Evolution of XDR-TB and the associated proteome

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Declaration

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Summary

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis*, carries a substantial health burden worldwide and in South Africa. Efforts towards prevention and effective treatment of this disease is essential to meet the END TB goals. The disease is exacerbated by HIV co-infection and drug resistance. Multidrug-resistant (MDR) TB (resistance to at least rifampicin and isoniazid) places enormous resource constraints on TB control programs with poorer treatment outcomes. More recently, extensively drug-resistant (XDR) TB (MDR-TB with additional resistance to a fluoroquinolone and an injectable) has become a global concern. XDR-TB develops through the acquisition of mutations in the *gyrA* and *rrs* genes.

The aim of this work was to investigate aspects of the evolution and physiology of XDR-TB, in particular acquisition of mutations and their impact on protein abundance.

Assessment of the impact of nucleoside reverse transcriptase inhibitors on the mutation rate of *Mycobacterium smegmatis* indicated no significant effect, suggesting that antiretroviral treatment does not contribute to the overlap of HIV infection and drug-resistant TB. Analysis of spontaneous ofloxacin-resistant mutants indicated that a Beijing clinical isolate acquired high level drug resistance mutations more readily than H37Rv, providing a possible reason for the association of Beijing with drug resistance. Analysis of spontaneous moxifloxacin resistant mutants showed that *gyrA* mutations at codons 88 and 94 were associated with resistance (defined as minimum inhibitory concentration (MIC) of ≥ 2 μg/ml). Despite the presence of *gyrA* mutations, moxifloxacin significantly impeded bacterial growth, supporting its continued use for the treatment of ofloxacin-resistant *M. tuberculosis*.

In an attempt to determine whether the fluoroquinolone MIC could be increased we selected spontaneous mutants from a fluoroquinolone resistant clone on higher concentrations of the fluoroquinolone. Sequencing of *gyrA* and *gyrB* identified additional mutations suggesting that double mutations are responsible for increasing the MIC. We could not find any involvement of efflux pump activity in modulating the MIC.

To determine the influence of the *gyrA* Asp94Gly and *rrs* A1401G mutation on the physiology of the pathogen we assessed their effect on protein abundance. A strong signature of differentially abundant proteins common to both clones, expressed from the ESX-5 cluster, suggested either the presence of a common unknown genetic variant (in unmapped genomic

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regions) or common physiological changes related to drug resistance. The *gyrA* mutant uniquely demonstrated decreased abundance in transport proteins, suggesting decreased cell wall permeability and increased drug tolerance. Changed abundance of proteins involved in transcription/translation was also observed, suggesting impaired functionality of the mutated gyrase. The *rrs* mutant displayed lowered abundance of stress proteins belonging to the DosR/DevR regulon, potentially impacting the mutant's response to dormancy. Ofloxacin treatment of the *gyrA* mutant resulted in increased abundance of proteins involved in iron acquisition and differential abundance of proteins indicating decreased cell division and growth. We hypothesize that increased abundance of iron acquisition proteins relates to chelation of iron by ofloxacin. Amikacin treatment of the *rrs* mutant decreased ribosomal protein abundance and increased proteins involved in tRNA-related processes. We hypothesise that this relates to increased degradation of ribosomes and a mechanism to compensate for reduced translational fidelity.

This study improved our understanding of the physiological factors contributing to the emergence of XDR-TB. Furthermore, our results suggest that the physiology of XDR *M. tuberculosis* differs from susceptible strains. These changes in physiology could inform further research on drug targets and optimal treatment regimens.

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Opsomming

Tuberkulose (TB), wat veroorsaak word deur die bakterie *Mycobacterium tuberculosis*, plaas aansienlike druk op gesondheidstelsels wêreldwyd en in Suid-Afrika. Pogings tot voorkoming en effektiewe behandeling van hierdie siekte is van kardinale belang om die Wêreld Gesondheidsorganisasie se doelwitte van "End TB“ te bereik. Die siekte word vererger deur MIV mede-infeksie en middelweerstandigheid. Multi-middelweerstandige (MDR) TB (weerstandig teen isoniasied en rifampisien) plaas geweldige hulpbron-beperkings op TB behandelingsprogramme met swakker behandelingsuitkomste. Meer onlangs het ekstensiewe middelweerstandige (XDR) TB (MDR TB met bykomende weerstand tot ‘n fluorokinoloon en ‘n tweede-linie middel wat as inspuiting toegedien word) ‘n wêreldwye kommer geword. XDR TB ontwikkel deur die verkryging van mutasies in die *gyrA* en *rrs* gene.

Die algemene doel van hierdie projek was om aspekte van die evolusie en fisiologie van XDR TB te ondersoek en spesifiek, die verkryging van mutasies en hulle impak op relatiewe kwantitatiewe proteïen-verskille.

Assessering van die impak van nukleosied trutranskriptase inhibeerders op die mutasie tempo van *Mycobacterium smegmatis* het geen beduidende effek gewys nie, wat daarop dui dat antiretrovirale behandeling nie bydra tot die dikwelse oorvleueling van MIV en middelweerstandige TB nie. Analise van spontane ofloksasien-weerstandige mutante het gewys dat ’n kliniese isolaat van die Beijing stamfamilie van *M. tuberculosis* meer geredelik mutasies wat hoë-vlak weerstandigheid veroorsaak, verkry het as die laboratorium stam, H37Rv, wat ’n moontlike rede kan wees waarom die Beijing stamfamilie met middelweerstandigheid geassosieer word. Analise van spontane moxifloksasien-weerstandige mutante het gewys dat *gyrA* mutasies in kodons 88 en 94 met weerstandigheid (gedefineër as ‘n minimum inhiberende konsentrasie (MIK) van ≥ 2 µg/ml) geassosieer kon word. Ten spyte van die teenwoordigheid van *gyrA* mutasies het moxifloksasien bakteriële groei beduidend verminder, wat ondersteunende bewyse is vir die voortgaan van gebruik van die middel vir ofloksasien-weerstandige tuberkulose behandeling.

In ‘n poging om te bepaal of die fluorokinoloon MIK verhoog kon word, is spontane mutante gekies vanaf ‘n fluorokinoloon-weerstandige kloon met hoër konsentrasie van die middel. Volgordebepaling van *gyrA* en *gyrB* het bykomende mutasies geïdentifiseer, wat daarop dui

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dat dubbele mutasies verantwoordelik is vir die verhoogde MIK. Geen betrokkenheid van effluks pompe by die modulering van die MIK is waargeneem nie.

Om die invloed van die *gyrA* Asp94Gly en *rrs* A1401G mutasies op die fisiologie van die patogeen te bepaal, is relatiewe kwantitatiewe proteïen-verskille gemeet. Daar was 'n sterk aanduiding van gemeenskaplike proteïen-verskille in die twee klone, spesifiek uitgedruk vanaf die ESX-5 geengroepgebied, wat moontlik toegeskryf kan word aan 'n gemene genetiese variant (in genomiese gebiede waarvan die volgorde nie bepaal kon word nie) of 'n gemene fisiologiese verandering verwant aan middelweerstandigheid. Die *gyrA* mutant het unieke veranderinge getoon, waaronder verminderde hoeveelhede van vervoer- proteïene, wat op verminderde deurlaatbaarheid van die selwand en potensieël verhoogde middeltoleransie dui. Verskille in proteïene betrokke by transkripsie en proteïen-sintese is ook waargeneem, wat dui op verswakte funksie van die gemuteerde girase. Die *rrs* mutant het verlaagde hoeveelhede van stres- proteïene gereguleer deur DosR/DevR getoon, wat potensieël die mutant se reaksie op dormansie kan beïnvloed. Behandeling van die *gyrA* mutant met ofloksasien het 'n vermeerdering van proteïene betrokke by ysterverkryging tot gevolg gehad, sowel as proteïenverskille wat dui op verminderde selverdeling en groei. Daaruit het die hipotese ontstaan dat vermeerdering van ysterverkrygingsproteïene verwant is aan chelaatvorming van ofloksasien met yster. Behandeling van die *rrs* mutant met amikasien het gelei tot verminderde vlakke van ribosoom- proteïene en vermeerde vlakke van proteïene betrokke by oordrag-RNA prosesse. Daaruit het die hipotese ontstaan dat die veranderinge verwant is aan verhoogde afbreking van ribosome en 'n kompenseringsmeganisme vir die verminderde akkuraatheid van proteïensintese.

Hierdie studie het gelei tot verbeterde kennis van die fisiologiese faktore wat bydra tot die opkoms van XDR TB. Verder het die resultate daarop gedui dat die fisiologie van XDR *M. tuberculosis* verskil van middel sensitiewe stamme. Hierdie veranderings in fisiologie kan kennis bydra tot verdere navorsing op teikens vir antibakteriese middels en optimale behandelingsplanne.

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List of abbreviations

°C	degrees Celsius
2-D	two-dimensional
3R	recombination, replication, repair
A	deoxyadenosine
ADC	Albumin-dextrose-catalse
ADP	adenosine diphosphate
ANOVA	analysis of variance
ART	antiretroviral
ATP	adenosine triphosphate
AZT	azidothymidine
C	deoxycytosine
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleid acid
dNTP	deoxy nucleoside triphosphate
ESX	ESAT-6 secretion system
FDR	false discovery rate
G	deoxyguanosine
GC	guanine-cytosine
HIV	Human immunodeficiency virus
LFQ	label-free quantification
MDR	Multidrug-resistant
MGIT	mycobacterial growth indicator tube
MIC	Minimum inhibitory concentration
mRNA	messenger ribonucleic acid
N	normal
nm	nanometer
nmol	nanomoles

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NRTI	nucleoside reverse transcriptase inhibitors
OADC	oleic albumin-dextrose-catalase
OD	optical density
PCR	polymer chain reaction
pmol	picomole
QRDR	quinolone resistance-determining region
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
RRDR	Rifampicin resistance-determining region
SNP	single nucleotide polymorphism
T	deoxythymidine
TB	Tuberculosis
TP	triphosphate
tRNA	transfer ribonucleid acid
WHO	World Health Organisation
XDR	Extensively drug-resistant
µ	micro

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Contents

Declaration i

Summary ii

Opsomming iv

Acknowledgments vi

List of abbreviations..viii

Contents..x

List of figures..xvi

List of tables..xvii

Chapter 1 Introduction xx

1.1 The tuberculosis (TB) epidemic 1

1.2 The acquisition of XDR in *M. tuberculosis* 3

1.2.1 Chromosomal mutation 3

1.2.2 Mutation rate 4

1.2.3 Selection of resistance-conferring mutations 5

1.2.4 Compensatory evolution 6

1.2.5 Modulation of drug resistance 6

1.3 Physiology of XDR 7

1.3.1 Consequences of drug resistance mutations 7

1.3.2 Consequences of treating a resistant strain with antibiotic 8

1.4 Hypotheses and aims 8

1.4.1 Hypothesis 1 8

1.4.2 Hypothesis 2 9

1.4.3 Hypothesis 3 9

Stellenbosch University https://scholar.sun.ac.za

Chapter 2 Literature review 20
2.1 Abstract 19
2.2 Introduction 19
2.3 *M. tuberculosis* mutates at a low rate in vitro 23
2.4 Generation of drug resistance-conferring mutations in *M. tuberculosis* during infection 25
2.5 The mycobacterial replication rate 27
2.6 A role for constitutive mutator strains? 29
2.7 Transient mutagenesis 32
2.8 Environmental mutagens 34
2.8.1 Antibiotics 34
2.8.2 Antiretroviral drugs 35
2.8.3 TB pathogenesis and the host environment 36
2.8.4 Smoking and air pollution 38
2.9 Conclusion 38
Chapter 3 The effect of nucleoside reverse transcriptase inhibitors on the mycobacterial mutation rate 53
3.1 Abstract 53
3.2 Introduction 53
3.3 Materials and methods 55
3.3.1 Preparation of stocks for drugs 55
3.3.2 Determination of minimum inhibitory concentration of ARVs for *M. smegmatis* 56
3.3.3 *M. smegmatis* fluctuation assays 56
3.3.4 Determination of mutation rate 57
3.3.5 PCR and sequencing 58
3.3.6 Calculation of intracellular concentrations of AZT-TP and 3TC-TP 58
3.3.7 Statistical analysis 58

Stellenbosch University https://scholar.sun.ac.za

3.3.8 Raw data ... 59

3.4 Results ... 59

3.4.1 Effect of nucleoside analogues on the growth of *M. smegmatis* ... 59

3.4.2 Treatment with 3TC and selection on 100 µg/ml rifampicin ... 60

3.4.3 Modification of fluctuation assay ... 63

3.4.4 Mutation rate in the presence of AZT ... 64

3.4.5 Serum concentrations of activated AZT and 3TC ... 65

3.4.6 Mutation rate in the presence of AZT-TP ... 66

3.4.7 Mutation rate in the presence of activated 3TC ... 68

3.5 Discussion ... 69

3.6 Conclusion ... 72

Chapter 4 Characterisation of *in vitro*-generated fluoroquinolone-resistant *Mycobacterium tuberculosis* mutants ... 81

4.1 Abstract ... 80

4.2 Introduction ... 80

4.3 Materials and methods ... 82

4.3.1 Preparation of stocks for drugs ... 82

4.3.2 General culturing of *M. tuberculosis* isolates ... 82

4.3.3 Strains used to select spontaneous mutants ... 82

4.3.4 Generation of spontaneous mutants ... 82

4.3.5 Selection of *M. tuberculosis* spontaneous mutants ... 83

4.3.6 Calculation of fitness score ... 83

4.3.7 PCR and sequencing of *gyrA* and *gyrB* ... 84

4.3.8 Statistical analyses ... 84

4.3.9 Mutation rate assay ... 85

4.3.10 Calculation of mutation rate ... 85

4.4 Results and Discussion ... 85

Stellenbosch University https://scholar.sun.ac.za

4.4.1 Mutations selected *in vitro* during the evolution of ofloxacin resistance 85

4.4.2 The rate of mutation to ofloxacin resistance 89

4.5 Conclusion 90

Chapter 5 Moxifloxacin retains anti-mycobacterial activity in the presence of *gyrA* mutations 95

5.1 Abstract 95

5.2 Manuscript text 95

Chapter 6 Mechanisms modulating the level of fluoroquinolone resistance 104

6.1 Abstract 104

6.2 Introduction 104

6.3 Materials and methods 105

6.3.1 Strains used in this study 105

6.3.2 Preparation of stocks for drugs 105

6.3.3 Determination of BO1 and K636 ofloxacin MIC 105

6.3.4 Selection of mutants exhibiting increased MICs to ofloxacin........ 106

6.3.5 Determination of minimum inhibitory concentrations for ofloxacin in the presence of verapamil and CCCP 106

6.3.6 Determination of minimum inhibitory concentrations for ofloxacin in the presence of reserpine 107

6.4 Results and discussion........ 109

6.4.1 Fluoroquinolone B01 MIC is modulated by additional mutations in *gyrA/gyrB* 109

6.4.2 The primary mechanism of fluoroquinolone resistance *in vitro* is *gyrA* mutation........ 109

6.5 Conclusion 113

Chapter 7 Deciphering the impact of mutations conferring extensive drug resistance on the physiology of *Mycobacterium tuberculosis*........ 116

Stellenbosch University https://scholar.sun.ac.za

7.1 Abstract ... 116

7.2 Introduction ... 116

7.3 Materials and methods ... 119

7.3.1 Isolates used in our study ... 119

7.3.2 PCR and sequencing ... 120

7.3.3 Growth curves ... 121

7.3.4 Preparation of stocks for drugs ... 121

7.3.5 Culturing for extraction ... 121

7.3.6 Protein extraction ... 122

7.3.7 Protein precipitation ... 123

7.3.8 Quantification of protein ... 123

7.3.9 Trypsin digest ... 123

7.3.10 Liquid chromatography ... 123

7.3.11 Mass spectrometry ... 124

7.3.12 Data analysis ... 124

7.3.13 Next generation sequencing ... 126

7.4 Results and discussion ... 127

7.4.1 Growth curves ... 127

7.4.2 Whole genome sequencing of *in vitro* mutants ... 127

7.4.3 Overview of proteomics ... 129

7.4.4 Proteomic changes common to a *gyrA* Asp94Gly and *rrs* A1401G mutant of *M. tuberculosis* ... 130

7.4.5 The proteome of a fluoroquinolone-monoresistant strain of *M. tuberculosis* ... 135

7.4.6 Fluoroquinolone treatment of a *gyrA* mutant of *M. tuberculosis* ... 141

7.4.7 The proteome of an *rrs* A1401G mutant of *M. tuberculosis* ... 145

7.4.8 Amikacin treatment of an *rrs* mutant of *M. tuberculosis* ... 146

Stellenbosch University https://scholar.sun.ac.za

7.4.9 The *rrs* mutation and amikacin treatment synergise to impact translation 146

7.5 Overall conclusion 152

Chapter 8 Conclusions and future work 162

8.1 Conclusions 162

8.2 Future work 164

8.2.1 The effect of nucleoside reverse transcriptase inhibitors on the mycobacterial mutation rate 164

8.2.2 Characterisation of *in vitro*-generated fluoroquinolone-resistant *M. tuberculosis* mutants 165

8.2.3 Evolution of moxifloxacin resistance in *Mycobacterium tuberculosis* 165

8.2.4 Mechanisms modulating the level of fluoroquinolone resistance 166

8.2.5 Deciphering the impact of mutations conferring extensive drug resistance on the physiology of *M. tuberculosis* 166

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List of figures

Figure 3.1 Lamivudine/3TC activation pathway in the mammalian host. MP=monoposphate, DP=diphosphate, TP=triphosphate (Whirl-Carrillo *et al.* 2012). 55

Figure 3.2 The effect of 3TC (left) and AZT (right) on the growth of *M. smegmatis* as determined by the broth microdilution method. The drug diluent was water. Resazurin was used as a colorimetric indicator of growth. Dark blue colour indicates no growth, while magenta indicates growth. The concentration employed in further experiments is outlined. 60

Figure 3.3 The effect of AZT-TP (left) and 3TC-TP (right) on the growth of *M. smegmatis* as determined by the broth microdilution method. Resazurin was used as a colorimetric indicator of growth. Dark blue colour indicates no growth, while magenta indicates growth. The concentrations employed in further experiments are outlined. 67

Figure 4.1 Overall fitness scores for different mutations selected on 2 µg/ml ofloxacin....... 88

Figure 5.1 Comparison of growth of fluoroquinolone-resistant *M. tuberculosis gyrA* mutants and their sensitive progenitor in the presence and in the absence of antibiotics. (A) Gly88Cys (B) Asp94Tyr (C) Asp94Asn (D) Asp94His (E) Asp94Gly. For each culture, the optical density at 600 nm was measured daily in three independent experiments, and the average was plotted. MOX, moxifloxacin; OFL, ofloxacin. Error bars indicate standard deviations. 100

Figure 7.1 Distribution of *gyrA* mutations in pre-XDR and XDR clinical isolates in the Western Cape, South Africa (collected from 2001 to 2012). Data obtained from Lizma Streicher, Division of Molecular Biology and Human Genetics, Stellenbosch University) 119

Figure 7.2 Clones used for proteomic analyses 120

Figure 7.3 Details of proteomic analysis. W7 was exposed to the relevant antibiotic diluents for 24 hours, while the mutants were exposed either to antibiotic diluents or antibiotic for 24 hours at mid-log phase. Each experiment was done in quadruplicate. 122

Figure 7.4 Growth curves for W7, BO10 and BA23. Growth curves were done in modified Sauton's broth. A starter culture was grown by inoculating a 1 ml stock into 4 ml media. When the starter culture reached an optical density of 0.8-1.0 at 600 nm (OD), it was diluted to an OD of 0.05 in a total of 25 ml modified Sauton's broth

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and incubated at 37°C. Shown are average OD readings taken in three independent experiments, error bars indicate standard deviation. 128

Figure 7.5 Confirmation of homogeneity within W7, a single colony selected from K636, with respect to TB16.3 (genome position 2447198, which is indicated by the arrow) using Sanger sequencing. 129

Figure 7.6 Venn diagram of proteins commonly and uniquely differentially abundant by at least 1.5-fold in the *gyrA* mutant vs wild-type and the *rrs* mutant vs wild-type analyses. 131

Figure 7.7 ESX-5 region of *M. tuberculosis.* Members of the transmembrane complex utilised in protein export are in yellow, cytosolic components are in blue, the membrane component mycosin P5 is in green, secreted proteins are in red....... 132

Figure 7.8 Approach taken to elucidate proteome changes due to only the *gyrA* or *rrs* drug resistance mutations 135

Figure 7.9 Proposed model for the effect of the presence of ofloxacin on the uptake and storage of iron. Ofloxacin sequesters iron, creating an iron-limiting environment, which leads to the upregulation of several components involved in the uptake and storage of iron. ViuB/IrtB import ferrated siderophores.The Esx-3 complex, especially EccD3, is possibly involved in the export of EsxG/H, which may play a positive role in siderophore import (Siegrist *et al.* 2014). BfrA stores iron intracellularly. ViuB, EccD3, and BfrA were more abundant after ofloxacin treatment in our study. 142

Figure 7.10 Genes surrounding the origin of replication in *M. tuberculosis* H37Rv. Proteins expressed from genes shaded in blue were less abundant after ofloxacin treatment. 143

Figure 7.11 Steps of bacterial protein synthesis. 151

Stellenbosch University https://scholar.sun.ac.za

List of tables

Table 1.1 Antibiotics used in the treatment of TB 1

Table 1.2 Drug resistance mutations specifically relevant to XDR-TB[a] 4

Table 2.1 Published rates for evolution of drug resistance to various antibiotics in *Mycobacterium tuberculosis* 21

Table 2.2 Factors potentially affecting mutation rate in *M. tuberculosis* and corresponding knowledge gaps 28

Table 3.1 Mutation rates of *M. smegmatis* in the presence and absence of 3TC 61

Table 3.2 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with 3TC 62

Table 3.3 Size and morphology of *M. smegmatis* single colony isolates with and without mutation in the RRDR of *M. smegmatis rpoB* 64

Table 3.4 Mutation rates of *M. smegmatis* in the presence and absence of AZT 65

Table 3.5 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with AZT 65

Table 3.6 Mutation rates of *M.smegmatis* in the presence and absence of AZT-TP 67

Table 3.7 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with AZT-triphosphate (AZT-TP) 68

Table 3.8 Mutation rates of *M. smegmatis* in the presence and absence of 3TC-TP 68

Table 3.9 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with 3TC-triphosphate (3TC-TP) 69

Table 4.1 Primers 84

Table 4.2 Prevalence of mutations selected on ofloxacin in H37Rv and a Beijing strain. ... 86

Table 4.3 Mutation rate of a clinical Beijing strain to ofloxacin resistance 90

Table 5.1 Comparison between mutants selected on 0.5 µg/ml vs. ≥ 2 µg/ml moxifloxacin *in vitro* 97

Table 6.1 Setup of resazurin microtiter method in 96-well format 108

Table 6.2 Mutations increasing resistance of a *gyrA* Asp94Gly mutant to ≥ 16 µg/ml ofloxacin 110

Table 6.3 Effect of efflux pump inhibitors CCCP and verapamil on growth of K636 and BO1 in the presence of ofloxacin 111

Table 6.4 Changes in ofloxacin MIC in the presence and absence of reserpine in 96-well format 112

Stellenbosch University https://scholar.sun.ac.za

Table 7.1	Proteins changed in abundance in both the *gyrA* and *rrs* mutant when compared to wild-type. 131
Table 7.2	Proteins involved in lipid metabolism differentially abundant in an Asp94Gly *gyrA* mutant of *M. tuberculosis* when compared to wild-type. 137
Table 7.3	Proteins involved in transport and differentially abundant in the *gyrA* Asp94Gly mutant compared to wild-type 138
Table 7.4	Summary of proteins involved in cell division and growth, decreased in abundance after treatment of BO10 with ofloxacin 143
Table 7.5	Proteins involved in translational processes in *M. tuberculosis* differentially abundant as a result of amikacin resistance and/or treatment 148

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Chapter 1 Introduction

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1.1 The tuberculosis (TB) epidemic

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis*, is one of the leading causes of death globally (World Health Organization 2015) and the World Health Organisation (WHO) declared the disease a public health emergency in 1993 (WHO 2012a). Worldwide, an estimated 9.6 million new and relapse cases (together called incident cases) occurred during 2014 and 1.2 million people died from TB (World Health Organization 2015). In South Africa, TB is the leading cause of death (Statistics South Africa 2013) and according to the WHO (World Health Organization 2015), the incidence of TB was an estimated 834 per 100 000 population in 2014, making it one of the top ten countries in the world with the highest incidence of TB.

It is clear that worldwide, and in South Africa, the burden of this disease is substantial and efforts towards finding ways of preventing and effectively treating this disease is of the essence. A vaccine for prevention of TB is available, namely the BCG (Bacillus Calmette-Guérin) vaccine. However, this vaccine may be only about 50% effective in preventing TB and this is highly variable in different geographical regions (Colditz *et al.* 1994). For this reason, research into combating the disease partly focuses on new anti-TB vaccines (McShane 2011). TB is also treatable by antibiotics and those currently used are shown in Table 1.1. According to the WHO (World Health Organization 2015), antibiotic treatment is effective in about 85% of patients.

Table 1.1 Antibiotics used in the treatment of TB

Drug[a]
First-line drugs
Rifampicin
Isoniazid
Pyrazinamide
Ethambutol
Rifabutin
Second-line injectable drugs
Kanamycin
Amikacin
Capreomycin
Streptomycin
Fluoroquinolones (second-line drugs)
Ofloxacin

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Levofloxacin
Moxifloxacin
Second-line oral bacteriostatic agents
Ethionamide
Protionamide
Cycloserine
Terizidone
p-aminosalicylic acid
Drugs not recommended by WHO for routine TB treatment, but used as last resort
Linezolid
Clofazimine
Amoxicillin
Clavulanate
Thioacetazone
Imipenem
Cilastatin
Clarithromycin

[a]The list of drugs currently used in treatment are taken from the Global Tuberculosis Report, 2012 (WHO 2012a).

The TB epidemic is exacerbated by a number of factors. One of these is HIV co-infection. The HIV and TB epidemics overlap as shown by the fact that a significant proportion of HIV-related deaths are due to TB (Sester *et al.* 2010), while worldwide, about 12%, and in South Africa, 61% of TB patients tested for HIV, tested positive (World Health Organization 2015). HIV is the strongest risk factor for tuberculosis, increasing incidence rates and hampering achievement of the goal to eradicate TB by 2050 (World Health Organization 2015).

In addition to HIV, the emergence of drug-resistant *M. tuberculosis* strains compromises TB treatment. Treatment of resistant TB requires the use of less effective, more expensive, toxic drugs and long periods of treatment (World Health Organization 2015). Multidrug-resistant (MDR) TB (defined as resistance to at least rifampicin and isoniazid) and extensively drug-resistant (XDR) TB (defined as MDR-TB with additional resistance to a fluoroquinolone and an injectable) have poorer treatment outcomes and a higher probability of death (World Health Organization 2015). In a recent study in South Africa, 36% of XDR-TB patients died after initiation of treatment (Dheda *et al.* 2010). Totally drug-resistant (TDR) cases have also been described, but WHO has yet to give a precise definition for this form of drug-resistant TB (WHO 2012b). Globally, 3.3% of new cases and 20% of previously treated cases were estimated to have MDR-TB (World Health Organization

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2015). To date, 105 countries have reported at least one XDR-TB case and an estimated 9.7% of MDR-TB cases have XDR-TB (World Health Organization 2015). South Africa had proportions of MDR-TB of 1.4 to 2.3% and 5.4 to 8.2% in new and previously treated cases, respectively (World Health Organization 2015). An association between HIV and M(X)DR TB has been observed in some settings (Sanchez-Padilla *et al.* 2012; Wells *et al.* 2007; Andrews *et al.* 2010). The causes for this association, where it was observed, has not been clarified.

Drug resistance in TB can either be acquired or transmitted. Acquired drug resistance occurs when a patient is infected with a drug susceptible strain, where the same strain with a similar DNA fingerprint becomes drug-resistant during the course of treatment. Such a definition therefore excludes re-infection with a drug-resistant strain. Transmitted or primary resistance occurs when a patient is initially infected with a drug-resistant strain. Acquired drug resistance is often taken as drug resistance arising in patients with a history of TB treatment, a definition which does not exclude the possibility of re-infection with another drug-resistant strain or the lack of diagnosis of drug resistance in the previous episode (Van Rie *et al.* 2000).

Various studies in South Africa have demonstrated that the increase in MDR-TB is due to transmission of drug-resistant strains, rather than individual cases of acquired resistance (Cox *et al.* 2010; Ioerger *et al.* 2010; Klopper *et al.* 2013; Marais *et al.* 2013; Streicher *et al.* 2012). In contrast, the XDR epidemic is mainly as a result of acquisition of drug resistance mutations (Dheda *et al.* 2010; Ioerger *et al.* 2010; Klopper *et al.* 2013; Said *et al.* 2012; Streicher *et al.* 2012; Streicher *et al.* 2015). Extensive heterogeneity has been observed especially for mutations in *gyrA* (Ioerger *et al.* 2010; Klopper *et al.* 2013), which confer resistance to fluoroquinolones.

1.2 The acquisition of XDR in *M. tuberculosis*

1.2.1 Chromosomal mutation

Genetically encoded drug resistance in bacteria can be mediated by plasmids, phages, transposons and other mobile genetic elements, as well as by chromosomal mutations (Gillespie 2002). In *M. tuberculosis*, all such drug resistance arises through chromosomal mutation (Smith *et al.* 2013), rather than mobile genetic elements. Mutations in specific genes are strongly associated with resistance to specific drugs (Sandgren *et al.* 2009; Smith *et al.* 2013). Mutations specifically relevant to XDR are shown in Table 1.2.

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Table 1.2 Drug resistance mutations specifically relevant to XDR-TB[a]

Genetic region where mutation confers resistance	Gene name	Drug(s) that mutation confers resistance to
Gyrase subunit A	*gyrA*	fluoroquinolone
Gyrase subunit B	*gyrB*	fluoroquinolone
16S ribosomal RNA	*rrs*	amikacin, kanamycin, capreomycin, streptomycin
S12 ribosomal protein	*rpsL*	streptomycin
2'-O-methyltransferase	*tlyA*	capreomycin
Glucose inhibited division gene	*gidB*	amikacin, kanamycin, capreomycin, streptomycin
Promoter of enhanced cellular survival gene	*eis*	amikacin, kanamycin
WhiB7	*whiB7*	kanamycin

[a]Information taken from (Georghiou *et al.* 2012; Kaur *et al.* 2016; Mayer & Takiff 2014; Maruri *et al.* 2012; Sreevatsan *et al.* 1996).

Drug resistance mutations can therefore be used to predict resistance in *M. tuberculosis* in molecular drug susceptibility testing (Ritter *et al.* 2014). Knowledge of mutations conferring clinically meaningful resistance is essential and in some cases, lacking (Desjardins *et al.* 2016; Eilertson *et al.* 2016; Farhat *et al.* 2013). Moxifloxacin is a later-generation fluoroquinolone recommended as part of a treatment regimen for extensive drug-resistant TB (XDR-TB), even where ofloxacin resistance may exist (World Health Organisation 2008a). The appropriate clinical breakpoint indicating resistance where further treatment with moxifloxacin is not possible (Niward *et al.* 2016), as well as the mutations predicting such clinically meaningful resistance have not been defined (Chien *et al.* 2016).

1.2.2 Mutation rate

Chromosomal mutations occur spontaneously as a result of errors associated with DNA replication and repair (Delbrück 1945; Fijalkowska *et al.* 2012; Tippin *et al.* 2004) after which bacilli containing mutations are selected under antibiotic pressure to outcompete other bacilli without the advantageous mutation. There are many factors that may influence the rate at which errors are introduced into the genome of the organism. An increased mutation rate as a result of the deletion of DNA repair genes (Jain *et al.* 2007; Kurthkoti *et al.* 2010; Kurthkoti & Varshney 2010; Malshetty *et al.* 2010; Rock *et al.* 2015) or treatment with known mutagenic agents (Boshoff *et al.* 2003) has been shown to result in higher numbers of drug-resistant organisms *in vitro,* which can be expected

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to result in higher rates of drug resistance *in vivo*. For this reason, it is important to investigate any potential factors that may result in an increased mutation rate. The contribution of mutagens to the drug-resistant TB epidemic has not been investigated thus far.

1.2.3 Selection of resistance-conferring mutations

Even though the rate of mutation is important for generating genetic diversity, ultimately the type of mutations and frequency of occurrence of these mutations will be determined by whether they are selected. The fitness cost associated with a particular mutation, which may impair the function of an enzyme, impacts the probability for selection, which is demonstrated by the fact that low-cost mutations occur at higher frequencies among drug-resistant TB isolates (Gagneux, Long, *et al.* 2006; Müller *et al.* 2012). Fitness is currently defined as the competitive growth advantage/deficit of a mutant compared to a sensitive strain without a mutation (Sander *et al.* 2002; Spies *et al.* 2013; Von Groll *et al.* 2010). However, the relative fitness of different mutations that confer resistance to the same antibiotic may differ in the presence of antibiotic, while being identical in the absence of antibiotic or vice versa. The relative fitness of different mutations in the presence of antibiotic has not been investigated thus far, but it is expected to influence the rate at which some mutations are selected over others. Another factor impacting the spectrum and frequency of particular mutations is that of epistasis, whereby the phenotypic expression of one allele is dependent on that of another allele (Fenner *et al.* 2012; Koch *et al.* 2014). For example, Karunakaran and Davies (2000) showed that, in *Mycobacterium smegmatis,* rifampicin resistance mutations were selected at a lower rate in a streptomycin-resistant mutant, compared to the sensitive progenitor, suggesting that the streptomycin resistance mutation results in a fitness defect when a rifampicin resistance mutation is obtained. The converse was also true, while a high rate of reversion was observed when the streptomycin/rifampicin-resistant mutant was selected on rifampicin/streptomycin respectively. In a recent study (Borrell *et al.* 2013), positive epistasis was demonstrated between resistance-conferring *rpoB* and *gyrA* mutations *in vitro*. Combinations of double mutations resulting in no fitness deficit correspond to those found most frequently among MDR and XDR isolates from South Africa. Even more recently, Salvatore *et al.* (2016) used a household-based case control study to assess the effect of fitness of certain drug resistance mutations on their transmissibility. In this study, the combination of a *katG* Ser315Thr and *rpsL* Lys43Arg mutation occurred less frequently in multiple-case households, suggesting a negative epistatic interaction between these mutations. Similarly, Fenner *et al.* (2012) showed an association of certain drug resistance mutations with particular strain backgrounds, supporting the idea that resistance mutations may interact with other mutations that do not necessarily confer resistance within the genome. This interaction is also evident in a recent study by Reeves *et al.* (2015) where the strain background modulated the

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aminoglycoside minimum inhibitory concentrations (MICs; the lowest concentration of antibiotic that inhibits 90% of growth) of strains with drug resistance mutations. Nothing is currently known about epistasis between resistance-conferring and other alleles specific to certain lineages. However, it is possible that, if certain strains are more likely to obtain high-fitness mutations compared to other strains, this provides an explanation for the potential association of certain lineages, for example the Beijing lineage, with drug resistance (Glynn *et al.* 2002).

1.2.4 Compensatory evolution

As a result of the fitness cost associated with certain mutations, it has been shown in many bacterial species that mutations alleviating the cost occur elsewhere in the genome (Melnyk *et al.* 2015). Thus far, in *M. tuberculosis*, such mutations, compensating for the fitness cost associated with *katG, rrs, thyA* and *rpoB* mutations, have been suggested or described. Mutations in *ahpC* have been suggested to alleviate the fitness cost of a subset of *katG* mutations (Gagneux, Burgos, *et al.* 2006; Hazbón *et al.* 2006).The *rrs*1402 mutation was shown to restore fitness of a G1484T mutant (Shcherbakov *et al.* 2010) and *rpoA, rpoC* and mutations in the non-RRDR (rifampicin resistance-determining region) region of *rpoB* compensate for the fitness cost associated with *rpoB* mutations (Cohen *et al.* 2015; Comas *et al.* 2012; De Vos *et al.* 2013). A mutation upstream of *thyX* has also been suggested to restore fitness in a *thyA* mutant (Fivian-Hughes *et al.* 2012). To date, no such mutations have been described in association with *rrs* 1401 or *gyrA* mutations, although a recent study suggested their potential presence in the case of *rrs* 1401 (Reeves *et al.* 2015).

