

Nitrogen metabolism and the regulation thereof in *Mycobacterium smegmatis*

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Declaration

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Summary

The nitrogen metabolic pathway is essential for growth and survival of all living organisms including prokaryotes. Certain components of the pathway, such as the enzyme glutamine synthetase (GS), have been studied; however, little information is available regarding the pathway in the mycobacteria. Our *in silico* studies revealed that many of the components and mechanisms involved in the pathway appear to be conserved between closely related Actinomycetales. Therefore, we investigated three aspects of nitrogen metabolic control in *Mycobacterium smegmatis*; namely, transcriptional regulation of nitrogen metabolism-related genes, control of enzyme activity and the signalling cascade governing the nitrogen metabolic response.

At the transcriptional level, it was found that nitrogen metabolism-related genes were regulated in response to ammonium availability. Two possible transcriptional regulators, AmtR and GlnR, which are the regulators responsible for control of nitrogen-related gene transcription in *Streptomyces coelicolor* and *Corynebacterium glutamicum* respectively, were identified in *M. smegmatis*. Through generation of *amtR* and *glnR* deletion mutants, we found that both potential regulators played a role in the control of nitrogen-related gene expression in *M. smegmatis*. GlnR acted as both an activator and repressor of gene transcription whilst AmtR appeared to activate gene expression which is different to the role its homolog plays in *C. glutamicum*. On a protein level we found that both GS and glutamate dehydrogenase (GDH) were responsible for ammonium assimilation in *M. smegmatis* and were regulated in response to ammonium availability. Two GDH isoforms (NAD⁺- and NADP⁺-specific) were identified in *M. smegmatis* and whereas only an NAD⁺-GDH was detected in *M. tuberculosis*. The *M. tuberculosis* GDH also played a largely anabolic role with regard to ammonium assimilation which is in contrast to the belief that ammonium can only be assimilated via GS in this pathogen. The signaling cascade was investigated through generation of a *glnD* deletion mutant in *M. smegmatis*. We were able to show that this pivotal protein (GlnD) was able to relay the cellular nitrogen status to the transcriptional machinery as well as to GS.

The data presented in this study has advanced our understanding of the nitrogen metabolic pathway in the mycobacteria. Through elucidation of such pathways, our knowledge of mycobacterial physiology and thus infection and survival improves, which could ultimately lead to the discovery of novel mechanisms to aid in the eradication of the disease.

Opsomming

Stikstof metabolisme is noodsaaklik vir die oorlewing en groei van alle organismes, prokariote ingesluit. Sekere sellulêre komponente, soos die ensiem glutamine sintetase (GS), is al tevore bestudeer, maar baie min verdere inligting is beskikbaar oor stikstof metabolisme in die mycobacteria. Ons *in silico* studies het gewys dat baie van die komponente en meganismes gekonserveerd gebly het tussen nou-verwante Actinomycetales. Dus het ons drie aspekte in die beheer van stikstof metabolisme ondersoek; naamlik, die transkripsionele regulering van stikstof metabolisme-verwante gene, die beheer van ensiem aktiwiteit en die sein-meganisme wat die reaksie op stikstof konsentrasie reageer.

Op transkripsionele vlak het ons gevind dat stikstof metabolisme-verwante gene gereguleer word in reaksie op stikstof beskikbaarheid. AmtR en GlnR is twee moontlike transkripsie reguleerders wat verantwoordelik is vir transkripsionele beheer in onderskeidelik *Streptomyces coelicolor* en *Corynebacterium glutamicum*. Beide hierdie proteïene is geïdentifiseer in *M. smegmatis*. Deur die konstruksie van *amtR* en *glnR* mutante, het ons gevind dat beide potensiële reguleerders 'n rol gespeel het in die beheer van stikstof-verwante transkripsie in *M. smegmatis*. GlnR het opgetree as beide 'n aktiveerder en 'n onderdrukker van transkripsie terwyl AmtR net 'n aktiverende rol gespeel het. Die funksie van AmtR in *M. smegmatis* is dus verskillend van sy homolog in *C. glutamicum*. Op proteïen-vlak het ons gevind dat beide GS en glutamaat dehidrogenase (GDH) verantwoordelik was vir die assimilasië van ammonium in *M. smegmatis* en albei was gereguleer in reaksie op ammonium beskikbaarheid. Twee vorme van GDH (NAD⁺-spesifieke- en NADP⁺-spesifieke GDH) was geïdentifiseer in *M. smegmatis* terwyl net 'n NAD⁺-spesifieke GDH in *M. tuberculosis* gevind is. Die *M. tuberculosis* GDH het ook 'n anaboliesie rol gespeel met betrekking tot ammonium assimilasië wat in teenstelling is met die huidige opvatting dat ammonium alleenlik deur GS ge-assimileer kan word. Die sein-meganisme is ondersoek deur 'n *glnD* *M. smegmatis* mutant te konstrueer. Ons het bewys dat hierdie deurslaggewende proteïen (GlnD) die sellulêre stikstof status aan die transkripsionele masjinerie, en aan GS kon oordra.

Die data wat in hierdie studie voorgelê word, het ons kennis van stikstof metabolisme in die mycobacteria gevorder. Sodanige metaboliese studies verbreed ons kennis van mycobacteriële fisiologie en dus *M. tuberculosis* infeksie en oorlewing en kan uiteindelik lei tot die ontdekking van unieke teken meganismes om te help met die beheer van die siekte en nuwe middelontwikkeling.

Presentations and Publications

The author has made the following oral and poster presentations, as well as publications, of work contained in this dissertation:

1. Poster Presentations

- 1.1. Harper, C.J; Hayward, D, Wiid, I.J.F and van Helden, P.D; “Expressional analysis of candidate genes involved in mycobacterial nitrogen metabolism” Seventh International Conference on the Pathogenesis of Mycobacterial Infections, Saltsjobaden, Stockholm, Sweden 26 – 29 June 2008 and Annual Academic Year day, University of Stellenbosch Medical Faculty, 2008
- 1.2. Harper, C.J; Hayward, D, Wiid, I.J.F and van Helden, P.D; “Elucidation of the physiological roles of Glutamine synthetase and Glutamate dehydrogenase in *Mycobacterium smegmatis*” Medical Research Council, Research Day, 2009
- 1.3. Harper, C.J; Wiid, I; van Helden, P. “Identification of the transcriptional regulator of nitrogen related genes in *Mycobacterium smegmatis*” Annual Academic Year day, University of Stellenbosch Medical Faculty, 2010

2. Oral Presentations

- 2.1. Harper, C.J; Hayward, D, Wiid, I.J.F and van Helden, P.D; “Elucidation of the physiological roles of Glutamine synthetase and Glutamate dehydrogenase in *Mycobacterium smegmatis*” Annual Academic Year day, University of Stellenbosch Medical Faculty, 2009
- 2.2. Oral presentations were made at various departmental research meetings at the Department of Molecular Biology and Human Genetics, University of Stellenbosch, Medical Faculty, 2009-2011

3. Publications

- 3.1. Harper, C.J.; Hayward, D.; Wiid, I and van Helden, P. 2008. Regulation of Nitrogen Metabolism: A comparison of mechanisms in *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. IUBMB Life. Oct;60(10):643-50.
- 3.2. Harper CJ, Hayward D, Kidd M, Wiid I, van Helden P. Glutamate dehydrogenase and glutamine synthetase are regulated in response to nitrogen availability in *Mycobacterium smegmatis*. BMC Microbiol. 2010 May 11; 10:138.

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“But ask now the beasts, and they shall teach thee; and the fowls of the air, and they shall tell thee: Or speak to the earth, and it shall teach thee: and the fishes of the sea shall declare unto thee.” ~Job 12:7-8. Almighty Lord, thank you.

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List of Abbreviations

(NH₄)₂SO₄ – ammonium sulphate
Amp – ampicillan
Amp^R – ampicillan resistance
Asn – asparagine
bp – base pairs
BSA – bovine serum albumin
°C – degrees Celcius
cDNA – complementary DNA
CFU – colony forming units
cm – centimetre
Ct – threshold value
DCO – double cross over
dH₂O – double distilled water
DNA – deoxyribonucleic acid
DNase – deoxyribonuclease
dNTP – deoxynucleotide triphosphate
F – Faraday
FBS – foetal bovine serum
GDH – glutamate dehydrogenase
Glu – glutamate/glutamic acid
Gln – glutamine
GOGAT – glutamine oxoglutarate aminotransferase
GS – glutamine synthetase
hr(s) – hour(s)
HRM – high resolution melt
Hyg – Hygromycin
Hyg^R – hygromycin resistance
IPTG – isopropyl-β-D-thiogalactopyranoside
Kan – kanamycin
Kan^R – kanamycin resistance

kb - kilobase

kV - kilovolts

LA – Luria-Bertani Agar

LB – Luria-Bertani medium

μ F – micro-Faraday

M – molar

M. – *Mycobacterium*

μ g – microgram

MgCl₂ – magnesium chloride

min - minute

μ l – microliter

μ M – micro Molar

mM – millimolar

MSO - L-methionine-S-sulfoximine

NAD - nicotinamide adenine dinucleotide

NADP - nicotinamide adenine dinucleotide phosphate

ng – nanogram

NH₃ - ammonia

NH₄⁺ - ammonium

(NH₄)₂SO₄ – ammonium sulphate

OADC – oleic acid/albumin/dextrose/catalase

OD – optical density

Ω - Ohms

ONPG – o-nitrophenol- β -D-galactoside

PCR – polymerase chain reaction

PMA - phorbol 12-myristic 13-acetate

pmol - picomole

RE – restriction enzyme

RNA – ribonucleic acid

RNase – ribonuclease

rpm – revolutions per minute

RT-PCR – real time polymerase chain reaction/reverse transcriptase polymerase chain reaction

SAP – shrimp alkaline phosphatase

SB – di-Sodium tetraborate decahydrate buffer

SCO – single cross over

SDS- sodium dodecyl sulphate

sec – second

SNP – single nucleotide polymorphism

TB – tuberculosis

TCA – Tricarboxylic acid cycle

T_m – annealing temperature

U – units

V – volts

X-gal – 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

CHAPTER ONE

Introduction

NOTE: Sections of the literature review presented in the following chapter were published as:
“Regulation of nitrogen metabolism in *Mycobacterium tuberculosis*: a comparison with mechanisms in *Corynebacterium glutamicum* and *Streptomyces coelicolor*.” Harper, C.J., Hayward, D., Wiid, I., van Helden, P. *IUBMB Life*, 2008 Oct; 60(10):643-50.

1.1 Preamble:

Mycobacterium tuberculosis, the aetiological agent of the disease tuberculosis, infected approximately 9.4 million people in the year 2009, which is more than any other year recorded in history (Koul *et al.*, 2011;WHO, 2010). It is considered to be one of the most common causes of death due to an infectious agent worldwide, with approximately 1.3million deaths in HIV-negative individuals in 2009 (WHO, 2010). Tuberculosis is a disease which correlates well to socio-economic circumstances with the majority of incident cases found in low income countries, especially those in Africa, which accounted for more than 30% of incident cases, and Asia (more specifically, south-east Asia) which accounted for approximately 55% of global incident cases (Figure 1) (WHO, 2010). The current epidemic can be attributed to any number of reasons including increased urbanization, immigration, increased trade, HIV infection and globalization (Zhang, 2005). There are major confounding factors influencing the successful treatment of tuberculosis which include the aforementioned HIV/AIDS pandemic and the emergence of drug resistant *M. tuberculosis* strains. In the light of these factors the WHO declared tuberculosis a global health emergency in 1993 (WHO, 1993).

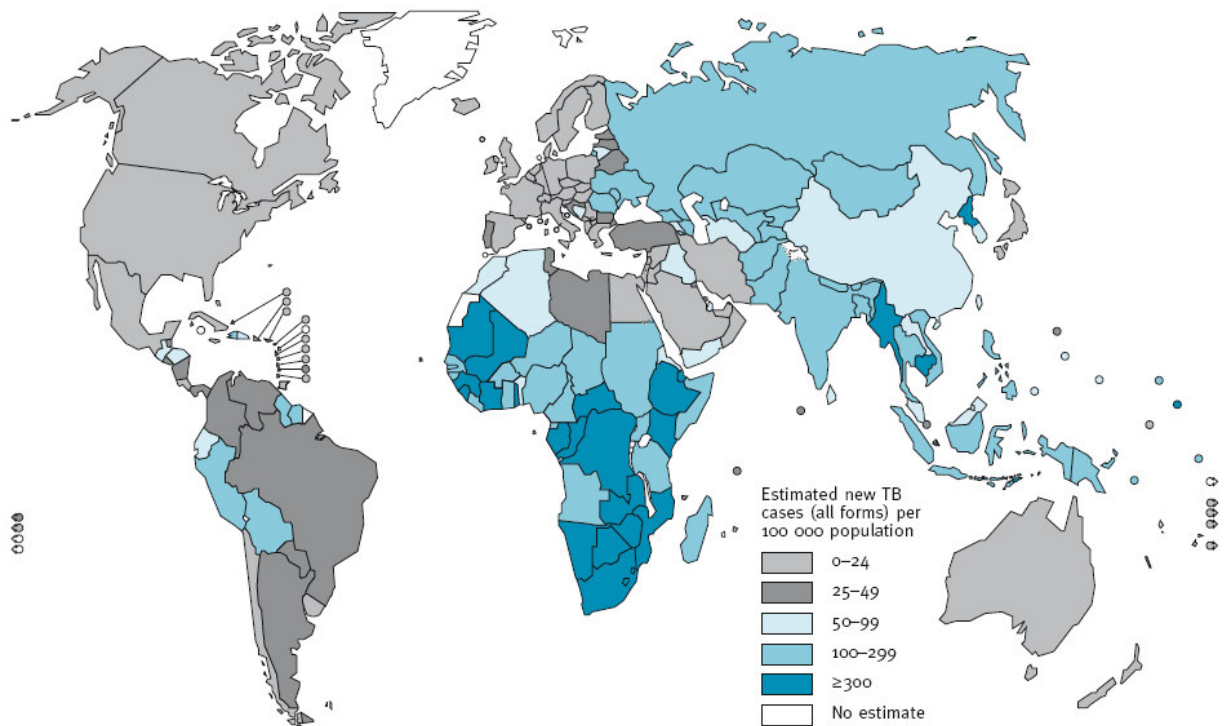


Figure 1: Estimated tuberculosis incidence cases per country in 2009. (Source: WHO, 2010 report)

1.2 A Brief History of *Mycobacterium tuberculosis*:

Tuberculosis has plagued human-kind for millennia. Evidence for the infection in the form of a gibbus as well as spinal deformities, typical of tuberculous infection of the vertebrae (also known as Potts disease or tuberculous spondylitis), was found dating as far back as pre-dynastic Egyptian mummies some 3000BC (Figure 2A) (Daniel *et al.*, 1994). The first written record of a tuberculous-type disease was formulated by a Babylonian monarch who documented a chronic lung disease in cuneiform script (the oldest known writing system) on a stone pillar approximately 2000 years before Christ. At the time of the famous Greek physician, Hippocrates (460-370BC), scholars described the most common condition of its day as ‘phthisis’ which is derived from the Greek for “wasting away”. The disease was later colloquially known as ‘consumption’ or ‘white plague’ due to the same observed effects. Although Hippocrates identified the first tubercles in animal tissues, they were at the time not connected to pulmonary phthisis and it was believed that the disease was hereditary in nature. At about the same time, Aristotle identified ‘scrofula’ (now known as tuberculous cervical lymphadenitis, Figure 2B) on the skin of pigs infected with tuberculosis which occurred as a result of inflammation of the lymph nodes in the neck. Aristotle believed that phthisis was contagious; however, it would take centuries before his theory would be recognised.

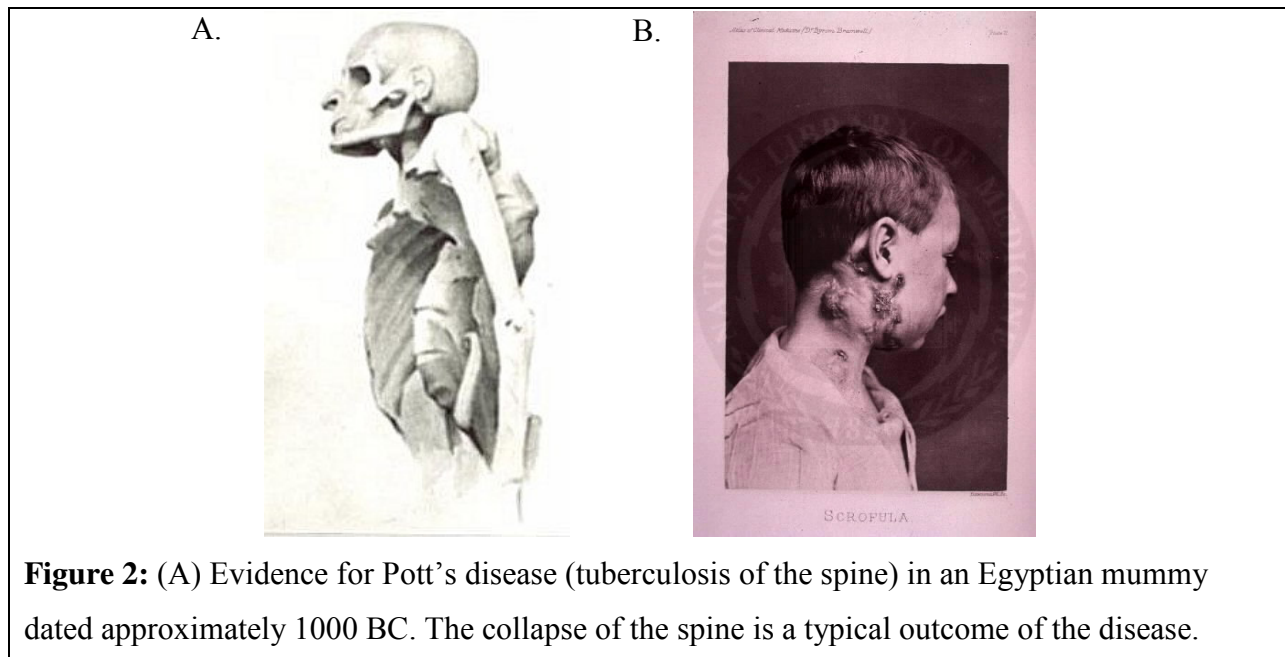


Figure 2: (A) Evidence for Pott's disease (tuberculosis of the spine) in an Egyptian mummy dated approximately 1000 BC. The collapse of the spine is a typical outcome of the disease.

(Smith and Ruffer, 1921) (B) Photograph of the condition historically known as ‘scrofula’. The swelling of the lymph nodes in the neck is attributed to *Mycobacterium tuberculosis* infection. (Source: <http://en.wikipedia.org>)

The historical period before the Renaissance saw many descriptions of phthisis by ancient Roman, Indian and Greek scholars (Herzog, 1998). With the exception of the Greek physician Galen (131-216AD) and Persian physician Avicenna (988-1037AD), who correctly deduced the contagious nature of phthisis, little advancement was made in understanding the disease during this period. The dawning of the renaissance and modern eras saw an increased following for the theory of the contagious nature of consumption as well as more accurate clinical descriptions of the disease, which may largely be attributed to the development of post-mortem techniques. In the 15th century, the Italian Fracastorius of Verona noted that consumption came from an ulcer in the lung and he proposed that the disease was transmitted by an ‘invisible virus’ which could survive in the clothing of a consumptive for up to two years. This resulted in the burning of all patients’ belongings upon their passing. In the 17th century, Franciscus Sylvius in his *Opera Medica* of 1679, finally noted that tubercles were characteristic of the lungs and other organs of consumptive patients. He also made the link between scrofula and phthisis. As a result of extraordinary insights by numerous scholars such as the physician Benjamin Marten (1704-1722BC), by end of the 17th century it was believed that consumption was contagious, that the disease may be spread by ‘small living creatures’ which when inside the body could result in the lesions and symptoms of the disease.

A rapid expansion in the knowledge of consumption occurred during the 19th century. It was during this time that consumption was called ‘the Great White Plague’ and was greatly romanticised due to the belief that sufferers had a ‘heightened sensitivity’. Women would whiten their skin with cosmetics to take on the appearance of consumptive patients. During this period, the infectious nature of the disease was scientifically demonstrated by the German, Philip Klenke (1813-1881), who took material from a tubercule and injected it into mice. The mice died of generalised tuberculosis (a term which was coined around the time to describe ‘affliction with tubercles’). In similar experiments, Jean Antoine Villemin (a French military surgeon, 1827-1892) showed that blood from consumptive rabbits, when injected into other laboratory animals,

could produce active disease. Due to the advent of microscopy in the previous century and consequent knowledge of the existence of protozoans, he thought that the disease could be caused by a single micro-organism. However, the aetiological agent of tuberculosis remained elusive and due to the myriad of symptoms, many still did not believe that it was a single disease. In 1882, Robert Koch presented to the Berlin Physiological Society for the first time his revolutionary findings that micro-organisms he named tubercule bacilli were present in the tubercule tissue. Koch was able to identify the microbes by a special staining technique he'd developed that was later adapted by Franz Ziehl and Friedrich Neelsen. He demonstrated in an experiment similar to the one performed by Philip Klenke, that the bacillus he identified (*Mycobacterium tuberculosis*), upon isolation and inoculation into lab animals, was the causative agent of tuberculosis disease. Koch was also able to generate a glycerine extract of the bacilli which he claimed had curative properties. This, alas, was untrue but he had developed 'tuberculin', a purified protein derivative which is now used in the Tuberculin Skin Test (TST) to identify infected individuals. Amongst his numerous achievements, Koch was the first to culture microbes on solid media and is considered, together with Louis Pasteur, to be one of the founders of bacteriology. He was awarded the Nobel Prize in 1905 for his tuberculosis findings.

1.3 A brief history of tuberculosis treatment:

Prior to the 19th century and discovery of the *M. tuberculosis* bacillus by Robert Koch, a phthisitic diagnosis was associated with death. Ancient attempts to treat the disease included quarantine, burning of a consumptive's clothes and possessions, a healthy diet, fresh air, milk, sea voyages, regular bloodletting and exercise (such as horse-riding), amongst others. In Medieval Europe, it was believed that consumption could be cured by the touch of a king as they were considered to have magical or curative powers due to the position bestowed upon them by divine right. The idea that fresh air and a healthy diet could treat consumption persisted well into the 19th century with the consequent introduction of sanatoria. Herman Bremmer thought that it would be best to bring consumptive patients to an 'immune place' which he described as being free of known tuberculosis cases and with the emphasis on strict rest and healthy diet regimes, it was assumed that the disease would be cured. The first sanatorium was established by Bremmer in 1856 in the Sudeten Mountains of Silesia with numerous others founded in remote locations in

subsequent years. Although the condition of the patients improved under these conditions, it was later found that the treatment was by no means curative and additional more invasive treatments were introduced. In the 1930's lung collapse therapy, artificial pneumothrax, phrenicectomy or thoracoplasty was applied to literally collapse and thereby immobilise the diseased lung to provide localised rest for the infected areas (Sharpe, 1931). These rather extreme methods of treatment often caused severe side effects and were abandoned soon after the discovery of anti-tuberculosis drugs.

Early in the 20th century, the French duo: Albert Calmette (1863 – 1933) and Camille Guérin (1872 – 1961) continued with attempts to develop a vaccine for tuberculosis. They were able to show that after more than 230 passages of *Mycobacterium bovis* on a medium consisting of potato slices and ox gall, that the bacterium was no longer able to create lesions in laboratory animals (Mahairas *et al.*, 1996). This attenuated strain was known as *M. bovis* BCG or bacille Calmette-Guérin and was first used in Paris in 1921 to immunize children against *M. tuberculosis* with great success. The vaccine became popular in the rest of Europe; however, it suffered a setback in 1930 in the form of the 'Lübeck disaster' where babies were inadvertently administered with live bacilli causing 73 fatalities. Although the protection afforded by BCG vaccination against pulmonary tuberculosis in adults is variable (Dockrell *et al.*, 2008), the efficacy of the vaccine in preventing disseminated forms of disease especially in children has resulted in an 80% global coverage today.

The middle of the Second World War saw the dawning of the anti-tubercular drug era with the discovery of streptomycin by Selman A. Waksman (1888-1973) and his student Albert Schatz (Schatz *et al.*, 1973; Waksman and Schatz, 1943). Although numerous other scientists had previously observed the inhibitory effect of metabolic products from organisms such as *Bacterium termo*, *Bacterium prodigiosum* and *Aspergillus fumigates*, they were not able to develop their ideas into a meaningful treatment application (Herzog, 1998). Waksman had been studying the inhibitory effect of certain soil fungi of the Actinomycetale bacterial group which lead to the isolation of a potent antibiotic, actinomycin, which was too toxic for human or animal use. In 1943, however, he and Schatz succeeded in isolating streptomycin from cultures of the Actinomycetale *Streptomyces griseus*. The drug was able to inhibit the tubercule bacilli and was

relatively non-toxic to humans, thus it was introduced as a therapy in 1944. For his work, Waksman was awarded the Nobel Prize in 1952 (Herzog, 1998). The following 15 years saw the rapid discovery and development of anti-tubercular compounds. These included thiosemicarbazone by Gerhard Domagk (1885-1964) in 1940 and para-aminosalicylic acid (PAS) discovered by Jorgen Erik Lehman (1898-1989) in 1946. Streptomycin was combined with PAS as a combination therapy in 1949 to combat emerging streptomycin resistant strains resulting from streptomycin monotherapy. One of the major first-line drugs, isoniazid, was developed in 1912 (Meyer and Malley, 1912) but its anti-tubercular activity was only described in the 1950's. Thereafter followed pyrazinamide (PZA, 1954), cycloserine (1955), and ethambutol (1962). Rifampin is also a first-line drug used in the treatment of tuberculosis and is derived from rifamycins which are molecules with antibiotic properties isolated from *Nocardia mediterranei*. A stable synthetic compound, rifampin (or rifampicin) was later developed by Lepetit Laboratories in Italy and introduced as a potent anti-tubercular drug in 1968 (Sensi, 1983). More recently, the aminoglycosides such as capreomycin, viomycin, kanamycin, and amikacin, and the newer quinolones (e.g. moxifloxacin, levofloxacin, ofloxacin, and ciprofloxacin) have been developed to augment anti-tuberculosis therapies.

Table 1: Daily and three-times weekly dose as recommended by the World Health Organization (WHO)*

Drug	Recommended Dose and Range (mg/kg body weight)	
	Daily	Three times weekly
Isoniazid	5 (4 – 6)	10 (8 – 12)
Rifampin	10 (8 - 12)	10 (8 – 12)
Pyrazinamide	25 (20 – 30)	35 (30 – 40)
Ethambutol	15 (15 – 20)	30 (25 – 35)
Streptomycin	15 (12 – 18)	15 (12 – 18)

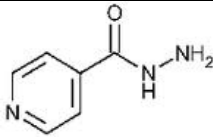
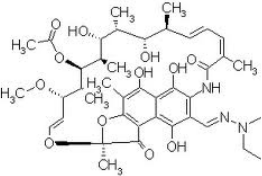
*(WHO, 2009)

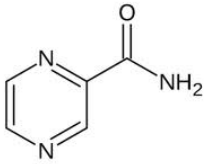
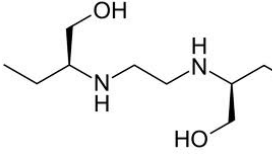
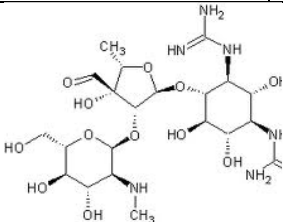
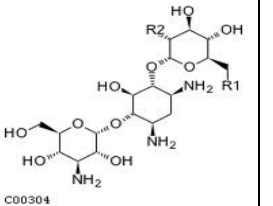
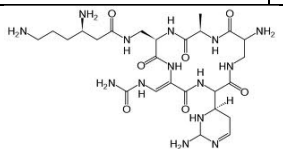
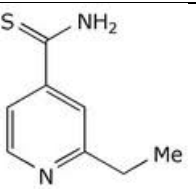
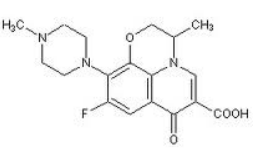
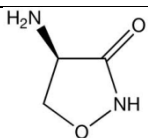
The current treatment regime for tuberculosis consists of a two-phase multi-drug approach. The multi-drug discipline was introduced to overcome the ability of *M. tuberculosis* to rapidly

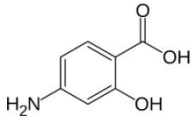
develop resistance to drugs used in mono-therapy. The first phase is an intensive one in which most (90%) of the bacilli are killed and the second phase, known as the continuation phase, is the period in which the last persistent bacilli are eradicated (Schluger, 2008). The major drug arsenal used to treat the disease are the so-called first-line drugs which display the highest bactericidal activity. These include isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin.

Isoniazid appears to be largely responsible for the rapid killing of the bacteria in the intensive phase whilst rifampin seems to target persistent bacteria during the continuation phase (Zhang, 2005). For patients infected with fully susceptible *M. tuberculosis*, a 6-month short course regimen called DOTS (Directly Observed Treatment, Short-course), recommended by the WHO, is administered which is comprised of rifampin and isoniazid, pyrazinamide and either ethambutol or streptomycin for two months followed by isoniazid and rifampin for the remaining 4 months (Table 1 and 2; Tuberculosis, a comprehensive clinical reference). This regimen has an approximate 95% success rate in curing pulmonary tuberculosis provided that patients fully comply. In the event that a patient develops drug resistant tuberculosis or is infected with a drug-resistant strain, second-line drugs (Table 2), which are generally bacteriostatic and have adverse side-effects, are employed to treat the disease in the DOTS-PLUS regime, which can last up to 24 months.

Table 2: First and second-line drugs currently used in the treatment of tuberculosis are given with their respective targets, structures, modes of action and the genes in which mutations can occur to confer resistance.

Drug		Structure	Target	Mode of Action	Genes in which mutations confer drug resistance
First-line drugs	Isoniazid		Cell wall	Activated INH inhibits mycolic acid biosynthesis.	<i>katG</i> , <i>inhA</i> , <i>ahpC</i> , <i>ndh</i> , <i>kasA</i>
	Rifampicin		RNA synthesis	Binds to RNA polymerase to prevent mRNA synthesis and consequent protein production.	<i>rpoB</i>

	Pyrazinamide		Unknown	Direct mode of action unknown, however, accumulation of the drug may cause non-specific damage.	<i>pncA</i>
	Ethambutol		Cell wall	Prevents arabinogalactan synthesis.	<i>embCAB</i> gene cluster
	Streptomycin		Translation	Binding of drug to ribosomes inhibits protein synthesis.	<i>rrs, rpsL</i>
Second-line drugs	Kanamycin and Amikacin (aminoglycosides)		Translation	Binding of drug to ribosomes inhibits protein synthesis.	<i>rrs</i>
	Capreomycin and Viomycin		Translation	Inhibits protein synthesis by binding to 30S and 50S ribosomal subunits.	<i>rrs</i>
	Ethionamide and Prothionamide		Cell wall	Pro-drug which when activated inhibits mycolic acid biosynthesis similar to isoniazid.	<i>inhA</i> promoter, <i>ethA</i> , <i>ethR</i>
	Ofloxacin and Ciprofloxacin (fluoroquinolones)		DNA structure replication	Inhibits DNA gyrase and DNA topoisomerase IV to prevent DNA supercoiling and replication.	<i>gyrA</i> , <i>gyrB</i>
	Cycloserine		Cell wall	Inhibits cell wall synthesis, however, direct mode of action is unknown.	<i>alrA</i> promoter, <i>Ddl</i>

	p- Aminosalicylic acid		Folate metabolism	The direct mode of action remains obscure but it has been shown to disrupt intracellular folate levels.	<i>thyA</i>
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1.4 The Mycobacteria:

The mycobacteria are nonmotile, rod shaped bacilli which are characterised by the presence of mycolic acids in their cell walls, by being acid-fast and by the rather rich G+C content of their genomes (61-71%) (Shinnick and Good, 1994). Although unable to be stained effectively with crystal violet, the mycobacteria are classified as Gram positive as they do not possess an outer cell membrane. Instead, they have a thick, complex, hydrophobic cell wall composed of peptidoglycan, arabinogalactan, mycolic acid and acylated trehalose layers with associated molecules such as lipoarabinomannan and mycosides (Grange, 2009). The prefix ‘myco’ is derived from Latin, meaning ‘fungus or waxy’ and in this context, the name describes the waxy appearance or texture of the bacterium which is attributed to the lipid content of their cell walls. The composition of the mycobacterial cell wall contributes to the resilience of the organism and many current therapeutic agents target cell wall biosynthetic processes.

The mycobacteria belong to the prokaryotic phylum Actinobacteria which consists principally of the so-called CMN cluster (*Corynebacteriaceae*, *Mycobacteriaceae* and *Norcardiaceae*), *Pseudonocardineae*, *Streptosporangineae*, *Streptomycineae*, and *Micrococcineae* (see figure 3). The Actinobacteria are a physiologically diverse phylum with many bacterial morphologies and metabolic pathways potentially important to man (e.g. the *Streptomycetaceae* are major antibiotic producers for pharmaceutical purposes) and the environment (e.g. decomposition and humus formation)(Gao and Gupta, 2005). In addition, members of this phylum such as *Corynebacterium diphtheria* (diphtheria), *Mycobacterium tuberculosis* (tuberculosis) and *Propionibacterium acnes* (acne) are significant human pathogens.

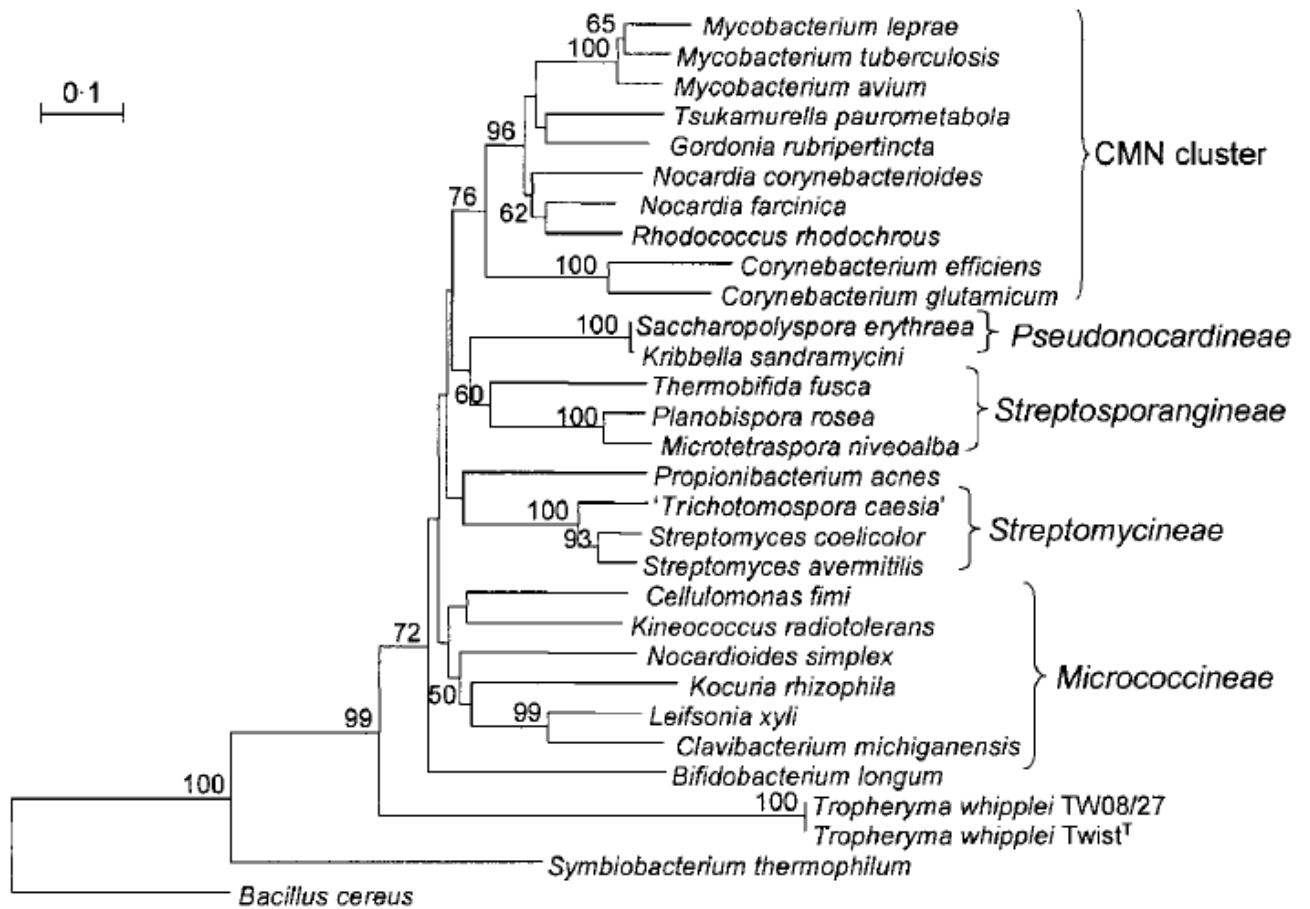


Figure 3: Evolutionary relationship between various genera of the prokaryotic Actinobacteria phylum (modified, Gao and Gupta, 2005). The *Corynebacteriaceae*, *Mycobacteriaceae* and *Norcardiaceae* belong to the CMN cluster.

Since the advent of molecular methods for identification, more than a hundred different mycobacterial species have been identified which are known to occupy a variety of habitats. The genus can be divided into those which inhabit a host (obligate pathogens) and those which live in the environment. These are often referred to as environmental mycobacteria, non-tuberculous-mycobacteria (NTM), atypical mycobacteria or ‘mycobacteria other than *M. tuberculosis*’ (MOTT). These bacteria have been isolated from water and soil sources and in the last 60 years, some have been recognised as a cause of human disease, the most common being chronic lung disease (Daley and Heifets, 2011). The *Mycobacterium* genus can also be divided into the slow-growers and the rapid-growers. The latter are generally non-pathogenic, however, they are regarded as opportunistic pathogens for humans (Daley and Heifets, 2011). The rapid-growers

are defined as those mycobacteria that are visible after 1 week of culture (e.g. *Mycobacterium smegmatis*). The slow-growers tend to be pathogenic and those that cause tuberculosis disease in mammals are grouped within the *M. tuberculosis* complex (see Table 3). Other animals such as birds (*M. avium*) frogs and turtles (*M. chelonae*) and reptiles (*M. fortuitum*) are known to be infected by mycobacterial species.

Table 3: Members of the *Mycobacterium tuberculosis* complex are given with their respective mammalian host species.

Mycobacterial species	Host
<i>M. tuberculosis</i>	Human
<i>M. bovis</i>	Cattle, deer, elk, bison, African buffalo, badger, opossum, human
<i>M. caprae</i>	Goat
<i>M. africanum</i>	Human
<i>M. microti</i>	Vole
<i>M. canetti</i>	Human
<i>M. pinnipedii</i>	Seal
<i>Dassie bacillus</i>	Dassie or hyrax

1.5 *Mycobacterium tuberculosis* infection and progression to disease:

Mycobacterium tuberculosis infection is in most cases due to the inhalation of an aerosolised droplet containing as few as one to three bacilli (Dharmadhikari and Nardell, 2009). The droplets are generally produced by respiratory movements such as coughing, sneezing, singing and speech. In rare cases, infection can occur via the gastro-intestinal tract when contaminated material such as milk is consumed. Upon contact with the mucosal surfaces of the lungs, the bacilli are phagocytosed by alveolar macrophages and a rapid inflammatory response is initiated. This innate immune response is mediated by the release of numerous cytokines and chemokines which in turn results in the recruitment of a variety of immune cells to the site of infection. If the invading pathogen is not eliminated, the specific or adaptive immune response is initiated and with time a granuloma is formed at the infection site. The granuloma is characterised by infected

macrophages surrounded by epithelioid (activated) lymphocytes and macrophages and this structure is thought to aid in the containment or localization of the infection. The granuloma may disappear and leave a small scar (or calcification) upon successful elimination of the infection by the specific immune response (Abebe *et al.*, 2011). In approximately 5% of cases, containment of the infection by the granuloma is unsuccessful and bacterial replication results in excessive macrophage death known as caseous necrosis (Hanekom *et al.*, 2007). The tissue destruction caused by necrosis can allow for the movement of *M. tuberculosis* into the lumen of the lung via cavitation (destruction of the lung lumen) (Abebe *et al.*, 2011). Once the disease has progressed to this stage, the patient is infectious and can spread the pathogen by aerosol. Extensive necrosis can also result in the migration of *M. tuberculosis* into the blood vessels (bacteraemia) which may lead to the infection of multiple organs; a condition known as miliary tuberculosis (Hanekom *et al.*, 2007). In addition, bacteraemia may lead to *M. tuberculosis* infection of the meninges which, if not controlled, can cause tuberculosis meningitis (Hanekom *et al.*, 2007). Miliary disease and tuberculosis meningitis are disseminated forms of the disease and can be particularly severe in small children.

M. tuberculosis is uniquely adapted to survive within host macrophages since they have evolved numerous mechanisms to interfere with nearly every step of the host immune response (Adebe *et al.*, 2010). Those individuals infected with *M. tuberculosis* and who did not immediately clear the infection are said to be latently infected. This is estimated to be the case for approximately one third of the world's population (Koul *et al.*, 2011). Latently infected individuals are regarded as non-infectious and asymptomatic which is a result of successful, but possibly temporary, containment of the infection by the host immune system. The bacilli probably enter a dormant state in which the bacilli are viable but non-replicating. There is, however, a risk of developing the disease arising from re-activation of the infection, which affects approximately 5% of latently infected individuals (Hanekom *et al.*, 2007). Re-activation can occur in cases where infected individuals become immuno-compromised or immune deficient, as well as the involvement of a range of pathogen-specific factors (Hanekom *et al.*, 2007).

1.6 The current tuberculosis crisis:

Not long after the introduction of chemotherapy (streptomycin) as a treatment for tuberculosis, bacilli resistant to the therapeutic agent were observed. Resistance to a drug arises from rare, spontaneous mutations that enable the bacteria to survive in its presence. This selective pressure can allow the resistant strain to become dominant in the infected host, which would result in treatment failure in the case of continued mono-therapy. The use of multi-drug therapies was established to curb the generation of drug-resistant strains, however, in recent years an increase in strains resistant to numerous drugs has occurred. This observation can be ascribed many reasons, the foremost of which is the accumulation of a series of mutations in a single strain due to the selective pressure of inadequate drug therapy (Shin *et al.*, 2008). Access to and administration of adequate therapy is a global problem and is complicated by patient non-compliance. Due to the extended time period required to treat tuberculosis and adverse side-effects from the chemotherapy, many patients (especially in resource poor settings) prematurely abort therapy which leads to the acquisition of drug-conferring mutations (Zhang, 2005). Bacteria resistant to both isoniazid and rifampin are considered to be multi-drug resistant (MDR-TB) and patients infected with these strains should be treated with a cocktail of second-line drugs in a specialised treatment regime. An alarming development is the relatively recent identification of extensively drug-resistant tuberculosis (XDR-TB). These are MDR-TB strains which are resistant to any fluoroquinolone and at least one of the injectable second-line drugs, making these strains virtually untreatable.

The HIV/AIDS pandemic has massive implications for global tuberculosis control. Individuals living with HIV are immuno-suppressed and as such are at high risk for tuberculosis. They are susceptible to the reactivation of latent bacilli and progress more rapidly to active disease after infection, a fact which has contributed to the resurgence of tuberculosis in recent years.

Tuberculosis is responsible for approximately one in four deaths that occur among HIV positive patients worldwide and it is the most common cause of death by opportunistic infection in HIV positive people in low income countries (Koul *et al.*, 2011). In addition, diagnosis of tuberculosis in HIV infected patients is made more difficult by the atypical presentation of the disease (Ginsberg, 2008). A further complication is that current tuberculosis and HIV/AIDS therapies,

when co-administered, can display intolerability and toxicity (Ginsberg, 2008). Co-infection with tuberculosis also increases the rate of HIV replication which increases the risk of mortality in these patients (Lawn and Bekker, 2009).

1.7 Nitrogen metabolism:

The challenges outlined above emphasise the necessity for new anti-tubercular chemotherapy. In the light of emerging drug resistance, therapeutic agents are required that target novel aspects of mycobacterial physiology to eliminate the possibility that existing resistance mechanisms could render any new drug ineffective. In addition, new drugs are needed which, whether administered on their own or in conjunction with existing therapies, could drastically shorten therapy time and minimize the potential for drug-drug interactions. The drugs commonly used for treatment today are generally active against growing or replicating bacilli. Phenotypically resistant bacteria include those that are in the stationary phase of growth, those that are dormant (in the case of latent infection) and those that are not killed during chemotherapy (persisters) (Zhang, 2005). We know so little about these phenomena that they may, in reality, be the same state. New drugs are required to target these bacterial populations to eliminate the possibility of re-activation of disease.

With these issues in mind, research into the physiology of the mycobacteria is essential, not only for the betterment of our understanding of the survival strategies employed by the organism but also to uncover potentially novel drug targets which could aid in current chemotherapy regimes. Of central importance to bacterial survival is the nitrogen metabolic pathway. The pathway is an essential one as it utilizes a number of specific mechanisms to assimilate nitrogen into some of the most important biological macromolecules (such as proteins, nucleic acids and cell wall constituents) of any cell. The essential nature of this metabolic pathway makes it an attractive system to study as it could provide insight into survival strategies employed by the mycobacteria as well as uncovering potentially novel drug targets.

The regulatory network governing nitrogen metabolism in the enteric bacterium, *Escherichia coli*, has been elucidated and has generally served as the prokaryotic model organism for many

years. More recently, research into the nitrogen metabolic pathway of other prokaryotes, notably those utilized for industrial purposes, has been done and it was found that diverse strategies exist between bacterial species (Amon *et al.*, 2010) with regard to both the regulation and function of the pathway. However, relatively little is known about the components of the pathway as well as the regulation thereof among members of Actinomycetale order. Of exception are two extensively studied organisms, namely: *Corynebacterium glutamicum* and *Streptomyces coelicolor*, whose nitrogen metabolic pathways are of particular interest due to their industrial application. *C. glutamicum* is a soil dwelling Actinomycetale which was identified in a screening program for L-glutamate producing microorganisms (Kinoshita *et al.*, 2004). Various strains are now well known for their ability to produce large quantities of L-glutamate (1 500 000 tonnes a year) and L-lysine (560 000 tonnes a year) as well as smaller amounts of other amino acids such as L-proline, L-isoleucine and L-alanine (Burkovski, 2006; Burkovski, 2003). The nitrogen metabolic pathway in *S. coelicolor* is of great importance as the pathway is utilized in both the formation of and fine-tuning of secondary metabolite production (Tiffert *et al.*, 2011a). This soil-dwelling, mycelium-producing Actinomycetale family is used to produce approximately two-thirds of known antibiotics in use today (Reuther and Wohlleben, 2007). In contrast, only a limited amount of work regarding nitrogen metabolism in the *Mycobacteriaceae* family has been done. Therefore, the closely related prokaryotes, *C. glutamicum* and *S. coelicolor*, have been used as models for the elucidation of the nitrogen metabolic pathway in the mycobacteria (Muller *et al.*, 2006).

Bacteria have developed complex mechanisms to regulate their nitrogen metabolic pathways in accordance with the availability of nitrogen sources in their exogenous environment. An intricate interplay between transporter proteins, signalling proteins, transcriptional regulators and effector enzymes takes place to sense the cellular nitrogen status and to direct the metabolic response when nitrogen concentrations change. In general, the metabolic pathway is regulated by two principle mechanisms; namely by control of nitrogen related gene expression and by the post-translational control of enzymes and signalling proteins involved in the pathway. These mechanisms allow for governance of the mode and rate of nitrogen uptake into the cell, control of the assimilation of various nitrogen sources via a number of different enzymes and ultimately, allow interaction with other metabolic pathways to maintain bacterial homeostasis.

1.7.1 Nitrogen uptake:

Prokaryotes are able to utilize an array of nitrogen sources which range from simple inorganic compounds such as dinitrogen and nitrate to complex organic compounds such as urea and amino acids (Merrick and Edwards, 1995). A variety of proteins are synthesized by bacteria to facilitate the transport and metabolism of these nitrogen sources for the generation of ammonium (NH_4^+), which is necessary for the biosynthesis of the required nitrogen-containing biological macromolecules. Ammonium, therefore, is the end product of the catabolism of nitrogen sources and, on its own, can sustain a higher rate of growth than any other nitrogen-containing compound, which makes it the favoured nitrogen source of most bacteria (Dodsworth and Leigh, 2007; Merrick and Edwards, 1995). Ammonia (NH_3) in its non-ionized form is lipophilic and can easily diffuse across cell membranes, provided that an ammonia concentration gradient exists across the membrane. The diffusion of ammonia into the cell is, however, not enough to support growth when environmental ammonia conditions become limiting and in this case, specialised transmembrane ammonium carriers are synthesised. These transporters belong to the Amt/Mep protein family and seem to rely on membrane potential to carry ammonium into the lumen of the cell (Van Drommelen *et al.*, 2001). Proteins belonging to the Amt/Mep family have been identified in all major phylogenetic kingdoms (Van Drommelen *et al.*, 2001) and most prokaryotic genomes encode for at least one *amt* gene.

Similarly to *E. coli*, the *C. glutamicum* genome encodes for two Amt/Mep paralogues (Meier-Wagner *et al.*, 2001; Jakoby *et al.*, 1999). It was shown that AmtA functions as a methylammonium transporter whereas AmtB acts as an ammonium-specific permease (Meier-Wagner *et al.*, 2001). BLAST analyses revealed that the *M. smegmatis* genome also encodes for both *amtA* and *amtB* homologues, whereas the *S. coelicolor* and *M. tuberculosis* genomes only have an *amtB* gene. The *amtB* gene is located in an apparent operon together with *glnK* and *glnD*, which is a highly conserved genetic arrangement among the mycobacteria (Amon *et al.*, 2008b). The genetic association between *amtB* and *glnK* is more widespread and can be found in a diverse range of Archaea and Eubacteria (Thomas *et al.*, 2000). In addition, this genetic association implies a functional relationship, which was confirmed and is explained in section

1.7.3 below. The expression of *amtA* and the *amtB-glnK-glnD* operon is strictly regulated in response to nitrogen availability in both *C. glutamicum* and *S. coelicolor* (Fink *et al.*, 2002; Jakoby *et al.*, 2000). The transcription of these genes is down-regulated during nitrogen sufficiency to prevent an energy wasting, futile cycle of active ammonium transport into the cell, counteracted by passive ammonium diffusion of ammonium out of it (Meier-Wagner *et al.*, 2001). Conversely, upon nitrogen limitation, transcription of the ammonium transporter genes is up-regulated to synthesize additional permeases which are incorporated into the cellular membrane to scavenge ammonium from the extracellular milieu.

1.7.2 Nitrogen assimilation:

Intracellular ammonium is assimilated into glutamate and glutamine which are the major nitrogen donors for biosynthetic reactions. Glutamate is the substrate for most transaminases (Reitzer, 2003) and is a source for the production of approximately 85% of nitrogen-containing compounds within a cell (Fisher, 1999). Glutamine, on the other hand, provides nitrogen for the production of purines, pyrimidines, glucosamine, ρ -benzoate and a variety of amino acids (Reitzer, 2003). There are two main mechanisms through which ammonium assimilation occurs, namely via the glutamine synthetase (GS)/glutamate synthase (glutamine oxoglutarate aminotransferase or GOGAT) pathway and/or via glutamate dehydrogenase (GDH).

1.7.2.1 GS/GOGAT:

Glutamine synthetase enzymes (EC 6.3.1.2) are ubiquitous and occur in at least four forms which are classified according to the number of subunits present and whether or not the enzyme is post-translationally regulated. GS type-1 (GSI) are prokaryote-specific enzymes which have 12 subunits, are post-translationally modified and occur in two possible isoforms, namely the GSI- α and - β subtypes. GS type-2 (GSII) enzymes are octamers which are largely associated with eukaryotic organisms, however, they have been identified in some bacterial genera as well e.g. *Streptomyces*, *Agrobacterium* and *Rhizobium* (Merrick and Edwards, 1995). A hexameric GS type-3 (GSIII) has been isolated from *Bacteriodes fragilis* and homologous sequences identified in *Butyrivibrio fibrisolvens* and *Synechocystis* strain PCC 6803 (Merrick and Edwards, 1995). An

octomeric GS type-4 (GSIV) has been identified in *Rhizobium leguminosarum* and *Rhizobium meliloti* and appears to be very different from previously described GS enzymes (Merrick and Edwards, 1995; Shatters *et al.*, 1993). In *C. glutamicum*, both GSI- α (GlnA2) and - β (GlnA1) have been characterized and it was found that GlnA1 is subject to post-translational regulation and is essential for *in vitro* survival (Jakoby *et al.*, 1997). In contrast, GlnA2 did not appear to contribute to ammonium assimilation as GS activity could not be detected in the absence of GlnA1, and it was determined that the enzyme is not essential for *in vitro* bacterial survival (Nolden *et al.*, 2001a). The physiological role of GlnA2 remains unclear. The genomes of both *S. coelicolor* and *M. tuberculosis* encode for four homologous GSI type proteins and, similar to *C. glutamicum*, it was found that GlnA1 was essential for the *in vitro* growth and survival of these organisms (Rexer *et al.*, 2006; Harth *et al.*, 2005).

The GS/GOGAT (from here forth GS refers to GlnA1, which is described above) system is a tightly regulated cyclic mechanism which is largely active when exogenous nitrogen concentrations are limiting. GS has a high affinity for its substrate, ammonium, and is the only effective mechanism available for ammonium assimilation when exogenous ammonium concentrations are less than 0.1mM (Merrick and Edwards, 1995). GS catalyses the production of glutamine from glutamate and ammonium, whereas GOGAT converts glutamine to glutamate (Figure 4). The system is regulated by transcriptional and post-translational mechanisms owing to the energy/ATP cost associated with the formation of glutamine by GS. For every mole of glutamine produced, one mole of ATP is consumed and it is thought that GS utilizes approximately 15% of the cellular ATP requirement (Reitzer, 2003). Therefore, in order to prevent a futile energy wasting cycle under conditions of ammonium excess, GS activity is down-regulated via the reversible adenylylation of GS subunits at a conserved tyrosine residue by the adenylyltransferase, GlnE in many organisms including *E. coli* (Woolfolk *et al.*, 1966), *C. glutamicum* (Nolden *et al.*, 2001a), *S. coelicolor* (Fink *et al.*, 1999) and *M. tuberculosis* (Pashley *et al.*, 2006). Adenylylated GS is able to catalyse the transferase reaction in which a glutamyl moiety from glutamine is transferred to hydroxylamine to produce γ -glutamylhydroxamate and ammonium. This reaction mechanism is often used in an assay to quantify GS activity (Stadtman *et al.*, 1979). In addition to adenylylation, GS activity can be regulated by oxidative modification (Fisher and Stadtman, 1992), by negative feedback of various products of glutamine metabolism (Woolfolk and Stadtman, 1967) as well as via control of *glnA1* expression (Jakoby *et al.*, 1997; Harth *et al.*, 1994; Fisher and Wray, Jr., 1989).

GS has formed a focal point of study with regard to nitrogen metabolism in *M. tuberculosis* since the enzyme has been associated with virulence and pathogenicity of the organism (see section 1.7.6). However, little is known about the physiological role and regulation of GS in other mycobacteria such as *M. smegmatis*. In contrast to GS regulation, there is little evidence of post-translational control of GOGAT activity and it would appear that transcriptional control is the main regulatory mechanism governing the enzyme's activity (Beckers *et al.*, 2001).

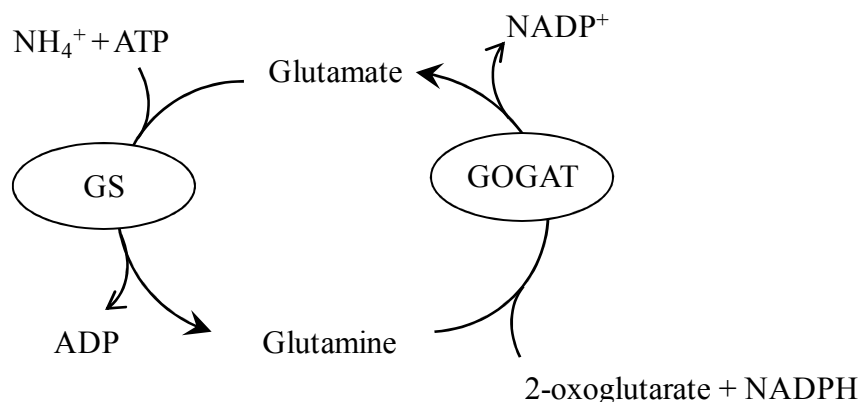


Figure 4: Energy dependant assimilation of ammonium via the cyclic glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway.

1.7.2.2 Glutamate dehydrogenase:

In addition to GS, ammonium can be assimilated via the reversible amination of 2-oxoglutarate to glutamate by GDH enzymes with a comparatively low energy cost. The disadvantage, however, is that GDH has a relatively low affinity for ammonium (K_m of approximately 1mM) in comparison with GS and, in many bacteria, is primarily active when exogenous ammonium is abundant (Merrick and Edwards, 1995). GDH assimilates ammonium by catalyzing the reversible amination of 2-oxoglutarate to form glutamate with the concomitant reduction of its cofactor, NAD(P)H (Figure 5). The enzymes also play a role in anapleurotic processes as they act as metabolic branch enzymes which can modulate the flux of intermediates such as 2-oxoglutarate between the Krebs cycle and nitrogen metabolism (Britton *et al.*, 1992).

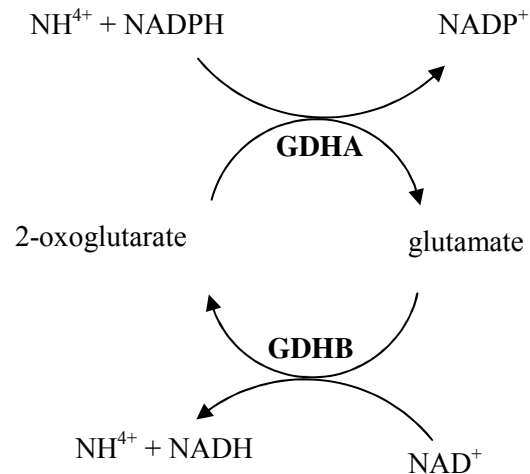


Figure 5: Schematic representation of the reaction mechanisms catalysed by the NADP⁺-specific glutamate dehydrogenase (GDHA) and the NAD⁺-specific glutamate dehydrogenase (GDHB). (Source: Harper *et al.*, 2010)

The GDH enzymes identified in prokaryotes usually function with either NADP⁺ (EC 1.4.1.4) or NAD⁺ (EC 1.4.1.2) as co-factors, whilst in higher eukaryotes the enzymes have dual co-factor specificity (Minambres *et al.*, 2000). GDH's utilizing NADP⁺ as co-factors are considered to be anabolic enzymes since they are primarily involved in the assimilation of ammonium via amination of 2-oxoglutarate for the production of glutamate (Duncan *et al.*, 1992) (Figure 5). These enzymes appear to be regulated in response to a number of growth conditions which include carbon and nitrogen limitation (Hanssler *et al.*, 2009; Antonopoulos *et al.*, 2003; Schwacha and Bender, 1993). In contrast, NAD⁺-specific GDH's are thought to perform a predominantly catabolic role as they catalyze the breakdown of glutamate to 2-oxoglutarate and ammonium (deamination) (Consalvi *et al.*, 1991; Miller and Magasanik, 1990; Rice *et al.*, 1985) (Figure 5). These enzymes do not appear to be regulated by ammonium limitation (Chavez and Candau, 1991; Stuart Shapiro, 1989). The GDH enzymes described thus far are multimeric proteins which can be divided into three groups according to their ultrastructures. A large number of NADP⁺ and NAD⁺-specific GDH's from a variety of organisms are made up of six subunits which are approximately 50kDa in size (Britton *et al.*, 1992). A subset of NAD⁺-specific GDH's belong to a second class of enzymes which are tetrameric structures whose subunits have an approximate molecular mass of 115kDa (Veronese *et al.*, 1974). More recently, a third class was identified (L₁₈₀ class)

comprising NAD⁺-specific GDH's, whose subunit size is approximately 180kDa (Minambres *et al.*, 2000). Enzymes belonging to the L_180 class have been described in *Streptomyces clavuligerus* (Minambres *et al.*, 2000), *Pseudomonas aeruginosa* (Lu and Abdelal, 2001), *Psychrobacter* sp. TAD1 (Camardella *et al.*, 2002) and *Janthinobacterium lividum* (Kawakami *et al.*, 2007) however, the physiological roles of these enzymes have not been well characterized. Due to the oligomeric structure, subunit size and hierarchical homology, four different classes of GDH exist; namely the α_6 -50_I and α_6 -50_{II} class of small GDHs and the α_6 -115 and α_6 -180 class of large GDHs.

In *C. glutamicum*, an NADP⁺-specific GDH is present and has been shown to play a largely anabolic role with regard to nitrogen assimilation (Bormann *et al.*, 1992). The gene encoding NADP⁺-GDH in *C. glutamicum* also appears to be regulated by a variety of conditions including ammonium availability (Hanssler *et al.*, 2009). The *S. coelicolor* genome, on the other hand, encodes for both an L_180 class NAD⁺-specific GDH as well as an NADP⁺-specific GDH (Minambres *et al.*, 2000). Activity of the latter enzyme was detected and found to be regulated in response to ammonium concentrations, similar to the NADP⁺-GDH found in *C. glutamicum* (Fisher, 1989). The *M. smegmatis* genome also encodes for both an NADP⁺-GDH and a L_180 class NAD⁺-specific GDH enzyme which is in contrast to *M. tuberculosis* which has a sequence for a L_180 class NAD⁺-specific GDH only (Harper *et al.*, 2010). Very little is known about GDH enzymes in the mycobacteria as a group and the physiological significance of the presence of both an anabolic- and a catabolic-type GDH within a single organism remains unclear.

1.7.3 Signalling:

In most bacteria, the co-ordination of post-translational and transcriptional regulation of nitrogen metabolism is controlled by small, highly conserved, trimeric signalling proteins known as PII proteins (Arcondeguy *et al.*, 2001). In enteric bacteria such as *E. coli*, two PII proteins known as GlnK and GlnB are present and are uridylylated (GlnB/K-UMP) at a highly conserved tyrosine residue (Tyr51) by another cascade protein, GlnD, in response to nitrogen limitation (Jiang *et al.*, 1998). GlnB-UMP signals nitrogen limitation to GlnE (Arcondeguy *et al.*, 2001) which, in turn, de-adenylylates GS to increase its activity and allow nitrogen assimilation under those conditions. In addition, GlnB-UMP is able to interact with NtrB, a sensory histidine kinase in *E. coli* which forms part of a two-component signal

transduction system in this organism to regulate transcription of nitrogen-related genes (see section 1.7.4). GlnK has been shown to be able to complement GlnB function, but less effectively (Reitzer, 2003; Arcondeguy *et al.*, 2001). When nitrogen sufficient conditions return, GlnD de-uridylylates GlnB and GlnK in order to down-regulate GS activity and modulate gene transcription.

