceptors. Under aerobic conditions, oxygen is used in the final electron transfer steps, and under anaerobic conditions, nitrate or fumarate can be used (Figure 2.1). Electron transport in mycobacteria is initiated through the activity of various NADH dehydrogenases (NDH) and succinate dehydrogenases (SDH), which transfer electrons to menaquinone, a lipophilic redox carrier (7, 13, 14). Electrons are then passed to various cytochrome oxidases, which are dependent on oxygen availability (Figure 2.1) (7, 15, 16). Of particular note is the fact that the mycobacterial ETC, like many bacterial ETCs, is branched and displays an extensive capacity to utilize numerous electron donors and acceptors for adapting to decreasing levels of oxygen tension and the availability of different reducing equivalents. The roles of various components of the ETC are detailed below.

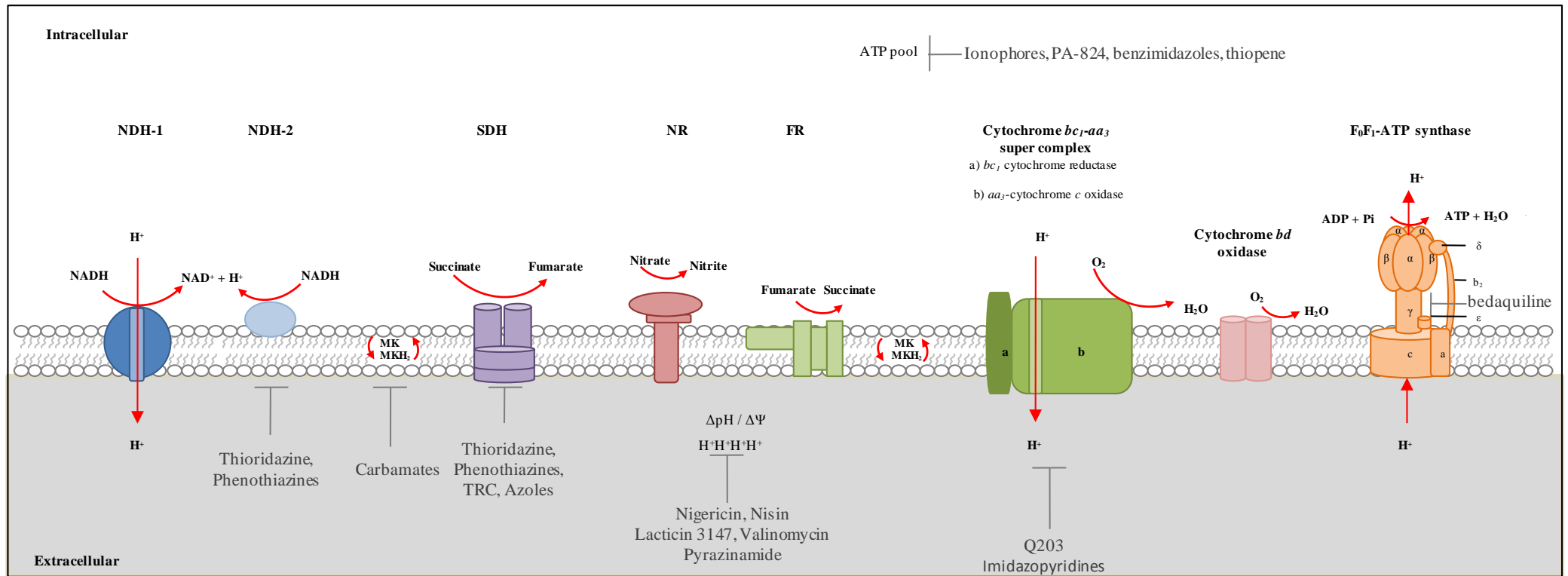


Figure 2.1 The mycobacterial electron transport chain. Proposed aerobic and hypoxic/anaerobic pathways are shown (7, 9). NDH-1, NDH-2, and succinate dehydrogenase (SDH) are electron donors which reduce menaquinone (MK) to menaquinol (MKH₂). Subsequently, MKH₂ becomes oxidized, transferring electrons to the terminal electron acceptors through the activity of cytochrome oxidases, nitrate reductase (NR), and fumarate reductase (FR). ATP production via the F₁F₀-ATP synthase is fuelled by translocation of protons (H⁺). The F₁F₀-ATP synthase consists of two regions, namely, the hydrophobic integral membrane region (F₀) composed of 3 subunits designated a, b, and c and the hydrophilic region (F₁), which extends into the cytoplasm and is composed of 5 subunits designated α , β , δ , γ , and ϵ (7, 45–47). Energy production may be targeted by anti-TB compounds at multiple points involved in ATP synthesis as shown. These include the transmembrane proton gradient (ΔpH)—targeted by compounds such as nigericin, nisin, lacticin 3147, valinomycin, and pyrazinamide (PZA), resulting in disruption of ATP homeostasis. Compounds resulting in a depletion of the ATP pool include phenothiazines, ionophores (shown in the diagram), PA-824, benzimidazoles, thiophene, and imidazopyridines (Q203)/bedaquiline—through direct/indirect effects on the ETC. Other components of energy metabolism targeted by compounds include NDH-2 (thioridazine and phenothiazine), SDH (thioridazine, phenothiazine, triclosan, and azoles), MK biosynthesis (carbamates), Q203, and the F₁F₀-ATP synthase (bedaquiline). Vertical red arrows indicate proton pumping components of the ETC that directly translocate protons into the extracellular matrix.

Electron donors

Analysis of the genome sequence of *M. tuberculosis* H37Rv reveals two types of NDHs in mycobacteria, namely, type I NDH (NDH-1), encoded by the *nuoABCDEFGHJKLMN* operon, and type II NDH (NDH-2), encoded by either the *ndh* gene or the *ndhA* gene (17, 18). In mycobacteria, the reduction in expression of *nuoB*, encoding a subunit of NDH-1, during oxygen deprivation suggests that NDH-1 is required preferentially under aerobic conditions (19), while use of NDH-2 is favoured under anaerobic/nonreplicating conditions (20). Treatment of mycobacteria with NDH-2-specific inhibitors, such as trifluoperazine, results in blockage of initiation of the ETC under anaerobic conditions, implying that NDH-2 is the dominant NDH involved in anaerobic respiration under the conditions tested (20). However, the dispensability of the entire *nuoABCDEFGHJKLMN* operon under aerobic conditions (20) suggests that NDH-2 can operate as an electron donor irrespective of oxygen availability. The NuoG subunit of NDH-1 has been identified in a forward genetic screen to be essential for inhibiting macrophage apoptosis and in virulence in the murine model of TB infection (21). The precise mechanism of apoptosis inhibition is not yet understood, but more-recent work suggests that it involves the neutralization of NOX2-derived reactive oxygen species (22). In this regard, the suppression of apoptosis leads to reduced distribution of tubercle bacilli between cells found within the lung and this results in decreased proliferation of *M. tuberculosis*-specific naive T cells (23). Transposon mutagenesis has identified NDH-1 to be dispensable for growth *in vitro*, and in this context, the apparent essentiality of NuoG for macrophage apoptosis does not seem to be directly related to its role in energy metabolism (24). Saturating transposon mutagenesis predicts that NDH-1 and NDH-2 are dispensable for growth of *M. tuberculosis in vitro* (24). Furthermore, an *ndhA* mutant is able to colonize mouse lungs, confirming that this enzyme is not essential for pathogenesis (25).

SDH, or succinate:menaquinone oxidoreductase, is another enzyme responsible for the donation of electrons to the quinone pool (13). Transposon mutagenesis confirmed that SDH is essential for survival of *M. tuberculosis in vitro* (24). This enzyme predominantly acts in the citric acid cycle, where it oxidizes succinate to fumarate, thereby donating electrons to the quinone pool (26, 27). It has been recently demonstrated that, through remodelling of the tricarboxylic acid cycle, the activity of SDH is essential for the metabolic adaptation to hypoxia, maintenance of membrane potential, and ATP synthesis, indicating that this is a key enzyme for persistence and therefore represents a potential new drug target (27).

Menaquinone

Quinones are lipid soluble electron carriers that are responsible for the transfer of electrons between the components of the ETC (7, 28). In mycobacteria, the predominant quinones are menaquinones (MK), in contrast to the case in *Escherichia coli*, which utilizes both menaquinone and ubiquinone (14, 29–31). In *M. tuberculosis*, MK is synthesized from the precursor chorismate through a series of reactions catalyzed by enzymes encoded by the *menABCDEFG* operon. Recently, an array of MenA inhibitors which display bactericidal activity against nonreplicating *M. tuberculosis* was developed (32). These observations suggest that MK needs to be continuously resynthesized to maintain membrane potential. Of the compounds developed, carbamates have shown significant bacteriostatic activity against nonreplicating *M. tuberculosis* (Figure 2.1) (32). Due to the importance of the MK pool in maintaining the ETC and subsequent ATP synthesis, this biosynthetic pathway represents an important target for anti-TB drug design.

Electron acceptors

Electrons from reduced menaquinol can be transferred to either of two terminal oxidases: (i) the cytochrome *bd*-type menaquinol oxidase or (ii) the *aa₃*-type cytochrome *c* oxidase (via the cytochrome *bc₁* reductase complex). It has been suggested that the mycobacterial *aa₃*-type cytochrome *c* oxidase and cytochrome *bc₁* reductase form a supercomplex (7, 16). The *M. tuberculosis* genome encodes a putative cytochrome *bd* oxidase which, in *Mycobacterium smegmatis*, has been shown to play an important role in the adaptation to a reduced oxygen environment (15, 17). The *M. tuberculosis* genome also encodes a membrane-bound respiratory/assimilatory *narGHI*-encoded nitrate reductase (NR) which is responsible for nitrate-associated respiration and assimilation (17, 33–36). Analysis of clinical *M. tuberculosis* isolates revealed that NarG plays an important role in fitness in macrophages (37), consistent with the detection of transcripts from this operon within granulomas from lungs of TB patients (38) and in the guinea pig model of TB infection (39). The NR is also required for survival of *M. tuberculosis in vitro* under anaerobic conditions of nonreplicating persistence (40) and for the protection of *M. tuberculosis* against acidic conditions during hypoxia (41). These data suggest that respiration using nitrate may be an important mechanism for adaptation under various conditions of stress. In support of this hypothesis, the *nirBD*-encoded nitrite reductase has recently been shown to play an important role in nonreplicating persistence of *M. tuberculosis* (34, 42). Considering other alternate electron acceptors, transcriptional analysis

revealed that *frdA*, a gene encoding the flavoprotein subunit of the fumarate reductase, is upregulated under hypoxic conditions in *M. tuberculosis* and plays a vital role in maintenance of an energized membrane (43).

ATP synthesis and the F₁F₀-ATP synthase

Substrate-level phosphorylation involves the production of ATP by the utilization of free energy produced during various steps in metabolic pathways and provides a fast source of ATP through a process that is not dependent on external electron acceptors (44). In contrast, during oxidative phosphorylation, ATP is produced through the activity of the F₁F₀-ATP synthase enzyme, which is coupled to the PMF. *M. tuberculosis* has been classified as an obligate aerobe; as such, it would be dependent on oxidative phosphorylation for growth and survival during pathogenesis. The structure of F₁F₀-ATP synthase in various prokaryotes is conserved and consists of two regions, namely, the hydrophobic integral membrane region (F₀) and the hydrophilic region (F₁), extending into the cytoplasm (Figure 2.1) (45–47). The F₀ region is composed of 3 subunits designated a, b, and c, whereas the F₁ region is composed of 5 subunits designated α , β , δ , γ , and ϵ (45, 46). ATP is synthesized by the $\alpha_3\beta_3$ -hexamer through rotation of the γ - ϵ pair (48). Rotation of subunits ϵ and γ is coupled to rotation of the c-ring upon proton translocation (Figure 2.1) (48, 49). The mycobacterial F₁F₀-ATP synthase is encoded by a single operon, *Rv1303-atpBEFHAGDC-Rv1312* (17). Recently, BlaI (Rv1846c) was identified as a transcriptional regulator of this operon (50). Treatment with ATP synthase inhibitors results in increased expression of *blaI*, suggesting a role for this regulator in response to stress (51). The mode of regulation of *blaI* in *Staphylococcus aureus* has been elucidated. BlaI has a structure that is similar to that of penicillin binding proteins and is able to act as a transcriptional repressor in response to antibiotic treatment (52). In this regard, it has been shown that BlaI (Rv1846c) in *M. tuberculosis* responds to antibiotic treatment and is released from its cognate operator sequences to allow gene expression (50). BlaR (Rv1845c), a zinc-dependent metalloprotease, has been hypothesized to play a role in cleaving itself and BlaI during derepression of the operon (50, 52). More recently, transcriptional regulators *Rv1773c* and *Rv3405c* have been identified as regulators for *Rv1303*, the first gene of the *atpBEFHAGDC* operon (53).

Drugs that target the ETC

Recent drug discovery efforts have led to numerous compounds which have shown great promise in the treatment of TB due to their ability to eliminate *M. tuberculosis* in various preclinical models and early clinical trials. Among these is the discovery of TMC207 (bedaquiline), which kills *M. tuberculosis* by inhibition of the membrane-bound F₁F₀-ATP synthase complex, resulting in depletion of cellular ATP levels and eventual death of the organism (49, 54–56). Use of this drug, commonly known as bedaquiline, resulted in decreased time to smear conversion during a phase IIb randomized trial (54, 57). Bedaquiline kills *M. tuberculosis* by interacting with the hydrophobic region of subunit c, as well as with subunit ε (48), of the F₁F₀-ATP synthase and does not cross-react with the human ATP synthase complex (56, 58). Inhibition of c-ring rotation due to disruption in the c-ring:ε subunit interaction results in inhibition of ATP production and subsequent cell death (48). The efficacy of bedaquiline in clinical trials confirms that targeting energy metabolism during TB infection may be promising, particularly for nonreplicating organisms, as there is documented evidence that ATP is essential for the viability of nonreplicating persistent mycobacteria (20, 55, 59). Moreover, the ability to eliminate subpopulations of persisting organisms provides an opportunity for tissue sterilization, thereby minimizing the risk for recrudescence through reactivation of persisting bacteria. The ability of these organisms to maintain an energized membrane potential in the face of prolonged quiescence is critical to their survival and highlights the importance of understanding the physiology of *M. tuberculosis* with regard to energy metabolism (9). In this context, ATP synthesis has become a focus area for the identification of new drug targets in mycobacteria (60).

The PMF is an important aspect in the final production of ATP via the F₁F₀-ATP synthase (20). The PMF is established through the development of the transmembrane proton gradient (ΔpH) which occurs when electrons move through the ETC and results in the establishment of membrane potential ($\Delta\psi$) (reviewed in references 6 and 7). The proton gradient generated through oxidative phosphorylation drives ATP synthesis via the F₁F₀-ATP synthase which is responsible for the conversion of the electrochemical potential energy generated by the PMF into chemical energy in the form of ATP (45). Since oxidative phosphorylation is the main source of energy production in mycobacteria, ATP synthase represents the key enzyme involved in ATP production in mycobacteria.

Studies of valinomycin and nigericin treatment (inhibitors of $\Delta\psi$ and ΔpH , respectively) revealed that in both actively replicating and hypoxic nonreplicating bacilli, death occurs via decreased ATP levels in a dose-dependent manner (20). Nisin, a lantibiotic produced by *Lactococcus lactis*, has been shown to dissipate $\Delta\psi$ and ΔpH , thereby disrupting energy metabolism (20, 61). Lantibiotics such as nisin and lactacin 3147 form pores in the mycobacterial cell membrane which result in dissipation of $\Delta\psi$ (Figure 2.1) (several studies have investigated the effect of these compounds in mycobacteria [61–65]). Although nisin is able to dissipate the membrane potential and decrease ATP levels in mycobacteria, the MIC values for various mycobacterial strains are very high and not comparable to those of current anti-TB drugs (62). This poor inhibitory activity of nisin has been attributed to its low solubility at pH 7, in contrast to lactacin 3147, which is soluble under such conditions and demonstrates greater activity against mycobacteria, thus warranting further investigation as a potential anti-TB drug (62). Another compound shown to target $\Delta\psi$ is pyrazinamide (PZA), where treatment results in a decrease in ATP levels, most likely the resulting effect of diminished membrane potential (66). Unlike current first- and second-line anti-TB drugs, PZA has been shown to be active against both replicating and nonreplicating mycobacteria (67). This was highlighted in a recent study, where nutrient-starved *M. tuberculosis* displayed increased susceptibility to PZA due to the decreased membrane potential (68). Furthermore, PZA treatment of mice infected with *M. tuberculosis* significantly reduces the release of proinflammatory cytokines and chemokines, suggesting that PZA has important host-directed effects (69).

Due to the importance of ATP for cellular viability, the components involved in the process of ATP production represent viable drug targets which, in combination with current anti-TB drugs, could be used for effective treatment. A number of existing compounds deplete cellular ATP levels and have subsequent bactericidal effects on replicating and nonreplicating mycobacteria (Figure 2.1). These compounds include *n*-decanesulfonylacetamide (DSA) and nisin. DSA is the lead compound of the β -sulfonylacetamide class of antimicrobials and has been shown to be active *in vitro* against replicating *M. tuberculosis* as well as against anaerobic *M. bovis* BCG (70–73). It has been proposed that DSA interferes with components of the respiratory chain, thereby disrupting energy metabolism (73).

Another compound targeting energy metabolism is PA-824, which is currently in human clinical trials as an anti-TB drug (57, 74–76). PA-824, a bicyclic nitroimidazole, has the ability to kill both replicating and hypoxic, nonreplicating *M. tuberculosis* through a multifaceted

mechanism that involves inhibition of mycolic acid biosynthesis and respiratory poisoning through intracellular release of nitric oxide, which is postulated to inhibit the final stages of electron transfer in cytochrome *c* oxidase (76).

More recent efforts have yielded a novel class of imidazo[1,2-*a*]pyridine amide (IPA) compounds (77, 78) that prevent proliferation of *M. tuberculosis* by inhibition of the cytochrome *bc*₁ reductase complex in the mycobacterial respiratory chain (77, 79) (Figure 2.1). These compounds bind the QcrB subunit and induce bacterial cell death by abrogating electron flow through the ETC, resulting in reduced ATP synthesis under aerobic and anaerobic conditions (79). Two independent studies identified QcrB as the target for IPAs through the generation of spontaneous resistant mutants carrying various substitutions at the Thr313 residue (77, 79). The lead compound from this series, Q203, displays potent killing of *M. tuberculosis* in axenic culture, in macrophages, and in the murine model of TB infection, with a spontaneous mutation rate in the order of 10⁻⁸ (79). Q203 is well tolerated in mice and now awaits further analysis in clinical trials.

Phenothiazines, such as chlorpromazine and thioridazine (THZ) (Figure 2.1), are a group of clinically relevant compounds that are predicted to target the ETC through inhibition of NDH-2 (18). THZ is an old neuroleptic agent that has demonstrated activity in killing drug-susceptible and drug-resistant *M. tuberculosis* in various model systems *in vitro* and *ex vivo* and in the murine model of TB infection (80–84). In the promising development, THZ demonstrated therapeutic benefit in treatment of XDR-TB patients in Argentina and is currently being used in trials in India (reviewed in reference 85). In a recent study, THZ activity was shown to be independent of the bacterial growth phase; i.e., THZ is effective against actively replicating bacilli, semi-dormant bacilli, and nonreplicating persisters (86). That study also demonstrated a low mutation frequency in *M. tuberculosis*, suggesting a delay in the development of THZ resistance (86). While retaining the ability to inhibit the ETC in mycobacteria, THZ also has the potential to directly inhibit efflux of drugs (87). In addition to phenothiazines, which target the mycobacterial NDH-2, clofazimine (CFZ)—a rhiminophenazine—is subject to reduction by NDH-2 and, upon subsequent oxidation, leads to the formation of reactive oxygen species (88), which presumably contributes to its efficacy in mice (89).

Numerous studies have investigated the efficacy of novel combinations of drugs by incorporating new and existing compounds which have demonstrated activity against bacterial

targets involved in energy metabolism. These combinations have demonstrated efficacy that is comparable or enhanced in comparison to the current anti-TB regimens, and the potential to shorten the current treatment period has been highlighted. A recent 14-day early bactericidal activity (EBA) study demonstrated the benefit of adding PA-824 to a regimen containing moxifloxacin (MXF) and PZA to shorten treatment duration (89). Bedaquiline–PZA–PA-824 and bedaquiline–PA-824–MFX combinations have demonstrated greater efficacy than rifampin (RIF)-isoniazid (INH)-PZA in reducing CFU (90). Tasneen *et al.* also demonstrated that drug combinations containing bedaquiline showed higher efficacy with respect to relapse prevention in a mouse model (89). In a separate study, numerous combinations of bedaquiline, PA-824, CFZ, and PNU-100480 were shown to be more effective than a RIF-INH-PZA combination (90).

Energy metabolism and drug efflux

Active transport of drugs and xenobiotics through the activity of efflux pumps (EPs) is well documented in bacteria and points to an important role for this process in phenotypic drug tolerance and the subsequent emergence of drug resistance due to reduced intracellular drug concentrations (5). A key feature of these systems is their dependence on the PMF or the availability of ATP, which inextricably links drug efflux to energy metabolism and the ETC (5, 91, 92). EPs adopt a diversity of structures in bacteria and can be classified into different families based on overall secondary structure and the molecules transported. These include, but are not limited to, the major facilitator superfamily (MFS), small multi-drug resistance (SMR) family, resistance/nodulation/cell division (RND) family, the ATP-dependent ABC-type superfamily of transporters, and the multidrug and toxic compound extrusion (MATE) family (91, 93, 94).

The widely distributed P-glycoprotein, encoded by the *mdr* gene, is one of the first ABC-type transporters implicated in drug efflux and has been implicated in the extrusion of numerous drugs in different organisms (91). The members of this family of transporters require the energy of ATP hydrolysis for active efflux of drugs (Figure 2.2) (95, 96). In contrast, the MFS, SMR, RND, and MATE-type EPs require an energized membrane and the PMF (ΔpH and $\Delta\Psi$) for activity (reviewed in reference 91). The pumps often operate as drug-metabolite/proton symporters, antiporters, or uniporters. Schematic representations of these EPs are shown in Fig. 2. The MFS-type EPs can be characterized into 6 evolutionarily related subfamilies, including those containing 14 or 12 transmembrane segments, sugar importers, phosphate ester

antiporters, and other transporters (97–99). It has been proposed that these large membrane-associated proteins have evolved via intragenic tandem gene duplication to give rise to a widely distributed, structurally diverse family of proteins (97). MFS EPs require the presence of protons in the periplasm and couple efflux with proton translocation to the cytoplasm (100) (Figure 2.2). However, these systems differ in their dependence on the ΔpH and $\Delta\Psi$; for example, the *bmr*-encoded multidrug EP in *Bacillus subtilis* requires a strong ΔpH to drive the extrusion of ethidium bromide through a electroneutral drug/proton antiport mechanism (91).

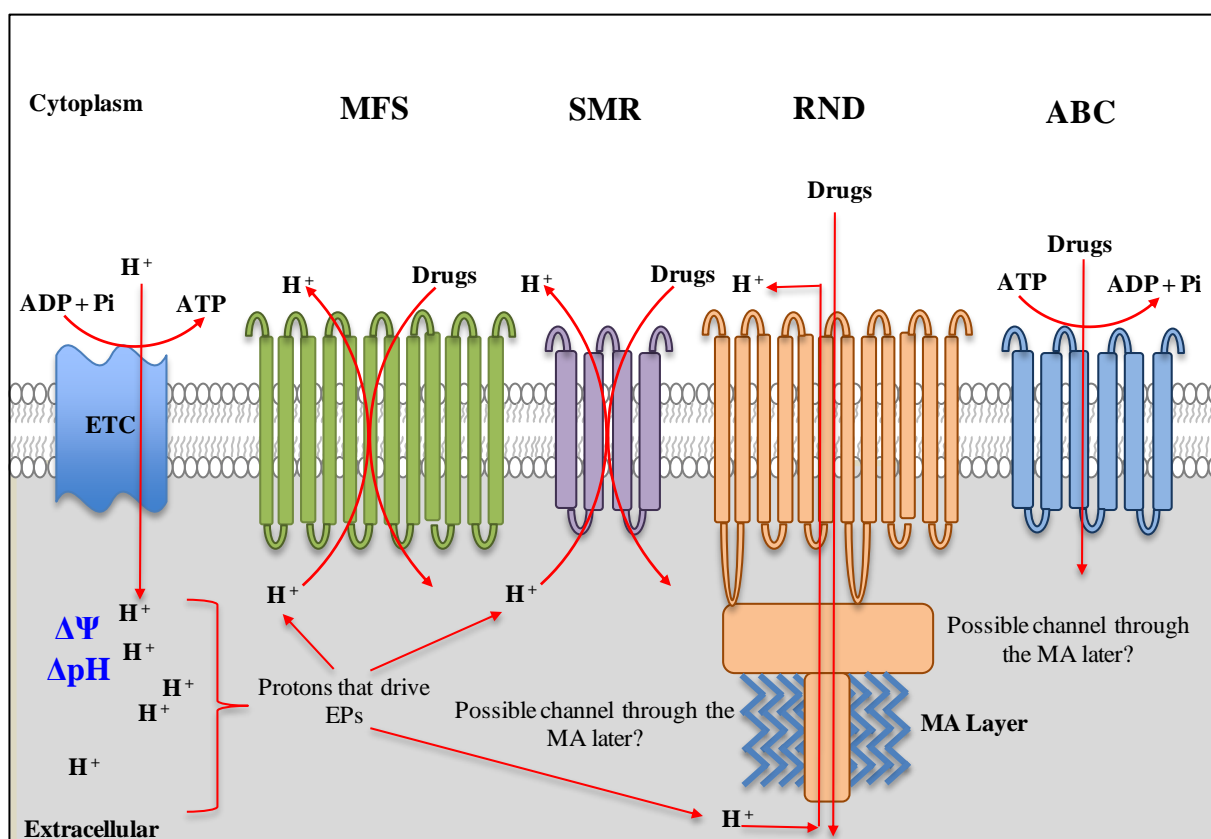


Figure 2.2 Efflux pumps in *M. tuberculosis*. The EPs dependent on the PMF and ATP are shown. The mycobacterial ETC generates a transmembrane proton gradient (ΔpH) resulting in membrane potential ($\Delta\Psi$); ΔpH and $\Delta\Psi$ together constitute the PMF. Protons translocated into the pseudoperiplasmic space are used by EPs to extrude drugs. The major facilitator superfamily (MFS) EPs are made of integral membrane proteins with 12 to 14 transmembrane regions, while the small multidrug resistance (SMR) EPs contain 4 to 6 transmembrane domains. Both systems use protons from the periplasm, and the members differ in their requirements for ΔpH and $\Delta\Psi$ (see the text for details). The resistance nodulation/cell division (RND) EPs are also integral membrane proteins, and members of this family from Gram-negative bacteria associate with other proteins to form a multisubunit complex that spans both the inner and outer membranes. Hence, there is a possibility that a similar structure occurs in mycobacteria, where the members of this group of EPs span the pseudoperiplasmic space and associate

with the mycolic acid (MA) layer, possibly through OmpA (outer membrane protein A)-like homologues. RND proteins also require protons—although these may originate from outside the cell—and the PMF. ABC transporters require ATP for the active drug extrusion and, as such, are also dependent on energy production in the cell. Not shown in the figure are the members of the fifth class of EPS, termed the multidrug and toxic compound extrusion (MATE) proteins; while some of these are dependent on PMF and protons, the majority of characterized members operate via sodium influx (93).

The Smr staphylococcal multidrug efflux protein and *E. coli* EmrR represent well-characterized EPs that belong to the SMR family, which is constituted of four transmembrane-containing transporters (91, 101). Smr from *Staphylococcus aureus* requires the ΔpH and $\Delta\Psi$ for efficient transport of a wide range of drugs and other small compounds (102). Reconstitution experiments with both Smr and Emr confirm that these EPs require the PMF and operate through a drug/proton antiport mechanism (103). The RND proteins constitute the third family of broad substrate EPs that require the PMF for activity and are structurally more complex than SMR EPs, containing 12 transmembrane domains and various loop regions (104, 105). Unlike the MFS family of EPs, these proteins are phylogenetically closely related, suggesting that they evolved from a single founding member (91). In addition to being autonomous transporters, the members of the RND family of proteins interact with other transport proteins in Gram-negative bacteria such as membrane fusion proteins (MFPs) and outer membrane factors (OMFs) to facilitate the transport of a variety of substrates (Figure 2.1) (105–107).

The genome of *M. tuberculosis* retains multiple homologues of the major families of PMF-dependent EPs described above and, in addition, encodes numerous ABC-type transporters or hypothetical proteins with homology to transporters (17, 108, 109). This complex multiplicity of EPs, detailed in Table 2.1, illustrates the ability of the tubercle bacillus to transport a variety of toxic compounds or antibiotics and has important implications for drug resistance in TB infection. The key feature of note is that many of these transport systems require either the PMF or ATP, thus linking their activity with metabolic flux through the ETC and the maintenance of an energized membrane.

Table 2.1 Putative efflux pumps in *M. tuberculosis*.

