The influence of growth conditions on the susceptibility of *Mycobacterium tuberculosis* to antibiotics

By

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A thesis submitted to University College London in fulfilment of the requirement for the degree of Doctor of Philosophy

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Declaration

I, Alice Ann Neville Marriott confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: 

Abstract

Mycobacterium tuberculosis is the causative agent of tuberculosis disease which is a global health emergency. Lengthy treatment is required due to the intrinsic resistance of *M. tuberculosis* to most classes of antibiotics and continued survival of drug tolerant persisting populations. This tolerance and persistence may be because of pre-existing populations of bacteria recalcitrant to treatment through phenotypic mechanisms such as slow growth or non-replicating persistence; or through genotypic alterations resulting in resistance phenotypes. In order to improve treatment, effective *in vitro* modelling of these populations and factors affecting efficacy is essential.

Cell density, growth rate and growth phase were all found to affect the efficacy of isoniazid and rifampicin in this study. *M. tuberculosis* grown at a high cell density was shown to be less susceptible to antibiotic exposure, this was particularly true for rifampicin. Growth phase was also considered, in the host, a bacterium is likely to encounter nutrient depletion and enter stationary and non-replicating phases. Isoniazid and rifampicin were both less effective the longer *M. tuberculosis* was in culture. This work demonstrated the need for consideration of multiple factors when designing and modelling treatment of tuberculosis.

Using continuous culture *M. tuberculosis* was grown at a fast (23.1 hours) and slow (69.3) doubling times, with and without isoniazid exposure aiming to elucidate the different responses these populations may have to antibiotic. Genotypic analyses showed that slow growing bacilli developed resistance to isoniazid through mutations specifically in *katG* codon Ser^{315} which are present in approximately 50–90% of all isoniazid-resistant clinical isolates. The fast growing bacilli persisted as a mixed population with *katG* mutations distributed throughout the gene. Phenotypic differences were detected between the populations at the two growth rates including expression of efflux mechanisms and the involvement of antisense RNA/small RNA in the regulation of a drug-tolerant phenotype.

Acknowledgements

I would like to sincerely thank both of my supervisors Dr Joanna Bacon at PHE, Porton Down and Professor Tim McHugh at University College London for all their help, support, patience and guidance during my research and compilation of this thesis. Special thanks to my principal supervisor Dr Joanna Bacon who through the years has provided help whenever it was needed and gone out of her way to support my studies and decisions which I very much appreciate.

Thanks to Dr Cath Arnold at PHE, Colindale who helped with the pyrosequencing and who made me very welcome by being so friendly and supportive and the staff within her team for including me in the lab.

I would like to acknowledge the Health Protection Agency (now Public Health England) for providing funding to make my studies possible.

I would like to thank a number of colleagues within the TB Research Group at Porton down for their scientific contributions to this research. Dr Rose Jeeves for her processing of the RNA samples and analysis, I am extremely grateful for the permission to use this data in chapter 6 and for your help and guidance. Dr Irene Freire-Martin for her permission to use the sequencing work that was carried out. Mr Jon Allnutt for the technical assistance with running batch and continuous cultures of *M*. *tuberculosis*, gardening tips, company in the lab and for all the snippets of valuable information I hope to have assimilated. I am very grateful for being able to absorb some of your years of experience with fermenters which I have no doubt has helped me immensely in my further career.

Thanks of course to 'the syndicate' who along with scientific guidance throughout my years at Porton Down have kept me sane, allowed me to talk and become firm friends. Special mention to Sam, for 2 years of amazing company in the CL3 and for all the banter in and out of Porton and to Yper Hall, partner fighting crime and true inspiration, having saved me from 'burnout' a number of times.

I am also very grateful to my current colleagues at the University of Bath for support, friendship and guidance through the tricky time of being a post-doc and writing up.

I would like to thank friends and family outside of science. Thank you to my parents for always supporting me no matter what I choose to do, for being so proud of anything I do and for sacrificing so much and working so hard so that I could have the opportunities I hope to have taken advantage of. I am grateful to mine and Dan's parents and family that have supported me,

special thanks to Marie and my mum for all the times you have put your lives on hold to provide childcare in order for me to write.

Finally and certainly not least I would like to thank Dan and Ava. Thank you for being so supportive Dan, allowing me to spend time with Ava but also continue my studies whilst you were the main care provider for many weekends, allowing me to complete this work. I hope that in years to come, Ava will understand what this thesis means and be proud of me despite all the weekend working.

I would like to dedicate this thesis to Kim Hatch who sadly passed away. She was an accomplished scientist, an amazing mother and a kind, considerate friend. She is a continuing inspiration for me and much of this work would not have been possible without her scientific and personal input. I hope one day to be as dedicated and highly regarded as she was to her work, colleagues and family.

Publications from this research

Jeeves, R. E.*, **Marriott, A. A. N**.*, Pullan, S. T., Hatch, K. A., Allnutt, J. C., Freire-Martin, I., Hendon-Dunn, C., Watson, R., Whitney, A.A., Tyler, R.A., Arnold, C., Marsh, P.D., McHugh, T.D., Bacon, J., (2015), '*Mycobacterium tuberculosis* Is Resistant to Isoniazid at a Slow Growth Rate by Single Nucleotide Polymorphisms in *katG* Codon Ser315.', *PloS one*., **10**(9): e0138253.

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Abbreviations

μ_{max}	Maximum growth rate
ACP	Enoyl-acyl carrier protein
AIDs	Acquired Immunodeficiency Syndrome
asRNA	Antisense RNA
CDC	Centers for Disease Control
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CO_2	Carbon dioxide
DAVID	Database for Annotation, Visualization and Integrated Discovery
DIM	phthiocerol dimycocerosate
DNA	Deoxyribonucleic acid
DOT	Dissolved oxygen tension
DOTS	Directly Observed Treatment Shortcourse
ECM	Extracellular material
FASII	Fatty acid synthase type II system
FG	Fast growth rate culture
GTC	Guanidine thiocyanate
HFS	Hollow fibre system
HIV	Human Immunodeficiency Virus
HPA	Health Protection Agency
IGRA	Interferon gamma release assay
INH	Isoniazid
IS	Insertion sequence
KARs	Ketoacyl reductase
LAM	liparabinomannan
MBC	Minimum bactericidal concentration
MDR	Multi-drug resistant
MGIT	Mycobacterial growth inhibition assay
MGT	Mean generation time
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
MSCIII	Microbiological safety cabinet class III

NAD	Nicotinamide adenine dinucleotide
NRP	Non-replicating persistence
OD	Optical density
ORF	Open reading frame
PAS	Para-amino salicylic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	pharmacodynamic
PHE	Public Health England
PK	pharmacokinetic
QRDR	Quinolone resistance determining region
REMA	Resazurin microtitre assay
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RRDR	Rifampicin resistance determining region
rRNA	Ribosomal ribonucleic acid
RTq PCR	Real time quantitative polymerase chain reaction
SG	Slow growth rate culture
SNP	Single nucleotide polymorphism
TA	Toxin-antitoxin
TB	Tuberculosis
TE Buffer	Tris-EDTA
TNF	Tumour necrosis factor
TST	Tuberculin skin test
TVC	Total viable count
XDR	Extensively drug resistant

1. Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis diseases and is a global health emergency. A treatment is available but consists of at least four antibiotics and is lengthy due to the intrinsic resistance of *M. tuberculosis* to most classes of antibiotic, resistance is further compounded due to non-compliance. Populations of bacteria may be recalcitrant to treatment through phenotypic mechanisms such as slow growth or genotypic alterations resulting in resistance. *In vitro* modelling of these populations and factors affecting efficacy of this treatment is essential in order to improve the current treatment regime and evaluate novel antibiotics.

1.1 Introduction to *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is an aerobic, pathogenic, slow growing bacilli. Humans are the only reservoir for *M. tuberculosis* and Robert Koch discovered in 1882 it is the causative agent of tuberculosis (TB). *M. tuberculosi*s infection usually affects the lungs leading to pulmonary tuberculosis and has been described throughout history, having been referenced by Hippocrates as 'phithsis'. The symptoms of active tuberculosis disease are a persistent cough, fever, weight loss, heavy night sweats, fatigue and loss of appetite, TB Alert recommends if a person has 3 or more of these symptoms that they should visit their doctor (TBAlert, 2010). Among the infected individuals, most do not develop active disease; their immune systems fail to eradicate the bacteria, which remain in a dormant state in granulomas for life (Józefowski *et al.*, 2008) . Latent tuberculosis infection is defined as infection with *M. tuberculosis* as manifested by a positive immunological test result but without evidence of active disease including symptoms, progressive radiographic changes or microbiological evidence of replicating organisms (Lobue and Menzies, 2010). This latent phase of tuberculosis can develop into active disease at any point and around 10% of people with a dormant tuberculosis infection develop active disease and become sources of new infections, infecting on average 10-15 people per year. The progression of disease varies according to numerous factors (figure 1.1) and the importance of an intact immune response in eradicating the infection cannot be ignored, whether this is through age or due to co-infection with other agents.

Tuberculosis was the principal cause of death by the end of the 19th and beginning of the 20th century throughout the world (Ducati *et al.*, 2006). In the twentieth century chemotherapy for tuberculosis became available, beginning with the discovery of Para-amino salicylic acid (PAS) and thiosemicarbazone in 1943 leading to a treatment for TB; although these agents turned out to be bacteriostatic rather than bactericidal. The year 1944 resulted in the use of streptomycin

which was the first antibiotic and first bactericidal agent effective against *Mycobacterium tuberculosis*. These advances were swiftly followed by the introduction of isoniazid and the rifamycins in the 1950s leading to the closure of most of the sanatoria used for the treatment of tuberculosis in the past. In the late 1970s and early 1980s, tuberculosis rates were declining and became regarded by many as a conquered disease (Harries, 2008).

Today tuberculosis is a global health emergency, there were 6 million new TB cases notified to national laboratories in 2014 although it was estimated 9.6 million fell ill with the disease and 1.5 million people died as a result of TB disease; more than 2 billion people, equal to one-third of the world's population, are infected with TB bacilli and the lifetime risk of developing active tuberculosis once infected is around 10% (WHO, 2015). Currently, at least one-third of the 35.3 million people living with HIV worldwide are infected with latent tuberculosis and persons co-infected with tuberculosis and HIV are 29.6 times more likely to develop active tuberculosis disease than persons without HIV. Tuberculosis Is the most common presenting illness and leading cause of death in persons living with HIV (WHO, 2013) and HIV co-infection is the most powerful known risk factor of progression of *M. tuberculosis* to active disease and increases the risk of latent TB reactivation 20-fold (Pawlowski *et al.*, 2012). Cell mediated immunity, including $CD4^+$ T-cells, has been shown to be essential for control of *M. tuberculosis* infection on humans and mice after experimental infection (Cooper, 2009) and along with tumour necrosis factor (TNF) have been shown to be important in maintaining granuloma organisation. Depletion of $CD4^+$ cells is a main feature of acquired immune deficiency syndrome (AIDs) and granuloma formation may fail in individuals with such a compromised immune system (Diedrich and Flynn, 2011), containment of infection is therefore reduced leading to an increased risk of extrapulmonary TB with diffuse lesions across multiple organs.

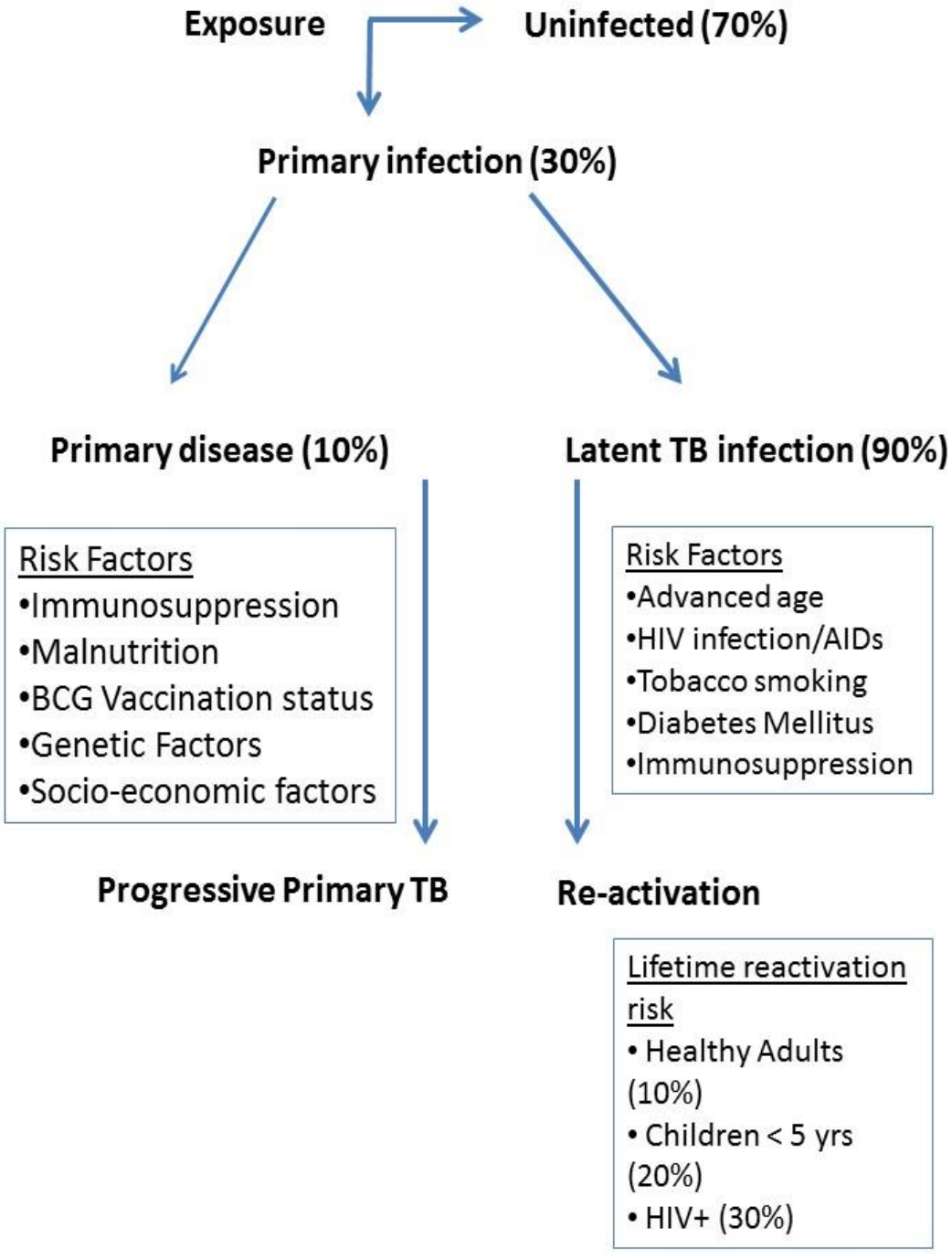


Figure 1.1 Progression of *M. tuberculosis* infection. Adapted from (Sharma and Mohan, 2013)

1.2 Treatment of Tuberculosis

1.2.1 Current Regimes

Mortality rates from tuberculosis infection significantly declined from the early to mid-20th century but funding for research dwindled and between 1970 and 1990, drug and vaccine developments slowed. With the onset of an AIDs pandemic and resistant strains emerging the rates of tuberculosis began to rise and with it, a renewed interest in research and drug development (Fogel, 2015). Today the standard chemotherapy for tuberculosis infection consists of at least four drugs prescribed for six to nine months (Vilcheze *et al.*, 2007). In the first two months of treatment isoniazid, rifampicin, pyrazinamide and ethambutol are prescribed followed by a further 4 months of just isoniazid and rifampicin. This regime has not changed for decades although the Directly Observed Treatment Short-Course (DOTS) was introduced in 1998 to address multi-drug resistant tuberculosis (Comas and Gagneux, 2009), the treatment algorithm set by the CDC is shown in figure 1.2 demonstrating the use of a combination of antibiotics for at least 6 months.

Currently the standard course of treatment for latent tuberculosis infection is 9 months of isoniazid or rifampicin mono treatment for 4 months when the strain is shown to be resistant to isoniazid. A course of isoniazid and rifampicin combined for 3 months has been shown to be superior to the 9 month isoniazid mono therapy regime with excellent treatment compliance (Spyridis *et al.*, 2007). Chemotherapy has two aims, to prevent the emergence of drug resistance and to shorten the period of treatment by killing the bacterial population quickly (D. A. Mitchison, 2000), this is complicated in *M. tuberculosis* due to its physiology the *in vivo* environments in which it can survive, it has been speculated that at least four significant subpopulations of bacteria exist.

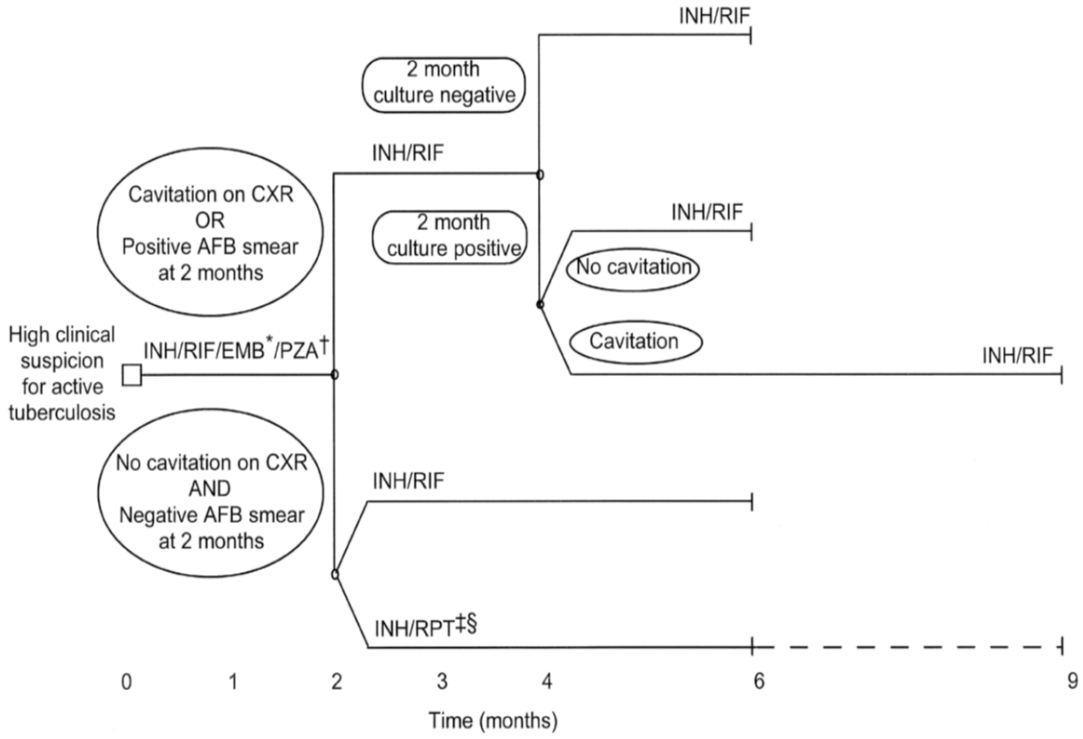


Figure 1.2 The treatment algorithm for active tuberculosis used by the Centers for Disease Control and Prevention (CDC) (Centers for Disease Control and Prevention, 2003). CXR – Chest X-Ray, AFB – Acid Fast Bacilli, INH – Isoniazid, RIF – rifampicin, PZA – pyrazinamide.

These sub-populations include active growers that may be killed by isoniazid, those with sporadic metabolic bursts that could be killed by rifampicin, a population with low metabolic activity that is considered likely to experience acidic surroundings and hypoxia that may be susceptible to pyrazinamide and finally dormant bacilli that are not killed by any current agents (Dover and Coxon, 2011).

1.2.1.1 Isoniazid

Isoniazid (INH) enters the cell through passive diffusion and kills only dividing bacteria, its action is bacteriostatic for the first 24 hours after which the action becomes bactericidal (Jindani *et al.*, 2003). In the same time frame the mycobacterium cells lose their acid fastness and morphological changes such as loss of internal structure, appearance of surface wrinkles and bulging occur (Vilcheze *et al.*, 2007)

Isoniazid is a prodrug that is activated by the catalase-peroxidase enzyme (KatG) encoded by the *katG* gene (Zhang *et al.*, 1992) that generates a range of highly reactive species which then attack multiple targets in *M. tuberculosis.* The reactive species produced by KatG mediated isoniazid activation include both reactive oxygen species such as superoxide, peroxide and hydroxyl

radical, nitric oxide and reactive oxygen species such as isonicotinic-acyl radical or anion and cation electrophilic species. The primary target of isoniazid inhibition is thought to be the InhA enzyme (enoyl-acyl carrier protein reductase), involved in elongation of fatty acids in mycolic acid synthesis. The active species (isonicotinic-acyl radical or anion) derived from KatG-mediated INH activation reacts with NAD(H) to form an INH-NAD adduct and then attacks InhA (Zhang and Yew, 2009)(figure 1.3)

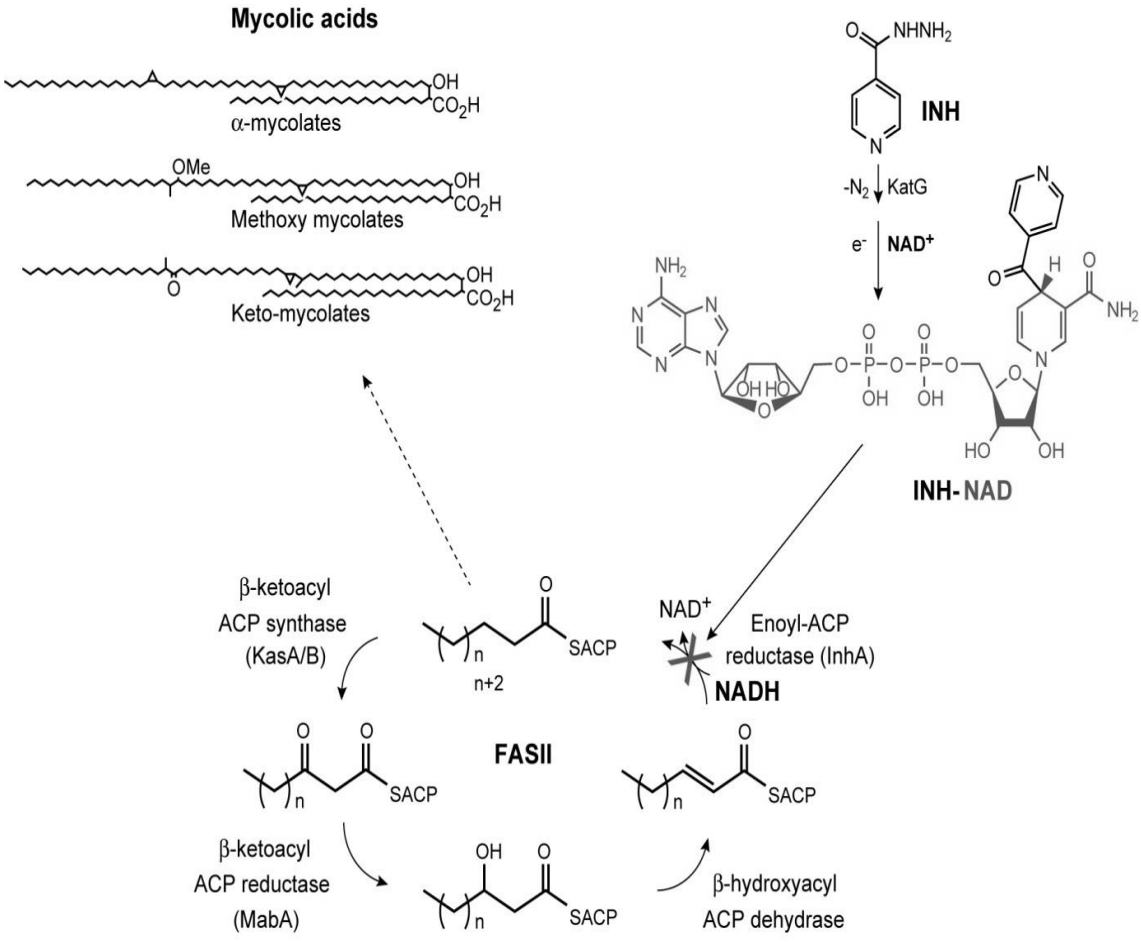


Figure 1.3. Mechanism of action of INH in *M. tuberculosis*. INH is activated by KatG to form the INH-NAD adduct. The adduct inhibits InhA, the enoyl-ACP reductase of the fatty acid synthase type II system (FASII), which synthesizes mycolic acids (representation of three mycolic acid classes). This inhibition results in the inhibition of mycolic acid biosynthesis and ultimately cell death. (Vilcheze *et al.*, 2007)

Isoniazid is most active within the first 2 days of treatment when treating active disease, it appears that isoniazid works most effectively against 'fast growing' actively dividing populations. When the disease being treated was in a more 'chronic' stage with a higher proportion of slow

growing bacilli, the early bactericidal activity of isoniazid was reduced and the kill prolonged (Mitchison 2000). This decline in isoniazid activity after 2 days of exposure was presumed to be due to the depletion of the exponentially growing population which isoniazid is most active against. Gumbo *et al* tested this hypothesis and found that the cessation of isoniazid activity was due to emergence of resistance to isoniazid not depletion of the exponentially growing population. In the guinea pig model of tuberculosis infection it was hypothesised that the biphasic killing and decline in early bactericidal activity was due to the emergence of drug tolerant persisters and not genotypically mutated bacilli (Ahmad *et al.*, 2009)

1.2.1.2 Rifampicin

Rifampicin was introduced as a treatment for tuberculosis in 1968 and greatly shortened the duration of the treatment regimen. Rifampicin diffuses freely into tissues, living cells, and bacteria, making it extremely effective against intracellular pathogens like *M. tuberculosis.*

The bactericidal activity of rifampicin stems from its interaction with the β-subunit of the RNA polymerase enzyme from prokaryotic organisms to inhibit transcription. This interaction physically blocks the elongation of the RNA chain (Hartmann *et al.*, 1967)

Rifampicin is used alongside isoniazid as a bactericidal treatment and is a key anti-mycobacterial in the regimen due to its activity against both growing and stationary phase bacilli with low metabolic activity, the latter activity is related to its high sterilising activity *in vivo* correlating with its ability to shorten treatment from 12-18 months to 6-9 months (Zhang and Yew 2009). The activity of rifampicin is concentration dependent and is strongly time dependent, the early bactericidal activity is less than isoniazid but rifampicin is responsible for sterilisation throughout the course of treatment (de Steenwinkel *et al* 2010). As discussed, the activity of isoniazid decreases after the first two days of treatment hypothesised to be due to the emergence of phenotypic and genotypic isoniazid resistance. The inclusion of rifampicin, with its continuing sterilising activity, is to prevent the emergence of resistance by killing the sub-populations of isoniazid mutants arising after the initial bactericidal response.

1.2.1.3 Pyrazinamide and Ethambutol

The activity of pyrazinamide, unlike any other anti-tuberculosis drug, is low against rapidly growing bacilli but increases as the metabolic activity of the bacilli decreases (Mitchison and Coates 2004). Pyrazinamide has very good sterilizing activity *in vivo* but is not active against *M. tuberculosis* under normal culture conditions at close to neutral pH. The pH has to be acidic (Zhang and Mitchison, 2003) therefore making studies investigating the efficacy *in vitro* difficult. Pyrazinamide is converted to the active form, pyrazinoic acid and it was widely accepted that

both pyrazinamide and pyrazinoic acid did not have a specific target but rather that cellular acidification causes inhibition of major processes. Recent studies have shown that both pyrazinamide and pyrazinoic acid have several different targets interfering with diverse biochemical pathways, especially in the NAD^+ and energy metabolism (Stehr *et al.*, 2015) and inhibition of the recycling of stalled ribosomes (Shi *et al.*, 2011)

Ethambutol is a drug that was initially reported to have activity against *M. tuberculosis* in 1961; similar to isoniazid, ethambutol primarily kills actively multiplying bacilli but has very poor sterilising activity. The main target of ethambutol appears to be the arabinogalactan biosynthesis pathway through inhibition of cell wall arabinan polymerisation (Mikusova *et al.*, 1995) and has also been reported to inhibit other pathways which include RNA metabolism, transfer of mycolic acids into the cell wall and phospholipid synthesis (Kolyva, Anastasia and Karakousis, 2012). The bacteriostatic nature of the activity of ethambutol means that it is mainly included within the treatment regimen to prevent emergence of resistance to other drugs.

1.2.1.4 Drug development

The current regime is far from ideal, with lengthy treatment times, non-compliance and a range or side effects and antagonism with therapies for other diseases. The TB drug development pipeline is active with a number of strategies including re-evaluation of existing TB drugs to optimize their utility; repurposing of drugs registered for non TB indications as components of TB drug combinations; development of improved analogues of compounds or drugs with some known but limited value for TB; and development of novel chemical entities with new modes of action against TB (Mdluli *et al.*, 2015). A new regimen would ideally show rapid bactericidal activity and achieve stable cure through potent sterilisation in a shorter time frame than the typical 6 months of the current situation. The differing sub-populations present in an *M. tuberculosis* infection, due to differing replication rates, should all be killed. Novel modes of action may need to be incorporated to ensure effectiveness against strains that are currently resistant to existing drugs. It is also important that any new antibiotics introduced into the regime be compatible with anti-HIV agents and remain low cost to be accessible to all high burden countries.

Currently the quinolone class of antibiotics is present in numerous clinical trials, moxifloxacin and gatifloxacin are being investigated in Phase III clinical trials as part of a 4 month regime for drug sensitive tuberculosis. Ciprofloxacin has demonstrated early bactericidal activity and is used in the treatment of multi-drug resistant tuberculosis (MDR-TB) however moxifloxacin has a superior activity against pulmonary TB and is less likely to select for resistance, leading to further evaluation in clinical studies (O'Sullivan, Hinds, *et al.*, 2008; Wiles *et al.*, 2010) The

quinolones target DNA gyrase in *M. tuberculosis,* an enzyme required for important cellular processes such as replication and transcription*,* resulting in lethal double stranded DNA breaks. Bedaquiline along with a nitroimadazole, delamanid are being evaluated as additions to background therapy for MDR-TB. Both these classes of drugs are also in trials with pyrazinamide against sensitive and MDR-TB tuberculosis (Mdluli *et al* 2015). There has also been interest in the rifamycin class of antibiotics of which rifampicin is a member. Currently there is a lack of compounds entering phase I of the clinical trial process and several target areas have been identified including iron acquisition and storage, cholesterol metabolism, central carbon metabolism and reactive oxygen species (ROS) generation. The area of ROS is of particular interest when considering the persister populations in disease, it has been reported that a small change in dissolved oxygen can affect killing of bacterial persisters. The bacteria can be phenotypically resistant to most antibiotics but still sensitive to high quantities of radicals which is critical within the hypoxic core of the granuloma environment (Grant *et al* 2012)

1.2.1.5 Focus on Isoniazid and Rifampicin

Isoniazid and rifampicin are the two 'frontline' antibiotics against *M. tuberculosis*, present in treatment regimens for at least 6 months. This is due to the fact that isoniazid reduces the bacterial load by around 95% over the first 2 days of treatment, essential for decreasing the risk of transmission of tuberculosis. Isoniazid has excellent early bactericidal activity but its effectiveness decreases after this initial period. Rifampicin then becomes important and is thought to be active against sporadically active *M. tuberculosis*, an action crucial for preventing relapses; the continued presence of isoniazid serves to limit the emergence of rifampicin resistance (Dover and Coxon 2011; Mitchison 2000).

Pyrazinamide is important within the regimen as it has an ability to kill a subset of bacteria not killed by other drugs, those bacteria that are subject to a hypoxic and possibly acidic environment. The inclusion of pyrazinamide has shortened the length of treatment from 9 months to the current 6 months. There is no denying the importance of this drug but it is difficult to model *in vitro* due to the acidic conditions necessary in order for it to become active.

The high bactericidal activity of isoniazid and rifampicin and the subsequent resistance has made these two drugs particularly important, in order to further understand how to treat tuberculosis either with novel therapies or existing ones these two antimicrobials need to be understood. This study focusses on isoniazid and rifampicin for that reason and the difficulties in working with pyrazinamide.

The differing modes of action between isoniazid and rifampicin are highlighted in sections 1.2.1.1 and 1.2.1.2.

1.3 Physiology of *M. tuberculosis* and intrinsic antibiotic resistance

The current inefficient treatment regime available reflects the intrinsic ability of *M. tuberculosis* to resist most common antibiotics. The intrinsic properties of *M*. *tuberculosis* leads to drug tolerance which is the ability to evade the bactericidal activity of antimicrobial drugs with the hallmark of tolerance being bacteriostasis with reduced and delayed bactericidal activity (Wallis *et al.*, 1999). The persistence of tuberculous disease and likelihood of relapse may in part be determined by drug tolerance. Drug tolerance in *M. tuberculosis* has traditionally been attributed to the impermeability of the cell wall however the situation is more complex.

1.3.1 The Mycobacterial cell wall

The mycobacterial cell wall is unique and its low permeability cannot be ignored and undoubtedly contributes to the intrinsic resistance. The exterior of the cell wall is comprised of a network of glycopeptidolipids and trehalose dimycolates.

The cell wall comprises of peptidoglycan as in many bacterial species but with a linked arabinogalactan layer and this arabinogalactan layer could serve to limit entry to hydrophobic molecules. A bilayer of lipophilic mycolic acid is covalently linked to the arabinogalactan layer, mycolic acids are C_{60}-C_{90} long chain carboxylic acids and this layer limits the penetration of hydrophilic molecules. Beyond the hydrophobicity of the mycolic acids, their unusual length and characteristic unsaturations of the alkyl chain also contribute to the permeability barrier along with unusual cross-linking in the peptidoglycan layer. The different types of unsaturation, which may vary across species, likely contribute to the higher or lower permeability of the cell walls of different mycobacteria (Wivagg *et al.*, 2014).

The cell wall cannot be entirely impermeable as nutrients need to enter the cell and waste products be excreted meaning there must be other mechanisms being used. Hydrophobic compounds penetrate by dissolving into and through the lipophilic cell wall but are slowed by the low fluidity and unusual thickness of the unique cell wall (Sarathy *et al.*, 2012). It has been hypothesised that this leaves time for activation of resistance related genes (Nguyen and Thompson, 2006)

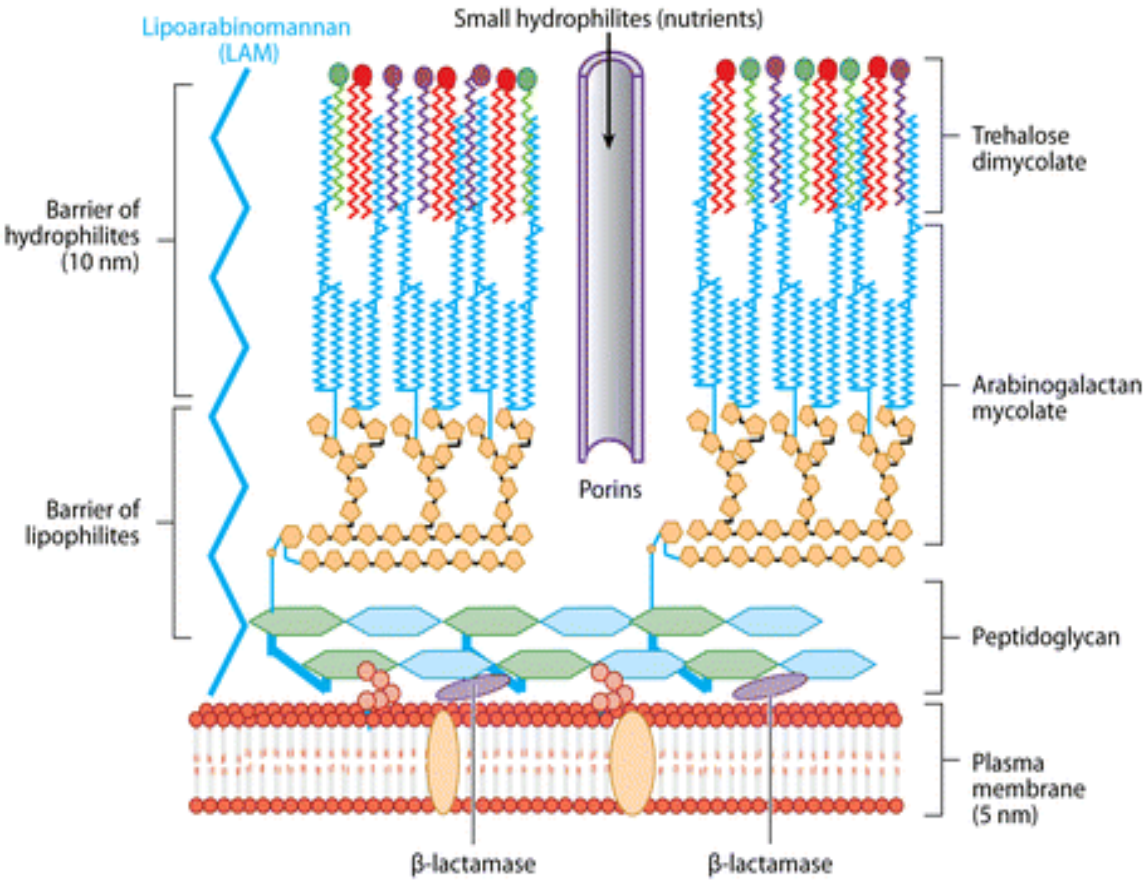


Figure 1.4 Schematic structure of mycobacterial cell wall, and the synergy of cell wall exclusion and internal defence mechanisms in controlling intrinsic antibiotic resistance in mycobacteria. Intercalation of hydrophilic arabinogalactan and hydrophobic mycolate containing layers creates an extremely impermeable envelope for antibiotic penetration. Small molecules and nutrients are transported through porin channels that are deposited through these layers. Taken from (Nguyen and Pieters, 2009)

Porin like channels are present in the mycobacterial cell wall for nutrient uptake and waste excretion, these channels have emerged convergently across mycobacterial species, specific to the nature of the organism. These porins have also been shown to be important in transport of antibiotics. In *Mycobacterium tuberculosis*, porins limit the growth rate and therefore antibiotic transport, the transport of hydrophilic compounds is also largely facilitated by porins (Sarathy *et al.*, 2012). A porin encoding gene from *M. smegmatis*, *mspA*, has been shown to increase sensitivity to isoniazid, ethambutol and β-lactams when expressed in *M. tuberculosis* (Stephan *et al* 2004).

Along with the unique structure of the cell wall, important enzymes are also present, *M. tuberculosis* has strong constitutive β-lactamase activity which combined with the highly

impermeable cell wall results in a high resistance to this class of widely used antibiotics. It has been shown however that if meropenem is combined with the β-lactamase inhibitor clavalanate some activity is evident (Wivagg et al 2014), opening up this class of antibiotics would be particular useful in treating drug resistant *M. tuberculosis*. There is also evidence of efflux pumps which use β-lactams as their substrate in *M. tuberculosis* (Almeida Da Silva and Palomino, 2011)

1.3.2 Efflux pumps

Efflux pumps are also a widely studied mechanism for intrinsic and acquired drug resistance in both prokaryote and eukaryote species. In *M. tuberculosis* the role of efflux pumps in intrinsic resistance varies depending on antibiotic, around 30% of isoniazid resistant clinical isolates cannot be explained compared to 5% of rifampicin resistant isolates (da Silva *et al.*, 2011). Not all currently available anti-tuberculous drugs are considered as substrates of efflux mechanisms which have a narrow spectrum in *M. tuberculosis*. This substrate range is however something that can rapidly change due to point mutations, as seen in *Bacillus subtilis* (Klyachko and Neyfakh, 1998). Bioinformatic data, particularly transcriptional studies, has shown that some efflux pumps are over-expressed in the presence of drugs such as isoniazid and rifampicin (Jiang *et al.*, 2008). Simultaneous expression of multiple genes involved in efflux of isoniazid, ethambutol and streptomycin signalled probable importance in multi drug resistant tuberculosis (Gupta *et al.*, 2010) and these expression profiles have aided in the association of specific efflux pumps with their substrates .

1.3.3 Antibiotic modification

Mycobacteria are able to inactivate antibiotics through chemical modifications. The aminoglycosides are a group of antibiotics that includes streptomycin. Aminoglycosides can be modified using phosphotransferases, nucleotidyltransferases and acetyltransferases, which are present across mycobacterial species including *M. tuberculosis*. Rifampicin can also be inactivated by many actinobacteria through glycosylation, phosphorylation and ribosylation (Nguyen and Pieters, 2009). Protection against oxygen toxicity through oxidative stress in most bacteria is provided by a reducing thiol, glucathione. Actinobacteria produce mycothiol and this appears to be unique to this order of bacteria, mycothiol provides resistance to several antibiotics such as vancomycin, rifamycin, macrolides, and β-lactams, which is hypothesised to be through direct interaction (Buchmeier *et al.*, 2003; Rawat *et al.*, 2002)

Modification of antibiotic targets has been reported in *M. tuberculosis*, macrolides such as erythromycin and lincosamide antibiotics inhibit bacterial protein synthesis. *M. tuberculosis* has a methyl transferase that methylates the 23S rRNA and lowers the affinity of the antibiotic for

ribosomes (Buriánková *et al.*, 2004) causing an intrinsic resistance to these classes of antibiotic. Mimicry of an antibiotic target has been highlighted as a mechanim that has potential to lower the efficacy of the fluoroquinolone class of antibiotics, MfpA is a molecule that resembles DNA and could serve to sequester fluoroquinolones and protect mycobacterial DNA from attack. Fluoroquinolones are an entirely synthetic antibiotic and this discovery provides a striking example of how evolution has provided bacteria with a perfect adaptability to environmental pressures, including human interventions (Nguyen and Pieters, 2009).

1.3.4 Growth rate and growth phase

One of the important factors in how *M. tuberculosis* has become a formidable pathogen is the inherent slow growth rate. Doubling times of 16 hours are achievable in optimal laboratory conditions but in reality doubling times vary depending on the site and stage of infection, often being much slower (Beste *et al.*, 2009). Since virtually all antibiotics preferentially kill rapidly replicating bacteria, a slow growth rate poses a problem for treatment. When the cell wall itself contains the primary target for drug action a reduction in the relative abundance of the target material may reduce the overall susceptibility of the cells. Altering the porin availability of the cell wall through reduced growth rate can also slow down the transport of hydrophilic agents (Brown *et al.*, 1990). Slowing down replication is an effective way to induce drug tolerance against many of the antibiotics that target cell division and cell wall synthesis.

Tuberculosis has two distinct phases, an acute phase in which bacteria are actively growing and a persistent phase in which the bacteria are very slow growing or in a non-replicating state. *M. tuberculosis* can remain in this dormant state for many years leading to latent disease. The mechanism behind the ability of *M. tuberculosis* to remain in a host with a competent immune system for decades had puzzled researchers for many years, the observation in mouse models that the bacteria causing infection were replicating very slowly or not replicating at all supported the hypothesis that it is persistent non-replicating organisms that cause latent tuberculosis disease (Rees and Hart, 1961). In this state, the organism is refractory to immune clearance but primed for reactivation; treatments are largely inactive against this population of non-dividing bacteria allowing the disease to become latent. After reactivation this population becomes infectious once again. It has been shown that switching from fast to slow growth is a carefully controlled process in mycobacteria involving a unique set of genes and not simply acceleration or deceleration of the same cellular processes (Beste *et al.*, 2009)

Growth phase can also affect the efficacy of antibiotic in similar ways to the growth rate. Bacteria that are exponentially growing and actively dividing are generally the most susceptible phase to

antibiotics with cell wall and replication based targets. As populations enter stationary and then death phase the proportion of the population that is rapidly dividing or dividing at all decreases and therefore the antibiotic susceptibility decreases. *In vitro* experiments have demonstrated that the number of persister cells in a population is directly proportional to the number of stationary phase cells inoculated into the culture (Balaban *et al.*, 2004), giving some indication as to the importance of the growth phase of an organism when studying antibiotic effects. Stationary phase cells of *M. tuberculosis* have been shown to reduce the sterilising activity of isoniazid nearly 1000 fold (Wallis *et al.*, 1999) and whilst rifampicin tends to be more active than isoniazid against this population the rates of killing are slowed (Paramasivan *et al.*, 2005).

1.4 Tolerance and persistence

There is variability in patient response to therapy even when drug sensitive bacteria are isolated; some are cured quickly but in others viable organisms persist in sputum for many months. This persistence is despite drug susceptibility *in vitro* and the risk of relapse is increased in people in which sputum sterilisation is delayed (Wallis *et al.*, 1999).

Antibiotic resistance results in the need for a higher concentration of antibiotic to prevent growth; that is, the minimum inhibitory concentration (MIC) is increased. Once the higher concentration is achieved, growth of the organisms will cease and killing will begin.