Both *C. glutamicum* and *S. coelicolor* possess only a single signal transduction protein, GlnK, whose encoding gene (*glnK*) is located in an operon together with the genes transcribing the ammonium transporter AmtB (*amtB*) and the cascade protein, GlnD (*glnD*). In contrast to the enteric bacteria, GlnK has been shown to be reversibly adenylylated rather than uridylylated by GlnD in response to nitrogen limitation in *C. glutamicum* (Strosser *et al.*, 2004) and *S. coelicolor* (Hesketh *et al.*, 2002). In *E. coli* and *C. glutamicum* it has been shown that native or un-adenylylated GlnK remains bound to the ammonium transporter AmtB to prevent unnecessary transport of ammonium into the cell under conditions of nitrogen sufficiency (Javelle *et al.*, 2004; Strosser *et al.*, 2004). Upon exposure of the bacteria to nitrogen starvation conditions, GlnK is adenylylated by GlnD and released from AmtB to enable enhanced uptake of ammonium. In *C. glutamicum*, adenylylated GlnK (GlnK-AMP) is subsequently relocated to the cytoplasm where it interacts with the global regulator of nitrogen-related gene transcription, AmtR, to remove the DNA-bound repressor and allow transcription to occur (Strosser *et al.*, 2004; Nolden *et al.*, 2001b; Jakoby *et al.*, 2000) (see section 1.7.4.1). The sequence of events is reversed under conditions of nitrogen excess: GlnK is de-adenylylated by GlnD and is subsequently bound to AmtB whilst allowing transcriptional repression by AmtR (Figure 6). In *S. coelicolor*, the reversible adenylylation of GlnK by GlnD also occurs during nitrogen starvation (Hesketh *et al.*, 2002), however, the physiological significance of this event has yet to be described as there is little evidence of the involvement of the GlnD/GlnK signalling cascade in transcriptional regulation in this organism.

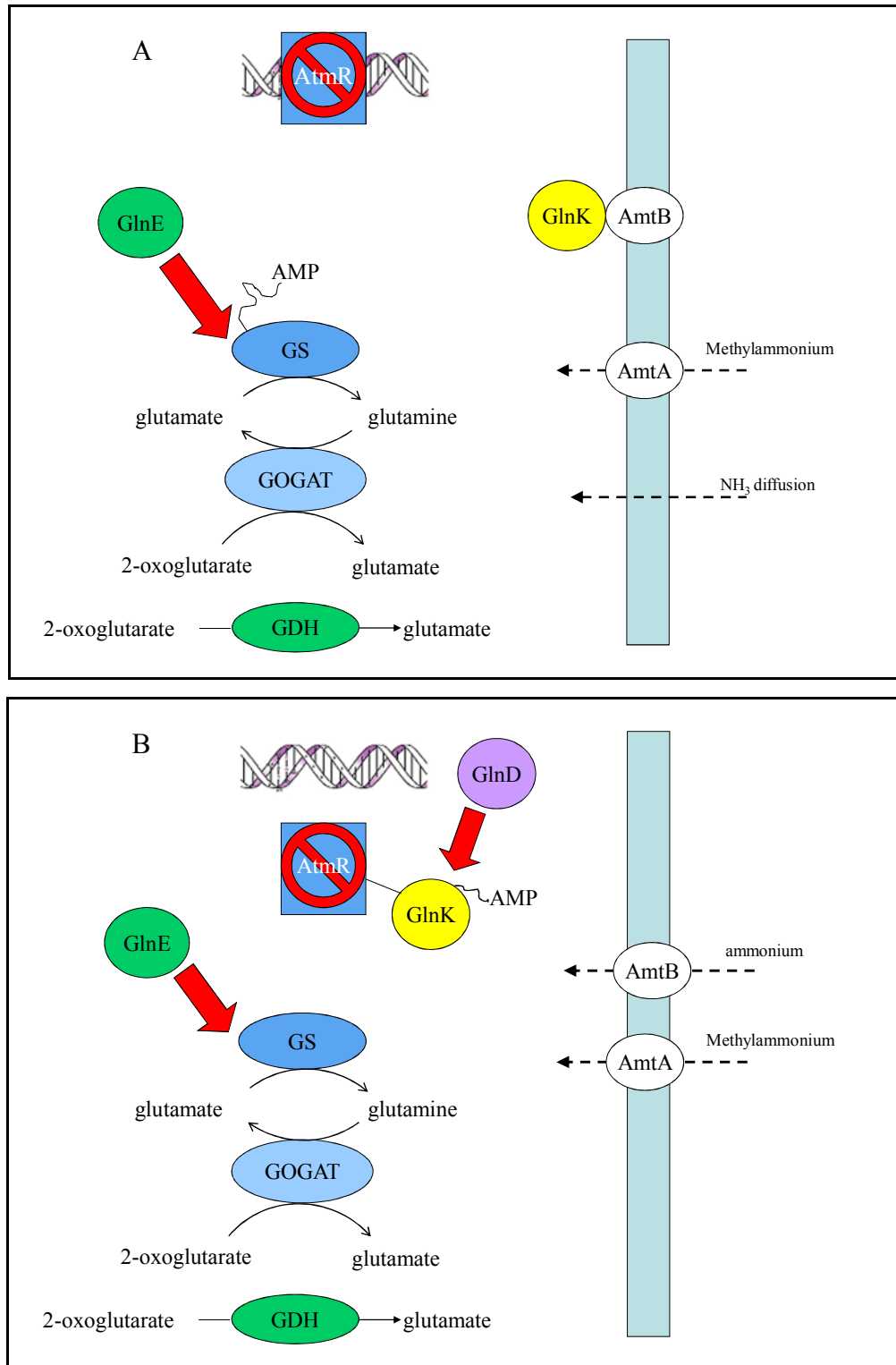


Figure 6: Cascade of events which occur during uptake and regulation of nitrogen in *C. glutamicum* under conditions of (A) nitrogen excess and (B) nitrogen limitation. During

nitrogen excess (A) ammonia enters the cell by passive diffusion and is assimilated via GDH pathway as glutamine synthetase activity is down-regulated by adenylylation by GlnE. The PII protein, GlnK, remains bound to AmtB to prevent facilitated transport of ammonium into the cell. AmtR remains bound to target DNA sequences to prevent transcription of nitrogen controlled genes. During nitrogen starvation, (B) the cellular nitrogen status is recognised by an unknown sensory mechanism and GlnD is activated to adenylylate GlnK. The GlnK-AMP is released from AmtB allowing facilitated transport of ammonium into the cell. GlnK-AMP binds AmtR and removes the transcriptional repression of nitrogen controlled genes. GlnE is signalled to deadenylylate glutamine synthetase via an unknown mechanism which allows ammonium assimilation via the high affinity GS/GOGAT pathway. (Source: Harper *et al.*, 2008)

Unlike enteric bacteria, the regulation of GS activity requires neither GlnK nor GlnD in either *C. glutamicum* (Burkovski, 2003) or *S. coelicolor* (Hesketh *et al.*, 2002), which indicates that cellular nitrogen status is not transferred to GlnE via the GlnK/GlnD system. The implication is that GlnE in actinomycetes is different from that of enteric bacteria and may be post-translationally modified by an unknown mechanism or that GlnE may act as a sensor of nitrogen availability. *M. smegmatis* and *M. tuberculosis* have, as do *C. glutamicum* and *S. coelicolor*, genes encoding for a single PII protein, *glnK* which is located in a operon together with *amtB* and *glnD* (Harper *et al.*, 2008). It has yet to be determined whether GlnD performs a reversible uridylylating (as in enteric bacteria) or adenylylating (as in *S. coelicolor* and *C. glutamicum*) function in the mycobacteria. Read *et al.*, (2007) generated an *M. tuberculosis glnD* knockout mutant, and it could be deduced from experimental work that a similar GlnD/GlnK-independent type of regulation of GS activity occurs compared to the Actinomycetale model organisms. Aside from these deductions, the physiological events that follow GlnK modification by GlnD in the mycobacteria are poorly understood.

1.7.4 Transcriptional regulation:

The increase in expression of approximately 100 genes in response to nitrogen limitation in *E. coli* is known as the nitrogen-regulated response (Ntr) which is largely governed by a two-component signal transduction system (Reitzer, 2003). Depending on the PII uridylylation state, the signaling proteins are able to interact with NtrB, a sensory histidine kinase which is

stimulated to phosphorylate and/or dephosphorylate NtrC, a σ^{54} -dependant transcription factor which regulates gene transcription (Reitzer, 2003). Under conditions of nitrogen sufficiency NtrB interacts with unmodified GlnB/K which is then stimulated to dephosphorylate NtrC and thereby prevent DNA binding and ensuing transcription. When nitrogen becomes limiting, the interaction between NtrB and GlnB/K is abolished by PII uridylylation, resulting in the phosphorylation of NtrC by NtrB which permits DNA binding and subsequent activation of gene transcription (Leigh and Dodsworth, 2007; Arcondeguy *et al.*, 2001). Although two-component signal transduction systems do exist in the Actinomycetes, a very different mode of nitrogen-related transcriptional control is found in these prokaryotes.

1.7.4.1 In *Corynebacterium glutamicum*:

Jakoby *et al.*, (2000) found that a global or master regulator was responsible for the transcriptional repression of nitrogen-related genes in *Corynebacterium glutamicum* under conditions of nitrogen surplus. The regulator was identified as belonging to the TetR-type family of transcriptional repressors with a signature helix-turn-helix DNA binding motif (Ramos *et al.*, 2005), and was designated AmtR (Jakoby *et al.*, 2000). The DNA sequence upstream of regulated genes, to which AmtR binds (termed the AmtR binding box) was characterized and found to consist of a palindromic sequence separated by up to 4 variable nucleotides (tttCTATAN₄AtAGt) (Beckers *et al.*, 2005; Jakoby *et al.*, 2000). The signaling cascade is essential for the adaptation of the transcriptional response to varying nitrogen availability conditions in *C. glutamicum* (Nolden *et al.*, 2001b). GlnK is adenylylated by GlnD during nitrogen limitation, which in turn interacts with AmtR to remove the repressor from its binding box and thereby allowing transcription to occur (Figure 6)(Beckers *et al.*, 2005). A number of studies investigating nitrogen-related gene expression in *C. glutamicum*, have allowed the characterization of the AmtR regulon (Buchinger *et al.*, 2009; Beckers *et al.*, 2005) which includes at least 36 genes which have been shown to be repressed when nitrogen excess conditions prevail (see Table 4 for a selection of genes belonging to the AmtR regulon).

Table 4: Genes encoding the major proteins involved in nitrogen metabolism in *C. glutamicum*, *S. coelicolor*, *M. tuberculosis* and *M. smegmatis*.

Transcriptional regulation of genes in *C. glutamicum* and *S. coelicolor* by the global transcriptional regulators, AmtR and GlnR respectively, are also indicated.

Protein	Description	Gene in <i>C. glutamicum</i>	Transcriptional regulation in <i>C. glutamicum</i>	Gene in <i>S. coelicolor</i>	Transcriptional regulation in <i>S. coelicolor</i>	Gene in <i>M. tuberculosis</i>	Gene in <i>M. smegmatis</i>
AmtA	methylammonium permease	<i>cg1785</i>	yes	-	-	-	<i>msmeg_6259 (putative)</i>
AmtB	high affinity ammonium permease	<i>cg2261</i>	yes	<i>SCO5583</i>	yes	<i>Rv2920c (putative)</i>	<i>msmeg_2425 (putative)</i>
GlnK	PII-type signal transduction protein	<i>cg2260</i>	yes	<i>SCO5584</i>	yes	<i>Rv2919c (putative)</i>	<i>msmeg_2426 (putative)</i>
GlnD	adenylyltransferase	<i>cg2258</i>	yes	<i>SCO5585</i>	yes	<i>Rv2918c (putative)</i>	<i>msmeg_2427 (putative)</i>
GlnE	adenylyltransferase	<i>cg2446</i>	no	<i>SCO2234</i>		<i>Rv2221c</i>	<i>msmeg_4293 (putative)</i>
GlnA	glutamine synthetase type-1	<i>cg2429</i>	yes	<i>SCO2198</i>	yes	<i>Rv2220</i>	<i>msmeg_4290</i>
NAD ⁺ - GDH	NADP-specific glutamate dehydrogenase	<i>cg2280</i>	yes	<i>SCO4683 (putative)</i>	yes	-	<i>msmeg_5442</i>
NAD ⁺ - GDH	NAD-specific glutamate dehydrogenase	-	-	<i>SCO2999 (putative)</i>	Not known	<i>Rv2476c (putative)</i>	<i>msmeg_4699 (putative)</i> <i>msmeg_6272 (putative)</i>
AmtR	TetR-type repressor	<i>cg0986</i>	no	-	-	<i>Rv3160c (putative)</i>	<i>msmeg_4300 (putative)</i>
GltB	glutamate synthase (large subunit)	<i>cg0229</i> or <i>(cgR_0263)</i>	yes	<i>SCO2026</i>		<i>Rv3859c</i>	<i>msmeg_3225 (putative)</i>
GltD	glutamate synthase (small subunit)	<i>cg0230</i> or <i>(cgR_0264)</i>	yes	<i>SCO2025</i>		<i>Rv3858c</i>	<i>msmeg_3226 (putative)</i>
GlnR	OmpR-type regulator	-	-	<i>SCO4159</i>	yes	<i>Rv0818c (putative)</i>	<i>msmeg_5784 (putative)</i>
UreA	Urease gamma subunit	<i>cg0113</i>	yes	<i>SCO1236 (putative)</i>	Yes	<i>Rv1848</i>	<i>msmeg_3627</i>
NirB	Nitrite reductase large subunit	-	-	<i>SCO2487 (putative)</i>	Yes	<i>Rv0252</i>	<i>msmeg_0427</i>

1.7.4.2 In *Streptomyces coelicolor*:

An OmpR-type regulator with a typical winged helix-turn-helix motif, designated GlnR, was found to regulate the transcription of nitrogen-related genes in *S. coelicolor* (Wray, Jr. and Fisher, 1993; Wray, Jr. *et al.*, 1991). Transcriptional studies of *glnR* mutant strains have shown that GlnR directly regulates the expression of at least 12 genes in this organism which includes well known nitrogen metabolism-related genes (Table 4) as well as some with unknown or hypothetical functions (Tiffert *et al.*, 2008). GlnR has been shown to both activate (*glnA*, *glnII*, *nirB*, *amtB*) and repress (*gdhA*, *ureA*) transcription under conditions of nitrogen limitation, however the transcriptional response to nitrogen excess is not known. More recently, an analysis of protein expression by two-dimensional polyacrylamide gel electrophoresis in an *S. coelicolor glnR* mutant strain was done and it was found that more than 50 proteins were differentially regulated in comparison to the wild type strain under conditions of nitrogen limitation (Tiffert *et al.*, 2011b). Many of these proteins are implicated in carbon and amino acid metabolism as well as nitrogen metabolism which suggests that GlnR plays a global metabolic role in *S. coelicolor*.

The signal communicating nitrogen starvation or excess condition to GlnR remains obscure. It is known that GlnK in *S. coelicolor* is adenylylated by GlnD; however it is not known whether GlnK-AMP relays the message to GlnR. Typical OmpR-type regulators are most often subject to phosphorylation by a genetically linked kinase sensor (similar to NtrB/C in *E. coli*) (Reuther and Wohlleben, 2007). The gene encoding for GlnR in *S. coelicolor* does not appear to be co-localized with a sensory kinase gene, yet a potential phosphorylation site has been identified in the GlnR sequence (Asp⁵⁰) (Wray, Jr. and Fisher, 1993). Thus GlnR may be regulated by a sensory kinase which is transcribed from a genetic location distant from its own (Reuther and Wohlleben, 2007).

1.7.4.3 In the mycobacteria:

Up until recently, the components playing a role in the transcription of nitrogen related genes in the mycobacteria have remained elusive. A study conducted by (Tiffert *et al.*,

2008) showed that *S. coelicolor* GlnR could bind to a conserved sequence upstream of a number of genes involved in nitrogen metabolism in most Actinomycetales including *M. tuberculosis*. A putative GlnR regulator is encoded by both the *M. tuberculosis* and *M. smegmatis* genomes which share 55% (MSMEG_5784) and 65 % (Rv0818) amino acid identity with the *S. coelicolor* GlnR respectively. It was also shown that GlnR appears to be conserved among the Actinomycetales with the exception of *Corynebacterium glutamicum* (Amon *et al.*, 2010). It was demonstrated through deletion of the putative GlnR homolog in *M. smegmatis*, that the protein was responsible for the transcriptional regulation of some of the genes involved in nitrogen metabolism in this organism (e.g. *glnA* and *amtB*)(Amon *et al.*, 2008a). However, there appears to be far fewer genes regulated by GlnR in *M. smegmatis* compared to the collection of genes controlled by GlnR in *S. coelicolor* (Tiffert *et al.*, 2008). A GlnR binding site upstream of a number of other genes involved in nitrogen metabolism in *M. smegmatis* could not be identified which included genes encoding for glutamate dehydrogenase, the urea operon and nitrite reductase and the GOGAT operon (Amon *et al.*, 2008b). A putative TetR-type regulator (Rv3610c) was identified in *M. tuberculosis* that shared only a 27.9% homology with the AmtR repressor in *C. glutamicum* which suggested that the existence of this protein in *M. tuberculosis* is unlikely. However, the genome of *M. smegmatis* does encode an AmtR-type regulator which shares a 42% amino acid identity with *C. glutamicum* AmtR. Similarly to *M. smegmatis*, the genomes of other closely related Actinomycetales such as *Nocardia farcinica*, *Rhodococcus sp.*, *Clavibacter michiganensis* and *Kineococcus radiotolerans* also encode for both AmtR and GlnR homologs; however the function of this co-occurrence remains unknown (Amon *et al.*, 2010). With regard to those genes that do not appear to be regulated by GlnR in *M. smegmatis* (Amon *et al.*, 2008a), AmtR may play an important regulatory role.

1.7.5 Nitrogen sensing:

Studies have indicated that intracellular glutamine and 2-oxoglutarate concentrations are the principle metabolites controlling the signalling cascade in *E. coli* (Leigh and Dodsworth, 2007; Reitzer, 2003). These metabolites are sensed by the

uridylyltransferase, GlnD, and the PII signalling proteins respectively (Ninfa and Jiang, 2005; Jiang *et al.*, 1998; Jiang *et al.*, 1997). During nitrogen starvation, the 2-oxoglutarate concentration rises and is bound by the PII protein, GlnB (Reitzer, 2003). Both 2-oxoglutarate and ATP are required at micromolar quantities for PII function, however, upon increased 2-oxoglutarate concentrations, PII function is inhibited thereby preventing the interaction of these proteins with NtrB and GlnE (Leigh and Dodsworth, 2007). A lack of PII interaction with NtrB would increase the phosphorylation state of NtrC and promote gene transcription, whereas a lack of PII interaction with GlnE would inhibit the adenylylation function of this protein and thereby increase GS activity. Although 2-oxoglutarate does not play a role in altering the PII uridylylation state, the metabolite is still considered a sensor of nitrogen limitation (Leigh and Dodsworth, 2007).

Glutamine, on the other hand, appears to be the metabolite flagging nitrogen sufficiency in *E. coli* since, when nitrogen is in excess, glutamine levels rise and are sensed by both GlnD and GlnE (Reitzer, 2003). The PII-uridylylation activity of GlnD is repressed (Arcondeguy *et al.*, 2001) to prevent the unnecessary activation of transcription and GS activity. Glutamine is also able to stimulate the adenylylating activity of GlnE, in a PII independent fashion, to down-modulate GS activity. Both GlnD and GlnE are constitutively produced which highlights their importance in sensing the cellular nitrogen status (Reitzer, 2003). Although GlnB, GlnD and GlnE are considered to be nitrogen sensory proteins, GlnD may be the principle sensor since it is able to directly activate the signalling cascade.

C. glutamicum accumulates large intracellular pools of amino acids such as glutamate, which respond slowly to changes in nitrogen availability and therefore make them poor signals for cellular nitrogen status (Burkovski, 2006; Nolden *et al.*, 2001b). Although intracellular glutamine concentrations did vary in response to nitrogen availability in *C. glutamicum*, the cellular response did not alter which suggests that this metabolite is not a signal for nitrogen status (Muller *et al.*, 2006). Protein sequence analysis of GlnD in *C. glutamicum* indicated that the protein does not have a ligand binding domain as found in other bacteria, such as *E. coli*, which implies that it does not have nitrogen sensory

properties (Tondervik *et al.*, 2006). In addition, transcription of the gene encoding for GlnD is not constitutive and varies in response to nitrogen availability, which makes it a poor sensory mechanism (Nolden *et al.*, 2001b). It was later shown that intracellular 2-oxoglutarate and ammonium levels altered rapidly in response to changes in nitrogen availability in *C. glutamicum*, which corresponded well to GlnK adenylation and the transcriptional response (Muller *et al.*, 2006). Thus, both ammonium and 2-oxoglutarate may play a role as indicators of cellular nitrogen status; however, experimental evidence regarding the nitrogen sensors is required.

Very little is known about the markers of cellular nitrogen status and the sensors involved in *S. coelicolor* and the mycobacteria; however, since these organisms possess similar metabolic machinery to *C. glutamicum*, it may be that similar sensory mechanisms are employed.

1.7.6 Nitrogen metabolism and *M. tuberculosis* pathogenicity:

Glutamine synthetase (GS) has formed the main focus of study with regard to nitrogen metabolism in *M. tuberculosis* since it has previously been demonstrated that a knockout of *glnA1* (gene encoding for GS) creates glutamine auxotrophy, which highlights the central role that GS plays in this metabolic pathway (Tullius *et al.*, 2003). In addition, GS mutants are attenuated for intracellular growth in human THP-1 macrophages and are unable to infect guinea pigs (Tullius *et al.*, 2003), which indicates that this enzyme is critically important for *M. tuberculosis in vivo*. It has been shown that GS is produced in large quantities by *M. tuberculosis* and the enzyme has been detected extracellularly as well as intracellularly in pathogenic mycobacterial species (Tullius *et al.*, 2001; Harth and Horwitz, 1999; Harth *et al.*, 1994). Up to one third of total GS activity detected could be attributed to extracellular GS (Harth *et al.*, 1994). It has been speculated that extracellular GS may play a role in *M. tuberculosis* pathogenicity by enhancing the ability of the bacillus to survive within the macrophage by manipulating phagosomal pH and thereby preventing lysosome-phagosome fusion (Tullius *et al.*, 2001; Miller and Shinnick, 2000). In addition, extracellular GS has been implicated in the production of a cell wall polymer, poly-L-glutamine-glutamate which can constitute up to 10% of total cell wall mass

(Hirschfield *et al.*, 1990). The polymer is found only in pathogenic mycobacteria and may play a role in cell wall integrity which could promote bacterial survival *in vivo* (Tullius *et al.*, 2001; Hirschfield *et al.*, 1990). Large quantities of GS are produced and it has been found that the adenylation function of GlnE is essential for the survival of *M. tuberculosis* (Carroll *et al.*, 2008; Parish and Stoker, 2000a), which is not the case for other bacteria, such as *C. glutamicum* or *S. coelicolor*. This adenylation function is thought to be important in the maintenance of intracellular glutamate to glutamine ratios and to prevent depletion of intracellular ATP levels (Carroll *et al.*, 2008). Due to its apparent essential physiological role, GlnE has become a source of interest for drug development (Carroll *et al.*, 2008).

Intensive study of GS in *M. tuberculosis* led to the discovery of L-methionine-S-sulfoximine (MSO), a glutamate analogue which, when phosphorylated by GS remains irreversibly bound to the active site thereby preventing substrate binding (Carroll *et al.*, 2008). MSO has been shown to selectively bind to and inhibit extracellular GS which in turn affects the biosynthesis of poly-L-glutamate/glutamine, resulting in diminution of the cell wall structure of pathogenic mycobacteria and consequent bacteriostasis (Harth and Horwitz, 1999). The administration of MSO to guinea pigs and human THP-1 cells infected with *M. tuberculosis* yielded bacteriostatic results (Harth and Horwitz, 1999; Harth and Horwitz, 2003). However, when co-administered with antibiotics such as INH and/or RIF, MSO acted synergistically and could reduce the MIC's of these first-line drugs to approximately one-tenth of their established MIC (Harth and Horwitz, 2003). This may be due to the loss of cell wall integrity as a result of a reduction in the production of poly-L-glutamate/glutamine by MSO treatment which in turn, would facilitate the movement of other antibiotics across this barrier.

MSO is an epileptogenic agent. In mice, it has been shown that MSO can traverse the blood-brain barrier and inhibit glutamine synthetase activity in the nerve endings of the brain (Griffith and Meister, 1978). It was thought that this may result in chemical imbalances which could affect neurotransmission and thereby bring about the convulsions that are observed. The sensitivity of animal species to MSO appears to vary quite considerably, with dogs being unusually sensitive, whilst monkeys and rats are relatively insensitive to MSO treatment (Harth and Horwitz, 2003). The sensitivity of

humans to MSO treatment remains unknown, but since MSO can inhibit both prokaryotic as well as eukaryotic glutamine synthetases (albeit to a lesser extent), its use as a therapeutic agent in humans is unlikely. There is, however, ongoing work being conducted to identify alternative compounds with greater specificity for mycobacterial glutamine synthetase and other extracellular proteins.

1.8 Study Design and Objectives of this Thesis:

This study can be divided into three main parts which focus on the major aspects of nitrogen metabolism. These include investigating: (i) the transcriptional control of nitrogen-related genes and their associated regulatory mechanisms, (ii) the physiological role and regulation of the major enzyme effectors and (iii) the function of the signalling cascade in control of enzyme activity and transcription. This dissertation is divided into three main objectives which explore the major aspects of nitrogen metabolism in *Mycobacterium smegmatis* as outlined above. The fast-growing and non-pathogenic *M. smegmatis* is often used as a model for the study of mycobacterial genetics, metabolic and regulatory pathways of *M. tuberculosis* (Titgemeyer *et al.*, 2007; Bange *et al.*, 1999; Pelicic *et al.*, 1998). With regard to nitrogen metabolism, in depth *in silico* analyses (Amon *et al.*, 2008b; Harper *et al.*, 2008) suggest that similar mechanisms exist between these closely related mycobacterial species. Therefore this study is based upon the use of *M. smegmatis* as a tool for the elucidation of the nitrogen metabolic pathway in *M. tuberculosis*.

1.8.1 Objective 1: To determine the relative change in the expression of genes putatively involved in nitrogen metabolism in *M. smegmatis* and to assess the regulatory roles of GlnR and a putative AmtR on the transcription these genes.

Comparable transcriptional regulation of nitrogen-related genes in *C. glutamicum* and *S. coelicolor* in response to nitrogen availability has been shown. It is hypothesised that homologous genes in *M. smegmatis* are similarly regulated in response to nitrogen starvation or excess. The expression profiles of the genes investigated in *M. smegmatis*

would indicate their possible involvement in the nitrogen metabolic pathway and whether the gene products play an analogous role to those in the Actinomycetale model organisms. GlnR has been shown to regulate a plethora of genes which are involved in carbon, amino acid and nitrogen metabolism in *S. coelicolor* (Tiffert *et al.*, 2011c; Tiffert *et al.*, 2008) and a reduced number of genes in *M. smegmatis* (Amon *et al.*, 2008a). There remain members of the pathway that are not apparently regulated by GlnR in *M. smegmatis* and the transcriptional regulation of these genes in response to varying nitrogen conditions has not yet been experimentally determined. It also remains to be shown whether AmtR could affect the transcription of genes already shown to be regulated by GlnR in response to nitrogen availability in *M. smegmatis*.

1.8.1.1 Hypothesis for Objective 1:

AmtR may play a role in the transcriptional regulation of nitrogen metabolism-related genes not found to be regulated by GlnR.

1.8.1.2 Goals for Objective 1:

- 1: To determine the relative change in expression of genes putatively involved in nitrogen metabolism in *M. smegmatis* under conditions of nitrogen starvation and excess.
- 2: To determine the promoter activity of the two possible transcriptional regulators in *M. smegmatis*, namely AmtR and GlnR, under conditions of nitrogen starvation and nitrogen excess.
- 3: To determine the regulatory roles of an AmtR-type and/or GlnR-type transcriptional regulator on transcription of nitrogen metabolism-related genes in *M. smegmatis* through the generation *amtR* and *glnR* deletion mutants.
- 4: To analyze the effect of an *amtR* deletion and a *glnR* deletion on *M. smegmatis* growth and survival *in vitro* and *in vivo*.

1.8.2 Objective 2: To investigate the possible physiological roles of the enzyme effectors of nitrogen metabolism in *M. smegmatis* which include the NADP⁺-specific and NAD⁺-specific glutamate dehydrogenases as well as glutamine synthetase.

In *C. glutamicum*, NADP⁺-GDH activity is regulated in response to nitrogen availability and nitrogen source. It was found that GDH specific activity increased upon nitrogen starvation (Hanssler *et al.*, 2009) and exposure to a variety of nitrogen sources. It has, until recently, been assumed that the activity of NAD⁺-GDH in prokaryotes is not regulated. However, O'Hare and colleagues (O'Hare *et al.*, 2008) showed an interaction between the protein GarA and the NAD⁺-GDH in *M. smegmatis* which could alter the specific activity of NAD⁺-GDH. The conditions under which the protein interaction and subsequent regulation of activity occurs is not clear. Glutamine synthetase is deemed the major nitrogen assimilatory enzyme, especially under conditions of nitrogen limitation. GS activity is known to be regulated in response to nitrogen availability; however, glutamine synthetase activity studies in *M. smegmatis* are lacking and the degree to which GS activity is regulated is not clear.

1.8.2.1 Hypothesis for Objective 2:

It is hypothesised that NADP⁺-GDH, NAD⁺-GDH and GS specific activity is regulated in response to nitrogen availability in *M. smegmatis*.

1.8.2.2 Goals for Objective 2:

1. To investigate the possible physiological roles and regulation of the NADP⁺-specific and NAD⁺-specific GDH enzymes in *M. smegmatis* under conditions of nitrogen starvation and excess.
2. To investigate *M. smegmatis* glutamine synthetase activity under the same conditions of nitrogen starvation and excess.

3. To investigate whether the glutamate dehydrogenase enzymes are regulated in response to the presence of alternative nitrogen sources.
4. To determine whether NADP⁺-specific and/or NAD⁺-specific GDH activity can be detected in *Mycobacterium tuberculosis*.

1.8.3 Objective 3: To study the control of expression of nitrogen metabolism-related genes and enzyme activity by the signalling cascade through mutagenesis analyses.

GlnD is, in many bacteria, a pivotal point in the nitrogen signalling cascade since it reversibly modifies the PII signalling protein, GlnK, by uridylylation or adenylylation in response to changes in nitrogen availability. It is not known whether this mechanism is required in *M. smegmatis* for signalling of the cellular nitrogen status to transcriptional regulators (as in *C. glutamicum*) or enzyme effectors (as in *E. coli*). Previously published reports suggest that the GlnD/GlnK mechanism is not important for cellular nitrogen signalling in *M. tuberculosis* (Read *et al.*, 2007).

1.8.3.1 Hypothesis for Objective 3:

It is hypothesised that, similarly to *S. coelicolor* and *M. tuberculosis*, GlnD/GlnK is not required for nitrogen signalling in *M. smegmatis*.

1.8.3.2 Goals for Objective 3:

1. To analyse the relative change in expression of genes known to be regulated by nitrogen availability (e.g. *glnA1* and *glnK*) in an *M. smegmatis* *glnD* knockout and wild type strains under conditions of nitrogen limitation and excess.
2. To determine whether there is a difference in GDH and GS specific activity in the *glnD* mutant vs wild type *M. smegmatis* strains when cultured under conditions of nitrogen limitation and excess.

CHAPTER TWO

Transcriptional control of nitrogen metabolism-related genes

NOTE: Sections of the following chapter have been submitted for publication as:

“Regulation of nitrogen-related gene transcription in *Mycobacterium smegmatis*: a role for GlnR and AmtR” Catriona Harper, Bienyameen Baker, Ray-Dean Pietersen,

Paul van Helden, Ian Wiid.

2.1 INTRODUCTION:

In silico analyses of the published *M. smegmatis* mc²155 and *M. tuberculosis* genomes, together with information available from the well-studied and closely related model organisms, *Corynebacterium glutamicum* and *Streptomyces coelicolor* has allowed the identification of genes involved in nitrogen metabolism in these mycobacteria. From these analyses, it is clear that the *M. smegmatis* genome boasts numerous additional copies of most of the nitrogen-related genes (Table 1). These genes appear to have been originally acquired through horizontal gene transfer as many have a high degree of homology to sequences from *Burkholderia*, *Agrobacterium*, *Synechococcus* and *Pseudomonas* species (Amon *et al.*, 2008b). The range of genes present in *M. smegmatis* correlates well to those present in the model Actinomycetes, thus it is thought that *M. smegmatis* is able to assimilate nitrogen from a variety of sources. The pathogenic mycobacterial species, however, display a reduced number of nitrogen-related genes in comparison to *M. smegmatis* (Table 1). This can be attributed to the phenomenon termed ‘reductive evolution’ in which obligate intracellular bacteria, such as *M. tuberculosis* and *M. leprae*, have lost a number of genes which are not deemed essential for survival as host processes are able to supply the nutrients required.

Table 1: Genes involved in nitrogen metabolism in *M. smegmatis*, *M. tuberculosis* and *M. leprae*.*

Protein description	Gene	Gene in <i>M. smegmatis</i>	Gene in <i>M. tuberculosis</i>	Gene in <i>M. leprae</i>
Methylammonium permease	<i>amtA</i>	<i>msmeg_6259</i> <i>msmeg_4635</i>	n/a	n/a
Ammonium permease	<i>amtB</i>	<i>msmeg_2425</i>	<i>Rv2920c</i>	<i>ML1627</i>
PII protein	<i>glnK</i>	<i>msmeg_2426</i>	<i>Rv2919c</i>	<i>ML1626</i>
Uridyltransferase/adenyltransferase	<i>glnD</i>	<i>msmeg_2427</i>	<i>Rv2918c</i>	<i>ML1625</i>
Adenyltransferase	<i>glnE</i>	<i>msmeg_4293</i>	<i>Rv2221c</i>	<i>ML1630</i>
TetR regulator	<i>amtR</i>	<i>msmeg_4300</i>	<i>Rv3160c</i>	n/a
OmpR regulator	<i>glnR</i>	<i>msmeg_5784</i>	<i>Rv0818c</i>	<i>ML2194</i>
Nitrite reductase	<i>nirB</i>	<i>msmeg_0427</i>	<i>Rv0252</i>	n/a
	<i>nirD</i>	<i>msmeg_0428</i>	<i>Rv0253</i>	n/a
Glutamate synthase	<i>gltB</i>	<i>msmeg_3225</i> <i>msmeg_5594</i>	<i>Rv3859c</i>	<i>ML0061</i>

		<i>msmeg_6459</i>		
	<i>gltD</i>	<i>msmeg_3226</i> <i>msmeg_6458</i>	<i>Rv3858c</i>	<i>ML0062</i>
NADP ⁺ - specific Glutamate dehydrogenase	<i>gdhA</i>	<i>msmeg_5442</i>	n/a	n/a
NAD ⁺ - specific Glutamate dehydrogenase	<i>gdhB</i>	<i>msmeg_4699</i> <i>msmeg_6272</i>	<i>Rv2476c</i>	n/a
Urease	<i>ureA</i>	<i>msmeg_3627</i> <i>msmeg_1093</i>	<i>Rv1848</i>	n/a
	<i>ureB</i>	<i>msmeg_3626</i>	<i>Rv1849</i>	n/a
	<i>ureC</i>	<i>msmeg_3625</i> <i>msmeg_1094</i>	<i>Rv1850</i>	n/a
	<i>ureF</i>	<i>msmeg_3624</i> <i>msmeg_1092</i>	<i>Rv1851</i>	n/a
	<i>ureG</i>	<i>msmeg_3623</i> <i>msmeg_1095</i>	<i>Rv1852</i>	n/a
	<i>ureD</i>	n/a <i>msmeg_1096</i>	<i>Rv1853</i>	n/a
Glutamine synthetase type-1	<i>glnA1</i>	<i>msmeg_4290</i>	<i>Rv2220</i>	<i>ML0925</i>
Glutamine synthetase type-2	<i>glnA2</i>	<i>msmeg_4294</i>	<i>Rv2222c</i>	n/a
Glutamine synthetase type-3	<i>glnA3</i>	<i>msmeg_3561</i>	<i>Rv1878</i>	n/a
Glutamine synthetase type-4	<i>glnA4</i>	<i>msmeg_2595</i>	<i>Rv2860c</i>	n/a
Unknown	<i>glnA</i>	<i>msmeg_1116</i> <i>msmeg_3827</i> <i>msmeg_3828</i> <i>msmeg_5374</i> <i>msmeg_6693</i>	n/a	n/a

*Those *M. smegmatis* genes that are highlighted in the table were studied in this chapter.

(Source: modified Amon *et al.*, 2008b)

The expression of most nitrogen-related genes in response to nitrogen availability in *M. smegmatis* remains unknown. In *C. glutamicum*, AmtR is responsible for the repression of nitrogen related genes under conditions of nitrogen sufficiency, whereas in *S. coelicolor*, GlnR is able to both activate and/or repress gene transcription in response to exogenous nitrogen concentrations. Interestingly, *M. smegmatis* appears to be the only mycobacterium to encode both transcriptional regulators, however, the functional

significance thereof is unknown. Although GlnR has been shown to regulate a subset of genes in *M. smegmatis* there remain a number of nitrogen related genes which do not appear to be controlled by this regulator (Amon *et al.*, 2008a). The paucity of knowledge regarding the transcriptional regulation of nitrogen-related genes in the mycobacteria prompted the study presented in this chapter. *M. smegmatis* genes displaying the greatest homology to genes with known functions in closely related organisms (such as *M. tuberculosis*, *C. glutamicum* and *S. coelicolor*) were chosen for study. In addition, the genes encoding for AmtR and GlnR homologs in *M. smegmatis* were rendered non-functional by generation of *amtR* and *glnR* deletion mutants in order to elucidate the roles of these proposed regulators.

2.1.1 Study Objectives:

Objective 1: To determine the relative change in expression of genes putatively involved in nitrogen metabolism in *M. smegmatis*, under conditions of nitrogen starvation and excess.

Objective 2: To determine the promoter activity of the two possible transcriptional regulators in *M. smegmatis*, namely AmtR and GlnR, under conditions of nitrogen starvation and nitrogen excess.

Objective 3: To determine the regulatory roles of AmtR and GlnR on transcription of nitrogen metabolism-related genes in *M. smegmatis* through the generation *amtR* and *glnR* deletion mutants.

Objective 4: To analyze the effect of an *amtR* and a *glnR* deletion on *in vitro* and *in vivo* growth and survival of *M. smegmatis*.

2.2 RESULTS:

2.2.1 Analysis of the relative change in expression of nitrogen-metabolism related genes in response to ammonium limitation and excess.

M. smegmatis was cultured in enriched 7H9 medium to midlog phase ($OD_{600} \approx 0.8$) and transferred to Kirchner's minimal medium in order to pre-condition the bacilli to ammonium limitation (3mM $(NH_4)_2SO_4$) and excess (60mM $(NH_4)_2SO_4$) for 1hr. Bacilli that were cultured in ammonium limiting conditions were exposed to an ammonium pulse for 0.5hrs. The opposite was true for bacteria grown under conditions of ammonium excess; namely, they were transferred to Kirchner's medium containing 3mM $(NH_4)_2SO_4$ for 0.5hrs, 1hrs, 2hrs and 4hrs. At each time interval, total mycobacterial mRNA was extracted; converted to cDNA and the relative change in gene transcription determined by real-time PCR. *16S rRNA* was used as an internal control. The PCR efficiencies of the primers used are given in Table 2 below. Each experiment was repeated at three times and each PCR was done in triplicate.

Table 2: PCR efficiencies of all primers used in this study as calculated from a standard curve by the REST©384 Version 1 software. Maximal PCR efficiency, as defined by the REST software, has been given a value of 2.

Gene name	<i>16S rRNA</i>	<i>glnA</i>	<i>amtB</i>	<i>glnK</i>	<i>glnD</i>	<i>gltB</i>	<i>glnE</i>	<i>msmeg_5442</i>	<i>msmeg_4699</i>	<i>msmeg_6272</i>	<i>nirD</i>	<i>ureA</i>
PCR Efficiency	1.65	1.94	1.82	1.89	1.71	1.76	1.89	1.96	1.93	2	1.76	1.81

The expression of *glnA*, *amtB* and *gltB* was significantly up-regulated ($p < 0.05$) in response to ammonium starvation throughout the time course study. Within 0.5hrs of exposure to nitrogen limitation, *glnA* transcription was up-regulated 3.3 fold and increased to an 8.6 fold maximum after 2hrs culture under nitrogen starvation conditions (Table 3). The expression of *amtB* under these conditions was similar with a 2 fold up-regulation of transcription within the initial 0.5hr phase followed by a steady increase in expression to an 8.4 fold maximum after 4hrs exposure to nitrogen limiting conditions

(Table 3). The regulation of *gltB* expression followed a similar trend with an up-regulation of transcription within 0.5hrs exposure to ammonium limiting conditions (5.3 fold) followed by an increase to 13.1 fold after 2hrs. The degree of *gltB* transcriptional up-regulation was slightly higher than for any of the other genes studied. Upon exposure to an ammonium pulse, there appeared to be a down-regulation in the expression of *glnA* (2.5 fold) and *amtB* (2 fold) (Table 3). In contrast, *gltB* transcription appeared to up-regulated (3 fold) under nitrogen excess conditions as well as under nitrogen starvation conditions (Table 3). Expression of *glnE*, encoding for the GS regulatory adenylyltransferase, was up-regulated (2 fold) after 2hrs exposure to ammonium limiting conditions; however, the expression of this gene was not regulated in response to an ammonium pulse (Table 3). Transcriptional regulation of the genes encoding for the glutamate dehydrogenase enzymes (*msmeg_5442*, *msmeg_6272* and *msmeg_4699*) in response to ammonium availability was investigated in a separate study and the results are presented in Chapter 3.

Table 3: Relative fold change in expression of nitrogen-metabolism related genes in response to ammonium starvation and excess.*

[(NH ₄) ₂ SO ₄]	Time	Gene					
		<i>glnA</i>	<i>amtB</i>	<i>glnK</i>	<i>glnD</i>	<i>gltB</i>	<i>glnE</i>
3mM	0.5hrs	3.3 (2.1 - 5.5)	2.0 (0.8 - 5.4)	0.3 (0.2 - 0.5)	0.7 (0.4 - 1.4)	5.3 (2.9 - 11)	1.5 (0.4 - 5.1)
	1hrs	3.5 (1.3 - 10.1)	3.9 (1.1 - 12.2)	0.4 (0.3 - 0.6)	0.8 (0.4 - 1.7)	4.7 (1.9 - 11.2)	1.1 (0.5 - 2.8)
	2hrs	8.6 (4.9 - 15.5)	6.4 (3.1 - 14.5)	0.3 (0.3 - 0.4)	1.0 (0.7 - 1.6)	13.1 (7.5 - 25.9)	2.0 (0.7 - 4.7)
	4hrs	6.2 (3.9 - 16.9)	8.4 (2.4 - 26.7)	0.3 (0.2 - 0.3)	0.7 (0.5 - 1.0)	11.6 (4.8 - 27.1)	1.1 (0.2 - 4.1)
60mM	0.5hrs	0.4 (0.3 - 0.6)	0.5 (0.4 - 0.7)	0.7 (0.4 - 1.3)	0.9 (0.5 - 1.7)	2.9 (1.9 - 4.6)	1.6 (0.3 - 7.9)

* Values that are highlighted represent a statistically significant change in transcription ($p < 0.05$) as calculated by REST-2009 V2.0.13. The standard error (SE) is also given.

In contrast to the regulation of *glnA*, *amtB*, *gltB* and *glnE*, it was found that *glnK* expression was significantly repressed (2.5 to 3fold) in response to ammonium limitation (Table 3). The transcriptional repression occurred within 0.5hrs exposure to nitrogen limiting conditions and was maintained for the remainder of the study. *glnD* transcriptional activity did not appear to alter significantly in response to ammonium limitation; however, there was a slight repression (approximately 1.4fold) of transcription after 4hrs culture under starvation conditions (Table 3). The differential expression of *amtB*, *glnK* and *glnD* was unexpected as these genes are thought to be located within an operon and thus co-transcribed. Therefore, it was also investigated whether a single mRNA species could be detected by PCR amplification across the junction between *amtB* and *glnK* and between *glnK* and *glnD* (Figure 1A). The results presented in Figure 1B show that an *amtB* and *glnK* mRNA transcript (1407bp; lanes 1 and 2) as well as a *glnK* and *glnD* mRNA species (680bp; lanes 5 and 6) could be detected, which suggests that these genes may be co-transcribed.

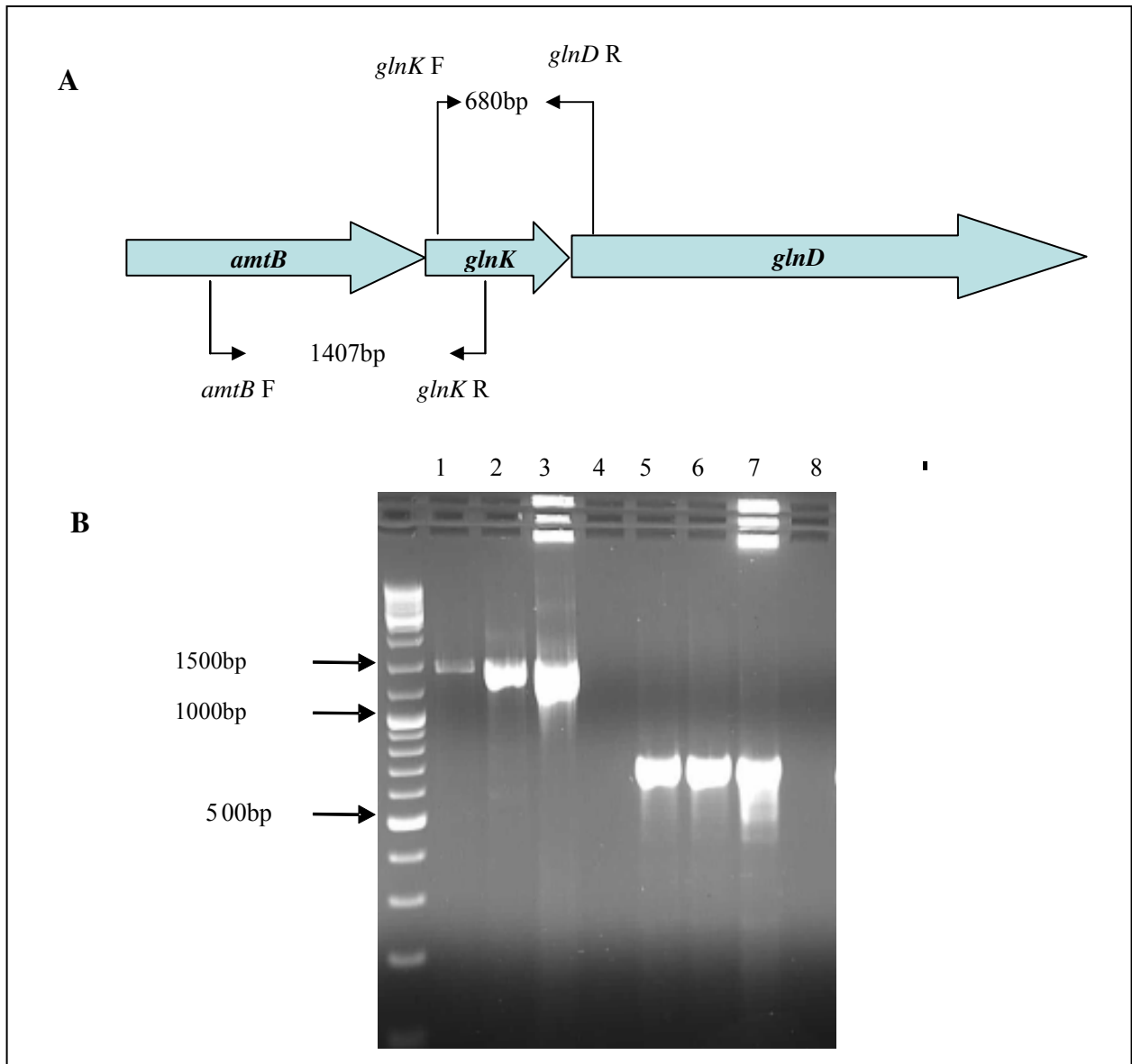


Figure 1: Investigation into *amtB/glnK/glnD* co-transcription by (A) PCR amplification across the junction between *amtB* and *glnK* as well as between *glnK* and *glnD* using cDNA as PCR template material. Primer sequences are given in Materials and Methods, Table 2. (B) Electrophoretic separation of PCR products spanning the *amtB/glnK* (Lanes 1 and 2) and *glnK/glnD* (Lanes 5 and 6) intergenic regions. Lanes 3 and 7 are positive controls for each PCR reaction and lanes 4 and 8 are non-template controls for each PCR reaction.

2.2.2 Analysis of *glnR* and *amtR* expression via promoter activity determination under conditions of ammonium starvation and excess.

The activity of possible promoter regions directly upstream of *glnR* (Figure 2B) and *amtR* (Figure 2A) were determined by β -galactosidase assays in response to ammonium limitation and excess (culture conditions are as described in section 2.2 above). Since only 8bp and 12bp separate *amtR* from its respective down- and up-stream flanking genes, it may therefore be located within an operon. Thus, the activity of the promoter region directly upstream of *amtR* (465bp genomic fragment upstream of the *amtR* gene) and the first gene in the potential operon (*msmeg_4301*; 352bp region upstream of the gene) were analyzed. The gene encoding for *glnR* may also be genetically linked with an acetyltransferase (*msmeg_5783*); however, *glnR* appears to be the first gene in the operon, therefore only the activity of the promoter region directly upstream of *glnR* (206bp fragment upstream of the *glnR* gene) was analyzed.

Upon selection of *M. smegmatis* strains successfully transformed with pJEM15 fused to the promoter region of interest, it was found that only the *glnR* (pJEM15-*glnR*) and *msmeg_4301* (pJEM15-*msmeg_4301*) promoter regions resulted in blue colonies on X-gal selection plates, which indicated that these two regions had active promoters (Figure 3C and D). *M. smegmatis* transformed with native pJEM15 acted as a negative control and, similar to the pJEM15-*amtR* transformants, appeared as white colonies on selection plates (Figure 3A and B). The promoter activities were quantified using the β -galactosidase assay and it was found that *glnR* and *msmeg_4301* promoter activity could be detected under all the conditions tested (Table 4). Analysis of the promoter region directly upstream of *amtR*, revealed that the promoter activity was very low, with similar values as obtained for the negative control (Table 4), which suggests that the existence of an intergenic promoter between *msmeg_4301* and *amtR* is unlikely.

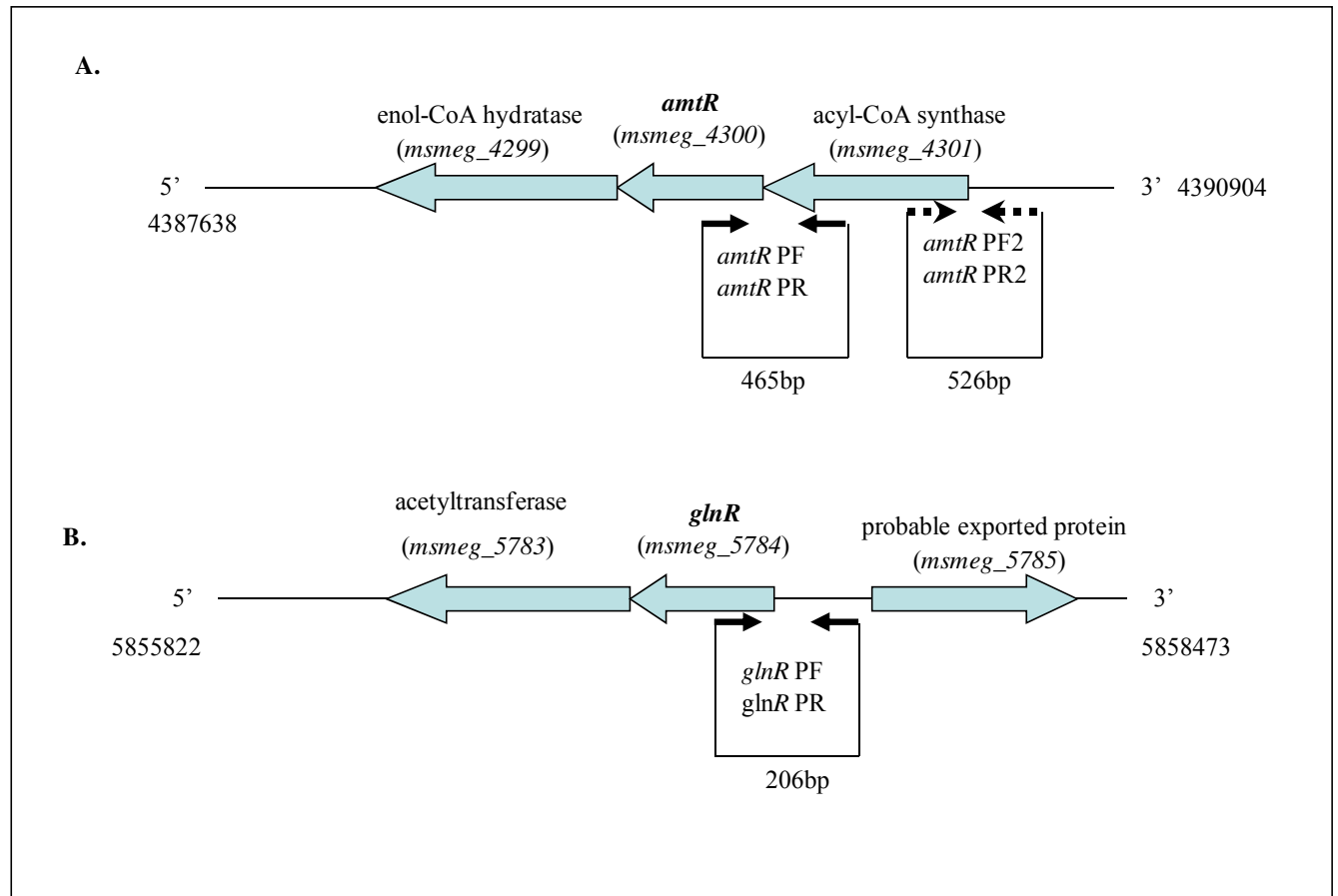


Figure 2: Diagrammatic representation of the genomic regions flanking (A) *amtR* and (B) *glnR*. The promoter regions analyzed in this study, including the primers used to amplify these regions are also shown.

It was observed that *glnR* promoter activity increased significantly during *M. smegmatis* exposure to ammonium limiting conditions (Table 4). A total increase in *glnR* promoter activity of approximately 46.5% was observed following culture under nitrogen limiting conditions for a period of 4hrs. An initial increase of approximately 210 units (from 941.1 – 1150.9 units) occurred within 0.5hrs culture under nitrogen starvation conditions and was followed by a second sharp increase of approximately 584 units after 2hrs exposure to these conditions (Table 4). When the bacteria were subjected to an ammonium pulse, an unexpected increase of 105.7 units (from 748.6 – 854.3 units) in *glnR* promoter activity was also observed (Table 4). Upon analysis of the activity of the promoter region upstream of *msmeg_4301* in response to nitrogen availability, it was found that the average promoter activity was approximately 3 fold greater than that found

for the *glnR* promoter region. Although the activity of the promoter did not alter initially in response to exposure to ammonium starvation conditions, it was found that an increase of approximately 170 units (from 3342.4 – 3512.5 units) occurred after 2hrs exposure to these conditions (Table 4). Upon subjection to an ammonium pulse, no significant change in promoter activity could be observed.

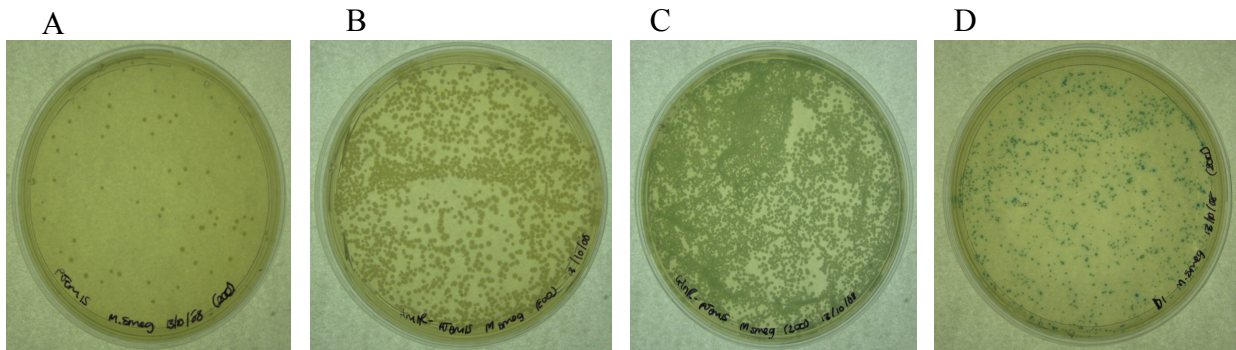


Figure 3: Photograph of *M. smegmatis* transformants containing (A) native pJEM15 (B) pJEM15-*amtR* and (C) pJEM15-*glnR* and (D) pJEM15-*msmeg_4301*.

Table 4: Activity, determined by β -galactosidase assays, of the potential promoter regions directly upstream of *glnR* and *amtR* as well as upstream of a possible operon wherein *amtR* may be located.

NH_4^+ concentration (mM)	Time	β -galactosidase Activity (units)*			
		pJem15	GlnR	upstream of AmtR	upstream of <i>msmeg_4301</i>
3	0	9.4 \pm 2.2	941.1 \pm 82.8	4 \pm 3.9	3359.3 \pm 52.0
	0.5	3.1 \pm 1.9	1150.9 \pm 54.8	-1.9 \pm 1.6	3385.3 \pm 31.0
	1	-0.6 \pm 0.5	1051.4 \pm 16.9	0.7 \pm 1.8	3342.4 \pm 16.6
	2	-4.6 \pm 1.5	1634.9 \pm 107.9	-3.3 \pm 1.8	3512.5 \pm 14.2
	4	-3.0 \pm 5.4	1759.9 \pm 27.3	8.4 \pm 0.9	3464.2 \pm 27.8
60	0	26.0 \pm 3.5	748.6 \pm 10.6	13.0 \pm 3.6	3411.0 \pm 23.9
	0.5	37.4 \pm 1.8	854.3 \pm 42.4	14.1 \pm 1.1	3434.3 \pm 19.9

*One unit is defined as the production of 1 μ Mole o-nitrophenol per minute per milligram protein. The standard deviation (SD) is also given.

2.2.3 In vitro growth of *glnR* and *amtR* knockout strains

Genetic deletions within *glnR* and *amtR* in the *M. smegmatis* genome were introduced to render the genes and their products non-functional. Using techniques described by (Parish and Stoker, 2000b), the functional form of the gene was replaced by a deleted form via homologous recombination to generate *glnR* (GlnRKO) and *amtR* (AmtRKO) knockout strains (see Appendix 2A and 2B for knockout methodology and PCR screening techniques). The growth and viability of these knockout strains were investigated by culture on enriched solid media as well as by growth in enriched liquid media. In addition, the ability of the knockout strains to grow in minimal medium with varying nitrogen source availability was also investigated.

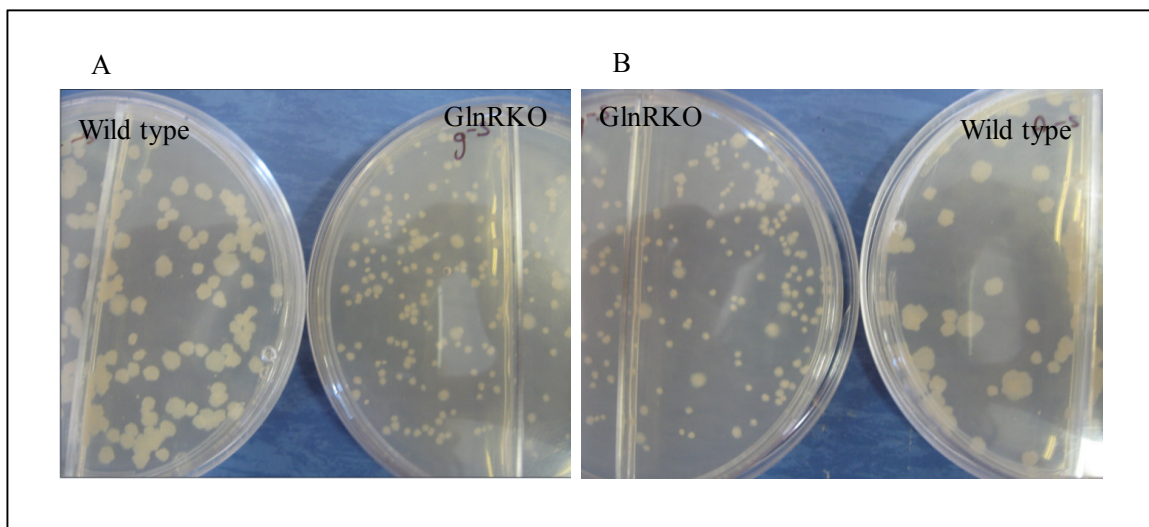
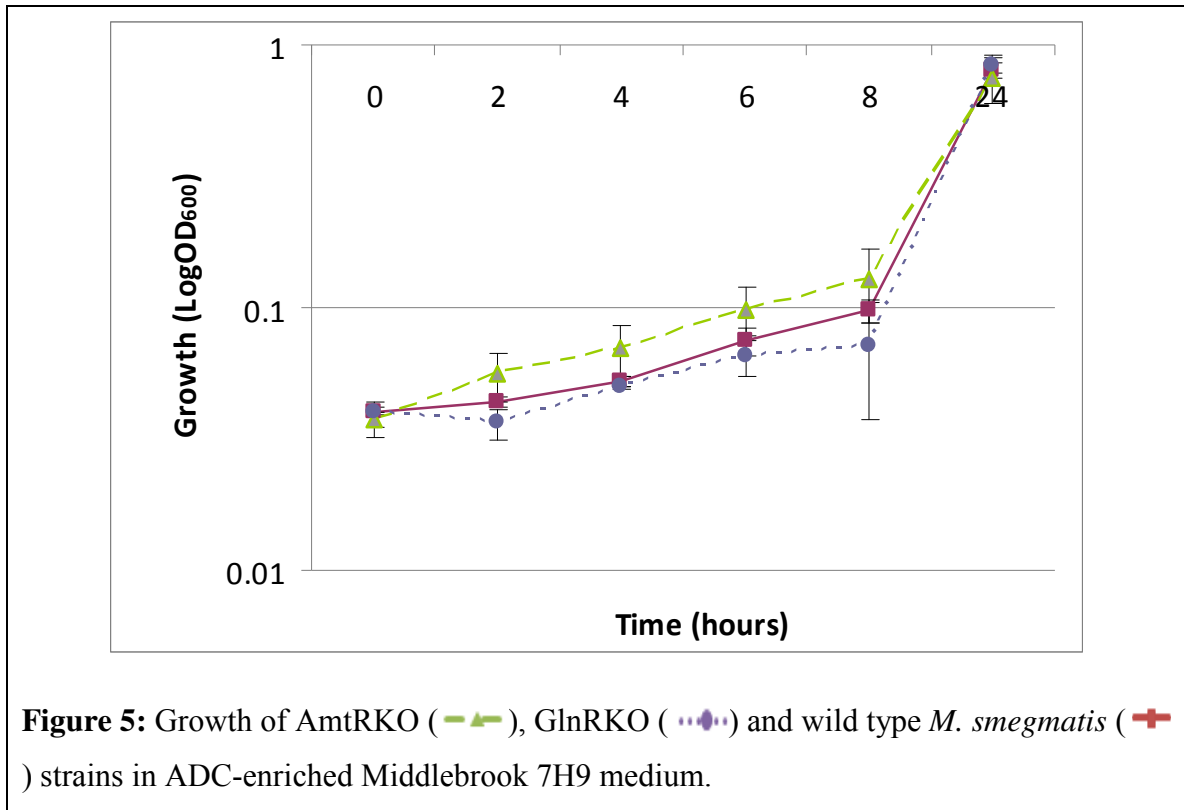


Figure 4: Wild type, GlnRKO and AmtRKO *M. smegmatis* strain growth on OADC-enriched 7H10 agar. In (A), the morphology of GlnRKO colonies (right) is compared to wild type *M. smegmatis* (left). In (B), GlnRKO colony morphology (left) is compared to AmtRKO colonies (right).