Gene	Description	Transporter Family	Energy Source	Drugs effluxed	Differentially regulated in clinical isolates	Differentially regulated by drugs	References
<i>Rv0037c</i>	Probable conserved integral membrane protein	MFS	ATP		Yes		(110)
<i>Rv0194</i>	Drugs-transport transmembrane ATP-binding protein	ABC	ATP				(17, 111)
<i>mmpS5</i> (<i>Rv0677c</i>)	Membrane protein mmpS5	RND	PMF	TET			(17, 112)
<i>Rv0849</i>	Probable conserved integral membrane protein	MFS	PMF	β -lactams			(113)
<i>Rv1218c</i>	Probable tetronasin-transport ATP-binding	ABC	ATP	β -lactams			(113)
<i>Rv1250</i>	Probable drug transport integral membrane protein	MFS	PMF		Yes		(110)
<i>Rv1258c</i>	Conserved membrane transport protein	MFS	PMF	INH, RIF, EMB, OFL, β -lactams	Yes	INH, RIF, OFL	(17, 110, 114-116)
<i>Rv1272c</i>	Probable drugs-transport transmembrane ATP-binding protein	ABC	ATP				(17, 118)

<i>Rv1273c</i>	Probable drugs-transport transmembrane ATP-binding protein	ABC	ATP	Yes		(17, 110, 118)
<i>itrA</i> (<i>Rv1348</i>)	Probable drugs-transport transmembrane ATP-binding protein	ABC	ATP			(17, 118)
<i>itrB</i> (<i>Rv1349</i>)	Probable drugs-transport transmembrane ATP-binding protein	ABC	ATP			(17, 118)
<i>Rv1410c</i>	Aminoglycosides/tetracycline-transport integral membrane protein	MFS	PMF	Yes	INH, RIF	(110, 115)
<i>Rv1456c- Rv1458c</i>	Integral membrane proteins	ABC	ATP	Yes (in drug resistant isolates)		(17, 119)
<i>Rv1463</i>	Conserved transmembrane ATP binding protein	ABC	ATP			(17)
<i>Rv1634</i>	Drug efflux membrane protein	MFS	PMF	Yes		(17, 109, 110, 120, 121)
<i>Rv1686c</i>	Probably conserved ATP-binding protein	ABC	ATP	Yes (in drug resistant isolates)		(122)

Rv1687c	Probably conserved ATP-binding protein	ABC	ATP		Yes	(110)
Rv1747	Conserved transmembrane ATP binding protein	ABC	ATP	INH		(17, 118)
Rv1877	Conserved membrane protein	MFS	PMF	TET, KAN, erythromycin		(17, 118, 120, 121)
bacA (Rv1819c)	Drugs-transport transmembrane ATP-binding protein	ABC	ATP		INH, RIF	(17, 115, 123, 124)
Rv2209	Probable conserved integral membrane protein	??	??		OFL	(124)
Rv2333c	Conserved membrane transport protein	MFS	PMF	TET, spectinomycin	Yes	(17, 110, 120, 125)
jefA (Rv2459)	Conserved integral membrane transport protein	MFS	PMF		INH, EMB	(17, 120, 124, 126, 127)
Rv2477c	Probable macrolide-transport ATP-binding protein	ABC	ATP		OFL	(124)
Rv2686c	Antibiotic-transport membrane leucine and alanine and valine rich protein	ABC	ATP	CIP		(17, 118, 128)
Rv2687c	Antibiotic-transport membrane leucine and valine rich protein	ABC	ATP	CIP		(17, 118, 128)
Rv2688c	Antibiotic-transport ATP-binding protein	ABC	ATP	CIP	STR	(17, 118, 124, 128)

<i>Rv2994</i>	Conserved membrane protein	MFS	PMF			STR	(17, 120, 121, 124)
<i>Rv3000</i>	Possible conserved transmembrane protein	ABC	ATP		Yes		(110)
<i>Rv3239c</i>	Conserved integral membrane transport protein	MFS	PMF			RIF	(17, 120, 129)
<i>Rv3728</i>	Conserved two-domain membrane protein	MFS	PMF			INH, RIF, EMB	(17, 120, 124)
<i>DrrA</i> (<i>Rv2936</i>)	Daunorubicin-dim-transport ATP-binding protein ABC transporter <i>drrA</i>	ABC	ATP	TET, STR, EMB, RIF	Yes		(17, 110, 118, 130, 131)
<i>DrrB</i> (<i>Rv2937</i>)	Daunorubicin-dim-transport membrane protein ABC transporter <i>drrB</i>	ABC	ATP	TET, STR, EMB	Yes		(17, 110, 118, 130, 131)
<i>DrrC</i> (<i>Rv2938</i>)	Daunorubicin-dim-transport membrane protein ABC transporter <i>drrC</i>	ABC	ATP	TET, STR, EMB		STR, EMB	(17, 110, 118, 130, 131)
<i>efpA</i> (<i>Rv2846c</i>)	Integral membrane efflux protein	MFS	PMF	possibly INH		INH	(17, 121, 124, 132, 133)

emrB (Rv0783)	Possible multidrug resistance integral membrane efflux protein emrB	MFS	PMF	RIF	Yes	(17, 110, 132, 134)
iniA (Rv0342)	Isoniazid inducible gene protein iniA	membrane protein		INH, EMB	Yes	(17, 110, 135, 136)
iniB (Rv0341)	Isoniazid inducible gene protein iniB	membrane protein		INH		(17, 135, 136)
iniC (Rv0343)	Isoniazid inducible gene protein iniC	membrane protein		INH		(17, 135, 136)
mmpL3 (Rv0206c)	Probable conserved transmembrane transport protein mmpL3	RND	PMF			(17, 137)
mmpL4 (Rv0450c)	Probable conserved transmembrane transport protein mmpL4	RND	PMF		Yes	(110)
mmpL5 (Rv0676c)	Probable conserved transmembrane transport protein mmpL5	RND	PMF	TET		(17, 112)
mmpL7 (Rv2942)	Probable conserved transmembrane transport protein mmpL7	RND	PMF	INH	Yes	(17, 110, 128, 137)

<i>mmpL11</i> (Rv0202c)	Probable conserved transmembrane transport protein mmpL11	RND	PMF			(17, 137)
<i>mmr</i> (Rv3065)	Integral membrane efflux protein	SMR	PMF	Erythromycin, β - lactams	INH, EMB	(17, 109, 113, 121, 124)
<i>pstB</i> (Rv0933)	Phosphate-transport ATP binding protein	ABC	ATP	INH, RIF, EMB, CIP		(17, 117, 118, 130, 138, 139)

Transporter families were identified by literature searches (relevant cases are cited in the text) and from the genomic annotation/comparisons

(<http://genolist.pasteur.fr/TubercuList/> and <http://tuberculist.epfl.ch/>)

Further associations between the PMF and drug efflux in mycobacteria have been made through various studies which demonstrated that dissipation of the PMF by treatment with efflux pump inhibitors (108, 114, 126, 140) can reverse low levels of resistance to TB drugs. Early work with ^{14}C -labeled RIF suggests that this drug may be extruded from mycobacteria by the activity of PMF-dependent efflux pumps—a process that can be marginally reversed by the addition of reserpine, an EP inhibitor (141). In drug-resistant strains, exposure to efflux pump inhibitors such as verapamil, reserpine, Phe-Arg- β -naphthylamide (PA β N), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and 2,4-dinitrophenol (DNP) restores susceptibility to anti-TB drugs such as RIF and ofloxacin (OFL) (129, 142, 143). Inclusion of verapamil in drug susceptibility assays results in a marked decrease in the MIC for bedaquiline and clofazimine (144). Knockout studies have further implicated efflux pumps such as Rv1218c, Rv3065, Rv0849, and Rv1258c as important mechanisms of resistance to various chemical classes of drug compounds in *M. tuberculosis* (145, 146). A recent study demonstrated increased expression of 15 EP-encoding genes, from various classes, in drug-susceptible and drug-resistant strains compared to reference, laboratory grown strains (110). These data suggest that infection in the human host presumably drives expression of these genes to transport/detoxify noxious compounds during pathogenesis, with the concomitant benefit of drug extrusion. Similarly, the expression of 10 EP-encoding genes—*Rv3065* (*mmr*), *Rv2938* (*drrC*), *Rv1819c* (*bacA*—recently implicated in vitamin B₁₂ acquisition [123]), *Rv2209*, *Rv2459*, *Rv2477*, *Rv2688*, *Rv2846* (*efpA*), *Rv2994*, and *Rv3728*—is differentially induced during *in vitro* drug treatment of clinical isolates with standard TB drugs (124), as detailed further in Table 2.1, suggesting a complex induction pattern of these genes during short-course chemotherapy. While the regulatory mechanisms governing these gene expression changes have not been completely described, there is some evidence for transcriptional regulation of EPs. In this regard, it has been shown that WhiB7, a redox-sensitive transcriptional activator, plays an important role in mediating intrinsic drug resistance in mycobacteria, in some cases through direct regulation of EPs (147–149). Similarly, resistance to INH has been associated with the IniB-IniA-IniC efflux system (Table 2.1), which is regulated by the MtrAB two-component system (150), and the *mmr*-encoded EP is regulated by a TetR-type repressor (151). With respect to the evolution of drug resistance, transcriptional profiling of longitudinal isolates from drug-compliant patients, with MANU1, CAS, and Beijing spoligotypes, revealed upregulation of various EPs/multidrug resistance proteins, including Rv3065 (*mmr*), Rv2936 (*drrA*), Rv2397, Rv1686c, and Rv1687c, pointing to a role for these proteins in the evolution of drug resistance during treatment (122). Increased

expression of genes encoding various efflux pumps upon exposure to RIF has been observed in *M. tuberculosis* which, in some cases, has been coupled with an increase in RIF and OFL tolerance. This phenomenon could be reversed by the addition of efflux pump inhibitors, highlighting the role of efflux in drug resistance (114–116, 129, 152). A similar result was observed during macrophage infection, where efflux pump activity was coupled to RIF and INH tolerance in *M. tuberculosis* (153). In RIF mono-resistant and susceptible *M. tuberculosis* isolates, initial low-level resistance to INH (reversible by the addition of efflux pump inhibitors) preceded the development of drug resistance-conferring mutations (126). Recently, it has been demonstrated that two EP-encoding genes, those encoding Rv2936 and Rv0783 (Table 2.1), are associated with RIF resistance in RIF-mono-resistant clinical isolates. Over expression of these two genes, but not of that encoding Rv0933, resulted in increased RIF resistance in *E. coli* (130). Similarly, low-level efflux-induced azithromycin resistance in *M. avium* was followed by reversible high-level resistance, thought to be due to acquisition of mutations (140).

These data suggest that the initial efflux activity allows antibiotic tolerance in mycobacteria, enabling resistance-causing mutations to arise and/or be selected for. In this context, the production of ATP via the F_1F_0 -ATP synthase would be essential for the function of these systems. Since efflux pumps seem to play an important role in contributing to drug tolerance, which may then lead to drug resistance, the driving energetic force behind these pumps represents an additional point of vulnerability with respect to targeted drug design in mycobacteria. Studies using PMF and ATP synthase inhibitors have demonstrated the role of the PMF and ATP in INH efflux in *M. smegmatis* (135). Although efflux has been studied in *M. tuberculosis*, the role of the driving force of these pumps in drug resistance remains poorly investigated. Direct coupling of PMF and ATP to drug efflux—and consequent drug resistance—has been investigated in *E. coli*, *B. subtilis*, *L. lactis*, *Streptococcus pneumoniae*, and a host of other organisms (reviewed in references 91, 97, 104, 105, 154, 155, 156, 157, and 158). Considering the success in targeting the ETC in TB drug development, together with the demonstrated importance of efflux as an active process contributing to drug resistance, the role of energy metabolism in drug resistance and inherent susceptibility in *M. tuberculosis* merits further investigation. The recent demonstration that iron-sulfur (Fe-S) cluster biogenesis is related to intrinsic susceptibility of bacteria to aminoglycosides due to the use of an alternate pathway for Fe-S biogenesis, which results in perturbations in the ETC, leading to reduced PMF and altered drug uptake (159), is consistent with this. Little is known about the energy

requirements of mycobacteria during infection, although it has been demonstrated that ATP levels are important for survival in actively replicating as well as nonreplicating mycobacteria, demonstrating the importance of maintaining ATP levels under conditions of growth and survival (20, 55). These ATP levels and associated PMF are essential for *M. tuberculosis* to extrude drugs via PMF-dependent EPs.

The presence of numerous potential regulators involved in the control of cellular ATP levels suggests that there may be different mechanisms involved in the regulation of ATP synthesis. It has been demonstrated that, although ATP levels drop significantly during the shift down to a nonreplicating state, the decreased levels of cellular ATP are essential for viability since treatment with bedaquiline resulted in a loss of viability. *M. smegmatis* displays up regulation of F₁F₀-ATP synthase in response to antibiotic stress. For example, an increase in F₁F₀-ATP synthase abundance was observed upon the exposure of *M. smegmatis* to ethambutol (EMB). Furthermore, among the other proteins up regulated in response to EMB, 23% were related to energy metabolism (160). Similarly, treatment with β -lactam antibiotics resulted in increased expression of F₁F₀-ATP synthase in *M. tuberculosis* (51). These data suggest that antibiotic treatment may inflict physiological stresses on bacteria that impose a requirement for greater energy production. In the case of β -lactam antibiotics, it is unclear whether their effect on the cell wall results in a perturbation of the PMF which is compensated for by an up-regulation of the genes encoding the ATP synthase. There have been no extensive studies showing the response of pathways involved in energy metabolism to anti-TB treatments such as RIF; this could provide novel insight into cellular responses or adaptations occurring within the cell leading to drug resistance or identification of targets for drug development.

Concluding remarks

The ETC and associated PMF are essential components for energy production through the generation of ATP, which is required for metabolic processes within the cell. The ETC has gained recent prominence in TB drug development through the discovery of numerous compounds that target this pathway such as bedaquiline and Q203. However, in addition to the obvious effects of inhibiting the ETC, a secondary effect of targeting this pathway would be a reduction in the activity of the various PMF/ATP-dependent EPs present in *M. tuberculosis*. These effects may accelerate cell death through higher intracellular concentrations of drugs and reduced extrusion of toxic metabolites, with an added benefit of reducing transient drug tolerance and consequent drug resistance. Various studies now point to potentially positive

therapeutic effects of using EP inhibitors such as verapamil to increase the potency of drugs and limit the acquisition of drug resistance. Energy metabolism, including the regulation thereof, represents an ideal component of metabolism to mine for new drug targets.

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CHAPTER 3

Materials and methods

3.1. Strain selection

Stellenbosch University, Department of Biomedical Sciences has an extensive *Mycobacterium tuberculosis* strain culture bank containing approximately 20 000 isolates, collected from 2001 to present. These isolates represent all culturable drug resistant isolates from the Western Cape region of South Africa. A large proportion of isolates have been genotyped by spoligotyping, representing numerous clades and lineages. Approximately 70% of the drug resistant epidemic in the Western Cape is driven by 4 strain families: Beijing/W-like (28%), Low Copy Clade (LCC) (26%), F11 (12%) and F28 (5%) (1). Resistance profiles range from mono-resistant to totally drug resistant (TDR) (in the sense that these isolates are resistant to all available drugs used to treat TB in South Africa); a subset of which reflect intra-patient evolution (*in vivo* evolution). For the purpose of this study the *M. tuberculosis* strain culture bank was interrogated to select a sample set where *in vivo* evolution of drug resistance was observed.

Briefly, patients with more than one clinical sample present in the strain culture bank were selected; multiple samples per patient were analysed to determine if *M. tuberculosis* had acquired additional drug resistance during the course of infection i.e. an initial sample was mono-resistant while a follow up sample has gained resistance to an additional anti-TB drug. The final sample set selected for this study samples consisted of isolates that demonstrate intra-patient evolution i.e. samples from individual patients where an initial sample shows to be rifampicin mono-resistant, and a follow-up sample multi-drug resistant (MDR). A total number of 66 isolates originating from 31 patients were selected for strain characterization (Appendix 2, Table A2.1).

Isolates shown to demonstrate intra-patient evolution of drug resistance from rifampicin mono-resistant to MDR were used to investigate the role of efflux pumps in the evolution of drug resistance. These isolates were characterised in Chapter 4. In addition, a separate panel of well characterised rifampicin mono-resistant *M. tuberculosis* clinical isolates were selected from the culture bank for this study (Table 3.1). These isolates represent numerous genetic backgrounds and *rpoB* mutations causing rifampicin resistance.

A set of *in vitro* generated rifampicin resistant *M. tuberculosis* mutants as well as their pan susceptible progenitor were also selected for analysis (Table 3.1). The *in vitro* *M. tuberculosis* rifampicin resistant mutants were previously generated in our department by Dr M de Vos, to provide a sample set with a “clean” genetic background to further investigate rifampicin

resistance mechanisms in *M. tuberculosis*. The *in vitro* mutants selected for this study had been fully characterized and analysed by whole genome sequencing (WGS) analysis (Table 3.2).

Table 3.1 *M. tuberculosis* isolates selected to investigate the role of efflux pumps in the evolution of drug resistance.

	Isolate	Genetic background	<i>rpoB</i> mutation*	Drug resistance profile
Clinical isolates	R966	Beijing	His526Tyr	Rifampicin mono-resistant
	R5608	EAI	Ser531Leu	Rifampicin mono-resistant
	R160	LCC	Ser531Leu	Rifampicin mono-resistant
<i>in vitro</i> mutants	K636	Beijing	-	Pan-susceptible
	K.531	Beijing	Ser531Leu	Rifampicin mono-resistant
	K.526	Beijing	His526Tyr	Rifampicin mono-resistant

* Amino acid change according to the *Escherichia coli rpoB* gene sequence

Table 3.2 Novel genetic variants unique to *M. tuberculosis* rifampicin resistant *in vitro* generated mutants relative to the pan-susceptible progenitor.

	Locus	Gene	Amino acid change	Gene description	Functional group
k.531	Rv0667	<i>rpoB</i>	Ser531Leu*	DNA-directed RNA polymerase beta chain	Information pathways
	Rv2185c	TB16.3	Gly101Gly	Conserved hypothetical protein	Conserved hypothetical protein
k.526	Rv0667	<i>rpoB</i>	His526Tyr*	DNA-directed RNA polymerase beta chain	Information pathways

* Amino acid change according to the *Escherichia coli rpoB* gene sequence

3.2. Mycobacterial strain culture

The selected clinical *M. tuberculosis* isolates are all preserved as coated glass bead stocks at -80°C within the Department of Biomedical Sciences. BACTEC™ Mycobacterial Growth

Indicator tubes (MGIT™ 960) were supplemented with OADC and inoculated with a single glass bead. Each inoculated MGIT was incubated in the BACTEC™ MGIT™ 960 instrument at 37°C. Following an indication of growth positivity, each MGIT was incubated at 37°C for an additional 5 days to allow for optimal mycobacterial growth units. A volume of 500µl of positive culture was then used to inoculate a starter culture in 10ml of 7H9 Middlebrook medium (Becton, Dickinson Microbiology system, Sparks, USA) (see appendix 1), supplemented with 10% albumin-dextrose-catalase (ADC), 0.2% (v/v) glycerol (Merck Laboratories, Saarchem, Gauteng, SA) and 0.1% Tween80 (Becton, Microbiology systems, Sparks, USA). Cultures were then grown in filtered screw cap tissue culture flasks (Greiner Bio-one, Maybachstreet, Germany) without shaking at 37°C until an optical density (OD₆₀₀) of 0.6-0.8 was reached. Ziehl-Neelsen (ZN) staining and plating cultures onto blood agar plates were done to assess contamination. An aliquot of *M. tuberculosis* culture was boiled and stored separately for subsequent PCR screening. An additional aliquot was plated on 7H10 media supplemented with OADC for subsequent DNA extraction. Glass bead and glycerol stocks (1:1 v/v, 500µl culture and 500µl 50% glycerol) were prepared and stored at -80°C.

3.3. Strain characterization

The 66 prospective *M. tuberculosis* isolates demonstrating *in vivo* evolution of drug resistance were screened for genotypic drug resistance using targeted PCR and Sanger sequencing of known drug resistance causing genes. In addition, these isolates were subjected to spoligotyping to determine strain family/genetic background. A refined list of isolates was subjected to phenotypic drug susceptibility testing for rifampicin and isoniazid to confirm the observed genotypes.

3.3.1. Targeted PCR and Sanger sequencing

PCR amplification of the known drug resistance associated genes (Table 3.3) was done by adding a 2µl of the boiled culture to a PCR reaction mixtures containing 1X Q buffer, 1X PCR buffer, 2mM MgCl₂, 0.4mM dNTPs, 50µM of each drug resistance associated gene primers (Table 3.3) and 1.25U Hot Star Taq polymerase (Qiagen, San Diego, CA, USA). Amplification was done under the following conditions: 95°C for 15 minutes; 40 cycles of 95°C for 30s, primer T_m for 30s (Table 3.1), and 72°C for 2 minutes; one cycle of 72°C for 5 minutes. Amplification for the *rpoB* gene was performed using a touchdown PCR. Reaction conditions are as follows: 95°C for 15 minutes, 2 cycles of 94°C for 1 minute, 72°C for 1 minute and 72°C

for 1 minute, 2 cycles of 94°C for 1 minute, 71°C for 1 minute and 72°C for 1 minute, 2 cycles of 94°C for 1 minute, 70°C for 1 minute and 72°C for 1 minute and 40 cycles of 94°C for 1 minute, 69°C for 1 minute and 72°C for 1 minute, with a final extension step of 72°C for 10 minutes.

PCR products were electrophoretically fractionated in a 2% agarose gel (100 volts, 1 hour) and visualised after ethidium bromide staining. Subsequently, the amplified products were sequenced (ABI PRISM DNA sequencer Model 377, Perkin Elmer) using the gene specific primers (Table 3.3). Sequences were aligned to the genome sequence of *M. tuberculosis* H37Rv (www.genolist/tuberculist/) using BioEdit Sequence Alignment Editor software (Ibis Biosciences, Carlsbad, CA, USA) and Basic Local Alignment Search Tool (BLAST) analysis (www.ncbi.nlm.nih.gov/BLAST/).

Table 3.3 Primer sequences used to amplify drug resistance associated genes in *M. tuberculosis*.

Gene		Primer sequence (5'-3')	T _m (°C)	Product length (bp)
<i>rpoB</i>	Forward	TGGTCCGCTTGCACGAGGGTCAGA	78	437
	Reverse	CTCAGGGGTTTCGATCGGGCACAT	76	
<i>katG</i>	Forward	TGGCCGCGGCGGTTCGACATT	62	419
	Reverse	GGTCAGTGGCCAGCATCGTC		
<i>inhA</i> promoter	Forward	CGCAGCCAGGGCCTCGCTG	55	246
	Reverse	CTCCGGTAACCAGGACTGA		
<i>gyrA</i>	Forward	TGACATCGAGCAGGAGATGC	62	324
	Reverse	GGGCTTCGGTGTACCTCATC		
<i>embB</i>	Forward	CGGCATGCGCCGGCTGATTC	64	260
	Reverse	TCCACAGACTGGCGTCGCTG		
<i>rrs</i>	Forward	TGCTACAATGGCCGGTACAA	62	290
	Reverse	CTTCCGGTACGGCTACCTTG		
<i>pncA</i>	Forward	GCTGGTCATGTTCCGCGATCG	62	700
	Reverse	CGCCGCCAACAGTTCATCC		

3.3.2. Spoligotyping

M. tuberculosis isolates were genotyped according to the internationally standardized method of spoligotyping as previously described (2).

3.3.3. Drug susceptibility testing

This method was adapted and modified from Siddiqi *et al.*, 2012 (3). A BACTEC™ MGIT™ 960 vial was supplemented with 0.8ml OADC, and subsequently inoculated with 0.1ml Freezer stock of the respective *M. tuberculosis* clinical isolates. The MGIT was incubated in the BACTEC™ MGIT™ 960 instrument at 37°C. Following indication of growth positivity, 0.1ml of the MGIT positive culture was sub-cultured into a fresh MGIT vial, supplemented with 0.8ml OADC. Once this vial was indicated positive growth, drug susceptibility tests (DSTs) were set up for RIF and INH. Briefly, 0.8ml OADC was added to each MGIT vial. For the rifampicin DST 0.1ml of rifampicin was added to a final concentration of 1µg/ml (critical concentration as defined by the WHO). For isoniazid two DSTs were set up per sample, where 0.1ml of isoniazid was added to a final concentration of 0.1 and 1µg/ml isoniazid to differentiate between low and high level INH resistance, respectively. The prepared MGIT vials were inoculated with 0.5ml of the positive MGIT culture. In addition 0.5ml of a 1:100 dilution of the MGIT positive culture was inoculated into an OADC supplemented MGIT vial in the absence of antibiotic as a growth control. Inoculated MGIT vials were incubated in the BACTEC™ MGIT™ 960 instrument at 37°C; the growth unit (GU) was monitored until the growth control reached a GU of 400. An isolate was defined as resistant if the experiment tube (containing drug) had a GU of 100 or more when the GU of the growth control reached 400. Conversely, an isolate was scored as sensitive if no growth was observed i.e. no change in GU, or the GU had not reached 100 when the growth control had reached a GU of 400.

3.4. DNA extraction

DNA was extracted according to standard protocols (4). Briefly, *M. tuberculosis* isolates cultured on 7H10 agar supplemented with OADC were heat killed at 80°C for one hour before being scraped into a 50ml tube containing glass beads and extraction buffer (Appendix 1), and vigorously vortexed. The bacilli were then treated by incubation at 37°C for 2 hours in the presence of 100mg/ml of lysozyme (Roche, Germany) and 50mg RNase A (Roche Germany). The bacilli were subsequently digested with 1.5mg proteinase K (Roche, Germany) in the presence of 1X proteinase K buffer (Appendix 1) at 42°C for 16 hours. To remove pretentious material an equal volume of phenol:chloroform;isoamyl alcohol (25:24:1) was added to each digest and inverted every 30 minutes, for 2 hours. Phase separation was achieved by centrifugation at 3000rpm for 20 minutes at room temperature. Thereafter the aqueous phase was transferred to a clean 50ml tube containing an equal volume of chloroform:isoamyl alcohol

(24:1). Following inversion the mixture was separated by centrifugation at 3000rpm for 20 minutes at room temperature. DNA from the resultant aqueous phase was precipitated following the addition of 1/10 volume of 3M sodium acetate (pH5.2) and an equal volume of isopropanol. The precipitated DNA was collected on a glass rod, washed in 70% ethanol for 10 minutes and left to air dry at room temperature before being dissolved in TE (Appendix 1).

3.5. RFLP DNA fingerprinting

DNA was subjected IS6110 RFLP fingerprinting according to the internationally standard protocol (4).

3.6. Illumina sequencing

WGS of *M. tuberculosis* isolates demonstrating *in vivo* evolution of drug resistance was done at the Next Generation Sequencing Facility (NGFS) at the University of the Western Cape and at the Centre for Proteomic and Genomic Research (CPGR), Cape Town, South Africa. DNA was sequenced on the IlluminaMiSeq platform using the MiSeq Reagent kit v3 (Illumina, CA, USA) generating paired end reads.

3.7. Bioinformatic analyses

Bioinformatic analysis of all Illumina sequencing data was done in collaboration with Dr M de Vos, Dr R van der Merwe and Dr A Dippenaar in the Department of Biomedical Sciences, Stellenbosch University. All Illumina raw sequencing data were in the fastq file format, which was used as a starting point for subsequent bioinformatic analysis. The approach followed for the analysis of each isolate is depicted in Figure 3.1. The raw sequencing reads were run through an automated pipeline developed by Dr R van der Merwe (manuscript in preparation). The steps involved in this pipeline are described briefly below.

3.7.1. Quality assessment

Raw sequencing reads for each *M. tuberculosis* isolate were evaluated using FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). This software validates the raw sequencing reads and generates quality scores based on the quality of the sequences, the content of the sequences as well as the level of duplication which occurred during sequencing. In addition the following data is generated for each isolate: read length, number of reads processed and the GC content of the reads.

3.7.2. Alignment and mapping to *M. tuberculosis* H37Rv reference genome

Trimmomatic version 0.32 (5) was used to identify and remove adapter sequences used for Illumina sequencing. In addition, Trimmomatic-0.32 removes low quality sequences by utilising a sliding window to assess the base quality across each read. It has been shown that using Trimmomatic-0.32 for pre-processing of Illumina reads improves the quality of the down-stream analyses steps (5).

Trimmed Illumina reads were subsequently aligned to the *M. tuberculosis* H37Rv reference genome using 3 aligners, namely: Novoalignv3-02-04 (<http://www.novocraft.com/main/index.php>), Burrows-Wheeler Alignment tool (BWA) v0.7.8 (6) and SMALT v0.7.5 (https://www.sanger.ac.uk/resources/software/smalt/#t_1). Utilisation of three independent aligners, each one using a different algorithm, minimises the identification of false positive variants being called due to mis-alignment. Each aligner used in this study required the reference genome to be indexed. The *M. tuberculosis* H37Rv genome used for this study was accessed from <http://www.ncbi.nlm.nih.gov/nucleotide/AL123456.3>.

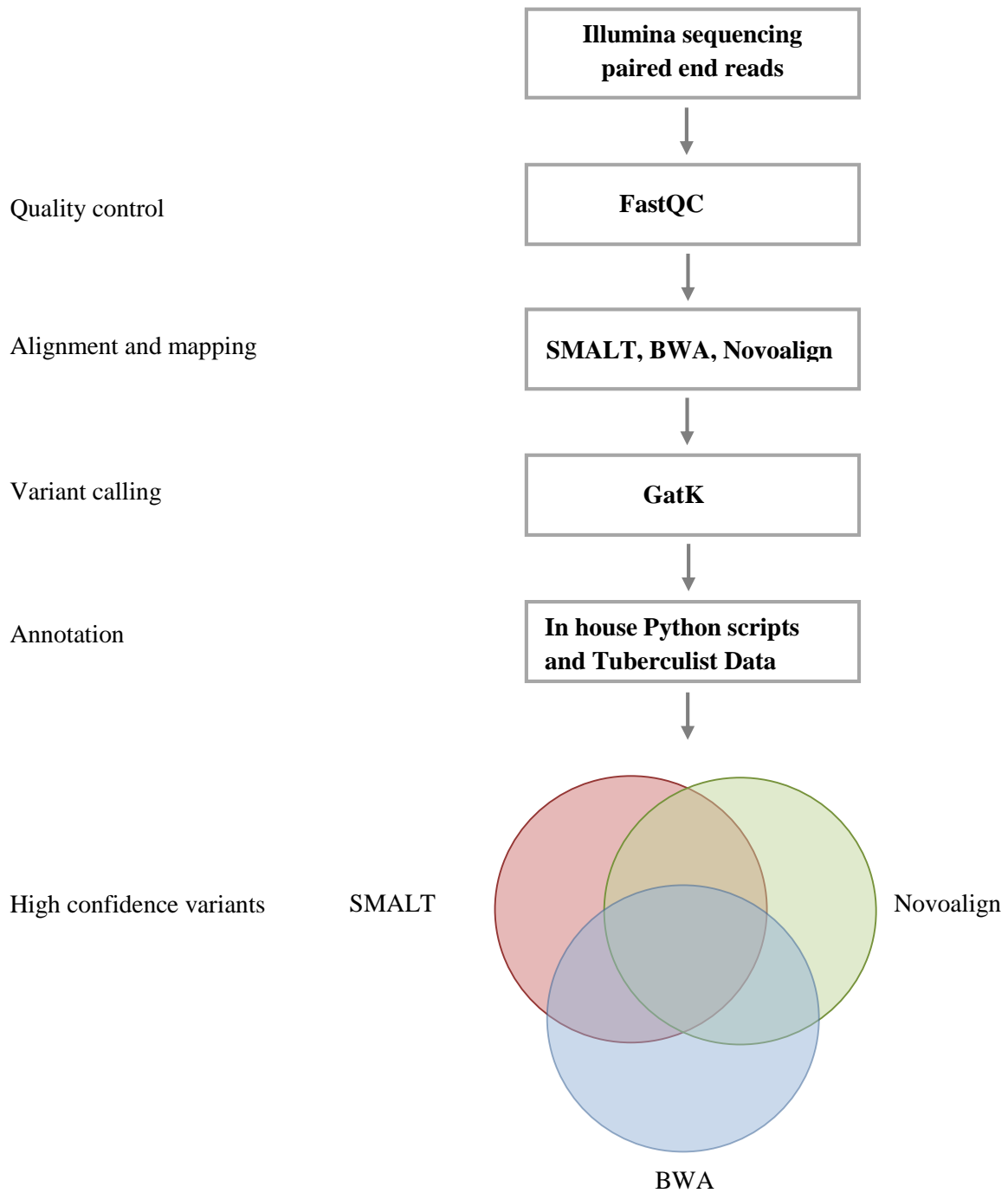


Figure 3.1 Schematic representation of the bioinformatics pipeline used in this study to assess Illumina sequencing data.