Tolerant organisms show only small or no changes in MIC but upon exposure to a higher than MIC concentration of antibiotic, viability is lost only very slowly (Handwerger and Tomasz, 1985). Drug tolerant bacteria respond to an otherwise bactericidal antibiotic as though it were bacteriostatic. In the presence of antibiotic the tolerant bacteria cease growing, they do survive and can initiate replication once the antibiotic is removed (Levin and Rozen 2006). *M. tuberculosis* has an intrinsic tolerance to most available drugs, using drug degradation and modification (e.g. β-lactamases), target modification (methyl-transferases) and target mimicry (MfpA resembles DNA and could sequester fluroquinolones) along with the unique cell wall structure which functions as an effective barrier (Nguyen and Pieters 2009) as discussed in section 1.3.

1.4.1 Persistence

Persistence is a non-inherited resistance and the term ‘microbial persistence’ describes a phenomenon whereby microorganisms which are drug-susceptible when tested outside the body are nevertheless capable of surviving within the body despite intensive therapy with the appropriate antimicrobial drug (McDermott, 1958). The rate and extent of killing of bacteria

depend on the genetic constitution of the bacterium and also the physiological state (e.g. growth rate) of the cells at the time the encounter the antibiotic (Handwerger and Tomasz 1985).

Persister cells do not remain persisters indefinitely but can spontaneously switch back to the normal, non-persistent state and regain the usual sensitivity to antibiotics. Upon switching back, they appear, according to previous experimental data to be indistinguishable from normal cells (Kussell *et al.*, 2005). It is thought that persistence may be a type of bacterial 'insurance policy' against stress. In a stressful environment the population either risks proliferation and then death or the option to suppress growth and be protected, for example in the presence of isoniazid. Reversible adoption of a slowly growing or non-growing state is regarded as a general strategy for persistence, this adaptation has been shown at the single cell level in *M. tuberculosis* (Wakamoto *et al*., 2013). Intrinsic factors such as noisy expression of genes influencing cell fate under antibiotic stress such as *katG* in *M. tuberculosis* may influence persistence, along with extrinsic factors such as oxidative stress due to reactive oxygen species (Balaban, Gerdes et al. 2013)

The assumption that persisters are pre-existing in a population before antibiotic exposure is based on a single example in *E. coli* (Balaban *et al.,* 2004) where it was hypothesised that there are two types of persister cells. Type I are non-growing cells generated in stationary phase and are pre-existing, these persister cells have a negligible spontaneous switching rate from wild type to persister during exponential growth. In a batch culture it is thought that the number of persister cells is directly proportional to the number of stationary phase cells inoculated into the culture. Type II persisters do not originate from passage through stationary phase and the number of persisters is determined by the total number of cells and not by the size of the inoculums from stationary phase (Balaban *et al.*, 2004).

If this hypothesis is true in *M. tuberculosis* populations, both types of persisters will be present, the combination of type I and type II ensures a continuous generation of persisters in the wild type population during exponential phase and an increase in number after passage through stationary phase. Bacteria exit lag phase asynchronously therefore populations will also include a subset of cells that have not begun to replicate, bacteria that are in the process of repairing DNA damage do not divide whilst the repair processes are underway (Levin and Rozen, 2006). This is another source of cells that may be able to persist through stress despite being genotypically drug sensitive.

A small persister sub-population within the larger antibiotic susceptible population has been shown to have differential susceptibility to oxidative stress, and was able to be eradicated through stimulation of reactive oxygen species (Grant *et al.*, 2012). This work demonstrates that

efforts are being made to study and also combat these persisting populations, if successful, the length of treatment along with the number of antibiotics required could be reduced.

Inherent tolerance to many classes of antibiotic make *M. tuberculosis* difficult to treat which is further compounded by its ability to lay dormant in a persistent state brought about by a decrease in growth rate and changes in the environment, metabolism and cell wall. Genetic resistance to frontline and emerging treatments is also a challenge and focus of much research which complicates the development of new therapeutics and regimes.

1.5 Resistance to frontline treatments

The regime to treat tuberculosis is complex consisting of multiple antibiotics over a long time period. Most common antibiotics are ineffective against *M. tuberculosis*, due to the bacterium being naturally resistant to many antibiotics and chemotherapeutic agents. These include β-lactams, owing to the presence of hydrolytic and drug modifying enzymes such as periplasmic β-lactamases and amino glycoside acetyltransferases, drug efflux systems and a hydrophobic cell wall that acts as a barrier, making treatment more difficult (Ducati *et al*, 2006)

Unlike the situation in other bacteria where acquired drug resistance is generally mediated through horizontal transfer by mobile genetic elements, in *M. tuberculosis* acquired drug resistance is caused mainly by spontaneous mutation in chromosomal genes producing the selection of resistant strains during sub-optimal therapy (Almeida Da Silva and Palomino, 2011). Despite drug susceptibility testing being part of the protocol for tuberculosis case management, resistance is still occurring due to previous treatment, non-compliance and improper or inadequate regimens. In 2013 3.5% of new and 20.5% of previously treated tuberculosis cases were caused by MDR-TB (WHO, 2014) and extensively drug resistant tuberculosis (XDR-TB) has been reported by 100 countries, on average 9% of people with MDR-TB have XDR-TB.

In 2012 it was estimated that less than 25% of MDR-TB cases were detected worldwide (WHO 2013). Further understanding of the mechanisms behind resistance is not only key to understanding the bacterium and developing alternative regimes and treatments but also to increase detection of resistant isolates so that they can be properly managed.

1.5.1 Isoniazid

Isoniazid resistance develops at a rate of 10^{-5}-10^{-7} mutation/cell division *in vitro* (Riska *et al.*, 2000) which is more frequently than for most anti-tuberculosis drugs but has been suggested to be lower (~10^{-8} mutations/cell division) *in vivo* (Bergval *et al.*, 2009)

A decrease in or total loss of catalase/peroxidise activity as the result of *katG* mutations are the most common genetic alterations associated with isoniazid resistance and so far more than a hundred mutations in *kat*G have been reported, with missense and nonsense mutations, insertions, deletions, truncations and more rarely full gene deletion leading to mutants with a variety of MICs (Almeida Da Silva and Palomino, 2011).

The most common mutation in the *M. tuberculosis katG* gene correlating with resistance to isoniazid is a single nucleotide polymorphism (SNP) at codon 315, resulting in a coding change from the wild type serine (AGC) to threonine (ACC). This mutation greatly reduces the affinity of wild type KatG for isoniazid whilst the enzyme maintains reasonably good steady state catalytic rates. Poor binding of the drug to the enzyme limits drug activation and brings about isoniazid resistance (S. Yu *et al.*, 2003). It has been postulated that this mutation does not incur a fitness cost, unlike some other isoniazid resistance mutations, it has been shown that strains with the *katG* S315T mutation were found to be in clusters almost as frequently as susceptible strains showing no transmissibility difference (van Soolingen *et al.*, 2000). Downregulation of transcription of *kat*G expression has also been shown to be associated with resistance to isoniazid (Ando *et al.*, 2011). It has been estimated that globally, 64% of all observed phenotypic isoniazid resistance is associated with the *kat*G315 mutation and 19 % in the *inhA* promoter region (Seifert *et al.*, 2015).

InhA is an enoyl-acyl carrier protein (ACP) reductase, mycobacteria utilise the products of InhA catalysis to create mycolic acids, important components of mycobacterial cell walls (Rozwarski *et al.*, 1998) and can be seen in figure 1.3.

An open reading frame (ORF) has been found immediately upstream of *inhA* in all mycobacteria tested thus far. This ORF, a strong homologue of many 3-ketoacyl reductases (KARs) was previously designated *orf1* and later renamed *mabA,* due to its probable involvement in mycolic acid biosynthesis. Mutations that alter binding affinity of InhA to NAD(H) also ultimately result in isoniazid resistance. Overexpression of *mabA* does not confer the ability to titrate isoniazid, this rules out the possibility of MabA being a direct target *in vivo* for an active isoniazid like isonicotinic acyl NADH/NADPH. This conclusion is consistent with the fact that until now no mutations have been mapped to the *mabA* ribosome binding site (RBS) with high frequency and also in the *inhA* ORF with lower but significant frequency (Banerjee *et al.*, 1998). Mutations in the putative promoter region of *inhA* have been demonstrated to result in hyper-expression and could lead to resistance due to titration of active isoniazid (isonicotinic acyl-NADH). An *M. tuberculosis* mutant carrying a mutation at -15 (C-15T) has been proposed as inducing overexpression of *inhA*, this mutation has been shown to produce a 20-fold increase in *inhA*

mRNA levels and subsequent InhA protein levels, showing the C-15T *inhA* promoter mutation mediates enhanced transcription of *inhA* mRNA levels, resulting in isoniazid resistance .

katG and *inhA* are the only two genes that have been shown to confer greater than fivefold increased susceptibility or resistance to *M. tuberculosis* upon gene transfer (Vilcheze *et al.*, 2005) The mutation described in *katG* is recessive in nature i.e. restoration of catalase peroxidise activity and isoniazid susceptibility is achieved when replaced or complemented with the wild-type gene. This is consistent with the fact that KatG activates isoniazid. In contrast, overexpressed *inhA* alleles or alleles causing amino acid substitutions within the structural gene have been shown to confer isoniazid resistance in a dominant fashion i.e. conferring isoniazid resistance when the mutant alleles replace or complement the wild-type gene. This dominant nature is consistent with the hypothesis that *inhA* encodes the target of isoniazid (Vilcheze *et al.*, 2005).

Oxidative stress mechanisms have also been identified as an area of interest for isoniazid resistance. The OxyR regulon in members of the family *Enterobacteriaceae* and other organisms is a sophisticated oxidative stress regulatory pathway activated in response to environmental challenges (Sreevatsan, Pan, Zhang, Deretic*, et al.*, 1997). *M. tuberculosis* has numerous frameshift mutations and deletions that render it naturally inactive (Deretic *et al.*, 1995) therefore the sensitivity of *M. tuberculosis* to isoniazid is due at least partly to a mutated OxyR regulon. Expression of alkyl hydroperoxidase (AhpC) is under the control of this regulon along with KatG and it has been hypothesised that upregulation of *ahpC* could partially or entirely compensate for a defective OxyR regulon and in the case of *katG* mutants, a defective catalase hyperoxidase. Mutations in the *ahpC* promoter sequences in resistant *M. tuberculosis* may account for resistance to isoniazid in those isolates with no mutations in *katG* or the promoter region of *inhA*. Mutations in *ahpC* have been detected in isolates with *katG* mutations and it has been suggested that changes to the *ahpC* promoter are not casually involved in isoniazid resistance, rather they act as compensatory mutations occurring as a consequence of loss of catalase peroxidase activity (Sherman *et al.*, 1996). Changes in this region are rare however and not obligatory in isoniazid resistant organisms, it has been hypothesised that mutations at codon 315 of *katG* may still retain adequate residual enzyme activity and therefore do not require compensatory AhpC for increased survival. A recent study showed no single mutation in the *ahpC-oxyR* intergenic region had an individual frequency above 1.3%; rather multiple mutations (at positions -48, -39, -15, -12, -10, -9, and -6,) in the *ahpC-oxyR* intergenic region occurred at a total cumulative frequency of 5.4% among phenotypically resistant isolates and

demonstrated that inclusion of this region increased the detection capacity of isoniazid resistance in *M. tuberculosis* (Seifert *et al.*, 2015).

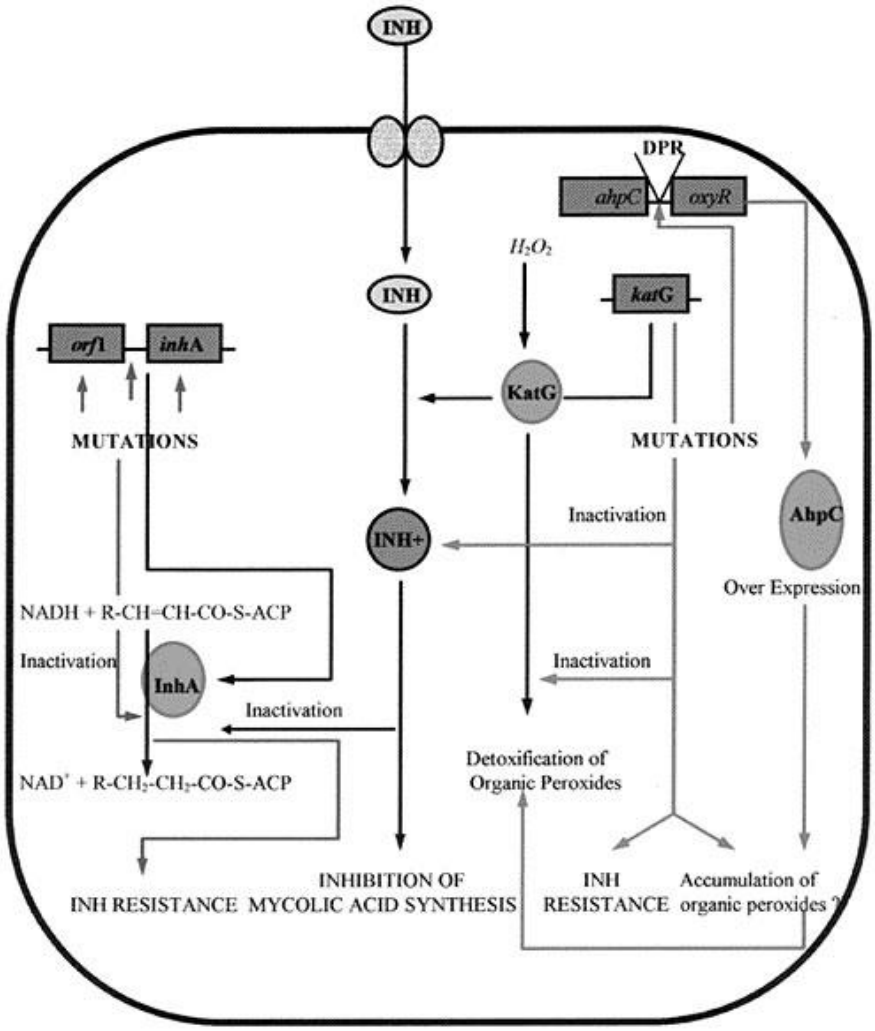


Figure 1.5 The mechanism of action of isoniazid (INH), acquisition of resistance and combating oxidative stress. DPR; divergent promoter region. Taken from (Rattan *et al.*, 1998)

1.5.2 Rifampicin, Pyrazinamide, Ethambutol and fluoroquinolone resistance

These antibiotics have been grouped together not because they are less important than isoniazid but because unlike isoniazid the resistance mutations to these antibiotics tend to be less complex. Resistance to these antibiotics tends to occur within a defined region of one gene unlike the complicated situation in isoniazid where mutations in multiple genes and regions have been shown to contribute to resistance.

In greater than 95 % of rifampicin resistant *M. tuberculosis* isolates mutations are located within an 81 base pair section of the *rpoB* gene (Seifert *et al.*, 2015) these mutations confer conformational changes leading to defective binding of the drug and consequently resistance. Mutations are dominated by single nucleotide changes, resulting in single amino acid

substitutions although in-frame deletions and insertions also occur at low frequencies. This region is known as the rifampicin resistance determining region (RRDR). Mutations at positions 531, 526 and 516 being the most frequent and causing high level resistance (Zhang and Yew, 2009) with Ser531 and His526 being documented in more than 70% of rifampicin resistant isolates (Rattan *et al.*, 1998). The molecular mechanism of resistance in 4% of the rifampicin resistant *M. tuberculosis* isolates that lack RRDR changes is currently unknown. Resistance to rifampicin can be a surrogate marker for multi-drug resistant tuberculosis as can resistance to pyrazinamide with an estimated 16.2% of tuberculosis cases and more than half of all MDR-TB cases resistant to pyrazinamide globally (Whitfield *et al.*, 2015). Pyrazinamide resistant isolates lose pyrazinamidase activity caused by mutations in *pncA*, the gene encoding pyrazinamidase. These changes include missense mutations, upstream presumed regulatory mutations, nucleotide insertions and deletions and termination mutations (Sreevatsan, Pan, Zhang, Kreiswirth, *et al.*, 1997). The worldwide burden of resistance to pyrazinamide is poorly understood and the pyrazinamide drug susceptibility test is technically challenging and rarely performed routinely. This is an important antibiotic within the regime as it is thought to be active against bacteria with low metabolic rates, an understanding and detection of resistance to this antibiotic is important to adequately treat MDR-TB cases.

As with pyrazinamide, resistance to ethambutol is not as clearly understood or reported as with isoniazid and rifampicin. Resistant isolates have been reported to have mutations on *embB*, a gene within the *embCAB* operon involved in arabinan biosynthesis, an important constituent of the mycobacterial cell wall. The ethambutol resistance determining region in *embB* is located in a cytoplasmic loop and mutations in this region are associated with around 70% of ethambutol resistant isolates of *M. tuberculosis.* Mutations in the *embC-embA* intergenic region have also been shown to contribute significantly to ethambutol resistance and inclusion of this region in susceptibility testing increased the rate of detection of ethambutol resistance.

One group of antibiotics, the quinolones, have been successfully used against *M. tuberculosis*. There are still concerns with this class of antibiotic as resistance is already occurring. A multi-drug efflux pump is thought to contribute to resistance mechanisms (Jacobs, 1999) along with a quinolone resistance determining region (QRDR). Resistance is mainly due to point mutations in the QRDR of the *gyrA* gene (codons 74 to 113), accounting for nearly 90 % of fluoroquinolone resistance in *M. tuberculosis*, these mutations lead to high level phenotypic cross resistance to fluoroquinolones in clinical isolates (Miotto *et al.*, 2015).

1.6 *In vitro* models for studying *Mycobacterium tuberculosis*

It is important to be able to model *M. tuberculosis in vitro* to enable studies of the interactions between the bacilli and compounds that may affect their growth. Being able to control environmental conditions gives the ability to study the effect on the growth, metabolism and susceptibility to antibiotics of bacteria. No single model will accurately capture all aspects of tuberculosis and in the same way there is no single model that can be used to demonstrate the efficacy of novel anti-tuberculous therapies. *In vitro* models tend to fall into two categories with respect to drug evaluation, static and dynamic. Static models evaluate *M. tuberculosis* responses to fixed concentrations of drugs and measures of response include colony-forming unit (CFU) counts or surrogates, such as optical density or time to positivity in liquid culture systems. The output from static models is most often the minimum inhibitory concentration (MIC) or less often the minimum bactericidal concentration (MBC), and even less often time-kill curves (Gumbo *et al.*, 2009). Dynamic models represent the changing concentration of antibiotic over time; the depletion of antibiotic *in vivo* is an important determinant in the microbial kill and emergence of resistance.

The hollow fibre system (HFS) has been used under a variety of conditions to study the pharmacokinetic/pharmacodynamic (PK/PD) properties of antibiotics (Gumbo *et al.*, 2009).
The HFS was originally developed as an *in vivo* device allowing tumour cells to be grown in a mouse, allowing the effect of the host on cell replication to be studied as it allows soluble factors including proteins to distribute in the host; the tumour cells remain within the system, allowing them to be retrieved and studied (Hollingshead, Alley *et al*. 1995). The two compartment kinetic model was described and aimed to simulate human pharmacokinetics and to expose bacterial cultures to changing drug concentrations, avoiding some of the limitations of conventional antibiotic testing at constant drug levels (Blaser *et al.*, 1985). This system does not require large culture volumes, allows multiple cultures to be run simultaneously, mimics the timecourse of antibiotic concentration in extravascular infections sites and can study the antibacterial effects of two antibiotics with differing properties.
Nutrients are supplied along with drugs to bacteria in controllable culture conditions and waste is taken away through a constant cycle of medium. Bacteria are said to be maintained in exponential growth due to this supply and recycling of media. As well as looking at time-kill kinetics under favourable growth conditions, this model has also been used to generate drug tolerant populations in *M. tuberculosis* due to the stress of low pH which is particularly important in studying pyrazinamide (Gumbo *et al.*, 2009)

One area of particular interest in *M. tuberculosis* research has been hypoxic environments, this condition has been modelled in order to further understand the environment of the granuloma. This is the environment in which it is thought *M. tuberculosis* enters a non-replicating persistent state and understanding not only the mechanisms behind this phase but also the effect it has on the organism, the host response and tolerance to antibiotics is of great interest. *In vitro* models of hypoxia aim to reduce the oxygen concentration available and induce microaerophilic conditions over time. Some *in vitro* models are adapted glass tubes or flasks that can be sparged with gases to induce hypoxia like stress (Deb *et al.*, 2009) (Wayne and Hayes, 1996) and these can be agitated or left to settle into an oxygen gradient (Wayne and Lin, 1982). The hollow fibre system has also been used by placing the system in an anaerobic chamber to induce hypoxia and the action of combinations of rifampicin and moxifloxacin were studied under these conditions (Drusano *et al.*, 2010). Hu *et al* developed the hundred day static culture system which involves gradual nutrient depletion and self-generated hypoxia by incubating the cultures for long time periods with no agitation (Hu *et al.*, 2006). After this prolonged period of incubation the cultures are resuscitated and exposed to antibiotic to study the phenotypic tolerance.

In order to study the effects of nutrient starvation Betts *et al* developed a model in which exponentially growing cells were washed and then re-suspended in phosphate buffered saline (PBS) in micro-titre plates or standing flasks (Betts *et al.*, 2002). Methylene blue was added to measure oxygen depletion and yielded interesting results but this is not a particularly quantitive process. This model was based on the Loebel model which was developed to investigate environmental conditions encountered by *M. tuberculosis* particularly the available of nutrients to convert to energy as early as 1933 (Loebel *et al.*, 1933).

1.6.1 Batch culture using fermenters

A batch culture in a fermenter is an advantageous tool when compared to a flask or tube culture. Using probes and a controller with defined set points parameters can be monitored and tightly controlled if necessary. Aeration can be controlled via the addition of relevant gases, either directly into the culture or into the headspace, an alternative or additive way of control is to increase or decrease the stirrer or impellor speeds to alter agitation of the culture. Dissolved oxygen is usually the measure of the amount of aeration in a culture and this is done via a pO_2 probe which measures the proportion of oxygen dissolved within the liquid. Once levels reach 0% dissolved oxygen, a redox (reduction oxidation) or oxidation reduction potential (ORP) probe can be used to measure the reducing potential of the medium, this is particularly useful in cultures running under micro-aerophilic or anaerobic conditions.

Temperature is able to be monitored or controlled via a temperature probe, the control can be via a water bath surrounding the vessel, a heat mat either underneath or wrapped around the culture or a water jacket around the vessel. Acid and base can be added as required to control pH, the set point can be altered throughout the culture to investigate the effect of pH meaning multiple pH values can be investigated within one culture experiment.

Aside from the multiple parameters that can be both monitored and controlled another advantage to using fermenter cultures is that they can be sampled over a long time course due to the larger volume. In a flask or tube experiment, multiple samples can reduce the volume in the culture that may result in all of the volume being used in a short amount of time. By removing volume, the aeration parameters can change also as these are not controlled, the headspace and therefore surface area increases allowing more oxygen transfer; as more samples are taken the aeration increases which may not be ideal when for example, studying the effects of hypoxia. Fermenters are sterile and closed systems, samples are drawn through a sampling port which introduces a far lower risk of contamination than sampling flasks or tubes. Due to the larger volume of a fermenter culture multiple samples can be taken over a long timecourse without affecting the experiment. As the volume decreases, the dissolved oxygen can be controlled via the methods mentioned previously, maintaining the desired aeration parameter despite decreasing volume.

Batch culture fermentation of *M. tuberculosis* has been used to study the effect of nutrient limitation (Bacon *et al.*, 2014; Hampshire *et al.*, 2004). By controlling air saturation it was possible to determine the effects of nutrient depletion rather than have the added variable of hypoxia induced stress. The aim was to look at NRP phase induced by nutrient depletion and these cultures were run for at least 100 days, the batch fermenter allowed multiple samples to be taken without compromising the aeration and also without contamination. This *in vitro* model has also been used to study stationary phase *M. tuberculosis* gene expression (Hampshire *et al.*, 2004) and extended to include non-replicating persistence (Bacon *et al.*, 2014).

1.6.2 Continuous culture

Batch cultures are useful tools allowing control and monitoring of physiological conditions as described in section 1.6.1. A controlled batch culture is an important *in vitro* model for studying the natural growth phases and life cycle of *Mycobacterium tuberculosis* and determining the effects of long term growth in simulated *in* vivo environments.

A limitation of batch cultures arises when specific variables such as growth rate want to be studied or controlled. Also the environment is constantly changing with time as a result of bacterial metabolism. The excretion of by-products and depletion of nutrients from the medium

result in an undefined environment, which has an unpredictable impact on the physiology and growth rate of the organism (Beste *et al.*, 2005)

Continuous culture is a method that enables control over the growth rate of an organism, once established, the influx of sterile medium from a reservoir is balanced by the efflux of spent medium, living cells and cell debris, allowing growth to occur at an equilibrium, with growth of new cells being balanced by those washed out (Hoskisson and Hobbs, 2005). The rate of growth, or doubling time, of the bacteria is equal to the rate at which the culture is being diluted, which is defined by the dilution rate, D=F/V (where D is the dilution rate, F= flow rate and V= volume of culture). The dilution rate is the flow of medium over the volume of the culture in the bioreactor. When there is a low number of cells within the vessel the growth rate of the organism can exceed the dilution rate as the nutrient is not limiting. Once the cell concentration becomes too high, the amount of cells that are removed from the vessel cannot be replenished by growth as the addition of the limiting nutrient is not sufficient to maintain this maximum growth rate, rates at or higher than the maximum growth rate result in wash out of the culture, that is dilution of the cells.

The vessel in which this takes place is named the chemostat (Figure 1.6), the system and term were described by Novick and Szilard (Novick and Szilard, 1950) and Monod (Monod, 1950) in the 1950s. Most chemostat systems in the literature used to study *M. tuberculosis* are carbon limited, in that it is the flow rate which determines the amount of carbon available to the organism with everything else available in excess, controlling growth rate. Nitrogen limited cultures have also been used to study the nitrogen regulated transcriptome of *M*. *smegmatis* using RNA sequencing analysis (Petridis *et al.*, 2015)

Using continuous culture allows control of the growth rate and therefore the mean generation time of the bacilli is also controlled, allowing study of the effect of changes in the environment on the physiology and genetics of the bacteria. A culture can be maintained in steady state (the growth of the bacteria at a constant rate and under constant environmental conditions) allowing individual parameters to be varied independently so that the direct effect of a single stimulus on the physiology and molecular genetics of an organism can be established (Bacon and Hatch, 2009).

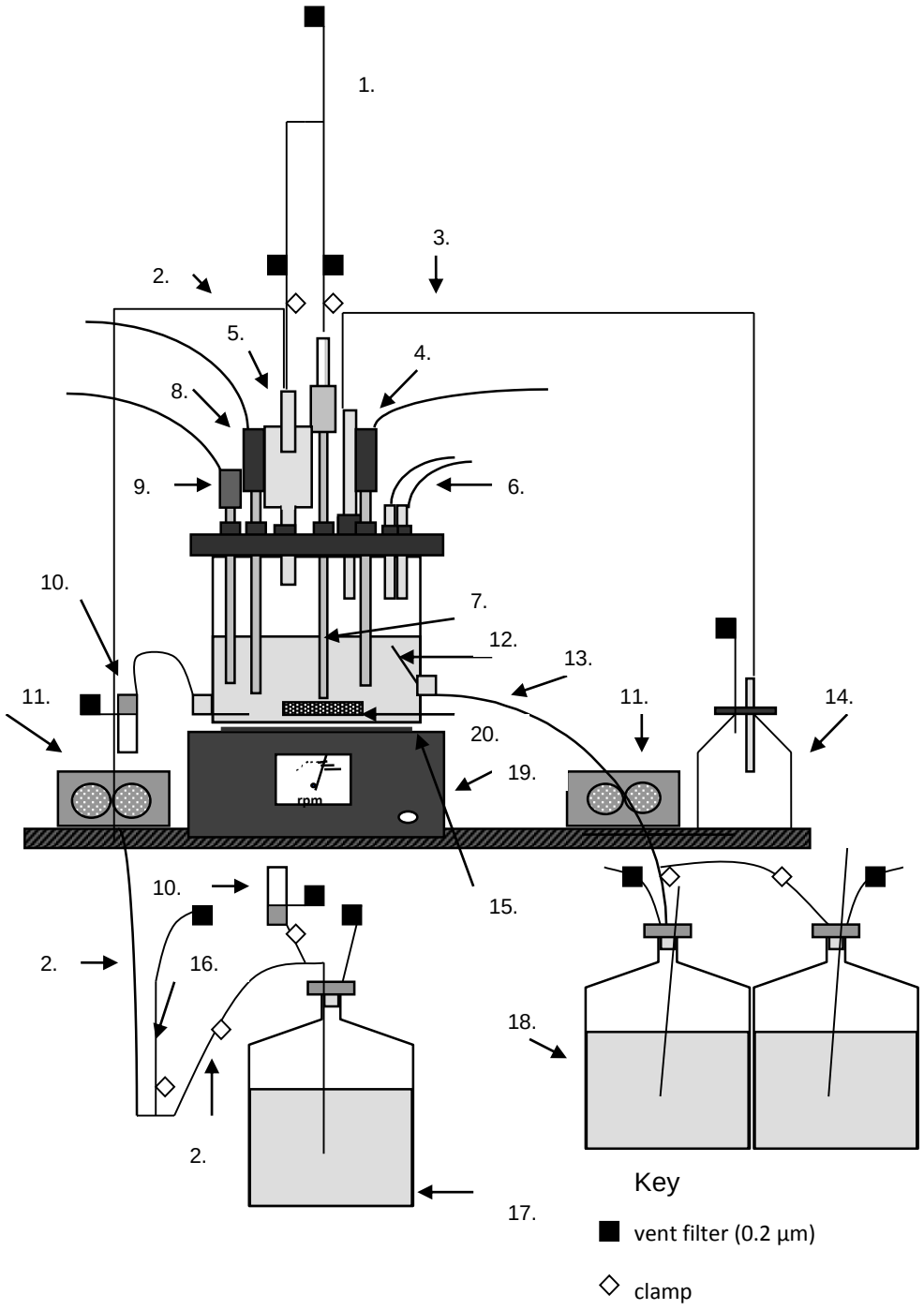


Figure 1.6 The chemostat system used for continuous culture experiments as described in sections 2.2.3.1 and 2.2.3.6. The labels are as follows: **1**. Air inlet **2.** Medium supply **3.** Air outlet/off-gas **4.** pH probe **5.** Anti-grow back device as part of medium inlet **6.** Spare ports for addition of acid and alkali **7.** Air inlet tube 8.Temperature probe **9.** Oxygen probe **10.** Sample port **11**. Peristaltic pumps **12**. Effluent port **13.** Effluent line **14.** Off-gas condensate **15.** Heat pad **16**. Glass pipette for flow rate measurement **17**. Medium supply **18.** Effluent waste pots or cell collection pots **19.** Stirrer **20**. Magnetic flea. Taken from (Bacon and Hatch, 2009)

The influence of reduced oxygen availability on pathogenicity and gene expression was studied using continuous culture (Bacon *et al.*, 2004) allowing investigation of oxygen parameters independent of other variables such as nutrient depletion which the batch models of hypoxia are lacking. The effect of environmental stress on the mutation rate of *M. tuberculosis* was measured by following the mutant frequency and distribution of mutations in the RRDR of the *rpoB* gene using continuous culture, where the pH of the culture was reduced (Bacon *et al.*, 2010; Jenkins *et al.*, 2009). Methods for determining mutation rate (the rate at which a mutation occurs over time) have often been complicated and require large numbers of parallel cultures. Most commonly quoted is the mutant frequency, which is the number of mutants in a population. Continuous culture allows multiple samples to be taken over a long timecourse from a high titre population which is required to accurately measure the mutation rate and the effect on growth rate itself in the absence of antibiotic can be studied. Mutation rates of novel compounds can be assessed and compared to current therapeutics and evaluated as to their suitability for inclusion into a new regime.

Continuous culture has been used as a powerful technique to study metabolic flux in *M. tuberculosis*, confirming the ability to fix carbon from carbon dioxide (CO_2) and identifying that deletion of one of two isocitrate lyase genes impairs replication of *M. bovis* BCG at a slow growth rate, making both these processes possible drug targets (Beste *et al.*, 2011). Metabolic flux analysis using labelled carbon is ideal for continuous culture as the amount of carbon available to the bacteria is known and controlled via the dilution rate in carbon limited cultures. The macromolecular composition of mycobacteria at slow and fast growth rates was also elucidated using continuous culture and found evidence for growth rate-mediated regulation of ribosome biosynthesis and lipid metabolism (Beste *et al.*, 2005). These two growth rates were also used to study the transcriptomic response associated with slow growth, showing association of slow growth with induction of expression of several genes of the dormancy survival regulon (Beste *et al.*, 2007a). This work also found high levels of similarity between the slow growing Mycobacteria in the chemostat and the transcriptional response of *M. tuberculosis* to growth in the macrophage, validating the use of continuous culture and these growth rates for the study of *M. tuberculosis.* Transposon site hybridisation selection was then used to define the genetic requirements of switching from a fast growth rate (23 hour doubling time) to a slow growth rate (69 hour doubling time) (Beste *et al.*, 2009).

To study antibiotic efficacy and the PK/PD of novel and current therapeutics, compounds can be added to the chemostat in a constant delivery at a known concentration or in pulses, this can be done in with one drug or in combination with another. This allows the study of drug interaction

with the bacilli but also how antibiotics work in combination in the current regime to combat tuberculosis infection. With continuous systems the volume of the culture is maintained so samples can be taken over a time course without disturbing the equilibria of the system. This also allows for samples for total viable count, optical density, and genomic study to be removed whilst knowing the precise readings of the culture and controlling certain aspects. Controlling the growth rate of *M*. *tuberculosis* using continuous culture allows the efficacy of antibiotics to be studied with respect to doubling time, to try to determine the mechanisms used by different sub-populations during infection.

The work presented in this thesis has used a variety of batch and continuous culture models to elucidate differing mechanisms of antibiotic resistance and persistence with respect to growth rate and phase. Using slow and fast growth rates of 69 hours and 23 hours respectively the response of these two populations to isoniazid has been studied using continuous culture (Jeeves *et al.*, 2015).

1.7 Objectives and Aims of Thesis

The main aim of this thesis was to establish whether persisting populations of *M. tuberculosis* in the presence of antibiotic have adapted through a genotypic mechanism i.e. mutation leading to resistance and persistence through treatment or phenotypic mechanisms i.e. physiological changes allowing survival through treatment but maintaining wild type genetics. Other factors that may affect antibiotic efficacy with *in vitro* systems were also investigated.

- Determine whether a high titre of cells in a system such as a chemostat reduces the efficacy of isoniazid and rifampicin.
    - The aim was to show titres that are routinely tested in laboratories in susceptibility tests do not represent *in vivo* titres and the minimum inhibitory concentration of an antibiotic can be affected by this.

- Determine the susceptibility profile of *M. tuberculosis* growing at different growth rates and in different growth phases
    - The aim was to show slow growing cells and cells that are not dividing are more recalcitrant to cells dividing at a faster growth rate or within exponential phase. This work aimed to address the different populations found within a *M*. *tuberculosis* infection and show the differing actions of antibiotics

- Determine whether slow growing *M. tuberculosis* is adapted to isoniazid using phenotypic or genotypic mechanisms.
    - The aim was to compare two growth rates, controlled in continuous culture, with the hypothesis being that the slow growing *M. tuberculosis* was adapted intrinsically to isoniazid exposure. This was investigated using sequencing of colonies to investigate genotypic changes in known isoniazid resistance loci and transcriptomic data to investigate phenotypic adaptations.

Chapter 2. Materials and Methods

2.1 Preparation of media for the culture of *Mycobacterium tuberculosis*

2.1.1 Preparation of CAMR *Mycobacterium* Media MOD2

The first two ingredients from table 2.1 were added to 500 ml of water, the remaining ingredients and CAMR Mycobacterium (CMYCO) solutions were added in the order listed and stirred to dissolve. The pH was adjusted to 6.5 with 20% KOH and the conductivity checked. The medium was filter sterilised and stored at 2˚C to 8˚C.

CMYCO solutions 1, 2 and 3 were prepared by adding the ingredients listed in tables 2.1 a, b and c to water and stirring to dissolve. CMYCO Solutions 1 and 2 were kept between 2 °C and 8 °C for up to six months and solution 3 was used within 1 week of production. CMYCO solutions 4 and 5 were made fresh each time they were required by adding the ingredients in the quantities specified in tables 2.1 d and e**,** these solutions were stirred to dissolve the ingredients. The CAMR *Mycobacterium* MOD2 medium (James *et al.*, 2000) was prepared by the Health Protection Agency, Microbiological Services Porton, Media department.

CAMR Mycobacterium Medium MOD 2		
Ingredients	**Amount for 1L**	**Units**
ACES BUFFER	10.000	g
KH_2PO_4	0.220	g
MILLI Q WATER	500.000	ml
CMYCO SOLUTION 1	10.000	ml
CMYCO SOLUTION 2	10.000	ml
CMYCO SOLUTION 3	100.000	ml
CMYCO SOLUTION 4	10.000	ml
BIOTIN 10 µg/ml SOLUTION *	10.000	ml
$NaHCO_3$	0.042	g
GLYCEROL	0.750	g
CMYCO SOLUTION 5	10.000	ml
TWEEN 80	2.000	ml
MILLI Q WATER TO	1,000.000	ml

*Biotin 10 µg/ml solution = 0.01 g in 1000 ml Milli Q water

Table 2.1 Ingredients of CAMR Mycobacterium Medium MOD2

CMYCO SOLUTION 1		
Ingredients	**Amount for 1L**	**Units**
$CaCl_2.2H_2O$	0.05550	gm
$MgSO_4.7H_2O$	21.40000	gm
$ZnSO_4.7H_2O$	2.87500	gm
MILLI Q WATER	1,000.00000	ml

CMYCO SOLN 2		
Ingredients	**Amount for 1L**	**Units**
COBALT CHLORIDE $6H_2O$	0.47600	gm
$CuSO_4.5H_2O$	0.00250	gm
$MnCl_2.4H_2O$	0.00200	gm
conc HCL	0.50000	ml
MILLI Q WATER	1,000.00000	ml

CMYCO SOLN 3		
Ingredients	**Amount for 1L**	**Units**
L-SERINE	1.00000	gm
L-ALANINE	1.00000	gm
L-ARGININE	1.00000	gm
L-ASPARAGINE	20.00000	gm
L-ASPARTIC ACID	1.00000	gm
L-GLYCINE	1.00000	gm
L-GLUTAMIC ACID	1.00000	gm
L-iso LEUCINE	1.00000	gm
L-LEUCINE	1.00000	gm
MILLI Q WATER	1,000.00000	ml

CMYCO SOLN 4		
Ingredients	**Amount for 1L**	**Units**
PYRUVIC ACID SODIUM SALT	100.00000	gm
MILLI Q WATER	1,000.00000	ml

CMYCO SOLN 5		
Ingredients	**Amount for 1L**	**Units**
$FeSO_4.7H_2O$	1.00000	gm
conc HCL	0.50000	ml
MILLI Q WATER	1,000.00000	ml

Table 2.1 a-e Ingredients of CMYCO solutions 1- 5

2.1.2 Preparation of CAMR Mycobacterium medium MOD6

The ingredients listed in table 2.2 were added to water and the pH adjusted to 6.5 with 20% KOH. This medium was prepared by the Health Protection Agency, Microbiological Services Porton, Media department.

CAMR Mycobacterium Medium MOD 6		
Ingredients	**Amount for 1L**	**Units**
ACES BUFFER ($C_4H_{10}N_2O_4S$)	10.00000	g
KH_2PO_4	0.22000	g
MILLI Q WATER	500.00000	ml
CMYCO SOLUTION 1	10.00000	ml
CMYCO SOLUTION 2	10.00000	ml
L-ASPARAGINE	2.00000	g
BIOTIN 10 µg/ml SOLUTION *	10.00000	ml
$NaHCO_3$	0.04200	g
CMYCO SOLUTION 5	10.00000	ml
TWEEN 80	2.00000	ml
ALBUMIN BOVINE FRACTION V	5.00000	g
MILLI Q WATER TO	1,000.00000	ml

Table 2.2 Ingredients and amounts for CAMR Mycobacterium MOD6

2.2 Culture of *Mycobacterium tuberculosis*

All work with *Mycobacterium tuberculosis* was carried out within a containment level 3 facility using class III microbiological safety cabinets (MSCIII).

2.2.1 Inoculation of a Seed Flask of *Mycobacterium tuberculosis*

A seed vial of *M. tuberculosis* H37Rv (ATCC 25618™) strain was removed from -70°C storage, plated evenly onto Middlebrook 7H10 Agar (Becton Dickinson 262710) plus OADC supplement (Becton Dickinson 212351) and incubated for three weeks at 37 °C until single colonies were evident. 200 ml of CAMR *Mycobacterium* Media MOD2 (section 2.1.1) was placed into a 1 litre

flask and inoculated with half a plate of this seed stock then left shaking at 37°C at 200 rpm for 72 hours.

2.2.2 Turbidity measurement and total viable counts of *Mycobacterium tuberculosis*

For a real time measurement of *M. tuberculosis* growth a 1 ml sample was removed from the culture pipetted into a cuvette and placed into a spectrophotometer, and an optical density (OD) measurement was made at 540 $_{nm}$. The viability of cultures was measured by a total viable count (TVC) method, a sample was removed from the culture and 1 ml was transferred to a micro-centrifuge tube. The cells were spun at 2415 x g for 5 minutes, the supernatant was removed, and the pellet was resuspended in phosphate buffered saline (PBS). This process was repeated, to wash any remaining antibiotic from the sample. A dilution series of the cells was prepared by adding 100 µl of the neat sample to 900 µl of PBS. 100 µl of the required dilutions (dictated by the turbidity of the culture) was plated onto Middlebrook 7H10 agar and spread. These plates were then incubated at 37°C for three weeks. To calculate the colony forming units per ml (cfu/ml) of culture, the number of colonies evident after 3-4 weeks incubation was recorded as was the dilution factor. Equation 2.1 was then used to calculate the cfu/mL.

$$\frac{Cfu}{ml} = Number\ of\ colonies \times 10 \times Dilution\ Factor$$

Equation 2.1 Calculation of cfu/ml

Total viable counts performed using the Miles and Misra method (Mile*s et al*, 1938) follow the method described above with the following modification: the plate was divided into quadrants for the dilutions. In each section, three 20 µl aliquots of the appropriate dilution were spotted, then left to dry at room temperature inside a MSCIII until no visible liquid could be seen. Plates were then incubated at 37°C for three weeks. Colonies could be counted after three weeks (figure 2.1). To calculate the cfu/ml of culture, the number of colonies evident after 2.5 - 3 weeks incubation was recorded as was the dilution factor. Equation 2.2 was used to calculate cfu/mL.

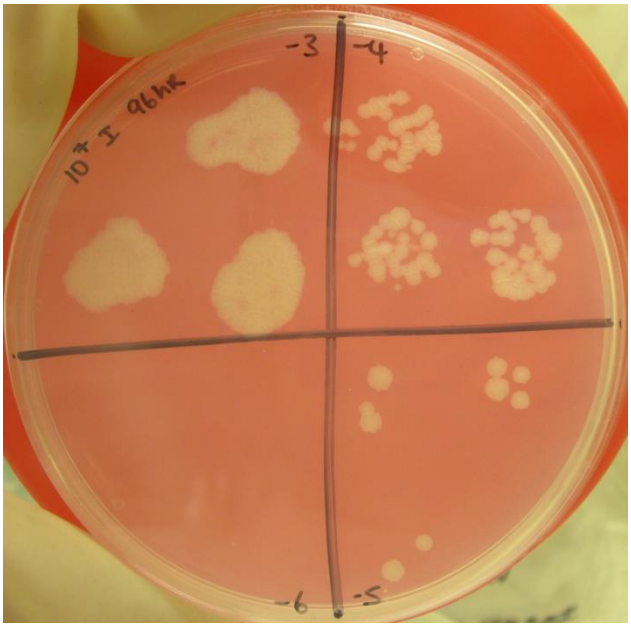


Figure 2.1 *Mycobacterium tuberculosis* colonies grown on Middlebrook 7H10 agar using the Miles and Misra method.

$$\frac{Cfu}{ml} = Number\ of\ colonies\ \times 50\ \times Dilution\ factor$$

Equation 2.2 Calculation of cfu/ml using the Miles and Misra method

2.2.3 Chemostat culture of Mycobacterium tuberculosis

2.2.3.1 Assembly of a chemostat for continuous culture

A 1 litre glass chemostat vessel (DASGIP 78903189) was rinsed with distilled water and oxygen and pH probes were inserted into ports in the chemostat head plate. The effluent and medium lines were assembled using silicon tubing and connected to the vessel along with the air inlet, off-gas condensate bottle, and sample port. Vent filters (0.2 µm) were added to the air inlets and outlets, sample port side arms, waste bottles, collection bottles and medium bottles to maintain sterility and prevent the buildup of pressure in the vessel as can be seen in figure 1.6. An electronic stirrer, two peristaltic pumps (medium and effluent), two autoclaved effluent reservoirs and the medium source (CAMR Mycobacterium MOD2, section 2.1.1) were placed inside an MSCIII. A magnetic flea and 600 ml of water were added to the chemostat vessel and the vessel was placed on top of the stirrer on a heat pad. The probes were then connected to the controller, which was used to maintain culture conditions. The signal of the oxygen probe was calibrated using a rough span of between 0% dissolved air saturation (via the addition of nitrogen) and 100% dissolved air saturation (via the addition of air) could be achieved. The pH

probe was calibrated with pH 4.0 and pH 7.0 buffers (Sigma 33665 and 33666) The acidity of the cultures was not controlled but was monitored. The water in the vessel was heated to 37°C using the heat pad and the temperature probe was calibrated using a hand held thermometer. The temperature in the vessel was measured until it reached 37°C, this reading was then set on the controller. The vessel was pressure tested by submerging it in water, ensuring the probe fittings were under the level of the water but that the vent filters remained dry. The air inlet and outlet clamps were removed and the air outlet tubing was extended past the vent filter using additional tubing. The end of this tubing was placed below the surface of the water. A syringe was connected to the air inlet line and air was pumped in and bubbles were seen coming out of the air outlet. The air outlet was clamped and this process was repeated, to check that no air bubbles exited the chemostat. The vessel was autoclaved at 121°C for 30 minutes with the air inlets re-clamped and air outlet un-clamped.

