

**FUNCTIONAL AND GENETIC STUDY OF *M.*
TUBERCULOSIS GLUTAMINE SYNTHETASE (GS) AND
OTHER FACTORS POSSIBLY INVOLVED IN GS
METABOLISM**



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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work, and has not, to my knowledge, previously in its entirety or in part been submitted at any university for a degree.

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Date

Summary

Sequence analysis showed that the essential *glnA1* gene of *Mycobacterium tuberculosis* might be closely related to an actinomycetes progenitor sequence and that this sequence may have undergone duplication into other non-essential GS encoding genes in some Actinobacteria, notably the mycobacteria. Also, the *M. tuberculosis glnA1* sequence remains conserved throughout the evolution of *M. tuberculosis*. It was also shown that *glnA1* is widely expressed in *M. tuberculosis* infected human pulmonary tissue, where *M. tuberculosis* might be present in altered phenotypes. These results imply that *glnA1* is under selective pressure against evolutionary change.

At transcriptional level it was shown that *M. tuberculosis glnA1* might be expressed from two alternate promoter sites, but that these promoter sites may not be controlled by environmental nitrogen (in the form of ammonium) variation. We also showed that *M. tuberculosis* GS is effectively exported by *M. smegmatis* to the cell wall, but that GS secretion into the exogenous environment does not occur. Also, evidence has been presented which suggested that *M. tuberculosis* GS might be modified at the C-terminus, probably as part of a mechanism that retains GS in the cytosol. This hypothesis was further substantiated where it was demonstrated that two GS isoforms of different size (short isoform in cytosol, longer isoform in cell wall) are present in *M. bovis* BCG. It is unknown what causes this modification, since it couldn't be observed in *M. smegmatis*, but it was suggested that it might be through the action of a *cis*- or *trans*-acting element present in proximity of the *M. tuberculosis glnA1* gene. It was also shown that a cluster of genes found immediately downstream of the *M. tuberculosis glnA1* sequence might be regulated in an operonic fashion under conditions of elevated environmental nitrogen concentrations. Two of the genes (*glnE* and *glnA2*) in this operon arrangement have been previously shown to be involved in nitrogen and glutamine metabolism. The function of the other gene, Rv2223c, is unknown. It was shown that Rv2223c homologs are mostly found in the mycobacteria and that it may encode an exported protease. It was hypothesised that this sequence and its adjacently located progenitor sequence, Rv2224c, might be involved in *M. tuberculosis* GS mediated metabolism. It was showed that over-expression of Rv2223c and Rv2224c may be toxic to *E. coli* and mycobacterial hosts, such as *M. smegmatis*, but that inhibition of transcription of these genes may be fatal to *M. bovis* BCG and *M. tuberculosis* H37Rv. It was also shown that Rv2223c is widely expressed in *M. tuberculosis* infected human tissue, which was comparable to that of *glnA1*.

The results presented in this study shed more light on the distribution and transcriptional regulation of GS in mycobacteria and has identified new genes that might be involved in GS regulation. These results may present new approaches to tuberculosis control and thereby contribute to alleviate the burden of the disease.

Opsomming

Genetiese en proteïen volgorde analise het aangedui dat die *glnA1* (kodeer vir glutamien sintetase (GS), 'n essensiele proteïen) geen van *Mycobacterium tuberculosis* die naaste verwant is aan 'n actinomyces voorloper volgorde wat duplikasie ondergaan het om die ander nie-essensiele GS koderende gene in sommige Actinobakterië te vorm, veral in die mikobakterië. Voorts het die *glnA1* geen geneties gekonserveerd gebly gedurende die evolusie van *M. tuberculosis*. Dit is ook aangetoon dat volop transkripsie van die *glnA1* geen voorkom in die *M. tuberculosis* geïnfecteerde pulmonêre weefsel waar *M. tuberculosis* moontlik mag voorkom.

Op transkripsionele vlak is dit aangetoon dat die *M. tuberculosis glnA1* geen vanaf twee onderskeie promotors uitgedruk mag word, maar dat hierdie twee promotors nie deur variasies in die konsentrasie van stikstof (in die vorm van ammonium) in die omgewing beïnvloed word nie. Daar is ook aangedui dat *M. tuberculosis* GS effektief deur *M. smegmatis* oor die selmembraan na die selwand vervoer word, maar dat daar nie GS sekresie na die ekstracellulêre omgewing geskied nie. Ook is bewyse gevind dat *M. tuberculosis* GS modifikasie aan die C-terminus mag ondergaan wat waarskynlik dien om GS beweging uit die sitosol te verhoed. Hierdie hipotese is versterk deurdat twee isoforms van verskillende grootte (klein in sitosol en groter in die selwand) gevind is in *M. bovis* BCG. Dit modifikasie meganisme is onbekend, maar vind moontlik nie plaas in nie-patogeniese mikobakterië soos *M. smegmatis* nie en mag moontlik deur *cis*- of *trans*-werkende elemente gefasiliteer word. Daar is aangedui dat 'n groepering van vier gene lanksaan die *glnA1* lokus in 'n operoniese meganisme gereguleer mag word onder variërende konsentrasies van stikstof in die omgewing. Dit is bekend dat twee van die gene in die operon (*glnE* en *glnA2*) betrokke in stikstof en glutamien metabolisme is.

Die funksie van die ander twee gene (Rv2223c en Rv2224c) is onbekend. Daar is aangetoon dat volgordes soortgelyk aan Rv2223c beperk is tot die mikobakterië en dat die geen 'n protease, wat moontlik gesekreter word vanuit die sitosol, kodeer. Daar is aangetoon dat die oorproduksie van die Rv2223c en Rv2224c proteïne toksies is vir *E. coli* en mikobakterië,

soos *M. smegmatis*, maar dat transkripsie inhibisie hierdie gene dodelik is vir *M. tuberculosis* H37Rv en *M. bovis* BCG. Daar is ook angedui dat die ekspresie van hierdie gene volop verspreid is in *M. tuberculosis* geïnfecteerde menslike weefsel, soortgelyk aan dié van *glnA1*.

Die resultate vervat in hierdie studie werp meer lig op die verspreiding en transkripsionele regulasie van GS in mikobakteriee en nuwe gene is ontdek wat betrokke by GS regulasie mag wees. Hierdie resultate mag bydra tot nuwe maniere om tuberkulose te bekamp en daardeur die voorkoms van die siekte te beperk.

Presentations

The author has made oral and poster presentations of work contained or related to this thesis on the following occasions:

1. Poster presentations:

1.1.1. Don Hayward; Colin Kenyon; Paul van Helden; Ian Wiid; 'Glutamine synthetase in *M. tuberculosis*: is protein modification coupled to its export mechanism?' Gordon Research Conference on Tuberculosis Drug Development, University of New England, Boston, USA 3-8 July 2005

1.1.2. D Hayward; B Page; I Wiid; P van Helden; D Loots; 'Oxidative Stress in Tuberculosis Disease and Treatment'; Keystone Symposia on Tuberculosis: Integrating Host and Pathogen Biology; Whistler, British Columbia, Canada April 2 - April 7, 2005

1.1.3. Hayward, D.; Wiid, I.; van Helden, P.D; 'The Role Of Mycothiol in Mycobacterial Survival and Adaptation to Oxidative Stress' 1st International Union of Biochemistry and Molecular Biology (IUBMB) and South African Society of Biochemistry and Molecular Biology (SASBMB) Special Meeting on Biochemical and Molecular Basis of Disease, Cape Town, South Africa, November 2001

1.1.4. Hayward, D.; Wiid, I; van Helden, P.D; Kenyon, C. "Unique distribution of glutamine synthetase in the mycobacteria" Keystone Symposia on Tuberculosis: From lab to field trails; Vancouver, British Columbia, Canada March 20 – March 25, 2007

1.1.5. Don Hayward; Paul van Helden; Ian Wiid: 'Is glutamine synthetase posttranslationally modified in *M. tuberculosis*?' Academic Year day, US Medical Faculty, 2004

1.1.6. Don Hayward; Colin Kenyon; Paul van Helden; Ian Wiid; 'Glutamine synthetase in *M. tuberculosis*: is protein modification coupled to its export mechanism?' Academic Year day, US Medical Faculty, 2005

1.2 Oral Presentations:

1.2.1. *M. tuberculosis* Biology of glutamine synthetase; DST GS Consortium at CSIR, Pretoria 2003

1.2.2. Evidence for a unique export system for glutamine synthetase in *M. tuberculosis*, Academic Year day, 2003

1.2.3. *M. tuberculosis* Biology of glutamine synthetase, DST GS Consortium at CSIR, Pretoria 2004

1.2.4. GS secretion mechanism of *M. tuberculosis*, Experimental Biology Group meeting, 2005

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"But thou, O Lord, are a shield for me; my glory, and the lifter of my head. I cried onto the Lord with my voice, and he heard me out of his holy hill." (Psalms 3: 3-4)

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List of Abbreviations Used In the Text

ABC	ATP-binding cassette
ADC	albumin/dextrose/catalase
AIDS	acquired immune-deficiency syndrome
Amp	Ampicillin
asp	asparagine
ATP	adenosine tri-phosphate
BCG	Bacille de Calmette et Guerin
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CF	culture filtrate

CMI	cell mediated immune response
CMN	<i>Corynebacteria, Mycobacteria and Nocardia</i>
3, 4-DCI	3, 4-dichloroisocoumarin
°C	degrees Celsius
DNA	deoxyribonucleic acid
DOTS	direct observed therapy short-course
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMB	ethambutol
ETZ	electron transparent zone
G+C	guanine + cytosine
g/l	grams per liter
GS	glutamine synthetase
GOGAT	glutamate synthetase
HA	hemagglutinin
HCl	hydrochloric acid
his	histidine
HIV	human immunodeficiency virus
hr	hours
HRPO	horseradish peroxidase
Hyg	Hygromycin
IFN- γ	interferon gamma
INH	isoniazid
IS	insertion sequence
ISH	<i>in situ</i> hybridisation
Kan	Kanamycin
kbp	kilobasepairs
kDa	kilodalton
LB	Luria-Bertani (medium)
M	molar
M.	<i>Mycobacterium</i>
Mb	megabases
MDR	multi drug resistance

µg	microgram
MHC	major histocompatibility complex
min	minutes
µl	microliter
µM	micromolar
MOTTs	mycobacteria other than tuberculosis
MTBC	<i>Mycobacterium tuberculosis</i> complex
ng	nanogram
nm	nanometer
OADC	oleic acid/albumin/dextrose/catalase
OD	optical density
ONPG	o-nitrophenyl- β -D-galactoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAS	para-aminosalicylic acid
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	proline-glutamic acid
%	percent
PhoA	bacterial alkaline phosphatase
PMSF	phenylmethanesulfonyl fluoride
PS-ODNs	phosphorothioate antisense oligonucleotides
PZA	pyrazinamide
^R	resistance
RBS	ribosome binding site
RIF	rifampin
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
ser	serine
ST-CF's	short-term culture filtrates
subsp.	subspecies

TAE	tris/acetic acid/EDTA buffer
TCA	trichloroacetic acid
3D	three-dimensional
TIGR	The Institute for Genomic Research
U	units
WCL	whole cell lysate
WHO	World Health Organisation
XDR	extremely drug resistant
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

CHAPTER ONE

INTRODUCTION

"The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!", but "That's funny..."

Isaac Asimov

1.1 PREAMBLE

“if the number of victims which a disease claims is the measure of its significance, then all diseases, particularly the most dreaded infectious disease, must rank far behind tuberculosis. Statistics teach that one seventh of all human beings die of tuberculosis, and that if one considers only productive middle-aged groups, tuberculosis carried away one-third and often more of these...”

These were the words of Robert Koch standing before an audience in Berlin during 1882 pointing out the devastating effect that tuberculosis has on the world's population, which still holds true more than two centuries later (Figure 1.1). Tuberculosis is still one of the leading causes of mortality due to an infectious agent (Ehlers, 1993), despite the availability of anti-tubercular drugs and the widespread use of a live, attenuated vaccine (NIAID, 2001). The disease is easily transmitted through inhalation of contaminated aerosol droplets that carries the pathogen *Mycobacterium tuberculosis* into the lungs of an individual (Nicas, 1995), where it encounters cells of the innate immune system (such as alveolar macrophages). The success of *M. tuberculosis* as a pathogen lies in its ability to evade destruction by, and continue to survive and replicate within these immune cells (Pethe *et al.*, 2004). It has been estimated that over 80% of tuberculosis cases present in the form of chronic pulmonary infection, which leads to symptoms such as coughing, fever, weight loss, tiredness and coughing up blood in the latter stages of disease progression (Hopewell, 1994).

Although it is true that only about 5% - 10% of non-immunosuppressed infected individuals will progress to active disease during their lifetimes, co-infection with the human immunodeficiency virus (HIV) significantly increases the risk to develop active disease to 10% per year (Dye *et al.*, 2002). It has been estimated by the World Health Organisation (WHO) that in the 20 years between the turn of the millennium and the year 2020, an additional 200 million individuals will develop active tuberculosis, while around 35 million infected individuals will have succumbed to the disease (WHO, 2002). But due to the rise in HIV co-infections, the number of mortalities may rise significantly, which would have profound implications on world economies, besides untold human suffering. Therefore, it is as important now as it was in 1882 to understand the agent of tuberculosis in order to effectively combat the disease.

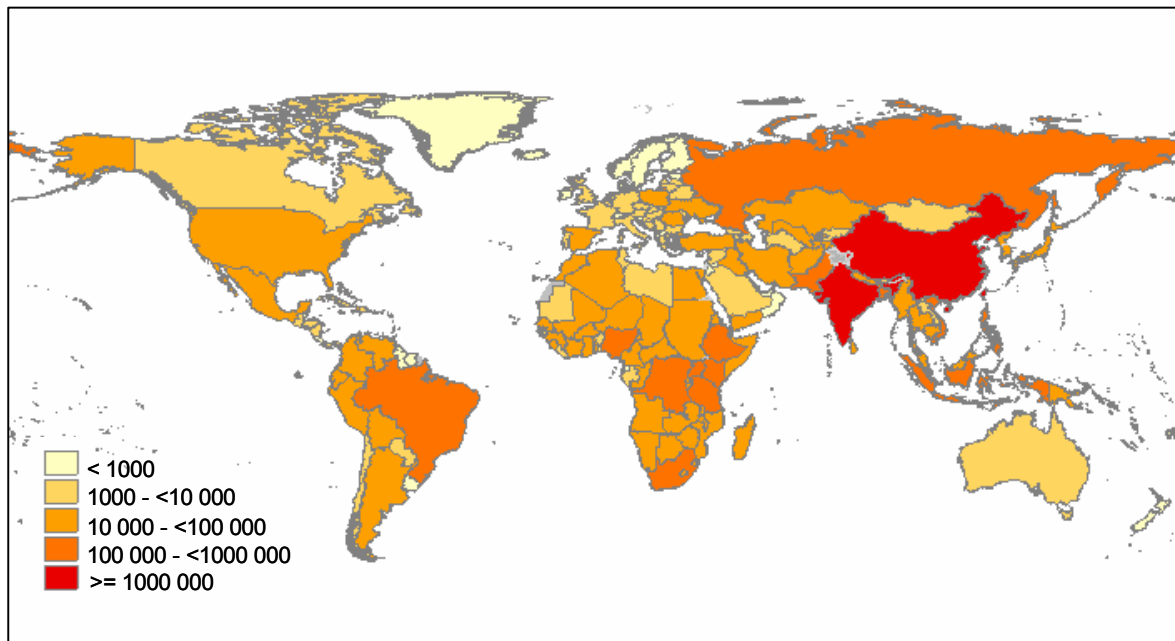


Figure 1.1. Despite modern medical advances, tuberculosis is still devastating countries with low gross national products in Africa, Asia, and Latin America. The WHO estimates that annually 3 million individuals die from tuberculosis while another 8 million are infected, with 95% of cases occurring in developing countries. The map shows the estimated tuberculosis cases per annum (available data as of 21 March 2005; source: WHO Stop TB Department)

1.2 THE MYCOBACTERIA

1.2.1 Ancestral taxonomy: the Actinobacteria

Currently, over forty different species of mycobacteria are recognised. These mycobacteria include pathogens from a variety of hosts other than humans, including fish, birds, rodents and animals, most notably agricultural animals such as dairy cows. The mycobacteria are classified as members of the gram-positive prokaryote phylum *Actinomycetales*. This phylum represents one of the major groups of the Regnum *Bacteria* and its members are all characterised by 61% to 71% guanine + cytosine (G+C) content of their genomes. The Division Actinobacteria include the CMN cluster of mycolic acid containing bacteria (*Mycobacteriaceae*, *Nocardiaceae* and *Corynebacteriaceae*), *Pseudonocardineae*, *Streptosporangineae*, *Streptomycineae*, *Micrococccineae* and some out-groups, such as the *Tropheryma*, *Symbiobacterium* and *Bacillus* spp. (Figure 1.2), which have a diverse range of

morphological appearances, such as coccoid (*Micrococcus*), fragmenting hyphae (*Nocardia*) and branched mycelia (*Streptomyces*). The bacterial families of the Actinobacteria are commonly found in terrestrial and aquatic ecosystems, where they may play an integral role in biodegradation of organic material and humus formation. Some, such as the *Nocardia*, are plant symbionts and function as nitrogen fixators. Some of the Actinobacteria, other than some members of the mycobacterial lineage, are also known to cause human infection, such as *Corynebacterium diphtheria* (diphtheria) and *Propionibacterium acnes* (acne).

1.2.2 Genus *Mycobacterium*

The genus *Mycobacterium* (*Myces*, Gr. meaning 'a fungus'; *bakterion*, Gr. 'a small rod'; *Mycobacterium* 'a fungus rodlet') was first introduced by Lehmann and Neumann (1896) to describe the infectious agents of leprosy and tuberculosis, based on their morphological and staining characteristics. These infectious agents have previously been known as *Bacterium leprae* and *B. tuberculosis* after Robert Koch first observed them in 1882. Today, the genus *Mycobacterium* includes a host of species defined as non-motile aerobic rod-shaped prokaryotic Actinobacteria that form colonies or bio-films consisting of straight or slightly curved rods that may in some cases branch. The *Mycobacteria* are further considered as Gram-positive bacilli with acid-fast cell walls, because of the presence of lipid components unique to the mycobacteria, namely the mycolic acids. According to modern taxonomy that uses defined protein families for phylogenetic tree constructions, the *Streptomyces* appears to be the out-group from which the mycobacteria and *Corynebacteria* have evolved (Bern and Goldberg, 2005; Gao and Gupta, 2005).

In general, the mycobacteria can be divided into two major groups, i.e. slow and fast growers (Figure 1.3). In culture, slow growing mycobacteria usually have replication times of 13 to 20 hours, while the fast growing mycobacteria have replication times ranging from 2 to 5 hours (David, 1973). It is interesting to note that most of the mycobacteria that cause disease in humans are of the slow growing species, and this is probably the reason for the chronic nature of mycobacterial infection and disease.

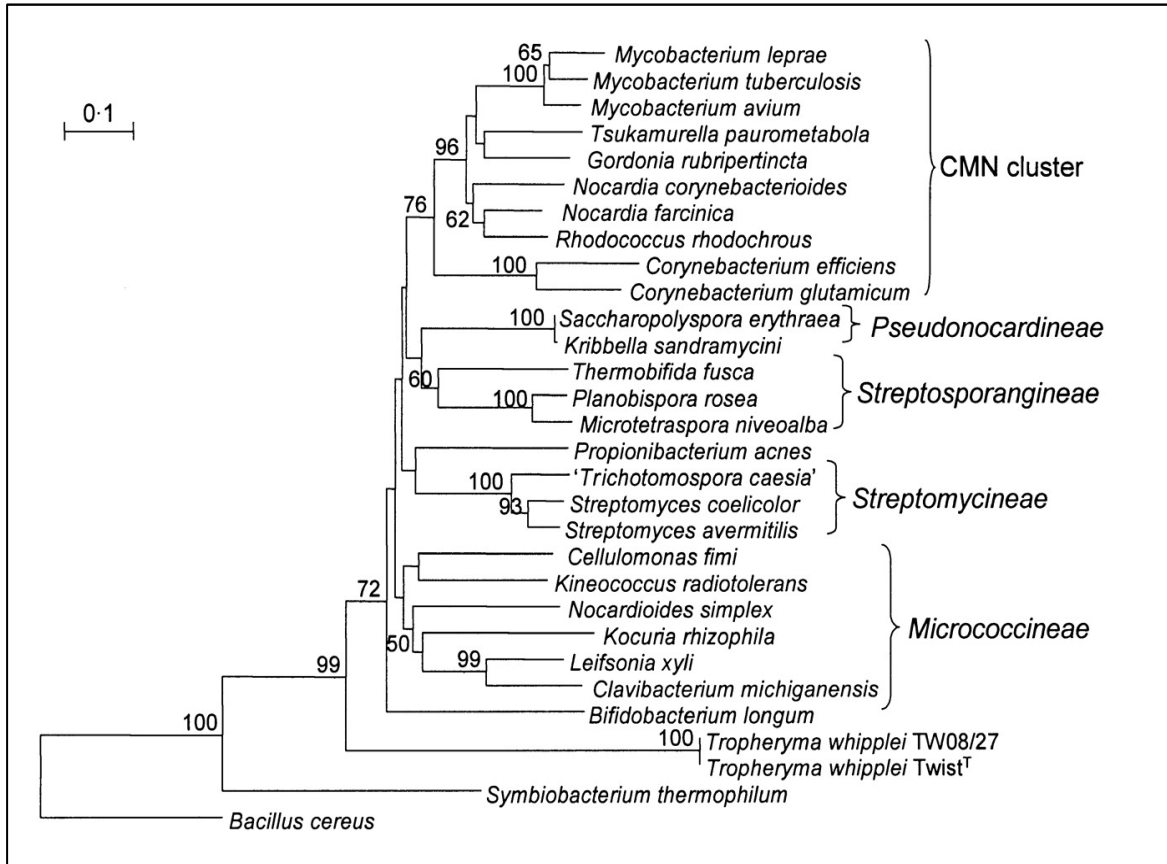


Figure 1.2. Bacterial phylogeny of the evolutionary relation between the various genera of the Division Actinobacteria (modified, Gao and Gupta, 2005).

Mycobacteria usually form tight, compact colonies with most of the organisms coagulating instead of forming a plaque on solid media, which may be due to the lipid content and hydrophobic nature of their cell wall constituents (Section 1.4). Mycobacteria are prototrophic and have minimal nutritional requirements (Barclay and Wheeler, 1989). It can survive on minimal media with basic carbon (such as glucose, glycerol or acetate) and nitrogen (ammonium or ammonium- containing amino acids such as asparagines) sources. Growth could be stimulated by addition of lipids and fatty acids, and a previous study suggested that lipids and fatty acids might be essential for mycobacterial survival in the stationary growth phase (McKinney *et al.*, 2000).

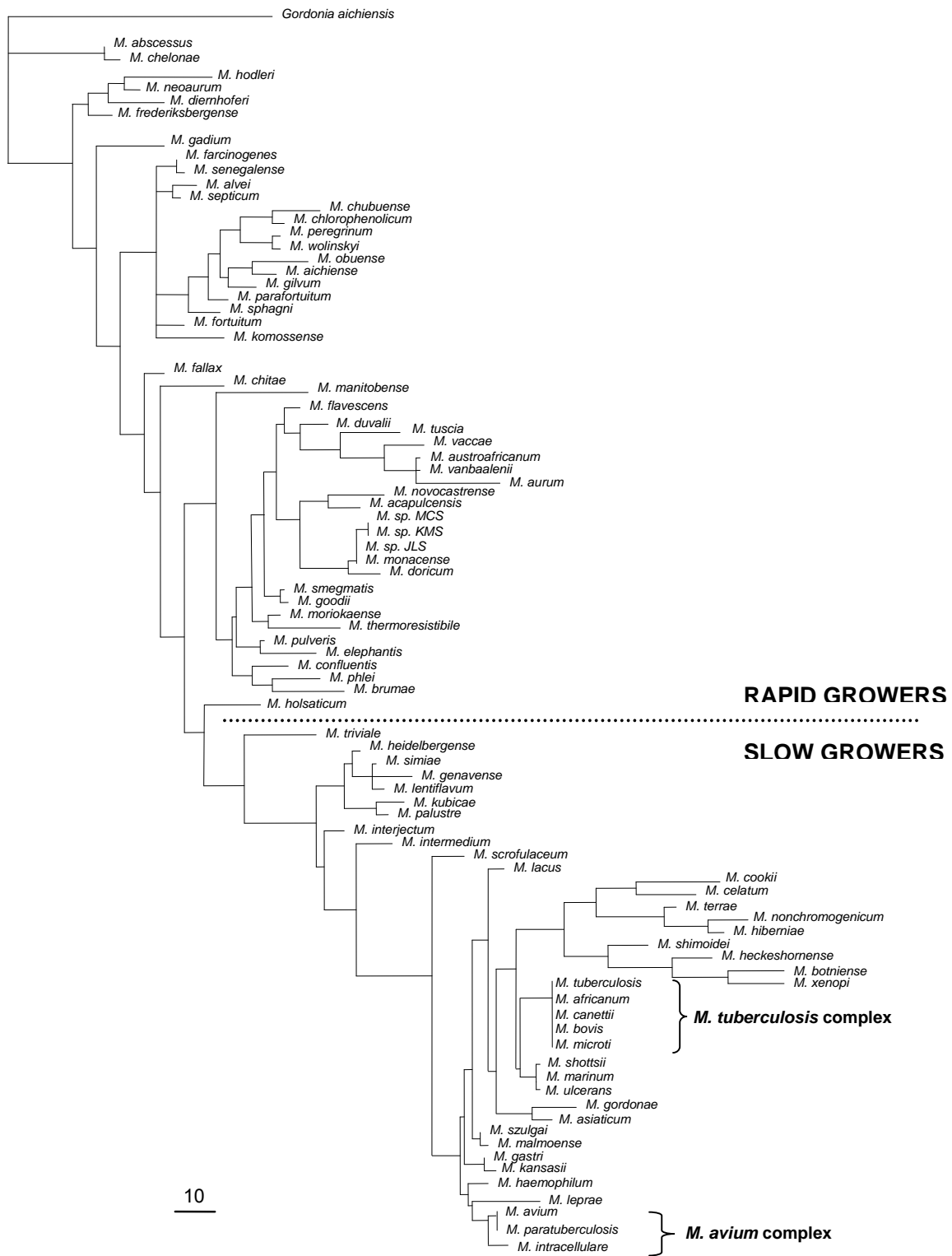


Figure 1.3. The mycobacterial phylogeny showing the evolutionary relation between the slow (usually pathogenic) and fast growing mycobacteria (Adapted with kind permission N. Gey van Pitteus).

Besides the etiological agents of human tuberculosis (*M. tuberculosis*) and leprosy (*M. leprae*), other usually harmless environmental saprophytic mycobacteria are emerging as infectious agents (Table 1.1). A growing body of evidence shows that these MOTTs (“mycobacteria other than tubercle bacilli”) may be involved in some opportunistic pulmonary and non-pulmonary infections especially in immunocompromised individuals (Horsburgh, Jr., 1996; Dobos *et al.*, 1999; Buijtsels *et al.*, 2005; Lai *et al.*, 2005b; Koh *et al.*, 2006). This may in part be due to the lipid rich mycobacterial cell wall (Section 1.4) and certain cell surface moieties (Katoch, 2004) affording a protective barrier to the bacteria, but mainly because of impaired immune responses of the HIV-infected individual, which would otherwise be able to contain the infection (von Reyn *et al.*, 1994). The infection and pathogenesis mechanisms of MOTTs are as yet not clear, although MOTTs have been reported to cause either localised or disseminated (i.e. not localised to a specific tissue or organ) disease depending on the extent of the immune deficit of the individual (Wayne and Sramrek, 1992).

1.3 MYCOBACTERIUM TUBERCULOSIS: A BRIEF HISTORY

Tuberculosis is one of the leading causes of mortality in the world despite the availability of anti-tubercular drugs and the widespread use of the live, attenuated *M. bovis* BCG vaccine. Although > 90% of individuals exposed to tuberculosis will not develop active disease, tuberculosis still accounts for more than two million deaths and eight million new cases globally each year (WHO, 2002). However, tuberculosis, caused by the *Mycobacterium tuberculosis* bacillus, is a disease of antiquity and may have been the cause of human suffering from the earliest times. Tuberculosis may have been well-known to the ancient Babylonians, judging from cuneiform inscriptions describing symptoms of the disease. These inscriptions are of the earliest known human records of tuberculosis. The oldest pathological evidence was observed in the skeleton of a 5400-year-old pre-dynastic Egyptian showing the typical kyphotic spinal deformity associated with mycobacterial infection (Crubezy *et al.*, 1998). A DNA fragment was retrievable from the skeleton and DNA sequencing showed that it was of mycobacterial origin, however, due to the antiquity of the sample it was not possible to distinguish between *M. tuberculosis* and *M. bovis*.

Table 1.1. Summary of MOTT infections in mostly aged or immunocompromised individuals.

Mycobacterium	Type of infection	Reference
<i>M. avium</i>	pulmonary	(Kuwabara <i>et al.</i> , 2004;Kirschner <i>et al.</i> , 1992)
<i>subsp. paratuberculosis</i>	Crohn's disease (regional ileitis)	(Chacon <i>et al.</i> , 2004)
<i>M. intracellulare</i>	pulmonary	(Meissner and Falkinham, III, 1986; Kobashi <i>et al.</i> , 2005; Kirschner <i>et al.</i> , 1992)
<i>M. kansasii</i>	pulmonary	(Zhang <i>et al.</i> , 2004)
<i>M. scrofulaceum</i>	pulmonary; disseminated	(Rodriguez <i>et al.</i> , 2004; Hsueh <i>et al.</i> , 1996)
<i>M. simiae</i>	pulmonary; disseminated	(Samra <i>et al.</i> , 2005;Yoshimura <i>et al.</i> , 2005; Marrache <i>et al.</i> , 2004)
<i>M. habana</i>	disseminated; extra pulmonary	(Chaturvedi and Gupta, 2002; Valdivia, 1973)
<i>M. interjectum</i>	lymphadenitis	(Springer <i>et al.</i> , 1993;De Baere <i>et al.</i> , 2001)
<i>M. xenopi</i>	pulmonary	(Gazzola <i>et al.</i> , 2004)
<i>M. heckeshornense</i>	pulmonary	(van Hest <i>et al.</i> , 2004;Richter <i>et al.</i> , 2001;Roth <i>et al.</i> , 2000)
<i>M. szulgai</i>	arthritis; pulmonary; skin ulcers	(Hamada <i>et al.</i> , 2005;Hakawi and Alrajhi, 2005;Tappe <i>et al.</i> , 2004)
<i>M. fortuitum</i>	cutaneous; pulmonary	(Palwade <i>et al.</i> , 2006;Inoue <i>et al.</i> , 2004)
<i>M. immunogenum</i>	ulceritis, hypersensitivity pneumonitis	(Loots <i>et al.</i> , 2005;Wallace, Jr. <i>et al.</i> , 2002)
<i>M. chelonae</i>	corneal ulceritis	(Bottone and Cho, 2005)
<i>M. marinum</i>	skin ulcers; pulmonary	(Contios <i>et al.</i> , 2005;Lai <i>et al.</i> , 2005)
<i>M. genavense</i>	disseminated	(Monill <i>et al.</i> , 2001;Hillebrand-Haverkort <i>et al.</i> , 1999)
<i>M. bohemicum</i>	cervical lymphadenitis	(Tortoli <i>et al.</i> , 2000)
<i>M. haemophilum</i>	cutaneous	(Tan <i>et al.</i> , 2004)
<i>M. celatum</i>	pulmonary	(Piersimoni <i>et al.</i> , 2003;Shahdad <i>et al.</i> , 2005)
<i>M. conspicuum</i>	disseminated	(Springer <i>et al.</i> , 1995)
<i>M. malmoense</i>	pulmonary	(Handrick <i>et al.</i> , 2005)
<i>M. ulcerans</i>	Buruli ulcer disease	(Bretzel <i>et al.</i> , 2005;Siegmond <i>et al.</i> , 2005)
<i>M. smegmatis</i>	male genitalia	(Wallace, Jr. <i>et al.</i> , 1988)
<i>M. wolinskyi</i>	post-surgical wound infections	(Brown <i>et al.</i> , 1999)
<i>M. goodii</i>	post-surgical wound infections	(Brown <i>et al.</i> , 1999;Spencer, Teske, and Bernstein, 2005)
<i>M. thermoresistibile</i>	cutaneous	(Cummings <i>et al.</i> , 2000)
<i>M. neoaurum</i>	urinary tract; possible meningoencephalitis	(Heckman <i>et al.</i> , 2004;Zanetti <i>et al.</i> , 2001)
<i>M. vaccae</i>	cutaneous; pulmonary	(Hachem <i>et al.</i> , 1996)
<i>M. palustre</i>	cervical lymphadenitis	(Torkko <i>et al.</i> , 2002)
<i>M. elephantis</i>	pulmonary	(Potters <i>et al.</i> , 2003)
<i>M. septicum</i>	pulmonary	(Adekambi and Drancourt, 2005)
<i>M. nonchromogenicum</i>	pulmonary; chronic tenosynovitis	(Sawai <i>et al.</i> , 2006)
<i>M. lentiflavum</i>	disseminated; pulmonary	(Suffys <i>et al.</i> , 2006;Niobe <i>et al.</i> , 2001;Tortoli <i>et al.</i> , 2002)
<i>M. abscessus</i>	pulmonary	(Chung <i>et al.</i> , 2005)
<i>M. tilburgii</i>	pulmonary	(Kolditz <i>et al.</i> , 2005)
<i>M. saskatchewanense</i>	bronchiectasis	(Turenne <i>et al.</i> , 2004)

Other evidence suggests that *M. tuberculosis* infection might actually have been common in lower class as well as upper-class ancient Egyptians, as *M. tuberculosis* DNA could be detected in the remains of Egyptian mummies dating back to as early as 3000 B.C (Zink *et al.*, 2001; Zink *et al.*, 2003). These mummies were from different regions of Egypt, suggesting that the infection was not only common, but also widespread. Evidence also suggest that tuberculosis spread by means of human hosts to other continents, since lesions

were found in Iron-age skeletons from Britain (Mays *et al.*, 2002) and South-Eastern Asia (Tayles and Buckley, 2004).

The first clear reference to tuberculosis disease is attributed to Hippocrates in 460 B.C., when he identified "phthisis" (a Greek word meaning consumption) as the most prevalent disease of the times. Aristotle, a contemporary of Hippocrates, noted that the Greeks generally believed phthisis to be contagious. Hippocrates observed that nearly everyone suffering from phthisis was killed by the disease and even went so far as to warn other physicians not to visit patients in late stages of the disease due to the danger of contracting it themselves. Interestingly, the Hippocratic School believed in the benefits of a change of residence, a thought later endorsed by Aretaeus (50 BC), who believed in the efficacy of sea voyages and country air. This manner of treatment was later refined by Galen (A.D 131 – 201) who defined phthisis as an ulceration that could be cured by drying the secretion, a process that could be encouraged through living in the high land of Phrygia (Western Part of Asia Minor in the region of Turkey).

Although the symptoms described by Hippocrates in 460 B.C were not elaborated on for many centuries, the pathology of phthisis was first described by the Leyden professor of medicine, Dr. Sylvius (Franciscus de la Boe) in his 1679 "*Opera Medica*". Therein were described the tubercles, abscesses and cavities associated with phthisis as a characteristic change of the lungs and other parts of the body in patients suffering from phthisis. Doctor Sylvius regarded the tubercles as enlarged lymph glands of the lung and believed in the contagiousness of the disease. The English physician Richard Morton (1637-1698) brought the tubercle as the cause of the disease to the foreground in his landmark book (1689) "*Phthisiologia, seu exercitationes de Phthisi tribus libris comprehensae* " (Trail, 1970). This book was widely known to English physicians of the time, since the Bills of Mortality for London in the 1700s showed that of the 19433 annual deaths from causes other than suicide, phthisis accounted for 2819 (18.5%) making it a very prominent disease. Notably, Morton believed in a hereditary predisposition to phthisis, an idea that would survive even after the causative agent of phthisis was discovered. Most noteworthy is the work by Gaspard Laurent Bayle (1774-1816) and Réne Theophile Hyacinthe Laennaec (1781-1826), who performed hundreds of post mortems on patients that complained of pain in the chest and night time sweats. Bayle and Laennaec defined the present day knowledge of the pathology (tubercle formation in the lungs), signs and symptoms of tuberculosis disease. Perhaps not surprisingly, Laennaec himself contracted phthisis and died in 1826, after

having undergone panacea (bleeding), in that time a common treatment for the disease. But his and Bayles' work showed that nodules in the lungs (termed "tubercles") were common to all patients succumbing to phthisis, hence the term "tuberculosis" coined by Johann Lukas Schönlein in 1839.

1.3.1 The identification of *Mycobacterium tuberculosis*

By the 1870s, the Dutch scientist Antoni van Leeuwenhoek (1623 – 1723) had already described the existence of microbes, which the pioneering work of Louis Pasteur (1822 – 1895) incorporated into the "germ theory of disease", which proposed that micro organisms were the cause of many diseases. Initially, many still adhered to the philosophy that germs were too ubiquitous to be the cause of disease, and that host factors such as heredity were the major cause of disease contractibility and progression. The pathways of infection by *M. tuberculosis* were inferred from the many intravenous (leading to miliary tuberculosis) and inhalation infection experiments. The search for the specific infectious agent began, notably through the experimentation of Edwin Klebs (1834 – 1913). In 1877 Klebs saw transference of the germ (via passaging on egg albumin media) and identified *Monas tuberculosum*, which he wrongly assumed to be the etiological agent of tuberculosis. Other researchers included Emanuel Aufrecht (in 1881) and Paul Clemens von Baumgarten (10 days after Koch, 1882), who independently described bacilli located in the tubercles, but owing to lack of culturing and staining methods, failed to identify these bacilli as the etiological agents.

The breakthrough in identifying *Mycobacterium tuberculosis* as the etiological agent of tuberculosis came in 1882 by Robert Koch (1843 - 1910) after he discovered a special staining technique that allowed him to see the (mycobacterial) bacillus (Koch, 1882). Koch's staining technique was later modified by the German doctors Franz Ziehl and Friedrich Neelsen, which became known as the acid-fast stain or Ziehl-Neelsen stain, which is still used today. Koch also observed the reaction of the skin to inoculation of *M. tuberculosis*, when the host has been previously exposed to the pathogen. This observation led to the development of the Mantoux skin test by Charles Mantoux in 1907, which is still in use today.

1.4 THE MYCOBACTERIAL CELL WALL

The success of the mycobacteria as a pathogen is probably due to the complex cell envelope or cell wall, demonstrated by the ability of dead mycobacteria to persist in macrophages for two weeks after phagocytosis (Clemens, 1997). Besides being the cellular exoskeleton that offers defence against mechanical and osmotic lysis, the cell envelope is also the interphase of physical interaction between the mycobacterial bacillus and its host-cell environment, protecting the bacillus from the harsh environment created by the human immune system. Also, it may serve as a barrier that impedes the diffusion of macromolecules, such as anti-mycobacterial drugs, into the bacillus (Raynaud *et al.*, 1999). The mycobacterial cell envelope plays host to a wide variety of molecules that serve a multitude of functions, most of which are critical to the viability of the bacillus. It is imperative to understand the nature and function of this complex envelope structure to enable understanding of the function of current anti-tuberculosis drugs and allow rational drug design.

The *M. tuberculosis* cell envelope can be divided into three regions, being the electron transparent capsule, the underlying electron dense cell wall core and cell membrane (Figure 1.4) (Daffe and Draper, 1998). The capsular region and cell wall core are probably also involved in *M. tuberculosis* virulence and may contain cell wall constituents unique to human-disease causing mycobacteria (which will be discussed in subsequent sections) which may be absent from non-virulent mycobacteria, such as *M. smegmatis*.

1.4.1 The capsule

The ability of pathogenic mycobacteria to grow in host cells has long been linked to a surrounding electron transparent zone (ETZ), or mycobacterial capsule, since this structure may be the site of physical interaction of the pathogen with the host-cell environment (Draper and Rees, 1970). The existence of the ETZ was first described in 1959 as a non-stainable membrane-enclosed space (“capsular space”) surrounding *M. lepraemurium* bacilli residing in mouse spleen cells (Chapman *et al.*, 1959). In that study, the authors speculated that the ETZ might be of host-cell origin due to a lack of an organised structure of the capsular space and since the enclosing membrane may become more distantly located from the mycobacterial bacillus. In 1961 it was concluded that the chemical and physical

characteristics of the ETZ was of mycobacterial rather than host origin (Hanks, 1961) and results presented in other studies supported that conclusion (Frehel *et al.*, 1986). Interestingly, the presence of an ETZ appears to be a characteristic of pathogenic mycobacteria, such as *M. tuberculosis* and *M. avium*, while it appears to be absent in non-pathogens such as *M. smegmatis* and *M. aurum* (Frehel *et al.*, 1988).

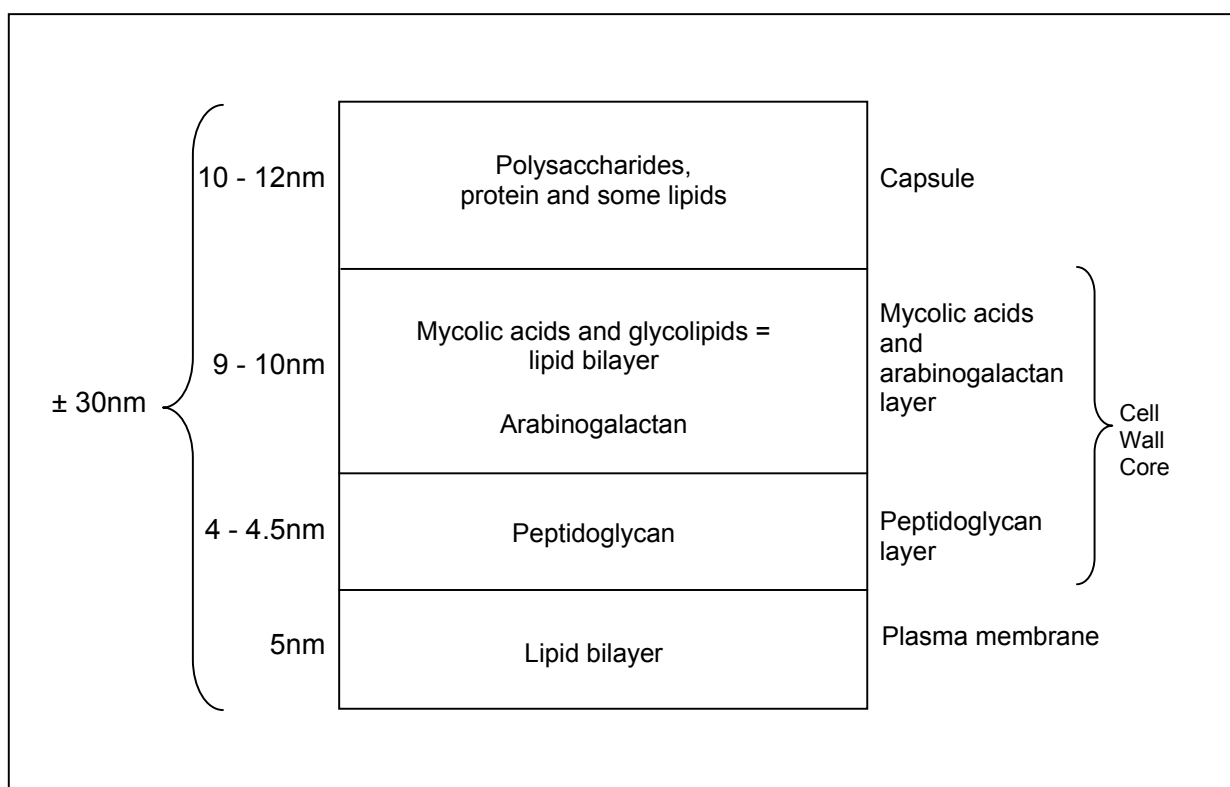


Figure 1.4. Schematic representation of the mycobacterial cell envelope showing the main constituents and predicted thickness of each layer.

In more recent studies it was shown that the capsular region of *M. tuberculosis* consists of a mixture of non-covalently bound polysaccharides, proteins and some lipids of which some may be involved in *M. tuberculosis* pathogenicity (Rastogi and Hellio, 1990; Ortalo-Magne *et al.*, 1995; Lemassu *et al.*, 1996). The polysaccharides consist mainly of glucan, arabinomannan (AM) and mannan (Daffe and Draper, 1998). The glucan is composed of five to six repeating 4- α -D-glycosyl residues with a mono- or di-glucosyl substitution on the sixth residue (Ortalo-Magne *et al.*, 1995). It has also been observed that, although similar in structure, capsular glucan may be of smaller molecular size than the *M. tuberculosis* cytosol-

associated glucan. Arabinomannan (D-arabino-D-mannan) is composed of mannan chains linked with short arabinan side chains (Misaki *et al.*, 1977). The arabinan termini of the arabinomannan were found to be extensively capped with mannosyl residues (Lemassu and Daffe, 1994; Ortalo-Magne *et al.*, 1996), although the amount of mannosyl capping does not appear to play a role in selective phagocytosis of virulent mycobacteria (Ortalo-Magne *et al.*, 1996) as previously thought.

It has been determined that most of the ~205 proteins found in the early growth-phase culture filtrate of *M. tuberculosis* (Sonnenberg and Belisle, 1997) may come from the outermost layer of the *M. tuberculosis* cell envelope (Ortalo-Magne *et al.*, 1995; Lemassu *et al.*, 1996). These proteins may be a complex mixture of actively secreted and cell envelope-associated or cytoplasmic polypeptides (Wiker *et al.*, 1991; Sonnenberg and Belisle, 1997; Navarre and Schneewind, 1999; Rosenkrands *et al.*, 2000; Mattow *et al.*, 2003). Most of the actively secreted proteins are identified by the presence of a *sec*-translocase leader or signal peptides in their amino acid sequence. However, several proteins were observed in the culture filtrate that apparently lacked any translocase recognition sequences and would therefore be expected to be restricted to the *M. tuberculosis* cytoplasm. Examples of exported proteins lacking apparent translocase recognition sequences are superoxide dismutase (Zhang *et al.*, 1991), alanine dehydrogenase (Andersen *et al.*, 1992), glutamine synthetase (Harth *et al.*, 1994) and ESAT-6 (Sorensen *et al.*, 1995). The observation of such proteins in the exogenous environment of *M. tuberculosis* has led to speculation that *M. tuberculosis* might employ other mechanisms of protein export than the known protein export systems. Interestingly, many of the exogenous *M. tuberculosis* proteins (including the examples named above) could be functionally linked to virulence and the ability of *M. tuberculosis* to survive the host immune onslaught (Daffe and Etienne, 1999).

1.4.2 The cell wall core

The structural definition of the cell wall core has been ongoing since the early 1960s and it can now be described as a mycolyl arabinogalactan-peptidoglycan complex (mAGP). The *M. tuberculosis* cell wall is composed of an soluble segment composed of long and short free fatty acids (lipids), cell wall proteins, phosphatidylinositol mannosides (PIMs), phthiocerol containing lipids, lipomannan (LM) and lipoarabinomannan (LAM) all intercalated into the lipid environment of the mycolic acids. The mycolic acids, together with arabinogalactan

(AG) and peptidoglycan (PG) form the “lower” insoluble segment of the cell wall core (Figure 1.5) (Daffe and Draper, 1998).

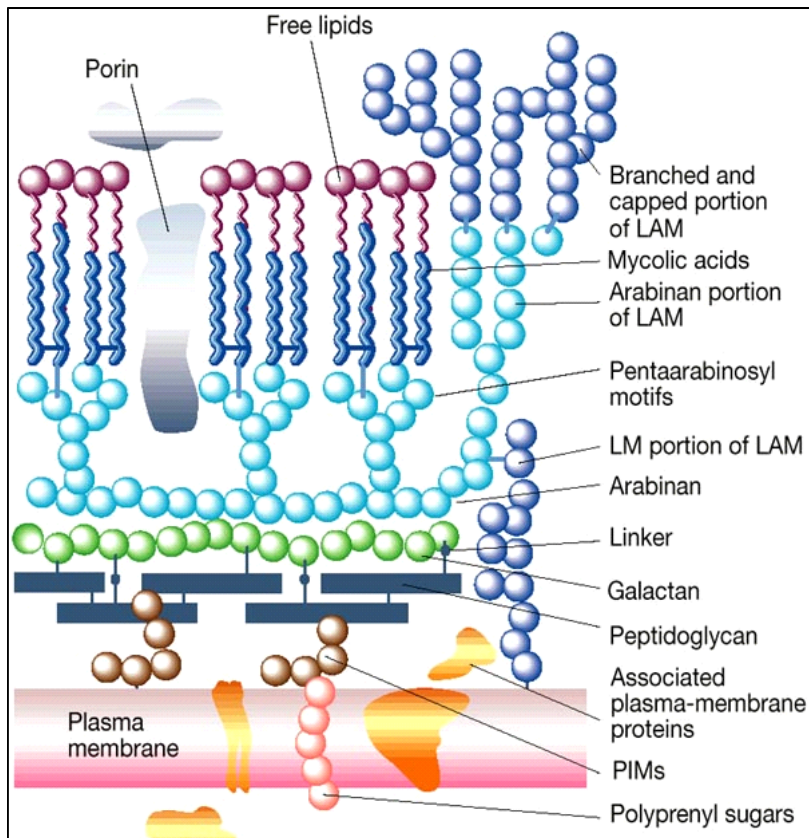


Figure 1.5. The mycobacterial cell wall is a complex structure of various long- and short chain fatty acids that lends many unique features to the mycobacteria. This cell wall is relatively impermeable to antibiotics. See text for definition of abbreviations. (Picture from T. Mahmud, Oregon University)

The insoluble fragment of the cell wall core may be essential for the viability of *M. tuberculosis*, while the compounds contained in the soluble fraction may be involved as effectors in the tuberculosis disease progression. The PG forms a vast matrix of alternating N-acetylglucosamine (GlcNAc) units and modified muramic acid residues (N-acylated with glycolic acid, MurNGly) over the *M. tuberculosis* cell membrane, a double lipid membrane similar to that found in most cells (Adam *et al.*, 1969; Petit *et al.*, 1969). The MurNGly residues may be specific to mycobacterial cell walls and serve as the site of covalent attachment of PG to the galactan region of AG via a special disaccharide-phosphate linkage [L-Rhap-(1→3)-D-GlcNAc-(1→P)] (McNeil *et al.*, 1990). The AG is a major cell wall polysaccharide of mycobacteria (Besra *et al.*, 1995). The most recent structural evidence suggests that AG is composed of a linear stretch of non-branched galactanfuranose (~ 23 units) with non-branched arabinan chains attached close to the reducing end of the galactan chain (Besra *et al.*, 1995). Furthermore, the non-reducing termini of the arabinan chains may constitute a branched penta-arabinofuranosyl structural motif at the terminal end where the mycolic acids may be located in clusters of four.

The mycolic acids are large α -alkyl branched and β -hydroxylated fatty acids. Mycolic acids can be divided into the non-oxygenated aliphatic mycolate, and oxygenated methoxy- and ketomycolate structural subclasses and it has been demonstrated that the absence of the oxygenated mycolic acids leads to *M. tuberculosis* cell envelope alteration and increased cell wall permeability, resulting in *M. tuberculosis* attenuation in mice (Dubnau *et al.*, 2000).

Delipidated and proteolytically digested cell walls of *M. tuberculosis* and *M. bovis* (also BCG) contain non-peptidoglycan poly-L-glutamic acid (Wietzerbin *et al.*, 1975a; Phiet *et al.*, 1976; Hirschfield *et al.*, 1990). This polymer may account for as much as 10% of the dry weight of the *M. tuberculosis* cell wall (Hirschfield *et al.*, 1990) but is completely absent from the cell walls of avirulent mycobacteria, such as *M. smegmatis* and *M. phlei* (Wietzerbin *et al.*, 1975b). The study by Wietzerbin *et al.* (1975) also showed that the poly-L-glutamic acid content of the *M. bovis* BCG strains varies between 0.6 and 2%, except in the *M. bovis* BCG Montreal sub-strain where it is completely absent. The exact nature of this polymer is unclear, but the study by Wietzerbin *et al.* (1975) showed that the α -carboxyl group of the L-glutamic acid residues could be involved in most of the peptide links and that the residues of the complete native polymer may be amidated. The function of poly-L-glutamic acid and

whether the presence of this polymer could be correlated with mycobacterial virulence is unknown.

The soluble region of the cell wall core consists of several lipid compounds that may play a role in *M. tuberculosis* virulence. Trehalose dimycolate (TDM), or cord factor, a mycolic acid-glucose compound (Noll and Bloch, 1955) has been shown by various studies using mice and rabbits to induce granuloma formation and inflammation of lung, liver and spleen tissues (Yamagami *et al.*, 2001; Saita *et al.*, 2000). Evidence has also been presented which suggests that TDM production elicits an effect on *M. tuberculosis* morphology (Hunter *et al.*, 2005). In that study, the virulence of *M. tuberculosis* isolates could be correlated with TDM production, being either rough mycobacterial colonies (high TDM production, virulent *M. tuberculosis*) or smooth colonies (low TDM production, avirulent *M. tuberculosis*). This correlation between virulence and colony morphology has also been demonstrated in other infectious mycobacteria (Howard *et al.*, 2006).

The major lipid component in the soluble segment of the *M. tuberculosis* cell wall core was discovered in 1936, and was referred to as a “unsaponifiable wax” (Stodola and Anderson, 1936). This lipid is phthiocerol dimycocerosate (DIM, C₃₆H₇₂O₃), a highly non-polar lipid due to the methyl or methylene nature of the C-35 backbone, and the two hydroxy groups that are esterified with two mycocerosic acids (composed of 28 methyl or methylene groups). Phthiocerol dimycocerosate has been implicated in the virulence of *M. tuberculosis* due to the two independent observations that *M. tuberculosis* H37Rv transposon mutants, which are unable to synthesise DIM were attenuated for growth in the mouse lung (Camacho *et al.*, 2001; Sirakova *et al.*, 2003). The attenuation of these DIM-less *M. tuberculosis* mutants were ascribed to an increased permeability of the cell wall and a subsequent increase in susceptibility to the reactive oxygen and nitrogen response elicited by the host immune response.

1.5 TUBERCULOSIS CHEMOTHERAPY: THEN AND NOW

In the previous centuries before Koch identified *M. tuberculosis* as the etiological agent of consumption or phthisis, as tuberculosis disease was then known, treatment usually resulted in tales of severe tragedy and/ or disappointment. In many cases, brutal treatment methods such as purging through bleeding (panacea) might have actually increased mortality. Other

more benign methods included herbology and dietary interventions or the well-known “benefits” of rest and climatic prescriptions that aids the body’s defences against *M. tuberculosis* infection. These measures were implemented in sanatoria, especially in Europe and the USA, which also had the function of removing and isolating the sick individuals from the general population.

During and around the 1840s, it was generally accepted that phthisis was heritable and treatment was mainly aimed at diminishing discomfort or to temporarily prolong life in order to afford the body time to overcome the disease by itself, since this can occur in some patients. During these times, the use of cod-liver oil as treatment was widely applied with a high rate of success, as symptoms improved in 63% of patients treated this way. In 18% of the patients, the progression of the disease was arrested, while 19% of patients showed non-responsive disease progression while on cod-liver oil treatment. Another great benefit of this treatment was that it lead to a weight increase in most patients. Other remedies favoured in the treatment of phthisis was potassium arsenate (Fowlers’ solution, $\text{KH}(\text{AsO}_2)_2$), which was in some cases (especially by phthisiologists such as William Osler, 1845 – 1919) promoted over the use of cod-liver oil. Indeed, in the 1906 play “A Doctors Dilemma” by the Irish-British Nobel Laureate George Bernard Shaw, tuberculosis treatment in Britain was described as “a huge commercial system of quackery and poison”.

The hypothesis of tuberculosis heritability survived even after Koch’s observation of the mycobacterial bacillus, which paved the way for pathogen directed treatment. Koch himself was the first person to have investigated medication that had a growth inhibitory effect on *M. tuberculosis*, since he believed that growth inhibition alone would be effective in the treatment of the disease. Therefore Koch tested a wide variety of compounds *in vitro*, which included β -naphthylamine, xylidine, fuchsin, gentian violet, methylene blue, quinoline violet, aniline yellow, auramine, mercury vapour and gold cyanide. Compounds of gold cyanide became the standard regimen for tuberculosis treatment before the advent of streptomycin in the 1940s. Interestingly, Koch observed that these compounds would inhibit *M. tuberculosis* growth *in vitro*, but not *in vivo*. The reason became clear through the studies of Paul Ehrlich (1854 – 1915), who, incidentally, coined the term chemotherapy to describe the antimicrobial effect of drugs. Ehrlich observed that gold cyanide was less effective *in vivo* because of the higher affinity of gold cyanide for host enzymes than for *M. tuberculosis*.

