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Structure-Function Studies of a Putative Ribonuclease HI from *Mycobacterium tuberculosis*.

Michelle Lesley Thomsen

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science.

March 1999

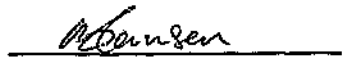
ABSTRACT

Bacterial Ribonuclease HI, which ensures that initiation of DNA replication occurs at the unique *oriC* locus, is encoded by *rnhA*. The *rnhA* gene from *Mycobacterium smegmatis* encodes a protein that is closely related to other bacterial RNases HI (Dawes *et al.*, 1995). Activity gel analysis detected RNase HI activity associated with proteins in whole-cell extracts of *Mycobacterium tuberculosis* in the 14-25 kDa size range. A putative *rnhA* homologue was identified in *M. tuberculosis* and sequence analysis revealed that the *rnhA* open reading frame contains an apparent fusion of two genes (Cole *et al.*, 1998). The 5'-region of the ORF corresponds to an *rnhA* homologue, whereas the 3'-region contains a gene, annotated herein as *pgm*, which encodes a protein belonging to the phosphoglycerate mutase (PGM) family of proteins. The full-length ORF, as well as the individual *rnhA* and *pgm* segments, were cloned into the pMAL-c2 expression vector and recombinant proteins were overexpresssed in *E. coli* as maltose binding fusion proteins. Recombinant proteins were purified and rabbit polyclonal antisera raised against each one were used to probe whole cell extracts of *M. tuberculosis*. Cross-reaction with polypeptides of unknown identity was observed. Limited proteolysis of the recombinant proteins suggest an instability of folding in *E. coli*. Functional investigation of the *M. tuberculosis* RNase HI included complementation of an *E. coli* RNase HI-defective mutant, and an *M. smegmatis* strain carrying a defective *rnhA* allele integrated at its *rnhA* locus, with *M. tuberculosis rhnA-pgm* supplied *in trans*. No complementation in either hosts was observed. Upon completion of the genome sequence of H37Rv (Cole *et al.*, 1998), it became apparent that the *rnhA-pgm* ORF is the fourth gene in an operon which includes a gene known to be involved in cobalamin biosynthesis. Significant homlogy of the PGM to CobC phosphatase of *Salmonella typhimurium*

implicates a role for *rmlA pgm* in the cobalamin biosynthetic pathway of *M. tuberculosis*.

DECLARATION

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



16·03·1999

Michelle Lesley Thomsen

Date

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ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Professor Valerie Mizrahi, for her support, guidance, direction, and time, which was readily put aside for discussion throughout this project.

I would like to thank Dr. W.R. Jacobs, Jr. of the Howard Hughs Medical Institute, U.S.A., for providing the pYUB328::H37Rv library that was used for this research.

Thank you to all my friends and co-workers in the Molecular Biology Unit for their assistance, advice, motivation and encouragement. Special thanks to Dr. Stephanie Dawes and Helena Boshoff for their availability, and willingness to assist.

I would like to thank my mom for her endless support and inspiration. Her gift to me has been a priceless one.

I would like to thank David for his continual encouragement and patience, and for being so supportive.

Thanks to Kerstein who, even across the miles, has always been there for me in so many ways.

I acknowledge and greatly appreciate the financial assistance given to me by The University of the Witwatersrand, The Foundation for Research and Development, and the South African Institute for Medical Research.

TABLE OF CONTENTS

TITLE PAGE

ABSTRACT i

DECLARATION iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS v

LIST OF FIGURES ix

LIST OF TABLES xi

1. INTRODUCTION 1

1.1 THE IMPACT OF TUBERCULOSIS 1

Current treatment and prevention 2

New approaches 2

1.2 THE BIOLOGY OF MYCOBACTERIA 5

Pathogenesis, immunity and dormancy 6

Growth rate regulation and chromosomal DNA replication 8

1.3 RIBONUCLEASES H 11

Prokaryotic, retroviral and eukaryotic RNases H 12

RNase HI of *E. coli* 13

Cellular roles of *E. coli* RNase HI 14

Genetic context of *E. coli rnhA* 15

E. coli rnhA mutants 15

Mycobacterial RNases HI 16

1.5 AIMS OF THIS STUDY 18

2. MATERIALS AND METHODS 20

2.1 CHEMICAL REAGENTS 20

2.2 BACTERIAL STRAINS AND GROWTH CONDITIONS 21

2.3 BACTERIAL TRANSFORMATIONS 24

Chemical transformation of *E. coli* strains 24

Transformation by Electroporation 25

2.4 PREPARATION OF MYCOBACTERIAL CELL LYSATES 26

2.5 DNA MANIPULATIONS 27

DNA extractions 27

Polymerase Chain Reaction (PCR) amplification of DNA 30

Restriction enzyme digests 31

Blunt Ending 31

DNA electrophoresis 32

Gel purification of DNA fragments 32

Ligations 32

DNA sequencing 33

Southern blot analysis 33

2.6 INDUCTION OF GENE EXPRESSION 35

MBP:: fusions 35

His tag:: fusions 35

2.7 PURIFICATION OF MBP FUSION PROTEINS 36

2.8 PROTEIN ELECTROPHORESIS 36

2.9 PRODUCTION OF POLYCLONAL ANTIBODIES 37

2.10 PROTEOLYSIS OF RECOMBINANT PROTEINS 37

2.11 WESTERN BLOT ANALYSIS 38

3. RESULTS 40

3.1 CONSTRUCTION OF EXPRESSION CASSETTES 40

a) Expression vector 40

b) Construction of RNase HI-PGM expression cassette 43

c) Construction of the PGM expression cassette 46

d) Construction of the RNase HI expression cassette 48

3.2 INDUCTION OF GENE EXPRESSION 51

3.3 PROTEIN PURIFICATION BY AFFINITY CHROMATOGRAPHY 52

3.4 PRODUCTION OF POLYCLONAL ANTIBODIES 53

3.5 PROTEIN CHARACTERISATION BY PROTEOLYTIC CLEAVAGE 53

(a) Factor Xa 53

(b) Limited proteolysis with trypsin 55

3.6 WESTERN BLOT ANALYSIS 56

(a) Western blot analysis of purified proteins 56

(b) Western blot analysis of mycobacterial cell lysates 58

3.7 ALTERNATIVE EXPRESSION STRATEGY 60

3.8 GENETIC COMPLIMENTATION EXPERIMENTS 62

(a) Complementation of *E. coli rnhA*- mutants 62

(b) Complementation of *M. smegmatis* 63

4. DISCUSSION 72

4.1 BIOCHEMICAL ANALYSIS 72

Protease treatment 73

Polyclonal antibodies and Western blot analysis 73

4.2 FUNCTIONAL ANALYSIS 75

4.3 FUTURE RESEARCH PROSPECTS 85

5. APPENDICES 86

APPENDIX I: Media and general solutions 86

APPENDIX II: DNA markers 89

APPENDIX III: List of abbreviations 90

6. REFERENCES 92

LIST OF FIGURES

Fig 1. Restriction map of *rnhA-pgm* 17

Fig 2. RNase HI multiple sequence alignment 19

Fig 3. Restriction map of pMAL-c2 41

Fig 4. Schematic diagram of *rnhA-pgm* ORF 41

Fig 5. DNA and protein sequence of *rnhA-pgm* ORF 42

Fig 6. Construction of the pMBP-RP expression cassette. 43

Fig 7. Restriction analysis of pMBP-RP 44

Fig 8. Confirmatory restriction digests and Southern blot of pMBP-RP 45

Fig 9. Sequence of the junction sites of pMBP-RP 45

Fig 10. Construction of the pMBP-P expression cassette 46

Fig 11. Restriction analysis and Southern blot of pMBP-P 47

Fig 12. Sequence of the junction sites of pMBP-P 48

Fig 13. Construction of the pMBP-R expression cassette 49

Fig 14. Restriction analysis and Southern blot of pMBP-R 50

Fig 15. Sequence of the junction sites of pMBP-R 50

Fig 16. Induction of gene expression 51

Fig 17. Purified recombinant proteins 52

Fig 18. Schematic diagram of MBP fusion proteins 54

Fig 19. Factor Xa cleavage of recombinant proteins 55

Fig 20. Proteolytic cleavage with trypsin 56

Fig 21. Western blot analysis of purified recombinant proteins 57

Fig 22. Western blot of mycobacterial cell extracts (anti-MBP antisera, anti-MBP. RNase HI-PGM antisera) 59

Fig 23. Western blot of mycobacterial cell extracts (anti-MBP::PGM antisera) ...59

Fig 24. Western blot of mycobacterial cell extracts

(anti-MBP::RNase HI antisera) 60

Fig 25. Genotype of mc^{2}155::pUSHδB SCO 64

Fig 26. Types of DCO events 64

Fig 27. Restriction maps of pRCX-R1 and pRCX-R4 65

Fig 28. Restriction analysis of pRCX-R1 and pRCX-R4 66

Fig 29. Flow diagram of the procedure used for complementation of

M. smegmatis. 67

Fig30. Southern blot analysis of mc^{2}155::pUSHδB(pRCX-R4) 68

Fig 31. Southern blot analysis of mc^{2}155::pUSHδB(pRCX-R1) 69

Fig 32. Western blot of WT and SCO transformants 70

Fig 33. CobC operon in *M. tuberculosis* and *M. leprae* 77

Fig 34. CobC substrate: α-Ribazole-P 81

Fig 35. CobC Alignment 83

LIST OF TABLES

Table 1 : Bacterial strains 22

Table 2 : Antibiotic solutions 23

Table 3 : Plasmids used in this study 28

Table 4 : Synthetic oligodeoxyribonucleotides 30

Table 5 : Southern blot wash procedures 34

Table 6 : Western blot analysis of mycobacterial cell extracts 58

Table 7 : Results of DCO events 66

Table 8 : Types of RNases H in bacteria, eukarya, and archaea 79

1. INTRODUCTION

1.1 THE IMPACT OF TUBERCULOSIS.

Mycobacterium tuberculosis (*M. tuberculosis)* is responsible for tuberculosis, a disease that has never ceased to be a global health problem (Bloom and Murray 1994). It is by far the most infectious disease worldwide, killing more people each year (3 million) than any other caused by a single infectious agent (Deshpande *et al.*, 1997; Lowrie *et al.*, 1997). It has been estimated that the number of people who develop active tuberculosis each year is likely to rise from 7.5 million in 1990, to over 10 million by the year 2 000 (Dolin *et al.*, 1994; Snider *et al.*, 1994).

The current global re-emergence of tuberculosis can be attributed to several factors. Firstly, the HIV-AIDS epidemic is having a major impact on global tuberculosis. In 1990, 4.2% of all tuberculosis cases were associated with HIV, and it is predicted that in the year 2000, an estimated 13.8% of all tuberculosis cases maybe associated with HIV (Dolin *et al.*, 1994). Because of its ability to destroy the immune system, HIV has emerged as the most significant risk factor for the progression of dormant tuberculosis infection to clinical disease, particularly in developing countries. Secondly, due to social dislocations, poverty, overcrowding, failure to invest public health infrastructure, failure of drug compliance and thus ineffective treatment, tuberculosis is a major health problem in developing countries (Snider *et al.*, 1994). The third confounding factor is the emergence of multidrug-resistant (MDR) tubercle bacilli. MDR tuberculosis is emerging as a major infectious disease problem throughout the world. It is defined as a case of tuberculosis caused by a strain of *M. tuberculosis* that is resistant to two or more antituberculosis drugs and arises under the

selective pressure of inadequate chemotherapy as a direct result of inappropriately administered therapy and poor compliance by patients (Riley, 1993).

Current treatment and prevention. Initial treatment of active tuberculosis currently involves administration of three of the first line drugs, rifampicin, isoniazid, pyrazinamide and ethambutol (or streptomycin). While the bacterial load is high, new cases should receive at least three drugs. It is then necessary to continue treatment with two drugs, usually rifampicin and isoniazid to ensure th[illegible]acteria are eliminated and the risk of relapse is minimized. The duration of treatment is from six to nine months. The principle underlying the current strategy for tuberculosis treatment is to reduce the likelihood of having spontaneous resistance developing, the selective proliferation of these organisms, and their subsequent transmission (Taylor, 1997). The intrinsic resistance of *M. tuberculosis* to antibiotics is due mainly to the highly hydrophobic cell envelope acting as a permeability barrier (Brennan and Draper, 1994). However, many potential resistance determinants are encoded in the genome and *M. tuberculosis* has also developed resistance to drugs by accumulating point mutations (Lety *et al.*, 1997; Scorpio *et al.*, 1997; Meier *et al.*, 1994; Morris *et al.*, 1995; Nash and Inderlied, 1995; Alangaden *et al.*, 1995). The live attenuated bacillus Calmette-Guérin (BCG) vaccine for the prevention of disease associated with *M. tuberculosis* was derived from the closely related virulent tubercle bacillus, *M. bovis* (Mahairas *et al.*, 1996). The efficacy of BCG remains unknown, yet it is currently administered to over 100 million children each year (Gulera *et al.*, 1996).

New approaches. One of the goals of research in the field of mycobacteriology is development of new methods that will improve and expedite the diagnosis, prevention and treatment of tuberculosis. New drug therapies are under investigation (Young and Duncan,

1995) and approaches to generating these include, for example, investigating an existing drug, which may be chemically modified to improve its anti-mycobacterial activity (Brogden *et al.*, 1994). Alternatively, new drugs can be developed from existing lead molecules. In this area fluoroquinolones and quinolones show promising anti-tuberculosis activity (Yew *et al.*, 1994; Cambau *et al.*, 1994). A third strategy involves the discovery of completely new molecules, such as enzyme inhibitors, either by random screening or by rational design. Two compound classes with promising *in vitro* and *in vivo* activity against *M. tuberculosis* are the oxazolidinones, and nitroimidazoles (Ashtekar *et al.*, 1991). There is, however, a demand for the identification of new drug targets in the mycobacterial cell for more effective chemotherapeutic intervention (Zurenko *et al.*, 1996; Efferen, 1997).

With respect to preventive measures, renewed attention has been focused on the search for effective vaccines. A clear strategy for mycobacterial vaccine development would be to start with a virulent isolate of *M. tuberculosis* and to generate a series of attenuated mutants with defects in genes required for intracellular survival and virulence. Many attempts are being made to identify targets for attenuation, with the hope of producing a rationally attenuated vaccine (Gupta and Tyagi, 1993; Jacobs and Bloom, 1994; Plum and Clark-Curtis, 1994). Development of efficient mutagenesis protocols is an essential element of vaccine strategies based on rational attenuation. Transposon mutagenesis, which has often been used for the identification of bacterial virulence factors, is one of the most powerful methods of mutagenesis. It involves using a mobile element to disrupt genes randomly in the chromosome upon transposition. Another method for creating mutants is by allelic exchange mutagenesis, where, by homologous recombination, the functional allele is replaced with an inactivated copy. Allelic exchange mutagenesis is a more targeted form of mutagenesis, as opposed to the random approach of transposon mutagenesis. Initially, attempts at

mutagenesis of *M. tuberculosis* were limited, in part, because of the inefficiency of the delivery systems. First attempts involved using a conventional strategy of employing a suicide delivery vector, where low frequency allelic exchange was demonstrated in the *M. tuberculosis* complex (Reyrat *et al.*, 1995; Azad *et al.*, 1996). Subsequently, a system enabling the positive selection of insertional mutants having lost the delivery vector was developed. This strategy employs the ts-*sacB* vectors, which combine the counterselective properties of the *sacB* gene and a mycobacterial thermosensitive origin of replication, and can therefore be efficiently counterselected on sucrose at 39°C (Pelicic *et al.*, 1997). The application of a ts-*sacB* vector has led to targeted allele replacement in *M. tuberculosis* (Pelicic *et al.*, 1997) and allowed the construction of *M. tuberculosis* transposition mutant libraries (Pelicic *et al.*, 1997). Development of conditionally replicating shuttle plasmids from the mycobacteriophages D29 and TM4 has also enabled efficient delivery of transposons into both fast- and slow-growing mycobacteria (Bardarov *et al.*, 1997). Infection of mycobacteria at the nonpermissive temperature results in highly efficient transposon delivery to the entire population of mycobacterial cells. As well as contributing to the construction of rationally attenuated vaccines, advancements in mutagenesis will open the way to studying the roles in pathogenicity of defined mycobacterial genes that may or may not present similarities to known virulence factors from other bacterial pathogens.