1.2.5 Modulation of drug resistance

A well known drug tolerance mechanism that may contribute to drug resistance in *M. tuberculosis*, is efflux. The activation or induction of efflux pumps have been shown to modulate the MIC of various drugs (Louw *et al.* 2009), including fluoroquinolones (Escribano *et al.* 2007; Singh *et al.* 2011; Sun *et al.* 2014), in drug-resistant *M. tuberculosis*. Evidence for this exists in the observations that the MICs for ofloxacin in ofloxacin-resistant isolates were reduced in the presence of efflux pump inhibitors (Escribano *et al.* 2007; Singh *et al.* 2011; Sun *et al.* 2014). A recent study (Sun *et al.* 2014) also showed that ofloxacin-resistant *M. tuberculosis* clinical isolates exhibiting ofloxacin MICs $\geq$ 16 µg/ml, showed a higher-fold decrease in MIC in the presence of efflux pump inhibitors reserpine, verapamil and CCCP when compared to to isolates with lower MICs. In addition, isolates with low MICs ($\leq$ 2 µg/ml) showed little modulation of MIC by efflux pump inhibitors. From this, the authors concluded that higher levels of resistance to ofloxacin may be associated with higher levels of efflux pump induction. Therefore efflux may represent a major mechanism for modulation of fluoroquinolone MICs. It is also clear that not all fluoroquinolone-resistant clinical isolates

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exhibit efflux pump activity (Eilertson *et al.* 2016; Singh *et al.* 2011). The genetic mechanisms that drive this drug tolerance mechanism are not well understood.

1.3 Physiology of XDR

1.3.1 Consequences of drug resistance mutations

Drug-resistant *M. tuberculosis* strains do not necessarily display identical physiology to their sensitive progenitor strains, even in the absence of antibiotic. The resistance-conferring mutations may result in physiological differences. Many of the drugs used in the treatment of TB target processes central to metabolism, such as transcription (*e.g.* rifampicin), DNA supercoiling (*e.g.* fluoroquinolones) and translation (*e.g.* aminoglycosides) (Musser 1995). Even though in many cases, mutations in genes involved in central metabolic processes have a negligible effect on the growth of the organism (Nessar *et al.* 2011), they may still have an effect on the functioning of the enzyme. Mutations in *gyrA*, which confer resistance to fluoroquinolones may interfere with the process of DNA supercoiling. Changes in supercoiling density affect transcription efficiency (Peter *et al.* 2004; Rovinskiy *et al.* 2012), therefore leading to differential expression. Mutations in *gyrA* relevant to fluoroquinolone resistance have also been shown to directly affect the expression profile in *Escherichia coli* (Bagel *et al.* 1999; Jeong *et al.* 2004; Reckinger *et al.* 2007; Steck *et al.* 1993; Sternglanz *et al.* 1981) and *S. typhimurium* (Fàbrega *et al.* 2009). Similarly, the *rrs* A1408G mutation in *E. coli,* which is equivalent to the *rrs* A1401G mutation in *M. tuberculosis* (Maus *et al.* 2005) has been shown to decrease the ribosome's affinity for initiation factor 1 (IF1) binding, resulting in spurious translation initiation (Qin & Fredrick 2009) and mistranslation. Mistranslation of proteins can be expected to have an effect on protein levels. Most importantly, recent publications have highlighted the possibility that certain drug resistance mutations, which include *gyrA,* may actually confer a selective advantage even in the absence of drug pressure (Baker *et al.* 2013; Han *et al.* 2012; Koch *et al.* 2014; Singh *et al.* 2011; Webber *et al.* 2013). In addition, there is evidence that the clinically relevant *gyrA* Asp87Gly substitution in *Salmonella* reduces susceptibility to other, unrelated drugs through an altered expression profile (Webber *et al.* 2013). The effect of *gyrA* and *rrs* mutations on the expression profile of *M. tuberculosis* has not been investigated thus far. However, given observations from other organisms, it is essential to investigate how pre-XDR and XDR strains may differ in their physiology compared to sensitive and MDR strains and how this could impact how they respond to other second-line drugs used to treat pre-XDR (MDR with additional resistance to only a fluoroquinolone or only an injectable) and XDR-TB and their propensity to develop additional resistance which leads to "TDR". Moreover, it would be essential to understand whether and how *gyrA* or *rrs* mutations may impact fitness of

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Mycobacterium tuberculosis in a positive manner, increasing transmission of strains harbouring these mutations (Koch *et al.* 2014; Miskinyte & Gordo 2013).

1.3.2 Consequences of treating a resistant strain with antibiotic

Resistant *M. tuberculosis* strains are often still exposed to the antibiotics they are resistant to within the patient, since routine drug susceptibility testing is either not performed or is delayed by weeks or months (Louw *et al.* 2011; World Health Organisation 2008b). A recent study in our laboratory has shown that treatment of MDR *M. tuberculosis* strains for 7 days with rifampicin *in vitro* conditioned the strains to become resistant to ofloxacin (Louw *et al.* 2011). This decrease in susceptibility was as a result of the induction of efflux pumps, which can be seen as a mechanism conferring cross-resistance or tolerance to other antibiotics. The conditioning effect may also apply to fluoroquinolones and aminoglycosides; it is conceivable that treatment with a fluoroquinolone may activate or upregulate the expression of efflux pumps, which may then also extrude other drugs. This conditioning effect, with respect to efflux or any other drug tolerance mechanism, has not been investigated for fluoroquinolones or aminoglycosides in *M. tuberculosis.* However, it is of the essence to investigate the possibility of a conditioning effect, to improve on drug regimens for the treatment of pre-XDR and XDR-TB.

The induction of tolerance to other drugs may not be the only result of treating a resistant strain with the antibiotic it is already resistant to. A study (Brunelle *et al.* 2013) in *Salmonella enterica* serovar Typhimurium has shown that treatment of tetracycline-resistant strains with tetracycline resulted in increased invasiveness, a process involved with virulence. Similarly, treatment of moxifloxacin-resistant *Clostridium difficile* with moxifloxacin resulted in upregulation of colonisation factor, which may contribute to increased colonisation fitness (Denève *et al.* 2009). It is therefore conceivable that treatment of pre-XDR and XDR *M. tuberculosis* with drugs it is already resistant to, may lead to increased virulence or fitness, a concept which has thus far not been investigated.

1.4 Hypotheses and aims

In this study, various aspects involving the evolution and physiology of extensive drug resistance in *M. tuberculosis* were investigated.

1.4.1 Hypothesis 1

Nucleoside reverse transcriptase inhibitors cause an increase in the mutation rate of mycobacteria.

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1.4.1.1 Aim 1

The aim of this part of the study was to calculate the mutation rate of *M. smegmatis* to acquire mutations in the rifampicin resistance-determining region of *rpoB,* with and without prior treatment with the triphosphate forms of the cytidine analogue 3TC and the thymidine analogue AZT.

1.4.2 Hypothesis 2

Mutations specifically conferring XDR or resistance to second-line fluoroquinolones and injectables would impact the physiology of *M. tuberculosis.*

1.4.2.1 Aim 1

Generate spontaneous mutants *in vitro,* monoresistant to second-line fluoroquinolones and injectables, and characterise them with respect to mutation.

1.4.2.1.1 Sub-aim 1

Generate spontaneous mutants conferring high-level resistance to the later-generation fluoroquinolone moxifloxacin (World Health Organisation 2011).

1.4.2.1.2 Sub-aim 2

Generate spontaneous mutants with increased fluoroquinolone MIC in order to investigate the possibility of an efflux-related mechanism modulating the MIC.

1.4.2.2 Aim 2

Compare differences in protein abundance, as a measure of the physiology of the bacterium (Cook *et al.* 2009), between mutants resistant to second-line fluoroquinolones and injectables and their wild-type progenitor.

1.4.3 Hypothesis 3

Treatment of mutants monoresistant to second-line fluoroquinolones and injectables with critical concentration of the drugs they are resistant to, will impact the physiology of the mutants.

1.4.3.1 Aim 1

Compare differences in protein abundance, as a measure of the physiology of the bacterium (Cook *et al.* 2009), between resistant mutants treated and untreated with second-line fluoroquinolones and injectables.

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Chapter 2 Literature review

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2.1 Abstract

The emergence and spread of multidrug-resistant strains of *Mycobacterium tuberculosis* remains a major concern of tuberculosis control programmes worldwide, as treatment depends on low-efficacy, toxic compounds that often lead to poor outcomes. *M. tuberculosis* develops drug resistance exclusively through chromosomal mutations, in particular single-nucleotide polymorphisms. Moreover, in laboratory assays the organism exhibits a spontaneous mutation rate that is at the lower end of the bacterial spectrum. Despite this, whole-genome sequencing technology has identified unexpected genetic diversity among clinical *M. tuberculosis* populations. This suggests that the mycobacterial mutation rate may be modulated within the host and, in turn, implies a potential role for constitutive and/or transient mutator strains in adaptive evolution. It also raises the possibility that environmental factors might act as key mutagens during *M. tuberculosis* infection. Here we consider the elements that might influence the mycobacterial mutation rate *in vivo* and evaluate the potential roles of constitutive and transient mutator states in the generation of drug resistance mutations. In addition,we identify key research questions that will influence future efforts to develop novel therapeutic strategies for a disease that continues to impose a significant global health burden.

2.2 Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) in humans. TB remains a massive global health problem and the emergence of multi- (MDR) (resistance to rifampicin and isoniazid) and extensively drug-resistant (XDR) (MDR-TB with added resistance to a fluoroquinolone and an injectable second-line agent) strains, coupled with the lack of new effective drugs to combat the disease, is of major concern (WHO 2012a). Recently, totally drug-resistant (TDR) cases have been described, but WHO has yet to define this form of drug-resistant TB (WHO 2012b). Treatment of MDR and XDR-TB requires prolonged chemotherapy with expensive and less efficient drugs that often lead to undesirable side effects (WHO 2010). TDR-TB might be untreatable with currently available drugs. For this reason, a greater understanding of how drug resistance arises and might be prevented is of critical importance.

In *M. tuberculosis*, genetically-encoded drug resistance arises exclusively through chromosomal mutations (Gillespie 2002; Huitric *et al.* 2010), the majority of which are single nucleotide polymorphisms (SNPs) (Sandgren *et al.* 2009). In general, mutations occur

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spontaneously (Delbrück 1945) as a consequence of errors that arise during DNA replication at a rate of one mutation per 10 000 to 100 000 base pairs per round of replication (Tippin *et al.* 2004). Correction by proofreading exonucleases reduces the rate approximately 2 log-fold (Tippin *et al.* 2004), with post-replicative DNA mismatch repair ensuring a further 3 log-fold reduction (Fijalkowska *et al.* 2012). The bacterial mutation rate is, in turn, defined as the probability of a mutation occurring per cell division, and so is determined per bacterium per generation (Pope *et al.* 2008). The calculated rates for the *in vitro* evolution of resistance to various antibiotics are shown for *M. tuberculosis* and related mycobacteria in Table 2.1.

In this review, we discuss the different factors that might contribute to the emergence of drug resistance mutations in *M. tuberculosis* during host infection. In particular, we evaluate whether spontaneous errors during DNA replication and repair are sufficient to drive the evolution of drug-resistant *M. tuberculosis* strains within the host or if additional factors contribute to the emergence of these mutant strains. Understanding the mechanisms and pathways that influence the mutation rate might identify targets for novel agents designed to prevent the development of drug resistance (Warner 2010) or to potentiate the activity of existing antituberculars (Kohanski, Dwyer, *et al.* 2010). In addition, it could aid the identification of environmental or host immune factors that could be manipulated (Schwegmann & Brombacher 2008), as well as inform the design of new drug regimens for the treatment of *M. tuberculosis.*

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Table 2.1 Published rates for evolution of drug resistance to various antibiotics in *Mycobacterium tuberculosis*

Antibiotic	**Strain**[a]	**Mutation Rate**[b]	**References**
Isoniazid	H37Rv	2.56×10^{-8}	(David 1970)
Isoniazid	MTB72 (Haarlem laboratory strain)	3.2×10^{-7}	(Bergval *et al.* 2009)
Rifampicin (1 mg/L)	H37Rv	2.25×10^{-10} to 3.32×10^{-9}	(David 1970)
Rifampicin (2 mg/L)	H37Rv	2.9×10^{-9} to 2.4×10^{-7}	(Kana *et al.* 2010; Werngren & Hoffner 2003; O'Sullivan, McHugh, *et al.* 2008)
Rifampicin (2 mg/L)	Harlingen	1.4×10^{-8}	(Werngren & Hoffner 2003)
Rifampicin (2 mg/L)	Beijing clinical isolates	7.9×10^{-9} to 1.3×10^{-8}	(Werngren & Hoffner 2003)
Rifampicin (2 mg/L)	Erdman	2.1×10^{-9}	(Ford *et al.* 2011)
Rifampicin (2 mg/L)	H37Rv with reduced catalase and peroxidise activity and no mutation in *katG*	8.76×10^{-7} to 2.24×10^{-6}	(O'Sullivan, McHugh, *et al.* 2008)
Rifampicin (2 mg/L)	H37Rv with *katG* mutation or deletion	2.7×10^{-7} to 3.3×10^{-6}	(O'Sullivan, McHugh, *et al.* 2008)

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Rifampicin (2 mg/L)	H37Rv with *katG* mutation or deletion, exposed to H_2O_2	$4x10^{-7}$ to $6x10^{-7}$	(O'Sullivan, McHugh, *et al.* 2008)
Rifampicin (2 mg/L)	H37Rv mutants with DinB1, DinB2 and double knockouts	$2.3x10^{-9}$	(Kana *et al.* 2010)
Rifampicin (5 or 10 mg/L)	H37Rv	$6x10^{-10}$	(Billington *et al.* 1999)
Rifampicin (8 mg/L)	MTB72 (Haarlem laboratory strain)	$9.81x10^{-9}$ to $6.45x10^{-8}$	(Bergval *et al.* 2009; Bergval *et al.* 2012)
Rifampicin (8 mg/L)	Typical Beijing strains (*ogt,muT2,mutT4* mutations)	$7.73x10^{-8}$ to $2.49x10^{-7}$	(Bergval *et al.* 2012)
Rifampicin (8 mg/L)	Atypical Beijing strain	$5.76x10^{-8}$ to $1.21x10^{-7}$	(Bergval *et al.* 2012)
Rifampicin (8 mg/L)	LAM strain (*Ag85C* mutation)	$8.18x10^{-8}$	(Bergval *et al.* 2012)
Rifampicin (8 mg/L)	LAM strain (*Ag85C* mutation and *katG* mutation	$6.28x10^{-8}$ to $7.2x10^{-8}$	(Bergval *et al.* 2012)
Rifampicin (8 mg/L)	T1 spoligotype family	$2.85x10^{-8}$	(Bergval *et al.* 2012)
Rifampicin (8 mg/L)	T1 spoligotype family (with *katG* mutation)	$1.33x10^{-8}$	(Bergval *et al.* 2012)
Rifampicin (8 mg/L)	MTB72 with *katG* deletions at amino acid 315 and 463	$9.69x10^{-8}$	(Bergval *et al.* 2012)

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Rifampicin (8 mg/L)	MTB72 with partial *katG* deletion	$5.71x10^{-7}$	(Bergval *et al.* 2012)
Ethambutol	H37Rv	$1x10^{-7}$ to $6.4x10^{-7}$	(David 1970)
Streptomycin	H37Rv	$2.95x10^{-8}$	(David 1970)
D-cycloserine	H37Rv	$9x10^{-11}$	(David 1970)
R207910/TMC207 (diarylquinolone ATP synthase inhibitor) at 0.3 mg/L	H37Rv and clinical isolates	$4x10^{-7}$ to $8.9x10^{-9}$	(Huitric *et al.* 2010)
R207910/TMC207 (diarylquinolone ATP synthase inhibitor) at 0.9 mg/L	H37Rv and clinical isolates	$2.4x10^{-9}$ to $3.9x10^{-8}$	(Huitric *et al.* 2010)
Ciprofloxacin	*M. fortuitum*	$5.1x10^{-9}$	(Gillespie *et al.* 2005)
Levofloxacin	*M. fortuitum*	$3.8x10^{-9}$	(Gillespie *et al.* 2005)
Moxifloxacin	*M. fortuitum*	$4.2x10^{-9}$	(Gillespie *et al.* 2005)
Pyrazinamide	*M. tuberculosis* clinical isolates	$1x10^{-5}$ [c]	(Stoffels *et al.* 2012)

[a]All strains are of *Mycobacterium tuberculosis* unless stated otherwise

[b]Units are per bacterium per generation

[c]Mutation frequency, the proportion of mutants within a bacterial population at a given time point. No data was available on mutation rate.

2.3 *M. tuberculosis* mutates at a low rate in vitro

M. tuberculosis does not exhibit an elevated mutation rate relative to most other bacteria (David 1970): under *in vitro* conditions, it is estimated that the organism makes a mutational error at 2 bases in every 10 000 genomes copied (Ford *et al.* 2011). This is despite the fact that the DNA repair complement of *M. tuberculosis* does not include homologues of

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known mismatch repair (MMR) enzymes (Mizrahi & Andersen 1998). *E. coli* mutants lacking MMR components are considered strong heritable mutator strains (Miller 1996); however, this does not apply to *M. tuberculosis.* In this review, the term "mutator" is applied both to a strain that exhibits a higher mutation rate compared to its progenitor, as well as a gene which, when mutated, confers a higher mutation rate on the organism. Instead, it has been proposed that MMR deficiency may, in fact, exert a selective pressure on *M. tuberculosis,* resulting in a more stable genome over time (Wanner *et al.* 2008), as is suggested by the lower than expected degree of polymorphism amongst simple sequence repeats (SSRs) in the *M. tuberculosis* genome. SSRs (also called microsatellites) consist of repeats of 1-6 base pair units and are particularly prone to polymerase slippage, which can lead to frameshift mutations (Kumar & Nagarajaram 2012; Sreenu *et al.* 2007; Wanner *et al.* 2008). Long tracts comprising more than seven repeats of the microsatellite unit are more prone to slippage than short repeats, and may contribute to genome instability. A lack of MMR would, therefore, be expected to result in an elevated number of polymorphic SSRs (Kumar & Nagarajaram 2012). However, *M. tuberculosis* contains fewer long sequence repeats than might be predicted on the assumption that nucleotides are randomly distributed (Wanner *et al.* 2008), and in comparison to *E. coli* (Sreenu *et al.* 2007). Consistent with the inference that the *M. tuberculosis* genome is under selective pressure to limit features that might contribute to inherent variability, Machowski *et al.* (2007) showed that PGRS repeats – found among PE_PGRS members of the highly conserved PE protein family, which is characterized by the presence of Proline-Glutamate residues in the N-terminal region – are not associated with a higher rate of mutation relative to base substitutions.

The absence of MMR in *M. tuberculosis* might nevertheless contribute to genetic variation. Amongst other functions, MMR is known to prevent recombination between non-identical sequences (Güthlein *et al.* 2009) during repair of double-stranded DNA breaks in other bacterial systems. In *M. smegmatis*, abrogation of nucleotide excision repair (NER) function results in an increased number of gene conversion events arising from mismatch errors during homologous recombination, suggesting that NER may (partially) compensate for the loss of MMR in mycobacteria (Springer *et al.* 2004). However, the fact that NER does not distinguish between the parent and daughter strands, whereas MMR does, raises the possibility that mutation fixation – rather than avoidance – might be a common outcome (Mizrahi & Andersen 1998). The lack of an MMR system might also promote exchange of divergent DNA sequences, thus driving genome evolution (Springer *et al.* 2004), since

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evidence suggests that recombination can occur in *M. smegmatis* when divergent loci are flanked by sequences of perfect homology (Springer *et al.* 2004). The mechanisms ensuring genomic stability in *M. tuberculosis* therefore represent an important area for future research.

2.4 Generation of drug resistance-conferring mutations in *M. tuberculosis* during infection

As noted above, *M. tuberculosis* does not display an elevated *in vitro* mutation rate compared with other bacteria. For this reason, drug-resistant mutants of *M. tuberculosis* are expected to be rare, and are predicted to arise as a consequence of spontaneous errors in DNA replication which are subsequently selected under applied drug pressure. Multiple factors could influence the rate of selection of drug-resistant mutants in the host, including the relative fitness of individual mutants (Blower & Chou 2004; Cohen & Murray 2004), patient compliance with prescribed drug regimens (Lipsitch & Levin 1998), pharmacokinetic variability amongst patients (Srivastava *et al.* 2011), spatial heterogeneity in drug distribution (Hermsen *et al.* 2012; Zhang *et al.* 2011), and the size of the infecting bacterial population (Colijn *et al.* 2011). The rate of selection of a particular mutation should, however, not be confused with the basal mutation rate. The fundamental principle remains: errors in DNA replication must occur in order to generate the base substitutions and other types of mutations that lead to drug resistance. It is not entirely clear, though, whether a relatively low mutation rate is sufficient to account for the elevated rates of acquired drug resistance observed clinically. Is the mutation rate within the host modulated in some way to bring about sufficient diversity from which drug resistance can be selected?

In a pioneering study, Sarah Fortune and colleagues applied whole-genome sequencing (WGS) technology to detect the mutational events that arose in *M. tuberculosis* during experimental infection of non-human primates (Ford *et al.* 2011). Using a flexible range of predicted mycobacterial replication rates, the authors estimated the rate at which the observed mutations must have arisen *in vivo*. Unexpectedly, this estimated *in vivo* mutation rate was very similar to that which was inferred from *in vitro* fluctuation analyses. and, moreover, did not differ for active versus latently infected animals. In other words, *M. tuberculosis* does not appear to enter into a hypermutable state within the host. Work by Saunders *et al.* (2011) reinforced the idea that drug resistance mutations are rare. In this case, the authors sequenced the genomes of serial isolates obtained from a TB patient over 12 years, a period which correlated with the emergence of strains resistant to isoniazid and, subsequently, rifampicin.

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WGS analysis suggested that only two SNPs - in *katG* and *rpoB* - differentiated the drug-resistant isolate from the susceptible strain, indicating that the mutation rate in the host is very low. The reference genome employed in this study was assembled from pooled data from all the serial isolates. It is possible, therefore, that other SNPs present at low frequencies were not detected. In addition, the authors estimated the bacillary population to be in the order of 10^{13} cells/lung, which far exceeds predicted values for *M. tuberculosis* infections (Canetti *et al.* 1960).

In contrast, Sun *et al.* (2012) utilized more sensitive WGS technology to track genomic changes in serial sputum samples obtained from three patients over the course of anti-TB treatment. In this case, the authors reported a high degree of diversity in the serial clinical specimens, an observation that is consistent with the idea that mutation rates *in vivo* might be higher than previously proposed; for example, between 8 and 41 SNPs arose during treatment in each sample, and as many as 34 SNPs were unique to a single sample. Interestingly, the majority of the SNPs were detected at frequencies below 20% in each sample, indicating that the sputum bacillary population was characterized by significant microheterogeneity at each time point. These observations are supported by other studies which employed Sanger sequencing to investigate the resistance-determining regions of specific target genes. For example, serial *M. tuberculosis* isolates obtained from patients over a period of 12 years revealed a diversity of mutations within *rpsL* (Mariam *et al.* 2011; Meacci *et al.* 2005). Similarly, Mariam *et al.* (2011) detected different *rpsL* and *rrs* mutations in serial isolates obtained from a single patient. These authors also reported transient co-existence of some mutations as well as successive clonal sweeps of other mutations, suggesting a dynamic interplay between the genetic variability of the infecting bacillus and the selective pressures associated with host infection.

The extent to which the sputum bacillary population can be considered representative of the total infecting mycobacterial population is not known. For this reason, the above studies might be limited in their dependence on organisms obtained from sputum. In a key study, bacilli isolated from discrete pulmonary lesions in patients with chronic TB were shown to possess different drug resistance alleles (Kaplan *et al.* 2003). It seems likely, therefore, that the microenvironments represented by each lesion may contribute to the heterogeneity observed within the TB population in the host. However, WGS analyses in the non-human primate model revealed significant heterogeneity among *M. tuberculosis* bacilli isolated within single lesions (Ford *et al.* 2011).

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2.5 The mycobacterial replication rate

The levels of genetic diversity identified in the studies above imply that *M. tuberculosis* might have an elevated mutation rate within the host compared with that calculated *in vitro*. However, it is important to remember that the fixation of spontaneous mutations occurs during replication; the mutation rate is, therefore, dependent on the rate of replication. This raises the possibility that the replication rate may be higher in the host than previously thought. Until recently, the prevailing dogma held that the rate of acquisition of dual resistance (required for MDR-TB) was the product of the individual mutation rates for rifampicin and isoniazid (Dye 2009; Martinez & Baquero 2000); that is, in the order of 10^{-16}. This requires that, for the evolution of MDR strains, a total population of at least 10^{16} bacilli must be present within any infected individual *prior to* the initiation of treatment. A recently proposed mathematical model (Colijn *et al.* 2011) suggests otherwise. In developing the model, Colijn and colleagues allowed for the possibility that a single drug-resistant mutant may arise early following infection, and could replicate to a large enough population from which the probability of the emergence of a second drug-resistance mutation would not be so low. Critically, they also considered the possibility that a greater number of replication events might occur during the initial infection period than has previously been assumed from *in vitro* estimates, an idea that is supported by recent evidence from experimental infections of macrophages (Rohde *et al.* 2012) and mice (Gill *et al.* 2009). Applying this set of revised parameters (Colijn *et al.* 2011), Colijn and colleagues showed that the likelihood of emergence of drug resistance prior to initiation of anti-TB therapy is much higher than previously expected, even when basing the calculation on published *in vitro* mutation rates for specific TB drugs.

Human TB can be usefully divided into three broad phases: active, chronic and reactivated TB (Murray 1999). Based on data from animal models of infection, it is assumed that the total number of *M. tuberculosis* bacilli remains stable during chronic TB and, that these are in a state of slow or non-replication (Muñoz-Elías *et al.* 2005; Rees & Hart 1961). Recent evidence suggests, however, that the apparently stable bacillary numbers conceal a population that is cycling continuously between active replication and death. Utilizing a "clock" plasmid which is lost from daughter cells during division, Gill *et al.* (2009) demonstrated that, during chronic infection in the mouse model, a balance is established between bacillary replication and death. This implies that the replication rate may be higher than previously thought during chronic TB in humans and is consistent with an emerging

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appreciation of latent TB as a continuum of mycobacterial growth states (Barry *et al.* 2009; Robertson *et al.* 2012). To date, the clock plasmid has only been assessed in the mouse model (Gill *et al.* 2009), which fails to recapitulate key features of human disease (Aly *et al.* 2006; Via *et al.* 2008). It will be interesting, therefore, to see whether an alternative model such as the non-human primate (Ford *et al.* 2011; Mehra *et al.* 2012) yields similar results.

Is the mycobacterial mutation rate altered during host infection, and might this explain the observed rates of acquired drug resistance? From the above discussion, it is clear that additional research is required to address this question. There are many factors that might influence the mutation rate; these are considered below and summarised in Table 2.2, together with the corresponding knowledge gaps.

Table 2.2 Factors potentially affecting mutation rate in *M. tuberculosis* and corresponding knowledge gaps

Factors affecting mutation rate	Knowledge gaps
Cellular mechanisms	
Lack of mismatch repair	Does the lack of this system play a role in generating genetic variation in *Mtb*?
Simple sequence repeats	Are simple sequence repeats hypermutable?
	Does the location of simple sequence repeats in *Mtb* have any effect on mutation rate?
Mutations in 3R[a] genes	Do genetically stable mutator strains occur within populations of TB in the host?
	Do sublineages of certain spoligotype families have varying mutation rates?
	How do the polymorphisms detected in 3R genes of various *Mtb* strains (Dos Vultos *et al.* 2008) affect their mutation rate?
	If certain spoligotype families are associated with drug resistance, why are they better able to adapt to drug pressure?
Existing drug resistance-conferring mutations	Do different drug-resistant *Mtb* mutants have different mutation rates?
Mistranslation	Does *Mtb* use mistranslation as a means to adapt to environmental stress and if so, how does it do so?

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Transcriptional mutagenesis	Does transcriptional mutagenesis play a role in the emergence of drug resistance in *Mtb?*
	Does Mfd, or the process of transcription-coupled repair, play a role in the emergence of drug resistance?
Error-prone DNA polymerases	How important a role does DnaE2 play in the emergence of drug resistance in the host?
External factors	
Antibiotics	Do certain antibiotic combinations/regimens increase the probability for acquired drug resistance?
	Do antibiotics, other than fluoroquinolones, used to treat *Mtb*, increase its mutation rate *in vitro?*
Antiretroviral drugs	Do antiretrovirals, especially NRTIs, increase the mutation rate of *Mtb in vitro* or *in vivo?*
The host environment	Is the exposure of *Mtb* to UV radiation long enough to effect upregulation of the SOS response?
	Is desiccation a relevant stress to acquired drug resistance in *Mtb?*
	Does oxidative stress drive the evolution of drug resistance?
	How could the downregulation of DNA repair enzymes contribute to the upregulation of the mutation rate?
	Do different DNA damaging stresses in the host contribute to the upregulation of the mutation rate, eg. alkylative stress, low pH, hypoxia?
Smoking	Is there a strong association between burning of fossil fuels/smoking with drug resistance?
	Does tobacco smoke affect the mutation rate of *Mtb?*

[a]3R genes are involved in DNA replication, recombination and repair

2.6 A role for constitutive mutator strains?

Individual cells within an apparently homogeneous bacterial population are unlikely to share an identical mutation rate. Spontaneous mutations in genes coding for DNA metabolic proteins can lead to the emergence of mutator strains which carry a short-term selective advantage owing to their capacity to produce a higher number of adaptive mutations (Denamur & Matic 2006). In some cases, these mutators are maintained owing to the linkage

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of the mutator allele with other beneficial mutations, but only as long as the fitness gain counterbalances (or exceeds) the cost inherent in the increased risk of generating deleterious mutations. For this reason, mutations associated with elevated mutation rates are expected to be lost from bacterial populations over time. The adaptive mutations may, however, become fixed if the mutator alleles revert to wild type, or if suppressor mutations occur (Denamur & Matic 2006).

There has been considerable interest in the potential association of mutator alleles with the emergence of drug resistance in *M. tuberculosis* (Couce *et al.* 2013). For example, mutations in *mutT4* and *mutT2* appeared to define certain sublineages of Beijing isolates (Ebrahimi-Rad *et al.* 2003). Beijing strains have been linked with the emergence of drug resistance in some settings (Glynn *et al.* 2002), however, the apparent association of specific strains with drug resistance could reflect transmission (Yang *et al.* 2012), a possibility that is consistent with the fact that no direct link has been demonstrated between the reported DNA repair mutations and the emergence of drug resistance (Chang *et al.* 2011; Ioerger *et al.* 2010; Lari *et al.* 2006). Instead, biochemical evidence suggests that the observed mutations might not impact DNA repair function at all (Moreland *et al.* 2009). Moreover, although *mutT2* and *mutT4* are annotated as DNA repair-type Nudix hydrolases, the substrate specificity of these enzymes *in vitro* suggests a role separate from DNA repair (Dos Vultos *et al.* 2006; Moreland *et al.* 2009), perhaps in regulating nucleotide availability (Moreland *et al.* 2009). Consistent with this idea, MutT2 (Dos Vultos *et al.* 2006; Sang & Varshney 2013) and MutT4 (Dos Vultos *et al.* 2006) do not function as antimutator proteins. It is possible, however, that the observed mutations confer other phenotypes which indirectly affect the acquisition of drug resistance, but not via a DNA repair pathway – for example, the G58R mutation in *mutT2* has been proposed to increase replication rate in macrophages (Moreland *et al.* 2009).

The evidence for the association of Beijing strains with an elevated mutation rate is mixed (De Steenwinkel *et al.* 2012a; De Steenwinkel *et al.* 2012b; Werngren & Hoffner 2003). In one study, various Beijing strains as well as non-Beijing strains were subjected to fluctuation analyses to determine their respective *in vitro* mutation rates on the assumption that mutator strains would exhibit at least a 10-fold higher mutation rate than corresponding "non-mutator" strains; however, the authors observed no difference between Beijing and non-Beijing isolates (Werngren & Hoffner 2003). It has subsequently been suggested that the Beijing strains employed in this analysis were not adequately defined (Dos Vultos *et al.* 2006) specifically in terms of the presence of putative mutator alleles (Dos Vultos *et al.*

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2009) and so were not true mutators. This limitation has not been addressed directly. It is also possible that Beijing strains are not characterized by elevated mutation rates *in vitro*, but are instead dependent on specific factors *in vivo* - such as increased oxidative stress and/or exposure to antitubercular drugs - to elicit an elevated mutation rate. In support of this idea, a recent study (De Steenwinkel *et al.* 2012b), demonstrated that Beijing strains were associated with significantly higher mutation frequencies compared to the East African Indian (EAI) strains when cultured in the presence of rifampicin. This effect was not observed with other antibiotics, implying that global mutation rates were not affected. The same strains were then applied in a mouse model of TB to determine the impact of treatment non-compliance on the acquisition of resistance *in vivo* (De Steenwinkel *et al.* 2012a). Notably, drug-resistant mutants arose in mice infected with the Beijing strain following exposure to the most severe conditions of noncompliance, and did not emerge in mice infected with the EAI strain. However, an acknowledged limitation of this study was that it utilized Beijing strains that were likely to have been closely related, so the results might not be applicable to all Beijing strains. For this reason, it may be more useful to consider sublineages of Beijing strains. For example, studies from Japan and Taiwan reported that specific Beijing sublineages differed in their associations with drug-resistant TB (Yang *et al.* 2012). Moreover, a comparative genomics analysis (Dos Vultos *et al.* 2008) of polymorphisms in 3R genes revealed that a specific Beijing strain had an accumulation of nonsynonymous mutations in genes encoding DNA glycosylase family enzymes.

The potential association of Beijing strains with acquired drug resistance could be ascribed to factors other than mutation rate, such as better developed efflux systems, a higher rate of replication (Moreland *et al.* 2009), or an increased ability to adapt to the fitness cost of resistance (Fenner *et al.* 2012). In the study by De Steenwinkel and colleagues (De Steenwinkel *et al.* 2012a), the resistant mutants selected under the model of non-compliance with isoniazid treatment carried no mutations in *katG* or *inhA*, the usual sites of INH resistance. It seems, therefore, that Beijing strains might be prone to acquiring mutations in genes other than target genes, enabling them to better adapt to drug stress (De Steenwinkel *et al.* 2012a).

A compelling case for the phenomenon of strain-specific mutation rates was made recently by Ford *et al.* (2013), who reported that *M. tuberculosis* strains from the East Asian lineage acquired drug resistance more rapidly than strains from the Euro-American lineage. Their results suggest that this is not due to an increased ability to adapt to antibiotic pressure, nor to

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an increased number of possible mutations. Instead, it appears that an elevated mutation rate in the absence of antibiotic pressure might contribute to the observed difference, although the mechanism(s) underlying the inferred difference in mutation rate remains to be determined.

There is also some evidence indicating that the presence of resistance-conferring mutations impacts the basal mutation rate. For example, a rifampicin-resistant mutant carrying an S522L mutation in *rpoB* was associated with a higher frequency of mutants than the progenitor, rifampicin-susceptible strain when selected on rifabutin (Anthony *et al.* 2005). In a follow-up study (Bergval *et al.* 2007) which investigated the induction in *rpoB* mutants of *recA* and *dnaE2* – encoding the principal regulator and key mutagenic polymerase of the mycobacterial SOS response, respectively – expression of *dnaE2* was moderately but significantly upregulated in S522L, H526D and S531W *rpoB* mutants, with S522L showing the highest constitutive expression. This is an intriguing observation; however, given that DnaE2 depends on the activity of the *imuA*-' and *imuB*-encoded accessory factors – which, like *dnaE2*, are included in the mycobacterial SOS regulon (Warner *et al.* 2010) – the functional consequences of increased levels of DnaE2 alone remain unclear. In other work, the same authors demonstrated that an isoniazid-resistant Haarlem strain harbouring a S315T mutation in *katG* was also associated with an increased mutation rate (Bergval *et al.* 2012). Again, this is an intriguing observation, and suggests that other unidentified strain-dependent mutations could interact with drug resistance-conferring mutations to confer a mutator phenotype.