However, in the years preceding Ehrlich's observations and despite Koch's findings in the 1890s, compounds of gold cyanide were widely promoted and became the most used chemotherapeutic drugs for the treatment of tuberculosis. In 1912 gold cyanide was retested in rabbits and given in various dosages to human subjects. Sub-mortal treatment with gold cyanide resulted in some successes, which led to the question whether it was the gold or the cyanide exhibiting the antimicrobial effect. Adolf Feldt, a German pharmacologist, decided that the active ingredient was gold, since gold cyanide was bactericidal in greater dilutions than that elicited by potassium cyanide. Interestingly, Feldt also observed the appearance of *M. tuberculosis* strains that were resistant to gold cyanide. These strains were recovered from *M. tuberculosis* infected rabbits that were treated with gold cyanide for only short periods. Feldt will also be remembered in history as the creator of gold cyanide compounds such as krysolgan and sanocrysin (Figure 1.6), of which the latter became the first line antibiotic against *M. tuberculosis* infection in the years preceding streptomycin. The side effects of these compounds were most unpleasant, and included menorrhagia and spontaneous abortion (krysolgan); anorexia, diarrhoea and fever (sanocrysin).

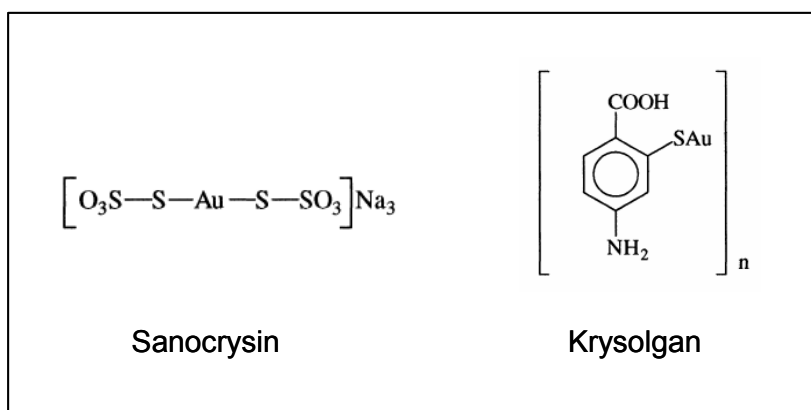


Figure 1.6. Chemical structures of Adolf Feldt's gold compounds Sanocrysin (sodium aurothiosulphate) and the gold (I) complex of 4-amino-2-mercaptobenzoic acid (Krysolgan) commonly used as anti-tuberculosis drugs preceding streptomycin.

1.5.1 Modern chemotherapy

The first practical breakthrough in modern *M. tuberculosis* chemotherapeutic treatment was made in 1943 through the discovery of streptomycin. Albert Schatz, a post-graduate student of a prominent soil biologist, Selman Waksman, isolated a compound from *Streptomyces*

griseus with high antimicrobial activity to gram-negative bacteria but low toxicity to animals (Schatz *et al.*, 1944). Schatz named the compound streptomycin (Mistiaen, 2002), a derivative of the generic name '*Streptomyces*' and due to its similarity to streptothricin (Schatz *et al.*, 1944). It seemed that the potential and implications of streptomycin in the chemotherapeutic treatment of tuberculosis was at first not realised by Waksman, since the antibacterial activity of streptomycin on *M. tuberculosis* was merely mentioned in the results in the paper by Schatz, Bugie and Waksman in 1944, as well as in a subsequent paper (Jones *et al.*, 1944) and no suggestion or speculation that it might be used in tuberculosis treatment was noted. Feldmann and Hinshaw first described the effect of streptomycin on infected guinea pigs in preliminary reports in 1944 and again in 1945 (H. Corwin Hinshaw Papers, American Philosophical Society; Comroe, Jr., 1978a). The first human to be treated with streptomycin was a 21-year old female on 20 November 1944 (Comroe, Jr., 1978). The result was, for that time, astonishing in that very shortly after administering streptomycin, the progression of the tuberculosis disease was halted, *M. tuberculosis* was cleared from the sputum and the patient healed. Streptomycin was also found to be effective against bubonic plague, cholera and typhoid fever. With the advent of streptomycin, the use of all gold compounds were scrapped virtually everywhere with immediate effect because the risk to the patient exceeded the possible therapeutic efficacy and also because streptomycin proved effective to extra-pulmonary tuberculosis.

However, *M. tuberculosis* strains showing resistance to streptomycin became apparent shortly after the introduction of streptomycin as a mono-therapy. In 1943, the Swedish pharmacologist Jorgen Lehmann tested Para-aminosalicylic acid (PAS) on *M. bovis* BCG cultures and discovered that PAS inhibited mycobacterial growth in lower concentrations than any other compound previously tested (Lehmann, 1964). Lehmann designed PAS as a sodium salicylate analog on the basis of previous work by other biologists; principally Bernheim (Bernheim, 1940), who hypothesised that sodium salicylate may be essential for continued mycobacterial growth. Interestingly enough, the first tests done by Lehmann with PAS on mycobacterial cultures were unsuccessful. He quickly realised that Bernheim used virulent mycobacteria in his studies, and subsequently found that PAS does not have an inhibitory effect on non-virulent mycobacteria, such as it has on virulent strains. Therefore, by the end of 1946 physicians had two drugs to combat tuberculosis, which greatly reduced the occurrence of drug resistant *M. tuberculosis* strains.

The next breakthrough in tuberculosis treatment came with the re-discovery of isoniazid (INH), which was first synthesised by Meyer and Mally in 1912 (Meyer and Mally, 1912). The efficacy of INH as an inhibitor of *M. tuberculosis* was first described in 1952 (Bernstein *et al.*, 1952). Soon after INH was first introduced as mono-therapy against tuberculosis disease, INH resistant *M. tuberculosis* strains began emerging. But adding INH to PAS and streptomycin (so-called triple therapy) resulted in a 90 – 95% cure rate of patients over the extremely long continuous treatment periods of 24 months (MRC, 1955). However, through triple therapy the emergence of acquired drug resistant *M. tuberculosis* phenotypes was effectively suppressed. Since INH, a host of compounds have been included in the chemotherapy of tuberculosis, mainly aiming to reduce the emergence of drug resistant phenotypes, shorten therapy duration and lessen toxicity and side effects.

During the early 1960s, ethambutol (EMB) was observed to clear *M. tuberculosis* in mice (Thomas *et al.*, 1961) and subsequently replaced PAS, since EMB was less toxic and resulted in a reduction in treatment period to 18 months (Doster *et al.*, 1973). The next compound to be introduced was rifampicin (RIF), which is a bactericidal derivative of rifamycin from *Streptomyces mediterranei* (Lancini and Hengeller, 1969). Rifampicin, administered in combination with INH, streptomycin and EMB resulted in a cure rate of >95% after a treatment period of only 8 – 9 months (Angel *et al.*, 1976; WHO 1976). Although known since the 1950s to exert anti-tuberculosis effects (Yeager *et al.*, 1952), Pyrazinamide (PZA) was introduced during the 1990s after the observation that similar cure rates could be obtained to that of RIF, streptomycin, EMB and INH tetra-therapy in only 6 months when combined with INH and RIF (Fox *et al.*, 1999).

Currently, treatment of tuberculosis disease involves a two-month combination therapy of INH, RIF, PZA and EMB, followed by a four-month combination of INH and RIF (Banerjee *et al.*, 1994; Palandez *et al.*, 2003). Various studies have shown that the 6-month regimens could be administered three times a week throughout the duration of chemotherapy (Hong Kong Chest Service, 1982; Hong Kong Chest Service, 1991), or twice weekly following a two-week daily induction phase (Cohn *et al.*, 1990). Failure in cure rates arose, however, due to non-compliance or abandonment of treatment. Therefore the WHO endorsed a direct observed therapy short-course (DOTS) that aimed to circumvent the cure failures due to the above-mentioned problems (WHO, 1994). The DOTS programme has certain drawbacks, such as relying on sputum microscopy for diagnosis, but has made an impact on cure rates

particularly in first world countries such as the USA where a decline in tuberculosis case rates of 7.8% per year over a five year period has been observed (Centres for Disease Control and Prevention, 2000).

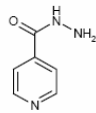
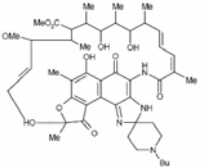

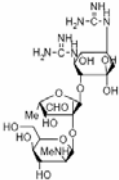

Compound	Mode of action	Target	MIC in vitro (mg/ml)
 Isoniazid	Mycolic acid biosynthesis inhibition; exact mode of action unclear but may act through free-radical intermediates	Cell wall	0.01 - 0.05
 Rifampin	Acts on DNA-dependent RNA polymerase thereby blocking protein synthesis	Transcription	0.05 - 1.0
 Pyrazinamide	Metabolism inhibition	Unknown	6.2 (pH = 5) to >400 (pH ? 6.0)
 Streptomycin	Protein synthesis inhibition through binding to and inhibition of ribosomes	Translation	0.5 - 2.5
$\text{C}_2\text{H}_5-\underset{\text{CH}_2\text{OH}}{\text{CH}}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\underset{\text{CH}_2\text{OH}}{\text{CH}}-\text{C}_2\text{H}_5$	Mode of action unclear, but may inhibit arabinogalactan biosynthesis	Cell wall	2.0 - 5.0
 p-Aminosalicylic acid	Unknown, but thought to disrupt folate biosynthesis	Folate metabolism	1.0 - 5.0

Figure 1.7. Summary of so-called first-line drugs, which are the drugs of choice for brief periods, usually taken in combination of two or more, of which at least two are isoniazid and rifampin (WHO-DOTS programme).

1.5.2 Drug Resistance

As mentioned in Section 1.5.1, one of the factors that has confounded the effective treatment of tuberculosis was the emergence of *M. tuberculosis* strains that do not respond to existing anti-tuberculosis drug treatment (referred to as drug resistant *M. tuberculosis*). Anti-tuberculosis drugs have been described as a “two-edged sword” which at the same time kill *M. tuberculosis* and also select for drug resistant phenotypes (Johnson *et al.*, 2006). The anti-tuberculosis drugs currently used exert their bactericidal action through the inhibition of a variety of essential cellular processes (see Figure 1.7). However, drug resistance is a man-made problem and seems to be mainly the result of the acquisition of mutations in the various genes encoding the targets of the anti-mycobacterial drugs through natural selection (Frothingham *et al.*, 2005). The slow doubling time of *M. tuberculosis* (~ 23 hours) and its ability to remain latent for prolonged periods (Wayne, 1994; reviewed in Parrish *et al.*, 1998) require that antibiotics must be taken over prolonged periods (a minimum of 6 months). During latent periods, micro-organisms enter a metabolically inactive state during which drugs that are usually bactericidal only have a bacteriostatic effect (Handwerker and Tomasz, 1985). These factors, together with unpleasant side effects and incompatibility with other chemotherapeutics, further exacerbates the rise in drug resistance because it leads to poor patient compliance during therapy, which promotes the evolution of drug resistant *M. tuberculosis* phenotypes (Gillespie, 2002).

Table 1.2 illustrates the extent of the drug resistant *M. tuberculosis* phenotypes globally. A recent report by the WHO showed that amongst these MDR-TB cases there is a rising incidence of so-called extensively drug resistant (XDR) *M. tuberculosis* infections (Wright *et al.*, 2006). Extensively drug resistant *M. tuberculosis* phenotypes are defined as multidrug-

resistant *M. tuberculosis* isolates (resistant to at least INH and RIF) with additional resistance to any fluoroquinolones and at least one injectable second-line drug (such as capreomycin, kanamycin and amikacin) (WHO Global Task Force on XDR-TB, 2006), allowing these isolates not to be killed by current front- and second-line drugs. Population based data in the report by Wright *et al.* (2006) showed that of the MDR *M. tuberculosis* isolates obtained from the USA (1993 to 2004), Latvia (2000 – 2002) and South Korea (2004), 4%, 19% and 15% respectively were XDR *M. tuberculosis*. In South Africa, 53 cases of XDR *M. tuberculosis* infections have been reported, of which 52 patients succumbed to the infection within 25 days (Koenig, 2006), although these cases were all HIV positive.

Table 1.2. Estimates of individuals infected with MDR-tuberculosis (Dye *et al.*, 2002)

Country	All cases	MDR-TB cases	%
Pakistan	273099	26201	0 - 21.6
Estonia	935	131	10.5 - 17.6
Latvia	2783	250	7.0 - 11.0
China (non-DOTS)	650502	49844	6.3 - 9.0
Russia	97223	5864	4.5 - 7.6
India	1864390	63136	1.6 - 5.2
Mozambique	86558	3023	2.4 - 4.6
China (DOTS)	650502	18520	2.0 - 3.7
Bangladesh	308271	4351	0 - 3.3
Peru	54310	1666	2.3 - 3.1
South Africa	215943	3267	0.6 - 2.4
USA	15123	183	1.0 - 1.4
England and Wales	6947	55	0.5 - 1.1

These observations highlight the need for new anti-tuberculosis chemotherapeutic compounds and strategies to effectively combat the transmission of the new drug-resistant *M. tuberculosis* strains or shorten the standard treatment course. The main objectives for the development of new anti-tuberculosis drugs includes a faster mode of action in order to shorten therapy, drugs that can counter the current drug resistant *M. tuberculosis* phenotypes and drugs that are non-toxic in order to limit side effects. A further objective is the sterilisation of latent or persistent forms of *M. tuberculosis* (Ormerod, 2005). These objectives led to a renewed interest in the biology of *M. tuberculosis* to find potential and unique metabolic factors that might be developed as future targets of drug intervention (Cohn *et al.*, 1997; Pablos-Mendez *et al.*, 1998).

1.6 GLUTAMINE SYNTHETASE

One of the *M. tuberculosis* molecules that emerged as a potential drug target is glutamine synthetase (GS) (EC 6.3.1.2, also known as L-glutamate: ammonia ligase). This enzyme plays a central role in nitrogen metabolism through its dual involvement in both ammonia assimilation and glutamine synthesis. Nitrogen is one of the major components of cell structures such as proteins, nucleic acids and coenzymes and therefore living organisms' ability to assimilate inorganic nitrogen is essential. Glutamine synthetase is involved in nitrogen assimilation under conditions of nitrogen starvation by acting in sequence with glutamate synthetase (GOGAT) in the GS/GOGAT pathway (Figure 1.8) (Nolden *et al.*, 2001). Ammonia is used in the synthesis of glutamine from glutamate and then the amide nitrogen of glutamine is transferred to α -ketoglutarate resulting in the formation of glutamate. The glutamate molecule may then act as an amino donor in transaminase reactions, using ammonia in the synthesis of all amino acids (when the GS/GOGAT pathway is present).

The role of GS in this pathway also serves as a means of an ammonia detoxification of cells, which together with CO_2 are by-products of urea hydrolysis. High levels of ammonia are toxic to most living cells, and there is evidence that the GS enzyme may have evolved to fulfil an ammonia-detoxifying role even in the first primitive pre-prokaryotes (Kumada *et al.*, 1993).

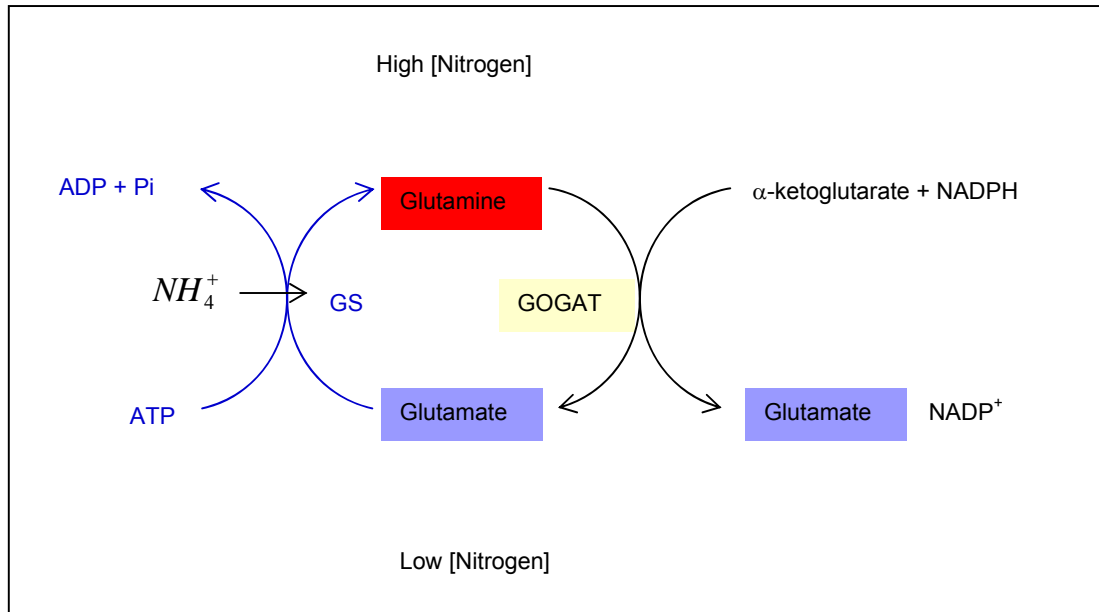


Figure 1.8. Ammonia incorporation through the synthetic action of glutamine synthetase (GS) and glutamate synthetase (GOGAT).

1.6.1 Structure and biochemistry

Glutamine synthetase is found in most living organisms in the form of three different isoforms: GS type I (GSI, found only in bacteria), GS type II (GSII, found in prokaryotes and eukaryotes) and GS type III (GSIII, some bacterial species). These GS isoforms differ with respect to regulation (see Section 1.6.3) and ultra-structure (see Figure 1.9).

Early electron microscopy of *E. coli* GS (Valentine *et al.*, 1968) as well as the crystal structure of unadenylylated *Salmonella typhimurium* GS (Janson *et al.*, 1984), showed that the GS enzyme exists as a petal-shaped dodecameric structure composed of two inverted hexameric rings of identical subunits.

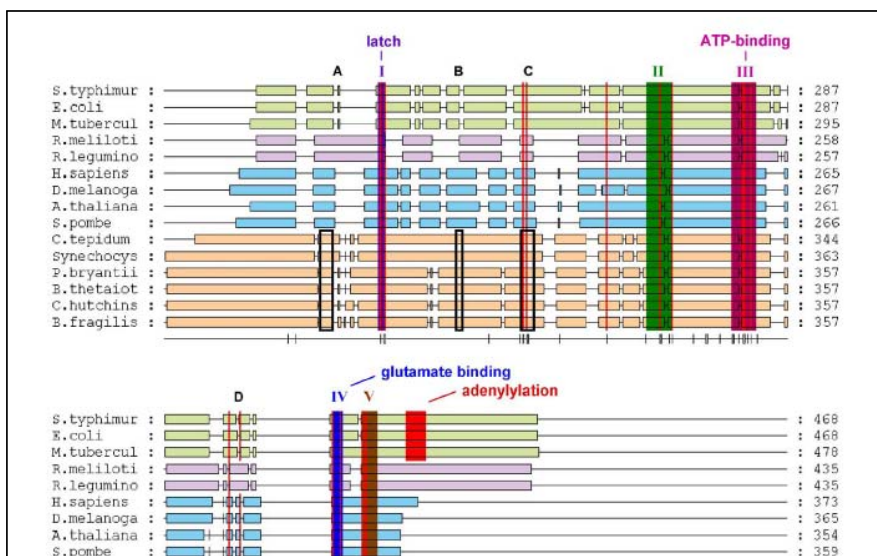


Figure 1.9. A visual comparison of GS protein domains shows the high degree of homology in functional regions and amino acids implicated in catalytic function. Note the presence of an adenylation site in the GSI enzymes, which is lacking in the other GS classes. (Source: van Rooyen *et al.*, 2006)

The molecule has a central channel into which the central loops of each GS sub-unit protrude (Figure 1.10). The two hexameric rings are held together by hydrophobic and hydrogen bond interactions between the helical N- and C-termini of each eclipsed sub-unit (Almassy *et al.*, 1986). These interactions are facilitated by the formation of a “hydrophobic hole” by the N-terminal of one sub-unit, into which the C-terminal α -helix (called a “helical thong”, see Figure 1.10) of the eclipsed sub-unit on the opposite hexameric ring inserts (Almassy *et al.*, 1986).

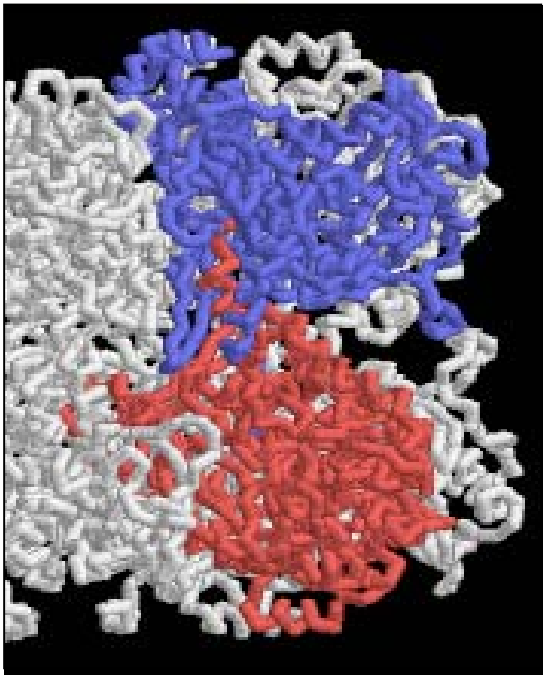


Figure 1.10. Backbone diagram showing the interaction of the C- and N-termini of two eclipsed GS-subunits. The C-terminal helical thong of the red sub-unit can be seen protruding into the blue sub-unit, thereby binding the two hexameric rings into a dodecameric structure. (Source: PDB <http://www.rcsb.org/pdb/home/home.do>)

A striking feature of the GS molecule is that the 12 enzymatic active sites are formed through the interaction of the C-terminus β -strands of one sub-unit with the N-terminus β -strands of a neighbouring sub-unit (Almassy *et al.*, 1986). These sites have been likened to a bi-funnel wherein ATP and glutamate bind at the opposite ends and two divalent cations bind at the bi-funnel joint. The cation binding sites, n1 and n2, can be occupied by either Mg^{2+} or Mn^{2+} for catalysis. These metal ions are each connected by four ligands to the protein backbone (Almassy *et al.*, 1986) and may have a regulatory effect on GS activity through inducing structural changes on binding. In the less active, or adenylylated state, GS prefers Mg^{2+} to Mn^{2+} for optimal activity (see Table 1.3 and Section 1.6.3). The n1 cation binding site plays a role in the binding of glutamate and stabilising role active GS (Shapiro and Ginsburg, 1968). The n2 cation binding site is involved in phosphoryl transfer (Hunt *et al.*, 1975) and interestingly has a 50 times lower affinity for metal ions than the n1 binding site (Hunt and Ginsburg, 1980).

Three-dimensional structures of *M. tuberculosis* GS sub-units have revealed that catalytic and regulatory amino acid residues may be situated on various mobile loops (Gill *et al.*, 2002). These include the Glu-327 flap (residues 324 – 329), the Asp-50 latch (residues 50 – 64) and the Tyr-397 adenylylation loop (residues 388 – 411).

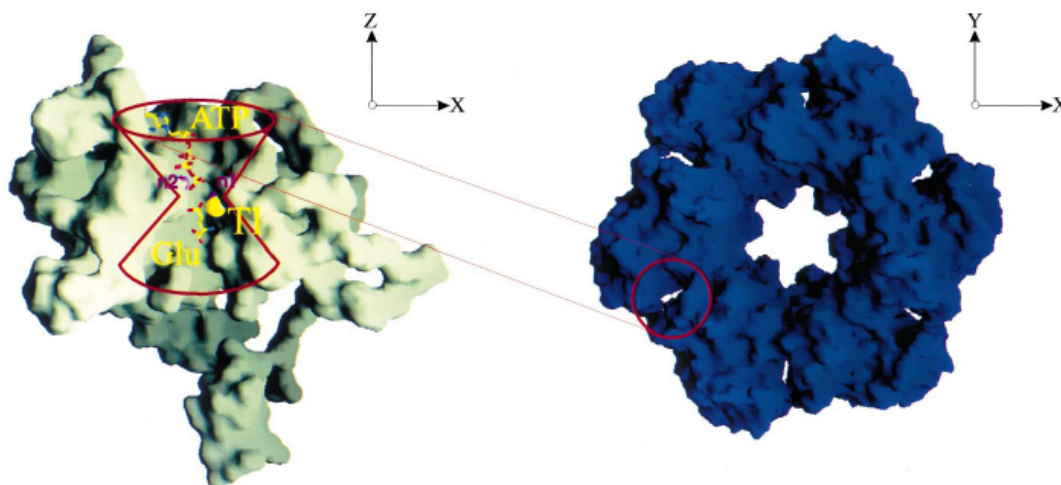
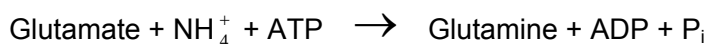


Figure 1.11. Structure of a sub-unit (left) and hexameric ring of sub-units (right) of bacterial glutamine synthetase (Eisenberg *et al.*, 2000). The helical thong formed by the C-terminal can be seen extending down the Z-axis in the representation of a single GS sub-unit.

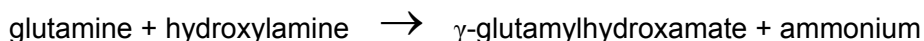
1.6.2 Reaction mechanism

The most important reaction catalysed by GS is the biosynthetic reaction that is the ATP-dependent condensation of glutamate with ammonium to form glutamine:



In this reaction, GS uses either Mg^{2+} or Mn^{2+} as co-factor.

The adenylylated form of the GS enzyme (see Section 1.6.3) catalyses the reverse or transferase reaction, which is the transfer of a glutamyl moiety of glutamine to hydroxylamine:



In this reaction, the adenylylated GS is active in the presence of Mn^{2+} (Luo *et al.*, 2005), and has a pH optimum of 6.9 to 8.0 as the state of adenylylation increases from 0 to 12 (Stadtman *et al.*, 1970).

The reaction mechanism possibly involves ATP and glutamate binding in their substrate sites at opposite ends of the active site in the initiating step (Eisenberg *et al.*, 2000). The γ -carboxylate group of glutamate attacks the γ -phosphate atom of ATP producing an activated γ -glutamyl phosphate intermediate and ADP (Liaw and Eisenberg, 1994). The ADP induces the movement of the Asp-50 latch to bind ammonium and subsequently deprotonates it to form active ammonia (Alibhai and Villafranca, 1994). In the second step of the reaction mechanisms, the ammonia ion attacks the δ -carbon of γ -glutamyl phosphate and forms a tetrahedral transition state intermediate. This intermediate is stabilised and protected from hydrolysis through the action of the Glu-327 flap, which closes the active site and thereby excludes water (Gill and Eisenberg, 2001). The Glu-327 residue accepts a proton from the transition state intermediate to form glutamine (Liaw *et al.*, 1995). The glutamine escapes the active site at the bottom half of the bi-funnel where glutamate entered in the first step of the reaction.

1.6.3 Glutamine synthetase regulation through adenylylation

One of the main differences between the three GS classes is that GSI can be regulated through the reversible esterification of AMP to the phenolic group of a tyr-residue (tyr-405 in the *M. tuberculosis* GS enzyme, Krajewski *et al.*, 2005) in a process termed adenylylation (see Figure 1.9 for sequence comparison). The adenylylation and deadenylylation of GS is catalysed by the bi-functional GS adenylyltransferase (E.C 2.7.7.42), encoded by *glnE* (Rhee *et al.*, 1985; Berlett *et al.*, 1998). Adenylylation of GS may proceed at a rate to match GS activity (and therefore glutamine production) with cellular glutamine utilisation, the enzyme being fully adenylylated in the presence of high glutamine concentrations and fully deadenylylated when glutamine levels are low. The mechanism explaining the effect of adenylylation has been described from data inferred from crystallographic models of *E. coli* GS (Figures 1.12 A and B), and suggests that the tyr-397 residue is situated on a mobile adenylylation loop which is in close proximity to the GS catalytic pocket (Almassy *et al.*,

1986; Gill *et al.*, 2002). Adenylation of tyr-397 causes the adenylation loop to move to a closer position to the glu-327 loop, enabling glu-327 to bind the adenyl group of AMP (Figure 12 B). This bond immobilises the glu-327 latch disabling the completion of the catalytic pocket and changing the affinity of divalent metal ion binding in the catalytic site (Gill *et al.*, 2002). In support of this model, a recent report has demonstrated that GS fails to be adenylylated when tyr-397 is substituted with either a serine or alanine residue (Luo *et al.*, 2005). In other bacteria, many of the genes involved in the regulation cascade of GS adenylylation (such as PII signalling molecules) are situated in operons in the vicinity of the *glnA1* gene (Merrick *et al.*, 1995; Schulz *et al.*, 2001; Burkovski, 2003). It is not known if the same mechanisms control the GS-adenylylation cascade in *M. tuberculosis*.

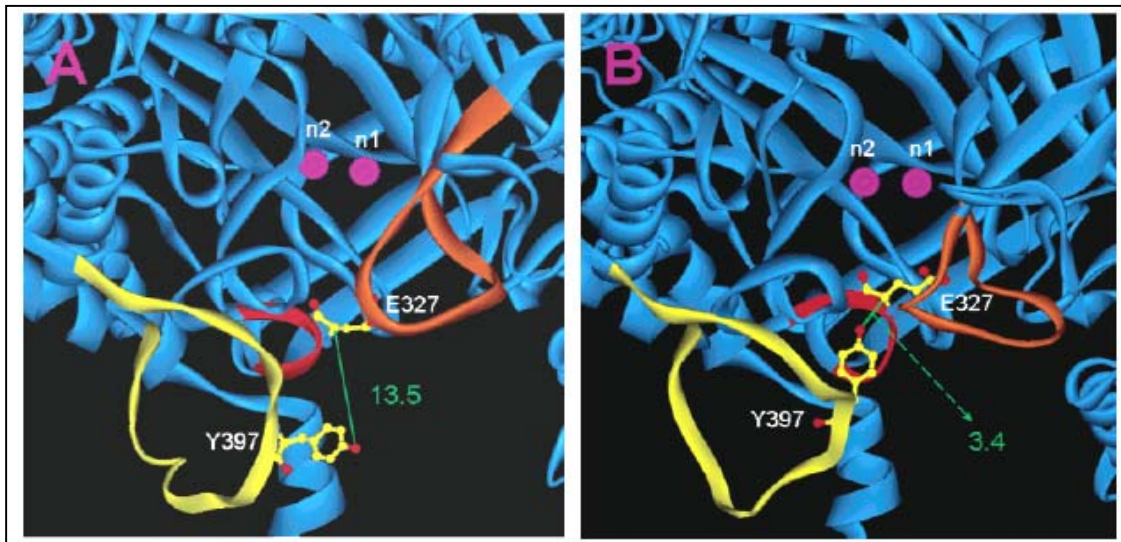


Figure 1.12. The mechanism of GS inhibition through the adenylation of tyr-397 involves the movement of the adenylation loop (yellow) towards the catalytic pocket of GS, where the α -carbon of glu-327 binds the adenyl group thereby preventing completion of the active site. Intermolecular distances between the participating atoms of glu-327 and tyr-397 (depicted as ball and stick structures) are shown in Å (Figure from Luo *et al.*, 2005).

1.6.4 Regulation through feedback inhibition

Glutamine synthetase is also subject to feedback inhibition by several end products of glutamine metabolism, namely histidine, tryptophan, AMP, CTP, carbamyl-phosphate, glucosamine-6-phosphate, and NAD⁺, and also by glycine and alanine (Woolfolk and Stadtman, 1964). All these compounds interact with different allosteric sites of the enzyme, since saturation of the GS enzyme with any one inhibitor only partially inhibits its function, whereas mixtures of inhibitors at a physiological concentration, effectively inhibit GS enzymatic function up to 90% (Shapiro and Stadtman, 1970). However, feedback inhibition is also influenced by the adenylation state of the sub-units of the enzyme (Kingdon and Stadtman, 1967). Fully deadenylylated enzyme appears insensitive to feedback inhibition (Shapiro *et al.*, 1967).

Table 1.3 Comparison of the characteristics of deadenylylated and adenylylated GS (Luo *et al.*, 2005).

Characteristic	deadenylylated	adenylylated
Mg ²⁺ -dependent biosynthetic activity	yes	no
Mn ²⁺ -dependent biosynthetic activity	no	yes
Mg ²⁺ -dependent γ -glutamyltransferase activity	yes	no
Mn ²⁺ -dependent γ -glutamyltransferase activity	yes	yes
pH optimum for Mn ²⁺ -dependent γ -glutamyltransferase activity	8	6.9
feedback inhibition sensitivity	absent	enhanced
effect of 4,5 M urea	none	inactivated

1.6.5 Glutamine synthetase of *M. tuberculosis*

The structure of *M. tuberculosis* GS and many aspects of its physiological role has been the subject of various studies. However, there still remain many features unique to *M. tuberculosis* GS of which the mechanisms have not been elucidated.

Glutamine synthetase is encoded by the *glnA* genes of which four homologues (*glnA1*, *glnA2*, *glnA3* and *glnA4*) have been identified in the *M. tuberculosis* genome (Cole *et al.*, 1998). It is thought that *glnA1* encodes a GSI enzyme, while *glnA2* encodes a GSII enzyme. The *glnA3* and *glnA4* gene sequences encode probable GSI and GSII enzymes, however

the amino acid residues usually associated with GS enzymes are less conserved in these protein sequences. It has recently been shown that the above *glnA* genes encode functional GS enzymes, with the GS encoded by *glnA1*, *glnA3* and *glnA4* synthesising L-glutamine, while the *glnA2* GS synthesises D-glutamine and D-isoglutamine (Harth *et al.*, 2005). However it was also shown that only *glnA1*, and not the *glnA2*, *glnA3* and *glnA4* genes, were able to complement a *M. smegmatis* glutamine auxotroph, suggesting that the main GS encoding locus is *glnA1* (Harth *et al.*, 2005). In support of this finding, it has previously been demonstrated through transposon site hybridisation studies that a functional *glnA1* is essential for the growth and survival of *M. tuberculosis* (Sasseti *et al.*, 2003).

The *glnA1* gene is situated in close proximity to the genes encoding the adenylyl-transferase (*glnE*, see Section 1.4.2) and *glnA2* (see Chapter 4, Figure 4.1) (Cole *et al.*, 1998). The adenylyl-transferase is involved in the reversible inhibition of GSI enzymes (Section 1.6.3) and it has been shown (via *glnE* mutation) that a functional adenylyl-transferase is essential for *M. tuberculosis* survival (Parish *et al.*, 2000). In this report, the authors speculated that *glnE* mutation resulting in absence of adenylyl-transferase activity could lead to a depletion of cellular glutamate through the un-regulated action of GS. It has also been shown that *glnA2* may be essential for *M. bovis* growth in minimal media (Collins *et al.*, 2002). In that study, it has also been shown that *glnA2* mutants lost the ability to cause infection in guinea pigs, however, the role of *glnA2* during infection has not been clarified.

1.6.5.1 Glutamine synthetase is a major exogenous protein of *M. tuberculosis*

Glutamine synthetase activity is normally limited to the cytosolic fraction of cells, but GS activity has been detected in the exogenous environment of infectious mycobacteria, specifically *M. tuberculosis* and *M. bovis*, including the *M. bovis* BCG strains (Harth and Horwitz, 1994). In *M. tuberculosis*, GS is one of the most abundant of the approximately 200 protein species found in the *M. tuberculosis* culture filtrate (Harth *et al.*, 1994; Sonnenberg and Belisle, 1997) suggesting that active GS could be exported to the exogenous environment of *M. tuberculosis*.

This observation led the hypothesis that exogenous GS activity may be a unique feature and probably a virulence factor of *M. tuberculosis*, as other non-infectious mycobacteria such as

M. smegmatis, *M. phlei*, *M. fortuitum* and *M. kansasii* do not show this phenomenon (Raynaud *et al.*, 1998).

Table 1.4 Comparison of the endo- vs. exogenous GS activity of various bacterial species (Harth and Horwitz, 1994).

	GS activity		
	Cell extract (A)	Culture supernatant (B)	Ratio B/A
<i>M. tuberculosis</i>			
Erdmann	63.7	28.7	1: 1.2
H37Rv	42.3	18.1	1: 2.3
H37Ra	36.5	16.4	1: 2.2
<i>M. bovis</i> BCG	49.6	11.4	1: 4.3
<i>M. smegmatis</i>	6.3	0.06	1: 105
<i>M. phlei</i>	6.9	0.06	1: 115
<i>E. coli</i> DH5 α	2.1	≤ 0.01	$\leq 1: 210$
<i>L. pneumophila</i>	2.1	≤ 0.02	$\leq 1: 211$
<i>B. subtilis</i>	2.8	≤ 0.03	$\leq 1: 280$

In addition, irreversible inhibition of exogenous GS with a non-covalently binding glutamate analog such as L-methionine-S-sulfoximine which is specific for GS and is unable to cross the mycobacterial cell wall (Harth and Horwitz, 1999), has a bacteriostatic effect on *M. tuberculosis* growth in both liquid culture and in *M. tuberculosis* infected human monocyte cell lines (Harth and Horwitz, 1999). In this study, it was also observed that the bacteriostatic effect was obtained in *M. tuberculosis* and *M. bovis* cultures only and that L-methionine-S-sulfoximine (an inhibitor of GS, Manning *et al.*, 1969) had no effect on cultures of other mycobacteria that do not exhibit exogenous GS activity.

The mechanism of export and the function that GS fulfils in the exogenous *M. tuberculosis* environment is unknown, although it has been speculated that exogenous GS might play a role in delaying lysosome fusion by inhibiting phagosomal acidification (Miller and Shinnick, 2000). This hypothesis was based on the enhanced survival (up to 6 fold over a 24 hour period) of recombinant *M. smegmatis* (a non-infectious species of the mycobacterial genus) expressing *M. tuberculosis glnA1* in human macrophages. Previous studies have shown that

the expression of *M. tuberculosis glnA1* in *M. smegmatis* yields a recombinant protein identical to the native *M. tuberculosis* GS protein and that this GS activity could also be detected in the *M. smegmatis* transformants' culture filtrate (Harth and Horwitz, 1997). From this observation, it has been hypothesised that GS is not actively exported by *M. tuberculosis*, and that the high concentration of GS in the exogenous environment of *M. tuberculosis* is due to mycobacterial lysis and the exogenous stability of the GS protein in the exogenous *M. tuberculosis* environment (Tullius *et al.*, 2001).

1.6.6 Why is exogenous GS essential for *M. tuberculosis* survival?

The reason for the observed bacteriostatic effect by L-methionine-S-sulfoximine described earlier is still unclear, but it is thought that exogenous GS might also be involved in cell wall biosynthesis of the pathogenic mycobacteria *M. tuberculosis* and *M. bovis*. This hypothesis is based on the observation that poly-L-glutamic acid, a polymer unique to the cell walls of *M. tuberculosis* and *M. bovis*, coincides with exogenous GS activity (Harth *et al.*, 2002). In the study by Harth *et al.* (2002), it was also hypothesised that GS may play a direct role in the synthesis of poly-L-glutamic acid, as it was shown that the concentration of this polymer decreases if GS is inhibited (Harth and Horwitz, 1999) or *glnA1* activity is inhibited (Harth *et al.*, 2000). The physiological and functional role and synthesis mechanism of poly-L-glutamic acid in the *M. tuberculosis* cell wall is still unknown. Besides playing a possible role on the cell wall integrity of *M. tuberculosis* through poly-L-glutamic acid synthesis, it was also hypothesised that GS might play a role in the disruption of phagosome-lysosome fusion through its involvement in nitrogen metabolism and ammonium production (Harth and Horwitz, 1994).

1.7 PROBLEM STATEMENT

Because of the essential role of GS in *M. tuberculosis* intracellular growth and survival, GS has been identified as a potential target for new anti-tuberculosis antibiotics. However, not much is known about the evolutionary stability of the GS encoding locus, *glnA1*, in *M. tuberculosis* or its expression during active disease. Furthermore, other factors may play a role in the physiological properties of *M. tuberculosis*, such as its export and secretion into the exogenous environment, which may be unique to *M. tuberculosis*. These mechanisms are still poorly understood and may reveal other areas for potential antibiotic intervention.

1.8 OBJECTIVES OF THE CURRENT STUDY

Objective 1: To investigate the evolution and genetic diversity of *M. tuberculosis* GS and its encoding gene, *glnA1*.

It is hypothesised that the genome of *M. tuberculosis* is not stable and continues to evolve and that this genomic evolution may impact on strain fitness and virulence (Warren *et al.*, 2000). There are a number of genetically diverse strains of *M. tuberculosis* that are known to vary in terms of virulence, as some may be more infectious than others (Manca *et al.*, 2001; Lopez *et al.*, 2003). In the light of these hypotheses, it is necessary to evaluate the genetic stability of *glnA1*, as mutations in this gene may be reflective of altered GS structure and function between the different *M. tuberculosis* strain families. Such mutations may also complicate antibiotic design and contribute to the emergence of resistance.

Objective 2: To investigate *glnA1* expression during active disease

Mycobacterium tuberculosis is a facultative intracellular pathogen that resides within the granuloma, a structure formed through the activation of the immune system after infection (Schlesinger *et al.*, 1990) where it may be exposed to varying aerobic (Wayne & Sohaskey, 2001) and toxic (Russell, 2001) conditions not conducive to bacterial growth. Not much is known about the mechanisms enabling *M. tuberculosis* to survive in these conditions. Therefore the expression of *glnA1* in *M. tuberculosis* infected human tissues may indicate the importance of GS *in vivo*, as the conditions in the granuloma during active disease may differ from conditions created in the laboratory.

Objective 3: To investigate the regulation of GS synthesis and other factors that might be involved in GS secretion and export.

Mycobacterium tuberculosis (as well as *M. bovis*, including the *M. bovis* BCG strains) is unique in having functional GS in its exogenous environment (Harth *et al.*, 1994). Therefore we hypothesise that there may be factors unique to *M. tuberculosis* that might enable the mycobacterium to actively or passively transport GS over its cell wall. Understanding these mechanisms may identify other unique mechanisms, which can be exploited as possible anti-tubercular drug targets. Therefore the regulation of *M. tuberculosis* GS expression and export will be studied in an *M. smegmatis* host, which does not secrete its own GS (Harth and Horwitz, 1994). Furthermore, the co-regulation of genes situated in gene cluster (*glnE*; *glnA2*; Rv2223c and Rv2224c) downstream of *glnA1* will be investigated to establish their functional relationship to GS transport.

CHAPTER TWO

Evolutionary ancestry and stability of *M. tuberculosis glnA1*

“Man is the only creature that consumes without producing. He does not give milk, he does not lay eggs, he is too weak to pull the plow, he cannot run fast enough to catch rabbits. Yet he is lord of all the animals”

Eric Arthur Blair (George Orwell), *Animal Farm*

(Born June 25, 1903; died of tuberculosis January 10, 1950)

Note: Parts of the results presented in the following chapter have been submitted for review publication as: ” **Evolution and stability of *M. tuberculosis glnA1***. Hayward, D; van Helden, P and I Wiid. Submitted to BMC Evolutionary Biology (2007)

2.1 INTRODUCTION

The essential role of the gene encoding glutamine synthetase (GS β , hereafter referred to as GS), *glnA1*, in the growth and survival of *M. tuberculosis* has been described in several reports (Harth *et al.*, 2000; Lee *et al.*, 2006). These studies made use of methods that disrupt GS synthesis, such as either *glnA1* mutation or translation inhibition through anti-sense technology. These studies imply that other *glnA* genes (*glnA2*, *glnA3* and *glnA4*) residing on the *M. tuberculosis* chromosome are unable to complement *glnA1* disruption (see Section 1.6.5). In this regard, a recent study has shown that all the *M. tuberculosis* *glnA1* homologs encode functional, but non-essential GS enzymes that are not as abundantly expressed as *glnA1* (Harth *et al.*, 2005). Therefore *glnA1* may be the major GS-producing locus in *M. tuberculosis*, which is similar to observations of *glnA1* deletion in other high G+C genome mycolic acid containing *Actinomyces*, such as *Corynebacterium glutamicum*. Similar to *M. tuberculosis*, deletion of the *C. glutamicum* *glnA1* results in the complete loss of GS activity and subsequent glutamine auxotrophy despite the presence of *glnA2* in *C. glutamicum* (Jakoby *et al.*, 1997). Therefore, it appears that *glnA2* is unable to complement deletion of the *C. glutamicum* *glnA1* and relieve glutamine auxotrophy. This observation, together with that of GS export by pathogenic mycobacteria (see Section 1.6.5.1) may all point to GS playing other roles in *M. tuberculosis* pathogenesis, which may not be present in non-pathogenic mycobacteria. This has been demonstrated by increased survival rates of *M. smegmatis* expressing *M. tuberculosis* *glnA1* in THP-1 macrophages (Miller and Shinnick, 2000). In that study it was speculated that *M. tuberculosis* GS might be involved in delaying phagosome acidification, thereby delaying lysosome fusion. These differences may be due to information contained in the *glnA1* and GS-protein sequences and structure.

2.1.1 Study aims:

2.1.1.1 To study *glnA* evolution in the *Actinobacteria*. The ancestry of *glnA1* might indicate the importance of this locus and why it is the main GS-producing locus in *M. tuberculosis*. This study may also indicate the distribution and importance of other GS encoding loci.

2.1.1.2 To investigate the similarity of the GS sequence between the closely related mycolic acid producing CMN-subgroup (*Corynebacteria* sp., *Mycobacteria* sp. and *Nocardia* sp.) of the *Actinobacteria*. The extent of evolutionary change in the GS protein sequence during

bacterial diversification may indicate the probability of changes that would allow GS to accomplish other functions during *M. tuberculosis* growth and survival.

2.1.1.3 To investigate the genetic stability of the *glnA1* locus and its immediate 5'- and 3'-flanking DNA sequences through *M. tuberculosis* strain diversification. Changes in nucleotide sequence may indicate the mutation tolerance of GS and may be of importance for antibiotic design and usage.

2.2 RESULTS

2.2.1 *Actinobacteria* GS phylogeny

The ancestry of the *glnA* sequence was investigated through phylogenetic analysis of the *glnA*-like orthologs present in the *Actinobacteria* (see Section 1.2.1). These orthologous protein sequences were obtained through BLAST comparison (Materials and Methods 2.3) of the *M. tuberculosis* H37Rv GS proteins encoded by the *glnA1*, *glnA2*, *glnA3* and *glnA4* open reading frames to all available actinobacterial genomes. Sequences of high similarity to *M. tuberculosis* GS I (encoded by *glnA1*) and GS II (encoded by *glnA2*) were detected in most of the available *Actinobacteria* genomes (Table 2.1). The GS I and GS II sequences were of comparable amino acid length and were more than 60% identical to the corresponding sequences in *M. tuberculosis* H37Rv, the only exceptions being the GS I and GS II sequences of *Rubrobacter xylanophilus* (GS I = 34% similar; GS II = 52% similar) and *Symbiobacterium thermophilum*, which had one GS sequence with 50% amino acid homology to *M. tuberculosis* GS I. The GS protein sequences encoded by *M. tuberculosis* H37Rv *glnA3* and *glnA4* had lower overall homology to orthologous sequences in the other *Actinobacteria*. One (most notably *glnA3*) or both of these protein sequences were absent in some *Actinobacteria*. The members of the *Micrococccineae* suborder (*Bifidobacterium longum*, *Leifsonia xyli* subspecies *xyli*, *Nocardiodes*, *Kineococcus radiotolerans* and *Brevibacterium linens*) (Stackebrandt *et al.*, 1997) have no *glnA3* and *glnA4* encoded GS protein sequences. The bacteria in the *Streptomycineae* suborder (*S. avermitilis* and *S. coelicolor*) have three GS sequences that show the highest homology to the proteins encoded by the *M. tuberculosis* *glnA1*, *glnA2* and *glnA4* genes. All members of the *M. tuberculosis* complex had four GS sequences sharing 100% homology to each other (see

Table 2.1). Some mycobacteria, such as *M. leprae* and *M. ulcerans*, had only *glnA1* and *glnA2* encoded GS orthologs.

The orthologous GS protein sequences obtained by sequence BLAST were aligned according to amino acid similarity index using ClustalW software (Materials and Methods 2.3.1). Phylogenetic analysis of these ClustalW alignments was carried out using the program Paup 4.0b4a (Swofford, 1998), with which a consensus tree was generated from 1000 bootstrapped trees using the majority formula. Branch lengths from the consensus trees of all of the above were drawn using the program Treeview 1.5 (Page, 1996). The GS I sequence of *Symbiobacterium thermophilum* was chosen as out-group for the phylogenetic reconstruction because *S. thermophilum* is most closely related to an *Actinobacteria* ancestor (see Figure 1.2). Figure 2.1 shows that the orthologous GS protein sequences are clustered into separate clades depending on the encoding *glnA* gene sequence. In these clades, bacteria of the same genus are partitioned into separately evolving lineages that resemble accepted phylogenetic trees (see Figures 1.2 and 1.3).

Table 2.1 *GlnA* sequence orthologs and their distribution in the *Actinobacteria*. The percentage homology to the *M. tuberculosis* orthologs is indicated by means of amino acid identity. The accession number and amino acid (aa) length of the protein sequence is indicated for each sequence. Shaded sequence information indicates the same gene.

Organism	<i>glnA1</i>		<i>glnA2</i>		<i>glnA3</i>		<i>glnA4</i>	
	Identities	Sequence acc. nr	Identities	Sequence acc. nr	Identities	Sequence acc. nr	Identities	Sequence acc. nr
<i>Acidothermus cellulolyticus</i> 11B	343/475 (72%)	YP_872682 474 aa	311/452 (68%)	YP_872678 453 aa	103/341 (30%)	YP_872678 453 aa	273/445 (61%),	YP_873609 446 aa
<i>Arthrobacter</i> sp. FB24	300/475 (63%)	YP_947504 474 aa	296/449 (65%)	YP_947491 446 aa	97/332 (29%)	YP_831086 446 aa	119/379 (31%),	YP_831086 446 aa
<i>Bifidobacterium longum</i> NCC2705	296/475 (62%)	NP_696248 478 aa	268/444 (60%)	NP_696466 445 aa	92/335 (27%)	NP_696466 445 aa	132/449 (29%),	NP_696466 445 aa
<i>Brevibacterium linens</i> BL2	294/470 (62%)	ZP_00378605 474 aa	282/452 (62%)	ZP_00378066 452 aa	102/350 (29%)	ZP_00378066 452 aa	254/452 (56%),	ZP_00381218 454 aa
<i>Corynebacterium diphtheriae</i> NCTC 13129	319/471 (67%)	NP_939986 478 aa	286/444 (64%)	NP_940011 446 aa	87/340 (25%)	NP_940011 446 aa	129/447 (28%),	NP_940011 466 aa
<i>C. efficiens</i> YS-314	303/472 (64%)	NP_738714 477 aa	297/444 (66%)	NP_738737 516 aa	98/331 (29%)	NP_738737 516 aa	130/446 (29%),	NP_738737 516 aa
<i>C. glutamicum</i> ATCC 13032	334/474 (70%)	YP_226455 477 aa	293/444 (65%)	YP_226471 446 aa	93/318 (29%)	YP_226471 446 aa	132/446 (29%),	YP_226471 446 aa
<i>C. jeikeium</i> K411	340/477 (71%)	YP_250482 500 aa	319/445 (71%)	YP_250455 448 aa	94/318 (29%)	YP_250455 448 aa	133/451 (29%),	YP_250455 448 aa
<i>Frankia</i> sp. EAN1pec	316/472 (66%)	EAN12723 474 aa	296/451 (65%)	EAN12719 452 aa	173/453 (38%)	EAN17203 496 aa	246/437 (56%),	EAN15827 470 aa
<i>Janibacter</i> sp. HTCC2649	314/472 (66%)	EAP98744 474 aa	315/44 (70%)	EAP99396 445 aa	183/432 (42%)	EAP99483 446 aa	272/454 (59%),	EAP97657 461 aa
<i>Kineococcus radiotolerans</i> SRS30216	322/472 (68%)	EAM74067 474 aa	294/446 (65%)	EAM74062 447 aa	77/245 (31%)	EAM74062 447 aa	275/450 (61%),	EAM72715 460 aa
<i>Leifsonia xyli</i> subsp. xyli str. CTCB07	293/471 (62%)	YP_062980 474 aa	285/444 (63%)	YP_061977 445 aa	95/332 (28%)	YP_061977 445 aa	122/379 (32%),	YP_061977 445 aa
<i>Mycobacterium avium</i> 104	431/478 (90%)	YP_881471 478 aa	420/446 (94%)	YP_881448 446 aa	350/433 (80%)	YP_882016 450 aa	364/461 (78%),	YP_882894 468 aa
<i>M. bovis</i> AF2122/97	478/478 (100%)	NP_855893 478 aa	446/446 (100%)	NP_855895 446 aa	450/450 (100%)	NP_855562 450 aa	475/475 (100%),	NP_856530 457 aa
<i>M. bovis</i> BCG str. Pasteur 1173P2	478/478 (100%)	YP_978326 478 aa	446/446 (100%)	YP_978328 446 aa	450/450 (100%)	YP_978005 450 aa	475/475 (100%),	YP_978966 475 aa
<i>M. flavescens</i> PYR-GCK	403/478 (84%)	EAS12379 478 aa	398/446 (89%)	EAS12360 446 aa	286/433 (66%)	EAS10635 453 aa	338/454 (74%),	EAS09445 469 aa
<i>M. leprae</i> TN	439/478 (91%)	NP_301707 478 aa	418/448 (93%)	NP_302123 448 aa	90/333 (27%)	NP_302123 448 aa	135/455 (29%),	NP_302123 448 aa
<i>M. smegmatis</i> str. MC2 155	402/478 (84%)	YP_888567 478 aa	396/446 (88%)	YP_888571 446 aa	280/433 (64%)	YP_887864 453 aa	336/451 (74%),	YP_886932 457 aa
<i>Mycobacterium</i> sp. KMS	407/478 (85%)	YP_939366 478 aa	398/446 (89%)	YP_939374 446 aa	205/432 (47%)	YP_936250 437 aa	334/449 (74%),	YP_938091 455 aa
<i>M. tuberculosis</i> CDC1551	478/478 (100%)	NP_336749 478 aa	446/446 (100%)	NP_336751 446 aa	450/450 (100%)	NP_336385 450 aa	475/475 (100%),	NP_337439 457 aa
<i>M. tuberculosis</i> F11	478/478 (100%)	ZP_01685137 478 aa	446/446 (100%)	ZP_01685139 446 aa	450/450 (100%)	ZP_01684789 450 aa	475/475 (100%),	ZP_01685769 462 aa
<i>M. tuberculosis</i> H37Rv	478/478 (100%)	NP_216736 478 aa	446/446 (100%)	NP_216738 446 aa	450/450 (100%)	NP_216394 450 aa	100% 457/457,	NP_217376 457 aa
<i>M. ulcerans</i> Agy99	430/477 (90%)	YP_905364 478 aa	417/446 (93%)	YP_905360 446 aa	90/331 (27%)	YP_905360 446 aa	139/455 (30%),	YP_905360 446 aa
<i>M. vanbaalenii</i> PYR-1	411/ 478 (85%)	YP_954385 478 aa	395/446 (88%)	YP_954396 446 aa	202/432 (46%)	YP_953732 442 aa	326/450 (72%),	YP_953098 459 aa
<i>Nocardia farcinica</i> IFM 10152	371/478 (77%)	YP_117877 478 aa	372/446 (83%)	YP_117870 446 aa	94/331 (28%)	YP_117870 446 aa	144/458 (31%),	YP_117870 446 aa
<i>Nocardioides</i> sp. JS614	339/ 472 (71%)	YP_923487 474 aa	290/439 (66%)	YP_923242 455 aa	95/331 (28%)	YP_923242 455 aa	265/449 (59%),	YP_923778 464 aa
<i>Propionibacterium acnes</i> KPA171202	316/473 (66%)	YP_055385 473 aa	283/444 (63%)	YP_055378 468 aa	102/333 (30%)	YP_055378 468 aa	125/407 (30%),	YP_055378 468 aa
<i>Rhodococcus</i> sp. RHA1	384/474 (81%)	YP_701142 478 aa	376/446 (84%)	YP_701152 446 aa	203/430 (47%)	YP_701692 433 aa	150/442 (33%),	YP_705251 451 aa
<i>Rubrobacter xylanophilus</i> DSM 9941	169/484 (34%)	YP_643903 443 aa	231/441 (52%)	YP_643903 443 aa	101/326 (30%)	YP_643809 444 aa	286/448 (63%),	YP_645913 455 aa
<i>Salinispora tropica</i> CNB-440	331/472 (70%)	EAU21273 474 aa	311/453 (68%)	EAU21297 451 aa	99/339 (29%)	EAU21297 451 aa	138/459 (30%),	EAU21297 451 aa
<i>Streptomyces avermitilis</i> MA-4680	335/472 (70%)	NP_827182 469 aa	312/452 (69%)	NP_827131 453 aa	100/344 (29%)	NP_827131 453 aa	296/452 (65%),	NP_827901 454 aa
<i>S. coelicolor</i> A3(2)	339/472 (71%)	NP_626450 469 aa	316/452 (69%)	NP_626490 453 aa	98/341 (28%)	NP_626490 453 aa	290/452 (64%),	NP_625889 462 aa
<i>Symbiobacterium thermophilum</i> IAM 14863	283/475 (50%)	YP_074027 471 aa	158/147 (33%)	YP_074027 471 aa	55/190 (28%)	YP_074027 471 aa	105/320 (32%),	YP_074027 471 aa
<i>Thermobifida fusca</i> YX	323/472 (68%)	YP_289049 474 aa	311/452 (68%)	YP_289043 453 aa	95/342 (27%)	YP_289043 453 aa	131/458 (28%),	YP_289043 453 aa
marine actinobacterium PHSC20C1	304/471 (64%)	EAR23759 478 aa	278/446 (62%)	EAR25342 445 aa	100/370 (27%)	EAR25918 416 aa	315/449 (70%),	EAR26195 455 aa

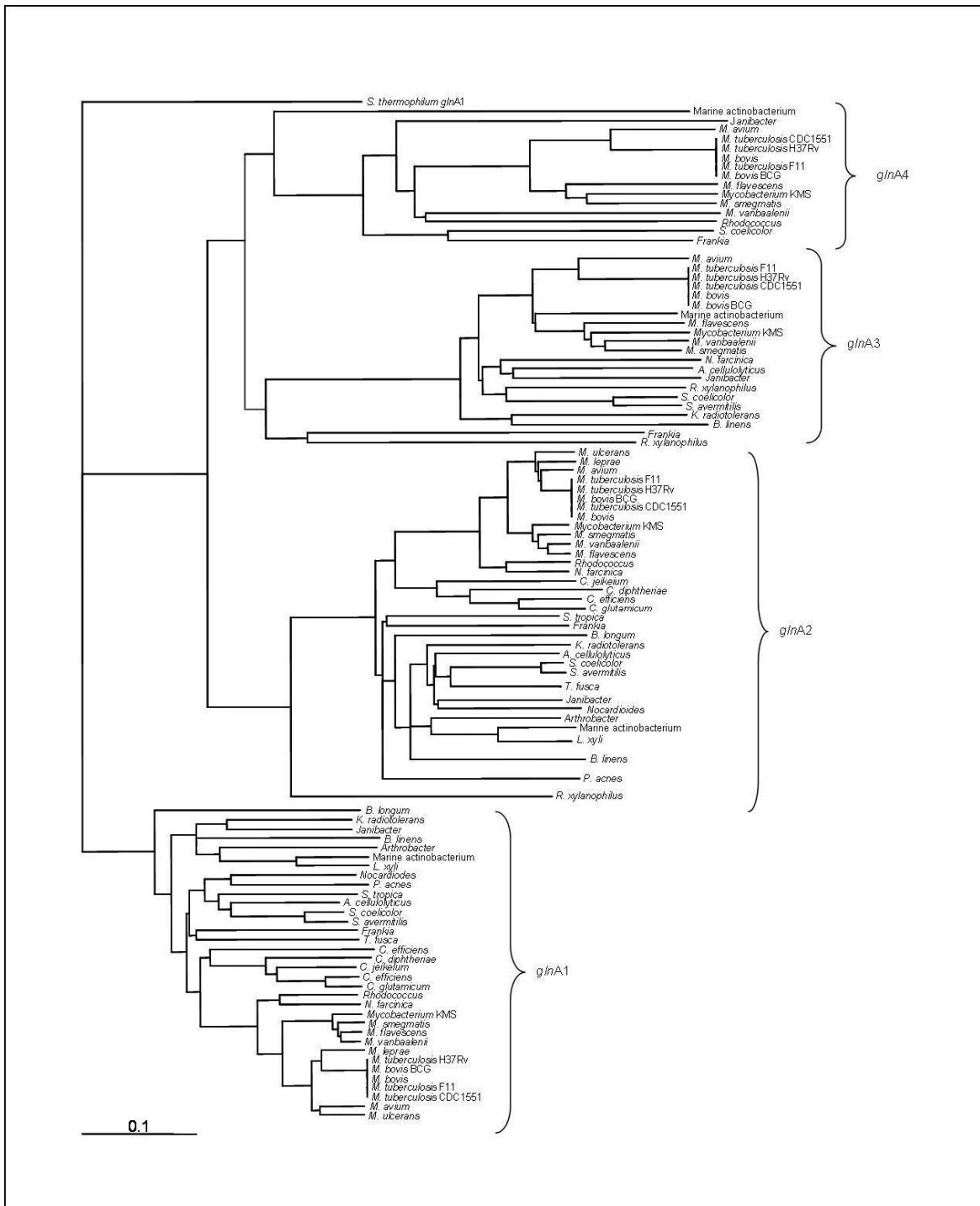


Figure 2.1. Phylogenetic reconstruction of GS sequence evolution in the *Actinobacteria*. Branch lengths are proportional to the number of evolutionary events (scale bar equals 0.1 evolutionary events) and branches with zero length were collapsed.

