The effect of glutamate homeostasis on the survival of *M. bovis* BCG

by

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Summary

Mycobacterium tuberculosis, the causative agent of the tuberculosis disease, is estimated to infect a third of the world's population and is therefore, arguably, the most successful human pathogen in recorded history. Immense efforts to understand the genetic factors and biochemical processes underlying the complex interactions between *M. tuberculosis* and its host cells have delivered staggering insights into the profound proficiency by which this bacterium establishes and maintains an infection. It is now clear that *M. tuberculosis* can interfere with the immune responses initiated by host cells in such a manner as to subvert the various bactericidal conditions established by these cells and thus eliminate the tubercle bacilli that infect them. Specific characteristics of *M. tuberculosis* which provide it with this ability include a nearly impenetrable cell wall, secretion systems which secrete special factors which directly interact with host immune factors. This enables *M. tuberculosis* to modulate the activities of the host environment and unique metabolic adaptations of *M. tuberculosis* allows the organism to survive in the hypoxic, oxidative, nitrosative, acidic and nutrient poor environment of immune cell phagosomes and to persist for decades in a quiescent state in otherwise healthy people. New observations into the pathways which constitute energy, carbon and central nitrogen metabolism, among others, in *M. tuberculosis*, suggest that a carefully orchestrated homeostasis is maintained by the organism which may modulate the concentrations and ameliorate the effect of molecules that are important to defensive strategies employed by host cells. Here we discuss various recent studies as well as new information provided by this study, focusing on central metabolism and its regulation in *M. tuberculosis*. We aim to highlight the importance of nitrogen metabolism in the subversive response employed by *M. tuberculosis* to survive, colonise and persist in the host. We argue that the homeostatic regulation of nitrogen metabolism in *M. tuberculosis* presents a profound vulnerability in the pathogen which should be exploited with compounds that inhibit the activities of various effector proteins found in this pathway and that are unique to the organism. Such compounds may provide valuable novel chemotherapies to treat tuberculosis patients and may alleviate the burden of multiple drug resistance which plagues tuberculosis treatments. Specifically, in this study we investigate the role of *M. bovis* BCG glutamate dehydrogenase (GDH) and glutamate synthase (GltS) by subjecting knockout mutants of the aforementioned gene products to various cellular stress conditions. Furthermore, we investigated how the genomes of each *M. bovis* BCG strain was affected post deletion of the

aforementioned protein products. The role of GDH was also tested in an murine macrophage model of infection to elucidate potential importance to colonisation and infection. This study provides novel results indicating an importance of GDH toward the resistance of nitrosative stress as well as a requirement for optimal persistence in RAW 264.7 macrophages. In addition, it was found that GltS is dispensable for resistance against nitrosative stress.

Opsomming

Mycobacterium tuberculosis, die organisme wat die aansteeklike siekte tuberkulose veroorsaak, infekteer ongeveer 'n dêrde van die wêreld populasie en is daarom, waarskynlik, een van die mees suksesvolle menslike patogene in geskiedenis. In die afgelope jare is daar noemenswaardige poging aangewend om genetiese faktore sowel as biochemiese prosesse te verstaan wat die komplekse interaksies tussen *M. tuberculosis* en sy gasheer selle verduidelik. Dit is nou voor die hand liggend dat *M. tuberculosis* kan inmeng met die reaksies van die immuun sisteem, om dus die bakteriosidiese omgewing wat geskep word deur die selle van hierde sisteem te vermy. Daar is spesifieke kenmerke van *M. tuberculosis* wat toelaat dat die bacilli so 'n omgewing kan weerstaan. Hierdie kenmerke is, onder andere, 'n byna ondeurdringbare selwand en uitskeiding sisteme wat spesiale faktore vrystel. Hierdie faktore het die vermoë om direk met die gasheer immuun sisteem 'n interaksie te hê wat dus die immuun sisteem moduleer. Verder, is *M. tuberculosis* se metabolisme aan gepas om die organisme te help teen die lae suurstof, hoë oksidatiewe en stikstof stress, lae pH en lae voedingswaarde omgewing te oorleef. *M. tuberculosis* het ook die vermoë om vir 'n onbeperkte tyd in 'n statiese toestand te oorleef, in gashere wat toon as gesond. Nuwe waarnemings in die energie, koolstof en sentrale stikstof metaboliese paaie stel voor dat 'n homeostase gehandhaaf word deur *M. tuberculosis,* wat die konsentrasies van verskeie molekules moduleer of die effek van molekules wat deur die gasheer vrygestel word as 'n verdedigings meganisme versag. In hierdie dokument bespreek ons verskeie studies, asook nuwe inligting voortgebring deur hierdie studie, wat fokus op sentrale metabolisme en sy regulering in *M .tuberculosis*. Ons raak aan die vermoë van *M. tuberculosis* om intrasellulêr te oorleef, koloniseer en voort te bestaan in 'n gasheer. Ons vemoed dat die homeostatiese regulering van stikstof metabolisme in *M. tuberculosis* n diepgaande kwesbaarheid in die patogeen skep wat die potensiaal het om uit gebuit te word. Molekules kan gesintiseer word wat die aktiwiteite van verskeie ensieme in hierdie padweg inhibeer en sodoende die organisme hinder. Sulke molekules mag dalk as waardevolle en oorspronklike medisynes ontwikkel word om tuberkulose patiënte meer suksesvol te behandel asook om die las van middelweerstandige bakterieë te verlig. Met betrokke tot hierdie spesifieke studie, het ons die rol van glutamaat dehidrogenase (GDH) en glutamaat sintase (GltS) van *M. bovis* BCG bestudeer deur om die uitslaan mutante van die genoemde geen produkte aan verskeie sellulêre stress toestande bloot te stel. Die effek van die verlore *gdh* en *gltBD* gene op die

evolusie van die genome van elke *M. bovis* BCG uitslaan mutant ras ten opsigte van die wilde tipe was ook bestudeer. Die rol van GDH was getoets in 'n muis makrofaag model van infeksie om te bepaal of GDH n funksie het in koloniseering en infeksie van *M. bovis* BCG. Hierdie studie het nuwe bevindinge voort gebring wat die belangrikheid van GDH in die weerstand teen stikstof oksied stress. Daar is verder bevind dat GDH n vereiste toon vir die suksessvolle oorlewing van *M. bovis* BCG in RAW 264.7 macrofage

Presentations and Publications

Poster presentation(s):

Poster presentation at the Stellenbosch University Faculty of Health Science Annual Academic Day 2015: Gallant J.L.; Viljoen, A.J.; Wiid, I.J.F.; van Helden P.D.; **NAD dependant glutamate dehydrogenase is required for protection of** *M. bovis* **BCG against nitrosative stress**.

Publication(s):

Gallant J.L.; Viljoen, A.J.; Wiid, I.J.F.; van Helden P.D.; **Glutamate dehydrogenase is required by M. bovis BCG for resistance to cellular stress**. Research article submitted to PloS one.

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"Yes, Knowledge harbours power but it does not bestow it upon the bearer. The wise know how to harness knowledge for they understand that there is always more to learn. Through this simple fact, they are ever learning and growing ever more powerful"- J.R.R Tolkien, The silmarillion

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List of Abbreviations and Terms

AAT	Aspartate aminotransferase
ADN	Albumin, Sodium chloride, Dextrose
ANOVA	Analysis of variance
AnsA	Aspariginase A
AnsP1	Asparagine/aspartate transporter 1
AnsP2	Asparagine/aspartate transporter 2
BAM	Binary alignment map
BCG	Bacillus Calmette–Guérin
BLAST	Basic Logic Alignment Search Tool
BWA	Burrows-Wheeler aligner
CFU	Colony forming units
CHP	Cumene hydroperoxide
CoA	Co-enzyme A
DETE/NO	Diethelenetriamine/Nitric oxide adduct
DlaT	Dihydrolipoamide acyltransferase
DMEM	Delbecco's modified Eagle's medium
DNA	Dioxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
Fd-gltS	Ferrodoxin glutamate synthase
GABA	Gamma aminobutaric acid
GAD	Glutamate decarboxylase
GarA	Glycogen accumulating regulator A
GDH	Glutamate dehydrogenase
gltS	Glutamate synthase
GS	Glutamine synthethase
HbN	Truncated hemoglobin
HLA	5-Hydroxylevulinate
HOA	Hydroxy-3-oxoadipate
IN/DEL	Insertion/deletion
Ino1	Myo-inositol-1-phosphate
KatG	Catalase-Peroxidase-peroxynitritase
KDH	Alpha ketoglutarate dehydrogenase
KGD	Alpha ketoglutarate decarboxylase
LPD	Dihydrolipoamide
MBDM	Murine bone derived macrophages
MIC	Minimum Inhibitory concentration
MOI	Multiplicities of Infection
MSG	Monosodium glutamate
MSO	L-methionine-S-sulfoxamine
NAD	Nicotine amide dinucleotide
NADH	Nicotine amide dinucleotide hydrate
NADP	Nicotine amide dinucleotide phosphate
NEB	New England Biolabs
NO	Nitric oxide
NOS	Nitric oxide Stress

Nos2	Nitric oxide synthase 2
OD	Optical density
PBS	Phosphate buffered saline
PDIM	Phthiocerol dimycocerosate
PflA	Pyruvate-formate lyase activating enzyme A
PknG	Protein kinase G
PknH	Protein kinase H
ppsB	Phenolpthiocerol synthesis type-1 polyketide synthase
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen stress
SAM	Sequence alignment map
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SSA	Succinic semialdehyde
STPK's	Serine/threonine protein kinases
TAE	Tris, acetate, EDTA
Tb	Tuberculosis
TCA	Tricarboxylic acid cycle
TraSH	Transposon site hybridisation
VCF	Variant call format

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Chapter 1

Study Background

1.1   Introduction

Tuberculosis (TB), an infectious pulmonary disease caused by the gram positive bacillus, *Mycobacterium tuberculosis*, remains a profound burden on developing countries. The development of TB chemotherapy which acts by novel mechanisms is required in order to improve treatment outcomes and prevent control the spread of drug resistant strains of *M. tuberculosis*. Molecular biologists have employed a range of mutagenesis techniques in order to search for genes of *M. tuberculosis* which play crucial roles in the ability of the organism to transmit and initiate an infection, to survive inside host immune cells, to maintain a dormant state during latent infection and to disseminate and grow actively during primary tuberculosis disease, with hopes to identify target molecules for the development of a set of completely new drugs active against the pathogen.

Elucidation of the full *M. tuberculosis* genomic sequence and the identification and annotation of approximately 4000 genes have allowed for the use of whole genome approaches to identify the genes that are required for growth of the bacillus *in vitro*, *ex vivo* and *in vivo* (1–5). It was revealed that a large percentage of genes involved in the transport and metabolism of nitrogenous molecules such as amino acids and nucleotides are required for *in vitro* growth of *M. tuberculosis* (2,6). More recently the transport of aspartate and biosynthesis of tryptophan were directly implicated as virulence mechanisms employed by the pathogen (7–9). Investigation of the metabolic systems which underlie nitrogen homeostasis in *M. tuberculosis* may allow novel insights which could be practically implemented in chemotherapy development. Glutamate is a central precursor in the production of most nitrogenous molecules (10–12). It is thus not surprising that a large number of observations in mycobacteria and related species, which will be discussed in this review, point to a crucial importance of the genes involved particularly in the metabolism of glutamate in the viability and growth of *M. tuberculosis*. Disruption of the pathways which regulate the levels of glutamate may present a profound vulnerability in the physiology of *M. tuberculosis*.

1.2   The biochemical pathways central to nitrogen homeostasis in *M. tuberculosis*

The major pathways of nitrogen metabolism involve the assimilation of ammonia/ammonium and the biosynthesis of glutamine and glutamate (Figure 1). In most prokaryotes, including the saprophytic *M. smegmatis*, inorganic ammonia/ammonium is assimilated through the activity of glutamine synthetase (GS), glutamate synthase (GltS, also known as glutamine oxoglutarate aminotransferase) and an anabolic glutamate dehydrogenase (NADPH-GDH) leading to the

production of glutamine and glutamate (13). In slow growing mycobacteria, such as *M. tuberculosis* and *M bovis* BCG, the anabolic GDH is not present and net glutamine and glutamate biosynthesis from inorganic ammonium occurs solely through the activity of GS and GltS (14). However, a second catabolic GDH (NAD^+-GDH) is present in all mycobacteria which is important in the deamination of glutamate (15–17). The *M. tuberculosis* genome contains a reduced set of genes in comparison to *M. smegmatis*, possibly as a result of the reductive evolutionary loss of genes involved in the biosynthesis of metabolites available in the host (18,19). Consequently, far fewer of the genes involved in central nitrogen metabolism in *M. tuberculosis* are redundant in comparison to the genome of *M. smegmatis* (14). The genes encoding for GS (*glnA1*), GltS (*gltB* and *gltD*) and GDH (*gdh*) were found to be required for optimal growth of *M. tuberculosis* in several transposon site hybridisation (TraSH) studies utilising media containing ammonium and glutamate (7H10) or ammonium and asparagine as nitrogen sources (1,2,5,6,20,21).
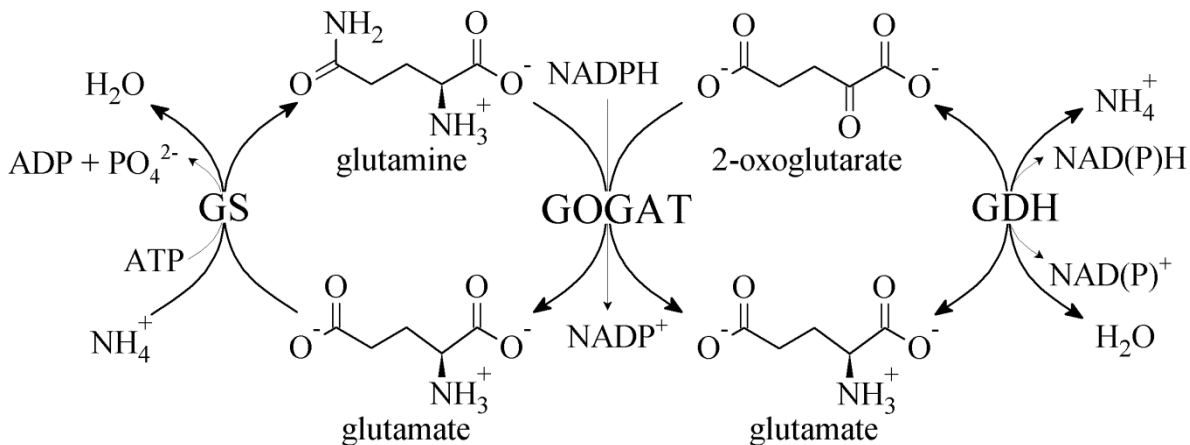
Figure 1: The major biochemical pathways responsible for ammonium assimilation and glutamine and glutamate production in mycobacteria.

1.3   The enzymes of Central nitrogen metabolism: Glutamine synthetase

The major types of prokaryotic GS enzymes (GS type I – III and T) have been identified based on differences in their posttranslational modifications (22). In *M. tuberculosis* all four annotated GS encoding genes (*glnA1-4*) are predicted to encode GS type I enzymes (23). GS type I is composed of 12 identical subunits that are arranged as two hexagonal rings on overlaying planes (24) and is subject to various regulatory mechanisms, including positive and negative substrate feedback (25), oxidative modification (26) and adenylylation (27). The major isoform of GS in *M. tuberculosis* is encoded by *glnA1* and was found to be abundantly expressed and released into the extracellular environment by pathogenic mycobacteria, but not by non-pathogens like *M. smegmatis* and *M. phlei*

or other non-mycobacterial genera (23,28–30). The extracellular release of GS in pathogenic mycobacteria was implicated in the biosynthesis of the poly-L-glutamate-glutamine structure present in the cell walls of pathogenic slow growing mycobacteria (31,32). Treatment of *M. tuberculosis* with the potent irreversible GS inhibitor, L-methionine-*S*-sulfoximine (MSO), inhibited growth *in vitro*, in human-like-macrophage cells (THP-1 cells) and in the guinea pig model of tuberculosis, yet this inhibitor had no effect on the growth of *M. smegmatis* (30,33,34). In addition, treatment of *M. tuberculosis* with antisense oligonucleotides to *glnA1* led to a marked reduction of growth *in vitro* and a deletion mutant strain of the gene was glutamine-auxotrophic, attenuated for growth in THP-1 cells and avirulent in guinea pigs and mice (32,35,36).

While *glnA2-4* was found to be non-essential to *M. tuberculosis* homeostasis (29), both *glnA1* and *glnA2* have been implicated in the pathogenicity of *M. bovis* (37,38). As a result of overwhelming data supporting the importance of GS in *M. tuberculosis* pathogenicity and virulence, this enzyme has been proposed as a target for development of novel anti-TB chemotherapy (39). Although MSO is a convulsive agent and *M. tuberculosis* gains resistance to it at a remarkably high rate (40–42), new inhibitors for GS which act by alternative mechanisms are being investigated which may circumvent these issues (43–46).

1.4   The enzymes of central nitrogen metabolism: Glutamate synthase

Three distinct types of GltS enzymes have been classified according to co-enzyme dependency, namely ferredoxin-GltS (Fd-GltS) which is found mostly in photosynthesizing organisms (cyanobacteria, algae, and chloroplasts of higher plants), NADPH-GltS which is mainly found in bacteria and NADH-GltS which is found in the non-green tissues of plants, fungi and lower animals (47,48). Fd-GltS and NADH-GltS are both monomeric enzymes of 150 kDa and 200 kDa polypeptide chains, respectively, while NADPH-GltS consists of a larger α polypeptide chain (≈ 150 kDa) and a smaller β polypeptide chain (≈ 50 kDa) arranged in an $(\alpha/\beta)_8$ hetero-octamer quaternary structure (47,48). In addition, to two putative operons, each containing a gene encoding for the α-subunit and a gene encoding for the β-subunit, multiple additional putative genes encoding for the α subunit are found in the *M. smegmatis* genome (14). In the slow growing *M. tuberculosis* and *M. bovis* BCG, however, only one putative operon contains the genes encoding for both the α (*gltB*) and β subunits (*gltD*). Notably, it was observed that azaserine, a known GltS inhibitor, inhibited *M. tuberculosis* growth with a minimum inhibitory concentration (MIC) < 1.0 µg/ml (49,50). No GltS enzyme has been detected in higher eukaryotes, including humans, and GltS has thus been identified as a possible novel anti-TB drug target (51). Notably, a marked deletion of the

gltBD operon has been shown to be viable in the closely related vaccine strain *M. bovis* BCG (17). This indicates a profound difference between *M. tuberculosis* and its relative in the accumulation of glutamate, taking into account that both the small and the large sub-units have been annotated as essential by transposon mutagenesis (1,2,5,6,20,21).

