DNA metabolism in mycobacteria

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I always wanted to be a dog
but I hesitated
for I thought they lacked certain skills.
Now I want to be a dog.

- Michael Ondaatje, ‘A dog in San Francisco’

DECLARATION

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

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Digby Francis Warner Date

ABSTRACT

Specialised mechanisms have evolved in pathogenic bacteria to enable adaptation to hostile and fluctuating host environments. Inducible mutagenesis, in particular, has been implicated in the emergence of antibiotic- and stress-resistant mutants. This thesis examined mycobacterial DNA metabolism with specific emphasis on the roles of multiple Y-family polymerases in the evolution of inter-strain variation and drug resistance in *M. tuberculosis*. The contribution of the *nrdZ*-encoded class II ribonucleotide reductase (RNR) to the maintenance of dNTP pools for replication and repair under hypoxic conditions was also explored. In addition, the co-factor requirement of NrdZ prompted an investigation into the biosynthesis and transport of adenosylcobalamin (AdoCbl) in *M. tuberculosis*.

The data suggest that the mycobacterial Y-polymerases are tightly regulated and restricted to specialised damage-free repair or replication restart. Disruptions in individual *M. smegmatis* mc^2155 DinB (pol IV) homologues resulted in novel antibiotic-resistance polymorphisms that were suggestive of non-redundant function. In contrast, abrogation of all error-prone polymerase activity failed to impair long-term competitive survival of mc^2155 *in vitro*. Similarly, heterologous overexpression of *M. tuberculosis* pol IV homologues did not increase spontaneous mutation rates in wild-type mc^2155, or complement damage hypersensitivity. However, treatment of *M. smegmatis* with gyrase inhibitors confirmed the differential induction of pol IV homologues in response to replication stalling and demonstrated elevated rates of spontaneous mutagenesis as a result of GyrB inhibition.

The class II RNR does not appear to play a significant role in mycobacterial pathogenesis. Specifically, NrdZ was unable to substitute for the class I RNR under aerobic conditions *in vitro*, and a *M. tuberculosis* Δ*nrdZ* deletion mutant was not impaired in its ability to adapt to hypoxia *in vitro*. Similarly, infection of immunocompetent mice suggested that *nrdZ* is not required for the survival or virulence of *M. tuberculosis in vivo*.

Disruptions in genes required for AdoCbl and methionine biosynthesis revealed that complex regulatory functions govern mycobacterial methionine and AdoCbl homeostasis. Loss of early (*cobK*) or late (*cobU*) stage AdoCbl biosynthetic enzymes had no effect on the growth of *M. tuberculosis* H37Rv *in vitro*. In contrast, deletion of the B_{12}-independent methionine synthase (*metE*) resulted in impaired growth on solid media that could be rescued by vitamin B_{12} but not L-methionine supplementation, simultaneously demonstrating the ability of *M. tuberculosis* to transport exogenous vitamin B_{12}. Significantly, double Δ*cobU*/Δ*metE* and Δ*cobK*/Δ*metE* deletion mutants in which all predicted methionine synthase activity was eliminated, were not impaired for growth in liquid minimal media, suggesting that *M. tuberculosis* H37Rv possesses alternative mechanisms for methionine generation. Finally, the attenuated virulence of the Δ*cobU* and Δ*metE* deletion mutants *in vivo* in immunocompetent mice indicated the relevance of AdoCbl biosynthesis to mycobacterial pathogenesis.

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Chapter 1

General Introduction

1.1 Introduction

Tuberculosis (TB) was declared a global health emergency by the World Health Organisation (WHO) in 1993. Over 1.5 million people died of TB during 1999 (WHO, 1999) and the disease currently claims approximately 2 million lives per annum, more than can be attributed to any other bacterial infection (Corbett *et al*., 2003). It is estimated that one-third of the world's population is infected with the causative agent, the obligate human pathogen *Mycobacterium tuberculosis* (Dye *et al*., 1999; Corbett *et al.*, 2003) and, based on prevailing incidence levels, a further 1000 million new infections will occur between 2002 and 2020, resulting in 150 million cases of active disease and 36 million deaths (WHO, 2004). Most initial infections are resolved by the cell-mediated immune response; however, TB immunity is static (Nathan & Shiloh, 2000) and a residual population of viable bacteria may survive for extended periods in a poorly understood state of clinical latency (Lillebaek *et al*., 2002). Spontaneous reactivation of latent TB infection occurs in approximately 5% of cases and is thought to account for almost half of all active pulmonary disease (Styblo, 1991). The massive reservoir of viable bacteria in the estimated 2 billion asymptomatically infected individuals worldwide is, therefore, of supreme importance for the epidemiology and control of TB. The potential of bacterial populations to occupy discrete lesions in a single host renders the disease complex, however (Boshoff & Barry, 2005), and targeting the intractable pathogen is further complicated by both the increasing emergence of multi-drug resistant (MDR) strains (Blower & Chou, 2004; Cohen & Murray, 2004) and the lethal synergy between *M. tuberculosis* and the human immunodeficiency virus (HIV; Selwyn *et al*., 1989).

1.2 Antimycobacterial Chemotherapy

The global TB control effort is focused on interrupting transmission of *M. tuberculosis* through chemotherapeutic intervention in active infectious disease. The cornerstone of this strategy, *d*irectly *o*bserved *t*herapy, *s*hort course (DOTS), became official policy of the World Health Organisation (WHO) in 1994, although modified forms were already in existence (Mitchison, 1998). DOTS is predicated on the supervised administration of combination therapy to sputum positive patients; in addition to ensuring individual compliance with the drug regimen, it was hoped that political commitment to TB treatment and the regular evaluation of programme performance would ensure success. Where fully implemented by well-funded national TB control programs, DOTS has improved treatment outcomes and limited development of drug-resistance (Cohn *et al*., 1990; Kim *et al*., 1997a; Reichmann, 1997; Dye *et al*., 1998); however, adoption of the DOTS programme has been slow in many areas and overall coverage remains inconsistent (Netto *et al*.,

1999). In addition, the efficacy of DOTS is eroded by non-compliance, the HIV pandemic and the emergence worldwide of drug-resistant *M. tuberculosis* strains (Selwyn *et al*., 1989, 1992; Whalen *et al*., 1997; Churchyard *et al*., 1999; De Cock & Chaisson, 1999; Kenyon *et al*., 1999; Kimerling, 2000; Espinal *et al*., 2001; Espinal, 2003). Paradoxically, recent mathematical modelling suggests that the emergence of 'hot zones' of MDR-TB is exacerbated in areas where well-functioning control programmes exert a selective pressure to acquire resistance while eliminating pansensitive wild-type *M. tuberculosis* strains (Blower & Chou, 2004; Cohen & Murray, 2004). Furthermore, a significant number of cases remain undiagnosed and therefore untreated (Dye *et al*., 2002), while the use of frontline drugs in preventive therapy is limited primarily to high-income settings with a small burden of disease (American Thoracic Society, 2000). Particularly sobering is the observation that there are currently more cases of active disease worldwide than when specific effective chemotherapy for TB was first introduced (Gillespie, 2002). The effectiveness of DOTS in reducing the prevalence of *M. tuberculosis* in the human population is, therefore, contentious and it has been argued that control will never be realised through treatment of active disease alone (Enarson, 2000; Suarez *et al*., 2001; Brewer & Heymann, 2004). Significantly, no chemotherapeutic strategy exists to eradicate clinically latent bacteria - if all transmission were immediately blocked, it is estimated that millions of cases would likely emerge over the next few decades from individuals with undiagnosed latent TB (Kaufmann, 2000). In addition, coinfection with HIV is associated with an increased likelihood of developing active TB and is the most important risk factor for reactivation (Selwyn *et al*., 1989; Perlman *et al*., 1999). As a result, there is a dire need for a sterilising drug to shorten the duration of chemotherapy and ultimately eradicate the massive reservoir of latent bacteria, both stated priorities of the Global Alliance for TB Drug Development (GATB). There are few candidates in the pipeline, however, although a recently discovered diarylquinolone offers considerable promise as a lead compound, both as a result of the potential to shorten the duration of chemotherapy and because the novel target - the mycobacterial ATP synthase – ensures that drug-sensitive and drug-resistant isolates are equally susceptible to its inhibitory effects (Andries *et al.*, 2004). Other advances include enhancements to rifampicin activity that have yielded rifapentine, and the adoption of several broad-spectrum antibacterials - particularly quinolones – in the treatment of MDR-TB (Duncan, 2004; Duncan & Barry, 2004). Differences in drug susceptibilities between replicating and non-dividing bacterial cells can be profound (Hobby & Lenert, 1957); therefore, perhaps the greatest limitation of current therapy is the dependence of all front-line antituberculars on actively replicating cells for their efficacy (Mitchison, 1985; Jindani *et al.*, 2003). In the hope of at least partially addressing this

problem, a small-molecule nitroimidazopyran active against anaerobic organisms is currently under development by GATB (Stover *et al*., 2000; Barry *et al*., 2004).

Frontline DOTS drugs

The current standard combination therapy requires the administration of four antimycobacterial drugs for a minimum period of six months – an "intensive phase" comprising two months isoniazid, rifampicin, and pyrazinamide therapy supplemented with streptomycin or ethambutol if drug resistance is likely, followed by a "continuation" or "consolidation" phase of a further four months of isoniazid and one other frontline drug, usually rifampicin were affordable (Mitchison, 1985). Each of the front-line drugs has been selected for its ability to target bacilli in various growth stages in disparate physiological states (Mitchison, 1985), with a bias towards interference in cell wall integrity. Oxidative activation of the isoniazid prodrug by the mycobacterial catalase-peroxidase *katG* yields a poorly understood active form that interrupts mycolic acid synthesis (Zhang *et al*., 1992; Heym *et al*., 1993). Similarly, pyrazinamide requires activation by the hydrolytic pyrazinamidase to pyrazinoic acid, which is thought to inhibit fatty acid synthesis (Zimhony *et al*., 2000) as well as mediating other specific and non-specific effects through intracellular acid accumulation. Ethambutol is another mycobacterial cell wall inhibitor, interfering in the synthesis of the arabinogalactan layer by blocking arabinose insertion. In contrast, rifampicin binds to the bacterial DNA-dependent RNA polymerase β subunit, disrupting transcription while, like other aminoglycosides, streptomycin inhibits protein synthesis.

The actively-dividing *M. tuberculosis* population in the lung cavity is key to transmission and emergence of drug resistance (Grosset, 2003; Kaplan *et al*., 2003), and is the principal target of therapy. Bacillary replication in pulmonary cavities resembles optimal *in vitro* aerobic growth, and the effectiveness of the key frontline drugs in treating acute TB is manifest in the rapid bacillary clearance within the first two months of chemotherapy (Grosset, 2003). In fact, the greatest benefit of short-course therapy derives from the bactericidal activity during the intensive phase, and results largely from the early killing effects of isoniazid (Slayden & Barry, 2000). However, despite the demonstration that high concentrations accumulate in all types of lesions (Barclay *et al*., 1953), isoniazid activity is transient and monoresistance common (WHO, 2004); therefore, pyrazinamide and especially rifampicin are required to supplement and sustain bactericidal effects. Like isoniazid, both rifampicin and pyrazinamide exhibit early bactericidal activity and are able to clear the sputum during therapy (Mitchison, 1985; Mitchison & Nunn, 1986).

Pyrazinamide mediates its antimycobacterial effects at low pH and so is ideally suited to the elimination of bacilli inside caseous necrotic foci, but appears to confer no benefit after the second month of therapy (Mitchison, 1985; Wayne, 1994). Another frontline drug, ethambutol, which has largely replaced streptomycin in standard regimens, also displays high bactericidal but no sterilising activity. It is significant that, although characterised by potent bactericidal activities, a common theme associated with the frontline drugs is their non-existent (isoniazid, streptomyin, ethambutol) or diminished (rifampicin, pyrazinamide) sterilising capability, considered a key factor in determining treatment duration (Mitchison, 1992).

Bacilli contained within macrophage phagosomes are thought to adapt to an altered, low-metabolic state in response to hypoxia, nutrient stress, and immune surveillance (Schnappinger *et al*., 2003). Treatment of this population is predominantly the function of rifampicin which is able to target slowly-metabolising organisms. Introduction of rifampicin in combination with pyrazinamide was responsible for the advent of short-course therapy, significantly decreasing the duration of the continuation, or sterilising, phase which is crucial to the eradication of drug-susceptible persisters and reduces reactivation risk to an acceptable level (Fox & Mitchison, 1975). Finally, there is an increasing awareness of the necessary interrelation of antimicrobial activity and host defences in determining the ultimate fate of tubercle bacilli (Gomez & McKinney, 2004), and evidence that elimination of pre-existing infection by antibiotic therapy, followed by repeated post-exposure vaccination, could prevent disease (Lowrie *et al*., 1999) suggests that immune modulation as an adjunct to antituberculous therapy might significantly enhance efficacy (Glassroth, 2000).

The cost of resistance

Propagation of a pathogen is dependent on the ability to survive, reproduce, and be transmitted, and is encapsulated in the concept of fitness (Cohen *et al*., 2003). Because antibiotics target genes or functions essential to the bacterium at some time during the infection process, a fitness cost has been attached to resistance mutations (Andersson & Levin, 1999), and has led to the assumption that removal of antibiotic selective pressure will favour reversion as a result of a competitive replicative disadvantage. Although fitness cost determines the stability and potential reversibility of the resistance mutation (Levin, 2002), there is increasing evidence that evolution in the absence of the selective antibiotic preferentially results in the acquisition of mutations that ameliorate the cost of resistance, rather than higher-fitness, drug-sensitive revertants (Schrag & Perrot, 1996; Björkman *et*

al., 1998, 2000; Levin *et al*., 2000; Reynolds, 2000; Björkholm *et al*., 2001; Nagaev *et al*., 2001). Of course, the larger mutational target (the chromosome) favours compensatory evolution over reversion (the individual gene; Andersson, 2003); therefore, the ability of bacteria to compensate genetically for the fitness cost will dictate the frequency of resistant mutants within a population. In addition, evidence suggests that although the most fit mutants will be selected in a large population, lower-fitness, compensated mutants might become fixed during bottlenecks if formed at a higher rate than fitter, susceptible revertants (Levin *et al*., 2000; Maisnier-Patin *et al*., 2002). Moreover, reversibility might be further reduced where genetic linkage exists between selected and non-selected resistance markers (Enne *et al*., 2001). Although information on the relative fitness of MDR isolates is limited (Davies *et al*., 2000), there is evidence that compensatory mutations can restore reproductive potential in monoresistant *M. tuberculosis* strains (Sherman *et al*., 1996; Heym *et al*., 1997; Telenti *et al.*, 1997; Li *et al*., 1998; Wilson *et al*., 1998; Slayden & Barry, 2000). Resistance phenotypes arising from separate mutation events can have different fitness costs (Li *et al*., 1998; Billington *et al*., 1999) and it has been demonstrated that clinically resistant strains are more frequently associated with low- than high-cost mutations (Billington *et al*., 1999; Sander *et al*., 2002). For example, isoniazid-resistant isolates most commonly contain a *katG* mutation that reduces prodrug activation while halving catalase-peroxidase activity (Rouse *et al*., 1996; Ramaswamy & Musser, 1998; Slayden & Barry, 2000). Furthermore, results of several studies investigating the effects of resistance on mycobacterial virulence (Middlebrook & Cohn, 1953, Ordway *et al*., 1995; Scorpio *et al*., 1997; Böttger *et al*., 1998; Billington *et al*., 1999; Pym *et al.*, 2002; Sander *et al*., 2002) are consistent with phenotypic heterogeneity (Li *et al*., 1998; Billington *et al*., 1999) and suggest that no direct correlation can be made between drug resistance and pathogenicity (Cohen *et al*., 2003). Significantly, there is evidence that relative fitness *in vitro* may depend not only on the particular resistance mutation, but also on the nature of the assay (Mariam *et al.*, 2004). Of course, in addition to altering the capacity to resist a drug, any resistance mechanism might impact on the ability of the pathogen to interact with the host environment. The apparent absence of a fitness cost could, therefore, reflect an inability to detect relevant growth defects *in vitro* (Andersson, 2003). Finally, while the potential effects on the fitness of *M. tuberculosis* are crucial to epidemiological predictions of the spread of resistance, particularly MDR isolates (Cohen *et al*., 2003), recent mathematical models suggest that, provided relative fitness of an MDR strain remains above a defined threshold, a subpopulation of the low-fitness MDR strain will outcompete both the drug-sensitive strains and other, less fit MDR strains where confronted by a functioning TB control programme (Blower & Chou, 2004;

Cohen & Murray, 2004). For this reason, the *distribution of fitness* (Cohen & Murray, 2004) among circulating *M. tuberculosis* strains is considered a more accurate predictive measure of resistance emergence, and has led to the proposal that DOTS policies necessarily be supplemented with anti-MDR strategies to limit resistance amplification (Blower & Chou, 2004; Cohen & Murray, 2004).

1.3 Molecular biological approaches to drug discovery: current trends

Detailed guidelines for the entire drug development process, including the requirements of a suitable drug target, have been established by GATB (http://www.tballiance.org); furthermore, the elucidation by the TB Structural Genomics Consortium of selected *M. tuberculosis* protein structures should further supplement drug discovery efforts (Goulding *et al*., 2002). It is significant, however, that a large proportion of research aimed at identifying and validating targets is performed almost exclusively in academic laboratories and, although the recent availability of the complete genome sequences of two *M. tuberculosis* strains (Cole *et al*., 1998; Fleischmann *et al*., 2002) has enabled post-genomic mycobacterial research, a function for 48% of the predicted proteins encoded by the *M. tuberculosis* genome cannot be assigned (Camus *et al*., 2002). The more salient approaches to target identification and validation are considered here briefly, although the use of RNA-based techniques is discussed further in subsequent sections.

Targeted gene knockout

Because target validation requires the demonstration of gene essentiality at some stage of the infection process, molecular biological approaches are dominated by the generation of mutants and phenotypic screening. Reverse genetic approaches are dominated by the use of targeted gene knockout to elucidate function, primarily driven by the desire to create rationally attenuated mutants as potential vaccine candidates (Jackson *et al*., 1999; Hondalus *et al*., 2000; Sambandamurthy *et al.*, 2002, 2005) or as a means to investigate specific genes, particularly those encoding potential drug targets (McKinney *et al*., 2000; Tullius *et al*., 2003). Of course, pre-selection of the target implies that this approach is hypothesis-driven and therefore potentially limited by prevailing dogma or preconception. While techniques for targeted mutagenesis have advanced considerably in the past few years - in particular, the use of suicide or conditionally replicating phage or plasmid vectors to deliver mutant alleles (Parish & Stoker, 2000; Bardarov *et al*., 2002) - the process remains time-consuming and inapplicable to large gene sets. However, the precise nature of the mutation introduced assures the abrogation of gene function; in addition, the same techniques employed to generate the mutant can be applied in

complementation analysis - central to the target validation process, or to establish essentiality of gene function in cases in which knockout mutants cannot be recovered.

Random (transposon) mutagenesis

Although targeted gene knockout is the method of choice for investigating individual gene function, random mutagenesis enables the generation and analysis of a large pool of mutants in a single experiment, effectively combining the identification and validation steps. No prior knowledge of individual gene function is required, so bias inherent in target selection is avoided. Initially applied in the screening of mutant libraries *in vitro*, the potential of this technique was fully realised when adapted for use *in vivo* by the addition of DNA sequence tags (Hensel *et al*., 1995; Cox *et al*., 1999). The application of signature-tagged mutagenesis (STM; Hensel *et al*., 1995) in the mouse infection model provides a powerful means of identifying and classifying mutants based on phenotypes revealed throughout the infection process (Cox *et al*., 1999), as well as revealing and validating immunomodulatory or persistence genes which would otherwise be impossible to predict (Hisert *et al*., 2004). A particularly innovative tool for target identification and validation in *M. tuberculosis* is the transposon site hybridisation (TraSH) method, in which mutant pools can be mapped by a DNA microarray-based approach (Sassetti *et al*., 2001; Sassetti *et al*., 2003; Sassetti & Rubin, 2003). The TraSH method was initially used to identify genes required for optimal mycobacterial growth *in vitro* (Sassetti *et al*., 2001, 2003); of the *M. tuberculosis* genes identified as essential, many are conserved in the *M. leprae* genome, which might be considered the minimal mycobacterial gene set, and a large proportion of these have no identifiable bacterial orthologs. Moreover, a significant proportion of essential genes as yet have no assigned function. The enormous utility of TraSH and related techniques for functional genomic screening during various stages of the infection process was confirmed by its successful application *in vivo* in a mouse model of infection (Sassetti & Rubin, 2003). Perhaps the most exciting finding of the *in vivo* study, however, was the demonstation that a significant subset of the genes required for growth *in vivo* are unique to mycobacteria and closely related species and, as such, hold considerable promise for new drug development. In recognition of its value as an information resource, the TraSH-based classifications have subsequently been added to the individual gene descriptions on the TubercuList WWW server (http://genolist.pasteur.fr/TubercuList/). Of course, the coverage of mutant libraries is dependent on the randomness of the insertion element and may be limited by peculiarities in DNA sequence (*e.g*. G+C content). In addition, the transposon insertion site must be determined and there is a risk that false conclusions may be drawn from disruptions that

are non-inactivating or confer polar effects on neighbouring genes. Mutants identified by certain randomised approaches (such as TraSH) cannot be isolated from the pool for further analysis in pure culture, necessitating follow-up by a targeted approach. However, modification of experimental models and application of TraSH in specific mutant backgrounds is expected to yield many more persistence targets as well as other classes of conditionally essential genes, genes of complementary function, and genes in related pathways.

Conditional mutagenesis

By definition, both targeted and random mutagenesis approaches are limited by the fact that the disruption – and therefore abrogation of gene function – is permanent. While this might not always affect the ability of a mutant to compete (or infect), there is the possibility that disruption *ab initio* is unfairly prejudicial, immediately invalidating the observed phenotype. A solution to this problem lies in the development of systems for conditional mutagenesis, perhaps allowing alternative persistence targets to be identified by switching genes off at a particular stage of infection. In addition, the availability of systems for conditional mutagenesis would facilitate the study of essential genes. Inducible expression of anti-sense RNA provides one possible approach, and has been validated to some extent in preliminary studies (Parish & Stoker, 1997). Methods for generating conditional knockouts are critically limited by the urgent need to identify and/or develop tightly regulatable promoters for use in mycobacteria. However, two recent studies have applied tetracycline-regulated expression systems to construct conditional deletion mutants of *M. tuberculosis in vitro* in liquid culture and macrophages (Blokpoel *et al.*, 2005; Ehrt *et al.*, 2005), successfully demonstrating the enormous potential of this technology for the control of mycobacterial gene expression in a variety of infection models.

Comparative genomics

Post-genomic mycobacterial research has been made possible by the completion of the genome sequence of the *M. tuberculosis* strain H37Rv in 1998 (Cole *et al*., 1998); in addition, several other mycobacterial genome-sequencing projects have subsequently been completed or are underway - *M. bovis* (Garnier *et al*., 2003), *M. leprae* (Cole *et al*., 2001), *M. avium*, *M. marinum, M. microti* and *M. smegmatis* (http://www.sanger.ac.uk; http://www.tigr.org), enabling whole-genome comparisons within and between closely related species (Marmiesse *et al*., 2004). The ability to identify patterns of evolutionary inheritance through the analysis of homologous or paralogous gene content, or to isolate those genes which have been inherited/lost together, can be used to infer essentiality of a

host of metabolic and virulence functions, or simply to cluster genes based on participation in the same or related biological pathways. In addition, inter-strain genomic comparisons provide a more dynamic sense of genome evolution than inter-species analyses. The genome sequences of clinical isolates - *M. tuberculosis* strains CDC1551 (Fleischmann *et al*., 2002) and 210 (http://www.tigr.org) - together with the genome sequence of the laboratory strain, H37Rv, are providing important insights into inter-strain variation (Fleischmann *et al*., 2002). Furthermore, the application of array-based technology enables genome-wide comparisons in the absence of whole-genome sequence data; for example, a recent study comparing a large subset of clinical isolates of *M. tuberculosis* with the H37Rv reference strain uncovered significant genome plasticity and revealed genes lost in clinical strains (Tsolaki *et al*., 2004). Of particular relevance is the potential to apply the knowledge gained from genomic studies of clinical isolates to further refine the list of genes comprising the essential virulence gene set, which could be used to restrict targets to those unique mycobacterial genes whose inactivation will not adversely affect normal intestinal flora – a problem that has plagued broad-spectrum antibiotics and is especially relevant in an HIV-prevalent context. In addition, the association of particular strains with host populations has been inferred from an analysis of clinical isolates, and could be used to elucidate the possible influences of genetic structure on TB prevalences, as well as uncovering the reasons for global variations in BCG efficacy (Hirsh *et al*., 2004).

Transcriptional profiling

Finally, a significant reduction in the duration of treatment will require the development of potent sterilising compounds; however, sterilising activity is difficult to measure and requires an appropriate model of persistence. In addition, conventional microbiological methods for resistance determination may be rendered inapplicable by the fact that persistence targets are likely not to be essential to the normal growth of the bacteria. All of the above considerations place an extremely high premium on the rapid development and validation of models for persistence in which sterilising compounds might be analysed. Bacterial mRNA analysis offers a real-time glimpse into the transcriptional response of the organism to the imposed environmental or physiological condition, and so is considered crucial to the identification and validation of metabolic fingerprints or sentinels associated with disease stage. Because RNA-based techniques are not reliant on the ability to detect a mutant phenotype, sentinel genes could be of particular use as surrogate markers for the rapid evaluation of new treatment regimens for persistent or latent TB infection. This

has the further potential of shortening the duration of clinical trials and thus removing a significant disincentive to new drug development. RNA-based analyses are, however, technically demanding and the results exquisitely sensitive to sample handling and preparation (Kendall *et al*., 2004a). Major technical hurdles remain to be overcome, particularly the current inability to extract sufficient high-quality RNA from *M. tuberculosis in situ*. In addition, the use of an appropriate standard in many situations is still to be satisfactorily resolved.

1.4 The BCG vaccine

TB infection and mortality rates are at current levels despite the co-ordinated delivery of an antitubercular vaccine - derived by serial *in vitro* passage of *Mycobacterium bovis* to yield a live attenuated strain, BCG (*b*acille *C*almette-*G*uérin, after its discoverers) safe for use in humans (Behr & Small, 1999) - to approximately 100 million newborns each year and more people over the last seventy years than any other vaccine (Kaufmann, 2000). Neonatal vaccination with BCG reduces the incidence of childhood TB, particularly the more severe forms associated with disseminated disease (Colditz *et al*., 1995); however, despite eliciting a vigorous antigen-specific immune response in most cases, BCG offers limited protective efficacy against adult pulmonary TB (Fine, 1995; Behr & Small, 1997; Brewer, 2000; Kaufmann, 2001). Critically, vaccination with BCG is unable to prevent infection (Fine, 1995) - analogous to the failure of a previous *M. tuberculosis* infection itself to thwart reinfection in high-prevalence areas (van Rie *et al*., 1999; Sonnenberg *et al*., 2001) - and it is estimated that the variable efficacy results in prevention of only a small percentage of all vaccine-preventable TB fatalities (Kaufmann, 2000). Failure of BCG in humans is in direct contrast to the reliable protection generally afforded in animal models (Smith, 1985), and has been attributed to many factors thought likely to impact on the immunogenicity of the vaccine. These relate primarily to differences in the delivered strains or vaccination protocols, and include variations ascribed to continual subculturing with resultant chromosomal deletions and loss of antigenicity, differential viabilities of the attenuated strains, routes of vaccine delivery, intervals between boosts, and effective dose (Mahairas *et al*., 1996, Behr & Small, 1997; Power *et al*., 1998; Behr *et al*., 1999; Gordon *et al*., 1999; Griffin *et al*., 1999; Jungblut *et al*., 1999; Turner *et al*., 2000). In addition, pre-exposure to naturally-occurring, nonpathogenic environmental mycobacteria is thought to antagonise the protective efficacy of the BCG vaccine (Fine, 1995; Brandt *et al*., 2002).

Prospects for a new vaccine

Precedent dictates that the global control and, ultimately, elimination of an infectious pathogen is dependent on the ability to prevent the acquisition of disease by susceptible individuals (Henderson, 1998; Enarson, 2000). The development of an effective prophylactic strategy for TB is therefore crucial. However, persistence of *M. tuberculosis* in the host implies a critical failure to induce maximum immune protection - a phenomenon profoundly realised in the post-primary reactivation and reinfection events that characterise high prevalence areas (van Rie *et al*., 1999; Sonnenberg *et al*., 2001) - and demands that a preventive vaccine induce a response superior to that evoked by *M. tuberculosis* itself (Kaufmann, 2000). Several key factors render the design of a new antitubercular vaccine unusual, however. For example, the prominent role of T cell-mediated immunity in controlling *M. tuberculosis* infection suggests that the recruitment of this subset should be prioritised, especially since the contribution of B cells or antibodies to control of TB remains controversial (Flynn, 2004). In addition, the enormous burden of clinically latent bacteria raises the option of immunotherapeutic vaccination, a prospect given further impetus by the potentially increasing requirement for vaccine therapy of co-HIV- or MDR-TB-infected cases (Blower & Chou, 2004; Cohen & Murray, 2004). Approaches to TB vaccine development are characterised by differences in the delivered immunogen and the response evoked. Subunit vaccines, comprising adjuvenated or expressed antigens, elicit strong $CD4^+$ T cell responses and allow prior selection of proteins of known antigenicity, but are limited by the restricted epitopic subset and T cell repertoires induced. Despite positive outcomes in murine models (Horwitz *et al*., 1995), subunit vaccines have consistently proved inferior to BCG in the more susceptible guinea pig model (Baldwin *et al*., 1998; Orme *et al*., 2001); recently, however, an antigen 85B/ESAT-6 fusion protein conferred protection equivalent to that afforded by BCG (Olsen *et al*., 2004). The fact that subunit vaccines are not compromised by prior exposure to environmental bacteria potentially recommends their use in settings in which BCG is ineffective; in addition, cell-free preparations are considered safer for use in immunocompromised individuals and may render BCG-equivalent levels of protection acceptable for further development.

Whole bacterial vaccines, comprising live-attenuated mycobacterial strains, benefit from an expanded epitopic repertoire and the broad stimulation of T cell populations (Kaufmann, 2000), while recombinant strains expressing additional antigens or immunomodulators could potentiate activity. It is likely that an *M. tuberculosis*-derived vaccine will offer increased immunogenicity over BCG (Behr & Small, 1997), however

serious safety concerns militate against using attenuated *M. tuberculosis* mutants or even recombinant BCG strains against a background of diminished immunocompetence (Talbot *et al*., 1997; Reynes *et al*., 2000). Furthermore, the heterogeneous immune mechanisms controlling infection and maintaining latent TB are incompletely understood (Flynn, 2004). Critically, unlike subunit vaccines, whole bacterial vaccines must exhibit significant improvement over the existing strain in order to be pursued. In addition, the demand that an *M. tuberculosis*-derived vaccine be at least as safe as BCG requires mutants sufficiently attenuated but not impaired in their immunogenicity or ability to confer long-term protection. Several attenuated *M. tuberculosis* strains offer marginal improvements over BCG in animal models (Jackson *et al*., 1999; Hondalus *et al*., 2000; Sambandamurthy *et al.*, 2002, 2005) and, although not all have been applied in protective assays, the characterisation of the *in vivo* phenotypes associated with vaccine candidates has provided significant insight into the host-pathogen interactions mediating the various stages of disease, including the mechanisms employed by *M. tuberculosis* in modulating innate and adaptive immune responses (Hingley-Wilson *et al*., 2003). In particular, the phenotypes associated with attenuated persistence (*per*) or pathology (*pat*) of disease have enabled identification of novel attenuations that might be more safely exploited in immune-deficient backgrounds or for prophylaxis against latent infection. Attempts at enhancing the existing BCG strain tend towards combination vaccines, and have included the construction of recombinant BCG overexpressing specific mycobacterial antigens (Horwitz *et al*., 2000; Horwitz & Harth, 2003), or complemented with the primary BCG attenuation deletion, *RD1* (Pym *et al*., 2003). Recently, *RD1*-complemented recombinant *M. microti* induced superior protective efficacy in the mouse and guinea pig models (Brodin *et al*., 2004). In addition, immunogens from a host of other organisms (Stover *et al*., 1991; Snewin *et al*., 2001), as well as enzymes conferring extrinsic activities (Hess *et al*., 1998), have been added to BCG with mixed efficacies. However, despite promising evidence of the potential of vaccine-mediated immunomodulation to maximise antitubercular activity (Lowrie *et al*., 1999), studies describing BCG strains secreting various cytokines have yielded disappointing results (O'Donnell *et al*., 1994; Kong & Kunimoto, 1995; Murray *et al*., 1996). Finally, the protacted periods inevitably required to assess the protective efficacy of a new pre-exposure vaccine are untenable and demand the identification of quantitative immunological correlates of protection (Kaufmann, 2000). In this respect, therapeutic vaccines constitute an attractive proposition since clinical trials in at-risk populations could proceed without compromising existing BCG vaccination programmes (Stewart *et al*., 2003). However, several vaccines of demonstrated efficacy as pre-exposure vaccines fail to reduce CFU when administered post-exposure (Turner *et*

al., 2000; Kaplan *et al*., 2003). Moreover, the risk that the resultant increase in immune activation might exacerbate pathology at sites of pre-existing infection (Moreira *et al*., 2002) requires that the effects of chronic immunostimulation, particularly the potential to aggravate occult disease or develop Koch toxicity, be minimised (Kaplan *et al*., 2003).

DNA vaccines

The reliance on cell-mediated immunity for the control of mycobacterial infections immediately suggests DNA vaccines as potential antitubercular immunoprophylactics. Bacterial DNA is both a natural adjuvant and mitogen, and DNA vaccines elicit strong humoral and cellular immune responses, making them attractive candidates for control of intracellular pathogens. The contribution of B cells or antibodies to protection against *M. tuberculosis* infection is, however, controversial (Flynn, 2004) and might be less significant than for pathogens with an extracellular stage in their replication cycle (Huygen, 2003). Interaction of an unmethylated CpG-containing DNA motif with receptors on the antigen presenting cell surface induces a T helper type 1 (T_H1) response (Klinman *et al*., 1996; Hemmi *et al*., 2000); in addition, both exogenous and endogenous antigen presentation pathways are stimulated by bacterial DNA, and vaccines can be formulated to target specific cellular compartments for antigen processing (Delogu *et al*., 2000). Nevertheless, the hallmark of DNA vaccines remains the strong $CD8^+$ T cell-mediated response elicited, closely resembling that induced by live pathogens and unlike the $CD4^+$ T cell response induced by vaccines based on protein antigens or killed pathogens (Huygen, 2003). DNA vaccines encoding a range of secreted, surface-associated, and cytosolic mycobacterial antigens have been described (Huygen *et al*., 1996; Tascon *et al*., 1996; Kamath *et al*., 1999; Li *et al*., 1999; Lowrie *et al*., 1999; Tanghe *et al*., 1999; Huygen, 2003). Strong humoral, cytotoxic T lymphocyte, and T_H1-based cellular immune responses have been reported, as well as an expansion in the epitopic repertoire of T cells in comparison with live *M. tuberculosis* or BCG (Denis *et al*., 1998; Tanghe *et al*., 2001; D'Souza *et al*., 2003), perhaps as a result subdominant epitopes (Huygen, 2003). However, none of the vaccines displays better protective efficacy than BCG, although coimmunisation with different DNA vectors in multisubunit vaccines has shown additive effects (Kamath *et al*., 1999; Delogu *et al*., 2002). In addition, vaccination regimens combining both BCG and DNA hold some promise; for example, prime-boost strategies (McShane *et al*., 2002) and immunisation with BCG adjuvenated in CpG oligondeoxynucleotides (Freidag *et al*., 2000). At the very least, the proven efficacy of DNA vaccines in eliciting an immune response (Huygen *et al*., 1996; Tascon *et al*., 1996; Lowrie *et al*., 1999) renders them useful candidates for the screening of potential protective antigens and characterisation of

dominant motifs, particularly in the absence of a referential framework governing the immunogenic potential of *M. tuberculosis* proteins (Kaufmann, 2000; Huygen, 2003).

A common theme that has emerged is the difficulty in ascertaining the reason for a particular DNA vaccine's failure. Of course, immunogenicity of the encoded peptide is primary, and epitopic repertoire is likely to depend on the structural integrity of the translated protein. However, several other variables have been implicated in determining relative protective efficacies (Huygen, 2003), including CpG content - specifically the proportion of immunostimulatory and neutralising motifs (Sato *et al*., 1996; Krieg *et al*., 1998), the physical structure of the plasmid vector (Huygen, 2003), the dose applied and mode of delivery, and the transfection efficiency and amount of antigen actually synthesised (Gurunathan *et al*., 2000). Comparisons between different vaccines are also dependent on the experimental methodology employed, such that the virulence of the *M. tuberculosis* strain, and route and interval between final immunisation and challenge, all impact on the measured outcome (Huygen, 2003). In addition, the relative effectiveness of the immune response evoked depends on the antigen, the genetic background of the host, and the stage of infection – factors of particular relevance for protective *versus* therapeutic vaccination (Huygen, 2003). Finally, post-exposure DNA vaccination offers the prospect of modifying, or augmenting, the immune mechanisms maintaining a persistent *M. tuberculosis* infection in check (Stewart *et al*., 2003; Flynn, 2004). Stimulation with alternative antigens might recruit additional T cell clones, thereby expanding the T cell repertoire or, at the very least, potentiating the existing response. However, the immune components interacting to contain a persistent infection are complex and modulation potentially fraught (Moreira *et al*., 2002; Taylor *et al*., 2003). Perhaps unsurprisingly, trials to date of DNA vaccines for immunotherapy of TB have proved controversial, and difficult to replicate (Lowrie *et al*., 1999; Turner *et al*., 2000; Repique *et al*., 2002). In addition, post-exposure vaccination might result in exacerbated Koch-type pathology (Turner *et al*., 2000; Taylor *et al*., 2003), although this is not a DNA vaccine-specific phenomenon and is likely caused by excess tumour necrosis factor (TNF)-α at the infection foci (Bekker *et al*., 2000; Moreira *et al*., 2002).

1.5 Antituberculous immunity

The tubercle bacillus is transmitted between human hosts almost exclusively through the respiratory tract. Airborne infectious droplet nuclei are inhaled and drawn deep into the lung alveoli where the bacilli lodge in terminal air spaces and are engulfed by resident alveolar macrophages (Rook & Bloom, 1994). Although able to destroy most potential

bacterial pathogens, alveolar macrophages are rendered impotent by several effective counter-mechanisms which enable *M. tuberculosis* to survive even the most potent antimicrobial effector functions (Russell, 2001). Unlike bacteria whose pathogenesis is dependent on the avoidance of phagocytosis, *M. tuberculosis* preferentially targets intracellular vacuoles and utilises multiple surface receptors to enter macrophages (Zimmerli *et al*., 1996; Schorey *et al*., 1997; Ernst, 1998; Aderem & Underhill, 1999), the choice of receptor likely determining the cellular response (Schlesinger *et al*., 1990; Wright & Silverstein, 1983). In addition, tubercle bacilli interfere in the normal maturation of phagosomes (Chua *et al*., 2004). After uptake by alveolar macrophages, intracellular replication of the bacilli ensues virtually unimpeded, culminating in the lysis of the infected cell and the subsequent engulfment of extracellular bacilli. The macrophages and monocytes attracted to the infection foci at this stage are equally incapable of effecting mycobacterial killing, and a second round of intracellular replication inside immature macrophages follows (Grosset, 2003). Bacillaemia enables the migration of bacteria inside dendritic cells (Geijtenbeek *et al.*, 2003; Tailleux *et al.*, 2003) to uninfected areas of the lung as well as extra-pulmonary organs (Monack *et al.*, 2004). However, the onset of acquired cell mediated immunity is triggered by the dissemination of bacilli to extra-pulmonary lymphoid organs, and is followed by a delayed-type hypersensitivity (DTH) reaction (Balasubramanian *et al.*, 1994; Kaufmann, 2001; Grosset, 2003). In most cases, the cell-mediated immune response effectively controls the infection and subsequent dissemination; of all infected individuals approximately 5-10% develop clinical disease, either within one or two years of the initial infection, or later, as a result of re-infection or reactivation of a latent population of viable bacilli persisting after the initial containment (Bloom & Murray, 1992). Therefore, although initial parasitisation of the human host is successful, primary infection is almost invariably asymptomatic. Critically, post-primary disease, comprising subsequent reactivation or reinfection, results from the failure of the immune system to exercise sterilising immunity. Surviving intra-phagosomal bacteria are believed to enter a state analagous to non-replicating persistence (NRP) *in vitro* until waning host immunity leads to reactivation from latency and the onset of disease (Wayne & Sohaskey, 2001). Fundamental questions therefore pertain to the ability of a population of viable *M. tuberculosis* bacilli to survive - and perhaps monitor (Boshoff & Barry, 2005) - the immune response, and several counterimmune and immunomodulatory mechanisms have been proposed. These include the evasion of active immune surveillance within tubercles; the localised subversion of the immune response, for example through interference in interferon (IFN)-γ signalling (Ting *et al*., 1999; Noss *et al*., 2000; Kincaid & Ernst, 2003) or down-regulation of immune modulators such as interleukin (IL)-12

(Hickman *et al*., 2002; Nau *et al*., 2002); the possible development of phenotypic tolerance of immune-mediated effectors as a result of stress regulon induction or metabolic alteration (Stewart *et al*., 2003); and the activity of detoxification or damage repair mechanisms. In addition, occupation of non-professional phagocytes lacking MHC II antigen presentation has been suggested as another persistence mechanism (Hernandez-Pando *et al*., 2000). Alternatively, it has been suggested that the stable maintenance of a low-level persistent infection could result from the auto-modulation of the immune response by the host to avoid pathological damage in excess of the risk posed by the residual population, while simultaneously maintaining immune memory (Stewart *et al*., 2003).

Cell-mediated immunity

Protective acquired immunity to *M. tuberculosis* is dominated by a T_H1 immune response mediated by $CD4^+$ T cells and accessory $CD8^+$, $\gamma\delta$ and CD1-restricted $\alpha\beta$ T cells, and characterised by the T_H1 cytokines IFN-γ and IL-12 (Murray, 1999; Flynn & Chan, 2001a; Kaufmann, 2001). Phagocytosis of *M. tuberculosis* bacilli by macrophages and dendritic cells induces a T_H1 profile (Henderson *et al*., 1997; Ladel *et al*., 1997b): $CD4^+$ T cells, stimulated by IL-12 and able to interact with peptides presented by Class II major histocompatibility (MHC) molecules, release IFN-γ which in turn activates macrophages (Flynn & Chan, 2001a). IFN-γ is a primary component of antimycobacterial immunity, mediating the upregulation of a large number of macrophage pathways (Ehrt *et al*., 2001), including the production by phagosome NADPH oxidase (phox) and inducible nitric oxide synthase (iNOS) of reactive oxygen (ROI) and nitrogen (RNI) intermediates, respectively (Nathan & Shiloh, 2000). Significantly, recent evidence suggests that the immune status of the macrophage (type-1 *versus* type-2) is critical to bacillary persistence, and that IFN-γ production might dependent on the production of IL-23 rather than IL-12 (Verreck *et al.*, 2004). Release of IFN-γ also induces expression of a vacuolar trafficking component required for maturation of *M. tuberculosis*-containing phagosomes (MacMicking *et al*., 2003). Stark evidence of the importance of $CD4^+$ T cells in protective immunity is provided by the pathogenic synergy between HIV and TB - it is estimated that HIV-positive PPD-positive patients have an 8-10% annual risk of developing active tuberculosis as opposed to a 10% lifetime risk in HIV-negative PPD-positive individuals (Selwyn *et al.*, 1989; Chaisson & Benson, 1995), while *in situ* analysis of lung lesions from incurable TB patients reveals a failure to recruit $CD4^+$ and $CD8^+$ T cells to cavity surfaces resulting in deficient macrophage activation and rampant bacillary replication (Kaplan *et al*., 2003).

The mycobacterial cell wall in immune modulation

A significant proportion of the *M. tuberculosis* genome is dedicated to putative lipid biosynthetic functions (Cole *et al*., 1998). Many cell wall-associated molecules have potent biological activity and are likely to be prominent in modulating the innate and adaptive immune responses (Brennan, 2003). For example, lipoarabinomannan inhibits IFN-γ-mediated macrophage activation *in vitro* (Sibley *et al*., 1988, 1990) and can induce production of TNF-α by macrophages (Chatterjee *et al*., 1992), while the interaction of mannose-capped lipoarabinomannan with dendritic cell receptors reduces antimycobacterial activity and stimulates the release of anti-inflammatory cytokines (Geijtenbeek *et al*., 2003; Tailleux *et al*., 2003). Recently, *M. tuberculosis* was shown to modulate apoptotic pathways in both macrophages and polymorphonuclear neutrophils (PMNs) – preventing apoptosis in infected macrophages (Sly *et al*., 2003), but promoting *in vitro* apoptosis of PMNs from patients with active TB (Alemán *et al*., 2004). The observation that virulent *M. tuberculosis* strains elicit different innate immune responses associated with differential infection outcomes (Manca *et al*., 1999b, 2001, 2004) further suggests that the signalling network induced by the interaction of host innate immune components with bacterial surface motifs might direct the subsequent course of infection (Stewart *et al*., 2003).

Immune-mediated tissue damage

A feature of TB is that the ("inappropriate") host immune response, rather than the inherent toxicity of the bacillus, defines the disease (Condos *et al*., 1998; Schluger & Rom, 1998; Dannenberg & Collins, 2001; Glickman & Jacobs, 2001), prompting the proposal that the organism's most virulent property might be the ability to induce the destructive lung pathology that likely aids transmission (Flynn, 2004). Consistent with the potentially active dynamic established between host immunity and *M. tuberculosis* is the demonstration that the efficacy of the protective response is reduced if challenged with dead as opposed to live bacilli (Bloch & Segal, 1955; Orme, 1988), and that antigenic composition is a function of growth phase (Shi *et al.*, 2004). Compelling evidence of the importance of host immune-mediated tissue damage in progression of pulmonary TB is provided by the pathology (*pat*) class of *in vivo* mutants (Hingley-Wilson *et al*., 2003), deficient in their ability to cause characteristic lung pathology despite exhibiting no growth defect in the presence of functional innate or acquired immune responses (Kaushal *et al*., 2002; Steyn *et al*., 2002). In particular, the observation that attenuation of virulence in *pat* mutants correlates with diminished immuno- and histopathology indicates that the

adaptive immune response induced by undefined, wild-type *M. tuberculosis* virulence mechanisms is the cause of typical tissue pathology and, ultimately, death. Further investigation of the mechanisms mediating the reduced pathology in these mutants, especially the potential induction of differential immunomodulatory profiles, is required to understand the immunopathological responses that characterise and determine disease progression (Hingley-Wilson *et al*., 2003).

1.6 Probing the *in vivo* environment

Recent advances in RNA-based technologies have shifted the focus of mycobacterial genetics to the analysis of gene expression profiles (Schnappinger *et al*., 2003). An unresolved (Kendall *et al.*, 2004b), but fundamental assumption underlying the use of expression analysis to probe the *in vivo* environment is that the differential expression of a gene provides direct evidence of its utility in that environment; that is, the encoded product *is necessary* for adaptation to the given condition. Based on this assumption, sentinel genes can be used as surrogate markers to probe prevailing physiological or environmental conditions. Such markers have already been employed to provide some measure of the appropriateness of particular model systems (Fenhalls *et al*., 2002b) and to investigate the *M. tuberculosis* response to environments encountered *in vitro* (Betts *et al*., 2002; Fisher *et al*., 2002; Ohno *et al*., 2003; Schnappinger *et al*., 2003; Voskuil *et al*., 2003, 2004), in mice (Shi *et al*., 2003; Timm *et al*., 2003) and in humans (Timm *et al*., 2003) using samples containing relatively large numbers of bacilli. Realisation of the full potential of mRNA analysis tools is completely reliant on the ability to isolate RNA from bacteria grown *in vivo*; however, current studies are critically limited by a technical inability to extract mycobacterial RNA of sufficient quantity and quality from infected lesions *in vivo* to enable whole-genome expression profiling by microarray although analysis by RT-PCR-based methods has yielded some insight (Timm *et al*., 2003). In a novel attempt to partially overcome the stated technical limitations, bacterial RNA extracted from isogenic mice that had been simultaneously infected was recently pooled for investigation (Talaat *et al*., 2004); however, *in situ* analysis of *M. tuberculosis* RNA transcripts in human lung tissue has demonstrated that each granuloma comprises a compact microenvironment characterised by a unique cytokine profile (Fenhalls *et al*. 2000; 2002a), therefore limiting the validity of pooling tissue samples from even a single individual. Clearly, a technological leap in RNA isolation methodologies and detection sensitivity will be required before expression profiling of samples obtained from the paucibacillary lesions of humans with latent TB infection could be considered, even at the single-gene level.

1.7 Tuberculous granulomas

In the presence of a functional adaptive immune system, TB bacilli are sequestered in granulomas comprising differentiated macrophages and surrounded by lymphocytes and other immune cells maintained in complex structures by extracellular matrix components (Flynn & Chan, 2001a; Cosma *et al*., 2003). Containment of bacilli within granulomas is essential for restriction of disease, limiting the spread of the infection and localising immune cell activity. A defective immune response, for example in HIV infection, is characterised by impaired granuloma integrity and more disseminated and lethal forms of TB (Lawn *et al*., 2002; Frieden *et al*., 2003). Inside granulomas, bacilli persist within macrophages or are found extracellularly in the central necrotic milieu typical of caseation (Grosset, 2003). In approximately 90% of immunocompetent individuals, cell mediated immunity results in the death of the bacilli within organised necrotising caseous lesions that are sealed off from surrounding tissue by fibrotic capsules and may eventually calcify and even ossify (Styblo *et al*., 1969; Sutherland, 1976; Chan & Kaufmann, 1994; Enarson & Rouillon, 1994). In some cases, no modification of the caseous centre occurs, and bacilli are thought to persist extracellularly within undefined lesions in states of low metabolic activity (Canetti, 1955; Rees & Hart, 1961; Hu *et al*., 2000; Bouley *et al.*, 2001; Grosset, 2003; Muñoz-Elías *et al.*, 2005). Alternatively, in the remaining minority of cases (between 5-10% of infected individuals), the caseum softens, and the release of bacilli into the oxygen-rich lung results in the progression of infection to pulmonary cavitation and clinical disease (Long, 1935; Canetti, 1955; Enarson & Rouillon, 1994; Bloom & Murray, 1992). Whatever the eventual outcome, at some time post initial infection bacilli in immunocompetent individuals are enclosed within granulomas likely characterised by hypoxia, low pH, nutrient deprivation, and awash in inhibitory organic acids (Loebel *et al*., 1933; Nyka, 1967, 1974; Schnappinger *et al*., 2003; Boshoff & Barry, 2005). In addition, through the course of the infection, the tubercle bacilli probably endure continual exposure to ROI and RNI produced by polymorphonuclear leukocytes and macrophages recruited and activated by the expanding T cell population (Nathan, 1992; Nicholson *et al*., 1996; MacMicking *et al*., 1997b; Lau *et al*., 1998; Wang *et al*., 1998; Chan & Flynn, 1999; Nathan & Shiloh, 2000).

While some resistance to antimicrobial defences is provided by the thick mycobacterial cell wall, survival in the hostile host environment – and therefore persistence of *M. tuberculosis* in the human population – is thought to depend on a number of mechanisms that have evolved to enable the TB bacillus to evade host immune detection, or subvert or detoxify antimicrobial defences. A possible codicil is provided by recent evidence

describing the inability of established granulomas to eradicate naïve superinfecting mycobacteria despite the specific, monocyte-directed trafficking of these bacilli to existing lesions (Cosma *et al*., 2004). This surprising finding suggests that, rather than the innate ability of *M. tuberculosis* to avoid host immune responses or adapt gradually to evolving host defence mechanisms, persistence and reinfection might be predicated on a fundamental inability of the host immune response to eradicate *M. tuberculosis* infection (Cosma *et al*., 2004). In particular, the observation that, despite a robust $CD4^+$ T cell response, all intracellular bacteria are not eliminated seems to imply a critical failure of that T cell subset to recognise or activate infected macrophages (Flynn, 2004), analogous to the decreased macrophage activation as a result of deficient $CD4^+$ and $CD8^+$ T cell recruitment to the cavity surface that has been implicated in uncontrolled human TB (Kaplan *et al*., 2003). Nevertheless, the failure of the host to exert a sterilising immunity requires the capacity of the bacilli to endure, in some cases for years or even decades. An apparently static balance is established between the host immune system and the pathogen's resistance (Rees & Hart, 1961; Muñoz-Elías *et al.*, 2005), the breakdown of which is evidenced in the increased incidence of reactivation disease in the face of HIV infection (Selwyn *et al*., 1989), immunosuppressive medication (Keane *et al*., 2001), malnutrition, alcohol and drug abuse, and old-age (Stead, 1967). Several possible bacillary survival tactics and metabolic adaptations are considered briefly below, with some emphasis on the types of DNA damage likely to be incurred by the bacilli during their occupation of granulomas, the possible mechanisms that have evolved to minimise or repair that damage, and the potential physiological and mutagenic consequences, particularly with respect to the emergence of drug resistant clinical isolates.

Phagosome maturation arrest

Persistence of *M. tuberculosis* hinges on its ability to survive (and replicate) in pathogen-friendly phagosomes in unactivated macrophages for extended periods. After phagocytic uptake, *M. tuberculosis* interrupts the normal maturation pathway (Clemens & Horwitz, 1995; Hasan *et al*., 1997; Via *et al*., 1997; Fratti *et al*., 2001; Fratti *et al*., 2003b; Chua *et al*., 2004), preventing phagosome-lysosome fusion (Armstrong & Hart, 1971; Mwandumba *et al*., 2004), and ensuring a *M. tuberculosis*-infected phagosome characterised by limited acidification and a deficiency of mature lysosomal hydrolases (Russell *et al*., 2002). Phagosome maturation arrest is a complex process, mediated primarily by the interference of mycobacterial lipid products in intracellular trafficking events (Chua *et al*., 2004). Despite the resultant incomplete acquisition of lysosomal compartments, the mycobacterial phagosome is not a static organelle (Clemens & Horwitz, 1996; Sturgill-

Koszycki *et al*., 1996), and recent studies have emphasised the importance of bioactive lipids in the mycobacterial cell envelope in continually modulating host cell membrane sorting and organelle biogenesis pathways (Fratti *et al*., 2001, 2003a, 2003b; Vergne *et al*., 2003; Chua *et al*., 2004). Importantly, the roles of these *M. tuberculosis*-derived glycophosholipids can be reconciled with other identified arrest mechanisms (Fratti *et al*., 2003a; Vergne *et al*., 2003). Of course, additional strategies might also be implicated and the range of genotypes observed in a recent screen of mutants impaired in their ability to cause phagosome maturation arrest suggests that the process is likely to be multifactorial (Pethe *et al*., 2004). Numerous studies have reported different *in vitro* susceptibilities of *M. tuberculosis* strains to ROI (Jackett *et al*., 1978; Laochumroonvorapong *et al*., 1997; Manca *et al*., 1999a; Firmani & Riley, 2002) and RNI (Friedman *et al*., 1997; O'Brien *et al*., 1994; Rhoades & Orme, 1997; Yu *et al*., 1999; Firmani & Riley, 2002). However, the observation that the virulence of different clinical *M. tuberculosis* isolates does not correlate with an ability to modulate the amount of NO generated within the macrophage, or with susceptibility to RNI *in vitro* or in tissue culture models (Rhoades & Orme, 1997), or even *in vivo* (Ordway *et al*., 1995), strongly suggests that virulence is asscociated with more effective interference in maturation pathways (Deretic & Fratti, 1999; Zahrt & Deretic, 2002). In addition, evidence of the ability of *M. marinum* to persist in phagolysosomes within granulomas (Bouley *et al.*, 2001) indicates that mycobacteria might have evolved at least two adaptive strategies for intramacrophage survival.

1.8 Intracellular survival and metabolism

The development of intervention strategies to reduce progression from latent to active disease in the estimated 2 billion individuals already infected is key to global control of TB. Novel therapeutics will, however, require an understanding of the mechanisms employed by the tubercle bacillus to survive for extended periods in immunologically educated hosts. Currently, there is a critical lack of a tractable, inexpensive animal model which replicates all aspects of latent disease (Flynn & Chan, 2001b; Boshoff & Barry, 2005). The murine model, in various modified forms (McCune & Tompsett, 1956; McCune *et al.*, 1956; de Wit *et al.*, 1995) is widely employed primarily because of its economy - relative to the more expensive, though potentially more relevant guinea pig, rabbit, or non-human primate models (Boshoff & Barry, 2005) - as well as the commercial availabilty of genetically and immunologically well-defined mice. However, while similarly reliant on a T_H1-type response for initial control of *M. tuberculosis* infection, the progression of the disease in mice *versus* humans is characterised by fundamental differences, particularly with respect to bacillary dissemination, granuloma formation and histopathology, and viable bacillary

counts during chronic persistence (McMurray *et al.*, 1996; Dannenberg & Collins, 2001; Duncan & Barry, 2004). In addition, while NO and RNI are the primary effectors mediating immune control during persistence in the mouse model (Chan *et al.*, 1995; MacMicking *et al.*, 1997a), *in vitro* attempts to stimulate iNOS expression in human mononuclear phagocytes have proved inconclusive (Shiloh & Nathan, 2000) although immunohistochemical studies have readily detected iNOS expression in inflamed or infected sites, as well as the increased exhalation of NO by tuberculosis patients (Nicholson *et al*., 1996; Wang *et al*., 1998; Weinberg, 1998). Oxygen availability within murine granulomas, too, might differ significantly from human TB, consistent with the disposability of *dosR* for virulence in a mouse model (Parish *et al.*, 2003a) and in contrast to observations in the guinea pig model (Malhotra *et al.*, 2004).

Despite the questionable relevance of existing animal and tissue culture models of infection to human TB (Graham & Clark-Curtiss, 1999; McKinney *et al*., 2001; Boshoff & Barry, 2005), wild-type and gene-deleted mutant mycobacterial strains have been profitably applied as bioprobes in several such models and *in vitro* to refine our understanding of the host environment, particularly as exists within the mycobacterial phagosome. Evidence suggests that persisting bacilli are metabolically active (Sever & Youmans, 1957; Hu *et al*., 2000; Bouley *et al.*, 2001) and, *in vitro*, adapt sufficiently to long-term non-replication to re-initiate cellular division (Hampshire *et al*., 2004). Re-activation and chromosomal division after long-term adaptation to starvation is suggested by the upregulation of genes involved in DNA repair and replication, as well as cell wall elongation (Hampshire *et al*., 2004). Several stresses have been applied to mimic the stimuli for entry into a state of NRP, including various models of nutrient starvation (Betts *et al*., 2002; Dahl *et al*., 2003), oxygen limitation (Wayne & Hayes, 1996; Boon & Dick, 2002), stationary-phase adaptation (Hampshire *et al*., 2004; Voskuil *et al.*, 2004) and exposure to low-dose nitric oxide (Voskuil *et al*., 2003). Analysis of gene expression in response to each of these conditions, as well as phenotypic and physiological characterisation, has validated the relevance of each to *in vivo* stress; for example, starvation in distilled water or PBS yields bacilli with altered morphology and acid-fast staining properties similar to *M. tuberculosis* isolated from lung lesions (Nyka, 1974), while oxygen-starved, stationary-phase cells are phenotypically antibiotic tolerant (Hu *et al*., 1998; Wayne & Sohaskey, 2001). Conversely, bacilli harvested from chronically infected mice exhibit enhanced thermal resistance characteristic of stationary-phase adaptation (Wallace, 1961). Selective *in situ* analysis of RNA expression levels in murine and human lung tissue has corroborated some of the *in vitro* models (Fenhalls *et al*., 2002b; Shi *et al*.,

2003; Timm *et al*., 2003; Talaat *et al*., 2004). Critically, the differential gene expression of selected *M. tuberculosis* genes in lung tissue sections of human TB patients has shown that *M. tuberculosis* responds to specific and variable microenvironments *in vivo* by adopting a variety of metabolic states which are probably defined primarily by host immune status and stage of infection (Fenhalls *et al*., 2002b; Kaplan *et al*., 2003; Timm *et al*., 2003).

1.9 Gene regulatory pathways

In an effort to identify those aspects most relevant to survival and adaptation of *M. tuberculosis* in the human host, *in vitro* models have tended to focus on the altered metabolism induced in response to hypoxia, the mechanisms employed to overcome nutrient starvation, and the ability of the organism to sustain and respond to structural and DNA damage mediated by antibacterial immune effectors such as toxic ROI and RNI. Comparison of the expression profiles characterising the response of *M. tuberculosis* to each of these challenges has revealed striking similarities between the transcriptomes induced by hypoxia, sublethal doses of nitric oxide, and macrophage infection (Sherman *et al*., 2001; Ohno *et al*., 2003; Schnappinger *et al*., 2003; Voskuil *et al*., 2003); in contrast, a distinct transcriptional profile is elicited by starvation or the stringent response (Betts *et al*., 2002; Dahl *et al*., 2003) indicating that both adaptation to hypoxia and starvation are relevant to survival *in vivo*, but that the relative importance of each is likely to depend on the stage of infection.

The DosR dormancy regulon

Accumulating evidence points to a prominent role for the DosR-DosS/DosT two-component response regulator (DosR regulon) in mediating the response of *M. tuberculosis* to host immune effectors, particularly NO and hypoxia, and adaptation to persistence in the human host (Sherman *et al*., 2001; Ohno *et al*., 2003; Park *et al*., 2003; Schnappinger *et al*., 2003; Shi *et al*., 2003; Voskuil *et al*., 2003; Karakousis *et al*., 2004; Roberts *et al.*, 2004). The DosR dormancy regulon comprises over 40 genes induced in response to non-toxic NO concentrations and hypoxia (Sherman *et al*., 2001; Ohno *et al*., 2003; Park *et al*., 2003; Voskuil *et al*., 2003; Kendall *et al*., 2004a; Roberts *et al*., 2004) and is required for long-term survival of mycobacteria *in vitro* in a low oxygen-induced dormant state (Boon & Dick, 2002; Voskuil *et al*., 2003). Included in the regulon are genes associated with anaerobic metabolism and stabilisation of cellular components. In addition, six dormancy genes contain UspA motifs; in *E. coli*, *uspA* expression is induced in response to DNA damage and during nutrient starvation (Nyström & Neidhardt, 1992,

1994; Ferianc *et al*., 1998; Diez *et al*., 2000; Kvint *et al*., 2000; Gustavsson *et al*., 2002), and disruption of *uspA* is associated with impaired survival under stress-induced growth arrest (Nyström & Neidhardt, 1993, 1994). It has been suggested (Voskuil *et al*., 2003) that the inclusion of USPs in the DosR regulon, together with the class II ribonucleotide reductase (*nrdZ*), is necessary for the synthesis and preservation of the duplicated chromosome characteristic of dormancy *in vitro* (Wayne, 1977). Although both hypoxia and low-dose NO cause *M. tuberculosis* growth arrest *in vitro* (Wayne & Hayes, 1996; Voskuil *et al*., 2003), evidence indicates that DosR-regulated dormancy *in vivo* is IFN-γ-, and therefore NO-, dependent (Schnappinger *et al*., 2003; Shi *et al*., 2003; Karakousis *et al*., 2004). An attractive hypothesis maintains that the host-mediated sequestering of tubercle bacilli in encapsulated avascular granulomas induces DosR-mediated bacteriostasis through a shift in the balance between oxygen tension and NO concentration (Park *et al*., 2003; Voskuil *et al*., 2003). Consistent with this model, increased transcription of DosR-regulon genes within wild-type murine lungs coincides with onset of immunity and bacterial growth arrest, and is delayed in $IFN^{-/-}$ mice; in addition, *in vivo* gene expression in this model implies the NO-induced transition of *M. tuberculosis* from active growth to non-replicating persistence (Shi *et al*., 2003). Moreover, the hypervirulence of a *dosR* mutant in SCID mice implicates T cell-mediated immunity in inducing DosR regulatory mechanisms (Parish *et al*., 2003a). Of course, the possibility remains that the dominant role of NO in inducing persistence might be unique to the mouse model (Boshoff & Barry, 2005); although it has been suggested that the inferred induction of NO synthases (Nicholson *et al*., 1996; Wang *et al*., 1998; Weinberg, 1998; Choi *et al*., 2002) and the presence of anti-α-crystallin antibodies in the majority of TB cases (Lee *et al*., 1992) likely indicate that NO is active in tuberculous granulomas and might induce a similar response (Voskuil *et al*., 2003), the relevance of the DosR regulon to human TB is currently circumstantial. As has been pointed out (Boshoff & Barry, 2005), induction of DosR genes on its own is not sufficient to enable adaptation to long-term NRP *in vitro* since survival is determined by the duration of the transition through the different stages of metabolic shutdown (Wayne & Sohaskey, 2001). Furthermore, transcriptional profiling has identified components that differentiate the response to oxygen limitation from that induced by exposure to low-dose NO (Boshoff *et al.*, 2004), suggesting that disruptions to respiratory pathways induce specific regulons (Boshoff & Barry, 2005). Evidence of the upregulation of DosR regulon genes in response to *in vitro* starvation (Hampshire *et al*., 2004) and other stresses (Kendall *et al*., 2004a) is consistent with the presence of independent signalling pathways, and raises the intriguing possibility that dormancy genes might be induced by other stimuli *in vivo*.

Other two-component regulatory systems

Persistence of *M. tuberculosis* in the human population requires an ability to sense and adapt to varying, mainly hostile, conditions. Eubacterial responses to environmental stimuli are mediated by two-component regulatory systems (2CRs), comprising a membrane-bound "sensor kinase" and a "response regulator", usually a transcription factor (Stock *et al*., 2000). In response to a particular stimulus, the sensor protein autophosphorylates and transfers the phosphate moiety to the effector, which then binds to a specific promoter sequence thereby regulating the co-ordinated transcription of a particular gene set; in some cases, interaction with additional 2CRs initiates a cascade of signalling (Hoch, 2000). Eleven paired 2CRs have been identified in the *M. tuberculosis* genome, as well as four isolated regulators and three isolated sensors (Cole *et al*., 1998). The importance of 2CRs to the ability of *M. tuberculosis* to sense and respond to different stimuli within the host is manifest in the involvement of signal transducers in both virulence, for example PhoP (Perez *et al*., 2001), and persistence, for example MprA (Zahrt & Deretic, 2001). Several studies have investigated the effects of 2CR abrogation on *M. tuberculosis* growth and virulence *in vivo* (Perez et al., 2001; Zahrt & Deretic, 2001; Ewann et al., 2002; Parish *et al*., 2003a , 2003b); however, with the exception of DosR (Ohno *et al*., 2003; Park *et al*., 2003; Voskuil *et al*., 2003; Kendall *et al*., 2004a; Roberts *et al*., 2004) and, to a lesser extent, MprA (He & Zahrt, 2005), the signals mediating 2CR induction, the regulons transcribed, and their mechanisms of action remain poorly understood. Nevertheless, the demonstration that mutations in different 2CRs are associated with both attenuation and hypervirulence is suggestive of the relevance of these systems to infection outcomes and, together with the demonstrated absence of 2CR homologues in higher organisms, recommends them as potential therapeutic targets (Parish *et al*., 2003a). Significantly, several virulence factors are included in 2CR-regulons (Dziejman & Mekalanos, 1995), while the *mprA*-encoded persistence regulator (Zahrt & Deretic, 2001) is located on a putative pathogenicity island absent in saprophytic mycobacteria (Talaat *et al.*, 2004). The hypervirulence associated with certain 2CR deletions might, therefore, indicate a suppressive regulatory function during initial stages of infection, consistent with immune modulation and persistence of the bacilli.