2.7 Transient mutagenesis

In contrast to constitutive mutator strains, a transient mutator phenotype results in a temporary increase in the mutation rate . There are a number of mechanisms by which this may occur: mistranslation of proteins, especially those involved in accurate replication and repair of DNA (Miller 1996); transcriptional mutagenesis (Brégeon *et al.* 2009; Clauson *et al.* 2010; Foster 2007; Han *et al.* 2008; Ross *et al.* 2006; Saxowsky & Doetsch 2006) and the up-regulation of error-prone DNA polymerases (Brégeon *et al.* 2009; Foster 2007). Consistent with the multiplicity of mechanisms which can generate this phenotype, it is likely that transient mutator strains are responsible for the majority of adaptive mutations in bacterial populations (Miller 1996; Drake 2007).

Transcriptional mutagenesis occurs when RNA polymerase bypasses DNA lesions, inserting incorrect nucleotides into the mRNA (Saxowsky & Doetsch 2006). If one of the resulting

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mutant proteins confers an altered growth phenotype, it may in turn lead to DNA replication past the mutagenic lesion, which could lead to the fixation of the mutation (Saxowsky & Doetsch 2006). Alternatively, the mRNA containing an error may encode a protein involved in DNA repair or replication, which could lead to a transient mutator phenotype. The transcription-repair coupling factor, Mfd, recruits the NER repair machinery to sites where RNA polymerase is stalled (Prabha *et al.* 2011). Mfd was shown to play a role in mutagenesis in *B. subtilis* (Ross *et al.* 2006) and to mediate point mutations conferring fluoroquinolone resistance in *Campylobacter jejuni* (Han *et al.* 2008). Currently, nothing is known about transcriptional mutagenesis in *M. tuberculosis*, suggesting this as a potentially useful area of future research.

Mistranslation describes any error that affects the accurate translation of mRNA into protein (Reynolds *et al.* 2010). A major mechanism by which this occurs is through mischarging of tRNAs (Bacher & Schimmel 2007), which can happen when the tRNA gene is mutated (Slupska *et al.* 1996) or when aminoacyl-tRNA synthetases are defective in their editing capabilities (Bacher & Schimmel 2007). Mistranslation was associated with an elevated mutation rate under conditions of stress in *C. albicans*, *Acinetobacter baylyi* (Reynolds *et al.* 2010) and *E. coli* (Miller 1996). To date, there are no published data on the impact of mistranslation on the mycobacterial mutation rate.

Perhaps the most intensively studied transient mutator system is the bacterial SOS response, which is induced by DNA damage. In most bacteria, the SOS response is regulated by the RecA/LexA system: during normal growth, SOS genes are negatively regulated by the LexA repressor protein. Following genotoxic damage, RecA protein binds to single-stranded DNA (ssDNA) at sites of DNA lesions and replicon arrest to form RecA-ssDNA filaments which can interact with LexA, stimulating its cleavage and de-repressing SOS regulon genes. Unlike bacteria such as *E. coli,* whose SOS regulons include multiple Y-family DNA polymerases capable of translesion synthesis (TLS), the DNA damage response in *M. tuberculosis* is limited to the *dnaE1*- and *dnaE2*-encoded catalytic (α) subunits of the C-family DNA polymerase III (Boshoff *et al.* 2003; Smollett *et al.* 2012; Wang *et al.* 2011; Warner *et al.* 2010). *M. tuberculosis* encodes two DinB-like Y-family polymerases (Kana *et al.* 2010), however neither is induced as part of the mycobacterial SOS response. Critically, deletion of *dnaE2* has been shown to reduce the frequency of rifampicin resistance emergence in the mouse model (Boshoff *et al.* 2003), thereby implicating the mutagenic cassette as a major mechanism driving adaptive evolution during chronic infection. It is tempting, therefore, to

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speculate that DnaE2-dependent transient mutagenesis functions in mycobacterial evolution; however, this requires further investigation.

Another low-fidelity repair system recently characterised in *M. smegmatis,* a model organism for *M. tuberculosis*, is that of non-homologous end joining (NHEJ) (Gong *et al.* 2005; Stephanou *et al.* 2007). Three proteins seem to be key to this process, including Ku, LigD and LigC (Gong *et al.* 2005). Even though NHEJ cannot strictly be considered a transient mutator system, it has the potential to elevate mutagenesis if upregulated under certain conditions (Gorna *et al.* 2010; Pitcher *et al.* 2007), a possibility that requires further investigation.

2.8 Environmental mutagens

2.8.1 Antibiotics

Antibiotics target essential functions and therefore impose a strong selective force for genetic resistance. At sub-inhibitory concentrations, antibiotics can also act as mutagens, driving the emergence of drug-resistant mutants, a phenomenon that has been demonstrated *in vitro* for different bacterial species and a variety of antibacterial classes (Baharoglu & Mazel 2011; Cohen & Walker 2010; Gutierrez *et al.* 2013; Kohanski, DePristo, *et al.* 2010; Nagel & Chan 2006; Thi *et al.* 2011; Veigl *et al.* 1991). *M. tuberculosis* may encounter sub-inhibitory drug concentrations as a result of patient non-compliance (Mitchison 1998), poor absorption by the gastrointestinal tract (Coupe *et al.* 1991), poor penetration of - or activity in - certain parts of the lung (Kjellsson *et al.* 2012), and host genetic factors which impact drug metabolism and clearance (Warnich *et al.* 2011). *M. tuberculosis* might also inadvertently be exposed to broad spectrum antibiotics (e.g., fluoroquinolones) in patients undergoing treatment for other respiratory infections (e.g., community-acquired pneumonia) (Long *et al.* 2009). Sub-inhibitory concentrations of ciprofloxacin, a fluoroquinolone whose mechanism of action results in the induction of the mycobacterial SOS response (O'Sullivan, Hinds, *et al.* 2008), have been shown to elevate *in vitro* mutagenesis in related mycobacterial species (Gillespie *et al.* 2005; Malik *et al.* 2012). Moreover, there is some evidence to suggest that fluoroquinolone use prior to diagnosis of TB may be associated with first-line drug resistance (Deutschendorf *et al.* 2012), especially if multiple prescriptions have been given (Long *et al.* 2009). However, this topic is controversial owing to conflicting evidence (Park *et al.* 2007).

It is likely too that antibiotics which do not act directly on DNA metabolic processes can lead to DNA damage. For example, multiple studies have reported that bactericidal antibiotics of

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very different classes – including β-lactams, fluoroquinolones and aminoglycosides – are united by a common mechanism of killing that results in the generation of hydroxyl radicals (Foti *et al.* 2012; Grant *et al.* 2012; Kohanski, DePristo, *et al.* 2010). Although very recent studies have questioned the general applicability of the model (Keren *et al.* 2013; Liu & Imlay 2013), there is some evidence implicating the antibiotic-dependent formation of reactive oxygen species (ROS) in an elevated mutation rate (Kohanski, DePristo, *et al.* 2010). In *M. tuberculosis*, the production of ROS has been directly correlated with the ability to eliminate an antibiotic tolerant bacillary population *in vitro* (Grant *et al.* 2012); however, to our knowledge, the link between ROS and mutagenesis has not been explored. Cirz *et al.* (2005) proposed an intriguing mechanism by which antibiotics other than fluoroquinolones could also lead to DNA damage (Cirz *et al.* 2005): on the assumption that disruption of metabolism could alter ATP/ADP ratios, it was argued that depletion of ATP – an essential energy source for DNA gyrase function – may result in stalling of replication forks and, potentially, SOS induction. Again, this possibility requires further investigation.

Genome-wide expression studies demonstrated that fluoroquinolones induce up-regulation of SOS clusters in *M. tuberculosis* (Boshoff *et al.* 2004), whereas inhibitors of translation do not (Boshoff *et al.* 2004). Consistent with the prediction that isoniazid might potentially generate ROS (Timmins & Deretic 2006), genome-wide studies have detected up-regulation of the SOS response (Chen *et al.* 2012) and *recA* (Waddell *et al.* 2004) following isoniazid exposure. Similarly, the *ligD* gene, essential for mycobacterial NHEJ (Gong *et al.* 2005), was also induced (Waddell *et al.* 2004). To date, however, the effect of subinhibitory concentrations of anti-TB antibiotics on the mutation rate of the organism has not been investigated *in vitro*, or in patients developing drug-resistant TB. Although challenging, an analysis of the drug regimens or drug combinations that could lead to an increased rate of the emergence of drug resistance in *M. tuberculosis*-infected patients could, therefore, be of great value in estimating the risk of acquisition of drug resistance during chemotherapy.

2.8.2 Antiretroviral drugs

It was previously suggested that antiretroviral drugs may affect the mutation rate of *M. tuberculosis* (Bradford *et al.* 1996). However, the evidence for an association between HIV status and *M. tuberculosis* drug resistance on an individual level is mixed (Sergeev *et al.* 2012). One class of antiretroviral comprises nucleoside reverse transcriptase inhibitors (NRTIs), which have been associated with mutagenic effects in animal models and humans

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(Walker *et al.* 2009). NRTIs are nucleoside analogues and may be genotoxic in bacteria owing to their ability to be incorporated into bacterial DNA, causing chain termination (Wutzler & Thust 2001) - which itself may lead to the induction of the SOS response (Boshoff *et al.* 2003; Mamber *et al.* 1990). Alteration of nucleotide pools and conformational changes in DNA polymerase might similarly also lead to reduced replication fidelity. However, while NRTIs are known to elevate the mutation rate of the HIV genome (Jewell *et al.* 2003) there are no published studies which have assessed their potential effects on *M. tuberculosis.*

2.8.3 TB pathogenesis and the host environment

The discrepancies observed between apparent mutation rates (and frequencies) *in vivo* and those measured *in vitro* suggest that the factors modulating the mutation rate during host infection might be very different from those operating under laboratory conditions. For example, a Beijing strain was associated with an elevated *in vitro* frequency of rifampicin resistance - but not isoniazid resistance - compared to EAI strains;(De Steenwinkel *et al.* 2012b) yet the converse was observed in a mouse model of infection (De Steenwinkel *et al.* 2012a). Similarly, Bergval *et al.* (2009) detected different types of isoniazid resistance-conferring mutations *in vitro* versus those generally observed clinically. Therefore, determining the mutagenic stimuli and DNA damaging events encountered *in vivo* may be critical to the identification of those factors that drive adaptive evolution and the emergence of drug resistance during host infection.

M. tuberculosis encounters many DNA-damaging influences during the infectious lifecycle (Gorna *et al.* 2010). For example, during aerosol transportation, bacilli are likely to encounter UV radiation and desiccation (Gorna *et al.* 2010). UV radiation induces expression of *lexA, dnaE2,* and *recA* (Boshoff *et al.* 2003), while *M. smegmatis* mutants defective in *ku* and *ligD* are susceptible to desiccation (Pitcher *et al.* 2007). Expression of Ku was also higher in granulomas than in broth (Rachman *et al.* 2006). In addition, the *mutT2* gene, which may be involved in hydrolysis of 8-oxo-dGTP (Dos Vultos *et al.* 2006), was down-regulated in response to lung surfactant (Schwab *et al.* 2009). This could lead to an increase in 8-oxo-dGTP in nucleotide pools and elevated mutagenesis (Jain *et al.* 2007).

Oxidative stress is a major mutagenic influence encountered by *M. tuberculosis* in the host. Macrophages, as an antibacterial defence mechanism, produce ROS and reactive nitrogen intermediates (RNI) (Ehrt & Schnappinger 2009). These reactive species interact with

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nucleotides, resulting in chemical modifications that can lead to base mispairing and DNA damage (Jain *et al.* 2007; Kurthkoti *et al.* 2010; Kurthkoti & Varshney 2010; Malshetty *et al.* 2010). DNA damage can, in turn, lead to an upregulation of error-prone DNA repair (Smollett *et al.* 2012). *M. tuberculosis* is well armed against oxidative stress; moreover, mechanisms to detoxify ROS and RNI are essential for the survival of this pathogen in the host (Ehrt & Schnappinger 2009). The high levels of redundancy in these and specific DNA repair (Warner 2010) pathways might, therefore, indicate a critical role in pathogenesis (Ehrt & Schnappinger 2009). The high GC content of the genome also suggests that *M. tuberculosis* may be especially vulnerable to oxidative damage (Kurthkoti & Varshney 2011; Wang *et al.* 2013). Similarly, observations from the non-human primate model (Ford *et al.* 2011) suggest a role for ROS in the spectrum of mutations observed.

In contrast, analyses by O'Sullivan *et al.* (2005) of mutations in *rpoB* and *pncA* in clinical isolates suggest a pattern inconsistent with oxidative stress as the major driver of bacillary evolution. Their study (O'Sullivan *et al.* 2005) was based on the assumption that resistance to isoniazid most often occurs before resistance to rifampicin (conferred by mutations in *rpoB)* or pyrazinamide (conferred by mutations in *pncA)*. Isoniazid resistance is conferred by mutations in *katG,* encoding an enzyme that functions in the oxidative stress response (Saint-Joanis *et al.* 1999). Mutations in *katG*, including the clinically dominant S315T mutation, have been shown to have a detrimental effect on the activity of this enzyme (DeVito & Morris 2003). Therefore *katG* mutants are expected to undergo mutagenesis driven by oxidative stress. However, O'Sullivan *et al.* (2005) did not observe the expected overall increase in G to A or C to T mutations in the genes analysed.

It is possible, though, that fitness plays a greater role in defining the mutation spectra of resistance genes in clinical isolates. This interpretation is consistent with evidence from another study which investigated the effect of lowering the pH on mutational events during *in vitro* culture of *M. tuberculosis* (Jenkins *et al.* 2009). Although the decrease in pH only impacted the mutation frequency slightly, there was a major effect on the mutation spectrum, in which rare, "less fit" mutations were observed. As discussed above, there is the possibility that less fit mutants are associated with upregulated expression of *dnaE2* (Bergval *et al.* 2007), which might provide the opportunity for the development of compensatory mutations, a possibility that requires further investigation.

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2.8.4 Smoking and air pollution

An association between drug resistance and smoking or tobacco use has been observed in some cases (Dalton *et al.* 2012; Ruddy *et al.* 2005). Cigarette smoke contains mutagenic chemicals (Fujita & Kamataki 2001; Yim & Hee 2001), and smoking and environmental pollutants could also alter the redox balance, in turn affecting the mutation rate (Kumar *et al.* 2011). A compound typically generated during combustion, 1,6-dinitropyrene (1,6-DNP), increased the incidence of drug resistance in *Pseudomonas aeruginosa* (Miyahara *et al.* 2011). Whether this compound, and other similar compounds, are important mutagens in the development of drug resistance in *M. tuberculosis* remains to be determined.

2.9 Conclusion

M. tuberculosis has a low mutation rate *in vitro*, yet seems capable of generating surprisingly high levels of genomic diversity within the host. However, determining whether the mutation rate is modulated during host infection is complicated by the fact that it is difficult to distinguish between the rate of selection for drug resistance and the rate at which the genotypic diversity for selection is itself generated. Moreover, the extent to which the mycobacterial replication rate itself is modulated during host infection remains unknown. Therefore, it is not clear whether the mutation rate of *M. tuberculosis* is higher in the host than *in vitro*, nor whether there are factors specific to the *in vivo* environment that might drive mutagenesis. Knowledge of the contribution made by transient and constitutive mutator strains to the mutation rate and, therefore, the evolution of drug resistance in *M. tuberculosis*, represents an important area for future research as part of continued efforts to inhibit - and prevent - the acquisition of drug resistance.

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Chapter 3 The effect of nucleoside reverse transcriptase inhibitors on the mycobacterial mutation rate

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3.1 Abstract

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis*, exhibits a high co-infection rate with HIV. Furthermore, an association between drug-resistant TB and HIV has been observed in some settings. The reasons for this are not well understood. Nucleoside reverse transcriptase inhibitors are included in HIV/antiretroviral treatment regimens and have shown the potential to be genotoxic, i.e. induce mutagenesis. The aim of this study was therefore to calculate the mutation rate of *Mycobacterium smegmatis*, as a model for *M. tuberculosis,* with and without prior treatment with nucleoside reverse transcriptase inhibitors 3TC and AZT. No significant increase in the mutation rate was detected after treatment with these drugs, as well as their active triphosphate forms. These results suggest that antiretroviral treatment does not lead to increased acquisition of drug-resistant TB as a result of their mutagenicity and that other factors play a role in the overlap of TB drug resistance with HIV infection.

3.2 Introduction

M. tuberculosis may encounter various mutagenic influences within the host, as discussed in Chapter 2, including various DNA damaging stresses and drugs. The TB epidemic is exacerbated by a high co-infection rate with HIV, and a significant proportion of HIV-related deaths are due to TB (Sester et al. 2010). It follows, therefore, that TB patients are often receiving antiretroviral therapy in addition to TB treatment. *M. tuberculosis* strains are therefore exposed to antiretroviral drugs.

Antiretroviral therapy may be of specific interest, since there is potential for antiretrovirals to increase the mutation rate of bacteria. Many of the currently used antiretroviral regimens include drugs that are nucleoside reverse transcriptase inhibitors - and therefore nucleoside analogues - such as lamivudine (3TC), emtricitabine (FTC), Tenofovir (TDF), Stavudine (d4T), Zidovudine (AZT), Abacavir (ABC) and didanosine (ddI). (Department: Health, Republic of South Africa 2010). These drugs have been subjected to routine toxicology tests to determine their genotoxocity or ability to cause cancer in humans (Wutzler & Thust 2001). All these drugs have shown genotoxicity in some, but not other, tests (Wutzler & Thust 2001).

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In particular, nucleoside analogues have been tested for mutagenicity in *Salmonella* and *Escherichia coli*. In fact, *Salmonella* is the organism used in a routine toxicology test, known as the Ames test, for carcinogenicity (Ballardin et al. 2005; Wutzler & Thust 2001). In this test, none of the nucleoside analogues increased the mutation rate (Ballardin et al. 2005; Wutzler & Thust 2001). Another nucleoside analogue, specifically a cytidine analogue called zebularine, showed potent induction of base substitutions in *E. coli* (Lee et al. 2004). However, tests for mutagenicity of nucleoside analogues have not been carried out in mycobacteria.

Previous results from the literature, however, suggest no effect of nucleoside analogues on the mutation rate of gram-positive bacteria or *M. tuberculosis* as gauged by the species-specific antibacterial activity of these compounds (Elwell et al. 1987). For example, AZT exhibited antibacterial activity against members of the family *Enterobacteriacea,* which includes *E. coli*, but not against gram-positive bacteria or *M. tuberculosis* and this was dependent on the phosphorylation of AZT to nucleotide level (Elwell et al. 1987). Similarly, Sandrini *et al.* (2007), showed that the deoxyribonucleoside kinases of different species of bacteria activate nucleoside analogues to different extents.

Nucleoside reverse transcriptase inhibitors are prodrugs. In mammalian cells, in order to activate these prodrugs, they are converted to the active triphosphate form in three phosphorylation steps (Whirl-Carrillo et al. 2012). For example, 3TC is phosphorylated to 3TC-monophosphate (3TC-MP) by deoxycytidine kinase, as shown in Figure 3.1. The second step by cytidine monophosphate/deoxycytidine monophosphate kinase results in the diphosphate form of the drug. Lastly, 3'-phosphoglycerate kinase or nucleoside diphosphate kinase converts the diphosphate form into 3TC-triphosphate (3TC-TP). The active forms may have genotoxic activity in bacteria, because they can be incorporated into bacterial DNA and cause chain termination (Wutzler & Thust 2001). Chain termination may lead to the induction of the SOS response (Mamber *et al.* 1990), which in turn could be responsible for an elevated mutation rate (Boshoff et al. 2003). Another proposed mechanism involves that of altering the balance of nucleotide pools (Wutzler & Thust 2001). If any one nucleotide or its equivalent phosphorylated nucleoside analogue is in excess compared to other nucleotides, this may lead to aberrant incorporation of the nucleotide/nucleoside analogue that is in excess.

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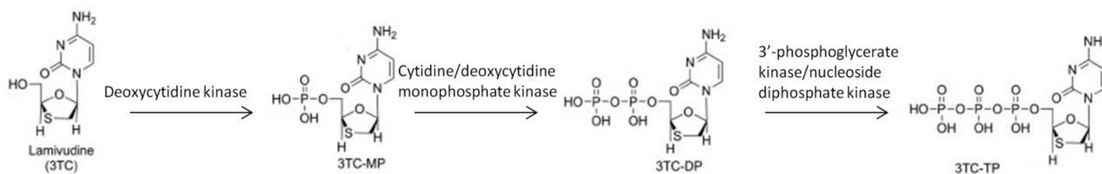

Figure 3.1 Lamivudine/3TC activation pathway in the mammalian host. MP=monoposphate, DP=diphosphate, TP=triphosphate (Whirl-Carrillo *et al.* 2012).

Because one of the proposed mechanisms for the induction of the mutation rate involves that the triphosphate form of the analogue is incorporated into the DNA (Mamber et al. 1990; Wutzler & Thust 2001), it follows that, in order for the nucleoside to exert its mutagenic effect, such kinases are necessary. So far, homologues of the kinases required in the first step of activation of 3TC and AZT (cytidine and thymidine kinase respectively) have not been found in *M. smegmatis* (Kapopoulou et al. 2011) or in *M. tuberculosis* (Kapopoulou et al. 2011; Lew et al. 2011; Pochet et al. 2003). It is thus likely that 3TC and AZT are not converted into 3TC-TP and AZT-TP respectively in mycobacteria *in vitro* and can therefore not exert their mutagenic effects. In addition, *M. tuberculosis* thymidylate kinase is deficient in its activity towards AZT monophosphate (Russell & Ostermeier 2014).

The possibility that the triphosphate forms of nucleoside reverse transcriptase inhibitors leads to an increase in mutation rate in mycobacteria has not been investigated thus far. This is relevant to *M. tuberculosis* in the host, where the active forms of the drug may be encountered. We therefore hypothesised that nucleoside reverse transcriptase inhibitors cause an increase in the mutation rate of mycobacteria. The aim of this study was to calculate the mutation rate of *M. smegmatis* to acquire mutations in the rifampicin resistance-determining region of *rpoB,* with and without prior treatment with the triphosphate forms of the cytidine analogue 3TC and the thymidine analogue AZT.

3.3 Materials and methods

3.3.1 Preparation of stocks for drugs

All drugs were obtained from Sigma-Aldrich. Rifampicin was made up to 4 mg/ml by adding the powder to one part dimethyl sulfoxide (DMSO), followed by 9 parts H_2O. The 3TC and AZT were made up to 1 mg/ml in H_2O. All stock solutions were stored at -20°C.

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3.3.2 Determination of minimum inhibitory concentration of ARVs for *M. smegmatis*

The MIC of the ARV's for *M. smegmatis* was determined by the broth microdilution method in a 96-well plate. *M. smegmatis* was grown in 7H9, supplemented with ADC, 0.2% glycerol and 0.05% Tween-80 to an OD_{600} of 0.5 to 0.6. This culture was then diluted 1000 times to a density of 10^4 cells/ml. Each plate contained one column of cells grown only with medium, one column with the drug diluent (H_2O), and a column containing a twofold dilution series of AZT or 3TC, ranging from 0.0625 to 16 µg/ml, or a twofold dilution series of AZT-TP ranging from 0.625 to 160 pmol/ml, or a twofold dilution series of 3TC-TP ranging from 0.015625 to 4 nmol/ml, in duplicate. It also included control wells with medium only, drug diluent only and drug only in duplicate. The plate was incubated for 3-4 days at 37°C, after which 10 µl of 0.02% (w/v) resazurin was added to each well and the plate incubated for a further 24 hours at 37°C. A magenta colour indicated growth, while blue indicated a lack of growth.

3.3.3 *M. smegmatis* fluctuation assays

3.3.3.1 3TC

The mutation rate in the presence and absence of 3TC was determined according to the method of Kurthkoti *et al.* (2008). Briefly, *M. smegmatis* was grown for 48 hours in 7H9 containing 0.2% glycerol, 0.1% Tween-80 and ADC, after which this starter culture was diluted 10^5 times in the same medium. To one half of this inoculum, 3TC was added to a final concentration of 1 µg/ml. The inoculum was then used to set up 25 independent cultures of 2 ml each for each treatment condition (thus 50 in total). The absence of mutants in the starting inoculum was confirmed by plating an equivalent number of cells as was inoculated per tube on 7H10 with 0.5% glycerol and ADC and 100 µg/ml RIF. The tubes were incubated with vigorous shaking at 37 °C for 6 days. Twenty of the twenty-five cultures were then centrifuged at 1811x g for 5 min, 1.7 ml of the supernatant was removed and the cells resuspended in the remainder of the liquid. The whole cell suspension was then spread onto 7H10 with glycerol, ADC and 100 µg/ml RIF. An aliquot was taken from the remaining 5 cultures and total viable counts determined by dilution plating on 7H10 with glycerol and ADC. The plates were incubated for 3-5 days at 37 °C after which the colonies were counted.

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3.3.3.2 AZT, AZT-TP and 3TC-TP

A single colony of *M. smegmatis* was inoculated into Middlebrook 7H9 supplemented with ADC, 0.05% Tween-80 and 0.2% glycerol and grown overnight at 37°C with vigorous shaking until an optical density at 600nm of 0.8-1.0. This starter culture was then diluted 16- to 20-fold to yield a suspension of approximately 10^6 cells/ml. A tenfold dilution series was then carried out to yield a suspension of approximately 10 cells/ml. To one half of the suspension AZT was added to a final concentration of 1 µg/ml, or AZT-TP to a final concentration of 20 pmol/ml or 3TC-TP to a final concentration of 2 nmol/ml. In the case of AZT, an equivalent volume of drug diluent (H_2O) was added to the other half of cells. In the case of the triphosphate compounds, these were diluted using Middlebrook 7H9 medium. Parallel tubes were set up with 1.5 ml culture each from stirring suspensions. An equivalent number of cells as were inoculated per tube were then spread out on Middlebrook 7H10, supplemented with ADC and 0.5% glycerol and containing 200 µg/ml RIF, to ensure the absence of pre-existing mutants in each culture. The parallel cultures were then incubated until an OD_{600} of about 0.8 was reached (approximately 48 hours). The incubation was at 37°C with shaking and with the caps loosened for improved aeration.

For 5 out of the total number of parallel tubes set up, the total final population was determined by preparing a dilution series of an aliquot taken from each tube. One hundred and fifty microliters from a range of dilutions were spread onto 7H10, supplemented with ADC and 0.5% glycerol. The rest of the tubes were centrifuged for 5 min at 1811x g to pellet the cells. Part of the supernatant (1.2ml) was removed, the pellet resuspended and the entire contents spread onto 7H10, supplemented with ADC, 0.5% glycerol and containing 200 µg/ml RIF. The plates were incubated for 5-6 days, after which the colonies were counted.

3.3.4 Determination of mutation rate

The Ma-Sandri-Sarkar maximum likelihood method was employed to calculate the mutation rate, using the web-based application available at www.mitochondria.org/protocols/FALCOR.html (Hall *et al.* 2009). The average total population size (Nt) across 5 cultures was employed as the value of Nt for each value of number of mutants (r). For determining the average Nt, only plates with colony counts 30-300 were included in the analysis for the sake of accuracy.

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3.3.5 PCR and sequencing

The sequences of the primers used to amplify the rifampicin resistance-determining region (RRDR) (Wanner *et al.* 2009) of *M. smegmatis rpoB* were as follows: SMrpoBFwd 5'-CATCGACCACTTCGGCAACC-3', SMrpoBRev 5'-CTCCTCGTCGGCGGTCAG-3'.

The components of the HotStar Taq DNA Polymerase kit (Qiagen) were used in each PCR reaction. Each reaction contained 1 x Q-solution, 1 x buffer, 3.5 mM Mg^{2+}, 1.6 mM dNTPs, 1 µM of each primer, and 0.025 units/µl of Taq. The reaction details were as follows:

95°C for 15 minutes

94°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute (x40)

72°C for 10 minutes

Boiled cultures for *M. smegmatis* were prepared by suspending a single colony in 50 µl of ddH_2O and boiling at 95°C for 25 minutes. Cells were pelleted by centrifugation and 1 µl of the supernatant used in the PCR reaction. The PCR products were purified and sequenced by the Central Analytical Facility at Stellenbosch University.

3.3.6 Calculation of intracellular concentrations of AZT-TP and 3TC-TP

The estimated concentrations of AZT-TP and 3TC-TP likely to be encountered within the alveolar macrophage, in units of moles per ml were calculated as follows: The maximum intracellular concentration of AZT-TP as observed from human tissue in the literature was found to be approximately 100 fmol/10^6 cells (Brody & Aweeka 1997; Dumond *et al.* 2008; Rodriguez-Torres *et al.* 2005; Rower *et al.* 2012; Wattanagoon *et al.* 2000). The volume of a human alveolar macrophage was previously determined to be 4990 μm^3 or 4.99 picolitre (Krombach *et al.* 1997). The volume of 10^6 alveolar macrophages would be approximately 5 µl. Therefore in 1 ml, 20 pmol of AZT-TP will likely be present. Similarly, the maximum intracellular concentration of 3TC-TP as observed from human tissue was found to be 10 pmol/10^6 cells (Dumond *et al.* 2008; Rodriguez-Torres *et al.* 2005; Rower *et al.* 2012). Therefore in 1 ml, 2 nmol of 3TC-TP will likely be present.

3.3.7 Statistical analysis

The proportions of particular types of nucleotide substitutions/indels selected after bacteria were treated or untreated with antiretroviral compounds were compared for statistical

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significance by a two-tailed Fisher's exact test. The confidence interval was 95%. GraphPad Prism 5.01 software was used in this analysis.

Confidence intervals for the fold change in mutation rate were calculated using Microsoft Excel.

3.3.8 Raw data

Raw data for these analyses can be found in the appendix.

3.4 Results

3.4.1 Effect of nucleoside analogues on the growth of *M. smegmatis*

The first step was to confirm that 3TC and AZT does not inhibit the growth of *M. smegmatis* at concentrations similar to those achieved in serum of patients treated with such drugs. To achieve this aim the minimum inhibitory concentration (MIC) of 3TC and AZT to *M. smegmatis* was determined across a range of 0.0625 to 16 µg/ml for both drugs. The concentration range of 3TC and AZT was chosen to fall within the window of concentrations achieved in serum when patients are treated with the drug (Bazzoli et al. 2011; Dumond et al. 2008; Else et al. 2012; Hemanth Kumar et al. 2009; Kumar et al. 2013; Li et al. 2010; Minzi et al. 2011; Narang et al. 2005; Wang et al. 1999). Results in Figure 3.2 showed that, as expected, no growth inhibitory effect was observed, even at the highest concentration.

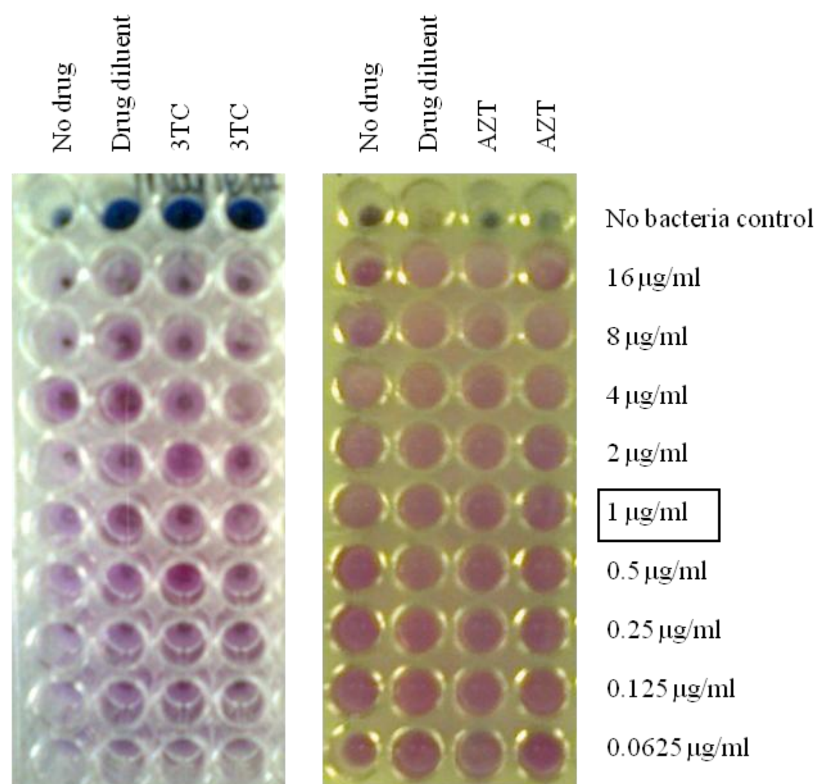

Figure 3.2 The effect of 3TC (left) and AZT (right) on the growth of *M. smegmatis* as determined by the broth microdilution method. The drug diluent was water. Resazurin was used as a colorimetric indicator of growth. Dark blue colour indicates no growth, while magenta indicates growth. The concentration employed in further experiments is outlined.

3.4.2 Treatment with 3TC and selection on 100 µg/ml rifampicin

Fluctuation assays were used to compare mutation rates in the presence and absence of 3TC. *M. smegmatis* was cultured for 6 days in the presence and absence of 1 µg/ml 3TC and mutants were subsequently selected on 100 µg/ml rifampicin. The method used was adapted from Kurthkoti *et al.* (2008), from which the length of culture time and the rifampicin selection concentration was taken. Three independent experiments were performed with 20 cultures for each treatment condition, thus 40 in total for each experiment. The number of mutation events ranged from 6 to 37, therefore 20 cultures were sufficient for calculating an accurate mutation rate using the MSS maximum likelihood method. This assumption was according to Rosche and Foster (2000), who previously determined the number of cultures required to achieve a theoretical precision of 20% when a specific number of mutation events are observed, using different estimators.

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When *M. smegmatis* was treated with 3TC, a significant decrease in mutation rate was observed. The mutation rate after treatment with 3TC was, on average, 0.7-fold that of the cultures left untreated (0.58-0.82, 95% confidence interval), as shown in Table 3.1. Therefore 3TC was not mutagenic to *M. smegmatis* under the conditions tested, which agrees with predictions from the literature.

Table 3.1 Mutation rates of *M. smegmatis* in the presence and absence of 3TC

Experiment	**Mutation rate ($x10^{-9}$ mutations/generation)**		**Fold change (3TC treated/untreated)**
	3TC-treated	**Untreated**	
1	2.91	4.20	0.69
2	5.94	7.37	0.81
3	17.53	29.17	0.60
Mean fold change			0.70
95% confidence interval			0.12

One of the ways by which nucleoside analogues could potentially alter the mutation rate is by altering the balance of nucleotide pools (Wutzler & Thust 2001). AZT has been shown to be a potent inhibitor of *M. tuberculosis* thymidine monophosphate kinase. Although such knowledge is not available for 3TC and cytidine monophosphate kinase, it is conceivable that nucleoside analogues inhibit enzymes involved in nucleotide synthesis. This may have an effect on availability of one or more nucleotides, which may influence the fidelity of DNA replication and repair (Mathews 2006; Wutzler & Thust 2001).

Samples treated and untreated with 3TC may therefore show a difference in mutation spectrum, even when no increase/decrease of the mutation rate is detectable. Therefore we selected one colony from each of a total of 120 rifampicin plates (pooling all three experiments). One colony from each plate was assumed to be representative of one independent mutation event (Morlock et al. 2000). It is important to select colonies proportionally to the number of plates, and not all the colonies, since a mutation event may have occurred early during culturing and expanded. This would bias proportions of independent mutation events.