2.2.2 Glutamine synthetase sequence similarity in the closely related CMN subgroup

To study the variation of the *glnA1* encoded GS I protein sequence in the closely related *Corynebacteria*, *Mycobacteria* and *Nocardia* (CMN) subgroup of the phylum *Actinobacteria*, the GS I protein sequences of all available mycobacteria as well as *Corynebacterium glutamicum*, *C. diphtheriae*, *C. efficiens*, *C. jeikeium* and *Nocardia farcinica* were compared by multiple sequence alignment with ClustalW (Materials and Methods 2.3.1). The GS I protein sequences of all the members of the *M. tuberculosis* complex (*M. tuberculosis* including the H37Rv, F11 and CDC1551 sub-strains, *M. bovis* and *M. bovis* BCG Pasteur) were 100% similar in every respect. The GS I sequences of the other mycobacteria were very similar to the GS I sequence of the members of the *M. tuberculosis* complex. All sequences were of equal length (478 amino acids) with amino acid homology between 84% (*M. smegmatis*) and 91% (*M. leprae*) (Table 2.1). The GS I sequences of the *Corynebacteria* showed some variation in protein length (between 477 and 500 amino acids) and were between 64% (*C. efficiens*) and 71% (*C. jeikeium*) similar to the *M. tuberculosis* complex GS I. The GS I sequence of *N. farcinica* had a length of 478 amino acids and was 77% similar to the *M. tuberculosis* complex GS I.

Figure 2.2 shows the ClustalW multiple sequence alignment of the CMN subgroup GS I sequences. A high degree of amino acid homology can be observed between all the sequences towards the C-terminal, while homology is less pronounced at the N-terminus. Some sequence variation has occurred in the *Corynebacteria* GS I adenylation and GS consensus signatures that differs from the classical signature sequences. In the *Corynebacteria*, leu (L) is substituted with asn (N) in the GS consensus signature (Figure 2.2), while val (K) is substituted with arg (R) in the adenylation consensus sequence (Figure 2.2). Leu (L) has been substituted with met (M) in the GS consensus sequence in *M. smegmatis*. In agreement with the phylogenetic reconstruction showing separate GS I sequence evolution between slow and fast growing mycobacteria (Figure 1.3), it can be seen that the GS I sequences of the fast- and slow growing mycobacteria share greater amino acid homology to each other respectively. Similarly, the GS I amino acid sequence evolution could be observed in the *Corynebacteria*, where the sequences of *C. efficiens* and *C. glutamicum* share higher homology to each other than to that of *C. jeikeium* and *C. diphtheriae*.

<i>M. tuberculosis</i>	-----VTEKTPDDVFKLAKDEKVEYVDVRFCDLPGIMQHFTIIPASAFDKSVFDDGLAFDGSIRGFQSIHESDMLLDPDPETARIDPFR
<i>M. leprae</i>	-----MTEKTSDDVLKAKDENIEFVDVQCDLPGIMQHFTIIPASAFDKSVFDDGLAFDGSIRGFQSIHESDMLLDPDPETARIDPFR
<i>M. avium</i>	-----MTETTPDDVFKLAKDEKVEYVDVRFCDLPGIMQHFTIIPVSVFDDSVFDDGLAFDGSIRGFQSIHESDMLLDPDPETAQIDPFR
<i>M. ulcerans</i>	-----MTDKTPDDVFKLAKDQVEYVDVRFCDLPGVMQHFTIIPVSVFDDSVFDDGLAFDGSIRGFQSIHESDMLLDPDPETAIDQFR
<i>M. flavescens</i>	-----MAEQTADDI IKLIKDESVYVDIRFCDLPGVQHFSPASAFDESVEFDDGLAFDGSIRGFQSIHESDMLLDPDPETARIDPFR
<i>M. smegmatis</i>	-----MAEKTSDDIFKLIKDENVEYVDIRFCDLPGVQHFSPASAFDESVEFDDGLAFDGSIRGFQSIHESDMLLDPDPETARIDPFR
<i>Mycobacterium KMS</i>	-----MADKTADDI IKLIKDESVYVDIRFCDLPGVQHFSPASAFDESVEFDDGLAFDGSIRGFQSIHESDMLLDPDPETARIDPFR
<i>M. vanbaalenii</i>	-----MAEKTADDI IKLIKDEQVEYVDIRFCDLPGVQHFSPASAFDESVEFDDGLAFDGSIRGFQSIHESDMLLDPDPETARIDPFR
<i>N. farcinia</i>	-----MTFSTADEVLIQYIKEDQYIYDIDIRFCDLPGVQHFSPAKAFPTADLAEGLAFDGSIRGFQSIHESDMLLDPDYSTARIDPFR
<i>C. jeikeium</i>	MCCFYILSQQFEIPTARNGRNTVSFKTAEVAKYIKDNDVEFVDVRFCDLPGIEQHFTIIPASEFDEDAEGLAFDGSIRGFQSIHESDMLLDPDLATAKIDPFR
<i>C. diphtheria</i>	-----MAFTSTEDVLIKIEENVEFVDIRFCDLPGIEQHFTIIPASMFTEAAEEDGFAFDGSIRGFQSIHESDMLLDPDLATAKIDPFR
<i>C. efficiens</i>	-----MAFNTPEEVTIKIDENVEFVDVRFCDLPGIEQHFTIIPASALDEDAIEEGLAFDGSIRGFQSIHESDMLLDPDLATAKIDPFR
<i>C. glutamicum</i>	-----MAFETPEEIVKFIKIDENVEFVDVRFCDLPGIEQHFTIIPASAFDADTIEEGLAFDGSIRGFQSIHESDMLLDPDLGTATLDPFR
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<i>M. tuberculosis</i>	AAKTLNINFFVHDPFTLEPYSRDPNRIARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRANGSFYEVDAISGWNMTGAATEADGSPNRYKVRHKGGFYFVA
<i>M. leprae</i>	AAKTLNINFFVHDPFTLEPYSRDPNRIARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRANGSFYEVDAISGWNMTGAATEADGSPNRYKVRHKGGFYFVA
<i>M. avium</i>	EAKTLNINFFVHDPFTLEPYSRDPNRIARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRANGSFYEVDAISGWNMTGSPNELDGSNRYKVRHKGGFYFVA
<i>M. ulcerans</i>	EAKTLNINFFVHDPFTLEPYSRDPNRIARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRANGSFYEVDAISGWNMTGAANEADGPNLGYKVRHKGGFYFVA
<i>M. flavescens</i>	AAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSKINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>M. smegmatis</i>	AAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSKINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>Mycobacterium KMS</i>	HAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSKINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>M. vanbaalenii</i>	AAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>N. farcinia</i>	AAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>C. jeikeium</i>	KAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>C. diphtheria</i>	KAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>C. efficiens</i>	KAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
<i>C. glutamicum</i>	KAKTLNINFFVHDPFTREAYSRDPRNVARKAENYLSTGIADTAYFGAAEFYIPDSVSPDSRINGTFFYEVDSGSGWNTGEPFEADGSANRYKVRHKGGFYFVA
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<i>M. tuberculosis</i>	PNDQYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHCHQSLWKDGLAPLMYDETYGA
<i>M. leprae</i>	PNDQYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>M. avium</i>	PVDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>M. ulcerans</i>	PVDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>M. flavescens</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>M. smegmatis</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>Mycobacterium KMS</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>M. vanbaalenii</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>N. farcinia</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>C. jeikeium</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>C. diphtheria</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>C. efficiens</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
<i>C. glutamicum</i>	PYDHYVLDLDRKMLTNLINSGLFLEKGGHVEVGGGQAEINQYFNSLLHAADDMLQYKIIKNTAWQNGKTVTFMFKPLFGDNGSGMHTHQSLSWKDGLAPLMYDETYGA
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<i>M. tuberculosis</i>	GLSDTARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>M. leprae</i>	GLSDTARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>M. avium</i>	GLSDTARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>M. ulcerans</i>	GLSDTARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>M. flavescens</i>	GLSDMARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>M. smegmatis</i>	GLSDIARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>Mycobacterium KMS</i>	GLSDTARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>M. vanbaalenii</i>	GLSDIARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>N. farcinia</i>	GLSDIARHYIGGLLHHAPSLLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>C. jeikeium</i>	GLSDIARHYIGGLLHHAGVLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>C. diphtheria</i>	GLSDIARHYIGGLLHHAGVLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>C. efficiens</i>	GLSDIARHYIGGLLHHAGVLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
<i>C. glutamicum</i>	GLSDIARHYIGGLLHHAGVLAFTNPTVNSYKRLVPGYFAPINLVYSQRNRSACVRIPIITGNNPKAKRLEFRCPDSSGNPYLAFSAAMLGLDGIKNEPQ
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<i>M. tuberculosis</i>	APVDRDYELPPEEAAIPQAPTQLASVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>M. leprae</i>	APVDRDYELPPEEAAIPQAPTQLASVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>M. avium</i>	APVDRDYELPPEEAAIPQAPTQLASVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>M. ulcerans</i>	APVDRDYELPPEEAAIPQAPTQLAAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>M. flavescens</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>M. smegmatis</i>	QPDVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>Mycobacterium KMS</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>M. vanbaalenii</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>N. farcinia</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>C. jeikeium</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>C. diphtheria</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>C. efficiens</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
<i>C. glutamicum</i>	APVDRDYELPPEEAAIPQAPTSLSAVIDRLEEDHEYLTEGGVFTDLIETWISFKRENEIMPVIRPHYPYFALYDYV
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Figure 2.2. Glutamine synthetase protein homology between different mycobacteria. The GS consensus signature [FYWL]-D-G-S-S-x(6,8)-[DENQSTAK]-[SA]-[DE]-x(2)-[LIVMFYA]-x(3,5)-[NPAT]-G-[GSTAN]-G-x-H-x(3)-S in grey and the adenylylation site consensus sequence K-[LIVM]-x(5)-[LIVMA]-D-[RK]-[DN]-[LI]-Y where Tyr (Y) is the site of adenylylation in green. Putative active site amino acid residues (Eisenberg *et al.*, 2002) are indicated in red. An asterisk (*) indicates identical sequence.

2.2.3 Genetic stability of *glnA1* during *M. tuberculosis* strain diversification

2.2.3.1 Evolutionary diversity of *M. tuberculosis* clinical isolates

Mycobacterium tuberculosis clinical isolates were typed by IS6110 RFLP genotyping methods and were grouped according to similarity index, calculated using the Dice coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic mean clustering method, GelCompar version 4.0) (Hermans *et al.*, 1995). The GelCompar presentation of the IS6110 banding patterns (Figure 2.3) shows that the clinical isolates are largely unrelated and diverse, although some may be grouped into families according to pattern similarities exceeding 65% (Warren *et al.*, 2000a). In this study, 54 *M. tuberculosis* isolates were selected, which included 9 low IS6110 copy ($n < 5$) and 45 high IS6110 copy number ($n \geq 5$) *M. tuberculosis* family groupings, with each having probably independently evolved from a mutual ancestral strain (Kremer *et al.*, 1999). Table 2.2 demonstrates that the isolates included in this study represent highly prevalent strain families, such as family 29 (Beijing families) and family 11 (Richardson *et al.*, 2002; Victor *et al.*, 2004), as well as representatives from less prevalent strain families (Richardson *et al.*, 2002).

Table 2.2*: *Mycobacterium tuberculosis* strain family prevalence in a high incidence setting from where the clinical isolates used in the current study were derived. *Adapted from Richardson *et al.*, 2002)

Strain Family	% of cases	Strain Family	% of cases
1	0.6	17	1.1
2	3.9	18	2.3
3	0.4	19	0.4
4	3.1	20	0.7
5	0.2	21	2.2
6	0.9	22	0.5
7	2.0	23	1.1
8	0.3	24	1.6
9	2.3	25	1.6
10	0.5	26	1.4
11	19.1	27	0.3
13	2.1	28	9.7
14	3.2	29	16.8
15	1.4	30	0.3
16	1.5		

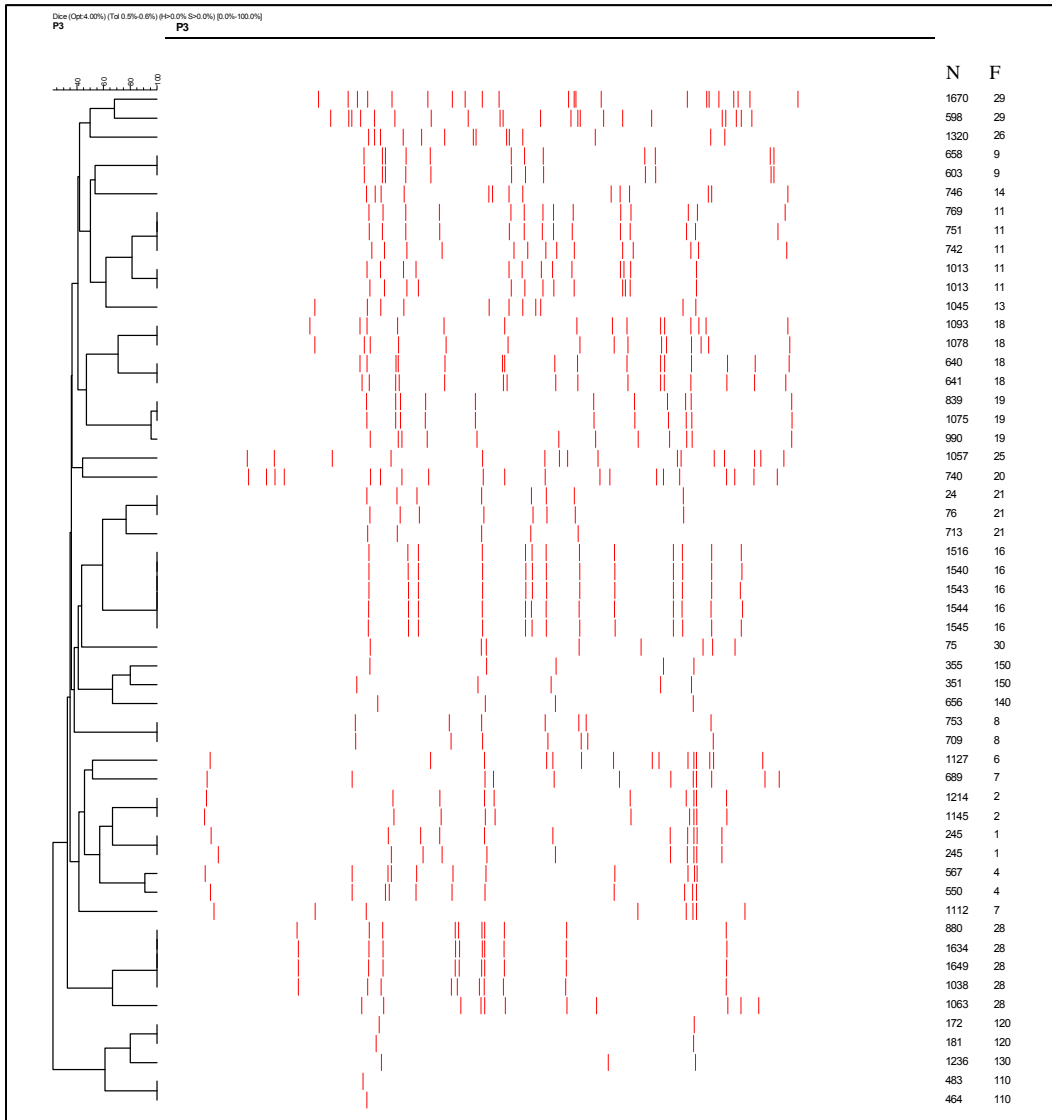


Figure 2.3. GelCompar-generated dendrogram illustrating the extent of genetic diversity between the *M. tuberculosis* clinical isolates included in the *glnA1* sequence comparison study. Each lane represents a single isolate of *M. tuberculosis*. The observed banding pattern is that of the IS6110 insertion element standard for the classification of *M. tuberculosis* genotypes into the appropriate *M. tuberculosis* strain families (Van Soolingen *et al.*, 1993). The Clinical isolate Number (N), and the *M. tuberculosis* strain family from which the isolate is derived (F) is shown.

Table 2.3: Characteristics of the clinical *M. tuberculosis* isolates used in this study. Included are the South African Western Cape (SAWC) database number, strain family and antibiotic susceptibility of these isolates with known sensitivity results. Blank spaces indicate that information is not available. (Table continued on next page).

SAWC	Family	IS6110_3	Resistance Tested	Isoniazid	Rifampin	Streptomycin	Emb	Pyrazinamide	Ethambutol	Kanamycin
24	21	95	Y	Sensitive	Sensitive		Sensitive			
75	30	482	Y	Sensitive	Sensitive		Sensitive			
76	21	95	Y	Sensitive	Sensitive		Sensitive			
172	120	191	Y	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
181	120	191	Y	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
245	1	141	Y	Sensitive	Sensitive	Sensitive	Sensitive		Sensitive	
355	150	338	N							
464	110	324	N							
483	110	324	N							
550	4	403	N							
567	4	403	N							
598	29	322	N							
603	9	76	Y	Sensitive	Sensitive					
640	18	121	N							
641	18	121	N							
656	140	381	N							
658	9	76	Y	Sensitive	Sensitive					
689	7	196	Y	Sensitive	Sensitive					
709	8	174	Y		Resistant					
713	21	171	N							
740	20	137	N							
742	11	34	Y	Sensitive	Sensitive					
746	14	374	Y	Sensitive	Sensitive					
751	11	34	Y	Sensitive	Sensitive					

SAWC	Family	IS6110_3	Resistance Tested	Isoniazid	Rifampin	Streptomycin	Emb	Pyrazinamide	Ethambutol	Kanamycin
753	8	174	Y		Resistant					
769	11	34	N							
839	19	109	Y	Resistant	Sensitive	Sensitive	Sensitive		Sensitive	
880	28	266	Y	Sensitive	Sensitive					
990	19	497	Y	Resistant	Sensitive	Sensitive	Sensitive		Sensitive	
1013	11	33	U							
1038	28	266	Y	Sensitive	Sensitive					
1045	13	79	Y	Sensitive	Sensitive					
1057	25	133	Y	Sensitive	Sensitive					
1063	28	288	Y	Sensitive	Sensitive					
1075	19	109	Y	Resistant	Sensitive	Sensitive	Sensitive		Sensitive	
1078	18	125	Y	Sensitive	Sensitive					
1093	18	125	Y	Sensitive	Sensitive					
1112	7	553	Y	Sensitive						
1127	6	131	N							
1145	2	190	N							
1214	2	190	N							
1236	130	326	Y	Sensitive	Sensitive					
1320	26	97	N							
1516	16	93	N							
1540	16	93	N							
1543	16	93	N							
1544	16	93	N							
1545	16	93	N							
1634	28	266	Y	Sensitive	Sensitive					
1649	28	266	Y	Sensitive	Sensitive					
1670	29	216	N							

The clinical isolates include some antibiotic-resistant and – sensitive *M. tuberculosis* phenotypes (Table 2.3). Drug sensitivity data of some of the isolates are not known, as some of the isolates have not been tested at the time of writing this manuscript.

2.2.3.2 Sequence comparison of *glnA1* from *M. tuberculosis* clinical isolates

The structural *glnA1* gene (1434 bp) and its 5' and 3' regions were PCR-amplified (Materials and Methods 3.1.2) from purified genomic DNA of the 54 *M. tuberculosis* clinical isolates described above. Oligonucleotides for PCR were designed to overlap by 46 to 116 bp to allow the accurate intermediate sequences to be constructed from incomplete sequencing data (Figure 2.4). Polymerase chain reactions were optimised for each oligonucleotide pair and single amplification products were obtained. These products were purified and submitted for direct automated sequencing. The complete *glnA1* sequences constructed in this manner were matched to the publicly available genome sequences of the *M. tuberculosis* H37Rv reference strain, *M. tuberculosis* CDC1551 and *M. tuberculosis* 210 (F11 clinical isolate) through BLAST comparison. Sequence comparisons showed no changes (mutation, deletion or insertion) in the *glnA1* structural gene and surrounding 3' and 5' regions in any of the *M. tuberculosis* clinical isolates. The 3' and 5' surrounding regions of the *glnA1* locus included all the regulatory elements described for *glnA1* transcription and regulation (Harth and Horwitz, 1997).

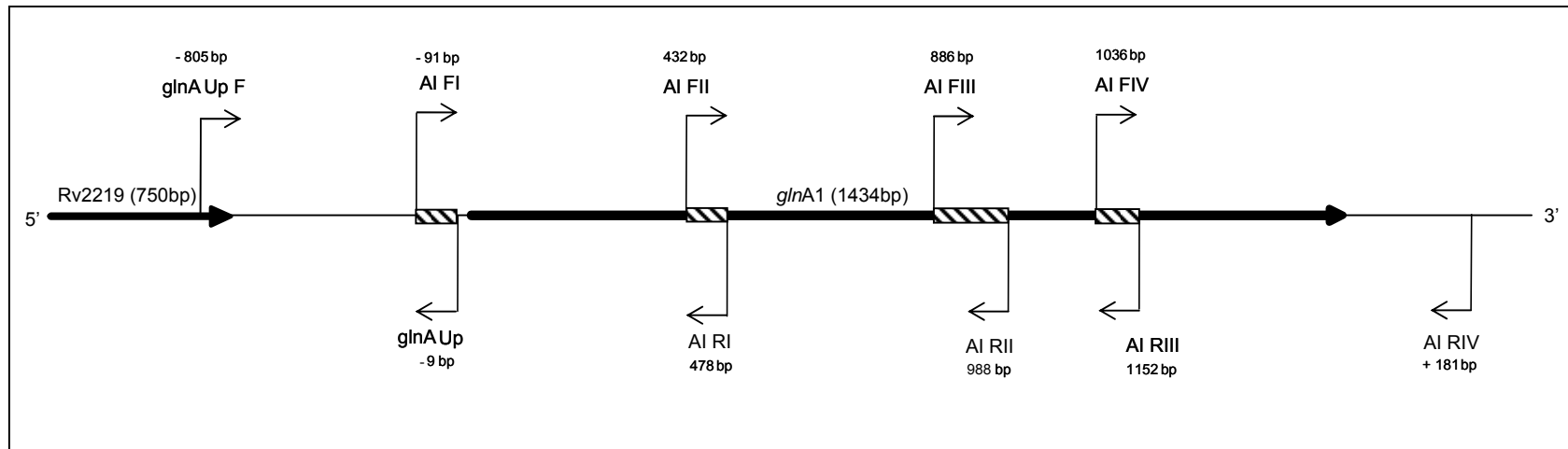


Figure 2.4. The *glnA1*-locus (Rv2219 and *glnA1* gene loci indicated in bold) of *M. tuberculosis* including its' 5'- and 3' surrounding regions were amplified with 5 PCR-oligonucleotide sets that were designed to amplify DNA fragments 294 bp to 796 bp in length. The PCR fragments had overlapping sequences (indicted as boxes) which facilitated the assembly of the full target region (-805bp to 1181bp). Priming positions relative to the *glnA1* start codon and primer orientations are indicated (arrows, see Materials and Methods, Table 4).

2.3 DISCUSSION

2.3.1. Glutamine synthetase evolution in the *Actinobacteria*

To investigate the evolutionary relationship and origins of the different *glnA* homologs present in the *M. tuberculosis* genome (*glnA1*, *glnA2*, *glnA3* and *glnA4*), the protein sequences they encode were investigated through phylogenetic analysis of all the GS encoding sequences present in genomes of the phylum *Actinobacteria*. All the *Actinobacteria* included in this study had GS I (encoded by *glnA1*) and GS II (encoded by *glnA2*) protein homologs. The phylogenetic reconstruction showed that the GS I sequence (encoded by *glnA1*) may be the most closely related to a actinobacterial GS progenitor that underwent a duplication event and diverged into the GS I and GS II (encoded by *glnA2*) sequences during the speciation of most of the members of the phylum. An exception was observed in *Symbiobacterium thermophilum*, a G+C rich obligate commensal thermophile that requires co-culture with an associating *Bacillus* strain to provide it with certain metabolites (Ohno *et al.*, 2000), such as carbon dioxide (Ueda *et al.*, 2004). *Symbiobacterium thermophilum* posses only a GS I encoding sequence and this sequence might be more closely related to the *Actinobacteria* GS progenitor sequence and might not have undergone duplication into the separately evolved GS I and GS II sequences. This observation is supported by the matching GS I phylogenetic tree to the actinobacterial phylogeny based on the CTP-synthetase and 16S rRNA gene sequences (Gao and Gupta, 2005), which suggest that the GS I sequence changed with its host organism. These observations are consistent with a previous study that investigated the divergence of GS enzymes during prokaryote and eukaryote evolution (Kumada *et al.*, 1993). In that study it was shown that GS I (present only in prokaryotes, see Section 1.6.1) and GS II may have shared a common pre-prokaryote ancestor that underwent a duplication event (Kumada *et al.*, 1993). The authors further speculated that these enzymes may have become specialised due to the central role of this enzyme in amino acid metabolism and nitrogen assimilation during speciation.

The role of GS I in bacterial growth has been the subject of various studies, but the role of the functionally different GS II enzyme (Section 1.6.1) is not well understood. It has

been shown that GS II plays an essential role in ammonia assimilation during nitrogen starvation in some *Actinobacteria*, such as the *Frankia* species (Tsai and Benson, 1989), but in other *Actinobacteria*, such as *M. tuberculosis*, conflicting evidence exists which suggests that GS II is either essential (Collins *et al.*, 2002) or non-essential (Sasseti *et al.*, 2003) for *M. tuberculosis* growth. Since the GS II enzyme might have been a duplication product of a GS I-like enzyme, it can be argued that GS II might play a minor role in prokaryotes, but became more specialised during speciation until it became the major GS enzyme in eukaryotes, in which GS I was subsequently lost. However, the phylogenetic reconstruction also suggests that either GS II or one of the other GS enzymes encoded by *glnA3* and *glnA4* could compensate for the loss of GS I in some *Actinobacteria* such as *Rubrobacter xylanophilus*. The *Rubrobacteriales* are extremophiles with high resistance to damage caused by ionising radiation (Asgarani *et al.*, 2000) and may be one of the deepest branches within the *Actinobacteria* phylum that preceded the emergence of the *Bifidobacteriales*, *Micrococcineae* and CMN subgroup (Gao and Gupta, 2005). Therefore GS I may have existed in *R. xylanophilus*, but became lost. It is interesting to observe that evidence has been presented that shows GS II to be more heat labile than GS I (Hillemann *et al.*, 1993) and probably became lost in the extreme habitat of the *Rubrobacteriales*.

The GS II sequence underwent a further duplication in some *Actinobacteria*, which may have given rise to the GS sequences encoded by *glnA3* and *glnA4*. The evolutionary distance between the clades formed by the *glnA3* and *glnA4* GS sequences suggest that the sequences diverged simultaneously at the time of the *Actinobacterial* speciation. In some *Actinobacteria*, *glnA2* diverged only into the GS sequence encoded by *glnA4* (*Brevibacterium linens*, *Kineococcus radiotolerans* and *Streptomyces* sp.), while the *glnA3* GS sequence is mainly restricted to the more recently speciated members of the CMN subgroup. It has been hypothesised that during evolution, non-essential genes may undergo change resulting in non-functional truncated products or even be “lost” due to the continual and reductive evolution of the bacterial genome, such as that of *M. leprae* and *M. ulcerans* (Cole *et al.*, 2001; Eiglmeier *et al.*, 2001; Sakharkar *et al.*, 2004; Stinear *et al.*, 2007). The absence of *glnA3* and *glnA4* may indicate that the GS enzymes encoded by these genes may have been redundant and therefore were deleted from these mycobacterial genomes. In support of this observation it has been shown that *glnA3* and *glnA4* are non-essential for the growth of other mycobacteria such

as *M. tuberculosis* (Sasseti *et al.*, 2003; Harth *et al.*, 2006; Lee *et al.*, 2006). However, the possibility that the paralogous GS enzyme sequences encoded by *glnA3* and *glnA4* have become specialised and necessary for growth in other *Actinobacteria* under certain conditions cannot be excluded.

2.3.2 Glutamine synthetase I evolution in the high G+C genome content actinobacteria

Genes needed for bacterial growth and survival, or essential genes, are mostly retained with minor sequence changes in genomes, suggesting that major genetic change in essential genes (leading to variation in the amino acid sequence resulting in change in enzyme expression or function) may be selected against during evolution (Sasseti *et al.*, 2003). The importance of GS I during bacterial growth is also reflected in the high degree of sequence homology observed between the species of the high G+C *Actinobacteria* CMN sub-group. The homology between actinobacterial GS I protein sequences is very pronounced in the conservation of amino acids putatively involved in the GS catalytic site and consensus signatures for ATP-binding and adenylylation regions. The high degree of homology, especially in amino acids which may play a role in the catalytic site structure and ATP- binding and adenylylation signatures, may suggest that changes in these sequences may be subjected to selective pressure.

2.3.3 Evolutionary stability of the *M. tuberculosis glnA1* locus

To further study the evolutionary stability of GS I, the *glnA1* locus of *M. tuberculosis* isolates has been studied through sequencing. The success of *M. tuberculosis* as a pathogen lies in its ability to adapt to a wide variety of changing environmental conditions during host infection. After *M. tuberculosis* infection, the host immune system becomes activated in an attempt to contain the bacterial infection through the formation of granulomas (Schlesinger *et al.*, 1990), which are aggregates of immune cells such as macrophages and lymphocytes. It has been hypothesised that adaptation to the constantly changing granulomatous environment might exert an evolutionary pressure resulting in genetic changes in the *M. tuberculosis* genome (Warren *et al.*, 2002a). These changes may impact on the survival fitness of *M. tuberculosis* strains (Warren *et al.*, 2000), which may subsequently have an influence on virulence due to phenotypic

and biological variance, causing some strains to be more infectious than others (Generozov *et al.*, 2000; Dormans *et al.*, 2004; Petrelli *et al.*, 2004; Janulionis *et al.*, 2005; Likhoshvai *et al.*, 2006). Genes have been identified of which mutations lead to an increase in *M. tuberculosis* virulence in mice, such as *pepD* (probable aminopeptidase) and *umaA1* (mycolic acid transferase) (McAdam *et al.*, 2002). Mutations in other genes, such as *erp* (Berthet *et al.*, 1998), *sigF* (Chen *et al.*, 2000) and *hma* (Dubnau *et al.*, 2000) may lead to *M. tuberculosis* attenuation in mice. Therefore the granuloma may provide *M. tuberculosis* with an environment that limits spread of the bacilli (Saunders *et al.*, 1999) but wherein they may remain viable for extended periods of time during which they may undergo significant evolutionary change (Warren *et al.*, 2002a). The evolutionary stability, or ability of *glnA1* to undergo genetic change during *M. tuberculosis* strain evolution, may be reflected in the *glnA1* sequence of separately evolved *M. tuberculosis* strains. It has been shown that disruptions in the *M. tuberculosis* *glnA1* gene results in glutamine auxotrophy, but it is not known if polymorphisms in the coding sequence or promoter regions may occur that might result in a slight alteration of *glnA1* activity. Here the *glnA1* locus of 54 evolutionary diverse *M. tuberculosis* clinical isolates was sequenced and no change was observed in the *glnA1* locus or its immediate 5'- and 3' flanking DNA regions. These strains included highly prevalent and less prevalent clinical strain families from high incidence settings in Cape Town, South Africa. It has been hypothesised that more prevalent strain families might possess increased virulence, or success in infection and causing disease, over less prevalent strain families (Richardson *et al.*, 2002). The lack of observed *glnA1* genetic diversity may imply that the *glnA1* DNA sequence is genetically stable and may imply that the evolutionary process may select against genetic change that can influence gene activity. It should also be noted that assumptions on similar enzyme activity cannot necessarily be made from the observation of genetic similarity, since it has been shown that variation in gene expression exists amongst different clinical *M. tuberculosis* isolates grown under the same conditions in liquid culture (Gao *et al.*, 2005). In that study it was shown that *glnA1* expression varied between 10 logarithmically growing *M. tuberculosis* clinical isolates. This observation implies that *glnA1* expression (perhaps also enzyme activity) may also differ between *M. tuberculosis* growth *in vivo* and *in vitro*.

CHAPTER THREE

***MYCOBACTERIUM TUBERCULOSIS* GLNA1 EXPRESSION REGULATION AND GS EXPORT**

“In all science, error precedes the truth, and it is better it should go first than last”

Hugh Walpole

Note: The experimental work (GS precipitation) was carried out in the Division of Bio-molecular sciences, Stellenbosch University under direction of C. Kenyon, DST.

3.1 INTRODUCTION

Structural studies of the gene encoding GS in *M. tuberculosis*, *glnA1*, could not identify any of the common features necessary for protein export (protein transport across the cell membrane to the cell wall) or secretion into the exogenous environment by known bacterial systems (Harth *et al.*, 1994; Tullius *et al.*, 2001). However, previous studies have shown the presence of GS activity in the *M. tuberculosis* culture filtrate (Harth *et al.*, 1994), or have identified GS in the culture filtrate of *M. tuberculosis* cultures (Sonnenberg and Belisle, 1997, Malen *et al.*, 2007). Structural studies of *glnA1* have identified two promoter regions upstream of *glnA1* that might regulate *glnA1* transcription under conditions of nitrogen excess, or during nitrogen starvation (Harth and Horwitz, 1997). It was shown that *M. tuberculosis glnA1* transcription was induced from a 45 bp upstream σ^{60} -like promoter element under conditions of nitrogen excess, while nitrogen limitation resulted in the inhibition of the 45 bp upstream promoter region and transcription of *glnA1* was initiated from a 116 bp upstream promoter region (Harth and Horwitz, 1997). It was shown that transcription initiated from either of these promoter regions resulted in mRNA molecules of different length, and it has been hypothesised that the un-translated mRNA leader of either of the mRNA molecules may encode the signal for GS secretion (Harth and Horwitz, 1997).

Alternatively, it has been hypothesised that GS is released by *M. tuberculosis* through the processes following cell death (Tullius *et al.*, 2003). It was hypothesised that large *sec*-secretion signal less proteins, such as GS and super oxide dismutase (SOD) might be present in the *M. tuberculosis* culture filtrate due to leakage from *M. tuberculosis* or autolysis of the bacilli. However, another study has shown that SOD is actively secreted by *M. tuberculosis* by the *secA2*-dependent secretion system (Braunstein *et al.*, 2003). It was shown that this secretion pathway does not require a *sec*-secretion signal peptide, but it was not determined if *M. tuberculosis* GS might be secreted by the *secA2*-dependant secretion pathway.

3.1.1 Study Aims:

The secretion mechanism of *M. tuberculosis* GS was studied in a heterologous mycobacterial expression host (*M. smegmatis*). This has been achieved through

investigating the sub-cellular compartmentalisation of a recombinant C-terminal tagged *M. tuberculosis* GS in the *M. smegmatis* expression host. The specific aims were:

3.1.1.1 To investigate if the 116 bp and 45 bp upstream promoter regions of *glnA1* play a role in *M. tuberculosis* GS secretion

3.1.1.2 To further investigate GS secretion due to *glnA1* transcription from the above promoter regions, GS sub-cellular distribution in response to varying conditions of nitrogen availability would have to be studied.

3.1.1.3 To investigate the role of cell death on *M. tuberculosis* GS secretion. This will be determined in *M. smegmatis* as expression host during the stationary phase of growth.

3.1.1.4 To determine if *M. tuberculosis* GS export could be mediated by the SecA2 – translocase. This will be determined through studying the protein-protein interaction of *secA2* with GS in an *E. coli*-based expression host.

3.1.1.5 To determine if GS is exported to the cell wall of slow-growing mycobacteria, GS will be studied in the cell wall fractions of *M. bovis* BCG.

3.1.1.5.1 To determine if differences in GS distribution are observed in *M. bovis* BCG. It will be attempted to study these differences in *M. smegmatis*. This will also be carried out through the stable integration of *M. tuberculosis* into the *M. smegmatis* chromosome.

3.2 RESULTS

3.2.1. *M. tuberculosis* GS export and secretion by *M. smegmatis*

To determine if the 116bp and 45bp promoter elements of *M. tuberculosis glnA1* are involved in GS secretion, *glnA1* expression constructs were generated that included either both the 116bp and 45bp promoter elements (“*glnA1* long” clone, Figure 3.1), or only the 45bp promoter element (“*glnA1* short” clone, Figure 3.1). To allow the detection of the *M.*

tuberculosis GS resulting from these expression constructs, both *glnA1* sequences were synthesised using an antisense PCR oligonucleotide that encoded a hexa-histidine sequence (6x his) and included a stop codon (Figure 3.1). Therefore, both recombinant GS proteins would be tagged with a 6x his sequence at the C-terminal (total theoretical molecular weight (MW) = 54.39 kDa). The *glnA1* “long” and “short” constructs were cloned into a mycobacterial expression vector (p19Kpro; Appendix C. 1) that contained a hygromycin B-resistance marker that allowed antibiotic selection of recombinant *M. smegmatis* cultures. These *M. tuberculosis glnA1* constructs were expressed in *M. smegmatis* cultured to the mid-logarithmic growth phase (OD_{600} of 0.3 - 0.4) in Kirchners’ minimal media alone (representing a low nitrogen medium) or Kirchners’ minimal media containing ammonium sulphate ($(NH_4)_2SO_4$) to a final concentration of 38 mM (high nitrogen medium) and hygromycin B. *Mycobacterium smegmatis* cultures transformed with the *glnA1* “long” and “short” showed comparable doubling times (as measured by OD_{600} readings) to control *M. smegmatis* transformed with an “empty” expression plasmid in both Kirchners’ minimal media or Kirchners’ minimal media containing 38 mM $(NH_4)_2SO_4$ indicating that the expression of the recombinant C-terminal tagged *M. tuberculosis* GS did not have an effect on the *M. smegmatis* growth rate.

To study the transport of the recombinant C-terminal tagged *M. tuberculosis* GS from the cytosol to other cellular structures of the *M. smegmatis* expression host, the *M. smegmatis* cultures expressing the recombinant *M. tuberculosis* GS proteins were fractionated into its sub-cellular fractions (cytosol, cell membrane, cell wall and culture filtrate) through differential centrifugation (Materials and Methods 9.1). Culture filtrate proteins were concentrated approximately 30x by exclusion centrifugation (Materials and Methods 9.1). The protein concentrations of the purified sub-cellular fractions were determined using the Bradford methods (Materials and Methods 9.2) and equal amounts of protein (6 μ g) from each sub-cellular fraction was Western blotted under denaturing conditions (SDS PAGE, Materials and Methods 4.1). Recombinant C-terminal tagged *M. tuberculosis* GS was detected on the Western blots using an anti-6x his primary antibody (Materials and Methods 10.1).

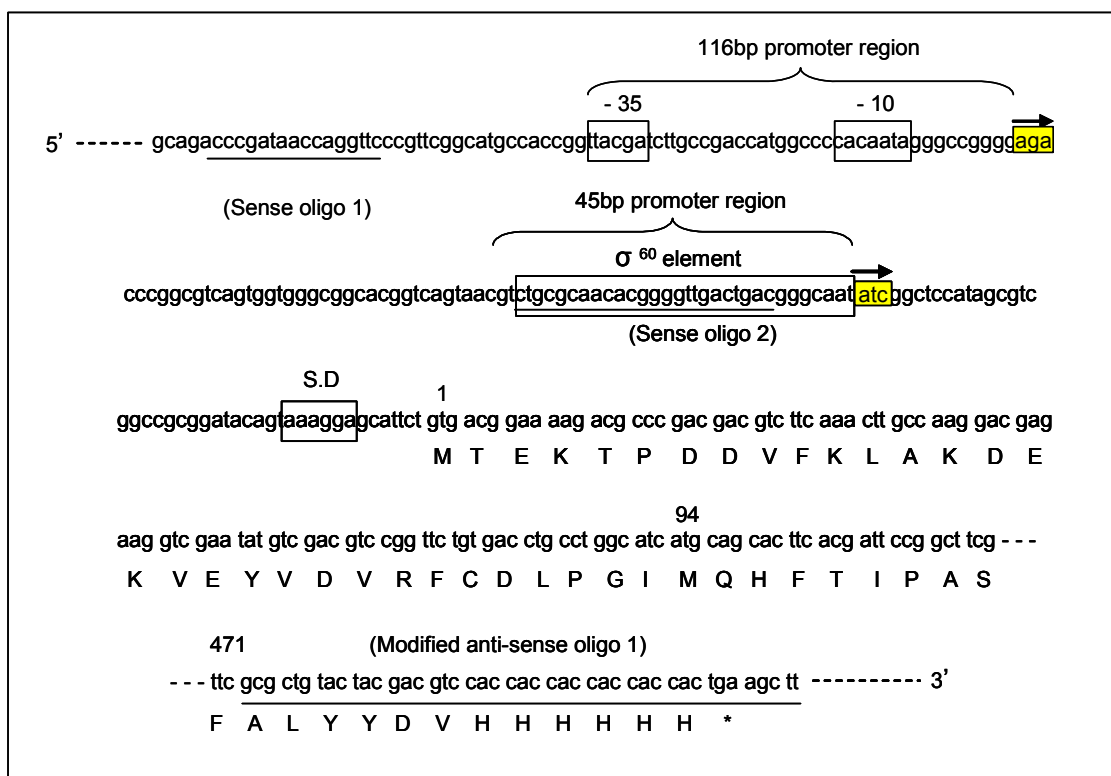


Figure 3.1. Genetic and protein sequences of *M. tuberculosis* *glnA1* “long” and “short” clones transformed into a *M. smegmatis* expression host. The upstream promoter regions of *glnA1* are indicated by boxes, with the transcription start sites following each promoter region indicated by arrows. The N- as well as modified C- terminal GS amino acid sequence is indicated with the corresponding DNA sequence. The *glnA1* “long” clone included both putative upstream promoter regions (PCR amplified using sense oligonucleotide 1, underlined), while the *glnA1* “short” construct included only the immediate upstream σ^{60} element (PCR amplified using sense oligonucleotide 2). The anti-sense PCR oligonucleotide was modified to include a 6x his encoding region followed by a stop codon and a *hindIII* restriction site.

Figure 3.2 shows that during growth in low nitrogen Kirchners’ media, recombinant C-terminal tagged *M. tuberculosis* GS (55 kDa) was exported from the cytosol to the cell membrane and cell wall fractions of *M. smegmatis* cultures transformed with the “*glnA1* long” construct. Under the same growth conditions, a low concentration of recombinant C-terminal tagged *M. tuberculosis* GS could be detected in the cell membrane fraction of *M. smegmatis* cultures transformed with the “*glnA1* short” constructs, suggesting that the expression levels

of the *glnA1* “short” construct was low during the *M. smegmatis* growth conditions used. The MW of the recombinant C-terminal tagged *M. tuberculosis* GS expressed from the *M. tuberculosis* *glnA1* “short” construct was similar to that detected in the sub-cellular fractions of the *M. smegmatis* culture transformed with the “*glnA1* long” construct, indicating that the GS expressed from either of the *M. tuberculosis* *glnA1* “long” and “short” constructs were of similar MW. No recombinant C-terminal tagged *M. tuberculosis* GS could be detected in the concentrated culture filtrates of *M. smegmatis* cultures expressing the *glnA1* “long” or *glnA1* “short” constructs, indicating that recombinant C-terminal tagged *M. tuberculosis* GS was not secreted by *M. smegmatis* during the mid-logarithmic growth phase in low nitrogen Kirchners medium.

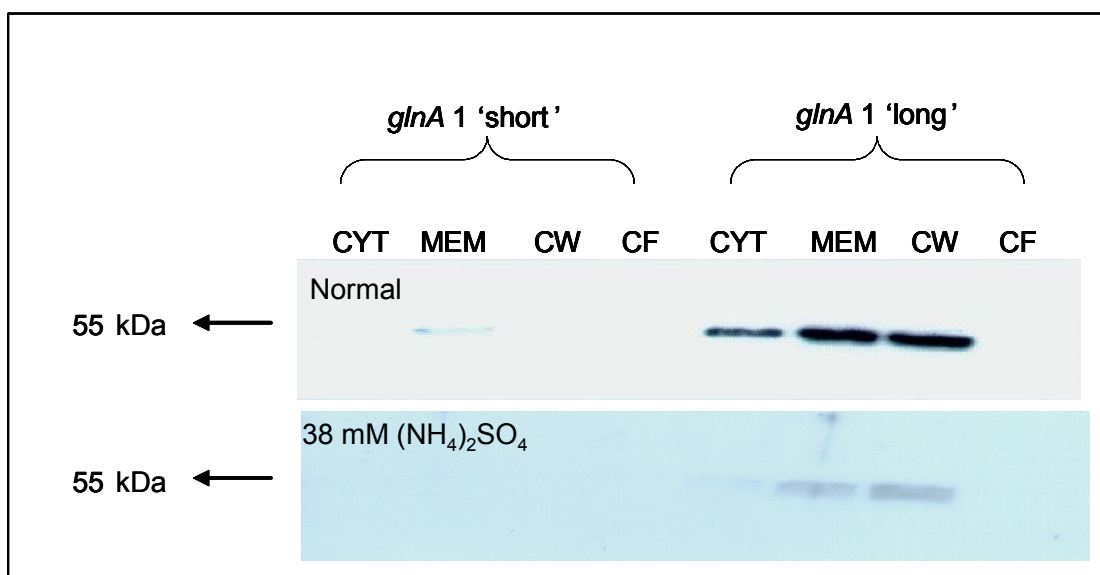


Figure 3.2. Sub-cellular location of recombinant *M. tuberculosis* GS in logarithmically growing *M. smegmatis* transformants expressing *M. tuberculosis* *glnA1* from either both *glnA1* upstream promoter regions (*glnA1* “long”), or only the immediate upstream promoter (*glnA1* “short”). These *M. smegmatis* cultures were grown in either Kirchner’s minimal medium (low nitrogen) or Kirchner’s minimal media containing ammonium sulphate to a final concentration of 38 mM (high nitrogen medium). The sub-cellular protein fractions included the cytosol (CYT), cell membrane (MEM), cell wall (CW) and concentrated culture filtrate (CF) proteins.

To investigate whether sub-cellular distribution of recombinant C-terminal tagged *M. tuberculosis* GS expressed from the 45 bp σ^{60} -like promoter element differed from the GS

expressed from the 116 bp promoter region, the *M. smegmatis* *glnA1* expression host was cultured in Kirchners medium containing $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 38 mM (high nitrogen medium). To minimise the effect of $(\text{NH}_4)_2\text{SO}_4$ assimilation during *M. smegmatis* growth, which may influence the expression of the recombinant C-terminal tagged *M. tuberculosis* GS due to lower effective $(\text{NH}_4)_2\text{SO}_4$ concentrations, an *M. smegmatis* start-culture was prepared in Kirchners media and cultured to a OD_{600} of 0.2. The *M. smegmatis* culture was collected through centrifugation and inoculated into the same volume of Kirchners media, with the added $(\text{NH}_4)_2\text{SO}_4$ as the original start-culture and grown to an $\text{OD}_{600} = 0.3 - 0.4$. It was attempted to limit a “temperature shock-response” of the *M. smegmatis* culture during the transfer from the original $\text{OD}_{600} = 0.2$ start-culture to the Kirchners medium containing the $(\text{NH}_4)_2\text{SO}_4$ by performing all steps (centrifugation and temperature of target media) at 37°C . The *M. smegmatis* cells were collected and 6 μg of each sub-cellular fraction were collected and Western blotted as before.

Figure 3.2 shows that the expression of recombinant C-terminal tagged *M. tuberculosis* GS was reduced in *M. smegmatis* cultures expressing both the *glnA1* “long” and “short” constructs. Contrary to expectations, no recombinant C-terminal tagged *M. tuberculosis* GS could be detected in any of the sub-cellular compartments or culture filtrate of the *M. smegmatis* host strain transformed with the “*glnA1* short” construct. Lower concentrations of recombinant C-terminal tagged *M. tuberculosis* GS were observed in the cell membrane and cell wall of *M. smegmatis* host strains transformed with the “*glnA1* long” construct. However, no recombinant C-terminal tagged *M. tuberculosis* GS could be detected in the concentrated culture filtrates of either *M. smegmatis* *glnA1*-expression host, indicating that (together with the above observations) increased nitrogen availability caused a down regulation of *M. tuberculosis* GS expression and did not result in the secretion of recombinant C-terminal tagged *M. tuberculosis* GS.

As the method of analysing the culture filtrate fraction described above may have certain technical shortcomings, such as masking of the recombinant C-terminal tagged *M. tuberculosis* GS when present in low amounts by other secreted *M. smegmatis* proteins, it was attempted to detect secreted *M. tuberculosis* GS in concentrated *M. smegmatis* culture filtrates with the γ -glutamyl transferase assay (Materials and Methods 9.4). The γ -glutamyl transferase assay provides an empirical measure of specific GS activity by a measurement of its adenylylated or de-adenylylated catalytic activity in reaction buffer containing Mg^{2+} or

Mn²⁺ (Stadtman *et al.*, 1979; see Section 1.6.2). Because any effects of the C-terminal 6x-his tag on the structure, and therefore activity, of the recombinant *M. tuberculosis* GS could not be determined, a copy of a “native” *M. tuberculosis*, which included a 805 bp region upstream and 181 bp region downstream of the *glnA1* structural sequence (see Chapter 2, Figure 2.4) was cloned into the p19Kpro mycobacterial expression vector (p19K*glnA1*) and expressed in *M. smegmatis*. *Mycobacterium smegmatis*, *M. smegmatis* expressing “native” *M. tuberculosis* GS and *M. smegmatis* expressing the recombinant C-terminal tagged *M. tuberculosis* GS was cultured in Kirchners media to the logarithmic growth phase. The cytosol fractions and concentrated culture filtrates were purified and assayed for GS activity using the γ -glutamyl transferase assay. Table 3.1 shows that although high specific GS activities could be detected in the *M. smegmatis* expression host cytosol fractions, no GS activity could be observed in the concentrated culture filtrates, which supports the hypothesis that *M. tuberculosis* GS is not secreted by logarithmic growing *M. smegmatis*.

Table 3.1. Glutamine synthetase specific activities (umoles/ min/ mg protein) in the cytosol and culture filtrates of *M. smegmatis* transformants cultured in Kirchners’ media. No GS activity could be detected in the culture filtrates of *M. smegmatis*, *M. smegmatis* expressing *M. tuberculosis* GS (p19K*glnA1*) or *M. smegmatis* expressing recombinant C-terminal tagged *M. tuberculosis* GS (*glnA1* “long”), despite high GS activity in the cytosol fractions. The OD₆₀₀ measurements of the *M. smegmatis* cultures are indicated. All GS activities were determined from the average of two independent γ -glutamyl transferase assays (see Materials and Methods 9.4).

Transformant	OD ₆₀₀	Cytosol GS		Culture filtrate GS	
		Reverse reaction (Mn ²⁺)	Forward reaction (Mg ²⁺)	Reverse reaction (Mn ²⁺)	Forward reaction (Mg ²⁺)
<i>M. smegmatis</i>	0.412	239.67	2.58	0	0
<i>M. smegmatis</i> p19K <i>glnA1</i>	0.396	253.64	0	0	0
<i>M. smegmatis glnA1</i> “long”	0.343	171.49	0	0	0

3.2.2. Glutamine synthetase secretion by *M. smegmatis* during the stationary growth phase

To investigate the hypothesis that *M. tuberculosis* GS is present in culture filtrates due to the processes following cell death, such as cell lysis, *M. smegmatis* transformed with both *M. tuberculosis* *glnA1*-constructs was cultured to the stationary growth phase ($OD_{600} > 1.2$) under similar conditions of nitrogen availability as described before (Section 3.2.1). Protein fractions of the cytosol and culture filtrate were prepared (Materials and Methods 9.1) and the presence of recombinant C-terminal tagged *M. tuberculosis* GS in these protein fractions was investigated through Western blotting (Materials and Methods, 10.1) of equal amounts of starting material (6 μ g of protein).

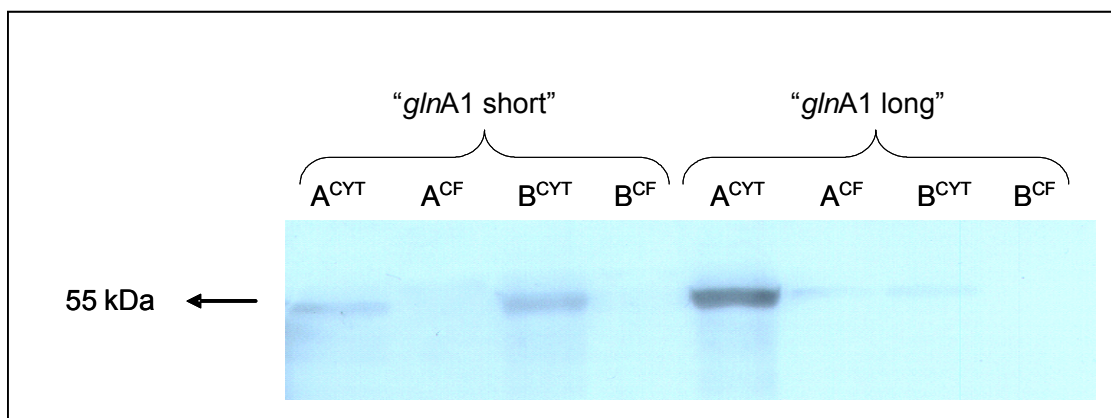


Figure 3.3. Detection of recombinant *M. tuberculosis* GS in the cytosol and culture filtrate of stationary phase *M. smegmatis* cultures. (A) Represents the cytosol and culture filtrate of *M. smegmatis* grown in Kirchners medium, while (B) represents the cytosol and culture filtrate of *M. smegmatis* grown in Kirchners media containing $(NH_4)_2SO_4$ to a final concentration of 38 mM.