The stringent response

RelA is the central regulator of the stringent response in most bacteria, catalysing the hyperphosphorylation of GTP to (p)ppGpp during amino acid and carbon source starvation (Cashel *et al*., 1996). Binding of the (p)ppGpp alarmone to the RNA polymerase

β subunit inhibits transcription of translation machinery components, stimulates amino acid biosynthesis and transport operons, and decreases transcription rates (Cashel *et al*., 1996; Barker *et al*., 2001; Chang *et al*., 2002; Wagner, 2002; Artsimovitch *et al*., 2004). In addition, (p)ppGpp affects the global transcriptional response to changing environmental conditions by mediating association of alternative σ factors with RNA polymerase (Jishage *et al*., 2002; Laurie *et al*., 2003). Accumulation of (p)ppGpp has been implicated in phenotypic tolerance of antibiotics (Tuomanen, 1986; Greenway & England, 1999; Keren *et al*., 2004), and in enhanced UV survival (McGlynn & Lloyd, 2000). Moreover, (p)ppGpp-mediated inhibition of transcription in wild-type *E. coli* triggers toxin/antitoxin-modulated programmed cell death (Aizenman, *et al*., 1996); induction of suicide modules in response to antibiotic treatment, DNA damage, oxidative and environmental stresses, is RelA-dependent (Sat *et al*., 2001, 2003; Hazan *et al*., 2004). In *M. tuberculosis*, the bifunctional Rel_{Mtb} (Avarbock *et al*., 1999) has been implicated in adaptation to nutrient stress *in vitro* (Betts *et al*., 2002; Hampshire *et al*., 2004), and inactivation of Rel_{Mtb} is associated with impaired long-term survival under starvation (Primm *et al*., 2000). Murine infection with a rel_{Mtb} mutant reveals a *per* phenotype, characterised by an inability to establish long-term infection and reduced histopathology (Dahl *et al*., 2003). Expression of a number of persistence and virulence factors appears to be affected by (p)ppGpp levels, including members of the antigen 85 complex, the *M. tuberculosis* complex-associated immunogen *esat6*, the *lpqH*-encoded 19 kDa lipoprotein (Dahl *et al*., 2003), mammalian cell entry (*mce*) proteins and several polyketide synthases (Hampshire *et al*., 2004). In addition, (p)ppGpp appears to interact with the ECF σ factor, σ^D, (Betts *et al*., 2002; Dahl *et al*., 2003), and evidence suggests that a subset of stringent response genes is σ^D-regulated (Raman *et al*., 2004). Finally, rel_{Mtb} is induced *in vitro* in response to hypoxia (Sherman *et al*., 2001), and a murine model of dormancy recently demonstrated that extracellular persistence of *M. tuberculosis* is Rel_{Mtb}-dependent (Karakousis *et al*., 2004). *In vitro*, microanaerobic stress elicits the regulated, sequential metabolic shut-down of DNA, most RNA, and protein synthesis by *M. tuberculosis* (Wayne, 1977; Wayne & Hayes, 1996; Hu *et al*., 1998); however, when conditions favour exit from dormancy, *M. tuberculosis* bacilli re-initiate synchronised protein and DNA synthesis almost immediately (Wayne, 1977; Hu *et al*., 1998). It has, therefore, been suggested that (p)ppGpp might function to effect *M. tuberculosis* entry into, and exit from, the nonreplicating state (Mizrahi *et al*., 2005).

Mycobacterial σ factors

The reversible interaction of σ factors with the prokaryotic RNA polymerase core enables modulation of gene expression profiles according to dynamic variations in the effective σ factor population (Manganelli *et al*., 2004b). The identification in *M. tuberculosis* of approximately 190 defined and putative transcriptional regulators, including σ factors (Cole *et al*., 1998; Fleischmann *et al*., 2002), is indicative of the complexity of stress response regulation, quite often involving the interplay of functionally overlapping and redundant regulons (Manganelli *et al*., 2004b). Evidence suggests that a hierarchy characterises σ factor interaction, with σ^H at the summit, although the recent identification of a putative promoter motif, upstream of *sigH*, regulating the RecA/LexA-independent DNA damage response pathway (Gamulin *et al*., 2004) might indicate an additional level of control. The prominent role of σ factors in mediating physiological adaptation, particularly in the face of hostile immune responses, is evident in the dramatic and varying phenotypes of σ factor mutants *in vivo* (Chen *et al*., 2000; Kaushal *et al.*, 2002; Steyn *et al*., 2002; Manganelli *et al*., 2004a; Raman *et al*., 2004). Moreover, six of the seven targeted or identified σ factor disruptions have revealed a role in virulence (*sigA*, *-C*, *-D*, *-E*, *-F*, *-H*, and not *sigB*; Manganelli *et al*., 2004b), and there is evidence that the same σ factor might mediate separate responses to distinct stages or sites of infection (Chan *et al*., 2002). A diverse range of *in vitro* stimuli induce σ factor responses, including starvation (Betts *et al*., 2002; Dahl *et al*., 2003; Hampshire *et al*., 2004), oxidative and environmental stresses (DeMaio *et al*., 1996; Gomez *et al.*, 1997; Hu & Coates, 1999, 2001; Manabe *et al*., 1999; Manganelli *et al*., 1999, 2004b; Michele *et al*., 1999) and adaptation to intraphagosomal survival (Graham & Clark-Curtiss, 1999; Manganelli *et al*., 2001; Schnappinger *et al*., 2003); it is expected that the advent of microarray-based expression profiling should facilitate identification and more precise characterisation of the various σ factor regulons and the anti-σ factors and anti-anti-σ factors providing post-translational regulation, as well as enabling the definition of specific consensus binding motifs (Raman *et al*., 2001, 2004; Kaushal *et al*., 2002; Manganelli *et al*., 2002, 2004b).

1.10 Emergence of drug resistance

Extended antibiotic chemotherapy is associated with evolution of multiple-antibiotic resistance in other bacteria (Baquero & Blázquez, 1997; Lipsitch *et al*., 2000; Livermore, 2000), a phenomenon likely to be replicated in *M. tuberculosis* infection. The long duration of the DOTS regimen, together with the toxic side effects of the front-line drugs and the temptation to cease therapy as symptoms subside, leads to patient non-compliance

(Mitchison, 1992); furthermore, recent epidemiological evidence of the increasing emergence of mono- and multidrug-resistant strains suggests that effective (or actual) monotherapy is common (WHO, 2004). In addition, several characteristics of the *in vivo* lifestyle render *M. tuberculosis* a difficult target for conventional antimicrobial chemotherapeutics (Pablos-Mendez, 2000). Typical of pathogenic mycobacteria, the tubercle bacillus has a long generation time and, in response to environmental conditions, is able to establish latent asymptomatic infection mediated by a strictly regulated shift to an alternative, decreased metabolic state (Wayne, 1994; McKinney *et al*., 2000; Schnappinger *et al*., 2003; Voskuil, 2004). Metabolic adaptation may result in specific antibiotics being selectively active against discrete sub-populations (Global Alliance for TB Drug Development, 2001); furthermore, there is increasing evidence that infecting populations occupy diverse microenvironments within the host (Canetti, 1955; Canetti *et al*., 1968; Jindani *et al*., 1980; Mitchison, 1985; Fenhalls *et al*., 2002b; Kaplan *et al*., 2003; Boshoff & Barry, 2005), many of which are likely to be recalcitrant to antibiotic penetration or refractory to activity (Iseman & Madsen, 1991; Elliott *et al*., 1995). Although unproven for *M. tuberculosis*, there is also the possibility that adaptation to nutrient stress via the stringent response might result in phenotypic tolerance of antimicrobials (Tuomanen, 1986; Greenway & England, 1999). Non-mutant, specialised survivor cells ("persisters"; Bigger, 1944) able to tolerate microbicidal antibiotics have been identified in biofilm and stationary-phase populations of several other bacterial pathogens and are thought to mimic *in vivo* persistence (Brooun *et al*., 2000; Lewis, 2001; Spoering & Lewis, 2001; Keren *et al*., 2004), perhaps mediated by the RelA-associated activity of toxin/antitoxin (TA)-modules (Lewis, 2000; Pedersen *et al*., 2002; Christensen *et al*., 2003). Although the function and regulation of the putative mycobacterial TA genes is still to be elucidated, resistance to several frontline antimicrobial compounds is observed during long-term *in vitro* adaptation and correlates with resistance in bacilli recovered from tuberculous lesions *in vivo* (Wayne, 1994; Wayne & Sramek, 1994; Herbert *et al*., 1996; Wallis *et al*., 1999). In addition, recent evidence from *E. coli* (Balaban *et al*., 2004; Miller *et al*., 2004) suggests that a switch to a persistent phenotype is favoured where bactericidal antibiotics are ineffective against slowly- or non-dividing cells (a characteristic of current front-line antituberculars) and is mediated by the SOS response (Miller *et al*., 2004).

Permeability and efflux

Altered permeability resulting from the stationary phase-associated thickening of the cell wall might be relevant to antimicrobial tolerance *in vivo* (Cunningham & Spreadbury, 1998). The concentration achieved by antibiotics inside bacterial cells is a function of cell

envelope permeability and the presence of efflux systems capable of extruding toxic compounds (Nikaido, 1994). *M. tuberculosis* is intrinsically resistant to many of the antibiotics and chemotherapeutics in current medical use (Jarlier & Nikaido, 1994), at least partially because of the relative impermeability of the mycolic acid-rich mycobacterial cell wall (Rastogi *et al*., 1981; Connell & Nikaido, 1994; Brennan & Nikaido, 1995). Hydrophilic diffusion is constrained by the limited number and restrictive structure of mycobacterial porins (Trias & Benz, 1994; Engelhardt *et al*., 2002); furthermore, it has been shown that the contribution of each of the various classes of mycolic acids to cell wall composition varies depending on growth phase and oxygen tension, and differs for *in vitro versus* intraphagosomal survival (Barry & Mdluli, 1996; Yuan *et al*., 1996, 1998). Reduced cell wall permeability accounts to only a certain degree for the inherent resistance of mycobacteria, however, and recent evidence indicates a role for efflux mechanisms in diminished intracellular drug accummulation (Piddock *et al*., 2000). Mycobacteria, including *M. tuberculosis*, contain a large number of putative drug efflux transporters (Cole *et al*., 1998; Garnier *et al*., 2003), including members of the major facilitator superfamily (MFS), as well as the multidrug and toxic extrusion, resistance-nodulation-cell division, small multidrug resistance (SMR), and ATP-binding cassette (ABC) families (Li & Nikaido, 2004). Although the contribution of drug transporters to the intrinsic resistance of mycobacteria is uncertain, overexpression of MFS transporter homologues (Liu *et al*., 1996; Takiff *et al*., 1996; Ainsa *et al*., 1998; Silva *et al*., 2001), results in low-level resistance to fluoroquinolones, aminoglycosides and tetracyclines; similarly, the *M. tuberculosis*-associated SMR-type pump, encoded by *mmr*, and the *drrAB*-encoded ABC transporter mediate resistance to several antimicrobials, including erythromycin and cationic dyes (De Rossi *et al*., 1998; Choudhuri *et al*., 2002). Expression of the *efpA*-encoded *M. tuberculosis* MFS drug transport homologue is induced after isoniazid treatment (Wilson *et al*., 1999), while disruption of specific efflux pump homologues renders *M. smegmatis* marginally more sensitive to antimycobacterials including isoniazid, rifamycins, fluoroquinolones, and erythromycin (Li *et al*., 2004); in addition, the partial dependence of mycobacterial β-lactam resistance on active extrusion has been demonstrated in a β-lactamase-deficient *M. smegmatis* strain (Li *et al*., 2004). Finally, removal of efflux gene regulation confers markedly increased resistance to several drugs, perhaps indicating a potential evolutionary mechanism for the development of multidrug resistance in pathogenic mycobacteria (Li *et al*., 2004).

Effective antibiotic concentrations

Mathematical modelling has identified the pre-antibiotic bacterial population size and poor adherence to therapeutic regimens as crucial components determining the risk of developing drug resistance, as well as resistance outcomes (Lipsitch & Levin, 1998). There is an increased probability of selecting resistance mutations during treatment with one antibiotic, necessitating the use of at least two antimycobacterials in antitubercular therapy. However, even when adhered to, limited efficacy of one or more drugs might result in effective monotherapy and thereby erode the potency of the combination, a situation exacerbated by bacilli occupying protected compartments in which an antibiotic might achieve only subminimum inhibitory concentrations (Lipsitch & Levin, 1998; Mitchison, 1998). Parallel evolution of a single founder population into heterogeneous, antibiotic-resistant sub-populations within isolated loci has recently been demonstrated in patients undergoing active antibiotic treatment for *E. coli* (Low *et al*., 2001) and *M. tuberculosis* (Kaplan *et al*., 2003). Furthermore, evidence from pathogenic infections suggests that low-level resistant variants (such as the extended-spectrum TEM β-lactamases) might evolve during occupation of particular niches within the host in which the locally available concentration of an antibiotic (subject to a chemical gradient) selects for the different variants (Baquero *et al*., 1998). In many cases, the specific antibiotic concentrations inhibiting the variant and wild-type strains may be extremely close, resulting in a narrow selective concentration range. Of course, the narrowness of the selective range often results in some low-level resistant mutants being considered clinically irrelevant; however, the ability of a given antibiotic concentration (even if very low) to select for a particular resistance mutation (even if almost neutral) has profound implications for the generation of diverse microbial populations in a single host (Baquero & Negri, 1997). In particular, the evolution of high-level resistant mutants may be enhanced *in vivo* in populations in which low-level resistant mutants have been selected and maintained in various compartments. The observation that the mutation rate for a given antibiotic may change depending on the effective concentration during selection, and that different rates and genotypes occur at discontinuous intervals along the spectrum of applied concentrations (Kohler *et al*., 1997; Zhou *et al*., 2000), has given rise to the concept “mutant prevention concentration” which has been used to assess differential antibiotic activities and efficacies (Dong *et al*., 2000; Sindelar *et al*., 2000). In addition, the correlation between antibiotic resistance levels and small increases in mutation rate is indicative of the stable evolution of resistant phenotypes (Gustafsson *et al*., 2003; Lindgren *et al*., 2003). Compelling evidence of the role of antibiotic exposure in eliciting, and selecting, resistance mutations is provided by the observation that naïve, wild-type *M.*

tuberculosis isolates are almost invariably antibiotic susceptible (Kochi *et al*., 1993). Preventing replication and spread of *M. tuberculosis* to relatively sheltered *in situ* compartments, and limiting adaptation of the organism to adverse conditions is therefore key, and depends heavily on frontline drugs with early bactericidal activity (Mitchison, 1993; Herbert *et al*., 1996; Wallis *et al*., 1999).

Molecular mechanisms of resistance

Bacterial diseases generally result from the multiplication of a single infecting pathogenic strain (Moxon & Murphy, 1978; Rubin, 1987) that subsequently acquires resistance through serial mutagenesis and only rarely through horizontal genetic transfer (Moxon *et al*., 1994). This is particularly true of pathogens whose preferential occupation of specific environments within the host restricts the opportunities for the acquisition of novel, transmissible genetic elements. The typical aetiology of TB - early, acute infection followed by extended immune-mediated isolation and latency - suggests that a similar situation might apply to *M. tuberculosis*. Recent evidence indicates that *M. tuberculosis* infections are clonal (Gutacker *et al*., 2002; Warren *et al*., 2004), and analyses of the genomes of clinical and laboratory mycobacterial strains have revealed only minor roles for horizontal gene transfer in the macroevolution of these pathogens (Cole *et al*., 1998; Cole *et al*., 2001; Kato-Maeda *et al*., 2001; Fleischmann *et al*., 2002; Hirsh *et al*., 2004; Tsolaki *et al*., 2004). Furthermore, *M. tuberculosis* does not possess epigenetic information in the form of plasmids, and no evidence exists for its natural competence (Derbyshire & Bardarov, 2000), although the demonstration of *RD1*-mediated regulation of conjugal transfer in *M. smegmatis* indicates the functional conservation of a secretory apparatus among mycobacteria (Flint *et al*., 2004). Nevertheless, in the absence of evidence to the contrary, these factors likely preclude the acquisition of genetic information by horizontal transfer. Resistance must, therefore, arise through the acquisition and maintenance of spontaneous chromosomal mutations in target or complementary genes or, rarely, from the inactivation of a target gene by a mobile genetic element such as IS*6110* (Dale, 1995; Lemaitre *et al*., 1999). Consistent with the genetic isolation of *M. tuberculosis* is the observation that all drug resistance determinants are chromosomally encoded (Ramaswamy & Musser, 1998). Specific mutations conferring monoresistance to the main antituberculous drugs have been identified in each of the target genes (Musser, 1995; Ramaswamy & Musser, 1998), and are identical in MDR strains (Morris *et al*., 1995): for example, rifampicin resistance correlates with mutations in *rpoB*, isoniazid with *katG*, *inhA*, *oxyR*, *ahpC*, *ndh* and *furA* mutations, streptomycin (*rrs*, *rpsL*), pyrazinamide (*pncA*, IS*6110* insertion), ethambutol (*embB*), and fluoroquinolones

(*gyrA*, *gyrB*). However, although the majority of mutations are associated with common resistance determining regions, not all drug resistant phenotypes can be mapped to particular alterations in target genes, but remain undefined (Musser, 1995; Ramaswamy & Musser, 1998).

Rates of resistance mutations

MDR strains constitute 1%-3% of total TB isolates and, although localised 'hot zones' of greater than 5% prevalence (Pablos-Méndez *et al.*, 2000) - or incidence (Farmer *et al.*, 1999) - characterise worldwide MDR-TB distribution, MDR-TB is a significant problem in every region under WHO surveillance (Espinal, 2003; Espinal *et al*., 2001; WHO, 2004). Based on *in vitro* measures of individual mutation rates to drug resistance (David, 1970), statistically the emergence of MDR-TB would appear to require a larger bacillary population than is usually present during infection. However, the assumption that the risk of developing multiple resistance equals the product of the individual mutation rates is likely an oversimplification considering the complex interplay *in vivo* of factors that might reduce treatment efficacy or increase mutagenesis, as discussed above. In addition, the pre-existence of heteroresistant organisms in a population prior to chemotherapy might accelerate emergence of drug resistance and has been reported for isoniazid and ethambutol (Rinder *et al*., 1999, 2001). Evidence from other chronic infectious pathogens, particularly *Psuedomonas aeruginosa*, indicates that a higher, stress-induced mutation rate could explain the apparent paradox (Alonso *et al*., 1999), and it has been shown that antibiotics independently select for mutator strains *in vivo* (Oliver *et al*., 2000; Gustafsson *et al*., 2003). Furthermore, *in vitro* evidence from *M. smegmatis* suggests that, in a stationary phase-associated hypermutable state, mutant antagonism between streptomycin- and rifampicin-resistance mutations enhances acquisition of resistance to both (Karunakaran & Davies, 2000). The applicability of this observation to *M. tuberculosis* infection is uncertain, but has fuelled research into the role of stationary-phase mutagenesis and mutator polymerases in the evolution of drug resistance in mycobacteria (Boshoff *et al.*, 2003).

Emergence of multidrug resistance

Infection with *M. tuberculosis* strains resistant to the two most commonly used frontline anti-TB drugs, isoniazid and rifampicin, is defined as multidrug-resistant TB (MDR-TB) and often culminates in incurable disease (Heifets & Cangelosi, 1999). Latest WHO estimates indicate that over 300 000 new cases of MDR-TB emerge worldwide each year, the most common pathway to multidrug resistance apparently beginning with

monoresistance to isoniazid or streptomycin (WHO, 2004). The prevalence of strains resistant to these single drugs correlates with those areas exhibiting the highest levels of multidrug resistance and, together with double isoniazid/streptomycin, triple isoniazid/streptomycin/rifampicin and quadruple isoniazid/streptomycin/rifampicin/ ethambutol resistant cases accounts for about 85% of resistance globally. Moreover, the "super strains" resistant to at least three of the four frontline TB drugs make up 79% of all MDR-TB cases. Treatment of MDR-TB is expensive, and the so-called "DOTS Plus" strategies that have been recommended for high-prevalence areas (Farmer & Kim, 1998; Gupta *et al*., 2001), comprise combinations of second-line drugs that are more expensive, more toxic, and less effective than the drugs used in standard therapy (Iseman, 1993). Furthermore, the observation that MDR-TB prevalences coincide with HIV infection rates is suggestive of a positive correlation between multidrug resistance and HIV seropositivity (Gillespie, 2002), although the identification of HIV as an independent risk factor for MDR-TB is contentious (WHO, 2004). Characteristic features of HIV/TB-associated clinical disease might, however, favour the emergence of resistance and it has been suggested that the relationship be closely monitored (WHO, 2004). It is likely, for example, that a functional immune system is required to potentiate drug activity and prevent resistance evolution (Gillespie, 2002; Gomez & McKinney, 2004) and that the large bacterial populations associated with immunocompromised individuals provide an expanded subset for selection and transmission of rare mutation events. Furthermore, factors implicated in effective monotherapy in immunocompetent individuals might be exacerbated in the absence of a functioning immune response. For example, uncontrolled replication and dissemination resulting in extra-pulmonary foci, pulmonary cavitation, and empyema could produce drug-inaccessible compartments. In addition, drug absorbtion might be compromised by HIV-associated clinical diseases such as gastrointestinal complaints arising from other chronic infections. Finally, the increase in TB incidence in high HIV-prevalence areas might indirectly lead to the spread increased spread of both susceptible and MDR strains; although potentially slower to emerge in immunocompetent individuals, infection with MDR strains might result in huge burdens of disease in the future (Gillespie, 2002).

1.11 *In vivo* stress

Pathogens are expected to encounter and adapt to many stresses during colonisation of the host. Occupation of an immature phagosomal compartment partially shields *M. tuberculosis* from the macrophage's most potent antimicrobial defence mechanisms, including acid, oxygenated lipids, fatty acids, lytic enzymes, and ROI and RNI (Aderem &

Underhill, 1999), enabling intraphagosomal replication during the early stages of infection. Even where maturation is stalled, however, the bacilli are exposed to a slight decrease in phagosomal pH (Deretic & Fratti, 1999), and the transcriptional response of *M. tuberculosis* to acid stress *in vitro* involves several genes involved in lipid metabolism, including *icl*, and the peroxynitrite reductase/peroxidase components *ahpC* and *ahpD* (Fisher *et al*., 2002). The glyoxylate shunt enzyme isocitrate lyase, encoded by *icl*, enables alternative energy production through the metabolism of fatty acids and is required for survival in macrophages and persistence in a mouse model of latent TB (McKinney *et al*., 2000). Induction of *icl* is associated with limiting oxygen levels *in vitro* (Wayne & Sohaskey, 2001) and entry into macrophages (Graham & Clark-Curtiss, 1999; Honer Zu Bentrup *et al*., 1999); in addition, *icl* expression was recently detected in a mouse model (Timm *et al*., 2003) and, according to tissue pathology, within macrophages in human tuberculous lung samples (Fenhalls *et al*., 2002b; Timm *et al*., 2003), all of which suggests that *in vitro* acid stress might mimic aspects of the *in vivo* environment. However, *icl* is also induced *in vitro* in a long-term nutrient starvation model (Hampshire *et al*., 2004), and in activated murine and human macrophages as part of a cohort of genes required for β-oxidation of fatty acids, likely indicating a switch to alternative (gluconeogenic) carbon metabolism in the phagosome (Dubnau *et al*., 2002; Schnappinger *et al*., 2003). Several genes potentially involved in fatty acid metabolism are common to both *in vitro* acid stress and intraphagosomal adaptation *in vitro* and *in vivo* (Dubnau *et al*., 2002; Fisher *et al*., 2002; Schnappinger *et al*., 2003), prompting the suggestion that low pH signals to *M. tuberculosis* the need to prepare for extended intraphagosomal survival (Fisher *et al*., 2002). *M. tuberculosis* is also likely to sustain damage to surface structures mediated by several antimicrobial defences including alveolar surfactant - a mild detergent with antibacterial activity, toxic peptides and proteins like granulysin (Dieli *et al*., 2001), and toxic free fatty acids secreted by macrophages within the mycobacterial phagosome and in the external environment (Akaki *et al*., 2000). Evidence for cell surface damage is provided by the upregulation in resting macrophages of *M. tuberculosis* genes associated with SDS treatment and cell wall maintenance (Schnappinger *et al*., 2003), including some involved in lipid biosynthesis and modification, as well as the *sigE*-encoded extracellular function (ECF) σ factor, essential for *M. tuberculosis* virulence in mice (Manganelli *et al*., 2004a) and implicated in survival of *M. tuberculosis* in macrophages (Manganelli *et al*., 2001) and in the *in vitro* response to detergent-induced surface stress, heat shock, and thiol-specific oxidation (Manganelli *et al*., 1999, 2002).

Hypoxia is an antimicrobial mechanism generally associated with inflammation and impaired vascularisation. As discussed above, induction of DosR in response to both hypoxic and NO-mediated signalling is considered relevant to latent infection (Schnappinger *et al*., 2003; Voskuil *et al*., 2003; Voskuil, 2004). A switch to anaerobic pathways and reduced energy generation characterises *M. tuberculosis* respiration inside the phagosome, with repression of genes encoding subunits of NADH dehydrogenase (NDH)-I, the ubiquinol-cytochrome C complex, and ATP synthase, and induction of alternative electron transporters including nitrate and fumarate reductases (Schnappinger *et al*., 2003). Components of NDH-1 are also downregulated in response to low pH (Fisher *et al*., 2002); in the murine phagosome, however, the partially compensating *ndh*-encoded NDH-2 is upregulated which might indicate adaptation to a low-energy intraphagosomal state, or a requirement for NAD^+ regeneration for the proposed β-oxidation reactions (Schnappinger *et al*., 2003). Expression of α-crystallin, a chaperonin encoded by *hspX* and included in the DosR dormancy regulon (Sherman *et al*., 2001; Boon & Dick, 2002; Ohno *et al*., 2003; Park *et al*., 2003; Voskuil *et al*., 2003), is strongly induced *in vitro* on entry into stationary phase (Yuan *et al*., 1996; Timm *et al*., 2003), and in response to hypoxia (Yuan *et al*., 1996; Sherman *et al*., 2001) and nitric oxide (Garbe *et al*., 1999; Ohno *et al*., 2003; Voskuil *et al*., 2003). The upregulation of *hspX* in activated macrophages (Yuan *et al*., 1998; Schnappinger *et al*., 2003), *in vivo* in mouse lungs (Schnappinger *et al*., 2003; Timm *et al*., 2003; Voskuil *et al*., 2003), and in lung specimens from human TB patients (Timm *et al*., 2003) might be in response to hypoxia or to NO, although the delayed expression in IFN-$\gamma^{-/-}$ mice (Shi *et al*., 2003) indicates likely NO mediation; in any event, upregulation of *hspX* under these various conditions suggests that similar stresses are imposed on *M. tuberculosis in vivo*. Moreover, members of the *mbt* operon are induced during microaerophilic growth *in vitro* (Bacon *et al*., 2004), and in quiescent and activated macrophages (Schnappinger *et al*., 2003).

M. tuberculosis is also likely to be deprived of nutrients and essential elements in its occupation of the phagosome and in granulomas. Recent *in vitro* models of starvation have identified broad metabolic adjustments thought likely to apply during persistence *in vivo* (Betts *et al*., 2002; Hampshire *et al*., 2004), and suggest that glucose deficiency and an abundance of fatty acids characterise the phagosome of activated macrophages (Boshoff & Barry, 2005); for example, genes involved in β-oxidation, the glyoxylate shunt, gluconeogenesis, amino acid/amine degradation, RNA synthesis and modification, and DNA transcription are induced in response to nutrient stress, while carbon degradative pathways, *de novo* ATP generation, and purine/pyrimidine synthesis are downregulated.

In addition, sulphur appears to be required for maintenance of redox balance and for translation initiation, and a number of genes required for sulphur metabolism are upregulated during starvation *in vitro* (Hampshire *et al*., 2004). Reduced intraphagosomal iron availability has been described (De Voss *et al*., 2000; Gold *et al*., 2001) and, in a mouse model, iron limitation was associated with growth arrest at sites of infection (Timm *et al*., 2003). In addtion, iron-scavenging siderophores are upregulated by *M. tuberculosis* in murine macrophages (Schnappinger *et al*., 2003) although this might be in response to oxidative stress (discussed below). Iron restriction is thought (Timm *et al*., 2003) to result from the downregulation of transferrin receptor expression in response to macrophage activation by cytokines (Mulero & Brock, 1999; Kim & Ponka, 2000) or from reduced intersection of mature mycobacterial phagosomes with iron-containing vesicles (Schaible *et al*., 1998; MacMicking *et al*., 2003). Decreased Mg^{2+} levels in the mycobacterial phagosome have also been reported, and are associated with increased *M. tuberculosis* acid sensitivity (Buchmeier *et al*., 2000). Gluconeogenesis is essential for virulence of *M. bovis* (Collins *et al*., 2002) and *M. bovis* BCG (Liu *et al*., 2003) in animal models, and expression of *M. tuberculosis pckA* (required for gluconeogenesis; Liu *et al*., 2003) is elevated in a mouse infection, consistent with reduced glucose availability *in vivo* (Timm *et al*., 2003). Transmission of aerosolised *M. tuberculosis* also incurs external environmental stresses such as nutrient starvation, exposure to UV/sunlight (to which it is particularly sensitive), dehydration, and temperature fluctuations (Corper & Cohn, 1933; Stewart *et al*., 2002).

Reactive immune effectors

M. tuberculosis is expected to sustain a variety of potentially DNA–damaging assaults *in vivo* (Mizrahi & Andersen, 1998; Boshoff *et al*., 2001), primarily from host-generated antimicrobial ROI and RNI (Adams *et al*., 1997; MacMicking *et al*., 1997a; Rich *et al*., 1997; Akaki *et al*., 2000; Nathan & Shiloh, 2000). Furthermore, the observation that *M. tuberculosis* is much more sensitive to acidic pH than other mycobacterial species (Gomes *et al*., 1999) suggests that the bacilli might incur DNA damage in phagosomes, probably mediated by RNI generated from acidified nitrite (Stuehr & Nathan, 1989; Sturgill-Koszycki *et al*., 1994; Oh & Straubinger, 1996; Foster & Moreno, 1999). DNA is a biological target for RNI and ROI (Wink *et al*., 1991; Burney *et al*., 1999), and interaction with toxic radicals is mutagenic (Zhuang *et al*., 2000). Furthermore, damage to cellular components required for the protection or propagation of DNA can indirectly affect chromosomal integrity, while detoxification reactions might themselves yield endogenous damaging adducts (Nathan & Shiloh, 2000). Additional endogenous reactive intermediates

are also likely to be generated by the switch between aerobic and anaerobic metabolism, and from the partial reduction of terminal electron acceptors during respiration (Boshoff & Barry, 2005). Although the relevance of tissue culture and animal models of infection to human TB is uncertain (Graham & Clark-Curtiss, 1999; McKinney *et al*., 2001; Boshoff & Barry, 2005), there is increasing evidence that the *in vivo* environment is DNA damaging (Boshoff *et al*., 2003; Sassetti & Rubin, 2003; Schnappinger *et al*., 2003). However, the expression and activities of putative detoxification and damage repair components in *M. tuberculosis* remains largely undefined (Mizrahi & Andersen, 1998; Mizrahi *et al*., 2000, 2005; Boshoff *et al*., 2001; Boshoff & Barry, 2005) though it is likely that, as in other organisms, some of the mechanisms implicated in antioxidant and antinitrosative defence function primarily to enable oxidative metabolism or in homeostatic signalling networks, but fulfil a vital role in tolerance of host immune effectors (Zahrt & Deretic, 2002; Nathan, 2003).

Exposure of *M. tuberculosis* to potent oxidative agents *in vitro* results in minimal differential gene expression (Deretic *et al*., 1995; Lee & Horwitz, 1995; Sherman *et al*., 1995; Garbe *et al*., 1996; Boshoff *et al*., 2003) and has been attributed to the impaired ability of the organism to mount a coherent oxidative stress response as a result of a natural deficiency in the *soxRS* and *oxyR* regulons (Deretic *et al*., 1995; Sherman *et al*., 1995; Dhandayuthapani *et al*., 1996; Garbe *et al*., 1996). Some oxidative stress response genes contained within the OxyR and SoxRS regulons in other bacteria are, however, expressed in *M. tuberculosis* in the absence of the central regulators, including the peroxiredoxin AhpC (Chen *et al*., 1998; Bryk *et al*., 2000; Springer *et al*., 2001; Schnappinger *et al*., 2003), superoxide dismutase (Wieles *et al*., 1995; Weldingh *et al*., 1998; Harth & Horwitz, 1999), thioredoxin (Raman *et al*., 2004), and the KatG catalase-peroxidase (Manca *et al*., 1999a; Master *et al*., 2001; Ng *et al*., 2004). A key question, though, pertains to the regulation (and therefore biological relevance) of many of the detoxifying mechanisms during NRP *in vivo*; for example, although superoxide dismutase levels decline in dormancy *in vitro* and after onset of the immune response in a mouse model, elevated *ahpD* expression has been identified as a marker for NRP (Wayne & Lin, 1982; Wayne, 1994; Hu *et al*., 1999; Shi *et al*., 2003; Voskuil, 2004). Damage repair is essential for the virulence and survival of other intracellular pathogens (Buchmeier *et al*., 1993; Spek *et al*., 2001); however, despite resulting in damage hypersensitivity *in vitro*, inactivation of mycobacterial alkylation reversal (*alkA::ogt*; Durbach *et al*., 2003) and recombinational repair (*recA*; Sander *et al*., 2001, 2003) pathways does not attenuate *in vivo* survival. Although it is possible that nitrosative or oxidative stresses do not induce

cytotoxic DNA damage in the mouse model, the virulence of isolates of the W-Beijing genotype (Rad *et al*., 2003) is similarly not impaired by the characteristic deficiency in elimination of damaged nucleotide pools (as a result of a *mutT* mutation) and/or in repair of alkylation damage (*ogt*) in these strains, nor do W strains exhibit a mutator phenotype (Werngren & Hoffner, 2003). It seems likely, therefore, that other repair pathways might preferentially function in mycobacteria; for example, a Ku-ligase system for DSB repair by nonhomologous end-joining (NHEJ) was recently characterised in *M. tuberculosis* (Weller *et al*., 2002; Della *et al.*, 2004) and might provide a possible alternative to homologous recombination for repair of such lesions (Mizrahi *et al*., 2005). In addition, *M. tuberculosis* contains homologues of a putative DNA repair system highly conserved in thermophilic Archaea and predicted to function in translesion synthesis (TLS) (Makarova *et al.*, 2002). Several base excision repair (BER) enzymes have also been identified that are required for growth *in vivo* (Sassetti & Rubin, 2003) but not *in vitro* under optimal conditions (Sassetti *et al.*, 2003) implying a role in virulence. Furthermore, mycobacteria including *M. leprae* possess several conserved Fpg/Nei-family DNA glycosylases (Cole *et al.*, 1998, 2001; Mizrahi & Andersen, 1998; Fleischmann *et al*., 2002); although the *in vivo* role of these homologues in BER remains uncertain, the activity of purified *M. tuberculosis* Fpg in repair of oxidised DNA (especially 8-oxo-G) was recently demonstrated (Guo *et al.*, 2004). Together with evidence that resistance to nitrosative stress in *M. smegmatis* is dependent on a functional uracil DNA glycosylase (Ung; Venkatesh *et al*., 2003), the demonstration that inactivation of proteasomal components (*mpa* and *paf*) and the *uvrB*-encoded excinuclease subunit is associated with elevated susceptibility to iNOS-generated RNI (Darwin *et al*., 2003), is suggestive of the increased importance of excision-based mechanisms such as BER and nucleotide excision repair (NER) in damage tolerance in mycobacteria (Mizrahi *et al*., 2005). Moreover, the upregulation of *uvrB* and several other DNA repair genes in response to certain damaging agents only, indicates that the type of lesion might determine the repair mechanism invoked (Boshoff *et al*., 2003).

Other mechanisms implicated in resistance against oxidative and nitrosative stress include the DosR-regulated α-crystallin (Garbe *et al*., 1999; Ehrt *et al*., 1997; Ruan *et al*., 1999; Sherman *et al*., 2001; Bryk *et al*., 2002; Ouellet *et al*., 2002), while some of the physical properties characteristic of mycobacteria have been implicated in the subversion of toxic ROI (Chan *et al*., 1989; Chan *et al*., 1991; Chan & Kaufmann, 1994; Barry & Mdluli, 1996; Flynn & Chan, 2001a). In addition, mycobacteria in a low oxygen-induced dormant state are highly resistant to mitomycin C (Peh *et al*., 2001), suggesting that some protection of chromosomal DNA is provided by the NO- or hypoxia-mediated induction of

M. tuberculosis UspA-like genes as part of the DosR regulon (Sherman *et al*., 2001; Ohno *et al*., 2003; Park *et al*., 2003; Voskuil *et al*., 2003). Finally, it has been suggested that several small molecules might act as biological traps for reactive intermediates, although their relevance in mycobacterial oxidative and nitrosative defence is unknown (Nathan & Shiloh, 2000).

1.12 DNA metabolism in mycobacteria

The mutational mechanisms underlying inter-strain variation and the adaptive evolution of drug resistance in *M. tuberculosis* are not well understood. However, it was hypothesised that the complex and variable environments encountered by *M. tuberculosis* during host colonisation and throughout the infective process might impose excessive demands on normal genetic regulatory systems. Furthermore, the likelihood that sub-clinical mycobacterial populations might be maintained in a state of prolonged stationary phase - either as a consequence of the specific ecological niche inhabited, or as a result of host immune effectors or antibiotic drugs - suggested that inducible mutator systems might function to enable adaptation and competitive survival. In particular, the presence of multiple genes encoding putative error-prone or Y-family DNA polymerases in sequenced mycobacterial genomes was strongly supportive of the idea that mycobacteria might possess the genetic means to modulate mutagenesis. This thesis aimed, therefore, to investigate mechanisms of mycobacterial DNA metabolism with specific emphasis on the role of multiple Y-family polymerases in genetic adaptation to stress. In addition, the role of the class II (oxygen-independent) ribonucleotide reductase (RNR) in the supply and maintenance of dNTP pools for replication and repair under hypoxia (also likely to prevail during long-term persistence *in vivo*) was considered. The vitamin B_{12} dependence of the class II RNR further demanded an investigation into the biosynthesis and transport of cobalamin (vitamin B_{12}), as well as the cobalamin requirements of other vitamin B_{12}-dependent isozymes. A similar methodology characterised the approach to each question and involved the construction of mutant strains with altered complements of genes of interest, and the phenotypic characterisation of the mutants, in terms of viability, survival, damage tolerance, and mutagenesis, where applicable. Although the thesis comprises three separate studies, the central hypothesis underlying the work presented is that, while dependent on genes with differential regulatory and functional mechanisms, as well as co-factor requirements, the metabolic pathways under investigation - genetic adaptation by chromosomal mutagenesis, dNTP pool maintenance for DNA synthesis or repair under hypoxic conditions, and biosynthesis of cobalamin for the vitamin B_{12}-dependent class II RNR - collectively enable *M. tuberculosis* to adapt to the complex and fluctuating host

environment. In summary, Chapter 3 explores the role of the mycobacterial Y-family polymerases in adaptive mutagenesis and long-term competitive fitness, with particular emphasis on the mycobacterial DinB (pol IV) homologues. The possible mutagenic consequences associated with the use of alternative gyrase inhibiting antibiotics for the treatment of *M. tuberculosis* infections are also considered. In Chapter 4, the contribution of the oxygen-independent class II RNR to hypoxic adaptation and virulence is assessed. Finally, in Chapter 5, the cobalamin requirement of *M. tuberculosis* is investigated in strains containing disruptions in the cobalamin biosynthetic pathway, or selectively manipulated to force utilisation of vitamin B_{12}-dependent isozymes. The role of cobalamin biosynthesis in the virulence of *M. tuberculosis* is also assessed.

Chapter 2

General materials and methods

2.1 GENERAL LABORATORY PROCEDURES

All general procedures and DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989; Sambrook & Russell, 2001). All culturing and molecular biological manipulations of mycobacteria were performed according to Jacobs *et al.* (1991), Larsen (2000), and Parish & Stoker (2001).

2.1.1 Bacterial strains and culture conditions

All bacterial strains used in this study were stored at -70°C in 30% glycerol (v/v) and are detailed in Table 2.1.

Table 2.1 Bacterial strains used in this study

Strain	Genotype/markers	Reference/source
Escherichia coli		
DH5α	*supE44 ΔlacU169* (*Φ80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)/ Promega
GM161	*dam-4 supE44 hsdS1*	E. R. Dabbs
Mycobacterium smegmatis		
mc^2155	*ept-1*, efficient plasmid transformation mutant of $mc^2$6	Snapper *et al.*, 1990
Mycobacterium tuberculosis		
H37Rv	ATCC 25618	London School of Hygiene and Tropical Medicine
CDC1551		H. Boshoff

Escherichia coli. All *Escherichia coli* strains were grown overnight at 37°C in Luria-Bertani broth (LB) with vigorous shaking (300-400 rpm) in a New Brunswick Series 25 Incubator Shaker, or on solid Luria-Bertani agar (LA). Antibiotics were added where appropriate.

Mycobacterium smegmatis. Unless otherwise indicated, all *M. smegmatis* strains were cultured in Middlebrook 7H9 medium (Difco) supplemented with 10% (v/v) ADC (Merck), 0.2% glycerol, and 0.10 % Tween80 (7H9M-ADC) or on Middlebrook 7H10 medium (Difco) supplemented with 10% (v/v) OADC (Merck) (7H10M-OADC).

Mycobacterium tuberculosis. All culturing of *M. tuberculosis* strains was performed in a Biosafety Level 3 laboratory. Manipulations of bacilli were performed in a Class II flow cabinet at negative pressure (170 kPa). Unless otherwise indicated, all strains were grown at 37°C in Middlebrook 7H9 medium supplemented with 10% (v/v) ADC (Merck), 0.2% glycerol and 0.05% Tween 80 (7H9M-ADC; Jacobs *et al.*, 1991) in roller bottles or as

stirred cultures, or on solid Middlebrook 7H10 agar (Difco) supplemented with 10% (v/v) OADC (Merck) (7H10M-ADC).

Antibiotics. For *E. coli*, liquid and solid media contained ampicillin (Amp) at a concentration of 200 µg/ml, kanamycin (Km) at 50 µg/ml, and hygromycin (Hyg) at 200 µg/ml. Antibiotics were included at the following concentrations for *M. smegmatis* and *M. tuberculosis* strains, respectively: Km, 25 µg/ml and 10 µg/ml; rifampicin (Rif), 200 µg/ml and 2 µg/ml; and Hyg at 50 µg/ml for both mycobacterial species.

Supplements. For counterselection of *E. coli* and mycobacterial clones harbouring the *sacB* gene, sucrose (Suc) was added to solid media to a final concentration of 5% (w/v) and 2% (w/v), respectively. For β-galactosidase activity (blue-white selection), 50 µl X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 20 mg/ml in dimethyl formamide) was included in 25 ml plates.

2.1.2 General cloning vectors

The general plasmids used are detailed in Table 2.2. Constructs specific to particular studies are detailed in the relevant chapter.

Table 2.2 Plasmids used in this study

Plasmid	Description	Reference/source
p2NIL	Cloning vector; Km^R	Parish & Stoker, 2000
pAINT	*E. coli*-Mycobacterium integrating shuttle vector; Km^R	Boshoff & Mizrahi, 2000
pBluescriptKS(+)	Cloning vector; Amp^R	Stratagene
pGEM3Z(+)f	Cloning vector; Amp^R	Promega
pGEM ®-T Easy	PCR cloning vector; Amp^R	Promega
pCR®2.1-TOPO®	PCR cloning vector; Amp^R Km^R	Invitrogen
pGOAL17	Plasmid carrying *lacZ* and *sacB* genes as a *Pac*I cassette; Amp^R	Parish & Stoker, 2000
pGOAL19	Plasmid carrying *hyg*, *lacZ* and *sacB* genes as a *Pac*I cassette; Amp^R	Parish & Stoker, 2000
pIJ963	Plasmid carrying *hyg* as a *Bam*HI-*Bgl*II cassette; Amp^R Hyg^R	Blondelet-Rouault *et al.*, 1997
pOLYG	*E. coli*-Mycobacterium multicopy shuttle vector, Hyg^R	O'Gaora *et al.*, 1997

2.2 TRANSFORMATION OF BACTERIA

2.2.1 Chemical transformation of *Escherichia coli*

Preparation of *E. coli* with rubidium chloride buffers

All *E. coli* cells were prepared with rubidium chloride buffers, according to a procedure obtained from Dr. P. Stolt. Briefly, a saturated culture of cells was used to inoculate Psi broth at a 1/100 dilution. The culture was grown to an OD_{600} of 0.6-0.7. The bacteria were chilled on ice for 15-30 minutes, harvested (1100 x g for 15 min at 4^{o}C), and then resuspended in RF-1 (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% glycerol v/v, pH 5.8) at 1/3 of the original volume. After 0.5-2 hours on ice, cells were harvested again, resuspended in RF-2 (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15 % glycerol v/v, pH 6.5) at 1/12.5 of the original culture volume, and left on ice for 15-30 minutes. Cell aliquots were then dispensed into pre-chilled 1.5 ml microcentrifuge tubes, and flash-frozen in an ethanol bath at -70^{o}C. Cells were thawed on ice when required.

Transformation with plasmid DNA

Competent cells (100-200 µl) were incubated with up to 1.0 µg DNA on ice for 1 hour, and then heat shocked for 90 seconds at 42 ^{o}C in a microcentrifuge tube. After chilling on ice for 1-2 minutes, 4 volumes of 2-TY were added to the cells, which were then incubated for 1 hour at 37^{o}C. Cells were plated on LA containing the appropriate antibiotics, and incubated overnight at 37 ^{o}C (Sambrook *et al.*, 1989; Sambrook & Russell, 2001) or, in the case of large or *Pac*I cassette-containing (Parish & Stoker, 2000) constructs, for two days at 30 ^{o}C.

2.2.2 Electroduction of *Escherichia coli*

Electrocompetent *E. coli* cells were prepared according the protocol set out in the Bio-Rad Gene Pulser manual. Briefly, 50 ml 2TY was inoculated with 1/100 volume of a saturated culture of *E. coli* DH5α and grown at 37^{o}C with vigorous shaking (300-400 rpm) until an OD_{600} of 0.6 was reached. Cells were placed on ice for 20 minutes and then harvested (1500 x g for 5 min). Cell pellets were resuspended in 50 ml ice-cold 10% glycerol, harvested as before, and washed again in 25ml and finally 1ml 10% glycerol. After the final wash, cells were resuspended in a final volume of 500 µl 10% glycerol and used immediately. For electroductions, a portion of a *M. smegmatis* colony was dispersed into 20 µl 1% glycerol and mixed with 100 µl electrocompetent DH5α cells. The mixture was transferred into an electroporation cuvette and pulsed once at 200 Ω, 35 µF, 2.5 kV. The

cells were rescued by the addition of 800 µl 2TY and incubated for I hour at 37^{o}C before plating on LA containing the appropriate selective antibiotics.

2.2.3 Transformation of mycobacteria by electroporation

All electroporations of *M. smegmatis* and *M. tuberculosis* were performed according to Larsen (2000), Gordhan & Parish (2001).

***Mycobacterium smegmatis*.** 100 ml cultures were grown overnight from a 1/100 dilution of saturated culture at 37^{o}C with shaking at 100 rpm. Cells were harvested by centrifugation (1100 x g for 10 minutes at 4^{o}C) and washed three times in 10% glycerol (v/v)pre-chilled at 4^{o}C. After the final wash, cells were resuspended in 2 ml 10% glycerol and used immediately. 400 µl aliquots were incubated briefly on ice with 50-1000 ng of plasmid DNA in microcentrifuge tubes. The cells were then transferred into pre-chilled electroporation cuvettes with 0.2 cm electrode gap, and pulsed once in a GenePulser^{TM} (Bio Rad) set at 2.5 kV, resistance 1000 Ω, capacitance 25 µF. Immediately after pulsing, 1 ml Middlebrook 7H9M-ADC was added to the cells which were transferred to a clean microcentrifuge tube and incubated for two hours at 37 ^{o}C. Electroporation reactions were plated on solid Middlebrook 7H10 agar containing the appropriate antibiotics and supplements and incubated for 3 days at 37^{o}C or 5 days at 30 ^{o}C.

***Mycobacterium tuberculosis*.** Electroporation competent *M. tuberculosis* cells were prepared according to essentially the same method as that described or *M. smegmatis*, except that all manipulations were performed at room temperature (Wards & Collins, 1996). Briefly, 300 ml stirred or rolling cultures were inoculated from freezer stocks, and grown with aeration for approximately 7 days until an OD_{600} of 0.8-1.0 was achieved. At least 6 hours before harvesting, glycine (30% w/v) was added to the cultures to a final concentration of 1.5% (w/v). Cells were harvested by centrifugation (1100 x g) for 15 minutes and then washed twice in 10% glycerol (v/v) before resuspending in 5 ml 10% glycerol. Cells were divided into 400 µl aliquots in electroporation cuvettes with 0.2 cm electrode gaps, and pulsed once in a GenePulser^{TM} (Bio Rad) set at 2.5 kV, resistance 1000 Ω, capacitance 25 µF. After pulsing, the cells were rescued in 1 ml Middlebrook 7H9M-ADC and incubated overnight at 37^{o}C without shaking. Electroporation reactions were plated on solid Middlebrook 7H10 agar containing the appropriate antibiotics and supplements and incubated for 21-28 days at 37^{o}C.

2.3 DNA EXTRACTION

2.3.1 Plasmid preparation from *Escherichia coli*

Small-scale preparation for screening cloned plasmids. Aliquots of saturated overnight cell cultures were transferred into 1.5 ml microcentrifuge tubes. Cells were pelleted by centrifugation at room temperature (15000 x g for 30 seconds), the supernatant discarded, and the cells lysed by alkaline lysis. Briefly, cells were resuspended in 100 µl Lysis Solution I (0.5 M Glucose, 50 mM Tris-HCl pH 8.0, 10mM EDTA), to which 200 µl Lysis Solution II (0.2 M NaOH, 1% SDS) was then added and gently mixed. After 5 minutes on ice, 150 µl Lysis Solution III (3 M Potassium Acetate, pH 5.5) was added and, after gentle mixing, centrifuged at 9000 x g for 5 minutes in a Heraeus microcentrifuge at 4^{o}C. The supernatant was isolated and treated with RNaseA (50 µg/ml) for 15 minutes at 42^{o}C. DNA was precipitated by adding 350 µl isopropanol, incubating at room temperature for 10 minutes, and then centrifuging for 10 minutes at 15000 x g. After discarding the supernatant, the DNA pellet was washed once in ice-cold 70% ethanol and dried at 42^{o}C in a vacuum centrifuge (SpeedVac, Savant, Farmingdale, NY, USA). DNA was then resuspended in 10-20 µl sterile water for immediate digestion by restriction enzyme, or was further purified by phenol/chloroform (1:1 v/v) extraction in 400 µl sterile water followed by a chloroform/isoamyl alcohol (24:1 v/v) extraction. DNA was then re-precipitated by adding 1/10 volume sodium acetate buffer (pH 5.3) and 2.5 volumes 100% ice-cold ethanol. After 30 minutes incubation at -20^{o}C, precipitated DNA was collected by centrifugation at 15000 x g at 4^{o}C, before a final ice-cold 70% ethanol wash and drying in a vacuum centrifuge.

Large-scale plasmid preparation. Cultures were grown overnight at 37^{o}C in 100 ml LB, with shaking at 300-400 rpm. Cells were harvested by centrifugation in a Beckmann J2-21 centrifuge using a JA-20 rotor for 10 minutes at 1100 x g and 4^{o}C. The remainder of the extraction procedure was the same as for the small-scale preparation described above, except that volumes were increased by a factor of 10.

2.3.2 Preparation of mycobacterial chromosomal DNA

Chromosomal DNA was isolated from *M. tuberculosis* and *M. smegmatis* using a modified CTAB (cetyltrimethylammonium bromide; ICN Biomedicals, Aurora, Ohio) method (Larsen, 2000). Mycobacterial cells were heat killed by incubating at 95^{o}C for 5 minutes. Cells were harvested by centrifugation at 13000 x g for 30 seconds in microcentrifuge tubes, and resuspended in 500 µl TE buffer (pH 8.0) with 50 µl lysosyme (10 mg/ml). After

1-2 hours at 37^{o}C, 70 µl 10% SDS and 6 µl proteinase K (10 mg/ml) were added and the samples incubated for an additional 2 hours at 65 ^{o}C before the addition of 100 µl 5M NaCl and 80 µl CTAB/NaCl solution (10% CTAB prepared in 0.7M NaCl). After a further 10 minutes incubation, the solution was extracted with chloroform/isoamyl alcohol (24:1 v/v) and the DNA precipitated with an equal volume isopropanol. The DNA precipitate was collected by centrifugation at 9000 x g and washed with 70% ice-cold ethanol, then vacuum dried and resuspended in sterile water.

2.4 DNA MANIPULATIONS

2.4.1 Agarose gel electrophoresis

Standard electrophoretic techniques were used (Sambrook *et al.*, 1989; Sambrook & Russell, 2001). Gels (0.8-2%) were prepared in 1xTAE from agarose powder (SeaKem). Low melting agarose powder (SeaPlaque GTG) was used for preparative gel electrophoresis of DNA. For the separation of low molecular weight DNA fragments (≤ 1kb), 4% gels were prepared using NuSieve® GTG® agarose (Cambrex Bio Science Rockland, Rockland, ME, USA). Gels were electrophoresed in a Hoefer HE 33 minigel horizontal submarine unit (Pharmacia Biotech, California, USA) at 75-100 volts. DNA molecular weight markers were from Roche Biochemicals (BMI-BMVI).

2.4.2 Recovery of DNA fragments from agarose gels

Agarase digestion of low melting temperature agarose gels

DNA was isolated from low melting temperature agarose gels as per manufacturer's instructions (Roche Biochemicals). Briefly, DNA fragments were excised from low melting agarose gels and heated for 10 minutes at 75^{o}C in 1 x agarase buffer. The tube was transferred to a 45^{o}C heating block, and incubated with 1 unit agarase enzyme per 100 µl for one hour. The mixture was chilled on ice for 15 minutes, then centrifuged (9000 x g for 10 minutes at 4^{o}C). The supernatant was isolated, and DNA recovered by ethanol precipitation before drying and resuspending in sterile water at a final concentration of 0.1-1 µg DNA/µl.

Recovery of DNA fragments by the GeneClean® II glass milk procedure

DNA was isolated as per manufacturer's instructions (Qbiogene). Briefly, weighed gel slices containing the fragment of interest were melted at 65^{o}C for 15 minutes with 3 volumes sodium iodide solution. 5 µl freshly resuspended glass milk was added, and DNA allowed to adhere by incubating at room temperature for 10 minutes. The glass milk was

then collected by centrifugation and washed with 700 µl New Wash solution. Finally, the pellet was air dried at room temperature for 10-20 minutes, and the DNA eluted in 10-15 µl sterile water. The glass milk was pelleted by centrifugation at 13000 x g for approximately 30 seconds, and the supernatant aspirated and transferred to a clean microcentrifuge tube.

2.4.3 Enzymatic modification of DNA

Restriction digests. All restriction enzymes were obtained from Roche Molecular Biochemicals, Amersham, or New England Biolabs, Inc. Up to 1 µg plasmid DNA was digested in 10 µl reactions for 1 hour at 37^{o}C (unless manufacturer's instructions indicated otherwise) with the appropriate buffer. Mycobacterial chromosomal DNA (up to 5µg) was digested overnight at 37^{o}C (unless manufacturer's instructions indicated otherwise).

Blunt-ending of 5' overhangs using the Klenow fragment of *E. coli* DNA polymerase I. Klenow enzyme and dNTPs were supplied by Roche Molecular Biochemicals. Reactions were performed in 10-20µl according to manufacturer's instructions, and incubated for 30 minutes at 37^{o}C.

Dephosphorylation of vector DNA. Calf intestinal (CIP) and shrimp (SAP) alkaline phosphatases were supplied by Roche Molecular Biochemicals. Reactions were performed according to the manufacturer's instructions for 30 minutes at 37^{o}C. DNA treated with CIP was purified by phenol/chloroform extraction and ethanol precipitation, whereas SAP was inactivated by heat denaturation at 65^{o}C for 15 minutes.

Ligations of DNA fragments. All DNA ligations were performed using the Epicentre® Fast-Link™ ligation kit as per the manufacturer's instructions. For cloning of plasmid constructs, vector and insert DNA were used at a maximum total amount of 200 ng/10 µl reaction. Approximately 1-5 µl of the ligation reaction was used to transform 100-200 µl competent *E. coli* cells.

Polymerase chain reaction (PCR). Oligonucleotide primers are listed in the materials and methods sections specific to each chapter. All standard PCR reactions were performed using the Roche FastStart Taq DNA Polymerase kit (Roche Molecular Biochemicals). For PCR of fragments required for knockout constructs, or for sequencing reactions, the Expand High Fidelity PCR System (Roche Molecular Biochemicals) was used. Standard 20 or 50 µl PCR reactions in 1 x supplied reaction buffer contained 5-100 ng plasmid or genomic DNA, 0.5-1.0 µM of each of the forward and reverse primers, 10 µM of each dNTP, 1.5 mM $MgCl_2$, and 10% DMSO or supplied GC-RICH solution. Cycling parameters were as follows: denaturation at 94^{o}C for 5 minutes, 30 cycles of denaturation (94^{o}C for 60 seconds), annealing (50-65^{o}C for 60 seconds), and extension (72^{o}C for 60

seconds), followed by a final extension at 72^{o}C for 7 minutes. PCR reactions were performed in an Eppendorf Mastercycler gradient. For construction of knockout or expression vectors, gel purified PCR products (2.4.2) were cloned into pGEM®-T Easy or TOPO 2.1 PCR cloning vectors (Table 2.2) according to manufacturer's instructions and sequenced before subsequent manipulations.

2.4.4 Southern blot analysis of mutant mycobacterial strains

Radiolabelling of probes. All probes for Southern blots were labelled using a random prime labelling technique. Briefly, probe DNA was dissolved in 14 µl sterile water and then heat-denatured at 96^{o}C for 10 minutes before rapid cooling on ice. The DNA was then labelled with [α^{32}P]-dCTP using the Random Primed Labelling Kit (Roche Biochemicals) according to the manufacturer's instructions. The labelling reaction was terminated by the addition of 50 µl 1 x TE buffer (pH 8.0), and labelled DNA separated from unincorporated nucleotides by passing through two pre-equilibrated G-25 Sephadex spin columns poured in 1 ml disposable syringes plugged with sterile glass wool. The purified probe was collected by centrifugation of the columns at 4400 x g for 3 minutes in an IEC clinical centrifuge. The purified probe was used immediately for Southern analysis, or stored at -20 ^{o}C for a maximum of 1 day.

Electroblotting. Chromosomal DNA (1-5 µg) digested with the appropriate restriction enzyme was separated by agarose gel electrophoresis (0.8-1.0%). The DNA was depurinated by soaking the gel in 0.25M HCl for 15 minutes, then denatured by soaking in 0.5M NaOH/1.5M NaCl solution for 15-20 minutes. The gel was briefly equilibrated in 1 x TBE buffer, overlayed with Hybond™-N nylon membrane, and sandwiched between two layers of pre-soaked 3MM Whatman filter and two sponges in a TE 22 Transphor cassette. The cassette was then inserted into a TE 22 Mini Transphor unit (Hoefer) and the DNA transferred at 0.5A for 2 hours at 4^{o}C. The DNA was then cross-linked to the nylon membrane by irradiation in a UV Stratalinker 1800 at 1200 mJ/cm^{2}.

Hybridisation. After cross-linking, membranes were incubated in pre-hybridisation solution (0.5% SDS; 6 x SSC; 5 x Denhardts; 50% deionised formamide) containing 10 µg/ml heat-denatured salmon-sperm DNA (Roche) at 42 ^{o}C for 2 hours in roller bottles in a Techne Hybridiser HB-1. The radiolabelled probe was denatured at 96^{o}C for 10 minutes and added to the pre-hybridisation buffer, and hybridisation allowed to occur overnight (12-16 hours) at 42 ^{o}C. The hybridisation buffer was then removed, and the filters washed twice in the hybridisation bottles with 2 x SSC; 0.1 % SDS for 15 minutes at 42^{o}C, then once with 0.5 x SSC; 0.1% SDS for 15 minutes at 42^{o}C, followed by one wash with 0.1 x

SSC; 0.1% SDS for 15 minutes at 42^{o}C, and finally a wash with 0.1 x SSC; 1% SDS for 30 minutes at 65^{o}C. The membranes were then exposed to X-ray film at -70^{o}C.

2.5 DNA SEQUENCING

All sequencing was performed off-site by Inqaba Biotech on a Spectrumedix 2410 Capillary Electrophoresis automated DNA sequencer using Big Dye Terminator V3.1 software from ABI for result analysis.

2.6 STATISTICAL ANALYSES

The statistical significance of differences between data sets was determined using GraphPad InStat® software. Analysis of mutation rates from fluctuation assay data was performed on a set of Microsoft Office Excel spreadsheets developed by E. Machowski, L. Machowski & S. Barichievy (unpublished) using algorithms adapted from Rosche and Foster (2000) and is described in Chapter 3.

Chapter 3

The mycobacterial Y-family polymerases: roles in genetic adaptation and long-term survival

3.1 ABSTRACT

SOS-inducible Y-family DNA polymerases have been implicated in the adaptive evolution of antibiotic- and stress-resistant mutants of pathogenic bacteria. In mycobacteria, induced base substitution mutagenesis to rifampicin and streptomycin resistance is dependent on DnaE2, a novel C-family polymerase. However, mycobacterial genomes encode several DinB (pol IV)-like Y-family members suggesting an alternative role in adaptation. Neither *dinP*- or *dinX*-encoded pol IV homologue is upregulated in the mycobacterial damage response. In contrast, *M. smegmatis* contains an additional pol IV homologue that is SOS-inducible. Disruption of all three pol IV homologues had no effect on the damage hypersensitivity of a *M. smegmatis dnaE2* deletion mutant, suggesting the dispensability of these polymerases in tolerance of UV-induced damage. Furthermore, overexpression of *M. tuberculosis dinP* and *dinX* did not increase spontaneous point or frameshift mutation rates in wild-type mc^{2}155 and did not restore damage tolerance in the hypersensitive mutant. Similarly, abrogation of all identifiable pol IV or EP polymerase activity in *M. smegmatis* mc^{2}155 failed to impair long-term competitive survival. Treatment of *M. smegmatis* with ofloxacin and novobiocin confirmed the differential induction of the pol IV homologues in response to replication stalling and, significantly, showed that elevated rates of spontaneous mutagenesis to rifampicin resistance are associated with GyrB inhibition. Genotypic analysis of these and other damage- and stress-induced rifampicin-resistant mutants identified a novel *rpoB* polymorphism that appears to be restricted to strains lacking one or more pol IV homologue. In combination, the data suggest that the mycobacterial pol IV homologues might not be individually redundant, but do not participate in adaptive mutagenesis. Rather, regulatory mechanisms might have evolved in mycobacteria to restrict Y-polymerase activity to error-free repair or replication restart in times of stress. Finally, attempts to disrupt *dinP* in *M. smegmatis* mc^{2}155 confirmed the presence of a duplicated chromosomal region. Furthermore, during the construction of an *M. smegmatis* deletion mutant lacking both copies of *dinP*, a mutant strain was fortuitously generated which appeared to have lost the duplicated region. The deletion mutation was found to have no discernable effect either on the growth rate of the organism or on its transformation efficiency, arguing against an association between the duplication in mc^{2}155 and its *ept* phenotype. Site-specific re-introduction of a wild-type *dinP* allele into this deletion mutant resulted in the creation of a strain isogenic to mc^{2}155 but lacking the duplicated region.

3.2 INTRODUCTION

The heavy reliance on chemotherapeutic intervention in active, infectious disease renders the global TB control effort especially vulnerable to the emergence of drug resistant *M. tuberculosis* strains. Furthermore, the observation (Boshoff *et al.*, 2003; Mizrahi *et al.*, 2005) that measured rates of antibiotic resistance of *M. tuberculosis in vitro* (David, 1970; David & Newman, 1971; Boshoff *et al*., 2003) do not correlate with the rapid emergence of MDR-TB clinical isolates worldwide (Espinal *et al*., 2000, 2003; WHO, 2004; Blower & Chou, 2004; Cohen & Murray, 2004) implies that a number of factors might act in combination to reduce treatment efficacy or increase mutation rates during parasitism of the human host. Considerable attention has recently been focused on the potential roles of stationary-phase and stress-induced mutagenesis in bacterial adaptation (Zambrano *et al*., 1993; Taddei *et al*., 1995; Kasak *et al.*, 1997; Zinser & Kolter, 1999; McKenzie *et al.*, 2000; Bhamre *et al*., 2001; Bull *et al*., 2001; Sung & Yasbin, 2002; Bjedov *et al.*, 2003; Sung *et al.*, 2003; Tegova *et al.*, 2004; Tenaillon *et al.*, 2004). In particular, the association of inducible mutation pathways with the emergence of drug-resistance isolates of pathogenic bacteria (Phillips *et al*., 1987; Ysern *et al*., 1990; Riesenfeld *et al*., 1997; Ren *et al*., 1999; Alonso *et al*., 1999; Oliver *et al*., 2000; Björkholm *et al*., 2001; Denamur *et al*., 2002; Visser, 2002) is considered especially relevant to the generation of antibiotic- and stress-resistance mutations in *M. tuberculosis*, whose microevolution within the hostile host environment is driven by genetic rearrangement and point mutations (Ramaswamy & Musser, 1998; Gutacker *et al.*, 2002). In most bacterial systems studied to date, adaptation to environmental stress is predicated on the activity of SOS-inducible, error-prone (EP) repair polymerases of the Y polymerase superfamily (McKenzie *et al.*, 2001; Yeiser *et al.*, 2002; Sung *et al.*, 2003; Tegova *et al.*, 2004). Members of the Y-family likely evolved to promote mutation avoidance and damage tolerance through a specialised ability to replicate across a variety of DNA lesions; however, the flipside of this ability is that the very properties enabling translesion synthesis (TLS) are implicated in mutagenesis (Friedberg *et al.*, 2001, 2002; Yang, 2003; Kunkel, 2004; Tippin *et al.*, 2004b).

3.2.1 The Y-polymerase superfamily

Efficient DNA replication requires both high speed and high fidelity (Friedberg *et al.*, 2002). Despite the presence of multiple DNA repair systems in most bacteria (Eisen & Hanawalt, 1999), some potentially replication-blocking DNA lesions escape detection, necessitating rapid TLS polymerase-mediated bypass (Goodman, 2002; Friedberg *et al.*,

2002, 2003; Tippin *et al.*, 2004b). Although it now seems obvious that specialised DNA polymerases might have evolved to replicate across lesions, the discovery that the replicative flexibility they provide can incur error rates two to four orders of magnitude greater than those of replicative polymerases (Kunkel, 2004) demanded a redefinition of traditional concepts of DNA synthesis fidelity. Moreover, the fact that many of these polymerases are upregulated in response to stress suggests a potential role in induced mutagenesis (Friedberg *et al.*, 2001; 2002). Most TLS polymerases fall into the Y-polymerase superfamily comprising a wide range of structurally-related proteins present in bacteria, archaea and eukaryotes (Ohmori *et al.*, 2001). Of these, the DinB subfamily – represented by *E. coli* pol IV (Wagner *et al.*, 1999) - is the most widespread (Ohmori *et al.*, 2001). Members of the Y-family catalyse low-fidelity synthesis on non-substrate templates such as undamaged DNA or non-cognate lesions, lack intrinsic 3'→5' proofreading exonuclease activity, adopt a distributive mode of DNA replication, and are able to support TLS of DNA lesions which might block replication by members of the other polymerase families (Friedberg *et al.*, 2001, 2002; Goodman, 2002). In particular, the identification of EP Y-family repair polymerases has raised significant questions pertaining to the structural relation of members of this family to the other main DNA polymerases, the features enabling faithful bypass of DNA lesions but low-fidelity synthesis of undamaged or non-cognate DNA and, finally, the co-ordination at a replication fork of multiple different polymerase classes (Friedberg *et al.*, 2001, 2002; Yang, 2003; Kunkel, 2004). Although Y-family polymerases share little sequence homology with the other polymerase families, structural analyses have confirmed a similar overall architecture (Ling *et al*., 2001; Silvian *et al*., 2001; Trincao *et al*., 2001; Zhou *et al*., 2001; Yang, 2003). Critically, the active site of Y-family polymerases is sufficiently flexible to allow bypass of lesions that might distort replicative polymerase geometry, but with a consequent reduction in stringency (Ling *et al.*, 2001; Friedberg *et al.*, 2002; Yang, 2003; Kunkel, 2004).

3.2.2 Heritable mutators

Mutation rates can be modified by heritable and environment-dependent mechanisms (Metzgar & Wills, 2000). Heritable mutator mechanisms are independent of the environment and are often associated with mutations in DNA replication or repair genes that confer constitutive mutator phenotypes (Horst *et al*., 1999). Alternatively, specific regions of a genome may perturb DNA polymerase activity, resulting in replication slippage errors comprising single-motif insertion and deletion events (InDels) (Kunkel & Bebenek, 2000). Such hotspots of mutation are typically associated with "contingency genes" encoding strongly selected structures such as surface antigens or

restriction/modification systems (Moxon *et al*., 1994; Field *et al*., 1999; Moxon & Wills, 1999). The inherent sequence repetition, together with evidence for frequent InDels in mycobacterial PE and PPE genes, for example, is suggestive of repeat expansions and contractions at these loci (Brosch *et al*., 2001; Banu *et al*., 2002). Small proportions of heritable mutators characterise commensal and chronic pathogenic bacterial populations *in vivo*, consistent with the idea that an elevated mutation rate may promote adaptation to the fluctuating host environment (Matic *et al*., 1997; Taddei *et al*., 1997b; Oliver *et al*., 2000; Björkholm *et al*., 2001; Denamur *et al*., 2002; Visser, 2002). However, the deleterious consquences of a constitutive mutator phenotype (Boe *et al*., 2000) ensure that maintenance of the mutator allele depends on genetic linkage to the resultant beneficial mutation (Boe *et al*., 2000; Visser, 2002) and, in general, regaining the wild-type genotype is essential to the long-term survival of the population (Giraud *et al*., 2001a, 2001b).