Considerable effort has also been invested over many decades in cataloguing mycobacterial components involved in immune responses, with the view to identification of candidates for subunit vaccines and for diagnostic kits (Andersen and Brennan, 1994; Ivanyi and Thole, 1994; Young *et al.*, 1992). The availability of cloned genes and suitable vectors has now opened a new avenue in which individual mycobacterial protein antigens are synthesized within transfected mammalian cells (Lowrie *et al.*, 1997). This is done by an alternative

vaccine strategy using DNA vaccines, which is still a fairly novel concept and in need of more thorough research with regards to protection against tuberculosis.

1.2 THE BIOLOGY OF MYCOBACTERIA

Mycobacteria are classified as Gram-positive bacteria, containing a high G+C content genome and a characteristic complex cell envelope. Like Gram-negative bacteria, mycobacteria form in their envelopes an impermeable outer layer that is distinct from the plasma membrane. The layers are, however, chemically quite different. That of Gram-negative bacteria is a free-standing bilayer of phospholipid and a characteristic lipopolysaccharide (Nikaido and Vaara, 1985), whereas the barrier in mycobacteria consists of a monolayer of very-long-chain fatty acids (mycolic acids) covalently linked to the remainder of the bacterial wall (Daffé *et al.*, 1998). The mycobacterial barrier certainly includes other lipids, and evidence that these are arranged to form a bilayer with the mycolic acids has been presented (Liu *et al.*, 1995).

Mycobacteria can be broadly classified into two groups on the basis of their growth rate. The slow-growing class includes pathogens such as *M. leprae*, *M. tuberculosis*, and *M. bovis*; while the fast-growing class includes environmental mycobacteria such as *M. phlei* and *M. smegmatis* (Clark-Curtiss, 1990), the latter of which, is used as a model organism in mycobacteriological research. The genus is a diverse one with mention here of only a few other commonly encountered organisms. More than 25 mycobacterial species other than *M. tuberculosis* can be found in specimens from humans (Heifets and Good, 1994). Some species have a high likelihood of being human pathogens, while pathogenicity for humans for some is questionable. Bacteria within the *M. avium* complex are prominent in the environment and are a source of serious disseminated infections in patients with HIV. *M.*

avium is a slow-growing, saprophytic mycobacterium, and an important opportunistic pathogen in immunocompromised conditions, such as HIV infected individuals. (Ellner *et al.*, 1991) *M. marinum* is an animal and human pathogenic organism (Huminer *et al.*, 1986; Ramakrishnan *et al.*, 1997), and is typical for skin lesions (Heifets and Good, 1994), while *M. paratuberculosis* causes paratuberculosis which is a widespread mycobacteriosis of ruminants (Cocito *et al.*, 1994).

M. tuberculosis is a facultative intracellular parasite, found within the mononuclear phagocytic cells of the host, with its characteristic high (65.6%) G + C content genome of 4.4 x 10^6 base pairs (Cole *et al.*, 1998). It has a relatively long mean generation time of 24 h and a chromosomal replication time period of 11 h (Hiriyana 1986), which may contribute to the chronic nature of the disease, imposes lengthy treatment regimens and represents a formidable barrier to research. Infections are initiated in the lung where the alveolar macrophages make initial contact with inhaled bacteria (Stokes *et al.*, 1993). Understanding the mechanisms of pathogenicity and identifying virulence factors of *M. tuberculosis* will assist in identifying novel drugs to counter resistance, and hopefully shorten the time course of treatment. In the case of *M. tuberculosis*, immunological, pathogenic, and cell-biological studies have revealed considerable information about the processes of infection and disease.

Pathogenesis, immunity and dormancy. The success *of M. tuberculosis* as a pathogen is dependent to a large extent on its ability to adapt to a wide range of conditions both within and outside the human host. Tubercle bacilli can survive intracellularly within macrophages, extracellularly in caseous tissues or liquefied cavities, and outside the human host in aerosols and in the soil. Central to understanding the pathogenesis of tuberculosis is the host-parasite relationship. Elucidating the uptake of the bacteria by the macrophage and

determining how the bacteria are able to survive and multiply within the macrophage are important areas of research in this field. The surface molecules of mononuclear phagocytes are receptors for a variety of pathogen-specific surface-ligand structures. It has been shown that complement receptor types 1,3 and 4 (CR1, CR3, and CR4) may mediate phagocytosis of *M. tuberculosis* organisms opsonized with complement component C3 (Schlesinger *et al.*, 1990; Hirsch *et al.*, 1994; Schorey *et al.*, 1997). Mannose receptors on human monocytes may also be important in uptake of virulent *M. tuberculosis* (Schlesinger, 1993).

A significant attribute of a pathogen with an intracellular lifestyle is its avoidance of, or resistance to the noxious environment of the secondary lysosome, a highly acidic compartment filled with hydrolases. *M. tuberculosis*, whose infectious cycle depends on entry and multiplication within mononuclear phagocytic cells, appears to prevent fusion of the mycobacterial phagosome with compartments containing lysosomal enzymes (Armstrong and Hart, 1971;1975). Phagosomes containing mycobacteria fail to acidify (Crowle *et al.*, 1991) at least in part because of removal or lack of recruitment of the proton ATPase (Sturgill-Koszycki *et al.*, 1994). The immune response to mycobacterial infection includes pathogenic as well as protective activities. It is possible that different types of immune responses are associated with recognition of different antigenic determinants (Ivanyi and Thole, 1994). Acquired resistance against tuberculosis rests on cell-mediated immunity, with the major factors being mononuclear phagocytes and the T lymphocytes. However, even in the face of coordinated T-cell-macrophage interactions, full eradication of the pathogen is frequently not achieved, so that the individual remains infected without developing active disease (Chan *et al.*, 1994). *M. tuberculosis* can persist within the human host for years without causing disease, in a syndrome known as latent tuberculosis (Parrish *et al.*, 1998). The state of dormancy of *M. tuberculosis* is an intriguing one. The bacillus

remains quiescent within the infected tissue, which may reflect metabolic shutdown due to a cell-mediated immune response that can contain, but not eradicate the infection. The dormant bacteria reactivate at the onset of immune suppression causing outbreak of disease sometimes many years after the initial infection (Chan *et al.*, 1994). Understanding its ability to remain quiescent for long periods of time and its re-emergence from this state is an area of active research in the field of *M. tuberculosis*.

Growth rate regulation and chromosomal DNA replication. As mentioned before, the vast majority of pathogenic strains of the Mycobacteria are slow-growing, while the fast-growing strains are mostly non-pathogenic, saprophytic organisms. However, even the fast-growing mycobacteria have doubling times that are six times slower than that of *E. coli*. The loose correlation that exists between pathogenicity and growth rate begs the question as to whether maintenance of slow growth is associated, in some way, with pathogensis (Young *et al.* 1990). Although the major factor limiting the growth rate of mycobacteria appears to be the rate of chromosomal DNA replication (Wheeler and Ratelege, 1994) the molecular basis of this effect is presently obscure.

DNA replication and cell division have been shown to be linked in many prokaryotes through the characterisation of DNA replication mutants. In *E. coli* chromosomal replication commences with the binding of the initiator protein, DnaA, to the origin of replication, *oriC*. (Kornberg and Baker, 1992; Skarstad and Boye, 1994; Messer and Weigel, 1996). The progression of steps in the initiation process involves the following:

1. The cooperative binding of approximately 20 DnaA protein molecules to *oriC* to form an initial complex.
2. A transition from the initial complex to an open complex in which a region of AT-rich 13-mer repeats denatures so that single-stranded (ss) DNA is exposed.

3. The formation of a prepriming complex upon the introduction of DnaB helicase into the forks of the melted DNA where it further expands the region of ssDNA.
4. Introduction of DnaG primase and subsequent formation of a mobile complex and sythesis of RNA primers.
5. The formation of a so-called "sliding clamp" onto the primed DNA, by loading of a homodimer of the β subunit of DNA polymerase (Pol) III, with the aid of the δ complex (a subassembly of Pol III) (Kelmann and O'Donnell, 1995; Herendeen and Kelly, 1996; Baker and Bell, 1998).
6. Synthesis of complementary DNA upon complete assembly of Pol III holoenzyme.

It has been indicated that the DnaA protein acts as an important molecular switch for initiation of chromosomal replication in *E.coli* (Katayama *et al.*, 1998).

Very little is known about the proteins involved in DNA replication in mycobacteria, and only one conditional-lethal mycobacterial DNA replication mutant has been described to date (Klann *et al.*, 1998). It is likely that the physiological processes such as DNA replication, transcription, and cell division are regulated differently in these unusually slow-growing microorganisms and the molecular mechanisms that govern the mycobacterial growth patterns are now the focus of several lines of investigation. DNA polymerase I (Huberts and Mizrahi, 1995), topoisomerase I (Yang, 1996), DnaA (Rajagopalan *et al.*, 1995), and *gyrA* and *gyrB* (Takiff *et al.*, 1994; Madhusudan *et al.*, 1994), from *M. tuberculosis* have been cloned and sequenced. Amongst some of the mycobacterial enzymes that have been characterised there are some differences relative to other bacterial species. Ribonucleotide reductase (RR), which catalyses the formation of dNTPs, and might therefore be rate-limiting in DNA replication has been purified from *M. tuberculosis*. This enzyme is encoded by homologues of the *nrdEF* genes of *E. coli*. *M. tuberculosis* is the only

bacterial species identified so far, in which class I RR subunits are not arranged in an operon (Yang *et al.*, 1997). The *dnaG*-encoded primase is an essential enzyme that recognizes specific nucleotide sequences and then synthesizes small primer RNAs that act as a substrate for the initiation of replication. In *E. coli* the *dnaG* gene has been shown to lie in the macromolecular synthesis operon (Lupski and Godson, 1984) which includes genes involved in regulating the initiation of synthesis of major informational macromolecules. The gene organisation has been shown to be strongly conserved in at least six diverse bacterial species (Versalovic *et al.*, 1993; Versalovic and Lupski, 1993) and was thought to possibly be universally conserved (Lupski and Godson, 1984). However, the genetic organisation at the *dnaG* locus, which is apparently conserved in slow- and fast-growing mycobacteria, is different from that observed in other bacteria, and this suggests that the mycobacteria exhibit a significant departure from the way other bacteria coordinate the synthesis of their macromolecules. However, characterisation of conditionally lethal *dnaG* mutants of *M. smegmatis* confirmed that there is linkage between DNA replication and cell division in this organism as observed in other prokaryotes (Klann *et al.*, 1998).

The origins of replication of *M. tuberculosis*, *M. smegmatis*, and *M. leprae* have been analysed and shown to contain a typical gram-positive pattern of genes, *rnpA-rnpH-dnaA-dnaN-recF-gyrB-gyrA* (Salazar *et al.*, 1996). The *oriC* region of *M. smegmatis* has been cloned and characterised (Rajagopalan *et al.*, 1995; Ming-Hui *et al.*, 1997) and the overall sequence organization, i.e., the size of *oriC*, total number of the AT-rich clusters, and the number and orientation of the putative DnaA boxes is different from that in *E. coli*. These differences tend to suggest the precise mechanism of replication initiation and possibly its regulation could be different from that reported for *E. coli* (Ming-Hui *et al.*,1997).

In light of this correlation that may exist between growth rate and pathogenicity, it was decided to study the RNase HI protein of *M. tuberculosis*. RNase HI is the specificity protein that ensures specificity of initiation of chromosomal replication at the *oriC* by preventing non-specific initiation at loci other than *oriC*, denoted *oriK*.

1.3 RIBONUCLEASES H

The RNase H family of enzymes endonucleolytically hydrolyses only the RNA strand of an RNA-DNA hybrid in a divalent metal-dependent fashion, to produce short oligonucleotides with 5'-phosphate and 3'-hydroxyl groups (Kanaya and Ikehara, 1993). There is strong conservation of key amino acid residues in prokaryotic and eukaryotic RNases HI and in the retroviral RNases (Itaya *et al.*, 1991; Mizrahi and Itaya, 1998; Wintersberger, 1990). This suggests a high degree of functional similarity in these enzymes at the level of overall three-dimensional structure, substrate binding and catalytic mechanism. RNase H activity has been shown to be associated with at least three RNases. RNases HI, encoded by *rnhA*, appears to constitute the primary RNase H function in *E. coli*. A second RNase H, designated RNase HII and encoded by the *rnhB* gene, was isolated from *E. coli* and found to have only 17% homology with *rnhA* in the first 155 aa (Itaya, 1990). RNase HII has been proposed to be universally present in various organisms (Ohtani *et al.*, 1999). Recently, a third enzyme was characterised in *B. subtilis* (Ohtani et al., 1999). It was designated RNase HIII (encoded by *rnhC*), and is believed to substitute for RNase HI function in organisms which lack a functional *rnhA*. This classifies the RNases H into three families. RNase HI activity is functionally redundant in *E. coli* as demonstrated by DNA polymerase I (PolI) and exonuclease III (ExoIII), which in addition to other activities, both show RNase HI activity (Mizrahi and Itaya, 1998).

Prokaryotic, retroviral, and eukaryotic RNases H. From studies using *E. coli rnhA* mutants, genes encoding RNase HI homologues from *Saccharomyces cerevisiae* (Cerritelli and Crouch, 1995), *Salmonela typhimurium*, and *Thermus thermophilus* HB8 (Itaya *et al.*, 1991) have been identified. A single RNase H enzyme was detected in extracts of *Streptococcus pneumoniae* and was designated RNase HII on the basis of sequence homology of three conserved motifs within the *E. coli rnhB* sequence (Zhang *et al.*, 1997). *Mycoplasma genitalium* and *Methanococcus jannaschii* both lack RNase HI (Fraser *et al.*, 1995; Bult *et al.*, 1996) suggesting its function is carried out by alternative enzymes. Three bacterial RNases H have been isolated and characterised from the Gram-positive bacteria *Bacillus subtilis*. The *rnhA* homologue (*ypdQ*) appears to have no RNase HI activity, while the other two are functional (Ohtani *et al.*, 1999). It seems that multiple RNase H-encoding genes per genome is a general feature of both eukaryotes and prokaryotes (Itaya, 1990).

Retroviral RNase H has been suggested to be responsible for the degradation of genomic viral RNA in the DNA-RNA hybrid so that minus-strand DNA is available as a template for synthesis of plus-strand DNA (Varmus, 1988). HIV-Reverse transcriptase (HIV-RT) is a multifunctional protein, containing both DNA polymerase and RNase H activity. The RNase H domain is a single 15kDa C-terminal domain, that has high sequence homology with the *E. coli* RNase HI, although when expressed independently is completely inactive (Keck and Marqusee, 1995). The HIV-RT has Mn^{2+}- dependent RNase H activity, and it is missing helix C and the following basic loop. This basic helix/loop is believed to be required for substrate recognition (Keck and Marqusee, 1995). Two RNases H of mammalian tissue have been described, RNase HI and RNase HII. RNase HI is the major mammalian enzyme representing around 85% of the total RNase H activity in the cell. The deduced sequence of the large subunit of human RNase HI displays significant homology to RNase HII from *E.*

coli, suggesting an evolutionary link between the mammalian RNases HI and prokaryote RNases HII (Frank *et al.*, 1998).

RNase HI of *E. coli*. The RNase HI enzyme of *E. coli* has been extensively studied and characterised and is used as a prototypical molecular model of the RNase HI enzyme (Carl, 1980; Kanaya *et al.*, 1990; Katayanagi *et al.*, 1990). Extensive knowledge of *E. coli* RNase HI includes its three dimensional structure, determined by x-ray chrystallography at 1.48Å resolution (Katayanagi *et al.*, 1992) and which amino acids are involved in the catalytic and substrate binding sites. It is a single-domain protein that contains no disulfide bonds (Dabora and Marqusee, 1994). The model for the enzyme-substrate complex proposes that a DNA-RNA hybrid binds to a groove-like depression by formation of hydrogen bonds between polar groups within the enzyme and 2'-OH groups of the RNA strand, thereby presenting the RNA to the catalytic site of the enzyme (Kanaya *et al.*, 1991). For the hydrolysis of the P-O3' bond of RNA by the enzyme, two alternative mechanisms have been proposed. One is a carboxylate-hydroxyl relay mechanism (Kanaya *et al.*, 1990), which is similar to that proposed for the DNase I activity (Suck and Oefner, 1986). The other is a two-metal ion mechanism (Yang *et al.*, 1990), which is identical with that proposed for the 3'-5' exonuclease activity of *E. coli* DNA polymerase I (Beese and Steitz, 1991). RNase HI seems to be similar to DNase I in that both enzymes act on double-stranded polynucleotides without much specificity for base sequence and both require metal cations for catalytic reactions (Suck and Oefner, 1986). The catalytic triad of RNase HI consists of Asp^{10}, Glu^{48}, Asp^{70}, with four important residues surrounding the catalytic site including, Ser^{71}, His^{124}, Asp^{130}, Asp^{134}. The $Mg2^{+}$- binding site, essential for activity, is located near this cluster of four acidic residues (Katayanagi *et al.*, 1990). Conservation of the residues surrounding the

catalytic site suggests a high degree of conservation at the secondary structure and mechanistic levels (Mirahi and Itaya, 1998).