A difference in bacterial survival and viability between the knockout strains and wild type *M. smegmatis* was studied by spreading equal volumes of culture, each grown to an $OD_{600} \approx 0.2$, onto OADC-enriched 7H10 plates and analyzing subsequent colony growth. After approximately four days, *M. smegmatis* colonies were visible and the colony

forming units (CFU) were counted. No significant difference in CFU count between the knockout strains and wild type *M. smegmatis* was found (data not shown). However, upon visual analysis of colony morphology, it was observed that the GlnRKO colonies were much smaller than either the wild type or AmtRKO colonies. The latter strains displayed approximately equal colony size (Figure 4).



No highly significant difference in growth rate between the knockout strains and wild type *M. smegmatis* could be detected when the bacteria were cultured in ADC-enriched 7H9 liquid medium (Figure 5). However, it was observed that the AmtRKO strain grew more rapidly than the wild type strain and in contrast, the GlnRKO strain divided slightly more slowly than wild type *M. smegmatis* (Figure 5). In order to determine the effect of ammonium availability on growth, the knockout strains were cultured in Kirchner's minimal medium containing $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source to final concentrations of 0.1mM, 3mM and 60mM. From Figure 6 it can be seen that AmtRKO strain growth did not differ significantly from wild type *M. smegmatis* growth under any of the

conditions tested. In contrast, however, it was found that GlnRKO strain growth was compromised when ammonium concentrations were limiting (Figure 6A and B). When ammonium was available in excess, the growth rate of the GlnRKO strain was comparable to that of wild type *M. smegmatis* (Figure 6C).

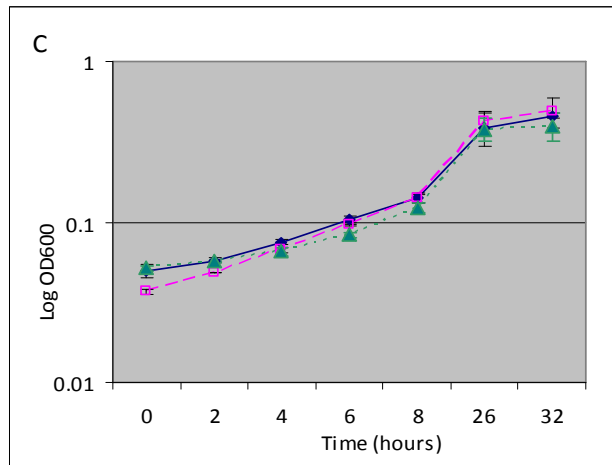
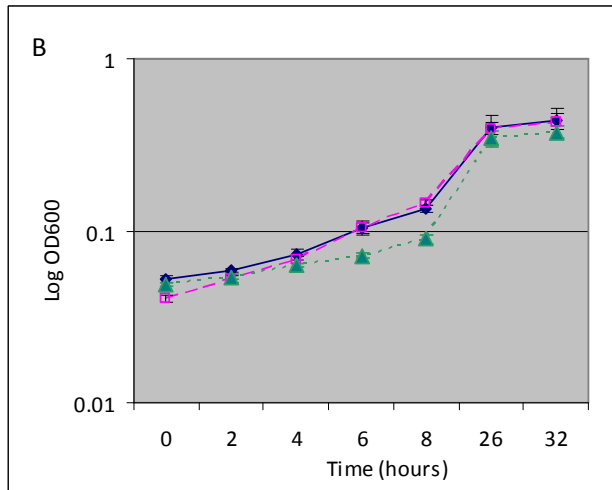
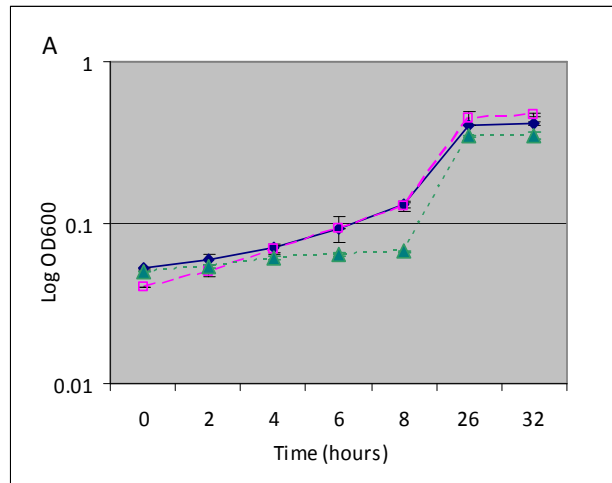


Figure 6: Growth of GlnRKO (.....), AmtRKO (-■-) strains and wild type (—●—) *M. smegmatis* strains in Kirchner's minimal medium containing ammonium sulphate as the sole nitrogen source to final concentrations of (A) 0.1mM, (B) 3mM and (C) 60mM.

2.2.4 Intracellular survival of GlnRKO and AmtRKO strains in comparison to wild type *M. smegmatis*

Bone-derived macrophages isolated from C57BL/6 mice were infected with wild type, GlnRKO and AmtRKO *M. smegmatis* strains in order to determine whether a difference in survival kinetics was apparent. A multiplicity of infection (MOI) of 1 or, more simply, a bacillus:macrophage ratio of 1:1 was used for infection. The macrophages were allowed to phagocytose the bacilli for 2hrs after which they were thoroughly washed to remove extracellular bacteria. In addition, a mild gentamycin treatment was applied to the cells to prevent extracellular bacterial growth. The macrophages were lysed 2hrs, 6hrs, 10hrs and 24hrs post infection to release any bacteria that had been phagocytosed. These were then diluted, plated onto 7H10 agar and the resulting CFU counted.

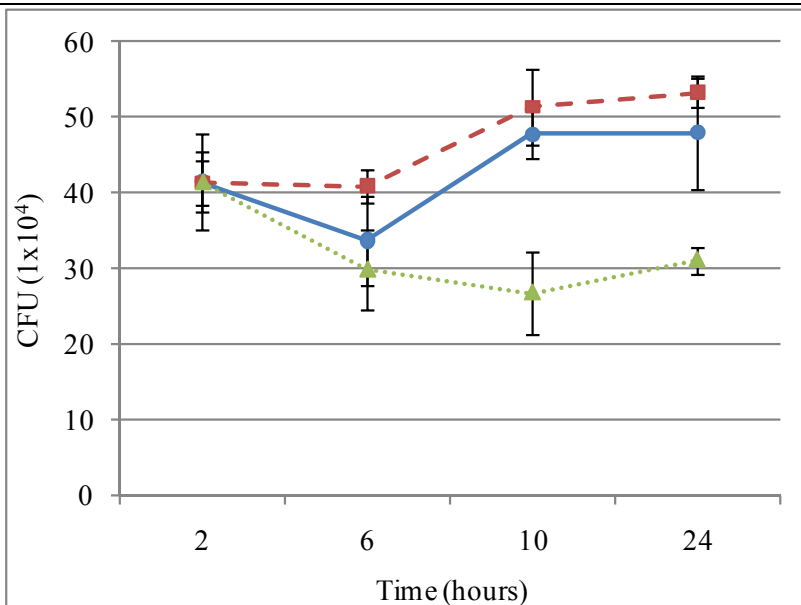


Figure 7: Intracellular survival of wild type *M. smegmatis* (—●—), AmtRKO (-■-) and GlnRKO (.....) deletion strains in C57BL/6 bone derived macrophages.

Wild type *M. smegmatis* and the AmtRKO strain showed similar patterns of intracellular survival. It was found that an initial killing phase occurred within the first 6hrs post-infection resulting in a decline in bacterial numbers (Figure 7). This phenomenon was more marked for wild type *M. smegmatis* than for the AmtRKO strain (Figure 7). The initial killing phase was followed by an absence of bacterial elimination by the macrophages which lead to intracellular growth of both wild type and AmtRKO *M. smegmatis* strains between 6hrs and 10hrs post-infection. The intracellular bacterial load did not increase significantly thereafter. In contrast, a relatively clear difference in intracellular survival between wild type *M. smegmatis* and the GlnRKO strain was observed (Figure 7). The initial killing phase (between 2hrs and 6hrs) was similar to that observed for wild type *M. smegmatis*, however, there appeared to be no intracellular growth of the GlnRKO strain in the subsequent 4hrs. The intracellular wild type *M. smegmatis* load remained significantly greater than that of the GlnRKO strain between 10hrs and 24hrs post-infection (Figure 7) which may reflect compromised intracellular survival capabilities of the GlnRKO strain.

2.2.5 Relative change in expression of nitrogen related genes in wild type, GlnRKO and AmtRKO *M. smegmatis* strains

The *glnR* and *amtR* knockout strains, as well as wild type *M. smegmatis* were cultured essentially as described in section 2.2.1; however, the bacteria were exposed to ammonium limiting and excess conditions for 0.5hrs and 1hr intervals only. Total bacterial mRNA was extracted, reverse transcribed to cDNA and the relative changes in gene expression analyzed by real-time PCR. The efficiencies of the primers used in this study are presented in Table 2.

The expression of *glnA* and *amtB* in response to ammonium limitation and excess in wild type *M. smegmatis* had previously been determined (section 2.2.1); however, was repeated in this study in order to ensure that the current experimental conditions did stimulate a nitrogen starvation/excess transcriptional response. It was found that *glnA* and *amtB* expression was up-regulated 6 fold and 3.21 fold respectively in response to

ammonium limitation whereas the expression of these genes were both down-regulated approximately 2 fold upon exposure to an ammonium pulse (Table 5). A similar regulation of *glnA* and *amtB* expression was observed previously (section 2.2.1). Based on these results, it was accepted that the culture conditions used did indeed produce a cellular response to nitrogen limitation and abundance. In addition to *glnA* and *amtB*, the transcriptional regulation of genes to be investigated in the knockout strains include those for which no consensus GlnR binding box could be identified in *M. smegmatis*; namely *ureA* (member of the urease operon), *nirD* (member of the nitrite reductase operon), *gltB* (member of the GOGAT operon), *msmeg_5442* (NADP(H)-dependant GDH), *msmeg_6272* and *msmeg_4699* (encoding for two possible NAD(H)-dependant GDH enzymes).

The regulation of *amtB* and *glnA* expression in the AmtRKO strain was similar to that observed for wild type *M. smegmatis*. There was a 4.17 fold increase in *amtB* transcription and a 3.46 fold increase in *glnA* expression after 1hr exposure to nitrogen limiting conditions (Table 5). In response to an ammonium pulse, *amtB* and *glnA* expression was significantly down-regulated by approximately 4 fold and 7.7 fold respectively (Table 5). The degree of down-regulation observed for these genes in the AmtRKO strain was greater than that observed in wild type *M. smegmatis*. It was observed that *glnA* and *amtB* expression was also up-regulated within 0.5hrs exposure to nitrogen limiting conditions in the GlnRKO strain (10.62 and 2.52 fold respectively); however, the degree of *glnA* up-regulation was greater than for either the wild type or AmtRKO *M. smegmatis* strains. In contrast to the wild type or AmtRKO strains, it was found that *glnA* and *amtB* expression was up-regulated by up to 10.27 fold and 1.4 fold respectively after exposure to ammonium excess conditions for 1hr (Table 5). These results suggest that GlnR may be involved in the transcriptional regulation of *glnA* and *amtB* in *M. smegmatis* which is in accordance with previously published reports (Amon *et al.*, 2008).

The transcriptional regulation of the urease operon, the GOGAT operon and the nitrite reductase operon in response to ammonium availability in *M. smegmatis* has not

previously been investigated. In this study, it was found that the expression of the first gene in the urease operon, *ureA*, in wild type *M. smegmatis* was up-regulated in response to both ammonium limitation (nearly 2 fold after 1hr) and excess (approximately 6 fold after 1hr) (Table 5). An up-regulation of *ureA* gene expression was also observed during ammonium starvation and excess in the GlnRKO strain; however, the degree of up-regulation was approximately 2 to 3 fold higher than observed for the wild type *M. smegmatis* strain (Table 5). In contrast, there was an apparent lack of *ureA* transcriptional regulation in the AmtRKO strain in response to varying ammonium concentrations which suggests that AmtR may play a role in the transcriptional regulation of this gene.

The first gene in the operon encoding for GOGAT, *gltB*, was found to be up-regulated in response to both ammonium starvation (approximately 6 fold after 1hr) and excess (approximately 13 fold after 1hr) (Table 5). In the AmtRKO strain, a similar increase in *gltB* transcription was observed during ammonium starvation (approximately 4 fold up-regulation); however there was complete lack of transcriptional regulation when the bacteria were transferred to ammonium excess conditions (Table 5). When the GlnRKO strain was exposed to ammonium limiting conditions, it was found that *gltB* expression increased up to 46.37 fold (Table 5) which is approximately 7 fold higher than the up-regulation observed for wild type *M. smegmatis*. Upon subjection to an ammonium pulse, *gltB* expression in the GlnRKO strain was also up-regulated (approximately 5 fold after 1hr). Thus it would appear that both AmtR and GlnR may be involved in the transcriptional control of the GOGAT operon.

Similarly to *gltB* expression, it was observed that the second gene in the nitrite reductase operon, *nirD*, was up-regulated in response to both ammonium limitation (approximately 2 fold after 1hr) and excess (approximately 3 fold within 0.5hrs) in wild type *M. smegmatis* (Table 5). A similar pattern of *nirD* expression occurred in the GlnRKO strain with a 2 fold up-regulation of expression within 1hr exposure to nitrogen limiting conditions and a 2 to 3 fold increase in transcription after culture in medium containing an excess of ammonium (Table 5). In contrast, there appeared to be a lack of *nirD* transcriptional control in the AmtRKO strain when the bacteria were exposed to

ammonium limitation. In addition, a significant down-regulation (2 to 3 fold) of expression was observed when the AmtRKO *M. smegmatis* strain was exposed to ammonium excess conditions (Table 5). These results indicate that AmtR could play a role in *nirB/D* regulation in response to ammonium availability.

Table 5: Relative quantification of gene expression in response to nitrogen limitation (3mM (NH₄)₂SO₄) and an ammonium pulse (60mM (NH₄)₂SO₄) in wild type *Mycobacterium smegmatis* as well as in the GlnRKO and AmtRKO deletion strains.

Time (hrs)	Gene	Ammonium sulphate concentration (mM)						Relative Fold Change*
		3			60			
		Wild type	GlnRKO	AmtRKO	Wild type	GlnRKO	AmtRKO	
0.5	<i>glnA</i>	6 ± 2.6	10.62 ± 5.26	3.28 ± 1.07	0.53 ± 0.16	5.21 ± 2.21	0.13 ± 0.06	
	<i>amtB</i>	3.21 ± 1.00	2.52 ± 0.59	3.92 ± 1.10	0.65 ± 0.11	1.41 ± 0.28	0.25 ± 0.07	
	<i>ureA</i>	0.8 ± 0.23	6.05 ± 3.06	1.17 ± 0.37	2.87 ± 1.01	5.47 ± 2.33	0.79 ± 0.21	
	<i>gltB</i>	3.64 ± 1.2	46.37 ± 26.01	3.92 ± 1.24	4.24 ± 1.29	2.27 ± 0.88	0.61 ± 0.21	
	<i>nirD</i>	1.26 ± 0.27	1.52 ± 0.40	2.43 ± 1.18	2.47 ± 0.69	2.24 ± 0.62	0.49 ± 0.13	
	<i>msmeg_4699</i>	0.5 ± 0.27	3.91 ± 1.87	0.96 ± 0.57	2.23 ± 0.75	4.81 ± 1.95	0.46 ± 0.17	
	<i>msmeg_5442</i>	0.88 ± 0.25	4.57 ± 1.30	1.32 ± 0.50	1.59 ± 0.45	5.48 ± 1.60	0.77 ± 0.20	
	<i>msmeg_6272</i>	0.9 ± 0.30	1.95 ± 0.74	0.41 ± 0.37	2.86 ± 0.75	5.21 ± 1.60	0.12 ± 0.06	
1	<i>glnA1</i>	4.96 ± 2.30	4.11 ± 2.00	3.46 ± 1.37	1.47 ± 0.46	10.27 ± 6.53	0.35 ± 0.09	
	<i>amtB</i>	3.64 ± 1.22	1.25 ± 0.27	4.17 ± 1.32	1.91 ± 0.46	1.4 ± 0.39	0.32 ± 0.08	
	<i>ureA</i>	1.92 ± 0.67	6.32 ± 3.66	0.76 ± 0.21	5.56 ± 2.63	11.52 ± 7.39	1.18 ± 0.39	
	<i>gltB</i>	5.93 ± 2.38	39.57 ± 22.57	3.45 ± 1.12	13.37 ± 5.61	4.83 ± 1.63	1.40 ± 0.45	
	<i>nirD</i>	1.66 ± 0.49	2.08 ± 0.65	1.07 ± 0.27	1.74 ± 0.47	3.00 ± 1.13	0.34 ± 0.08	
	<i>msmeg_4699</i>	1.91 ± 1.05	5.45 ± 3.19	0.55 ± 0.19	6.36 ± 2.13	9.47 ± 4.59	0.54 ± 0.19	
	<i>msmeg_5442</i>	1.64 ± 0.79	2.19 ± 0.71	1.24 ± 0.42	3.01 ± 1.06	8.68 ± 2.98	0.94 ± 0.24	
	<i>msmeg_6272</i>	1.33 ± 0.44	1.37 ± 0.54	0.18 ± 0.08	4.08 ± 1.06	6.58 ± 3.45	0.5 ± 0.15	

*All highlighted values represent a statistically significant change in transcription ($p < 0.05$) as calculated by REST-284 V1. The calculated standard deviation is also given.

An investigation into the transcriptional regulation of genes encoding for the glutamate dehydrogenase enzymes revealed that none of the genes were regulated in response to ammonium limitation in wild type *M. smegmatis*. However, when the bacilli were cultured in medium containing an excess of ammonium for 1hr, *msmeg_4699*, *msmeg_5442* and *msmeg_6272* expression were up-regulated by approximately 6, 3 and 4 fold respectively (Table 5). In the GlnRKO strain, *msmeg_4699* and *msmeg_5442* transcription appeared to up-regulated upon ammonium starvation and all three genes were up-regulated upon exposure to ammonium excess conditions. In the AmtRKO strain, *msmeg_4699* and *msmeg_6272* expression was down-regulated in response to both nitrogen limitation and excess. This study indicates that both GlnR and AmtR may play a role in the transcriptional regulation of the genes encoding for GDH enzymes. GlnR appears to play an important role in the regulation of *msmeg_4699* and *msmeg_5442* expression, particularly under conditions of nitrogen limitation whilst AmtR appears to affect the transcriptional control of all three genes, largely when the bacteria are exposed to ammonium excess conditions.

2.3 DISCUSSION:

2.3.1 Regulation of nitrogen metabolism-related gene expression in response to ammonium availability as determined by real-time PCR.

The transcriptional regulation of nitrogen metabolism-related genes has been extensively investigated in the Actinomycetale model organisms: *C. glutamicum* and *S. coelicolor*. It is known that AmtR, the global regulator of nitrogen-related gene transcription in *C. glutamicum* blocks the expression of at least 33 genes in this organism under conditions of nitrogen sufficiency (Buchinger *et al.*, 2009). Upon exposure to nitrogen limiting conditions, the transcriptional repression is removed, resulting in the up-regulation of the expression of genes which include the *amtB-glnK-glnD* operon (Jakoby *et al.*, 2000), *glnA* (Nolden *et al.*, 2001a) and the *gltB/D* operon (Beckers *et al.*, 2001). The expression of *glnE* does not appear to be regulated in response to nitrogen availability in *C. glutamicum* (Buchinger *et al.*, 2009). In *S. coelicolor*, GlnR is the global regulator of

nitrogen metabolism-related gene expression and is able to both activate and repress gene transcription. The known GlnR regulon consists of 12 genes in *S. coelicolor*, of which the transcription of *glnA* (Wray, Jr. and Fisher, 1993; Wray, Jr. *et al.*, 1991) and *amtB*, among others, is activated in response to nitrogen starvation (Tiffert *et al.*, 2008). The expression of *glnE* and the *gltB/D* operon in *S. coelicolor* has not yet been investigated.

It has previously been shown that an increase in NH_4^+ concentration from 3.8mM to 38mM resulted in a 10 fold reduction in *M. tuberculosis* GS activity (Harth *et al.*, 1994). The observed GS activity response to the change in NH_4^+ concentration indicated that bacteria exposed to 3.8mM NH_4^+ were starved of nitrogen. In addition to a change in activity, a response at the level of GS transcription was also observed (Harth and Horwitz, 1997). An $(\text{NH}_4)_2\text{SO}_4$ concentration of 3mM was thus used to induce nitrogen starvation in *M. smegmatis* whilst Kirchner's medium containing 60mM $(\text{NH}_4)_2\text{SO}_4$ was considered as nitrogen sufficiency or excess (Harper *et al.*, 2010). The hallmark feature of a cellular nitrogen starvation response in many bacterial species (Amon *et al.*, 2010; Burkovski, 2003; Merrick and Edwards, 1995) is the up-regulation of *glnA* and in many cases, *amtB* transcription. This was indeed found for *M. smegmatis* cultured in Kirchner's medium containing 3mM $(\text{NH}_4)_2\text{SO}_4$. In addition, a down-regulation of *glnA* and *amtB* transcription was observed upon exposure of the bacilli to an NH_4^+ pulse which is indicative that our experimental conditions did stimulate a cellular nitrogen starvation and excess type transcriptional response. Our results indicate a similar pattern of gene transcription with regard to *glnA* and *amtB* expression in *M. smegmatis* as in the model Actinomycetales, which suggests a similar physiological importance of these proteins; namely the efficient transport and assimilation of ammonium under nitrogen limiting conditions. The rapidity of the *glnA* and *amtB* transcriptional response suggests that *M. smegmatis* is relatively sensitive to changes in exogenous ammonium concentrations, which in turn highlights the importance of the nitrogen metabolic pathway for the maintenance of bacterial homeostasis.

The expression of the genes encoding for one of the key nitrogen metabolic enzymes, GOGAT, is under the control of AmtR in *C. glutamicum*, and is thus regulated by the cellular nitrogen status in this organism (Beckers *et al.*, 2001). In contrast, the *gltB/D* transcriptional response to nitrogen availability in *S. coelicolor* is unclear and remains unknown in the mycobacteria. It has previously been shown that GOGAT activity is regulated in response to nitrogen availability in *S. coelicolor* (Fisher, 1989), and, in the absence of known post-translational regulatory mechanisms, it may be that GOGAT activity is regulated at the transcriptional level in this organism. The *gltB* transcriptional response in *M. smegmatis* was surprising, as *gltB* expression appeared to increase under both nitrogen limiting and excess conditions. Under nitrogen starvation conditions, an increase in *gltB* expression is expected as GOGAT functions in a cyclic mechanism in conjunction with GS for the assimilation of nitrogen under these conditions. Under conditions of nitrogen excess, GDH is thought to perform the principle nitrogen assimilatory role due to its bio-energetically more favourable catalytic processes. The up-regulation of *gltB* transcription under conditions of nitrogen excess in *M. smegmatis* remains unclear as GOGAT does not play a direct role in ammonium assimilation; however, it may be that a requirement for GOGAT production during nitrogen excess conditions exists for the maintenance of intracellular metabolite pool levels and subsequent bacterial homeostasis. Although it is not known whether GlnR plays a direct role in *gltB/D* transcription in *S. coelicolor*, it has more recently been shown that *gltB* expression is under the control of the stress-response sigma factor, σ^H , in this organism (Kormanec and Sevcikova, 2002). Since the *M. smegmatis* genome also encodes for homologous stress-response σ^H proteins (Singh and Singh, 2009) it may be that *gltB* expression is under similar control by homologous sigma factors. Culture of *M. smegmatis* in Kirchner's minimal medium as well as exposure to varying ammonium concentrations may have induced a stress response which could result in the unbiased up-regulation of *gltB* transcription observed in this study.

The adenylyltransferase, GlnE, has formed a focus of study in *M. tuberculosis* due to the observation that its function is essential for *M. tuberculosis* survival (Parish and Stoker,

2000a). It is known that the enzyme is important for the modulation of GS activity and to prevent futile energy expenditure; however, it is also thought that its GS modulating function is critical for the maintenance of the intracellular glutamate/glutamine ratios which appear to be essential for *M. tuberculosis* survival (Pashley *et al.*, 2006; Parish and Stoker, 2000a). Analysis of *glnE* transcriptional regulation in *M. tuberculosis* showed that the gene was not significantly regulated in response to ammonium availability (Pashley *et al.*, 2006). An investigation into the transcriptional regulation of *glnE* in *M. smegmatis* yielded similar results as observed in its pathogenic relative. The expression of *glnE* in *M. smegmatis* was relatively insensitive to changes in ammonium sulphate concentration. A modest increase in transcription (2 fold) was observed after 2hrs exposure to ammonium limitation but was not maintained after 4hrs exposure to the same conditions. It was also observed that *glnE* expression could be detected via reverse-transcriptase PCR when *M. smegmatis* was cultured in enriched 7H9 medium, yet no mRNA transcript could be detected when *M. smegmatis* was cultured in Kirchner's medium containing asparagine (Asn) as the sole nitrogen source (data not shown). In *M. tuberculosis* it was found that *glnE* expression did appear to be regulated in response to a variation of nitrogen source as much higher levels of *glnE* promoter activity could be detected when the bacilli were cultured in the presence of ammonium rather than amino acid nitrogen sources (Pashley *et al.*, 2006). Since 7H9 contains a variety of nitrogen sources, including ammonium (40mg/l ferric ammonium citrate), it is not surprising that *glnE* expression levels were higher in this medium in comparison with Kirchner's medium containing the amino acid Asn as the sole nitrogen source. The physiological reason for this particular regulatory observation, however, remains obscure.

The order of genes located in operons which encode for essential cellular machinery such as cell division proteins and ribosomes as well as operons encoding for genes whose products physically interact appear to be conserved among distantly related eubacteria (Thomas *et al.*, 2000). The products of *glnK* and *amtB* do physically interact and the genetic association between *glnK* and *amtB* can be found in a diverse range of Eubacteria as well as Archaea (Thomas *et al.*, 2000). The gene encoding for the uridylyltransferase,

GlnD (encoded by *glnD*), is also often found clustered together with *amtB* and *glnK*, however, it is not always considered to be genetically linked due to the observation that *amtB* and *glnK* are up-regulated upon exposure to nitrogen limitation and *glnD* does not appear to be regulated in response to nitrogen availability in many bacteria (Nolden *et al.*, 2001b). In the Actinomycetes, however, the genetic organization appears to be conserved (Nolden *et al.*, 2001b). In *C. glutamicum*, *amtB*, *glnK* and *glnD* are transcribed as an operon (Jakoby *et al.*, 2000) and its expression is up-regulated in response to nitrogen starvation after removal of the DNA-bound repressor, AmtR (Nolden *et al.*, 2001b). These three genes are also co-transcribed in *S. coelicolor* and their expression is under the control of GlnR in response to nitrogen availability (Hesketh *et al.*, 2002). In *M. smegmatis*, the expression of each gene located in the potential operon was investigated in response to ammonium availability. It was found, in accordance with other studies (Amon *et al.*, 2008a), that *amtB* expression increased upon nitrogen starvation and was down-regulated upon exposure to an ammonium pulse. Contrary to our expectations, *glnK* expression was significantly repressed during nitrogen starvation conditions and did not appear to be affected by an ammonium pulse. In contrast, *glnD* transcription did not appear to be highly affected by changes in ammonium concentration. A previous study briefly investigated *glnK* and *glnD* expression in response to nitrogen limitation in *M. smegmatis*, but could not detect any significant regulation of *glnK* or *glnD* expression under the conditions tested (Nolden *et al.*, 2001b). This result suggests that *glnK* and *glnD* are not transcribed together with *amtB* in *M. smegmatis* and are not regulated in a similar fashion. In support hereof, the authors could not detect a common real-time PCR transcript between the three genes (Nolden *et al.*, 2001b).

More recently a study declared that a common transcript of *amtB*, *glnK* and *glnD* could be detected in *M. smegmatis* (Amon *et al.*, 2008a); however, the authors did not investigate the expression of each gene individually. We also investigated whether mRNA transcripts could be detected spanning the junctions between *amtB* and *glnK*, as well as between *glnK* and *glnD*, and it was found that such transcripts did indeed exist which suggests co-transcription of these genes in *M. smegmatis*. However, in the light of

the differences observed in the relative changes in gene expression in response to ammonium availability, co-transcription of these genes appears contradictory. It may be that a limited number of polycistronic mRNA transcripts are produced and are exponentially amplified by PCR which leads to the assumption that all three genes are similarly transcriptionally regulated. Although no regulatory DNA sequences could be detected between the three genes, it may be that as yet unidentified transcriptional regulators could play a role in the differential expression observed or that post-transcriptional mechanisms, such as mRNA degradation, may play a role in the transcriptional regulation of these genes.

The physiological roles of AmtB, GlnK and GlnD have been well studied in *C. glutamicum* and have been found to be essential for the transcriptional and GS activity adaptation to changing environmental nitrogen concentration conditions. However, little is known of the physiological significance of *glnK* and *glnD* in *S. coelicolor* as they do not appear to be required for the same functions they perform in *C. glutamicum*. Even less is known about the roles of GlnK and GlnD in the mycobacteria. The observed down-regulation of *glnK* expression under our experimental conditions remains obscure; however, the lack of *glnD* transcriptional regulation observed in *M. smegmatis* may indicate a possible nitrogen sensory role, similar to that of *E. coli*.

2.3.2 *glnR* and *amtR* expression.

The expression of *glnR* does appear to be regulated in response to nitrogen availability in *S. coelicolor* since there is an increase in transcription upon exposure to nitrogen limitation and a down-regulation of expression when nitrogen sufficient conditions return (Tiffert *et al.*, 2008). It is still unclear; however, as to which factors are responsible for the observed *glnR* transcriptional regulation in *S. coelicolor* (Reuther and Wohlleben, 2007). Previous work showed that the *glnR* homolog in *M. smegmatis*, which shares a 46% amino acid identity to *S. coelicolor glnR*, did not respond on a transcriptional level to nitrogen starvation conditions (Amon *et al.*, 2008a). However, in contrast, our results indicate an increase in *glnR* expression, which was observed as an increase in promoter

activity, upon exposure to nitrogen starvation conditions, similar to that observed for *S. coelicolor*. Since no GlnR-binding box could be identified upstream of *glnR* in *M. smegmatis* (Amon *et al.*, 2008a), it remains unclear as to which factors are responsible for the transcriptional regulation of this gene.

It has recently been shown that PhoP, the OmpR-type response regulator of the PhoR/P two component system in *S. coelicolor*, which regulates the transcription of genes in response to phosphate levels, is able to bind to operator sequences in the promoter region of *glnR* (Rodriguez-Garcia *et al.*, 2009). Although the regulation of *glnR* by PhoP in response to nitrogen availability has not yet been explored in *M. smegmatis*, it may be that *glnR* is regulated by mechanisms similar to that of PhoP.

In *C. glutamicum*, the gene encoding for the master regulator of nitrogen-related genes, *amtR*, appears to be constitutively transcribed with low amounts of *amtR* transcript expressed under various conditions of nitrogen availability (Jakoby *et al.*, 2000). Our analysis of the promoter activity directly upstream of the *amtR* homolog in *M. smegmatis* revealed that *amtR* expression is most likely not driven from a promoter in the area studied. In contrast, it appeared as though the promoter responsible for *amtR* expression was situated in the region upstream of the gene adjacent to *amtR*, *msmeg_4301*, which suggests that *amtR* is located in an operon together with *msmeg_4301*. Our results also indicated that the promoter activity upstream of *msmeg_4301* remained relatively constant when *M. smegmatis* was exposed to ammonium starvation and excess conditions which suggests that *amtR* expression is constant under varying ammonium concentration conditions, similar to *amtR* expression in *C. glutamicum*.

2.3.3 *In vitro* and *in vivo* growth of *glnR* and *amtR* knockout *M. smegmatis* strains.

The physiological roles of AmtR and GlnR were investigated in *M. smegmatis* by replacing the functional form of *amtR* or *glnR* with a deleted form of the gene. Initial characterization of the knockout strains involved the study of their growth on solid agar

and in liquid media in order to determine whether differences in growth rate and viability could be distinguished in comparison with wild type *M. smegmatis*.

The knockout strains and wild type *M. smegmatis* were cultured on enriched 7H10 agar in order to determine whether a difference in bacterial viability on solid media could be detected. Since no significant difference in CFU count between the wild type and knockout *M. smegmatis* strains could be observed, the result suggests that all strains could survive similarly on enriched solid agar. However, the GlnRKO colonies appeared to be much smaller than either the wild type or AmtRKO colonies. The diminished GlnRKO colony size may reflect a lower bacterial count than the larger colonies, which in turn suggests that the GlnRKO strain exhibits a slower rate of cell division on 7H10 agar in comparison to the wild type and AmtRKO *M. smegmatis* strains. Analysis of GlnRKO strain growth in enriched/complex liquid medium (7H9), however, revealed no deleterious effects on growth which is similar to the result obtained during growth analysis studies of a *glnR* knockout in *S. coelicolor* (Fink *et al.*, 2002) and *M. smegmatis* (Amon *et al.*, 2008a). Similarly, an *amtR* deletion did not have any effect on the bacterial growth rate in complex liquid medium. These are not surprising results as enriched 7H9 medium contains numerous carbon and nitrogen sources to support bacterial growth. Growth of the knockout strains in Kirchner's minimal medium containing various concentrations of ammonium sulphate as their sole nitrogen source revealed that only the GlnRKO strain was compromised in its ability to adapt and grow, particularly under ammonium starvation conditions which indicates that a *glnR* knockout affects the ability of the bacilli to assimilate ammonium under low ammonium availability conditions. In contrast, an *amtR* knockout did not appear to affect bacterial growth under any of the conditions tested which is in contrast to the retarded growth rate observed of a *C. glutamicum amtR* deletion mutant under analogous conditions (Takano *et al.*, 2008). Therefore, AmtR may not be critical for the assimilation of ammonium in *M. smegmatis*.

Initial work to compare the intracellular survival kinetics of knockout strains versus wild type *M. smegmatis*, made use of the human acute monocytic leukemia cell line known as THP-1 cells. A range of MOI's (1 to 5) were used to infect THP-1 cells, which had been

stimulated to differentiate into active macrophages. It was found, however, that a large number of extracellular bacteria were present after infection of the macrophages at the completion of the experiment, which confounded the results. The extracellular bacteria were able to grow and replicate in the medium bathing the THP1 cells and their presence may have originated through insufficient washing of the macrophages immediately after infection or through inadequate phagocytosis of the bacteria by the THP1 cells. THP-1 derived macrophages are often used as a model for the study of *M. tuberculosis* intracellular growth and infection (Theus *et al.*, 2004); however, for our study, it appeared to be a poor model for *M. smegmatis* infection. This may be due to the rapid *M. smegmatis* growth rate (3hrs) in comparison to *M. tuberculosis* (24hrs) which could overwhelm the phagocytic and killing ability of the THP-1 cells or it may be due to poor experimental execution. As an alternative approach, bone derived macrophages isolated from the C57BL/6 mouse strain were utilized for determination of intracellular *M. smegmatis* survival. Upon electron microscopy analysis, it was observed that these cells have larger and more numerous lysosomes in comparison with THP1 cells (personal communication, Chantelle de Chatelier) which suggests that they have a more potent killing ability. An initial MOI of 5 was used for these experiments, however, similar to what was found with the THP-1 cells, a large degree of extracellular bacterial growth was observed. An MOI of 1 was used to infect the murine macrophages and, together with thorough washing and a mild gentamycin treatment, it was found that extracellular bacterial growth could be prevented during the course of the experiment.

The killing of wild type *M. smegmatis* by C57BL/6 bone derived macrophages has previously been investigated and it was found that the bacilli were rapidly killed within the first 4hrs post-infection. Within the subsequent 4hrs, however, there was a swift increase in bacterial load to numbers greater than the initial MOI, due to apparent intracellular growth. Thereafter, the macrophages appeared to steadily eliminate the infection (Jordao *et al.*, 2008). It is thought that the observed intracellular macrophage killing and bacterial growth phases are induced by the doubling time of the infecting bacteria which, in this case, was every 3 to 4.5hrs (Jordao *et al.*, 2008). Our results indicated a similar trend, with a wild type *M. smegmatis* killing phase within the first 4hrs

followed by an intracellular bacterial growth phase within the next 4hrs. Earlier work has shown that macrophages, depending on their origin and unique killing mechanisms, require between 24hrs and 48hrs after infection to completely kill *M. smegmatis* (Jordao *et al.*, 2008), thus our relatively high bacterial numbers observed 24hrs post-infection are not unusual since complete eradication of the infection by the murine macrophages may occur only within the ensuing 24hrs. Our results indicate that an *amtR* deletion does not adversely affect intracellular survival as the knockout strain and wild type *M. smegmatis* displayed analogous survival curves. Although not statistically significant, it was interesting to note that the AmtRKO strain displayed a slightly elevated survival rate in comparison with wild type *M. smegmatis*. A similar trend was observed for AmtRKO strain growth in enriched 7H9 medium and Kirchner's minimal medium containing various ammonium sulphate concentrations as AmtRKO strain growth appeared to be slightly more rapid than for either the wild type or GlnRKO strains (section 2.2.4). It may be that an *amtR* knockout allows for a slightly enhanced rate of cell division. In contrast, a *glnR* deletion did appear to severely compromise the capability of the bacterium to survive intracellularly. Given the detrimental effect that this mutation has on the ability of the bacterium to assimilate nitrogen (as seen by the poor growth of the GlnRKO strain under ammonium limiting conditions, section 2.2.4), it may be that the GlnRKO strain is unable to survive due to the innate inability of the *glnR* knockout strain to assimilate nitrogen from the nutrient deficient phagolysosome (Lorenz *et al.*, 2002). In addition, the GlnRKO strain may not be able to adequately adapt its nitrogen metabolic response upon onslaught by reactive nitrogen species and other macrophage weaponry. Thus GlnR appears to be critical for intact ammonium assimilation processes which, in turn, is required for adequate growth both *in vitro* and *in vivo*.

2.3.4 Relative change in expression of nitrogen metabolism-related genes in wild type, GlnRKO and AmtRKO *M. smegmatis* strains.

2.3.4.1 *glnA* and *amtB* expression

Previous work has shown that GlnR in *M. smegmatis* is able to regulate *glnA* and *amtB* expression in response to nitrogen availability (Amon *et al.*, 2008a). In our GlnRKO strain, it was found that *glnA* and *amtB* expression was up-regulated in response to both nitrogen limitation and excess which is a surprising observation given that previous studies reported an absence of *glnA* and *amtB* transcriptional change when an *M. smegmatis glnR* knockout strain was cultured in modified 7H9 medium without a nitrogen source (Amon *et al.*, 2008a). Our results differ from this previous report, which may be owing to the effect of different experimental conditions. In our study the bacilli were cultured in a minimal medium, which could exert different biological pressures in comparison to enriched 7H9 medium. Our results thus point to the possible existence of an additional regulatory system(s), independent of GlnR, which may be active under our culture conditions. Our *glnA* and *amtB* transcriptional results also indicate a possible absence of gene repression by GlnR during nitrogen excess conditions.

2.3.4.2 *ureA* expression

The urease enzyme is able to metabolize urea to produce ammonia which can subsequently be assimilated via GS or GDH into glutamine or glutamate respectively. In *M. tuberculosis*, it was found that urease expression increased under asparagine limitation conditions presumably to enhance the ability of the bacterium to utilize alternative nitrogen sources in the environment (Clemens *et al.*, 1995). Interestingly, urease has been implicated as a possible virulence factor of *M. tuberculosis* since the production of ammonia from urea is thought to alkalinize the pathogen's microenvironment and thereby prohibit phagosome-lysosome fusion (Clemens *et al.*, 1995). The transcriptional response of the urease operon to ammonium availability in the mycobacteria is, however, unknown. Our results point to an unbiased up-regulation of *ureA* expression during both

ammonium limitation and excess conditions. The physiological reasons for the observed enhanced *ureA* expression under ammonium starvation conditions may be similar to that described for *M. tuberculosis* during asparagine limitation i.e. for the utilization of other possible nitrogen sources. However, the increase in *ureA* expression during ammonium excess conditions is rather obscure. It was found that an atypically large up-regulation of *ureA* expression occurred in the GlnRKO strain during exposure to both nitrogen limiting and excess conditions in comparison with wild type *M. smegmatis* which points to an absence of transcriptional repression under these conditions. In *S. coelicolor*, *ureA* forms part of the GlnR regulon and its transcription is repressed during nitrogen limiting conditions (Tiffert *et al.*, 2008). Although we did not observe any significant repression of *ureA* transcription in wild type *M. smegmatis* under our ammonium starvation conditions, we could show a de-regulation of *ureA* transcription in the GlnRKO strain, which may point to this regulator playing a possible repressive role, similar to its homolog in *S. coelicolor*. In contrast, a complete lack of *ureA* regulation was observed in the AmtRKO strain. In *C. glutamicum*, the expression of *ureA* is repressed by AmtR during nitrogen excess conditions and the repression is removed during nitrogen limitation (Beckers *et al.*, 2004); however, our results indicate that AmtR may enhance or activate *ureA* expression in *M. smegmatis* under both ammonium availability conditions. Therefore, if AmtR does indeed regulate *ureA* transcription in *M. smegmatis* then it would appear that the regulator plays a different physiological role from its *C. glutamicum* homologue.

2.3.4.3 *gltB* expression

The transcriptional regulation of the genes encoding for GOGAT, namely the *gltBD* operon, in response to ammonium availability was investigated earlier in the chapter (section 2.2.1). In the current study, the expression of *gltB* in wild type *M. smegmatis* was investigated in response to ammonium starvation and excess. Similar to our previous results, *gltB* expression was up-regulated during both ammonium starvation and excess conditions (possible reasons for this observation are outlined in section 2.3.1). In the GlnRKO strain, there appeared to be a very strong up-regulation of *gltB* transcription

under nitrogen limiting conditions, which suggests a lack of transcriptional control by GlnR under these conditions. In contrast, there appears to be an absence of *gltB* transcriptional regulation in the AmtRKO strain under conditions of ammonium excess. Thus, it may be that both regulators are involved in the fine-tuning of *gltB* expression under varying conditions of ammonium availability. Previous reports suggest that *gltB* expression is under the direct control of the stress response factor σ^H in *S. coelicolor* (Kormanec and Sevcikova, 2002). Thus it may also be that a genomic deletion of either *glnR* or *amtR* may cause certain cellular stresses which, in turn, may direct the observed *gltB* transcriptional response by homologous σ^H proteins in *M. smegmatis*.

2.3.4.4 *nirD* expression

A functional nitrate/nitrite assimilation pathway has been shown to be active in both *M. smegmatis* (Khan *et al.*, 2008) and *M. tuberculosis* (Malm *et al.*, 2009). Nitrate is reduced to nitrite by nitrate reductase which is encoded by the constitutively expressed *NarGHIIJ* operon. Nitrite is converted to ammonia by nitrite reductase (encoded by the *nirBD* operon) which can be assimilated via GS or GDH. It was observed that *nirBD* expression was up-regulated by GlnR under nitrate limiting conditions in *S. coelicolor* (Tiffert *et al.*, 2008); however no *nirBD* homolog in *C. glutamicum* could be identified (Malm *et al.*, 2009). In *M. tuberculosis* it was found that GlnR was responsible for the up-regulation of *nirBD* when nitrate was supplied as the sole nitrogen source at limiting concentrations (Malm *et al.*, 2009); however, it has not yet been investigated whether the *nirBD* genes are regulated in response to varying ammonium concentrations when it is supplied as the sole nitrogen source. An up-regulation of *nirD* expression in wild type *M. smegmatis* was observed, similar to *gltB* and *ureA*, under both nitrogen limiting and excess conditions. Comparable *nirD* transcriptional regulation occurred in the GlnRKO strain which could eliminate GlnR as a regulator of *nirBD* expression under our experimental conditions. In contrast, the absence of AmtR in *M. smegmatis* did appear to cause a de-regulation of *nirD* expression since there was a lack of transcriptional up-regulation under both experimental conditions. Similar to the regulation of *ureA* expression, it would seem that

in *M. smegmatis*, AmtR plays a different physiological role to its *C. glutamicum* counterpart.

2.3.4.5 GDH gene expression

All three genes encoding for possible GDH enzymes appeared to be regulated only in response to ammonium excess in wild type *M. smegmatis*. The observed up-regulation of GDH gene expression is not unreasonable since GDH enzymes are thought to play the predominant ammonium assimilatory role under conditions of nitrogen sufficiency (Fisher, 1989). Both AmtR and GlnR appear to be involved in the regulation of genes encoding for the GDH enzymes in *M. smegmatis*. The expression of *msmeg_4699* and *msmeg_5442* were up-regulated during nitrogen starvation in the absence of *glnR* whilst *msmeg_6272* expression appeared to remain unaffected. Thus GlnR may play a repressive role with regard to the transcriptional control of these two genes. The expression of *msmeg_4699* and *msmeg_6272* appeared to be down-regulated in the absence of AmtR during ammonium starvation conditions which implies that AmtR functions either as a transcriptional activator or that the protein prevents undue repression under these conditions. Exposure to ammonium excess conditions resulted in the transcriptional up-regulation of all three genes in both wild type *M. smegmatis* and in the GlnRKO strain which suggests that GlnR does not regulate transcription under these conditions. In contrast, a deletion of *amtR* resulted in either a down-regulation in gene transcription (*msmeg_4699* and *msmeg_6272*) or a complete absence of transcriptional control (*msmeg_5442*). These results hint at a rather complex mechanism of GDH transcriptional control which appears to be dependent on the gene investigated as well as the concentration of ammonium to which the bacilli are exposed. In summary, *msmeg_5442* expression appears to be controlled by GlnR during ammonium starvation whilst *msmeg_6272* seems to be under the control of AmtR. The expression of *msmeg_4699* on the other hand seems to be influenced by both potential regulators under ammonium limiting conditions. In contrast, AmtR appears to play a role in the transcriptional control of all three genes during exposure to ammonium excess conditions.

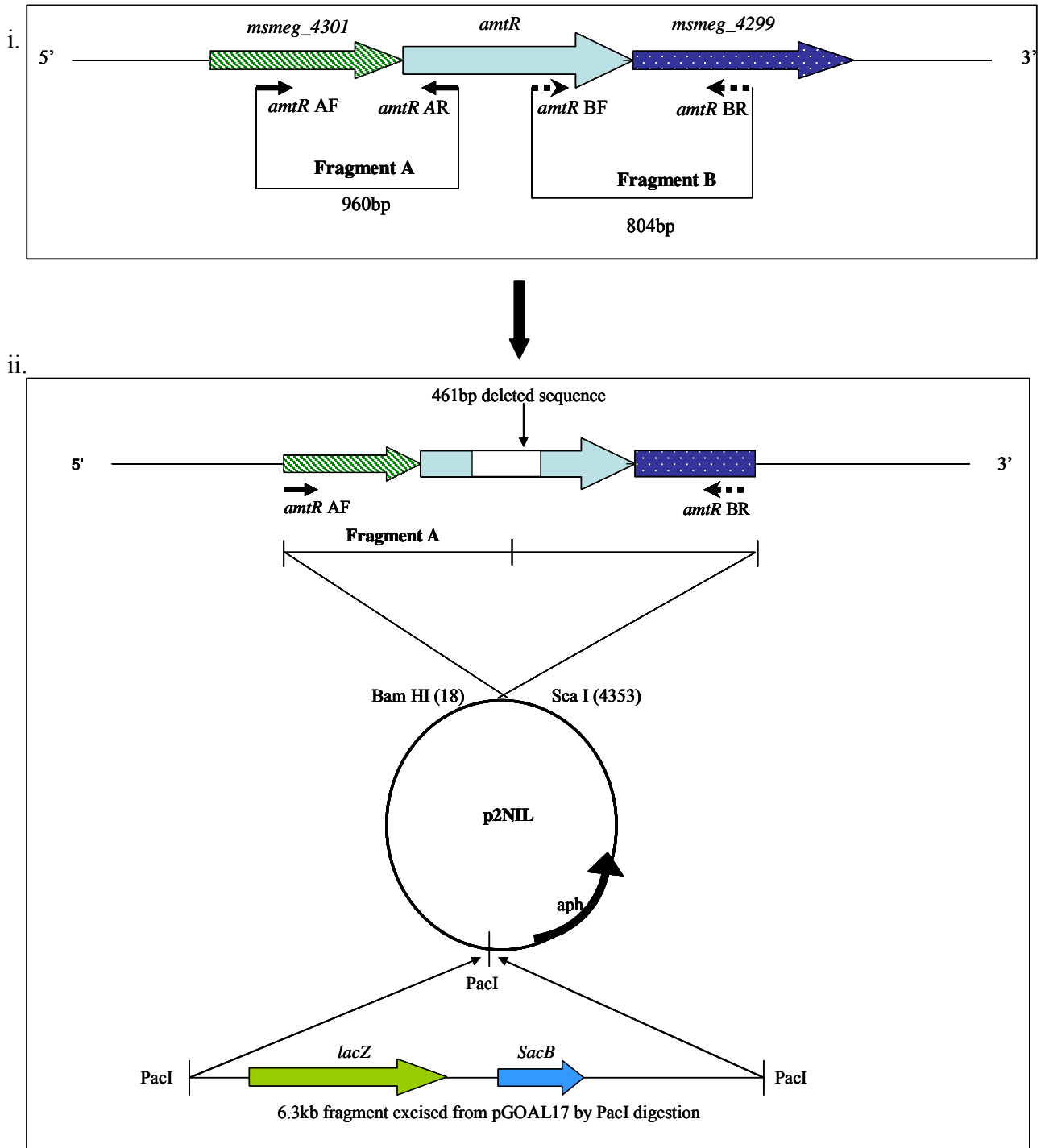
2.3.4.6 Synopsis of the role of GlnR and AmtR in nitrogen metabolism-related gene expression.

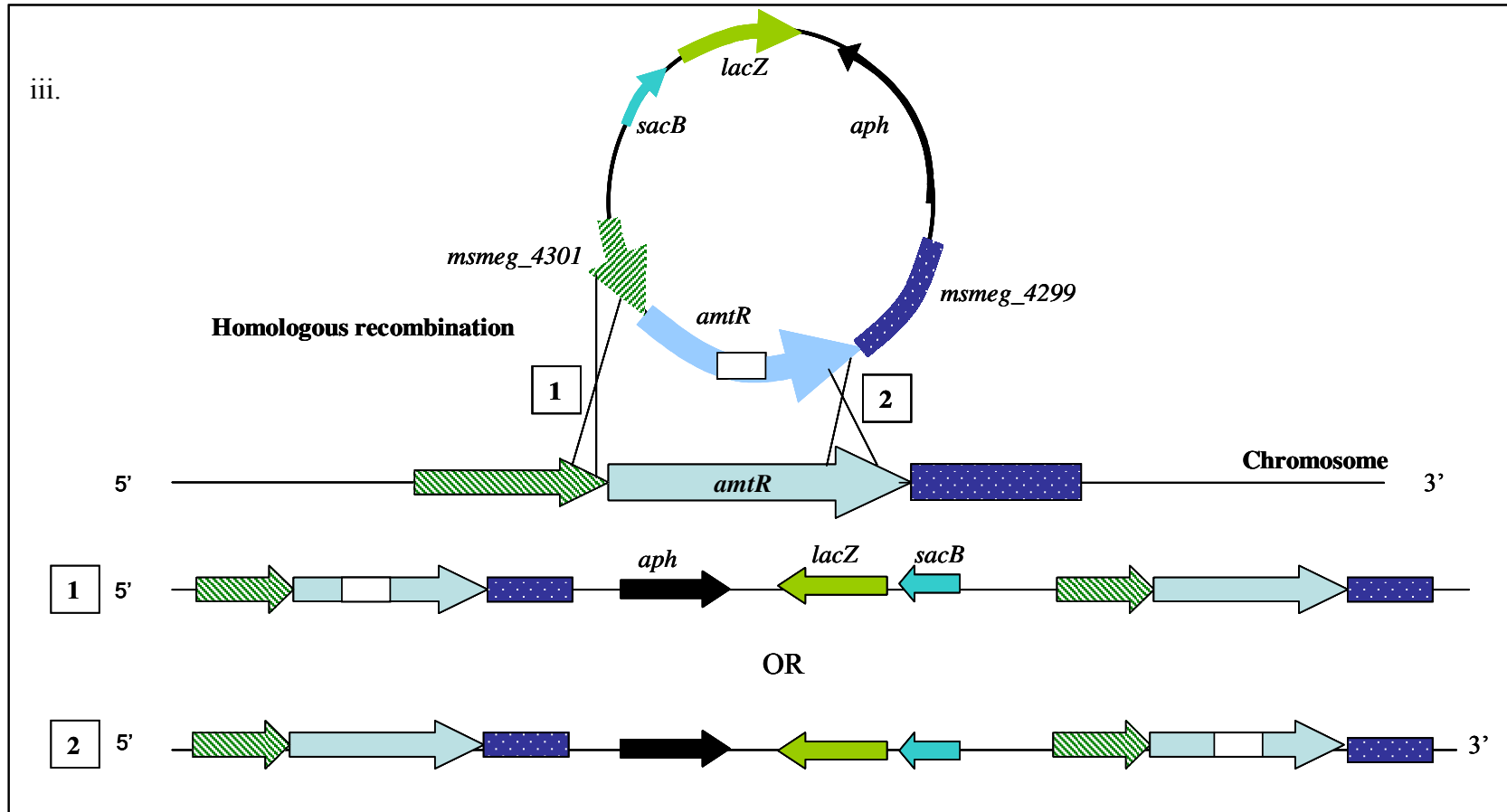
In summary, the results suggest that GlnR may play a role in the transcriptional control of *glnA*, *amtB*, *gltB*, *ureA*, *msmeg_4699* and *msmeg_5442* during both ammonium limitation and excess. GlnR did not seem to affect *msmeg_6272* transcriptional responses. In the case of *ureA*, *msmeg_5442* and *_4699*, a *glnR* deletion resulted in an up-regulation of gene transcription during nitrogen limitation which points to a repressive role played by GlnR under these conditions. Since it is known that GlnR is able to activate the expression of *glnA* and *amtB* during nitrogen limiting conditions (Amon *et al.*, 2008a), the observed up-regulation of *glnA*, *amtB* and *gltB* expression under the same conditions in the GlnRKO strain may point to the involvement of other factors, independent of GlnR, which could play a role in the transcriptional regulation of these genes. Upon exposure of the bacilli to an ammonium pulse, it was found that *glnA*, *ureA*, *msmeg_4699* and *msmeg_5442* expression was also atypically up-regulated when *glnR* was not present in the *M. smegmatis* genome which also points to a lack of transcriptional repression under these conditions. The absence of an up-regulation of *gltB* expression under these conditions in the GlnRKO strain indicates that GlnR may activate *gltBD* transcription during nitrogen sufficient conditions. AmtR appeared to play a role in the transcriptional response of *nirD*, *ureA*, *gltB* and all three GDH genes. In general, the expression of these genes appeared to be down-regulated or not regulated at all, in response to either ammonium limitation or excess conditions. In the case of *ureA* and *nirD* expression, there was a lack of up-regulation or activation of transcription during either nitrogen limitation or excess. In fact, the expression of some of the genes including *ureA*, *nirD*, *msmeg_4699* and *msmeg_6272* appeared to be repressed. These results indicate a possible inability to activate gene transcription or an inability to remove transcriptional repression in the absence of AmtR, which is in direct contrast to the repressive role which the AmtR homolog in *C. glutamicum* is known to fulfill.

Previous work could not identify GlnR binding sites upstream of *gltB/D*, *ureA* and the genes encoding for the GDH enzymes (Amon *et al.*, 2008a); however, our data does implicate GlnR in the transcriptional control of these genes. It could be that GlnR may bind to DNA sequences slightly dissimilar to the binding box already characterized (Amon *et al.*, 2008a) and thereby escape detection, or GlnR may control the expression of these genes in an indirect manner such as through transcriptional regulation of additional regulatory proteins. It has recently been shown that PhoP, the OmpR-type response regulator of the PhoR/P two component system in *S. coelicolor*, which regulates the transcription of genes in response to phosphate levels, is able to bind to operator sequences in the promoter regions of *glnR*, *amtB* and *glnA* (Rodriguez-Garcia *et al.*, 2009). The expression of nitrogen related genes is therefore under both direct and indirect control of an entirely different regulator in response to phosphate concentration in this organism. A PhoR/P two component system in *M. tuberculosis* has been shown to be essential for the production for virulence-associated lipids (sulpholipids, diacyltrehaloses and polyacyltrehaloses) (Walters *et al.*, 2006); however, this system does not appear to be involved in phosphate metabolism in this pathogen. Homologous PhoR/P genes are present in *M. smegmatis* but the transcriptional regulation of phosphate-related genes appears to be under the control of a different two-component system (SenX3/RegX3) (Glover *et al.*, 2007). The role of these different two-component systems with regard to the regulation of nitrogen-related genes in the mycobacteria remains to be investigated. The absence of a proposed regulator should, in theory, cause no change in target gene/s expression in response to varying nitrogen availability. Our results, however, do indicate transcriptional change in the absence of a regulator. For example, *glnA* expression is up-regulated upon nitrogen starvation and excess in the GlnRKO strain. This points to the presence of additional factors governing the regulation of nitrogen-related genes in *M. smegmatis* with interesting candidates being the response regulators of other two-component systems.

APPENDIX 2A: Schematic representation of the methodology used to introduce an *amtR* deletion into the *M. smegmatis* genome.

Unmarked deletion mutants in *Mycobacterium smegmatis* were generated using techniques described by Parish and Stoker, 2000 as well as by Goosens, 2008 (MSc thesis). The replacement of a functional copy of a target gene, such as *amtR*, with a deleted form of the gene was achieved by homologous recombination.





iv.

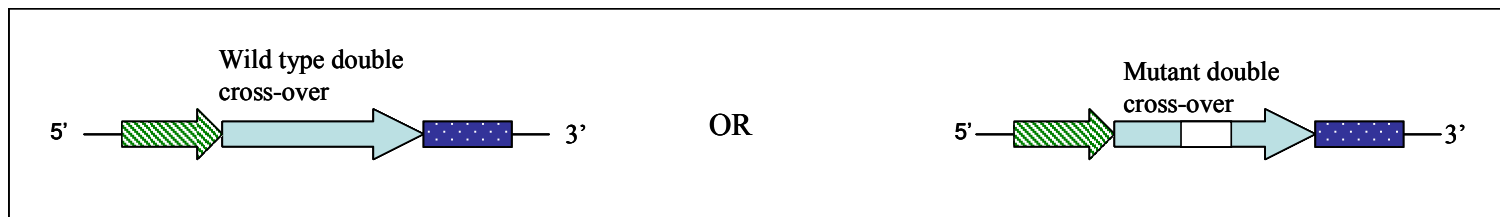


Figure 1: (i) PCR amplification of approximately 1000bp upstream (Fragment A) and 1000bp down-stream (Fragment B) of the desired deletion region. Primers used to amplify these fragments had unique restriction digestion sites for (ii) ligation of the two fragments (A and B) to generate a 1764bp region (Fragment A/B) which was homologous to the *M. smegmatis* chromosome but had a 461bp deletion in the *amtR* gene. Fragment A/B was cloned into unique restriction sites (BamHI and ScaI) of the p2NIL suicide plasmid. In addition, a 6.3kb fragment containing the *lacZ* selection marker and the *sacB* counter-selection markers were excised from the pGOAL17 plasmid by PacI digestion and cloned into a unique PacI site in p2NIL. (iii) Upon transformation of the knockout construct into *M. smegmatis*, two possible recombination events can occur. The single cross-over colonies were isolated and cultured on selection media to force a second recombination event which would result in either (iv) an *amtR* mutant genotype or reversion to the wild type genotype.

