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Function and expression of class I ribonucleotide reductase small subunit-encoding genes in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*

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A thesis submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg,
in fulfillment of the requirements for the degree of Doctor of Philosophy

December, 2008.

Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



30 March 2009

…….. ……..

Mohube Betty Mowa Date

Abstract

Ribonucleotide reductases (RNRs) are a class of enzymes catalyzing the de novo reduction of ribonucleotides to deoxyribonucleotides essential for DNA replication and repair. In addition to the class Ib RNR encoding genes, *nrdE* and *nrdF2*, *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* also contain a homologue of a Chlamydial class Ic small subunit-encoding gene, *nrdB*. *M. tuberculosis* also contains an alternate class Ib RNR small (R2) subunit, NrdF1. In *M. smegmatis* mc^2155, the class Ib RNR genes are located on a large chromosomal duplication. *M. tuberculosis nrdF2* has been previously demonstrated to be essential for in vitro growth. It was hypothesized that different class I RNR R2 subunits could be used by the tubercle bacilli to survive and persist in the host. To test this hypothesis, function and expression of the class I R2-encoding genes in *M. tuberculosis* and *M. smegmatis* was investigated. Arguing against a specialist role for the alternate R2 subunits was the finding that NrdB in both organisms and NrdF1 in *M. tuberculosis* are individually and collectively dispensable for growth and long-term survival in vitro, resistance to genotoxic stress, adaptation during RNR inhibition by hydroxyurea and virulence in mice. Further confirming the essentiality of NrdF2 in mycobacteria and that NrdB cannot substitute for NrdF2 function in vitro was the finding that *nrdF2* is essential for growth of a strain of *M. smegmatis* mc^2155 lacking the duplicated chromosomal region (ΔDRKIN). ΔDRKIN showed marked hypersensitivity to a wide range of compounds including hydroxyurea and mitomycin C, whereas deletion of only one copy of *nrdF2* in *M. smegmatis* mc^2155 resulted in a specific hypersensitivity to hydroxyurea. Through the construction of *nrdR*-deficient mutants of *M. tuberculosis* and *M. smegmatis*, the class Ib RNR genes were shown to be specifically regulated by an NrdR-type repressor, as evidenced by increase in *nrdE* and *nrdF2* transcript levels in *nrdR*-deficient mutants of *M. tuberculosis* and *M. smegmatis*. Interestingly, however, upregulation of these genes did not affect *M. smegmatis* or *M. tuberculosis* in vitro growth, DNA damage survival or resistance to hydroxyurea. Together, these observations identify a potential vulnerability in dNTP provision in mycobacteria, and thereby offer a compelling rationale for pursuing the class Ib RNR as a target for drug discovery.

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Table of Contents

Declaration ……..ii

Abstract ……...……iii

Acknowledgements ……..iv

Publications from this work ……..v

List of Figures……...xii

List of Tables…….....xiv

1. Introduction……. …...1

1.1 Tuberculosis…….. …...1

1.1.1 TB prevention……. …...1

1.1.2 Diagnosis of *M. tuberculosis* infection……... …...3

1.1.3. TB chemotherapy…….....4

Drug resistance…….4

Mechanisms of drug resistance……5

1.2 *M. tuberculosis* as a human pathogen……...10

1.2.1 *M. tuberculosis* pathogenesis and immunity……10

1.2.3 *M. tuberculosis* evasion of the immune defense……..13

1.3 New TB drug discovery and development……...16

1.3.1 Drugs in clinical trials……..16

1.3.2 Modeling the persistent *M. tuberculosis* infection……...19

In vitro models……19

Animal models……21

1.3.3 Genetic tools for TB drug discovery……22

Random mutagenesis……..23

Targeted gene knockout…….24

Conditional gene knockout…….25

Gene expression profiling……...26

Protein expression and interaction studies…….28

Comparative Genomics……..29
1.3.4 New targets/pathways for TB drug discovery……..29
Carbon metabolism…….30
Energy metabolism…….31
The proteasome……..31
1.4 Ribonucleotide reductases (RNRs)……32
1.4.1 Class I RNR……..35
1.4.2 Class II RNR…….39
1.4.3 Class III RNR……...39
1.4.4 RNR-encoding genes in mycobacteria……40
M. tuberculosis and *M. smegmatis* RNR encoding genes and their function…….42
1.4.5 Regulation of bacterial RNRs……...44
1.4.6 RNR as a druggable protein…….....47
1.5 Aims and Objectives…….....48

2. Materials and Methods……..50
2.1 Bacterial strains and growth conditions……...50
2.2 Plasmid vectors……..52
2.3 Bacterial transformation……..52
2.3.1 Chemical transformation of *E. coli*……..52
Preparation of competent cells……...52
Transformation……...52
2.3.2 Electroporation of mycobacteria……..53
2.4 DNA extraction and purification from bacteria……56
2.4.1 Small scale plasmid DNA isolation from *E. coli*…….56
2.4.2 Bulk plasmid DNA preparation from *E. coli*……57
2.4.3 Extraction of chromosomal DNA from mycobacteria…….57
2.4.4 Small scale extraction of chromosomal DNA from *E. coli* and mycobacteria for PCR screening…….58
2.4.5 Phenol-chloroform extraction and salt-ethanol precipitation of DNA……58

2.5 DNA manipulations……...58
2.5.1 Agarose gel electrophoresis……58
2.5.2 Purification of DNA fragments from agarose gels……..59
Agarase digestion of the gels……59
Purification by GeneClean II glass milk……59
Purification by NucleoSpin Extract II……59
2.5.3 Treatment of DNA with enzymes……60
Restriction digests……..60
De-phosphorylation……60
Blunting of 5' and 3' DNA overhangs……...60
Ligation of DNA fragments……60
2.6 Polymerase chain reaction (PCR)……..61
2.7 DNA sequencing……61
2.8 Southern blot analysis……..61
2.8.1 Synthesis and labelling of probes……61
2.8.2 Electroblotting……..62
2.8.3 Probe hybridization……..62
2.8.4 Detection of bound probe……63
2.9 Construction and genotypic characterization of *nrd* gene allelic exchange mutants and complemented counterparts in *M. smegmatis* and *M. tuberculosis*……..64
2.9.1 Targeted knockout of *nrd* genes in *M. tuberculosis*……65
2.9.2 Construction of *M. smegmatis nrd* genes deletion mutants……..66
2.9.3 Complementation of *M. smegmatis* Δ*nrdR*::*hyg* and Δ*nrdF2*::*hyg*……..68
2.10 Analysis of gene expression……...69
2.10.1 RNA isolation……69
2.10.2 Reverse Transcription (RT)……70
2.10.3 Quantitative RT-PCR Assay……...70
Real-Time, Quantitative RT-PCR Assay (qRT-PCR)……70
Semi-quantitative RT-PCR……71

2.11 Competitive in vitro growth and long-term survival assays……..71
2.12 Susceptibility testing of mycobacterial strains…….72
2.13 Sensitivity to UV irradiation…….73
2.14 Sensitivity to nitrosative stress……73
2.15 Assessment of UV-induced mutation frequencies……74
2.16 Luria-Delbrück fluctuation tests…….74
2.17 Infection of mice and determination of bacterial load……74
2.18 Statistical analysis……...75

3. Results……..76
3.1 The genomes of *M. tuberculosis* and *M. smegmatis* contain multiple RNR-encoding genes……...76
3.2 The role of class Ic RNR in mycobacteria……78
3.2.1 *nrdB* is dispensable for growth of *M. smegmatis* and *M. tuberculosis* in vitro……....78
3.2.2 *nrdB* is dispensable for competitive growth and long-term survival of *M. smegmatis* …….81
3.2.3 The role of *nrdB* in nitrosative stress survival……..83
3.2.4 The role of *nrdB* in RNR inhibition survival……85
3.2.5 Effect of *nrdB* loss on mycobacterial resistance to DNA damage……...86
3.2.6 The role of *nrdB* in *M. tuberculosis* growth, dissemination and survival in mice……...89
3.3 The function of the alternate small subunit of class Ib RNR in *M. tuberculosis*……91
3.3.1 *nrdF1* is dispensable for growth of *M. tuberculosis* in vitro……91
3.3.2 Requirement of *nrdF1* in DNA damage, RNR inhibition and translation inhibition survival……..93
3.3.3 Effect of *nrdF1* loss on *M. tuberculosis* growth, dissemination and survival in vivo……95

3.4 Collective roles of *nrdB* and *nrdF1* in *M. tuberculosis*……..97

3.4.1 Construction of a mutant strain of *M. tuberculosis* lacking both *nrdF1* and *nrdB*……...97

3.4.2 Comparative susceptibility of the Δ*nrdF1*Δ*nrdB* mutant of *M. tuberculosis* to DNA damage and HU……99

3.5 The role of the duplication in class Ib RNR-encoding genes in the physiology of *M. smegmatis* mc^{2}155……...101

3.5.1 The remaining copy of *nrdF2* in the ΔDRKIN strain is essential for growth……..101

3.5.2 One copy of *nrdF2* is dispensable for growth of *M. smegmatis* mc^{2}155…...102

3.5.3 Loss of the duplicated region of *M. smegmatis* mc^{2}155 affects susceptibility to DNA damage and HU……..104

3.5.4 The role of *nrdF2* duplication in *M. smegmatis* survival on exposure to HU and DNA damage……106

3.6 Expression of *nrd* genes in *M. tuberculosis* and *M. smegmatis* strains……...108

3.7 *nrdR* homologues and NrdR boxes are present in mycobacteria…….110

3.7.1 *nrdR* function in growth and long-term survival *of M. smegmatis* and *M. tuberculosis*……..112

3.7.2 Expression of *nrd* genes in the *M. tuberculosis* Δ*nrdR* and *M. smegmatis* Δ*nrdR*::*hyg* mutants…….116

3.7.3 Impact of up-regulation of class Ib RNR gene expression on resistance to RNR inhibition and to DNA damage……..118

3.8 Effects of altered class Ib RNR-encoding gene expression on mutagenesis…….118

4. Discussion…….121

4.1 NrdB does not play a significant role in dNTP provision in mycobacteria……..122

4.2 The alternate class Ib RNR (NrdEF1) does not play a significant role in dNTP supply in *M. tuberculosis*…….126
4.3 Effect of the 56-kb genomic duplication on the physiology of *M. smegmatis* mc^{2}155……...128
4.4 *M. tuberculosis* dNTP supply is provided exclusively by NrdEF2 activity……..130
4.5 NrdR is a negative regulator of class Ib RNR gene expression in mycobacteria……...132
4.6 Phenotypic effect of increased class Ib RNR encoding genes expression levels in mycobacteria……...134
4.7 Future studies……...137

5. Appendices……...138
Appendix 1: List of Abbreviations……138
Appendix 2: Culture media……140

6. References……142

List of Figures

Figure 1.1 De novo dNTP synthesis pathway catalised by class I RNR and its role in DNA metabolism……33

Figure 1.2 Structure of a class Ia RNR……. 36

Figure 1.3 Catalytic mechanism of class Ia RNR……37

Figure 3.1 Chromosomal context of RNR-encoding genes in *M. tuberculosis* H37Rv, *M. smegmatis* mc^2155, and ΔDRKIN and multiple protein sequence alignment of the NrdBs from *M. tuberculosis*, *M smegmatis* and *Chlamydia trachomatis*……77

Figure 3.2 Construction and growth kinetics of *M. tuberculosis* and *M. smegmatis nrdB* mutants……...80

Figure 3.3 Competitive growth and long-term survival of *M. smegmatis* Δ*nrdB*::*hyg*……82

Figure 3.4 Susceptibility of *M. smegmatis* Δ*nrdB*::*hyg* and *M. tuberculosis* Δ*nrdB* to nitrosative stress……84

Figure 3.5 Susceptibility of *M. tuberculosis* Δ*nrdB* and *M. smegmatis* Δ*nrdB*::*hyg* to HU……86

Figure 3.6 Sensitivity of *M. tuberculosis* Δ*nrdB* and *M. smegmatis* Δ*nrdB*::*hyg* to MTC and UV irradiation……...88

Figure 3.7 Growth and dissemination of *M. tuberculosis* Δ*nrdB* in mice……. 90

Figure 3.8 Construction and growth kinetics of *M. tuberculosis* Δ*nrdF1*. ……92

Figure 3.9 Susceptibility of *M. tuberculosis* Δ*nrdF1* to MTC, UV irradiation, STR and HU……..94

Figure 3.10 Growth and dissemination of the Δ*nrdF1* mutant of *M. tuberculosis* in mice……...96

Figure 3.11 Deletion of *nrdB* in the *M. tuberculosis* Δ*nrdF1* background and growth of the *M. tuberculosis* Δ*nrdF1*Δ*nrdB* double mutant in vitro……...95

Figure 3.12 Sensitivity of *M. tuberculosis* Δ*nrdF1*Δ*nrdB* to MTC , UV irradiation and HU……100

Figure 3.13 Deletion of *nrdF2* in the ΔDRKIN and mc^2155 strains of *M. smegmatis*…… 103

Figure 3.14 Sensitivity of ΔDRKIN to HU, MTC and UV irradiation……...105

Figure 3.15 Sensitivity of *M. smegmatis* Δ*nrdF2*::*hyg* to HU, MTC and UV irradiation……..107

Figure 3.16 Genomic organization of *nrdR* in *M. smegmatis* and *M. tuberculosis* in comparison to *S. coelicolor*, multiple protein sequence alignment of *M. tuberculosis* and *M. smegmatis* against *S. coelicolor* NrdR and putative NrdR boxes located upstream of mycobacterial *nrdHIE* gene cluster and the *nrdF2* gene……... 112

Figure 3.17 Deletion of *nrdR* in *M. smegmatis*, and *M. tuberculosis* and growth kinetics of the mutant strains…… 115

Figure 3.18 Long-term survival of the *M. smegmatis* Δ*nrdR*::*hyg* mutant in pure culture or in competition with mc^2155::pAINT……... 115

Figure 3.19 Semi-quantitative RT-PCR analysis of *nrdF2* and *nrdE* expression in *M. smegmatis* Δ*nrdR*::*hyg* and Δ*nrdR*::*hyg*::pNRDR……...117

Figure 3.20 Sensitivity of *M. smegmatis* Δ*nrdR*::*hyg* and *M. tuberculosis* Δ*nrdR* to HU, MTC and UV irradiation……....119

Figure 4.1 The role and regulation of *nrd* genes in *M. tuberculosis*……133

Figure 4.2 Three levels of class Ia RNR regulation in Streptomyces……..136

List of Tables

Table 1.1 Mechanisms of resistance of *M. tuberculosis* to first-line drugs…….. .9

Table 1.2 Division and properties of RNR enzyme classes and sub-classes……. 34

Table 1.3 Mycobacterial RNR gene complements…….41

Table 2.1 Bacterial strains used in this study…….50

Table 2.2 Plasmids vectors used in this study……54

Table 2.3 Oligonucleotide primers used for probe synthesis……63

Table 2.4 Probes, enzymes used and fragments detected in Southern blotting…. 64

Table 2.5 PCR primers and amplicons used to create knockout vectors for allelic exchange mutagenesis……...67

Table 2.6 PCR primers used to construct Δ*nrdR*::*hyg* complementation vector... 69

Table 2.7 Oligonucleotide primers used for RT-PCR……72

Table 3.1 Analysis of the remaining small subunit encoding *nrd* genes expression in mycobacterial mutant strains……109

Table 3.2 Levels of *nrd* gene transcripts in *M. tuberculosis* H37Rv during early logarithmic-phase aerobic growth in 7H9-OADC medium……..109

Table 3.3 qRT-PCR analysis of *nrd* gene expression in Δ*nrdR*::*hyg* and Δ*nrdR* relative to their parental wild-type strains…… 117

Table 3.4 Spontaneous mutation rates and UV-induced mutation frequencies of *M. tuberculosis* and *M. smegmatis* strains…….....120

1. Introduction

1.1 Tuberculosis

Tuberculosis (TB) is one of the leading causes of death globally, with 9.2 million new cases and 1.7 million deaths occurring in 2006 alone (WHO, 2008). Despite the estimation that 2 billion people are infected with the causative agent, *Mycobacterium tuberculosis*, worldwide (Gomez and McKinney, 2004), only 10 % of these individuals will develop active disease whilst the majority will remain in an asymptomatic state of latent TB infection (LTBI), which significantly contributes to the future burden of TB (Cardona and Ruiz-Manzano, 2004). HIV co-infection further complicates the efforts to combat the TB pandemic, serving as a major factor contributing to the high mortality rates amongst those infected (Kaufmann, 2004; Sharma *et al.*, 2005). Of the TB cases and deaths observed in 2006, 0.7 million cases and 0.2 million deaths were from HIV co-infected individuals (WHO, 2008). It is estimated that in high HIV/AIDS burden countries, TB prevalence will reach 609 per 100 000 population by 2015 (Dye *et al.*, 2005).

1.1.1 TB prevention

Currently, live attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is used as a vaccine for TB prevention, with nearly 100 million children estimated to receive BCG every year (Barreto *et al.*, 2006). BCG was developed by in vitro passaging of an isolate of *M. bovis* 230 times from 1908 to 1921. However, from 1921 to 1961 this isolate had been passaged 1173 times, resulting in a number of genotypically and phenotypically different daughter strains, collectively known as BCG (Behr and Small, 1999; Behr, 2001a, 2002). Despite its long-term use, BCG protective efficacy has been variable (ranging from 0 to 80 %), being more effective in protection against primary TB in children than adult pulmonary TB (Barreto *et al.*, 2006; Behr, 2002; Castillo-Rodal *et al.*, 2006; Lagranderie *et al.*, 1996; Wu *et al.*, 2007). Currently, there is no global consensus as to which strain of BCG has optimal efficacy for general

use. These findings have stimulated considerable interest in investigating the reasons underlying the variability in protective efficacy of BCG. Genetic alterations which occurred during the evolution of different BCG strains resulted in different phenotypes and hence, in different protective efficacy (Behr, 2001b, 2002; Leung *et al.*, 2008; Mostowy *et al.*, 2003). Host factors, such as pre-exposure to other mycobacterial species (Brandt *et al.*, 2002; Demangel *et al.*, 2005; Young *et al.*, 2007) and route of vaccination have also been found to affect BCG efficacy (Aldwell *et al.*, 2006; Chen *et al.*, 2004; Manabe *et al.*, 2002; Wang *et al.*, 2004). Most importantly, as a live vaccine, BCG also poses a health threat to immunocompromised individuals (Bustamante *et al.*, 2007).

Considerable effort has been made to find a vaccine that is safe and more effective in preventing pulmonary TB (Brennan *et al.*, 2007; Izzo *et al.*, 2005). Several studies have explored the possibility of enhancing the efficacy of BCG, with recombinant BCG vaccine (rBCG) candidates conferring better protection than BCG having been described (Fattorini, 2007). They include rBCG30 [BCG expressing antigen (Ag) 85B] (Horwitz *et al.*, 2000; Horwitz and Harth, 2003; Horwitz *et al.*, 2006) and rBCG::Δ*ureC-ll* O (a urease-deficient BCG mutant expressing lysteriolysin O) (Grode *et al.*, 2005). Several groups are also interested in finding a booster vaccine for individuals whose immune system has already been primed by mycobacterial infection or by BCG vaccination. Amongst the promising booster vaccine candidates identified is Mtb72F (subunit vaccine of a fusion molecule comprising the *M. tuberculosis* PPE family member, Rv1196, and a putative serine protease, Rv0125) (Brandt *et al.*, 2004; Tsenova *et al.*, 2006) and MVA-85A (a recombinant, replication-deficient vaccinia virus expressing Ag85A from *M. tuberculosis*) (Brookes *et al.*, 2008; Fletcher *et al.*, 2008; Horwitz *et al.*, 2005; McShane *et al.*, 2004; Williams *et al.*, 2005). Interest in eventually replacing BCG by a suitably attenuated strain of *M. tuberculosis* or *M. bovis* have yielded several vaccine candidates, albeit with less or equal protective efficacy to BCG (Aguilar *et al.*, 2007; Hotter *et al.*, 2005; Pavelka *et al.*, 2003; Sambandamurthy *et al.*, 2005; Senaratne *et al.*, 2007).

1.1.2 Diagnosis of *M. tuberculosis* infection

Despite its limitations (Kang *et al.*, 2005; Soysal *et al.*, 2008; Winje *et al.*, 2008), the tuberculin skin test, which involves intradermal injection of purified protein derivative (PPD) followed by measurement of delayed type hypersensitivity response, is still a commonly used diagnostic test for *M. tuberculosis* infection. Recently, PPD (QuantiFERON-TB) (Streeton *et al.*, 1998; Taggart *et al.*, 2004) or more specific *M. tuberculosis* stimulatory antigens ESAT-6 and CFP-10 proteins (QuantiFERON-TB Gold test) (Diel *et al.*, 2006; Liu *et al.*, 2004; Mori *et al.*, 2004; Ulrichs *et al.*, 2000; Weldingh and Andersen, 2008) have been used in whole blood assays as stimulatory antigens for IFN-γ release, whereby the IFN-γ is then quantified and used as an indication of *M. tuberculosis* infection. Application of more recently developed techniques for diagnosis of active TB infection and drug susceptibility testing (*e.g.* BACTEC, phage-based and various nucleic acid amplification assays) have provided more sensitive and rapid means of diagnosing active TB infection (Banaiee *et al.*, 2001; Banaiee *et al.*, 2003; Gali *et al.*, 2006; Katoch, 2004; Nahid *et al.*, 2006; Rodrigues *et al.*, 2007; Rusch-Gerdes *et al.*, 2006; Tevere *et al.*, 1996). Using a mathematical model of the TB epidemic currently raging in South African adults, it was estimated that simultaneous execution of culture and drug susceptibility testing in 37 % of new cases and 85 % of failed treatment cases may reduce TB mortality by 17 %, reduce multi-drug resistant (MDR)-TB incidence by 14 % and prevent 47 % of MDR-TB deaths from 2008 – 2017 (Dowdy *et al.*, 2008). However, the high cost of these new diagnostic assays, combined with inadequate diagnostic laboratory infrastructure limits their use in most developing countries (Glassroth, 2005).

1.1.3. TB chemotherapy

Following the 1993 declaration by the World Health Organization (WHO) that TB is a global health emergency, the directly observed therapy short-course (DOTS) program was implemented. Even though DOTS has improved the treatment and cure rates of TB significantly (Shargie and Lindtjorn, 2005), this disease still remains a massive global health challenge (Brewer and Heymann, 2004). More than five decades ago, the treatment of TB required 18-24 months' administration of streptomycin (STR) and p-aminosalicylic acid (PAS). The introduction of isoniazid (INH), followed by two sterilizing drugs, rifampicin (Rif) and pyrazinamide (PZA), shortened the duration TB chemotherapy to six months, leading to the development of "short-course" chemotherapy (Jasmer *et al.*, 2002; Jawahar, 2004; Mitchison, 2005a; Narita *et al.*, 2002; Torres, 1998). The current regimen comprises two phases: an intensive phase, which targets mainly the actively replicating bacilli and continuation phase, which target those organisms that persisted through the intensive phase. A regimen, starting with 2 months' intensive phase therapy with INH, Rif, STR and PZA, followed by 4 months' continuation phase treatment with INH and Rif, is used as the gold standard in first-line chemotherapy, although in some cases, STR is substituted with ethambutol (EMB) (Mitchison, 2005b).

Drug resistance

The current complicated and lengthy regimen for TB chemotherapy often result in noncompliance and inappropriate treatment (Jose *et al.*, 2007; Meacci *et al.*, 2005; Picon *et al.*, 2007), which can lead to development of drug resistance. About 460 000 new cases of MDR-TB, defined by the presence of disease causing strains that are resistant to two first-line drugs, INH and Rif (Ginsberg and Spigelman, 2007), are estimated to occur every year world wide (WHO, 2006), with 500 000 cases detected in 2006 alone (WHO, 2008). Drug resistance poses the biggest threat and challenge to the current TB interventions. Treatment of MDR-TB is more difficult, requiring 18-24 months of therapy with second-line drugs that have limited sterilizing capacity, are less effective and more toxic (Ginsberg and Spigelman, 2007; Kaufmann, 2004; Sharma and

Mohan, 2006). Most importantly, the high cost of MDR-TB treatment (Floyd and Pantoja, 2008; Resch *et al.*, 2006) is exacerbating the severity of the TB epidemic in developing countries. The magnitude of the threat presented by drug-resistant TB is underscored by the recent emergence and spread of extensively drug resistant (XDR) strains of *M. tuberculosis,* which are defined as MDR strains that are also resistant to a fluoroquinolone and at least one of the injectable drugs (Blaas *et al.*, 2008; Masjedi *et al.*, 2006; Migliori *et al.*, 2007; WHO, 2008). A devastating outbreak of XDR-TB in South Africa resulted in a rapid spread and high mortality rates in those co-infected with HIV (Gandhi *et al.*, 2006). A recent study found the highest percentage of South African XDR-TB cases studied to be acquired (Mlambo *et al.*, 2008), suggesting that most of the XDR-TB cases are a consequence of a failed therapy. These factors have emphasized the urgent need to develop new drugs that are active against both drug-susceptible and drug-resistant strains of *M. tuberculosis.*

The biological fitness of drug resistant strains and their impact on the epidemiology of MDR-TB is a topic that has attracted considerable attention (Billington *et al.*, 1999; Cohen and Murray, 2004; Gagneux *et al.*, 2006a; Pym *et al.*, 2002). In vitro studies have shown that drug resistance mutations may compromise the competitive growth of *M. tuberculosis* in liquid cultures and in macrophage cell lines (Billington *et al.*, 1999; Gagneux *et al.*, 2006b; Mariam *et al.*, 2004). Nonetheless, it has been suggested that even though the majority of the occurring MDR strains are less fit, a smaller proportion of highly fit MDR strains may outcompete less fit strains (Cohen and Murray, 2004; Gagneux *et al.*, 2006a). Supporting this notion is the finding that certain clinical, drug-resistant isolates from patients were found to have no significant fitness cost, which could be due to non-cost resistance mutations being favored (Pym *et al.*, 2002) or due to the emergence of compensatory mutations (Gagneux *et al.*, 2006b).

Mechanisms of drug resistance

Even though the intrinsic drug resistance of mycobacteria is most commonly attributable to the low permeability of the their complex lipid rich cell wall (Camacho *et*

al., 2001; Philalay *et al.*, 2004; Wang *et al.*, 2000), efflux systems have also been implicated in intrinsic drug resistance. They act by extruding a wide range of antibiotics from the cell (Amaral *et al.*, 2007; Danilchanka *et al.*, 2008; Li *et al.*, 2004; Pasca *et al.*, 2005; Ramon-Garcia *et al.*, 2007). There are four families of drug resistance transmembrane efflux proteins (Sharma and Mohan, 2004). Members of all of these families can be identified in *M. tuberculosis* genome. About 2.5 % of the *M. tuberculosis* genome is predicted to encode ATP-dependent ABC multidrug resistance transporters (Braibant *et al.*, 2000), including the fluoroquinolone resistance ABC pump encoded by the Rv2686c-Rv2687c-Rv2688c operon (Pasca *et al.*, 2004). The genome of *M. tuberculosis* encodes 13 transmembrane proteins predicted to be of the resistance nodulation division of transporters. These are proposed to act in drug efflux, specific to mycobacteria, and are designated as "mycobacterial membrane proteins large" (MmpL) (Cole *et al.*, 1998).

Non-intrinsic drug resistance in *M. tuberculosis* can be either phenotypic or genetic. Phenotypic resistance is non-heritable and can be due to slow growth or non-replication of bacteria which results in tolerance to drugs (persisters). Phenotypically resistant bacilli can be eradicated following relapse after a successful treatment (Connolly *et al.*, 2007; Sacchettini *et al.*, 2008). Genetic resistance is due exclusively to mutations which occur in the drug target or in the gene encoding a pro-drug activator, resulting in an irreversible loss of susceptibility to the drug. In *M. tuberculosis*, genetic resistance to antitubercular drugs is conferred exclusively by chromosomally borne mutations (Gillespie, 2002; Guo *et al.*, 2008; Sekiguchi *et al.*, 2007; Sharma and Mohan, 2004; Wang *et al.*, 2007). There is no evidence in this organism for the acquisition of drug resistance through horizontal gene transfer. Over the past few years, the molecular mechanisms of resistance to almost all known antitubercular drugs, including the recently discovered compounds, have been elucidated (Bamaga *et al.*, 2001; Ginsburg *et al.*, 2005; Manjunatha *et al.*, 2006a; Petrella *et al.*, 2006; Shi *et al.*, 2007). The frequency with which resistance arises in vitro differs depending on the specific antibiotic, with frequencies of resistance of *M. tuberculosis* to Rif and INH being 3.1 ×

10^{-8} and 3.5×10^{-6}, respectively. However the mutation frequency decreases to 9×10^{-14} when both Rif and INH are used in combination (Gillespie, 2002; Johnson *et al.*, 2006c).

Some of the characterized mutations conferring resistance of *M. tuberculosis* to antitubercular drugs are summarized in Table 1.1. Rif inhibits RNA polymerase function by binding to its β subunit encoded by *rpoB*. Different mutations in the *rpoB* gene conferring Rif resistance have been identified, with the majority (95 %) occurring within a small region of <100 bp – the Rif-resistance determining region (RRDR). Three substitution mutations, namely Ser531Leu, His526Tyr and Asp516Val occur in >70 % of the Rif resistant isolates that have been characterised, with the Ser531Leu mutation occurring even more frequently than His526Tyr and Asp516Val (Billington *et al.*, 1999; Guo *et al.*, 2008; Johnson *et al.*, 2006c; Telenti *et al.*, 1993). INH is a pro-drug which is activated by the *katG*-encoded catalase-peroxidase upon entry into the cell to produce toxic, reactive radicals including the isonicotinic acyl radical, which can damage several targets (DeVito and Morris, 2003; Timmins *et al.*, 2004a, b). The principal cellular target of INH is the NADH-dependent enoyl acyl carrier reductase, InhA, which plays an essential role in mycolic acid synthesis (Slayden and Barry, 2002; Vilcheze *et al.*, 2006). Although mutations in other genes can confer INH resistance (*e.g.*, *ndh*), approximately 70-80 % of INH resistant strains are associated with mutations in the *katG* and *inhA* genes with the most common being a Ser315Thr substitution in KatG and a -15C→T substitution in the promoter region of *inhA* (Guo *et al.*, 2006; Guo *et al.*, 2008; Johnson *et al.*, 2006c; Leung *et al.*, 2006; Parsons *et al.*, 2005).

PZA is a highly effective drug that does not have a specific target. After the conversion of this pro-drug into its active form, pyrazinoic acid (POA), through the action of the pyrazinamidase, PncA, this weak acid accumulates in the cell under acidic conditions, and acidifies the cytoplasm, so inhibiting cellular metabolism (Boshoff and Mizrahi, 1998, 2000; Boshoff *et al.*, 2002; Zhang *et al.*, 1999; Zhang and Mitchison, 2003). An

interesting feature of PZA is its greater sterilizing effect on stationary phase than actively growing cultures (Mitchison, 2005b) and the enhancement of its activity under hypoxic conditions and by energy metabolism inhibition (Gu *et al.*, 2008; Wade and Zhang, 2004). PZA resistance is mainly due to mutations in the *pncA* gene which abrogate the amidase activity of the PncA enzyme and thus preclude the production of POA (Bamaga *et al.*, 2001; Boshoff and Mizrahi, 2000; Huang *et al.*, 2003; Johnson *et al.*, 2006c; Louw *et al.*, 2006; Scorpio and Zhang, 1996).

EMB acts by inhibiting the arabinosyl transferase (EmbB) which is required for cell wall synthesis (Belanger *et al.*, 1996). The majority of EMB resistant mutants carry mutations in the *embB* gene. Mutations in the Met306 codon of EmbB resulting in substitution with Val, Leu or Ile account for resistance in 70-90 % of EMB resistant isolates, with Met306Leu and Met306Val conferring a higher level of resistance than Met306Ile (Johnson *et al.*, 2006b; Parsons *et al.*, 2005; Plinke *et al.*, 2006; Telenti *et al.*, 1997). STR inhibits translation by interacting with the 16S rRNA and S12 ribosomal protein (encoded by *rrs* and *rpsL*, respectively). Mutations in both *rrs* and *rpsL* confer STR resistance, with *rpsL* mutations conferring a higher level of resistance (Honore and Cole, 1994; Honore *et al.*, 1995; Johnson *et al.*, 2006c).

Table 1.1 Mechanisms of resistance of *M. tuberculosis* to first-line drugs

Drug	Mode of action	Target and encoding gene/s	Common mutated genes conferring resistance and encoded protein	Reference
Isoniazid	Mycolic acid biosynthesis inhibition	EnoylACP reductase, *inhA*	*inhA*, Enoyl-ACP reductase *katG*, Catalase peroxidase	(DeVito and Morris, 2003; Guo *et al.*, 2006; Leung *et al.*, 2006; Parsons *et al.*, 2005; Vilcheze *et al.*, 2006)
Rifampicin	Transcriptional inhibition	RNA polymerase β-subunit, *rpoB*	*rpoB*, RNA polymerase β-subunit	(Billington *et al.*, 1999; Guo *et al.*, 2008; Telenti *et al.*, 1993)
Pyrazinamide	Interference with pH homeostasis	No specific target	*pncA*, Pyrazinamidase	(Bamaga *et al.*, 2001; Boshoff and Mizrahi, 1998, 2000; Boshoff *et al.*, 2002; Huang *et al.*, 2003; Zhang *et al.*, 1999)
Ethambutol	Inhibition of arabinan synthesis	Arabinosyl transferase, *embB*	*embB*, Arabinosyl transferase	(Parsons *et al.*, 2005; Plinke *et al.*, 2006; Telenti *et al.*, 1997)
Streptomycin	Translational inhibition	S12 ribosomal protein, *rpsL* and 16S rRNA, *rrs*	*rpsL*, S12 ribosomal protein *rrs*, 16s rRNA	(Honore and Cole, 1994; Honore *et al.*, 1995)

1.2 *M. tuberculosis* as a human pathogen

M. tuberculosis is an aerobic, acid fast, slow growing bacillus with doubling time of *ac*. 24 hours (Hartmans *et al.*, 2006). It has got a genome size of 4.4 mb with 65.6 % GC content (Cole *et al*. 1998). Is the most virulent intracellular human pathogen amongst the *M. tuberculosis* complex (MTBC), which includes *M. bovis, M. microti, M. africanum, M. canetti* and *M. caprae* (Brosch *et al.*, 2002; Cole, 2002b; Smith *et al.*, 2006). It is characterized by its unique thick lipid rich cell wall, which is commonly implicated in pathogenesis (Alderwick *et al.*, 2007; Barry, 2001; Hotter *et al.*, 2005; Karakousis *et al.*, 2004a; Reed *et al.*, 2004; Stokes *et al.*, 2004; Zuber *et al.*, 2008). Although there are many important molecules in the *M. tuberculosis* cell wall, mycolic acids are the most abundant and most extensively studied (Behr *et al.*, 2000; Schroeder and Barry, 2001; Takayama *et al.*, 2005; Wang *et al.*, 2000; Yuan *et al.*, 1998; Zuber *et al.*, 2008). The most studied virulence-enhancing lipids produced by *M. tuberculosis* are the phthiocerol dimycocerosates (PDIMs). Since the identification of PDIMs as virulence factors, this class of lipids has attracted considerable interest in terms of its contribution to mycobacterial pathogenesis (Hotter *et al.*, 2005; Pinto *et al.*, 2004; Rousseau *et al.*, 2004; Sirakova *et al.*, 2003). A class of lipids structurally related to PDIMs – the phenolic glycolipids (PGLs) – has also been investigated and postulated to account for the virulence of the HN878 strain of *M. tuberculosis* in mice (Reed *et al.*, 2004). *M. tuberculosis* is an airborne pathogen, extremely slow growing and requires Biosafety level 3 conditions, hence, *Mycobacterium smegmatis*, a fast-growing, non-pathogenic saprophyte is usually used as a model organism in mycobacteriology (Kana and Mizrahi, 2004).

1.2.1 *M. tuberculosis* pathogenesis and immunity

In most cases, the TB bacillus enters the host by inhalation of the *M. tuberculosis*-containing aerosol and engulfment by alveolar macrophages before dissemination to other parts of the body. Usually, it leads to a pulmonary infection, which can develop into chronic disease and severe tissue destruction (Raja, 2004). Phagocytosis by alveolar macrophages via surface receptors, including Toll-like receptors (TLRs), complement receptors and mannose receptors, represents the first line of cellular defense against microbial invasion (Alagarasu *et*

al., 2007; Pieters, 2001; Smith, 2003; van Crevel *et al.*, 2002). Upon ingestion, *M. tuberculosis* is contained in the phagosome, an endocytic vacuole formed after phagocytosis. After phagosome-lysosome fusion (to form the phagolysosome), the bacilli are subjected to degradation by lysosomal enzymes. Bacilli taken up by macrophages are also subjected to the bactericidal activities of antibacterial agents such as reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNIs) and toxic peptides (Pieters, 2001; Smith, 2003). The bactericidal activities of hydrogen peroxide (H_2O_2), a ROI generated by macrophages via the oxidative burst, has been demonstrated in mice (Adams *et al.*, 1997; Nathan and Shiloh, 2000). Several lines of evidence implicate RNIs as the most potent antimicrobial agents in mice macrophages (MacMicking *et al.*, 1997; Olin *et al.*, 2008). RNIs are believed to play a similar role in human macrophages (Firmani and Riley, 2002; Flynn *et al.*, 1998; Nathan, 2006; Nicholson *et al.*, 1996). Supporting this notion is the findings that increase in expression of inducible NO synthase (iNOS), which catalyzes the production of NO (one of the RNIs), facilitated inhibition of *M. tuberculosis* growth in a human macrophage-like cell line (Liu *et al.*, 2006). NO is a potent RNI that can react with O_2 to yield a variety of antimycobacterial products, including NO_2^- and NO_3^-, and with O_2^- free radical to produce peroxynitrite ($ONOO^-$) (Nathan and Shiloh, 2000; Nathan, 2006). The central role of NO in restricting *M. tuberculosis* growth in mice was supported by the finding that knockout mice lacking iNOS, are markedly more susceptible to *M. tuberculosis* infection than wild-type mice (MacMicking *et al.*, 1997).

Mycobacteria that escape the first-line host defense mechanisms outlined above will multiply intracellularly, resulting in lysis of macrophages and concomitant recruitment of more phagocytes to the site of infection. The recruited phagocytes will ingest the invading mycobacteria, which grow exponentially in the intracellular environment. Two to three weeks after infection, further release of proinflamatory cytokines and chemokines (*e.g.* interleukin (IL)-12 and tumor necrosis factor (TNF)-α) by mycobacteria-harboring macrophages mediate the recruitment and activation of antigen-specific T cells. This results in the development of effective cellular immunity through a complicated series of events (Akahoshi *et al.*, 2003;

Kaufmann, 2002; Long and Gardam, 2003). Briefly, presentation of mycobacterial antigens by antigen presenting cells (*e.g.* macrophages) to the recruited antigen-specific $CD4^+$ and $CD8^+$ T cells via the surface major-histocompatibility complex (MHC) class II or I, respectively, results in further recruitment, stimulation and proliferation of antigen specific T-cells (Cowley and Elkins, 2003; Cowley *et al.*, 2005; Lazarevic and Flynn, 2002; Raja, 2004). Then, the effective type-1 T cells (Th1, *e.g.* $CD4^+$ cells) become the main source of the major immune effector molecules, IFN-γ and TNF-α. This leads to more production of cytokines, which feed back to phagocytic cells harboring replicative intracellular bacteria and increase their bacteriostatic and bactericidal functions. Mycobacterial growth is then arrested and the bacilli can be contained within the granuloma (lesions composed of macrophages in the center surrounded by T and B cells) (Ben-Ali *et al.*, 2004; Botha and Ryffel, 2003; Cowley and Elkins, 2003; Lopez-Maderuelo *et al.*, 2003; Ogus *et al.*, 2004; Ottenhoff *et al.*, 2005; Picard *et al.*, 2002). Despite the host's aggressive antimycobacterial activities, the bacilli may remain viable for extended periods of time within the granuloma to generate LTBI. The bacilli may later resuscitate when conditions are favorable, leading to cell necrosis and cavitation of the lung (Casanova and Abel, 2002; Raja, 2004; van Crevel *et al.*, 2002).

During mycobacterial pathogenesis, macrophages are the major antigen presenting partners for T-cells and a source of IL-12. However dendritic cells also represent the most professional antigen presenting cells for priming naïve T-cells and are an important source of IL-12. Dendritic cells can take up mycobacteria, they have bacteriostatic activity, they acquire antigen presenting capabilities for mycobacterial-derived antigens and hence, can trigger the adaptive immune response (Jiao *et al.*, 2002; Tailleux *et al.*, 2003a). Due to their migratory ability, they have also been shown to play a role in dissemination of *M. tuberculosis* (Humphreys *et al.*, 2006). Finally, the humoral immune response has also been shown to play a protective role against *M. tuberculosis* infection (de Vallie`re, 2005; Hamasur *et al.*, 2003; Maglione *et al.*, 2007; Teitelbaum *et al.*, 1998; Williams *et al.*, 2004) and dissemination (Costello *et al.*, 1992).

1.2.3 *M. tuberculosis* evasion of the immune defense

The success of *M. tuberculosis* as a pathogen centers on its ability to manipulate or avoid the host defense mechanisms for its own survival. There has been a high level of interest in this area and a variety of mechanisms have been suggested to contribute to the survival and interference of *M. tuberculosis* with the immune response. These include inhibition of phagocytosis (Torrelles *et al.*, 2008; Villeneuve *et al.*, 2003), inhibition of phagosome-lysosome fusion (Chua and Deretic, 2004; Hestvik, 2004; MacGurn and Cox, 2007; Malik *et al.*, 2000; Malik *et al.*, 2003), resistance to RNI (Davis *et al.*, 2007; Miller *et al.*, 2004) and inhibition of antimicrobial peptide synthesis (Rivera-Marrero *et al.*, 2004). The ability of the tubercle bacilli to arrest phagosome maturation, as an intracellular survival strategy, has been well demonstrated (Hestvik *et al.*, 2005; Pethe *et al.*, 2004; Vergne *et al.*, 2004a). Normally, phagosome maturation involves acquisition of the GTPase, Rab5, accumulation of the PI3P-binding protein, early endosome autoantigen1 (EEA1) and phosphorylation of phosphatidylinositol (PI) to generate PI-3-phosphate (PI3P) by the PI kinase VPS34 (Fratti *et al.*, 2001; Vieira *et al.*, 2003). Recruitment of Rab7 to the phagosome facilitates fusion with lysosomes (Harrison *et al.*, 2003; Hmama *et al.*, 2004). Within phagosomes, there is increased acidification from pH 5.5 to 4.5 facilitated by the vacuolar ATPase, which results in activation of the lysosomal hydrolases (Singh *et al.*, 2006b; Steinberg and Grinstein, 2008).

In contrast, phagosomes containing live pathogenic mycobacteria retain the early endosomal GTPase Rab5 and do not acquire EEA1 (Brumell and Scidmore, 2007; Fratti *et al.*, 2003a; Fratti *et al.*, 2001; Hmama *et al.*, 2004; Kelley and Schorey, 2003; Vergne *et al.*, 2004b). They also do not accumulate vacuolar ATPase, and hence, do not acidify below pH 6.3 (Vergne *et al.*, 2004a). *M. tuberculosis* mutants defective in counter-immune mechanisms, most importantly arresting phagosome-lysosome fusion, mutants hypersensitive to intraphagosomal acid stress and hence, attenuated for intraphagosomal survival, have been isolated. They include mutants in membrane-associated proteins, and lipid synthesis and transport proteins. The variety of these mutants suggests that modulation of phagosome

maturation is likely to represent a complex multigenic process (Hisert *et al.*, 2004; MacGurn and Cox, 2007; Pethe *et al.*, 2004; Stewart *et al.*, 2005; Vandal *et al.*, 2008).

The surface properties of the tubercle bacilli have been shown to have a significant influence on these processes, and several mycobacterial cell wall lipids and glycolipids have been implicated in altered phagosome biogenesis. The mycobacterial cell wall glycolipid, lipoarabinomannan (LAM), has been shown to inhibit EEA1 recruitment to the phagosomes (Fratti *et al.*, 2001) and disrupt delivery of lysosomal hydrolases (Fratti *et al.*, 2003b; Hayakawa *et al.*, 2007). Another *M. tuberculosis* glycolipid that has been shown to interfere with phagosomal maturation is phosphatidylinositol mannoside (PIM), a phosphatidylinositol analogue and a precursor of LAM. Several studies have demonstrated that PIM specifically facilitates fusion of early endosomes, retaining Rab5 GTPase, blocking acquisition of lysosomal constituents and inhibiting phagosomal acidification (Briken *et al.*, 2004; Brumell and Scidmore, 2007; Vergne *et al.*, 2004b). The mycobacterial cell wall does not only interfere with immune signaling pathways but also confers resistance to killing due to its relatively impermeable physical barrier to the hydrolytic enzymes encountered within macrophages (Camacho *et al.*, 2001).