The chemostat was placed inside a MSCIII and the medium source and effluent reservoirs were connected and the tubing was connected to the peristaltic pumps. All probes and the stirrer were connected to the controller. The oxygen probe was calibrated by warming the vessel to 37°C whilst stirring and oxygen and nitrogen were pumped in alternately until it was calibrated between 0% and 100% dissolved air saturation (DAT) which is equivalent to 0 and 20% dissolved oxygen tension (DOT). The water was drained from the chemostat and then it was filled with 400 ml of CAMR Mycobacterium media MOD2 (section 2.1.1) via the medium line and then warmed to 37°C, the maximum and minimum stirrer speeds were set (Bacon and Hatch, 2009). Flow rates were set by calibrating the medium pump by measuring the amount medium pulled from the medium source in a set amount of time. The pump setting could then be set to the specific flow rate required.

2.2.3.2 Calculation of the dilution rate and doubling time of bacteria within continuous culture

The dilution rate is the flow of medium divided by the volume of the culture in the bioreactor. It was calculated using equation 2.3

$$D = {}^{f}/_{v}$$

Equation 2.3 Dilution rate of a chemostat

D = dilution rate (hr^{-1})

f = flow of medium into the vessel (ml/hr)

v = volume of culture in vessel (ml)

Using the dilution rate value the doubling time of the bacteria was be calculated using equation 2.4

$$T_d = {\log_e 2}/{D}$$

D = dilution rate (hr^{-1})

T_d = Doubling time (hours)

$$\therefore D = {\log_e 2}/{T_d}$$

Equation 2.4 Calculation of doubling time of a bacterium in continuous culture.

2.2.3.3 Calculation of specific growth rate and doubling time in uncontrolled bacterial cultures

To calculate the doubling time of a population of cells in an experiment where the growth rate and doubling time are not controlled equations 2.5 and 2.5 b were used.

$$\ln N_t - \ln N_0 = \mu\,(t - t_0)$$

μ = Specific growth rate

N = Number of cells at time **t**

N_0 = Number of cells at time zero

t_0 = Time zero

The following conversion allows the for use of $\log_{10}$ values rather than natural logs

$$\mu = \left((\log_{10} N - \log_{10} N_0)2.303\right)/(t - t_0)$$

Equation 2.5 Calculation of growth rate.

$$T_d = (\log_{10} N_t - \log_{10} N_0)/\log_{10} 2$$

Equation 2.5b Calculation of doubling time

2.2.3.4 Calculation of theoretical washout of continuous culture

The theoretical washout was calculated based on the flow rate of the chemostat and the doubling time of the bacterium as controlled by the dilution rate as shown in equation 2.6.

$$Flow\ of\ medium\ per\ doubling\ time\ (mls) \Big/ Total\ volume\ of\ chemostat\ (mls)$$

Equation 2.6 Calculation of dilution factor for theoretical washout of bacteria from a continuous culture

This gives a dilution factor, the viable count data from each generation could then be multiplied by this dilution factor to show the rate at which non-growing bacteria would be diluted from the system.

2.2.3.5 Calculation of mutant frequency and mutation rate

The mutant frequency was calculated as shown below.

$$Number\ of\ colonies\ isolated\ on\ antibiotic\ plate \Big/ Total\ viable\ count$$

The mutation rate was calculated as shown in equation 2.7.

$$\mu = \left[\left(\frac{r_2}{N_2}\right)\right] - \left[\left(\frac{r_1}{N_1}\right)\right] \times \ln\left(\frac{N_2}{N_1}\right) = (f_2 - f_1) \times \ln\left(\frac{N_2}{N_1}\right)$$

μ = mutation rate
r1 = observed number of mutants at first time-point
r2 = observed number of mutants at last time-point
N1 and N2 = total numbers of viable cells at first and last time-points respectively
f1 and f2 = mutant frequencies at first and last time-points respectively

Equation 2.7 Calculation of the mutation rate of bacteria within continuous culture

2.2.3.5 Addition of isoniazid to continuous culture

A 1 mg/ml stock of isoniazid (Sigma - I3377) was made by dissolving 10 mg of isoniazid in 10 ml of molecular grade water (Sigma W4502). This stock was then filtered through a Minisart 0.2 µm syringe filter (Sigma 16534K) and stored at 4˚C. The isoniazid stock was diluted to 0.1 mg/ml and 10 ml of this stock was added aseptically to a 2 litre bottle of MOD2 medium (section 2.1.1) and mixed well. For addition of isoniazid directly to the chemostat vessel, a solution of isoniazid

containing 2.5 ml of a 0.1 mg/ml stock in approx 10 ml of MOD2 medium was prepared in a sterile glass universal. This allowed the antibiotic to be added via the sample port in a volume that would ensure complete addition of the antibiotic.

The media line of a steady state continuous culture (section 2.2.3.1) was drained back to the medium source by switching off the medium pump and removing the tubing from the medium pump. The media line of the chemostat was then connected to the medium bottle containing MOD2 and 0.5 µg/ml isoniazid. The medium line was primed up to the chemostat vessel using a hand held pump attached to the vent filter of the medium bottle; the medium line was clamped at the point just before the medium was to enter the chemostat vessel. The medium tubing was then replaced in the medium pump (which was still switched off), the clamp removed and the effluent pump switched off. The antibiotic was added via the sample port to a final concentration of 0.5 µg/ml, and the sample port washed through several times to ensure the addition of the entire volume of antibiotic. The medium and effluent pumps were switched back on to maintain continuous culture with a constant concentration of isoniazid throughout the experiment. The continuous culture system described can be seen in Figure 2.2. The flow rate of the vessel was checked as described in section 2.2.3.1.

2.2.3.6 Controlled batch culture to investigate nutrient starvation

A 2-litre glass vessel was assembled following the instructions for continuous culture (section 2.2.3.1) but no effluent or medium lines were required. The vessel was filled with MOD6 medium (section 2.1.2), pre-warmed and a fixed stirrer speed set. The dissolved oxygen tension (DOT) was maintained at 10% via addition of air and nitrogen as required. The vessel was left stirring once inoculated and no fresh nutrients were added or effluent removed. These cultures were set up by J. Allnutt, TB Research, Microbiology Services Porton, Health Protection Agency.

2.3 Recovery of isoniazid resistant colonies from the chemostat

Middlebrook 7H10 Agar plus OADC supplement was prepared and contained 1 µg/ml isoniazid (Sigma Aldrich I3377-5G), which was equivalent to 2 x MIC. 100 µl of the undiluted, 10^{-1} and 10^{-2} diluted cells that had previously been prepared for the TVC (section 2.2.2) were spread onto the agar in triplicate and the plates were incubated at 37°C for 3 – 4 weeks. The colonies were counted. Cells were also plated onto agar without antibiotic as a positive control to determine the viable count. Colonies growing on antibiotic were kept for further genotypic and phenotypic analysis by storing on agar at 4˚C.

2.4 Antibiotic Susceptibility testing of *M. tuberculosis* under different growth conditions

2.4.1 Susceptibility to antibiotics in liquid medium

The medium that the sample of *M. tuberculosis* being analysed was cultured in was aliquoted into 10 ml universal tubes (Sterilin 128A/P) and antibiotic was added in the volumes outlined in table 2.3

A 10 ml sample was removed from the nutrient starvation batch culture (section 2.2.3.6) at time points corresponding to the different mycobacterial growth phases (i.e. exponential, stationary, death and persistence) this was determined by $OD_{540\ nm}$ readings and viable counts when available. A 10 ml sample was removed from the continuous culture vessels (section 2.2.3.1) when steady state growth at the desired dilution rate had been established for 3-5 mean generations. Steady state this was determined by $OD_{540\ nm}$ readings and stable DOT, rpm and pH readings. The $OD_{540\ nm}$ of the sample removed from the source culture was measured. The universals were inoculated with *M. tuberculosis* to give a starting $OD_{540\ nm}$ of 0.05; this was calculated using equation 2.7. The cultures were mixed well and a 1 ml sample was removed for $OD_{540\ nm}$ measurement and a 1 ml sample was removed for TVC using the Miles and Misra method (described in section 2.2.2). The cultures were then left shaking at 37˚C at 200 rpm. Additional samples for $OD_{540\ nm}$ and TVC were removed immediately after inoculation and 24 hours post inoculation for both the nutrient starvation culture and the continuous culture. Samples were taken at 144 hours post inoculation from the nutrient starvation batch culture and 96 hours post inoculation for samples from the continuous culture.

Volume of stock to add (ml)	Conc of stock soln (µg/ml)	Total final volume	INH final conc µg/ml
0.025	100	10	0.25
0.05	100	10	0.5
0.1	100	10	1
0.2	100	10	2
0.4	100	10	4
0.8	100	10	8
0.32	1000	10	32

Volume of stock to add (ml)	Conc of stock (µg/ml)	Total final volume	RIF final conc (µg/ml
0	10	10	0
0.016	10	10	0.016
0.032	10	10	0.032
0.064	10	10	0.064
0.128	10	10	0.128
0.256	10	10	0.256
0.512	10	10	0.512
0.2048	100	10	2.048

Table 2.3 The volume and concentration of antibiotic added to 10 ml medium to give required final concentration.

$$\frac{Actual\ OD\ of\ sample}{Required\ starting\ OD\ (0.05)} = Dilution\ factor$$

$$\frac{Amount\ of\ medium\ (ml)}{Dilution\ factor} = Amount\ of\ culture\ to\ add\ to\ medium\ (mls)$$

Equation 2.8 Calculation to determine the amount of inoculum to add to give a starting $OD_{540\ nm}$ of 0.05

2.5. Heat killing of Mycobacterium tuberculosis for DNA extraction and inactivation

100 µl of Tris-EDTA Buffer (TE Buffer) pH 8.0 (Applied Biosystems AM9849) was aliquoted into a suitable RNase and DNase-free screw cap tube with an o-ring. Wooden toothpicks were autoclaved to sterilise them and the colony of interest was picked using the sterile toothpick from the agar and emulsified in the TE Buffer. 10 µl of this solution was spread onto a Middlebrook 7H10 agar plate to provide growth for glycerol stocks. Four colonies were spread onto each plate by dividing the plate into quarters. The tubes containing the remaining emulsified colony were then put in a heat block pre-warmed to 80˚C and heated for 20 minutes. The tubes were then stored at -80˚C. The plates were incubated at 37˚C until confluent growth was observed.

2.5.1. Preparation of glycerol stocks of Mycobacterim tuberculosis colonies

A solution of glycerol at a concentration of 20% (v/v) was prepared and autoclaved for 11 minutes at 126 °C to sterilise it. 1 ml of this solution was aliquoted into screw cap tubes with an o-ring. Growth from the 10 µl plated out prior to heat killing (section 2.5) was scraped into the glycerol and mixed with a 10 µl inoculating loop. These tubes were then stored at -80˚C.

2.6 RNA Extraction from Mycobacterium tuberculosis

2.6.1. Isolation of RNA

A 5M guanidine thiocyante (GTC) lysis solution was made by weighing out 591 g of GTC (Sigma Aldrich G9277), 5 g of N lauryl sarcosine (Sigma L9150), 7.3 g Trisodium citrate (Sigma S4641) and 5 ml of Tween 80 (Sigma P5188) and making up to 1 litre with nuclease free water. 1 volume of culture sampled was added to 4 volumes of GTC lysis solution. The GTC – sample mixture was incubated at room temperature for 1 hour. The sample mixture was spun down in a bench top centrifuge for 15 minutes at 1935 x g. The supernatant was then poured off leaving a small amount (<100 µl) of GTC in the bottom of the tube. The pellet(s) were re-suspended by pipetting

gently up and down, 1.2 ml of Trizol (Invitrogen 15596018) was added and the pellet(s) re-suspended thoroughly. The sample was then transferred to a 2 ml tube containing 0.5 ml 0.1 mm silica beads (Fisher Scientific MBR-247-105B) and these were lysed using a reciprocal shaker (FastPrep FP120) for 45 seconds at a speed of 6.5. The pink solution above the beads was then transferred into 240 µl of chloroform and shaken vigorously for 20 seconds. This solution was spun at 2415 x g for 10 minutes. The aqueous phase was then removed and added to 600 µl chloroform, shaken vigorously for 20 seconds and spun for 10 minutes at 2415 x g - this step was then repeated. The aqueous phase was then added to 600 µl isopropanol plus 60 µl sodium acetate (Sigma Aldrich S7899) and frozen at -70˚C at least overnight to aid precipitation.

2.6.2 Purification of Total RNA

Total RNA was isolated from the extractions detailed in section 2.6.1 using the *mir*Vana™ miRNA Isolation kit (Agilent AM1561). The sample was prepared by thawing it on ice and then adding 1.25 volumes of room temperature 100% ethanol to the extraction. A filter cartridge was placed into a collection tube (both provided in the kit) and the sample/ethanol mixture was applied to the filter. A volume of 700 µl was applied at a time, for sample volumes larger than this the mixture was applied in successive applications to the same filter. The cartridge assembly was then centrifuged for 15 seconds at 10,000 x g to allow RNA to bind to the filter cartridge. The flow through was discarded and this process repeated until all of the sample/ethanol mixture was through the filter. The collection tube was re-used for the following wash steps, 700 µl of miRNA Wash Solution 1 (provided with the kit) was applied to the filter cartridge and this was spun at 10,000 x g for 5-10 seconds. The flow through was discarded from the collection tube and the filter replaced into the same collection tube. 500 µl of Wash Solution 2/3 (provided with the kit) was applied to the filter cartridge and drawn through as in the previous step. This process was then repeated with a second 500 µl amount of Wash Solution 2/3. After discarding the flow through from the last wash the filter cartridge was replaced into the same collection tube and the assembly spun for 1 minute to remove residual fluid from the filter. The filter cartridge was then transferred into a fresh collection tube and 100 µl of pre-heated (95˚C) RNase-free water (Sigma W4502) was applied to the centre of the filter and the cap closed. The assembly was spun for 20-30 seconds at maximum speed (13,000 x g) to recover the RNA. The eluate was collected which contained the RNA and stored at -70˚C this was performed according to instructions from Invitrogen (http://tools.invitrogen.com/content/sfs/manuals/cms_055423.pdf).

2.6.3 Quantification of RNA

The concentrations of RNA prepared in sections 2.6.1 and 2.6.2 were determined by using the NanoDrop 2000 and NanoDrop 3000 (Thermo Scientific). The blank measurement was taken using RNase-free water; this was the same solution the RNA was eluted into at the end of the total RNA isolation procedure (section 2.6.2). Along with the RNA concentration, the 260/280 and 260/230 ratios of each sample were recorded to allow assessment of phenol or GTC contamination.

2.6.4 Analysis of RNA integrity

The Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA) was used to assess the integrity of the RNA isolated in section 2.6.2. The Agilent RNA 6000 Nano Kit (Agilent, 5067-1511) was used and the RNA 6000 Nano Quick Start Guide followed. Samples to be analysed were heat denatured at 70˚C for 2 minutes before loading them onto the bioanalyser chip.

To prepare the Agilent RNA 6000 ladder (with a range from 25 – 6000 nucleotides) it was heat denatured in an RNase free tube at 70˚C for 2 minutes and was then immediately cooled down on ice. Aliquots were prepared corresponding to daily usage and stored at -70˚C. To prepare the gel, 550 µl of RNA 6000 Nano gel matrix was pipetted into a spin filter (provided with the kit) which was centrifuged at 1500 x g for 10 minutes at room temperature. 65 µl of this filtered gel was aliquoted into RNase-free tubes and used within 4 weeks. Before preparation and loading of the gel-dye mix, the RNA 6000 Nano dye concentrate was allowed to equilibrate to room temperature for 30 minutes and was then vortexed for 10 seconds, spun down and 1 µl added to a 65 µl aliquot of filtered gel. This solution was then briefly vortexed to ensure mixing and centrifuged at 13000 x g for 10 minutes at room temperature.

To load the gel-dye mix, a new RNA 6000 Nano chip (provided with the Agilent 6000 Nano Kit) was placed on the chip priming station and 9 µl of gel-dye mix was pipetted into the well indicated by the protocol and symbols on the chip. The chip priming station was then closed and the plunger on the attached syringe pressed until it is held by the clip on the chip priming station, the clip was released after exactly 30 seconds. A further 9 µl of the filtered gel was then pipetted into the second and third gel wells on the chip. To load the RNA 6000 Nano Marker, 5 µl of marker was loaded into all 12 sample wells and in the well marked with a ladder symbol. 1 µl of prepared ladder (after thawing) was loaded into the well marked with the ladder symbol and 1 µl of each sample to be analysed was loaded into each of the sample wells required, any unused sample wells had 1 µl of RNA 6000 Nano Marker loaded. The chip was vortexed for 1 minute at 2400 rpm and the chip was run in the Agilent 2100 bioanalyser within 5 minutes by selecting the corresponding assay parameters.

The RNA quality was assessed by review of the integrity of the 16S and 23S rRNA peaks (Fig 2.3), ensuring that the trace showed a flat baseline punctuated with narrow peaks and a complete absence of broad, rounded peaks. The entire electrophoretic trace was used by the Agilent 2100 bioanalyzer software to calculate a RNA Integrity Number (RIN) allowing comparison between samples independent of sample concentration. The scale of the RIN is 1-10 with 1 being the lowest and 10 being the highest. A RIN of greater than 7 would indicate a sample of high enough quality for downstream transcriptomic analysis such as microarray.

The presence of small RNAs was also detected by a peak on the electropherogram, this peak appeared at around 25 seconds corresponding to RNA of 20-200 nucleotides, this represents the 5s region.

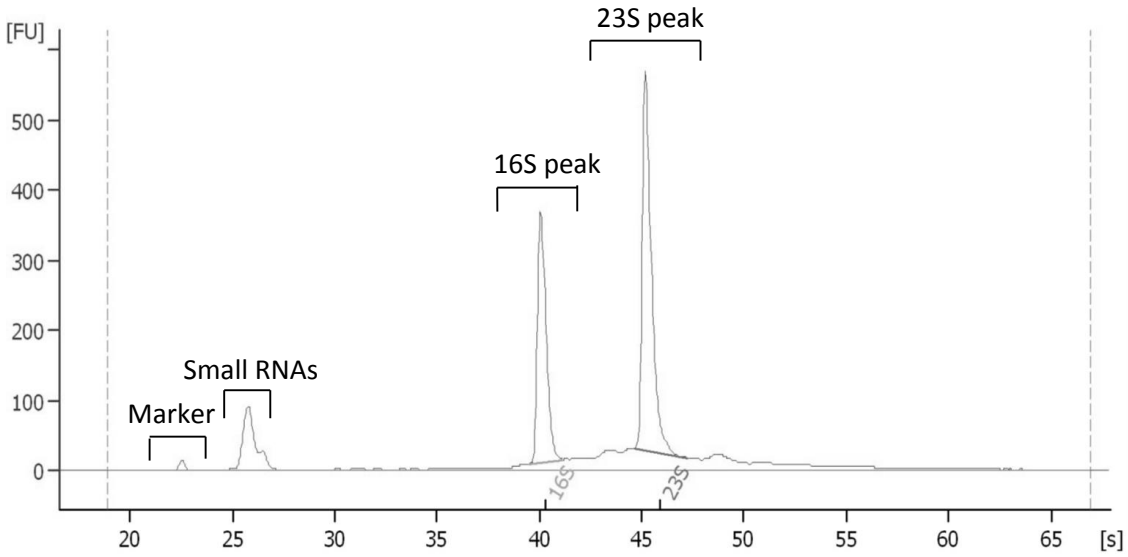


Figure 2.2 Example of a bioanalyser trace with a RIN of 10 including the presence of small RNAs. The x-axis represents time in seconds [s] in the assay and the y-axis represents fluorescence units absorbed [FU]. The 16S and 23S peaks are assigned by the Bioanalyzer software using the ladder as reference.

2.7 Transcriptomic analysis

The work in the following sections was performed by Dr Rose Jeeves, PHE, Porton Down, UK. The methods are taken from (Jeeves *et al.*, 2015)

2.7.1 RNA Labelling

RNA was labelled using the Kreatech ULS™ Fluorescent Labeling Kit for Agilent arrays (Kreatech EA-023). The labelled RNA was fragmented by adding 2 µl 10x fragmentation buffer, incubating

for 15 minutes at 70°C, then adding 2 µl stop solution (Ambion® AM8740). Labelled RNA (20 µl) was added to 27.5 µl Kreatech blocking reagent (Kreatech EA-023), 55 µl of 2x Hybridisation buffer and 7.5 µl of molecular grade water. Arrays were hybridised overnight at 65°C, then washed in Gene Expression wash buffer 1 (Agilent 5188-5327) for 1 minute at room temperature with agitation, then in Gene Expression wash buffer 2 for 1 minute at 37°C with agitation. Slides were scanned immediately using an Agilent Scanner.

2.7.2 Transcriptomic analyses

Whole genome gene expression analyses were performed. Microarray experiments were performed using a custom Agilent tiling array (ArrayExpress accession A-BUGS-47) with 180,000 60-mer oligos evenly tiled across the *M. tuberculosis* H37Rv genome. Features were extracted from the array images using Agilent Feature Extraction Software (v10.7) with local background correction. Probes were first filtered to only include those covering annotated genes. Intensity values were normalised and analysed using GeneSpring software (version 12.6 GX). Firstly, quantile normalisation was applied across the combined slow and fast growth rate datasets, followed by an averaging of the expression level of all probes across each open reading frame, on either the sense (S) or antisense (AS) strand. A 2-way ANOVA (using a Benjamini-Hochberg correction p-value of P = 0.05 and growth rate and MGT as conditions) was used to identify significantly differentially expressed genes between fast growth rates and slow growth rates either in the presence or absence of isoniazid. A further filter was applied to select genes with at least a two-fold change in gene expression. Gene lists derived from all pairwise comparisons can be found in the supporting information in (Jeeves *et al.*, 2015). Genes were assigned groups based on their entries in Tuberculist (http://tuberculist.epfl.ch/). For the analysis of the expression of small and non-coding RNAs, Limma (Ritchie *et al.*, 2015) analyses were performed following quantile normalisation between arrays to find differentially expressed regions (oligos) within the genome. Ratios of fluorescence intensity values across the genome were visualised using a custom version of the genome browser JBrowse (http://www.ncbi.nlm.nih.gov/pubmed/19570905) which included the novel tracks MultiBigWig and MultiXYPlot (Source code and data available at https://github.com/rtylerr/jbrowse1.11-MultiBigWig). The differentially expressed regions identified by Limma could then be visualised alongside the calculated ratio plots for manual curation.

2.7.3 Real –time quantitative PCR (RTq PCR)

PCR reactions utilised the 16S rRNA gene as an endogenous control. The ahpC primer and probe sequences were designed using Primer3 software (http://primer3.ut.ee/). Primers and probes were optimised to have a reaction efficiency of 90–110% to validate them for use with the ΔΔCt method of quantification; these data can be found in the supporting information (Jeeves *et al.*, 2015) along with primer and probe sequences. Reverse transcription took place in a total volume of 20 µl containing 100 ng total RNA, 300 ng random primers (Invitrogen™ Life Technologies), 10 mM DTT, 0.5 mM each of dCTP, dATP, dGTP and dTTP, and 200 units Superscript III (InvitrogenTM Life Technologies). The 20 µl reactions were assembled in a standard ABI 96-well plate in triplicate as follows; 1.8 µl of 10 µMahpC primers or 1 µlof10 µM16S primers, 0.5 µlof10 µMof probe, 10 µl of Taqman Universal Mastermix II with UNG (Applied Biosystems part No 4428175) and 1 µl of cDNA. 16S rRNA was used as an endogenous control. Reactions were performed in an ABI-7900 light- cycler with the program as follows; 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 55°C for 45 seconds. Data were analysed using the SDS 2.4 and RQ Manager 1.2.1 Software (ABI) to calculate ΔΔCt and relative quantification values.

2.8 Polymerase Chain Reaction (PCR)

PCR was performed with Readymix™ Taq PCR Reaction Mix (Sigma P4600) according to the manufacturer's instructions. Unless otherwise stated PCRs were performed with the following reaction conditions; 12 minutes at 94˚C; 40 cycles of 30 seconds at 94˚C, 30 seconds at 60˚C, 1 minute at 72˚C; 10 minutes at 72˚C. Colony PCR was performed by using 1 µl of the heat killed suspension described in section 2.5 as the template for PCR. PCR products were visualised by gel electrophoresis on 2% (w/v) TAE (4.84 g Tris, 1.14 ml acetic acid, 2 ml EDTA pH 8.0, per litre) agarose gels containing 1x (v/v) SYBR® safe DNA gel stain (Invitrogen) in 1x TAE buffer. TrackIt™ 1 kb Plus DNA ladder (Invitrogen) was used according to manufacturer's instructions and the gels were run at 80 v for 45 minutes before the DNA was visualised in a UVIdoc (UVITECH Cambridge) gel documentation imaging system.

The primers used to amplify the regions of interest of each gene are listed below in table 2.4 and shown in figures 2.4 and 2.5 along with the sequencing primer used in the pyrosequencing assay.

Gene	Forward primer	Reverse Primer	Sequencing Primer
katG	CGGTCACACTTTCGGTAAGA	B-CCGTACAGGATCTCGAGGA	GGACGCGATCACCA
inhA	B-GAGCGTAACCCCAGTGCGAAAG	CCAGGACTGAACGGGATACGAATG	TGGCAGTCACCCC

Table 2.4 Primers used for PCR amplification and pyrosequencing

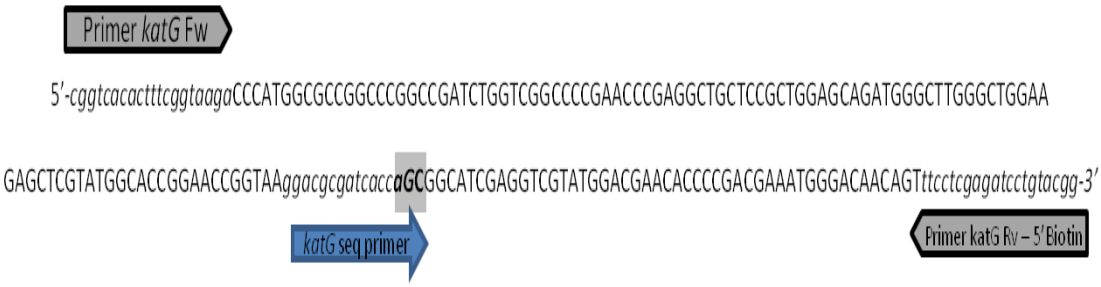


Figure 2.3 The amplicon of *katG* that was produced by using primers specified in Table 2.4. The region sequenced is directly after the sequencing primer. Codon 315 is highlighted with the single nucleotide polymorphism (SNP) being reported at the G position. The PCR primers and sequencing primers were taken from (Arnold *et al.*, 2005)

Figure 2.4 The amplicon of *inhA* that was produced by using primers specified in Table 2.4. The region sequenced is directly after the sequencing primer. Positions that SNPs have been reported at in clinical isolates are highlighted in bold. The PCR and sequencing primers were taken from (Garza-Gonzalez *et al.*, 2009)

2.9 Pyrosequencing of PCR amplicons

2.9.1 Preparation of sample

Once thawed, 20 µl of PCR product was mixed with 3 µl streptavidin-coated Sepharose beads, 40 µl binding buffer (Qiagen 979006) and 17 µl nuclease free water in a 96-well PCR plate. The plate was covered with sealing foil. This mixture was then incubated for 5-10 minutes at room temperature while shaking at 1400 rpm in a micro-titre plate shaker. A PSQ® plate (Qiagen 979002) was prepared by adding 45 µl of 0.3 µM sequencing primer (as detailed in Table 2.4) in Annealing buffer (Qiagen 979009) per well. The 'well layout' for the PSQ® plate corresponded to the layout of the PCR plate. To remove non-annealed primers and non-biotinylated product, the solution, with the beads in suspension, was aspirated using the Vacuum Prep Tool (Qiagen 9001740). The beads stuck to the filters of the tool's pins and the tool and PCR plate were kept together whilst tilting them to check that all the solution had been aspirated. The tool was then transferred to the first trough and 70% ethanol was aspirated for roughly 5 seconds. The tool was then transferred to the second trough and 0.2 M NaOH was aspirated for roughly 5 seconds to denature the DNA, then the tool was transferred to the third trough where it was aspirated with washing buffer (Qiagen 979008) for roughly 5 seconds. The vacuum was then released and the tool was placed in the PSQ plate containing 45 µl 0.3 µM sequencing primer in Annealing buffer and gently shaken to release the beads with the single-stranded PCR product attached. The sequencing primer was annealed to the PCR product by heating the plate at 80˚C for 2 minutes and then allowing it to cool down to room temperature by removing the plate and placing it on the bench. The Vacuum Prep Tool was shaken in the fourth trough containing MilliQ water, to release any leftover beads and then washed by aspirating water for 20-30 seconds.

2.9.1.2 Sequencing

The powdered enzyme and substrate mixes from the PyroMark Gold Q96 Reagents Kit (Qiagen 972804) were reconstituted in 620 µl nuclease free water each. A new SNP run was created and the details of the dispensation orders used are shown in table 2.5. The dispensation order was designed to cover the region of the single nucleotide polymorphism (SNP) directly after the sequencing primer and the region after this to confirm the presence of wild type sequence. To sequence the SNP, nucleotides A, C, T and G were added at this position.

Gene and Position of SNP	**Name of Dispensation order**	**Dispensation Order**
katG Codon 315	*katG*	ACTGCGCATCGA
katG Codon 315	*katG* plus	ACTGCGCATCGAGTCGTATG
katG Codon 315	*katG* extended	ACTGCGCATCGAGTCGTATG[actg]30
inhA promoter region	*inhA*	GACACTATCGTCTCGCGCGCATCGTATGTCAGCTAGCT

Table 2.5 Dispensation orders designed for detection of SNPs in *Mycobacterium tuberculosis* by pyrosequencing

The enzyme mix, substrate mix and the four deoxynucleotides were added to the PSQ® 96 cartridge (Qiagen 979004), the quantities to be added to the cartridge were calculated once the plate layout and dispensation order had been defined. The PSQ® plate and cartridge were loaded into the PSQ96 MA instrument and the sequencing run was started. Once finished, the plate and cartridge were removed from the instrument, the plate was disposed of and the cartridge washed with MilliQ water to be re-used.

Chapter 3. Factors affecting the efficacy of isoniazid and rifampicin against *Mycobacterium tuberculosis in vitro*

3.1 Introduction

In work performed by Kim Hatch within the Tuberculosis Research Group at Public Health England, Porton Down, found that the MICs of isoniazid and rifampicin used in a chemostat system did not perform as expected and this was particularly evident with rifampicin. The MICs had been established using a standard inoculum cultured in a low volume batch culture. It was noted that perhaps the high titre of bacteria in this system, akin to *in vivo* titres, was affecting the result. The factors that may have been affecting the efficacy of a drug were investigated to enable fuller interpretation of any results and to improve the modelling of *M. tuberculosis* interacting with antibiotics *in vivo.*

Most of the current drug susceptibility/efficacy methods for *M. tuberculosis* suffer from low predictability along with a clinical irrelevance and from unacceptably low reliability resulting from poor reproducibility (Kim, 2005). This is due to drug susceptibility testing methods and interpretation varying greatly. There are a variety of different conventional culture methods using agar based media are still the most widely utilised in many countries. The standard methods using Lowenstein–Jensen medium include the proportion method, the absolute concentration method and the resistant ratio method (Canetti *et al.*, 1963), which are fairly well standardised with clinical samples, at least for the major anti-tuberculosis drugs. Microscopic observation of growth with and without the addition of antibiotic is a low cost, low technology tool (Brady *et al.*, 2008) for assessing drug susceptibility in liquid culture. The resazurin microtitre assay (REMA) is a colourimetric, culture based drug susceptibility assay which has been endorsed by the World Health Organisation (Katawera *et al.*, 2014). These assays along with the widely used mycobacterial growth indicator tube (MGIT) (BD Diagnostics) are liquid culture methods used for rapid detection and drug susceptibility testing of *M. tuberculosis*. Molecular methods for detection of resistant isolates are also being developed and are attractive as they are rapid, are able to be standardised and can require less training. Non-molecular methods are still widely used however due to the cost of setting up molecular laboratories, particularly in areas with high tuberculosis incidence. Molecular methods do not completely eliminate the need for culture and require known loci and mutations in order to accurately detect resistance (Wilson, 2013).

Many *in vitro* models and experimental readouts, such as MIC (minimum inhibitory concentration), used to determine the efficacy of an antibiotic do not take into account a

number of realities in an infected host. The complex interactions between a host and a bacterium are not fully understood, it is necessary to develop models and experiments that take into account factors that contribute to an antibiotic's efficacy against a bacterium. Changes to the environmental conditions of the bacterium, such as pH, caused by natural bacterial growth processes may result in an environment that is not optimal for the action of the antibiotic (Udekwu *et al.*, 2009). The physiological state of the bacteria, such as the growth rate or the phase of growth is important as all antibiotics have a mode of action that targets cellular processes. In granulomas, formed during latent tuberculosis disease, the antibiotic may not be able to penetrate to reach the bacteria.

Bacteria that are not actively growing or grow slowly can be more recalcitrant to the effect of drugs, similarly persister sub-populations of bacteria can emerge within a population of exponentially growing cells. It has been found that the fraction of persister cells increases with decreased growth rate in continuous culture (Sufya *et al.*, 2003), indicating growth rate could have a significant impact on the efficacy of TB treatments. In the case of tuberculosis, depending on the stage of infection, some of the *M. tuberculosis* organisms are actively multiplying, very probably at a rate similar to that in a log phase culture, while at the other extreme, their metabolism and growth is completely inhibited. It is important to study and define the action of anti-tuberculosis therapies against slow growing and dormant bacteria as these are the thought to be the population of *M. tuberculosis* cells that are responsible for the re-activation of TB disease. Since virtually all antibiotics preferentially kill rapidly replicating bacteria, it has been hypothesized that the reduced growth and metabolic activity of these quiescent populations is responsible for the 'antibiotic tolerance' observed during infection (Baek *et al.*, 2011) Growth limitation by lack of available nutrients gives rise to cells with reduced growth rates and coincidentally radically altered cell envelopes. When the envelope itself contains the primary target for drug action a reflection in the relative abundance of the target material may reduce the overall susceptibility of the cells (Brown *et al.*, 1990) for example, reduced susceptibility to isoniazid has been characterised as one of the defining features of dormancy (Petros C. Karakousis, Ernest P Williams and William R. Bishai, 2007).

The cell density of a bacterial population is another major factor that may affect the efficacy of an antibiotic. I*n vitro* studies would ideally be carried out using inocula that are comparable to the titre of bacteria that would be seen in an infection *in vivo* because they might better predict the efficacy of an antibiotic (Davey and Barza, 1987). MICs are usually estimated over a short period, using optical density (OD_{540nm}) measurements in cultures inoculated with around $5x10^5$

cells ml^{-1} of exponentially growing bacteria. These experiments are carried out at temperatures and in media optimal for their growth and the action of the antibiotic (Udekwu *et al.*, 2009) A reduction in the effective concentration of the antibiotic (free active drug) in the medium due to antibiotic-denaturing enzymes or by binding of the antibiotic to bacterial cell components would reduce the efficacy of an antibiotic. The rate and extent at which this occurs would be proportional to the density of the bacteria exposed and therefore the concentration of enzymes and availability of cell components. It has been shown that in cultures with a low inoculum the level of antibiotic was higher than the MIC for 100% of the timecourse, whereas in cultures with a high inoculum, this level was only maintained for 38% of the same period of time (LaPlante and Rybak, 2004). The rate of killing or inhibition of growth may be proportional to the amount of antibiotic available to each bacterium at the time of exposure. This may be due to pharmacodynamic, pharmacokinetic and physiological reasons that are likely to vary among species of bacteria and antibiotics *in vivo.* This is something that needs to be considered *in vitro*, for example, for the same concentration of an antibiotic in the medium (1mg/L), for a culture containing 10^8 bacteria/ml, the amount of drug available *per bacterium* is 1/1000 as great as that in culture of 10^5 bacteria/ml. At both densities the number of antibiotic molecules per bacterium would be enormous but the ability of antibiotics to kill or inhibit the growth of bacteria may require a large number of molecules of the drug per cell or per target due to physiological factors meaning uptake or conversion of drug is not optimal (Udekwu *et al.*, 2009), this will vary depending on the target of the antibiotic.

In many infectious diseases including tuberculosis, the quantity of bacteria can far exceed the standard MIC inoculum, with levels of 10^8 cfu/ml of lung and above being isolated from the lungs of mice (De Groote *et al.*, 2011) and guinea pigs (Ordway *et al.*, 2010) infected with *M. tuberculosis*. MRI imaging of the lungs of infected non-human primates has also shown the formation of lesions with large numbers of bacteria at a high cell density in granulomas similar to the pathology presented in humans (Sharpe *et al.*, 2009, 2010a). The chemostat system allows for high titres of bacteria to be grown under controlled conditions in a continuous culture, there is a constant flow of nutrients. This is in contrast to a closed batch culture which does not allow for control of factors such as pH and oxygen level and allows an accumulation of bacterial waste and toxins and a depletion of available nutrient. In previous work (carried out at PHE, Porton Down – data not shown) using these chemostat systems we have found that an MIC calculated using a low titre (10^5 cfu/ml) batch culture is not entirely applicable, there are a number of factors that can affect the susceptibility of an organism or the effectiveness of a drug to be delivered and/or activated.

The work in this chapter aims to identify whether factors such as cell density, the doubling time of the bacteria and the phase of growth of a bacteria influence the susceptibility to isoniazid and rifampicin. This will then help to interpret results in future experiments and indicate whether the current MIC testing practices are a true reflection of the environment in the host that the treatment is going to be active in.

The specific objectives for this work were as follows:

1. To determine whether cell titre has an effect on the MIC of isoniazid and rifampicin against *M. tuberculosis*

2. To determine whether the growth phase of *M. tuberculosis* culture has an effect on the efficacy of Isoniazid and Rifampicin at a range of concentrations to include the MIC

3. To determine whether the growth rate i.e. doubling time of *M. tuberculosis* has an effect on the efficacy of Isoniazid and Rifampicin at a range of concentrations to include the MIC

3.2 Establishment of exponential growth in flask cultures for use as an inoculum

For experiments that were investigating the effect of cell density on the efficacy of isoniazid and rifampicin against *M. tuberculosis* a population of exponentially growing cells was required in order to provide the starting inoculums. By using cells that were still actively dividing, any effects on the results caused by cells being in stationary phase or being nutrient depleted could be minimised. Four seed flasks were set up as described in section 2.2.1, each inoculated with a quarter of a plate of confluent growth of *M. tuberculosis* seed stock that was 2-3 weeks old.

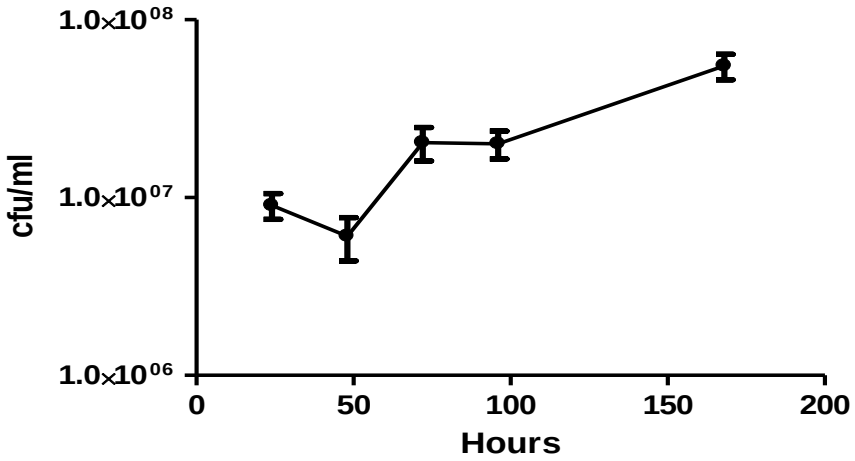


Figure 3.1 Growth of *M. tuberculosis* in flasks in MOD 2 medium (section 2.1.1) shaking at 200 rpm at 37°C to determine the optimum time to sample exponentially growing cells. These data comprise of four separate flask experiments, each with three viable count readings at each timepoint. The error bars represent the standard error of the mean (SEM).

The viable count data in figure 3.1 shows that the average titre of the flasks after 24 hours of growth was 9.0 x 10^6 cfu/ml. It was not possible to take a value at 0 hours as the inoculum from the plate is clumpy and does not disperse well immediately, making any sampling at this time point inaccurate. After 48 hours there was no significant difference in the titre compared to the previous timepoint, this could be due to the *M. tuberculosis* adapting to a new growth medium, having being cultured on agar before being inoculated into the flask leading to a lag phase. The 24 hour result could also be artificially high as it was noted that there were still clumps of bacteria in the flask, if one of these aggregates was sampled it could have led to a titre appearing artificially high. After 48 hours, the flasks all increased in cell titre and by 168 hours after inoculation had risen to an average titre of 5.5x10^7 cfu/ml.

Flasks were sampled at 72 hours of growth for use as an inoculum in cell density experiments, figure 3.1 showed that this would provide a cell sample that was exponentially growing and not

in a stationary or lag phase. It appeared that sampling at around 48 hours would not be ideal due to the lag phase.

3.3 The effect of cell density on the efficacy of Isoniazid

A flask was filled with approximately 300 ml of CMM MOD 2 medium (section 2.1.1) and inoculated with confluent growth of *M. tuberculosis* from agar plates that had been incubated for 2-3 weeks to provide exponentially growing cells at a high titre. The flask was left to grow for 72 hours, these cells were then used to inoculate two sets of flasks containing 50 ml of CMM MOD 2 medium each to a level of $1x10^7$ cfu/ml, $1x10^6$ cfu/ml and $1x10^5$ cfu/ml. A set without isoniazid to provide positive control data and a set containing 0.5 µg/ml isoniazid (1 x MIC) were produced. These were then sampled over a timecourse of 10 days to provide viable count and optical density data and to test the hypothesis that cells at a higher density will be killed more slowly than those at a lower cell density.

The results have been categorised as shown in table 3.1. These parameters were taken from (LaPlante and Rybak, 2004)

Result	**Definition of effect of antibiotic**
$\geq$ 3-$\log_{10}$ cfu/ml reduction	Bactericidal
< 3-$\log_{10}$ cfu/ml reduction	Bacteriostatic
No observed reduction	Inactivity

Table 3.1 Parameters by which the effect of an antibiotic on *M. tuberculosis* is defined.

3.3.1 The effect of a high cell density (1 x 10^7cfu/ml) of *Mycobacterium tuberculosis* on the efficacy of isoniazid

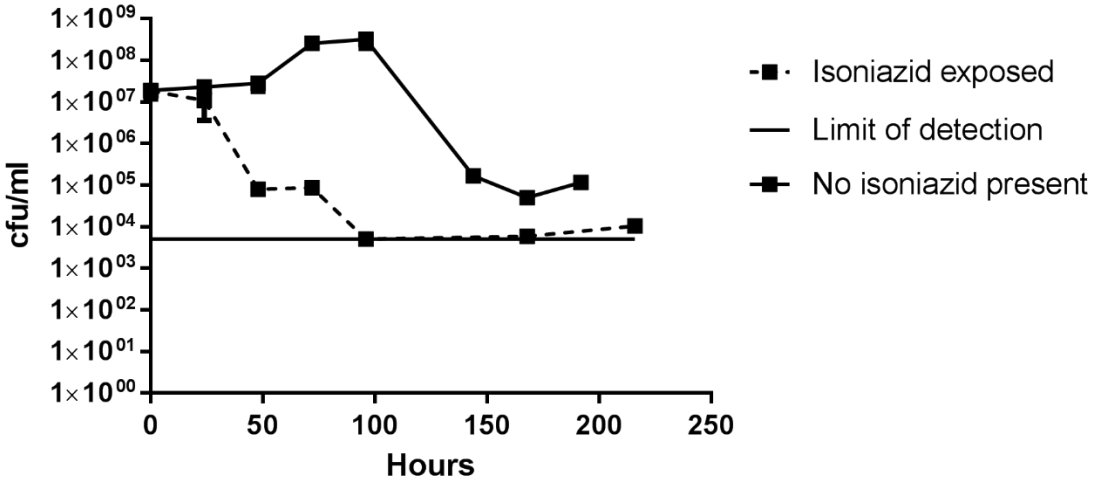


Figure 3.2 A graph showing cells growing at a cell density of 1 x 10^7 cfu/ml in the presence of 0.5 µg/ml isoniazid (MIC) and without the presence of isoniazid. The data is the result of 3 independent flasks with 3 viable counts for each timepoint. The error bars represent the SEM.