3.7.2.1. *Novoalign*

Novoalign utilises the Needleman-Wunsch algorithm to align Illumina reads to the reference genome.

3.7.2.2. BWA

BWA utilises Burrows-Wheeler Transform (BWT) to align short reads to a reference genome.

3.7.2.3. SMALT

SMALT utilises a banded Smith-Waterman algorithm to align Illumina reads to an indexed reference genome.

Each aligner generates an output file in a Sequence Alignment/Map format (SAM). Each SAM file generated by the different aligners is subsequently validated using Picard version 1.107 (<http://picard.sourceforge.net>). Sam tools was then used to convert the SAM files generated to binary format (BAM file), before sorting and indexing the resultant BAM file.

3.7.2.4. Conversion, sorting and realignment of BAM files

Following this conversion and sorting of the BAM files the Genome Analysis Toolkit (GatK) version 3.1-1 was used to realign misaligned reads by insertions and deletions (InDels) within the sequenced genome. Picard version 1.107 was subsequently used again to sort and index these realigned BAM files. The “Mark Duplicates” function of Picard version 1.107 was used to find and remove PCR duplicates which may have been generated during the sequencing reaction. Once all PCR duplicates were removed SAM tools was used to index this BAM file again.

3.7.2.5. Variant calling and annotation

The resultant BAM files were used to identify two types of variants, namely single nucleotide polymorphisms (SNPs) and InDels. The UnifiedGenotyper tool of GatK was used to call variants with a quality value above 50. Any variants with a quality score between 10 and 50 were still included for further analysis.

Three separate lists of variants were generated from this analysis, one for each aligner used. Polymorphisms which were present in all three lists were extracted using in-house python scripts (Dr R van der Merwe, manuscript in preparation) and considered to be high confidence polymorphisms (Figure 3.1). These variants were annotated using Tuberculist data (<http://genolist.pasteur.fr/TubercuList/>) and in-house python scripts.

3.7.2.6. Phylogenetic analysis

A molecular phylogenetic analysis was done to confirm that the *M. tuberculosis* isolates from the same patient cluster together in the correct lineages. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (7). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (8). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 20961 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (9).

3.7.3. Identification of polymorphisms unique to each rifampicin mono-resistant and MDR isolate

To identify polymorphisms associated with the evolution of drug resistance i.e. unique to each rifampicin mono-resistant and MDR isolate the lists of high confidence variants generated in section 3.7.2 were compared and a list of unique polymorphisms were generated for each isolate using in-house python scripts. In addition, a list of common polymorphisms shared between the rifampicin mono-resistant and MDR isolate was generated for each sample set.

3.7.4. Validation of polymorphisms

High confidence polymorphisms were manually inspected using Genomeview (www.genomeview.org) to ensure that a polymorphism considered unique to one isolate was indeed not present in the paired isolate. Polymorphisms were subsequently filtered based on their quality values (a quality score of 50 was used as a cut-off). A final list of polymorphisms for each isolate was validated using targeted PCR and Sanger sequencing. Briefly, an aliquot of genome DNA was added to the following reaction mix containing: 1XQ buffer, 1X PCR buffer, 2mM MgCl₂, 0.4mM dNTPs, 50µM of each primer (Table 3.4) and 1.25U Hot Star Taq polymerase (Qiagen, San Diego, CA, USA). Amplification was done under the following conditions: 95°C for 15 minutes; 40 cycles of 95°C for 30s, primer T_m for 30s (Table 3.4), and 72°C for 2 minutes; one cycle of 72°C for 5 minutes.

PCR products were electrophoretically fractionated in a 2% agarose gel (100 volts, 1 hour) and visualised after ethidium bromide staining. Subsequently, the amplified products were sequenced (ABI PRISM DNA sequencer Model 377, Perkin Elmer) using the gene specific primers (Table 3.4). Sequences were aligned to the genome sequence of *M. tuberculosis* H37Rv (www.genolist/tuberculist/) using BioEdit Sequence Alignment Editor software (Ibis Biosciences, Carlsbad, CA, USA) and Basic Local Alignment Search Tool (BLAST) analysis (www.ncbi.nlm.nih.gov/BLAST/).

Table 3.4 Primer sequences used to validate polymorphisms identified by whole genome sequencing analysis.

Isolate	Gene	Primer sequence (5'-3')	T _m (°C)	Product length (bp)
R721 + R807	<i>Rv0435c</i>	CGGAGACCGGGTCAAAG CGGCAGGCCAAGCTC	60	250
	<i>Rv0668</i>	GCGGTGAGGAAGTGGTCTAC GTCGTGGATCGACACACCTT	60	242
	<i>Rv0965c</i>	GGGTGATGTGGGCATCAAG GTGGTGTGACGTCAGCAAAG	62	249
	<i>Rv1850</i>	ACGGCTACCGTCTGGACAT CCGGTCTTCGGTGACCTC	61	250
	<i>Rv3218</i>	CTGATCGACTGCGGTGAG AAGTGCACTCCGGTAATTGG	59	218
	<i>Rv2004c</i>	GCCGATGACATCTTCTTGGT ACCACCGCGCGATAAG	60	233
	<i>Rv3563</i>	G TTCACCTGCGAGCATGAC CACAGCGATTCGTCGTAGC	60	230
	<i>Rv0667</i>	GCTGACCGCGGAGGA CGATCACGCCCTTGTTG	60	248
	<i>Rv3696c</i>	ACACCAACGCGCACCT AGAACCCGACCGCCAAG	60	249
R912 + R1210	<i>Rv1128c</i>	GTGCCTGCACATAGCATTCA CTCCTCCTCGGTGGATTGT	60	227
	<i>Rv2236c</i>	TGATCGGCTGCCTGCT AGCGCTGCCAGGTG	60	248
	<i>Rv2664</i>	GCCCGAAGTTCTACGACTACC CCTGGCGGGTGATGC	60	240
	<i>Rv2772c</i>	TTGAGCCCCTACTCGATCTG CCAATGACCGGCAGGAT	60	201
	<i>Rv2984</i>	CATCCGACTCAAGATGAATGC TGCCGATCCAGAACTCGT	60	227
	<i>Rv3391</i>	CGTGCCGGTCGACTATGT	60	235

		GGTGGCCGCCATGTT	
R6264 + R15312	<i>Rv3537</i>	TCTCGGAGCGCAACTCTC GAATCCAGCCACCTGCTC	60 238
	<i>Rv0534c</i>	GGCCGTGTGGTGGAAA CTGAGCAGCGCCAAAAC	60 236
	<i>Rv0669c</i>	GCATTCACCAGCGACTTAGG TGGTCAGGTTGTACAGCAGGT	60 246
	<i>Rv1459c</i>	ACCCTCATCGTCGGAATTTT CACGTGGAATCAGCGTAGG	60 242
	<i>Rv1484</i>	CTGATTCAGCGCATCACC GCATACGAATACGCCGAGAT	60 248
	<i>Rv1629</i>	GCATCACCGTGCTCTCC CTAGGGGTGAGCCCGTACTT	60 226
	<i>Rv2935</i>	GCGTCGTTTTCTGTTTCC GCCGAAAGTGTCGACCAAC	60 248
	<i>Rv1415</i>	CAAGGATGGTGGGGTTCTG TCAATGTGCTTCTCGTGCTT	60 241
	<i>Rv0932c</i>	CAGCGGTAAGCCTGTTGG ATCGCACGTAGGCATAGACG	61 189
	<i>Rv1362c</i>	TCAACACCGAAACCACTGAC GGGTCGGTATTGCTCAAGGT	60 248
	<i>Rv1844c</i>	CCCAACTGAAAGGCAGGAC AAGAAATTCGGGGATCGTTT	60 226
	<i>Rv1925</i>	CCGCGTTGCGATCCT TTGATCGATGACGAGGACTG	60 250
	<i>Rv2016</i>	GCATAGATAGCCCCAGACC CTGATGGTGCCAACAAGAGC	61 186
	<i>Rv2048c</i>	GACGGCACCGGATTCTC CCCCTACGGTGTTGAACG	60 992
<i>Rv3043c</i>	GGGTGGACCGCCTACAC CGTGAGCAGCGGGAAC	60 237	

3.8. Minimum Inhibitory Concentration (MIC) determination

This technique was adapted and modified from (10). Briefly, *M. tuberculosis* isolates were cultured in 7H9 supplemented with OADC to an OD₆₀₀ of 0.2-0.3 and thereafter a 1:100 dilution was prepared in 7H9 media. MIC plates were set up in 96-well round-bottom micro titre plates. A volume of 50µl 7H9 supplemented with OADC was added to each well in rows 2 to 12. No media was added to row 1.

In row 1, 100µl of media was added to the first well to serve as a control. An additional control of 10% DMSO (diluted in media) was added to the second. Rifampicin at a concentration 4 x greater than the highest desired rifampicin concentration was added to the remaining 6 wells

of row 1 (diluted in 10% DMSO in media). From row 1 through to row 12 a 1:2 dilution series was carried out, subsequently diluting rifampicin in each new row. A volume of 50µl of the 1:100 dilution of *M. tuberculosis* was added to every well in rows 2 to 12. The micro titre plates were then sealed and incubated at 37°C for 7 to 14 days. Plates were subsequently scored for growth or no growth to determine the rifampicin MIC. A range of concentration from 20 to 250µg/ml rifampicin was tested.

The MIC can be defined as the lowest anti-TB drug concentration where there was no visible *M. tuberculosis* growth (11).

3.9. Determination of the role of efflux pump in drug resistance in *M. tuberculosis* (inhibitor experiment)

3.9.1. Effect of efflux pump inhibitor verapamil on the growth of *M. tuberculosis* to rifampicin

Inhibitor experiments were carried out in the BACTEC™ MGIT™ 960 instrument as described above, and were adapted from Siddiqi *et al.*, 2012 (3). The following vials were set up for each *M. tuberculosis* isolate: a growth control (MGIT inoculated with a 1:100 dilution of the culture), an positive growth control (MGIT inoculated with undiluted culture), rifampicin control (MGIT inoculated with undiluted culture and containing a final concentration of 10µg/ml RIF), verapamil control (MGIT inoculated with undiluted culture and containing a final concentration of 50µg/ml verapamil) and an experimental vial (MGIT inoculated with undiluted culture and containing a final concentration of 10µg/ml rifampicin and 50µg/ml verapamil). Inoculated MGIT vials were incubated in the BACTEC MGIT™ 960 instrument at 37°C; the GUs were monitored until the growth control reached a GU of 400.

The percentage of susceptibility restored by efflux pump inhibitor verapamil was calculated at previously described (12), at the point in growth were the growth control reached a GU of 400.

$$\frac{((GU_{\text{DRUG}} - GU_{\text{EPI+DRUG}}) - (GU_{\text{CONTROL}} - GU_{\text{EPI}}))}{GU_{\text{DRUG}}}$$

Where: GU_{DRUG} = growth units in the presence of 10µg/ml rifampicin

GU_{EPI} = growth units in the presence of 50µg/ml verapamil

$GU_{EPI+DRUG}$ = growth units in the presence of 10 μ g/ml rifampicin and 50 μ g/ml and verapamil
 $GU_{CONTROL}$ = growth unit in the absence of any drug (undiluted growth control)

3.9.2. Effect of efflux pump inhibitor verapamil on the level of rifampicin resistance of *M. tuberculosis*

The MIC values for rifampicin were determined in the presence and absence of efflux pump inhibitor verapamil in the BACTEC™ MGIT™ 960 instrument as described above, and adapted from Siddiqi *et al.*, 2012 (3). The following vials were set up for each *M. tuberculosis* isolate: MGIT inoculated with a 1:100 dilution of the culture), a positive growth control (MGIT inoculated with undiluted culture), and vials for MIC determination. Each concentration used had 2 MGIT vials, one containing 50 μ g/ml verapamil, and one with no verapamil present.

For rifampicin concentrations a $\frac{1}{4}$ and $\frac{1}{2}$ MIC were used (based on the MIC determined for each isolate in section 3.8). For each rifampicin concentration 2 vials were used, one containing 50 μ g/ml verapamil, and one with no verapamil present. Inoculated MGIT vials were incubated in the BACTEC MGIT 960 instrument at 37°C; the MIC was determined when the 1:100 growth control reached a GU of 400. Experiments were monitored for a further 7 days.

3.10. Gene expression analysis

M. tuberculosis was inoculated into 5ml of 7H9 supplemented with ADC as an initial starter culture. At an OD_{600} of 0.8 *M. tuberculosis* was sub-cultured into 50ml 7H9 supplemented with ADC for the growth curve cultures. OD_{600} readings were measured over a period of 28 days to generate growth curves for each *M. tuberculosis* isolate in order to determine mid-log phase. Once the mid-log phase was determined, each *M. tuberculosis* isolate was cultured in 7H9 supplemented with ADC. Two cultures were set up for each isolate. At mid-log on culture of *M. tuberculosis* was exposed to rifampicin concentration equivalent to $\frac{1}{4}$ MIC (determined in section 3.8) for a period of 24 hours before RNA was extracted. The second culture served as an unexposed control culture.

3.10.1. RNA extraction

M. tuberculosis RNA was isolated using the FastRNA® Blue kit (MP Biomedicals LLC, CA, USA). This technique was adapted and modified from the manufacturer extraction protocol. Briefly, 5 culture volumes of GITC solution (Appendix 2) was added to each *M. tuberculosis*

culture before centrifugation at 4000rpm for 10 minutes at 4°C. Pellets were resuspended in 1ml of RNAPro solution before being transferred to a 2ml screw-cap containing Lysing Matrix B. Culture suspensions were subsequently homogenised in a FastPrep®-24 Instrument (MP Biomedicals LLC, CA, USA) using 4 runs of 25 seconds at a setting of 6.5W. Samples were cooled on ice for 1 minute between each run. Following decontamination the lysates were removed from the BSL-3 laboratory and centrifuged at 12 000 rpm for 10 minutes at 4°C. The supernatant was transferred to 300µl of chloroform (Sigma-Aldrich, St Louis, Germany), vortexed and incubated for 5 minutes at room temperature. The RNA containing aqueous phase was separated by centrifugation at 12 000 rpm for 10 minutes at 4°C, and transferred to a new RNase free Eppendorf tube containing 500µl RNase free absolute ethanol (Sigma-Aldrich, St Louis, Germany) (cooled to -20°C). Samples were inverted to allow for mixing to occur before incubation at -20°C for 1 hour. Nucleic acids were subsequently pelleted by centrifugation at 12 000 rpm for 20 minutes at 4°C before being washed with 500µl RNase free 75% ethanol (Sigma-Aldrich, St Louis, Germany) (cooled to -20°C). Following the wash step the supernatant was discarded and the nucleic acid pellet left to air dry for 5 minutes. Pellets were resuspended in a total volume of 50µl RNase free water and stored at -80°C until further use.

3.10.2. DNase treatment

RNA samples were treated with RNase-free DNase to remove any residual genomic DNA (gDNA). A volume of 10µl of RNA was added to the following reaction: 10µL of DEPC-treated H₂O, 4µL of DNase Buffer and 4µL of RQ1 DNase (Promega, WI, USA). The DNase treatment reaction mixture was incubated at 37°C for 30 minutes before RNase-free water was added to the reaction to a total volume of 200µl. An equal volume of cooled phenol:chlorophorm (4:1, v/v) (Sigma-Aldrich, St Louis, Germany) was added before mixing by vortexing and incubation on ice for 10 minutes. Samples were subsequently centrifuged at 12 000rpm for 10 minutes at room temperature to allow for phase separation. The upper aqueous phase was collected and added to 0.1 volumes of RNase-free sodium acetate (pH 5.2) (Sigma-Aldrich, St Louis, Germany) and 2.5 volumes cooled absolute ethanol. Following mixing by vortexing, samples were incubated overnight at 4°C. DNA-free RNA was pelleted by centrifuged at 12,000 rpm for 30 minutes at 4°C and the resulting pellet was washed with 500µL of cooled 75% ethanol. The 75% ethanol was aspirated and the RNA pellet was left to air-dry for 10 minutes before resuspending in a total volume of 13µl water.

3.10.3. Assessment of RNA

An aliquot of each RNA sample was removed for quality assessment. A volume of 1 µl DNase-treated RNA was added to the following PCR reaction mixture to assess gDNA contamination: 1X Q buffer, 1X PCR buffer, 2mM MgCl₂, 0.4mM dNTPs, 50µM of each forward and reverse *gyrA* primer (Table 3.1) and 1.25U Hot Star Taq polymerase (Qiagen, San Diego, CA, USA). Amplification was performed under the following conditions: 95°C for 15 minutes; 40 cycles of 95°C for 30s, primer 62°C for 30s, and 72°C for 2 minutes; one cycle of 72°C for 5 minutes. *M. tuberculosis* genomic DNA was added to a reaction mix to serve as a positive PCR control.

PCR products were electrophoretically fractionated in 2% agarose gel (100 volts, 1 hour) and visualised after ethidium bromide staining.

An aliquot of DNA-free RNA was sent to the Stellenbosch University Central Analytical Facility (CAF) for Bioanalyser analysis to determine the integrity and concentration of RNA. Bioanalyser analysis generates an RNA Integrity Number (RIN) score for evaluation of RNA integrity. A value of 10 indicates completely intact RNA, while a value of 5 indicates partial degradation. RNA samples with a RIN score greater than 7 were used for further analysis.

3.10.4. cDNA synthesis

The QuantiTect Reverse Transcriptase Kit (Qiagen, San Diego, CA, USA) was used to synthesise cDNA from purified RNA according to manufacturer's instructions. An amount of 0.5µg RNA was added to the following reaction mix to eliminate any residual gDNA: 2µl gDNA buffer and RNase-free water (to a final volume of 14µl). The gDNA wipeout reaction was incubated at 42°C for 2 minutes. Quantiscript RT buffer (1x), RT Primer mix (an optimised blend of oligo-dTs and random primers) and 1µl Reverse transcriptase. The reverse transcriptase mix was incubated at 42°C for 15 minutes, followed by incubation at 95°C for 3 minutes to inactivate the reverse transcriptase. A no Reverse transcriptase control was included for each sample i.e. a cDNA synthesis reaction was set up without the Reverse transcriptase enzyme.

3.10.5. Primer design for Quantitative Real Time PCR

Primers were designed to investigate the expression of an array of efflux pumps and energy metabolism genes in *M. tuberculosis* (Table 3.5). To investigate the response of energy

metabolism genes to rifampicin exposure one gene encoding each component of the electron transport chain was selected. To investigate the role of efflux pumps in drug resistance as well as in response to rifampicin exposure, numerous ABC transporter and MFS pumps were selected as well as one RND class pump.

Table 3.5 Primer sequences used to amplify cDNA for quantitative real time PCR analysis.

	Gene		Primer sequence (5'-3')	T _m (°C)	Product length (bp)	
Housekeeping	<i>sigA</i>	Forward	GTGCACATGGTCGAGGTGAT	62	114	
		Reverse	CGGGGTGATGTCCATCTCTT			
	16S	Forward	CTGGGTTTGACATGCACAGG	62	104	
		Reverse	ACCCAACATCTCACGACACG			
Efflux pump genes	<i>Rv1819c</i>	Forward	ATGTTCGATCGGGGTTTTTCAG	62	133	
		Reverse	CGAGCCAGTCTTGTGTGAGG			
	<i>DrrA</i>	Forward	GTATCTCGAGGAGGCCGGATG	62	127	
		Reverse	GGGCACTATTTTCGCAGAAGG			
	<i>EmrB</i>	Forward	ACATGACGATGACGGTGCTC	62	124	
		Reverse	AGGCCCTGTGGGATGATATG			
	<i>Rv3728</i>	Forward	TGCGCAAATCTACGAGCACT	62	129	
		Reverse	TCCTCCACCAAATGGTCTCC			
	<i>PstB</i>	Forward	CGCAGCATCTTCAACTACCG	62	106	
		Reverse	CGAGCACGTTGTCCATGATT			
	<i>Rv0676c</i> (<i>mmpL5</i>)	Forward	TCCCCAGAATGATCCGTACC	62	106	
		Reverse	ATCTGTCCGACCGTTTCCAG			
	Energy metabolism genes	<i>nuoA</i>	Forward	CGGATGTTGTTCATCGTCT	62	122
			Reverse	CGAACACCGTGAGCATGAAT		
<i>sdhA</i>		Forward	ACGAGTTTTACGCGCTGGAT	62	113	
		Reverse	CTTGGCGTGAAAGACATGGA			
<i>menA</i>		Forward	GCTGACGTTTCGTGCTAATGC	62	131	
		Reverse	CACGCAGTACCGGGATCAG			
<i>qcrC</i>	Forward	GTCCACAGAACATGCCGAAG	62	123		
	Reverse	ATCCGCCGAGTAGGTAACCA				
<i>ctaD</i>	Forward	TGGGAGAACTCGAAGCCATT	62	146		
	Reverse	GCCACCGATGAAGAAAAAGC				
<i>atpA</i>	Forward	TAGAAGCTTTCGCCGCTTTC	62	148		
	Reverse	GTGCCCAGGAAGATCGAAAC				

3.10.6. Quantitative Real Time PCR

The iScript™ Reverse Transcriptase Supermix was used to set up all Quantitative Real Time PCR (RT-qPCR) reactions as per manufacturer's instructions (Bio-rad Laboratories, CA, United States). Briefly 1µl of a 1:10 dilution of cDNA was added to 5µl SYBR Green mix, 10µM of each forward and reverse primer and water was added to a final volume of 10µl. A no template control was added to assess contamination. In addition, a no reverse transcription control was included. RT-qPCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-rad Laboratories, CA, United States). The following reaction conditions were used: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, 62°C for 30 seconds followed by 65°C for 5 minutes.

Each RT-qPCR experiment was done on triplicate biological samples that were each assayed in duplicate.

3.10.7. Statistical analysis

16S rRNA and *sigA* were included as housekeeping/reference genes for each RT-qPCR reaction. The level of gene expression of each individual gene was quantified by the delta-delta Ct calculation in which the relative abundance of the target gene was normalized relative to the levels of the housekeeping genes. Data analyses were done according to the delta-delta CT equation $R=2^{-(LCT\ sample - LCT\ control)}$. Only experiments with a standard deviation of <0.5 were included for analysis. Significant fold changes were identified based on The Relative Expression Software Tool -384 (REST-384©) that assigns significance with a significance level of 5% (13).

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CHAPTER 4

Evolution of drug resistance in *Mycobacterium tuberculosis*

4.1. Background

South Africa is ranked by the World Health Organisation as having one of the highest incidences of Tuberculosis (TB) globally. Control of this disease is threatened by the emergence of drug resistance. Multi-drug resistant (MDR) cases are classified as infection with *Mycobacterium tuberculosis* strains that are resistant to two of the first-line anti-TB drugs: isoniazid and rifampicin, while pre-extensively drug resistance (pre-XDR) is defined as MDR infection with additional resistance to a fluoroquinolone or to a second line injectable (kanamycin, amikacin or capreomycin), but not both. XDR is classified as infection with MDR with additional resistance to both a fluoroquinolone and a second line injectable (1, 2). Conventionally, drug resistance in *M. tuberculosis* has been associated with mutations in single target genes (1, 3). For example, a mutation in the *rpoB* gene causes rifampicin resistance. However, an increasing body of evidence suggests that additional mechanisms may be involved in the emergence and subsequent adaptation of *M. tuberculosis* phenotype prior to or following the evolution of drug resistance through a fixed mutation in a defined target gene. It has been shown that the activation of efflux may be the initial gateway mechanism enabling subsequent acquisition of resistance, but not necessarily be the sole cause of clinical resistance (4). Despite the role of efflux (discussed in Chapter 5), other mechanisms contributing to resistance as well as the physiological consequence of mutations has not been thoroughly investigated.

More recently, whole genome sequencing (WGS) has been applied to investigate the evolution of drug resistance based on the hypothesis that additional mutational events may precede or occur concurrently with known resistance conferring mutations (4). A well-known example of such a phenomenon is the observation of compensatory mutations in *M. tuberculosis* (5, 6). Numerous studies have suggested that the development of drug resistance causing mutations may incur a fitness cost in *M. tuberculosis* (7–9). However, *in vitro* culture and epidemiological studies have demonstrated that drug resistant *M. tuberculosis* is able to compensate for the fitness cost associated with the accumulation of drug resistance conferring mutations (7, 8). The mechanisms responsible for ameliorating fitness cost remain largely unknown. Secondary mutations in *rpoA* and *rpoC* have been associated with rifampicin resistance, suggesting that these mutations may play a compensatory role in restoring fitness (5). An epidemiological study in the South African setting recently showed the presence of *rpoA* and *rpoC* mutations in the context of transmission of strains harbouring *rpoB* mutations (6). Evidence suggests that

a subset of *rpoC* mutations are associated with a specific *rpoB* mutation i.e. *rpoB* Ser531Leu (6, 10). However neither of these studies has ruled out other compensatory mechanisms, possibly involved in rifampicin resistance. Similarly, mutations in the promoter region of *ahpC* have previously been associated with isoniazid resistance. These mutations have been suggested to compensate for the loss of katG activity (11).

WGS has also been used as a tool to identify novel mechanisms responsible for drug resistance. Numerous studies have reported on genetic diversity occurring in *M. tuberculosis* during the evolution of drug resistance (Reviewed by Trauner *et al.*, 2014) (4). While some studies have demonstrated genomic stability with low genetic diversity, more recent studies have shown high genetic diversity with the emergence of additional genetic variants above those observed in drug target genes (4, 12–14). These variants have been identified in several genes involved in lipid metabolism, cell wall biosynthesis, purine metabolism, and transcriptional control (4). However, the primary focus of the observed genetic variability has been on known drug resistance causing mutations. Some studies have demonstrated the acquisition of known mutations during the course of infection, highlighting selective evolution during drug treatment (15–17), others have investigated the longitudinal fluctuation in mutation frequencies for one drug resistance causing gene (18). That study highlighted different aspects of genetic variability, but an important conclusion was the importance of investigating sub-populations present within in a patient. The presence of sub-populations is a major concern when considering the sensitivity of standard genetic tests for the diagnosis of drug resistance in *M. tuberculosis* (19).

This study aimed to further investigate the *in vivo* evolution of drug resistance in *M. tuberculosis*, with the overall aim of identifying genetic markers associated with the evolution of drug resistance as well as investigating the power of deep sequencing to identify heterogeneity within *M. tuberculosis* populations for diagnostic purposes.

4.2. Hypothesis

During the evolution of resistance from rifampicin mono-resistant to MDR, the *M. tuberculosis* genome accumulates additional mutations altering its physiology.

4.3. Aims

This study aims to investigate the genetic variation between sequential *M. tuberculosis* isolates obtained from individual patients in a South African setting who developed MDR-TB during treatment of rifampicin mono-resistant TB.

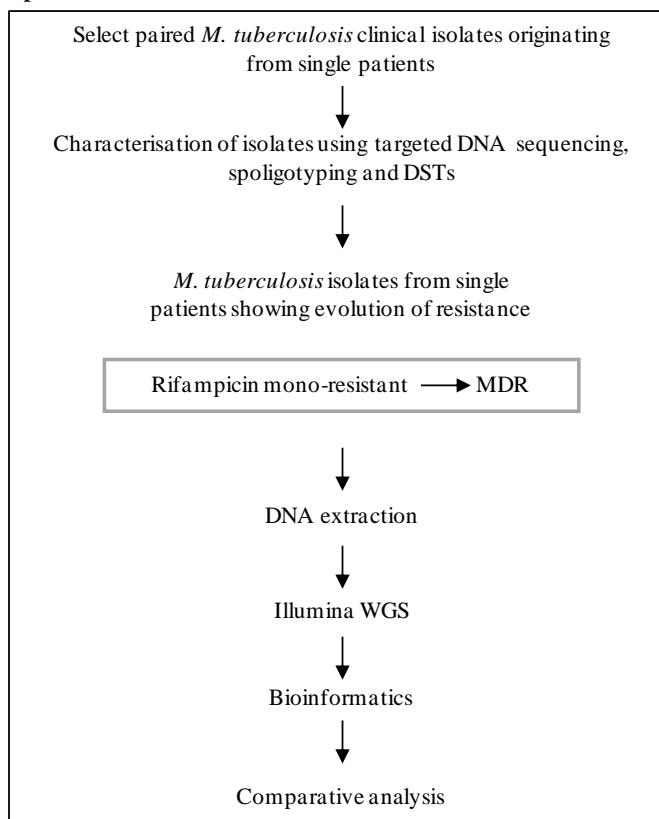
Specific aims:

1. To interrogate the Stellenbosch University *M. tuberculosis* strain bank to enable the identification of serial isolates demonstrating intra-patient evolution of drug resistance.
2. To use whole genome sequencing to investigate the accumulation of genomic mutations occurring during the evolution of MDR-TB.

4.4. Experimental approach

Methods used in this study have been detailed in Chapter 2. The experimental approach followed in this study is outlined in Figure 4.1 below.

Specific aim 1



OUTCOME:

1. Identify paired isolates where *M. tuberculosis* shows acquisition of MDR from rifampicin mono-resistance
2. Identification of variants in each isolate relative to *M. tuberculosis* H37Rv
3. Identification of variants unique to the MDR isolate

Figure 4.1 Approach to investigate the accumulation of genomic changes between sequential *M.*

tuberculosis isolates demonstrating evolution from rifampicin mono-resistance to MDR obtained from individual patients in a South African setting.

4.4. Results

4.4.1. Interrogation of the Stellenbosch University, Department of Biomedical Sciences *M. tuberculosis* strain bank

The Department of Biomedical Sciences, Stellenbosch University, has an extensive culture bank containing approximately 20 000 *M. tuberculosis* isolates (both drug resistant and sensitive), collected from 2001 to present. These clinical isolates have in part been characterised by spoligotyping, targeted DNA sequencing and routine DST. An analysis of the *M. tuberculosis* strain bank was performed based on the available sample information. Patients whose samples are available in the strain bank are represented with a unique patient identifier (ID); no information about patients is available for research purposes. Patients with more than one clinical isolate were selected and analysed to determine the patterns of infection, i.e. a patient was infected with a single strain which maintained the same drug resistance profile or the strain evolved drug resistance during the course of infection. Figure 4.2 shows the breakdown of the samples represented in the strain bank, where multiple samples are available for single patients.

A total of 3354 patients were identified to have multiple samples within the strain bank; together these patients have a total of 11286 samples. The drug resistance classification in the data base was used to discriminate patients whose samples retained the same drug resistance profile throughout infection from those who showed acquisition of drug resistance. Sixty-seven percent of patients (2250) with multiple serial isolates retained the same drug resistance profile at multiple time points (Figure 4.2). A large percentage of patients (19%) were shown to have either mixed infection or re-infection i.e. were infected with multiple strains across serial isolates (based on available spoligotyping results) during the course of infection, or they were unclassified within the strain bank i.e. no DST or genotypic results were available (Figure 4.2). For the purpose of this study the latter samples were excluded from further investigation.