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After selection of colonies, the rifampicin resistance-determining region (RRDR) of *rpoB* (Jain et al. 2007; Kurthkoti et al. 2008; Kurthkoti et al. 2010; Wanner et al. 2009) was amplified by PCR and the product was sequenced. There was no significant difference in the mutation spectrum between isolates treated or untreated with 3TC as seen in Table 3.2, not even when all mutations resulting in a substitution with C or G were combined. This suggests that 3TC does not perturb nucleotide pools leading to a bias in certain types of nucleotide substitutions over others.

Table 3.2 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with 3TC

Type of mutation	Number of isolates (%)		P-value[a]
	3TC-	3TC+	
A to C, T to G	1 (1.7)	2 (3.3)	1.000
A to G, T to C	12 (20.0)	8 (13.1)	0.3376
A to T, T to A	0 (0.0)	2 (3.3)	0.4959
C to A, G to T	0 (0.0)	3 (4.9)	0.2438
C to G, G to C	11 (18.3)	11 (18.0)	1.000
C to T, G to A	8 (13.3)	9 (14.8)	1.000
deletion from1285 to 1314	1 (1.7)	1 (1.6)	1.000
no mutation	27 (45.0)	25 (41.0)	0.7151
All mutations to C/G	24 (40)	21 (34.4)	0.5754

[a]Significant differences between the proportions were calculated by the Fisher's exact test (two-sided)

Importantly, 41-45% of the isolates did not harbour a mutation in the RRDR of *rpoB* (Table 3.2). This may imply that we are not assessing mutation rate, but rather epigenetic mechanisms of adaptation to the drug. However, since the percentage of isolates without a mutation were similar, we can assume that 3TC does not have an effect on the acquisition of rifampicin resistance by epigenetic means. Against this background there is therefore no increase in the mutation rate after treatment with 3TC.

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3.4.3 Modification of fluctuation assay

In Table 3.1, a tenfold variation in the mutation rate was observed across assays. Also, in Table 3.2, there was a high proportion of isolates without a known rifampicin resistance-conferring mutation. To reduce the variation observed in the assay and reduce the number of isolates without a known mutation, the fluctuation assay was modified in two ways. Firstly, we decided to assess the mutation rate of *M. smegmatis* in log phase rather than in stationary phase, as we did in the previous section, by growing a low-density inoculum for 48 hours to an OD_{600} of 0.8. There were other reasons for this modification: *M. smegmatis* may become hypermutable in stationary phase (Karunakaran & Davies 2000) and this could mask small mutation rate differences between cultures treated and untreated with nucleoside analogue. In addition, although the broth microdilution method to assess MIC showed no difference in growth between *M. smegmatis* treated and untreated with nucleoside analogue, growth differences across the growth curve from lag to stationary phase may still occur, which may not be detectable after 6 days of growth. This growth difference may have an effect on the apparent mutation rate, since the number of replication events may be underestimated due to an unknown number of death events. Clumping of very high-density cultures may also affect single colony formation on antibiotic selection plates.

Secondly, mutants were selected on 200 μg/ml rifampicin. The higher concentration should limit the proportion of rifampicin-resistant mutants lacking a mutation in the RRDR of *rpoB*. This was tested by growing 10 parallel cultures to log phase and plating the cultures onto 200 μg/ml rifampicin. The plates were incubated for 7 days. After 7 days, two types of colonies with distinct morphology and size could be seen. Colonies either were relatively big with a rough colony morphology, or relatively small with a smooth colony morphology. We selected 7 colonies for each morphology and sequenced the RRDR of *rpoB*. All 7 large, rough colonies had a mutation in the RRDR of *rpoB*, whereas all small, smooth colonies did not (Table 3.3)

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Table 3.3 Size and morphology of *M. smegmatis* single colony isolates with and without mutation in the RRDR of *M. smegmatis rpoB*

Colony name	Size and morphology	Nucleotide substitution	Nucleotide position in *M. smegmatis rpoB*
6b	Big, rough	C/T	1313
5b	Big, rough	A/G	1325
7b	Big, rough	A/G	1325
8b	Big, rough	C/T	1439
9b	Big, rough	A/G	1325
10b	Big, rough	A/G or C	1325
3b	Big, rough	C/T	1324
1s	Small, smooth	none	N/A
2s	Small, smooth	none	N/A
3s	Small, smooth	none	N/A
4s	Small, smooth	none	N/A
6s	Small, smooth	none	N/A
8s	Small, smooth	none	N/A
10s	Small, smooth	none	N/A

Taking these results into account, the colony morphology could be used to exclude wild-type colonies from the fluctuation assay, thus ensuring the assessment of mutation rate. Limiting growth of the plates to 5 days also limited the emergence of wild-type colonies. From this pilot experiment, the number of mutation events was estimated to be around 1, which would require 40 parallel cultures in such an experiment to accurately determine the mutation rate (Rosche & Foster 2000).

3.4.4 Mutation rate in the presence of AZT

Using the new conditions described above, the mutation rate of *M. smegmatis* was assessed in the presence and absence of 1 µg/ml AZT. Once again, this concentration is relevant in light of human serum concentrations (Bazzoli et al. 2011; Dumond et al. 2008; Kumar et al. 2013; Wang et al. 1999). Results shown in Table 3.4 indicate no increase in mutation rate of *M. smegmatis* treated with the nucleoside analogue, AZT, as predicted from the literature. On average, *M. smegmatis* treated with AZT displayed a mutation rate 1.17-fold that of bacteria left untreated (0.89-1.45, 95% confidence interval).

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Table 3.4 Mutation rates of *M. smegmatis* in the presence and absence of AZT

Experiment	Mutation rate ($x10^{-9}$ mutations/generation)		Fold change (AZT treated/untreated)
	AZT-treated	Untreated	
1	3.71	4.21	0.88
2	11.17	8.53	1.31
3	5.34	4.08	1.31
Mean fold change			1.17
95% confidence interval			0.28

One mutant was selected per plate (where mutants were present, since some plates did not select for mutants) and the RRDR of *rpoB* sequenced to determine a potential difference in mutation spectra for reasons already discussed in Section 3.4.2. The results of this are shown in Table 3.5. No significant differences in the types of mutations obtained were observed, even when all mutations to A or T were combined.

Table 3.5 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with AZT

Type of mutation	Number of isolates (%)		P-value[a]
	AZT-	AZT+	
A to C; T to G	3 (10.3)	2 (6.3)	0.6615
A to G; T to C	9 (31.0)	13 (40.6)	0.4282
C to A; G to T	2 (6.9)	4 (12.5)	0.6736
C to T; G to A	12 (41.4)	13 (40.6)	1.0000
wt	3 (10.4)	0 (0.0)	0.1015
All mutations to A/T	14 (48.3)	17 (53.1)	0.7997

[a]Significant differences between the proportions were calculated by the Fisher's exact test (two-sided)

3.4.5 Serum concentrations of activated AZT and 3TC

As described above in Section 3.1, AZT and 3TC are phosphorylated by host or human kinases to the active form, therefore AZT-triphosphate (AZT-TP) and 3TC-triphosphate (3TC-TP) occur within host cells. *M. tuberculosis* is likely to encounter the activated form, due to its lifestyle within alveolar macrophages (Flynn & Chan 2003).

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In order to mimic the *in vivo* conditions it was essential to determine the concentrations of AZT-TP and 3TC-TP that *M. tuberculosis* is likely to encounter. This was complicated by the fact that pharmacokinetic measurements of these metabolites are given in moles per million cells (Brody & Aweeka 1997; Dumond et al. 2008; Rower et al. 2012; Wattanagoon et al. 2000), where values for AZT-TP ranged from 25-50 fmol/10^6 cells and from 1-5 pmol/10^6 for 3TC-TP. Only one study has calculated the volume of a human alveolar macrophage (Krombach et al. 1997). Using this value, the volume of 10^6 cells could be estimated and a value for the concentration of AZT-TP/3TC-TP in moles/µl calculated (see Section 3.3.6). Therefore, in this study, *M. smegmatis* was treated with 20 pmol/ml AZT-TP and 2 nmol/ml 3TC-TP.

Under these conditions, the effect of AZT-TP and 3TC-TP on the growth of *M. smegmatis* was tested in the concentration ranges of 0.625 to 160 pmol/ml and 0.015625 to 4 nmol/ml respectively. From Figure 3.3 it is evident that neither AZT-TP nor 3TC-TP influenced the growth rate of *M. smegmatis*.

3.4.6 Mutation rate in the presence of AZT-TP

The effect of AZT-TP on the mutation rate of *M. smegmatis* after treatment with or without 20 pmol/ml of the compound was assessed. The results of three independent experiments are shown in Table 3.6, where the mean fold change was 1.60 (0.58-2.62, 95% confidence interval). No statistically significant increase of the mutation rate was therefore seen in the presence of AZT-TP at the concentration tested.

One mutant per plate from experiment 2 was selected and amplified and the RRDR of *rpoB* was sequenced. No significant difference in mutation spectra (shown in Table 3.7) was detected. However, when all mutations resulting in a substitution with thymidine or its complementary base, adenine, were combined, there was a higher percentage of such changes in cultures treated with AZT-TP than cultures left untreated. This proportion was not significantly higher.

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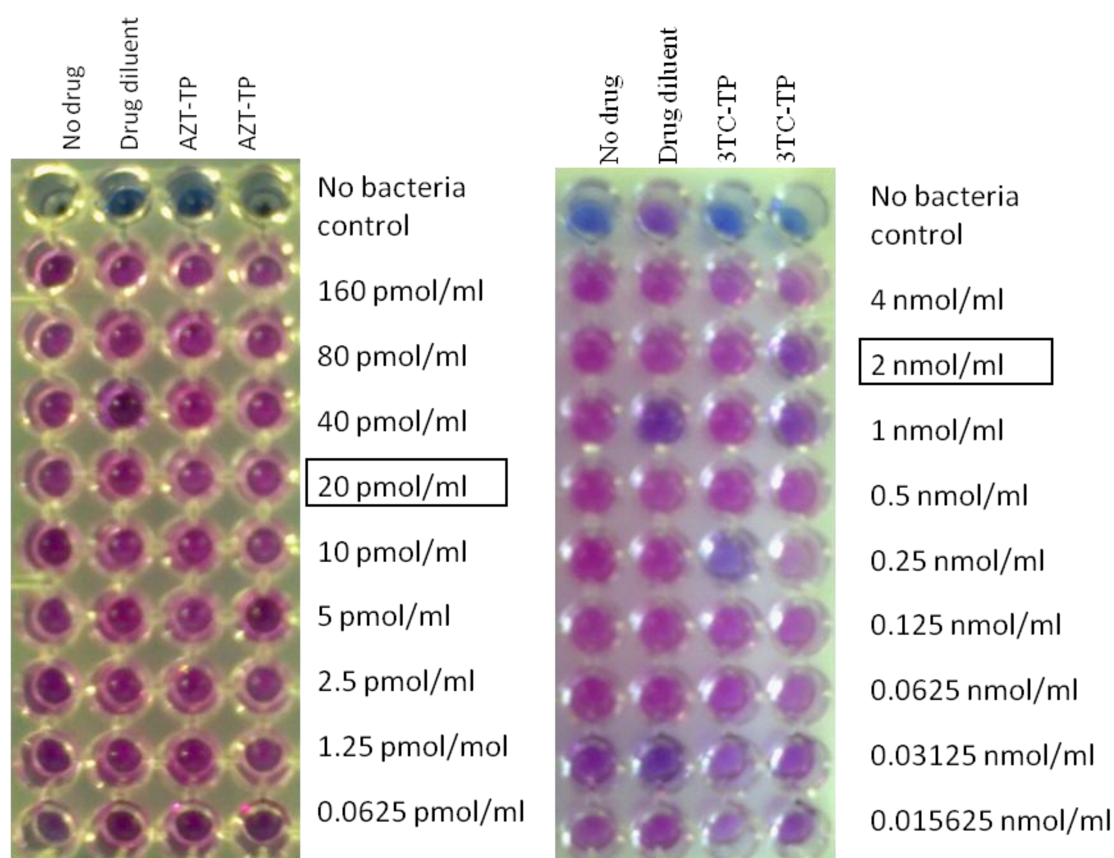

Figure 3.3 The effect of AZT-TP (left) and 3TC-TP (right) on the growth of *M. smegmatis* as determined by the broth microdilution method. Resazurin was used as a colorimetric indicator of growth. Dark blue colour indicates no growth, while magenta indicates growth. The concentrations employed in further experiments are outlined.

Table 3.6 Mutation rates of *M.smegmatis* in the presence and absence of AZT-TP

Experiment	**Mutation rate (x10^{-9} mutations/generation)**		**Fold change (AZT-TP treated/untreated)**
	AZT-TP-treated	**Untreated**	
1	3.87	4.58	0.85
2	8.83	6.51	1.36
3	9.13	3.51	2.60
Mean fold change			1.60
95% confidence interval			1.02

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Table 3.7 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with AZT-triphosphate (AZT-TP)

Type of mutation	Number of isolates (%)		P-value[a]
	AZT TP-	AZT TP+	
A to G; T to C	14 (46.7)	16 (48.5)	1.0000
A to T; T to A	0 (0.0)	3 (9.1)	0.2396
C to A; G to T	3 (10.0)	1 (3.0)	0.3396
C to T; G to A	8 (26.7)	13 (39.4)	0.4226
A to C; T to G	3 (10.0)	0 (0.0)	0.1022
C to G; G to C	1 (3.3)	0 (0.0)	0.4762
wt	1 (3.30	0 (0.0)	0.4762
All mutations to A/T	11 (36.7)	17 (51.5)	0.3118

[a]Significant differences between the proportions were calculated by the Fisher's exact test (two-sided)

3.4.7 Mutation rate in the presence of activated 3TC

Due to the expense of 3TC-TP, only 2 experiments with 5 parallel cultures each could be carried out. Therefore this experiment was regarded as a pilot experiment. No increase of the mutation rate was observed in the presence of 3TC-TP, in fact, on average, the mutation rate seemed to decrease, as shown in Table 3.8, where treated bacteria had a mutation rate 0.75-fold that of untreated bacteria (0.41-1.09, 95% confidence interval).

Table 3.8 Mutation rates of *M. smegmatis* in the presence and absence of 3TC-TP

Experiment	Mutation rate ($x10^{-9}$ mutations/generation)		Fold change (3TC-TP treated/untreated)
	3TC-TP-treated	Untreated	
1	1.87	3.27	0.57
2	2.16	2.34	0.92
Mean fold change			0.75
95% confidence interval			0.34

A total of 15 colonies each were selected and the *rpoB* RRDR amplified and sequenced. The colonies were selected in proportion to the number of plates, for example, if two colonies

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were selected per plate in experiment 1 in the presence of 3TC-TP, the same number were selected per plate in experiment 1 in the absence of 3TC-TP. Types of mutations are listed in Table 3.9. Although none of the differences were significant, there was an ~2-fold increase in guanine/cytidine substitutions under 3TC-TP treatment. It is possible, therefore, that 3TC-TP altered nucleotide pools. However, confirmation of this would require additional experimentation.

Table 3.9 Types of mutations observed in rifampicin-resistant isolates from cultures treated and untreated with 3TC-triphosphate (3TC-TP)

Type of mutation	**Number of isolates (%)**		**P-value[a]**
	3TC TP-	**3TC TP+**	
A to G; T to C	4 (26.7)	8 (53.3)	0.2635
C to A; G to T	3 (20.0)	1 (6.7)	0.5977
C to T; G to A	8 (53.3)	5 (33.3)	0.4621
deletion of CAC	0 (0.0)	1 (6.7)	1.0000

[a]Significant differences between the proportions were calculated by the Fisher's exact test (two-sided)

3.5 Discussion

No antibacterial activity, significant increase in the mutation rate or significant changes in mutation spectrum were observed when *M. smegmatis* was treated with AZT or 3TC (Table 3.1, Table 3.2, Table 3.4 and Table 3.5) as expected. However, the triphosphate forms of these drugs did not exhibit any antibacterial or mutagenic acitivity either. Firstly, this may be because such compounds cannot cross the cell wall and/or cell membrane of *M. smegmatis*, due to the polarity of this molecule (Owono Owono et al. 2013; Pochet et al. 2003). This possibility may be tested by liquid chromatography of whole cell lysate and extracellular medium to determine if these compounds are taken up by the cells or not. This was, however, outside the scope of our study. However, there was a slight difference in base substitutions between cultures treated and untreated with triphosphates. This suggests that these active drugs may be able to enter the cell, but since the differences were not significant, this is not clear. Increasing the sample size may result in significant differences. Secondly, the DNA polymerases of *M. smegmatis* may not utilise them as substrates for DNA synthesis. This

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explanation may not hold true though, since it has been shown that *M. tuberculosis* DNA polymerase I does utilise dideoxynucleotide 5'-triphosphates (Mizrahi & Huberts 1996). Lastly, it is possible that the concentration employed in this study is too low for the induction of the mutation rate. Differences in affinity of DNA polymerase/HIV-1 reverse transcriptase (Lim & Copeland 2001) may account for why the concentration is not sufficient.

One limitation we encountered in this study, was the system used to assess the increase in mutation rate. The acquisition of rifampicin resistance is widely used as a method to assess changes in mutation rate or frequency in *M. smegmatis* (Boshoff et al. 2003; Kana et al. 2010; Kurthkoti et al. 2008; Kurthkoti et al. 2010), but it is not ideal. As seen in Table 3.2, a large proportion of rifampicin-resistant *M. smegmatis* isolates did not harbour a mutation within the RRDR of *rpoB*. We cannot exclude the possibility that other mutations in *rpoB* outside the RRDR or elsewhere in the genome contributed to the resistance. However, it is not feasible to attempt to detect these mutations in all the isolates sequenced. We also cannot exclude the possibility that resistance was not due to the acquisition of a mutation, but to stochastic epigenetic responses (Wakamoto et al. 2013). *M. smegmatis* is more resistant to rifampicin than the slow grower *M. tuberculosis,* as a result of the *arr* gene, which encodes an enzyme that ribosylates and inactivates rifampicin (Alexander et al. 2003). It is therefore possible that changes in expression of Arr led to rifampicin resistance.

We attempted to overcome this by increasing the rifampicin selection concentration to 200 µg/ml. This decreased the proportion of mutations without a mutation in the RRDR, however, it also limited the variety of possible mutations. As seen in Table 3.5, Table 3.7 and Table 3.9, when compared to Table 3.2, increasing the concentration of rifampicin to 200 µg/ml resulted in a limited number of A/T to T/A and C/G to G/C mutations, although such mutations were still present. Sequencing a higher number of isolates should rectify this problem.

Fluctuation assays in *M. smegmatis* should perhaps be carried out using a different selection antibiotic, if the researcher is only interested in the rate of mutation. If there is also interest in the type of nucleotide substitution obtained, then acquisition of rifampicin resistance is advantageous, since there are so many possible, well characterised mutations that confer this in *M. smegmatis* (Telenti et al. 1993). Other antibiotics either do not lead to such a variety in the selection of known mutations or the mutations are not as well characterised (Karunakaran & Davies 2000; Revel et al. 1994). Future work could focus on developing a system

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involving a different antibiotic, for example ofloxacin. A wide variety of mutations confer ofloxacin resistance to *M. tuberculosis* (Sandgren et al. 2009) and these could be investigated as to whether they also confer resistance to *M. smegmatis.*

From Table 3.1, it is clear that fluctuation assays are highly variable and not always reproducible. This is why, in order to make an assumption about changes in mutation rate, the assay should be repeated at least 3 times (Personal communication, Anastasia Koch, University of Cape Town) and a consistent trend is taken as an up/downregulation of the rate. In our study, assessing and comparing mutation rates more than three times will decrease confidence intervals. For example, an average increase in mutation rate was seen in this study when *M. smegmatis* was treated with AZT-TP (Table 3.6), however, this was not significant due to the size of the confidence interval. In future, these assays should be repeated a number of times more to reach a more accurate answer.

It is possible that errors in the total population count affected the results. Clumping of bacilli may affect the number of colony forming units (cfu) obtained, where one colony may have started from 2 bacteria rather than one individual bacterium. *M. smegmatis* was cultured to mid-log phase in media containing Tween-80 and dilution series were performed in media containing Tween-80. Both culturing to mid-log phase and in the presence of detergent would have diminished this problem. However, to improve the probability of obtaining single bacilli, the culture can also be syringed a number of times, and quality control performed by acid-fast/Ziehl-Neelsen staining.

M. smegmatis was used as a model organism for *M. tuberculosis,* as a fast-growing, non-infectious member of the same genus. *M. smegmatis* has previously been used as a model to study changes in mutation rate and perform fluctuation assays (Kurthkoti et al. 2008; Kurthkoti et al. 2010), using the acquisition of *rpoB* mutations as a measure of mutation rate. Mycobacteria also share important commonalities in their DNA repair systems, notably the lack of a mismatch repair system (Cole et al. 1998). It is therefore likely that the lack of genotoxicity and antibacterial activity of AZT-TP and 3TC-TP will also apply to *M. tuberculosis.* Furthermore, retrospective clinical studies may suggest that antiretroviral drug use is not associated with drug resistant TB, suggesting that nucleoside reverse transcriptase inhibitors do not influence the spontaneous rate of mutation in *M. tuberculosis,* thereby leading to higher rates of resistance. In a South African study (Andrews *et al.* 2010), there was a significantly higher rate of ART among MDR/XDR patients, but the authors speculated

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that this was because previous treatment of TB was associated with drug resistance, and previously treated patients are routinely tested for HIV, making it more likely that such patients will receive ART.

Also, although HIV positivity may increase the prevalence of drug-resistant TB (Andrews et al. 2010; Flor de Lima & Tavares 2014; Mesfin et al. 2014; Mulisa et al. 2015; Sanchez-Padilla et al. 2012; Wells et al. 2007), there is controversy in the literature with regards to the association of drug resistance and HIV status as the association was not observed in all studies (Berhan et al. 2013; Cox et al. 2010; Espinal et al. 2001; Hoza et al. 2015; Lukoye et al. 2015; Sanchez-Padilla et al. 2012; Suchindran et al. 2009; van den Hof et al. 2013).

Where an association between drug-resistant TB and HIV exists, it could be due either to increased acquisition of resistance, a possibility that would apply if some antiretroviral drugs were mutagenic, or to accelerated transmission of drug-resistant TB (Sergeev et al. 2012). There are studies that have reported an association specifically between HIV seropositivity and acquired drug-resistant TB (Conaty et al. 2004; Robert et al. 2000), as well as acquired rifamycin resistance (Jenny-Avital 2002; Lutfey et al. 1996; March et al. 1997; Ridzon et al. 1998; Vernon et al. 1999). However, the association could be explained by malabsorption (Peloquin et al. 1993; Sahai et al. 1997) (as evidenced by the co-occurrence of diarrhoea) of anti-TB drugs in HIV-positive patients, which may lead to an inadequate treatment regimen, in turn leading to resistance (Ridzon et al. 1998). Secondly, prior use of rifabutin, which can cause cross-resistance to other rifamycins, such as RIF, may lead to the emergence of RIF mono-resistance in the as yet undiagnosed TB infection (Ridzon *et al.* 1998). Lastly, additional drugs added to a multiple anti-TB drug regimen may encourage non-compliance, leading to the emergence of resistance (Suchindran *et al.* 2009). There seems to be more evidence for an association of HIV status with transmission of drug-resistant strains, according to recent meta-analyses of global data (Mesfin et al. 2014; Suchindran et al. 2009), suggesting that a mutagenic influence, leading to a higher rate of spontaneous mutations and hence an increased rate of drug resistance, is not relevant.

3.6 Conclusion

In conclusion, we could find no evidence that the triphosphate, active forms of 3TC or AZT resulted in an increased mutation rate in *M. smegmatis,* suggesting that use of nucleoside

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reverse transcriptase inhibitors in the treatment of tuberculosis does not result in higher rates of resistance.

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Chapter 4 Characterisation of *in vitro*-generated fluoroquinolone-resistant *Mycobacterium tuberculosis* mutants

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4.1 Abstract

Fluoroquinolones and second-line injectables form the backbone of the multidrug-resistant (MDR) tuberculosis (TB) treatment regimen and resistance to these drugs defines extensive drug resistance (XDR). Resistance to fluoroquinolones and second-line injectables are mediated mainly by mutations in *gyrA* and *rrs*, genes encoding enzymes central to metabolism. The impact of such mutations on the physiology of *Mycobacterium tuberculosis* have not been studied. Studying the effects of such mutations is hampered by the lack of monoresistant clinical isolates without concomitant drug resistance mutations which are matched to an isogenic progenitor. The aim of this study was thus to select fluoroquinolone-monoresistant *M. tuberculosis* mutants *in vitro* from a pan-susceptible progenitor and characterise them with respect to mutation. Results showed that resistant mutants originating from a Beijing clinical isolate were more likely to harbour *gyrA/gyrB* mutations conferring high-level drug resistance to ofloxacin than the laboratory strain H37Rv, suggesting that epistasis between strain background and drug resistance mutations could contribute to the association of the Beijing strain with drug resistance. Furthermore, prevalence of mutations could be correlated with growth rate in the presence of antibiotic, suggesting that either the fitness cost of or the level of resistance imparted by a drug resistance mutation impacts the success of certain mutants in drug-resistant TB.

4.2 Introduction

Resistance to at least one fluoroquinolone and a second-line injectable (aminoglycosides such as amikacin, kanamycin and capreomoycin) (Zumla *et al.* 2015), forms the basis for definition of XDR-TB, where XDR-TB is defined as MDR-TB with added resistance to these drugs (World Health Organization 2015). Fluoroquinolones and second-line injectables form the backbone of the MDR-TB regimen and resistance to these drugs has serious implications in that it becomes difficult to construct an efficient regimen that can be tolerated by the patient (Falzon *et al.* 2013; World Health Organisation 2008).

Resistance to fluoroquinolones can mainly be attributed to mutations in the genes encoding subunits of the DNA gyrase enzyme of *Mycobacterium tuberculosis*, namely *gyrA* and *gyrB* (Maruri *et al.* 2012), although a large proportion of isolates have unknown mechanisms associated with resistance (Devasia *et al.* 2012). Efflux pump induction (Sun *et al.* 2014) and DNA mimicry (Hegde *et al.* 2005) have been suggested as possible mechanisms. Recently, a

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mutation in *eccC5* was shown to confer fluoroquinolone resistance (Eilertson *et al.* 2016). Resistance to aminoglycosides can be conferred by various mutations in various genes (see Chapter 1). However, amikacin or kanamycin are the aminoglycosides of choice when building a treatment regimen for MDR-TB, (World Health Organisation 2008) and resistance to these drugs are mediated mainly by the A1401G mutation in the 16S rRNA gene *rrs* (Georghiou *et al.* 2012).

Nothing is currently known about the impact of mutations in DNA gyrase or *rrs* on the physiology of *M. tuberculosis.* Mutations in enzymes central to metabolism, involved in DNA supercoiling or translation (Musser 1995), indicates the possibility of a profound negative effect on the organism's physiology. Furthermore, there are cases where drug resistance mutations have conferred an advantage, for example, the *gyrA* S83L mutation increased survival of *Escherichia coli* in macrophages (Miskinyte & Gordo 2013), a *gyrA* Asp87Gly mutant of *Salmonella enterica* acquired multidrug resistance and potentially an increased ability to survive under stress conditions (Webber *et al.* 2013), while mutations in *gyrA* were associated with increased virulence in *Pseudomonas aeruginosa* (Agnello & Wong-Beringer 2012).

We therefore hypothesised that acquisition of mutations conferring resistance to fluoroquinolone or aminoglycoside may change the physiology of *M. tuberculosis.* This change may impact the way the organism responds to stress, such as third-line antibiotic treatment, survival within the host and transmission. Understanding the physiology of XDR-TB may provide information on ideal drug treatment regimens.

An obstacle to studying the influence of *gyrA/gyrB/rrs* mutations on the physiology of *M. tuberculosis* lies in the lack of easily attainable monoresistant clinical isolates. Because fluoroquinolones and aminoglycosides are used to treat MDR-TB, clinical isolates exhibiting stepwise evolution of susceptibility to resistance generally harbour other drug resistance mutations, with the exception of fluoroquinolone-monoresistant isolates from patients exposed to fluoroquinolones prior to TB diagnosis (Van der Heijden *et al.* 2013). The presence of first-line drug resistance mutations may determine how the *gyrA/gyrB/rrs* mutation affects the physiology of the bacillus. In order to study the impact of only one drug resistance mutation in isolation, we therefore used a pan-sensitive clinical isolate of *M. tuberculosis* from which we selected *gyrA* and *rrs* mutations *in vitro.*

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The overall aim of our work was to study the impact of a mutation conferring fluoroquinolone or aminoglycoside resistance on the physiology of *M. tuberculosis.* In this chapter, we focus on the selection and characterisation of ofloxacin- (as a representative fluoroquinolone) monoresistant mutants from a pan-sensitive *M. tuberculosis* clinical isolate as a progenitor.

4.3 Materials and methods

4.3.1 Preparation of stocks for drugs

Ofloxacin was made up by first dissolving in 1 part 0.1 N NaOH and then adding 9 parts H_2O. The stock concentrations were 2.5 and 4 mg/ml respectively. Aliquots of the stock solution were stored at -20°C.

4.3.2 General culturing of *M. tuberculosis* isolates

M. tuberculosis isolates were either cultured in liquid medium, consisting of 7H9 supplemented with ADC, 0.05% Tween-80 and 0.2% glycerol, or on solid medium, consisting of 7H10 supplemented with ADC and 0.5% glycerol, at 37°C. ADC was prepared as a 10x solution as follows: 5% bovine serum albumin (w/v), 2% glucose monohydrate (w/v) and 0.2% of an aqueous suspension of bovine liver catalase from Sigma-Aldrich (catalogue number C3155).

Growth of the liquid cultures was measured as the optical density (OD) of the culture at 600 nm absorbance.

All cultures were confirmed to be mycobacteria and free of contamination with other bacteria by streaking on blood agar followed by incubation at 37°C or visualisation using Ziehl-Neelsen staining and light microscopy.

4.3.3 Strains used to select spontaneous mutants

Mutants resistant to ofloxacin were selected from a pan-susceptible clinical isolate belonging to the Beijing family (K636) as well as from H37Rv.

4.3.4 Generation of spontaneous mutants

M. tuberculosis mutants were selected according to the method of Morlock *et al.* (2000). Briefly, H37Rv or K636 were cultured in liquid media to an OD_{600} of ~0.8 to 1 and then subcultured in liquid media to an OD_{600} of 0.8. At this OD, these strains were previously

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determined, by dilution plating on Middlebrook 7H10 supplemented with ADC in our laboratory, to contain 10^8 cells/ml. The culture was then diluted to 10^3 cells/ml, a density at which the likelihood of pre-existing mutants would be very low. This diluted culture was then aliquoted into ninety to one hundred parallel cultures of 5 ml each, which were left to grow for 28 days at 37°C.

4.3.5 Selection of *M. tuberculosis* spontaneous mutants

After 28 days, the parallel cultures were centrifuged for 10 minutes at 1811x g in a benchtop centrifuge. The pellets were then resuspended in 2 ml 0.5% Tween-80:H_2O and the centrifugation step repeated. One milliliter of the supernatant was then discarded, the pellet resuspended, and 100 µl of this suspension spread onto plates containing critical concentration (2 µg/ml) ofloxacin (World Health Organisation 2008b). The plates were incubated for 32 days. Colonies representing resistant mutants were picked from the plates, their sizes relative to one another recorded and inoculated into 5 ml liquid media. When the cultures had grown to high turbidity, 1 ml of each culture was stored at -80°C and ~200 µl was boiled at 90 to 100°C for 25 minutes. The mutation conferring resistance was determined by PCR amplification and sequencing of the *gyrA* quinolone resistance-determining region (QRDR). Where strains exhibited no mutation in the *gyrA* QRDR, the *gyrB* QRDR was amplified and sequenced.

4.3.6 Calculation of fitness score

A fitness score associated with each mutation was calculated as a crude measure of the relative fitness associated with such a mutation. This method was the author’s own work. Briefly, colonies were assigned relative sizes as a measure of growth in the presence of ofloxacin, i.e. very small = 1, small = 2, small to medium = 3, medium = 4, medium to big = 5, big = 6.

For each mutant (*gyrA* or *gyrB*) a fitness score, where fitness is measured by growth in the presence of ofloxacin, was calculated as $(1n_1+2n_2+3n_3+4n_4+5n_5+6n_6)/y$

Where n_x is the number of colonies with colony size x.

and y = the proportion of each mutant, relative to the total number of mutants, after sequencing.

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4.3.7 PCR and sequencing of *gyrA* and *gyrB*

Primer sequences and annealing temperatures are shown in Table 4.1.

Each reaction contained 1x Q-solution, 1x buffer, 3.5 mM Mg^{2+}, 1.6 mM dNTPs, 1 µM of each primer, 0.025 units/µl of HotStar Taq DNA Polymerase (Qiagen) and 1 µl of the supernatant of a boiled culture was used in a 25 µl PCR reaction. The reaction details were as follows:

95°C 15 minutes

94°C for 1 minute, annealing temperature (see Table 4.1) for 1 minute and 72°C for 1 minute for 40 cycles

72°C for 10 minutes

The PCR products were visualized after electrophoretic fractionation in 1% agarose in Tris – borate-EDTA (TBE) buffer and staining with ethidium bromide.

The PCR products were purified and sequenced by the Central Analytical Facility at Stellenbosch University. Sequences were aligned to that of *gyrA/gyrB* of H37Rv, which were obtained from TubercuList (http://tuberculist.epfl.ch) (Lew *et al.* 2011). Alignment was carried out using BioEdit Alignment Sequence Editor (Hall 1999).

Table 4.1 Primers

Name	**Sequence**	**Annealing temperature (°C)**
***M. tuberculosis gyrA* QRDR**		
GyrA FOR	5’-TGACATCGAGCAGGAGATGC-3’	62
GyrA REV	5’-GGGCTTCGGTGTACCTCATC-3’	
***M. tuberculosis gyrB* QRDR**		
GyrB_F2	5'-GTATCGCGGCACGTAAGG-3'	53
GyrB_R2	5'-GCCACTTGAGTTTGTACAGC-3'	

4.3.8 Statistical analyses

The Fisher’s exact test (two-sided) was used to calculate whether the frequency of each mutation differs significantly between H37Rv and Beijing. This test was carried out using GraphPad Prism 5.

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4.3.9 Mutation rate assay

K636 was grown in liquid media to an optical density at 600 nm of 0.8. The culture was then diluted 16-fold, which should yield about 10^6 cells/ml (see section 4.3.4). The culture was then further diluted 10^4-fold and from this 15 parallel cultures of 5 ml each were aliquoted and incubated at 37°C for 28 days. A number of cells equivalent to the initial inoculum into each flask, was spread out onto solid media containing critical concentration of antibiotic (2 µg/ml ofloxacin) to confirm the absence of pre-existing mutants. After 28 days, 10 out of the 15 cultures were centrifuged at 1811x g for 10 minutes at room temperature, the supernatant discarded and the pellets resuspended into 300 µl 0.5% Tween-80. The entire volume of each flask was spread onto one plate of solid media containing critical concentration of antibiotic (2 µg/ml ofloxacin). Half of the total culture was subcultured on solid media containing antibiotic. For the remaining 5 cultures, the final population size was determined by preparing a tenfold dilution series from each culture, using an aliquot that was a defined portion of the culture, and spreading the 10^{-6} to 10^{-9} dilutions onto solid media without antibiotics. The plates were incubated for 28 days at 37°C and the colonies counted.

4.3.10 Calculation of mutation rate

The Ma-Sandri-Sarkar maximum likelihood method was employed to calculate the mutation rate, using the web-based application available at www.mitochondria.org/protocols/FALCOR.html (Hall *et al.* 2009). The average total population size (Nt) across 5 cultures was employed as the value of Nt for each value of number of mutants (r). Where only a portion of the culture was spread onto antibiotic-containing plate, the following equation was used to correct the number of mutation events for plating efficiency: $m_{act}=m_{obs}((z-1)/z \ln z)$, where m_{act} is the actual number of mutation events, m_{obs} is the number of mutation events observed, z is the fraction of the culture plated.