3.2.3 Adaptive evolution of mutation rates

Inducible (environment-dependent) mutators, on the other hand, act in a genome-wide manner to increase mutation rates specifically in response to applied stress (Metzgar & Wills, 2000). Those cells that survive produce progeny cells with normal mutation rates, thereby reducing the risk of unchecked mutagenesis. Whereas the acquisition of a mutator phenotype is a random event, it has been proposed that inducible mutagenesis is an adaptive response that has evolved by second-order selection to modulate mutation rates while limiting the costs associated with a constitutive mutator phenotype (Metzgar & Wills, 2000; Radman *et al*., 2000; Tenaillon *et al*., 2001, 2004). Alternatively, the pleiotropic hypothesis holds that stress-induced mutagenesis merely reflects the inevitable consequence of molecular constraints on survival mechanisms (Tenaillon *et al*., 2004). Whatever the selective force governing their evolution, the selection of inducible mutator mechanisms is inferred from the negative correlation between stress-induced mutagenesis and constitutive mutators, as well as by the observation that, where stresses are frequent or of long duration, inducible mutators are selected as efficiently as mutator alleles (Bjedov *et al*., 2003).

3.2.4 SOS-inducible polymerases

While MMR-deficient strains constitute nearly all identified natural mutators (LeClerc *et al*., 1996; Matic *et al*., 1997), several environment-dependent mutator systems have been implicated in induced mutagenesis (Taddei *et al*., 1995, 1997a; Hughes & Andersson, 1997; Bridges, 1998; Bjedov *et al*., 2003). Of these, the well-characterised *E. coli* SOS

response is considered the prototype. Significantly, the SOS regulon includes the EP repair polymerases, pol V (UmuD'C) and pol IV (DinB) (Kenyon & Walker, 1980; Bagg *et al.*, 1981; Walker *et al.*, 2000; Courcelle *et al.*, 2001). Pol V provides the dominant TLS activity following DNA damage in *E. coli* (Rangarajan *et al.*, 1999), the fidelity of synthesis depending on the nature of the lesion (Reuven *et al*., 1999; Tang *et al*., 1999, 2000; Maor-Shoshani *et al*., 2003a). In particular, pol V has been implicated in the high proportion of lesion-targeted, base substitution mutations arising after UV treatment of *E. coli* (Lawrence *et al*., 1990; LeClerc *et al*., 1991; Tang *et al*., 1999; 2000). Pol IV, on the other hand, possesses limited TLS ability (Napolitano *et al*., 2000; Tang *et al*., 2000; Kim *et al.*, 2001; Shen *et al*., 2002; Wagner *et al*., 2002). Furthermore, unlike pol V (Rangarajan *et al.*, 1999), deletion of pol IV does not result in damage hypersensitivity (Kim *et al.*, 1997). Similarly, although overexpression of pol IV dramatically increases spontaneous mutation rates (Kim *et al*., 1997, 2001; Wagner & Nohmi, 2000; Kobayashi *et al*., 2002), decreasing intracellular pol IV levels has a less profound effect (Strauss *et al*., 2000; Kim *et al*., 2001; McKenzie *et al*., 2001; Kuban *et al*., 2004; Wolff *et al*., 2004). Instead, pol IV has been proposed to re-initiate stalled replication forks by extending transiently misaligned primer-template strands (Wagner *et al*., 1999; Kim *et al*., 2001; Kobayashi *et al*., 2002; Goodman, 2002). Consistent with this role, *E. coli* pol IV generates small, non-targeted frameshift mutations *in vivo* (Brotcorne-Lannoye *et al*., 1986; Kim *et al*., 1997).

3.2.5 Stress-induced mutagenesis and stationary-phase adaptation

A common misconception is that spontaneous mutations arise as a consequence of errors made exclusively during DNA replication in actively growing cells. However, it is well established that mutations arise in apparently static (or non-dividing) bacterial populations subjected to non-lethal selective conditions (Ryan, 1955; Cairns *et al.*, 1988; Taddei *et al*., 1995; Hughes & Andersson, 1997; Karunakaran & Davies, 2000). Furthermore, the increased periods spent in stationary phase by most cellular populations ensures that even very low mutation rates can lead to the accumulation of high mutation frequencies per cell (Bridges, 1998). Prolonged exposure of bacterial populations to non-lethal selective pressure elevates mutation rates by a process known as adaptive or stationary-phase mutagenesis (Foster, 2000; McKenzie & Rosenberg, 2001). Among the mutation mechanisms reported are transposition events (Modi *et al*., 1992; Naas *et al*., 1994), substitution and frameshift mutations (Zambrano *et al*., 1993; Zinser & Kolter, 1999; Bhamre *et al*., 2001; Bull *et al*., 2001; Sung *et al.*, 2003; Tegova *et al.*, 2004), and gene amplifications (Sonti & Roth, 1989; Andersson *et al.*, 1998; Hastings *et al.*, 2000; Hendrickson *et al*., 2002). In general, stress is likely to arise as a consequence of the

specific ecological niche inhabited and is compounded by host immune effectors. However, the presence of antibiotic concentration gradients *in vivo* might result in lethal antibiotics causing non-lethal stress (Baquero & Negri, 1997; Baquero *et al.*, 1998). Moreover, the importance of stress-related mutagenesis in the emergence of resistant bacteria has been repeatedly demonstrated, and is particularly relevant to the application of bacteriostatic selective pressure (Phillips *et al*., 1987; Ysern *et al*., 1990; Riesenfeld *et al*., 1997; Ren *et al*., 1999; Alonso *et al*., 1999; Oliver *et al*., 2000; Björkholm *et al*., 2001; Denamur *et al*., 2002; Visser, 2002; Boshoff *et al.*, 2003).

Several lines of evidence implicate SOS-induced mutagenesis in adaptive mutation pathways. SOS genes are upregulated in stationary bacterial populations (Dri & Moreau, 1994; Taddei *et al.*, 1995) and endogenous DNA damage has been implicated in mutagenesis in apparently static cells (Benov & Fridovich, 1996; Hall, 1998). Both SOS induction, as well as SOS-regulated pol IV, are required for the generation of stationary-phase but not growth-dependent point mutations in *E. coli* (Cairns & Foster, 1991; Foster, 2000; McKenzie *et al.*, 2000, 2001, 2003). Furthermore, strains defective in the repair of DNA lesions exhibit increased rates of adaptive mutation (Rebeck & Samson, 1991; Bridges, 1995; Bridges *et al*., 1996; Pedraza-Reyes & Yasbin, 2004). In addition, *E. coli* pol IV is induced by the general stress-response σ factor, RpoS, during long-term starvation (Layton & Foster, 2003). Significantly, the involvement of EP repair polymerases in stationary-phase mutation is consistent with the emergence in restrictive environments of mutants of increased fitness (Zambrano *et al*., 1993; Zambrano & Kolter, 1993, 1996; Foster, 1999; Smeulders *et al*., 1999; Zinser & Kolter, 1999; Yeiser *et al.*, 2002). Furthermore, the detection of resistant phenotypes after prolonged exposure to a specific antibiotic is reminiscent of adaptive mutagenesis, and hints at the possible involvement of inducible mutator polymerases in such cases (McKenzie & Rosenberg, 2001; Rosenberg, 2001).

3.2.6 The stationary phase in mycobacteria

The physiological state adopted by *M. tuberculosis* during latent clinical infection is poorly understood despite being crucial to propagation and sustained cycles of infection (Parrish *et al*., 1998; Stewart *et al*., 2003; Gomez & McKinney, 2004). Although often referred to as dormant, in its strictest sense this term should be reserved for a “reversible state of low metabolic activity in which cells can persist for extended periods without cell division” (Kell *et al*., 1995). In particular, emergence from dormancy requires specific molecular signals – such as resuscitation promoting factors (Rpfs) (Mukamolova *et al*., 1998) - in contrast to

stationary-phase exit, which is induced by a favourable change in environmental conditions. Although possibly containing a proportion of dormant cells, clinically latent *M. tuberculosis* infections are thought to comprise heterogeneous populations of mostly metabolically-active bacilli maintained in a state of non-growth or prolonged stationary phase (Canetti, 1955; Rees & Hart, 1961; Hu *et al*., 2000; Bouley *et al.*, 2001; Grosset, 2003; Muñoz-Elías *et al.*, 2005) by a combination of nutrient limitation (McKinney *et al.*, 2000; Dahl *et al.*, 2003; Timm *et al.*, 2003) and environmental stress (Loebel *et al*., 1933; Nyka, 1967, 1974; Nathan & Shiloh, 2000; Fenhalls *et al*., 2002b; Shi *et al*., 2003). In such populations, a state of slow-replicating or nonreplicating persistence ensures limited cell turnover and a constant net viable count (Rees & Hart, 1961; Parrish *et al*., 1998; Muñoz-Elías *et al.*, 2005).

3.2.7 Inducible mutagenesis in mycobacteria

M. tuberculosis possesses the components of a functional LexA/RecA-regulated SOS response (Durbach *et al*., 1997; Movahedzadeh *et al*., 1997a, 1997b; Papavinasasundaram *et al*., 1997; Mizrahi & Andersen, 1998; Davis *et al.*, 2002a). In addition, there is evidence of hypermutability (Karunakaran & Davies, 2000) and adaptive mutation (Smeulders *et al*., 1999) during stationary-phase growth in *M. smegmatis*. The identification of genes encoding putative Y-family homologues (Cole *et al.*, 1998; Mizrahi & Andersen, 1998) was, therefore, strongly suggestive of the presence in mycobacteria of mutagenic processes under cellular control. However, since the inception of this study, several other groups have failed to observe the *M. tuberculosis* pol IV homologues in screens of DNA damage-response genes (Brooks *et al*., 2001; Boshoff *et al*., 2003; Rand *et al*., 2003). Instead, the *dnaE2*-encoded alternative catalytic (α) subunit of DNA polymerase III represents the only polymerase that is induced in the mycobacterial SOS regulon (Boshoff *et al.*, 2003). Furthermore, DnaE2 has been shown to be the central player in damage-induced base substitution mutagenesis in *M. tuberculosis* (Boshoff *et al*., 2003), identifying the mycobacterial polymerase as the founder member of a novel family of *dnaE*-type family C polymerases from Gram-positive bacteria that catalyse TLS synthesis (Tippin *et al*., 2004b). An *M. tuberculosis dnaE2* deletion mutant exhibits damage hypersensitivity *in vitro*, and is attenuated *in vivo* in a murine infection model (Boshoff *et al.*, 2003). Moreover, abrogation of DnaE2 activity *in vivo* is associated with reduced emergence of drug resistance mutations. Coupled with the induction of *dnaE2* during stationary infection, these observations implicate DnaE2-mediated DNA repair in virulence and in the adaptive evolution of drug resistance during persistent infection (Boshoff *et al.*, 2003). Furthermore, the phenotypes associated with loss of DnaE2 activity

suggest that this family C polymerase is functionally analogous to *E. coli* SOS-inducible pols IV and V in many respects and might, therefore, have usurped the predicted physiological roles of the identified mycobacterial pol IV homologues (Mizrahi *et al.*, 2005).

3.2.8 Aims and objectives of this study

At the inception of this work, the components of a functional mycobacterial LexA/RecA-dependent damage response had been identified and characterised (Mizrahi & Andersen, 1998; Durbach *et al*., 1997; Movahedzadeh *et al*., 1997a, 1997b; Papavinasasundaram *et al*., 1997). In particular, it was known that the *dinP*- and *dinX*-encoded mycobacterial DinB (pol IV) homologues were not included in the inducible polymerase complement of the SOS regulon (Davis *et al.*, 2002a). However, evidence implicating Y-polymerases (then referred to as the UmuC/DinB/Rev1/Rad30 polymerase superfamily; Gerlach *et al.*, 1999) in damage tolerance and adaptive evolution in other bacteria suggested a link between pol IV gene function, stress-induced mutagenesis, and the emergence of drug resistance in mycobacteria, which was explored in both *M. tuberculosis* and the non-pathogenic *M. smegmatis*. The specific objectives extended primarily to the genetic characterisation of the mycobacterial pol IV homologues, and were:

1. To investigate the mutagenic capacity of the pol IV homologues by determining whether overexpression of *M. tuberculosis dinP* or *dinX* resulted in increased rates of spontaneous mutagenesis.
2. To establish whether the mycobacterial pol IV homologues were implicated in adaptive, or stress-induced, mutagenesis.

During the course of this study, three significant findings were reported which impacted on the original stated aims: firstly, the characterisation of an alternative LexA/RecA-independent damage-response pathway (Rand *et al*., 2003) which, like the SOS response, did not include the *dinP*- and *dinX*-encoded pol IV homologues; secondly, and most importantly, the identifiction of the *dnaE2*-encoded C-family polymerase as the primary mediator of inducible mutagenesis in mycobacteria (Boshoff *et al.*, 2003); and, finally, evidence that the dinP-encoded pol IV homologue might be implicated in replication restart (Boshoff *et al.*, 2004). As a result of these data, the aims were modified to include the following:

3. Given the dominant role of *dnaE2*, to investigate whether the mycobacterial pol IV homologues contributed at all to damage tolerance, and whether the homologues were individually redundant.
4. An attempt to identify conditions under which the pol IV homologues were induced, if any.
5. An analysis of the role of the additional, Rv3394c-encoded SOS-inducible Y-family polymerase in damage tolerance.

3.3 MATERIALS AND METHODS

3.3.1 Mycobacterial strains, plasmids, and probes

The strains used in this study are detailed in Table 3.1, plasmids are listed in Table 3.2, probes in Table 3.3, and primers in Table 3.4.

Table 3.1 Strains used in this study

Strain	Description	Reference/Source
M. smegmatis		
dinP$^{+/-}$	Single *dinP* knockout mutant of mc^{2}155; Hyg^R	This study
dinP$^{-/-}$	*dinP* null mutant of mc^{2}155; Hyg^R	This study
ΔDR	Deletion mutant in duplicated region of mc^{2}155; derivative of *dinP*$^{+/-}$; Hyg^R	This study
*dinP*Kin	Deletion mutant in duplicated region of mc^{2}155 with reconstituted wild-type *dinP* allele; derivative of ΔDR; unmarked	This study
Δ*dinP3*	*dinP3* (MSMEG6405) deletion mutant of mc^{2}155; unmarked	S. Dawes
Δ*dinX*	*dinX* deletion mutant of mc^{2}155; Hyg^R	This study
dinP$^{-/-}$/*dinP3*	Double *dinP* and *dinP3* deletion mutant of mc^{2}155; derivative of *dinP*$^{-/-}$; Hyg^R	This study
dinP$^{-/-}$/*dinX*	Double *dinP* and *dinX* deletion mutant of mc^{2}155; derivative of *dinP*$^{-/-}$; Hyg^R	This study
ΔY	Triple *dinP*, *dinP3* and *dinX* deletion mutant of mc^{2}155; derivative of *dinP*$^{-/-}$/*dinP3*; Hyg^R	This study
ΔC	Quadruple *dinP*, *dinP3*, *dinX* and *dnaE2* deletion mutant of mc^{2}155; derivative of ΔY; Hyg^R Km^R	This study
M. tuberculosis		
ΔRv3394c	Rv3394c deletion mutant of *M. tuberculosis* H37Rv; Hyg^R	This study
ΔRv3394c/ ΔRv3395c	Double ΔRv3394c/ ΔRv3395c deletion mutant of *M. tuberculosis* H37Rv; Hyg^R	This study

Table 3.2 Plasmids used in this study

Plasmid		
Promoter analysis		
pAI*dinP*	pAINT containing 631 bp upstream plus 29 bp internal *M. tuberculosis dinP* sequence	H. Boshoff
pAI*dinX*	pAINT containing 1009 bp upstream plus 32 bp internal *M. tuberculosis dinX* sequence	H. Boshoff
pAI3055	pAINT containing 1009 bp upstream plus 36 bp internal *M. tuberculosis* Rv30055 sequence	H. Boshoff
pEM28w	RBS*lacZ* (promoterless) from pATB12 in pHINT; Hyg^R Amp^R	E. Machowski
pEM*dinP*	pEM28w containing 1815 bp *Asp*718-*Eco*RV fragment from pAI*dinP*	This study
pEM*dinX*	pEM28w containing 2214 bp *Eco*RV fragment from pAI*dinX*	This study
pEM3055	pEM28w containing 2213 bp *Eco*RV fragment from pAI3055	This study
Overexpression vectors		
pOLYGaa	Multicopy *E.coli–Mycobacterium* shuttle vector with inducible acetamidase promoter; Hyg^R	This study
pOLYGaa::*dinP*	Multicopy *E.coli–Mycobacterium* shuttle vector overexpressing *M. tuberculosis dinP* off the inducible acetamidase promoter; Hyg^R	This study
pOLYGaa::*dinX*	Multicopy *E.coli–Mycobacterium* shuttle vector overexpressing *M. tuberculosis dinX* off the inducible acetamidase promoter; Hyg^R	This study
***M. smegmatis* knockout constructs**		
p2Δ*dinP*	p2NIL carrying Δ*dinP* allele; Km^R	This study
p2Δ*dinP::hyg*	p2Δ*dinP* with *hyg* cassette from pIJ963 cloned in *Bam*HI site; Km^RHyg^R	This study
p2Δ*dinP*17	*hyg*-marked *dinP* knockout vector - p2Δ*dinP*::*hyg* containing *Pac*I cassette from pGOAL17; $Km^RHyg^RSuc^S$	This study
p2Δ*dinP*19	Unmarked *dinP* knockout vector - p2Δ*dinP* containing *Pac*I cassette from pGOAL19; $Km^RHyg^RSuc^S$	This study
p2*dinP*	p2NIL carrying 2749 bp TdinPF1R2 PCR fragment containing mc^2155 *dinP* plus 1660 bp flanking sequence; Km^R	This study
p2*dinP*Kin	Unmarked *dinP* "knock-in" vector – p2*dinP* containing *Pac*I cassette from pGOAL17; Km^RSuc^S	This study
p2Δ*dinX*	p2NIL carrying Δ*dinX* allele; Km^R	This study
p2Δ*dinX*::*hyg*	p2Δ*dinX* with *hyg* cassette from pIJ963 cloned in *Bgl*II site; Km^RHyg^R	This study
p2Δ*dinX*17	*hyg*-marked *dinX* knockout vector - p2Δ*dinX*::*hyg* containing *Pac*I cassette from pGOAL17; $Km^RHyg^RSuc^S$	This study
p2Δ*dinX*19	Unmarked *dinX* knockout vector - p2Δ*dinX* containing *Pac*I cassette from pGOAL19; $Km^RHyg^RSuc^S$	This study
*dinP3*ZHB	Knockout vector carrying unmarked deletion allele of mc^2155 *dinP3* (MSMEG6405) and *Pac*I cassette from pGOAL19; $Km^RHyg^RSuc^S$	S. Dawes
pGmsmE2KO	Knockout vector carrying *aph*-marked deletion allele of mc^2155 *dnaE2* and *Pac*I cassette from pGOAL17; $Amp^RKm^RHyg^RSuc^S$	H. Boshoff
***M. tuberculosis* knockout constructs**		
p2ΔRv3394c	p2NIL carrying ΔRv3394c allele; Km^R	This study
p2ΔRv3394c::*hyg*	p2ΔRv3394c with *hyg* cassette from pIJ963 cloned in *Bgl*II site; Km^RHyg^R	This study
p2ΔRv3394c17	*hyg*-marked Rv3394c knockout vector - p2ΔRv3394c::*hyg* containing *Pac*I cassette from pGOAL17; $Km^RHyg^RSuc^S$	This study
p2ΔRv3394/5c	p2NIL carrying ΔRv3394/5c allele; Km^R	This study
p2ΔRv3394/5c::*hyg*	p2ΔRv3394/5c with *hyg* cassette from pIJ963 cloned in *Bam*HI site; Km^RHyg^R	This study
p2ΔRv3394/5c17	*hyg*-marked Rv3394/5c knockout vector - p2ΔRv3394/5c::*hyg* containing *Pac*I cassette from pGOAL17; $Km^RHyg^RSuc^S$	This study

3.3.2 Construction of overexpression vectors

The pAGAN11 vector, carrying the 4.1 Kb inducible acetamidase gene of *M.smegmatis* as a *Bam*HI insert (Parish *et al.*, 1997), was obtained from Tanya Parish. The insert was excised as a *BamHI* fragment and cloned into pOLYG in the opposite direction to the *hyg* cassette in order to minimise possible read through effects, yielding a vector termed pOLYGaa (Table 3.2). The *M. tuberculosis dinP* and *dinX* regions were amplified from genomic DNA using oligonucleotide primers (Table 3.4) containing artificial *Eco*RV restriction sites, and cloned into the TOPO 2.1 PCR cloning vector. The 1091 bp DINPFR1 PCR product contained the entire *M. tuberculosis* H37Rv *dinP* gene plus 30 bp upstream sequence, while the the 1472 bp DINXFR1 fragment contained the entire *M. tuberculosis* H37Rv *dinX* and 65 bp upstream sequence. Sequenced PCR products were digested with EcoRV and cloned into the blunted *Cla*I site of the pOLYGaa vector, to generate inducible expression vectors overexpressing *M. tuberculosis dinP* (pOLYGaa::*dinP*) and dinX (pOLYGaa::*dinX*), respectively. Initial experiments were performed in liquid media supplemented with 2g/l acetamide (Sigma) to induce expression of the vector, or succcinate as a control (Sigma), as described (Parish *et al.*, 1997). However, semi-quantitative RT-PCR determined that the vector was leaky and that the relative expression in mc^2155 strains containing either pOLYGaa::*dinP* or pOLYGaa::*dinX* *versus* the vector control (pOLYGaa) was sufficient to invalidate the need for acetamide induction.

Table 3.3 Probes used in this study

Probe		
TdinPFR1	948 bp PCR product containing 45 bp 5' *dinP* coding sequence	This study
TdinPFR2	880 bp PCR product containing 44 bp 3' *dinP* coding sequence	This study
P3p	798 bp *Eco*Rv-*Xba*I fragment from *dinP3*ZHB	S. Dawes
MsXFR2	1744 bp PCR product containing 87 bp 3' *dinX* coding sequence	This study
MsmE2	780 bp *Asp*718 fragment from pGmsmE2KO	H. Boshoff
3395FR1	1763 bp PCR product containing 213 bp 3' *M. tuberculosis dinP* coding sequence	This study

3.3.3 Construction of reporter probes and measurement of β-galactosidase activities

Construction of reporter probes

Transcriptional reporters were constructed by cloning the promoter regions of *M. tuberculosis dinP* (pEM*dinP*) and *dinX* (pEM*dinX*) into a reporter construct (pEM28w; E.

Machowski, unpublished) containing a promoterless *lacZ* gene. The pEM*dinP* construct contained 631 bp sequence upstream of *dinP* plus 29 bp internal *dinP* sequence and was generated by cloning the 1815 bp *Asp*718-*Eco*RV fragment from pAI*dinP* (H. Boshoff, unpublished) into pEM28w. Both pEM3055 and pEM*dinX* were similarly created by cloning the 2214 bp *Eco*RV fragment from pAI*dinX* (H. Boshoff, unpublished) and the 2213 bp *Eco*RV fragment from pAI3055 (H. Boshoff, unpublished) into pEM28w to yield reporter constructs comprising 1009 bp upstream plus 37 bp internal *dinX* sequence (pEM*dinX*) and 1009 bp sequence upstream of Rv3055 plus 36 bp internal Rv3055 sequence (pEM3055), respectively.

Measurement of β-galactosidase activities

β-galactosidase assays were performed using modifications of a previously described method (Ghanekar *et al.*, 1999). Absorbance readings (OD_{600}) of individual *M. smegmatis* cultures were determined at each timepoint, and 2ml aliquots harvested in duplicate by centrifugation at 4^{o}C (5000 rpm for 5 min). Cell pellets were washed in 1ml pre-chilled Z-buffer (60mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$; 50 mM β-mercaptoethanol), resuspended in 900µl Z-buffer before transferring to a Lysing Matrix A tube (Qbiogene), and lysed in a ribolyser (Savant Fastprep FP120) for 2 cycles (15 seconds at speed 4) with cooling on ice for 2 minutes between pulses. The samples were then tansferred to clean microcentrifuge tubes, the cell debris pelleted by centrifugation, and the lysate aspirated and transferred to another clean microcentrifuge tube and stored on ice. To assess β-galactosidase activity in each sample, 100 µl of the above lysate was made up to 900 µl with Z-buffer, and 200 µl ONPG (2-nitrophenyl β-D-galactopyranoside, 8 mg/ml stock; Sigma) added. The mixture was incubated at 37°C for 80 min, before reading the absorbance of each sample at 420 nm (OD_{420}), using Z buffer as the blank. β-gal activity (Miller units) was calculated acording to the following equation:

$$\text{β-gal activity (Miller units)} = (OD_{420} \times 1000)/(OD_{600} \times T \times V)$$

where, T = time (min); V = volume (ml) of lysate

To determine specific activity, 200ul of the lysate was made up to a volume of 800ul with Z-buffer, and 200µl Bradford's reagent added and mixed gently by inversion. The mixtures were incubated at room temperature for approximately 10 minutes, and the absorbances read at 595 nm (OD_{595}). Protein concentrations were determined with reference to a standard curve which was established simulataneously by performing the same assay on an appropriate range of known concentrations of BSA (Roche). Specific activity (Miller units per mg of protein) was then determined by dividing calculated β-galactosidase activities by the relative protein concentrations.

3.3.4 Construction of the marked and unmarked suicide delivery vectors

***M. smegmatis* Δ*dinP*.** The sequences of the putative *M. smegmatis dinP* and *dinX* genes were obtained by BLAST homology search (Altschul *et al*., 1990) of the preliminary *M. smegmatis* genome sequence database at The Institute for Genomic Research (TIGR; http://www.tigr.org) using the respective *M. tuberculosis* gene sequences (TubercuList; http://www.pasteur.fr/Bio/TubercuList) as probes. Two primer sets, TdinPF1/TdinPR1 and TdinPF2/TdinPR2 (Table 3.4) were designed to amplify sequence upstream and downstream of the putative *M. smegmatis dinP* gene from wild-type mc^{2}155 genomic DNA. Ligation of the PCR products TdinPFR1 (948 bp) and TdinPFR2 (880 bp) eliminated 982 bp *dinP* coding sequence. Sequenced PCR products were cloned into p2NIL (Parish and Stoker, 2000) to yield p2Δ*dinP*. To mark the deletion allele, the *Bam*HI/*Bgl*II *hyg* cassette (1738 bp) from pIJ963 (Blondelet-Rouault *et al.*, 1997) was inserted into the deletion junction between upstream and downstream PCR fragments to generate p2Δ*dinP*::*hyg*. Finally, the 6359-bp *Pac*I fragment from pGOAL17 (*lacZ-sacB*; Parish and Stoker, 2000), or the 7939-bp *Pac*I fragment from pGOAL19 (*hyg-lacZ-sacB*; Parish & Stoker, 2000) was cloned into the corresponding site of the vector carrying the marked (deletion-insertion; p2Δ*dinP*::*hyg*) or unmarked (deletion; p2Δ*dinP*) allele, generating p2Δ*dinP*17 and p2Δ*dinP*19, respectively.

***M. smegmatis dinP*Kin.** PCR amplification of *M. smegmatis* mc^{2}155 genomic DNA using the TdinPF1 and TdinPR2 primers generated a 2749 bp fragment containing the entire mc^{2}155 dinP gene plus 877 bp upstream and 783 bp downstream sequence. The sequenced fragment was cloned into p2NIL (Parish and Stoker, 2000) to generate p2*dinP*, *and* the p2*dinP*Kin vector created by the addition of the 6359-bp *Pac*I fragment from pGOAL17 (*lacZ-sacB*; Parish and Stoker, 2000).

M. smegmatis* Δ*dinX. The *M. smegmatis* Δ*dinX* deletion allele was constructed in the same manner as the Δ*dinP* deletion allele. Briefly, two primer sets, MsXF1/MsXR1 and MsXF2/MsXR2 (Table 3.4) were used to amplify sequence upstream and downstream of the putative *M. smegmatis dinX* gene from wild-type mc^{2}155 genomic DNA. Ligation of the PCR products MsXFR1 (2107 bp) and MsXFR2 (1727 bp) eliminated 1185 bp *dinX* coding sequence. Sequenced PCR products were cloned into p2NIL (Parish and Stoker, 2000) to generate p2Δ*dinX*. To mark the deletion allele, the *Bam*HI/*Bgl*II *hyg* cassette (1738 bp) from pIJ963 (Blondelet-Rouault *et al.*, 1997) was inserted into the deletion junction between upstream and downstream PCR fragments, thereby creating p2Δ*dinX*::*hyg*. Finally, the 6359-bp *Pac*I fragment from pGOAL17 (Parish and Stoker, 2000), or the 7939-bp *Pac*I fragment from pGOAL19 (Parish & Stoker, 2000) was cloned into the relevant marked or unmarked deletion allele to generate p2Δ*dinX*17 and p2Δ*dinX*19, respectively.

Table 3.4 Oligonucleotide primers used in this study

Name	Sequence (5'-3')	Comments[†]
TaqMan analysis of *M. tuberculosis dinP* and *dinX*		
DINP-F	AAGCAGCGCGCCAAGA	forward primer
DINP-R	CGGTGAGCTGATAGATGCCA	reverse primer
DINP-PR	CGCTACCGGGTTGGCGAAACC	probe
DINX-F	TGACCACCATGGAGCAATTG	forward primer
DINX-R	CGTTGGTGCGCGTGC	reverse primer
DINX-PR	ACGAGGCGATCGACTCCATCGCT	probe
sigAF	GACGAGGAGATCGCTGAACC	forward primer
sigAR	TCGTCTTCATCCCAGACGAAA	reverse primer
sigA-PR	CCGAAAAGGACAAGGCCTCCGG	probe
***M. tuberculosis* polIV overexpression constructs**		
DINPF1	GGGGGGATATCGAAAACTGTGCGGACGATGAT	*Eco*Rv
DINPR1	CCCCCCTATAGATCCGGTCAAGATTGGCGTGA	*Eco*Rv
DINXF1	GGGGGGATATCGCCATAGCGAAAACTAGCTCG	*Eco*Rv
DINXR1	CCCCCCTATAGTGGCCAGCGGCTGCAGTAG	*Eco*Rv
***M. smegmatis* mc^{2}155 *dinP* knockout allele**		
TdinPF1	GCGGCGAAGCTTCGTTGTAGAACGGCACCT	*Hin*dIII
TdinPR1	CGGCCGGGATCCACCGAAGCCAGGAACTG	*Bam*HI
TdinPF2	CGGCGGGGATCCGGTCCGGTTGCTCGGGGT	*Bam*HI
TdinPR2	CGGCGGGGTACCCTGGCGCCGTCGAACGCG	*Asp*718
***M. smegmatis* mc^{2}155 *dinX* knockout allele**		
MSXF1	CGGCCCCATGGGCGCGTA	*Asp*718
MSXR1	GGGGGCTCTAGAGGGTGG	*Bgl*II
MSXF2	GGGCTCTAGACGGCGCGGCT	*Bgl*II
MSXR2	GGCGCTCTTCGAACCAGTGGT	*Hin*dIII
***M. smegmatis rpoB* sequencing**		
MsmrpoBF1	GCAGACCCTGATCAACATCC	forward primer
MsmrpoBR1	ACGTCGCGGACCTCGAGG	reverse primer
Semi-quantitative RT-PCR of *M. smegmatis* pol IV homologues		
*dinP*F	GGGATCAGTGACAACAAGCA	forward primer
*dinP*R	CCATCACCGTCATCCAGTTC	reverse primer
*dinX*F	GACGTCGAGGCGTTCTGT	forward primer
*dinX*R	TCTTGGCGATCTGTTTACCC	reverse primer
*dinP3*F	CGAGGCGTTCCTCGACGTAT	Courtesy R. Brackin
*dinP3*R	CACCAAGTTCCTGGCCAAGG	Courtesy R. Brackin
***M. tuberculosis* Rv3394c and Rv3395c knockout alleles**		
3395F1	GCCCCGTTCGAAAGCCTA	*Hin*dIII
3395R1	CCCCCTTCTAGAGGCGCCT	*Bgl*II
3395F2	GCCGGCTCTAGACGAGCCT	*Bgl*II
3395R2	GGCGCCCCATGGGCTCCA	*Asp*718
3395F3	GGGGCCGGATCCACCCTGGT	*Bam*HI
3395R3	GCGCGGGGTACCGGTTCTT	*Asp*718

[†]Restriction sites utilised for subsequent cloning of PCR fragments are underlined where appropriate, and the relevant enzyme detailed under Comments.

***M. tuberculosis* ΔRv3394c and ΔRv3394c/ΔRv3395c.** The *M. tuberculosis* ΔRv3394c and ΔRv3394c/ΔRv3395c deletion alleles were constructed using the same methods as those described above for *M. smegmatis.* Briefly, three primer sets - 3395F1/3395R1, 3395F2/3395R2, and 3395F3/3395R3 (Table 3.4) - were designed to amplify sequence upstream and downstream of wild-type *M. tuberculosis* H37Rv Rv3394c and Rv3395c. Ligation of the PCR products 3395FR1 (1745 bp) and 3395FR2 (1900 bp) eliminated 1014 bp Rv3394c coding sequence to create the ΔRv3394c allele; ligation of 3395FR1 (1745 bp) and 3395FR3 (1899 bp) eliminated the entire Rv3395c gene as well as 1371 bp Rv3394c coding sequence to create the ΔRv3394/5c allele. Sequenced PCR products were cloned into the relevant sites of suicide vector p2NIL (Parish and Stoker, 2000) to generate p2ΔRv3394c and p2ΔRv3394/5c, respectively. To mark the deletion allele, the *Bam*HI/*Bgl*II *hyg* cassette (1738 bp) from pIJ963 (Blondelet-Rouault *et al.*, 1997) was inserted into the deletion junction between upstream and downstream PCR fragments, thereby creating p2ΔRv3394c::*hyg* and p2ΔRv3394/5c::*hyg*. Finally, the 6359-bp *Pac*I fragment from pGOAL17 (*lacZ-sacB*; Parish and Stoker, 2000), was cloned into the marked deletion alleles to generate p2ΔRv3394c17 and p2ΔRv3394/5c17, respectively.

3.3.5 Mutation rate (μ) assessment by fluctuation assay

Rif^R mutations

Rates of forward mutations to Rif^R in *M. smegmatis* strains overexpressing *M. tuberculosis dinP* or *dinX* were determined by Luria-Delbrück fluctuation assay, and analysed according to Rosch & Foster (2000). Briefly, a single colony of each *M. smegmatis* strain was picked from a fresh electroporation plate, and used to inoculate a pre-culture (7H9M-ADC plus 50 μg/ml Hyg) which was grown to log phase (OD600 ~.1.0). Cells were harvested by centrifugation (13000 rpm for 1 min), diluted in 0.5 % Tween80, and used to inoculate a starting culture (7H9M-ADC plus 50 μg/ml Hyg) at a concentration of approximately 100 CFU/ml. Aliquots were plated in duplicate on plain 7H10M-OADC for initial CFU counts (N_0), and on 7H10M-OADC containing 200 μg/ml Rif for assessment of possible revertants in the starting culture. The starting culture was aliquoted into parallel 15 ml culture tubes to yield individual 2.5 ml cultures of approximately 100 CFU/ml. The number of tubes used was dependent on the *m* value (Rosche & Foster, 2000), and was determined over the course of the study to be satisfied by experiments comprising at least 7 parallel tubes. The tubes were incubated in a New Brunswick series 25 Orbital incubator (New Brunswick, Inc, USA) at 300-400 rpm at 37^{o}C until log phase (OD_{600} ~ 1.0) was reached. Before harvesting, Tween80 was added to each culture tube to a final concentration of 0.5%; and mixed by gently vortexing. Cultures were diluted serially in

0.5% Tween80, and plated on plain 7H10M-OADC or on 7H10M-OADC plus 200 ug/ml Rif to determine number of Rif^R mutants and final CFU (Nt) values, respectively.

Adapted protocol for assessment of frameshift reversion rates to histidine prototrophy

Freezer stocks of the *M. smegmatis* histidine auxotroph strains were streaked on 7H10 plates supplemented with 100 µg/ml histidine as well as 7H10 plates without histidine to confirm histidine auxotrophy before onset of the fluctuation assay. A single colony was inoculated into 10 mL Middlebrook 7H9 liquid medium containing 100 µg/ml histidine and grown to log phase (OD_{600} ~ 1.0). A 1 ml aliquot was harvested (13000 rpm for 1 min) and washed twice in 1ml 0.5% Tween80 (13000 rpm, 1 min) to remove any residual supplement. After the final wash, cells were re-suspended in 1ml 0.5% Tween80. A dilution series was established in 0.5% Tween80, and used to inoculate a starting culture (Middlebrook 7H9 without histidine supplement) at a concentration of approximately 100 CFU/ml, as per above. Aliquots were plated in duplicate on 7H10M-OADC without supplement for assessment of possible revertants in the starting culture, and on 7H10M-OADC containing 100 µg/ml histidine for initial CFU counts (N_0). The starting culture was aliquoted into parallel 15 ml culture tubes to yield 2.5 ml cultures, and incubated as per above. Before harvesting, Tween80 was added to each culture tube to a final concentration of 0.5%; and mixed by gently vortexing. The entire volume of each culture tube was aspirated into 2 ml microcentrifuge tubes, and cells harvested by centrifugation (13000rpm, 1min). Cells were then re-suspend in 500µl 0.5% Tween80, combined into single microcentrifuge tubes, and washed twice in 1ml 0.5% Tween80 to remove any residual supplement. After the final wash, cells were re-suspended in 1ml 0.5% Tween80, diluted serially, and plated on solid 7H10 agar with or without histidine supplement to determine Nt values or number of revertants, respectively.

Determination of mutation rates

Rates of frameshift reversion to histidine prototrophy or mutations to Rif^R were determined from at least three independent fluctuation assay experiments as per algorithms described in Rosche & Foster (2001). Briefly, the Lea-Coulsen Method of the Median (Rosche & Foster, 2000) was used to generate *m* values which were refined by iteration on a set of Microsoft Office Excel spreadsheets generated by E. Machowski, L.Machowski & S. Barichievy (unpublished). According to the MSS algorithm, the probability (p_r) of observing all the experimental values of the number of mutants (r) for a given *m* is calculated until an *m* is identified that maximises the likelihood function for r = 0 to150 (Ma *et al.*, 1992; Rosche & Foster, 2000). The mutation rate is then calculated by dividing *m* (the mean number of mutations per culture) by the total number of cells in the culture, N_t.

3.3.6 Damaging treatments of mycobacteria

Mycobacterium tuberculosis

For Taqman analyses of *M. tuberculosis dinP* and *dinX* expression in response to damage, duplicate 10 ml cultures were grown to an OD_{650} of 0.3–0.4 and were treated with 2mM *S*-nitrosoglutathione (GSNO; Sigma) or 4 mM H_2O_2 (Sigma) and incubated for 12 hours at 37^{o}C. For DNA damage by UV, cells were split into parallel 5 ml aliquots in 78 cm^2 dishes and open dishes irradiated with 20 mJ/cm^2 (UV Stratalinker 1800, Stratagene) before being recombined in 10 ml volumes and incubated at 37^{o}C for 24 hr.

Mycobacterium smegmatis

UV survival of *M. smegmatis* mc^{2}155 strains was determined according to Boshoff *et al.*(2003). Briefly, duplicate serial dilutions of log phase cultures of each strain were plated in triplicate on solid 7H10M-ADC without any antibiotic. Open plates were irradiated at the specific UV fluence (UV Stratalinker 1800, Stratagene) and then incubated at 30 ^{o}C for 3-4 days until visible CFUs were enumerated. For analysis of induced mutagenesis in response to UV damage, log phase cultures were harvested and resuspended in 6 ml aliquots in 78 cm^2 dishes and irradiated with 20 mJ/cm^2 (UV Stratalinker 1800, Stratagene). After irradiation of open dishes, cells were resuspended in fresh media and allowed to grow for specific intervals before aliquots were plated on solid 7H10M-ADC without antibiotic (for viable CFU assessment) or containing 200 µg/ml Rif (for determination of Rif^R frequencies). To monitor the mutation frequencies of *M. smegmatis* strains in response to novobiocin (NVB) treatment, log phase cells were exposed to specific NVB concentrations for defined time intervals before plating as above.

3.3.7 RNA isolation and first-strand cDNA synthesis

RNA was isolated according to the method of Betts *et al.* (2002). Briefly, 50 ml cultures were harvested by centrifugation (1100 x g for 10 minutes) and pellets resuspended in 1 ml TRIzol (Gibco-BRL) on ice. The cell suspensions were immediately transferred to tubes containing Lysing Matrix B (Qbiogene) and lysed in a ribolyser (Savant Fastprep FP120) for three cycles (20 seconds at speed 6) with cooling on ice for 1 min between pulses. Samples were then centrifuged (13000 rpm for 45 seconds), and the solution above the beads and cellular debris removed to a tube containing 200 ml of chloroform and Phase Lock gel (heavy; Sigma). Samples were inverted rapidly for 15 s and then periodically for 2 min before centrifugation in an Eppendorf 5415D microcentrifuge (13000 rpm for 5 minutes). The top aqueous layer was transferred to a fresh tube and an equal volume of isopropanol added and mixed well. Precipitated nucleic acids were collected by centrifugation (12000 x g for 20 min at 4^{o}C), and pellets washed with 70% ethanol and air

dried. Crude RNA samples were treated with DNase I (Ambion) by incubation at 37^{o}C for 20 min and purified further using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Samples were then subjected to a final DNase I treatment using the Ambion DNA-*free*™ kit, according to manufacturer's instructions. The quality of purified total RNA was assessed by gel electrophoresis (2% agarose; 0.1% SDS).

Reverse Transcription

All RT reactions were performed in an Eppendorf MasterCycler Gradient using the method modified from Manganelli *et al.* (1999). Briefly, 20 µl reactions were set up in DEPC-treated H_2O comprising 20 ng/µl of total RNA and 0.25 µM of each antisense primer. RNA was denatured at 94^{o}C for 90 seconds, and then annealing of the RNA and antisense primers alowed to occur at 65^{o}C for 3 minutes followed by 3 minutes at 57^{o}C. RNA was reverse transcribed in 20 µl reactions comprising 10 µL of the annealing mixture, 1x Avian Myeloblastoma Virus (AMV) RT Buffer (Sigma), 200 µM each dNTP (Sigma), 4 mM $MgCl_2$ (Sigma), 0.6 µl Dimethyl Sulphoxide (DMSO; Sigma) and 2 U Enhanced AMV RT (Sigma), which were incubated for 30 min at 60^{o}C, heated at 95^{o}C for 5 min and chilled to 4^{o}C. Control reactions containing no AMV RT were established in parallel to assess levels of contaminating DNA. Samples were then diluted with 70ml H_2O and stored at -70^{o}C.

3.3.8 Real-Time, Quantitative RT-PCR Assay (Taqman)

First-strand cDNA synthesis was performed as described above (3.3.7) and real-time quantitative PCR performed on the ABI Prism 7700 sequence detection system using the PE Applied Biosystems Taqman PCR core reagent kit. Taqman primer and probe sets were designed on the ABI Prism 7700 sequence detection system using Primer Express software and are described in Table 3.4. RNA levels were normalised to *sigA* gene expression (Manganelli *et al.*, 1999; Boshoff *et al.*, 2003). Gene expression was determined using two independent induction experiments using a control untreated cell sample for each time point. Each RNA sample was independently reverse transcribed twice and tested in triplicate for each probe-primer set.

3.3.9 Semi-quantitative RT-PCR of the mc^{2}155 response to gyrase inhibitors

Primers used for semi-quantitative RT-PCR were designed using the Primer3 design programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are detailed in Table 3.4. All primers were designed to amplify DNA fragments internal to the coding sequence of the relevant genes with the same annealing temperatures.

PCR amplifications

The standard 50 µl PCR reactions consisted of 1x FastStart PCR buffer without $MgCl_2$ (Roche), 4 mM $MgCl_2$ (Roche), 250 µM each dNTP (Roche), 5 µl DMSO (Sigma), 0.5 mg/ml BSA (Roche), 0.5 µM of each primer, 2.5 U FastStart Taq DNA Polymerase (Roche) and 2 µl cDNA from the RT reaction or genomic DNA. Cycling parameters were as follows: an initial heat activation step of 95^{o}C for 10 minutes and 15 cycles stringent amplification (95^{o}C denaturation for 30 seconds, 65^{o}C anealing for 30 seconds, and 72^{o}C polymerisation for 30 seconds) was followed by a second round of amplification (95^{o}C denaturation for 30 seconds, 57^{o}C anealing for 30 seconds, and 72^{o}C polymerisation for 30 seconds). The amount of contaminating chromosomal DNA was tested in parallel using the control samples. Samples were separated on 4% agrose gel (2.4.1).

3.3.10 Long-term competitive survival assays

Competitve survival of *M. smegmatis* mc^{2}155 strains was determined essentially according to Smeulders *et al.* (1999). Briefly, aerobic cultures comprising equal concentrations of *hyg*-marked deletion mutants and wild-type mc^{2}155 were set up in 50 ml liquid 7H9M-ADC without antibiotic in 500 ml Ehrlenmeyer flasks. Pure cultures of each strain were also set up in parallel in order to control for possible long-term survival defects. Cultures were incubated at 30^{o}C with slow rotation (100 rpm), and media were not refreshed at any stage during the course of the experiment. At each timepoint, aliquots were harvested in duplicated, and serial dilutions plated in triplicate on solid 7H10-MOADC containing no antibiotic (for assessment of total viable CFU) and 50 µg/ml Hyg (for assessment of mutant CFU). To avoid problems associated with clumping at later timepoints, aliquots were harvested and resuspended in 0.5% Tween80 before diluting in 0.5 % Tween80. Aliquots from the pure cultures were also plated on solid 7H10M-OADC containing 200 µg/ml Rif to allow determination of mutation frequencies.

3.3.11 Sequencing of the *rpoB* locus of Rif^R *M. smegmatis* mutants

Extraction of DNA for sequencing. DNA was extracted from Rif^R colonies using a small-scale extraction method. Briefly, Rif^R colonies were picked from Middlebrook 7H10 plates containing 200 µg/ml Rif and chromosomal DNA extracted by boiling each colony for 20 minutes in a solution of 40 µL chloroform and 20 µL of distilled water at 100^{o}C. Centrifugation followed for 5 minutes at 10000 x g. The supernatant was used immediately as PCR template or stored at -20^{o}C.

Amplification and sequencing. The *rpoB* region was amplified with the MsmrpoBF1/MsmrpoBR1 primer set and using the Expand High Fidelity PCR System

(Roche Molecular Biochemicals). For the PCR reaction, 2 µl of the boiled mixture was included in a 20 µl reaction comprising 1 x supplied reaction buffer, 1.0 µM of each of the forward and reverse primers, 10 µM of each dNTP, 1.5 mM $MgCl_2$, and 10% GC-RICH solution. Cycling parameters were as follows: denaturation at 95^{o}C for 5 minutes, 30 cycles of denaturation (95^{o}C for 60 seconds), annealing at 60^{o}C for 45 seconds, and extension at 72^{o}C for 45 seconds, followed by a final extension at 72^{o}C for 5 minutes. An aliquot (2µl) was removed for gel electrophoreses on a 4% agarose gel to confirm successful amplification as well as the approximate fragment size (183 bp in wild-type mc^2155). The remaining volume was dispatched for sequencing.

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3.4 RESULTS

3.4.1 The mycobacterial Y-family homologues

The crystal structures of various bacterial and eukaryotic Y-family homologues have revealed similar palm, finger and thumb domains characteristic of the catalytic cores of the high-fidelity replicative enzymes (Ling *et al*., 2001; Silvian *et al*., 2001; Trincao *et al*., 2001; Zhou *et al*., 2001). However, polymerases of the Y superfamily also contain a unique C-terminal "little-finger" (LF) domain (Ling *et al*., 2001; Silvian *et al.*, 2001; Trincao *et al.*, 2001) that is structurally conserved among Y-family members and has been shown to be essential for TLS *in vivo*, perhaps through an ability to bind regulatory co-factors (Haracska *et al*., 2001b; Kannouche *et al*., 2001). The interaction of *E. coli* pol IV with the β processivity factor, for example, is dependent on five residues at the extreme C-terminus of this domain (Figure 3.1) (Lenne-Samuel *et al.*, 2002) and is essential for pol IV-mediated bypass and mutagenesis (Becherel *et al*., 2002; Lenne-Samuel *et al.*, 2002). Significantly, conserved consensus pentapeptide motifs mediating β clamp binding have been identified in most eubacterial genomes (Dalrymple *et al.*, 2001; 2003) and have been implicated in the interaction of a number of protein families with β (Yang, 2000; López de Saro & O'Donnell, 2001; Dalrymple *et al.*, 2001, 2003; Ohmori *et al*., 2001; Noirot-Gros *et al*., 2002; Sung *et al*., 2003; Kurz *et al*., 2004).

The *M. tuberculosis* H37Rv genome contains two putative Y-family polymerases of the DinB sub-class (Figure 3.1, Figure 3.2) (Ohmori *et al.*, 2001), namely *dinP* (Rv3056) and *dinX* (Rv1537) (Cole *et al*., 1998). Consistent with the significant sequence homology DinP and DinX share with *E. coli* pol IV (Figure 3.1), these proteins are designated Pol IV 2 and Pol IV 1, respectively, on the TubercuList database (http://genolist.pasteur.fr/TubercuList/). In addition, based on predicted binding affinities for the β sliding clamp, *dinP* and *dinX* have been identified as DinB1.5 and DinB1.4 Y-family sub-class polymerases, respectively (Dalrymple *et al*., 2003). Interestingly, members of the DinB1.5 sub-class, including *M. tuberculosis* DinP (Figure 3.1), contain no identifiable β clamp binding region, suggesting that polymerases of this sub-class either do not bind the β clamp or bind through an alternative β-binding motif (Dalrymple *et al*., 2003). In addition to the pol IV homologues, the presence of a putative consensus β-binding motif recently identified *M. tuberculosis* Rv3394c as a novel DinB3-type Y-family polymerase (Dalrymple *et al.*, 2003). However, although containing putative DinB and IMS domains that are suggestive of a role in damage protection, Rv3394c lacks conserved acidic residues shown to be essential for polymerase activity (Figure 3.1). Therefore,

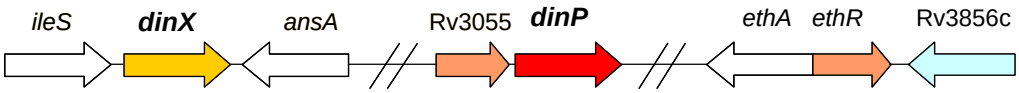

ecDinB.PRO
ssDpo4.PRO
mtdinP.PRO
mtdinX.PRO
msdinP3.PRO
mtRv3394c.PRO
ecUmuC.PRO

Figure 3.1 Sequence alignment of the mycobacterial Y-family polymerases. *M. tuberculosis* H37Rv DinP (mtdinP. PRO), DinX (mtdinX.PRO) and Rv3394c (mtRv3394c.PRO), and *M. smegmatis* mc^{2}155 DinP3 (msdinP3.PRO), are aligned with *E. coli* pol IV (ecDinB.PRO) and the UmuC subunit of *E. coli* pol V (ecUmuC.PRO). Based on structural data from *Sulfolobus solfataricus* P2 Dpo4 (ssDpo4.PRO), conserved residues known to be important for polymerase activity are boxed in red, and for structural integrity in green (Ling *et al.*, 2001). In addition, the little-finger domain secondary structures observed in *S. solfataricus* Dpo4 are represented by dark-blue arrows – representing β strands, and light-blue arrows – representing α-helices (Ling *et al.*, 2001). The consensus β-clamp-binding motifs (Dalrymple *et al.*, 2003) for *M. tuberculosis* DinX and Rv3394c, *M. smegmatis* DinP3, and *E. coli* pol IV (DinB) and UmuC, are contained within black boxes. *M. tuberculosis* DinP does not possess a β-binding motif (Dalrymple *et al.*, 2003). The absence in *M. tuberculosis* Rv3394c of conserved acidic residues required for polymerase activity should also be noted.

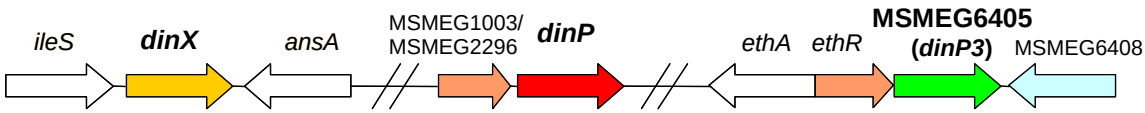
Rv3394c was excluded from the mycobacterial Y-polymerase complement for the purposes of this study.

The corresponding gene complement of *M. smegmatis* mc^{2}155 is more complex (http://www.tigr.org): there are two copies of *dinP* (MSMEG1002, and an unannotated ORF located between MSMEG2295 and MSMEG2296) as a result of a chromosomal duplication (see 3.4.4), as well as a single *dinX* homologue (MSMEG3178). In addition, the mc^{2}155 genome encodes another pol IV homologue (MSMEG6405) that is not present in *M. tuberculosis* H37Rv, but is found in *M. avium* subsp. *paratuberculosis* (http://www.tigr.org). For ease of reference, the additional mc^{2}155 pol IV homologue was provisionally designated *dinP3* (for third *dinP*-like gene; S. Dawes & R. Brackin, unpublished). *M. smegmatis* DinP3 belongs to the DinB1.2 Y-family sub-class containing a carboxy-terminal β-binding domain (Dalrymple *et al*., 2003; Figure 3.1). Significantly, bioinformatic analysis revealed the presence of a Cheo-type SOS box (Cheo *et al*., 1991) upstream of *M. smegmatis dinP3* (R. Brackin, unpublished). In addition, the LexA/RecA-mediated regulation of *dinP3* expression in response to DNA damage was recently confirmed (R. Brackin, unpublished). Critically, although sequence identities are strongly suggestive of DNA polymerase function (Figure 3.1), thus far of the mycobacterial Y-family homologues only *M. tuberculosis* DinX has been shown biochemically to possess polymerase activity (B. Kana, unpublished).

***M. tuberculosis* H37Rv**



***M. smegmatis* mc^{2}155**



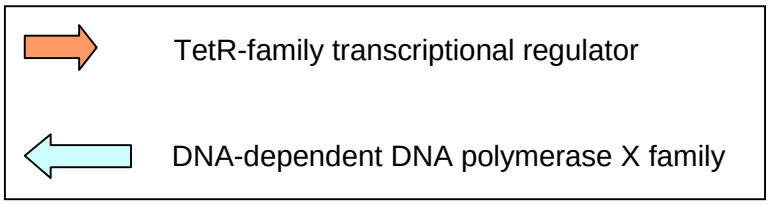


Figure 3.2 Genomic organisation of the mycobacterial pol IV homologues. *M. tuberculosis* H37Rv contains two pol IV homologues encoded by *dinP* and *dinX*. An additional, pol IV homologue, *dinP3* (MSMEG6405), is contained in the *M. smegmatis* mc^{2}155 genome. The absence of *dinP3* in *M. tuberculosis* distinguishes the two mycobacterial SOS responses (see Table 3.12). Rv3055-encoded TetR-family transcriptional regulators precede *dinP* in both genomes. An *ethR*-encoded TetR-family transcriptional regulator is situated directly upstream of *dinP3* in *M. smegmatis* mc^{2}155.

3.4.2 Regulation of *M. tuberculosis dinP* and *dinX*

The *M. tuberculosis* pol IV homologues are not DNA damage inducible

Previous studies have established that, despite the *din* or "*d*amage *in*ducible" designation, neither pol IV homologue (*dinP* or *dinX*) is upregulated following treatment of *M. tuberculosis* H37Rv with a variety of DNA damaging agents (Brooks *et al.*, 2001; Boshoff *et al.*, 2003). Although consistent with the dominant role of DnaE2 in SOS mutagenesis in mycobacteria (Boshoff *et al.*, 2003), the failure of DNA damage to cause upregulation of these genes contrasts with the SOS-inducibility of the Y-polymerases in other bacteria (Kenyon & Walker, 1980; Bagg *et al.*, 1981; Friedberg *et al.*, 1995; Walker *et al.*, 2000; Courcelle *et al.*, 2001; Sung *et al.*, 2003). Furthermore, the presence of multiple Y-family members in addition to *dnaE2*, coupled with evidence that recombinant DinX displays template-directed DNA polymerase activity *in vitro* (B. Kana, unpublished), raises several questions as to the function and regulation of these enzymes. In particular, evidence indicating a potential role for *M. tuberculosis dinP* following inhibition of DNA replication (Boshoff *et al.*, 2004) is reminiscent of a previous suggestion that regulation of mycobacterial damage response pathways might be lesion-specific (Boshoff *et al.*, 2003).

Table 3.5 Induction of *M. tuberculosis* pol IV homologues[†]

Treatment	*dinP*	*dinX*
GSNO (2 mM)	2.13 ± 2.36	0.50 ± 0.09
H_2O_2 (5 mM)	1.92 ± 0.99	0.35 ± 0.02
UV (20 mJ/cm^2)	2.04 ± 1.76	0.70 ± 0.09

[†]Fold induction relative to log phase expression and normalised to *sigA* gene

To confirm that neither Y-family homologue is induced in response to alternative DNA damaging agents, H37Rv was exposed to 2 mM *S*-nitrosoglutathione (GSNO) - an RNI source (St. John *et al.*, 2001), and 5 mM hydrogen peroxide (H_2O_2) – an ROI source (Firmani & Riley, 2002), as well as to UV irradiation, and expression of *dinP* and *dinX* determined by real-time, quantitative RT-PCR (Table 3.5). None of the DNA damaging treatments resulted in statistically significant alterations in *dinP* or *dinX* mRNA transcript levels relative to log phase, in accordance with previous reports (Brooks *et al.*, 2001; Boshoff *et al.*, 2003).

M. tuberculosis Rv3055 and *dinX* promoter regions are active in *M. smegmatis* during stationary phase *in vitro*

Genes encoding the SOS-inducible polymerases pol IV (*dinB*), pol II (*polB*) and pol V (*umuDC*) are transcribed during prolonged stationary phase in *E. coli* in the absence of exogenous DNA damaging agents (Yeiser *et al*., 2002). In addition, expression of *E. coli dinB* is further induced late in stationary-phase, potentially implicating pol IV in a global response to long-term starvation and stress (Layton & Foster, 2003, 2005). To investigate the expression profiles of the mycobacterial Y-family polymerases, transcriptional reporters were constructed in which the promoter regions of *M. tuberculosis dinP* (pEM*dinP*) and *dinX* (pEM*dinX*) were fused to a promoterless *lacZ* gene, and β-galactosidase activities monitored during *in vitro* growth of *M. smegmatis* mc^{2}155. The pEM*dinP* construct contained 631 bp sequence upstream of *dinP* plus 29 bp internal *dinP* sequence, while pEM*dinX* comprised 1009 bp upstream plus 37 bp internal *dinX* sequence. A putative TetR-like transcriptional regulator, encoded by Rv3055, is located immediately upstream of *dinP* in the H37Rv genome (Figure 3.2; Cole *et al.*, 1998). Therefore, another construct (pEM3055) was designed to examine expression of the putative regulator and contained 1009 bp sequence upstream of Rv3055 plus 36 bp internal Rv3055 sequence. Alignments of the *M. tuberculosis* promoter regions with the

A

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MtdinP:    46 CGCGAGG-CAA-GAGCCCGTACC--AG-AGCGGGGCGACGCGGCACGCAATCGTGCACTC 100
MsdinP:    28 CTCGAGGTCACCGACCGCGCGCCGCAGGAGCGCGGCGACGCCGCCCGGAACCGGGC-CGC 86

MtdinP:   101 C-TGTTGGAGGCGGCGCGCCGCCTGATCGCCCGAA-GCGGTGCGGACGCAATCACCATGG 158
MsdinP:    87 GATCCTCGACGCGGCGCGCCGGCTCATCGCC-GAGCGCGGACCCGACGCCGTGAGCACCG 145

MtdinP:   159 ACGACGTTGCCGCGGCCGCTGGCGTCGGCAAAGGCACCTTGTTTCGCCGCTTCGGCAGCC 218
MsdinP:   146 ACGACATCGCCGTCGAGGCCGGCGTCGGCAAGGGCACCCTGTTCCGCCGCTTCGGCACCC 205

MtdinP:   219 GTGCCGGCCTGATGATGGTGTTGCTCGATGAAGACGAGCGAGCCAGTCAGCAGGCCTTCC 278
MsdinP:   206 GCGCCGGGCTGATGATGGTGCTGCTCGACGAGGACGAGAAGGCCCACCAGGACGCGTTCA 265

MtdinP:   279 TGTTCGGCCCGCCACCGCTGGG-CCCGGATGCTCCGCCGCTGGACCGCCTGATCGCATTC 337
MsdinP:   266 TCTTCGGGCCGCCGCCGCTCGGTCCCGGG-GCCCCTCCCCTCGACCGTCTGCTCGCCTAC 324

MtdinP:   338 GGTCGGGAGCGAATGCGCTTCGTCCATGCCCATCAC-CAGCTGCTGTCGGAAGCCAACCG 396
MsdinP:   325 GGCCGCGAACGGATGAAGTTCGTCCGCGAGCACCACGCA-CTGCTCTCCGACGCCGCGCG 383

MtdinP:   397 GGATCCACAAACCCGCCACAGCGCGGCGCTATCGGTACTGCGCACCCATTTGCGGGTACT 456
MsdinP:   384 CGATCCGCAGGCACGCTTCGGCGGGCCGGACACGCTGCACCGGTCCCACGTCCGGATGCT 443

MtdinP:   457 GCTGGCCTCGGCGCCGACCACCGGCGACCTGGATGCCCAGACCGATGCCCTGCTAGCGCT 516
MsdinP:   444 GCTGCAGGCCGCGGGGACCACGGGCGACCTCGACGCACAGGCCGACGCGCTCGTCGCGCT 503

MtdinP:   517 GCTCGACGTCGACTATGTCGAGCACCAACTC-AACGCCGGCGGCCATACCCTGCAAAC-C 574
MsdinP:   504 GCTCGACGCCGACTACGTCGCCTACCAACTGGAACGCGGACG--CAG-C--TGCGAGGTC 558

MtdinP:   575 CTGGGCGACGCATGGGA-GAGCCTGGCGCGAAAACTGTGCGGAC 617
MsdinP:   559 CAGGCCGCCGCCTGGGCCGACACCG-CACGTAAGTTGTGTGGCC 601
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B

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Mt3055:   139 CAAGTCCGACATCAAAATCTTGGCCTTAGTGGGAAGCCTGCGCGCGGCGTCGTTCAACCG 198
Ms3055:    15 CATGGCCGAAATCAAGGTGTTGGTACTCGTCGGCAGCCTCCGGGCGGCGTCCATCAACCG 74

Mt3055:   199 CCAGATCGCCGAGCTGGCTGCCAAGGTCGCTCCGGACGGCGTC-ACCGTCACCATGTTCG 257
Ms3055:    75 CCAACTCGCCGAACTGGCAGTCGAGACCGCGCCCGACGGTGTGGACCTGCAGG-TGTTCG 133

Mt3055:   258 AGGGGCTGGGGGACCTGCCGTTCTACAACGAAGACATCGACACAGCGACGGAGGTGCCGG 317
Ms3055:   134 ACCGGCTGGCCGAGGTGCCGTTCTACAACGAAGACATCGACAC--CG-CTGATGTGCCCG 190

Mt3055:   318 CGCCGGTGAGCG-CGTTGCGGGAGGCCGCG-TCTGACGCGCACGCTGCCTTGGTGGTCAC 375
Ms3055:   191 AGCCGGCC-GCGGCGCTGCGTGCCGCGGCGGTC-GAGGCGGACGCAGCCCTGCTCGTCAC 248

Mt3055:   376 GCCGGAATACAACGGCAGCATTCCGGCCGTGATCAAGAACGCGATCGACTGGCTGTCCAG 435
Ms3055:   249 CCCCGAGTACAACGCCACCATCCCCGCCGTCCTCAAGAACGCCATCGACTGGCTGTCGCG 308

Mt3055:   436 GCCATTCGGCGATGGCGCGTTGAAGGACAAGCCGTTGGCCGTGATCGGCGGCTC-CATGG 494
Ms3055:   309 GCCGTACGGCAGTGGCGCGCTGCACGGCAAGCCGGCCGCCGTGATCGGCGCCGCGCACGG 368

Mt3055:   495 GCCGCTACGGCGGGGTATGGGCGCACGACGAGACTCGCAAGTCGTTCAGCATCGCTGGCA 554
Ms3055:   369 GC-GCTACGGCGGTGTGTGGGCGCACGACGAGACCCGCAAGTCGCTGGGCGTCGCAGGCC 427

Mt3055:   555 CGCGGGTGGTCGATG-CGATCAAACTGTCGGT-GCCGTTCCAAACTCTGG--G-CAAGTC 609
Ms3055:   428 CGCGCGTGGTCGAGGACG-TCAAGCTGTCGATCGCAGCGTCGA-CGCTGGACGACAAG-C 484

Mt3055:   610 GGTCGCG-GACGACGCCGGGCTGGCGGCGAATGTGCGCGACGCCGTCGGCAA-CTTG-GC 666
Ms3055:   485 ACCCGCGCGAGAACGCCGAGGTGACCGTGAGTGTCCGGGAAGCCGTCGCCAAGCTTGTGG 544

Mt3055:   667 CG-CTGAGGTCG-GCTGATC-CCTGGGCCG 693
Ms3055:   545 CGGCCGTCGCCGAGGTGCCCGCCGGGGTCG 574
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C

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MtdinX:     8 CCACCGAGATAATCTGGCGTGGTCTGACACGCGAGCGATCGGTGCACCTGACGGACTGGC 67
              |||||||| | |||||||| ||||||||  | |||||  |||||||||||| || |||||||
MsdinX:  2333 CCACCGAGGTGATCTGGCGGGGTCTGACGGGGGAGCGCTCGGTGCACCTCACCGACTGGC 2392

MtdinX:    68 CAGCGCCCGACCTGCTGCCGTCGGATGCCGACCTGGTCGCCGCGATGGACCAGGTCCGCG 127
              | |   ||||  |||||||   |||| | |||||||| |||||||||||||||||  ||||
MsdinX:  2393 CGGAAGCCGATGTGCTGCCCAAGGATCCGGACCTGGTGGCCGCGATGGACCAGGTGCGCG 2452

MtdinX:   128 ACGTGTGCTCGG-CGGCATCCTCGCTGCGCAAGGCCAAGAAGCTACGGGTGCGCCTGCCG 186
              | ||||||||||  |||| || ||||||||||||||||||||||| ||||||||||||||
MsdinX:  2453 AGGTGTGCTCGGTCGGC-TCGTCGCTGCGCAAGGCCAAGAAGCTGCGGGTGCGCCTGCCG 2511

MtdinX:   187 CTACCGAAACTCATTGTGGCAGTTGAGAATCCGCAACTTCTGAGGCCGTTCGTCGACCTC 246
              || ||||||||  |  ||||| ||  || ||||| |   |||| ||||||||| ||||||
MsdinX:  2512 CTGCCGAAACTGACCGTGGCCGTGCAGGATCCGGACAGTCTGCGGCCGTTCGCCGACCTG 2571

MtdinX:   247 ATTGGCGACGAGCTTAACGTGAAGCAGGTCGAACTGACCGATGCCATCGACACCTATGGC 306
              || | |||||||||  ||||| |||   ||||| || | ||| | | |   | |||| ||
MsdinX:  2572 ATCGCCGACGAGCTCAACGTCAAGGCCGTCGAGCTCAGCGACGACGTGCCCGCCTACGGG 2631

MtdinX:   307 CGATTCGAGCTCACGGTCAACGCCCGGGTAGCCGGACCACGGCTGGGCAAAGATGTGCAG 366
              || |||||||||  |||||||||||||||| ||||| || ||  | ||||| || ||||||
MsdinX:  2632 CGCTTCGAGCTCGCGGTCAACGCCCGGGTCGCCGGGCCGCGCATCGGCAAGGACGTGCAG 2691

MtdinX:   367 GCCGCCATCAAGGCGGTCAAGGCCGGCGACGGCGTCATAAACCCGGACGGCACCTTGTTG 426
              ||||| |||||||| |||||||| || || |||||  | ||||| ||||||||  |
MsdinX:  2692 GCCGCGATCAAGGCCGTCAAGGCGGGGGAGGGCGTGGTCAACCCCGACGGCACGCTCACC 2751

MtdinX:   427 GCGGGCCCCGCGGTGCTGACGCCCGACGAGTACAACTCCCGGCTGGTGGCCGCCGACCCG 486
              |||||||| | |||||||   ||||| ||||||| ||||  |||||| || |||||||||
MsdinX:  2752 GCGGGCCCTGTGGTGCTGCAACCCGAGGAGTACACCTCCAAGCTGGTTGCGGCCGACCCG 2811

MtdinX:   487 GAGTCCACCGCGGCGTTGCCCGACGGCGCCGGGCTGGTCGTTCTGGATGGCACCGTCACT 546
              || |  ||||||||| |||||||||| || || ||||| || || || ||||| || ||
MsdinX:  2812 GAATGGACCGCGGCGCTGCCCGACGGTGCGGGTCTGGTGGTCCTCGACGGCACGGTGACC 2871