For efficient delivery of NO, bacteria-containing phagosomes have been demonstrated to recruit iNOS. Exclusion of iNOS by mycobacteria-containing phagocytes has been associated with high resistance of *M. tuberculosis* to RNIs (Miller *et al.*, 2004, Davis *et al.*, 2007). Ca^{2+} has also been shown to be required for subsequent effector mechanisms of innate immunity, including the respiratory burst that generates ROI and the maturation of phagosomes to phagolysosomes (Connolly and Kusner, 2007; Kusner, 2005). However, *M. tuberculosis* prevents the elevation of host cytosolic Ca^{2+} levels by inhibiting sphingosine kinase, an enzyme required in the mediation of the increase of Ca^{2+} (Malik *et al.*, 2003), thus contributing to reduced phagosome-lysosome fusion and enhanced survival within human macrophages (Connolly and Kusner, 2007; Malik *et al.*, 2000).

To prevent activation of macrophages, *M. tuberculosis* interferes with antigen presentation and cytokine release following infection (Banaiee *et al.*, 2006; Gehring *et al.*, 2003; Kincaid and Ernst, 2003; Master *et al.*, 2008). Multiple mechanisms have been suggested to account for inhibition of antigen presentation (Chang *et al.*, 2005), including inhibition of antigen expression and processing (Pai *et al.*, 2004), MHC-II (Noss *et al.*, 2000; Noss *et al.*, 2001; Pai *et al.*, 2003), MHC-I expression (Tobian *et al.*, 2003), transport of MHC-peptide complexes to the cell surface and loading of immunodominant peptides onto MHC-II molecules (Ramachandra *et al.*, 2001). As described above, production of PGLs has been linked to virulence through downregulation of the host inflammatory response to *M. tuberculosis* infection (Reed *et al.*, 2004; Sinsimer *et al.*, 2008). It has been shown that *M. tuberculosis*-infected monocyte derived macrophages do not synthesize IL-12, resulting in a reduced ability to induce T-cell proliferation (Mariotti *et al.*, 2004). The ability of *M. tuberculosis* to induce a shift from an effective Th1 to an ineffective Th2 response has also been implicated in virulence (Manca *et al.*, 2001; Rook *et al.*, 2005). Mycobacterial LAM induces IL-12 and TNF-α production by macrophages in a TLR-2-dependent manner (Moller *et al.*, 2001; Quesniaux *et al.*, 2004), but it can also inhibit pro-inflammatory cytokines in a TLR-2-independent fashion (Briken *et al.*, 2004; Gagliardi *et al.*, 2005; Pathak *et al.*, 2005; Quesniaux *et al.*, 2004). LAM has also been shown to inhibit dendritic cell-mediated immune response (Geijtenbeek *et al.*, 2003) by specifically interacting with dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) (Pitarque *et al.*, 2005; Tailleux *et al.*, 2003b).

1.3 New TB drug discovery and development

No new TB drugs have been introduced for more than 40 years, largely as a result of the lack of activity in the field of TB drug discovery and development. However, the situation has changed significantly in recent years with the establishment of new initiatives such as the Global Alliance for TB Drug Development (TB Alliance; www.tballiance.org). The top priority of the TB Alliance is to shorten the duration of chemotherapy from the current 6-9 months to two months or less (www.tballiance.org). A study done in South East Asia estimated that a two-month regimen introduced by 2012 could prevent ~ 20 % of new cases and ~ 25 % of TB deaths (Salomon *et al.*, 2006). Because the extended chemotherapy is mainly believed to be attributable to the ability of the bacilli to enter into a non-replicating state, understanding the mechanisms used by the tubercle bacilli to enter this persistent state may contribute significantly in shortening TB chemotherapy (Connolly *et al.*, 2007). In the past few years, significant efforts have been made to develop new TB drugs and, despite the difficulties and challenges faced, several promising candidates have emerged. The discussion here will be focused on those which have reached phase II and phase III clinical trials.

1.3.1 Drugs in clinical trials

The high potency of fluoroquinolones against replicating and nonreplicating mycobacteria has attracted considerable attention and has led to their use as second-and third-line drugs (Keshavjee *et al.*, 2008; Moadebi *et al.*, 2007). These drugs include moxifloxacin (Moxi) and gatifloxacin (Gati) which are amongst the first "new" antitubercular drugs to be tested in phase III clinical trials and with a potential to be used in first-line chemotherapy (Check, 2007). Their high antimycobacterial activity in vitro (Ruiz-Serrano *et al.*, 2000), in the murine model of TB (Nuermberger *et al.*, 2004a; Nuermberger *et al.*, 2004b) and in humans (Johnson *et al.*, 2006a; Peloquin *et al.*, 2008; Rustomjee *et al.*, 2008b) has been demonstrated. In monotherapy, both drugs had high early bactericidal activity (EBA), greater than that of INH, and were well tolerated in pulmonary TB patients (Johnson *et al.*, 2006a; Peloquin *et al.*, 2008). A combination of either Moxi and Gati with INH+Rif+PZA in the first 2 months of

treatment improved the sterilizing activity in pulmonary TB patients (Rustomjee *et al.*, 2008b), underscoring the potential for using fluoroquinolones in first-line TB therapy.

The nitroimidazole, PA-824, is one of the most promising novel antitubercular drug candidates identified to date. Its biological target has been proposed to be an enzyme involved in mycolate biosynthesis, whereby it inhibits the oxidation of hydroxymycolates, a known precursor to cell wall ketomycolates. PA-824 is a pro-drug which requires bacterial activation catalyzed a nitroimidazo-oxazine specific nitroreductase Rv3547, which may interact directly with the pro-drug (Manjunatha *et al.*, 2006a; Manjunatha *et al.*, 2006b, Bashiri *et al.*, 2008; Stover *et al.*, 2000). Reduction of PA-824 by Rv3547 results in the production of bactericidal RNIs (including NO) (Singh *et al.*, 2008). It possesses high in vitro activity against replicating and nonreplicating organisms, and against MDR strains of *M. tuberculosi*s. It showed impressive activity against oxygen-starved cultures with a minimum inhibitory concentration (MIC) of 0.015-0.25 µg/ml in vitro. It is most effective by oral administration in mice at 25 mg/kg and in guinea pigs at 40 mg/kg (Stover *et al.*, 2000). The feasibility of including PA-824 in the current standard chemotherapy has been demonstrated in mice. PA-824 alone showed significant bactericidal activity during the first two months of treatment in mice similar to that of INH in human. When used in combination with INH, it inhibited the selection of INH resistant mutants (Tyagi *et al.*, 2005). Compared to mice treated with the standard regimen, which were culture positive after two months of treatment and relapsed after four months, substitution of INH by PA-824 resulted in culture negativity in mice lungs after two months of treatment and no relapse was observed after four months (Tasneen *et al.*, 2008).

The recent discovery of a diarylquinolone compound, R207910 (TMC207), which targets the *M. tuberculosis* ATP synthase proton pump, generated a lot of excitement (Andries *et al.*, 2005; Huitric *et al.*, 2007; Koul *et al.*, 2007; Petrella *et al.*, 2006). It has a high specific mycobactericidal activity with an MIC for *M. tuberculosis* of 0.03-0.12 µg/ml in vitro. It also showed activity against drug-resistant strains with an MIC of 0.06

µg/ml and an effective half-life of over 24 hours. When used as monotherapy, the bactericidal activity of R207910 in mice was higher than that of INH and Rif by 1 $\log_{10}$. One month of combination oral treatment with R207910+INH+PZA and R207910+Rif+PZA gave bactericidal activities similar to that obtained with Rif+INH+PZA therapy after 2 months (Andries *et al.*, 2005; Ballell *et al.*, 2005; Huitric *et al.*, 2007). Unlike INH, R207910 has the same killing efficiency against non-replicating (dormant) bacilli as aerobically growing bacilli (Koul *et al.*, 2008). Combination therapy of R207910 with amikacin, PZA, Moxi and ethionamide gave culture negativity after 2 months of treatment in mice (Lounis *et al.*, 2006). Most importantly, unlike INH which is effective only during the first week of treatment, the activity of R207910 increased from the 2nd week and continued in the last two weeks of therapy (Lounis *et al.*, 2008). Similarly, in TB patients, the bactericidal activity of R207910 monotherapy resulted in delayed bacterial killing, with increased activity being observed from day four, unlike INH and Rif which showed significant activity from first day of treatment. On the basis of these findings, R207910 is considered a good drug to target persisters, having the potential to shorten TB chemotherapy. Most importantly, R207910 was well tolerated by patients (Rustomjee *et al.*, 2008a).

Matsumoto and colleagues reported another promising lead compound, OPC-67683, a nitro-dihydro-imidazooxazole derivative and mycolic acid biosynthesis inhibitor (Matsumoto *et al.*, 2006). It is highly effective with an MIC of 0.006–0.024 µg/ml against both drug susceptible and resistant strains in vitro and at low doses in mice (0.625 mg/kg). OPC-67683 was shown to have high bactericidal activity against intracellular *M. tuberculosis* H37Rv at a concentration of 0.1 µg/ml, similar to that of Rif at 3 µg/ml. In combination therapy with Rif and PZA, mouse lungs were converted to culture negativity in four months as compared to the standard regimen of Rif, INH, EMB and PZA where Colony forming units (CFUs) could still be detected after six months of therapy (Matsumoto *et al.*, 2006; Saliu *et al.*, 2007).

1.3.2 Modeling the persistent *M. tuberculosis* infection

In vitro models

There has been considerable effort in trying to model the environment encountered by *M. tuberculosis* in human using in vitro models. It is believed that *M. tuberculosis* encounters nutrient starvation, hypoxia, nitrosative and oxidative stress during infection and it is this combination of conditions which drives the tubercle bacilli into a dormant, drug-tolerant state (Fenhalls *et al.*, 2002; Rachman *et al.*, 2006a; Timm *et al.*, 2003). In vitro models that may reflect the persistent or the dormant state of *M. tuberculosis* have been defined. Phenotypic studies, transcriptomic and proteomic analyses of *M. tuberculosis* under these conditions have been used to investigate the metabolism and physiology of *M. tuberculosis* in the various models.

Stationary phase cultures in which cells are characterised by high resistance to a variety of stresses, have been widely used as a simple and an inexpensive way to model *M. tuberculosis* persistence (Hampshire *et al.*, 2004; Hu and Coates, 2001; Smeulders *et al.*, 1999; Voskuil *et al.*, 2004). An in vitro dormancy model developed by Wayne and Hayes demonstrated the usefulness of in vitro stationary cultures to model persistence. It is based on the generation of a dormancy-inducing oxygen gradient generated by gentle stirring of cultures. In this model, the first stage of nonreplicating persistence (NRP), designated as NRP1, induced by dissolved oxygen levels of ~ 1 % saturation is followed by NRP2 at oxygen levels below ~ 0.06 % saturation (Wayne and Hayes, 1996). The dormant state of cells in this model is characterized by antibiotic tolerance and dramatic decrease in DNA, RNA, and protein synthesis (Gomez and McKinney, 2004; Wayne and Hayes, 1996). Using this model in combination with transcriptional profiling, a range of genes essential for hypoxia survival were described (Hampshire *et al.*, 2004; Muttucumaru *et al.*, 2004; Voskuil *et al.*, 2004). The Wayne model was recently extended to use in vivo by infecting mice with *M. tuberculosis* cells in NRP2, which were unable to replicate for the first three weeks post infection. This demonstrated the potential of the model to reflect some aspects of LTBI in the human host and as a tool for developing drugs against dormant bacilli (Woolhiser *et al.*, 2007).

The usefulness of the Wayne model was demonstrated by the discovery of the dormancy regulon, which is highly induced during NRP2 and also by exposure to low levels of NO (Voskuil *et al.*, 2003; Voskuil *et al.*, 2004). This regulon is comprised of approximately 50 genes under the control of *dosR/S/T* two-component regulatory system (Kendall *et al.*, 2004a; Roberts *et al.*, 2004; Schnappinger *et al.*, 2003). Intriguingly, several DosR-regulated genes were found to be constitutively upregulated in strains belonging to the Beijing lineage, consistent with the 50-fold higher level of DosR observed in these strains compared to non-Beijing controls (Reed *et al.*, 2007).

The nutrient starvation model, whereby cultures are grown under limited nutrient availability has also been employed to study mycobacterial persistence. The nutrient starvation model employed by Loebel *et al*. involves growth of cultures in phosphate-buffered saline (PBS) for an extended period of time. Bacilli in this model are characterised by drug resistance, no or little replication and dramatic reduction in aerobic respiration. However, the bacilli are able to recover when later transferred to a nutrient-rich media (Betts *et al.*, 2002; Loebel *et al.*, 1933a, b). Recently, Betts *et al*. used this model to characterize the transcriptome and proteome changes during starvation, which provided evidence for slow-down of energy metabolism, transcription, lipid biosynthesis and cell replication by the bacilli under nutrient- limiting conditions. Another major finding was the induction of *M. tuberculosis* stringent response in this model (Betts *et al.*, 2002). The stringent response is mediated by increased levels of hyperphosphorylated guanine nucleotides [(p)ppGpp], which bind to the β-subunit of the RNA polymerase to induce a specific pattern of bacterial gene expression in which most genes are switched off while a subset of genes are upregulated (Crosse *et al.*, 2000; Gong *et al.*, 2002; Primm *et al.*, 2000; Wendrich *et al.*, 2002), affecting more than 80 genes in *E. coli* (Block and Haseltine, 1975; Braedt and Gallant, 1977; Pao and Gallant, 1979). Unlike in Gram negative bacteria where two proteins (RelA and SpoT) are responsible for the synthesis of (p)ppGpp (Balzer and McLean, 2002; Gong *et al.*, 2002), mycobacteria possess only one stringent response regulator, Rel (Avarbock *et al.*, 2005; Avarbock *et al.*, 1999). Interestingly, a *M. tuberculosis* rel_{MTb} knockout

mutant was found to be impaired for normal growth in vitro, long-term survival under in vitro starvation, anaerobic conditions and establishment of chronic infection in mice (Dahl *et al.*, 2003; Dahl *et al.*, 2005; Primm *et al.*, 2000).

Animal models

The mouse model of TB infection has contributed extensively to the current knowledge base regarding *M. tuberculosis* pathogenesis. This model is characterized by a progressive increase in organ bacterial loads in the lungs during the acute phase of infection followed by a stable number of CFUs during the chronic phase which occurs 3-4 weeks after infection due to the onset of the acquired immune response (Flynn, 2006). However, whether the stable number of bacteria in the chronic phase is static, with little or no replication, or is dynamic, where continuous bacterial division is balanced by bacterial death, remains a controversial question. An interesting study by Muñoz-Elías and colleagues defined viable counts as CFUs and total counts as chromosome equivalents by quantitative real-time PCR. Their data supported the hypothesis that the stable number of bacterial CFUs in the lungs during chronic infection represents a static equilibrium (Muñoz-Elías *et al.*, 2005), however the bacilli are metabolically active (Talaat *et al*. 2007). Because of the availability of reagents, and of genetically modified, and inbred strains of mice, which are relatively easy to house, the mouse model is the most commonly used animal model in TB research although the differences in pathology in the mouse infection compared to humans are well recognised (Aly *et al.*, 2006; Flynn, 2006; Muñoz-Elías *et al.*, 2005; Smith, 2003; Ulrichs and Kaufmann, 2006; Via *et al.*, 2008). The Cornell mouse model of latent TB, which is based on the artificial induction of latency by antibiotic treatment, has been used to model certain aspects of LTBI (McCune *et al.*, 1956). This was followed by the development of two modified versions of this model: in the first, reactivation was induced by an immune suppressor (Flynn *et al.*, 1998) and in the second, low-dose variant, very low number of bacilli are used to infect mice, such that latency is induced by the host control of the infection (Botha and Ryffel, 2002; Radaeva *et al.*, 2005).

The use of other models which mimic human infection more closely has been limited by their difficulty to house animals under BSL3 conditions, high cost and reagent availability. The guinea pig model has been useful for studying dissemination and secondary granuloma formation and is commonly used as an important model for preclinical studies in new vaccines and drug interventions due to their intrinsically increased susceptibility to TB infection (Brandt *et al.*, 2004; Hoff *et al.*, 2008; Lenaerts *et al.*, 2007; Lenaerts *et al.*, 2008; Orme, 2005). Pulmonary TB in rabbits recapitulates all five stages in human infection and it has been commonly used as a model for meningeal TB (Tsenova *et al.*, 2005; Tsenova *et al.*, 2006). In non-human primates, the disease pathology is almost indistinguishable from humans, confirming that this is the best available for all aspects of human disease (Flynn, 2006; Gupta and Katoch, 2005; Via *et al.*, 2008).

1.3.3 Genetic tools for TB drug discovery

One approach for TB drug discovery is to identify the compound with high bactericidal activity against *M. tuberculosis* by high-throughput screening of a compound library in a whole-cell assay, followed by identification of its target(s) and optimization of the active compound. Whole-genome sequencing of mutant strains resistant to the identified active compound has proven to be a useful method for target identification (Andries *et al.*, 2005; Sacchettini *et al.*, 2008). However, this is costly and not always successful as certain antitubercular compounds have no defined targets (Matsumoto *et al.*, 2006; Stover *et al.*, 2000). Also, whole-cell screens may yield highly toxic compounds and the lack of cellular target may negatively affect lead optimization (Sacchettini *et al.*, 2008). An alternate approach to drug discovery, which was also the major focus of this study, is to describe the potential drug target using genetic and biochemical approaches and then identify an inhibiting agent. High-throughput screens can also be used in enzyme inhibition assays to identify agents with inhibiting activities against an identified target. However this approach also comes with its limitations; for example, the agent might not display whole-cell activity, possibly as a result of a lack of permeability (Sacchettini *et al.*, 2008).

The development and application of powerful genetic tools, combined with the availability of whole-genome sequence data, has led to the identification of (conditionally) essential genes in *M. tuberculosis* and hence, to the identification of a large number of potential targets for drug discovery (Cole *et al.*, 1998; Cole *et al.*, 2001; Garnier *et al.*, 2003; Machowski *et al.*, 2005; Stinear *et al.*, 2007). Discussed below are some of the currently available genetic approaches used for new drug target identification.

Random mutagenesis

Random mutagenesis using transposable elements represents a powerful tool for drug target identification. This forward genetic approach is based on the random insertion of a transposable element into a genome to create a library of mutants. By identifying the location of individual insertions within the library of mutants, gene essentiality under the conditions tested can be inferred from the absence of insertions in the gene (McAdam *et al.*, 2002; Sassetti *et al.*, 2003; Sassetti and Rubin, 2003). A powerful adaptation of this approach is in signature tagged mutagenesis (STM), which enabled the identification of genes crucial for virulence in macrophages and animal models of TB (Camacho *et al.*, 1999; Collins *et al.*, 2005; Cox *et al.*, 1999; Hisert *et al.*, 2004; Rosas-Magallanes *et al.*, 2007). In this technique, pools of transposon mutants are generated in which each member of the pool is labeled with a unique genetic tag that can be detected using a combination of PCR, DNA hybridization and sequencing (Holden and Hensel, 1998). Pools recovered a few hours after infection are compared to those recovered several weeks post-infection in order to identify those mutants that are missing from the recovered pool, and are thus assumed to be defective for growth in the host (Collins *et al.*, 2005; Murry and Rubin, 2005; Rosas-Magallanes *et al.*, 2007; Ruley *et al.*, 2004). Using STM, several genes involved in mycobacterial survival in vivo were identified by screening mutant libraries in vitro (Vandal *et al.*, 2008), human macrophage (Rosas-Magallanes *et al.*, 2007), goldfish (Ruley *et al.*, 2004), mouse

(Camacho *et al.*, 1999; Cox *et al.*, 1999; Hisert *et al.*, 2004) and guinea pig (Collins *et al.*, 2005) model of infection.

Transposon pools can also be screened using more advanced and effective approaches based on microarray hybridization called designer arrays for defined mutant analysis (DeADMAn) and transposon site hybridization (TraSH). DeADMAn uses the same mutant pool size as STM, while in TraSH, the saturating levels of mutagenesis are reached (Murry and Rubin, 2005; Murry *et al.*, 2008). Both TraSH and DeADMAn have been used to identify mutants attenuated for growth in mouse lungs (Lamichhane *et al.*, 2005; Sassetti *et al.*, 2003; Sassetti and Rubin, 2003) and in guinea pigs (Jain *et al.*, 2007). Analysis of mutant pools allows identification of potential drug targets; however, gene functions that are complemented by the presence of other cells carrying wild-type alleles will not be identified. This necessitates the use of targeted gene mutations to validate the results from pooled mutants analysis (Murry and Rubin, 2005).

Targeted gene knockout

Targeted gene knockout by homologous recombination involves the delivery of the inactivated allele on a suitable vector, such as a suicide plasmid, conditionally replicating plasmid or conditionally replicating mycobacteriophage (Bardarov *et al.*, 2002; Guilhot *et al.*, 1992; Machowski *et al.*, 2005; Parish and Stoker, 2000). This study explored the use of suicide plasmid delivery of the mutant allele. In this case, single crossover (SCO) recombinants are first identified from which double crossover (DCO) are selected using appropriate selectable and counterselectable markers. The first homologous recombination event between a suicide vector and the chromosome occurring on either side of the mutation gives rise to a partial merodiploid SCO recombinant carrying the wild type allele, the mutant allele and the vector sequence. Depending on which side of the mutation the second crossover event occurs, the excision of the vector from the chromosome will result in the SCO recombinant strain either reverting to wild type or producing an allelic exchange (DCO) mutant (Machowski *et al.*, 2005; Parish *et al.*, 1999). For selection/counter selection of allelic

exchange recombinants, SCO clones are subcultured and incubated without selection before plating on selective media. If a mutant allele is marked with a drug resistance gene, the resistant recombinants obtained from the second recombination event are most likely to represent allelic exchange mutants. If the resistance cassette is placed on the suicide plasmid, it will be excised together with the suicide vector during the second recombination event, and the resulting clones may include both allelic exchange mutants and wild-type revertants (Kana and Mizrahi, 2004; Machowski *et al.*, 2005; Parish *et al.*, 1999; Parish and Stoker, 2000; Pavelka and Jacobs, 1999).

Conditional gene knockout

Genes that are essential for the growth of *M. tuberculosis* in vitro represent the most attractive drug targets. However, conditionally essential genes, such as those encoding proteins that are specifically required for growth in vivo or persistence during chronic infection, also represent attractive drug targets (Boshoff *et al.*, 2003; Dahl *et al.*, 2003; McKinney *et al.*, 2000; Sambandamurthy *et al.*, 2002). Most genetic tools are only useful for functional analyses of non-essential genes. Conditional gene silencing methods are required to validate (conditionally) essential targets. Construction of conditional mutants is normally achieved by using inducible promoters, which can be switched on and off under defined conditions, enabling analyses of essential mycobacterial genes. Antisense-based conditional gene knockout systems relied on the use of the inducible acetamidase gene from *Mycobacterium smegmatis* (Greendyke *et al.*, 2002; Narayanan *et al.*, 2000; Parish *et al.*, 1997; Parish and Stoker, 1997). However this system does not allow a tight regulation of mycobacterial gene expression, thereby resulting in basal expression of the repressed gene. The recent description and utilization of tetracycline (Tet) responsive elements, which allow regulation of bacterial gene expression both in vitro and in vivo, have created a powerful set of tools for use in conditional gene silencing in mycobacteria (Blokpoel *et al.*, 2005; Ehrt *et al.*, 2005). Tet repressor (TetR) proteins regulate the expression of a family of tetracycline-exporting proteins. In the absence of Tet, TetR tightly binds to *tet* operators (*tetO*) in the promoter of the *tet*A gene encoding the Tet exporter and

suppresses transcription. As Tet becomes available, it binds TetR and induces a conformational change that results in dissociation of TetR from *tet* operators and thus induces expression of TetR controlled genes (Berens and Hillen, 2003; Carroll *et al.*, 2005). The recent demonstration that *M. tuberculosis* proteasome is essential for in vitro growth and virulence in mice using the Tet system, further confirmed its utility in studying genes with (conditionally) essential functions (Gandotra *et al.*, 2007).

Gene expression profiling

Transcriptional profiling of *M. tuberculosis* from the human host or under conditions that are believed to mimic the conditions encountered by the pathogen in the human host during the various stages of infection has been very widely applied as a tool for describing the physiology and metabolism of the organism and their adaptation to different environmental conditions (Boshoff and Manjunatha, 2006). Whole-genome expression profiling has been used as a tool to predict functions of differentially regulated genes (Kendall *et al.*, 2004b; Murry and Rubin, 2005). There has been a considerable amount of work on transcriptome analysis of *M. tuberculosis* including studies in macrophages (Ehrt *et al.*, 2001; Fontan *et al.*, 2008; Schnappinger *et al.*, 2003; Tailleux *et al.*, 2008), mice (Mollenkopf *et al.*, 2006; Shi *et al.*, 2005; Shi *et al.*, 2008) and humans (Rachman *et al.*, 2006b). In vitro studies have focused on *M. tuberculosis* in stationary phase cultures (Hampshire *et al.*, 2004; Voskuil *et al.*, 2004) and acidic pH (Fisher *et al.*, 2002), and following exposure to NO or hypoxia (Bacon *et al.*, 2004; Muttucumaru *et al.*, 2004; Voskuil *et al.*, 2004) or inhibitors of metabolism (Boshoff *et al.*, 2004; Waddell *et al.*, 2004). *M. tuberculosis* expression patterns in wild type and NOS2-deficient macrophages were first examined by Schnappinger *et al*. (Schnappinger *et al.*, 2003). Their data suggested a metabolic shift in *M. tuberculosis* as it adapts and persists in the intracellular environment (Schnappinger *et al.*, 2003). To understand the host-pathogen interaction, Tailleux *et al*. analysed the transcription profiles of both *M. tuberculosis* and human macrophages or dendritic cells simultaneously. The finding that the gene expression profile of *M. tuberculosis* in macrophages reflected that of a growing organism whereas in dendritic cells, it reflected

that of a highly stressed cell, suggested that macrophages might be more permissive for mycobacterial growth (Tailleux *et al.*, 2008).

Talaat *et al*. compared changes in the transcription profile of *M. tuberculosis* during exponential growth in liquid culture to that in SCID and Balb/c mice, which allowed in-vivo-specific responses to be identified (Talaat *et al.*, 2004). When the same group studied the transcription profile of *M. tuberculosis* during chronic infection and reactivation following immune suppression in mice, they identified genes which may have an important role in the revival of the bacilli (Talaat *et al.*, 2007). Genome-wide expression analysis of *M. tuberculosis* from human lung has also been reported. These studies revealed upregulation of lipid biosynthesis, DNA repair, transport of amino acids, anaerobic respiration, PE and PPE genes, indicating that *M. tuberculosis* does encounter DNA damage, nutrient starvation and hypoxia in human lungs and implicating changes in cell envelope as a mechanism of persistence (Rachman *et al.*, 2006a). Recently, transcriptional analysis was done on bacilli directly isolated from sputum samples. Contrary to the belief that mycobacteria in sputum samples are actively replicating, this study revealed a transcriptional profile consistent with a slow or non-growing bacillus (Garton *et al.*, 2008).

Although these studies have been highly informative, differential expression of a gene under a particular condition does not necessarily imply that the gene plays an essential role under that condition (Gordhan *et al.*, 2006; Rengarajan *et al.*, 2005). Another limitation of transcription profiling is that genes that are constitutively expressed and posttranscriptionally regulated genes are not identified, although these could be of greatest importance (Kendall *et al.*, 2004b).

Protein expression and interaction studies

Proteomic analysis directly identifies and measures levels of expressed proteins in response to a particular stimulus (Rosenkrands *et al.*, 2002; Yuan *et al.*, 1996). This method provides a powerful adjunct to transcriptome analysis for understanding the pathogen response to the stimulus (Betts *et al.*, 2002; Rao *et al.*, 2008a; Rosenkrands *et al.*, 2002). Wang and colleagues studied the proteome of *M. smegmatis* in response to INH, EMB and 5-chloropyrazinamide (an analogue of PZA). By identifying pathways that are responsive to drug treatment, the possible target(s) for 5-chloropyrazinamide could be inferred, which included carboxylic acid, amino acid, organic acid and nitrogen compound metabolism (Wang and Marcotte, 2008). An interesting study used a biotin-switch enrichment method in combination with mass spectrometry to identify the S-nitroso proteome of *M. tuberculosis* which include the major targets for RNIs. Most S-nitroso proteins identified were enzymes involved in intermediary metabolism, lipid metabolism and antioxidant defense against RNIs (Rhee *et al.*, 2005).

Other studies have used global protein expression analysis and computational methods to construct response networks. These networks are then used to identify functionally related proteins that may work together in an organizational structure and to predict function of proteins based on those in the same network with known functions (Mawuenyega *et al.*, 2005). Identifying interacting proteins also plays an important role in predicting protein function. A simple and robust system called mycobacterial protein fragment complementation (M-PFC), designed to identify protein-protein interactions in a mycobacterial host, was recently developed (Singh *et al.*, 2006a). It is based on the gain of trimethoprim resistance by the functional reconstitution of two murine dihydrofolate reductase (DHFR) domains independently fused to two interacting proteins (Singh *et al.*, 2006a).

Comparative Genomics

Genome comparisons among different mycobacterial strains and between different strains of the same species have provided a wealth of information about the pathogenesis, physiology and biochemistry of mycobacteria, so opening new avenues for research in drug target and antigen discovery (Brosch *et al.*, 2000; Cole, 2002a, b; Fleischmann *et al.*, 2002; Murry and Rubin, 2005; Sharma and Tyagi, 2007; Stinear *et al.*, 2008; Vishnoi *et al.*, 2007). The identification of differences in several chromosomal regions [region of difference (RD)] between members of the MTBC has greatly accelerated the development of more specific diagnostic tests and new vaccine candidates (Brosch *et al.*, 2002; Cockle *et al.*, 2002; Cole, 2002b). It is been proposed that if the 3.27 Mb genome of *M. leprae*, which carries only 1605 protein-coding genes, was once similar in size and coding capacity to those of other mycobacteria (> 4.3 Mb), then the gene deletion and decay that occurred during the reductive evolution of this organism may have naturally defined the minimal gene set essential for intracellular growth and pathogenesis (Cole *et al.*, 2001; Cole, 2002b).

1.3.4 New targets/pathways for TB drug discovery

As described above, the prolonged duration of TB chemotherapy is mainly attributable to the ability of *M. tuberculosis* to persist in a non-replicating, drug tolerant state (Gomez and McKinney, 2004; McKinney, 2000). As a result, most current new drug discovery and development programs are aimed at identifying drugs that have potential to shorten TB chemotherapy (Duncan and Barry, 2004; Sacchettini *et al.*, 2008). As a result, considerable effort has been placed on identifying, validating and ranking such targets. In one study, a computational tool was developed and used to rank different *M. tuberculosis* proteins as drug targets based on a number of criteria (Hasan *et al.*, 2006). Some of the targets/pathways that are being actively explored are discussed below.

Carbon metabolism

The switch to using fatty acids as a carbon source was the first validated example of a persistence mechanism in *M. tuberculosis* (McKinney *et al.*, 2000). Under glucose-limiting conditions, *M. tuberculosis* decreases glycolysis and induces the glyoxylate shunt, which enables it to assimilate C_2 compounds (acetate) produced via the breakdown of fatty acids (McKinney *et al.*, 2000). Isocitrate lyase (Icl) is the first enzyme in the glyoxylate pathway required by *M. tuberculosis* to live on fatty acids as the major source of carbon. *M. tuberculosis* strains express either one or two Icl enzymes (Icl1 and Icl2) (Lorenz and Fink, 2002; Muñoz-Elías and McKinney, 2005). The *icl1* gene is upregulated upon entry into stationary phase (Bacon *et al.*, 2004; Voskuil *et al.*, 2004), by hypoxic conditions (Bacon *et al.*, 2004; Muttucumaru *et al.*, 2004; Voskuil *et al.*, 2004) and in activated macrophages (Schnappinger *et al.*, 2003). An *icl1* mutant of *M. tuberculosis* Erdman grows normally during the acute phase of infection, but is unable to persist in the chronic phase (McKinney *et al.*, 2000). A Δ*icl1*Δ*icl2* double mutant of the Erdman strain is significantly impaired for intracellular replication in macrophages and growth in the mouse lung, suggesting that the two enzymes have a joint function in fatty acid metabolism (Muñoz-Elías and McKinney, 2005). The second enzyme in the glyoxylate bypass is malate synthase. Antibodies against this enzyme have been detected in TB patients (Singh *et al.*, 2005). The importance of the glyoxylate cycle for persistence of *M. tuberculosis* and the absence of this pathway in mammals has made Icl and malate synthase very attractive drug targets (Anstrom and Remington, 2006; Sharma *et al.*, 2000; Smith *et al.*, 2003). High-throughput compound screens for inhibitors of both Icl (Sacchettini *et al.*, 2008) and malate synthase have been conducted and which have led to the identification of promising malate synthase inhibitors (http://www.tballiance.org).

Energy metabolism

The finding that de novo ATP synthesis is essential for *M. tuberculosis* survival under hypoxia and non replicating persistence makes the ATP synthase a very attractive target for drugs against persistent bacilli. ATP levels in hypoxic, non-replicating bacilli are 5-6-fold lower than aerobic replicating *M. tuberculosis*, making the organism highly susceptible to a further reduction in levels of ATP (Rao *et al.*, 2008b). This idea is supported by high activity of the ATP synthase inhibitor, R207910, against non-replicating mycobacteria (Koul *et al.*, 2008). The second major drug target in energy metabolism is NADH-menaquinone oxidoreductase II (Ndh2), which catalyzes the first step in the electron transport chain. *M. tuberculosis* Ndh2 is essential for growth in vitro (Teh *et al.*, 2007; Weinstein *et al.*, 2005; Yano *et al.*, 2006) and has been shown to be the target for phenothiazine drugs (Yano *et al.*, 2006), which have a high antitubercular activity in vitro and in mice (Weinstein *et al.*, 2005).

The proteasome

Proteasomes are large multi-subunit proteases which facilitate several cellular processes including degradation of damaged (*e.g.* oxidized, nitrated or nitrosated) proteins (Bochtler *et al.*, 1999). The *M. tuberculosis* proteasome core contains α and β subunits encoded by the operonic *prcA* and *prcB* genes, respectively. These genes are essential for growth in vivo, and in vitro and resistance to nitrosative, and oxidative stress (Darwin *et al.*, 2003; Gandotra *et al.*, 2007; Rhee *et al.*, 2005; Sassetti *et al.*, 2003). The essentiality of the *M. tuberculosis* proteasome was supported by the finding that two proteasome associated genes Rv2115c (*mpa*) and Rv2097c (*pafA*) are essential for *M. tuberculosis* growth in mice and resistance to nitrosative and oxidative stress (Darwin *et al.*, 2003). The *mpa* gene encodes the ATPase, which may be required for substrate unfolding and transfer into the proteasome core (Darwin *et al.*, 2005), whereas *pafA* may be important for the recognition of the target protein (Festa *et al.*, 2007). Similar to eukaryotes, *M. tuberculosis* proteasome targets are modified by a prokaryotic ubiquitin-like protein (Pup) encoded by Rv2111c, which is operonic with *prcAB*, as a signal for degradation (Pearce *et al.*, 2008). The essentiality and the availability of *M. tuberculosis*

proteasome structures (Hu *et al.*, 2006; Lin *et al.*, 2006) make it a potential drug target (Gandotra *et al.*, 2007).

1.4 Ribonucleotide reductases (RNRs)

Ribonucleotide reductases (RNRs) are a class of enzymes that play an essential role in nucleotide metabolism. They catalyze the reduction of all four ribonucleotides to their corresponding deoxyribonucleotides (dNTPs) by a radical-dependent redox reaction, whereby NADPH serves as a final reductant. Phosphorylation catalyzed by deoxynucleoside-diphosphate kinases and conversion of dUTP to dTTP catalyzed by thymidylate synthase comprise the final steps in the de novo biosynthetic pathway (Figure 1.1) (Eklund *et al.*, 2001; Mathews and Van Holde, 1996). The essentiality of RNR function for growth and survival of all living organisms has led to a lot of interest in elucidating the structure, function, and regulation of RNRs. There are three main classes of RNR (class I, class II and class III), which differ in their subunit composition, co-factor use and oxygen requirements (Table 1.2). The catalytic mechanism of RNR involves a complex series of redox reactions, which is conserved among all the classes. All classes of enzyme share an essential cysteine residue at the active site (Kolberg *et al.*, 2004; Nordlund and Reichard, 2006). Unlike the class II enzyme, the class I and class III RNRs cofactors cannot interact directly with the active site cysteine to produce a thiyl radical important for downstream reactions. In these cases, a radical is generated and transferred instead through a long radical transfer chain from the small subunit radical site to the large subunit active site (Katterle *et al.*, 1997; Kolberg *et al.*, 2004; Saleh and Bollinger, 2006).

Class I enzymes reduce ribonucleoside 5'-diphosphates (NDPs) to deoxyribonucleoside 5'-diphosphates (dNDPs) (Figure 1.1), while the majority of class II and all class III enzymes reduce ribonucleoside 5'-triphosphates (NTPs) to deoxyribonucleoside 5'-triphosphates (dNTPs) (Kolberg *et al.*, 2004). A class IV enzyme, in which the small subunit contains a manganese center instead of a diiron center, was postulated to exist

(Oehlmann and Auling, 1999), but was shown to be an artifact (Fieschi *et al.*, 1998; Huque *et al.*, 2000).

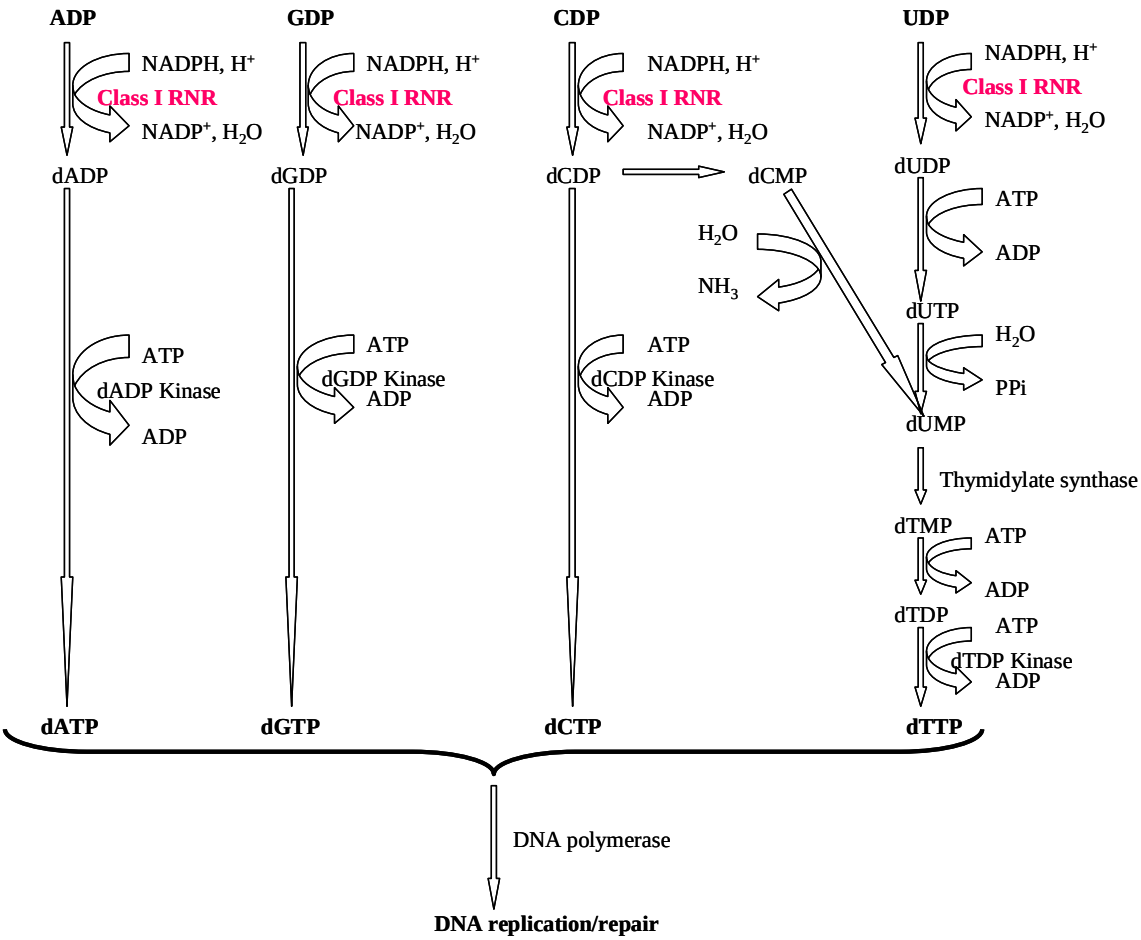


Figure 1.1 De novo dNTP synthesis pathway catalised by class I RNR and its role in DNA metabolism. Class I RNR reduces NDP substrates to dNDPs, which are then phosphorylated by deoxynucleoside-diphosphate kinases (dRNK) to yield dNTP products. For the synthesis of dTTP, several steps are essential before the final kinase reaction to convert either dCDP or dUDP to dTDP. dCDP is dephosphorylated to form dCMP, which is then deaminated to form dUMP, while dUDP is first phosphorylated to produce dUTP as a substrate for dephosphorylation to generate dUMP. dUMP is then converted to dTMP by thymidylate synthase and phosphorylated twice to form dTTP (Eklund *et al.*, 2001; Mathews and Van Holde, 1996).

Table 1. 2 Division and properties of RNR enzyme classes and sub-classes

Properties	**CLASS IA**	**CLASS IB**	**CLASS IC**	**CLASS II**	**CLASS III**
Genes	*nrdAB*	*nrdEF*	*nrdAB*	*nrdJ*/*nrdZ*	*nrdDG*
Subunit structure	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$	α_2 or α	$\alpha_2 + \beta_2$
Metal cofactor	Fe-O-Fe	Fe-O-Fe	Mn-O-Fe Fe-O-Fe	Adenosyl cobalamin	[Fe-S]S-adenosin-methionine
Oxygen requirements	Aerobic	Aerobic	Aerobic	Oxygen independent	Anaerobic
Active radicals	Tyrosyl Cysteinyl	Tyrosyl Cysteinyl	Cysteinyl	Adenylyl Cysteinyl	Glycyl Cysteinyl
Substrate	NDP	NDP	NDP	NDP/ NTP	NTP
Reductant	Thioredoxin Glutaredoxin	NrdH-redoxin Glutaredoxin	Thioredoxin Glutaredoxin	Thioredoxin	Formate
Allosteric sites	2	1	2	1	2
dATP inhibition	Yes	No	Yes	No	Yes
Occurrence	Eukaryotes Eubacteria Bacteriophages Viruses	Eubacteria	Eubacteria	Archaebacteria Eubacteria Bacteriophages	Archaebacteria Eubacteria Bacteriophages

1.4.1 Class I RNR

Oxygen-dependent, class I enzymes constitute the most widely studied RNRs, and occur within the highest to the lowest living systems. The structure of class I RNR is a tetramer comprising two dimeric subunits with a quaternary $\alpha_2\beta_2$ organization of the holoenzyme (Figure 1.2) (Nordlund and Reichard, 2006). However, the exception is the *Saccharomyces cerevisiae* heterodimeric enzyme, which has a unique subunit composition of αα'ββ', where only one of the β subunits can bear a diiron center (Perlstein *et al.*, 2005; Sommerhalter *et al.*, 2004). Until recently, class I enzymes were subdivided into class Ia and class Ib based on allosteric regulation and utilization of different electron donors. The class Ia RNR large (R1/α) subunit consists of two allosteric sides, a redox site and a catalytic site, while the small (R2/β) subunit contains a tyrosyl radical and a diiron center per monomer, essential for enzyme activity (Figure 1.2) (Nordlund and Reichard, 2006). Class Ia enzymes are found in all eukaryotes except for the unicellular *Euglena gracilis*, which has a class II enzyme (Torrents *et al.*, 2006a), in prokaryotes, viruses, and in bacteriophages. These enzymes are characterized by the fact that they possess two allosteric sites, with the *E. coli* and mouse enzyme being the prototype for prokaryotes and eukaryotes, respectively (Kolberg *et al.*, 2004).

The *E. coli* class Ia RNR-based mechanism of ribonucleotide reduction by RNRs is depicted in Figure 1.3. Upon substrate binding at the catalytic site, the tyrosyl radical from the R2 subunit abstracts a hydrogen atom from cysteine 439 (C439), so converting it to a thiyl radical, which then results in the reduction of the tyrosine (Step 1). The thiyl radical is then used to abstract the hydrogen atom from carbon 3 (C-3') of the substrate, producing a free radical at that position. The C-3' substrate radical then facilitates the protonation of the hydroxyl at C-2 by C225 and its release as water (Steps 2 and 3). The C225 anion then forms a disulphide bond with C462, transferring a hydrogen atom to C-2'. The C-3' radical then abstracts its original hydrogen from C439 (Step 4). The product is released and C439 abstracts its hydrogen atom from the tyrosine in the R2 subunit, so regenerating the tyrosine radical (Step 5).