1.5   The enzymes of central nitrogen metabolism: Glutamate dehydrogenase

GDH enzymes have very diverse evolutionary, structural and functional properties and four distinct types of GDHs have been identified ($S50_I$, $S50_{II}$, L115 and L180). The $S50_I$ and $S50_{II}$ GDHs are mainly homohexameric enzymes with ≈ 50 kDa polypeptide chains, are specific for NADPH, NADH or have a dual co-factor specificity, function mainly in the assimilation of ammonia and are found in eukaryotes and eubacteria ($S50_I$) or distributed among all domains of life ($S50_{II}$) (52). In contrast, the L115 GDH's are homotetramers with ≈ 115 kDa polypeptide chains, are specific for NAD^+, function in the catabolism (deamination) of glutamate and have mostly been found in lower eukaryotes (53). Similar to the L115 class, the L180 GDHs are NAD^+ specific and function in the catabolism of glutamate, however these GDHs have ≈ 180 kDa polypeptide chains arranged as homohexamers (54–56) or homotetramers (57) and are only found in bacterial genomes. *M. smegmatis* has genes encoding for $S50_I$, L180 and possibly L135 GDH (58), while only a gene for L180 GDH is present in the *M. tuberculosis* and *M. bovis* BCG genomes (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The unique properties of L180 GDH compared to other characterised GDHs, including a very large subunit size, exclusive NAD^+ co-enzyme specificity, apparent function in the deamination of glutamate, exclusive distribution among bacteria and allosteric activation by asparagine and aspartate may have positive implications for the potential of L180 GDH as a specific anti-TB drug target (54–57). A protein BLAST of *M. tuberculosis* NAD dependent GDH against the non-redundant protein sequences for common intestinal bacterial genera, including *Bacteroides*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Staphylococcus*, *Lactobacillus* and *Clostridium* delivered no homologues, which may further qualify L180 GDH as a specific anti-TB drug target (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

1.6   Regulation of central nitrogen metabolism

The regulation of the major effector enzymes of central nitrogen metabolism (GS, GltS and GDH) is well studied in *Escherichia coli*, *Bacillus subtillis*, *Streptomyces coelicolor* and *Corynebacterium glutamicum* because of their biotechnological or industrial utility as well as in the saprophytic *M. smegmatis* (58–61). In *E. coli,* high-ammonium affinity GS activity is increased and low

ammonium affinity anabolic NADPH-GDH activity is decreased when nitrogen availability is limiting (61,62). Under nitrogen-rich growth, NADPH-GDH can efficiently assimilate ammonia and the molecule of ATP consumed in the production of glutamine by GS is an unnecessary expenditure, anabolic NADPH-GDH activity is increased and GS activity decreased. Similar regulation of GS in response to nitrogen-rich and nitrogen-limited conditions has been observed for other prokaryotes, including *C. glutamicum* (63), *S. coelicolor* (64), *B. subtillis* (65) and *M. smegmatis* (58). While fewer studies exist on the regulation of GltS, there is evidence that the enzyme is strongly up-regulated, like GS, in response to nitrogen limitation (63,66,67). A similar trend in the regulation of anabolic NADPH-GDH to that observed in *E. coli* was found in *S. coelicolor* (68), but not in *C. callunae* (69), *C. glutamicum* (66) or *M. smegmatis* (58) cultured under high or low nitrogen conditions.

A unique feature of the mycobacteria is the exceptionally high level at which they express GS. In one study it was observed that mycobacterial species exhibited approximately 20-fold more total GS activity on average than members of the Gram-negative and Gram-positive bacteria (23). In addition, slow growing mycobacteria, including *M. tuberculosis*, had approximately 10-fold more total GS activity on average than did fast growing mycobacteria. Unlike *E. coli* and *M. smegmatis* in which GS is only highly expressed when nitrogen is limiting ($[NH_3] < 0.1$ mM), in *M. tuberculosis* and other slow growing mycobacteria GS enzymatic activity is high even in the standard mycobacterial growth medium 7H9 which contains at least 7.6 mM NH_4^+. However, *M. tuberculosis* GS activity was observed to be decreased as much as 10-fold in response to a 10-fold increase in the nitrogen source $(NH_4)_2SO_4$, from 3.8 mM (normally present in 7H9) to 38 mM (23).

As is the case with the effectors of central nitrogen metabolism, its regulatory mechanism in *M. tuberculosis* contains a reduced set of components in comparison to *M. smegmatis*. However, similarities between the *M. smegmatis* and *M. tuberculosis* genomes include elements of a signal transduction cascade involved in GS regulation in other actinomycetes, namely *glnB, glnD* and the GS-adenylyl transferase, *glnE* (14). In addition, the *M. tuberculosis* genome appears to contain *glnR*, the global transcriptional regulator of nitrogen metabolism present in many Actinomycetes including *M. smegmatis*. The *glnR* nitrogen-response regulon was recently determined in a genome wide analysis for *M. smegmatis* and it was found that GlnR controls the expression of more than 100 genes in response to nitrogen limitation (70). The role of GlnR has recently been elucidated in *M. tuberculosis* (71). It was found that *M. tuberculosis* had fewer GlnR binding sites compared to *M. smegmatis* and only eight of these binding site locations were shared between *M. tuberculosis*

and *M. smegmatis* (69,71). Furthermore, GlnR regulates gene expression in response to nitrogen starvation and in response to nitric oxide stress (71). It was demonstrated that GlnR directly controls both nitrate reductase (*narGHJI*) and nitrite reductase (*nirBD*) in response to nitric oxide stress, yet in *M. smegmatis* GlnR is not responsible for *narGHJI* regulation (71). Interestingly, *glnR* was found to be non-essential for optimal growth of *M. tuberculosis* in all of the TraSH studies to date which could suggest a role for other transcriptional regulators in the control of nitrogen metabolism in this bacterium (1,2,5,49,72). It was observed that *glnA1* is defined by two transcriptional initiation sites in *M. tuberculosis*, one producing a short transcript which is more abundant under standard growth conditions and the other a long transcript which is more abundant under nitrogen-rich growth conditions (28). However, the transcriptional regulator responsible for expression from either proximal or distal GS transcription initiation sites remains unknown. The conversion of ammonia to glutamine is an energy intensive process, thus by adenylylation of GS *via* GlnE, GS is inhibited which conserves energy. Furthermore, GlnE is subject to additional regulation whereby activation of GlnE is stimulated by a rise in ammonia levels (73).

Interestingly, despite no requirement for *glnB* or *glnD* for *in vitro* growth of *M. tuberculosis*, *glnE*, which may be under control of the products of the aforementioned genes, was shown in an early genetic study of *M. tuberculosis* to be an essential gene and was also identified as required for *in vitro* growth in all of the TraSH studies (1,2,5,6,20,74). More recently, it was shown that the *glnA1-glnE-glnA2* operon in *M. tuberculosis* could be replaced with an antibiotic marker to generate a glutamine auxotroph and that a *glnE* deletion mutant could only be generated when the growth medium was supplemented with both glutamine and MSO, the absence of adenylylation by GlnE results in levels of GS activity that is toxic to the bacteria (35,75). Whether this toxicity is as a result of the effect of GS on cytosolic levels of ammonia or glutamine remains to be determined.

Another mechanism for homeostatic control of glutamine and glutamate metabolism in *M. tuberculosis* has been elucidated through the activity of the serine/threonine protein kinase G (PknG). It was observed that PknG phosphorylates glycogen accumulation regulator A (GarA), a small protein containing a forkhead associated domain near the C-terminal and bearing homology to the *C. glutamicum* protein oxoglutarate dehydrogenase inhibitor protein I (OdhI) (75,76). In *M. tuberculosis*, GarA functions by predominantly inhibiting GDH while simultaneously activating GltS, subsequently inducing a state that promotes the biosynthesis of glutamate (16,77–79). Upon phosphorylation of GarA at the threonine residue 21 by PknG, the effect of GarA is abrogated resulting in activation of GDH and inhibition of GltS activity. It was speculated that this type of

control would result in a decrease in intracellular glutamate levels (78). Although *garA* is an essential gene in *M. tuberculosis,* a deletion mutant could be generated in *M smegmatis* (80). It was observed that this mutant suffered a growth defect in particular in media containing carbon sources that do not enter the TCA cycle through glycolysis, such as acetate or succinate (80). While results suggested that the mutant was deficient in levels of glutamate or asparagine, since additional supplementation with these amino acids improved the growth of the mutant (80). Moreover, it was observed in the *ΔgarA* mutant complemented with mutant *garA* copies that express GarA which cannot bind GDH, yet is able to bind either KDH or to GltS, that growth phenotypes observed with this strain could be restored to full and intermediate levels of wild type, respectively (80). Thus indicating that regulation of GDH by GarA is critical to sustain homeostasis of glutamate levels in *M. smegmatis*, although the case could be different in slow growing pathogenic mycobacteria. Indeed, it was observed that a conditional *garA* mutant of *M. tuberculosis*, in which expression of *garA* is inhibited in the presence of anhydrotetracycline, could be chemically complemented by supplementation of growth media with 10 mM glutamate, glutamine or asparagine (80). This result confirms the importance of GarA in the regulation of the metabolism of these amino acids in *M. tuberculosis* and that inhibition of GDH and stimulation of GltS by GarA are likely essential processes in the pathogen. The serine/threonine kinase, PknG, has been shown to be important to growth of *M. tuberculosis* in *ex vivo* and *in vivo* infection models and is being investigated for it's potential as a promising drug target (81–84). Although it is thought that PknG phosphorylates host proteins and thereby plays its part in the arrest of phagosomal maturation (85), no such host-protein substrates of PknG have been identified. It is likely that part of PknG's role in pathogenicity has implications in the maintenance of glutamine/glutamate homeostasis. Studies in enteric bacteria have found that the glutamine to glutamate ratio is of central importance to cellular homeostasis and this may well be the case for *M. tuberculosis* (86–88).

1.7   *M. tuberculosis* nitrogen homeostasis *in vivo*

Pathogenic mycobacteria may reside in various environments in the host organism, ranging from the phagosomal or even cytosolic compartments of a macrophage cell to the hypoxic necrotic cavity of a caseous granuloma. The nutritional context is likely to be different in each of the microenvironments encountered by *M. tuberculosis* (89,90). There is not much known about the exact nutritional context within the macrophage and especially within the macrophage phagosome. The concentrations of the 20 common amino acids were measured in THP-1 cells cultured under standard conditions and it was found that while all amino acids were present at concentrations

higher than 0.1 mM, the most abundant amino acids were glutamate (18.56 mM), glutamine (4.45 mM), aspartate (7.16 mM), glycine (3.82 mM), and asparagine (1.28 mM) (36). However, the authors observed that growth of a glutamine-auxotrophic *glnA1 M. tuberculosis* mutant in the THP-1 cells was markedly impaired, while its growth was chemically complemented by at least 2 mM glutamine in axenic culture. This suggests that the phagosome membrane has a very low permeability for small molecules such as amino acids. A source of nitrogen in the phagosome may become available through the activity of host defence mechanisms which produce reactive nitrogen species like nitric oxide (NO) which in turn leads to availability of nitrite, nitrate and ammonium (91,92). Moreover, the genome wide expression profile of *M. tuberculosis* in conditions resembling the environments encountered by the pathogen in the human host and the genes that are required for optimal growth of the organism *in vitro*, *ex vivo* and *in vivo* suggests that the chief nitrogen sources that are utilized by the intracellular mycobacterium include ammonium (possibly obtained from nitrate by the activity of nitrite reductase and nitrate reductase or through deamination of amino acids), glutamine, glutamate, asparagine, aspartate and glycine (1,2,5,6,20,21).

At least 22 genes encoding for proteins involved in the transport of a nitrogen source could be identified in the *M. tuberculosis* genome (1,2,5,6,20,21). These include transporters for ammonium, glutamine, asparagine, arginine, glycine, nitrate and nitrite. Interestingly, no homologue of the glutamate permeases found in *C. glutamicum* (*GluABCD*, *gltP* and *gltS*) or *M. smegmatis* (*gluD*) are found in *M. tuberculosis* or *M. bovis* BCG despite observations that these strains will grow in medium containing glutamate as a sole nitrogen source (73,93). However, the requirement of the genes that encode both subunits of GltS for growth of *M. tuberculosis* in 7H10 medium which contains 3.4 mM L-Glu, suggests that the glutamate uptake (whether by passive diffusion or a yet unknown system) is inadequate to meet the cellular demand for the amino acid in the absence of the GltS biosynthetic pathway. *M. tuberculosis* and *M. bovis* BCG also do not have homologues to any of the genes assigned to encode urea transporters in *M. smegmatis* (14), however the urease of both *M. tuberculosis* and *M. bovis* BCG have been implicated in the alkalisation of the phagosome suggesting both the presence of urea as a nitrogen source and urea uptake during infection (94–96).

Surprisingly, only four genes encoding for putative nitrogen uptake systems in *M. tuberculosis*, were found to be essential for optimal growth *in vitro* or *in vivo* using TraSH. These included *glnQ* and *glnH* (both encode proteins involved in glutamine transport), a gene encoding for a putative amino acid permease and *proV* (a putative gene encoding for an osmoprotectant/glycine importer) which was non-essential to *in vitro* growth, but found to be essential *in vivo* (1,2,5,6,20,21,72).

Essentiality of the transporters of glutamine illustrates the importance of glutamine production by extracellular GS to homeostasis. Glutamine and glutamate can act as precursors or nitrogen donors in the biosynthesis of all other nitrogenous molecules in the bacterial cell (10–12,97,98). Observations that auxotrophic mutants deficient in pathways involved in the biosynthesis of branched-chain amino acids, leucine, arginine, methionine, proline and tryptophan perform poorly *in vivo* further illustrates the importance of glutamine and glutamate and regulation of their levels by central nitrogen metabolism to the pathogenicity of *M. tuberculosis* (99–104).

Recently, it was found that a putative asparagine/aspartate transporter (AnsP1) is able to transport aspartate across the cell envelope (7,15). Deletion of *ansP1* resulted in impaired virulence of *M. tuberculosis* in a mouse model, despite no growth defect *in vitro*, suggesting that the assimilation of aspartate by *M. tuberculosis* is required for virulence (8). The *M. tuberculosis* genome encodes a second asparagine permease, AnsP2, which is able to successfully transport asparagine across the cell envelope (8). Strangely, deletion of *ansP2* was unable to attenuate virulence as no growth defect was observed *in vivo* (7). This observation suggests that asparagine is not a required nutrient for virulence and survival of *M. tuberculosis*. Asparagine does however remain an important amino acid in the context of combating host defences. Asparaginase (AnsA) readily converts L-asparagine and water to L-aspartate and ammonia. Knock out studies of *M. tuberculosis ansA* revealed that loss of AnsA results in an *in vivo* growth defect (8). Interestingly, AnsA is secreted and it was speculated by the authors that the conversion of asparagine to aspartate is necessary for virulence due to the release of ammonia to the *M. tuberculosis* extracellular environment (7). Ammonia may in turn alkalise the phagosome and circumvent acidification and maturation as well as crucial processes of macrophage defence against intracellular infection (7). By following such an approach, *M. tuberculosis* can initiate a quick response against the host defences while simultaneously gaining nutrition in the form of nitrogen and carbon through the assimilation of aspartate. The *M. tuberculosis* genome has been predicted to contain genes coding for at least three aspartate aminotransferases (AAT); *aspB*, *aspC* and *Rv3722c*, all of which were predicted essential by TraSH (1,2,5,6,15,105). AAT catalyses the reversible transfer of the α-amino group of aspartate to α-ketogluterate to produce glutamate and oxaloacetate in the presence of pyridoxal-5-phosphate. The direction of these reactions are usually controlled by substrate availability, therefore an increase in aspartate from host derived asparagine may promote a condition that favours glutamate production *via* AAT.

The nitrogen sources that are utilized by the infecting mycobacterium may not only be determined by the available nitrogen sources, but may, to a considerable extent, be a consequence of the metabolic response initiated by the bacilli to subvert host defence mechanisms. For example, alanine and glycine degradation is implicated in entry into the dormancy phase (106–110), the metabolism of ammonium, urea, glutamate, aspartate and asparagine may be important for resistance of the mycobacteria to the acidification of the maturing phagosome (8,86,95,111). Furthermore, nitrite production from nitrate (which is a natural product of NO) is likely to be important in low oxygen environments, such as the granuloma, where this compound may act as an alternative electron acceptor to oxygen (92,112).

1.8   The role of glutamine and glutamate in *M. tuberculosis*

The important roles of glutamine and glutamate as precursors or intermediate molecules in the synthesis of other nitrogenous molecules were discussed in previous sections. However, an interesting observation was the remarkably high level to which glutamate is accumulated in *M. tuberculosis* and it could be speculated that the high intracellular pool of glutamate may act as a reservoir of nitrogen in environments where nutrients are limiting (113). This high level of glutamate accumulation may also be testimony to the importance of this amino acid in various processes which may be linked to *M. tuberculosis* virulence (Figure 2).

1.9   The interplay between central nitrogen metabolism and carbon metabolism

Both glutamine and glutamate are gluconeogenic amino acids and may be utilised as sources of carbon and energy, although this may vary between different organisms (114). It was observed in early studies of *M. tuberculosis* respiratory metabolism that glutamate is one of only three amino acids (the others being glycine and sarcosine) that may be utilized as an energy and carbon source by *M. tuberculosis* (115,116). In addition to a role in the regulation of GltS and GDH, GarA was also found to interact with α-ketoglutarate decarboxylase (KGD) which plays an important role in central carbon metabolism in *M. tuberculosis* (16). In *M. tuberculosis* KGD forms along with dihydrolipoamide acyltransferase (DlaT) and dihydrolipoamide dehydrogenase (Lpd) the α-ketoglutarate dehydrogenase (KDH) complex responsible for the production of the tricarboxylic acid cycle (TCA) intermediate succinyl Co-enzyme A (CoA) (117,118).
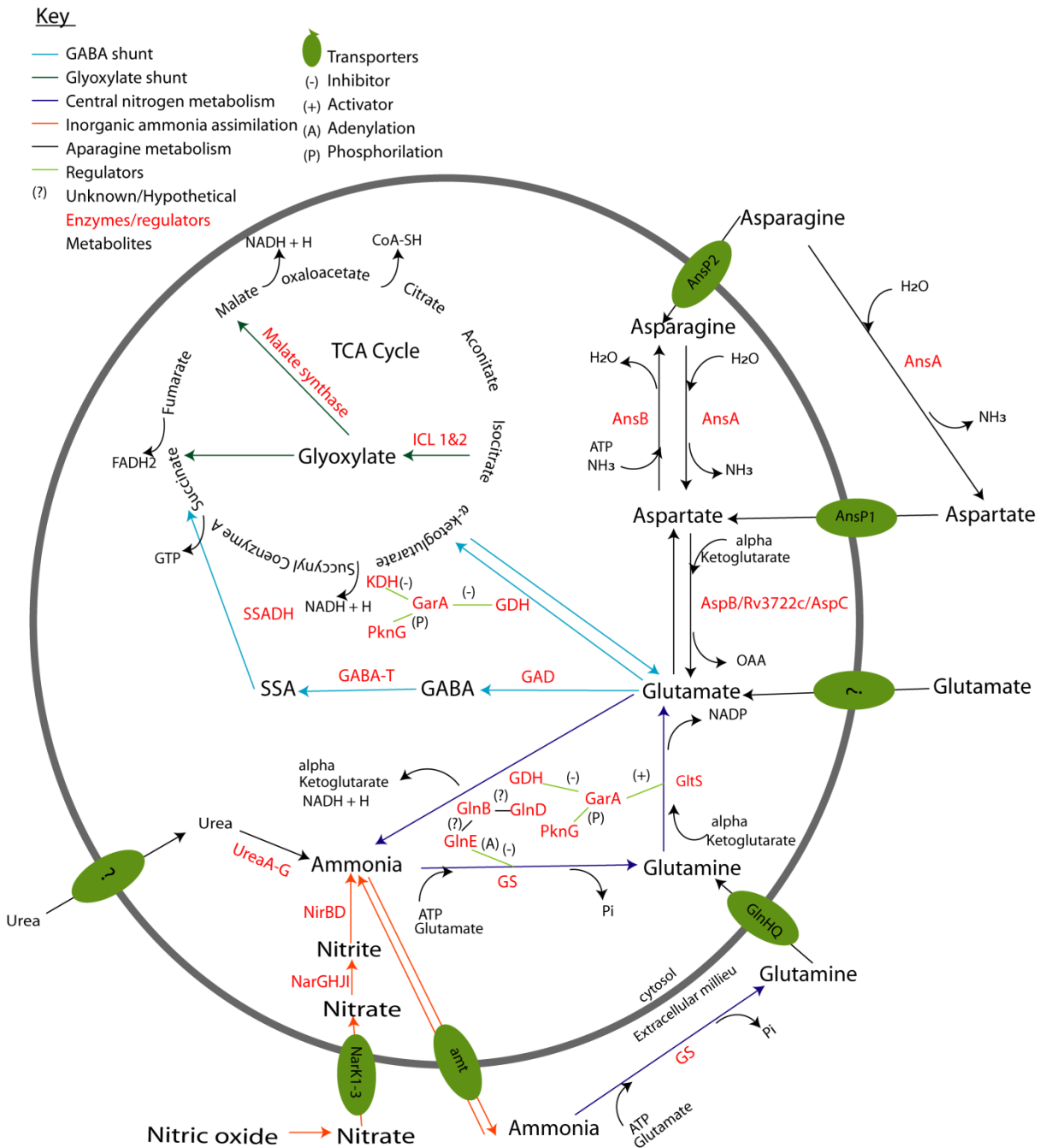
Figure 2: *M. tuberculosis* **depends upon central nitrogen metabolism and the production of L-glutamine, and L-glutamate for efficient utilization of the nitrogen sources acquired by the pathogen from the host cell.** Metabolism of glutamine and glutamate is regulated possibly to maintain a homeostatic ratio of the cytosolic glutamine to glutamate pools and/or a high glutamate concentration, although the specific stimulus for a regulatory response is unknown. The roles that the highly versatile glutamate may play during infection are of special interest and should become the topic of more investigations.