MtdinX:   547 GCCGAACTCGAAGCCGAGGGCTGGGCCAAAGATCGCATCCGCGAACTGCAAGAGCTGCGT 606
              |  || ||||| |||||||||||||||||| || |||||||| |||||||| || |||||
MsdinX:  2872 GAGGAGCTCGAGGCCGAGGGCTGGGCCAAGGACCGCATCCGTGAACTGCAGGAACTGCGG 2931

MtdinX:   607 AAGTCGACCGGGCTGGACGTTTCCGACCGCATCCGGGTGGTGATGTCGGTGCCTGCGGAA 666
              |||||||||||  ||||| || || |||||||||  |||    || |||||||| || |||
MsdinX:  2932 AAGTCGACCGGCCTGGAGGTGTCGGACCGCATCTCGGTACGCATCTCGGTGCCCGCCGAA 2991

MtdinX:   667 CGCGAAGACTGGGCGCGCACCCATCGCGACCTCATTGCCGGAGAAATCTTGGCTACCGAC 726
              | |||  | ||||| ||||| || ||||| ||||| || || || ||| | || |||
MsdinX:  2992 CACGAGCAGTGGGCACGCACGCACCGCGATCTCATCGCAGGCGAGATCCTCGCGACCAGT 3051

MtdinX:   727 TTCGAATTCGCCGACCTCGCCGATGGTGTGGCCATCGGCGACGGCGTGCGGGTA-AGCAT 785
              |||||||||||||  |  |||| |||| ||  ||||||||||||||||||||  || ||
MsdinX:  3052 TTCGAATTCGCCGAGGTGACCGACGGTGCGGAGATCGGCGACGGCGTGCGGGTCGAG-AT 3110