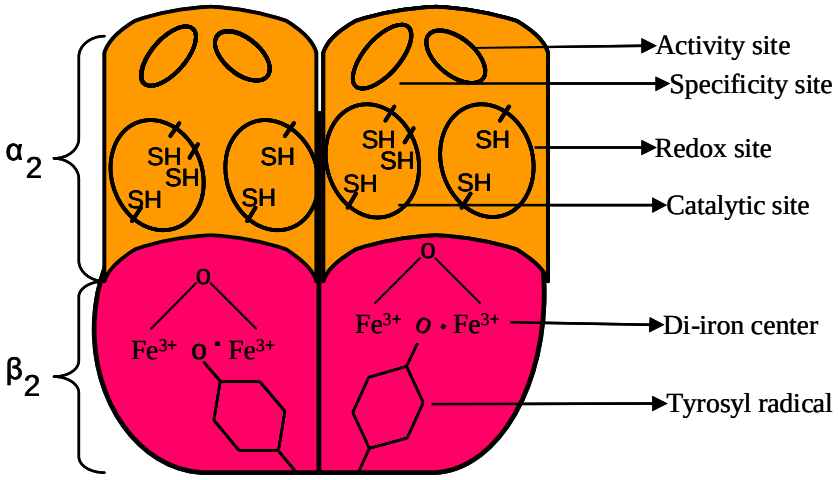


Figure 1.2 Structure of a class Ia RNR. The structure is a tetramer with two dimeric subunits. Each monomer of the R1 subunit consist of two allosteric sides: activity and specificity sites, a redox site and a catalytic, while the R2 subunit monomer contains a tyrosyl radical and a diiron center (Kolberg *et al.*, 2004; Mathews and Van Holde, 1996; Nordlund and Reichard, 2006).

The external co-factor (glutaredoxin/thioredoxin) is used to reduce the disulfide bond formed, hence regenerating the active form of the enzyme for recycling (Step 6). The oxidized glutaredoxin/thioredoxin is then reduced by the glutaredoxin/thioredoxin reductase which can then be reduced by NADPH (Eklund *et al.*, 2001; Kolberg *et al.*, 2004; Nordlund and Reichard, 2006).

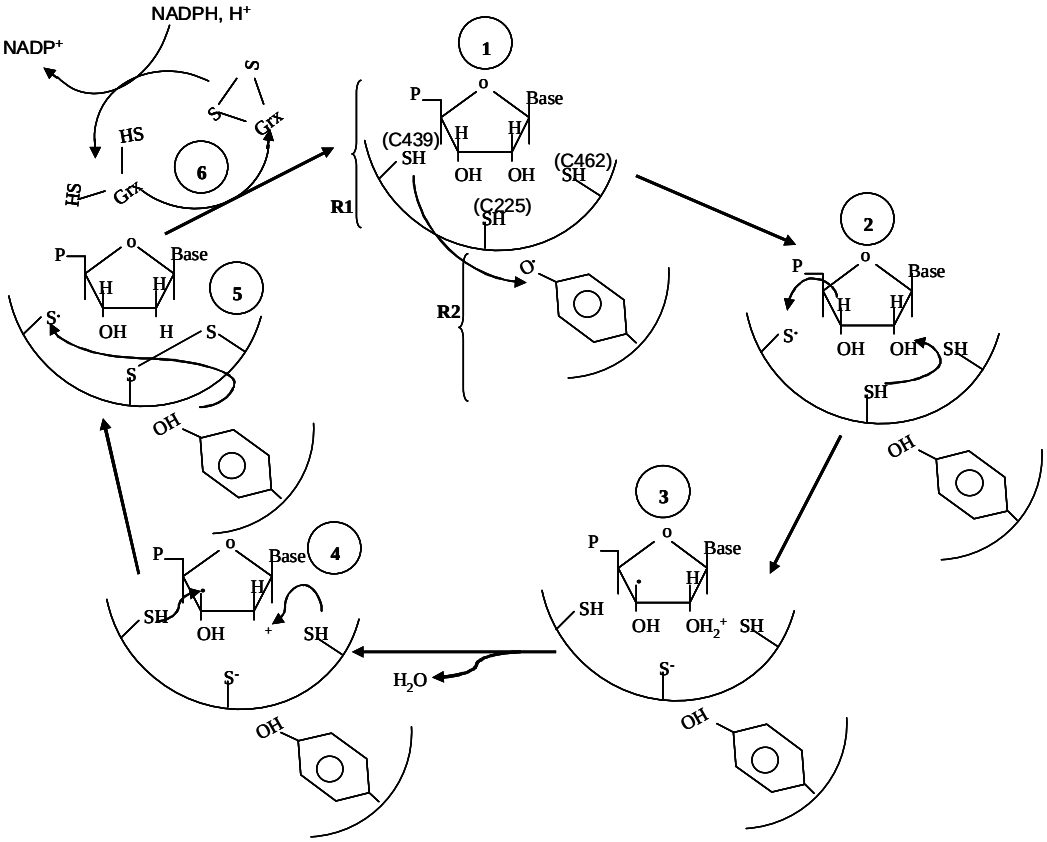


Figure 1.3 Catalytic mechanism of class Ia RNR. The mechanism involves a series of free radical dependent redox reactions initiated by the tyrosine radical in the R2 subunit and facilitated mainly by three cysteine residues (C439, C225 and C462) in the catalytic site of the R1 subunit. Glutaredoxin (Grx)/thioredoxin serves as a final reductant of the enzyme for recycling (Kolberg *et al*., 2004; Mathews and Van Holde, 1996; Nordlund and Reichard, 2006).

In contrast to class Ia RNRs, class Ib enzymes have only one allosteric site (Eliasson *et al.*, 1996), and lack the activity site (Figure 1.2) for ATP/dATP binding for allosteric, on/off switching of the enzyme. This form of the enzyme is only found in prokaryotes, with the RNR from *Salmonella typhimurium* being the most widely studied class Ib enzyme (Galander *et al.*, 2006; Uppsten *et al.*, 2003a; Uppsten *et al.*, 2003b). Unlike the class Ia enzyme which uses thioredoxin or glutaredoxin (Gon *et al.*, 2006b; Koc *et al.*, 2006; Ortenberg *et al.*, 2004), class Ib RNR uses the NrdH protein as an electron donor (Jordan *et al.*, 1997a).

In bacteria, the class Ia RNR large (R1) subunit is encoded by *nrdA* and the small (R2) subunit by *nrdB*, whereas the class Ib large subunit is encoded by *nrdE* and the small subunit by *nrdF*. Both the large and small subunits from the two subclasses show low (<30 %) protein sequence identity; however, the essential catalytic residues are conserved (Jordan *et al.*, 1994). In *E. coli*, the operonic *nrdEF* genes are transcribed together with *nrdI* and *nrdH*, located immediately upstream of *nrdEF* (Monje-Casas *et al.*, 2001), where *nrdI* encodes the NrdF di-iron cluster reductant (Cotruvo and Stubbe, 2008; Roca *et al.*, 2008) and *nrdH* encodes the NrdE disulfide bond reductant (Jordan *et al.*, 1997a).

A third type of class I enzyme – the class Ic RNR – was recently identified in *Chlamydia trachomatis* (Högbom *et al.*, 2004). The class Ic RNR is distinguished from class Ia and Ib enzymes by the unique structural and biochemical features of its small subunit, which has also been designated as NrdB. Its most interesting feature is that the tyrosine residue which is involved in the catalytic activity of class Ia and class Ib enzymes is substituted by phenylalanine, and yet the enzyme retains activity (Högbom *et al.*, 2004; Roshick *et al.*, 2000). In the classic class I RNR mechanism, a di-iron cofactor (Fe^{II}-Fe^{II}) reacts with oxygen to form Fe^{III}-Fe^{IV} which oxidizes the tyrosyl residue to generate the stable tyrosine radical in the R2 subunit that oxidizes the cysteine residue in the R1 subunit to generate the cysteinyl radical (Fig. 1.3). The class Ic enzyme by-passes the need for the tyrosine residue by using Fe^{III}-Fe^{IV} to directly oxidize a cysteine residue in the R1 subunit (Högbom *et al.*, 2004; Voevodskaya *et al.*, 2005; Voevodskaya *et al.*, 2006; Voevodskaya *et al.*, 2007a). Recently, an interesting study showed that in the presence of manganese, the enzyme uses a manganese-iron cofactor (Mn^{IV}- Fe^{III}) instead of Fe^{III}-Fe^{IV} to generate the cysteinyl radical, which represents a more active form than a di-iron associated enzyme (Jiang *et al.*, 2007a; Jiang *et al.*, 2007c; Voevodskaya *et al.*, 2007b).

1.4.2 Class II RNR

Class II enzymes are oxygen independent, comprise a single polypeptide in a monomeric or dimeric form (α or α_2), and use adenosylcobalamin as a radical generator (Gleason and Olszewski, 2002; Jordan *et al.*, 1997b; Tauer and Benner, 1997). Hemolytic cleavage of the adenosylcobalamin generates an adenosyl radical that interacts directly with an active site cysteine to form the reactive cysteinyl radical (Eklund *et al.*, 2001). Like class I enzymes, class II RNRs use thioredoxin or glutaredoxin as electron donors. Furthermore, like class Ib RNRs, class II enzymes are not inhibited by dATP. They are found commonly in eubacteria, with the best studied example being the class II RNR of *Lactobacillus leichmanni* (Eliasson *et al.*, 1999; Sintchak *et al.*, 2002), and were recently also identified in eukaryotes (Torrents *et al.*, 2006a). In bacteria, class II RNRs are encoded by *nrdJ* (Borovok *et al.*, 2002; Jordan *et al.*, 1997b), which has been designated as *nrdZ* in *M. tuberculosis* (Cole *et al.*, 1998) (Table 1.2).

1.4.3 Class III RNR

Class III enzymes are only found in strict or facultative anaerobic bacteria and some bacteriophages, with the T4 enzyme serving as the prototype (Andersson *et al.*, 2000; Logan *et al.*, 2003). The large subunit is encoded by *nrdD* and the small subunit by *nrdG* (Nordlund and Reichard, 2006; Sun *et al.*, 1995; Torrents *et al.*, 2001). Class III enzymes also adopt an $\alpha_2\beta_2$ quaternary structure in which the large subunit dimer contains the glycyl radical and binding sites for the allosteric effectors (Torrents *et al.*, 2001) and the small subunit contains an essential iron-sulfur cluster (Sun *et al.*, 1995; Sun *et al.*, 1996). Class III enzymes use a glycyl radical to generate a cysteinyl radical, produced in the large subunit by the hemolytic cleavage of S-adenosyl methionine, and facilitated by the small subunit/activase iron-sulfur cluster (Gambarelli *et al.*, 2005; Kolberg *et al.*, 2004; Ollagnier *et al.*, 1997). Once the glycyl radical is formed, the R1 subunit catalyses the reaction independent of the R2 subunit, unlike the situation in class I RNR in which continuous interaction between the large and small subunits is required for catalysis (Nordlund and Reichard, 2006; Sun *et al.*, 1995; Torrents *et al.*, 2001). Whereas the bacterial class III enzyme is inhibited by dATP (Torrents *et al.*, 2000), the viral enzyme is not

(Andersson *et al.*, 2000). In contrast with class I and class II RNRs, class III enzymes use formate as a reductant (Mulliez *et al.*, 1995; Mulliez *et al.*, 2001; Padovani *et al.*, 2001).

1.4.4 RNR-encoding genes in mycobacteria

The availability of whole-genome sequences of a number of mycobacterial species (Brosch *et al.*, 2007; Cole *et al.*, 1998; Cole *et al.*, 2001; Fleischmann *et al.*, 2002; Garnier *et al.*, 2003; Stinear *et al.*, 2007; Stinear *et al.*, 2008) has allowed their complements of RNR-encoding genes to be identified (http://rnrdb.molbio.su.se). Most bacterial genomes, including mycobacteria, contain genes encoding more than one RNR class (Borovok *et al.*, 2002; Dawes *et al.*, 2003; Jordan *et al.*, 1999), which are expressed in response to different environmental stimuli (Borovok *et al.*, 2002; Garriga *et al.*, 1996; Masalha *et al.*, 2001; Monje-Casas *et al.*, 2001). All mycobacteria possess a class Ib RNR encoded by *nrdE* and *nrdF* genes (designated herein as *nrdF2*). As observed in *E. coli* (Monje-Casas *et al.*, 2001), the mycobacterial *nrdE* is operonic with *nrdH* and *nrdI*. Interestingly, *nrdI* is a pseudogene in *M. smegmatis*. Mycobacteria other than *M. leprae* and *M. ulcerans* also possess an R2 subunit-encoding gene homologous to that of the chlamydial class Ic RNR, designated as *nrdB*. However, *nrdE* is the only R1 subunit-encoding gene found in these organisms. The genomes of *M. tuberculosis* and *M. bovis* are distinguished from those of other mycobacteria by the presence of both an alternate class Ib R2 subunit-encoding gene, *nrdF1*, as well as a class II RNR-encoding gene, *nrdZ*. *M. smegmatis* mc^2155, on the other hand, is unusual in that the *nrdH*, *nrdI*, *nrdE* and *nrdF2* genes are located on a duplicated region of the chromosome, and hence, are present in duplicate copies (Table 1.3) (Warner *et al.*, 2006).

Table 1. 3 Mycobacterial RNR gene complements

Organism	**Class Ib**					**Class Ic**	**Class II**
	R1	**R2**	**R2[a]**	**R1 reductase**	**R2 reductase**		
M. tuberculosis H37Rv	*nrdE*	*nrdF2*	*nrdF1*	*nrdH*	*nrdI*	*nrdB*	*nrdZ*
M. tuberculosis CDC155	*nrdE*	*nrdF2*	*nrdF1*	*nrdH*	*nrdI*	*nrdB*	*nrdZ*
M. smegmatis mc^2155	*nrdE* [c]	*nrdF2* [c]	-	*nrdH* [c]	*nrdI* [bc]	*nrdB*	-
M. leprae	*nrdE*	*nrdF2*	-	*nrdH*	*nrdI*	*nrdB* [b]	*nrdZ* [b]
M. bovis sups.bovis	*nrdE*	*nrdF2*	*nrdF1*	*nrdH*	*nrdI*	*nrdB*	*nrdZ*
M. bovis BCG	*nrdE*	*nrdF2*	*nrdF1*	*nrdH*	*nrdI*	*nrdB*	*nrdZ*
M. marinum	*nrdE*	*nrdF2*	-	*nrdH*	*nrdI*	*nrdB*	-
M. ulcerans	*nrdE*	*nrdF2*	-	*nrdH*	*nrdI*	N/A	
M. avium 104	*nrdE*	*nrdF2*	-	*nrdH*	*nrdI*	*nrdB*	-
M. avium sups.paratub -erculosis	*nrdE*	*nrdF2*	-	*nrdH*	*nrdI*	*nrdB*	-

-, Absent
a. Alternate class Ib RNR small subunit
b. Pseudogene
c. Two copies

M. tuberculosis and *M. smegmatis* RNR encoding genes and their function

The mechanisms of DNA metabolism in *M. tuberculosis* are currently subjects of considerable interest. They have an impact on the understanding of genome evolution, acquisition of drug resistance by chromosomal mutagenesis and maintenance of genome integrity, which allows the dormant bacilli to reactivate in a viable form after prolonged periods of non-replicating persistence (Mizrahi *et al.*, 2000). Induction of *M. tuberculosis* RNR genes in the artificial granuloma model (Karakousis *et al.*, 2004b) and in human lungs (Rachman *et al.*, 2006b) provides evidence for the requirement of de novo dNTP synthesis for DNA replication and repair in these compartments.

In addition to class Ib RNR genes *nrdE* (Rv3051c) and *nrdF2* (Rv3048c), *M. tuberculosis* H37Rv genome also contains *nrdF1* (Rv1981c) which encodes the alternate class Ib small subunit, NrdF1 (Cole *et al.*, 1998; Yang *et al.*, 1994; Yang *et al.*, 1997). In this organism, both *nrdF*2 and *nrdE* are essential under in vitro growth conditions (Dawes *et al.*, 2003). NrdF1 contains key catalytic residues (Yang *et al.*, 1997) and can form a complex with NrdE, albeit with a lower affinity than NrdF2 (Uppsten *et al.*, 2004). The NrdF1 and NrdF2 proteins have 71 % amino acid identity and both are expressed in vitro (Dawes *et al.*, 2003; Yang *et al.*, 1997). The residues essential for catalysis and structural organization are conserved in both proteins with the exception of substitutions of the Met189 and Phe258 residues in NrdF2 by Leu and Tyr, respectively, in NrdF1 (Uppsten *et al.*, 2004). However, recombinant NrdEF2 displayed RNR activity in vitro, whereas NrdEF1 did not (Yang *et al.*, 1997). Interestingly, translational inhibition and DNA damage in *M. tuberculosis* resulted in the upregulation of both *nrdF1* and *nrdF2* (Boshoff *et al.*, 2004). Dawes *et al*. also showed that the two genes are expressed at a similar level under the in vitro growth conditions tested (Dawes *et al.*, 2003). Based on the above findings, it is conceivable that NrdF1 might play a role in dNTP supply under certain environmental conditions (Boshoff *et al.*, 2004).

A class II RNR encoded by *nrdZ* (Rv0570) in *M. tuberculosis* (Cole *et al.*, 1998; Dawes *et al.*, 2003) is part of the DosR/DevR regulon (Voskuil *et al.*, 2004). *M.*

tuberculosis nrdZ expression was found to be 10-fold lower than *nrdF2* and *nrdF1* under normal in vitro growth conditions, but upregulated 8-fold as the culture approached anaerobiosis (Dawes *et al.*, 2003). However, unlike in Streptomyces (Borovok *et al.*, 2004), the lack of phenotype of *nrdZ* knockout mutant in vitro under aerobic and microaerobic conditions and for growth and persistence in vivo suggests that *nrdZ* does not play a major role in dNTP supply, at least under the conditions tested (Dawes *et al.*, 2003).

In contrast to *M. tuberculosis*, the genome sequence of *M. smegmatis* mc^2155 (http://www.tigr.org) suggests that this organism possesses a much simpler complement of RNR-encoding genes than its pathogenic, slow-growing counterpart. *M. smegmatis* does not possess a class II RNR-encoding gene (*nrdZ*) or the alternate class Ib small subunit-encoding gene, *nrdF1*. Until recently, the only recognizable RNR-encoding genes in *M. smegmatis* were *nrdE* and *nrdF2* (http://www.tigr.org/tigr-scripts/CMR2/). However, both of these genes are located on a 56 kb region of the chromosome of mc^2155 that is duplicated and flanked by IS*1096* elements (Galamba *et al.*, 2001; Warner *et al.*, 2006). Wild type *M. smegmatis* mc^2155 therefore contains two identical copies of each of these genes (MSMEG2299 and MSMEG1019 for *nrdE* and MSMEG2313 and MSMEG1033 for *nrdF2*). Importantly, a mutant strain of *M. smegmatis* lacking the entire duplicated region was recently constructed (ΔDRKIN) (Warner *et al.*, 2006). This deletion mutant strain was found to be indistinguishable from mc^2155 with respect to growth, transformation efficiency (*ept* phenotype) and cell surface characteristics and was thus proposed as an attractive alternative to mc^2155 for use in studying *M. smegmatis* genes located in the duplicated region (Warner *et al.*, 2006).

Interestingly, a homologue of the chlamydial R2-encoding gene is also found in *M. smegmatis* (MSMEG0349) and *M. tuberculosis* (Rv0233) (Cole *et al.*, 1998). This raises the possibility that these mycobacteria may use a class Ic RNR under certain environmental conditions for the provision and maintenance of adequate levels of dNTPs to serve the DNA synthesis and repair requirements of the organism.

1.4.5 Regulation of bacterial RNRs

Even though there are different transcriptional or translational RNR regulatory mechanisms in different organisms, the allosteric regulation of the enzyme is conserved across all living systems (Nordlund and Reichard, 2006). By allosteric control of the enzyme, bound ATP or dATP at the activity site turns the enzyme on or off (Birgander *et al.*, 2004; Birgander *et al.*, 2005; Kasrayan *et al.*, 2004). A recent study showed that binding of dATP at the active site inhibits the enzyme by inducing the formation of an $\alpha_4\beta_4$ holocomplex (Rofougaran *et al.*, 2008). Binding of ATP or dNTP at the specificity site determines the specificity for each of the four substrates (Kolberg *et al.*, 2004; Nordlund and Reichard, 2006). ATP/dATP binding to the specificity site induces pyrimidine deoxynucleotide (dTTP and dCTP) synthesis. The resulting dTTP then binds, leading to the production of the purine deoxynucleotide dGTP, which will also bind to induce dATP synthesis (Andersson *et al.*, 2000; Eliasson *et al.*, 1996, 1999; Hofer *et al.*, 1998; Larsson *et al.*, 2004).

While emphasis has been placed on understanding the allosteric regulation of the RNR enzymes (Reichard, 2002), relatively little is known about the transcriptional mechanisms that regulate expression of RNR-encoding genes. The reason behind the presence of more than one class of RNR in some organisms is still a question of considerable interest, with further complexity provided by the coexistence of more than one enzyme belonging to the same class or subclass (Monje-Casas *et al.*, 2001) or more than one large or small subunits belonging to the same class (Bracchi-Ricard *et al.*, 2005; Huang and Elledge, 1997; Tanaka *et al.*, 2000; Uppsten *et al.*, 2004). Regulation mechanisms of different RNRs under a variety of environmental conditions have therefore been used to probe the specific roles of these enzymes. The induction of RNR genes by DNA damage (Boshoff *et al.*, 2004; Brooks *et al.*, 2001; Filatov *et al.*, 1996; Hakansson *et al.*, 2006; Mulder *et al.*, 2005) and hydroxyurea [HU (Masalha *et al.*, 2001)] has been well documented.

There has been significant progress in studying RNR gene regulation systems in yeast compared to bacterial systems, with most information coming from studies in *S. cerevisiae* (Chabes *et al.*, 1999; Fu and Xiao, 2006; Huang *et al.*, 1998; Yao *et al.*, 2003; Zhao and Rothstein, 2002). The discussion here will focus on some of the

RNR gene regulation work done in bacterial systems. *E. coli* contains genes encoding class Ia, class Ib and class III enzymes (Fontecave *et al.*, 1989; Kolberg *et al.*, 2004). Transcription of the *nrdHIEF* operon is upregulated during early exponential phase and in response to oxidative stress. Under oxygen limitation, the anaerobic *nrdDG* is highly expressed to functionally substitute for the aerobic *nrdAB*. DNA damage induces only *nrdAB*, while HU induces both *nrdAB* and *nrdEF* (Monje-Casas *et al.*, 2001; Nordlund and Reichard, 2006; Reichard, 1993). Recently, a regulatory mechanism in *E. coli*, which couples DNA synthesis and dNTP synthesis, was suggested. The authors proposed that ATP-bound DnaA, essential for DNA replication initiation, represses *nrdAB* transcription. Based on the fact that ATP-DnaA represses *nrdAB* transcription more strongly than ADP-DnaA, they suggested that conversion of ATP-DnaA to ADP-DnaA at the end of replication initiation increases *nrdAB* expression (Gon *et al.*, 2006a).

Corynebacterium ammoniagenes contains only a class Ib RNR, encoded by non-operonic *nrdE* and *nrdF* genes. Both are highly expressed in early log phase and induced by HU and H_2O_2 (Torrents *et al.*, 2003). *Lactococcus lactis* (Jordan *et al.*, 1996) and *Staphylococcus aureus* (Masalha *et al.*, 2001) contain both class Ib and class III RNRs for aerobic and anaerobic growth, respectively. Upregulation of the *L. lactis* class Ib enzyme under microaerophilic conditions implicates this enzyme in dNTP provision under hypoxia where it may compensate for the inactivation of the class III enzyme by low levels of oxygen (Jordan *et al.*, 1996; Torrents *et al.*, 2000; Torrents *et al.*, 2001). *Pseudomonas aeruginosa* contains all three different classes of RNR, with class Ia expressed highly in exponential phase and class II in stationary phase. *P. aeruginosa nrdJ* can support growth in the absence of the class I enzyme activity (Jordan *et al.*, 1999; Torrents *et al.*, 2005a). The only RNR in *B. subtilis*, encoded by *nrdEF*, was recently implicated in anaerobic growth. In this organism , anaerobic *nrdEF* expression was found to be dependent on the ResDE two-component redox regulatory system with two potential ResD binding sites identified upstream of the *nrdEF* transcriptional start site (Hartig *et al.*, 2006).

Borovok and colleagues have intensively studied RNR gene regulation in Streptomyces. Streptomyces *spp.* (Borovok *et al.*, 2002), like *M. tuberculosis*

(Dawes *et al.*, 2003), contain both class I and class II enzymes. However, unlike in *M. tuberculosis* where only class Ib can support aerobic growth (Dawes *et al.*, 2003), Streptomyces use either class of RNR for aerobic growth (Borovok *et al.*, 2004). In *S. coelicolor*, class Ia is regulated by adenosylcobalamin, whereby binding to a B_{12}-riboswitch upstream of the untranslated region of *nrdAB* represses the expression. This was confirmed by the observation that deletion of a cobalamin (B_{12}) biosynthetic gene *cobN*, results in high levels of *nrdAB* transcripts (Borovok *et al.*, 2004; Borovok *et al.*, 2006). A third gene (*nrdS*) belonging to the AraC family of transcription regulators was identified in *Streptomyces coelicolor* as part of an *nrdABS* operon (Borovok *et al.*, 2004), but its function is unknown.

A gene designated as *nrdR*, which is operonic with *nrdJ* in *S. coelicolor*, was also identified and reported to be involved in the regulation of the transcription of *nrdJ* (Borovok *et al.*, 2002; Borovok *et al.*, 2004). Deletion of *nrdR* in *S. coelicolor* resulted in 20-fold increase in transcription of *nrdJ* and, to a lesser extent, *nrdABS* (Borovok *et al.*, 2004). It was suggested that upon intracellular accumulation of dATP, NrdR complexes with dATP via its ATP cone domain, resulting in conformational changes, and binds to two 16-bp direct repeats (NrdR boxes) upstream of the *nrdJ* and *nrdABS* promoter by its zinc finger domain to repress gene expression (Borovok *et al.*, 2004; Grinberg *et al.*, 2006). This model implies that dATP can serve both as an allosteric and transcriptional regulator. Supporting this observation is the fact that mutations in the ATP cone decreased the DNA binding ability of NrdR (Grinberg *et al.*, 2006, Grinberg *et al.*, 2008). In several bacteria, *nrdR* genes are found to be mostly clustered with RNR genes or with genes involved in DNA replication, such as *dnaB, dnaI* and *polA*. The NrdR box consensus sequence in Actinobacteria was reported as 'acaCwAtATaTwGtgt'. NrdR boxes are highly conserved across different bacterial species and found upstream of most operons encoding RNRs from all three classes (Rodionov and Gelfand, 2005). *E. coli* NrdR was recently characterized and found to directly repress all three classes of RNR with its deletion resulting in high transcription of class Ib and, to a lesser extent, class Ia and class III (Torrents *et al.*, 2007).

RNR gene regulation in mycobacteria is poorly understood. NrdZ is part of the DosR regulon induced under microaerophilic conditions (Voskuil *et al.*, 2004). *M. tuberculosis nrdE*, *nrdF1* and *nrdF2* were reported to be highly expressed in log phase (Dawes *et al.*, 2003), and upregulated by genotoxic stress and translational inhibition (Boshoff *et al.*, 2004). Interestingly, all sequenced mycobacterial genomes contain an *nrdR* homologue, although its role in regulating RNR-encoding gene expression has not been investigated.

1.4.6 RNR as a druggable protein

RNR has been investigated as a potential drug target for anticancer, antibacterial and antiviral agents (Cerqueira *et al.*, 2007; Shao *et al.*, 2006; Wakisaka *et al.*, 2005). Iron chelators, substrates analogues and radical scavengers are potent RNR inhibitors, with radical scavengers such as HU and hydroxylamine being the most commonly used RNR inhibitors (Eklund *et al.*, 2001; Shao *et al.*, 2005; Torrents *et al.*, 2005b). HU and its derivatives are classical RNR radical scavengers and have been commonly used for cancer treatment (Chou *et al.*, 1977; van't Riet *et al.*, 1979). Because the interaction between the small and large subunits of the enzyme subunit is critical for catalytic activity (Coves *et al.*, 1995; Kasrayan *et al.*, 2004; Uppsten *et al.*, 2006), short peptides which interact with the C-terminus of the smaller subunit to prevent holoenzyme complex formation have been investigated as potential antiproliferative agents (Xu *et al.*, 2006). Inhibitors which span the active site and the specificity site and compounds which bind at the interface of the subunits to disturb radical transfer also hold promise as potent RNR inhibitors (Coves *et al.*, 1996; Eklund *et al.*, 2001). Structural studies suggest that structure-based design of compounds that specifically inhibit bacterial RNR without affecting the mammalian enzyme may be possible (Eklund *et al.*, 2001; Kolberg *et al.*, 2004; Strand *et al.*, 2004). The C-terminus residues of *M. tuberculosis nrdF1* and *nrdF2* differ from other R2s in that they are more hydrophilic and more negatively charged (Yang *et al.*, 1997). Supporting the prioritization of *M. tuberculosis* NrdE as a druggable protein (Hasan *et al.*, 2006) is the observation that short peptides derived from the C-terminus of *M. tuberculosis* R2 subunit potently inhibited NrdEF2 enzyme activity in an vitro enzyme assay (Nurbo *et al.*, 2007).

1.5 Aims and Objectives

The presence of three class I RNR small subunit-encoding genes in *M. tuberculosis* might be an indication of an inherent metabolic flexibility that allows the bacilli to adapt to grow and survive under the conditions exerted by the host defense mechanisms. It is evident from prior work that the NrdF2 subunit is indispensable for growth under standard, aerobic culture conditions in vitro (Dawes *et al.*, 2003). However, the roles of the alternate small subunits are unknown. Even though no discernable enzymatic activity was observed for NrdEF1 (Yang *et al.*, 1997), the finding that *nrdF1* is transcriptionally responsive to genotoxic and translational stress (Boshoff *et al.*, 2004) suggests that it may serve a specialized role in survival of the organism under such conditions. Similarly, the properties of the Chlamydial-type R2 subunit, which may render the class Ic RNR resistant to RNIs (Högbom *et al.*, 2004) suggests that *nrdB* may also serve a specialist role in dNTP provision in *M. tuberculosis* in vivo. Finally, the identification of *nrdR* in mycobacteria suggests that mycobacterial RNRs may also be regulated by NrdR. Against this background, the overall aim of this study was to elucidate the molecular mechanisms adopted by mycobacteria for the RNR-catalyzed provision of dNTPs under a variety of stressful conditions, with particular emphasis on the function and expression of the class I R2-encoding genes in *M. tuberculosis* and *M. smegmatis.* To achieve this aim, the following objectives were set:

1. To investigate the role of class 1c RNR in mycobacteria by constructing and phenotyping *M. smegmatis* and *M. tuberculosis nrdB* mutant in terms of virulence in mice and/or growth in vitro, nitrosative, and genotoxic stress survival and HU sensitivity.
2. To evaluate the ability of class 1c RNR to functionally substitute for the class 1b NrdEF2 enzyme in *M. smegmatis* by knocking out the class Ib R2 encoding gene, *nrdF*2.
3. To elucidate the role of NrdF1 in growth of *M. tuberculosis* in vitro and in vivo, adaptation during genotoxic stress, translation inhibition and survival in the presence of HU, by knocking out *nrdF1* and assessing the mutant's behavior under those conditions.

4. To determine if there is functional redundancy or interplay between NrdF1 and NrdB in *M. tuberculosis* by constructing a mutant with deletions in both *nrdF1* and *nrdB*, followed by phenotypic characterization in terms of in vitro growth, sensitivity to DNA damage and HU.
5. To study the role of NrdR in mycobacteria by constructing a knockout mutant of *M. smegmatis* and *M. tuberculosis* disrupted in the *nrdR* gene and assessing the effect of *nrdR* loss on expression of all the *nrd* genes, in vitro growth, sensitivity to HU, and DNA damaging agents and to mutagenesis.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

All bacterial strains used in this study are described in Table 2.1

Table 2.1 Bacterial strains used in this study

Name	Description	Source
E. coli		
DH5α	*supE44 ÄlacU169* (*F80 lacZÄM15*) *hsdR17 recA1 endA1 yrA96 thi-1 relA1*	Promega
M. tuberculosis		
H37Rv	ATCC 25618, virulent laboratory strain	Laboratory collection
Δ*nrdB*	Derivative of H37Rv carrying an unmarked deletion in *nrdB*	This work
Δ*nrdF1*	Derivative of H37Rv carrying an unmarked deletion in *nrdF1*	This work
Δ*nrdF1*Δ*nrdB*	Derivative of H37Rv carrying an unmarked deletions in *nrdF1* and *nrdB*	This work
Δ*nrdR*	Derivative of H37Rv carrying an unmarked deletion in *nrdR*	This work
M. smegmatis		
mc^2155	*ept-1*, efficient plasmid transformation mutant of mc^26	(Snapper *et al.*, 1990)
mc^2155::pAINT	mc^2155 derivative carrying Km resistance plasmid vector (pAINT, Table 2.2) integrated at the *attB* locus	This work
ΔDRKIN	Derivative of mc^2155 lacking the 56 kb chromosomal duplication	(Warner *et al.*, 2006)
ΔDRKIN::pAINT	ΔDRKIN derivative carrying pAINT plasmid integrated at the *attB* locus	This work
Δ*nrdB::hyg*	Derivative of ΔDRKIN carrying a *hyg* marked deletion in *nrdB*	This work
Δ*nrdF2::hyg*	Derivative of mc^2155 carrying a *hyg* marked deletion in *nrdF2*	This work
Δ*nrdF2*::*hyg*::pNRDF2	Derivative of Δ*nrdF2::hyg* carrying the *nrdF2* gene from *M. tuberculosis* integrated at the *attB* locus	This work
ΔDRKINSCO	Single cross-over recombinant between ΔDRKIN and p2ΔSMF2KO (Table 2.2); Hyg^R, Km^R	This work
ΔDRKINSCO::pNRDF2	Single cross-over integrant of ΔDRKIN carrying p2ΔSMF2KO (Table 2.2) integrated at the *nrdF2* locus and the *nrdF2* from *M. tuberculosis* integrated at	This work

	the *attB* locus	
ΔDRKIN*nrdF2*::*hyg*::pNRDF2	Derivative of ΔDRKIN carrying a *hyg* marked deletion in the remaining chromosomal copy of *nrdF2* and pNRDF2 (Table 2.2); Hyg^R, Gm^R	This work
Δ*nrdR*::*hyg*	Derivative of mc^2155 carrying a *hyg* marked deletion in *nrdR*	This work
Δ*nrdR*::*hyg*::pNRDR	Derivative of Δ*nrdR*::*hyg* carrying *M. smegmatis nrdR* integrated at the *attB* locus (via pNRDR, Table 2.2); Hyg^R, Km^R	This work
*dnaE*2::*aph*	*M. smegmatis* mc^2155 *dnaE2* deletion mutant hypersensitive to UV damage	(Boshoff *et al.*, 2003)

E. coli

The growth of *E. coli* on solid and in liquid media was carried out according to standard protocols (Sambrook *et al.*, 1989; Sambrook and Russell, 2001). All strains were grown in Luria Bertani broth (LB) or Luria Agar (LA) overnight at 37 ^oC supplemented with relevant antibiotics when necessary. Liquid cultures were shaken at 300 rpm in a New Brunswick Series 25 Shaker Incubator. Strains transformed with knockout constructs were incubated at 30 ^oC and, in the case of liquid cultures, shaken at 100 rpm to minimise plasmid rearrangement. Ampicillin (Amp), kanamycin (Km), hygromycin (Hyg) and gentamycin (Gm) were used at a final concentration 100, 50, 50 and 10 μg/ml, respectively.

M. smegmatis

Liquid cultures were grown in LB or Middlebrook 7H9 broth (Difco) supplemented with 0.2 % glycerol (v/v), 0.05 % (v/v) Tween 80 and either 0.085 % NaCl (w/v) and 0.2 % glucose (w/v) (7H9-GS), 10 % Middlebrook albumin-dextrose-catalase (ADC, 7H9-ADC) or Middlebrook oleic acid-albumin-dextrose-catalase (OADC, 7H9-OADC) at 37 ^oC and shaking at 300 rpm. Plating was performed on LA or Middlebrook 7H10 supplemented with 0.2 % glycerol, 0.085 % NaCl and 0.2 % glucose (7H10-GS) or 10 % Middlebrook OADC (7H10-OADC) and incubated at 37 ^oC. All *M. smegmatis* strains were stored at -70 ^oC in 30 % glycerol (v/v). Km (25 μg/ml), Hyg (50 μg/ml), Gm (10 μg/ml) and Rif (200 μg/ml) were added to the media where necessary.

M. tuberculosis

M. tuberculosis strains were cultured in a Biosafety Level III laboratory and all manipulations carried out in a BioFlow Class II biological safety cabinet at 180 kPa negative pressure. All strains were grown in 7H9-ADC or 7H9-OADC in roller bottles or stationary in tissue culture flasks. Strains were also grown on 7H10-OADC. All cultures were incubated at 37 ^{o}C. Media was supplemented with Hyg (50 μg/ml) where necessary.

2.2 Plasmid vectors

Plasmid vectors used are described in Table 2.2.

2.3 Bacterial transformation

2.3.1 Chemical transformation of *E. coli*

Preparation of competent cells

An overnight culture was diluted one hundred fold in LB and left to grow to an OD_{600} of 0.6-0.7. The cells were then incubated on ice for 15-30 min, centrifuged in a Beckmann J2-21 centrifuge using a JA-20 rotor at 3000 rpm for 10 min at 4 ^{o}C and the supernatant discarded. The pellet was then re-suspended in 1/3 of the original volume with RF-1 buffer (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15 % glycerol (v/v), pH 5.8) and incubated for 0.5-2 h on ice. The cells were then centrifuged, re-suspended in RF-2 buffer (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15 % glycerol (v/v), pH 6.5) at 1/12 of the original culture volume and left on ice for 15-30 min. Cells were then aliquoted into ice-cold microcentrifuge tubes, flash-frozen in ethanol and stored at -70 ^{o}C.

Transformation

Competent cells were thawed on ice and 100-200 μl of cells were added to pre-chilled DNA in a microcentrifuge tube followed by incubation on ice for 10 min. The cells were heat shocked at 42 ^{o}C for 90 s followed by the addition of 1 ml of 2×TY rescue media to the cells before incubation for 1 h at 37 ^{o}C to allow for the

expression of genes encoding for antibiotic resistance. Cells were then plated on LA supplemented with the relevant antibiotic and incubated overnight at 37 ^{o}C.

2.3.2 Electroporation of mycobacteria

Electroporations of *M. smegmatis* and *M. tuberculosis* were performed according to published protocols (Gordhan and Parish, 2001).

M. smegmatis

An overnight preculture was diluted 100-fold in 100 ml of 7H9-GS and grown overnight at 37 ^{o}C and 100 rpm orbital shaking. The culture was then centrifuged in a Beckmann J2-21 centrifuge using a JA-20 at 3000 rpm for 10 min at 4 ^{o}C and supernatant discarded. The cells were washed three times with ice-cold 10 % glycerol (v/v) and re-suspended in 2 ml of 10 % glycerol. Then 400 µl of freshly prepared cells were mixed with an ice-cold 1-3 µg plasmid DNA in microcentrifuge tubes and briefly incubated on ice. The cells were then transferred into pre-chilled electroporation cuvettes and pulsed at 2.5 kV, resistance 1000 W, capacitance 25 µF in a BioRad GenePulserTM. Immediately, 1 ml of 7H9-GS was added to the cells, transferred to a new microcentrifuge tube and incubated at 37 ^{o}C. After 3 h of incubation, electroporated cells were plated on 7H10-GS containing the appropriate supplements and incubated for 3-5 d at 37 ^{o}C.

Table 2. 2 Plasmids vectors used in this study

Name	Description	Source
p2NIL	*E. coli* cloning vector; Km^R	(Parish and Stoker, 2000)
pGEM3Z(+)f	*E. coli* cloning vector; Amp^R	Promega
pGEM-T Easy	*E. coli* PCR TA cloning vector; Amp^R	Promega
pCR2.1-TOPO	*E. coli* PCR TA cloning vector, Amp^R Km^R	Invitrogen
pAINT	*E. coli*-Mycobacterium integrating shuttle vector; Km^R	(Boshoff and Mizrahi, 2000)
pMV306K	*E. coli*-Mycobacterium integrating shuttle vector; derivative of pMV306 carrying an *aph* gene; Km^R	H. Boshoff
pGOAL17	Plasmid carrying the *lacZ* and *sacB* genes as a *Pac*I cassette, Amp^R	(Parish and Stoker, 2000)
pGOAL19	Plasmid carrying the *lacZ* , *sacB* and *hyg* genes as a *Pac*I cassette, Amp^R	(Parish and Stoker, 2000)
pIJ963	Plasmid carrying the *hyg* resistance gene, Amp^R Hyg^R	(Blondelet-Rouault *et al.*, 1997)
pOLYG	*E. coli*-Mycobacterium multicopy shuttle vector, Hyg^R	(O'Gaora *et al.*, 1997)
pNRDF2	Derivative of pGINT carrying the *nrdF2* gene from *M. tuberculosis*; Gm^R Amp^R	(Dawes *et al.*, 2003)
p2ΔSMB	p2NIL carrying *M. smegmatis* Δ*nrdB* allele; Km^R	This work
p2ΔSMB::*hyg*	p2ΔSMB with *hyg* cassette from pIJ963 cloned in *Bgl*II site; $Km^R Hyg^R$	This work
p2ΔSMBKO	Knockout vector for creating a *hyg* marked deletion in *nrdB* of *M. smegmatis*; Km^R Hyg^R	This work
p2ΔTBB	p2NIL carrying *M. tuberculosis* Δ*nrdB* allele; Km^R	This work
p2ΔTBBKO	Knockout vector for creating an unmarked deletion in *nrdB* of *M. tuberculosis*; Km^R Hyg^R	This work
p2ΔSMF2	p2NIL carrying *M. smegmatis* Δ*nrdF2* allele; Km^R	This work

p2ΔSMF2::*hyg*	p2ΔSMF2 with *hyg* cassette from pIJ963 cloned in *Bgl*II site; $Km^R Hyg^R$	This work
p2ΔSMF2KO	Knockout vector for creating a *hyg* marked deletion in *nrdF*2 in *M. smegmatis*; $Km^R Hyg^R$	This work
pGEMTBF1	pGEM3Z(+)f carrying *M. tuberculosis* 8.4 kb *Bam*HI BAC fragment containing *nrdF1* ; Amp^R	This work
pGNRDF1	Derivative of pGEM3Z(+)f carrying an 883 bp deletion in the *M. tuberculosis nrdF1* gene and 3'- and 5'-flanking sequences; Amp^R	This work
p2ΔTBF1	p2NIL carrying *M. tuberculosis* Δ*nrdF1* allele; Km^R	This work
p2ΔTBF1KO	Knockout vector for creating an unmarked deletion in *nrdF1* in *M. tuberculosis*; $Km^R Hyg^R$	This work
p2ΔTBR	p2NIL carrying *M. tuberculosis* Δ*nrdR* allele; Km^R	This work
p2ΔTBRKO	Knockout vector for creating an unmarked deletion in *nrdR* in *M. tuberculosis*; $Km^R Hyg^R$	This work
p2ΔSMR	p2NIL carrying *M. smegmatis* Δ*nrdR* allele; Km^R	This work
p2ΔSMR::*hyg*	p2ΔSMR with *hyg* cassette from pIJ963 cloned in *Bgl*II site; $Km^R Hyg^R$	This work
p2ΔSMRKO	Knockout vector for creating a *hyg* marked deletion in *nrdR* in *M. smegmatis*; $Km^R Hyg^R$	This work
pNRDR	Derivative of pMV306K carrying the *nrdR* gene from *M. smegmatis*; Km^R	This work

M. tuberculosis

A log-phase culture was diluted 100-fold in fresh 7H9-ADC or 7H9-OADC media and grown to an OD_{600} of 0.8-1.0. Glycine was added to the culture to a final concentration of 1.5 % (w/v) followed by overnight incubation at 37 ºC. The cells were then centrifuged at 3000 rpm for 10 min at room temperature and the supernatant discarded. The cells were washed twice in 10 % glycerol (v/v) and then re-suspended in 2-5 ml 10 % glycerol. Four hundred µl of re-suspended cells were mixed with 1-3 µg/ml of DNA in electroporation cuvettes at room temperature and pulsed at 2.5 kV, resistance 1000 W, capacitance 25 µF in a BioRad GenePulserTM. One ml of 7H9-ADC or 7H9-OADC was added to the cells, transferred to fresh microcentrifuge tubes and incubated overnight at 37 ^{o}C. Cells were then plated on 7H10-OADC agar containing the appropriate supplements and incubated at 37 ^{o}C for 3-4 weeks.