It was found that KDH is inhibited by GarA in *M. tuberculosis* and this effect was abrogated upon phosphorylation by PknG (78,80,82). The mode of GarA function suggests a basal inhibitory state of KDH and GDH while GltS remains active (78). Only upon phosphorylation by PknG is GDH and KDH activated which may explain why it was for long believed that *M. tuberculosis* possessed no KDH and thus no TCA cycle (117) (Figure 2). The fact that GarA is essential to the survival of *M. tuberculosis* (80) and PknG is required for growth in macrophages as well as in mice (80)(85)(82) emphasizes the important link between nitrogen and carbon homeostasis provided by glutamate in *M. tuberculosis* pathogenesis. Strikingly, it was shown recently that glutamate is able to modulate carbon flux in *Francisella*, also a facultative intracellular pathogen. Mutants of *Francisella* lacking a glutamate permease displayed a decreased load of TCA cycle intermediates compared to wild type. Interestingly, succinate, fumarate and α-ketogluterate had a significantly lower yield in the mutant and citrate levels were comparable to wild type. Thus it was shown that glutamate transport, and therefore glutamate, is able to fuel the TCA cycle in this organism (119). Although KGD is responsible for the production of succinic semialdehyde (SSA), a precursor to the TCA cycle intermediate succinate (113), it was recently shown that this enzyme couples the decarboxylation of α-ketoglutarate with the condensation of glyoxalate leading to the eventual production of 2-hydroxy-3-oxoadipate (HOA) which then spontaneously decarboxylates to 5-hydroxylevulinate (HLA) (120). The authors speculated that HOA and HLA may be precursors to an essential mycobacterial compound. In addition, the same authors proposed in a more recent communication that instead of KGD, the γ-aminobutyric acid (GABA) shunt pathway may be a more likely route of SSA production in this organism (121). Glutamate is a substrate of the first step in the GABA shunt pathway, which is catalysed by glutamate decarboxylase (GAD, *gadB*) and could be speculated to present an important link in an alternative pathway for SSA production in the TCA cycle of *M. tuberculosis* when the conventional KDH pathway is inhibited. Cholesterol has been identified as a favoured carbon source for *M. tuberculosis* during infection (122). In a recent TraSH study *gdh* failed to be identified as required for growth in minimal medium with cholesterol as carbon source by just missing statistical significance ($p = 0.07$, significance set at $p = 0.05$ in the experiment) (2). This may suggest that the adaptation of metabolism to utilize cholesterol as a carbon source has an effect on the metabolism of glutamate and thus also on the homeostasis of central nitrogen metabolism.

1.10   The role of central nitrogen metabolism in cellular stress

Glutamine and glutamate are osmoprotectants and compatible solutes in bacteria (87,88,123,124). Compatible solutes are organic solutes found in the cell, which when present at a high concentration, facilitate the efficient functioning of enzymes (125). The high intracellular pool of glutamate may thus also be related to the compatible solute qualities of the amino acid. In *E. coli*, decarboxylation of glutamate to γ-aminobutyrate (GABA) by GAD consumes a proton, which is then removed from the cytosol through a glutamate/GABA antiporter and thus plays an important role in acid resistance (86). Although *M. tuberculosis* does not possess the genes for the glutamate/GABA antiporters normally involved in the function of the intracellular GAD system, the presence of a GAD-encoding gene, *gadB*, suggests a possible function of glutamate metabolism in acid resistance (1,2,5,6,119), which may be particularly beneficial to *M. tuberculosis* in the phagosome, where the pH has been estimated to range between 4.5 and 5.5 (126,127). It was demonstrated, in *E. coli,* that under severe acid stress (pH 2.5) glutamate conveys the best protection against oxidative stress (1 mM H_2O_2 challenge) compared to pH 5.5 and pH 7.0. Interestingly, the addition of 2 mM GABA was unable to convey adequate protection against oxidative stress under severe acid stress challenge. Thus the authors showed that the GABA shunt requires the glutamate transporter, and glutamate decarboxylase to convey protection in *E. coli* (128). In *E. coli*, glutamate in conjunction with arginine conveys protection against acidic stress by increasing internal pH and reversing membrane potential from intracellular negative to positive (129). Thus Bradley *et al* speculates that the increase in internal pH in the presence of either glutamate or arginine compared to the acid challenge in their absence may decrease the global cellular stress experienced, subsequently increasing the survival rate. Glutamate has also been implicated in phagosomal escape and resistance to oxidative stress in *Francisella* and *E. coli* O157:H7 (119,128). It has been suggested that glutamate may provide protection against oxidative stress, through the use of downstream molecules such as glutathione and α-ketoglutarate to act as anti-oxidants. Furthermore, glutamate transport is specifically required for defence against intracellular reactive oxygen stress (119).

1.11   Conclusion and future considerations

While some features regarding the regulators of the enzymes in central nitrogen metabolism have been elucidated, including the importance of GlnE and PknG to pathogenicity and the interactions between these proteins and their respective substrates, the stimuli which result in changes in the expression and activities of GDH and GltS specifically, have not been fully elucidated. It is not known if these enzymes are regulated by the same fluctuations in nitrogen supply which regulate GS. Of significant importance may be the high intracellular levels of glutamate, which may be involved in homeostasis of central nitrogen metabolism, homeostasis of central carbon metabolism, efficient enzyme functioning, osmoprotection, acid resistance and which could act as a reservoir of nitrogen when nutrients become limiting. Although a disturbance in glutamine and glutamate levels was observed in a PknG deficient mutant of *M. tuberculosis*, which was avirulent in mice (130), it has not been established whether the role of PknG in pathogenicity of *M. tuberculosis* has to do with its interaction with host proteins or its control of glutamine/glutamate homeostasis. Although studies linking glutamate metabolism specifically to cellular stress and phagosomal escape have been demonstrated in other organisms (86,119,128,129) the potential role of glutamate in these processes have not been investigated in the pathogenicity of mycobacteria. Moreover, the contribution of the enzyme central to glutamate production, GltS, and the enzyme which may play a decisive role in the catabolism of glutamate, GDH, to survival and growth of *M. tuberculosis* in macrophages should be determined experimentally. These enzymes may offer unique and specific avenues for development of novel chemotherapeutic intervention strategies because of their central roles in nitrogen homeostasis in *M. tuberculosis*.

1.12   Study design, aims and objectives

In this study we aim to address questions described in Chapter 1, sections 1.8-1.10 with specific aims and objectives listed below. All specific questions were addressed by experimental enquiry and conclusions were extrapolated thereof. Throughout this study, *M. bovis* BCG Pasteur 1733p2 was used as a model organism for *M. tuberculosis*. These two organisms are closely related and share 99.9 % homology on a genetic level (131). The close genetic relation of *M. bovis* BCG to *M. tuberculosis* allows for a more accurate investigation of biochemical processes addressed in this specific study. In many cases saprophytic *M. smegmatis*, the alternative *M. tuberculosis* model, lacks the biochemical machinery which is under investigation compared to *M. bovis* BCG. This

study builds upon a previous study in which the mutants controlling glutamate homeostasis were generated (17)

Aim 1:

Investigate the effect of glutamate deregulation in *M. bovis* BCG on a genomic level and identify which genes are affected in the process.

Objectives:
1. Identify mutations that may have occurred in the genome of *M. bovis* BCG as a direct result of deleting GDH.
2. Indicate which pathways are most vulnerable to the loss of glutamate homeostasis.
3. Investigate the phenomenon of a compensatory mutation(s) that arose when *M. bovis* BCG lacking GDH is grown in media with glutamate as the sole nitrogen source using a whole genome approach.

Aim 2:

Investigate the role of GDH and GltS of *M. bovis* BCG in the survival of the bacilli during *in vitro* cellular stress and when exposed to the environment of a murine macrophage.

Objectives:
1. Examine the role of GDH and GltS in response to (a) osmotic stress, (b) reactive oxygen and acidic stress as well as (c) nitrosative stress.
2. Investigate the ability of a promising mutant strain, observed from aim 2, objective 1 a-c, to survive within a murine macrophage model of infection

Chapter 2

Results and Discussion:

The effect of glutamate deregulation

2   Introduction

M. bovis BCG is widely used as a model organism for *M. tuberculosis* due to the close relation (99.9% sequence identity) of the two strains on the genomic level (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (131). The genes involved in the regulation of glutamate, *gdh, gltB and gltD,* are predicted to be essential to the growth of *M. tuberculosis* in standard 7H9 media by TraSH (1,2,6,20). However, despite its high genomic similarity to *M. tuberculosis*, another study, upon which this study builds, was previously able to generate mutants of *gdh* and *gltBD* in *M. bovis* BCG Pasteur (17). We thus speculated that the shift in essentiality of *gdh* and *gltBD* between pathogenic *M. tuberculosis* and non-pathogenic *M. bovis* BCG may be as a result of a metabolic adaptation which is specifically associated with the more virulent lifestyle of *M. tuberculosis*. Interestingly, while PknG, (a regulator of both GDH and GltS, see chapter 1; section 1.6) was found to be essential in the control of glutamine levels in *M. tuberculosis*, it was observed that this was not the case in *M. bovis* BCG (78,82,132). However, it is also possible that suppressor mutations in our *gdh* and *gltBD* mutants allowed survival of these mutants at the selection steps in mutagenesis. We performed whole genome sequencing to test this hypothesis. Furthermore, it was found that extensive incubation of *M. bovis* BCG *Δgdh* in glutamate as the sole nitrogen source results in the amelioration of a observed growth deficit after three weeks (17). This effect was proved to be as a result of unknown suppressor mutation(s) by selecting and isolating single colonies from three week old cultures and testing their growth in medium containing glutamate as the sole nitrogen source (17).

In this study, we sequenced the genomes of the progenitor *M. bovis* BCG Pasteur strain used to generate *gdh* and *gltBD* mutants, as well as the *Δgdh* and *ΔgltBD* mutant strains to rule out the contribution of suppressor mutations during genetic manipulation to the survival of these mutants, as well as to further explore any effects of GDH or GltS loss on the genome of *M. bovis* BCG. In addition, it was attempted to identify the suppressor mutation by re-evaluating the growth profile of the *gdh* mutant on glutamate as the sole nitrogen source followed by whole genome sequencing.

2.1   Results and discussion

2.1.1   Next generation sequencing: Evaluating the loss of GDH and GltS on the genome of *M. bovis* BCG.

Next generation sequencing allows for high throughput analysis of entire genomes. This technology may be harnessed to assess the effects the loss of an enzyme on the entire genome of an organism or to detect subtle differences between two strains such as SNP's or IN/DEL's.

The genomes of *M. bovis* BCG Δ*gdh; ΔgltBD; Δgdh* complement and *ΔgltBD* complement as well as the *M. bovis* BCG wild type progenitor strain were sequenced on an Illumina Miseq platform. Initially, a low number of total SNP's were expected as an organism may potentially generate point mutations after each cell division. However, we observed that the *ΔgltBD* mutant had the highest percentage of SNP's with a total of 58 whilst the wild type had 4 and *Δgdh* had 8 compared to the published reference (Figure 3).
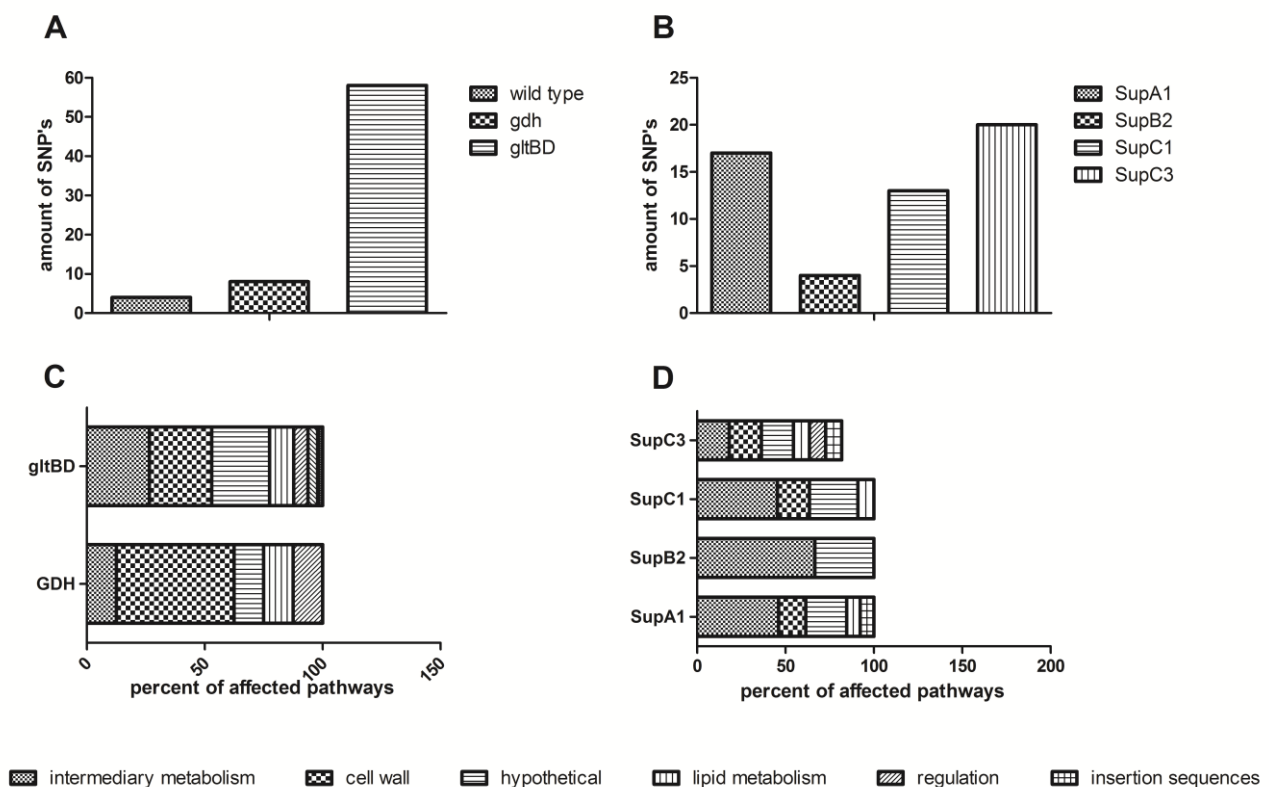
Figure 3: Metrics after analysis of whole genome sequencing data. Data is indicative of putative SNP's A) Amount of putative SNP's detected in wild type, mutant and complement

strains compared to published wild type *M. bovis*. Single nucleotide polymorphisms were additionally filtered by mapper sum = 3. **B)** Amount of putative SNP's detected in suppressor mutant strains. SNPS were filtered by mapper sum = 3. **C)** Metabolic pathways in which SNP's were found associated with *Δgdh* and *ΔgltBD* mutants. Single nucleotide polymorphism distribution is represented as a percentage of total SNP's found in each mutant after filtering. **D)** Metabolic pathways in which SNP's were found associated with SupA1, SupC1, SupB2 and SupC3 compensatory mutants. Single nucleotide polymorphism distribution is represented as a percentage of total SNP's found in each mutant after filtering

Post alignment and initial bioinformatics analysis (Section 5.5.1-5.5.4) of the sequenced strains (Table 5 [Chapter 5, Section 5.1], excluding *RAW 264.7* ATCC TIB-71) metrics are displayed in Figure 3 A and B. Interestingly, *ΔgltBD* had a large amount of putative SNP's compared to wild type and *Δgdh* strains (Figure 3 A). Variant calling of the compensatory mutant strains yielded unexpected results. Although there were similar putative SNP's between the four sequenced strains (Table 2), the number of SNP's varied among each strain (Figure 3 B). In order to determine metabolic which pathways were most affected through either the generation of our mutant strains by knock out or by natural mechanisms, SNP distribution and affected genes were mapped to each genes specific function (Figure 3 C and D). It was found that the *Δgdh* mutant contained the most SNP's in genes associated with cell wall and cell processes (50%). The highest distribution of SNP's found in *ΔgltBD* was shared among genes associated with intermediary metabolism and respiration as well as cell wall and cell wall and cell processes (27%) followed closely by unknown hypothetical proteins (24%) (Figure 3 C). Overall putative SNP's were mostly found in genes associated with intermediary metabolism and respiration (average 44%) in the compensatory mutants. In addition, conserved hypothetical proteins of unknown function had the second highest SNP distribution at 23% (Figure 3 D).

It is of interest to note the difference between affected gene distribution between *Δgdh* and *ΔgltBD* (Figure 3 C). The largest possible metabolic adaptions to the loss of GDH seem to arise in cell wall metabolism (Figure 3 C). This was expected considering the control of both GDH and GltS on glutamate and glutamine levels, respectively. The loss of GltS directly influences the glutamine levels which increases and limits the levels of glutamate, while GDH is responsible for the combustion of glutamate to ammonia (17). It has been shown that glnA1 is directly responsible for the glutamine levels and therefore the biosynthesis of poly glutamine/glutamate features of the mycobacterial cell wall (30,36). It is thus of interest to observe a relatively even distribution of variants found in *ΔgltBD* genes responsible for the respiration and cell wall pathways while GDH is mostly implicated in cell wall metabolism (Figure 3 C). This however, is in line with additional

findings made by this study. During cellular stress, such as nitrosative stress, cell wall metabolism was found to be upregulated (71).