Cellular roles of eubacterial RNase HI. From work with *rnh* (RNase H- defective) mutants of *E. coli*, the following physiological functions of RNase H have become evident.

1. Initiation of Chromosomal replication at *oriC*. In *E. coli* initiation of chromosomal replication occurs at a specific locus; the *oriC*. Mutants defective in RNase HI function have been shown to initiate replication from a number of other sites on the chromosome, denoted *oriK* (Asai and Kogoma, 1994). It has been demonstrated that there are at least four different alternative initiation sites (origins), from which constitutive stable DNA replication (cSDR) originates in *rnh* mutant cells (von Meyenburg *et al.*, 1987). This alternative chromosomal replication pathway which operates in the absence of RNase H, is independent of the two main elements of normal replication initiation, the DnaA protein and the *oriC* (von Meyenburg *et al.* 1987). It does however, require the recombinase activity of RecA (Kogoma *et al.* 1985). RNase HI appears to prevent non-specific initiation of chromosomal replication at loci other than *oriC* by destabilising R-loops (von Meyenburg *et al.*, 1987).

2. Removal of RNA primers from Okazaki fragments during lagging strand synthesis. DNA polymerases require a free 3'-hydroxyl group to extend a DNA chain, so initiation of replication requires the use of RNA primers. These RNA primers are important in initiating Okazaki fragments for lagging strand synthesis. The RNase HI enzyme performs subsequent removal of these RNA primers (Ogawa and Okazaki, 1984).

3. Initiation of replication of Col E1-type plasmids (Itoh and Tomizawa, 1980; Selezer and Tomizawa, 1982). In this plasmid, the precursor form of the RNA primer is transcribed by RNA polymerase from a site 550 bp upstream of the replication origin. This transcript, after forming a hybrid with DNA at the origin site, is subjected to degradation by RNase HI and

the resultant RNA acts as an active RNA primer for DNA synthesis by DNA polymerase I (Nishitani *et al.*, 1993).

Genetic context of *E. coli rnhA*. Gene cloning and DNA sequence analysis has revealed that the *rnhA* gene of *E. coli* maps in the immediate vicinity of the *dnaQ* gene encoding the ε-subunit of DNA polymerase III, and that the two genes are separated by only 64 bp and are oriented in opposite directions. The genes are divergently transcribed, and the significance of this in the regulation of gene expression is not yet understood (Quinones *et al.*, 1987). In *S. typhimurium*, as in *E. coli*, the *dnaQ* (*mutD*) gene is adjacent to the *rnhA* gene (Maki *et al.*, 1983; Cox and Horner, 1986), and the structural relationship between the two genes is the same in both species (Itaya *et al.*, 1991). When the SOS regulon is expressed constitutively, transcription from the *rnh* gene decreases to approximately 20% of the initial level (Quinones *et al.*, 1987).

***E. coli rnhA* mutants** As mentioned previously, RNase HI-defective *E. coli* cells exhibit cSDR. In the absence of normal replication, cSDR allows the cells to grow at a 30 to 40% reduced growth rate and with a twofold-decreased DNA content (von Meyenburg *et al.*, 1987). Stable DNA replication (iSDR) can be induced in rnh^+ cells after SOS-inducing treatments (Kogoma *et al.*, 1975; 1979). *rnhA* mutants also constitutively express the SOS response due to persisting ssDNA arising as DNA is displaced by the formation of R-loops (Kogoma *et al.*, 1993). Some *E. coli rnhA* defective strains have shown a lethal phenotype, when combined with other disrupted genes for example, a non-functional RecG helicase and defective RNase HI. Lethality here is probably due to persisting R-loops (Hong *et al.*, 1995). An *rnhA* mutation in conjunction with *recB* or *recC* mutation is lethal (Kanaya and Crouch, 1984; Itaya and Crouch, 1991a). This is due to a defect in the repair function of these

enzymes. *recB*270(Ts), or *recC*271(Ts), in conjunction with *rnhA*-339::*cat* renders *E. coli* temperature sensitive for growth (Itaya and Crouch, 1991b). In the present study, the *rnhA*-339::*cat*-recC271(Ts) was employed to investigate complementation by *M. tuberculosis* RNase HI. A combination of an *rnhA* mutation and either *polA* (Kogoma *et al.*, 1993; Hong *et al.*, 1995), or *topA* (Drolet *et al.*, 1995) also present lethal phenotypes. It is noted that defects in DNA gyrase can correct several phenotypes associated with a mutation in the *rnhA* gene (Drolet *et al.*, 1995).

Mycobacterial RNases HI. The *M. smegmatis* RNase HI shares 50% amino acid identity with the *E. coli* RNase HI and contains the three invariant acidic residues comprising the catalytic triad, and the four invariant residues surrounding the active site. It is closely related to other bacterial RNases HI at the level of protein structure and function. The chromosomal context of the *M. smegmatis rnhA* gene differs from its Gram-negative counterpart implying that the function that may be served by coordinate regulation of the *dnaQ-rnhA* transcriptional unit in Gram-negative bacteria has no parallel in mycobacteria. RNase H activity was biochemically detected by activity gel analysis in *M. tuberculosis* at approximately 17 kDa and was thus assigned as an RNase HI. However, gene cloning attempts failed to identify an *rnhA* homologue in this organism, suggesting a significant degree of sequence divergence of RNase HI within the genus Mycobacteria (Dawes *et al.*, 1995).

A putative *rnhA* homologue was identified in *M. tuberculosis* by genome sequencing (Cole *et al.*, 1998). The *M. tuberculosis* open reading frame (ORF), Rv2228c, encodes a hypothetical protein, 364 amino acids (aa) in length, which can be separated into two distinct segments on the basis of sequence alignments. The N-terminal amino acid residues 1-133

encode the putative RNase HI, while the C-terminal residues 163-364 encode a polypeptide exhibiting strong homology to proteins of the phosphoglycerate mutase (PGM) family. Homology based analysis showed that the PGM domain is of expected length (*ca.* 200 aa) and contains residues known to be essential for catalysis. The *rnhA* and *pgm* sequences are separated by an intervening spacer region of 80-85 bp with the lack of apparent stop and start codons. The *rnhA-pgm* ORF is illustrated in Figure 1. An analogous *rnhA-pgm* ORF (371 aa) was also identified in *M. leprae*. (EMBL accession number L78818) with the only significant difference being an additional seven residues between the domains in the *M. leprae* ORF.

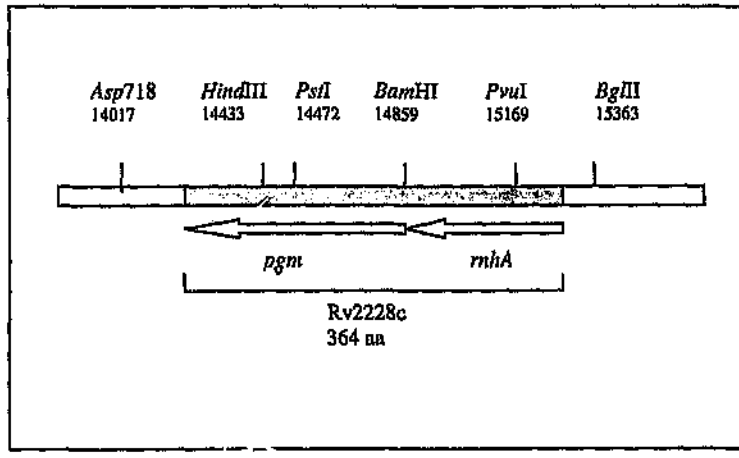


Figure 1: Restriction map of the region encoding *rnhA-pgm* (Cole *et al.*, 1998). Numbering corresponds to that of Genbank accession number Z70692 (cosmid Y427).

The N-terminal domain has been assigned as a putative RNase HI on the basis of its significant sequence homology to the RNases HI from *Bacillus subtilis* (Genbank accession number L77246) and *Enterococcus faecalis* (annotated *ebsB*; Bensing and Dunny, 1993). These RNase HI have no basic helix-loop hinge region thus are relatively short in length,

134-135 aa respectively, relative to 150-158 aa of other bacterial RNases HI (Stahl *et al.*, 1994; Keck and Marqusee, 1995). In this respect the Gram-positive subfamily resembles the retroviral RNases H (Stahl *et al.*, 1994). All the catalytically important residues are present, except for the counterpart of His ^{124} of *E. coli* RNase HI, which is substituted by a tryptophan. There is speculation that the Gram-positive RNase HI might retain Mn^{2+}-dependent activity.

Neither N- nor C- terminal fusions have been previously identified in RNases HI from eubacteria. However, eukaryotic RNases H of known sequence are composed of an RNase H domain similar in size and sequence to that of *E. coli* RNase HI and additional domains of unknown function. The RNase HI of *Saccharomyces cerevisiae* has such an RNase H domain at its C-terminus (Cerritelli and Crouch, 1995). RNases H from *Schizosaccharomyces pombe* and *Crithidia fasciculata* also contain this non-RNase H domain with a common motif (Ceritelli *et al.*, 1998). A sequence alignment of the *M. tuberculosis rnhA* sequence with other *rnhA* sequences is shown in Figure 2.

1.4 AIMS OF THIS STUDY

The functional implications of the conserved *rnhA-pgm* linkage observed in *M. tuberculosis* and *M. leprae* are unknown. Since this unexpected finding is potentially significant with respect to understanding the cellular role RNase HI might serve in the slow-growing mycobacteria, a study aimed at characterising the *rnhA-pgm* ORF from *M. tuberculosis* was undertaken. The specific objectives were (1) to clone, overexpress, and purify recombinant *M. tuberculosis rnhA-pgm*-encoded polypeptides in *E. coli.* (2) To raise polyclonal antibodies against the individual RNase HI and PGM domains for use in identifying these

polypeptides in *M. tuberculosis*. (3) To functionally characterise the *rnhA-pgm* by genetic complementation in *E. coli* and *M. smegmatis* host strains.



Figure 2: Multiple sequence alignment of the RNases HI of Gram-positive bacteria *M. tuberculosis* (MtrnhA; Cole *et al.*, 1998), *M. leprae* (MlrnhA; Genbank Acc. No. L78818), *Enterococcus faecalis* (EfrnhA; Bensing and Dunny, 1993), *Bacillus subtilis* (BsrnhA; Iwakura *et al.*, 1988), and *M. smegmatis* (MsrnhA; Dawes *et al.*, 1995), with those of the Gram- negative bacteria *Thermus thermophilus* (TtrnhA; Itaya and Kondo, 1991), *E. coli* (EcrnhA; Kanaya and Crouch, 1983), *Salmonella typhimurium* (StrnhA; Itaya *et al.*, 1991), and *Buchnera aphidicola* (BarnhA; Munson *et al.*, 1993),

2. MATERIALS AND METHODS

2.1 CHEMICAL REAGENTS

All reagents were analytical grade or higher. Restriction endonucleases and restriction enzyme buffers, agarase, DNase I, Klenow enzyme, RNase A, calf intestinal phosphatase, Taq DNA polymerase, T4 polymerase, bovine serum albumin (BSA), fish sperm DNA, dithiothreitol (DTT), isopropyl-α-D-galactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-α-D-thiogalactopyranoside (X-gal), DNA molecular weight markers, deoxyribonucleotide triphosphates (dNTP's), PVDF membrane, trypsin, and antibiotics chloramphenicol, hygromycin B, kanamycin, and ampicillin were from Boehringer Mannheim, Mannheim, Germany. Ammonium acetate, calcium chloride, Coomassie brilliant blue, dimethylsulphoxide ($DMSO_4$), isoamyl alcohol, dichlorodimethylsilane, glucose, glycerol, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES), magnesium chloride, phenol, polyethyleneglycol (PEG) 6000, potassium acetate, potassium chloride, potassium dihydrogen phosphate, sodium acetate, sodium dihydrogen phosphate, and 1,1,1-trichloroethane were from Merck, Darmstadt, Germany. Amberlite resin beads, ethylenediaminetetraacetic acid (EDTA), maltose, polyoxyethylenesorbitan monooleate (Tween 80), Ponceau S, Sephadex G-25, rubidium chloride, Tris-borate-EDTA (TBE) buffer, Trizma base, and urea were obtained from Sigma, St Louis, MO, USA. Dimethyl formamide, ethanol, formamide, β-mercaptoethanol, sodium citrate, and Triton X-100 were from BDH, Poole, UK. Bromophenol blue was obtained from Gurr Ltd., London, UK. Chloroform was obtained from Holpro Lavasz, Midrand, South Africa. Hybond-N nylon filters, [α-^{35}S] deoxyadenosine triphosphate (1 000 Ci/mmol), [α-^{32}P] deoxycytosine triphosphate (3 000 Ci/mmol) and some restriction

endonucleases with restriction enzyme buffers were from Amersham International., UK. Glasswool and Sodium dodecyl sulphate (SDS) were from Fluka Chemie AG, Buchs, Switzerland. 3M XDA Trimax X-ray film was obtained from X-ray Imaging Services, Rustenburg, South Africa. Filter paper and 30 000 NMWL Ultrafree MC filter units were obtained from Whatman International, Maidstone, UK. Gel drying paper was from Hoefer Scientific Instruments, San Francisco, CA, USA. Methanol was from SMM Chemicals, Alberton, South Africa. Sodium chloride was obtained from Associated Chemical Enterprises, Johannesburg, South Africa. Agar, bacto-agar, and Middlebrook 7H9 broth were from Difco, Detroit, MI, USA. Yeast extract and tryptone were obtained from Oxoid Ltd, Basingstoke, UK. N,N,N',N',-tetramethylethylenediamine (TEMED), ammonium persulphate, acrylamide, N,N'-methylene-bis-acrylamide (Bis), SDS-PAGE molecular weight standards, xylene cyanol, and Protein Assay Kit were from Bio-Rad, Hercules, CA, USA. Sodium hydroxide, isopropanol and hydrochloric acid were from Labchem, Boksburg, South Africa. SeaKem®LE agarose and SeaPlaque®GTG® low melting agarose were obtained from FMC Bioproducts, Rockland, ME, USA. Microcentrifuge tubes were from Eppendorf, Hamburg, Germany. Amylose resin and Factor Xa was from New England Biolabs Inc., Beverly, CA. Sterivex-GS 0.22 µm filters were from Millipore S.A., Molsheim, France.

2.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains used in this study are shown in Table 1. Strains were stored in 50% glycerol at –70°C, and recovered on plates of the preferred medium using antibiotics at the concentrations shown in Table 2. Infectious supernatants of phage were stored at 4°C.

Table 1: Bacterial strains.

BACTERIAL STRAINS	GENOTYPE	REFERENCE OR SOURCE
	E. coli	
BL21(DE3)	*hsdS gal* (*λIts*857 *ind 1 sam7 nin5 lac UV5-t7* gene 1)	Studier and Moffatt (1986)
DH5α	*supE*44*ΔlacU*169(*Φ80 lacZΔM*15)*hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi-relA*1	Hanahan (1983)
JM101	*supE thiΔ*(*lac-proAB*)F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZΔM*15]	Yanisch-Perron *et al.* (1985)
TB1	*araΔ*(*lac proAB*) *rpsL* (*Φ80 lacZΔM*15) *hsdR*	Johnston *et al* (1986)
MIC3037	F *supE*44, *supF*58, *lacY1*or*Δ*(*laclZY*)6, *trpR*55, *galK*2, *galK*2, *galT*22, *metB*1, *hsdR*14 ($r^-_k m^+_k$), *rnhA*-339::*cat, recC*271	Itaya and Crouch (1991,b)
	M. smegmatis	
mc^2155	A highly transformable derivative of mc^26	Snapper *et al.* (1990)
mc^2155::pUSHδB	Derivative of mc^2155 carrying a copy of pUSHδB integrated at *rnhA* locus.	Dawes (1998)
	M.tuberculosis	
H37Rv	*M. tuberculosis* laboratory strain	TB Reference Laboratory SAIMR

E. coli strains were cultured in liquid media, with vigorous shaking (350-400 rpm) at 37°C with the respective antibiotic as required, under which conditions most *E. coli* strains reached mid-logarithmic growth stage (for *E. coli* OD_{600}≈0.4) in 2-3 h. Plates were incubated overnight at 37°C. DH5α, BL21(DE3) and TB1 cells were

Table 2: Antibiotic Solutions.