2.4 DNA extraction and purification from bacteria

All DNA extraction procedures were performed according to standard protocols (Sambrook *et al*., 1989; Sambrook and Russell, 2001).

2.4.1 Small scale plasmid DNA isolation from *E. coli*

Overnight cultures of *E. coli* grown in 1 ml of LB were transferred into 1.5 ml microcentrifuge tubes, centrifuged in an Eppendorf 5415D microcentrifuge at 13000 rpm for 30 s at room temperature and the supernatant discarded. The cells were then re-suspended in 100 µl Solution I (0.5 M Glucose, 50 mM Tris·HCl pH 8.0, 10 mM EDTA), and lysed with the addition of 200 µl Solution II (0.2 M NaOH, 1 % (w/v) SDS) with gentle mixing. The lysates were incubated for 5 min at room temperature. Thereafter, 150 µl of Solution III (3 M potassium acetate, pH 5.5) was added to neutralize the solution and this was followed by centrifugation at 13000 rpm for 5 min at 4 ^{o}C. The supernatants were then transferred to new microcentrifuge tubes, RNase A added to a final concentration of 50 µg/ml and incubated at 42 ^{o}C for 30 min. To precipitate DNA, 350 µl of isopropanol was added followed by incubation at room temperature for 5 min and then centrifugation for 10 min at 13000 rpm at room temperature. The supernatants were discarded and the pellets washed once

with 1 ml of ice cold 70 % ethanol and dried in a SpeedVac (Savant, USA). The DNA was then re-suspended in 50-150 µl sterile distilled water.

2.4.2 Bulk plasmid DNA preparation from *E. coli*

Overnight cultures in 100 ml LB were harvested by centrifugation for 10 min at 5000 rpm at 4 ^{o}C. Plasmid DNA was isolated similarly as described above for small scale purification, except that all volumes were increased by a factor of 10. Bulk plasmid DNA preparation and purification by an anion-exchange based Nucleobond kit was conducted as per manufacturer's instructions (Macherey-Nagel, Germany).

2.4.3 Extraction of chromosomal DNA from mycobacteria

A modified method of CTAB (cetyltrimethylammonium bromide; ICN Biomedicals, Ohio) extraction of chromosomal DNA from *M. tuberculosis* and *M. smegmatis* was used throughout this study (Larsen, 2000). Cultures of mycobacteria were dispensed in microcentrifuge tubes, heat-killed for 30 min at 95 ^{o}C and centrifuged at 13000 rpm for 30 s. The cells were re-suspended in 500 µl of TE buffer (10 mM Tris·HCl, 10 mM EDTA, pH 8.0) containing 50 µl of a solution of lysozyme (10 mg/ml) and incubated at 37 ^{o}C for 2 h. Thereafter, 70 µl of 10 % SDS and 6 µl of proteinase K (10 mg/ml) were added and incubated for 2 h at 65 ^{o}C. This was followed by the addition of 100 µl of 5 M NaCl and 80 µl of 10 % pre-warmed CTAB/NaCl (10 % CTAB prepared in 0.7 M NaCl) and incubation for further 10 min. An equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the mixture, followed by centrifugation at 13000 rpm for 5 min. The DNA containing aqueous phase was then transferred to fresh microcentrifuge tubes and equal volume of isopropanol was added to precipitate the DNA. The DNA was harvested by centrifugation at 13000 rpm, washed with 70 % ice-cold ethanol, dried in a SpeedVac (Savant, USA) and finally re-suspended in sterile distilled water.

2.4.4 Small scale extraction of chromosomal DNA from *E. coli* and mycobacteria for PCR screening

Colonies were picked from plates, re-suspended in 20 µl of distilled water followed by the addition of 40 µl of chloroform. The cells were then incubated for 20 min at 100 ^{o}C and centrifuged for 5 min at 13000 rpm. The DNA containing aqueous phase was then directly used as a template for PCR.

2.4.5 Phenol-chloroform extraction and salt-ethanol precipitation of DNA

To remove excess salt or inhibitors from prepared DNA samples, the volume of DNA containing solution was made up to 300 µl with TE. Thereafter, 1/3 of the volume of TE-saturated phenol (10 g phenol, 10 ml TE) was added, followed by mixing and centrifugation for 5 min at 13000 rpm at room temperature. The aqueous phase was then transferred to a fresh sterile microcentrifuge tube and a 1/3 volume of chloroform/isoamyl alcohol (24:1 v/v) was added followed by room temperature centrifugation for 30 s at 13000 rpm. The aqueous phase was transferred to new tubes and DNA precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volumes of 100 % ice-cold ethanol. After incubation for 30 min at -20 ^{o}C, precipitated DNA was harvested by centrifugation at 13000 rpm at 4 ^{o}C, washed with ice cold 70 % ethanol, dried in a SpeedVac and re-suspended in sterile distilled water.

2.5 DNA manipulations

All DNA manipulations procedures were performed according to standard protocols (Sambrook *et al.*, 1989; Sambrook and Russell, 2001).

2.5.1 Agarose gel electrophoresis

Agarose gels were prepared by dissolving electrophoresis grade agarose powder (Sigma, USA), with boiling, in 30 ml 1 × TAE (40 mM Tris·HCl, 1 mM EDTA, pH 8, 0.1 % glacial acetic acid) to a final concentration of 0.8-2 %. Thereafter 3 µl of 2 % (w/v) ethidium bromide (Sigma, USA) was added for visualization of DNA, poured on to a gel casting tray (Hoeffer, Amersham Pharmacia, USA) and left to set. Low-melting agarose powder (SeaPlaque GTG) was used for purification of DNA

fragments. DNA samples and molecular weight markers (Roche Biochemicals, Germany) were mixed with loading dye (0.025 % bromophenol blue, 30 % glycerol) and loaded onto gels. Electrophoresis of gels was performed in gel tanks (Hoeffer, Amersham Pharmacia, USA) filled with 1 × TAE buffer connected to a power pack (BioRad, South Africa) at 80-100 V. Gels were visualized using a GelDoc 2000 system (BioRad, South Africa).

2.5.2 Purification of DNA fragments from agarose gels

Agarase digestion of the gels

Agarase digestions were performed according to the manufacturer's instructions (Fermentas, Lithuania). Briefly, DNA fragments were cut from low-melting agarose gels and melted at 65 ^{o}C in microcentrifuge tubes. The molten agarose was then cooled to 45 ^{o}C, 1 unit of agarase enzyme per 100 µl was added and the mixture incubated for a further 1 h at 45 ^{o}C. The tubes were then incubated on ice for 15 min and centrifuged at 13000 for 10 min at 4 ^{o}C to remove any undigested agarose. The supernatants were transferred to fresh tubes and DNA was precipitated by adding 2.5 volumes of 100 % ice-cold ethanol and harvested by centrifugation. The pellets were dried under vacuum and re-suspended in sterile distilled water.

Purification by GeneClean II glass milk

Purification of DNA fragments using a salt concentration dependent procedure in which DNA binds to the silica matrix (glass milk) at high salt concentration was performed using the GeneCleanII Kit according to manufacturer's instructions (Qbiogene, USA).

Purification by NucleoSpin Extract II

A NucleoSpin Extract II kit, which uses the same principle as above, was also used to purify DNA fragments following the manufacturer's instructions (Macherey asdrtyyNagel, Germany).

2.5.3 Treatment of DNA with enzymes

Restriction digests

Restriction enzymes used in this study were obtained from Amersham Pharmacia Biotech (USA), Roche Biochemicals (Germany) or New England Biolabs, Inc (England). Enzyme restriction reactions were performed according to the manufacturer's specifications. Plasmid DNA digestions were performed in the appropriate buffer for 3 h at the specified temperature. Genomic DNA digestions were carried out overnight in the appropriate buffer.

De-phosphorylation

To remove 5' phosphate groups from DNA, digested DNA was treated with calf intestinal alkaline phosphatase according to the manufacturer's instructions (Roche Biochemicals, Germany). The reactions were carried out for 30 min at 37 ^{o}C and DNA purified by phenol-chloroform extraction and ethanol precipitation (Section 2.4.5).

Blunting of 5' and 3' DNA overhangs

The 5' cohesive overhangs resulting from restriction digestion of DNA were filled in using the Klenow enzyme and dNTPs from Roche according to manufacturer's instructions. Reactions were performed in the appropriate buffer for 30 min at 37 ^{o}C. Blunting of 3' overhangs was carried out using T7 DNA polymerase following manufacturer's instructions (Promega, USA). The reactions were incubated in the supplied buffer for 10 min at 37 ^{o}C.

Ligation of DNA fragments

The Fast-Link^{TM} ligation kit (Epicentre Technologies, USA) was used for ligations of DNA according to the manufacturer's instructions. Ligations of vector and inserts were carried out in 10 µl reaction volumes with incubation at room temperature for 2 h. The total reaction volume was used to transform 100-200 µl of *E. coli* competent cells.

2.6 Polymerase chain reaction (PCR)

Standard PCR reactions were performed using the Roche FastStart Taq DNA Polymerase kit as per manufacturer's instructions (Roche Biochemicals, Germany). For amplification of fragments for use in homologous recombination, the Expand High Fidelity PCR System (Roche Biochemicals, Germany) or Phusion PCR system (New England Biolabs, England) was used. Amplifications from plasmid and genomic DNA were performed with 10-50 and 50-100 ng of template DNA, respectively, in 50 µl reaction volumes. Thermal cycler settings were: denaturation at 94 ^{o}C for 5 min followed by 40 cycles with each cycle consisting of denaturation at 94 ^{o}C for 30 s, annealing at 58-65 ^{o}C for 60 s, extension at 72 ^{o}C for 0.5-2 min, followed by a final extension at 72 ^{o}C for 10 min. All PCR reactions were performed using Eppendorf Mastercycler gradient (Eppendorf, Germany) or Hybaid PCR Express (Hybaid, UK) thermocyclers.

2.7 DNA sequencing

All sequencing was performed by Inqaba Biotech (South Africa) on a Spectrumedix 2410 Capillary Electrophoresis automated DNA sequencer using Big Dye Terminator V3.1 software from ABI for sequence analysis.

2.8 Southern blot analysis

2.8.1 Synthesis and labelling of probes

All DNA probes for Southern blotting were synthesised and labelled using the PCR DIG Probe synthesis kit (Roche Biochemicals, Germany) as described by the manufacturer. Briefly, PCR reactions were carried out in a final volume of 50 µl with 1× supplied PCR buffer containing $MgCl_2$. 2.5 µl PCR DIG labelling mix, 2.5 µl dNTP stock solution and 10-100 pg plasmid or 10-100 ng genomic DNA template were used during the labelling reaction. Thermal cycler parameters were as follows: denaturation at 95 ^{o}C for 2 min and 40 cycles with each cycle consisting of denaturation at 95 ^{o}C for 30 s, annealing at 60 ^{o}C for 30 s, extension at 72 ^{o}C for 40 s, followed by a final extension at 72 ^{o}C for 7 min. Approximately 5 µl of the resulting PCR product was run on a 0.8 % gel without ethidium bromide and stained for 5 min in 20 ml 1× TAE buffer containing 20 µl of 2 % (w/v) ethidium bromide.

The products were used directly as probes or stored at - 20 ^{o}C for a maximum period of 1 year.

2.8.2 Electroblotting

Restriction enzyme digestions of 1-5 µg chromosomal DNA were carried out overnight and the reactions separated on 0.8 % agarose gel by electrophoresis (Section 2.5.1) and the gels were photographed with a ruler using a GelDoc system (BioRad, South Africa). The DNA was then de-purinated by immersing the gels in 0.25 M HCl for 15 min followed by denaturation of DNA by incubation in 0.5 M NaOH/1.5 M NaCl solution for 15-20 min. The gels were then briefly equilibrated in 1 × TBE buffer (0.178 mM Tris·HCl; 17.8 mM boric acid; 2 mM EDTA, pH 8.0) before they were overlaid with HybondTM-N nylon membranes (Roche Biochemicals, Germany) and sandwiched between two 3 MM Whatman filter papers and two sponges in a TE 22 Transphor cassette (Hoefer Scientific, USA). The cassettes were then inserted into a TE 22 Mini Transphor unit (Hoefer Scientific, USA) and DNA electroblotted in 1 × TBE at 4 º C for 2 h at 0.5 A. To cross-link the DNA to the nitrocellulose, membranes were UV irradiated in a UV Stratalinker 1800 (Stratagene, USA) at 1200 mJ/cm^2. Membranes were hybridised immediately or stored at room temperature.

2.8.3 Probe hybridisation

All probes used were synthesized using primers outlined in Table 2.3 and are described in Table 2.4. Probe hybridization was carried out in DIG-Easy-pre-hybridisation buffer (10 ml) (Roche Biochemicals, Germany) which was added to roller bottles containing membranes and incubated by rolling in a Techne Hybridiser HB-1 at 42 ^{o}C for 30-120 min. The DIG-labelled probe was then denatured by incubation at 95 ºC for 10 min. The pre-hybridisation buffer was then substituted with a fresh 42 ^{o}C pre-warmed DIG-Easy-hybridisation buffer containing the denatured probe (1 µl of probe per 1 ml of buffer) and hybridization was then carried out overnight at 42 ^{o}C.

Table 2. 3 Oligonucleotide primers used for probe synthesis

Name	Sequence (5'→3')	Region Amplified
M. smegmatis		
smnrdB-F2	TTCGGGAAGATCTGCAGCGCACGTGGGCGC	142 bp of the 3' of *nrdB* and
smnrdB-R2	CGGTGCGGTACCGCAGTCCGTGACGGTCAA	878 bp of downstream homologous sequence
smnrdF2-F1	ATGATCGCGGCGGTGGCAAGCTTGATGGCG	175 bp of the 5' end of
smnrdF2-R1	TCGGTGAGGGTGTGCCAGATCTGGATGTCG	*nrdF*2 and 752 bp of upstream homologous sequence
smnrdR-F1	CACAGGAGCGAATACGCCGGACGAAAGGC	123 bp 5' end of *nrdR* and
smnrdR-R1	CGAGCACCGAGATCTCGACCGTGGTGAAAC	758 bp of upstream homologous sequence
M. tuberculosis		
tbnrdB-F1p	CGTCGAGATCGACGGTACCGTGTTGCCCAC	114 bp of the 5' of *nrdB* and
tbnrdB-R1p	GTCGATGTCGGCCGGATGCCAGATCTT TGC	813 bp of upstream homologous sequence
tbnrdF1-F1p	CGACCACCGCACCAAGCTTGTCTAGCAGGG	96 bp of the 3' of *nrdF*1 and
tbnrdF1-R1p	CGGTGCAGGGGGGATCCACGACTTTTTCTC	916 bp of downstream homologous sequence
tbnrdR-F2	CTACGGTGGTGGATCCTCGTCCACATTCGG	123 bp of the 5' end of *nrdR*
tbnrdR-R2p	GTCTACCGTTTTGCGCGTGCGACACGCTTC	and 770 bp of upstream homologous sequence

2.8.4 Detection of bound probe

Following overnight hybridisation of probe DNA to the target DNA, hybridisation buffer was discarded and membranes washed twice with 2 × SSC; 0.1 % SDS for 5 min at room temperature, then twice with pre-warmed 0.5 × SSC; 0.1 % SDS for 15 min at 68 ^{o}C. The membranes were then transferred to a container and briefly washed once with 1× Wash buffer [1× Maleic acid buffer (0.1M Maleic acid, 0.15M NaCl, pH7.5); 0.3 % Triton] at room temperature followed by incubation in 1× Blocking buffer (1 × Maleic acid buffer; 1 × Roche Blocking buffer) for 30 min at room temperature. Thereafter, the membranes were incubated for a further 30 min in fresh 1× Blocking buffer containing Anti-DIG-Alkaline phosphatase conjugate (Roche Biochemicals, Germany) and then washed twice with 1 × Wash buffer for 15 min. The membranes were then equilibrated in detection buffer (50 mM $MgCl_2$; 0.1 M pH 9 Tris·HCl; 1 M NaCl), incubated at 37 ^{o}C with an alkaline phosphatase substrate, CSPD (Roche Biochemicals, Germany) for 15 min and exposed to 3MM medical X-ray film in a developing cassette at room temperature for 1-3 h. X-Ray films were then developed in an XP400 developer (Peromac Medical Services, South Africa).

Table 2.4 Probes, enzymes used to digest genomic DNA and fragments detected in Southern blotting

Name	Description	Enzyme	Fragments size detected (kb)	
M. smegmatis			Mutant allele	Wild- type allele
smnrdB-F2R2	1020 bp amplicon amplified using smnrdB-F2 and smnrdB-R2 (Table 2.3) used to probe for *nrdB*	*Nru*I	2.4	3.3
smnrdF2-F1R1	927 bp amplicon amplified using smnrdF2-F1 and smnrdF2-R1 (Table 2.3) used to probe for *nrdF*2	*Nru*I	0.8	2.1
smnrdR-F1R1	881bp amplicon amplified using smnrdR-F1 and smnrdR-R1 (Table 2.3) used to probe for *nrdR*	*Bam*HI	3.1	2.3
M. tuberculosis				
tbnrdB-F1pR1p	927 bp amplicon amplified using tbnrdB-F1p and tbnrdB-R1p (Table 2.3) used to probe for *nrdB*	*Sma*I	3.9	4.6
tbnrdF1-F1pR1p	1120 bp amplicon amplified using tbnrdF1-F1p and tbnrdF1-R1p (Table 2.3) used to probe for *nrdF*2	*Sac*I	3.4	4.3
tbnrdR-F2R2p	893 bp amplicons amplified using tbnrdR-F2 and tbnrdR-Rp (Table 2.3) used to probe for *nrdR*	*Sal*I	3.2	1.3

2.9 Construction and genotypic characterization of *nrd* gene allelic exchange mutants and complemented counterparts in *M. smegmatis* and *M. tuberculosis*

The sequences of the putative *M. smegmatis* and *M. tuberculosis nrd* genes under investigation in this study were obtained from the complete genome sequences at http://www.tigr.org/tigr-scripts/CMR2/ and http://www.pasteur.fr/Bio/TubercuList, respectively. Targeted gene knockout in mycobacteria was carried out by homologous recombination with two-step selection using the p2NIL/pGOAL-based suicide plasmid system, as previously described (Parish and Stoker, 2000). Upstream and downstream homologous fragments were obtained by PCR amplification using pairs of primers containing restriction sites (Table 2.5) for ease of cloning. In some cases of *M. tuberculosis*, fragments were cloned directly from the Bacterial Artificial Chromosome (BAC) library of strain H37Rv that was kindly provided by Prof. Stewart Cole (EPFL, Lausanne). To avoid inadvertent mutations, all PCR amplicons for deletion mutagenesis were first cloned into pGEM3Z(+)f, pGEM-T Easy or pCR2.1-TOPO (Table 2.2) and sequenced (Section 2.7) before

cloning into p2NIL (Table 2.2) (Parish and Stoker, 2000). All vectors, primers and probes used for targeted gene knockout are outlined on Table 2.2, Table 2.5 and Table 2.4 respectively. For blue-white selection of both *E. coli* and mycobacterial strains, solid media was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to a final concentration of 50 μg/ml. To counter-select against clones carrying the *sacB* gene, sucrose (Suc) was added to solid media to a final concentration of 5 % (w/v) for *E. coli* or 2 % for mycobacterial strains.

2.9.1 Targeted knockout of *nrd* genes in *M. tuberculosis*

To construct the suicide vector for targeted knockout of the *M. tuberculosis nrdB* gene, a 1.997-kb fragment containing the 5-terminal 67 bp of the gene and upstream flanking sequence and a 1.96-kb fragment containing the 3'-terminal 156 bp of *nrdB* and flanking sequence were amplified using the tbnrdB-F1/ tbnrdB-R1 and tbnrdB-F2/ tbnrdB-R2 primer pairs, respectively (Table 2.5). Both fragments were simultaneously cloned into the *Asp*718-*Hind*III-digested p2NIL, creating the p2ΔTBB vector, which carries a 722 bp deletion within the *nrdB* gene.

To create a suicide substrate for deletion mutagenesis of the *nrdF1* gene in *M. tuberculosis*, a 8.4 kb *Bam*HI BAC fragment containing the *M. tuberculosis nrdF1* gene and flanking sequences was cloned into the *Bam*HI site of pGEM3Z(+)f to produce pGEMTBF1. A 2.48 kb *Sna*BI-*Asp*718 fragment containing 47 bp of the 3' end of *nrdF1* and a 1.99 kb *Mfe*I-*Bam*HI fragment containing 39 bp of the 5'-end of *nrdF1* were purified from this vector and subcloned into *Asp*718/*Bam*HI-digested pGEM3Z(+)f to yield pGNRDF1. An *Eco*RI-*BamH*I fragment (4.48 kb) carrying the Δ*nrdF1* allele (883 bp deletion in the *nrdF1* gene) from pGNRDF1 was then cloned into the *Sca*I/*BamH*I sites of p2NIL creating p2ΔTBF1. Site-specific deletion of *nrdR* in *M. tuberculosis* was achieved by first amplifying a 1.89 kb fragment containing the 5'-terminal 123 bp of *nrdR* and flanking sequence and a 1.88 kb containing the 3'-terminal 127 bp of *nrdR* and flanking sequence using the tbnrdR-F2/ tbnrdR-R2 and tbnrdR-F1/ tbnrdR-R1 primer pairs (Table 2.5), respectively. Both fragments were then cloned in p2NIL yielding p2ΔTBR, so eliminating 215 bp within the *nrdR* gene.

Thereafter, a 7.94 kb *hyg-lacZ-sacB Pac*I cassette from pGOAL19 (Parish and Stoker, 2000) was cloned into the *Pac*I site of p2ΔTBB, p2ΔTBF1 and p2ΔTBR generating the p2ΔTBBKO, p2ΔTBF1KO and p2ΔTBRKO (Table 2.2) knockout vector respectively. All constructs were individually electroporated into *M. tuberculosis* H37Rv to generate the Δ*nrdB*, Δ*nrdF1* and Δ*nrdR* mutants of *M. tuberculosis*, respectively. The p2ΔTBBKO vector was subsequently electroporated into the Δ*nrdF1* mutant strain to create the double Δ*nrdF1*Δ*nrdB* mutant strain. Deletion mutants were phenotypically isolated by two-step selection using previously described methods (Gordhan and Parish, 2001; Parish and Stoker, 2000). *Sma*I, *Sac*I and *Sal*I were used to digest genomic DNA for *nrdB*, *nrdF1* and *nrdR* (Table 2.4) mutant genotyping by Southern blot analysis using tbnrdB-F1pR1p, tbnrdF1-F1pR1p and tbnrdR-F2R2p (Table 2.4) as probes, respectively.

2.9.2 Construction of *M. smegmatis nrd* genes deletion mutants

To construct a knockout vector for deletion of the *nrdB* gene in *M. smegmatis*, a 1006 bp fragment containing the 5'-terminal 249 bp of *nrdB* and flanking sequence was amplified using the smnrdB-F1 and smnrdB-R1 primers and a 1020 bp fragment containing the 3'-terminal 142 bp of *nrdB* and flanking sequence was amplified using smnrdB-F2 and smnrdB-R2 primers (Table 2.5). Both amplicons were simultaneously ligated into *Asp*718-*Hind*III-digested p2NIL generating p2ΔSMB (Table 2.2) in which 571 bp of the coding sequence of *nrdB* was eliminated. A homologous recombination substrate for deletion of the *nrdF*2 gene in *M. smegmatis* was created by amplifying a 920 bp fragment containing the 5'-terminal 175 bp of *nrdF2* and flanking sequence and a 1007 bp fragment containing the 3'-terminal 133 bp of *nrdF*2 and flanking sequence using the primer pairs smnrdF2-F1/smnrdF2-R1 and smnrdF2-F2/smnrdF2-R2, respectively. The fragments were cloned into p2NIL, so deleting a 678 bp internal segment of *nrdF2* and yielding p2ΔSMF2. To construct a vector for knockout of *M. smegmatis nrdR*, the primer pairs smnrdR-F1/ smnrdR-R1 and smnrdR-F2/ smnrdR-R2 were used to amplify an 881 bp fragment carrying the 5'-terminal 123 bp of *nrdR* and an 808 bp fragment carrying the 3'terminal 113 bp of *nrdR* and flanking sequences. Fragments were cloned in p2NIL yielding p2ΔSMR, so eliminating 217 bp of the *nrdR* gene.

Table 2. 5 PCR primers and amplicons used to create knockout vectors for allelic exchange mutagenesis

Gene	Mutant allele	Size of internal deletion	Knockout vector	Primer	Oligonucleotide pairs used for vector construction		
					Sequence (5'→3')	Enzyme Site	Region amplified
M. smegmatis							
nrdB	Δ*nrdB*::*hyg*	571 bp	pΔ2SMBKO	smnrdB-F1	CTGCTCGTCGTGGCCGCGATaaGCTTCGGGG	*Hind*III	249 bp of the 5' end of *rdB* and
				smnrdB-R1	CGCGCATCGCCGACATGAACGGCTaGAtCTC	*Bgl* II	757 bp of upstream homologous sequence
				smnrdB-F2	TTCGGGAaGATCtGCAGCGCACGTGGGCGC	*Bgl* II	142 bp of the 3' end of *nrdB*
				smnrdB-R2	CGGTGCGGtACcGCAGTCCGTGACGGTCAA	*Asp*718	and 878 bp of downstream homologous sequence
*nrdF*2	Δ*nrdF*2::*hyg*	678 bp	pΔ2SMF2KO	smnrdF2-F1	ATGATCGCGGCGGTGGCAAgCtTGATGGCG	*Hind*III	175 bp of the 5' end of *nrdF*2
				smnrdF2-R1	TCGGTGAGGGTGTGCCAGatCTGGATGTCG	*Bgl* II	and 752 bp of upstream homologous sequence
				smnrdF2-F2	CCGACGTCAACCCGGaGATCtTCTCGGCGC	*Bgl* II	133 bp of the 3' end of *nrdF*2
				smnrdF2-R2	TTTGACGAGGTGATCGGCCGCGgTaCCCCG	*Asp*718	and 867 bp of downstream homologous sequence
nrdR	Δ*nrdR*::*hyg*	217 bp	pΔ2SMRKO	smnrdR-F1	CACAGGAGCGAATACGCCGGACGAAAGGC	*Asp*718	123 bp of the 5' end of *nrdR*
				smnrdR-R1	CGAGCACCGaGaTCTCGACCGTGGTGAAAC	*Bgl* II	and 758 bp of upstream homologous sequence
				smnrdR-F2	GCGTGAACTCGACGAGaTCtCCTACCTGCG	*Bgl* II	113 bp of the 3' end of *nrdR*
				smnrdR-F2	CCGTGTTGCaAGcTTTCACCGACTCCGACG	*Hind*III	and 695 bp of downstream homologous sequence
M. tuberculosis							
nrdB	Δ*nrdB*	722 bp	pΔ2TBBKO	tbnrdB-F1	CCCAGCACGAGCGGTAccACCTGATCAAG	*Asp*718	67 bp of the 5' end of *nrdB*
				tbnrdB-R1	GCTTCAGTGGCAGGCTCGCCCAaTTGAGTC	*Mfe*I	and 1930 bp of upstream homologous sequence
				tbnrdB-F2	GCAATtgTCGACCGACAAGGGAATGCGCCG	*Mfe*I	156 bp of the 3' end of *nrdB*
				tbnrdB-R2	CCTTTCCCGCAAgCTtCGCCTCGGTATGTG	*Hind*III	and 1804 bp of downstream homologous sequence
nrdR	Δ*nrdR*	215 bp	pΔ2TBRKO	tbnrdR-F1	GTAAGGCCATCGGTACCACCAGCTTGGGGC	*Asp*718	127 bp of the 3' end of *nrdR*
				tbnrdR-R1	GTGGCCTACCTACGGaTCcCGTCGGTCTAC	*BamH*I	and 1762 bp of downstream homologous sequence
				tbnrdR-F2	CTACGGTGGTGgATCcTCGTCCACATTCGG	*BamH*I	123 bp of the 5' end of *nrdR*
				tbnrdR-R2	CCCGTCAAAGCCAGTGTaAGCTTCGGCCTG	*Hind*III	and 1774 bp of upstream homologous sequence

* Restriction sites within the primer sequences utilized for subsequent cloning of PCR fragments are underlined and changed bases to create restriction sites are in lower case, in red is the enzyme with its specific site not in the primer sequence, but downstream of it, F1/F2 represent a forward primer, R1/R2 represent a reverse primer

Thereafter, the 1738-bp *hyg* cassette from pIJ963 (Blondelet-Rouault *et al.*, 1997) (Table 2.2) carried on a *Bgl*II fragment was cloned in the *Bgl*II site located at the junction of the upstream and downstream fragments giving rise to the plasmids p2ΔSMB::*hyg*, p2ΔSMF2::*hyg* and p2ΔSMR::*hyg* (Table 2.2). A 6.36-kb *lacZ-sacB* *Pac*I cassette from pGOAL17 (Parish and Stoker, 2000) was then cloned into the *Pac*I site of each of these vectors to generate p2ΔSMBKO, p2ΔSMF2KO and p2ΔSMRKO, the knockout vectors for *M. smegmatis nrdB*, *nrdF*2 and *nrdR*, respectively (Table 2.2).

The p2ΔSMBKO vector was electroporated into the ΔDRKIN mutant of *M. smegmatis* mc^2155 (Warner *et al.*, 2006), and p2ΔSMRKO was electroporated in mc^2155 to create Δ*nrdB*::*hyg* and Δ*nrdR*::*hyg* respectively. p2ΔSMF2KO was electroporated into mc^2155 to create Δ*nrdF2*::*hyg* and into ΔDRKIN strain to create ΔDRKINΔ*nrdF2*::*hyg*::pNRDF2 following electroporation of a single crossover recombinant (ΔDRKINSCO, Table 2.1) with pNRDF2 (Table 2.2), so creating ΔDRKINSCO::pNRDF2 (Table 2.1). Mutant strains were phenotypically isolated by two-step selection, as previously described (Gordhan and Parish, 2001; Parish and Stoker, 2000). Genomic DNA from single cross-over (SCO), double cross-over (DCO) and parental strains was digested with *Nru*I for both *nrdB* and *nrdF2* and with *Bam*HI for *nrdR* mutant genotyping using smnrdB-F2R2, smnrdF2-F1R1 and smnrdR-F1R1 (Table 2.4) as probes for Southern blot analysis.

2.9.3 Complementation of *M. smegmatis* Δ*nrdR*::*hyg* and Δ*nrdF2*::*hyg*

The primer pair, smnrdRC1/smnrdRC2 (Table 2.6), was designed to amplify a 967 bp fragment containing the putative *M. smegmatis nrdR* gene plus 311 bp upstream and 203 bp downstream flanking sequence from wild-type mc^2155 genomic DNA. The fragment was then sequenced and cloned into the *Asp*718 and *Hind*III sites of the integrative vector, pMV306K that was kindly provided by Dr. Helena Boshoff to create pNRDR (Table 2.2). Δ*nrdF2*::*hyg* was complemented using pNRDF2 (Dawes *et al.*, 2003). Both pNRDR and pNRDF2 vectors were electroporated into the Δ*nrdR*::*hyg* and

Δ*nrdF2*::*hyg* strain respectively. Transformants were screened for site-specific integration at the *attB* chromosomal locus using a forward (attBS2) and reverse (attL4) PCR primer set to amplify a 320 bp fragment spanning the *attL* region, while a forward (attL2) and reverse (attBS1) PCR primer set was used to amplify a 282 bp fragment spanning the *attR* region (Barichievy, S., MSc dissertation, University of the Witwatersrand, 2005).

Table 2. 6 PCR primers used to construct Δ*nrdR*::*hyg* complementation vector

Gene	**Vector**	**Oligonucleotide pairs used for vector construction**		
M. smegmatis nrdR	pNRDR	Primer name	Sequence (5'-3')*	Amplicon properties
		smmrdRC1	CAGTGAACTGGCC GTCGTGCAGGTaCc GTC	967-bp amplicon containing *M. smegmatis nrdR* with 311 bp 5' (upstream) and 203 bp 3' (downstream) sequence
		smnrdRC2	CCCGCATGTTCGC GACGAAgCTtGGC ATCC	

* Restriction sites used for cloning are underlined and bases changed to introduce restriction sites are in lower case

2.10 Analysis of gene expression

2.10.1 RNA isolation

RNA was isolated as previously described (Downing *et al.*, 2004). Briefly, cultures of mycobacteria were grown to an OD_{600} of 0.3 and the bacteria then harvested by centrifugation at 1000 rpm for 10 min and cells re-suspended in 1 ml of TRIzol (Sigma, USA). The cells were lysed with Lysing Matrix B (Qbiogene, USA) in a ribolyzer (Savant Fastprep FP120) for three cycles at speed setting of 6 for 20 s with cooling on ice for 2 min between pulses. Lysates were then centrifuged at 13000 rpm for 45 s. The supernatants were then transferred to tubes containing Phase Lock gel (Merck, Germany) and 200 µl of chloroform followed by vigorous mixing for 15 s, and then periodically for 2 min. The resulting suspension was centrifuged at 13000 rpm for 5 min and the aqueous phase transferred to a fresh microcentrifuge tube. An equal volume of isopropanol was added and the samples were then centrifuged at 10000 rpm for 20 min

at 4 ^{o}C. Pellets were washed with 70 % ethanol, air-dried and re-suspended in DEPC-treated H_2O. Contaminating genomic DNA in the RNA preparation was then digested with DNase I (Ambion, USA) and samples purified using an RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. RNA samples were then subjected to a second round of DNase treated using Turbo DNase according to manufacturer's instructions (Ambion, USA). The quality of the RNA was assessed by electrophoresis on a 2 % agarose gel containing 0.1 % SDS and the purity gauged by the A_{260}/A_{280} ratio, whereby a ratio of 1.9-2.1 indicated a pure RNA.

2.10.2 Reverse Transcription (RT)

Reverse transcription of RNA was carried out as previously described (Downing *et al.*, 2004). To anneal primers to RNA, 20 µl annealing reactions consisting of 1 µg RNA and 0.25 µM of each reverse primer were set up by first denaturing the RNA at 94 ^{o}C for 90 s before annealing at 65 ^{o}C for 3 min followed by 3 min at 57 ^{o}C. Thereafter 10 µl of the annealing mixture was mixed with 10 µl of a master mix consisting of 1×RT Buffer (Sigma, USA), 200 µM each dNTP mix (Sigma, USA), 4 mM $MgCl_2$ (Sigma, USA), 0.6 µl dimethyl sulphoxide (DMSO; Sigma) and 2 U Enhanced Avian Myeloblastoma Virus (AMV) RT (Sigma, USA). The RNA was then reverse transcribed using Eppendorf Mastercycler at the following parameters: reverse transcription at 60 ^{o}C for 30 min, then denaturation at 95 ^{o}C for 5 min and final cooling at 4 ^{o}C. To determine the amount of DNA contamination, control reactions that contained no RT were run in parallel.

2.10.3 Quantitative RT-PCR Assay

Real-Time, Quantitative RT-PCR Assay (qRT-PCR)

Primers used for quantitative RT-PCR were designed using the Primer3 design programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are detailed on Table 2.7. All primers were designed to amplify ~ 90-150 bp DNA fragments that were internal to the open reading frames of genes of interest. Primers used for the quantification of *sigA* in *M. tuberculosis* were the same as those described by Dawes *et*

al. (Dawes *et al.*, 2003). qRT-PCR was performed using a Roche LightCycler system and a LightCycler Fast start DNA Master SYBR Green I kit (Roche Biochemicals, Germany) according to the manufacturer‘s instructions. For absolute quantification of mRNA levels, a linear or polynomial standard curve that was based on ten-fold serial dilutions of the wild-type genomic DNA was set up using the LightCycler software (version 4.0). Thereafter, the absolute amount of mRNA in test reactions were determined by extrapolation from standard curves and these absolute transcript numbers were normalized to the number of *sigA* transcripts in the same sample. The normalized data from mutant strains were compared to normalized transcript levels in the wild type control. These analyses were performed in triplicate biological samples, each in duplicate.

Semi-quantitative RT-PCR

cDNA was synthesized as above, using the primers described above. Two-fold serial dilutions of the cDNA were prepared and 2 µl from each dilution was used as the template in a 50 µl PCR reaction. The PCR reaction mixture contained 4 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 0.4 µM primers, 0.5 mg/ml BSA, 10 % DMSO, reaction buffer and 2.5 U of FastStart Taq polymerase (Roche Biochemicals, Germany). PCR reaction parameters were as follows: Denaturation at 94°C for 10 min was followed by cycling for 14 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 30 s and 24 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. The PCR products were then analyzed on a 2 % agarose gel.

2.11 Competitive in vitro growth and long-term survival assays

To assess competitive growth of *M. smegmatis* strains deficient in particular *nrd* genes, equal amounts of Hyg-resistant mutant strains, and Km-resistant parental strains, generated by electroporating *M. smegmatis* mc^2155 or ΔDRKIN with pAINT (mc^2155::*aph*, ΔDRKIN::*aph*, Table 2.2) and selecting Km-resistant transformants, were co-cultured in a final volume of 100 ml without antibiotic. At each time point, aliquots were plated on solid 7H10-ADC containing Hyg (50 µg/ml) or Km (25 µg/ml)

for enumeration of mutant and parental strain CFUs, respectively. Competitive long-term survival assays were set up and assessed similarly as for competitive growth, except that cultures were incubated at 30 ^{o}C with shaking at 100 rpm for at least 5 months. To avoid early clumping of cultures, the concentration of Tween 80 in the media used for these experiments was increased to 0.1 %

Table 2.7 Oligonucleotide primers used for RT-PCR

Name	Sequence (5'- 3')	Region targeted
RTtbnrdF2-F1	GGTCTGGCGTTGGTTGAC	Position 631-718 in *M. tuberculosis nrdF2*
RTtbnrdF2-R1	CCACCTCGTTGTCGTAGAGC	
RTtbnrdF1-F1	AGTTCCACCGAGCAGCAG	Position 154-283 in *M. tuberculosis nrdF*1
RTtbnrdF1-R1	TCAGGACCGCCTCTTCGT	
RTtbnrdB-F1	CGGACGACGAACGTGACTAC	Position 149-229 in *M. tuberculosis nrdB*
RTtbnrdB-R1	GCTGGATGTCCTCGGTCA	
RTtbnrdE-F1	GTTGCTGGAGGATGCGTTC	Position 645-740 in *M. tuberculosis nrdE*
RTtbnrdE-R1	CGGTAGATGTCGGGGTGATG	
RTtbnrdZ-F1	GGCTGGTGTTTCTCGACACG	Position 1055-1154 in *M. tuberculosis nrdZ*
RTtbnrdZ-R1	TAAGGCAGCAGTGGGACCTC	
RTsmnrdF2-F1	CGAGGAGAACCCGAACCT	Position 402-522 in *M. smegmatis nrdF*2
RTsmnrdF2-R1	GCCCGAGTAGAACAGGAAGC	
RTsmnrdE-F1	GAGCCCAAGACCGACAAG	Position 1651-1744 *M. smegmatis nrdE*
RTsmnrdE-R1	GCACCGACTCCTTCAACTG	
RTsmnrdB-F1	CTGTGCGCGGAGTTCATC	Position 178-288 in *M. smegmatis nrdB*
RTsmnrdB-R1	CTGCGTCAGGTACATCTCGTC	
RTsmSigA-F1	GGGCGTGATGTCCATCTCCT	Position 367-488 in *M. smegmatis sigA*
RTsmSigA-R1	GTATCCCGGTGCATGGTC	

2.12 Susceptibility testing of mycobacterial strains

Sensitivity of strains to mitomycin C (MTC; 0.01–0.1 μg/ml), HU (1-80 mM) and STR (0.025–10) μg/ml were determined by plating, in duplicate, serial dilutions of stationary and log-phase cultures on media containing different concentrations of each compound. Plates were then incubated at 37 ^{o}C until visible CFUs could be enumerated. Sensitivity to novobiocin (Novo, 1-100 μg/ml), moxifloxacin (Moxi 0.1-2 μg/ml) and ciprofloxacin (Cipro, 1–20 μg/ml) was determined by spotting 10 μl of serial dilutions of stationary

and log-phase cultures in duplicate, on media containing different concentrations of each compound followed by Cfu enumeration. The minimum inhibitory concentrations (MICs) of MTC, HU, Novo, STR and ofloxacin (Oflox) were determined using the microbroth dilution technique in 96-well microtitre plates, as previously described (Lee *et al.*, 2003). MTC and HU susceptibility testing in liquid cultures was determined by first diluting stationary-phase and log-phase *M. smegmatis* cultures in fresh media supplemented with different MTC or HU concentrations. Cultures were incubated and growth followed for at least 48 h. Survival in the presence of the MTC or HU was assessed by adding the compounds to the undiluted stationary-phase cultures to different final concentrations and CFUs assessed over 48 h post compound addition.

2.13 Sensitivity to UV irradiation

Assessment of bacterial viability after exposure to UV irradiation was conducted using previously described procedures (Boshoff *et al.*, 2003). Briefly, serial dilutions of log-phase cultures were plated in duplicate on 7H10-OADC media and the plates were then irradiated at UV fluences ranging from 0-40 mJ/cm^2 in a Stratalinker 1800. CFUs were enumerated and the proportion of surviving bacteria was scored relative to untreated controls.

2.14 Sensitivity to nitrosative stress

Sensitivity to S-Nitrosoglutathione (GSNO, Sigma) as an NO donor was assessed by incubating liquid cultures (OD_{600} ~ 0.02) with increasing concentrations of GSNO ranging from 0-16 μg/ml for 24 h and CFUs were enumerated on solid media. Survival in the presence of acidified nitrite was determined according to Firmani and colleagues (Firmani and Riley, 2002). Briefly a ten fold dilution mid-log-phase (OD_{600} ~ 0.6) cultures were incubated in 7H9-OADC at pH 5.3 supplemented with $NaNO_2$ at concentrations ranging from 0-48 mM for 24 h. Serial dilutions of cultures were then plated on solid media to enumerate CFUs

2.15 Assessment of UV-induced mutation frequencies

UV-induced mutation frequencies were determined as previously described (Boshoff *et al.*, 2003). Briefly, 40 ml of log-phase cultures were harvested and the bacterial cells were then re-suspended in 5 ml of fresh 7H9-OADC media, followed by UV irradiation at 25 mJ/cm^2. Thereafter, the culture volume was adjusted back to the original volume before incubating at 37 ^{o}C to allow for recovery. Serial dilutions were then plated on 7H10-OADC to determine viable cell counts post irradiation and 1 ml of undiluted culture was plated on media containing Rif 200 μg/ml at times ranging from 0-24 h post irradiation. Mutation frequencies were determined by dividing the number of Rif resistant mutants by total viable cell counts post irradiation.

2.16 Luria-Delbrück fluctuation tests

Spontaneous mutation rates were determined using the Luria-Delbrück fluctuation assay as previously described (Rosche and Foster, 2000). Briefly, 35 cultures each containing approximately 100 cells/ml in a final volume of 2.5 ml were set up and incubated in a 37 ^{o}C rotary shaking incubator. After 7 d, the total volume from 30 cultures was individually plated on media containing Rif at 200 μg/ml to determine the number of resistant mutants arising. Serial dilutions from the 5 remaining cultures were plated to enumerate the total number of viable cells. The number of mutations per culture was calculated using P_0 method of Luria and Delbrück or Lea-Coulson Method of the Median, from which the mutation rate was then calculated.

2.17 Infection of mice and determination of bacterial load

All mouse model experiments were done at the Public Health Research Institute (Newark NJ, USA) under the supervision of Prof. Gilla Kaplan (Laboratory of Mycobacterial Immunity and Pathogenesis). The protocol for these experiments was approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey (Newark, NJ, USA). Eight to ten week-old female B6D2/F_1 mice from Jackson Laboratories (Bar Harbor, ME) were aerosol

infected with wild type or mutant strains of *M. tuberculosis* by exposure to aerosol particles in a nose-only infection apparatus as previously described (In Tox Products, Albuquerque, MN). This resulted in the seeding of ~ 2.3 $\log_{10}$ bacteria within the mouse lungs. Three mice were sacrificed per time point over a period of 126 d where the lungs, liver and spleens of infected animals were harvested, homogenized and serial dilutions plated to enumerated organ bacillary loads (Moreira *et al.*, 1997; Tsenova *et al.*, 1997).