Table 1: Single nucleotide polymorphisms found in *M. bovis* BCG *Δgdh* compared to wildtype and complement strains.

Gene name	*Syn/non syn	AA change	Nucleotide change	Orientation	Position in genome	Function
Ino1	Non-syn	I120T	A:G	[‡](-)	50750	MYO-INOSITOL-1-PHOSPHATE SYNTHASE
ctpI	Syn	-	G:C	(-)	126079	PROBABLE CATION-TRANSPORTER ATPASE
Mb0600c	Non-syn	L150V	T:C	(-)	685068	PROBABLE CONSERVED INTEGRAL MEMBRANE PROTEIN
PknH	Non-syn	V375L	T:A	(-)	1412864	PROBABLE TRANSMEMBRANE SERINE/THREONINE-PROTEIN KINASE H PKNH
ppsB	Non-syn	A821T	G:A	(+)	3210105	PHENOLPTHIOCEROL SYNTHESIS TYPE-I POLYKETIDE SYNTHASE
Mb3114	Non-syn	A158T	G:A	(+)	3409943	CONSERVED HYPOTHETICAL PROTEIN
Mb3865	Non-syn	N79Y	A:T	(+)	4245554	PROBABLE CONSERVED MEMBRANE PROTEIN
Mb3924c	Syn	-	G:A	(-)	4312872	POSSIBLE CONSERVED MEMBRANE PROTEIN

Data representative of single nucleotide polymorphisms annotated by three independent mappers (BWA, SMALT, Novo align). SNP's were filtered by removing polymorphisms common to wild type BCG and *M .bovis Δgdh* complement.
*Syn: Synonymous; non-syn: Non-synonymous
[‡](+):   sense strand; (-):   anti-sense strand

The specific SNP's unique to *Δgdh* are shown in Table 1. All mappers calculate a phred scaled quality score to determine the probability of the alignment being incorrect. All depicted SNP's were high confidence SNP's mapped by all three mappers used for alignment (Chapter 5, section 5.5.2). Unfortunately, most of the high confidence SNP's are found within coding regions annotated as hypothetical by Tuberculist offering no information about their possible functional effects (http://tuberculist.epfl.ch/). Of the eight high confidence SNP's, only three were found within coding regions of studied genes, i.e. myo-inositol 1-phosphate synthase (*ino1*); Protein kinase H (*pknH*) and phenolpthiocerol synthesis type-1 polyketide synthase (*ppsB*) (Table 1). The gene product of Ino1 is involved in the phosphatidylinositol biosynthetic pathway and catalyses the conversion of D-glucose 6-phosphate to 1L-myo-inositol 1-phosphate. Ino1 is predicted to be non-essential by transposon mutagenises (133).

Inositol is rarely found within prokaryotes, yet the mycobacterial envelope contains a significant amount of phosphatidylinositol that is anchor linked to arbinomannan and mannan. Therefore, it has been speculated that inositol plays a role in host-pathogen interaction and may be required for the survival of the bacilli in the host (134). PpsB is involved in the biosynthesis of phenolpthiocerol and phthiocerol dimycocerosate (PDIM), both these lipids form integral parts of the mycobacterial cell wall. The role of these lipids has been shown to be essential for virulence, furthermore PpsB has been shown to be essential in *M. tuberculosis* by transposon mutagenesis (1,2,5,6,135–137).

Slow growing mycobacteria possess 12 eukaryotic like serine/threonine kinases (STPK's) which include PknH. As with all other STPK's, PknH is involved in signalling transduction *via* a phosphorylation cascade. These cascades are one of the main mechanisms involved in the translation of external stimuli to adaptive gene expression. Previous studies have attributed STPK's to the modulation of virulence in *M. tuberculosis* (130,138–142). Interestingly, PknH has been shown to be non-essential for growth in standard 7H9 media through transposon mutagenesis, yet is required for infection in BALB/c mice. Along with enhanced *in vivo* bacilli load for the PknH mutant it was observed that PknH displayed an increased resistance to acidified nitrate stress. This may suggest a potential role of PknH in the detection of external nitric oxide (1,2,5,6,143). Furthermore, knockout mutants of *pknH* resulted in attenuation of PDIM synthesis as well as differential expression in the PDIM biosynthetic pathway (144).

M. bovis BCG *Δgdh* contains two SNP's in genes which contribute to the biosynthesis of the mycobacterial cell wall, while one SNP was detected in a gene which has a role in the detection of nitric oxide. *In silico* analysis of the non-synonymous point mutations (see Chapter 5, Section 5.5.5) have predicted that the SNP's found in both PknH and PpsB have no overall effect on the functionality of the protein. However, the free energy of PknH (-0.87 Kcal/mol) and PpsB (-0.51 Kcal/mol) suggest a potential negative shift of Gibbs free energy toward a state that favours instability of both proteins (Table 3). Studies in enteric bacteria have found the ratio of glutamine to glutamate is of importance to cellular homeostasis, this may be true for slow growing mycobacteria as well (30,33,86,87,145,146). As discussed previously, the maintenance of glutamine and glutamate is controlled by prominent virulence factors such as GlnA1 and PknG and also directly by GltS and GDH (28,140). Furthermore, it has been shown that GS, involved in the production of glutamine and ammonia, has an extracellular role in the biosynthesis of poly L-glutamine/glutamate component of the cell wall (36). It could be speculated that the loss of GDH may disrupt the ratios of glutamine and glutamate and in so doing disturb the downstream flux of glutamate throughout various synthetic pathways. This may require the cell to compensate by mutating genes that have roles in the biosynthesis of the cell wall.

2.1.2   Next generation sequencing: Evaluating SNP's of the compensatory mutants.

Compensatory mutants of *Δgdh* were generated by a previous study, these mutant phenotypes reverted back to wild type when exposed to glutamate as the sole nitrogen source for an extended period of time (3 weeks) (17). It speculated by the authors that a metabolic shift may have occurred on a genetic level which results in the amelioration of the knock out phenotype. We were unable to pinpoint such a mutation(s) due to the variability of the amount of putative SNP's (Figure 3 B). Yet, there is a clear trend in the role of the genes in which SNP's were most common. Nearly half off all putative SNP's were found to be in genes associated with intermediary metabolism and respiration (Figure 3 D). It is possible for *M. bovis* BCG to utilise L-glutamate as both a carbon and nitrogen source, yet the *Δgdh* mutant fails to grow when glutamate is the sole nitrogen source (17,147). The lack of growth is indicative of a loss in respiratory function thus it is sensible that the most profound changes are found within respiratory metabolism. It is reasonable to assume that instead of a single major mutation reverts the phenotype of *Δgdh* to wild type it is a cohort of various mutations found within the respiratory pathways of *M. bovis* BCG that results in *Δgdh* regaining wild type phenotypes under this specific condition.

Next generation Illumina Miseq sequencing and subsequent bioinformatics analysis was used to identify SNP's unique to the strains harbouring a potential compensatory mutation(s). A total of 17 SNP's were identified that are unique to *M. bovis* BCG *Δgdh*: SupA1; 4 SNP's unique to *Δgdh*: SupB2; 13 SNP's unique to *Δgdh*: SupC1 and 20 SNP's unique to *Δgdh*: SupC3 (Figure 4). Unexpectedly, the amount of SNP's were wide spread between the 4 mentioned strains that displayed the ameliorated phenotype explained previously (17).

In an attempt to estimate which SNP may potentially be involved in ameliorating the poor growth profile of *Δgdh* when grown on glutamate as the sole nitrogen source (17), SNP's present in all four or three of the four strains were pooled. Of the nine SNP's present in three of the four strains only two of these were present in all four strains. These SNP's were *Mb2287c*, annotated as a hypothetical protein and *Mb2823* which is annotated as a possible hydrolase (Table 2).

Table 2: Single nucleotide polymorphisms found in compensatory mutant strains after comparison to Δ*gdh*.

Gene name	*Syn/non syn	AA change	Nucleotide change	Orientation	Position in genome	Function
[A]*Mb2287c*	Syn	-	C:G	-	2516163	conserved hypothetical proline rich protein
[A]*Mb2823*	Syn	-	C:T	+	3066230	possible hydrolase
[B]*ctpI*	Syn	-	A:G	-	128301	probable cation-transporter atpase i ctpi
[B]*Mb0978c*	Non-syn	T50R	T:C	-	1065253	possible oxidoreductase
[B]*pks7*	Non-syn	T977A	A:G	+	1863618	probable polyketide synthase pks7
[B]*Mb2647*	Non-Syn	H57R	A:G	+	2911002	conserved hypothetical protein
[B]*pflA*	Non-syn	S257F	C:T	+	3460073	pyruvate formate lyase activating protein
[B]*pflA*	Non-syn	L270P	T:C	+	3460112	pyruvate formate lyase activating protein
[B]*Mb3532c*	Syn	-	G:C	-	3867127	probable short-chain type dehydrogenase/reductase
[B]*Mb3943*	Syn	-	C:T	+	4335108	hypothetical alanine rich protein

Data represented are pooled Snp's common in either all 4 strains[(A)] or three out of the four compensatory mutant strains[(B)]. Snp's presented are unique to compensatory strains only.

Table 3: *In silico* SNP analysis of non-synonymous single nucleotide polymorphisms confirmed by Sanger sequencing

Gene	Amino acid change	[1]SNAP	[2]RI	[3]SIFT	[4]score	[5]I-mutant (stability)	[6]Gibbs (Kcal/mol)
M. bovis BCG *Δgdh*							
PknH	V375L	Neutral	2	Tolerated	0.42	decreased	-0.87
ppsB	A821T	Neutral	5	Tolerated	0.41	Decreased	-0.51
Mb3114	A158T	Neutral	6	Tolerated	0.96	Decreased	-0.78
Mb3865	N79Y	Non-neutral	0	affects function	0.01	Increased	0.23
Mb0600c	L150V	Neutral	5	Tolerated	0.35	Decreased	-1.52
Ino1	I120T	Neutral	2	Tolerated	0.07	Decreased	-2.37
M. bovis BCG *Δgdh*:supA_1;B_2;C_1;C_3							
Mb0978c	T50R	Non-neutral	0	Affects function	0.00	Increased	-0.29
Pks7	T977A	Neutrtal	4	Tolerated	0.11	Decreased	-0.84
Mb2647	H57R	Neutral	2	ND		Decreased	-0.19
pflA	S257F	Neutral	8	Tolerated	0.05	Increased	0.37
pflA	L270P	Neutral	8	Tolerated	0.11	Decreased	-1.51

1:   Online freeware for the prediction of non-synonymous point mutations on the functional effect of proteins

2:   Reliability index of SNAP results. The default is 0 and the highest is 9, the greater the RI value the greater the confidence of the prediction.

Neutral: The point mutation is not functionally discernible from the wild type

Non-neutral: The point mutation is functionally discernible from the wild type

3:   SIFT is a homology based freeware tool that distinguishes tolerant from intolerant amino acid substitutions in a given sequence

4:   If the substitution is predicted to be damaging to protein function the score is less or equal to 0.05. Alternatively if the substitution is tolerated the score is greater than 0.05.

5:   I-mutant calculates the stability of the protein with a substation relative to the wild type.

6:   Gibbs free energy calculated in kcal/mol. G = 0, the protein is in its most stable form. G<O, decrease in protein stability. G>O increase in protein stability.

ND: Not determined

These SNP's were synonymous point mutations, thus the lack of a change in amino acid suggests that the protein would function as wild type. There is still a possibility that a synonymous mutation could have a transient effect on the transcription of the specific genes. Although the genomes of *M. tuberculosis* and *M. bovis* BCG have been deciphered, for many of the coding regions only crude homology based annotations provide clues toward the function of the protein product (133). It is thus not practical to draw meaningful conclusions or speculations on the potential role of hypothetical proteins on cellular physiology. The diversity of SNP's found within the 4 compensatory mutations provide an opportunity to assess SNP's that are found in three of the four

strains. These SNP's were more abundant, with 8 SNP's present in three out of four compensatory mutant strains, excluding the SNP's found in all four (Table 2).

The *M. bovis* BCG *Δgdh*: supA1, B2, C1, C3 SNP's with non-synonymous point mutations found within three of the four compensatory mutant strains are summarised in Table 3. Using *in silico* prediction methods (Chapter 5, section 5.5.5.), the probable effect of an amino acid change on the function of the protein product could be determined. Of the five SNP's found to have a non-synonymous point mutation only one, Mb0978, is predicted to affect the function of the protein product (Table 3).

Furthermore, the stability of the non-synonymous SNP's was determined by calculating the free energy associated with the amino acid substitution (Chapter 5, section 5.5.5.). Two of the five point mutations favoured an increase in stability while the rest favoured a decrease (Table 3). Mb0987c codes for a probable oxidoreductase and was the only protein with a non-synonymous SNP to predict an effect in function. Interestingly, the stability of the protein is predicted to be increased from the wild type. Pyruvate formate lyase activating enzyme A (PflA) has two non-synonymous point mutations. Both mutations were predicted to have no effect on the protein function. However, one of the SNP's, S257F, increases protein stability whilst the other, L270P, severely decreases protein stability (Table 3). The increase in protein stability brought forth by S257F is negligible with a ΔG of 0.37, when considering the ΔG of L270P is -1.51. Proteins and their surrounding environment are regulated by the laws of thermodynamics and are driven to decrease enthalpy while simultaneously increasing entropy (148). Thus proteins strive for the lowest free energy conformation or most ordered state. The increase in enthalpy resulting from the L270P substitution is great enough to offset the increase in entropy provided by S257F (Table 3). This shift toward a state promoting enthalpy naturally decreases the stability of the protein (148). This may in turn result in many adverse effects on the chemistry of the protein such as loss of electrostatic interactions or backbone strain.

Chapter 3

Results and Discussion

The Role of GDH on Cellular Stress

3   Introduction

M. tuberculosis, a facultative intracellular pathogen, has adapted to survive the hostile environment of the macrophage. In order to persist and replicate certain defensive measures are required by the bacilli to combat host defences. Studies have shown that both *M. tuberculosis* and *M. bovis* BCG are able to circumvent the formation of a phagolysosome which directly stops certain macrophage killing mechanisms (30,85,149). It has further been shown that pathogenic mycobacteria are able to escape phagosomes to the cytosol and counteract many of the free radicals produced by the macrophage *via* enzymatic activity (145,150–153). In addition, pathogenic mycobacteria are able to persist in the cell by reducing metabolic activity through dormancy (110,154).

Central nitrogen metabolism concerns the biochemical patterns involved in the uptake, production and conversion of ammonia, glutamate and glutamine into all other nitrogenous molecules found in the cell (11,12) (Figure 2). These pathways are subject to strict regulation as has been demonstrated for various mycobacteria, including *M. tuberculosis*, both at transcriptional and translational level (chapter 1, section 1.2), which may indicate that the levels of the different substrates that they act upon fluctuate or are manipulated by the mycobacteria in the different microenvironments that they encounter. One such microenvironment is the phagosome where infecting pathogenic mycobacteria are exposed to various stresses as outlined in previous paragraphs. Studies have indeed hinted to the role of central nitrogen metabolism and its effect on ammonium, glutamate and glutamine levels as a defensive mechanism against various environmental stressors that the cells may experience (119,123,128,155).

Here it is hypothesised that glutamate homeostasis, which is controlled by GDH and GltS, is required for the protection of *M. bovis* BCG against cellular stress. This was tested *in vitro* by simulating potential stress conditions that may be experienced by the bacilli through the use of knockout *gdh* and *gltBD M. bovis* BCG Pasteur strains.

3.1   Results and discussion

3.1.1   Osmotic stress

Osmotic stress is defined as a high or low osmolarity that exerts pressure on the cell and this phenomenon is characterised by the influx or efflux of water from the cell. A change in osmolarity

occurs when there is a sudden change of solute concentration in the surrounding environment. When confronted with a high solute concentration of extracellular salts water is drawn from the cell through osmosis, subsequently dehydrating the cell. Alternatively, a low concentration of salts in the extracellular environment causes water molecules to enter the cell through osmosis which could, in some cell types, directly result in cell death of the specific cell under stress.

Glutamate, the substrate of NAD-dependent GDH and product of GltS, has been shown to be a compatible solute. Compatible solutes are small molecules, which at very high concentrations in the cytosol, have inert characteristics and are thus unable to affect the activity of enzymes. However, compatible solutes are still able to establish a gradient across the cell membrane which offers protection against osmotic upshock (156). It has further been demonstrated that *E. coli* rapidly accumulates glutamate *via* GltS in the presence of high extracellular salt concentrations when ammonia is the sole nitrogen source (157). This mechanism allows *E. coli* to rapidly increase its intracellular solute concentration to combat the high extracellular salt concentration.