4.4 Results and Discussion

4.4.1 Mutations selected *in vitro* during the evolution of ofloxacin resistance

Ofloxacin-resistant mutants were selected from both H37Rv and a Beijing strain commonly observed in the TB epidemic in the Western Cape, South Africa, namely K636. As shown in Table 4.2, ten different types of mutations were selected for. All these mutations are also commonly observed in clinical strains of *M. tuberculosis* (Maruri *et al.* 2012). However, the widely reported Gly88Ala mutation in *gyrA* was not observed *in vitro*.

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Table 4.2 Prevalence of mutations selected on ofloxacin in H37Rv and a Beijing strain.

Mutation	Number of colonies (%)[a]		p value[b]
	H37Rv	Beijing	
gyrA Gly88Cys	9 (10)	7 (8)	0.7946
gyrA Ala90Val	20 (22)	10 (11)	0.0715
gyrA Ser91Pro	6 (7)	1 (1)	0.1179
gyrA Asp94Ala	1 (1)	0 (0)	1.000
gyrA Asp94Asn	5 (5)	10 (11)	0.1871
gyrA Asp94Gly	16 (17)	9 (10)	0.1972
gyrA Asp94His	6 (7)	12 (13)	0.1423
gyrA Asp94Tyr	17 (18)	41 (46)	0.0001***
All codon 94	45 (49)	72 (80)	< 0.0001***
gyrB Asp461Asn	1 (1)	0 (0)	1.000
gyrB Asp461His	11 (12)	0 (0)	0.0007***
All *gyrB*	12 (13)	0 (0)	0.0003***

[a]Percentage that specific mutation makes up of the total number of isolates selected per strain.

[b]The Fisher's exact test (two-sided) was used to calculate whether the proportion of each mutation differs significantly between H37Rv and Beijing. Significant values are indicated by asterisks ($p < 0.05$).

The reason for not observing the Gly88Ala mutation can be attributed to the MIC generally associated with this mutation (Maruri *et al.* 2012; Matrat *et al.* 2006). This mutation seems to confer low-level resistance with an MIC of 2 µg/ml for ofloxacin. Since the concentration of 2 µg/ml ofloxacin was used to select mutants, this would have inhibited the growth of this mutant. Similarly, the low prevalence of certain mutations, such as Asp94Ala, Ser91Pro and certain *gyrB* mutations, may be attributed to the fact that, in clinical isolates, their MICs are close to 2 µg/ml (Maruri *et al.* 2012; Zhang *et al.* 2014). This may mean that 2 µg/ml ofloxacin represents a borderline concentration, at which these mutants survive less well, and they are therefore selected against. In order to observe a wider spectrum of mutants, the selection concentration could be lowered to 1 µg/ml. This concentration may be a better measure of the critical concentration, as was shown by others (Angeby *et al.* 2010).

We did not obtain any clones without mutations in the quinolone resistance-determining region (QRDR) of *gyrA* or *gyrB*, that were resistant to ≥ 2 µg/ml ofloxacin. However, in

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previous analyses of fluoroquinolone-resistant clinical isolates, up to 58% did not harbour a mutation in the QRDR of *gyrA* or *gyrB* (Ginsburg *et al.* 2003). This may be because mechanisms of resistance other than *gyrAB* mutations only confer low-level resistance of a two- to fourfold increase in MIC over wild-type (Kocagöz *et al.* 1996). Therefore lowering the selection concentration to 1 µg/ml ofloxacin in our study may lead to the selection of wild-type isolates. However, wild type isolates with MICs > 2 µg/ml have also been observed (Duong *et al.* 2009), which suggests that this is not the only reason. It may be that wild-type isolates with high MICs occur at very low frequencies, therefore increasing the numbers of mutants selected may lead to the selection of such mutants. Lastly, *in vitro* mutants are not subjected to the same conditions as those within the host, which may further affect the type of resistance mechanism selected for. Bottleneck events in the host (Warner 2010) may lead to the selection of rare, low-fitness mutations that would not be detected *in vitro.*

Unexpectedly, there were significant differences in the spectrum of mutations selected when H37Rv was compared to Beijing, as shown in Table 4.2. Almost half (46%) of the Beijing isolates harboured a *gyrA* Asp94Tyr mutation compared to only 18% in H37Rv ($p = 0.0001$). Similarly, no *gyrB* mutations were observed against a Beijing background, while the *gyrB* Asp461His mutation occurred in 12% of H37Rv isolates ($p = 0.0007$). Mutations in codon 94 were more likely to be selected in the Beijing strain compared to H37Rv ($p < 0.0001$), while *gyrB* mutations were more likely to be selected in H37Rv ($p = 0.0003$). These results are intriguing, since they may suggest that genetic background influences the selection of drug resistance mutations. An interaction between mutations is termed epistasis (Borrell & Gagneux 2011), which has been suggested to play a role in the emergence and spread of drug-resistant tuberculosis. Indeed, it has been shown that certain lineages of *M. tuberculosis* favour certain isoniazid resistance-conferring mutations (Fenner *et al.* 2012). Moreover, this has also been shown for *gyrA*, in isolates known to stem from different epidemics - thereby eliminating the possibility of clonal expansion of one mutation - where Beijing strains were more likely to harbour codon 94 mutations in *gyrA* (Duong *et al.* 2009; Mokrousov *et al.* 2008). Interestingly, in Vietnam (Duong *et al.* 2009), Beijing strains were shown to be more likely to have high-level fluoroquinolone resistance, together with being more likely to harbour codon 94 mutations. In addition, a meta-analysis (Maruri *et al.* 2012) has shown that codon 94 mutations in general lead to higher level fluoroquinolone resistance in *M. tuberculosis.* This leads to the possibility that Beijing strains favour mutations that confer high-level resistance, which may contribute to its association with the MDR-TB epidemic

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(see Chapter 2). Fenner *et al.* (2012) have also shown this for isoniazid, where Lineage 2 (which includes Beijing strains) was more likely to harbour the *katG* S315T mutation and less likely to harbour *inhA* promoter mutations. The first confers high-level isoniazid resistance, while the latter confers low-level (Fenner *et al.* 2012).

We also observed that colonies selected on ofloxacin varied in size. We therefore recorded the relative sizes of various colonies to see if there was any correlation between the size and the type of mutation. The size of the colony could be linked to the growth rate of a particular mutant in the presence of antibiotic and therefore its ability to survive (*in vitro* fitness) in the presence of antibiotic. A fitness score was calculated for each mutant (Figure 4.1).

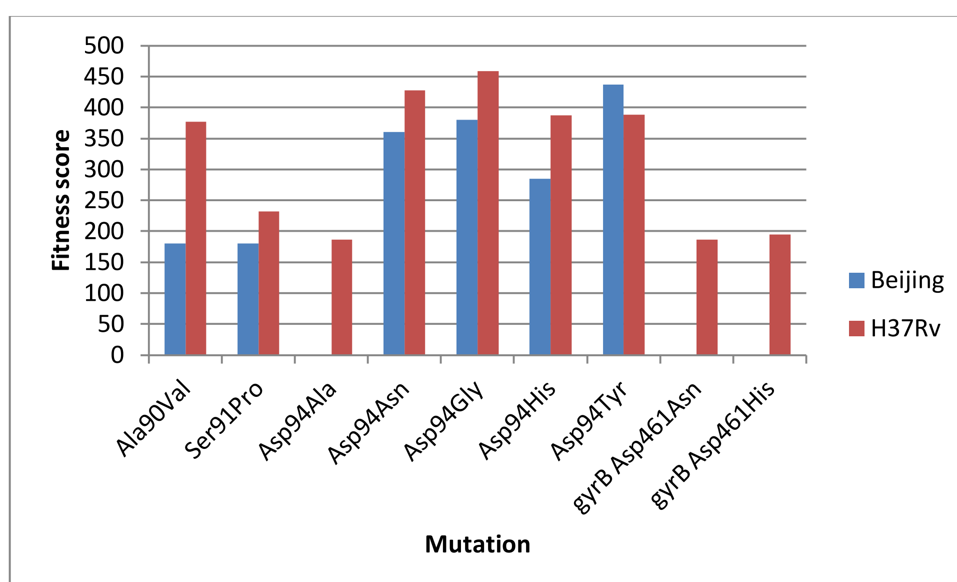

Figure 4.1 Overall fitness scores for different mutations selected on 2 µg/ml ofloxacin

Mutations in codon 94 of *gyrA* (except for the Asp94Ala mutation) resulted in higher growth in the presence of antibiotic and this was the case for both strains. In general, colonies that were better able to adapt to antibiotic pressure, as evidenced by larger colony sizes, also occurred more frequently (see Table 4.2). For example, the *gyrA* Asp94Tyr mutation in the Beijing strain had the highest fitness score and was also the mutation selected most frequently. Mutations with low fitness scores also had low frequencies; for example, the *gyrA* Ser91Pro mutation was the sixth-highest in frequency in both strains and had the sixth- to seventh-highest fitness scores respectively. However, there were some outliers. The *gyrB* Asp461His mutation which occurred 12 times more often than the *gyrA* Asp94Ala and *gyrB* Asp461Asn mutations in H37Rv, had a similar fitness score (194 vs 186) to these mutations.

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Also, the *gyrA* Ala90Val mutation occurred 11 times more often in the Beijing strain than the *gyrA* Ser91Pro mutation, yet had an identical fitness score.

Our study differs from more traditional fitness assays which measure the relative fitness cost as a growth deficit when compared with a wild type progenitor of the mutant, competitively or non-competitively, in the absence of antibiotics *in vitro* (Billington *et al.* 1999; Mariam *et al.* 2004; Sander *et al.* 2002; Spies *et al.* 2013; Von Groll *et al.* 2010). In these studies it has been shown that the fitness cost of a particular type of mutant can be negatively correlated with the frequency of occurrence of that mutant amongst clinical isolates (Shcherbakov *et al.* 2010). However, this does not take into account the relative ability of various mutants resistant to the same antibiotic to grow in the presence of that antibiotic. Different mutants resistant to the same antibiotic may have different levels of resistance, which, in addition to the inherent fitness cost imparted by the mutation, may also impact the ability of the mutant to survive. In our study, we did not perform competition assays to assess the fitness of the mutants in the absence of the antibiotic, therefore we do not know whether the fitness cost also determined the relative ability of mutants to survive in the presence of antibiotic, or whether this was as a result of the level of resistance imparted by the mutation. This is a limitation of our study. However, it was clear that different *gyrA* mutants did not grow at the same rate in the presence of ofloxacin and that there was some correlation of growth rate with the frequency at which these mutants was observed *in vitro*.

4.4.2 The rate of mutation to ofloxacin resistance

The rate of mutation for the Beijing strain employed in these studies to resistance to 2 µg/ml ofloxacin was estimated as 1.14×10^{-8} (range 0.83 to 1.48×10^{-8}). The number of mutation events and the total population count used to calculate the rate as well as 95% confidence intervals are shown in Table 4.3. The rate of mutation to ofloxacin resistance is about double that reported for other fluoroquinolones in *M. fortuitum* (Gillespie *et al.* 2005).

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Table 4.3 Mutation rate of a clinical Beijing strain to ofloxacin resistance

Antibiotic	Number of mutation events	Total population	Mutation rate (per 10^9 bacteria)	Upper difference (95% CI)	Lower difference (95% CI)
Ofloxacin[a]	32	2.79×10^9	11.42	34.18	30.74

[a]2 µg/ml was the concentration employed for selection of mutants.

4.5 Conclusion

In conclusion, we were able to select ofloxacin-resistant mutants harbouring mutations commonly observed in clinical isolates. There were significant differences in the distribution and prevalences of ofloxacin resistance-conferring mutations between H37Rv and Beijing, suggesting epistatic interactions between strain background mutations and *gyrA/B* mutations and suggesting the possibility that Beijing strains are more prone to acquisition of mutations that confer high-level resistance to ofloxacin. There was a correlation between prevalence of *gyrA/B* mutations and growth rate in the presence of ofloxacin. This suggests differing abilities for specific mutants to tolerate ofloxacin, which could be ascribed to either the fitness cost incurred by acquisition of the mutation or the level of resistance imparted as a result of the mutation.

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Chapter 5 Moxifloxacin retains anti-mycobacterial activity in the presence of *gyrA* mutations

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5.1 Abstract

Moxifloxacin-resistant *Mycobacterium tuberculosis* mutants were selected *in vitro* using different concentrations of moxifloxacin. *gyrA* mutations at codons 88 and 94 were associated with resistance (defined as an MIC of ≥ 2 g/ml) (P < 0.0001 and P=0.0053, respectively). Despite the presence of *gyrA* mutations, moxifloxacin significantly impedes bacterial growth, supporting its use for the treatment of ofloxacin-resistant *M. tuberculosis*.

5.2 Manuscript text

Moxifloxacin, a third-generation fluoroquinolone (World Health Organisation 2011), is significantly more potent than earlier generation fluoroquinolones, such as ofloxacin, which has been widely used in treatment of tuberculosis (TB) (World Health Organisation 2010). Moxifloxacin shows improved *in vitro* activity against ofloxacin susceptible *Mycobacterium tuberculosis* (Angeby *et al.* 2010). Cross-resistance exists between fluoroquinolones, and mutations that emerge under ofloxacin treatment are usually associated with moxifloxacin minimum inhibitory concentrations (MICs) above the WHO-defined critical concentration of 0.25 µg/ml (World Health Organisation 2008b). However, 84-98% of isolates with mutations conferring fluoroquinolone resistance have MICs of ≤ 2 µg/ml for moxifloxacin, which does not necessarily reflect clinical resistance (Angeby *et al.* 2010; Cheng *et al.* 2004; Kam *et al.* 2006; Nosova *et al.* 2013; Sirgel *et al.* 2012; Von Groll *et al.* 2009). Based on this, an alternative critical concentration of 2 µg/ml has been suggested (Kam *et al.* 2006; Poissy *et al.* 2010; Sirgel *et al.* 2012).

The WHO has recommended substituting moxifloxacin for ofloxacin in the treatment of multidrug-resistant TB (MDR-TB), defined as being resistant to at least isoniazid and rifampicin, and extensively drug-resistant TB (XDR-TB), defined as MDR with additional resistance to a fluoroquinolone and an injectable (Sirgel *et al.* 2012; World Health Organisation 2008a). Moxifloxacin as a first-line drug to treat susceptible TB is also currently undergoing trials (Clayden P *et al.* 2012; Conde *et al.* 2009) and has shown promise to shorten treatment duration (Conde *et al.* 2009). Since the use of moxifloxacin for the treatment of TB is not yet widespread, it is not clear which mutations could emerge. Mutations that facilitate increased moxifloxacin resistance (MICs ≥ 2 µg/ml) have clinical relevance, given that the current standard dose leads to serum concentrations of 1.3 to 6.9 µg/ml (Almeida *et al.* 2007; Kam *et al.* 2006; Manika *et al.* 2012; Poissy *et al.* 2010; Sirgel *et al.* 2012). Knowledge of such mutations is critical to optimize rapid molecular methods for

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the detection of moxifloxacin resistance. This study therefore aimed to identify spontaneous moxifloxacin resistant mutants selected in the presence of ≥ 2 µg/ml moxifloxacin.

A pan-susceptible *M. tuberculosis* Beijing genotype strain was used as progenitor for the selection of a wide range of independent mutants according to Morlock *et al.* (2000). Briefly, four sets of thirty to thirty-five low-inoculum parallel cultures were set up in enriched (supplemented with albumin-dextrose-catalase, 0.2% glycerol and 0.05% Tween-80) 5 ml 7H9 medium and cultured at 37°C for 28 days. Subsequently, the entire culture was plated on 7H10 media (supplemented with albumin-dextrose-catalase and 0.5% glycerol) containing 3 and 4 µg/ml moxifloxacin, while approximately 10% of the culture was plated onto 7H10 containing 0.5 and 2 µg/ml moxifloxacin. Where possible, two colonies were picked per plate and subcultured in 5 ml enriched 7H9. The *gyrA* gene was subsequently amplified and sequenced from boiled aliquots of the respective cultures (Sirgel *et al.* 2012). For isolates with a wild-type *gyrA* gene, the *gyrB* was amplified and sequenced using primers 5'-GTATCGCGGCACGTAAGG-3' and 5'-CCACTTGAGTTTGTACAGC-3' using a similar protocol as for *gyrA* (Sirgel *et al.* 2012), except that the annealing temperature was adjusted to 53°C.

For 0.5, 2, 3 and 4 µg/ml moxifloxacin, 56, 55 70 and 69 colonies were selected and analysed respectively. Analysis of sequencing results identified 9 *gyrA* and 4 *gyrB* mutations (Table 5.1). Two novel mutations, *gyrA* Ala90Lys (GCG/AAG) and Asp94Lys (GAC/AAA), were identified. Isolates harbouring mutations in codons 89 and 90 of *gyrA* or any codon of *gyrB* were selected only on media that contained 0.5 µg/ml of moxifloxacin. *GyrA* Gly88Cys and codon 94 mutations were associated with exposure to media containing ≥ 2 µg/ml moxifloxacin ($p < 0.0001$ and $p=0.0053$, respectively), which are similar to those observed in a previous study (Ginsburg *et al.* 2005). The proportion of clones with a Gly88Cys mutation increased as the concentration of moxifloxacin increased. The association of this mutation with resistance to new-generation fluoroquinolones was also shown in work on DC-159a (Sekiguchi *et al.* 2011).

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Table 5.1 Comparison between mutants selected on 0.5 µg/ml vs. ≥ 2 µg/ml moxifloxacin *in vitro*

Mutation		Data for moxifloxacin at concn (µg/ml) of:						
Gene	Amino acid change	0.5 µg/ml	2 µg/ml		3 µg/ml		4 µg/ml	
		N (%)[a]	N (%)	P-value[b]	N (%)	P-value	N (%)	P-value
gyrA	Gly88Cys	0 (0.0)	32 (58.2)	**< 0.0001**	48 (68.6)	**< 0.0001**	61 (88.4)	**< 0.0001**
gyrA	Asp89Asn	1 (1.8)	0 (0.0)	1	0 (0.0)	0.4444	0 (0.0)	0.4480
gyrA	Ala90Lys	1 (1.8)	0 (0.0)	1	0 (0.0)	0.4444	0 (0.0)	0.4480
gyrA	Ala90Val	1 (1.8)	0 (0.0)	1	0 (0.0)	0.4444	0 (0.0)	0.4480
gyrA	Asp94Asn	5 (8.9)	10 (18.2)	0.1757	14 (20.0)	0.1315	7 (10.1)	1
gyrA	Asp94Gly	5 (8.9)	3 (5.5)	0.7163	4 (5.7)	0.5093	0 (0.0)	**0.0163**
gyrA	Asp94His	3 (5.4)	1 (1.8)	0.6182	0 (0.0)	0.0852	0 (0.0)	0.0872
gyrA	Asp94Lys	0 (0.0)	0 (0.0)	N/A[c]	0 (0.0)	N/A	1 (1.4)	1
gyrA	Asp94Tyr	14 (25)	9 (16.4)	0.3497	4 (5.7)	**0.0038**	0 (0.0)	**< 0.0001**
gyrB[d]	Asn499Asp	2 (3.6)	0 (0.0)	0.4955	0 (0.0)	0.1956	0 (0.0)	0.1987

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gyrB	Asn499Lys	17 (30.4)	0 (0.0)	**< 0.0001**	0 (0.0)	**< 0.0001**	0 (0.0)	**< 0.0001**
gyrB	Thr500Asn	6 (10.7)	0 (0.0)	**0.0271**	0 (0.0)	**0.0066**	0 (0.0)	**0.0069**
gyrB	Glu501Asp	1 (1.8)	0 (0.0)	1	0 (0.0)	0.4444	0 (0.0)	0.4480
Total		56	55		70		69	

[a]Percentage of all mutants selected at the indicated moxifloxacin concentration; [b]Significance of proportion compared to that at 0.5 µg/ml by Fisher's exact test, values in bold type are significant ($p < 0.05$); [c]N/A, not applicable; [d]Numbering for *gyrB* according to Maruri *et al.* (2012)

In our study, clones with the Gly88Cys mutation were not observed at 0.5 µg/ml moxifloxacin, suggesting a high fitness cost associated with this mutation. Conversely, clones with codon 94 mutations were observed on all moxifloxacin concentrations tested, suggesting a low fitness cost of these mutations. This may explain why codon 94 mutations but not Gly88Cys were selected during moxifloxacin treatment of mice (Almeida *et al.* 2007; Ginsburg *et al.* 2005) or emerged after treatment of patients with new-generation fluoroquinolones (which included moxifloxacin) prior to diagnosis of TB (Van der Heijden *et al.* 2013). Interestingly, the *gyrA* Asp94Asn mutation emerged in mice treated with moxifloxacin at a dose which produced similar pharmacodynamic/pharmacokinetics in patients (Almeida *et al.* 2007), while in our study, this mutation was observed at equal proportions across all concentrations.

As a next step, we compared the mutation rate of moxifloxacin to ofloxacin. A fluctuation assay was set up according to Morlock *et al.* (2000). Briefly, two sets of 15 parallel 7H9 cultures of the Beijing genotype strain were grown for 28 days at 37°C and, for ten of these, the entire culture was plated on 7H10 media containing 2 µg/ml of either ofloxacin or moxifloxacin. The average final population size was determined using the other 5 cultures after plating a tenfold dilution series in 0.5% Tween-80 on 7H10 media without antibiotic. Mutation rate was calculated using the Ma-Sandri-Sarkar-maximum likelihood method (Hall *et al.* 2009). The spontaneous mutation rates for moxifloxacin and ofloxacin resistance were within the same logarithmic range (2.3 and 1.1 per 10^8 bacteria, respectively). This suggests that resistance to new fluoroquinolones is attained as easily as resistance to older fluoroquinolones.

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However, in the first experiment (Table 5.1) colonies with a reduced diameter were observed on solid media containing 2, 3 and 4 µg/ml moxifloxacin compared with 0.5 µg/ml, suggesting that *gyrA* mutants are partially susceptible to ≥ 2 µg/ml moxifloxacin. To further investigate this, we monitored the growth rate of isolates habouring *gyrA* Gly88Cys, Asp94Asn, Asp94Tyr, Asp94His and Asp94Gly mutations, cultured in stationary cultures containing enriched 7H9 and in the absence or presence of 2 µg/ml ofloxacin or moxifloxacin. We also monitored the growth of the progenitor strain without antibiotic. The mutants were selected randomly. For each isolate, a frozen 1ml stock was inoculated into 9ml enriched 7H9. When growth reached an optical density (OD) of 0.8 to 1.0, the starter cultures were diluted to an optical density of 0.05 in a total of 18ml enriched 7H9 with or without the respective antibiotics. The optical density of each culture was measured daily at 600 nm, using a visible light spectrophotometer. Each experiment was done in triplicate. Figure 5.1 shows that the growth of all mutants was significantly impeded by moxifloxacin but not by ofloxacin ($p < 10^{-5}$) using the double repeated measures analysis of variance statistical test Figure 5.1(A) to (E)). The isolate with a Gly88Cys mutation was the least susceptible to moxifloxacin Figure 5.1(A)), followed by the isolate with an Asp94Asn mutation in *gyrA* (Figure 5.1(C)). The remaining three mutants were almost completely inhibited.

In conclusion, *gyrA* Gly88Cys and Asp94Gly/His/Tyr/Asn/Lys mutations are associated with high-level moxifloxacin resistance *in vitro* and may confer reduced susceptibility to moxifloxacin in patients treated with this drug. Rapid molecular drug resistance testing should therefore include these mutations. Encouragingly, however, reduced growth rates *in vitro* of mutants in 2 µg/ml moxifloxacin suggests that growth of these mutants in patients is likely to be suppressed at attainable moxifloxacin serum concentrations (Almeida *et al.* 2007; Kam *et al.* 2006; Manika *et al.* 2012; Poissy *et al.* 2010; Sirgel *et al.* 2012). This supports a previous meta-analysis, which showed that moxifloxacin was still effective for the treatment of XDR-TB despite the presence of fluoroquinolone resistance (Jacobson *et al.* 2010).

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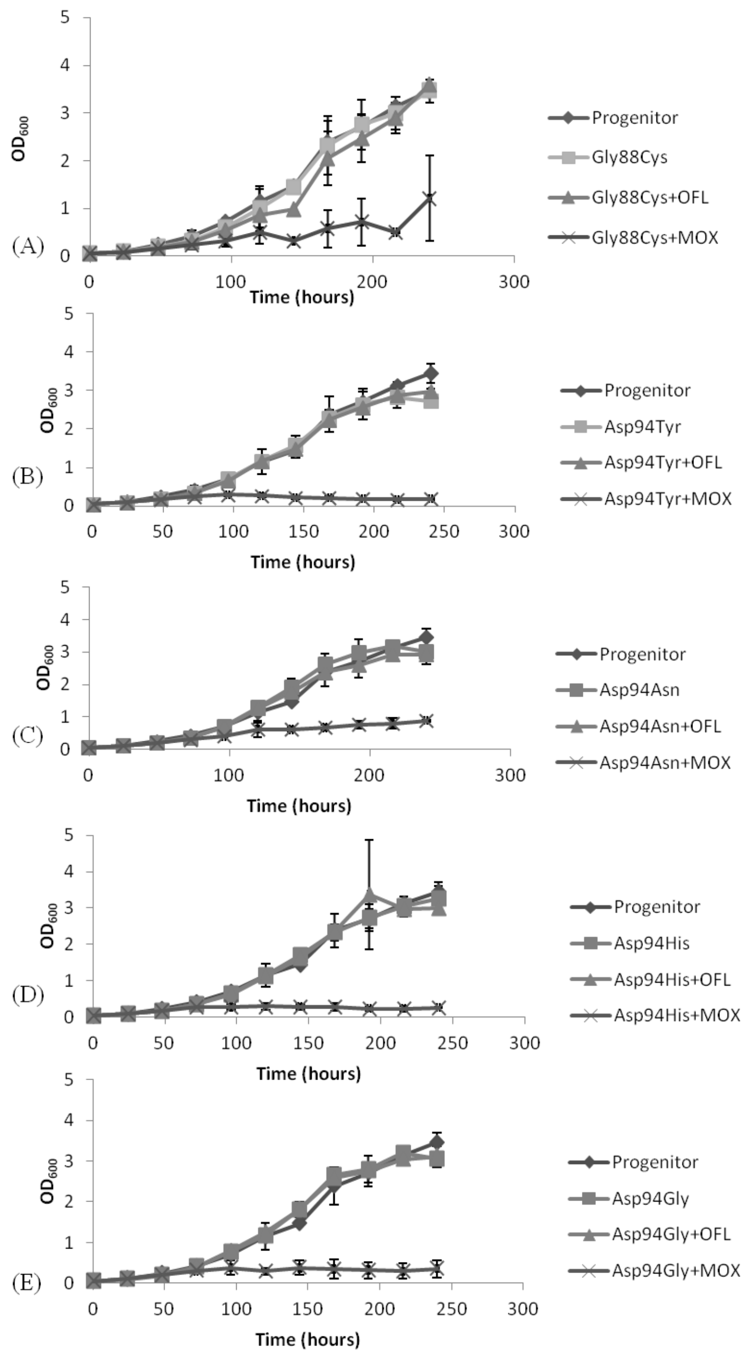

Figure 5.1 Comparison of growth of fluoroquinolone-resistant *M. tuberculosis gyrA* mutants and their sensitive progenitor in the presence and in the absence of antibiotics. (A) Gly88Cys (B) Asp94Tyr (C) Asp94Asn (D) Asp94His (E) Asp94Gly. For each culture, the optical density at 600 nm was measured daily in three independent experiments, and the average was plotted. MOX, moxifloxacin; OFL, ofloxacin. Error bars indicate standard deviations.

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Chapter 6 Mechanisms modulating the level of fluoroquinolone resistance

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6.1 Abstract

Drug resistance in *Mycobacterium tuberculosis* is associated with mutations in drug target genes and in the case of fluoroquinolones, with mutations in *gyrA* or *gyrB*. However, the level of drug resistance as measured by minimum inhibitory concentration (MIC) is not purely a product of *gyrA* or *gyrB* mutation, but is modulated by other factors, such as efflux. The aim of this study was therefore to increase the MIC of a fluoroquinolone-resistant clone of *M. tuberculosis* by selection of spontaneous mutants on higher concentrations of fluoroquinolone and to characterise the resistance mechanism involved. Sequencing of *gyrA* and *gyrB* identified additional mutations suggesting that double mutations are responsible for increasing the MIC. No involvement of efflux pump activity in modulating the MIC was detected in our *in vitro* mutants, suggesting that other, additional factors may play a role in acquisition of drug resistance *in vivo*.

6.2 Introduction

The prevailing dogma regarding drug resistance in *Mycobacterium tuberculosis* is that drug resistance is a product of mutations in drug target genes (Louw *et al.* 2011; Smith *et al.* 2013). However, this may be an oversimplified view, as is evident from studies on fluoroquinolone-resistant *M. tuberculosis* clinical isolates. A meta-analysis of the mutations encoding fluoroquinolone resistance and their association with minimum inhibitory concentration (MIC) showed a wide range of concentrations required to inhibit growth of isolates harbouring the same drug resistance mutation (Maruri *et al.* 2012). This suggests an interplay of various factors modulating this level of resistance. Furthermore, a recent study (Sun *et al.* 2014), showed that *M. tuberculosis* clinical isolates with ofloxacin MICs ≥ 16 µg/ml exhibited a higher-fold decrease in MIC in the presence of efflux pump inhibitors (reserpine, verapamil and CCCP) when compared to isolates with lower MICs. Furthermore, isolates with low MICs (≤ 2 µg/ml) showed little modulation of MIC by efflux pump inhibitors. From this the authors concluded that higher levels of resistance to ofloxacin may be associated with higher levels of efflux pump induction. Taken together, it suggests that increased efflux could be the main mechanism modulating the level of fluoroquinolone resistance in *M. tuberculosis.*

Higher levels of efflux pump activity could conceivably be due either to mutations in as yet unknown regions of the genome (Singh *et al.* 2011) or increased expression and activity of

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efflux pumps (Chatterjee *et al.* 2013; Louw *et al.* 2011; Willemse 2013). If efflux modulates the level of resistance, the clinical implication would be that efflux pump inhibitors could be investigated as co-treatment with fluoroquinolones to potentiate efficacy of the antibiotic (Gupta *et al.* 2013). We hypothesised that it would be possible to increase the MIC of a fluoroquinolone-resistant isolate of *M. tuberculosis* and that the increase would be related to an efflux mechanism. To test this hypothesis we aimed to further increase the MIC of an already fluoroquinolone-resistant mutant by selection on concentrations of antibiotic higher than its MIC and to characterise the resistance mechanism involved.

6.3 Materials and methods

6.3.1 Strains used in this study

BO1, a *gyrA* Asp94Gly mutant resistant to ofloxacin was generated *in vitro* from the progenitor K636, a pan-sensitive clinical isolate belonging to the Beijing family, and characterised as described in Chapter 4.

6.3.2 Preparation of stocks for drugs

Ofloxacin was made up by first dissolving in 1 part 0.1 N NaOH and then adding 9 parts H_2O. The stock concentration was 2.5 mg/ml. Aliquots of the stock solution were stored at -20°C.

Reserpine and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) stock solutions were made to 4 mg/ml and 8 mg/ml respectively in DMSO and aliquots stored at -80°C. Verapamil hydrochloride stock solution was made up to 8 mg/ml in H_2O and aliquots stored at -80°C.

6.3.3 Determination of BO1 and K636 ofloxacin MIC

The ofloxacin MICs for K636 and BO1 were determined using the automated BACTEC MGIT 960 instrument (BD Biosciences) according to the manufacturer's instructions. *M. tuberculosis* was grown to an OD of 0.4 in liquid media and 100 µl of this inoculated into MGIT tubes supplemented with 10% Oleic Albumin Dextrose Catalase Growth Supplement (OADC). The culture was incubated in the MGIT instrument at 37°C until a positive growth reading was reached. *M. tuberculosis* was then subcultured by addition of 100 µl to a fresh MGIT, supplemented with 10% OADC. When positivity was reached, each MGIT was incubated for a further two days at 37°C. A 1:100 dilution of the culture in sterile saline was prepared and 500 µl of this dilution was inoculated into a new MGIT tube. This MGIT

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culture would serve as the growth control. The undiluted MGIT culture (500 µl) was inoculated into MGIT tubes containing a range of antibiotic concentrations. In the case of the sensitive strain, K636, two fold dilutions from 0.25 to 4 µg/ml were tested, while for BO1, a range of 8 to 32 µg/ml ofloxacin was tested. The MGIT cultures were placed in a BACTEC MGIT 960 instrument and EpiCentre (version 5.75A) TBeXist software (BD Bioscience) was used to monitor the culture growth for approximately 14 days and to analyse the data generated by the MGIT 960 instrument.

6.3.4 Selection of mutants exhibiting increased MICs to ofloxacin

In order to generate fluoroquinolone-resistant mutants with a high MIC (> 16 µg/ml), BO1 was employed as the progenitor strain and mutants were selected according to the method of Morlock *et al.* (2000) with modifications. Briefly, seven parallel B01 cultures were set up in 7H9 media supplemented with ADC. Twenty percent of 3 of the cultures was plated on 7H10 supplemented with OADC, 0.5% glycerol and either 10, 12, 16 or 20 µg/ml ofloxacin. The entire volume of the remaining 4 cultures was spread onto 7H10 supplemented with OADC, 0.5% glycerol and either 24, 28, 32 or 64 µg/ml ofloxacin. After 28 days of incubation at 37°C, one colony was selected off plates containing 16, 20, 28 and 64 µg/ml. After another 28 days of incubation, two colonies were selected each off plates containing 20, 28, 32 and 64 µg/ml. Selected colonies were cultured in 7H9 media supplemented with ADC. An aliquot of each culture was heat inactivated and both *gyrA* and *gyrB* QRDRs were amplified and sequenced as previously described (Section 4.2.7).

6.3.5 Determination of minimum inhibitory concentrations for ofloxacin in the presence of verapamil and CCCP

The same method as detailed above was used to determine the MICs of K636 and BO1 in the presence and absence of efflux pump inhibitors (EPIs) verapamil and CCCP. A diluted growth control was included as above, as well as an undiluted control containing no EPI or drug. An undiluted control containing only EPI was also included. The isolates were assayed across a range of ofloxacin concentrations in the presence or absence of EPI. Ofloxacin concentrations were 0.125, 0.25 and 0.5 µg/ml for K636, and 0.5, 2, 4, 8 and 16 for BO1. These concentrations were chosen as it was hypothesised that the efflux pump inhibitors would lower the MIC. A final concentration of 10 µg/ml verapamil and 4 µg/ml CCCP was used per MGIT, concentrations that have been shown to inhibit efflux in other studies (Eilertson *et al.* 2016; Louw *et al.* 2011; Pule 2013; Singh *et al.* 2011).

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6.3.6 Determination of minimum inhibitory concentrations for ofloxacin in the presence of reserpine

Since reserpine is not compatible with the media in MGIT tubes, we determined the MIC of ofloxacin for K636 and BO1 in the presence and absence of reserpine by the broth microdilution or resazurin microtiter method (Palomino *et al.* 2002). Briefly, two-fold dilutions of ofloxacin were prepared in duplicate generating a concentration ranging from 0.016 to 16 µg/ml for BO1, and from 0.0005 to 0.5 µg/ml for K636. Controls included: one well containing liquid media only, one well containing ofloxacin diluent (0.01N NaOH) only, and 11 wells containing only cells without drug. Two fold dilutions of the drug diluent similar to the drug dilutions, were also done in a single replicate. Therefore where a dilution series of the diluent was done in the presence of reserpine, this represented a control assessing the possible inhibitory effect of reserpine. This assay as described was done in the presence and absence of 80 µg/ml reserpine, a concentration shown to inhibit efflux previously (Louw *et al.* 2011; Pule 2013). Table 6.1 shows the setup for this experiment. K636 and BO1 were grown to an optical density at 600 nm of 0.6 in liquid media, after which they were diluted 1:100 (which yields ~10^4 cells/ml). Fifty microliters of this dilution were added per well, yielding a total of 100 µl per well. Assays were done in triplicate.