Figure 3.3 shows that no recombinant C-terminal tagged *M. tuberculosis* GS expressed from the *glnA1* "short" construct could be detected in the culture filtrate of *M. smegmatis* in the stationary growth phase under varying nitrogen availability in Kirchners media or Kirchners media containing $(NH_4)_2SO_4$. A slight signal could be detected in the culture filtrate of *M. smegmatis* expressing recombinant C-terminal tagged *M. tuberculosis* GS from the *glnA1* "long" construct during the stationary growth phase (Figure 3.3 *glnA1* long A^{cf}), although this signal is less than that detected in the cytosol of the same *M. smegmatis* culture (Figure 3.3

glnA1 long A^{cyt}). Therefore it may be possible that *M. tuberculosis* GS might be present in the stationary growth phase culture filtrate due to liberation from the cytosol through cell death, although it may only be detectable when expression levels in the cytosol is high.

Intriguingly, Figure 3.3 also shows that the expression of recombinant C-terminal tagged *M. tuberculosis* GS from the *glnA1*-short construct was up regulated during the stationary growth phase of *M. smegmatis* in Kirchners media and Kirchners media to which (NH₄)₂SO₄ has been added. This upregulation was not observed in *M. smegmatis* cultures expressing recombinant C-terminal tagged *M. tuberculosis* GS from the *glnA1* “long” construct, where almost undetectable amounts of recombinant C-terminal tagged *M. tuberculosis* GS was synthesised during growth in Kirchners media containing (NH₄)₂SO₄ (compare figure 3.3 *glnA1* short B^{cyt} and *glnA1* long B^{cyt}). This is an unanticipated observation, since it would be expected that because the *glnA1* “long” construct includes the *glnA1* “short” promoter region (see Figure 3.1), the same recombinant C-terminal tagged *M. tuberculosis* GS expression would be observed. This difference in recombinant C-terminal tagged *M. tuberculosis* GS may indicate that other regulators of transcriptional activity may be present in the region upstream of *M. tuberculosis glnA1*.

3.2.3. Glutamine synthetase export and the mycobacterial *secA2* helper translocase

In Section 3.2.1 it was shown that *M. smegmatis* could export high concentrations of a recombinant *M. tuberculosis* GS containing a C-terminal 6x his-tag to its cell wall. However, no secretion of the protein into the *M. smegmatis* culture filtrate could be observed during mid-logarithmic growth. This finding was substantiated by the lack of GS activity (Section 3.2.1) in the culture filtrates of *M. smegmatis* expressing *M. tuberculosis* GS (in its native form). This observation suggests that an export mechanism responsible for the translocation of GS from the cytosol to the cell wall may exist, and may also be present in mycobacteria other than the slow-growing pathogenic mycobacteria (such as *M. tuberculosis*, *M. bovis*, including the *M. bovis* BCG strains, see Section 1.6.5.1).

To investigate whether the translocation of the recombinant *M. tuberculosis* GS could be facilitated by the *secA2* transport system described by Braunstein *et al.* (2003), the genes encoding *M. tuberculosis secA2* (Rv1821) and GS (*glnA1*) were cloned into bacterial-2-hybrid target- and bait expression plasmids (Materials and Methods, Addendum C.5). The

vector constructs containing *secA2* (pUT*secA2*) and *glnA1* (pKT*glnA1*) were simultaneously transformed into an *E. coli* JM109 host strain (Materials and Methods 1.1). *Escherichia coli* transformants containing both vector constructs were selected on LB-media containing X-gal and the appropriate antibiotics (Materials and Methods 1.3). Controls included *E. coli* transformed with non-associating vector constructs (pKT25 and pUT18; Addendum C.5), as well as associating constructs (pKTZip and pUTZip, Karimova *et al.*, 1998).

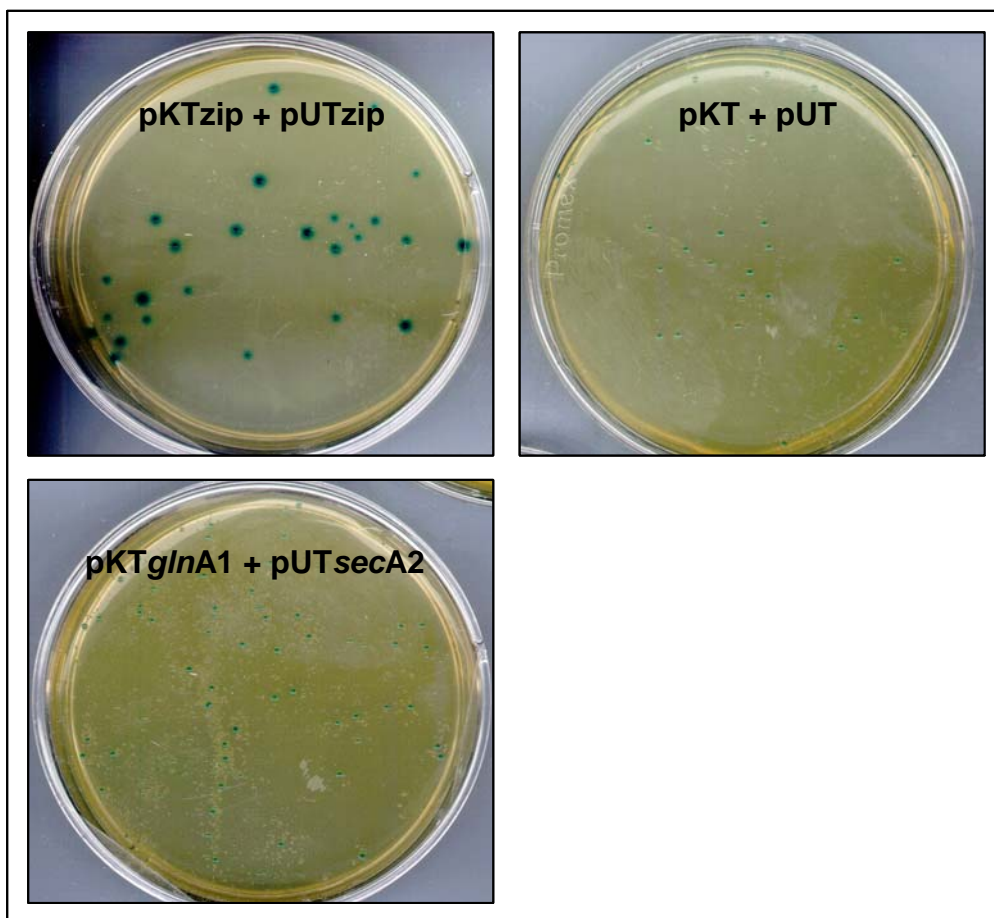


Figure 3.4. *glnA1* and *secA2* protein-protein interactions were studied with the bacterial-2-hybrid system in *E. coli* as interaction-expression host. The positive control (interacting proteins encoded by pKTzip and pUTzip) showed blue colonies on X-gal containing LB medium, while the negative control (pKT + pUT) showed white colonies. White colonies showing no protein-protein interaction were observed in *E. coli* transformants expressing *M. tuberculosis glnA1* and *secA2* (pKT*glnA1* + pUT*secA2*).

Figure 3.4 show that the positive controls (pKTZip and pUTZip) formed blue colonies on X-gal containing LB-medium, while the non-associating controls (pKT and pUT) formed white colonies. The *E. coli* transformants containing the pUTsecA2 and pKTglnA1 vector constructs formed white colonies, indicating that secA2 and GS does not interact on protein-level and that secA2 might therefore not be responsible for *M. tuberculosis* GS export to the mycobacterial cell wall.

3.2.4. Glutamine synthetase in the *M. bovis* BCG cytosol and cell wall

To investigate whether the observed export of *M. tuberculosis* GS (as described in Section 3.2.1) is present in slow growing mycobacteria (such as *M. bovis* BCG), it was attempted to show the presence of recombinant C-terminal 6x his-tagged GS in the sub-cellular fractions of *M. bovis* BCG similar to Section 3.2.1. However, detection of recombinant C-terminal 6x his-tagged GS could not be established. The failure to express recombinant C-terminal 6x his-tagged GS could not be explained, but it may be due to instability of either the hygromycin B antibiotic, or the hygromycin B resistance marker of the plasmid over the long duration of culturing times for slow-growing mycobacteria on agar medium. Therefore the presence of GS in the cell wall of slow-growing GS-secreting mycobacteria, such as *M. bovis* BCG, was investigated by GS isolation through $(\text{NH}_4)_2\text{SO}_4$ precipitation (Materials and Methods 9.3).

Glutamine synthetase was isolated from the cell wall and cytosol fractions of a 1.5L *M. bovis* BCG culture. The cell wall fraction was recovered by gentle abrasion of the *M. bovis* BCG cell pellet with glass beads prior to cell disruption through sonication (Materials and Methods 9.3). After two steps of GS $(\text{NH}_4)_2\text{SO}_4$ precipitation, protein samples were analysed by SDS-PAGE electrophoresis (Materials and Methods 4.1). Figure 3.5 shows that two proteins of differing molecular weight (MW) could be purified to single band purity from the *M. bovis* BCG cell wall and cytosol fractions. A higher MW protein (56 - 58 kDa) was associated with the *M. bovis* BCG cell wall, while a lower MW protein (53 – 55 kDa) was detected in the cytosol. This was surprising, as GS protein species of differing MW was not observed in the cell wall and cytosol fractions after recombinant *M. tuberculosis* GS expression in the *M. smegmatis* heterologous expression host (Section 3.2.1).

This result suggests that GS is exported to the *M. bovis* BCG cell wall, but that the protein may undergo post-translational modification (such as binding of another low MW peptide) that does not occur during recombinant C-terminal tagged *M. tuberculosis* GS export in a fast-growing heterologous mycobacterial expression host, such as *M. smegmatis*, where changes in *M. tuberculosis* GS MW was not observed (see Section 3.2.1).

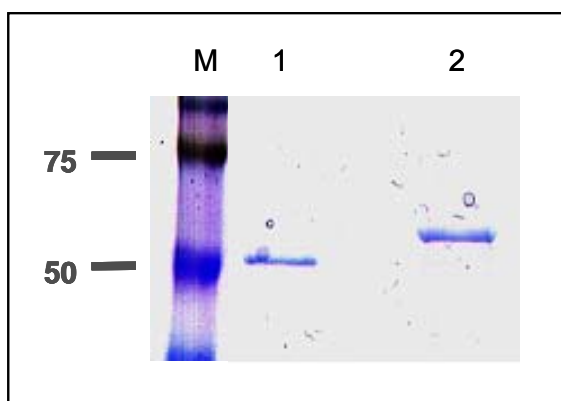


Figure 3.5. Two isoforms (proteins of ~54 and ~57kDa) of GS were detected in *M. bovis* BCG Pasteur. The 54 kDa GS isoform is associated with the cytosol (Lane 1), while a 57 kDa GS isoform is present in the cell wall (Lane 2) of *M. bovis* BCG Pasteur. Molecular weight of the protein standard (Lane M) is indicated.

3.2.5. Integration of the *M. tuberculosis glnA1*-containing chromosomal domain into *M. smegmatis*.

To investigate the difference in MW observed in the cytosol and cell wall fractions of *M. bovis* BCG, the chromosomal segment of the *M. tuberculosis* chromosome containing the *glnA1* locus was stably integrated into the *M. smegmatis* genome, resulting in an “*M. smegmatis glnA1* integral” strain able to express an unaltered *M. tuberculosis* GS in addition to its own GS.

The chromosomal segment containing *glnA1* and its surrounding sequences was derived from an *M. tuberculosis* cosmid library maintained in *E. coli* (Bange *et al*, 1999). *Escherichia coli* colonies were screened with an enhanced chemiluminescent labelled (ECL) probe specific for *glnA1* (Materials and Methods 8; Materials and Methods 11). The presence of an undisrupted *glnA1* sequence was determined by a PCR reaction that amplified an 805 bp upstream region of *glnA1* (therefore containing all necessary promoter elements) and a gene

(Rv2224c) located in a gene cassette (containing *glnE*, *glnA2*, Rv2223c and Rv2224c) downstream of the *glnA1* gene (Figure 3.6 A). The stable integration of the selected cosmid into the *M. smegmatis* genome was confirmed by the same PCR screen as described above after a few generations of the cosmid-transformed *M. smegmatis* that had been cultured in Middlebrook 7H9 medium without antibiotic selection (Figure 3.6 B).

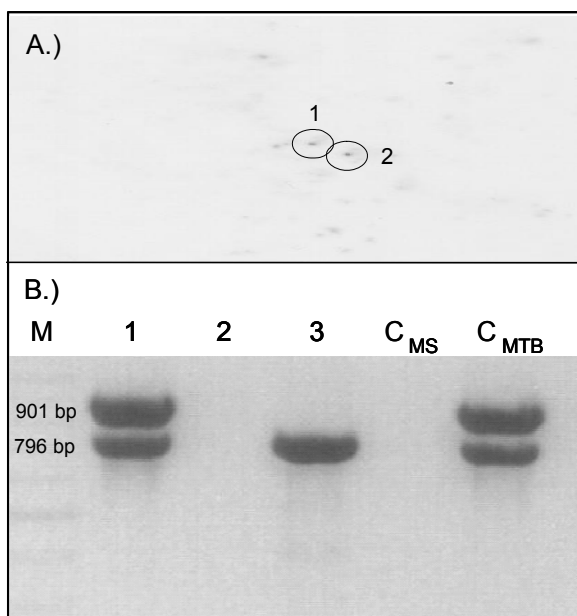


Figure 3.6. ECL detection of *E. coli* colonies harbouring the chromosomal region of *M. tuberculosis* that contains *glnA1*. Two colonies that showed hybridisation of an ECL labelled probe complementary to an internal region of *M. tuberculosis glnA1* were selected (A). The presence of *glnA1* and its upstream and downstream regions was determined through PCR (Panel B). Through PCR amplification a 796 bp product representing the region immediately upstream of *glnA1*, as well as a 901 bp product representing the region upstream of Rv2224c was observed in Colony 1 (Panel B, lane 1) indicating that all the genes between and including *M. tuberculosis glnA1* and Rv2224c were present in the clone, while Colony 2 (Panel B, lane 3) only contained the *glnA1* locus.”. *Mycobacterium smegmatis* (C_{MS}) and *M. tuberculosis* (C_{MTB}) DNA controls included showed the expected sizes of PCR products and that no cross-hybridisation PCR products were formed. Lane 2 contains a negative control for PCR contamination.

The *M. smegmatis glnA1* integral strain was transformed with the *glnA1* “long” construct and fractionated into sub-cellular fractions (similar to those obtained in Section 3.2.1). However,

no recombinant C-terminal 6x his-tagged *M. tuberculosis* GS could be detected in any of the fractions (result not shown). To determine if the lack of recombinant C-terminal 6x his-tagged *M. tuberculosis* GS was due to transcriptional down-regulation of *glnA1* due to the presence of more than two copies of *glnA1* (that of *M. smegmatis* and *M. tuberculosis*), it was attempted to detect the recombinant *M. tuberculosis* GS in larger whole lysate protein preparations by means of dot-blotting (Materials and Methods 10.2). It was also attempted to enrich recombinant C-terminal 6x his-tagged *M. tuberculosis* GS from whole lysates of the *M. smegmatis glnA1* integral strain transformed with the “*glnA1* long” construct through 6x his-affinity purification (Material and Methods 9.6). Figure 3.7 shows that anti-6x his antibody binding could be detected in whole lysates of the *M. smegmatis glnA1* integral strain transformed with the “*glnA1* long” construct (Figure 3.6 lane 3), as well as affinity purified protein samples (Figure 3.7 lane 4). Low background signal of anti-body binding could be observed in the *M. smegmatis* (Figure 3.7 lane 1) and *M. smegmatis glnA1* integral (Figure 3.7 lane 2) whole lysate preparations, probably due to non-specific binding due to high protein concentrations.

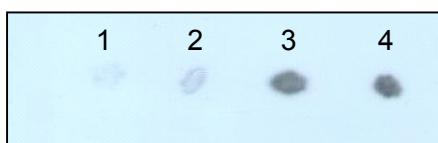


Figure 3.7. Dot-blot detection of recombinant C-terminal tagged *M. tuberculosis* GS in the *M. smegmatis glnA1* integral strain. Low levels of background signal were detected in negative controls consisting of *M. smegmatis* (lane 1) and *M. smegmatis glnA1* integral strain (lane 2) whole lysates. Hybridisation signal were detected in whole lysates of *M. smegmatis glnA1* integral strains transformed with the “*glnA1* long” construct (lane 3), as well as affinity purified preparations (lane 4) from the whole lysates represented in lane 3.

These results suggest that the recombinant C-terminal tagged *M. tuberculosis* GS protein is in fact expressed, although at low levels in the *M. smegmatis glnA1* integral strain. Therefore a more sensitive 6x his detection method was employed to detect recombinant C-terminal 6x his-tagged *M. tuberculosis* GS on Western blots (Materials and Methods 10.2) of whole lysate proteins, which showed the presence of recombinant *M. tuberculosis* GS (Figure 3.8 Panel A, lane 2) in the whole lysate of the *M. smegmatis glnA1* integral strain. A low concentration of another small molecular weight protein (~ 13 kDa) was observed that was

not present in the untransformed *M. smegmatis* integral whole lysate (Figure 3.8 Panel A, lane 1), suggesting that the 6x his tag might have been cleaved from recombinant GS sub-units. A low concentration affinity purified recombinant C-terminal tagged *M. tuberculosis* GS protein was recovered from the *M. smegmatis glnA1* integral strain transformed with the *M. tuberculosis* “*glnA1* long” construct (Figure 3.8 Panel B, lane 1). The affinity purified protein migrated as a single band with a MW of approximately 60 kDa, indicating that in the presence of a “native” *M. tuberculosis glnA1* copy, recombinant C-terminal tagged *M. tuberculosis* GS may have a larger MW. No corresponding protein could be detected in the affinity-purified *M. smegmatis glnA1* integral whole lysate controls that was not transformed with the *M. tuberculosis glnA1* “long” construct (result not shown).

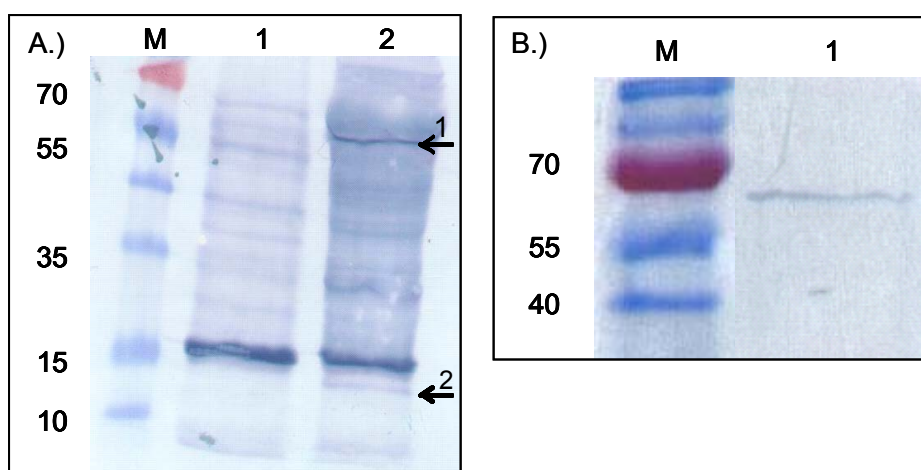


Figure 3.8. Recombinant *M. tuberculosis* GS detection in *M. smegmatis glnA1* integral strains was detected by anti-6x his colorimetric staining. Panel A shows that 2 protein bands (55 kDa – 60 kDa, arrow 1 and 13 kDa, arrow 2) could be detected in the whole lysate of the *M. smegmatis glnA1* integral strain (Panel A, lane 2) that was not present in the *M. smegmatis glnA1* integral control whole lysate (Panel A, lane 1). Panel B shows the 6x his affinity purified protein migrated as a single band of approximately 60 kDa (Panel B, lane 1). Molecular weight is indicated in kDa (Molecular weight marker, Panel A and B, lane M).

3.3 DISCUSSION

The sub-cellular compartmentalisation of recombinant *M. tuberculosis* GS was investigated in a heterologous mycobacterial host that does not secrete its own GS, namely *M. smegmatis* (Harth and Horwitz 1994). However, *M. tuberculosis* GS is putatively expressed

from two alternative promoter sites located 116bp and 45bp upstream of the *glnA1* start codon, which may regulate *glnA1* expression in response to differences in environmental nitrogen limitation or abundance (Harth and Horwitz 1994). To allow the investigation of the differences in GS expression and translocation using the alternative *M. tuberculosis glnA1* promoter regions, the expression of *M. tuberculosis glnA1* in *M. smegmatis* was carried out from either both the promoter regions (116 bp and 45 bp), or only one (45 bp) under conditions of variable nitrogen availability. The results have shown that *M. tuberculosis* GS is preferentially expressed under the direction of the 116 bp promoter region during growth in Kirchners' media, while little amounts of recombinant GS was expressed from the 45 bp promoter region alone under similar culturing conditions. When environmental nitrogen concentrations were increased through the addition of $(\text{NH}_4)_2\text{SO}_4$, GS production was significantly reduced in both constructs and totally absent when the 116 bp upstream promoter region is not present. This suggests that GS synthesis may also be controlled at transcriptional level by nitrogen availability. Down-regulation of *glnA1* transcriptional activity under conditions of nitrogen excess has also been observed in the closely related *Corynebacteria* (Nolden *et al.*, 2001). This down-regulation of *glnA1*, as observed in the *Corynebacteria*, may be due to the presence of a *tetR*-type repressor sequence upstream of the *glnA1* locus (Jakoby *et al.*, 2000). In that study the *tetR* repressor protein has been identified as AmtR, which is responsible for the repression of gene transcription under conditions of nitrogen excess in the *Corynebacteria*. In another study it was shown that AmtR may control the expression of many of the genes involved in nitrogen metabolism, and may therefore be a master regulator of nitrogen control in the *Corynebacteria* (Beckers *et al.*, 2005). In other prokaryotes (such as *Bacillus subtilis*), *glnA* is regulated at the transcriptional level by the *glnA*-specific *glnR* regulator under conditions of nitrogen excess (Wray, Jr. and Fisher, 1993). It is not known whether these mechanisms of nitrogen metabolism and *glnA1* transcription control are present in *M. tuberculosis*, as many of the genes involved in the GS regulation cascades have been identified through sequence homology only. However our results indicate that GS may be regulated by mechanisms other than post-translational modification (through adenylation) under conditions of nitrogen excess. In support of this hypothesis, results of a separate quantitative study by our group that is investigating *glnA1* expression under varying nitrogen conditions, have shown that *glnA1* may be totally down-regulated under conditions of nitrogen excess (60 mM NH_4^+ added to Kirchners cultures), while being abundantly expressed under conditions of nitrogen starvation (3 mM NH_4^+) (Harper, C.; personal communication). Whether there are

transcriptional repressors that could disable *M. tuberculosis* GS synthesis in *M. smegmatis*, or if the *glnA1* promoter region itself plays a role in the down-regulation of transcription is not known.

It was also observed that large concentrations of recombinant *M. tuberculosis* GS are translocated from the *M. smegmatis* cytoplasm to the cell membrane and cell wall. Glutamine synthetase had a MW of approximately 55 kDa, indicating that no post-translational protein modifications, such as N-terminal cleavage, have taken place during GS translocation from the *M. smegmatis* cytosol to the cell wall. Post-translational modification of *M. tuberculosis* GS was not expected, since the GS sequence does not possess an N-terminal secretion signal peptide necessary for *sec*-dependant export (Pugsley, 1993) and no N-terminal modification of the secreted GS sequence was observed in previous studies (Harth and Horwitz, 1997; Sonnenberg and Belisle, 1997). However, a *sec*-like protein export pathway (mediated by a *secA2* helper translocase) that does not require the cleavage of a N-terminal *sec*-signal sequence was shown to be present in mycobacteria, including *M. tuberculosis* and *M. smegmatis* (Braunstein *et al.*, 2003). In the study by Braunstein *et al.* (2003) it was shown that other N-terminal *sec*-secretion signal sequence lacking proteins predominant in the *M. tuberculosis* culture filtrate, such as SodA, might be secreted through the SecA2-dependant mechanism. Since this mechanism might explain the export of recombinant *M. tuberculosis* GS by *M. smegmatis*, the protein-protein interaction of *M. tuberculosis* GS and SecA2 was investigated in an *E. coli*-based bacterial-2-hybrid system. The results showed that no protein-protein interaction between GS and SecA2 could be detected, suggesting that *M. tuberculosis* GS is probably not exported by this mechanism. However, the results presented here show that the mechanism required for GS export is present in *M. smegmatis*, despite the observation of a previous study that showed that *M. smegmatis* does not export its native GS (Harth and Horwitz, 1997). It could also be argued that a possible confounding factor in this study was the fact that *E. coli* was used as an expression host and that both proteins were fusion constructs. Subtle alterations in target protein folding and structure may influence the recognition and interaction sites between the two *M. tuberculosis* proteins (Baneyx and Mujacic; 2004; Garcia-Fruitos *et al.*, 2005; Petersson *et al.*, 2004).

The high concentrations of recombinant *M. tuberculosis* present in the *M. smegmatis* cell wall may suggest a physical association between GS and the mycobacterial cell wall. This

observation is supported by the lack of recombinant GS in the logarithmic growth phase culture filtrates of the *M. smegmatis* expression host, despite the high level of expression observed in the cytosol. Furthermore, low concentrations of GS could be detected in the culture filtrate of *M. smegmatis* cultures that had entered the stationary growth phase. It is believed that many cytosol - associated proteins are liberated into the bacterial culture medium following cell membrane disintegration after cell death (Wiker, 2001). Increases in the concentration of such cytosolic proteins have been observed during the entry into the stationary growth phase of *M. tuberculosis*, suggesting extensive lysis of dying bacilli (Andersen *et al.*, 1991). Since it could not be determined whether the apparently low concentrations of recombinant *M. tuberculosis* GS detected in the culture filtrate of stationary growth phase *M. smegmatis* cultures were derived from the cytosol, it cannot be argued that GS was actively secreted during the stationary *M. smegmatis* growth phase.

To investigate whether GS is similarly associated with the cell walls of GS-secreting mycobacteria, such as *M. bovis* BCG, GS was isolated through ammonium sulphate precipitation from cytosol and cell wall protein preparations of *M. bovis* BCG. The cell wall proteins were prepared by gentle abrasion of the *M. bovis* BCG biomass that would result in the removal of the outermost layer of the cell wall of the mycobacterium. This method of gentle mechanical removal of cell wall proteins has been used previously and showed that although the outermost layer of the bacilli were removed, minimal lysis of bacilli occurred (Ortalo-Magné *et al.*, 1995). The GS proteins from both the *M. bovis* BCG cytosol and cell wall fractions were purified to single band purity which both had γ -glutamyl transferase activity. Two GS proteins of differing molecular weight (MW) was observed that were restricted to the cell compartment from which they were isolated. A lower MW GS (corresponding to the predicted MW of GS sub-units of ~ 54 kDa) was obtained from the cytosol of *M. bovis* BCG. From the cell wall, a higher MW GS sub-unit (~ 58 kDa) was isolated. The difference in MW is counter-intuitive, since modifications of exported proteins would usually be expected to be of a smaller MW than the cytosolic form due to post-translational modifications, such as peptide cleavage (Palmer and Berks, 2003). Glutamine synthetase subunits of higher MW (56 to 58 kDa) than predicted have been observed in *M. smegmatis* cultures expressing *M. tuberculosis glnA1* from a constitutive plasmid construct (Harth and Horwitz, 1997). This discrepancy in the observed and predicted MW of GS could not be explained, although it was shown that the observed increase in GS MW was not due to the adenylation state or post-translational modifications such as glycosylation or

lipidation. In other studies the molecular weight of GS has been determined as 53.57 kDa (Gu *et al.*, 2003) or 54 kDa (Rosenkrans *et al.*, 2000). It has been suggested that observed differences in molecular weight could be ascribed to transcriptional effects, such as transcription from the two alternative *M. tuberculosis glnA1* promoter regions (as described in Section 3.2.1) that may result in GS of different molecular weight. However, our results do not support this hypothesis, since recombinant *M. tuberculosis* GS expressed from either or only one promoter region showed GS of homogenous MW, similar to that of the predicted MW (54.39 including the 6x his C-terminal tag). It may therefore be argued that an additional polymer responsible for the observed increase in MW was purified together with GS from the *M. bovis* BCG cell wall. This polymer might not be present in *M. smegmatis*, or have a different affinity for *M. tuberculosis* GS.

A candidate for such an additional polymer may be that of poly-L-glutamic acid. It has been shown that GS may be directly involved in the synthesis of poly-L-glutamic acid (Harth *et al.*, 2000). This polymer has been observed in the cell walls of pathogenic mycobacteria only, such as *M. bovis* (including some of the *M. bovis* BCG sub-strains; Phiet *et al.*, 1976) and *M. tuberculosis* (Harth *et al.*, 1994), although probably not *M. leprae* (Melancon-Kaplan *et al.*, 1988). It has been shown that the poly-L-glutamic acid polymer might be non-covalently associated with the peptidoglycan layer of the *M. tuberculosis* cell wall of which it may constitute as much as 10% of the dry weight (Hirschfield *et al.*, 1990). The function of this polymer is unknown, although it has been speculated that it might function as an internal nitrogen and carbon reservoir (Hirschfield *et al.*, 1990), or in maintaining the integrity of the *M. tuberculosis* cell wall (Wietzerbein *et al.*, 1975). However, it has been shown that transcriptional inhibition of *M. tuberculosis glnA1* results in a decline in poly-L-glutamic formation in the cell wall (Harth *et al.*, 2000), suggesting that active GS is required for its formation. Such a hypothesis might explain the observed bactericidal effect of compounds such as methionine-L-sulfoximine (MSO) on *M. tuberculosis* (Tullius *et al.*, 2003). It has been shown that MSO cannot cross the *M. tuberculosis* cell wall into the cytosol (Harth and Horwitz, 1997), thereby implying that the compound is exerting its bactericidal effect on the GS located in the *M. tuberculosis* cell wall. Following from these observations it could therefore be hypothesised that the poly-L-glutamic acid may be associated with GS during its synthesis. The additional MW of this polymer might then explain the observed size difference in the larger cell wall associated GS. In addition, the GS observed in the cell wall fraction of *M. bovis* BCG was of one MW, which implies that the poly-peptides associated

with GS may be of equal size. It could not be shown that the recombinant *M. tuberculosis* GS expressed in *M. smegmatis* is active and therefore it may be argued that poly-L-glutamic acid could probably not be synthesised by the recombinant GS after expression in *M. smegmatis*.

To the hypothesis that a difference in *M. tuberculosis* GS MW may be observed in *M. smegmatis* if a functional *M. tuberculosis* is present, an *M. tuberculosis* chromosomal fragment containing the *glnA1* locus was integrated into the *M. smegmatis* chromosome. The *M. smegmatis* integral strain was consequently transformed with the *glnA1* long construct to enable detection of *M. tuberculosis* GS. Intriguingly, hybridisation signal of anti-6x his antibody could not be detected in the whole lysates of the *M. smegmatis* integral strain transformed with the “*glnA1* long” construct. Dot blots showed that an epitope was present in spots of concentrated proteins, which may suggest that transcription rates of the “*glnA1* long” construct might have been very low due to the presence of three *glnA1* genes (those of *M. smegmatis*, *M. tuberculosis* and the *M. tuberculosis* “*glnA1* long” construct). A more sensitive method of 6x his epitope detection (based on colorimetric staining) showed the presence of a ~ 60 kDa protein, as well as a ~ 13 kDa product not present in *M. smegmatis* integral control lysates. The identity and MW of the recombinant GS was confirmed through affinity purification, which showed a single protein band with a MW of ~ 60 kDa. This result was intriguing, since it showed that the recombinant *M. tuberculosis* GS produced in the *M. smegmatis* integral strain had a higher MW than when expressed in *M. smegmatis* without the integrated cosmid (MW ~ 55 kDa). This observation may imply that the recombinant *M. tuberculosis* may be modified (by addition of another low MW moiety) in the presence of a functional copy of *M. tuberculosis glnA1*, and therefore presumably functional *M. tuberculosis* GS. If this is true, it may also imply that GS does not catalyse the addition-event, since the recombinant GS protein may not be active.

The smaller ~ 13 kDa product could not be observed after affinity purification, although it cannot be excluded that it may be present in very low and undetectable amounts. This product may imply that the C-terminus of *M. tuberculosis* GS is modified in the *M. smegmatis* integral strain. The consequences of such a C-terminal modification are unknown, since this terminus is involved in the dodecamerisation of the GS hexameric rings (see Section 1.6.1). However, these results imply that other *cis*- or *trans*- acting factors might be involved in GS

modification or association. These factors might be located in close proximity to the *glnA1* locus integrated into the *M. smegmatis* chromosome in the *M. smegmatis* integral strain.

3.3.1 Conclusion

Although it has been shown that the chemical requisites necessary for the forward catalytic reaction of GS, such as ATP (Harth *et al.*, 2000) and ammonium (Clemens *et al.*, 1995), are present in the culture filtrate of *M. tuberculosis*, GS may not necessarily have a specific function in the culture filtrate, but may fulfil an integral role in maintaining cell wall integrity through the formation of poly-L-glutamic acid. Although the method of concentrating culture filtrate proteins used in this study may not have been effective in enriching low abundance proteins, the results of the sub-cellular compartmentalisation of *M. tuberculosis* GS strongly suggest that although *M. tuberculosis* GS may be exported by *M. smegmatis*, it is not secreted into the culture filtrate, suggesting that GS activity detected in mycobacterial culture filtrates by previous studies might be due to other reasons, such as mechanical damage of mycobacterial cell walls resulting in a release of the cell wall bound GS. It may however also be possible that other factors involved in GS secretion may exist in *M. tuberculosis*, but not in non-pathogenic mycobacteria, such as *M. smegmatis*.

CHAPTER FOUR

Operon structure of other genes in close proximity to *glnA1*

*“When it rained down sorrow it rained all over me
Cause my body rattles like a train on that old SP
I've got the TB blues
I've got that old TB I can't eat a bite
Got that old TB I can't eat a bite
Got me worried soul I can't even sleep at night”*

James Charles (Jimmie) Rodgers, T.B Blues
(Born September 8, 1897 – died of tuberculosis May 26, 1933)

Note: Some of the results presented in the following chapter were submitted for peer review and publication as: **“Transcriptional regulation and essentiality of genes downstream of *M. tuberculosis glnA1***. Hayward, D; van Helden, P; Kenyon, C and I Wiid to the Journal of Bacteriology on 07-07-2007

4.1 INTRODUCTION

In Chapter 3 we showed that substantial quantities of *M. tuberculosis* GS subunits are exported over the cell membrane and into the cell wall of a mycobacterial expression host, such as *M. smegmatis*. This result indicated that an export mechanism might exist in mycobacteria that aids this export. We hypothesised that retention of GS in the cytosol might involve C-terminal modification and that the factor(s) performing this task may be located in proximity to the *glnA1* locus. Gene clusters in prokaryotic genomes are typically composed of functionally related genes which are situated adjacent to each other on the same DNA strand with intergenic gaps of no more than 300 bp (Overbeek *et al.*, 1999). Clusters of genes adjacently located and transcribed in the same direction are a common feature of the *M. tuberculosis* genome (Cole *et al.*, 1998). Examples of gene clusters containing functionally related genes include the genes involved in lactose utilisation (*lac*), galactose utilisation (*gal*), as well as genes involved in histidine (*his*) and tryptophan biosynthesis (*trp*) (Lawrence and Roth, 1996). Such gene clusters were also observed in the genome of *M. tuberculosis*, and include examples such as the ESAT-6 gene clusters (Gey van Pittius *et al.*, 2001), the *mce* operon and various operons in which the ATP-binding cassette (ABC) transporters are situated (Gioffre *et al.*, 2005).

A cluster of four genes is present downstream of the coding sequence of *M. tuberculosis* glutamine synthetase, *glnA1* (Figure 4.1). Two of the genes in this cluster, *glnE* and *glnA2*, were previously shown to be essential for *M. tuberculosis* growth, since viable deletion strains of these sequences could not be established (Parish and Stoker, 2000; Collins *et al.*, 2002). The other two gene sequences in this arrangement, Rv2223c and Rv2224c, are annotated as serine proteases of unknown function.

4.1.1 Study aims

4.1.1.1. To investigate the promoter activity of the intergenic regions of the gene cluster downstream of *glnA1*

4.1.1.2. To investigate the two DNA sequences downstream of *glnE* and *glnA2*, Rv2223c and Rv2224c in terms of protein sequence distribution

4.1.1.3. *In silico* functional analysis of the proteins encoded by Rv2223c and Rv2224c

4.2 RESULTS

4.2.1 The chromosomal region surrounding *glnA1*

The genes in the *M. tuberculosis* chromosomal region surrounding the *glnA1* locus were retrieved from TubercuList (Materials and Methods 2.1). Figure 4.1 shows the location of genes surrounding *glnA1* on the *M. tuberculosis* chromosome, while the predicted functions, positions, sizes are summarised in Table 4.1. In Table 4.1 it can be seen that the functions of many of the genes are unknown or that a putative annotation has been ascribed due to the presence of sequence domains common to known functional protein classes. The functions of some genes, namely *glnA1*, *glnE* and *PanB* have been determined experimentally (Harth and Horwitz, 1997; Parish and Stoker, 2000; Sugantino *et al.*, 2003). The Rv2219A open reading frame (ORF) is situated 199 bp downstream of *glnA1* on the obverse DNA strand and is annotated as a conserved membrane protein. The *glnE* gene locus is situated 318 bp upstream of *glnA1* on the divergently transcribed negative DNA strand. The *glnE* gene encodes a glutamine synthetase adenylyltransferase, which is responsible for the reversible regulation of GS through differential adenylylation of the individual sub-units (Section 1.6.3). The *glnE* sequence is located in close proximity (48 bp) to *glnA2*, which encodes a GS type II enzyme responsible for the synthesis of D-glutamine and D-isoglutamine (Harth *et al.*, 2005). The *glnA2* locus is separated by 95 bp from an open reading frame, Rv2223c, that may encode a exported protease. The Rv2223c locus is located 62 bp upstream of a similar ORF, Rv2224c, also annotated as a putatively exported protease-encoding sequence.

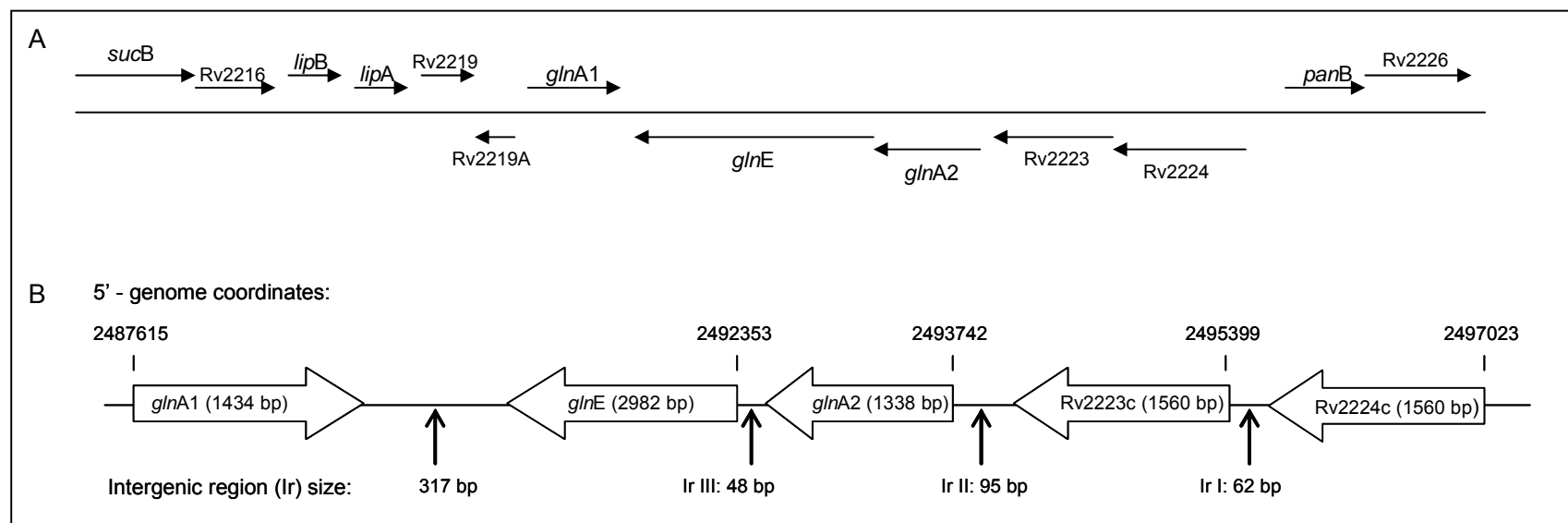


Figure 4.1. The *M. tuberculosis* chromosomal domain containing *glnA1*

Table 4.1. Summary of the sizes, functions and products of the genes situated in the immediate chromosomal vicinity of the *glnA1* locus.

Gene	Size	Function	Product	Coordinates	Reference
<i>lipB</i>	693 bp (230 aa)	lipoate biosynthesis	lipoate biosynthesis protein B (EC 6.-.-.)	2484584 - 2485276	
<i>lipA</i>	936 bp (311 aa)	lipoate biosynthesis	lipoate biosynthesis protein A	2485273 - 2486208	
<i>Rv2219</i>	753 bp (250 aa)	unknown	conserved transmembrane protein	2486235 - 2486987	
<i>Rv2219A</i>	423 bp (140 aa)	unknown	conserved membrane protein	2486994 - 2487416	
<i>glnA1</i>	1437 bp (478 aa)	glutamine synthesis	glutamine synthetase (EC 6.3.1.2)	2487615 - 2489051	(Harth and Horwitz, 1997)
<i>glnE</i>	2985 bp (994 aa)	glutamine synthetase regulation	glutamine synthetase adenylyltransferase (EC 2.7.7.42)	2489369 - 2492353	(Parish and Stoker, 2000)
<i>glnA2</i>	1341 bp (446 aa)	glutamine synthesis	glutamine synthetase (EC 6.3.1.2)	2492402 - 2493742	
<i>Rv2223c</i>	1563 bp (520 aa)	function unknown	exported protease (EC 3.4.-.-)	2493837 - 2495399	
<i>Rv2224c</i>	1564 bp (520 aa)	function unknown	exported protease (EC 3.4.-.-)	2495461 - 2497023	
<i>panB</i>	846 bp (281 aa)	pantothenate biosynthesis	3-methyl-2-oxobutanoate hydroxymethyltransferase (EC 2.1.2.11)	2497742 - 2498587	(Sugantino <i>et al.</i> , 2003)
<i>Rv2226</i>	1542 bp (513 bp)	unknown	conserved hypothetical protein	2498832 - 2500373	

4.2.2 Operon structure and regulation

To investigate the functional relationship between the four genes in the 3' domain of *glnA1*, the co-expressed of *glnE*, *glnA2*, Rv2223c and Rv2224c was investigated through reverse transcription (RT) of the intergenic regions linking these genes. It has been previously observed that internal promoter elements may reside between genes co-expressed as an operon as an additional means of transcriptional control (Torres *et al.*, 2001; Matsumoto *et al.*, 2005; Li *et al.*, 2006; Oyamada *et al.*, 2007). Therefore the relative promoter activity of the intergenic regions were investigated under conditions of varying nitrogen availability.

4.2.2.1 Reverse transcription

To determine whether the genes described above are transcribed as a single polygenic mRNA species, mRNA representing the intergenic regions was amplified through reverse transcription (Materials and Methods 3.2) and PCR (Materials and Methods 3.1.2) from total mRNA prepared from a mid-logarithmic growing *M. tuberculosis* H37Rv culture (Materials and Methods 7.3). To minimise the chance of obtaining PCR artefacts from mRNA read-through products (Wernisch *et al.*, 2003), anti-sense PCR oligonucleotides were designed that would prime approximately 100 bp into the 5'-start of the structural genetic region of *glnE*, *glnA2* and Rv2223c (Materials and Methods 2.2). Figure 4.2 shows that RT-products could be obtained for the intergenic regions located between *glnE* and *glnA2* (350 bp, lane 4) and *glnA2* and Rv2223c (300 bp, lane 3). The sizes of the PCR products were similar in size to that obtained using *M. tuberculosis* H37Rv DNA as PCR template. No RT-product could be detected representing the intergenic region between Rv2223c and Rv2224c (lane 2). Relative to the control reactions, no residual *M. tuberculosis* H37Rv mRNA could be detected in total mRNA samples (negative control, lane 1). Controls in the PCR amplification of cDNA produced through RT indicated that there was no contamination of the PCR reaction that could have resulted in false positive results.

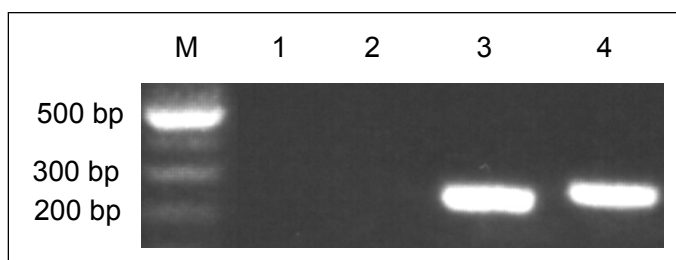


Figure 4.2. Co-transcription of the four genes in the chromosomal domain was detected through RT-PCR using primers that span the intergenic regions. Lane 1 represents the reverse transcription negative control, lane 2; the intergenic region between Rv2224c and Rv2223c, lane 3; a 259 bp amplification product of the intergenic region between Rv2223c and *glnA2* and lane 4; the intergenic region between *glnA2* and *glnE*.

4.2.2.2 Specific promoter activity of the intergenic regions

To investigate whether promoter activity may be present in the genetic regions separating the genes described in 4.2.2.1, the relative promoter activity of the intergenic regions between Rv2224c and Rv2223c (ir I, Figure 4.1B); Rv2223c and *glnA2* (ir II, Figure 4.1B) and *glnA2* and *glnE* (ir III, Figure 4.1B) were determined. These intergenic regions were cloned into a promoterless mycobacterial expression vector containing a functional *lacZ* sequence (Materials and Methods 5.1 and Addendum C.3). These constructs were transformed into *E. coli* as plasmid amplification host, and colonies were selected from LB-Kanamycin agar plates containing X-gal. All *E. coli* transformants (even *E. coli* transformed with an insert-less pJEM15 plasmid) were blue on X-gal containing LB agar plates. This was also observed in a study by Timm *et al.* (1994) who concluded that white-blue screening should be carried out in a mycobacterial host. All pJEM15 constructs were transformed into *M. smegmatis* (Materials and Methods 6.2) as a mycobacterial expression host (1). Promoter activity in the intergenic regions resulted in measurable β -galactosidase activity in the transformed *M. smegmatis* cultures. Figure 4.3 shows transformed *M. smegmatis* cultured on mycobacterial agar containing X-gal and it can be seen that the *M. smegmatis* transformed with the pJEM15 constructs containing the upstream region of Rv2224c (pJem4Up) and the intergenic sequence between *glnA2* and Rv2223c (Ir II) formed blue colonies (indicative of β -galactosidase activity). The blue colour of these *M. smegmatis* colonies were comparable to a positive control construct that contains the P_{AN} – IS900 transposase promoter region (Murray *et al.*, 1992). In contrast, the *M. smegmatis* transformed with the pJEM15 constructs containing the intergenic regions between *glnE* and *glnA2* (Ir III) and Rv2223c and

Rv2224c (Ir I) were white. These colonies were comparable to a negative control consisting of *M. smegmatis* transformed with an control pJEM15 plasmid.

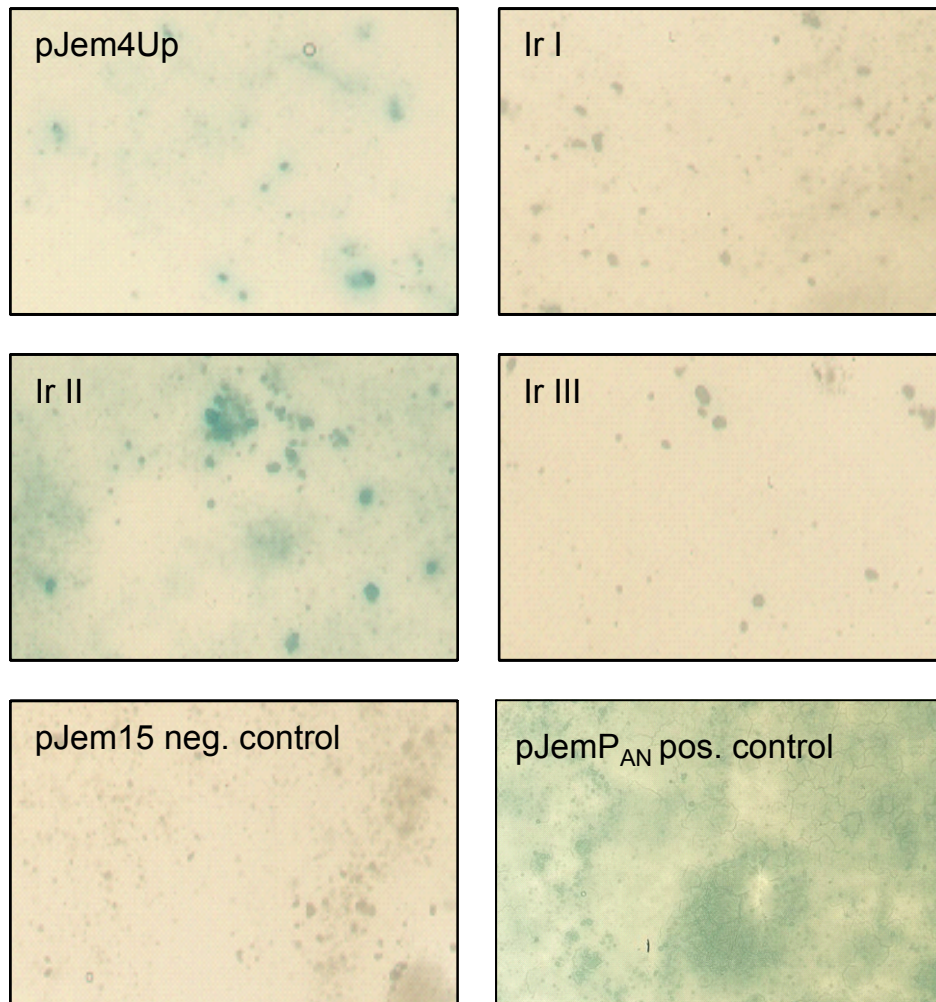


Figure 4.3. *Mycobacterium smegmatis* transformed with promoter constructs were plated on X-Gal containing mycobacterial agar in the presence of Kanamycin. *Mycobacterium smegmatis* cultures transformed with constructs including the Rv2224c upstream region (pJem4Up), the intergenic region between Rv2223c and *glnA2* (Ir II) and the positive control (pJemP_{AN}) exhibited β -galactosidase activity, while *M. smegmatis* transformed with constructs including the intergenic regions between Rv2223c and Rv2224c (Ir I), *glnA2* and *glnE* (Ir III) as well as a negative pJEM15 control showed no activity. Blue colonies indicate promoter activity due to the expression of the *lacZ*, while white colonies indicates a lack of promoter activity.

The β -galactosidase activity of the *M. smegmatis* transformants were quantitatively determined using the ONPG β -galactosidase spectrophotometric assay (Pardee *et al.*, 1959; Materials and Methods 9.5) during the logarithmic (OD600 = 0.4 – 0.6) and stationary (OD600 > 1.2) growth phases under conditions of varying nitrogen availability. The β -galactosidase activity could be directly translated to the relative strength of promoter activity of the sequences that were cloned at the 5'-end of the lacZ locus contained in the pJEM15 plasmid. Figure 4.4 shows the relative promoter activity of the 5'-regions of *M. tuberculosis* Rv2224c (pJem4Up), Rv2223c (Ir I), *glnA2* and *glnE* during the logarithmic and stationary growth phases of *M. smegmatis*. In Table 4.2 it can be seen that background β -galactosidase activity was detected in the pJem15 negative control assays. Since the β -galactosidase activity demonstrates relative promoter activity, the data presented in Figure 4.4 were standardised to the β -galactosidase activity of the pJem15 negative control assays. The non-standardised means and deviations are listed in Table 4.2. β -galactosidase activity could be detected in the 5'-regions of Rv2224c and *glnA2*, while the activity for 5'-regions of Rv2223c and *glnE* were comparable to that of the negative control. Promoter activity changed during the *M. smegmatis* stationary growth phase, in that increased β -galactosidase activity was observed in the 5'-regions of all the genes, as slight increase in promoter activity could also be observed in the 5'-region of Rv2223c (pJem Ir I, Figure 4.4) and *glnE* (pJem Ir III, Figure 4.4). An up-regulation of promoter activity in the 5'-region of the Rv2224c (pJem4Up, Figure 4.4) and *glnA2* (pJem Ir II, Figure 4.4) was also observed during the stationary phase of *M. smegmatis* growth.

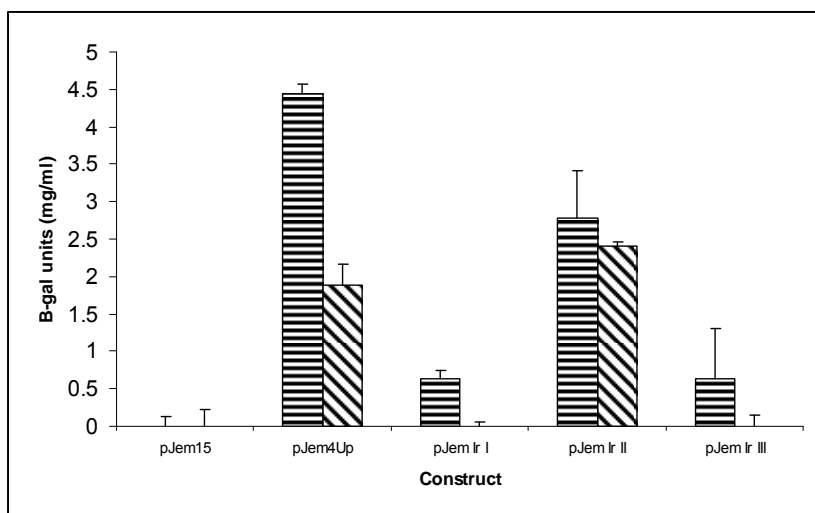


Figure 4.4. Relative promoter activity of the putative promoter regions upstream of Rv2224c (pJem4Up), Rv2223c (pJem Ir I), *glnA2* (pJem Ir II) and *glnE* (pJem Ir III) during the mid logarithmic (▨) and stationary growth phases (■) of *M. smegmatis*.

Plasmid (Promoter)	β-galactosidase activity			
	Logarithmic growth	Stationary growth	5mM (NH ₄) ₂ SO ₄	50mM (NH ₄) ₂ SO ₄
pJEM15	0.47 ± 0.21	0.35 ± 0.13	-0.27 ± 0.27	-0.09 ± 0.05
pJEM 4upstream	2.35 ± 0.28	4.79 ± 0.13	2.26 ± 0.16	-0.07 ± 0.02
pJEM irI	0.34 ± 0.06	0.98 ± 0.12	-0.01 ± 0.03	0.88 ± 0.09
pJEM irII	2.87 ± 0.06	3.13 ± 0.64	2.07 ± 0.15	2.3 ± 0.06
pJEM irIII	0.41 ± 0.15	0.99 ± 0.68	0.3 ± 0.88	0.19 ± 0.08

Table 4.2. β-galactosidase activity detected in ribolysed cell extracts of transformed *M. smegmatis* cultures grown to different growth phases and under different conditions of nitrogen availability in the logarithmic growth phase. The results represent the means and standard deviations of three independent assays.

To study the effect of nitrogen availability on promoter activity, the *M. smegmatis* transformants were grown in Kirchners medium to the mid logarithmic growth phase ($OD_{600} = 0.4 - 0.6$), collected by centrifugation, and incubated for one growth division (~4 hours) in Kirchners medium in which asparagine was substituted by 5 mM (NH₄)₂SO₄ or 50 mM (NH₄)₂SO₄ as nitrogen source. Figure 4.5 shows that promoter activity in the 5'-regions of Rv2224c and Rv2223c were influenced by the nitrogen concentration in the growth medium. Promoter activity could not be detected in the 5'-region of Rv2224c (pJem4Up, Figure 4.5) at high concentrations of nitrogen availability, but was upregulated under conditions of nitrogen starvation. In contrast, promoter activity was

upregulated in the 5'-region of Rv2223c (pJem Ir I, Figure 4.5) under high nitrogen availability, but was observed to be down regulated under low nitrogen conditions. The activity of the *glnA2* and *glnE* 5'-regions remained unchanged during fluctuations in nitrogen availability.