Both *M. tuberculosis* and *M. bovis* BCG have an extremely high intracellular concentration of glutamate (135). It is therefore hypothesised that a disruption of glutamate homeostasis may result in increased sensitivity to osmotic stress in the form of high extracellular salt concentration. To test this hypothesis both *gdh* and *gltBD* knockout mutants were exposed to increasing concentrations of NaCl and the effect on growth was monitored.
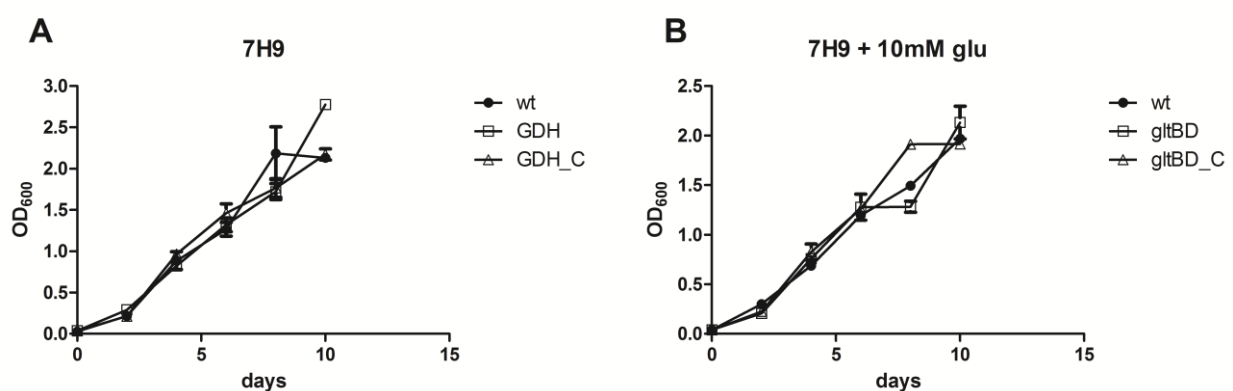
Figure 4: Optical density measurements indicating growth profiles of wild type *M. bovis* BCG, Δ*gdh*, Δ*gltBD*, Δ*gdh* complement, Δ*gltBD* complement in Middlebrook 7H9. A) Growth of *M. bovis* BCG, Δ*gdh* and Δ*gdh* complement in standard culture media. **B**) Growth of *M. bovis* BCG, Δ*gltBD* and Δ*gltBD* complement in 7H9 supplemented with 10mM glutamate. Data presented are means and standard errors calculated from three independent growth curve experiments (n = 3). In some cases error bars are smaller than the symbols.
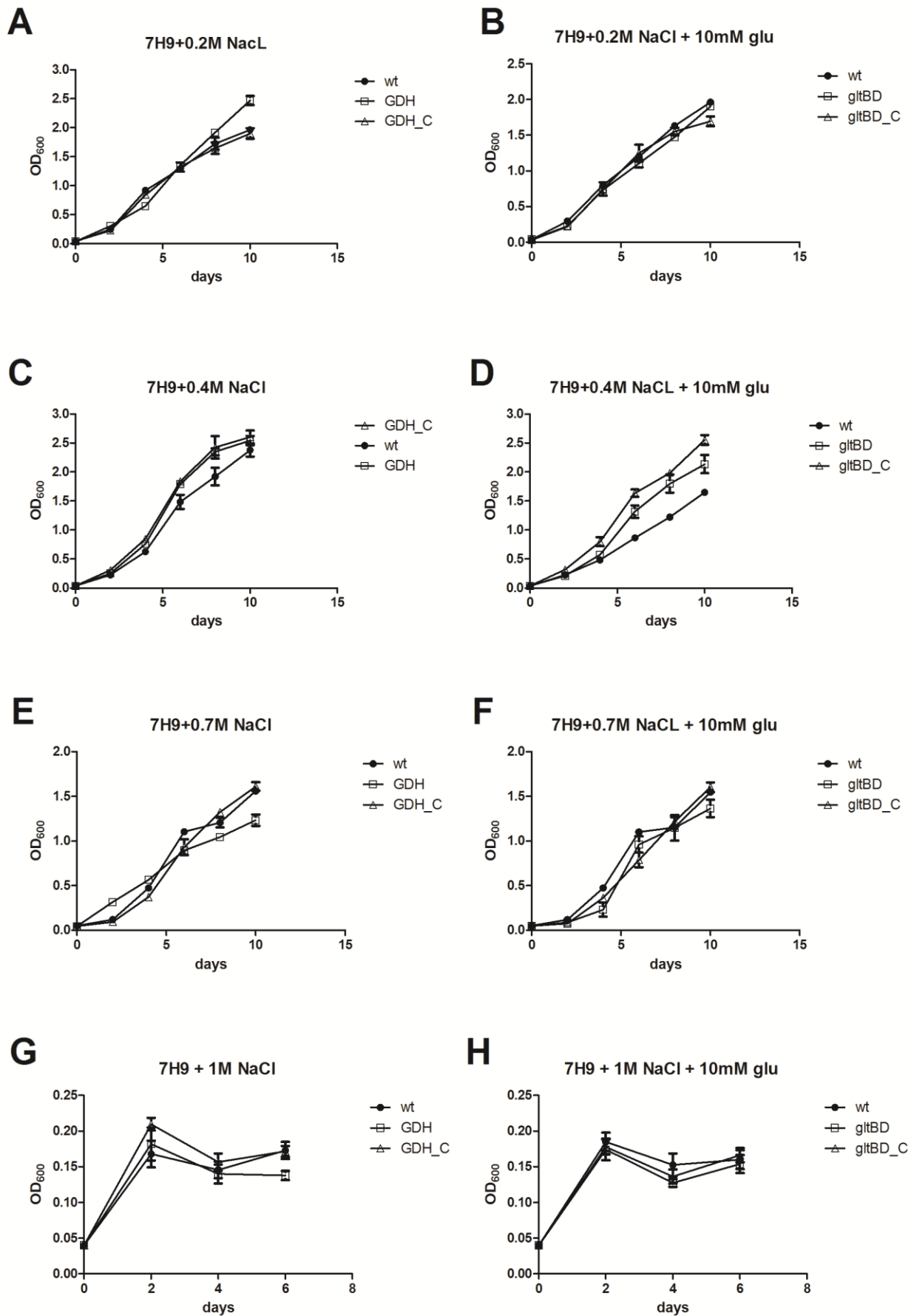
Figure 5: Growth of wild type *M. bovis* BCG, Δ*gdh*, *ΔgltBD*, *Δgdh* complement, *ΔgltBD* complement when exposed to 7H9 with excess salt concentrations or 7H9 and exposed 7H9 supplemented with 10mM L-glutamate. In all relevant cases, L-glutamate was supplemented in 7H9 to a final concentration of 10mM specifically to compensate the growth defect of *ΔgltBD* in 7H9. **A**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, Δ*gdh* and *Δgdh* complement in 7H9 exposed with 0.2M NaCl. **B**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, *ΔgltBD* and *ΔgltBD* complement in 7H9 supplemented with 10mM glutamate and 0.2M NaCl. **C**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, Δ*gdh* and *Δgdh* complement in 7H9 exposed with 0.4M NaCl. **D**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, *ΔgltBD* and *ΔgltBD* complement in 7H9 supplemented with 10mM glutamate and 0.4M NaCl. **E**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, Δ*gdh* and *Δgdh* complement in 7H9 exposed with 0.7M NaCl. **F**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, *ΔgltBD* and *ΔgltBD* complement in 7H9 supplemented with 10mM glutamate and 0.7M NaCl. **G**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, Δ*gdh* and *Δgdh* complement in 7H9 exposed with 1M NaCl. **H**) OD_{600} measurements indicating growth over time of *M. bovis* BCG, *ΔgltBD* and *ΔgltBD* complement in 7H9 supplemented with 10mM glutamate and 1M NaCl. Data is presented are means and standard errors calculated from three independent growth curve experiments (n = 3). In some cases error bars are smaller than the symbols.

There was no observable growth defect between the mutants, complement and wild type *M. bovis* BCG strains in standard culture media (Figure 4; Addendum A; Table S1). It was previously reported that a *M. bovis* BCG knockout of *gltBD* experienced a severe growth defect when cultivated on standard 7H9 (17). However, it was reported in the same study that the growth defect may be alleviated by addition of 10 mM L-glutamate to the 7H9. Therefore, to allow growth of the *ΔgltBD* mutant culture media used to investigate the effect of osmotic stress on this mutant and it's complement were always supplemented with 10 mM glutamate as reported before, no differences in growth profile were observed between the *ΔgltBD* mutant and the wild type strain in 7H9 supplemented with 10 mM glutamate (Figure 4 B, p=0.99). The addition of NaCl at high concentrations clearly had a marked retarding effect on the growth of all the strains used in this study (Figure 5). There was a decrease in OD across all strains as NaCl increased from an OD_{600} of 2.5 in standard 7H9 (Figure 4) to an OD_{600} of 0.25 in 7H9 supplemented with 1 M NaCl at the final day of measurement (Figure 5 G & H; Addendum A; Table S2) However, the addition of NaCl, even at high concentrations such as 1 M, had no statistically significant effect on the growth of either the mutant strains investigated compared to the wild type (Table 4). Despite not testing statistically significant, wild type cultures appeared to be slightly less dense than mutant cultures at later time points in medium containing 0.4 M NaCl (Figure 5 C & D).

Table 4: P-values of ANOVA and Bonferroni post-test's on each of the growth curves indicating significance

	ANOVA	*Significant*	*Bonferroni*	*Significant*
7H9	*P=0.9935*	*NO*	*Wt vs gdh*	*NO*
			Wt vs comp	*NO*
			gdh vs comp	*NO*
7H9 +10 mM L-glu	*P=0.9782*	*NO*	*Wt vs gltBD*	*NO*
			Wt vs comp	*NO*
			gltBD vs comp	*NO*
7H9+0.2M NaCl	*P=0.9646*	*NO*	*Wt vs gdh*	*NO*
			Wt vs comp	*NO*
			gdh vs comp	*NO*
7H9+0.2M NaCl+ 10mM L-glu	*P=0.9800*	*NO*	*Wt vs gltBD*	*NO*
			Wt vs comp	*NO*
			gltBD vs comp	*NO*
7H9+0.4M NaCl	*P=0.9232*	*NO*	*Wt vs gdh*	*NO*
			Wt vs comp	*NO*
			gdh vs comp	*NO*
7H9+0.4M NaCl+10mM L-glu	*P=0.6262*	*NO*	*Wt vs gltBD*	*NO*
			Wt vs comp	*NO*
			gltBD vs comp	*NO*
7H9+0.7M NaCl	*P=0.9788*	*NO*	*Wt vs gdh*	*NO*
			Wt vs comp	*NO*
			gdh vs comp	*NO*
7H9+0.7M NaCl+10mM L-glu	*P=0.9598*	*NO*	*Wt vs gltBD*	*NO*
			Wt vs comp	*NO*
			gltBD vs comp	*NO*
7H9+1M NaCl	*P=0.9132*	*NO*	*Wt vs gdh*	*NO*
			Wt vs comp	*NO*
			gdh vs comp	*NO*
7H9+1M NaCl+10mM L-glu	*P=0.9716*	*NO*	*Wt vs gltBD*	*NO*
			Wt vs comp	*NO*
			gltBD vs comp	*NO*

Data representing calculated p-values for all growth curves pertaining to osmotic stress. All ANOVA and Bonferroni analysis was done with a p-value >0.05. Wild type *M. bovis* BCG; Δ*gdh;* *ΔgltBD, Δgdh* complement/ *ΔgltBD* complement.

Although it was previously reported that L-glutamate affects the tolerance of enteric bacteria to osmotic stress, we were unable to detect any significant difference between the growth of the *gdh* and *gltBD* mutant strains and wild type over time when exposed to increased extracellular solute concentrations (157) (Figure 5, Table 4). However, it should be taken into consideration that addition of a relatively high concentration of glutamate (10 mM) to *ΔgltBD* cultures is a unavoidable confounding element in the investigation of the osmoprotective role of glutamate in the strain. Ideally, glutamate should be omitted from culture media to test the effect of the amino acid on osmotic stress (17).

Furthermore, when McGlaggens *et al* 1994 displayed protection against osmotic stress the *E. coli* cells were grown in ammonia as the sole nitrogen source, it is not possible to grow *M. bovis* BCG *ΔgltBD* in media containing ammonia as the sole nitrogen source as described previously (17). Therefore, we were unable to force the glutamate production pathway through central nitrogen metabolism. In addition, we were unable to see any significant effect when testing *M. bovis* BCG vs *M. bovis* BCG Δ*gdh* (Figure 5). This may be due to the lack of anabolic GDH in slow growing mycobacteria. *M. bovis* BCG has a single GDH which converts glutamate to ammonia and α-ketoglutarate in the presence of NAD. Thus glutamate cannot be synthesised by this pathway which could explain the lack of observable difference when *M. bovis* BCG and *M. bovis* BCG Δ*gdh* is exposed to high concentrations of NaCl.

In conclusion, osmotic stress does affect the growth rate of *M. bovis* BCG, the growth of which is dramatically reduced at 1 M NaCl. However, both GltS and GDH in *M. bovis* BCG are dispensable to cellular survival when confronted with hyper osmolarity. These results suggest that cytosolic glutamate levels are not regulated by GDH or GltS when the bacteria is under osmotic stress as reported in *E. coli* (128), possibly due to the maintenance of glutamate at a high level regardless of intracellular/extracellular solute concentrations. Future studies may be devoted to determining whether osmotic stress exacerbates the growth defect of *ΔgltBD* in medium with low glutamate content or in which glutamate is completely absent. Alternatively, future studies could investigate whether depletion of glutamate in the cytosol of *ΔgltBD* in medium devoid of glutamate, renders this strain hyper sensitive to osmotic stress compared to wild type.

3.1.2   Reactive oxygen stress

During infection, the host defends against the intrusion of foreign cells by deploying reactive oxygen intermediates (ROI). These compounds are highly reactive and form complexes with other compounds; this in turn induces a stress response against the foreign cell termed reactive oxygen stress (ROS). Being a facultative intracellular pathogen *M. tuberculosis* has adapted to survive the reactive oxygen burst by natural mechanisms such as superoxide dismutase (SOD), catalase-peroxidase-peroxynitritase (KatG) and peroxynitrite reductase enzymatic reactions as well as mechanisms to block the fusion of the phagosomes with lysosomes (158). It has previously been demonstrated in *E. coli* that GDH has a protective effect against ROS when under extreme acidic stress (pH 2-3) (128).

Typically *in vivo, M. tuberculosis* cells experience acidic conditions when confronted by acidic stress in the macrophage at a pH of 4.5 (159). The effect of sub-lethal concentrations of cumene hydroperoxide (CHP) (500 uM) (160) on wild type and mutant strains was therefore tested at this pH.
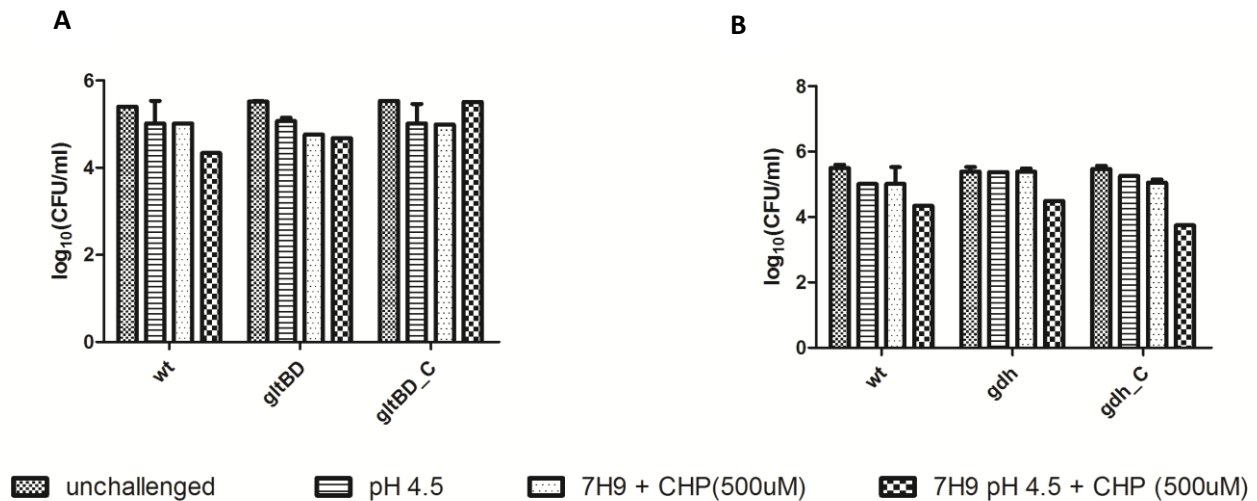
Figure 6:Graphs indicate the effect of ROS and/or acidic stress on wild type, mutant and compliment strains of *M. bovis* BCG under after 2 hours exposure. Bars indicated as unchallenged represent a 0 hour time point for each strain. A) Average Log CFU/ml of wild type *M. bovis* BCG, *ΔgltBD* and *ΔgltBD* complement either uncontested (unchallenged) or contested and measured after two hours of induction. Strains were induced with CHP and/or HCL set to a final pH of 4.5 in 7H9. **B)** Average Log CFU/ml of *M. bovis* BCG, Δ*gdh* and *Δgdh* complement either uncontested (unchallenged) or contested and measured after two hours of induction. Strains were induced with CHP and/or HCL set to a final pH of 4.5 in 7H9. Error bars are representative of SEM from 2 independent experiments. Variance was analysed by two-way ANOVA subject to a Bonferroni post-test.

There was no significant difference between both mutant strains compared to wild type after 2 hour exposure to CHP, indicating that after 2 hour exposure, neither *Δgdh* nor *ΔgltBD* experiences a deficit in cell viability when exposed to either CHP or CHP and low pH 4.5 (Figure 6 A & B). The treatment did however have a suspected significant effect on survival of the bacteria expressed as log CFU (p=0.0086) indicating an expected vulnerability of all strains to the ROS treatment. Previous studies have reported a protective effect associated with GDH in *E. coli* when exposed to ROS and acidic stress simultaneously. This phenomenon was witnessed under severe acidic stress (pH 2.5) during which glutamate provided the best protective response. At pH 5.5 and 7.0 *E. coli* RpoS was responsible for protection against ROS (128). It was also found that over a period of 15 days at pH 4.5 *Δgdh* experienced a marked decrease in cell viability while *ΔgltBD* had a higher cell viability measurement compared to wild type BCG. Indeed a decrease in cell viability of the *Δgdh* mutant was only observed after 5 days of cultivation at pH 4.5 (Personal communication, Viljoen *et al* 2013.). Thus it can be concluded that neither GDH nor GltS plays a pivotal role in the early protection (2 hours) against ROS or acid stress, yet GDH is required for the persistent survival of BCG over larger time frames (15 days).

3.1.3   Nitric oxide stress

Similar to reactive oxygen compounds, nitric oxide is found naturally in all living cells as a by-product of urea metabolism. Due to the toxic nature of free radical compounds such as NO, bacteria maintain a strict homeostasis and regulation of nitric oxide to detoxify cells.

As part of innate defence against invading pathogens, immune cells such as macrophages are able to release a burst of NO to sterilise the environment of unwanted pathogens, the exact mechanism of killing intracellular mycobacterial cells is however not yet known. It is theorised that mycobacterial cells combat the nitrosative burst by entering dormancy (4,90,110,154,161) or through detoxification by truncated haemoglobin (HbN) (162,163). Nitric oxide triggers the dormancy regulon when used as a minimal electron acceptor, effectively inhibiting aerobic respiration. Interestingly, this is only the case when NO is present at a low concentration (164,165). When *M tuberculosis* cells are treated with higher concentrations than necessary for dormancy, stress related gene responses are induced (4). It has previously been shown that during dormancy *M. bovis BCG* GDH is down regulated. However, when the regulatory dormancy genes Dos S/T

were deleted it was found that GDH was up regulated when challenged with NO, and remained unchanged when nitric oxide was omitted (165). Tight regulation of GDH by PknG suggests that GDH is active during infection due to the critical role of PknG to establish infection *in vivo* (138).
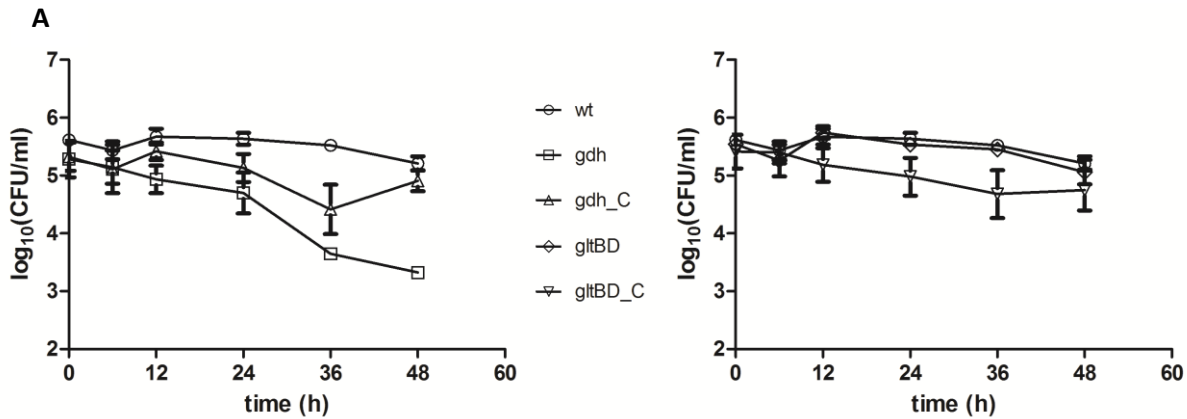
Figure 7: These graphs depict the cell viability as measured by log CFU/ml over time of *M. bovis* BCG wild type complement and mutant strains challenged with sub-lethal concentrations of diethylenetriamine/nitric oxide adduct (DETE/NO). A) The effect of DETE/NO on *M. bovis* BCG wild type, *M. bovis* BCG *Δgdh* and *M .bovis Δgdh* complement over a 48 hour period. **B)** The effect of DETE/NO on *M. bovis* BCG wild type, *M. bovis* BCG *ΔgltBD* and *M. bovis* BCG *ΔgltBD* complement over a 48 hour period. Error bars are representative of SEM, p = 0.05 data was analysed with a two-way ANOVA and Bonferroni post-test. Both graphs are representative of at least three independent experiments.