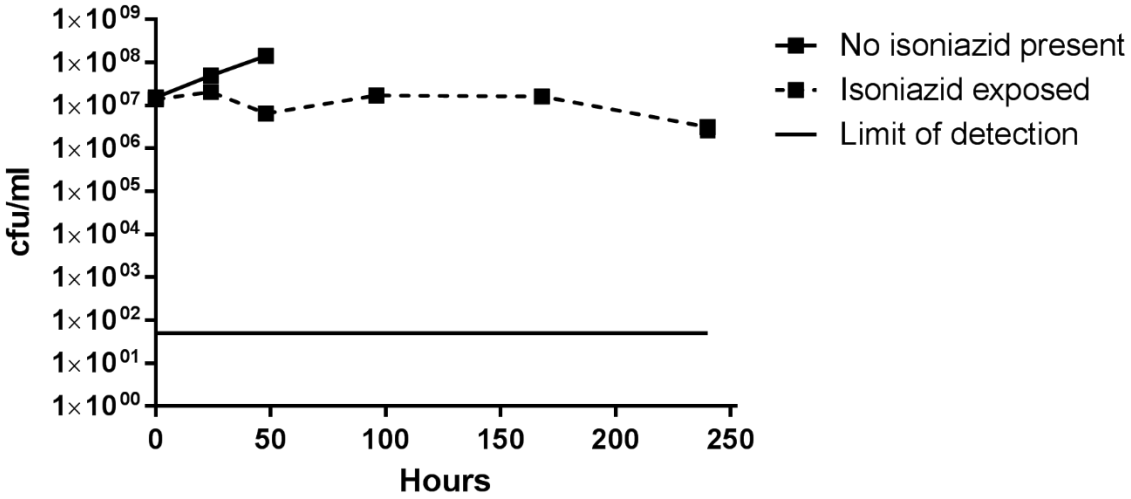


Figure 3.3 A graph showing cells growing at a cell density of 1 x 10^7 cfu/ml in the presence of 0.5 µg/ml isoniazid (MIC) and without the presence of isoniazid. This is a replicate experiment of Figure 3.2, each symbol is the mean of three viable count measurements.

Figures 3.2 and 3.3 show that the flasks were inoculated with an average starting titre of $1.67x10^7$ cfu/ml in the control flasks (without isoniazid) and $1.03x10^7$ cfu/ml in the flasks containing isoniazid.

The control cultures in both experiments grew, doubling after less than 24 hours to a cell titre of $2.1x10^7$ cfu/ml at the 24 hour timepoint, showing conditions were favourable for growth of *M. tuberculosis*. After 48 hours one of the control flasks became contaminated and no further data was available but this flask was showing continuing exponential growth, reaching a peak of $1.43x10^8$ cfu/ml.

Exponential growth continued to 72 hours post-inoculation (in the remaining control flask), reaching a peak cell titre of $1.4x10^8$ cfu/ml. After this point the viable count declined. This is possibly due to a typical bacterial growth curve occurring within the flask with the cells entering a stationary and then death phase 100 hours post inoculation and beyond. The starting titre was intentionally high and could have limited the amount of growth possible due to nutrient depletion and bacterial toxins building up within a batch system such as a flask. A lag phase was seen in Fig 3.2 which lasted for around 48 hours. This lag phase needs to be considered when analysing the data from the flask with isoniazid exposure, any apparently bacteriostatic action of the drug within this first 48 hours could just be a mirroring of this lag phase.

The two independent replicate flasks of *M. tuberculosis* exposed to 0.5 µg/ml isoniazid (1 x MIC) added showed different profiles to each other. The results from these experiments were not combined and averaged as the profiles showed differing trends.

After 48 hours exposure to isoniazid there is a significant decrease in viable cells ($p=0.005$), dropping to $7.6x10^4$ cfu/ml (figure 3.2). This level of viability then remains constant at 72 hours with no further killing occurring until 96 hours when a drop in viability to the limit of detection is seen ($5x10^3$ cfu/ml), this value is then maintained until 216 hours after exposure with isoniazid when a viable count of $1x10^4$ cfu/ml was detected. This indicates that there were still viable cells present in the culture throughout the exposure to isoniazid. The effect of the isoniazid appears to bactericidal throughout the experiment.

There was no significant change in the viable cell number after 24 hours ($p=0.100$ using Mann Whitney) (figure 3.3). This is in contrast to the flask containing no isoniazid acting as a control culture and shows the effect of isoniazid after 24 hours exposure appears to be bacteriostatic. This flask shows no further change after 96 hours ($p=0.2683$), with the viable count remaining

stable at around $1x10^7$ cfu/ml. At the end timepoint of the experiment the cell titre is still high at $1.6x10^7$ cfu/ml, showing no significant difference (p=0.2683) to the starting cell titre at 0 hours. The action of isoniazid appears to remain bacteriostatic throughout this culture as would be expected from an MIC dose, neither causing cell death nor allowing growth of *M. tuberculosis*, using the definitions in table 3.1, isoniazid in this Fig 3.3 would be defined as inactive, if this were an infection there would be a very high titre of bacteria still remaining. This effect appeared to be immediate as there was no increase in cell numbers after 24 hours which would be slightly more than the doubling time for *M. tuberculosis in vitro.* This did not reflect the result seen in figure 3.2 in terms of cfu/ml but both flasks showed a population of cells that persist through treatment of isoniazid. Previous work using these techniques has found the MIC of isoniazid to be 0.5 µg/ml which was reflected in the results shown in figure 3.3, this could indicate that these results are the true result and that the results obtained in figure 3.2 are anomalous. The limit of detection was also high in the results shown in figure 3.2 and so for the repeat of this work (the results seen in figure 3.3) the design of the method was altered by plating out more dilutions to achieve a lower limit of detection. The results at the lower cell densities may also give an indication as to which set of results reflects the true nature of the effect of cells growing at 10^7 cfu/ml on the efficacy of isoniazid.

3.3.2 The effect of intermediate and low cell densities (1 x 10^6 cfu/ml and 1x 10^5 cfu/ml) of *Mycobacterium tuberculosis* on the efficacy of isoniazid

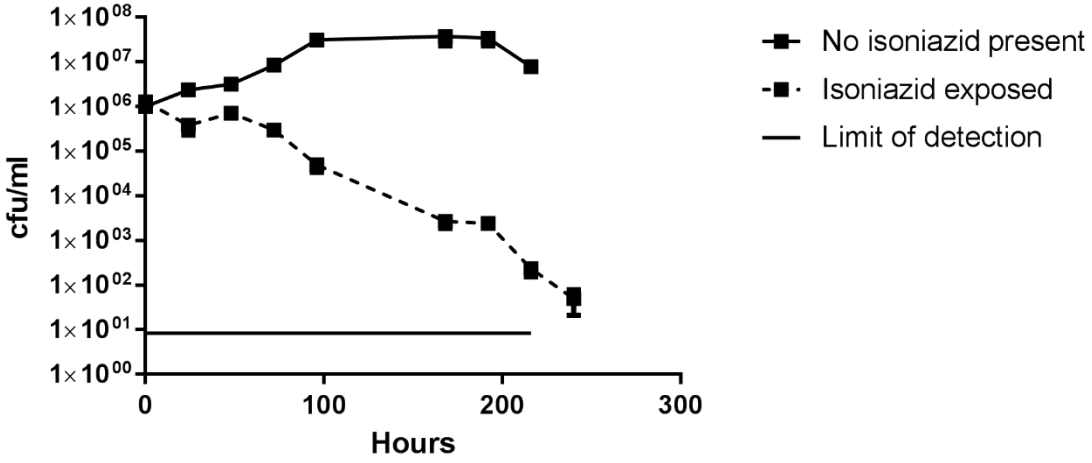


Figure 3.4 The effect of cell density on cells at 10^6 cfu/ml with and without 0.5 µg/ml (1 x MIC) Isoniazid. The lines represent the average of at least two independent flasks with 6 viable counts from each flask at each timepoint, error bars are present denoting the SEM. The limit of detection is represented by the solid reference line.

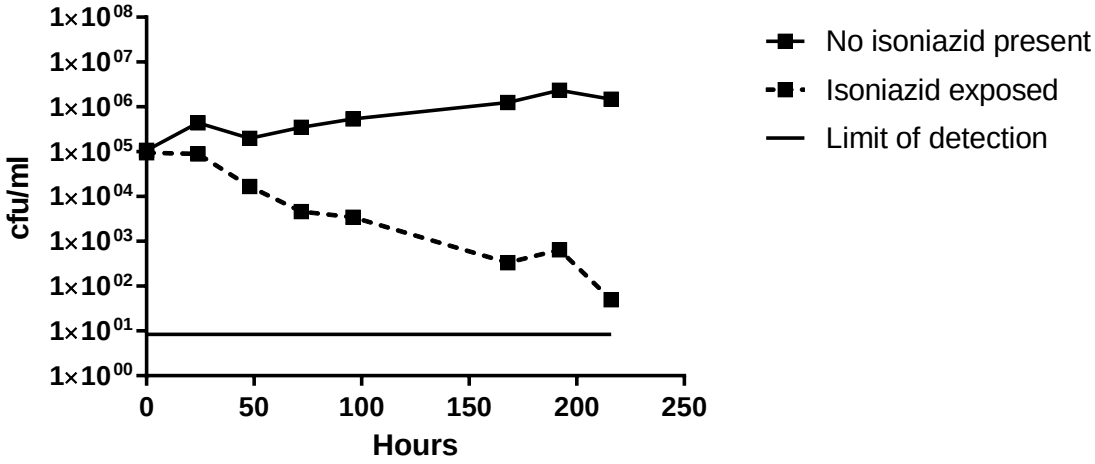


Figure 3.5 The effect of cell density on cells at 10^5 cfu/ml with 0.5 µg/ml (1 X MIC) isoniazid and without isoniazid. The lines represent the average of two independent flasks, with 6 viable counts performed on each flask at each timepoint. Error bars were applied denoting the SEM, in cases of very small error these may not be visible due to the scale of the y-axis. The limit of detection is represented by the solid horizontal reference line

The growth of *M. tuberculosis* at a titre of 10^6 cfu/ml (figure 3.4) showed reproducibility and the data was averaged to increase the statistical power and reliability of the results. The flasks were inoculated with the desired starting cell titre of 1x10^6 cfu/ml, with an average of 1x10^6 cfu/ml in

the control cultures and $1.3x10^6$cfu/ml in the cultures containing isoniazid. The control cultures grew exponentially between 0 and 96 hours reaching a peak viable count of $3.1x10^7$ cfu/ml, indicating conditions were favourable for growth of *M. tuberculosis*. There was no decline in this viability up to 192 hours post inoculation indicating any decline in viability seen in the flask containing isoniazid would be due to the presence of antibiotic rather than unfavourable growth conditions.

The flasks inoculated at a titre of $1x10^5$ cfu/ml (figure 3.5) were to represent the standard titre that is usually used in assessing drug efficacy, such as MIC experiments and determining susceptibility of organisms to antibiotics (Udekwu *et al.*, 2009). The data in figure 3.5 shows that the control flasks were inoculated with an average cell titre of $1x10^5$cfu/ml and the flasks containing isoniazid were inoculated with an average cell titre of $9.5x10^4$ cfu/ml. The control cultures grew exponentially, reaching a peak $2x10^6$ after 192 hours post inoculation, showing that at this starting titre the *M. tuberculosis* is able to proliferate and that the growth conditions were favourable.

At 24 hours of growth and drug exposure the cultures containing isoniazid at the intermediate cell density (figure 3.4) showed a decrease in viability which was statistically significant ($p=0.0028$) when compared to the 0 hour value. This is in contrast to the low cell density experiment results seen in figure 3.5 which shows a delay in the reduction of the viable count until after the 24 hour timepoint as there is no significant difference between this cell titre and the 0 hour value ($p=0.5242$)

After 24 hours the viable cell number began to decline steadily at both intermediate and low cell densities. In the intermediate cell density experiment the cells with a starting titre of $1x10^6$ cfu/ml showed a decrease in viability of 60% between 48 and 72 hours post inoculation and exposure, which increases to 83% between 72 hours and 96 hours. The difference in the viable cell numbers between 96 and 168 hours showed a decrease in viable cell count of 94%, this is a reduction in the rate of killing when compared to between 72 and 96 hours as this drop in viability is over a shorter time period. The low cell density cultures resulted in a drop in titre after 48 hours to $1.7x10^4$ cfu/ml and continued to fall to $3.3x10^2$ cfu/ml by 168 hours

In the intermediate cell density experiment (figure 3.4) after 168 hours exposure to isoniazid the rate of death slowed considerably with 90% of the population that were viable at 168 hours remaining viable 24 hours later, this period is then followed by a further increase in killing at 216 hours resulting in a final viable cell titre of $2.3x10^2$ cfu/ml. In the low cell density experiment a

slight increase in viable count was seen at 192 hours which also occurred in the high and intermediate cell density experiments (figure 3.2 and 3.4), followed by a period of more rapid killing than seen throughout the timecourse, resulting in a viable count of $5x10^1$ cfu/ml.

There is a clear difference in the response of the control and drug exposed cultures at both intermediate and low cell density experiments indicating that the cell death seen in the drug exposed cultures was due to the presence of isoniazid. The overall trend of these results shows that 0.5µg/ml isoniazid results in a bactericidal killing trend when the culture is inoculated with either $1x10^6$ cfu/ml or $1x10^5$ cfu/ml. The bactericidal action of isoniazid seems to be delayed however as death rates at the beginning of the time course are much lower than after 48 hours exposure in both the intermediate and low cell density experiments.

It is worth noting that the half life of isoniazid has been calculated to be between 1 and 5 hours *in vivo*, with variation due to the speed of acetylation in the liver of a patient, the half life of rifampicin is around 3.5 hours and it is also metabolised in the liver (Arbex *et al.*, 2010). In experiments such as the ones performed in this chapter, antibiotic is not re-added to the cultures so the half life becomes important. Within the liquid culture however, the enzymes responsible for the metabolism of isoniazid *in vivo* are not present. Nevertheless, the MIC of isoniazid and rifampicin was tested after culture in medium for a prolonged period at PHE, Porton Down (data not shown) and the MIC was found to be unaffected. This means that the antibiotics used in prolonged experiments was still active within the medium.

3.4. The effect of different cell densities on the efficacy of rifampicin against *Mycobacterium tuberculosis*

These experiments were set up as described in section 3.3 but the antibiotic added was rifampicin at a concentration of 8 ng/ml. This was the MIC calculated previously within the tuberculosis research group at PHE, Porton Down. The hypothesis was that cells at a higher density would be more recalcitrant to the effects of rifampicin within the medium.

3.4.1 High cell density

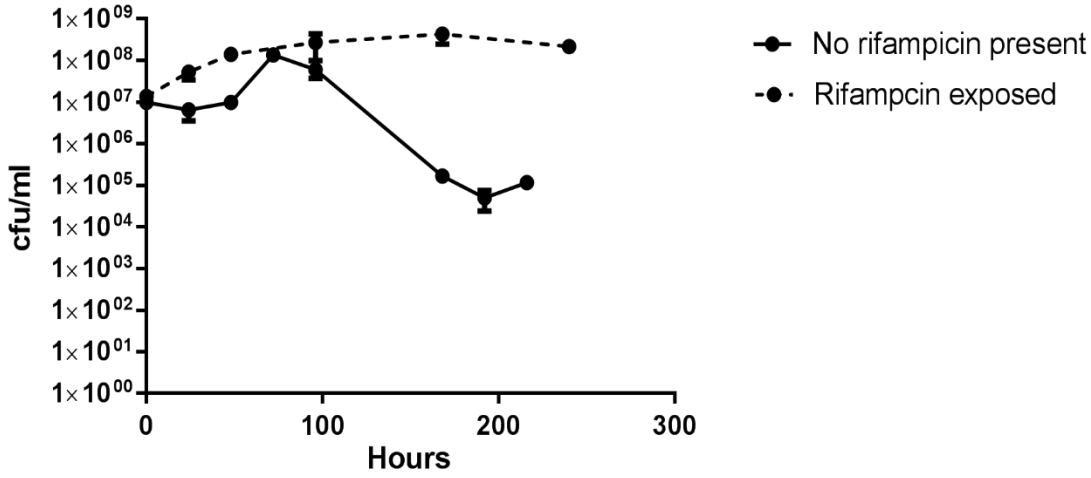


Figure 3.6 The effect of cell density on cells at 10^7 cfu/ml with 8 ng/ml (1 x MIC) rifampicin and without rifampicin. The lines represent the average of two independent cultures, with each having 3 viable counts at each timepoint (n=6) error bars are present denoting the standard deviation.

The culture with rifampicin added in figure 3.6 showed the inoculum was $1.4x10^7$ cfu/ml. This culture showed an increase in viability between the 0 hour and 168 hour final time point. The resulting viable count was $4.3x10^8$ cfu/ml. The viable cells then doubled in number between 24 and 48 hours, 48 and 96 hours and 96 and 168 hours. The doubling time of the *M. tuberculosis* cells exposed to rifampicin increased throughout the course of the experiment from 24 hours to 48 hours to a final doubling time of 72 hours, indicating that the rifampicin was having an effect on the growth rate but not the viability of the cells.

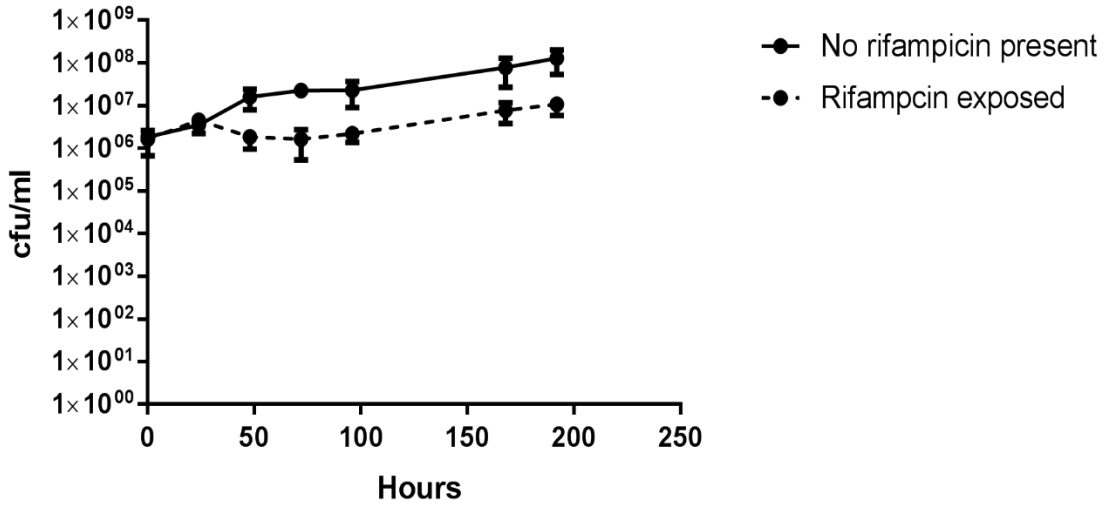


Figure 3.7 The effect of cell density on cells at 10^6 cfu/ml with 8 ng/ml (1 x MIC) rifampicin and without rifampicin. The lines represent the average of two independent cultures, with 9 viable counts at each timepoint (n=18) error bars are present denoting the standard deviation.

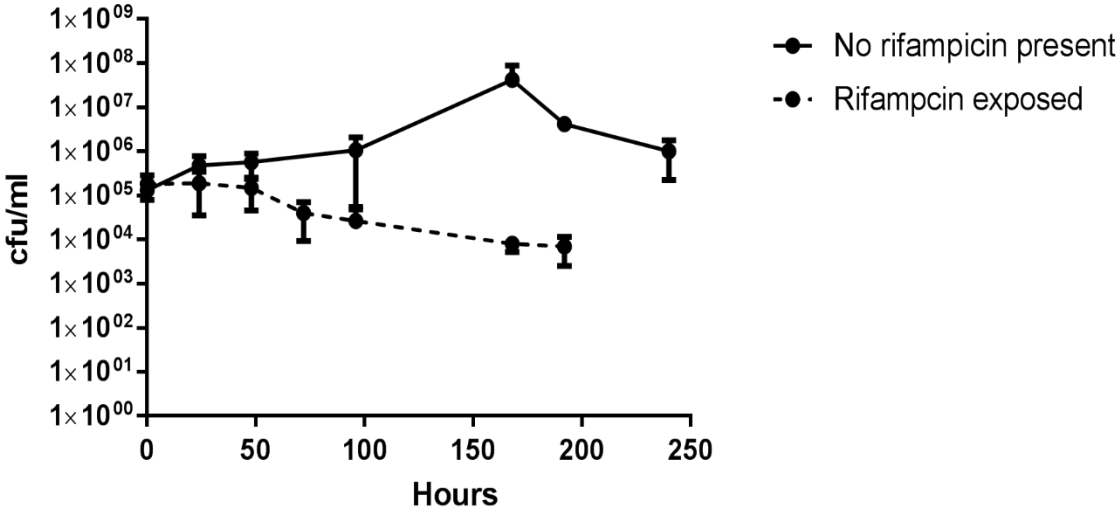


Figure 3.8 The effect of cell density on cells at 10^5 cfu/ml with 8 ng/ml (1 x MIC) rifampicin and without rifampicin. The lines represent the average of two independent cultures, with at least 6 viable counts at each timepoint (n=≥ 12). Error bars are present denoting the standard deviation.

3.4.2. Intermediate cell density

The intermediate cell density data (figure 3.7), shows that the control cultures increased in viability to a final viable count of $1.28x10^8$ cfu/ml, indicating conditions were favourable for growth from a starting inoculum of $1x10^6$ cfu/ml. The average inoculum at 0 hours for the control cultures was $1.8x10^6$ cfu/ml and for the cultures containing rifampicin it was $1.6x10^6$ cfu/ml and there was no significant difference between these numbers (p=0.1605 Mann Whitney). At 24 hours after inoculation, there was no significant difference seen between the control and drug containing cultures, both showing an increase in viable cell numbers. This indicates that the rifampicin is not acting on the *M. tuberculosis* cells within the first 24 hours of exposure, this is similar to the high cell density experiment seen in Fig 3.6. At 48 hours the rifampicin flasks are showing a significantly lower cell titre than the control (p=<0.0001) indicating the rifampicin was inhibiting the growth of the bacteria. Evidence of rifampicin having an effect was also seen in the high density experiment but in contrast there is a loss of viable cells in the intermediate cell density rather than a slowing of the growth rate as seen in the high density experiment.

No additional effect of rifampicin was seen after 48 hours of exposure, the control culture and culture containing rifampicin both maintained a viable count, showing the rifampicin appears to have reduced the viable count by 1 log in the first 48 hours when compared to the control culture. Between 96 and 168 hours the viable count increases to $7.9x10^7$ cfu/ml in the control flask and $7.8x10^6$ cfu/ml in the rifampicin containing flasks continuing the log difference between the flasks and showing that the *M. tuberculosis* is adapting to the presence of rifampicin, with a doubling time of approximately 36 hours. This doubling time is slower than the observed *in vivo* and *in vitro* value of around 24 hours and as also seen in the high density experiment, it is showing that a reduction in growth rate could be reducing the effect of rifampicin.

3.4.3 Low cell density

The average starting titre of the control cultures was $1.3x10^5$ cfu/ml and $1.8x10^5$ cfu/ml for the cultures containing rifampicin, this difference was not statistically significant (p0.2896) (figure 3.8)

By 24 hours the control cultures had increased to $4.8x10^5$ cfu/ml and the rifampicin containing cultures maintained a population at $1.9x10^5$ cfu/ml, signifying that rifampicin is having a bacteriostatic effect on the *M. tuberculosis* cells, that continues through to 48 hours of incubation with no significant difference between the 24 and 48 hour viable counts (p=0.3181). At 72 hours there is a drop in viability within the cultures containing rifampicin to $4x10^4$ cfu/ml indicating the rifampicin is having an effect, this is confirmed by the control flasks, containing no

rifampicin, increasing in viability. This drop continues to the end timepoint when an average of $7x10^3$ cfu/ml were recovered from the rifampicin treated cultures. There is still a population of cells remaining in the flasks after 192 hours and the killing slowed between 168 and 192 hours but these viable counts were on or very close to the limit of detection so the reliability of these numbers is perhaps not as high as earlier in the timecourse. This is in contrast to the higher cell densities where growth was seen in the presence of rifampicin by the end of the time course.

These results indicate that an MIC dose of rifampicin added to cells growing at 1 x 10^5 cfu/ml, as would be the case in a clinical setting (Udekwu *et al.*, 2009) result in the expected growth curve, bacteriostasis and some death. At higher, perhaps more clinically relevant bacterial titres (De Groote *et al.*, 2011; Ordway *et al.*, 2010; Sharpe *et al.*, 2009, 2010a) the effect of the rifampicin is diminished. Along with the findings in this chapter and work done in the chemostats at PHE, Porton Down by members of the TB research group, it appeared that 8 ng/ml was not a high enough concentration of rifampicin for an MIC within high titre cultures. Further MIC experiments were performed using cultures grown in flasks, allowing a high cell density to be achieved, to determine an appropriate concentration to be used. The results showed that 32 ng/ml rifampicin was the concentration that yielded the response expected of an MIC (bacteriostasis) (figure 3.9). This result was true regardless of the length of exposure and this concentration (32 ng/ml) was used in subsequent experiments in this thesis as an MIC for rifampicin.

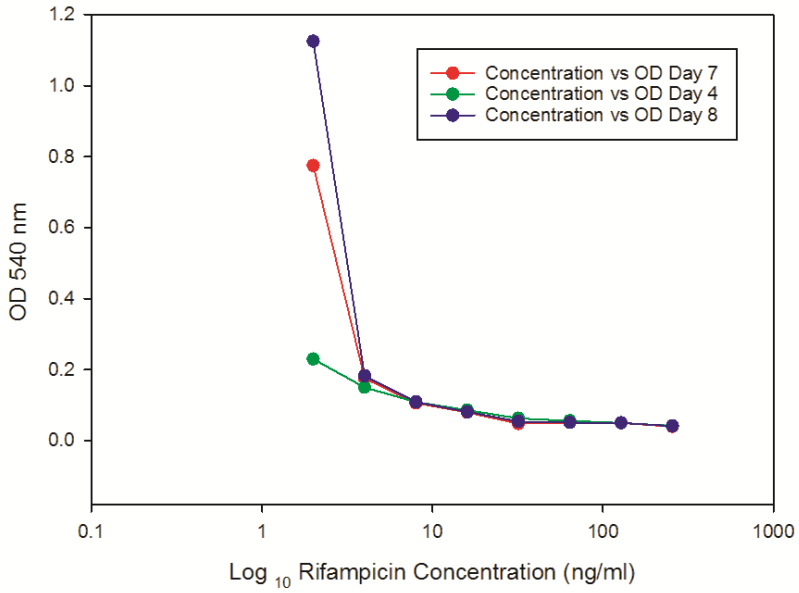


Figure 3.9 The OD_{540} of *M. tuberculosis* cultures after 4, 7 and 8 days of exposure to varying levels of rifampicin (0-256 ng/ml). The MIC was taken as the first concentration with no increase in growth (32 ng/ml)

3.5 The impact of the growth rate of *M. tuberculosis* on the efficacy of isoniazid and rifampicin

It has been shown that growth rate can have an effect on the number of persister cells in a population (Sufya *et al.*, 2003). *M. tuberculosis* is thought to grow at different rates *in vivo* with slow growing cells thought to reduce the efficacy of treatment which as with many antibiotics preferentially kill rapidly dividing bacteria. The chemostat allows control of growth rate, using this model doubling times of 23.1 hours, 69.3 hours and 138.6 hours were chosen to represent actively dividing, slow growing and very slow growing populations of *M. tuberculosis* that might be encountered by isoniazid and rifampicin. These experiments were set up as described in section 2.4 and the cells used as an inoculum, were sampled from continuous cultures at the aforementioned doubling times. The readout for antibiotic efficacy was the growth ratio of the cultures. This was calculated by dividing the viable count at 96 hours post-inoculation by the initial viable count at 0 hours (inoculation), the method is described in section 2.4. When cells are referred to having an MGT of 23.1, 69.3 or 138.6 hours in the following sections, this refers to the MGT of the chemostat the samples of cells were taken from, the results presented in the following sections represent these chemostat samples used as inoculums for batch cultures exposed to isoniazid.

3.5.1 *M. tuberculosis* at varying growth rates exposed to isoniazid

To assess whether the differences in the susceptibility to isoniazid between growth rates was apparent after just 24 hours, analyses were performed on viable counts taken 24 and 96 hours after the initial antibiotic exposure and the growth ratio was calculated. This would then inform as to how quickly the antibiotic acted on the bacterial cells and also how early on in the timecourse the effects of growth rate could be seen to affect the susceptibility. The drug concentrations in these experiments are static, however, by using a wide range of concentrations and assessment of mycobacterial killing at different time points during drug

exposure, insight can be provided into the dynamic killing capacity of fluctuating drug concentrations (de Steenwinkel *et al.*, 2010).

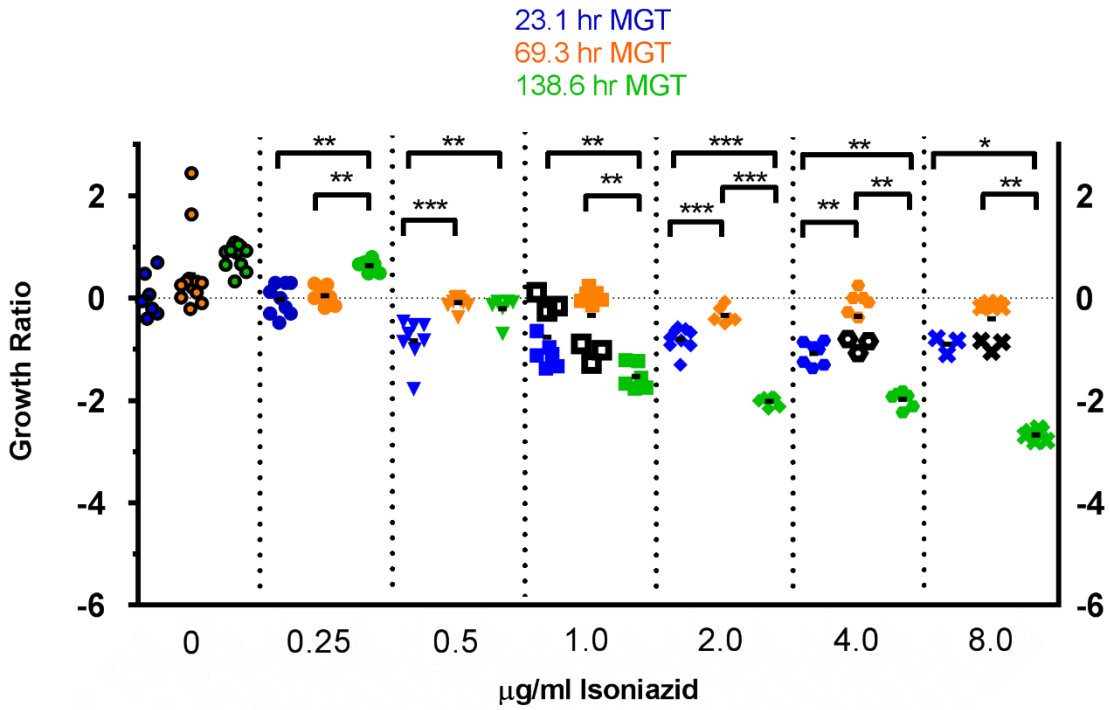


Figure 3.10. *M. tuberculosis* exposed to varying concentrations of isoniazid (MIC 0.5 µg/ml) over 24 hours. Cells were removed from continuous culture in which the doubling time had been controlled at either 23.1 hours, 69.3 hours or 138.6 hours and used as inocula for smaller cultures with varying levels of isoniazid present. P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05. Each symbol represents a growth ratio of a viable count taken at 0 and 24 hours. Two independent chemostat experiments were run for each growth rate, where the results are bimodal the results have been separated (colour and black) based on the chemostat the inoculum originated from.

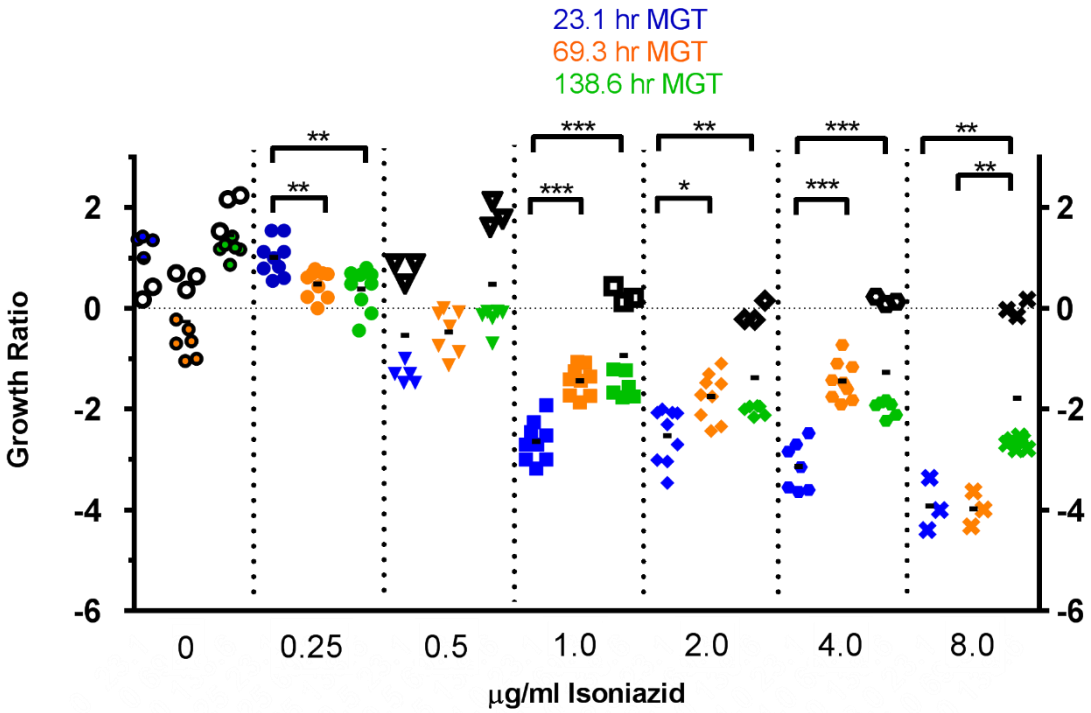


Figure 3.11. *M. tuberculosis* exposed to varying concentrations of isoniazid (MIC 0.5 µg/ml) over 96 hours. Cells were removed from continuous culture in which the doubling time had been controlled at either 23.1 hours, 69.3 hours or 138.6 hours and used as inocula for smaller cultures with varying levels of isoniazid present. P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05. Each symbol represents a growth ratio of a viable count taken at 0 and 96 hours. Two independent chemostat experiments were run for each growth rate, where the results are bimodal the results have been separated (colour and black) based on the chemostat the inoculum originated from. These results are from the same experiment as the earlier timepoint of 24 hours seen in Figure 3.10

When the results were analysed after 24 hours of antibiotic exposure (Figure 3.10) there was no difference between the fastest growth rates (23.1 hour MGT and 69.3 hour MGT) in the susceptibility of cells to sub-MIC levels (0.25 µg/ml) of isoniazid, with both showing positive growth ratios (an increase in the number of viable cells). The slowest growing cells also showed a positive growth ratio but this was significantly higher than the cells growing at the two faster growth rates indicating reduced susceptibility to isoniazid at a very slow growth rate. After 96 hours of exposure (as seen in Fig 3.11) the slowest growing cells were the most susceptible.

At MIC levels of isoniazid there was no growth rate effect at 96 hours, there was a similar pattern after 24 hours of exposure. The data is more spread at 96 hours however, this suggests there may be more heterogeneity in the populations of *M. tuberculosis* after exposure to isoniazid for 96 hours.

At levels of isoniazid above the MIC at 96 hours (figure 3.11) the cells with an MGT of 69.3 hours were significantly less susceptible to isoniazid than those at the fastest growth rate, there was no discrimination between cells with an MGT of 69.3 and 138.6 hours. The effect of the isoniazid is concentration dependent up to 2 x MIC, increasing the concentration above this does not have an effect until 16 x MIC. Conversely, at 24 hours the effect of isoniazid was concentration independent at the two fastest growth rates when all levels of isoniazid appeared to be inhibiting growth rather than killing. This lag in kill was also seen in the cell density results in sections 3.3.1 – 3.4. This difference in susceptibility between the two fastest growth rates was generally apparent after 24 hours of exposure, the slowest growing cells however were the most susceptible at levels of isoniazid at MIC and above at 24 hours which is in contrast to the result after 96 hours.

These very slow growing cells appeared to be affected quickly by isoniazid at 24 hours and the effect was concentration dependent until 2 x MIC, following a similar pattern to the faster growth rates at 96 hours. The control data (0 µg/ml isoniazid) at 24 and 96 hours (figures 3.10 and 3.11) showed the cells with an MGT of 138.6 hours had grown the most and appeared to have adapted to batch culture more successfully than the two faster growth rates. The *M. tuberculosis* with an MGT of 138.6 hours did not appear to have a lag phase and consequently the isoniazid acted within 24 hours resulting in a similar pattern of results seen at 96 hours with the two faster growth rates. During the timecourse of this experiment between 24 and 96 hours a population of very slow growing cells emerged that were adapted and therefore less susceptible than the faster growth rates to very high levels of isoniazid.

3.5.2 The effect of the growth rate of *M. tuberculosis* on the efficacy of rifampicin

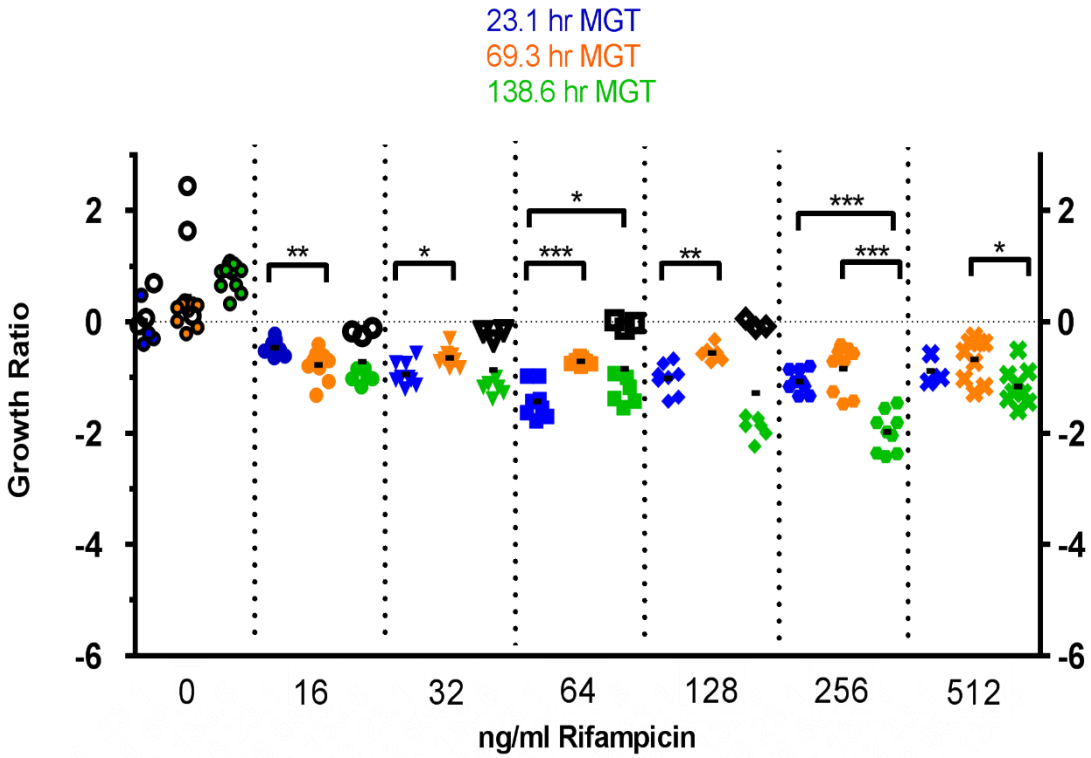


Figure 3.12 *M. tuberculosis* exposed to varying concentrations of rifampicin (MIC 32 ng/ml) over 24 hours. Cells were removed from continuous culture in which the doubling time had been controlled at either 23.1 hours, 69.3 hours or 138.6 hours and used as inocula for smaller cultures with varying levels of rifampicin present. P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05. Each symbol represents a growth ratio of a viable count taken at 0 and 24 hours. Two independent chemostat experiments were run for each growth rate, where the results are bimodal the results have been separated (colour and black) based on the chemostat the inoculum originated from.

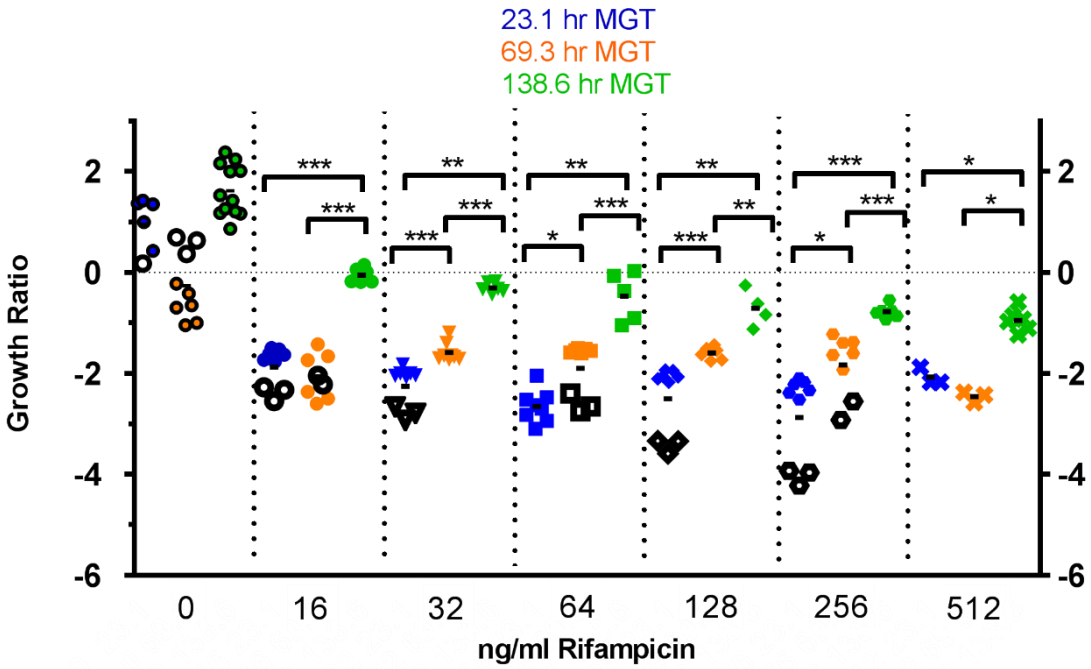


Figure 3.13 *M. tuberculosis* exposed to varying concentrations of rifampicin (MIC 32 ng/ml) over 96 hours. Cells were removed from continuous culture in which the doubling time had been controlled at either 23.1 hours, 69.3 hours or 138.6 hours and used as inocula for smaller cultures with varying levels of rifampicin present. P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05. Each symbol represents a growth ratio of a viable count taken at 0 and 96 hours. Two independent chemostat experiments were run for each growth rate, where the results are bimodal the results have been separated (colour and black) based on the chemostat the inoculum originated from.

The results from the cell density work indicated that an MIC of rifampicin of 8 ng/ml was unsuitable for high titre cultures and the MIC was adjusted to 32 ng/ml (section 3.4.2). At a sub-MIC level (16 ng/ml) of rifampicin there was a decline in viability after 96 hours in the cells with an MGT of 23.1 and 69.3 hours with no difference between these growth rates. The growth of the slowest growing cells was inhibited showing a bacteriostatic effect of this level of drug. The discrimination between growth rates is obvious after 96 hours of exposure (figure 3.13) but at 24 hours this effect is reduced.