2.18 Statistical analysis

The independent Student's *t* test or paired *t* test was used to assess statistical significance of pair-wise comparisons using GraphPad Prism Software (http://www.graphpad.com/quickcalcs/ttest1.cfm).

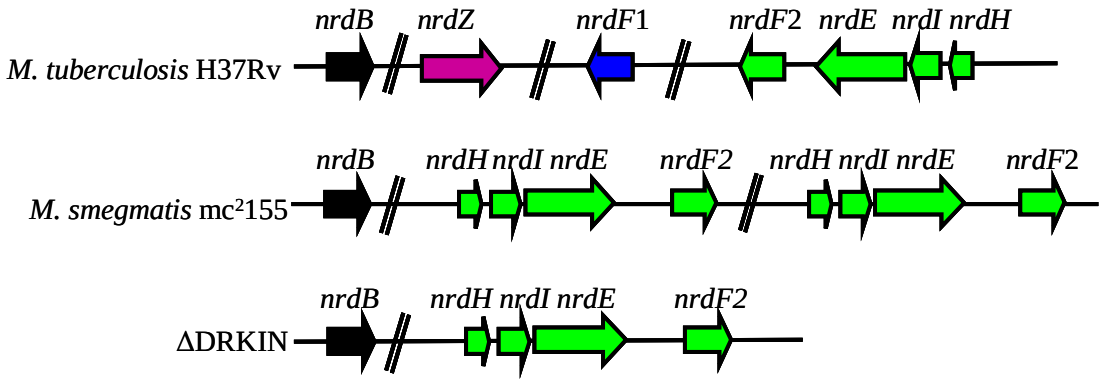
3. Results

3.1 The genomes of *M. tuberculosis* and *M. smegmatis* contain multiple RNR-encoding genes

Unlike eukaryotes, most bacteria contain genes encoding more than one class of RNR (Kolberg *et al.*, 2004) and mycobacteria are not an exception. Similar to other mycobacteria (http://rnrdb.molbiol.su.se), in addition to the class Ib RNR encoding genes *nrdE* (downstream and operonic to *nrdHI*) and *nrdF2*, both *M. tuberculosis* (Cole *et al.*, 1998) and *M. smegmatis* (http://www.tigr.org/tigr-scripts/CMR2/) possess a small subunit-encoding gene homologous to that of the Chlamydial class Ic RNR (Högbom *et al.*, 2004), designated as *nrdB* (Figure 3.1A). *M. smegmatis* and *M. tuberculosi*s NrdB have the essential residues of the Chlamydial protein, including the iron ligands, second coordination sphere ligands to histidines and the phenylalanine in place of the normal, radical-harboring tyrosine residue conserved (Figure 3.1B) (Högbom *et al.*, 2004). Like in *M. bovis* (Brosch *et al.*, 2007; Garnier *et al.*, 2003), *M. tuberculosis* also contains an alternate class Ib R2 subunit-encoding gene, *nrdF1* as well as the class II RNR-encoding gene, *nrdZ* (Cole *et al.*, 1998; Dawes *et al.*, 2003), whereas *M. smegmatis* mc^2155 has duplicate copies of the *nrdHIE* and *nrdF2* genes, which reside on an IS*1096*-flanked 56 kb duplication (Warner *et al.*, 2006) (Figure 3.1A).

The deletion of *nrdZ* had no effect on growth or survival of *M. tuberculosis* under conditions of hypoxia in which expression of the gene is induced (Voskuil *et al.*, 2003), or on virulence in mice (Dawes *et al.*, 2003). The presence of multiple class I RNR small subunit encoding genes in these mycobacteria suggests that they may be able to modulate RNR subunit composition under various environmental conditions. Of these, only *nrdF2* has been demonstrated to be essential for aerobic growth of *M. tuberculosis* H37Rv in vitro (Dawes *et al.*, 2003).

A



B

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                                                                                  ▼
M. tuberculosis                  ---------MTRTRSGSLAAGGLNWASL----------PLKLFAGGNAKF
M. smegmatis                     ---------MTRTHFDSIRAGGLNWSSL----------PLKLFAGGNAKF
Chlamydia trachomatis            MQADILDGKQKRVNLNSKRLVNCNQVDVNQLVPIKYKWAWEHYLNGCANN

M. tuberculosis                  WHPADIDFTRDRADWEK--LSDDERDYATRLCTQFIAGEEAVTEDIQPFM
M. smegmatis                     WDPADIDFSRDRADWEA--LTEREREYATRLCAEFIAGEEAVTKDIQPFM
Chlamydia trachomatis            WLPTEIPMGKDIELWKSDRLSEDERRVILLNLGFFSTAESLVGNNIVLAI
                                     ♦
M. tuberculosis                  SAMRAEGRLADEMYLTQFAFEEAKHTQVFRMWLDAVGISEDLHR------
M. smegmatis                     SAMRAEGRLGDEMYLTQFAFEEAKHTQVFRMWLDAVGVTDDLHS------
Chlamydia trachomatis            FKHVTN--PEARQYLLRQAFEEAVHTHTFLYICESLGLDEKEIFNAYNER

M. tuberculosis                  -YLDDLPAYRQIFYAELPECLNALSADPSPAAQVRASVTYNHIVEGMLAL
M. smegmatis                     -LIEEVPAYVQIFCEELPAALEALTSDPSPAAQVRASVVYNHVVEGMLAL
Chlamydia trachomatis            AAIKAKDDFQMEITGKVLDPNFRTDSVEGLQEFVKNLVGYYIIMEGIFFY