MtdinX:   786 CGAAAAGACCTGA 798
               |  |||  ||||
MsdinX:  3111 TGCGAAGGTCTGA 3123
```

Figure 3.3 **Nucleotide sequence alignments of promoter regions.** BLAST alignment (Altschul *et al.*, 1990) of the putative *M. tuberculosis* promoter regions with the corresponding *M. smegmatis* mc^{2}155 upstream regions. **A.** *M. tuberculosis dinP* (MtdinP) aligned with mc^{2}155 *dinP* (MSMEG1002; MsdinP); **B.** *M. tuberculosis* Rv3055 (Mt3055) aligned with mc^{2}155 MSMEG1003 (Ms3055); and **C.** *M. tuberculosis dinX* (MtdinX) aligned with mc^{2}155 *dinX* (MSMEG3178; MsdinX).

corresponding *M. smegmatis* regions confirmed significant sequence homology (Figure 3.3): the *M. tuberculosis dinP* promoter region (pEM*dinP*) shares 69% sequence identity in 584 bp overlap with *M. smegmatis* mc^{2}155, the Rv3055 promoter region (pEM3055) 73% identity in 570 bp overlap, and the dinX promoter region (pEM*dinX*) 78% in 793 bp overlap. The reporter plasmids as well as the promoterless control (pEM28w) were delivered into wild-type mc^{2}155 and integration at the *attP* locus confirmed by PCR. During exponential growth in liquid media, both Rv3055 and *dinX* were highly expressed in comparison to *dinP* or the control, as determined by specific β-galactosidase activities (Figure 3.4). The activity of the *dinP* promoter construct did not differ significantly from the vector control throughout the assay. After two weeks in stationary phase, expression ofboth Rv3055 and *dinX* was well above the *dinP* construct and the vector control, and had increased above the corresponding levels achieved during exponential growth (Figure 3.4). The failure to observe any activity associated with the *dinP* promoter construct indicated that *dinP* might be operonic with the Rv3055-encoded transcriptional regulator. Furthermore, the activity of the *M. tuberculosis dinX* and Rv3055 promoter constructs in *M. smegmatis* mc^{2}155 suggested the potential stationary-phase induction of these genes. However, neither pol IV homologue was identified in global transcriptional profiles of *M. tuberculosis* H37Rv maintained in prolonged stationary-phase (Hampshire *et al.*, 2004), raising the possibility that the regulation of these genes differs in stationary-phase *M. smegmatis* mc^{2}155. Therefore, although the degree of homology between *M. tuberculosis* and *M. smegmatis* promoter sequences (Figure 3.3) was suggestive of the utility of *M. smegmatis* as a surrogate for expression analysis of *M. tuberculosis* genes, the relevance of these data requires confirmation by transcriptional analysis.

3.4.3 Overexpression of *M. tuberculosis dinP* and *dinX* has no effect on rates of spontaneous point or frameshift mutagenesis

Spontaneous mutagenesis is significantly increased in *E. coli* strains overexpressing *dinB* and is characterised primarily by elevated frequencies of frameshift mutations, particularly –1 frameshifts, but also by increased levels of base substitutions (Kim *et al*., 1997, 2001; Wagner & Nohmi, 2000; Kobayashi *et al*., 2002). To determine whether overexpression of the mycobacterial Y-family homologues results in increased untargeted mutagenesis, *M. tuberculosis dinP* (pGaa::*dinP*) and *dinX* (pGaa::*dinX*) were placed under the control of an inducible *M. smegmatis* acetamidase promoter (Mahenthiralingam *et al.*, 1993; Parish *et al.*, 1997; Narayanan *et al.*, 2000) in a pOLYG-based replicating plasmid (Figure 3.5). The constructs, as well as a vector control (pOLYGaa), were delivered into *M. smegmatis* mc^{2}155 by electroporation, and successful transformants confirmed by electroduction.

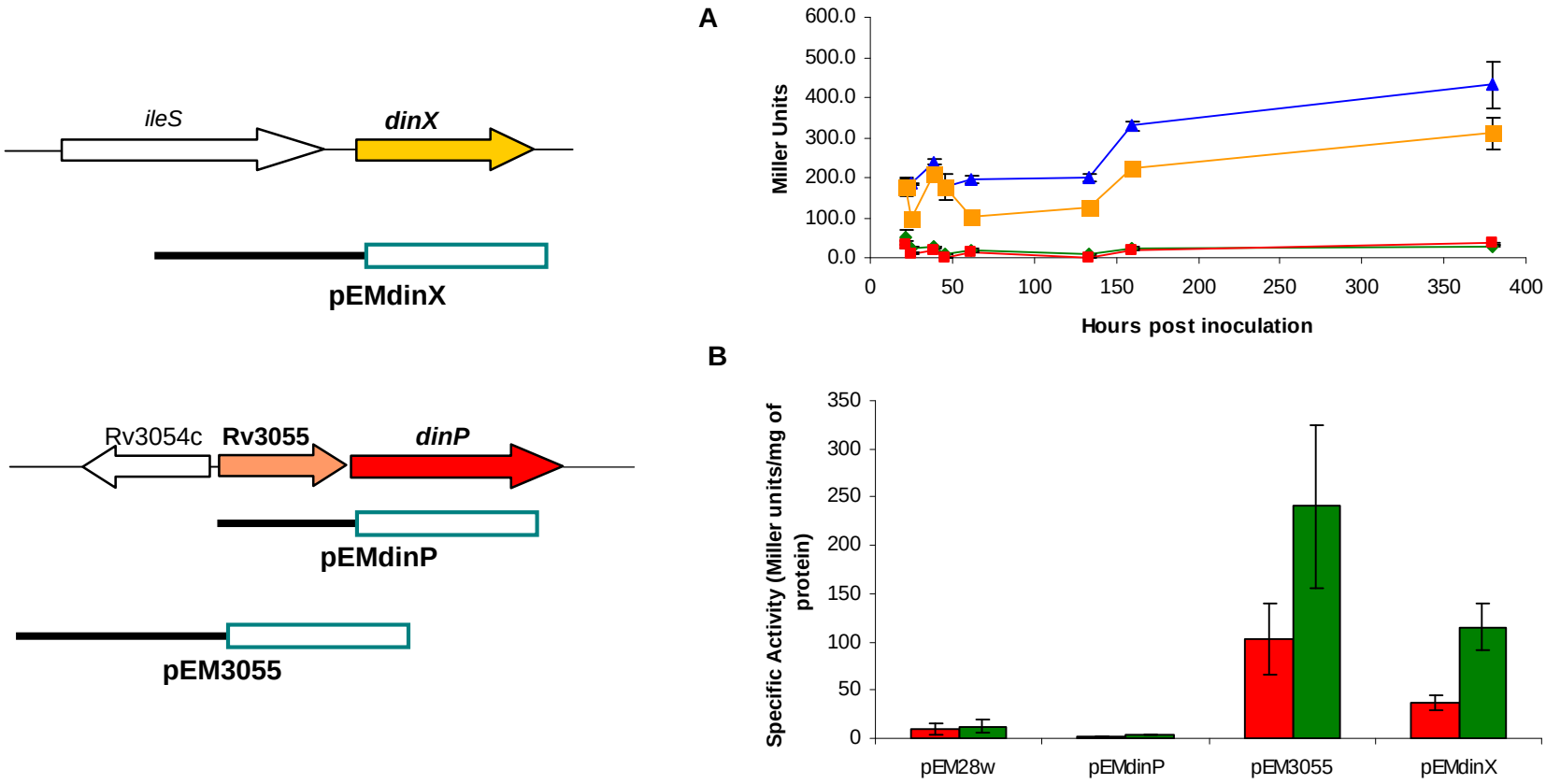


Figure 3.4 Stationary-phase upregulation of *M. tuberculosis* pol IV homologues. β-galactosidase activities of *M. smegmatis* mc^{2}155 strains carrying *lacZ* transcriptional fusions of the promoter regions of *M. tuberculosis dinP* (pEM*dinP*), *dinX* (pEM*dinX*), and Rv3055 (pEM 3055), as well as the vector control (pEM28w), were monitored during long-term growth *in vitro*. The schematic diagram on the left shows the locations of the relevant promoter regions which were cloned upstream of a promoterless *lacZ* gene (blue rectangular box). A. Miller Units indicated both Rv3055 and *dinX* were highly expressed during exponential growth and remained elevated throughout the 380 hr incubation. In contrast, expression of *dinP* did not differ significantly from the vector control at any time during the assay. B. Normalisation of Miller Unit values for protein concentration confirmed the differential activities of the Rv3055 and *dinX* promoter constructs *versus dinP* and the vector control in logarithmic phase (red bars) and after two weeks stationary phase incubation (green bars). Data are representative of at least two independent experiments. Each timepoint shows the mean of duplicate samples, and error bars indicate standard deviations

Transcriptional upregulation of *dinP* and *dinX* in the presence or absence of acetamide was assessed by semi-quantitative RT-PCR using *M. tuberculosis*-specific oligonucleotide primers (Figure 3.5). Expression of both *dinP* and *dinX* was significantly induced after addition of acetamide. However, elevated mRNA levels were also evident in the absence of acetamide treatment, and likely resulted from the leaky acetamide promoter compounded by the presence in each bacillus of multiple copies of the pOLYG-based shuttle vector (O'Gaora *et al*., 1997).

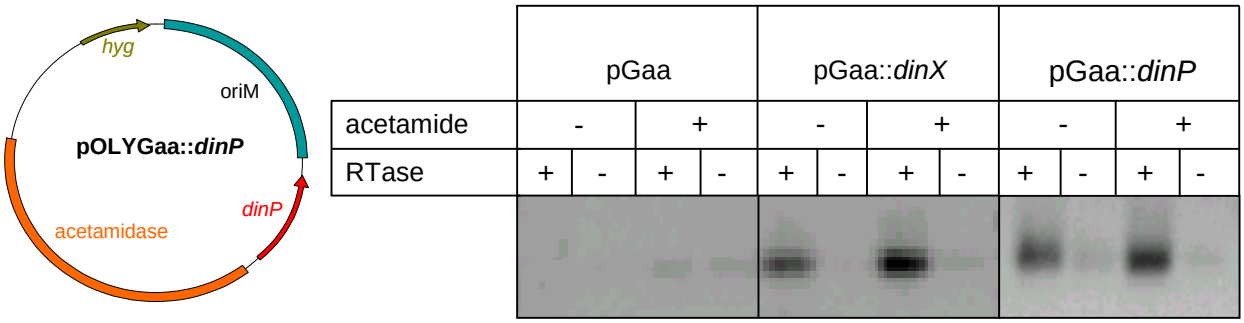


Figure 3.5 Inducible expression of the *M. tuberculosis* pol IV homologues. Upregulation of *M. tuberculosis dinP* (pGaa::*dinP*) and *dinX* (pGaa::*dinX*) under the control of an inducible *M. smegmatis* acetamidase promoter (Mahenthiralingam *et al.*, 1993; Parish *et al.*, 1997; Narayanan *et al.*, 2000) was assessed by semi-quantitave RT-PCR in the presence or absence of acetamide. Expression levels are compared with the vector control (pOLYGaa) and potential DNA contamination excluded by performing the reaction with or without reverse transcriptase (RTase). Although expression of both *dinP* and *dinX* was induced after addition of acetamide, significant mRNA levels were also evident in the absence of acetamide treatment, perhaps indicating a leaky acetamide promoter or the presence of elevated transcript levels in cells harbouring multiple copies of the expression vector (see text).

Spontaneous mutagenesis in the mc^{2}155 strains harbouring the overexpression vectors was assessed by determining rates of forward mutations to rifampicin resistance (Rif^R) in a Luria-Delbrück fluctuation assay (Luria & Delbrück, 1943). Mutations in an 80 bp region of the *rpoB* gene encoding the RNA polymerase β subunit account almost exclusively for Rif^R isolates of *M. tuberculosis* (Telenti *et al.*, 1993, 1997; Musser, 1995; Ramaswamy & Musser, 1998; Morlock *et al*., 2000), of which almost 70% result from point mutations in the codons for Ser531 and His526, with a Ser531Leu substitution (TCG to TTG) the most common mutation encountered clinically and *in vitro* (Telenti *et al.*, 1993, 1997; Billington *et al.*, 1999; Morlock *et al.*, 2000; Boshoff *et al.*, 2003; Mariam *et al.*, 2004), probably because that mutation is associated with the lowest fitness cost *in vitro* (Billington *et al.*, 1999; Morlock *et al.*, 2000; Mariam *et al.*, 2004). Using the MSS algorithm (Ma *et al.*, 1992; Rosche & Foster, 2000), the mutation rate in wild-type mc^{2}155 was calculated at approximately 5x10^{-8} mutations per cell per generation (Table 3.6), consistent with previous reports (Boshoff *et al.*, 2003). Significantly, rates of mutations to Rif^R in strains

carrying either the empty vector (pOLYGaa) or the *M. tuberculosis dinP* (pOLYGaa::*dinP*) or *dinX* (pOLYGaa::*dinX*) overexpression vectors were indistinguishable from wild-type mc^2155, irrespective of acetamide induction (2-9 x 10^{-8} mutations per cell per generation; Table 3.6). To eliminate the possibility that heterologous overexpression of the *M. tuberculosis* homologues in mc^2155 is inherently flawed despite increased transcript levels, the overexpression vectors were recently applied in *M. tuberculosis* H37Rv. Preliminary data of Rif^R mutation frequencies from these studies have confirmed the inability of *dinP* and *dinX* overexpression to elevate spontaneous mutagenesis (data not shown).

Table 3.6 Mutation rates for *M. smegmatis* strains overexpressing *M. tuberculosis dinP* and *dinX*[a].

Strain	Rif mutation rate (x 10^{-8})	hisD5T frameshift mutation rate (x 10^{-8})
mc^2155	4.90 ± 1.4	NA
pGaa	3.22 ± 1.2	4.79 ± 2.0
pGaa::dinP	8.43 ± 1.5	8.74 ± 1.7
pGaa::*dinX*	2.55 ± 1.0	6.25 ± 2.5

[a] The table indicates the mutation rate calculated by the MSS algorithm.

The essentiality of RpoB for mycobacterial survival (Sassetti *et al.*, 2003) demands that only mutations that do not abrogate gene function are detected, and raises the possibility that an assay based on the emergence of Rif^R mutations will be too proscriptive in terms of the mutational event tolerated, and so will result in an underestimation of mutagenesis. Previous studies have identified frameshift mutations as the characteristic pol IV-mediated mutational event (Kim *et al*., 1997, 2001; Wagner & Nohmi, 2000; Kobayashi *et al*., 2002) with fourfold increases in *dinB* expression having no effect on base substitution rates (Wolff *et al.*, 2004). Therefore, in order to ascertain whether overexpression of the *M. tuberculosis* pol IV homologues mediates increased frameshift mutations, the overexpression vectors (as well as the vector control) were introduced into an *M. smegmatis* reporter strain carrying a frameshift mutation in the *hisD* gene (*hisD*5T; E. Machowski, unpublished). Reversion to histidine prototrophy is restored by a -1 frameshift at the position of an artificial 5T run, ctttttg→cttttg (LeuPhe205**→LeuLeu205), allowing frameshift mutations to be scored by plating on unsupplemented media. Again, the number of His^+ revertants arising during a Luria-Delbrück fluctuation assay was analysed using the MSS method (Rosche & Foster, 2000). Based on this assay, the rate of spontaneous -1 frameshift mutations in wild-type mc^2155 containing the vector control

(pGaa) was estimated as approximately 4.8 x 10^{-8} mutations per cell per generation (Table 3.6). As observed for mutations to Rif^R (Table 3.6), no significant difference in frameshift mutation rate was associated with overexpression of either of the *M. tuberculosis* pol IV homologues with or without acetamide induction. The failure of *dinP* or *dinX* overexpression to increase spontaneous mutagenesis is similar to results observed with *M. tuberculosis dnaE2* (Boshoff *et al.*, 2003) and adds further impetus to the suggestion that additional factors might be required to facilitate access of the non-replicative polymerases to undamaged DNA (Boshoff *et al*., 2003; Tippin *et al.*, 2004b).

3.4.4 A chromosomal duplication in *M. smegmatis* mc^2155

To investigate the potential physiological roles of the mycobacterial pol IV homologues in DNA repair, genetic diversification, and long-term survival, a *dinP* deletion mutant of *M. smegmatis* mc^2155 was generated by allelic exchange. *M. smegmatis* has been widely used to investigate mycobacterial physiology and genetics and, despite significant limitations to its applicability (Reyrat & Kahn, 2001; Barry III, 2001), is often employed in preliminary experiments to determine the value of pursuing a particular line of research in the more intransigent pathogenic mycobacteria. The two-step homologous recombination method employed to generate the *dinP* knockout mutant (Boshoff & Mizrahi, 1998; Parish & Stoker, 2000) was standard to the creation of all gene deletion mutants of *M. tuberculosis* and *M. smegmatis* discussed in this thesis, and so will be described in some detail here. Flanking genomic sequence upstream and downstream of *M. smegmatis dinP* was amplified by PCR using oligonucleotide primers with engineered restriction sites in the respective 5' termini to facilitate subsequent cloning and, in the case of marked insertion-deletion alleles, the insertion of an antibiotic resistant cassette. All PCR amplifications were performed using the EXPAND^{TM} high-fidelity PCR system and the products sequenced to confirm sequence integrity. Ligation and cloning of the *dinP* upstream (TdinPFR1) and downsteam (TdinPFR2) fragments into p2NIL resulted in a deletion allele that effectively eliminated 943 bp internal *dinP* sequence (p2Δ*dinP*). The *Pac*I cassette from pGOAL19 - containing a hygromycin resistance gene (*hyg*), as well as the *lacZ* and *sacB* genes to enable blue/white and sucrose counterselection, respectively - was then cloned into p2Δ*dinP* to yield an unmarked *dinP* knockout construct (p2Δ*dinP*19; Figure 3.6A). In order to create a marked *dinP* deletion allele, the *hyg* cassette was inserted into p2Δ*dinP* at the site of the deletion (p2Δ*dinP*::*hyg*). Cloning of the *Pac*I cassette from pGOAL17 - containing the *lacZ* and *sacB* genes but lacking *hyg* - into p2Δ*dinP*::*hyg* yielded a knockout construct containing a *hyg*-marked *dinP* deletion-insertion allele (p2Δ*dinP*17; Figure 3.6A).

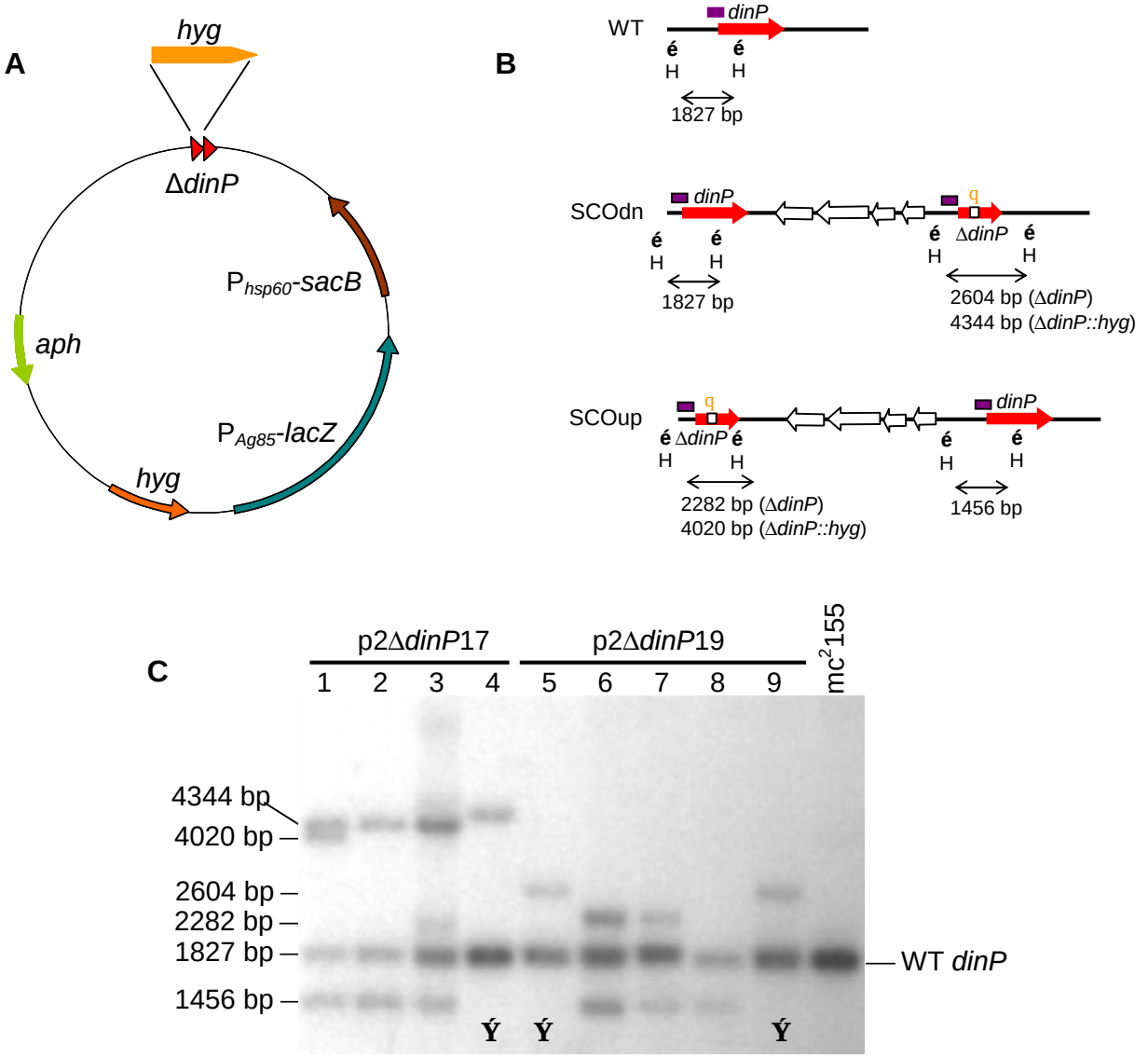


Figure 3.6 Construction of a *dinP* deletion mutant strain of *M. smegmatis* mc^{2}155.
A. Schematic representation of the p2Δ*dinP*17 and p2Δ*dinP*19 delivery vectors in which 982 bp *M. smegmatis* mc^{2}155 *dinP* coding sequence is eliminated. The deletion junction into which the hygromycin resistance gene (*hyg*) was inserted to generate the marked deletion-insertion allele, is indicated. (*aph*, kanamycin resistance gene; P_{Ag85a}-*lacZ*, *b*-galactosidase driven by the mycobacterial antigen 85A promoter; P_{hsp60}-*sacB*, sucrose sensitivity gene driven by the mycobacterial Hsp60 promoter). B. Site-specific integration of either the marked (p2Δ*dinP*17) or unmarked (p2Δ*dinP*19) knockout delivery vector to give an upstream (SCOup) or downstream (SCOdn) SCO should result in only two bands (H, *Hin*dIII restriction sites; open arrows indicate the *lacZ*, *sacB*, *aph* and *hyg* genes carried on the relevant deletion vectors; TdinPFR1 probe represented by a purple box; *hyg* represented by q). C. Southern blot analysis of wild-type *M. smegmatis* mc^{2}155 (mc^{2}155) and putative *dinP* single cross-over recombinants (SCOs). Three of the putative *dinP* SCOs (indicated by a **Ý**) appeared to give the expected banding pattern: 1827 bp and 4344 bp for a marked downstream cross-over (lane 4) and 1827 bp and 2604 bp for an unmarked downstream cross-over (lanes 5 and 9). However, the deeper intensity of the wild-type band (WT *dinP*) in these lanes and mc^{2}155 was suggestive of an extra *dinP* copy.

Wild-type mc^{2}155 was separately electroporated with p2Δ*dinP*17 and p2Δ*dinP*19 and plated on solid medium containing Km, Hyg, and X-gal. Putative single cross-over (SCO) recombinants that were Km^R, Hyg^R and blue in colour were analysed by Southern hybridisation (Figure 3.6C). Briefly, genomic DNA from each of the putative SCOs was subjected to *Hin*dIII restriction digest and probed with the radio-labelled upstream PCR fragment (TdinPFR1). Site-specific integration of the deletion construct should result in only two bands – the intact wild-type gene and the disrupted allele carried on the vector. Only three of the putative SCOs recovered from the two electroporation experiments gave bands of the expected sizes and, in all three cases, the knockout vector had integrated downstream of the chromosomal *dinP* (Figure 3.6C; lanes 4, 5, and 9). However, the intensity of the 1827-bp wild-type band (WT *dinP*) in the downstream SCOs was noticably deeper (lanes 4, 5, and 9). In addition, the remaining clones (excluding lane 1) showed composites of the banding patterns expected of a single wild type gene (1827 bp) and an upstream SCO (1456 bp and 4020 bp for the marked construct and 1456 bp and 2282 bp for the unmarked construct; lanes 2, 3, 6 and 7). During the course of this work, Galamba *et al.* (2001) reported the identification of a duplication region in mc^{2}155 carrying the *adhC* gene. This finding, together with the unexpected complexity in the SCO banding patterns, immediately suggested the possible existence of an extra copy of *dinP* in mc^{2}155 and, further, that *dinP* might be contained within the duplicated region. Consistent with this proposal, the banding pattern in lane 1 could be resolved into two separate SCO events at duplicate *dinP* loci.

As per the established two-step protocol, the downstream SCOs were cultured in liquid media containing no antibiotic (for the unmarked deletion allele) or Hyg only (for the marked deletion-insertion allele) to allow the second cross-over event to occur. Serial dilutions of each culture were then plated on solid media containing X-gal and 2% sucrose to enable phenotypic counterselection of putative double-cross over (DCO) recombinant mutants. White, sucrose-resistant colonies were selected for analysis by Southern blot. *Sal*I digested genomic DNA from each putative DCO mutant was probed with the downstream PCR fragment (TdinPFR2; Figure 3.7). Ten strains (eight from p2Δ*dinP*17 and two from p2Δ*dinP*19) were identified with the genotype expected of the relevant DCO mutation in addition to an intact wild-type copy. The presence of the both the wild-type *dinP* and a deletion allele in a single strain confirmed that mc^{2}155 is merodiploid for *dinP.* A representative mutant (*dinP*$^{+/-}$) carrying both wild-type and Δ*dinP*::*hyg* deletion-insertion alleles is shown in Figure 3.7.

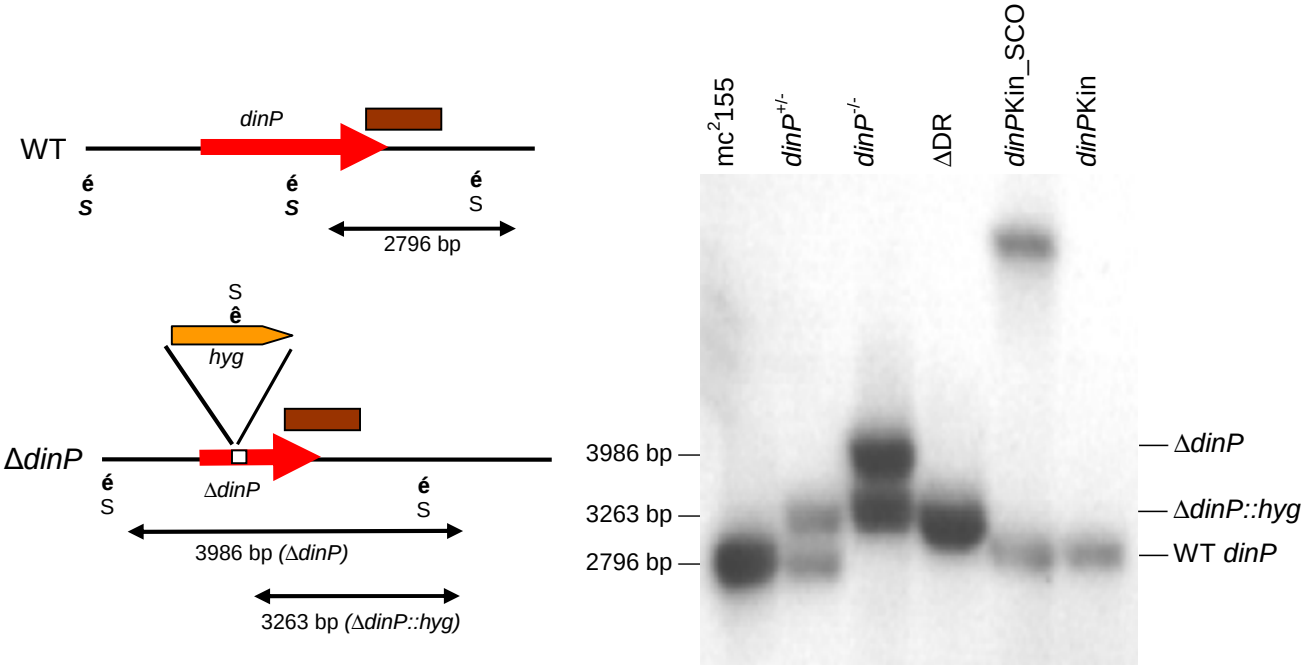


Figure 3.7 Construction of the *M. smegmatis dinP*Kin mutant. Hybridisation of the TdinPFR2 probe to *Sal*I-digested DNA from wild-type *M. smegmatis* mc^{2}155 (mc^{2}155), the single *dinP* knockout mutant (*dinP*$^{+/-}$), the *dinP* null mutant (*dinP*$^{-/-}$), and the region-deleted mutant (ΔDR), as well as dinPKin SCO and DCO recombinant strains. The genotypes of the relevant *dinP* deletion mutants and wild-type mc^{2}155 are illustrated in the schematic diagram (S, *Sal*I restriction sites; probe represented by a brown box). Elimination of 982 bp *dinP* sequence results in the loss of the internal *Sal*I site; however, an additional *Sal*I site is present in the *hyg* cassette. The single *dinP* knockout mutant (*dinP*$^{+/-}$) produced two bands (WT *dinP* and Δ*dinP::hyg*, 2796 bp and 3263 bp) indicating that mc^{2}155 is merodiploid for *dinP*. Both copies of *dinP* have been disrupted in the null mutant (*dinP*$^{-/-}$) by replacement with marked (Δ*dinP::hyg*, 2796 bp) and unmarked (Δ*dinP*, 3986 bp) deletion alleles. Absence of the unmarked deletion allele (Δ*dinP*, 3986 bp) in the ΔDR mutant (ΔDR) indicated the probable loss of the duplicated region in this strain. Finally, replacement of the Δ*dinP::hyg* allele in the ΔDR mutant with a wild-type *dinP* allele resulted in the creation of a novel *M. smegmatis* strain (*dinP*Kin) that is isogenic with mc^{2}155 but lacks the duplicated region.

M. smegmatis mc^{2}155 *dinP* is dispensable for growth *in vitro*

To eliminate the second copy of *dinP*, the *dinP*$^{+/-}$ strain was transformed with p2Δ*dinP*19. It was reasoned that maintaining the *dinP*$^{+/-}$ strain under Hyg selection throughout the two-step method might serve to minimise the risk of homologous recombination at the same locus, which would result in the futile replacement of the marked deletion allele (Δ*dinP*::*hyg*) with its unmarked counterpart (Δ*dinP*). Following this approach, a *dinP* null mutant of mc^{2}155 was constructed in which both copies of the gene had been replaced – one by a marked deletion-insertion (Δ*dinP*::*hyg*) and the other by an unmarked *dinP* deletion allele (Δ*dinP*). This strain was designated *dinP*$^{-/-}$ (Figure 3.7). As expected, loss of DinP activity was not associated with a growth phenotype under standard *in vitro* conditions (data not shown).

Isolation of a *dinP* deletion mutant lacking the duplicated region

During the process of generating *dinP*$^{-/-}$ from *dinP*$^{+/-}$, an unusual mutant was identified which seemed, on genotypic analysis, to have lost entirely the second copy of *dinP* and its flanking sequence (ΔDR; Figure 3.7). Growth of the ΔDR deletion mutant was indistinguishable from wild-type mc^{2}155 under standard *in vitro* conditions (data not shown). Furthermore, the fact that ΔDR was isolated from the same precursor strain (a SCO mutant obtained after transforming *dinP*$^{+/-}$ with p2Δ*dinP*19), and in the same counterselection step of the two-step procedure as gave rise to *dinP*$^{-/-}$, strongly suggested that the second *dinP* copy might have been lost as a result of a homologous recombination event that eliminated the entire duplicated region. That possibility was recently confirmed by the release of the *M. smegmatis* mc^{2}155 genome sequence (http://www.tigr.org) which established that the duplicated regions are approximately 52 kb in size and are situated 1.2 Mb apart on the mc^{2}155 chromosome (Figure 3.8). Significantly, each region is flanked by two IS*1096* elements (http://www.tigr.org) that likely acted as substrates for the homologous recombination event that gave rise to the original duplication. Consistent with this proposal, subsequent work has confirmed the loss of the chromosomal duplication, as well as the retention of only a single IS*1096* element, in the ΔDR mutant (Figure 3.8; Galamba *et al*., unpublished).

Transformation efficiency of the ΔDR strain

M. smegmatis mc^{2}155 was originally isolated as an efficient plasmid transformation (*ept*) mutant derived from the *M. smegmatis* reference strain ATCC 607 (mc^{2}6; Snapper *et al*., 1990). Given recent evidence that the chromosomal duplication is absent from mc^{2}6 and must have occurred during the evolution of *M. smegmatis* mc^{2}155 from the parental strain (Galamba *et al*., unpublished), the potential role of the duplication (or at least a region or gene contained therein) in the differential transformability of the mc^{2}155 and mc^{2}6 stains was considered worth investigating. Therefore, both wild-type mc^{2}155 and the ΔDR mutant were electroporated with decreasing concentrations of an integrating plasmid (pAINT) and transformants scored by plating on selective media. If the duplicated region were somehow associated with increased transformation efficiency, loss of the duplication in the ΔDR mutant should result in the reduction of transformability to levels associated with mc^{2}6. However, no significant difference in transformation frequencies was observed (data not shown), eliminating the duplication as a candidate for the *ept* phenotype.

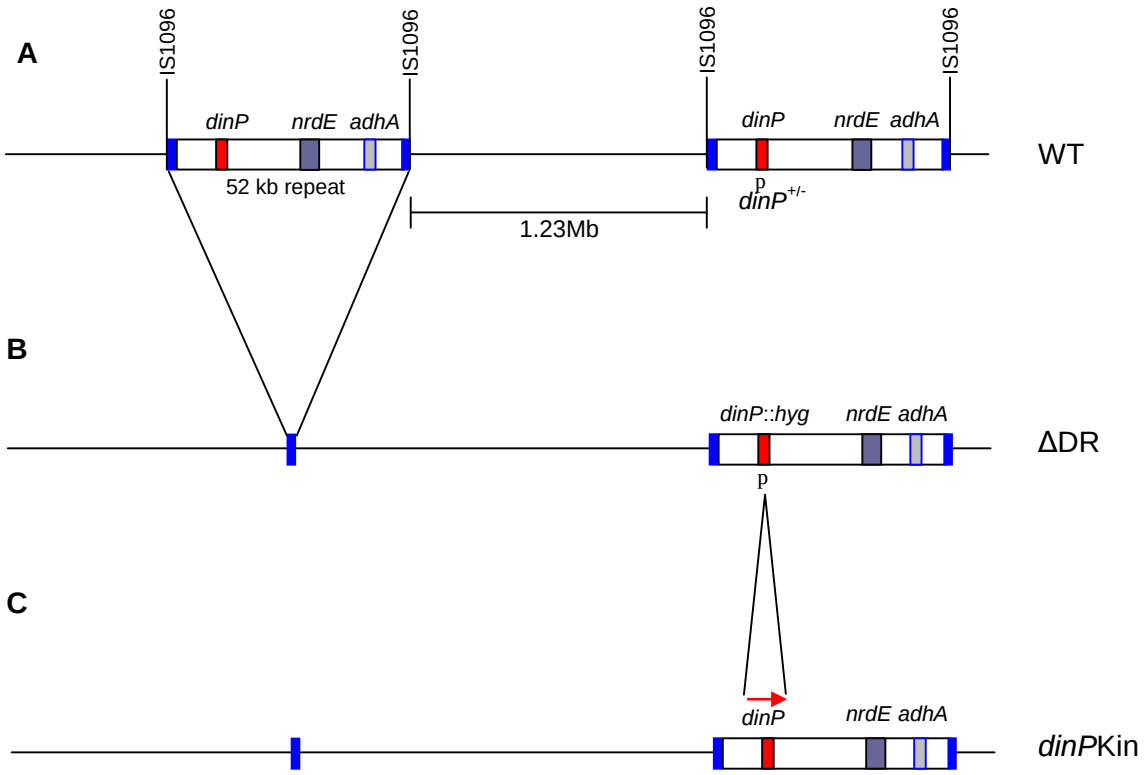


Figure 3.8 The *M. smegmatis* mc^{2}155 duplicated region. A. Homologous recombination between flanking IS*1096* repeat elements during the evolution of *M. smegmatis* mc^{2}155 (WT) from the parental mc^{2}6 strain resulted in the duplication of a 52 kb chromosomal region (Galamba *et al.*, unpublished; www.tigr.org). In addition to *dinP*, approximately 49 predicted ORFs are located in the duplicated region, including *nrdE* and *adhA* (originally called *adhC*; Galamba *et al.*, 2001). **B**. During construction of a mc^{2}155 *dinP*$^{-/-}$ null mutant, the duplication was fortuitously lost via another IS*1096*-mediated homologous recombination event that left behind a single IS*1096* copy at the site of the duplication. Loss of the duplication resulted in the creation of the ΔDR mutant strain containing a *hyg*-marked (p) *dinP* deletion allele. **C**. Site-specific replacement of the *dinP*::*hyg* allele in ΔDR with a wild-type mc^{2}155 *dinP* gene yielded the *dinP*Kin mutant.

Restoration of a wild-type *dinP* allele in ΔDR

The *M. smegmatis* mc^{2}155 genome sequence revealed the presence of 50 predicted ORFs in the duplicated region (http://www.tigr.org), prompting speculation that the higher gene dose in mc^{2}155 versus mc^{2}6 might manifest in altered physiology. Coupled with the fact that multiple sequential gene knockout steps are required to create null mutants of any one of the fifty duplicated genes in mc^{2}155, it was proposed that the ΔDR mutant might prove useful as an alternative to mc^{2}155 in studies aiming to disrupt genes with corresponding copies located in the duplicated region. However, the presence of a *hyg*-containing *dinP* deletion allele in the ΔDR mutant would necessarily limit the biological relevance of this strain and derivatives. In order to create a mutant containing the full

genetic complement of wild-type mc^{2}155, but lacking the duplicated region (Figure 3.8), a functional *dinP* allele (*dinP*Kin) was re-introduced into the ΔDR mutant by the same two-step method (Boshoff & Mizrahi, 1998; Parish & Stoker, 2000). In this case, however, the presence of a *hyg* marker at the site of the *dinP* deletion in ΔDR raised the possibility that recombination might occur at the *hyg* locus rather than between homologous chromosomal and vector-delivered *dinP* ORFs. A "knock-in" construct was therefore created comprising the intact mc^{2}155 *dinP* flanked by 877 bp upstream and 783 bp downstream sequence, and the *Pac*I cassette from pGOAL17 lacking the *hyg* cassette (p2*dinP*Kin). Southern analysis of the putative SCO mutants confirmed site-specific integration of p2*dinP*Kin at the *dinP* locus (*dinP*Kin_SCO; Figure 3.7). After further phenotypic selection, a DCO mutant was isolated in which the Δ*dinP*::*hyg* allele had been replaced by an intact copy of the wild-type mc^{2}155 *dinP* gene (*dinP*Kin; Figure 3.7). The re-introduced allele and flanking regions were sequenced to confirm sequence integrity of the *dinP*Kin strain (data not shown). No growth phenotype was associated with loss of the duplicated region under standard *in vitro* conditions (data not shown) and, although still to be tested, it is unlikely that the re-introduction of a functional *dinP* allele will affect the wild-type transformability of the ΔDR mutant. Furthermore, it was recently shown that cell-wall physiology in the mutant is indistinguishable from wild-type mc^{2}155 (Gilles Etienne, personal communication), providing further support for the potential utility of *dinP*Kin.

3.4.5 *M. smegmatis* mc^{2}155 EP polymerases are not required for long-term survival *in vitro*

M. smegmatis Y-family polymerases are dispensable for growth *in vitro*

To determine the effects of the putative Y-family polymerases on mycobacterial fitness and mutagenesis, strains of mc^{2}155 were generated that contained individual deletions in the *M. smegmatis dinP*, *dinX*, and *dinP3* genes (Figure 3.9). Again, all knockout mutants were generated by two-step allelic mutagenesis (Boshoff & Mizrahi, 1998; Parish & Stoker, 2000). A *hyg*-marked insertion-deletion allele eliminated 1185 bp of 1354 bp *dinX* sequence (Δ*dinX*::*hyg*; Figure 3.10C). Similarly, an mc^{2}155 *dinP3* mutant was created in which 1106 bp of 1239 bp *dinP3* sequence was deleted (S. Dawes, unpublished; Figure 3.10B). The presence in *M. smegmatis* of multiple pol IV homologues raised the possibility that the remaining, alternative polymerases might complement, and therefore mask, lost activity in the *dinP*$^{-/-}$, *dinX*, and *dinP3* single gene deletion mutants. Therefore, a programme of sequential gene deletion was adopted to create combined deletion mutants in the *dinP*$^{-/-}$ background. The *hyg*-marked *dinP* deletion-insertion in *dinP*$^{-/-}$ excluded the

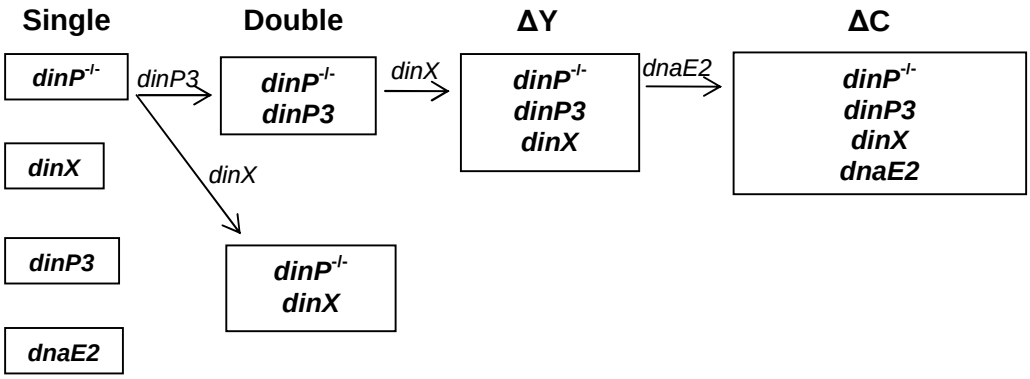


Figure 3.9 ***M. smegmatis*** **mc^{2}155 pol IV and EP polymerase knockout strains.** The flow diagram illustrates the sequential generation of pol IV and EP polymerase deletion mutants of *M. smegmatis* mc^{2}155. The single Δ*dinP3* and Δ*dnaE2* mutants were provided courtesy of Dr. S. Dawes and Dr. H. Boshoff, respectively. Construction of the additional single and multiple deletion mutants is described in the text. Genotypes are illustrated in Figure 3.9.

use of *hyg*-marked knockout constructs owing to the risk of unwanted cross-over events at that locus. Therefore, an unmarked *dinX* deletion allele was constructed that was identical to Δ*dinX*::*hyg* except for the absence of the inserted *hyg* marker (Figure 3.10). The unmarked *dinP3* and *dinX* knockout constructs were then applied to disrupt the corresponding genes in the *dinP*$^{-/-}$ background, thereby generating double mutants characterised by *dinP*$^{-/-}$/*dinP*3 and *dinP*$^{-/-}$/*dinX* genotypes, respectively (Figure 3.10). Deletion of *dinX* in the double *dinP*$^{-/-}$/*dinP*3 mutant yielded a strain of mc^{2}155 in which all putative Y-family activity was disrupted, designated ΔY (*dinP*$^{-/-}$/*dinP3*/*dinX*). Elimination of each of the Y-family genes in ΔY was confirmed by Southern blot using the appropriate gene-specific probe (Figure 3.10). None of the single (*dinP*$^{-/-}$, *dinX*, *dinP3*), double (*dinP*$^{-/-}$/*dinP3*, *dinP*$^{-/-}$/*dinX*) or ΔY deletion mutants displayed any growth defect under standard *in vitro* conditions, indicating the individual and collective dispensability of these genes for normal growth (data not shown).

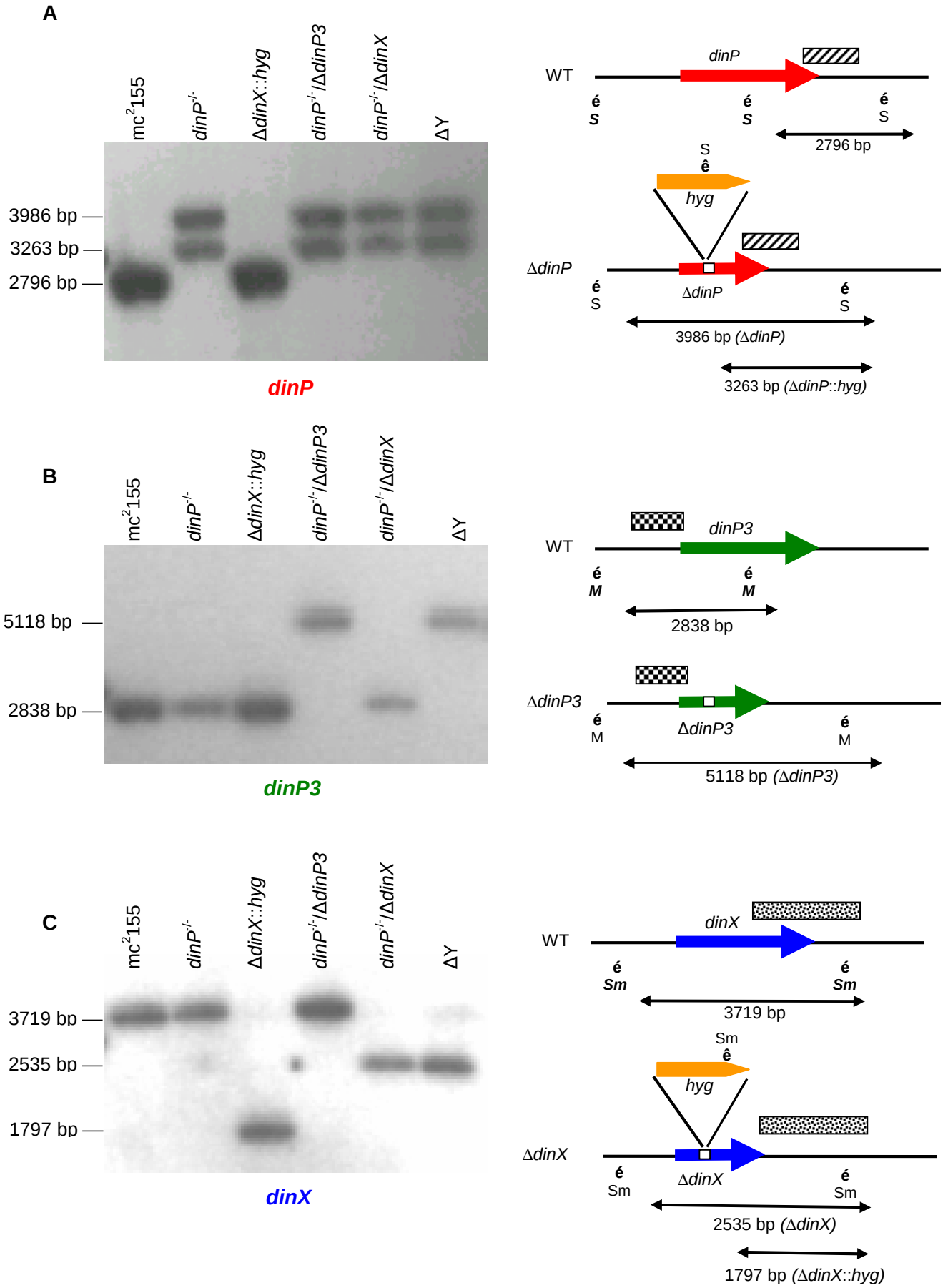

A
mc²155
dinP⁻/⁻
ΔdinX::hyg
dinP⁻/⁻/ΔdinP3
dinP⁻/⁻/ΔdinX
ΔY
3986 bp
3263 bp
2796 bp
dinP
WT
dinP
S
S
S
2796 bp
hyg
ΔdinP
ΔdinP
S
S
3986 bp (ΔdinP)
3263 bp (ΔdinP::hyg)
B
mc²155
dinP⁻/⁻
ΔdinX::hyg
dinP⁻/⁻/ΔdinP3
dinP⁻/⁻/ΔdinX
ΔY
5118 bp
2838 bp
dinP3
WT
dinP3
M
M
2838 bp
ΔdinP3
ΔdinP3
M
M
5118 bp (ΔdinP3)
C
mc²155
dinP⁻/⁻
ΔdinX::hyg
dinP⁻/⁻/ΔdinP3
dinP⁻/⁻/ΔdinX
ΔY
3719 bp
2535 bp
1797 bp
dinX
WT
dinX
Sm
Sm
3719 bp
hyg
ΔdinX
ΔdinX
Sm
Sm
2535 bp (ΔdinX)
1797 bp (ΔdinX::hyg)

Figure 3.10 Construction of multiple sequential pol IV deletion mutants of mc^{2}155.
DNA from wild-type *M. smegmatis* mc^{2}155 (mc^{2}155), the single *dinP* (*dinP*$^{-/-}$) and *dinX* (Δ*dinX*::*hyg*) mutants, the double *dinP*$^{-/-}$/*dinP3* (*dinP*$^{-/-}$/Δ*dinP3*) and *dinP*$^{-/-}$/*dinX* (*dinP*$^{-/-}$/Δ*dinX*) mutants, and the ΔY deletion mutant was digested by the relevant restriction enzyme and probed with each of the three pol IV-specific probes. Restriction maps of the various strains are schematically illustrated in the line drawings adjacent to each Southern blot (not drawn to scale). **A**. *Sal*I-digested DNA hybridised to the TdinPFR2 probe. Construction of the Δ*dinP* allele eliminates 982 bp of *dinP* coding sequence, and results in the loss of the internal *Sal*I restriction site. However, an additional *Sal*I restriction site is introduced by the *hyg* cassette in the Δ*dinP*::*hyg* allele (S, *Sal*I restriction sites; WT, wild-type; probe represented by a hatched box). **B**. *Mlu*I-digested DNA hybridised to the P3p probe. The Δ*dinP3* allele eliminates 1371 bp of *dinP3* coding sequence (M, *Mlu*I restriction sites; WT, wild-type; probe represented by a chequered box). **C**. *Sma*I-digested DNA hybridised to the MsXFR2 probe. The Δ*dinX* allele eliminates 1186 bp dinX coding sequence. An additional *Sma*I restriction site is introduced by the *hyg* cassette in the Δ*dinX*::*hyg* allele (Sm, *Sma*I restriction sites; WT, wild-type; probe represented by a spotted box).

Loss of pol IV activity does not impair long-term competitve fitness of mc^{2}155

Maintenance of a population in a constant environment with limiting amounts of essential nutrients tends to select for fitter mutants (Novick & Szilard, 1950; Atwood *et al.*, 1951; Dykhuizen, 1990; Zambrano *et al.*, 1993; Zambrano & Kolter, 1996; Finkel & Kolter, 1999; Yeiser *et al.*, 2002) which are thought to arise as a result of a random mutation that confers a competitive advantage on the cell (Dykhuizen, 1990). The enhanced fitness of the mutant enables near complete population takeover by cells of the specific genotype in a process referred to as “periodic selection” (Novick & Szilard, 1950; Atwood *et al.*, 1951; Dykhuizen, 1990). Specifically, incomplete takeover results in the coexistence of multiple adaptive mutants, and is considered especially relevant to the evolution of diversity in natural populations (Finkel & Kolter, 1999). *E. coli* possesses three damage-induced polymerases, each of which is dispensable for *in vitro* growth in pure culture (Yeiser *et al*., 2002). However, inactivation of one or more of the Y-family polymerases impairs *E. coli* for long-term stationary-phase survival *in vitro* (Yeiser *et al*., 2002).

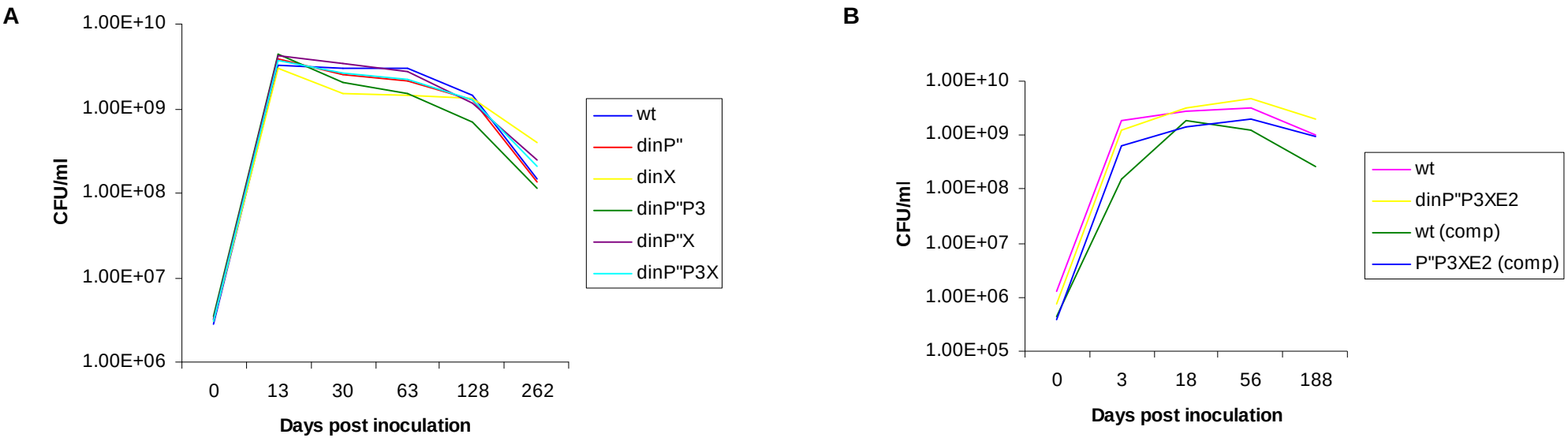


Figure 3.11 Long-term competitve growth of the mc^{2}155 Y-family and EP polymerase deletion mutants.
A. Composite growth and survival patterns of the *dinP*$^{-/-}$ null (dinP'') and *dinX*::*hyg* (dinX) single deletion mutants, the *dinP*$^{-/-}$*/dinP3* (dinP''P3) and *dinP*$^{-/-}$*/dinX* (dinP''X) double deletion mutatns, as well as ΔY (dinP"P3X) grown in competitive co-culture with wild-type mc^{2}155 (wt).
B. Wild-type *M. smegmatis* mc^{2}155 (wt) and the ΔC mutant (dinP''P3XE2) were grown in pure culure and in competition [P''P3XE2 (comp) and wt (comp)].

Furthermore, the reduced fitness of the mutant is evident only in competition with wild-type, suggesting the involvement of damage-inducible polymerases in the growth advantage in stationary phase (GASP) phenotype (Zambrano *et al*., 1993; Zambrano & Kolter, 1993, 1996; Zinser & Kolter, 1999). To determine the role of the Y-family polymerases in long-term fitness of *M. smegmatis*, a survival assay (LTG1) was established (Smeulders *et al.*, 1999) in which selected *hyg*-marked single (*dinP*$^{-/-}$, *dinX*::*hyg*) and double (*dinP*$^{-/-}$/*dinP3*, *dinP*$^{-/-}$/*dinX*) gene deletion mutants, as well as ΔY (*dinP*$^{-/-}$/*dinP3*/*dinX*), were grown in co-culture with wild-type mc^{2}155 (Figure 3.11A). Pure cultures of each of the strains were also assessed as a control. Loss of one or any combination of mycobacterial Y-family polymerases had no effect on long-term survival of any of the mutant strains in pure culture (data not shown). In addition, none of mutant strains, including ΔY, was compromised in its ability to compete with wild-type mc^{2}155 over the 262 day duration of the experiment (Figure 3.11A).

***M. smegmatis* dnaE2 does not confer a fitness advantage during stationary-phase**

The failure to observe a competitive disadvantage associated with loss of the putative mycobacterial Y-family members contrasted with the severe impairment observed in *E. coli* SOS polymerase mutants (Yeiser *et al*., 2002) and was particularly surprising in the case of the ΔY mutant, which had been stripped of all putative pol IV activity. However, given the dominant role of DnaE2 in mediating damage-induced mutagenesis in mycobacteria (Boshoff *et al*., 2003), it was conceivable that the intact *M. smegmatis dnaE2* gene might complement abrogated Y-family function in ΔY. Therefore, an additional gene deletion mutant was constructed in which *dnaE2* was eliminated in the ΔY background. The fact that the ΔY mutant carried a Δ*dinP*::*hyg* allele again required a knockout construct lacking the *hyg* cassette in order to minimise the potential for spurious recombination. To this end, a marked *dnaE2* deletion-insertion allele (Boshoff *et al.*, 2003) carrying a kanamycin (Km) resistance cassette (*aph*) was delivered into ΔY by electroporation. Putative white DCO mutants were selected in a single step by plating on media containing Km, 2% sucrose, and X-gal. Disruption of *dnaE2* in ΔY resulted in a mutant mc^{2}155 strain denuded of all predicted EP activity. This strain was designated ΔC (for "complete deletion") and was characterised by a *dinP*$^{-/-}$/*dinP3*/*dinX*/*dnaE2* genotype (Figure 3.12). Growth of the ΔC mutant was indistinguishable from wild-type mc^{2}155 under standard *in vitro* conditions (data not shown). To assess the effects of complete EP loss on long-term survival of mc^{2}155, a competitive assay (LTG2) was established in which the ΔC strain was maintained alone, or in co-culture with wild-type mc^{2}155, for 188 days (Figure 3.11B). Remarkably, complete abrogation of predicted EP function conferred

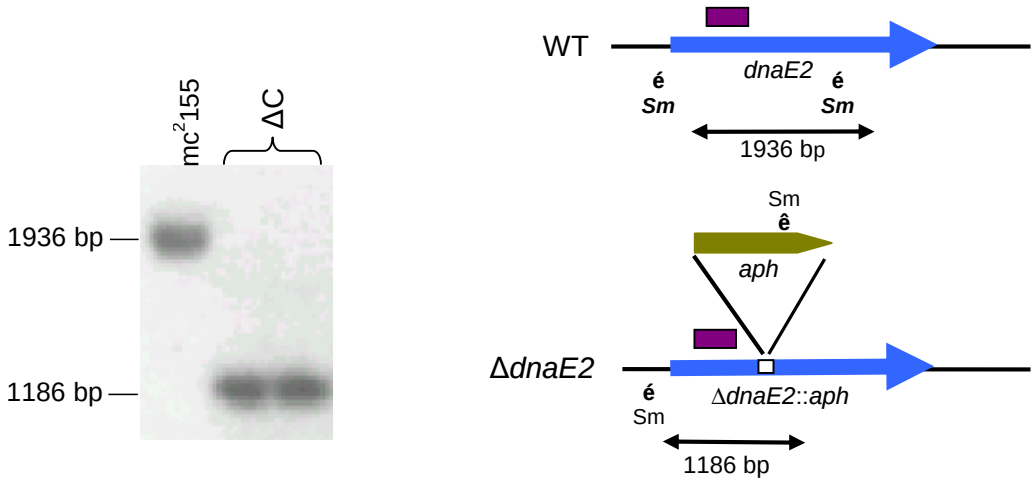


Figure 3.12 An EP polymerase-free strain (ΔC) of *M. smegmatis* mc²155. Disruption of dnaE2 in the ΔY (*dinP*$^{-/-}$/*dinP3*/*dinX*) background (see Figure 3.7) results in a multiple dinP$^{-/-}$/dinP3/dinX/dnaE2 deletion mutant (ΔC). The Southern blot shows *Sma*I-digested DNA from wild-type (mc²155) and the ΔC mutant probed with MsmE2 (purple box). Restriction maps of the relevant genotypes for wild-type and mutant strains are schematically illustrated in the line drawings adjacent to the blot (not drawn to scale; Sm, *Sma*I; WT, wild-type).

no significant growth or survival phenotype on the ΔC mutant, either in pure culture or in competition with wild-type mc²155.

M. smegmatis does not exhibit stationary-phase mutagenesis *in vitro*

Evidence implicating induced mutagenesis in long-term *in vitro* survival of *E. coli* under starvation conditions (Yeiser *et al*., 2002) raised the posssibility that the Y-family homologues might mediate the hypermutable state previously reported in stationary-phase mycobacterial populations (Karunakaran & Davies, 2000). To assess the contribution of the Y-family members, as well as DnaE2, to stationary-phase mutagenesis in *M. smegmatis*, the emergence of rifampicin resistant (Rif^{R}) mutants of each of the strains in pure culture was scored for the first 128 days during both long-term survival assays. That is, Rif^{R} mutants of wild-type mc²155, *dinP*$^{-/-}$, *dinX*, *dinP*$^{-/-}$/*dinP3*, *dinP*$^{-/-}$/*dinX*, and ΔY were isolated during LTG1, and of wild-type mc²155 and ΔC during LTG2. Mutation frequencies in each of the single (*dinP*$^{-/-}$ and *dinX*), and double (*dinP*$^{-/-}$/*dinP3*, *dinP*$^{-/-}$/*dinX*) gene deletion mutants, as well as the ΔY and ΔC multiple deletion mutants, did not differ significantly from wild-type mc²155 in both LTG1 and LTG2 (Figure 3.13). Furthermore, no increase in mutation frequency was observed in wild-type mc²155 or

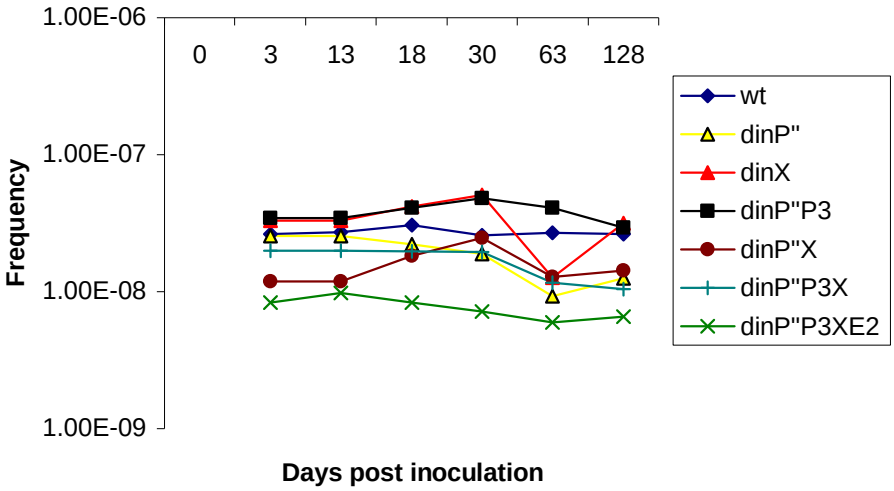


Figure 3.13 Rifampicin resistance fequencies of *M. smegmatis* strains during long-term growth *in vitro*. The emergence of rifampicin resistant (Rif^R) mutants of each of the *M. smegmatis* pol IV and EP polymerase mutant strains was scored for the first 128 days during the LTG1 and LTG2 long-term survival assays.

mutant strains at any timepoint during either assay (Figure 3.13). Although contradicting previous evidence of stationary-phase hypermutability in *M. smegmatis* mc^26 (Karunakaran & Davies, 2000), the mutation frequencies observed under the conditions tested are supported by parallel point and frameshift mutation rate assessments by fluctuation assay (S. Barichievy & R. Brackin, unpublished).

The identification of novel *rpoB* mutations in *M. smegmatis* mc^2155 in stationary phase

Base substitutions and small frameshift deletions constitute the most abundant TLS polymerase-mediated mutations (Friedberg *et al.*, 2001, 2002; Goodman, 2002; Tippin *et al.*, 2004b). However, patterns or "fingerprints" (Wolff *et al*., 2004) of nucleotide incorporation or deletion have been identified that are specific to the nature of the DNA template (or lesion if damaged) and the polymerase involved (Kim *et al*., 1997, 2001; Reuven *et al*., 1999; Tang *et al*., 1999, 2000; Wagner & Nohmi, 2000; Kobayashi *et al*., 2002; Shen *et al*., 2002; Goodman, 2002; Wagner *et al*., 2002; Tippin *et al*., 2004a). Despite the apparent dispensability of the mycobacterial EP polymerases for stationary-phase mutagenesis, it was postulated that loss of one or more TLS polymerases might alter the mutational profile during long-term persistence. To investigate this possibility, the *rpoB* resistance determining regions (Musser, 1995) of Rif^R mutants arising during LTG1

Table 3.7 *rpoB* mutations in Rif-resistant mutants derived from *M. smegmatis*[a] in LTG1.

		dinP$^{-/-}$		*dinX*		*dinP*$^{-/-}$*P3*		*dinP*$^{-/-}$*X*		ΔC	
		63	128	63	128	63	128	63	128	63	128
Mutation	Substitution	(12)	(12)	(9)	(6)	(14)	(13)	(14)	(11)	(11)	(11)
cac→cgc	His526→Arg	66.7	58.3	44.4	50	42.8	54	43	36	64	36
cac→ccc	His526→Pro							14	9	9	18
cac→tac	His526→Tyr	16.7	33.3	33.3	16.7	21.4	31	36	55	18	46
cac→gac	His526→Asp		8.3								
tcg→ttg	Ser531→Leu	8.3									
tcg→ttc	Ser531→Phe						15				
tcg→tgg	Ser531→Trp									9	
cag→aag	Gln513→Lys	8.3		11.1	16.7	21.4					
cag→cgg	Gln513→Arg					7.1					
cag→ctg	Gln513→Leu				16.7						
30 bp deln.	Gln513→Ser522			11.1		7.1		7			

[a] The table indicates the percentage frequency of each mutation recovered from a particular strain after 63 or 128 days growth in LTG1. The total number of mutants at each timepoint is shown in brackets.

and LTG2 were amplified by PCR and sequenced. Sequence analysis of the Rif^R genotypes isolated at specific timepoints during LTG1 (Table 3.7) and LTG2 (Table 3.8) revealed no obvious mutation fingerprint that might be assigned to an individual mycobacterial Y-family polymerase. In addition, the prevalence of Ser531 and His526 mutations in mediating Rif^R in mycobacteria was confirmed. However, in contrast to the *rpoB* mutational profiles described in *M. tuberculosis* (Telenti *et al.*, 1993, 1997; Musser, 1995; Ramaswamy & Musser, 1998; Billington *et al.*, 1999; Morlock *et al.*, 2000; Boshoff *et al.*, 2003; Mariam *et al.*, 2004), point mutations in the His526 codon constituted the dominant mc^{2}155 Rif^R genotype. Mutations at His526 accounted for all Rif^R mutants isolated from the Y-family gene deletion mutants after 128 days in LTG1 (Table 3.7), as well as the majority of Rif^R mutants of wild-type mc^{2}155 and ΔC after 56 days in LTG2. Furthermore, it was possible to discern a progressive trend in both LTG1 (Table 3.7) and LTG2 (Table 3.8) from diverse Rif^R genotypes at the earlier timepoints to almost exclusive His526 and Ser531 substitutions later on. The prevalence of these mutations suggests that His526 substitutions might incur the least fitness cost in *M. smegmatis*.

Table 3.8 *rpoB* mutations in Rif-resistant mutants derived from *M. smegmatis*[a] in LTG2.

		WT				ΔC			
		0	3	18	56	0	3	18	56
Mutation	Substitution	(0)	(8)	(20)	(19)	(1)	(16)	(5)	(18)
cac→cgc	His526→Arg		12.5	10	16		6		28
cac→ccc	His526→Pro			5	11		19		33
cac→tac	His526→Tyr		50	10	26	100	38	20	28
cac→gac	His526→Asp			5					
tcg→ttg	Ser531→Leu		12.5	35	31		13	20	5.5
tcg→tgg	Ser531→Trp						6	20	
cag→aag	Gln513→Lys								5.5
cag→cgg	Gln513→Arg						6		
cag→ctg	Gln513→Leu			15	16				
cgt→ctt	Arg529→Leu		25	5					
12 bp deln.	Gln513→Asp516						6		
30 bp deln.	Gln513→Ser522						6	40	
Unknown[b]				15					

[a] The table indicates the percentage frequency of each mutation recovered from a particular strain after 0, 3, 18, or 56 days growth in LTG2. The total number of mutants isolated at each timepoint is shown in brackets.
[b] Rif^R mutants which did not map to the sequenced *rpoB* amplicon.

Base substitutions in the codon for Gln513 served as another source of Rif^R mutants, consistent with similar observations in *M. tuberculosis* (Musser, 1995). However, Arg529Leu mutants were identified (Table 3.8) which have not been described previously for *M. tuberculosis* (Musser, 1995). Finally, although the contribution of individual polymerases to the mutational spectrum was not detectable in this analysis, two novel *rpoB* deletions conferring rifampicin resistance were identified. The largest of these comprised a 30 bp deletion spanning the residues Gln513 to Ser522 (Figure 3.14) and was found in Rif^R mutants isolated during both LTG1 – in *dinX*, *dinP*$^{-/-}$/*dinP3*, and *dinP*$^{-/-}$/*dinX* strains (Table 3.7); and LTG2, in the ΔC mutant (Table 3.8). In addition, Rif^R ΔC mutants were isolated containing a smaller 12 bp deletion that eliminated Gln513 to Asp516 (Table 3.8). While they could not be attributed to the loss of a specific individual polymerase, it was notable that deletions arising in both LTG1and LTG2 were restricted to mutant strains deficient in one or more Y-family members. Moreover, the identification of

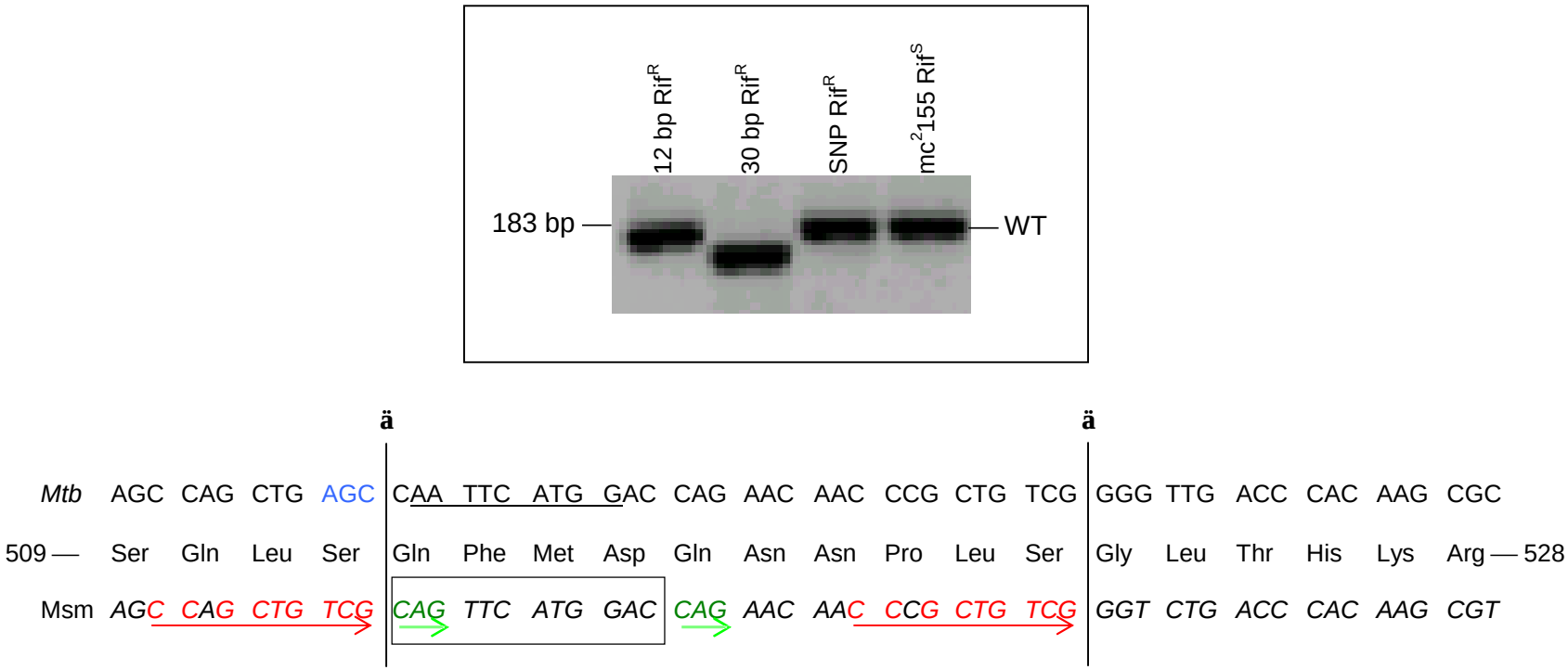


Figure 3.14 Deletions in the *rpoB* gene of *M. smegmatis* mc^{2}155 conferring Rif^R. The novel 30 bp in-frame deletion eliminates 10 aa from Gln513 to Ser522 (marked by **ä**). The 9 bp direct repeat sequences that might stabilise a hairpin structure or recombination intermediate (see Discussion) and so result in the deletion of the intervening DNA sequence are highlighted in red and underlined (→). Although sharing 100% amino acid identity (Hetherington et al, 1995), the *M. tuberculosis* (*Mtb*) and mc^{2}155 (*Msm*) coding sequences differ at Ser512 (highlighted in blue), thought likely to prevent the emergence of similar Rif^R deletion mutants in *M. tuberculosis* isolates. The location of the 12 bp deletion in the *M. smegmatis* ΔC Rif^R isolate is also shown (boxed region), and might depend on identical flanking Gln513 and Gln 517 codons (highlighted in green and underlined, →). A smaller 9 bp deletion found in the same locus in *M. tuberculosis* clinical isolates (Musser, 1995) is underlined in black. The codon numbering system is as previously described (Telenti *et al.*, 1993; Musser, 1995). The inset shows a 3% agarose gel comparing the *rpoB* PCR amplicons from Rif^S wild-type mc^{2}155 (mc^{2}155 Rif^S) with a Rif^R SNP mutant (SNP Rif^R), as well as the 12bp and 30bp Rif^R deletion mutants.

the 30 bp deletion in ΔC (lacking all Y-family homologues as well as *dnaE2*) as well as individual Y-family gene deletion mutants indicated that the polymorphism arose independently of DnaE2.

3.4.6 The Y-family polymerases in TLS

The *M. smegmatis* pol IV homologues are not required for UV tolerance

Loss of Y-family activity in other bacteria is associated with damage hypersensitivity (Rangarajan et al., 1999; Sung et al., 2003) and is consistent with the demonstrated TLS ability of these polymerases (Reuven *et al*., 1999; Tang *et al*., 1999, 2000; Wagner *et al*., 1999, 2002; Napolitano *et al*., 2000; Wagner & Nohmi, 2000; Kim *et al*., 2001; Kobayashi *et al*., 2002; Shen *et al*., 2002; Maor-Shoshani *et al*., 2003a). To assess the collective contribution of the mycobacterial Y-family homologues to damage tolerance, a UV survival assay was performed (Figure 3.15). Mycobacteria are highly sensitive to direct UV exposure, and that sensitivity is profoundly increased in *M. smegmatis* and *M. tuberculosis dnaE2* deletion mutants (Boshoff *et al*., 2003). Because of the dominant role of DnaE2, it was expected that any contribution of the Y-family members to UV tolerance would be small at best, and perhaps undetectable. However, the fact that UV survival data had been established for wild-type mc^{2}155 and the *dnaE2* deletion mutant (Boshoff *et al.*, 2003) meant that the same strains could be used as benchmarks to confirm UV killing efficiencies, as well as to elucidate the potentially minor role of the Y-family members in damage tolerance. To this end, UV survival of the ΔC strain (lacking all predicted EP activity) was compared with the Δ*dnaE2* mutant (possessing the full mycobacterial Y-family complement; Boshoff *et al*., 2003) and wild-type mc^{2}155. Irradiation with a range of UV fluences (0-45 mJ/cm^2) confirmed the previously reported (Boshoff *et al.*, 2003) differential susceptibilities of wild-type mc^{2}155 and the *dnaE2* mutant to UV exposure (Figure 3.15). In addition, although percentage survival values appeared to be approximately one log fold higher in our hands for both strains, the overall trend in UV-mediated killing was faithfully replicated. The discrepancy was therefore not considered sufficient to invalidate the assay, and likely resulted from differences between effective UV doses. Survival of the ΔC strain appeared to be slightly reduced in comparison to the Δ*dnaE2* mutant over the range of UV fluences tested but was not statistically significant relative to the standard error (Figure 3.15). To confirm that the mycobacterial DinP and DinX homologues do not participate in UV-induced damage tolerance, post-UV survival of mc^{2}155 strains containing the inducible *M. tuberculosis dinP* and *dinX* overexpression vectors (see Figure 3.5) was assayed. Heterologous overexpression of the *M.*

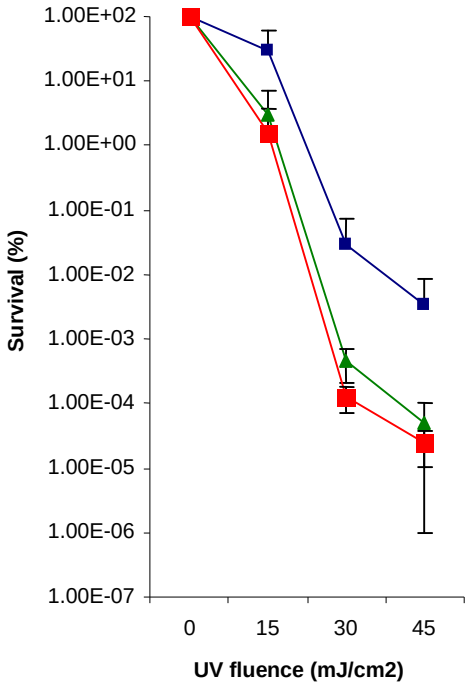


Figure 3.15 Effect of UV irradiation on survival of *M. smegmatis*. Serial dilutions of wild-type *M. smegmatis* mc^{2}155 (- ¢ -), the ΔdnaE2 mutant (- p -) and ΔC (- ¢ -) were plated in triplicate and irradiated at different energy fluences. No significant difference in survival frequencies was observed between the Δ*dnaE2* and ΔC mutant strains. Results represent the mean values of at least three independent experiments with standard errors.

tuberculosis Y-family homologues in this system did not result in enhanced UV tolerance in comparison with wild-type mc^{2}155, with or without acetamide induction (data not shown).

rpoB fingerprinting of Rif^R mutants from UV-irradiated cultures

The failure to identify a contribution of the mycobacterial Y-family homologues to UV survival suggested that damaged-induced mutation frequencies in the ΔC strain, lacking all EP activity, should be indistinguishable from the *dnaE2* mutant, containing the full putative mycobaterial Y-family complement. The measurement of UV-induced mutation frequencies was, however, complicated by the profound UV sensitivity associated with loss of *dnaE2* in the Δ*dnaE2* and ΔC mutants (Boshoff *et al.*, 2003; Figure 3.15). Therefore, as expected, no significant difference could be detected by scoring Rif^R mutants arising after UV irradiation of the Δ*dnaE2* and ΔC strains (data not shown). To confirm that the mycobacterial pol IV homologues do not participate in error-prone TLS (and so are not mutagenic), an indirect strategy was applied in which the *rpoB* mutational profiles arising in Rif^R Δ*dnaE2* and ΔC mutants post-UV irradiation were compared. Both Δ*dnaE2* and ΔC strains were exposed to a single, UV dose and allowed to recover in

liquid culture for specific time intervals before plating on Rif-containing media. Recovery was necessary to allow potentially mutagenic TLS repair of UV damage, and an intermediate fluence of 25 mJ/cm^2 was applied to ensure a sufficient population of surviving bacilli for selection. Genotypic analysis of the *rpoB* locus of all Rif^R mutants arising in both Δ*dnaE2* and ΔC at each recovery timepoint revealed no mutational bias between the two strains (Table 3.9). However, Rif^R mutants of ΔC were again isolated that contained the 30 bp deletion (Gln513→Ser522) in *rpoB*, thereby confirming the relevance of this polymorphism to the emergence of Rif^R in strains lacking one or more of the Y-polymerases.

Table 3.9 *rpoB* mutations in Rif-resistant mutants derived from UV irradiated *M. smegmatis*[a].

		Δ*dnaE2*			ΔC		
		2.5 hr	5 hr	24 hr	2.5 hr	5 hr	24 hr
Mutation	Substitution	(n=9)	(n=6)	(n=20)	(n=6)	(n=6)	(n=19)
cac→cgc	His526→Arg			5		33.3	10.5
cac→ccc	His526→Pro			30			10.5
cac→tac	His526→Tyr	33.3	16.7	25	33.3	33.3	42
ccac→ttac	ThrHis526→ThrTyr	11.1		10			
tcg→ttg	Ser531→Leu	33.3	50	20		16.7	32
tcg→ttc	Ser531→Phe			10			
gac→gag	Asp516→Glu	11.1					
gac→ggc	Asp516→Gly		16.7				
ggac→aaac	MetAsp516→IleAsn		16.7				
tcg→ttg	Ser522→Leu				16.7		
30 bp deln.	Gln513→Ser522						5
Unknown[b]		11.1			50	16.7	

[a] The table indicates the percentage frequency of each mutation recovered from a particular strain after 2.5, 5, or 24 hours recovery following UV irradiation. The total number of mutants isolated at each timepoint is shown in brackets.
[b] Rif^R mutants which did not map to the sequenced *rpoB* amplicon.

Significantly, the same deletion was not observed at any timepoint in the *dnaE2* deletion mutant (Table 3.9), consistent with previous evidence suggesting that the appearance of *rpoB* deletion mutants was *dnaE2*-independent. In addition, it was notable that the

mutation conferring Rif^R in half of all Rif^R ΔC mutants (3/6) isolated after 2.5 hrs recovery could not be mapped to the 81 bp resistance determining region, or to flanking sequence in the approximately 150 bp portion of the 183 bp PCR amplicon that gave readable sequence. One Rif^R *dnaE2* mutant was isolated at the initial timepoint that similarly could not be mapped to the analysed *rpoB* region. A small percentage of Rif^R mutations in clinical *M. tuberculosis* isolates do not contain mutations in the 81 bp *rpoB* core region and, in some cases, resistance cannot be mapped to mutations in *rpoB* at all (Musser, 1995). The origin of resistance in these strains remains unknown; however, it would appear that the undefined mutation might incur a significant fitness cost since this "genotype" was gradually cleared from the ΔC population over the course of recovery and was not evident after 24 hrs in either strain.

A cac→tac transition, resulting in a His→Tyr substition at His526, constitutes the most frequent UV-induced mutation in *M. tuberculosis* (Boshoff *et al.*, 2003), and was similarly dominant in both *M. smegmatis* Δ*dnaE2* and ΔC Rif^R mutants (Table 3.9). The characteristic mutation associated with UV-induced mutagenesis in wild-type *M. tuberculosis* is, however, a double ccac→ttac (ThrHis526→ThrTyr) transition that is very rarely present in non-irradiated H37Rv (Boshoff *et al.*, 2003). This double transition was completely absent in an *M. tuberculosis* Δ*dnaE2* deletion mutant, implying probable DnaE2-dependent mutagenic TLS across a cyclobutane pyrimidine dimer or a pyrimidine pyrimidone (6-4) photoproduct (Boshoff *et al.*, 2003). In contrast to *M. tuberculosis*, the ccac→ttac resistance mutation was evident in the *M. smegmatis* Δ*dnaE2* mutant strain, albeit at low frequencies (Table 3.9). However, the same mutation was not present in any of the ΔC Rif^R isolates, again suggesting the possible involvement of one or more of the pol IV homologues in mutagenic repair synthesis. Of the possible candidate polymerases, it is significant that *dinP3* is found in *M. smegmatis* but not *M. tuberculosis* and, further, that *dinP3* is inducible as part of the SOS response in mc^{2}155 (Figure 3.16). It is likely, therefore, that the functional DinP3 in the *M. smegmatis dnaE2* strain might partially compensate for loss of DnaE2-mediated TLS activity. However, the profound lethality associated with loss of *dnaE2* indicates that DinP3 activity barely complements DnaE2-mediated TLS.

3.4.7 Gyrase inhibition and induced mutagenesis

Mycobacterial Y-family polymerases are differentially induced in response to gyrase inhibitors

Considerable attention has been focused on the potential supplementation of TB chemotherapy with broad-spectrum antimicrobial agents, particularly the fluoroquinolones (FQs) (Tsukamura, 1985; Grosset, 1992; Cambau *et al.*, 1994; Crofton *et al.*, 1997; Ji *et al.*, 1998; Blumberg *et al.*, 2003; Fattorini *et al*., 2003; Gosling *et al*., 2003; O'Brien, 2003; Veziris *et al*., 2003; Aubry *et al.*, 2004; Nuermberger *et al*., 2004; Pletz *et al*., 2004). However, there have been reports of the rapid emergence of FQ-resistant *M. tuberculosis* clinical isolates (Perlman *et al*., 1997; Ginsburg *et al*., 2003). FQs block bacterial replication and transcription by targeting the type II topoisomerases, DNA gyrase and topoisomerase IV. Binding of topoisomerase IV and the GyrA subunit of DNA gyrase in complex with DNA results in the formation of stable ternary structures that cause potentially lethal DSBs (Drlica & Zhao, 1997) and potently induce the SOS response (Gudas & Pardee, 1976; Phillips *et al*., 1987; Piddock & Wise, 1987; Walters *et al.*, 1989; Piddock *et al.*, 1990; Ysern *et al*., 1990; Gmuender *et al.*, 2001). Mycobacterial SOS and damage repair genes including *dnaE2* are upregulated following exposure of H37Rv to FQs *in vitro* (Boshoff *et al*., 2004). Of particular interest, however, was the observation that *M. tuberculosis dinP* is induced in response to treatment with a related replication and transcription inhibitor of the coumarin class of antibiotics, novobiocin (NVB) (Boshoff *et al.*, 2004). NVB is bacteriostatic, and binds the DNA gyrase GyrB subunit without causing DSBs (Maxwell, 1993; Gmuender *et al.*, 2001; Boshoff *et al*., 2004). Although exposure to NVB fails to cause DSBs – and, therefore, does not result in mycobacterial SOS induction - *dnaE2* expresion is also upregulated in *M. tuberculosis* following NVB treatment (Boshoff *et al*., 2004). The SOS-independent induction of *dnaE2* contradicts previous evidence that *dnaE2* is solely LexA/RecA regulated (Boshoff *et al.*, 2003; Rand *et al.*, 2003) and is suggestive of an alternative damage-independent regulatory mechanism that might operate during replication stalling. To confirm the differential transcription of the *M. smegmatis* Y-family polymerases in response to alternative classes of gyrase inhibitor, wild-type mc^2155 was exposed to NVB and the fluoroquinolone, ofloxacin (OFX). Expression analysis by semi-quantitative RT-PCR revealed that neither *dinP* or *dinX* was upregulated after treatment with OFX for one or four hours (data not shown), confirming similar observations in *M. tuberculosis* (Boshoff *et al.*, 2004).

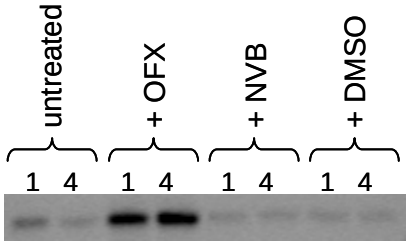


Figure 3.16 Transcriptional response of *M. smegmatis dinP3* to stalled replication. RT-PCR analysis of *dinP3* expression in *M. smegmatis* mc^{2}155 after exposure to alternative classes of gyrase inhibitor. Cells were treated with novobiocin (NVB) or ofloxacin (OFX) for one or four hours, and expression compared with untreated or solvent (DMSO) controls.

In contrast, transcription of *dinP3* was highly induced after only one hour OFX exposure (Figure 3.16), consistent with a postulated role for the additional *M. smegmatis* Y-family homologue in the LexA/RecA-mediated mycobacterial SOS regulon. Furthermore, treatment of *M. smegmatis* with NVB did not result in elevated *dinP3* expression (Figure 3.16).

The role of Y-family polymerases in survival and mutagenesis after gyrase inhibition

The unique upregulation of *dinP* in mycobacteria in response to transcriptional inhibition by NVB but not OFX (Boshoff *et al.*, 2004) was suggestive of a potential role of this Y-family homologue in mediating replication re-start following NVB-mediated replication fork arrest. In *E. coli* , for example, SOS-inducible Pol II (*polB*) is required for error-free rescue of stalled replication forks on damaged DNA (Rangarajan *et al*., 1999, 2002), perhaps followed by pol V-mediated lesion bypass (Friedberg *et al*., 1995; Rangarajan *et al*., 1999; Goodman & Woodgate, 2000; Pham *et al*., 2001). To determine whether loss of *dinP* was associated with increased susceptibility to gyrase inhibitors, wild-type mc^{2}155, as well as *dinP*$^{-/-}$, Δ*dinP3* and ΔC strains, were treated with OFX and NVB and cell densities and plating efficiencies determined at a series of timepoints extending over 24 hours. The ΔC and the *dinP*$^{-/-}$ mutants were utilised because it was felt that comparative susceptibilities of these two strains to different gyrase inhibitors might reveal the possible roles of the other mycobacterial pol IV (DinX and DinP3) and C-family EP (DnaE2) polymerases. Similarly, the *dinP3* mutant was included because of the demonstrated SOS-mediated upregulation of *dinP3* expression in response to OFX treatment (Figure 3.16). In addition, to elucidate any subtle effects of loss of the Y-family and EP polymerases, a range of NVB and OFX concentrations was employed spanning the known MICs of those compounds (Aubry *et*

al., 2004; Boshoff *et al.*, 2004). However, the susceptibilities of both the Y-family (*dinP*$^{-/-}$ and *dinP3*) and EP (ΔC) mutants to either drug were indistinguishable from wild-type mc^{2}155 throughout the 24 hour treatment (data not shown).

The demonstration that FQ treatment results in an SOS-mediated elevation in the mutation rate in other organisms (Gudas & Pardee, 1976; Phillips *et al*., 1987; Piddock & Wise, 1987; Walters *et al.*, 1989; Piddock *et al.*, 1990; Gmuender *et al.*, 2001) suggested that an evaluation of susceptibility might not detect subtle differences manifest in re-growth post antibiotic treatment. In particular, in the absence of published data on the mutagenicity of NVB, it was postulated that interference in DNA replication might result in an increased mycobacterial mutation rate despite a failure to induce the SOS response, potentially as a pleiotropic effect of DinP-mediated replication re-start. The presence of numerous Y-family homologues in mc^{2}155, however, posed the problem of redundant function. To eliminate the possible confounding effects of the other mycobacterial Y-family polymerases, NVB-induced mutation frequencies of wild-type mc^{2}155 and ΔY were compared by scoring the appearance of Rif^R mutants of each strain at specific timepoints following treatment. In addition, to determine the potential contribution of DnaE2, the ΔC mutant was included in the assay (Table 3.10). Susceptibilities of the ΔY and ΔC mutants to NVB (25 μg/ml) did not differ significantly from wild-type mc^{2}155, irrespective of treatment duration (data not shown). Exposure of mc^{2}155 to NVB resulted in an approximately 10-fold increase in frequency of Rif^R mutants throughout the 24 hr treatment period and, surprisingly, was indistinguishable in both ΔY and ΔC strains.

Table 3.10 Mutation frequencies following NVB treatment.

	Rif mutation frequency (x 10^{-8})	
Strain	untreated	+ NVB
mc^{2}155	6.04 ± 2.7	70.9 ± 15.2
ΔY	6.15 ± 2.0	54.5 ± 24.6
ΔC	9.67 ± 3.7	54.2 ± 20.5

However, it was noted that the Rif^R mutants arising in the ΔC and ΔY strains seemed overall to exhibit altered colony morphotypes - specifically smaller size – and were delayed in their appearance (data not shown). To confirm the increased Rif^R mutation frequencies, streptomycin resistant (Str^R) mutants of mc^{2}155, ΔY and ΔC were isolated in

parallel at the timepoints described above. The frequency of Str^R mutations in untreated wild-type mc^2155 as well as the ΔY and ΔC mutants was estimated at $7.0 \pm 4.0 \times 10^{-9}$, in line with previously reported values (Boshoff *et al.*, 2003). In contrast to the Rif analysis, however, treatment with NVB did not result in an increased Str^R mutation frequency in any of the strains throughout the 24 hr period (data not shown).

Novobiocin-induced mutagenesis

The elevated Rif^R frequency was indicative of induced mutagenesis, consistent with the observed upregulation of *dinP* expression after NVB treatment (Boshoff *et al.*, 2003). However, analysis of Str^R frequencies did not corroborate the hypermutability inferred from the emergence of Rif^R mutants. Furthermore, the failure to detect differential Rif^R mutation fequencies in wild-type mc^2155 *versus* either the ΔY or ΔC strains suggested that the phenomenon was independent of either the Y-family polymerases or DnaE2. To further investigate the contribution of mycobacterial Y-family homologues, as well as DnaE2, to NVB-induced mutagenesis, Rif^R mutants isolated after 3 hr NVB exposure were analysed genotypically (Table 3.11). Sequencing of a region of *rpoB* (Figure 3.14) from each of twenty Rif^R mutants of mc^2155, ΔY and ΔC revealed starkly contrasting mutational profiles (Table 3.11). Eighty-five percent (17/20) of Rif^R mutants arising in wild-type mc^2155 after 3hr NVB treatment contained substitutions at the common His526 or Ser531 loci, the majority of which resulted from a cac→cgc (His526→Arg) transition mutation. Of the remaining fifteen percent (3/20), one Rif^R mutant did not map to the sequenced *rpoB* region, and another possessed a ctg→cgg (Leu533→Pro) transversion previously identified in Rif^R *M. tuberculosis* isolates (Musser, 1995). The final mc^2155 Rif^R mutant was characterised by separate mutations in two sequential codons (accc→gcca), resulting in a double 519AsnPro520→519SerGln520 substitution, a mutation that has not been described in *M. tuberculosis* (Musser, 1995). Novel Rif^R mutations were also identified in the ΔY and ΔC strains, including two substitutions at Arg529 in ΔY and at His542 in ΔC. Most revealing, however, was the finding that 55% of Rif^R ΔC and 70% of Rif^R ΔY mutants could not be mapped to the sequenced *rpoB* region. In addition, 30 bp *rpoB* deletion mutants of ΔC and ΔY were again isolated. No deletions were observed in wild-type mc^2155, consistent with previous data suggesting that the deletion might be unique to strains partially or completely deficient in Y-family homologues.

Table 3.11 *rpoB* mutations in Rif-resistant mutants after treatment of *M. smegmatis*[a] with NVB.

		WT	ΔY	ΔC
Mutation	Substitution	(n=20)	(n=20)	(n=20)
cac→cgc	His526→Arg	60	5	5
cac→ccc	His526→Pro	5	5	
cac→tac	His526→Tyr	5	5	
tcg→ttg	Ser531→Leu	10		
tcg→tgg	Ser531→Trp	5		5
cag→ccg	Gln513→Pro			5
gac→ggc	Asp516→Gly			5
ctg→atg	Leu521→Met			5
cgt→tgt	Arg529→Cys		5	
cgt→cat	Arg529→His		5	
ctg→cgg	Leu533→Pro	5		10
cac→ctc	His542→Leu			5
aac→agc/ ccg→cag	Asn519→Ser/ Pro520→Gln	5		
30 bp deln.	Gln513→Ser522		5	5
Unknown[b]		5	70	55

[a] The table indicates the percentage frequency of each mutation identified in selected Rif^R isolates arising after 3 hr NVB exposure. Twenty mutants were isolated at each timepoint (ishown in brackets).
[b] Rif^R mutants which did not map to the sequenced *rpoB* amplicon.

3.4.8 The role of Rv3394c in damage protection

Transcriptional profiling of the *M. tuberculosis* response to various DNA damaging agents *in vitro* revealed the upregulation of two additional putative DNA polymerases, encoded by Rv3856c and Rv3394c (Boshoff *et al.*, 2003). Induction of Rv3856c is apparently LexA/RecA-independent (Davis *et al.*, 2002a), suggesting alternative damage-response regulation (Rand *et al.*, 2003; Gamulin *et al.*, 2004). Furthermore, although Rv3856c contains both phosphodiesterase and X-family polymerase domains (http://genolist.pasteur.fr/TubercuList/), the X-family polymerases consitute a large family of nucleotidyl transferases, many of which are not DNA polymerases (Aravind & Koonin, 1999). Similarly, although identified as the founder member of the novel DinB3 subfamily of putative eubacterial Y-family polymerases (Dalrymple *et al.*, 2003), the predicted amino acid sequence of Rv3394c indicates loss of catalytic residues required for polymerase

activity (Figure 3.1). However, the involvement of DinB3 and DnaE2 family members in the same replication or repair pathway is implied by their frequent co-localisation in sequenced eubacterial genomes (Dalrymple *et al.*, 2003); in *M. tuberculosis*, for example, *dnaE2* (Rv3370c) and Rv3394c are separated by 24.7 kb. Furthermore, and consistent with damage induction (Boshoff *et al.*, 2003), a Cheo-type SOS box (Cheo *et al.*, 1991) has been identified upstream of the coding region of a predicted ORF, Rv3395c, that appears to be operonic with Rv3394c (Davis *et al.*, 2002a).

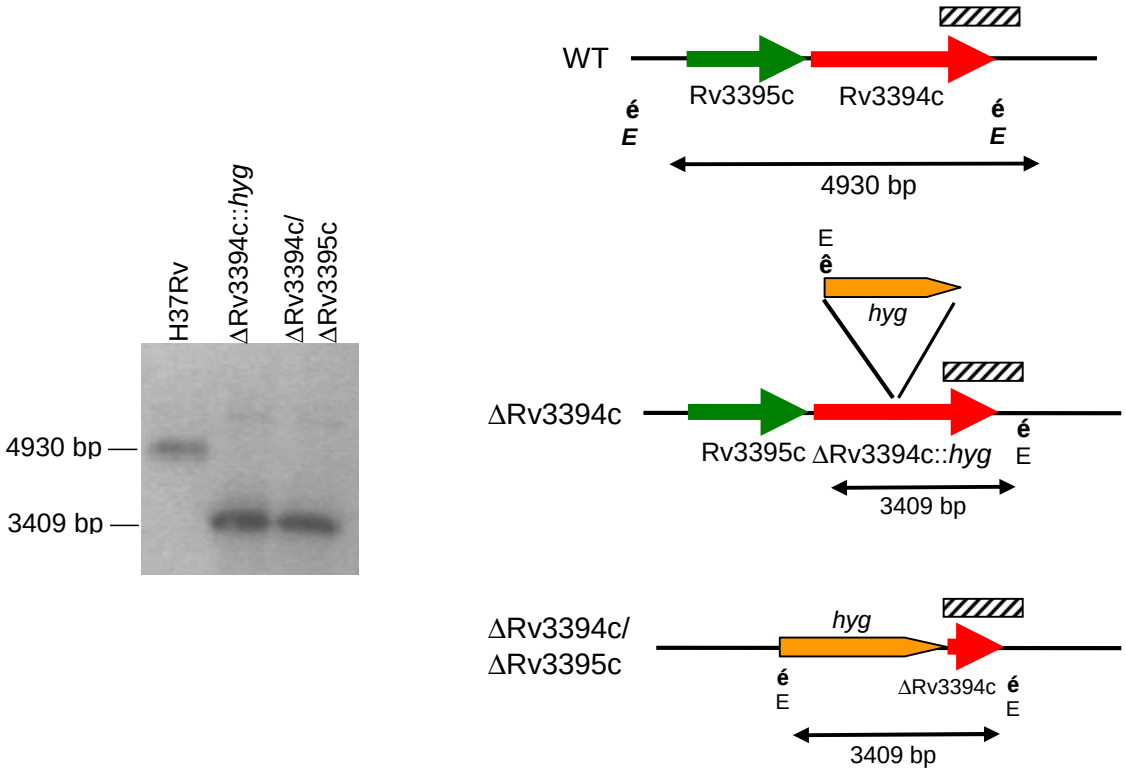