Levels of rifampicin at MIC and above showed a pattern, the longer the MGT of the cells, the less susceptible they were to rifampicin after 96 hours (figure 3.12). This pattern continued until 16 x MIC (512 ng/ml) when the two fastest growth rates are indistinguishable from each other but the slowest growth rate was significantly less susceptible. The effect of rifampicin appeared to be concentration independent at all growth rates at 96 hours which is in contrast to the isoniazid results (figure 3.10) which showed a concentration dependent effect up to 2 x MIC.

The clear distinction between growth rates observed after 96 hours of exposure could not be observed after 24 hours of exposure. There is a significant difference between the 23.1 and 69.3 hour MGT populations, with the latter being slightly less susceptible, the range of killing was small and there was no difference between 69.3 and 138.6 hour MGT populations until 8 x MIC. This is in contrast to the isoniazid results in section 3.5.1 which showed the slowest growing cells to be the most susceptible after 24 hours, this was only true at the highest levels of rifampicin. Rifampicin affected the slowest growing cells after 24 hours but did not have any further effect as evidenced in figure 3.12, the cells with an MGT of 23.1 and 69.3 hours seemed to lose most of their viability after 24 hours of exposure. This delay in killing of the faster growth rates is a similar pattern to the isoniazid results.

The results for isoniazid and rifampicin are not the same with differences at sub-MIC and MIC levels of antibiotic being particularly striking. Cells growing in sub-MIC levels of isoniazid showed growth which was in contrast to the decline in viability seen when the cells were grown in rifampicin. The highly significant difference seen between growth rates when cells were exposed to rifampicin (the slower the growth, the less susceptible to cells) was not seen when exposed to MIC levels of isoniazid. At levels of antibiotic above the MIC the susceptibility profiles of the bacteria to both isoniazid and rifampicin were very similar with a high level of killing with the slowest growth rate being the least susceptible. This population of very slow growing cells (MGT 138.6 hours) appears the most susceptible to both antibiotics at 24 hours but there is no further killing of this population over the timecourse, it appears there may be two populations, one that

is highly susceptible to isoniazid and rifampicin that is killed within 24 hours and another that is recalcitrant and able to persist through the presence of drug.

3.6 The effect of the growth phase of *M. tuberculosis* on the efficacy of isoniazid and rifampicin

The aim of this work was to establish whether the phase of growth of *M. tuberculosis* has an effect in the susceptibility to isoniazid and rifampicin. Most antibiotics target actively dividing cells which are exponentially growing. *M. tuberculosis* can reside in the host in a variety of states including in granulomas where it is thought to be dormant or non-replicating. This population of bacteria is very difficult to treat and this work aims to use a culture modelling different phases of growth as a source of cells to determine the effect of growth phase on the effectiveness of isoniazid and rifampicin.

The experiments were set up as described in section 2.4, using growth ratio of the cultures as a readout for antibiotic efficacy. This was calculated by dividing the viable count at 144 hours post-inoculation by the initial viable count at 0 hours (inoculation), the method is described in section 2.4.1.

The cells used as an inoculum, were growing in an extended controlled batch culture as described in section 2.2.3.6 and (Bacon *et al.*, 2014). The aim was to remove cells at different growth phases; exponential, stationary, death phase and non-replicating persistence caused by nutrient starvation. The total viable count data for the batch culture can be seen in figure 3.14 with annotation to describe when in the culture the samples were removed.

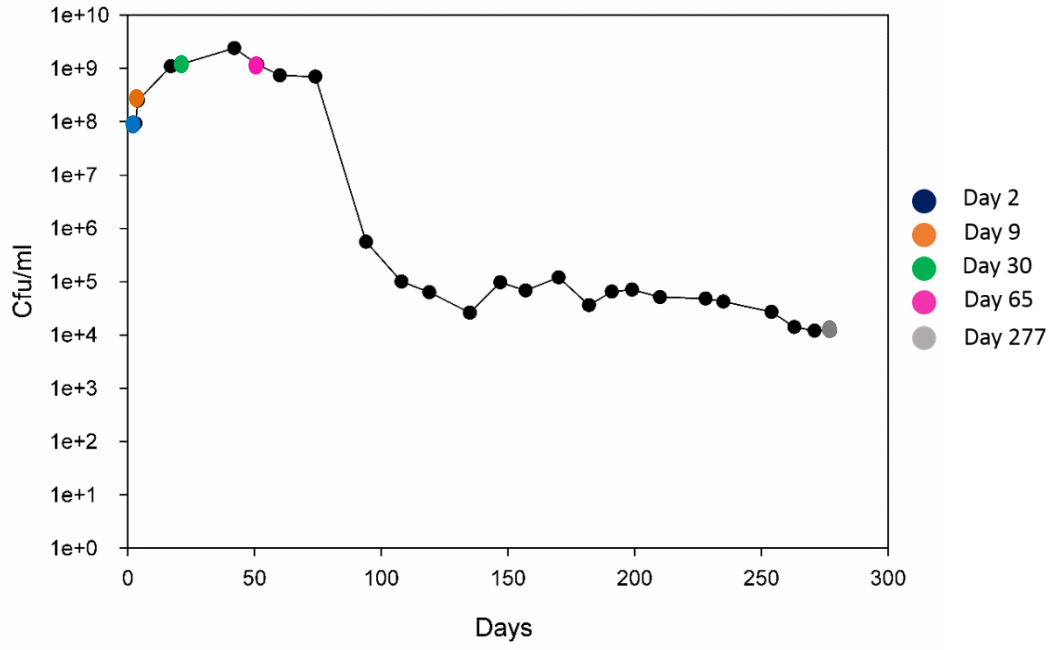


Figure 3.14 *M. tuberculosis* grown in batch culture as described in section 2.2.3.6. Samples were taken for antibiotic susceptibility testing at days 2, 9, 30, 65 and 277.

Samples taken at days 2 and 9 were in the exponential phase of growth (figure 3.14). It appears that the exponential phase slowed after day 9 leading to stationary phase which was sampled at day 30, this phase lasted around 40 days. The sample taken at day 65 was in late stationary phase and also coincided with the beginning of a death phase, where there was a decline in viable count over the course of 60-70 days as the culture became nutrient depleted. The sample taken at the end of the culture (day 277) was when the culture was in a non-replicating persistent (NRP) phase, characterised by a slow growing or non-replicating dormant-like state, in this culture, this phase lasted for at least 60 days.

3.6.1 The effect of growth phase of M. tuberculosis on the efficacy of isoniazid

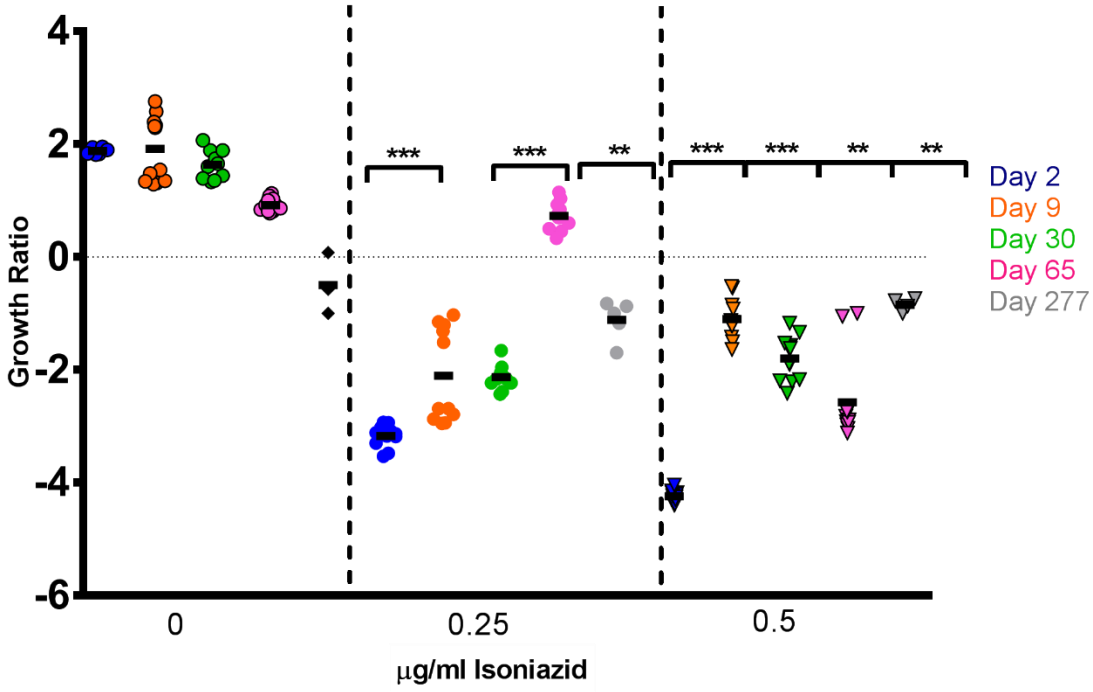


Figure 3.15 *M. tuberculosis* grown in batch culture and sampled at either 2 days, 9 days, 30 days, 65 days, or 277 days. These samples were used as inocula for smaller cultures exposed to varying concentrations of isoniazid (MIC 0.5 µg/ml) over a 144 hour period. This graph shows levels of isoniazid from 0 µg/ml to 0.5 µg/ml). P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05 (n=≥3).

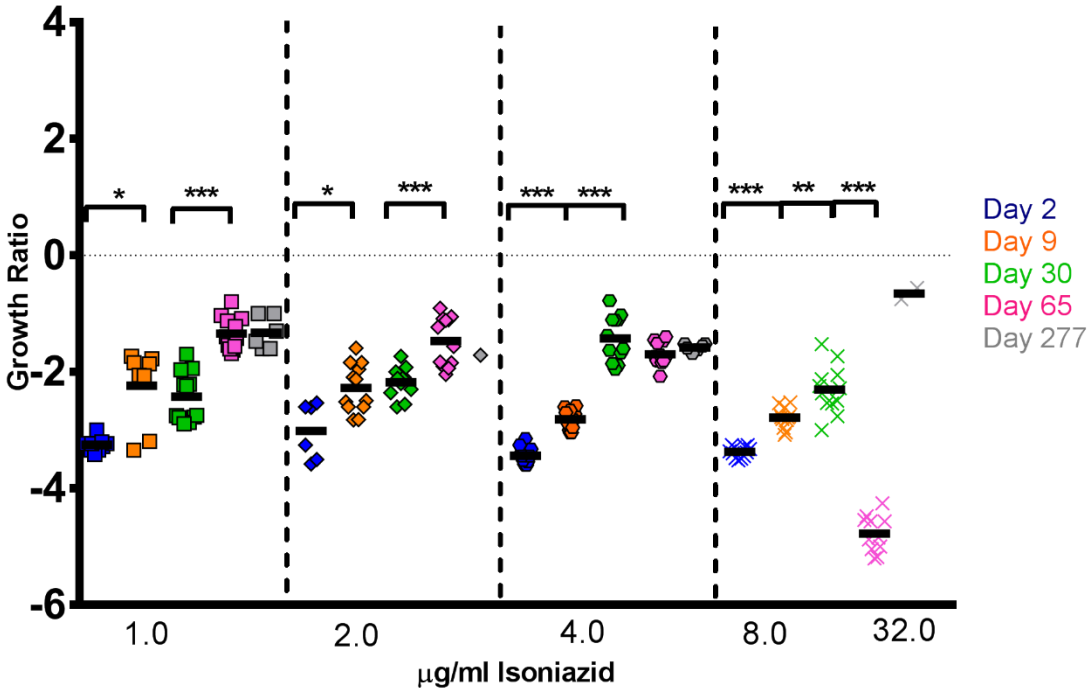


Figure 3.16 *M. tuberculosis* grown in batch culture and sampled at either 2 days, 9 days, 30 days, 65 days, or 277 days. These samples were used as inocula for smaller cultures exposed to varying concentrations of isoniazid (MIC 0.5 µg/ml) over a 144 hour period. This graph shows levels of isoniazid from 1.0 µg/ml to 32 µg/ml). P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05 (n=≥3).

At a sub-MIC level of isoniazid (0.25 µg/ml) cells cultured for 65 days were the least susceptible to isoniazid (figure 3.15). There was no significant difference between cells that were cultured for 9 and 30 days, perhaps showing that day 9 was the start of stationary phase as evidenced in figure 3.13. The cells taken from the non-replicating persistence phase were more susceptible to isoniazid than the cells removed at 65 days of culture but the non-replicating persistence sample was still less susceptible than all other time points earlier than day 65.

The exponentially growing cells (day 2 sample) were the most susceptible to an MIC level of isoniazid with the NRP cells being the least susceptible. An increase in susceptibility was seen between days 9 and 65 (from early to late stationary/death phase). At levels of isoniazid above the MIC (figure 3.16), the overall trend was a decrease in susceptibility the longer the bacteria were cultured for. As was seen with a sub-MIC level of isoniazid, at 2 x MIC and 4 x MIC there was no significant difference between cells removed from culture at day 9 and day 30, indicating that these cells could be in a similar growth phase.

When samples were removed at 65 days (late stationary/early death phase) and from the NRP phase, the level of isoniazid was increased from 16 x MIC to 64 x MIC to try to determine what level of isoniazid would be highly effective against these cells, due to a large proportion of them remaining viable at the lower isoniazid concentrations. There was a high level of kill seen in the cells removed in the late stationary/early death phase (day 65), this was the highest level of kill seen throughout this experiment. Cells in the NRP phase showed a small level of kill similar to the lower concentrations of isoniazid, appearing much less susceptible to high levels of isoniazid than cells taken earlier in the culture. It must be noted that only two data points (i.e. comparable viable counts at the beginning and end of the experiment) are available at this concentration so the data is less reliable than earlier time points and lower antibiotic concentrations.

The control data for the cells removed at the NRP phase also needs to be discussed. The overall growth ratio is negative, due to poor growth of the control samples. A sample was taken from the NRP phase prior to this experiment to determine how long it would take for these *M. tuberculosis* cells to re-grow (using OD_{540nm} as a measurement), the result being that it took 12-14 days to see an increase in $OD_{540nm.}$. Based on this finding, the antibiotic susceptibility assay was left for a further 192 hours (a total of 336 hours) but when the samples were plated out they were found to be contaminated. The poor growth of the control could be due to a lag phase indicated by the amount of time it took to see an increase in $OD_{540nm.}$

3.6.2 The effect of growth phase of *M. tuberculosis* on the efficacy of rifampicin

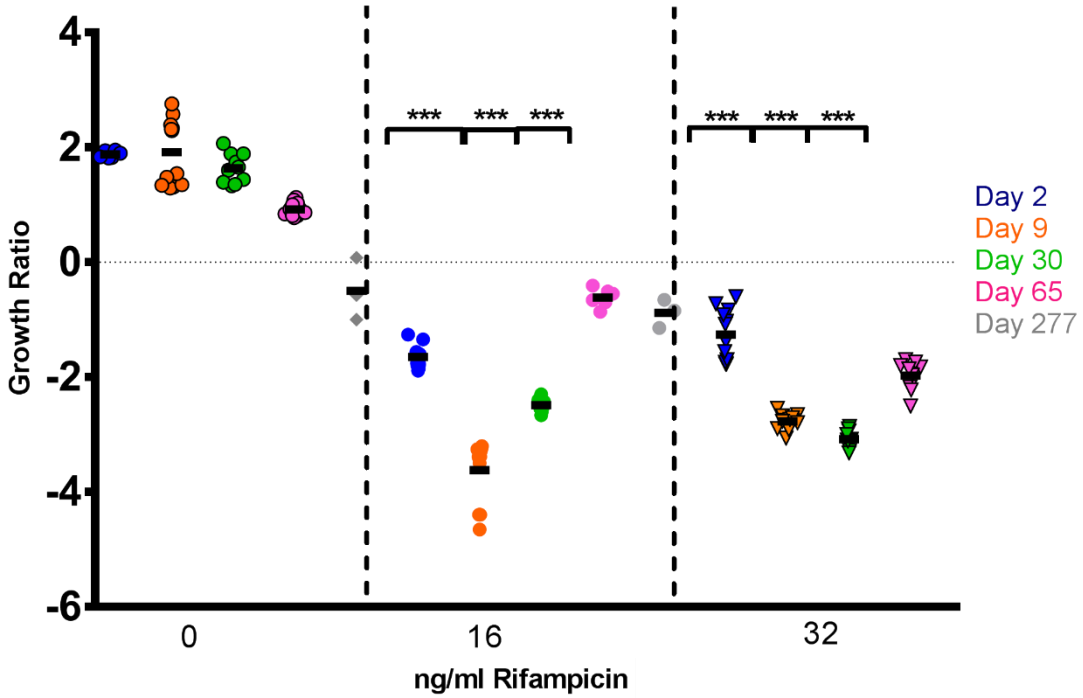


Figure 3.17 *M. tuberculosis* grown in batch culture and sampled at either 2 days, 9 days, 30 days, 65 days, or 277 days. These samples were used as inocula for smaller cultures exposed to varying concentrations of rifampicin (MIC 32 ng/ml) over a 144 hour period. This graph shows levels of rifampicin from 0 ng/ml to 32 ng/ml). P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05 (n=≥3).

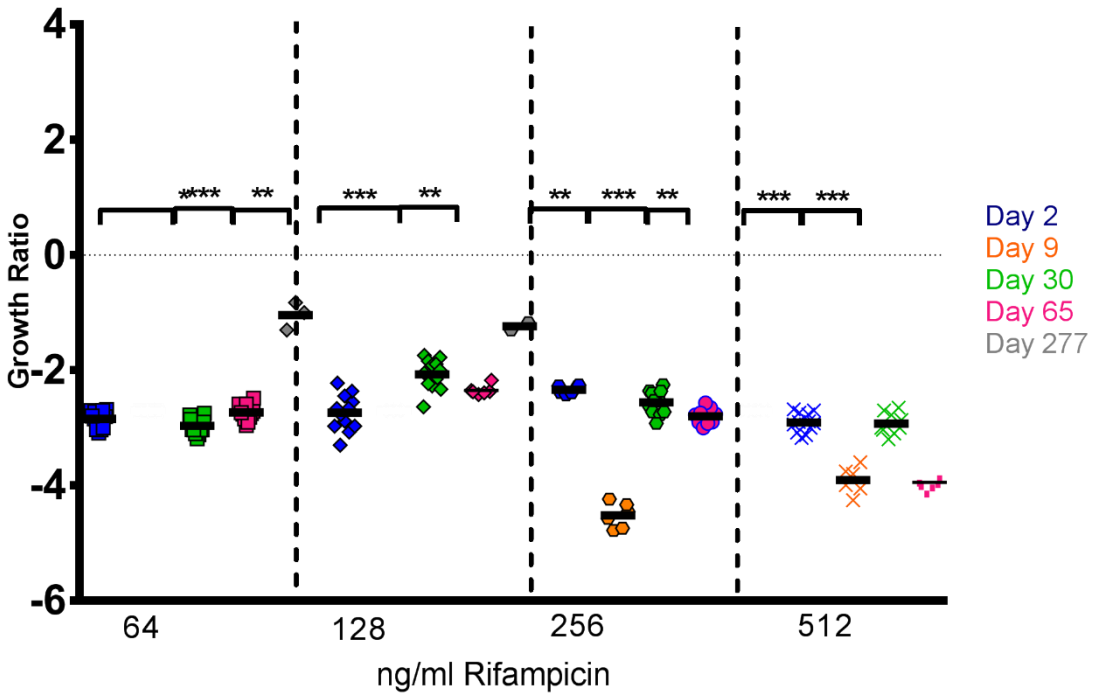


Figure 3.18 *M. tuberculosis* grown in batch culture and sampled at either 2 days, 9 days, 30 days, 65 days, or 277 days. These samples were used as inocula for smaller cultures exposed to varying concentrations of rifampicin (MIC 32 ng/ml) over a 144 hour period. This graph shows levels of rifampicin from 64 ng/ml to 512 ng/ml). P-values (calculated using Mann-Whitney t-test in Graph Pad Prism 5) are equal to *** <0.001 ** 0.001 to 0.01 or *0.01 to 0.05 (n=≥3).

At a sub-MIC level of rifampicin the *M. tuberculosis* cells became less susceptible to rifampicin the longer they were cultured for (figure 3.17). This is true for all the growth phases apart from the exponential phase cells (day 2) that were significantly less susceptible than the stationary phase samples. When the rifampicin concentration was increased to an MIC level, the exponential phase cells were once again less susceptible than the stationary and late stationary/early death phase samples. The samples removed at days 9 and 30 were similar in their susceptibility profiles, this was also seen in the isoniazid data (section 3.7.1). The susceptibility to rifampicin began to decrease in the cells removed in the late stationary/early death phase (day 65).

At 2 x MIC and 4 x MIC (64 ng/ml and 128ng/ml) there was more cell death than at MIC levels of rifampicin in the exponential phase cells, there was a similar level of killing in the stationary and late stationary/early death phase cells (days 30 and 65) (figure 3.18). The difference between the samples taken at day 30 and 65 was more pronounced at lower levels of rifampicin. The NRP cells were significantly less susceptible than the cells growing in the exponential or stationary

phases of growth. Increasing the concentration of rifampicin to 8 and then 16 x MIC did not result in the growth ratio of the exponential phase cells decreasing, this is also true of the stationary phase (day 30) data and is true of the late stationary/early death phase cells at 8 x MIC. At the highest level of antibiotic used (512 ng/ml 16 x MIC) the late stationary/early death phase cells showed a marked decline in viability compared with the lower rifampicin concentrations indicating this is the level required to observe further killing than was seen at 2 x MIC (64 ng/ml)

The data from the sample taken 9 days post inoculation from the batch culture is incomplete due to the plating out of the susceptibility assay at 2 x MIC and 4 x MIC resulting in no colonies growing on agar, this was true even when the sample was plated undiluted. It is unclear as to whether this result is an anomaly; if it is a genuine result then it appears that the cells from the day 9 sample are the most susceptible to rifampicin. The data from the day 9 cultures in sub-MIC, 8 x MIC and 16 x MIC support this observation. In order to confirm this, repeat cultures would need to be sampled and assayed. There are also results missing from the NRP sample at 256 ng/ml and 512 ng/ml rifampicin, this was also due to no colony growth at any dilution that was plated out.

The differences between the effect of isoniazid and rifampicin was seen at an MIC level, the exponentially growing cells were the most susceptible to isoniazid but the least susceptible to rifampicin. Above MIC levels of drug the susceptibility to isoniazid decreased the longer the cells had been in batch culture for which was a clear pattern, the link between growth phase and susceptibility to rifampicin was not as clear although the NRP cells were the least susceptible. There was no difference in the susceptibility of cells that were in culture for 65 days and those from the NRP phase at levels of isoniazid above MIC, showing that cells in the NRP phase have no susceptibility advantage to isoniazid over those in the late stationary phase. Overall there was more kill with rifampicin, particularly with the day 65 cultures, reflecting that these cells were not dividing. It therefore appears that isoniazid was less effective but that the cells were still synthesising RNA enabling rifampicin to have more of an effect even if metabolism is slowed.

3.7 Discussion

Cell density was one of the factors investigated in this chapter because it is thought that there are differences in the number of bacteria throughout the host during infection. MICs of antibiotics have been shown to increase with the increasing number of bacteria exposed (Udekwu *et al.*, 2009), this suggests that an antibiotic could clear the bacteria growing at a low concentration of cells but leave a populations of high concentrations of cells that could act as a

reservoir of infection. The results in sections 3.3 and 3.4 show that the action of both isoniazid and rifampicin are affected by cell density. It has been suggested that a reason for a cell density effect would be that in a high cell density population of bacteria there would ultimately be a lack of nutrients at the core of an infection (LaPlante and Rybak, 2004). In a batch culture, nutrients would certainly be depleted faster if more bacteria growing and it could be that the metabolism of high cell density bacteria is lower due to the decreasing availability of nutrients. Antibiotics such as isoniazid and rifampicin acting on cellular processes may become less effective as the metabolism slows. The results in these sections showed that the doubling time of the *M. tuberculosis* cells exposed to rifampicin was increased. Also, at a high cell density a reduction in the effective concentration of the antibiotic (free active drug) may well be bound to the cell structure of killed as well as viable bacteria and/or other structural or chemical components (Udekwu *et al.*, 2009)

Isoniazid appears to be effective killing low and intermediate density populations with a bactericidal effect but the high cell density results were inconclusive due to variation between replicates. The results from the susceptibility testing of antibiotics against other bacteria (Davey and Barza, 1987; LaPlante and Rybak, 2004; Udekwu *et al.*, 2009) suggest the findings in this chapter that show ineffectiveness of isoniazid at a high cell density (figure 3.3) are supported by evidence in other systems. An increase in MIC was seen at a high cell density for six antibiotics tested against *Staphylococcus aureus*, these antibiotics included vancomycin, daptomycin and oxacillin (Udekwu *et al.*, 2009). These antibiotics disrupt cell wall synthesis and the cytoplasmic membrane of bacteria in a similar mode of action to isoniazid against *M. tuberculosis* and the cell density effect was most apparent at cell titres of 10^7 cfu/ml and above. The result in figure 3.3 showed inactivity of isoniazid at a high cell density of 10^7 cfu/ml, in the context of other work this is evidence of cell density affecting the efficacy of isoniazid.

Rifampicin showed a more striking cell density effect with inactivity at high and intermediate cell densities and a bacteriostatic effect at a low cell density. Rifampicin has been shown to form a drug-enzyme complex with RpoB within 10 minutes of incubation (Gumbo *et al.*, 2007) whereas isoniazid requires conversion to an active form mediated by the enzyme KatG. In a high density population the rifampicin available may run out faster than isoniazid due to rapid binding. A reduction in metabolism caused by the limiting of nutrients at a high density may affect isoniazid less quickly than rifampicin. Isoniazid pro-drug can accumulate intracellularly before conversion to an active form so a reduction in metabolism may slow the conversion. Uptake however may have already occurred and efflux pumps may be slowed. These

adaptations may also allow time for a drug tolerant population to emerge as in a high titre population there is also more chance of a spontaneous mutation occurring.

The different activities of isoniazid and rifampicin against high cell density populations of bacteria highlight the need for a multi-drug approach in the treatment of *M. tuberculosis* infection. These results have also served to inform that in experiments aiming to model infection that has a high bacterial load, the effect of different titres needs to be considered, especially when assessing rifampicin susceptibility. This result then led to an increase of the MIC of rifampicin used in future experiments.

Cells with an MGT of 69.3 hours were less susceptible to isoniazid and rifampicin compared to cells with the fastest doubling time (23.1 hours) at all concentrations up to 16 x MIC (section 3.5). This shows a clear relationship between growth rate and susceptibility to antibiotics; the slower the doubling time the less effective an antibiotic is. This is probably due to rifampicin and isoniazid acting on cellular processes. It is interesting to see this effect in *M*. *tuberculosis* as it is naturally a slow growing bacterium; the reduction at even slower growth rates highlights the challenge in treating this disease. Both antibiotics showed a concentration independent killing effect and this was true at all growth rates for rifampicin and between 2-8 x MIC for isoniazid. This suggests that increasing the concentration of the antibiotic does not have any advantage, perhaps indicating that there is a sub-population of cells that are preferentially killed, the remaining cells appear recalcitrant to the antibiotic. Isoniazid took longer to affect the slow growing cells than rifampicin; the result of exposure for 24 hours did not reflect the result of exposure for 96 hours for the slowest growing cells. The slowest growing cells were shown to be least susceptible to isoniazid and rifampicin overall, however after 24 hours of exposure this population was the most susceptible. The slowest growing cells showed the most growth when grown in no drug, perhaps they had more nutrients remaining intracellularly and were able to actively divide with no lag phase, allowing isoniazid to act on these cells more so than the faster growing cells, which may have been in a metabolic lag (which was also seen in the cell density work). This result highlights the need to continue experiments such as this, with slow growing bacteria for longer than 24 hours of drug exposure as the growth ratio at 96 hours for isoniazid was contradictory to the 24 hour result for the very slow growing *M. tuberculosis*. The inclusion of this time-point has allowed analysis on the exposure times required to see the effect of the antibiotic.

Growth phase also affected the efficacy of isoniazid and rifampicin, particularly for isoniazid. The longer the cells were in batch culture, the less susceptible they were to both antibiotics, which

followed a similar pattern to the effect of growth rate. This pattern was seen at sub-MIC levels of rifampicin but overall there was a less clear link between growth phase and susceptibility to rifampicin. This result could reflect the differing actions of isoniazid and rifampicin. Rifampicin targets RNA synthesis and so even if the cells are not dividing or are dormant they still have low levels of RNA synthesis and this process is still essential. Rifampicin also acts quickly against its target and is not reliant on conversion by bacterial enzymes to be effective. In contrast, isoniazid requires bacterial KatG to convert it to an active form and acts on the cell wall, this process would be perhaps more affected by a slowing or cessation of growth, less cell wall turnover would also weaken the effectiveness of isoniazid by reducing the available target site (Bardou *et al.*, 1998; Petros C. Karakousis, Ernest P Williams and William R. Bishai, 2007). It has also been shown that cells from this culture and others accumulated an extracellular material (ECM) as time went on and there was an upregulation of LAM (lipoarabinomannan) (Bacon *et al.*, 2014). Rifampicin is lipophilic and so is able to enter the *M. tuberculosis* cell wall even in times of ECM accumulation, this may be why there did not seem to be such a strong association of susceptibility to rifampicin to growth phase when compared to isoniazid; the accumulating ECM may have posed more of a barrier to isoniazid than rifampicin. This observation is supported by work showing that pyrazinamide, isoniazid and rifampicin have lower lesion concentrations than in plasma *in vivo* and isoniazid had the lowest coefficient of penetration in both healthy lung and lesions (Kjellsson *et al.*, 2012).

The difference between isoniazid and rifampicin activity was also seen in the day 65 (late stationary) and NRP cells, there was no significant difference in the response of these two cell populations when exposed to isoniazid but the day 65 cells were more susceptible to rifampicin than the NRP cells (section 3.6.2) . If there was no turnover of the cell wall and no division at late stationary phase and NRP phase the isoniazid would likely be ineffective. At day 65 (stationary phase) and in the NRP phase (day 277) there was still RNA synthesis occurring allowing rifampicin to act on those cells that were still viable but not necessarily dividing. Using this culture, transcriptomic analyses showed upregulation of gene expression in stationary phase and in the NRP phase (Bacon *et al.*, 2014) demonstrating active RNA synthesis. One of those upregulated genes was *mmr* a multidrug efflux pump, revealing one of the possible mechanisms of drug tolerance of non-replicating *M. tuberculosis* to both isoniazid and rifampicin.

The effect of isoniazid also highlighted that there was no difference in the response to isoniazid between the day 9 (exponential phase) and day 30 (early stationary phase) cells suggesting that they were in the same stationary phase of growth in terms of the amount of cell wall synthesis

but that the amount of RNA synthesis may be different between these two time points. It was shown that there was an induction of mycolic acid synthesis at day 30 (Bacon *et al.*, 2014), this may have led to an increase in KatG activity which could have led to an accumulation of isonicotinic acid intracellularly. If enough isonicotinic acid was accumulating, the transmembrane pH difference may have been disrupted leading to diffusion of isonicotinic acid out if the *M. tuberculosis* cell (Bardou *et al.*, 1998). This diffusion would lead to an apparent reduction in activity of isoniazid which may help to explain why the day 65 cells were less susceptible to isoniazid than previous time points despite an observed induction of mycolic acid synthesis. When a much higher concentration of isoniazid was used (64 x MIC) there was a large difference between the viability of the day 65 and NRP cells, a difference which had not been seen at lower concentrations of isoniazid, the day 65 cells were far more susceptible than the NRP cells. Increasing the concentration of isoniazid in the medium may have restored the concentration gradient with isonicotinic acid intracellularly allowing passive diffusion into the cell once more.

It appears that cell density, growth rate and phase all affect the efficacy of antibiotics against *M. tuberculosis*. Generally, as cells become slower growing either through lack of nutrients (as seen in the cell density and growth phase work) or through a slower doubling time, they become less susceptible. These experiments have also served to highlight the differences between isoniazid and rifampicin, showing rifampicin to be quicker at killing but showing more discrimination between growth phases. Isoniazid appears effective against fast growing cells but does not affect late stationary cells differently to NRP phase cells. The evidence that these factors clearly affect antibiotic efficacy both in terms of the bacterial adaptation and the antibiotic mechanism of action, will aid in the interpretation of the isoniazid chemostat experiment results in the chapter 4.

Chapter 4. Effect of growth rate on the response of *Mycobacterium tuberculosis* to Isoniazid

4.1 Introduction

Isoniazid has been shown to prevent the progression of tuberculosis and prevent reactivation in people with a positive tuberculin skin test (TST) or interferon gamma release assay (IGRA) and no symptoms of active disease (Lobue and Menzies, 2010). Isoniazid is therefore an important therapeutic used at all stages of disease. Resistance and persistence to isoniazid therapy is a prevalent problem, globally in 2014 480,000 people developed multi-drug resistant TB (MDR-TB), leading to 190,000 deaths (WHO, 2015).

It is thought that during isoniazid treatment distinct populations of bacilli are present; those in the lining of open cavities with an abundant supply of oxygen are probably growing at a fast rate whilst those in closed lesions deprived of oxygen are likely to be growing slowly. Previous studies that plotted the viable counts of *M. tuberculosis* obtained from the sputum of patients undergoing treatment with isoniazid showed an initial rapid kill followed by a slower killing (figure 4.1). This supports the theory that there is heterogeneity in the rate of growth of the initial population (D A Mitchison and Coates, 2004)

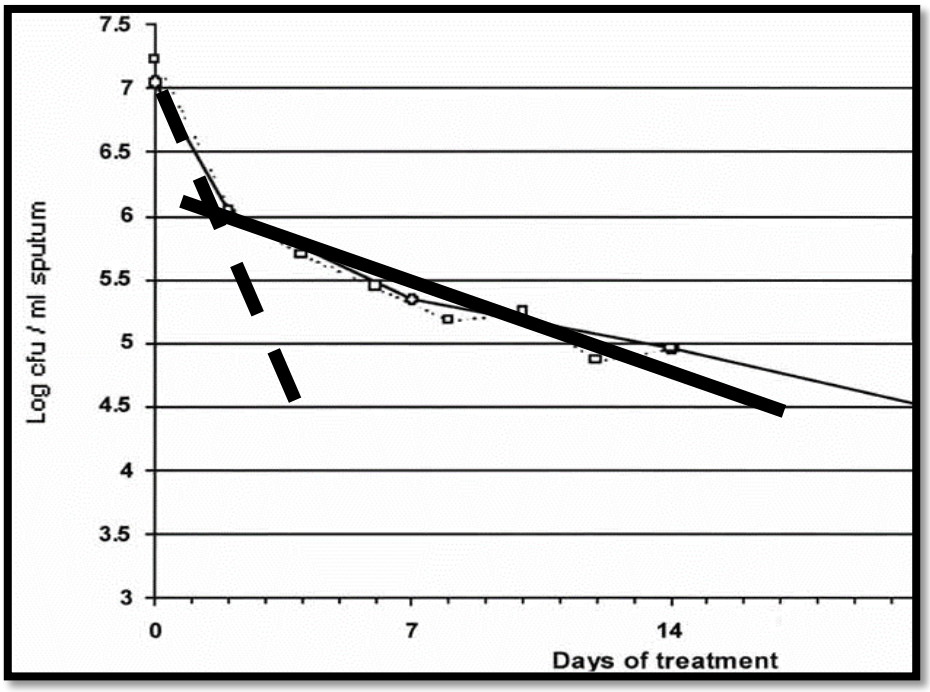


Fig 4.1 Viable counts of *M. tuberculosis* per ml sputum during the first month of treatment with regimens containing isoniazid (Taken from (D A Mitchison and Coates, 2004)) The solid and dashed lines overlaid show a suggested heterogeneity in the population with the solid line representing hypothesised fast growing bacilli and the dashed representing slow growing bacilli.

When the four drugs used to treat tuberculosis are combined (isoniazid, rifampicin, pyrazinamide and ethambutol) isoniazid has a high initial early bactericidal activity during the first 2 days of treatment (Jindani *et al.*, 2003) when it is presumed that rapidly growing bacteria are killed. After 2 days, virtually all of the sterilising activity is due to rifampicin and other drugs in the regimen such as pyrazinamide and is not influenced by the continued presence of isoniazid; Leading to the hypothesis that the bactericidal success of isoniazid is influenced by the growth rate of the bacilli. The inclusion of isoniazid beyond the initial treatment phase is to prevent any reactivation of disease and prevent resistance to the other antibiotics in the regime.

Continuous culture is the only culture system for bacteria that allows the growth rate (i.e. mean generation time) of the bacteria to be controlled via limitation and dilution rate of a key nutrient (section 1.6.2). Continuous culture was used to investigate the differences between *M. tuberculosis* growing at a slow growth rate or fast growth rate by analysing viable count data. Samples were also taken for sequencing and for gene expression studies to enable an analysis of the effect of growth rate on the response of *M. tuberculosis* to isoniazid

The metabolism and cell composition of *M. bovis* BCG with mean generation times of 23.1 hours and 69.3 hours has been studied previously, with correlations in RNA content, ribosome production and proportions of lipids to the growth rate (D J Beste *et al.*, 2005). These mean generation times represent a growth rate close to that seen in human disease and a rate that is 3 times slower, representing a slow growing population. It is clear that there is an effect of growth rate on the physiology of the bacteria which also influences the response of *M. tuberculosis* to isoniazid, cells removed from continuous culture in Chapter 3 showed that isoniazid was less efficacious against cells that had been grown at slow growth rate. This difference was also seen when the effect of growth phase was investigated; early exponential cells sampled after two days of culture were more susceptible to isoniazid than those cultured for longer periods.

By adding isoniazid directly to a continuous culture at a controlled growth rate and removing samples over a time-course, rather than removing cells and investigating susceptibility in small batch cultures as in Chapter 3, the response to isoniazid can be studied under reproducible defined and controlled conditions. This will allow the hypothesis to be tested that fast growing *M. tuberculosis* is preferentially killed by isoniazid and that slow growing bacilli are less susceptible to the effects of isoniazid and are the population most likely able to persist through drug treatment.

The specific objectives for this work were as follows:

1. To model the growth characteristics of *M. tuberculosis* in the presence of isoniazid under fast and slow culture conditions

2. To determine the effect of mean generation time on isoniazid susceptibility

3. To characterise temporal changes in susceptibility to MIC levels of isoniazid during continuous culture at fast and slow growth rates.

4.2 *Mycobacterium tuberculosis* growing in continuous culture at a mean generation time of 69.3 hours

Mycobacterium tuberculosis was grown in continuous culture as described in section 2.2.3 with a dilution rate of 0.01 h^{-1} (mean generation time of 69.3 hours). Samples were removed from the cultures after each mean generation time through-out a steady-state period. Control cultures were run without the addition of isoniazid, to establish the viable count data, transcriptomic and antibiotic resistance of cultures in steady-state that were not exposed to antibiotic as a baseline for further experiments. Further cultures were established, once these cultures were confirmed to be in steady–state using readouts such as turbidity, pH and dissolved oxygen tension (section 2.2.2) isoniazid was added at 0.5 µg/ml (MIC) (section 2.2.3.5). The response to isoniazid was explored by studying the viability, gene expression changes and the development of isoniazid resistance (sections 2.2.2, 2.6, 2.3 and 2.5).

4.2.1 *Mycobacterium tuberculosis* growing in continuous culture at a mean generation time of 69.3 hours with the addition of MIC level of Isoniazid (0.5 µg/ml)

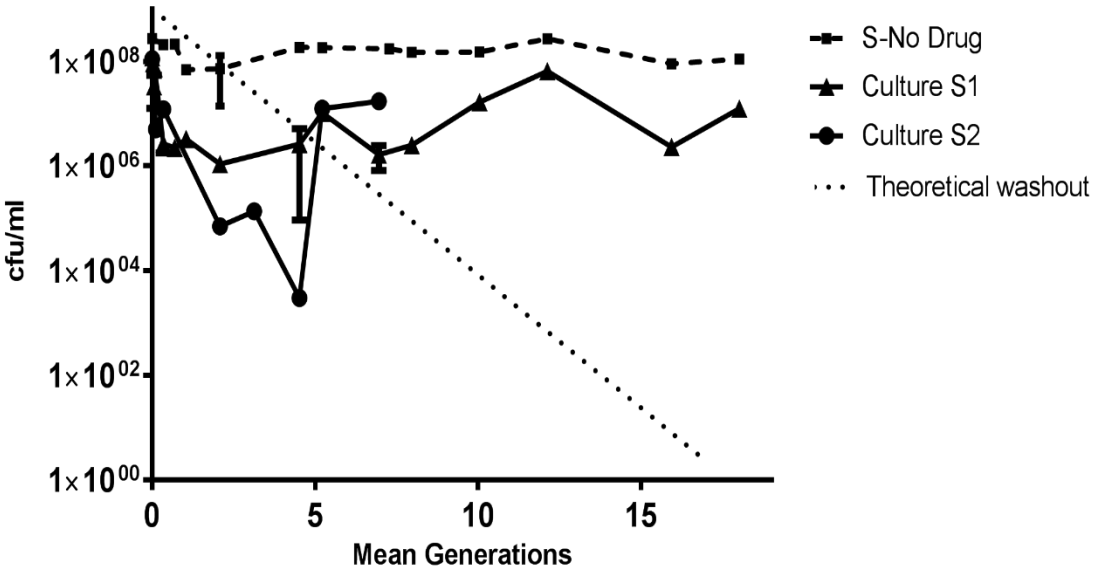


Figure 4.2 Viable count data from *M. tuberculosis* growing in continuous culture at a dilution rate of 0.01 h^{-1}, a doubling time of 69.3 hrs. The cultures were in steady state with no isoniazid addition (S-No drug) and replicate cultures with 0.5 µg/ml isoniazid addition (Culture S1 and Culture S2) at 0 mean generations. The theoretical washout line is represented by the dotted diagonal line. Error bars represent the SEM (N=3)

The cultures in figure 4.2 were cultured for 3 to 5 mean generations to establish steady state at a dilution rate of 0.01 h^{-1} (MGT of 69.3 hours), once this steady state was established, isoniazid was added to both the medium reservoir and chemostat vessel at a concentration of 0.5 µg/ml (section 2.2.6), this was taken as the zero hour time-point.

The total viable count (TVC) data in both cultures growing with a doubling time of 69.3 hours showed a loss of viability within the first few hours after drug addition, this loss of viability was demonstrated by a 1 log drop in the total viable count. The control culture that had not been exposed to isoniazid (S-No Drug figure 4.2) shows a maintenance of steady-state viability indicating isoniazid that was causing the decrease in cfu/ml in the drug treated cultures rather than an inability of *M. tuberculosis* to maintain a viable population at this growth rate in the chemostat system. The bactericidal effect observed seems to occur at different rates in the two slow growth cultures. In culture S2 (figure 4.2) there was a period of killing within the first 2 mean generations that reduced viable cell numbers from $2.01x10^8$ to $7x10^4$ cfu/ml. Between 2 mean generations and 4.5 mean generations there was another period of killing with a reduction in viable cell numbers to $1x10^3$ cfu/ml, which represents the lowest viable count reading. This shows a loss of 99.997% of the viable population of *M. tuberculosis*. This is showing an increase in the rate of death (0.036 h^{-1}) that is higher than the theoretical wash out of the culture, which is equivalent to a bacteriostatic responses therefore showing that an MIC level of isoniazid is bactericidal in our system (0.017 h^{-1}). After 4.5 mean generations there was a rapid rise in viability in the culture in the presence of isoniazid. The value at 4.5 mean generations was close to limit of detection of the plating at this timepoint, it is possible that this result at 4.5 mean generations is anomalous, due to the fact that *M. tuberculosis* is not known to divide as quickly as indicated by these results, either *in vivo* or *in vitro* . This culture did not continue past 7 mean generations but it appeared that it was establishing and maintaining a population of cells at 1 x 10^7 cfu/ml in the presence of isoniazid after the initial bactericidal kill.

Culture S1 in figure 4.2 showed the same bactericidal kill over the initial hours after isoniazid addition at 0 mean generations, with a 99.75 % reduction in viable cells by 2 mean generations after isoniazid addition. This replicate culture reached a maximum titre after isoniazid addition of 6.3 x 10^7 cfu/ml at 12 mean generations, after a period of re-growth. This re-growth period from 2 mean generations onwards shows fluctuating viable counts, dropping to around 1 x 10^5 cful/ml followed by an increase to 1 x 10^7 cfu/ml.

The lowest viable count was higher in the replicate culture (S2) than the lowest viable count for the first culture (S1) and also occurs earlier on in culture.

Both cultures followed a pattern of rapid killing followed by re-growth of the population resulting in an establishment of *M. tuberculosis* growing in the presence of isoniazid; the culture that was continued until 18 mean generations (Culture S1 figure 4.2) showed maintenance of an apparently isoniazid-tolerant population.

4.3 *Mycobacterium tuberculosis* growing in continuous culture at a mean generation time of 23.1 hours

Fast growing chemostat cultures were established (as described in section 4.2) with a dilution rate of 0.03 h^{-1} which equates to a mean generation time of 23.1 hours, a doubling time similar to the natural rate seen *in vivo* and *in vitro* and a rate that has been previously studied in mycobacteria (Beste *et al.*, 2005). Two steady-state cultures were set up and isoniazid added at a concentration of 0.5 µg/ml and a third culture was maintained in steady state with no isoniazid added to provide control data.