ANTIBIOTIC	STOCK SOLUTION		WORKING CONCENTRATION	
	CONCENTRATION	STO AGE	*E. COLI*	*M. SMEGMATIS*
Ampicillin	35 mg/ml in 50% ethanol	-20°C	100 µg/ml	
Chloramphenicol	35 mg/ml in 50% ethanol	-20°C	35 µg/ml	
Hygromycin	200 mg/ml (supplied in PBS)	4°C	200 µg/ml	50 µg/ml
Kanamycin	50 mg/ml in water	4°C	50 µg/ml	10 µg/ml

cultured in Luria-Bertani (LB) liquid media and plated on Luria-Bertani agar (LA) plates. For induction of gene expression TB1 cells were grown in LB containing glucose at 2 g/L. MIC3037 cells were grown in LB, and plated on LA with a modified NaCl content of 5 g/L. MIC3037 is a temperature sensitive strain, its permissive temperature being 30°C (Itaya and Crouch, 1991a,b) In order to test complementation, transformed cells were grown at 30°C and 42°C instead of 37°C. JM101 cells were cultured in minimal media.

M. smegmatis strains were grown in Middlebrook-Tween medium or LB (TWEEN 0.05% v/v) with gentle shaking at 100 rpm at 37°C under which conditions, mid-logarithmic growth (for *M. smegmatis* OD=0.8) was reached after *ca.* 18 h (Jacobs *et al.*, 1991). Plates were incubated for 3-5 d at 37°C. The *M. tuberculosis* strain was grown in Middlebrook 7H9 (TWEEN 0.05%) with OADC enrichment, at 37°C using magnetic stirring of 1 rpm for aeration, under which conditions, mid-logarithmic growth was reached after 10-14 d. Media compositions are given in Appendix 1.

2.3 BACTERIAL TRANSFORMATIONS

(a). Chemical Transformation of *E. coli* Strains. For the preparation of competent cells all techniques were carried out aseptically.

Rubidium Chloride Method. (This procedure was based on the method of Dr. P. Stolt, personal communication, and used for DH5α, TB1, BL21(DE3), MIC3037 *E. coli* strains.) A fresh overnight culture was used to inoculate 100 ml of LB (1:100) and was grown at 37°C in a shaking incubator until cells reached mid-logarithmic phase. Cells were transferred to 50 ml culture tubes, chilled on ice for 15 min, and were harvested at 4 000 xg for 15 min at 4°C. Each pellet was resuspended in 17 ml of solution RF1 (Appendix 1), stored on ice for 30 min, and harvested again at 4 000 xg for 15 min at 4°C. Each pellet was resuspended in 4 ml of solution RF2 (Appendix 1) and the cell suspension measured out in 400 µl aliquots. These aliquots were flash frozen and stored at –70°C and 200 µl was used per transformation.

For transformation of TB1, DH5α, MIC3037, BL21(DE3) strains, (Sambrook *et al.*, 1989) DNA was added to 200 µl of chemically competent cells and the mixture was kept on ice for 30 min. With the exception of temperature-sensitive MIC3037, all other *E. coli* strains were then heat shocked at 42°C for 90 s. MIC3037 underwent heat shock at 37°C for 5 min. In order to allow for phenotypic expression of the antibiotic resistance gene, 1 ml of LB was immediately added to the heat-shocked cells and the culture was incubated at 37°C for 1 h. The cells were then harvested at 10 000 xg for 2 min, resuspended in 50 µl of LB and plated on LA containing the appropriate antibiotic, and incubated at 37°C overnight or at 42°C for complementation of MIC3037.

For the preparation of competent JM101 cells and their subsequent transformation, a modified protocol was used as follows. A fresh overnight culture of JM101 was used to inoculate 50 ml of LB (1:100) and the culture grown in a shaking incubator at 37°C until mid-log phase. Cells were chilled on ice for 10 min, then harvested at 4000 xg for 10 min at 4°C. The pellet was resuspended in 10 ml of 0.1 M $MgCl_2$, stored on ice for 30 min, and centrifuged again at 4 000 xg for 10 min at 4°C. Pellets were resuspended in 1 ml of 0.1 M $CaCl_2$ and measured out in 400 µl aliquots. Cells were stored at 4°C for up to 1 week only and 50 µl was used per transformation. For transformations into JM101 cells, the replicative forms of M13 phage were added to 50 µl of freshly prepared $CaCl_2$ competent cells and the cells incubated on ice for 30-40 min. Cells were heat-shocked at 42°C for 90 s just prior to plating. Before plating, 3 ml aliquots of molten salt agar (0.9% NaCl/ 0.75% DIFCO agar) was kept at 56°C in a sterile culture tube. A 200 µl aliquot of JM101 cells at mid-logarithmic phase, 50 µl of 100 mM IPTG, 50 µl of X-gal (2% solution), and the transformation mix were added to the salt agar and plated onto a pre-warmed LA plate. Once the top agar was set, plates were incubated at 37°C overnight.

(b) Transformation by Electroporation. For the preparation of electrocompetent *M. smegmatis* (Jacobs *et al.*,1991), a fresh overnight culture was used to inoculate 100 ml of LB, which was incubated at 37°C until the culture reached mid-logarithmic phase. Cells were harvested at 5000 xg for 10 min, washed several times in 1% glycerol or sterile water with wash volumes of 50 ml, 25 ml, 10 ml, and 2 ml respectively. Final resuspension was in 400 µl and 80 µl of fresh electrocompetant cells was used for

each electroporation. Aliquots of 80 µl were flash frozen, and stored at -70°C, and washed with 1% glycerol when thawed out.

For the preparation of electrocompetent *E.coli* cells (as detailed in the Bio-Rad Gene Pulsar manual), 100 ml of LB was inoculated with a fresh overnight culture. This was grown, with shaking, at 37°C until mid-logarithmic growth phase. Cells were harvested at 5 000 xg for 10 min, and washed in 10% glycerol in the following wash volumes: 50 ml, 25 ml and 1 ml. Final resuspension was in 150 µl and 40 µl of fresh electrocompetent cells were used per electroporation, or electroduction. Aliquots of 40 µl were flash frozen and stored at –70°C.

A Bio-Rad Gene Pulser TM (Bio-Rad Laboratories, Hercules, CA) was used for electroporating competent cells and performing electroductions. Transformations with replicating plasmids required 10-20 ng of DNA. Immediately after electroporation, cells were recovered with 1 ml of LB and incubated at 37°C to allow for phenotypic expression of the antibiotic resistance gene, (3 h for *M. smegmatis* and 1 h for *E. coli*), after which cells were plated. For electroductions, a colony of *M. smegmatis*, containing the plasmid of interest, was mixed with electrocompetent *E. coli* cells and electroductions performed under *E. coli* electroporation conditions (Baulard *et al.*, 1992). The settings for electroporating DNA into mycobacteria were 1000Ω, 25uF, 2.5kV, while those for *E.coli* were 200Ω, 25uF, 2.5kV.

2.4 PREPARATION OF MYCOBACTERIAL CELL LYSATES

Mycobacteria, from 200 ml of culture in mid-logarithmic growth p... ...ere harvested at 8 000 xg for 10 minutes at 4°C. Cells were resuspended in 4 ml of PBS,

transferred into Eppendorf tubes, and following harvesting at 10 000 xg for 10 min, were washed again in PBS. The cells were finally concentrated into a total volume of 1.4 ml of PBS. A volume of 0.7 ml was transferred to the lysing matrix, for mechanical lysis in the BIO-101 Fast Prep 120 at a speed of 5.5 for 2 x 15 s. The cell extract was transferred to an Eppendorf tube, and debris removed by centrifugation at 10 000 xg for 15 min. Total protein content of the extract was quantified by the mini Bradford assay as supplied in Bio-Rad Protein Assay Kit, using BSA as a protein standard. Cell extracts were stored at –20°C.

2.5 DNA MANIPULATIONS

The plasmids used in this study are described in Table 3 and the synthetic oligonucleotides are shown in Table 4.

(a) DNA extractions

Plasmid isolation (Sambrook *et al.*, 1989). Rapid plasmid isolations were performed for routine minipreps of plasmid DNA. Cells were harvested at 10 000 xg for 5 min at 4°C from 1.5 ml of overnight culture (grown in LB). They were lysed and treated with 100 µl of Solution I (50 mM Tris-HCl, pH 8.0 / 10 mM EDTA), 200 µl of solution II (0.2 M NaOH /1% SDS), and 150 µl of solution III (3 M KOAc, pH 5.5). Bacterial debris was removed by centrifugation at 10 000 xg for 10 min, and the supernatant treated with RNase A, final concentration of 100 µg/ml. Incubation with RNase A was carried out at 42°C for 15 min. This was followed by one extraction with 400 µl of 1:1 phenol:chloroform and one extraction with 200 µl of chloroform. DNA was precipitated with 1 ml of 98% ethanol and pelleted by centrifugation at 10 000 xg for 25 min at 4°C. The pellet was washed with 1 ml of 70% ethanol, vacuum dried and resuspended in 10 µl water.

Table 3: Plasmids used in this study.

PLASMIDS	FEATURES	REFERENCE
pMAL-c2	*E. coli* expression vector, *malE lacZ bla*	New England Biolabs, Beverly, MA
pET-15b	*E. coli* expression vector, *bla*	Novagen, Madison, WI
pGEM3Zf(+)	*E. coli* cloning vector, pUC derivative, *bla,*	Promega, Madison, WI
pRCX3	Multicopy *E. coli-M. smegmatis* shuttle vector, *aph, xylE*	Curcic *et al.*, 1994
M13mp18/19 (RF)	*E.coli* M13 phage cloning vectors	Amersham, UK.
pADM8	Derivative of pGEM-3Zf(+) carrying 3.65 kb (*Xba*I-*Eco*RI) containing *rnhA-pgm* ORF, from cosmid MTCY427	A.De Meyer, unpublished
pADM7	Derivative of pGEM-3Zf(+) carrying 1.35 kb (*Asp*718-*Bgl*II) fragment containing *rnhA-pgm* ORF from cosmid MTCY427	A. De Meyer, unpublished
pSD1	3.6 kb *Pst*I fragment containing 350 bp of *rnhA* (*M.smegmatis*) and upstream sequence cloned into pGEM3Zf(+)	Dawes (1998)
pUSH	*sacB* cloned into pBluescript (Stratagene, La Jolla, CA), with *hyg*	Dawes (1998)
pPS	720 bp *Pst*I-*Sac*I fragment of pSD1 cloned in pGEM3Zf(+)	Dawes (1998)
pUSH-δP	720 bp *Eco*RI-*Hind*III fragment from pPS, with the *Bam*HI site blunt-ended and religated, cloned in the *Sma*I site of pUSH	Dawes (1998)
pMBP-R	432 bp *rnhA* PCR fragment cloned as a translational fusion with the *malE* in (*Xmn*I-*Hind*III)-digested pMAL-c2	This work
pMBP-P	678 bp *pmg* PCR fragment cloned as a translational fusion with the *malE* in (*Xmn*I-*Eco*RI)-digested pMAL-c2	This work
pMBP-RP	1113 bp *rnhA-pgm* PCR fragment cloned as a translational fusion with the *malE* in (*Xmn*I-*Eco*RI)-digested pMAL-c2	This work
pET-RNH	434 bp *rnhA* PCR fragment cloned in (*Nde*I-*Bam*HI)-digested pET-15b	This work
pRCX3-R1	*Dra*I-digested pRCX3, containing 3.65 kb (*Eco*RI-*Xba*I) fragment from pADM8	This work
pRCX3-R4	pRCX3 *Dra*I-digested, containing 3.65 kb (*Eco*RI-*Xba*I) fragment from pADM8, cloned in opposite orientation relative to pRCX-R1	This work
M13mp18/19 SB-475	M13mp18/19 carrying 475 bp *Sac*I-*Bam*HI fragment from pMBP-RP	This work
M13mp18/19 BE-685	M13mp18/19 carrying 685 bp *Bam*HI-*Eco*RI fragment from pMBP-RP	This work
M13mp18/19 SE-725	M13mp18/19 carrying 725 bp *Sac*I-*Eco*RI fragment from pMBP-P	This work
M13mp18/19 SH-478	M13mp18/19 carrying 478bp *Sac*I-*Hind*III fragment from pMBP-R	This work

For plasmid digests 1-2 µl of DNA was sufficient for screening purposes. Qiagen columns (Boehringer Mannheim, Germany) were used for large-scale purification of plasmid DNA.

Chromosomal DNA isolation from *M.smegmatis* (De Wit, 1990). Cells were harvested at 10 000 xg for 5 min at 4°C from 2 ml of culture grown to early stationary phase. Pellets were resuspended in 500 µl of TES (10 mM Tris-HCl pH 8.0 / 0.1 mM EDTA / 150 mM NaCl) buffer, and incubated at 70°C for 30 min. To this mixture, 90 µl of 10% SDS, and 600 µl of phenol was added and samples allowed to shake horizontally for 3 h at 37°C. Bacterial debris was removed by centrifugation at 10000 xg for 5 min at room temperature and the supernatant was extracted twice with 1 ml of chloroform:isoamylalcohol (24:1). DNA was precipitated with 1 ml of 98% ethanol and 0.1 volumes of 3 M NaOAc by centrifugation at 10 000 xg for 20 min at 4°C, washed with 1 ml of 70% ethanol and vacuum-dried. Chromosomal DNA was then gently resuspended in 100 µl water, and an equal volume of 25% PEG 6000 /2.5 M NaCl solution added. DNA was re-precipitated on ice for 10 min, pelleted by centrifuged at 10000 xg for 15min at 4°C, washed in 1 ml 70% ethanol, vacuum-dried, resuspended in 10 µl water and stored at –20°C.

Single stranded (ss) DNA isolation (Yanisch-Peron *et al.*, 1985). Plaques were picked off plates and resuspended in 2 ml of 2TY with JM101 cells (1:100 inoculation). The phage was grown up, shaking at 37°C, for approximately 5 h, for ssDNA isolation. Cells were harvested at 10 000 xg for 5 min, and the supernatant recovered. DNA was precipitated with 200 µl of 20% PEG 6000 / 2.5M NaCl at 10 000 xg for 20 min, resuspended in 100 µl of TE (10 mM Tris pH8.0; 1 mM EDTA)

buffer, with subsequent phenol:chloroform extraction, and standard ethanol precipitation. DNA was resuspended in 10 µl water and 1 µl was run on a gel to check for the presence of inserts while 4 µl was used for a sequencing reaction. Replicative form (RF) DNA of the phage was extracted from the cell pellet by the standard plasmid isolation protocol.

Table 4: Oligonucleotides used in this study.

NAME	SEQUENCE 5'-3'	FEATURES
TB-RNH F2	GTGAAAGTTGTCATCGAA	Start codon (bold) for *rnh-pgm*, sense (+) stand. Position: 15281- 15263 *
TB-RNHNDE-F1	CCCGCATATGAAAGTTGTC ATCGAAG	G/C clamp, followed by *Nde* I site, and start codon (bold), (+) strand. Position: 15288-15264
TB-PGM-F2	ATGGTTGCGACCGAGTC	Start codon for *pgm* (+) strand (bold). Position : 14846-14829
TB-RNH- R3	CAGACAGGCTATCTATAG ATGGCGTGCGAATTCCGGG	Stop codon (bold), *Eco*RI site, G/C clamp antisense (-) strand. Positions: 14202-14168
TB-RNH-R2	AATCGGCTGCGGCGGATT AGAAGCTTCGGG	Stop codon for *rnhA* domain (bold), *Hind*III site, G/C clamp (-) strand. Positions: 14879-14849
TB-RNHBAM-R	AATCGGCTGCGGCGTAGG CGGATCCCGGG	Stop codon (bold), and *Bam*HI site, G/C clamp (-) strand. Positions: 14878-14850

* Numbering according to Genbank accession number Z70692 (cosmid Y427).

(b) Polymerase Chain Reaction (PCR) amplification of DNA

PCR reactions (50 µl) containing about 10 ng of template plasmid DNA, 1 µM of each primer, (forward and reverse) 200 µM of dNTPs, 5 µl of 10 x Buffer (final concentration of $MgCl_2$: *ca.* 15 mM) and 0.5 U of Taq DNA polymerase were carried out in a Hybaid Omnigene Temperature Cycler. The PCR involved one cycle at 94°C

(5 min), followed by 30 cycles of 94°C x 1 min, 46°C x 1 min and 72°C x 1 min, and one final cycle of 72°C for 5 min. PCR products were polished with 1 U of Klenow enzyme in a $MgCl_2$-containing buffer. For each set of primers used for PCR, control reactions were performed. These included (i) a negative control, to which no template DNA was added, (ii) template DNA and the forward primer only, (iii) template DNA and the reverse primer only, and (iv) the positive control reaction, with both forward and reverse primers in the presence of template DNA.