Figure 3.17 Construction of Rv3394c and Rv3395c deletion mutants of *M. tuberculosis* H37Rv. *Eco*RI-digested DNA from H37Rv, the ΔRv3394c deletion mutant, and the ΔRv3394c/ΔRv3395c deletion mutant probed with 3394FR1 (shaded box). Construction of the ΔRv3394c allele eliminates 1014 bp of Rv3394c coding sequence; the ΔRv3394c/ΔRv3395c allele eliminates 1371 bp of Rv3394c coding sequence and Rv3395c entirely. However, an additional *Eco*RI site is introduced by the *hyg* cassette (E, *Eco*RI; WT, wild-type).

To investigate the potential role of Rv3394c in mediating damage tolerance induced mutagenesis in *M. tuberculosis*, two *hyg*-marked deletion-insertion mutants of H37Rv were generated by the two-step method. The first of these, ΔRv3394c, eliminated 1014 bp (of 1584 bp) Rv3394c sequence (Figure 3.17), while in the other (ΔRv3394c/ΔRv3395c) a segment comprising the entire Rv3395c gene plus 1371 bp Rv3394c sequence was

deleted (Figure 3.17). Neither strain was impaired *in vitro* under normal growth conditions (data not shown). Rv3394c contains a conserved IMS (*impB*/*mucB*/*samB*) domain that, in other bacteria, has been implicated in UV protection and induced mutagenesis (Lodwick *et al.*, 1990; Gruz *et al.*, 1996, 1998; Runyen-Janecky *et al.*, 1999). The induction of Rv3394c in response to UV and H_2O_2 exposure was, therefore, strongly suggestive of a role in SOS-regulated damage repair (Boshoff *et al.*, 2003). To determine whether loss of Rv3394c resulted in increased susceptibility to DNA damaging agents, survival of wild-type H37Rv and the ΔRv3394c mutant were compared after 6 hours treatment with 5 mM H_2O_2. No difference in plating efficiencies was observed between strains at either H_2O_2 concentration (data not shown). Similarly, exposure of both wild-type H37Rv and ΔRv3394c to a range of UV fluences from 0-45 mJ/cm^2 did not reveal increased damage susceptibility in the Rv3394c mutant (Figure 3.18).

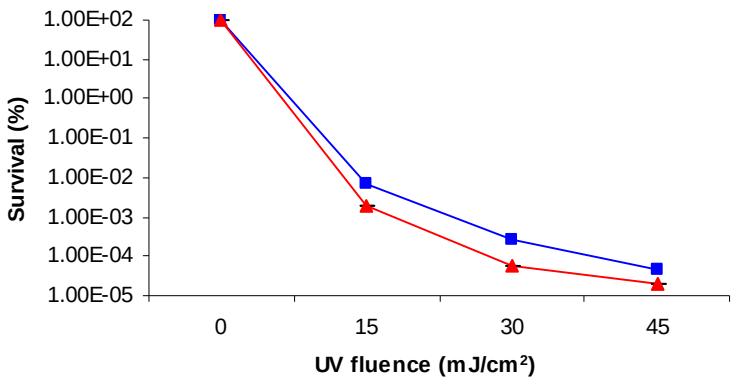


Figure 3.18 Damage survival of *M. tuberculosis* strains. Serial dilutions of wild-type *M. tuberculosis* (-¢-) and the ΔRv3394c mutant (-p-) were plated in triplicate and irradiated at different energy fluences. No significant difference in survival frequencies was observed between the two strains. Results represent the mean values of two independent experiments with standard errors.

3.5 DISCUSSION

Prokaryotic Y-family polymerases share several characteristic genetic features, including their induction in most bacterial SOS responses (Kenyon & Walker, 1980; Bagg *et al.*, 1981; Friedberg *et al.*,1995; Becherel & Fuchs, 1999; Walker *et al.*, 2000; Courcelle *et al.*, 2001; Duigou *et al.*, 2004), as well as the demonstration that their overexpression results in elevated rates of untargeted (Kim *et al.*, 1997; Duigou *et al.*, 2004) and UV-targeted mutagenesis (Duigou *et al.*, 2004). Members of the Y-family have been implicated in two broad, but intricately linked, cellular processes – damage tolerance through the rescue of stalled replication forks by specialised lesion bypass, and the generation of targeted and untargeted mutations that contribute to adaptive evolution. Preliminary experiments with recombinant enzyme have established that *M. tuberculosis* DinX exhibits template-directed DNA polymerase activity (B. Kana, unpublished). In contrast, previous reports have shown that neither *M. tuberculosis* pol IV homologue – encoded by *dinP* and *dinX* – is upregulated in response to DNA damage (Brooks *et al.*, 2001; Boshoff *et al.*, 2003). The absence of pol IV homologues from the mycobacterial SOS regulon (as well as the LexA/RecA-independent damage response; Rand *et al.*, 2003; Gamulin *et al.*, 2004) represents the first departure from classic Y-family behaviour. That observation was extended in the work presented here to a demonstration that the mycobacterial homologues fail, with limited exceptions, to fulfil additional genetic requirements of prototypical Y-polymerase activity. Specifically, heterologous overexpression of *M. tuberculosis dinP* and *dinX* does not increase *M. smegmatis* mc^2155 mutation rates irrespective of DNA damage (Table 3.6). Moreover, loss of the *M. smegmatis* pol IV complement has no effect on UV susceptibility (Figure 3.15), and overproduction of the *M. tuberculosis dinP*- and *dinX*-encoded pol IV homologues does not restore damage tolerance even partially. Conversely, abrogation of all identifiable pol IV (ΔY) or EP polymerase activity (ΔC) fails to impair the long-term survival of mc^2155 (Figure 3.11), suggesting that none of these polymerases – including the SOS-inducible pol IV encoded by *dinP3* – confers a competitive fitness advantage during stationary phase adaptation.

EP polymerases in Gram-positive *versus* Gram-negative bacteria

The recent identification of DnaE2 as the dominant player in damage tolerance and induced mutagenesis in *M. tuberculosis* (Boshoff *et al*., 2003) was particularly surprising given the mycobacterial Y-family complement (Table 3.12). In *E. coli*, the *dnaE*-encoded C family polymerase provides the main, high-fidelity replicative polymerase activity (McHenry & Kornberg, 1977; McHenry, 1988; Kornberg & Baker, 1992), while pol II and the EP pols IV and V are strongly upregulated during SOS (Kenyon & Walker, 1980; Bagg

et al., 1981; Iwasaki *et al.*, 1990; Woodgate & Ennis, 1991; Friedberg *et al.*, 1995; Bonner *et al*., 1988; Kim *et al.*, 1997, 2001; Qiu & Goodman, 1997; Walker *et al.*, 2000; Courcelle *et al.*, 2001). In contrast, two distinct subclasses of C-family polymerases have been identified in Gram-positive bacteria (Bruck & O'Donnell, 2000): class I (*dnaE*-type) which lack intrinsic proofreading ability; and class II (*polC*-type), which contain both polymerase and 3'→5' proofreading exonuclease activities on one polypeptide chain. Significantly, recent evidence has shown that the complement and function of the C-family polymerases varies among these organisms (Dervyn *et al*., 2001; Inoue *et al*., 2001; Boshoff *et al.*, 2003; Bruck *et al*., 2003). In *B. subtilis* and *S. aureus*, for example, both *polC* and *dnaE* polymerase subclasses are essential, separately catalysing leading and lagging strand synthesis, respectively (Dervyn *et al*., 2001; Inoue *et al*., 2001). Furthermore, *B. subtilis dnaE* has been implicated in SOS-induced mutagenesis (Le Chatelier *et al.*, 2004), suggesting a role in both replication and EP TLS depending on differential protein interactions (Tippin *et al.*, 2004b). *Streptococcus pyogenes*, on the other hand, encodes essential class I and class II polymerases, with PolC providing sole replicative function and DnaE EP polymerase activity (Bruck & O'Donnell, 2000; Bruck *et al*., 2003). *M. tuberculosis* does not contain a *polC*-type C-family polymerase but, instead, two *dnaE*-type polymerases separately fulfil essential replicative (*dnaE1*) and non-essential EP (*dnaE2*) polymerase roles (Boshoff *et al.*, 2003). Although unusual, the presence of two *dnaE*-type C-polymerases is not unique, and several other Gram-positive bacterial species have been shown to contain multiple *dnaE* genes (Boshoff *et al.*, 2003; Tippin *et al.*, 2004b). Of course, the presence of one or more Y-family homologues in addition to the expanded *dnaE* complement raises compelling questions regarding the function of the Y-polymerases in mediating damage tolerance and induced mutagenesis in those organisms (Tippin *et al*., 2004b). In particular, the unusual induction of the specific polymerase types in *M. tuberculosis* and some other Gram-positive organisms (Y-family polymerases are not SOS-regulated, C-family polymerases are) has prompted speculation that distinct LexA/RecA-dependent damage responses might have evolved in Gram-negative versus Gram-positive organisms involving discrete polymerase families (Tippin *et al*., 2004b).

The mycobacterial SOS and damage-inducible polymerase complement

M. tuberculosis does not possess homologues of either *E. coli* SOS-inducible pols II or V (Cole *et al.*, 1998; Mizrahi & Andersen, 1998). Instead, in addition to DnaE2, the *M. tuberculosis* SOS regulon includes another putative DNA polymerase, Rv3394c (Davis *et al.*, 2002a; Boshoff *et al.*, 2003). Despite lacking conserved acidic residues required for

Table 3.12 Y-family and EP polymerase complements and properties.

Polymerase	Expression			UV tolerance	UV mutagenesis	β clamp[‡]	Reference
	SOS	NVB	Stat. phase				
E. coli							
pol II	ü	?	ü	ü	û	ü	Kenyon & Walker, 1980; Bagg *et al.*, 1981;
pol IV	ü	?	ü	û	û	ü	Walker *et al.*, 2000; Courcelle *et al.*, 2001;
pol V	ü	?	ü	ü	ü	ü	Yeiser *et al.*, 2002; Layton & Foster, 2003
B. subtilis[Ý]							
DnaE	ü	?	?	û	ü	ü	Le Chatelier *et al.*, 2004
pol IV (YqjH)[†]	û	?	?	û	û	ü	Duigou *et al.*, 2004
pol V (YqjW)	ü	?	?	û	ü	ü	Duigou *et al.*, 2004
M. smegmatis							
DnaE2	ü	ü	?	ü	ü	û	Boshoff *et al.*, 2003; This work
pol IV							
DinP	û	ü	?	û	û	û	
DinX	û	û	?	û	û	ü	This work
Rv3394c	ü	ü	?	û	û	ü	R. Brackin & E. Machowski, unpublished
DinP3	ü	û	?	û	û	ü	
M. tuberculosis							
DnaE2	ü	ü	?	ü	ü	û	Brooks *et al.*, 2001; Boshofff *et al.*, 2003
pol IV							
DinP	û	ü	?	û	û	û	Brooks *et al.*, 2001; Davis *et al.*, 2002a;
DinX	û	û	?	û	û	ü	Boshoff *et al.*, 2003;
Rv3394c	ü	û	?	û	û	ü	Rand *et al.*, 2003

[‡] The presence of a consensus β-clamp binding motif is as per Dalrymple *et al.* (2001, 2003).
[Ý] In addition to *yqjH* and *yqjW*, the *B. subtilis* genome contains putative *yozK-yobH* and *uvrX*-encoded Y-family DNA polymerases which are part of integrated prophages (Kunst *et al*., 1997). The study cited, by Duigou *et al.* (2004), employed a *B. subtilis* strain cured of its prophages.
[†] *B.subtilis* YqjH and YqjW are loosely designated homologues of *E. coli* pols IV and V, respectively, based on the genetic analysis of Duigou *et al.* (2004).

polymerase activity (Figure 3.1), Rv3394c contains putative DinB and IMS domains (http://genolist.pasteur.fr/TubercuList/) that are suggestive of a role in damage tolerance. In addition, a putative β clamp binding motif classifies Rv3394c as a novel DinB3-type Y-family polymerase (Dalrymple *et al.*, 2003). Expression of Rv3394c is regulated by an SOS-box located immediately upstream of Rv3395c (Davis *et al.*, 2002a), and is strongly upregulated following DNA damage (Davis *et al.*, 2002a; Boshoff *et al.*, 2003) and OFX-mediated gyrase inhibition. However, in this study it was demonstrated that Rv3394c activity is not required for survival of oxidative (H_2O_2) or UV-induced DNA damage (Figure 3.18). The dispensability of Rv3394c for damage tolerance in *M. tuberculosis* is consistent with the dominant role of DnaE2 (Boshoff *et al.*, 2003). However, it is possible that loss of Rv3394c does not affect survival but instead translates into altered damage-induced mutation profiles. The contribution of Rv3394c to induced mutagenesis might, therefore, be better elucidated by comparing Δ*dnaE2* and Δ*dnaE2*/ΔRv3394c double mutants. It is notable, for example, that the cac→tac (His526→Tyr) transition that constitutes the most frequent mutation in UV-irradiated wild-type *M. tuberculosis* H37Rv (Boshoff *et al.*, 2003) was also the dominant Rif^R mutation in both *M. smegmatis* Δ*dnaE2* and ΔC Rif^R strains after UV irradiation (Table 3.9), suggesting that some other TLS activity might be present in strains deficient in DnaE2 and pol IV-mediated EP polymerase activity.

The *M. smegmatis* mc^2155 damage-inducible regulon is distinct from *M. tuberculosis* (Table 3.12) in that it includes a LexA/RecA-dependent pol IV homologue encoded by *dinP3* (MSMEG6405). Treatment of mc^2155 with the known mycobacterial SOS inducer, OFX (Boshofff *et al.*, 2004), confirmed the potent upregulation of *dinP3* in response to DNA damage (Figure 3.16) and pointed to a possible role in damage tolerance, although this was not supported by the indistinguishable UV susceptibilities of the Δ*dnaE2* and ΔC mutant strains (Figure 3.15). However, some evidence of a possible role for DinP3 was provided by the Rif^R profiles of UV-irradiated mc^2155 strains. In particular, genotypic analysis of the core *rpoB* resistance-determining region in Rif^R mutants of UV-treated mc^2155 Δ*dnaE2* identified the ccac→ttac (ThrHis526→ThrTyr) double transition mutation that is the hallmark of UV-irradiated *M. tuberculosis* cells (Table 3.9). Although detected at low frequencies, the presence of the double transition genotype in the *M. smegmatis* Δ*dnaE2* mutant contrasted with previous evidence of the elimination of that mode of lesion bypass in *M. tuberculosis* Δ*dnaE2* (Boshoff *et al.*, 2003). Furthermore, the same mutation was not present in any of the UV-induced Rif^R isolates of *M. smegmatis* ΔC, again implicating one or more of the mc^2155 Y-family homologues (*dinP3*, *dinP* or *dinX*) in

mutagenic repair synthesis. Of these, the SOS-inducible *dinP3* uniquely distinguishes *M. tuberculosis* and *M. smegmatis* and is, therefore, the most compelling candidate. Of course, the profound damage susceptibility associated with loss of *dnaE2* indicates that DinP3 cannot complement the absent DnaE2, but may play a minor role in UV-induced TLS.

The failure of *M. tuberculosis dinP* or *dinX* overexpression to enhance UV tolerance in mc^2155 is also consistent with the dispensability of the mycobacterial pol IV homologues in repair of UV-induced damage. However, it is possible that access of the overexpressed *M. tuberculosis* pol IV homologues to the template DNA might be restricted by the full replicative and EP polymerase complement in wild-type mc^2155 (explored below). In *E. coli*, for example, pol IV interacts with RecA, and the $UmuD_2$ homodimer, to form a complex that stabilises stalled replication forks (Godoy *et al.*, 2004). Interestingly, if UmuD is supplied, rates of adaptive mutagenesis in strains overexpressing pol IV are reduced (Godoy *et al.*, 2004), which suggests that previous reports of hypermutation as a result of pol IV overproduction likely reflected artificial "flooding" of regulatory interactions. Similarly, although genotypic analysis hinted at a role for the pol IV homologues in TLS, it is possible that the effect is not biologically relevant, but instead reflects artificially increased access to the damaged DNA template in the absence of DnaE2. Therefore, it might be interesting to investigate the potential competition among the mycobacterial polymerases for the replication fork. To this end, spontaneous *hisD* frameshift mutation and point mutation rates, as well as post-UV survival, will be assessed in *M. smegmatis* ΔC mutants overexpressing *M. tuberculosis dinP* and *dinX*. The Hyg^R genotype of ΔC precludes the use of the acetamidase-inducible expression vectors (which are also *hyg*-marked); therefore, alternative, Km^R vectors have been constructed in which *M. tuberculosis dinP* and *dinX* are constitutiveley expressed off the mycobacterial Hsp60 promoter (data not shown). In addition, an investigation of potential mycobacterial pol IV interactions - using a yeast two-hybrid system, for example – should also reveal auto- or cross-modulatory binding.

Mycobacterial EP polymerases do not contribute to long-term fitness

Mutants of increased fitness emerge in bacterial populations subject to prolonged stationary phase or starvation (Novick & Szilard, 1950; Helling *et al.*, 1987; Cairns & Foster, 1991; Zambrano *et al*., 1993; Zambrano & Kolter, 1993, 1996; Lenski & Travisano, 1994; Zinser & Kolter, 1999; Sung & Yasbin, 2002; Elena & Lenski, 2003; Pedraza-Reyes & Yasbin, 2004; Tegova *et al.*, 2004). Similarly, long-term maintenance of *M. smegmatis*

in vitro results in the generation of strains that are better adapted to the limiting conditions, out-competing exponential phase-adapted bacilli in stationary-phase culture (Smeulders *et al*., 1999). Mutants are thought to arise during an induced state of stationary-phase hypermutability in mc^{2}155 (Karunakaran & Davies, 2000), reminiscent of adaptive mutations in other model systems (Foster, 2000; Rosenberg, 2001; Sung & Yasbin, 2002; Hastings *et al.*, 2004). Together with the requirement for DnaE2 for persistence of *M. tuberculosis in vivo* and, particularly, emergence of antibiotic resistance isolates, these observations were strongly suggestive of the relevance of adaptive mutagenesis to mycobacterial pathogenesis. Significantly, pol IV homologues have been implicated more than any other SOS polymerase in stationary-phase mutagenesis in several bacterial systems (Sung *et al.*, 2003; Tegova *et al.*, 2004; Hastings *et al.*, 2004), although the precise contribution remains unresolved (Strauss *et al*., 2000; McKenzie *et al*., 2000, 2001, 2003; Kim *et al*., 2001; Hastings *et al.*, 2004; Kuban *et al*., 2004; Wolff *et al*., 2004).

Deletion of all pol IV function (other than the possible contribution of the Rv3394c homologue) in ΔY (*dinP*$^{-/-}$/*dinP3*/*dinX*) failed to impair competitive fitness of *M. smegmatis* mc^{2}155 during extended stationary-phase adaptation *in vitro* (Figure 3.11A). This result was consistent with the failure of individual (*dinP*$^{-/-}$, *dinX*) and double (*dinP*$^{-/-}$/*dinP3*, *dinP*$^{-/-}$/*dinX*) pol IV deletion mutants to impact survival (Figure 3.11A) but was surprising given the activity (Figure 3.4) of the *M. tuberculosis* Rv3055 (directly upstream of *dinP*; Figure 3.2) and *dinX* promoter regions in mc^{2}155 during stationary-phase. In *E. coli*, for example, although all three SOS polymerases are required for competitive fitness and are constitutively transcribed throughout long-term survival (Yeiser *et al*., 2002), pol IV alone is induced late in stationary-phase subject to RpoS and GroE regulation (Layton & Foster, 2003, 2005), perhaps in combination with RpoS-mediated MMR downregulation (Tsui *et al*., 1997; Bjedov *et al*., 2003). Furthermore, although overexpression of *dinP* and *dinX* failed to influence mutagenesis in mc^{2}155 (Table 3.6), it was felt that the conditions (or factors) required for enhanced access of any or all of the pol IV homologues to the DNA template might prevail during stationary phase - the *dnaN*-encoded β-processivity factor (discuused below), for example, is induced during early adaptation of *M. tuberculosis* to starvation *in vitro* (Betts *et al.*, 2002). DnaE2 similarly fails to elevate rates of spontaneous mutagenesis when overexpressed *in vitro* (Boshoff *et al.*, 2003) but is implicated in adaptive evolution of drug resistance. However, deletion of *dnaE2* as well as the three pol IV homologues did not impair the ability of ΔC to survive in co-culture with wild-type (Figure 3.11B). Although this observation correlated with the failure to observe stationary-phase hypermutability in any mc^{2}155 strain including wild-type during either LTG1 or

LTG2, the dispensability of *dnaE2*, in particuar, was difficult to reconcile with the demonstrated *in vivo* role of *M. tuberculosis dnaE2* (Boshoff *et al.*, 2003). It is possible, however, that *M. smegmatis* does not incur sufficient damage during stationary-phase survival under the conditions tested and that the mycobacterial SOS damage response pathway (Brooks *et al.*, 2001; Boshoff *et al.*, 2003) is not induced. Alternatively, Rv3394c might be able to complement the loss of the pol IV homologues during long-term persistence, a possibility which requires further investigation.

Restricted access of mycobacterial EP polymerases to the replication fork?

The demand for high speed and high fidelity of DNA replication requires that the TLS polymerases be maintained in close proximity to the replicative complex but with their mutagenic activity under control, and prompts an unresolved question regarding the ability of cells to co-ordinate the activities of multiple different polymerases at a single replication fork (Friedberg *et al.*, 2002; Yang, 2003; Kunkel, 2004). In particular, the observation that the replicative flexibility provided by the TLS polymerase is counter-balanced by poor processivity and reduced fidelity on undamaged DNA or non-cognate lesions suggests that the expression of these enzymes, and their interaction with the DNA template, must be closely regulated (Friedberg *et al.*, 2002; Dalrymple *et al.*, 2003; Yang, 2003; Rattray & Strathern, 2003; Tippin *et al.*, 2004b). Overexpression of *E. coli* pol IV on multi-copy plasmids profoundly increases rates of spontaneous mutagenesis, resulting particularly in –1 frameshifts, but also base substitutions (Kim *et al*., 1997, 2001; Wagner & Nohmi, 2000; Kobayashi *et al*., 2002). In contrast, overexpression of *M. tuberculosis dinP* and *dinX* in *M. smegmatis* mc^2155 did not increase rates of spontaneous point (Rif^R) or frameshift (*hisD*5T) mutations above wild-type mc^2155 (Table 3.6), despite the confirmation of increased transcript levels (Figure 3.5). This result was analagous to the failure of *M. tuberculosis dnaE2* overproduction to elevate mutation frequencies in the absence of DNA damage (Boshoff *et al.*, 2003

As has been repeatedly emphasised, the *M. tuberculosis* genome encodes two pol IV homologues (*dinP* and *dinX*) plus Rv3394c (Cole *et al.*, 1998); *M. smegmatis* mc^2155, on the other hand, contains a *dinP3*-encoded Y-family member and an extra copy of *dinP* (Figure 3.7), in addition to homologues of all three *M. tuberculosis* Y-polymerases. Combined with the essential, high-fidelity C-family replicative polymerase encoded by *dnaE1* and the novel TLS C family polymerase encoded by *dnaE2* (Boshoff *et al.*, 2003), as well as the mycobacterial pol I (*polA*; Mizrahi & Huberts, 1996), *M. tuberculosis* H37Rv therefore contains at least six defined or putative polymerases (Cole *et al.*, 1998; Mizrahi

et al., 2000), and *M. smegmatis* eight (http://www.tigr.org). Together with the prospect that these numbers might be expanded by further polymerase homologues (Makarova *et al.*, 2002), it seems likely that additional factors might be required to facilitate access of the non-replicative polymerases to undamaged DNA in mycobacteria (Boshoff *et al*., 2003; Tippin *et al.*, 2004b).

The β clamp and mycobacterial polymerase switching

The current TLS model proposes that a stalled replicative polymerase is temporarily replaced by one or more TLS polymerases to prevent replication fork collapse and to ensure efficient lesion bypass and strand elongation (Friedberg *et al.*, 2002; Goodman, 2002; Yang, 2003; Kunkel, 2004). On completion of TLS, the specialised polymerases dissociate from the DNA, allowing the main replicative enzyme to resume high-fidelity processive replication. Recent evidence implicates the *dnaN*-encoded eubacterial β_2 processivity factor in modulating the recruitment and operation of many proteins involved in various steps of DNA replication, recombination, and repair (Naktinis *et al*., 1996; Warbrick, 2000; Yang *et al*., 2000; Dalrymple *et al*., 2001, 2003; López de Saro & O'Donnell, 2001; Ohmori *et al*., 2001; Lenne-Samuel *et al.*, 2002; Noirot-Gros *et al*., 2002; Bunting *et al.*, 2003; López de Saro *et al.*, 2003a, 2003b; Sung *et al*., 2003; Burnouf *et al.*, 2004; Kurz *et al.*, 2004; Sutton, 2004; Wijffels *et al*., 2004). According to the model, polymerase "switching" is facilitated by the action of processivity factors which function as molecular "tool-belts" to interchangeably attach replicative and TLS polymerases to the primer-template junction, perhaps without disruption of the Pol III core assembly (Pagès and Fuchs, 2002, 2003). Significantly, all five characterised *E. coli* DNA polymerases interact with the β clamp (Hughes *et al*., 1991; Naktinis *et al*., 1995; Kim & McHenry, 1996; Wagner *et al*., 2000; López de Saro & O'Donnell, 2001; Sutton *et al*., 2001a, 2002; Lenne-Samuel *et al*., 2002), and β binding increases the processivity and efficiency of pols II, IV and V (Hughes *et al*., 1991; Bonner *et al*., 1992; Rangarajan *et al*., 1999; Sutton *et al*., 1999; Wagner *et al*., 1999; Tang *et al*., 2000). Furthermore, biochemical and structural suggest that the β clamp might fulfil the dual role of maintaining pol IV near the primer-template junction as well as ensuring that the polymerase remains bound to DNA sufficiently long to allow replication through intractable template sequences, but not long enough to generate a lethal mutational load (Bunting *et al*., 2003; Bertram *et al*., 2004).

Homology to the β-binding consensus motif is the sole determinant of binding strength and defines permissable (and competitive) protein interactions. On this basis, DnaE1 – containing a sequence that closely matches the consensus (Dalrymple *et al.*, 2001) – is

likely to assume primary position among the polymerases in the mycobacterial β-binding hierarchy. A consensus β-binding sequence is absent from DnaE2, a feature which appears to be common in organisms possessing multiple *dnaE* copies (Boshoff *et al.*, 2003). Of the mycobacterial pol IV homologues, DinX is classified as a DinB1.4-type polymerase containing a carboxy-terminal β-binding motif, whereas DinP lacks the consensus (Dalrymple *et al*., 2003). *M. smegmatis* DinP3, on the other hand, is included in the DinB1.2 sub-family (Dalrymple *et al*., 2003), representatives of which have been shown to bind β (Tang et al., 2000; Wagner et al., 2000; Lenne-Samuel et al., 2002; Noirot-Gros et al., 2002). Although β-binding affinities of each of the mycobacterial pol IV homologues require biochemical confirmation, it is submitted that *dnaE1* might prove difficult to displace under normal replicative conditions (discussed below), and that polymerase exchange might be restricted exclusively to replication stalling as a result of damage or other inhibitors (Wijffels *et al*., 2004). A mutation in *E. coli dnaE*, for example, results in higher-fidelity synthesis (Fijalkowska *et al.*, 1993) and has been shown to reduce adaptive mutagenesis (Foster *et al.*, 1995; Harris *et al.*, 1997), perhaps by inhibiting access of pols IV and V to DNA termini (McKenzie *et al.*, 2001; Foster, 2004). Similarly, evidence suggests that, in *E. coli*, pol II competes with pol IV to limit mutagenesis (Foster *et al.*, 1995): rates of adaptive mutagenesis are elevated in *E. coli polB* mutants (Escarceller *et al.*, 1994) - apparently as a result of increased pol IV expression (Foster, 2000; Layton & Foster, 2003) - and in mutants in which pol II proofreading but not polymerase ability has been eliminated (Foster *et al.*, 1995; Foster, 2000).

Of course, a limitation inherent in the defined β-binding hierarchies is that the consensus motif is the sole determinant of binding strength and priority. Since a small percentage of all proteins in a eubacterial cell would inevitably contain pentapeptide sequences matching the observed or permissible variants of the consensus motif, it is likely that interactions in the replisome governing DNA access involve many other protein-protein and protein-DNA interactions intricately regulated by a series of switches. However, evidence that some β-binding partners interact with β_2 outside the common peptide-binding site (López de Saro *et al*., 2003a, 2003b) is consistent with the proposal that the cell effectively imposes a hierarchy of competitive interactions by forcing β-binding proteins (including replicative and TLS polymerases) to use a combination of unique and common contacts with the clamp, thereby ensuring that certain partners only are able to compete effectively with a particular β-partner-DNA complex for access to the restriction fork during various DNA transactions (Sutton, 2004). Finally, it has recently been

proposed that, in *E. coli*, the $UmuD_2C$ (pol V) complex might function as a prokaryotic DNA damage checkpoint control by binding and sequestering the β-clamp (Opperman *et al.*, 1999; Murli *et al.*, 2000; Sutton *et al.*, 2001a; Sutton & Walker, 2001). Similarly, the *B. subtilis* pol IV homologue (YqjH), has tentatively been implicated in the transient growth inhibition of stationary-phase cells, possibly subject to stress (Duigou *et al.*, 2004). It is conceivable that either of the *M. tuberculosis* pol IV homologues functions in an analogous manner; however, the absence of an identified β-binding motif in DinP seems to favour DinX as a potential checkpoint protein. Therefore, it would be interesting to compare the growth re-start kinetics of *dinP* and *dinX* null mutants with wild-type, either exiting from stationary-phase, or after treatment with a replication inhibitor such as NVB.

Induced mutagenesis and gyrase inhibition

The observation that *M. tuberculosis dinP* is upregulated, together with *dnaE2*, in response to NVB-mediated gyrase inhibition (Boshoff *et al.*, 2004), was significant since it constituted the first reported evidence of induced *dinP* expression and, further, suggested that an alternative SOS- and damage-independent regulatory mechanism might operate to rescue stalled replication. In *E. coli*, pols II and V are proposed to act sequentially after DNA damage - pol II providing the initial catalyst for replication restart, to be followed later by pol V-mediated lesion bypass at persisting damage sites (Friedberg *et al*., 1995; Rangarajan *et al*., 1999; Goodman & Woodgate, 2000; Pham *et al*., 2001; Goodman, 2002). The superficial (functional) similarities of *M. tuberculosis* DnaE2 to *E. coli* pol V were noted previously (Boshoff *et al.*, 2003). Therefore, the co-induction of *dinP* and *dnaE2* after NVB treatment hinted at a loosely analogous role for the mycobacterial polymerases in replication restart, with DinP and DnaE2 fulfilling the roles of pols II and V, respectively. Furthermore, the differential transcriptional responses to OFX- and NVB-mediated stalling (Boshoff *et al.*, 2004) - in particular, the induced polymerase complement - seemed to be indicative of tight regulation. That observation was confirmed, and extended, in this study with the demonstration that *M. smegmatis dinP3* is included in the OFX-induced SOS response but is not induced by NVB. In addition, evidence was presented showing that NVB exposure is mutagenic (Table 3.10). Previous studies have established that, in other bacteria, treatment with OFX results in SOS induction and elevated mutation rates (Gudas & Pardee, 1976; Phillips *et al*., 1987; Piddock & Wise, 1987; Walters *et al.*, 1989; Piddock *et al.*, 1990; Gmuender *et al.*, 2001). While FQ-induced SOS upregulation in *M. tuberculosis* (Boshoff *et al.*, 2003) suggests a link between induced mutagenesis and the rapid emergence of FQ resistance (Perlman *et al*., 1997; Ginsburg *et al*., 2003), the data presented here indicate that replication inhibition

might be mutagenic *per se*, though mediated by different mechanisms. Of course, the *in vitro* analysis of the mutagenicity of compounds with antibacterial activity raises several technical issues, in particular the fact that concentrations approaching the MIC eliminate the majority of the bacteria, including mutants. In addition, compounds mediating killing effects by targeting DNA might inevitably cause damage at lower concentrations that will elevate mutagenicity. However, the suggested link between prolonged, low-dose antibiotic exposure and mutations to resistance in *M. tuberculosis* demands investigation. Significantly, although NVB does not induce a classic SOS response (Boshoff *et al.*, 2003), gyrase inhibition-induced mutagenesis is consistent with evidence that disruptions in genes involved in DNA metabolism result in constitutive SOS upregulation (O'Reilly & Kreuzer, 2004). Furthermore, the relevance of SOS-independent pol IV induction in response to antibiotic treatment was recently given additional impetus by the demonstration that β-lactam-mediated inhibition of cell wall synthesis in *E. coli* results in LexA/RecA-independent *dinB* expression and elevated rates of frameshift mutagenesis (Pérez-Capilla *et al.*, 2005).

The indistinguishable frequencies of Rif^R mutations in NVB-treated mc^2155 *versus* ΔY and ΔC strains appeared to indicate DnaE2- and pol IV-independent mutagenesis (Table 3.10), and were consistent with the inability to detect altered NVB susceptibilities associated with loss of either TLS polymerase class. In contrast, NVB-induced *rpoB* mutational profiles (Table 3.11) implicated one or more pol IV homologue in error-free replication restart (explored below). Although still to be validated, the potential morphological differences between Rif^R isolates arising in wild-type and mutant strains suggest that the mechanism of resistance might differ in strains lacking the full pol IV complement, a possibility further strengthened by the high proportion of unmapped Rif^R mutations in ΔY and ΔC. While Str^R frequencies failed to corroborate NVB-induced mutagenesis, it is possible that the assay does not tolerate the mutational events that result from NVB treatment. Whatever the source of the resistance, the distinct "genotypes" seem to implicate the mycobacterial pol IV homologues in differential NVB-induced mechanisms. It has recently been shown that certain *E. coli umuC* mutants are resistant to hydroxyurea (HU), a replication inhibitor which functions by limiting the supply of dNTPs (Godoy *et al.*, 2004). Intriguingly, HU treatment in those strains results in pol IV-dependent induced mutagenesis, suggesting that the low Km of the TLS polymerase for dNTPs (Bertram *et al*., 2004) might enable pol IV-mediated EP synthesis in limiting dNTP levels. Whether elevated mutation rates are associated with HU treatment of *M. tuberculosis* is unknown; however, it would be interesting to investigate the capacity of HU-mediated

replication stalling to induce *dinP* expression (analogous to NVB induction) and elevate mutagenesis.

The role of the Y-family homologues in the novel *rpoB* deletion

Genotypic analyses of Rif^R isolates of wild-type mc^2155 and the various pol IV deletion mutants arising under numerous applied stresses failed to assign a distinct mutagenic profile to any of the individual *M. smegmatis* pol IV homologues. However, novel 12 bp and 30 bp *rpoB* deletions were identified that were restricted to strains carrying deletions in one or more pol IV or EP polymerase homologues. Although the apparent absence of the deletion polymorphism from wild-type mc^2155 might be reflective of selection bias, the prevalence of this genotype in low fequency mutant populations (such as ΔC post-UV), as well as the repeated identifiction of deletion polymorphisms under various imposed stresses, likely eliminate that possibility. The restriction of the deletion polymorphisms to strains lacking one or more pol IV homologues seemed to indicate the non-redundancy of the individual genes. However, the precise mechanism for the generation of the deletions in *rpoB* remains unclear. It is possible that, in the case of the 30 bp deletion, the codons for the flanking LeuSer motifs (CTG TCG) act as substrates for inappropriate recombination or slipped-strand mispairing events that result in the looping out of the intervening sequence (Figure 3.14). The direct homology between nine of the ten nucleotides extending from the 3' base of Ser509 to the 3' base of Ser513, and the corresponding sequence from the 3' base of Asn519 to the 3' base of Ser522, might be predicted to stabilise a recombination or hairpin-like intermediate. The flanking Gln513 and Gln517 codons could function in an analogous manner in the smaller 12 bp deletion (Figure 3.14). *M. smegmatis* and *M. tuberculosis* share perfect amino acid sequence identity over the core 81 bp resistance determining region of *rpoB* (Hetherington *et al.*, 1995). However, codon usage differs between the two mycobacterial species (Figure 3.14). It is possible that the non-homologous Ser512 codon in *M. tuberculosis rpoB* provides a natural block to any looping out that might occur in the corresponding *M. smegmatis rpoB* region. Consistent with this hypothesis, neither polymorphism – 12 bp deletion or 30 bp deletion - has been reported in *M. tuberculosis* clinical or laboratory isolates. Several smaller insertion and deletion events are, however, localised to the region between Gln513 and Asn519 (Telenti *et al.*, 1993, 1997; Musser, 1995; Ramaswamy & Musser, 1998; Morlock *et al*., 2000) that, together with the observed deletions in *M. smegmatis rpoB*, are likely indicative of the relative mutational flexibility this region provides in mediating Rif^R while maintaining RNA polymerase function.

It was recently shown that *E. coli* pols IV and V are able to replicate across foreign, non-DNA segments lacking all fundamental features and inherent chemical properties of DNA, the profile of the bypass products depending on the nature of the insert and the polymerase involved (Maor-Shoshani *et al*., 2003b). Significantly, bypass of hydrocarbon chains of 12 methylene residues resulted in the elimination of the insert, and, in the case of pol IV, an adjacent nucleotide. The authors proposed that the deletions occured via a looping out mechanism that required TLS polymerases to "hop" across potentially replication-blocking adducts (Maor-Shoshani *et al*., 2003b), consistent with previous evidence of synthesis across DNA hairpins (Viswanathan *et al.*, 1999) and template switching (Pääbo *et al.,* 1990). Furthermore, it has been shown that RNA polymerases co-operate during transcription elongation to read-through intrinsic and extrinsic obstacles in the DNA template (Ephstein *et al.*, 2003; Ephstein & Nudler, 2003), and to extend across gaps (Liu & Doetsch, 1996). In effect, multiple RNA polymerases initiating from the same promoter reactivate and "push" backtracked elongation complexes at arrest sites, perhaps in combination with trailing ribosomes. Intriguingly, such co-operative behaviour has recently been implicated in transcriptional mutagenesis (Brooks & Marietta, 2004), including deletions of 5 to 22 nt downstream of a lesion. Furthermore, 12 nt deletions in this system are predominantly associated with synthetic thymine dimers characteristic of UV-induced damage. Although such a mechanism has not been demonstrated for any of the DNA polymerase families, it is tempting to speculate that, in the absence of the full cellular Y-polymerase complement, the replicative polymerase subunits might mediate similar lesion avoidance. Otherwise, alternative repair mechanisms might operate to overcome the stalling of the replication fork at sites of unrepaired DNA damage or as a result of NVB-mediated gyrase inactivation. The identification among Rif^R Y polymerase mutants of deletions in *rpoB* between loci of microhomology (Tables 3.7, 3.8, 3.9, 3.11; Figure 3.14), is suggestive of the activity of recombinational DNA repair (Lusetti & Cox, 2002). Alternatively, the recently identified mycobacterial NHEJ system might mediate damage tolerance under certain circumstances (Weller *et al.*, 2002; Della *et al.*, 2004). NHEJ constitutes the major pathway for the repair of DSBs in mammalian cells (Krejci *et al.*, 2003), and has been proposed to operate in tandem with nucleases and/or polymerases to anneal directly repeated sequences flanking DSBs with a resultant loss of intervening sequence (Rattray & Strathern, 2003). Of course, not all repair mechanisms require sequence homology and it is possible that the mutational events identified represent the only sizeable polymorphisms that retain essential RpoB activity. Smaller deletions that introduce frameshifts or nonsense mutations might also occur that are not

commensurate with cell viability. That is, the frequency of the 30 bp deletions might reflect a selection bias rather than a mechanistic preference.

Why so many mycobacterial pol IV homologues?

Fundamental questions pertain to the presence in mycobacteria of so many polymerase classes and sub-classes in addition to the replicative polymerase. It is possibe that each has evolved for the specialised bypass of a particular lesion(s), or for the rescue of replication forks at specific slipped-base mispairs (Goodman, 2002). The prevalence of Rif^R deletion polymorphisms in mc^2155 mutants carrying single and multiple pol IV deletion alleles, in particular, suggests that the individual pol IV homologues are not redundant, and is supported by recent evidence indicating that loss of *dinP3* in *M. smegmatis* mc^2155 results in elevated rates of spontaneous -1 frameshift mutations (R. Brackin & E. Machowski, unpublished). The failure of this study to identify a definite phenotype associated with loss of a particular pol IV homologue might, therefore, reflect the inherent inability of artificial, exogenous damaging agents to mimic *in vitro* the lesions which constitute the cognate template of each polymerase *in vivo*. For example, although UV irradiation has been utilised profitably to demonstrate EP TLS by *E. coli* pol V (Reuven *et al.*, 1999; Tang *et al.*, 1999) the observation that pol V homologues are more often associated with enteric bacteria (Tegova *et al.*, 2004) raises questions as to the dominant lesions in that environment. Similarly, the read-out – *rpoB* resistance or *hisD* frameshift mutations - limits the locus and mechanism of allowable mutagenic events and so underestimates both rates and functional requirements. In addition, the binary nature of *in vitro* assays (resistant *versus* susceptible; auxotroph *versus* prototroph) is likely to overlook subtleties in phenotype. The heterogeneity of environments colonised during an infection, for example, suggests that varying degrees of antibiotic resistance might emerge. The broad range of antibiotic susceptibility profiles in *P. aeruginosa* strains isolated from the sputa of cystic fibrosis patients (Mereghetti *et al.*, 1998) provides some support for the notion that a wider variety of antibiotic resistant mutants are selected during infection than can be estimated from *in vitro* assays. However, the absence of any evidence implicating DinP or DinX in mutagenic TLS, coupled with the failure to associate any of the mycobacterial pol IV homologues with a competive survival advantage *in vitro*, suggests that a predicted role for these polymerases in adaptive mutagenesis is invalid.

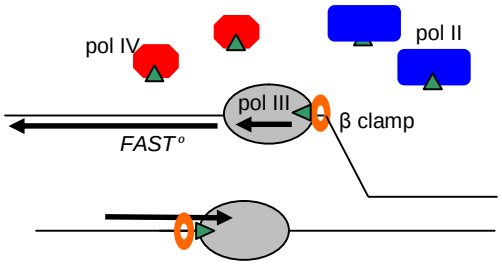
A model for polymerase activity

The variation in strength, frequency and nature of inducible mutagenesis mechanisms is thought to be reflective of the dynamic response of different pathogens to specific local

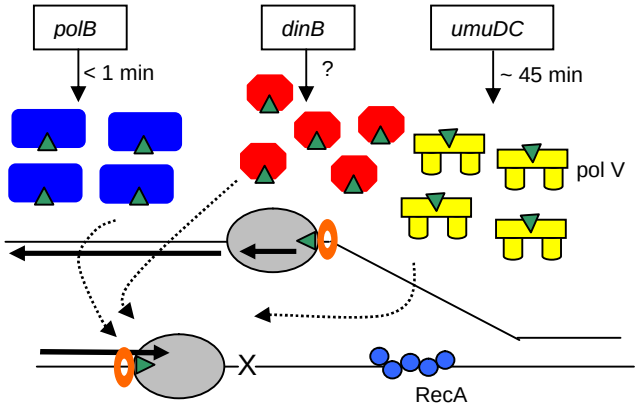
environments (Tenaillon *et al*., 2004). Stable, acquisitive evolution results from minimal disturbances to established bacterial pathways and host-pathogen interactions (Wright, 2004) - a concept reinforced by recent evidence of the adaptation of separate *M. tuberculosis* lineages to particular host populations (Hirsh *et al*., 2004). These observations suggest that the long-term selection or counter-selection of small-effect mutators is likely to exert greater influence on bacterial evolution (Taddei *et al*., 1997). As stated earlier, two distinct but interrelated biological functions characterise the Y-family polymerases - damage tolerance and adaptive mutagenesis. Although the potential for mutagenesis classifies Y-family members as error-prone or "sloppy" (Goodman, 2002), it has been argued that the specialised TLS ability of these polymerases demands their re-designation as "high-fidelity" on lesions or classes of lesions that constitute their preferred, or cognate, templates (Friedberg *et al.*, 2002). Extending this argument - and without invalidating the contention that evolution has not tended to higher fidelity systems since the relative biological cost (mutation *versus* survival) is small (Tenaillon *et al*., 2004) - it is submitted that mechanisms might have evolved in mycobacteria to restrict the Y-family (pol IV) homologues to specialised high-fidelity polymerase function, thereby eliminating a role in mutagenesis.

The model proposed (Figure 3.19) is based on the idea that the dual biological functions of Y-family polymerases (TLS and adaptive mutagenesis) might be thought of as being regulated on two distinct levels – gene expression, and access to the replicative complex. The second of these, access, is explored first. Recent evidence has emphasised the role of the β-clamp in mediating competitive polymerase switching (and other DNA interacting proteins) at the replication fork. As discussed above, a restricted number of mycobacterial polymerases contain consensus β-binding motifs. Based on homology to the consensus, it appears that high-affinity binding by the *dnaE1*-encoded, high-fidelity replicative polymerase might occlude access of the other polymerases to the replication complex under normal growth conditions. Furthermore, although expression analysis has failed to detect increased *dnaE1* transcript levels following damage (Boshoff *et al.*, 2003), the identification upstream of *dnaE1* of a putative consensus motif (RecA-NDp; Gamulin *et al.*, 2004) regulating the mycobacterial LexA/RecA-independent damage response raises the possibility that the high-fidelity subunit might continue to modulate access of the alternative polymerase classes to the template after damage. A combination of increased (damage-induced) expression, and enhanced affinity for the holoenzyme complex (perhaps in the presence of additional damage-induced factors or subunits) would, therefore, be required for DnaE2 to access the replication fork at a replication-blocking

E. coli



Normal replication

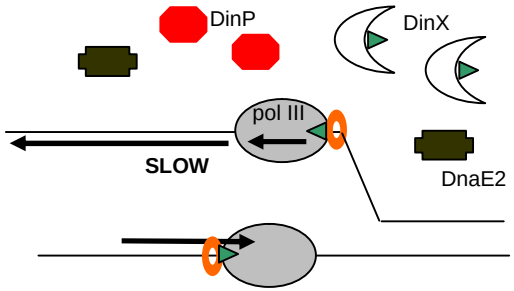


Replication stalling

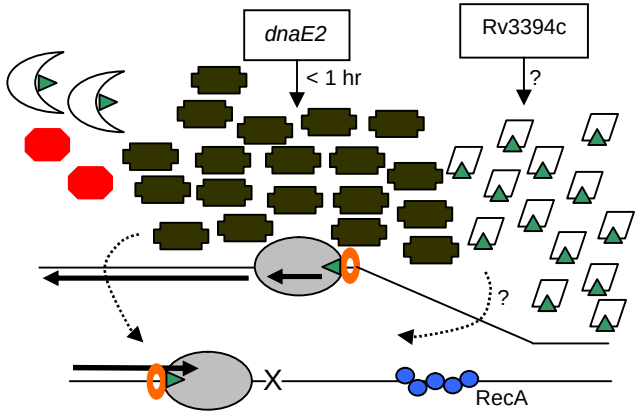
?

NVB treatment

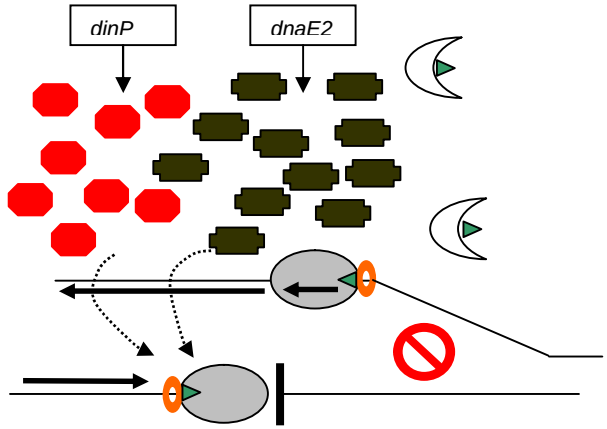
M. tuberculosis



Normal replication



Replication stalling



NVB treatment

Figure 3.19 A model for polymerase dynamics in fast- *versus* slow-growing organisms. During normal replication in ***E. coli***, pol II (*polB*) and pol IV (*dinB*) are constitutively expressed to ensure rapid replication restart following routine, spontaneous inactivation of the replication fork. If the replication fork is not immediately re-activated, or if stalled by a replication-blocking lesion (**X**), a RecA nucleoprotein filament can assemble on the unwound, single-stranded DNA, thereby inducing the SOS response. SOS upregulates expression of pol II (*polB*), pol IV (*dinB*), and pol V (*umuDC*) – all of which contain consensus β-clamp binding motifs (represented by p) and so are able to displace the replicative polymerase (pol III). Elevated expression of the three polymerases ensures greater damage tolerance but might also be subject to second-order selection as a result of the potential for mutagenesis. Pol V, in particular, is highly regulated both transcriptionally and post-translationally and is induced late in SOS. In ***M. tuberculosis***, the high-affinity binding of the *dnaE1*-encoded high-fidelity replicative polymerase likely limits access of other polymerases to the replication complex under normal growth conditions. Furthermore, slow polymerisation by the replicative polymerase might reduce the need for rapid, pol IV-mediated replication restart. Significantly, in addition to DnaE1, only DinX and Rv3394c contain putative β-binding motifs. In response to damage, or as a result of replication fork collapse, expression of *dnaE2* is highly elevated. Of the *M. tuberculosis* pol IV homologues, only Rv3394c is upregulated following damage, but its function is unknown. The faster growing *M. smegmatis* possesses an additional pol IV homologue (DinP3) which is SOS-inducible. Treatment of *M. tuberculosis* with NVB, a gyrase B inhibitor, causes replication arrest (**X**) and upregulates expression of *dnaE2* and *dinP*, which might combine in error-free replication restart. The response of *E. coli* to NVB is unknown.

lesion (Boshoff *et al.*, 2003). The *B. subtilis* pol V homologue (YqjW), for example, despite containing a consensus β-binding motif, mediates UV-induced mutagenesis independently of the β-clamp, suggesting alternative protein interactions when replicating undamaged and UV-damaged templates (Duigou *et al.*, 2004). Finally, it has been suggested that, in *E. coli*, mismatch repair proteins might be required to mediate access of Pol IV to the 3' OH end of the DNA strand in the presence of a functional replicative polymerase (Strauss *et al*., 2000) – a mechanism the naturally MMR deficient mycobacteria have eliminated (Mizrahi *et al.*, 2000). The second regulatory activity pertains to expression. A genetic characteristic of the Y-family polymerases relates to their induction in the canonical bacterial SOS response. However, as discussed above, the mycobacterial pol IV homologues are excluded from both SOS- and LexA/RecA-independent damage response regulons. In contrast, the importance of *dnaE2* for TLS is suggested by its upregulation in at least two different damage responses – SOS (Davis *et al.*, 2002a; Boshoff *et al.*, 2003) and NVB-mediated replication stalling (Boshoff *et al.*, 2004).

During high speed replication in fast-growing organisms, extension by the replicative polymerase competes with exonucleolytic proofreading mechanisms as well as polymerase dissociation from the 3' terminus (Fijalkowska & Schaaper, 1995). In particular, the efficiency of proofreading is thought to be limited by the decreased residence time of mismatched termini in the active site of the proofreading subunit. Therefore, multiple post-replicative repair pathways are required to maintain genomic

integrity (Eisen & Hanawalt, 1999). During aerobic growth of *E. coli*, replication forks undergo routine, spontaneous inactivation as often as once per round of replication (Cox *et al.*, 2000). If the replication fork is not immediately re-activated, or if stalled by a replication-blocking lesion, the continued unwinding of DNA ahead of the stalled replication complex creates a stretch of single-stranded DNA that can support assembly of a RecA nucleoprotein filament and results in SOS induction (Figure 3.19; Sassanfar & Roberts, 1990). Significantly, *E. coli dinB* (pol IV) is expressed at high constitutive levels – approximately 250 molecules of pol IV per cell (Kim *et al*., 1997), compared with about 50 molecules of pol II, 30 molecules of pol III, and fewer of the pol III holoenzyme (McHenry & Kornberg, 1977; Bonner *et al*., 1988; Qiu & Goodman, 1997). The relatively high basal expression of *E. coli* pol IV, in particular, is thought to be required for rapid replication restart under normal replicative conditions (Goodman, 2002). The proposed housekeeping role is further supported by evidence of the wide distribution of pol IV homologues among most organisms (Ohmori *et al.*, 2001; Goodman, 2002). In contrast, the other *E. coli* TLS polymerase, pol V, is limited to prokaryotic genomes (Ohmori *et al.*, 2001; Goodman, 2002). Furthermore, in *E. coli*, pol V is tightly controlled being essentially undetectable in uninduced cells, but increasing to ~ 200 copies per cell post-SOS induction (Woodgate & Ennis, 1991). Consistent with the inherent error-prone nature of the enzyme, pol V activity is highly regulated both transcriptionally - as part of the LexA/RecA regulon (Bagg *et al*., 1981), and post-translationally - through Lon and ClpXP protease-mediated processing (Frank *et al*., 1996; Gonzalez *et al*., 1998; Goodman & Woodgate, 2000; Sutton *et al.*, 2001b; Neher *et al*., 2003), and access of pol V to DNA might further be limited by late SOS induction (Sommer *et al.*, 1998; Tippin *et al*., 2004b). In addition, it has been proposed that subunit exchange between UmuD and UmuD' in the UmuD'$_2$C complex inactivates pol V following TLS, reducing the potential for mutations on undamaged DNA during SOS (Shen *et al*., 2003). Both pols II and IV are also induced in response to damage – pol II about seven-fold (Bonner *et al*., 1988; Qiu & Goodman, 1997) and pol IV about ten-fold (Kim *et al*., 2001). Interestingly, despite SOS induction, no obvious repair defects have been associated with loss of pol IV in *E. coli* (Rattray & Strathern, 2003); however, *E. coli dinB* mutants are defective in untargeted mutagenesis (Brotcorne-Lannoye *et al.*, 1986). Therefore, beyond the repair requirement, the mechanism of pol IV induction (as part of the SOS response) has likely also been subject to second-order selection since it is intricately linked to genetic adaptation and diversification.

In contrast, it has been proposed (Mizrahi *et al.*, 2000) that the decreased extension rate of *M. tuberculosis* pol III might improve proofreading efficiency by increasing access of 3'-

exonucleases to mismatched primer termini, thereby ensuring fidelity in the absence of several post-replicative DNA repair pathways (Mizrahi & Andersen, 1998). In addition, slower replication will inevitably limit the need for rapid, pol IV-mediated replication fork rescue (Figure 3.19). Although one or more pol IV homologues could be constitutively expressed under normal growth conditions (a possibility requiring further investigation), access to the replication complex might be restricted by apparently non-existent (*dinP*) or limited (*dinX* or Rv3394c) β-clamp binding affinity. Instead, it is proposed that the damage lesion itself might select for, and enable access of, the specialised polymerase(s) to the template. That is, the preference for damaged DNA templates or stalled replication complexes, together with the relative processivity of the main mycobacterial replicative polymerase, will inevitably limit the opportunities for the pol IV homologues to access the replisome. Following damage, expression is not upregulated because the pol IV homologues have been selectively eliminated from the mycobacterial SOS (Davis *et al.*, 2002a; Boshoff *et al.*, 2003) or alternative (Rand *et al.*, 2003; Gamulin *et al.*, 2004) damage response regulons. *M. smegmatis*, on the other hand, which has a faster growth rate, possesses a damage-inducible pol IV homologue (*dinP3*) with a β-binding motif (Dalrymple *et al.*, 2003). Although difficult to reconcile with the presence of a *dinP3* homologue in *M. avium* subsp. *paratuberculosis*, it is tempting to speculate that SOS-inducibility of pol IV homologues might be linked to growth rate.

Of course, the proposed evolutionary balance between mutagenesis and replication rate will also dictate the damage-inducible polymerase complement in an organism. As stated above, *B. subtilis* - like mycobacteria - encodes an SOS-inducible EP DnaE (Table 3.12; Le Chatelier *et al.*, 2004); however, unlike the mycobacterial DnaE2, *B. subtilis* DnaE is essential - catalysing lagging strand synthesis during normal replication (Dervyn *et al*., 2001) – and, moreover, contains a consensus β-binding motif (Dalrymple *et al.*, 2001). In a further departure from the mycobacteria, genetic analyses have shown that *B. subtilis* possesses a damage-inducible Y-polymerase complement that is loosely analogous to the SOS polymerases in *E. coli* (Table 3.12). Specifically, *B. subtilis* contains *yqjH*- and *yqjW*-encoded pol IV and pol V homologues, respectively (Duigou *et al.*, 2004), both of which contain consensus β-binding motifs (Dalrymple *et al.*, 2001). During normal growth, YqjH (pol IV) is constituvely expressed, whereas YqjW (pol V) is almost undetectable, increasing significantly in response to UV damage (Duigou *et al.*, 2004). In contrast to *E. coli*, however, loss of YqjW (pol V) function in *B. subtilis* does not confer damage hypersensitivity (Duigou *et al.*, 2004). Together with evidence that decreasing *dnaE* transcript levels similarly does not increase damage susceptibility in *B. subtilis* (Table

3.12; Le Chatelier *et al.*, 2004), the apparent dispensability of YqjW (pol V) for damage tolerance seems to indicate the potential for DnaE and pol V to provide complementing post-UV TLS activity. Furthermore, expression of YqjH (pol IV) is not damage inducible (Duigou *et al.*, 2004), suggesting that increased access of DnaE and pol V to the damaged template might negate the role of pol IV-mediated mutagenesis in that organism. Significantly, the failure to detect upregated pol IV expression in both *M. tuberculosis* and *B. subtilis* in response to damage implies the dispensability of inducible pol IV function in organisms possessing EP C-family polymerase subunits.

The evolution of drug resistance in *M. tuberculosis*

The exclusion of the mycobacterial pol IV homologues from induced mutagenesis appears to nominate the *dnaE2*-encoded pol III subunit as the sole player in the evolution of drug resistance in *M. tuberculosis*, as suggested (Boshoff *et al.*, 2003). However, given the stated disparity between *in vitro* mutation rates and the rapid emergence of drug-resistant clinical isolates, a fundamental question remains unanswered: is EP replication the only mechansism by which chromosomally-encoded drug resistance mutations arise? Recent evidence suggests that the evolution of metabolic pathways (Wright, 2000, 2003, 2004), as well as somatic hypermutation (Wright *et al.*, 2004), might be driven by the transcriptional response of an organism to an applied stress. Stress-directed mutagenesis - as the process is termed (Wright, 2004) - is distinct from adaptive or inducible mutation mechanisms in its strict insistence that the stress causing the mutation also constitutes the selective pressure. Essentially, transcription of genes derepressed in response to starvation or other stresses is thought to create localised supercoiling and DNA secondary structures, such as stem loops, that can stabilise conformations containing an unpaired base in a loop or at the base of the stem (Wright, 2003, 2004; Wright *et al.*, 2004). Inherent thermodynamic instability as well as an increased likelihood of being modified by other intracellular enzymes renders unpaired bases vulnerable to mutation (Lindahl, 1993). Subsequent replication, especially in (mismatch) repair-deficient organisms, can fix chromosomal mutations, and therefore targets mutations to the very genes *required* to mutate to alleviate the stress (Wright, 2000, 2003, 2004).

It is tempting, therefore, to speculate that two alternative (but overlapping) mutagenic mechanisms might operate in combination in *M. tuberculosis* depending on the stimulus (Figure 3.20). The first of these is the classic, damage-induced or SOS-type which, in *M. tuberculosis*, is mediated by DnaE2 and is, perhaps, amplified by stationary-phase physiology. A range of host immune effectors and other environmental damaging agents,

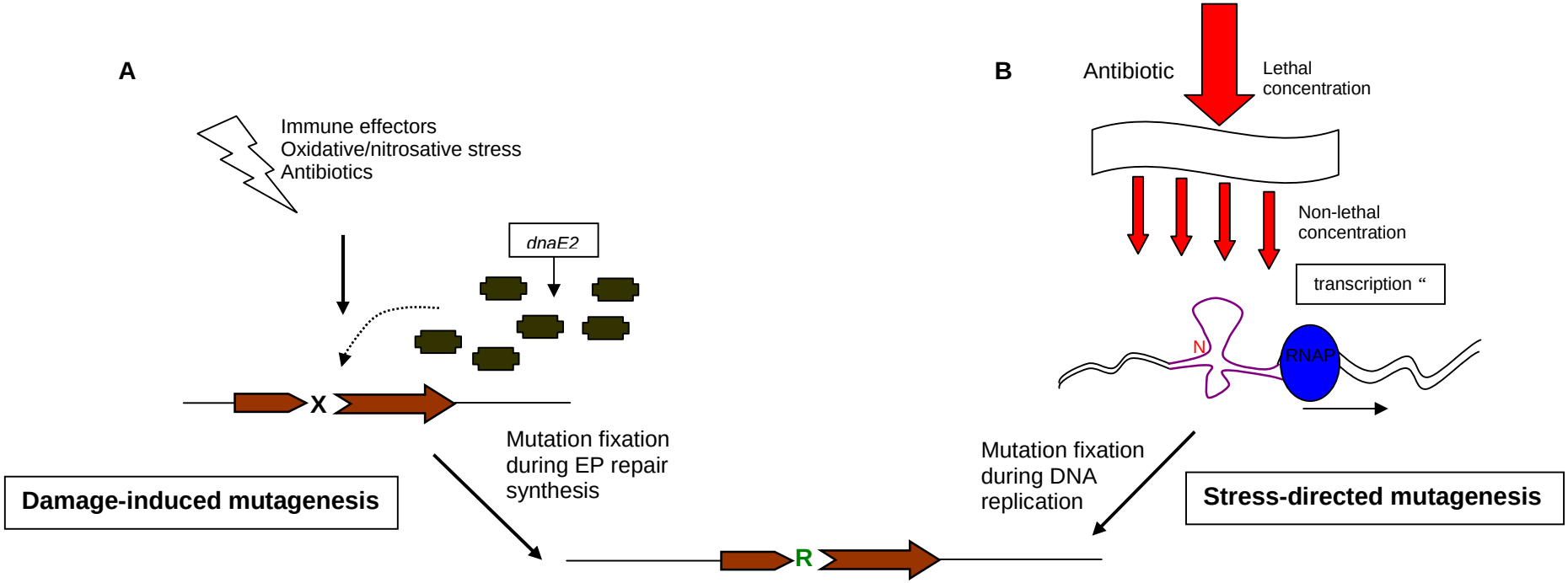


Figure 3.20 The *in vivo* evolution of drug resistance in *M. tuberculosis*. Two mechanisms are proposed to mediate inducible mutagenesis in *M. tuberculosis* during host infection. **A**. A damage lesion (**X**) might be caused by a range of host immune effectors and other environmental damaging agents, as well as endogenous oxidative and nitrosative metabolic stresses, or antibiotics. Stalled replication at the lesion induces *dnaE2* expression. In some cases, EP repair synthesis by DnaE2 fixes a mutation at the site of the damage. Where the mutation confers antibiotic resistance (**R**), it is selected. **B**. Alternatively, antibiotic concentration gradients *in vivo* might expose bacilli to non-lethal antibiotic selection, and so result in upregulated transcription of the target gene(s). Localised supercoiling and DNA secondary structures in the wake of the RNA polymerase (RNAP) stabilise conformations with an unpaired base (N) in a loop or at the base of a stem (Wright, 2004). Unpaired bases are thermodynamically unstable and vulnerable to enzymatic modification (Lindahl, 1993). Mutated bases are fixed during DNA replication, especially in (mismatch) repair-deficient organisms, and those conferring antibiotic resistance are selected. Stress-directed mutagenesis (Wright, 2004) therefore targets mutations to the gene(s) required to mutate to alleviate the stress (Wright, 2000, 2004).

as well as endogenous oxidative and nitrosative metabolic stresses, or antibiotics, might induce damage lesions. Stalled replication at a lesion induces *dnaE2* expression either as part of the mycobacterial SOS response, or by unknown regulatory mechanisms analogous to NVB-mediated *dnaE2* induction. EP repair synthesis by DnaE2 might fix mutations in chromosomal DNA at the site of the damage, in some cases conferring antibiotic resistance which is then selected. Secondly, it is proposed that stress-directed mutagenesis might operate in sequestered environments *in vivo* (Mitchison, 1985). Specifically, the effective antibiotic concentration (subject to a chemical gradient) is thought likely to expose bacilli to non-lethal antibiotic selection (Baquero & Negri, 1997; Baquero *et al.*, 1998) which might result in upregulated transcription of the particular antibiotic target or compensatory pathway gene(s), increasing the possibility of mutations – and, therefore, resistance mutations. Significantly, the mutation rate for a given antibiotic has been shown to change depending on the effective concentration during selection and, further, that different mutation rates and resistant mutants might be obtained at discontinuous intervals along the spectrum of applied concentrations (Kohler *et al.*, 1997). An obvious flaw in this proposal is that, apart from translational inhibitors, exposure of *M. tuberculosis* to most classes of antibiotic does not necessarily induce expression of specific target or drug-resistance genes (Boshoff *et al.*, 2004). However, most studies are conducted using antibiotic concentrations that – even if not at the specific MIC – are likely at levels far in excess of those that might impose non-lethal selective pressure *in vivo*. Furthermore, evidence of cross-induction – that is, treatment with one antibiotic upregulates expression of genes implicated in resistance to another, unrelated antibiotic (Boshoff *et al.*, 2004) – is consistent with the recent demonstration that well-implemented TB control programmes may actually exacerbate the emergence of MDR-TB isolates (Blower & Chou, 2004; Cohen & Murray, 2004).

Finally, the evolution of low-level drug resistant variants in other infecting bacterial populations (such as the extended-spectrum TEM β-lactamases) implies that the locally available concentration of an antibiotic within particular host environments selects for the different variants (Baquero *et al.*, 1998). In addition, the specific antibiotic concentrations inhibiting the variant and wild type strains have been shown to be extremely close in many cases, resulting in a narrow selective concentration range (Baquero *et al.*, 1998). The narrowness of the selective range often results in some low-level resistant mutants being considered clinically irrelevant. However, the ability of a given antibiotic concentration (even if very low) to select for a particular resistance mutation (even if almost neutral) has profound implications for the generation of diverse microbial populations (Baquero &

Negri, 1997). The evolution of high-level resistant mutants may be enhanced *in vivo* in populations in which low-level resistant mutants have been selected and maintained in various compartments; in fact, the role of low-level resistant variants may be analogous to that of multidrug efflux pump systems in the emergence of antibiotic resistant mutants. Expression of the efflux system genes is usually down-regulated, and even when constitutively expressed produces only minor variations in the MIC values of antibiotics. However, the idea that mutations in efflux pump systems, in combination with mutations in other antibiotic targets, are the basis of clinically relevant antibiotic resistant phenotypes has recently gained credence, as demonstrated in the acquisition of quinolone resistance (Martinez *et al.*, 1998).

Future studies

In order to validate many of the claims of the polymerase model proposed, it is critical that the relative and absolute expression levels of all mycobacterial polymerases be determined during log- and stationary-phase growth. Furthermore, biochemical analyses of the catalytic activity of the individual polymerases are required to elucidate possible redundant (or non-redundant) function with particular emphasis on the lesions bypassed and relative polymerase processivities. Studies are already underway to measure indirectly the possible competition between pol IV and EP polymerases for the replication fork by overexpressing the *M. tuberculosis* pol IV homologues in the *M. smegmatis* ΔC mutant. However, as suggested, a parallel investigation into potential mycobacterial pol IV interactions to elucidate auto- or cross-modulatory binding - using a yeast two-hybrid system, for example – is required. Furthermore, the potential interaction(s) of the various polymerases with regulatory factors such as the mycobacterial β-clamp also demand investigation. Heterologous promoter assays hinted at the operonic expression (and induction) of Rv3055 and *dinP* during stationary phase; therefore, the potential regulatory role of Rv3055 in modulating *dinP* activity is considered worth pursuing. Finally, the contribution of additional putative polymerases and polymerase classes to damage tolerance needs to be examined, particularly the roles of the SOS-inducible mycobacterial Rv3394c and *dinP3* gene products.

Chapter 4

Ribonucleotide reduction in *Mycobacterium tuberculosis*

4.1 ABSTRACT

M. tuberculosis encodes both oxygen-dependent (class I) and –independent (class II) ribonucleotide reductase (RNR) subunits, suggesting that the organism might modulate deoxyribonucleotide triphosphate (dNTP) biosynthesis in response to oxygen availability. In particular, evidence of the induction of the *nrdZ*-encoded class II RNR under microaerophilic conditions potentially implicated NrdZ in the survival of *M. tuberculosis* in the hypoxic environment thought to prevail *in vivo*. To investigate the contribution of NrdZ to the growth and persistence of *M. tuberculosis*, a Δ*nrdZ* null mutant was constructed by allelic exchange mutagenesis. Treatment of the Δ*nrdZ* mutant with hydroxyurea, a class I-specific inhibitor, confirmed the inability of NrdZ to substitute for the *nrdEF2*-encoded class I RNR under aerobic conditions. In addition, survival of the Δ*nrdZ* mutant strain was not impaired under hypoxic conditions *in vitro*. Moreover, bacterial loads in the lungs of B6D2/F1 mice infected with the Δ*nrdZ* mutant were comparable to those of lungs infected with the parental wild-type strain, suggesting that *nrdZ* might not be required for the virulence of *M. tuberculosis* in the mouse model.

4.2 INTRODUCTION

4.2.1 Ribonucleotide reductases (RNRs)

DNA synthesis and repair are dependent on the adequate supply of deoxyribonucleotides (dNTPs) to replicative and repair DNA polymerases. Ribonucleotide reductases (RNRs) catalyse the *de novo* synthesis of all four dNTPs from their ribonucleotide precursors, thereby performing an essential role in nucleotide cycling and dNTP pool modulation (Reichard, 1993, 1997; Chimploy *et al.*, 2000). Significantly, RNR expression and activity are induced following DNA damage in both eukaryotes and prokaryotes (Gaudet & Tsang, 1999; Courcelle *et al.*, 2001; Chabes *et al.*, 2003), and disruptions in several genes involved in nucleotide pool maintenance are associated with constitutive SOS gene upregulation (O'Reilly & Kreuzer, 2004). Since the activity of RNRs constitutes the first committed step in DNA synthesis, these enzymes are potentially rate-limiting and so have been recognised as attractive targets for novel antiviral, antiparasitic and antiproliferative chemotherapeutics (Moss *et al*., 1993; Rubin *et al*., 1993; Nocentini, 1996; Szekeres *et al*., 1997; Ingram *et al*., 1999) as well as potential subunit vaccines (Fagan *et al*., 2001).