4.3.1 *Mycobacterium tuberculosis* grown in continuous culture at a mean generation time of 23.1 hours

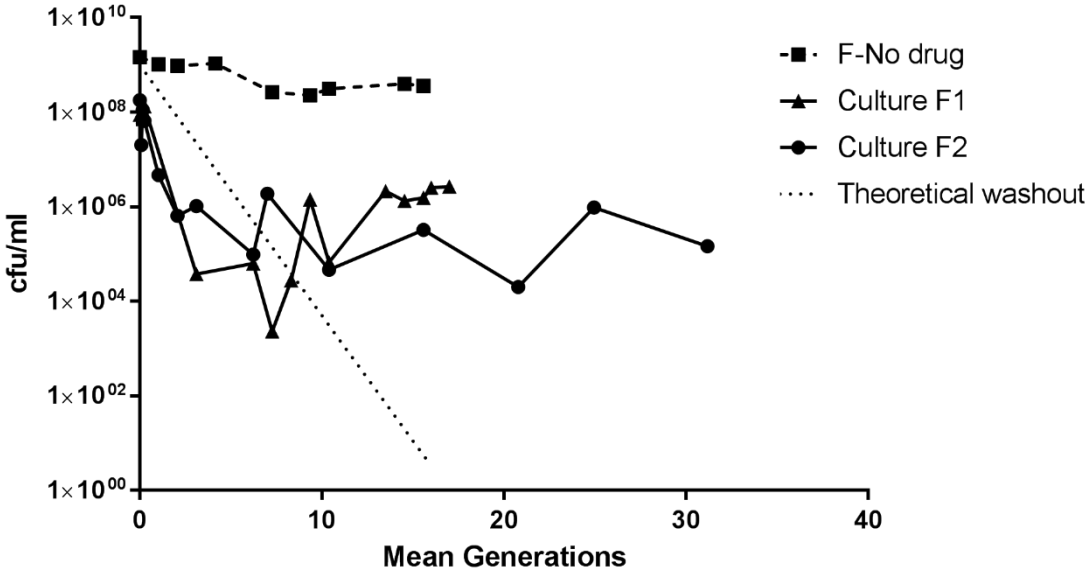


Figure 4.3 Viable count data from *M. tuberculosis* growing in continuous culture at a dilution rate of 0.03 h^{-1}, a doubling time of 69.3 hrs. The cultures were in steady state with no isoniazid addition (F-No drug) and replicate cultures with 0.5 µg/ml isoniazid addition (Culture F1 and Culture F2) at 0 mean generations. The theoretical washout line is represented by the dotted diagonal line. Error bars represent the SEM (N=3)

The viability data in figure 4.3 shows cultures with an imposed mean generation time of 23.1 hours. In the absence of isoniazid there is maintenance of a viable population of exponentially

growing *M. tuberculosis* cells at 1 x 10^9 cfu/ml for the duration of the experiment (17 mean generations)

The addition of isoniazid to the fast growing cultures in figure 4.3 resulted in an immediate decrease in viability at a rate greater than the theoretical washout indicating a bactericidal effect. This killing action appeared to last until 7 mean generations in both cultures which was in contrast to the slow growing experiments in figure 4.2 where the bactericidal effect appeared to cease earlier in the time-course. In the fast growing cultures there was maintenance of an exponentially growing population of *M. tuberculosis* at a titre of 1 x 10^5 cfu/ml for the duration of the cultures, in culture F1 in figure 4.3 this rose to 1 x 10^6 cfu/ml after 9 mean generations which was then maintained. In culture F2 there was a fluctuation between 1 x 10^5cfu/ml and 1 x 10^6 cfu/ml over the last 20 mean generations of the experiment, this fluctuation was similar to the pattern in the slow growing culture, Culture S1 (figure 4.2). From these chemostat experiments it appears that the slow growing *M. tuberculosis* (MGT 69.3 hours) was rapidly killed after addition of isoniazid as seen in the slow growing experiment. The slow growing cells then re-established a population growing in the presence of isoniazid which reached a titre similar to the initial steady state level prior to isoniazid addition indicating an adaptation to the presence of isoniazid allowing re-growth. The fast growing *M. tuberculosis* maintained an exponentially growing population but at a lower titre than the initial titre, illustrating a difference between the slow and fast growing *M. tuberculosis*.

4.4 Determination of changes in susceptibility to isoniazid of *M. tuberculosis* cultured in the chemostat using cell density

To determine whether the susceptibility of the *M. tuberculosis* in the cultures represented in figures 4.2 and 4.3 changes over time, cell samples were removed as an inoculum for medium containing various levels of isoniazid. Using optical density provides a fast readout and allows the calculation of the MIC of isoniazid at various time points in the chemostat cultures, which may indicate whether the bacteria are adapting to the presence of antibiotic.

Cells were removed from the chemostat and added to 25 mL universal tubes containing various levels of isoniazid were inoculated to a starting OD_{540nm} of 0.05 as described in section 2.4.1, this method was followed with OD_{540nm} measurements taken daily for at least 96 hours post inoculation and no TVC data was collected. The data has been represented as a growth ratio (the initial 0 hr OD_{540nm} measurement / final OD_{540nm} measurement) to allow the amount of growth to be plotted as one column, this ratio was then log_{10} transformed to create a scale that is easily

visible on one graph and allows reduction in turbidity (i.e. cell death) to be visualised with a negative value.

A total viable count would be a more accurate measure of the viability of the cells, optical density measurements can only measure the density of a population of cells and cannot distinguish between live and dead cells, or changes to the cell wall which may alter the light absorption or scattering properties of the cell. Despite these limitations it is a widely used method and a relationship between optical density and viable counts has been demonstrated in *M. tuberculosis* (Peñuelas-Urquides *et al.*, 2013). One of the major factors in using optical density is also the slow growing nature of *M. tuberculosis* causing a lengthy incubation time, optical density provides a result which can be virtually instant giving information on an experiment whilst it is still running.

Control cultures with no isoniazid were included at each timepoint to visualise growth and to reduce the discussion on the variables that could have caused a reduction in optical density in the presence of isoniazid. Viable count data has been taken from these cultures also (figures 4.2 and 4.3) and susceptibility data from before drug addition was also plated out (chapter 3) meaning that the conclusions from this work are not solely based on optical density data.

4.4.1 Susceptibility of slow growing (MGT 69.3 hours) *M. tuberculosis* after chemostat culture

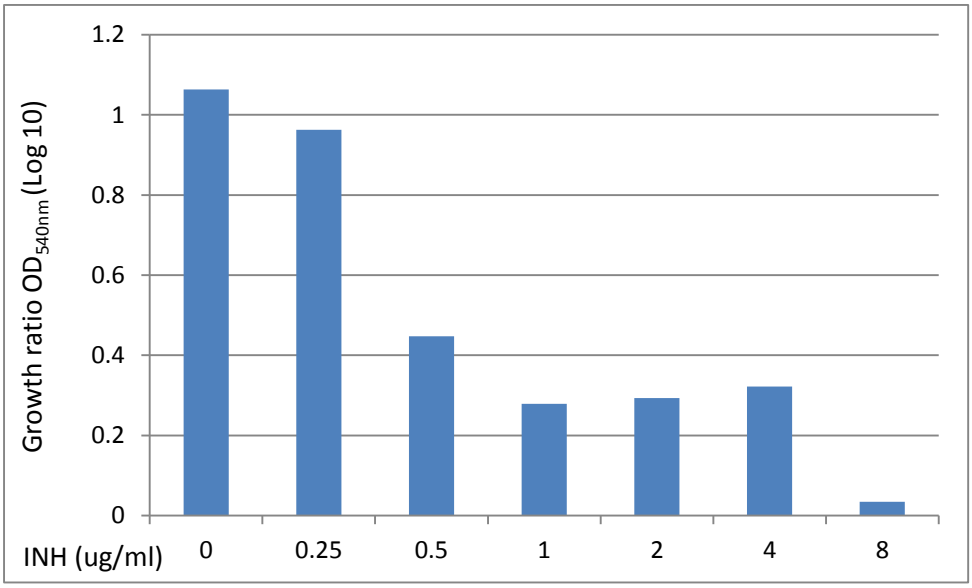


Figure 4.4 The changing susceptibility of *M. tuberculosis* cells after chemostat culture at a slow growth rate (MGT 69.3 hours). Cells were removed from the S-No Drug culture seen in Figure 4.2 at the 0 mean generation timepoint after establishment of steady state. These growth ratio results were calculated after 96 hours of culture.

After 96 hours of culture in medium containing no isoniazid, *M. tuberculosis* removed from a slow growing chemostat grew, with the OD_{540nm} reading increasing over 10-fold (figure 4.4). The *M. tuberculosis* also grew at the higher concentrations of isoniazid in the range tested with a marked drop off in growth at 0.25 µg/ml isoniazid. At levels of 0.5 µg/ml (MIC) to 4 µg/ml there was a doubling in the OD_{540nm} reading at 96 hours compared with the reading from the 0 hour timepoint and this growth ratio was similar for all concentrations, showing a concentration independent pattern. At 8µg/ml there was very little growth with less than 10% increase in turbidity, this reading could have indicated no growth from the start of the experiment as the actual reading was only 0.005 of an increase, which is negligible especially when this is compared to the increases in turbidity seen at lower concentrations of isoniazid.

This result could indicate that the MIC is higher than previously established at 0.5µg/ml although there is a 46% increase in killing between 0.25 µg/ml and 0.5 µg/ml. The original MIC experiments were carried out using cells from a fast growing chemostat, this result shows that the MIC of isoniazid is higher in slow growing *M. tuberculosis* even before isoniazid exposure and shows growth rate is a factor to be accounted for when determining MICs of antibiotics in continuous culture.

4.4.1.2 Susceptibility of slow growing (MGT 69.3 hours) *M. tuberculosis* after chemostat culture in the presence of isoniazid

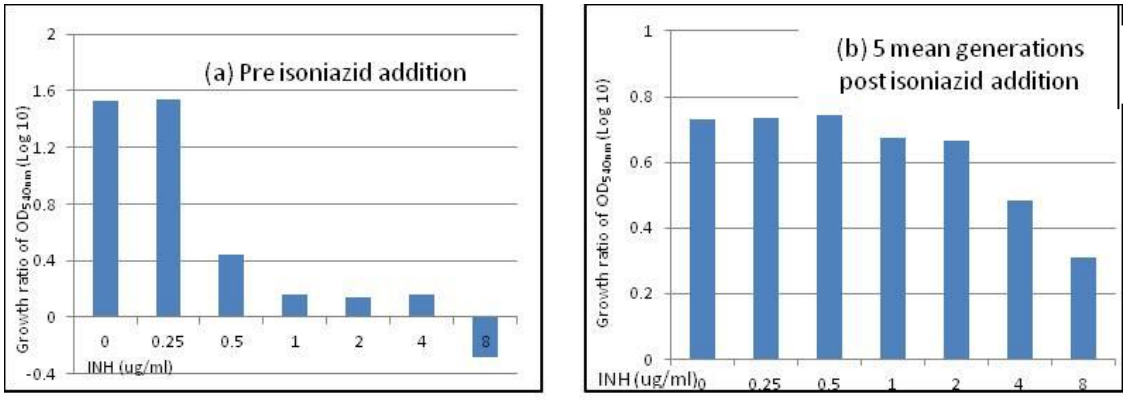


Figure.4.5 The changing susceptibility of *M. tuberculosis* cells after chemostat culture in the presence of isoniazid (0.5 µg/ml) at a slow growth rate (MGT 69.3 hours). Cells were removed from culture S1 seen in figure 4.2 before isoniazid addition (a) and after 5 MG of isoniazid exposure (b)

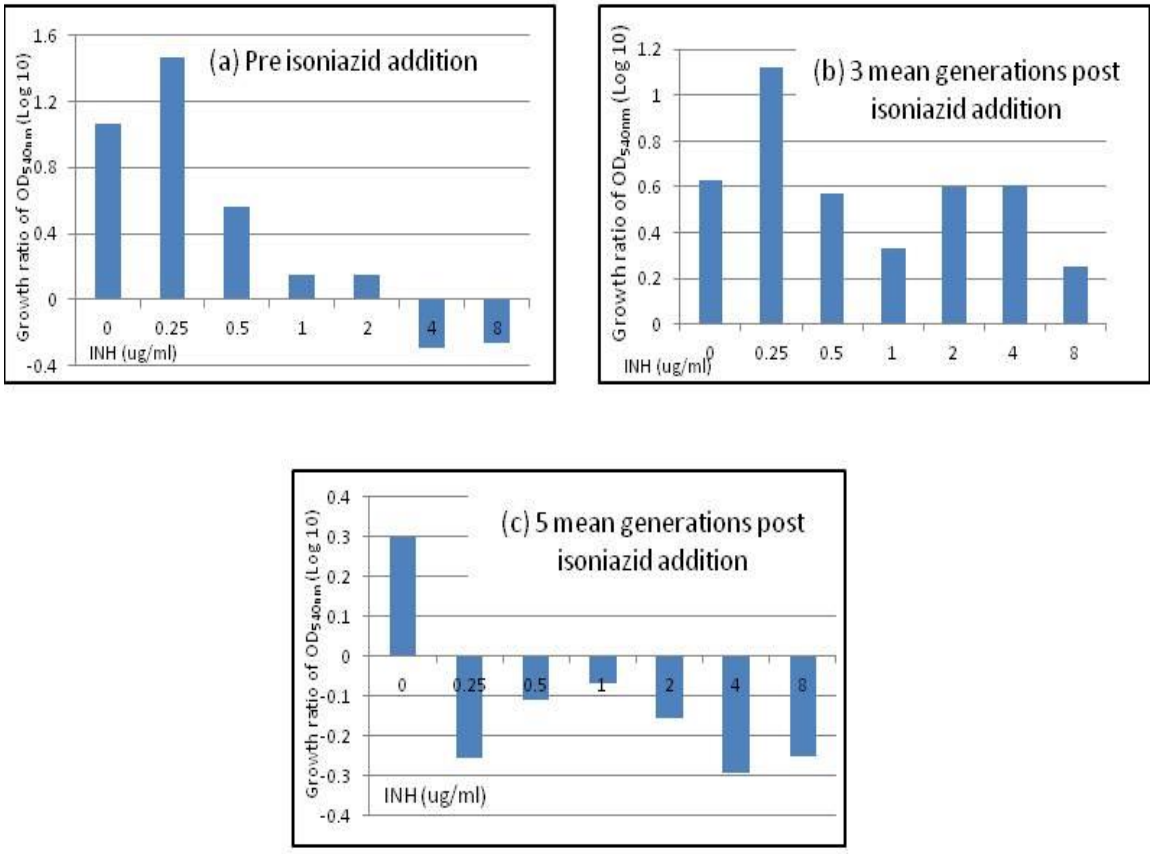


Figure 4.6 The changing susceptibility of *M. tuberculosis* cells after chemostat culture in the presence of isoniazid (0.5 µg/ml) at a slow growth rate (MGT 69.3 hours). Cells were removed from culture S2 seen in figure 4.2 before isoniazid addition (a), after 3 MG of isoniazid exposure (b) and after 5 MG of isoniazid exposure (c)

At 3 mean generations post isoniazid exposure in the chemostat (figure 4.2) there was a marked difference in the profile of the response to isoniazid. When a sample was tested for drug susceptibility from a slow growing culture (culture S2 figure 4.2) growth was enhanced at all levels of isoniazid (figure 4.5b), indicating an adaptation to isoniazid by *M. tuberculosis* which was observed in culture S2 in figure 4.2.

At 5 mean generations of exposure there were differing results (in figures 4.5c and 4.6b). The results do however reflect the difference in the slow growing chemostat cultures (figure 4.2). In culture S2 there was an increased rate of killing between 3 and 5 mean generations with the lowest viable count occurring at 5 mean generations (figure 4.2). The cells removed after 5 MGT in culture showed an increase in their susceptibility to isoniazid with a decrease in optical density

at every concentration of isoniazid (figure 4.6c). The control (0 µg/ml isoniazid) showed the cells at this timepoint had the ability to grow; the growth of the control however was lower than in previous time points and gave an indication that the *M. tuberculosis* at 5 mean generations in culture S2 was perhaps compromised by the presence of isoniazid within the chemostat the sample was taken from (figure 4.2). After removal from the chemostat, cells were washed before the MIC experiments but the viability due to being cultured in the presence of isoniazid may have been affected.

Figure 4.5b shows at 5 mean generations of exposure the cells in this experiment were able to grow in the presence of isoniazid at all concentrations; there is a decreased susceptibility to isoniazid in these cells which is in contrast to figure 4.6c. This reflects the maintenance of a large population of *M. tuberculosis* in the chemostat in culture S1 at 5 mean generations indicating in this slow growth experiment these cells were adapted earlier than the cells in culture S2 to the presence of isoniazid (figure 4.2)

4.4.2 Susceptibility of fast growing (MGT 23.1 hours) *M. tuberculosis* after chemostat culture

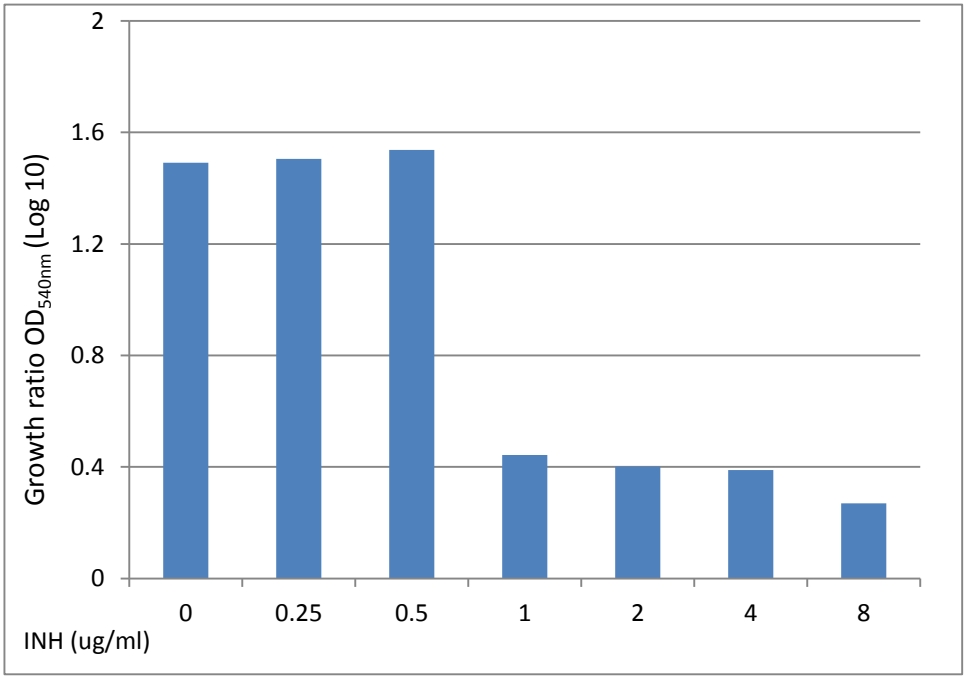


Figure 4.7 The changing susceptibility of *M. tuberculosis* cells after chemostat culture at a fast growth rate (MGT 23.1 hours). Cells were removed from the F-No Drug culture seen in Figure 4.3 at the 0 mean generation timepoint after establishment of steady state. These growth ratio results were calculated after 96 hours of culture

The cells removed from the fast growth control culture (figure 4.3) grew well in levels of isoniazid up to 1 µg/ml (figure 4.7). There was a large reduction in the growth of *M. tuberculosis* after 0.5

µg/ml as has been seen in the results presented in section 4.4.1 and 4.4.1.2. There was a concentration independent effect between 1 µg/ml and 4 µg/ml with these concentrations showing between a 2.4 and 2.7 fold increase in optical density. The highest concentration of isoniazid used (8 µg/ml) showed some discrimination when compared to the lower concentrations with a 1.8 fold increase in growth but this was still higher than most of the results from the slow growing cultures at this concentration (with the exception of figure 4.5b)

This result shows a decreased susceptibility in fast growing cells compared to the slow growth culture at the lower levels of isoniazid (figure 4.4) and a similar susceptibility profile for the two growth rates at concentrations at or above 1 µg/ml isoniazid. It appears that the MIC may be around 1 µg/ml for this fast growth culture, although there was a doubling in the OD_{540nm} reading at this concentration of isoniazid this measurement was taken after 96 hours of culture, in effect showing a doubling time of 96 hours which is far from optimal for *M. tuberculosis*.

4.4.2.1 Susceptibility of fast growing (MGT 23.1 hours) *M. tuberculosis* after chemostat culture in the presence of isoniazid

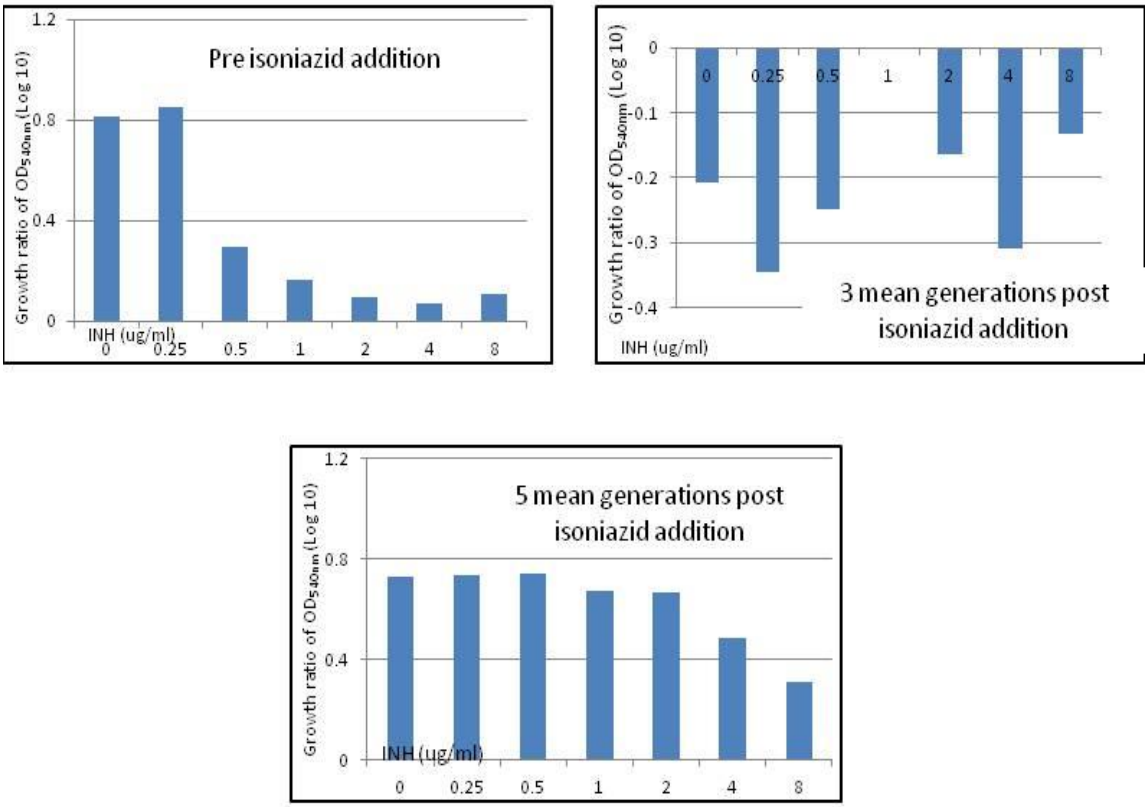


Figure 4.8 The changing susceptibility of *M. tuberculosis* cells after chemostat culture in the presence of isoniazid (0.5 µg/ml) at a fast growth rate (MGT 23.1 hours). Cells were removed from culture F1 seen in figure 4.3 before isoniazid addition (a), and after 3 MG of isoniazid exposure (b)

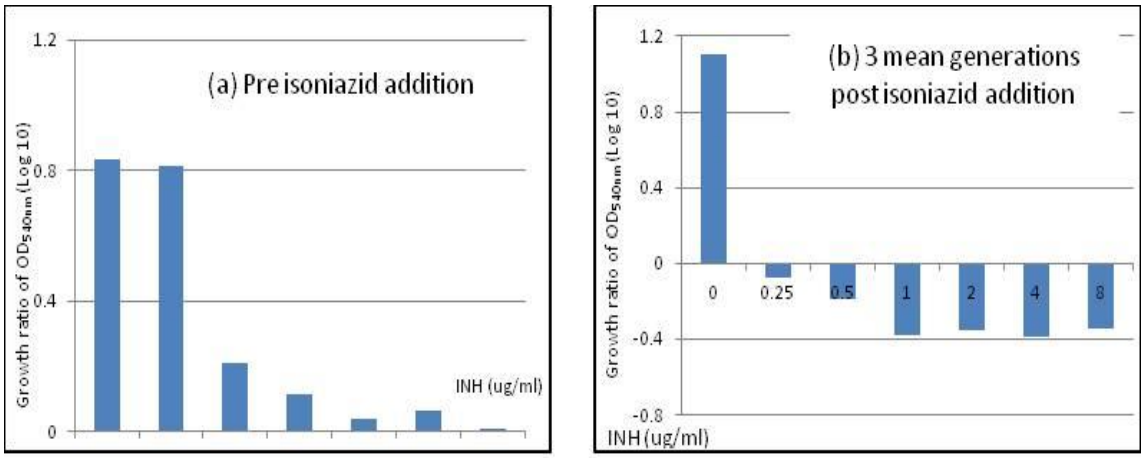


Figure 4.9 The changing susceptibility of *M. tuberculosis* cells after chemostat culture in the presence of isoniazid (0.5 µg/ml) at a fast growth rate (MGT 23.1 hours). Cells were removed from culture F2 seen in figure 4.3 before isoniazid addition (a), after 3 MG of isoniazid exposure (b)

The samples removed from the fast growing chemostats after 3 mean generations of isoniazid exposure showed a marked difference in the susceptibility of *M. tuberculosis*. There was a decrease in optical density at every concentration of isoniazid (figures 4.8b and 4.9b), indicating an increased susceptibility to isoniazid. At the timepoint in the culture the samples were removed from (3 mean generations) (Cultures F1 and F2 figures 4.3) the period of rapid bactericidal killing was coming to an end. Titres were at 1 x 10^6 cfu/ml (started at 1 x 10^8 cfu/ml) and 3 x 10^4 cfu/ml (started at 3 x 10^8 cfu/ml) respectively, with these figures illustrating the rapid bactericidal action of isoniazid in these chemostats. It appears at 3 mean generations the cells left in the chemostat were still highly susceptible to isoniazid and probably compromised as even a sub-MIC level (0.25 µg/ml) of isoniazid inhibited growth, this was reflected in the susceptibility results (figures 4.8b and Fig 4.9b). The cells do not appear to have adapted to the antibiotic presence in the chemostat culture at 3 mean generations. It is also of note that the control culture in figure 4.8b did not grow. It is likely this is due to the compromised nature of the cells not being able to withstand the re-inoculation and at a lower titre meaning perhaps there was not a sufficient population to establish in the susceptibility experiment.

After 5 mean generations of isoniazid exposure (figure 4.8c), the cells had decreased in susceptibility when compared to 3 mean generations of exposure (figure 4.8b). There was a 5 fold increase in the optical density across the concentration range to 4 µg/ml isoniazid, at and above 4 µg/ml there was still at least a doubling in optical density reading (figure 4.8b). This indicated that even though growth at these concentrations may be much slower, it is not inhibited altogether, showing an increase in MIC from around 0.5 µg/ml to greater than 8 µg/ml

isoniazid. At 5 mean generations in the continuous cultures with isoniazid added (Cultures F1 and F2 figure 4.3) there was a very slight increase in viable count, indicating that it is this point at which the cells become adapted to the presence of isoniazid in the chemostat. After 5 mean generations of isoniazid exposure in both cultures there is a trend for an increase in viable cells and a cessation of overall killing with a population adapted to growth in the chemostat maintaining at a titre of around 1x 10^6 cfu/ml (figure 4.3)

4.5 Discussion

The results in this chapter confirm a difference in the response of *M. tuberculosis* to isoniazid based on growth rate. In continuous culture (section 4.2.1 and 4.3.1) after a period of bactericidal killing at both growth rates, the slow growing cells were able to re-establish a population at a high titre whereas the fast growing bacteria maintained a population at a lower titre. The bactericidal response seen at both growth rates has been observed previously with isoniazid exposure in a number of studies (de Steenwinkel *et al.*, 2010; Gumbo *et al.*, 2007; D A Mitchison and Coates, 2004; Paramasivan *et al.*, 2005) This result also mimics the result seen *in vivo*, demonstrating rapid bactericidal kill in the first few days of treatment (figure 4.1 (D A Mitchison and Coates, 2004)). The results in this chapter have allowed a clear difference to be seen between growth rates by controlling mean generation time using continuous culture, whereas other studies have investigated the theory of differing growth rates of *M. tuberculosis* using metabolism based assays and growth phase in uncontrolled batch cultures (de Steenwinkel *et al.*, 2010; Paramasivan *et al.*, 2005) or stationary phase cells (Mitchison and Coates, 2004; Paramasivan *et al.*, 2005). Studies using continuous culture controlling the growth rates at 23.1 and 69.3 hours, as in this study, have shown differences in metabolic flux, the genetic requirements and mechanisms for pyruvate dissimilation between slow and fast growing mycobacteria (Beste *et al.*, 2007b; Beste *et al.*, 2009, 2011) but has not investigated the effect of growth rate on the efficacy of antibiotics.

The susceptibility results in this chapter show that the MIC of isoniazid is around the expected 0.5 μg/ml for both the slow and fast growing cultures that have been exposed to isoniazid. The slow growing (MGT 69.3 hours) *M. tuberculosis* adapted to the presence of isoniazid faster than the fast growing (MGT 23.1 hours) *M. tuberculosis* with a decrease in susceptibility seen after 3 mean generations of exposure in the slow growing bacteria compared to 5 mean generations of exposure in the fast growing *M. tuberculosis*.

The difference in the rate of adaptation could be the reason for the different responses seen in the fast and slow growth chemostat cultures with the slow growing population re-establishing

a population more quickly and at a higher titre than the fast growing *M. tuberculosis*. The results in chapter 3 showed the slow growing cells were less susceptible to levels of isoniazid above the MIC when compared to the fast growing cells. This effect was apparent after just 24 hours of culture in isoniazid. At an MIC level of isoniazid the results indicated that the slow growing cells took longer to be affected by the isoniazid. The susceptibility results in this chapter showed a similar MIC prior to isoniazid exposure but this was based on turbidity as a quick read out rather than viable counts as seen in chapter 3 which is a more accurate measure of an antibiotic's effect on viability.

The substantial drop in cell biomass at both growth rates would have led to an excess of glycerol (the limiting carbon source) in the medium. This would have meant that there would be no longer carbon limited control of growth rate and provide an environment suitable for an increase in growth rate. Only the slow growing cells were able to increase their growth rate and re-establish cell titres close to original value before addition of isoniazid. It is possible that at a controlled mean generation time of 23.1 hours, the fast growing population that was established after the bactericidal kill was growing at close to the maximum growth rate of *M. tuberculosis* in this system (μ_{max}). Previous work in the TB Research group at PHE, Porton Down determined that the mean generation time at μ_{max} was close to 23.1 hours (data not shown). The fast growing cells would not have been able to grow any faster than this maximum growth rate and therefore not increase in biomass even in the presence of excess glycerol. The slow growing cells however would have had the opportunity to grow at or near the maximum growth rate because of the excess glycerol and increase their biomass as seen in the re-establishment of a high titre population.

The excess glycerol allowing the slow growing cells to re-establish a population at a high titre in the presence if isoniazid helps to explain the differences between the two growth rates in response to isoniazid but the susceptibility results in this chapter and chapter 3 show that the slow growing cells are in general less susceptible to isoniazid. Data was collected from the chemostat cultures presented in this chapter on the mutation rate and mutant frequency (sections 5.2, 5.3 5.4), colonies that were able to grow on isoniazid containing medium were sequenced at loci of known isoniazid resistance genes (5.2.1, 5.3.1, 5.4.1) This will aim to determine whether the difference between the slow and fast growing bacteria is due to a genetic element or whether the slow growing bacteria are phenotypically adapted to isoniazid exposure underpinning a genotypic adaptation once exposed to isoniazid.

Chapter 5. Investigation into the genotypic responses of *Mycobacterium tuberculosis* to isoniazid at differing growth rates

5.1 Introduction

The results in Chapter 4 showed a difference in the response of *M. tuberculosis* to isoniazid based on growth rate. In order to investigate whether this was due to an increase in mutation rate, the mutant frequency and subsequently the mutant genotypes of *M. tuberculosis* were measured throughout the chemostat cultures presented in Chapter 4. The differences in the responses could be due to a difference in mechanism of adaptation, for example, a phenotypic change such as changes in expression levels of key isoniazid related genes or a genotypic change, leading to selection of mutations in isoniazid resistance genes. It is often thought that the slow growth is a phenotypic persistence mechanism against antibiotic encounters (M. R. Brown *et al.*, 1990; Kussell *et al.*, 2005) allowing a natural tolerance to many antibiotics. It may be that slowing the growth rate of *M. tuberculosis* even further using the chemostat may elucidate changes that explain the emergence of resistance and persistence in the face of isoniazid therapy that are not seen at the natural doubling time of around 17-24 hours.

5.1.1 Determining mutation rate

The methods for calculating mutation rates are dependent on the model of expansion of mutant clones originally described by Luria and Delbrück (Luria and Delbruck, 1943). During exponential growth, every cell has a low probability of sustaining a mutation during its lifetime and this probability is the mutation rate. The mutant frequency is simply the proportion of cells in a population that are a mutant, and this depends on factors such as population size or the time when the first mutation appeared (Couce and Blazquez, 2011).

Mutant frequencies can be good indicators of the rate at which mutations are induced by DNA damaging agents but they are not adequate for investigating spontaneous mutations, this is because the population of mutants is composed of clones, each of which arose from a cell that sustained a mutation (Foster, 2006) . One of the disadvantages of averaged mutant frequencies is their low reproducibility (that is, the probability of repeated measurements displaying the same results under the same conditions) rather than their poor accuracy (Couce and Blazquez, 2011).

It is preferable to measure the mutation rate as the relationship between mutant frequency and the rate at which mutations occur is uncertain. If a mutation arises early in the culture period, then a large number of mutant progeny occur and this would be represented by a higher mutant frequency than if the mutation had occurred later in the culture (Pope *et al.*, 2008). The exponential growth of a bacterial mutant population then leads to a low probability event occurring early during the growth of a population having huge consequences.

In many experiments mutation rates are low and the number of bacteria that can be worked with is relatively small; in chemostat systems the number of bacteria is large (billions). This method is based on the idea of growing bacteria exponentially until probability dictates that a mutant will be present. This method is very accurate but is often referred to in the literature as complicated and time consuming (Foster, 2006; Snyder and Champness, 2007), this is because the culture needs to be sampled at multiple time points in order to gain the information that is required, something that is achievable within the chemostat system. If the number of mutants and total number of bacterial cells are known at each time point, then the mutation rate can be calculated from the slope of the line describing the relationship between the fraction of mutants against generation number based on the assumption that the higher the mutation rate the faster the proportion of mutants will increase. With the chemostat model of tuberculosis at two different growth rates established, it was decided that mutant accumulation would fit these studies well.

5.1.2 Target genes for sequencing

katG is a gene in *Mycobacterium tuberculosis* encoding a catalase peroxidise enzyme that activates isoniazid, also known as isonicotinylhydrazine (INH) (Zhang *et al.*, 1992). The most common mutation in the *M. tuberculosis katG* gene correlating with resistance to isoniazid is a SNP at codon 315, resulting in a coding change from the wild type serine (AGC) to threonine (ACC). This mutation greatly reduces the affinity of wild type KatG for isoniazid whilst the enzyme maintains reasonably good steady state catalytic rates. Poor binding of the drug to the enzyme limits drug activation and brings about isoniazid resistance (Yu *et al.*, 2003). Other coding changes have also been detected at this codon resulting in varying resistances and catalase-peroxidase activity (Marttila *et al.*, 1998; Ramaswamy and Musser, 1998). It has been summarised that 50-95% of isoniazid resistant strains are the result of mutations in the *katG* gene (Garza-Gonzalez *et al.*, 2009) and between 75-90% of these are located in codon 315 (Ramaswamy and Musser, 1998).

InhA is an enoyl-acyl carrier protein (ACP) reductase, this class of molecule catalyses the nicotinamide adenine dinucleotide (NADH)-dependent reduction of a growing fatty acid chain linked to ACP, this is an enzymatic activity that is common to all known fatty acid biosynthetic pathways. Mycobacteria utilise the products of InhA catalysis to create mycolic acids (Rozwarski *et al.*, 1998). Mutations in the *inhA* gene account for 20-25% of isoniazid resistant strains (Garza-Gonzalez *et al.*, 2009; Ramaswamy and Musser, 1998) one such area of this gene is in the putative promoter region.

These two regions were chosen to cover the largest frequency of mutations that have been found in clinical and previous research studies. Focussing on these regions allows a large number of colonies to be screened from multiple time points of cultures at different growth rates with and without exposure to isoniazid.

The specific objectives for this work were as follows:

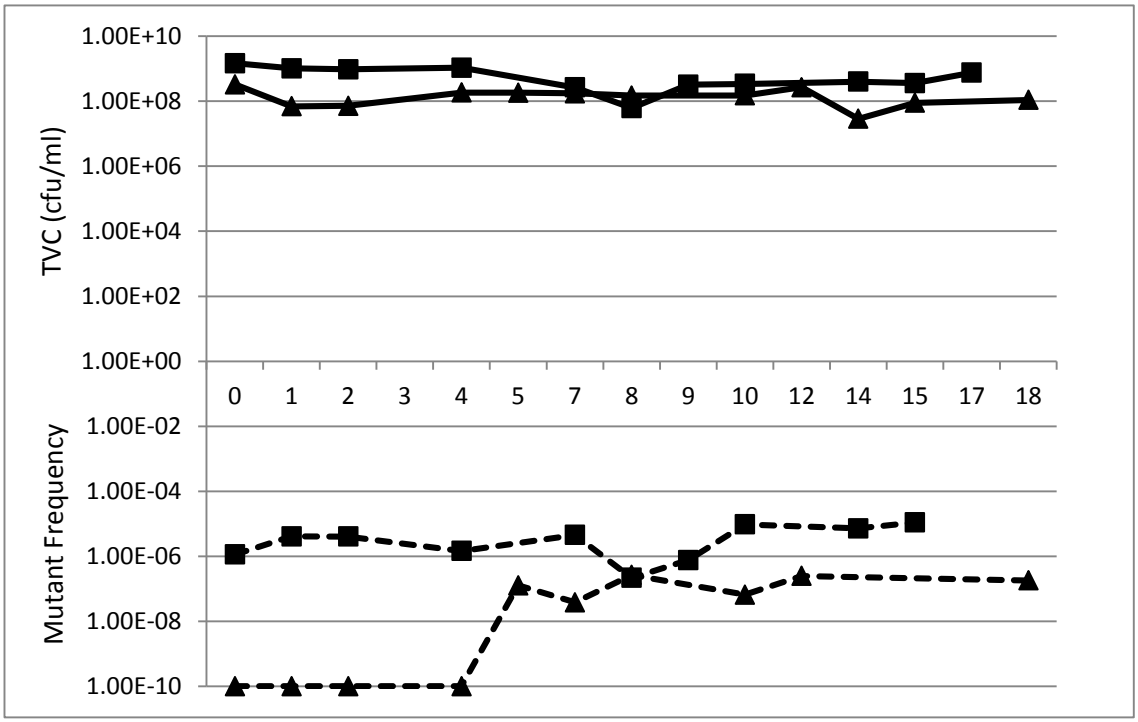
1. To recover isoniazid-resistant mutants growing in the chemostat at slow and fast growth rates and measure the mutant frequency.

2. To apply the mutant accumulation method to determine if there were any differences in the mutation rates between the slow and fast growing *M. tuberculosis* cultures.

3. To sequence regions of genes known to be involved in isoniazid resistance to determine whether *M*. *tuberculosis* is adapting genotypically and/or phenotypically to the presence of isoniazid and whether slow and fast growing cells are adapting to isoniazid exposure via different mechanisms.

5.2. Mutant frequency and mutation rate to isoniazid in the absence of selection pressure

To assess the mutation rate to isoniazid in the absence of selection pressure, chemostats were set up (Section 2.2.3) at fast and slow grow rates with no isoniazid added. The viable count data showing maintenance of steady state has already been presented in sections 4.2.1 and 4.3.1. Samples from these cultures were plated onto 2 X MIC (1 µg/ml) isoniazid to study mutant frequency and mutation rate.



Mean generation	Mutation rate of fast	Mutation rate of slow
1	2.47×10^{-6}	
2	1.15×10^{-6}	
4	2.64×10^{-8}	
5		2.5×10^{-8}
7	2.5×10^{-7}	5.19×10^{-9}
10	4.63×10^{-7}	6.41×10^{-9}
12		2.05×10^{-8}
15	4.25×10^{-7}	
18		9.92×10^{-9}

Figure 5.1 The viable count data for a fast growth (square symbols solid line) and slow growth culture (triangle symbols solid line). The lower half of the axis shows the mutant frequency data for the same fast (square symbols broken line) and the slow (triangle symbols broken line) growing cultures. The mutant frequency value of 1×10^{-10} has been plotted to indicate samples were plated but no colonies were isolated. The table shows the mutation rate data; this was calculated using the pre-drug timepoint as a reference

The viable count data for both the fast and slow growth cultures in the absence of isoniazid is very similar as discussed in chapter 4. The mutant frequency and rate remain stable for the length of the culture and is equivalent to the natural mutation rate of *M.* tuberculosis to isoniazid ($1 \times 10^{-6} – 2 \times 10^{-8}$) (Bergval *et al.*, 2009; Riska *et al.*, 2000) This was also true of the slow growth culture after 5 mean generations of culture; before this time point there were no colonies isolated on 2 x MIC isoniazid from the culture. This result could indicate a change in the slow growth population after 5 mean generations of culture in the chemostat, the result at 5 mean generations and beyond is within the natural mutation rate and the reason for this variation has not been determined. Colonies from these cultures were picked and DNA extracted for pyrosequencing at *katG* codon 315 and the promoter region of *inhA*, two regions that are frequently mutated in isoniazid resistant strains of *M. tuberculosis* to determine whether any genetic changes were occurring in the absence of isoniazid

5.2.1 Pyrosequencing of colonies isolated from fast and slow growth rate chemostat cultures have not been exposed to isoniazid

This sequencing data represents the *M. tuberculosis* colonies that grew on 2 x MIC isoniazid (1 µg/ml) after culture in the chemostats containing no isoniazid (figure 5.1) Colonies were sequenced using pyrosequencing at *katG* codon 315 and in the promoter region of *inhA*, the regions amplified and the method for pyrosequencing can be found in sections 2.7 and 2.8.

The chemostat growing at a slow growth rate that had not been exposed to isoniazid to act as control data produced very few colonies that exhibited the ability to grow on 2 x MIC isoniazid. A very small number of colonies i.e.< 10, were recovered at 10, 12 and 16 mean generations after the experiment started but not analysed as the sample size was too small. The colonies that did grow were small and difficult to obtain DNA for PCR from and so there is no pyrosequencing data for this culture due to the lack of ability to grow on 2 x MIC isoniazid.

The data available in figure 5.2 shows the fast growing *M. tuberculosis* population was comprised of a majority wildtype genotype at all time points (69.5% - 98%). The most variation in the genotype of the population was at 4 mean generations, 31% of the population tested were mutated at *katG* codon 315. The mutation rate throughout this culture was no higher than the natural rate and the emergence of these mutants in small numbers is to be expected. It is clear to see that of the colonies isolated from the fast growth chemostat with the ability to grow on 2 x MIC isoniazid, the majority were wild type at *katG* codon 315 (figure 5.2) indicating that these colonies are naturally able to grow on isoniazid likely due to a phenotypic adaptation.