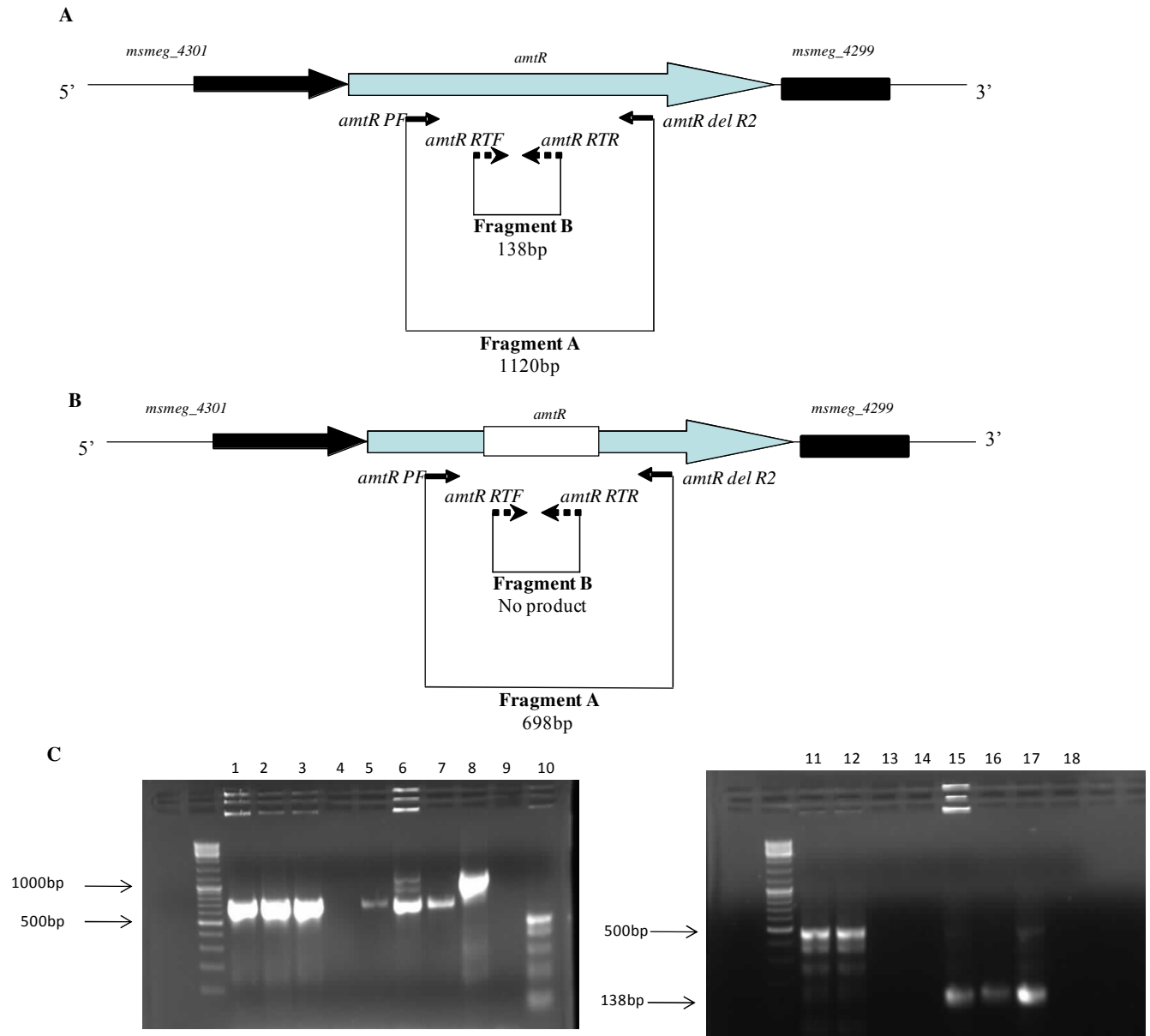
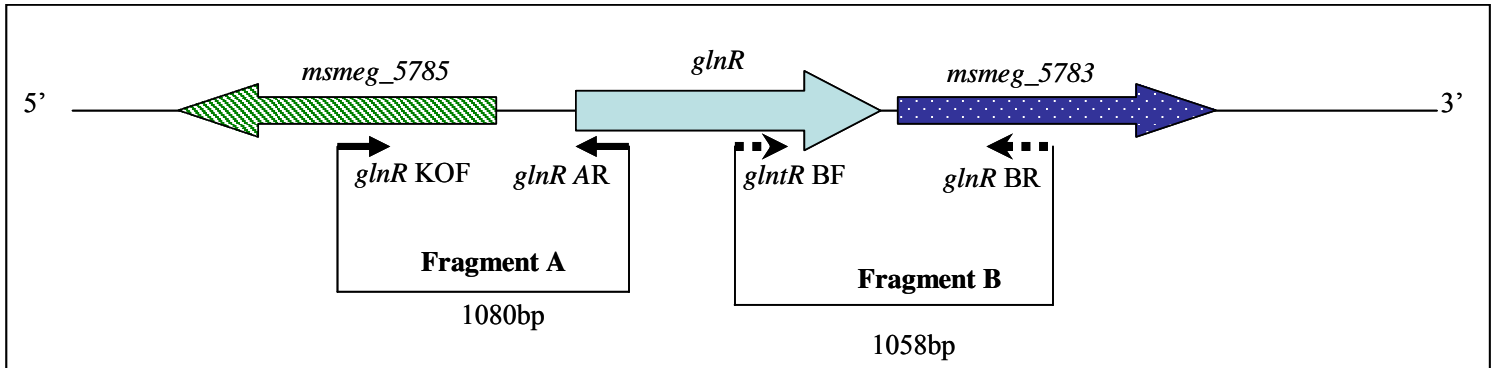


Figure 2: Schematic representation of the PCR reactions used to identify (A) wild type *M. smegmatis* and (B) *amtR* knockout *M. smegmatis*. The PCR products obtained (Fragments A and B) as well as the primers used to amplify those products are given. (C) Gel image of the electrophoretic separation of Fragments A (lanes 1-7) and B (lanes 10-16) amplified from 7 potential *amtR* knockout strains. Lanes 8 and 17 are positive controls for Fragments A and B respectively. Lanes 9 and 18 are negative controls for Fragments A and B respectively.

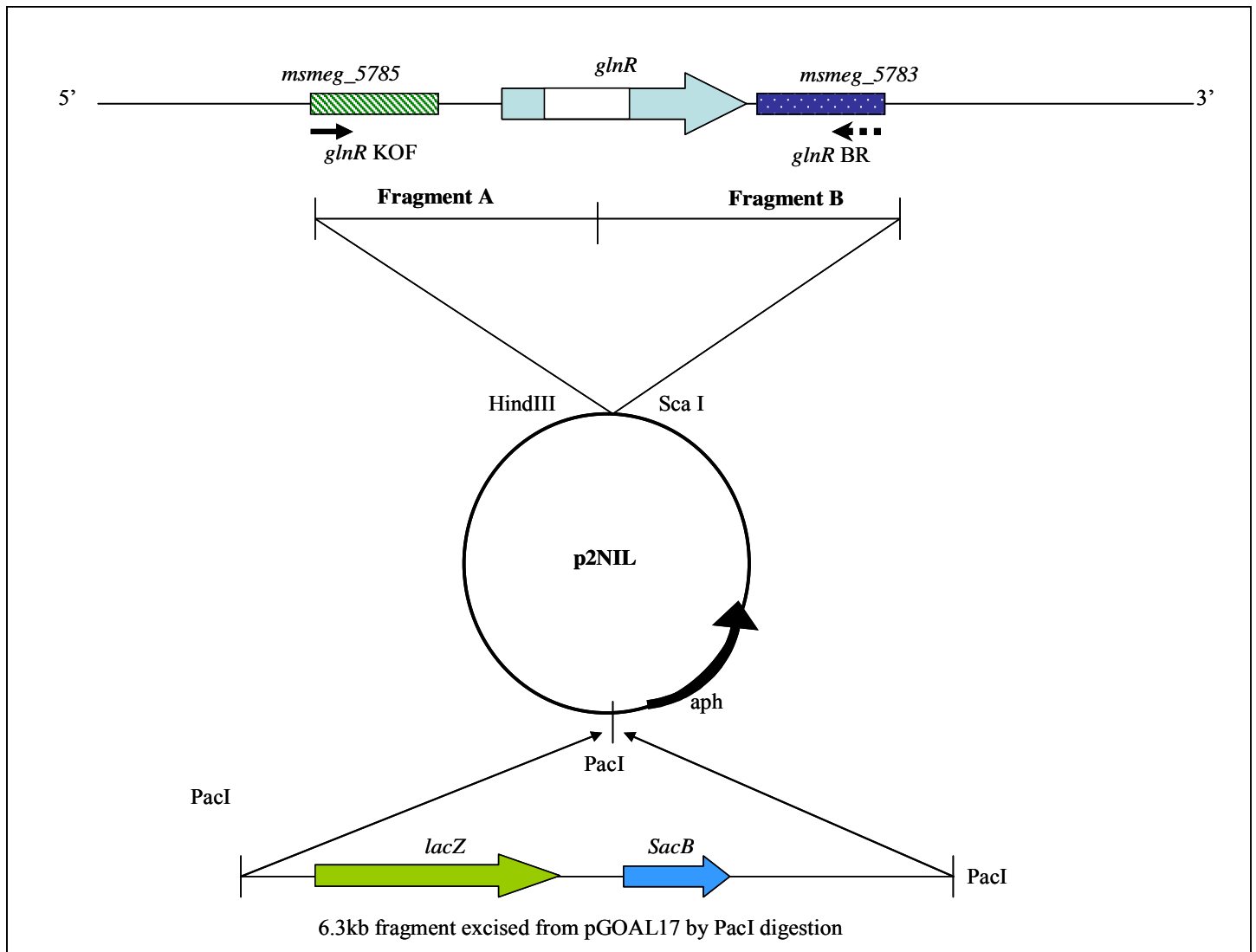
It can be seen from Figure 2A that, in the absence of a deletion in the *amtR* gene, the size of Fragment A would be 1120bp. The positive control for the amplification of Fragment A yielded a PCR product of approximately 1120bp which was an expected result, as purified DNA from wild type *M. smegmatis* cells was added to the reaction as DNA template. With the exception of the colony represented by the PCR reaction in Figure 2C, lane 4, the amplification of Fragment A from the 7 colonies screened yielded a smaller PCR product of approximately 700bp (Figure 2C, lanes 1-7) which coincided with the size of Fragment A when a copy of the deleted form of *amtR* was present in the cell (Figure 2B). Upon amplification of Fragment B (Figure 2C, lanes 10-16), non-specific PCR amplicons were observed after PCR of the colonies analysed in lanes 10-12. In contrast, no PCR product was found after amplification of Fragment B from colonies represented by lanes 13 and 14. PCR amplicons from colonies represented in lanes 15-16 yielded products (approximately 138bp) which correspond to Fragment B in wild type *M. smegmatis* (Figure 2A). From these results, it is clear that the colonies analysed in lanes 6 and 7 (and lanes 15 and 16) have undergone a single crossover homologous recombination event as a copy of both the deleted and undeleted form of *amtR* is present. The results obtained for colonies represented in lanes 4 and 5 (and lanes 13 and 14) were repeated and it was found that these were also single cross-over colonies. The colonies represented by lanes 1-3 (and lanes 1-12) had, however, undergone a double cross-over event to create a deletion within the *amtR* gene sequence. Although PCR products were obtained for the PCR amplification of Fragment B in these colonies, the products were, however, as a result of non-specific amplification. All PCR products obtained were submitted for sequencing in order to confirm the results obtained by PCR screening.

APPENDIX 2B: Schematic representation of the methodology used to introduce a *glnR* deletion into the *M. smegmatis* genome.

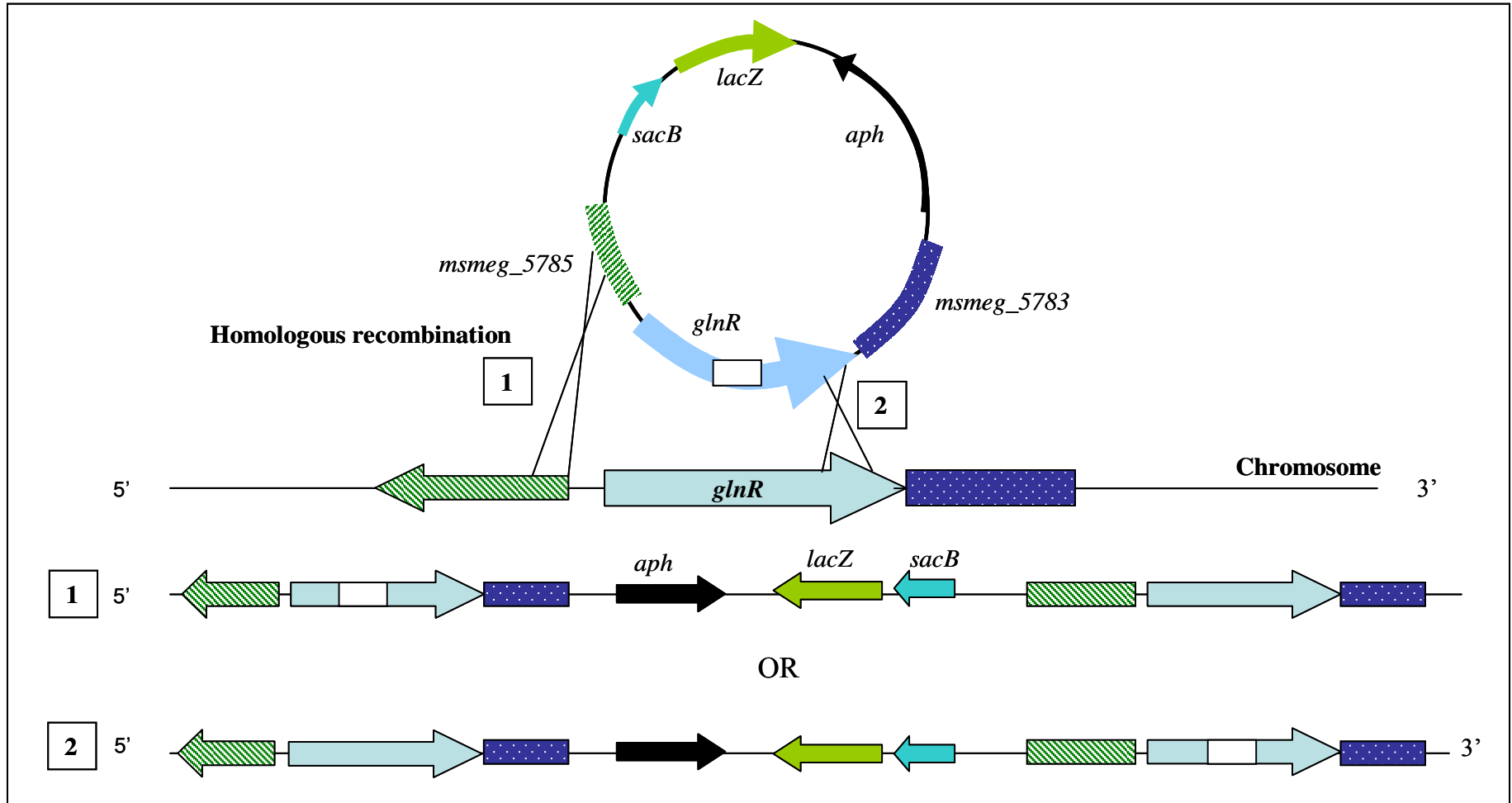
i.



ii)



iii)



iv)

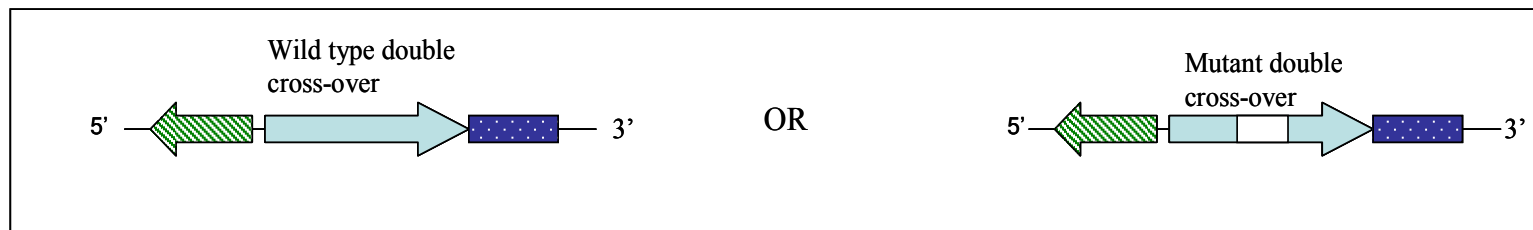
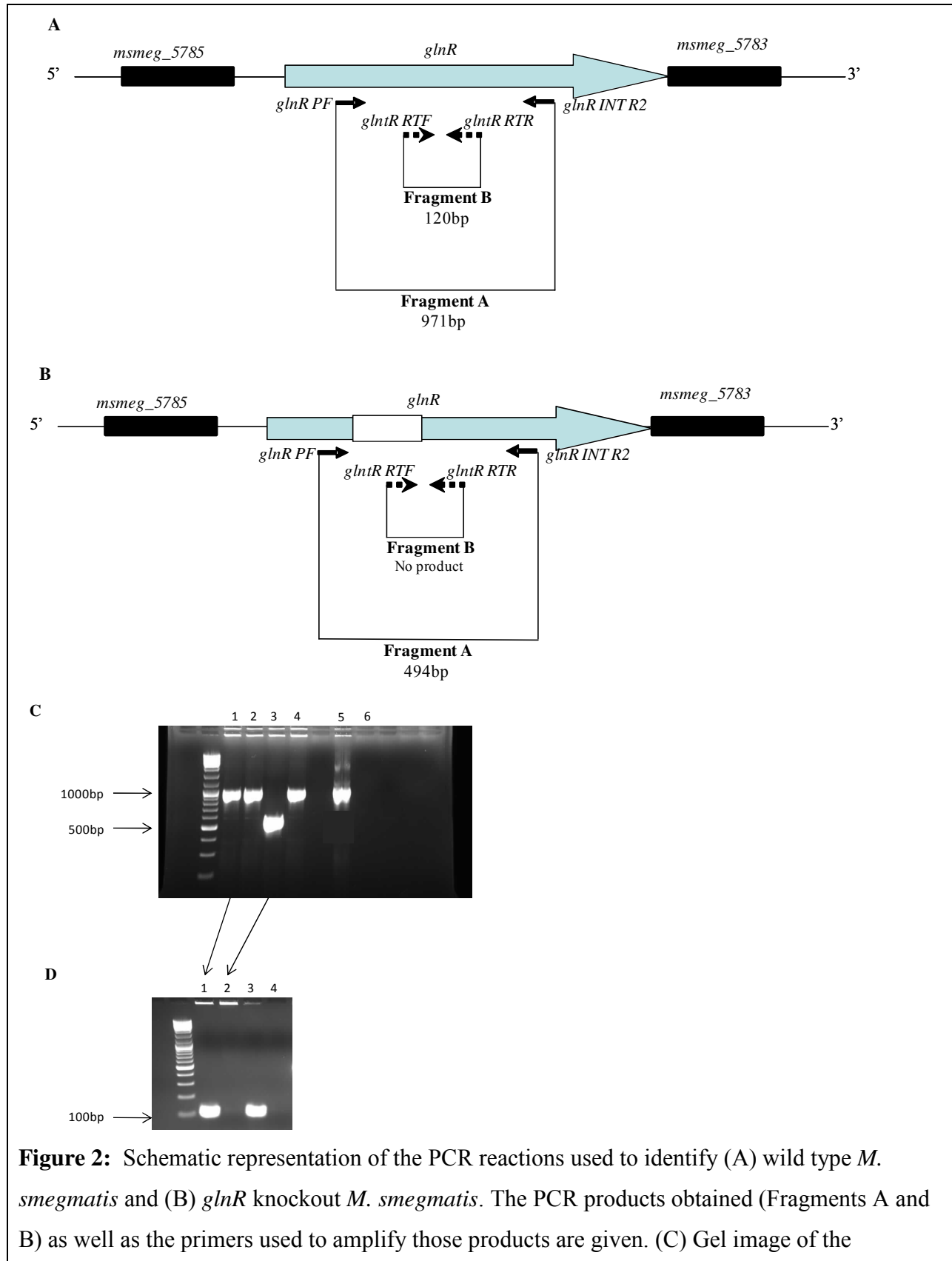


Figure 1: (i) PCR amplification of 1080bp upstream (Fragment A) and 1058bp down-stream (Fragment B) of the desired deletion region. Primers used to amplify these fragments had unique restriction digestion sites for (ii) ligation of the two fragments (A and B) to generate a 2138bp region (Fragment A/B) which was homologous to the *M. smegmatis* chromosome but had a 400bp deletion in the *amtR* gene. Fragment A/B was cloned into unique restriction sites (HindIII and ScaI) of the p2NIL suicide plasmid. In addition, a 6.3kb fragment containing the *lacZ* selection marker and the *sacB* counter-selection markers were excised from the pGOAL17 plasmid by PacI digestion and cloned into a unique PacI site in p2NIL. (iii) Upon transformation of the knockout construct into *M. smegmatis*, two possible recombination events can occur: the single cross over colonies were isolated and cultured on selection media to force a second recombination event which would result in either (iv) an *glnR* mutant genotype or reversion to the wild type genotype.



electrophoretic separation of Fragment A from 4 colonies (lanes 1-4). Lanes 5 and 6 are positive and negatives controls, respectively. (D) Gel image of the electrophoretic separation Fragment B from 2 colonies (lanes 1-2). Lanes 3 and 4 are positive and negatives controls, respectively

In the absence of a copy of the deleted form of the *glnR* gene, PCR amplification of Fragment A would yield a product of 971bp (Figure 2A). This product was indeed obtained for the amplification of wild type *M. smegmatis* DNA (Figure 2C, lane 5). A PCR product corresponding to wild type Fragment A was also amplified in the colonies screened in lanes 1, 2 and 4 (Figure 2C) which indicated that these colonies did not have a deleted form of the *glnR* gene. The amplification of Fragment A from the colony represented in lane 3 (approximately 500bp, Figure 2C) did, however, show a PCR amplicon size similar to that which would be present in a *glnR* knockout strain (Figure 2B). Upon amplification of Fragment B from the colonies represented in lanes 1 and 3 in Figure 2C, it was found that no PCR product was produced (lane 2) in the colony which gave a smaller Fragment A product. This colony was found to have undergone a double cross-over event resulting in the presence of a deleted form of *glnR* in the *M. smegmatis* genome. These PCR results were confirmed by sequencing.

CHAPTER THREE

Effectors of Nitrogen Metabolism

NOTE: Sections of the following chapter were published as: “**Glutamate dehydrogenase and glutamine synthetase are regulated in response to nitrogen availability in *Mycobacterium smegmatis***” Harper, C.J., Hayward, D., Kidd, M., Wiid, I., van Helden, P. *BMC Microbiol.* 2010 May 11;10:138.

3.1 INTRODUCTION:

Comparatively little is known about the major enzymatic effectors of nitrogen metabolism in *Mycobacterium smegmatis*. Glutamine synthetase has been well studied in *M. tuberculosis* due to its implication in the virulence and pathogenicity of the organism and *M. smegmatis* has often been used as a heterologous host for the study of GS in pathogenic mycobacterial species (Harth *et al.*, 2005; Harth and Horwitz, 1997). However, studies regarding the regulation and function of native *M. smegmatis* GS are few in number. In addition to GS, the *M. smegmatis* genome also encodes for three possible glutamate dehydrogenase enzymes which include an NADP⁺-GDH (*msmeg_5442*), a putative NAD⁺-GDH enzyme (*msmeg_6272*) and an L_180 class NAD⁺-GDH (*msmeg_4699*). A functional NADP⁺-GDH was isolated from *M. smegmatis* and its enzyme kinetics were characterized Sarada *et al.*, (1980); however the physiological role and regulation of the enzyme was not investigated. The NADP⁺-GDH of *M. smegmatis* has a subunit size of approximately 49kDa (Table 1) which is characteristic of the class of small GDH's typically found in most bacterial species (Minambres *et al.*, 2000). More recently, an NAD⁺-GDH (MSMEG_4699) was isolated from *M. smegmatis* during a study which aimed to identify proteins that interact with a small signaling protein, GarA (O'Hare *et al.*, 2008). An analysis of the molecular mass of MSMEG_4699 (approximately 183kDa, Table 1) indicated a subunit size similar to that of the L_180 class NAD⁺-GDH from *Streptomyces clavuligerus*, which was previously characterized (Minambres *et al.*, 2000). A third putative NAD⁺-GDH (MSMEG_6272) is also encoded by the *M. smegmatis* genome, but it is not known whether the gene is transcribed or whether a functional protein product is synthesized.

Table 1: *In silico* analysis of nucleotide and peptide sequences encoding for the NADP⁺-GDH (MSMEG_5442) and NAD⁺-GDH enzymes (MSMEG_4699 and MSMEG_6272) in *M. smegmatis*.

Characteristic	Protein		
	MSMEG_5442	MSMEG_4699	MSMEG_6272
Co-factor	NADP ⁺	NAD ⁺	NAD ⁺
Gene size (bp)	1350	4785	3213
Amino acids	449	1613	1070
Subunit mass (kDa)	49	183	118

Table 2: Identification of GDH protein sequences from mycobacterial genomes which display homology to any of the three different GDH amino acid sequences found in *M. smegmatis*.

Organism	% identity to MSMEG_5442*	% identity to MSMEG_4699*	% identity to MSMEG_6272*
<u>Slow growers</u>			
<i>M. tuberculosis</i>	n/a	71	53
<i>M. bovis</i>	n/a	72	53
<i>M. marinum</i>	n/a	72	53
<i>M. ulcerans</i>	n/a	n/a	n/a
<i>M. kansasii</i>	n/a	72	53
<i>M. leprae</i>	n/a	70	52
<i>M. avium</i>	n/a	72	52
<i>M. paratuberculosis</i>	n/a	72	
<i>M. intracellulare</i>	n/a	72	52
<u>Rapid growers</u>			
<i>M. abscessus</i>	74	65	52
<i>M. vanbaalenii</i>	84	76	51
<i>M. sp MCS</i>	82	76	52
<i>M. sp. KMS</i>	82	70	52
<i>M. sp. JLS</i>	82	76	52
<i>M. glivum</i>	80	77	52
<i>M. parascrofulaceum</i>	n/a	72	53

*Blastp (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis using completely sequenced mycobacterial genomes from the publicly available Genbank database.

With the exception of *M. parascrofulaceum*, it would appear that the genomes of the rapidly growing mycobacteria contain genes encoding for both an NADP⁻- and an NAD⁺-GDH (Table 2). In contrast, the slow growing and generally pathogenic mycobacterial species have only an NAD⁺-GDH which tends to display a high degree of similarity to the L_180 GDH class GDH found in *M. smegmatis*. This may reflect the phenomenon of reductive evolution in these

pathogenic species (Ahmed *et al.*, 2008). The gene encoding for GDH in *M. tuberculosis* (*Rv2476c*) shares a 71% amino acid identity with MSMEG_4699 (Table 2) and has a subunit size of approximately 177kDa which is very similar to the subunit size of the L_180 class of GDH's. From Table 2, it is also apparent that none of the mycobacterial genomes investigated has a gene with any similarity to *msmeg_6272*. According to an *in silico* determination of MSMEG_6272 subunit molecular mass, it appears as though the protein has an approximate subunit size of 118kDa (Table 1) which is similar to those enzymes belonging to the class of large GDH's characterized by a subunit mass of 115kDa. This class of large GDHs has thus far only been identified in protozoans and fungi (Minambres *et al.*, 2000; Britton *et al.*, 1992) and Blast analyses revealed no significant similarity of these GDH's to MSMEG_6272.

Considering the importance of GS in mycobacterial virulence and pathogenicity, it is surprising that so little is known of the function of the alternative ammonium assimilatory enzyme, GDH, in the mycobacteria. The importance of these enzymes for the nitrogen assimilatory process is poorly understood. It is not known whether the GDH enzymes play a predominantly anabolic or catabolic role with regard to nitrogen metabolism, nor is it known whether regulatory mechanisms exist to control GDH activity or transcription. Therefore, in addition to an investigation into the regulation of GS in *M. smegmatis*, this study attempts to unravel the physiological roles and regulation of the NADP⁺-specific and NAD⁺-specific GDH enzymes in *M. smegmatis*. In this Chapter, NAD⁺-GDH activity is briefly investigated in *M. tuberculosis* in order to determine whether a functional enzyme is present. Considering that the *M. tuberculosis* genome encodes only for a single GDH enzyme, the gene is sequenced in a number of *M. tuberculosis* clinical isolates in order to determine whether mutations exist, which could potentially contribute to the fitness of certain strain families.

3.1.1 Study Objectives:

Objective 1: To determine whether the specific activities of GS and the NADP⁺- and NAD⁺-GDH enzymes in *M. smegmatis* are regulated in response to ammonium availability.

Objective 2: To quantify the relative change in transcription of the genes encoding for GS, the NADP⁺-GDH and both the NAD⁺-GDH genes in response to ammonium availability in *M. smegmatis*.

Objective 3: To investigate NADP⁺-GDH and NAD⁺-GDH specific activity in *M. smegmatis* and *M. tuberculosis* upon culture of the bacilli in an enriched medium with various nitrogen sources. In parallel, the effect of GS inhibition on GDH activity in *M. tuberculosis* will be investigated.

Objective 4: To determine whether differences in the sequence encoding for the GDH gene between *M. tuberculosis* clinical isolates are present.

3.2 RESULTS:

3.2.1 Glutamine synthetase specific activity in response to ammonium limitation and excess

M. smegmatis was grown in enriched 7H9 medium to an OD₆₀₀ ≈ 0.8 and transferred to Kirchner's medium containing either a limiting (3mM (NH₄)₂SO₄) or an excess (60mM (NH₄)₂SO₄) amount of ammonium. The bacilli were incubated at 37°C for 1hr in order to pre-condition the bacteria to these conditions (time point zero). Bacteria that had been exposed to ammonium limitation for 1hr were subsequently transferred to Kirchner's minimal medium containing an excess of ammonium for 0.5hrs and 1hr. In parallel, *M. smegmatis* that had been cultured under ammonium excess conditions were transferred to Kirchner's medium containing a limited amount of ammonium for 0.5hrs, 1hr, 2hrs and 4hrs. At each time interval, the cells were lysed, the crude protein extract isolated and the total GS activity determined using the γ -glutamyl transferase assay (Hubbard and Stadtman, 1967). Through use of this assay, the percentage adenylation of GS subunits could also be calculated (Stadtman *et al.*, 1979) to give an indication of post-translational control by GlnE under the conditions tested. All experiments were repeated at least three times and the γ -glutamyl transferase assays were done in triplicate.

It was found that the activity of the high ammonium affinity GS enzyme in *M. smegmatis* increased significantly (by approximately 12 units; p=0.01) within 0.5hrs exposure to ammonium limiting conditions (Table 3). The measured GS activity continued to increase to a maximum of 103 units after 4hrs exposure to ammonium limiting conditions, which was approximately 2.5

fold greater than the activity determined at 0hrs (Table 3). Although not statistically significant, it was observed that the degree of adenylylation of GS subunits was reduced approximately 1.5 fold between 0hrs and 4hrs exposure to ammonium limitation (Table 3). It was found that a significant reduction in GS activity (1.5 fold; $p=0.00$) occurred within 0.5hrs exposure of the bacilli to an ammonium pulse. A concomitant increase (10%) in GS adenylylation was observed; however, this result was not found to be statistically significant (Table 3).

Table 3: Total *M. smegmatis* glutamine synthetase activity under conditions of ammonium limitation (3mM $(\text{NH}_4)_2\text{SO}_4$) and excess (60mM $(\text{NH}_4)_2\text{SO}_4$), as determined by the γ -glutamyl transferase assay.

$(\text{NH}_4)_2\text{SO}_4$ Concentration (mM)	Time (hours)	Specific activity (U)	p-value*	Adenylylation (%)	p-value*
3	0	45.1 ± 17.2		43 ± 17	
	0.5	57.4 ± 12.2	0.01	33 ± 12	0.17
	1	62.5 ± 11.9	0.27	35 ± 13	0.78
	2	78.2 ± 16.6	0.00	34 ± 5	0.88
	4	103.1 ± 17.2	0.00	29 ± 4	0.49
60	0	75.9 ± 2.3		35 ± 5	
	0.5	50.1 ± 1.2	0.00	45 ± 4	0.23
	1	46.6 ± 5.8	0.08	40 ± 3	0.42

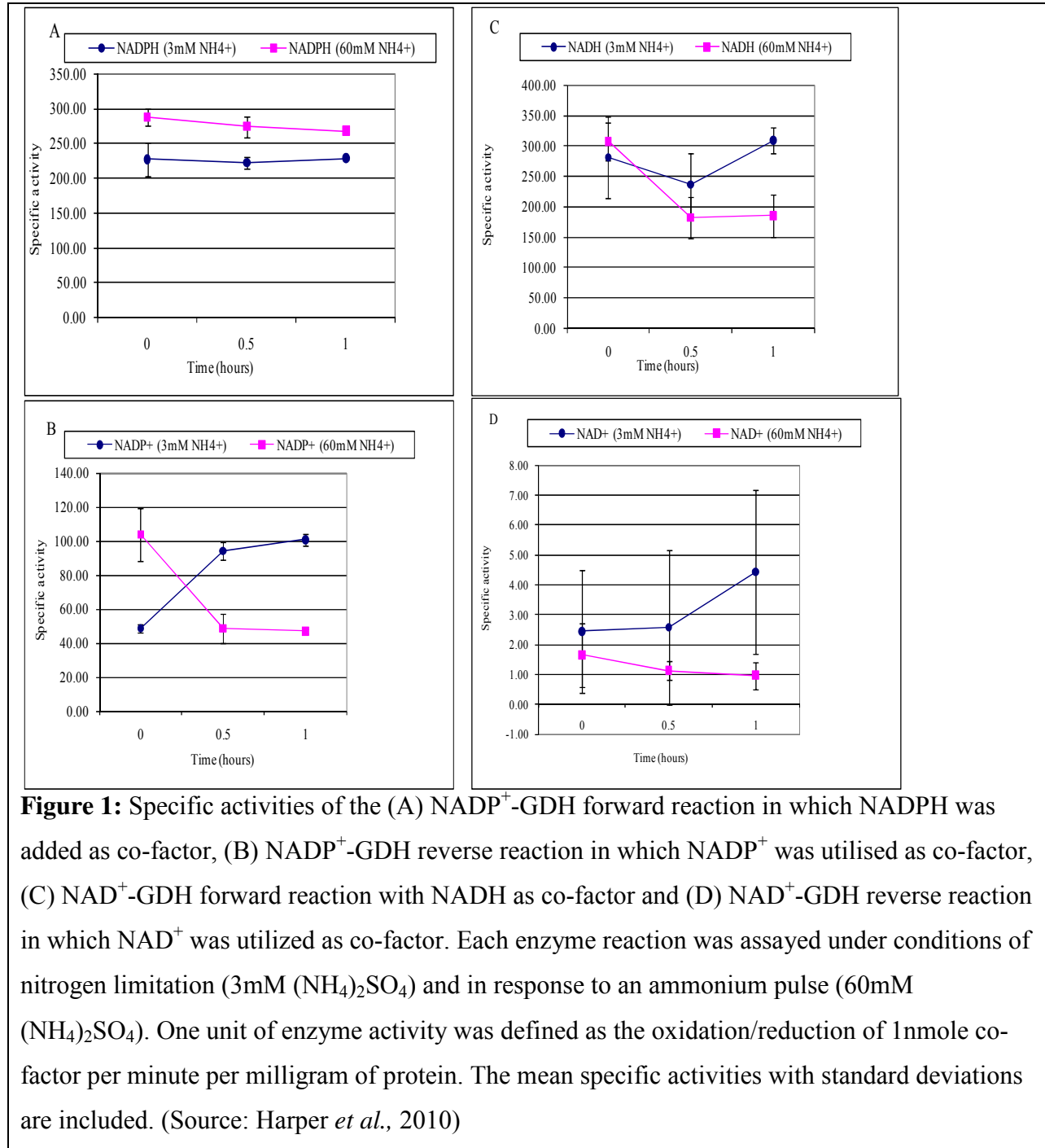
* $p < 0.05$ indicates a statistically significance change in GS specific activity between time points.

A two-way ANOVA model with the appropriate nested terms was used to calculate the significance values (Source: modified Harper *et al.*, 2010).

3.2.2 NADP⁺-specific and NAD⁺-specific glutamate dehydrogenase specific activity in response to ammonium limitation and excess conditions.

M. smegmatis was cultured under the conditions as described in section 3.2.1 above. The crude protein extract was isolated from bacilli that had been exposed to ammonium limitation (3mM $(\text{NH}_4)_2\text{SO}_4$) and excess (60mM $(\text{NH}_4)_2\text{SO}_4$) culture conditions and the specific activities of both the forward (reductive amination) and reverse reactions (oxidative deamination) of the NADP⁺-

specific and NAD⁺-specific GDH enzymes were determined. The experiments were repeated at least three times and all enzyme assays were done in triplicate.



The specific activity of the NADP⁺-GDH aminating, or forward, reaction activity in *M. smegmatis* did not significantly alter in response to ammonium availability (Figure 1A). Nor did

the NADP⁺-GDH aminating activity change upon prolonged (up to 4hrs) exposure to ammonium starvation conditions (Table 4). From Figure 1A, it would appear that, during ammonium starvation, the NADP⁺-GDH aminating reaction activity remained greater than when the bacilli were exposed to ammonium excess conditions. This observation, however, could be misleading since at certain points during each experiment, *M. smegmatis* was exposed to the same conditions of ammonium availability. For example, the bacilli were cultured under ammonium excess conditions (60mM (NH₄)₂SO₄) for 1hr at time point 0hr before exposure to ammonium limitation (preconditioning stage) and after 1hr subsequent to transfer from ammonium limiting media. The discrepancy between NADP⁺-GDH specific activity at such time points may be due to experimental differences (for example: slight differences in the amount of starting material used, differences in assay conditions and absorbance readings during the enzyme activity assays).

Table 4: Specific activity of the aminating (NADPH and NADH) as well as the deaminating (NADP⁺ and NAD⁺) reactions catalyzed by the NADP⁺-specific and NAD⁺-specific glutamate dehydrogenase enzymes under conditions of ammonium limitation.

Time (hours)	Specific Activity (U)							
	NADPH	p-value*	NADP ⁺	p-value*	NADH	p-value*	NAD ⁺	p-value*
0	227.5±24.5		48.6±2.4		281.4±67.4		0.02±3.5	
0.5	222.3±8.9	0.76	94.1±5.0	0.01	263.7±51.3	0.01	2.6±2.6	0.99
1	228.7±2.0	0.71	100.9±3.6	0.69	309.1±21.2	0	4.4±2.8	0.91
2	230.7±9.7	0.91	103.5±9.4	0.8	309.5±41.5	0.98	2.5±1.9	0.91
4	208.6±11.2	0.2	102.4±25.4	0.85	356.1±19.9	0.01	0.2±3.5	1

*p<0.05 (in bold print) were regarded as statistically significant changes in specific activity between time points. A two-way ANOVA model with the appropriate nested terms was used to calculate the significance values (Source: modified Harper *et al.*, 2010).

In contrast to NADP⁺-GDH aminating activity, it was found that the reverse reaction or NADP⁺-GDH deaminating activity was significantly affected by ammonium availability. Upon exposure to ammonium limiting conditions, an approximate 2 fold up-regulation in deaminating reaction activity was observed (Figure 1B and Table 4, p=0.001) which remained relatively constant in response to prolonged exposure to these conditions (Table 4). When *M. smegmatis* was subjected

to an ammonium pulse, a rapid, approximately 2-fold decrease in activity was observed within 0.5hrs (Figure 1B), which remained relatively unchanged for the following 0.5hrs (Figure 1B).

Total NAD⁺-GDH forward or aminating activity was also found to be regulated in response to ammonium availability. When *M. smegmatis* was incubated in ammonium limiting medium, it was observed that an initial decrease in total NAD⁺-GDH aminating activity occurred within 0.5hrs (approximately 18 units; p=0.01, Table 4). This was followed by a 14% increase in activity within the subsequent 0.5hrs (Figure 1C and Table 4, p=0.00). Total NAD⁺-GDH aminating activity increased significantly once again between 2hrs and 4hrs exposure to ammonium limiting conditions (approximately 50 units, p=0.01; Table 4). Upon exposure to an ammonium pulse, it was found that total NAD⁺-GDH aminating activity was down-regulated approximately 2 fold, (Figure 1C) which is similar to the regulation observed for NADP⁺-GDH deaminating or reverse reaction (Figure 1B). Analysis of the *M. smegmatis* NAD⁺-GDH deaminating reaction revealed very low specific activity levels which did not appear to be influenced by exogenous ammonium concentrations.

In summary, our results indicate that only the NADP⁺-GDH deaminating reaction and the NAD⁺-GDH aminating reaction activities are regulated in response to ammonium availability. Under our experimental conditions, total NAD⁺-GDH deaminating reaction activity appears to be much lower (as much as 80 fold, Table 4) than its aminating activity. Similarly, the NADP⁺-GDH aminating reaction activity appears to be greater than the de-aminating reaction activity (as much as 4.6 fold, Table 4).

3.2.3 Transcriptional control of GDH genes.

M. smegmatis was cultured as described in section 3.2.1 above. At each time point, total bacterial mRNA was extracted, reverse transcribed to cDNA and the relative change in transcription of the genes encoding for the GDH enzymes in *M. smegmatis* were determined either by semi-quantitative real-time PCR (*msmeg_4699* and *msmeg_5442*) or by reverse transcriptase PCR (*msmeg_6272*). Since the pattern of *glnA* transcription is known under these specific culture conditions in *M. smegmatis* (Chapter two, section 2.2.1), the expression of *glnA* was also

investigated by real-time PCR to act as an internal control for this study. The relative change in transcription was calculated as a ratio of target gene expression to the expression of *sigA*, which was used as an internal control or housekeeping gene (Liu *et al.*, 2008; Hu and Coates, 1999). The PCR efficiencies of the primer pairs used were previously determined (Chapter 2, Table 2). In the current study, the expression of *msmeg_6272* was determined by reverse transcriptase PCR which excluded the need for calculation of the PCR efficiency for the primer pair used (MSMEG_6272F/R, Materials and Methods, Table 2).

Table 5: Relative quantification^a of the expression of *msmeg_4699* (L_180 class NAD⁺-GDH), *msmeg_5442* (NADP⁺-GDH) and *glnA* (GS), by Real-time PCR, after exposure of *M. smegmatis* to conditions of ammonium limitation (3mM (NH₄)₂SO₄) and excess (60mM (NH₄)₂SO₄).

(NH ₄) ₂ SO ₄ concentration (mM)	Time (hours)	Gene					
		<i>msmeg_4699</i>	P- value*	<i>msmeg_5442</i>	P- value*	<i>glnA</i>	P- value*
3	0.5	0.31 ± 0.06	0.001	0.44 ± 0.08	0.001	3.33 ± 1.34	0.003
	1	0.58 ± 0.05	0.001	0.5 ± 0.075	0.001	3.53 ± 1.65	0.009
	2	12.8 ± 4.58	0.001	1.49 ± 0.93	0.901	8.56 ± 3.92	0.001
	4	18.03 ± 15.62	0.001	2.68 ± 2.62	0.272	9.79 ± 6.34	0.003
60	0.5	1.02 ± 0.15	0.997	0.96 ± 0.16	1	0.31 ± 0.11	0.001

^aThe relative change in transcription at each time point was compared to gene expression at time point zero; namely after 1hrs pre-conditioning of *M. smegmatis* under conditions of either ammonium limitation or excess. Values >1 reflect an up-regulation of gene expression whereas values <1 represent a down-regulation of expression in relation to the non-regulated internal reference, *sigA*. *A statistically significant change in gene transcription (p<0.05) from time point zero as calculated by the Relative Expression Software Tool (REST-384 V1). (Source: modified Harper *et al.*, 2010).

Upon introduction of *M. smegmatis* to ammonium limiting culture conditions, it was found that *glnA* expression was significantly up-regulated within 0.5hrs exposure to these conditions (3.33 fold; $p=0.003$, Table 5). The degree of *glnA* transcriptional up-regulation appeared to increase after 2hrs exposure to these conditions to reach a maximal up-regulation of approximately 10 fold after 4hrs (Table 5). A reciprocal regulation of *glnA* expression was observed after exposure of nitrogen starved *M. smegmatis* to an ammonium pulse; namely a 3 fold down-regulation (calculated by $1/\text{expression ratio}$) in *glnA* transcription levels (Table 5).

The expression of the genes encoding for the L_180 class NAD^+ -GDH (*msmeg_4699*) and the NADP^+ -GDH (*msmeg_5442*) in *M. smegmatis* were significantly down-regulated (approximately 3 and 2 fold respectively) within 0.5hrs exposure to ammonium limiting conditions (Table 5). After 1hr, the expression of *msmeg_5442* and *msmeg_4699* was also repressed approximately 2 fold and *msmeg_5442* transcription levels did not vary significantly thereafter (Table 5). In contrast, it was observed that *msmeg_4699* expression increased significantly (approximately 13 fold, $p=0.001$; Table 5) after 2hrs exposure to ammonium limiting conditions. In the current study, the expression of neither *msmeg_5442* nor *msmeg_4699* appeared to vary significantly in response to an ammonium pulse (Table 5).

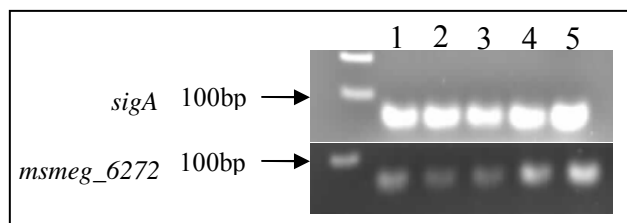


Figure 2: Gel image following reverse transcriptase PCR of *msmeg_6272* after *M. smegmatis* was cultured under ammonium limiting conditions (3mM $(\text{NH}_4)_2\text{SO}_4$). Lane (1) 0hrs, at which point *M. smegmatis* had been pre-conditioned to ammonium excess (60mM $(\text{NH}_4)_2\text{SO}_4$) conditions for 1hr (2) 0.5hrs ammonium limitation (3) 1hr ammonium limitation (4) 2hrs ammonium limitation (5) 4hrs ammonium limitation. *sigA* was amplified as an internal control. (Source: Harper *et al.*, 2010).

The expression of the putative NAD⁺-GDH encoded by *msmeg_6272* was analyzed by reverse transcriptase PCR due to difficulties in obtaining a single PCR product using a real-time PCR platform. Subsequent to PCR amplification of *msmeg_6272* in a bench-top PCR machine, the products were separated on a 1% agarose gel and quantified, relative to a *sigA* internal control, by densitometric analysis of the gel image (Yuan *et al.*, 2007). Under our experimental conditions, an *msmeg_6272* mRNA transcript could be detected and visual analysis of the gel image indicated that expression of the gene was regulated in response to ammonium availability (Figure 2). From densitometric analyses of the gel image, it was found that *msmeg_6272* expression was down-regulated (approximately 2 fold, Table 6) within 0.5hrs exposure to ammonium limiting conditions, however, after 2hrs exposure to these conditions, there appeared to be a 2 fold increase in gene transcription which did not appear to alter thereafter (Table 6).

Table 6: Relative quantification of *msmeg_6272* expression in *M. smegmatis* in response to ammonium limitation (3mM (NH₄)₂SO₄) and excess (60mM (NH₄)₂SO₄), as determined by reverse transcriptase PCR.

(NH ₄) ₂ SO ₄ concentration	Time (hrs)	Fold Increase (+) or Decrease (-) in expression*
3mM	0.5	--
	1	no change
	2	++
	4	no change
60mM	0.5	no change

* The relative increase/decrease in expression was determined relative to the previous time point.

(Source: Harper *et al.*, 2010)

3.2.4 NAD⁺-GDH and NADP⁺-GDH specific activities in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* during culture in enriched 7H9 medium containing different nitrogen sources .

M. tuberculosis H37Rv and *M. smegmatis* were cultured in OADC-enriched 7H9 medium to an OD₆₀₀ ≈ 0.6. L-Asparagine (Asn; 35mM), L-glutamine (Gln; 10mM) and ammonium sulphate ((NH₄)₂SO₄; 30mM) were added to the medium in separate experiments. The bacteria were

cultured in the presence of the various nitrogen sources for roughly two generations (2 days for *M. tuberculosis* and 6 hours for *M. smegmatis*) to allow for adaptation to the manipulated media. Thereafter, the cells were harvested by centrifugation, the crude protein extract from each culture was isolated and GDH enzyme activity assays were performed. To investigate the effect of GS inhibition on the GDH activity of *M. tuberculosis* cultures, l-methionine-S-sulfoximine (MSO; 55 μ M) was added where appropriate. These experiments were performed at least twice and enzyme assays were done in duplicate.

Upon addition of $(\text{NH}_4)_2\text{SO}_4$ or glutamine to 7H9 medium, it was found that NADP^+ -GDH aminating reaction activity in *M. smegmatis* was reduced by approximately 10 units (Figure 3), whereas the presence of asparagine did not significantly alter aminating enzyme activity. The activity of the NADP^+ -GDH reverse or deaminating reaction was not significantly altered upon addition of the various nitrogen sources to 7H9 medium (Figure 3). Neither the aminating nor the deaminating reaction activity of the NAD^+ -GDH was altered upon addition of various nitrogen sources to 7H9. Under all the conditions tested, it was found that NAD^+ -GDH aminating reaction activity remained greater than NADP^+ -GDH aminating reaction activity.

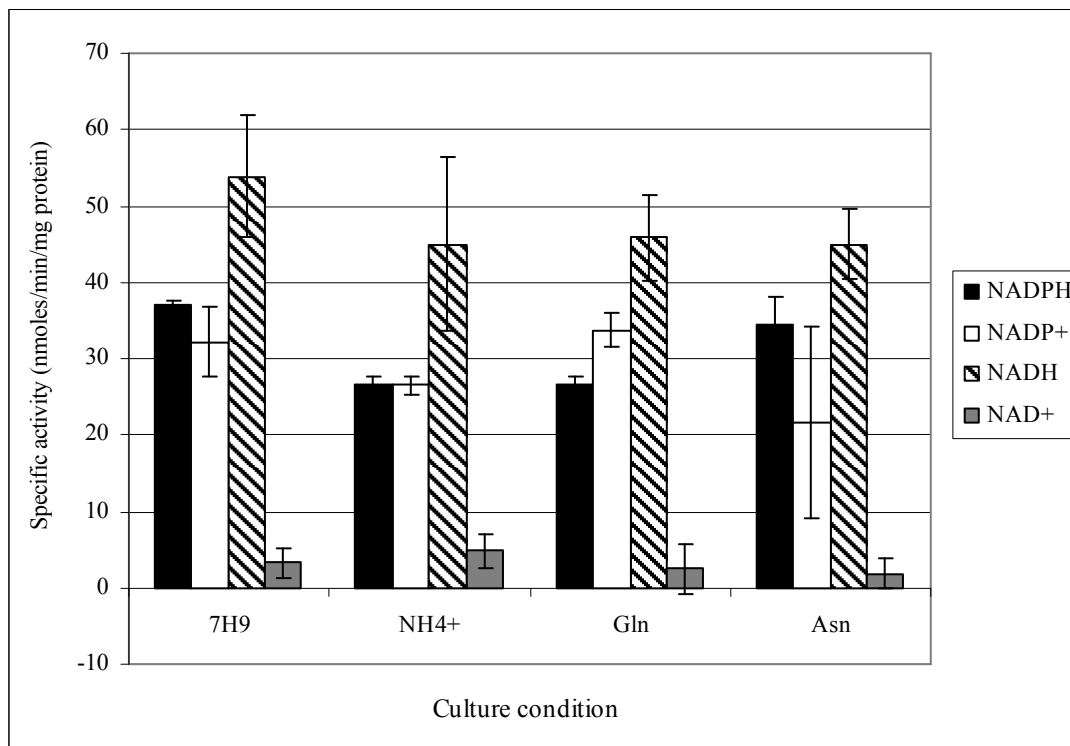


Figure 3: Specific activity of the aminating and deaminating reactions catalyzed by both the NADP⁺-specific and NAD⁺-specific glutamate dehydrogenases in *Mycobacterium smegmatis*.

NADP⁺-specific GDH activity could not be detected in *M. tuberculosis* culture filtrates which supports *in silico* studies that point to the absence of the enzyme in the slow-growing mycobacteria. We could, however, detect NAD⁺-specific GDH activity under all the culture conditions tested. It was found, similar to the case in *M. smegmatis*, that NAD⁺-GDH aminating reaction activity was greater than the deaminating reaction activity under the conditions tested (Table 7). The NAD⁺-GDH aminating reaction activity in *M. tuberculosis* appeared to be slightly repressed upon addition of 10mM Gln and a 10 fold increase in (NH₄)₂SO₄ concentration in 7H9 medium (Table 7), which is similar to the observed repression of NADP⁺-GDH aminating activity in *M. smegmatis* (Figure 3). In contrast, the addition of Asn to 7H9 medium (35mM final concentration) caused an approximately 7 fold increase in NAD⁺-GDH aminating reaction activity (Table 7). The NAD⁺-GDH deaminating reaction activity did not appear to be significantly affected by exposure to the various culture media (Table 7).

We were also interested in the effect of the absence of GS activity on NAD⁺-GDH enzyme activity in *M. tuberculosis*. Therefore, the potent GS inhibitor, MSO, was added to culture media and GDH enzyme activity assays were performed. The addition of 55μM MSO did not have a significant effect on NAD⁺-GDH aminating reaction activity in *M. tuberculosis* when Gln and (NH₄)₂SO₄ were added to standard 7H9 medium (Table 7). Neither did the addition of MSO have any effect on NAD⁺-GDH deaminating reaction activity under any of the culture conditions tested. However, there did appear to be an approximate 25 unit reduction in NAD⁺-GDH aminating reaction activity upon addition of MSO to a culture containing 35mM Asn (Table 7). In summary, these results indicate that a functional NAD⁺-GDH does exist in *M. tuberculosis* and that, similar to its *M. smegmatis* counterpart, the enzyme appears to favor the aminating or anabolic reaction. In addition, the NAD⁺-GDH aminating reaction appears to be sensitive to changes in nitrogen source.

Table 7: Specific activity of the aminating and deaminating reactions catalyzed by the NAD⁺-specific GDH in *M. tuberculosis* during culture in enriched 7H9 medium with various nitrogen sources.

Culture condition	Specific activity (units)	
	NADH	NAD ⁺
7H9	28.94 ± 6.82	14.47 ± 9.09
(NH₄)₂SO₄	20.10 ± 3.41	4.82 ± 5.9
(NH₄)₂SO₄+MSO	21.70 ± 1.14	6.43 ± 4.55
Gln	17.68 ± 0.98	14.47 ± 2.27
Gln+MSO	16.88 ± 1.14	12.06 ± 3.41
Asn	188.10 ± 6.82	12.06 ± 12.51
Asn+MSO	163.99 ± 4.55	19.29 ± 13.64

3.2.5 Sequencing of the NAD⁺-GDH in *M. tuberculosis* clinical isolates

The GDH domain includes the core region of the GDH enzyme which consists of two subdomains that are responsible for the catalytic function of the enzyme (Minambres *et al.*, 2000) (see Appendix 3A). The gene sequence encoding for the GDH domain of the NAD⁺-GDH enzyme in *M. tuberculosis* (Rv2476c) was determined through multiple sequence alignments with a previously characterized enzyme belonging to the same class (Minambres *et al.*, 2000)(Appendix 3B). The GDH domain was PCR amplified from purified DNA previously extracted from 40 *M. tuberculosis* clinical isolates which represent each of the known *M. tuberculosis* strain families (based on ISO6110 RFLP analysis)(Richardson *et al.*, 2002). The GDH domain was amplified in two parts/fragments and the PCR primers (Materials and Methods, Table 2) were designed to overlap by 129bp to allow accurate interpretation of incomplete sequencing data. The fragments amplified were purified and submitted for automated sequencing. The resulting sequence data was aligned using ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). No differences in the domain sequences of the clinical isolates could be detected.

3.3 DISCUSSION

3.3.1 Glutamine synthetase specific activity in response to ammonium limitation and excess conditions

A variety of bacteria show a similar type of GS regulation as found in *M. smegmatis*; namely, an up-regulation of GS activity upon exposure to nitrogen limiting conditions and a down-modulation of GS activity when nitrogen sufficient conditions return (Merrick and Edwards, 1995). These results indicate that *M. smegmatis* did sense 3mM (NH₄)₂SO₄ as an ammonium limiting condition since GS activity was up-regulated in order to efficiently assimilate nitrogen under these conditions. GS activity was down-regulated upon exposure of *M. smegmatis* to 60mM (NH₄)₂SO₄, which indicated that the bacilli did sense this condition as one of nitrogen sufficiency. The down-regulation observed was most likely in order to limit futile energy use by GS under these conditions and to maintain appropriate intracellular glutamine/glutamate ratios (Figure 4). In *M. tuberculosis*, it was found that a similar up-regulation (approximately 3.7fold)(Carroll *et al.*, 2008) and down-regulation (Harth *et al.*, 1994) of GS activity occurred under analogous experimental conditions.

In *M. tuberculosis*, as well as a number of other organisms, the observed control of GS activity has been ascribed to both transcriptional regulation (Harth and Horwitz, 1997) and post-translational adenylylation by the essential enzyme, GlnE (Carroll *et al.*, 2008; Parish and Stoker, 2000c). In order to accurately calculate the adenylylation state of GS using the γ -glutamyl transferase assay, the pH at which the activity of completely deadenylylated and completely adenylylated GS, in the presence of the co-factor Mn²⁺, is equal (iso-activity pH) has to be determined. This is a requirement for the adenylylation assay as the activity of GS varies at different pH levels depending on the average adenylylation state of the enzyme and on the divalent cation used (Mn²⁺ or Mg²⁺). To calculate the number of adenylylated GS subunits in a sample, both divalent cations are utilized in the assay system, therefore, the assays need to be carried out at the iso-activity pH. It was attempted to determine the iso-activity pH of GS in *M. smegmatis* crude protein extracts; however, this was not successful. Thus accurate determination of the iso-activity pH of *M. smegmatis* GS required purified enzyme, which we were not able to obtain. In our γ -glutamyl transferase assay a pH of 7.15 was used which is the GS iso-activity pH

determined for *E. coli* (Stadtman *et al.*, 1979). Therefore, our data reflects only a tendency or trend concerning the adenylylation state of *M. smegmatis* GS which may explain why we did not observe a statistically significant alteration in the degree of GS adenylylation upon a change in exogenous ammonium concentration. The trend was, however, that under ammonium starvation culture conditions, the extent of *M. smegmatis* GS adenylylation (see section 3.2.1 for assay of extent of adenylylation) decreased which would allow for a concomitant increase in GS activity. The opposite was observed for *M. smegmatis* which was exposed to an ammonium pulse. Our previous work has shown an up-regulation in the expression of the gene encoding for GS (*glnA*) upon exposure to ammonium limitation, as well as the reciprocal regulation of *glnA* expression during conditions of ammonium excess (Chapter 2, section 2.2.1). The *de novo* synthesis of GS via *glnA* transcriptional control could also contribute to the observed regulation of GS specific activity when exogenous ammonium concentrations were altered.

3.3.2 NADP⁺-specific and NAD⁺-specific glutamate dehydrogenase specific activity in response to ammonium limitation and excess conditions

3.3.2.1 Regulation of the NADP⁺-GDH in response to ammonium limitation and excess

The NADP⁺-specific GDH enzymes present in prokaryotes are thought to predominantly play an anabolic role with regard to nitrogen metabolism; namely, the formation of glutamate from ammonium and 2-oxoglutarate (Britton *et al.*, 1992; Consalvi *et al.*, 1991). In general, due to the relatively low affinity of the enzyme for its substrates, it is thought that these enzymes are largely active under conditions of nitrogen sufficiency. The NADP⁺-specific GDH enzymes have been well characterized in a number of bacteria and although most studies claim to assess the aminating and deaminating reactions, only the results of the aminating reaction activities are given (Camardella *et al.*, 2002; Lu and Abdelal, 2001; Duncan *et al.*, 1992; Miller and Magasanik, 1990). The regulation of NADP⁺-specific GDH aminating activity in response to nitrogen availability appears to be variable amongst bacterial species. Studies in *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Bacillus subtilis* (Meers *et al.*, 1970) and *Klebsiella*

aerogenes (Brenchley *et al.*, 1973) have indicated a significant down-regulation in NADP⁺-GDH aminating specific activity upon ammonium limitation. Other studies have indicated a complete lack of regulation under analogous conditions (Muller *et al.*, 2006; Camarena *et al.*, 1998; Patterson and Hespell, 1985). Our results indicated that NADP⁺-GDH aminating activity was present but was not regulated in response to ammonium availability in *M. smegmatis*. This is in contrast with a recent study in the closely related Actinomycetale, *C. glutamicum*, which showed that NADP⁺-GDH aminating activity was significantly increased in response to nitrogen starvation (Muller, 2005). Sarada *et al.*, (1980) briefly described an increase in NADP⁺-GDH activity in *M. smegmatis* upon increased NH₄⁺ concentrations; however, it is not clear as to whether it is the aminating or the deaminating reaction to which the authors refer.

In contrast, it was found that NADP⁺-GDH deaminating reaction activity was regulated in response to ammonium availability in *M. smegmatis* (Figure 4). The GDH enzymes catalyze reversible reactions; therefore it is interesting to note that only the deaminating reaction activity appeared to be regulated in response to nitrogen availability in this organism. The functional or physiological significance of this observation remains unclear. The deaminating reaction catalyses the hydrolysis of glutamate to ammonium and 2-oxoglutarate; therefore, it may be that the regulation of glutamate catabolism serves to maintain intracellular glutamate/2-oxoglutarate as well as glutamate/glutamine ratios. These ratios are known to play important roles in growth rate modulation (Ikeda *et al.*, 1996), protection from high osmotic environments (Tian *et al.*, 2005; Kempf and Bremer, 1998) and maintenance of intracellular potassium pools (Yan, 2007), amongst others. It is thought that the mycobacteria have large intracellular glutamate pools (Tian *et al.*, 2005) which could serve to buffer changes in nitrogen and energy source availability. An increase in glutamate catabolism under nitrogen limiting conditions may serve to release ammonium which could be assimilated by GS for essential glutamine production. The decrease in glutamate catabolism upon exposure of *M. smegmatis* to ammonium excess conditions may be due to a negative feedback effect on the deaminating reaction due to the formation of glutamate under these conditions by the NADP⁺-GDH and NAD⁺-GDH anabolic reactions (Tian *et al.*, 2005).

3.3.2.2 Regulation of the NAD⁺-GDH in response to ammonium limitation and excess

Initial studies done to detect total NAD⁺-GDH activity in *M. smegmatis* whole cell lysates utilized a 100mM Tris buffer (tris(hydroxymethyl)aminomethane) in the GDH enzyme activity assay. In these studies, the aminating NAD⁺-GDH specific activities were detectable but were up to 8 fold lower than when a phosphate buffer was used (data not shown). Regardless of which buffer was used, total NAD⁺-GDH deaminating reaction specific activities remained approximately the same. The apparent inhibition of NAD⁺-GDH activity by Tris appears to be typical of the L_180 class of GDH enzymes (Minambres *et al.*, 2000) which points to the existence of a functional L_180 class GDH in *M. smegmatis* (i.e. MSMEG_4699). Minambres *et al.*, (2000) reported a 100% inhibition of the *S. clavuligerus* L_180 GDH in 80mM Tris. Since we observed an approximate 87% inhibition of total NAD⁺-GDH aminating activity by assay in 100mM Tris buffer, it may be that an additional functional GDH such as MSMEG_6272, which is not sensitive to Tris, was able to catalyze the reaction (this has been investigated in Appendix 3A). In *M. smegmatis*, the total NAD⁺-GDH aminating activity was found to be up-regulated upon ammonium starvation, presumably to assist nitrogen assimilation under these conditions. Conversely, it was found that the aminating NAD⁺-GDH activity was down-regulated during an ammonium pulse which may be attributed to the presence of a constitutively active NADP⁺-specific GDH which could adequately assimilate nitrogen under ammonium excess conditions (Figure 4). From our data, it would appear that at least one of the NAD⁺-GDHs found in *M. smegmatis* plays a largely anabolic or assimilatory role, which is in contrast to the belief that these enzymes are normally involved in glutamate catabolism.