M. tuberculosis                  TGYYAWHKICVERAILPGMQELVRRIGDDERRHMAWGTFTCRRHVAADDA
M. smegmatis                     TGYYAWHRICVDRGILPGMQELVRRIGDDERRHMAWGTFTCRRHVAADDA
Chlamydia trachomatis            SGFVMILSFHRQ-NKMIGIGEQYQYILRDETIHLNFGIDLINGIKEENPG
                                                                                  ▼
M. tuberculosis                  NWT-VFETRMNELIPLALRLIEEGFALYGDQPPFDLSKDDFLQYSTDKGM
M. smegmatis                     NWA-VFETHMNELIPVALRLTQEGFALYGDDIPFGLEEGEFLQYSSDRGM
Chlamydia trachomatis            IWTPELQQEIVELIKRAVDLEIEYAQDCLPRGILGLRASMFIDYVQHIAD
                                   ♦
M. tuberculosis                  RRFGTISNARGRPVAEIDVDYSPAQLEDTFADEDRRTLAAASA-------
M. smegmatis                     RRFGTISSARGRPLAEIDVDYTPLQLEDTFADEDERALTAVKAAAAAAN-
Chlamydia trachomatis            RRLERIG-LKPIYHTKNPFPWMSETIDLNKEKNFFETRVIEYQHAASLTW
```

Figure 3.1 Chromosomal context of RNR-encoding genes in *M. tuberculosis* H37Rv, *M. smegmatis* mc2155, and ΔDRKIN (A, not drawn to scale) and multiple protein sequence alignment of the NrdB from *M. tuberculosis*, *M. smegmatis* and *Chlamydia trachomatis* (B, http://align.genome.jp/sit-bin/clustalw). A: The genes are denoted by arrows, with same color denoting homologous genes. The gene annotations are taken from Tuberculist (http://genolist.pasteur.fr/Tuberculist/) and are shown above the genes. B: The position of the phenylalanine which substitutes for the radical-harboring tyrosine residue found in other class I (Nordlund and Reichard, 2006) small subunits is highlighted in red, iron ligands are highlighted in yellow and highlighted in pink are the second coordination sphere ligands to histidines (Högbom *et al*., 2004). The triangles indicate the positions of the deletion in the *M. tuberculosis*

Δ*nrdB* mutant, whereas diamonds shows the positions of the deletion in the *M. smegmatis* Δ*nrdB*::*hyg* mutant, as described in Section 3.2 below.

An *M. smegmatis* mc^2155 derivative lacking the entire duplicated region (ΔDRKIN), hence containing only one copy of the class Ib RNR encoding genes (Figure 3.1A), had no in vitro growth defect (Warner *et al.*, 2006). However, the effect of the 56 kb duplication in mc^2155 and its loss on the growth and/or physiology of the ΔDRKIN mutant were not further investigated. Moreover, the function of *nrdB* and *nrdF1* in mycobacterial DNA metabolism under diverse stressful conditions has never been studied. Understanding the regulation mechanisms of these genes might also facilitate understanding their specialized roles, if any, in mycobacterial DNA metabolism. Moreover, with the exception of *M. tuberculosis nrdZ*, which belongs to a group of "dormancy" genes under the control of the DosR/S/T two-component regulator system (Roberts *et al.*, 2004; Voskuil *et al.*, 2003), little is known of the mechanisms that regulate the transcription of the other *nrd* genes in mycobacteria. To investigate these issues, a genetic approach to analyze the function of the *nrdF1* and *nrdB* genes and the regulation of all *nrd* genes in *M. tuberculosis* and *M. smegmatis* was adopted, as described below.

3.2 The role of class Ic RNR in mycobacteria

3.2.1 *nrdB* is dispensable for growth of *M. smegmatis* and *M. tuberculosis* in vitro

Unlike in Chlamydia (Roshick *et al.*, 2000), mycobacteria contain a gene encoding a classical class Ib R2, NrdF2, in addition to one encoding a Chlamydial-type class Ic R2, NrdB. The inability to delete the *nrdF2* gene in *M. tuberculosis* in the absence of a second (complementing) copy of this gene (Dawes *et al.*, 2003) suggested that *nrdB* cannot substitute for *nrdF2* function in this organism under the conditions tested. During pathogenesis, *M. tuberculosis* encounters host antibacterial agents such as RNIs and ROIs, which may have a DNA damaging or bactericidal effects by inhibiting essential enzymes like RNR (Flynn *et al.*, 1998; Fontecave, 1998). Given the biochemical characteristics of the Chlamydial class Ic RNR (Jiang *et al.*, 2008b) and the lifestyle of *M. tuberculosis* as an intracellular pathogen, it is tempting to speculate that

NrdB may serve a specialized role in providing dNTPs under the particular conditions encountered in vivo, in particular, nitrosative stress. To test this possibility, *nrdB* was targeted for deletion in *M. tuberculosis* H37Rv by using the p2ΔTBBKO suicide vector (Table 2.2) for two-step allelic exchange to replace the wild type *nrdB* allele with a deletion allele. Inactivation of this gene yielded an unmarked deletion mutant (Δ*nrdB*) in which 722 bp of internal coding sequence was removed, resulting in elimination of the phenylalanine residue at the normal radical-harboring tyrosine position and all of the iron ligands residues essential for Chlamydial enzyme activity (Roshick *et al.*, 2000; Voevodskaya *et al.*, 2006) (Figure 3.1B). To eliminate any complication that the class Ib *nrd* gene duplication may have on determining the phenotypic effects of *nrdB* gene loss in *M. smegmatis*, a *hyg*-marked deletion-replacement mutant of *M. smegmatis* (Δ*nrdB*::*hyg*) was constructed in the ΔDRKIN strain rather than in wild type mc^2155 using p2ΔSMBKO (Table 2.2) as a substrate for homologous recombination. This mutation deleted a 571 bp internal segment of the gene, also bearing the phenylalanine residue at the normal radical harboring tyrosine position and all the iron ligand residues, and replaced this segment with a Hyg resistance marker (Figure 3.1B).

Mutants were phenotypically selected by blue-white color selection followed by sucrose counter-selection (Parish and Stoker, 2000) and genotypically confirmed by Southern blot analysis. Genomic DNA from *M. tuberculosis* SCO (tb*nrdB*SCO) and DCO (Δ*nrdB*) recombinants and the wild type strain produced 5.3 kb and 3.9 kb, 3.9 kb and 4.6 kb cross-hybridizing bands, respectively, on a Southern blot (Figure 3.2A and Figure 3.2B), while the *M. smegmatis* SCO (s*nrdB*SCO), DCO (Δ*nrdB*::*hyg*) and parental strain (ΔDRKIN) produced 5.7 kb and 2.4 kb, 2.4 kb and 3.3 kb fragments, respectively (Figure 3.2C and Figure 3.2D). Successful deletion of *nrdB* in both organisms confirms the dispensability of this gene for normal growth in both *M. tuberculosis* and *M. smegmatis*. The growth rates in liquid culture of the *M. tuberculosis* Δ*nrdB* (Figures 3.2E) and *M. smegmatis* Δ*nrdB*::*hyg* (Figures 3.2F) mutants were indistinguishable from wild type.

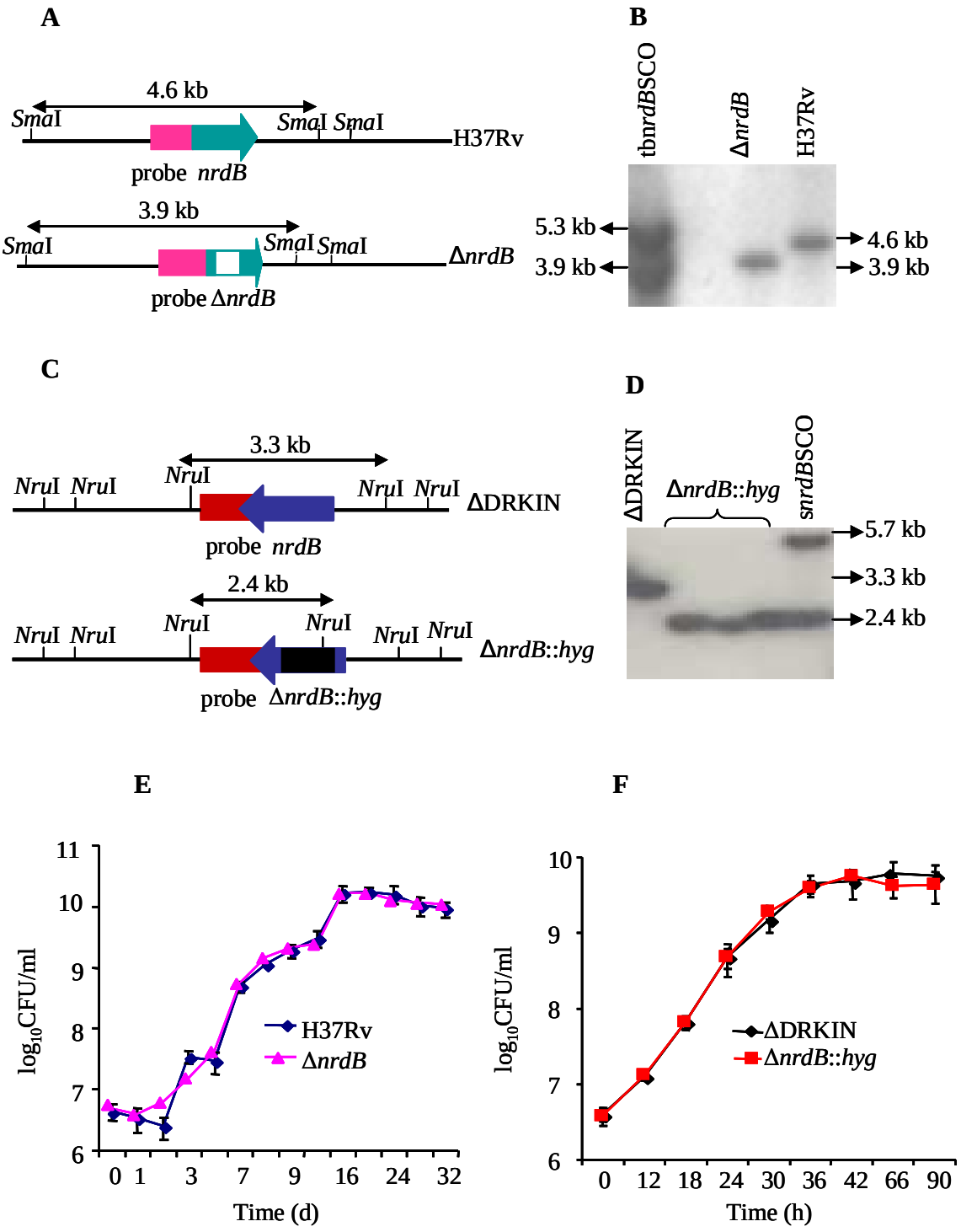


Figure 3.2 Construction and growth kinetics of *M. tuberculosis* and *M. smegmatis nrdB* mutants· A and C: Schematic representation of parental and the mutant alleles showing the restriction enzyme sites and probes used for Southern blot analysis. B: Southern blot analysis of genomic DNA samples isolated from *M. tuberculosis* parental (H37Rv), SCO (tbnrdBSCO) and

DCO (Δ*nrdB*) strains digested with *Sma*I and hybridized with tbnrdB-F1pR1p probe (Table 2.4). D: Southern blot analysis of genomic DNA samples isolated from *M. smegmatis* parental (ΔDRKIN), SCO (s*nrdB*SCO) and DCO (Δ*nrdB*::*hyg*) strains digested with *Nru*I and hybridized with smnrdB-F2R2 probe (Table 2.4). E: Growth curve of Δ*nrdB* in comparison with H37Rv under standard in vitro growth conditions. F: Growth curve of Δ*nrdB*::*hyg* in comparison with ΔDRKIN under standard in vitro growth conditions. For growth studies, low inoculum (OD600 ~0.02) cultures were prepared by diluting stationary phase (OD600 ~3) pre-cultures in fresh media and growth followed by determining viable cell counts (CFUs) over a period of 32 d (E) or 90 h (F). Data represent average CFUs from three biological replicates and error bars indicate standard deviations between the three cultures.

3.2.2 *nrdB* is dispensable for competitive growth and long-term survival of *M. smegmatis*

To determine the effect of *nrdB* loss on long-term survival of *M. smegmatis*, individual cultures of Δ*nrdB*::*hyg* and its parent, ΔDRKIN were set-up in triplicate and CFUs enumerated for a period of over 5 months. Both the mutant and the parental strain survived equally through 175 d (Figure 3.3A). Hence, no defect in long-term survival of Δ*nrdB*::*hyg* in pure culture was observed. The contribution of *nrdB* to the fitness of *M. smegmatis* for competitive growth and long-term survival was then investigated by co-culturing, in the absence of antibiotic selection, Δ*nrdB*::*hyg* and its parental strain that had been marked by integration of a Km resistance-encoding plasmid vector (pAINT, Table 2.2, Table 2.1, ΔDRKIN::pAINT) at the *attB* chromosomal locus. Aliquots were then plated on solid media supplemented with Hyg or Km for mutant and parental strain CFU enumeration, respectively. Both strains grew (Figure 3.3B) and survived (Fig 3.3C) equally well in competition (Figure 3.3B and 3.3C). Therefore, *nrdB* loss did not result in any fitness cost for competitive growth and long-term survival of *M. smegmatis*.

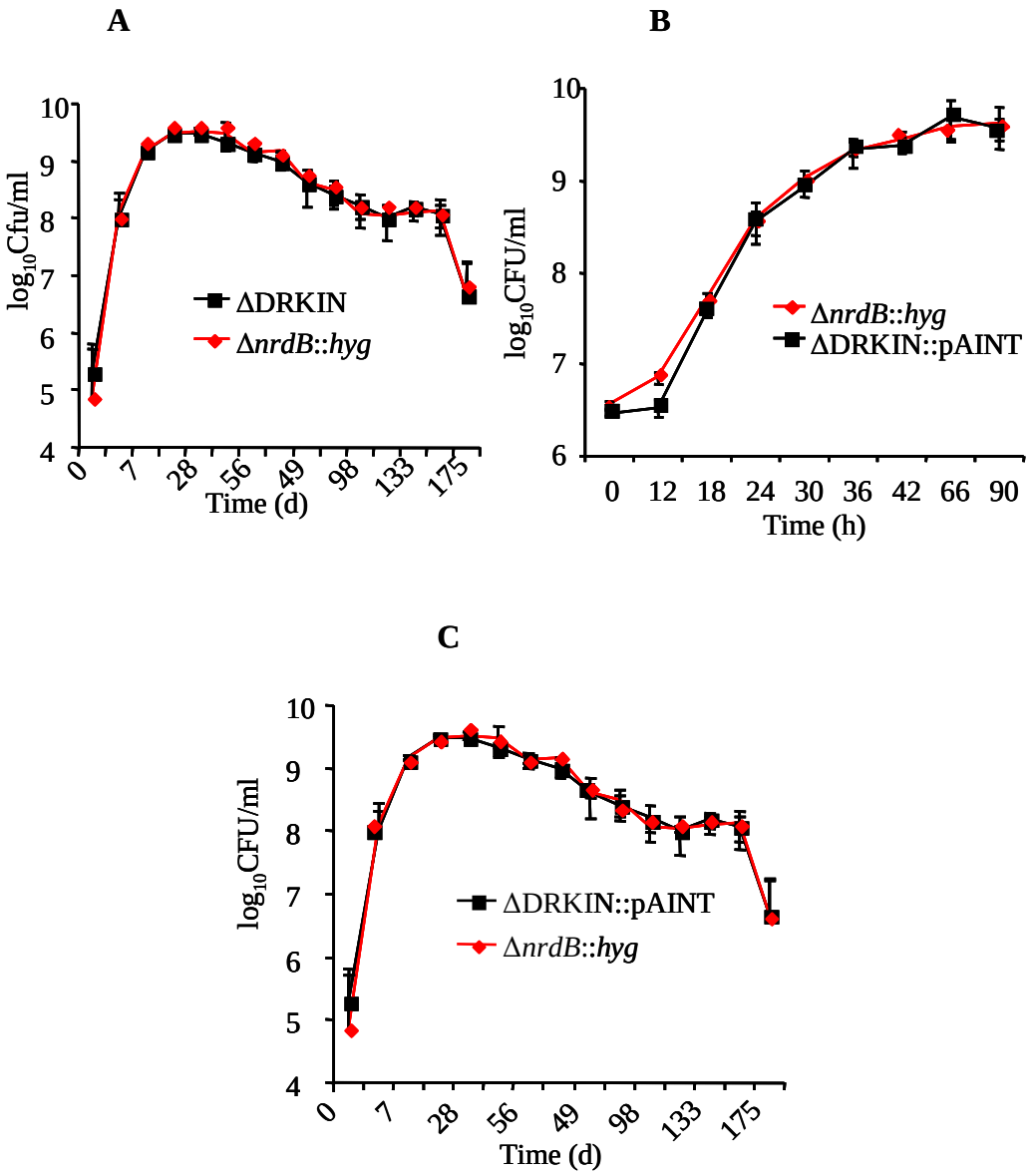


Figure 3.3 Competitive growth and long-term survival of *M. smegmatis* Δ*nrdB*::*hyg*. A: Δ*nrdB*::*hyg* and ΔDRKIN were cultured individually and CFUs assessed at different time points over a period of 175 d. B and C: Equal starting inoculum of Δ*nrdB*::*hyg* and ΔDRKIN::pAINT were co-cultured in 7H9-GS media and CFUs determined for over 90 h (B) or 175 (C) d. Each data point data represent an average of CFUs from three biological culture replicates. Error bars indicate standard deviations between the three cultures.

3.2.3 The role of *nrdB* in nitrosative stress survival

A unique catalytic mechanism of NrdB (Voevodskaya *et al.*, 2005) has been proposed to be responsible for the high tolerance to nitrosative stress in bacterial pathogens carrying the class Ic RNR (Högbom *et al.*, 2004). To test this hypothesis in mycobacteria, the effects of NO on growth or survival of the *M. tuberculosis* Δ*nrdB* and *M. smegmatis* Δ*nrdB*::*hyg* mutants and their parental strains were assessed. To monitor mycobacterial growth in the presence of NO, log-phase cultures of *M. smegmatis* Δ*nrdB*::*hyg* and ΔDRKIN were diluted in fresh 7H9-GS media supplemented with different concentrations of an NO donor, GSNO, and growth assessed after 24 h of incubation. Survival of Δ*nrdB* and Δ*nrdB*::*hyg* in the presence of acidified $NaNO_2$ was assessed by incubating cultures in media containing increasing concentrations of acidified $NaNO_2$ (Firmani and Riley, 2002). Growth inhibition for both ΔDRKIN and Δ*nrdB*::*hyg* could be observed for GSNO concentrations of 2 μg/ml and higher, with 2.7-3.2 $\log_{10}$ growth inhibition observed at a concentration of 12-16 μg/ml (Figure 3.4A). However, no deferential growth in the presence of GSNO was observed between the two strains (Figure 3.4A).

Both ΔDRKIN and Δ*nrdB*::*hyg* showed a similar trend in susceptibility to acidified nitrite with 52 % survival at 24 mM $NaNO_2$ and 0.01 % survival at 48 mM $NaNO_2$ confirming that Δ*nrdB*::*hyg* was not impaired for survival during exposure to acidified nitrite (Figure 3.4B). The survival of H37Rv in the presence of acidified nitrite was similar to that reported previously, with 35 % survival observed at a concentration of 6 mM (Firmani and Riley, 2002). Arguing further against a role for NrdB in nitrosative stress survival in *M. tuberculosis* is the similarity in the survival of H37Rv and the Δ*nrdB* mutant over the range of acidified nitrite concentrations tested (Figure 3.4C).

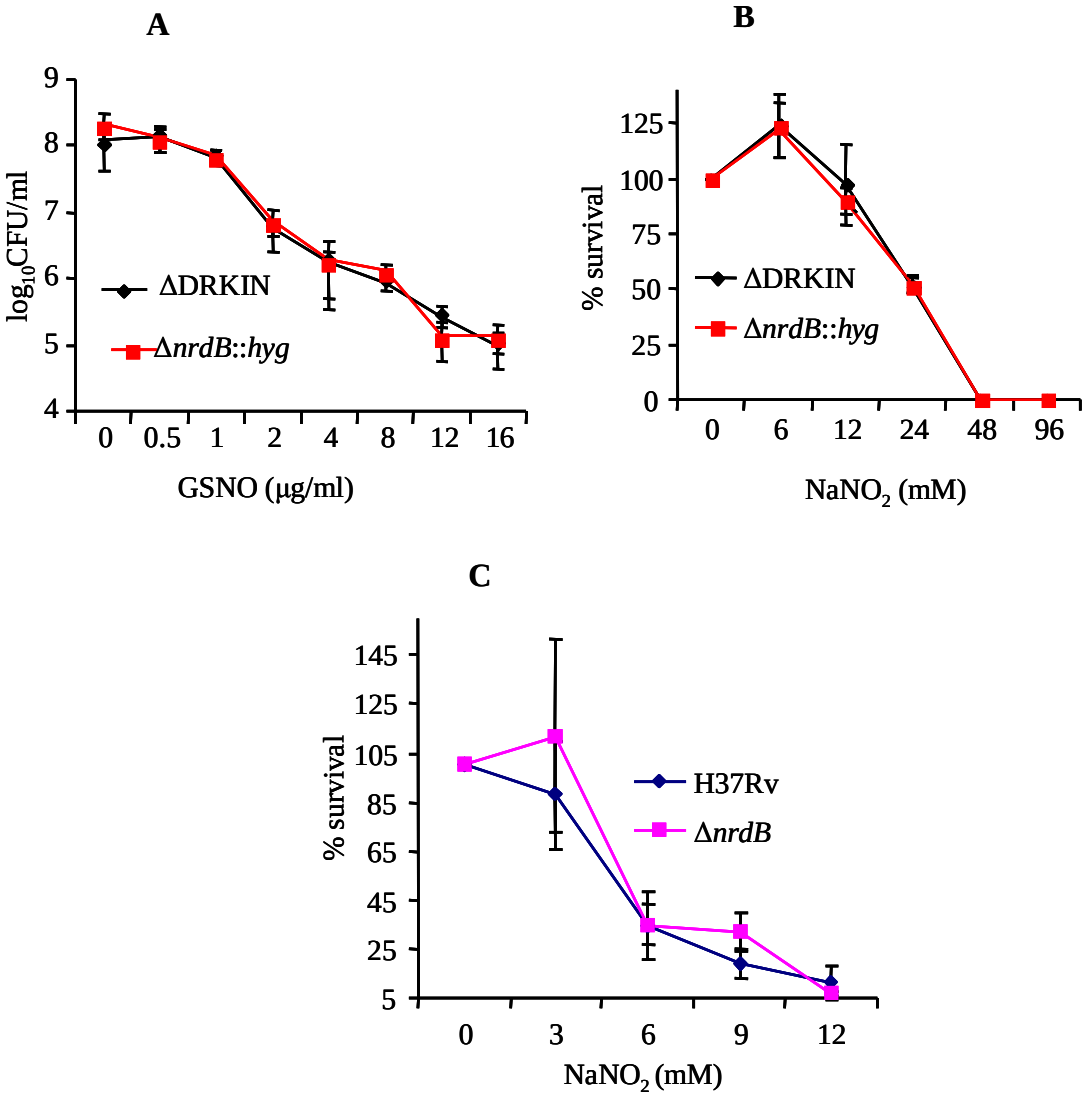


Figure 3.4 Susceptibility of *M. smegmatis* Δ*nrdB*::*hyg* (A and B) and *M. tuberculosis* Δ*nrdB* (C) to nitrosative stress. Growth of Δ*nrdB*::*hyg* in GSNO was assessed by growing cultures in fresh 7H9-GS media supplemented with different concentrations of GSNO for 24 h before plating for CFU enumeration (A). Survival of Δ*nrdB*::*hyg* (B) and Δ*nrdB* (C) in acidified nitrite was assessed by incubating cultures for 24 h in 7H9-GS/7H9-OADC media at pH 5.3 supplemented with different concentrations of NaNO2, followed by plating and CFU enumeration. Results represent an average from three biological culture replicates, with standard deviations indicated by error bars.

3.2.4 The role of *nrdB* in RNR inhibition survival

HU inhibits class I R2 activity by scavenging the tyrosine radical in the R2 subunit (Akerblom *et al.,* 1981) and inhibition of the class Ib RNR (NrdEF) by HU has been well documented (Torrents *et al.,* 2005b; Yang *et al.,* 1997). Even though the inhibition of the Chlamydial class Ic enzyme by HU has also been reported (Roshick *et al.,* 2000), the lack of a tyrosine catalytic radical in NrdB (Högbom *et al.,* 2004) makes the question of whether the class Ic RNR is less sensitive to HU as compared to class Ib enzyme an intriguing one. Hence, the role of NrdB in mycobacteria could potentially be to serve the demand for dNTPs under the conditions were NrdF2 activity is completely eliminated by tyrosine radical scavengers. To test this, the sensitivity of both the Δ*nrdB* and Δ*nrdB*::*hyg* mutants to HU was assessed by plating stationary-phase (not shown) and/or log-phase (Figure 3.5) cultures on solid media supplemented with increasing concentrations of HU, followed by CFU enumeration.

When included in solid media at a concentration of 9 mM, HU resulted in a 5.6 $\log_{10}$ CFU reduction of *M. tuberculosis* H37Rv (Figure 3.5A), whereas HU at 10 mM resulted in 3.5 $\log_{10}$ CFU reduction of ΔDRKIN (Figure 3.5B). In both mycobacterial species, deletion of *nrdB* had no effect on susceptibility to HU. Although the ΔDRKIN strain was subsequently shown to be hypersensitive to HU relative to mc^2155 (see Figure 3.14A below), deletion of *nrdB* in the ΔDRKIN background had no effect on HU sensitivity (Figure 3.5B). *nrdB* was also found to be entirely dispensable for HU susceptibility in *M. tuberculosis* (Figure 3.5A). When MIC values for HU were determined by broth microdilution described by Lee *et al.* (Lee *et al.,* 2003), values of 760 μg/ml were observed for both Δ*nrdB* and H37Rv, compared to 190-380 μg/ml for Δ*nrdB*::*hyg* and ΔDRKIN. These findings were further supported by the lack of differential HU sensitivity between Δ*nrdB*::*hyg* and ΔDRKIN strains when growth and survival in the presence of HU was assessed by CFU determination after 48 h incubation in liquid media supplemented with different concentrations of HU (Section 2.12, not shown).

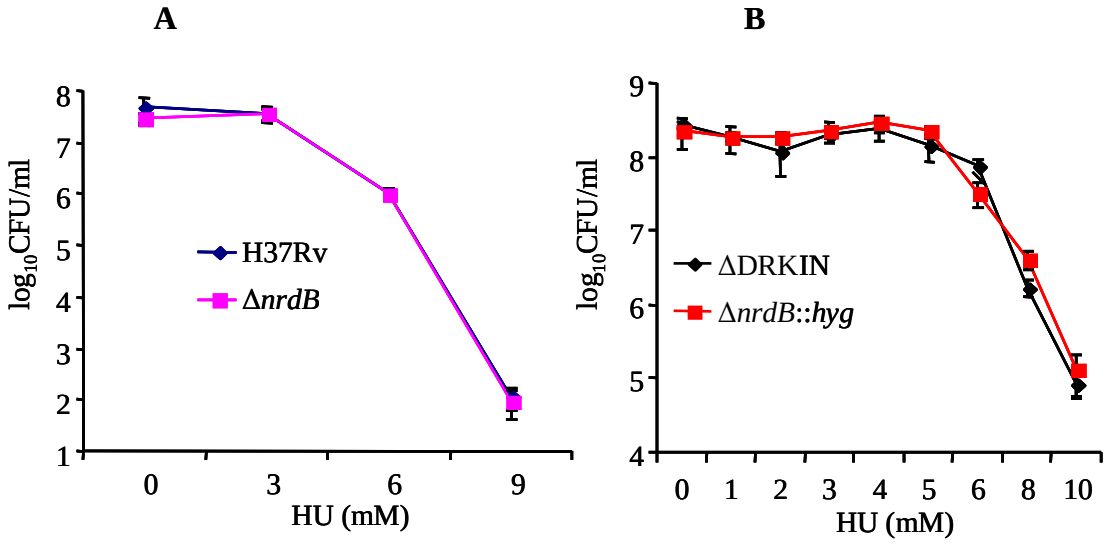


Figure 3.5 Susceptibility of *M. tuberculosis* Δ*nrdB* (A) and *M. smegmatis* Δ*nrdB*::*hyg* (B) to HU. Log-phase cultures of the mutant strains and their parental wild type strains were plated on solid media supplemented with different concentrations of HU and incubated until CFUs could be enumerated. The data represent an average from three biological replicates, with standard deviations between the cultures indicated by the error bars.

3.2.5 Effect of *nrdB* loss on mycobacterial resistance to DNA damage

The requirement of dNTPs for DNA synthesis during DNA repair makes RNR an essential enzyme to meet this demand. To investigate whether mycobacteria use a class Ic enzyme for the supply of dNTPs for DNA repair synthesis, the sensitivity of the *M. tuberculosis* Δ*nrdB* and *M. smegmatis* Δ*nrdB*::*hyg* mutants to MTC as a generalized DNA damaging agent and their survival after UV irradiation were assessed. Susceptibility testing to MTC was done by plating stationary-phase (not shown) and/or log-phase (Figure 3.6A and Figure 3.6B) cultures on solid media supplemented with different concentrations of MTC followed by CFU enumeration (Section 2.12). Survival following DNA damage induced by UV irradiation was assessed by irradiating plated serial dilutions of log-phase cultures at different UV fluencies and determination of the percentage survival of irradiated cells vs. untreated controls by CFU assessment.

MTC treatment reduced the viability of both H37Rv and *M. smegmatis* strains by approximately 3.5 (Figure 3.6A) and 4.5 (Figure 3.6B) $\log_{10}$, respectively, at a concentration of 0.1 μg/ml. Similarly, when MIC values were determined using the

broth microdilution protocol, values of 0.06 and 0.004-0.015 μg/ml were obtained for wild type *M. tuberculosis* (H37Rv) and *M. smegmatis* ΔDRKIN. Although the parental strain of the *M. smegmatis* Δ*nrdB*::*hyg* mutant, ΔDRKIN, was subsequently shown to be hypersensitive to MTC relative to its parent, mc^2155 (see Figure 3.14B below), deletion of *nrdB* in the ΔDRKIN background did not exaggerate the phenotype (Figure 3.6B).

Lack of differential susceptibility to MTC between mutants and parental strains observed by the plate sensitivity assay and by MIC determination was further corroborated by the observation that growth and survival of the Δ*nrdB*::*hyg* mutant in liquid media supplemented with different concentrations of MTC was comparable to that of the wild type, as measured by CFU assessment (not shown). The effect of loss of *nrdB* function on survival of *M. smegmatis* or *M. tuberculosis* strains following exposure to UV irradiation was then assessed (Figure 3.6C and 3.6D). Exposure of the parental and Δ*nrdB*::*hyg* mutant of *M. smegmatis* to UV irradiation at different fluencies resulted in a survival pattern similar to that of *M. smegmatis* mc^2155 reported previously (Boshoff *et al.*, 2003). However, unlike *dnaE2*::*aph*, which was significantly impaired for UV survival (Figure 3.6D), in agreement with previous findings (Boshoff *et al.*, 2003), no significant difference in UV survival was observed for *M. tuberculosis* Δ*nrdB* (Figure 3.6C) or *M. smegmatis* Δ*nrdB*::*hyg* (Figure 3.6D) when compared to their respective parental strains.

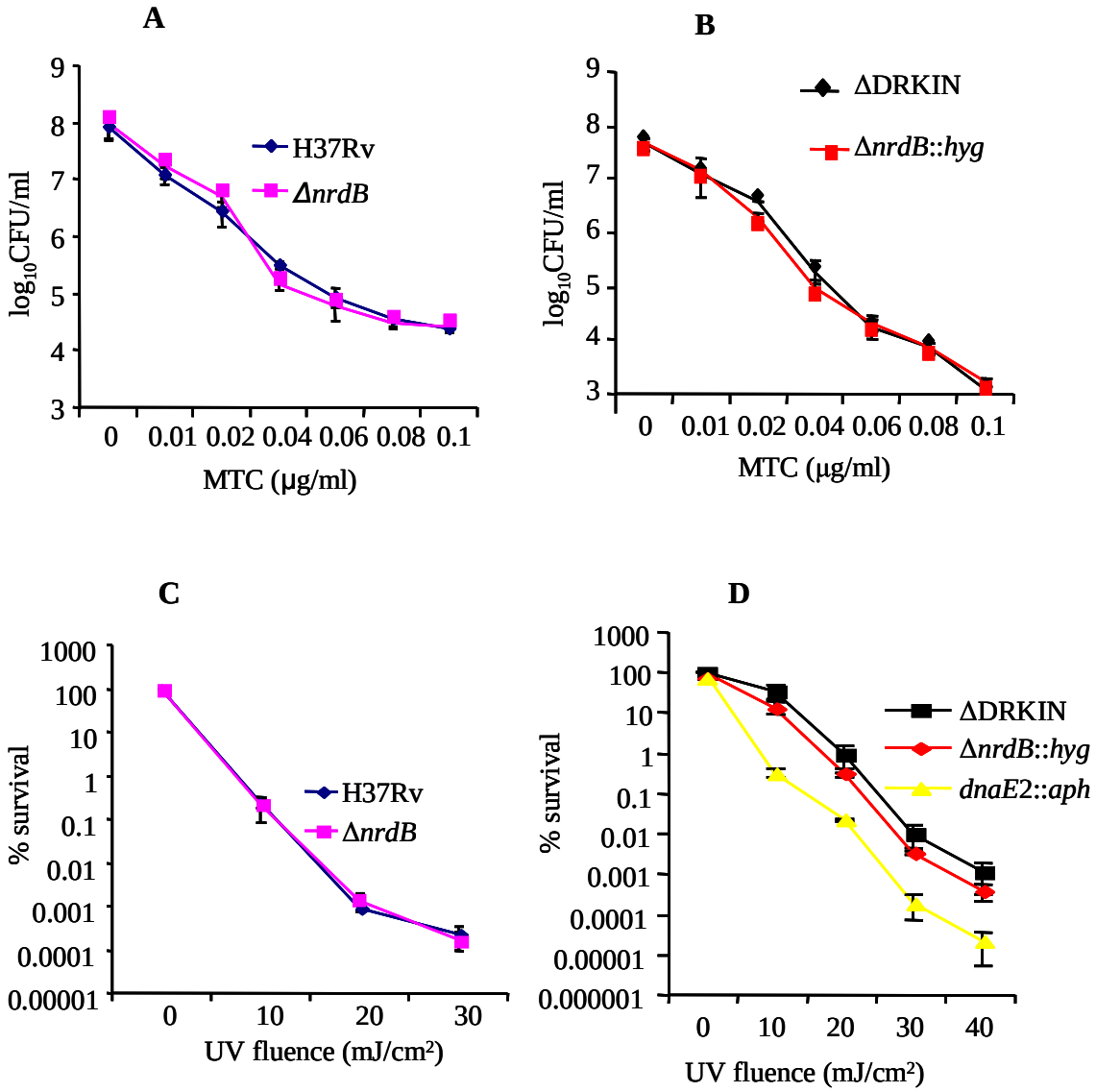


Figure 3.6 Sensitivity of *M. tuberculosis* Δ*nrdB* and *M. smegmatis* Δ*nrdB*::*hyg* to MTC (A and B) and UV irradiation (C and D). A and B: *M. tuberculosis* (A) and *M. smegmatis* (B) log-phase cultures were plated on solid media supplemented with different concentrations of MTC. C and D: Open plates on which serial dilutions of *M. tuberculosis* (C) and *M. smegmatis* (D) cultures were plated were UV irradiated in a Stratalinker (Stratagene) at increasing UV fluences up to 40 mJ/cm^2. The *dnaE2*::*aph* mutant was included in the *M. smegmatis* assay as a UV-hypersensitive control (Boshoff *et al.*, 2003). All plates were incubated until CFUs could be enumerated. The data shown are from three biological culture replicates plated in duplicates. Error bars indicate standard deviations between the three cultures.

3.2.6 The role of *nrdB* in *M. tuberculosis* growth, dissemination and survival in mice

Understanding the mechanisms of *M. tuberculosis* growth and survival in the host is of utmost importance. Because NO is a key mediator of bacterial killing in the mouse model of infection (MacMicking *et al.*, 1997), a murine model of pulmonary tuberculosis was used to investigate whether *nrdB* contributes to the growth and survival of *M. tuberculosis* during the various stages of infection in vivo. After a group of immunocompetent B6D2/F1 mice were infected by the nose-only aerosol inhalation route with the wild type and Δ*nrdB* strains, bacillary loads were followed in the lungs, livers and spleens over a period of 126 d. From an initial lung bacillary count of *ca*. 2.3 $\log_{10}$, the bacterial loads for both strains increased progressively to reach a steady-state level of *ca*. 6.0 $\log_{10}$ (Figure 3.7A). Dissemination to the spleen could be detected from 14 d post infection, from which bacillary load increased from between 2-2.7 $\log_{10}$ to a maximum of *ca*. 4 $\log_{10}$ for both the wild-type and the mutant (Figure 3.7B). Both strains also showed dissemination to the liver from 14 d post-infection (Figure 3.7C). Therefore, no attenuation for colonization of the mouse lung, defects in chronic infection in the lungs (Figure 3.7A) or in dissemination to the spleen (Figure 3.7B) or liver (Figure 3.7C) was observed for the Δ*nrdB* mutant. In addition, no differences in gross pathology were observed between the lungs infected with the mutant and wild-type strain (not shown).

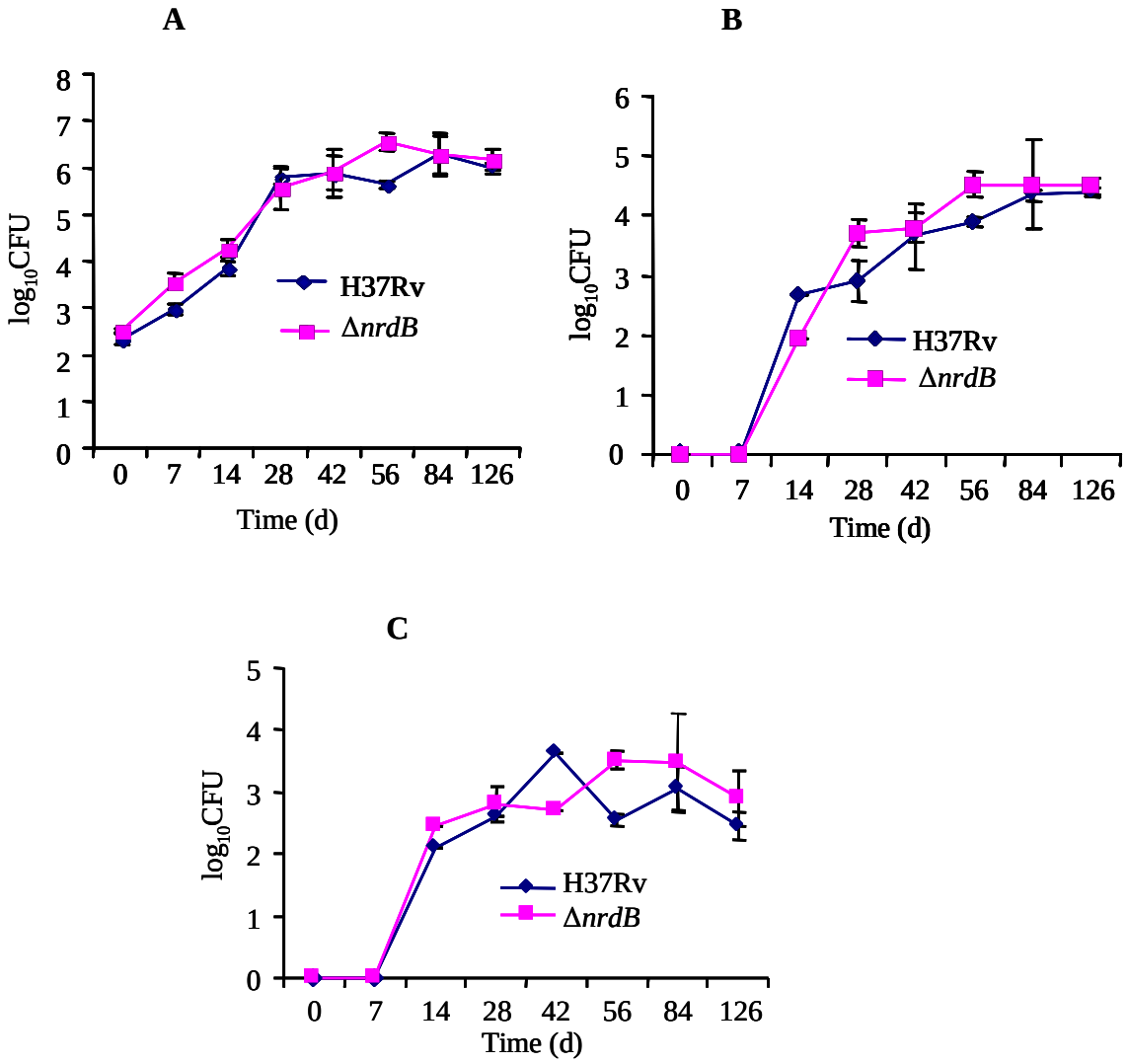


Figure 3.7 Growth and dissemination of *M. tuberculosis* Δ*nrdB* in mice. Mice were infected with wild type *M. tuberculosis* H37Rv and the Δ*nrdB* by aerosol inhalation. Bacillary loads in the lungs (A), spleens (B) and livers (C) were determined over 126 d of infection period. Each time point represents average CFUs from three mice and error bars indicate standard deviations of bacillary count from three mice.

3.3 The function of the alternate small subunit of class Ib RNR in *M. tuberculosis*

3.3.1 *nrdF1* is dispensable for growth of *M. tuberculosis* in vitro

In addition to the class Ib RNR small subunit, NrdF2 (Dawes *et al.*, 2003), *M. tuberculosis* also contains an alternate small subunit, NrdF1 (Yang *et al.*, 1997). It has been shown that the NrdF2 subunit is essential for growth in vitro, leading to the speculation that *nrdF1* cannot substitute for *nrdF2* function in *M. tuberculosis* (Dawes *et al.*, 2003). However the requirement of *nrdF1* under the various environmental conditions encountered by the bacilli during its pathogenesis has never been investigated. Even though no apparent biochemical activity of NrdF1 was observed (Yang *et al.*, 1997), the finding that *nrdF1* is expressed in vivo (Yang *et al.*, 1997), is highly expressed during exponential growth (Dawes *et al.*, 2003), and is induced by DNA damage and translational inhibition (Boshoff *et al.*, 2004) suggested that NrdF1 might play a role in RNR function under specific conditions. To elucidate the function of *nrdF1* in *M. tuberculosis*, an *nrdF1* null mutant with 883 bp sequence carrying the radical-bearing tyrosine and electron path residues deleted, was constructed by homologous recombination using p2ΔTBF1KO knockout construct (Table 2.2). A genomic DNA digest from DCO (Δ*nrdF1*) produced 3.4 kb and 1.5 kb fragments compared to H37Rv which produced 4.3 kb and 1.5 kb and the SCO (*nrdF1*SCO) with 8 kb, 3.4 kb and 1.5 kb fragments on the Southern blot (Figure 3.8A and Figure 3.8B). Successful deletion of *nrdF1* supports the prediction that it is not essential for growth in vitro (Dawes *et al.*, 2003). Dispensability of *nrdF1* for in vitro growth of *M. tuberculosis* was further validated by the indistinguishable growth kinetics of Δ*nrdF1* and H37Rv when the growth was followed by OD (not shown) and CFU enumeration over a period of 32 d (Figure 3.8C).

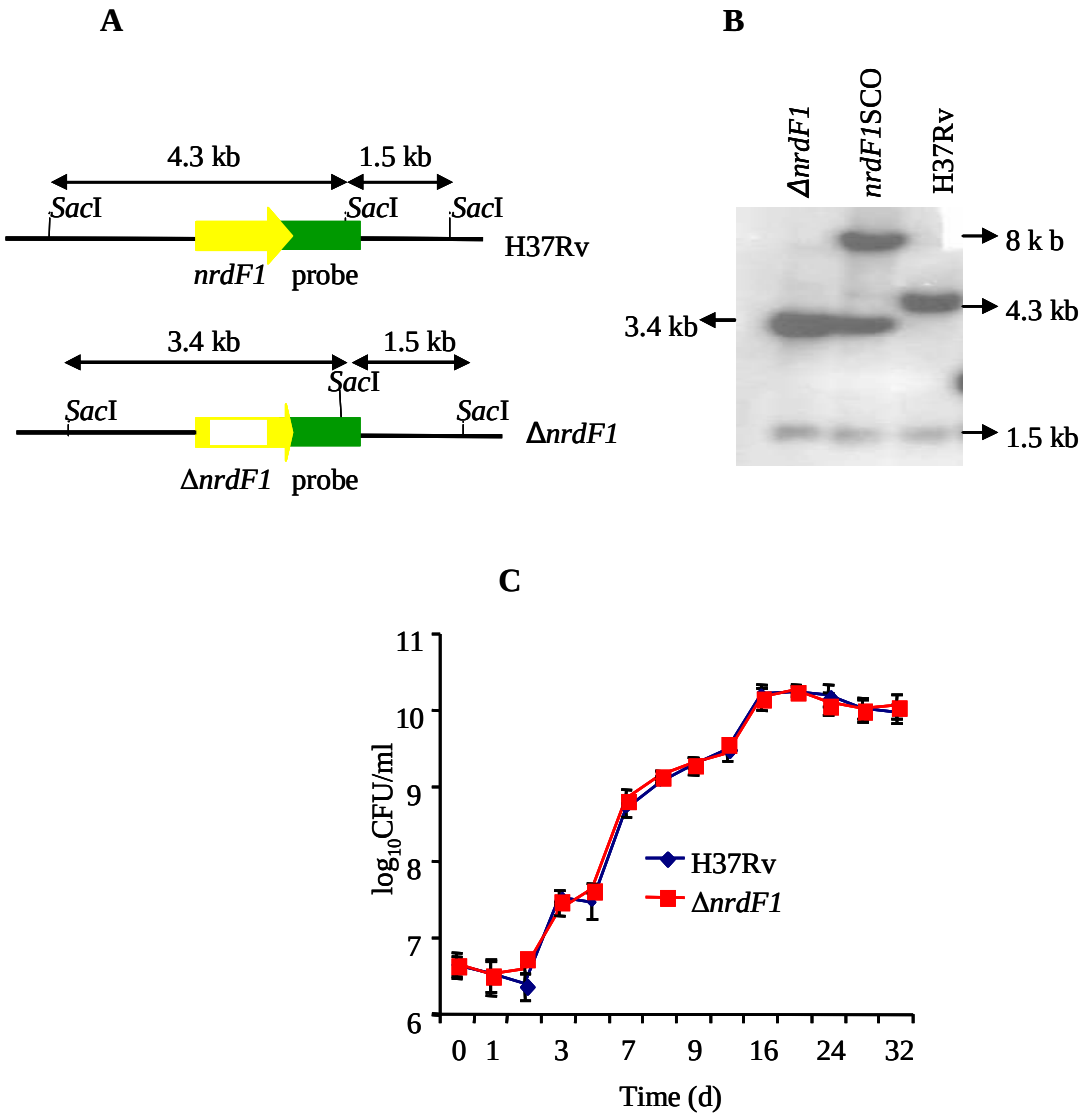


Figure 3.8 Construction and growth kinetics of *M. tuberculosis* Δ*nrdF1*. A: Schematic representation of parental and the mutated alleles showing the restriction enzyme sites and probe used for Southern blotting. B: Southern blot analysis of genomic DNA samples isolated from H37Rv, SCO (*nrdF1*SCO) and DCO (Δ*nrdF1*) strains digested with *Sac*I and hybridized with the tbnrdF1-F1pR1p probe (Table 2.4). C: Growth of Δ*nrdF1* in vitro. The same wild-type data as on Figure 3.2E was used. Each time-point represents an average from three biological replicate cultures. Error bars indicate the standard deviations between three biological replicates.

3.3.2 Requirement of *nrdF1* in DNA damage, RNR inhibition and translation inhibition survival

Upregulation of *nrdF1* in response to DNA damage (Boshoff *et al.*, 2004) led to the speculation that NrdF1 may be used for dNTP provision during DNA repair in *M. tuberculosis*. To investigate this, Δ*nrdF1* was tested for susceptibility to MTC and UV irradiation as compared to the wild type strain using previously described methods. In contrast, no significant differential susceptibility was observed between the mutant and the wild-type strain to either MTC (Figure 3.9A) or UV irradiation (Figure 3.9B) under all the concentrations or fluences tested. *nrdF1* has also been demonstrated to be upregulated by treatment with fluoroquinolones (Boshoff *et al.*, 2004). Therefore, sensitivity of Δ*nrdF1* to Moxi, Cipro and Novo was determined by spotting assay, where serial dilutions of cultures were spotted on plates containing increasing concentrations of either compounds, followed by incubation until the CFUs could be enumerated (Section 2.12). The experiment was performed at least twice for each compound. Similarly, no differential sensitivity to either Moxi, Cipro or Novo was observed between Δ*nrdF1* and H37Rv (not shown). Confirming the lack of Δ*nrdF1* phenotype upon exposure to MTC and fluoroquinolones were the equivalent MIC values for MTC, oflox and Novo for the mutant and H37Rv strains, which were found to be 0.06, 1.25 and 3.12 μg/ml, respectively.

Upregulation of *nrdF1* in the presence of a translation inhibitor, STR (Boshoff *et al.*, 2004) was suggestive of an important function of NrdF1 in mycobacterial translation inhibition survival. Therefore, to determine if this is the case, Δ*nrdF1* sensitivity to STR was determined by plating and the broth microdilution MIC assay (Section 2.12) compared to the wild type strain. However, after plating on different concentrations of STR and enumerating CFUs, no differential growth inhibition was observed between the mutant and the wild-type strain (Figure 3.9C). Similarly both the mutant and the wild-type had equivalent MIC values for STR (1.25 μg/ml).