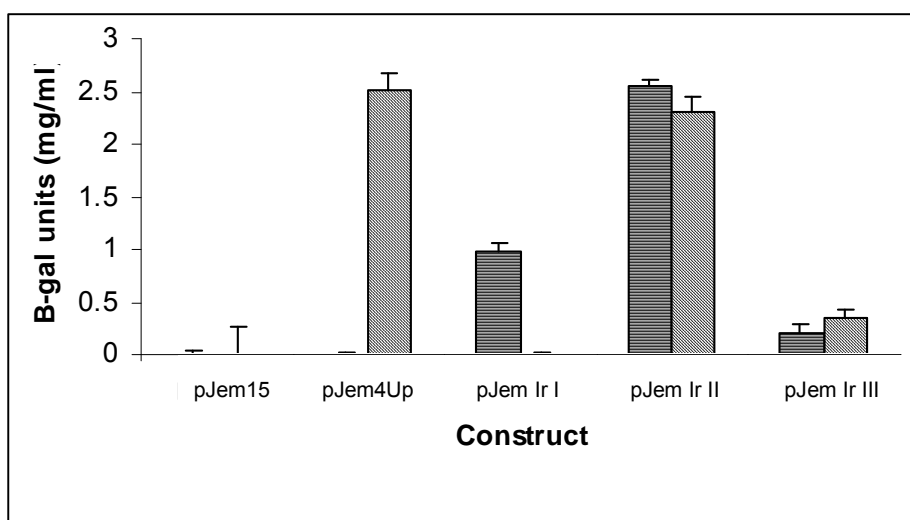


Figure 4.5. Relative promoter activity of the putative promoter regions under conditions of nitrogen starvation: 5 mM (NH₄)₂SO₄ (▨) or nitrogen excess: 50 mM (NH₄)₂SO₄ (■).

4.2.3 *In silico* analysis of the sequences encoded by Rv2223c and Rv2224c

The results shown in Section 4.2.2 suggest that the expression of the enzymes encoded by Rv2223c and Rv2224c in *M. tuberculosis* may be regulated in conjunction with other genes involved in *M. tuberculosis* GS regulation and metabolism. To gain insight into the possible function that the proteins encoded by these ORFs might perform, the amino acid sequences of the proteins were analysed using *in silico* methods (Methods and Materials 2.3 and 2.4). Examination of the protein sequences of the Rv2223c and Rv2224c and paralogs thereof by means of *in silico* analysis might impart information helpful in determining what the functions of these proteins might be.

4.2.3.1 Distribution of homologous sequences

The function of the enzymes encoded by the ORFs' Rv2223c and Rv2224c are unknown, however they are annotated as putative exported proteases. To investigate

whether protein paralogs with known function may be present in other organisms, the amino acid sequences of Rv2223c and Rv2224c were compared by sequence BLAST to 570 fully sequenced bacterial genomes available on the NCBI genome web-server (Materials and Methods 2.3). Both the *M. tuberculosis* Rv2224c and Rv2223c ORFs may encode peptides of 520 amino acids. Figure 4.6 shows that these protein sequences share a similarity of 46%. These homologous amino acids are dispersed over the whole sequence, with the least homologous region being the amino acid sequences contained in the protein N-terminal. Some stretches of conserved amino acid sequences can be observed in particular regions of the protein sequence, suggestive of potential enzymatic functional sites.



Figure 4.6. Alignment of the protein sequences of the putative proteases encoded by Rv2223c and Rv2224c in *M. tuberculosis* H37Rv. The homology between the two protein sequences was determined as 46%.

Sequences from organisms that share an amino acid similarity higher than 35% to the *M. tuberculosis* Rv2223c and Rv2224c sequences are summarised in Table 4.3. From the data presented in Table 4.3, it can be seen that homologous sequences to the *M. tuberculosis* Rv2223c and Rv2224c amino acid sequences are not widely distributed through the bacterial species whose genome sequences are available on the NCBI sequence-BLAST webpage. Sequence paralogs with a homology higher than 35% were only detected in the Actinobacteria. In bacteria other than the *Mycobacteria*, one sequence paralog per bacterial genome was observed (Table 4.3), while the mycobacterial genomes contained paralogous sequences to both Rv2223c and Rv2224c.

Table 4.3. Rv2223c and Rv2224c paralogs obtained through protein sequence BLAST to all bacterial genomes present on NCBI web server. The sequence accession numbers and annotated functions of these sequences together with the percentage of identical amino acids contained in each sequence compared to the *M. tuberculosis* Rv2223c and Rv2224c sequences are indicated. In the organisms where only one sequence homolog for either the Rv2223c and Rv2224c sequences could be obtained, a open space was left in the column indicating sequence accession number and function, although homology to the paralogous *M. tuberculosis* sequence is shown.

Organism	Rv2223c				Rv2224c			
	Sequence acc. nr.	Identities	Function	Sequence length	Sequence acc. nr.	Identities	Function	Sequence length
<i>M. tuberculosis</i> H37Rv	NP_216739	520/520 (100%)	exported protease	520	NP_216740	520/520 (100%)	probable exported protease	520
<i>M. tuberculosis</i> CDC1551	NP_336752	520/520 (100%)	proteinase	520	NP_336753	507/507 (100%)	putative proteinase	507
<i>M. tuberculosis</i> F11	ZP_01685140	520/520 (100%)	hypothetical protein	520	ZP_01685141	518/518 (100%)	hypothetical protein	518
<i>M. bovis</i> AF2122/97	NP_855896	520/520 (100%)	probable exported protease	520	NP_216740	520/520 (100%)	probable exported protease	520
<i>M. ulcerans</i>	YP_905359	384/477 (80%)	exported protease	522	YP_905357	457/518 (88%)	exported protease	518
<i>M. avium</i>	YP_881447	375/475 (78%)	hydrolase	519	YP_881445	438/520 (84%)	putative hydrolase	520
<i>M. leoprae</i>	NP_302124	373/479 (77%)	possible hydrolase	511	NP_302125	441/520 (84%)	possible secreted protease	535
<i>Mycobacterium</i> sp. KMS	YP_939375	325/464 (70%)	TAP domain protein	508	YP_939376	349/493 (70%)	TAP domain protein	511
<i>M. smegmatis</i>	YP_888572	320/474 (67%)	hydrolase	500	YP_888573	332/492 (67%)	protease	510
<i>M. vanbaalenii</i> PYR-1	YP_954397	306/464 (65%)	TAP domain protein	505	YP_954398	314/495 (63%)	TAP domain protein	516
<i>Rhodococcus</i> sp. RHA1	YP_701153	254/469 (54%)	possible proteinase	566		248/474 (52%)		
<i>Nocardia farcinica</i>	YP_117869	247/467 (52%)	Putative hydrolase	555		253/475 (53%)		
<i>Arthrobacter</i> sp. FB24	YP_830213	178/467 (38%)	TAP domain protein	524		181/476 (38%)		
<i>Streptomyces avermitilis</i> MA-4680	NP_825801	190/488 (38%)	protease	533		185/492 (37%)		
<i>Thermobifida fusca</i> YX	YP_290836	172/463 (37%)	putative proteinase	500		192/484 (39%)		
<i>Streptomyces coelicolor</i> A3	NP_627738	178/462 (38%)	secreted proteinase	539		186/488 (38%)		
marine actinobacterium	ZP_01131087	178/475 (37%)	peptidase	512		175/490 (35%)		
<i>Leifsonia xyli</i> supsp. <i>xyli</i>	YP_061517	170/480 (35%)	peptidase	518		172/487 (35%)		
<i>Kineococcus radiotolerans</i>	ZP_00616853	179/485 (39%)	A/B hydrolase	515		195/483 (40%)		
<i>Nocardioides</i> sp. JS614	YP_921618	184/487 (37%)	TAP domain protein	521		180/485 (37%)		
<i>Janibacter</i> sp. HTCC2649	ZP_00994153	179/501 (35%)	putative secreted proteinase	515		178/481 (37%)		

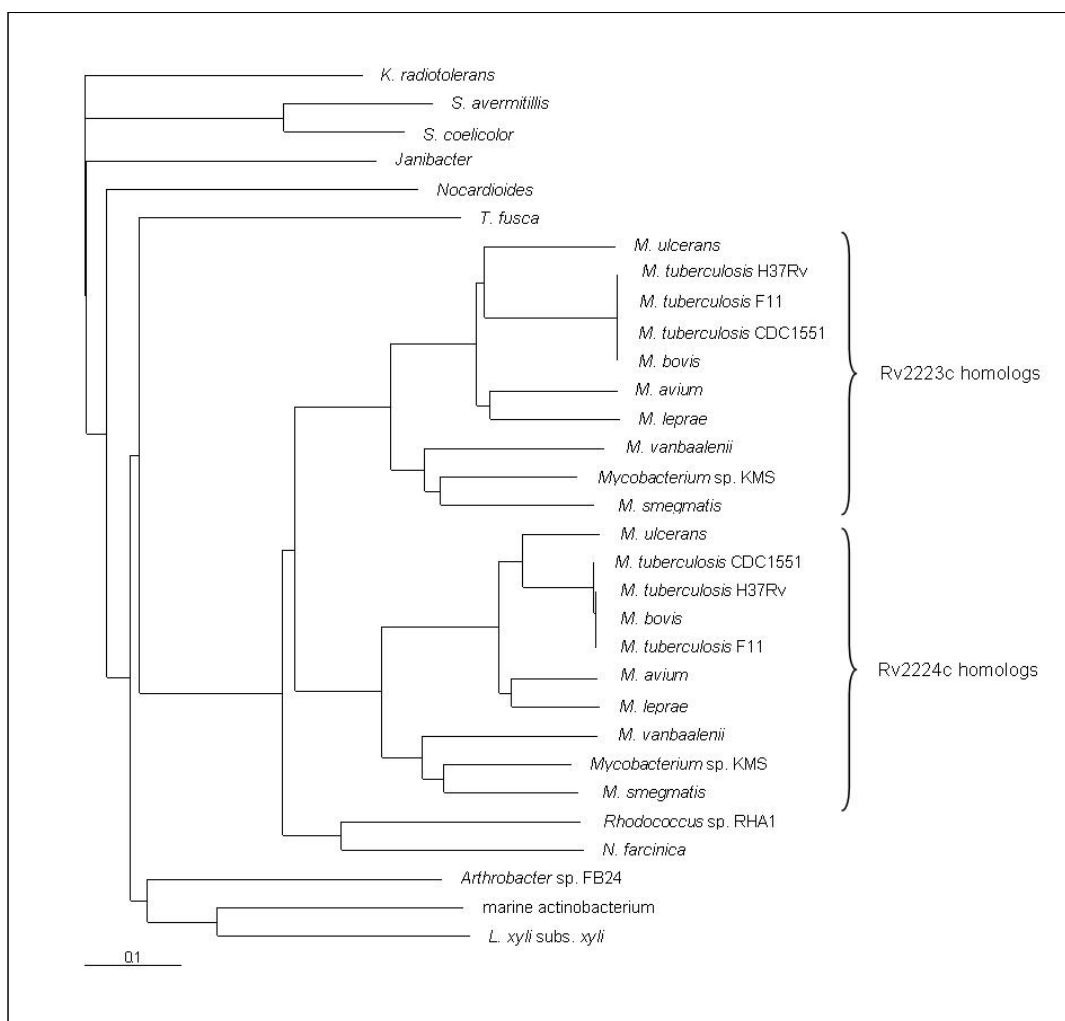


Figure 4.7. The phylogenetic reconstruction demonstrating the evolution of the *M. tuberculosis* Rv2223c and Rv2224c protein sequences shows that these sequences might be derived from a single ancestral progenitor, but that might have evolved separately. The scale bar indicates an evolutionary distance of 0.1.

To investigate whether the Rv2223c and Rv2224c sequence homologs of the mycobacteria could have arisen through a duplication event, a phylogenetic reconstruction was done using all homologous sequences (Materials and Methods, 2.3.1). The *Kineococcus radiotolerans* sequence paralog was chosen as out-group for the construction of the phylogenetic tree, since this organism evolved prior to the speciation of the other actinobacteria in the *Actinobacterial* phylogenetic tree (see Figure 1.2). The phylogenetic tree representing the evolution of the *M. tuberculosis* Rv2223c and Rv2224c sequences is shown in Figure 4.7. It can be seen that an ancestral progenitor sequence has diverged into separate clades (Rv2223c homologs and

Rv2224c homologs, Figure 4.7) containing different lineages in the mycobacteria. In these mycobacterial clades, the slower growing mycobacteria (*M. tuberculosis*, *M. avium* and *M. leprae*) and rapid growers (*M. flavescens*, *M. vanbaalenii*, *M. smegmatis* and *Mycobacterium* sp. KMS) are clustered similar to mycobacterial species diversification (see Figure 1.3). Together with the data summarised in Table 4.3, no conclusion could be made as to which open reading frame sequence bears the closest relation to the progenitor sequence, but that the duplication event might only be present in the mycobacteria.

4.2.3.2 *In silico* functional analysis of Rv2223c and Rv2224c

Protein sequence homologs of the *M. tuberculosis* Rv2223c and Rv2224c sequences were detected in all the mycobacterial genomes included in this study. A high degree of protein sequence homology is observed between these sequences from the different mycobacteria, with the sequences being identical in the *M. tuberculosis* strains and *M. bovis*. The sequence homology between the *M. tuberculosis* strains and the other mycobacteria is shown in Table 4.3. The multiple sequence alignment of the mycobacterial sequences in Figure 4.8 shows the high degree of amino acid conservation in these sequences, particularly with regard to amino acids that might play a role in the structural conformation and folding of the final protein product, such as cysteines (cys) which may form disulphide bonds through the interaction of thiol side-groups which may stabilise global protein folding (Creighton, 1975; Baud *et al.*, 1993). From the sequence alignment represented in Figure 4.8, it can be seen that the cys residues are remarkably conserved in both sequences in all the mycobacterial homologs included in this study. All the *M. tuberculosis* Rv2223c and Rv2224c amino acid homologous sequences contain 10 cys residues situated close to or in conserved amino acids domains, which may lead to the formation of 5 disulphide bridges in the folded protein.

All the mycobacterial sequences aligned in Figure 4.8 possess a typical lipase serine protease gly-X₁-ser-X₂-gly consensus motif, where X₁ is usually either tyr or his (Brady *et al.*, 1990). The active ser is usually part of a ser - his - asp catalytic triad, which is normally close to the enzyme surface but not completely exposed to solvents. Through inspection of the sequence alignment in Figure 4.8, gly₃₂₁, ser₂₃₂ and gly₃₂₅ (numbering based on the residue position in the *M. tuberculosis* Rv2223c amino acid sequence) are

conserved together with the tyr₃₂₄ residues through all the sequences. Four conserved his (his₁₈₁, his₃₀₆, his₃₇₈ and his₄₈₈) residues were observed, with his₃₇₈ and his₄₈₈ located in the most homologous amino acid stretches. A number of conserved asp residues can be observed. To determine which his and asp residues may form part of the catalytic triad, the amino acid sequences of Rv2223c and Rv2224c were compared to the MEROPS database (Materials and Methods 2.4). Through this comparison it was determined that his₄₈₈ and asp₄₆₁ were the most likely residues participating in the ser - his - asp lipase serine protease catalytic triad with ser₂₃₂ as the likely active ser residue. The linear order of nucleophile, acid and his in these potential catalytic triads identifies the Rv2223c and Rv2224c proteins as belonging to the α/β hydrolase fold proteins (Ollis *et al.*, 1992).

A further *in silico* analysis was carried out with the aim to predict the position of possible enzyme active sites and the functional class to which the enzymes encoded by Rv2223c and Rv2224c may belong through protein motif searches using the ExPasy web-server (Materials and Methods 2.4). Sequences with a high level of occurrence were excluded. Through this search, it was determined that both the protein sequences encoded by Rv2223c and Rv2224c may belong to the serine proteases of the tripeptidyl-peptidase B type (tripeptidyl aminopeptidase (II), tripeptidyl peptidase II or TPP), clan SC, family S33, and code S33.006. The serine lipase active site-sequence (PS00013) pattern was located at amino acid position 226 - 235 (**InYLGYSyGT**) in the Rv2223c sequence, and at amino acid position 222 – 231 (**LtYLGYSyGT**) in the Rv2224c sequence (Figure 4.8). A prokaryotic membrane lipoprotein lipid attachment site (PS00013) pattern was predicted to be located at amino acid position 21 – 31 (**LAVALVLVGC**) in the Rv2223c sequence. Lipoprotein lipid attachment sites are usually precursor signal peptides that might be recognised by lipoprotein signal peptidases (Class II), which may cut the polypeptide upstream of the cys₃₁. No lipoprotein lipid attachment site could be detected in the Rv2224c sequence.

<i>M. tuberculosis</i> Rv2223c	SQMAGFQTAFNDYAADCARS	PACPLGTDSAQWVNR	YHALVDPLVQK-----	PGKTS	DP	PRGLSYADATTGT	INALYS	SPQ	342	
<i>M. avium</i>	NQMAGFQTAFNDYATDCARS	PGCPLGTDPAQFVNR	YHALVDPLVTR-----	PGRTAD	PRGLSYADATTGT	INALYTPA			344	
<i>M. leprae</i>	QQMAGFQIAFTDYAADCAR	SASCPLGTDP	SQWVNR	YHALIDPLVTK-----	PGRTSD	PRGLGYADATTGT	INALYTPV		333	
<i>M. vanbalenii</i>	NQQAGFQKAFEYAADCAES	VGCP	LGTDPAQFVAR	FHQVLDPLVTR-----	PAATS	DP	PRGLGYQDAITGT	ASGLYSQR	329	
<i>M. flavescens</i>	TQNTGFORAFERYAADCAK	SAGCPLGTDP	TQFVAR	FHQVLDPLAVR-----	PAATS	DP	PRGLGYQDAITGT	VVSALYSQR	329	
<i>M. sp. KMS</i>	AQQAGFQRAFDAYAADCAK	SAGCPLGTDPAQFVAR	YHQVLDPLVAR-----	PAATS	DP	PRGLSYQDAITGT	TGNALYTPR		378	
<i>M. smegmatis</i>	RQMAAFQKAFDAYAADCGK	LWGCPLGNDPAQYVNR	FHQVLDPLVAR-----	PGATS	DP	PRGLSYQDAITGT	TGNALYSPR		390	
<i>M. tuberculosis</i> Rv2224c	RQAKGFQDAFNNYAADC	AKNAGCPLGADPAKAVE	VYHSLVDPLVDPDNP	RISR	PARTK	DP	PRGLSYSDAIVGT	IMALYSPN	345	
<i>M. avium</i>	RQAKGFQDAFNNFAAEC	AKQPCPLGTDPAKAV	DVYHSLVDPMPVDP	PN	PMVGR	PIPTN	DP	PRGLSYSDAIVGT	IMALYSPN	345
<i>M. leprae</i>	RQAKGFQDAFNNYAADC	CARKLSCPLGADPAKAV	DVYHNLVNDPNN	PMVSR	PAPT	KDP	PRGLSYSDAIVGT	IMTYLSP	360	
<i>M. vanbalenii</i>	RQAAAFQQAFDDYATDCA	IDEDCPLGTDPAKAV	DVYRSLVWPLVDA-----	PADTT	DP	PRGLSYNDAVVGT	ILPLYSPG		341	
<i>M. flavescens</i>	RQAAAFQQAFDDYAADCA	IDDDCPLGTDPAKAVE	VYHSLVWPLVDE-----	PAETS	DP	PRGLSYNDAVVGT	ILPLYSPA		320	
<i>M. sp. KMS</i>	RQAAAFQTAFNDYAADC	AKQPCPLGTDPAKAVE	VYKSLVDPVLEN-----	PAKT	DP	PRGLSYSDAIVGT	IQTYLSPN		361	
<i>M. smegmatis</i>	RQAAAFQKAFDDYAADCA	SPDCPLGTDPAKAV	DVYKSIDPLVEN-----	PAKTR	D	GRGLSYSDATVGT	ILPLYSPN		335	
Clustal Consensus	* .** ** :	5.	6*** *:: *	:: :: ** :	*	* * * ** .*	** .** ** :		145	
<i>M. tuberculosis</i> Rv2223c	RWKYLTSGLLGLQRGSDA	GDLLVLADDDYGRDAD	GHYSNDQDAFNAVRCV	DAPTPADPA	AAVWAADQR	IRQVAP	FFLSYGQF		422	
<i>M. avium</i>	HWKYLTSGLLGLARGTD	DAGDLLALADDDYQGR	DRNGHYSNDQDAFNAVRCV	DAPNPTDQAS	WVSADQK	IRQAAP	FFLSYGPF		424	
<i>M. leprae</i>	HWKYLTSGLLGLQRGTD	DAGDLLLADDDYNGR	DRNGHYTNDQDAFNAIRC	VDAPVPTDSAS	WVSADQQ	IRQAAP	FFLNYGQF		413	
<i>M. vanbalenii</i>	YWFYLTSGLLGLQRGTD	PGDLLLADDDYQQR	DRNGHYKNRQDAFNAIRC	VDAPVPTDSAP	WVEADRR	SREVA	FFLSYGPF		409	
<i>M. flavescens</i>	FWFYLTSGLLGLQRGTD	PGDLLLADDEYQRR	DRGHYSNDQDAFTAIRC	VDAPVPTDPA	VWADARR	SRAAV	FFLAYGQF		409	
<i>M. sp. KMS</i>	LWAYLTSGLLGLQRGTD	PGDLLLADDEYQRR	DASGHYANRQDAFTAIRC	VDAPYPTDPA	AWVQARR	FREAA	FFLSYGPF		458	
<i>M. smegmatis</i>	YWFYLTSGLLGLERGT	DAEDLLLADDDYQHR	DRNGHYQNQDAFTAIRC	VDDVYPTDPA	VWAEDR	QIREVA	FFFAYGGF		470	
<i>M. tuberculosis</i> Rv2224c	LWQHLLDGLSELVDN	-RGDTLLALADMYMRR	DSHGRYNNSGDARVA	INCVDQPPV	TDR	DKVIDED	RRAREIA	FFMSYGKF	424	
<i>M. avium</i>	LWHHLTDGLSELVDH	-RGDTLLALADMYMRR	DAHGHYTNATDARVA	INCVDQPPIT	DR	AKVIDED	RRSREIA	FFMSYGQF	424	
<i>M. leprae</i>	LWHHLTDGLSELVDH	-RGDTLLALADMYMRR	DSGHYTNATDARVA	INCVDQPPIT	DR	AKVIDED	RRSREIA	FFMSYGKF	439	
<i>M. vanbalenii</i>	LWRHLTQALTEIKKG	-RGDTMLALSDLYMGR	DAQGHYSNDTDSRVA	NCVDPKPAIT	DR	ATLVEQ	DRRVREA	FFMAYGEF	420	
<i>M. flavescens</i>	LWRHLTQALGEVKEG	-KGDMLALADLYMER	NAQGRYTNSTDVRI	AVNCVDPKPAIT	DR	ATLVEQ	DRRVREA	FFMAYGEF	399	
<i>M. sp. KMS</i>	LWRYLTQGLTELEAG	-RGDTMLALADLYMRR	DAQGHYNSDARVA	NCVDRPPV	KDR	DKIVEQ	DRKREVA	FFMSYGEF	440	
<i>M. smegmatis</i>	LWRHLTDALTALKKG	-DGNLMLALADLYMGR	DAKGHYNSDARVA	NCMDKPAIK	DKATVVE	QDKR	REVA	FFMSYGEF	414	
Clustal Consensus	* .**.* :	:	* * * * * :	* * * * *	:	* * * * *	:: : * .** : ** *		179	
<i>M. tuberculosis</i> Rv2223c	TGSAPRDL	CALWVPV	PATSTPHPAAP	AGAGKVVVVSTTH	DPATPYQ	SGVDLARQLG	APLITFDGTQ	TAVFDGN	QCVD	502
<i>M. avium</i>	TGNAPRDL	CALWVPV	PATSAPHAAPP	PAGKVVVVSTTH	DPATPYQ	AGVNLARQLG	GPLITFDGTQ	HAVFNGD	QCVD	504
<i>M. leprae</i>	TGNAPRDL	CALWVPV	PATSMHPAPP	VAPGKVVVVSTTH	DPATPYQ	AGVDLAREL	SSPLITFDGTQ	HAVFNGD	QCVD	493
<i>M. vanbalenii</i>	TGFAPRDL	CAMWVPV	PPTPVVGVASS	PGPGKVVVVSTTG	DPATPYQ	AGVNLARQLG	GPLITFDGTQ	HAVFNGD	ACVD	489
<i>M. flavescens</i>	TGFAPRDL	CAMWVPV	PPTSVVGAATS	PGPGKVVVVSTTG	DPATPYE	AGIALADQLD	QLSFTGTQ	HAVFNGD	ACVD	489
<i>M. sp. KMS</i>	TGYAPRDL	CVGLWVPV	PATSAPRPAT	SPGPGKVVVVSTTG	DPATPYE	AGVALARQMG	ASLITFE	GKQHT	VVFNGD	538
<i>M. smegmatis</i>	TGFAPRDL	CAFVWPV	PATSTARPV	TGPGPGKVVVVSTTG	DPATPYQ	AGVDLARQMG	AALLSFD	GQHT	VVFNGD	550
<i>M. tuberculosis</i> Rv2224c	TGDAPLGT	CAFVWPV	PPTSQPHAVS	APGLVPTVVVSTTH	DPATPYK	AGVDLANQLR	GSLLT	FDGTQ	HAVFNGD	504
<i>M. avium</i>	TGNAPLGT	CAFVWPV	PPTSKPHIVS	APGLVPTVVVSTTH	DPATPYK	AGVDLANQLR	GSLLT	FDGTQ	HAVFNGD	504
<i>M. leprae</i>	TGDAPLGT	CAFVWPV	PPTSKPHIVS	APGLVPTVVVSTTH	DPATPYK	AGVDLANQLR	GSLLT	FDGTQ	HAVFNGD	519
<i>M. vanbalenii</i>	TGLAPMT	TCGFVWPV	PPTSVPH	ELDVSGLPPILVISTT	NDPATPYQ	AGVDLARQLD	GLT	LVTFEGT	QHTVSLQ	500
<i>M. flavescens</i>	TGLAPMT	TCGFVWPV	PPTSKPHEIS	VGLPPILVISTT	NDPATPYQ	AGVNLARQLG	GGALVTFEGT	QHTVAL	QGD	479
<i>M. sp. KMS</i>	TGHAPLST	CAFVWPV	PPTSEPH	ELDVGLPPILVISTT	NDPATPYQ	AGVDLARQLG	GGMLT	FE	EGTQHTVVFQ	520
<i>M. smegmatis</i>	TGDAPLGT	CAFVWPV	PPTSEPH	ELKVEGLPPILVISTT	NDPATPYQ	AGVDLARQLG	GGT	LVTFEGT	QHTVVFQ	494
Clustal Consensus	** ** *	8.:	****.* :	:	** ** *	*****:::	** :: . :::: * ** .	::* : 9.*		225
<i>M. tuberculosis</i> Rv2223c	MHYFLD	GTLPP	TSLR	CAP	520					
<i>M. avium</i>	VRVYFAGT	PPP	ASYRC	QS	522					
<i>M. leprae</i>	VHYFVDE	TLL	PASL	QCQP	511					
<i>M. vanbalenii</i>	VEFLVNST	PPP	AGLRC	--	505					
<i>M. flavescens</i>	VAFLTNST	PPP	SGLRC	--	505					
<i>M. sp. KMS</i>	VRFLVDG	VVPP	NGLRC	--	554					
<i>M. smegmatis</i>	LRFLTDL	QS	PPGNLAC	--	566					
<i>M. tuberculosis</i> Rv2224c	TAYLIGGT	TPPS	GAKC	--	520					
<i>M. avium</i>	TAYLVGGT	TPPS	GAKC	--	520					
<i>M. leprae</i>	TEYLIGGT	TPPS	GAKC	--	535					
<i>M. vanbalenii</i>	VRYLVDV	VVPP	DTRC	--	516					
<i>M. flavescens</i>	VPYLIDGS	VVPP	DTRC	--	495					
<i>M. sp. KMS</i>	ARYLIDV	VVPP	DTRC	--	537					
<i>M. smegmatis</i>	ATYLIDV	EVPP	ADTRC	--	510					
Clustal Consensus	::	*	10	229						

Figure 4.8. Sequence alignment of the mycobacterial homologs of the *M. tuberculosis* Rv2223c and Rv2224c amino acid sequences. Conserved cys-residues are numbered (1 – 10) and residues potentially involved in the formation of the enzymatic active site indicated by rectangular frame in the *M. tuberculosis* sequence. In the Clustal consensus sensus, an asterisk (*) represents identical amino acids, a semi-colon (:):conserved and a stop (.) semi-conserved.

In order to study the N-termini of proteins encoded by Rv2223c and Rv2224c for leader peptide signals, protein sequences were analysed on the SignalP leader peptide prediction server available on the Centre for Biological Sequence Analysis (CBS) web page (Materials and Methods, 2.4). SignalP predictions were made using networks based on gram-positive bacterial data. The protein sequence was then analysed for the presence of transmembrane helices on the CBS TMHMM server (Materials and Methods 2.4). The prediction gives the most probable location of trans-membrane helices in the sequence and is found by an algorithm called N-best which sums over all paths through the model with the same location and direction of the helices. The data generated in this way were compared with the hydrophobicity profiles of the sequences, which allows a visual probability of membrane spanning and surface exposed protein domains.

The signal peptide prediction identified a possible cleavage site between amino acids 34 and 35 (AGA – TE) in the Rv2223c protein sequence, which led to the (statistical) conclusion that the sequence is a leader peptide signal (Figure 4.9). Analysing the protein on the TMHMM server predicted that one trans-membrane helix, consisting of 17 amino acids from position 13 to 35 relative to the first amino acid, may be encoded in the sequence (Figure 4.9). The total probability that the N-terminus of the protein could be found in the cell cytoplasm was predicted as 0.80423. Of the remaining amino acids in the protein sequence, it was predicted that the amino acids from position 1 to 12 would form the cytoplasmic part of the protein, while the amino acids from position 36 to 520 would be present outside the cell after cleavage. Similarly, the amino acid sequence of Rv2224 may encode one trans-membrane helix (Figure 4.9). It was predicted that the first to twelfth amino acid might be present on the cytosol side of the cell membrane, while amino acids 13 to 35 might form the trans-membrane helix. The most likely enzymatic cleavage site was predicted to present between met₄₁ and ala₄₂. The rest of the peptide (amino acids 42 to 520) may be present outside the cell membrane.

This prediction was tested through the analysis of the hydrophobicity profiles of the sequences. These profiles are based on the Kyte-Doolittle and Hopp-Woods (Kyte and Doolittle, 1982; Hopp and Woods, 1983) scales, which determines the hydrophilicity / hydrophobicity of a peptide chain based on the amino acid side groups contained therein. On the Kyte-Doolittle scale, values of ≥ 1.6 typically indicates hydrophobic membrane spanning protein domains and as can be seen in Figure 4.10, the profiles of both the Rv2223c and Rv2224c protein sequences indicates a sharp increase in

hydrophobicity of the N-termini to a peak at around position 12 in the amino acid sequences. The Hopp-Woods scale shows the hydrophilicity of the peptide. According to this scale, a value > 0 would suggest that the particular domain is likely to be exposed on the surface of the folded protein. Therefore this result is in concordance with the transmembrane helix prediction server profile, which calculated the first amino acids to be hydrophilic (therefore more likely associated with the cytosol) followed by a hydrophobic peptide. This result furthermore agrees with the result obtained from the signal peptide prediction server, as the predicted cleavage site (amino acid 34 to 35) is outside the region of the protein (amino acid 36 to 520) predicted to be on the exogenous side of the membrane.

Another observation from the Kyle-Doolittle and Hopp-Woods profiles is the positions of the putative ser₂₃₂, his₄₈₈ and asp₄₆₁ catalytic triad residues indicated in Figure 4.10. These residues may all be situated on the transition cusp between the hydrophobic interior and hydrophilic exterior of the protein. Therefore the catalytic site may be accessible, but not exposed to the exogenous environment of the enzyme.

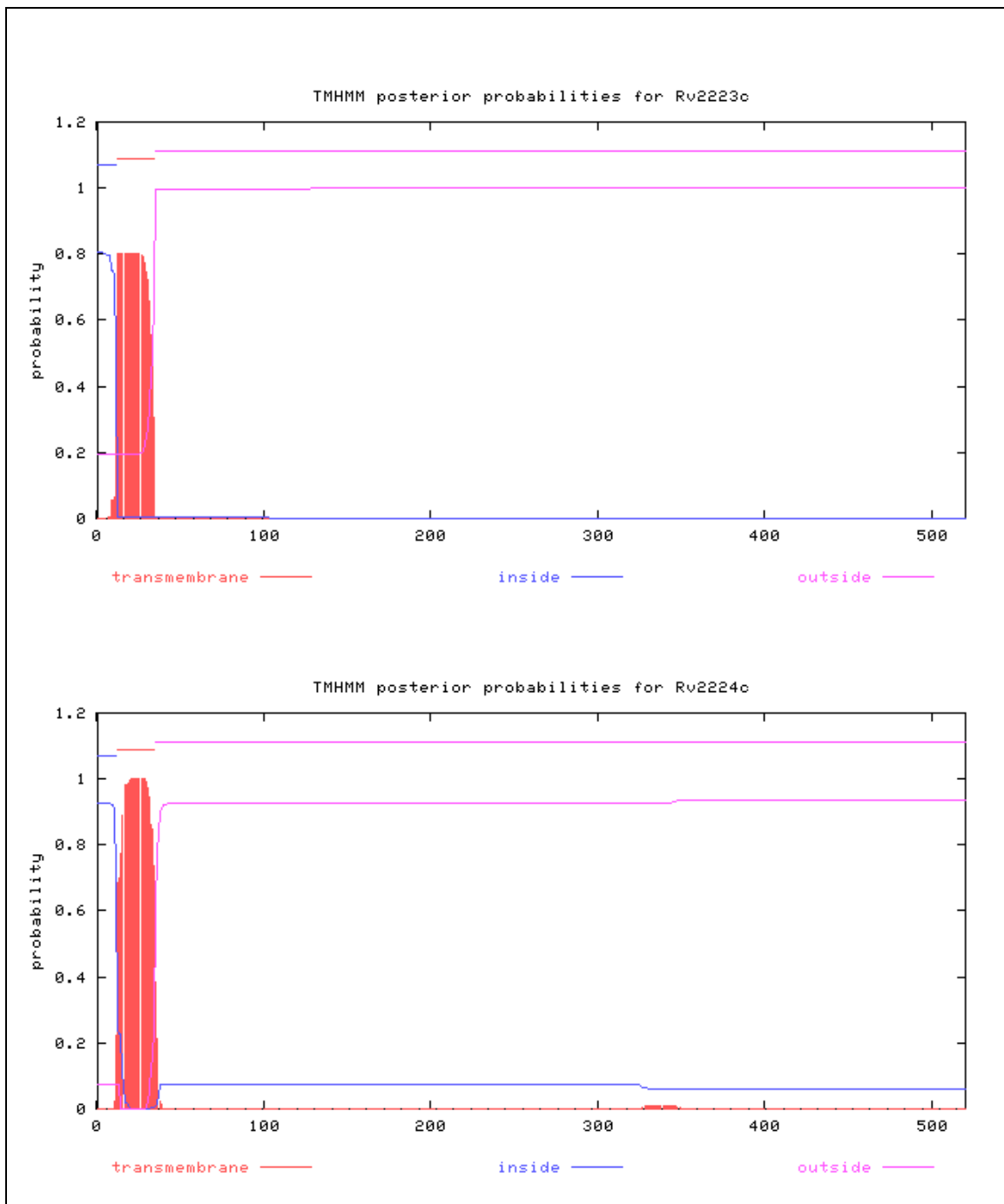


Figure 4.9.: The hydrophobicity profiles of the proteins encoded by Rv2223c and Rv2224c demonstrates that a region near the N-terminus of these proteins may be associated with the mycobacterial cell wall, suggesting that the proteins may be exported.

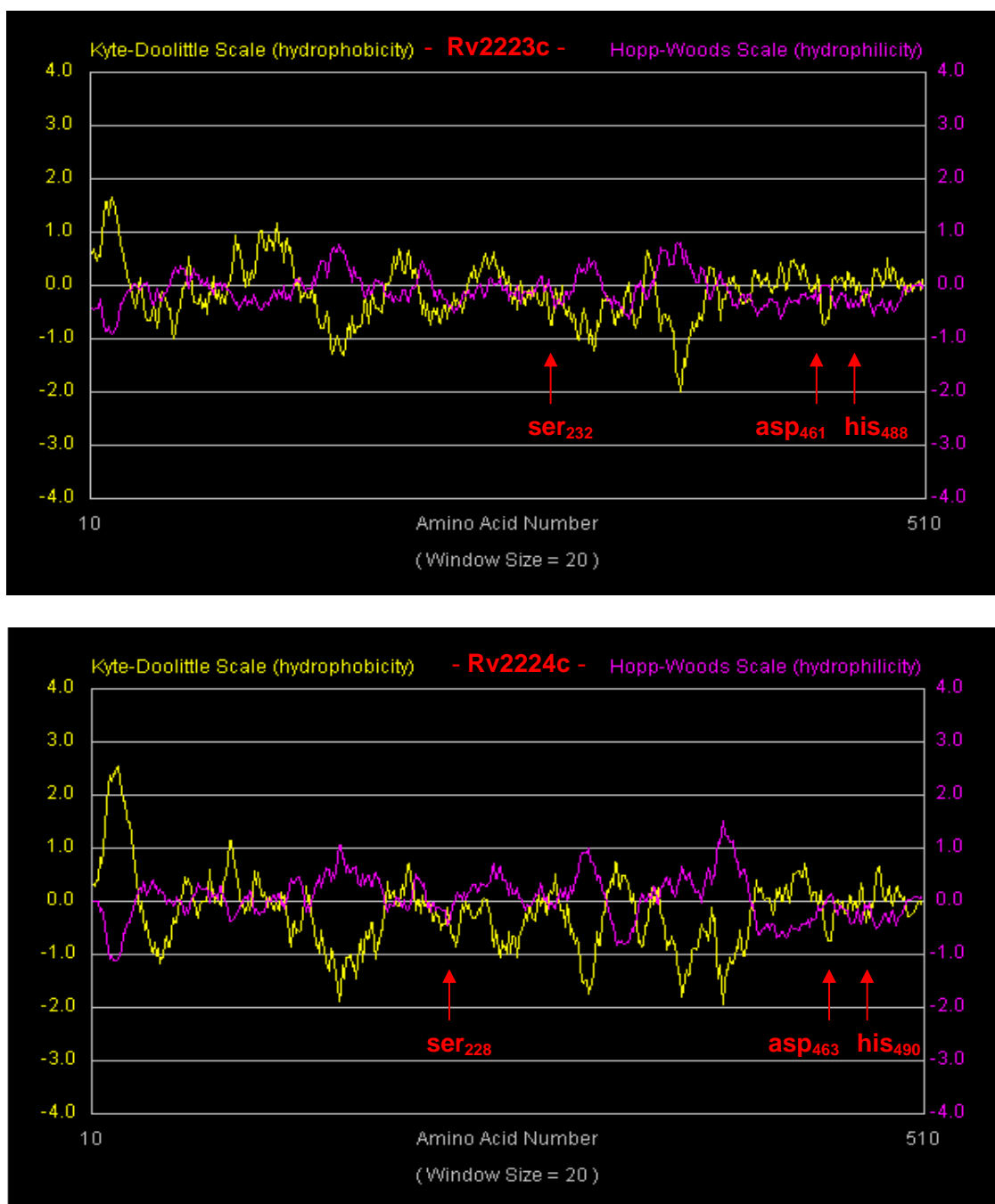


Figure 4.10. Kyte-Doolittle and Hopp-Woods hydrophobicity and hydrophilicity plots of the Rv2223c and Rv2224c sequences. The position of putative active site amino acids is indicated on the plots.

4.3 DISCUSSION

In Chapter 3 it was suggested that GS exported to the *M. tuberculosis* cell wall may be post-translationally modified by as yet unknown mechanisms due to size differences in GS sub-units isolated from different cellular compartments of *M. bovis* BCG. Size differences in GS sub-units have been noted before, but not described. One report has however shown that differences in GS sub-unit size may not be due to effects such as methylation or adenylation (Harth and Horwitz, 1997). We have shown that GS peptides of differing MW is not observed when *M. tuberculosis* GS was produced through *glnA1* expression in other non-GS secreting mycobacteria, such as *M. smegmatis* (see Section 3.2.1). Therefore it was hypothesised that *M. tuberculosis* may possess some unique mechanism of GS modification, which may be coupled with GS secretion.

4.3.1 Operon structure and promoter activity of the genes located downstream of *glnA1*

Although the nitrogen-signalling cascade has been extensively studied in *Corynebacteria*, relatively little is known about the influence of nitrogen availability on the regulation of *M. tuberculosis* genes that are thought to be involved in nitrogen metabolism. In this study, the co-expression and promoter activity of a cluster of genes located downstream of the glutamine synthetase encoding *glnA1* sequence in mycobacteria was investigated to see if the other two genes of unknown function located in this region may play a role in nitrogen metabolism.

Our results show an inverse correlation between the regulation of the promoters upstream of Rv2223c and Rv2224c under conditions of nitrogen availability. The method of investigating promoter activity used in this study is not quantitative, but the results suggest that Rv2224c may be expressed from a promoter with constitutive activity during logarithmic growth, while Rv2223c may only be expressed at very low levels. The transcription of both Rv2223c and Rv2224c may increase during the later stages of mycobacterial growth. However, Rv2223c transcription may be up-regulated during conditions of nitrogen excess, while Rv2224c transcription is down-regulated. The promoter activity of the Rv2223c upstream region also suggests that Rv2223c transcription may be down-regulated during nitrogen starvation. The presence of

differential promoter activity was also reflected by the absence of a RT-PCR product representing the 62bp intergenic region between Rv2224c and Rv2223c, which suggests that these two genes may not be transcribed as a single polygenic mRNA. The differential regulation of these open reading frames may suggest that they may have different functions, despite being similar in amino acid sequence.

The function of the proteins encoded by Rv2224c and Rv2223c are unknown, but it has previously been shown that the expression of Rv2224c may be up regulated during *M. tuberculosis* infection of human macrophages (Dubnau *et al.*, 2002), indicating that the protein encoded by Rv2224c may also be necessary during *M. tuberculosis* pathogenesis. Both the proteins encoded by these genes are annotated to have leader sequences and may therefore be exported to the *M. tuberculosis* cell membrane by a *sec*-dependent mechanism. A previous study showed that the Rv2224c protein could be detected in *M. tuberculosis* cell membranes, but not the Rv2223c sequence (Sinha *et al.*, 2005). The results of the current study suggest that Rv2224c may be constitutively expressed during *M. tuberculosis* growth in culture and under nitrogen starvation, but that Rv2223c is expressed during later stages of the *M. tuberculosis* growth cycle and up-regulated under conditions of nitrogen excess. This observation may explain the lack of B-galactosidase activity of *M. smegmatis* transformed with the Rv2223c upstream reporter construct grown on X-gal containing agar plates. The absence of Rv2223c in the *M. tuberculosis* membrane fractions may therefore be explained due to culturing conditions and it is expected that Rv2223c may locate to the mycobacterial cell wall.

Our results are equivocal about the presence of a promoter in the upstream region of the adenylyl transferase encoding *glnE* locus. There are conflicting reports regarding the co-expression of *glnA2* and *glnE* as a polygenic mRNA species (Collins *et al.*, 2002; Pashley *et al.*, 2006). In this study, RT-PCR products representing the intergenic regions of Rv2223c: *glnA2* and *glnA2*: *glnE* were detected, suggesting that Rv2223c, *glnA2* and *glnE* may be co-transcribed as a poly-cistronic mRNA. This mRNA species may be present during specific growth conditions, since the promoter upstream of Rv2223c may not have constitutive activity. This observation would explain the lack of a longer mRNA transcript by Northern analysis, as described in a previous report (Pashley *et al.*, 2006). Similar to that report, results from this study showed constitutive promoter activity in the intergenic region upstream of *glnA2*, which is not influenced by conditions of varying nitrogen availability. This is also observed in the closely related *Corynebacteria*, where

glnA2 is similarly situated adjacent to the *glnE* locus and the expression are not influenced by nitrogen levels (Nolden *et al.*, 2001). However, this observation does not exclude the presence of a *glnE* promoter, as operons with weaker internal promoters that are activated under certain conditions have been detected in *M. tuberculosis*, such as that in the phosphate transport (Torres *et al.*, 2001) and DNA gyrase operon (Unniraman *et al.*, 2002). It is believed that these weaker internal promoters may aid the enhancement of gene transcription under certain conditions. It could be speculated that the up-regulation of the Rv2223c promoter may lead to an increased production of *glnA2* and *glnE* under conditions of nitrogen excess. This might be a mechanism leading to increased synthesis of the *glnE*-encoded adenylyltransferase necessary to control GS activity through adenylylation under conditions of nitrogen excess.

4.3.2 Rv2223c and Rv2224c evolution

The sequence BLAST comparisons of the *M. tuberculosis* Rv2223c and Rv2224c protein sequences against the available bacterial genomes revealed that only the mycobacterial species have both sequences encoded by different genes in their genomes. Other actinobacterial genomes contain one protein ortholog of these sequences. This observation, together with the extensive protein sequence homology between the protein sequences of Rv2223c and Rv2224c, suggests that the two genes arose from an ancestral progenitor gene by duplication and subsequent evolutionary divergence. It could not be determined which *M. tuberculosis* gene is most closely related to the progenitor sequence. Gene duplication is a crucial mechanism of evolutionary innovation through increasing both gene number and gene complexity (He and Zhang, 2005). However progenitor and duplicate genes (or inparalogs) might undergo evolutionary changes at different rates (He and Zhang, 2005). The difference in the rate of evolutionary change may result in considerable sequence variation from the progenitor. It has been established that in many cases, the sequence least homologous to the progenitor sequence retained the original function, and therefore assumptions based on homology to the closest relative orthologous sequence may not reflect closest functional relative (Notebaart *et al.*, 2005; Aravind *et al.*, 2006).

The function of the proteins encoded by Rv2223c and Rv2224c could not be inferred from the sequence BLAST comparisons, since very few sequence orthologs with known function could be identified in other bacteria. The specific functions of the homologous

genes in all the bacteria used in this study are unknown. Because of the high degree of homology surrounding the catalytic serine gly – ser - gly consensus sequence (Brady *et al.*, 1990), most of the sequences are annotated as serine proteases. The presence of these putative amino acids involved in forming the catalytic triad in regions of the sequence that might be exposed to the environment lends support to this hypothesis.

CHAPTER FIVE

Expression Analysis of Rv2223c and Rv2224c

*“Science! true daughter of Old Time though art!
Who alterest all things with thy peering eyes.”*

Edgar Allan Poe, Sonnet to Science

(Born January 19, 1809, died of tuberculosis October 10, 1849)

5.1 INTRODUCTION

When the full *M. tuberculosis* H37Rv genome sequence was first made available by Cole *et al.* in 1998, specific function could only be ascribed to around 40% of the 3 924 predicted open reading frames. Most of the functions ascribed to these genes were based on bioinformatic approaches that depend on sequence similarity to sequences of known function in other bacteria, which has been shown in some cases to be incorrect (Watkins *et al.*, 2006; Johnston *et al.*, 2003). Another study showed that 9.4% of the genomic sequences were specific to mycobacteria and do not have any known homologs (Camus *et al.*, 2002). For example, the importance of Rv2223c and Rv2224c in *M. tuberculosis* growth and survival is unknown. In Chapter 4 it was shown that the Rv2223c and Rv2224c open reading frames might encode proteins with functions related to GS metabolism. These functions may be involved in the export mechanism of *M. tuberculosis* GS (Chapter 3).

5.1.1 Study aims

5.1.1.1 To express and purify the enzyme encoded by Rv2224c in *E. coli*.

5.1.1.2 To analyse the phenotype of after inhibition of Rv2223c and Rv2224c in *M. bovis* BCG, which may indicate if Rv2223c and Rv2224c are essential for *M. tuberculosis* or *M. bovis* BCG growth.

5.1.1.3 To analyse the *in vivo* expression of Rv2223c and *glnA1* in *M. tuberculosis* infected human tissue

5.1.1.4 To analyse protein-protein interaction of *M. tuberculosis* Rv2223c and GS.

5.2 RESULTS

5.2.1 Expression of *M. tuberculosis* Rv2223c in *E. coli*

In order to express the enzyme encoded by the *M. tuberculosis* Rv2223c open reading frame for purification and enzymatic studies, the sequence was cloned into a L-arabinose inducible *E. coli* expression vector (Materials and Methods 5, Appendix C.7). The *M. tuberculosis glnA1* sequence was cloned in a similar manner as a control. An *E. coli* araBA and araC deletion strain (Materials and Methods 1.2) was transformed with an Rv2223c or *glnA1* vector construct and positive transformants were cultured in liquid media until an OD₆₀₀ of 0.3 – 0.4 was reached. Expression of recombinant proteins was induced by the addition of various concentrations of L-arabinose (0.002% to 20%; Figure 5.1) and cells were incubated at 30⁰C or 37⁰C for 4 hours. It was observed that *E. coli* cultures transformed with the Rv2223c construct did not increase in turbidity after the addition of L-arabinose. For the detection of recombinant protein, the *E. coli* transformants were disrupted by different methods, such as lysis through osmotic shock, ribolisation and sonication. Figure 5.1 shows that recombinant Rv2223c could not be detected in whole lysates at the expected size (55 kDa) on a 12% poly-acrylamide gel after induction with L-arabinose at a final concentration of 20%, 2% and 0.2% respectively. In lanes 1 to 3 (Figure 5.1), *M. tuberculosis* GS could be observed as a single band migrating around 55 kDa. The overnight culturing of transformed *E. coli* at 30⁰C did not result in growth in cultures expressing recombinant Rv2223c. No recombinant Rv2223c could be detected by Western blot detection (Materials and Methods 10) using anti-6 x his-tag antibodies (results not shown).

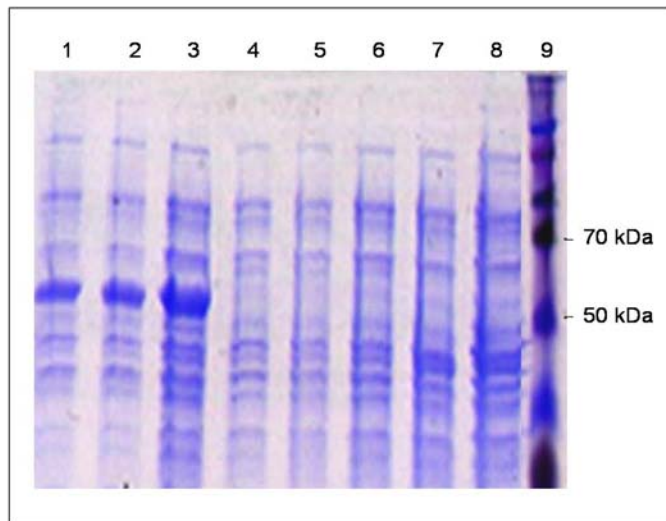


Figure 5.1. Attempted expression of recombinant *M. tuberculosis* GS (lanes 1 to 3) and Rv2223c (lanes 4 to 8) at varying final concentrations of L-arabinose. Expression of recombinant GS were induced with 20% (lane 1); 2% (lane 2) and 0.2% L-arabinose (lane 3). Rv2223c expression could not be induced with L-arabinose to a final concentration of 20% (lane 4); 2% (lane 5); 0.2% (lane 6); 0.02% (lane 7) and 0.002% (lane 8).

In order to investigate whether the recombinant Rv2223c protein may have an effect on the *E. coli* expression hosts' growth, *E. coli* transformed with the Rv2223c and *glnA1* constructs was cultured on agar media containing L-arabinose to a final concentration of 0.2% or agar media containing no L-arabinose. Agar plates were incubated at 30°C for 2 days. Figure 5.2 shows that both the Rv2223c and *glnA1* transformed *E. coli* grew on agar that does not contain L-arabinose, but that only the *E. coli* transformed with the *glnA1* construct grew in the presence of 0.2% L-arabinose. No growth of *E. coli* transformed with the Rv2223c construct could be detected on agar plates containing 0.2% L-arabinose after 2 days.

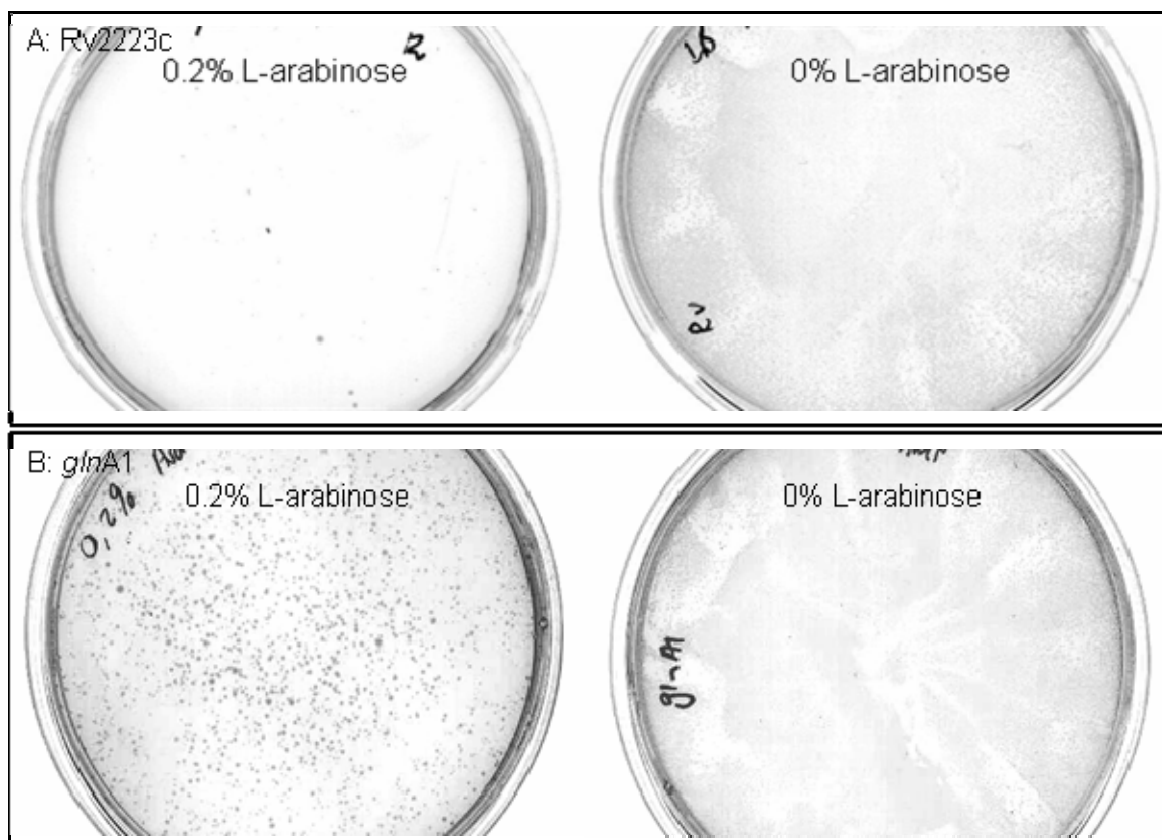


Figure 5.2. *Escherichia coli* colonies were observed on solid media when *E. coli* transformed with expression plasmids carrying *M. tuberculosis* Rv2223c (A) and *glnA1* (B) under conditions where expression of the recombinant proteins were not induced. When protein expression was induced through addition of 0.2% L-arabinose, *E. coli* colonies could be observed for cultures expressing recombinant *M. tuberculosis glnA1*, but not Rv2223c.

5.2.2 Inhibition of Rv2223c and Rv2224c expression in *M. tuberculosis* H37Rv and *M. bovis* BCG

The efficacy of antisense oligodeoxynucleotides (ODNs) to inhibit the expression of specific gene targets has been shown in various organisms (Lisziewicz *et al.*, 1993; Barker *et al.*, 1996; Andratschke *et al.*, 2001). Due to the hydrophobic nature of the mycobacterial cell wall, the passage of hydrophilic compounds (Rastogi *et al.*, 1981; Rastogi *et al.*, 1989) is prevented. To minimise degradation of ODNs by bacterial mechanisms, ODNs were modified by synthesising all inter-nucleoside linkages as phosphorothioates, which leads to higher lipophilicity and therefore uptake by

mycobacterial cells (Harth *et al.*, 2002). In order to study the effect of Rv2223c and Rv2224c translation inhibition on the phenotype of *M. tuberculosis* H37Rv, BACTEC cultures were treated with sequence specific PS-ODNS (Section 5.2.2.1). To overcome the limitations of PS-ODN half-life and entry into the mycobacterial cell, a vector based anti-sense delivery system was constructed (Section 5.2.2.2). This anti-sense delivery system made use of a mycobacterial expression plasmid containing an acetamide inducible promoter (Parish *et al.*, 1997). The synthesis of anti-sense mRNA could thereby be controlled through the induction of the plasmid promoter.