RNRs are classified into three major classes according to both sequence homology and mechanisms for free radical generation (Reichard, 1993, 1997). Although the members of each class appear unrelated - their amino acid sequences differ, they utilise different

means to generate the protein radicals required for substrate activation, and usage of the essential metal co-factors has not been evolutionarily conserved - all RNRs employ a cysteine thiyl free-radical mechanism to catalyse essentially the same chemistry at the ribonucleotide level (Stubbe & van der Donk, 1995; Reichard, 1997; Sjöberg, 1997; Eklund *et al*., 2001; Stubbe *et al*., 2003). In addition, RNRs of all classes regulate their substrate specificity by allosteric mechanisms to ensure balanced production of each of the four dNTPs, and have retained critical residues in their protein sequences, an indication that despite large differences in primary structure, some similarity in tertiary structure has been preserved which is suggestive of divergent RNR evolution (Jordan & Reichard, 1998; Eklund *et al*., 2001; Reichard, 2002). For this reason, it has been proposed that ribonucleotide reduction constituted a crucial step in the ancient transition from RNA to DNA genomes (Reichard, 1993, 2002; Riera *et al.*, 1997).

4.2.2 RNRs and adaptation to oxygen availability

An essential difference between the classes of RNR is the relation of the free radical to dioxygen. Whereas class I enzymes are limited to aerobic conditions (oxygen forms part of the system that generates the tyrosyl radical; Fontecave *et al*., 1992), class III RNRs are strictly anaerobic and undergo rapid destruction of the glycyl radical in the presence of oxygen (Gedde-Dahl, 1952); the process of radical formation by class II enzymes, however, is neither oxygen dependent nor oxygen sensitive, affording enzymes in this class the flexibility to operate in both aerobic and anaerobic organisms (Jordan & Reichard, 1998). The presence of more than one class of RNR in many organisms (Jordan *et al*., 1994, 1996, 1999), coupled with evidence of the altered expression of RNR genes in response to changes in oxygen tension (Garriga *et al*., 1996; Vollack *et al*., 1999; Masalha *et al*., 2001), suggests that RNRs function principally to allow adaptation to varying oxygen levels in the environment (Poole *et al*., 2002; Reichard, 2002) and probably evolved to facilitate survival during different stages of the life cycle (Jordan & Reichard, 1998; Jordan *et al*., 1999; Torrents *et al*., 2000, 2002). However, different RNR classes have been shown to be simultaneously active in some organisms during aerobic growth (Jordan *et al*., 1999; Borovok *et al*., 2002, 2004), and a number of bacterial genomes contain more than one enzyme of the same class or subclass (Jordan *et al*., 1994; Kunst *et al*., 1997; Monje-Casas *et al*., 2001). In addition, there is increasing evidence that functional redundancy should not be inferred from the presence of multiple alternative RNR subunits (Huang & Elledge, 1997; Dawes *et al.*, 2003).

4.2.3 The mycobacterial RNR complement

A loose correlation has been observed between growth rate and pathogenicity in mycobacteria (Young, 1990). Coupled with the demonstration that chromosomal replication is the primary determinant of the mycobacterial growth rate (Jacobs *et al.*, 1993; Wheeler & Ratledge, 1994; Hiriyanna *et al.*, 1996), the position of RNRs at the potentially rate-limiting step of DNA synthesis has prompted several investigations into the activity of the *M. tuberculosis* RNR homologues (Yang *et al.*, 1994, 1997; Dawes *et al.*, 2003). *M. tuberculosis* contains genes for subunits of both class I (*nrdE/F1/F2*) and class II (*nrdZ*) RNRs (Yang *et al*., 1994, 1997; Cole *et al*., 1998), but does not possess a strictly anaerobic class III RNR. Class I RNRs are cell cycle-regulated, tetrameric enzymes, comprising homodimers of two large subunits containing substrate and allosteric regulatory sites, and two small subunits which enclose an iron(III)/tyrosyl radical redox centre (Reichard, 1997; Jordan & Reichard, 1998). Enzymes of the class I form have traditionally been divided into two subclasses, Ia and Ib, based on allosteric regulation and utilisation of different electron donors (Jordan *et al*., 1996), the active enzyme in *M. tuberculosis* falling into class Ib (Yang *et al*., 1997). In most organisms, the large and small subunits of enzymes in this class are encoded by the well-characterised *nrdEF* system. However, in *M. tuberculosis*, *nrdF2* encodes the active small subunit (Yang *et al*., 1994, 1997) and, despite containing key catalytic residues, NrdF1 is unable to associate with NrdE to form a functional RNR (Yang *et al*., 1997), as confirmed by the demonstration that *nrdF2* is essential for growth *in vitro* (Dawes *et al*., 2003; Sassetti *et al.*, 2003). The *M. tuberculosis* class I RNR complement was recently expanded with the identification of an alternative class Ic subfamily in which the tyrosyl radical site is absent and replaced by an iron-coupled radical (Högbom *et al*., 2004). Significantly, comparative genomics suggests that class Ic enzymes are restricted to intracellular pathogens, including *M. tuberculosis* and *M. bovis*, and archaeons and/or thermophiles, prompting speculation that the independence of the stable tyrosyl radical in the class Ic subunit confers increased resistance to the antimicrobial effects of iNOS-generated NO (Högbom *et al*., 2004).

4.2.4 The *nrdZ*-encoded class II RNR

The oxygen-independent class II RNRs require 5'-deoxyadenosylcobalamin (coenzyme B_{12}) for radical generation, and are composed of a single subunit homologous to the large subunit of the class I enzymes (Reichard, 1993, 1997; Jordan & Reichard, 1998). The inclusion of *nrdZ* and a putative transcriptional regulator (Rv0569 - located 29 bp

upstream of *nrdZ*) in the DosR dormancy regulon that is highly induced in response to NO and hypoxia (Sherman *et al*., 2001; Park *et al*., 2003; Voskuil *et al*., 2003; Muttucumaru *et al*., 2004) prompted speculation that the *M. tuberculosis* class II RNR might enable dNTP biosynthesis under conditions of low oxygen availability (Voskuil *et al.*, 2003). In particular, the presence in the *M. tuberculosis* genome of a putative *nrdZ*-encoded class II RNR in addition to the oxygen-dependent class Ib RNR fuelled speculation that NrdZ might function in the supply of dNTPs for replication or repair during long-term persistence *in vivo*, and so might be implicated in the growth or survival of the bacillus in the microaerophilic environment thought to prevail in a clinically dormant lesion (Mizrahi *et al*., 2000; Voskuil *et al.*, 2003).

4.2.5 Aims and objectives of this study

When this study was initiated, the potential contibution of the *nrdZ*-encoded class II RNR to mycobacterial pathogenesis and hypoxic persistence was unknown. A parallel study established the essentiality of NrdF2 (Dawes *et al.*, 2003), implying the essentiality of the oxygen-dependent class Ib RNR for normal aerobic growth. However, the contribution, if any, of NrdZ to normal aerobic growth and under conditions of limitiing oxygen availability remained to be determined. Therefore, the specific objectives extended to the phenotypic characterisation of an *nrdZ* deletion mutant, and were:

1. To create a null mutant of *nrdZ* in *M. tuberculosis* H37Rv.
2. To determine whether NrdZ contributed to RNR activity under aerobic conditions.
3. To investigate the role of NrdZ during hypoxic adaptation and persistence *in vitro*.
4. To determine whether *nrdZ* was required for virulence of *M. tuberculosis in vivo*

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains and plasmids

The strains, plasmids, primers and probes used in this study are detailed in Table 4.1.

Table 4.1 List of strains, plasmids, primers and probes

Strain	Description	Reference/source
Δ*nrdZ*	*nrdZ* knockout mutant of H37Rv	This study
Plasmid		
pDWZ04	Subclone from gridded library of *M. tuberculosis* cloned in pBluescript carrying *M. tuberculosis nrdZ* gene and flanking sequences; Amp^R	S. Andersen
p2Δ*nrdZ*	p2NIL carrying Δ*nrdZ* allele; Km^R	S. Andersen
p2Δ*nrdZ*19	Unmarked *nrdZ* knockout vector - p2Δ*nrdZ* containing *Pac*I cassette from pGOAL19; Km^R Hyg^R Suc^S	This study
pMV306K	*E. coli*-Mycobacterium integrating shuttle vector, Km^R	H. Boshoff
pMV*nrdZ*	pMV306K containing *M. tuberculosis nrdZ* plus 800 bp upstream sequence	This study
Primers		
attBS1	ACGTGGCGGTCCCTACCG	S. Barichievy
attBS2	ACAGGATTTGAACCTGCGGC	S. Barichievy
attL2	CTTGGATCCTCCCGCTGCGC	S. Barichievy
attL4	AATTCTTGCAGACCCCTGGA	S. Barichievy
Probe		
nrdZp	2011 bp *Sal*I-*Pvu*II fragment from pDWZ04 containing 865 bp 3' *nrdZ* sequence	This study

4.3.2 Site-specific knockout of *M. tuberculosis nrdZ*

Construction of the Δ*nrdZ* deletion allele

A PCR-generated *nrdZ* amplicon was used to probe a gridded plasmid library of the *M. tuberculosis* genome (kindly provided by M. Everett, GlaxoSmithKline, Stevenage, United Kingdom). The pDWZ04 clone was found to contain the entire 2076-bp *nrdZ* gene plus 3286-bp sequence downstream of the stop codon and 1260 bp upstream of the start codon. A 1568-bp *Pst*I-*Sal*I fragment and a 2205-bp *Sal*I-*Kpn*I fragment were excised from pDWZ04 and cloned into the corresponding sites of p2NIL to obtain a vector (p2Δ*nrdZ*; S. Andersen) containing a Δ*nrdZ* allele lacking the internal 814-bp *Sal*I fragment that encodes three of the five cysteine residues shown to be essential for RNR activity in *Lactobacillus leichmannii*

(Booker *et al.*, 1994). The *hyg-lacZ-sacB* cassette from pGOAL19 (Parish & Stoker, 2000) was cloned into the *Pac*I site to generate p2Δ*nrdZ*19.

Genotypic characterisation of Δ*nrdZ* deletion mutants

The p2Δ*nrdZ*19 knockout construct was electroporated into *M. tuberculosis* H37Rv and deletion mutants isolated as described (2.2.3). Both SCO and DCO recombinants were confimed genotypically by Southern blot (2.4.4). Briefly, chromosomal DNA digested with *Mlu*I was probed with the 2011 bp *Sal*I-*Pvu*II fragment from pDWZ04 (nrdZp; Table 4.1).

Integration of pMV*nrdZ* into *M. smegmatis* mc^{2}155

pMVnrdZ was delivered into wild-type *M. smegmatis* mc^{2}155 by electroporation as described (2.2.3). To ensure correct integration of pMV*nrdZ* into *M. smegmatis* mc^{2}155 host strains, PCR primers were designed to flank the *attL* and *attR* sites. A forward (attBS2) and reverse (attL4) PCR primer set was used to amplify a 320 bp sized fragment spanning the *attL* region while a forward (attL2) and reverse (attBS1) PCR primer set was used to amplify a 282 bp sized fragment spanning the *attR* region. PCR products were analysed by agarose gel electrophoresis.

4.3.3 Hydroxyurea assay

MICs of hydroxyurea (HU) for parental and Δ*nrdZ* strains were determined by using BACTEC 460-TB methodology (Rastogi *et al.*, 1995) and BACTEC 12B vials (Becton-Dickinson, Towson, Md.) containing cyanocobalamin (CNCbl; Sigma-Aldrich, St. Louis, Mo.) at a concentration of 1 µg/ml and concentrations of HU (Sigma-Aldrich) ranging from 1 to 50 mM.

4.3.4 Wayne model conditions

Stirred Wayne model conditions were reproduced in round-bottom Pyrex tubes (capacity 15 ml) filled with 10 ml Dubos albumin broth (Difco) containing 0.05% Tween 80, as described (Wayne & Sohaskey, 2001), generating a headspace-to-volume ratio of 0.5. The Wayne model for adaptation of *M. tuberculosis* to hypoxia induces a sequential shiftdown of the organism through two stages of nonreplicating persistence (NRPI and NRPII) in response to a self-generated, temporal O_2 gradient (Wayne & Hayes, 1996). In this model, the sealed culture replicates in a logarithmic fashion, but as O_2 becomes limiting (concentration, <1%), replication ceases and, although the optical density continues to increase, the CFU counts do not (NRPI). The culture enters NRPII at O_2 concentrations less than 0.06%, when no further increase in optical density is observed. All tubes used were inoculated with a 1/100 dilution of

a log-phase culture in fresh Dubos medium and sealed with Parafilm internally and externally. Duplicate indicator tubes containing 0.5 µg of methylene blue per ml were established for each experiment, as described (Wayne & Lin, 1982; Wayne & Hayes, 1996). All cultures were stirred slowly with 5 mm magnetic stir bars (Fisher Scientific, Pittsburgh, Pa.). For the settled Wayne model, parallel cultures were established as above but without agitation (Wayne & Lin, 1982). Individual cultures were removed at various timepoints for CFU assessments and discarded. Where appropriate, metronidazole (MTZ; Sigma) was added to cultures prior to sealing with Parafilm.

4.3.5 Infection of mice, determination of bacterial load, and survival

Ten-week-old female B6D2/F1 mice (Charles River Laboratories, Wilmington, Mass.) were infected via the respiratory route, as previously described (Moreira *et al.*, 1997). Mice were inoculated by exposure to an aerosolized suspension of the parental or Δ*nrdZ* strains of *M. tuberculosis* using a nose-only exposure system (In-Tox Products, Albuquerque, N.M.) (Tsenova *et al.*, 1997). This procedure resulted in implantation of approximately 100 organisms into the lungs of each mouse, which was confirmed by plating lung homogenates on Middlebrook 7H11 plates 3 h post infection. At specific timepoints four mice from each group were sacrificed, and organs harvested. Lungs, livers, and spleens were homogenized and plated to determine bacterial loads. A portion of the upper right lung was used for histological analysis. Five mice from each group were monitored for survival

4.4 RESULTS

4.4.1 The contribution of NrdZ to aerobic RNR activity

The *nrdZ*-encoded class II RNR is dispensable for growth *in vitro*

The demonstration that the *nrdF2*-encoded alternative small subunit is essential for *M. tuberculosis* viability *in vitro* (Dawes *et al*., 2003; Sassetti *et al.*, 2003) implied that the class II RNR, NrdZ, does not provide sufficient RNR activity during normal aerobic growth to complement even partially the loss of the functional class I enzyme. However, the possible function of NrdZ under aerobic growth conditions, as well as the predicted requirement for *nrdZ* in microaerophilic growth or adaptation to anoxia (Mizrahi *et al.*, 2000), remained unexplored. To this end, a Δ*nrdZ* allele was constructed in which the 814 bp internal *Sal*I fragment containing three of the five highly-conserved cysteine residues (Figure 4.1) shown to be essential for coenzyme B_{12}-dependent class II RNR activity in other organisms (Booker *et al*., 1994; Tauer & Benner, 1997) was eliminated. The unmarked deletion allele (p2Δ*nrdZ*19) was delivered into wild-type H37Rv by electroporation. Using two-step selection (Parish & Stoker, 2000), marked and unmarked Δ*nrdZ* DCO mutants were obtained, and the unmarked deletion mutant selected for further manipulation. The genotypic confirmation by Southern blot of the unmarked deletion is shown in Figure 4.2. The successful generation of allelic exchange mutants indicated that *nrdZ* is dispensable for viability of *M. tuberculosis in vitro*. In addition, the *nrdZ* deletion mutant was indistinguishable from wild-type H37Rv under normal *in vitro* growth conditions (data not shown).

NrdZ does not contribute to tolerance of hydroxyurea in *M. tuberculosis*

The potent class I RNR inhibitor, hydroxyurea (HU), inhibits DNA synthesis by specifically destroying the essential class I RNR free radical (Sinha & Snustad, 1972; Jordan *et al*., 1994). HU was previously shown to abrogate *M. tuberculosis* NrdEF2 function *in vitro* (Yang *et al*., 1997), although the seclusion of the tyrosyl free radical in the interior of the protein (Liu *et al*., 1998) ensures the relative resistance of *M. tuberculosis* to radical scavengers (Elleingand *et al*., 1998). To ascertain the potential for NrdZ to catalyse RNR activity under aerobic conditions, as well as detect any possible contribution of the class II RNR to aerobic growth, class I RNR activity was inhibited by treating the Δ*nrdZ* mutant and the isogenic parental strain with HU, and radiometrically determining comparitive growth kinetics using the BACTEC method. The results reported represent data from this study and a collaboration with Dr. S. Dawes. HU inhibited the growth of *M. tuberculosis* at an MIC of 5 mM which was

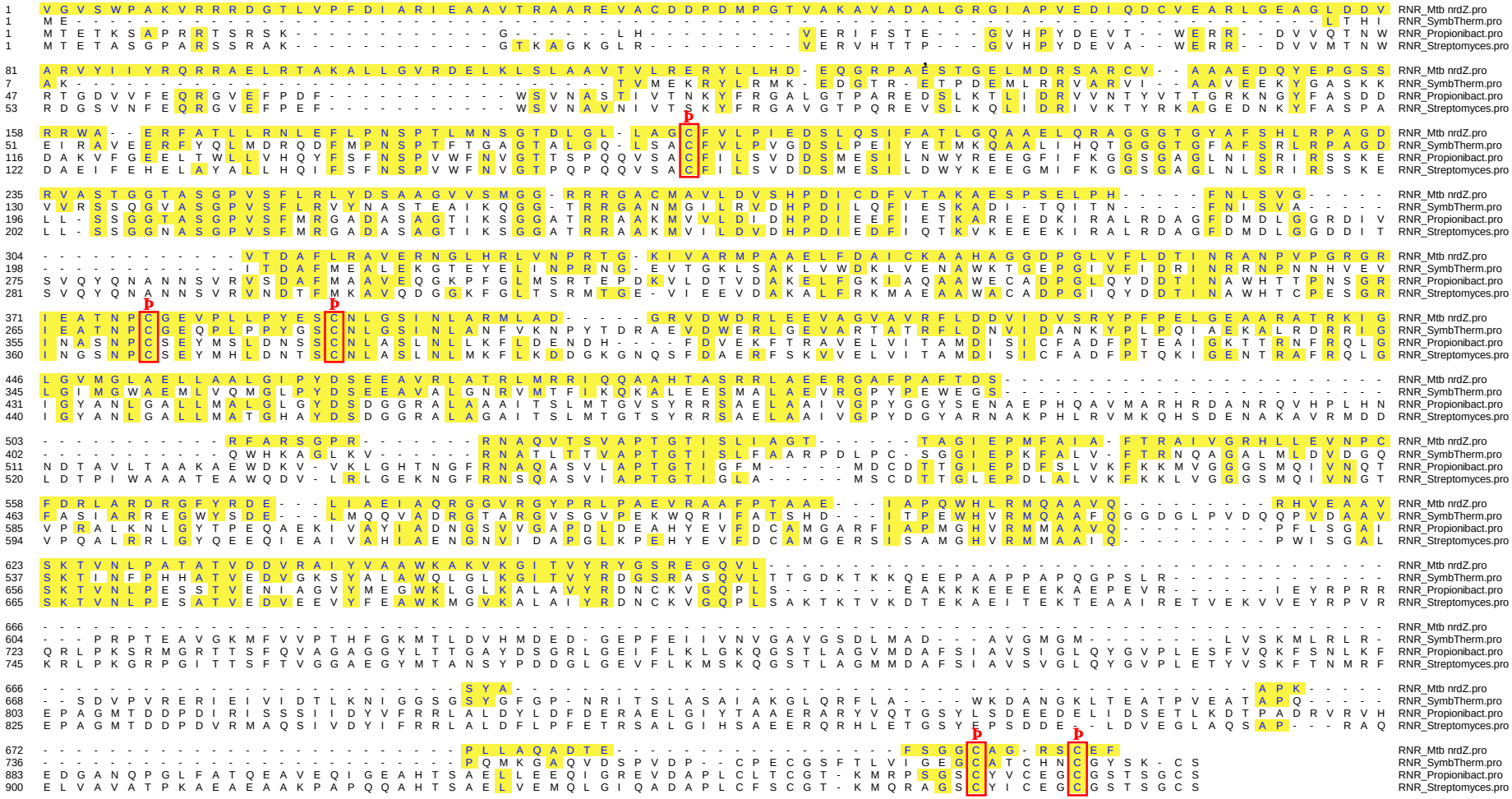


Figure 4.1 Alignment of *M. tuberculosis* NrdZ with other class II RNRs. The *nrdZ*-encoded class II RNR from *M. tuberculosis* H37Rv (RNR_Mtb nrdZ.pro) aligned with class II RNRs from selected actinomycetes, including *Symbiobacterium thermophilum* (RNR_SymbTherm.pro; YP076589.1), *Propionibacterium acnes* (RNR_Propionibact.pro; YP055736), and Streptomyces coelicolor A3(2) (RNR_Streptomyces.pro; NP629929). Accession numbers are included in brackets. Amino acid identity is highlighted in yellow. The five conserved cysteine residues are boxed in red and marked with an asterisk (Þ). Arrows (,) mark the position of the 814-bp deletion in *nrdZ* which eliminated three of the active cysteine residues.

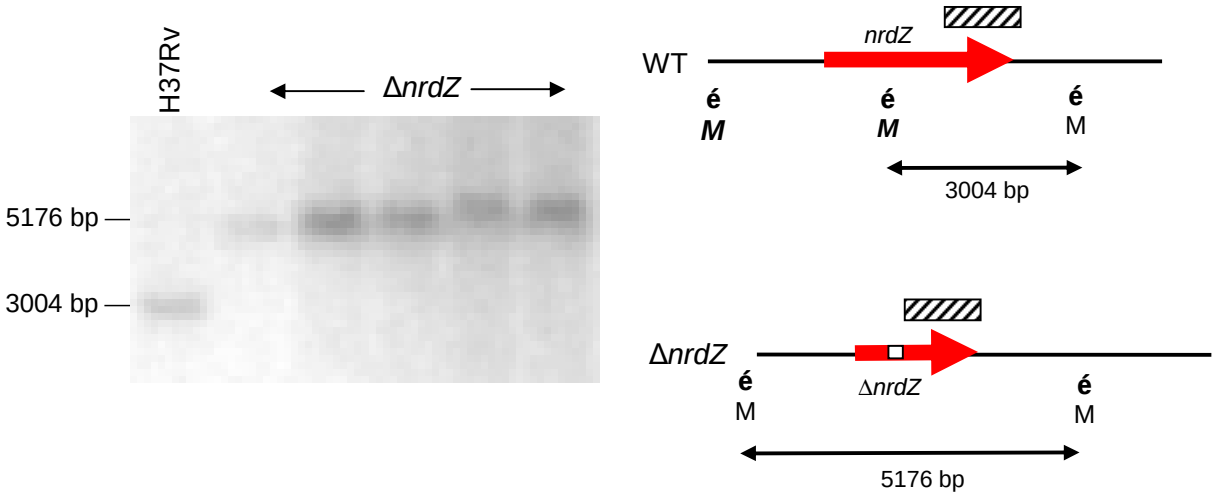


Figure 4.2 Construction of an *nrdZ* deletion mutant of *M. tuberculosis* H37Rv. *Mlu*I-digested DNA from H37Rv and the Δ*nrdZ* mutants probed with nrdZp. Restriction maps of the various clones are schematically illustrated in the line drawings adjacent to the Southern blot (not drawn to scale). Construction of the Δ*nrdZ* allele by elimination of 814 bp of *nrdZ* coding sequence results in the loss of the internal *Mlu*I site (M, *Mlu*I; WT, wild-type; probe is represented by a hatched box).

consistent with the previously reported susceptibility of recombinant NrdEF2 to HU inhibition (Yang *et al.*, 1997). However, no increased susceptibility in the Δ*nrdZ* strain to HU was observed over the range tested (data not shown). Class II RNRs require coenzyme B_{12} (adenosylcobalamin; AdoCbl) for radical generation (Reichard, 1993, 1997; Jordan & Reichard, 1998). Although the ability of mycobacteria to synthesise AdoCbl is established (Peterson & Pope, 1951; Aithal & Sirsi, 1963, 1964; Karasseva *et al*., 1977), the regulation of AdoCbl biosynthesis in *M. tuberculosis* is not well defined. To eliminate the possibility that a vitamin B_{12} deficiency might impair NrdZ function in wild-typ H37Rv and result in the failure to detect differential HU susceptibilities, the above assay was repeated, this time providing both wild-type and ΔnrdZ strains with exogenous vitamin B_{12}. Again, however, no differential susceptibility was observed in the *nrdZ* mutant *versus* wild-type (Figure 4.3).

4.4.2 NrdZ is not reqired for adaptation to hypoxia

The Δ*nrdZ* deletion mutant is not impaired for survival under microaerophilia

Basal expression of *nrdZ* in aerated *in vitro* cultures was shown by a colleague (Dr. S. Dawes) to be at levels at least ten-fold lower than the other *nrd* genes. In contrast, under

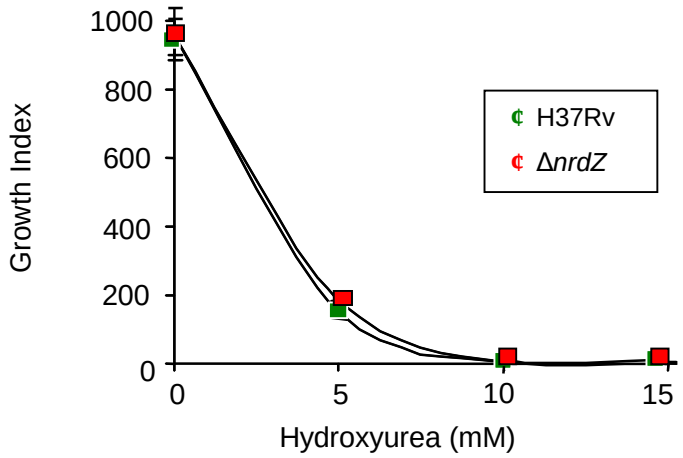


Figure 4.3 Inhibition of *M. tuberculosis* strains by HU. Wild-type *M. tuberculosis* H37Rv and the Δ*nrdZ* mutant were inoculated into Middlebrook 7H12 test medium supplemented with cyanocobalamin (vitamin B_{12}) and growth monitored radiometrically by the BACTEC method for ten days after exposure to different concentrations of HU (courtesy Dr. S. Dawes).

Wayne model conditions (Wayne & Hayes, 1996; Wayne & Sohaskey, 2001), *nrdZ* was significantly upregulated (approximately eight-fold) during adaptation to limiting oxygen levels through the NRPI and NRPII phases of nonreplicating persistence (NRP) and, unlike the other *nrd* genes, exhibited a net increase in transcription in comparison with unstressed growth. The responsiveness of *nrdZ* to reduced oxygen availability prompted an investigation of the role of *nrdZ* in survival of *M. tuberculosis* under microaerophilic conditions. Parallel cultures of wild-type H37Rv and the Δ*nrdZ* mutant were subjected to gradual oxygen depletion under both the settled and stirred models of adaptation to NRP, and anaerobiosis confirmed by observing decolourisation in a monitor tube containing *M. tuberculosis* and methylene blue (Wayne & Lin, 1982; Wayne & Hayes, 1996). Plating efficiencies at 1 week post-NRPII in the stirred Wayne model or 6 months post-NRPII in the settled model revealed that the *nrdZ* mutant was not impaired for survival in comparison with the isogenic parental strain (Figure 4.4). Furthermore, no difference in colony size or morphology was observed which might otherwise have indicated an impaired ability to re-initiate growth on exit from dormancy *in vitro*.

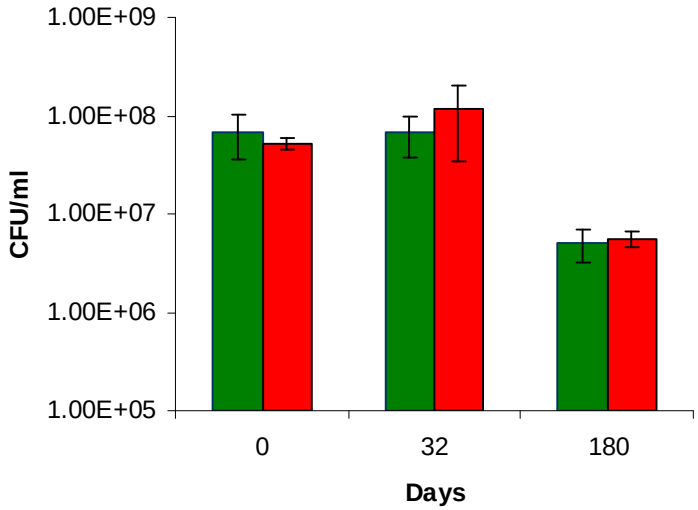


Figure 4.4 Hypoxic survival of *M. tuberculosis* strains. Wild-type *M. tuberculosis* H37Rv (green bars) and the Δ*nrdZ* mutant (red bars) subjected to gradual oxygen depletion under the settled model of adaptation to NRP (Wayne & Lin, 1982) were plated at specific timepoints following entry into NRPII. Data represent mean values from duplicate platings at each timepoint and error bars standard deviations.

Loss of *nrdZ* does not attenuate *M. tuberculosis* for survival *in vivo*

To determine if *nrdZ* is required for *in vivo* survival of *M. tuberculosis*, a collaboration was set up in which immunocompetent mice were infected with wild-type H37Rv and the Δ*nrdZ* mutant. The results discussed comprise mouse work done by Dr. L. Tsenova & Dr. S. Dawes. The predicted role of NrdZ in providing dNTPs for replication or repair during microaerophilic growth recommended the use of the relatively *M. tuberculosis*-resistant B6D2/F_1 mouse strain (Medina & North, 1998). Mice were infected by the aerosol route, and bacillary load assessed in lung, spleen and liver up to 14 months post infection. From an initial lung bacillary count of approximately 100 CFU, the mycobacterial load increased progressively before attaining a maximal level of 10^6 CFU at around 90 days post infection (Figure 4.5). Thereafter, high numbers of viable bacilli persisted for the remainder of the 14 month infection period. Significantly, loss of *nrdZ* did not impair the ability of *M. tuberculosis* to establish infection, or to survive for the 14 month infection period, as determined by lung CFU counts (Figure 4.5). Both spleen and liver counts were similarly unaffected by loss of *nrdZ* (data not shown). In addition, histological analysis revealed no significant difference in disease pathology between the *nrdZ* mutant and wild-type H37Rv at 90 days and nine months post infection (data not shown).

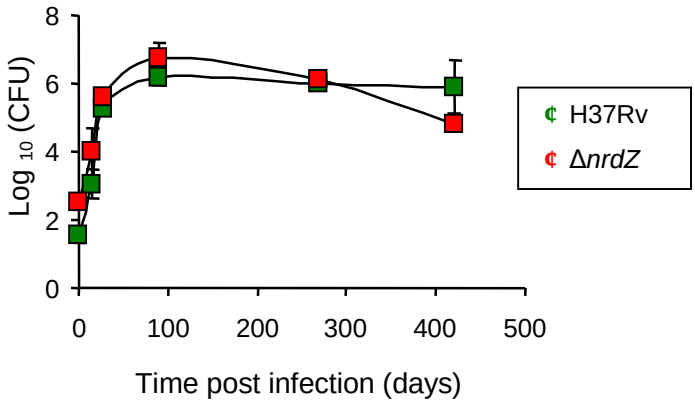


Figure 4.5 Growth of *M. tuberculosis* strains in B6D2/F1 mice. Mice were infected with wild-type *M. tuberculosis* H37Rv and the ΔnrdZ mutant by aerosol, and bacillary loads in the lungs determined over a 14-month infection period. Each timepoint represents the mean bacillary count from four mice, and error bars indicate standard deviations (courtesy Dr. L. Tsenova and Dr. S. Dawes).

4.4.3 NrdZ and damage tolerance under microaerophilia

Despite the demonstrated upregulation of *nrdZ* on transition between NRPI and NRPII (Dawes *et al*., 2003), the Δ*nrdZ* mutant displayed no survival phenotype *in vivo* in the mouse model and *in vitro* under oxygen limitation. These results were suggestive of a possibile inability of plating efficiencies to detect subtle effects on DNA replication. Synthesis of DNA ceases as the oxygen tension falls below 1% under Wayne model conditions (Wayne & Hayes, 1996). However, evidence implicating *dnaE2*-mediated error-prone DNA synthesis in the emergence of drug resistant mutants (Boshoff *et al*., 2003) is highly suggestive of on-going repair activity during long-term persistence, and is consistent with the predicted role of NrdZ in the provision of dNTPs for repair of DNA damage *in vivo* under microaerophilic conditions (Mizrahi *et al*., 2000). It has been shown that metronidazole (MTZ), a DNA-damaging nitroimidazole that specifically kills anaerobes (Edwards, 1979), preferentially targets that subpopulation of *M. tuberculosis* which has adapted to nonreplicating persistence via sequential metabolic shiftdown *in vitro* in the Wayne model (Wayne & Sramek, 1994). Although the precise mechanism of action of MTZ in mycobacteria is unknown (Brooks *et al*., 1999; Lim & Dick, 2001), studies in other bacterial systems indicate that the active metabolite causes DNA strand breaks which prevent replication (Edwards, 1979). To investigate the potential requirement for a functional NrdZ in survival of MTZ-mediated DNA damage under Wayne model conditions, wild-type and Δ*nrdZ* strains were treated with 64 $\mu g.ml^{-1}$ MTZ and plated 14 days after onset of anaerobiosis. The complete inactivity of MTZ against aerobically grown bacilli, even at concentrations well in excess of the MIC (Wayne & Sramek, 1994),

enabled the addition of MTZ at inception of the experiment, and so eliminated the need to manipulate the cultures during incubation, avoiding any unintended alteration to the oxygen tension. Preliminary data indicated that the mean survival of the *nrdZ* mutant appeared to be slightly depressed in comparison with wild-type H37Rv under these conditions, although the increased susceptibility was not statistically significant. Furthermore, addition of exogenous vitamin B12 to the cultures had no discernible effect (data not shown).

Analysis of the complete and unfinished genomes of other mycobacteria revealed the presence of an identical *nrdZ* gene in *M. bovis* (Garnier *et al*., 2003), but no homologues within the genomes of *M. leprae* (Cole *et al*., 2001), *M. avium*, or *M. smegmatis* (http://www.sanger.ac.uk; http://www.tigr.org). Despite the absence of a class II RNR, however, *M. smegmatis* displays similar susceptibility to MTZ activity *in vitro* in models of dormancy (Dick *et al*., 1998; Lim & Dick, 2001). Therefore, as an alternative approach to abrogation of *nrdZ* gene function in *M. tuberculosis*, it was felt that the effect of conferring NrdZ activity on *M. smegmatis* should be investigated. Specifically, it was reasoned that the presence of the functional *M. tuberculosis* class II enzyme might improve the ability of *M. smegmatis* to survive MTZ-induced damage under Wayne conditions. A pMV206-based integrating plasmid (Mdluli *et al*., 1996) - containing the *M. tuberculosis nrdZ* gene plus 800 bp upstream (specifically including the putative Rv0569-encoded transcriptional regulator) and 400 bp downstream sequence - was delivered into *M. smegmatis* mc^2155 by electroporation and integration at the *attP* locus confirmed by PCR. MTZ treatment of wild-type mc^2155 and the *nrdZ*-containing strain again yielded no differential susceptibility, with or without exogenous vitamin B_{12} (data not shown).

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4.5 DISCUSSION

This study formed part of a broader genetic analysis of the essentiality and differential expression of the putative *M. tuberculosis* RNRs (Dawes *et al*., 2003), and extended to an attempt to identify a potential physiological function for the *nrdZ*-encoded class II RNR. Underpinning all manipulations was the knowledge that the functionality of NrdZ had not been biochemically demonstrated, but was inferrred from the conservation in *M. tuberculosis* of the residues shown to be essential for catalytic activity in well-characterised archaeal and eubacterial class II RNRs (Figure 4.1; Booker & Stubbe, 1993; Booker *et al*., 1994). Similarly, little was known regarding the regulation or activity of the vitamin B_{12} biosynthetic pathway in *M. tuberculosis* - essential for class II RNR radical generation (Reichard, 1993, 1997; Jordan & Reichard, 1998) - or of the organism's ability to transport exogenous vitamin B_{12} or convert the supplement to the necessary coenzyme B_{12} form. The deficiency in published data on mycobacterial cobalamin metabolism prompted a parallel investigation that is presented in Chapter 5.

The class Ib RNR is essential and provides dominant aerobic RNR activity

The succesful disruption of *nrdZ* implied the non-essentiality of the *M. tuberculosis* class II RNR for viability *in vitro*. *M. tuberculosis* contains multiple RNR genes encoding subunits of class Ib (*nrdE/F1/F2*) and class II (*nrdZ*) enzymes (Yang *et al*., 1994, 1997; Cole *et al*., 1998), as well as a recently identified class Ic homologue (Högbom *et al.*, 2004). In the related actinomycete *S. coelicolor*, the class Ia (NrdAB) and class II (NrdJ) RNRs are individually dispensable, and the simultaneous inactivation of both enzymes is required for lethality (Borovok *et al*., 2004). This observation is suggestive of the ability of the two enzyme classes to substitute functionally during *in vitro* aerobic growth of that organism (Borovok *et al*., 2004). The oxygen-independent class II RNR has further been shown to provide dominant RNR activity during vegetative growth in some organisms containing both class II and class Ib enzymes (Jordan *et al*.,1997; Borovok *et al*., 2002). The failure to isolate Δ*nrdF2* deletion mutants of *M. tuberculosis* without supplying an intact copy of the *nrdF2* allele *in trans* (Dawes *et al.*, 2003) was, therefore, surprising since it implied the inability of NrdZ to complement - even partially - class Ib RNR function during normal *in vitro* growth. The subsequent demonstration of *nrdE* and *nrdF2* essentiality by high-density mutagenesis confirmed this observation (Sassetti *et al.*, 2003).

Abrogation of the dominant RNR acitivity, either through genetic disruption or chemical inhibition, has been shown in some organisms to result in increased transcription of alternative RNR classes in order to maintain the nucleotide pool balance (Masalha *et al*., 2001; Borovok *et al*., 2004). For example, deletion of the class II RNR in *S. coelicolor* results in the constitutive elevation of class Ib expression, and is indicative either of cross-regulation of the class Ia and class II RNRs (Borovok *et al*., 2004) or of possible allosteric regulatory mechanisms (Thelander & Reichard, 1979; Hofer *et al.*, 1998). Little is known of the regulation of the different RNR genes in *M. tuberculosis*, however. The two class Ib subunits appear to be co-ordinately regulated (Dawes *et al*., 2003), and RNR gene expression is induced after exposure to chemical inhibitors of transcription or translation (Boshoff *et al*., 2004). Evidence that transcription of *nrdZ* under aerobic conditions is approximately ten-fold lower than that of the *nrdF2*-encoded alternative subunit (Dawes *et al*., 2003) suggested that, in the absence of direct or allosteric upregulation, reduced NrdZ expression might account for the inability to complement loss of the class Ib RNR. Therefore, to identify any contribution whatever of NrdZ to RNR activity under aerobic conditions, class Ib RNR function in the *nrdZ* mutant and the isogenic parental strain was chemically abrogated by treatment with HU (Figure 4.3). It was expected that exposure to a range of concentrations spanning the dose shown previously to inhibit recombinant NrdEF2 (Yang *et al*., 1997) might enable detection of subtle differences in HU sensitivities in the Δ*nrdZ* mutant *versus* wild-type. However, the susceptibilities of H37Rv and Δ*nrdZ* to HU were indistinguishable, indicating that NrdZ does not contribute to aerobic growth of *M. tuberculosis*, and that the activity of the class II RNR is not stimulated following repression of the functional class Ib enzyme. Moreover, the demonstrated inhibition of the chlamydial class Ic RNR by HU (Roshick *et al.*, 2000) eliminates the possibility that the recently identified mycobacterial class Ic homologue (Högbom *et al.*, 2004) might provide residual activity during HU treatment. It was recently demonstrated that, during oxidative stress, the *E. coli* cobalamin-independent methionine synthase (MetE) is inactivated by glutathionylation of a key active-site cysteine residue (Hondorp & Matthews, 2004). The dependence of class II RNRs on five highly-conserved cysteines for catalytic activity (Booker *et al*., 1994; Tauer & Benner, 1997), raises the possibility that enzymes of this class might be similarly inactivated. While oxidative inactivation might conceivably be applied to explain the apparent failure of NrdZ to contribute RNR activity during aerobic gowth, it is difficult to reconcile this contention either with the fact that inactivation of *E. coli* MetE required treatment with exogenous oxidative agents or, more

importantly, with evidence that class II RNRs provide dominant RNR activity during aerobic growth of some other organisms (Jordan *et al*.,1997; Borovok *et al*., 2002).

Regulation of mycobacterial RNR activity

The apparent cross-regulation of RNR activity in *S. coelicolor* is thought to be at least partially mediated by the *nrdR*-encoded transcriptional regulator (Borovok *et al.*, 2004). Unlike in *Streptomyces* spp., however, the *M. tuberculosis* NrdR homologue (Rv2718c) and the *nrdZ*-encoded class II RNR are unlinked. Furthermore, an unrelated transcriptional regulator (Rv0569), lacking the ATP-cone domain characteristic of class I and class III RNR allosteric activity sites (Aravind *et al*., 2000), is situated directly upstream of *nrdZ*. The proximal location of Rv0569 to *nrdZ*, as well as the presence of two putative DosR binding motifs upstream of Rv0569 (Park *et al*., 2003) suggests that the two genes are operonic. Both Rv0569 and *nrdZ* have been included in the DosR regulon (Sherman *et al*., 2001; Park *et al*., 2003; Voskuil *et al*., 2003), although some studies have detected increased Rv0569 transcription in response to low-oxygen in the absence of *nrdZ* induction (Bacon *et al.*, 2004). Interestingly, there is evidence that Rv0569 might be down-regulated by HspR during the heat-shock response in *M. tuberculosis* (Stewart *et al*., 2002). Together with evidence that the recently identified class Ic RNRs are well-represented in thermophilic organisms as well as intracellular pathogens (Högbom *et al*., 2004), the possible HspR-mediated repression of *M. tuberculosis* Rv0569 raises the possibility that expression of the various RNR classes might be regulated in response to environmental stimuli other than oxygen tension. The potential role of Rv0569 in the regulation of RNR expression is considered worth investigating, and an Rv0569 deletion mutant of H37Rv has been constructed to this end (data not shown).

Is NrdZ dispensable for hypoxic adaptation?

The inclusion of *nrdZ* in the DosR dormancy regulon (Park *et al*., 2003; Voskuil *et al*., 2003) suggested that the class II RNR might be required to modulate dNTP biosynthesis under conditions of low oxygen availability (Voskuil *et al.*, 2003). However, disruption of *nrdZ* had no effect on the survival of *M. tuberculosis* in either the settled or stirred Wayne models of adaptation to *in vitro* hypoxic dormancy. The absence of a survival phenotype in the Δ*nrdZ* strain contrasted with the profoundly impaired survival of a *M. bovis* BCG *dosR* mutant under the same conditions (Boon & Dick, 2002). However, DosR regulates a concerted shift in metabolic activity to enable bacillary adaptation to long-term hypoxic persistence (Park *et al*.,

2003; Voskuil *et al*., 2003) and it is considered unlikely that loss of a single component would impair survival as dramatically. In addition, although *nrdZ* is included in the DosR regulon, the observation that DNA synthesis terminates as the oxygen tension falls below 1% in NRPI (Wayne & Hayes, 1996) possibly implies that the class II enzyme is more likely to function in the low-level maintenance of dNTP pools for repair, or in preparation for exit from the dormant state. Consistent with this hypothesis, an *S. coelicolor* mutant strain lacking a functional class II RNR was recently shown to be impaired in its ability to recover from prolonged anaerobic incubation (Borovok *et al*., 2004). Of particular interest, however, was the observation that loss of the class II enzyme was not lethal: despite exhibiting a significant delay before resuming growth following re-exposure to oxygen, the mutant strain ultimately attained wild-type viable counts (Borovok *et al*., 2004). Although no alterations in colony morphology were observed in the Δ*nrdZ* mutant after exit from the Wayne model, it is possible that the three to four week incubation period necessary for unaided detection of visible colonies on plates might have afforded the mutant strain sufficient time to reduce any lag. The prolonged generation time of *M. tuberculosis* could have rendered a recovery phenotype undetectable by colony size alone, and might demand the application of a microscope to analyse colony forming ability during the outgrowth period (Wayne & Hayes, 1996).

RNRs constitute the only known *de novo* pathway for the biosynthesis of dNTPs (Jordan & Reichard, 1998) and have traditionally been considered essential components of a minimal bacterial genome. However, complete loss of RNR activity might not be lethal where deoxyribonucleosides are externally available, however, and exogenous supplementation with deoxyribonucleosides has been shown to restore anaerobic growth *in vitro* (Shearer *et al.*, 1999; Folmsbee *et al.*, 2004). It is theoretically possible, therefore, that limited cell turnover might release sufficient DNA and dNTP precursors into the medium to enable complementation of the *nrdZ* deletion during *in vitro* persistence. Although no evidence exists for the natural competence of *M. tuberculosis* (Derbyshire & Bardarov, 2000), uptake of DNA as a carbon and energy source has been reported in starving populations of other bacteria not otherwise competent (Finkel & Kolter, 2001). An additional possibility holds that either the *M. tuberculosis* class Ib or recently identified class Ic enzymes might operate under severe oxygen limitation (Jordan *et al*., 1996; Masalha *et al*., 2001), and remains to be explored.

Mycobacteria in hypoxia-induced dormancy under the Wayne model are diploid, and resumption of growth on re-exposure to oxygen is characterised by the synchronous initiation of RNA synthesis, followed by cell division and then finally DNA replication (Wayne, 1977; Wayne & Hayes, 1996; Wayne & Sohaskey, 2001; Lim *et al*., 1999). Although unlikely to be significantly involved in the chromosomal duplication that occurs in NRPI (the class Ib RNR should operate at this oxygen tension; Dawes *et al.*, 2003), the observed maximal expression of *nrdZ* late in NRPII as the bacilli enter anaerobiosis (Dawes *et al*., 2003) was suggestive of a role for NrdZ in the *completion* of the final round of chromosomal replication, thereby enabling synchronous exit. Of course, the caveat remains that *M. smegmatis* too displays synchronised replication on exit from Wayne model conditions (Dick *et al*., 1998) despite lacking a class II homologue. Unfortunately, the phenomenon of synchronous growth has proven a challenging model to reproduce, frustrating any comparison between wild-type and Δ*nrdZ* mutant strains.

DNA damage under limiting oxygen

It is likely that anerobioisis on its own does not cause extensive DNA damage, and might explain the failure to identify an impaired ability of the *nrdZ* mutant to survive or adapt to in hypoxia *in vitro*. In eukaryotes, survival of DNA damage has been shown to depend on elevated RNR activity and a resultant expansion in dNTP pools, and is intimately linked with cell cycle regulation (Elledge & Davis, 1990; Tanaka *et al*., 2000; Chabes *et al*., 2003). Similarly, class I RNR genes are strongly upregulated in *E. coli* following UV exposure (Courcelle *et al.*, 2001) and, in some organisms, class I RNR induction is specific to novobiocin (NVB)-mediated gyrase inhibition (Gmuender *et al.*, 2001). In *M. tuberculosis*, the class Ib RNR genes as well as *nrdF1* are induced in response to transcriptional inhibition by fluoroquinolones (FQs) and coumarins (Boshoff *et al*., 2004), as well as by the DNA damaging agent mitomycin C (Rand *et al.*, 2003). Treatment of *M. tuberculosis* with FQs and mitomycin C induces the SOS regulon (Davis *et al.*, 2002; Boshoff *et al*., 2003, 2004) whereas NVB does not (Boshoff *et al.*, 2004). The upregulation of the class I RNR genes in response to NVB treatment is, therefore, suggestive of an SOS-independent regulatory mechanism, analogous to the strong, LexA-independent induction of *nrdAB* in *E. coli* (Courcelle *et al.*, 2001). Significantly, the promoter region of *nrdF2* contains motifs for LexA binding as well as the dominant LexA/RecA-independent mycobacterial damage response pathway (Gamulin *et al.*, 2004). To elucidate the potential role for the class II RNR in

provision of dNTPs for DNA repair synthesis under hypoxia *in vivo*, a relatively resistant mouse strain was infected with wild-type and mutant *nrdZ M. tuberculosis* strains and proliferation and survival under immune surveillance determined by CFU assessment over a 14 month infection period. No phenotype was associated with loss of NrdZ activity during both acute and chronic stages of infection, suggesting that *nrdZ* does not contribute significantly to *in vivo* infection in the murine model. However, in contrast to the attenutaion observed in the guinea pig (Malhotra *et al.*, 2004), loss of *dosR* is associated with hypervirulence in the mouse model (Parish *et al.*, 2003a), suggesting that oxygen availability and dominant immune effectors within murine granulomas might differ significantly from human TB.

The lack of a phenotype in the mouse model prompted an investigation into the effects of DNA damage on survival of *M. tuberculosis* in the Wayne model. Preliminary data suggesting that the Δ*nrdZ* mutant is not more susceptible than wild-type H37Rv to MTZ-mediated DNA damage are unexpected; however, the activity of DSB or other energy-intensive repair mechanisms during effective metabolic shutdown is uncertain, and NrdZ might simply provide dNTPs for repair on resumption of growth. Therefore, as with the recovery phenotype, lack of the class II enzyme might translate into delayed repair and therefore outgrowth, and could demand microscopic analysis of colony forming ability. Alternatively, the critical role played by RNRs in modulating dNTP levels (Elledge *et al*., 1992) suggests that loss of NrdZ under these conditions might result in unbalanced dNTP pool compositions (Chimploy *et al*., 2000). Excessive dNTP concentrations are associated with elevated mutagenesis (Chabes *et al*., 2003) and mutations in genes involved in maintaining balanced nucleotide pools result in constitutive upregulation of the SOS response (O'Reilly & Kreuzer, 2004). It is, therefore, postulated that comparing mutagenesis in the mutant and isogenic wild-type strains after hypoxia-induced dormancy might prove a more revealing assay.

MTZ was selected for its known anaerobic killing effects, and also because its demonstrated inactivity against *M. tuberculosis* under aerobic conditions (Wayne & Sramek, 1994) ensures minimal manipulation of the experimental setup. However, the technical difficulties notwithstanding, it is possible that another damaging agent that does not result in DSBs might be more effectively employed. Of course, an assessment of damage survival assumes on-going (or at least sporadic) DNA synthetic activity under hypoxia. It is thought that the

damage likely incurred during long-term survival in the human host necessitates some degree of DNA repair during latent infection. The demonstration that loss of the *dnaE2*-encoded error-prone repair polymerase impairs *M. tuberculosis* for long-term survival *in vivo* (Boshof *et al.*, 2003) provides additional evidence of DNA repair during prolonged infection, a process likely to demand dNTP pool replenishment. However, the minimal effect of MTZ on bacillary survival in the Cornell model (Brooks *et al*., 1999) suggests that anaerobiosis may not characterise murine infection, and might explain the observation that the Δ*nrdZ* mutant is not impaired for survival *in vivo* (Dawes *et al*., 2003). It was recently reported that *M. tuberculosis* adapts sufficiently to long-term, starvation-induced non-replication *in vitro* to re-initiate cellular division (Hampshire *et al*., 2004). Of particular interest was the observation that DNA repair genes are upregulated prior to re-activation of growth, and that no alteration in environmental conditions is required to stimulate this process (Hampshire *et al*., 2004). Significantly, these data suggest that *M. tuberculosis* might actively repair chromosomal DNA during long-term persistence *provided* the bacilli are adequately adapted.

NrdZ and transmission

Finally, an analysis of large sequence polymorphisms in a cohort of clinical *M. tuberculosis* isolates recently identified a deleted region (RD 121) comprising *nrdZ* and two downstream ORFs, implying the non-essentiality of these genes in causing disease (Tsolaki *et al*., 2004). Significantly, RD121 clusters with another deletion (RD 174) containing additional members of the DosR regulon. Loss of regulatory mechanisms in *M. tuberculosis* is associated with dramatic phenotypes *in vivo* (Kaushal *et al*., 2002; Steyn *et al*., 2002; Parish *et al*., 2003a), prompting speculation that the absence of genes implicated in metabolic shutdown in the face of the immune response (Schnappinger *et al*., 2003; Shi *et al*., 2003; Voskuil *et al*., 2003; Karakousis *et al*., 2004) might inevitably result in altered disease progression, perhaps favouring active disease and transmission (Tsolaki *et al*., 2004).

Chapter 5

Biosynthesis of adenosylcobalamin in *Mycobacterium tuberculosis*

5.1 ABSTRACT

The biosynthesis of adenosylcobalamin (AdoCbl), the precursor of several cobalamin co-factors including methylcobalamin (MeCbl) and vitamin B_{12} (cyanocobalamin; CNCbl), is energetically demanding and relies on a complex network of enzyme-catalysed reactions. Previous studies have established the ability of mycobacteria to synthesise AdoCbl, consistent with the presence of at least three putative AdoCbl or MeCbl-dependent isozymes in the *M. tuberculosis* H37Rv genome. To investigate the effects of abrogated AdoCbl biosynthetic function on *M. tuberculosis*, mutants were constructed containing deletions in genes required during early (*cobK*) or late (*cobU*) stages of the biosynthetic pathway. Loss of CobU and CobK activity had no effect on the growth of *M. tuberculosis* H37Rv in liquid minimal media. However, differential growth phenotypes in parental and Δ*cobU* mutant strains following sudden CNCbl withdrawal suggested that disruptions to the biosynthetic pathway might affect cobalamin homeostasis. The relevance of AdoCbl biosynthesis or B_{12}-dependent isozymes to mycobacterial pathogenesis was further suggested by the apparent attenuation of the Δ*cobU* deletion mutant *in vivo* in a mouse model, despite exhibiting no survival phenotype in human or murine macrophage models of infection. A *M. tuberculosis* H37Rv mutant containing a deletion in the B_{12}-independent methionine synthase (*metE*) was impaired for growth *in vitro* and could not be rescued by L-methionine supplementation. Significantly, exogenous CNCbl complemented growth of the Δ*metE* mutant, simultaneously demonstrating the ability of *M. tuberculosis* to transport CNCbl despite encoding no identifiable B_{12}-specific transport system. The demonstration that methionine auxotrophy in the Δ*metE* mutant could be rescued by supplementation with CNCbl but not L-methionine was suggestive of separate but interlinked regulatory functions governing mycobacterial methionine metabolism and cobalamin homeostasis. Finally, *M. tuberculosis* double deletion mutants in which CobU or CobK function was abrogated in addition to MetE were not attenuated for growth in liquid minimal media, possibly indicating the presence in *M. tuberculosis* of an alternative mechanism for methionine synthesis.

5.2 INTRODUCTION

The adenosylcobalamin (AdoCbl) requirement of the *nrdZ*-encoded class II RNR (Chapter 4) prompted an investigation into the biosynthesis of, and requirement for, AdoCbl by *M. tuberculosis*. Furthermore, the proposed role of NrdZ in maintaining nucleotide pools for repair of DNA damage suggested that the synthesis (or transport) of AdoCbl by *M. tuberculosis* during chronic persistence might be relevant to the long-term maintenance of

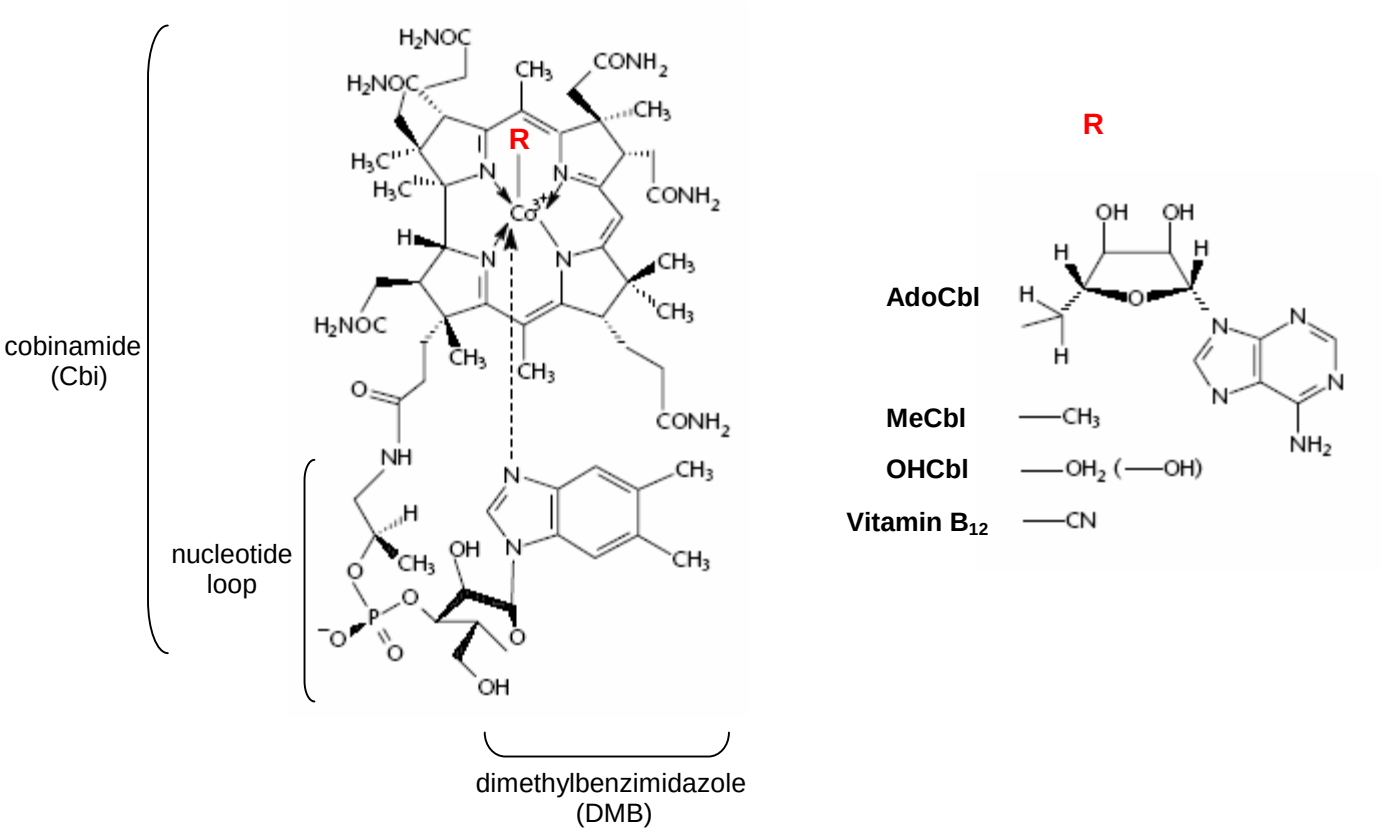

Figure 5.1 **Structures of cobalamin co-factors and derivatives.** Cobalamin co-factors are organometallic corrinoid derivatives comprising a central cobalt atom ligated equatorially by four nitrogen atoms contained within a tetrapyrrolic corrin ring. An intramolecular nucleotide, dimethylbenzimidazole (DMB), occupies the lower axial position. The upper axial position (R) constitutes the site of cofactor reactivity in cobalamin-dependent enzymes, and is occupied by a methyl or a deoxyadenosyl group in methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), respectively. Vitamin B12 (or cyanocobalamin, CNCbl) was the first cobalamin to be isolated (Rickes *et al.*, 1948; Smith, 1948), the cyano group being an artifact of the extraction process. (Adapted from Banerjee & Ragsdale, 2003)

genomic integrity, and to the function of the various mycobacterial DNA polymerases during stationary-phase adaptation and survival. In particular, it was expected that loss of the class II RNR, either *in vitro* or *in vivo*, would impair adaptation of *M. tuberculosis* to hypoxia and, further, that the phenotype might be replicated by creating a mutant strain defective in the ability to synthesise the necessary cobalamin-derived co-factor. However, while attempting to determine the capacity of NrdZ to function during aerobic growth and, therefore, complement (even partially) the loss of the dominant class Ib RNR, it became clear that little was known regarding either the ability of *M. tuberculosis* to synthesise *sufficient* AdoCbl for class II RNR activity, or to transport exogenous cobalamin. Specifically, although several investigators have previously established by biological assay that mycobacteria synthesise AdoCbl (Peterson & Pope, 1952; Aithal & Sirsi, 1963, 1964; Karasseva *et al*., 1977), none has determined the regulation of AdoCbl biosynthesis

in *M. tuberculosis*, the enzymes in that organism dependent on cobalamin-derived co-factors for activity, or the consequences of the loss of AdoCbl biosynthetic ability on viability or virulence. In addition, the failure to identify putative mycobacterial B_{12}-specific transporters (Rodionov *et al.*, 2003) raised the possibility that *M. tuberculosis* might rely exclusively on *de novo* AdoCbl synthesis.

5.2.1 Cobalamin co-factors

Cobalamin is a general term describing a group of organometallic corrinoid derivatives that includes two versatile, biologically-active co-factors - AdoCbl and methylcobalamin (MeCbl), according to the methyl or deoxyadenosyl group occupying the site of co-factor reactivity (Figure 5.1; Banerjee & Ragsdale, 2003). Cyanocobalamin (CNCbl; vitamin B_{12}) is produced exclusively by prokaryotes (Warren *et al.*, 2002), although the cyano- ligand is artificial and results from the extraction procedure (Rickes *et al.*, 1948; Smith, 1948). However, to avoid confusion, throughout the course of this chapter the term 'B_{12}-dependent' will be applied to denote a process or enzyme utilising a cobalamin co-factor. Although required in a range of metabolic processes, the actual number of B_{12}-dependent enzymes is limited, and probably explains the minute quantities of AdoCbl necessary for survival (Raux *et al*., 2000). The largest subset of B_{12}-dependent enzymes requires an AdoCbl cofactor, and includes methylmalonyl-CoA mutase and the class II RNR (Banerjee, 1997; Martens *et al*., 2002). AdoCbl-associated enzymes generally catalyse isomerase reactions, although the specific pathway of radical propagation differs in each case (Licht *et al*., 1996; Padmakumar *et al*., 1997). MeCbl, on the other hand, mediates B_{12}-dependent methyl transfer reactions, for example in methionine and methane biosynthesis (Banerjee, 1997; Ludwig & Matthews, 1997). As well as NrdZ, the *M. tuberculosis* proteome contains two enzymes predicted to require cobalamin-derived co-factors (Rodionov *et al.*, 2003): the B_{12}-dependent methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase, MetH), and methylmalonyl CoA mutase (MutAB) which is required for the conversion of succinyl CoA to methylmalonyl CoA, a crucial substrate in polyketide synthesis (Rainwater & Kalattukudy, 1985). Both NrdZ and MetH have corresponding B_{12}-independent isozymes catalysing similar functions, namely the class Ib and newly-identified (Högbom *et al*., 2004) class Ic RNR enzymes, and the B_{12}-independent methionine synthase (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, encoded by *metE*), respectively. In addition, *M. tuberculosis* contains several genes encoding putative propionyl CoA carboxylase α and β subunits that might function to produce methylmalonyl

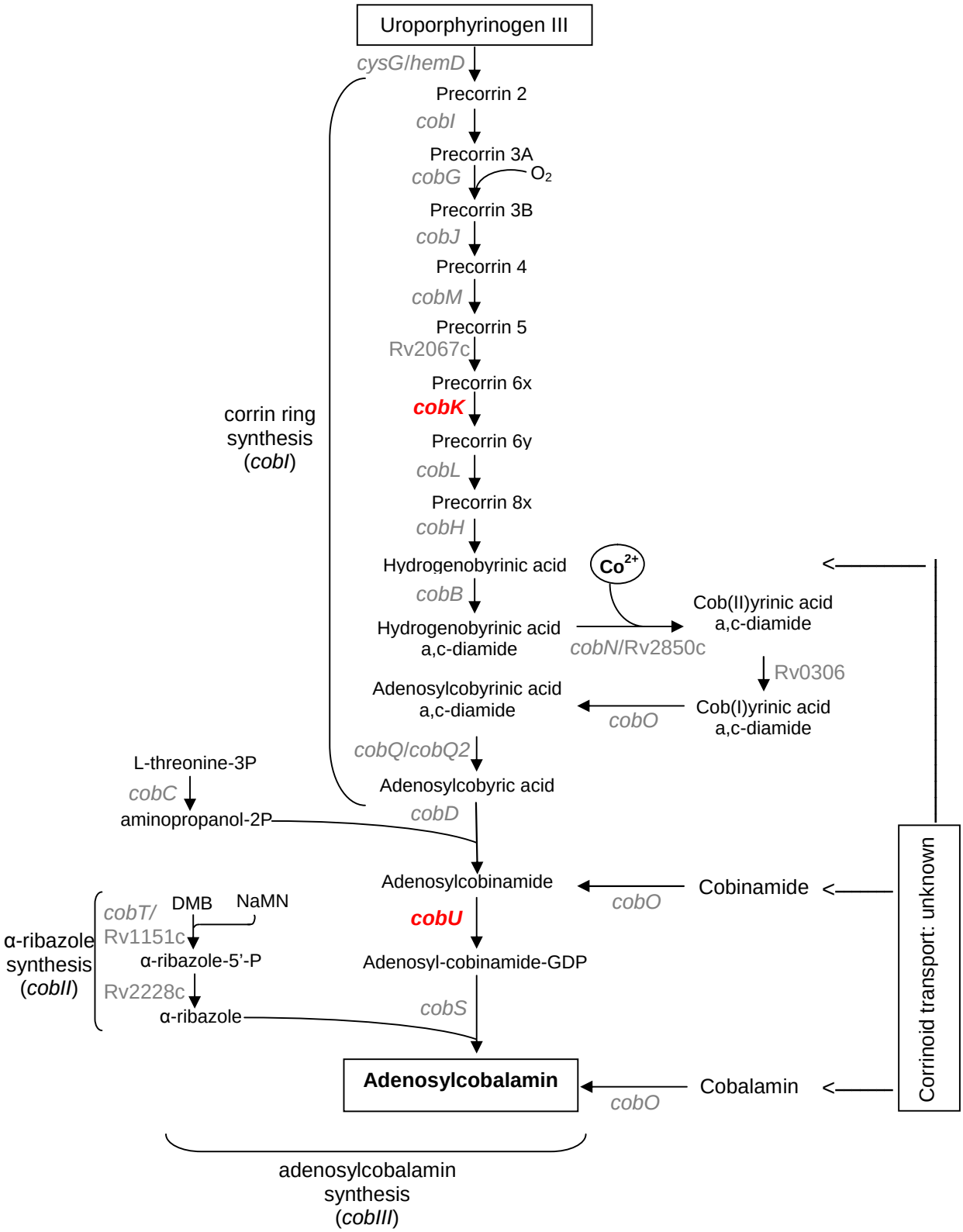

Figure 5.2 The putative *M. tuberculosis* adenosylcobalamin biosynthetic pathway. Shown is a construction of the pathway as predicted to occur in *M. tuberculosis* H37Rv based on sequence homology with known cobalamin biosynthetic genes. The aerobic pathway is characterised by late cobalt insertion. The genes relevant to this study are highlighted in red. Gene names are from Tuberculist (http://genolist.pasteur.fr/TubercuList/). (Adapted from Roth *et al.*, 1993; Rodionov *et al.*, 2003; S. Dawes, unpublished)

CoA independently of AdoCbl via carboxylation of propionyl CoA (Rodriguez & Gramajo, 1999; Rainwater & Kalattukudy, 1985), two of which are essential for optimal *in vitro* growth (Sassetti *et al.*, 2003). In *E. coli*, the B_{12}-independent methionine synthase (MetE) is approximately 100-fold less active than the B_{12}-dependent enzyme (MetH) but the reduced activity is compensated for by significantly increased levels of *metE* transcription (Whitfield *et al.*, 1970; Gonzalez *et al.*, 1992; Greene, 1996; Bandarian & Matthews, 2001). However, with the partial exception of class Ib and class II RNRs (Dawes *et al.*, 2003; Boshoff *et al.*, 2004) little is known of the regulation or activities of the various mycobacterial B_{12}-dependent and B_{12}-independent isozymes.

5.2.2 The AdoCbl biosynthetic pathway

AdoCbl has the most complex structure of any biological co-factor (Figure 5.1; Battersby, 1994). Its intricate and energetically expensive synthesis from uroporphyrinogen III (UroIII, the common precursor of various tetrapyrrolic co-factors; Warren & Scott, 1990; Warren *et al.*, 2002) requires over 25 enzyme-catalysed reactions (Figure 5.2), and is unique among vitamins in being confined to certain microorganisms (Marsh, 1999; Raux *et al*., 2000; Warren *et al*., 2002; Rodionov *et al*., 2003). The prototypical aerobic and anaerobic AdoCbl biosynthetic pathways are defined by those occurring in *Pseudomonas denitrificans* (Stamford, 1994) and *S. typhimurium* (Roth *et al.*, 1993), respectively, and have traditionally been divided into three main steps (Roth *et al*., 1993; Lawrence & Roth, 1995) - part I (referred to as *cobI*) entailing the conversion of UroIII to the complex intermediate adenosylcobinamide (AdoCbi); part II (*cobII*) comprising α-ribazole synthesis from dimethylbenzimidazole (DMB) and a phosphoribosyl group donated by nicotinate mononucleotide (NaMN) (Warren *et al.*, 2002); and, finally, part III (*cobIII*) in which AdoCbi and α-ribazole are covalently linked to produce AdoCbl. The classification of distinct aerobic and anaerobic biosynthetic pathways, however, has recently favoured the division of AdoCbl biosynthesis into two major stages (O'Toole *et al*., 1994; Rodionov *et al*., 2003); the first of these, corrin ring synthesis, differs for aerobic and anaerobic pathways, critically in the timing of cobalt insertion ("early" in anaerobic biosynthesis, "late" in aerobic) whereas the second, comprising adenosylation, aminopropanol attachment, and nucleotide loop assembly, is common to both (Warren *et al*., 2002). Consistent with the classification according to oxygen dependency, several genes have been identified that are considered sentinels of the presence in a particular organism of the either aerobic or anaerobic pathways (Debussche *et al*., 1992, 1993; Raux *et al*., 1997, 2002; Rodionov *et al.*, 2003). However, this distinction is not rigid and there is growing evidence that some

bacteria are able to synthesise AdoCbl under both conditions (Shearer *et al.*, 1999; Raux *et al.*, 2000).

5.2.3 The mycobacterial AdoCbl biosynthetic gene complement

A complete pathway for *de novo* AdoCbl biosynthesis, including putative non-orthologous substitutes, has been constructed for *M. tuberculosis* based on bioinformatic analysis (Figure 5.2; Rodionov *et al.*, 2003; S. Dawes, unpublished) and is suggestive of the aerobic type: the mycobacterial cobaltochelatase - predicted to comprise *cobN*- and Rv2850c-encoded subunits (Rodionov *et al.*, 2003) - is characteristic of 'late' (aerobic) cobalt insertion; the *cobG*-encoded mono-oxygenase is oxygen-dependent and specific to aerobic synthesis (Debussche *et al.*, 1993); and the CobK and CobJ orthologues contain residues typical of aerobic organisms (Shearer *et al.*, 1999). Homologues of nearly all of the *M. tuberculosis* genes are found in the complete and uncompleted genomes of *M. smegmatis*, *M. bovis*, and *M. avium* with some notable differences (S. Dawes, unpublished); for example, *cobF* is present in *M. smegmatis* and *M. avium* but a non-orthologous enzyme is predicted to function in *M. tuberculosis* and *M. bovis* (Rodionov *et al*., 2003). In addition, although *M. bovis* has retained the ability to synthesise AdoCbl *in vitro* (Karasseva *et al.*, 1977), the effect of the RD9 deletion - eliminating the 5'-terminal region of *cobL* and the two upstream genes in other members of the *M. tuberculosis* complex (Brosch *et al*., 2001; Garcia-Pelayo *et al.*, 2004) - on the expression and activity of the *cobLKM* operon is unknown. Both *cobK* and *cobL* were recently identified in a limited set of genes whose expression is unique to replication in immunocompetent mice (Talaat *et al.*, 2004) and disruption of *cobL* has been shown to impair *M. tuberculosis* for *in vivo* survival in the murine model (Sassetti & Rubin, 2003). Similarly, high density mutagenesis has identified two additional putative AdoCbl biosynthetic genes, *cobC* – required for both *in vitro* and *in vivo* survival (Sassetti *et al.*, 2003; Sassetti & Rubin, 2003), and *cobQ2*, which is necessary for optimal growth *in vitro* (Sassetti *et al.*, 2003). Interestingly, *M. tuberculosis* possesses paralogous pairs of genes associated with AdoCbl biosynthesis, comprising *cobQ* (encoding the corrin amidase) and *cobQ2*, as well as *cysG* (encoding a multifunctional uroporphyrin III methyltransferase) and *hemD* (Cole *et al.*, 1998), the roles of which remain to be elucidated. Although the essentiality of *cobQ2* is suggestive of non-redundant activity, it is likely that, as with *cobC* - encoding an aminotransferase involved in aminopropanol synthesis - these genes function in additional cellular processes beyond AdoCbl synthesis. Finally, the biological relevance of AdoCbl to pathogenesis of *M. tuberculosis* might be inferred, albeit loosely, from the significant representation of genes associated with AdoCbl biosynthesis or encoding B_{12}-

dependent enzymes among the ranks of genes predicted to be under strong negative evolutionary selection for amino acid substitutions (Plotkin *et al.*, 2004).

5.2.4 Regulation of AdoCbl metabolism and transport

AdoCbl post-transcriptionally regulates expression of a number of AdoCbl biosynthetic and transport genes (Lundrigan *et al*., 1991; Richter-Dahlfors and Andersson, 1992). Regulation is dependent on the formation of alternative RNA structures (Richter-Dahlfors *et al*., 1994; Franklund & Kadner, 1997; Ravnum & Andersson, 1997, 2001; Nou & Kadner, 1998; Nahvi *et al*., 2002), and is thought to be mediated by a so-called *B12*-element, a motif highly conserved on both sequence and structural levels (Vitreschak *et al*., 2003). Although widely distributed among 5'-untranslated regions of cobalamin-related genes (Rodionov *et al*., 2003; Vitreschak *et al*., 2003), regulation of gene expression by the *B12*-element is postulated to occur by different mechanisms depending on the bacterial taxon. For example, it is proposed that inhibition of translation initiation regulates AdoCbl biosynthesis and transport in actinomycetes, whereas transcriptional attenuation predominates in some other groups (Vitreschak *et al*., 2003). Comparative genomics recently identified several additional genes potentially regulated by the *B12*-element, including cobalt transporters and chelatases, and corrin ring methyltransferases (Rodionov *et al*., 2003). In addition, B_{12}-independent methionine synthases and RNRs are usually associated with *B12* elements in genomes containing corresponding B_{12}-dependent isozymes (Rodionov *et al*., 2003). However, while *M. tuberculosis* contains two putative *B12*-element-binding motifs located upstream of a predicted *cbtG*-type cobalt transporter as well as the B_{12}-independent methionine synthase (*metE*), a *B12*-regulatory motif is not associated with *nrdZ* (Rodionov *et al.*, 2003).

5.2.5 Cobalamin and cobalt transporters

Most bacteria that utilise AdoCbl, but do not synthesise their own, possess a combination of *B12*-element-regulated B_{12}-specific and ferric siderophore transporters to actively transport cobalamins, cobinamide or other corrinoid precursors (Rodionov *et al*., 2003). The most well-characterised B_{12}-specific transport system is found in enteric bacteria and consists of a TonB-dependent outer membrane receptor, BtuB, that operates in complex with a *btuFCD*-encoded ABC transporter (Cadieux *et al*., 2002). Transport of cobalt and other heavy metal ions is generally mediated by the unspecific CorA transport system (Smith *et al*., 1993), although high-affinity, AdoCbl synthesis-specific cobalt transporters have been identified (Roth *et al*., 1993) that are usually preceded by regulatory *B12* elements (Rodionov *et al.*, 2003). As stated above, mycobacteria possess a putative *B12*-

regulated, *cbtG*-type cobalt transporter (Rodionov *et al.*, 2003); furthermore, cobalt supplementation has been shown to increase AdoCbl biosynthesis *in vitro* (Karasseva *et al.*, 1977). There is, however, a complete or partial lack of *btu*-type B_{12}-specific transporters in sequenced mycobacterial genomes (Rodionov *et al*., 2003), although the expression and activities of two FecB homologues (ABC-type cobalamin/Fe^{3+} siderophore transporters) remain to be determined (S. Dawes, unpublished).

5.2.6 Aims and objectives of this study

This study was initially instigated by the need to generate a *M. tuberculosis* mutant strain impaired in its ability to synthesise the AdoCbl co-factor required for NrdZ activity, in the hope of mimicking any observed *nrdZ* phenotype. To this end, the aims comprised:

1. the construction of a *M. tuberculosis* Δ*cobU* deletion mutant
2. analysis of the ability of the Δ*cobU* mutant to adapt to hypoxia *in vitro*

During the course of this work, however, the enigmatic phenotypes associated with disruptions to cobalamin homeostasis, coupled with the demonstrated inability of NrdZ to complement class Ib RNR activity and the apparent dispensability of NrdZ for adaptation to hypoxia *in vitro*, prompted the following revised aims:

1. the demonstration of vitamin B_{12} auxotrophy in a *M. tuberculosis* mutant strain restricted to the utilisation of alternative, B_{12}-dependent metabolic pathways
2. an assessment of the role of AdoCbl biosynthesis and B_{12}-dependent metabolism in survival and virulence of *M. tuberculosis in vivo.*

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains and plasmids

The mycobacterial strains, plasmids and probes used in this study are detailed in Table 5.1. All *cob* and *met* deletion mutants of *M. tuberculosis* were maintained in the presence of 1 µg/ml CNCbl (Sigma) unless otherwise indicated.

Table 5.1 List of strains, plasmids, and probes used in this study

Strain	Description	Reference/source
Δ*cobU*	*cobU* deletion mutant of H37Rv	This study
Δ*metE*	*metE* deletion mutant of H37Rv	This study
Δ*cobK*	*cobK* deletion mutant of H37Rv	K. Downing/This study
Δ*cobU*/Δ*metE*	Double *cobU*/*metE* deletion mutant of H37Rv	This study
Δ*cobK*/Δ*metE*	Double *cobK*/*metE* deletion mutant of H37Rv	This study
Plasmid		
p2Δ*cobU*	p2NIL carrying Δ*cobU* allele; Km^R	This study
p2Δ*cobU*19	Unmarked *cobU* knockout vector - p2Δ*cobU* containing *Pac*I cassette from pGOAL19; $Km^R Hyg^R Suc^S$	This study
p2Δ*metE*	p2NIL carrying Δ*metE* allele; Km^R	This study
p2Δ*metE*::*hyg*	p2Δ*metE* with *hyg* cassette from pIJ963 cloned in *Bam*HI site; $Km^R Hyg^R$	This study
p2Δ*metE*17	*hyg*-marked *metE* knockout vector - p2Δ*metE*::*hyg* containing *Pac*I cassette from pGOAL17; $Km^R Hyg^R Suc^S$	This study
p2Δ*metE*19	Unmarked *metE* knockout vector - p2Δ*metE* containing *Pac*I cassette from pGOAL19; $Km^R Hyg^R Suc^S$	This study
p2Δ*cobK*19	Unmarked cobK knockout vector containing *Pac*I cassette from pGOAL19; $Km^R Hyg^R Suc^S$	K. Downing
Probe		
cobUFR2	1750 bp PCR product containing 113 bp 3' *cobU* coding sequence	This study
metEFR2	1740 bp PCR product containing 562 bp 5' *metE* coding sequence	This study
cobKp	1162 bp *Xho*I/*Sal*I fragment from p2Δ*cobK*19	This study

5.3.2 Construction of the marked and unmarked suicide delivery vectors

***M. tuberculosis* Δ*cobU*.** Two primer sets, cobUF1/cobUR1 and cobUF2/cobUR2 (Table 5.2), were designed to amplify sequence upstream and downstream of *cobU* from wild-type *M. tuberculosis* H37Rv genomic DNA. Ligation of the PCR products cobUFR1 (2058 bp) and cobUFR2 (1732 bp) eliminated 280 bp *cobU* coding sequence to create the Δ*cobU* allele. Sequenced PCR products were cloned into the relevant sites of suicide vector p2NIL (Parish and Stoker, 2000) to generate p2Δ*cobU*. Finally, the 7939-bp *Pac*I fragment from pGOAL19 (*hyg-lacZ-sacB*; Parish & Stoker, 2000) was cloned into the corresponding site of p2Δ*cobU* to generate p2Δ*cobU*19.

Table 5.2 Primers used in this study

Name	Sequence (5'-3')	Comments†
cobUF1	GGCGGCAAAGCTTCGGGTACCTGGT	*Hin*dIII
cobUR1	GCCGGGGGATCCCAATCGGGATCGCT	*Bam*HI
cobUF2	GGGGCGGGATCCCATTCTTCGGGCGT	*Bam*HI
cobUR2	GGCGGCGGTACCAAGGGCGAGCTGAAGT	*Asp*718
metEF1	GGGGTTCGGTACCATCCGGCTGCCG	*Asp*718
metER1	GGCGCCCGGATCCACACGCATCTGT	*Bam*HI
metEF2	GGGGGCCGGATCCAACTCTTCGAG	*Bam*HI
metER2	GCGCGGGAAGCTTCAACTTCGGGCA	*Hin*dIII

†Restriction sites utilised for subsequent cloning of PCR fragments are underlined, and the relevant enzyme detailed under Comments.