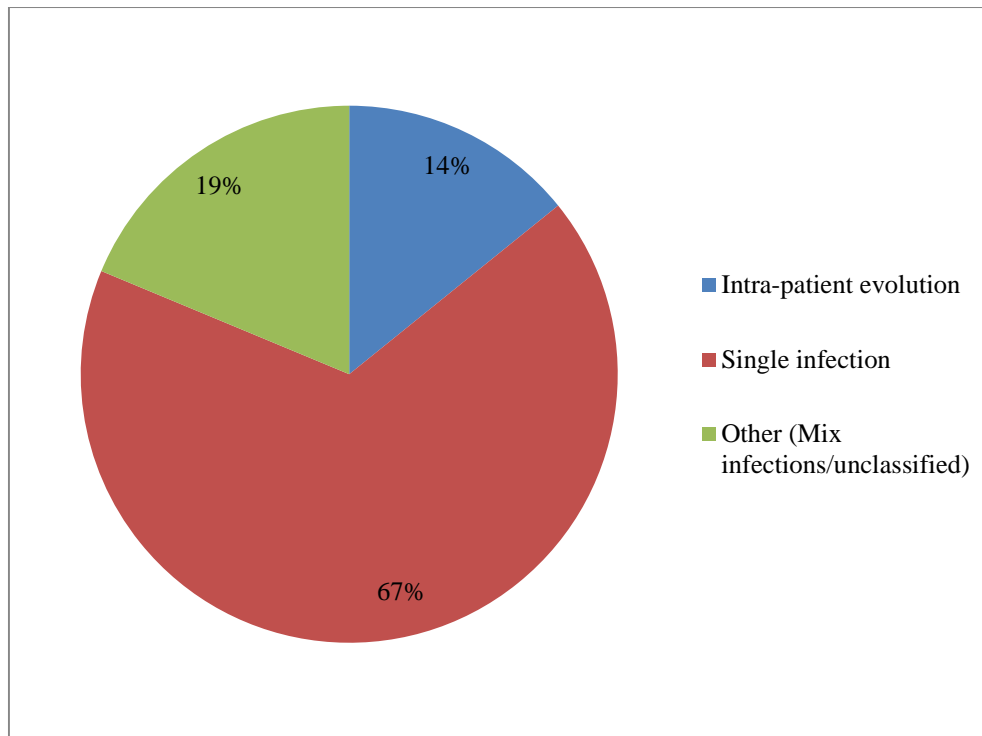


Figure 4.2 Breakdown of the types of sample sets represented in the Stellenbosch University *M. tuberculosis* strain bank. Approximately 67% of patients with sequential samples represented in the strain bank were infected with a strain whose drug resistance profile remained constant during the course of infection. A further 19% of patients were shown to have mixed infection or a reinfection with a different strain, based on spoligotyping results. Approximately 14% of patients were shown to be infected with strains which acquired additional drug resistance during the course of infection.

Approximately 14% of the patients represented in the data base were shown to be infected with strains which acquired resistance to additional anti-TB drugs during the course of infection (476 patients); a total number of 2830 *M. tuberculosis* samples are available for these patients (Figure 4.2). A breakdown of the types of drug resistance acquisition in clinical isolates observed in these patients is shown in Table 4.1. The classification of the drug resistance profile is based on DSTs and in some cases line probe assays. The predominant pattern of evolution was found to be from MDR to pre-XDR (51.89%). The percentage of patients with strains evolving to XDR was 10.71%, while 9.24% of the patient isolates showed the evolution from MDR to pre-XDR to XDR.

Samples demonstrating evolution of resistance from rifampicin mono-resistant to MDR were selected and characterised to confirm the reported phenotypes. A total number of 66 samples originating from 31 patients were selected for characterisation (Appendix 2, Table 1).

Table 4.1 Patterns of drug resistance evolution represented within the Stellenbosch University strain bank from individual patients.






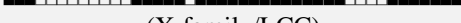
Pattern of evolution	Samples represented in the strain bank % (number of patients)
Rifampicin mono-resistant to MDR	7.35 (35)
Isoniazid mono-resistant to MDR	9.66 (46)
MDR to pre-XDR	51.89 (247)
MDR to XDR	10.71 (51)
MDR to pre-XDR to XDR	9.24 (44)
Pre-XDR to XDR	11.13 (53)

4.4.2. Strain characterisation

Selected *M. tuberculosis* isolates were characterised using PCR and Sanger sequencing of known drug resistance causing mutations, as well as spoligotyping. The initial screen included analysed the *inhA* promoter, and *rpoB* and *katG* genes for resistance-causing mutations (Appendix 2, Table A2.1). Eighteen sample sets (38 isolates) with discrepant genotypes (according to PCR and sequencing) and phenotypes (according to database) were eliminated from further screening since they no longer represented isolate sets demonstrating evolution from rifampicin mono-resistant to MDR. Specifically, these isolates were originally classified as rifampicin mono-resistant, but contained isoniazid resistance-conferring *inhA* promoter or *katG* mutations. The remaining 28 isolates were further characterised by PCR and Sanger sequencing of the *embB*, *gyrA*, *rrs* and *pncA* genes. Following this, a further 16 isolates were eliminated due to the presence of either *gyrA*, *embB*, *rrs* or *pncA* mutations (Appendix 2, Table A2.2). The remaining 16 isolates were subjected to rifampicin and isoniazid DST, to confirm the drug resistant phenotype (Appendix 2, Table A2.3). A total of 6 isolates showed to have the correct drug resistance phenotype, 3 rifampicin mono-resistant isolates, each with a paired MDR isolate. Serial isolates where the initial isolate was rifampicin mono-resistant and the follow-up sample MDR were subjected to spoligotype analysis to confirm that the isolates in each set were of the same strain family (Appendix 2, Table A2.4). Serial isolates were eliminated if the strain family differed between the rifampicin mono-resistant and MDR isolates since this suggested either mixed infections or exogenous re-infection with a different strain and the original *M. tuberculosis* strain responsible for infection had not evolved additional resistance to isoniazid.

A total of 6 isolates, from 3 patients demonstrated *in vivo* evolution of isoniazid resistance. Genotyping of *rpoB*, *katG* and *inhA* promoter as well as the spoligotype for each isolate are shown in Table 4.2. Resistance to isoniazid was conferred by mutations in the *katG* gene or *inhA* promoter for patients 1 and 2 respectively. In contrast, the MDR isolate from patient 3 (R15213) had no *katG* or *inhA* promoter mutations despite phenotypic isoniazid resistance suggesting that an alternative mechanism of isoniazid resistance was present (Appendix 2, Table A2.4). Table 4.2 summarises the genotypic characteristics of the serial isolates from the three patients where intra-patient evolution of isoniazid resistance was observed.

Table 4.2 Genotypic and phenotypic characterisation of 6 *M. tuberculosis* isolates originating from 3 patients demonstrating *in vivo* evolution of drug resistance.

Patient	Isolate name	Phenotypic resistance	<i>rpoB</i> *	<i>katG</i>	<i>inhA</i> promoter	Spoligotype
1	R721	Rifampicin	Ser531Leu	-	-	 (Beijing)
	R807	Rifampicin Isoniazid	Ser531Leu	Gly309Val	-	 (Beijing)
2	R912	Rifampicin	His526Tyr	-	-	 (EAI)
	R1210	Rifampicin Isoniazid	His526Tyr	-	-15	 (EAI)
3	R6264	Rifampicin	Leu511Pro	-	-	 (X-family/LCC)
	R15213	Rifampicin Isoniazid	Leu511Pro	-	-	 (X-family/LCC)

*Amino acid change according to the *Escherichia coli rpoB* gene sequence

Analysis of the respective spoligotype patterns suggested that the *M. tuberculosis* strains remained the same during the course of the disease. However, given the low discriminatory power of spoligotyping for Beijing genotypes as well as the high prevalence of this genotype in the Western Cape of South Africa, IS6110 DNA fingerprinting was done to ensure that the strain in isolate R721 and R807 were identical and from the same Beijing sub-lineage (Figure 4.3). The non-Beijing isolates were included in the analysis to confirm spoligotyping results.

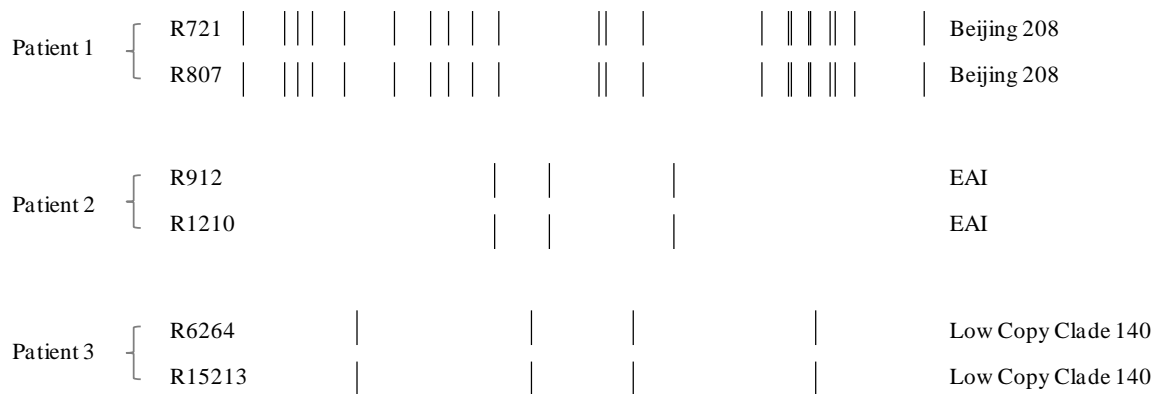


Figure 4.3 RFLP DNA fingerprinting confirming the paired rifampicin mono-resistant and MDR *M. tuberculosis* isolate from each patient are of the same genetic background.

In summary, of the 66 samples (from 31 patients) screened a total of 3 pairs of isolates were confirmed to show *in vivo* evolution of MDR (*sensu stricto*). The 6 isolates comprising these 3 sets are summarised in Table 4.4.

Table 4.4 *M. tuberculosis* isolates demonstrating *in vivo* evolution from rifampicin mono-resistance to MDR.

Patient	Isolate name	Collection date	Drug resistance profile	Strain family
1	R721	22/10/2003	Rifampicin mono-resistant	Beijing
	R807	19/05/2004	MDR	Beijing
2	R912	15/09/2004	Rifampicin mono-resistant	EAI
	R1210	12/08/2005	MDR	EAI
3	R6264	05/07/2009	Rifampicin mono-resistant	LCC
	R15213	03/08/2011	MDR	LCC

4.4.3. Whole genome sequencing

4.4.3.1. Initial whole genome sequencing analysis and quality control

To investigate the accumulation of genetic variants during the evolution of drug resistance, the 6 *M. tuberculosis* clinical isolates (Table 4.3) were sequenced on the IlluminaMiSeq platform. FastQC reports (Appendix 3) were generated for each isolate to assess the quality of the

sequencing data. The genomes of the 6 selected *M. tuberculosis* isolates (Table 4.3) were aligned to *M. tuberculosis* H37Rv reference genome using 3 mappers. Qualimap reports were generated to assess the quality of the alignment and mapping to *M. tuberculosis* H37Rv reference genome (summarised in Table 4.6). The Qualimap report showed the GC content of the mapped reads ranged from 64.97 to 66.17%, which correlates to the GC content of *M. tuberculosis* (20). The percentage of mapped reads was high, ranging from 95.46 to 98.47% indicating successful alignment and mapping of the Illumina reads to the reference genome.

Table 4.6 Evaluation of the quality of the mapped sequencing data generated by the IlluminaMiSeq platform.

	R721	R807	R912	R1210	R6264	R15213
GC Content (%)	64.97	64.86	65.13	65.71	65.66	66.17
Mapping Coverage (%)	95.46	96.98	98.34	98.29	98.46	98.47
Number of Reads Mapped	3 799 676	2 930 788	2 851 935	2 430 795	3 175 563	3 490 840
Mean Read Length (bp)	181.88	177.94	186.72	180.73	168.52	176.63
Coverage Depth (fold)	143.94	112.25	120.36	98.94	120.85	138.97

The number of variants identified relative to *M. tuberculosis* H37Rv using each mapper are shown in Table 4.7. A polymorphism was considered to be high confidence if it was identified in all 3 mappers. Polymorphisms not detected by all three mappers were excluded from this study.

Table 4.7 Number of polymorphisms identified by the 3 mappers used in this study relative to *M. tuberculosis* H37Rv.

	Patient 1		Patient 2		Patient 3		
	R721	R807	R912	R1210	R6264	R15213	
SNPs	BWA	2877	2762	3573	3852	2045	2219
	Novoalign	2047	2033	2853	2887	1500	1526
	SMALT	3367	3508	4618	4634	2625	2745
	High confidence*	1703	1722	2532	2543	1204	1227
	BWA	184	211	260	281	141	140
InDels	Novoalign	204	206	249	253	143	140
	SMALT	193	223	254	268	143	144
	High confidence*	134	134	161	164	92	81
	BWA	184	211	260	281	141	140

*Variants identified in all 3 mappers were considered to be high confidence variants and used for further analyses

4.4.3.2. Identification of high confidence variants unique to rifampicin mono-resistant and MDR isolates

In order to confirm that the *M. tuberculosis* isolates collected from the respective patients were closely related a phylogenetic tree was constructed using whole genome sequencing data of these isolates together with well characterised *M. tuberculosis* isolates with known assigned lineages (Figure 4.3). This phylogenetic analysis showed that the isolates from each patient clustered together, confirming clonality as well as the genetic lineage. R721 and R807 clustered with isolates belonging to lineage 2 which is also known as the East Asian lineage and includes the Beijing genotype (21). R912 and R1210 clustered with isolates belonging to lineage 1 (Indian Oceanic), which includes isolates belonging to the East Africa India lineage. Similarly, R6264 and R15213 clustered with isolates known to belong to lineage 4, classified as Euro-American. Sub-lineages of lineage 4 include isolates from the following families: Latin American Mediterranean (LAM), Family 11 (F11), Low Copy Clade (LCC), T family as well as *M. tuberculosis* reference H37Rv.

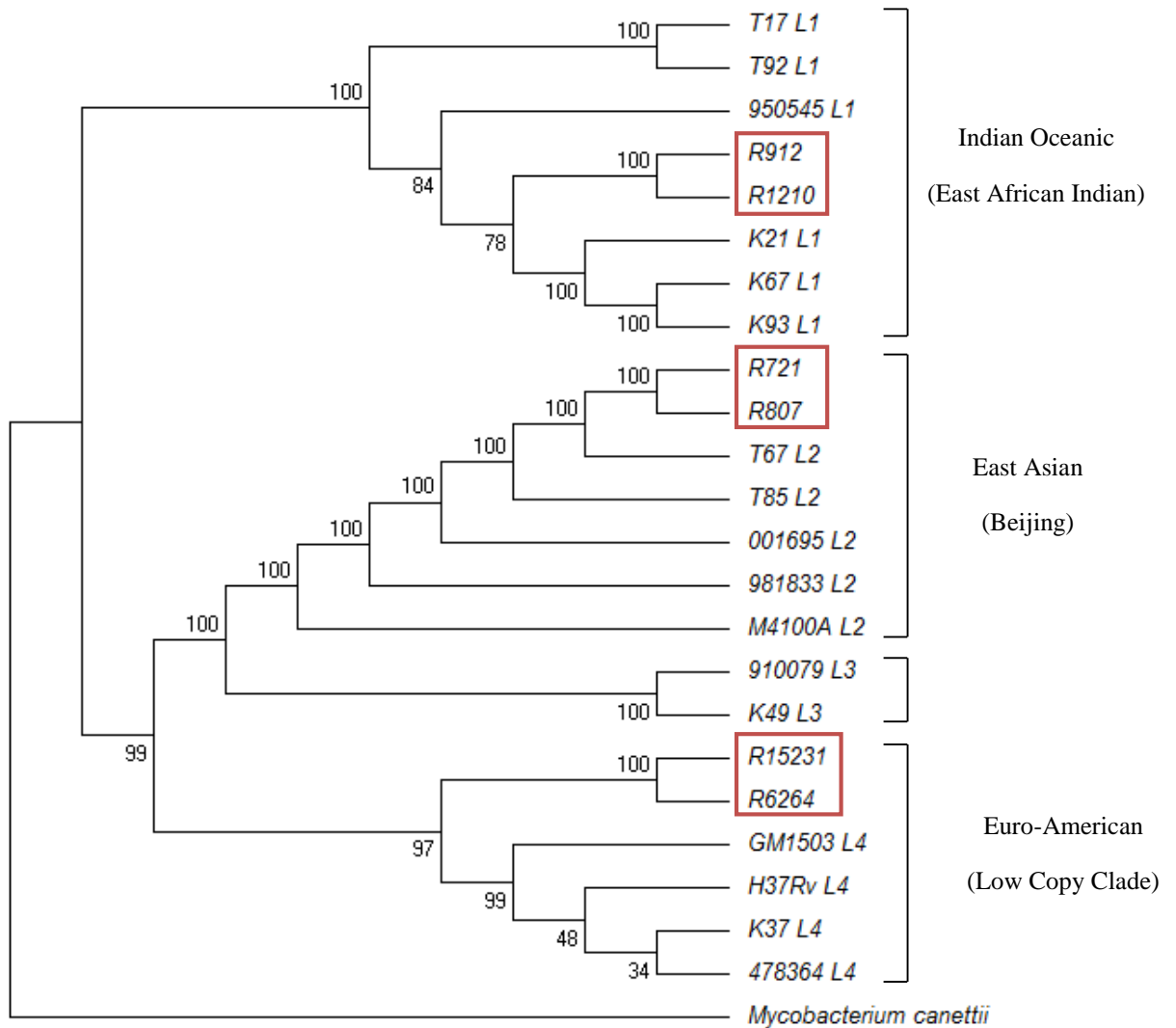


Figure 4.3 Molecular Phylogenetic analysis by Maximum Likelihood method. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

To identify polymorphisms occurring concomitantly with the evolution of isoniazid resistance the genome sequence of the rifampicin mono-resistant isolate was compared to the MDR isolate for each patient sample set, i.e. the polymorphisms for each isolate relative to *M. tuberculosis* H37Rv (Table 4.7) for the rifampicin mono-resistant isolate were compared to those identified in its paired MDR isolate. Polymorphisms of interest were those found to be unique to either the rifampicin mono-resistant or MDR isolate (Table 4.8).

Table 4.8 Number of variants identified to be unique to each isolate within a patient sample set relative to *M. tuberculosis* H37Rv.

		Patient 1		Patient 2		Patient 3	
		R721	R807	R912	R1210	R6264	R15213
SNPs	BWA	656	494	347	570	253	470
	Novoalign	117	61	45	85	34	48
	SMALT	464	565	369	348	222	264
	High confidence*	42	10	16	15	4	14
InDels	BWA	8	29	21	38	15	13
	Novoalign	8	12	8	13	6	4
	SMALT	14	1	18	35	17	25
	High confidence*	3	1	0	2	0	0

*Variants identified in all 3 mappers were considered to be high confidence variants

High confidence polymorphisms were manually inspected using Genomeview (www.genomeview.org) to ensure that a polymorphism considered unique to one isolate was indeed not present in the paired isolate. Polymorphisms were subsequently filtered based on their quality values. In addition, polymorphisms identified in *pe_ppe* genes were excluded from further analysis since these repetitive regions are GC rich and difficult to map efficiently during mapping and alignment to the *M. tuberculosis* H37Rv reference genome.

Polymorphisms identified as unique to either the rifampicin mono-resistant or MDR isolates were validated using targeted PCR and Sanger sequencing (Table 4.9, Table 4.10 and Table 4.11). Tables 4.9, 4.10 and 4.11 show that the majority of the variants identified were only present in a portion of the population with mutant variants being present in as little as 11% of the Illumina reads. Sanger sequencing was used to investigate what percentage of a mutant population would still be visible on a chromatogram. Figure 4.4 shows representative chromatograms demonstrating the ability of Sanger sequencing to detect mutant populations present at low frequency within a population.

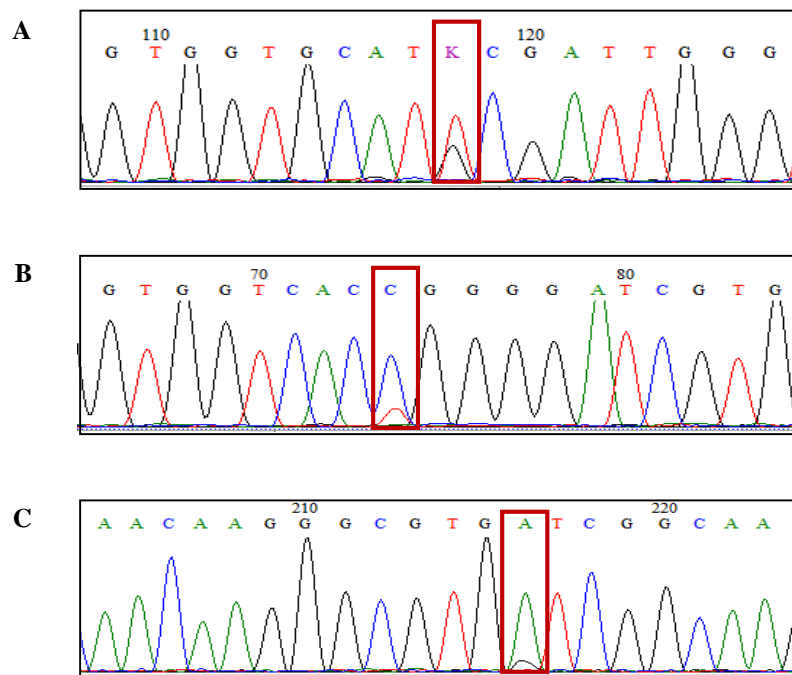


Figure 4.4 Sanger sequencing of wild type and mutant alleles present at varying proportions within the *M. tuberculosis* population. (A) Sanger sequencing chromatogram for *Rv1418*, where 70% of Illumina reads contained the mutant allele. Sanger sequencing scored this as a hetero-variant, denoted by ‘K’ in the sequence. (B) Sanger sequencing chromatogram for *Rv1629*, where 45% of Illumina reads contained the mutant allele. Sanger sequencing scored this as the wild type allele. (C) Sanger sequencing chromatogram for *Rv0667*, where 11% of Illumina reads contained the mutant allele. Sanger sequencing scored this as the wild type allele.

For the purpose of this study a polymorphism was considered a polymorphism as fixed when it represented in 95% of the Illumina sequencing reads, while a heterogeneous polymorphism was defined by a base pair change, insertion or deletion in less than 95% of the reads. For patient 1, only one fixed polymorphism was seen in the *katG* gene in isolate R807, a known isoniazid resistance causing mutation. This polymorphism was present in the original screen confirming that R807 was an MDR isolate. Numerous heterogeneous polymorphisms were observed in both R721 and R807 in genes involved in cell wall metabolism, information pathways, intermediary metabolism and respiration as well as in genes encoding conserved hypothetical proteins (Table 4.9). For patient 2, while R912 showed to have only fixed polymorphisms, the paired MDR isolate, R1210, had only heterogeneous polymorphisms. It is interesting to note that the polymorphisms unique to R912 were subsequently not present in the population after a period of time (approximately 11 months) (Table 4.10). These polymorphisms were also seen in genes involved in cell wall metabolism, information pathways, intermediary metabolism and respiration as well as in lipid metabolism.

Heterogeneous SNPs were also seen in genes encoding conserved hypothetical proteins. For patient 3 only heterogeneous polymorphisms were observed to be unique to the respective isolates.

Table 4.9 Variants identified to be unique during the comparison of the rifampicin mono-resistant and MDR *M. tuberculosis* isolates of patient 1 during the *in vivo* evolution of drug resistance.

Isolate	Variant	Locus	Gene	Amino acid change	Coverage of variant (%)	Gene description	Functional category [§]
R721*		Rv0435c		I397L	50	Putative conserved ATPase	Cell wall and cell processes
		Rv0435c		D395Y	50	Putative conserved ATPase	
		Rv0668	<i>rpoC</i>	V1039A	30	DNA-directed RNA polymerase RpoC (RNA polymerase beta' subunit).	Information pathways
		Rv0965c		syn (132)	12.5	Conserved hypothetical protein	Conserved hypotheticals
	SNPs		<i>Upstream of Rv1482c</i>		18.5	Intergenic (upstream of a conserved hypothetical)	
		Rv1850	<i>ureC</i>	Q11K	48	Urease alpha subunit UreC (urea amidohydrolase)	Intermediary metabolism and respiration
		Rv1850	<i>ureC</i>	Q11R	49	Urease alpha subunit UreC (urea amidohydrolase)	
		Rv3218		Y174H	66	Conserved protein	Conserved hypotheticals
		Rv2004c		A - AAG	43	Conserved protein	Conserved hypotheticals
	InDels		Rv3563	<i>fadE32</i>	A-AC	40	Probable acyl-CoA dehydrogenase FadE32
		Rv3696c	<i>glpK</i>	A-AC	73	Probable glycerol kinase GlpK (ATP:glycerol 3-phosphotransferase)	Intermediary metabolism and respiration
R807[#]		Rv1908c	<i>katG</i>	G309V	100	Catalase-peroxidase-peroxynitritase T KatG	Virulence, detoxification, adaptation
	SNPs	Rv0667	<i>rpoB</i>	I889V	11	DNA-directed RNA polymerase RpoB (RNA polymerase beta subunit)	Information pathways
		Rv3696c	<i>glpK</i>	T91I	80	Probable glycerol kinase GlpK (glycerokinase) (GK)	Intermediary metabolism and respiration

*Rifampicin mono-resistant; #MDR; §Functional category as classified by Tuberculist (<http://genolist.pasteur.fr/TubercuList/> and <http://tuberculist.epfl.ch/>)

Table 4.10 SNPs identified to be unique during the comparison of the rifampicin mono-resistant and MDR *M. tuberculosis* isolates of patient 2 during the *in vivo* evolution of drug resistance.

Isolate	Locus	Gene	Amino acid change	Coverage of variant (%)	Gene description	Functional category ^s
R912*	Rv1128c		G430S	98	Conserved hypothetical protein	Insertion seqs and phages
	Rv2236c	<i>cobD</i>	L269S	97	Probable cobalamin biosynthesis transmembrane protein CobD	Intermediary metabolism and respiration
	Rv2664		H22Q	99	Hypothetical protein	Conserved hypotheticals
	Rv2772c		E149*	97	Probable conserved transmembrane protein	Cell wall and cell processes
	Rv2984	<i>ppk1</i>	P631A	96	Polyphosphate kinase PPK (polyphosphoric acid kinase)	Intermediary metabolism and respiration
	Rv3391	<i>acrA1</i>	syn (248)	99	Possible multi-functional enzyme with acyl-CoA-reductase activity AcrA1	Lipid metabolism
	Rv3537	<i>kstD</i>	syn (378)	98	Probable dehydrogenase	Intermediary metabolism and respiration
R1210#		<i>inhA</i> promoter	-15	45		
	Rv0534c	<i>menA</i>	G219D	20	1,4-dihydroxy-2-naphthoate octaprenyltransferaseMenA	Intermediary metabolism and respiration
	Rv0669c		D574N	17	Possible hydrolase	Intermediary metabolism and respiration
	Rv1459c	<i>mptA</i>	R36C	25	Possible conserved integral membrane protein	Cell wall and cell processes
	Rv1484	<i>inhA</i>	S94A	70	NADH-dependent enoyl-[acyl-carrier-protein] reductase InhA (NADH-dependent enoyl-ACP reductase)	Lipid metabolism
	Rv1629	<i>polA</i>	syn (146)	45	Probable DNA polymerase I PolA	Information pathways

Rv2935	<i>ppsE</i>	C582R	69	Phenolphthiocerol synthesis type-I polyketide synthase PpsE	Lipid metabolism
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*Rifampicin mono-resistant; #MDR;

[§]Functional category as classified by Tuberculist (<http://genolist.pasteur.fr/TubercuList/> and <http://tuberculist.epfl.ch/>)

Table 4.11 SNPs identified to be unique during the comparison of the rifampicin mono-resistant and MDR *M. tuberculosis* isolates of patient 3 during the *in vivo* evolution of drug resistance.

Isolate	Locus	Gene	Amino acid change	Coverage of variant (%)	Gene description	Functional category [§]
R6264*	Rv1415	<i>ribA2</i>	K170I	7	Probable riboflavin biosynthesis protein RibA2	Intermediary metabolism and respiration
	Rv0932c	<i>pst2</i>	A333E	16	Periplasmic phosphate-binding lipoprotein PstS2	Cell wall and cell processes
	Rv1362c		syn (163)	8	Possible membrane protein	Cell wall and cell processes
	Rv1844c	<i>gnd1</i>	G459D	13	Probable 6-phosphogluconate dehydrogenase Gnd1	Intermediary metabolism and respiration
R15231#	Rv1925	<i>fadD31</i>	E126A	11	Probable acyl-CoA ligase FadD31 (acyl-CoA synthetase)	Lipid metabolism
	Rv2016		G1A	11	Hypothetical protein	Conserved hypotheticals
	Rv2048c	<i>pks12</i>	G1638R	16	Polyketide synthase Pks12	Lipid metabolism
	Rv3043c	<i>ctaD</i>	syn (382)	11	Probable cytochrome C oxidase polypeptide I CtaD (cytochrome AA3 subunit 1)	Intermediary metabolism and respiration

*Rifampicin mono-resistant; #MDR

[§]Functional category as classified by Tuberculist (<http://genolist.pasteur.fr/TubercuList/> and <http://tuberculist.epfl.ch/>)

GO enrichment analysis was done using the Rv numbers of the genes where polymorphisms were observed in each individual isolate. No common GO terms were specifically enriched in each isolate. Similarly when the Rv numbers of the polymorphisms seen in all the rifampicin mono-resistant isolates or all the MDR isolates were analysed no GO terms were found to be significantly enriched. However, the number of genes of interest may be too small for an efficient GO enrichment. In addition, the GO terms may be too refined to identify a pattern on a larger scale. Therefore the functional groups used for classification by Tuberculist (<http://genolist.pasteur.fr/TubercuList/> and <http://tuberculist.epfl.ch/>) were used to analyse the type of polymorphisms identified in this study (Table 4.12).

Table 4.12 Functional characterisation of high confidence variants unique to rifampicin mono-resistant and MDR *M. tuberculosis* isolates.

Functional category	Total number of genes	Number of genes unique to rifampicin mono-resistant isolates (%)	Number of genes unique to MDR isolates (%)
Cell wall and cell processes	5	2 (40)	3 (60)
Information pathways	2	1 (50)	1 (50)
Conserved hypotheticals	4	3 (75)	1 (25)
Intermediary metabolism and respiration	9	5 (56)	4 (44)
Virulence, detoxification, adaptation	1	0 (0)	1 (100)
Insertion seqs and phages	1	1 (100)	0 (0)
Lipid metabolism	5	1 (20)	4 (80)

The number of genes observed in each functional category for this analysis was low. However, when comparing the genes containing polymorphisms unique to each isolate it was seen that polymorphisms were predominantly observed in MDR isolates in genes involved in cell wall and cell processes and lipid metabolism. The highest number of polymorphisms observed in the rifampicin mono-resistant isolates was in genes involved in intermediary metabolism and respiration; however the proportion of polymorphism in the rifampicin mono-resistant and MDR isolates is very similar.