5.2.2.1 Rv2223c and Rv2224c inhibition with PS-ODNs

In order to obtain an indication of the importance of Rv2223c and Rv2224c in *M. tuberculosis* H37Rv liquid cultures, *M. tuberculosis* H37Rv was cultured in BACTEC to a growth index of 600 – 700, which represents bacilli in the early logarithmic growth phase (Roberts *et al.*, 1983). At this stage, bacilli would still be in homogeneous suspension and clumping would be minimal. These actively growing *M. tuberculosis* H37Rv cultures were incubated overnight with PS-ODN to a final concentration of 10 μ M before being re-inoculated into BACTEC (Materials and Methods 1.3.2). Growth of *M. tuberculosis* cultures incubated with PS-ODN complementary to Rv2223c and Rv2224c mRNA were compared to control cultures that were incubated with 10 μ M of random non-specific PS-ODN (no BLAST-sequence homology to the *M. tuberculosis* genome). Figure 5.3 A and B shows that gene knockdown of Rv2223c and Rv2224c resulted in a ~ 80% inhibition of *M. tuberculosis* H37Rv growth compared to controls. Growth curves of *M. tuberculosis* H37Rv control cultures treated with non-specific random PS-ODN shows that the PS-ODN had almost no growth inhibitory effect compared to the Rv2223c and Rv2224c specific PS-ODN, as the control cultures grew to a maximum measurable growth index of 999. A normal downward trend at the end of the logarithmic growth phase (~ Day 7 – 8, Figure 5.3 A and B) due to nutritional starvation was also observed. Time to positivity for cultures treated with Rv2223c anti-sense PS-ODN was 5 to 6 days (Figure 5.3 A), while those of cultures treated with Rv2224c anti-sense PS-ODN was 4 to 5 days. The growth curves of both cultures also show that when growth positivity has been reached (i.e. the inhibitory effect of the PS-ODN has been overcome), both *M. tuberculosis* cultures grew exponentially.

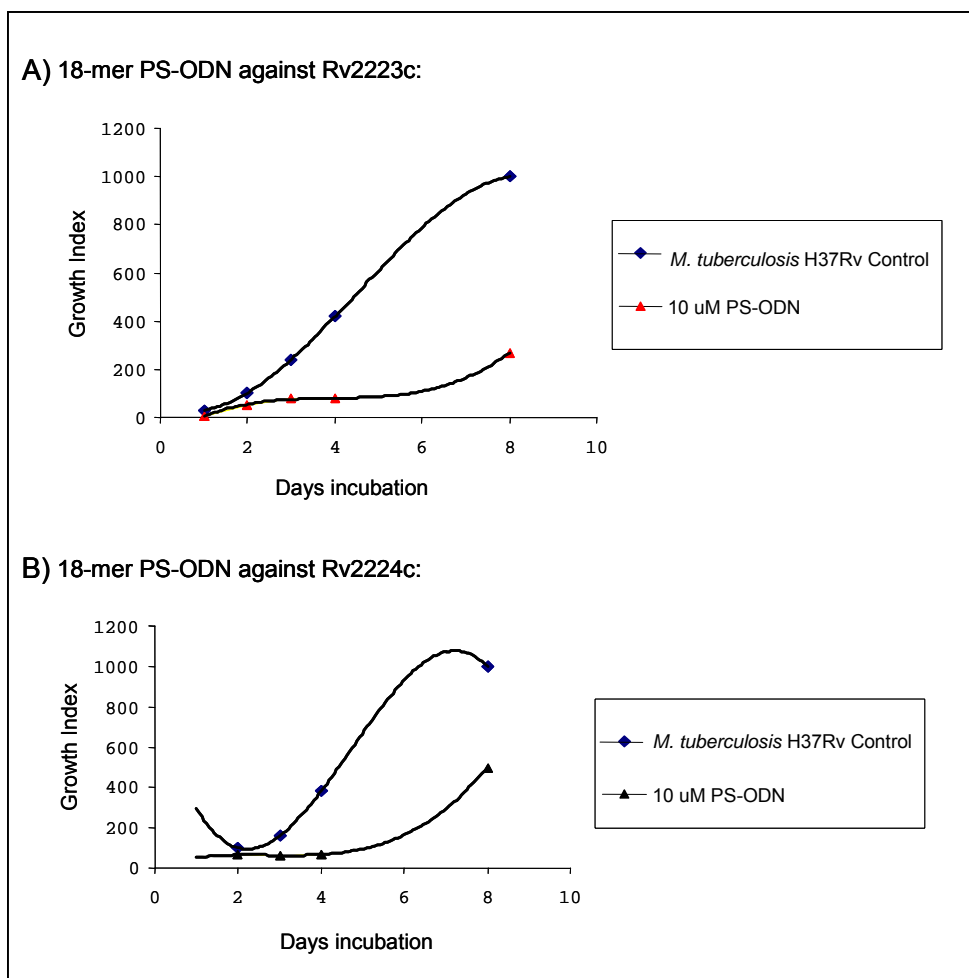


Figure 5.3. BACTEC growth indices of *M. tuberculosis* H37Rv cultures treated with anti-sense PS-ODN targeting Rv2223c (A) and Rv2224c (B) compared to control cultures treated with non-specific PS-ODN.

5.2.2.2 Vector-based anti-sense expression

Because the effect of PS-ODNs can be overcome by bacterial nuclease digestion, the effects of longer-term gene knockdown of Rv2223c and Rv2224c could not be studied. To overcome the limited up-take efficiency and half-life of anti-sense PS-ODN, a vector-based approach was followed to investigate the mycobacterial Rv2223c and Rv2224c knockdown phenotype in *M. bovis* BCG. *Mycobacterium bovis* BCG was chosen as expression host due its genetic similarity to *M. tuberculosis* and safety considerations of working with *M. tuberculosis* electro-transformations. Therefore an acetamide-inducible mycobacterial expression vector was modified to produce mRNA molecules

complementary to Rv2223c (492 bp), Rv2224c (460 bp) and *glnA1* (517 bp) (Materials and Methods 5.4). *Mycobacterium tuberculosis glnA1* was chosen as a control experiment due to the known bactericidal phenotype of *glnA1* transcription inhibition (Harth *et al.*, 2000).

Figure 5.4 shows the *M. bovis* BCG phenotypes of *M. bovis* BCG transformed with constructs containing a anti-sense clone of *glnA1* (pANT-*glnA1*), Rv2223c (pANT-Rv2223c) and Rv2224c (pANT-Rv2224c). As a control for plasmid stability and phenotypic effect alone, a plasmid construct without any cloned insert (pANT-0) was transformed into *M. bovis* BCG. These *M. bovis* BCG transformants were selected on 7H11 mycobacterial culture plates containing hygromycin B and 7H11 agar plates containing hygromycin B and acetamide to a final concentration of 0.01%. Table 5.1 and Figure 5.4 show that in all transformed *M. bovis* BCG cultures (Figure 5.4 A – C), bacteria grew well in comparison with control cultures (Figure 5.4 D) in the absence of acetamide. Upon induction of anti-sense mRNA synthesis by the addition of 0.01% acetamide to the solid media, no *M. bovis* BCG growth could be observed for *M. bovis* BCG transformed with pANT-*glnA1*, pANT-Rv2223c or pANT-Rv2224c. On the pANT-Rv2223c and pANT-Rv2224c solid media, one or two *M. bovis* BCG revertant colonies could sometimes be observed. Control cultures of *M. bovis* BCG cultured on 7H11 media containing 0.01% acetamide showed that the acetamide content in substituted 7H11 media had no effect on colony forming units.

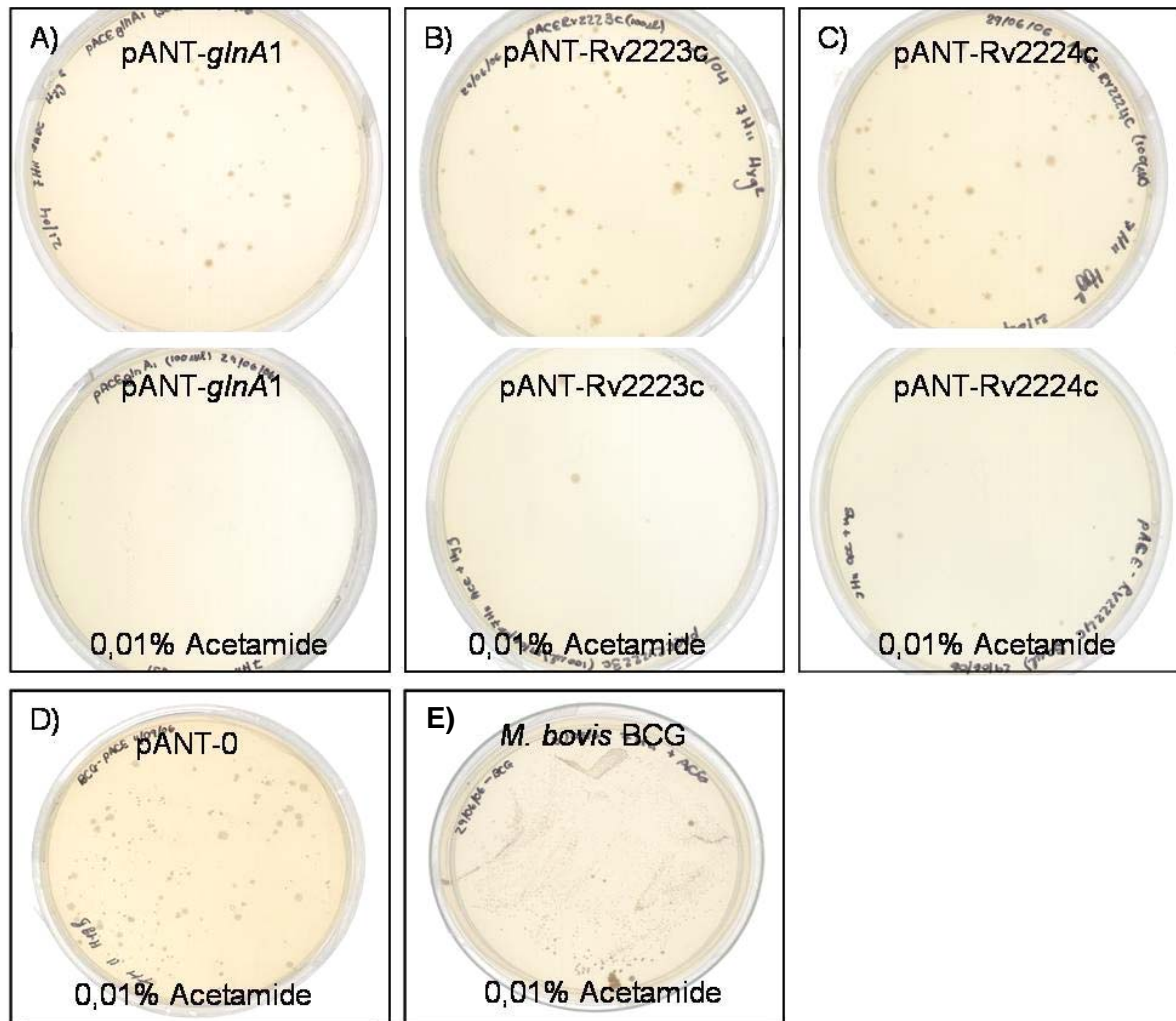


Figure 5.4. Growth phenotypes of *M. bovis* BCG cultures transformed with pANT-*glnA1* (Panel A), pANT-Rv2223c (Panel B) and pANT-Rv2224c (Panel C) and cultured on agar or agar containing acetamide to a final concentration of 0.01%. No, or very few colonies could be observed on agar containing acetamide when compared to controls without acetamide. Control cultures included *M. bovis* BCG transformed with pACE alone (Panel D) and non-transformed *M. bovis* BCG (Panel E).

Table 5.1. Colonies of *M. bovis* BCG transformed with constructs producing anti-sense mRNA to *glnA1*, Rv2224c and Rv2223c formed on 7H11 agar and agar containing 0.01% acetamide as a inducing agent for anti-sense mRNA synthesis.

	Colonies per agar plate	
	0% Acetamide	0.01% Acetamide
pANT- <i>glnA1</i>	33	2
pANT-Rv2224c	45	3
pANT-Rv2223c	37	5
pANT-0	>50	>50
<i>M. bovis</i> BCG	>100	>100

5.2.3 Bacterial-2-hybrid analysis of GS and Rv2223c interaction

Direct protein-protein interaction between *M. tuberculosis* GS and the putative protease encoded by Rv2223c were investigated with an *E. coli* based bacterial-2-hybrid system. The coding sequences of *glnA1* and Rv2223c were cloned into two separate target vectors that contained the two subunits of the catalytic domain of adenylate cyclase (see Section 3.2.2). Interaction between two cloned target sequences would result in β -galactosidase activity on X-gal containing LB media, therefore presenting colonies as blue or colourless. In Figure 5.5 A, blue colonies indicative of β -galactosidase activity could be observed for *E. coli* colonies doubly transformed with expression plasmids containing interacting proteins (pKTzip and pUTzip). Figure 5.5 B shows that this β -galactosidase activity is only present as background in *E. coli* colonies transformed with non-interacting controls. Figure 5.5 C indicates that, compared to the control experiments, no interaction between GS and the protein encoded by Rv2223c could be observed. Some background false-positive Lac⁺ colonies could be observed on both the negative and control experiments. These false positive colonies are usually due to endogenous upstream promoter mutations occurring at an approximate frequency of 10^{-7} to 10^{-8} (Karimova *et al.*, 1998). The same results were obtained when *E. coli* transformants were cultured at 30°C in order to aid recombinant protein expression and correct target protein folding. Both sequences were analysed for *E. coli* codon-bias.

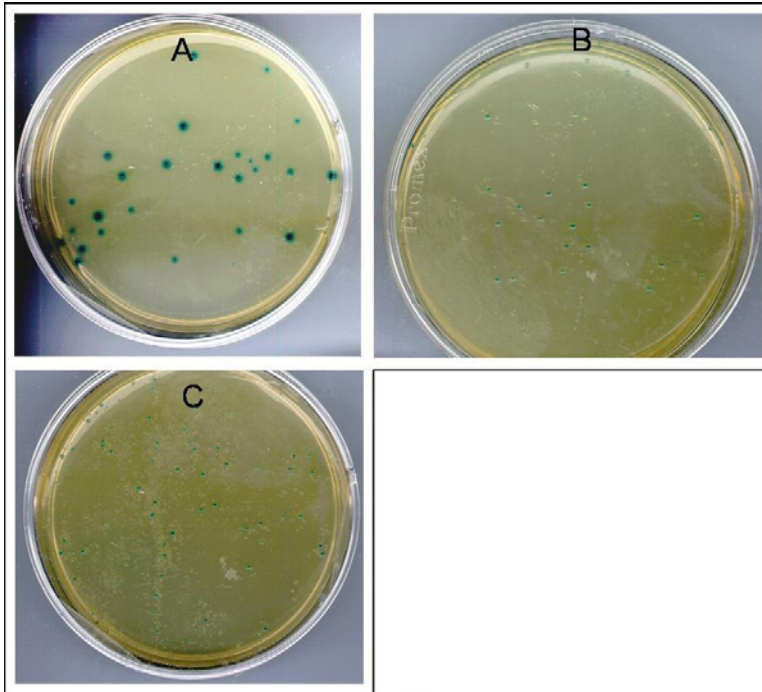


Figure 5.5. *Escherichia coli* based Bacterial-2-hybrid indicates interacting proteins by β -galactosidase activity (blue on X-gal containing media), as can be seen for positive controls transformed with constructs (pKTzip and pUTzip) encoding interacting proteins (A). Colonies transformed with constructs (pKT25 and pUT18) encoding non-interacting proteins remain white, although some light blue background colonies were observed (B). Colouring of colonies transformed with constructs encoding *M. tuberculosis* GS and Rv2223c were comparable to the negative controls (C).

5.2.4 *In situ* Hybridisation

In order to gain some indication of the importance of Rv2223c during *M. tuberculosis* host infection, the localisation of Rv2223c expression was studied through RNA: RNA *in situ* hybridisation (ISH; Materials and Methods 12) to granulomatous human lung biopsies. The expression of *M. tuberculosis glnA1* (encoding GS) was investigated by the same method as an indication of the distribution of expression of an essential gene.

As shown in Figures 5.6 – 5.9, all infected tissues analysed were positive for *glnA1* and Rv2223c expression in cellular regions of mature and immature granulomas. The highest intensities of *glnA1* and Rv2223c positive staining was evident in regions

containing high amounts of cellular structures, such as the tissue at the periphery and surrounding both immature (non-necrotic) and mature (necrotic) granulomas.

The necrotic granuloma contained giant cells and a lymphocyte cuff (consisting of lymphocytes and macrophages) that were positive for *glnA1* and Rv2223c expression. A transition zone, weakly positive for *glnA1* and Rv2223c expression, was seen extending into the central region of caseous necrosis that was negative for *glnA1* and Rv2223c mRNA, as indicated by the blue signal. No positive hybridisation signal of the sense mRNA riboprobe of either genes to sections originating from the same tissue samples was observed (Negative controls, Figures 5.6 – 5.9), demonstrates that the hybridisation of the anti-sense probe was specific to mRNA sequences of *M. tuberculosis glnA1* and that the hybridisation conditions were sufficiently stringent to ensure specific probe binding.

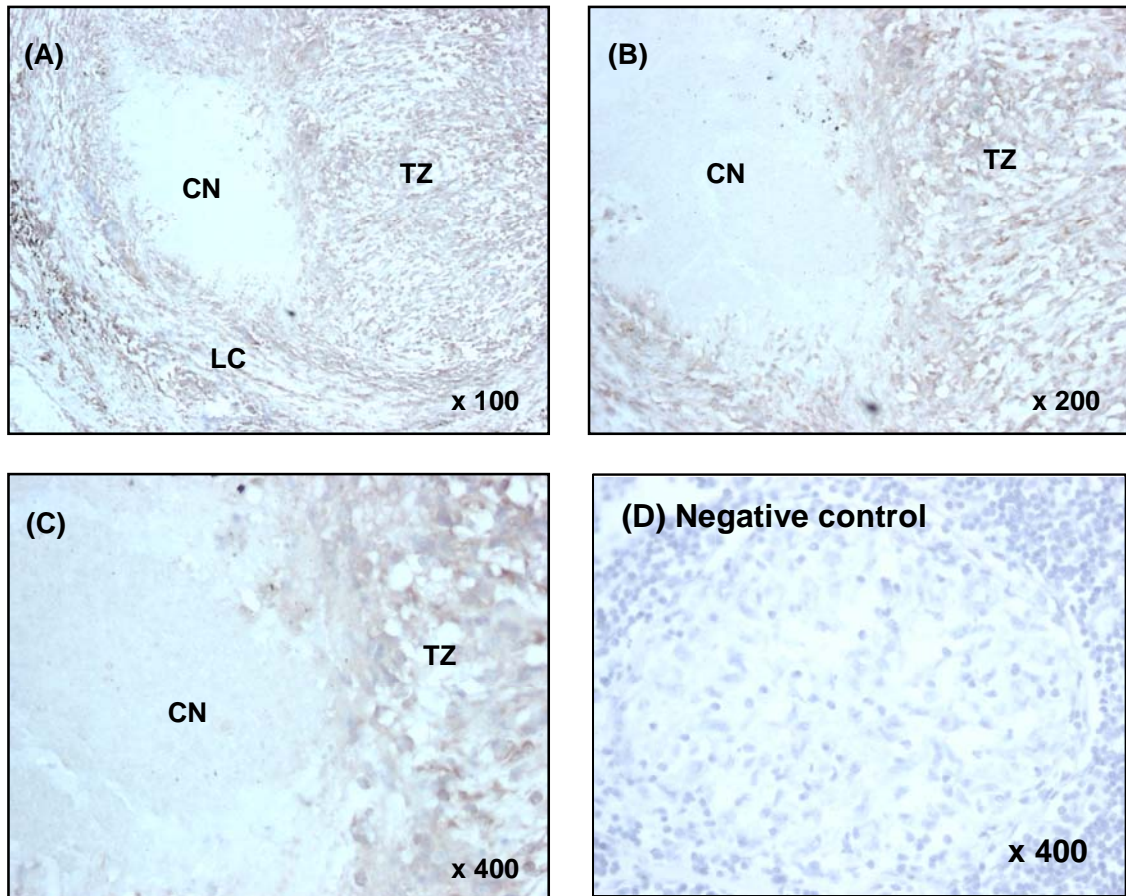


Figure 5.6. *In situ* hybridisation of a *M. tuberculosis glnA1* mRNA specific probe in a necrotic granuloma. Positive hybridisation (brown signal indicates hybridisation, whereas blue indicates the non-binding of the mRNA probe) demonstrated that *glnA1* was expressed in the lymphocyte cuff (LC) and the transition zone (TZ) surrounding the area of caseous necrosis (CN), where no *glnA1* expression could be detected. Higher power magnification shows that *glnA1* was expressed on the periphery of the necrotic region (B + C). The negative control shows that non-specific binding of the probe did not occur in these tissues (D).

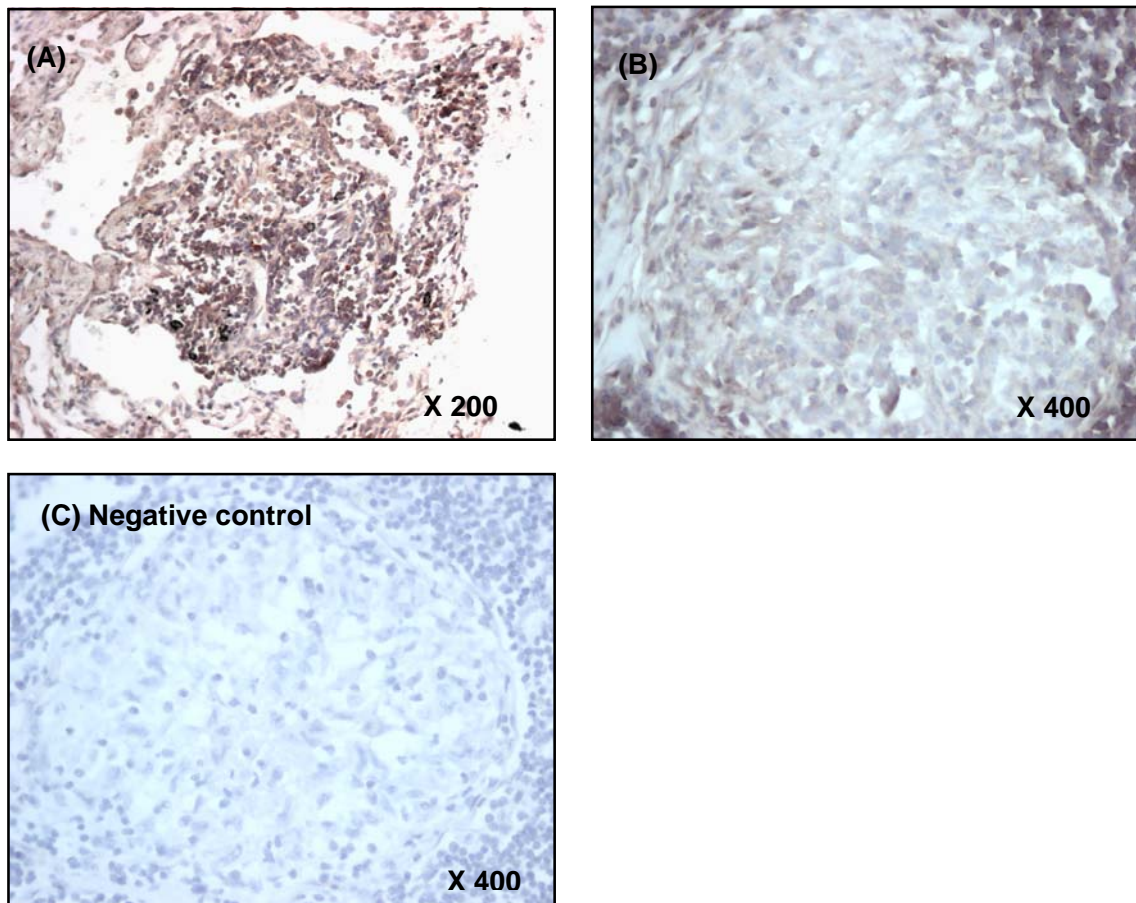


Figure 5.7. *Mycobacterium tuberculosis glnA1* is abundantly expressed in non-necrotic granulomas and surrounding tissues, as demonstrated by the dark brown hybridisation signal (brown signal indicates hybridisation, whereas blue indicates lack of binding of the mRNA probe). Higher power magnification of the internal region of the granuloma shows even distribution of *glnA1* expression, as well as macrophages and lymphocytes, which were negative for *glnA1* hybridisation. The negative control shows that non-specific hybridisation of the probe did not occur.

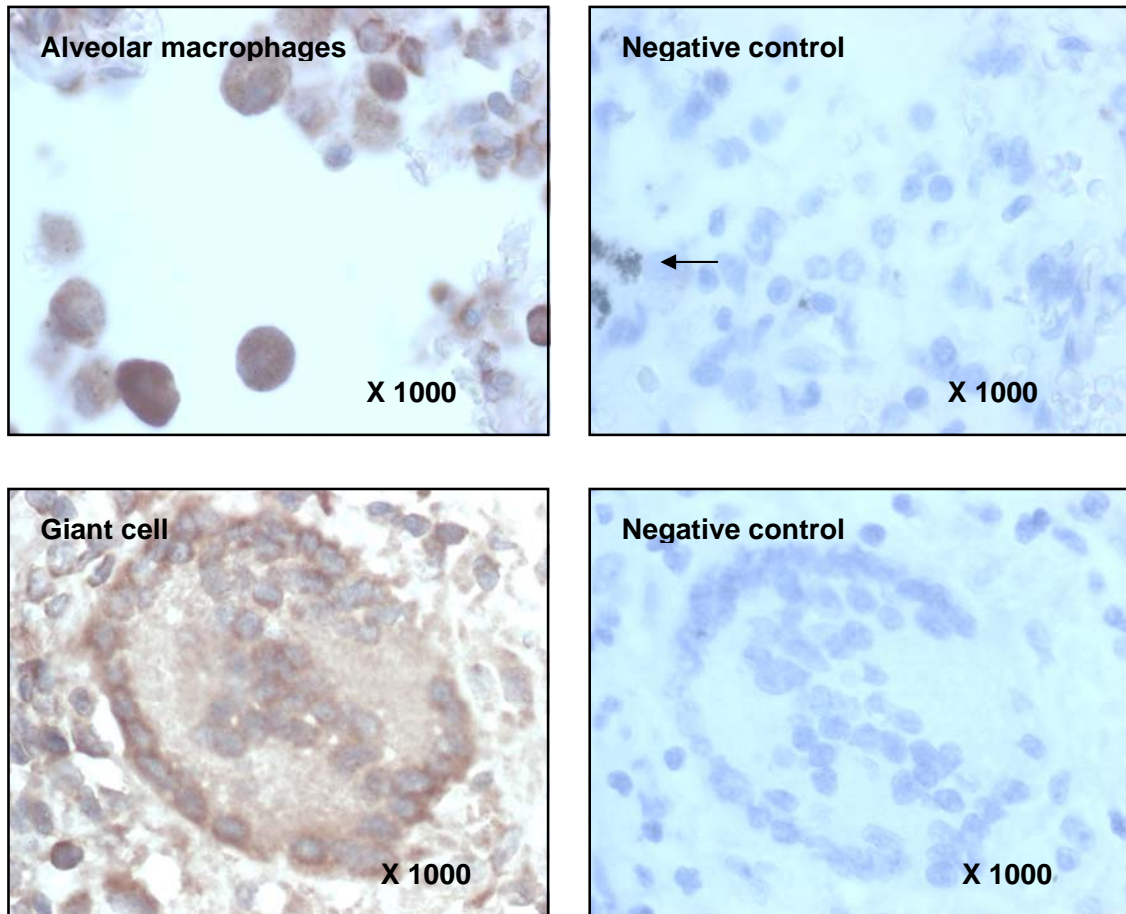


Figure 5.8. *Mycobacterium tuberculosis glnA1* expression could be associated with host immune cells and structures found in the lung granuloma, such as alveolar macrophages and giant cells. Black carbon deposits are indicated on the negative control with an arrow.

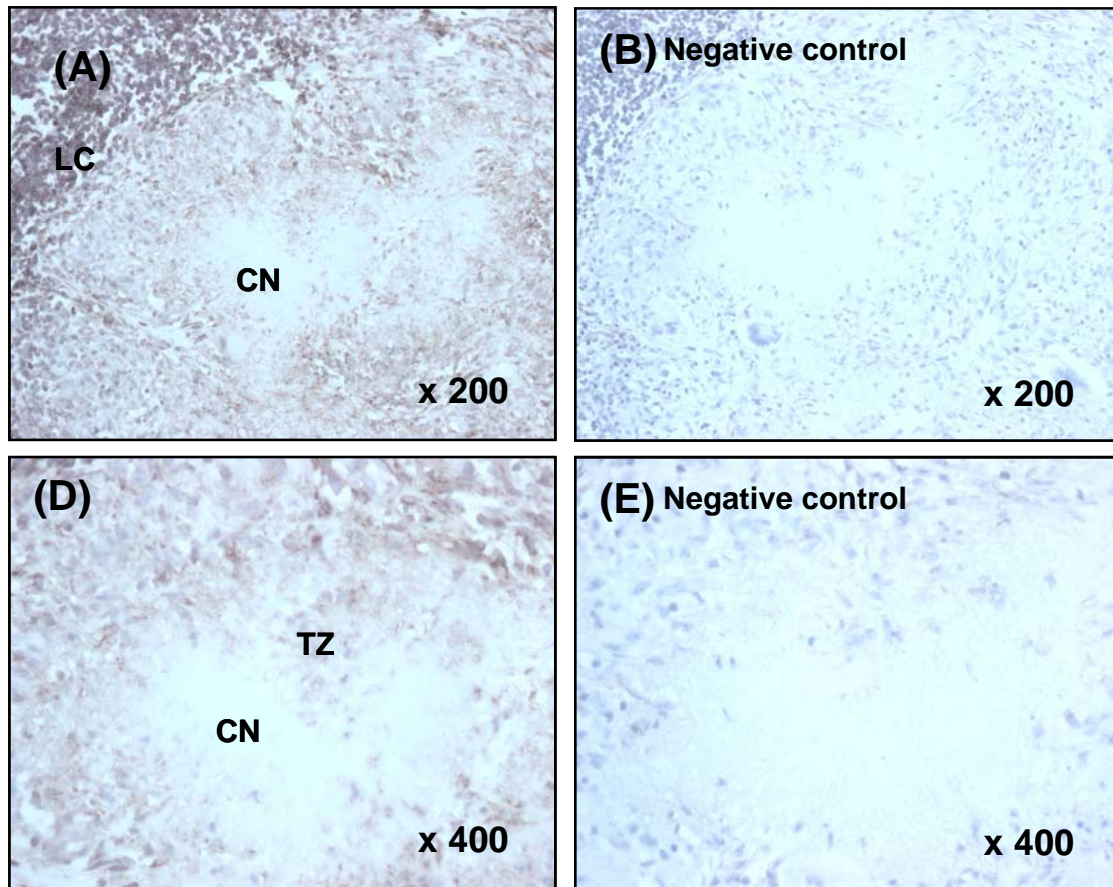


Figure 5.9. *In situ* hybridisation of a *M. tuberculosis* Rv2223c mRNA specific probe to a necrotic pulmonary granuloma. Positive hybridisation as indicated by brown signal (blue signal indicates non-binding of the mRNA probe) demonstrated that Rv2223c was expressed in the lymphocyte cuff (LC) and the transition zone (TZ) surrounding the area of caseous necrosis (CN), where no Rv2223c expression could be detected. Higher power magnification shows that Rv2223c is expressed on the periphery of the necrotic region (D). Negative controls indicate that no non-specific binding of the probes occurred in the tissues (B and E).

5.3 DISCUSSION

5.3.1 The Rv2223c encoded protein may be toxic to *E. coli*

In order to produce recombinant Rv2223c protein in *E. coli*, the coding sequence was cloned into a L-arabinose inducible *E. coli* hexa-histidine fusion vector. Recombinant *M. tuberculosis* GS has been synthesised stably and in active form in previous studies in *E. coli* (Singh *et al.*, 2004), and therefore *M. tuberculosis glnA1* was cloned in a similar manner as an expression control experiment. However, *E. coli* cultures expressing recombinant Rv2223c failed to grow after expression induction, even after longer incubation periods at low induction agent concentrations (which might allow the *E. coli* host cells time to overcome the growth inhibitory effect), suggesting that the Rv2223c protein might be toxic to *E. coli*. Previous reports have demonstrated that some expression vectors might be intrinsically toxic to the hosts, but usually such transformed hosts present as small, slow growing colonies on selection plates lacking recombinant protein expression inducing agent (Miroux and Walker, 1996). Much of the toxic effects of recombinant protein expression in *E. coli* host strains are ascribed to transcription surpassing the cells' translation ability, resulting in high cytosol concentrations of unstable naked mRNAs (lost and Dryfus, 1995; Makarova *et al.*, 1995). It has also been suggested that the cell might invest most of its protein translation capacity into the production of the recombinant protein, thereby not synthesising sufficient concentrations of normal cellular proteins (Dong *et al.*, 1995). However, through the induction of *M. tuberculosis glnA1* it has been observed that altering the final concentration of the arabinose-inducing agent may control the rate of transcription. The synthesis of the recombinant Rv2223c protein has been attempted at various final concentrations with no success. From these observations it might be argued that even low levels of the Rv2223c protein product itself may be toxic to *E. coli* cultures. Because the specific function and substrate of the Rv2223c protein is unknown, it could not be determined why the toxic effect was observed. However, it might be speculated that if the Rv2223c protein is expressed in a functional form it might disrupt normal cellular processes. This observation might also lead to enable speculation that during the bacterial-2-hybrid

studies, non-functional Rv2223c might have been expressed. Therefore the results of this study might be considered inconclusive.

5.3.2 Inhibition of *M. tuberculosis* and *M. bovis* BCG Rv2223c and Rv2224c

The use of anti-sense mRNA has been shown to be an effective manner to suppress the translation of target gene mRNA (Harth *et al.*, 2000; Li *et al.*, 2007). To study the knockdown phenotype of Rv2223c and Rv2224c, anti-sense molecules administered in the form of PS-ODNs or expression plasmid based have been done in *M. tuberculosis* and *M. bovis* BCG. In both cases, a significant effect on viability of the mycobacteria was observed. When *M. tuberculosis* H37Rv was treated with PS-ODNs targeting Rv2223c and Rv2224c, a marked reduction in growth was observed compared to control cultures. An increase of growth could be observed after the duration of incubation, which may indicate that *M. tuberculosis* may overcome the effect of inhibition, or that some viable mycobacteria may have remained in the culture that has not taken up sufficient amounts of PS-ODN to cause a death. This result may also indicate that inhibition of Rv2223c and Rv2224c may have a bacteriostatic effect that cause slower growth, and not a bactericidal effect.

To investigate the longer-term effects of Rv2223c and Rv2224c inhibition, a plasmid based anti-sense delivery construct was generated by using an inducible mycobacterial expression plasmid. The effects of anti-sense mediated suppression were done in *M. bovis* BCG as expression host (for bio-safety reasons), which is 99% similar to *M. tuberculosis* on a genetic level. To control for the efficacy of this system, control *M. bovis* BCG cultures transformed with anti-sense constructs targeting *glnA1* were produced. It has been shown that anti-sense mediated suppression of *glnA1* has a bactericidal effect (Harth *et al.*, 2000). No growth could be observed in *M. bovis* BCG cultures transformed with the anti-sense constructs targeting Rv2223c, Rv2224c and *glnA1*. This effect was stable over months, suggesting that the effect of Rv2223c and Rv2224c inhibition may be bactericidal and that the inhibition could not be overcome to allow growth, suggesting that the proteins encoded by Rv2223c and Rv2224c may be essential for *M. bovis* BCG growth. Because of the lethal phenotype shown by mycobacterial cultures treated with either method of anti-sense mediated gene suppression, the levels of mRNA could not be determined by methods such as reverse transcription- or real-time PCR. At the

present time, a plasmid-based anti-sense delivery approach has not been used to inhibit mRNA translation in mycobacteria. The results of this study shows that long-term effects of gene inhibition could be studied by using inducible expression plasmids as vectors for anti-sense encoding DNA sequences. Also the use of longer anti-sense mRNA sequences to increase specificity for target molecules may not influence the efficacy of a plasmid-based anti-sense construct.

These results also suggest that all the genes in the genetic cassette downstream of *glnA1* may be essential. In Chapter 4 it was shown that a polygenic mRNA species containing the coding information for Rv2223c, *glnA2* and *glnE* might be present under some growth conditions. However, the methods employed in this study should not affect the translation of *glnA2* and *glnE*, because it was shown that only the regions of double stranded mRNA are degraded (Robbins *et al.*, 1998; Thonberg *et al.*, 2004). Also, *glnA2* may have a promoter sequence (see Chapter 4), which suggests that a ribosomal binding site should also be present. Therefore translation of the *glnA2* and *glnE* genes might not be affected by the breakdown of Rv2223c due to RNase H activity (Thonberg *et al.*, 2004).

5.3.3 *In situ* hybridisation of Rv2223c and *glnA1* in *M. tuberculosis* infected tissue

The importance of Rv2223c for *M. tuberculosis* growth (as discussed in Section 5.3.2) was further investigated in human tissue derived from pulmonary *M. tuberculosis* infection. The precise micro-environment inside the granuloma is unknown, but it is assumed that *M. tuberculosis* might be exposed to varying aerobic (Wayne and Sohaskey, 2001) and toxic (Russell, 2001) conditions not conducive to mycobacterial growth. The mechanisms enabling *M. tuberculosis* to survive and proliferate in these conditions are still poorly understood, but some genes encoding virulence factors, or factors that enable *M. tuberculosis* to infect and adapt to the host environment, have been identified (Triccas and Gicquel, 2000). Disruption of these virulence genes result in *M. tuberculosis* attenuation, while complementation of the disrupted genes restores virulence. Many of these genes may be involved in intermediary metabolism (Cole *et al.*, 1998), which not only enable *M. tuberculosis* to subsist on alternative energy sources (Segal, 1984; Segal and Bloch, 1956), but also enter a state of metabolic inactivity or latency under conditions of low oxygen tension (Wayne and Hayes, 1998).

Various studies have attempted to elucidate which genes are essential for *M. tuberculosis* survival under different growth conditions *in vitro* or in animal models (Li *et al.*, 1998; Camacho *et al.*, 1999; Rodriguez *et al.*, 1999; Ravn *et al.*, 1999). The expression of most of the genes investigated under those conditions has not been correlated with expression in humans during infection, which may differ (Florczyk *et al.*, 2001; Mariani *et al.*, 2000). Glutamine synthetase has also been implicated in the virulence of *M. tuberculosis* (Tullius *et al.*, 2003) due to its potential contribution to cell wall homeostasis through poly-L-glutamic acid synthesis and/ or involvement in altering the pH and ammonia concentration of host cell phagocytes (Harth *et al.*, 1994). It has been shown that the activity of the *glnA1* may vary between evolutionary different *M. tuberculosis* strains grown *in vitro* under similar conditions (Goa *et al.*, 2005), although it is assumed that *glnA1* may be active in the early stages of *M. tuberculosis* growth due to observed GS activity in such cultures (Harth *et al.*, 1994). Localisation of *glnA1* expression in *M. tuberculosis* induced host granulomas may suggest the importance of GS *in vivo* during active growth and survival of *M. tuberculosis*, since the conditions in the granuloma during disease may differ substantially from controlled *in vitro* conditions. Therefore comparing the distribution of Rv2223c expression in the same tissues with that of *glnA1* might give an indication of the importance of Rv2223c during *M. tuberculosis* host infection. Previous studies have shown that the activity of some genes may be influenced by host factors by virtue of showing transcriptional activity only in host macrophages and granulomas, but not *in vitro* (Ramakrishnan *et al.*, 2000). Therefore the distribution of *glnA1* and Rv2223c expression during active *M. tuberculosis* disease was investigated through *in situ* hybridisation of a *glnA1* specific mRNA probe to human pulmonary granuloma tissue, as well as other forms of lung diseases where *M. tuberculosis* may be involved, such as sarcoidosis.

In situ hybridisation showed a high level of *glnA1* and Rv2223c expression in cells associated with immature non-necrotic granulomas, suggesting that during the active stages of infection GS and the enzyme encoded by Rv2223c may be required for survival during the initial phase of the host immune response. As the granuloma matures, a necrotic centre develops which may either calcify or become caseous (liquefied). The central area of caseous necrosis is a-cellular, although it is thought (by virtue of the presence of intact *M. tuberculosis* genomic DNA) that *M. tuberculosis* might

survive in these zones in a dormant or metabolically inactive state of persistence (Fenhalls *et al.*, 2002a). In the mature granuloma, positive ISH binding signal for *glnA1* and Rv2223c was observed in the lymphocyte cuff and the transition zone up to the periphery of caseous necrosis. No hybridisation signal was observed in the caseous necrosis regions of the granuloma. It is unknown if *M. tuberculosis* is viable or just metabolically inactive in caseous regions, but a previous report has shown that nutrient and oxygen limiting conditions, such as that found in the central necrotic region of a granuloma, had a suppressing effect on gene expression (Hu *et al.*, 1998). Therefore the observed lack of *glnA1* or Rv2223c expression in the necrotic granuloma could suggest that transcriptional activity of these genes are more repressed in restricted areas of the granuloma, although it would not imply that functional GS or Rv2223c protein is also absent. These results are in agreement with previous studies that demonstrated an increased survival of recombinant *M. smegmatis* expressing high levels of *M. tuberculosis* GS from an expression plasmid in THP-1 macrophages (Miller and Shinnick, 2000). The reason for the increased *M. smegmatis* survival has not been determined, though the study by Miller and Shinnick disputed the role of GS in delaying phagosome acidification and subsequent lysosome fusion. In a later study, it was demonstrated that a *glnA1* *M. tuberculosis* mutant generated through allelic exchange was incapable to grow in THP-1 macrophages and infection of *M. tuberculosis*-susceptible guinea pigs (Tullius *et al.*, 2003). In that study it was suggested that active GS was essential for intracellular growth and virulence of *M. tuberculosis* due to its central role in glutamine production and potential involvement in the detoxification of the ammonium-rich environment of *M. tuberculosis* in host cells. These results also give a further indication that the protein encoded by the Rv2223c gene may be important for the survival of *M. tuberculosis*. Intriguingly, we previously showed (see Chapter 4) that the expression of Rv2223c might be up regulated during the abundance of nitrogen (possibly in the form of ammonia). It has long been hypothesised that the production of ammonia by intracellular *M. tuberculosis* might play a role in the inhibition of phagosome maturation, possibly through influencing phagosome pH (Clemens *et al.*, 1995). Therefore the results of the current study may also reflect that high concentrations of ammonia may be present in exogenous environment of intracellular *M. tuberculosis*, which may cause an up regulation of Rv2223c expression.

It was also observed that the amount of positive riboprobe-binding signal for both genes are very abundant in the human lung tissues used. It is unknown if all the cells surrounding the granulomas are infected by *M. tuberculosis*, since ZN staining of infected lung tissue generally shows a few scattered bacilli in these tissues. This abundant presence of *M. tuberculosis* gene expression has been detected before in granulomas using ISH (Fenhalls *et al.*, 2002a), and it may be argued that the ISH technique is oversensitive due to non-specific binding of the riboprobes. However, the apparent absence of *M. tuberculosis* in these regions may also be due to the ability of mycobacteria, such as *M. tuberculosis* (Chandrasekhar and Ratnam, 1992; Wang and Chen, 2001) and *M. paratuberculosis* (Wall *et al.*, 1993), to enter an altered growth-phase dependent state during which they lose their acid-fastness (Gillespie *et al.*, 1986). The report of Wall *et al.* suggests that cell wall deficient (and therefore non-acid-fast) mycobacteria may be in the non-metabolically active stationary growth phase. It is possible that mRNA of certain genes may still be present in detectable amounts in stationary growth phase or dormant mycobacteria (Smeulders *et al.*, 1999; Hu and Coates, 2001). Therefore ZN staining may not readily detect an abundance of *M. tuberculosis* bacilli in infected host tissues and their associated cells (Ulrichs *et al.*, 2005) that may be detected through *in situ* hybridisation techniques (Hulten *et al.*, 2000a; Hulten *et al.*, 2000b) and DNA-DNA hybridisation techniques (Fenhalls *et al.*, 2002b).

5.3.4 *Mycobacterium tuberculosis* GS and Rv2223c may not physically interact

The bacteria-2-hybrid system was designed that two complementary components (T25 and T18; Ladant *et al.*, 1989) of the *Bordetella pertussis* adenylate cyclase are expressed as target - and bait fusion proteins from two independent expression plasmids in an *E. coli* adenylate cyclase-deficient strain (Karimova *et al.*, 1998). Expression of T25 and T18 alone cannot reconstitute adenylate cyclase, but co-expression as fusion proteins to interacting proteins results in functional dimerisation and therefore cAMP synthesis. Cyclic AMP regulates the transcription of the *lac* and *mal* operons, and thereby enabling *E. coli* to ferment lactose and maltose, which is therefore easily detectable on indicator media (Ullmann *et al.*, 1979; Danchin *et al.*, 1981). To investigate whether GS and the Rv2223c protein may interact through direct protein-protein interaction, coding sequences were cloned into the T25 and T18 fusion vectors and transformed into a adenylate cyclase-deficient *E. coli* strain. Because the protein product

of Rv2223c potentially possesses a N-terminal leader peptide that might be cleaved by the SecA encoded bacterial translocase protein export system (Economou and Wickner, 1994), this protein was cloned as a C-terminal T18-fusion protein, while *glnA1*, which is considered leader-peptideless (Harth and Horwitz, 1997) was cloned as a N-terminal T25-fusion protein. However, functional complementation of β -galactosidase activity could not be observed in *E. coli* transformants harbouring both fusion vectors, implying that GS and Rv2223c do not physically interact.

A limitation of the bacterial-2-hybrid system is that other factors confounding protein-protein interaction, such as steric hindrance or correct peptide folding could not be controlled for. The T25 (224 amino acids) and T18 (114 amino acids) fusion tags were in the correct proximal arrangement to associate. The reciprocal influence the GS (474 amino acids) and Rv2223c (518 amino acids) fusions have on the 3D-structure of the T25 and T18 peptides are unknown. Changes in 3D structure of either protein might influence the binding affinity between the two interacting proteins. Also, it is unknown if position of the N- and C- termini of the target and bait-proteins would facilitate the association of T25 and T18.

CHAPTER SIX

Conclusion

“Ours is essentially a tragic age, so we refuse to take it tragically”

David Herbert Richards (DH) Lawrence, *Lady Chatterley's Lover*
(Born September 11, 1885, died of tuberculosis February 2, 1930)

6.1 FINAL CONCLUSIONS AND FUTURE PROSPECTS

In this study it was shown that *M. tuberculosis* GS may be an attractive target for the development of new antibiotics, since it is widely expressed during *M. tuberculosis* host infection, and that its encoding sequence, *glnA1*, is an essential gene and arguably does not tolerate evolutionary change. This implies that the GS encoded by *glnA1* should be homogenous in all *M. tuberculosis* strains and that resistance to antibiotics due to changes in the enzyme may not readily occur. Other genes, such as the putative exported proteases encoded by Rv2223c and Rv2224c, may be involved in GS export regulation. Although the function of the proteins encoded by these genes was not delineated, this study showed that inhibition of the synthesis of the gene products may be lethal to *M. tuberculosis*. Therefore these genes, which are not widely distributed in the Bacterial Kingdom, may also be exploitable for antibiotic intervention and in fact be better targets than GS, which also occurs in the host.

Future prospects include an extended study of Rv2223c and Rv2224c, and other factors involved in the regulation of nitrogen metabolism. There are several signalling and effector molecules involved in the regulation of GS and GOGAT in the actinomycetes that differs from other systems, such as *E. coli* (Burkovski, 2003a). These systems are poorly described in *M. tuberculosis* and might reveal novel mechanisms and pathways of regulation, since not all of the genes encoding the factors involved in the control of GS and GOGAT are present in *M. tuberculosis* (reviewed in Burkovski, 2003b). Such a study may further the knowledge of how *M. tuberculosis* adapts to its host environment, since nitrogen (in the form of ammonia, for instance) may play an important role in host-immune response evasion (Clemens and Horwitz, 1995). The deeper understanding of the expression and control of the *M. tuberculosis* GS may lead to new interventions which again would contribute to the control and alleviation of the burden of the tuberculosis disease.

MATERIALS AND METHODS

“Don’t go around saying the world owes you a living; the world owes you nothing – it was here first”

Mark Twain (1835 -1910)

1. BACTERIAL STRAINS AND CULTURING METHODS

1.1 Bacterial strains and culture media

Escherichia coli JM109 (ATCC 53323) was used as a host for all cloning experiments. *Mycobacterium tuberculosis* H37Rv (reference strain, ATCC 25618), *M. bovis* BCG sub-strains Pasteur (ATCC 35734) and Montreal (ATCC 35746, a gift from Dr. Marcel Behr, Montreal General Hospital, Montreal, Canada) and *M. smegmatis* mc²155 (ATCC 700084; Snapper *et al.*, 1990) were routinely used as study material and heterologous mycobacterial cloning and expression hosts.

E. coli was grown on solid or liquid Luria-Bertani (LB) agar (Appendix A.2.4) or liquid (Appendix A.2.3) medium as described by Sambrook *et al.* (1989). Mycobacterial strains were grown at 37°C for 2 days with shaking (200 rpm, *M. smegmatis*) or 14 days with stirring (*M. tuberculosis* and *M. bovis* BCG) in Middlebrook 7H9 Mycobacterial medium (Appendix A.2.5) supplemented with ADC (Appendix A.2.1) and containing 0.05% (v/v) Tween 80 (Sigma). For transformant selection and β -galactosidase activity detection on solid media, *M. smegmatis* cells were grown on Middlebrook 7H11 Mycobacterial agar (Appendix A.2.6) supplemented with OADC (Appendix A.2.7) and 0.001% (w/v) 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, Appendix A.3.26).

1.2 *Escherichia coli* strains for protein expression

For the expression of recombinant *M. tuberculosis* proteins in *E. coli*, the *E. coli* TOP10 strain was used which is capable of transporting L-arabinose, but not metabolizing it (Guzman *et al.*, 1995). This was important in the protein expression studies as the level of L-arabinose will be constant inside the cell and not decrease over time. The *E. coli* TOP10 strain is *araBADC*-. It has both *araBA* and *araC* deleted, and the gene for *araD* has a point mutation in it, making it inactive (Invitrogen).

1.3 Culturing methods

All bacterial strains were cultured at 37°C. Starter cultures were prepared by spreading a bacterial suspension on LB agar media (Appendix A.2.4) containing a final concentration of 5 µg/ml of the appropriate antibiotics (Appendix A.1) when required (see Table 1).

Table 1. Cloning and expression plasmids used in this study. All plasmids used in mycobacterial expression applications were *E. coli* shuttle plasmids. The selection markers are indicated. See Appendix C for plasmid maps.

Plasmid (see App. C)	Bacterial Host	Selection marker	Reference
pGemt-Easy	<i>E. coli</i>	Ampicillin	Promega
pJEM15	<i>E. coli</i> / Mycobacterial	Kanamycin	Timm <i>et al.</i> , 1994
p19Kpro	<i>E. coli</i> / Mycobacterial	Hygromycin B	De Smet <i>et al.</i> , 1999
PBAD/gIII	<i>E. coli</i>	Ampicillin	Invitrogen
pKT25	<i>E. coli</i>	Kanamycin	Karimova <i>et al.</i> , 2000
pUT18	<i>E. coli</i>	Ampicillin	Karimova <i>et al.</i> , 2000
pACE	<i>E. coli</i> / Mycobacterial	Hygromycin B	De Smet <i>et al.</i> , 1999

Liquid cultures were prepared by inoculating a single bacterial colony into the appropriate liquid media (Table 2). Liquid cultures of mycobacteria were incubated with constant stirring with a magnetic stirrer-bar, while *E. coli* was cultured with constant shaking in an incubator on a orbital shaking platform to allow even aeration and minimise coagulation. Kanamycin (50 µg/ml, Roche), Ampicillin (50 µg/ml; Roche, Appendix A.1.1) and Hygromycin B (50 µg/ml for *E. coli*; 100 µg/ml for mycobacteria, Roche) were added to bacterial liquid cultures when antibiotic selection was required. All work on *M. tuberculosis* H37Rv was performed in a Biosafety Level III facility. Culture growth was monitored by spectrophotometric measurement of culture turbidity at a wavelength of 600 nm (OD₆₀₀ value).

Table 2. Bacterial strains and culture media used in this study

Organism	Culture Media	
	Liquid	Solid
Mycobacteria: <i>M. tuberculosis</i> <i>M. bovis</i> BCG <i>M. smegmatis</i>	1.) 7H9 Broth + 10% (v/v) ADC supplement 2.) Kirchners minimal media	7H11 Agar + 10% (v/v) OADC supplement
<i>Escherichia coli</i> : DH5 α TOP10 (Δ araBA and Δ araC; Invitrogen)	Luria Berthani (LB media)	LB Agar

1.3.1 Blue/ white colony selection

In order to screen for β -galactosidase activity in transformed bacterial cells, transformed *M. smegmatis* or *E. coli* cells were selection-plated onto agar-indicator plates containing 5 μ g/ml (10 μ g/ml Hygromycin B for mycobacterial cultures) of the appropriate antibiotic, 0.001% (w/v) X-Gal (Appendix A.3.26) and 0.2 mM isopropyl β -D-thiogalactopyranoside (IPTG, Appendix A.3.15). Colonies that had β -galactosidase activity were blue when cultured on X-gal-containing indicator plates.

1.3.2 Mycobacterial culture using BACTEC 460TB system

A 5 ml *M. tuberculosis* H37Rv culture was prepared in Middlebrook 7H9 medium (Appendix A.2.5) enriched with ADC (Appendix A.2.1). At a culture density of approximately $A_{600} = 0.6$, 100 μ l of the *M. tuberculosis* starter culture was inoculated into a BACTEC 12B medium vial (Libonati *et al.*, 1988). Cultures in BACTEC were grown until an appropriate growth index (GI) of 500 was reached. This culture was used as starter culture for BACTEC evaluation of antisense oligonucleotides on *M. tuberculosis*. Sterility of mycobacterial cultures was monitored on 7H9 broth/bacteriological agar plates and by Ziehl-Neelsen acid fast staining (Section 1.4). The starter cultures above represented *M. tuberculosis* in exponential phase of growth. Five hundred micro litres of starter culture was added to a sterile 1.5 ml eppendorf

tube containing a sterile micro stirrer bar. The culture was brought to a final concentration of 10mM Phosphorothioate-linked 18-mer antisense oligonucleotide and the culture stirred for 72 hours, which represents 3 doubling times for *M. tuberculosis*. After 72 hours, 100ul of cultures were added to a fresh BACTEC vial and the growth monitored every 24 hours (one doubling time for *M. tuberculosis*). Phosphorothioate oligonucleotides (Table 4) with no BLAST sequence homology (Section 2.3) to the *M. tuberculosis* genome sequence (Tuberculist, Table 3) were used as controls to evaluate the effect of such oligonucleotides on mycobacterial growth.

1.4 Ziehl-Neelsen (ZN) staining of mycobacterial cultures

This method could be used to selectively stain mycobacteria in tissue samples as well as from liquid cultures. The method depends on the presence of mycolic acids. Briefly, cells were immobilised on a microscope slide with fixing solution (supplied by SAMAR) and incubated at 100°C on a hot plate for 30 min. Slides were stained with carbol-fuchsin (Diagnostic Media Products) for 10 min with intermittent flaming (boiling must be prevented). Thereafter samples were destained through rinsing with 5% acid-alcohol (Appendix A.3.1) and counterstained with methylene blue (Diagnostic Media Products). The slides were rinsed with H₂O and allowed to dry. Thereafter samples were viewed under a light microscope at 100x magnification and oil immersion. Figure 1 shows typical results of ZN-staining, where acid-fast bacteria stains pink to red, and non-acid-fast bacteria stains blue to black)

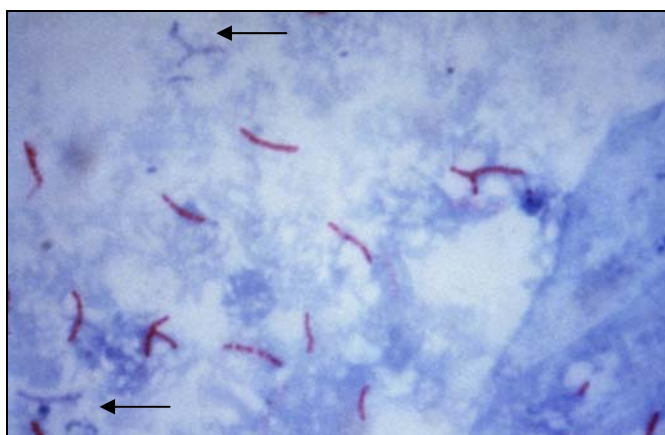


Figure 1. Ziehl-Neelsen stained *M. smegmatis* culture showing the mycobacterial bacilli (red) and what may be non-mycobacterial contaminant bacilli (blue bacilli indicated by arrows).

2. BIO-INFORMATICS

Bioinformatics and computational biology involve the use of computer-based techniques for the analysis of biological data to solve biological problems at the molecular level. Bioinformatics were used here for DNA and protein sequence alignment, gene finding, protein structure alignment, protein structure prediction, prediction of gene expression, protein-protein interactions, and the modelling of evolution.