***M. tuberculosis* Δ*metE*.** The *M. tuberculosis* Δ*metE* deletion allele was constructed using essentially the same method as that described above for Δ*cobU.* Briefly, two primer sets, metEF1/metER1 and metEF2/metER2 (Table 5.2), were designed to amplify sequence upstream and downstream of *metE* from wild-type *M. tuberculosis* H37Rv genomic DNA. Ligation of the PCR products metEFR1 (1720 bp) and metEFR2 (1969 bp) eliminated 1367 bp *metE* coding sequence to create the Δ*metE* allele. Sequenced PCR products were cloned into the relevant sites of suicide vector p2NIL (Parish and Stoker, 2000) to generate p2Δ*metE*. To mark the deletion allele, the *Bam*HI/*Bgl*II *hyg* cassette (1738 bp) from pIJ963 (Blondelet-Rouault *et al.*, 1997) was inserted into the deletion junction between upstream and downstream PCR fragments, thereby creating p2Δ*metE*::*hyg*. Finally, the 6359-bp *Pac*I fragment from pGOAL17 (*lacZ-sacB*; Parish and Stoker, 2000), or the 7939-bp *Pac*I fragment from pGOAL19 (*hyg-lacZ-sacB*; Parish & Stoker, 2000) was cloned into the relevant marked or unmarked deletion allele to generate p2Δ*metE*17 and p2Δ*metE*19, respectively.

5.3.3 Macrophage infections

THP-1 infections

THP-1 (ATCC TIB-202) cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal calf serum, 0.45% glucose, 0.15% sodium pyruvate, and 4 mM L-glutamine. Cultures were maintained at concentrations of approximately 5 x10^5 cells/ml, and the cells were induced to the macrophage-like state by 24 h pre-treatment with 40 nM 12-*O*-tetradecanoylphorbol-13-acetate (PMA) (Tsuchiya *et al.*, 1982). Cells were infected with *M. tuberculosis* strains at multiplicity of infection (MOI) of 1 bacillus to 5

macrophages; infections were allowed to occur for approximately 2 hours before washing with phosphate-buffered saline and replacement of the medium with fresh medium. Intracellular bacteria were isolated at each timepoint after lysis of the macrophages with 0.05% SDS, and plated on solid 7H10 M-OADC supplemented with 1 µg/ml CNCbl.

J774 infections

The murine macrophage-like cell line J774 was cultured as per established methods (Mueller-Ortiz *et al.*, 2001). Adherent monolayers at a concentration of 10^6 cells/ml were established in 24-well plates 1 day prior to infection with *M. tuberculosis* strains. Suspensions of *M. tuberculosis* H37Rv and the Δ*cobU* mutant were added to Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 4 mM L-glutamine. Mycobacteria were incubated at 37^{o}C for 1 hour before being added to the J774 cells, which had been washed twice with warm PBS, in duplicate wells at a MOI of 1 to 5. After 1 hour, the monolayers were washed three times for 5 min at 37°C with gentle shaking with warm PBS to remove nonadherent bacteria. At each timepoint, the J774 cells were lysed with 0.10% SDS, and serial dilutions of the lysates plated on 7H10-OADC agar plates containing 1 µg/ml CNCbl for CFU assessment.

5.3.4 Infection of mice, determination of bacterial load, and survival

Six-to-eight-week old female C57BL/6 mice (Taconic) were infected with approximately 50 CFU of MTb via the aerosol route and infection monitored by plating of lung and spleen homogenates from 3–5 mice at different times post-infection (Manca et al., 2001; Boshoff *et al.*, 2003). Time-to-death analysis was performed on the remaining mice. Mice were euthanized when they became moribund.

5.4 RESULTS

5.4.1 Disruption of AdoCbl biosynthesis in *M. tuberculosis*

Generation of a Δ*cobU* deletion mutant of H37Rv

The final stage of AdoCbl biosynthesis is characterised by nucleotide loop assembly, and comprises the activation and joining of cobinamide and DMB (Warren *et al.*, 2002). In *S. enterica*, a *cobU*-encoded cobinamide kinase/cobinamide phosphate guanylyltransferase converts AdoCbi to AdoCbi-GDP, which then combines with α-ribazole to produce AdoCbl (Figure 5.2; Roth *et al.*, 1993).

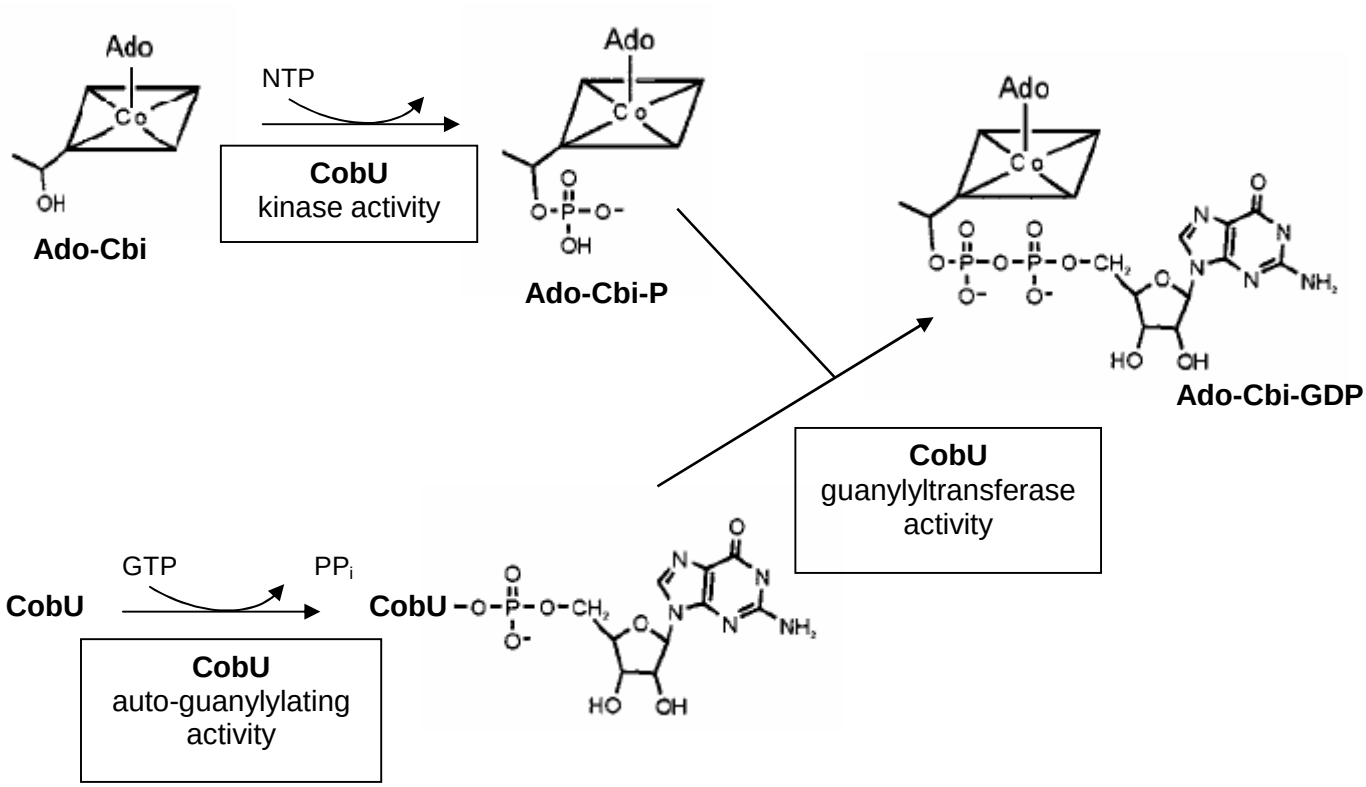

Figure 5.3 Reactions catalysed by the CobU enzyme. CobU autoguanylylation induces the catalytically active conformation that phosphorylates the hydroxyl group of the aminopropanol moiety of adenosylcobinamide (AdoCbi). Either ATP or GTP (shown as NTP) can function as γ-phosphate donor (O'Toole & Escalante-Semerena, 1995). Transfer of the GMP moiety attached to CobU onto AdoCbi-P generates AdoCbi-GDP, which is combined with α-ribazole to produce adenosylcobalamin (AdoCbl) in a reaction catalysed by CobS (not shown). (Adapted from Warren *et al.*, 2002.)

Despite its small size (180 amino acids in *Salmonella*, 174 amino acids in *M. tuberculosis*; Cole *et al.*, 1998), CobU is a multifunctional enzyme that catalyses sequential reactions comprising its own auto-guanylylation and the phosphorylation of AdoCbi to AdoCbi-P and, finally, transfer of the guanylyl group to AdoCbi-P to yield AdoCbi-GDP (Figure 5.3; O'Toole & Escalante-Semerena, 1995; Thomas *et al.*, 2000). The crucial role of CobU late

in AdoCbl biosynthesis, together with the identification of orthologues in several other bacterial species (O'Toole & Escalante-Semerena, 1995; Raux *et al.*, 2000; Rodionov *et al.*, 2003), recommended *cobU* as an initial target for disruption in order to abrogate AdoCbl biosynthesis in *M. tuberculosis*. To this end, an unmarked Δ*cobU* deletion allele was constructed in which 280 bp (of 522 bp) internal *cobU* sequence was eliminated (Figure 5.4A), and delivered into H37Rv by electroporation. The possibility that loss of AdoCbl biosynthetic ability in *M. tuberculosis* might result in CNCbl auxotrophy demanded that media be supplemented with 1 µg/ml CNCbl throughout the process of generating the Δ*cobU* mutant. Putative DCO mutants were obtained by the two-step method (Parish & Stoker, 2000) and confirmed genotypically by Southern blot (Figure 5.4A).

Possible B_{12} dysregulation in the Δ*cobU* mutant

The successful isolation of an unmarked Δ*cobU* deletion mutant on media supplemented with CNCbl suggested that either AdoCbl was not required for normal *in vitro* growth, or that *M. tuberculosis* is able to transport CNCbl. To examine whether loss of *cobU* impacted on unsupplemented growth of *M. tuberculosis in vitro*, parallel cultures of wild-type H37Rv and the Δ*cobU* mutant were established in Middlebrook 7H9 liquid media with (7H9+B_{12}) and without (7H9-B_{12}) exogenous CNCbl and aliquots plated after 168 hours incubation. In order to establish the CNCbl requirements of both strains throughout the assay, as well as to avoid possible growth artefacts arising from different media or residual CNCbl supplement, bacilli were harvested and washed twice in 10% glycerol before plating onto solid 7H10 agar both with (7H10+B_{12}) and without (7H10) CNCbl supplement (Figure 5.5). The growth kinetics of wild-type H37Rv and the Δ*cobU* mutant, as determined by optical density, were indistinguishable in liquid culture irrespective of the presence of exogenous CNCbl (data not shown). In addition, plating efficiencies of wild-type H37Rv on supplemented (7H10+B_{12}) and unsupplemented (7H10) solid agar suggested that AdoCbl availability does not affect normal *in vitro* growth of wild-type H37Rv (data not shown). In contrast (Figure 5.5), growth of the Δ*cobU* mutant on unsupplemented solid agar (7H10) was impaired if bacilli had been harvested from liquid media containing CNCbl (+B_{12}). However, no effect on plating efficiency was observed if the Δ*cobU* mutant had been cultured in liquid media lacking CNCbl supplement (-B_{12}).

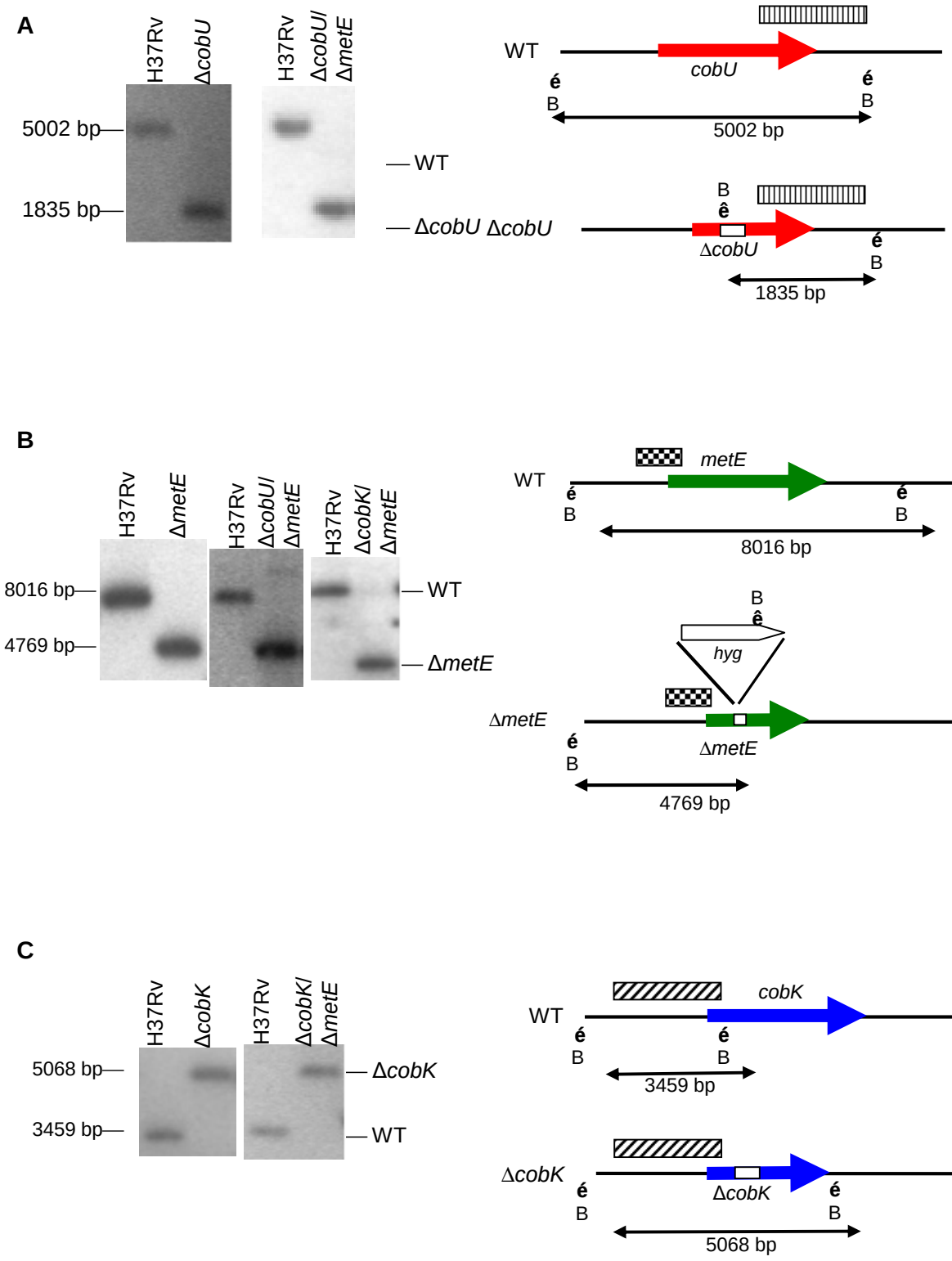
A
H37Rv
ΔcobU
H37Rv
ΔcobU/
ΔmetE
5002 bp
1835 bp
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Figure 5.4 Construction of *M. tuberculosis* mutants disrupted in cobalamin and methionine biosynthetic pathways. DNA from wild-type *M. tuberculosis* H37Rv, the single Δ*cobU,* Δ*cobK*, and Δ*metE* mutants, and the double Δ*cobU*/Δ*metE* and Δ*cobK*/Δ*metE* deletion mutants was digested with *Bam*HI and probed with each of the three probes specific to *cobU*, *cobK* and *metE*. Restriction maps of the various gene deletions are schematically illustrated in the line drawings adjacent to each Southern blot (B, *Bam*HI; WT, wild-type; not drawn to scale). **A**. *Bam*HI-digested DNA hybridised to the *cobU*p probe. Construction of the Δ*cobU* allele eliminates 280 bp of *cobU* coding sequence. An additional *Bam*HI restriction site is introduced in the Δ*cobU* deletion allele (probe represented by a striped box). **B**. *Bam*HI-digested DNA hybridised to the metEFR2 probe. The Δ*metE*::*hyg* deletion-insertion allele eliminates 1367 bp of *metE* coding sequence. An additional BamHI site is introduced by the *hyg* cassette in the Δ*metE*::*hyg* allele (probe represented by a chequered box). **C**. *Bam*HI-digested DNA hybridised to the *cobK*p probe. The Δ*cobK* allele eliminates 214 bp *cobK* coding sequence, including an internal *Bam*HI site (probe represented by a hatched box).

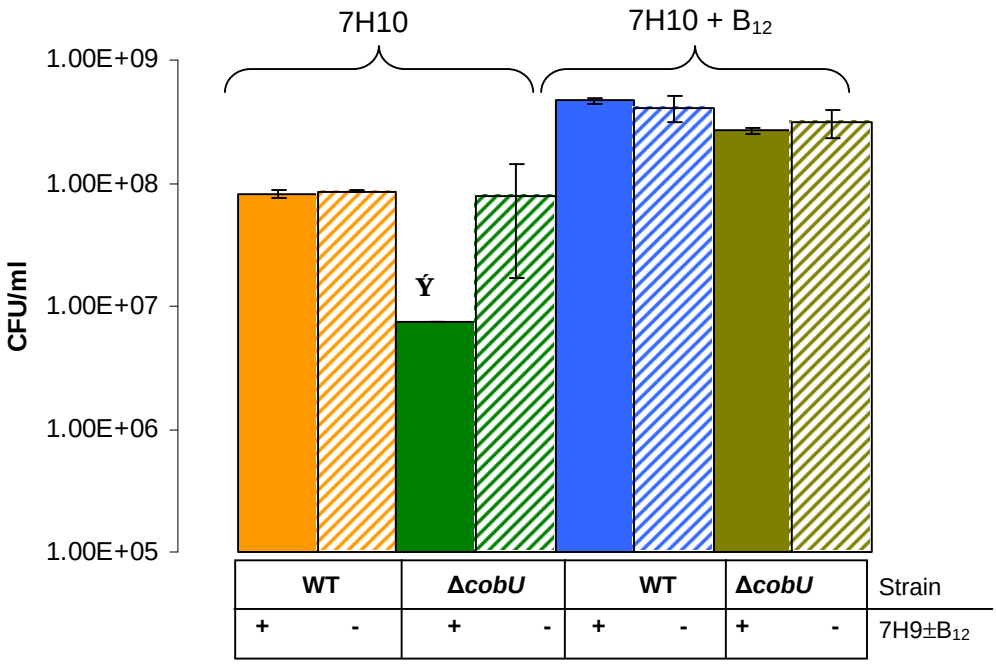


Figure 5.5 The vitamin B_{12} "shock" phenotype in *M. tuberculosis* Δ*cobU*. Plating efficiencies of the *M. tuberculosis* Δ*cobU* deletion mutant were determined by scoring CFUs on CNCbl-supplemented (7H10+B_{12}) and unsupplemented (7H10) 7H10 agar after 168 hours growth in 7H9 liquid media with (+B_{12}) and without (-B_{12}) exogenous CNCbl. Data represent mean CFU values from duplicate platings in at least three idependent experiments and error bars indicate standard deviations. An asterisk (**Ý**) denotes the significant difference between mean CFU values ($p<0.0001$).

M. tuberculosis cobU is not required for survival *in vitro* in macrophage models

The upregulation of putative AdoCbl biosynthetic genes in wild-type H37Rv in response to CNCbl withdrawal seemed to imply the operation of at least one B_{12}-dependent metabolic pathway. To determine whether functional AdoCbl biosynthetic activity was required for pathogenesis of *M. tuberculosis*, intracellular survival of the Δ*cobU* mutant was compared with wild-type H37Rv in the macrophage model of infection. Both murine (J774) and PMA-stimulated human (THP-1) macrophage cell lines were infected in parallel with Δ*cobU* and

wild-type H37Rv, and intracellular survival of the bacilli determined by CFU assessments at 24 hour timepoints extending over 8 days. Plating efficiencies of the Δ*cobU* mutant did not differ significantly from wild-type H37Rv in the human macrophage model (data not shown). Similarly, deletion of *cobU* did not impair survival of *M. tuberculosis* H37Rv in murine macrophages (Figure 5.6).

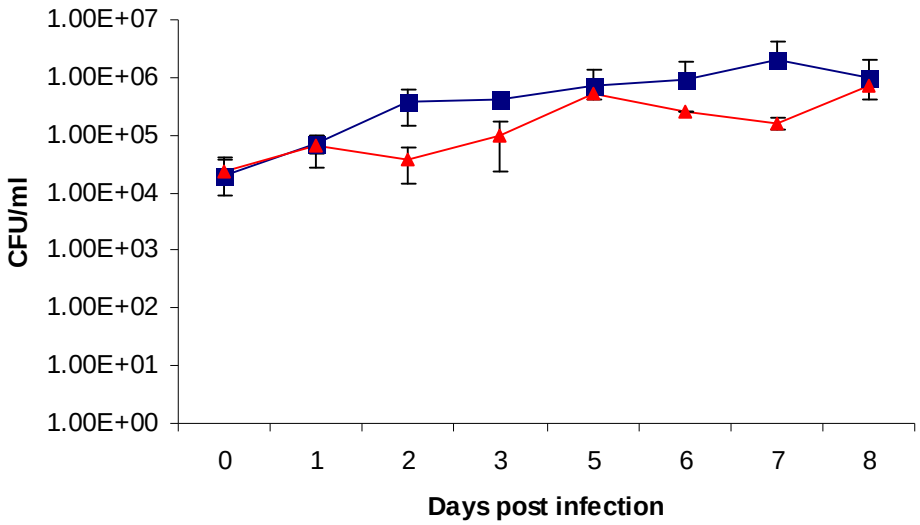


Figure 5.6 Survival of *M. tuberculosis* strains in murine macrophages. A J774 murine bone-marrow macrophage cell line was infected with wild-type H37Rv (- ¢ -) and the ΔcobU deletion mutant (- p -), and intracellular survival of the bacilli determined by CFU assessment on 7H10 agar supplemented with 1 µg/ml CNCbl. Each timepoint represents the mean bacillary count from at least two dilutions plated in triplicate, and error bars indicate standard deviations.

5.4.2 The B_{12}-independent methionine synthase (MetE)

Disruption of *metE* impairs normal growth of *M. tuberculosis in vitro*

The inability to detect a definite *in vitro* phenotype associated with loss of CobU activity prompted the genetic manipulation of *M. tuberculosis* to force the utilisation of putative B_{12}-dependent isozymes. The specific AdoCbl biosynthetic abilities of enteric bacteria – *S. typhimurium* synthesises AdoCbl *de novo* under anaerobic conditions only (Jeter *et al.*, 1984) while *E. coli* requires a cobinamide precursor (Ford *et al.*, 1955) – ensure that mutant strains containing disruptions in the B_{12}-independent methionine synthase (*metE*) are methionine auxotrophs under aerobic conditions *in vitro*. As an alternative to methionine supplementation, Δ*metE* mutants of *S. typhimurium* and *E. coli* can be rescued with exogenous CNCbl, which stimulates activity of the B_{12}-dependent methionine synthase (*metH*) and so restores growth rates to normal levels (Davis & Mingioli, 1950; Smith, 1961). The enforced reliance of Δ*metE* mutants on the MetH apoprotein for methionine synthase activity has, therefore, become an established method to elucidate

AdoCbl biosynthetic pathways in these organisms, using methionine auxotrophy as an indirect readout (Jeter *et al.*, 1984). It was felt, therefore, that a Δ*metE* mutant of H37Rv might provide a useful background to elucidate the AdoCbl biosynthetic requirements of *M. tuberculosis*. To this end, *hyg*-marked and unmarked deletion alleles of *M. tuberculosis metE* were delivered into H37Rv by electroporation and putative DCO mutants selected by the two-step homologous recombination method (Boshoff & Mizrahi, 1998; Parish & Stoker, 2000). The deletion allele eliminated 1367 bp (of 2280 bp) *metE* coding sequence. Surprisingly, no unmarked DCO mutants could be isolated – all white colonies obtained after sucrose selection were wild-type revertants; in addition, the marked construct yielded only minute colonies after a considerable period of outgrowth (in excess of four weeks) on unsupplemented media. Based on the knowledge that *M. tuberculosis* synthesises AdoCbl, it had not been predicted that disruption of the *metE*-encoded B_{12}-independent methionine synthase *on its own* would impair normal *in vitro* growth. The sucrose selection step was, therefore, considered flawed and so was repeated. To eliminate the possibility that *M. tuberculosis* might not produce AdoCbl in sufficient quantity to enable MetH to complement fully the absent MetE function under normal aerobic conditions, CNCbl was added to all media. Again, only wild-type revertants were obtained with the unmarked *metE* deletion allele; however, the putative Hyg^R DCO mutants exhibited normal, wild-type colony size and morphology on the CNCbl-supplemented selective media. Genotypic analysis of these clones revealed the successful replacement of wild-type *metE* with the marked deletion allele (Figure 5.4B). The requirement for exogenous CNCbl supplementation was confirmed by patching the Δ*metE* mutant on unsupplemented media and media containing CNCbl (Figure 5.7A). Significantly, the ability of exogenous CNCbl supplement to complement the Δ*metE* mutant simultaneously demonstrated the ability of *M. tuberculosis* to transport vitamin B12.

Generation of a Δ*cobU*/Δ*metE* double knockout mutant

The partial CNCbl auxotrophy exhibited by the Δ*metE* mutant - despite retaining full AdoCbl biosynthetic ability - suggested that MetH-mediated methionine synthesis was insufficient to complement loss of *metE* or, alternatively, that *de novo* AdoCbl synthesis was unable to meet the co-factor requirements of the B_{12}-dependent isozyme. Whatever the cause, it was predicted that abrogation of AdoCbl biosynthetic ability in the Δ*metE* mutant would cripple MetH function, and so result in complete CNCbl (or methionine) auxotrophy. Because the Δ*metE* mutant carried a *hyg*-marked *metE* deletion allele, a strategy was adopted in which the marked Δ*metE*::*hyg* knockout constuct was delivered

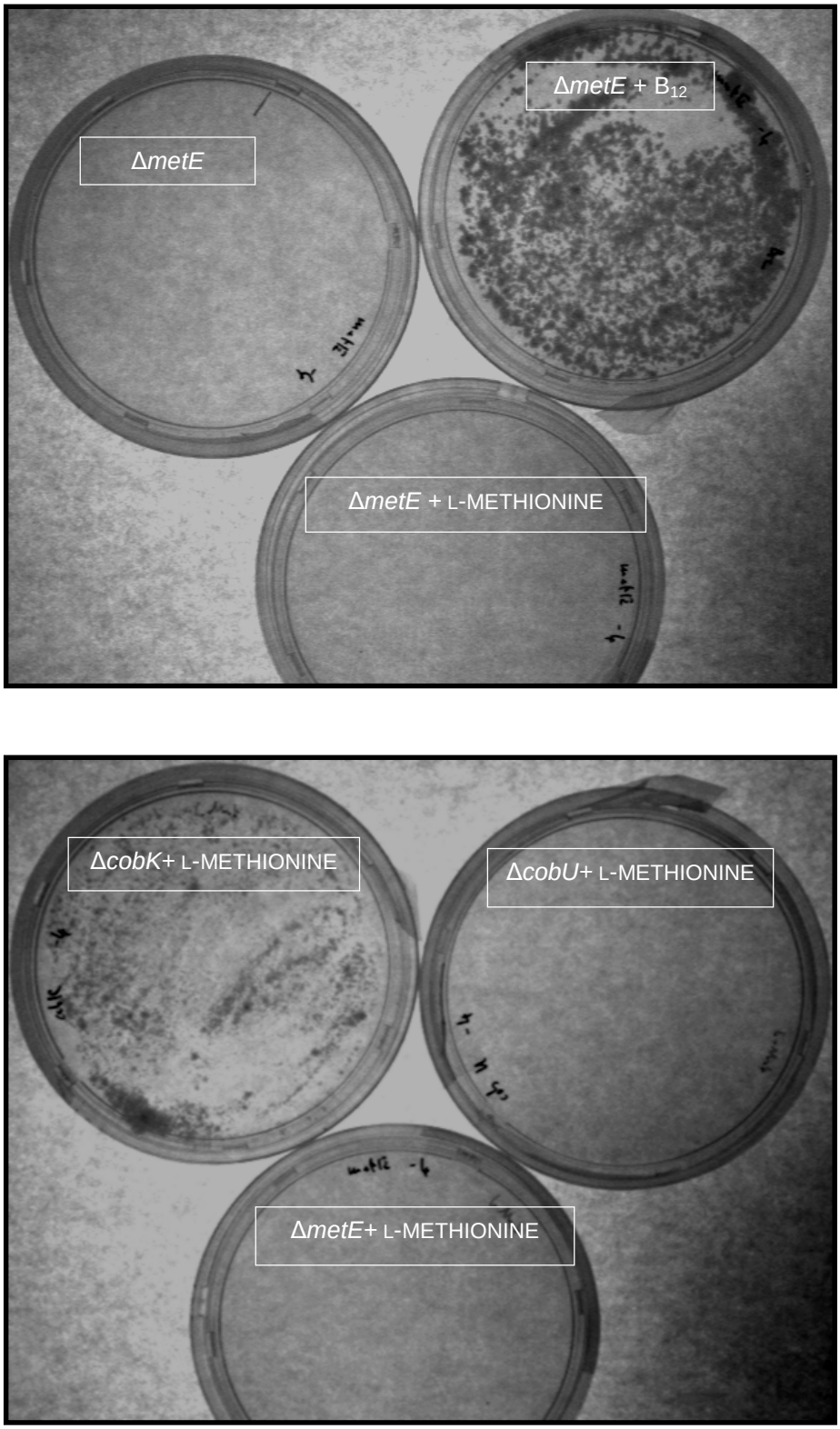


Figure 5.7 Substrate utilisation by *M. tuberculosis* mutants disrupted in cobalamin and methionine biosynthetic pathways. A 50 µl aliquot of a log-phase culture of each of the mutant strains was streaked onto solid 7H10 agar supplemented with the appropriate substrate and growth scored after 21 days incubation. **A.** The *M. tuberculosis* Δ*metE* mutant exhibited wild-type growth on 7H10 agar supplemented with 1 µg/ml CNCbl (Δ*metE* + B_{12}), but was unable to grow on unsupplemented 7H10 agar (Δ*metE*) or on 7H10 agar containing 1 mg/ml L-methionine (Δ*metE* + L-METHIONINE). **B.** Growth of the *M. tuberculosis* Δ*cobK* and Δ*cobU* mutant strains was compared with the Δ*metE* mutant on 7H10 agar containing 1 mg/ml L-methionine.

into the unmarked Δ*cobU* mutant. After electroporation, DCOs were obtained by two-step selection (Parish & Stoker, 2000) on media containing CNCbl supplement. Southern blot analysis confirmed the Δ*cobU*/Δ*metE* double deletion genotype (Figure 5.4A,B). Surprisingly, growth of the Δ*cobU*/Δ*metE* double mutant in liquid 7H9 media was indistinguishable from wild-type whether provided with exogenous CNCbl supplement or not (data not shown). However, on solid 7H10 agar, the Δ*cobU*/Δ*metE* double mutant exhibited the same partial CNCbl auxotrophy that characterised the single Δ*metE* mutant (data not shown).

5.4.3 Functionality of *M. tuberculosis* CDC1551 MetH

The cobalamin moiety in B_{12}-dependent methyltransferases is estimated to undergo oxidative inactivation once every 2000 turnovers (Drummond et al, 1993), necessitating its reductive reactivation for re-entry into the catalytic cycle. In *E. coli*, MetH is a monomer comprising four distinct domains consistent with the partial reactions catalysed - an N-terminal homocysteine-binding domain, an N^5-methyl-tetrahydrofolate-binding domain, a cobalamin-binding domain, and a C-terminal *S*-adenosylmethionine-binding domain required for reactivation (Drummond *et al.*, 1993; Goulding *et al.*, 1997). Sequence analysis revealed that the *M. tuberculosis* H37Rv MetH shares significant homology with the *E. coli* enzyme and possesses key residues associated with each of the four domains (S. Dawes, unpublished). In *M. tuberculosis* CDC1551, the C-terminal region of *metH* containing the reactivation domain - in particular, the essential *S*-adenosyl-L-methionine (SAM) binding motif (Dixon *et al.*, 1996) - is truncated (Fleischmann *et al*., 2002), suggesting that the B_{12}-dependent methionine synthase is disabled. To ascertain whether the absence of this domain necessarily abrogates MetH activity, the *hyg*-marked deletion allele used to disrupt H37Rv *metE* was delivered into CDC1551 by electroporation. It was predicted that loss of MetE should not be lethal in the presence of a functional metH. Despite readily generating SCO recombinants, post-sucrose selection has yielded only Hyg^R mutants among almost fifty putative DCO mutants, even when supplemented with exogenous CNCbl (data not shown). Although a conclusive complementation analysis has not yet been performed, the data suggest that *metE* is essential in CDC1551 and therefore, that the truncated MetH is defective. *M. tuberculosis* CDC1551 is a highly infectious clinical isolate that has been implicated in a number of tuberculosis outbreaks (van Embden *et al.*, 1993; Valway *et al.*, 1998). The deletion of two of the three putative B_{12}-dependent enzymes (NrdZ and MetH) in individual *M. tuberculosis* clinical isolates (Fleischmann *et al.*, 2002; Tsolaki *et al.*, 2004) is, therefore, potentially significant and suggests that loss of B_{12}-dependent isozymes might favour transmission and active

disease - analogous to the deletion of DosR components in other clinical isolates (Tsolaki *et al.*, 2004).

5.4.4 Targeting an earlier step in AdoCbl biosynthesis

Generation of Δ*cobK* and Δ*cobK*/Δ*metE* double deletion mutants

The failure to associate CNCbl auxotrophy with the Δ*cobU* mutant *in vitro* raised the possibility that another enzyme(s) was substituting for absent CobU activity. A non-orthologous protein has recently been shown to fulfil cobinamide kinase activity in archaea, for example (Thomas & Escalante-Semerena, 2000). Therefore, it was decided that targeting an earlier step in AdoCbl biosynthesis might prove more instructive. The *cobK*-encoded mycobacterial precorrin-6x reductase homologue functions in part I (*CobI*) of the predicted AdoCbl biosynthetic pathway, dedicated to AdoCbi synthesis (Figure 5.2; Blanche *et al.*, 1992). Significantly, loss of *cobK* in *Paracoccus denitrificans* eliminates the activity of the B_{12}-dependent RNR and so is lethal under anaerobic conditions (Shearer *et al.*, 1999). An unmarked Δ*cobK* deletion mutant of *M. tuberculosis* H37Rv was generated in collaboration with Dr. K. Downing (Figure 5.4C). The Δ*cobK* allele eliminated 214 bp (of 735 bp) *cobK* sequence. Growth kinetics of the Δ*cobK* mutant were indistinguishable from wild-type H37Rv under standard conditions (data not shown), corroborating the *in vitro* dispensability of AdoCbl biosynthesis previously inferred from the Δ*cobU* mutant. To confirm the inability of combined Δ*cobU*/Δ*metE* deletions to confer CNCbl auxotrophy on *M. tuberculosis*, a double Δ*cobK*/Δ*metE* deletion mutant was also generated (Figure 5.4B,C). As with the single Δ*metE* and double Δ*cobU*/Δ*metE* deletion mutants, the Δ*cobK*/Δ*metE* strain exhibited partial CNCbl auxotrophy on solid 7H10 agar, but was not impaired in liquid 7H9 (data not shown).

The *M. tuberculosis* AdoCbl and methionine biosynthetic pathway mutants are not strict CNCbl auxotrophs

The failure to demonstrate complete CNCbl auxotrophy in any of the *M. tuberculosis* mutants raised the possibility that trace amounts of CNCbl might be present in solid 7H10 and liquid 7H9 media. To eliminate the potential effects of contaminating CNCbl, parallel cultures of each of the single (Δ*cobU*, Δ*cobK*, Δ*metE*) and double (Δ*cobU*/Δ*metE*, Δ*cobK*/Δ*metE*) deletion mutants were established in unsupplemented Sauton's minimal media and growth compared with wild-type H37Rv (Figure 5.8). Initial bacillary counts were determined by plating on solid 7H10 agar supplemented with 1 µg/ml CNCbl, and growth assessed by measuring absorbances at 600 nm (OD_{600}) at specific timepoints extending over 216 hours. In addition, final OD_{600} readings were confirmed by plating

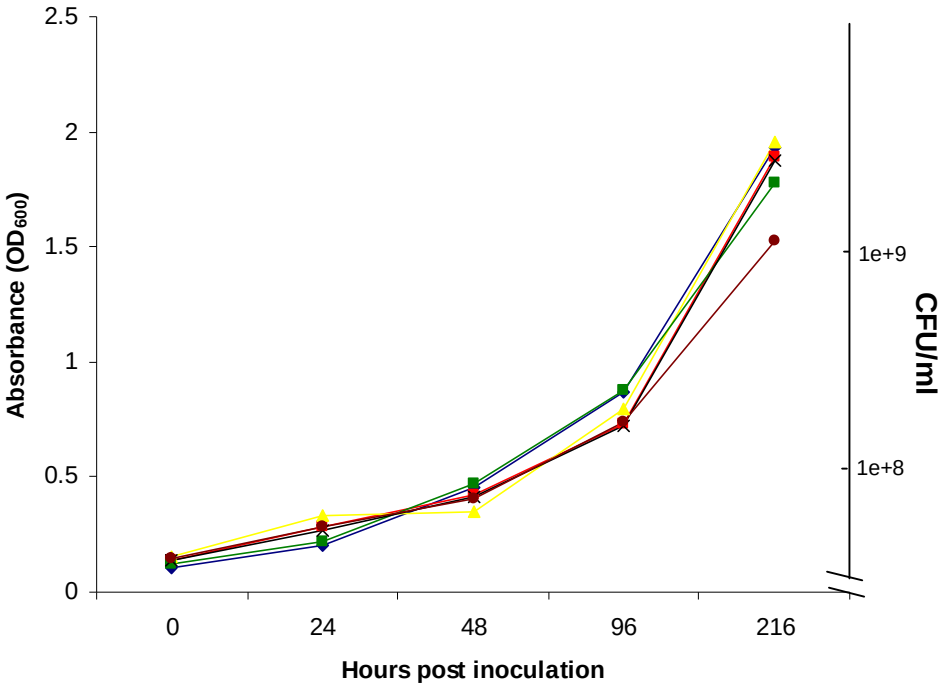


Figure 5.8 Growth kinetics of *M. tuberculosis* strains *in vitro* in minimal media. Growth of wild-type *M. tuberculosis* H37Rv (-®-) in unsupplemented Sauton's liquid minimal media was determined by absorbance (OD_{600}) and compared with the single Δ*cobU* (-¢-), Δ*cobK* (-þ-), and Δ*metE* (-¢-) deletion mutants, as well as the double Δ*cobU*/Δ*metE* (-®-) and Δ*cobK*/Δ*metE* (-Î-) mutant strains. Absorbance measurements were confirmed by plating aliquots of the individual cultures on 7H10 agar supplemented with 1 µg/ml CNCbl at 0 hours, 48 hours and 216 hours, and scoring CFUs.

CFUs at the 216 hour timepoint. Based on both absorbance readings and CFU analysis, growth of each of the deletion mutants from an intial OD_{600} ~ 0.100 to a final OD_{600} > 1.5 was indistinguishable from wild-type H37Rv.

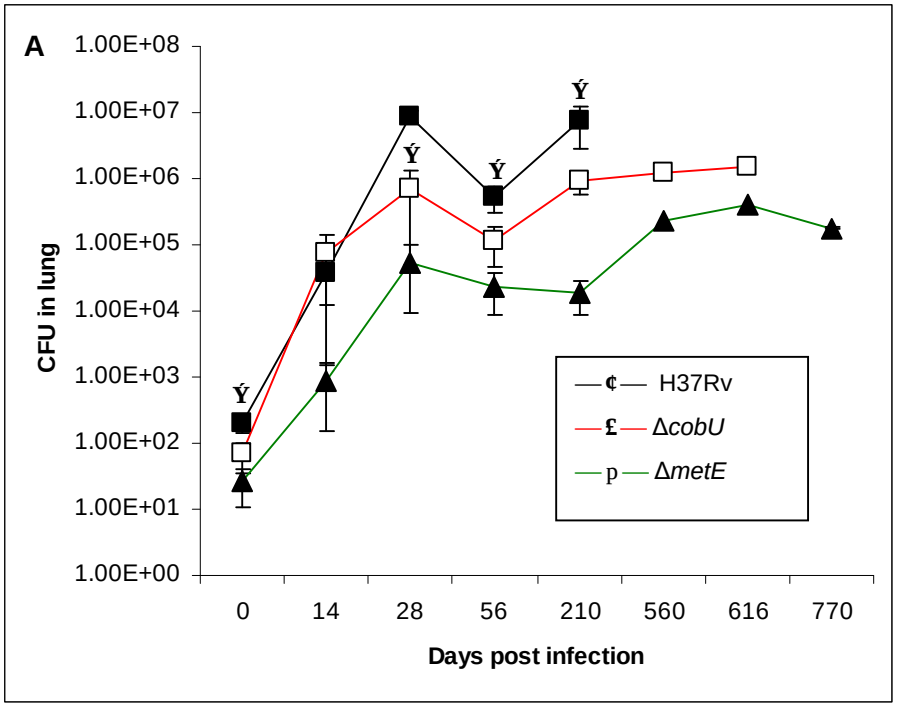
Methionine supplementation does not rescue growth of *M. tuberculosis* Δ*metE*

Although *S. typhimurium* and *E. coli* Δ*metE* strains have largely been used to define steps in AdoCbl biosynthesis, methionine auxotrophy in those mutants can be complemented by supplementing growth media with exogenous methionine (Davis & Mingioli, 1950; Smith, 1961; Jeter *et al.*, 1984). To assess the ability of methionine supplementation to complement loss of MetE activity in *M. tuberculosis* , aliquots of all mutant strains as well as wild-type H37Rv were harvested from unsupplemented minimal media, washed, and plated on solid 7H10 agar containing no supplement, 1 µg/ml CNCbl, and 1 mg/ml L-methionine (Figure 5.7B). Growth of wild-type H37Rv was identical on all media (data not shown). Furthermore, all mutants exhibited wild-type growth on 7H10 containing CNCbl. However, growth of mutants containing deletions in *metE* (Δ*metE*, Δ*metE*/Δ*cobU*, and Δ*metE*/Δ*cobK*) was impaired on unsupplemented 7H10 and, surprisingly, was not rescued

by plating on 7H10 supplemented with L-methionine. In addition, the Δ*cobU* mutant – which achieved wild-type growth levels in the presence of CNCbl supplement – did not grow on L-methionine-supplemented plates (Figure 5.7B). In contrast, the Δ*cobK* mutant grew equally well on CNCbl- and L-methionine-supplemented media.

5.4.5 *M. tuberculosis* Δ*cobU* and Δ*metE* mutants are attenuated *in vivo*

The varied responses of wild-type H37Rv and the Δ*cobU* and Δ*metE* deletion mutants to perturbations in cobalamin homeostasis - manifest in the altered growth phenotypes (Δ*cobU* and Δ*metE*) and transcriptional profiles (H37Rv and Δ*cobU*) resulting from manipulations of exogenous CNCbl availability as well as intrinsic AdoCbl biosynthetic and B_{12}-related metabolic activity – suggested that AdoCbl biosynthesis might play a role *in vivo* during host infection. To assess the requirement for functional AdoCbl metabolic activity *in vivo*, immunocompetent C57BL/6 mice were infected with wild-type H37Rv and the Δ*cobU* and Δ*metE* deletion mutants (Figure 5.9). All mouse infections and early CFU analyses were performed in the laboratory of Dr. Clif Barry III with the assistance of Dr. H. Boshoff; subsequent CFU and time-to-death assessments are courtesy Dr. H. Boshoff. Mice were infected by the aerosol route, and bacillary loads assessed in lung and spleen up to 210 days post infection. For the Δ*cobU* and Δ*metE* strains, bacillary load assessments were extended throughout the course of the time-to-death analysis. The median survival time of mice infected with H37Rv was 290 days, consistent with previous assessments (Boshoff *et al.*, 2003). However, the prolonged median survival times observed in mice infected with either the Δ*cobU* (460 days) or Δ*metE* (534 days) mutant strain, indicated that disruptions to AdoCbl biosynthetic (Δ*cobU*) or B_{12}-independent methionine synthase (Δ*metE*) activity impair the virulence of *M. tuberculosis* (Figure 5.9B). Two weeks after infection, lung counts for the Δ*cobU* mutant ($7.36 \pm 7.20 \times 10^4$) were not significantly different from corresponding wild-type levels ($3.68 \pm 2.41 \times 10^4$; $P>0.3$). However, after four weeks, lung counts for the Δ*cobU* mutant differed significantly from wild-type by approximately one log ($8.76 \pm 1.86 \times 10^6$ for H37Rv *versus* $6.84 \pm 6.36 \times 10^5$ for Δ*cobU*; $P<0.001$) and, after thirty weeks, remained at depressed levels ($9.22 \pm 3.48 \times 10^5$ for Δ*cobU* *versus* $7.71 \pm 4.93 \times 10^6$ for H37Rv; $P<0.1$). Lung CFU counts for the Δ*metE* mutant after two weeks ($9.00 \pm 7.43 \times 10^2$) were two log-fold lower than either Δ*cobU* or wild-type H37Rv, and reached a maximum of $1.92 \pm 1.02 \times 10^4$ bacilli after thirty weeks.



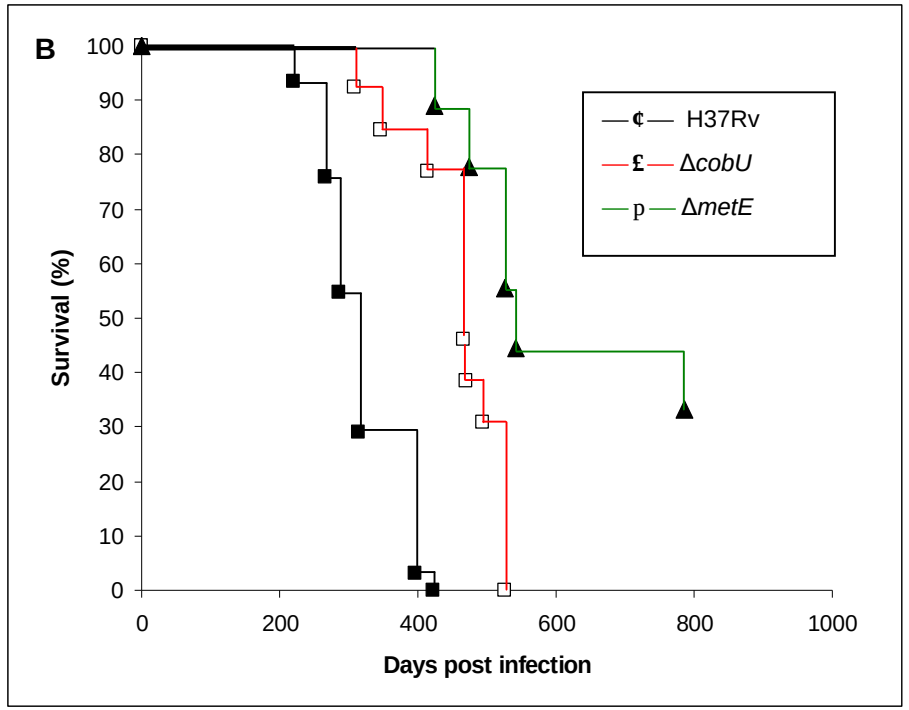


Figure 5.9 **Virulence of *M. tuberculosis* strains in C57BL/6 mice. A.** Lung CFU counts determined at different times post-infection for wild-type H37Rv, and the Δ*cobU* and Δ*metE* deletion mutants. Each timepoint represents the mean bacillary count from three to four mice, and error bars indicate standard deviations. Asterisks (**Ý**) indicate significant differences between mean bacillary counts from mice infected with wild-type H37Rv versus the Δ*cobU* deletion mutant. **B. T**ime-to-death analysis in mice infected by aerosol with wild-type H37Rv, and the Δ*cobU* and Δ*metE* mutants. Fifteen mice were used for time-to-death studies of each strain.

5.5 DISCUSSION

Precedent for the relevance of vitamin metabolism to mycobacterial pathogenesis was recently provided by evidence of the essentiality of genes encoding pantothenate (vitamin B_5) biosynthetic subunits (Sambandamurthy *et al.*, 2002; Sassetti *et al.*, 2003). A functional pantothenate biosynthetic pathway is required for persistence of *M. tuberculosis in vivo* in the murine model (Sambandamurthy *et al.*, 2002), and *M. tuberculosis* pantothenate auxotrophs have been succesfully applied as live attenuated vaccine candidates either alone or in combination with additional attenuating mutations (Sambandamurthy *et al.*, 2002, 2005; Sampson *et al.*, 2004). The work presented here represents an attempt to investigate the role of AdoCbl in mycobacterial pathogenesis, and has shown that disruptions to AdoCbl biosynthesis are associated with altered metabolism and disease progression (Table 5.3). Specifically, deletions in putative early (*cobK*) or late (*cobU*) stages of the mycobacterial AdoCbl biosynthetic pathway are not lethal and do not affect growth *in vitro* in unsupplemented liquid minimal media. However, despite having no effect on survival in either murine or human macrophage models of infection, loss of *cobU* impaired the ability of *M. tuberculosis* H37Rv to achieve wild-type bacillary loads *in vivo* in immunocompetent mice, as well as attenuating virulence. Disruption of the B_{12}-independent methionine synthase, MetE, was similarly associated with attenuation of survival and virulence *in vivo*; in contrast to the AdoCbl biosynthetic mutants, however, loss of MetE function on its own resulted in partial CNCbl auxotrophy *in vitro* on solid media. Significantly, the growth defect was not exacerbated by disruptions to AdoCbl biosynthetic function in the Δ*cobU*/Δ*metE* and Δ*cobK*/Δ*metE* double deletion mutants and, moreover, could not be replicated in any of these strains in liquid minimal media, therefore suggesting the complex interaction of AdoCbl, methionine and, potentially, sulphur metabolic pathways in mycobacterial pathogenesis (discussed below).

AdoCbl biosynthesis in mycobacteria

The presence in *M. tuberculosis* of homologues of the majority of enzymes required for AdoCbl biosynthesis (Rodionov *et al*., 2003; S. Dawes, unpublished) is highly suggestive of the conservation in mycobacteria of at least one essential B_{12}-dependent metabolic pathway. Additional support for the proposed relevance of AdoCbl to mycobacterial pathogenesis might also be obtained from evidence of the dramatic survival and virulence phenotypes associated with the loss of putative AdoCbl biosynthetic genes *in vitro* (Sassetti *et al.*, 2003) and *in vivo* (Sassetti & Rubin, 2003), as well as the inferred significance of related AdoCbl biosynthetic pathway genes in infection of immunocompetent mice (Talaat *et al.*, 2004). Significantly, the *M. leprae* genome

sequence (Cole *et al.*, 2001) revealed homologues of both B_{12}-dependent (MetH) and -independent (MetE) methionine synthases, as well as putative adenosyltransferase and B_{12}-dependent methylmalonyl-CoA homologues (Rodionov *et al.*, 2003). The conservation of B_{12}-dependent pathways in *M. leprae*, in particular - despite the wholesale loss of AdoCbl biosynthetic pathway components (Cole *et al.*, 2001) - strongly suggests that a selective advantage is associated with the retention of B_{12}-dependent isozymes and, further, that the organism (and perhaps other pathogenic mycobacteria) might be able to obtain cobalamin from the host. Moreover, although only tenuously pertaining to mycobacterial AdoCbl synthesis, clinical data describing a high incidence of tuberculosis among strictly vegetarian Indians - thought likely to result from chronic vitamin B12 deficiency (Chanarin & Stephenson, 1988) – provide an alternative perspective on the multiple roles cobalamin co-factors might fulfil in disease progression. In particular, although multiple host immune and physiological pathways are likely impacted by a chronic lack of vitamin B_{12}, an intriguing hypothesis holds that vitamin B_{12} deficiency results in a failure to convert methylmalonic acid to succinic acid - a methylmalonyl CoA mutase-catalysed reaction requiring AdoCbl - and so results in methylmalonic acid build-up, thereby providing a favourable environment for mycobacterial replication (Chanarin & Stephenson, 1988).

The mycobacterial *cob* genes and cobalamin homeostasis

The failure to induce obvious CNCbl auxotrophy in either Δ*cobU* or Δ*cobK* deletion mutant was consistent with the presence in the *M. tuberculosis* genome of alternative, B_{12}-independent isozymes. Furthermore, the recent demonstration that a non-orthologous protein provides cobinamide kinase activity in archaea (Thomas & Escalante-Semerena, 2000) raises the possibility that an unidentified protein(s) might complement deficient CobU activity in the *M. tuberculosis* Δ*cobU* mutant. Of course, *cobK*-encoded precorrin-6x reductase function might similarly be complemented in the Δ*cobK* strain, and therefore demands that AdoCbl production in the Δ*cobU* and Δ*cobK* mutants be directly analysed in order to confirm abrogated biosynthetic ability. Significantly, the differential growth phenotypes of wild-type H37Rv and the Δ*cobU* mutant in response to CNCbl supplementation semed to indicate that, although not necessary for normal growth, AdoCbl might regulate an aspect of metabolism. In particular, the apparent CNCbl “shock” phenotype of the Δ*cobU* mutant (Figure 5.5) implies that loss of the full AdoCbl biosynthetic complement might result in the dysregulation of B_{12}-dependent pathways, or an impaired ability to adapt to CNCbl availability.

Table 5.3 Growth characteristics of *M. tuberculosis* mutants containing disruptions in AdoCbl and methionine biosynthetic pathway genes[†]

	In vitro						***In vivo***	
STRAIN	7H10	7H10 + CNCbl	7H10 + L-Methionine	Liquid minimal media	J774	THP-1	Growth	Virulence
H37Rv	**ü**	**ü**	**ü**	**ü**	**ü**	**ü**	**ü**	**ü**
Δ*cobU*	**ü** (“shock”)	**ü**	**û**	**ü**	**ü**	**ü**	**û**	**û**
Δ*cobK*	**ü**	**ü**	**ü**	**ü**	NA	NA	NA	NA
Δ*metE*	**û** (impaired)	**ü**	**û**	**ü**	NA	NA	**û**	**û**
Δ*cobU*/Δ*metE*	**û** (impaired)	**ü**	**û**	**ü**	NA	NA	NA	NA
Δ*cobK*/Δ*metE*	**û** (impaired)	**ü**	**û**	**ü**	NA	NA	NA	NA

[†] A tick (**ü**) indicates wild-type growth or virulence, a cross (**û**) impaired growth or attenuation.

The regulation of AdoCbl metabolism in *M. tuberculosis*

M. tuberculosis possesses putative *B12*-element-binding motifs that are thought to regulate cobalt transport as well as the activity of the B_{12}-independent methionine synthase (Rodionov *et al.*, 2003). The inferred inability of the Δ*cobU* mutant to respond to CNCbl availability (Figure 5.5) has recently prompted an investigation into the capacity of *M. tuberculosis* to adapt to CNCbl levels and, perhaps, regulate B_{12}-dependent metabolism. Preliminary microarray analyses of the transcriptional responses of both wild-type H37Rv and the Δ*cobU* mutant to sudden CNCbl withdrawal, in particular, have failed to detect distinct B_{12}-associated regulons in either wild-type H37Rv or the Δ*cobU* mutant (data not shown). However, the transcriptional profiles of wild-type *M. tuberculosis* H37Rv grown under various conditions of CNCbl availability have revealed the differential regulation of several genes associated with AdoCbl biosynthesis, providing tentative support for hypothesis that wild-type *M. tuberculosis* regulates AdoCbl production. Specifically, with the exception of *cobI* - encoding the mycobacterial SAM:precorrin-2 methyltransferase required for the generation of precorrin-3A (Figure 5.2), expression of each of the enzymes catalysing the initial steps from δ-aminolaevulinic acid (ALA) condensation to precorrin-3B production is consistently upregulated in wild-type H37Rv within 6 hours of CNCbl withdrawal. In contrast, whole-genome expression profiles of the Δ*cobU* mutant under the same condtions have failed to identify an obvious B_{12}-dependent response, again suggesting dysregulation of cobalamin homeostasis in strains containing disruptions in the biosynthetic pathway.

Methionine biosynthesis in *M. tuberculosis*

The successful disruption of *metE* was significant since it demonstrated that loss of B_{12}-independent methionine synthase activity in *M. tuberculosis* H37Rv is not lethal. Although not essential, the growth phenotype associated with loss of *metE* confirms the inclusion of *metE* among genes required for optimal growth of *M. tuberculosis in vitro* (Sassetti *et al.*, 2003), the nature of the TraSH technique necessarily excluding any mutant too defective to satisfy even the moderate “intermediate growth rate” criteria (Sassetti *et al.*, 2003). Furthermore, the profound growth impairment and partial CNCbl auxotrophy of the Δ*metE* deletion mutant provide support for the proposal that a complex regulatory network likely governs the interaction of AdoCbl, methionine and, potentially, sulphur metabolic pathways in mycobacteria, consistent with the surprising inability of L-methionine to complement growth of Δ*metE*. Considerable attention has recently been focused on the construction of amino acid auxotrophs of mycobacteria, particularly for their possible application as live-attenuated vaccines (Hinshelwood & Stoker, 1992; McAdam *et al.*,

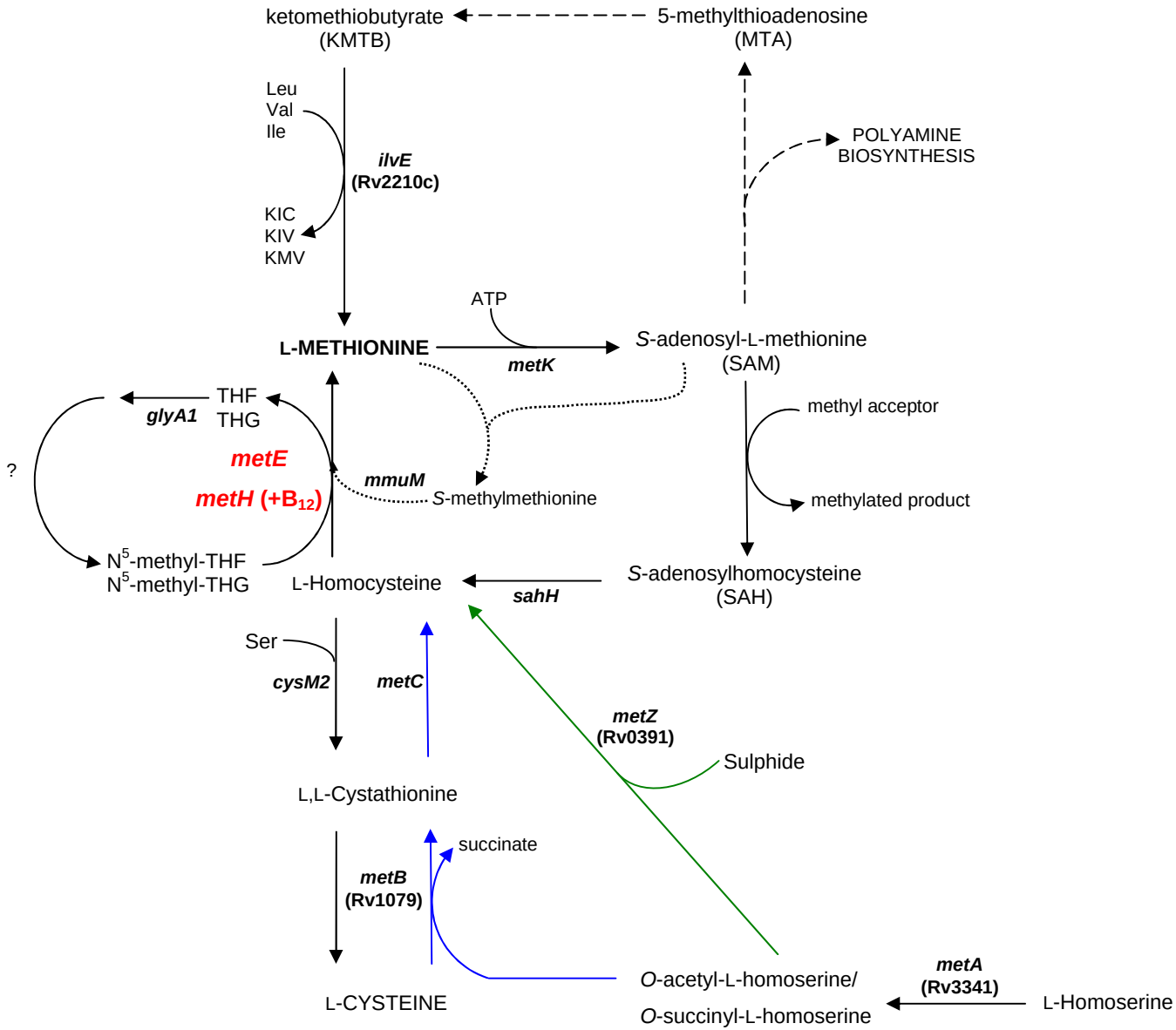

ketomethiobutyrate
(KMTB)
5-methylthioadenosine
(MTA)
Leu
Val
Ile
ilvE
(Rv2210c)
KIC
KIV
KMV
POLYAMINE
BIOSYNTHESIS
ATP
L-METHIONINE
metK
S-adenosyl-L-methionine
(SAM)
THF
THG
glyA1
methyl acceptor
metE
metH (+B12)
mmuM
S-methylmethionine
methylated product
?
N5-methyl-THF
N5-methyl-THG
L-Homocysteine
sahH
S-adenosylhomocysteine
(SAH)
Ser
cysM2
metC
metZ
(Rv0391)
Sulphide
L,L-Cystathionine
succinate
metB
(Rv1079)
L-CYSTEINE
O-acetyl-L-homoserine/
O-succinyl-L-homoserine
metA
(Rv3341)
L-Homoserine

Figure 5.10 L-Methionine biosynthesis in *M. tuberculosis*. Alternative B_{12}-dependent (MetH) and B_{12}–independent (MetE) methionine synthase isozymes (highlighted in red) catalyse *S*-methyltransfer from 5-methyltetrahydrofolate (N^5-methyl-THF) and 5-methyltetrahydropteroyltri-L-glutamate (N^5-methyl-THG) to L-homocysteine to generate L-methionine. *M. tuberculosis* H37Rv does not encode a 5,10-methylenetetrahydrofolate reductase (MetF) homologue and the enzyme(s) catalysing regeneration of 5-methyltetrahydrofolate (N^5-methyl-THF) and 5-methyltetrahydropteroyltri-L-glutamate (N^5-methyl-THG) in the folate pathway are unknown. Blue arrows indicate the transsulphuration and green arrows the direct sulfhydrylation pathways of sulphur incorporation. Broken arrows represent conversions with multiple intermediate steps. The dotted lines indicate the putative *mmuM*-mediated pathway of L-methionine synthesis from L-homocysteine and *S*-methylmethionine; the alternative pathway, also requiring *mmuM* activity, utilises SAM directly as methyl donor in the methylation of homocysteine (not shown). *M. tuberculosis metB* (Rv1079) encodes bifunctional cystathionine γ-synthase and cystathionine γ-lyase activities, and therefore functions in both transsulphuration and reverse transsulphuration pathways (Wheeler *et al.*, 2004). The mycobacterial branched-chain amino acid aminotransferase (BCAT) is encoded by *ilvE* (Rv2210c) and preferentially utilises isoleucine (Ile), leucine (Leu), and valine (Val) as amino donors (Venos *et al.*, 2004). KIC, ketoisocaproate; KIV, ketoisovalerate; KMV, ketomethylvalerate.

1995; Chambers *et al.*, 2000; Hondalus *et al.*, 2000; Gordhan *et al.*, 2002; Pavelka *et al.*, 2003; Sampson *et al.*, 2004). The impaired growth of Δ*metE* mutants on unsupplemented media is, however, surprising given the presence in *M. tuberculosis* of a B_{12}-dependent methionine synthase (MetH), and suggests a dominant role for the B_{12}-independent isozyme in meeting bacillary methionine requirements. The inability of the B_{12}-dependent methionine synthase (MetH) to completely restore growth in the *metE* mutant on unsupplemented media might be ascribed to insufficient AdoCbl production to satisfy the co-factor requirements of the B_{12}-dependent enzyme, or to the rapid oxidative inactivation of AdoCbl under aerobic conditions (Drummond *et al.*, 1993). The potential insufficiency of AdoCbl production in *M. tuberculosis* is, however, difficult to reconcile with evidence of significant AdoCbl biosynthesis during growth *in vitro* (Peterson & Pope, 1952; Aithal & Sirsi, 1963, 1964). Microbiological assays of AdoCbl biosynthesis by *M. tuberculosis* have traditionally relied on liquid culturing of bacilli and, importantly, have shown that the bulk of AdoCbl activity is associated with the culture filtrate. It is possible, though, that *M. tuberculosis* might not produce similar quantities of AdoCbl on solid media, consistent with recent evidence of the inability to isolate AdoCbl from bacilli grown on solid 7H10 agar (S. Dawes, personal communication).

An alternative methionine biosynthetic pathway in *M. tuberculosis*?

The *M. tuberculosis* B_{12}-dependent (MetH) and B_{12}-independent (MetE) methionine synthase isozymes are predicted to catalyse the final, *S*-methyltransfer reaction in methionine biosynthesis (Figure 5.10). The demonstration that loss of AdoCbl biosynthetic ability (*cobU* or *cobK*) in the Δ*metE* mutant was not lethal in unsupplemented liquid minimal media was significant because it suggested that *de novo* methionine biosynthesis

is not essential for mycobacterial growth *in vitro* or that an alternative mechanism(s) might exist in *M. tuberculosis* to generate methionine. Methionine functions as the universal N-terminal amino acid and so is required for the initiation of peptide chain synthesis (*N*-formylmethionyl-tRNA^{fMet}; Adams & Cappecchi, 1966; Webster *et al.*, 1966), as well as providing the precursor for the biosynthesis of *S*-adenosylmethionine (SAM; Figure 5.10), the principal methyl donor in one-carbon reactions (Matthews, 1996). Consistent with the essentiality of the *metK*-encoded SAM synthase (Sassetti *et al.*, 2003), SAM has been implicated in the synthesis of polyamines (Sekowska *et al.*, 1998; Figure 5.10) – thought to regulate transcription (Sarkar *et al.*, 1995) and to stabilise DNA during cell division (Marton & Pegg, 1995) – and is also required for mycobacterial persistence as a result of its donor function in various methyltransferase reactions during mycolic acid biosynthesis (Dubnau *et al.*, 2000; Glickman *et al.*, 2000). Folate metabolism is intrinsically linked to methionine biosynthesis, raising the potential for genomic instability and mutagenesis as a result of excessive uracil incorporation into DNA (Fenech, 2001). In addition, recent evidence suggests that methionine might constitute the preferred sulphur source of *M. tuberculosis* in the host (Wheeler *et al.*, 2004).

Methionine biosynthesis in micro-organisms has been shown to occur via two alternative mechanisms – the canonical *E. coli* transsulphuration pathway that uses cysteine as a sulphur source (Sekowska *et al.*, 2000), and direct sulfhydrylation - that incorporates inorganic sulphur (Thomas & Surdin-Kerjan, 1997; Belfaiza *et al.*, 1998). Interestingly, actinomycetes are included among the few organisms able to utilise both transsulphuration and direct sulfhydrylation pathways: *Corynebacterium glutamicum*, for example, has been shown to synthesise methionine by both routes (Lee & Hwang, 2003), and recent evidence suggests that mycobacteria possesses the same ability (Figure 5.10; Wheeler *et al.*, 2004, 2005). In particular, both *M. tuberculosis* (Cole *et al.*, 1998) and *M. leprae* (Cole *et al.*, 2001) encode an *O*-acetylhomoserine sulfhydrylase homologue (MetZ; Rv0391) that, in *C. glutamicum*, has been shown to catalyse the conversion of *O*-acetylhomoserine to homocysteine using sulphide as the sulphur source (Hwang *et al.*, 2002). Furthermore, a functional reverse transsulphuration pathway was recently characterised in *M. tuberculosis* that enables the incorporation of sulphur from methionine into cysteine (Wheeler *et al.*, 2004). However, while sulfhydrylation and transsulphuration offer alternative routes for the incorporation of sulphur into the methionine precursor L-homoocysteine (Figure 5.10), both are futile in the absence of key *S*-methyltransfer activity, suggesting that the Δ*metE*/Δ*cobK* and Δ*metE*/Δ*cobU* double mutants should be impaired for growth in the absence of exogenous CNCbl.

The energy required for methionine biosynthesis ensures that many organisms do not possess *de novo* methionine biosythetic ability and instead rely on transport and salvage pathways to maintain methionine homeostasis (Rodionov *et al.*, 2004). It has recently been shown, for example, that both Gram-positive (Trackman & Abeles, 1983; Furfine & Abeles, 1988; Myers *et al.*, 1993; Wray & Abeles, 1993; Wray & Abeles, 1993; Cornell *et al.*, 1996; Heilbronn *et al.*, 1999) and Gram-negative (Murphy *et al.*, 2002; Sekowska & Danchin, 2002; Ashida *et al.*, 2003) bacteria are able to recycle methionine from the polyamine byproduct methylthioadenosine. The complete pathway has not been identified in *M. tuberculosis*; however, a functional branched-chain amino acid aminotransferase (*ilvE*; Rv2210c) has been characterised (Venos *et al.*, 2004), consistent with the predicted existence of methionine salvage in *M. tuberculosis* (Wheeler *et al.*, 2004, 2005). In addition, the genomes of both *M. tuberculosis* and *M. leprae* contain a YagD-type *S*-methylmethionine-homocysteine methyltransferase (*mmuM*; Figure 5.10) which, in *E. coli*, has been shown to synthesise methionine from homocysteine using either *S*-methylmethionine or SAM as methyl donors (Neuhierl *et al.*, 1999). *E. coli* does not encode the enzymes necessary for *S*-methylmethionine synthesis but is reliant on YkfD-mediated transport of exogenous supplement; whether *M. tuberculosis* possesses *S*-methylmethionine biosynthetic ability is unknown, and no mycobacterial YkfD homologues have been identified. However, coupled with evidence (S. Dawes, unpublished) of strict CNCbl auxotrophy in an *M. smegmatis* mc^2155 double Δ*cobK*/Δ*metE* deletion mutant (in contrast to the partial CNCbl auxotrophy of the analogous *M. tuberculosis* mutant), the absence of an MmuM homologue in *M. smegmatis* mc^2155 (www.tigr.org) suggests that *M. tuberculosis* MmuM might function in methionine recycling. According to this predicted role, deletion of *mmuM* in the *M. tuberculosis* double Δ*cobU*/Δ*metE* or Δ*cobK*/Δ*metE* mutants should be lethal.

Cross-regulation of B_{12}-dependent and B_{12}-independent methionine synthase activity?

The observation that supplementation of solid media with L-methionine does not restore growth of any of the *M. tuberculosis* strains containing a disrupted *metE* allele - namely the Δ*metE*, the Δ*metE*/Δ*cobU*, and the Δ*metE*/Δ*cobK* mutants, raises several possibilities. Firstly, growth of both wild-type H37Rv and the *cobK* mutant on L-methionine eliminates any suggestion that methionine supplementation *on its own* might be toxic to *M. tuberculosis*, consistent with previous reports of methionine supplementation in members of the *M. tuberculosis* complex (McAdam *et al.*, 1995; Mougous *et al.*, 2002; Wooff *et al.*, 2002; Wheeler *et al.*, 2004). Similarly, the possibility that *M. tuberculosis* is immune to L-

methionine supplementation through a failure to access or transport exogenous methionine on solid media is invalidated by the differential growth characteristics of the Δ*cobU* and Δ*cobK* mutants on L-methionine-supplemented media. Significantly, growth of both Δ*cobU* and Δ*cobK* is indistinguishable on plain as well as CNCbl-supplemented 7H10 agar, but L-methionine supplementation inhibits the Δ*cobU* mutant. Furthermore, although genes encoding mycobacterial methionine transporters have not been identified (Cole *et al.*, 1998), active methionine transport has been demonstrated in *M. tuberculosis* (Wheeler *et al.*, 2004) and is thought to be mediated by any of the predicted ABC amino acid transporters (Ayling *et al.*, 1979; Isnard *et al.*, 1996; Seth & Connell, 2000). Instead, it is submitted that the differential effects of adding L-methionine to the various mutants strains might be better explained with reference to the potential cross-regulation of AdoCbl and methionine biosynthetic pathways. Both the Δ*cobU* and Δ*cobK* mutants contain functional B_{12}-dependent and B_{12}-independent methionine synthases, but are distinguished by the respective locations of their AdoCbl biosynthetic pathway disruptions. Because CobK activity is required early on in the *cobI* stage of AdoCbl synthesis (Figure 5.2), disruption of *cobK* should result in prematurely aborted corrin ring synthesis, and accumulation of precorrin-6x. In contrast, CobU is active during the final stages of AdoCbl biosynthesis (Figure 5.2), and modifies the *completed* AdoCbi substrate. The *M. tuberculosis* genome contains two putative *B12*-element-binding motifs located upstream of a predicted cobalt transporter and the B_{12}-independent methionine synthase, *metE* (Rodionov *et al.*, 2003). It is possible that accumulation of AdoCbi in the Δ*cobU* mutant represses *metE* transcription by binding to the *B12*-element, with L-methionine simultaneously inhibiting *metH* expression. Under these circumstances, methionine metabolism would be halted, perhaps resulting in cysteine toxicity (Reed, 1995; Wheeler *et al.*, 2004), especially on solid media (Chen *et al.*, 2003).

The role of sulphur metabolism in the *M. tuberculosis* Δ*metE* phenotype

Disruption of *metE* in *Corynebacterium glutamicum* similarly results in methionine auxotrophy despite the presence of alternative *metE*- and *metH*-encoded methionine synthases (Rückert *et al.*, 2004). In contrast to *M. tuberculosis* Δ*metE*, however, growth of the *C. glutamicum* Δ*metE* mutant can be rescued by supplementing media with either L-methionine or CNCbl (Rückert *et al.*, 2004). Significantly, sulphate is also able to restore growth of the *C. glutamicum* Δ*metE* mutant but is effective only in liquid media (Rückert *et al.*, 2004), suggesting that additional pathways of sulphur metabolism might be restricted on solid agar. Both Sauton's minimal medium and solid Middlebrook 7H10 agar contain magnesium sulphate as a standard component. Therefore, the unimpaired growth of the

M. tuberculosis Δ*metE* mutant (as well as the Δ*metE*/Δ*cobK* and Δ*metE*/Δ*cobU* strains) in unsupplemented Sauton's minimal medium (Figure 5.8) but not on solid Middlebrook 7H10 agar (Figure 5.7A) might similarly indicate that *M. tuberculosis* is restricted to the assimilation of exogenous sulphate in liquid media. A mycobacterial sulphate transport system has been characterised, and is encoded by the *cysTWA subI* genes (Wooff *et al.*, 2002); however, sulphate uptake *on its own* fails to account for the ability of the Δ*cobK*/Δ*metE* and Δ*cobU*/Δ*metE* double mutants to grow in the absence of a functional methionine synthase. Nevertheless, evidence that sulphate supplementation complements methionine auxotrophy implies that the phenotypes putatively ascribed to impaired methionine metabolism might be dominated by disruptions to sulphur homeostasis. In contrast to *M. tuberculosis* mutants containing Δ*metE* deletion alleles, several methionine auxotrophs of the *M. tuberculosis* complex can be rescued by methionine supplementation (McAdam *et al.*, 1995; Mougous *et al.*, 2002; Wooff *et al.*, 2002). However, methionine auxotrophy in those strains mapped to sulphate transport (McAdam *et al.*, 1995; Wooff *et al.*, 2002) and reduction (Mougous *et al.*, 2002) pathways and not to either of the methionine synthase isozymes. Furthermore, the combined loss of both sulphate transport and *metC*-encoded cystathionine β-lyase activity (Cole *et al.*, 2001) is thought to result in the natural methionine auxotrophy of *M. leprae* (Wheeler *et al.*, 2004). Interestingly, inspection of the genome reveals that, apart from the loss of *metC*, *M. leprae* possesses the full complement of mycobacterial methionine metabolic genes: there are homologues of MetA and MetZ (required for direct sulfhydrylation; Wheeler *et al.*, 2005; Figure 5.10); CysM2 and MetB (required for reverse transsulfuration; Wheeler *et al.*, 2004, 2005); MetE and MetH (methionine synthesis); MmuM (putative methionine recycling); and IlvE (methionine formation from ketomethiobutyrate; Venos *et al.*, 2004). It is proposed, therefore, that *M. leprae* might require exogenous methionine solely to fulfil sulphur requirements and, further, that the presence of multiple methionine pathway genes is indicative of the significance of methionine homeostasis in mycobacteria.

The differential ability of *C. glutamicum* – and, potentially, *M. tuberculosis* - to utilise sulphate in liquid *versus* solid media might also be applied to explain the variable growth phenotype associated with the *cobU* mutant. It is possible that, during growth in liquid 7H9 media supplemented with CNCbl, excess CNCbl represses *metE* transcription by binding to the upstream *B12*-element (Rodionov *et al.*, 2003; Vitreschak *et al.*, 2003), simultaneously supporting sufficient MetH activity to maintain growth at wild-type levels. If a naïve Δ*cobU* culture is grown under these conditions from a starting OD_{600} of ~0.050 to

an early log-phase OD_{600} of approximately 1.0, and is then harvested, washed, and immediately plated on solid 7H10 agar lacking CNCbl supplement, growth might resemble that of the *metE* mutant as a result of the inability of MetH to maintain wild-type growth in the absence of the necessary B_{12} co-factor. In addition, residual CNCbl-mediated suppression of *metE* might compound delayed MetE activity. Of course, as with the Δ*metE* mutant, the phenotype is completely absent where solid media contain CNCbl supplement since full activity of MetH maintains wild-type growth. If, on the other hand, a liquid culture of the Δ*cobU* mutant is subjected to serial passage and sub-culturing, it is submitted that the gradual (or intermittent) depletion of the CNCbl supplement results in the utilisation of both B_{12}-dependent and B_{12}-independent methionine synthases so that subsequent washing and plating does not necessitate the sudden switch to full MetE activity, thereby circumventing the 'shock' phenotype. It is vital, therefore, that transcription of the alternative methionine synthase isozymes is compared during growth in CNCbl-supplemented and -unsupplemented media.

AdoCbl biosynthesis in *M. tuberculosis in vivo*

The relevance of AdoCbl biosynthesis to mycobacterial pathogenesis was suggested by the attenuation of the Δ*cobU* deletion mutant in the mouse model of infection (Figure 5.9). Although bacillary loads in the lungs of mice infected with wild-type H37Rv and the Δ*cobU* mutant were indistinguishable at 14 days post infection, the Δ*cobU* mutant failed to match the growth of H37Rv and did not achieve wild-type levels at any stage thereafter during the infective process, consistent with the prolonged median time-to-death of mice infected with the mutant strain. Loss of AdoCbl activity might exert pleiotropic effects on *M. tuberculosis* metabolism over and above the predicted influence on the function of the various B_{12}-dependent isozymes. The *in vivo* growth phenotype of the Δ*cobU* mutant did not mimic the Δ*nrdZ* mutant, for example (Chapter 4); however, a time-to-death analysis was not performed in that study precluding a comparison of virulence attenuation. Significantly, the *in vivo* growth and virulence phenotypes of the Δ*metE* strain were more profound. However, although not achieving wild-type bacillary loads, the ability of the Δ*metE* deletion mutant to survive and replicate *in vivo* suggests that that the ready availability of sulphate in tissues and body fluids (Wooff *et al.*, 2002) might complement growth. Alternatively, exogenous L-methionine might complement deficient MetE activity *in vivo*; a methionine auxotroph of *M. bovis* BCG unable to transport sulphate is able to survive as well as wild-type *in vivo* (Wooff *et al.*, 2002), for example, thereby providing compelling evidence of the ability of mycobacteria to transport methionine *in vivo*. Specifically, despite the inability of L-methionine-supplemented solid media to support

growth of the Δ*metE* mutant, it is possible that exogenous methionine might only prove inhibitory to the Δ*metE* mutant on media (or under conditions) where AdoCbl biosynthesis is repressed, consistent with the inability to detect AdoCbl production in wild-type H37Rv on solid media (S. Dawes, personal communication). Finally, a potential caveat to the *in vivo* phenotypes of the ΔcobU and ΔmetE mutants is provided by the initial colony counts (Figure 5.9A). Lungs of mice infected with the Δ*metE* mutant (26 ± 15) contained approximately seven-fold fewer CFU than wild-type H37Rv (194 ± 54). Similarly, initial counts from Δ*cobU* infected lungs (69 ± 34) were three-fold lower than wild-type, a difference which was statistically significant ($P<0.01$). Spleen counts confirmed the reduced bacillary loads in the mutant strains (data not shown). Stocks of all *M. tuberculosis* strains including wild-type H37Rv were maintained at the same titre, implying that the reduced bacillary loads associated with the Δ*cobU* and Δ*metE* mutants reflect poor aerosol delivery. Mouse TB is a chronic disease of the lungs (Pierce *et al.*, 1953; Dunn & North, 1995; Dannenberg & Collins, 2001); therefore, it possible that the effective starting lung CFU might dictate disease progression (H. Boshoff, personal communication). Assessments of resistance in different mouse strains, for example, have demonstrated the differential infection outcomes associated with inoculum size (Smith, 1985; Medina & North, 1998). Although the difference in starting lung CFU was maintained throughout the course of infection in the case of the Δ*metE* deletion mutant, it is perhaps significant that bacillary loads in the lungs of the H37Rv- and Δ*cobU*-infected mice were indistinguishable at two weeks post infection.

Transport of vitamin B_{12} in mycobacteria

M. tuberculosis H37Rv does not encode BtuFCD-type cobalamin transporters and no alternative mycobacterial B_{12}-specific transport mechanisms have been identified (Rodionov *et al.*, 2003). The demonstration that CNCbl partially complements growth of the H37Rv *metE* mutant is, therefore, significant since it implies the ability of *M. tuberculosis* to transport vitamin B_{12} as well as both MetH functionality. In contrast to *M. tuberculosis*, the *M. smegmatis* genome contains a putative BtuC homologue (MSMEG 4553), but lacks homologues of the remaining transport components (www.tigr.org). An *M. smegmatis* mc^2155 double Δ*cobK*/Δ*metE* deletion mutant is a CNCbl auxotroph (S. Dawes, unpublished). Significantly, disruption of *btuC* in the mc^2155 Δ*cobK*/Δ*metE* mutant is not lethal (data not shown), reinforcing the likelihood that alternative vitamin B_{12} transport mechanisms might function in mycobacteria. Whole-genome transcriptional analysis of either *M. tuberculosis* Δ*cobU* or wild-type H37Rv strains subjected to CNCbl supplementation or withdrawal failed to reveal any obvious transport candidates. In

humans, mutations in *MMAA* - a putative AAA ATPase-type mitochondrial vitamin B_{12} transporter - define the *cblA* complementation group that is associated with methylmalonic acidemia (Dobson *et al.*, 2002). Interestingly, a possible *M. tuberculosis MMAA* homologue (Rv1496) was recently identified in the *M. tuberculosis* H37Rv genome (S. Bosnjak, unpublished). As with a number of sequenced microbial genomes (Dobson *et al.*, 2002), Rv1496 is located in a predicted operon including the mycobacterial *mutAB* genes (Cole *et al.*, 1998). Although the functionality and regulation of this putative alternative vitamin B_{12} transporter in *M. tuberculosis* remains to be determined, it was noteworthy that expression of the individual genes within the operon clustered under both *in vitro* and *in vivo* conditions (Talaat *et al.*, 2004). Furthermore, preliminary transcriptional analysis by microarray suggests that expression of both Rv1496 and *mutAB* might be elevated in wild-type H37Rv as well as the Δ*cobU* mutant during growth in CNCbl-supplemented media (data not shown). It is, therefore, tempting to speculate that Rv1496 and *mutAB* constitute orthologous components of a vitamin B_{12}-dependent system that has been conserved through evolutionary endosymbiosis (Margulis, 1975).

An immunomodulatory role for AdoCbl?

Previous *in vitro* assays of the relative AdoCbl biosynthetic abilities of pathogenic and non-pathogenic mycobacteria reported significant amounts of cobalamin in the culture filtrate (Aithal & Sirsi, 1964) and, further, that the distribution of B_{12}-dependent activity between culture filtrate and bacterial mass differed according to mycobacterial species (Aithal & Sirsi, 1963). The relevance of cobalamin extrusion to *in vivo* infection is uncertain; however, the demonstrated high-affinity interaction between NO and both cobinamide and cobalamin in various oxidation states - in particular, the low pH-specific interaction of NO with cobalamin (III) (Sharma *et al.*, 2003) – raises the intriguing possibility that cobalamin might constitute an indirect mycobacterial immunomodulatory mechanism. As stated previously, pathways implicated in tolerance of reactive intermediates are thought to have evolved primarily to function in normal cellular metabolism and signalling, but may be subject to second-order selection for additional roles in defence against host immune mechanisms (Zahrt & Deretic, 2002; Nathan, 2003). The possible dominant role of NO as a murine immune effector (Boshoff & Barry, 2005) suggests that attenuation of the *cobU* mutant *in vivo* might result from an inability to modulate the interaction with the host immune system. Evidence in support of this theory is scant, although it is notable that maximal *cobK* expression is observed at 21 days *in vivo* (Talaat *et al.*, 2004) and coincides with immune activation and, further, that several genes of the putative *M. tuberculosis* AdoCbl biosynthetic pathway are associated with

survival in immunocompetent mice (Sassetti & Rubin, 2003; Talaat *et al.*, 2004). In *E. coli*, MetE is inactivated by oxidised glutathione (GSSG) during periods of oxidative stress (Hondorp & Matthews, 2004). Furthermore, GSSG-mediated inactivation is cysteine residue-specific and might represent a deliberate and reversible mechanism to modulate MetE activity while preventing damage to the active site. In light of the postulated role of cobalamin in mycobacterial defence, it is tempting to speculate that the upregulation of vitamin B_{12} production upon immune activation - to enable MetH to assume dominant methionine synthase activity - could simultaneously increase cobalamin export and so augment immune tolerance. Significantly, it has been shown that expression of *metE* is downregulated in activated macrophages (Schnappinger *et al.*, 2003), whereas genes involved in sulphate assimilation are induced in *M. tuberculosis* in response to oxidative stress (Pinto *et al.*, 2004). Of course, such speculation must be tempered by the possible NO-mediated inactivation of the B_{12}-dependent methionine synthase itself (Danishpajooh *et al.*, 2001). Furthermore, studies so far have not identified *metH* among those genes significantly upregulated *in vitro* in macrophage models or during *in vivo* infection (Schnappinger *et al.*, 2003; Talaat *et al.*, 2004); however, it is possible that the higher efficiency of the B_{12}-dependent isozyme (Greene, 1996; Bandarian & Matthews, 2001) might invalidate the need for increased transcription and that maintenance of homeostasis could, instead, rely on increased vitamin B_{12} biosynthesis.

Future studies

Possible cross-regulation of AdoCbl and methionine biosynthetic pathways was suggested by the data presented in this study (Table 5.2), and demands further investigation. Significantly, preliminary follow-up studies utilising transcriptional analysis by microarray have pointed to the responsiveness of *M. tuberculosis* to available CNCbl. However, direct quantification of the expression levels of several sentinel AdoCbl biosynthetic genes, as well as the B_{12}-dependent and B_{12}-indepedent methionine synthase isozymes in wild-type and mutant strains in response to a variety of conditions should provide a more definitive view of the possible interaction of different metabolic pathways. In addition, expression analyses might enable characterisation of the conditions under which MetE and MetH operate, perhaps explaining the apparent inability of the B_{12}-dependent isozyme to fully complement abrogated B_{12}-independent metionine synthase activity in the Δ*metE* mutant. In this regard, genetic manipulation of the proposed alternative methionine biosynthetic or salvage pathways – suggested by the presence of the *mmuM*-encoded YagD homologue as well as IlvE – in the various *M. tuberculosis* and *M. smegmatis* mutant strains should prove enlightening. Finally, analysis at the mRNA

level of the apparent CNCbl “shock” phenotype might yield novel insights into the regulation of AdoCbl, methionine, and sulphur metabolism in *M. tuberculosis*, as well as the specific mechanisms adopted by mutant strains in adapting to biosynthetic deficiencies.

Appendices

Appendix 1: List of abbreviations

Abbreviation	Abbreviated term/phrase
ADC	Albumin-dextrose complex supplement for Middlebrook 7H9
AdoCbi	Adenosylcobinamide
AdoCbl	Adenosylcobalamin
Amp	Ampicillin
aph	Gene encoding aminoglycoside phosphotransferase
ATCC	American Type Culture Collection
BCG	bacille Calmette-Guérin
bla	Gene conferring resistance to ampicillin
bp	base pair(s)
BSA	Bovine serum albumin
CFU	Colony forming unit
CNCbl	Cyanocobalamin (vitamin B_{12})
DCO	Double cross over
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOTS	Directly observed therapy, short-course
DSB	Double strand break
EP	Error-prone
GASP	Growth advantage in stationary phase
HIV	Human immunodeficiency virus
Hyg	Hygromycin B
hyg	Gene conferring resistance to hygromycinB
IFN	Interferon
IL	Interleukin
kb	Kilo base pair(s)
Km	Kanamycin
K_m	Michaelis constant
LA	Luria-Bertani agar
lacZ	Gene encoding β-galactosidase
LB	Luria-Bertani broth

LJ	Lowenstein-Jensen
MIC	Minimum inhibitory concentration
MMR	Mismatch repair
MTZ	Metronidazole
NO	Nitric oxide
NVB	Novobiocin
OADC	ADC with oleic acid, supplement for Middlebrook 7H10
OD_{600}	Optical density at 600 nanometre wavelength
OFX	Ofloxacin
ONPG	2-nitrophenyl β-D-galactopyranoside
ORF	Open reading frame
PCR	Polymerase chain reaction
^{R}	Resistant/resistance
RBS	Ribosome binding site
Rif	Rifampicin
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RT	Reverse transcription/transcriptase
^{S}	Sensitive/sensistivity
S.A.	Specific actvity
sacB	Gene encoding levansucrase
SCO	Single cross over
SDS	Sodium dodecylsulphate
ss	Single stranded
Suc	Sucrose
TB	Tuberculosis
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
Tween	Polyoxyethylene sorbitan monooleate
U	Units
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 litre with deionised water, and sterilised by autoclaving at 121^{o}C for 20 minutes, unless otherwise stated.

2TY broth

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

Luria-Bertani broth (LB)

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

Luria-Bertani agar (LA)

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

Middlebrook-ADC (7H9M-ADC)

4.7 g Middlebrook 7H9 broth base; 2 ml glycerol.

100ml ADC supplement added after autoclaving.

Middlebrook-OADC plates (7H10M-OADC)

19 g Middlebrook 7H10 agar powder; 2ml glycerol.

100 ml OADC supplement added after autoclaving.

Psi broth

5g yeast extract; 20g tryptone; 5g magnesium sulphate

pH 7.6 with potassium hydroxide

Sauton's minimal media

4 g asparagine; 0.5 g magnesium sulphate; 2 g citric acid; 0.5 g potassium dihydrogen orthophosphate; 0.05 g ammonium ferric citrate; 48 ml glycerol

pH 7.2; sterilised by filtration.

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