4.4.4. Compensatory mutations

The whole genome sequencing data generated was used to investigate the presence of compensatory mutations in each *M. tuberculosis* isolate (Table 4.13). Isolates R912 and R1210 retained the same *rpoC* mutation (Ala172Val) during the course of infection, as well as 2 synonymous SNP at codons 61 and 173. Similarly, R6264 and R15231 retained the same *rpoC* mutation during the course of infection. These mutations were fixed within the population i.e. occurring in more than 95% of the Illumina reads. R721 was found to have an *rpoC* Val1039Ala mutation in 30% of the Illumina reads. This mutation was not present in the subsequent MDR isolate (R807), which had acquired an additional *rpoB* polymorphism (I889V) in 11% of the Illumina reads.

Table 4.13 Mutations identified in the *rpoB* and *rpoC* genes in rifampicin mono-resistant and paired MDR *M. tuberculosis* isolates.

Patient	Isolate	<i>rpoB</i>	<i>rpoC</i>
1	R721 [§]	Ser531Leu*	Val1039Ala
	R807 [#]	Ser531Leu*; I889V; 1075 (syn)	-
2	R912 [§]	His526Try*; 1075 (syn)	Ala172Val; 61 (syn); 173 (syn)
	R1210 [#]	His526Try*; 1075 (syn)	Ala172Val; 61 (syn); 173 (syn)
3	R6265 [§]	Leu511Pro*	Gly594Glu
	R15231 [#]	Leu511Pro*	Gly594Glu

*Amino acid change according to the *Escherichia coli rpoB* gene sequence

[§]Rifampicin mono-resistant; [#]MDR

4.4.5. Department of Biomedical Sciences Genome bank analysis

The Department of Biomedical Sciences has a genome bank of approximately 400 *M. tuberculosis* clinical isolates (at the time of submission) which have been whole genome sequenced. This collection includes both drug sensitive and resistant isolates. The genes of interest identified in this study (Tables 4.9, 4.10 and 4.11) were investigated in this genome bank to determine the possible clinical relevance of polymorphisms in these genes. The majority of the polymorphisms identified in this study were only present in the *M. tuberculosis* isolates investigated, with the exception of the polymorphism identified in *Rv0932 (pst2)* (Ala333Glu). This polymorphism was present in 11 *M. tuberculosis* isolates in the genome

bank, all of which are isoniazid resistant. In addition, the polymorphism identified in *Rv1484* (*inhA*) (Ser93Ala) was shown to be present in 6 MDR *M. tuberculosis* isolates. However, an additional 15 isoniazid resistant *M. tuberculosis* isolates were found to have a different polymorphism in this gene (Ile194Thr). A final gene of interest highlighted in this analysis was *Rv0534*. The polymorphism identified in this study (Table 4.10) was only present in R1210, however a polymorphism at codon 219 (Gly219Asp) of this gene was seen in 33 drug resistant isolates and no drug sensitive isolates.

4.5. Discussion

Alarming, 14% of the patients represented in the data base showed to be infected with strains which showed the evolution of additional drug resistance during the course of infection. However, this may be an under-estimate given that routine drug susceptibility testing is limited to only 4 anti-TB drugs and many of the cases had only a single cultured isolate. In addition, there may be patients who never returned for follow up sample collection, these would therefore be excluded from this 14%. This clearly indicates that treatment may be inefficient in numerous patients, given that additional resistance is acquired during the course of infection. Mixed infection was also observed in a large number of patient isolates indicating possible exogenous re-infection. This finding reflects the high infection pressure of drug resistant TB in communities ravaged by TB as well as nosocomial transmission in healthcare facilities. Analyses of the patient isolates showing acquisition of drug resistance allowed for the generation of a list of *M. tuberculosis* isolates which may be used to investigate the *in vivo* evolution of drug resistance, focusing on numerous evolutionary paths i.e. mono-resistance evolving to MDR, MDR evolving pre-XDR and subsequent XDR as well as MDR evolving XDR. This study focused on the evolution of MDR *M. tuberculosis* isolates from rifampicin mono-resistant progenitor isolates.

Our analysis of rifampicin resistant cases which progressed to MDR-TB highlighted the inaccuracy of routine DST. Our strain bank was shown to have a total of 66 isolates from 31 patients demonstrating evolution from rifampicin mono-resistance to MDR infection. However, subsequent confirmation of genotypic and phenotypic drug resistance, as well as spoligotyping (in a few instances) highlighted the gross inaccuracy of the data for the samples represented in the strain bank – at least for isoniazid which is one of the easier drugs to test. This highlights the potential laboratory error and inaccuracy of the standardised diagnostics and/or data capturing error. Our observation that many of the isolates had resistance to

additional drugs beyond MDR is of additional concern as these patients would have received in appropriate MDR treatment. This would impact the period of infectiousness as well as treatment outcome with the potential for developing XDR-TB.

The results of this study highlight the importance of including genetic testing in the diagnosis of TB disease. Genotypic analysis resulted in the identification of a number of isolates with additional resistance. The simplicity of genetic testing would enable routine laboratories to process higher number of samples with less biohazard exposure.

In depth interrogation of the genomes of the three pairs of serial isolates that the genome of *M. tuberculosis* is dynamic during the course of infection. Our hypothesis expected that the MDR isolates would contain unique polymorphisms relative to the rifampicin mono-resistant isolates, however numerous polymorphisms were observed to be unique in each mono-resistant isolate. The disappearance of these polymorphisms during the emergence of resistance suggests they were not providing a selective advantage during the growth of *M. tuberculosis* under the selective pressure of the host and drug. If these patients were on first-line therapy and thereby were exposed to isoniazid, individual bacilli within the *M. tuberculosis* population which harboured either a *katG* or *inhA* mutation would be expected to dominate. Consequently, polymorphisms observed in the rifampicin mono-resistant isolate were 'lost' from the population and polymorphisms unique to the bacilli that gained isoniazid resistance were enriched. The treatment information for each patient at the time of sample collection is not available, however based on standard treatment protocols it can be assumed that the patients remained on first line treatment after the collection of the initial rifampicin mono-resistant sample. The policy guidelines in place for the management of Directly Observed Treatment Short course (DOTS) state that patients infected with mono-resistant *M. tuberculosis* should remain on standard first-line therapy (<http://www.sahealthinfo.org/tb/mdrtbguidelines.pdf>). If this was the case for these 3 patients, it reflects the incorrect implementation of the treatment guidelines with the consequence of amplification of drug resistance.

M. tuberculosis responsible for disease in Patient 1 acquired a *katG* mutation conferring isoniazid resistance, while the causative *M. tuberculosis* isolate in patient 2 acquired an *inhA* promoter mutation. Both of these mutations are regarded as high confidence mutations conferring isoniazid resistance. Interestingly, the *M. tuberculosis* MDR isolate for Patient 3 did not acquire any mutations in *katG* or the *inhA* promoter region. Phenotypic tests showed this isolate to be resistant to isoniazid using a critical concentration of 1 µg/ml. The paired isolates

from patient 3 were therefore included for whole genome sequencing analysis with the aim of identifying the mechanism of isoniazid resistance. No known isoniazid resistance causing mutations were observed (*ahpC*, *ndhA*, *kasA*) in the sequencing data of R15213. However a polymorphism was observed in *pst2* which has previously been associated with isoniazid resistance in *M. tuberculosis* isolates with no known resistance causing mechanism (22). Furthermore, analysis of approximately 400 *M. tuberculosis* genomes showed that the polymorphism identified in this study is present in 11 other isoniazid resistant *M. tuberculosis* clinical isolates. Of these, approximately half the isolates contained either a *katG* or *inhA* promoter mutation. This observation supports the potential role of a mutation in *pst2* in contributing to isoniazid resistance. Further studies will be required to determine whether this *pst2* polymorphism does indeed result in isoniazid resistance.

Whole genome sequencing analysis was used to identify compensatory mutations in the *rpoC* gene in the *M. tuberculosis* isolates analysed in this study. For the *M. tuberculosis* isolates from patients 2 (R912 and R1210) and 3 (R6264 and R15231) the *rpoC* mutation remained constant during the course of infection. However, the mutations present in *rpoC* in these isolates (Ala172Val and Gly594Glu) were previously shown to be present in both rifampicin sensitive and resistant isolates and thereby are not considered to be compensatory mutations (8). In addition, R912 and R1210 both harboured an additional polymorphism in *rpoB* (A1075A), which has also been identified as a phylogenetic marker and therefore has no role in compensating for loss of fitness (8). For patient 1 the rifampicin mono-resistant isolate (R721) was shown to have an *rpoC* (Val1039Ala) mutation in approximately 30% of the Illumina reads. This mutation was not observed in the paired MDR isolate (R807); the MDR isolate contained an additional *rpoB* mutation (Ile889Val) in 11% of the Illumina reads. Neither of these mutations has previously been identified in literature. Similarly, analysis of the Department of Biomedical Sciences genome bank revealed that neither of these polymorphisms are present in the available clinical *M. tuberculosis* isolates, suggesting that these polymorphisms are transient and do not allow for any fitness advantage in *M. tuberculosis*.

A principle finding of this study was the range of genes with polymorphisms which were observed to emerge and disappear in during the course of infection. Previous studies have reported additional mutations occurring concurrently with drug resistant causing mutations (4, 12–14). These variants have been identified in several genes involved in lipid metabolism, cell wall biosynthesis, purine metabolism, and transcriptional control (4). Numerous polymorphisms have previously been identified to be ancillary to drug resistance i.e. provide

an optimal physiological environment for a particular drug resistance phenotype (4). For example, genes involved in polyketide synthesis have been hypothesised to be ancillary to rifampicin resistance (4, 23). In this study polymorphisms were seen in *ppsE* (encoding Phenolphthiocerol synthesis type-I polyketide synthase PpsE) and *pks12* (encoding polyketide synthase 12). *Mycobacterium avium-intracellulare* and *Mycobacterium bovis pks12* knockout strains have been shown to be more susceptible to multiple anti-TB compounds (24), while *ppsA* (a component in the PDIM biosynthetic pathway) has been shown to be up regulated in rifampicin resistant isolates. *ppsE* is proposed to be involved in the same pathway as *ppsA*. In this study it was also noted that polymorphisms unique to the MDR isolates were predominantly seen in genes involved in cell wall and cell processes or lipid metabolism. Farhat *et al* recently suggested that remodeling of the cell wall may play an important role in stable drug resistance phenotypes (23). Our findings support this idea, demonstrating the acquisition of mutations in genes involved in cell wall remodeling.

While the findings of these studies may all identify the acquisition of additional mutations during the course of infection, this is the first study to identify heterogeneous polymorphisms in genes involved in many metabolic processes. Heterogeneity has recently been investigated for drug resistance causing genes, focusing on the implications for genetic testing. Sun *et al* demonstrated a fluctuation in the mutation frequency of various drug resistance causing genes as well as in genes not associated with drug resistance (18). In a separate study the presence of sub-populations was highlighted using a whole genome sequencing approach (15), and the present study complements these findings. In this study high genetic diversity was seen between serial isolates, during the evolution of drug resistance. While some fixed polymorphisms were observed, there were many heterogeneous polymorphisms. This highlights the presence of sub-populations that are present within a patient at a single time period, and how these populations are adapting and changing over time. Based on these findings we propose a model for the evolution of drug resistance in *M. tuberculosis* (Figure 4.5), where numerous genes may contain heterogeneous polymorphisms at time point 1, with 1 fixed drug resistance causing mutation. The physiological consequences of the heterogeneous polymorphisms remain unknown. During the course of infection, under host pressure, some polymorphisms may emerge, while others are lost from the population. This variation in genome may depend on the environmental pressure encountered within the host. Under selective drug pressure, drug resistance causing mutations may emerge. This would result in a

different individual predominating in the population at a second time point. Again, numerous polymorphisms may emerge or disappear while *M. tuberculosis* best adapts to its surroundings.

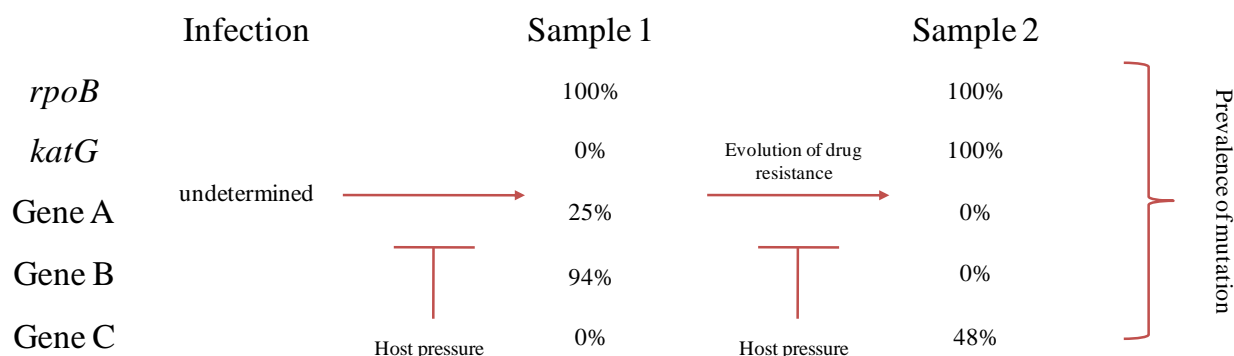


Figure 4.5 Proposed model for the evolution of *M. tuberculosis*. After the onset of infection, host pressure acts on *M. tuberculosis*. Certain polymorphisms emerge or disappear, never becoming fixed within the *M. tuberculosis* population while others become fixed e.g. drug resistance causing mutations. It remains unknown how many individual populations are present at one time point or if specific polymorphisms occur together with other mutations or alone i.e. mutations in gene A and B may occur within the same individual within a population.

While this study highlighted that the *M. tuberculosis* genome is dynamic, it was unable to identify markers associated with the evolution of drug resistance. All three sample sets were of a different genetic background and direct comparison between each set was therefore not possible. It is likely that a larger set of isolates including numerous samples from each strain family will increase the likelihood of identifying markers for the evolution of drug resistance. However, the pattern of evolution observed in all three isolates was the same i.e. polymorphisms emerge and disappear during the course of infection regardless of strain family. Additional samples for each patient would also have been advantageous, providing the means to determine if there are specific heterogeneous polymorphisms which may become fixed in the population or if the genome remains in a dynamic state. This limitation may be overcome by investigating a different pattern of drug resistance evolution. This study has identified samples from patients which show multiple evolutionary patterns which may be used for future studies. For example, there are 375 samples originating from 44 patients demonstrating evolution from MDR to pre-XDR and subsequent XDR. In addition, a more comprehensive study which includes proteome and transcriptomic analysis may provide information on the physiological consequences of the genomic changes observed during the evolution of resistance.

In conclusion, this study has demonstrated the dynamics of the genome of *M. tuberculosis* during the course of infection and during the evolution of drug resistance. The identification of heterogeneous polymorphisms present in the *M. tuberculosis* population at low frequencies highlights the quantitative capacity of deep sequencing, a tool which should be exploited for the design of future diagnostic tests.

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CHAPTER 5

The role of efflux pumps in the evolution of drug resistance in *Mycobacterium tuberculosis*

5.1. Background

Active efflux is commonly understood to contribute to drug resistance in numerous bacteria and has been thoroughly reviewed (1–3). There is also an increasing body of evidence highlighting the contribution of efflux pump systems in *Mycobacterium tuberculosis* to drug resistance and drug tolerance (discussed in Chapter 2) (4). Tolerance represents a physiological state which enables bacterial survival in the presence of antibiotic treatment; following removal of the drug(s), active growth is resumed as is fully drug susceptibility (5). While poorly understood, it is generally thought that drug tolerance is a result of a physiological response to external stimuli, rather than as a result of resistance causing mutations (6).

It is known that exposure to certain anti-TB drugs induces tolerance in *M. tuberculosis*; the mechanism of which has been linked to efflux pump activity (7–10). However, a recent study demonstrated that macrophage infection induced tolerance to anti-TB drugs (7). Upon further investigation it was shown that this tolerance in this model could be reversed with the addition of efflux pump inhibitors, confirming the role of efflux pump activity in the induction of tolerance. More alarming are the results of a follow-up study where it was observed that macrophage-induced tolerance negatively affected the activity of newer anti-TB compounds (favourable for their ability to shorten treatment regimens) such as moxifloxacin, PA-824, linezolid and bedaquiline (11). In addition, recent studies have highlighted the contribution of efflux pump activity to low level phenotypic drug resistance, or tolerance i.e. increased levels of resistance with no known drug resistance causing mutations (12, 13). This low level drug tolerance provides an opportunity for *M. tuberculosis* to develop drug resistance causing mutations, resulting in clinical resistance. Efflux pump activity has therefore been suggested to be a gateway mechanism for the development of drug resistance (discussed in detail in Chapter 2) (4). Furthermore, recent studies have investigated the role of efflux pump activity in cross-resistance between anti-TB compounds (14–16). This phenomenon requires additional research in an era where resistance to anti-TB drugs is developing at rapid rates, especially when considering that efflux-mediated cross resistance has been shown for promising new anti-TB drugs such as bedaquiline (14, 17).

With knowledge of the contribution of efflux pump activity to drug resistance and/or tolerance in *M. tuberculosis* there has been an increased focus on the use of efflux pump inhibitors as a complementary TB treatment. Numerous studies have shown the efficacy of including efflux pump inhibitor verapamil in treatment regimens; this calcium channel antagonist has been

shown to shorten treatment of susceptible and MDR infection in mice (11, 18, 19). It has been shown that due to the induction of CYP3A4 system by rifampicin, verapamil serum concentrations may be reduced by up to 10-fold (20, 21), fuelling the investigation into verapamil analogues. Recently verapamil analogues have been shown to have increased synergy with rifampicin and comparable activity with verapamil (18).

The influence of the genotypic characteristics of *M. tuberculosis* on efflux pump inhibitor efficacy has previously been considered. In a study investigating ofloxacin tolerance in *M. tuberculosis*, Louw *et al.* hypothesised that the differential response seen between isolates was due to the presence of different *rpoB* mutations, that *M. tuberculosis* isolates harbouring different *rpoB* mutations may have different transcriptomes and certain genes may be differentially expressed (22). A follow-up study by our research group showed a substantial difference in transcriptional profiles when comparing *rpoB* Ser531Leu and His526Tyr *in vitro* generated mutants (J du Plessis, M.Sc thesis 2013). In a separate study, Adams *et al.* hypothesised that the efflux pump inhibitors may be more effective on strains over-expressing efflux pumps and their efficacy may therefore be strain-dependent (11). There is therefore a gap in the knowledge on the efficacy of efflux pump inhibitors on strains with different genotypic profiles. No studies have compared the response of *M. tuberculosis* isolates from different genetic backgrounds to efflux pump inhibitor treatment. A broader approach has previously been followed with respect to the role of efflux pumps in rifampicin resistance specifically. Numerous studies have investigated the transcriptional response of efflux pumps to rifampicin exposure in *M. tuberculosis* isolates with the most prominent *rpoB* mutations (Ser531Leu and His526Tyr), however no direct comparison has been made between *M. tuberculosis* isolates harbouring an *rpoB* Ser531Leu and His526Tyr with respect to the role of efflux pumps in rifampicin resistance.

In addition, it is unknown whether other genomic changes may also alter the efficacy of efflux pump inhibitors i.e. does the acquisition of mutations alter the physiology of *M. tuberculosis*? Recent studies have investigated the genomic changes that occur in serial patient isolates (discussed in Chapter 4), but there has been little investigation into the physiological consequences of these genetic changes. One such study compared the whole transcriptome of paired susceptible and MDR *M. tuberculosis* isolates from single patients; up-regulation of specific efflux pumps, ABC transporters and membrane proteins was shown in the MDR isolates when compared to a susceptible ‘progenitor’ isolate (23). This up-regulation of transporters was coupled with down-regulation of general cellular metabolism, allowing the

authors to hypothesise that this may be responsible for low level resistance to numerous anti-TB drugs and that further drug exposure may select for drug resistance causing mutations (23). These findings highlight the potential role of efflux pumps in the evolution of drug resistance. However, little research has expanded on these recent findings or investigated the physiological changes associated with only the acquisition of one additional drug resistance causing mutation. For example, is this phenomenon associated with acquisition of single mutations or will up-regulation only be observed in an *M. tuberculosis* isolate which has accumulated numerous mutations?

5.2. Hypotheses

The contribution of efflux pump activity to drug resistance differs between *M. tuberculosis* isolates with different genetic backgrounds and *rpoB* mutations. In particular we hypothesise that:

1. Efflux pump activity contributes to the level of rifampicin resistance in *M. tuberculosis*.
2. The contribution of efflux pump activity to drug resistance is altered during the acquisition of drug resistance in *M. tuberculosis*.
3. Rifampicin resistant *M. tuberculosis* isolates with different rifampicin resistance causing mutations may display differential efflux pump activity.

5.3. Aims

This study aims to determine the role of efflux pump activity in drug resistant *M. tuberculosis* isolates.

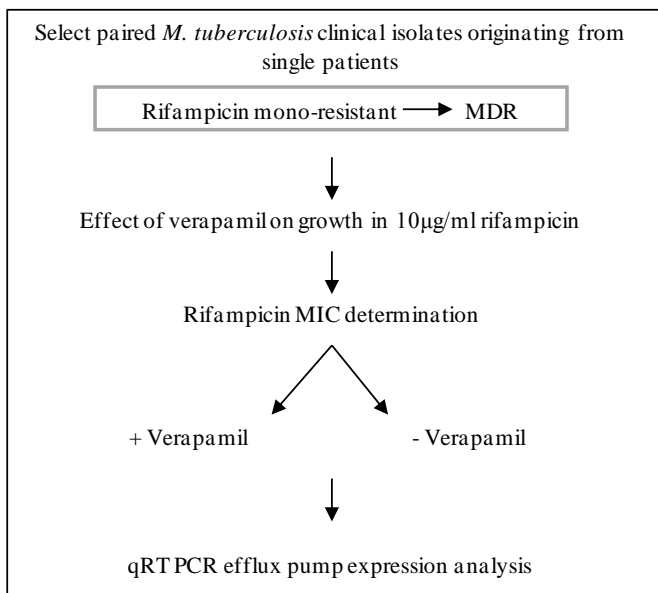
Specific aims:

1. To investigate the role of efflux activity in the evolution of drug resistance.
2. To determine if genetic background or specific *rpoB* mutations influence the role of efflux pumps in drug resistance (i.e. the activity of efflux pumps and/or the influence of verapamil on the level of drug resistance).
3. To investigate the influence of different *rpoB* mutations on efflux pump activity in *M. tuberculosis*, with specific focus on rifampicin and ofloxacin levels of resistance.

5.4. Experimental approach

Methods used in this study have been detailed in Chapter 3. The experimental approach followed in this study is outlined in Figure 5.1 below.

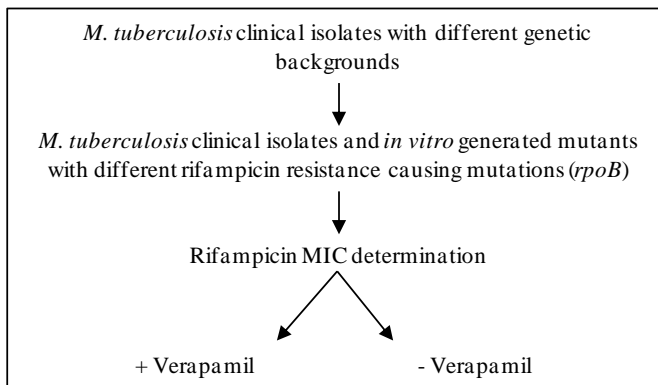
Specific aim 1



OUTCOME:

1. To determine the influence of efflux pump inhibitor verapamil on the growth of rifampicin resistant *M. tuberculosis* isolates at a clinically relevant rifampicin concentration
2. Determine if efflux pump activity changes during the acquisition of drug resistance
3. To determine if the constitutive expression of efflux pumps changes during the acquisition of additional drug resistance

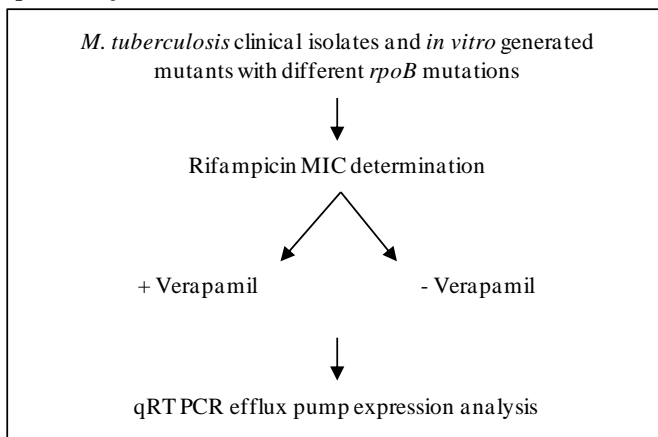
Specific aim 2



OUTCOME:

1. Determine if genetic background influences the response to verapamil i.e. the activity of efflux pumps
2. Determine if specific *rpoB* mutations influence the response to verapamil i.e. the activity of efflux pumps

Specific objective 3



OUTCOME:

1. Determine if specific *rpoB* mutations influence the role of efflux pumps in rifampicin levels of resistance
2. To compare the constitutive expression of efflux pumps in *M. tuberculosis* isolates with different *rpoB* mutations

Figure 5.1 Approach to elucidate the role of efflux pumps in drug resistance in closely related *M. tuberculosis* isolates

5.5. Results

5.5.1. The role of efflux pump activity in the evolution of drug resistance in *M. tuberculosis*

M. tuberculosis isolates identified in Chapter 4 were selected to represent the evolution/acquisition of drug resistance from rifampicin mono-resistant to MDR in this study (Table 5.1).

Table 5.1 Characteristics of *M. tuberculosis* selected to show acquisition of drug resistance.

Patient	Isolate name	Genetic background	Drug resistance profile	<i>rpoB</i> mutation	INH resistance causing mutation
1	R721	Beijing	Rifampicin mono-resistant	Ser531Leu	-
	R807	Beijing	MDR	Ser531Leu	<i>katG</i> Gly309Val
2	R912	EAI	Rifampicin mono-resistant	His526Tyr	-
	R1210	EAI	MDR	His526Tyr	-15 <i>inhA</i> promoter

*Amino acid change according to the *Escherichia coli rpoB* gene sequence

The growth of each isolate was monitored during exposure to 10µg/ml rifampicin, in the presence and absence of verapamil (Figure 5.2 and 5.3). The selected rifampicin concentration of 10µg/ml reflects a clinically relevant concentration as serum concentrations have been shown to range from 8 to 24µg/ml in some patients (24–27). *M. tuberculosis* growth in the BACTEC MGIT™ 960 vials was measured by an increase in fluorescence, as an oxygen quenched fluorophore is released during oxygen consumption (28).

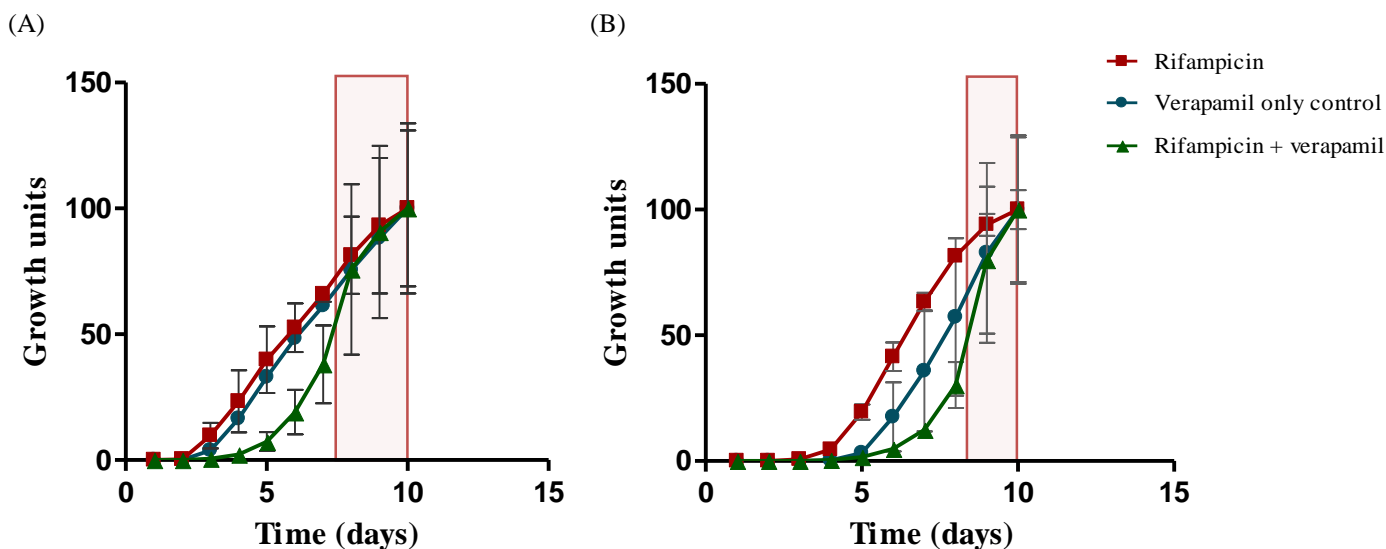


Figure 5.2 Growth of closely related (A) rifampicin mono-resistant (R721) and (B) MDR (R807) *M. tuberculosis* clinical isolates from patient 1, in the presence and absence of verapamil. The highlighted regions indicate the time at which the 1:100 growth controls measured as 400 growth units, the point at which the percentage of susceptibility restored by verapamil was calculated.

Figures 5.2A and 5.3A show that verapamil treatment delays the onset of growth (as a reflection of oxygen consumption) of *M. tuberculosis* isolates R721 and R921 in the presence of rifampicin, respectively. This suggests that verapamil does not restore susceptibility to rifampicin indefinitely. A similar effect was observed for the MDR isolates R807 and R1210 (Figure 5.2B and 5.3B) which evolved from R721 and R921, respectively.