2.1 Sequence retrieval

Annotations and descriptions of individual genes of *Mycobacterium tuberculosis* and protein sequences were obtained from the Pasteur *M. tuberculosis* database (Table 3). The DNA or protein sequences were used in various applications (Figure 2).

2.2 Oligonucleotide design

Oligonucleotides for PCR (Section 3) were designed to be homologous to unique sequences flanking the genomic area to be amplified, and were carefully designed to make the PCR reaction as specific as possible. All oligonucleotides for PCR used in this study were designed using DNA sequence data obtained from the Pasteur Tuberculist website (Table 3). Once the sequence to be amplified was identified, PCR oligonucleotides were designed using DNAMAN software (Lynnon Biosoft). Primers were between 18 and 24 bases, with a GC content of 50% – 60% (see Table 4). The melting temperature (T_m) representing the temperature, at which the oligonucleotide/ DNA template duplex dissociates, was restricted to a difference of within 2⁰C of each oligonucleotide pair. Melting point temperature was calculated as described in Equation B.2.

When oligonucleotides were designed with internal restriction enzyme (RE) recognition sites, these oligonucleotides were checked for self-annealing sequences using Primer Premier software (Premier Biosoft International). This software predicts binding energy and non-homologous bases in the PCR oligonucleotide that may cause mispriming and/or non-specific binding. The sequence of each oligonucleotide was checked for binding specificity by sequence BLAST (Section 2.3) on the Tuberculist website. All oligonucleotides were synthesised by Integrated DNA technologies (IDT, USA). All oligonucleotides longer than 30

bp were quality checked by MALDI-TOF analysis (IDT, USA) to ensure truncated synthesis products were not present.

2.2.1 Phosphorothioate antisense oligonucleotides (PS-ODNs)

Eighteen-mer PS-ODNs (University of Cape Town) targeting the mRNA sequence of Rv2223c and Rv2224c (Table 4) were designed using the same *M. tuberculosis* genome sequence data (Tuberculist) and software (DNAMAN) as in Section 2.2. As a negative control, a PS-ODN was designed that does not have a sequence homolog in the *M. tuberculosis* genome.

2.3 Sequence comparisons

Basic Local Alignment Search Tool (BLAST) searches identified homologous DNA and protein sequences through the comparison of a known sequence (obtained from the Pasteur Tuberculist web-site, Table 3) to either *M. tuberculosis* genomic data, or the genome sequences of other bacteria available on the National Centre for Biotechnology Information (NCBI, Table 3) or the Institute for Genomic Research (TIGR, Table 3). The BLAST-program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

Table 3. Web-site address and authors of the various software applications used in bioinformatic approaches. The superscript reference numbers refers to Figure 2.

Application	Software or Web-page	Software author or Web-page addresses
Sequence retrieval ⁽¹⁾	Tuberculist <i>M. tuberculosis</i> H37Rv genome database	http://genolist.pasteur.fr/TubercuList/
Oligonucleotide design ⁽²⁾	DNAMAN ver. 4.1	Lynnon BioSoft
	Primer Premier ver. 5.0	Premier BioSoft International
Sequence comparison	Clustal W multiple sequence alignment tool ⁽³⁾	http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi
	BioEdit sequence alignment editor ver. 5.0.9	North Carolina State University
	PAUP ver 4.0 beta 10 phylogenetics software ⁽⁴⁾	Sinauer Associates, Inc Publishers
	TreeView ver. 1.6.6 phylogenetic tree editor	http://www.ebi.ac.uk/clustalw/
	Bacterial genomic BLAST ⁽⁵⁾	http://taxonomy.zoology.gla.ac.uk/rod/rod.html
	<i>M. smegmatis</i> genomic DNA data	http://www.tigr.org
Protein structure and function analysis	GelCompar ver 4.0 ⁽⁶⁾	Applied Maths, Kortrijk, Belgium
	Hydrophobicity profiling ⁽⁹⁾	http://www.cbs.dtu.dk/services/TMHMM-2.0/
	ExPasy Protein motive searches ⁽⁸⁾	http://au.expasy.org/
	CBS Signal peptide prediction server ⁽¹⁰⁾	http://www.cbs.dtu.dk/services/SignalP/
	CBS TMHMM transmembrane helices prediction ver. 2.0 ⁽¹¹⁾	http://www.vivo.colostate.edu/molkit/hydropathy/index.html

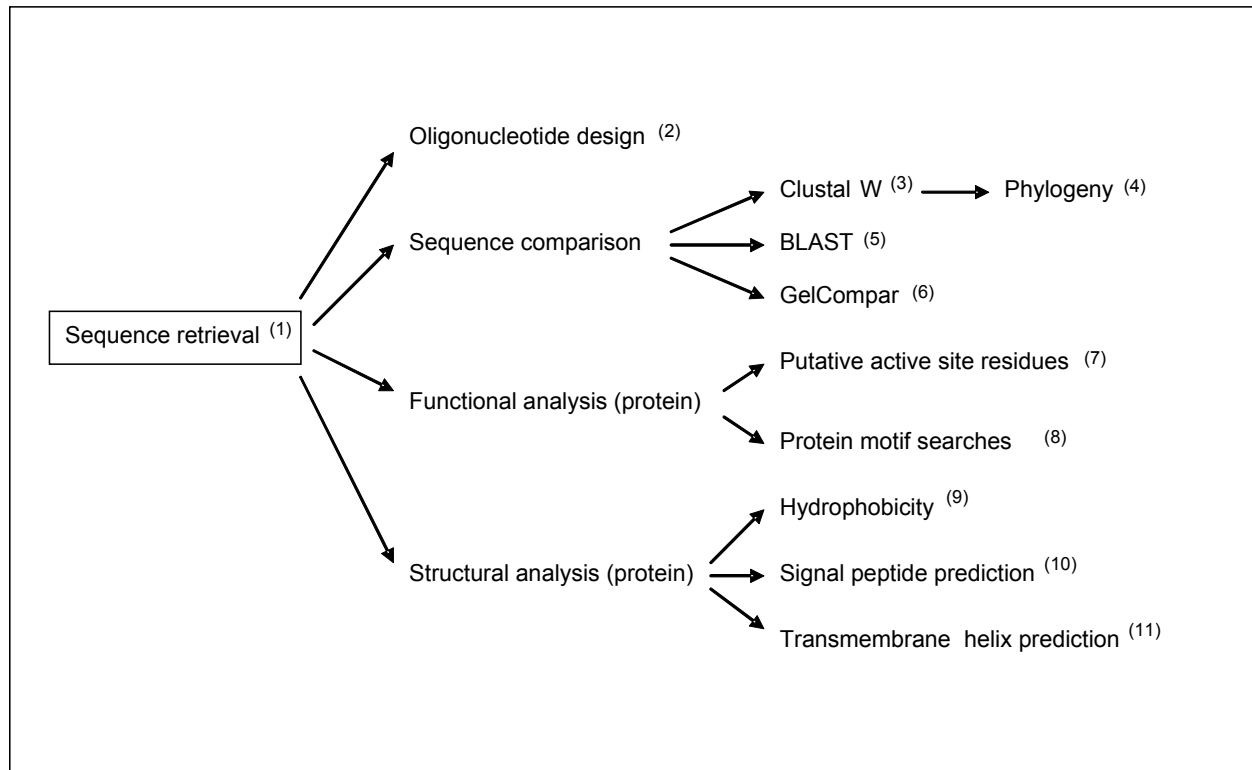


Figure 2. Retrieved DNA and protein data were analysed and manipulated with various bioinformatic software applications. These applications were used in PCR oligonucleotide design, as well as predicting the possible structure and function of a non-described polypeptide (such as Rv2223c and Rv2224c) from amino acid sequences. The superscript numbering refers to Table 3, where the software or online web-site addresses of the various applications are described.

Table 4: List of PCR oligonucleotides, including the length of the amplification product (fragment length), the theoretical annealing temperature (T_m , see Equation B.2), the chromosomal priming coordinates, internal restriction enzyme sites (RE sites, underlined in the oligonucleotide sequence), the application for which the amplification product was used as well as a Chapter reference.

Name	Sequence (5' - 3')	Frag size	Tm	Genome Coordinate	RE site	Section
AI FI	CACGGTCAGTAACGTCTGC	550 bp	55	2487524	none	2.2.3.2
AI RI	TCCACCTCGTAGAAGGAGC		55	2488081	none	2.2.3.2
AI FII	TTCGATTCGGTGAGCTTC	574 bp	57	2488029	none	2.2.3.2
AI RII	GCCGCTGTAGGAGTTCA		57	2488602	none	2.2.3.2
AI FIII	ACGACGAGACGGGTTATG	294 bp	54	2488483	none	2.2.3.2; 5.2.4
AI RIII	ATCAGCATGGCCGAGAAC		54	2488768	none	2.2.3.2; 5.2.4
AI FIV	TGGTCTATAGCCAGCGCA	597 bp	56	2488633	none	2.2.3.2
AI RIV	GAGATGATTGCCAAGCGG		56	2489229	none	2.2.3.2
glnA Up F	AGATGGACACGGTGGAGT	796 bp	55	2486860	none	2.2.3.2; 3.2.5
glnA Up R	CTTTACTGTATCCGCGGC		55	2487605	none	2.2.3.2; 3.2.5
Long F	CCCGATATCCAGGTTCCCGTTCGGC	1622 bp	82	2487425	EcoRV	3.2.1; 3.2.5
Short F	CGTCTGCGCAACACGGGATCCCTG		80	2487552	BamH1	3.2.1
glnA16his R	<u>AAGCTT</u> CAGTGGTGGTGGTGGTGGGACGTCGTAGTACAGCGCGA	1495 bp		2489047	HindIII	3.2.1; 3.2.5
GS pKT25 F	CATT <u>CTGCAG</u> CGGAAAAGACGCCCGAC	1425 bp	56	2487619	Pst I	3.2.2; 5.2.3
GS pKT25 R	GAGTCCT <u>GGTACC</u> TCGTAGTACAGCGCGAA		56	2489044	Kpn I	3.2.2; 5.2.3
SecA2 pUT18F	ATTGCG <u>GCATGC</u> GACGATGGCGGCCATGTG	570 bp	98	2067667	Sph I	3.2.6
SecA2 pUT18R	CGCGCA <u>CTCTAG</u> ACTCGTCGGCGGCAA		90	2068237	Xba I	3.2.6
Rv2224c utF	GGCCTTCCATCTCTGTAGG	901 bp	60	2497834	none	3.2.5
Rv2224c utR	AGCCCAAGAACCAACG		56	2496933	none	3.2.5
ir II RNAF	CGTGACGGCGTATTTAATC	290 bp	56	2495515	none	4.2.2.1
ir II RNAR	GGCAGCTGTTCCAACCTCTG		60	2495225	none	4.2.2.1
ir III RNAF	ATGGCAACCAAGTGTGTGG	259bp	56	2493922	none	4.2.2.1
ir III RNAR	ACCGAGACGTCTGTGAA		52	2493663	none	4.2.2.1
ir IV RNAF	CGAGAATTGCCGTCCAGT	295 bp	56	2492578	none	4.2.2.1
ir IV RNAR	GGGTCAACTAATCCGAGCC		60	2492283	none	4.2.2.1

(Table 4 continued from previous page)

Name	Sequence (5' - 3')	Frag size	Tm	Genome Coordinate	RE site	Section
ToUp irF	GGCCTTCATCTCTGTAGG	247 bp	58	2497834	none	4.2.2.2
ToUp irR	CAGCCCACAGGTACCAACGCCACGGC		58	2496932	Kpn I	4.2.2.2
ToI r IF	CGGTGGGATCCAGGGCGACAGCTGCA	288 bp	58	2495552	Bam H1	4.2.2.2
ToI r IR	CGAGCAGGTACCCGAAGGACAGCAG		58	2495336	Kpn I	4.2.2.2
ToI r IIF	ACGGGGCCCAACACACTGCGGTGT	203 bp	58	2493943	Apa 1	4.2.2.2
ToI r IIR	AACCGGATCCGGACGAAGCGGATGT		58	2493680	Bam H1	4.2.2.2
ToI r IIIF	CAAGGGCCCGGAGTGGGCGAACTA	157 bp	58	2492466	Apa 1	4.2.2.2
ToI r IIIR	GCTGGGTACCGAGTTTGGTCACGAC		58	2492335	Kpn I	4.2.2.2
GS Ex F	GCATTCCATGGCGGAAAAGACGCCCGAC	1425 bp	56	2487619	Nco I	5.2.1
GS Ex R	AGTCCTTCTAGATCGTAGTACAGCGCGA		56	2489044	Xba I	5.2.1
Ex2224 f	GCAGCACCATGGGCATGAGGCTGT CTC	1575 bp	56	2497022	Nco I	5.2.1
Ex2224 r	GTTTCGTCGACCCGGTAGCACTTGGCGCCG		56	2495447	Sal I	5.2.1
Rv2223 PS-ODN	CGTAGCTGTATCCGAGGT	--	--	2494700	none	5.2.2.1
Rv2224 PS-ODN	GCCGTACGAGTAGCCCAG	--	--	2496334	none	5.2.2.1
PS-ODN negative	GGGTGCCGTACGAGTAGCCCAGGT	--	--	--	none	5.2.2.1
pACE glnA1 F	CCACGGATCCGACATGTTGC	571 bp	64	2487825	Bam H1	5.2.2.2
pACE glnA1 R	GCATCGATTTCAACTGGTAGT		58	2488300	Cla 1	5.2.2.2
pACE Rv2224c F	ACAGGATCCGTTTCGATGGTCATAAA	460 bp	51	2496709	Bam H1	5.2.2.2
pACE Rv2224c R	CACCATCGATAATCATTGCC		58	2496286	Cla 1	5.2.2.2
pACE Rv2223c F	GTGGGATCCATGCGAAGTTGGC	492 bp	66	2495122	Bam H1	5.2.2.2
pACE Rv2223c R	ACATCGATACCTAACCGTTCCAG		62	2494676	Cla 1	5.2.2.2
RV pUT18 F	ATTTCGCGCATGCGACGATGGCGGCCATGTG	1550 bp	56	2495403	Sph I	5.2.3
RV pUT18 R	CGCGCACICTAGACTCGTCGGCGGCCAA		56	2493853	Xba I	5.2.3
Rv ISH F	CACGTCGAACAGGTCTACCG	120 bp	64	2494859	none	5.2.4
Rv ISH R	CTAACGCTTGGCGAACCA		56	2494739	none	5.2.4
SP6	ATTTAGGTGACACTATAG	--	48	--	--	Materials and Methods 12.5
T7	TAATACGACTCACTATAGGGAGA	--	64	--	--	Materials and Methods 12.5

2.3.1 Phylogenetic trees

In order to evaluate the evolutionary relationship between sequences, similar DNA or protein sequences from various bacteria were identified through BLAST searches. These sequences were aligned by Clustal W (Table 3), which produces biologically meaningful multiple sequence alignments of divergent sequences by calculating the best match for the selected sequences, and lined them up so that the identities, similarities and differences can be seen. Non-homologous DNA or amino acid stretches were removed from the Clustal W alignments using the BioEdit sequence alignment editor (Table 3). The edited Clustal W alignments were saved as a text (.txt) file and analysed by bootstrapping resampling using Phylogenetic Analysis Using Parsimony (PAUP) software. The PAUP software required a user-defined run-code to specify operation parameters (Figure 3), such as resampling number of the sequence data sets. An outgroup-sequence was specified, which was selected based on the relationship of the organism from which the sequence originated to *M. tuberculosis*. Using these parameters, the PAUP software constructed a preliminary tree (alignment.tre), which was resampled 1000 times by bootstrapped analysis (nreps = 1000). The consensus trees were drawn using the Treeview (Table 3) software.

```
;
endblock;

begin paup;

log file = C:\PAUP\alignment.txt;

set criterion = distance;

outgroup USER DEFINED [/ONLY] ;

bootstrap file=C:\PAUP\alignment.tre nreps=1000 bseed=15 search=Nj;

gettrees file=C:\PAUP\alignment.tre;

contree all/strict=no majrule=yes LE50=yes file=C:\PAUP\alignment.tre;

gettrees file=C:\PAUP\alignment.tre;

describe / chglist=yes BrLens=yes;

savetrees file=C:\PAUP\Trees\ alignment.tre brLens=yes;

log stop;

end;
```

Figure 3. The user-defined run-code required for the PAUP software defines the Clustal W data input (alignment.txt), an data out-group as well as the number of resampling (nrep) to be done. The software saves the generated data as a phylogenetic tree (alignment.tre), which can be viewed and manipulated using Treeview software.

2.4 Functional and structural analysis

Protein sequence data was used for the identification of putative active site amino acids and protein motif searches. Structural data such as hydrophobicity profiles, the presence of export signal peptides and transmembrane helices could be obtained using computational algorithms embedded in specialised software (see Table 3). In all these applications, amino acid sequences were entered in either raw or FASTA format for the software to be able to function.

3. POLYMERASE CHAIN REACTION (PCR)

The PCR technique provides a rapid means of exponentially amplifying a specific target region of DNA flanked by unique regions to which PCR oligonucleotides are designed. These oligonucleotides prime the DNA-polymerase mediated 5' – 3' synthesis of two complementary strands of the desired DNA segment, effectively doubling its concentration with every round of amplification. Products of PCR reactions were analysed by gel-electrophoresis (Section 4.2).

3.1 Reaction conditions

The different *Taq*-DNA polymerases used in this study necessitated the preparation of different reaction mixtures for optimal enzyme function. Due to the nature and length of some of the DNA PCR target sequences, stabilisers such as DMSO were added to a final concentration of 2% - 4% to some reactions. All PCR amplification reactions were performed in a GeneAmp PCR system 2400 (Perkin Elmer). To control for contamination, all PCR procedures included a reaction where template DNA was omitted.

3.1.1 Long Range PCR

The Expand Long Range PCR (Roche) was used when amplification products exceeded 1000 bp, or where the target sequences were non-amplifiable by the conventional PCR method due to fragment length (Section 3.1.2). Reaction mixtures contained 100 ng of *M. tuberculosis* H37Rv DNA template, 25 pmols of each oligonucleotide primer (Table 4), 1x Reaction Buffer 3 (Roche), and 5 U Expand Long Range *Taq* DNA polymerase (Roche). The

Taq DNA polymerase has proofreading ability and therefore does not create 3'- adenine overhangs on amplification products. Deoxyribonucleic acid products synthesised using this *Taq* – DNA polymerase cannot be used in TA-cloning (Section 5) applications without preparation prior to cloning. Cycling conditions included an initial 2 min *Taq* activation and DNA denaturation step at 94°C, followed by 30 cycles at 94°C for 30 sec, 30 sec at the required oligonucleotide pair T_m (Table 4) and elongation at 68°C for a time of 1 min per Kb to be amplified. Adding DMSO to a final concentration of not more than 2% increased primer specificity should too many spurious products form.

Long oligonucleotides that were engineered to include a hexa-histidine encoding sequence, stop codon and RE site (Table 4) had regions of lower homology to the target DNA sequence. Therefore, after the initial denaturation step, 10 cycles of PCR were done using 50°C as annealing temperature. For the remaining 20 cycles, an annealing temperature of 60°C was used.

3.1.2 Routine PCR

For the amplification of PCR products up to 1000 bp, HotStart *Taq*-polymerase (Qiagen) was used. The *Taq*-polymerase included in this system is a non-proofreading DNA polymerase that synthesises DNA fragments with 3'- adenine overhangs which could be directly used in TA-cloning applications (Section 5). Each reaction contained 25 pmol of each oligonucleotide (Table 4), 100 ng DNA template, 10 µM of each dNTP, 1x reaction buffer (supplied), 20 mM MgCl₂, 1x Q-solution (supplied additive that enables efficient amplification of GC-rich templates), 1U *Taq*-polymerase and sterile dH₂O to a final volume of 50 µl.

3.2 Reverse transcription (RT) PCR

The PCR amplification of mRNA was divided into two steps, consisting of cDNA first-strand synthesis (reverse transcription) and subsequent PCR amplification of the RT-synthesised cDNA. Each 50 µl RT reaction contained 200 ng purified mRNA (Section 7.3); 50 pmol of reverse PCR oligonucleotide; 200 mM of each dNTP (Qiagen); 2.5 mM 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane (DTT, Merck); 10 U RNase inhibitor (Invitrogen) and 1 U of Titan reverse transcriptase enzyme mix (Roche) in a 1 x final concentration reaction buffer

(Roche). Coding-DNA synthesis was carried out at 50°C for 1 hour. Controls for the RT reaction included a RT reaction wherein mRNA template was omitted.

The PCR amplification of the cDNA was done as described in Section 3.1.2. A control PCR reaction was included with 200 ng of purified mRNA to control for DNA contamination.

4. ELECTROPHORESIS

Proteins and DNA were routinely fractionated by gel electrophoresis. The gel acts as a molecular sieve that separates DNA fragments according to size in an electrical field.

4.1 SDS-PAGE electrophoresis

Protein samples were routinely fractionated under denaturing conditions in a 7.5% – 15% (depending on the molecular weight of the proteins) poly-acrylamide resolving gel (Table 5). The reagents specified in Table 5 are sufficient for the preparation of two 7 x 10 cm gels, 0.75 mm – 1 mm thick. The ammonium persulphate and TEMED were added to the acrylamide solution just prior to pouring the gel. The acrylamide solution was poured into a Protean II gel-cast assembly (BioRad) and overlaid with a thin layer of hydrated iso-propanol to prevent the top of the gel from dehydrating. The acrylamide gel was allowed to polymerise for 30 min at RT.

After the resolving gel has polymerised, the iso-propanol was decanted and the acrylamide gel surface rinsed with ddH₂O to remove all iso-propanol. The ddH₂O was decanted and the top of the gel carefully blotted with Whatman 3M blotting paper to remove excess dH₂O immediately before the 3% stacking gel solution (Table 5) was added. The gel comb was positioned into the stacking gel, and the gel was allowed to polymerise for approximately 30 minutes. At least 1 cm of stacking gel was present between the bottom of the loading wells and the resolving gel. After the stacking gel has polymerised, the casting assembly was disassembled, the gel comb removed and the gel was placed in the Protean II electrophoresis apparatus as described by the manufacturer.

Table 5. Component reagents for the preparation of acrylamide gel grades (%) for two 0.75 mm mini-slab assemblies.

Component	3%	7.5 %	10%	12%	15%
Acrylamide/ bisacrylamide (40% stock)	650 μ l	2.5 ml	3.33 ml	4.0 ml	5.0 ml
1.5 M Tris-HCl (pH 8.8)	417 μ l	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS	50 μ l	0.1 ml	0.1 ml	0.1 ml	0.1 ml
H ₂ O	3.9 ml	4.85 ml	4.0 ml	3.35 ml	2.35 ml
TEMED	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l
10% (w/v) ammonium persulphate	50 μ l	50 μ l	50 μ l	50 μ l	50 μ l

Gel electrophoresis was carried out in SDS-PAGE running buffer (Appendix A.3.21). Prior to loading, protein samples were denatured for 10 minutes at 95^oC in 1x reducing buffer (Appendix A.3.20) and subsequently snap-cooled on ice to prevent protein refolding. Loading wells were carefully rinsed with glycine running buffer and approximately 6 μ g (see Section 9.2 for determination of protein concentration) of protein was loaded onto the wells. Seven μ l Rainbow molecular weight marker (Amersham, UK), or 7 μ l pre-stained protein marker (Inqaba Biotechnologies) was loaded in the outside lanes. The gel was electrophoresed at 80V (constant voltage) until the loading buffer dye migrated approximately 1 cm into the resolving gel. Thereafter the gel was electrophoresed at 200V (constant voltage) until the loading buffer dye had just migrated off the gel. Thereafter the protein samples were visualised through 20 min Coomassie staining (Section 4.1.1), or Western blotted onto a Nylon membrane for anti-body detection of specific proteins (Section 10).

4.1.1 Coomassie staining of SDS-PAGE gels

Following SDS-PAGE electrophoresis, the acrylamide gel was placed in a dish containing Coomassie stain (Appendix A.3.9) to completely cover the gel. The gel was stained at RT for a minimum of 30 min, but not longer than 3 hours, by gentle agitation on an orbital shaker. After staining, the Coomassie stain was decanted and the gel rinsed once with destain solution (Appendix A.3.11). This was replaced by another volume of destain solution and the

gel was allowed to destain at RT by agitation on a orbital shaker. The destain solution was changed regularly (approximately 3x) until the background was clear. Alternatively, gels were destained overnight by incubation at 4⁰C in a large volume (approximately 50 mL) of destain solution.

4.2 Agarose gel electrophoresis

Deoxyribonucleic acid samples were analysed by electrophoresis in TBE (Appendix A.3.23) agarose gels. Gels (5 cm x 10 cm) were prepared by the suspension of agarose powder at a concentration of 0.8% - 1.2% m/v (depending on DNA fragment length to be resolved) in 1x TBE buffer. The suspension was gently heated until the agarose was completely dissolved. Ethidium bromide was added to a final concentration of 10ug/ml (Appendix A.3.12) and the agarose was poured into the casting apparatus. Samples were prepared for electrophoresis by the addition of a 1x loading dye (Appendix A.3.17). The gel was run at 100 V (constant voltage) until the dye front reached approximately 2 cm from the end of the gel. The DNA was visualised on a transilluminator and photographed.

5. CLONING

All cloning steps were done essentially as described by Sambrook *et al.* (1989). Target DNA sequences generated by PCR were cloned either by T/A ligation into a suitable cloning vector (such as pGemt-Easy, Promega), or restriction enzyme digestion and subsequent sticky- or blunt-end ligation into a similarly RE-digested plasmid.

All T/A cloning was performed using the pGemt-Easy cloning vector system (Promega), which is supplied in a linear form containing T-overhangs. Reactions were prepared in a 10µl total volume according to the manufacturers instructions (Promega). One µl of T4 ligase was added to a reaction containing 3 µl purified DNA insert, 5 µl 2x ligation buffer (supplied) and 1 µl (50 pMoles) linearised pGemt-Easy vector. Ligation was done O/N at 4⁰C.

5.1 Plasmid and insert preparation by RE-digestion

In order to facilitate directional cloning or sub-cloning into bacterial host-specific expression vectors (Table 2), target DNA (insert and vector) had to be prepared through restriction

endonuclease (RE-) digestion. Various restriction endonucleases or combinations thereof were used depending on the fragment to be cloned. Briefly, RE reactions were prepared in a solution containing a 1x final concentration of the required RE-buffer, target DNA (insert or vector) and 10 U RE/ ug DNA. Restriction enzyme digestions were incubated at 37⁰C for a minimum of 1 hour, followed by insert and linearised plasmid DNA recovery through gel-electrophoresis or mini-prep purification (Section 7.2)

5.2 Partial RE-digestion

In some instances where a specific RE recognition site was not unique and it was necessary to digest DNA at only one specific restriction site, partial RE digestion was carried out. All following steps, unless stated otherwise were carried out on ice. A 100 µl reaction mixture containing DNA (4 – 10 µg final concentration) and 1x of the appropriate RE buffer was prepared on ice. The reaction mixture was divided into 5 reaction tubes, such that reaction tube 1 contained 30 µl, tubes 2 to 4 contained 20 µl each, and tube 5 contained 10 µl reaction mixture. The appropriate RE was added to a final concentration of 3 U per µg DNA to reaction tube 1. For the following steps it was necessary to use each pipette tip only once between steps. Ten µl of the mixture from reaction tube 1 was transferred to reaction tube 2. The tube was gently mixed and 10 µl was then transferred to reaction tube 3. This serial dilution was repeated so that the five reaction tubes finally contained 20 µl of the reaction mixture. The five reaction tubes were then incubated at the appropriate RE working temperature for 15 min. The reactions were stopped by 10 min RE inactivation at 65⁰C. Samples were then fractionated by electrophoresis (Section 4.2) and subsequently purified from the gel (Section 7.2.2).

5.3 Removal of 5' phosphate groups from linearised vector DNA

For sticky- or blunt-end ligation of the RE digested vector to a DNA fragment, the phosphate groups of 5' DNA overhangs of the vector were removed by treatment of the RE digested vector DNA with shrimp alkaline phosphatase (SAP; Roche). The phosphatase reaction was carried out in a 1x SAP reaction buffer with 0.5 U of SAP per µg DNA. The reaction mixture was incubated at 37⁰C for 1 – 2 hours, followed by a 15 min inactivation of the enzyme at 65⁰C.

Five units of T4 Ligase (Promega) were added to 50 ng of prepared vector mixed with the required amount of insert DNA to a molar ratio of 3:1 (Equation B.1) in a 1x ligation buffer (Promega). The ligation reaction was incubated O/N at 4^oC to ensure maximum vector to insert ligation. Recombinant constructs were purified by phenol/chloroform extraction (Section 7.2.3) and resuspended in H₂O to a final volume 10 µl. Two µl of the purified vector construct mixture was used for electro-transformation (Section 6.1.1 and Section 6.2.1).

5.4 Preparation of antisense clones

To produce expression plasmid-based antisense mRNA to mycobacterial gene-transcripts, 460 bp – 570 bp DNA sequences complementary to the target sequences were synthesised through PCR (Section 3.1.2). Forward and reverse PCR oligonucleotides (see Table 4) were designed to include suitable RE-recognition sites to allow directional cloning of the target DNA sequences into the MCS of the acetamide inducible vector, pAce (Appendix C.2). The forward PCR oligonucleotide also included a ATG codon as a transcriptional initiator sequence, while the reverse PCR oligonucleotide included a translational stop-codon (TGA or TAG) in addition to the RE-recognition sites. Through directional cloning, the target sequence was cloned in the antisense direction, which would therefore produce an mRNA molecule complementary to the mRNA transcript of the mycobacterial target gene.

6. PREPARATION OF COMPETENT CELLS AND ELECTRO-TRANSFORMATION

6.1 *Escherichia coli*

Escherichia coli cells (all strains in Section 1 were prepared in the same manner) from a frozen stock culture were streaked onto a LB-agar plate (Section 1.1) containing 50 µg/ml tetracycline (Appendix A.1.4) and the culture was incubated at 37^oC O/N. A single colony was inoculated into 50 ml LB-media (Appendix A.2.3) containing 50 µg/ml tetracycline and cultured O/N at 37^oC in a shaking incubator. One litre LB-medium containing 50 µg/ml tetracycline was pre-warmed to 37^oC and then inoculated with 10 ml of the O/N starter culture. This culture was incubated at 37^oC in a shaking incubator until the OD₆₀₀ reached 0.5 – 0.8. The culture was cooled for 30 min on ice and all subsequent steps were strictly performed at 4^oC.

The *E. coli* cells were collected by 15 min centrifugation at 1150 x g (4°C). The culture supernatant was removed and the cells were washed 3 times with 1 l sterile 10% (v/v) glycerol. Following each wash, the biomass was collected by 15 min centrifugation at 1150 x g (4°C). Finally, all washed *E. coli* suspensions were pooled and collected by 20 min centrifugation at 1150 x g (4°C). The cells were re-suspended in sterile 10% (v/v) glycerol to a final volume of 2 – 3 ml. Fifty-µl aliquots of the final *E. coli* suspension were used immediately or flash frozen in liquid nitrogen and stored at -80°C.

6.1.1 Electro-transformation

Frozen stocks of competent *E. coli* cells were placed on ice to thaw. Two µl of purified plasmid construct (Section 7.2) were gently mixed with the 50 µl thawed competent cell suspension. This mixture was carried over to a 0.2 cm electroporation cuvette (BioRad, pre-cooled to 4°C) and incubated on ice for 1 min. The Gene Pulser (BioRad) was set to a constant voltage of 2.5 kV, a capacitance of 125 F, a secondary capacitance of 25 µF, and a resistance of 200 Ω. The cuvette was mounted in the Gene Pulser chamber and pulsed once. One ml of freshly prepared SOC medium (Appendix A.2.10) was added to the contents of the cuvette and mixed. The cell suspension was transferred to a sterile micro-centrifuge tube and incubated at 37°C for 1 hour with vigorous shaking. One hundred µl of the electro-transformed cell suspension was plated on selection plates containing 50 µg/ml of the required antibiotic (Table 1) and cultured at 37°C O/N.

6.2. Preparation of mycobacterial competent cells

Mycobacteria were cultured in ADC-enriched 7H9 mycobacterial medium (Section 1) to an OD₆₀₀ of approximately 0.5. Cultures were routinely checked for contamination by ZN-staining (Section 1.4). The mycobacterial cells were collected through 10 min centrifugation at 1150 x g (4°C) and washed 3x with 10% glycerol the same as with *E. coli* cells (Section 6.1). One hundred µl aliquots of the final suspensions were made. Electro-competent *M. bovis* BCG cells were used fresh as it was observed that transformation efficiency was greatly reduced if -80°C stocks were used. Electro-competent *M. smegmatis* cells were used fresh, however -80°C stocks were still electro-competent although slightly less efficiently than fresh preparations.

6.2.1. Electro-transformation

Competent (mycobacterial) bacilli were kept on ice during all preparations. Two μl of purified plasmid construct (Section 7.2) was gently mixed with 100 μl competent bacilli suspension. This mixture was carried over to a sterile 0.2 cm electroporation cuvette (BioRad, pre-cooled to 4^oC) and then incubated on ice for 1 min. The Gene Pulser (BioRad) was set to a constant voltage of 2.5 kV, a capacitance of 125 F, a secondary capacitance of 25 μF , and a resistance of 1000 Ω . The cuvette was mounted in the Gene Pulser chamber and pulsed once. One ml of freshly prepared OADC enriched 7H9 medium (Appendix A.2.5 and Appendix A.2.7) was added to the contents of the cuvette and mixed. The cell suspension was transferred to a sterile micro-centrifuge tube and incubated at 37^oC for 3 hours (fast-growing mycobacteria) or 4 hours (slow-growing mycobacteria) with vigorous shaking. The mycobacterial cells were collected by 30 s centrifugation in a Eppendorf bench-top microfuge at maximum speed (RT), resuspended in 100 μl OADC-enriched 7H9 and plated on selection plates containing 100 $\mu\text{g}/\text{ml}$ of the required antibiotic (Table 1). Cultures were incubated at 37^oC until growth appeared (~ 2-3 days for fast-growers; ~ 21 days for slow-growers).

7. NUCLEIC ACID PURIFICATION

7.1 Plasmid preparations from *E. coli* cultures

7.1.1 Mini-prep plasmid DNA purification

Small-scale purification of plasmid DNA from *E. coli* cultures was essentially carried out with the Promega DNA mini-prep system following the manufacturers instructions with slight modifications. *E. coli* cells were collected by 10 min centrifugation at 1150 x g (4^oC) and resuspended in 200 μl Resuspension Buffer (provided) followed by the addition of 200 μl Lysis Buffer (provided). The lysis-suspension was neutralised with 250 μl Neutralising Buffer. The DNA-containing lysate was cleared of cellular debris by centrifugation at 15 000 x g for 5 min (RT). The DNA in the cleared lysate was immobilised on a purification column (provided) and washed with 700 μl 70% ethanol (RT). Excess ethanol was removed from the purification column by a 2min centrifugation at 15 000 x g (RT). Immobilised DNA was eluted

into a sterile 1.5 ml micro centrifuge tube through the addition of 50 μ l dH₂O (65⁰C) to the column and a 1 min centrifugation at 15 000 x g (RT).

7.1.2 Maxi-prep plasmid DNA purification

Large-scale isolation of recombinant plasmid DNA from *E. coli* was carried out following the protocol essentially as described by the manufacturer of the Nucleobond Kit (Machery-Nagel) with slight modifications. Briefly, a starter culture was prepared by selecting a clonal colony of electro-transformed *E. coli* from selection agar plates (Section 1) and inoculation into 10 ml LB media (Appendix A.2.3) containing the appropriate antibiotic to a 50 μ g/ml final concentration (Table 1). This inoculum was incubated O/N in a 37⁰C shaking incubator. One ml of the starter culture was inoculated into 100 ml sterile pre-warmed LB medium containing the appropriate selection antibiotic to a 50 μ g/ml final concentration and cultured O/N at 37⁰C in a shaking incubator. The cells were collected by centrifugation at 1150 x g (4⁰C) and resuspended in 4 ml S1 buffer (supplied) pre-cooled to 4⁰C. The cells were lysed by the addition of 4 ml S2 lysis buffer and gentle mixing by inversion. To allow complete *E. coli* cell disruption, the mixture was incubated for 5 min (RT) followed by neutralisation of the lysis mixture by the addition of 4 ml buffer S3 (supplied). The mixture was incubated at 4⁰C for 5 min.

The bacterial lysate was cleared by filtration (provided or Whatman No. 2 filtration paper). Plasmid DNA was immobilised from the filtrate in a pre-equilibrated (2 ml N2 buffer) Nucleobond AX 100 column by gravity flow through. The column was washed twice with 5 ml buffer N3. Plasmid DNA was eluted from the column with 2 ml buffer N5 and precipitated with 0.7 volumes of iso-propanol (equilibrated to RT). The plasmid DNA was immediately collected by 30 min centrifugation at 15 000 x g (4⁰C) and washed with ice-cold 70% ethanol. Plasmid DNA was collected after 10 min centrifugation at 15 000 x g (4⁰C). The 70% ethanol was aspirated and the plasmid DNA allowed to dry (65⁰C). Finally, the plasmid DNA was resuspended in either TE buffer (Appendix A.3.24) or dH₂O depending on further applications. Plasmid DNA was quantified using the Nanodrop system (Inqaba Biotechnologies).

7.2 DNA preparations

7.2.1 Mini-column DNA purification

Polymerase chain reaction amplified DNA was purified using the Promega SV-column system. The manufacturer's instructions were essentially followed and briefly involved mixing the PCR reaction with an equal volume of membrane binding solution (supplied). The DNA was immobilised on a SV DNA binding column (supplied), washed twice at RT with a 70% ethanol solution and dried by a 5-min centrifugation at top speed in a microfuge. To elute the DNA, 50 µl nuclease free dH₂O (Promega), pre-warmed to 65⁰C, was added to the column. The DNA was collected through 1 min centrifugation at 15 000 x g (RT). The DNA was quantified with the Nanodrop DNA quantification system (Inqaba Biotechnology).

7.2.2 Purification of DNA from TBE agar

Deoxyribonucleic acid products were recovered from mixtures of DNA molecules of differing size through electrophoresis in a 1% TBE agar gel (Section 4.2). The desired DNA fragment(s) were excised from the gel and purified using the Promega mini-prep system. Briefly, the excised gel fragment was weighed and DNA binding buffer (supplied) was added to a final volume of 1 µl/mg of TBE agar. This preparation was incubated at 65⁰C until the TBE agar gel was completely melted. The solution was loaded onto a SV column (supplied) and DNA was purified using the same procedure as described in Section 7.2.1.

7.2.3 Plasmid DNA purification by chloroform/ isoamylalcohol extraction

The phenol/ chloroform purification method was followed to enrich or purify small quantities of target DNA, or small DNA fragments that are not retained by the DNA purification matrices of most purification systems (usually DNA fragments smaller than 100 bp). Briefly, DNA samples were made up to a volume of 100 µl or greater to which an equal amount of chloroform/ isoamylalcohol (24:1) was added. The suspension was carefully mixed and centrifuged for 30 min at 4⁰C. The aqueous layer was recovered to which a 1/10 volume of 3 M NaAc (pH 5.5) was added. Two volumes of 95% ethanol (-20⁰C) was added and DNA precipitated at -20⁰C O/N. Precipitated DNA was collected after 30 min centrifugation at 15 000 x g (4⁰C). The supernatant was carefully aspirated and the DNA precipitate washed

once with 70% ethanol (-20°C) to remove all excess salt and then allowed to air-dry. The pellet was resuspended in 10 µl nuclease free dH₂O (Promega) pre-warmed to 65°C.

7.3 Ribonucleic acid (RNA) isolation and purification

Mycobacterium tuberculosis was cultured in 7H9 liquid media (Section 1) to an OD₆₀₀ of 0.6. The cells were collected by 15 min centrifugation at 1150 x g (4°C), the culture supernatant was discarded, and the bacterial pellet suspended in 0.85 ml TRIZOL (Gibco BRL) per 10 ml original *M. tuberculosis* culture. The bacterial suspension was lysed in a BioLab riboliser at speed setting 6.5 for 45 sec using conical 2.0 ml centrifugation tubes filled with 0.5 mm glass beads to a equal volume of the original cell pellet. This step was repeated twice with a 1 min cooling on ice between the steps. After the final cycle, samples were incubated for 5 mins at RT to allow complete dissociation of nucleoprotein complexes.

Chloroform was added to each sample at 0.2 ml per 0.75 ml TRIZOL before homogenisation. Samples were mixed by low speed vortexing for 15 sec to allow mixing of the phases, followed by 5-min incubation at RT. Aqueous and organic phases were separated by 15 min centrifugation at 15 000 x g (4°C). The aqueous phase was recovered and nucleic acids precipitated by the addition of 0.5 ml iso-propanol per 0.75 ml TRIZOL originally used and 10 min incubation at room temperature. Precipitated nucleic acids were collected by 10 min centrifugation at top speed in a micro centrifuge (4°C). The supernatant was carefully aspirated and the nucleic acid pellet washed once with 1 ml ice cold 70% ethanol. The ethanol was removed and the sample was left to air-dry at room temperature.

The nucleic acid precipitate was resuspended in 80 µl nuclease free H₂O to which 10 µl of 10x DNase buffer, 40U RNase Inhibitor and 25U DNase was added (the total volume of the reaction was 100 µl). The reagents were mixed and incubated for 30 min at 37°C. The reaction was stopped by a 10-min incubation at 65°C. Total mRNA was recovered by a final purification step using a RNA Miniprep clean-up kit (Qiagen) essentially following the manufacturers instructions. Three hundred and fifty µl Buffer RLT (supplied) was mixed with the 100 µl reaction and then 250 µl 95% ethanol was added. This mixture was loaded onto an RNeasy mini column, which was assembled in a 2 ml collection tube (supplied). The column assembly was centrifuged for 15 sec at 15 000 x g (RT). The RNeasy mini column was assembled into another 2 ml collection tube (supplied) and 500 µl Buffer RPE (supplied)

was added to the mini column. The mini column assembly was centrifuged for 15 sec at 15 000 x g (RT). The flow-through was discarded and another 500 µl RPE was added to the mini column. The mini column assembly was centrifuged for 2 min at 15 000 x g (RT) and the mini column was assembled in a 1.5 ml collection tube (supplied). The mRNA was eluted from the mini column by adding 50 µl nuclease free ddH₂O and 1 min centrifugation at 15 000 x g (RT). Ribonucleic acid was quantified using the Nanodrop system (Inqaba Biotechnologies).

8. COLONY BLOTTING

To identify *E. coli* colonies harbouring *M. tuberculosis* H37Rv genome library cosmids containing target library inserts, the *E. coli* clones were spread onto LB-agar plates containing Ampicillin (50 µg/ml, Roche) and allowed to grow at 37°C for 16 h. Bacterial colonies were transferred to dry Hybond-N⁺ membrane filters (Amersham) by placing the membrane (does not require prior treatment) onto the colonies and incubating for one minute (room temperature). Orientation of the disc with respect to the colonies on the LB-agar was marked by simultaneously piercing the membrane and agar with a syringe needle dipped in India ink (local stationery store). The membrane was then placed, colonies facing upwards, on Whatmann 3MM paper soaked in 10% SDS (m/ v) for 5 min. The membrane was subsequently transferred to Whatmann 3MM paper saturated with denaturing solution (Appendix A.3.7) for 10 mins. Thereafter the membrane was placed for 5 mins on Whatmann 3MM paper soaked in neutralising solution (Appendix A.3.8). Finally the membrane was rinsed in 2x SSC (pH 7.0, Appendix A.3.22) on an orbital shaker for 3 mins. The DNA was fixed to the membrane by baking for 2 hours at 80°C in a vacuum oven.

Colonies harbouring cosmids with the *M. tuberculosis* genomic region that contains the *glnA1* to Rv2224c ORFs were identified by ECL direct nucleic acid labelling and detection (Amersham, UK). After baking, the membranes were pre-hybridised in ECL hybridisation buffer (supplied) for 1 hour at 42°C with gentle agitation. Thereafter the membranes were probed by the addition of an ECL labelled probe (10 ng/ml; Section 11), which was complementary to an internal region of Rv2224c (Table 4) to the ECL hybridisation buffer. Probe hybridisation was carried out at 42°C with gentle agitation for 4 hours. After hybridisation, the membrane was washed for 20 min with gentle agitation in 2 – 5 ml Primary Wash buffer (pre-heated to 42°C, Appendix A.3.19) per cm² membrane. A further 20 min

wash was carried out under the same conditions with fresh Primary Wash buffer. Thereafter the membrane was washed twice in an excess volume of 2x SSC for 5 min at RT with gentle agitation. The ECL-hybridisation positive clones were visualised by autoradiography on Kodak X-ray film. Following agar plate orientation, corresponding positive colonies were picked (as the cosmid sizes are around 40 000 kb, positive clones were obtained with a frequency of $\pm 1:300$) and positive clones were confirmed by PCR of the gene used as probe (Rv2224c, as well as *glnA1* that are situated downstream, see Chapter 4, Figure 4.1). Finally, the isolated cosmids were sequenced on an automated sequencer using T3 and T7 sequencing primers to confirm the correct DNA region as well as to determine the exact start and stop of insert DNA.

9. PROTEIN PURIFICATION AND ACTIVITY ASSAYS

9.1 Cell fractionation

In order to monitor the expression of recombinant hexa-histidine tagged proteins, crude protein extracts were prepared from host cultures (*E.coli*, *M. smegmatis*, *M. bovis* BCG). Briefly, cultures were collected by 20 min centrifugation at 1181 x g (4°C). The cell pellet was resuspended in pre-cooled phosphate buffered saline (equal to 2x the cell pellet) containing 1 mM Phenylmethylsulphonylfluoride (PMSF, Roche) and carried to a sterile 2.0 ml Apex tube. The Apex tube was prepared with glass beads (0.5 mm diameter) of an approximately equal volume to the cell pellet. These preparations were stored at -80°C or used immediately. The mixture was ribolysed for 45 sec at speed setting 6.5 in a FastPrep riboliser (Bio101) and centrifuged at 15 000 x g for 2 min (RT). The cleared lysate was aspirated and stored in SDS-PAGE denaturing loading buffer (Appendix A.3.20) at -80°C or used immediately for acrylamide gel electrophoresis and Western Blotting (Section 10)

To monitor the distribution of recombinant hexa-histidine tagged proteins amongst cellular compartments, a more refined cell fractionation was performed (Figure 4). For this purpose, the expression host-cells were grown in Kirchners medium (Appendix A.2.2) and, after cell collection, the cleared culture supernatant was first sterilised by filtration through a 0.22 μ m syringe filter unit with low protein affinity (Millipore, USA) and then the proteins were concentrated using Amicon Ultra centrifugal filter devices with a >15 kDa exclusion (Millipore, USA). The cell pellet was resuspended in PBS containing 1 mM PMSF pre-cooled

to 4°C and ribolysed as described before. The resulting lysate was cooled on ice for 5 min and then centrifuged for 10 min at 15 000 x g (4°C). The pellet was retained for cell wall protein isolation.

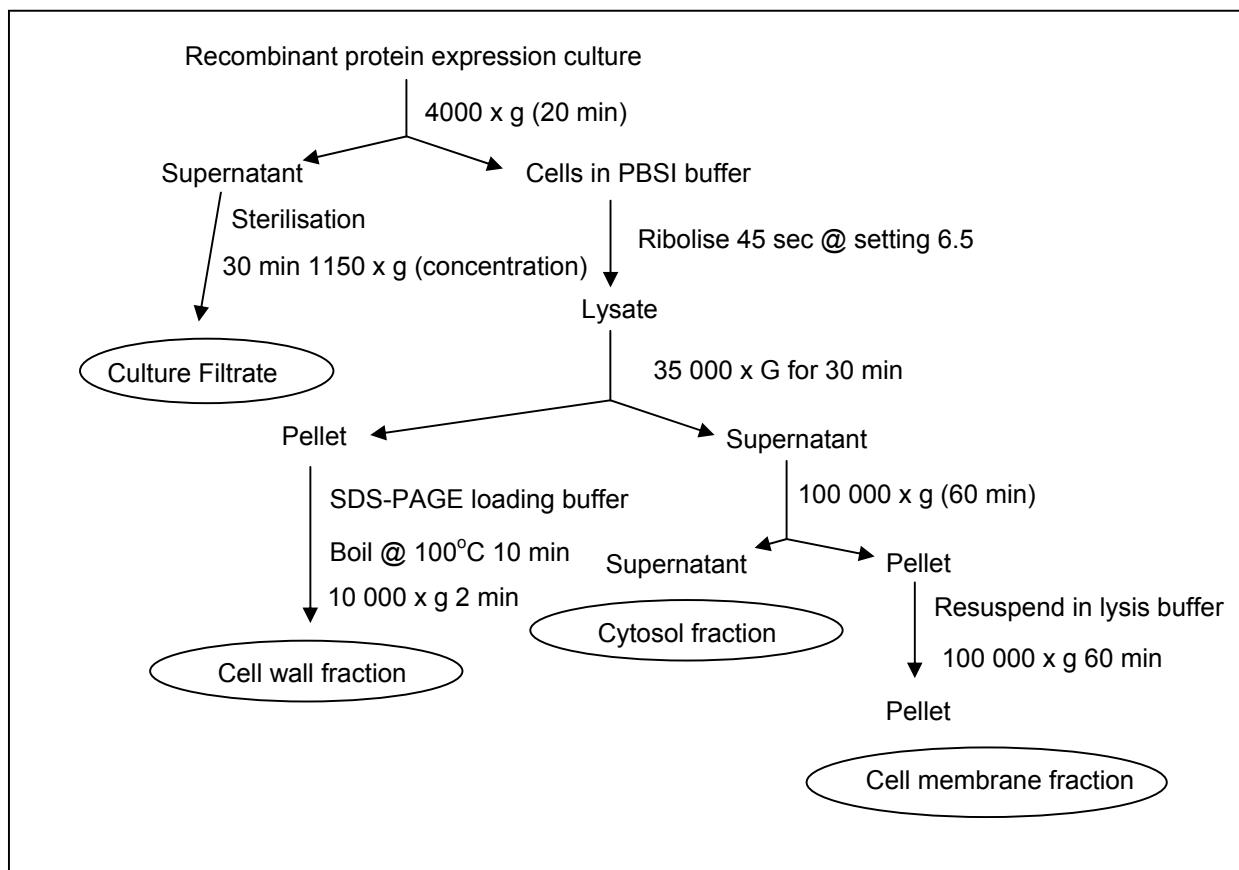


Figure 4. Flow diagram describing the method of bacterial fractionation into different sub-cellular compartments.

The resulting cleared lysate was collected and centrifuged for 1 h at 100 000 x g (4°C) in a Beckmann ultra-centrifuge to collect the membrane fraction (visible as a thin orange coloured film at the bottom of the centrifuge tube). The supernatant contained the cytosol-associated proteins. The membrane fraction was washed once by re-suspension in 200 µl PBS (pH 7.0) containing 1 mM PMSF and collected by ultra centrifugation as before.

Cell wall associated proteins were extracted from the lysate pellet by first washing the pellet twice in PBS (4°C) containing 1 mM PMSF to remove any residual cytosol proteins. The pellet was collected by 15 min centrifugation at 15 000 x g (4°C) and resuspended in a 1x

volume SDS-PAGE reducing loading buffer (Appendix A.3.20) and incubated for 10 min at 100°C. The suspensions were cleared by 15 min centrifugation at 15 000 x g and the supernatant used in SDS-PAGE electrophoresis and Western blotting (Section 10).

9.2 Protein concentration determination

Protein concentrations were determined with the Bradford method (Bradford, 1976) using the BioRad Protein Assay. The dye reagent was prepared by diluting dye with ddH₂O (1:4). Five dilutions of a bovine serum albumin (BSA) standard were prepared in a linear range of 0.2 to 0.9 mg/ml in ddH₂O. A blank reaction was also prepared by mixing equal volumes of ddH₂O with suspension buffer (PBS-PMSF) or culture media (if the culture media used did not contain protein-based supplements). Samples were prepared by mixing 10 µl of sample with 10 µl ddH₂O. Twenty µl of each BSA standard and protein sample preparation was mixed with 980 µl diluted reaction dye. The reaction was incubated for 5 min at room temperature and then read at 595 nm using a spectrophotometer. A standard curve was prepared and the sample concentrations were inferred from the standard curve.

9.3 Glutamine synthetase purification

Glutamine synthetase was purified from *M. bovis* BCG Pasteur essentially as described by Shapiro and Stadtman (1970) with slight modifications. *Mycobacterium bovis* BCG was cultured in Kirchners medium (Appendix A.2.2) and when cultures reached an OD₆₀₀ > 1.0, the bacteria were pelleted by 10 min centrifugation at 10 000 x g at 4°C. The cells were resuspended in 30 ml Resuspending Buffer A (RBA, Appendix A.3.13) and vortexed gently (approx 400-500 rpm) 3 times for 1 min each with 5 millimetre diameter glass beads (enough to be equivalent to the biomass volume). This step was carried out to remove the cell surface GS. The cells were collected by 10 min centrifugation at 10 000 x g at 4°C. The supernatant was retained for purification of the cell surface GS, while cytosol associated GS was purified from the cell pellet.

9.3.1 Cell lysis and removal of nucleic acids

For the preparation of cytosol GS purification, the cell pellet was resuspended in 50 ml RBA. The cell suspension was sonicated (CupSonics) for a total of 15 minutes at a 50% duty

cycle. These cycles consisted of a 1 sec on cycle, followed by a 0.1 second off cycle at maximum power. After every minute of sonication, the cell suspension was cooled on ice for 1 min. After sonication, the suspension was centrifuged for 10 minutes at 10 000 x g (4°C). The protein concentration of the supernatant was determined spectrophotometrically using Equation B.5. The supernatant proteins were adjusted to a 20 – 30 mg/ ml final concentration with RBA. To precipitate nucleic acids from the supernatant, a 10% v/ v of a 10% m/ v streptomycin sulphate solution was added to the supernatant. This suspension was stirred for 15 min (4°C) using a magnetic stirrer and then centrifuged at 20 000xg for 10 minutes (4°C). The supernatant was retained.

9.3.2 First ammonium sulphate precipitation

Glutamine synthetase was purified from the supernatants of the cell wall surface and cytosol GS preparations by ammonium sulphate precipitation. The pH of the suspension was adjusted to 5.15 with 0.5 M sulphuric acid (H₂SO₄) and stirred for 15 min (at 4°C). The precipitate was collected by 15 min centrifugation at 20 000 x g (4°C). The supernatant was retained to which a 30% volume of a saturated solution of ammonium sulphate [(NH₄)₂SO₄] pre-cooled to 4°C was added. The pH was adjusted to pH 4.6 with 0.5 M H₂SO₄ and stirred for 15 min at 4°C. The suspension was centrifuged at 20 000xg for 15 min and the pellet resuspended in 50 ml RBA. The pH of the suspension was adjusted to pH 5.7 with 1 M ammonium hydroxide (NH₄OH) by titration until all the acid-ammonium sulphate precipitate was evenly suspended. After the precipitate was resuspended it was stirred for 8-12 hours (4°C), ensuring that the pH remained at pH 5.7. The presence of GS activity was determined in the supernatant as well as the precipitate by the γ -glutamyl transferase assay (Section 9.4).

9.3.3 Second ammonium sulphate precipitation

Glutamine synthetase was precipitated from the cleared supernatant by a second round of (NH₄)₂SO₄ precipitation as described above, except that the pH was adjusted to pH 4.4 with 0.5 M H₂SO₄. After centrifugation at 20 000 x g for 15 min (4°C), the pellet was resuspended in 20 ml RBA. The pH of the solution was then adjusted to 5.7 with 1 M NH₄OH and incubated at 4°C for a further 8 - 12 hours with stirring. The suspension was centrifuged at 20 000 x g for 10 minutes and the cleared supernatant was retained. The presence of GS

was determined in the supernatant, as well as the precipitate, by the γ -glutamyl transferase assay (Section 9.4).

Glutamine synthetase purity was then determined through SDS-PAGE gel electrophoresis (Section 4.1). If there were still large amounts of other proteins in the sample, GS was precipitated in a third round of $(\text{NH}_4)_2\text{SO}_4$ precipitation, as described in Section 9.3.2, except that 10% by volume of a saturated $(\text{NH}_4)_2\text{SO}_4$, pre-cooled to 4°C , was added to the supernatant and the pH was adjusted to pH 5.15 with 0.5 M H_2SO_4 . The suspension was centrifuged for 15 min at $20\,000 \times g$ (4°C), the supernatant was retained and the pH was adjusted to 4.4 with 0.5 M H_2SO_4 . The solution was equilibrated at this pH for 15 minutes and then centrifuged at $20\,000 \times g$ for 10 minutes (4°C). The pellet was resuspended in 5 ml RBA (pH 5.7) and centrifuged at $20\,000 \times g$ for 10 minutes (4°C). To increase purity this step could be repeated several times without any significant loss in GS activity. The enzyme was stored at 4°C in RBA (pH 7.0).

9.4 γ -glutamyl-transferase activity assay

Glutamine synthetase activity was assayed using the transferase assay. This reaction gives a colour reaction that can be measured spectrophotometrically. For each sample, an assay series was prepared consisting of 1 blank reaction and two duplicates of an Mn^{2+} , Mg^{2+} and $\text{Mn}^{2+} + \text{Mg}^{2+}$ combination. Reactions were prepared as in Table 6.