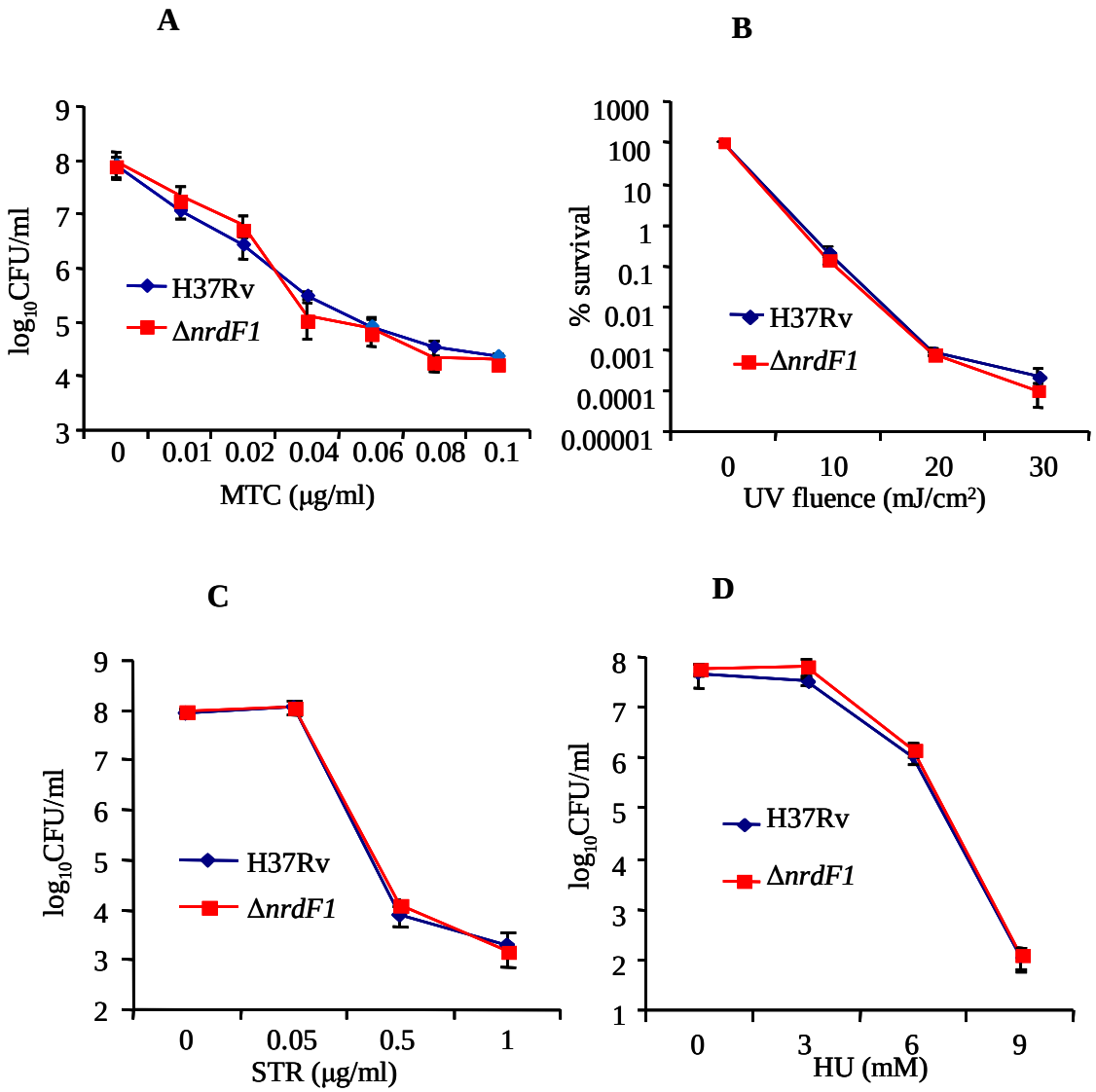


Figure 3.9 Susceptibility of *M. tuberculosis* Δ*nrdF1* to MTC, UV irradiation, STR and HU. Serial dilutions of log-phase cultures were plated on solid media supplemented with different concentrations of MTC (A), STR (C) or HU (D). B: Sensitivity to UV damage. Open plates on which serial dilutions were plated were UV irradiated and incubated until CFUs could be enumerated. The wild-type data on A, B and D is the same data as on Figure 3.6A, Figure 3.6C and Figure 3.5A respectively. Data represent CFU averages from three biological replicate cultures plated in duplicate. Error bars indicate standard deviations.

To investigate whether *nrdF1* plays any role in dNTP supply, the ability of Δ*nrdF1* to grow under RNR inhibiting conditions was assessed by plating on HU-containing plates at different concentrations and by MIC determination. However, both the mutant and the wild-type strain showed no differential susceptibility to HU on the plating assay (Figure 3.9D) and MIC determination, with both having a HU MIC of 760 μg/ml.

3.3.3 Effect of *nrdF1* loss on *M. tuberculosis* growth, dissemination and survival in vivo

The possibility of *nrdF1* being used for adequate dNTP supply during the pathogenesis of *M. tuberculosis* in vivo remained to be unexplored. In this work, a mouse model of pulmonary tuberculosis using immunocompetent B6D2/F1 mice was used to address this question. Mice were infected through the respiratory route with the H37Rv and Δ*nrdF1* strains. Ability to initiate an infection, growth, survival and dissemination of the bacilli in the lungs, spleen and livers were monitored over a period of 126 d by CFU assessment. The bacterial load increased from an initial lung bacillary count of approximately 2.3 $\log_{10}$ to *ca* 6 $\log_{10}$ CFUs/lung (Figure 3.10A). CFUs in to the spleen could be detected from 14 d post infection, and increased from 2.7 to the maximum of *ca* 4 $\log_{10}$ CFUs/spleen (Figure 3.10B). Dissemination in to the liver was detected from 14 and 28 d post infection for H37Rv and Δ*nrdF1* respectively (Figure 3.10C). However, deletion of *nrdF1* did not attenuate *M. tuberculosis* for initiation of infection, growth and survival in the lungs (Figure 3.10A) or in dissemination to the spleen (Figure 3.10B) or liver (Figure 3.10C). No differences in gross pathology were observed between the lungs infected with the mutant and wild-type strain (not shown).

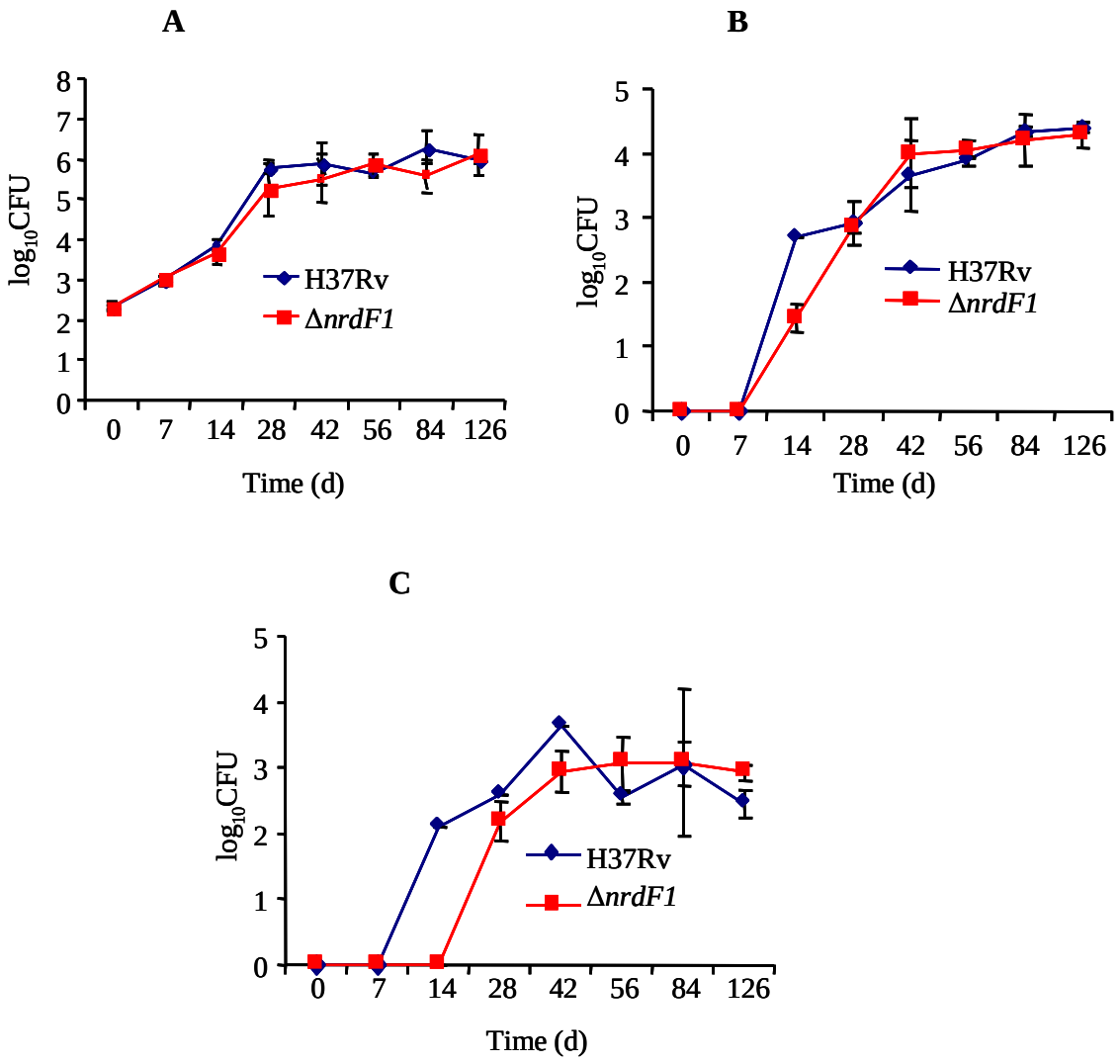


Figure 3.10 Growth and dissemination of the Δ*nrdF1* mutant of *M. tuberculosis* in mice. Mice were infected through the respiratory route with H37Rv or Δ*nrdF1* and organ bacillary loads were determined over a period of 126 d. Each time point represents the average bacillary counts in the lungs (A), Spleen (B) and livers (C) from three mice. The same wildtype data as in Figure 3.7 was used. Error bars indicate standard deviations between three mice.

3.4 Collective roles of *nrdB* and *nrdF1* in *M. tuberculosis*

3.4.1 Construction of a mutant strain of *M. tuberculosis* lacking both *nrdF1* and *nrd*B

The lack of phenotype of the Δ*nrdB* and Δ*nrdF1* mutants under all the conditions tested led to the speculation that both genes could be fully substituting one another's function in *M. tuberculosis*, and hence, obscuring the possible effects of individual disruption. In *S. cerevisiae*, the RNR small subunit is a heterodimer of two polypeptides, RNR4 and RNR2, which complex with one another to form a functional enzyme with the large subunit (Huang and Elledge, 1997; Perlstein *et al.*, 2005). To investigate whether NrdF1 and NrdB may be functionally redundant or functionally related, a mutant of *M. tuberculosis* with deletions in both *nrdB* and *nrdF1* was constructed by electroporating p2ΔTBBKO into the Δ*nrdF1* mutant and isolating a Δ*nrdF1*Δ*nrdB* double mutant by two-step selection, as previously described (Parish and Stoker, 2000). The *nrdB* allele was genotyped by Southern blot analysis (Figure 3.11A and Figure 3.11B), and deletion of *nrdB* confirmed by the presence of a 3.9 kb cross-hybridising band (as observed in the Δ*nrdB* single mutant, Figure 3.2D), as opposed to a 4.6 kb band, characteristic of the wild type *nrdB* allele. The disruption of *nrdF1* in the Δ*nrdF1*Δ*nrdB* double mutant was also re-confirmed by Southern blot analysis (not shown). As observed in the single mutants, disruption of both *nrdF1* and *nrdB* did not have any effect on *M. tuberculosis* growth in vitro (Figure 3.11C).

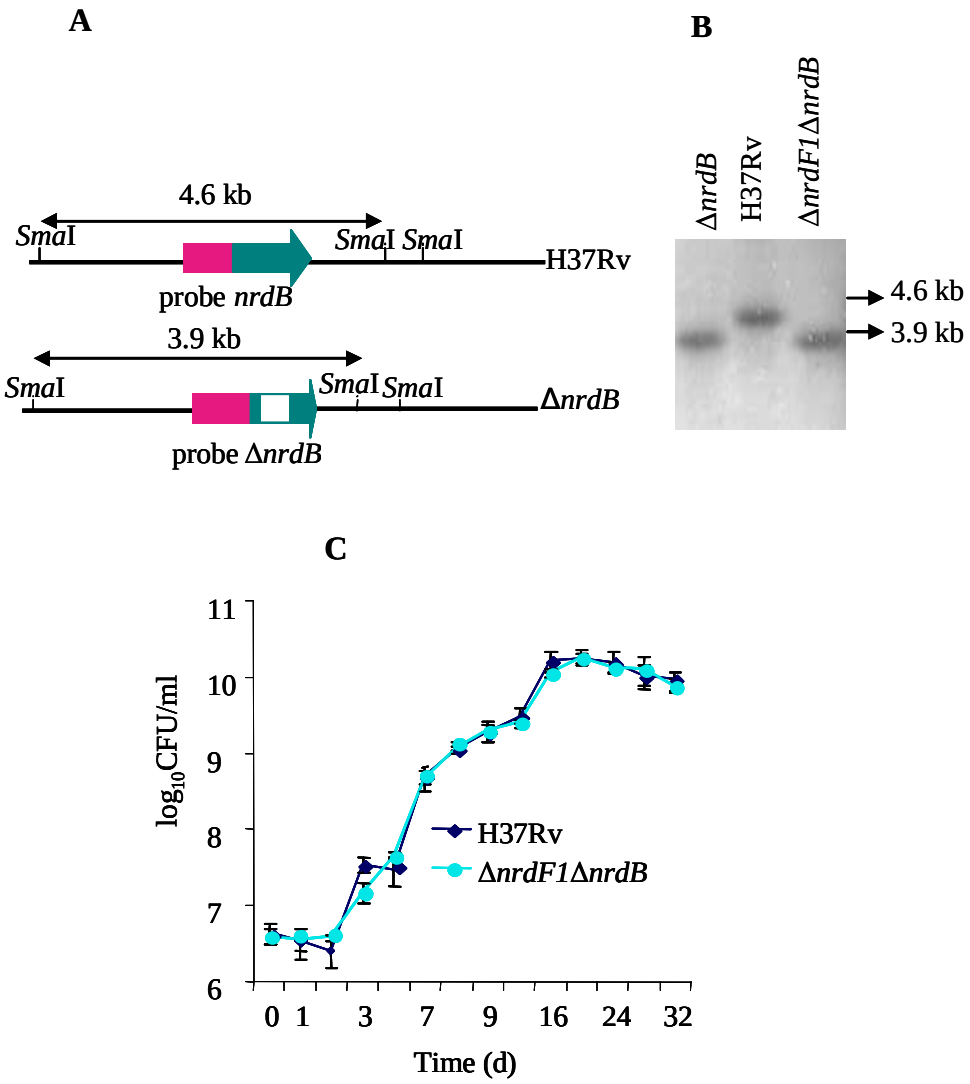


Figure 3.11 Deletion of *nrdB* in the *M. tuberculosis* Δ*nrdF1* background and growth of the Δ*nrdF1*Δ*nrdB* double mutant in vitro. A: Schematic representation of *nrdB* parental and deleted alleles showing the restriction enzyme sites and probes used for Southern blotting. B: Southern blot analysis of genomic DNA samples isolated from H37Rv, Δ*nrdB* and Δ*nrdF1*Δ*nrdB* digested with *Sma*I and hybridized with the tbnrdB-F1pR1p probe (Table 2.4). C: A growth curve showing the growth kinetics of Δ*nrdF1*Δ*nrdB* in comparison with H37Rv. The same wild-type data as on Figure 3.8C was used. CFU averages from three biological replicate cultures plated in duplicate were used to plot the graphs and error bars indicate standard deviations between the three cultures.

3.4.2 Comparative susceptibility of the Δ*nrdF1*Δ*nrdB* mutant of *M. tuberculosis* to DNA damage and HU

The question of whether the *nrdF1* and *nrdB* genes have redundant roles in the survival of *M. tuberculosis* following DNA damage was a major focus of this study. Susceptibility of the Δ*nrdF1*Δ*nrdB* mutant to DNA damage was assessed by monitoring its sensitivity to MTC and UV irradiation. However, disruption of both genes did not result in any defects in growth in the presence of mitomycin (Figure 3.12A) or survival post UV irradiation (Figure 3.12B). To further phenotype this double mutant, the redundancy of *nrdF1* and *nrdB* function in *M. tuberculosis* survival under RNR inhibiting conditions was assessed by comparing the HU susceptibility of Δ*nrdF1*Δ*nrdB* to that of the wild-type strain. However, no significant difference in CFUs between H37Rv and the mutant strain was observed over the range of drug concentrations tested (Figure 3.12C).

The fact that individual or combined loss of both alternate class I small subunits, NrdB and NrdF1 (this study) or loss of the class II RNR, NrdZ (Dawes *et al.*, 2003) did not impair *M. tuberculosis* for DNA damage survival, and/or RNR inhibition survival, in vitro and in vivo growth, argue against the hypothesis that the alternate RNRs play a significant role in dNTP supply under the conditions tested and suggest instead that the class Ib enzyme, NrdEF2, alone can serve this need.

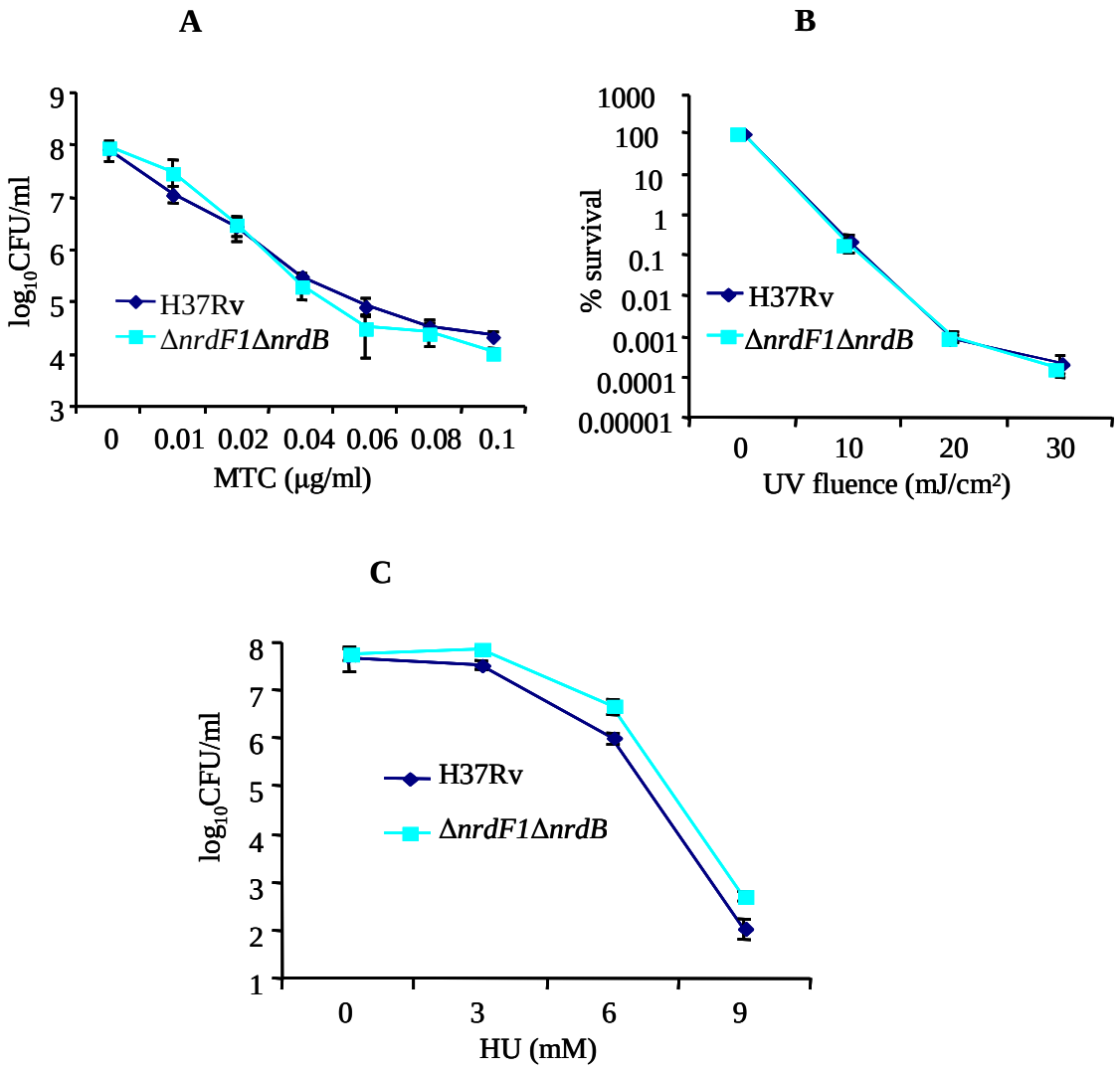


Figure 3.12 Sensitivity of *M. tuberculosis* Δ*nrdF1*Δ*nrdB* to MTC (A), UV irradiation (B) and HU (C). Serial dilutions of log-phase cultures were plated on solid media supplemented with different concentrations of MTC (A) or HU (C). B: Open plates on which serial dilutions were plated were UV irradiated and then incubated until CFUs could be enumerated. The wild-type data on A, B and C is the same as in Figure 3.9A, B and D respectively. The plots represent data from an average of three biological replicates with standard deviations indicated by the error bars.

3.5 The role of the duplication in class Ib RNR-encoding genes in the physiology of *M. smegmatis* mc^{2}155

3.5.1 The remaining copy of *nrdF2* in the ΔDRKIN strain is essential for growth

To investigate the role of *nrdF2* in mc^{2}155, we attempted to construct a *hyg*-marked knockout mutant in ΔDRKIN with an internal segment of 678 bp encoding the radical-bearing tyrosine and most of the electron path residues of the *nrdF2* gene eliminated by allelic exchange mutagenesis using p2ΔSMF2KO (Table 2.2) as a substrate. However, all 89 white colonies obtained from several selections on sucrose plus X-gal where found to be *sacB-lacZ* mutants. The fact that *M. smegmatis* mc^{2}155 NrdF2 shares 93.4 % sequence identity with the *M. tuberculosis* H37Rv homologue (http://cmr.tigr.org/cgi-bin/CMR/) suggests that they are likely to be functionally equivalent. To determine whether failure to obtain DCOs was due to the essentiality of the single *nrdF2* gene remaining in the ΔDRKIN mutant background, a SCO recombinant (ΔDRKINSCO, Table 2.1) was complemented with a copy of the *M. tuberculosis nrdF2* gene with its promoter delivered on the integrating vector, pNRDF2 (Table 2.2) (Dawes *et al.*, 2003). This resulted in the construction of ΔDRKINSCO::pNRDF2 strain (Table2.1), which was then grown in liquid culture without selection before selection for DCO mutants by plating on antibiotic-containing plates (Parish and Stoker, 2000).

The DCO (ΔDRKIN*nrdF2*::*hyg*::pNRDF2, Table 2.1), ΔDRKIN and the ΔDRKINSCO::pNRDF2 strains were analyzed by Southern blot analysis. ΔDRKIN*nrdF2*::*hyg*::pNRDF2 produced 1.4 kb and 0.8 kb cross-hybridising bands corresponding to the *hyg*-marked deletion allele, and a 4 kb band corresponding to the complementing gene compared to 2.1 kb and 1.4 kb bands in ΔDRKIN, which correspond to the wild-type allele, and 4.5, 4, 3.1, 1.4, and 0.8 kb bands in the complemented single crossover strain, ΔDRKINSCO::pNRDF2 (Figure 3.13A and Figure 3.13B). This analysis confirmed the deletion of the remaining chromosomal copy of *nrdF2* in ΔDRKIN in the presence of functional *nrdF2* gene located elsewhere on the chromosome. The fact that DCO mutants of ΔDRKIN could only be obtained in

the presence of a complementing *nrdF2* allele are consistent with previous observations in *M. tuberculosis* H37Rv, in which complementation was used to demonstrate that *nrdF2* is essential under normal in vitro growth conditions (Dawes *et al.*, 2003). These findings also confirm that the alternate, class Ic R2-encoding gene, *nrdB*, is unable to substitute for *nrdF2* function for growth of *M. smegmatis*.

3.5.2 One copy of *nrdF2* is dispensable for growth of *M. smegmatis* mc^{2}155

To investigate the effect, if any, of the duplication in class Ib-encoding RNR genes in *M. smegmatis* mc^{2}155, a knockout mutant lacking only one copy of *nrdF2* was constructed using p2ΔSMF2KO as a substrate for homologous recombination. The presence of a *hyg*-marked deletion allele (Δ*nrdF2*::*hyg*) represented by a 0.8 kb cross-hybridising fragment in the Southern blot, in addition to bands of 2.1 kb and 1.4 kb in size (Figurer 3.13A and Figure 3.13C), which correspond to the wild type *nrdF2* allele, confirmed the genotype of the Δ*nrdF2*::*hyg* mutant (Table 2.1). The genotype of this mutant was further confirmed by qRT-PCR analysis of the level of *nrdF2* expression using RNA samples from three biological replicates of early log-phase (OD_{600} ~0.3) cultures. This analysis confirmed that, similar to ΔDRKIN, which showed a relative *nrdF2* expression level of 0.6 ($P < 0.1$) normalized to *sigA*, Δ*nrdF2*::*hyg* showed a relative *nrdF2* expression level of 0.5 ($P < 0.1$, Table 3.1). This level is approximately half of that observed in mc^{2}155, which carries two functional copies of *nrdF2*. The successful disruption of one of the copies of *nrdF2* in mc^{2}155 suggests that the *nrdF2* duplication of class Ib RNR-encoding genes has no apparent effect on growth. This finding is consistent with the fact that loss of the entire 56-kb duplication (which resulted in the formation of the ΔDRKIN mutant from mc^{2}155) had no effect on growth (Warner *et al.*, 2006).

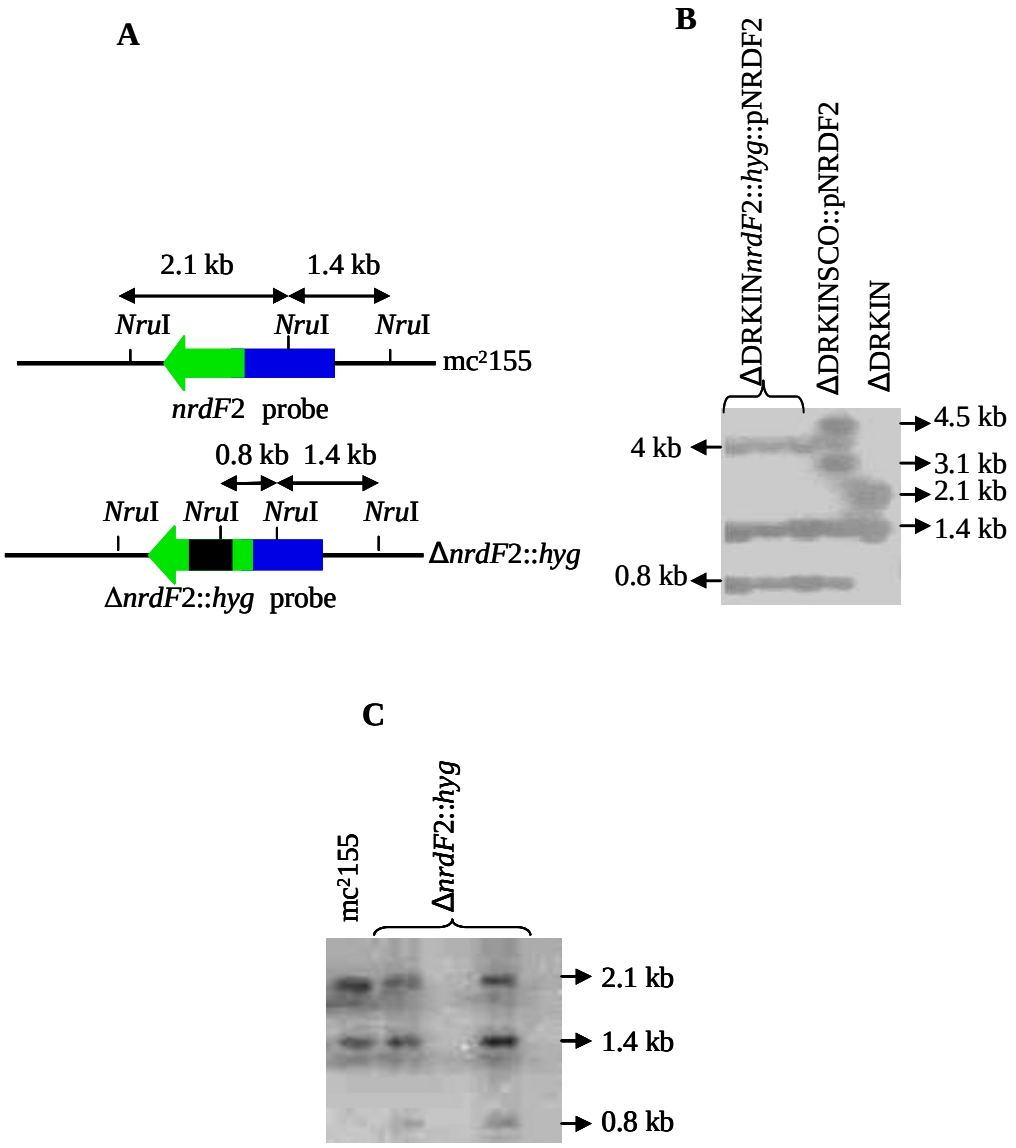


Figure 3.13 Deletion of *nrdF2* in the ΔDRKIN and mc^{2}155 strains of *M. smegmatis*. A: Schematic representation of parental and *hyg*-marked deleted alleles of *nrdF2* showing the restriction enzyme sites and probes used for Southern blotting. B and C: Southern blot analysis of genomic DNA samples isolated from parental, SCO and DCO strains digested with *Nru*I and hybridized with the smnrdF2-F1R1 probe (Table 2.4).

3.5.3 Loss of the duplicated region of *M. smegmatis* mc^{2}155 affects susceptibility to DNA damage and HU

It has been previously shown that the genome duplication in mc^{2}155, which contains 50 genes other than those for the class Ib RNR, does not play any significant role in growth of *M. smegmatis* under normal conditions (Warner *et al.*, 2006). To further investigate the physiological effect of loss of the duplicated region, the ΔDRKIN strain was assessed in terms of sensitivity to RNR inhibition by HU and to DNA damage by MTC treatment and UV irradiation, as described previously. Treatment with MTC at 0.1 μg/ml resulted in a 2.6 $\log_{10}$ kill of mc^{2}155 (Figure 3.14B). Treatment of mc^{2}155 with HU at 9 mM had no effect on viability, whereas 12 mM HU resulted in a ca. 0.4 $\log_{10}$ kill (Figure 3.14A). Interestingly, in comparison with mc^{2}155, the ΔDRKIN showed marked hypersensitivity both to HU and MTC, with a 2.8 $\log_{10}$ increase in killing by 9 mM HU (Figure 3.14A, $P<0.0001$) and 2.2 $\log_{10}$ increase in killing by 0.1 μg/ml MTC (Figure 3.14B, $P<0.01$) being observed. This was supported by the reduction in the MIC values for HU and MTC of ΔDRKIN from the wild-type values of 760 to 190-380 μg/ml (2-4 fold reduction) and 0.03-0.06 to 0.004-0.015 μg/ml (4-16 fold reduction), respectively. Unlike the UV-hypersensitive *dnaE2*::*aph* control (Boshoff *et al.*, 2003), the ΔDRKIN strain displayed no hypersensitivity to the cytotoxic effects of UV irradiation compared to its parental wild type (Figure 3.14C). However, preliminary data have shown that the ΔDRKIN strain is also hypersensitive to other agents including Rif, oflox and Novo (data not shown).

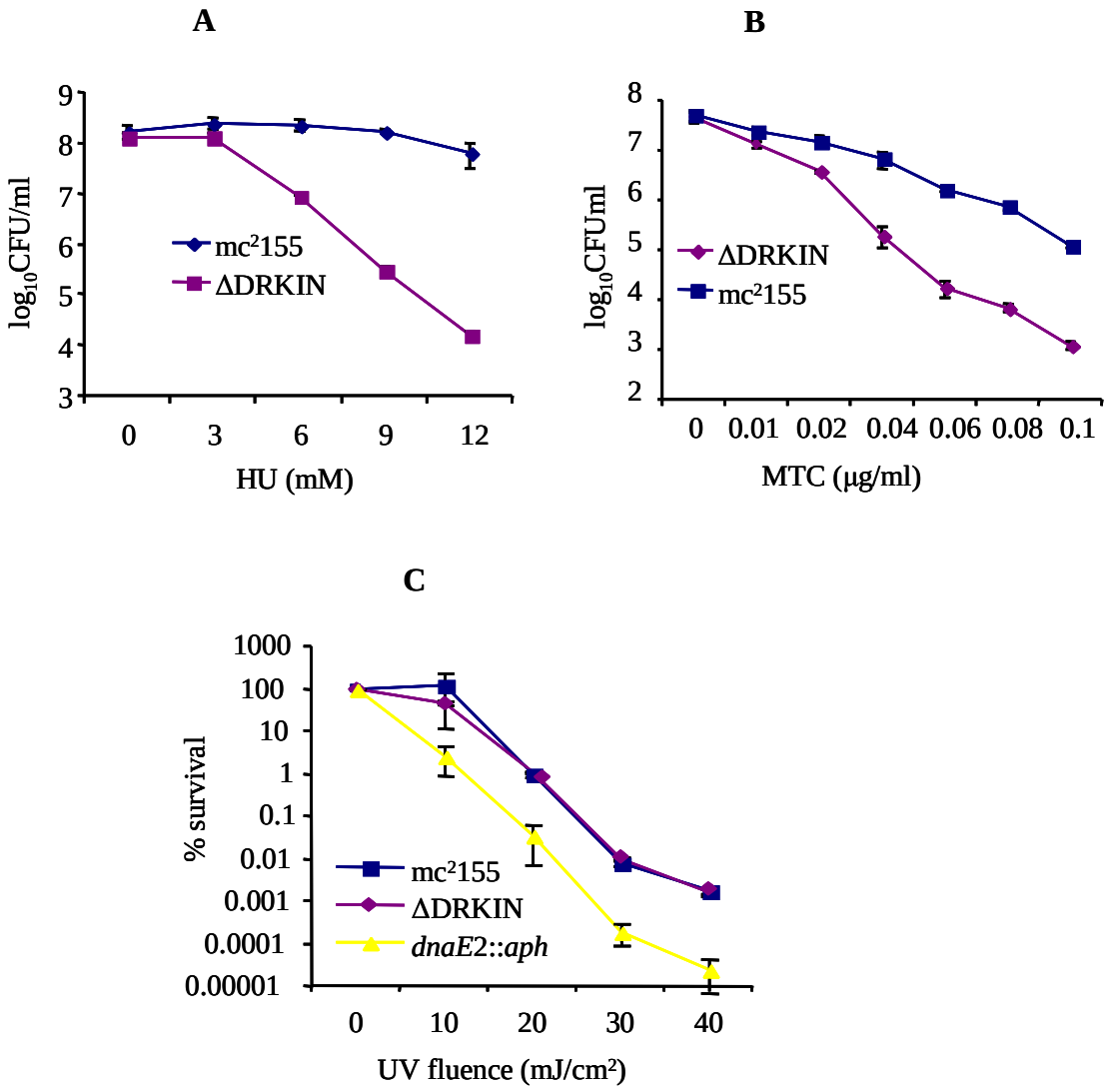


Figure 3.14 Sensitivity of ΔDRKIN to HU (A), MTC (B) and UV irradiation C. For the HU and MTC sensitivity assays, CFUs were enumerated after plating dilutions of log-phase cultures on 7H10-GS supplemented with different concentrations of each compound. For UV survival, open plates on which serial dilutions were plated were irradiated at different UV fluences before CFU counts determination. The *dnaE2*::*aph* mutant was included in this assay as a UV-hypersensitive control (Boshoff *et al.*, 2003). Data represent average CFU counts from three biological culture replicates. Error bars indicate the standard deviations.

3.5.4 The role of *nrdF2* duplication in *M. smegmatis* survival on exposure to HU and DNA damage

To investigate whether the observed hypersensitivity of ΔDRKIN to HU could be explained by the loss of one set of class Ib RNR genes, the Δ*nrdF2*::*hyg* mutant was assessed for sensitivity to HU by the plating assay. Importantly, as observed for ΔDRKIN (Figure 3.14A), the Δ*nrdF2*::*hyg* mutant showed hypersensitivity to HU with 2.1 $\log_{10}$ increase in killing compared to mc^2155 observed at a drug concentration of 9 mM (Figure 3.15A, P<0.0001). As described above, previous work had confirmed that the *M. tuberculosis nrdF2* expressed from its own promoter in pNRDF2 was able to complement *nrdF2* gene function in *M. smegmatis* (Figure 3.13B). Therefore, to confirm whether the HU hypersensitivity of the Δ*nrdF2*::*hyg* mutant was due to insertional inactivation of the *nrdF2* gene, a vector carrying *M. tuberculosis* H37Rv *nrdF2* homologue (pNRDF2, Table 2.2, Dawes *et al.*, 2003) was integrated at the *attB* locus of Δ*nrdF2*::*hyg* to produce Δ*nrdF2*::*hyg*::pNRDF2 (Table 2.1), which was assessed for HU susceptibility. Partial complementation of the HU hypersensitivity of Δ*nrdF2*::*hyg* was observed (Figure 3.15A). In addition, the 2-4 fold reduction in the HU MIC for Δ*nrdF2*::*hyg* (190-380 μg/ml) compared to the wild type strain (760 μg/ml) was also partially reversed by genetic complementation with the *M. tuberculosis nrdF2* gene via pNRDF2 (MIC = 380 μg/ml).

To investigate whether two copies of *nrdF2* are important to meet the dNTP demand during DNA repair, the Δ*nrdF2*::*hyg* mutant was also tested for sensitivity to MTC and UV irradiation using the plating assay (Section 2.12 and Section 2.13). However, unlike ΔDRKIN, which was also hypersensitive to MTC (Figure 3.14B), the susceptibility of Δ*nrdF2*::*hyg* to MTC was indistinguishable from that of the wild-type strain (Figure 3.15B). Therefore, the hypersensitivity of ΔDRKIN to MTC could not be linked specifically to loss of the second copy of *nrdF2*. However, like ΔDRKIN, no UV survival phenotype was observed with Δ*nrdF2*::*hyg* as compared to the wild type and *dnaE2*::*aph* controls (Figure 3.15C).

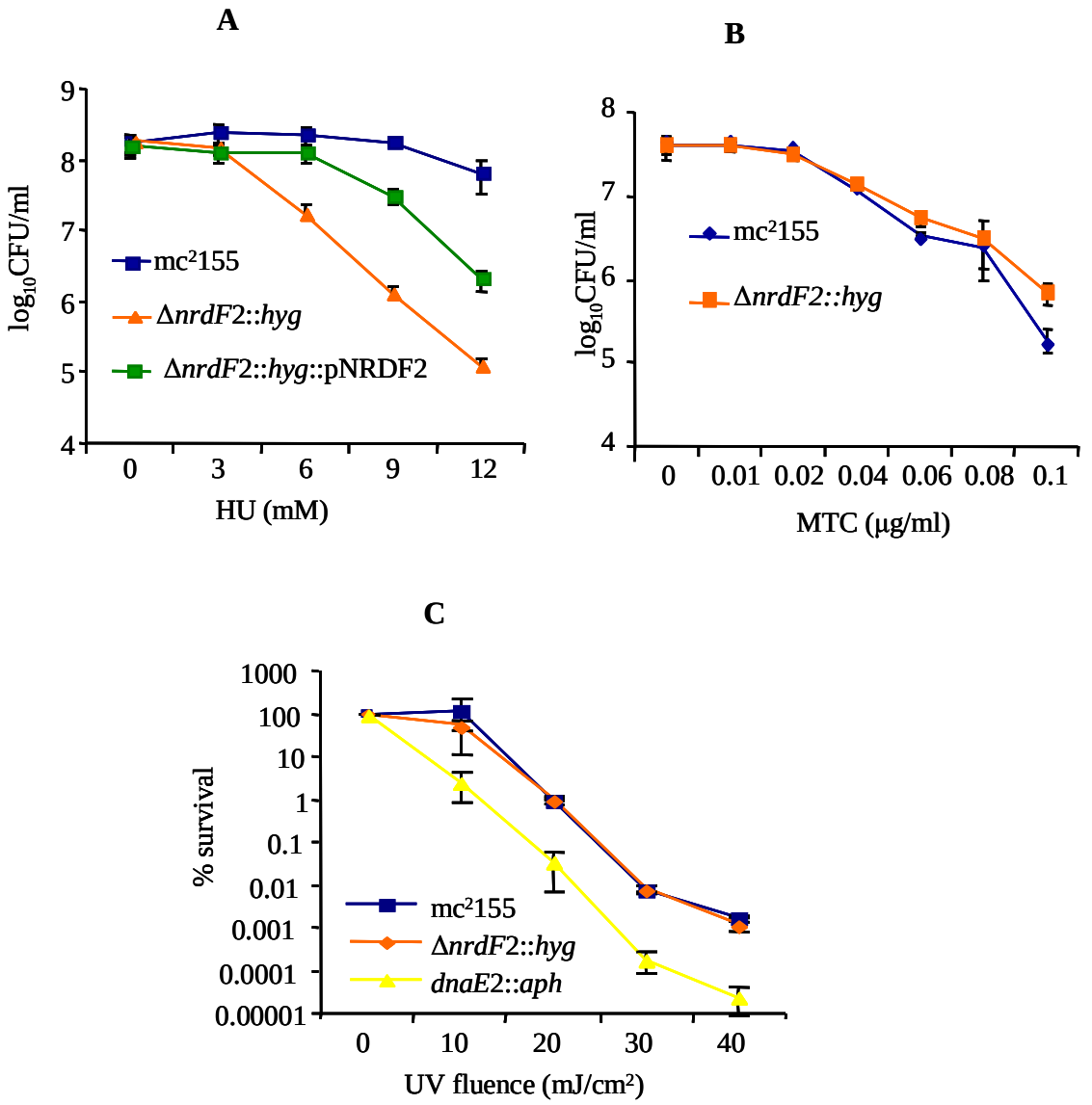


Figure 3.15 Sensitivity of *M. smegmatis* Δ*nrdF2*::*hyg* to HU (A), MTC (B) and UV irradiation (C). A and B: Log-phase cultures were plated on 7H10-GS media supplemented with different concentrations of either HU or MTC. C: Open plates on which serial dilutions were plated were UV irradiated and *dnaE2*::*aph* was used as a positive control. The *dnaE2*::*aph* and/or wild-type data on A and C is the same as on Figure 3.14A and C respectively. The plots represent data from average CFUs from three biological replicates with standard deviations between them indicated by the error bars.

3.6 Expression of *nrd* genes in *M. tuberculosis* and *M. smegmatis* strains

In Streptomyces, *nrdAB* was upregulated 13-fold in response to *nrdJ* deletion, and was consequently proposed to compensate for *nrdJ* loss (Borovok *et al.*, 2004). The lack of discernable growth phenotypes for the Δ*nrdB*::*hyg*, Δ*nrdF2*::*hyg* mutants of *M. smegmatis* and Δ*nrdB*, Δ*nrdF1*, and Δ*nrdF1*Δ*nrdB* mutants of *M. tuberculosis* was suggestive of the presence of a regulatory response affecting the expression of the remaining R2-encoding genes. This was investigated by qRT-PCR analyses to determine the expression levels of *nrdF2*, *nrdF1* and *nrdB* , normalized to *sigA*, in the various mutants under standard growth conditions. In contrast to the observation in Streptomyces, individual or combined deletion the RNR small subunit-encoding genes did not affect the expression of the remaining genes in *M. tuberculosis* (Table 3.1). Similarly, loss of *nrdB* or one copy of *nrdF2* did not affect the expression of the remaining gene in *M. smegmatis* (Table 3.1). This observation nullifies the hypothesis that mycobacteria may obscure the effect of *nrdB* or *nrdF1* loss by transcriptionally regulating the remaining small subunit encoding genes.

Despite the occurrence of three distinct R2-encoding genes in *M. tuberculosis*, there is only one class I RNR large subunit encoding gene in this organism, namely, *nrdE* (http://rnrdb.molbiol.su.se). This suggests that all three R2s, NrdF2, NrdF1 and NrdB, must compete with one another for access to the large subunit, NrdE, to form different class I RNRs. To determine whether NrdE levels may be a limiting factor in this regard, comparative transcript levels of the various *nrd* mRNAs in wild type *M. tuberculosis* during early log-phase growth were determined by real-time qRT-PCR. The *nrdE* gene served as target sequence for the *nrdHIE* gene cluster which is likely to constitute an operon in this organism (Fig. 3.1A). As shown in Table 3.2, the *nrdE* and *nrdF2* genes were expressed at comparable levels to one another during this phase of *M. tuberculosis* growth. In contrast, the levels of expression of *nrdF1* and *nrdB* were considerably lower than *nrdF2* (4- and 6-fold, respectively).

Table 3.1 Analysis of the remaining small subunit encoding *nrd* genes expression in mycobacterial mutant strains

Strains	**Normalized gene expression relative to wild type**		
	nrdF1	*nrdF2*	*nrdB*
M. tuberculosis			
H37Rv	1	1	1
Δ*nrdF1*	ND	1.6 ± 0.7	1.2 ± 0.6
Δ*nrdB*	1.2 ± 0.6	2.1 ± 0.7	ND
Δ*nrdF1*Δ*nrdB*	ND	1.1 ± 0.4	ND
M. smegmatis			
mc^2155	N/A	1	1
Δ*nrdB*::*hyg*	N/A	0.5 ± 0.2*	ND
Δ*nrdF2*::*hyg*	N/A	0.5 ± 0.1*	1.0 ± 0.2

*Significantly different (P<0.1). The statistical significance is based on a pair-wise comparison using the unpaired *t*-test, ND-Not done, N/A-Not applicable

Table 3.2 Levels of *nrd* gene transcripts in *M. tuberculosis* H37Rv during early logarithmic-phase aerobic growth in 7H9-OADC medium

Organism	**Level of *nrd* gene transcript relative to *sigA*[a]**			
	nrdE	*nrdF2*	*nrdF1*	*nrdB*
M. tuberculosis H37Rv	5.2 ± 0.9	5.1 ± 0.2	1.4 ± 0.1	0.91 ± 0.34

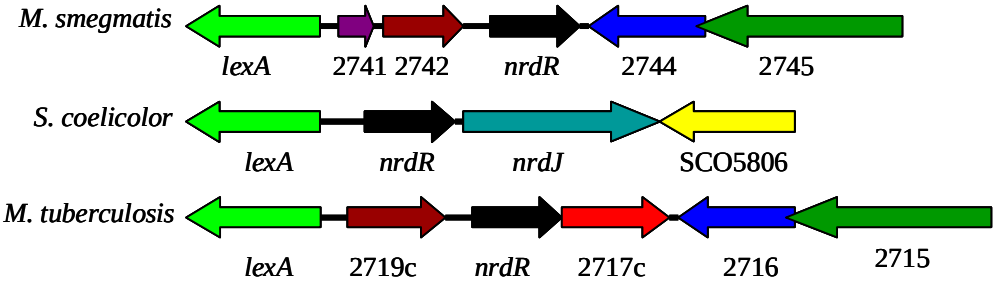
[a]Expression levels were measured in cultures at OD_{600} = 0.3

3.7 *nrdR* homologues and NrdR boxes are present in mycobacteria

Since its identification in Streptomyces, homologues of *nrdR* have been identified in other organisms and the function of its encoded protein, NrdR, as a negative regulator of *nrd* gene expression demonstrated (Borovok *et al.*, 2002; Rodionov and Gelfand, 2005; Torrents *et al.*, 2007). To identify *nrdR* homologues in *M. smegmatis* and *M. tuberculosis*, *S. coelicolor nrdR* was used as a query sequence in a BLAST search against the *M. smegmatis* mc^2155 and *M. tuberculosis* H37Rv genome sequences (http://tigrblast.tigr.org/cmr-blast/). NrdR homologues with 65.8 % and 66.9 % amino acid sequence identity to *S. coelicolor* NrdR were found in *M. smegmatis* (MSMEG_2743) and *M. tuberculosis* (Rv2718c), respectively (Figure 3.16A). Homologues were also identified in all sequenced mycobacterial genomes including *M. leprae* (http://cmr.tigr.org/cgi-bin/CMR). Multiple protein sequence alignment of the NrdRs from *M. tuberculosis* and *M. smegmatis* against the *S. coelicolor* NrdR reference clearly shows that both two essential streptomyces NrdR domains, namely, the Zn ribbon and ATP-cone are well conserved (Figure 3.16B) (http://align.genome.jp/sit-bin/clustalw). However, there are notable differences in the genomic context of *nrdR* between Streptomyces and mycobacteria. Unlike in *S. coelicolor* where *nrdR* is immediately adjacent to *lexA*, in *M. tuberculosis* and *M. smegmatis*, a LexA-regulated gene is located between *lexA* and *nrdR* (Rv2719c in *M. tuberculosis* and MSMEG_2742 in *M. smegmatis*). Another distinguishing feature of the mycobacterial homologues is that they are not proximal to other *nrd* genes, unlike the organization in Streptomyces, where *nrdR* is immediately upstream of the class II RNR-encoding *nrdJ* gene (Fig. 3.16A).

Bioinformatic analyses (Rodionov and Gelfand, 2005) suggested that both the *nrdHIE* gene cluster and the *nrdF2* gene in mycobacteria may be regulated by NrdR given the presence of canonical NrdR boxes upstream of *nrdH* and *nrdF2*, which are highly conserved among the mycobacteria. Interestingly, the mycobacterial NrdR boxes were specifically associated with class Ib-encoding RNR genes (Fig. 3.16C and 3.16D) and were not found upstream of *nrdB*, *nrdF1* or *nrdZ* in any of the sequenced mycobacteria harboring one or more of these genes.

A



B

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                                                                  ♦
                                                                  ▼
M.tuberculosis      MHCPFCRHPDSRVIDSRETDEGQAIRRRRSCPECGRRFTTVETAVLAVVK
M.smegmatis         MHCPFCRHPDSRVVDSRETDEGQAIRRRRSCPECGRRFTTVETAVLAVVK
S.coelicolor        MHCPFCRHPDSRVVDSRTTDDGTSIRRRRQCPDCSRRFTTVETCSLMVVK

M.tuberculosis      RSGVTEPFSREKVISGVRRACQGRQVDDDALNLLAQQVEDSVRAAGSPEI
M.smegmatis         RSGVSEPFSREKVIRGVRRACQGRDVDDDALNVLAQKVEDAVRGLGTPEI
S.coelicolor        RSGVTEPFSRTKVINGVRKACQGRPVTEDALAQLGQRVEEAVRATGSAEL
                                ♦
                                ▼
M.tuberculosis      PSHDVGLAILGPLRELDEVAYLRFASVYRSFSSADDFAREIEALRAHRNL
M.smegmatis         PSHEVGLAILGPLRELDEVAYLRFASVYRSFSSAEDFEREIEALRAHRGA
S.coelicolor        TTHDVGLAILGPLQELDLVAYLRFASVYRAFDSLEDFEAAIAELRETTGH

M.tuberculosis      SAHS----------------------------
M.smegmatis         --------------------------------
S.coelicolor        PGEEDDTGAGSQENDRGPTGAGQVPEPAGAAD
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C

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M. tuberculosis     CCAGGGAATTTCGAAAATGTTAT TCAGAACATCTTGTAT CTCTTCTCCGTGCCACCCCCT
M. bovis            CCAGGGAATTTCGAAAATGTTAT TCAGAACATCTTGTAT CTCTTCTCCGTGCC-------
M. leprae           CCAGGGAATTTCGTAACTGTTGT TCAGAACATCTTGTAT CTATTCTCCTTGCC-------
M. ulcerans         CCAGGGAATTTCAAAAATGTCGT TCCGAACATCTTGTAT CTCTTCTCGTTGTC-------
M. marinum          CCAGGGAATTTCAAAAATGTCAT TCCGAACATCTTGTAT CTCTTCTCGTTGTC-------
M. smegmatis        CCTGGGAATTTCAGAAATGTTAT TCAGAACATCTTGTAT GGCTTCTTC-TGTGG------
                    ** *********  ** ***  *** *************   ****   **

M. tuberculosis     AGGTGCCAC CCCCTAGGTGTAGTGT TTTCGAGTACCGGCAGATCCCAGGTTCACCAGGTC
M. bovis            -------AC CCCCTAGGTGTAGTGT TTTCGAGTACCGGCAGATCCCAGGTTCACCAGGTC
M. leprae           -------AC CCACTAGGTGTAGTGT TTTAAGGTGCCGGCAGATCCCAGGTTCAC TA-CGC
M. ulcerans         -------AC CCCCTAGGTGTAGTGT GTCGAGG-ACCGACAGAACTCAGGTTCACCGGTTC
M. marinum          -------AC CCCCTAGGTGTAGTGT TTCGAGG-ACCGACAGAACCCAGGTTCACCGGTTC
M. smegmatis        -------GG CCACTAGGTGTAGTGT CTGAGAG-ACCGACAGGCCACC ACAGTTCGGGAGC
                              ** ************* *    *  *** ***  * *       *     *
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D