Mean generation	Genotype	AGC*	AAC	AGA	ACC	ATC	AGG	Mix	Total
	0	51	4	1	0	0	1	0	**57**
	2	82	0	1	0	0	1	0	**84**
	4	16	2	4	0	0	1	0	**23**
	10	57	1	0	0	0	0	0	**58**
	14	34	2	0	0	0	0	0	**36**

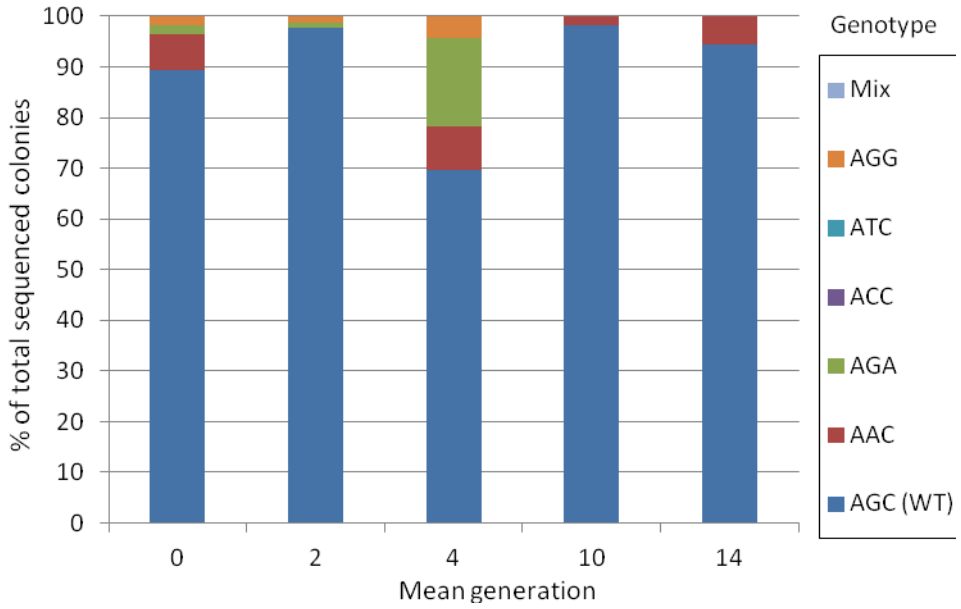
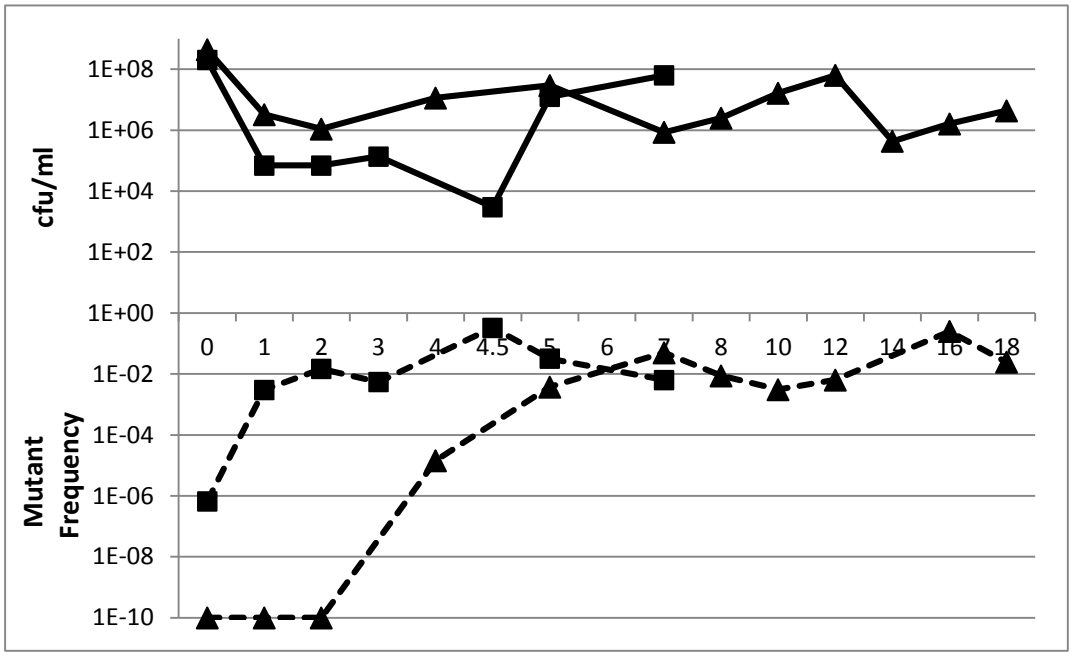


Figure 5.2 The genotype of the colonies recovered on 2 x MIC isoniazid isolated from a chemostat growing with a doubling time of 23.1 hours with no isoniazid present. The genotype shown is at *katG* codon 315 * denotes the wild type genotype. The total number of colonies sequenced is shown in the far right column. The graph is a visualisation of the data

All samples (from all growth rates and conditions) sequenced in the promoter region of *inhA* were wild type.

There could be mutations at other isoniazid relevant loci but the probability of this is low as the 70-80% of isoniazid resistant isolates have mutations in these loci (Rattan *et al.*, 1998)

5.3 Mutant frequency and mutation rate to isoniazid of slow growing *M. tuberculosis* exposed to isoniazid



Mean generation	Mutation rate culture 1	Mutation rate culture 2
1	1.10E-03	
2	6.38E-03	
3	1.50E-03	
4		3.60E-06
5	6.40E-03	7.48E-04
7	9.07E-04	7.14E-03
8		1.09E-03
10		3.08E-04
12		5.29E-04
16		1.55E-02
18		1.36E-03

Figure 5.3 The viable count data for two biological replicate slow growth cultures with isoniazid added, Slow Growth 1 (SG1) (square symbols solid line) and Slow Growth 2 (SG2) (triangle symbols solid line). The lower half of the axis shows the mutant frequency data for SG1 (square symbols broken line) and SG2 (triangle symbols broken line). The mutant frequency value of 1 x 10^{-10} has been plotted to indicate samples were plated but no colonies were isolated. The table shows the mutation rate data, this was

Both slow growth cultures showed an increase in the number of mutants up to 5 mean generations after isoniazid addition, the data shows culture SG1 maintained a stable mutation rate through this period – this pattern is due to the bactericidal action of isoniazid over the corresponding time period which can be seen in the TVC data (figure 5.3). The point at which the mutant frequency is at its highest corresponds to the lowest TVC in both slow growth cultures indicating that at these time points the population is comprised of *M. tuberculosis* with the ability to grow on 2 x MIC isoniazid; the mutation rate at these time points (5 and 7 mean generations) is very similar in both cultures (6.4×10^{-3} and 7.1×10^{-3} respectively). The overall trend of both slow growth cultures was an increase in the number of colonies able to grow on isoniazid followed by a plateau and maintenance of this population in the culture. In culture SG2 no colonies were isolated on isoniazid until 4 mean generations after isoniazid addition to the chemostat. The corresponding TVC data shows less kill by isoniazid in the first 2 mean generations, this data could suggest the cells within this culture were using more phenotypic adaptation within the chemostat but this adaptation was not maintained when grown on solid medium containing isoniazid where the growth rate was not controlled. The slow growth control data also showed a lack of colonies able to grow on isoniazid until 4 mean generations and very few after this, indicating the slow growing culture is less able to grow on isoniazid naturally than the fast growth culture and perhaps takes time to adapt.

Colonies that were isolated from these cultures on 2 x MIC isoniazid were sequenced at codon 315 of *katG* and in the promoter region of *inhA* (sections 2.7 - 2.8) to determine whether any genetic mutation was present.

5.3.1 Pyrosequencing of colonies isolated from slow growth rate (69.3 hr MGT) chemostat cultures exposed to isoniazid

Mean generation	Genotype	AGC*	AAC	AGA	ACC	ATC	AGG	Mix	Total
	Culture: Slow Growth 1								
	1	9	1	0	0	0	0	0	**10**
	2	4	28	3	1	2	0	0	**38**
	3	9	7	1	0	0	0	0	**17**
	Culture: Slow Growth 2								
	4	22	6	1	0	0	0	0	**24**
	7	8	36	3	17	18	0	12	**94**
	12	4	33	6	30	19	0	5	**97**

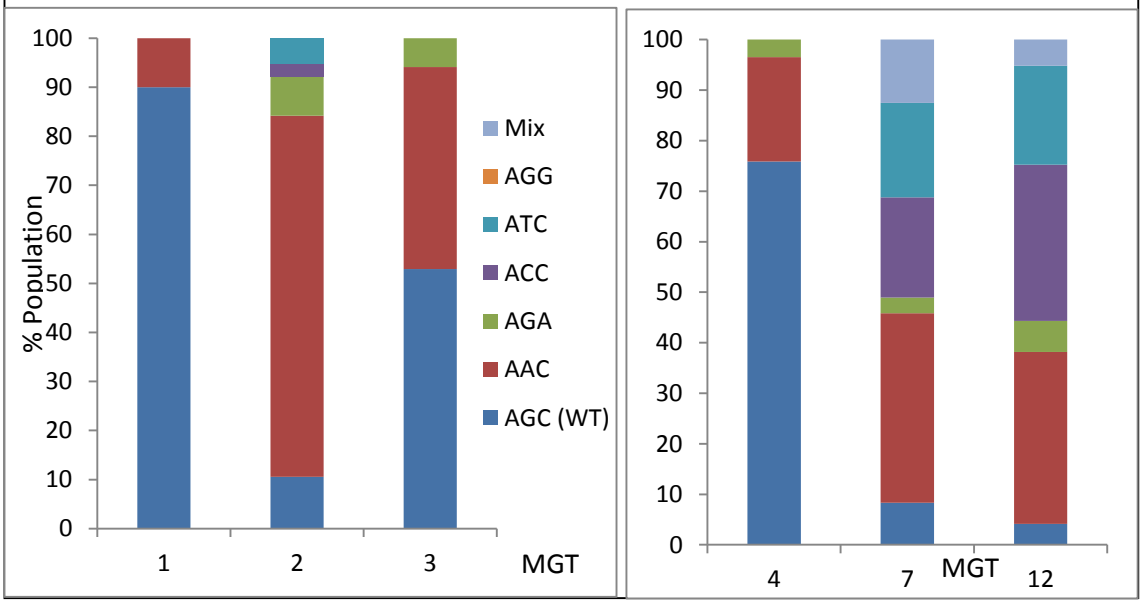


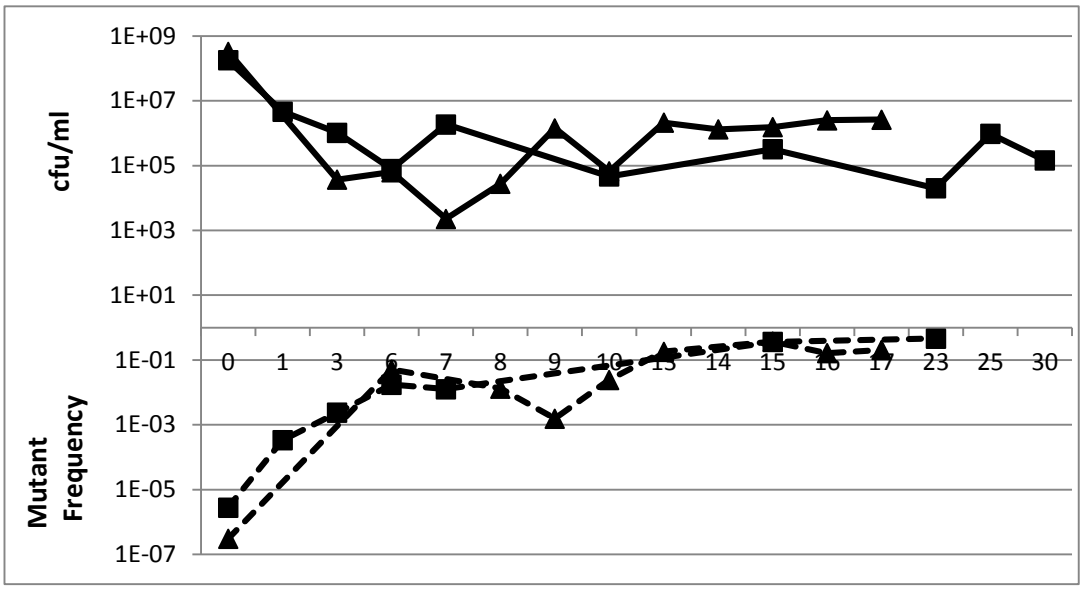
Figure 5.4 The genotype of the colonies recovered on 2 x MIC isoniazid isolated from cultures SG1 and SG2 (figure 5.3). The genotype shown is at *katG* codon 315 * denotes the wild type genotype. The total number of colonies sequenced is shown in the far right column. The graphs below the table are a visualisation of the data in the table as a percentage.

The majority (90%) of the population in culture SG1 was wild type (AGC) codon 315 of *katG* after 1 mean generation of culture with isoniazid (figure. 5.4). Similar proportions can be seen in the 0 mean generation time point for the fast growth control (figure 5.2) indicating that no adaptation or selection had occurred due to the presence of isoniazid in SG1 at this point. The variety of mutations increased as did the proportion of the population that was mutated at *katG* codon Ser^{315} at 2 mean generations post isoniazid addition. This was at a point in the culture where there had been over a 3-log drop in viable cell number, suggesting the wild type cells were preferentially killed. It is worth noting that this data shows cells that are wild type at *katG* codon Ser^{315} but still had the ability to grown on isoniazid, showing the slow growing *M. tuberculosis* is using strategies other than mutation at this loci such as mutation at other loci or phenotypic adaptation. The dominant mutation throughout the sequencing data available for culture SG1 is *katG* codon Ser^{315} *AGC*>AAC and this mutation is also present throughout the data for SG2, this mutation does not outcompete any of the other mutations though. At the first timepoint mutants were recovered in culture SG2 there is a majority wild type population, as the mutant frequency and rate continue to increase (figure 5.3 SG2) so does the proportion of the population that is mutant, with a large variety in mutation seen, this population was then maintained. The viable count data between 7 and 12 mean generations shows an increase in number (figure 5.3 SG2) indicating that the mutant population was increasing in number with no fitness cost associated with mutation.

It appears that both slow growing cultures were adapting genotypically to the presence of isoniazid and a pattern emerged in both cultures of a majority wild type population being replaced with a majority mutant population. One of the mutations occurring frequenctly through the timecourse was *katG* codon Ser^{315} AGC>AAC, resulting in a coding change to threonine. This mutation is the most common seen in clinical samples (Haas *et al.*, 1997a; Heym, 1997; Lipin *et al.*, 2007; Musser *et al.*, 1996). The AGC>AAC mutation results in a coding change to asparagine which has been seen in samples of MDR *M. tuberculosis* isolated in Africa as has the AGC>ATC mutation resulting in a change to isoleucine (Haas *et al.*, 1997a).

This variation not only shows the clinical relevance of these mutations and this model but also shows that all SNPs were maintained within the chemostat without one outcompeting the others. This may be that at a controlled MGT of 69.3 hours, any fitness cost is nullified due to an imposed slow growth rate.

5.4 Mutant frequency and mutation rate to isoniazid of fast growing *M. tuberculosis* exposed to isoniazid



Mean generation	Mutation rate FG 1	Mutation rate FG 2
1	2.28E-04	
3	6.33E-04	
6	1.85E-03	8.16E-03
7	1.78E-03	
8		1.20E-03
9		1.66E-04
10		2.29E-03
13		1.34E-02
15	2.39E-02	2.46E-02
16		1.02E-02
17		1.21E-02
23	1.89E-02	

Figure 5.5 The viable count data for two biological replicate fast growth cultures with isoniazid added. Fast Growth 1 (FG1) (square symbols solid line) and Fast Growth 2 (FG2) (triangle symbols solid line). The lower half of the axis shows the mutant frequency data for FG1 (square symbols broken line) and FG2 (triangle symbols broken line). The table shows the mutation rate data, this was calculated using the pre-drug timepoint as a reference.

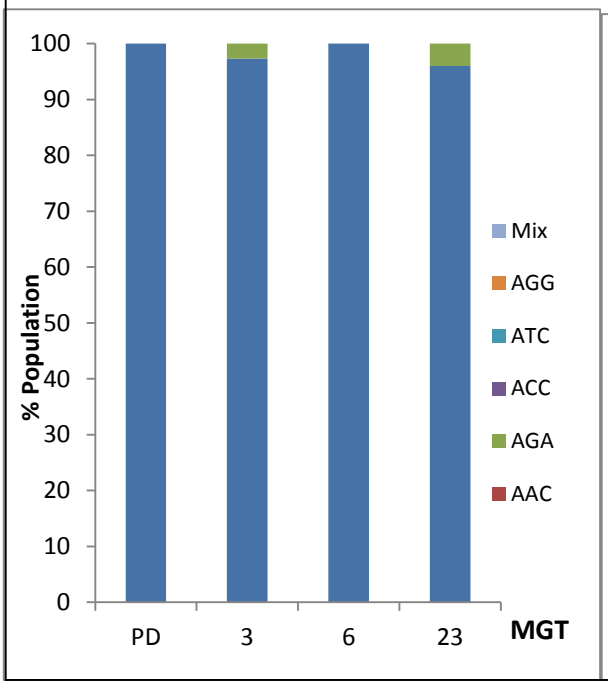
Both fast growing cultures showed a steady increase in the number of colonies able to grow on 2 x MIC isoniazid over the first 6 mean generations of isoniazid exposure. The mutation rate at this point was around 1 x 10^{-3} in both cultures, much higher than the natural reported mutation rate of *M. tuberculosis* to isoniazid. It was at this timepoint also that the viable count was approaching its lowest point, after 6 mean generations of exposure both cultures maintained a mutation rate around 1 x 10^{-2} and this heightened mutation rate to isoniazid was reflected in the high frequency of mutants (figure 5.5). The viable count remained stable from around 10 mean generations post isoniazid exposure with no real increase in viable population from the lowest viable count after the initial bactericidal killing period. The slow growth cultures maintained a population at a higher titre (figure 5.3) than these fast growth cultures indicating that the fast growing *M. tuberculosis* were able to proliferate in the presence of isoniazid but may have incurred a fitness cost. The mutation rate was generally higher in the fast growing culture and there were more mutants certainly in the latter part of the time-course. The slow growing culture contained mutants with SNPs in *katG* codon Ser^{315} (figure 5.4). Colonies isolated on 2 x MIC isoniazid from the fast growing cultures were also sequenced at the same loci to determine whether any isoniazid resistant mutations could be found and whether the population also predominantly consisted of *katG* codon Ser^{315} mutations as with the slow growers (figure 5.4)

5.4.1 Pyrosequencing of colonies isolated from fast growing *M. tuberculosis* exposed to isoniazid

Throughout both fast growth cultures there were very few SNPs at *katG* codon 315 that arose. The population of fast growing *M. tuberculosis* in the chemostat remained wild type at *katG* codon 315 during continual isoniazid exposure. There were less mutants when isoniazid was present in the cultures than in the culture that was not exposed to isoniazid (figure 5.2) but the majority of the population in both culture conditions was wild type at this loci.

The colonies sequenced were isolated on 2 x MIC isoniazid and the population in the chemostat was clearly able to grow exponentially in the presence of isoniazid (figure 5.5) although not as successfully as the slow growing cultures (figure 5.3). This indicates that the fast growing *M. tuberculosis* were using a mechanism other than mutation at *katG* codon 315 or the promoter region of isoniazid.

Mean generation	Genotype	AGC*	AAC	AGA	ACC	ATC	AGG	Mix	Total
	Culture: Fast Growth 1								
	Pre-drug	12	0	0	0	0	0	0	**12**
	3	36	0	1	0	0	0	0	**37**
	6	28	0	0	0	0	0	0	**28**
	23	24	0	1	0	0	0	0	**25**
	Culture: Fast Growth 2								
	Pre-drug	17	0	0	1	0	0	0	**18**
	6	60	0	0	0	0	0	0	**60**
	13	88	2	5	1	1	1	0	**98**



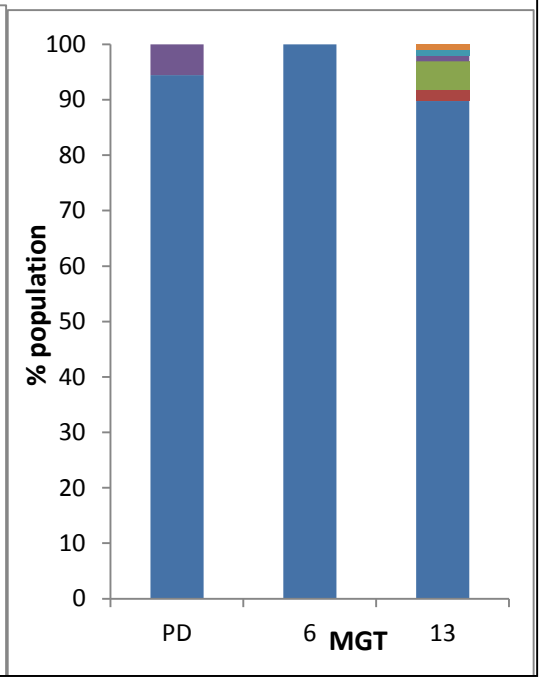


Figure 5.6 The genotype of the colonies recovered on 2 x MIC isoniazid isolated from cultures FG1 and FG2. The genotype shown is at *katG* codon 315 * denotes the wild type genotype. The total number of colonies sequenced is shown in the far right column. The graphs below the table are a visualisation of the data in the table as a percentage.

5.5 Sanger sequencing of the *katG* gene from fast and slow growing *Mycobacterium tuberculosis*

Sequencing of the *katG* gene after 13 MGT of exposure to isoniazid (sequenced by Dr Irene Freire-Martin , Jeeves et al., 2015) was performed to confirm that nearly 90 % of fast growing colonies were wild type at *katG* codon 315 at this time-point (figure 5.6) (Jeeves *et al.*, 2015). Of the 77 colonies sequenced that were wild type, 65 had alternative mutations in the *katG* gene (table 5.1), some of which have been seen previously and most of which were deleterious (Rouse *et al.* 1996; Ramaswamy *et al.* 2003;Heym *et al*. 1993; Haas *et al*. 1997a; Hazbón *et al*. 2006). Five of the colonies contained a double mutation in *katG* (table 5.2), some of which included a mutation in codon 315, most of the alternative mutations caused a frameshift upstream of *katG* codon 315. This work confirms that there were very few mutations at *kat*G codon 315 in the fast growing population but a number of frameshift and deletion mutations within *katG* would have disrupted the function of *katG* entirely (Heym *et al.*, 1995).

Mutation	AA change
G1C	M1L
T2C	M1A
G3A	M1V
G3C	M1V
G3T	M1V
3Gdel	Frameshift
7GAdel	Frameshift
18Ains	Frameshift
19Cins	Frameshift
G28T	E10*
54Cins	Frameshift
78GAAATACins	Frameshift
G91T	E31*
G113A	W38*
140Tins	Frameshift
G241T	E81*
G264T	Q88H
G2095A	G699R
295Gdel	Frameshift
G311A	R104Q
G320A	W107*
355Cins	Frameshift
G356C	R119P
G362A	G121D
367Gins	Frameshift
371Gdel	Frameshift
A413G	N138S
A425C	D142A

Mutations continued	AA change continued
G451T	V151F
G452A	W198*
[597Gins]	Frameshift
628Ains	Frameshift
633Gins	Frameshift
T685G	Y229D
[G944A]	S315N
[G944C]	S315T
[G944T]	S315I
C945G	S315R
[C945A]	S315R
A970C	T324P
G983T	W328L
1102Tins	Frameshift
C1153T	R385W
G1282C	G428R
G1282T	G428W
[G1283T]	G428V
{1317Gdel}	Frameshift
A1316G	Q439R
1559Cdel	Frameshift
1701Tins	Frameshift
G1795T	G599*
T1985G	L662R
T2002C	W668R

Table 5.1 Colonies picked from slow or fast growth cultures (Culture SG2 and Culture FG2) at 12 or 13 MGT were subjected to Sanger sequencing. The notation used for each mutation is as follows: original nucleotide followed by the nucleotide number and finally the new nucleotide. The corresponding amino acid changes or effects at each position are also indicated. In cases where an amino acid alteration is indicated by an asterisk there was a truncation of the gene. The square brackets indicate that the mutation was found in both growth rates, curly brackets indicate a mutation in slow growth and no brackets indicate a mutation in fast growth. Taken from (Jeeves *et al.*, 2015)

Culture name	Alternative mutation-nucleotide position & (315 codon)	No. of colonies with this mutation	Amino acid change or effect
SG2;12MGT	1317Gdel (ATC)	1	Frameshift (S315I)
	G1283T (ATC)	1	G428V (S315I)
	597Gins (AAC)	3	Frameshift (S315N)
FG2; 13MGT	A1316G & G1795T	1	Q439R & G599*
	1559Cdel (AGA)	1	Frameshift (S315R)
	597Gins (AAC)	1	Frameshift (S315N)
	597Gins (AAC)	1	Frameshift (S315N)
	355Cins & G264T	1	Frameshift & Q88H

Table 5.2 Double nucleotide polymorphisms within the *katG* gene in colonies isolated from slow or fast growth cultures (Culture SG2 and Culture FG2) at 12 MGT or 13 MGT, respectively, were identified by Sanger sequencing. The notation used for each mutation is as follows: original nucleotide followed by the nucleotide number and finally the new nucleotide. Some of the *katG* codon Ser^{315} mutations will have shifted by a nucleotide because of a deletion ("del") or insertion ("ins") upstream of the *katG* codon Ser^{315}. Corresponding amino acid changes or effects at each position are also indicated. In cases where an amino acid alteration is indicated by an asterisk there was a truncation of the gene. Taken from (Jeeves *et al.*, 2015)

5.6 Susceptible colonies

Colonies isolated from chemostats that were not exposed to antibiotic (either in the control culture from a pre-drug sample of the drugged cultures) were taken and cultured on isoniazid containing plates to determine their susceptibility. The aim was to pick a range of colonies from different growth rates however many of the colonies became contaminated, the results shown are from the slow growth no drug control.

Those colonies that were susceptible i.e. did not grow on isoniazid were then sequenced to confirm a wild type genotype at *katG* codon 315 and in the promoter region of *inhA*. Of the colonies sequenced 100 % were wild type at both codons (table 5.3)

Mean Generation Time	Days after inoculation	No. of colonies	Susceptible (1 µg/ml isoniazid)	*katG* codon 315	*inhA* promoter region
69.3	31	5	Yes	Wild Type	
	41	10	Yes		
	43	3	Yes		
	51	8	Yes		
	53	4	Yes		

Table 5.3 Colonies sequenced at *katG* codon 315 after being determined susceptible to 2 x MIC (1 µg/ml) isonazid.

5.7 Discussion

Both cultures demonstrated equivalent mutation rates which reflected previous findings showing little differences in the mutation rates of active latent and reactivated disease samples from non-human primates (Ford *et al.*, 2011). Mutation rates in latent infection in humans have however been reported to be lower than in actively replicating samples, these mutation rates were calculated by sequencing the outbreak and control strains recovered during the study (Colangeli *et al.*, 2014). The slow growing cultures seemed less able to grow on isoniazid initially (the first 4 mean generations) in both the control and drugged cultures. The mutant frequency increased as the TVC declined and the pyrosequencing shows a genetically adapted population with a variety of SNPs at *katG* codon 315.

The SNPs seen were clinically relevant mutations in response to isoniazid and have been reported previously (Haas *et al.*, 1997a; Hazbón *et al.*, 2006; Lipin *et al.*, 2007), this high percentage of mutations at codon 315 of *katG* and the occurrence of different non-synonymous substitutions demonstrate the importance of this codon for the development of isoniazid resistance (Haas *et al.*, 1997b), further strengthening the reasoning for the choice of this loci for analysis. The slow and fast growth mutants were wild type in the promoter region sequenced of *inhA* but it has been shown that *inhA* promoter mutations are inversely associated with mutations in *katG* (Hazbón *et al.*, 2006). Resistance to isoniazid via *inhA* promoter mutations is due to over-expression of *inhA.* However, this is an unlikely to occur as a primary mutation as expression of *inhA* has been shown to be tightly regulated (Banerjee *et al.*, 1994). Therefore

overexpression could be too much of an energy cost to the bacterium and so *katG* mutations are likely to be selected for over *inhA* promoter mutations.

The fast growth cultures that were not exposed to isoniazid showed a similar result to the drugged cultures in that *M. tuberculosis* at this growth rate appeared to be phenotypically adapted when *katG* codon 315 sequencing results were analysed (figure 5.2). Further sequencing was done on these colonies and showed that the majority of the resistant colonies that were wild type at *katG* codon 315 had mutations elsewhere within the *katG* gene (Jeeves *et al.*, 2015). Many of these mutations resulted in frameshifts or deletions that would have disrupted the function of *katG* entirely.

The previous results in Chapters 3 and 4 showed the slow growing *M. tuberculosis* adapted quicker and earlier to isoniazid exposure in terms of the viable count and susceptibility and it was thought this was due to a natural phenotypic adaptation. The results in this chapter indicate that the slow growing *M. tuberculosis* was adapting genotypically at *katG* codon 315. A mutation that occurs with increasing frequency the more drug resistant an isolate becomes (Hazbón *et al.*, 2006) and because of catalase peroxidase activity retention, confers a high level of resistance (Somoskovi *et al.*, 2001). The quicker adaptation of the slow growers compared with the fast growers could be due to the retention of KatG activity compared to the loss of KatG function the fast growers would have encountered. The slow growth rate conditions could have been selecting against the maintenance of mutants with alternative sites of mutation and loss of KatG activity. The fast growing population with disrupted KatG activity did survive through isoniazid treatment, indicating there was a requirement for a functional KatG in the slow growers but not in the fast growing population. The dependency on a functional KatG in the slow growers could be due to a need to protect against the additive effects of free radical isoniazid intermediates (Wengenack and Rusnak, 2001) and increased exposure to free radicals through a longer replicative lifespan. *In* vivo During infection, an important part of human immune defences is the production of anti-microbial oxygen and nitrogen radicals by phagocytes, a functioning KatG is essential in the oxidative stress network of *M. tuberculosis* (Nambi *et al.*, 2015).

Fitness is the capability of a genotype or individual cell to survive and reproduce, genetic mutations associated with antibiotic resistance have been shown to reduce the fitness of a bacteria (Andersson and Hughes, 2010). One such consequences of a reduction in fitness is a reduction in the growth rate of an organism (Lenski, 1998; Schulz zur Wiesch *et al.*, 2010). The imposed slow growth rate in culture may have been slow enough to nullify any fitness cost caused by mutations in *katG* codon 315. The increased growth rate in the slow growing cultures

after the initial bactericidal kill is likely to have still been slower than the fast growth rate which was close to μ_{max} in our system otherwise we would start to see a more diverse range of katG mutations (as seen in the fast growth) starting to accumulate.

Transcriptomic analyses were performed (in Chapter 6) from RNA samples removed from the slow and fast cultures described in this chapter. The transcriptomics analyses (Chapter 6) aimed to determine any differences in phenotypic adaptation between the two growth rates and whether there was upregulation of any genes in response to the differing mutations in *katG*. In populations with a defective KatG, upregulation of *ahpC* could compensate for the loss of oxidative stress response, these compensatory mutations have been described previously (Sherman *et al.*, 1996). Phenotypic adaptation to antibiotic exposure can include upregulation of efflux pumps which has been described previously in response to isoniazid exposure (Gupta *et al.*, 2010; Jiang *et al.*, 2008). It will also be interesting to determine the differences in transcriptomic profiles between fast and slow growth before isoniazid exposure.

Chapter 6. Transcriptomic analysis of fast and slow growing *Mycobacterium tuberculosis* exposed to isoniazid

6.1 Introduction

Results have been presented in chapters 4 and 5 showing the differing responses of *M. tuberculosis* to the presence of isoniazid in the chemostat based on doubling time. Slow growing (69h MGT) bacilli appear to be adapting to the effects of isoniazid exposure by mutation of *katG* codon 315 as shown by the pyrosequencing results in chapter 5. The fast growing bacilli (23h MGT) did not appear to be adapting at either *katG* codon 315 or in the region of the promoter that controls *inhA* expression levels but did show mutations in other regions of the *katG* gene. Both growth rates resulted in a population of bacteria surviving through drug exposure (MIC levels of 0.5 µg/ml) in continuous culture but the mechanisms that were used to achieve this appear to be different. Resistance is a term usually preserved for bacteria that have a genetic adaptation to the presence of antibiotic and can be identified using sequencing methods. Persisters are a population of bacteria able to survive antibiotic exposure but are formed through a number of independent parallel mechanisms (Lewis, 2008), these include phenotypic mechanisms such as transcriptional regulation. Persisters are generally classed as phenotypic variants of the wild type (Kere*n et al*, 2011) meaning that sequencing methods will not identify any differences in these cells to the wild type in traditional antibiotic resistance loci.

In order to investigate phenotypic adaptation, RNA was extracted from the continuous culture systems as described in (section 2.6) to compare the expression profiles of the slow and fast growing *M. tuberculosis* in the presence of isoniazid. Genome wide expression analysis using microarray provides a powerful means to study the adaptive survival strategies of bacteria in response to a variety of stress conditions (Bacon and Marsh, 2007; Karakousis *et al.*, 2008; Kendall *et al.*, 2004). These strategies include regulatory changes resulting in changes to the physiology of the bacterium. Regulatory adaptations inform the bacterium as to the genomic changes that maximises the chance of adaptation and evolutionary success (Ryall *et al.*, 2012). A regulatory change in genes involved in a DNA repair mechanism could lead to a change in mutation rate. A downregulation in DNA repair could result in mutations, such as single nucleotide polymorphisms (SNPs), these could confer an advantageous phenotype which is then selected for.

Previous studies investigating transcriptional responses of *M. tuberculosis* in a variety of environments gives an indication of the groups of genes that may be important in the

interpretation of the results in chapters 4 and 5. In a previous study looking at the gene expression of both slow and fast growing *Mycobacteria* it was found that expression of genes belonging to the PE and PPE families, insertion sequence (IS) elements and genes associated with biosynthesis of the cell envelope were significantly up-regulated in the slow growing bacteria whereas genes involved in the synthesis and modification of macromolecules were down-regulated (Beste *et al.*, 2007b). Some of these genes could also alter the response of the bacterium to isoniazid. Isoniazid regulatory genes (IRGs) have found to be up-regulated in *M. tuberculosis* in the mid-exponential phase of growth (Karakousis *et al.*, 2008) , comparable to the fast growth rate in the experiments in this thesis. These IRGs include genes of the FASII operon, genes involved in mycolate transfer and fatty acid β-oxidation and genes involved in the detoxification of the toxic consequences of isoniazid and antibiotic efflux (Wilson *et al.*, 1999) Another interesting group of genes that are important for growth through isoniazid exposure are those that are involved in the conversion of isoniazid from its pro-drug to active form, for example differential expression of *ndh* can contribute to isoniazid resistance in isolates that do not contain mutations in *katG, inhA* or *kasA* (Karakousis *et al.*, 2008) In general, lack of IRG expression by organisms following exposure to isoniazid correlated with reduction in drug activity.

By comparing the gene expression profiles of the fast and slow growth *M. tuberculosis* exposed to isoniazid, a list of significantly up or down regulated genes was produced with the hypothesis that fast and slow growing *M. tuberculosis* were persisting through isoniazid treatment using differing mechanisms. The slow growers were accumulating mutations in *katG* codon 315 and the fast growers accumulating mutations disrupting the function of KatG. The transcriptomic data was interrogated with the aim of determining whether additional mechanisms to survive isoniazid exposure, such as compensatory regulation was occurring in these populations.

The specific objectives of this work were as follows:

- To identify genes involved in phenotypic adaptation to isoniazid and whether these differ between slow and fast growing *M. tuberculosis*

- To investigate whether there is evidence of compensatory regulation of genes in the fast growth experiments to compensate for a defective KatG due to mutation.

6.2 The effect of isoniazid addition on gene expression in *M. tuberculosis*

M. tuberculosis grown in the chemostat shows a differing response to isoniazid, based on growth rate (figure 6.1). Samples were taken for RNA before isoniazid was added to the steady-state cultures and then at 1 MGT and 2 MGT post-isoniazid addition. This study focused on the transcriptional changes occurring at 2 MGT because this was a point at which the profiles of the cultures began to differ from each other in their response to isoniazid exposure.

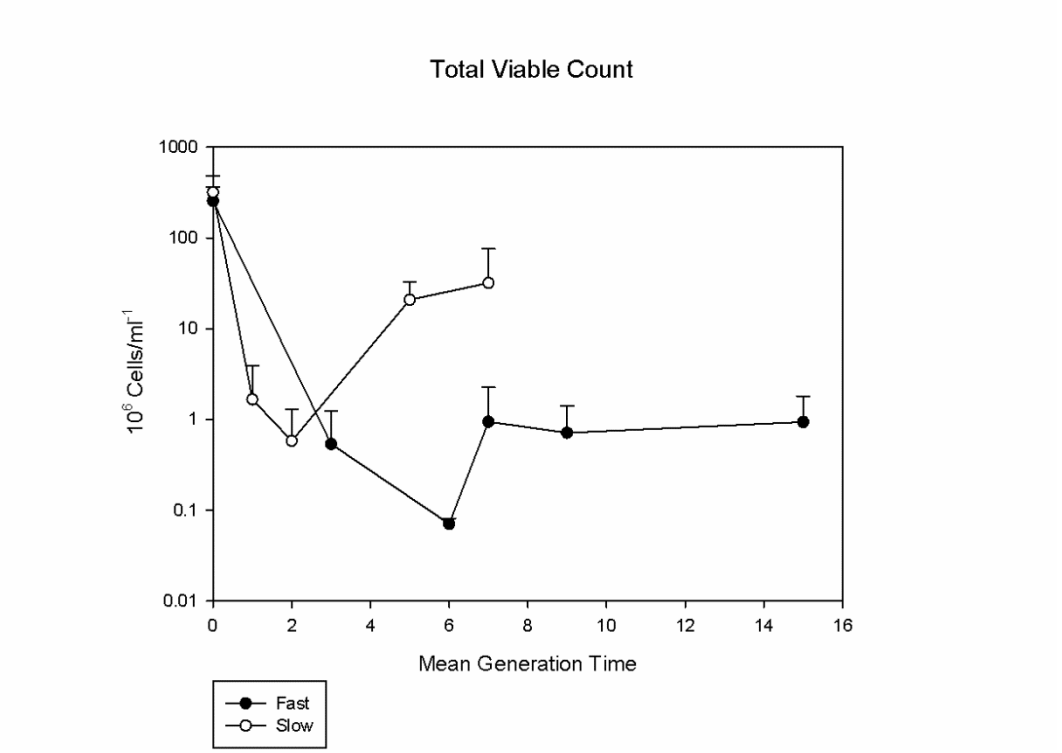


Figure 6.1 Total Viable Counts of *Mycobacterium tuberculosis* H37Rv in response to isoniazid in a continuous culture.

The total RNA collected was then hybridised to a custom designed tiling array from Agilent with 180 x coverage of the *Mycobacterium tuberculosis* H37Rv genome (section 2.7). The gene expression intensity values for cultures with or without drug exposure were normalised and analysed using GeneSpring software. Firstly, quantile normalisation was applied across the combined slow and fast datasets, followed by an averaging of the expression levels of all the probes for each open reading frame.

Significance analyses were applied to find genes that were significantly differentially expressed between fast and slow growth rates either in the absence or absence of isoniazid by adopting a pairwise comparison method using 2-way ANOVA with a Benjamini-Hochberg correction (P=0.05) A further filter was applied to select genes with at least a two-fold change in gene expression. All microarray processing, statistics, PCR validation and compilation of gene lists

were performed by Dr Rose Jeeves at Public Health England, Porton Down. Full geneand can be found in (Jeeves *et al.*, 2015).

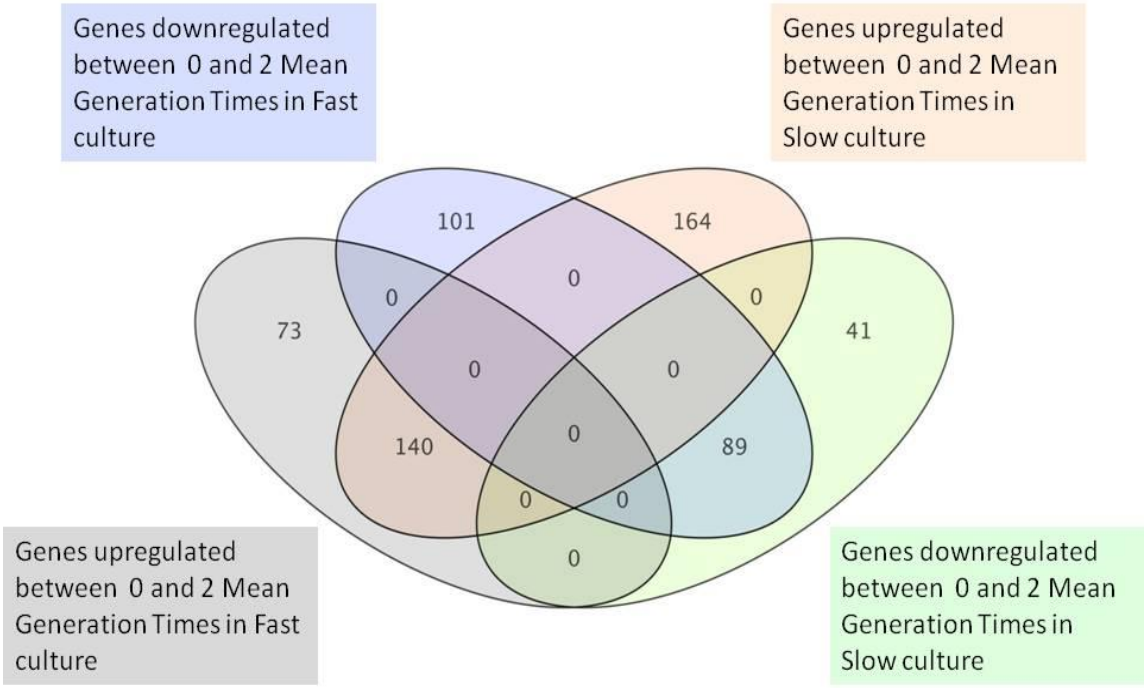


Figure 6.2 Venn diagram depicting the number of genes differentially regulated between 0 and 2 mean generation times from isoniazid addition in fast and slow growth rate continuous cultures.

There were 164 genes up-regulated in the slow growing culture alone between 0 and 2 mean generations (figure 6.2), the number uniquely up-regulated in the fast growing culture is almost half this amount (73). When looking at down-regulated genes the opposite result is true with 101 genes in the fast growth culture alone down-regulated over time, more than double the number in the slow growth culture alone (41). There was a tendency towards up-regulation of the genes differentially expressed in both slow and fast cultures (140) when compared to the shared down-regulated genes (89).

6.2.1 Differentially expressed genes in both fast and slow growing *Mycobacterium tuberculosis*

This data describes genes that were significantly differentially expressed in both slow and fast growing *M. tuberculosis* over time after the addition of isoniazid, representing expression in response to isoniazid addition, independent of growth rate. Lists of differentially expressed genes, which were filtered as described in section 6.2, were submitted to the DAVID Functional

Annotation Bioinformatics tool (http:// david.abcc.ncifcrf.gov/) (Huang *et al.*, 2009a, 2009b) to identify statistically significantly enriched functional groups of genes. Gene functions were identified by Uniprot accession number and significant percentage enrichment was identified using a Benjamini multiple testing correction. The data discussed is from samples taken after 2 mean generations of isoniazid exposure compared to expression levels prior to isoniazid addition when the cultures were in steady-state.

There were 89 genes that were down-regulated over the course of 2 mean generations in both the fast and slow growth cultures. There were several major functional groups of genes identified using DAVID analysis. Genes involved in aerobic respiration were down-regulated and these included a group of NADH dehydrogenases of complex I (Rv3145-6, Rv3148-9, Rv3151-3, Rv3155-7) which catalyse the transfer of electrons. Downregulation of this regulon results in less free NADH within the cell, which would slow the catalysis of isoniazid pro-drug, indicating that this was a general response to the presence of isoniazid. Essential ATP synthases (Rv1307-10) were also down-regulated under both growth conditions and have been previously shown to be down-regulated under stress conditions (Betts *et al*., 2002), this correlates with the presence of isoniazid as it would be expected that aerobic respiration would be down-regulated in the presence of a bactericidal antibiotic due to cell death, which can be seen in the viable count data in Chapter 4.

Alongside these aerobic respiration genes was the down-regulation of secreted proteins Rv2780 and Rv1886c. Rv2780 encodes for Ald, which is a secreted L-alanine dehydrogenase, this protein is thought to be linked to cell wall synthesis as alanine is an important constituent of peptidoglycan. Rv1886c encodes secreted antigen FbpB (also known as antigen 85-b) and this protein is involved in cell wall mycolation and possesses activity required for the biogenesis of 'cord factor', a dominant structure necessary for maintaining cell wall integrity. In addition to these cell wall related genes, two cell division genes were also down regulated Rv2147c and Rv2150c encoding SepF and FtsZ respectively. Polymerisation of Fts into a circular structure at mid-cell, the Z ring, initiates cytokinesis in bacteria. This Z ring functions as a scaffold for a group of conserved proteins that execute the synthesis of the division septum. SepF is one of these proteins and is an essential part of cell division (Hamoen *et al*., 2006). The down-regulation of these groups of genes suggests a reduction in cell wall metabolism and cell division in both fast and slow growing mycobacteria in the presence of isoniazid.