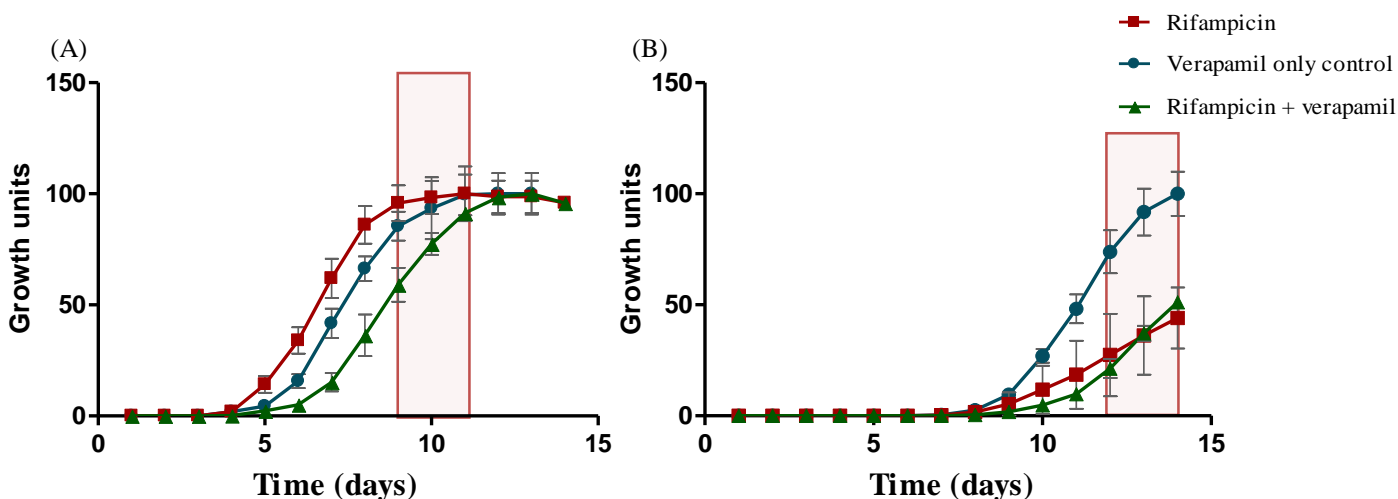


Figure 5.3 Growth of closely related (A) rifampicin mono-resistant (R912) and (B) MDR (R1210) *M. tuberculosis* clinical isolates from patient 2, in the presence and absence of verapamil. The highlighted

regions indicate the time at which the 1:100 growth controls measured as 400 growth units, the point at which the percentage of susceptibility restored by verapamil was calculated.

The percentage of restored susceptibility to rifampicin, due to verapamil treatment ranged from 13 to 72% for the rifampicin mono-resistant isolates and 0 to 47% for the MDR isolates (Table 5.2, Figure 5.4). This value was calculated at the time that the 1:100 growth control of the experiment reached 400 growth units, the time at which an experiment is evaluated for standard DST (indicated in Figures 5.2 and 5.3). Verapamil restored susceptibility to rifampicin by up to 72% in the rifampicin mono-resistant isolate in patient 1 (R721), while only restored susceptibility by up to 13% in the rifampicin mono-resistant isolate from patient 2 (R912). Similarly, verapamil had a larger effect on the MDR isolate from patient 1 (R807) when compared to that of patient 2 (R1210).

Table 5.2 Percentage of susceptibility to rifampicin by verapamil in *M. tuberculosis* clinical isolates.

	Isolate	Restored susceptibility (%)
Patient 1	R721	72.88
	R807	47.65
Patient 2	R912	13.33
	R1210	0

When comparing the effect of verapamil treatment on clinical isolates from a single patient it was observed that there was no significant difference between the rifampicin mono-resistant and MDR isolates. This was the case for isolates from both patient 1 and 2. However, a differential response to verapamil between rifampicin mono-resistant isolates R721 and R912 was found to be significant at the time point when a standard DST would be scored (Figure 5.4). Similarly, the percentage of rifampicin susceptibility restored by verapamil was significantly different between the two MDR isolates, namely R807 and R1210 (Figure 5.4).

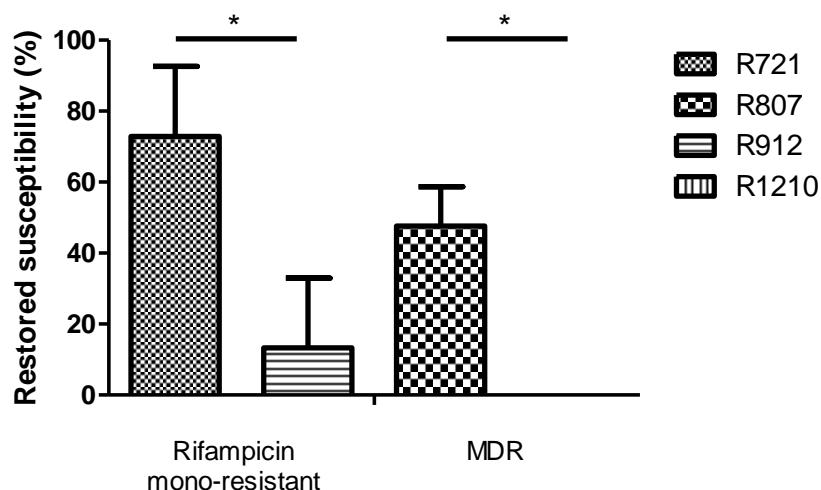


Figure 5.4 Restoration of rifampicin susceptibility by efflux pump inhibitor verapamil in rifampicin mono-resistant and MDR *M. tuberculosis* clinical isolates. *statistically significant difference ($p < 0.05$)

The rifampicin MIC was subsequently determined for each isolate in the presence and absence of verapamil (Table 5.3) to determine the influence of verapamil on the level of rifampicin resistance.

Table 5.3 Change in the level of rifampicin resistance in closely related *M. tuberculosis* isolates in the presence of efflux pump inhibitor verapamil.

Patient	Isolate name	Drug resistant profile	Rifampicin MIC ($\mu\text{g/ml}$)	Rifampicin MIC in the presence of verapamil ($\mu\text{g/ml}$)	Fold change in MIC
1	R721	Rifampicin mono-resistant	120	30	4
	R807	MDR	120	60	2
2	R912	Rifampicin mono-resistant	100	100	0
	R1210	MDR	100	100	0

The MIC values for each isolate originating from the same patient remained unchanged during the acquisition of isoniazid resistance: $120\mu\text{g/ml}$ for R721 and R807 and $100\mu\text{g/ml}$ for R912 and R1210. However, the level of resistance to rifampicin was different between the rifampicin mono-resistant isolate (R721) and MDR isolate (R807) from patient 1 in the presence of verapamil. Verapamil treatment resulted in a 4-fold decrease in MIC for R721, but only a 2-

fold decrease in R807. Verapamil treatment showed no effect on the rifampicin MIC for the *M. tuberculosis* isolates from patient 2.

The expression levels of a panel of efflux pump encoding genes were investigated to compare the levels of gene expression between the rifampicin mono-resistant and MDR paired isolates from each patient. Table 5.4 shows no significant differential expression of efflux pump genes between rifampicin mono-resistant and MDR isolates from individual patients (where significance is defined at a fold change >2 and a p value < 0.05). While *Rv1819c* and *mmpL5* were shown to be up regulated by 5.50 and 3.72 fold respectively, this result was not considered significant since the p value was not <0.05.

Table 5.4 Differential expression of efflux pump genes between rifampicin mono-resistant and MDR paired isolates represented as a fold change in expression.

	<i>rpoB</i> mutation*	<i>EmrB</i>	<i>Rv3728</i>	<i>PstB</i>	<i>DrrA</i>	<i>Rv1819c</i>	<i>mmpL5</i>
Patient 1	Ser531Leu	1.59	-0.47	0.42	1.33	1.50	1.41
Patient 2	His526Tyr	-1.42	1.83	-1.98	1.62	5.50	3.72

*Amino acid change according to the *Escherichia coli rpoB* gene sequence

These results show that verapamil delays the onset of growth in the presence of rifampicin, at a clinically relevant concentration. In addition it was observed that verapamil has a more pronounced effect on certain isolates, decreasing their MIC by up to 4-fold, whereas in others there is no fold change. There was no statistically significant fold change in the expression of efflux pump genes between the rifampicin mono-resistant and MDR isolates.

5.5.2. Determination of the role of genetic background and *rpoB* mutation type on efflux pump activity in *M. tuberculosis*

In order to investigate the role of genetic background and *rpoB* mutations on efflux pump activity a panel of rifampicin mono-resistant *M. tuberculosis* clinical isolates from the Beijing and EAI strain genotypes were selected. Two isolates were selected for each genotype, each with a different *rpoB* mutation (Table 5.5).

Table 5.5 Characteristics of *M. tuberculosis* isolates selected to investigate the role of genetic background and *rpoB* mutations on efflux pump activity.

Isolate	Genetic background	<i>rpoB</i> mutation*	Drug resistance profile
R721	Beijing	Ser531Leu	Rifampicin mono-resistant
R966	Beijing	His526Tyr	Rifampicin mono-resistant
R5608	EAI	Ser531Leu	Rifampicin mono-resistant
R912	EAI	His526Tyr	Rifampicin mono-resistant
R160	LCC	Ser531Leu	Rifampicin mono-resistant

*Amino acid change according to the *Escherichia coli rpoB* gene sequence

The rifampicin MIC was determined for each isolate in the presence and absence of verapamil (Table 5.6 and 5.7). One LCC rifampicin mono-resistant *M. tuberculosis* isolate with an *rpoB* Ser531Leu mutation was included in this study (Table 5.5). A LCC rifampicin mono-resistant isolate with an *rpoB* His526Tyr was not available for use in this study.

Table 5.6 Change in the level of rifampicin resistance in *M. tuberculosis* clinical isolates from different genetic background with an *rpoB* Ser531Leu mutation in the presence of efflux pump inhibitor verapamil.

<i>M. tuberculosis</i> isolate	Genetic background	Rifampicin MIC (µg/ml)	Rifampicin MIC in the presence of verapamil (µg/ml)	Fold change in MIC
R721	Beijing	120	30	4
R5608	EAI	80	20	4
R160	LCC	120	60	2

Each of the *M. tuberculosis* isolates with an *rpoB* Ser531Leu mutation showed a response to verapamil treatment, with a 2- to 4- fold decrease in MIC. Conversely, *M. tuberculosis* isolates with an *rpoB* His526Tyr mutation showed no response to verapamil treatment with regards to a decrease in MIC (Table 5.7).

Table 5.7 Change in the level of rifampicin resistance in *M. tuberculosis* clinical isolates from different genetic backgrounds with an *rpoB* His526Tyr mutation in the presence of efflux pump inhibitor verapamil.

<i>M. tuberculosis</i> isolate	Genetic background	Rifampicin MIC (µg/ml)	Rifampicin MIC	
			in the presence of verapamil (µg/ml)	Fold change in MIC
R966	Beijing	50	50	0
R912	EAI	100	100	0

The data presented in Tables 5.3, 5.6 and 5.7 suggest that *rpoB* mutation type and not genetic background may be important in defining the response to verapamil treatment. These clinical isolates are not closely related and their similar response to verapamil across different genetic backgrounds supports the notion that the *rpoB* mutation defines the response to verapamil treatment.

5.5.3. Influence of different *rpoB* mutations on efflux pump activity in *M. tuberculosis*

To further investigate the role of different *rpoB* mutations on efflux pump activity the effect of verapamil on the rifampicin MIC of *in vitro* generated rifampicin mono-resistant *M. tuberculosis* isolates was determined (Table 5.8). The isolates containing an *rpoB* Ser531Leu and His526Tyr mutation were generated from a common progenitor *M. tuberculosis* clinical isolate. This strategy allows for a “clean” experiment, excluding the influence of other genetic factors which may play a role in clinical *M. tuberculosis* isolates.

Table 5.8 Change in the level of rifampicin resistance in *M. tuberculosis in vitro* generated rifampicin resistant mutants with different *rpoB* mutations in the presence of efflux pump inhibitor verapamil.

<i>M. tuberculosis</i> isolate	<i>rpoB</i> mutation	Rifampicin MIC (µg/ml)	Rifampicin MIC	
			in the presence of verapamil (µg/ml)	Fold change in MIC
K636.1	Ser531Leu	80	40	2
K636.2	His526Tyr	200	200	0

Confirming the results seen in Tables 5.6 and 5.7, Table 5.8 shows that verapamil results in a 2-fold decrease in rifampicin MIC in the *in vitro* generated mutant with an *rpoB* Ser531Leu mutation, while it has no influence on the level of resistance of the *rpoB* His526Tyr mutant.

These results support the notion that specific *rpoB* mutations result in a differential response to efflux pump inhibitor verapamil, where *M. tuberculosis* isolates with an *rpoB* Ser531Leu mutation are more susceptible to verapamil treatment when compared to *M. tuberculosis* isolates with an *rpoB* His526Tyr mutation.

To further investigate this observation the expression of a panel of efflux pumps was analysed using RT-qPCR, comparing the isolate with the *rpoB* Ser 531Leu mutation and the *rpoB* His526Tyr mutation with the common pan susceptible progenitor (Table 5.9).

Table 5.9 Differential gene expression of *M. tuberculosis in vitro* generated rifampicin resistant mutants compared to their shared pan-susceptible progenitor isolate.

	<i>EmrB</i>	<i>Rv3728</i>	<i>PstB</i>	<i>DrrA</i>	<i>Rv1819c</i>	<i>mmpL5</i>
K636.1	-1.37	-7.57*	-1.79	-2.42	1.20	-2.46
K636.2	-2.22*	-1.80	-1.94	-2.41	-1.47	1.46

*P<0.05

In comparison to the pan-susceptible progenitor, no significant up-regulation of efflux pump genes was observed. One efflux pump, *Rv3728*, was significantly down-regulated in K636.1. Significant down-regulation was defined as a fold-change of >2 and a p-value <0.05. This isolate harbours a Ser531Leu *rpoB* mutation. Similarly, one efflux pump, *EmrB*, was significantly down-regulated in K636.2 (harbouring a His526Tyr *rpoB* mutation). These results do not correlate with observed effect of verapamil on rifampicin MIC, suggesting that the expression of efflux pumps contributing to the MIC is a response to the presence of rifampicin and is not due to constitutive efflux expression i.e. rifampicin exposure induces the up-regulation of efflux pump activity subsequently influencing the rifampicin MIC.

These results confirm the observation verapamil has a differential effect on *M. tuberculosis* isolates harbouring different *rpoB* mutations. In addition, it was observed that there is little significant difference in efflux pump expression between *in vitro M. tuberculosis* mutations harbouring different *rpoB* mutations.

5.6. Discussion

Due to the increasing emergence and spread of drug resistant *M. tuberculosis* there is an increased focus on identification of new anti-TB drug targets, as well as design of optimised treatment regimens for improving the length and efficacy of TB treatment. One approach has been to develop new compounds with novel targets; however bedaquiline is the first new anti-TB drug to be approved for used in patients in 40 years (29). Alternately repurposing of compounds traditionally used elsewhere has shown great success. Verapamil, a calcium channel antagonist, has traditionally been used for treatment of cardiovascular disease but have recently shown great promise as a complementary treatment for the current anti-TB regimens. While numerous efflux pump inhibitors have been used to investigate efflux activity in *M. tuberculosis*, verapamil is the most promising inhibitor under consideration for inclusion in treatment regimens. Adams *et al.* state that verapamil is the most appealing efflux pump inhibitor since it is already approved by the FDA and is well tolerated in humans (11, 17).

Verapamil had been shown to inhibit bacterial efflux systems (3), which have been the focus of a large body of research in recent years due to their role in defining the levels of drug resistance, as well their role in drug tolerance (22,31). Numerous studies have shown the efficacy of verapamil in improving the treatment regimens, in *in vitro* studies and *in vivo* mice studies (11, 18, 19, 30).

Major findings demonstrated the power of verapamil in inhibiting efflux mediated macrophage induced tolerance, a mechanism considered to be a gate-way mechanism to the development of drug resistance causing mutations (4, 11). In addition, verapamil has been shown to shorten the treatment period of the standard regimen with regards to bactericidal and sterilizing activity (18, 19). The results of our study complement these findings, demonstrating that at clinically relevant rifampicin concentrations, the onset of growth is delayed in the presence of verapamil. This may have implications during treatment, with the delay in growth caused by verapamil allowing anti-TB compounds time to act on their targets. This is an important observation since numerous compounds have been suggested to be extruded from the cell by efflux pumps, allowing the mycobacterial cell to stabilise in the presence of drugs during the development of resistance causing mutations (12, 13).

However, while this study demonstrated the ability of verapamil to delay the onset of growth at clinically relevant concentrations of rifampicin a startling observation was the differential

effect this treatment had on *M. tuberculosis* rifampicin resistant isolates with different *rpoB* mutations. Previous studies in our research group have focused on the difference between *M. tuberculosis* isolates harbouring different *rpoB* mutations with respect to varying levels of rifampicin resistance; the main *rpoB* mutations of interest on the most prominent clinical isolates in South Africa: Ser531Leu and His526Tyr. A main observation has been the delay in the induction of ofloxacin tolerance in isolates with an *rpoB* 526 mutation when compared to a 531 mutation. Induction of tolerance was observed in *M. tuberculosis* isolates harbouring an *rpoB*531 mutation after only 24 hours of rifampicin exposure, while isolates with an *rpoB*526 mutation showed only a minor change in ofloxacin MIC after a 7 day period (22). These findings led to additional studies which demonstrated that *M. tuberculosis in vitro* generated mutants harbouring different *rpoB* mutations had a vastly different transcriptional profile with genes involved in intermediary metabolism and respiration as well as cell wall and cell processes significantly differentially expressed between isolates with an *rpoB* Ser531Leu and His526Tyr mutation (J du Plessis, M.Sc thesis 2013).

The results of the current study complement the findings of previous work in our lab; here it was demonstrated that verapamil has a larger effect on *M. tuberculosis* clinical isolates harbouring an *rpoB* Ser531Leu mutation compared to an *rpoB* His526Tyr. Verapamil restored susceptibility to rifampicin (at 10µg/ml) by up to 72% in a rifampicin mono-resistant isolate, and 47% in an MDR isolate harbouring an *rpoB* ser531Leu mutation. Conversely, susceptibility was only restored by up to 13% in a rifampicin mono-resistant isolate harbouring an *rpoB* His526Tyr mutation, while no restoration was observed in the MDR isolate.

When considering *M. tuberculosis* paired isolates from a single patient, it was observed that there is a large difference in response to verapamil, especially in the case of patient 1 where verapamil decreases the rifampicin MIC by 4-fold in the rifampicin mono-resistant isolate, but only results in a 2-fold decrease in the paired MDR isolate. Subsequent gene expression analysis comparing the expression levels of six efflux pump genes between the rifampicin mono-resistant and MDR isolate revealed no statistically significant differential expression. However, in patient 1 five of the six efflux pump genes investigated showed to be up-regulated by less than 2-fold. Two of the genes investigated in patient 2 showed a fold change greater than 2, but with no statistical significance. When considering the number of efflux pumps encoded for by the *M. tuberculosis* genome it may be possible to hypothesise that there may be a biological significance if numerous efflux pumps are differentially expressed. A biological

effect of a larger scale of efflux pumps being up-regulated may account for the difference observed between the rifampicin mono-resistant and MDR isolate. A whole transcriptome approach would be beneficial for understanding the differences observed between the paired rifampicin mono-resistant and MDR isolates, especially considering that whole genome sequencing analysis demonstrated no genetic difference between these isolates which may account for the phenotypic difference observed (Chapter 4).

To further investigate the differential response to verapamil observed between the initial isolates selected with different *rpoB* mutations, the rifampicin MIC was determined for a larger sample set. Since the isolate harbouring a Ser531Leu mutation was a Beijing isolate, and the His526Tyr isolate was from the EAI lineage, the new isolates selected represent a paired isolate for each lineage i.e. an additional Beijing isolate with an *rpoB* His526Tyr and an EAI isolate with a Ser531Leu mutation were selected. This allowed for the determination of either genetic background or *rpoB* mutation as a causative mechanism for the observed differential response. In addition a LCC isolate with an *rpoB* Ser531Leu mutation was selected. Our results confirmed that the differential response to verapamil was in fact due to a difference in *rpoB* mutation, and was independent of genetic background. Verapamil was shown to be more effective on *M. tuberculosis* isolates harbouring an *rpoB* Ser531Leu mutation. This finding was subsequently confirmed using *in vitro* generated *rpoB* mutants originating from the same progenitor, with a “clean” genetic background. It can therefore be concluded that additional genetic changes are not contributing to the observed phenotype.

Numerous studies investigating efflux pump activity have been done in *M. tuberculosis* isolates with different *rpoB* mutations. These studies demonstrated the role of efflux pumps in defining the level of resistance or causing drug tolerance in *M. tuberculosis* isolates with *rpoB* Ser531Leu or *rpoB* His526Tyr using efflux pump inhibitors such as verapamil to confirm the role of drug efflux. However, this is the first study demonstrating a difference in response to verapamil treatment between isolates with different *rpoB* mutations. The causative mechanism of this observed difference is however unknown. Future studies such as whole transcriptomic and proteomic analysis may be able to identify differential expression of genes involved in this response and identify the mechanism of the increase sensitivity of *rpoB* Ser531Leu mutants to verapamil. This study demonstrated that in both the *in vitro* generated mutants efflux pump genes were down-regulated which does not correlate to the efficacy of an efflux pump inhibitor. This suggests that the efflux pump activity is activated by the presence of rifampicin. This

hypothesis is supported by numerous studies where efflux pump genes have been shown to be up-regulated in response to rifampicin exposure, reviewed in Chapter 2 (4). However, that lack of up-regulation between the rifampicin mono-resistant *in vitro* mutants and their pan-susceptible progenitor is a unique finding since studies have demonstrated differential efflux pump gene expression between sensitive and MDR *M. tuberculosis* clinical isolates (23). However, *in vitro* generated mutants may differ from clinical resistant isolates since *M. tuberculosis* was not exposed to host pressure and additional anti-TB drugs during the selection of the resistant mutant. Again, only a select number of genes were analysed and a whole transcriptomic approach may reveal a more prominent pattern since there are numerous efflux pumps encoded by the mycobacterial genome.

An additional limitation of this study is that only one anti-TB drug was investigated as a model. It is unknown whether the observed response to verapamil based on the *rpoB* mutation will carry through to other anti-TB drugs. This is important to consider since efflux based mechanisms have been shown to contribute to bedaquiline resistance and subsequent cross-resistance to clofazimine (14, 17). Bedaquiline is currently used as a treatment option for MDR patients who are already harbouring an *rpoB* mutation. If the effects observed in this study, as a result of *rpoB* mutation types, extend to the efflux activity associated with bedaquiline and clofazimine resistance levels, there may be serious consequences for treatment outcomes.

In conclusion, this study confirmed the contribution of efflux pump activity to rifampicin resistance in *M. tuberculosis* by demonstrating a change in the level of resistance in response to efflux pump inhibitor verapamil. In addition this study showed a difference in the response of *M. tuberculosis* isolates with different *rpoB* mutations to verapamil treatment. This finding may have implications for the consideration of the inclusion of efflux pump inhibitors in treatment regimens. Inclusion of verapamil may be less efficient when treating isolates with an *rpoB* His526Tyr mutant, depending on the concentrations used. It has been observed that it is unclear if the amount of verapamil used in *in vitro* studies is attainable in patients undergoing treatment (11). It may be important to consider this since verapamil is known to have negative side effects on patients who do not suffer from cardiovascular problems.

However verapamil is still a valuable treatment option for *M. tuberculosis* since it may inhibit efflux-induced tolerance which provides a stable environment for the development of resistance causing mutations (12, 13, 31). This study highlights the delayed onset of growth of *M. tuberculosis* in the presence of verapamil.

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CHAPTER 6

The role of energy metabolism and drug efflux in rifampicin resistance in *Mycobacterium tuberculosis*

6.1. Background

It has previously been commonly understood that mutations in the rifampicin resistance determining region (RRDR) of the *rpoB* gene (encoding RNA polymerase) are the sole cause of rifampicin resistance in *Mycobacterium tuberculosis* and the level of drug resistance may correlate to specific *rpoB* mutations (1, 2). This view of drug resistance in general, and more specifically rifampicin resistance has changed in recent years with an increasing amount of studies focusing on the contribution of efflux pump systems to drug resistance. The use of efflux pump inhibitors, as well as gene expression analysis in response to anti-TB drug exposure is frequently used to investigate the role of efflux pumps in drug resistance, identifying the involvement of numerous pumps in rifampicin resistance (3). For example, over expression of Rv1258c has been associated with resistance to isoniazid, rifampicin, ethambutol, ofloxacin and β -lactams while over-expression of the *Rv0559c-Rv0560c* gene cluster has been associated with rifampicin resistance (4–6). Studies using efflux pump inhibitors have implicated drug efflux in resistance to compounds such as rifampicin, isoniazid and ofloxacin (7–10). More recently, studies with efflux pump inhibitor verapamil have implicated drug efflux in cross resistance to clofazamine and bedaquiline (11, 12).

Previous studies in our group have demonstrated that closely related *M. tuberculosis* isolates harboring the same *rpoB* mutations displayed a wide range of minimum inhibitory concentrations; efflux pumps were shown to be involved in defining the level of rifampicin resistance (9). Subsequent investigation into the proteomic response to rifampicin exposure identified an array of proteins up-regulated in response to rifampicin (Figure 6.1) (M.Sc Thesis, Margaretha Bester).

From Figure 6.1 it is evident that proteins involved in energy metabolism were differentially expressed in response to rifampicin exposure. Of particular interest were the AtpA and AtpH subunits of the F₁F₀-ATP synthase. In a separate proteomic study the AtpA and AtpB subunits were shown to be up-regulated in response to ethambutol treatment in *M. smegmatis* (13) confirming the involvement of this enzyme in response to antibiotic exposure. This enzyme is the final enzyme involved in the production of ATP in mycobacteria (14) and is of particular interest as a drug target in *M. tuberculosis*. Along with AtpA and AtpB, cytochrome *c* oxidase has also been to be up-regulated in response to ethambutol exposure (13).

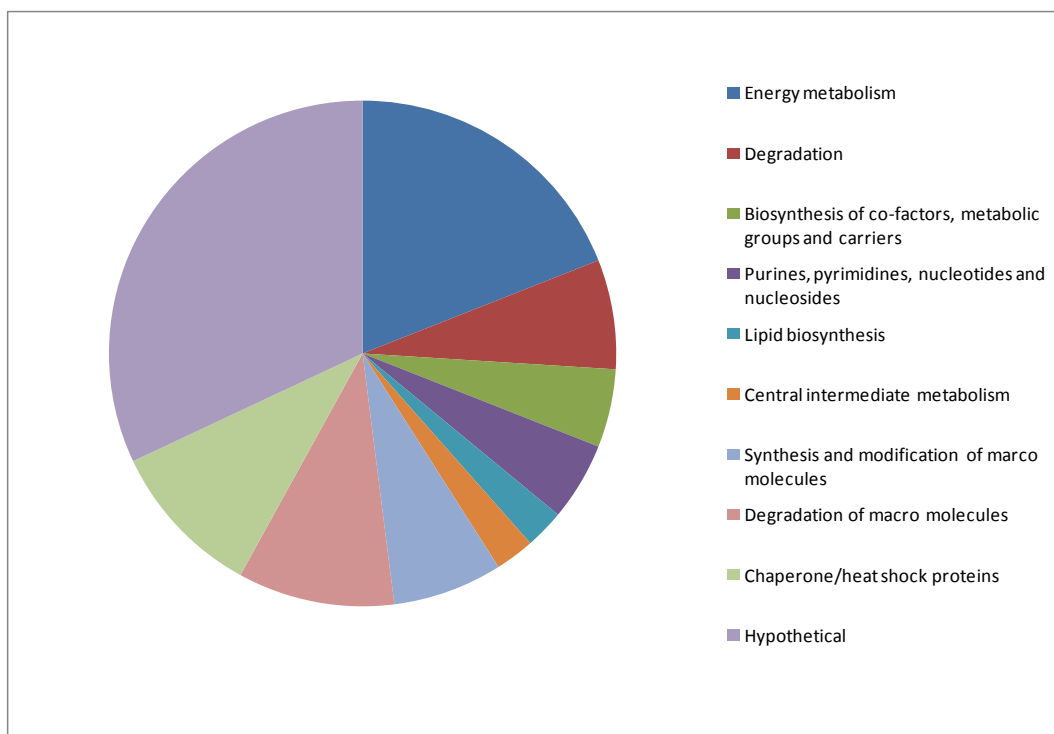


Figure 6.1 Differentially regulated proteins in response to rifampicin exposure in *M. tuberculosis* closely related clinical isolates (M.Sc Thesis, Margaretha Bester).

F₁F₀-ATP synthase is a unique target for bedaquiline, the first new anti-TB treatment approved by the FDA in 40 years (15). Bedaquiline has many desirable qualities, including decreased time to smear conversion of MDR-TB cases, a lack of cross resistance to current first-line anti-TB drugs, *in vitro* activity against resistant and sensitive isolates, as well as against non-replicating bacilli (16–19).

Beyond the role of the F₁F₀-ATP synthase as a drug target, it is important to investigate this and other components involved in energy metabolism since both ATP and the proton motive force are driving factors of efflux pumps (3). Components involved in the production of energy metabolism are attractive drug targets due to their essentiality for survival, as well as their role in fuelling efflux pumps. It is therefore important to ascertain the role of energy metabolism as well as drug efflux in drug resistant.

6.2. Hypothesis

Rifampicin exposure induces a signalling cascade in rifampicin resistant *M. tuberculosis* isolates which culminates in the up-regulation of numerous genes such as those encoding efflux pumps as well as those encoding components involved in energy metabolism.

6.3. Aims

This study aims to investigate the role of energy metabolism and drug efflux in rifampicin resistant *M. tuberculosis* clinical isolates.

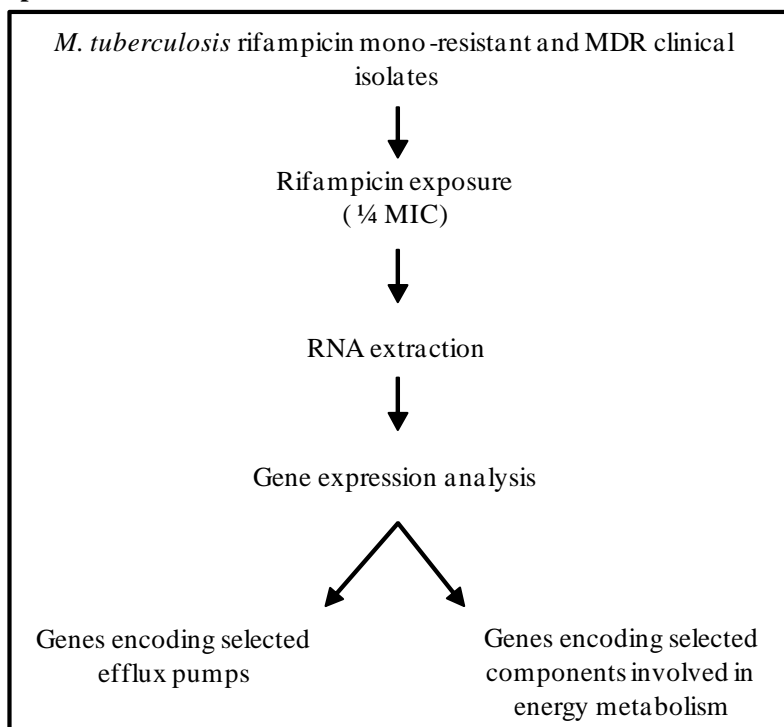
Specific aims:

1. To determine the expression levels of components involved in energy metabolism genes in response to rifampicin exposure in rifampicin resistant *M. tuberculosis* clinical isolates.
2. To determine the expression levels of a subset of efflux pump genes in response to rifampicin exposure in rifampicin resistant *M. tuberculosis* clinical isolates.