M. bovis BCG *Δgdh* experiences a sharp decrease in cell viability at 24 hours (P<0.05) hours compared to *M. bovis* BCG wild type under nitrosative stress (Figure 7 A; Addendum A, Table S3). At the 36 hour and 48 hour time point there is a significant loss of viability of *Δgdh* compared to wild type ((Figure 7 A, (p<0.0001)). The phenomenon is not observed for the *gltBD* mutant (Figure 7 B; Addendum A, Table S3). The complemented *Δgdh*, does experience a decrease in cell count overtime, however this decrease is non-significant when compared to wild type (P>0.05). No significant differences were observed between *M. bovis* BCG wild type, *M. bovis* BCG *ΔgltBD* and *M. bovis* BCG *ΔgltBD* complement over the entirety of the 48 hour exposure to DETE/NO ((Figure 7 B; Addendum A, Table S3), indicating that GltS is dispensable when cells are under NO stress. In order to investigate whether the protective effect of GDH under nitrosative stress is due to the ammonium releasing activity of associated with the GDH enzymatic reaction, wild type, *Δgdh* and its complement were cultured under high concentrations of ammonium sulphate to load the bacilli with ammonium prior to exposure of DETE/NO. This ammonium priming resulted in a significant

increase in the survival of *M. bovis* BCG *Δgdh* when exposed to both 20 mM and 30 mM ammonium sulphate compared to *M. bovis* BCG *Δgdh* exposed to DETE/NO only (Figure 8; Addendum A, Table S4). The killing effect of 48 hours of NO exposure on *Δgdh* is thus ameliorated when excess ammonia is present inside the cell prior to the onset of NO exposure (Figure 7 A, Figure 8) It could thus be concluded that the hydrolysis of glutamate by GDH is required for resistance against nitrosative stress *in vitro*. Since it was previously shown that GDH is down regulated during dormancy, it could be speculated that GDH is required for the initial survival of the bacilli upon nitrosative burst and in this fashion allows the bacilli to switch on other stress defence mechanisms as it enters dormancy. This is likely, taking into account the nature of GDH regulation. It is safe to assume that GDH is active during infection and gltS is inactive due to regulation by PknG-GarA regulatory complex (Chapter 1, Section 1.5).
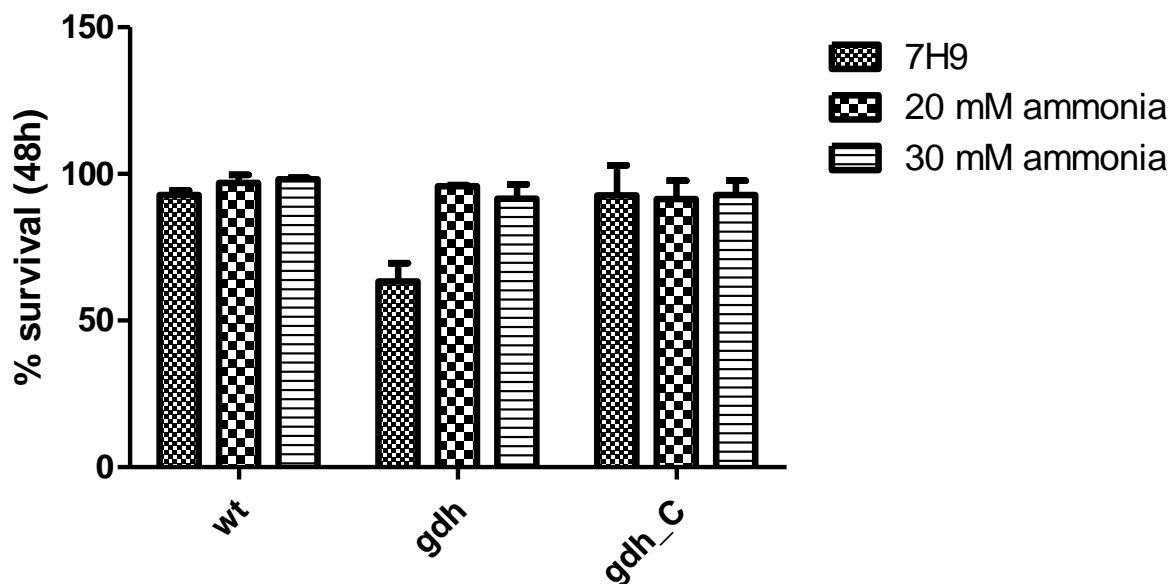
Figure 8: Percentage survival of *M. bovis* BCG wild type complement and mutant strains primed for either 20 mM or 30 mM (NH$_4$)$_2$SO$_4$. All cultures were washed by centrifugation at least four times and challenged with 500 uM DETE/NO in 7H9+ADN for 48 hours. Percent survivability was determined by the difference in log CFU/ml counts between 0 hours and 48 hours. Data is representative of SEM and analysed by two-way ANOVA and Bonferroni post-test. P-value was set to 0.05. Data is representative of at least two independent experiments.

Although *Δgdh* bacteria primed with high ammonium concentrations were washed extensively to remove any remaining extracellular ammonium prior to NO exposure, it remained possible that cytosolic ammonium could diffuse out of the cells exerting their effect on NO molecules in the extracellular milieu. Therefore, to gain more insight on whether ammonium liberated by the action

of GDH primarily exerted a protective effect against NO within in the cytosol or within the medium, total intracellular and extracellular nitrite was determined using a Griess assay. The concentrations of nitrite alludes to the amount of NO that was once present, since NO is rapidly converted to nitrite. The internal nitrite concentrations was observed to vary between strains where higher concentrations of nitrite was found within the *Δgdh* mutant compared to the wild type when cells were not primed with high cytosolic ammonium prior to NO exposure (Figure 9 A, p = 0.0422), whilst external concentrations remained relatively similar. This suggests that ammonium released by GDH activity has its primary protective effect against NO stress within the cytosol. In all cases, nitrite levels were below the detection limit of the Griess assay in cultures that were not challenged with DETE/NO.
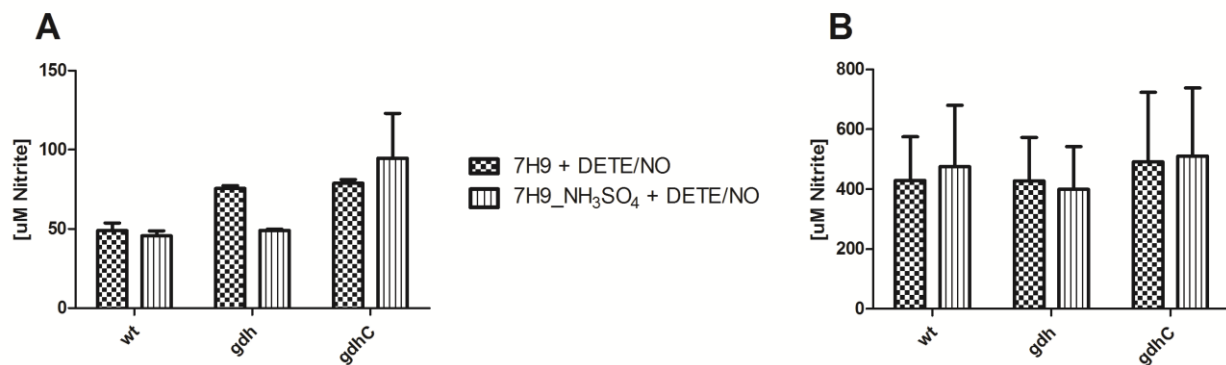
Figure 9: Griess assay estimating the concentration of Nitrite in either the whole cell lysate (A) or the supernatant (B). A) Intracellular levels of nitrite measured by Griess assay in whole cell lysates of *M. bovis* BCG wild type, mutant and complement strains by spectrophotometry OD_{600} 540 nm. **B)** Extracellular levels of nitrite measured by Griess assay in the supernatants of *M. bovis* BCG wild type, mutant and complement strains. Error bars are representative of at least two independent experiments. Two-way ANOVA was used to determine variance between strains. P-value was set to 0.05.

This study provides the first experimental evidence to implicate the role of NAD dependent GDH in resistance against nitrosative stress. Low ammonia conditions results in a upregulation of the *amt* transporter in *M. tuberculosis* (166) and *C. glutamicum* (167), it is possible that the GDH deficient mutant internalises free ammonia molecules through the amt transporter to compensate for the lack of GDH, and subsequently releases the ammonia in the extracellular environment upon exposure to nitric oxide. It should be mentioned that AnsA is involved in ammonia production in the extracellular environment by the conversion of asparagine to aspartate (7,8). Therefore, it could be speculated that AnsA increases the extracellular ammonia pool to combat against cellular stressors

such as acidification and ROS/NOS bursts and GDH is used to combat the reactive nitrogen molecules that are able to transverse the cell wall of the bacilli during early stages of infection.

3.1.4   Macrophages

Both *M. bovis* BCG and *M. tuberculosis* are able to persist when infecting macrophages by blocking the maturation of the phagolysosome. The killing mechanism in murine macrophages is largely dependent upon the use of NO (149,168,169). Mice deficient in nitric oxide synthase succumb rapidly to infection indicating that NO has a pivotal role in murine defence against tubercle infection (101,170). Vulnerabilities involving GDH in an *ex vivo* setting has been hinted in previous studies and demonstrated in murine bone derived macrophages (MBDM, personal communication, *Viljoen et al*). As mentioned previously, PknG plays a pivotal role in the regulation of GDH and mycobacterial virulence (82,84). *M. bovis* BCG *ΔpknG* is attenuated for virulence in bone marrow derived macrophages (MBDM) (85). In addition, PknG binds to GarA which in turn activates GDH and KDH while inhibiting GltS (140,171). Previously, a decrease in cell viability was observed when MBDM were infected with *M. bovis* BCG *Δgdh* (personal communication, *Viljoen et al* 2013). This implicates the importance of GDH for optimal survival of *M. bovis* BCG in MBDM. Here it was aimed to further corroborate decreased growth and survival of the *Δgdh* mutant in macrophages by using another *in vitro* macrophage infection system, RAW 267.4 macrophages.

There was no observable difference in the starting colony counts between the wild type, mutant and complement strains (Figure 10, P<0.05). Thus, all strains are phagocytosed equally by RAW 267.4 macrophages. It is therefore likely that any decrease in bacterial viability that occurs later than the 0 hour (T_0) is due to killing by the macrophage.
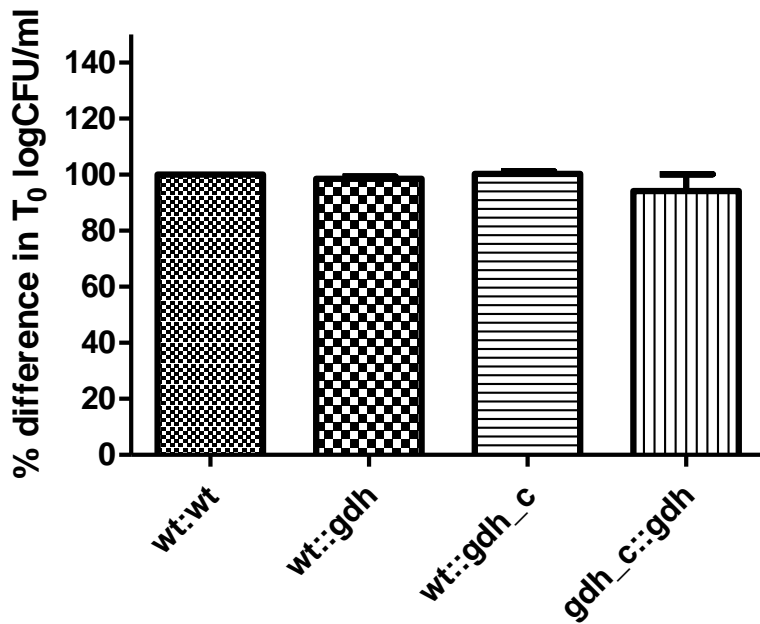
Figure 10: The percent T_0 log CFU/ml counts of strains relative to each other. This graph indicates the count of colony forming units directly after infection and washing relative to other strains. Where **(wt::wt)** is represented as % CFU/ml T_0 of *M. bovis* BCG wild type relative to its self. **(wt::gdh)** is representative of %CFU/ml T_0 *M. bovis* BCG wild type relative to % CFU/ml T_0 *M. bovis* BCG *Δgdh*. **(wt::gdh_c)** is representative of % CFU/ml T_0 *M. bovis* BCG wild type relative to % CFU/ml T_0 *M. bovis Δgdh* complement. **(gdh::gdh_c)** is representative of % CFU/ml T_0 *M. bovis* BCG *Δgdh* relative to % CFU/ml T_0 *M. bovis Δgdh* complement. Data presented of at least three independent experiments and error bars as presented as a function of SEM. Data was analysed by one-way ANOVA with p = 0.05.

M. bovis BCG *Δgdh* intracellular survival is compromised in RAW 267.4 macrophages compared to wild type *M. bovis* BCG over a 6 day period. *M. bovis* BCG is avirulent and thus remains static, which is expected (Figure 11). *M. bovis* BCG *Δgdh* experiences significant loss of cell viability compared to wild type on days 4 and 6 (p<0.01). However, the phenotype was only partially restored to wild type levels in the genetically complemented *Δgdh* strain (Figure 11; Addendum A, Table S5). These observations are in line with observations made in an earlier study in our lab in murine bone derived macrophages (personal communication, *A.J. Viljoen*). The NAD dependent GDH of *M. bovis* BCG is required for optimal persistence in murine macrophages.
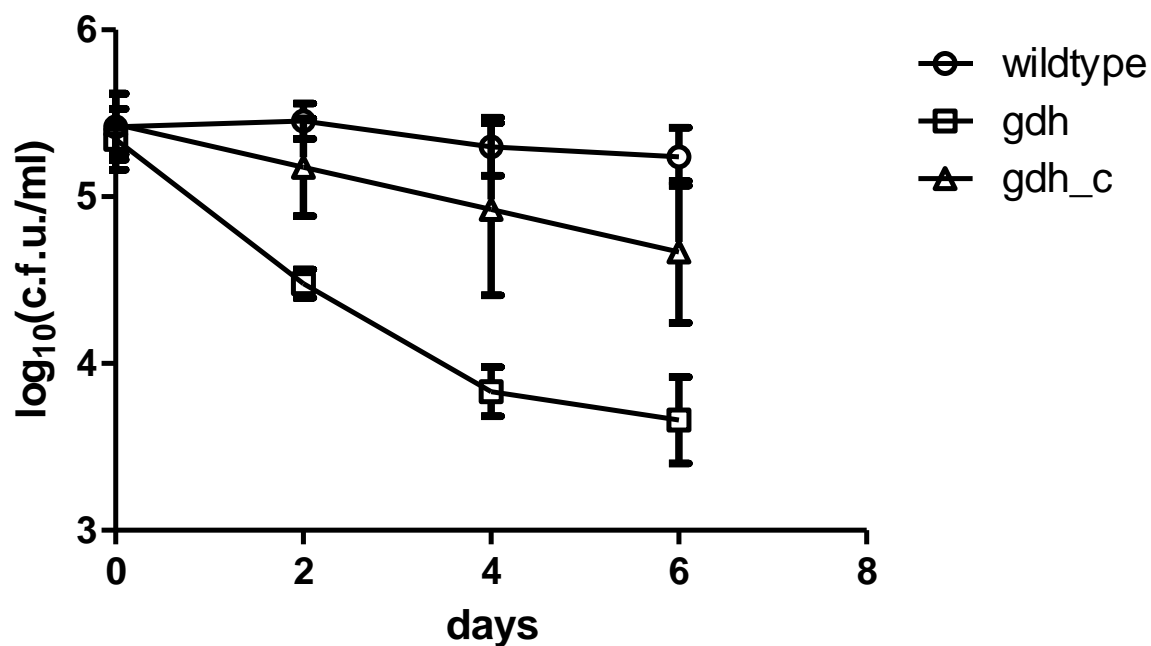
Figure 11: Infection of RAW 264.7 murine cell line with *M. bovis* BCG wild type, complement and mutant strains. Macrophages were infected at a MOI of 5:1. Infected macrophages were treated with penicillin/streptomycin (1:100) and plated every second day for a total of six days. In certain cases error bars are smaller than the symbols. Error bars are representative of SEM. Data presented is of at least three independent experiments. Data was analysed by two-way ANOVA and a Bonferroni post-test with p-value set to 0.05.

Although the complementation is sub optimal there is a 2.5 log CFU/ml decrease in cell viability of *M. bovis* BCG *Δgdh* compared to the wild type strain. Furthermore, statistically, it was found by two way ANOVA that the complement displayed no significant difference to the wild type across the entirety of the experiment whilst significant difference was detected between the complement and *Δgdh* at day 4 (p<0.05) and day 6 (p<0.05) (Figure 11; Addendum A, Table S5). It is likely that the roles of NAD dependent GDH with regard to resistance against NO (Figure 7) and acidic stress (17) is responsible for the decline in survival of *M. bovis* BCG *Δgdh* in RAW 264.7 macrophages. It has however, been shown that murine cell lines experience increased production of nitric oxide compared to their human counterparts (172). However, it has also been shown that nitric oxide synthase 2 (NOS2) was more frequently expressed in the lungs of patients infected with TB than the healthy controls (173), suggesting the relevance for the murine macrophage model to study the nitrosative context that *M. tuberculosis* bacilli encounter in patients. Furthermore, it has been shown that NOS2 is co-localised in the phagosome, indicating the availability of NO in the absence of a lysosome fusion (174). Studies have demonstrated the protective role of nitric oxide produced by

murine macrophages against mycobacterial infections (170). It was further found that the levels of nitric oxide produced by interferon gamma activated macrophages were not enough to entice a bactericidal effect. Co-localisation and the low concentration of nitric oxide radicals to the phagosome may explain the nitric oxide cue for dormancy (164,174). Interestingly, NO released by human macrophages enhanced the growth of *M. tuberculosis,* possibly by use of oxidised NO_2^- /NO_3^- (174). Deletion mutants of *narG* were unable to replicate in interferon gamma activated human macrophages and the result was a static *M. tuberculosis* culture (174).