(c) Restriction enzyme digests

Restriction enzyme digests of plasmid DNA were carried out in 10 µl or 20 µl volumes, at the enzyme's optimal temperature for 1 h, with the corresponding restriction enzyme buffer. If necessary, vectors were treated with calf intestinal alkaline phosphatase after digestion for 1 h at 37°C for removal of the 5'-phosphate residues. Chromosomal DNA was digested in 20-30 µl volumes at the optimal temperature for 3-16 h. Reaction mixtures were extracted once with 1:1 phenol: chloroform, and the DNA precipitated by the standard ethanol precipitation method described above, for further manipulation.

(d) Blunt-ending

For filling in of 5'-overhangs, DNA was treated with Klenow enzyme (1 U), in Klenow buffer and 200 µM of dNTP's for 1 h at 37°C. T4 DNA polymerase (1 U) was used, in its supplied buffer, with 200 µM of dNTP's for removal of 3'-phosphoryl groups. Incubation with T4 DNA polymerase was for 15 min at 37°C.

(e) DNA electrophoresis

Standard 0.8%-1.5% agarose gels were used for routine examination of DNA (Ausubel *et al.*, 1989). The concentration of ethidium bromide in the agarose gel was 0.3 μg/μl. DNA samples, re-suspended in water or TE were mixed with DNA loading dye (0.004% bromophenol blue (BPB), 5% glycerol) prior to loading and electrophoresis was carried out in TAE (0.04 M Tris-acetate/ 0.001 M EDTA) buffer.

(f) Gel purification of DNA fragments

DNA fragments were excised and purified from low melting agarose gels by treatment with agarase enzyme (Boerhinger Mannheim, Germany). The band of interest was excised and weighed, and 4 μl of 25 x concentrated agarose buffer was added per 100 mg of gel. The gel was melted at 65°C for 15min. Agarase enzyme was added (2 U per 100 mg of gel) and incubated at 45°C for 2 h. Samples were centrifuged at 10 000 xg for 15 min, the supernatant recovered and the DNA precipitated with 98% ethanol and 3 M NaOAc (pH5.5), washed with 70% ethanol, vacuum dried and resuspended in an appropriate amount of water for ligations.

(g) Ligations

The Fast-Link DNA ligation kit (Epicentre Technologies, Madison, WI) was used for all ligation reactions. Molar ratios of vector and insert were optimized and ligated according to the manufacturers' instructions. Ligations were carried out at room temperature for 1 h (cohesive-end) or 3 h (blunt-end ligations).

(h) DNA Sequencing (Sanger *et al.*, 1977).

DNA to be sequenced was subcloned into M13mp18 and M13mp19, and single stranded (ss) DNA was isolated for sequencing using the Sanger dideoxy-mediated chain termination method using a Sequenase Version 2.0 DNA sequencing kit (Amersham International, UK) with the M13 universal primer. Due to high G+C content of *M. tuberculosis* DNA, both dGTP and dITP reactions were performed for resolution of compressions. Reactions were terminated with denaturing loading dye (95% formamide/ 20 mM EDTA/ 0.025 % BPB/ 0.025 % xylene cyanol). Samples were loaded on a 6% denaturing PAG in TBE buffer (0.178 M Tris base/ 17.8 mM Boric acid/ 0.4 mM EDTA) and electrophoresis was carried out at 1.5-2 kV (*ca.* 1.5 h for a short run, and *ca.* 3.5 h for a long run). The gel was maintained at an optimal temperature of about 50°C throughout. Sequence analysis was carried out using the Lasergene Sequence Analysis Software (DNASTAR Inc., Madison, WI) and database searches were carried out using the BLAST family of algorithms resident on the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/) (Altschul, 1997).

(i)Southern Blot Analysis

Random-primed labeling of probe. DNA (resupended in water) was first denatured at 95°C for 10 min and snap-cooled on ice for 1 min. DNA was labeled with [α-^{32}P] dCTP, by Klenow enzyme, using the Random Primed Labeling Kit according to the manufacturers' protocol (Boehringer Mannheim, Germany). The reaction was incubated at 37°C for 30 min, and terminated by the addition of 180 µl of TE buffer. Unincorporated dNTPs were removed by spinning through a column of Sephadex G-25. The column was prepared with 1 ml of G-25 Sephadex, which was poured into a 1 ml syringe, plugged with silanized glass wool. The column was equilibrated with 200

µl of TE buffer. This volume was retrieved by centrifugation of the column at 4 000 rpm for 3 min in an IEC clinical centrifuge. The labeled reaction was then loaded onto the column and centrifuged as above, with the labeled DNA collected in the flow-through, and stored at –20°C until used.

Transfer. Following agarose gel electrophoresis, DNA was depurinated in 0.25 M of HCl, denatured in 0.5 M NaOH /1.5 M NaCl and then electrophoretically tranfered onto Hybond-N membrane, in TBE buffer, at 0.5 A for 2h. DNA was cross-linked onto the membrane by UV-irradiation using a Stratagene UV cross-linker.

Hybridisation. Membranes were pre-hybridised in 10ml hybridisation buffer (0.5% SDS / 6x SSC / 5x Denhardt's / 50% deionised formamide), with 10 ug/ml of heat-denatured fish sperm DNA, at 42°C for 2 h. Denatured, labeled probe was then added and the membrane hybridised overnight (≈17 hours) at 42°C, and then subsequently washed in SSC/SDS buffers, with increasing stringencies as described in Table 5. The blot was then exposed onto X-ray film and developed.

TABLE 5: Southern blot wash procedures.

SOLUTION COMPOSITION	TIME	REPEATS	TEMP.
2 x SSC/ 0.1% SDS	15 min	x2	42°C
0.5 x SSC/ 0.1% SDS	15 min	x1	42°C
0.1 x SSC/ 0.1% SDS	15 min	x1	42°C
0.1 x SSC/ 1.0% SDS	30 min	x1	65°C

2.6 INDUCTION OF GENE EXPRESSION

(a) MBP fusion proteins. (NEB, Beverly, MA.) MBP recombinant proteins were induced in *E. coli* TB1 cells. For small-scale analysis of induction, 20 ml of rich broth and ampicillin were inoculated with 200 µl of a fresh overnight culture. An aliquot (1 ml) was extracted at mid-logarithmic phase, denoted as time point zero, and harvested at 10 000 xg for 5 min at 4°C. IPTG, to a final concentration of 0.3 mM, was added to the remaining culture to induce expression, and 1 ml aliquots of cells were extracted at 30 min, 90 min, 120 min, and 180 min after its addition. Cells were harvested as before, and all pellets resuspended in 1X SDS PAGE sample loading buffer (t_0:20 µl, t_{60}: 30 µl, t_{90}:50 µl, t_{120}: 80 µl, t_{180}: 100 µl). A 10 µl aliquot of each sample was directly loaded onto the SDS-PAG for electrophoresis.

For large scale production of proteins, 100 ml of culture was induced with IPTG and cells were harvested, in 50 ml aliquots, at 4 000 xg for 20 min at 4°C. Each pellet was resuspended in 5 ml of column buffer (10 mM Tris-HCl / 200 mM NaCl / 1 mM EDTA/ 1 mM DTT) and frozen overnight at –20°C. Once freeze-thawed, cells were lysed by sonication (Power 14 for 15 s pulses, for 3,5 –4,0 min) and samples were centrifuged at 10 000 xg for 30 min at 4°C. The supernatant (crude extract) was decanted and made up to a total volume of 45 ml with column buffer, in order to dilute the protein, for affinity chromatography.

(b) His –tag fusion proteins (Novagen, Madison, WI.). Induction of pET-15b clones was carried out in *E. coli* BL21 (DE3) cells. Small-scale inductions were carried out as described above for the pMAL-c2 constructs, although cultures were induced with IPTG at a final concentration of 1 mM.

2.7 PURIFICATION OF MBP FUSION PROTEINS

MBP fusion proteins were purified by one-step affinity chromatography over an amylose column according to the manufacturers' instructions (NEB, Beverly, MA.). The amylose resin was poured into a 50 ml syringe, plugged with silanized glass wool, washed and loaded with the crude extract. MBP fusion proteins were eluted with column buffer and 10 mM maltose, and collected in 3 ml fractions. Peak protein-containing fractions, monitored by OD_{280}, were pooled. Proteins were concentrated by centrifugation over Ultrafree ML filters (Millipore) and stored either in column buffer, or an enzyme storage buffer at –20°C. Protein concentration was determined by the mini Bradford assay as supplied in the Bio-Rad Protein Assay Kit, using BSA as a protein standard. Phosphate buffered saline (PBS) was the chosen column buffer when purifying protein for production of antibodies in order to ensure that no EDTA was present in the immunization solution.

2.8 PROTEIN ELECTROPHORESIS

All protein electrophoresis (Laemmli, 1970) was carried out on a Hoeffer mini-gel system or Bio-Rad Mighty-small Unit using 12% SDS-polyacrylamide gels (30:0.8 Acrylamide: bis-acrylamide). Protein samples were denatured in 3 x SDS-PAGE loading dye (0.1875 M Tris-HCl pH 6.8, 6% SDS, 15% β-mercaptoethanol, 30% glycerol, 0.006% BPB) at 90°C for 5 min before loading. Gels were initially run at 80 V for 30 min, and then at 100 V for 2.5-4 h. and were either set up for electrophoretic Western blot transfer or stained with Coomassie Brilliant Blue (0.5% Coomassie Brilliant Blue in a 5:5:2 water:methanol:glacial acetic acid solution) for 30-60 min and subsequently destained in a 30 % methanol / 10 % acetic acid solution.

2.9 PRODUCTION OF POLYCLONAL ANTIBODIES

New Zealand White, adult rabbits were immunized in order to produce polyclonal antibodies against the recombinant proteins. Prior to immunization (Day 1), 5 ml of blood was taken from each rabbit for extraction of pre-immune serum. Blood samples were centrifuged at 4 000 rpm for min in a clinical centrifuge and the serum carefully drawn off with a pipette, and stored at –20°C. Each rabbit was then inoculated, either sub-cutaneously (s.c.) or intravenously (i.v.) with 0.2 mg of protein made up in a volume of 600 µl of PBS. Since these recombinant proteins could be produced in milligram amounts, adjuvants were not required. A booster inoculation was given on Day 7 (0.2 mg recombinant protein), and again on Day 14 (0.2 mg of protein). On Day 21, 2.5 ml of blood was extracted from each rabbit in order to assess antibody titres, and another 0.2 mg booster inoculation given. After ensuring that antibody titres were sufficiently high for Western blot analysis, the rabbits were exsanguinated (Day 30) and the serum recovered was stored at –20°C.

2.10 PROTEOLYSIS OF RECOMBINANT PROTEINS

All proteolysis reactions were carried out in PBS, with varying protease:substrate ratios. For preparation of the protease stock solutions, 1.97 mg of trypsin was resuspended in 197 µl protease storage buffer (500 mM NaCl/ 20 mM Hepes pH 8.0/ 50%glycerol) to give a 10 mg/ml stock solution of trypsin. Factor Xa was supplied as a 1 mg/ml stock solution in the NEB buffer (500 mM NaCl/ 20 mM Hepes pH 8.0/ 2 mM $CaCl_2$/ 50% glycerol). Proteolysis reactions were performed in 20-30 µl volumes with 20-30 µg of recombinant protein substrate. Reactions were carried out at room temperature and 5 µl aliquots were taken from each sample at different time intervals,

(1 h, 3 h, 8 h) and run on a 12% SDS polyacrylamide gel for fractionation of cleavage products.

2.11 WESTERN BLOT ANALYSIS

Proteins were fractionated by SDS-PAGE (12% SDS-Polyacrylamide) and transferred onto a microporous polyvinylidene difluoride (PVDF) membrane. Electrophoretic transfer was carried out at 90 V for 2 h at 4°C. Transfer buffer contained 10% methanol, 14.4 g/L glycine, 3 g/L Tris-base) Prior to Western blotting, membranes were stained, for 1 min, with 0.1% Ponceau S stain (0.1% w/v Ponceau/ 0.1% v/v Acetic acid) for visible detection of markers. Western blot analysis was carried out using a Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Boehinger Mannheim, Germany). This kit utilizes POD-labeled secondary antibodies for chemiluminescent detection. All incubations were performed at room temperature, in a roller bottle system. The membrane was blocked for 1 h in 1% blocking solution. (10 ml per 100 cm^2 blot). Primary antibody incubation was carried out in 10 ml of 0.5% blocking solution for 1 h, at an optimal serum dilution for the antisera used. The membrane was washed twice in approximately 40 ml of TBST (50 mM Tris base; 150 mM NaCl; 0.1% Triton X) for 15 min, and twice in 15 ml of 0.5% blocking solution for 10 min. Secondary antibody incubation was carried out in 10 ml of 0.5% blocking solution for 30 min, using an antibody dilution of 1:10 000. This was followed by four washes, each in 40 ml of TBST for 20 min. For the detection reaction, 3 ml of detection solution (Solution A : solution B; 100:1) was used per blot. The membrane was exposed to the detection reaction for 60 s and then exposed to X-ray film. This was an optimal length of time to detect the signal. Exposures ranged from 10 s - 5 min, within 20 min of adding detection solution. For checking antibody titres, protein (1-2 µg)

was spotted onto PVDF membrane, and the Western blot protocol followed as described above. This procedure gave a qualitative comparison between the pre-immune and post-immune sera.

3. RESULTS

3.1. CONSTRUCTION OF EXPRESSION CASSETTES

An *M. tuberculosis* cosmid containing the *rnhA* homologue was isolated from a pYUB328::H37Rv library (Balasubramanian, 1995). The *rnhA* gene, carried on a 1.35 kb *Asp*718-*Bgl*II fragment (Figure 1) was subcloned into pGEM3Z(+)f. This construct (pADM7) was used as template DNA to amplify the sequence encoding the putative RNase HI from *M. tuberculosis* for construction of expression cassettes. The pMAL-c2 expression vector was employed for cloning of the entire ORF (*rnhA-pgm*) as well as the *rnhA* and *pgm* regions of DNA independently of each other.

(a) Expression Vector

The pMAL-c2 expression vector contains the *malE* gene from *E.coli*, which encodes a maltose-binding protein (MBP). The gene of interest is cloned downstream from *malE*, resulting in high-level expression, off the "tac" promoter, of an MBP fusion protein that can be purified in a one-step purification method using the affinity of MBP for maltose (Maina *et al.*, 1988; Duplay *et al.*, 1984; Kellerman *et al.*, 1982). pMAL-c2 expresses the *malE* gene, without its signal sequence, fused to the *lacZ*α gene. The multiple cloning site is positioned between *malE* and *lacZ*α, so inserting the coding sequence of interest inactivates the β-galactosidase α-fragment activity of the *malE- lacZ*α fusion. When constructs are transformed into the TB1 strain of *E. coli* (Johnston *et al.*, 1986), which serves as an α-complementing host, colonies which contain the inserted sequence do not appear blue, but rather white when patched onto plates containing X-gal. Figure 3 shows the restriction map of pMAL-c2.

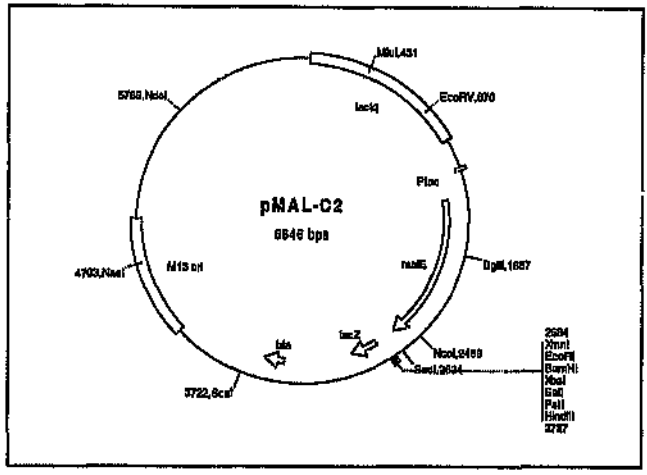


Figure 3: Restriction map of pMAL-c2 expression vector.

Figures 4 is a schematic diagram, showing the *rnhA-pgm* ORF and the position of primers designed to amplify DNA segments, while Figure 5 shows the complete DNA sequence of the ORF and details the features of primers used to amplify DNA for cloning in pMAL-c2.