In addition to the observation that the NAD⁺-GDH enzymes in *M. smegmatis* may play a predominantly assimilatory role in nitrogen metabolism, it would appear that at least one of these enzymes is regulated in response to ammonium availability. Indeed, it was found that the activity of the L_180 class NAD⁺-GDH in *M. smegmatis* was affected by the binding of a small protein, GarA (O'Hare *et al.*, 2008). This protein plays a role in glycogen metabolism in *M. smegmatis* (Belanger and Hatfull, 1999), hence the name Glycogen accumulation factor, or GarA. This molecule appears to be highly conserved amongst the Actinomycetes. GarA possesses a fork-head associated domain (FHA) as well as a conserved N-terminal phosphorylation motif which enables GarA to mediate protein-protein interactions and to be regulated via phosphorylation. The phosphorylation motif contains a highly conserved threonine residue, which may be

phosphorylated by serine/threonine kinase B (pknB) (Villarino *et al.*, 2005) or serine/threonine kinase G (pknG) (O'Hare *et al.*, 2008) which implicates GarA in phosphorylation dependant regulation mechanisms (Niebisch *et al.*, 2006). OdhI is the GarA ortholog in *C. glutamicum* (71% amino acid identity) and it has been shown to be phosphorylated by pknG in this organism. Native OdhI is able to interact with 2-oxoglutarate dehydrogenase, an important enzyme in the tricarboxylic acid cycle (TCA), and cause a reduction in its activity. The protein interaction and subsequent down-modulating effect on enzyme activity is removed by the phosphorylation of OdhI by pknG (Niebisch *et al.*, 2006). An analogous scenario has been observed in *M. smegmatis*. Unphosphorylated or native GarA has been shown to interact with both the L_180 class NAD⁺-GDH (MSMEG_4699) and α -ketoglutarate decarboxylase (a member of the TCA cycle) and cause a down-modulation of enzyme activity through a reduction in the affinity of the enzyme for its substrate (O'Hare *et al.*, 2008). The binding of GarA to either enzyme is abolished by phosphorylation by pknG. Although it is not yet known under which conditions pknG is stimulated to phosphorylate or dephosphorylate GarA, it may be that the GarA/pknG mechanism may play an important role in the observed regulation of NAD⁺-GDH enzyme activity.

Previous studies of the L_180 class GDH in *S. clavuligerus*, found an induction of the catabolic or deaminating reaction activity under certain nutritional conditions, including ammonium limitation (Minambres *et al.*, 2000). Under our experimental conditions, the activity of the NAD⁺-GDH deaminating reaction in *M. smegmatis* remained very low and did not significantly change in response to ammonium availability. Recently, O'Hare *et al.*, (2008) were able to purify the *M. smegmatis* L_180 class GDH (MSMEG_4699) and it was found that the enzyme exhibited half maximal deaminating reaction activity at 83mM glutamate whilst intracellular glutamate concentrations in the mycobacteria have been estimated to be approximately 80mM (Tian *et al.*, 2005). In our study, NAD⁺-GDH enzyme deaminating activity was assayed using 100mM glutamate, which is slightly higher than physiological concentrations, yet only very low levels of activity could be detected. In contrast to the study conducted by O'Hare *et al.*, (2008), the NAD⁺-GDH deaminating activity in *M. smegmatis* was not analyzed using purified protein, but rather in a total crude protein extract. It may be that allosteric regulators of GDH activity present in the crude protein extract (or whole cell lysate) could affect the activity of the NAD⁺-GDH deaminating reaction. In addition, it was found that the NAD⁺-GDH deaminating reaction

activity in an *M. bovis* BCG crude protein extract only increased significantly after the addition of approximately 500mM glutamate (Personal communication, B. Viljoen, PhD student) which far exceeds physiological glutamate concentrations. These studies suggest, therefore, that NAD^+ -GDH has a very low affinity for glutamate and that under physiological or *in vivo* conditions, the deaminating NAD^+ -GDH reaction is not active in *M. smegmatis* and possibly not either in *M. bovis* BCG.

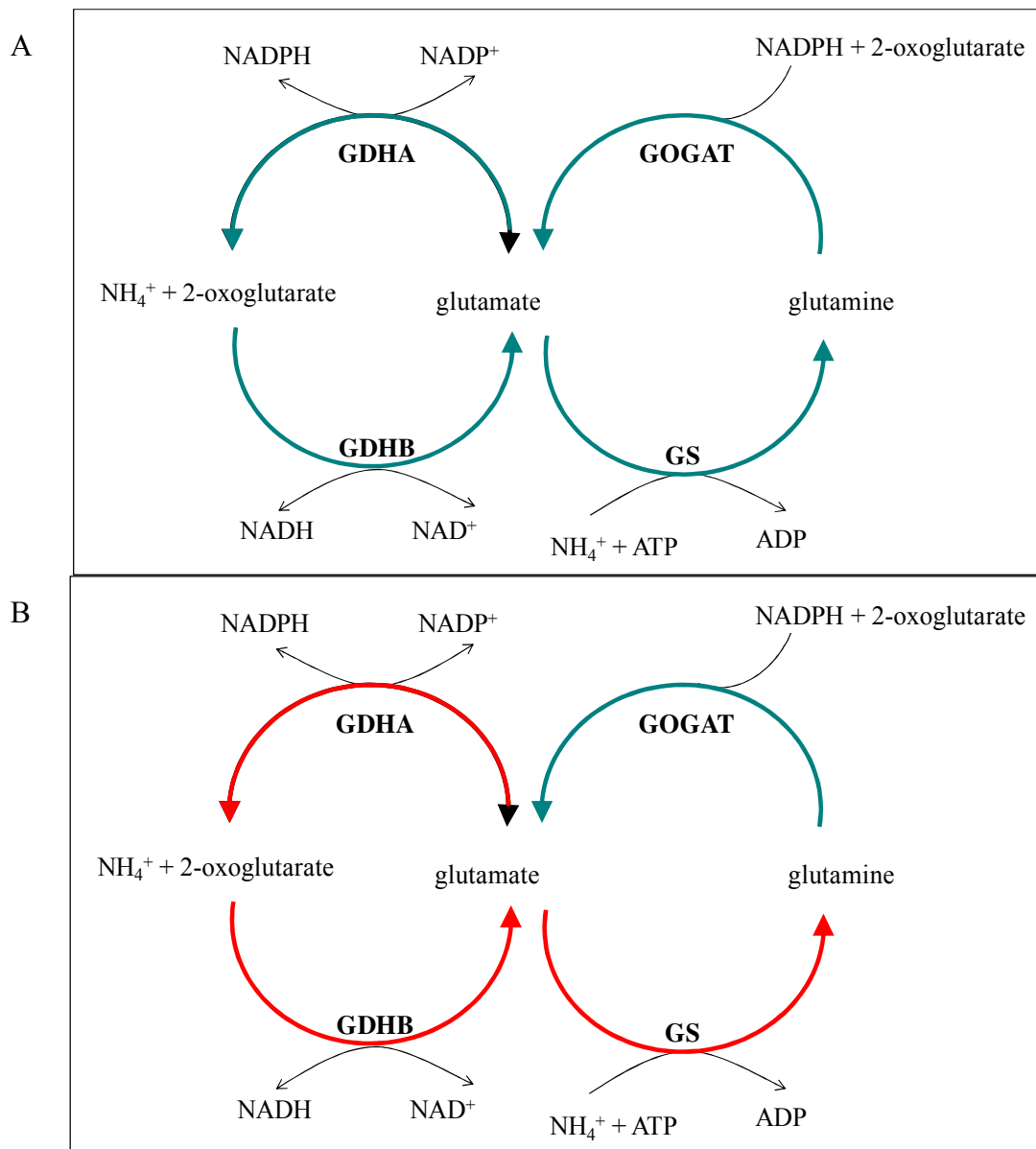


Figure 4: Schematic representation of the reactions catalysed by the major enzyme effectors of nitrogen metabolism under conditions of (A) ammonium limitation and (B) ammonium excess in

M. smegmatis. Down-regulation of enzyme activity is indicated by a red arrow whilst an up-regulation of enzyme activity is indicated by a blue arrow. A black arrowhead indicates no change in enzyme activity. GDHA and GDHB represent NADP⁺-GDH and NAD⁺-GDH respectively. In (A) GDHA deaminating activity is up-regulated to catabolise glutamate for the production of ammonia. The ammonia produced can be assimilated by GS or GDHB whose activities are up-regulated to increase nitrogen assimilatory capacity. The regulation of GOGAT enzyme activity data is implied from *gltB/D* expression data and the possible up-regulation of GOGAT activity could replenish glutamate stores. In (B) GDHA deaminating, GDHB aminating and GS activities are down-regulated. GOGAT activity could possibly be up-regulated to restore internal metabolite pools.

3.3.3 Relative quantification of the expression of genes encoding for GS and the GDH enzymes in *M. smegmatis* in response to ammonium limitation and excess

The down-regulation of *glnA* expression observed during exposure to ammonium limitation and the up-regulation of expression after an ammonium pulse was similar to previous studies done (Chapter 2, section 2.2.1), which indicated that our experimental conditions did stimulate a nitrogen starvation and excess physiological response. The degree of *glnA* transcriptional up-regulation within the first hour following culture in ammonium limiting medium was not as pronounced as the up-regulation of *glnA* transcription seen after 2hrs and 4hrs exposure to the same conditions. This result probably reflects an increasing cellular requirement for the *de novo* synthesis of GS due to prolonged exposure to nitrogen limitation, in order to efficiently assimilate ammonium under these conditions. *M. tuberculosis* is known to have large intracellular glutamate pools which could serve as a buffer for fluctuations in extracellular nitrogen concentrations (Tian *et al.*, 2005). It may be that, similar to its pathogenic relative, *M. smegmatis* also has these large intracellular nitrogen resources which would be depleted after prolonged ammonium limitation and therefore create a heightened state of intracellular nitrogen starvation. This could possibly account for the significant increase in *glnA* expression seen after 2hrs and 4hrs exposure to ammonium starvation conditions. A relatively constant increase in GS activity (section 3.2.1) was observed under ammonium starvation conditions which could be attributed to both an up-regulation in *glnA* transcription and post-translational control by GlnE.

In our studies, we did not observe a statistically significant change in the degree of adenylylation of GS subunits; however, there did appear to be a decrease in adenylylation with prolonged exposure to ammonium limitation (section 3.2.1). In the absence of any significant change in adenylylation, it would appear that transcriptional control may be the main regulatory mechanism governing an increase in intracellular GS activity levels in *M. smegmatis* during ammonium limitation. It has recently been shown that the adenylylation function of GlnE is essential for *M. tuberculosis* survival (Carroll *et al.*, 2008). This pathogen is known to produce excessively large quantities of GS, which is rapidly adenylylated to prevent depletion of intracellular glutamate and ATP reserves. This pool of inactive GS in *M. tuberculosis* has been shown to remain relatively constant and, similar to what was found in *M. smegmatis*, the increase in GS activity in response to decreased ammonium availability was ascribed to an increase in total GS levels (Carroll *et al.*, 2008). In the absence of a statistically significant change in the degree of GS adenylylation in *M. smegmatis*, it may be that similar to *M. tuberculosis*, the increase in GS activity upon exposure to ammonium starvation conditions could be attributed largely to *de novo* synthesis of the enzyme and therefore to transcriptional control.

The transcriptional regulation of genes encoding for the GDH enzymes in the mycobacteria has, to our knowledge, not yet been investigated. Our results indicate a down-regulation of *msmeg_5442* transcription in response to ammonium limitation in *M. smegmatis* which is similar to the observed transcriptional regulation of the NADP⁺-GDH in *S. coelicolor* (encoded by *SCO4683*) under analogous conditions (Tiffert *et al.*, 2008). The regulation of *msmeg_5442* expression did not, however, directly correlate with the observed regulation of NADP⁺-GDH specific activity under ammonium limiting conditions. Within 0.5hrs exposure to these conditions, a down-regulation of *msmeg_5442* expression was observed whilst an increase in NADP⁺-GDH aminating reaction activity was detected (section 3.2.2, Table 4). This result suggests that *de novo* synthesis of NADP⁺-GDH is not the sole mechanism regulating enzyme activity in response to ammonium availability; therefore, alternative regulatory mechanisms may be involved in the regulation of NADP⁺-GDH activity. Post-translational modification of GDH enzymes in the form of phosphorylation by kinases has been implicated in the control of GDH enzyme activity in *E. coli* (Lin and Reeves, 1994) and in higher mammals such as *Spermophilus*

richardsonii (Richardson's ground squirrel) (Bell and Storey, 2010). It may be that such mechanisms are conserved and may play a role in NADP⁺-GDH regulation in *M. smegmatis*.

Our studies show a slightly different trend with regard to the regulation of genes encoding for the NAD⁺-GDH enzymes in *M. smegmatis*. An initial down-regulation in *msmeg_4699* and *msmeg_6272* expression was observed (section 3.2.3, Table 5) during exposure to ammonium limiting conditions which coincided with an initial decrease in NAD⁺-GDH specific activity (section 3.2.2, Table 4). However, between 0.5hrs and 1hr there was a significant increase in NAD⁺-GDH aminating activity but no up-regulation of the genes encoding for the NAD⁺-GDH enzymes, which hints at the presence of additional regulatory mechanisms governing NAD⁺-GDH activity. After 2hrs and 4hrs exposure to ammonium limiting conditions, however, an up-regulation in *msmeg_4699* and *msmeg_6272* expression was once again mirrored by an increase in NAD⁺-GDH aminating reaction activity. Therefore, it would appear that both transcriptional control as well as other regulatory mechanisms, such as possibly the GarA/pknG regulatory system, contributes to the governance of NAD⁺-GDH activity. A post-translational regulatory mechanism, such as the GarA/pknG system, may also account for the decrease in NAD⁺-GDH activity observed in response to an ammonium pulse, as a change in transcription of the NAD⁺-GDH genes could not be detected under those conditions. In Chapter two, section 2.2.6 (Table 5), it was found that an up-regulation of all three genes encoding for the GDH enzymes occurred in response to 1hr exposure to ammonium excess conditions. The discrepancy in these two findings may lie in slight experimental differences, for example, in the current study the expression of *sigA* was used as an internal reference gene whilst in the previous study, the expression of *16SrRNA* was employed to normalize the data. In addition, in this study, the expression of *msmeg_6272* was analyzed by reverse transcriptase PCR which is a rather more crude measure of relative expression in comparison to real-time PCR. However, despite the differences between the experiments, the results of this study can not be discredited as the enzyme assays and transcriptional work were performed on the same biological cultures thereby drawing a very narrow link between gene expression and enzyme activity.

3.3.4 NAD⁺-GDH and NADP⁺-GDH specific activities in *M. smegmatis* and *M. tuberculosis* during culture in enriched 7H9 medium containing different nitrogen sources

Enriched 7H9 medium is a complex medium containing a variety of nitrogen sources such as 3.78mM $(\text{NH}_4)_2\text{SO}_4$, 0.04g/l ferric ammonium citrate ($\text{C}_6\text{H}_8\text{O}_7\text{nFe}\cdot\text{nH}_3\text{N}$) and 3.4mM L-glutamic acid. In addition, enriched 7H9 medium contains a variety of carbon and energy sources such as oleic acid, glycerol, albumin, Tween80 and dextrose. Since GDH enzymes are considered to be important branch enzymes linking carbon and nitrogen metabolism, the activity of the forward and reverse reactions of both enzymes was investigated in an enriched medium (7H9) with additional nitrogen sources. In *M. smegmatis*, it was observed that the aminating and deaminating reaction activities of NADP^+ -GDH and the aminating activity of NAD^+ -GDH was greatly reduced in bacilli that were cultured in enriched 7H9 medium in comparison with Kirchner's minimal medium. The latter medium does not contain the plethora of carbon/energy and nitrogen sources which complex 7H9 medium contains, thus growth in Kirchner's minimal medium could create a number of stresses involving a variety of metabolic pathways. It would appear from our results that there is a greater prerequisite for GDH function under conditions of nitrogen and/or carbon stress. In addition to a role in nitrogen assimilation, the higher levels of total GDH enzyme activity observed in Kirchner's minimal medium may be due a requirement for increased control of the 2-oxoglutarate flux between the Krebs cycle and nitrogen metabolism in order to satisfy carbon metabolism requirements. It has been shown that both GDH isoforms are regulated by ammonium availability in *M. smegmatis* (section 3.2.2), however, since these enzymes also play a role in carbon/energy metabolism, the regulation and physiological roles of the glutamate dehydrogenase enzymes appears to be quite complex. In the presence of adequate carbon and nitrogen sources available in 7H9, it was observed that neither the aminating nor deaminating activity of NAD^+ -GDH was affected by the presence of additional nitrogen sources in the medium. However, it was found that an addition of $(\text{NH}_4)_2\text{SO}_4$ and Gln to 7H9 medium resulted in a reduction in NADP^+ -GDH aminating activity. A similar decrease in NADP^+ -GDH aminating activity in response to ammonium addition has previously been described in *M. smegmatis* cultured under analogous conditions (Ahmad *et al.*, 1986). These results are in contrast to the lack of NADP^+ -GDH regulation seen when $(\text{NH}_4)_2\text{SO}_4$ concentrations were varied in Kirchner's minimal medium (section 3.2.2). Although the reasons for the observed down-regulation in activity are obscure, it may be that ammonium could be

adequately assimilated by an apparently constitutively active NAD⁺-GDH under these conditions.

An analysis of GDH activity in *M. tuberculosis* indicated that only an NAD⁺-GDH is found in this pathogen as no NADP⁺-GDH activity could be detected. Similar to the NAD⁺-GDH in *M. smegmatis*, it was observed that the aminating reaction activity of the sole GDH in *M. tuberculosis* was greater than the deaminating reaction activity which possibly indicates a greater role for ammonium assimilation rather than glutamate catabolism. It was found that the aminating reaction activity was repressed upon addition of (NH₄)₂SO₄ and Gln to 7H9 medium which suggests that the NAD⁺-GDH aminating reaction is regulated in response to nitrogen source. In *M. smegmatis*, no repression of NAD⁺-GDH aminating activity was observed upon addition of (NH₄)₂SO₄ or Gln to 7H9 medium; however, a similar repression of NAD⁺-GDH aminating reaction activity was observed when *M. smegmatis* was cultured in the presence of excess ammonium (section 3.2.2) in Kirchner's minimal medium. The difference in regulation observed may be due to the presence of an additional GDH in *M. smegmatis*. The large scale up-regulation in NAD⁺-GDH aminating reaction activity in *M. tuberculosis* in response to the presence of Asn was surprising; however, in a previous study it was found that Asn acted as an allosteric activator of the L_180 class GDH found in *S. clavuligerus* (Minambres *et al.*, 2000). An analogous activation of NAD⁺-GDH activity in *M. smegmatis* did not occur which may indicate a difference in NAD⁺-GDH structure and function between the rapid and slow growing mycobacteria. Upon addition of MSO to the culture media, it was anticipated that, in the absence of GS function, GDH activity would be enhanced. This, however, was not found to be the case and may be because GS activity was not required in 7H9 medium since the bacteria were not starved of nitrogen.

GS has been implicated in *M. tuberculosis* virulence and pathogenicity and is an essential nitrogen assimilatory enzyme. It is thought that under conditions of nitrogen limitation, GS plays the dominant nitrogen assimilatory role due to the high affinity of the enzyme for ammonium; however, its function is coupled to the hydrolysis of cellular ATP. The exact nutritional conditions to which *M. tuberculosis* is exposed *in vivo* (such as in the phagolysosome or granulomatous lesions) remains unclear, but it is generally considered to be a nutrient poor

environment (Betts *et al.*, 2002) which may therefore be deficient in nitrogen sources. This would imply that GS plays the major nitrogen assimilatory role *in vivo*. We have, however, shown that a functional NAD⁺-GDH does exist in *M. tuberculosis* which appears to play a largely anabolic or assimilatory role in nitrogen metabolism. Since the energy cost associated with ammonium assimilation by NAD⁺-GDH is much lower than that of GS, it may be that NAD⁺-GDH plays an important role in nitrogen assimilation in bacilli that do not have large energy reserves e.g. *M. tuberculosis* which is in a dormant or persistent state. Taking into account that differences in virulence among *M. tuberculosis* strain families exist (Johnson *et al.*, 2010) and in light of the fact that NAD⁺-GDH activity could be detected in *M. tuberculosis* culture filtrates we undertook to analyze the genetic sequence encoding for the GDH domain of NAD⁺-GDH in *M. tuberculosis* clinical isolates. We could not, however, detect any genetic changes (mutations/insertions/deletions) in the strains investigated which implies a conservation of NAD⁺-GDH function among these bacilli. This lack of observed genetic variation is not unusual as a previous study sequenced the gene encoding for glutamine synthetase (*glnA*) in the same 40 clinical isolates and could not either detect any genetic changes (Hayward, 2007). Although no variation in the genetic sequence of the NAD⁺-GDH in *M. tuberculosis* could be detected, it may be that differential expression of these genes among the clinical isolates may confer an altered phenotype and therefore a competitive advantage. This hypothesis remains to be investigated. A lack of genetic diversity in the NAD⁺-GDH domain sequence of the clinical isolates investigated indicates that these enzymes are able to catalyze reactions typical of GDH enzymes. The genetic regions flanking the GDH domain were not, however, investigated. Although the function of these flanking regions remains unknown, this region may contain genetic differences which could result in differential regulation of NAD⁺-GDH in the clinical isolates and thereby affect the fitness of the pathogen.

APPENDIX 3A: In silico characterisation of the NAD⁺-specific glutamate dehydrogenase enzymes in *Mycobacterium smegmatis*

Introduction:

The *M. smegmatis* genome encodes for two possible NAD⁺-GDH enzymes, namely MSMEG_4699 and MSMEG_6272. The former has been isolated (O'Hare *et al.*, 2008) and shown to have GDH catalytic ability, however, it is not known whether MSMEG_6272 encodes for a functional GDH enzyme. An analysis of MSMEG_4699 subunit size (Chapter 3, Table 1) and evidence from our studies suggests that it belongs to the L_180 class of GDH enzymes, whilst an estimation of MSMEG_6272 subunit size (Chapter 3, Table 1) places it in the L_115 class of NAD⁺-GDH's which, until now, have only been identified in protozoa and fungi (Minambres *et al.*, 2000; Britton *et al.*, 1992). In *S. clavuligerus*, the activity of the L_180 class NAD⁺-GDH identified in this organism was completely inhibited by the presence of 80mM Tris (Minambres *et al.*, 2000) which was used as a buffer in the assay to detect enzyme activity. In *M. smegmatis* we found a large inhibition of total NAD⁺-GDH activity in the presence of 100mM Tris, however, NAD⁺-GDH activity could still be detected (Chapter three, section 2.3.2). It may be that the L_180 class NAD⁺-GDH in *M. smegmatis* is different to its homolog in *S. clavuligerus* and therefore does not display complete inhibition of activity by 100mM Tris, however, it may also be that an additional or alternative functional NAD⁺-GDH, such as MSMEG_6272, is synthesised and able to catalyse the oxidative reduction of 2-oxoglutarate and ammonium in the presence of Tris. These observations prompted an *in silico* analysis of key GDH residues in NAD⁺-GDHs from a number of organisms in order to determine whether MSMEG_6272 may encode for a functional GDH.

In a study conducted by Minambres *et al.*, (2000), a comprehensive analysis of the amino acid sequence encoding for the L_180 class NAD⁺-GDH from *S. clavuligerus* was done. The analysis was based on a comparison with GDH sequences from thoroughly studied organisms such as *Clostridium symbiosum*, *P. furiosus*, *Thermotoga maritime*, *Thermococcus litoralis*, *E. coli* and *B. taurus*. The tertiary structures of the GDH enzymes from the aforementioned organisms had previously been elucidated and critical amino acid residues for GDH function were identified

which, in turn, allowed for the identification of these residues in the L_180 class NAD⁺-GDH of *S. clavuligerus*. In our study, the amino acid sequences encoding for L_180 class NAD⁺-GDH enzymes from *M. smegmatis*, *M. tuberculosis*, *Pseudomonas aeruginosa* PAO1 and *S. clavuligerus* were aligned with MSMEG_6272 in order to elucidate whether the latter protein sequence may encode for a functional enzyme.

Results:

Protein sequences encoding for NAD⁺-GDH enzymes from *S. clavuligerus*, *M. smegmatis*, *M. tuberculosis* and *P. aeruginosa* were obtained from the Genbank database. The protein sequences from *S. clavuligerus* (Minambres *et al.*, 2000) and *P. aeruginosa* (Lu and Abdelal, 2001) are known to encode for L_180 NAD⁺-GDH enzymes whilst it is thought that the sole GDH in *M. tuberculosis* (Rv2476c) and MSMEG_4699 from *M. smegmatis* also belong to this class of enzymes. The amino acid sequences were aligned using ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and critical amino acid residues were identified.

Streptomyces	MQTKLDEAKAELLARAARVAENSPGGGHLPTGSESGRRPDQDTLLGYLQRYLLHTAPEDL	60
MSMEG_4699	-MTREG---RQVGDGRGGLASPEMIRRLSVAFLSTYRGPQADAPGVTSTGPLAVAAHDDL	56
MSMEG_6272	-----	
Rv2476c	-MTIDPGAQDVEAWTTFASADIPDWISKAYIDSYRGRPRDDSS-EATKAAEASWLPASL	58
Pseudomonas	MAFFTAASKADFQHQQLQTALAQHLGDKALP-----QVTLFAEQFFSLISLDEL	48
Streptomyces	TDRDPVDIYGAAYSHYRLAENRPQGTANVRVHTPTVEENGWTCSHSVVEVTTDDMPFLVD	120
MSMEG_4699	VSD-----DLVAAHYRLASMRAPGETKAAVYPGD-----AGSGAALQIVTDQAPMLVD	104
MSMEG_6272	-----	
Rv2476c	LTP-----AMLGAHYRLGRHRAAGESCVAVYRADDP----AGFGPALQVVAEHGGMLMD	108
Pseudomonas	TQRRLSDLVGCTLSAWRLLEFRDRDQPEVRVYNPDYEKHGWQSTHTAVEVLHPDLFFLVD	108
Streptomyces	SVTNELSRQGRGIHVVIHPQVTVRRDVTGKLEIHLHTDRHTPVKSSALPHDALVESWIHV	180
MSMEG_4699	SVTVLLHRHGIAYTAIMNPVFRVRRGLDGELLDVRPAEEAAPG-----DGADECWILV	157
MSMEG_6272	-----	
Rv2476c	SVTVLLHRLGIAYAAIILTPVFDVHRSPTGELLRIEPAEGTS-----PHLGEAWMHV	160
Pseudomonas	SVRMELNRRGYSIHTLQTNVLSVRRSAKELKEILPKGSQG-----KDVSQESLMYL	160
Streptomyces	EIDRETDRADLKQITADLLRVLSDVREAVEDWEKMRREALRIAEELPREPVASDLADQEV	240
MSMEG_4699	PITAAADGEALTEATRLVPGILAEARQIGLDSGAMIAALHGLANDLATDLEG-HFPNAER	216
MSMEG_6272	-----	
Rv2476c	ALSPAVDHKGLAEVERLLPKVLADVQRVATDATALIATLSELAGEVESNAGG-RFSAPDR	219
Pseudomonas	EIDRCAHAGELRALEKAIIEVLGVEVRVTVADFEPMKAKARELLTWLGKAKLK--VPAEEL	218
Streptomyces	EEARELLRWLAADHFTFLGYREYELTGSDALAAVPGTGLGILRSDDLHSEDEDHPVSPSF	300
MSMEG_4699	KEVAALLRWLADGHFVLLGYQCCVVDGNAEV-DPASRLGVLRLR-----	260
MSMEG_6272	-----	
Rv2476c	QDVGELLRWLGDGNFLLGYQRCRVADGMVYG-EGSSGMGVLRGR-----	263
Pseudomonas	KEVRSYLEWLLDNHFTFLGYEEFVVADEADGGRMVYDEKSFGLTRLRAG----LSKDD	274

Streptomyces	SRLPADARAKAREHRLLVLTkansratVHRPSYLDYVGKFFDADGNVIGERRFLGLFSS	360
MSMEG_4699	---NDVLPPLTSDDLLVLAQATMPsYLRyGAYPIVVVRES-PGASRVIEHRFVGLFTV	316
MSMEG_6272	-----	
Rv2476c	---TGSRPRLTDDDKLLVLAQARVGSYLRyGAYPIAVREY-VDGS-VVEHRFVGLFSV	318
Pseudomonas	LHIEDYAVAYLREPVLLSFAKAHPsrVHRPAYPDVVSIRELDGKGRVIRECRFMGLFTS	334
Streptomyces	AAYTESVRRVPVIRRKVAEVLGDAGFSPNSHDGRDLLQILETYPRDELFTQTPVDQLRSIV	420
MSMEG_4699	AAMNANALEIPLISRRVEEALAMAHDP-SHPGQLLRDI IQTIPRPELFASSKQLLEMA	375
MSMEG_6272	-----	
Rv2476c	AAMNADVLEIPTISRRVREALAMAESDP-SHPGQLLLDVIQTVPRPELFLSAQRLLTMA	377
Pseudomonas	SVYNESVNDIPFIRGKVAEVMRRSGFDTKAHLGKELAQVLEVLPRDDLFTQTPVDELFTSA	394
Streptomyces	TSVLYLQERRRLRLYLrqDEYGRYSALVYLPRDRYTTGVRRLRIDILKEELGGTSVDF	480
MSMEG_4699	LAVVDLGSRRRTLLFLRADHLAHFVSLVYLPRDRYTTAVRLEMQDILVRELGGAGIDYS	435
MSMEG_6272	-----	
Rv2476c	RAVVDLGSQRQALLFLRADRLQYFVSLVYMPDRYTTAVRMQFEDILVREFGGTRLEFT	437
Pseudomonas	LAIVRIQERNKIRVFLRKDPYGRFCYCLAYVPRDYVSTETRLKIQQVLMERLQASDCEFW	454
Streptomyces	AWNTESILSRHFVVRVPSG-----TELPHLTADTERIEARLVEAARSWADGFSDA	532
MSMEG_4699	ARVSESPWAVVHFTVRLP-----EGTAADSVDTsLENESRIQDLLTEATRNGWRMISA	489
MSMEG_6272	-----	
Rv2476c	ARVSESPWALMHFMVRLPEVGVAGEAAPPVDVSEANRIRIQGLL TEAARTWADRLIGA	497
Pseudomonas	TFFSESVLARVQF ILRVDP-----KSRIDIDPARLEEEVIQACRSWQDDYSS	502
Streptomyces	LNAELGEERAAELLRRYGAAFPEGYKADTPRAAVADLVHIEALAGSGRDFALSLEYEVP	592
MSMEG_4699	AAAA-SISPAA--LEHYAHAFPEYKQAFAPQDAIADISLIEALQDSSVKLVLDATA---	543
MSMEG_6272	-----MSTAFFNGEELDLAGDEITVRLHHPVHPHEPLRFVLA-----	38
Rv2476c	AAAAGSVGQAD--AMHYAAAFSEAYKQAVTPADAIGDIAVI TELTDDSVKLVFSERD---	552
Pseudomonas	VVENLGEAKGTNVLADFPKGFPAgyRERFAPHFAVVDLQHLLSLSLSEQ-RPLVMSFYQPLA	561
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Streptomyces	AGPGERRFKIYRTGEQVSLSAVLPVLRQLGCEVVDERPYELRGADRSLAWIYDFGLRMPV	652
MSMEG_4699	-EDRVWKLWYLGGSASLSSELLPMLQSMGVVLEERPF TLRRTDGLPVWYQFKISPHP	602
MSMEG_6272	-----AGQPVP LRRMLPALESMDLEVIN EHTTSPTRDGSVCHVYELVDPG-	85
Rv2476c	-EQGVAQLTWFLGGRTASLSQLLPLMQSMGVVLEERPF SVTRPDGLPVWYQFKISPHP	611
Pseudomonas	QGEQQLHCKLYHADTPLALSVDVLP ILENLGLRVLGEFPYRLRHQNGREYWIHDFAFYAE	621
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Streptomyces	GSGNGEYLGDDAR--ERFQDAFAAVWTGAaENDGFNSLVLGAGLNWRQAMVLRAYAKYLR	710
MSMEG_4699	SIPHAPDAEAQRDTAQRFADAVTAIWHGRVEIDRFNELVMRAGLTWQQVVVLRAYAKYLR	662
MSMEG_6272	TAGAQQFARDWNRAQDQICDTFRAIWSDRIEADRLNALIPTAGLDWRQVAVLRSYSRYLR	145
Rv2476c	TIPLAPTVAERAATAHRFAEAVTAIWHGRVEIDRFNELVMRAGLTWQQVLLRAYAKYLR	671
Pseudomonas	G---LDVDIQQLN--EILQDAFVHIVSGDAENDAFNRLVLTANLPWRDVALLRAYARYLK	676
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Streptomyces	QAGSPFSQDYMEDTLRTNVHTRLVLVSLFEARMAPERQRAGTELDG---LLEELD GALD	767
MSMEG_4699	QAGFPYSQSHIESVLNENPHTRSLIDLFEALFD--PSQE-TDGRRDAQAAAAVAADID	719
MSMEG_6272	QLPLPYGQGRIQVLLDNPAATTAVALFEARFARQSVSGAVRERDMAAADQRLEAEID	205
Rv2476c	QAGFPYSQSYIESVLNEHPATVRSVLVDFEALFV--PVPSGSASNRDAQAAAAVAADID	729
Pseudomonas	QIRLGFDLGYIASALNAHTDIARELVRLFkTRFYLARKLTAEDLEDKQKLEQAILGALD	736
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Streptomyces	QVASLDEDRILRSFLTVIKATLRNFFQGTGDGVDGRHGYSMKFDPQAIPDL PPRPA	827
MSMEG_4699	ALVSLDTRVLRAFANLIEATLRNIFYVAR--PDSARARNVLAFLKNPLVIKELPLPRPK	777
MSMEG_6272	RVVHIDADRILRTYRSLVNATVRTNAFTPE--ALTPKAP-YLVHKFDASAIDELQPRL	262
Rv2476c	ALVSLDTRILRAFASLVQATLRNIFYVTR--QGSARCRDVLALKLNAQLIDEPLPRPR	787
Pseudomonas	EVQVLNEDRILRRYDLLIKATLRNIFYQ---PDGNGQNKSYFSKFNPKAIPELPRPVPK	793
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Streptomyces	YEIwVYSPRVEGVHLRFgkVARGGLRWSDRREDFRTEILGLVKAQMVKN TVIVPV GAKGG	887
MSMEG_4699	FEIFVYSPRVEGVHLRFgfvA RGGLR WSDRREDFRTEILGLVKA Q AVK N AVIVPV GAKGG	837
MSMEG_6272	SEIFVYAPQFEGHLRFGLVA RGGLR WSDRHDDYRTEVLGLVKA Q AVK N AMIVPA GAKGV	322
Rv2476c	YEIFVYSPRVEGVHLRFgPVA RGGLR WSDRREDFRTEILGLVKA Q AVK N AVIVPV GAKGG	847
Pseudomonas	YEIFVYSPRVEGVHLRGGKVA RGGLR WSDREDFRTEVLGLVKA Q VK N AVIVPV GAKGG	853
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Streptomyces	FVAKQLPD---PSVDRDAWLAEGIACYRTFISALLDITDN--MVGGEVVPADVVRHDG	941

MSMEG_4699	FVVKRPPTLTGDAADREATRAEGVECYRLFISGLLDVTDNVDKATGAVVTPPEVVRD	897
MSMEG_6272	FVVKPP-----SSRTEGMRCYKQFVSALLDVVDN--AVSDEPPAPEGVVCHDG	369
Rv2476c	FVVKRPLPTGDDPAADRDATAEAGVACYQLFISGLLDVTDNVDHATASVNPPEVVRD	907
Pseudomonas	FVPRRLP-----LGGSRDEIQAEATACYRIFISGLLDITDN--LKEGEVPPANVVRHDE	906
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Streptomyces	DDTYLVV AAD KGTASF S DIANEVALAYGFWLGDFAFASGG S AGYDHKMGIT T ARGAWESVE	1001
MSMEG_4699	EDAYLVV AAD KGTATF S DIANEVAKSYGFWLGDFAFASGG S IGYDHKAMGIT T AKGAWESVK	957
MSMEG_6272	PDPYLVV AAD KGTATF S DIANAVALDRGYWLGDFAFASGG S AGYDHKAMGIT T ARGAWVSGD	429
Rv2476c	DDAYLVV AAD KGTATF S DIANDVAKSYGFWLGDFAFASGG S VGYDHKAMGIT T ARGAWAVK	967
Pseudomonas	DDPYLVV AAD KGTATF S DIANGIAAEYGFWLGDFAFASGG S AGYDHKMGIT T AKGAWVSQ	966
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Streptomyces	RHFRELGHDTQTQ DF TVV GVGDMS GDVFGNGM LLS E H IRLVAA F DHRHIFIDPAPDAATS	1061
MSMEG_4699	RHFREMGVDTQTQ DF TVV GVGDMS GDVFGNGM LLS SK H IRLVAA F DHRHIFIDPNPDAGR	1017
MSMEG_6272	SHLQELGIDSAT DF RVV GVGDMS GDVFGNGM LLR RGIRLVAA F DHRHIFVDPDPSTQAA	489
Rv2476c	RHFREIGIDTQTQ DF TVV GVGDMS GDVFGNGM LLS SK H IRLVAA F DHRHIFIDPNPDAAVS	1027
Pseudomonas	RHFREREGIDVQKD NISVI GVGDMS GDVFGNG LLMS DK LQ LVAA F N H MHIFIDPNPDAASS	1026
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Streptomyces	YAERRRLELPRSSWADYNKELLSAGGGIHPRTAKSIPVNAQVRAALGIEAGITK---M	1117
MSMEG_4699	WDERRRLFDLPRSSWADYDKSLISEGGGVYSRQOKSIPISPVQVTRALGLDADVE---EL	1073
MSMEG_6272	YRERQRLFALARSSWDDYDRAVISAGGGVWPRTAKRIPVSEQMRATLGISGEIT---SV	545
Rv2476c	WAERRRMFLPRSSWSDYDRSLISEGGGVYSRQOKAIPLSAQVRAVLGIDGSDVGGAAAM	1087
Pseudomonas	FVERQRLFNLPSSWADYDAKLISAGGGIFLRSKSAIATPEMKARFDIQA--DR---L	1080
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Streptomyces	TPAELMRAILKAPVDLLWNRGIGTYVKASSESNADVGDKANDP IRVNGDELRVKVVGE GG	1177
MSMEG_4699	TPPALIKAILKAPVDLLWNGGIGTYIKAE TEADADVGD RANDQIRVCGNQVRKAVIGE GG	1133
MSMEG_6272	TPTELIRHILCAPVDLLFNNGGIGTYIKASDEQHGDVGDVNDNVRVDANQVRKAVIVE GG	605
Rv2476c	APPNLIRAILRAPVDLLFNNGGIGTYIKAESESDADVGD RANDPVRVNAQVRKAVIGE GG	1147
Pseudomonas	DPTYLHALLKAPVDLLWNGGIGTYVKSSKETHADVGDKANDGLRVDGRELRAKVVGE GG	1140
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Streptomyces	N LGLTQLGRIEFDRNGGKVNTDAID N SAG V DT S D H EVNIKILLNALVTEGDLTLKQRN I	1237
MSMEG_4699	N LGVLTALGRIEFDLAGGRINTDALDN S AG V DC S D H EVNIKILIDS AVTAGKVTPEERTEL	1193
MSMEG_6272	N LGVSPRGRIEFARNGGYINTDAIDNAAG V DC S D H EVN L KILL----AGRIRGTERNTL	660
Rv2476c	N LGVLTALGRVEFDLGGGRINTDALDN S AG V DC S D H EVNIKILIDS SVSAGTVKADERTQL	1207
Pseudomonas	N LGM T QLARVEFGLHGGANNTDFIDNAG V DC S D H EVNIKILLNEVVQAGDMTEKQRNAL	1200
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Streptomyces	LAQMTDEVGALVLRNNYAQNVALANAVSQSTSLVHAHQRYLRKLVDRDGLDRSLEFLPTD	1297
MSMEG_4699	LLSMTDEVEGLVLDNRDQNDLMTSRANAASLLSVHARMKDLVDNRGLNRELEALPSE	1253
MSMEG_6272	LADMTDEVAHVLANRAHNRLLRDARSNAQMVSVHARMTALERD-GLVRELEHLPSA	719
Rv2476c	LESMTDEVAQLVLDNEDQNDLMTSRANAASLLPVHAMQIKYLVARGVNRLEALPSE	1267
Pseudomonas	LVKMTDAVGALVVGNNYKQTQALSQAQRARERIAEYKRLMGDLEARGKLDRALEFLPSD	1260
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Streptomyces	RQIRDLLNNGRGLSQPELAVLLAYTKITVADELIGTSLPDDPYLLRLLHAYFPKPLLERY	1357
MSMEG_4699	KEIRRRADAGIGLTSPELATLMAHVKLALKDDVLAASDLPDQEVFASRLPYFPTRLREEL	1313
MSMEG_6272	EEFAAMASAGEGLTRPQLATLMAHTKLGKADLLAADDFFDPPYFTAALHRYFPATLRRRL	779
Rv2476c	KEIARRSEAGIGLTSPELATLMAHGLKKEVLATELDPDQDVFAASRLPRYFPQALRERF	1327
Pseudomonas	EELAERISAGQGLTRAELSVLISYSKIDLKESLLKSLVPDDDYLTRDMETAFFALLAEKF	1320
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Streptomyces	PEAVDHHALRREIITTVLVNDTVNTGGSTFLHRLREETGASIEEVVRAQTAARAFIDLGA	1417
MSMEG_4699	HGEIRSHQLRREIITTMLVNDLVDTAGISYAYRITEDVGVGPVDAVRSYVAINAIFGIGD	1373
MSMEG_6272	GDRLTEHPLRREIVATAVNVHLVATSGLYAFRLVEETDAGTSEIVRAHAVASEVFELDA	839
Rv2476c	TPEIRSHQLRREIVTTMLINDLVDTAGITYAFRIAEDVGVTPIDAVRTYVATDAIFGVGH	1387
Pseudomonas	GDAMRRHRLKREIVSTQIANDLVNHMGITFVQRLKESTGMSAANVAGAYVIVRDVHFLPH	1380
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Streptomyces	VWDAVEALDN-QVSAEVQTRMRLHSRRLVERGTRWLLGNRPQPLELAETVEFFAERVEQV	1476
MSMEG_4699	VWRRIRAAAGDAGVPTSVTDRMTLLRRLVDRAGRLLNRYRQPLAVGAEINRFGAKVAAL	1433
MSMEG_6272	LWHDLHAAG---LSPQLTDSLIEHGKLLDRASRWFLLNRPQPLSIAAEFAARFQA-VAML	895
Rv2476c	IWRRIRAAAN---LPIALSDRLTLDRLLDRAGRLLNRYRQPLAVGAEINRFAAMVKAL	1444
Pseudomonas	WFRQIENLDY-QVPADIQLTLMDELMRLGRRATRFWLRSRRNELDAARDVAHFGRPAAAL	1439
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Streptomyces	WSRLPKMLRGADREWYESLYRELTA VGVPEELAVRVAGFSSAFP TLDIVA IADRTKKEPL	1536
MSMEG_4699	TPRMSEWLRGDDKAIVSKEAGDFASHGVPEDLAYHIATGLYQYSLLDVIDIADIVDREP	1493

(Minambres *et al.*, 2000). An analysis of NAD⁺-GDH sequences from *M. smegmatis*, *M. tuberculosis* and *P. aeruginosa* revealed that the core regions displayed a higher degree of homology in comparison to the entire amino acid sequence when a Blastp was done using the *S. clavuligerus* NAD⁺-GDH sequence (Table 1). This result suggests that the core NAD⁺-GDH regions are more highly conserved than the flanking regions. It was found that the core region of MSMEG_6272 displayed a lower degree of homology (73% positives; Table 1) to the core region of *S. clavuligerus* than MSMEG_4699 (81% positives; Table 1). In addition, it can be seen from Figure 1 that the major dissimilarity between the MSMEG_6272 protein sequence and the other GDH enzymes investigated is the absence of amino acid residues at the amino-terminal side of the protein flanking subdomain I.

Table 1: Analysis of the degree of homology between NAD⁺-GDH enzymes from *M. tuberculosis*, *P. aeruginosa* and *M. smegmatis* in comparison with the L_180 class NAD⁺-GDH from *S. clavuligerus*. Both an exact amino acid match (% identity) and degree of homology due to conserved substitutions (% positives) are given for the entire GDH amino acid sequence as well as for the GDH core region.

Organism	% Identity		% Positives	
	complete GDH sequence	GDH core region	complete GDH sequence	GDH core region
<i>S. clavuligerus</i>	100	100	100	100
<i>M. tuberculosis</i>	46	66	61	81
<i>P. aeruginosa</i>	46	68	63	80
<i>M. smegmatis</i>				
MSMEG_4699	47	67	62	81
MSMEG_6272	46	61	62	73

An analysis of the amino acid residues playing a role in the catalytic mechanism of the GDH enzymes revealed that in all the sequences analyzed, the critical residues involved in glutamate and dinucleotide binding were conserved (Figure 1 and Table 2). In addition, the classical βαβ fold or Rossmann fold for dinucleotide binding (Figure 1, residues highlighted in yellow) appeared to be largely conserved in the organisms studied. Within the Rossmann fold, however, there are two amino acid residues encoded by MSMEG_6272 which represent non-conserved

amino acid substitutions (Arg-445 and Arg-463) but it is unclear as to whether these substitutions would render the dinucleotide binding fold non-functional. The conserved GXGXX(G/A/S) motif (Figure 1, residues highlighted in green) which dictates the dinucleotide helix turn has been shown to be typically either GXGXXS or GXGXXA in the L_180 GDH class. In all of the NAD⁺-GDH sequences studied including MSMEG_6272, the motif appeared to be typical of that of the L_180 NAD⁺-GDH class (Figure 1).

Table 2: Comparative analysis of the amino acid residues known to play a critical role in L_180 class NAD⁺-GDH function in *S. clavuligerus* with MSMEG_6272.

Amino acid residue in <i>S. clavuligerus</i>	Amino acid in MSMEG_6272	Function of amino acid residue*
Lys-875	Lys-310	Recognises the 1-carboxyl group of glutamate
Lys-885	Lys-320	Enhances nucleophilicity of the water molecule
Asp-951	Asp-379	Proton transfer to and from glutamate
Arg-853	Arg-348	Forms salt bridge with Asp-951 to stabilize active site structure
Arg-849	Arg-344	Interacts with glutamate
Gly-850	Gly-285	Binds to glutamate side chain atoms
Ala-950	Ala-378	
Val-1207	Val-635	
Ser-1210	Ser-638	Interacts with glutamate and influences glycosidic bond of nicotinamide ring
Gly-851	Gly-346	Influences the shape of the active site pocket
Gly-883	Gly-318	
Gly-1206	Gly-634	
Thr-992	Thr-420	Conformation of glycosidic bond
Ser-978	Ser-406	Interacts with glutamate
Ala-949	Ala-377	Interacts with side chain atoms

*Functions described by Minambres *et al*, 2000.

Discussion:

From an analysis of the amino acid residues essential for GDH function, it would appear that these amino acid residues are conserved in the MSMEG_6272 protein sequence which suggests that the protein may possess GDH catalytic ability. With the exception of two amino acid residues in the Rossmann fold, it would appear that core region of MSMEG_6272 is very similar to the other organisms studied.

In contrast, the amino acid residues in the regions beyond the core differ dramatically. The purpose of the extensive amino acid sequence surrounding the core region of the L_180 and L115 class of GDH enzymes remains completely unknown (Minambres *et al.*, 2000). Similarly, an *in silico* analysis of the sequences flanking the core regions of both NAD⁺-GDH enzymes in *M. smegmatis* could not detect any functional motifs or membrane spanning regions. These regions have, however, been thought to be involved in allosteric control of GDH activity similar to the control of GDH activity in *Chlorella sorokiniana* (Saitou, 1996) and *Homo sapiens* (Peterson and Smith, 1999). Allosteric regulation of the L_180 NAD⁺-GDH (MSMEG_4699) in *M. smegmatis* has previously been shown as binding of the small protein, GarA, is able to down-modulate GDH activity (O'Hare *et al.*, 2008). In a screening program for possible GarA interactor proteins, only MSMEG_4699 was identified which points to differential regulation of MSMEG_6272 and MSMEG_4699. This may be due to the large differences observed in amino-terminal sequences flanking the core regions of these enzymes. Since the tertiary structure of L_180 class GDH enzymes has not yet been elucidated, it is not possible to determine whether there are amino acid residues that are characteristic of this class. Apart from an estimate of protein subunit size, there is little information to allow discrimination between the L_180 class and L_115 class of GDH enzymes at an amino acid residue level.

In summary, it would seem from the analysis of critical amino acid residues in the GDH core region that MSMEG_6272 may encode for a functional NAD⁺-GDH protein. However, since the regions flanking the core are different to MSMEG_4699, it may be that MSMEG_6272 belongs to a different class of enzymes and is not regulated in a similar fashion to its counterpart.

APPENDIX 3B: PCR primer design for sequencing of the GDH domain of the NAD⁺-GDH in *M. tuberculosis* clinical isolates

Streptomyces Rv2467c	TCGAGGCCGGATGGCGCCGAGCGGCAGCGTGCGGGCACCGAGC-TGAC 2252 TCGAAGCGCTTTTCGTTCCGGTGCCGTCGGGGTCGGCGAGCAATCGCGAT 2148 **** *
Streptomyces Rv2467c	GGACGGGCTCCTCGAAGAGCTG--GACGGGGCGCTCGACCAGGTGCCAG 2300 GCCAAGCGGCCGCTGCCGCTGTCGCCGCGGACATCGACGCGCTGGTGA 2198 *
Streptomyces Rv2467c	TCTCGACGAGGACCGCATCCTGCGCTCCTTCCTCACCCTCATCAAGGCCA 2350 CCTGGACACCGACCGGATCCTGCGCGCCTTCGCGTCTGGTTCAGGCA 2248 *
Streptomyces Rv2467c	CTCTGCGGACCAACTTCTCCAGGGCACCAGGGTGACGGGGTGGACGGC 2400 CGTTGCGACCAATTACTTTGTG-----ACACGCCAGGGTTCGGCCGCG 2292 *
Streptomyces Rv2467c	CGCCACGGCTATGTGTCGATGAAGTTCGACCCGACGGCGATCCCCGATCT 2450 TGCCGTGATGTATTGGCGCTGAAACTCAATGCCAGTTGATCGACGA 2342 *
Streptomyces Rv2467c	GCCCGCGCCCGCCCGCCTACGAGATCTGGGTGACTCCCCCGGGTGC 2500 TCCGCTGCGCGTCCCGGTACGAGATTTTGTGTATTCGCCCGCGTCG 2392 *
Streptomyces Rv2467c	AGGGCGTGCATCTGCGCTTCGGCAAGGTCGCGCGCGGGTCTGCGCTGG 2550 AAGGCGTGCATCTGAGGTTTCGGCCAGTTGCGCGTGGCGGGTTCGCTGG 2442 *
Streptomyces Rv2467c	TCCGACCGGCGCGAGGACTTCCGTACGAGATCCTCGGCTGGTCAAGGC 2600 TCGGACCGCGTACGATTTCCGAACCGAGATTCCTCGGATTGGTCAAGGC 2492 *
Streptomyces Rv2467c	GCAGATGGTCAAGAACACGGTGATCGTGCCGGTCGGGCCAAGGGCGGT 2650 GCAAGCGGTGAAGAACGCCGTCATCGTGCCGGTCGGGCCAAGGGCGGT 2542 *
Streptomyces Rv2467c	TCGTGCGCAAGCAGTCCCG-----GACCCCTCCGTGGACCGT 2688 TCGTGGTCAAGCGTCCCCGCTGCCACCGGCGACCCCGCGCCGACCGC 2592 *
Streptomyces Rv2467c	GACGCCTGGCTCGCCGAGGGCATCGCCTGTACCGCACCTTCATCTCCGC 2738 GACGCCACCGCGCCGAAGGGTTCGCTGCTATCAGCTGTTTCATCTCCGC 2642 *
Streptomyces Rv2467c	CCTGCTCGACATCACCGACAACATGGT-----CGGCGGCGAG-GTGGTGC 2782 GTTGCTCGATGTCACCGACAACGTCGATCATGCGACCGCAAGCGTCAAC 2692 *
Streptomyces Rv2467c	CCCCGGCGGACGTCGTACGCCACGACGGTGACGACACCTATCTCGTCGTC 2832 CCCCCGCCGAGGTGGTGCACGTGACGGCGACGACGCTACTTGGTAGTG 2742 *
Streptomyces Rv2467c	GCCGCCGACAAGGGCACCGCTCCTTCTCCGACATCGCCAACGAGGTGCG 2882 GCCGCCGACAAGGTACTGCCACCTTTTCCGATATCGCCAACGATGTCG 2792 *
Streptomyces Rv2467c	CCTCGCCTACGGCTTCTGGCTCGGCGACGCCTTCGCCCTCCGGCGGCTCCG 2932 CAAGTCTATGGGTTCTGGCTGGGCGACGCGTTTCGCCCTCCGGCGGATCGG 2842 *
Streptomyces Rv2467c	CCGGATACGACCACAAGGCCATGGGCATCACCGCCCGGGCGCCTGGGAG 2982 TGGGCTACGACCACAAGGCCATGGGCATCACCGCCCGGGGGGCTGGGAG 2892

Streptomyces Rv2467c	<p>*** ***** ** *****</p> <p>TCGTGGAGCGCCACTTCCGCGAGCTGGGCCACGACACCAGACCCAGAGGA 3032 GCGTAAACCGCATTTCCGAGAGATCGGGATCGACA CCAGACCCAGGA 2942 **** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>CTTACCCTCGTCCGGTGGGGACATGTCCGGCGACGTCTTCGGCAACG 3082 TTCACCGTCTGGGAATCGGCCACATGAGCGCGACGTATTCCGCAACG 2992 ***** ** * ***** *****</p>
Streptomyces Rv2467c	<p>GCATGCTGCTCAGCGAGCACATCCGGCTGGTCCGCCCTTCGACCACCGG 3132 GCATGTTGCTGAGCAAGCACATCCGGCTGATCGCCGCTTCGACCAC GC 3042 ***** ** * ***** *****</p>
Streptomyces Rv2467c	<p>CACATCTTCATCGACCCCGCCCGGACCGGCCACCTCGTACGCCGAGCG 3182 CACATCTTCCTCGACCCCAACCCGATGCCGGGTGTCTGGGCCGACG 3092 ***** ***** ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>CCGCCGGCTCTTCGAGCTGCCCGTTCGAGCTGGGCCGACTACAACAAG 3232 CCGGCGGATGTTGAGTTCGCCCGTCCAGCTGGAGCGACTATGACAGGT 3142 ** * ** * ***** ***** ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>AACTGCTGTCCGGGGCGGGCATCCACCCCGTACCGCCAAGTCGATC 3282 CTCTGATCAGCGAGGGCGGGGTGTACAGCCGGAACAGAAAGCCATC 3192 *** * * ** ***** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>CCCGTCAACGCGCAGGTGCGGGCCGCGCTCGGGATCGAGGCC----- 3324 CCGCTCAGCGCGCAGGTCCGCGCGTCTCGGCATCGACGGCTCAGTTGA 3242 ** * ** * ***** ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>---GGGATACCAAGATGACCCCGCCGAGCTGATGCGGGCGATCCTCA 3370 CGGGGGGCGCCGAGATGCCCCGCCAACCTATTCCGGCGATTCTGC 3292 *** * ** * ***** ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>AGGCCCGCTCGACCTGTGTGGAACGGCGGCATCGGGACGTATGTCAAG 3420 GGGCGCGGTGGACCTGTGTCAACGGCGGCATCGGCACTTACATCAAG 3342 *** * ** * ***** ***** ***** ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>GCGTCGTCGAGTCCAACCGCGGACGTCCGGCACAAGCCAACGACCCAT 3470 GCCGAGTCAGAGTCGGATGTGTGTCGGCGATCGGCCAACGATCCGGT 3392 ** * ** * ***** * ** * ** * ***** ***** ***** ** * ** *****</p>
Streptomyces Rv2467c	<p>CCGGGTCAACGGCGACGAACTGCGGGTCAAGGTCGTCGGCGAGGGCGCA 3520 GCGAGTCAACCGCAATCAAGTCCGCGCAAGGTCATTGGCGAAGGTGGCA 3442 * ** * ** * * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>ACCTGGGGCTGACCCAGCTCGGCCGATCGAGTTCGACCGCAACGGCGGC 3570 ATCTCGGAGTACGGCGTGGGCGGTGTCGAATTCGATCTGTCCGGCGGC 3492 * ** * ** * ** * * ** * ** * ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>AAGGTCAACACCGACGCGATCGACAACAGCGCCGGTGTGGACACATCCGA 3620 CGGATCAACACCGACGCGCTGGACAACCTCCGCCGGCTGGACTGTCTCGGA 3542 * ***** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>CCACGAGGTCAACATCAAGATCTGCTGAACGCGCTGGTACCAGGGCGG 3670 CCACGAGGTCAACATCAAGATCTGATCGACTCGTGGTGAGCGCCGGCA 3592 ***** ***** * ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>ACCTCACCTCAAGCAGCGCAATATCTGCTGGCGCAGATGACCGACGAG 3720 CGGTCAAAGCCGACGAACGCACACAGCTGCTCGAGTCAATGACCGACGAG 3642 *** * * * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>GTCGGCGCTGGTGTGTCGCAACAACACGCGCAGAACGTCGCCCTGGC 3770 GTAGCGC AACTGGTGTCTCGCGACAACGAAGATCAGAACGACTGTATGGG 3692 ** * ***** * ** * ** * ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces Rv2467c	<p>CAACGCCGTCAGCCAGTCCACCTCCCTCGTCCAGCCAC-CAGCGCTAT 3819 CACCAGTCGCGCAACCGCGCCAGCCTGCTGCCGGTGCACGCAATGCAGA 3742 ** * * * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *****</p>
Streptomyces	<p>CTGCGCAAGCTGGTCCGGACGGGGCGTGGACCGCTCCCTGGAGTTCCT 3869</p>

Rv2467c	TCAAGTAT-TTGGTGGCTGAGCGCGGGTCAACCGCAATTGGAAGCGCT	3791
	* * * * * * * * * * * * * * * * * *	
Streptomyces	GCCACCGACCGGCAGATCCGGGACCTGCTCAACAACGGCAGGGCCTGA	3919
Rv2467c	GCCGTCGGAGAAGGAGATTGCCCGGCGCTCCGAGGCCGGCATCGGGCTCA	3841
	* * * * * * * * * * * * * * * * * *	

Figure 1: Multiple sequence alignment of the genes encoding for NAD⁺-GDH in *Streptomyces clavuligerus* (Streptomyces) and in *M. tuberculosis* (Rv2476c). The GDH domain is highlighted in grey. PCR primers used to amplify fragments A and B are highlighted in yellow and blue respectively.

CHAPTER FOUR

GlnD and the Signaling Cascade

4.1 INTRODUCTION:

In *E. coli*, GlnD plays a central role in the nitrogen regulatory system (Ntr) by sensing and signaling the cellular nitrogen status to various cellular components which adjust enzyme activity and transcription of nitrogen-related genes accordingly. In most prokaryotes studied to date, GlnD acts as a uridylyltransferase which reversibly modifies the PII signaling protein(s) (GlnB and/or GlnK) in response to nitrogen availability. Under nitrogen limiting conditions, GlnD uridylylates the PII (PII-UMP) proteins in *E. coli* which are then able to interact with GlnE and activate the de-adenylation of GS in order to increase GS specific activity (Jaggi *et al.*, 1997). PII-UMP is not able to interact directly with the response regulator, NtrB, and the absence of this interaction stimulates its kinase activity which results in the phosphorylation of, and subsequent activation of the transcriptional regulator, NtrC (Merrick and Edwards, 1995; Arcondeguy *et al.*, 2001). The converse cascade of events occurs upon exposure of *E. coli* to conditions of nitrogen sufficiency; namely, GlnD de-uridylylates PII which then interacts with GlnE to stimulate the adenylation of GS to reduce its activity. Native PII also interacts with NtrB in order to stimulate its phosphatase activity which causes dephosphorylation and deactivation of NtrC to ultimately abolish transcription of nitrogen metabolism-related genes (Atkinson *et al.*, 1994).

During exposure of *E. coli* to nitrogen starvation conditions, the intracellular level of 2-oxoglutarate has been shown to rise and, together with ATP, is bound by the PII proteins. The conformation of the PII protein is altered upon binding of 2-oxoglutarate and ATP, which allows for the association of the PII protein with GlnD and consequent activation of its uridylylating activity (Jiang *et al.*, 1998). When nitrogen is in excess, glutamine levels are increased in *E. coli*, which interacts directly with GlnD and inhibits its uridylylating activity whilst stimulating the de-uridylylation reaction (Jiang *et al.*, 1998). GlnD enzymes from a variety of organisms appear to have a similar structure to that of *E. coli*, which includes an N-terminal nucleotidyltransferase domain (NT), a PII-uridylyltransferase domain (UT), an HD domain (so-called due to the conserved histidine and aspartate residues) and two aspartokinase-chorismate mutase-TyrA (ACT) domains (Tondervik *et al.*, 2006). The nucleotidyltransferase domain has been shown to be essential for the uridylylation activity of the GlnD protein whilst the PII-uridylyltransferase domain is a motif found in GlnD-like uridylyltransferases, but has not been assigned a specific

function (Tondervik *et al.*, 2006). It was thought that the NT domain was also responsible for de-uridylylation of the PII proteins (Jiang *et al.*, 1998). It was found, however, that a truncated GlnD protein, in which the C-terminal portion of the protein was deleted in both *C. glutamicum* (Strosser *et al.*, 2004) and *S. coelicolor* (Hesketh *et al.*, 2002), was able to modify GlnK but was unable to demodify the PII protein. These results suggested that two different domains were responsible for the two different reactions. It was recently shown that de-uridylylation of the PII proteins is catalyzed by the HD domain of GlnD in *Rhodospirillum rubrum* (Zhang *et al.*, 2010), which supports the observations made in the aforementioned Actinomycetes. GlnD is thought to be the primary sensor of intracellular nitrogen status in *E. coli* as it directly senses fluctuating glutamine levels. This sensory ability has been ascribed to the ACT domains of GlnD, since this domain has been implicated in the control of enzyme activity after binding to particular amino acids in other organisms (Zhang *et al.*, 2010). Sequence analysis of GlnD in *M. smegmatis* (Pfam database: <http://pfam.sanger.ac.uk>) (Finn *et al.*, 2010) revealed that the protein encodes for all of the domains described for *E. coli*, which suggests that a functional protein may be present in this organism. Analysis of the GlnD protein in *C. glutamicum* revealed that the ACT domain is absent, which could account for the absence in cellular response upon fluctuations in intracellular glutamine concentrations (Nolden *et al.*, 2001b). The fact that a cellular response to nitrogen availability did occur in *C. glutamicum* suggests that a different sensor of nitrogen status may be present.