Here we provide experimental evidence implicating the role of GDH in host colonisation, we were able to show that this enzyme is indeed required by *M.* bovis BCG to persist in a macrophage model of infection. It could be speculated that GDH is required by *M. bovis* BCG to increase the intracellular pool of ammonia during infection. This increase of ammonia serves the dual purpose of protection against acid stress by alkalising the environment as well as protecting against free nitric oxide radicals. The mechanism that ammonia uses to decrease the efficacy of toxic levels of NO still remains unknown.

Chapter 4

Study Conclusions and Considerations

Central nitrogen metabolism, and the regulation thereof, has been implicated in the virulence of *M. tuberculosis* and prominent drug targets have been identified (33,36,78,84,130,146). The protein products of *gdh* and *gltBD* remain relatively understudied, yet many observations have indirectly implicated these genes in virulence (78,79). Both these genes have been annotated as essential by various TraSH studies (1,5,6,21,72). Interestingly, knock out mutants of these genes were generated in the closely related strain, *M. bovis* BCG (17). This achievement suggests a profound difference between the pathogenic *M. tuberculosis* and non-pathogenic *M. bovis* BCG. The regulators of these proteins have shown to be required for sustained infection in both *M. tuberculosis* and *M. bovis* BCG (82,84). Although these mutants are not required for growth in standard mycobacterial culture media we were able to demonstrate the requirement of GDH for optimal survival against nitrosative stress and within murine macrophages.

Whole genome sequencing identified a wide variety of SNP's only found within the *Δgdh* strain. The most prominent genes that underwent mutations were involved in cell signalling and cell wall biosynthesis. Of the non-synonymous SNP's detected by sequencing those found in *pknH* and *ppsB* were of interest. The role of PknH has been demonstrated in Balb/c mice and it was found PknH is most likely required for sensing environmental nitric oxide. It was further shown that the downstream substrates for PknH are proteins required for cell wall biosynthesis (143). Interestingly, PpsB is involved in the biosynthesis of the cell envelope component, phenolpthiocerol (136). It was recently shown that PDIM and peptidoglycan biosynthesis is up-regulated in the presence of nitric oxide (71). It is likely that the loss of GDH has an effect on cell wall biosynthesis by deregulating glutamate homeostasis. It was previously shown that the levels of glutamine and glutamate are strenuously regulated to maintain the cell wall of *M. tuberculosis* (22,35,82,175).

Pathogenicity of an organism is a complex phenomenon and is based upon an intricate physiology that allows the survival of the organism in a harsh environment. Nitric oxide is a prominent defensive mechanism against tubercle infection in mice (154,170). It remains a controversial topic due to the low concentrations observed in human macrophages (172,174). It is of interest to note that low nitric oxide concentration is required for the activation of the dormancy regulon in *M. tuberculosis* (4,154). This physiological state allows the pathogen to survive for extended periods of time within an infected host cell. Observations have been made that indicate a possible role for nitric oxide, apart from dormancy induction in humans, in later stages of infection (176). The physiology and unique metabolism of *M. tuberculosis* allows the bacilli to modulate the host immune response by detoxifying nitric oxide radicals (145,153,162,177). Yet it is clear that nitric

oxide still has a substantial killing effect which indicates that other mechanisms of action are responsible for optimal protection. We were able to identify a novel mechanism for resistance against nitric oxide stress through the action of *M. bovis* BCG GDH. Mutants lacking GDH were unable to survive sub-lethal concentrations of nitric oxide *in vitro*. In addition, the effect was ameliorated when excess ammonia was present within the cell. It is likely that GDH combusts glutamate to ammonia in order to avoid the effect of nitric oxide within the cell. This is an interesting phenomenon considering that GDH has a low affinity for glutamate and is basally inhibited (78,80). It is tempting to speculate that free ammonia is generated intracellularly in the presence of nitric oxide, sensed by PknH, which subsequently induces the nitrate/nitrite reductase genes (71). These genes are responsible for nitrate and nitrite cycling and provide a method of detoxification of nitric oxide and link internal ammonia to the reaction (92). Further investigation is required in the recycling of nitric oxide by method of redox to substantiate our speculation.

M. tuberculosis is able to replicate within macrophages while non-pathogenic mycobacteria simply persist. We investigated the role of *M. bovis* BCG GDH in RAW 264.7 macrophages and found the enzyme to be required for optimal survival within murine macrophages. This phenomenon is in line with previous studies investigating the role of GDH in murine bone derived macrophages (personal communication, *Viljoen et al*). Our data suggest that the increased production of nitric oxide associated with murine macrophages is responsible for the decrease in cell viability observed in mutants of *M. bovis* BCG lacking a functional GDH.

We have demonstrated the requirement of *gdh* in the optimal survival of *M. bovis* BCG in RAW 264.7 macrophages. To understand this phenotype we investigated the susceptibility of this mutant to nitric oxide stress, results indicated a profound vulnerability of *M. bovis* BCG mutants lacking *gdh*. This is in line with published data that has demonstrated the use of nitric oxide as one of the main killing mechanisms by murine macrophages to combat invading mycobacteria (154,170,172). We are thus able to conclude, from our observations, that the *Δgdh* mutant failed to grow optimally in murine macrophages due to the observed susceptibility to nitric oxide. To understand why *M. bovis* BCG mutants lacking GDH is sensitive to NO, and subsequently susceptible to macrophage killing mechanisms, we used whole genome sequencing to identify possible point mutations. We found two genes unique to *Δgdh* with non-synonymous point mutations that have direct influence on both cell wall biosynthesis and nitric oxide stress. These polymorphisms may have arisen in response to the loss of GDH in the *M. bovis* BCG genome. Mutations are able to emerge randomly in response to changes in the environment as a part of natural evolution. It is not

unlikely that the loss of GDH has forced the generation of point mutations in strategic genes to negate the loss of this enzyme, bearing in mind that GDH is the only enzyme of its kind in the *M. bovis* BCG genome. Based on past publications it is known that the ratios of glutamine and glutamate have important roles in the biosynthesis of the cell wall and disruption of these levels lead to decreased virulence of *M. tuberculosis* (28,35,36,88). Owing to the deregulation of glutamate levels by *Δgdh* it is obvious that cell wall synthesis will be affected, hence the presence of the mutation. It is thus not surprising that the largest amount of putative SNP's found in the *Δgdh* mutant is found within genes associated with cell wall metabolism. In addition, glutamate deregulation affects the level of ammonia, and subsequently interferes with nitrate/nitrite cycling. Transcription of nitrate/nitrite cycling enzymes is largely based on substrate dependency, low oxygen environments, NO and regulation by GlnR (71,92,154,178). It is known that *M. tuberculosis* does not readily scavenge ammonium from the environment yet it is required for resistance virulence and against nitric oxide stress (57,60,95), a phenomenon that has also been suggested by recent published data (69) and this study. It was previously reported that ammonia assimilation is required for the virulence of *M. tuberculosis* through GlnA1 in THP-1 macrophages (36). It may be deduced from our observations as well as Chandra *et al* 2009 that ammonia produced by GDH activity increases the ammonia pool, which acts as a direct substrate for GlnA1. In our *gdh* mutant ammonia is no longer produced, inevitably causing a nett depletion in ammonia levels, and thus is unable to sustain the demands of GS, resulting in reduced survival of the *Δgdh* strain compared to wild type BCG. Although GS is able to function extracellularily (29), the enzyme action is required for sustained growth within the cell as well (29,165), thus indicating a niche requirement for intracellular ammonia biosynthesis. It is known that the urease activity of slow growing mycobacteria generates intracellular ammonia, yet it is not required for growth of *M. tuberculosis* in macrophages (180). Although the urease of *M. bovis* BCG attenuates major histocompatibility class II trafficking to the macrophage (181), it was also shown that *M. bovis* BCG urease does not have a striking contribution to the survival of BCG in macrophages (182). It is tempting to address the role of ammonia assimilation in the combat of nitric oxide stress with context to our observations in murine macrophages as well as the role of ammonia to survival. However, it is not possible to extrapolate a viable hypothesis without further investigation into the role of central nitrogen metabolism and resistance to cellular stress in slow growing mycobacteria.

In conclusion, findings in this study contribute to the fundamental knowledge of the unique metabolism of slow growing mycobacteria. In addition, this study has attributed previously unknown roles for NAD dependent GDH and intracellular ammonia in the protection against nitric

oxide stress. We have demonstrated a profound vulnerability of *M. bovis* BCG mutants lacking GDH when infected macrophages. This study also provides experimental evidence in the support of GDH as a potential novel target for chemotherapeutic intervention against *M. tuberculosis* infection.

Chapter 5

Materials and Methods

5.1   Strains used in this study

All strains are listed in Table 5**.** Mutant strains of *M. bovis* BCG were generated previously (17)

Table 5: Bacterial and eukaryotic strains used in this study

Strains	Description	Source
M. bovis BCG-wild type	Wild type *M. bovis* BCG str. Pasteur 1743P2. Progenitor bacterial strain to all other *M. bovis* BCG strains used in this study	Laboratory collection
Δgdh	Mutant *M. bovis* BCG strain, deletion of 2487 bp *NruI* fragment spanning the GDH domain within the *gdh* gene	(17)
ΔgltBD	Mutant *M. bovis* BCG strain, allelic exchange mutant. The *gltBD* operon is replaced with a hygromycin cassette	(17)
Δgdh attB::pGCgdh	Mutant *M. bovis* BCG strain, *Δgdh* complemented strain. Carries the plasmid pGC with gentamycin resistance cassette	(17)
ΔgltBD attB::pGCgltBD	Mutant *M. bovis* BCG strain, *ΔgltBD* complemented strain. Carries the plasmid pGC with gentamycin resistance cassette	(17)
Δgdh supA1	Compensatory mutant able to grow on excess glutamate. This mutant was generated from *Δgdh*	(17)
Δgdh supB2	Compensatory mutant able to grow on excess glutamate. This mutant was generated from *Δgdh*	(17)
Δgdh supC1	Compensatory mutant able to grow on excess glutamate. This	(17)

	mutant was generated from *Δgdh*	
Δgdh supC3	Compensatory mutant able to grow on excess glutamate. This mutant was generated from *Δgdh*	(17)
RAW 264.7 ATCC TIB-71	Macrophage cell line originated from *Mus musculus.* Cell type: Abelson murine lukemia induced virus transformed macrophage	Laboratory supply

5.2   Cultivation and culture maintenance

All strains, prokaryotic and eukaryotic as well as media used in this study are listed in Table 5. For the routine maintenance of *M. bovis* BCG wild type and mutants, cells were cultured without agitation in Middlebrook 7H9 media supplemented with Middlebrook OADC (oleic acid, albumin, dextrose, catalase) 10% v/v (BD scientific, USA) in 25 cm^2 (5ml)) and 75 cm^2 tissue culture flasks (30ml) (Nunc, Denmark. Frozen bacterial stocks were prepared for long term storage of viable *M. bovis* BCG and mutants in 15% glycerol stored at -80 ^{O}C. For routine maintenance of *M. bovis* BCG *ΔgltBD*, this specific mutant strain was supplemented with 10 mM glutamate for routine cultivation. All further maintenance procedures of this strain are similar to the procedures mentioned above. All strains were routinely tested for contamination by Ziehl-Neelson staining and microscopy as described previously (183). *M .bovis* BCG *Δgdh* complement and *ΔgltBD* complement were maintained under 25 µg/ml gentamycin (Sigma-Aldrich, USA) antibiotic resistance for all cultivation experiments.

RAW 264.7 Macrophages were maintained in DMEM (Sigma-aldrich, USA) supplemented with 10% Fetal bovine serum (FBS) (Sigma-aldrich, USA), this media will be referred to as D10. Frozen macrophages were maintained within this media from frozen seed lots in 25 cm^2 (5 ml) and 75 cm^2 (30 ml) tissue culture flasks without agitation at 37 ^{O}C in a CO_2 controlled (5 % CO_2) bio-oven. Cells were replaced with fresh media every second day.

Table 6: Culture media and media supplements used in this study and their composition

Media	Components
7H9	$(NH_4)_2SO_4$,0.5 g/L; L-Glutamate, 0.5 g/L; sodium citrate, 0.1 g/L; pyridoxine, 1 mg/ml; biotin, 0.5 mg/L; disodium phosphate, 2.5 g/L; monopotassium phosphate, 1 g/L; ferric ammonium citrate, 40 mg/L; magnesium sulphate, 50 mg/L; calcium chloride, 0.5 mg/L; zinc sulphate, 1 mg/L; copper sulphate, 1 mg/L; glycerol 0.2% v/v ; Tween 80, 0.05% v/v
7H9:Nitrogen limiting	Sodium citrate, 0.1 g/L; pyridoxine, 1mg/ml; biotin, 0.5 mg/L; disodium phosphate, 2.5 g/L; monopotassium phosphate, 1g/L; ferric citrate, 40 mg/L; magnesium sulphate, 50 mg/L; calcium chloride, 0.5 mg/L; zinc sulphate, 1 mg/L copper sulphate, , 1mg/L; glycerol 0.2% v/v; Tween 80, 0.05% v/v.
7H11	Pancreatic digest of casein, 1 g/L; ammonium sulphate, 0.5 g/L; monosodium glutamate, 0.5 g/L; disodium phosphate, 1.5 g/L; monopotassium phosphate, 1.5 g/L; ferric ammonium citrate, 40 mg/L; copper sulphate, 1 mg/L; calcium chloride, 0.5 mg/L; bacteriological agarose, 13.5 g/L; glycerol 0.5% v/v; Tween80 0.05% v/v
OADC	Oleic acid, 0.06% v/v; bovine serum albumin fraction V, 50 g/L; D-dextrose, 20 g/L; sodium chloride, 8.5 g/L; catalase 0.04g/L

ADN	Bovine serum albumin fraction V, 50g/L; D-dextrose,20 g/L; sodium chloride, 8.5 g/L.
DMEM/High	Slight variations from the original Delbucco media: Glucose, 4500 mg/L; Sodium bicarbonate, 4500 mg/L; pyridoxal replaced with pyridoxine
D10	Slight variations from the original Delbucco media: Glucose, 4500 mg/L; Sodium bicarbonate, 4500 mg/L; pyridoxal replaced with pyridoxine and additional 10% Fetal bovine serum fraction V

5.3:   Cellular stress

5.3.1   Preparations for cellular stress

M. bovis BCG wild type and mutant strains were pre-cultured in 5 ml Middlebrook 7H9 media enriched with ADN in $25cm^2$ tissue culture flasks from frozen seed lots until a OD_{600} 0.8-1.0. Pre-cultures of *ΔgltBD* were supplemented with 10 mM glutamate and complement strains, *ΔgltBD* complement and *Δgdh* complement, were supplemented with gentamycin to select for cells containing the gentamycin resistance cassette associated with pGC. Cultures were subsequently sub-cultured to a starting OD_{600} 0.05 and cultured to OD_{600} 0.5-0.8 under the same specifications as above.

5.3.2   Nitrosative stress

5.3.2.1 DETE/NO challenge

Upon reaching mid logarithmic phase (17) the cultures were diluted to an OD_{600} 0.0005 in 6 ml 7H9, 0.05% Tween 80 and enriched with ADN, no other supplements were added at this stage. The strains were challenged with sub-lethal concentrations (500 uM) diethlenetriamine/nitric oxide adduct (DETE-NO) (Sigma-Aldrich, USA) for 48h. All strains were plated at 0h, 6h, 12h, 24h, 36h,

and 48h time points on 7H11 agar (BD scientific, USA). Plates containing the challenged strains were incubated at 37°C for 2-3 weeks or until colonies formed. Colonies were subsequently counted and recorded.

5.3.2.2 The effect of ammonia on nitric oxide stress

M. bovis BCG wild type, *M. bovis* BCG *Δgdh* and *M. bovis* BCG *Δgdh* complement were pre-cultured in Middlebrook 7H9 supplemented with 20 or 30 mM ammonium sulphate, ADN and 25 µg/ml Gentamycin where appropriate. Cultures were incubated at 37°C without agitation until an OD$_{600}$ 1.0 and subsequently sub-cultured under the same conditions until an OD$_{600}$ 0.8-1.0. Mycobacterial suspensions were washed with Phosphate buffered saline (PBS) (Sigma-Aldrich, USA) and transferred to 7H9 mycobacterial culture medium (without additional ammonia) supplemented with ADN and 25 µg/ml Gentamycin where appropriate to a theoretical OD of 0.0005. All strains were challenged with nitric oxide as stated in 5.3.2.1 and plated on 7H11 at 0 hours and 48 hours. Plates were incubated at 37°C for 2-3 weeks or until colonies form. Cell viability was determined by CFU and expressed as a percentage cell death relative to *M. bovis* BCG wild type

5.3.2.3 Griess assay

Initially seed lots were inoculated in 5 ml 7H9 supplemented with 10% v/v ADN, 0.05% Tween 80 and when necessary 20 mM ammonium sulphate and 25 µg/ ml gentamycin. Cultures were transferred to cell culture flasks at a starting OD of 0.05 and allowed to grow without shaking at 37°C until an OD$_{600}$ of 0.8-1.0. Cultures containing ammonium sulphate $_4$ was washed 4 times with PBS to remove extracellular ammonia before transfer to fresh media. Optical density was determined by spectrophotometry and all cultures were set to an OD$_{600}$ of 0.8 in 6 ml 7H9. Where required cultures were challenged with 500 µM DETE/NO, both challenged and unchallenged cultures were incubated for 48 hours at 37°C without shaking. After the incubation period cells were separated from media by centrifugation (4000 rpm, 4°C, 15 minutes). Supernatants were kept for analysis. Harvested cells were re-suspended in 500 µl sonication buffer (Tris-HCL pH 7.4 and 0.1% Tween 80) and sonicated (1 minute, amplitude of 50) to retrieve intracellular metabolites. Post sonication, cellular debris was removed by centrifugation in a bench top microfuge (15 000 rpm, 4 °C, 15 minutes) and intracellular metabolites were kept for further analysis.

The total intracellular and extracellular concentration of nitrite was determined *via* Griess assay as stipulated by the manufacturer (Sigma-Aldrich, USA). Briefly, sodium nitrite standard solutions were prepared in a range of 0 µm to100 µM. Total reaction volume was set to 200 µl with the reaction buffer provided in flat bottom microtiter plates (Greiner Bio one, Austria). Sulfanalamide was added to samples and buffer followed by 5 min incubation at room temperature (25 °C). Subsequent addition of an equal volume of naphthylethylenediamine was added to each well and incubated for 10 minutes at room temperature. Absorbance was read by spectrophotometry (BMG labtech, Germany) at OD_{540} to determine nitrite concentrations.

5.3.3   Oxidative stress and acidic stress

Tests for oxidative stress follow a similar approach to that of nitrosative stress. Cells were cultivated as stated in 5.3.1. Media was set to pH 4.5 with hydrogen chloride to test the effect of oxidative stress at lower pH levels. Upon reaching mid log phase, 0.5-0.8, cultures were diluted to an OD_{600} of 0.0005 in 4 ml 7H9 containing 0.05% Tween 80 and enriched with 10 % v/v ADN. Cultures were challenged with cumene hydroperoxide (Sigma-Aldrich, USA) at sub-lethal concentrations (500 µM) for a total of 2 hours. Cells were plated at 0h and 2h and incubated at $37^{O}C$ for 2-3 weeks or until colonies formed. The colonies were counted and recorded.