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Table 6.1 Setup of resazurin microtiter method in 96-well format

	With reserpine				**Without reserpine**			
	A	B	C	D	E	F	G	H
1	Media only	Diluent only	Drug only	Drug only	Drug only	Drug only	Diluent only	Media only
2	Media	Diluent 1x[a]	16 or 0.5 µg/ml +1x diluent	16 or 0.5 µg/ml +1x diluent	16 or 0.5 µg/ml +1x diluent	16 or 0.5 µg/ml +1x diluent	Diluent 1x	Media
3	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
4	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
5	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
6	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
7	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
8	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
9	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
10	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
11	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media
12	Media	Diluent	Drug	Drug	Drug	Drug	Diluent	Media

[a]Shaded blocks indicate a twofold dilution series from wells 2 to 12.

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6.4 Results and discussion

6.4.1 Fluoroquinolone B01 MIC is modulated by additional mutations in *gyrA/gyrB*

To test the hypothesis that the MIC of a strain harbouring a known fluoroquinolone resistance-conferring mutation could be increased by subsequent selection on solid media containing ofloxacin at a concentration higher than the predetermined MIC, we plated BO1 onto solid media containing > 16 µg/ml ofloxacin. Sequencing of the *gyrA* and *gyrB* genes showed the presence of additional mutations either in *gyrA* codon 90, or in *gyrB* in all the cultures tested (Table 6.2). Our results demonstrated that double mutations in *gyrA* and/or *gyrB* can co-exist within one strain and that the presence of these multiple mutations increased the fluoroquinolone MIC.

This is in line with a previous study which observed double mutations, (i.e. two mutations in *gyrA* only or one mutation in *gyrA* and one mutation in *gyrB*) in *M. tuberculosis* fluoroquinolone-resistant *in vitro* mutants (Kocagöz *et al.* 1996). At the time the authors hypothesised that double mutation could be the way in which the bacteria could increase level of fluoroquinolone resistance (Kocagöz *et al.* 1996). However, analysis of serial isolates from the same patient suggests an alternative hypothesis where two or more strain populations co-exist with different resistance-conferring alleles (Duong *et al.* 2009; Mokrousov *et al.* 2008; Streicher *et al.* 2012). Interestingly, double mutations, whether as a result of heterogeneity or not, increase the MIC of a *M. tuberculosis* population in clinical isolates according to a meta-analysis of fluoroquinolone resistance mutations (Maruri *et al.* 2012).

6.4.2 The primary mechanism of fluoroquinolone resistance *in vitro* is *gyrA* mutation

To determine whether efflux contributed to the level of ofloxacin resistance in our study, we assayed for a reduction in the ofloxacin MIC of BO1 in the presence of efflux pump inhibitors. Since the MIC of BO1 was known to be 16 µg/ml, we tested growth of this isolate at a range of concentrations lower than 16 µg/ml in the presence of three different efflux pump inhibitors. The inhibitors were verapamil and carbonyl cyanide m-chlorophenyl hydrazone (CCCP), for which results are shown in Table 6.3, and reserpine, shown in Table 6.4. As a control, K636 was also assayed for growth in the presence and absence of the three efflux pump inhibitors at concentrations of ofloxacin ≤ 0.5 µg/ml.

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Table 6.2 Mutations increasing resistance of a *gyrA* Asp94Gly mutant to ≥ 16 µg/ml ofloxacin

Ofloxacin selection concentration (µg/ml)	**Additional amino acid change in *gyrA***	**Additional amino acid change in *gyrB***
16	None	Thr500Asn
20	None	Asp461Asn
20	None	Asp461His
20	None	Asp461Val
28	None	Asp461Asn
28	None	Asn499Lys
28	None	Asn499Lys
32	None	Asp461Asn
32	None	Asn499Lys
64	None	Asp461Asn
64	None	Asp461Asn
64	Ala90Val	None

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Table 6.3 Effect of efflux pump inhibitors CCCP and verapamil on growth of K636 and BO1 in the presence of ofloxacin

BO1					
CCCP (µg/ml)	**Verapamil (µg/ml)**	**Ofloxacin (µg/ml)**	**Experiment 1[a]**	**Experiment 2**	**Experiment 3**
0	0	0	+	+	+
4	0	0	+	+	+
4	0	0.5	+	+	+
4	0	2	+	ND	+
4	0	4	ND	+	+
4	0	8	+	+	+
4	0	**16[b]**	+/-	-	-
0	10	0	+	+	+
0	10	0.5	+	+	+
0	10	2	+	+	+
0	10	4	+	+	+
0	10	8	+	+	+
0	10	**16**	-	+/-	+
K636					
CCCP (µg/ml)	**Verapamil (µg/ml)**	**Ofloxacin (µg/ml)**	**Experiment 1**	**Experiment 2**	**Experiment 3**
0	0	0	+	+	+
4	0	0	+	+	+
4	0	0.125	+	+	+
4	0	0.25	+	+	+
4	0	**0.5**	-	-	-
0	10	0	+	+	+
0	10	0.125	+	+	+
0	10	0.25	+	+	+
0	10	**0.5**	-	-	-

[a]+, growth; ND, not determined; -, no growth; +/-, intermediate, defined as exhibiting growth units < 100 at the time when the growth control reached 400, but > 100 14 days after.

[b]MICs in the absence of efflux pump inhibitor are in bold

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Table 6.4 Changes in ofloxacin MIC in the presence and absence of reserpine in 96-well format

Isolate	MIC	
	With reserpine	**Without reserpine**
K636	0.25-0.5	0.5
BO1	8	8

There was no reduction in the MIC of BO1 in the presence of CCCP and verapamil (Table 6.3). In fact, for BO1, adding these compounds to cultures treated with 16 µg/ml ofloxacin showed mixed results. In the presence of CCCP, in two out of three experiments, BO1 was inhibited by 16 µg/ml ofloxacin, but had intermediate growth in the third. In the presence of verapamil and 16 µg/ml ofloxacin, BO1 also showed mixed results suggesting that the MIC was very close to this concentration of ofloxacin. In addition, there was also no reduction in the MIC of BO1 or K636 in the presence of reserpine (Table 6.4). This suggests that the ofloxacin MIC of this *in vitro*-generated mutant was independent of efflux pumps modulated by CCCP, verapamil or reserpine.

To confirm that additional resistance was conferred by mutations in *gyrA/gyrB* and not by efflux, we subcultured a selection of the double mutants on various concentrations of ofloxacin in the presence of efflux pump inhibitors (Table 6.2). Mutants reflective of the various ofloxacin MICs were tested in this assay (*gyrB* Thr500Asn and Asp461Val mutants, as well as *gyrB* Asp461Asn mutants selected on 28 and 64 µg/ml respectively). The concentrations of ofloxacin ranged from 8 µg/ml (½xMIC of BO1) to the concentration on which the double mutant was originally selected. Results (not shown) indicated that the addition of efflux pump inhibitors reserpine, verapamil and CCCP did not result in the inhibition of growth of any of these mutants, not even at the highest concentration tested. This suggested that efflux did not play a role in modulating the level of ofloxacin resistance in these *in vitro* mutants.

The absence of any effect of efflux pump inhibitors on BO1 in our study may be because ofloxacin resistance was selected for *in vitro,* which does not reflect intermittent therapy, as is the case clinically (Schmalstieg *et al.* 2012; Srivastava *et al.* 2010). It is probably not as a result of the specific *gyrA* mutation of this isolate, as there was no association between mutation and efflux pump inhibition in another study (Singh *et al.* 2011), or the fact that our

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strain was monoresistant, as efflux could be inhibited in fluoroquinolone-monoresistant isolates in another study (Eilertson *et al.* 2016). The presence of efflux pump activity in only a portion of clinical isolates, even when cultured under *in vitro* conditions (which excludes the possibility that efflux is only induced *in vivo)* and high antibiotic concentrations therefore suggests the presence of genetic mechanisms that evolved during intermittent drug exposure that are not well understood. Alternatively, this mechanism is epigenetic, a possibility that has not been explored for mycobacteria (Shell *et al.* 2013).

6.5 Conclusion

In conclusion, the ofloxacin MIC could be increased in an ofloxacin-resistant *in vitro* generated *gyrA* Asp94Gly mutant by acquisition of additional *gyrA/gyrB* mutations co-occurring with the mutation in codon 94. Efflux-related drug resistance mechanisms were not detected in the mutants with increased MIC or the *gyrA* Asp94Gly progenitor. Further work is required to understand how efflux may evolve as mechanism modulating the level of resistance in *M. tuberculosis.*

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Chapter 7 Deciphering the impact of mutations conferring extensive drug resistance on the physiology of *Mycobacterium tuberculosis*

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7.1 Abstract

The physiology of *Mycobacterium tuberculosis* can inform new therapeutic strategies. Current knowledge of *M. tuberculosis* is based largely on analysis of drug susceptible strains, however, drug-resistant strains may differ in their physiology. Studies in other organisms have shown physiological implications of drug resistance mutations beyond resistance. In addition, antibiotics may have effects unrelated to mechanism of action. The aim of our study was therefore to assess the individual impacts of mutations responsible for extensive drug resistance (XDR). The impact of *gyrA* Asp94Gly and *rrs* A1401G mutations, as well as ofloxacin or amikacin treatment of the respective mutants, were assessed using mass spectrometry-based label-free relative quantitative proteomics. A strong signature of differentially abundant proteins common to both clones, involving the ESX-5 cluster, suggested either a common unknown genetic variant or physiological changes related to drug resistance. The *gyrA* mutant may display decreased cell wall permeability and increased drug tolerance due to reduced levels of transport proteins and may have impaired gyrase functionality as evidenced by differential abundance of transcription/translation proteins. The *rrs* mutant displayed lowered abundance of DosR-regulated proteins, suggesting a changed response during dormancy. Ofloxacin treatment resulted in increased iron acquisition proteins, possibly related to iron chelation by ofloxacin, and differential abundance of proteins indicating decreased cell division and growth. Amikacin treatment decreased ribosomal protein abundance and increased proteins involved in tRNA-related processes. We hypothesise that this relates to increased degradation of ribosomes and compensating for reduced translational fidelity. Results suggest that the physiology of XDR *M. tuberculosis* differs from susceptible strains. Changes in physiology could inform further research on drug targets and optimal treatment regimens.

7.2 Introduction

Understanding the physiology of *Mycobacterium tuberculosis* is vital in light of the extent of the TB epidemic (World Health Organization 2015). Knowledge of its interaction with the host, factors that determine virulence, its ability to persist and cause chronic infection and its ability to adapt to antibiotic exposure and acquire antibiotic resistance could provide a promising approach to discover new therapeutic strategies, important in light of the slow progress made in discovery of new antibiotics for TB (Baer *et al.* 2015).

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"Omics" analyses, involving mainly transcriptomics and proteomics, provide a promising approach towards studying the physiology of *M. tuberculosis.* The transcriptome provides insight into transcription of certain genes, measured by mRNA levels in response to different conditions. This, however, does not take into account post transcriptional and post translational regulatory events, which determine the efficiency whereby RNA is translated into protein, which in turn may also play an important role in the physiology of the cell. Because proteomics involves identification and (relative) quantification of proteins within the cell, measuring these levels provides a closer absolute picture of the biology of the organism. Relative levels of proteins indicate which metabolic pathways are more or less active in an organism. (Cook *et al.* 2009)

Our current knowledge of the physiology of *M. tuberculosis* is largely based on the analysis of drug susceptible strains, and in particular the laboratory strain, H37Rv, which may not reflect clinically relevant *M. tuberculosis* strains, as genomic differences seen in clinical isolates could determine their physiological properties (Devasundaram & Raja 2016). Currently, it is assumed that the only effect of the presence of drug resistance mutations is to confer resistance, however, these mutations may have physiological implications beyond their ability to survive in the presence of an antibiotic. Such mutations often occur in enzymes central to metabolism (Musser 1995), which, on the one hand, in many bacteria, lead to a fitness defect for the strain involved (Andersson & Levin 1999). On the other hand, they could even be beneficial to the organism. For example, *rpoB* mutations increase antibiotic production in *Streptomyces coelicolor* (Hosaka *et al.* 2009; Hu *et al.* 2002; Xu *et al,* 2002). Furthermore, *gyrA* mutations were shown to lead to increased virulence in *Pseudomonas aeruginosa* (Agnello & Wong-Beringer 2012), increased survival of *Escherichia coli* in macrophages (Miskinyte & Gordo 2013) and multidrug resistance in *Salmonella enterica* (Webber *et al.* 2013).

In addition, it is assumed that antibiotics only act on their drug target and that a mutation conferring resistance to the drug results in no effect on the physiology of the bacterium when it is treated with an antibiotic. However, antibiotics may have pleiotropic effects. It is well known that antibiotics act as signalling molecules (Heeb *et al.* 2011; Romero *et al.* 2011), especially since they are based on compounds synthesised naturally by micro-organisms. The clinical relevance of exposing rifampicin resistant strains to rifampicin was recently highlighted as it showed that this treatment reduced susceptibility to ofloxacin (Louw *et al.* 2011). Resistant *M. tuberculosis* strains are often still exposed to the antibiotics to which they

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are resistant within the patient, as resistance is not immediately diagnosed (Louw *et al.* 2011). The clinical implication of this is that, unless drug resistance is detected prior to initiation of treatment, treatment of resistant *M. tuberculosis* strains with the antibiotic that they are resistant to, may potentiate further drug resistance.

Since XDR-TB is defined as multidrug-resistant (MDR, resistance to at least rifampicin and isoniazid) TB with additional resistance to a fluoroquinolone and a second-line injectable/aminoglycoside (World Health Organization 2015), it follows that the emergence of XDR-TB is associated with the acquisition of mutations conferring resistance to fluoroquinolones and aminoglycosides. In the case of fluoroquinolones, these are, in the majority of cases, *gyrA* mutations (Maruri *et al.* 2012). When building a treatment regimen for MDR-TB, amikacin or kanamycin are the aminoglycosides of choice (World Health Organisation 2008). These two antibiotics have high levels of cross-resistance (World Health Organisation 2008), mediated mainly by the A1401G mutation in the *rrs* gene (Georghiou *et al.* 2012).

The proteomes of amikacin-resistant and ofloxacin-monoresistant clinical isolates of *M. tuberculosis* have been compared to pan-sensitive isolates (Kumar *et al.* 2013; Lata *et al.* 2015). However, the 2-D gel methodology used in those studies limited the number of proteins that could be identified and introduced bias, as the extracted proteins were fractionated by hydrophobicity, pI and molecular weight (Zhu *et al.* 2010). In addition, the susceptible and resistant isolates included in the study on amikacin-resistant isolates (Kumar *et al.* 2013) may have had many other genetic differences, including strain-specific and drug resistance-encoding variants, making it difficult to distinguish between proteome changes conferred by a single specific drug resistance mechanism. Similarly, in the study by Lata *et al.* (2015), although the isolates were monoresistant, no information was provided as to how they were related genetically to the pan-susceptible isolates they were compared to. To overcome these limitations, our study aimed to investigate differences in protein abundance in isogenic, pan-susceptible ofloxacin-monoresistant (*gyrA* Asp94Gly) and amikacin-monoresistant (*rrs* A1401G) *M. tuberculosis* clones using liquid chromatography fractionation methods associated with mass spectrometry and label-free relative quantification. In addition we aimed to investigate the effect of exposing the ofloxacin-monoresistant or amikacin-monoresistant *M. tuberculosis* clones to sublethal concentrations of the corresponding antibiotics to determine whether this exposure would alter the expression of proteins which could potentiate resistance/tolerance to other anti-TB drugs.

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7.3 Materials and methods

7.3.1 Isolates used in our study

BO10, a *gyrA* Asp94Gly mutant selected from the pan-sensitive Beijing clinical isolate, K636, was generated as in Sections 4.3.4 and 4.3.5. BA23, an *rrs* A1401G mutant, was generated using similar methodology, except that this clone was selected on 4 µg/ml amikacin. Both clones were characterised by PCR and sequencing as detailed below.

Clones harbouring mutations conferring either resistance to a fluoroquinolone or aminoglycoside were selected to reflect the most prominent mutation seen in the XDR-TB epidemic in the Western Cape, South Africa. The most prevalent mutation known to confer ofloxacin resistance in pre-XDR and XDR-TB is the *gyrA*Asp94Gly mutation, as shown in Figure 7.1. Only one (known) mutation is associated with amikacin resistance in the Western Cape, which is the *rrs* A1401G mutation (personal communication, Lizma Streicher, Division of Molecular Biology and Human Genetics, Stellenbosch University).

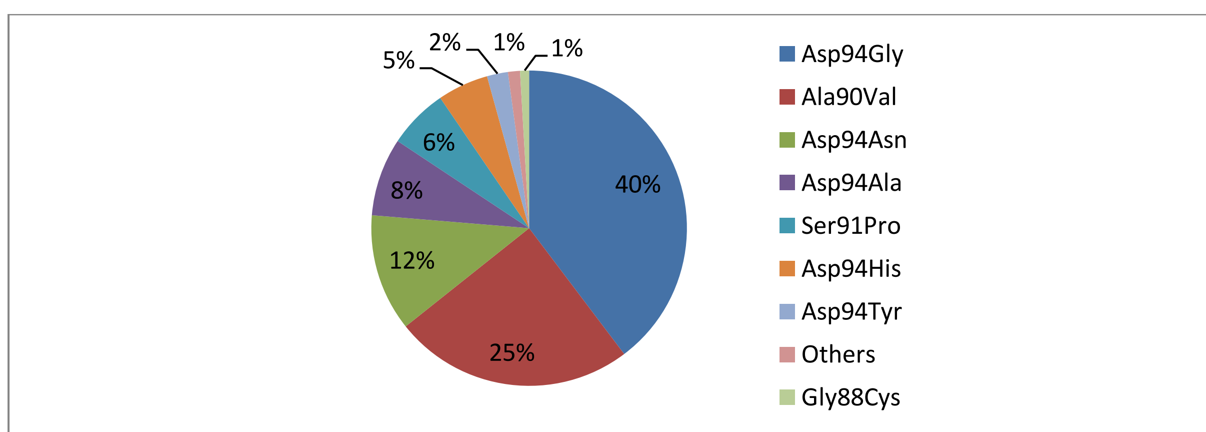

Figure 7.1 Distribution of *gyrA* mutations in pre-XDR and XDR clinical isolates in the Western Cape, South Africa (collected from 2001 to 2012). Data obtained from Lizma Streicher, Division of Molecular Biology and Human Genetics, Stellenbosch University)

W7 was selected as a single colony isolate from K636 as in Section 4.3.4 and 4.3.5, except that, after 28 days of growth of the parallel liquid cultures, a 50 µl aliquot was taken from one of the cultures and streaked onto solid media without antibiotics. The plate was incubated for 28 days. Colonies (W1 to W9) were selected and inoculated into 5 ml liquid media. When the cultures had grown to high turbidity, 1 ml of each culture was stored at -80°C. W7 was confirmed not to harbour drug resistance-conferring mutations in *gyrA* and *rrs* as determined by PCR and whole genome sequencing as detailed below.

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Figure 7.2 summarises how the different clones used in proteomic analyses were generated.

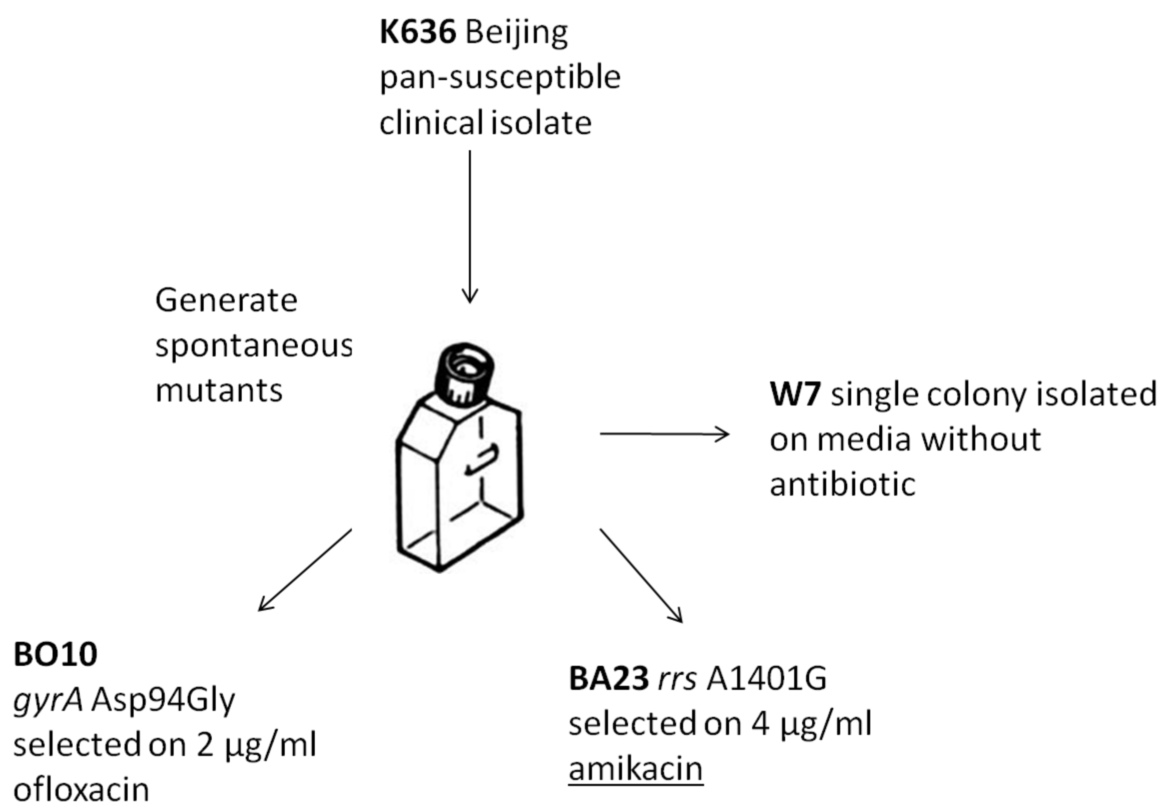

Figure 7.2 Clones used for proteomic analyses

Stocks were made of BO10, BA23 and W7 as follows: Frozen stocks made in Section 4.3.4 were added to 9 ml liquid media and incubated at 37°C. When the OD_{600} reached 0.8-1.0, the cultures were diluted to an OD_{600} of 0.05 in a total volume of 20 ml. After 6 days of incubation at 37°C, 1 ml stocks were prepared and stored at -80°C.

7.3.2 PCR and sequencing

The mutation conferring resistance to amikacin was determined as in Section 4.3.7 by PCR amplification of the *rrs* gene (nt 1224-1514) using the primers rrs290_F (5'-TGCTACAATGGCCGGTACAA-3') and rrs290_R (5'-CTTCCGGTACGGCTACCTTG-3') with an annealing temperature of 62°C followed by Sanger sequencing. The mutation conferring resistance to ofloxacin in BO10 was determined as in Section 4.3.7 for the ofloxacin-resistant clones.

PCR and sequencing of a heterogeneous variant of Rv2185c/TB16.3 in K636 (see Section 7.4.2) was performed as in Section 4.3.7. 1 µl of genomic DNA diluted to 25 ng/µl in water

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was used per reaction for K636, while a single colony of W7 was resuspended into 50 µl ddH_2O and boiled for 20 minutes at 95°C. One microliter of the supernatant was used in the PCR reaction. TB16.3 primer sequences were 5'-GATCCAGGCGAGGTGATG-3' (TB16.3 Fwd) and 5'-CGGTGGATACCACCAACACT-3' (TB16.3 Rev) with an annealing temperature of 54°C.

7.3.3 Growth curves

Growth curves were done to determine the OD range defining mid-log phase. Growth curves were done in modified Sauton's broth, made up of the following: 0.4% L-asparagine, 0.4% glucose, 0.2% citric acid, 0.05% monopotassium phosphate, 0.05% magnesium sulphate, 0.005% ferric ammonium citrate, 0.0001% zinc sulphate and 0.05% Tween-80. The pH was adjusted to 7.0 and the medium was filter-sterilised. A starter culture was grown by inoculating a 1 ml stock into 4 ml media. When the starter culture reached an optical density of 0.8-1.0 at 600 nm (OD), it was diluted to an OD of 0.05 in a total of 25 ml modified Sauton's broth and inclubated at 37°C. OD readings were taken on days 2, 4, 7, 9, 11, 14, 16, 18 and 21 and these were plotted on the y-axis against number of days. The experiment was done in biological triplicate.

7.3.4 Preparation of stocks for drugs

All drugs were obtained from Sigma-Aldrich. Amikacin sulphate salt was made up to a stock solution of 2.5 mg/ml in H_2O. Ofloxacin was made up to 4 mg/ml by first dissolving in 1 part 0.1 N NaOH and then adding 9 parts H_2O. All stock solutions were stored at -20°C.

7.3.5 Culturing for extraction

All experiments were carried out in quadruplicate and these were referred to as biological replicates. Culturing for protein extraction was performed in modified Sauton's broth. For each biological replicate, 1 ml of stock of W7, BO10 and BA23, made in Section 7.3.4, was inoculated into 4 ml media as starter cultures. When the cultures reached an OD of 0.8-1.0, cells were diluted in 25 ml modified Sauton's broth to an OD of 0.05. This was done in duplicate for each clone. These cultures were grown until an OD of 0.8-1.0. At this time, either antibiotic diluent or antibiotic was added to the cultures as follows: Ofloxacin was added to a final concentration of 2 µg/ml to one culture of BO10, while the same volume of diluent (0.01N NaOH) was added to the other BO10 culture, as well as one of the W7 cultures. Amikacin was added to a final concentration of 1 µg/ml to one of the BA23 cultures, while the same volume of antibiotic diluent (H_2O) was added to the other BA23 culture, as

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well as the remaining W7 culture. The cultures were then incubated at 37°C for 24 hours, after which the cells were pelleted by centrifugation at 1800x g and washed twice in cold 1x Dulbecco's PBS (Sigma). The concentrations of and exposure times to ofloxacin or amikacin were chosen to reflect the exposure conditions used in previous work in our laboratory (Louw *et al.* 2011) that led to the hypothesis in this study (See Section 1.3.2 and 1.4.3). Cell pellets were stored at -20°C until protein extraction.

Figure 7.3 sets out the structure of our proteomics analysis with respect to culturing of strains and how they were compared.

Wild type treated 24 hrs with antibiotic diluent		Mutant treated 24 hrs with antibiotic diluent		Mutant treated 24 hrs with antibiotic	
Ofloxacin experiment			Amikacin experiment		
W7+ diluent	BO10+ diluent	BO10+ 2µg/ml ofloxacin	W7+ diluent	BA23+ diluent	BA23+ 1 µg/ml amikacin

ANOVA, multiple comparisons correction, post-hoc Tukey test (Ofloxacin experiment)

ANOVA, multiple comparisons correction, post-hoc Tukey test (Amikacin experiment)

Figure 7.3 Details of proteomic analysis. W7 was exposed to the relevant antibiotic diluents for 24 hours, while the mutants were exposed either to antibiotic diluents or antibiotic for 24 hours at mid-log phase. Each experiment was done in quadruplicate.

7.3.6 Protein extraction

Pellets were thawed and resuspended in 300-500 µl modified RIPA buffer, containing protease inhibitor (complete protease inhibitor cocktail, Roche). Modified RIPA buffer contained 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630 and 0.1% sodium deoxycholate. The cells were transferred to 2 ml cryogenic tubes with O-rings containing 0.1 mm glass beads and 10 µl RNase-free DNAse (2 U/ml, Benzonase®) was added to each tube. Pellets were ribolysed for 6 cycles of 4 m/s for 20 sec, the samples were

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put on ice for 1 min after every second cycle. The whole cell lysate was then centrifuged at 12000 x g for 10 min at room temperature. The supernatant was transferred to a new tube and centrifugation repeated. The whole cell lysate was then filter-sterilised with a 0.22 µm PVDF syringe filter and stored at -20°C.

7.3.7 Protein precipitation

Proteins were precipitated using acetone. Briefly, 4 volumes of cold acetone were added to 1 volume of protein solution. Samples were kept at -20°C overnight. Proteins were then pelleted by centrifugation at maximum speed, using a benchtop centrifuge. Pellets were air-dried and resuspended in denaturation buffer (6M urea, 2M thiourea in 10 mM Tris pH 8.0).

7.3.8 Quantification of protein

Proteins were quantified using a modified Bradford assay in 96-well format. To make the solution compatible with the high pH of urea, samples were diluted in 0.1% HCl.

7.3.9 Trypsin digest

After quantification, 40 µg of proteins in denaturation buffer were reduced using 1 mM dithiothreitol for 1 hour at room temperature. Proteins were then alkylated with 5.5 mM iodoacetamide for 1 hour at room temperature in the dark. Lys-C protease, which cleaves on the C-terminal of lysine residues, was added in a w/w ratio of 1:100 (Lys-C:sample) and samples incubated for 3 hours at room temperature. Samples were then diluted with 4 volumes 20 mM ammonium bicarbonate, after which trypsin was added in a w/w ratio of 1:50 (trypsin:sample) and incubated for 18 hours at room temperature. Samples were then dried under vacuum. Dried peptides were sent for liquid chromatography and mass spectrometry at the Central Analytical Facility at Stellenbosch University.

7.3.10 Liquid chromatography

Liquid chromatography was performed on a Thermo Scientific Ultimate 3000 RSLC equipped with a 2 cm x 100 µm C_{18} trap column and a 35 cm x 75 µm in-house manufactured C18 column (Luna C18, 5 µm; Phenomenex) analytical column. The solvent system employed was as follows: Loading: 2% acetonitrile:water and 0.1% formic acid (FA); Solvent A: 2% acetonitrile:water and 0.1% FA and Solvent B: 100% acetonitrile:water. The samples were loaded onto the trap column using loading solvent at a flow rate of 5 µL/min from a temperature-controlled autosampler set at 7°C. Loading was performed for 10 min before the sample was eluted onto the analytical column. Flow rate was set to 350 nL/min

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and the gradient generated as follows: 2.0% Solvent A for 5 min; 2%-4% Solvent B from 5-10 minutes, 4%-10% from 10-20 min, 10%-40% from 20-95 min using Chromeleon non-linear gradient 7, 40-80% Solvent B and from 95-100 min. Thereafter the column was washed for 10 min with 80% Solvent B followed by equilibration. Chromatography was performed at 50°C and the outflow delivered to the mass spectrometer through a stainless steel nano-bore emitter.

7.3.11 Mass spectrometry

Mass spectrometry was performed using a Thermo Scientific Fusion mass spectrometer equipped with a Nanospray Flex ionization source. The sample was introduced through a stainless steel emitter. Data was collected in positive mode with spray voltage set to 2kV and ion transfer capillary set to 275°C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. MS1 scans were performed using the orbitrap detector set at 12 000 resolution over the scan range 350-1650 with AGC target at 3 E5 and maximum injection time of 40 ms. Data was acquired in profile mode.

MS2 acquisitions were performed using monoisotopic precursor selection for ion with charges +2-+6 with error tolerance set to +/- 10ppm. Precursor ions were excluded from fragmentation once for a period of 30 s. Precursor ions were selected for fragmentation in HCD mode using the quadrupole mass analyzer with HCD energy set to 35%. Fragment ions were detected in the ion trap mass analyzer using rapid scan rate. The AGC target was set to 1E4 and the maximum injection time to 45 ms. The data was acquired in centroid mode.

All samples were run in duplicate (termed technical duplicates).

7.3.12 Data analysis

Peptide and protein identification and quantification was done separately for the two sets relating to ofloxacin and amikacin, respectively, as shown in Figure 7.3. Set 1:W7 exposed to H_2O, BA23 exposed to H_2O and BA23 exposed to 1 µg/ml amikacin in H_2O. Set 2:W7 exposed to 0.01N NaOH, BO10 exposed to 0.01N NaOH and BO10 exposed to 2 µg/ml ofloxacin in 0.01N NaOH.

Raw files were analysed using MaxQuant 1.5.3.30 (Tyanova *et al.* 2015). The Andromeda search engine (Cox *et al.* 2011) was used to search all tandem mass spectra against a customised concatenated reference database. This database was created containing all genomic sequences and annotational information of *M. tuberculosis* H37Rv, as well as

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genomic variants found in K636, BO10 and BA23. The database was created as follows: Variants were identified as described in Section 7.3.13. Variants were annotated using annotation data from TubercuList (Lew *et al.*, 2011). The consensus sequence for coding DNA sequences (CDSs) of each strain was created and the strain-specific versions of CDSs were translated and used for peptide searches. Peptides had to be at least 7 amino acids in length, fully digested with trypsin, allowing two missed cleavages. The initial allowed mass tolerance was set to 20 ppm at the MS level and 0.5 Da at the MS/MS level. Variable modifications were N-terminal N-acetylation and methionine oxidation, while the fixed modification was carbamidomethylation of cysteine. False discovery rates for peptide-spectrum matches and protein identification were set to 1%. We used the match between runs algorithm, where the match time window was 0.7 min and the alignment time window 20. Results of the MaxQuant analysis are in files called proteinGroups.txt.

Relative quantitative analysis was performed using the label-free quantification option in MaxQuant and LFQ intensity values in the proteinGroups.txt files were analysed using Perseus 1.5.3.0 (Tyanova *et al.* 2016). Only proteins identified by at least two unique peptides were considered, while those identified by site, matched against the reverse database or identified as a potential contaminant were not considered. Zero values of label-free quantitiative (LFQ) intensity were converted to NaN (not a number). LFQ intensity values were then averaged for each set of two technical duplicates. Therefore, where a protein was detected in one run, but not the other for the same sample, it was assumed that the missing value would be equal to the intensity observed in the matching run. The highest possible value was therefore imputed for the missing value. Average LFQ intensities for each biological replicate were then further filtered so that at least 3 valid values were present for each strain. Average LFQ intensities were then transformed (log_2X). ANOVA, followed by a multiple comparisons correction involving the software's built-in permutation-based false discovery rate (FDR) algorithm, was used to identify significant differences in average LFQ intensity between three groups (W7, mutant untreated, mutant treated). The multiple comparisons correction FDR was set to 5% with 250 randomisations. To determine which means were significantly different between the three groups, the post-hoc Tukey test was carried out using the Microsoft Excel add-in Daniel's XL toolbox version 6.51 (Kraus 2014).

TubercuList (http://tuberculist.epfl.ch/index.html) (Lew *et al.* 2011) and PATRIC (Pathosystems Resource Integration Center) (Wattam *et al.* 2014), accessed at https://www.patricbrc.org/portal/portal/patric/Home, were used to assign function to proteins

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significantly different in abundance between groups. A literature search for each protein was also carried out in Pubmed.

7.3.13 Next generation sequencing

7.3.13.1 Culturing for DNA extraction

One hundred microliters of a log-phase culture (OD_{600}=1-2) for W7, K636, BO10 and BA23 were spread onto 7H10 supplemented with ADC and 0.5% glycerol. ADC was prepared as a 10x solution as follows: 5% bovine serum albumin (w/v), 2% glucose monohydrate (w/v) and 0.2% of an aqueous suspension of bovine liver catalase from Sigma-Aldrich (catalogue number C3155). Cultures were incubated for 3 weeks at 37°C.

7.3.13.2 DNA extraction

Genomic DNA was extracted from W7, K636, BO10 and BA23 according to the method detailed in Warren *et al.* (2009).

7.3.13.3 Sequencing and library preparation

For K636 and BA23, sequencing and library preparation was done in collaboration with Drs Arnab Pain and Abdallah M. Abdallah from the King Abdullah University of Science and Technology (KAUST). Sequencing was done on the Illumina HiSeq2000 (Illumina, California, USA) platform, using a paired-end approach. Read lengths for K636 and BA23 were 100 bp and 84 bp, respectively. The depth of coverage ranged from 40-113 times for K636, depending on the mapping algorithm used. Different mapping algorithms influence how different regions are mapped, in turn impacting the average coverage. Depth of coverage was 110 times for BA23.

For BO10, sequencing and library preparation was done by the Agricultural Research Council in South Africa. Sequencing was done on the Illumina MiSeq, v3 chemistry, pair end 300x300bp. Read length for BO10 was 301 bp, while depth of coverage was ~73 times.