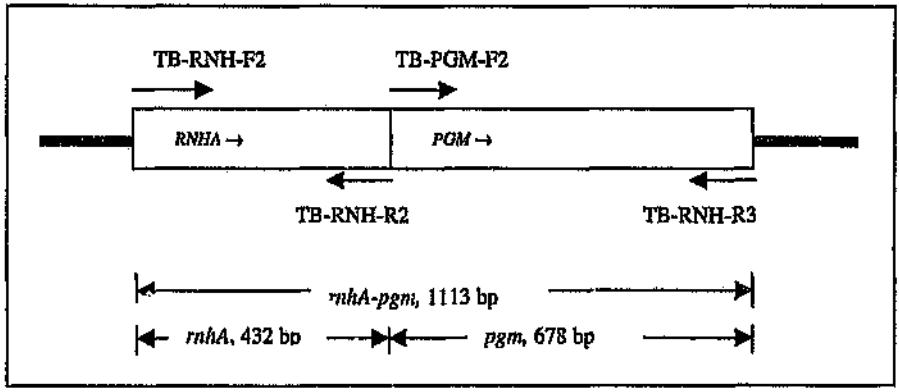


Figure 4: Schematic diagram of *rnhA-pgm* ORF, and the primers used for PCR amplification of DNA.

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*Bgl*II

```
cccagatctcggcggccgccgaggatgaagtggtgcgctgcccggaatgcggagcgatcttgttgcggctcgaaggattt 15286

                TB-RNH-F2
                gtgaaagttgtcatcgaa
gaggagtgaaagttgtcatcgaagccgacggcggatcgcggggcaatcccggaccggccggatacggcgcggtggtgtgg 15207
      V K V V I E A D G G S R G N P G P A G Y G A V V W
accgccgatcactccaccgtgctggccgagtccaagcaggcgatcggccgggcgacgaacaacgtcgccgaataccgcgg 15127
 T A D H S T V L A E S K Q A I G R A T N N V A E Y R G
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  L I A G L D D A V K L G A T E A A V L M D S K L V V

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tttcgcaggatcaactacgagtgggttccgcgtgcccggaacacgtatgcagaccggcttggccaatgacgcgatggacgc 14887
 F R R I N Y E W V P R A R N T Y A D R L A N D A M D A
                                        TB-PGM-F2
                                        atggttgcgaccgagtc
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        ttagccgacgccgcctaatcttcgaagccc
                TB-RNH-R2
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G A R G T P T R L L L L R H G Q T E L S E Q R R Y S G
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 E L H R R W L Q D T S I T P P G G E S F D D V L R R V
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  R R G R D R I I V G Y E G A T V L V V S H V T P I K
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                                        gtctgtccgatagatatctaccgcacgcttaaggccc
                                                        *  TB-RNH-R3
 E F Y A D G A S S V R L V N Q T G Y L . 364
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                                                                              KpnI
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Figure 5: DNA and protein sequence of the *Asp*718-*Bgl*II fragment from cosmid MTCY427, showing the primers used to amplify DNA for cloning in pMAL-c2. Restriction sites are shown in bold and stop codons are indicated with an asterisk. Numbering corresponds to Genbank accession number Z70692.

(b) Construction of RNase HI-PGM expression cassette.

The *rnhA-pgm* ORF was amplified by PCR from the cosmid subclone pADM7 with primers TB-RNH-F2 and TB-RNH-R3. This reverse primer was designed to incorporate an *Eco*R1 site several base pairs downstream of the stop codon. As shown in Figure 6, a fragment of the expected size (1113 bp) was generated. The PCR product was treated with Klenow enzyme, digested with *Eco*RI and subsequently gel purified for cloning in (*Xmn*I-E*co*RI)-digested pMAL-c2. The ligation product was transformed into competent *E.coli* TB1 cells and plated onto LA-Amp. Colonies were then patched onto LA-Amp plates with IPTG and X-gal for positive selec putative clones.

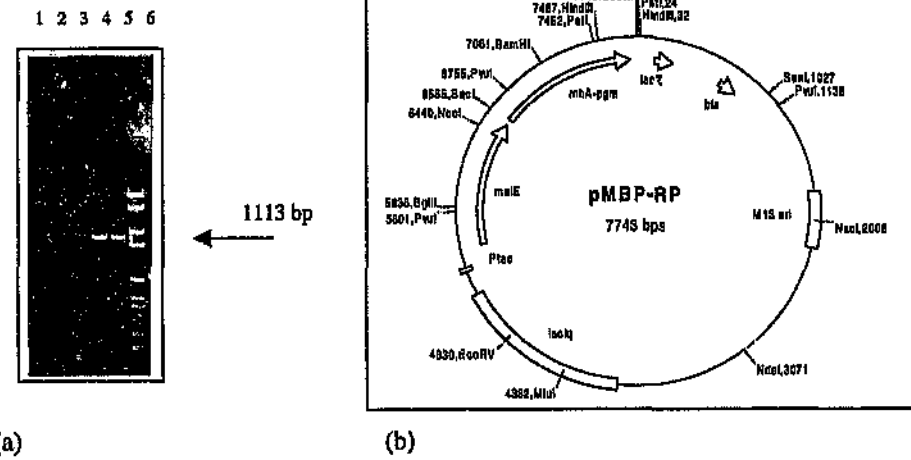


Figure 6: Construction of the pMBP-RP expression cassette. (a) PCR amplification of *rnhA-pgm* from pADM7. Lane 1: negative control, no template DNA, lane 2: TB-RNH-F2 control; lane 3: TB-RNH-R3 control; lanes 4 and 5: product (1113 bp) formed with both TB-RNH-F2 and TB-RNH-R3 in the presence of template; Lane 6: DNA marker λVI. (b) Restriction map of pMBP-RP.

White colonies were selected for plasmid screening by restriction enzyme analysis. Clones with the correct restriction map were selected and the presence of the insert DNA was confirmed by Southern blot analysis, using the PCR-generated *pgm* fragment (678 bp) as a probe. These results are shown in Figures 7 and 8.

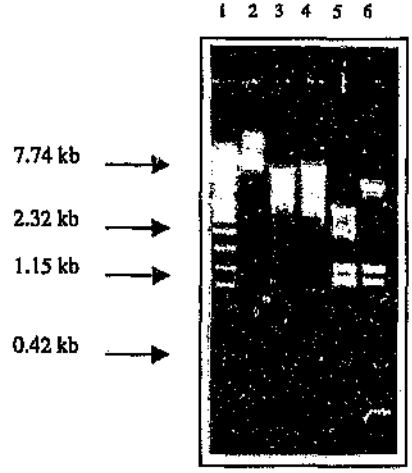


Figure 7: Restriction analysis of pMBP-RP. Lane 1: DNA marker λIV; lane 2: pMBP-RP undigested; lanes 3, 4, 5, 6: pMBP-RP, *HindIII*; *PstI*; *PstI*/*NdeI*; *PstI*/ *NcoI*-digested respectively.

A positive clone, designated pMBP-RP was subsequently subcloned into M13 for confirmatory DNA sequence analysis. Due to the length of the ORF, the insert sequence was excised from pMBP-RP in two fragments for subcloning in M13. The plasmid pMBP-RP was digested with *Sac*I and *Bam*HI to give a 475 bp product which was cloned in M13mp18 and M13mp19 for sequencing the 3'-terminus of the ORF. For sequencing the 5'-terminus, pMBP-RP was digested with *Bam*HI and *Eco*RI, and the resulting 685 bp product was cloned in M13mp18 and M13mp19. Single stranded (ss) DNA was isolated and sequenced using the universal primer. Figure 9 shows the in-frame start (GTG) and stop codon of the final construct.

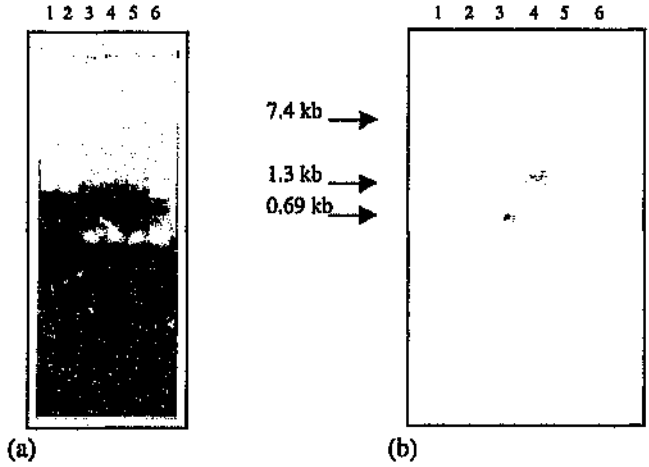


Figure 8: Restriction analysis (a) and confirmatory Southern blot analysis (b) of pMBP-RP, probed with fragment *pgm*. (a) and (b) Lane 1: DNA marker λIV; lane 2: pMAL-c2 *Eco*RI; lanes 3, 4, 5, 6: pMBP-RP *Bam*HI; *Eco*RI-*Nco*I; *Hind*III; *Pst*I-digested respectively.

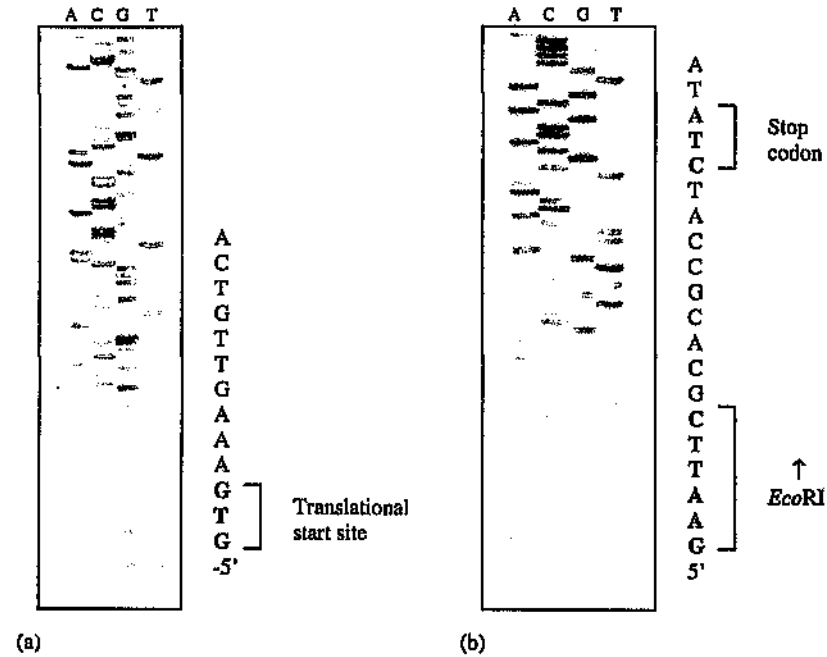
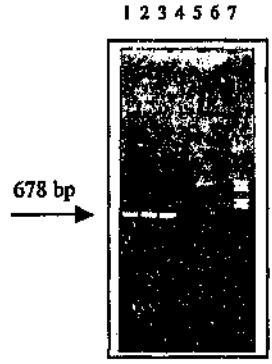


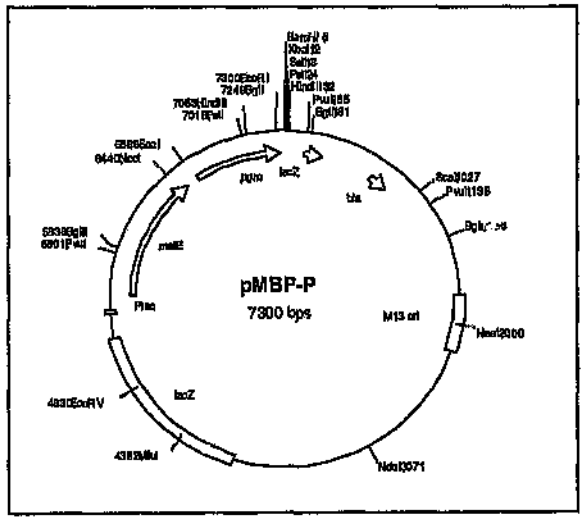
Figure 9: Sequence of the *Xmn*I and *Eco*RI junction sites in pMBP-RP. (a) Sequence generated by sequencing M13mp19-BE685 and (b) M13-SB475 with the universal primer.

(c) Construction of the PGM expression cassette.

The *pgm* coding region of the ORF was amplified by PCR independently of the *rnhA* region with primers TB-PGM-F2 and TB-RNH-R3, yielding a product 678 bp in length (Figure 10). The forward primer was designed with an in frame start codon, while the reverse primer contains an artificial stop codon and an engineered *Eco*R1 site to facilitate cloning. The 678 bp PCR product was polished with Klenow enzyme, digested with *Eco*RI, and gel purified for cloning in (*XmnI-Eco*RI)-digested pMAL-c2



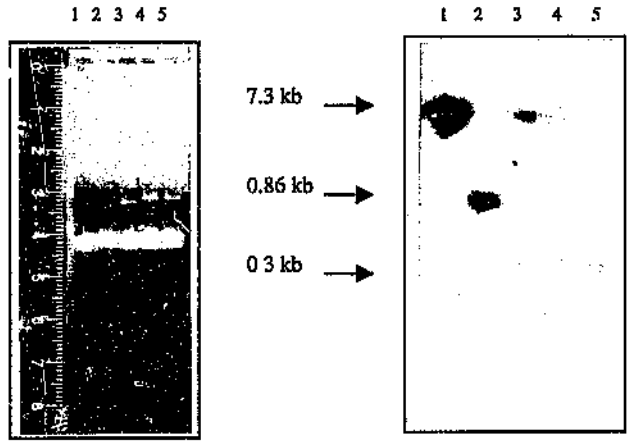
(a)



(b)

Figure 10: Construction of pMBP-P expression cassette.(a) PCR amplification of *pgm* from pADM7. Lanes 1, 2 and 3: product formed with both TB-PGM-F2 and TB-RNH-R3 (678 bp) in the presence of the template; lane 4: negative control, no template DNA; lane 5: TB-PGM-F2 control reaction; lane 6: TB-RNH-R3 control reaction; lane 7 DNA marker λVI. (b) Restriction map of pMBP-P.

After transformation into TB1 cells and patching onto LA-Amp plates with IPTG and X-gal, putative clones were analysed by restriction analysis. Clones with the correct restriction map were analysed by Southern blot using the PCR-generated *pgm* fragment as a probe (Figure 11). pMBP-PGM was digested with *Sac*I and *Eco*RI and the resulting 725 bp fragment carrying the entire *pgm* region was cloned in M13mp18 and M13mp19 for confirmatory sequence analysis. Figure 12 shows the in-frame start (ATG) and stop codon of the final construct.



(a) (b)

Figure 11: Restriction analysis (a) and confirmatory Southern blot analysis (b) of pMBP-P, probed with fragment *pgm*. (a) and (b); Lane 1: pMBP-P *Eco*RI-digested; lane 2: *Eco*RI/ *Nco*I-digested; lane 3: *Hind*III-digested; lane 4: *Pst*I-digested; lane 5: *Pst*I/ *Nco*I-digested..

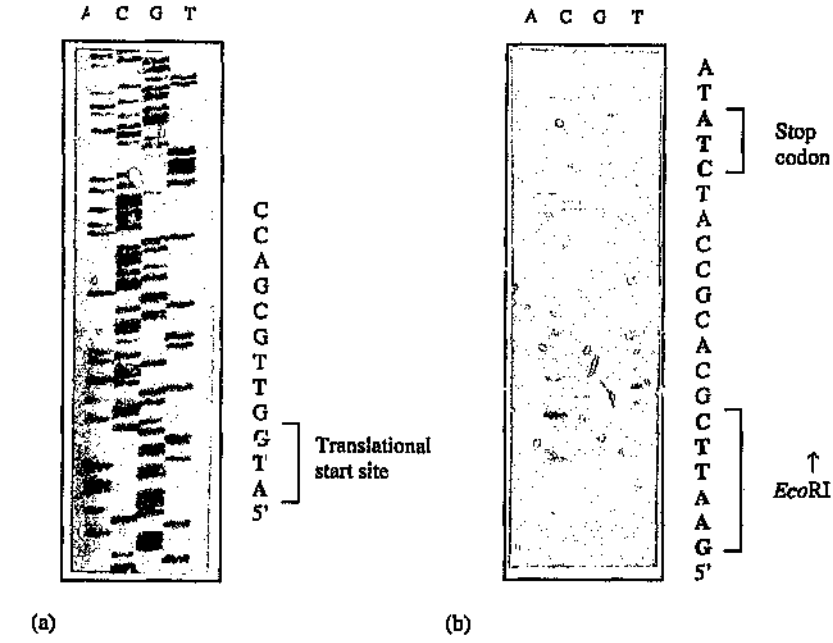


Figure 12: Sequence of the *Xmn*I and *Eco*RI junction sites in pMBP-P. (a) sequence generated by sequencing M13mp18-SE725 and (b) M13-SE725 with the universal primer.

(d) Construction of the RNase HI expression cassette.

The *rnhA* coding region of the ORF was amplified by PCR, independently of the *pgm* region with primers TB-RNH-F2 and TB-RNH-R2 to yield a 432 bp product (Figure 13). TB-RNH R2 contained an engineered stop codon, as well as a *Hind*III site to facilitate cloning. The PCR product was polished with Klenow, digested with *Hind*III and gel purified for cloning in (*Xmn*I-*Hind*III)-digested pMAL-c2. As described previously, ligation products were transformed into *E.coli* TB1 cells, and putative positive clones were screened by restriction analysis.

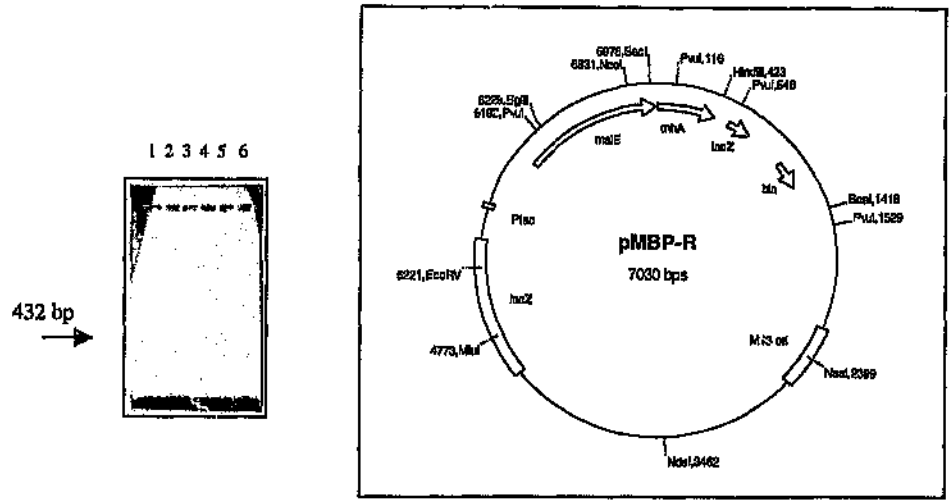


Figure 13: Construction of pMBP-R expression cassette. (a) PCR amplification of *rnhA* from pADM7. Lane 1: DNA marker λVI; lanes 2 and 3: product formed (432 bp) with both TB-RNH-F2 and TB-RNH-R2 in the presence of template; lane 4: negative control, no template DNA; lane 5: TB-RNH-R2 control reaction; lane 6: TB-RNH-F2 control reaction. (b) Restriction map of pMBP-R