There were 141 genes up-regulated in the cultures in response to isoniazid. Rv3181c and Rv1561 encode for the VapB45 antitoxin and VapC11 toxin respectively. Toxin-antitoxin (TA) systems

have been implicated in the formation of persister cells leading to antibiotic tolerance in an otherwise susceptible population (Keren *et al*., 2011). The VapBC TA systems are a member of the largest TA systems in *M. tuberculosis* and are thought to be associated with ribonuclease function and have been shown to block translation and mRNA cleavage in *M. smegmatis* (Robson *et a*l., 2009). Transient activation of these mRNAses may allow the bacteria to adapt to stress not only by inhibiting replication but also by degrading existing transcripts allowing a rapid change in the metabolic program of the bacteria (Ramage *et al*., 2009). It appears that both the fast and slow growing bacteria have responded to the stress of isoniazid addition by trying to quickly adapt to the environment through regulation of TA systems, of which there are a high number in *M. tuberculosis*.

Another possible adaptation that can be seen in this expression data was the up-regulation of Rv3065 (mmr) which encodes for a multi-drug efflux pump. Bacterial efflux pumps are membrane proteins that are capable of actively transporting a broad range of substrates including drugs, from the cytoplasm to the exterior of the cell. Increased expression of efflux pump genes confers a low-level resistant phenotype and mmr has been shown to be overexpressed in strains of *M. tuberculosis* exposed to isoniazid (Machado *et al.*, 2012; Rodrigues *et al.*, 2012).

The increase in expression of mmr in both the slow and fast growing cultures over the first two mean generations of this experiment showed a response to the presence of isoniazid which allowed the possibility of continued replication enabling the chance of a mutation conferring drug resistance to occur.

6.3 The effect of growth rate on gene expression in *Mycobacterium tuberculosis*

6.3.1 Gene expression prior to isoniazid addition

In the absence of antibiotic, the expression profiles of the slow and fast cultures were very similar with only 7 genes being differentially expressed (Table 6.1). This is a small number of genes that are different between the cultures when compared to work in *M. bovis* (BCG), which found 84 genes exclusively required for slow growth (Beste *et al.*, 2009). An important group of genes that may have been expected to be more highly expressed in the fast growing cultures would be ribosomal related genes, as evidence of growth rate mediated regulation of ribosome biosynthesis has been presented also in *M. bovis* (BCG) (Beste *et al.*, 2005).

Four of these were more highly expressed in the fast growing culture when compared to slow growing culture (Rv1592c, Rv0196, Rv2429 and Rv2428) and 3 genes were more highly

expressed in the slow growing culture (Rv2638, Rv3572, Rv3089) compared to the fast growing culture data.

Higher expression in Fast cultures		
Gene	**Description**	**P-value (Growth Rate)**
Rv1592c-S	Probable inactive lipase	0.007014
Rv0196-S	Uncharacterized HTH-type transcriptional regulator	0.006672
Rv2429-S	Alkyl hydroperoxide reductase AhpD	0.006672
Rv2428-S	Alkyl hydroperoxide reductase subunit C AhpC	0.006528
Higher expression in Slow cultures		
Gene	**Description**	**P-value (Growth Rate)**
Rv2638-S	SpoIIAA family protein	0.001306
Rv3572-S	Putative uncharacterized protein	0.007448
Rv3089-S	Fadd13 possible Fatty Acid-CoA-Ligase	0.007014

Table 6.1 Genes found to be differentially expressed in slow and fast growing *M. tuberculosis* in chemostat culture before addition of isoniazid. These results are from 2 replicates with an ANOVA performed from the larger data set.

The fast growing cultures show a higher level of expression of *ahpC* and *ahpD* (Rv2428 and Rv2429) which encode an alkyl hydroperoxide reductase. In *M. tuberculosis*, OxyR, the central regulator of bacterial oxidative and nitrosative stress responses is inactive (Deretic *et al.*, 1995). Despite this loss, several other oxidative stress response genes are preserved. One of the genes under the control of OxyR is *ahpC* which is suggested to protect mycobacteria from the deleterious effects of the combinatorial products of reactive oxygen and nitrogen intermediates (Pagán-Ramos *et al.*, 2006). KatG is the only peroxide inducible gene in *M. tuberculosis* in the absence of functional OxyR. It has been shown that in the absence of KatG, (for example due to mutation), overexpression of the *ahpC* gene occurs, AhpC can act as an antioxidant; protecting DNA and other proteins from oxidative stress (Chauhan and Mande, 2002). Levels of AhpC are low in wild type *M. tuberculosis*. Increased expression of *ahpC* could perturb peroxidative

Term	P-Value	Genes	Fold Enrichment	Benjamini correction
fatty acid metabolism	0.000294	Rv0905-S, Rv2930-S	29.87058824	0.014001601
lipid metabolism	0.000393	Rv0905-S, Rv2930-S	27.15508021	0.009389622
antibiotic resistance	0.001705	Rv1846c-S, Rv0667-S	16.59477124	0.0269263

Table 6.2 Results from the DAVID Functional Annotation Bioinformatics tool *(http://* david.abcc.ncifcrf.gov/*)* showing statistically significantly enriched groups of genes in the fast growing (MGT 23.1 hours) *M. tuberculosis*. Significant percentage enrichment was identified using a benjamini multiple testing correction.

requirement for KatG activity. A clinical isolate has been studied that possessed an upregulated promoter region of *ahpC* and it is thought this could compensate for any loss of KatG activity (O'Sullivan, McHugh, *et al.*, 2008). If the fast growing *M. tuberculosis* in this experiment was expressing *ahpC* at a level higher than the slow growing *M. tuberculosis* then perhaps the requirement for *katG* expression was reduced, this could lead to less isoniazid being converted to pro-drug and apparent resistant/persistent phenotype without a mutation.

6.3.2 Differential gene expression specific to fast growing Mycobacterium tuberculosis in the presence of isoniazid

There were 101 genes uniquely down-regulated in the fast growing *M. tuberculosis* over the course of the first 2 mean generations of the experiment post isoniazid-addition (figure 6.2). Most of these down-regulated genes were classed as oxidoreductases, including dyhydrofolate reductase, cytochrome P450 and aldehyde dehydrogenase, which are essential genes involved in processes such as glycolysis, oxidative phosphorylation, and amino acid metabolism. The presence of isoniazid appears to be causing a down-regulation of the essential metabolism of the cell, which is reflected in figure 6.1 by the decline in titre over the first 2 mean generations of isoniazid exposure. Another group of genes up-regulated in the presence of isoniazid were lipoproteins, which have a functional role in the transport of nutrients through the cell wall. The role of lipoproteins in the adaptation to antibiotic exposure is still unclear but two of these genes were up-regulated in a previous study upon isoniazid exposure (Waddell *et al.*, 2004).

Membrane proteins including genes encoding DNA translocases such as *ftsK* and *ftsW*, both involved in cell division were also down-regulated over time.

The results of the DAVID analysis described how groups of genes that were enriched only in the fast growing *M. tuberculosis* at 2 mean generations (table 6.2); the viable count data (figure 6.1) suggested this was the timepoint at which the two growth rates began to differ in their responses to isoniazid.

Genes involved in fatty acid metabolism and lipid metabolism were enriched 30 fold and 27 fold respectively in the fast growing *M. tuberculosis*. Gene Rv2930 (*fadD26*) is involved in phthiocerol dimycocerosate (DIM) biosynthesis, which is a major cell wall lipid of the tubercle bacillus. It is a highly apolar wax and with the exception of one species of mycobacteria, all DIM containing species are pathogenic, leading to the idea that DIM is implicated in virulence (Brennan, 2003). Mutants without DIM have been shown to be more permeable and hypersensitive to antibiotics (Camacho *et al.*, 2001; J. Yu *et al.*, 2012) and so the enrichment of genes in the fast growth when compared to slow growth involved in fatty acid and lipid metabolism, including DIM biosynthesis, in the presence of isoniazid could indicate a defence mechanism against entry of isoniazid into the cell by trying to maintain the cell wall and the natural low permeability of the mycobacterial cell wall. This is an indication of a phenotypic adaptation by the fast growing mycobacteria. There was also a 17 fold enrichment of genes classified to be involved in antibiotic resistance. Rv1846c encodes BlaI which is a transcriptional repressor, BlaC governs β-lactamase production in response to beta-lactam treatment and causes de-repression of the BlaI regulon and up-regulation of ATP synthase transcription, indicating a potential regulatory loop between cell wall integrity and ATP production (Sala *et al.*, 2009). Gene, Rv0607 encodes for RpoB which catalyses the transcription of DNA to RNA, *rpoB* is the site of mutations that confer resistance to the rifamycin antibacterial agents, such as rifampicin. Mutations in *rpoB* that confer resistance to rifamycins do so by altering residues of the rifampicin binding site on RNA polymerase (Campbell *et al.*, 2001).

6.3.3 Differential gene expression specific to slow growing *Mycobacterium tuberculosis* in the presence of isoniazid

There were more than double the number of genes upregulated over 2 mean generations in the slow growing *M. tuberculosis* when compared to the fast growing *M. tuberculosis*. Two toxins, part of a TA system, VapC24 and VapC39 were both upregulated in slow growth. There were TA genes up-regulated in both the fast and slow growth (section 6.2.1) (VapB45 antitoxin, VapC11 toxin). The additional up-regulation of toxins in the slow growing bacteria perhaps indicates a more varied and more prominent role of TA systems in this population. As a result of

bioinformatics analysis of *M. tuberculosis*, 38 potential toxins (23 VapC homologues) were identified (Pandey and Gerdes, 2005). It was also found that *M. leprae* appears to have lost all functional toxin genes and this is thought to be due to the unchanging nature of the niche *M. leprae* occupies in the human host, as opposed to the free-living slow growing *M. tuberculosis* (Pandey and Gerdes, 2005).

At 2 mean generations of culture in isoniazid, NADH dehydrogenases and oxidoreductases were enriched in slow growth indicating a possible increase in metabolism and a response to oxidative stress. The expression of putative transposases (Rv1199c, Rv3348and Rv3844) was also up-regulated in slow growth cultures. Insertion-sequence (IS) elements are known to be segments of bacterial DNA that can move from one position on a chromosome to a different position on the same chromosome or on a different chromosome. When IS elements appear in the middle of genes, they interrupt the coding sequence and inactivate the expression of that gene. Owing to their size and in some cases the presence of transcription and translation termination signals, IS elements can also block the expression of other genes in the same operon if those genes are downstream from the promoter of the operon (Griffiths AJF, Miller JH, Suzuki DT, 2000). Transposition, mediated by transposases, is known to account for some of the genetic diversity in mycobacteria as this event can cause mutation and IS6110, a *Mycobacterium tuberculosis* complex species-specific insertion element, is used in DNA fingerprinting of *M. tuberculosis* strains (Thorne *et al.*, 2011).

There were very few down-regulated genes in the slow growth culture, genes involved in mycolic acid biosynthesis were down-regulated including Rv0469 (*umaA* a probable mycolic acid synthase), this could be a response to the presence of isoniazid and a slowing of cell division.

6.3.4 Differential expression of *ahpC* in fast and slow growing *M. tuberculosis*

The expression profiles of the slow and fast growing *M. tuberculosis* prior to addition of isoniazid were very similar, indicating differing growth rate does not cause major expression changes. This is in contrast to other work using these growth rates in chemostats where 84 genes were exclusively required for slow growth (Beste *et al.*, 2009). The main difference was the significant enrichment in expression of *ahpC* and *ahpD* in the fast growing culture and not the slow. It appears the fast growing *M. tuberculosis* has a natural higher expression of the alkyl hydroperoxide reductase which is an important oxidative stress response gene. The fast growing *M. tuberculosis* in these results was used to model the actively growing state of the bacteria as opposed to the slow growing *M. tuberculosis* which is likely to be the state the bacteria are in when entering the latent phase of the disease once infection has been established in the host.

These actively growing cells may have a disposition to increased *ahpC* expression to combat the increased threat of oxidative stress caused by free radical attack. Actively growing cells are more likely to encounter hyperoxic environments, for example in the airway after infection or in the sputum prior to expulsion, which can lead to the production of reactive oxygen species. The immune system of the host also relies on the production of oxygen radicals to kill pathogens and it is likely that it is actively growing *M*. *tuberculosis* cells in a newly infected host that encounter these defences. A natural increased expression of *ahpC* in fast growing *M. tuberculosis* would indicate the phenotypic ability to survive these environments of likely free radical attack, this could also contribute to the greater involvement of phenotypic resistance seen in the fast growing cultures (section 5.3.1.2). Expression levels of *ahpC* following isoniazid exposure increased in both growth rates but were higher in the slow growing bacteria by 2 mean generations compared to the fast growers, the differential expression of *ahpC* was confirmed using qRT-PCR as can be seen in figure 6.3.

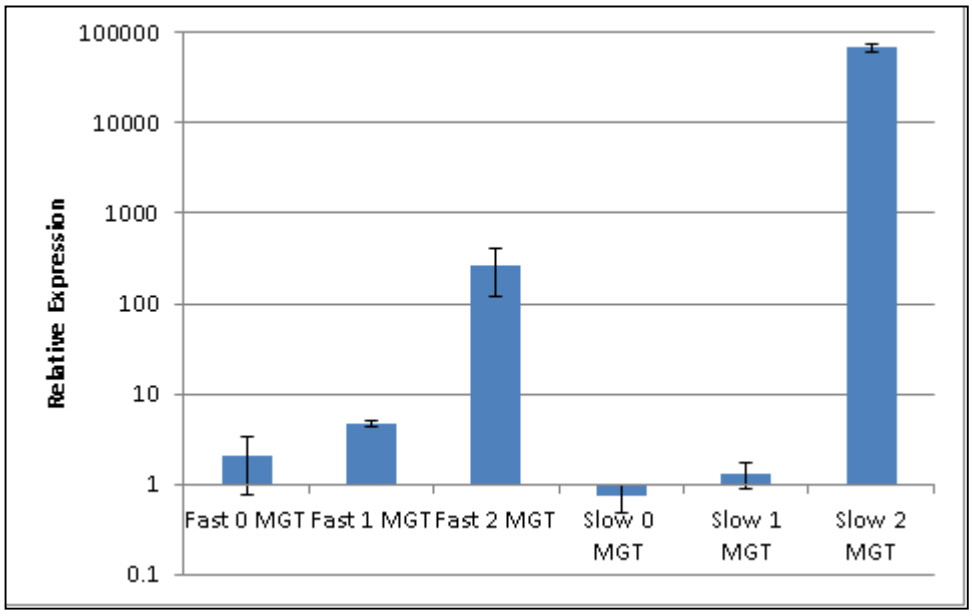


Figure 6.3 Relative expression levels, as confirmed by qRT-PCR, of the *ahpC* gene of *M. tuberculosis* in fast and slow growing cultures across 2 mean generations of isoniazid exposure. Data provided by Dr Rose Jeeves.

It has been hypothesised that *M. tuberculosis* maintains a peroxidative homeostasis (Heym *et al.*, 1997) that is maintained via regulation of *ahpC* and *katG.* An increase in *ahpC* expression following exposure to isoniazid may be due to *katG* being sequestered by isoniazid. The increase in expression of *ahpC* in the slow growing bacteria could be to compensate for partial loss of function of KatG, compromised by the mutations seen in *katG* codon 315 in section 5.3.1 The

mutants in the fast growing population (section 5.4.1) are likely to have a more compromised KatG, the increase in expression of *ahp*C could compensate for some of this but it may be that the increase in efflux pump expression may be another compensatory response to the loss of KatG function.

6.4 Antisense RNA

In this study, tiling arrays were used as a means of investigating expression. These arrays are high density arrays composed of oligonucleotide probes that span the entire genome of an organism. This is particularly useful as they do not require a fully annotated genome and provides an unbiased approach to interrogate the genome for areas of novel non-protein coding RNA (Yazaki *et al.*, 2007)

Antisense RNA (asRNA) is a single stranded RNA that is complementary to a messenger RNA (mRNA) strand transcribed within a cell. asRNA provides a mechanism of gene expression regulation through transcriptional interference. The use of tiling arrays has revealed unexpectedly large numbers of closely spaced promoters, dramatically increasing the potential for transcriptional interference through a number if methods, including; collision of two divergently elongating RNA polymerase complexes, occlusion of the promoters by an already elongating RNA polymerase and removal of bound RNA polymerase by the collision of an already elongating RNA polymerase complex. An RNA duplex can also generate a specific processing site, which may lead to a translationally inactive mRNA (Georg and Hess, 2011)

A full analysis and interpretation of the asRNA data is outside of the scope of this study but there were some results of note. There was a general up-regulation of asRNA expression in both the fast and slow growth cultures over the first two mean generations of culture in the presence of isoniazid. In the slow growing *M. tuberculosis* however, 71% of the total significantly up-regulated genes were antisense compared to 42% in the fast growth. This indicates that perhaps the slow growing bacteria are using antisense RNA as a level of transcriptional regulation more than the fast growing *M. tuberculosis.*

6.5 Discussion

In response to isoniazid, in both fast and slow growing *M .tuberculosis,* there was a down-regulation in aerobic respiration and core metabolic processes, which is in agreement with previous results obtained in a nutrient starvation model (Betts *et al.*, 2002). Cell wall constituents and genes involved in cell wall mycolation along with cell division genes were also down-regulated. Isoniazid acts by inhibiting mycolic acid synthesis and requires NADH to form

an intermediary complex; by down-regulating respiration including NADH pathways, there may be less free NADH in the cell which could slow the catalysis of isoniazid pro-drug to its active form.

There appears to be a general stress response indicated by the up-regulation of toxin-antitoxin (TA) systems and two TA genes were up-regulated suggesting a response to isoniazid. TA systems allow a rapid change in the metabolic program of the bacteria (Ramage *et al.*, 2009) and have been implicated in persistence (Lewis, 2012). It has been suggested in *M. tuberculosis* that RelE toxins are involved in determining the fraction of drug tolerant persisters *in vitro* and this overexpression was drug specific (Singh *et al.*, 2010) although this result could not be replicated *in vivo*. The upregulation of TA systems also indicates an adaptation to a changing environment, whether that be isoniazid exposure or the changing host conditions, *M. tuberculosis* has a large number of TA systems allowing this adaptation (Pandey and Gerdes, 2005) possibly faciliting the maintenance or generation of persister cells.

Further evidence of a stress response was seen with the up-regulation of a multidrug efflux pump (*mmr)*, this has been attributed to emergence of high level resistance. In previous studies, overexpression of mmr, showed no effect on the MIC of isoniazid against M. tuberculosis in response (Rodrigues *et al.*, 2013). It is thought the overexpression of mmr and other efflux pumps may be due to a general stress response caused by exposure to isoniazid and would also serve to transport any other bacterial metabolites either derived from isoniazid itself or generated in response to isoniazid-induced damage. It has also been suggested that sub-therapeutic antibiotic stress induces efflux pumps in mycobacteria and this is the first step in acquiring higher level resistance (Schmalstieg *et al.*, 2012). The efflux pump induction allows protection from the antibiotic allowing replication and increasing the chances of mutation as part of the stochastic process. The induction of efflux pumps preceding emergence of high level resistance is thought to be part of the same process and not two independent events.

There was a general trend of antisense up-regulation in both fast and slow growing *M. tuberculosis*. With the advent of new technologies the analysis of transcription of individual cells is possible. It has become clear that transcription is not a continuous smooth process and is irregular with periods of inactivity followed by strong periods of activity know as transcriptional bursting. This phenomenon may occur under new environmental conditions and has been shown to lead to significantly higher intrinsic 'noise' in asRNA based regulation than in transcription factor based regulation (Mehta *et al.*, 2008). This 'noise' is due to random fluctuations in gene expression and can establish cell-to-cell differences in an isogenic

population of bacteria (Casadesús and Low, 2013). This could increase the phenotypic heterogeneity of cells within a population and antisense expression is associated with genes of larger expression variability. Small RNAs also allow cells to respond rapidly to large changes in input signals, such as environmental conditions, which suggests a niche for these RNAs in allowing cells to transition quickly between different states (Mehta *et al.*, 2008), this is often why genes involved in the stress response or environment specific genes are often enriched for in antisense expression (Xu *et al.*, 2011).

6.5.1 Fast Growth

Genes enriched at 2 mean generations on their function only in fast growth also included genes involved in fatty acid and lipid metabolism including DIM biosynthesis genes. It has been shown that mutants without DIM are more permeable to antibiotics (Camacho *et al.*, 2001) so enrichment of these genes shows a phenotypic adaptation by the fast growing mycobacteria by maintaining the cell wall and its natural low permeability. This enrichment, occurring only in the fast growing bacteria could indicate this population is suffering more cell wall damage in the presence of isoniazid. Lipoproteins were also enriched and these non-covalently linked lipids have also been implicated in contributing to the virulence of some mycobacterial species, enrichment of these genes in the fast growth may indicate a response to isoniazid that involves an increase in virulence, perhaps as a method of survival.

Genes involved in antibiotic resistance indicate a shift to an up-regulation in cell metabolism, Rv1846c (BlaI) could be present as a response to cell wall damage caused by the isoniazid resulting in up-regulation of ATP synthase. The presence of *rpoB* on the list could be less to do with its links to rifampicin resistance but more to the essential role it plays in transcription, indicating that the fast growing cells are responding to the presence of isoniazid by altering transcription and ATP synthesis.

A multi-drug efflux pump (*mmr)* was upregulated in both fast and slow growing *M. tuberculosis* which appears to be a general stress response. The fast growing bacteria also upregulated *efpA* an efflux pump which has been shown to be involved in isoniazid efflux (M. Wilson *et al.*, 1999). This additional method of isoniazid efflux could indicate a gene that allows a phenotypic adaptation and a means of persistence in the fast growing bacteria.

6.5.2 Slow Growth

In the slow growing *M. tuberculosis* there was more upregulation of genes, including toxin-antitoxin genes, this could indicate a more varied gene regulation pattern in this population in

response to isoniazid and alternative phenotypic adaptation which could lead to generation of persisters. Of the genes upregulated, 71% were antisense, indicating that the slow growers are using an alternative gene expression regulation system that is able to fine tune transcription regulation. Transcription factors often control regulons consisting of many genes and operon structures are generally transcribed as one unit; asRNA implements adjustment of mRNA amounts from genes within an operon (Georg and Hess, 2011). Putative transposases were upregulated in the slow growth cultures, showing another possible variation in gene expression control methods. Transposons can also cause genetic mutation and in general, the more transposition, the more genetic instability occurs. Transposition has been shown to occur more readily from transcriptionally active locations, a stable strain may develop more rapid variation due to transposition into an active site (Wall *et al.*, 1999). There were more than double the number of genes upregulated between 0 and 2 mean generations in the slow growing cultures compared to the fast, indicating the slow growing *M. tuberculosis* could be more susceptible to genetic change due to more transposition because of the increased number of transcriptionally active locations.

6.5.4 Conclusions

It appears that the fast growing *M. tuberculosis* in this experiment are adapting by increasing fatty acid and lipid metabolism as has been seen previously when microarrays were performed on exponentially growing *M. tuberculosis* exposed to isoniazid (Wilson *et al.*, 1999). Along with the upregulation of *efpA* involved in isoniazid efflux this suggests a number of phenotypic adaptations by the fast growing bacteria. An up-regulation of *ahpC* at 2 mean generations of isoniazid exposure could be a mechanism to manage the toxic consequences of any isoniazid activation and compensate for loss of KatG function in those mutants with disruptive mutations in *katG*.

The transcriptomic results suggest the main difference between the growth rates is the method of transcriptional regulation. The slow growers have a more varied and fine-tuned response involving antisense RNA and toxin-antitoxin systems; this combined with the up-regulation of mobile genetic elements could provide an environment for the surviving bacteria to be more suited to the niche environment and able to respond to the changing conditions more quickly, whilst accumulating advantageous genetic mutations within *katG* codon 315. The upregulation of TA systems at both growth rates indicates a mechanism to allow formation of persisters (Lewis, 2012) and adapt to changing environments (Pandey and Gerdes, 2005) the fast growing

mycobacteria appear to have capitalised on this with phenotypic adaptations along with the deleterious mutations in KatG.

Chapter 7. Discussion

This study set out to investigate growth rate differences in *M*. *tuberculosis* and the effect these phenotypic states may have on antibiotic efficacy. It has been discussed previously that the environment in which bacteria grow is not always optimal for the action of an antibiotic (Udekwu *et al.*, 2009). *In vitro* studies have demonstrated that the number of persister cells in a population is directly proportional to the number of stationary phase cells inoculated into the culture (Balaban *et al.*, 2004). Also, decreasing the growth rate of *E. coli* in continuous culture has been shown to increase the fraction of persister cells (Sufya *et al.*, 2003). Growth rate is the change in cell number per unit time and growth phase refers to the metabolic state of the bacterium and a population could have a slower growth rate but still be in the exponential phase of growth. These two factors are not synonymous and are both effective indicators of the fitness of a population and also give clues to adaptive techniques. Reduced growth rate has been hypothesised to be responsible for antibiotic tolerance (Baek *et al.*, 2011) , almost all antibiotics preferentially kill rapidly replicating bacteria so the slowing of metabolic processes such as cell wall synthesis and RNA transcription results in a reduction in antibiotic target. In many infectious diseases including tuberculosis, the quantity of bacteria can far exceed standard MIC testing inoculums and so antibiotic concentrations derived from these tests may be insufficient. Understanding how these factors can influence the effectiveness of an antibiotic treatment is essential. Titres *in vivo* are far higher than those tested clinically and poor treatment outcome that is based on low titre susceptibility testing could be contributing to the tuberculosis burden and not reflecting the true pharmacokinetics of the antibiotic in situ (Udekwu *et al.*, 2009). Continuous culture has allowed cell density effects to be investigated because of the ability to grow and maintain high titre populations of bacteria in a controlled and defined environment over a timecourse. The effectiveness of an antibiotic can be investigated in an environment which reflects *in vivo* conditions; more so than in a small scale flask culture.

7.1 Cell density influences antimicrobial efficacy

Cell density experiments (section 3.3) highlighted rifampicin's ineffectiveness at titres ranging from 10^5 cfu/ml to 10^7 cfu/ml; titres that are clinically relevant (De Gro*ote et al*, 2011; Ordway *et al*, 2010; Sharpe *et al*, 2009, 2010b) and found *in vivo* during infection. Rifampicin was more effective at lower titres that are often used as an MIC testing standard (10^5 cfu/ml) (Udekwu *et al.*, 2009) (figure 3.8). Isoniazid was shown to be effective at higher cell densities than rifampicin but still showed reduced killing at high cell densities (figure 3.5) which has been seen previously (Davey and Barza, 1987; Gilbert *et al.*, 1987; LaPlante and Rybak, 2004; Udekwu *et al.*, 2009). *In*

vitro data using *Staphylococcus aureus* showed that in low titre populations the antibiotic was at the MIC or higher for the full timecourse. In high titre populations the concentration of antibiotic was effectively diluted by the number of cells, showing high titre inoculums to have a significant impact on the efficacies of nafcillin and vancomycin (LaPlante and Rybak, 2004). It was also noted in (Udekwu *et al.*, 2009) that the cell density effect differed depending on the mode of action of the antibiotic. At a high bacilli titre the nutrients are depleted by bacterial growth faster than in a comparably low titre culture, this reduction would lead to a reduction in the growth rate of the population which could slow the action of antibiotics, this was demonstrated in *Streptococcus pyogenes* in stationary phase when penicillin binding proteins 1 and 4 were undetectable in a high inoculum therefore leading to the primary targets being absent (Stevens *et al.*, 1993). For isoniazid at a high cell density it could be that a reduced growth rate leads to a reduction in mycolic acids due to a slowing of cell division and therefore a reduction in target for isoniazid. Uptake of isoniazid is via passive diffusion and a disruption in proton motive force or reduction in available ATP does not affect this; it has been shown that KatG activity is the limiting factor in isoniazid activity so when growth rate is decreased due to nutrient limitation the activity of KatG and subsequent conversion of the isoniazid pro-drug will also decrease (Bardou *et al.*, 1998)**.**

The effectiveness of rifampicin has been shown to be concentration dependent (T Gumbo et al., 2007) and time dependent (de Steenwinkel *et al*., 2010; Jayaram *et al.*, 2003) intracellularly. An increase in the concentration of bacilli would effectively lower the concentration of rifampicin available per cell and perhaps lead to less effectiveness at a higher cell density. Rifampicin does not require conversion to an active form unlike isoniazid so once within the cell the amount of time required to act is shorter possibly leading to quicker depletion extracellularly due to passive diffusion based on the concentration gradient.

As well as demonstrating a cell density effect this work also served to demonstrate the differences in modes of action of rifampicin and isoniazid and also introduced the ideas of growth rate and phase affecting antibiotic efficacy.

7.2 Slow growing *M. tuberculosis* is better adapted to antimicrobial exposure

It has generally been accepted that the slower the doubling time of a bacterium the less bactericidal kill is afforded by an antibiotic (Brown and Williams, 1985; Brown *et al.*, 1990; Gilbert and Brown, 1978; Sufya *et al.*, 2003). This was seen in this study with isoniazid and rifampicin and appeared independent of drug concentration. This was important as doubling times in bacterial infections have been shown to be slower than batch culture *in vitro*

experiments (Cozens *et al.*, 1986) To study this slow growth phenotype is important in order to determine antibiotic efficacy at more relevant growth rates. The requirement for a multi-drug approach to treatment of tuberculosis disease was also modelled in section 3.5 as the different efficacies of isoniazid and rifampicin were revealed. Isoniazid appeared more slow acting than rifampicin as the results from the very slow growing bacteria after 24 hours of exposure were not reflective of the amount of action after 96 hours of isoniazid exposure (figures 3.9 and 3.10). These initial experiments served to highlight the importance of considering bacterial physiological factors such as growth rate and phase in the design of new tuberculosis therapies and in the design of experiments aiming to model their action in infection. The requirement for new antimicrobials that are effective against all growth rates has also been highlighted as the efficacy of the two frontline antibiotics for tuberculosis treatment dramatically decreased with slowing growth rate. Bedaquiline belongs to a novel class of antibiotics, which are bactericidal against slow growing and non-replicating mycobacteria (Lakshmanan and Xavier, 2013), targeting the mycobacterial ATP synthase (de Jonge *et al.*, 2007). This new drug has been incorporated into the treatment regime although due to adverse effects is seen as a treatment for use for patients with MDR-TB with no other treatment options (Worley and Estrada, 2014), there is obviously still a need for new therapies

7.3 Effect of growth rate on the activity of isoniazid

The study was then continued (chapter 4) using isoniazid as it is one of the frontline therapies used for both active infections and latent infections (Centers for Disease Control and Prevention, 2003). Previous studies have shown that the decrease in the *M. tuberculosis* population in sputum during therapy was biphasic, showing isoniazid caused a rapid bacillary decrease during the first two days (Jindani *et al.*, 2003; Mitchison, 1979). The theory from this previous work was that the bi-phasic kill was due to depletion of exponentially growing organisms and it was the growth rate of the population that determined the effectiveness of the antibiotic. An understanding of the action of isoniazid on different populations and the emergence of resistance is still important information that could inform the usage of this antimicrobial.

The observation that growth rate led to a reduction in effectiveness of isoniazid was further modelled and investigated by adding isoniazid directly to continuous cultures with controlled doubling times (sections 4.2.1 and 4.3.1). It has previously been demonstrated that the biphasic killing of tuberculosis by isoniazid was accounted for by emergence of resistance, due to a heterologous population (Gumbo *et al.*, 2007), the cessation of early bactericidal kill by isoniazid corresponded with the drug resistant isolates in exponential growth exceeding the population

of susceptible cells. This study in Gumbo *et al* used the Hollow Fibre Model which allows pharmacokinetic and pharmacodynamic (pk-pd) studies of drugs with the absorption and elimination kinetics of the drug being closely controlled (Cadwell, 2012). This model does not control the doubling time of the bacteria and although a very valuable model the continuous culture experiments in this study were focussed on the effect of growth rate. By using continuous culture, the organisms were maintained in exponential phase so the effect of the growth rate can be studied to determine whether it has an effect on the emergence of resistance. This population can then be interrogated and the different aspects such as mutation rate and phenotypic adaptation investigated.

7.4 Slow growing *M. tuberculosis* is adapted to isoniazid with mutations in *KatG* codon 315

It was hypothesised that the difference in the responses of slow and fast growing *M. tuberculosis* to isoniazid were due to different mechanisms of adaption. The fast growing bacteria were adapting less successfully than the slow growing *M. tuberculosis* and it was thought this was due to a natural phenotypic adaptation brought about by slow growth rate. The chemostat culture result (sections 4.2 and 4.3) confirmed that growth rate has an impact on isoniazid activity; the slow growing mycobacteria were able to increase their growth rate and re-establish a high titre population after an initial period (2-3 MGT) of bactericidal kill whereas the faster growing mycobacteria maintained a lower titre population after the same magnitude of bactericidal kill. An increase in growth rate occurred after bactericidal kill, the reduction of the steady state population in the chemostat will have led to an increase of the carbon source, glycerol, allowing for growth rates higher than the imposed dilution rate. The slow growing bacteria may have been able to take advantage of this in the presence of isoniazid more so than the fast growers as the imposed fast growth rate (23.1 hour MGT) was close to μ_{max} in this system.

When the mechanism behind this relationship was investigated it was found that the two growth rates had very similar mutation rates, agreeing with work from other authors (Ford *et al.*, 2011). The reason behind the ability of *M. tuberculosis* to grow in the presence of isoniazid was however different depending on growth rate. The majority of the mutant population recovered from the slow growing population of *M. tuberculosis* was mutated at the clinically relevant codon 315 of *katG* whereas only a small number of the fast growers possessed this mutation, the majority of the mutations in the fast growers were elsewhere in *katG*. This mutation at *katG* codon 315 did not appear to be imposing a fitness cost at the controlled doubling time of 69.3 hours as the culture was able to grow and maintain a very high titre population within the chemostat. Pyrosequencing of isoniazid relevant codons of *katG* and *inhA* allowed a rapid

method to screen for SNPs that may be contributing to the ability of *M. tuberculosis* to grow on isoniazid. The range of non-synonymous substitutions found in codon 315 of *katG* highlighted the importance of this gene and regions high in non-synonymous mutations have been shown to be strongly associated with antibiotic resistance (Shekar *et al.*, 2014). This heteroresistance has also been demonstrated in clinical samples (Rinder *et al.*, 2001). The absence of any SNPs in the region of *inhA* sequenced was in agreement with an observation that mutations in this gene are inversely associated with mutations in *katG* (Banerjee *et al.*, 1994) as *inhA* over-expression may be at significant cost to the bacterium.

The presence of *katG* codon 315 mutations predominantly in the slow growing population suggests that this was a mutation selected for during slow growth. It could be that this mutation does incur a fitness cost, perhaps the reduction in catalase activity and ability to tolerate oxidative stress, reducing the growth rate of the bacterium. This fitness cost may have reduced the ability of *M. tuberculosis* to grow at the faster doubling time but the slower doubling time of 69 hours may have been sufficient to nullify the fitness cost. The majority of mutations in the slow growers were in this codon of *katG* and not dispersed throughout the gene as in the fast growers. In contrast to the slow growing bacteria the fast growing *M. tuberculosis* was more susceptible to the concentrations of isoniazid above the MIC. The pyrosequencing results showed genetically wild type regions of *katG* codon 315 and the promoter region of *inhA*, the two regions that were sequenced were chosen because the frequency of mutations occurs in these regions at 70-80 %, in this study they have served to highlight the difference between growth rates. More recently, work done by Dr Irene Freire-Martin, has shown that 65 out of 77 isoniazid-resistant colonies that were wild type in *KatG* codon 315 isolated from the fast growing population (after 12-13 MGT) contained mutations elsewhere within the *katG* gene (Jeeves *et al* 2015) (section 5.4.1). This suggests that the KatG codon 315 mutation was selected for by slow growth and perhaps other alternative more deleterious mutations throughout the gene were lost (selected against), indicating that slow growing *M. tuberculosis* requires a functional KatG.

7.5 Slow growing *M. tuberculosis* has more varied transcriptional regulation

The continuous culture experiments also allowed RNA to be collected for transcriptomic analyses (chapter 6), which showed that there was more gene upregulation in general in the slow growing bacteria, including toxin-antitoxin genes, possibly serving as an alternative mechanism of transcription regulation. Further evidence for alternative regulation was the high ratio of upregulated antisense RNA, a mechanism enabling bacteria to rapidly adapt to changing

environmental conditions (Mehta *et al.*, 2008). Antisense RNAs along with other non-coding RNA based mechanisms have been discovered through the use of high density tiling arrays and RNA sequencing (RNA-seq). In *Mycobacterium tuberculosis* 65 % of the genes have an antisense component corresponding to >10% of the coding transcript during exponential growth and this increases to >90% in stationary phase, the extent of this antisense transcription suggests that it may represent a common component of gene regulation in *M*. *tuberculosis* (Arnvig and Young, 2012) and also other bacterial species such as *Helicobacter pylori, Salmonella enterica and Pseudomonas aeruginosa* to name a few (Sesto *et al.*, 2012). Successful pathogenesis relies on the ability to adapt to unfavourable environments and to thrive in environments suited to bacteria replication, accumulation and detection of sRNAs linked to different stress regulons can inform on the response of the bacteria to these environments and support rational targeting of improved therapies (Arnvig and Young, 2012).

In a study of the genetic requirements of fast and slow growth in mycobacteria it was shown that transcriptional regulators were responsible for control of growth rate, many of which have been implicated in virulence and persistence (Beste *et al.*, 2009). The slow growing mycobacteria from the chemostats in this thesis adapted to isoniazid exposure via upregulation of methods of transcriptional regulation (section 6.4) such as antisense RNA and toxin-anti-toxin (TA) genes. TA systems allow a rapid change in the metabolic program of the bacteria (Ramage *et al.*, 2009) and have been implicated in persistence (Lewis, 2012). Non-coding RNA in *M. tuberculosis* has been shown to have an association with genes implicated in pathogenesis and riboregulation may also be involved in the generation of persister cells (Arnvig and Young, 2012) perhaps it is this sub-population that are the most well adapted to virulence and persistence *in vivo,* a strategy that has been highlighted previously (Beste *et al.*, 2007a). This transcriptional data along with the clinically relevant SNPs seen in *katG* codon 315 indicate that the slow growing population in this study are perhaps the most clinically relevant in terms of adaptation to the host environment and their response to isoniazid exposure. The chemostat has proven to be a method allowing isolation of clinically relevant populations of *M. tuberculosis* that can be thoroughly investigated over a timecourse at the high titres often seen in *in vivo.*

7.6 Fast growing *M. tuberculosis* adapts with phenotypic adaptation along with more varied *katG* mutation

Many of the mutations (frameshifts and deletions) found in the fast growers entirely disrupted the function of KatG, suggesting that a functional *katG* encoded catalase peroxidise is a requirement for slow growth but not fast growth. The transcriptomic data (sections 6.2.1 and

6.3.2) showed enrichment of gene expression involved in increasing the strength of the cell wall (*fadD26, echA6*), this could be a response due to the fast growing cells undergoing more cell wall damage by isoniazid or it could be an adaptation to decrease the permeability of the cell wall, making the entry of the isoniazid pro-drug more difficult. Further evidence for a phenotypic adaptation was upregulation efflux pumps (*mmr*, *efpA*) which have been shown to be involved in isoniazid efflux (Wilson *et al.*, 1999) and efflux pump mediated resistance has also been demonstrated in *M. tuberculosis* (Gumbo *et al.*, 2007). The faster growth rate imposed may itself have been advantageous in that the turnover of cell wall may have been fast enough to diminish some of the effect of isoniazid, the fast growers may have been more susceptible than the slow growers but were still able to adapt and maintain a population in the presence of isoniazid. Efflux pump mediated resistance may be transient and it is thought that this mechanism appears to serve only as a 'stepping stone' to high level resistance (Trauner *et al.*, 2014). To fully investigate the role of efflux pumps in this population, an efflux pump inhibitor such as reserpine could be introduced as has been done to elucidate the mechanism of efflux pump mediated drug tolerance of *M. tuberculosis* previously (Colangeli *et al.*, 2005). The possible loss of KatG function in the fast growers with frameshift/deletion mutations would prevent the conversion of the isoniazid pro-drug and lead to a mechanism of resistance, the combination of this strategy along with efflux pump upregulation and cell wall strengthening allowed the fast growers to persist through isoniazid exposure. The loss of KatG function would leave this population open to oxidative attack and perhaps this is the reason that the slow growing population was able to thrive rather than persist; they retained the catalase peroxidise activity of KatG enabling them to withstand oxidative attack.

At both growth rates expression of *ahpC* and *ahpD* (alkyl hydroperoxide reductase subunits C and D) increased over time which could be hypothesised to be compensating for reduction or elimination of the function of KatG caused by mutation. The majority of the slow growing population had mutations at *KatG* codon 315 which as discussed earlier leads to lack of isoniazid activation but maintains some catalase peroxidise activity. At 2 mean generations after exposure transcription of *ahpC* was higher in slow growth than fast growth, this combination of a partially functional *katG* with *ahpC* may have given the slow growers an effective mechanism to combat oxidative attack. Mutations in *ahpC* have been detected in isolates with *katG* mutations and it has been suggested that changes to the *ahpC* promoter are not casually involved in isoniazid resistance, rather they act as compensatory mutations occurring as a consequence of reduction or loss of catalase peroxidase activity (Sherman *et al* 1996).

7.7 Future studies

Isoniazid resistant mutants have also been shown to revert to wild type in the absence of selection pressure (Richardson *et al.*, 2009). Further work in plating of the colonies isolated in the presence of antibiotic onto medium containing no antibiotic may show reversion to a wild type phenotype. Replacing the medium in the continuous culture with medium containing no antibiotic would also allow measurement of the changes to the population at a phenotypic and genotypic level and further add to the understanding of the nature of the resistance of both the slow and fast growing populations.

Whole genome sequencing would determine whether there are any compensatory mutations for loss of KatG function in the fast growers and would allow other key isoniazid resistance genes such as *inhA* (Vilcheze *et al.*, 2006) and *ahpC* (Seifert *et al.*, 2015; Sherman *et al.*, 1996) be visualised. Determining whether the fast growing populations have mutations in isoniazid resistance genes or in previously unidentified areas of the genome may highlight an alternative mechanism of resistance or indeed an epistatic relationship with the detected mutations. In many bacterial strains compensatory evolution has been shown to mitigate fitness costs associated with drug resistance mutations and it has been seen in rifampicin resistant isolates of *M.* tuberculosis (Borrell and Gagneux, 2009; Eldholm *et al.*, 2015). Interrogation of the whole genome would give an unbiased read-out of mutation rates and compensatory mutations.

Future work that would further elucidate the methods used by differing growth rate populations of *M. tuberculosis* would involve a investigating a range of drug concentrations within the chemostat, to look at the populations that would arise with a fluctuating level of isoniazid, similar to the exposure *in vivo* in patients treated for tuberculosis. It would also be interesting to model combinations of antibiotics within the chemostat to further elucidate the differing effects of isoniazid and rifampicin on *M. tuberculosis* and how they might work synergistically to clear specific populations of bacteria. This would be useful to model in both batch and continuous cultures to investigate the effects of growth phase and look at growth rate also.

Continuous culture could also be powerful tool for characterising clinical mutants, it would be interesting to characterise clinically relevant mutations at different growth rates in the presence of single and combination therapy. This could help inform under what conditions these mutants arise, whether they thrive at a slow growth rate, in a particular environment such as low pH which has been demonstrated to be permissive to a wide range of mutations in *rpoB* causing resistance to rifampicin (Jenkins *et al.*, 2009) or if a certain threshold of antibiotic concentration causes increased mutation. The ability to model and replicate clinical mutations strengthens the

chemostat model for investigating mechanisms of antibiotic persistence and resistance in clinically relevant bacterial strains. Using *M. tuberculosis* that has been isolated directly from patients may help characterise the effect of genetic background on resistance phenotypes as it has been shown that the genetic background of a strain of *M. tuberculosis* can influence the capacity of transmission even in strains with the same resistance mutations (Fenner *et al.*, 2012)

7.8 Conclusion

The main aim of this thesis was to establish whether persisting populations of *M*. *tuberculosis* in the presence of antibiotic had adapted differently due to growth rate and whether the adaptation seen was due to phenotypic or genotypic mechanisms. Susceptibility profiles of *M. tuberculosis* did show a difference based on growth rate with the slower growing cells generally more recalcitrant to the effect of an antibiotic.

- The reduced susceptibility of the slow growing bacteria appears to be due to specific genetic mutation at *katG* codon 315 and differing transcriptional regulation.
    - Any fitness cost associated with mutations in *katG* codon 315 were nullified initially by the imposed slow growth rate, it is possible that there was a large enough mutant population to survive even when the growth rate increased due to excess glycerol. The slow growers may have been conserving energy by fine tuning transcriptional regulation as a stress response which may have led to increased genetic heterogeneity and could have served as a mechanism of survival for *M. tuberculosis* in the presence of isoniazid
- Efflux pump upregulation and maintenance of KatG activity may help to explain the success of the fast growing population.
    - The upregulation of efflux pumps in the fast growing population in this study could be an indication of how the higher level resistance occurs. Efflux pump mediated resistance can be transitional and a stepping stone for a higher level resistance by allowing mutants to establish a population. *In vivo,* the faster growing bacteria with efflux pump mediated resistance could facilitate a slower growing population to emerge with a genetic mutation; the genetic mutation in the slow growing population appears more specific and retains KatG activity perhaps explaining the success of this population in the presence of isoniazid.

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