```
M. tuberculosis     GACGG-TCGCTGCGGCGAACTAGCCGGCGAAACAGGCGAGCGGATTCGCGACACGCAAAC
M. bovis            GACGG-TCGCTGCGGCGAACTAGCCGGCGAAACAGGCGAGCGGATTCGCGACACGCAAAC
M. leprae           GCCGC-TCACGGTGGCTAATTAGGTGGC----TGGGCTAGTCGACACGCAA------AAC
M. ulcerans         CCTG--TAGC-GCGGCTGCCCTGGATTC----CGGGCCGCTTGGTTGGCGACACGCAAAC
M. marinum          CCTGG-TAGC-GCGGCTGCCCTGGATTC----CGGGCCGCTTGGTTGGCGACACGCAAAC
M. smegmatis        GGTGAAACGCCACGTCGCGCTTGCGGAG----TTCGCGTGTGAACGCGACACGCCCAACC
                       *     *   * *      *            **          *  *      * *

M. tuberculosis     ACAACTTCTTGTGTTGCAGTACCTTGTCGGACCCCAGGGGTAGTGTTTGAGGCCTAGC--
M. bovis            ACAACTTCTTGTGTTGCAGTACCTTGTCGGACCCCAGGGGTAGTGTTTGAGGCCTAGC—
M. leprae           ACTACTTCTTGTGTTGCGGCGTTATGTCGGACCCCAAGGGTAGTGTTTAAGGTCTAAGTA
M. ulcerans         ACAACTTCTTGTGTTGCGCCGCCTTGTCGGCCCCTACTGGTAGTGTTTGTGGCCTAAGCA
M. marinum          ACAACTTCTTGTGTTGCGCCGCCTTGTCGGCCCCTACGGGTAGTGTTTGTGGCCTAAGCA
M. smegmatis        ACAACATCTCGGGGAGCGCCGCGAACTTTCCCACCAGTTGTAGTGTTGGTATCGTCGCCG
                    ** ** *** * *  **         *    * * *   ********       *
```

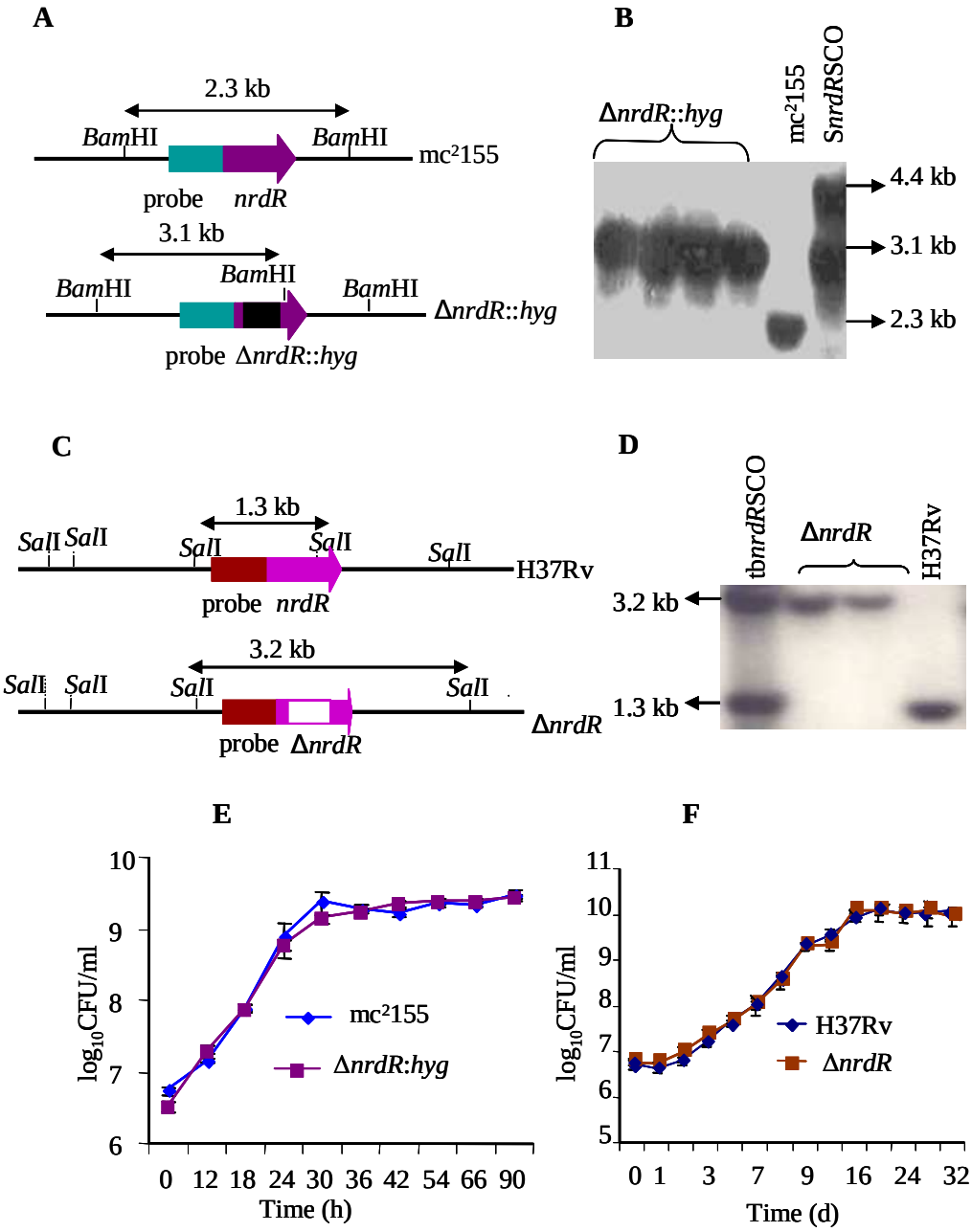
Figure 3.16 Genomic organization of *nrdR* in *M. smegmatis* and *M. tuberculosis* in comparison to *S. coelicolor* (A), multiple protein sequence alignment of *M. tuberculosis* and *M. smegmatis* against *S. coelicolor* NrdR (B) and putative NrdR boxes located upstream of mycobacterial *nrdHIE* (C) gene cluster and the *nrdF2* gene (D). A: The genes are denoted by arrows and the annotation (http://tigrblast.tigr.org/cmr; http://genolist.pasteur.fr/Tuberculist/) is shown below the genes. The same fill colors indicate homologues in both organisms (http://tigrblast.tigr.org/cmr-blast/), with *nrdR* denoted by a solid black fill effect. B: Deletion positions in *M. smegmatis* (♦) and *M. tuberculosis* (▼) are shown. Highlighted in pink is the Zn ribbon domain with conserved two pairs of cystein and four consecutive arginine residues. Highlighted in red is the ATP cone. C and D: Putative NrdR boxes located upstream of mycobacterial *nrdHIE* (C) gene cluster and the *nrdF2* (D) gene are bold and highlighted and were identified based on the consensus palindromic sequence, acaCwAtATaTwGtgt (Rodionov and Gelfand, 2005).

3.7.1 *nrdR* function in growth and long-term survival of *M. smegmatis* and *M. tuberculosis*

To date, *nrdR* homologues have been identified in several microbes, but *nrdR* gene function has only been well studied in Streptomyces (Borovok *et al.*, 2004; Grinberg *et al.*, 2006) and *E. coli* (Torrents *et al.*, 2007). To determine NrdR function in mycobacteria, its encoding gene was targeted for deletion in *M. smegmatis* and *M. tuberculosis* using the p2ΔSMRKO and p2ΔTBRKO constructs, respectively (Table 2.2). Southern blot analysis of the *M. smegmatis* knockout mutant (Δ*nrdR*::*hyg*) revealed the presence of a 3.1 kb cross-hybridising band compared to a 2.3 kb band in the wild type and the 4.4 kb and 3.1 kb bands in the SCO (S*nrdR*SCO) (Figure 3.17A and Figure 3.17B). This genotypic analysis confirms the deletion of 217 bp of the *M. smegmatis* gene, so eliminating most of the ATP cone domain residues (Figure 3.16B), and the insertion of a *hyg* resistance marker. Southern blot analysis of the *nrdR* mutant of *M. tuberculosis* (Δ*nrdR*) revealed a 3.2 kb cross-hybridising band from the DCO in comparison to a 1.3 kb from wild-type and both 3.2 kb and 1.3 kb bands from the SCO (tb*nrdR*SCO) (Figure 3.17C and 3.17D). These data

confirmed the deletion of a 215 bp segment of *nrdR* that encodes the ATP cone (Figure 3.16B). Successful deletion of *nrdR* in two mycobacterial species confirms its dispensability for growth under the conditions tested. Both mutant strains (Δ*nrdR*::*hyg* and Δ*nrdR*) displayed growth indistinguishable from their parental wild type strains (Figure 3.17E and 3.17F). In addition, when Δ*nrdR*::*hyg* was co-cultured with mc^2155 that had been marked with a Km-resistance gene delivered on the pAINT vector (mc^2155:pAINT, Table 2.1), it did not show any competitive growth disadvantage (Figure 3.17G).

To determine the role of *nrdR* in long-term survival, individual cultures of the Δ*nrdR*::*hyg* and mc^2155 or mixed cultures of Δ*nrdR*::*hyg* and mc^2155::pAINT were grown and CFUs enumerated periodically over a period of *ca*. 5 months. Survival of the Δ*nrdR*::*hyg* was similar to that of mc^2155 or mc^2155::pAINT in pure culture (Figure 3.18A) and in competition (Figure 3.18B), suggesting that the mutant showed no long-term survival defect either in pure culture or in competition with the wild-type strain.


A
2.3 kb
BamHI
BamHI
mc²155
probe nrdR
3.1 kb
BamHI
BamHI
BamHI
ΔnrdR::hyg
probe ΔnrdR::hyg
B
ΔnrdR::hyg
mc²155
SnrdRSCO
4.4 kb
3.1 kb
2.3 kb
C
1.3 kb
SalI SalI
SalI
SalI
SalI
H37Rv
probe nrdR
3.2 kb
SalI SalI
SalI
SalI
ΔnrdR
probe ΔnrdR
D
tbnrdRSCO
ΔnrdR
H37Rv
3.2 kb
1.3 kb
E
log10CFU/ml
10
9
8
7
6
mc²155
ΔnrdR:hyg
0 12 18 24 30 36 42 54 66 90
Time (h)
F
log10CFU/ml
11
10
9
8
7
6
5
H37Rv
ΔnrdR
0 1 3 7 9 16 24 32
Time (d)

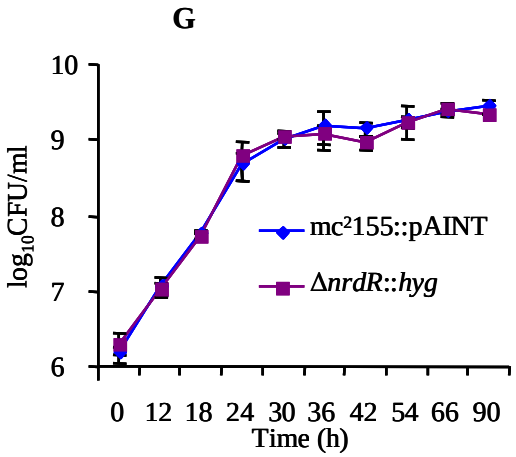


Figure 3.17 Deletion of *nrdR* in *M. smegmatis*, and *M. tuberculosis* and growth kinetics of the mutant strains. A and C: Schematic representation of parental alleles and the mutant allele in *M. smegmatis* and *M. tuberculosis* showing the restriction enzyme sites and probes used for Southern blotting. B: Southern blot analysis of genomic DNA samples isolated from mc^{2}155, SCO (s*nrdR*SCO) and DCO (Δ*nrdR*::*hyg*) strains digested with *BamH*I and probed with smnrdR-F1R1(Table 2.4). D: Southern blot analysis of genomic DNA samples isolated from H37Rv, SCO (tb*nrdR*SCO) and DCO (Δ*nrdR*) strains digested with *Sal*I and hybridized with the tbnrdR-F2R2p (Table 2.4). E and F: In vitro growth of Δ*nrdR*::*hyg* (E) and Δ*nrdR* (F). G: Competitive growth of Δ*nrdR*::*hyg* with mc^{2}155::pAINT.

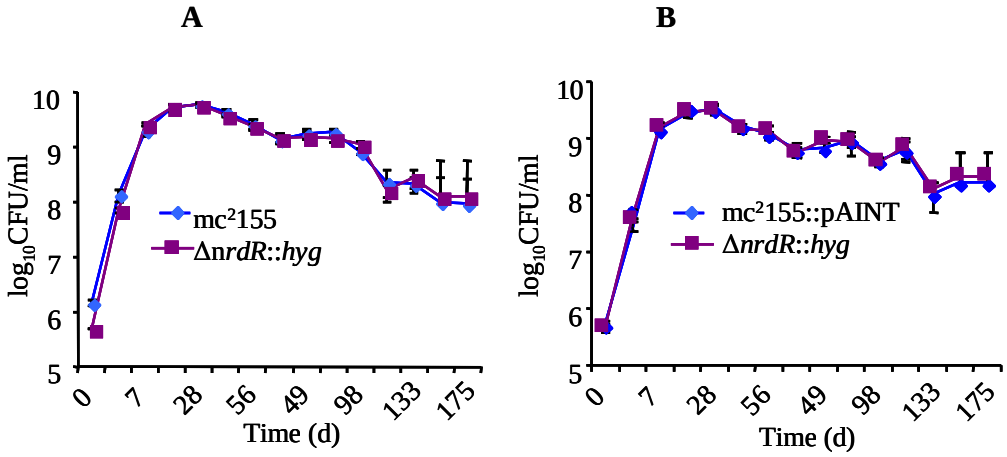


Figure 3.18 Long-term survival of the *M. smegmatis* Δ*nrdR*::*hyg* mutant in pure culture (A) or in competition with mc^{2}155::pAINT (B). To determine the long term survival and competition fitness of the Δ*nrdR*::*hyg* mutant, this strain was grown in pure culture and in co-culture with mc^{2}155::pAINT. Samples were withdrawn periodically over a 175 d and CFUs (differentially) enumerated. The data represent three biological culture replicates. Error bars indicate standard deviations between the three cultures.

3.7.2 Expression of *nrd* genes in the *M. tuberculosis* Δ*nrdR* and *M. smegmatis* Δ*nrdR*::*hyg* mutants

As described above, NrdR has been shown to serve as a negative transcriptional regulator of *nrd* gene expression in other bacterial species (Borovok *et al.*, 2004; Grinberg *et al.*, 2006; Torrents *et al.*, 2007). To investigate its role in the transcriptional regulation of *nrd* genes in mycobacteria, the expression levels of all *nrd* genes in Δ*nrdR*::*hyg* and Δ*nrdR* strains were determined by real-time qRT-PCR. mRNA levels in total RNA samples isolated from early log-phase (OD_{600} ~ 0.3) cultures were analyzed and normalized to *sigA* copy numbers. Loss of *nrdR* function in both *M. tuberculosis* and *M. smegmatis* resulted in a significant increase in the expression of *nrdE* (2.8 and 4.9 fold in *M. tuberculosis* and *M. smegmatis*, respectively) and *nrdF2* (3.1 and 3.7 fold in *M. tuberculosis* and *M. smegmatis*, respectively). Increased expression levels of both *nrdE* and *nrdF2* in the *M. smegmatis* Δ*nrdR*::*hyg* mutant could be reversed to approximately the same levels as observed in mc^2155 by integration of the full length *M. smegmatis nrdR* gene expressed from its own promoter via pNRDR (Table 2.2, Figure 3.19) to generate Δ*nrdR*::*hyg*::pNRDR (Table 2.1). Loss of *nrdR* function did not have any effect on *nrdB* expression in either mycobacterium, or on *nrdF1* and *nrdZ* expression in *M. tuberculosis* (Table 3.3). This observation confirms the prediction based on bioinformatic analysis that NrdR is a transcriptional repressor of only *nrdHIE* and *nrdF2* expression in these organisms.

Table 3.3 qRT-PCR analysis of *nrd* gene expression in Δ*nrdR*::*hyg* and Δ*nrdR* relative to their parental wild-type strains

Strain	Normalized gene expression relative to wild type				
	nrdF1	*nrdF2*	*nrdE*	*nrdB*	*nrdZ*
M. tuberculosis					
H37Rv	1	1	1	1	1
Δ*nrdR*	1.00 ± 0.03	3.1 ± 0.3 **	2.8 ± 0.4**	1.1 ± 0.2	1.2 ± 0.5
M. smegmatis					
mc^2155	N/A	1	1	1	N/A
Δ*nrdR*::*hyg*	N/A	3.7 ± 0.7*	4.9 ± 0.8**	0.7 ± 0.2	N/A

Statistically significant differences are denoted by asterisks. *:P < 0.01; ** - P < 0.001. The statistical significance is based on the pair-wise comparison, by unpaired *t*-test, of the *sigA*-normalized expression level of the gene of interest in the mutant strain *vs*. its parental wild-type strain.

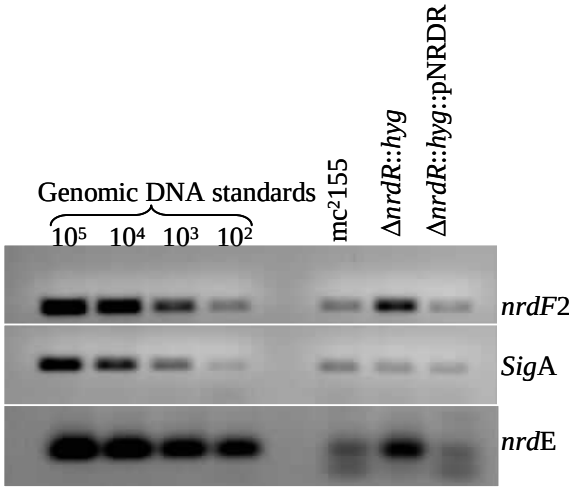


Figure 3.19 Semi-quantitative RT-PCR analysis of *nrdF2* and *nrdE* expression in *M. smegmatis* Δ*nrdR*::*hyg* and Δ*nrdR*::*hyg*::pNRDR. cDNA for *nrdF2* and *nrdE* quantification was diluted 32× and 512× respectively, and was used neat for *sigA* quantification. A 2-µl aliquot was used as the template in a 50 µl PCR reaction. Ten µl samples of the PCR products were then analyzed by electrophoresis on a 2 % agarose gel. Genomic DNA standards represent 10-fold serial dilutions of *M. smegmatis* mc^2155 genomic DNA.

3.7.3 Impact of up-regulation of class Ib RNR gene expression on resistance to RNR inhibition and to DNA damage

Increasing the expression of RNR-encoding genes in bacterial cells has been suggested to compensate for RNR inhibition, thereby rescuing the cells from dNTP starvation (Masalha *et al.*, 2001; Torrents *et al.*, 2003). As described above, loss of NrdR resulted in up-regulation of class Ib RNR gene expression in *M. smegmatis* and *M. tuberculosis*. To determine whether this conferred any advantage in terms of dNTP starvation survival, the HU susceptibilities of the Δ*nrdR*::*hyg* and Δ*nrdR* mutants were compared to their respective wild type strains. However, CFU counts from the surviving cells showed no significant differential sensitivities to HU (Figure 3.20A and Figure 3.20B). Moreover, all strains showed the same MIC for HU (760 μg/ml). The phenotypic effect of de-repression of class Ib RNR gene expression on sensitivity to genotoxic stress was then investigated by evaluating the sensitivity of the mutants to MTC and UV irradiation. However, no differential sensitivity to genotoxic stress was observed for the *nrdR* mutants (Figure 3.20C, D, E and F).

3.8 Effects of altered class Ib RNR-encoding gene expression on mutagenesis

Imbalances in dNTP pools have been shown to confer mutagenic effects in other organisms (Gon *et al.*, 2006a; Wheeler *et al.*, 2005). To investigate if altered levels of expression of class Ib RNR genes have any impact on mutagenesis in mycobacteria, the rates of spontaneous mutation and/or frequencies of UV-induced mutation to Rif resistance were determined in the *M. smegmatis* Δ*nrdR*::*hyg*, Δ*nrdF2*::*hyg* and ΔDRKIN mutants and the *M. tuberculosis* Δ*nrdR* mutant and compared to those of their respective wild type strains. As shown in Table 3.4, mutation rates and/or mutation frequencies were very similar across all strains, with the exception of the *dnaE2*::*aph* control, which was defective in UV-induced mutation to Rif resistance, as expected (Boshoff *et al.*, 2003).

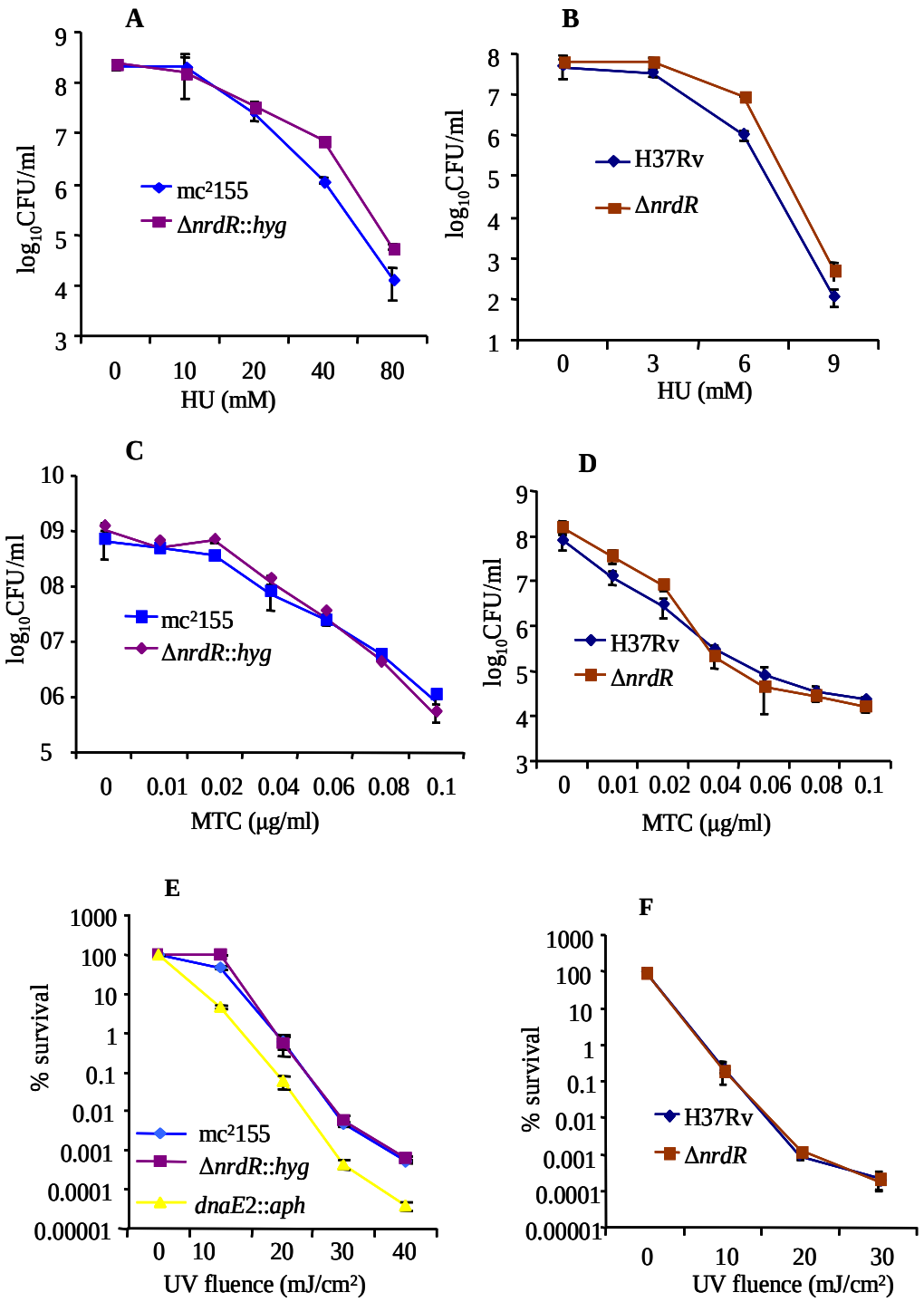


Figure 3.20 Sensitivity of *M. smegmatis* Δ*nrdR*::*hyg* and *M. tuberculosis* Δ*nrdR* to HU (A and B), MTC (C and D) and UV irradiation (E and F). A-D: *M. smegmatis* (A and C) and *M. tuberculosis* (B and D) log-phase cultures were plated on solid media supplemented with different concentrations of HU or mitomycin. E-F: Open plates on which serial dilutions of *M. smegmatis* (E) and *M. tuberculosis* (D) strains were plated were UV irradiated. All plates were incubated until CFUs could be enumerated. The wild-type data on B, D and F is the same as on Figure 3.12C, A and B respectively. A: Data is from one representative of three experiments with averages and standard deviations between three technical replicates. The

data in panel B-F is a representation of an average of three biological replicates, with standard deviations between the cultures indicated by the error bars.

Table 3.4 Spontaneous mutation rates and UV-induced mutation frequencies of *M. tuberculosis* and *M. smegmatis* strains

Strain	**Mutation rate (probability of mutations/cell/ generation)**	**Mutation frequency**	
		Untreated	Measured 24 h post UV irradiation
M. smegmatis			
mc^2155	5.5×10^{-9}	4.8×10^{-7}	3.2×10^{-6}
ΔDRKIN	6.3×10^{-9}	1.7×10^{-7}	3.3×10^{-6}
Δ*nrdF2*::*hyg*	4.4×10^{-9}	2.8×10^{-7}	2.3×10^{-6}
Δ*nrdR*::*hyg*	8.2×10^{-9}	2.3×10^{-7}	3.0×10^{-6}
dnaE2::*aph*	ND	1.4×10^{-7}	6.1×10^{-7}
M .tuberculosis			
H37RV	ND	1.6×10^{-8}	6.8×10^{-6}
Δ*nrdR*	ND	1.7×10^{-8}	6.5×10^{-6}

ND - Not done

Mutation rates are representatives from at least two experiments. Mutation frequencies are an average of one experiment done in three biological replicates.

4. Discussion

The occurrence of multiple RNR encoding genes in a single organism has led to the speculation that different RNR isoenzymes are used to fine-tune the provision of dNTPs for DNA replication and repair under different environmental conditions (Borovok *et al.*, 2002; Borovok *et al.*, 2004; Jordan *et al.*, 1999; Masalha *et al.*, 2001). This is clearly exemplified in other organisms such as *E. coli* where the *nrdHIEF*-encoded class Ib RNR has been speculated to operate under conditions of oxidative stress and iron starvation survival, while *nrdDG*-encoded class III RNR functionally substitutes for the essential, *nrdAB*-encoded class Ia RNR under oxygen limitation (Monje-Casas *et al.*, 2001; Reichard, 1993). The *Lactococcus lactis* class Ib enzyme has similarly been suggested to substitute the class III enzyme under hypoxic conditions (Jordan *et al.*, 1996; Torrents *et al.*, 2000). Most intriguing is the RNR system of *Pseudomonas aeruginosa*, which contains genes encoding all three classes of RNR. Even though the class III enzyme did not show any evidence of activity (Jordan *et al.*, 1999), the class Ia RNR has a demonstrated role in oxygen-rich conditions and the class II enzyme operates under limiting oxygen and upon class I RNR inhibition (Jordan *et al.*, 1999; Torrents *et al.*, 2005a; Torrents *et al.*, 2006b). Another interesting finding was the discovery of *nrdAB* genes encoding an oxygen-dependent class I enzyme in the anaerobe, *Bacteroides fragilis*, which led to speculation that this form of RNR may be important for oxidative DNA damage survival (Smalley *et al.*, 2002).

The purpose of this study was to use a genetic approach to investigate the functional significance of the multiplicity of class I RNR small subunit-encoding genes in mycobacteria in terms of growth in vitro and in vivo, and in survival under a variety of different stress conditions. Furthermore, a mechanism of transcriptional regulation of RNR-encoding encoding genes by NrdR was studied in two mycobacterial species.

4.1 NrdB does not play a significant role in dNTP provision in mycobacteria

As a first line of defense against pathogens, the host immune system produces several antibacterial agents including RNIs (MacMicking *et al.*, 1997; Nathan and Shiloh, 2000; Nicholson *et al.*, 1996; Shiloh and Nathan, 2000) and ROIs (Adams *et al.*, 1997; Nathan and Shiloh, 2000) which serve to kill the invading pathogen. In addition to the 29 enzymes identified (Rhee *et al.*, 2005), the bactericidal effects of RNIs include inhibition of *M. tuberculosis* class I RNR enzyme (by targeting the tyrosine radical in the small subunit) (Fontecave, 1998; Roy *et al.*, 1995). *M. tuberculosis* also appears to sustain DNA damage in the human host (Rachman *et al.*, 2006b), which could also be due to the action of RNIs (Darwin *et al.*, 2003). Supporting this notion is the demonstration that a nucleotide excision repair gene *uvrB* is required for *M. tuberculosis* RNI resistance and DNA damage tolerance in vitro (Darwin *et al.*, 2003; Darwin and Nathan, 2005). Most importantly, deletion of *uvrB* resulted in attenuation of *M. tuberculosis* for growth in mice and the phenotype was reversed in iNOS deficient mice (Darwin and Nathan, 2005). The base excision repair genes *xthA*, *ung* and *end* were also shown to be required for *M. tuberculosis* survival in mice (Sassetti and Rubin, 2003). Further evidence supporting the exposure of the tubercle bacilli to DNA damaging agents in vivo is the requirement of an SOS-regulated gene, *dnaE2*, which encodes a specialized DNA polymerase that is involved in DNA damage tolerance and persistent infection in mice (Boshoff *et al.*, 2003). Together, these observations underscore the need for an adequate supply of dNTPs for DNA repair synthesis by *M. tuberculosis* in vivo.

Identification and classification of the only RNR in Chlamydia as a new type of class I enzyme (class Ic RNR) based on its unique enzymatic features (Högbom *et al.*, 2004; Roshick *et al.*, 2000) added to the complexity of RNRs, and generated considerable interest in this sub-type of class I RNR (Jiang *et al.*, 2007b; Voevodskaya *et al.*, 2005; Voevodskaya *et al.*, 2006; Voevodskaya *et al.*, 2007a; Voevodskaya *et al.*, 2007b). In the class Ic RNR, the catalytic radical-bearing tyrosine residue in the small subunit, NrdB, is substituted by phenylalanine. Moreover, unlike the normal setting in the class Ia and Ib enzymes whereby the

diiron cofactor (Fe^{III}- Fe^{IV}) is used to oxidize the tyrosyl residue in the R2 subunit, which then oxidizes the cysteine residue in the R1 subunit, the class Ic R2, NrdB, uses Fe^{III}- Fe^{IV} to directly oxidize a cysteine residue in the large subunit for the initiation of substrate reduction (Högbom *et al.*, 2004; Voevodskaya *et al.*, 2005; Voevodskaya *et al.*, 2006; Voevodskaya *et al.*, 2007a). More recently, the class Ic enzyme was reported to also use Fe^{III}- Mn^{IV} as a cofactor, which is more effective than a diiron cluster (Jiang *et al.*, 2007b; Jiang *et al.*, 2008a; Voevodskaya *et al.*, 2007b). The unique mechanism employed by the Chlamydial class Ic RNR was hence suggestive of a potential survival strategy against host-mediated nitrosative stress in all organisms harboring a class Ic enzyme (Högbom *et al.*, 2004).

Unlike Chlamydia, which only possess a class Ic-type R2 subunit (Roshick *et al.*, 2000), mycobacteria with the exception of *M. leprae* possess a Chlamydial-like R2 homologue encoded by the *nrdB* gene in addition to at least one classical, class Ib R2 subunit. The *nrdB* genes of *M. tuberculosis* and *M. smegmatis* encode proteins that contain all of the essential residues of a class Ic R2 and expression analysis confirmed that both are expressed albeit at a lower transcript abundance than the class Ib R2 subunits. Together, these findings suggested that *nrdB* might encode a functional R2 subunit that could associate with NrdE to form a class Ic RNR (NrdEB) in mycobacteria. When *nrdB* was targeted for deletion by homologous recombination, DCO mutants were successfully obtained in both *M. smegmatis* and *M. tuberculosis*. Both mutants grew equally well in comparison with their respective parental strains under normal in vitro growth conditions. Hence, these observations distinguish the mycobacterial NrdB from the essential Chlamydial protein (Roshick *et al.*, 2000).

Considerable efforts have been directed at trying to model conditions encountered by the bacilli in vivo, with the aim of understanding the persistence of pathogenic mycobacteria (Betts *et al.*, 2002; Flynn, 2006; Gupta and Katoch, 2005; Hampshire *et al.*, 2004; Wayne and Hayes, 1996). Several adaptive mechanisms have been proposed, which include reduction of energy demand (Dahl *et al.*, 2003; Kusner, 2005; Primm *et al.*, 2000; Shi *et al.*, 2005) and a switch to the use of fatty acids as a sole source of carbon (Kusner, 2005; Muñoz-Elías and McKinney, 2005;

Schnappinger *et al.*, 2003). The observation that loss of *nrdB* did not have any impact on the long-term survival of *M. smegmatis* suggests that NrdB does not play any role in stationary phase adaptation of *M. smegmatis*. When cell populations are exposed to unfavorable conditions, the most fit are likely to outcompete the unfit population (Cohen and Murray, 2004; Gagneux *et al.*, 2006a; Gagneux *et al.*, 2006b). The fact that abrogation of NrdB function did not impair *M. smegmatis* for growth or long-term survival in competition with the wild type, argues against a significant role for NrdB in fitness for growth and long-term survival.

In this study, the possibility of mycobacteria using class Ic enzyme to survive nitrosative stress exerted by the host was investigated in *M. tuberculosis* and *M. smegmatis* by assessing the effect of *nrdB* loss to mycobacterial sensitivity to NO. However, both Δ*nrdB* and Δ*nrdB*::*hyg* sensitivities to GSNO and/or acidified nitrite were indistinguishable from those of the parental strains. Similarly, determination of the effect of *nrdB* deficiency on *M. smegmatis* and *M. tuberculosis* susceptibility to genotoxic stress caused by MTC or UV irradiation resulted in no differential sensitivity between the mutant and the wild type strains. These findings argued against a significant role for the putative class Ic RNR in nitrosative and genotoxic stress tolerance in mycobacteria. HU is a classical class I RNR inhibitor, which acts by scavenging the enzyme's catalytic tyrosine radical. The absence of the tyrosine radical in the class Ic RNR small subunit (Högbom *et al.*, 2004; Voevodskaya *et al.*, 2005; Voevodskaya *et al.*, 2006) raises a compelling question as to whether the intrinsic resistance of mycobacteria to HU may be influenced by the presence of a class Ic enzyme. However, the *nrdB* mutants showed no differential susceptibility to HU as compared to their respective wild-type strains arguing against a significant role for NrdB in dNTP supply under the conditions in which the class Ib enzyme is inhibited.

Despite the hostile environment provided by the host immune system, *M. tuberculosis* is able to subvert the otherwise lethal effects of immune effector mechanisms to ensure its own survival (Hestvik *et al.*, 2005; Rengarajan *et al.*, 2008; Vergne *et al.*, 2004b). Studies investigating mechanisms involved in *M. tuberculosis* survival and persistence in vivo have provided crucial information on

M. tuberculosis pathogenesis (Downing *et al.*, 2005; McKinney *et al.*, 2000; Pandey and Sassetti, 2008; Stokes *et al.*, 2004). Considering the difference between the class Ic and the essential class Ib RNR catalytic mechanism (Voevodskaya *et al.*, 2005; Voevodskaya *et al.*, 2006; Voevodskaya *et al.*, 2007b), it was tempting to speculate that *M. tuberculosis* may utilise a class Ic RNR for dNTP provision when exposed to hostile host defense mechanisms, in particular, nitrosative stress, which is generated after the onset of the acquired immune response (MacMicking *et al.*, 1997; Nathan and Shiloh, 2000; Nicholson *et al.*, 1996; Shiloh and Nathan, 2000). However, when an *nrdB* mutant of *M. tuberculosis* was used to infect immunocompetent mice and bacillary loads followed, the mutant did not show any defects in establishing acute or chronic infection in the lungs and dissemination to the spleen and liver. Because nitrosative or genotoxic stresses are expected to prevail in vivo, lack of a growth and survival phenotype of the *M. tuberculosis nrdB* mutant in mouse lung was consistent with the lack of phenotype under nitrosative stress and DNA damaging conditions in vitro. Together, these findings argue against a significant role for NrdB, and hence, for the putative class Ic RNR, NrdEB, in mycobacterial survival in vivo. These observations could be due to the fact that during the acute phase of infection there is little, if any, nitrosative stress (Nathan and Shiloh, 2000; Smith, 2003), so during this time NrdEF2 may be fully active to provide adequate dNTPs required for growth. Nitrosative stress becomes abundant after the onset of acquired immune response and thus, during the chronic phase of infection (Nathan and Shiloh, 2000; Smith, 2003). However, during this time there is little if any DNA replication (Muñoz-Elías *et al.*, 2005). Therefore, even though NrdEF2 activity might be reduced by the effect of RNIs, the residual activity might be enough to serve the relatively limited dNTP requirement for DNA replication and repair synthesis during the chronic phase of infection.

In *E. coli*, the class Ia and class Ib RNRs contain distinct large subunits, NrdA and NrdE, which associate with their respective small subunits to form a functional enzyme (Jordan and Reichard, 1998). Despite the multiple class I small subunit-encoding genes in mycobacteria, there is only one detectable large subunit-encoding gene, *nrdE*. Whether the class Ic NrdB subunit can compete with the class Ib NrdF2 for association with NrdE remains an intriguing question. To date, no functional

studies on NrdB to probe its interaction with NrdE and ability to form a functional class Ic enzyme have been performed in mycobacteria. The lack of phenotype of the *nrdB* mutant strains of *M. smegmatis* and *M. tuberculosis* under any of the conditions tested may be due to poor/inadequate association of NrdB with NrdE or a complete lack thereof. Despite the conservation of all of the essential residues of a class Ic R2, there is relatively weak homology and no chromosomal context similarity between the NrdB of *Chlamydia trachomatis* and that of *M. smegmatis* (23 % protein identity) and *M. tuberculosis* (31 % protein identity). Instead, the Chlamydial NrdB shows stronger homology to the mycobacterial NrdF2 with 46 and 45 % identity with NrdF2 from *M. smegmatis* mc^2155 and *M. tuberculosis* H37Rv, respectively. However, unlike *M. tuberculosis* NrdF1, which shares 71 % amino acid identity with NrdF2 (Yang *et al.*, 1997), the mycobacterial NrdB proteins show little homology to the NrdF2 counterparts, raising further questions regarding the ability of the mycobacterial NrdB to associate or form a functional enzyme with NrdE. The finding that in *M. tuberculosis*, *nrdE* transcript levels were relatively similar to those of *nrdF2* whereas the expression levels of *nrdB* were 6-fold lower than that of *nrdF2*, suggests that NrdB may be out-competed for interaction with NrdE by NrdF2, resulting in the NrdEF2 form of the enzyme predominating. Finally, the chlamydial class Ic RNR has been reported to use manganese as a more effective cofactor than iron (Jiang *et al.*, 2007b; Jiang *et al.*, 2007c; Voevodskaya *et al.*, 2007b). Therefore, the lack of phenotype of the *nrdB* mutants could also be due to an insufficiency of manganese in the systems used.

4.2 The alternate class Ib RNR (NrdEF1) does not play a significant role in dNTP supply in *M. tuberculosis*

A distinguishing feature of *M. tuberculosis* is the presence in this organism of genes encoding two distinct class Ib R2 subunits, namely, NrdF1 and NrdF2 (Yang *et al.*, 1997). The transcriptional up-regulation of *nrdF1* in response to treatment with DNA damaging agents and translational inhibitors (Boshoff et al., 2004) suggested that this alternate R2 subunit may serve a specialist role in dNTP provision, for example, for DNA repair synthesis. Precedent for this idea exists from recent studies in mammalian system, which identified a second mammalian R2 subunit (p53R2) as

a DNA damage inducible protein with 80-90 % identity to the normal R2 (Byun *et al.*, 2002; Nakano *et al.*, 2000) and showed that it is involved in DNA repair (Guittet *et al.*, 2001; Kimura *et al.*, 2003; Tanaka *et al.*, 2000; Yamaguchi *et al.*, 2001). The alternate R2 subunit, p53R2, can interact with the only R1 subunit at the same site as R2 to form a functional enzyme (R1p53R2) (Guittet *et al.*, 2001; Qiu *et al.*, 2006; Shao *et al.*, 2004), albeit with less affinity and hence, less activity (Qiu *et al.*, 2006; Yen *et al.*, 2006).

The successful deletion of *nrdF1* from the genome of *M. tuberculosis* and lack of phenotype of the resulting Δ*nrdF1* mutant for growth in vitro confirmed the dispensability of *nrdF1* in *M. tuberculosis*, thus differentiating it from the essential *nrdF2* gene (Dawes *et al.*, 2003). However, unlike in mammalian cells where the disruption of p53R2 resulted in hypersensitivity to DNA damage (Zhou *et al.*, 2003), and despite the upregulation of *nrdF1* by DNA damage treatments (Boshoff *et al.*, 2004), the Δ*nrdF1* did not show any defects in DNA damage survival. Mammalian R1p53R2 was reported to be less sensitive to HU than R1R2 (Shao *et al.*, 2004; Yen *et al.*, 2006). Whether NrdF1 could be used to compensate for inhibition of the essential NrdEF2 enzyme by HU was determined by assessing the effect of *nrdF1* gene loss on the susceptibility of *M. tuberculosis* HU. However, no differential sensitivities were observed between the mutant and the wild type strains. Similarly, infection of mice with the Δ*nrdF1* mutant resulted in no differential virulence between the mutant and the wild-type strain arguing against a specialised role for NrdF1 in dNTP provision under the conditions prevailing in vivo. Finally, loss of *nrdF1* had no effect on the sensitivity of *M. tuberculosis* to streptomycin despite its up-regulation in response to treatment with translational inhibitors (Boshoff *et al.*, 2004).

Similar to mammalian p53R2 (Guittet *et al.*, 2001; Qiu *et al.*, 2006; Shao *et al.*, 2004; Yen *et al.*, 2006), NrdF1 has been demonstrated to interact with NrdE in vitro, albeit with a weaker association than NrdF2 (Yang *et al.*, 1997). However, unlike p53R2, which associates with R1 subunit to form a functional RNR enzyme (Guittet *et al.*, 2001; Qiu *et al.*, 2006), NrdEF1 did not exhibit any enzyme activity when tested (Yang *et al.*, 1997). In addition to that, the lower level of *nrdF1* expression in

comparison to that of *nrdF2* observed in this study may reduce the chances of an NrdEF1 enzyme predominating compared to the NrdEF2. Therefore, some or all of these observations may account for the lack of phenotype of Δ*nrdF1* under the conditions tested.

4.3 Effect of the 56-kb genomic duplication on the physiology of *M. smegmatis* mc^{2}155

The effect (if any) of the 56-kb genomic duplication on the physiology of *M. smegmatis* mc^{2}155 is a subject of considerable interest, but has not been studied to any significant extent. In this study, the ΔDRKIN strain, which is an *M. smegmatis* mc^{2}155 derivative lacking the entire duplicated region (Warner *et al.*, 2006), and a knockout mutant lacking one copy of a single gene in the duplicated region, namely *nrdF2*, were used to investigate the effect of duplication of class Ib RNR-encoding genes on the physiology of *M. smegmatis* mc^{2}155. Although one copy of *nrdF2* could be disrupted in *M. smegmatis* mc^{2}155, the same was not true in the ΔDRKIN mutant. The inability to inactivate the single *nrdF*2 gene remaining in ΔDRKIN without a complementing copy of the *M. tuberculosis nrdF2* gene suggested that *nrdF*2 is essential for growth in this background. Supporting this finding was the demonstrated essentiality of *nrdF2* in *M. tuberculosis* (Dawes *et al.*, 2003). Therefore, as in *M. tuberculosis*, the *nrdB* gene is unable to substitute for *nrdF*2 function in *M. smegmatis*.

The observation that deletion of the entire duplicated region rendered mc^{2}155 hypersensitive to a wide range of compounds including Rif and fluoroquinolones, implicated the duplication in enhanced survival of *M. smegmatis* mc^{2}155 under diverse conditions of stress. The hypersensitivity of ΔDRKIN to HU and MTC triggered an investigation into the role of the duplication of class Ib RNR genes in this phenotype. Interestingly, insertional inactivation of only one copy of *nrdF2* in mc^{2}155 resulted in specific hypersensitivity to HU, suggesting that the duplication of class Ib RNR genes allows for increased survival under conditions of dNTP starvation resulting from HU-mediated inhibition of RNR. The incomplete reversion of HU hypersensitivity in *nrdF2* mutant by the complementing copy could result

from the use of a heterologous gene which may not be equivalent to *M. smegmatis nrdF2* in terms of expression and function. It could also be due to the fact that the complementing copy is out-of-chromosome context of expression at the *attB* locus, resulting in expression levels not restored to the parental levels. Although the *nrdF2* gene is induced in *M. tuberculosis* by MTC treatment (Boshoff *et al.*, 2004; Rand *et al.*, 2003), halving the *nrdF2* gene dosage did not affect the sensitivity of *M. smegmatis* to this compound. Therefore, unlike HU, the hypersensitivity of ΔDRKIN to MTC could not be attributed to a reduction in RNR expression/activity. It is possible that the hypersensitivity of ΔDRKIN to MTC is attributable to halving the dosage of another gene(s) carried on the duplicated region of the mc^2155 chromosome (Warner *et al.*, 2006). One possible candidate in this regard is *dinP* as this gene encodes a putative PolIV (DinB)-type, Y-family DNA polymerase whose orthologues are involved in translesion synthesis (TLS) across replication-blocking lesions in other organisms (Jarosz *et al.*, 2007).

In *Saccharomyces cerevisiae* there are two RNR large subunit-encoding genes, RNR1 and RNR3 (encoding Y1 and Y3, respectively, sharing ~ 80 % amino acid identity) (Elledge and Davis, 1990) and two small subunit encoding genes, RNR2 and RNR4 (encoding Y2 and Y4 respectively, with 56 % amino acid identity) (Huang and Elledge, 1997; Wang *et al.*, 1997). RNR1 is essential and while RNR3 is not, it is highly inducible by DNA damage and can complement RNR1 loss when over-expressed (Domkin *et al.*, 2002; Elledge and Davis, 1990). The small subunit is a heterodimer of Y2 and Y4 (Perlstein *et al.*, 2005; Sommerhalter *et al.*, 2004). Y4 is 50 amino acid shorter than Y2 and lacks 6 of 16 residues including three iron binding residues essential for catalysis and highly conserved in most R2 proteins (Huang and Elledge, 1997; Wang *et al.*, 1997). However, Y4 is required for the assembly of the diiron-tyrosyl radical cofactor in Y2 (Nguyen *et al.*, 1999; Sommerhalter *et al.*, 2004; Wang *et al.*, 1997). In a recent study, RNR4 was shown to be important for induced mutagenesis, corroborated by reduced mutation frequencies post UV irradiation in RNR4 null mutant and by HU treatment of the wild-type strain. This was suggested to be a consequence of reduced dNTP pools in the mutant (Lis *et al.*, 2008; Strauss *et al.*, 2007). Several other studies in viruses (Ji and Mathews, 1991; Sargent and Mathews, 1987) and eukaryotic cells (Dare *et al.*,

1995; Hyodo *et al.*, 1984; Song *et al.*, 2003) have also showed that dNTP pool imbalances affect DNA mutagenesis. Most importantly, dramatic reduction of dNTP pools in eukaryotic cells results in DNA repair inhibition and hence increased levels of mutations (Snyder, 1988). In contrast to the findings in other organisms, halving the level of *nrdF*2 expression in ΔDRKIN and Δ*nrdF*2::*hyg* did not result in change in either spontaneous mutation rates or UV-induced mutation frequencies. This could be because the one NrdF2 remaining copy can adequately balance and maintain the dNTP pool essential for DNA repair. Corroborating this is the observation that the 56 kb duplicated region in mc^2155 is dispensable for DNA replication during *M. smegmatis* growth (Warner *et al.*, 2006), under which dNTP pools demand is expected to be higher than during DNA repair.

4.4 *M. tuberculosis* dNTP supply is provided exclusively by NrdEF2 activity

In *S. cerevisiae*, loss of RNR4 results in the overexpression of the second small subunit encoding gene RNR2 and the formation of a homodimer $(Y2)_2$ which interact with the large subunit homodimer $(Y1)_2$ to form a less active $(Y1)_2(Y2)_2$ (Perlstein *et al.*, 2005). An analogous situation exists in Streptomyces whereby deletion of the class Ia enzyme results in the compensatory up-regulation of class II RNR-encoding gene expression (Borovok *et al.*, 2002). Similarly, in mammalian cells, disruption of p53R2 results in the increase in R2 expression levels, which was suggested to substitute for p53R2 function in DNA repair (Lin *et al.*, 2004; Zhou *et al.*, 2003). In stark contrast to these findings, no differential expression of the remaining RNR genes was observed in the Δ*nrdB*, Δ*nrdB*::*hyg*, Δ*nrdF2*::*hyg*, Δ*nrdF1* or Δ*nrdF1*Δ*nrdB* mycobacterial mutant strains. This observation argues against any regulatory cross-talk between the two or three R2-encoding genes in *M. smegmatis* and *M. tuberculosis* under normal in vitro growth respectively.

The observation that *S. coelicolor nrdJ* mutant lacking a class II RNR, but not an *nrdB* mutant lacking the class Ia enzyme failed to grow on HU-containing media, led to the conclusion that under class I RNR inhibiting conditions, NrdJ is able to substitute for class Ia RNR function (Borovok *et al.*, 2004). In contrast, an *M.*

tuberculosis nrdZ mutant lacking the putative vitamin B_{12}-dependent class II RNR did not display hypersensitivity to HU relative to the wild type strain even in the presence of exogenous vitamin B_{12} supplement, arguing against a significant role for NrdZ in dNTP provision (Dawes *et al.*, 2003). Recently, mutants of *M. tuberculosis* lacking *nrdF1* and/or *nrdB* in the *nrdZ* background were constructed, confirming that the class II RNR-encoding gene (*nrdZ*) and the two alternate class I RNR R2-encoding genes (*nrdF1* and *nrdB*) are collectively dispensable for growth (data not shown). However, it remains to be determined whether, and to what extent, the combined loss of function of these genes (*nrdB*, *nrdF1* and *nrdZ*) affects the HU susceptibility of *M. tuberculosis*. Based on the findings reported in this and a previous study (Dawes *et al.*, 2003), it is tempting to speculate that *M. tuberculosis* may depend exclusively on NrdEF2 activity to supply dNTPs for DNA synthesis and repair.

By arguing against specialized roles for NrdZ, NrdF1 and NrdB in the provision of dNTPs during DNA repair and replication in *M. tuberculosis* under the conditions of oxygen restriction, genotoxic and nitrosative stress encountered in vivo, these findings differentiate *M. tuberculosis* from organisms which utilize a multiplicity of RNRs to adapt to environmental conditions that may be variable and hostile. This study has thus revealed a potential vulnerability in dNTP provision in *M. tuberculosis* (Figure 4.1), which provide a compelling rationale for pursuing the NrdEF2 form of the RNR enzyme as a target for anti-tubercular drug discovery (Nurbo *et al.*, 2007; Yang *et al.*, 1997). However, it is worth noting that *Bacillus mojavensis* and *Bacillus subtilis* have been shown to use externally supplied deoxyribonucleosides for anaerobic growth (Folmsbee *et al.*, 2004). Even though there is no evidence in support of this suggestion, it is possible that *M. tuberculosis* may scavenge deoxyribonucleosides from the host. This possibility will have to be addressed in order to further validate the NrdEF2 enzyme as a drug target. The availability of powerful new tools for conditional gene silencing in *M. tuberculosis* suggests that these could be used to address this question by investigating the effects of conditional knockdown of NrdEF2 on growth and persistence in the mouse model (Blokpoel *et al.*, 2005; Ehrt *et al.*, 2005; Gandotra *et al.*, 2007).

4.5 NrdR is a negative regulator of class Ib RNR gene expression in mycobacteria

Consistent with the presence of canonical NrdR boxes upstream of *nrdHIE* and *nrdF2*, disruption of *nrdR* in both *M. tuberculosis* and *M. smegmatis* resulted in a significant upregulation of both *nrdF*2 and *nrdE*. However, the specific signals that lead to de-repression of the *nrdR*-regulated *nrdHIE* and *nrdF2* genes in mycobacteria are yet to be established. Consistent with the lack of identifiable NrdR boxes upstream of *nrdB, nrdF*1 and *nrdZ*, loss of *nrdR* did not affect the expression of these genes, implicating NrdR as a specific mycobacterial class Ib RNR negative regulator (Figure 4.1). This finding differentiates mycobacteria from other organisms in which the function of the NrdR regulator has been investigated. In *E. coli*, for example, NrdR negatively regulates the expression of all three classes of RNR, although deletion of the *nrdR* gene has a much greater effect on expression of the class Ib RNR genes (*nrdHIEF*) than the class Ia (*nrdAB*) or class III (*nrdDG*) genes (Torrents *et al.*, 2007). In *S. coelicolor*, NrdR regulates both the class II RNR-encoding *nrdJ* gene with which it is operonic, and the *nrdABS* operon, with *nrdJ* being more highly induced by loss of NrdR function than *nrdABS* (Borovok *et al.*, 2004).

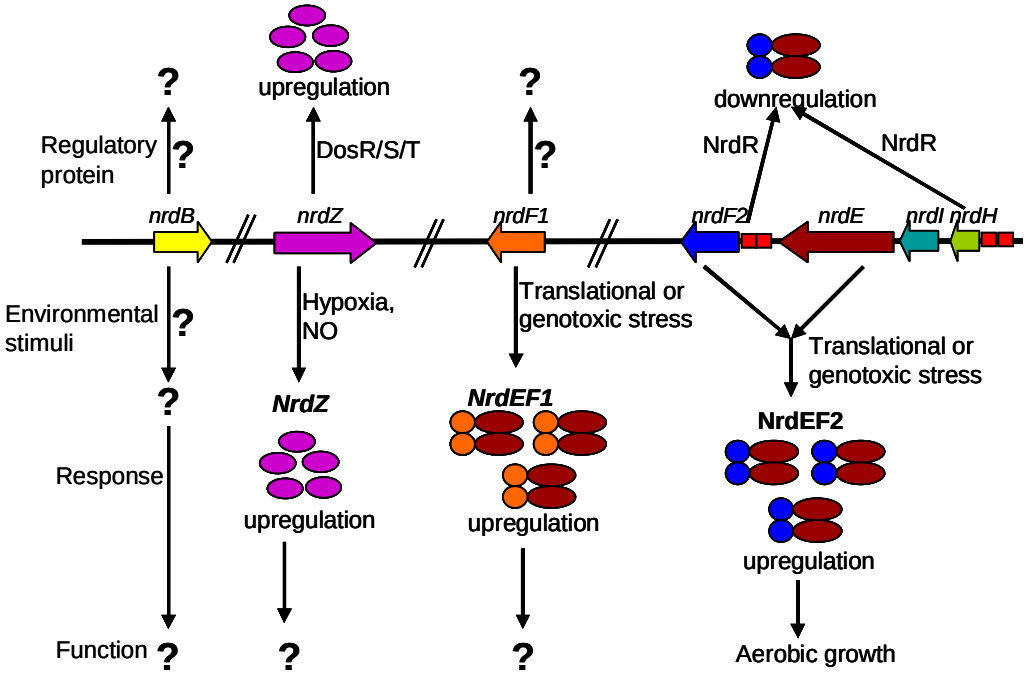


Figure 4.1 The role and regulation of *nrd* genes in *M. tuberculosis*. NrdEF2 is essential for aerobic growth of *M. tuberculosis* in vitro (Dawes *et al.*, 2003) and its encoding genes are upregulated in response to translational inhibition and genotoxic stress (Boshoff *et al.*, 2004). *nrdHIE* and *nrdF2* are negatively regulated NrdR (this work). *nrdF1* is also upregulated by translation inhibition or genotoxic stress (Boshoff *et al.*, 2004) and NrdF1 can interact with NrdE to form NrdEF1 (Yang *et al.*, 1997). *nrdB* is expressed under normal in vitro growth conditions in *M. tuberculosis* (this work) but nothing is known about the regulatory mechanisms governing its expression and whether it can interact with NrdE to form a functional enzyme. The roles of *nrdF1* and *nrdB*, if any, in dNTP provision have yet to be established. *nrdZ* is induced by hypoxia and low-dose NO (Roberts *et al.*, 2004; Voskuil *et al.*, 2003), but is dispensable for growth under hypoxia and for growth and survival in mice (Dawes *et al*., 2003).

4.6 Phenotypic effect of increased class Ib RNR encoding genes expression levels in mycobacteria

Unlike in human cancer cells, where elevated levels of R2 lead to faster growth (Fan *et al.*, 1996), increased expression of *nrdE* and *nrdF2* caused by loss of NrdR function did not affect growth of *M. tuberculosis* and *M. smegmatis* in vitro. The HU hypersensitivity resulting from halving the expression levels of *nrdF*2 in *M. smegmatis* implicated the dosage of class Ib RNR-encoding genes in HU susceptibility, and thus confirmed that the class Ib RNR is the major target for HU in this organism. In bacterial (Roshick *et al.*, 2000; Tipples and McClarty, 1991), mammalian (Akerblom *et al.*, 1981; Choy *et al.*, 1988; Yen *et al.*, 1994; Zhou *et al.*, 1995), viral (Jiang *et al.*, 2004) and insect (Gerenday *et al.*, 2001; Shotkoski *et al.*, 1999) cells, increased expression of class I RNR-encoding genes leads to enhanced resistance to HU. Very similar to NrdR is Crt1, a negative regulator of yeast RNR gene expression. Increased levels of RNR genes due to deletion of CRT1 in *S. cerevisiae* conferred enhanced resistance to HU (Fu and Xiao, 2006; Huang *et al.*, 1998). In contrast to these findings, over-expression of *nrdHIE* and *nrdF2* resulting from loss of NrdR function had no significant effect on HU sensitivity in both *M. tuberculosis* and *M. smegmatis*.

Deletion of a yeast RNR inhibitor Sml1 (Chabes *et al.*, 1999; Zhao *et al.*, 1998; Zhao *et al.*, 2000; Zhao *et al.*, 2001), Crt1 (Fu and Xiao, 2006; Huang *et al.*, 1998) and another *nrd* transcriptional repressor, Crt10 (Fu and Xiao, 2006), resulted in increased levels of dNTP pools and concomitant resistance to DNA damage. Similarly, disruption of the yeast large subunit allosteric site for dATP inhibition resulted in increased dNTP pools and enhanced DNA damage resistance (Chabes *et al.*, 2003). However, constitutive increase in dNTP pools can also result in growth retardation and hypersensitivity to DNA damage (Chabes and Stillman, 2007). Contrary to these reported findings in other organisms, in this study, increased expression of *nrdE* and *nrdF2*, due to loss of regulation, did not have any effect on *M. tuberculosis* and *M. smegmatis* sensitivity to DNA damage. Like dNTP pool imbalances (Dare *et al.*, 1995; Hyodo *et al.*, 1984; Ji and Mathews, 1991; Sargent and Mathews, 1987; Song *et al.*, 2003), proportional increases in dNTP levels may

also be mutagenic (Chabes *et al.*, 2003; Wheeler *et al.*, 2005). Nontheless, induction of the class Ib RNR by de-repression of the *nrdHIE* and *nrdF2* genes did not confer hypermutabilty in *M. smegmatis* and *M. tuberculosis*.

Due to the RNR's fundamental importance in every living system, cells have evolved complex surveillance mechanisms to regulate RNR activity in both a cell cycle and environmental conditions dependant manor. This ensures adequate and balanced dNTP pools for high fidelity in DNA replication and repair. In addition to the allosteric regulation of the enzyme, both eukaryotic and prokaryotic RNRs are regulated by one or several mechanisms at more than one level of gene expression. The best studied example of a tightly regulated RNR system is that in *S. cerevisiae*. Crt1 represses transcription of *S. cerevisiae* RNR genes by binding on the promoter sequences (Huang *et al.*, 1998) while Sml1 inhibit RNR enzyme activity by binding to the large subunit (Chabes *et al.*, 1999; Zhao *et al.*, 1998). In addition, *S. cerevisiae* RNR activity is regulated by subcellular localization of the small subunits, which are predominantly localized in the nucleus and translocate to the cytoplasm to co-localize with the bigger subunit upon DNA damage (An *et al.*, 2006; Yao *et al.*, 2003; Zhang *et al.*, 2006).

Another example of a tightly controlled RNR activity is in *S. coelicolor*, where class Ia RNR activity is regulated transcriptionally and translationally and at the protein level by allosteric regulation. Depicted on Figure 4.2 are the three levels of *S. coelicolor* NrdAB regulation. Transcription of both class II and class I is inhibited by NrdR-dATP complex, which binds to the NrdR boxes upstream of the target genes (Borovok *et al.*, 2004; Grinberg *et al.*, 2006). In addition, the *S. coelicolor* class Ia enzyme is regulated by adenosylcobalamin, whereby binding to a B_{12}-riboswitch element in the upstream-untranslated region of *nrdAB* represses the translation of the mRNA (Borovok *et al.*, 2004; Borovok *et al.*, 2006).

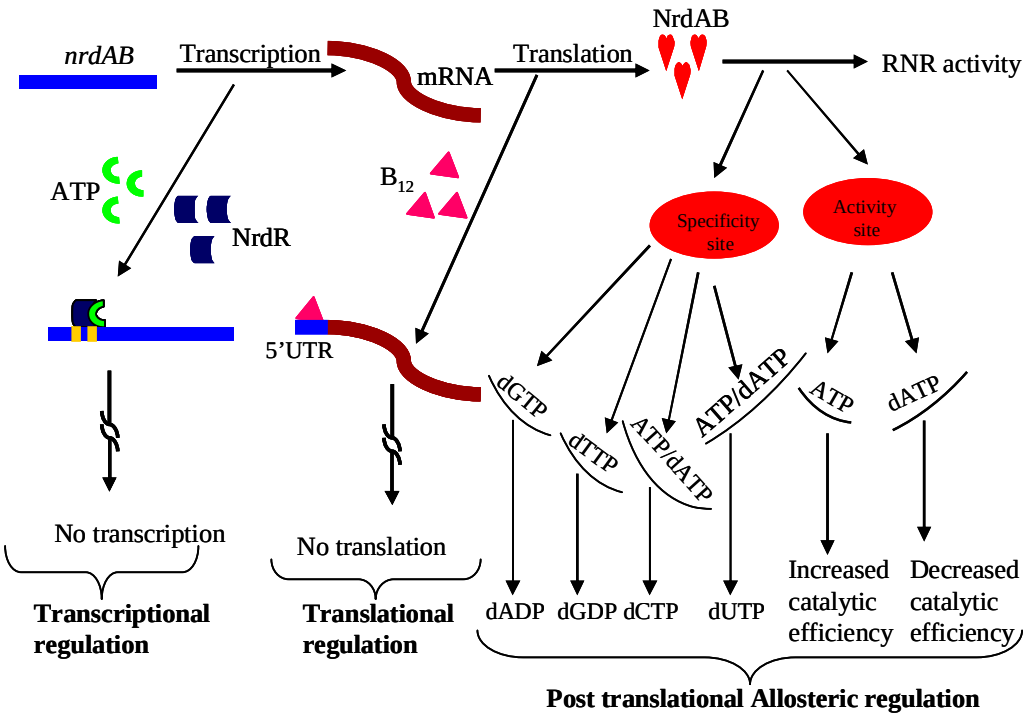


Figure 4.2 Three levels of class Ia RNR regulation in Streptomyces. Transcription is negatively regulated by binding of ATP-NrdR complex to the NrdR boxes (■) upstream of *nrdAB*. During translational regulation by riboswitch mechanism, vitamin B_{12} bind to the B_{12}-riboswitch element in the 5'-untranslated region (5'-UTR) of the *nrdAB* transcript, hence inhibiting translation (Borovok *et al.*, 2004; Borovok *et al.*, 2006). Allosteric regulation of the enzyme involves binding of ATP or dATP to the activity site to activate or inactivate the enzyme respectively and binding of different dNTPs/ATP at the specificity site to regulate the specificity of the enzyme (Reichard, 2002).

Prior to this study, the only known *nrd* regulatory mechanism in *M. tuberculosis* was the regulation of *nrdZ* by DosR/S/T regulatory system (Figure 4.1) (Roberts *et al.*, 2004; Voskuil *et al.*, 2003). Although *M. tuberculosis* also contains a vitamin B_{12}-dependent RNR (NrdZ), no riboswitches were identified upstream of other RNR-encoding genes (Warner *et al.*, 2007) which suggests that regulation of RNR gene expression by vitamin B_{12} does not occur in this organism. The reasons underlying the lack of observable phenotypes in the *nrdR* mutants are unclear. However, considering the potentially deleterious effects associated with increased dNTP pools (Chabes and Stillman, 2007), there might be an existing post-transcriptional mechanisms that regulate RNR function to modulate dNTP pool increase in mycobacteria. Supporting this speculation is the work by Chabes and Thelander in

mammalian cells, which suggested that production of dNTPs is primarily regulated by relative levels of dATP/ATP (Chabes and Thelander, 2000).

4.7 Future studies

The questions concerning the roles of both NrdB and NrdF1 in mycobacterial dNTP provision still remains unanswered. The relationship between all RNR subunits in *M. tuberculosis* is poorly understood. Regulation mechanisms that govern the expression of *nrdB* and the effects of RNR genes expression level to dNTP pool levels in mycobacteria remains to be investigated. Based on the transcript level quantification data reported in this study, it was speculated that lack of phenotype in both the *nrdB* and *nrdF1* mutants of *M. tuberculosis* may be due to restricted access of NrdB or NrdF1 to NrdE and/or an inability of NrdE to form functionally active RNRs by association with these alternate small subunits. Further biochemical studies are required to determine the ability of these additional small subunits in *M. tuberculosis* and *M. smegmatis* to access and interact with NrdE and to measure the strength of interaction. The availability of improved methods for directly determining nucleotide concentrations should allow variations in dNTP pools resulting from altered levels of mycobacterial RNR gene expression to be monitored and correlated with changes in the physiological state of these organisms. Finally, investigating the role of additional small subunits in the absence of the class II RNR enzyme by phenotypically characterizing *M. tuberculosis* mutants lacking the *nrdF1* and/or *nrdB* genes in Δ*nrdZ* background will clarify whether there is a redundancy between in *nrdB* or *nrdF1* and *nrdZ* function.

5. Appendices

Appendix 1: List of Abbreviations

ADC	Albumin-dextrose complex supplement for Middlebrook 7H9
OADC	ADC with oleic acid, supplement for Middlebrook 7H10
Amp	Ampicillin
aph	Gene encoding aminoglycoside phosphotransferase
ATCC	American Type Culture Collection
BER	Base excision repair
BCG	Bacille Calmette-Guérin
bp	Base pairs
BSA	Bovine serum albumin
CFU	Colony forming unit
Cipro	Ciprofloxacin
d	Days
DCO	Double cross over
DMSO	Dimethylsulphoxide
DOTS	Directly observed therapy, short-course
dRNK	Deoxynucleoside-diphosphate kinase
EMB	Ethambutol
GSNO	S-Nitroso glutathione
h	Hours
HIV	Human immunodeficiency virus
Hyg	Hygromycin B
hyg	Gene conferring resistance to hygromycin B
HU	Hydroxyurea
INH	Isoniazid
kb	Kilo base pair(s)
Km	Kanamycin
LA	Luria-Bertani agar
lacZ	Gene encoding β-galactosidase
LB	Luria-Bertani broth

LTBI	Latent TB infection
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
min	Minutes
Moxi	Moxifloxacin
MTBC	*M. tuberculosis* complex
MTC	Mitomycin C
NER	Nucleotide excision repair
NO	Nitric oxide
Novo	Novobiocin
OD_{600}	Optical density at 600 nanometre wavelength
Oflox	Ofloxacin
ORF	Open reading frame
PAS	P-aminosalicylic acid
PCR	Polymerase chain reaction
POA	Pyrazinoic acid
PZA	Pyrazinamide
r	Resistant/resistance
rBCG	Recombinant BCG
Rif	Rifampicin
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RT	Reverse transcription/transcriptase
s	Seconds
sacB	Gene encoding levansucrase
SCO	Single cross over
SDS	Sodium dodecylsulphate
STR	Streptomycin
Suc	Sucrose
TB	Tuberculosis
Tris	Tris(hydroxymethyl)aminomethane
Tween	Polyoxyethylene sorbitan monooleate
XDR	Extensively drug resistant

X-gal 5-bromo-4-chloro-3-indolyl-α-D-thiogalactopyranoside

Appendix 2: Culture media

All media made up to a final volume of 1 liter with deionised water, and sterilised by autoclaving at 121 ^{o}C for 20 minutes, unless otherwise stated.

2-TY Broth

16 g tryptone powder;
10 g yeast extract;
5 g sodium chloride.

Luria-Bertani broth

10 g tryptone powder
5 g yeast extract
10 g sodium chloride.

Luria-Bertani agar

10 g tryptone powder
5 g yeast extract
10 g sodium chloride
15 g DIFCO agar powder

Middlebrook-Glucose-Salt (7H9-GS)

4.7 g Middlebrook 7H9 broth base
2 ml glycerol
10 ml glucose-salt [0.085 % NaCl (w/v) and 0.2 % glucose (w/v)] supplement added after autoclaving

Middlebrook-ADC (7H9-ADC)

4.7 g Middlebrook 7H9 broth base
2 ml glycerol
100 ml ADC supplement added after autoclaving.

Middlebrook-Glucose-Salt plates (7H10-GS)

19 g Middlebrook 7H10 agar powder

2 ml glycerol

10 ml glucose-salt [0.085 % NaCl (w/v) and 0.2 % glucose (w/v)] supplement added after autoclaving

Middlebrook-OADC plates (7H10-OADC)

19 g Middlebrook 7H10 agar powder

2 ml glycerol

100 ml OADC supplement added after autoclaving

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