6.4. Experimental approach

Methods used in this study have been detailed in Chapter 3. The experimental approach followed in this study is outlined in Figure 6.2 below.

Specific aims 1 and 2



OUTCOME:

1. To define the response of components involved in energy metabolism in *M. tuberculosis* to rifampicin exposure.
2. To determine the influence of rifampicin exposure on the expression of a panel of efflux pumps in *M. tuberculosis*.

Figure 6.2 Approach to elucidate the role of efflux pumps in drug resistance in closely related *M. tuberculosis* isolates

6.5. Results

6.5.1 RNA extraction and quality assessment

In order to characterize the growth characteristics of the individual *M. tuberculosis* isolates, growth curves were plotted to determine the number of days required for each isolate to reach mid-log phase following a standard inoculum (Figure 6.3). This analysis showed that there was no significant difference in growth characteristics of the different isolates during log phase. However, the OD₆₀₀ readings decrease after day 14 for isolates R912 and R1210 due to the formation of clumps in the liquid culture. Mid-log phase was defined by an OD₆₀₀ of 0.8.

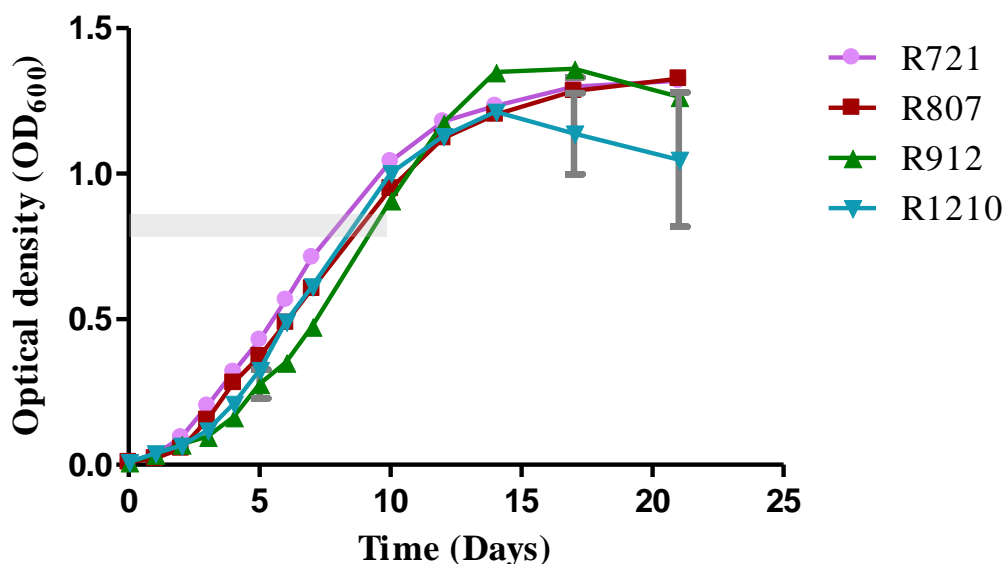


Figure 6.3 Growth curves for *M. tuberculosis* isolates used in this study. Error bars denote standard deviation between duplicate experiments.

In order to determine the transcriptional response of a panel of efflux pump and energy metabolism genes to rifampicin exposure mid-log *M. tuberculosis* cultures were exposed to rifampicin at a concentration equivalent to $\frac{1}{4}$ MIC for each isolate (determined in Chapter 5, Table 5.2). *M. tuberculosis* isolates were exposed for a period of 24 hours before RNA was extracted. An unexposed control (cultured in the absence of rifampicin) was included for each isolate.

Following DNase treatment, the RNA samples were subjected to PCR analysis to ensure there was no residual DNA (Figure 6.4 A). No PCR products were present on the gel confirming the

absence of DNA contamination. Subsequently the integrity of the isolated RNA as well and the concentration of RNA was assessed using the Bioanalyser. Figure 6.4 B shows a representative Bioanalyser Prokaryotic RNA gel. Concentrations ranged from 41ng/ μ l to 665ng/ μ l.

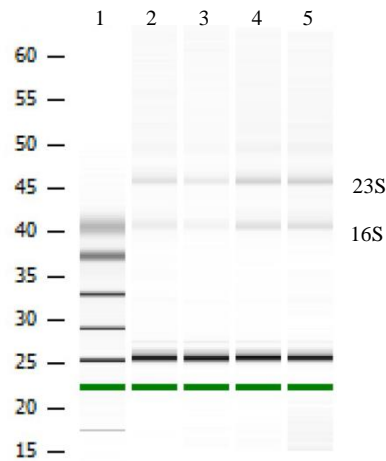


Figure 6.4 RNA quality assessment. Representative Bioanalyser fractionation of RNA samples indicating the presence of 16S and 23S RNA. Lane 1: Ladder, lanes 2 to 5: DNase treated RNA samples.

6.5.2. The response of components involved in energy metabolisms to 24 hours rifampicin exposure in *M. tuberculosis* clinical isolates

Representative genes from various components involved in energy metabolism were evaluated with RT-qPCR to determine whether rifampicin exposure altered the level of expression of these selected genes (Table 6.1). The energy metabolism components evaluated included the F₁F₀-ATP synthase (*atpA*), succinate dehydrogenase (*sdhA*), *bc₁* cytochrome reductase (*qcrC*), *aa₃* cytochrome *c* oxidase (*ctaD*), NADH dehydrogenase (*nuoA*) and MenA (*menA*) (involved in menaquinone biosynthesis). Genes were considered to be significantly differentially expressed if they were differentially expressed by a factor of 2-fold or more (relative to the unexposed control), with a p value < 0.05.

Table 6.1 Differential gene expression in response to rifampicin exposure of components involved in energy metabolism in *M. tuberculosis*.

	Beijing		EAI	
	Rifampicin mono-resistant	MDR	Rifampicin mono-resistant	MDR
	R721	R807	R912	R1210
<i>atpA</i>	-1.25599	-1.13506	1.042479	-0.43952
<i>sdhA</i>	-0.33717	-1.03303	1.042971	1.207413
<i>qcrC</i>	-1.47021	-1.49675	-1.05158	-1.20143
<i>ctaD</i>	-1.08467	0.018845	1.085799	-0.53016
<i>nuoA</i>	1.081016	-1.32605	1.077796	-1.02839
<i>menA</i>	1.090646	-1.67803	0.01951	-0.76938

This study shows no differential response in gene expression between isolates from different lineages or with different drug resistance profiles, indicating that the components involved in the electron transport chain and therefore energy metabolism are not influenced at the level of transcription in these *M. tuberculosis* isolates in response to rifampicin exposure at a concentration of 1/4 MIC.

6.5.3. The response of efflux pump genes to 24 hours rifampicin exposure in *M. tuberculosis* clinical isolates

A panel of efflux pump genes were evaluated with RT-qPCR to identify differential gene expression in response to rifampicin exposure (Table 6.3). The characteristics of the efflux pumps evaluated are shown in Table 6.2. Genes were considered to be significantly differentially expressed if they were differentially expressed by a factor of 2-fold or more (relative to the unexposed control), with a p value < 0.05.

Table 6.2 Characteristics of selected efflux pumps for the investigation into the transcriptional response to rifampicin exposure in *M. tuberculosis*.

Efflux pump	Gene	Description	Transporter family
EmrB	<i>Rv0783</i>	Possible multidrug resistance integral membrane efflux protein EmrB	MFS
Rv3728	<i>Rv3728</i>	Conserved two-domain membrane protein	MFS
PstB	<i>Rv0933</i>	Phosphate transport ATP-binding protein	ABC
DrrA	<i>Rv2936</i>	Daunorubicin-dim (dimycocerosate) transport ATP-binding protein	ABC
bacA	<i>Rv1819c</i>	Drug transport transmembrane ATP-binding protein, vitamin B12	ABC
mmpL5	<i>Rv0676c</i>	Probable conserved transmembrane transport protein MmpL5	RND

For the majority of the efflux pumps investigated in this study, no differential expression was observed in response to rifampicin exposure. However *mmpL5* was significantly up-regulated in all *M. tuberculosis* isolates independent of their genetic background, namely Beijing or EAI. *mmpL5* was up-regulated in both rifampicin mono-resistant and MDR isolates.

Table 6.3 Differential gene expression of efflux pump genes in *M. tuberculosis* in response to rifampicin exposure.

	Beijing		EAI	
	Rifampicin mono-resistant R721	MDR R807	Rifampicin mono-resistant R912	MDR R1210
<i>EmrB</i>	0.315294	-1.35808	-0.66023	1.96942
<i>Rv3728</i>	0.588727	-0.05518	-0.4481	0.994989
<i>PstB</i>	0.365759	-1.30298	-0.51266	-0.05814
<i>DrrA</i>	0.001703	-1.21936	-1.20799	1.216266
<i>bacA</i>	-1.21698	-1.30537	-0.38066	-1.2373
<i>mmpL5</i>	10.62143*	7.363803*	5.082641*	10.7345*

*Significant differential expression

Whole genome sequencing data was available for all isolates used in this study and was therefore used to determine if genomic changes could explain the results observed in Table 6.3. The upstream regions of each efflux pumps gene was investigated for the presence of polymorphisms which may influence the regulation of each gene. No polymorphisms were observed in any of the putative promoter regions investigated in this study. In addition, the efflux pump genes were investigated for the presence of polymorphisms which may influence the activity of the candidate efflux pumps (Table 6.4).

The genome sequences of the Beijing isolates (R721 and R807) (originating from a single patient) showed polymorphisms in two efflux pump encoding genes, *bacA* and *mmpL5*. Similarly, the genome sequences of the EAI isolates (R912 and R1210) identified polymorphism in four of the six efflux pump encoding genes investigated in this study, namely *Rv3728*, *DrrA*, *bacA* and *mmpL5*. The polymorphisms occurring in *bacA* are not the same between the isolates from different genetic backgrounds. The *mmpL5* gene showed to have the same polymorphism in all four isolates used in this study. It is unknown whether these polymorphisms influence the activity of the efflux pumps.

Table 6.4 Polymorphisms identified in efflux pump genes in *M. tuberculosis* clinical isolates.

Efflux pump	Beijing		EAI	
	Rifampicin mono-resistant	MDR	Rifampicin mono-resistant	MDR
	R721	R807	R912	R1210
EmrB	-	-	-	-
Rv3728	-	-	Ala608Thr	Ala608Thr
PstB	-	-	-	-
DrrA	-	-	His309Asp	His309Asp
bacA	Trp153Ser	Trp153Ser	Ile603Val	Ile603Val
mmpL5	Ile948Val	Ile948Val	Ile948Val	Ile948Val

The Department of Biomedical Sciences has a genome bank of approximately 400 *M. tuberculosis* clinical isolates (at the time of submission) which have been whole genome sequenced. This collection includes both drug sensitive and resistant isolates. The polymorphisms identified in this study in efflux pump genes (relative to *M. tuberculosis* H47Rv) (Table 6.4) were investigated in this genome bank to determine the possible clinical

relevance of polymorphisms in these genes. The polymorphisms identified in *mmpL5* (Ile948Val) and *bacA* (Trp153Ser) were present in 330 and 13 isolates, respectively. These polymorphisms occurred in both drug sensitive and resistance isolate indicating that they are not involved in drug resistance. The remaining polymorphisms identified in R912 and R1210 were shown to be present in an additional 5 *M. tuberculosis* isolates, all of which belong to the EAI lineage, indicating that this polymorphism may be a phylogenetic marker.

Together these results indicate *mmpL5* is significantly up-regulated in response to rifampicin exposure in both rifampicin mono-resistant and MDR isolates from different genetic backgrounds. *EmrB*, *Rv3728*, *PstB*, *DrrA* and *bacA* were not significantly differentially expressed in response to rifampicin exposure. The up-regulation of *mmpL5* confirms that the rifampicin exposure influences gene expression and that the absence of differential expression in the energy metabolism genes and the remaining efflux pump genes was not due to experimental error.

6.5.4. Investigation into transcriptional regulators associated with the candidate efflux pumps in this study

Recent ChIP-Seq technology has allowed for the identification of putative regulatory interactors for numerous genes in *M. tuberculosis*. The data generated from this experiment was used to identify predicted regulatory interactors of the efflux pump genes investigated in the study (Table 6.5). Many transcriptional regulators were identified to interact with each efflux pump.

Table 6.5 Predicted regulatory interactors for efflux pumps in *M. tuberculosis*.

Efflux pump	Predicted regulatory interactors
EmrB	<i>Rv0023, Rv0757, Rv0767c, Rv0967, Rv1033c, Rv1404, Rv1816</i>
Rv3728	<i>Rv0023, Rv0047c, Rv0081, Rv0135c, Rv1033c, Rv1353c, Rv1990c, Rv2021c, Rv2034, Rv2250c, Rv2324, Rv2506, Rv3736</i>
PstB	<i>Rv0081, Rv0967, Rv1816</i>
DrrA	<i>Rv0023, Rv0047c, Rv0135c, Rv0273c, Rv0302, Rv0757, Rv0767c, Rv0967, Rv1353c, Rv1473A, Rv1556, Rv1719, Rv1816, Rv2034, Rv2887, Rv2989, Rv3219, Rv3597c</i>
bacA	<i>Rv0081, Rv0324, Rv1816, Rv3133c, Rv3574, Rv3597</i>
mmpL5	<i>Rv0047c, Rv0081, Rv0678, Rv0691c, Rv0967, Rv1473A, Rv1719, Rv2011c, Rv3133c, Rv3249c, Rv3597c, Rv3736</i>

The genomes for each isolate used in this study were subsequently investigated to identify polymorphisms in the predicted regulatory interactors identified in Table 6.5. For R721 and R807 polymorphisms were identified in three candidate transcriptional regulator encoding genes, namely *Rv0324*, *Rv0302* and *Rv1719* (Table 6.6). Polymorphisms were also identified in three candidate transcriptional regulator encoding genes in R912 and R1210, namely *Rv1816*, *Rv0324* and *Rv3736* (Table 6.6). A polymorphism in one candidate transcriptional regulator, *Rv0324*, was present in all four isolates in the study.

Table 6.6 Polymorphisms identified in predicted regulatory interactors in *M. tuberculosis*.

Candidate transcriptional regulator	Beijing		EAI	
	Rifampicin mono-resistant	MDR	Rifampicin mono-resistant	MDR
	R721	R807	R912	R1210
Rv1816	-	-	Leu191Arg	Leu191Arg
Rv3736	-	-	Gly144Arg	Gly144Arg
Rv0324	Thr168Ala	Thr168Ala	Thr168Ala	Thr168Ala
Rv0302	His84Asp	His84Asp	-	-
Rv1719	Asp104Tyr	Asp104Tyr	-	-

The presence of polymorphisms in candidate transcriptional regulators (Table 6.6) suggests that the transcriptional regulators may have altered function and depending on the response of their predicted interactors (Table 6.7) to rifampicin, it may be possible to associate a

transcriptional regulator with the response of *M. tuberculosis* to rifampicin exposure. However, these may also represent phylogenetic markers. A larger subset of isolates would need to be investigated to determine the role of these polymorphisms. Rv1816 and Rv0302 are classified as transcriptional repressors, while Rv3736 is a transcriptional activator. Rv0324 and Rv1719 may act as a transcriptional activator or repressor (<http://genolist.pasteur.fr/TubercuList/>; The Pfam protein families database (20)).

Table 6.7 Efflux pumps predicted to interact with candidate transcriptional regulators observed to have a polymorphism.

Candidate transcriptional regulator	Family	Regulatory function	Predicted interactor	<i>M. tuberculosis</i> isolates containing polymorphism
Rv1816	TetR	Repressor	PstB, DrrA, EmrB, BacA	R912; R1210
Rv3736	Probable araC/xyls	Activator	mmpL5, Rv3728	R912; R1210
Rv0324	Probable arsR	Activator and/or repressor	BacA	R721; R807; R912; R1210
Rv0302	TetR/acrR	Repressor	DrrA	R721; R807
Rv1719	Similar to IclR regulators	Activator and/or repressor	mmpL5, Rv3728	R721; R807

This study identified five predicted transcriptional interactors of interest. Each transcriptional regulator contained a polymorphism which may influence its regulatory activity, and therefore play a role in the response to rifampicin exposure.

6.6. Discussion

Numerous previous studies in our group have investigated the mechanisms involved in defining the level of rifampicin resistance in *M. tuberculosis*. Findings of these studies culminated in the hypothesis that exposure of rifampicin resistant *M. tuberculosis* isolates to rifampicin results in the activation of signalling response pathways which cause an up-regulation of numerous efflux pump genes and the F₁F₀-ATP synthase.

Previous work demonstrated an up-regulation of the AtpA and AtpH proteins in response to rifampicin exposure in *M. tuberculosis* clinical isolates. The AtpA and AtpH for part of the F₁F₀-ATP synthase enzyme, the final enzyme involved in oxidative phosphorylation and is responsible for the conversion of electrochemical potential energy generated by the proton motive force into chemical energy in the form of ATP phosphoanhydride bonds (3, 21, 22). However, it can be assumed that an up-regulation in the amount of protein encoding the final enzyme involved in energy metabolism may impose a pressure on the cell to up-regulate other components involved in energy metabolism to ensure optimisation of the whole system since energy production is not a single step process. Therefore numerous genes involved in energy metabolism in *M. tuberculosis* were selected as candidates for this study.

Our investigation into the response of components involved in energy metabolism to rifampicin exposure demonstrated that there was no significant differential expression of genes involved in energy metabolism in *M. tuberculosis*. This included the *atpA* gene, encoding the AtpA subunit which was previously shown to be up-regulated in response to rifampicin exposure (M.Sc Thesis, Margaretha Bester). This discrepant result may be attributed to post-transcriptional processing. An increase in protein abundance may not equate to an increase in gene expression; numerous studies in other organisms have noted a negative correlation between mRNA and protein abundance (23). Negative correlation has been attributed to the extended half-life of proteins; as well difference in post-transcriptional processing and regulation (23, 24). In addition, it has been highlighted that the synthesis and degradation of mRNA transcripts need to be better understood to understand the correlation between mRNA and protein abundance (24).

In addition to the *atpA* gene, no significant differential expression of components involved in energy metabolism in *M. tuberculosis* was observed in response to rifampicin exposure. Together these results indicate the level of transcription of genes involved in energy metabolism was not altered in response to rifampicin exposure. However, this does not rule out an increase in flux through the electron transport chain. The activity of F₁F₀-ATP synthase has previously been shown to be regulated by the presence of inhibitor ADP. In addition it has been noted that a change in the proton motive force correlates with a change in enzyme activity (22). Considering this, and the findings of this study it may be more beneficial to investigate the levels of ATP or the NADH/NAD⁺ ratios in response to rifampicin exposure to better understand the influence of this anti-TB drug on energy metabolism. In addition, understanding

the response of ATP levels and NADH/NAH⁺ ratios to drug exposure may allow for a better understanding of efflux pump activity considering that efflux pumps largely rely on ATP hydrolysis or the proton motive force for functioning (discussed in Chapter 2) (3).

Of the six efflux pump genes investigated in this study, only *mmpL5* was observed to be up-regulated in response to rifampicin exposure (Table 6.3). This up-regulation was observed across all isolates used in this study, however there was no significant difference between the level of expression in rifampicin mono-resistant and MDR isolates. The same observation was made when comparing between isolates of different lineages. This suggests that the up-regulation of *mmpL5* in response to rifampicin exposure is a general occurrence, not limited to specific *M. tuberculosis* genetic backgrounds. The *mmpL5* gene encodes a probable conserved membrane transport protein of the same name, and is a member of the resistance/nodulation/cell division (RND) family of transporters (25). This membrane protein forms an efflux system with a protein encoded by an adjacent gene, *mmpS5*. This *mmpS5-mmpL5* efflux system has previously been associated with azole resistance, but more recently has been associated with bedaquiline resistance (11, 25). In addition, over-expression of this efflux pump system has been demonstrated to be the causal mechanism of cross resistance between bedaquiline and clofazamine, a renewed drug of interest for TB treatment (11, 26). Over-expression of the *mmpS5-mmpL5* efflux system and the subsequent increase in resistance levels to these compounds is due to a mutation in a repressor of the genes encoding these proteins, Rv0678 (11, 25, 26). Whole genome sequencing of the *M. tuberculosis* isolates used in study revealed no mutation in the gene encoding this repressor, suggesting the presence of alternate regulatory mechanisms for this efflux pump system. Indeed, ChIP-seq analysis has identified an additional 12 predicted regulatory interactors for *mmpL5*. Due to the importance of bedaquiline in treatment of MDR and XDR infection, as well its ability to shorten treatment periods it is important to further investigate the efflux based resistance mechanism (17, 19, 27, 28). Identification of additional mutations in regulators of this efflux system is vital for the development of rapid genotypic diagnostic tests. While efflux based cross-resistance has been demonstrated between bedaquiline and clofazamine, no studies have investigated cross resistance between first-line anti-TB drugs and bedaquiline and /or clofazamine. Initial studies into the efficacy of bedaquiline demonstrated no cross-resistance between first-line drugs and bedaquiline with respect to mode of action and resistance causing mutations i.e. *M. tuberculosis* isolates resistant to first-line drugs did not develop bedaquiline resistance at a higher rate than pan-susceptible isolates (16, 17). Additionally susceptibility testing of a clofazamine resistant

mutant (due to an *Rv0678* mutation) demonstrated no cross resistance with rifampicin (11). Together these findings suggest an alternate regulatory pathway to be involved in the up-regulation of *mmpL5* in response to rifampicin exposure.

Interestingly, while *mmpL5* was observed to be up-regulated, none of the other efflux pump encoding genes investigated in this study showed a significant response to rifampicin exposure. The efflux pump genes investigated in this study have previously differentially expressed in response to rifampicin exposure. For example, *Rv3728* was shown to be up-regulated 2-fold in response to rifampicin exposure at $\frac{1}{2}$ MIC in 3 out of 5 *M. tuberculosis* isolates investigated (29). In addition *drvA* and *pstB* differential expression was observed in response to sub-inhibitory concentrations of rifampicin (30–32). It is therefore possible that the rifampicin concentration used in this study ($\frac{1}{4}$ MIC) was not sufficient to elicit a response. This may be an important observation since depending on the MIC of specific *M. tuberculosis* isolates, during infection *M. tuberculosis* may never be exposed to a rifampicin concentration as high $\frac{1}{2}$ MIC. *bacA* was demonstrated to be up-regulated in response to rifampicin in the same study, under the same conditions. In a separate study *bacA* was shown to be up-regulated by exposure to $\frac{1}{4}$ MIC (33); however our study did not show the same response.

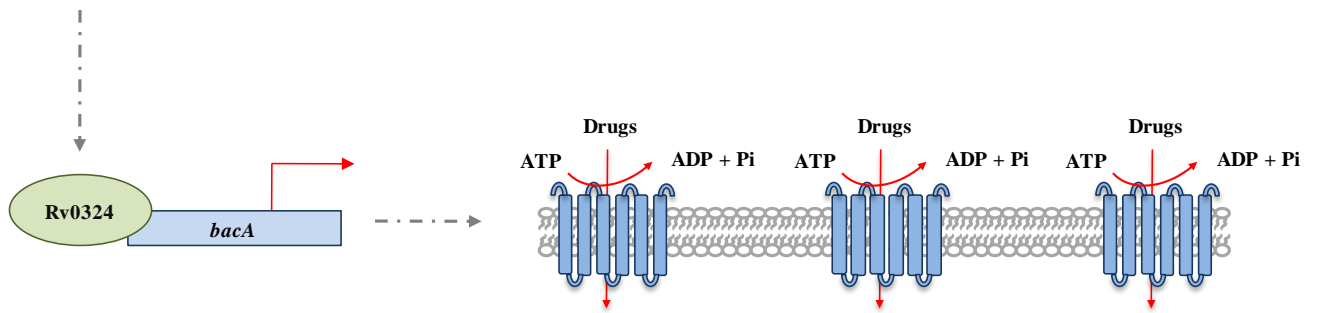
To investigate these results further, available ChIP-seq data was used to identify predicted regulatory interactors for each of the efflux pump encoding genes used in this study (Table 6.5). Whole genome sequence data for the *M. tuberculosis* isolates used in this study was subsequently used to identify polymorphisms in the predicted regulatory interactors. Polymorphisms were identified in six of the predicted interactors (Table 6.6) however only one predicted regulator was observed to have a mutation in all *M. tuberculosis* isolates used in this study. *Rv0324* is a probable transcriptional regulator belonging to the ArsR family of transcriptional activators and repressors (<http://genolist.pasteur.fr/TubercuList/>; The Pfam protein families database). Based on the observed up-regulation of *bacA* in response to rifampicin exposure in literature, and the absence of up-regulation in the isolate used in this study containing a polymorphism in a predicted regulator of *bacA* this study proposes that *Rv0324* may act as a transcriptional activator in response to rifampicin exposure (Figure 6.5).

We propose a model whereby in response to rifampicin exposure transcriptional regulator *Rv0324* binds to *bacA*, up-regulating *bacA* expression resulting in an increase in the amount of BacA ABC transporter in the cell membrane and a subsequent increase in the efflux of

rifampicin from the cell (Figure 6.5A). In the case of the isolates used in this study, a polymorphism prevents the activation of *BacA* by *Rv0324* and no up-regulation of this gene is observed in response to rifampicin exposure (Figure 6.5B).

Further investigation into the genomes available in the Department of Biomedical Sciences identified the Thr168Ala polymorphism in *Rv0324* to be present in 321 *M. tuberculosis* clinical isolates, 57 of which were susceptible. In order to confirm the hypothesis that the polymorphism in *Rv0324* prevents the activation of expression of *bacA* in response to rifampicin exposure futures studies include investigating the response of *bacA* to rifampicin exposure in numerous isolates which have a wild type *Rv0324* gene. An additional finding in literature is that while *bacA* is over-expressed in response to rifampicin exposure, a *bacA* deficient *M. tuberculosis* mutant demonstrated no change in sensitivity to rifampicin (34). This indicates that *bacA* is not responsible for contributing to the level of rifampicin resistance, but the expression thereof is altered in the presence of drug.

While the response to rifampicin observed in this study was minimal, only a small subset of genes was analysed. More information may be acquired with an RNA-seq experiment, where all genes differentially expressed in response to rifampicin exposure may be observed. In addition, a limitation of this study was the small sample size; only four *M. tuberculosis* isolates were investigated. A larger study where a range of rifampicin concentrations are used may also shed light on the discrepant results seen between this study (where $\frac{1}{4}$ MIC was used) and those of studies where $\frac{1}{2}$ MIC was used.

Rifampicin

B

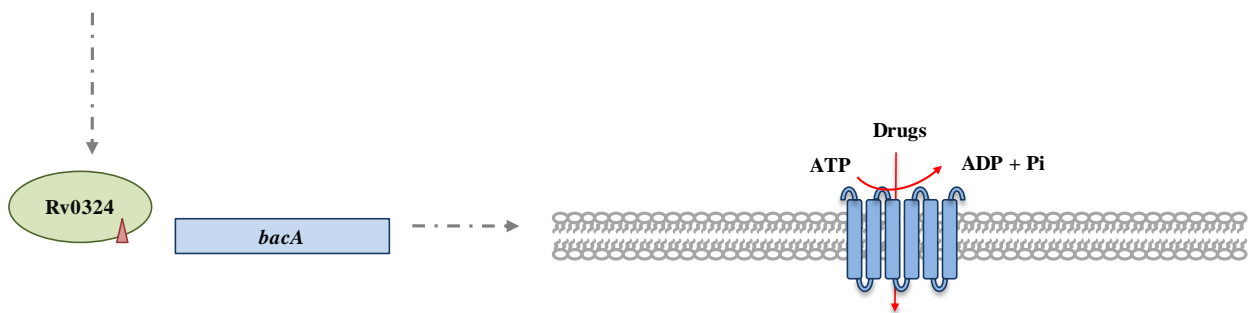
Rifampicin

Figure 6.5 Schematic representation of the proposed regulation of *bacA* in response to rifampicin exposure in *M. tuberculosis*. In the presence of rifampicin Rv0324 activates transcription of *bacA* resulting in increased expression of this gene and subsequently an increase in the amount of BacA deposited in the cell membrane (A). In the case of a polymorphism in Rv0324, *bacA* expression remains unchanged as does the amount of ABC transporter deposited in the cell membrane (B).

In conclusion, this study is the first to demonstrate the up-regulation of *mmpL5* in response to rifampicin exposure. Furthermore a putative transcriptional regulator acting in response to rifampicin exposure was identified. A model for the response to rifampicin exposure has been proposed, however further studies are needed to substantiate this hypothesis.

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CHAPTER 7

Conclusion

This study aimed to investigate mechanisms contributing to drug resistance in *M. tuberculosis* by focusing on three main aspects namely: the *in vivo* evolution of drug resistance from rifampicin mono-resistance to multi-drug resistance (MDR), the role of efflux pumps in rifampicin resistance with a specific focus on the effect of *rpoB* mutations on the response to efflux pump inhibitor verapamil and lastly, the response of energy metabolism and efflux pump genes to rifampicin exposure.

Interrogation of the strain bank housed in the Department of Biomedical Sciences allowed for the identification of individual patients with multiple samples present in the strain bank which demonstrate potential evolutionary patterns with respect to drug resistance i.e. numerous drug resistance evolution patterns. This is an important resource which may be used for future studies to further investigate the *in vivo* evolution of drug resistant in *M. tuberculosis*. This study investigated a small subset of the available samples, representing the evolution of MDR from rifampicin mono-resistance. A total of 6 isolates originating from 3 patients were identified and confirmed to have evolved MDR during the course of treatment. Whole genome sequencing successfully demonstrated the genome of *M. tuberculosis* is dynamic during the course of infection, with numerous genetic changes emerging and disappearing between 2 time points. This finding challenges available literature which has shown the stability of the *M. tuberculosis* genome over time, while supporting more recent findings that the *M. tuberculosis* genome is highly dynamic and diverse (1–7). In addition, deep sequencing allowed for the identification of sub-populations present within a patient at a given time point, highlighting the capacity of deep sequencing. This finding has implications when considering the design of rapid genotypic diagnostics. For instance, current genetic testing may not be sensitive enough to detect an *rpoB* mutation present in only 10% of the population and when put on standard first-line treatment a patient infected with this isolate would have a high likelihood of developing rifampicin resistance, weakening the 4 drug regimen. Deep sequencing technologies should therefore be exploited for the design of new diagnostic tests. This conclusion is complementary to recent commentaries based on whole genome sequencing studies (7, 8).