For W7, both sequencing and data analysis was performed at the Central Analytical Facility at Stellenbosch University, using the Ion Torrent platform and associated software for data analysis. Depth of coverage was not calculated for W7.

7.3.13.4 Data sources

Sequence data were provided in FASTQ file format and shared via a secure file transfer protocol (FTP). The reference genome *M. tuberculosis* H37Rv (accession number

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AL123456.3) in FASTA format, genome summary information and summary of gene sequences were downloaded from NCBI at http://www.ncbi.nlm.nih.gov/nuccore/AL123456.3

7.3.13.5 Quality assessment

The quality of the whole genome sequences for K636, BO1 and BA23 were assessed using FASTQC. This program is available from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ and was used to validate the quality of the reads.

7.3.13.6 Analysis

Next generation sequence data of K636, BO10 and BA23 were processed using the USAP (universal sequence analysis pipeline) software developed in the Division of Molecular Biology and Human Genetics, Stellenbosch University by Dr Ruben van der Merwe (described in Black *et al.* 2015). Default variant filtering parameters were used as described in Black *et al.* 2015. A command line interface in Linux was used to run USAP using python 2.7.

7.3.13.7 Comparison of SNP/indel differences between strains

USAP was used to identify SNPs/indels unique to any of the strains. The .vcf file received from CAF after data analysis for W7 was edited manually before comparison to BO10 and BA23 in USAP.

7.4 Results and discussion

7.4.1 Growth curves

Growth curves for W7, BA23 and BO10 are shown in Figure 7.4. There was no difference in growth between the three strains.

7.4.2 Whole genome sequencing of *in vitro* mutants

The progenitor strain, K636, as well as BO10, BA23 and W7 were subjected to whole genome sequencing to confirm monoresistance and to characterise any additional genetic differences between the strains that may affect the proteomic analysis.

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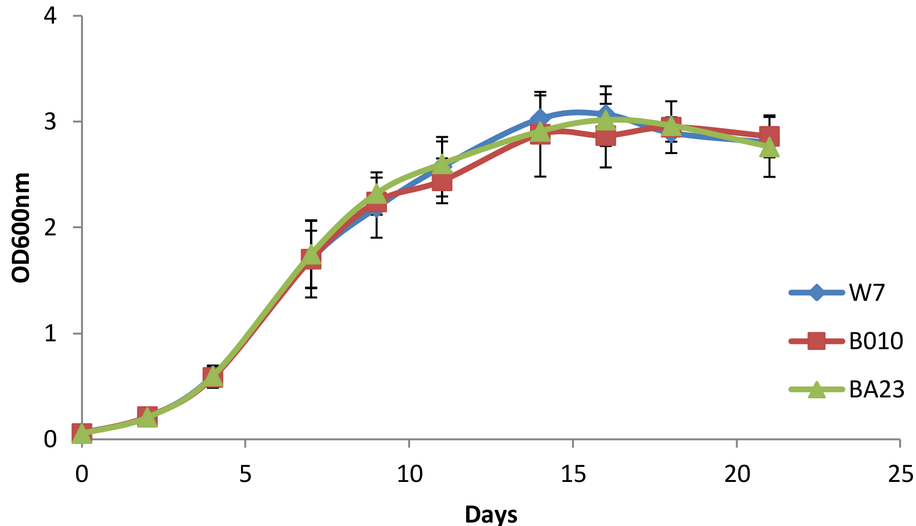

Figure 7.4 Growth curves for W7, BO10 and BA23. Growth curves were done in modified Sauton's broth. A starter culture was grown by inoculating a 1 ml stock into 4 ml media. When the starter culture reached an optical density of 0.8-1.0 at 600 nm (OD), it was diluted to an OD of 0.05 in a total of 25 ml modified Sauton's broth and incubated at 37°C. Shown are average OD readings taken in three independent experiments, error bars indicate standard deviation.

We compared the three sequences generated by the Illumina platform, since they were directly comparable, under the assumption that W7 would be genetically identical to K636, since it was a single colony derived from the strain (Results shown in the Appendix). We were able to confirm the unique (i.e. not in BA23 or K636) presence of an A/T to G/C mutation at genome position 7582 (relative to H37Rv) in BO10, which translated to an Asp94Gly mutation in the *gyrA* gene. Similarly, we were able to confirm the unique (i.e. not in BO10 or K636) presence of an A/T to G/C mutation at genome position 1473246 (relative to H37Rv) in BA23, which translated to an A1401G mutation in the *rrs* gene. We also observed that the K636 strain was actually a mixture of two strains, where reads harbouring both G and T were detected at genome position 2447198 (relative to H37Rv). This translated to a synonymous mutation in codon 101 of Rv2185c/TB16.3. However, BO10 and BA23 were homogenous, with only a T at this position. We confirmed by Sanger sequencing that W7 was identical to BO10 and BA23 in this respect as shown in Figure 7.5.

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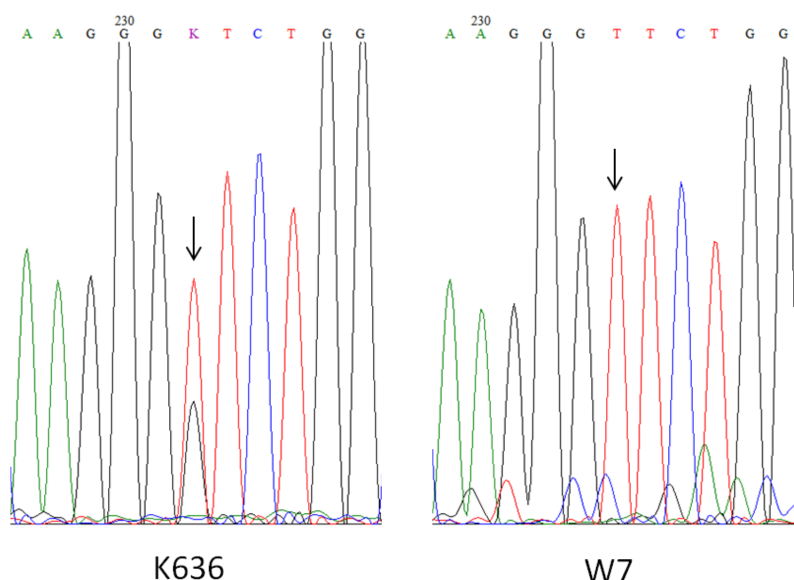

Figure 7.5 Confirmation of homogeneity within W7, a single colony selected from K636, with respect to TB16.3 (genome position 2447198, which is indicated by the arrow) using Sanger sequencing.

Whole genome sequencing also indicated the presence of a SNP at genome position 3537599 (relative to H37Rv) in BO10. This SNP did not occur in K636 or BA23. Comparison of ion torrent-derived data indicated that this SNP was also not in W7 or BA23, but did occur in BO10. This SNP translated to a Glu121Gly mutation in Rv3169 and may have an effect on protein expression in BO10. This is discussed further in 7.4.5.1.

7.4.3 Overview of proteomics

The proteinGroups.txt files for each experiment can be viewed in the Appendix.

Data analysis was approached as follows (see Figure 7.8 below): Proteins were identified by the presence of at least 2 unique peptides. Only proteins where at least three biological replicates had intensity values, after averaging of technical duplicates, were considered for relative quantitative analysis by ANOVA, followed by a permutation-based multiple comparisons correction with 5% FDR. An ANOVA was carried out, because it is statistically more rigorous than t-tests comparing two groups at a time. Performing two-way comparisons between the three groups would have resulted in a three-fold inflated error rate. After ANOVA, a post-hoc Tukey test was performed to see which means were significantly different between groups. Furthermore, biological significance was also considered, where proteins more or less abundant by at least 1.5-fold were considered to have more impact on the physiology of the bacterium than proteins with lower changes in abundance (McCarthy &

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Smyth 2009). In this study we focused on proteins altered by at least 1.5-fold in abundance, followed by proteins significantly altered in abundance performing similar functions or belonging to the same pathways/regulons/operon.

For the comparison between W7, BO10 untreated and BO10 treated with ofloxacin (see Figure 7.3), 1972 proteins were identified by at least 2 peptides. After filtering for proteins with at least 3 valid values in each group, 1412 proteins were considered for relative quantitative analysis by ANOVA, followed by a permutation-based multiple comparisons correction with FDR of 5%. This yielded 317 proteins significantly differentially abundant between the three groups compared. Lists of proteins significantly different in abundance can be viewed in the Appendix.

For the comparison between W7, BA23 untreated and BA23 treated, 2079 proteins were identified by at least two unique peptides. After filtering for proteins with at least three valid values in each group, 1267 proteins were considered for ANOVA and a permutation-based multiple comparisons correction with FDR 5%. There were 166 proteins significantly differentially abundant between the three groups considered. Lists of proteins significantly different in abundance can be viewed in the Appendix.

7.4.4 Proteomic changes common to a *gyrA* Asp94Gly and *rrs* A1401G mutant of *M. tuberculosis*

Proteome analysis led to the discovery that the *rrs* and *gyrA* mutants shared common differences to the wild-type clone as shown in Table 7.1 and Figure 7.6. Altered protein abundances were highly significant and more than 1.5-fold. Furthermore, all proteins shared between these analyses were always concordant with respect to being more or less abundant when the mutant was compared to the wild-type clone.

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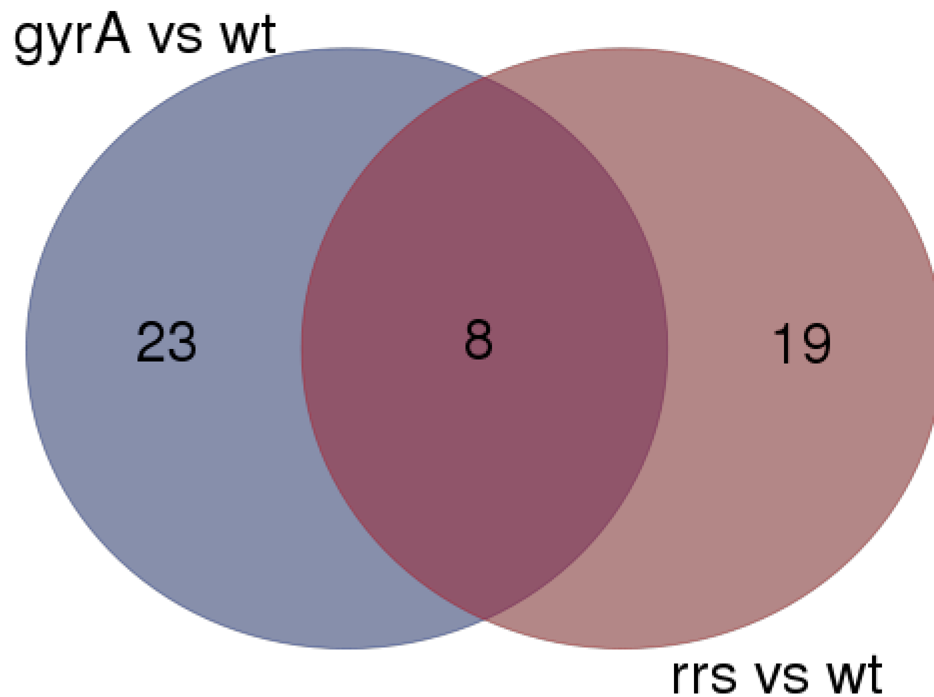

Figure 7.6 Venn diagram of proteins commonly and uniquely differentially abundant by at least 1.5-fold in the *gyrA* mutant vs wild-type and the *rrs* mutant vs wild-type analyses.

Table 7.1 Proteins changed in abundance in both the *gyrA* and *rrs* mutant when compared to wild-type.

Proteins increased in abundance	**Fold change in *rrs* mutant**	**Fold change in *gyrA* mutant**
Rv2558	1.5	1.7
Proteins decreased in abundance	**Fold change in *rrs* mutant**	**Fold change in *gyrA* mutant**
EccC5 (Rv1783)	1.6	1.6
EspG5 (Rv1794)	2.7	2.3
EccD5 (Rv1795)	1.6	2.3
MycP5 (Rv1796)	1.5	2.1
Rv2005c	2.9	1.7
Ald (Rv2780)	3.1	2.6
Lat (Rv3290c)	2.2	1.8

Of note are several proteins expressed from the ESX-5 cluster, indicated in Figure 7.7. Also of note is alanine dehydrogenase (Ald). Both the ESX-5 cluster and Ald share a common regulatory mechanism, namely the SenX3-RegX3 regulatory system (Elliott & Tischler 2016a; Elliott & Tischler 2016b; Roberts *et al.* 2011), which is sensitive to phosphate limitation (Elliott & Tischler 2016a).

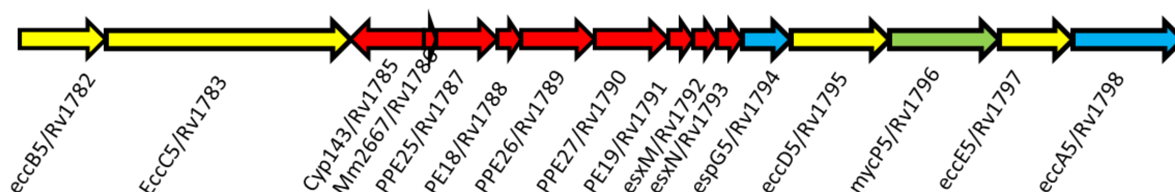

Figure 7.7 ESX-5 region of *M. tuberculosis.* Members of the transmembrane complex utilised in protein export are in yellow, cytosolic components are in blue, the membrane component mycosin P5 is in green, secreted proteins are in red.

The most likely explanation for the commonly altered protein abundance is a common genetic variant. It is likely that W7, the sensitive single colony isolate of the progenitor strain, K636, harboured an unknown mutation which resulted in increased ESX-5 expression. We assumed W7 to be identical to K636, except for the heterogeneity with respect to the TB16.3 synonymous SNP (see Figure 7.5). However, we cannot rule out that W7 may have acquired additional spontaneous mutations during subsequent subculturing processes, especially selection of a single colony on a plate. Whole genome sequencing of W7 was performed using Ion Torrent technology and resulting SNP/indel differences to H37Rv were compared to the differences observed for BO10 and BA23 after Illumina sequencing. Ion Torrent sequencing of *M. tuberculosis* yields many false positives (personal communication, Dr Anzaan Dippenaar, Division of Molecular Biology and Human Genetics, Stellenbosch University). Therefore comparing SNP/indel differences among W7 and the Illumina results yielded results of low confidence.

The clear lowered abundance of the entire ESX-5 cluster led to an analysis of the lists of SNP/indel differences between W7 and BO10, as well as between W7 and BA23. These were analysed for the presence of variants in genes and regions surrounding these genes that could explain lowered ESX-5 protein abundance, which would be shared between the comparisons. Results are in the Appendix. This analysis included ESX-5, *SenX3-RegX3* and *pst* genes. *Pst* genes were included because ESX-5 was shown to be regulated in response to phosphate starvation and deletion of a phosphate transporter, PstA1, and this affected how RegX3

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controlled ESX-5 expression (Elliott & Tischler 2016b). This analysis yielded no genetic variants that could explain increased ESX-5 expression in W7.

Importantly, whole genome sequencing via the Illumina platform also has limitations in terms of providing a full picture of genomic differences between W7 or K636, BA23 and BO10, since the isolates used in our study originated from a Beijing clinical isolate, while H37Rv was used as the only available reference strain in bioinformatic analyses of whole genome sequencing. There are large-scale genomic differences between clinical isolates and H37Rv (Fleischmann *et al.* 2002), which means that there are regions of the Beijing genome that cannot be aligned to the reference strain. Any polymorphisms in such regions will be missed. In addition, the mycobacterial genome has a high GC-content and harbours many repeats, especially in PE/PPE/PGRS proteins. This means that some regions cannot be amplified by PCR, while other regions display low mappability. Polymorphisms in such regions will also remain undetected, as these regions are filtered out when controlling for quality (Galagan 2014).

Future work is needed to investigate any potential genomic variants between W7, BO10 and BA23. W7 is currently being analysed by sequencing on the Illumina platform to enable direct comparison to BO10 and BA23. A proteogenomic approach could also be followed, where peptide sequences obtained during mass spectrometry could be used to detect variants. Importantly, for future work involving whole genome sequencing, it is essential for a reference strain more closely related to clinical strains circulating in the region to be assembled. This would enable the identification of more genomic variants. Identification of genomic variants in concert with proteomics provides a promising approach for further understanding of the physiology of drug-resistant *M. tuberculosis.*

We cannot exclude the possibility that both the *rrs* and *gyrA* mutants acquired an unknown genetic variant during the evolution of drug resistance that would explain commonly altered protein abundance. This is particularly relevant given the recent discovery of a SNP in EccC5 that confers fluoroquinolone resistance (Eilertson *et al.* 2016). Eilertson *et al.* (2016) suggested that the mutation leads to reduced functionality of ESX-5 and reduced uptake of ofloxacin across the cell envelope. This is plausible since ESX-5 is involved in the uptake of nutrients (Ates *et al.* 2015). They showed that it is probably not as a result of efflux - a likely hypothesis in light of *Rv1782/1783* constituting an ATPase - since efflux pump inhibitors did not reduce the ofloxacin MIC of EccC5 mutants in the study by Eilertson *et al.* (2016). A

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recent study (Ates *et al.* 2015) also showed that ESX-5 plays an essential role in controlling the outer membrane permeability of *Mycobacterium marinum*, a conclusion that emerged from the observation that essentiality of *eccC5* and *mycP5* could be circumvented when the permeability of the outer membrane was increased. The permeability of the outer membrane was increased by heterologous expression of the porin, MspA, which rescued growth of the *esx-5* deletion mutants, and transposon mutants deficient in genes involved in biosynthesis of phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGLs) were able to tolerate the deletion of *eccC5* or *mycP5*. Deficiencies in PDIM and PGL biosynthesis likely affects the integrity of the cell envelope negatively, since these transposon mutants were more sensitive to various antibiotics (Ates *et al.* 2015). Taking this into account, the complete loss of ESX-5 may result in an impermeable outer membrane, which is detrimental to growth of *M. marinum.* However, a reduction in expression, as observed in our study, may be tolerated by the bacterium and even become beneficial to enable drug tolerance. Whether downregulation of ESX-5 evolved as a common drug resistance mechanism during the process of selecting for drug resistance mutations will require further investigation.

The other proteins in Table 7.1 are of interest, since they may be co-regulated with ESX-5 by the same mechanism that still remains to be elucidated. Ald (Rv2780) is involved in alanine metabolism and specifically in the pathway where alanine is incorporated into peptidoglycan and loss of function of this protein was shown to confer D-cycloserine resistance (Desjardins *et al.* 2016). The universal stress protein Rv2005c has not been well characterised, however, its expression is increased during hypoxia (Hingley-Wilson *et al.* 2010). Deletion of the gene did not affect survival of *M. tuberculosis* under various stress conditions or intracellular infection and it was suggested that the protein may be redundant (Hingley-Wilson *et al.* 2010). Lat and Rv2558 may play roles in persistence (Duan *et al.* 2016; Luthra *et al.* 2008). However, this has been challenged for Rv2558 (Gordhan *et al.* 2006). The function of Rv0036c is currently unknown.

Due to the strong possibility that shared protein abundance changes could be due to a mutation other than *gyrA* or *rrs,* we excluded these proteins from further analyses in order to elucidate the unique impact of the *gyrA* or *rrs* mutations on the proteome, as shown in Figure 7.8.

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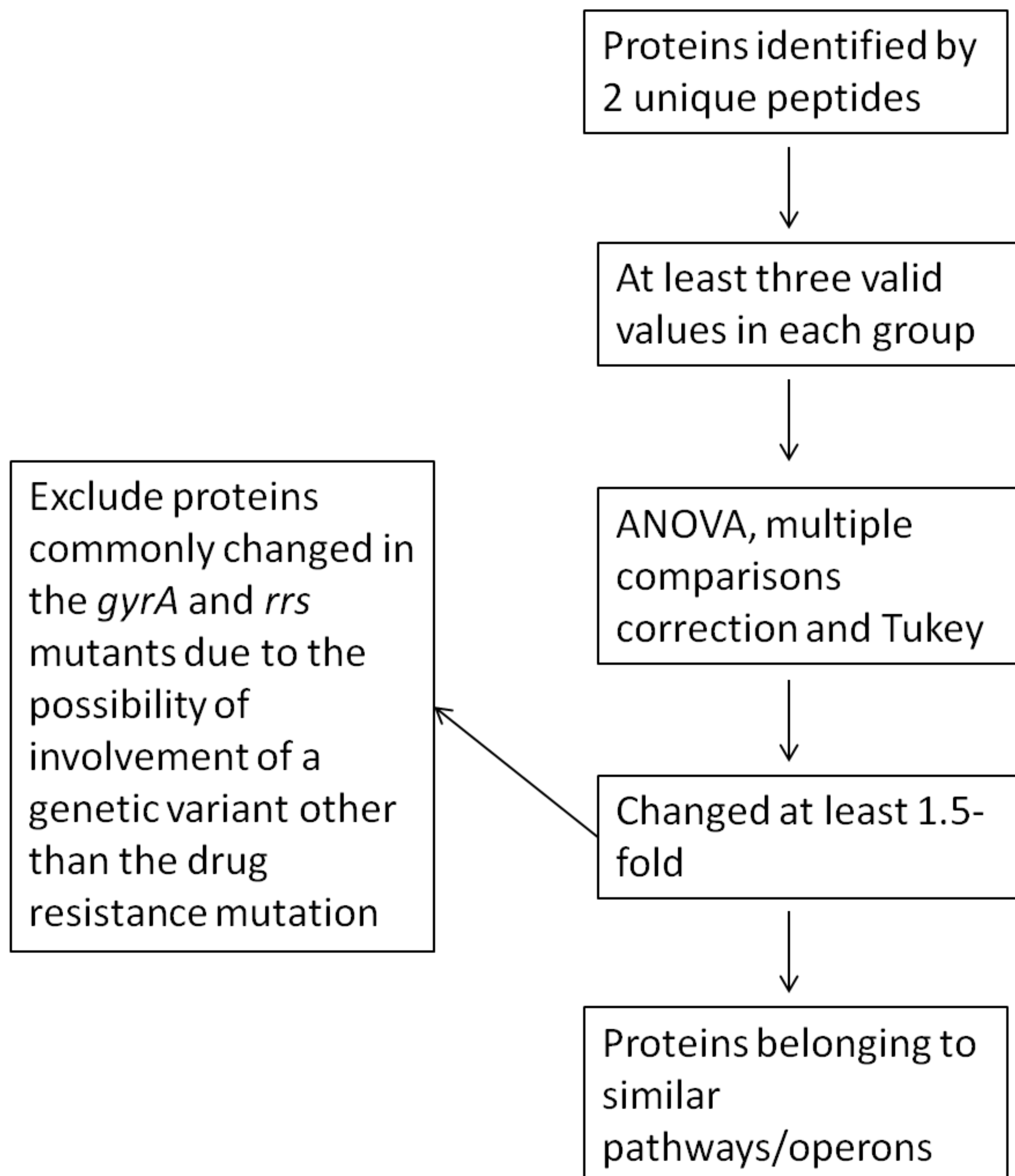

Figure 7.8 Approach taken to elucidate proteome changes due to only the *gyrA* or *rrs* drug resistance mutations

7.4.5 The proteome of a fluoroquinolone-monoresistant strain of *M. tuberculosis*

Comparison between W7 (treated with antibiotic diluent) and BO10 (*gyrA* Asp94Gly mutant treated with antibiotic diluent), showed 113 proteins which were significantly more abundant - of which 9 were at least 1.5-fold higher - and 33 proteins which were significantly less abundant - of which 22 were at least 1.5-fold lower - in the strain with the *gyrA* mutation.

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Seven proteins decreased and one protein also increased in abundance in the *rrs* mutant were excluded from the analysis.

7.4.5.1 Potential impact of Rv3169 mutation

The mutation in *Rv3169* is a spontaneous mutation that may have been selected for two reasons: 1). It may be a mutation compensating for loss of fitness due to the *gyrA* mutation (which could explain why no difference in growth was observed between BO10 and W7), which would include mechanisms for further increasing resistance to fluoroquinolones. 2) It may simply be a neutral spontaneous mutation not affecting the growth of this mutant under *in vitro* conditions. After analysis of the Glu121Gly SNP in Rv3169, using PROVEAN (Choi *et al.* 2012), which predicts the functional effects of SNPs and indels, this mutation was predicted to be deleterious. In accordance with this, Rv3169 was significantly less abundant by 1.9-fold in the *gyrA* mutant, which suggests that it is degraded as a missense peptide. The function of Rv3169 is not known, but it is known that the protein contains an AttH-like domain (Mao *et al.* 2012), a domain which likely plays a role in lipid metabolism, specifically lipid biosynthesis (Gough *et al.* 2001). Many enzymes involved or possibly involved in lipid metabolism were differentially abundant in the *gyrA* mutant, as shown in Table 7.2, however, all except Rv0504c were less than 1.5-fold changed in abundance, suggesting that the impact was not significant (see 7.4.3). These proteins may be regulated as a result of loss of function by Rv3169, however, this remains to be demonstrated.

There is some evidence that the decreased abundance of Rv3169, which in turn may have become genetically fixed as a result of the acquisition of fluoroquinolone resistance, may be drug resistance-related. Rv3169 was decreased in abundance in three MDR clinical isolates when compared with susceptible clinical isolates (Phong *et al.* 2015). It is not clear how and if the resistant and susceptible strains were related, i.e. whether the susceptible isolates were progenitors of the resistant isolates, therefore this change could be strain-specific, but could also be as a result of the evolution of drug resistance. The mutation in Rv3169 has not thus far been reported as positively selected in drug-resistant isolates in recent studies (Desjardins *et al.* 2016; Farhat *et al.* 2013).

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Table 7.2 Proteins involved in lipid metabolism differentially abundant in an Asp94Gly *gyrA* mutant of *M. tuberculosis* when compared to wild-type.

Proteins more abundant in *gyrA* mutant	**Fold change**	**Function**
FabH (Rv0533c)	1.3	Mycolic acid synthesis
Rv0504c (possibly hadA)	1.6	Mycolic acid synthesis
CmaA1 (Rv3392c)	1.2	Mycolic acid synthesis
FadD21 (Rv1185c)	1.3	Formation of diacyltrehaloses
LppX (Rv2945c)	1.3	PDIM transport
Rv0281	1.4	Possible S-adenosylmethionine-dependent methyltransferase involved in polyketide biosynthesis
Rv0731c	1.2	May be O-methyltransferase involved in polyketide biosynthesis
EchA16 (Rv2831)	1.3	β-oxidation of fatty acids
FadE15 (Rv1476c)	1.2	β-oxidation of fatty acids
EchA6 (Rv0905)	1.4	β-oxidation of fatty acids
Rv0813c	1.4	May bind fatty acids
Proteins less abundant in *gyrA* mutant		
EchA15 (Rv2679)	1.3	β-oxidation of fatty acids
Rv1866	1.3	May be involved in lipid degradation

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7.4.5.2 Transport across the cell envelope

Several proteins involved in transport across the cell envelope were reduced in abundance in the *gyrA* mutant. These are summarised in Table 7.3.

Table 7.3 Proteins involved in transport and differentially abundant in the *gyrA* Asp94Gly mutant compared to wild-type

Protein	Fold change	More/less abundant	Secretion system
EccD3 (Rv0290)	2.4	Less	ESX-3
$EccCa_1$ (Rv3870)	1.3	Less	ESX-1
YajC (Rv2588c)	2.6	Less	Sec
EspA (Rv3616c)	1.9	More	ESX-1
PPE68 (Rv3873)	1.5	More	ESX-1
Rv3104c	2.1	Less	Potassium efflux
Rv0178	2.0	Less	Mce1-associated protein, involved in lipid transport
Rv2326c	1.7	Less	Probable ABC transporter

ESX-3 secretes virulence factors and plays an important role in the uptake of iron (Tufariello *et al.* 2016). The ESX-1 system is regarded as one of the most important virulence determinants, secreting important virulence factors such as EsxA, EsxB and Esp proteins (Blasco *et al.* 2012) which interact with the host cell. ESX-1 may also play a role in cell wall stability, however. An ESX-1-associated protein previously reported to affect cell wall integrity (Garces *et al.* 2010) is EspA. Decreased cell wall integrity was inferred from greater sensitivity to SDS and other detergents (Garces *et al.* 2010) when EspA was mutated in such a way as to abolish its ability to dimerise via disulphide bond formation. Deletion of the whole *espACD* region as well as ESX1, resulted in the same trend, suggesting that ESX1 and its associated proteins are involved with cell wall integrity in some way (Garces *et al.* 2010). However, in our study, $EccCa_1$ was less abundant, but since EspA is a substrate for the ESX-1 secretion system (Garces *et al.* 2010), our results may indicate that downregulation of ESX-1 components leads to accumulation of EspA in the cytosol. This leads to the question whether EspA would still exert its effect on cell wall integrity if it is not secreted. Garces *et al* (Garces *et al.* 2010) showed that EspA was not secreted in cells grown without detergent,

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while others (Sani *et al.* 2010) showed that ESX-1 substrates are not secreted, but associated with the mycobacterial capsule in minimally disturbed cultures. Therefore, given that the cultures in our study were grown in the presence of low concentrations of detergent, it is possible that preventing loss of EspA by secretion may strengthen the cell wall. However, this remains to be demonstrated. Another ESX1 substrate, PPE68 (Rv3873) was 1.5-fold more abundant in the *gyrA* mutant. This protein has been suggested to act as an ESX-1 gating protein (Brodin *et al.* 2004; Sampson 2011), where, if *ppe68* was deleted, increased secretion of EsxA (ESAT-6) and EsxB (CFP-10) was observed.

Two proteins possibly involved in lipid transport were decreased in abundance in the *gyrA* mutant, namely Rv0178 and Rv2326c. Rv0178 forms part of the *mce1* operon (Casali *et al.* 2006), which was suggested to re-import mycolic acids (Forrellad *et al.* 2014). Rv2326c, a probable ABC transporter, was shown to be regulated by Rv0494, a lipid-responsive transcriptional regulator (Yousuf *et al.* 2015).

YajC is a preprotein translocase which increases the efficiency of Sec transport, responsible for the export of unfolded peptides across the membrane. The Sec system is also involved in inserting proteins into the membrane (Feltcher *et al.* 2010). Rv3104c is an uncharacterised transmembrane protein that may be involved in potassium efflux (Wattam *et al.* 2014).

The decreased abundance of transport proteins may suggest a phenotype of decreased cell envelope permeability, as was suggested for ESX-5 (Eilertson *et al.* 2016). Whether this is the case, and whether this represents a drug resistance mechanism, would require further investigation. However, where YajC was less abundant in the untreated *gyrA* mutant (see Table 7.3), it was substantially (3.5-fold) more abundant when the *gyrA* mutant was treated with ofloxacin compared to the untreated mutant (data not shown). This may suggest that decreased abundance of this protein is not relevant to drug resistance. ESX-1 and its associated proteins may be involved in cell wall stability, resulting in increased resistance to antibiotics. The theory that a fluoroquinolone-resistant mutant could be cross-resistant to other antibiotics, especially those targeting the cell wall, is plausible (Suzuki *et al.* 2014). An enoxacin-resistant (fluoroquinolone-resistant) laboratory-generated mutant of *E. coli* exhibited increased MICs to β-lactam antibiotics, which target the cell wall.

7.4.5.3 Direct effects of the *gyrA* mutation on supercoiling and related processes

In *Salmonella enterica,* the *gyrA* Asp87Gly mutation, frequently seen in fluoroquinolone-resistant *Salmonella* strains and equivalent to the *gyrA* Asp94Gly mutation in *M. tuberculosis*

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(when the sequences are aligned, data not shown), resulted in a decrease in supercoiling (Webber *et al.* 2013). A decrease in supercoiling density affects transcription, DNA replication, chromosome segregation, recombination and transposition (Rovinskiy *et al.* 2012). Furthermore, it was shown in *Salmonella* that there is fine-tuning of the rates of coupled transcription-translation and supercoiling density, ensuring that the rates are matched. (Rovinskiy *et al.* 2012). Therefore, the negative effects of gyrase mutations on supercoiling density could be alleviated by slowing of transcription and translation (Rovinskiy *et al.* 2012). Proteins involved with the transcription/translation process include Gre factors and ribosomes amongst others (Rovinskiy *et al.* 2012). In agreement with this, our study showed 1.8-fold increased abundance of Rv3788, assigned as potential transcription elongation factor GreB (Mao *et al.* 2012). Gre proteins are necessary for rescuing stalled transcription complexes by cleavage of the nascent transcript (China *et al.* 2011). However, Rv3788 did not show any mRNA cleavage activity (China *et al.* 2011). Instead, Rv3788 was shown to be an inhibitor of transcription, independent of sequence (China *et al.* 2012). The increased abundance of Rv3788 may therefore play a role in regulation of the rate of transcription. Two ribosomal subunits were 2- and 1.8-fold less abundant respectively in BO10, namely RplK (Rv0640) and RplJ (Rv0651). Their decreased expression may also be related to a slowing of the transcription-translation process.

Our results suggested the possibility that the mutation in *gyrA* leads to a fine-tuning in rates of transcription and translation. However, this contrasts with results from a recent study which show that the Asp94Gly mutation does not affect supercoiling or cleavage activity of *GyrA* in *M. tuberculosis* (Aldred *et al.* 2016). It also contrasts with our results, where no difference in growth rate between the *gyrA* mutant and the wild-type clone was observed as shown in Figure 7.4. Perhaps the differences were too small to be measurable by the assays employed in the study by Aldred *et al.* (2016) or in our study. This requires further investigation.

7.4.5.4 Conclusion

In conclusion, the *gyrA* Asp94Gly mutant when compared to the wild-type clone originating from the progenitor displayed differential abundance of proteins potentially involved in decreasing cell envelope permeability. There were transport and associated proteins that may have decreased permeability of the cell wall and increased cell wall stability. Although controversial, there was some indication that the *gyrA* mutation affected supercoiling of the *M. tuberculosis* genome as evidenced by the slowing of transcription and translation.

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7.4.6 Fluoroquinolone treatment of a *gyrA* mutant of *M. tuberculosis*

Comparison between BO10 (treated with 2 µg/ml ofloxacin) and BO10 (treated with diluent only), yielded 115 proteins more abundant – of which 38 were at least 1.5-fold more - and 102 less abundant – of which 22 were at least 1.5-fold less.

7.4.6.1 Iron metabolism

Fluoroquinolones have been shown previously to be potent iron chelators (Badal *et al.* 2015). We therefore propose that ofloxacin treatment of *M. tuberculosis* leads to an iron-limiting environment, which leads to the induction of proteins involved in iron uptake. This is supported by our observation that the presence of ofloxacin led to a significant increase in the abundance of proteins ViuB (Rv2895c, 1.6-fold), BfrA (Rv1876, 1.7-fold), EccD3 (Rv0290, 1.7-fold) and HemC (Rv0510, 2.3-fold) involved in iron acquisition. ViuB, together with IrtB, were shown to constitute a siderophore importer, where siderophores are iron-chelating molecules used to scavenge iron from the host (Farhana *et al.* 2008). BfrA was shown to be an iron storage protein (Reddy *et al.* 2012), while EccD3 forms part of the ESX-3 secretion system, which is important in the uptake of siderophore-associated iron (Tufariello *et al.* 2016). Taken together, the over-abundance of these genes may indicate the increased uptake of extracellular iron in response to iron limitation. The increased abundance of BfrA contradicts a previous study which showed that iron limitation did not lead to the upregulation of iron storage proteins in medium with low levels of iron, while it did in macrophages (Reddy *et al.* 2012). Therefore it is not clear whether its increased abundance is as a result of iron limitation. Bacterioferritins, like BfrA, contain heme, and HemC may be involved in porphyrin biosynthesis (Lew *et al.* 2011), although this protein has not been characterised in *M. tuberculosis* to our knowledge. A model for how ofloxacin treatment may affect iron metabolism is shown in Figure 7.9.