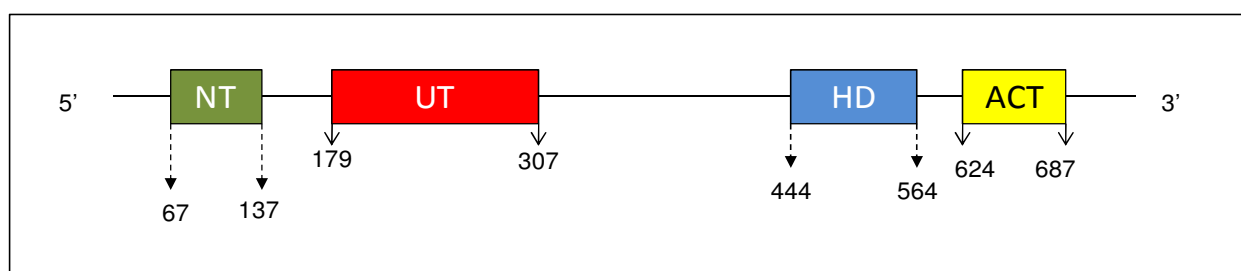


Figure 1: Schematic representation of the nucleodityltransferase (NT), GlnD PII-uridylyltransferase (UT), Histidine-Aspartate (HD) and aspartokinase-chorismate mutase-TyrA (ACT) domains of GlnD in *M. smegmatis*. The amino acid residues encoding for the domains are given.

In contrast to *E. coli*, it has been shown that *C. glutamicum* and *S. coelicolor* have only a single PII protein, GlnK (Strosser *et al.*, 2004; Hesketh *et al.*, 2002) which is adenylylated rather than uridylylated by GlnD in these Actinomycetes. Blast analyses indicate that only a single PII protein, GlnK, is present in *M. smegmatis* and *M. tuberculosis* (Harper *et al.*, 2008). It is not known whether GlnD adenylylates or uridylylates the PII protein in these organisms; however, it has been hypothesized that adenylylation of PII proteins by GlnD may be a conserved mechanism in the Gram-positive Actinomycetes (Burkovski, 2007). In *C. glutamicum*, the adenylylation of GlnK has been shown to be required for the removal of AmtR-induced repression of nitrogen-related genes, however, it does not appear to be necessary for modulation of GS activity via GlnE in response to nitrogen availability (Burkovski, 2007; Burkovski, 2003). A similar phenomenon was observed in *S. coelicolor*, since GlnK was found to be adenylylated by GlnD, however, post-translational control of GS via GlnE remained unaffected by the absence of either GlnD or GlnK (Hesketh *et al.*, 2002). The role of GlnD regarding the control of transcription of nitrogen metabolism-related genes in *S. coelicolor* has not yet been determined.

The function of GlnD in *M. tuberculosis* was investigated by generating a chromosomal deletion of the gene encoding for the putative uridylyltransferase (Read *et al.*, 2007). It was found that a deletion of *glnD* was not lethal and that the knockout strain was not compromised in its ability to utilize a number of nitrogen sources. Although the effect of such a deletion on the transcriptional control of nitrogen-metabolism related genes was not investigated, it was found that the deletion caused a de-regulation of GS activity, and in particular, of extracellular GS in *M. tuberculosis* (Read *et al.*, 2007). In the aforementioned study it was also found that total GS activity increased upon an increase in ammonium concentration in wild type *M. tuberculosis* cell free extracts (Read *et al.*, 2007), which is in contrast with previous studies (Harth *et al.*, 1994). In the light of the differences between the slow and rapidly growing mycobacteria as well as the rather contradictory results presented by Read *et al.*, (2007), this Chapter aims to elucidate the role of GlnD in *M. smegmatis*.

4.1.1 Study Objectives

Objective 1: To generate a *glnD* knockout in *M. smegmatis* (GlnDKO) and to determine the effects of the deletion on growth under various conditions of ammonium availability.

Objective 2: To determine the role of GlnD in the transcriptional control of nitrogen-related genes by comparing the relative change in expression of *glnA* and *amtB* in wild type and GlnDKO *M. smegmatis* strains.

Objective 3: To investigate the role of GlnD in the signaling of cellular nitrogen status to the major enzyme effectors of the nitrogen metabolic pathway in *M. smegmatis* by analyzing the difference in specific activities of GS and GDH enzymes in wild type and GlnDKO *M. smegmatis* strains under varying conditions of nitrogen availability.

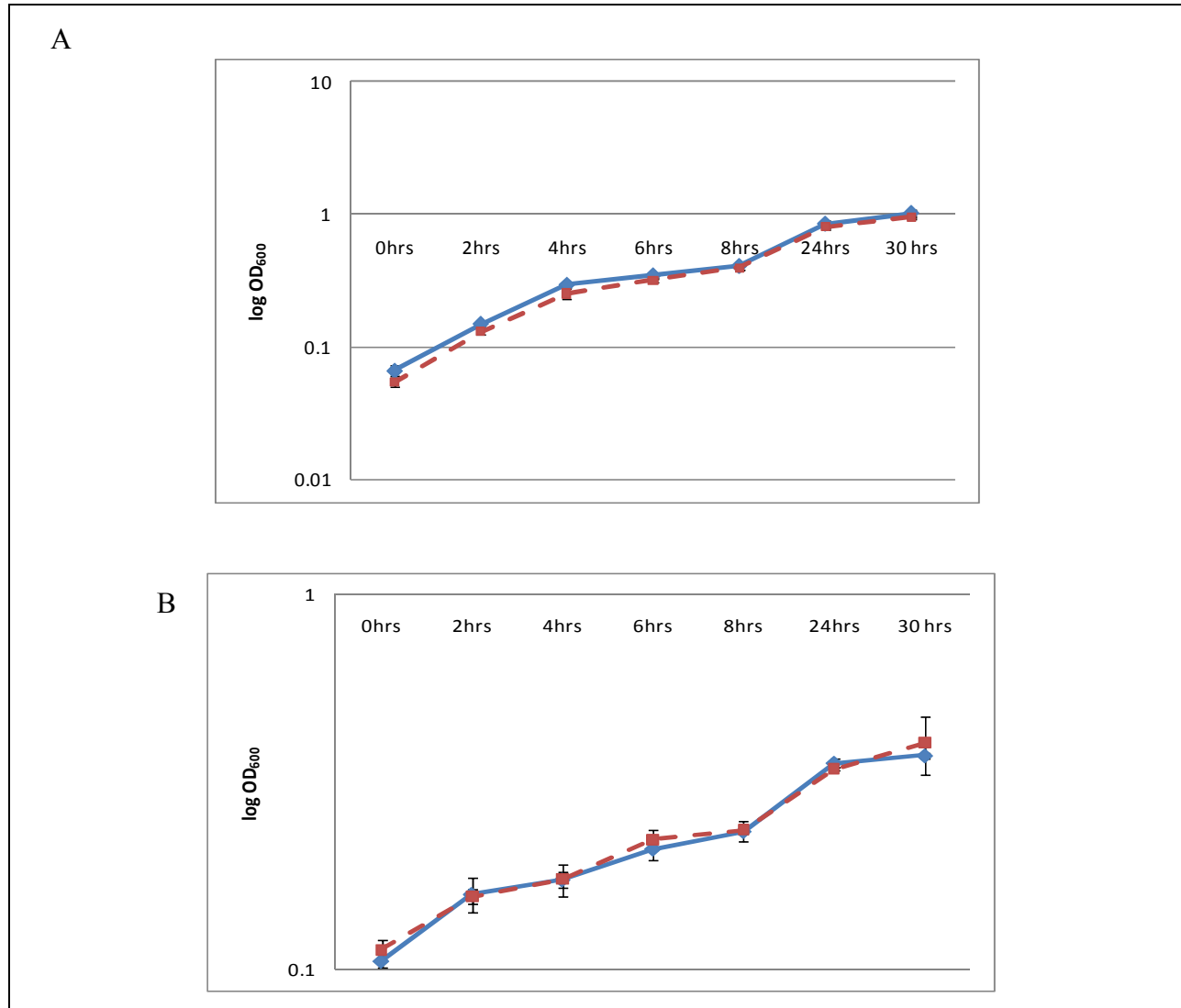
4.2 RESULTS:

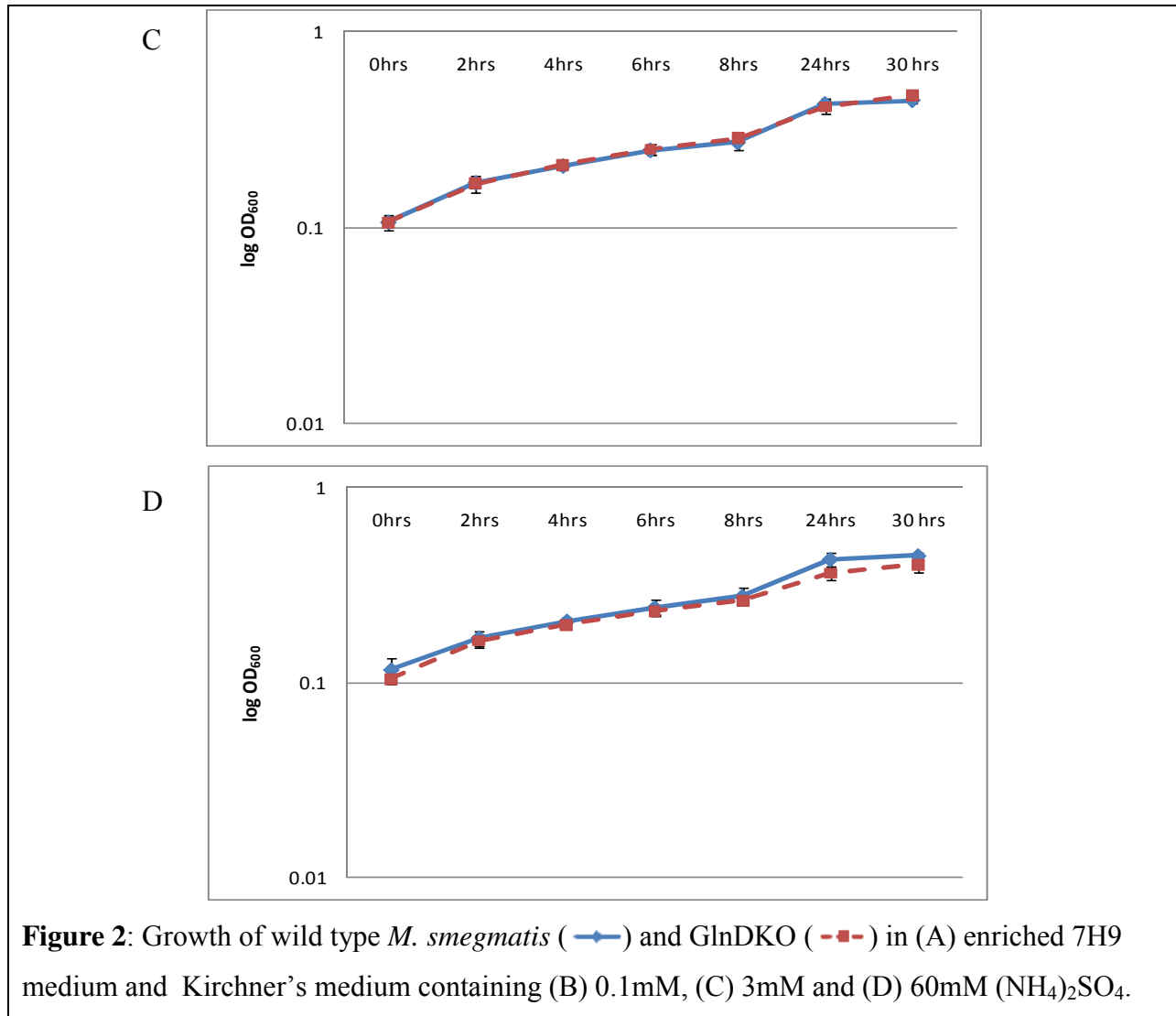
4.2.1 In vitro comparison of growth between wild type *M. smegmatis* and the *glnD* deletion mutant *M. smegmatis* strain

A deletion in the central portion of the *glnD* gene in *M. smegmatis* was generated using the same techniques as described in Chapter two, namely, the replacement of the wild type copy of the gene with a deleted form thereof via homologous recombination (Parish and Stoker, 2000b) (see Appendix A). The growth and viability of the GlnDKO strain was analyzed by culture in OADC-enriched 7H9 liquid medium as well as on OADC-enriched 7H10 agar plates. In addition, the ability of the GlnDKO strain to grow in Kirchner's minimal medium containing various concentrations of ammonium sulphate (0.1mM, 3mM or 60mM) as the sole nitrogen source was analyzed.

Wild type *M. smegmatis* and the GlnDKO *M. smegmatis* strain were cultured in OADC-enriched 7H9 broth until an approximate $OD_{600} \approx 0.5$. A 10-fold dilution series of each culture was made and 100 μ l of each dilution spread onto an OADC-enriched 7H10 agar plate. This experiment was repeated twice. After 3 days the colonies were counted and there appeared to be no significant difference in growth, survival or colony morphology of the GlnDKO strain in comparison with wild type *M. smegmatis* (data not shown). In addition, no significant difference in growth rate between wild type *M. smegmatis* and the GlnDKO strain could be observed when the bacteria

were cultured in OADC-enriched 7H9 medium (Figure 2A) or in Kirchner's minimal medium containing either 0.1mM (Figure 2B), 3mM (Figure 2C) or 60mM (Figure 2D) ammonium sulphate.





4.2.2 Effect of a *glnD* deletion on the transcriptional control of nitrogen metabolism-related genes in *M. smegmatis*

Wild type *M. smegmatis* and the GlnDKO strain were cultured essentially as described in Chapter 3, section 3.2.1. Briefly, the bacteria were cultured in 7H9 medium to mid-log phase and transferred to Kirchner's minimal medium containing either 60mM (excess) or 3mM (starvation) ammonium sulphate as the sole nitrogen source. After a pre-conditioning period of 1hr, bacteria that had been exposed to the 60mM ammonium sulphate concentration condition were transferred to Kirchner's medium containing 3mM ammonium sulphate for 0.5hrs. The opposite procedure was performed for bacteria that had been pre-conditioned in a 3mM ammonium

sulphate concentration condition. Total bacterial RNA was extracted at time point zero (time point zero was regarded as directly after pre-conditioning) and after 0.5hrs incubation in medium containing either an excess or a limited amount of ammonium. The RNA was reverse transcribed to cDNA and the relative expression of nitrogen metabolism-related genes was determined by Real-time PCR. *16SrRNA* was used as an internal control (housekeeping gene) to normalize gene expression data. Each experiment was repeated at least three times and every PCR reaction was done in triplicate. The PCR efficiencies of the primers used had previously been determined (Chapter 2, Table 2).

The transcriptional regulation of genes encoding for GS (*glnA*) and the ammonium transporter, AmtB (*amtB*), in response to ammonium availability, have been extensively characterized in a variety of organisms including *C. glutamicum*, *S. coelicolor* and *M. smegmatis*. The expression of these two genes was investigated in the GlnDKO strain to determine whether the absence of the putative uridylyltransferase had an aberrant effect on the regulation of gene transcription. In most of the prokaryotes studied to date, *glnA* and *amtB* expression is up-regulated upon exposure to ammonium limitation and conversely regulated when exogenous ammonium concentrations are in excess. This was indeed found to be the case in the wild type *M. smegmatis* strain (Table 1), which displayed an increase in *glnA* (6 fold) and *amtB* (3 fold) expression during ammonium starvation conditions. A 2 fold down-regulation of both genes occurred under conditions of ammonium excess (Table 1; this data is also discussed in Chapter 2). In contrast, the expression of *glnA* and *amtB* were up-regulated upon exposure of the GlnDKO strain to both ammonium starvation (13 and 6 fold respectively) and excess (13 and 3 fold respectively) conditions (Table 1). The regulation of these two genes in the GlnDKO strain resembles the pattern of expression observed in the GlnRKO strain (Chapter 2, Table 5), namely an up-regulation of gene transcription during exposure to either ammonium limitation or excess culture conditions (Table 1).

Table 1: Relative change in expression of *glnA* and *amtB* in the GlnDKO, GlnRKO and wild type *M. smegmatis* strains under conditions of ammonium limitation (3mM (NH₄)₂SO₄) and excess (60mM (NH₄)₂SO₄)^a

Gene	(NH ₄) ₂ SO ₄ concentration					
	3mM			60mM		
	Wild type ^b	GlnDKO	GlnRKO ^b	Wild type ^b	GlnDKO	GlnRKO ^b
<i>glnA</i>	6 ± 2.6	13.0 ± 9.5	10.62 ± 5.26	0.53 ± 0.16	13.3 ± 7.5	5.21 ± 2.21
<i>amtB</i>	3.21 ± 1.0	6.8 ± 3.6	2.52 ± 0.59	0.65 ± 0.11	2.9 ± 0.7	1.41 ± 0.28

^aHighlighted values represent a statistically significant (p<0.05) change in gene expression as compared to time point zero.

^bExpression data obtained from Chapter two, Table 5

4.2.3 Effect of a *glnD* deletion on GDH activity under conditions of ammonium limitation and excess

To investigate whether GlnD may play a role in the regulation of GDH activity in response to ammonium availability, wild type *M. smegmatis* and the GlnDKO strain were cultured as described in Chapter 3, sections 3.2.1 and 3.2.2. A crude protein extract was made and the specific activity of the NADP⁺-GDH and NAD⁺-GDH aminating reactions, as well as the NADP⁺-GDH deaminating reaction was determined after exposure of the bacteria to ammonium limiting and excess conditions (Figure 3). The experiments were repeated at least three times and the enzyme assays were done in triplicate. The deaminating reaction activity of the NAD⁺-specific GDH isoform was excluded from the analysis since the activity of this reaction has been shown to be very low and insensitive to changes in ammonium concentration (Chapter 3).

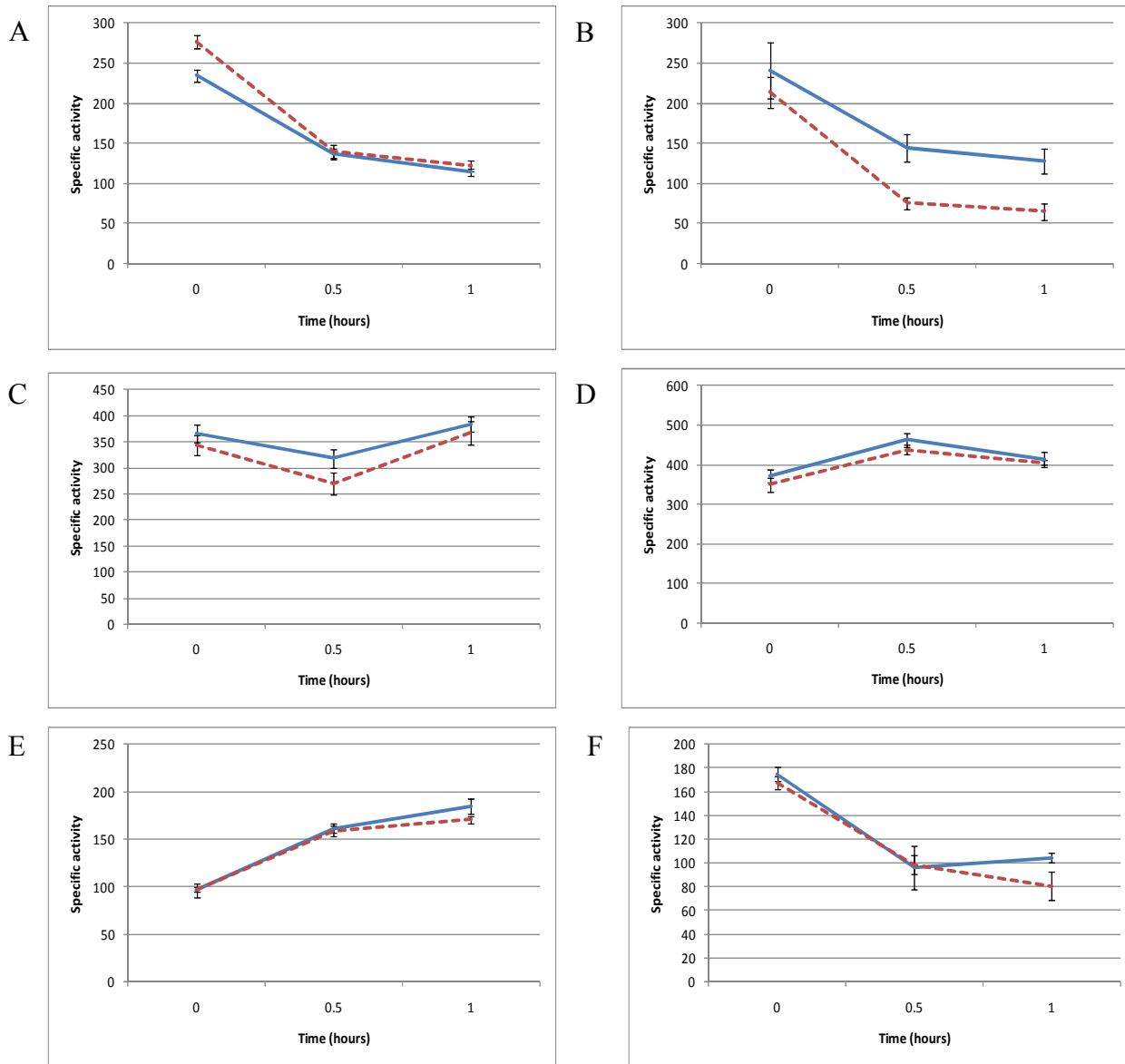


Figure 3: Specific activity (1nmol NAD(P)(H)/min/mg protein) of the NAD⁺-GDH aminating reaction under conditions of ammonium (A) limitation and (B) excess, the NADP⁺-GDH aminating reaction under conditions of ammonium (C) limitation and (D) excess and deaminating reaction under conditions of ammonium (E) limitation and (F) excess in wild type *M. smegmatis* (solid line) and in the GlnDKO *M. smegmatis* strain (dashed line).

It was generally found that the pattern of regulation of both GDH isoforms in the GlnDKO strain was similar to that of wild type *M. smegmatis* (Figure 3). NAD⁺-GDH aminating activity was down-regulated by approximately 100 units and 140 units within 0.5hrs exposure to ammonium

starvation conditions in wild type and GlnDKO *M. smegmatis* respectively (Figure 3A). Upon exposure to an ammonium pulse (Figure 3B), NAD⁺-GDH aminating reaction activity was also reduced in both the wild type (approximately 100 units) and the GlnDKO strains (approximately 140 units). The degree of down-regulation of NAD⁺-GDH aminating reaction activity upon exposure to both ammonium starvation and excess conditions appeared to be greater for the GlnDKO strain within the first 0.5hrs than for wild type *M. smegmatis*.

It was observed that the NADP⁺-GDH aminating reaction was moderately decreased within 0.5hrs exposure to ammonium starvation conditions in both the wild type (by approximately 50 units) and GlnDKO (by approximately 75 units) *M. smegmatis* strains (Figure 3C). The initial down-regulation was followed by an increase in NADP⁺-GDH aminating reaction activity within the ensuing 0.5hr in both strains (Figure 3C). The increase in NADP⁺-GDH aminating reaction activity between 0.5hrs and 1hr was about the same as the initial decrease in activity in both the GlnDKO and wild type *M. smegmatis* strains. The opposite pattern of NADP⁺-GDH aminating reaction activity was observed upon exposure of both the wild type and GlnDKO *M. smegmatis* strains to an ammonium pulse (Figure 3D). Upon analysis of the NADP⁺-GDH deaminating reaction (Figure 3E), it was observed that an increase in activity occurred within 0.5hrs exposure to ammonium starvation conditions in both the wild type (65 units) and GlnDKO (62 units) *M. smegmatis* strains. The converse regulation of NADP⁺-GDH activity was found when the two strains were exposed to ammonium excess conditions, namely; a down-regulation of activity in both wild type (78 units) and GlnDKO (69 units) *M. smegmatis* strains (Figure 3F).

4.2.4 Effect of a *glnD* deletion on GS activity under conditions of ammonium limitation and excess

The γ -glutamyl transferase assay was used to determine total GS activity in the crude protein extracts isolated from wild type and GlnDKO *M. smegmatis* which had been cultured in Kirchner's minimal medium containing either an excess or a limited amount of ammonium (section 4.3.2 above). These experiments were repeated at least three times and the γ -glutamyl transferase assays were done in triplicate.

In the wild type *M. smegmatis* strain, a moderate increase in total GS activity (approximately 12 units or 22% increase, Table 2) was observed after exposure of the bacteria to ammonium limiting conditions for 1hr. This was similar to our previous results which indicated an approximately 17 units (27%) increase under the same experimental conditions (Chapter 3, Table 3). Upon introduction of excess amounts of ammonium to previously starved wild type bacilli, a significant reduction in total GS activity of approximately 163 units (76% decrease) occurred within 0.5hrs (Table 2). Although the degree of down-regulation of GS activity differed, it was found that in both this experiment and our previous work (Chapter 3, Table 3) that GS activity was significantly reduced when the bacilli were exposed to ammonium excess conditions.

Table 2: Total GS specific activity as determined by the γ -glutamyl transferase assay in wild type *M. smegmatis* and in the *glnD* knockout strain in response to ammonium limitation (3mM $(\text{NH}_4)_2\text{SO}_4$) and excess (60mM $(\text{NH}_4)_2\text{SO}_4$).

$(\text{NH}_4)_2\text{SO}_4$ concentration	Time (hours)	Specific Activity ($\mu\text{moles}/\text{min}/\text{mg Protein}$)	
		Wild type	GlnDKO
3mM	0	37.92 \pm 2.44	105.72 \pm 1.48
	0.5	42.96 \pm 1.93	102.96 \pm 0.83
	1	49.02 \pm 5.95	109.92 \pm 0.9
60mM	0	215.92 \pm 5.9	186.60 \pm 28.83
	0.5	52.49 \pm 7.24	161.41 \pm 33.5
	1	47.81 \pm 3.07	165.37 \pm 24.35

The absence of a functional GlnD protein appeared to have a significant effect on the regulation of total GS activity in *M. smegmatis*. A slight increase in GS activity in response to ammonium limitation was observed (approximately 7 units or 2.6%, Table 2); however, it was found that total GS activity in the GlnDKO strain was at least 2 fold higher than in the wild type *M. smegmatis* strain (Figure 4). Within 0.5hrs after exposure of the GlnDKO strain to an ammonium pulse, a slight decrease in total GS activity was observed; however, this did not appear to be statistically significant (Figure 4 and Table 2). Therefore, the absence of GlnD appeared to abolish the down-regulation of GS activity normally observed in wild type *M. smegmatis*, upon exposure to ammonium excess conditions.

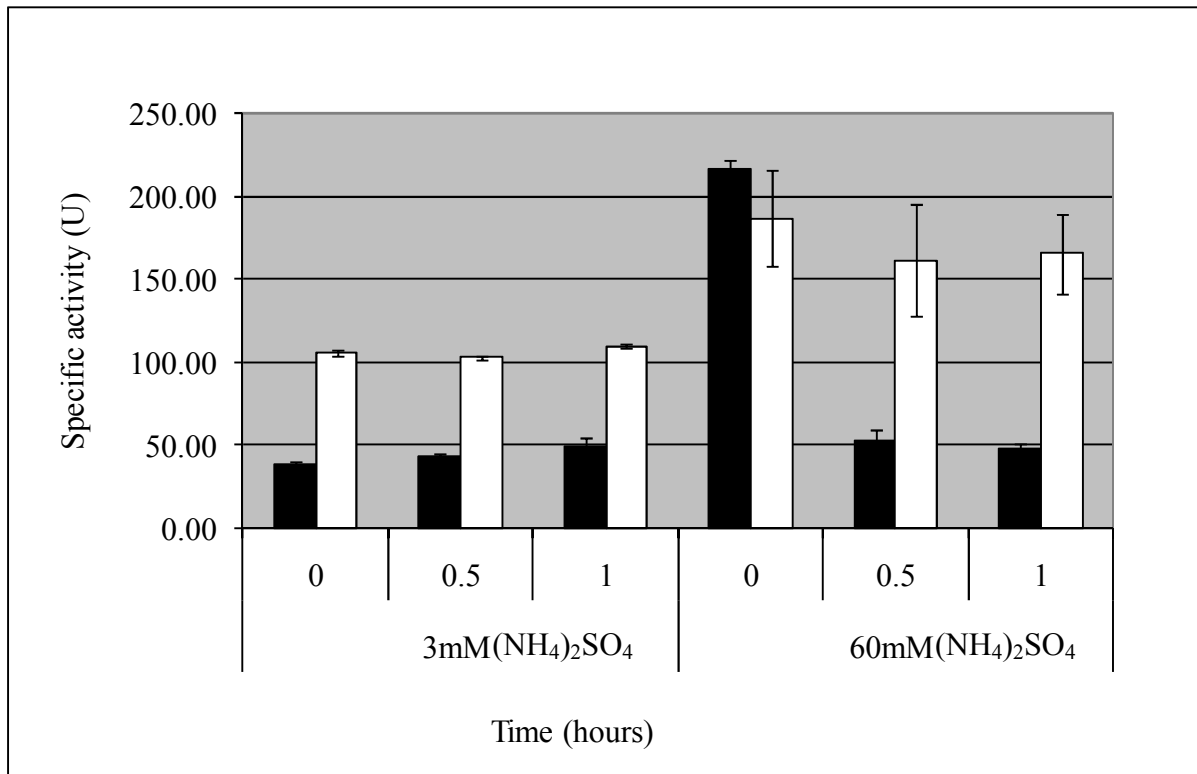


Figure 4: Total GS specific activity ($1\mu\text{mol } \gamma\text{-glutamyl hydroxamate}/\text{min}/\text{mg}$) as determined by the γ -glutamyl transferase assay in wild type *M. smegmatis* (black bars) and in the *glnD* knockout strain (white bars) in response to ammonium limitation (3mM (NH₄)₂SO₄) and excess (60mM (NH₄)₂SO₄).

4.3 DISCUSSION:

In initial attempts to generate a *glnD* knockout mutant, a technique known as mycobacterial recombineering was used (van Kessel and Hatfull, 2008b). The method is a plasmid-based system which encodes for mycobacteriophage recombination proteins whose expression is under the control of an inducible acetamidase promoter (van Kessel and Hatfull, 2008a). An *M. smegmatis* recombineering strain was generated by transformation of the plasmid (pJV75amber), encoding for the mycobacteriophage proteins, into wild type *M. smegmatis*. In order to introduce a point mutation, the recombineering strain is transformed with a short single stranded DNA oligonucleotide which is homologous to the region of interest, with exception of the single base target. In order to generate a *glnD* knockout, an oligonucleotide with a single base deletion, was designed to be homologous to the 5' end of the gene. Upon induction of the acetamidase

promoter and transformation of the oligonucleotide into the recombineering strain, a DNA recombination event, promoted by the mycobacteriophage recombination proteins, would introduce the point mutation into the *M. smegmatis* genome. The single base pair deletion in the *glnD* gene was designed to produce a stop codon as well as a frame-shift mutation and thereby render the protein product non-functional. The screening of potential knockout colonies was done using PCR amplification, high resolution melt analysis and DNA sequencing (see Appendix 4B). Although this method greatly simplifies the time-consuming process of generating a knockout mutant in the mycobacteria, we were not successful in recovering the desired point mutation in *M. smegmatis*. It was found that *M. smegmatis* struggled to grow in the medium designed to induce the expression of the mycobacteriophage recombineering proteins which may be one of the reasons that the desired mutant was never recovered. In the light of the difficulties that arose through use of the recombineering method, the three-way cloning method developed by Parish and Stoker (2000) to generate a *glnD* deletion mutant was employed.

4.3.1 *In vitro* growth of the GlnDKO *M. smegmatis* strain

It has previously been shown that a *glnD* deletion in *M. tuberculosis* had little effect on the growth of the organism in comparison with the wild type strain (Read *et al.*, 2007). A similar result was obtained when *glnD* was inactivated in *S. coelicolor* (Hesketh *et al.*, 2002). In contrast, deletion of *glnD* in *C. glutamicum* had a negative impact on growth under ammonium limiting culture conditions (Nolden *et al.*, 2001b). Since GlnD is essential for the removal of AmtR-induced repression of gene transcription in *C. glutamicum*, it was thought that one of the reasons for the poor growth rate observed was due to a decrease in the synthesis of ammonium transporters and, therefore, a decrease in ammonium uptake (Nolden *et al.*, 2001b). The growth of our *M. smegmatis* GlnDKO strain was similar to that of the *glnD* knockout strains generated in *M. tuberculosis* and *S. coelicolor*, which may indicate a greater similarity in the nitrogen regulatory cascade among these Actinomycetes in comparison with *C. glutamicum*. The master regulator of nitrogen metabolism-related gene transcription in the *Corynebacteria* is AmtR, whilst only GlnR has been identified in *S. coelicolor* and *M. tuberculosis*. *M. smegmatis* has both proteins yet its growth appeared to be similar to those bacteria whose master regulator of nitrogen metabolism-related gene transcription is GlnR. This may indicate that AmtR plays a

rather different role with regard to nitrogen metabolism in *M. smegmatis*, in comparison with its homolog in *C. glutamicum*. A similar conclusion was drawn from an analysis done on the regulatory role of AmtR in Chapter two.

4.3.2 Effect of a *glnD* deletion on the transcriptional control of *amtB* and *glnA*

In most of the prokaryotes studied to date, GlnD is able to uridylylate or adenylylate the PII protein(s) which in turn relays the intracellular nitrogen signal to other components within the cell. Although modification of the PII protein by GlnD has not been experimentally verified in the mycobacteria, the probability of such a reaction is high as the genomic arrangement and reaction mechanism is well-conserved across several bacterial genera (Thomas *et al.*, 2000). Following the modification of GlnK by GlnD under conditions of ammonium limitation in a number of organisms (such as *E. coli*, *B. subtilis* and *C. glutamicum*), GlnK-U/AMP is able to interact with the appropriate transcriptional regulator to bring about an increase in the expression of *amtB* and *glnA*. Conversely, GlnD is able to de-modify GlnK under conditions of nitrogen excess which subsequently signals the transcriptional regulators to down-regulate the expression of those genes. GlnR has been shown to activate *glnA* and *amtB* expression in *M. smegmatis* under conditions of nitrogen limitation (Amon *et al.*, 2008a) and repress the expression of these genes when nitrogen sufficient conditions return (Chapter two, section 2.2.6). An analysis of the transcriptional regulation of *glnA* and *amtB* in the GlnDKO *M. smegmatis* strain indicated a de-regulation of transcriptional control, namely; an up-regulation of *glnA* and *amtB* expression under conditions of both ammonium limitation and excess in the GlnDKO strain. The observed pattern of transcriptional regulation in the GlnDKO strain was similar to the effects observed in the GlnRKO strain, which suggests that GlnD may be involved in the transduction of cellular nitrogen status to the nitrogen metabolism-related transcriptional regulatory machinery. Typical OmpR-type proteins are the response regulators of two-component regulatory systems and normally function together with sensory histidine kinases (Kenney, 2002). In *S. coelicolor*, a potential phosphorylation site (Asp⁵⁰) in the OmpR-type regulator, GlnR, has been identified (Wray, Jr. and Fisher, 1993); however, regulation of GlnR by a sensory kinase has yet to be shown. An alignment of the *M. smegmatis*, *M. tuberculosis* and *S. coelicolor* GlnR protein sequences revealed that the potential phosphorylation site appears to be conserved in these

mycobacteria which suggests that they too could be regulated in a similar manner. In the absence of experiment evidence regarding the phosphorylation of GlnR by a sensory kinase it may be that GlnK is able to interact with GlnR directly or that the PII protein can interact with a third party, such as a sensory histidine kinase, which can signal GlnR to modulate the expression of nitrogen metabolism-related genes.

The up-regulation of *glnA* and *amtB* expression under conditions of ammonium starvation in the GlnDKO strain was unexpected since, in the absence of GlnD, only native or unmodified GlnK would be present in the cell which, in turn, should bring about a repression of gene transcription (Figure 5). An analysis of the deletion region within *glnD* revealed that a portion of the nucleotidyltransferase domain was not deleted (Appendix A). The gene could thus possibly produce a truncated protein product which retains its uridylylating or adenylylating ability.

Upon exposure of the GlnDKO strain to an ammonium pulse, *amtB* and *glnA* expression was increased in a manner similar to the GlnRKO strain. This result suggests that a signal to GlnR regarding an increase in intracellular ammonium concentrations was lacking. This then implies that the truncated GlnD protein was not able to de-modify the PII protein to eventually bring about transcriptional repression. An analysis of our knockout region indicated that the majority of the HD domain was deleted (Appendix A) which would explain the inability of the truncated GlnD protein to de-modify GlnK. These results also indicate that the uridylylating and de-uridylylating functions of GlnD are not performed by the same domain.

4.3.3 Effect of a *glnD* deletion on the activity of the major nitrogen assimilatory enzymes: GDH and GS.

The regulation of GDH activity in response to ammonium availability in *M. smegmatis* was investigated in Chapter three. The results obtained indicated that the specific activity of both GDH isoforms was indeed regulated and we were therefore interested in whether GlnD could play a role in the signaling of cellular nitrogen status to the GDH enzymes. The aminating reaction activity of both the NAD⁺-GDH and NADP⁺-GDH enzymes, as well as the deaminating reaction activity of the NADP⁺-GDH enzyme was investigated in the GlnDKO strain. No

significant differences in the regulation either GDH isoform could be detected in the absence of GlnD which indicates that the GDH enzymes are not regulated by the GlnD-GlnK signaling system.

Some of the results presented in the current study differ somewhat from previous work (Chapter three). For example: we could not previously detect a change in NADP⁺-GDH aminating reaction activity in response to ammonium availability (Chapter three, Figure 1A), whereas in the current study we observed a slight modulation in aminating reaction activity within 0.5hrs exposure to either ammonium limitation or excess. An additional discrepancy between the two studies includes the observation that NAD⁺-GDH aminating reaction activity decreased when *M. smegmatis* was exposed to both ammonium limitation and excess conditions in the current study, whilst previously, an initial decrease in aminating reaction activity was found under ammonium limiting conditions which was followed by an increase in reaction activity (Chapter three, Figure 1C). In the former study, the enzyme assays were performed manually, by addition of the reaction substrates to microcuvettes and subsequent spectrophotometric measurement (done by hand) of the change in absorbance at short time intervals. In contrast, the enzyme assays done in the current study were set up and executed in microtitre plates and the change in absorbance measured simultaneously by a microtitre plate reader. The margin for human error by using the former method is significantly greater than the latter method which may account for the differences observed between studies.

In *E. coli*, the PII proteins are modified by GlnD in response to nitrogen availability and are then able to interact with GlnE to control the adenylylation state of GS. In Actinomycetes such as *C. glutamicum* and *S. coelicolor*, however, GS was found to be adenylylated by GlnE in response to ammonium availability in a GlnD-GlnK independent fashion (Harper *et al.*, 2008; Strosser *et al.*, 2004; Burkovski, 2003; Hesketh *et al.*, 2002). In this study, an analysis of the regulation of GS activity in the GlnDKO strain indicated that GlnD does play a role in the signaling of the cellular nitrogen status to this enzyme in *M. smegmatis*. A slight increase in GS activity upon exposure to ammonium limiting conditions in the GlnDKO strain was observed which is consistent with our *glnD* mutant having retained its ability to modify the PII protein as modified PII could interact with GlnE to signal the deadenylylation GS (Figure 5). Upon exposure of the GlnDKO strain to

an ammonium pulse, no significant change in GS activity was observed which indicated that the truncated GlnD protein was unable to de-modify GlnK (this is consistent with previous results, see section 4.4.2). In wild type *M. smegmatis* cells, unmodified GlnK could interact with GlnE to bring about an increase in GS adenylylation and a subsequent down-regulation of GS activity (Figure 5). The reduction in GS activity observed in the wild type strain may be due to a combination of adenylylation of GS by GlnE and down-modulation of *glnA* expression (see section 4.4.2). It was observed that GS activity in the GlnDKO strain remained higher than the activity detected in wild type cells which could be due to the *de novo* synthesis of GS as seen by high *glnA* expression levels in the GlnDKO strain under conditions of both ammonium limitation and excess (see section 4.4.2). GS activity in the GlnDKO strain which was exposed to ammonium excess conditions appeared to be greater than when the GlnDKO strain was exposed to ammonium limiting conditions. This may be due to the fact that in the former experiment, the bacteria were pre-conditioned in ammonium limiting media for 1hr which could allow for the modification of GlnK by the truncated GlnD protein, followed by the interaction of modified GlnK with GlnE to bring about the deadenylylation of GS in order to increase its activity. These high GS activity levels could not be reduced by GlnE due to an inability of the truncated GlnD protein to de-modify GlnK.

Since GlnD has been shown to play a role in the adaptation of nitrogen metabolism- related gene expression and GS activity, it is noteworthy that the GlnDKO strain was able to survive and grow at similar rates to the wild type *M. smegmatis* strain under various ammonium concentration culture conditions. The GlnDKO strain was not severely impaired in its ability to adapt to ammonium limitation conditions due to the observation that the truncated GlnD protein may have retained its ability to modify GlnK. This could explain the relatively normal rate of growth of the GlnDKO strain observed under ammonium starvation conditions. In *M. tuberculosis*, it has been shown that GlnE is essential as it is required to reduce GS activity which is expressed to a very high level in this pathogen. High levels of active GS could lead to the depletion of intracellular ATP and glutamate stores which would severely affect growth (Read *et al.*, 2007). It may be that the *de novo* synthesis of unmodified GlnK in the GlnDKO *M. smegmatis* strain, when exposed to ammonium excess conditions, could be sufficient to signal the adenylylation of GS to allow for normal growth.

A study in which *glnD* was deleted in *M. tuberculosis* showed that total intracellular GS activity in the wild type and mutant strains did not differ significantly when the bacteria were cultured either under ammonium limiting or excess conditions (Read *et al.*, 2007). It was, however, found that the activity of extracellular GS was largely abolished in the *glnD M. tuberculosis* strain. The authors also showed that intracellular GS activity was up-regulated upon exposure of the wild type and mutant strain to ammonium excess (30mM ammonium sulphate) culture conditions (Read *et al.*, 2007). Considering that GS is not exported in *M. smegmatis*, that a difference in GS activity between the wild type and GlnDKO *M. smegmatis* strains was observed under all conditions tested and that GS activity was down-regulated upon exposure of *M. smegmatis* to ammonium excess conditions, it may be that the role of GlnD in *M. smegmatis* and its pathogenic relative are quite different.

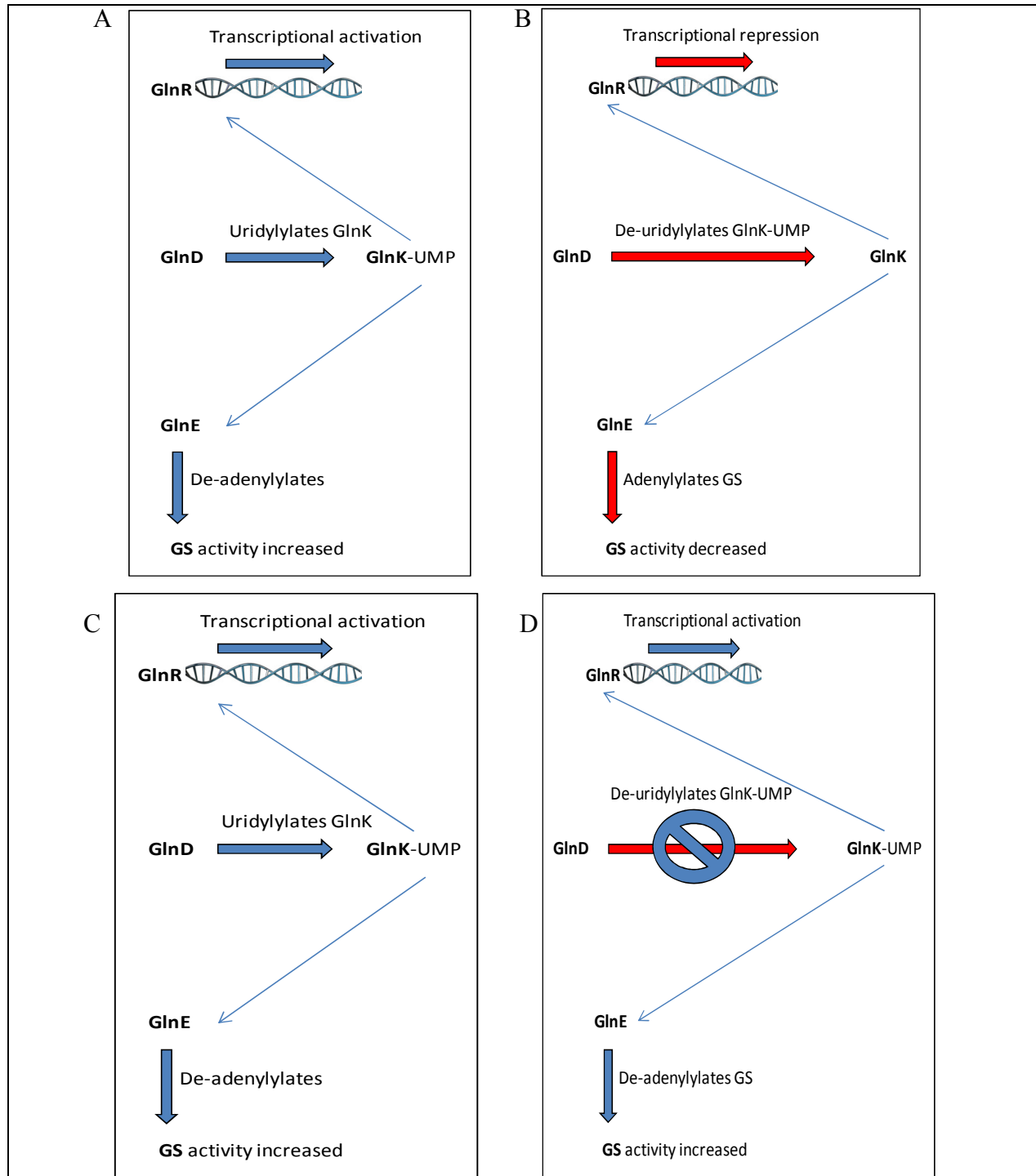
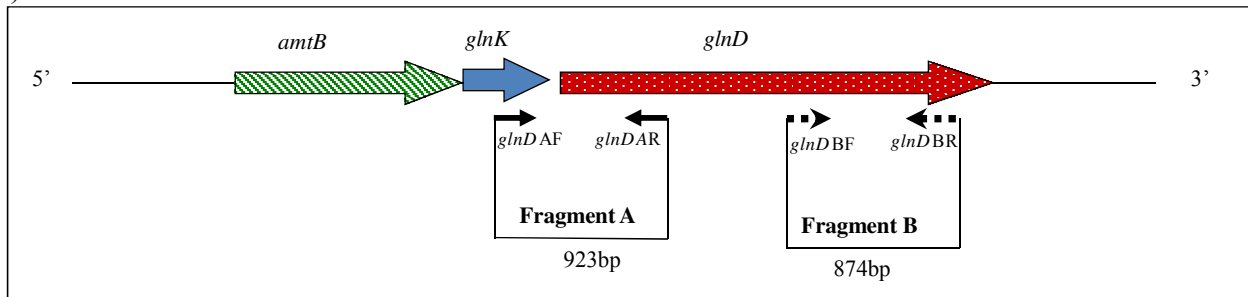


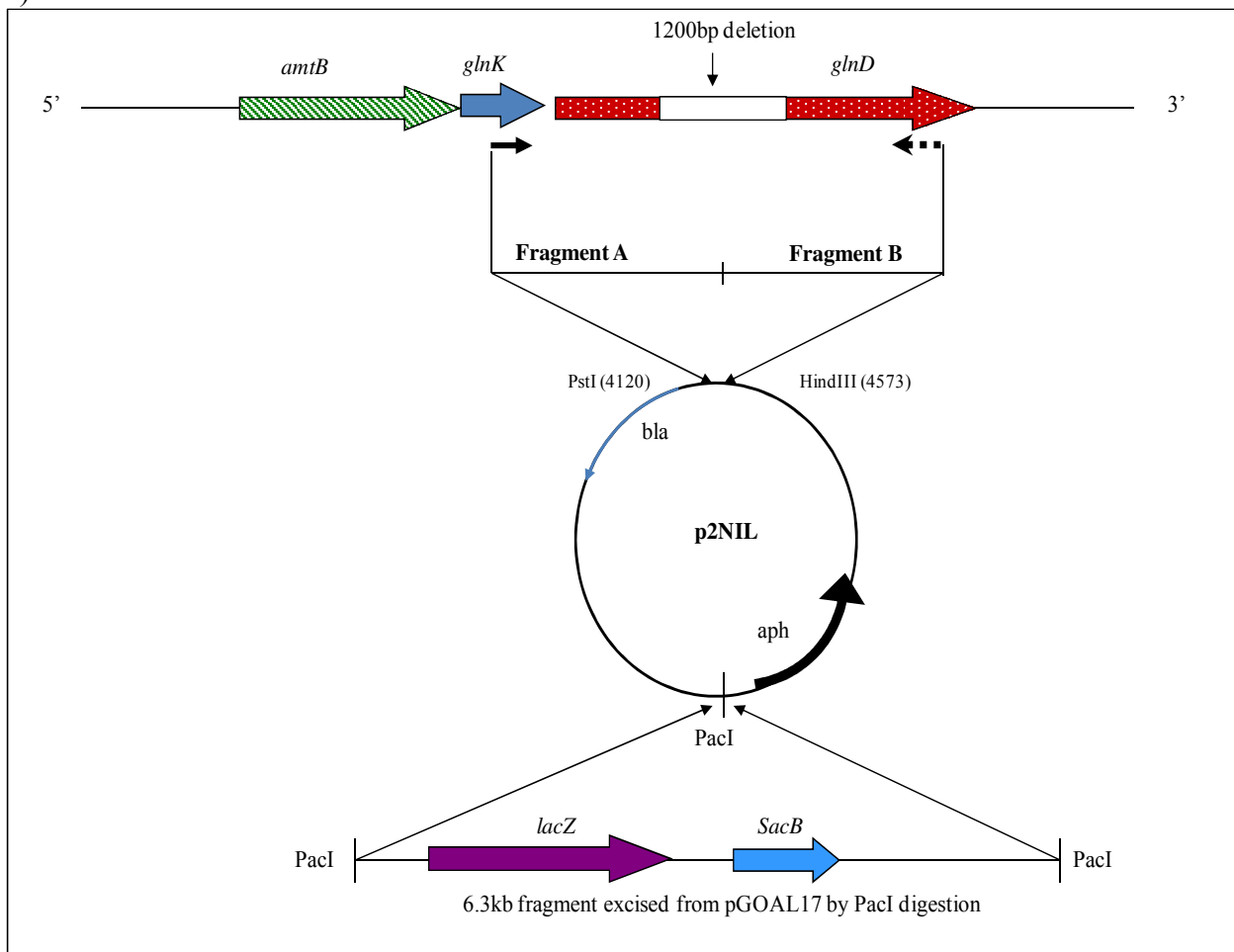
Figure 5: Model of GlnD function in wild type *M. smegmatis* under conditions of (A) nitrogen limitation and (B) excess, as well as the GlnDKO strain under conditions of (C) nitrogen limitation and (D) excess.

APPENDIX 4A: Schematic representation of the methodology used to introduce a *glnD* deletion into the *M. smegmatis* genome.

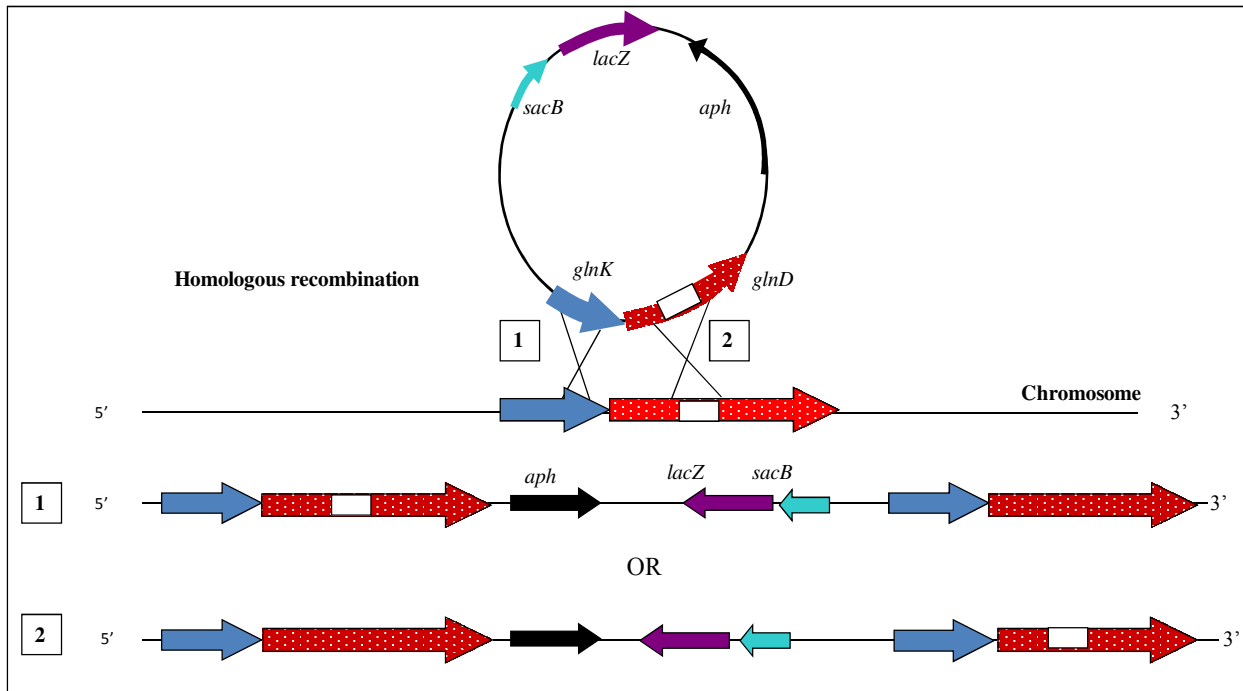
i)



ii)



iii)



iv)

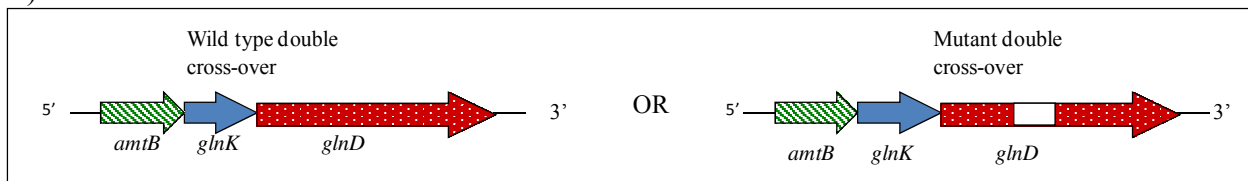
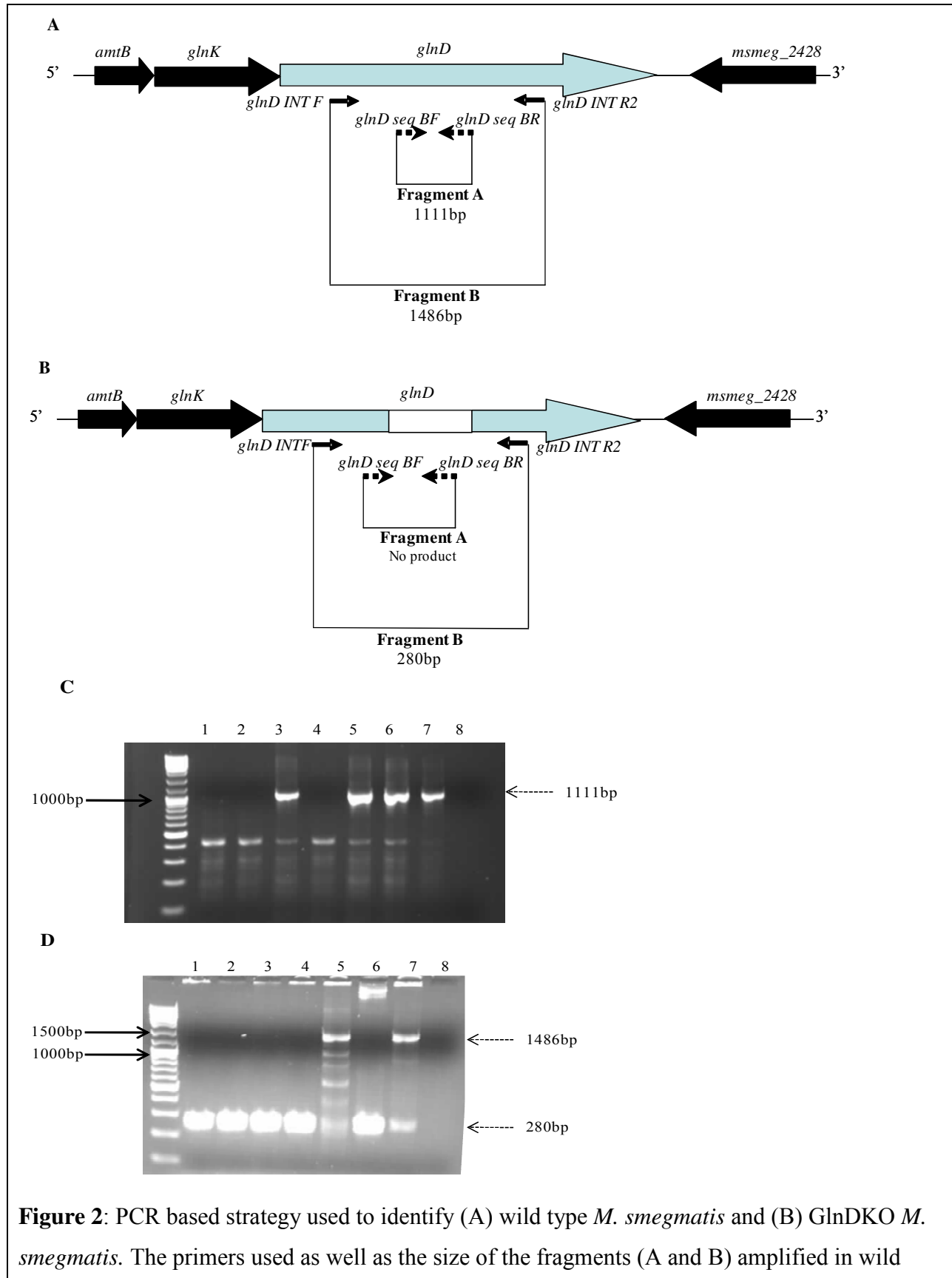


Figure 1: (i) PCR amplification of approximately 1000bp upstream (Fragment A) and downstream (Fragment B) of the desired deletion region. The primers used to amplify the two fragments had unique restriction sites which could allow for (ii) ligation of Fragments A and B. This resulted in a 1761bp fragment which, with the exception of a 1200bp deletion in the centre, was homologous to the *M. smegmatis* chromosome. Fragment A/B was cloned into the p2NIL suicide vector through use of unique restriction sites (PstI and HindIII). A 6.3kb DNA fragment excised from the pGOAL17 vector which contained a *lacZ* selection marker and a *sacB* counter-selection marker was also cloned into p2NIL via a unique PacI restriction site. (iii) After transformation of the knockout construct into *M. smegmatis*, single cross-over colonies were isolated and cultured on selection media to induce a second recombination event which would result in either (iv) a *glnD* mutant genotype or reversion to the wild type genotype.



type and GlnDKO *M. smegmatis* strains are given. (C) Electrophoretic separation of Fragment A following colony screening of six colonies (Lanes 1-6). Lanes 7 and 8 are positive and negative PCR controls respectively. (D) Electrophoretic separation of Fragment B following the identification of five potential DCO colonies (Lanes 1-6) as determined by the PCR amplification of Fragment A. Lanes 6 and 7 are positive PCR controls in which the deletion construct and wild type *M. smegmatis* DNA was added as template respectively. Lane 8 is a negative PCR control.

Mycobacterium smegmatis was transformed with the construct depicted in Figure 1.ii, namely; p2NIL containing the deleted form of the *glnD* gene as well as the *lacZ* selectable marker and the *sacB* counter-selectable marker. The 1200bp region that was deleted from *glnD* (Figure 1.ii) included a portion of the nucleotidyltransferase (NT) and HD domains as well as the entire PII-uridylyltransferase (UT) domain (Figure 3). Upon transformation of *M. smegmatis* with the deletion construct, a single cross-over event could occur whereby the entire construct was inserted into the genome via homologous recombination (Figure 1.iii). The bacteria that had undergone a single cross event were forced to perform an additional recombination event in order to survive on selection media; in which case the bacteria could have reverted to the wild type genotype or the required double cross-over genotype (Figure 1.iv). In order to differentiate between SCO and DCO colonies as well as to determine which DCO event occurred, two PCR reactions were carried out. In Figure 2C, colonies potentially having undergone a DCO event were screened using primers which would only produce a product (Figure 2A, Fragment A, 1111bp) if a wild type copy of the gene was present in the genome. It was found that colonies represented by the PCR products separated in Lanes 1, 2 and 4 did not yield the desired PCR product (1111bp) which indicated that a wild type copy of *glnD* was not present in the genome. The colonies represented by the bands separated in Lanes 3, 5 and 6, however, could be either wild type *M. smegmatis* or bacilli that had undergone a SCO event due to the presence of the 1111bp PCR product. An additional confirmatory PCR reaction, using primers flanking the deletion region (Fragment B), was done. A larger PCR amplicon (Fragment B, 1486bp) would be produced in the presence of a wild type copy of *glnD* (Figure 2A), whilst a much smaller PCR product (Fragment B, 280bp) would be formed if a deleted form of the gene was solely present (Figure 2B). In Figure 2D, five potential DCO colonies, as identified by the absence of Fragment

A, were screened using primers for the amplification of Fragment B. The colony represented by the bands in Lane 5 had undergone a second recombination event, however, it had reverted to the wild type genotype as a large PCR amplicon (1486bp), similar to the wild type positive control (Lane 7), was produced. The four remaining colonies had a deleted form of the *glnD* gene present, as the smaller PCR product (280bp) was produced.