Table 6. The reactions comprising the transferase assay of GS activity. All reagents in the assay were prepared as described in Addendum A.3.14. Hydroxylamine and Na-arsenate were prepared fresh before each reaction.

REAGENTS	BLANK (μl)	Mn (μl)	Mg (μl)	Mn/ Mg (μl)
Imidazole buffer	390	230	230	190
Glutamine	80	80	80	80
Hydroxylamine	40	40	40	40
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	40	40	-	40
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	-	-	40	40
Na- ADP	-	60	60	60
Na-arsenate	-	100	100	100
Total volume of assay	550	550	550	550

The reaction was started by the addition of 50 μ l of the GS enzyme preparation to each reaction preparation. Reactions were incubated at 37°C for 30 min, where after the reaction was stopped by the addition of 900 μ l stop mix (Addendum A.3.14.7). The addition of the stop mix resulted in a colour change of the reaction. The reaction was centrifuged at 15 000 x g for 2 min (RT) to pellet residual ferric chloride. The cleared supernatant was decanted into a 1 ml plastic spectrophotometer cuvette (Merck) and read at a wavelength of 540 nm in a spectrophotometer.

9.5 β -Galactosidase assays

β -Galactosidase activity was quantitatively assayed as described (Pardee *et al.*, 1959; Timm *et al.*, 1994) using sonicated total cellular extracts of *M. smegmatis* transformed with different pJEM constructs (Appendix C.3). The *M. smegmatis* cellular extracts were prepared by sonication for a total time of 5 minutes (15 sec bursts with 30 sec intervals) at 4.5 setting in a Misonix sonicator and using a micro tip probe. Protein concentrations of sonicated extracts were determined spectrophotometrically using the BioRad protein concentration assay (Section 9.2). The assay is based on the β -galactosidase substrate *o*-nitrophenyl- β -D-galactoside (ONPG) and one unit of β -galactosidase is defined as producing 1 μ Mole *o*-nitrophenol per minute from *o*-nitrophenyl- β -D-galactoside at 28°C, pH 7.0 (1 μ Mole/ ml *o*-nitrophenol has an optical density at 420 nm of 0.0075). β -Galactosidase activity was determined using 6.6 μ g of protein and 30 min reaction incubation at 28°C. All activity assays were performed in triplicate. The units of β -galactosidase activity were calculated as described by Timm *et al.* (1994) and Berthet *et al.* (1998) (see Equation B.4).

9.6 MagnaHis affinity purification of 6x his-tagged proteins

The MagnaHis protein purification system (Promega) was used for small-scale purification of 6 his-tagged proteins. This system makes use of paramagnetic pre-charged nickel particles and are effective under non-denaturing or denaturing (by addition of urea to a final concentration of 6 M) conditions. Hexa-histidine-tagged protein were purified from whole cell lysates that were prepared either by sonication (see Section 9.5) or ribolisation (see Section 9.1). Thirty μ l of MagnaHis Ni-particles were added to 1 ml of cell lysate and mixed by inversion (room temperature). The suspension was incubated at room temperature for 2 min,

where after the Ni-particles were collected using a locally produced magnetic stand (Cor Wytenburg). The supernatant was removed by aspiration and 200 μ l of Binding/ Wash buffer (supplied) added to the Ni-particles. The Ni-particles were resuspended by inversion and the suspension was incubated at room temperature for 2 min. The Ni-particles were collected using the magnetic stand and the supernatant removed by aspiration. This wash-step was repeated three times using 200 μ l of Binding/ Wash buffer. To elute proteins, 100 μ l MagnaHis Elution Buffer (supplied) was added. The Ni-particles were resuspended using a pipette and incubated at room temperature for 2 mins. There after the Ni-particles were collected using the magnetic stand and the supernatant (containing eluted proteins) removed by aspiration. Proteins were analysed by SDS-PAGE electrophoresis (Section 4.1) or Western blotting (Section 10).

10. WESTERN BLOTTING

Protein samples were fractionated according to molecular size by SDS-PAGE electrophoresis (Section 4.1). A Hybond-P PVDF membrane (Amersham, UK) was cut to the size of the poly-acrylamide gel and re-hydrated by rinsing in 95% methanol until the membrane was a homogeneous light-grey colour. Since the membrane has high affinity for proteins, it was carefully handled with tongs and wearing nitrile gloves at all times. The methanol treated membrane was pre-soaked; together with 2 x Western blot pads (BioRad) and 2 x Whatman 3M blotting paper sheets (prepared to the size of the Western bolt pads) in Western blot transfer buffer (Appendix A.3.25) for 15 min. The gel was taken from the electrophoresis apparatus and soaked in Western blot transfer buffer for 5 min. The blotting apparatus (BioRad Protean II) was assembled in the order prescribed by the manufacturer. The assembly of the blotting apparatus was carried out in a shallow tray filled with transfer buffer to avoid drying of the membrane.

The assembly was slotted into the transblotter and the reservoir was filled with Western blot transfer buffer pre-cooled to 4⁰C. A frozen ice pack (supplied with trans-blotting apparatus) was inserted into the trans-blotter (to keep the transfer buffer cool) and a magnetic stirrer was added to the apparatus reservoir (prevents buffer hot spots during transfer). Trans-blotting was carried out for 1 hour at a constant voltage of 150 V in a cold room (4⁰C). The trans-blotting apparatus was disassembled and the membrane was blocked O/N in a BSA-Tween 20 blocking solution (Appendix A.3.2) at 4⁰C. The transfer of the pre-stained protein

molecular weight marker (Fermentas), which was used during SDS-PAGE electrophoresis, served as a visual indicator of successful protein transfer. Following transblotting, the membrane was blocked overnight at 4⁰C in a 2% BSA solution prepared in PBS-T (Appendix A.3.18). Specific proteins were detected through antibody mediated ECL detection (Section 10.1) or nickel-NTA conjugated colorimetric assays (Section 10.2).

10.1 Chemiluminescent Western detection

The blocked membrane was carefully rinsed once in phosphate buffered saline/ Tween 20 (PBS-T) buffer (Appendix A.3.18). The primary anti-hexa-histidine antibody (Invitrogen) was diluted 1: 5 000 in PBS-T buffer and added to the membrane in a clean plastic petri-dish. The membrane was incubated at RT in the primary antibody preparation for an hour with gentle agitation. The primary antibody preparation was decanted (can be stored at -20⁰C and re-used) and the membrane was quickly rinsed once in PBS-T buffer and then washed twice for 15 min each in PBS-T buffer with gentle agitation. The membrane was then incubated for one hour in a 1:10 000 PBS-T dilution of horseradish-peroxidase (HRPO) linked secondary antibody (Invitrogen) at RT with gentle agitation. Thereafter the secondary antibody preparation was decanted; the membrane quickly rinsed once in PBS-T and washed three times for 15 min each in PBS-T with gentle agitation. The HRPO-linked secondary antibody detection was performed through ECL (Section 11.1).

10.2 Colorimetric Western detection

The blocked membrane was transferred into 20 mL PBS-T (Appendix A.3.18) buffer to which 2 µl HisDetector Nickel-HRP (Promega) was added. This membrane was incubated at room temperature for 1 hour with gentle agitation. Following the hybridisation, the membrane was washed three times for 5 min each with PBS-T. Thereafter the membrane was developed by a 10 min to 15 min incubation in 10 mL TMB Membrane Substrate (Promega) at room temperature. The reaction was stopped by rinsing the membrane in ddH₂O.

11. DIRECT ENHANCED CHEMILUMINESCENCE LABELING (ECL) OF DNA PROBES

Chemiluminescent labelling (Amersham) was carried out essentially as recommended by the manufacturer. The DNA (PCR products and purified as in Section 7.2.1) to be labelled was diluted to a concentration of 10 ng/ μ l with sterile dH₂O. One hundred ng DNA (10 μ l) was denatured by 5 minutes incubation at 95^oC in a solid-state heating block and then snap-cooled on ice for 5 min. The tube was briefly spun to collect the condensate. An equal volume of DNA labelling reagent (10 μ l) was added to the denatured DNA and the mixture was briefly mixed. A volume of glutaraldehyde solution, equal to the DNA labelling reagent (10 μ l), was added and the suspension was mixed thoroughly. The mixture was incubated for 10 minutes at 37^oC and used immediately or within 10 – 15 min.

11.1 ECL signal detection (DNA and HRPO-linked antibodies)

An equal volume of Detection Reagent 1 (Amersham) was mixed with Detection Reagent 2 (Amersham) to a solution volume sufficient to cover the whole blot. The DNA or protein side of blots was exposed to the Detection Reagent solution for 1 min at RT. The excess detection reagent was drained by gently blotting the “back” or non-DNA/ protein bound side with a paper towel. The blot was placed in a plastic sleeve and exposed for 1 – 10 min to a sheet of x-ray film (Kodak). The film was developed in an automatic X-ray film developer.

12. *IN SITU* HYBRIDISATION (ISH)

12.1 Brief description

The *in situ* hybridisation method was designed for non-radioactive detection of mRNA and DNA in tissue preparations. This method provides a sensitive detection system for biotinylated probes that takes advantage of the very high affinity of biotin for streptavidin (a non-glycosylated 60 kDa protein with near neutral pI). These complexes are also extremely stable over a wide range of temperatures and pH. In brief, a biotin labelled DNA probe is hybridised to target mRNA in cells or tissues, which are fixed on a microscope slide (see Figure 5). A signalling group (eg. alkaline phosphatase and horseradish peroxidase), covalently attached to streptavidin, is then bound to the biotinylated probe. The hybridised probe is then detected by incubating the samples with dye substrates for alkaline

phosphatase. Formation of a brown signal indicates the location of the hybridised probe, which could be visualised under a light microscope.

The variables influencing the sensitivity of ISH are dependant on the effect that tissue preparation has on the retention and accessibility of target mRNA or DNA, the type of probe construction and labelling, the sensitivity of the method used for signal detection and the effect of hybridisation conditions on the efficiency of hybridisation.

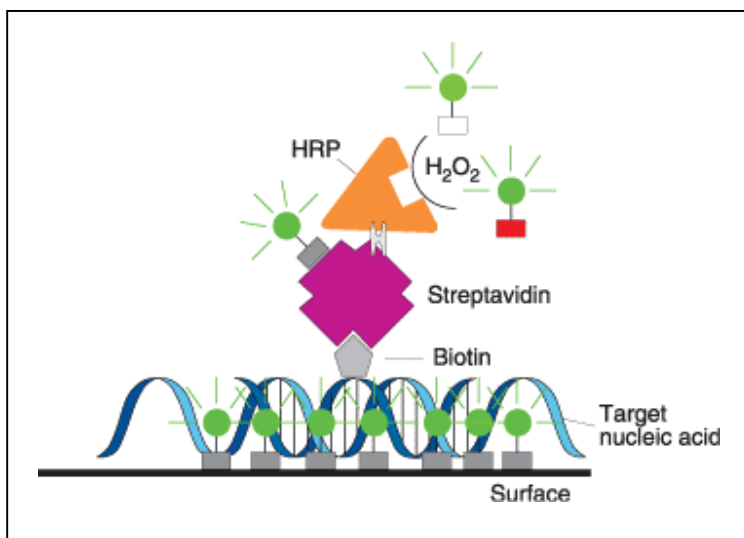


Figure 5. An 18-mer biotinylated riboprobe complementary to a specific target mRNA molecule (fixed on a microscope slide) can be detected through an enzyme reaction detecting a substrate such as horseradish peroxidase (HRP) covalently linked to streptavidin

12.2 Patient selection and tissue specimens

Lung tissue biopsy material was collected from patients who had an episode of tuberculosis disease. Biopsy tissue was collected by the Department of Anatomical Pathology (Stellenbosch University) and immersed in saline in a clean, sterile container. The biopsy tissue was fixed in formalin, processed and embedded in wax as described in Fenhalls *et al.* 2002a. Sections of each tissue were prepared and used for histological analysis and ZN-staining (Section 1.4) to confirm the presence of *M. tuberculosis*. Informed consent was

obtained from all the patients and the study was approved by the Stellenbosch University Ethical Review Committee (Amendment of project 94/107).

12.3 Slide preparation

Glass microscope slides were pre-treated with an adhesive agent so that tissue does not detach during the protocol. This was carried out by pre-cleaning the microscope slides with O/N immersion in chromic acid (Appendix A.3.6), followed by 5 successive washes and a final 30 min incubation in ddH₂O. After the washes with ddH₂O, the slides were immersed in acetone (Merck) for 30 min followed by a 30 min immersion in 2% 3-aminopropyltriethoxysilane (Sigma) in acetone. The slides were dried by baking at 70°C for 1 h and then incubated in diethyl-pyrocabonate (DEPC; Sigma) treated ddH₂O (Appendix A.3.10) for 2 hours to remove RNases. The slides were dried by baking at 70°C overnight.

12.4 Pre-treatment of tissue samples for ISH

Paraffin embedded lung biopsy tissue was cut into 5 µm thick sections with a microtome. The tissue sections were applied to prepared RNase free glass microscope slides (Section 12.3). Paraffin was removed from the tissue sections by 2 times 10 min incubations in xylene at room temperature. Thereafter, the xylene-treated sections were re-hydrated by 2 x 2 min immersions in absolute (95%) ethanol, followed by 90% ethanol and finally 70% ethanol. Slides were then immersed in 0.1 M PBS (pH 7.0) for 5 min. The slides were incubated in a proteinase / DNase digestion preparation (Appendix A.3.16) for 20 min in a 37°C humidified chamber. The slides were then washed with 0.1 M PBS for 5 min and immersed in 0.4% (v/v) paraformaldehyde for a further 5 min to inhibit residual protease activity and paraformaldehyde also to re-fix digested tissue. The slides were subsequently washed with DEPC-treated ddH₂O (Appendix A.3.10) for 2 min and acetylated (to limit non-specific riboprobe hybridisation) for 10 min in a 1:400 (v/v) triethanolamine (Merck) DEPC-treated ddH₂O solution. The slides were rinsed with DEPC-treated ddH₂O for 5 min and re-hydrated through 2 times 2 min immersions in absolute (95%) ethanol, followed by 90% ethanol and finally 70% ethanol. The sections were air-dried at RT for 40 – 60 min before being used for ISH.

12.5 Biotinylated riboprobe preparation

Deoxyribonucleic acid fragments which were complementary to a 120 bp region of *M. tuberculosis glnA1* and a 120 bp region of Rv2223c was generated through PCR amplification (see Table 4 and Section 3.1.2) and cloned into the Promega pGEMt-Easy cloning plasmid system (Section 5). Insert orientation relative to the pGEMt-Easy T7 promoter (Appendix C.8) was determined by PCR screening of the vectors using the T7 and SP6 sequence specific PCR oligonucleotides (Table 4). Plasmids were purified (Section 7.1.1) and sequenced to confirm the insert sequence and orientation. Subsequently the vectors were amplified in an *E. coli* JM109 host-strain (Section 6.1) and purified for use in riboprobe synthesis.

Sense (3' – 5') probes that were used for the detection of target mRNA were synthesised from the T7 promoter of the pGEMt-Easy construct using T7 RNA polymerase (GibcoBRL), while anti-sense probes were synthesised from the 5' – 3' cloned insert DNA strand using SP6 RNA polymerase (GibcoBRL). The anti-sense probes would therefore be complementary to and hybridise to its target mRNA, while the sense probe would be unable to form a hybrid and would therefore serve as a control. The biotinylated riboprobe synthesis was carried out under RNase free conditions which entailed preparation in a sterile 1.5 mL DEPC treated Eppendorf microfuge tube using 1 µg DNA, 1x T7 or SP6 transcript buffer, 1 mM of each dATP, dGTP, UTP and Biotin-14-CTP, 0.5 mM dCTP, 5 mM DTT, 120 U RNase inhibitor (GibcoBRL, Switzerland), 100 U T7 polymerase or 52.2 U SP6 polymerase and RNase free (Promega) or DEPC treated ddH₂O to a final volume of 50 µl. The contents were gently mixed and condensate briefly collected by centrifugation and the transcription reaction carried out for 90 min at 37⁰C. The riboprobes were precipitated by addition of 150 µl 95% ethanol and precipitation at -20⁰C O/N. Biotinylated riboprobes were collected through 30 minute centrifugation at 15 000 x g (4⁰C). The riboprobe pellet was washed once with 70% ethanol (4⁰C) and collected with a 10 min centrifugation as above. The ethanol supernatant was aspirated and the precipitate dried in a Speed Vac Concentrator (Savant, USA) for 5 min. The dried riboprobes were resuspended in 100 µl RNase free ddH₂O (Promega) or DEPC treated ddH₂O and allowed to completely go into suspension by incubation at 37⁰C for 30 min. If not used immediately, riboprobes were stored at -20⁰C.

Biotinylated riboprobes were confirmed by spotting 5 – 10 µL onto Hybond N+ membranes (Amersham, UK) followed by detection with Streptavidin-conjugated alkaline phosphatase in conjunction with the NBT/BCIP substrate (GibcoBRL, Switzerland), as discussed in Section 12.6.

12.6 RNA: RNA *in situ* hybridisation

In situ hybridisation was carried out using an ISH kit (Gibco) and the procedure was followed essentially as described by the manufacturer. Pre-treated slide-mounted tissue samples (Section 12.3) were covered with a hybridisation mixture consisting of 20 µl of a 20% dextran sulphate solution, 20 µl 2 x hybridisation buffer (supplied) and anti-sense or sense biotinylated riboprobes (see Section 12.5) to a final concentration of 5 µg/ ml. These hybridisation preparations were then incubated for 16 – 18 h in a 50°C humidified chamber. After hybridisation, the slides were washed twice for 15 min in 2 x SSC (Appendix A.3.22) to remove non-hybridised riboprobe. The slides were then immersed for 20 min (RT) in 100 µl Blocking solution (supplied) to inhibit possible endogenous enzyme activity, which may influence enzyme-based detection methods. The blocking solution was removed by capillary action (touching absorbent paper to the edge of each slide). A working conjugate mixture consisting of 10 µl streptavidin-alkaline phosphatase conjugate (ISH Kit) and 90 µl conjugate dilution buffer (ISH Kit) was applied to each slide after which the slides were incubated at RT in a humidified chamber for 15 min. A control slide was prepared by omitting the streptavidin-alkaline phosphatase conjugate treatment for both the *M. tuberculosis glnA1* and Rv2223c riboprobes that would indicate endogenous enzyme activity and therefore give a false positive signal. The streptavidin-alkaline phosphatase conjugate-treated slides were washed twice for 15 min each in Buffer A (Appendix A.3.4) at RT and then incubated for 5 min in pre-warmed Buffer B (Appendix A.3.5) at 37°C. Levamisole, a potent inhibitor of endogenous alkaline-phosphatases, was added to this preparation at a final concentration of 200 mg/ ml. Finally, the slides were incubated in a 4-nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolylphosphate (NBT/ BCIP) solution (Gibco ISH Kit) at 37°C until the desired level of signal was achieved. Rinsing the slide in ddH₂O stopped the reaction.

Counterstaining of the probed tissue that allowed visualisation of cells with no hybridised probe were carried out by 5 min incubation in methyl green (DAKO, USA), a basic dye that reacts with anionic groups, such as the phosphate groups of nucleic acids. After methyl

green treatment, slides were briefly rinsed with 95% ethanol and then dehydrated through 30 sec immersion in graded alcohols (2 x 90% ethanol, 2 x 95% ethanol) and final 2 min incubation in 100% xylene (Merck). Slides were then mounted in a permanent mounting medium (Entellen, Merck). A positive hybridisation signal was observed as a brown colour, while a blue colour indicated negative hybridisation.

Photographic images were captured using a Zeiss microscope (Axioscope 2) fitted with a Sony 3CCV video camera. Images were saved using the Axiovision software (Zeiss, Germany). It is important to note that ISH is an empirical staining technique and should not be taken as a quantitative measure for bacterial load (see Chapter 5 Discussion).

APPENDIX A: SOLUTIONS

A.1 Antibiotic stock solutions:

A.1.1) Ampicillin

50 mg/ ml in ddH₂O, filtered sterilised (0.22 µm filter, Millipore, USA) and stored at – 20°C

A.1.2) Kanamycin

50 mg/ ml in ddH₂O, filtered sterilised (0.22 µm filter, Millipore, USA) and stored at – 20°C

A.1.3) Hygromycin B

50 mg/ ml in ddH₂O, filtered sterilised (0.22 µm filter, Millipore, USA) and stored at – 20°C

A.1.4) Tetracyclin

50 mg/ ml in ethanol, filtered sterilised (0.22 µm filter, Millipore, USA) and stored at – 20°C

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 0.22 µm filtration unit (Sterilin) ADC was stored at 4°C.

A.2.2) Kirchners medium:

21.13 mM Na₂HPO (anhydrous); 29.4 mM KH₂PO₄ (anhydrous); 4.34 mM MgSO₄·7H₂O; 8.5 mM Trisodiumcitrate; 33 mM L-Asparagine monohydrate and 16.66 % v/ v Glycerol.

Dissolve the reagents, except for the glycerol, 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 121°C. Nitrogen limiting conditions were created by preparing Kirchners omitting the Asparagine.

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 (LB) agar:

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

A.2.5) 7H9 Mycobacterial medium:

Suspend 4.7 g 7H9 medium powder (Difco) in 900 ml dH₂O containing 2 ml glycerol or 0.5 g Tween 80 (Sigma). Autoclave for 15 min at 121°C

A.2.6) 7H11 Mycobacterial agar:

Suspend 21 g 7H11 agar (Difco) in 900 ml dH₂O containing 5 ml glycerol. Autoclave for 15 min at 121°C.

A.2.7) OADC mycobacterial solid 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 0.22 µ vacuum filtering unit (Sterillin). OADC was stored at 4°C and added to 7H11 Mycobacterial agar at 30°C – 45°C.

A.2.8) Sautons Media:

3.67 mM KH₂PO₄; 2.0 mM MgSO₄.7H₂O; 10.41 mM Citric acid; 0.2 mM Ammonium iron (3+) citrate; 27 mM L-Asparagine monohydrate and 16.66 % v/ v Glycerol.

Dissolve the reagents, except for the glycerol, in half the final volume dH₂O using heat. Once dissolved, add the glycerol and make up to final volume with dH₂O. Adjust to pH 7.4 with 1 M NaOH. Autoclave for 15 min at 121°C.

A.2.9) SOB medium:

Dissolve 2.0% w/ v Bacto-tryptone , 0.5% w/ v Bacto-yeast extract, 8.5 mM NaCl, add KCl to 2.5 mM, pH 7.0. Autoclave for 15 min at 121°C

A.2.10) SOC medium:

Add glucose (20 mM final concentration) and MgCl_2 (10 mM final concentration) to SOB medium prior to use.

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 $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and sterile dH_2O to 10 ml. Prepare fresh, or store aliquots at -20°C for not longer than 1 month.

A.3.3) BSA-Tween 20 Blocking buffer:

2% w/ v bovine serum albumin (BSA) in PBS-T buffer

A.3.4) Buffer A:

100 mM Tris-HCl (pH 7.5), 150 mM NaCl

A.3.5) Buffer B:

100 mM Tris-HCl (pH 9.5), 50 mM MgCl_2 , 100 mM NaCl

A.3.6) Chromic acid (H_2CrO_4):

1.76 M $\text{K}_2\text{Cr}_2\text{O}_7$, 2.62 M H_2SO_4

A.3.7) Colony blotting denaturing solution:

1.5 M NaCl, 0.5 M NaOH

A.3.8) Colony blotting neutralising buffer:

1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 0.001 M Na_2EDTA

A.3.9) Coomassie blue staining solution:

0.25% m/ v Coomassie Blue (Merck), 45% v/ v methanol, 10% acetic acid. Filter before use.

A.3.10) DEPC ddH₂O:

10% v/ v DEPC (Merck) in ddH₂O. Autoclave for 15 min at 121°C.

A.3.11) Destaining solution:

0.1% v/ v Glycerol, 7.5% v/ v Glacial acetic acid, 5% v/ v Methanol.

A.3.12) Ethidium bromide stock solution:

10 mg/ ml in TE buffer (pH 8.0)

A.3.13) Glutamine synthetase Resuspension Buffer A (RBA):

10 mM Imidazole-HCl, 10 mM MnCl₂.4H₂O; pH 7.0

A.3.14) γ -glutamyl-transferase activity assay:

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

A.3.14.1) 112,9 mM Glutamine

A.3.14.2) 70.72 mM MnCl₂.4H₂O

A.3.14.3) 913.33 mM MgCl₂.6H₂O

A.3.14.4) 900 mM NH₂O

A.3.14.5) 180 mM Na₂HAsO₄

A.3.14.6) 4.45 mM Sodium ADP

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

A.3.14.7) Stop mix: 1 M FeCl₃, 201 mM CCl₃COOH and 5.2 % v/ v absolute HCl in distilled water. Mix before use.

A.3.15) IPTG (Isopropyl - β -D-thiogalactopyranoside) stock solution:

100 mM in ddH₂O, sterilise by 0.22 μ m filter filtration

A.3.16) ISH simultaneous proteinase and Dnase digestion preparation:

10 mM Tris-HCl (pH = 7.5); 5 mM EDTA; 1 μ g/ ml Proteinase K (GibcoBRL) and 10 μ g/ ml DNase I (GibcoBRL).

A.3.17) 6x Loading Buffer for agarose gels

30% Glycerol, 0.25% w/ v bromophenol blue, 0.6% w/v SDS in TE buffer (pH 8.0)

A.3.18) PBS-T Wash Buffer:

10 mM Phosphate buffered saline (PBS, Sigma), 0.2% v/ v Tween 20

A.3.19) Primary wash buffer:

6 M urea, 0.4% w/ v SDS, 0.5x SSC

A.3.20) 2 x Reducing sample buffer:

34% v/ v 1 M Tris-HCl (pH 6.8), 20% Glycerol, 30% v/ v SDS (20% m/ v), 0.5% v/ v Bromophenol Blue (0.75%), 0.2% EDTA (0.5 M) and 10% β -mercaptoethanol. Store at 4⁰C or – 20⁰C if not to be used for longer periods.

A.3.21) SDS-PAGE Running buffer:

25 mM Tris, 192 mM Glycine and 0.1% SDS

A.3.22) 20 x SSC:

0.3 M Tri-sodiumcitrate, 3 M NaCl; pH 7.0. 2x SSC working concentrations are prepared by a 10x dilution with dH₂O

A.3.23) 10x Tris-Borate-EDTA (TBE) Buffer:

0.98 M Tris, 0.98 M Boric Acid, 20 mM EDTA, pH 8.3

A.3.24) Tris-EDTA buffer (TE):

10 mM Tris, 1 mM EDTA, pH 8.0

A.3.25) Western blot transfer buffer:

25 mM Tris, 192 mM glycine and 20% v/ v methanol

A.3.26) X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) stock solution:

50 mM in dimethylformamide (DMF) or dimethylsulfoxide (DMSO)

Appendix B: Mathematical equations

Equation B.1: Required insert concentration (ng) = $\frac{3(50ng)(Insert(kB))}{Vector(kB)}$

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

Equation B.3: To covert pmol to μg = N pmol

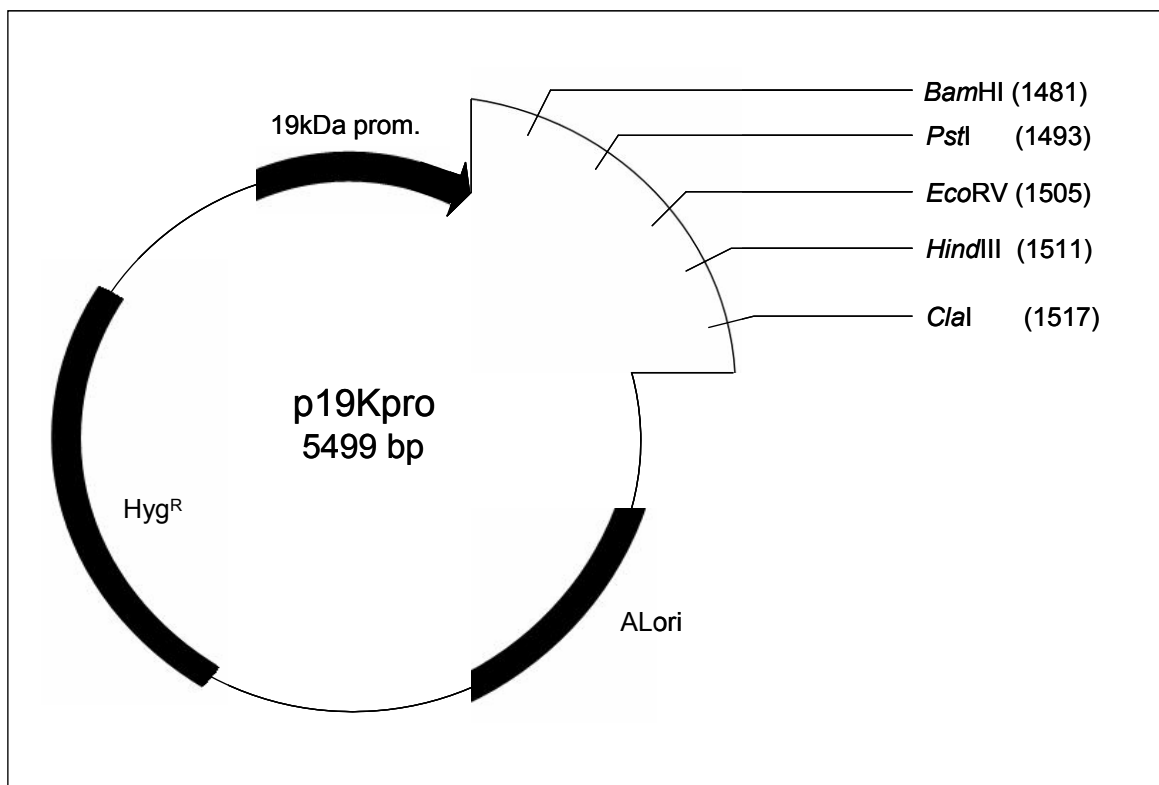
Equation B.4: 1Unit = $200 \times OD_{420}/\text{mg}$ of protein/minute

Equation B.5: Protein Conc (mg/ml) = $[(OD_{280} \times 1.55) - (OD_{260} \times 0.76)] \times \text{dilution}$

Equation B. 6: Solution Volume= $\frac{\text{Protein Conc (mg/ml)} \times \text{Current Solution Volume}}{\text{Required Concentration (mg/ml)}}$

APPENDIX C: PLASMID/ CLONING VECTOR MAPS

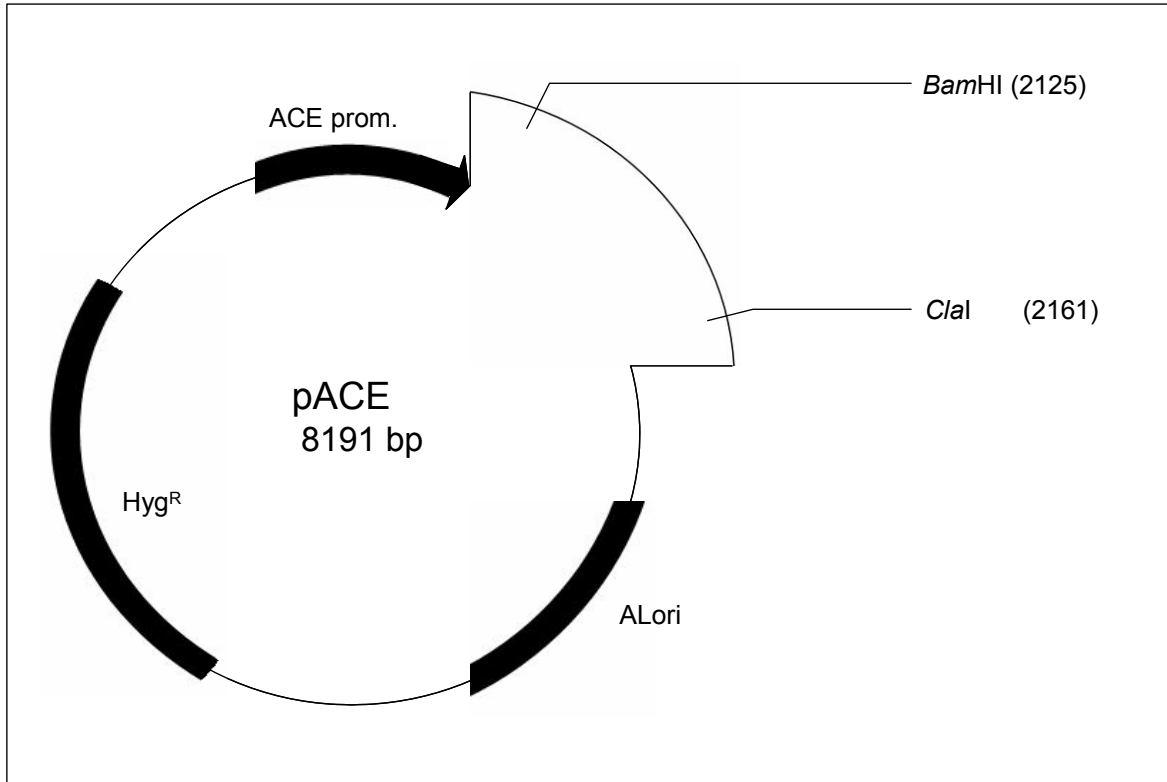
C.1) p19Kpro Mycobacterial expression plasmid



The p19Kpro mycobacterial expression plasmid was used for the constitutive expression of recombinant *M. tuberculosis* proteins under direction of the 19kDa *M. tuberculosis* antigen promoter (19kDa prom.) in other mycobacterial hosts, such as *M. smegmatis* and *M. bovis* BCG. The origin of replication (ALori) is derived from the *M. fortuitum* pAL5000 plasmid and is recognised by both *E. coli* and *mycobacteria*, enabling the amplification of vector constructs in fast-growing *E. coli*. The p19Kpro plasmid carries a hygromycin B resistance (Hyg^R) selection marker. Unique RE-recognition sites located in the multiple cloning site together with their plasmid coordinates are shown.

The p19Kpro plasmid was constructed by Dr. Koen de Smet (Imperial College Medical School at St Mary's) and was used with permission from Dr. Douglas Young (Imperial College Medical School at St Mary's)

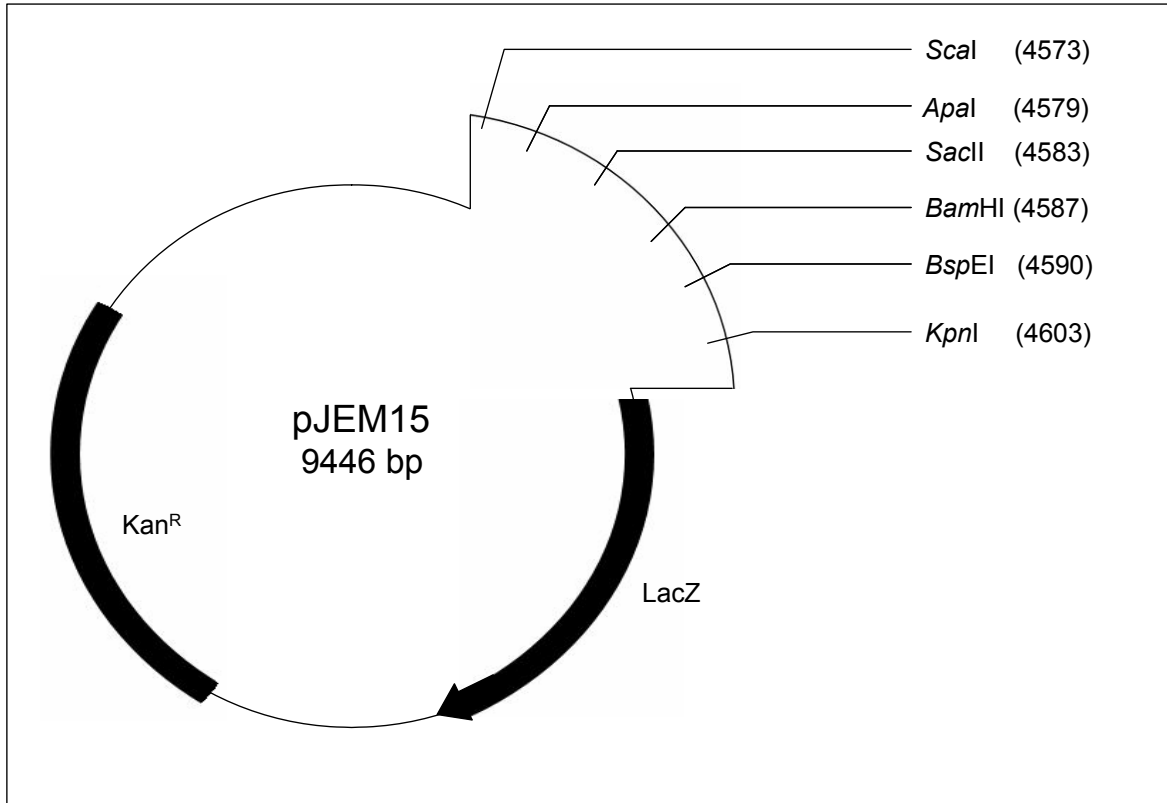
C.2) pACE acetamidase operon promoter plasmid



The pACE acetamidase operon promoter plasmid was used for the concentration dependant acetamide-inducible expression of anti-sense mRNA molecules in mycobacterial hosts such as *M. smegmatis* and *M. bovis* BCG. The origin of replication (ALori) is derived from the *M. fortuitum* pAL5000 plasmid and is recognised by both *E. coli* and *mycobacteria*, enabling the amplification of vector constructs in fast-growing *E. coli*. The pACE acetamidase operon promoter plasmid carries a hygromycin B resistance (Hyg^R) selection marker. Unique RE-recognition sites (only *Bam*HI and *Cla*I) located in the multiple cloning site together with their plasmid coordinates are shown.

The pACE acetamidase operon promoter plasmid was constructed by Dr. Koen de Smet (Imperial College Medical School at St Mary's) and was used with permission from Dr. Douglas Young (Imperial College Medical School at St Mary's)

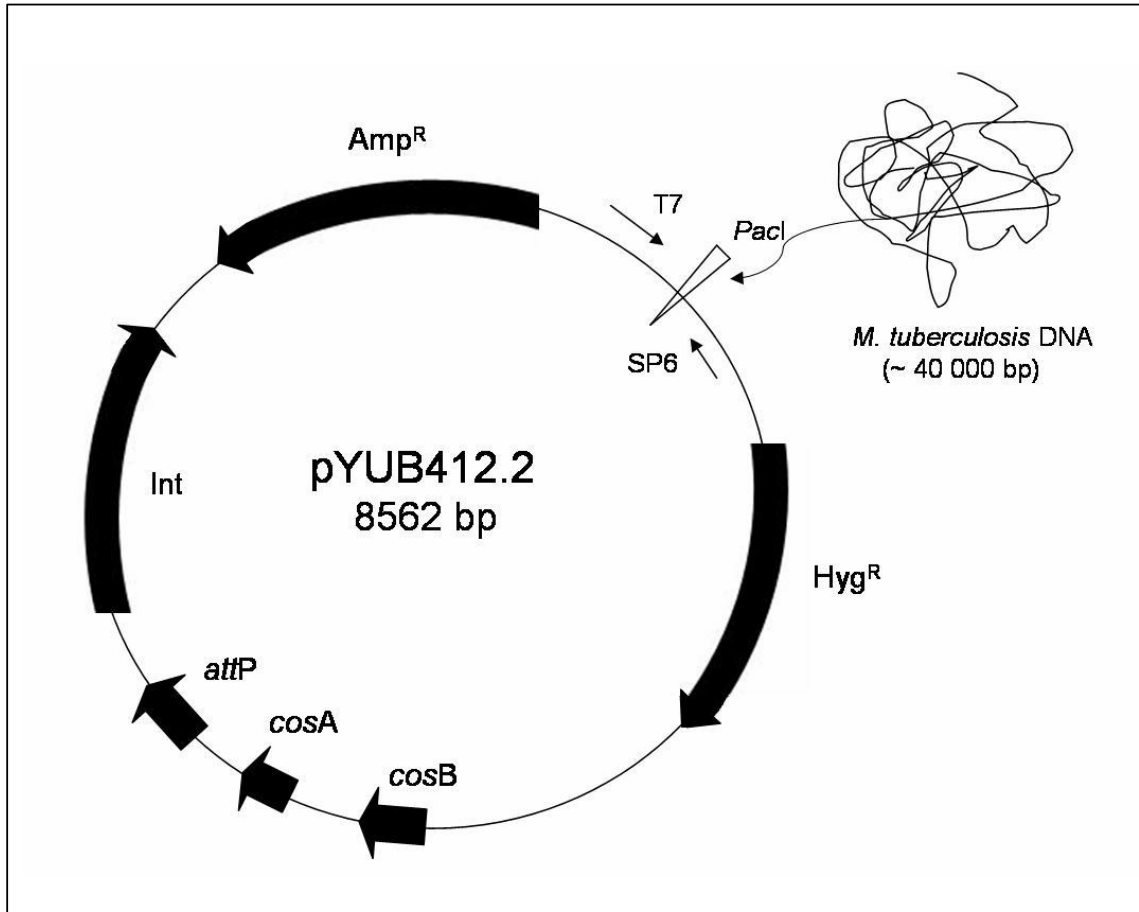
C.3) pJEM 15 promoter probe plasmid



The pJEM 15 promoter probe plasmid was used for the relative quantification of promoter activity in *M. smegmatis* as a mycobacterial expression host. The genetic region that was investigated for promoter activity was cloned directly upstream of a functional (but promoter less) *lacZ* gene. Existence of promoter activity in the cloned insert resulted in *lacZ* production, of which the relative activity could be determined by β -galactosidase activity assay. The pJEM 15 promoter probe plasmid carries a kanamycin resistance (Kan^R) selection marker. Unique RE-recognition sites located in the multiple cloning site together with their plasmid coordinates are shown.

The pJEM 15 promoter probe plasmid was obtained from, and used with permission from Dr. Brigitte Gicquel (Institute Pasteur, Paris, France)

C.4) pYUB412.2 *M. tuberculosis* cosmid library vector construct



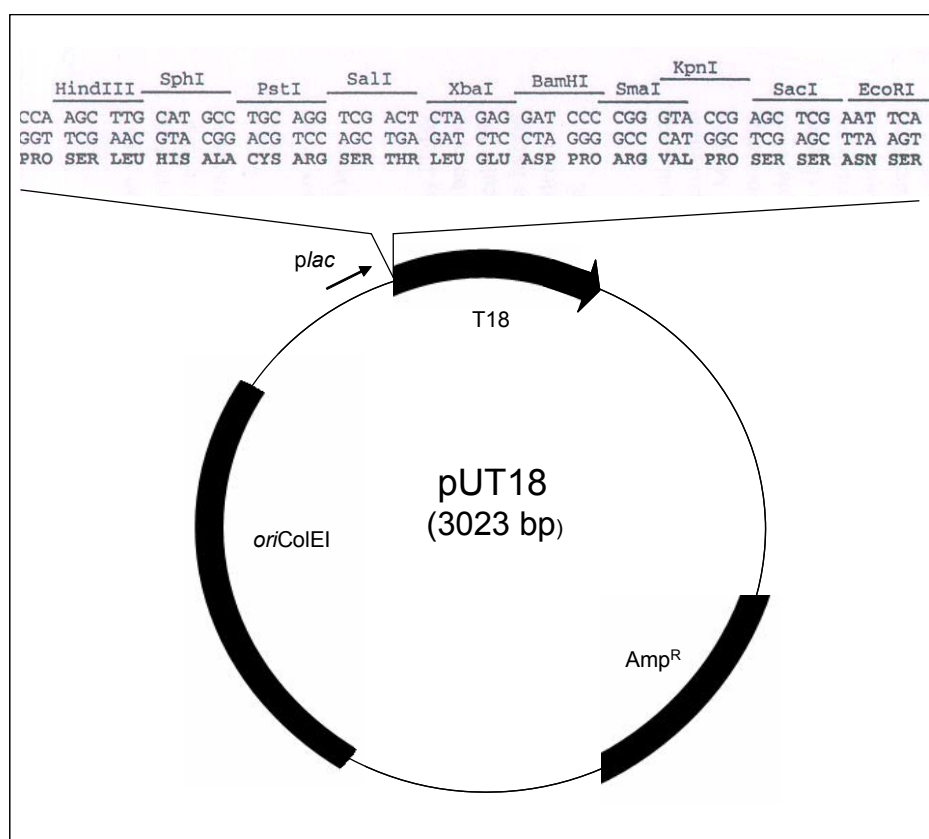
The pYUB412.2 *PacI*-excisable cosmid cloning vector was used for the permanent integration of *M. tuberculosis* genomic DNA into *M. smegmatis*. The *M. tuberculosis* genomic library was constructed by cloning *Sau3A* genomic DNA fragments (approximately 40 000 kb) into the *PacI* site of the pYUB412.2 vector. This vector construct cannot replicate in mycobacteria (i.e. a suicide plasmid), but has the origin of replication for *E. coli* (*oriE*, not shown). The vector confers resistance to ampicillin (Amp^R) in *E. coli* and hygromycin B (Hyg^R) in mycobacteria. The cosmid vector has the mycobacteriophage L5 attachment site (*attP*), the L5 integrase gene (*int*). This vector efficiently integrates into the mycobacteriophage L5 attachment site (*attB*) of the mycobacterial chromosome and is stable (Lee *et al.*, 1991).

The *M. tuberculosis* H37Rv genomic DNA integrating cosmid library (Bange *et al.*, 1999) was a gift from Dr. F.C. Bange (Medizinische Hochschule, Hannover, Germany).

C.5) Bacterial-2-hybrid cloning plasmids

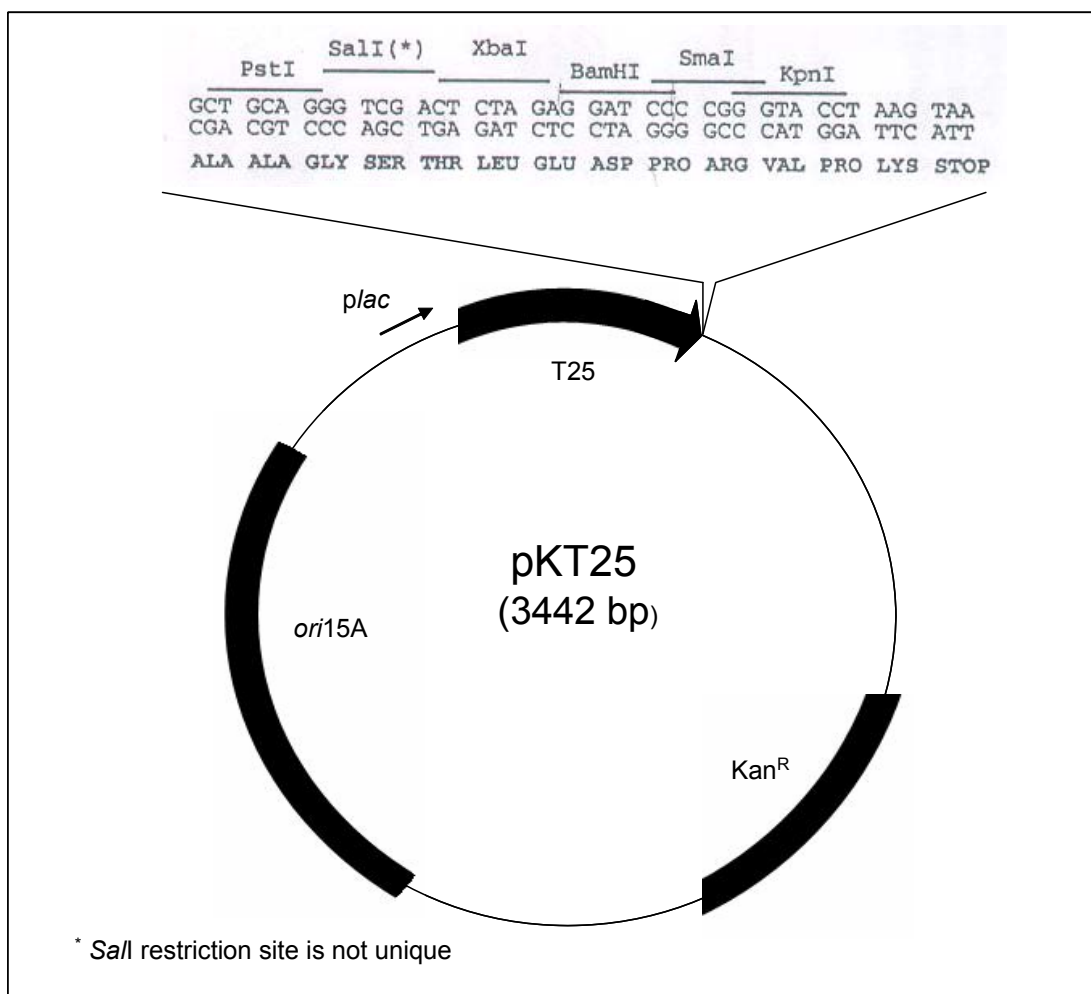
The bacterial-2-hybrid system is based on the cAMP signalling cascade and both the plasmids described below (C.5.1 and C.5.2) are required in a double electro-transformation of an adenylate cyclase deficient *E. coli* DMH1 strain. Material transfer agreements (MTA) were signed between Dr. Ian Wiid (Stellenbosch University) and Dr. Daniel Ladant (Pasteur Institute, Paris, France) for the use of this system.

C.5.1) pUT18 C-terminus fusion plasmid



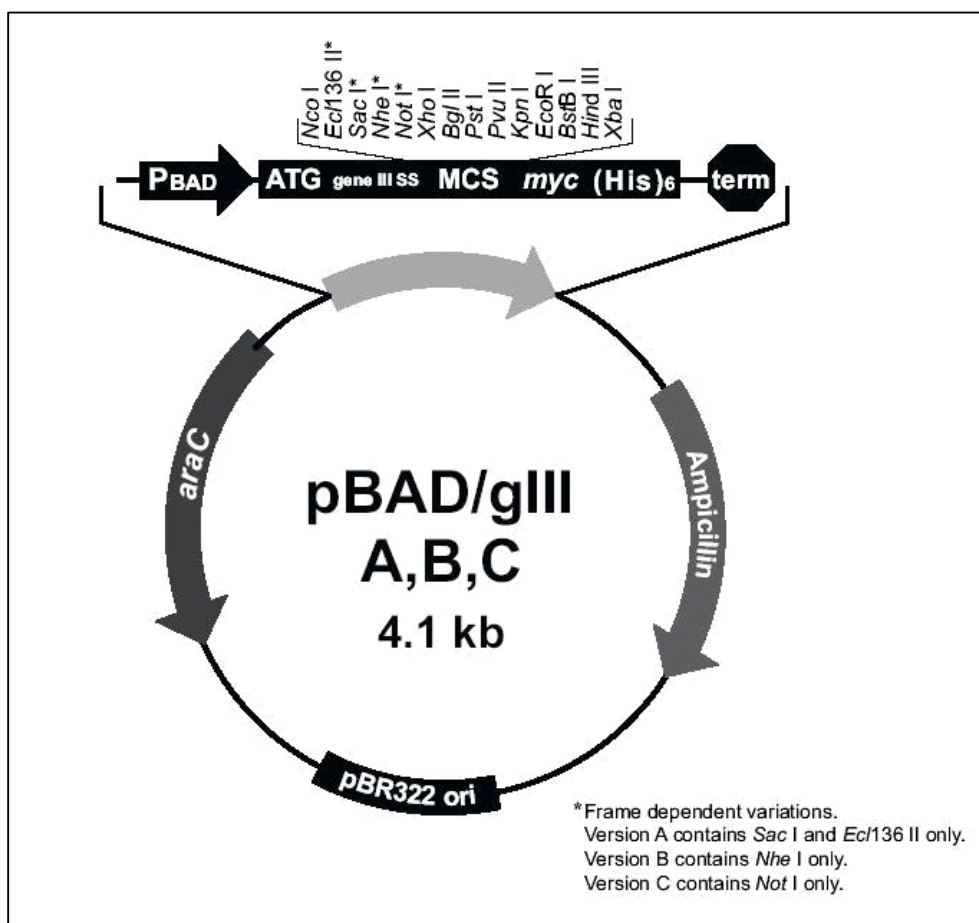
The pUT18 cloning plasmid was used to create recombinant *M. tuberculosis* proteins fused at the C-terminal with the T18 sub-unit of adenylate cyclase (amino acids 225 – 399). The T18 open reading frame is situated downstream of the multiple cloning site containing 9 unique RE-recognition sites. To create successful C-terminal fusion proteins, the insert had to be cloned without a stop-codon and in-frame with the 5' – end of the T18 open reading frame. Expression of the fusion-T18 construct is driven by the *lac* promoter (*plac*). pUT18 is a high copy number plasmid (*oriColEI*) with an ampicillin resistance selection marker (*Amp^R*).

C.5.2) pKT25 N-terminus fusion plasmid



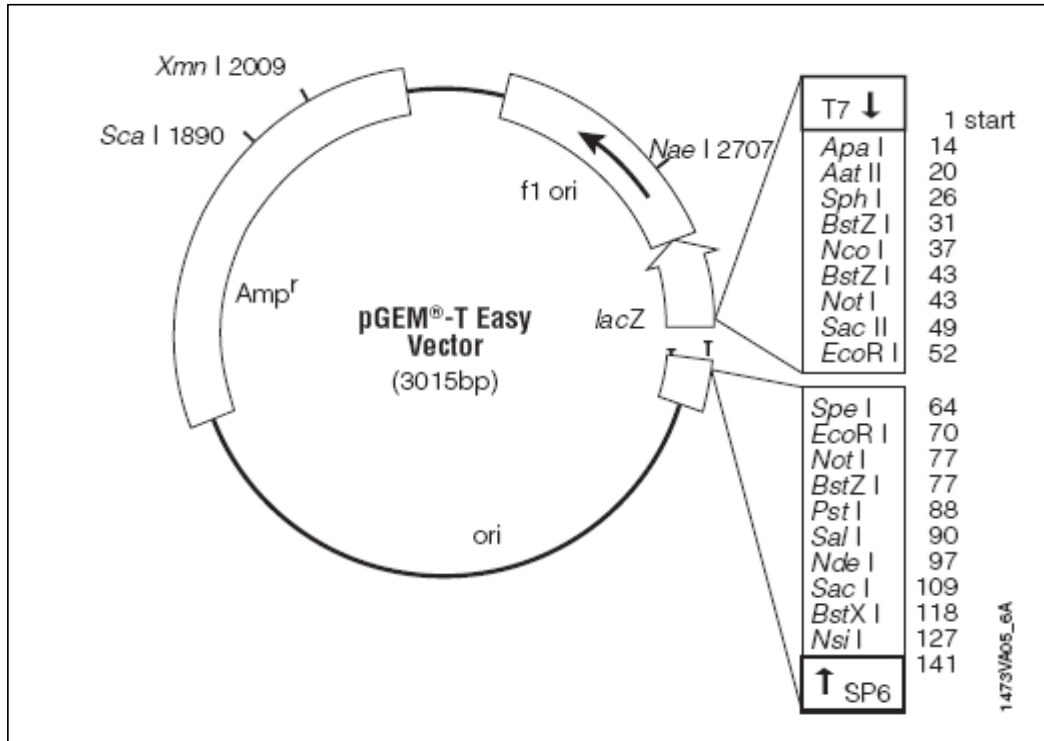
The pKT25 cloning plasmid was used to create recombinant *M. tuberculosis* proteins fused at the N-terminal with the T25 sub-unit of adenylate cyclase (amino acids 1 - 224). The multiple-cloning site is situated at the 3'-end of the T25 open reading frame and contains 5 unique RE-recognition sites (the *Sali* RE-site is not unique). Expression of the T25-fusion construct is driven by the *lac* promoter (*plac*). To create successful N-terminal fusion proteins, the insert had to be cloned in-frame with the 3'-end of the T25 open reading frame. pKT25 is a low copy number plasmid (*ori15A*) with an kanamycin resistance selection marker (Kan^R).

C.6) pBAD/gIII C-terminus hexa-histidine fusion vectors (Invitrogen)



The pBAD/gIII, pBR322-derived cloning vector was used to express recombinant *M. tuberculosis* proteins as a C-terminus hexa-histidine fusion protein in an *E. coli* expression host. Expression of the fusion-protein is under the control of a arabinose-inducible promoter (P_{BAD} or *araBAD*). Also, the vector has an intrinsic transcription initiation codon (ATG), as well as a *rmB* transcription termination region (term). The vector contains a ampicillin resistance selection marker and also the open reading frame of *araC*, which encodes a regulatory protein responsible for the tight regulation of the P_{BAD} promoter (Lee *et al.*, 1980; Lee *et al.*, 1992).

C.7) pGEMt-Easy (Promega)



The high-copy number pGEM-T Easy plasmid was used for the direct cloning of PCR products synthesised by DNA-polymerases without proofreading activity. The PCR product synthesised by such a DNA polymerase is amplified with 3' deoxyadenine overhangs (DNA template-independent), which could ligate to the 3' Thymine overhangs (T) of the pGEM-T plasmid. pGEM[®]-T Easy Vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the peptide coding region of the enzyme β -galactosidase. Insertional inactivation of the β -galactosidase peptide allows recombinant clones to be directly identified by colour screening on X-Gal containing indicator plates (Materials and Methods 1.3). The pGEM-T Easy also contains an ampicillin resistance (Amp^R) marker.

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