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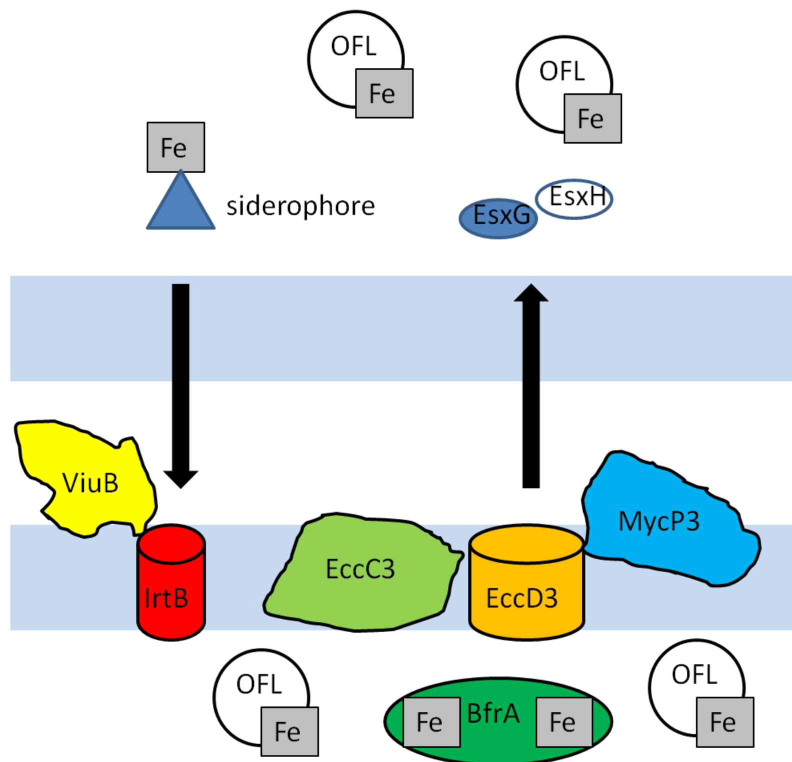

Figure 7.9 Proposed model for the effect of the presence of ofloxacin on the uptake and storage of iron. Ofloxacin sequesters iron, creating an iron-limiting environment, which leads to the upregulation of several components involved in the uptake and storage of iron. ViuB/IrtB import ferrated siderophores.The Esx-3 complex, especially EccD3, is possibly involved in the export of EsxG/H, which may play a positive role in siderophore import (Siegrist *et al.* 2014). BfrA stores iron intracellularly. ViuB, EccD3, and BfrA were more abundant after ofloxacin treatment in our study.

7.4.6.2 Growth rate

Changes in abundance of specific proteins suggested a downregulation of the growth rate in response to ofloxacin treatment. These proteins are shown in Table 7.4. Firstly, SigA has been shown to mediate growth of *M. tuberculosis in vivo*, since recombinant strains of *M. tuberculosis* expressing antisense transcripts to *sigA* showed decreased growth in mice lungs and in macrophages (Wu *et al.* 2004). However, this was not the case *in vitro.* Several proteins encoded by genes surrounding the origin of replication (Stewart *et al.* 2004), as shown in Figure 7.10, were altered in abundance. CwlM has been shown to affect surface growth, cell division, bacterial pathogenesis, adhesion and invasion of host cells (Deng *et al.* 2005), while the elimination and overexpression of ParB results in growth inhibition in *M.smegmatis,* suggesting an important role in cell cycle progression (Maloney *et al.* 2009). DnaJ1 may have been responsible for the downregulation of genes surrounding the origin of

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replication, although this is not clear. Its overexpression in *M. bovis* resulted in increased expression of genes surrounding the origin of replication, many of them involved in DNA replication (Stewart *et al.* 2004). In our study, the decreased abundance of DnaJ1 may have led to the decreased expression of several genes surrounding the origin of replication. However, the relationship between decreased abundance of DnaJ1 and other proteins encoded by genes surrounding the origin of replication may not be that simple, since it was shown that changing the stoichiometric ratio of this protein to interacting partners may be more important than whether it is more or less abundant (Stewart *et al.* 2004).

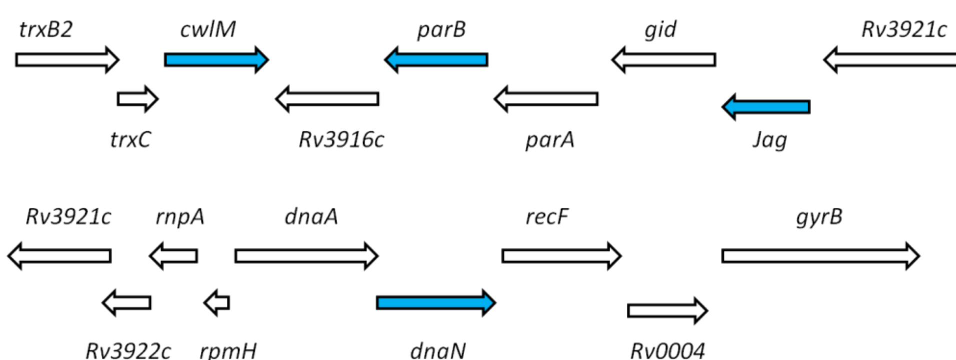

Figure 7.10 Genes surrounding the origin of replication in *M. tuberculosis* H37Rv. Proteins expressed from genes shaded in blue were less abundant after ofloxacin treatment.

Table 7.4 Summary of proteins involved in cell division and growth, decreased in abundance after treatment of BO10 with ofloxacin

Protein	Fold change decreased abundance	Function
SigA (Rv2703)	1.6	Primary sigma factor that regulates growth rate
CwlM (Rv3915)	2.1	Essential N-acetylmuramoyl-L-alanine amidase
ParB (Rv3917c)	2.0	Chromosome partitioning
Jag (Rv3920c)	1.3	Unknown

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DnaN (Rv0002)	1.2	Beta chain of DNA polymerase III, responsible for the initiation of replication
DnaJ1 (Rv0352)	1.8	Co-chaperone
Wag31 (Rv2145c)	1.4	Essential protein that mediates cell division
PbpA (Rv0016c)	1.5	Peptidoglycan synthesis
Rv3677c	1.6	Beta-lactamase
VapB10 (Rv1398c)	2.9	Antitoxin
EspJ (Rv3878)	1.9	ESX-1 associated protein

Apart from the proteins expressed from the genes in Figure 7.10, loss of PbpA (Rv0016c) resulted in a division defect in *M. smegmatis* (Pavelka *et al.* 2014). Rv3677 is a beta-lactamase possibly involved in peptidoglycan metabolism and therefore potentially in the modification of the cell wall during cell division (Pavelka *et al.* 2014). Toxins and antitoxins play roles in the regulation of the growth rate as evidenced by how the inducible overexpression of toxins in *M. smegmatis* and *M. tuberculosis* led to growth inhibition (Ahidjo *et al.* 2011). A decrease in antitoxin would lead to increased free cognate toxin, which may lead to the downregulation of the growth rate (Ahidjo *et al.* 2011). Overexpression of vapB antitoxin in *M. smegmatis* (Demidenok *et al.* 2014) prevented this organism from entering dormancy, therefore the decrease in antitoxin may also lead to a propensity for the *gyrA* mutant to enter dormancy when under ofloxacin treatment. Lastly, EspJ has been suggested to play a role in growth rate regulation (Singh *et al.* 2015).

Taken together, we observed decreased abundance of proteins involved in replication, cell division and chromosome partitioning. We propose that ofloxacin treatment leads to a decrease in growth rate, which may mean a greater propensity for the bacterium to enter a persistent, non-replicating state. This would require further investigation. However, we did not observe decreased growth of a similar mutant under ofloxacin treatment (see Figure 5.1(E)). In our study, the antibiotic was added at lag phase in that case, whereas in the proteomics experiment, the antibiotic was added in mid-log phase and growth was not monitored after addition of antibiotic. In addition, strains were cultured in rich 7H9 medium

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in Chapter 5, whereas proteomics experiments were performed in minimal media. It is also possible that ofloxacin, despite resistance of the *gyrA* mutant, still results in a stress response as evidenced by a downregulation of the growth rate.

7.4.6.3 Conclusion

In conclusion, ofloxacin may sequester iron leading to iron limited conditions. The increased abundance of proteins involved in iron uptake after ofloxacin treatment of BO10 indicated this possibility. As such, this adaptive response constitutes a compensatory fitness mechanism. The implication is that the availability of iron may impact ofloxacin resistance in some way and that drugs targeting the uptake or availability of iron may synergise with ofloxacin treatment. Such drugs are currently under development (Meneghetti *et al.* 2016). Furthermore, ofloxacin treatment may result in decreased growth of a *gyrA* mutant.

7.4.7 The proteome of an *rrs* A1401G mutant of *M. tuberculosis*

Comparison between W7 (treated with antibiotic diluent) and BA23 (*rrs* A1401G mutant treated with antibiotic diluent), showed 54 proteins which were significantly more abundant - of which 7 were at least 1.5-fold higher - and 29 proteins which were significantly less abundant - of which 20 were at least 1.5-fold lower - in the strain with the *rrs* mutation. Eight proteins decreased and three proteins also increased in abundance in the *gyrA* mutant were excluded from the analysis.

7.4.7.1 Stress proteins and the DosR/DevR regulon

Proteins belonging to the DosR (also known as DevR) regulon were less abundant in the *rrs* mutant compared to the wild-type clone. These included the universal stress proteins Rv1996 (1.9-fold), Rv2005c (2.9-fold) and Rv2623 (2.5-fold). The stress protein HspX (Rv2031c) was also 2.7-fold less abundant, as well as the uncharacterised Rv2629 (1.7-fold). Although Rv2005c was also less abundant in the *gyrA* mutant, this protein was increased in abundance by a higher fold in the *rrs* mutant than in the *gyrA* mutant (2.9- vs 1.7-fold). These stress proteins, in a previous study, were induced by hypoxia, nitric oxide and adaptation to low oxygen *in vitro*, resulting in an *in vitro* dormant state (Voskuil *et al.* 2003). It is not clear what the impact of the decreased abundance of these proteins would be. Rv1996 and Rv2005c were previously suggested to be functionally redundant in *M. tuberculosis*, since mutation of these genes did not impact survival of the organism under hypoxic and other stress conditions, including infection of macrophages (Hingley-Wilson *et al.* 2010). There is some evidence that Rv2623 may be involved in regulating the growth rate and induction of

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dormancy – its overexpression reduces growth (Drumm *et al.* 2009). Since the other universal stress proteins also belong to the dormancy regulon (Voskuil *et al.* 2003), they may play a similar role. Further work is required to understand how and if acquisition of an *rrs* mutation could impact the growth rate and the ability to enter a dormant state of *M. tuberculosis* in any way.

7.4.8 Amikacin treatment of an *rrs* mutant of *M. tuberculosis*

Treatment of an *rrs* A1401G mutant of *M. tuberculosis* with 1 µg/ml amikacin resulted in 17 proteins more abundant - of which none were more than 1.5-fold abundant – and 10 proteins less abundant – of which 4 proteins were decreased by more than 1.5-fold. Amikacin treatment therefore had very little impact on protein abundance and this could be ascribed to the high MIC of > 512 µg/ml imparted by the *rrs* mutation (data not shown). Three of the four proteins significantly impacted by amikacin treatment were ribosomal proteins RplD (Rv0702), RpmD (Rv0722) and RpsO (Rv2785c), which will be discussed further below. The fourth protein was Rv3519 of unknown function, but predicted to be in the KstR regulon, which controls lipid degradation (Kendall *et al.* 2007).

7.4.9 The *rrs* mutation and amikacin treatment synergise to impact translation

ANOVA of summed intensities for proteins across the three groups, namely wild type, *rrs* mutant untreated and *rrs* mutant treated with 1 µg/ml amikacin for 24 hours, yielded differential abundance of proteins involved in translation. It was not possible to distinguish whether this change was due to the presence of the *rrs* mutation, or whether as a result of treatment. In some cases, abundance was only significantly different when the treated mutant was compared to the untreated wild type, for example in the case of RplJ, RplV and RplD (see Table 7.5). In other cases, either the *rrs* mutation or amikacin treatment were responsible, as is the case for Rv1301 and TrmD, respectively. Thirdly, in some cases, the *rrs* mutation and amikacin treatment had a cumulative effect, for example as seen for RplP. Therefore we concluded that the *rrs* A1401G mutation and amikacin treatment had a similar impact on translation, which, in some cases, was cumulative.

Many structural ribosomal proteins were less abundant in the analysis, and some of these were subunits specifically involved in formation of the peptidyl-transferase centre, or binding of 16S rRNA. In contrast, enzymes involved in tRNA modification, tRNA charging and tRNA synthesis, binding of aminoacyl-tRNA to the A-site and translocation of the nascent

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peptide from the A- to the P-site were more abundant. Table 7.5 provides a list of these proteins.

First, it would be of value to understand amikacin action. Amikacin is a semi-synthetic 2-deoxystreptamine aminoglycoside (Wilson 2014). This particular class of aminoglycosides, which includes the natural antibiotic paromomycin (Wilson 2014), inhibits translation at particular steps of bacterial protein synthesis (shown in Figure 7.11) in five potential different ways: 1. inhibiting the translocating reaction 2. Inhibiting ribosome subunit recycling 3. reducing fidelity of translation by allowing binding of non-cognate tRNAs to mRNA (Wilson 2014) 4. Inhibiting 30S and 50S subunit assembly and 5. Inhibition of initiation factor 2 binding to formyl-methionyl-tRNA, the tRNA molecule that initiates protein synthesis (Evans *et al.* 2003).

It was striking that many of the ribosomal proteins less abundant in our study were specifically involved in the process impacted by amikacin, which, in turn, would also be impacted by the presence of the *rrs* A1401G mutation. This mutation, generally designated as A1408G in most organisms, was shown to decrease mobility of *rrs* A1492 and A1493 by forming hydrogen bonds (Romanowska *et al.* 2011). The mobility of the latter two residues is important for ensuring binding of cognate tRNAs (Romanowska *et al.* 2011). We propose therefore that the drug resistance mutation A1401G in our strain may impact the fidelity of the process of translation, but not sufficiently to confer a fitness defect. The effects of the mutation and the antibiotic are therefore at the A-site of the ribosome. RplD, RplV and RplF bind 23S rRNA, the ribozyme responsible for peptidyltransferase activity, while RpsO binds 16S rRNA (See Table 7.5). RplS may play a role in the function of the A-site. RplJ is involved in the functioning of the L7/L12 stalk of the ribosome, where tRNA enters the A-site (Bashan & Yonath 2008).

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Table 7.5 Proteins involved in translational processes in *M. tuberculosis* differentially abundant as a result of amikacin resistance and/or treatment

Protein ID	Protein name	Fold change in mutant vs wt	Fold change in treated mutant vs untreated mutant	Fold change in treated mutant vs untreated wild type	More/ less abundant	Function
Rv0429c	Def	NS[a]	NS	1.5	More	Deformylase involved in translation
Rv0684	FusA1	1.2	1.1	1.4	More	Promotes translocation.
Rv0685	Tuf	NS	NS	1.2	More	Promotes aminoacyl-tRNA binding to A-site
Rv1301	Rv1301	1.5	NS	1.6	More	May be TsaC protein (YrdC domain) required for threonyl-carbamoyl-adenosine t(6)A37 modification in tRNA
Rv1649	PheS	1.6	NS	1.7	More	Phenylalanyl-tRNA synthetase alpha chain
Rv1650	PheT	1.4	NS	1.5	More	Phenylalanyl-tRNA synthetase beta chain

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Rv2555c	AlaS	1.2	NS	1.2	More	Alanyl-tRNA synthetase
Rv2906c	TrmD	NS	NS	1.5	More	Methylates guanosine-37 in various TRNAS
Rv3580c	CysS1	NS	NS	1.3	More	Cysteinyl-tRNA synthetase
Rv0651	RplJ	NS	NS	1.5	Less	50S ribosomal protein L10
Rv0702	RplD	NS	NS	2.0	Less	50S ribosomal protein L4 which binds directly to 23S rRNA.
Rv0706	RplV	NS	NS	2.0	Less	50S ribosomal protein L22, specifically binds 23S rRNA, important during re-constitution of 50S.
Rv0708	RplP	1.3	1.4	1.9	Less	50S ribosomal protein L16, directly binds 23S rRNA and is located at the A-site of the peptidyl-transferase center.

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Rv0716	RplE	NS	NS	1.4	Less	50S ribosomal protein L5, mediates attachment of the 5S RNA into the large ribosomal subunit.
Rv0719	RplF	1.2	1.3	1.6	Less	50S ribosomal protein L6, binds directly to 23S rRNA and is located at the A-site of the peptidyl-transferase center.
Rv0722	RpmD	NS	5.0	12.8	Less	50S ribosomal protein L30.
Rv2785c	RpsO	NS	1.5	1.6	Less	30S ribosomal protein S15, binds 16S rRNA.
Rv2904c	RplS	1.3	1.3	1.7	Less	50S ribosomal protein L19, located at the 30S-50S ribosomal subunit interface. May play a role in the structure and function of the A-site.

[a]NS, not significant

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The reasons for the decreased expression of these, and other ribosomal proteins, are not clear. It may be that 16S rRNA plays a regulatory role in expression of some of the operons encoding ribosomal protein genes and that the mutation changes their regulation. Alternatively, the mutation in 16S rRNA may affect the rate of degradation of ribosomal proteins. There is some evidence that mutations in the active site of the ribosome may lead to degradation of the ribosome (Paier *et al.* 2015), although mutation of residue 1492 in 16S rRNA did not have this result in *E. coli.* It is also possible that the inhibition of ribosome assembly by amikacin, as discussed above, may lead to increased degradation of ribosomes, since it has been shown that disassembled 50S and 30S subunits are more prone to degradation in *E. coli* (Zundel *et al.* 2009). These hypotheses need to be tested.

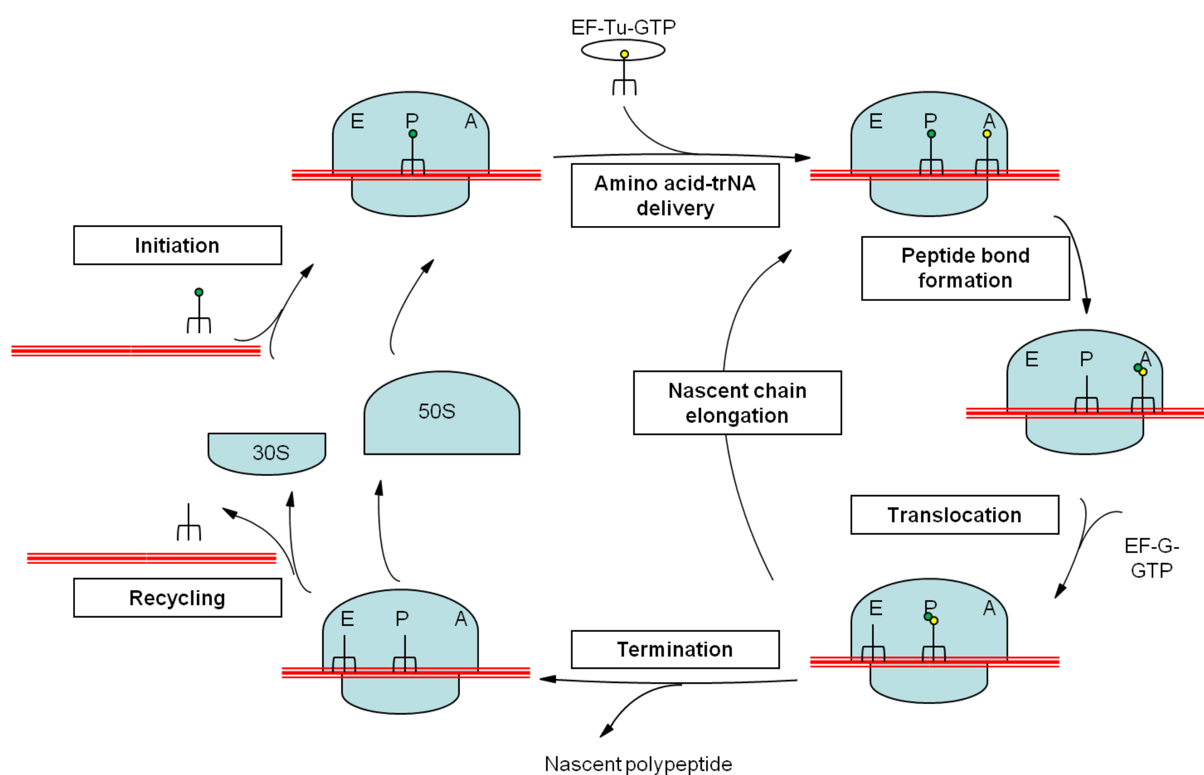

Figure 7.11 Steps of bacterial protein synthesis.

The decrease in abundance of these ribosomal proteins may explain why enzymes involved in synthesis of tRNAs were in turn more abundant (see Table 7.5). This may represent a compensatory mechanism to combat the slower rate of protein synthesis, specifically the slower rate of the peptidyltransferase reaction. The increase in proteins involved in tRNA modification may, in turn, represent a mechanism to compensate for reduced binding of cognate tRNAs, potentially brought about by the *rrs* mutation and/or amikacin treatment as

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explained above. Of note are Rv1301, an essential, but as yet uncharacterised protein in *M. tuberculosis,* which was predicted to be a threonyl-carbamoyl AMP synthase (Mao *et al.* 2012) involved in modification of tRNA, and TrmD, an essential tRNA methyltransferase. Modifications to tRNA is essential for accurate decoding of mRNA (Thiaville *et al.* 2015). This also indicates the need for studying and characterising Rv1301.

7.4.9.1 Conclusion

To conclude the effect of amikacin resistance and treatment on the proteome of *M. tuberculosis:* differential abundance of proteins involved in translation reflected the specific processes impacted by the *rrs* mutation and/or amikacin treatment. It is possible that the mutation in 16S rRNA plays a regulatory role in expression of ribosomal proteins involved in the peptidyltransferase reaction. Alternatively, the mutation or amikacin treatment may lead to increased ribosomal degradation. The decrease in ribosomal proteins and therefore translation may be compensated for by increased tRNA synthesis. The 16S rRNA mutation and/or amikacin treatment may also lead to a slight loss in translation fidelity, which may be compensated for by increased tRNA modification.

7.5 Overall conclusion

Relative quantitative proteomic comparisons of the wild type susceptible strain to *gyrA* Asp94Gly and *rrs* a1401G mutants of *M. tuberculosis* respectively, led to proteins commonly altered in both mutants. The differences were highly significant and clearly involved several proteins expressed from the same cluster, namely the ESX-5 cluster (Ates *et al.* 2015) amongst other proteins. This indicated strong possibilities that 1) W7 harbours an unknown mutation driving this difference 2) both mutants acquired a common variant during evolution of drug resistance. There is some indication that decreased expression of ESX-5 could play a role in fluoroquinolone resistance (Eilertson *et al.* 2016), providing some evidence for event 2). Further work is required to accurately detect genomic variants between W7, BO10 and BA23 and Illumina sequencing of W7 is therefore currently under way. Our study also indicated the need for a reference strain that is more reflective of the genomic diversity of clinical strains circulating in our region, which would further improve accuracy in detecting genomic variants.

To elucidate the individual contributions of each drug resistance-conferring mutation on proteomic changes, we therefore excluded the commonly altered proteins. Our results showed that the *gyrA* Asp94Gly or *rrs* A1401G mutations did have an impact on protein abundance in

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M. tuberculosis. Many proteins involved in transport, specifically protein export, were less abundant in the Asp94Gly mutant, suggesting a less permeable cell envelope. Altered proteins also involved ESX-1-associated proteins which suggested an increase in cell wall stability. Whether these mechanisms are relevant to drug resistance, needs to be tested further. The *gyrA* mutation may also have affected transcription-translation coupling, suggesting that it may affect the function of the gyrase enzyme, another possibility that requires further testing. The *rrs* mutant compared to wild-type had decreased abundance of several stress proteins belonging to the DosR regulon. The reason for this is not clear, but these proteins have been linked to dormancy and growth under hypoxia.

The *rrs* mutation and amikacin both impacted the proteome in a similar manner and acted synergistically to impact translation. This change involved a decrease in abundance in structural ribosomal proteins, with a concurrent increase in enzymes involved in tRNA-related processes. A role for both the *rrs* mutation and amikacin treatment resulting in increased ribosomal protein degradation was suggested, while the increase in tRNA-related processes was suggested to be a mechanism compensating for decreased rates and fidelity of translation.

In this study, treatment of a *gyrA* mutant with critical concentration ofloxacin resulted in significantly altered protein abundance. In contrast, treatment of the *rrs* mutant with critical concentration amikacin had very little impact on protein abundance. Ofloxacin treatment led to the increased abundance of proteins involved in iron acquisition, which could be explained by the possibility that ofloxacin chelates this metal. Ofloxacin treatment also decreased abundance of proteins involved in growth and replication of *M. tuberculosis*, which suggests that ofloxacin treatment still impacts growth rate in some manner.

Our study generated many viable hypotheses that could be validated in further work.

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Chapter 8 Conclusions and future work

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8.1 Conclusions

In this study, we investigated various aspects involving the evolution and physiology of extensive drug resistance in *M. tuberculosis*. Since acquisition of drug resistance by *M. tuberculosis* is mediated by spontaneous occurrence of mutations in drug target genes as a result of errors during DNA replication (Gillespie 2002; Delbrück 1945), it follows that the rate at which mutations occur plays an important role in this process. We therefore summarised the literature in an effort to understand how and to what extent this error rate contributes to the emergence of drug resistance *in vivo*. Various knowledge gaps were highlighted, which represent important areas for future research. In particular, there are indications that the mutation rate could be influenced by the presence of phylogenetic and drug resistance mutations, or be induced transiently as an adaptive mechanism during stress, or by DNA damage brought about by environmental factors encountered by the bacillus.

We then focused on the possibility that one of the environmental factors encountered by *M. tuberculosis* within the host, antiretroviral drugs, could impact the rate of mutation. This is especially relevant in light of the overlap of the HIV and TB epidemics (Raviglione & Sulis 2016; Sester *et al.* 2010). We employed *M. smegmatis* as a model organism for these studies and showed that the nucleoside reverse transcriptase inhibitors azidothymidine (AZT), also known as Zidovudine, and 3TC (known as Lamivudine) and their active triphosphorylated forms did not result in an elevated mutation rate.

The next step was to select spontaneous mutants monoresistant to drugs that form the backbone of treatment of MDR - i.e. resistance to these drugs define XDR – namely fluoroquinolones and second-line injectables or aminoglycosides (World Health Organization 2015). Monoresistant clones were generated *in vitro* since such clones would likely be isogenic with their progenitor and contain no additional drug resistance mutations. During this process, ofloxacin-monoresistant clones were selected from both H37Rv and a Beijing clinical isolate. We showed a significant difference in the prevalence of certain SNPs between these two strains. In particular, Beijing strains were more likely to acquire mutations in codon 94 of *gyrA,* which are also associated with higher MICs, than H37Rv, suggesting a possible reason for the association of Beijing with drug resistance, as discussed in Chapter 2. Furthermore, mutations in codon 94, excluding the mutation to alanine, were associated with a higher growth rate in the presence of antibiotic, as measured by colony size.

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Spontaneous mutants resistant to the second-generation fluoroquinolone, moxifloxacin, were also selected and characterised. In particular, the aim was to characterise mutations conferring resistance to ≥ 2 µg/ml moxifloxacin, an alternative and clinically more relevant critical concentration for this drug (Kam *et al.* 2006; Poissy *et al.* 2010; Sirgel *et al.* 2012), as opposed to 0.5 µg/ml. It was shown that Gly88Cys and mutations in codon 94 of *gyrA* conferred high-level moxifloxacin resistance. In addition, moxifloxacin still significantly impeded *in vitro* growth of *M. tuberculosis* harbouring these mutations, suggesting that moxifloxacin could be used to treat XDR or pre-XDR tuberculosis, i.e. where fluoroquinolone resistance exists.

The potential mechanisms modulating the level of fluoroquinolone resistance, and whether these were related to efflux, were also investigated by second-step selection on concentrations of ofloxacin higher than the MIC of the progenitor clone, a *gyrA* Asp94Gly mutant. Additional mutations in *gyrA* or *gyrB* were obtained, which was interesting, since, where such double mutations have been observed clinically, they were assumed to be as a result of heterogeneity. By testing for changes in MIC in the presence of efflux pump inhibitors, we showed that efflux did not modulate the level of ofloxacin resistance either in a subset of the double mutants or in the progenitor Asp94Gly mutant.

We investigated if and how the acquisition of mutations that commonly confer second-line resistance in clinical isolates would impact the physiology of *M. tuberculosis* by relative quantification of protein abundance between the resistant strains and their progenitor. In this analysis spontaneous mutants generated *in vitro* were employed, since they were more likely to be isogenic to the progenitor and not harbour additional resistance mutations as would be the case in clinical isolates. *gyrA* Asp94Gly and *rrs* A1401G mutants were therefore selected for this analysis, resistant to ofloxacin and amikacin respectively. Proteomic analysis showed a strong common signature of altered protein abundance shared between the two mutants. This common signature involved proteins expressed from the ESX-5 cluster (Ates *et al.* 2015), which were less abundant in both mutants. However, the two mutants also displayed unique significant alterations in protein abundance. In the *gyrA* Asp94Gly mutant several proteins involved in transport across the cell envelope were less abundant, suggesting that such a mutant could have a less permeable cell wall. In addition, results suggested that the mutation could impact gyrase functionality in such a way as to slow transcriptional and translational rates. The *rrs* mutant displayed lowered abundance of stress proteins belonging

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to the DosR/DevR regulon. This regulon is important in regulation of the growth rate and for survival when encountering gaseous stresses in granulomas (Gautam *et al.* 2011).

Lastly, changes in protein abundance, after treatment of the two mutants with critical concentration of the drug each was resistant to, were assessed. The *gyrA* mutant was treated with critical concentration ofloxacin, while the *rrs* mutant was treated with critical concentration amikacin. Ofloxacin treatment of a *gyrA* mutant resulted in increased abundance of proteins involved in iron acquisition and this could be related to reports of this drug chelating iron (Badal *et al.* 2015). Drugs targeting uptake or availability of iron could therefore synergise with fluoroquinolones. Ofloxacin treatment also had a major impact on cell division proteins and proteins involved in regulation of the growth rate.

Translation was impacted by both the presence of the *rrs* mutation and amikacin treatment and it was not possible in our study to separate the individual contributions of these factors to this effect. Ribosomal proteins were decreased in abundance, while proteins involved in tRNA-related processes were increased. These results may be explained by a similar impact of the *rrs* mutation and amikacin treatment on translation, where, firstly ribosome subunit assembly is reduced, leading to increased degradation of the ribosomes, and secondly, translation fidelity is affected negatively, leading to increased abundance of proteins involved in tRNA modification. This theory would need experimental verification.

8.2 Future work

8.2.1 The effect of nucleoside reverse transcriptase inhibitors on the mycobacterial mutation rate

- In order to confirm the possibility that AZT-TP and 3TC-TP did not induce the mutation rate due to impermeability of the cell envelope to these molecules, liquid chromatography of whole cell lysate and extracellular medium could be carried out.
- Our work also suggested that much future work should be done to improve the reproducibility and accuracy of and to standardise fluctuation assays in *M. smegmatis.* This organism may prove a valuable tool in screening for mutagenic compounds or conditions, since it is non-pathogenic and fast-growing. Future work should focus on developing fluctuation assays using an antibiotic such as ofloxacin, which would yield a variety of nucleotide substitutions, while eliminating resistance mechanisms not encoded by mutations in the drug resistance region of the relevant antibiotic resistance

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gene. Future work should also focus on reducing clumping within cultures, to obtain more accurate results with respect to colony count.

- The size of confidence intervals in our study when mutation rates were compared in the presence and absence of compound can be decreased by repeating the assay a number of times more. This will clarify whether the average increase in the mutation rate after treatment with AZT-TP was significant.

8.2.2 Characterisation of *in vitro*-generated fluoroquinolone-resistant *M. tuberculosis* mutants

- Future work could be carried out to investigate whether epistasis plays a role in the success of the Beijing lineage globally and its association with drug resistance (Hanekom *et al.* 2011). The possibility that the genetic background of these lineages results in the preferential acquisition of mutations conferring high-level resistance could be confirmed by repetition of our study as well as by selecting on other drugs.
- Fitness may be more complex in the presence of antibiotic pressure. Future work should therefore focus on developing a method to assess the competitive advantage of strains harbouring certain mutations in the presence of antibiotic. For example, two mutant strains could be co-cultured in the presence of antibiotic, and selected on this antibiotic, after which a rapid method, such as high-resolution melting could be employed to screen large numbers of colonies to determine the relative proportions of mutants. Such a measure of fitness could further enhance our understanding of the dynamics of the emergence of drug resistance of *M. tuberculosis* within the host.

8.2.3 Evolution of moxifloxacin resistance in *Mycobacterium tuberculosis*

- Results from our study suggested that the *gyrA* Gly88Cys mutation in *M. tuberculosis* carries a fitness cost. Future work should include competitive fitness assays to confirm this. Competitive fitness assays in the presence of 0.5 µg/ml moxifloxacin should also be carried out to confirm that the absence of the *gyrA* Gly88Cys mutation in strains selected on this concentration is due to a fitness cost.
- If the *gyrA* Gly88Cys mutation carries a fitness cost future work could include whole genome sequencing of both *in vitro* mutants and clinical strains harbouring a *gyrA* Gly88Cys mutation to determine the possible presence of compensatory mutations.

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8.2.4 Mechanisms modulating the level of fluoroquinolone resistance

- Further work is needed to understand how efflux evolves as a mechanism modulating the level of drug resistance in *M. tuberculosis* and why this mechanism exists in some clinical isolates and not others. Clinical isolates that have displayed efflux pump inhibitor-mediated modulation of MIC could be subjected to whole genome sequencing and compared to isolates not displaying this modulation.

8.2.5 Deciphering the impact of mutations conferring extensive drug resistance on the physiology of *M. tuberculosis*

- Illumina sequencing of W7 is required to characterise any genomic differences between this clone and BA23 and BO10 that may have impacted protein expression. In addition, PacBio sequencing (Benjak *et al.* 2016) could be performed to obtain better coverage of repeat regions.
- For whole genome sequencing, a reference strain more closely related to clinical strains circulating in the region should be assembled. This would enable detection of more variants. A proteogenomic approach (De Souza *et al.* 2011) could also be taken whereby regions in our clinical strain missing from H37Rv and additional amino acid polymorphisms could be identified.
- Proteomics results in general could be validated by quantitative real time PCR (q-RT-PCR), although levels of mRNA and protein may not always be correlated. Therefore a lack of concordance between these results should be interpreted with caution. Western blot analyses or selected reaction monitoring mass spectrometry where absolute copy numbers of proteins are determined could be used to validate results.
- The possibility that the *gyrA* fluoroquinolone-monoresistant mutant could be more tolerant to other antibiotics, especially those targeting the cell wall, as a result of a less permeable cell wall could be investigated by growth in the presence of subinhibitory antibiotic concentrations. The propensity to acquire resistance to other antibiotics could also be investigated by assessing mutation frequency in the presence of other antibiotics.
- Experimental work is needed to show that ofloxacin does indeed chelate iron and that this has an impact on *M. tuberculosis.* The potential relationship between fluoroquinolone MIC and iron limitation could be investigated or growth of *M.*

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tuberculosis in iron-limited and iron-rich conditions in the presence of fluoroquinolone could be explored.

- Further work is required to determine whether the *gyrA* mutation affects the rate of transcription or translation.
- How ofloxacin impacts growth rate of a *gyrA* mutant and if this is indeed the case, needs to be investigated further. If no growth phenotype can be observed, the significance of decreased abundance of various proteins involved in cell division and replication should be investigated further.
- Further work is required to understand how and if acquisition of an *rrs* mutation could impact the growth rate and the ability to enter a dormant state of *M. tuberculosis* in any way.
- Characterisation of Rv1301, a protein that may be involved in tRNA modification and may play an important role in amikacin resistance.

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