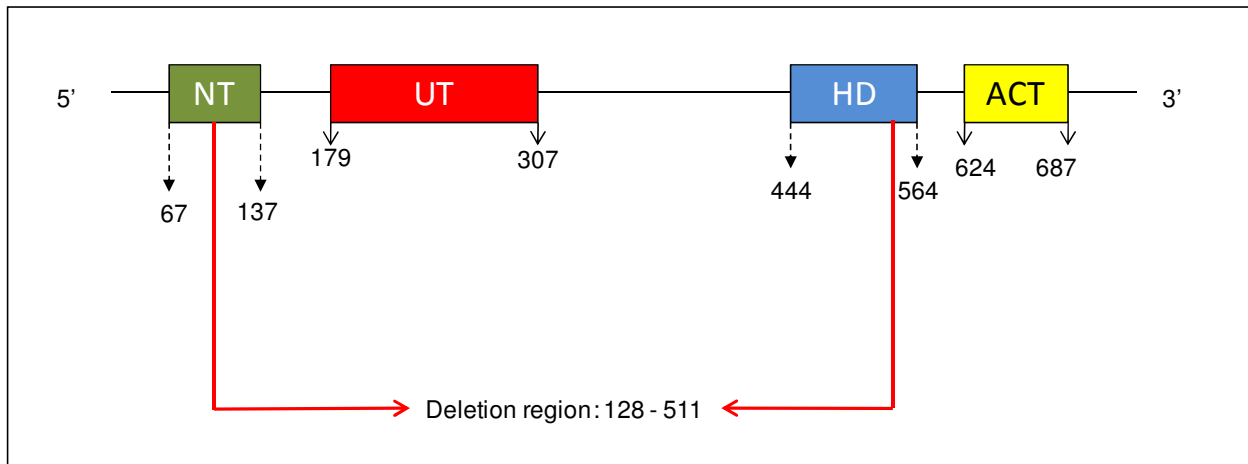


Figure 3: Schematic presentation of the various domains comprising the GlnD protein of *M. smegmatis*. The 1200bp region which was deleted from GlnD is indicated. The functions of the domains are given in Chapter 4.

Figure 1: Multiple sequence alignment of representative PCR fragments amplified from potential mutant colonies (1-5) and wild type *M. smegmatis* using the Kapa2G Robust PCR kit. The reference DNA sequence from the Genbank database is also given. The position of desired deletion is the cytosine residue is marked with an arrow. The undesired adenosine deletion is highlighted.

In order to confirm the results obtained with the KAPA2G Robust Polymerase kit, the PCR reactions were repeated with an alternative PCR kit, namely the HotStarTaq Polymerase kit (Qiagen). The desired cytosine deletion was not found using this kit (Figure 2). A similar undesired adenosine deletion was observed in colonies 2, 3 and 5 located 2bp downstream of the desired mutation site (Figure 2), however, the mutation was not identified in the same colonies as obtained by the Kapa2G Robust Polymerase kit (Figure 1). The conflicting results presented by the use of the two different kits prompted the analysis of a larger PCR fragment spanning the deletion region.

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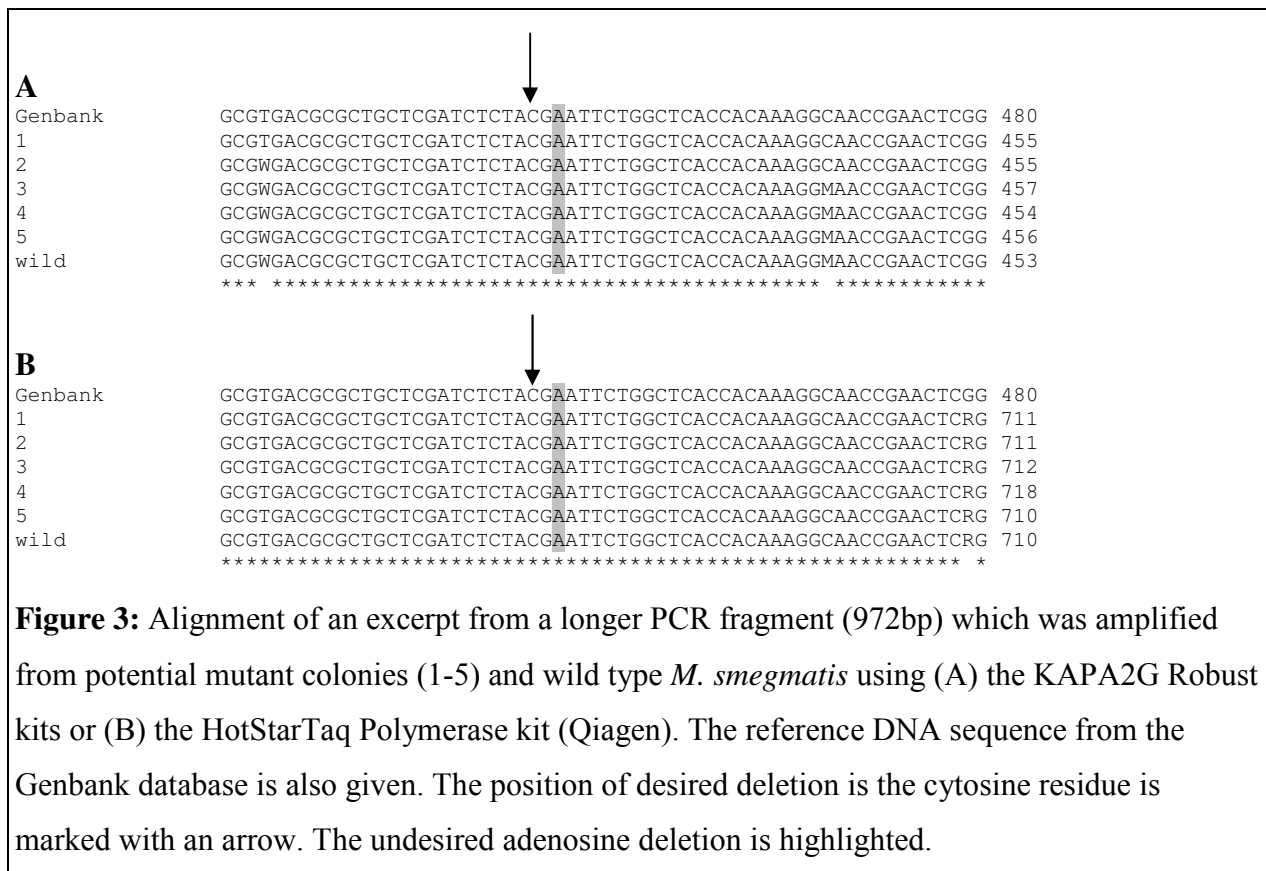
                                     ↓
Genbank      GCGGCAACTGGATTTCGGCCGCGCTGCGTGACGCGCTGCTCGATCTCTACGAATTCTGGCT 60
1            -----GTCCYWRSCCKCTCGATCTCTACGAATTCTGGCT 34
2            -----GAGGYGCK-CTCGATCTCTACG-ATTCTGGCT 30
3            -----CGCYWWSCTGCTCGATCTCTACG-ATTCTGGCT 32
4            -----ACMSSSGKWWMSTKSKCGRTCTCTACGAATTCTGGCT 38
5            -----GGCGKWWGCKCCTCGAWCTCTACG-ATTCTGGCT 33
wild type    -----ACMSSSGKWWMSTKSKCGRTCTCTACGAATTCTGGCT 38
                                     .  *.*  *****  *****