5.3.4   Osmotic stress

Cells were pre-cultured for testing osmotic stress as stated in 5.3.1. Upon reaching an OD of 1.0 the cells are diluted to an OD_{600} of 0.05 in modified 7H9 containing sodium chloride at a specified range, 0.05% tween as well as 10 % v/v ADN. The cultures were allowed to incubate for a total of 10 days at $37^{O}C$. The *ΔgltBD* strain and in certain cases wild type *M. bovis* BCG was supplemented with 10 mM glutamate. Antibiotics (25 µg/ml gentamycin) were added to complement strains in order to maintain the plasmids during cultivation under osmotic stress. Osmotic up-shock was induced by addition of sodium chloride to the media at 0.2 mM, 0.4 mM, 0.7 mM and 1 M final concentration while standard 7H9 culture medium acts as a control. Optical density of the cultures were measured every second day to track growth over time. The OD was measured on a BMG omega microplate reader, path length correction was set to 5.88 mm with 10 flash readings per well as well as shaking at 300 rpm before reading.

5.4   Isolation of DNA

For whole genome sequencing high quality DNA was isolated from *M. bovis* BCG wild type, *M. bovis* BCG *Δgdh, M. bovis* BCG *ΔgltBD, M. bovis* BCG *Δgdh* complement, *M. bovis* BCG *ΔgltBD* complement and *M. bovis* BCG *Δgdh::*supA1; supB2; supC1; supC3 as previously described (121). Briefly, *M. bovis* BCG strains were grown on 7H11 agar plates until a bacterial lawn forms, plates containing sufficient amounts of bacteria were incubated at 80°C for one hour. Bacterial cells were dislodged from the plate by addition of 3 ml extraction buffer (5% MSG, 50 mM tris-HCl, 25 mM EDTA, pH7) and scraped into a 50 ml falcon tube (Greiner, USA) containing approximately 5 ml of glass balls with a 5 mm diameter. Any remaining bacteria were transferred to the falcon tube by the same approach as mentioned previously with an additional 3 ml of extraction buffer. Bacterial clumps were removed by method of vortex for 2 min and lysed by the addition of 500 µl lysozyme and 2.5 µl RNaseA (Roche, Germany) followed by incubation at 37°C for 2 hours. After lysis, 600 µl proteinase K buffer (Roche, Germany) and 150 µl proteinase K (Roche, Germany) was added to the falcon tube and incubated 45°C for a total of 16 hours. Organic molecules were subsequently separated by phase *via* addition of 5 ml phenol/chloroform/isoamylalcohol at a ratio of 25:24:1, this suspension was mixed by inversion in 30 minute intervals for a total of 2 hours. The tubes were subsequently centrifuged at 1800 x G for 20 minutes at room temperature to aid the separation of organic phases. The lower organic phase contains the cellular debris and proteins whilst the upper organic phase contains the desired nucleic acids. The top aqueous phase, containing the genomic DNA, was transferred to a new 50 ml falcon tube followed by DNA precipitation by addition of 600 µl sodium acetate (3 M, pH 5.5) and 7 ml of ice cold isopropanol. DNA precipitates immediately, the DNA was subsequently collected on a sterile thin glass rod then transferred to a 1.5 ml microcentrifuge tube (Axygen, USA). The microcentrifuge tubes contains 1 ml 70% ethanol and the DNA is allowed to incubate in solution for 10 minutes, the glass rod was then transferred to a fresh 1.5 ml microcentrifuge tube and allowed to dry at room temperature for 2-3 hours. DNA was dislodged from the glass rod by addition of 50 µl nuclease free water and allowed to reconstitute overnight at 4 °C.

5.5   Whole genome sequencing

5.5.1   Next generation sequencing platform

Samples of extracted genomic DNA (see section 5.4) from *M. bovis* BCG wild type, *M. bovis* BCG *Δgdh, M. bovis* BCG *ΔgltBD, M. bovis* BCG *Δgdh* complement, *M. bovis* BCG *ΔgltBD* complement and *M. bovis* BCG *Δgdh::*supA1; supB2; supC1; supC3 was submitted for whole genome sequencing. Sequencing was performed on an Illumina MiSeq platform at the University of Western Cape (South African National Bioinformatics Institute) with the use of an Illumina Miseq reagent kit V2 (Illumina, USA).

5.5.2   Bioinformatics analysis of sequencing data

Bioinformatics analysis was done by use of an in-house pipeline for the analysis of next generation sequencing data. This pipeline was constructed within the department where the study was done and is named ASAP. Briefly, all sequencing samples analysed using FASTQC to determine the quality of sequenced reads prior to trimming. Trimmomatic (184) was subsequently used to remove unwanted or low quality sequence data, a Phred score of 33 was used as is accustomed when analysing Miseq data. The trimmed sequence files were subsequently aligned to the *M. bovis* AF 2122/97 using a total of three alignment programs namely, BWA (185); Novo align (http://www.novocraft.com/) and SMALT (http://sourceforge.net/projects/smalt/). Picard tools (http://picard.sourceforge.net.) and SAMtools (http://samtools.sourceforge.net/) are used to sort the aligned sequences as well as index the sequence alignment map (SAM) files. SAM files are converted to binary alignment map (BAM) files using SAMtools and subsequently indexed. Sequences were realigned around insertion/deletion (IN/DEL) areas with the use of genome analysis toolkit version 2.7.4 and subsequently sorted with picard (http://picard.sourceforge.net). The realigned BAM files are indexed with SAMtools and PCR duplicates were removed with picard tools version 1.84. The BAM files were reindexed using SAMtools followed by single nucleotide polymorphism (SNP) and IN/DEL calling with the use of genome analysis toolkit software version 2.7.4 to generate a variant call format (VCF) file. Genome coverage statistics were generated with bed tools. All analysis and manipulations of whole genome sequences was done using linux OS.

5.5.3   Post alignment analysis of VCF files

The VCF files generated by the process explained above (Chapter 5, Section 5.5.2) contain either a set of SNP's or IN/DEL's. These variants are indicative of SNP's or IN/DELS found between each respective strain and the published *M. bovis* AF 2122/97 wild type. Each of the strains variants were subjected to a further filtering of unwanted variants by comparing the VCF files of the mutant, complement and compensatory mutation strains to that of the VCF files generated for the *M. bovis* BCG Pasteur in-house laboratory strain. For this comparison an in-house file comparison program was used to compare the VCF files in order to generate ordered excel files. These new files contain SNP's and IN/DEL's that are unique to the mutant, complement or compensatory strain and are thus not found within the laboratory wild type or published wild type strain. In order to compare the compensatory mutant strains to the knockout *gdh* strain the excel files were subsequently manipulated to regain VCF files. This was done by removing unwanted parameters within the excel files and converted to VCF which allows the comparison script to once again recognise the excel files as VCF files. The SNP's and IN/DEL's unique to *Δgdh*_SupA1, *Δgdh*_SupB2, *Δgdh*_SupC1 and *Δgdh*_SupC3 were found by rerunning the comparison script. Further filtering to the set of SNP's and IN/DEL's was applied by removing any intergenic variants, variants that were not called by Novo align, SMALT and BWA and variants that had a quality score below 300.

5.5.4   Confirmation of single nucleotide polymorphisms

Single nucleotide polymorphisms were confirmed by Sanger sequencing (inqaba biotec™). Genomic regions encompassing the polymorphism were amplified by polymerase chain reaction (PCR) using Phusion® high fidelity polymerase (NEB, USA) and the resulting amplicons were visualised by agarose gel electrophoresis (1% TAE, 120 V, 2 hours) with the use of a 1 Kb DNA ladder (Fermentas, USA).

Table 7: Primers used for the amplification of genomic regions associated with SNP's

Gene name	Primer name	Sequence	Tm
SNP's unique to GDH			
Protein kinase H	*PknH_F*	3'GCGCAGTTCTTCCATTTGGTC5'	60.4°C
	PknH_R	5'CCGCAGCCAAGAATCCA3'	59.61°C
phenolpthiocerol synthesis type-I polyketide synthase	*ppsB_F*	3'AAGCGTTAATCGCGGACTAC5'	60.4°C
	ppsB_R	5'CTTAATTGCTGCCCGGATCTT3'	60.4°C
SNP's associated with compensatory mutants			
Hypothetical Hydrolase	*Mb2823_F*	3'GAATCGTTGGACTGGTTGGA5'	60.4°C
	Mb2823_R	5'TCTATCCGTTTCAGGTGTTGTT3'	58.95°C
Polyketide synthase 7	*Pks7_F*	3'CTACGGGTGAACCGATTGT5'	60.1°C
	Pks7_R	5'AACACCGCCGGATGAAT3'	57.19°C
Possible oxidoreductase	*Mb0978c_F*	3'GTAACTTCACCGGCTCGTC5'	62.32°C
	Mb0978c_R	5'CGATTCGATCAACCACGCTA3'	60.4°C
Probable pyruvate formate lyase activating protein	*pflA_1_F*	3'TTCACCTACAACGACCCAAC5'	60.4°C
	pflA_1_R	5'ATAGCGGACCACCCTATCTTA3'	60.6°C
Probable pyruvate formate lyase activating protein	*pflA_2_F*	3'TTCACCTACAACGACCCAAC5'	60.4°C
	pflA_2_R	5'ATAGCGGACCACCCTATCTTA3'	60.61

Table 8: Cycling conditions used for the amplification of targeted genomic regions

Cycle	Temperature in Celsius	time	
Pre denaturation	98°C	30 seconds	
Denaturation	98°C	5 seconds	
Annealment	63°C	15 seconds	35 cycles
Extension	72°C	15 seconds	
Final extension	72°C	5 minutes	
Hold	4°C	∞	

5.5.5 *In silico* analysis of single nucleotide polymorphisms

The chosen SNP's (chapter 2, section 2.1) were subject to *in silico* analysis to determine whether a non-synonymous point mutation would have a significant effect on the function of the proteins. Established servers were used to assess the structural integrity through SNAP (186) and SIFT (187–

190) while changes in Gibbs free energy was calculated by I-mutant (191). Gibbs free energy calculation is used to determine whether proteins will fold in a more or alternatively, less stable tertiary structure.

5.6   Tissue culture cultivation and infection

5.6.1   Cultivation and preparation of bacterial and macrophage strains

RAW 264.7 ATCC TIB-71 macrophages were cultured in 11 ml of D10 (DMEM + FBS) using 75 cm^2 tissue culture flasks until confluent and maintained by splitting until the day of infection. Viable RAW macrophages were quantified by staining with trypanblue (1:5) and counted in a haemocytometer. Penetration of trypanblue is indicative of dead or damaged macrophages, subsequently these macrophages were omitted from the final count. Macrophages were seeded to a final total of 5 x 10^5 RAW 264.7 in 24 well plates (Nunc, Germany) containing D10. Macrophages were incubated without agitation at 37 °C, 5% CO_2 overnight to allow the cells to adhere.

Mycobacterial strains were grown to an OD_{600} of 0.8-1.00 as mentioned in section 5.2, mycobacterial cells were harvested by centrifugation (4000 rpm, 10 min, 25 °C). Mycobacterial cells were subsequently resuspended in equal volumes of D10 and adjusted to an OD_{600} of 1.0. *M. bovis* BCG wild type, mutant and complement strain was further diluted to a final concentration of 2.5×10^7/100 µl prior to infection.

5.6.2   Macrophage infection

M. bovis BCG wild type, *M. bovis* BCG *Δgdh* and *M. bovis* BCG *Δgdh* complement suspensions were added to RAW 264.7 ATCC TIB-71 cells adhered monolayers to a final MOI of 5:1 and incubated without agitation at 37 °C, 5% CO_2 for 3 hours. Supernatant was subsequently removed and replaced with fresh D10 media supplemented with penicillin/streptomycin (penstrep) antibiotic cocktail (1:100) and incubated for an additional hour at 37 °C, 5% $CO_2.$ Macrophage cells infected with *M. bovis* BCG were subsequently washed with PBS and reconstituted with fresh D10.

5.6.3   CFU determination

RAW 264.7 ATCC TIB-71 cells infected with *M. bovis* BCG wild type, *M. bovis* BCG *Δgdh* and *M. bovis* BCG *Δgdh* complement were lysed by the addition of 18 mΩ water and scraping. The infected monolayers were mixed by pipetting and serially diluted to a ratio of 1:10, dilutions 10^{-3}; 10^{-4} and 10^{-5} were plated on Middlebrook 7H9 media and incubated at 37 °C for two weeks or until colonies formed. Colonies were subsequently counted to determine CFU/ml.

To determine the percentage of log CFU/ml of one strain relative to another time 0 (T_0) counts were used. The equation for the calculation of percent log CFU/ml is as follows $[X = \left(\frac{Y_1}{Y_2}\right) 100]$. Where X is equal to percent Log CFU/ml, Y_1 is equal to a specific strain and Y_2 is equal to another. This is used to establish whether there are any differences between the number of cells initially taken up by the macrophage.

5.7   Statistical analysis

All statistical analyses were done with the statistics software GraphPad Prism 5 version 5.01 (GraphPad software). Repeated experiments were regarded as randomized block designs and assessed by repeated measures one-way or two-way ANOVA tests. Additional Bonferroni post-tests were used to compare pairs of columns. Probabilities of $P < 0.05$ were considered significant.

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Addendum A: Supplementary Tables

Table S1: Optical density of wild type *M. bovis* BCG, mutant and compliment cultures in standard 7H9.

Day	Wild type	Δgdh		ΔgltBD		Δgdh complement		ΔgltBD complement	
	Average OD	Average OD	Significance	Average OD	Significance	Average OD	Significance	Average OD	Significance
0	0.04	0.04	ns, p>0.05	0.04	ns, p>0.05	0.04	ns, p>0.05	0.04	ns, p>0.05
2	0.30	0.29	ns, p>0.05	0.21	ns, p>0.05	0.21	ns, p>0.05	0.23	ns, p>0.05
4	0.68	0.82	ns, p>0.05	0.76	ns, p>0.05	0.97	ns, p>0.05	0.83	ns, p>0.05
6	1.19	1.32	ns, p>0.05	1.28	ns, p>0.05	1.46	ns, p>0.05	1.26	ns, p>0.05
8	1.49	1.72	ns, p>0.05	1.28	ns, p>0.05	1.77	ns, p>0.05	1.91	ns, p>0.05
10	1.96	2.78	S, p<0.01	2.13	ns, p>0.05	2.17	ns, p>0.05	1.92	ns, p>0.05

OD values are indicative of three independent experiments. In some cases media was supplemented with 10 mg/ml glutamate and/or 25 µg/ml gentamycin where necessary. Significance is determined by two way ANOVA and a Bonferroni post-test where "s" is significant and "ns" is not significant. Significance was set to p<0.05. all strains were compared to wild type

Table S2: Optical density of wild type *M. bovis* BCG, mutant and compliment cultures in 7H9 supplemented with 1M NaCl.

Days	Wild type	Δgdh		ΔgltBD		Δgdh complement		ΔgltBD complement	
	Average OD	Average OD	Significance	Average OD	Significance	Average OD	Significance	Average OD	Significance
0	0.04	0.04	ns, p>0.05	0.04	ns, p>0.05	0.04	ns, p>0.05	0.04	ns, p>0.05
2	0.17	0.18	ns, p>0.05	0.17	ns, p>0.05	0.21	ns, p>0.05	0.18	ns, p>0.05
4	0.15	0.14	ns, p>0.05	0.13	ns, p>0.05	0.16	ns, p>0.05	0.14	ns, p>0.05
6	0.17	0.14	ns, p>0.05	0.15	ns, p>0.05	0.17	ns, p>0.05	0.17	ns, p>0.05

OD values are indicative of three independent experiments. In some cases media was supplemented with 10 mg/ml glutamate and/or 25 µg/ml Gentamycin where necessary. Significance is determined by two way ANOVA and a Bonferroni post-test where "s" is significant and "ns" is not significant. Significance was set to p = 0.05. All strains were compared to wild type.

Table S3: Average log CFU/ml colony counts of *M. bovis* BCG, mutant and complement strains in the presence of DETE/NO.

hours	Wild type	*Δgdh*		*ΔgltBD*		*Δgdh* complement		*ΔgltBD* complement	
	Average OD	Average OD	Significance	Average OD	Significance	Average OD	Significance	Average OD	Significance
0	5.613	5.284	ns, p>0.05	5.544	ns, p>0.05	5.325	ns, p>0.05	5.414	ns, p>0.05
6	5.434	5.145	ns, p>0.05	5.260	ns, p>0.05	5.112	ns, p>0.05	5.399	ns, p>0.05
12	5.673	4.934	ns, p>0.05	5.746	ns, p>0.05	5.419	ns, p>0.05	5.184	ns, p>0.05
24	5.635	4.697	s, p<0.05	5.534	ns, p>0.05	5.130	ns, p>0.05	4.981	ns, p>0.05
36	5.524	3.646	s, p<0.001	5.453	ns, p>0.05	4.416	s, p<0.05	4.681	ns, p>0.05
48	5.209	3.322	s, p<0.001	5.060	ns, p>0.05	4.909	ns, p>0.05	4.746	ns, p>0.05

Colony forming units are indicative of three independent experiments. In some cases 25 µg/ml Gentamycin was added to culture media. Data was analysed by two way ANOVA followed by a Bonferroni post-test. Significance was set to p = 0.05. "s" is indicated as significant while "ns" is indicated as non-significant. All strains were compared to wild type

Table S4: Percentage survival of *M. bovis* BCG wild type, mutant and complement strains when exposed to either DETE/NO or DETE/NO with previous priming of ammonium sulphate.

Strain	Condition				
	7H9	7H9 + 20 mM $(NH_2)SO_4$		7H9 + 30 mM $(NH_2)SO_4$	
	% survival	Log CFU/ml	significance	Log CFU/ml	significance
Wild type	93.29	96.99	ns, p>0.05	98.15	ns, p>0.05
Δgdh	59.70	95.82	s, P<0.001	91.54	ns, p>0.05
Δgdh comp	86.90	91.41	ns, p>0.05	92.78	ns, p>0.05

Percentage survival was calculated as mentioned previously (Chapter 5, section 5.3.2.2). Data is representative of at least two independent experiments. Data was analysed by two way ANOVA with Bonferroni post testing. All strains were compared to wild type. Significance was set to p = 0.05. "s" is indicated as significant while "ns" is indicated as non-significant.

Table S5: Average log CFU/ml colony counts of *M. bovis* BCG wild type, mutant and complement strains post infection in RAW 264.7 ATCC TIB-71 cells.

Time	Wild type	*Δgdh*		*Δgdh* complement	
	Average log cfu/ml	Average log CFU/ml	Significance	Average log cfu/ml	Significance
0	5.42	5.34	ns, p>0.05	5.43	ns, p>0.05
2	5.45	4.48	ns, p>0.05	5.18	ns, p>0.05
4	5.30	3.83	s, P<0.01	4.92	ns, p>0.05
6	5.24	3.66	s, P<0.001	4.67	ns, p>0.05

Colonies were counted after plating of washed macrophage cells as mentioned previously (chapter 5, section 5.6.1-5.6.3). Data is indicative of at least three independent experiments and was analysed
by two-way ANOVA. P was set to $p = 0.05$. All strains were compared to wild type. "s" is indicated as significant while "ns" is indicated as non-significant