Three putative constructs were probed with the PCR-generated *rnhA* fragment to confirm the presence of the insert. From Figure 14 it is evident that all three constructs were identical. Only one was selected and designated pMBP-R. This clone was digested with *Sac*I and *Hind*III and the resulting 478 bp fragment carrying the entire region encoding the *rnhA* was cloned in M13mp18 and M13mp19 for confirmatory sequence analysis.

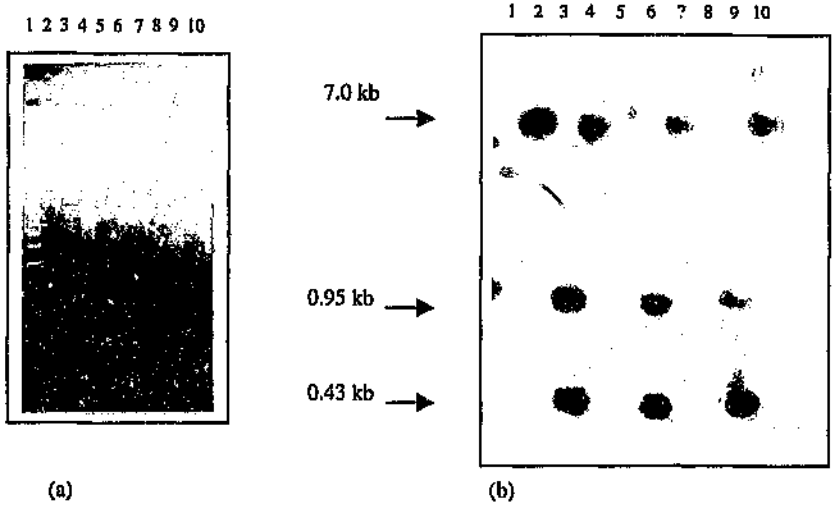


Figure 14: Restriction analysis (a) and confirmatory Southern blot (b) of three putative clones of pMBP-R, probed with fragment *rnhA*. (a) and (b) lane 1: DNA marker λIV, lanes 2,3 and 4,: pMBP-R1 *Hind*III, *Pvu*I, *Nco*I-digested; lanes 5,6 and 7,: pMBP-R2 *Hind*III, *Pvu*I, *Nco*I-digested; lanes 8,9 and 10: pMBP-R3 *Hind*III, *Pvu*I, *Nco*I-digested. pMBP-R1 was selected for sequencing, and subsequently designated pMBP-R.

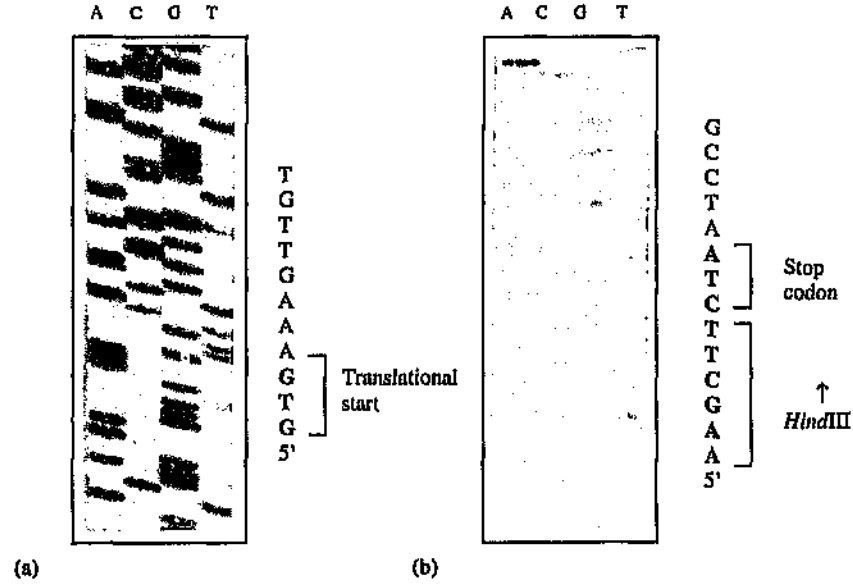


Figure 15: Sequence of the *Xmn*I and *Hind*III junction sites in pMBP-R. (a) Sequence generated by sequencing M13mp18-SH478 and (b) M13-SH478 with the universal primer.

3.2. INDUCTION OF GENE EXPRESSION

Expression of recombinant fusion proteins was induced, initially on a small scale, by adding IPTG to a final concentration of 0.3 mM during logarithmic growth of transformed TB1 cultures. Samples of uninduced cells were collected at time zero, and thereafter at hourly intervals for three hours. Cells were harvested and whole cell extracts were fractionated by SDS PAGE in order to monitor induction of gene expression. As shown in Figure 16 induction of pMBP-R resulted in overexpression of a protein, *ca.* 62 kDa in size, which corresponds to the expected size of the MBP::RNase HI fusion protein. Similarly, induction of pMBP-P resulted in overexpression of a protein of *ca.*70 kDa, which corresponds to the expected size of the MBP::PGM fusion protein, and induction of pMBP-RP yielded a protein of *ca.* 85 kDa, as expected for the MBP::RNase HI-PGM fusion protein.

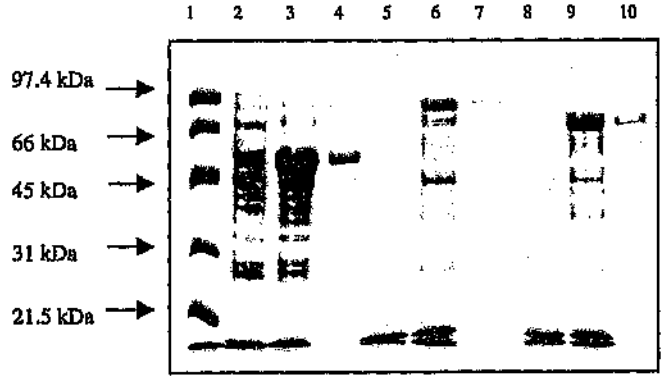


Figure 16: SDS-PAGE analysis of induced proteins. Lane 1: SDS-PAGE marker; lanes 2 and 3: whole cell lysates of *E. coli* (pMBP-R) uninduced and induced (3 h), respectively; lane 4: MBP::RNase HI (62 kDa) after purification by amylose affinity chromatography described in Materials and Methods; lanes 5 and 6: whole cell lysates of *E. coli* (pMBP-RP) uninduced and induced (3 h) respectively; lane 7: purified MBP::RNase HI-PGM (85kDa); lanes 8 and 9: whole cell lysates of *E.coli* (pMBP-P) uninduced and induced (3 h) respectively; lane 10: purified MBP::PGM (70kDa).

3.3. PROTEIN PURIFICATION BY AFFINITY CHROMATOGRAPHY

Recombinant fusion proteins were purified to homogeneity from large-scale induced cultures. As described in Materials and Methods the crude extract was separated from the insoluble fraction and loaded onto the amylose resin for purification. Following elution with maltose, recombinant fusion proteins were found to be most concentrated in fractions three to six (0.05-0.29 mg/ml) decreasing gradually to fraction ten (0.01 mg/ml). Samples of the purified recombinant fusion proteins are shown in Figure 17. Once purified, recombinant proteins were either stored in PBS for immunization of rabbits for the production of antibodies, or stored in column buffer for SDS PAGE analysis.

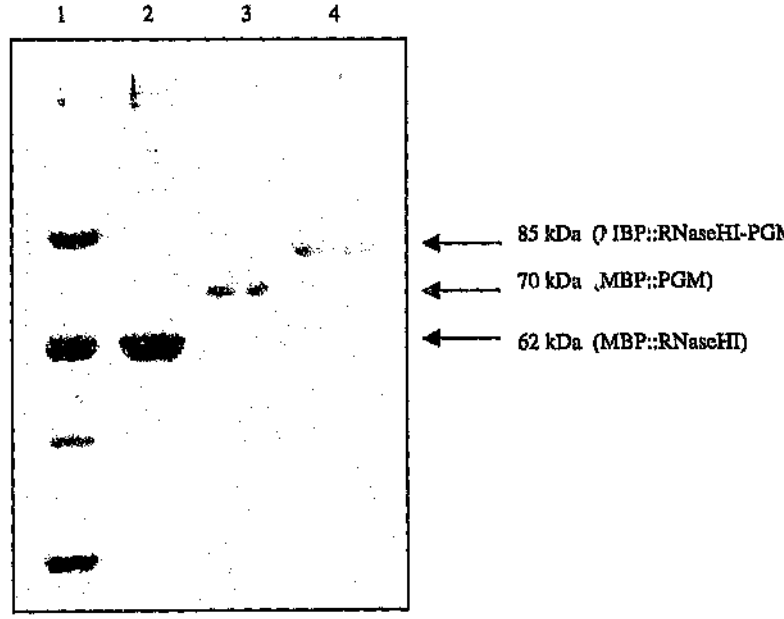


Figure 17: SDS-PAGE analysis of purified recombinant MBP: fusion proteins. Lane 1: SDS-PAGE markers; lane 2: MBP::RNase HI (62 kDa); lane 3: MBP::PGM (70 kDa); lane 4: MBP::RNase-PGM (85 kDa).

As shown in Figure 17 the MBP::RNase HI recombinant protein gave the best yield of homogeneous product following elution from the amylose column. By comparison, the fractions of MBP::PGM and MBP::RNase HI-PGM proteins gave lower yields and were contaminated. In order to investigate whether these contaminating bands correspond to proteolytic degradation products by *E. coli* proteases, cells were treated with protease inhibitors prior to lysis. After column purification, these fractions were analysed, by SDS-PAGE, against untreated controls, but no difference was observed.

3.4. PRODUCTION OF POLYCLONAL ANTIBODIES

Polyclonal antibodies were raised against the recombinant fusion proteins, MBP::RNase HI, MBP::PGM, and MBP::RNase HI-PGM for the purpose of producing probes for monitoring the presence of RNase HI, PGM and/or derivatives thereof in extracts of *M. tuberculosis*. In order to assess antibody titres of the antisera, Western blot analysis was performed against known amounts of each purified recombinant protein, which were spotted onto PVDF membrane. Relative intensities of the chemiluminescent reaction gave an indication of the relative strengths of antibodies. In some instances the pre-immune sera showed an insignificantly low background activity with the purified proteins, while each post-immune sera showed definite cross-reactivity with all recombinant fusion proteins. Qualitative analysis of antibody titres showed no distinct difference between the i.v. and s.c. routes of immunization (data not shown).

3.5. BIOCHEMICAL CHARACTERISATION BY PROTEOLYTIC CLEAVAGE

(a) Factor Xa.

The pMAL-c2 vectors contain the recognition sequence for the specific protease Factor Xa which is located directly upstream of the *Xmn*I site, and therefore,

theoretically, allows for the MBP to be cleaved from the protein of interest (Nagia and Thøgersen, 1987). Upon treatment with Factor Xa, only partial cleavage of the recombinant proteins was ever observed. The N-terminal MBP domain which was cleaved from the fusion proteins, was visible on Coomassie-stained SDS-PAGE gels at *ca.* 47 kDa, however, no defined C-terminal cleavage products of the expected sizes were visible on SDS-PAGE. Cleavage of a positive control fusion protein, MBP::paramyosin, into its two distinct domains confirmed positive activity of Factor Xa.

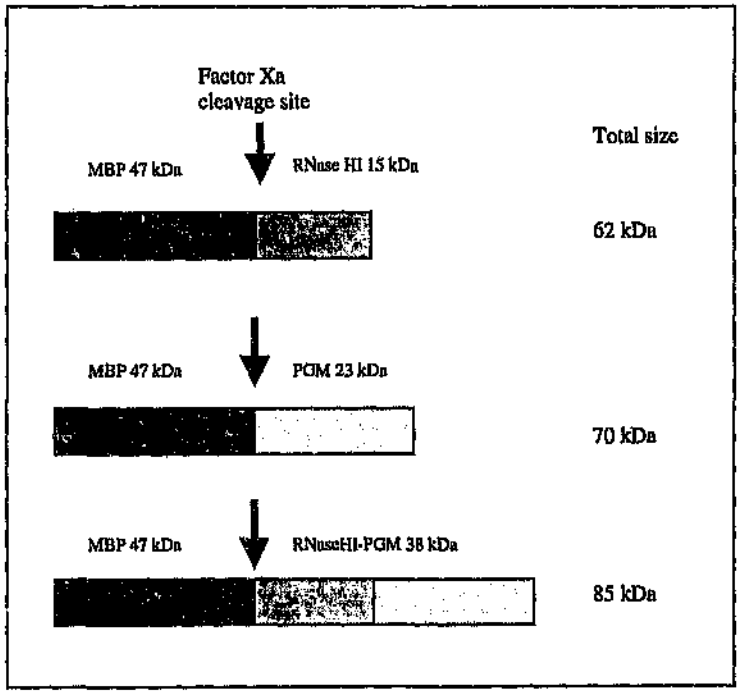


Figure 18: Schematic diagram of recombinant fusion proteins

As seen in Figure 19 the MBP::PGM protein showed a cleavage product of *ca.* 30 kDa, and the MBP::RNase HI-PGM showed a cleavage product, amongst others, of

ca. 36.5 kDa. Sequence analysis showed that the amino acid sequence of *rnhA-pgm* contained no additional Factor Xa recognition sequences, so it would be expected that each recombinant protein would only be cleaved once, separating it from the MBP domain.

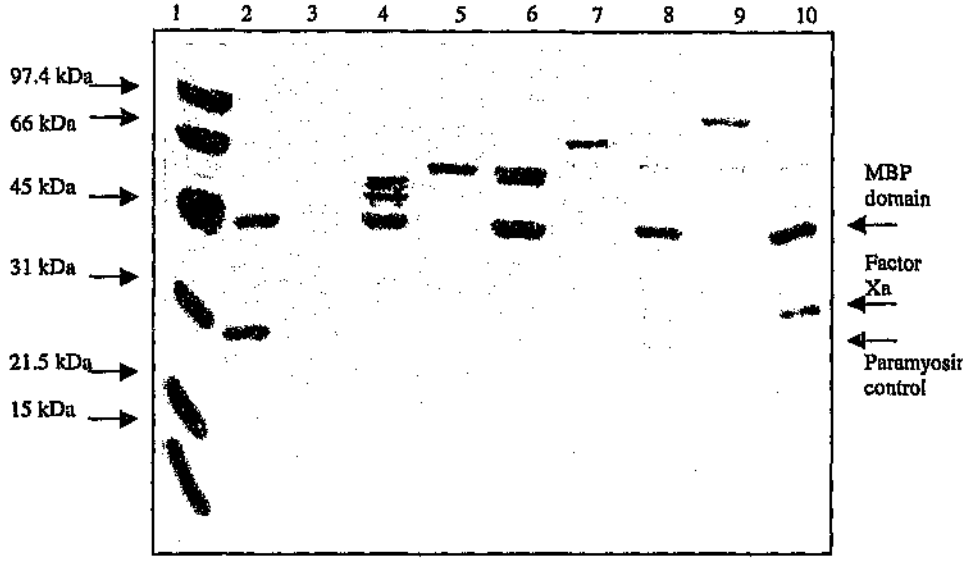


Figure 19: SDS-PAGE analysis of proteolytic cleavage of recombinant proteins with Factor Xa. Protease: substrate ratio of 1: 50. Lane 1: SDS-PAGE marker; lanes 2: MBP-paramysin cleaved with Factor Xa, positive control; lanes 3 and 4: MBP-LacZ, uncleaved and cleaved, respectively; lanes 5 and 6: MBP::RNase HI, uncleaved and cleaved; lanes 7 and 8: MBP::PGM, uncleaved and cleaved; lanes 9 and 10: MBP::RNase HI-PGM, uncleaved and cleaved.

(b) Limited proteolysis with trypsin.

Upon treatment with trypsin, identical cleavage products were obtained from each MBP fusion protein and showed the same degradation pattern as MBP alone. This

result suggests that the visible products were all derived from the MBP domain. In contrast, cleavage products specific to the RNase HI and/ or the PGM domains were not detected by Coomassie staining.

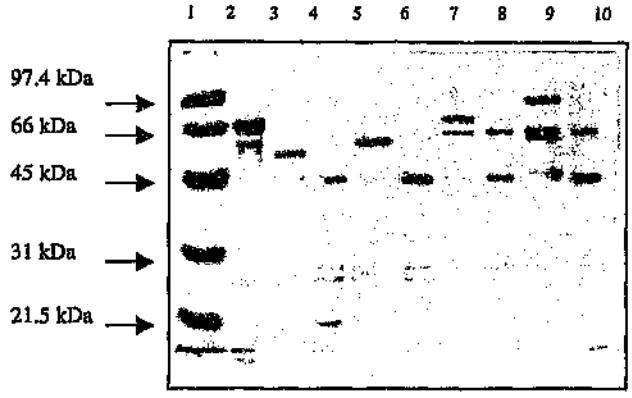


Figure 20: SDS-PAGE analysis of proteolytic cleavage of recombinant proteins with trypsin. Protease:substrate ratio of 1:20. Lane 1: SDS-PAGE marker; lane 2: BSA cleaved with trypsin, positive control; lanes 3 and 4: MBP::LacZ, uncleaved and cleaved, respectively; lanes 5 and 6: MBP::RNase HI, uncleaved and cleaved, lanes 7 and 8: MBP::PGM, uncleaved and cleaved; lanes 9 and 10: MBP::RNase HI-PGM, uncleaved and cleaved.

3.6. WESTERN BLOT ANALYSIS

(a) Western blot analysis of purified proteins.

The antibodies raised, were used to probe whole cell extracts of induced *E. coli* (pMBP-P) and *E. coli* (pMBP-RP) cultures, as well as purified recombinant fusion proteins fractionated by SDS PAGE. Each different antibody cross-reacted with each recombinant fusion protein as well as the MBP-LacZ protein, suggesting strong