However, this study was limited by the small number of available samples confirmed to demonstrate evolution of resistance from rifampicin mono-resistance to MDR. Future studies include deep sequencing of a larger sub-set of samples demonstrating evolution of drug resistance from MDR to pre-extensively drug resistant (XDR) and XDR. In addition, whole

genome sequencing will be coupled with whole proteomic analysis to investigate the physiological consequences of any genetic changes observed.

This study went on to investigate the role of efflux pump activity in rifampicin resistance, focusing on the effect of *rpoB* mutations on the response to verapamil treatment. It was demonstrated that verapamil has a differential effect on *M. tuberculosis* isolates with different *rpoB* mutations. *M. tuberculosis* isolates with an *rpoB* Ser531Leu mutation causing rifampicin resistance were more susceptible to verapamil treatment when compared to an isolate with an *rpoB* His526Tyr mutation. These findings suggest differential efflux pump activity between isolates however RT-qPCR was unable to identify substantial differential expression of efflux pump genes accounting for this response. Future studies include whole transcriptomic and/or proteomic analysis to identify proteins or regulatory pathways altered by the presence of different *rpoB* mutations. The differential response to verapamil observed due to the presence of different *rpoB* mutations highlights the importance of personalised treatment. Considering potential side-effects of verapamil on patients with no cardiovascular problems, it may be important to determine if an *M. tuberculosis* isolate will sufficiently respond to verapamil treatment. Additional studies are needed to determine if the presence of specific *rpoB* mutations will influence the use of verapamil to counter act the efflux mediated level of resistance to bedaquiline and clofazamine. Recent studies have highlighted the concurrent use of verapamil and bedaquiline to potentiate bedaquiline treatment. It has also been suggested that since verapamil lowers the MIC of bedaquiline, lower concentrations may potentially be considered to diminish the toxic effect of treatment (9, 10). The findings of this study should serve as the basis for future studies to investigate the importance of the pathogens genetic background on the efficacy of inhibitor treatment.

Lastly, this study investigated the response of *M. tuberculosis* to rifampicin exposure. This study demonstrated that in response to rifampicin exposure there is no significant change in gene expression of selected components involved in energy metabolism in *M. tuberculosis*. In addition, little change was seen in response to rifampicin exposure with regards to efflux pump expression. This may be due to the concentration of rifampicin used; previous studies used sub-inhibitory concentrations of rifampicin ($\frac{1}{2}$ MIC). In addition, our study only investigated a small subset of efflux pump genes. Furthermore, gene expression analysis is not a measure of efflux pump activity, or flux through energy metabolism pathways. Future studies involve measuring the presence of metabolites in response to drug exposure e.g. measuring the levels

of cellular ATP or the NADH/NAD⁺ ratios. In addition future studies include investigating the response to rifampicin exposure using whole transcriptomic and proteomic analysis.

While little response to rifampicin exposure was observed, *mmpL5* was shown to be significantly up-regulated in response to rifampicin exposure. This is the first study to show the response of this gene to rifampicin exposure. This gene encodes the mmpL5 protein, forming part of the mmpS5-mmpL5 efflux system, recently associated with bedaquiline and clofazamine resistance (9, 11). This efflux based resistance mechanism is founded on the presence of a mutation in the Rv0678 repressor of this efflux system (9, 11). Susceptibility testing of a clofazamine resistant mutant (due to an Rv0678 mutation) demonstrated no cross resistance with rifampicin (11). The *M. tuberculosis* isolates in this study did not have any mutations in this repressor, indicating a different regulatory pathway is responsible for the response of *mmpL5* to rifampicin exposure. Future studies include elucidating if the response of this efflux pump to rifampicin exposure is associated with rifampicin resistance, or if this up-regulation serves a different metabolomic purpose since this efflux system has been proposed to be involved in iron acquisition (11).

In addition, this study identified a predicted transcriptional regulator of the BacA efflux pump. We propose that transcriptional regulator Rv0324 may be involved in the response to rifampicin exposure, based on the observation that *M. tuberculosis* isolates in this study with a mutation in this regulator showed no up-regulation of *bacA*, which has been shown in literature to be up-regulated in response to rifampicin exposure (12). The response to rifampicin exposure of BacA needs to be investigated in *M. tuberculosis* isolates with and without an Rv0324 mutation to confirm whether a wild type Rv0234 will respond to rifampicin exposure resulting in up-regulation of BacA. In addition, should this hypothesis be found to be true, additional interaction partners of this regulator need to be determined to identify a regulatory pathway which acts in response to rifampicin exposure.

In order to identify additional regulatory mechanisms in response to rifampicin exposure, a proposed future study is to do a forward genetic screen using transposon mutagenesis. Briefly, a library of transposon mutants generated in a rifampicin resistant *M. tuberculosis* clinical isolate (previously generated and housed in the Department of Biomedical Sciences) will be exposed to varying concentrations of rifampicin. A negative screen using WGS will allow for the identification of genes essential for the response to rifampicin exposure.

In conclusion this study demonstrated that the *M. tuberculosis* genome is dynamic during infection. This study highlights the importance of coupling phenotypic studies with whole genome sequencing analysis. Knowledge of the genome sequences of the *M. tuberculosis* isolates used in this study allowed us to associate the *rpoB* mutation type to the differential response to rifampicin, since no other genetic changes could clearly be associated with this observation. This study demonstrated the importance of the *rpoB* mutation on efflux pump activity and the subsequent response to verapamil treatment. In addition this study identified *mmpL5* to respond to rifampicin exposure; however the consequence of this observation remains unknown. A proposed transcriptional regulator responding to rifampicin exposure was also identified in this study. A multi-study approach allowed for a broader understanding of the mechanisms involved in drug resistance in *M. tuberculosis*.

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APPENDIX 1

Solutions and Media

7H9 Broth

4.7g Middlebrooke 7H9

0.2% Tween 80

2.5ml glycerol

900ml water

Autoclave to sterilise

Supplement with 100ml OADC

7H10 Plates

19g Middlebrooke 7H10

5ml glycerol

900ml water

Autoclave to sterilise

Supplement with 100ml ADC

Extraction buffer

5% sodium glutamate

50mM Tris-HCl [pH 7.4]

25mM EDTA

Proteinase K buffer

5% sodium dodecyl sulfate

100mM Tris-HCl [pH7.8]

50mM EDTA

TE Buffer

10mM Tris

1mM EDTA

GITC

4M guanidium thiocyanate

25mM sodium citrate [pH7]

0.5% sarcosyl

0.1M β -mercaptoethanol

APPENDIX 2

Additional results

Table A.2.1 *M. tuberculosis* isolates selected from the strain bank to show evolution from rifampicin mono-resistance to MDR and re-classified using *rpoB*, *katG* and *inhA* promoter sequencing.

Patient	Isolate name	<i>rpoB</i>	<i>inhA</i> promoter	<i>katG</i>	Original classification	New classification
1	R721	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R807	531TTG	wt	309GTT	MDR	MDR
2	R3301	531TTG	-15	wt	Rifampicin mono	MDR
	R3552	531TTG	-15	wt	MDR	MDR
3	R13112	Del (507-508)	wt	wt	Rifampicin mono	Rifampicin mono
	R13235	Del (507-508)	wt	wt	Rifampicin mono	Rifampicin mono
	R13855	Del (507-508)	wt	wt	MDR	Rifampicin mono
4	R912	526TAC	wt	wt	Rifampicin mono	Rifampicin mono
	R1210	526TAC	-15	wt	MDR	MDR
5	R12218	511CCG	wt	wt	Rifampicin mono	Rifampicin mono
	R12275	511CCG	wt	wt	MDR	Rifampicin mono
6	R6264	511CCG	wt	wt	Rifampicin mono	Rifampicin mono
	R15213	511CCG	wt	wt	MDR	Rifampicin mono
7	R1435	531TTG		315ACC	Rifampicin mono	MDR
	R1801	531TTG	wt	315ACC	MDR	MDR
8	R8916	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R12523	531TTG	wt/-15	315AGC/ 315ACC	MDR	Mix
9	R1093	wt	wt	wt	Rifampicin mono	sensitive
	R1233	531TTG	wt	315ACC	MDR	MDR
10	R1141	526CGC	-15	wt	Rifampicin mono	MDR

	R1191	526TAC	-15	wt	MDR	MDR
11	R1235	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R20213	wt	wt	wt	MDR	Sensitive
12	R2951	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R7974	531TTG	-15	wt	MDR	MDR
13	R3324	wt	wt	285GTC	Rifampicin mono	INH mono
	R3389	wt	wt	wt	MDR	sensitive
14	R20760	wt/516GTC	wt	295CCG	Rifampicin mono	Mix
	R20743	516GTC	wt	Wt/295CCG	MDR	Mix
15	R293	516GTC	wt	315ACC	Rifampicin mono	MDR
	R302	516GTC	wt	315ACC	MDR	MDR
16	R2080	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R2081	531TTG	wt	wt	unclassified	Rifampicin mono
	R2083	531TTG	wt	wt	unclassified	Rifampicin mono
17	R12390	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R12706	531TTG	wt	wt	Unclassified	Rifampicin mono
18	R800	533CCG	-15	wt		MDR
	R873	533CCG	-15	wt	MDR	MDR
19	R1976	531TTG	wt	wt	Rifampicin mono	rifampicin mono
	R2541	531TTG	wt	315AAC	MDR	MDR
20	R21542	526CAA and Del (508-509)	wt	wt	Rifampicin mono	Rifampicin mono
	R21686	526CAA and Del (508-509)	wt	wt	MDR	Rifampicin mono
21	R1605	526TAC	wt	wt	Rifampicin mono	Rifampicin mono
	R1932	-	-	-	MDR	
22	R2135	531TTG	wt	wt	Rifampicin mono	Rifampicin mono

	R2387	531TTG	-15	wt	MDR	MDR
23	R3289	531TTG	-15	wt	Rifampicin mono	MDR
	R3806	531TTG	-15	wt	MDR	MDR
24	R2376	531TTG	-15	wt	Rifampicin mono	MDR
	R2581	531TTG	-15	wt	MDR	MDR
25	R7252	531TTG	-17	wt	Rifampicin mono	MDR
	R8790	531TTG	-17	wt	Rifampicin mono	MDR
	R10359	531TTG	-17	wt	MDR	MDR
26	R3172	wt	-15	wt	Rifampicin mono	INH mono
	R5196	531TTG	-15	wt	MDR	MDR
27	R4324	533CCG	wt	294GAA	Rifampicin mono	MDR
	R6866	wt	wt	wt	MDR	sensitive
28	R16529	wt	wt	315ACC	Rifampicin mono	INH mono
	R16775	wt	wt	315ACC	MDR	INH mono
29	R5608	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R15828	531TTG	wt	wt	MDR	Rifampicin mono
30	R8707	wt	wt	315AAC	Rifampicin mono	Rifampicin mono
	R9817	516GTC	wt	315AAC	MDR	MDR
31	R12681	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R13347	531TTG	wt	wt	Rifampicin mono	Rifampicin mono
	R13680	-	-	-	MDR	Rifampicin mono

Table A2.2 PCR and Sanger sequencing results for *embB*, *gyrA*, *rrs* and *pncA*.

Sample set	Isolate name	<i>rrs</i>	<i>embB</i>	<i>gyrA</i>	<i>pncA</i>
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1	R721	wt	wt	wt	wt	Rifampicin mono
	R807	wt	wt	wt	wt	MDR
2	R13112	wt	wt	wt	wt	Rifampicin mono
	R13235	wt	wt	wt	wt	Rifampicin mono
	R13855	wt	wt	wt	wt	Rifampicin mono
3	R912	wt	wt	wt	wt	Rifampicin mono
	R1210	wt	wt	wt	wt	MDR
4	R12218	wt	wt	wt	wt	Rifampicin mono
	R12275	wt	wt	wt	wt	Rifampicin mono
5	R6264	wt	wt	wt	wt	Rifampicin mono
	R15213	wt	wt	wt	wt	Rifampicin mono
6	R2951	wt	wt	wt	wt	Rifampicin mono
	R7974	C1300T; G1321A; C1445T	wt	wt	wt	Mixed population
7	R2080	wt	wt	wt	wt	Rifampicin mono
	R2081	wt	wt	wt	wt	Rifampicin mono
	R2083	wt	wt	wt	wt	Rifampicin mono
8	R12390	wt	wt	wt	wt	Rifampicin mono
	R12706	wt	wt	wt	wt	Rifampicin mono
9	R1976	wt	wt	wt	wt	Rifampicin mono
	R2541	wt	306ATA	wt	wt	
10	R21542	wt		wt	10CAC	Poly resistant
	R21686	wt		wt	10CAC	Poly resistant

11	R2135	1300 C/T & 1321 G/A & 1445 C/T		91CCG	103TAG	Mixed population
	R2387	1401 A/G	wt	94GGC	-	XDR
12	R5608	wt	wt	wt	wt	Rifampicin mono
	R15828	wt	wt	wt	391 GG ins	Poly resistant
13	R12681	wt	306GTG	wt	97GAT	
	R13347	wt	306GTG	wt	97GAT	





Table A2.3 Rifampicin and isoniazid DST results

Sample set	Isolate name	RIF dst (1µg/ml RIF)	INH dst (0.1µg/ml INH)	Classification
1	R721	R	S	Rifampicin mono
	R807	R	R	MDR
2	R13112	R	R	MDR
	R13235	R	R	MDR
	R13855	R	R	MDR
3	R912	R	S	Rifampicin mono
	R1210	R	R	MDR
4	R12218	R	R	MDR
	R12275	R	R	MDR
5	R6264	R	S	Rifampicin mono
	R15213	R	R	MDR
6	R2080	R	S	Rifampicin mono
	R2081	R	S	Rifampicin mono
7	R2083	R	S	Rifampicin mono
	R12390	R	S	Rifampicin mono
	R12706	R	S	Rifampicin mono

R = resistant; S = sensitive

Table A2.4 Spoligotype analysis of *M. tuberculosis* isolates demonstrating evolution from rifampicin mono-resistance to MDR

	Classification	Spoligotype pattern	Spoligotype interpretation
1	Rifampicin mono		BEIJING
	MDR		BEIJING

2	R912	Rifampicin mono		EAI
	R1210	MDR		EAI
3	R6264	Rifampicin mono		X3 (LCC)
	R15213	MDR		X3 (LCC)

APPENDIX 3

FastQC results

The figures presented below are a representation of the FastQC results generated for all *M. tuberculosis* isolates sequenced.

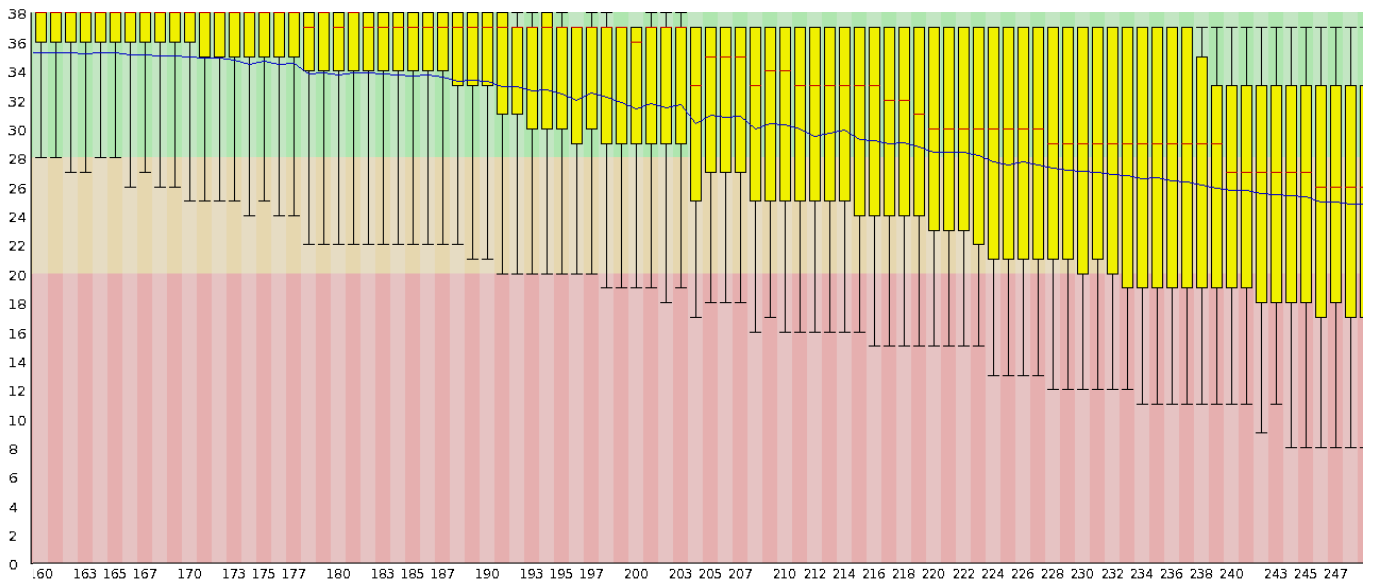


Figure A3.1 The distribution of quality scores across all bases. The blue line represents the mean quality, the red lines represent median values and the green and red regions of the plot indicate high and low quality scores, respectively.

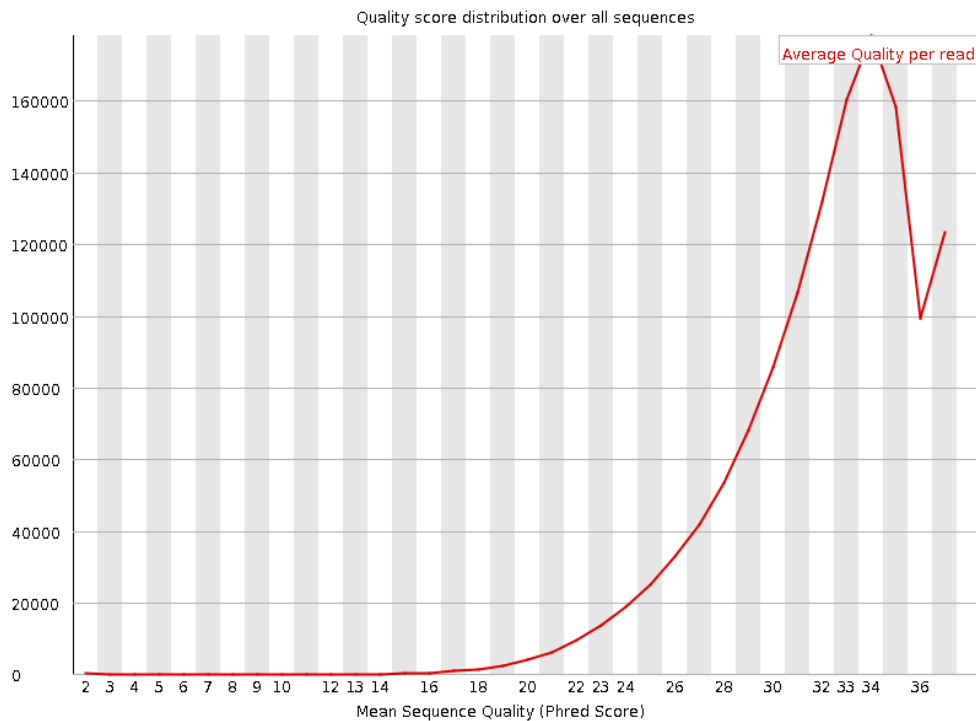


Figure A3.2 The distribution of quality across all sequences. Score used to assess whether a proportion of the sequences have universally low quality scores. 93

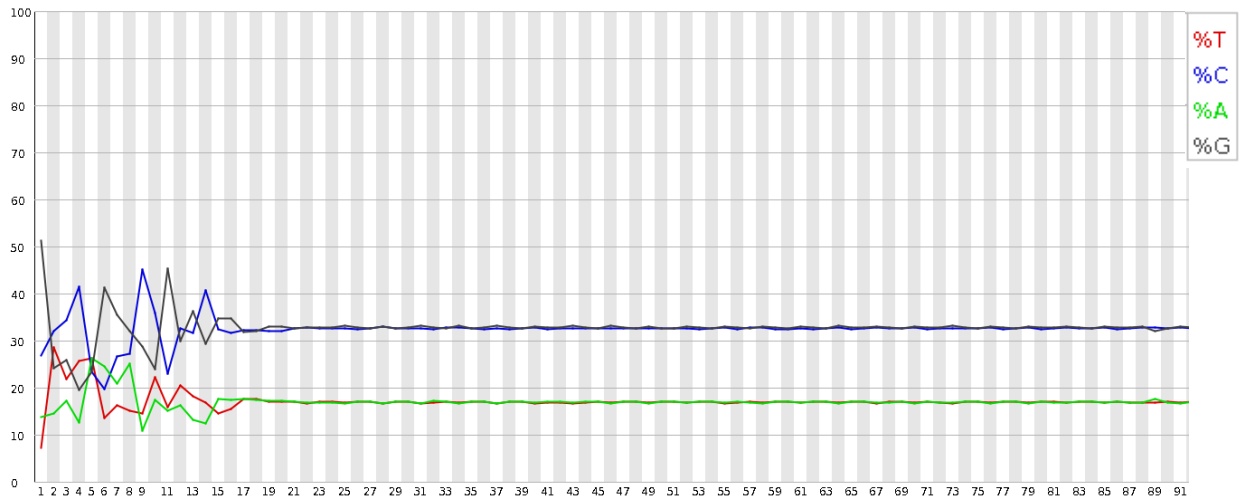


Figure A3.3 Sequence content across all bases. *M. tuberculosis* is expected to have a high GC content

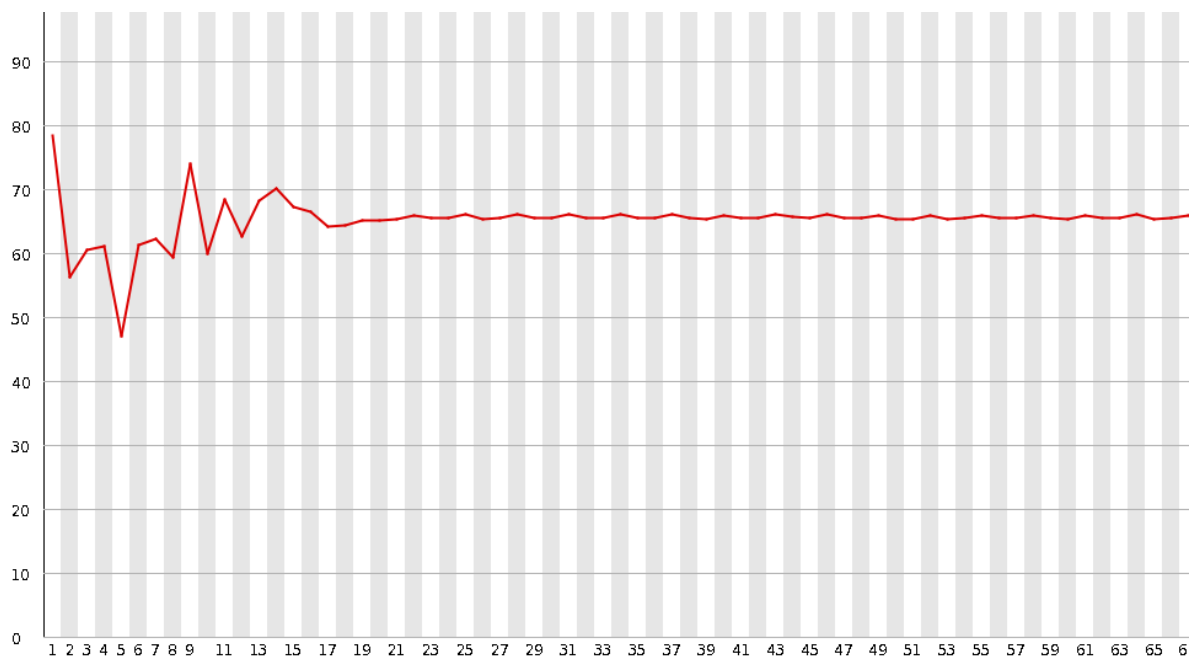


Figure A3.4 Representative of the mean percentage of GC content across all bases. The horizontal red line indicates minimal change in the average GC content for all bases. A GC content of above 60% is expected for *M. tuberculosis*.

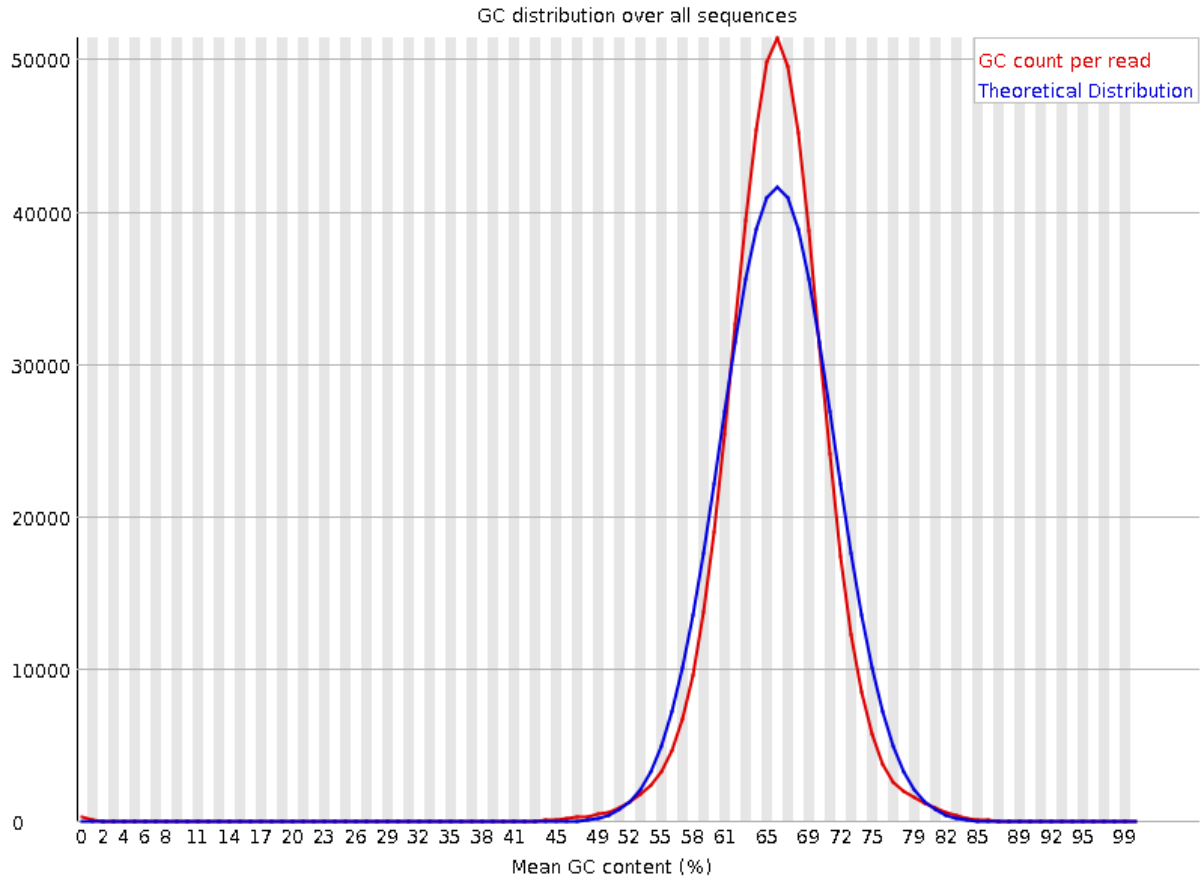


Figure A3.5 The mean distribution of the percentage GC content across all sequences. A normal distribution of the GC content is observed with the central peak corresponding to the overall GC content of this genome.

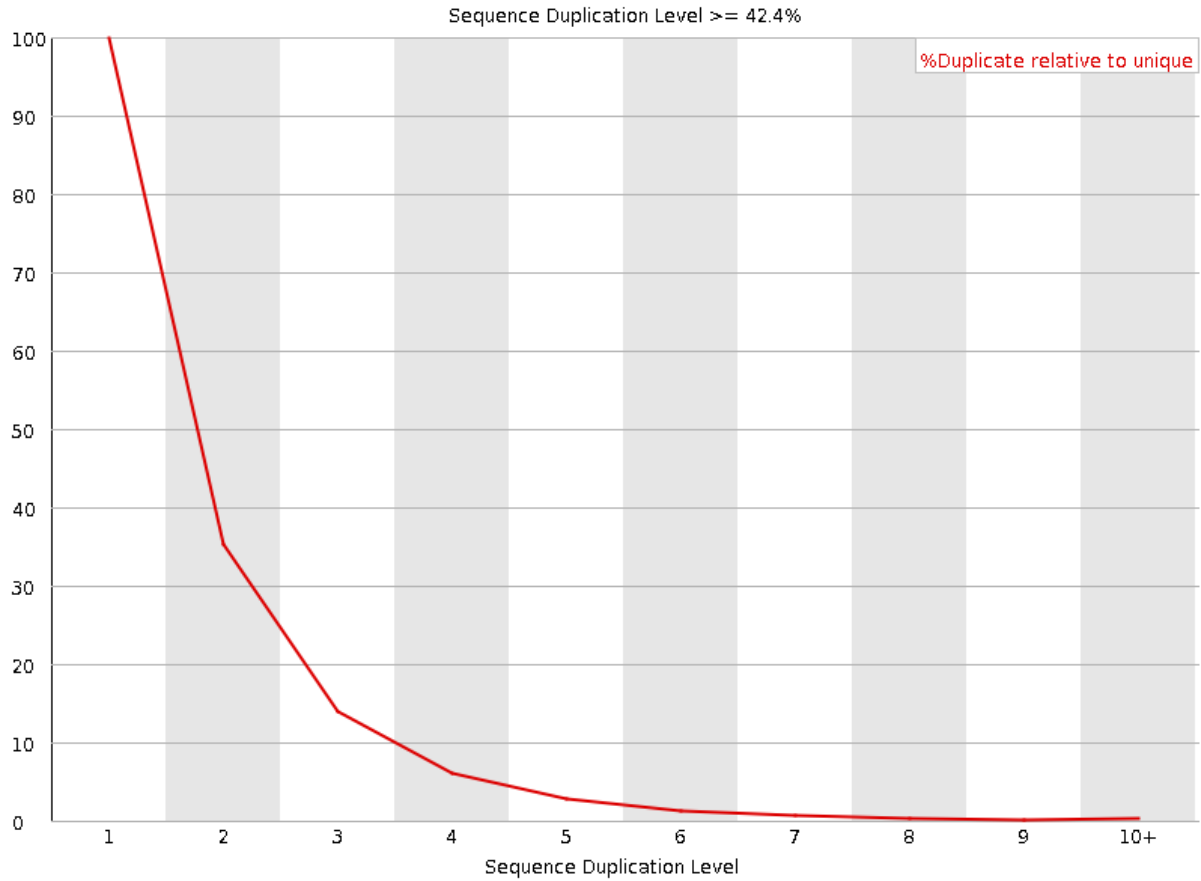


Figure A3.6 Sequence duplication levels in the library sequenced. A high percentage of duplicate sequences are present in the Illumina reads, however PCR duplicates are removed during bioinformatic analysis.