Genbank      CACCACAAAGGCAACCGAACTCGGCATCACCGCCGACAG- 99
1            CACCACAAAGGCAACCGAACTCGGCATCACCGCCGACAGC 74
2            CACCACAAAGGCAACCGAACTCGGCATCACCGCCGACAGC 70
3            CACCACAAAGGCAACCGAACTCGGCATCACCGCCGACAGC 72
4            CACCACAAAGGCAACCGAACTCGGCATCACCGCCGACAGC 78
5            CACCACAAAGGCAACCGAACTCGGCATCACCGCCGACAGC 73
wild type    CACCACAAAGGCAACCGAACTCGGCATCACCGCCGACAGC 78
*****

```

Figure 2: Multiple sequence alignment of representative PCR fragments amplified from potential mutant colonies (1-5) and wild type *M. smegmatis* using the HotStarTaq Polymerase kit (Qiagen). The reference DNA sequence from the Genbank database is also given. The position of desired deletion is the cytosine residue is marked with an arrow. The undesired adenosine deletion is highlighted.

A 972bp PCR fragment spanning the deletion region was amplified using both the KAPA2G Robust Polymerase kit as well as the HotStarTaq Polymerase kit. When the longer PCR fragment was amplified and analyzed after DNA sequencing, it was found that no undesired adenosine deletion was produced by either PCR kit (Figures A and B). Although the full length multiple sequence alignment is not shown here, it was found that the HotStarTaq Polymerase kit (Qiagen) gave consistently more accurate DNA sequencing results. In conclusion, the screening of colonies harboring potential point mutations should only be done if a positive control for the desired mutation is available. If no such positive control is available, screening for the mutation can be done by DNA sequencing, however, it would be recommended to PCR amplify larger sequences for more accurate DNA sequencing results.



Conclusions

Considering that biological systems would not be viable without nitrogen containing compounds, it is surprising that so little is known of this metabolic pathway in the mycobacteria.

Approximately 10 years ago, *M. tuberculosis* glutamine synthetase was found to be essential for the infection of human macrophages and guinea pigs (Tullius *et al.*, 2003) and was thus identified as a potential drug target. More recently, GlnE was also found to be essential for *M. tuberculosis* survival and the interaction between GS and GlnE has become a point of interest for drug development (Carroll *et al.*, 2008). A lot of work has been done on the validation of these potential drug targets; however, little attention has been paid to the rest of the nitrogen metabolic pathway. In order to assist in the eradication of the growing global tuberculosis crisis, an improved understanding of *M. tuberculosis* infection is required and therewith, an understanding of the physiology of the organism. In this dissertation, we attempted to broaden the scope of knowledge available on nitrogen metabolism in the mycobacteria by analyzing the three main aspects of nitrogen metabolism control; namely, the regulation of nitrogen metabolism-related gene expression, the control of enzyme effector activity and the signaling of the nitrogen status to various cellular components. *M. smegmatis* was used for these studies, largely due to the non-pathogenic nature of the organism as well as its rapid growth rate. In addition, *M. smegmatis* is often used as a heterologous host for the study of various aspects of bacterial metabolism in *M. tuberculosis*, therefore, an improved understanding of *M. smegmatis* may allow for more enlightened use of this organism as a model for the study of its pathogenic relatives.

In this work, the expression of nitrogen metabolism-related genes in response to ammonium availability was studied. In certain instances, it was found that the pattern of expression of genes (such as *amtB* and *glnA*) was similar to that of the Actinomycetale model organisms, *C. glutamicum* and *S. coelicolor*, which may indicate similar roles of the protein products in the nitrogen metabolic pathway. On the other hand, the expression of some of the genes, such as *gltB* and *ureA*, was very dissimilar to the pattern of expression in the model Actinomycetes which suggests that the protein products may play slightly different roles in the metabolic pathway. Although the function of each gene product was not investigated, we were interested in the possible regulatory mechanisms dictating their expression. Our results indicate that both of the possible transcriptional regulators, GlnR and AmtR, identified in *S. coelicolor* and *C.*

glutamicum respectively, are involved in the expression of nitrogen metabolism-related gene expression.

Our data suggests that the regulation of nitrogen metabolism-related genes in *M. smegmatis* is not as simple as previously thought, as other factors appear to play a role. It does not seem to be as uncomplicated as deleting a potential regulator and observing the anticipated response as the results obtained from such experiments can be misleading. There appears to be a multi-faceted control of expression, which is not surprising as metabolic pathways do not operate in isolation – there is a flux of metabolic intermediates, such as 2-oxoglutarate, between pathways which means that there may be cross-talk of regulatory proteins between pathways. An example of this is the role of PhoP in regulating *glnR* expression in *S. coelicolor* (Rodriguez-Garcia *et al.*, 2009). Also, σ^H factors were shown to be able to regulate *gltB/D* expression in *S. coelicolor* (Kormanec and Sevcikova, 2002). Nevertheless, our knockout studies have indicated that both GlnR and AmtR are implicated in the control of nitrogen metabolism-related gene expression (Figures 1, 2 and 3). GlnR in *M. smegmatis* appears to both activate and repress gene transcription which is similar to its function in *S. coelicolor*. AmtR in *M. smegmatis*, however, appears to activate gene transcription, which is different to its repressive function in *C. glutamicum* (Jakoby *et al.*, 2000). Future work would include a continuation of the current studies in order to determine the exact role of AmtR in *M. smegmatis*. In addition, the GlnR regulon in *M. smegmatis* could be characterized; however, microarray analyses and proteomics studies would be done in order to give a better indication as to the global role of these regulatory proteins.

On an enzymatic level, we have shown that glutamine synthetase in *M. smegmatis* is regulated in response to nitrogen availability; however, the control of enzyme activity appears to be largely due to the *de novo* synthesis of the enzyme rather than by post-translational modification mechanisms. We have also been able to show that both *M. smegmatis* and *M. tuberculosis* have functional GDH enzymes. In *M. smegmatis*, the activity of both an NADP⁺-specific and an NAD⁺-specific GDH could be detected, however, only an NAD⁺-GDH could be detected in *M. tuberculosis*. Our results suggest that both of these enzymes play an anabolic role with regard to nitrogen metabolism and that they are regulated in response to nitrogen availability. It was previously thought that GS was the sole mechanism available for the assimilation of nitrogen in *M. tuberculosis*; however, our work indicates that an alternative route via GDH is possible. A

limitation of the enzyme activity work was that whole cell lysates were used in order to perform the assays. These lysates may contain proteins/enzymes which could utilize the co-factors added to the assays in addition to the enzyme of interest or activators/inhibitors may be present which could, therefore, possibly confound the results. We, however, were not interested in absolute specific activity values but rather in comparing activities between culture conditions which was made possible by treating all samples in the same way. Whole cell lysates have been utilized successfully in a variety of previous studies (Harper *et al.*, 2010), therefore our results should not be disregarded. Future prospects include an in depth study of the nitrogen assimilatory role of GDH in pathogenic mycobacteria, particularly in those which are in a dormant or latent state, to assess the importance of this enzyme in an energy-deficient environment.

An analysis of the signaling cascade in *M. smegmatis* has indicated that GlnD plays a pivotal role in the control of gene transcription and GS activity in this organism (Figures 2 and 3). Although a physical interaction between GlnD and GlnK as well as between GlnK, GlnE and GlnR remain to be shown, our results suggest that these interactions do indeed occur. Therefore, future prospects include the identification of the type of GlnK modification which GlnD carries out, namely adenylation or uridylylation. Also an *M. smegmatis* strain lacking GlnK could be generated in order to show that the GlnD-GlnK interaction is important for the adjustment of gene transcription and GS activity in response to nitrogen availability. Through this future work, one could aim to identify the signal to GlnR; namely whether a direct interaction between GlnK and GlnR occurs or whether GlnK interacts with an as yet unidentified sensory kinase.

There are known differences regarding aspects of the nitrogen metabolic pathway between *M. smegmatis* and *M. tuberculosis*. One example is the observation that GS is secreted in *M. tuberculosis* and not in *M. smegmatis*. Another is that *M. smegmatis* possess two GDH isoforms whereas *M. tuberculosis* has only an NAD⁺-GDH. Lastly, *M. smegmatis* has both AmtR and GlnR as regulators of gene transcription whereas only GlnR appears to be present in *M. tuberculosis*. It may be that, due to the additional components involved, the nitrogen metabolic pathway in *M. smegmatis* may be more complicated than its pathogenic relative. Despite these differences, the core nitrogen regulatory and enzymatic features appear to be similar between closely related Actinomycetales. Thus, our understanding of the components involved in nitrogen metabolism in the mycobacteria have been enhanced through this body of work which

may serve to direct additional research into the pathway in *M. tuberculosis*. The elucidation of such pathways serves to better our knowledge of mycobacterial physiology, which in turn, sheds light on the ability of the pathogen to infect and survive within its host. These insights could ultimately lead to the discovery and development of novel means to aid in the eradication of the disease.

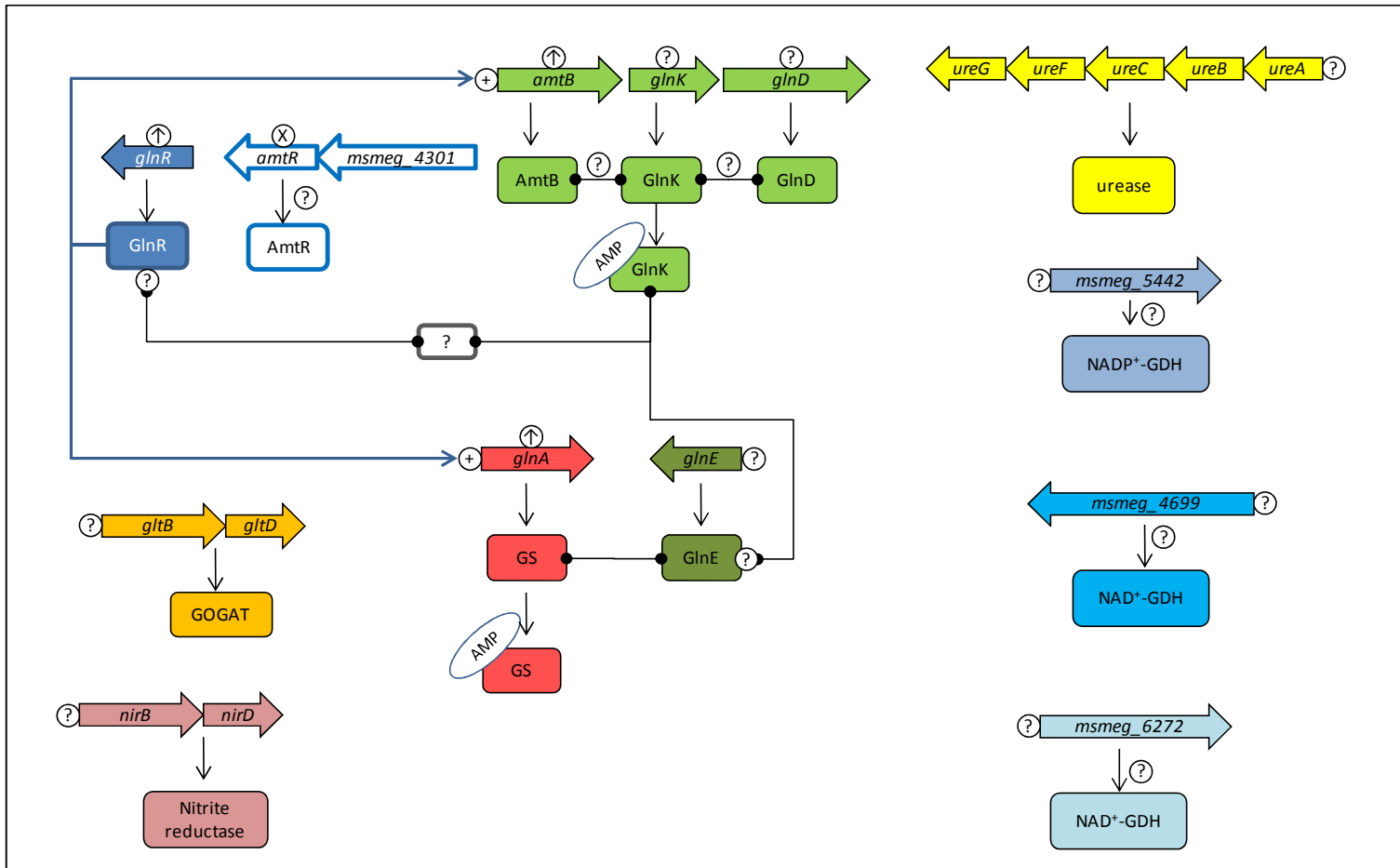


Figure 1: Schematic representation of the known interactions in *M. smegmatis* prior to this study. Solid lines indicate a positive effect (\oplus) on gene expression whilst a negative effect (\ominus) is indicated by a dashed line. Red lines (solid or dashed) represent transcriptional regulation by AmtR whilst blue lines show regulation by GlnR. An up-regulation (\uparrow), down-regulation (\downarrow) or absence (\otimes) of change in expression in wild type *M. smegmatis* is shown. Black connector lines (\bullet — \bullet) indicate a protein interaction.

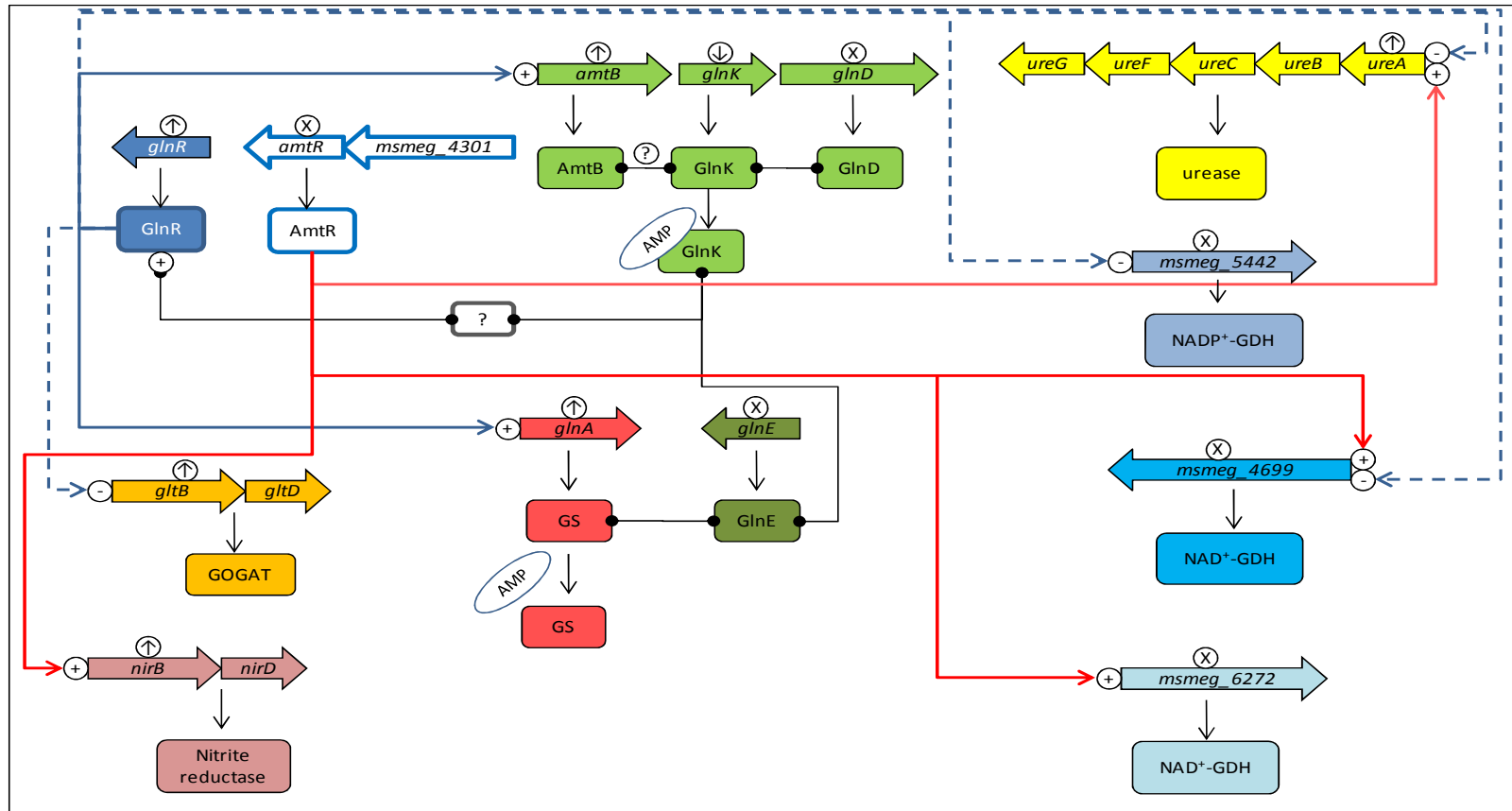


Figure 2: Schematic representation of the cascade of events which occur on a transcriptional and protein interaction level when *M. smegmatis* is exposed to conditions of ammonium limitation. Solid lines indicate a positive effect (\oplus) on gene expression whilst a negative effect (\ominus) is indicated by a dashed line. Red lines (solid or dashed) represent transcriptional regulation by AmtR whilst blue lines show regulation by GlnR. An up-regulation (\uparrow), down-regulation (\downarrow) or absence (\otimes) of change in expression in wild type *M. smegmatis* is shown. Black connector lines ($\bullet\text{---}\bullet$) indicate a protein interaction.

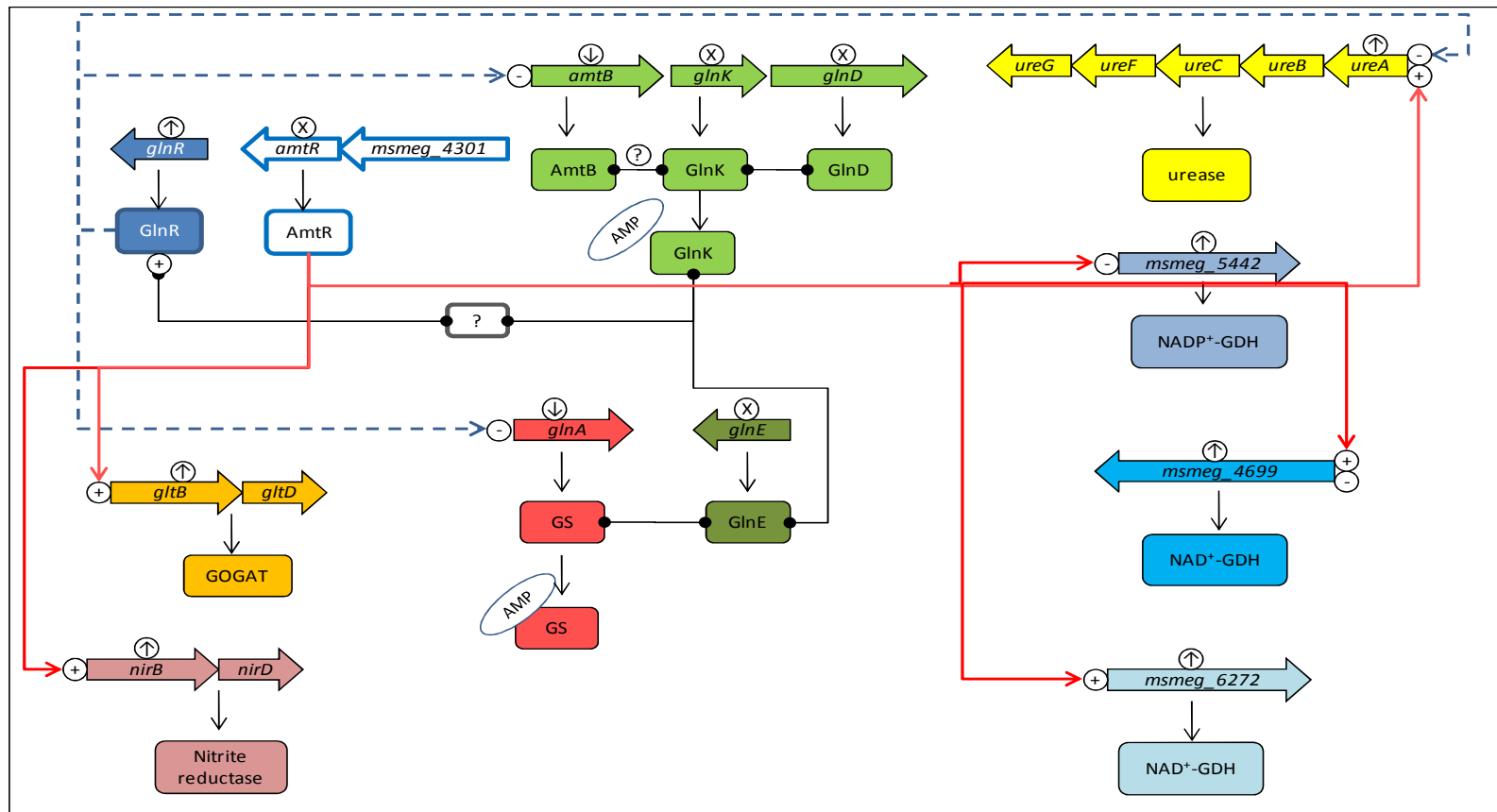


Figure: Schematic representation of the cascade of events which occur on a transcriptional and protein interaction level when *M. smegmatis* is exposed to conditions of ammonium excess. Solid lines indicate a positive effect (\oplus) on gene expression whilst a negative effect (\ominus) is indicated by a dashed line. Red lines (solid or dashed) represent transcriptional regulation by AmtR whilst blue lines show regulation by GlnR. An up-regulation (\oplus), down-regulation (\ominus) or absence (\otimes) of change in expression in wild type *M. smegmatis* is shown. Black connector lines ($\bullet\text{---}\bullet$) indicate a protein interaction.

Materials and Methods

1. BIOINFORMATICS

In silico, or computational tools were used to analyse genomic and molecular biological data. These bioinformatics tools allowed for the identification, retrieval, alignment and structural analysis of a number of DNA and amino acid sequences possibly involved in nitrogen metabolism in *Mycobacterium smegmatis*.

1.1 Sequence Retrieval and Alignment

Gene and protein sequences included in this study were identified and retrieved from the following internet based databases:

- i) The J. Craig Venter Institute Comprehensive Microbial Resource (JCVI-CMR, Table 1) (<http://cmr.TIGR.org/tigr-scripts/CMR/CmrHomePage.cgi>)
- ii) The National Centre for Biotechnology Information (NCBI, Table 1)
- iii) Tuberculist (Table 1)

The sequences retrieved were used in the search for homologous gene or amino acid sequences in a variety of organisms whose genome sequences were listed within the database. Both the JCVI-CMR and NCBI databases provided the Basic Local Alignment Search Tool (BLAST) search tool which allowed retrieval of homologous gene and/or amino acid sequences from different sources (Table 1). DNA and/or protein sequence similarity was analyzed using the ClustalW (Table 1) sequence alignment tool. Multiple sequences could be aligned to identify any differences that may have been present between the sequences.

1.2 Primer Design

Oligonucleotides (primers) for PCR amplification could be designed using three different software programs. Primers were routinely designed using the internet-based Primer3 version 0.4.0 program (Table 1) or alternatively using the Primer Premier Version 5.0 software (Table 1) or DNAMAN Version 4.1 software (Table 1). The primers were designed to be between 18 and

24bp, to have a G+C content between 50-60% and to anneal to unique sequences (all sequences obtained from NCBI or CMR databases, see section 1.2) within the target genome. Primer specificity was analyzed by an *in silico* PCR amplification programme (Table 1) to ensure that the desired PCR amplification product was generated and to determine the genetic co-ordinates of the amplified sequence. For any primer pair, a maximum melting temperature (T_m) difference of 2°C was allowed. The T_m for any primer was calculated according to Equation C.1 (Appendix C).

Where necessary, restriction enzyme cutting sites were designed into the primer sequence using Primer Premier. The software predicted binding energy and non-homologous bases in the PCR oligonucleotide that may cause mispriming and/or non-specific binding. The oligonucleotides were synthesised either by Integrated DNA technologies (IDT, USA) or Inqaba Biotechnology (South Africa). Alternatively, restriction sites were synthesised on the 5' end of primers to avoid restriction enzyme digestion within the primer sequence itself. This allowed subsequent use of the same primers for PCR screening purposes.

1.3 Protein Structure and Function Analysis

A number of proteomics bioinformatic tools were used to analyse protein or amino acid sequences. Both the Prosite and Pfam databases (Table 1) could be used to identify protein families, domains and possible active sites of a query amino acid sequence. Three dimensional modelling of amino acid query sequences was done using the SWISS-MODEL (Table 1) database.

1.4 Sequence analysis for High Resolution Melt (HRM)

The DINAmelt server (<http://www.bioinfo.rpi.edu/applications/hybrid/twostate-fold.php>) was used to predict possible melting profiles and secondary structures produced by a specific DNA sequence (Markham and Zucker, 2005). Melting temperatures as well as melting curves of nucleic acid sequences may be affected by the presence of secondary structures such as stem-loop arrangements. The position and sequence of primers for HRM analysis (section 9.3) was

optimised by the use of this server to avoid the presence of any secondary structures in the DNA sequence to be analysed.

1.5 Regulatory sequence analysis

Analysis of regulatory sequences was done using the Regulatory Sequence Analysis Tools database (Table 1). Whole genome searches for inputted regulatory sequences upstream of all genes within the desired genome were done using the genome-scale-pattern tool (http://crfb.univ-mrs.fr/rsaTools/genome-scale-dna-pattern_form.cgi). Alternatively, the search could be performed on a select number of genes of interest as chosen by the user (RSA-tools-DNA-pattern, <http://crfb.univ-mrs.fr/rsaTools/>). All gene sequences retrieved by the Regulatory Sequence Analysis Tool and used in this study were from the NCBI database (section 1.2).

Table 1: List of websites and software used in bioinformatic applications. The website addresses and software authors are also given.

<u>Application</u>	<u>Website/Software</u>	<u>Website address or software author</u>
Sequence retrieval	JCVI-CMR	http://cmr.TIGR.org/tigr-scripts/CMR/CmrHomePage.cgi
	NCBI	http://www.ncbi.nlm.nih.gov
	Tuberculist	http://genolist.pasteur.fr/TubercuList
Homology searches	NCBI-BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
	CMR-BLAST	http://blast.jcvi.org/cmr-blast
Sequence comparison	ClustalW	http://www.ebi.ac.uk/Tools/clustalw
Oligonucleotide design	Primer3 ver 0.4.0	http://frodo.wi.mit.edu/primer3
	Primer Premier ver 5.	PREMIER Biosoft International
	DNAMAN ver 4.1	Lynnon Biosoft

	In silico PCR amplification	http://insilico.ehu.es/PCR/
Protein structure and function	Prosite	http://au.expasy.org/prosite/
	Pfam	http://pfam.sanger.ac.uk
	SWISS-MODEL	http://swissmodel.expasy.org/
Regulatory sequence analysis	Regulatory	http://crfb.univ-mrs.fr/rsaTools
	Sequence Analysis Tools	

2. BACTERIAL STRAINS, GROWTH CONDITIONS AND CULTURING TECHNIQUES

2.1 Bacterial strains and general growth media

Escherichia coli (ATCC 53323) was routinely used in cloning experiments and were grown on either solid Luria-Bertani agar (LA, appendix A.2.4) or in liquid Luria-Bertani broth (LB, appendix A.2.3) supplemented with antibiotics when appropriate (see appendix A.1). The following antibiotics were routinely used: Ampicillin (Amps, 100µg/ml), Hygromycin (Hyg, 100µg/ml) and/or Kanamycin (Kan, 50µg/ml). Solid LA medium could, in addition to the various antibiotics, be supplemented with 40µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; appendix A.3.9), 0.2mM isopropyl-β-D-thiogalactopyranoside (IPTG; appendix A.3.6) and/or 5% sucrose (appendix A.3.15) when appropriate (Table 2).

Mycobacterium smegmatis mc²155 (ATCC 700084) and *Mycobacterium tuberculosis* H37Rv (ATCC 25618) were routinely cultured in liquid ADC- (appendix A.2.1) or OADC (see appendix A.2.5)-enriched Middlebrook 7H9 Mycobacterial medium (Difco, appendix A.2.6). The mycobacteria were also grown on solid OADC-enriched Middlebrook 7H10 or 7H11 Mycobacterial agar (Difco, appendix A.2.7). *M. smegmatis* was cultured in liquid LB broth and/or on solid LA medium during growth and selection of knockout *M. smegmatis* strains

(section 8). Where appropriate Kan (25µg/ml) and Hyg (50µg/ml) were added to the media and the solid agar media was additionally be supplemented with 40µg/ml X-gal and/or 5% sucrose where appropriate.

Table 2: Plasmids used in this study are given with their bacterial hosts, their genetic characteristics including plasmid size and selection markers. See Appendix B for plasmid maps.

Plasmid	Bacterial Host	Characteristics	Selection Marker	Size (bp)	Reference
pGemT-Easy	<i>E.coli</i>	Amp ^R , <i>lacZ</i> -alpha, <i>oriE</i>	Ampicillin	3015	Promega
p2nil	<i>E.coli</i> and <i>M. smegmatis</i>	Kan ^R , <i>oriE</i> , mycobacterial suicide plasmid	Kanamycin	4753	(Parish and Stoker, 2000c)
pGoal17	<i>E.coli</i> and <i>M. smegmatis</i>	Amp ^R , <i>oriE</i> , <i>lacZ</i> and <i>sacB</i> genes as a <i>PacI</i> cassette	Hygromycin Sucrose	8855	(Parish and Stoker, 2000b)
pJEM15	<i>E.coli</i> and <i>M. smegmatis</i>	Kan ^R , promoterless- <i>lacZ</i> reporter gene, <i>oriE</i> , <i>oriM</i>	Kanamycin	9446	(Timm <i>et al.</i> , 1994)
pJV75amber	<i>E.coli</i> and <i>M. smegmatis</i>	Kan ^R , Hyg ^S but becomes Hyg ^R upon homologous recombination, <i>oriE</i> , <i>oriM</i> , inducible acetamidase promoter,	Kanamycin Hygromycin	9009	(van Kessel and Hatfull, 2008b)

All cultures were maintained at 37°C. Liquid cultures were incubated with shaking (Labcon shaking incubator) or stirring to maximise aeration and exposure to nutrients as well as minimize bacterial clumping. *E. coli* was routinely grown overnight whilst *M. smegmatis* was cultured for 3 to 5 days. Starter cultures with a final volume of 5ml 7H9 supplemented with 100µl PANTA (PANTATMPlus Kit; Becton Dickinson, USA) and OADC were prepared when *M. smegmatis* was grown from frozen stocks (section 2.2). Mycobacterial cultures were routinely checked for contamination by Ziehl-Neelson (ZN) staining (section 2.3).

2.2 Bacterial Stocks

Frozen bacterial stocks were prepared by the addition of 850µl bacterial (OD600 ≈ 0.8) suspension to 150ml cooled, sterile glycerol. After thorough mixing, the bacteria were stored at -80°C and thawed on ice when required.

2.3 Ziehl-Neelsen (ZN) Staining

The detection of mycobacteria (acid fast bacteria) was achieved by the selective staining of mycolic acids found in the mycobacterial cell wall. A small aliquot of bacterial suspension was immobilized on a glass slide by heating at 65°C for 20min. The slide was then completely covered with 0.3% carbol-fuchsin (Becton Dickinson, USA) and heated until steaming (boiling should be prevented). After 5min incubation at room temperature (to allow staining of the mycolic acids), the carbol-fuchsin was rinsed off with water and the slide de-stained by flooding with 5% acid alcohol (appendix A.3.1) for 2min at room temp. The slide was rinsed with water and covered with 0.3% methylene blue counter-stain (Becton Dickinson, USA) and left for 1 to 2min at room temperature. Thereafter the slide was thoroughly rinsed with water and allowed to dry before analysis by light microscopy. Mycobacterial cells are acid-fast and were stained pink whilst non-mycobacterial (non-acid fast bacteria) cells were counterstained blue.

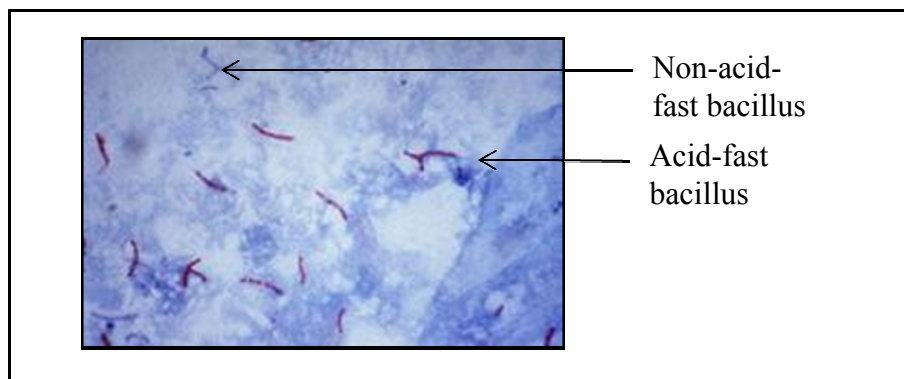


Figure 1: Ziehl-Neelsen stain of a *Mycobacterium tuberculosis* sputum sample which shows acid-fast mycobacterial cells (red) and non-acid-fast bacteria (blue).

2.4 Nitrogen starvation and/or excess culture conditions for *Mycobacterium smegmatis*

M. smegmatis was cultured in a minimal medium known as Kirchner's Broth (Howells and Holzhaus, 1954) containing asparagine as the sole nitrogen source. The media was modified by alteration of the asparagine (Asn) concentration to (i) 3.8mM (ii) 30mM and (iii) 60mM. Asn was replaced with ammonium sulphate ((NH₄)₂SO₄) as an alternative nitrogen source.

Mycobacterium smegmatis was cultured in Kirchner's broth containing 3.8mM Asn or 3mM (NH₄)₂SO₄ to simulate conditions of nitrogen starvation and conversely, growth in Kirchner's broth containing 60mM Asn or 60mM (NH₄)₂SO₄ created conditions of nitrogen excess. In order to study the effect on nitrogen starvation on *M. smegmatis*, the bacteria were first grown in OADC-enriched 7H9 medium to an OD₆₀₀ ≈ 0.8. The bacteria were transferred to Kirchner's Broth containing 60mM Asn or (NH₄)₂SO₄ for 1hrs and then placed in Broth containing only 3.8mM Asn or 3mM (NH₄)₂SO₄ in order to induce a nitrogen starvation response. Conversely, in order to study the effect of an excess of nitrogen, *M. smegmatis* was exposed to nitrogen starvation conditions (3.8mM Asn or 3mM (NH₄)₂SO₄) for 1hrs and then transferred to Kirchner's Broth containing 60mM Asn or (NH₄)₂SO₄. These culture conditions were used to determine the effect of nitrogen starvation and/or excess on gene expression, promoter activities, enzyme specific activities and other applications.

2.5 Bacterial titre determination

The bacterial titre of frozen *M. smegmatis* stocks (section 2.2) was determined prior to *in vivo* and *in vitro* growth studies. Two frozen bacterial stocks made from a single *M. smegmatis* culture were chosen at random and thawed on ice. The stocks were passed through a 1ml insulin syringe needle approximately 30 times in order to disperse bacterial clumps. A 10x dilution series of the bacteria was made using 7H9 medium. 100µl of each dilution was plated onto 7H10 agar plates and the colony forming units (CFU) were counted. The bacterial titre was determined as the number of CFU per millilitre liquid medium.

2.6 *In vitro* growth and viability determination

M. smegmatis growth in liquid media was determined by spectrophotometric measurement of culture turbidity at OD₆₀₀. A culture volume was inoculated with bacteria to an initial OD₆₀₀ ≈ 0.2. Thereafter, spectrophotometric readings were taken at 2hr intervals for 8hrs and again at 24hrs and 48hrs. The rate at which culture turbidity increased gave an indication of the growth rate of *M. smegmatis* in liquid media. All growth experiments were done in triplicate. *M. smegmatis* viability was determined by growth in Middlebrook 7H9 medium supplemented with OADC or ADC to an OD₆₀₀ of approximately 0.2. A 10-fold dilution series of the culture set up and 100µl of each dilution was spread onto Middlebrook OADC-enriched 7H10 agar. Each viability experiment was done in triplicate. The number of CFU's and colony morphology gave an indication of bacterial survival and viability.

3. POLYMERASE CHAIN REACTION (PCR)

PCR is used for the exponential amplification of specific DNA sequences. The reaction is carried out by DNA polymerases which are able to synthesise DNA in the 5' to 3' direction after the annealing of PCR primers to regions flanking the target DNA sequence. The DNA polymerases synthesise the complementary strands between bound oligonucleotides.

3.1 Routine PCR

HotStar *Taq* polymerase (Qiagen) was routinely used for DNA amplification of up to 2000 base pairs (bp). A typical PCR reaction contained 25pmol of each oligonucleotide (forward and reverse primers), 200µM of each deoxynucleotide triphosphate (dNTP, Qiagen), 1 x reaction buffer (supplied), 1 x Q-solution (supplied), 2mM MgCl₂, 1U *Taq*-polymerase, approximately 100ng DNA template and dH₂O to a final volume of 25µl. The general amplification protocol for HotStar *Taq* polymerase consisted of an initial 15min *Taq* activation step at 95°C, followed by 35 to 40 cycles of (i) a 30sec denaturation step (ii) a 30 sec annealing step at the required oligonucleotide annealing temperature (T_m) and (iii) an elongation or synthesis step (72°C) of one minute per kilobase (kb) to be amplified. A final elongation step of 10min at 72°C was carried out to ensure that any remaining single stranded fragments were fully extended. For PCR

colony screening of transformed cells, a small aliquot of bacterial cells was added to the PCR reaction. The high temperatures required for PCR amplification caused the bacterial cells to rupture and release genomic DNA which could then be used as a template for amplification by the *Taq* polymerase. All PCR experiments done included a negative control to which no DNA template was added. A positive control was included where necessary. The products of PCR amplification were analyzed by gel electrophoresis (section 3.3) in order to ascertain whether the desired amplicon was produced. For a full list of the PCR primers used in this study, please refer to Table 3.

3.2 Long Range PCR

When PCR fragments exceeded 1000bp or could not be amplified by routine PCR methods, the Long PCR Enzyme Mix (Fermentas) or the FastStart High Fidelity PCR System (Roche) was used. Each Long PCR Enzyme Mix (Fermentas) reaction contained 1 x Long PCR buffer; 1.5mM MgCl₂; 10μM of each dNTP; 25pmol of each oligonucleotide, approximately 100ng DNA template and dH₂O to a final volume of 50μl. The FastStart High Fidelity PCR System (Roche) reactions were prepared by adding 1X Reaction buffer; 1.8mM MgCl₂; 10μM of each dNTP; 25pmol of each oligonucleotide; roughly 100ng DNA template and dH₂O to a final volume of 50μl. The general cycling protocol was the same as for the routine PCR (section 2.3.1) with the exception that the *Taq* activation period at 94°C for both polymerases was 2min instead of 10min. PCR products were visualised by the methods outline in section 3.3.

3.3 Gel Electrophoresis

DNA samples and PCR products were fractionated on 0.6 – 1.2% agarose gels, depending on the size of the DNA fragments to be resolved. Gels were prepared by adding the appropriate amount of molecular grade agarose to a volume of 1 x SB buffer (see Appendix A.3.10). The suspension was gently heated until the agarose had completely dissolved. Ethidium bromide (Sigma, see Appendix A.3.3) was added to the gel to a final concentration of 10μg/ml and subsequently poured into a casting tray. Samples were loaded into the gel wells together with 1 x Loading dye

(Fermentas). A 3kb DNA ladder (GeneRuler™ DNA Ladder Mix, Fermentas) was loaded into a well adjacent to the samples in order to determine the size of fractionated DNA samples. Between 80V and 120V was applied across the gel until the loading dye front was approximately 1cm from the end of the gel. The resolved DNA was visualized under UV light supplied by a transilluminator and photographed.

Table 3: PCR primers including internal restriction enzyme sites (restriction enzymes names in brackets and their sites underlined in the oligonucleotide sequence), theoretical annealing temperature, chromosomal priming co-ordinates, the length of the amplification product and a reference to the section the oligonucleotide was used with this study.

Gene amplified	Sense primer (5'-3')	Tm	Genome Coordinates	Product size (bp)	Section
glnA F	GTCGACATTCGCTTCTGTGA	60	4374912	139	2.2.1
glnA R	ATTCGTGGATCGACTGGAAC	60	4375050		
glnK F	TCACGCTGGAAGATGTCAAG	60	2509695	113	2.2.1
glnK R	ACCGCGGTACACCTCAGTAT	62	2509807		
16S F	CAGCTCGTGTCTGAGATGT	62	3823944	148	2.2.1
16S R	AAGGGGCATGATGACTTGAC	60	3824091		
glnD F	ATTCTGGCTCACCACAAAGG	60	2510257	110	2.2.1
glnD R	CAGATCGAGGTCTGAGTACGG	66	2510367		
glnE F	gTCGACATTCGCTTCTGTGA	60	4380266	101	2.2.1
glnE R	GTTGAGTTCGTCCCAGTCGT	62	4380367		
amtB F	TTCTTCTACGGCGGACTTTC	60	2508389	112	2.2.1
amtB R	ACATCGAGTAGCCCCACAGT	62	2508501		
glnR F (MSMEG_5784)	GCCGCTGGATCTCACCTA	58	5856938	120	Appendix 2B
glnR R (MSMEG_5784)	CCGCCGAAGAAGTCGTAA	58	5857057		
GlnA1roche F	ATGTGCTGCTGTTCAAGT	52	4375573	66	2.2.5
GlnA1roche R	TGAAGGTGACGGTCTTGC	58	4375638		
SigAroche F	GACTCGGTTTCGCGCCTA	56	2827072	64	2.2.5 and 3.2.3
SigAroche R	CCTCTTCTTCGGCGTTG	54	2827135		
MSMEG_6272 F	TGATCCGCCACATCCTG	54	6338327	65	3.2.3
MSMEG_6272 R	GATGTAGGTGCCGATGC	54	6338391		

MSMEG_5442 F	AGATCATGCGGTTCTGTC	54	5526393	61	2.2.5 and 3.2.3
MSMEG_5442 R	GTGTATTCACCGATGTGCC	58	5526453		
MSMEG_4699 F	GTGAGGACTTCCGCACC	56	4784687	104	2.2.5 and 3.2.3
MSMEG_4699 R	CCGCTTGACGACGAATC	54	4784790		
msmeg_6272 F2	GTACAGCCTGCTCGACATCA	62	6339489	88	2.2.5
msmeg_6272 R2	TGCCGACAACTCGAAGTAGA	60	6339576		
ureA F	ATGGCAATATCGCCGTCTA	56	3689711	61	2.2.5
ureA R	ACGAGATCAGCAAACGATCC	60	3689771		
nirD F	GGCGTGTGCCTACGACTT	58	504074	97	2.2.5
nirD R	CGAACCGTCATCCAGCTT	56	504170		
gltB F2	GAGTCCATCGAGGACATGGT	62	6524516	114	2.2.5
gltB R2	CTTGACATCACCTTGAGCA	58	6524629		
GlnR KO F	CCAGGTCGAAGCTTACTCCCTCGCC(HindIII)	82	5858452	1090	Appendix 2B
GlnR AR	GTCGGCGGTACCCGTCTCCAA (KpnI)	70	5857363		
GlnR BF	TGTGGGGGTACCACTTCTTC (KpnI)	62	5856962	1058	Appendix 2B
GlnR BR	CTCGAAGTACTGCAGCACC (ScaI)	60	5855905		
GlnR INT R2	GGTGGAGGTCACTGACTGG	62	5857440		Appendix 2B
AmtR - AF	CAACGGGATCCACGAGGCATTC (BamHI)	70	4389950	960	Appendix 2A
AmtR - AR	TGCGGTACCCGTGGGTGGT (KpnI)	64	4388991		
AmtR - BF	TCCCTCCCGAGGTACCCTG (KpnI)	64	4388567	804	Appendix 2A
amtR - BR	GACGACAGCAGCAAGTACTT (ScaI - blunt)	60	4387764		
amtRdel F2	TACCTGGCCGAACTCCAG	58	4389145	754	Appendix 2A
amtRdel R2	ACGCGATGGCTTCATAGG	56	4388392		
AmtR - PF	CGACCTGGGCCCGTATCTGGG (ApaI)	72	4389512	465	2.2.2
AmtR - PR	CGGTCTGCCCCGGGATCCGCGGCTG (BamHI)	90	4389512		
GlnR - PF	GACATTGGGCCCCACGAGACC (ApaI)	70	5857671	206	2.2.2
GlnR - PR	TCGACGGTCGGTACCAGTAGATCCA (KpnI)	78	5857466		
MSMEG_6272 F	CGTGGCTACTGGCTGGGTGA	66	6337864	256	3.2.3
MSMEG_6272 R	CGACGAAGATGTGGCGGTGAT	66	6338119		

MSMEG_4699 F	CGAGTTGGGCGGACTACGA	62	4783900	225	3.2.3
MSMEG_4699 R	CCTCGGTCTCGGCTTTGATG	64	4784124		
MSMEG_5442 F	CGCAACGGCGGTGAGAAA	58	5526017	285	3.2.3
MSMEG_5442 R	GAAGCCCAGGAACTTGACGA	62	5526301		
Rv2476c AF	GCGTCGTTGGTTCAGGC	56	2779204	829	3.2.5
Rv2476v AR	GGTCGAGGAAGATGTGGC	58	2780032		
Rv2476c BF	CCCAGACCCAGGATTTCA	56	2778597	736	3.2.5
Rv2476c BR	TCGGCGAGCACCAGTT	52	2779332		
GlnD oligo	CTGCGTGACGCGCTGCTCGATCTCTAGAATTCTGGCTCACC	N	2510229	41	4.4
		A			
GlnD KOF2	CGGCAACTGGATTTCGG	52	2510207	99	Appendix 4B
GlnD KOR2	GCTGTCGGCGGTGATG	54	2510305		
GlnD AF PstI	CTGCAGGCTGGCGTGTGACAGATG (PstI site)	58	2509550	923	Appendix 4A
GlnD AR	GCTGTGATCGAGGCGAAT	56	2510472		
GlnD BF	AAGCTCGTGCGGTACCAC (KpnI site)	58	2511623	874	Appendix 4A
GlnD BR HindIII	AAGCTTGTTTCCTGACGCACCTGCT (HindIII site)	58	2512496		
GlnD INTF	CCACAAAGGCAACCGAAC	56	2510268	287	Appendix 4A
GlnD INTR2	AGCACGTCGAGCAGTTCC	58	2511753		
GlnD seq AF	GAGTACTCGGTGGACTTCGTG	66	2509811	972	Appendix 4B
GlnD seq AR	GTACACGTCGGCGAGTTGC	60	2510782		
GlnD seq BF	TCGAGGAACTCGTCGAACAC	62	2510631	111	Appendix 4A
GlnD seq BR	AGTCCAGCACCACCATGTC	62	2511741		

4. DNA ISOLATION AND PURIFICATION

4.1 Recovery of DNA from Agarose gels and PCR reactions

After gel electrophoresis, DNA fragments of the desired size were excised from agarose gels and purified using the Wizard SV Gel and PCR Clean-UP System (Promega) according to the manufacturer's instructions. Likewise, PCR amplified DNA could be purified from the reaction mix using the above-mentioned kit. The DNA was eluted from the mini-columns in 30-40µl dH₂O. The DNA was quantified with the Nanodrop DNA quantification system (Inqaba Biotechnology).

4.2 Purification of Plasmid DNA

Plasmid DNA was isolated from *E. coli* using the Wizard SV Miniprep system (Promega) according to the manufacturer's instructions. Plasmid DNA was eluted from the minicolumn in 40-50µl dH₂O. To increase DNA yields, an incubation period of 1min at room temperature was allowed after addition of 40-50µl dH₂O to the mini-column. Thereafter, the DNA was eluted by centrifugation at 16000×g in a benchtop centrifuge (Eppendorf Centrifuge 5415D) for 1min and quantified by the Nanodrop DNA quantification system (Inqaba Biotechnology).

4.3 Crude DNA extraction

M. smegmatis grown in liquid culture media was collected by centrifugation (16000 x g for 1min) and resuspended in a small volume of 7H9 (approximately 100µl). The cells were lysed by boiling at 100°C for 5min followed by rapid cooling on ice. Cellular debris was collected by centrifugation (16000×g for 1min) and the supernatant containing genomic DNA was aspirated and retained for down-stream applications such as PCR (section 3.1).

5. RNA MANIPULATIONS

5.1 RNA Isolation

M. smegmatis cells in liquid culture with an $OD_{600} \approx 0.8$ were collected by centrifugation (Eppendorf Centrifuge 5810R) at $2465 \times g$ ($4^{\circ}C$) for 10 minutes. The supernatant was discarded and the cells resuspended in 1ml Trizol (Invitrogen). The cell suspension was transferred to a 2.0ml screw cap microtube (Quality Scientific Plastics) containing 0.5mm glass beads and ribolyzed (Fastprep FP120, Bio101 Savant) at a maximum speed setting of 6.0 for 20sec. The tubes were immediately placed on ice for 1 minute to dissipate the heat caused by friction during the ribolyzing process. The homogenisation step was repeated 6 times with intermittent cooling and the cooled homogenate incubated at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. This was followed by the addition of 250 μ l chloroform to the tube containing the homogenized cells and glass beads. The mixture was rapidly inverted for the first 20 seconds and periodically thereafter for a further 5 minutes at room temperature. After thorough mixing of chloroform and the homogenate, the tubes were centrifuged (Hettich Mikro 200) at $18626 \times g$ ($4^{\circ}C$) for 10min to allow phase separation.

The clear aqueous layer was transferred to a 1.5ml tube. It was important to ensure that the inter- and organic phases were not disturbed as this would result in DNA contamination of the RNA sample. Twice the aqueous layer volume of 100% ethanol was added to the supernatant, mixed by inversion and incubated at $-20^{\circ}C$ overnight to allow nucleic acid precipitation.

The precipitated nucleic acids were collected by centrifugation at $18626 \times g$ ($4^{\circ}C$) for 20min and the supernatant carefully poured off. A volume of 1ml 70% ethanol was added to the pellet and the tube inverted several times to wash the RNA. This ethanol wash was again followed by centrifugation at $18626 \times g$ ($4^{\circ}C$) for 10 min to pellet the nucleic acid. The ethanol was then carefully aspirated and the tube inverted to allow the pellet to air-dry for approximately 10-15minutes. Care had to be taken to prevent excessive desiccation of the pellet as this would hamper RNA resuspension. The nucleic acids were resuspended in 30-50 μ l RNase-free water

(Ambion) by gentle pipetting. In some cases the water and pellet needed to be heated to 55-60°C for 10min to facilitate resuspension.

5.2 DNase-treatment of isolated RNA

The presence of contaminating DNA in the RNA sample could adversely affect downstream applications such as Real Time-PCR (section 5.5) and reverse-transcriptase PCR (section 5.7), thus a deoxyribonuclease (DNase) digestion (Turbo DNase, Ambion) was done to remove any remaining DNA. To 20µl resuspended RNA, the following was added: 0.1 volume 10X buffer (supplied); 2U Turbo DNase and 40U ribonuclease (RNase) inhibitor (RNasin Plus, Promega). The reaction was incubated at 37°C for 30min, after which an additional 2U Turbo DNase was added and incubated for a further 30min. The DNase was inactivated by the addition of 0.2 volumes of DNase Inactivation Reagent (supplied) and allowed to incubate at room temperature for 2min with intermittent mixing. The Inactivation Reagent (supplied) was pelleted by centrifugation at 18626×g for 2min at 4°C and the supernatant was removed and used for reverse transcription (section 5.4) or stored at -80°C until required.

5.3 RNA Analysis

After DNase digestion, RNA quality as well as the detection of DNA contamination was determined by electrophoresis on a 1% agarose gel. The presence of two defined bands, corresponding to the 16S and 23S ribosomal-RNA's respectively, were indicative of intact RNA and the absence of high molecular weight smears on the gel indicated that contaminating DNA in the RNA sample was unlikely. In addition, a routine PCR (section 3.1) reaction using 16SrRNA primers (Table 3) and 2µl of the RNA sample was done in order to determine whether contaminating DNA was present in the RNA sample. If DNA was found to be present, a second DNase digestion (section 5.2) was carried out. RNA purity, quality and total mRNA concentration was determined by the Experion system (Bio-Rad). The analysis was done using the RNA Standard Sense Analysis Kit (Bio-Rad) according to manufacturer's instructions; with the exception that 7µl loading buffer was added to each well instead of the recommended 5µl.

This ensured that the probes were thoroughly immersed in solution which was an absolute requirement for the successful analysis of the RNA samples.

5.4 Reverse Transcription

Reverse transcription is the synthesis of double stranded complementary DNA (cDNA) from an RNA template by RNA-dependant DNA polymerases. In this study, cDNA was synthesised from total isolated RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to manufacturer's instructions. Ideally, an amount of 1µg total mRNA was added to each 20µl reaction together with random hexamer primers (supplied) to provide high and relatively equal cDNA yields for all samples. This cDNA was then utilised for both Real Time-PCR (section 5.5) and reverse-transcription-PCR (section 5.7).

5.5 Real-time Polymerase Chain Reaction (RT-PCR)

RT-PCR reactions were done to quantify the relative changes in gene expression levels in *M. smegmatis* strains exposed to various culture conditions. The technique is based on the simultaneous amplification of a target mRNA sequence and the quantification thereof. In our studies, SYBR green was used as an intercalating dye which fluoresced upon binding with double stranded DNA which allowed the tracking of an increase in PCR product in "real time". The dye, however, could bind non-discriminatively to double stranded DNA sequences (such as primer-dimers and non-specific PCR products) which would prevent an accurate quantification of the target mRNA, thus PCR optimisation was imperative. The melting or dissociation curve of the PCR products were analysed by raising the temperature by small increments until the melting point of the double stranded DNA was obtained. During this process the fluorescent signal was monitored. Each PCR product had a unique melting curve due to amplicon length, base pair composition, secondary structures, etc. which allowed the detection of any non-specific PCR products, primer-dimers or contamination. All biological experiments as well as each PCR reaction were performed in triplicate.

The RT-PCR reactions were performed in the LightCycler version 1.5 (Roche Molecular Biochemicals) using either the LightCycler Master^{Plus} SYBR Green (Roche) or the Master SYBR Green kit (Roche). PCR master mixes (SYBR Green dye and Taq polymerase were supplied) were made up according to the manufacturer's instructions. The 20 μ l reaction, using the Master^{Plus} kit, contained 14 μ l nuclease-free water (Ambion), 2 μ l master mix, 1 μ l sense primer (5pmol/ μ l), 1 μ l antisense primer (5pmol/ μ l) and 2 μ l cDNA template (1/10 dilution) (section 5.4). When greater primer specificity was required, the Master kit was used in which MgCl₂ (25mM stock) was supplied separately from the master mix. This enabled MgCl₂ titrations to be performed to increase primer annealing specificity. The 20 μ l reaction contents were the same as for the Master^{Plus} kit with the exception that the volume of water was adjusted for the volume of MgCl₂ added.

The 7900HT Fast Real-Time System (Applied Biosystems) was also used to perform RT-PCR as it allowed for a greater number of samples (96 well plate format) to be analysed at any given time and thereby reduced possible inter-run variation. The LightCycler Master^{Plus} SYBR Green (Roche) and Master SYBR Green kit (Roche) were compatible with the 7900HT system and the 10 μ l reactions were set up as for the LightCycler with the exception that 0.08 μ l ROX dye (Sigma-Aldrich) was added to each reaction. The fluorescent ROX dye acted as an internal reference for normalization of the fluorescent signal and served to reduce inter-run variation caused by pipetting errors or fluorescence fluctuations.

5.6 Analysis of RT-PCR data

In this study, the method of relative gene quantification described by (Pfaffl *et al.*, 2002) was used to monitor relative changes in gene expression between control and sample groups. The PCR efficiency of each primer pair was calculated by a standard curve method using *M. smegmatis* DNA as template for PCR in order to correct for differences in PCR efficiencies between PCR reactions. One of two reference or housekeeping genes, namely *16S rRNA* and *sigA* (Sala *et al.*, 2008) were used as internal control transcripts for the normalization of mRNA quantity. For the mathematical model used, the threshold value (Ct) values for each transcript had to be determined. The threshold cycle, as defined by (Livak and Schmittgen, 2001), is the

cycle number at which amount of amplified target reaches a fixed threshold. A maximum standard deviation of 0.5 between Ct triplicates was allowed. All data was statistically analyzed using the REST©384 Version 1 software (Relative Expression Software Tool) which employed a model based on Equation C.2 (appendix C; (Pfaffl *et al.*, 2002).

5.7 Reverse Transcription PCR

Reverse transcriptase PCR reactions were carried out in the GeneAmp PCR System 9700 (Applied Biosystems) using HotStar Taq DNA Polymerase (Qiagen) according to manufacturer's instructions (section 3.1). A 1/10 and 1/100 dilution of the cDNA template (section 5.4) was made and equal volumes (2µl) were used in each PCR reaction. Together with the gene of interest, a housekeeping gene such as *16SrRNA* or *sigA* was also amplified from every cDNA sample in order to normalise expression of the target gene. The PCR protocol was the same as described in Materials and Methods section 3.1. Upon completion of PCR, the products were serially diluted (usually a 1:2 dilution series) and fractionated on a 1% agarose gel. Densitometric analysis of gel images (Un-Scan-It gel Automated Digitizing System, Version 5.1) was used to quantify gene expression (Yuan *et al.*, 2007).

6. PROTEIN MANIPULATIONS AND ENZYMATIC ASSAYS

6.1 Crude Protein Extraction

Mycobacterium smegmatis cells were grown to an of $OD_{600} \approx 0.8$ at which point they were collected by centrifugation at $3465 \times g$ for 10min at $4^{\circ}C$. The cells were resuspended in 1ml Tris-HCl buffer (pH 8.0) or Phosphate buffer (pH 7.00) and transferred to a 2.0ml screw cap microtube (Quality Scientific Plastics) containing 0.5mm glass beads and ribolyzed (Fastprep FP120, Bio101 Savant) at maximum speed for 20sec. This ribolyzing step was repeated 3 to 4 times with cooling on ice between cycles in order to ensure that greatest amount of cells were ruptured. The cell lysate was centrifuged at $18626 \times g$ for 10min at $4^{\circ}C$ and the protein

containing supernatant removed. The crude protein extract was used in glutamate dehydrogenase, glutamine synthetase and β -galactosidase activity assays.

6.2 Protein Concentration determination

The Bradford method was used to determine the concentration of soluble cytosolic proteins in crude protein extracts and the Protein Concentration Assay (Bio-Rad) was used for this purpose. A protein concentration standard curve was generated by making serial dilutions (ranging from between 0 and 1mg) of a bovine serum albumin (BSA) standard. The protein-binding dye (supplied) was diluted in a ratio of 1:4 with dH₂O and 980 μ l thereof was added to each 20 μ l BSA dilution. The mixture was mixed gently by pipetting and allowed to incubate at room temperature for 5min before quickly obtaining absorbance readings at 595nm with a spectrophotometer. The reaction containing 0 μ g BSA was used as the blank or reference reaction. For calculation of the protein concentrations in unknown samples, 3 μ l sample was added to 17 μ l water to which 980 μ l diluted dye was also added. The absorbance values obtained were compared to the standard curve and the resulting protein concentrations were divided by 3 to obtain the true protein concentrations in each sample.

6.3 Glutamate Dehydrogenase activity assays

There are two glutamate dehydrogenase enzymes encoded for by the *M. smegmatis* genome, namely a nicotinamide adenine dinucleotide phosphate (NADP⁺)-specific glutamate dehydrogenase (GDH) and a nicotinamide adenine dinucleotide (NAD⁺)-specific GDH. Each enzyme was assayed for both forward and reverse reaction activity. The forward reactions were assayed by spectrophotometric measurement of NADPH or NADH oxidation at 340nm. The reverse reactions were assayed by measuring the reduction of NADP⁺ or NAD⁺ at 340nm. Each biological experiment and all assay reactions were performed in triplicate.

i) NADP⁺-GDH

The NADP⁺-GDH activity was assayed essentially as done by Sarada *et al*, (1980). Forward reaction (reductive aminating activity) activity was assayed by preparation of a 1mL reaction

volume containing 100mM Tris HCl (pH 8.2), 100mM NH₄Cl; 10mM α -ketoglutarate and 0.1mM NADPH. The reverse reaction (oxidative deaminating activity) assay preparation consisted of 0.1mM Tris-HCl (pH 9.4); 200mM glutamate and 0.1mM NADP⁺. The reactions were initiated by the addition of 6 μ g *M. smegmatis* crude protein extract.

ii) NAD⁺-GDH

The activity of both the forward and reverse NAD⁺-GDH reactions were assayed using a combination of methods from (Loyola-Vargas and de Jimenez, 1984) and (Minambres *et al.*, 2000). The 1mL anabolic or forward reaction (reductive amination) assay consisted of 100mM Phosphate buffer (HK₂PO₄/H₂NaPO₄; pH 7.0); 100mM NH₄Cl; 10mM α -ketoglutarate and 0.16mM NADH. The 1mL catabolic or reverse reaction (oxidative deamination) was prepared by adding 100mM Phosphate buffer (pH 7.0); 100mM L-glutamate; and 2mM NAD⁺. The assay reactions were initiated by the addition of 10 μ g *M. smegmatis* crude protein extract.

Absorbance readings were taken immediately after addition of crude protein extract (time zero) and every 5min thereafter for 30min. Alternatively, the assay reactions were scaled down to a total volume of 200 μ l and the assays performed in a microtitre plate reader (Synergy HT, Bio-Tek). The assays in this case were initiated by the addition of the enzyme substrate. Specific enzyme activities were calculated using the NADPH extinction co-efficient of 6.22 cm²/ μ mole. One unit of enzyme activity was defined as 1nmole of coenzyme (NAD(P)H) oxidized or reduced per minute, per milligram of protein added.

6.4 Glutamine synthetase activity assay

Glutamine synthetase (GS) transferase activity was determined by the γ -glutamyl transferase activity assay which resulted in a colorimetric reaction that could be spectrophotometrically measured (Stadtman *et al.*, 1970). For each sample to be assayed, three different reactions were set up containing the co-factors manganese (Mn²⁺), magnesium (Mg²⁺) and the two in combination. Both the adenylylated and de-adenylylated forms of glutamine synthetase were active in the presence of Mn²⁺ thereby measuring total GS transferase activity (Shapiro and Stadtman, 1970). The substitution of Mn²⁺ with Mg²⁺ inhibited the activity of the adenylylated

form of GS thereby measuring only de-adenylylated GS activity. Therefore, the assay containing both Mn^{2+} and Mg^{2+} gave an indication of the activity of the adenylylated form of the enzyme as it formed the difference between total GS activity (in the presence of Mn^{2+}) and activity of the de-adenylylated enzyme (in the presence of both Mn^{2+} and Mg^{2+}). From these activities, the degree of glutamine synthetase adenylylation could be determined.

The 550 μ l assay reactions were prepared, in technical duplicate, according to Table 4 and GS transferase activity was initiated by the addition of 6 – 10 μ g crude protein extract.

Table 4: Preparation of the reactions required to determine glutamine synthetase activity using the γ -glutamyl transferase activity assay.

Reagents*	Blank	Mn^{2+}	Mg^{2+}	Mn^{2+}/Mg^{2+}
Glutamine	15mM	15mM	15mM	15mM
Hydroxylamine	60mM	60mM	60mM	60mM
$MnCl_2 \cdot 4H_2O$	0.3mM	0.3mM		0.3mM
$MgCl_2 \cdot 6H_2O$			60mM	60mM
Na- ADP		0.4mM	0.4mM	0.4mM
Na-arsenate		30mM	30mM	30mM
Final Volume (10mM Imidazole Buffer pH 7.15)	550 μ l	550 μ l	550 μ l	550 μ l

*See Appendix A.3.4 for the composition of stock solutions used.

After addition of crude protein extract, the reactions were incubated at 37°C for 30min. To halt the transferase reaction, 900 μ l Stop Mix (appendix A.3.5.7) was added and the reactions were centrifuged at top speed for 2min at room temperature on a benchtop centrifuge to collect any iron precipitate that may have formed. The supernatant was transferred to a 1ml spectrophotometric cuvette and absorbance readings were taken at a wavelength of 540nm. Alternatively, 200 μ l of each reaction could be transferred to a microtitre plate and absorbance readings taken by a microtitre plate reader (Synergy HT, Bio-Tek). One unit of γ -glutamyl transferase activity was defined as the amount of enzyme able to produce 1 μ mol of γ -glutamyl hydroxamate per minute (Shapiro and Stadtman, 1970). See equation C.3 for calculations (appendix C).

6.5 Promoter activity determination using β -galactosidase activity assays

The promoter activity of selected genes was determined essentially according to methods used by (PARDEE and Prestige, 1959) and (Timm *et al.*, 1994). Various promoter regions were cloned into pJEM15 plasmids (appendix B.2), transformed into *M. smegmatis* and grown on Middlebrook 7H11 agar containing X-gal and Kan (section 2.1). Colonies were picked and grown in 7H9 medium with Kan till an $OD_{600} \approx 1$ and then transferred to Kirchner's medium (appendix A.2.2) to induce nitrogen starvation and/or excess conditions (section 2.4). The cell free/crude protein extract was prepared according to section 6.1 and the concentration thereof determined as described in section 6.2. The promoter activity of target genes under conditions of nitrogen starvation and or excess was determined using the β -galactosidase assay. The assay was based on the hydrolysis of the chromogenic substrate o-nitrophenyl- β -D-galactoside (ONPG) to o-nitrophenol by β -galactosidase. A yellow colour reaction resulted upon addition of sodium carbonate (Na_2CO_3) which was measured spectrophotometrically at both 420 and 550nm (Figure 2). Each assay was initiated by the addition of 6-10 μ g crude protein extract and incubated at 37°C for 30min. All biological experiments as well as each assay were performed in triplicate. One unit of β -galactosidase activity was defined as the production of 1 μ Mole o-nitrophenol per minute at 28°C (at pH 7) as was calculated according to Equation C.4 (appendix C).

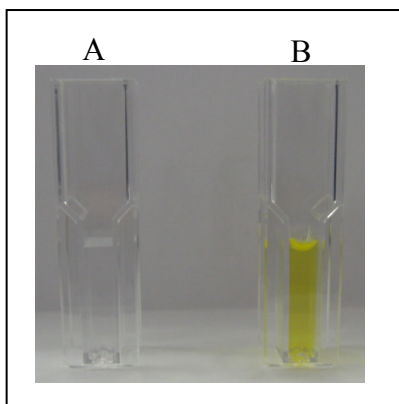


Figure 2: Colorimetric reaction which occurs when (B) an active promoter is assayed using the β -galactosidase assay. (A) represents a negative control assay.

7. CLONING

Molecular cloning of target DNA sequences into various vectors was done essentially as described by (Sambrook *et al.*, 1989). Target DNA sequences were cloned into vectors by simple T/A cloning or ligated into vectors after restriction enzyme digestion.

7.1 Ligation

All ligations were done with T4 DNA Ligase (Promega) as per the manufacturer's instructions. An insert to vector ratio of 3:1 was routinely used in ligation reactions and a final amount of 50ng vector per reaction was preferred. The amount of target or insert DNA required was calculated according to Equation C.5 (appendix C).

Ligation reactions were prepared by the addition of 1µl T4 DNA ligase and 1µl 10 x ligation buffer to the vector and insert and made up to a final volume of 10µl with nuclease free water. The reaction was incubated at room temperature for 1 to 2 hours or overnight at 4°C. The enzyme was inactivated by incubation at 65°C for 15min.

7.2 T/A cloning

The cloning of PCR sequences was done using the pGEM-T Easy vector (Promega) system (see appendix B.1). The vector was supplied as a linear sequence with a 3'-T-overhangs at the insertion site which was complementary to the adenosine overhang at the ends of PCR products generated by certain thermostable DNA polymerases. A 10µl ligation reaction was prepared as outlined in section 7.1. *E. coli* transformed (section 7.6) with pGemT-Easy vectors were grown on LA plates containing Amps, X-gal and IPTG and could be screened by the presence of blue or white colonies. Ligation of target DNA into the vector's multiple cloning site resulted in disruption of the Lac operon and colonies containing the desired insert were white. The presence of the desired insert was confirmed by colony screening PCR (section 3.1).

7.3 Cloning after Restriction Enzyme Digestion

Directional cloning of target sequences into appropriate vectors was done using restriction enzyme digestion. The digestion of both vector and insert sequence with the same enzyme allowed ligation into the vector in either direction. To prevent self-religation, the vector was de-phosphorylated with Shrimp Alkaline Phosphatase (SAP; section 7.4). The digestion of both vector and insert with two different enzymes allowed for ligation of the insert into the vector in a specific orientation. Restriction enzyme digestion reactions were prepared according the manufacturer's (Roche, Fermentas, Promega) instructions. Generally, between 2 and 4 μ g plasmid DNA or PCR product was digested with approximately 10U restriction enzyme in a final volume of 10 μ l-20 μ l containing 1 x enzyme-specific buffer (supplied). Reactions were incubated at temperatures required for optimal enzyme function (specified by manufacturer) for 1 hour. The digestion reactions were stopped by a 15min incubation period at temperatures that inactivated enzyme activity (also specified by manufacturer). Two restriction enzyme digests were done simultaneously (double digest) if the restriction enzyme buffers, incubation and inactivation temperatures were compatible. Ligation of restriction enzyme digested target DNA sequences into similarly digested vectors was done as described in section 7.1.

7.4 De-phosphorylation

The self re-ligation of vectors digested with a single RE was prevented by treatment with Shrimp Alkaline phosphatase (SAP, Fermentas). The enzyme catalyzed the removal of 5' phosphate groups from 5'-protruding, 5'recessive or 5'-blunt ends of the vector before ligation with target DNA sequences. De-phosphorylation reactions contained 1 x SAP Buffer and 2U SAP per 1 μ g DNA. The reaction was incubated at 37°C for 30min and the enzyme was subsequently inactivated at 65°C for 15min.

7.5 Preparation of Electro-competent *E. coli*

E. coli from frozen stocks was spread onto LA plates containing 10 μ l/ml tetracycline (see appendix A.1.4) and incubated overnight at 37°C. Single colonies were picked and inoculated

into 50ml LB starter culture containing 10µl/ml tetracycline and grown overnight at 37°C with shaking. One litre of LB medium containing 10µl/ml tetracylin was also prepared and prewarmed overnight at 37°C with shaking. The prewarmed LB was inoculated with 10ml of starter culture and incubated at 37°C for 3-4hrs with shaking until $OD_{600} \approx 0.7$. The culture flasks were then placed on ice for 30min to 1hr and all downstream procedures were performed under ice-cold conditions. *E. coli* cells were harvested by centrifugation in a GSA rotor at 1150 x g for 10min at 4°C. The supernatant was removed and the cells carefully resuspended in a volume of ice-cold 10% glycerol equal to the original culture volume. The cells were again spun at 1150 x g for 10min at 4°C and this wash step was repeated three times. After the final wash, the cells were pooled and collected by 20 min centrifugation at 1150 x g (4°C). The cells were resuspended in 2-4ml 10% glycerol and 100µl aliquots were either used directly for transformation or snap frozen in liquid nitrogen and stored at -80°C for later use.

7.6 Electro-transformation of *E. coli*

Frozen electro-competent *E. coli* cells were allowed to thaw on ice. A 50µl aliquot of cells was gently mixed with 2µl ligation reaction mix (section 7.1) or plasmid DNA or PCR product and transferred to a pre-cooled 0.2cm electroporation cuvette (Bio-Rad). Transformations were performed in the Gene Pulser (Bio-Rad) which was set to a voltage of 2.5kV, a capacitance of 125F, a secondary capacitance of 25µF, and a resistance of 200Ω. Immediately after transformation the cells were resuspended in 1ml SOC medium (see appendix A.2.8) and transferred to a sterile microcentrifuge tube. The cells were then incubated at 37°C for 1hrs with shaking before spreading 50-200µl cell suspension onto LA plates containing appropriate antibiotics (Table 1).

7.7 Preparation of Electro-competent *M. smegmatis*

M. smegmatis was grown in 7H9 with ADC to an $OD_{600} \approx 0.8$ and analyzed for contamination by ZN staining (section 2.3). The culture was placed on ice and allowed to cool for 1hrs before the cells were harvested by 10 min centrifugation at 3220×g (4°C). The cells were washed with 30ml ice-cold 10% glycerol followed by 10min centrifugation at 3220×g (4°C). This wash step was

repeated at least three times. As for *E. coli*, after the final wash the cells were pooled and collected by 10min centrifugation at 3220×g (4°C) and the cells gently resuspended in 2-4ml 10% glycerol. Aliquots of 100µl each were either used for transformation or snap frozen in liquid nitrogen for storage at -80°C.

7.8 Electro-transformation of *M. smegmatis*

Frozen electro-competent *M. smegmatis* stocks were thawed out on ice. Plasmid DNA (2-4µg) was added to 200µl cells and transferred to a pre-cooled 0.2cm electroporation cuvette (Bio-Rad). Transformations were done in the Gene Pulser (Bio-Rad) which was set to a voltage of 2.5kV, a capacitance of 125F, a secondary capacitance of 25µF, and a resistance of 1000Ω. A volume of 1ml LB (bacteria transformed with knockout plasmids) or OADC enriched 7H9 (all other *M. smegmatis* transformations) was added to transformed cells and transferred to a sterile microcentrifuge tube. The cells were incubated at 37°C for 3hrs before plating onto appropriately supplemented media (Table 1).

7.9 Selection of Transformed Cells

After transformation, starter cultures were prepared by spreading a volume of *E. coli* or *M. smegmatis* suspension to LB agar or Middlebrook 7H10/7H11 agar respectively. The agar contained appropriate antibiotics to allow for selection of the desired transformants (Table 1). Single bacterial colonies were picked and transferred to the appropriate liquid medium with continued antibiotic selection pressure. Bacterial growth was determined by spectrophotometric measurement of the cell culture at a wavelength of 600nm. Selection of desired transformants was also done by detection of β-galactosidase activity in transformed cells. This blue/white colony screening technique was used when cells were transformed with plasmids containing the *Lac* operon (pGemT Easy, see section 7.2) or *lacZ* gene (pJEM15 and pGoal17). Successfully transformed cells containing the desired insertion sequence in pGemT Easy appeared as white colonies on the selection plates whilst cells containing the desired insertion sequence in pJem15 and pGoal17 appeared as blue colonies. The presence of the desired DNA sequence within the vector was confirmed by routine PCR (section 3.1)

8. CONSTRUCTION AND IDENTIFICATION OF GENE REPLACEMENT MUTANTS **IN *M. smegmatis***

The gene knockout strategy used was based on previously described methods (Parish and Stoker, 2000b; Goosens, 2008). The strategy involved the replacement of the functional copy of a target gene with a deleted form of the gene by homologous recombination.

8.1 Construction of knockout vectors

The construction of the disrupted gene sequence was initiated by PCR (section 3) and involved the amplification of two PCR fragments. The first fragment (part A) included 1000bp flanking the 5' end of the target gene and a portion of the 5' end of the gene (approximately 150bp). The second fragment (part B) included a portion of the 3' end of the target gene (approximately 150bp) and 1000bp flanking the 3' end of the gene. The two PCR fragments were ligated to provide a deleted form of the gene with 1000bp flanking either end which was included to provide sufficient sequence identity for homologous recombination to occur.

The PCR amplified fragments were cloned separately into pGemT-Easy vectors via T/A cloning (section 7.2) and transformed into *E. coli* (section 7.6). After plasmid extraction (section 4.2) the fragments were excised from pGemT-Easy by restriction enzyme digestion (section 7.3) as specific restriction enzyme cutting sites had been designed into the primers (see section 1.3). This allowed for the ligation of the two PCR fragments which resulted in the deletion of a central portion of the gene. Restriction enzyme cutting sites designed into the outermost primers allowed site-specific ligation of the disrupted gene and flanking sequences into p2NIL (appendix B.3). The fragments were ligated into p2NIL by three way cloning. The presence of the deleted sequence was confirmed by PCR and sequencing (ABI PRISM DNA Sequencer 377, Perkin Elmer).

The vector pGoal17 (appendix B.4) was digested with PacI and a 6.3kb fragment containing the *sacB* and *lacZ* cassettes was isolated after gel electrophoresis (section 4.1). This fragment was

cloned into the unique Pac1 cutting site in p2NIL to create an unmarked knockout suicide vector containing *aph* (p2NIL) and *lacZ* (pGoal17) selectable markers and a *sacB* (pGoal17) counter-selectable marker. The *aph* gene conferred Kan resistance and the *lacZ* gene expressed β -galactosidase which hydrolyzed X-gal to form a blue colour. The *sacB* counter-selectable marker expressed levansucrase which catalyzed the conversion of sucrose to levan, a toxin which resulted in cell death.

The unmarked knockout suicide vectors were verified by transformation into *E.coli* prior to transformation into *M. smegmatis*. Transformed *E. coli* cells were grown on LA plates containing Kan and X-gal to test for Kan^R and β -galactosidase activity. Blue colonies were selected and grown on LA containing X-gal and sucrose to test for levansucrase activity and therefore sucrose sensitivity.

8.2 Identification of Single Cross Overs (SCO) colonies

The knockout vectors were transformed into *M. smegmatis* (section 7.8) and grown on LA plates containing Kan and X-gal for 4-7days. pJem15 was also transformed into *M. smegmatis* as a positive control for transformation efficiency. SCOs were identified as blue colonies and genotypically confirmed by PCR (section 3).

8.3 Identification of Double Cross Overs (DCO) colonies

The SCO colonies were passaged in LB medium without drugs or supplementation for 1-2days to allow for a second crossover event to occur. The cells were grown till mid-log phase and a dilution series made (1/50; 1/100; 1/150; 1/200). A volume of 50 μ l of each dilution was plated onto LA plates containing X-gal and sucrose (5%) and grown for 3-6days. White colonies that grew on sucrose plates had survived due to the loss of the vector sequence containing the *sacB* gene since they were unable to produce the levan toxin. Homologous recombination could have resulted in one of two genotypes (i) SCO cells that had reverted back to the wild type form of the gene when the second crossover occurred on the same side as the first or (ii) if the second crossover event occurred on the opposite side of the mutation, the deleted form of the gene was

integrated into the chromosome to give the desired DCO genotype. Replica plating (section 8.4) and PCR colony screening (section 3.1) were done in order to differentiate between the possible genotypes.

8.4 Replica Plating

A single *M. smegmatis* colony was grown in 500µl LB containing 0.1% Tween80 for 1-2days. Approximately 2-5µl of the bacterial suspension was dotted onto two LA plates; one containing Kan and X-gal and the other X-gal only. The plates were incubated for 2-3days and scored for growth or lack thereof. Colonies that failed to grow in the presence of Kan had lost the vector backbone and thus could have either the wild type or DCO genotype. Kan sensitive colonies were further screened by PCR methods (section 8.5).

8.5 PCR Screening for SCO and DCO

In order to screen for the presence of possible genotypes, two PCR reactions were done:

- i) Primers were designed to flank the deletion region and the difference in PCR amplicon size could distinguish between the larger wild type allele and/or the smaller deleted allele.
- ii) An additional confirmatory PCR was designed with a primer annealing to a sequence within the deleted region a primer flanking the deleted region. If a wild type copy of the gene was present then a PCR product would have been formed whereas no PCR product would be synthesised if only the deleted form of the gene was present.

Thus, in order to confirm the presence of a SCO, a deleted form of the gene should have been present and was confirmed by reaction (i). In addition, a wild type form of the gene should have been present and this was confirmed by reaction (ii). To screen for a DCO, the presence the deleted form of the gene was confirmed by reaction (i) and the absence of the wild type form of the gene was confirmed by the absence of a PCR product generated by reaction (ii).

9. CONSTRUCTION AND IDENTIFICATION OF *M. smegmatis* KNOCKOUT MUTANTS BY POINT MUTAGENESIS

The knockout technique used was based on a system developed by van Kessel and Hatfull (2007 and 2008) which relied on the creation of an *M. smegmatis* strain boasting elevated recombination frequencies due to the presence of the pJV75amber vector (Appendix 6B) encoding for mycobacteriophage recombination proteins whose expression is under the control of an acetamidase promoter. This technique was used to create an SNP at the 5' end of the gene which would result in a stop codon as well as a frame-shift and therefore generate a knockout of the gene of interest.

9.1 Formation of the recombineering *M. smegmatis* strain

Wild type *M. smegmatis* was cultured in 7H9 and the cells made electrocompetent as described (section 7.7). The electrocompetent cells were transformed (section 7.8) with pJV75amber (appendix B.5) and cultured on 7H10 agar plates containing 25µg/ml Kan for 3 to 5 days. Single *M. smegmatis* colonies were grown in 7H9 liquid medium supplemented with 10% ADC, 0.05% Tween80 and 25µg/ml Kan to an OD₆₀₀ of approximately 0.8 at which point frozen stocks (section 2.2) of the recombineering strain were made.

9.2 Construction of the *M. smegmatis* mutant strain

Frozen *M. smegmatis* recombineering strain stocks were thawed on ice and cultured in a 5ml starter culture containing 7H9 and 25µg/ml Kan until saturation. A volume of 100ml 7H9 containing Kan and 0.2% succinate (appendix 6A.3.14) was inoculated from the starter culture to an OD₆₀₀ ≈ 0.025 and grown to an OD₆₀₀ of approximately 0.4. The pJV75amber acetamidase promoter was induced to actively transcribe the mycobacteriophage recombination machinery by the addition of acetamide (see A.3.13) to a final concentration of 0.2%. The cells were incubated with shaking for 3hrs at 37°C at which point the cells were made electrocompetent (section 7.8).

An oligonucleotide (e.g. GlnD oligo, Table 3) was designed to contain a specific SNP flanked by approximately 20bp with homology to the target gene where a recombination event could occur. The oligonucleotide was HPLC purified after synthesis and approximately 100ng thereof was transformed into the induced *M. smegmatis* recombineering strain. As a selective marker, 500ng of a second oligonucleotide (Jcv198; Table 2) was co-transformed with the first which, after recombination with a mutated form of the Hyg resistance gene encoded by pJv75amber, would restore Hyg resistance. The transformed cells were recovered in 7H9 medium for 3hrs at 37°C, after which they were plated onto 7H10 agar containing 50µg/ml Hyg.

9.3 Detection of point mutations by high resolution melt (HRM) analysis

Colonies which grew on 7H10 agar plates containing Hyg were picked and cultured in 1ml 7H9 medium. A crude DNA extraction from 500µl of the culture was done (section 4.3) and subsequently used as a template for the PCR amplification of a DNA fragment flanking approximately 50bp either side of the desired SNP. The PCR reaction mix was essentially the same as described for Routine PCR reactions (section 3.1); however, 1µl Syto9 fluorescent dye (1/100 dilution; appendix 6A.3.16) was added to each 25µl reaction. For every unknown genotype or sample screened, wild type *M. smegmatis* DNA was also amplified. After amplification of the region of interest, 12.5µl of each sample amplification product was mixed with 12.5µl wild type amplification product and allowed to form duplexes by the following protocol: 95°C for 45sec; 40°C for 2min and cooling to 4°C indefinitely.

The melting curve of the resulting duplexes was studied via HRM analysis (Corbett Life Science, Australia). The PCR products containing the desired SNP formed heteroduplexes with wild type PCR products. The heteroduplexes could be identified by the difference in melting curve profiles when compared with wild type homoduplex melting curves. The formation of the desired polymorphism was confirmed by sequencing (ABI PRISM DNA Sequencer 377, Perkin Elmer).

10. INTRACELLULAR SURVIVAL OF *M. SMEGMATIS* IN THP1 CELLS

10.1 Growth of THP-1 cells

The human acute monocytic leukaemia derived cell line, THP-1, was utilised to determine the intracellular survival profile of *M. smegmatis* strains. THP1 cell stocks were thawed from -80°C cryopreservation and placed in 9ml complete medium for THP1 cells (appendix 6A.2.9). The 9ml cell suspension was centrifuged for 5min at 289×g (room temperature, Eppendorf centrifuge 5810R) in order to remove the supernatant containing toxic DMSO from the freezing procedure. The cell pellet was resuspended in 4.5ml complete medium and placed in a T45 tissue culture flask at 37°C in 5% carbon dioxide for growth overnight. An additional 4-5ml complete medium was subsequently added to the cells in order to increase the medium volume. The THP-1 cells were allowed to grow until they reached approximately 90% confluency at which point the cells were diluted (split) with complete medium. The cells were transferred to T75 tissue culture flasks and inspected daily by light microscopy until the cells again reached 90% confluency at which point they would be split once more. THP-1 cells could be differentiated for use in infection experiments between the 3rd and the 5th split.

10.2 Counting and Differentiation of THP-1 cells

All split THP-1 cell cultures were pooled and collected by centrifugation. The cell pellet was resuspended in approximately 20ml complete medium (appendix 6A.2.9) lacking antibiotics. In order to count the cells, 1ml resuspended THP1 cells was removed and added to 9ml complete medium. 50µl diluted cell suspension was mixed with 50µl Trypan blue, placed on a hemocytometer (Cascade Biologics) and the cells counted according to the manufacturers instructions.

A total of 3.75×10^6 cells in a volume of 2ml complete medium containing 100nM phorbol 12-myristic 13-acetate (PMA) were seeded into each of the 6 wells of a tissue culture plate (Nunc). The cells were incubated for 48hrs and allowed to differentiate due to stimulation by PMA. The differentiated THP-1 cells (dTHP-1) were washed twice with RPMI 1640 and once with

complete medium lacking antibiotics in order to remove the PMA. A final volume of 1ml complete medium without antibiotics was added to the dTHP1 cells which were rested for 24hours prior to infection.

10.3 Infection of dTHP-1 cells

Mycobacterium smegmatis frozen stocks whose bacterial titre had previously been determined (section 2.5), were thawed on ice and the cells collected by centrifugation at 16 000×g for 5min in a benchtop centrifuge. The cell pellet was resuspended in 1ml RPMI 1640 containing 10% FBS and passed through a 1ml syringe needle to disrupt bacterial clumps. *M. smegmatis* was added to dTHP-1 cells at a ratio of dTHP1:bacteria of 2:1 (multiplicity of infection or MOI) and incubated at 37°C in 5% CO₂ for 2hrs to allow for phagocytosis of the bacteria to occur (time point zero). At 0 and 4hrs, extracellular bacteria were removed by washing the plates three to four times with pre-warmed RPMI 1640 supplemented with 10% FBS and adding a final volume of 1ml complete medium lacking antibiotics to the washed cells. After 1hr, a 10 minute gentamycin treatment (20µg/ml) was applied to infected dTHP-1 cells in order to kill any extracellular bacteria (Plum *et al.*, 1997). The cells were washed 3 times with RPMI 1640 in order to remove any residual gentamycin and incubated in complete medium without antibiotics for the remainder of the infection study.

10.4 Recovery of *M. smegmatis* from macrophages and survival curve determination

Intracellular survival of *M. smegmatis* was determined at 0hrs (2hrs post-infection), 3hrs, 6hrs and 24hrs. The dTHP-1 cells at each time interval were lysed by the addition of 0.1% Triton X-100 for 1min after the medium bathing the cells was removed. The lysed cells were transferred to a safe-lock microfuge tube on ice and subsequently collected by centrifugation at 18626×g (4°C) for 5min. The supernatant was removed and the pellet containing liberated intracellular bacteria was resuspended in 1ml 7H9 medium (Middlebrook). A 10X dilution series of the resuspended bacteria was made and plated onto 7H10 agar plates. CFU were counted in order to determine the intracellular survival of *M. smegmatis*.

11. INTRACELLULAR SURVIVAL OF *M. SMEGMATIS* IN C57BL/6 MACROPHAGES

11.1 Macrophage isolation and culture

Bone marrow derived macrophages (BMDM) were cultured essentially as previously described (Frehel *et al.*, 1986). Precursor cells (stem cells) were extracted from the femur bone marrow of C57/Bl6 mice that were 6 to 8 weeks old. Undifferentiated murine macrophages were seeded onto 6-well tissue culture plates (Corning) such that each well contained 3×10^6 cells at the time of differentiation. The precursor cells were grown in RPMI-1640 medium supplemented with 10% FBS and stimulated to differentiate by the addition of 10% L-929 supernatant (appendix 6A.2 10). After 5 days incubation at 37°C in 5% CO₂, the cells were washed twice with RPMI-1640 medium in order to remove any undifferentiated cells and debris. Growth medium (appendix 6A.2.11) was added once again and replaced every two days.

11.2 Infection of C57/Bl6 macrophages

The bacterial titre of frozen *M. smegmatis* stocks was calculated as described in section 2.5. The total number of bacteria required for the infection of macrophages at a particular MOI was calculated and the desired volume of stock containing the bacteria was placed in sterile safe-lock tube. The bacteria were collected by centrifugation at 16000×g on a bench top centrifuge at room temperature. The supernatant was discarded and the bacteria were diluted with RPMI 1640 such that a volume of 100µl contained the desired number of bacteria for infection of macrophages in a well. The bacterial suspension was passed through a 1ml Tuberculin syringe at least 20 times in order to eradicate bacterial clumps.

A volume of 100µl diluted bacteria was added, drop by drop, to the 2ml growth medium present in the well bathing the macrophages. The bacteria were allowed to infect the macrophages for 2 hours. Thereafter the cells were washed four times with ice cold Phosphate Buffered Saline (Sigma) to remove any extracellular bacteria. In addition, the macrophages were subjected to a mild gentamycin treatment in order to kill any extracellular bacteria. The treatment involved removal of any medium in the wells and subsequent addition of 2ml pre-warmed growth medium

containing 20µg/ml gentamycin to the wells. The macrophages were incubated for 10min at 37°C, after which the gentamycin medium was removed and the cells washed with pre-warmed RPMI 1640 to remove the antibiotic. Pre-warmed growth medium was again added to the wells. Gentamycin treatments were repeated at 10hrs and 14hrs post infection.

After the first gentamycin treatment, macrophages were lysed (time point zero) by addition of 1ml 0.025% SDS (appendix 6A.3.17) at room temperature. The cells were incubated at room temp for 10min and then scraped off the bottom of the well and transferred to a sterile safe-lock tube. The bacterial cells were collected by centrifugation at 16000×g in a benchtop centrifuge and resuspended in 1ml 7H9. Thereafter a 10-fold dilution series of the bacteria was made and each dilution spread onto 7H10 agar plates. Macrophages not being harvested at time zero were incubated in 2ml growth medium for the remainder of the study. Infected macrophages were lysed and intracellular bacteria plated after 4hrs, 8hrs, 12hrs and 24hrs. The bacterial colonies were counted and a survival curve was plotted.

APPENDIX 6A: Solutions

A.1 Antibiotic stock solutions:

A.1.2) *Kanamycin*

50 mg/ml in ddH₂O, filtered sterilised (0.22µm filter, Millipore, USA) and stored at – 20⁰C

A.1.3) *Hygromycin B*

50 mg/ml in ddH₂O, filtered sterilised (0.22µm filter, Millipore, USA) and stored at – 20⁰C

A.1.4) *Tetracyclin*

5 mg/ml in ethanol, filtered sterilised (0.22µm filter, Millipore, USA) and stored at – 20⁰C

A.1.5) *Ampicillin*

50mg/ml in ddH₂O, filter sterilised (0.22µm, Millipore, USA) and stored at -20⁰C

A.2 Culture Media:

A.2.1) *ADC mycobacterial liquid culturing media supplement:*

0.5% w/v Bovine serum albumin (BSA) Fraction V; 0.2% v/v glucose; 0.015% v/v catalase (Merck). The supplement was prepared in ddH₂O and was sterilised through a 0.22µm filtration unit (Sterilin) ADC was stored at 4⁰C.

A.2.2) *Kirchners medium:*

21.13mM Na₂HPO₄ (anhydrous); 29.4mM KH₂PO₄ (anhydrous); 4.34mM MgSO₄.7H₂O; 8.5mM Tri-sodiumcitrate; 16.66 % v/v Glycerol. Nitrogen donors added were 3.8mM, 30mM or 60mM L-Asparagine monohydrate or 3mM and/or 60mM (NH₄)₂SO₄.

Dissolve the reagents, except for the glycerol and nitrogen donors, in half the final volume dH₂O using heat. Once dissolved, add the glycerol and make up to final volume with dH₂O. Autoclave for 15 min. at 121⁰C. After the broth has cooled, add nitrogen donors to desired concentrations.

A.2.3) Luria-Bertani (LB) Medium:

1% w/v Bacto-tryptone, 0.5% w/v Yeast Extract, 171 mM NaCl, pH 7.0. Autoclave for 15 min at 121°C.

A.2.4) Luria-Bertani (LA) agar:

1.5% w/v Bacto-agar in LB medium. Autoclave for 15 min at 121°C.

A.2.5) OADC mycobacterial liquid culturing media supplement:

0.005% v/v oleic acid (Merck), 0.5% w/v BSA Fraction V, 0.2 % v/v glucose, 0.02% v/v catalase (Merck), w/v 0.085% NaCl. The supplement was prepared in ddH₂O and filter sterilised using a 0.22µm vacuum filtering unit (Sterilin). OADC was stored at 4°C and added to 7H11 Mycobacterial agar at 30°C – 45°C.

A.2.6) 7H9 Mycobacterial medium:

Suspend 4.7g 7H9 medium powder (Difco) in 900ml dH₂O containing 2ml glycerol or 0.5g Tween80 (Sigma). Autoclave for 15min at 121°C

A.2.7) 7H10 or 7H11 Mycobacterial agar:

Suspend 19g 7H10 or 7H11 agar (Difco) in 900 ml dH₂O containing 5ml glycerol. Autoclave for 15min at 121°C.

A.2.8) SOC medium:

Add glucose (20mM final concentration) and MgCl₂ (10mM final concentration) to SOB medium prior to use.

A.2.9) Complete medium for THP1 cells:

RPMI medium (Sigma) containing 10% heat-inactivated foetal bovine serum (FBS; Gibco) and 2% penicillin-streptomycin solution (10000U penicillin and 10mg streptomycin; Sigma)

A.2.10) L-929 supernatant

The supernatant of L-929 cells grown in RPMI-1640 (with glutamine) supplemented with 10% FBS under high bicarbonate (3.7g/L) and 5% CO₂ was collected when the cells were at maximum confluency. The supernatant was filtered through a 0.2µm filter and stored at -20°C.

A.2.11) Growth medium for C57/Bl6 macrophages:

RPMI medium (Sigma) containing 10% heat-inactivated foetal bovine serum (FBS; Gibco) and 10% L-929 supernatant.

A.3 Buffers and Solutions:

A.3.1) 5% Acid alcohol (ZN-staining):

5% HCL in methanol

A.3.2) Ammonium persulphate (APS) (10% w/ v):

1g (NH₄)₂S₂O₈ and sterile dH₂O to 10ml. Prepare fresh, or store aliquots at – 20⁰C for not longer than 1 month.

A.3.3) Ethidium bromide stock solution:

10 mg/ml in TE buffer (pH 8.0)

A.3.4) γ-glutamyl-transferase activity assay:

Assay components prepared in 50mM Imidazole Buffer (pH 7.15):

A.3.4.1) 112,9mM Gln

A.3.4.2) 70.72mM MnCl₂.4H₂O

A.3.4.3) 913.33mM MgCl₂.6H₂O

A.3.4.4) 900mM Hydroxylamine (NH₂O)

A.3.4.5) 180mM Sodium arsenate (Na₂HAsO₄)

A.3.4.6) 4.45mM Sodium ADP

This mixture should be used fresh, as the sodium-ADP and hydroxylamine are unstable over long periods.

A.3.5.7) Stop mix: 1 M FeCl₃, 201mM trichloroacetic acid (CCl₃COOH) and 5.2 % v/v absolute HCl in distilled water. Mix before use.

A.3.6) *IPTG (Isopropyl -β-D-thiogalactopyranoside) stock solution:*

100mg/ml in ddH₂O, sterilise by 0.22μm filter filtration

A.3.7) *Primary wash buffer:*

6M urea, 0.4% w/v SDS, 0.5× SSC

A.3.8) *20× SSC:*

0.3M Tri-sodium citrate, 3M NaCl; pH7.0. 2× SSC working concentrations are prepared by a 10× dilution with dH₂O

A.3.9) *X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) stock solution:*

20mg/ml in dimethylformamide (DMF) or dimethylsulfoxide (DMSO)

A.3.10) *20× SB Buffer:*

19.1g di-Sodium tetraborate decahydrate dissolved in 500ml water.

A.3.11) *1M Tris-HCl buffer*

121.14g Tris (Sigma) was dissolved in 800mL water. Adjust pH to the desired value by adding concentrated HCl. The final volume was adjusted to 1L with water and sterilized by autoclaving.

A. 3.12) *0.1M Phosphate Buffer (NaH₂PO₄/K₂HPO₄)*

0.2M sodium phosphate (mono-sodium salt) was made by dissolving 13.8g NaH₂PO₄ in 250ml and adjusting the volume to 500ml with dH₂O. To make a 0.2M potassium phosphate (di-sodium salt), 19.2g K₂PO₄ was dissolved in 250ml water and the volume adjusted to 500ml with dH₂O. Approximately 40ml 0.2M sodium phosphate was placed in a glass bottle with a magnetic stirrer and titrated with 0.2M potassium phosphate until pH7 was reached. An equal volume of water was added to the titrated buffer to obtain 0.1M phosphate buffer.

A.3.13) 20% Acetamide

100g acetamide (Sigma) was dissolved in 500ml dH₂O. Stir until dissolved and filter sterilise (0.22 micron filter). 1ml thereof was added 99ml 7H9 to obtain a final concentration of 0.2% acetamide.

A.3.14) 20% Succinate

100g sodium succinate dibasic hexahydrate (Sigma) was dissolved in 500ml dH₂O. Stir until dissolved and filter sterilise (0.22 micron filter). 1ml thereof was added 99ml 7H9 to obtain a final concentration of 0.2% succinate.

A.3.15) 50% Sucrose

250g sucrose was dissolved in 500ml dH₂O. The solution was sterilised by it passing through a 0.22micron filter.

A.3.16) 1:100 Syto9

Syto9 fluorescent dye (Invitrogen) was diluted 100x by the addition of 1µl dye to 99µl dH₂O.

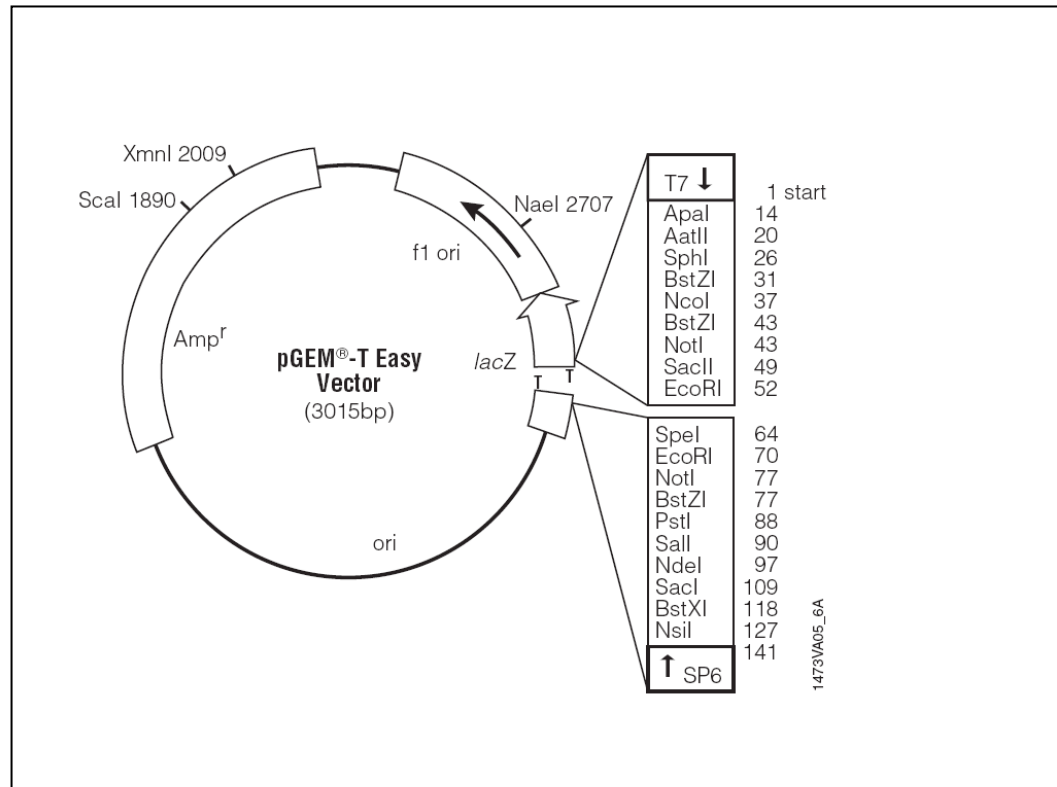
A.3.17) 0.025% SDS

A 10% SDS stock solution (Sigma) was diluted 400X by the addition of 1.25ml 10% SDS to 498.75ml dH₂O to obtain a 0.025% working solution.

APPENDIX 6B: Plasmid maps

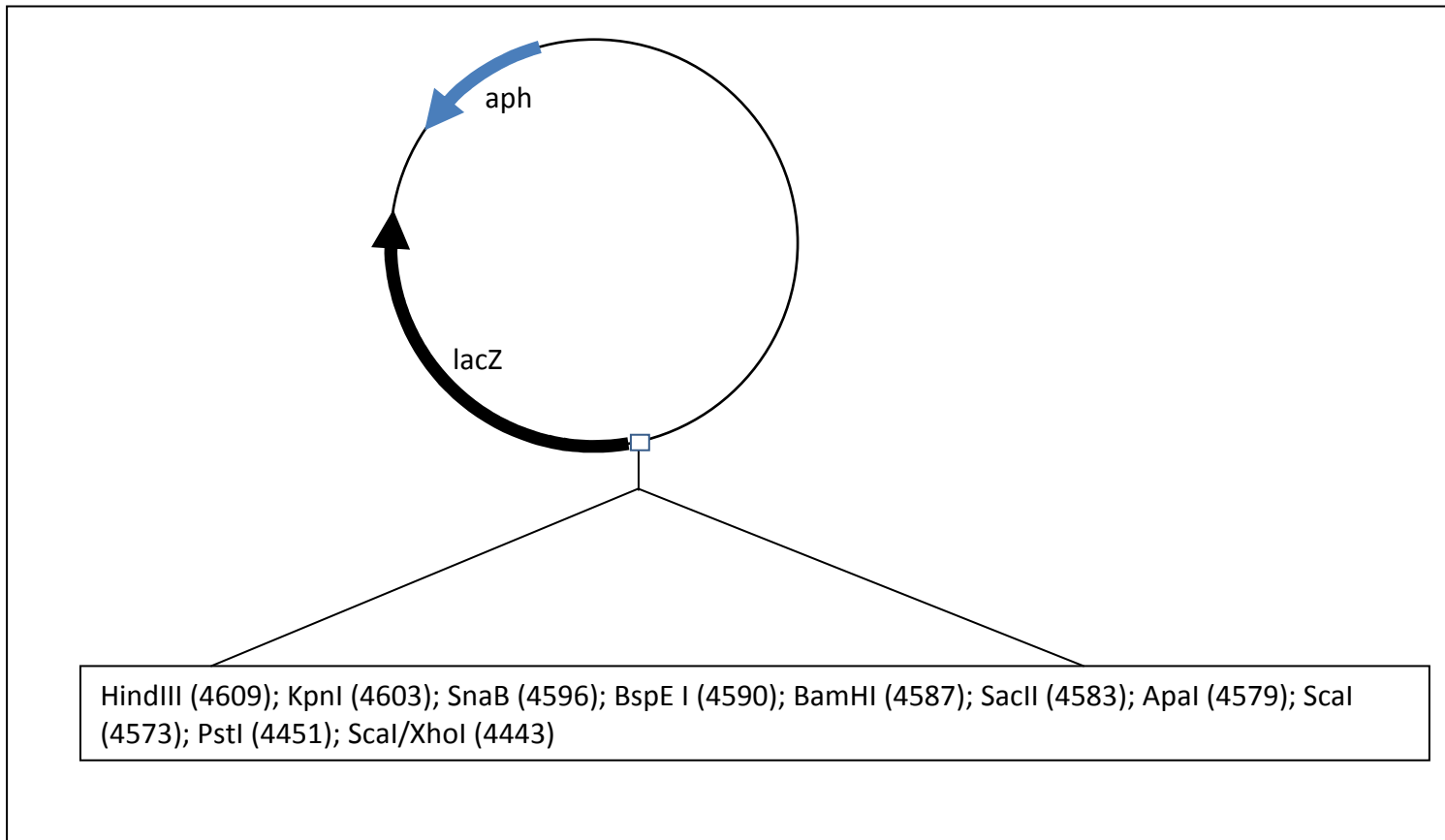
Refer to Table 2 for plasmid map references.

B.1) pGEM-T Easy Vector map



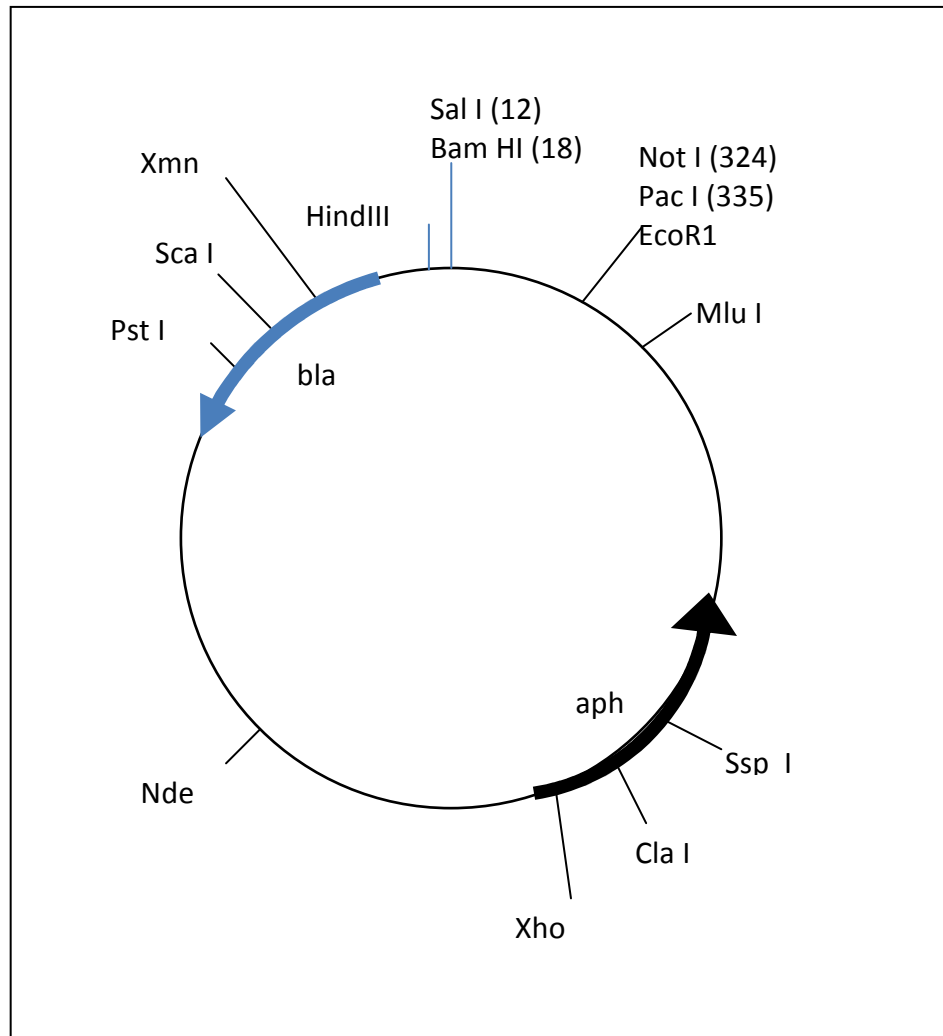
pGEM-T Easy vectors are commercially available (Promega), high copy number plasmids used for the direct cloning of PCR products synthesised by certain thermostable *Taq* polymerases. The vector has single 3'-thymine overhangs flanking the MCS site which are able to ligate to PCR products generated by *Taq* polymerases as they often add a single 3'-deoxyadenosine to their amplified products. The cloned PCR sequence disrupts the expression of the *lacZ* reporter gene, thus successful transformants are identified as white colonies on X-gal containing selection plates (see Materials and Methods section 7.2). The MCS has a number of unique restriction enzyme sites as well as specific enzyme restriction sites flanking the MCS thereby allowing the release of the insert by digestion with two different restriction enzymes or with a single restriction enzyme digestion respectively. The vector has an ampicillin resistance marker.

B.2) pJEM15 plasmid map



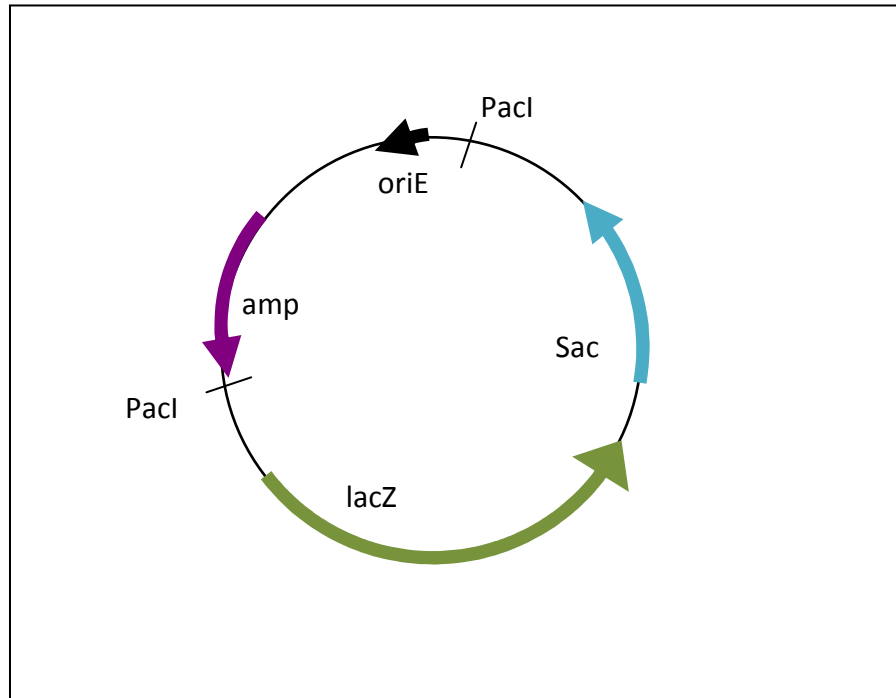
The pJEM15 plasmid (9446bp), a regulatory sequence probe vector (Timm *et al.*, 1994), was used for the relative quantification of promoter activity in *M. smegmatis*. The genetic regions containing promoters of interest were cloned upstream of the promoterless *lacZ* reporter gene in pJEM15. The cloning process was facilitated by a number of unique restriction enzyme cutting sites in the MCS of the vector. Upon ligation of the target sequence to the *lacZ* gene, the construct was transformed into *M. smegmatis* and successful transformants could be selected for by the presence of the *aph* gene encoded by the vector conferring kanamycin resistance. Potential promoter activity within the cloned genetic region could drive the transcription of the reporter gene resulting in β -galactosidase activity which could subsequently be assayed to quantitate the promoter activity of the target sequence.

B.3) p2NIL plasmid map



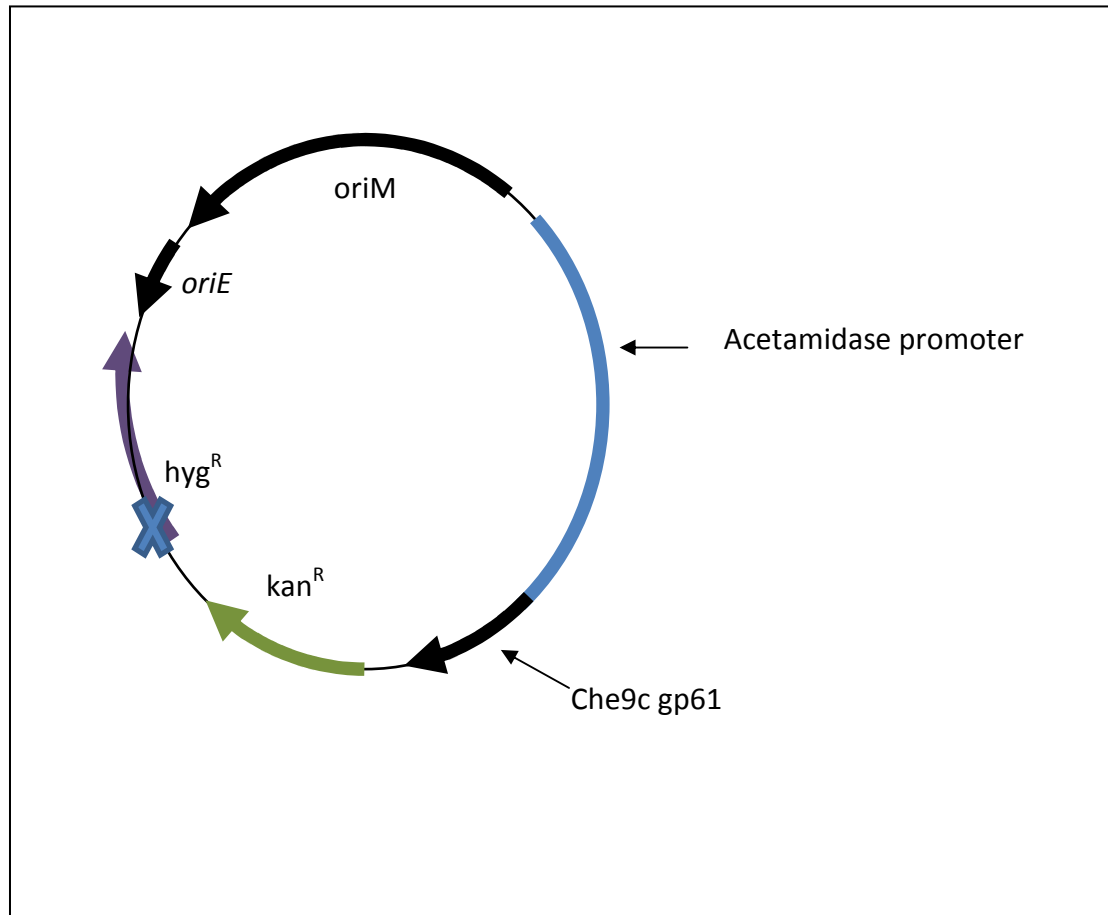
The p2NIL vector (4753bp) is a relatively simple cloning vector and was used for the manipulation of target sequences for the production of gene knockout strains in *M. smegmatis*. The vector is able to replicate within *E. coli* (*oriE*) and has a number of restriction enzyme sites to facilitate cloning of target sequences. p2NIL is a mycobacterial suicide plasmid which is unable to replicate within *M. smegmatis* but is integrated into the genome via homologous recombination. The vector encodes for a unique PacI restriction site which facilitates ligation with pGOAL17 as well as for an *aph* gene which confers kanamycin resistance as a selective marker.

B.4) pGOAL17 plasmid map



The pGOAL17 vector was used in the creation of gene deletion constructs for the production of gene knockouts via homologous recombination in *M. smegmatis*. The pGOAL vector series was designed to enable the creation and storage of marker cassettes (Parish and Stoker, 2000c). pGOAL17 contains a 6.3kb marker cassette flanked by two PacI restriction sites which includes the *lacZ* reporter gene and the *sacB* suicide gene. pGOAL17 also has an *oriE* for replication within *E. coli* and confers ampicillin resistance to select for successful *E. coli* transformants.

B.5) pJV75amber plasmid map



The pJV75amber plasmid was used for the production of single nucleotide polymorphisms (SNP; substitution, deletion or insertion) in *M. smegmatis* (van Kessel and Hatfull, 2008b). The vector encodes for the Che9c mycobacteriophage recombination protein gp61 which is under the control of an acetamide inducible promoter. Upon induction, the production of mycobacteriophage recombineering machinery greatly enhances the rate of homologous recombination. An SNP is generated through recombination with a 50bp synthetic oligonucleotide which is homologous to the target sequence and encodes for the SNP of interest. An additional oligonucleotide is co-transformed with the oligonucleotide of interest which is able to undergo recombination with a mutated *hyg^R* gene encoded by the plasmid. The recombination event restores hygromycin resistance and can thereby be used as a marker for a homologous

recombination event. The vector also encodes for the *aph* gene which confers kanamycin resistance as an additional selection marker. pJV75amber is able to replicate in both *E. coli* and *M. smegmatis* due to the presence of both an *oriE* and *oriM*.

APPENDIX 6C: Equations

Equation C.1) Melting point temperature (T_m) Celsius degrees = $2(A+T) + 4(G+C)$

Equation C.2) Expression ratio = $(E_{\text{target}})^{\Delta C_t \text{ target (Control-Sample)}} / (E_{\text{reference}})^{\Delta C_t \text{ reference (Control-Sample)}}$

E = PCR efficiency

Δ = difference

C_t = threshold cycle

target = gene being investigated

reference = internal reference gene

Control = control condition

Sample = sample condition

Equation C.3) Glutamine synthetase activity (umoles/min/mg protein) =

Total assay volume = $[(Ave \text{ OD}_{Mn^{2+}, Mg^{2+} \text{ or } Mn/Mg^{2+}} / \text{extinction coefficient}) / (\text{protein in assay}) / (\text{time})]$

Equation C.4) β -galactosidase activity (Units) = $[(OD_{420} - (OD_{550} \times 1.65)) \times 200] / \text{mg protein} / \text{min}$

Equation C.5) Amount of target DNA (ng) = $\frac{3(50)(\text{size of insert (bp)})}{\text{Size of vector (bp)}}$

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