recognition of the MBP domain (data not shown). Figure 21 shows cross-reactivity of the commercial Anti-MBP antibody with the recombinant fusion proteins.

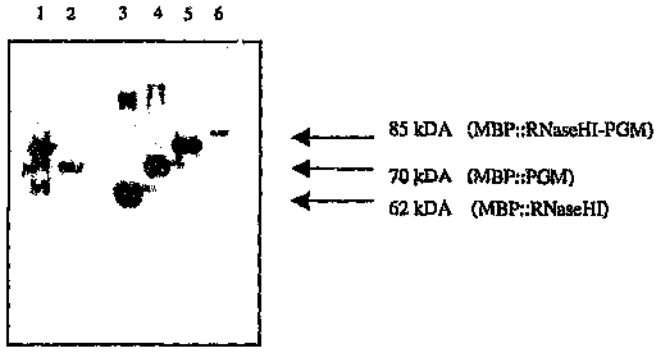


Figure 21: Western blot analysis of recombinant proteins with commercial Anti-MBP antiserum (1:10 000). Lanes 1 and 2: whole cell extracts of induced *E. coli* (pMBP-RP) and *E. coli* (pMBP-P) respectively; lane 3: purified MBP::RNase HI; lane 4: purified MBP::PGM; lane 5: purified MBP::RNase HI-PGM; lane 6: MBP::paramyosin. Anti-MBP Serum: (1:10 000)

Recombinant proteins cleaved partially with Factor Xa were also probed with Anti-MBP::RNase HI antisera. In each case the MBP domain (*ca.* 47 kDa) was detected, but the antisera failed to detect cleavage products of sizes corresponding to the RNase HI or RNase HI-PGM polypeptides. Similar results were seen in the case of proteins cleaved with trypsin, where all the MBP cleavage products were detected by Anti-MBP::RNase HI, showing extensive cross-reactivity of this serum with the MBP domain (data not shown).

(b) Western blot analysis of mycobacterial cell lysates.

In order to investigate expression or post-translational modification of the fusion protein, whole cell extracts of *M. tuberculosis* and *M. smegmatis* were probed with the polyclonal antibodies raised against recombinant fusion proteins. Western blots were optimised for concentration of lysate loaded on the gel and serum dilution for each of the antisera. For optimal results 10-40 μg of cell lysate was loaded on the gel and antisera dilutions varied according to the serum used. As shown in Figures 22, 23, and 24, and summarised in Table 6, Anti-MBP, Anti-MBP::PGM, and Anti-MBP::RNase HI-PGM antisera detected a protein of *ca.* 69 kDa in both *M. tuberculosis* and *M. smegmatis* lysates, which was assumed to be due to non-specific recognition of a cellular protein(s). The Anti-MBP::PGM antisera showed an extensive amount of background reaction which could not be reduced efficiently (Figure 23). In contrast, anti-MBP::RNase HI antisera cross-reacted with two distinct proteins in *M. tuberculosis* cell extracts (Figure 24). The larger protein was *ca.* 51 kDa in size, which appeared to be specific to *M. tuberculosis* as it was undetected in the *M. smegmatis* cell extract. The second cross-reactive protein was *ca.* 37 kDa in size, which is close to the expected size of the RNase HI-PGM fusion protein (38 kDa). This 37 kDa protein was also detected in the *M. smegmatis* cell extract.

Table 6: Western Blot analysis of mycobacterial cell extracts.

Antisera	H37Rv lysate	mc^2155 lysate
Anti-MBP	69 kDa	69 kDa
Anti-MBP::RNase HI	51 kDa, 37 kDa	37 kDa
Anti-MBP::PGM	69 kDa	69 kDa, 54 kDa,
Anti-MBP::RNaseHi-PGM	69 kDa	69 kDa

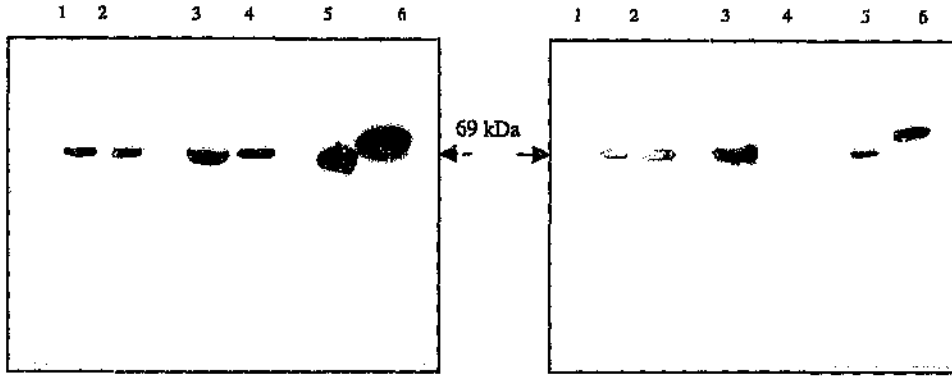


Figure 22: Western blot analysis of mycobacterial cell extracts. (a) H37Rv (lanes 1, 2, 3: 10, 20, 40 μg) and mc^2155 (lane 4: 20 μg); lanes 5, and 6: purified proteins MBP::RNase HI; MBP::RNase HI-PGM, blotted with Anti-MBP (1:2000). (b) H37Rv (lanes 1, 2, 3: 10, 20, 40 μg) and mc^2155 (lane 4: 20 μg); lanes 5,and 6: purified recombinant proteins MBP::RNase HI; MBP::RNase HI-PGM, blotted with Anti-MBP::RNase HI-PGM Serum 4 (1:200).

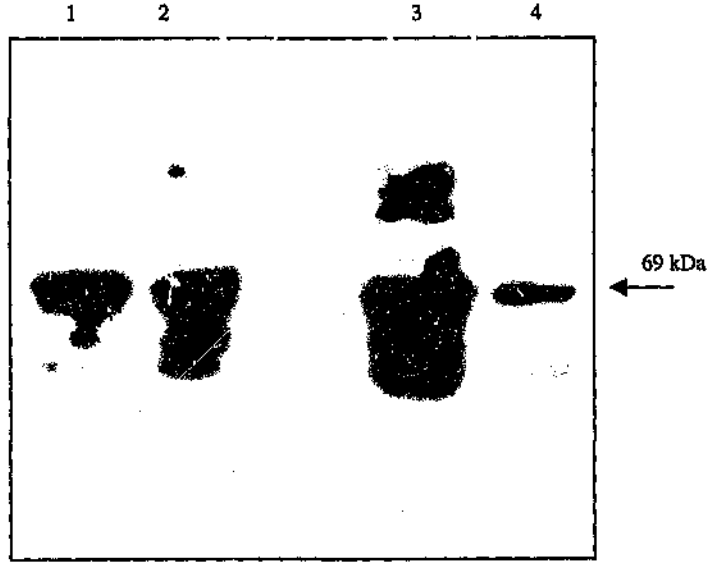


Figure 23: H37Rv (lanes 1, 2, 3: 10, 20, 40 μg) and mc^2155 (lane 4: 20 μg) blotted with Anti-MBP::PGM Serum 6 (1:200).

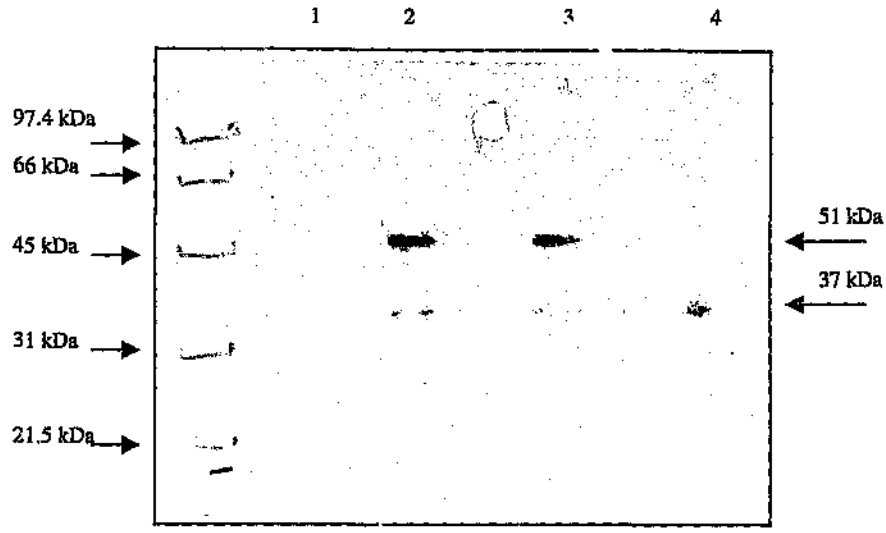


Figure 24: H37Rv (lanes 1, 2, 3: 10, 20, 40 µg) and mc^2155 (lane 4: 20 µg) blotted with Anti-MBP::RNase HI Serum 1 (1:200)

If the 37 kDa band detected in *M. tuberculosis* by anti-MBP::RNase HI antiserum is in fact the RNase HI-PGM polypeptide (predicted molecular weight of 38kDa), then results of Figure 24 would suggest that there might be a homologue of *rnhA-pgm* in *M. smegmatis*. Southern blot analysis of *Bam*HI-digested *M. tuberculosis* and *M. smegmatis* genomic DNA showed that the *rnhA-pgm* probe bound weakly to a 3.2 kb band in *M. smegmatis*. However, this band was also detected with the *pgm* probe, but not t' *hA* probe, which suggests that the cross-reactive band does not contain a . . . .ologue (data not shown).

3.7. ALTERNATIVE EXPRESSION STRATEGY

The Western blot analysis using antisera raised against MBP-fusion proteins suggested that the relatively large MBP domain was immunodominant. Moreover,

Factor Xa cleavage precluded recovery of intact recombinant proteins lacking the MBP domain (Fig. 19) for use as alternative immunogens. In an attempt to produce a recombinant protein with a smaller N-terminal fusion, with the aim of raising more specific antibodies against the *M. tuberculosis* proteins, an alternative expression strategy was employed. This strategy was based on the pET expression system, in which target genes are cloned in expression plasmids under the control of strong bacteriophage T7 transcription and translation signals to produce recombinant proteins tagged with six consecutive histidine residues. The *rnhA* coding region of the ORF was amplified by PCR independently of the *pgm* region, using TB-RNHNDE-F and TB-RNHBAM-R to yield a 434 bp product. This fragment was digested with *Nde*I and *Bam*HI, gel purified and subsequently cloned in (*Nde*I-*Bam*HI)-digested pET-15b. A positive clone was obtained by restriction mapping and was denoted pET-RNH, but difficulties arose in trying to subclone into M13 for sequence analysis. Furthermore, no overexpression was evident on SDS-PAGE analysis of induced pET-RNH cell lysates and when probed with anti-MBP::RNase HI antisera, RNase HI expresssion could not be detected. This construct could therefore not be utilized to produce protein for raising polyclonal antibodies

Using an analogous approach, attempts were made to clone the full-length *rnh-pgm* ORF into pET-15b. DNA was amplified by PCR with TB-RNHNDE-F and TB-RNH-R3 to yield the expected 1116 bp product. This fragment was treated with Klenow enzyme and subsequently digested with *Nde*I, to give a cohesive 5'-terminal end, for cloning in pET-15b. Several attempts of this cloning strategy were made, but persistent, clonal rearrangement was observed, thus precluding cloning of this fragment.

3.8. GENETIC COMPLEMENTATION EXPERIMENTS

(a) Complemention of *E. coli rnhA* mutants with *M. tuberculosis rnhA in trans*.

The generation of RNase HI mutants in *E.coli* has facilitated elucidation of some of the cellular roles of RNase HI (Horiuchi, 1984; Kogoma, 1986) and conditional lethal *E. coli rnhA* mutants have been used as hosts for functional complementation (Itaya and Crouch, 1991a; Itaya *et al.*, 1991). Since RNase HI-defective mutants require a functional RecBCD enzyme for growth (Kanaya and Crouch, 1884; Itaya and Crouch, 1991a) *rnh*-339::*cat recC271* (ts) (MIC3037) strains are temperature-sensitive for growth. In order to check for functional complementation of these *rnhA* mutants with putative RNase HI from *M. tuberculosis*, pMBP-R and pMBP-RP were introduced into MIC3037, and their effects on growth of MIC3037 at the non-permissive temperature were monitored. Chemically competent MIC3037 cells were transformed with pMAL-c2 as a negative control plasmid. The other constructs contained the gene encoding the RNase HI domain of *M. tuberculosis*. Cells were incubated and results showed that there was growth at the permissive temperature (30°C), but not at the restrictive temperature (42°C). The presence of plasmid in the transformants, was confirmed by restriction analysis of DNA recovered from two colonies from each plate. These cultures were subsequently plated out at serial dilutions and incubated at both 30°C and 42°C. IPTG was added to half of the plates in order to ascertain the effects of induced protein expression on the viability of transformants at the restrictive temperature. However, no complementation was observed with any of the constructs. Based on the negative results obtained in *E. coli*, it was decided to investigate functional complementation in a mycobacterial host.

(b) Genetic complementation of an *M.smegmatis* integrant with *M. tuberculosis rnhA* provided *in trans*.

The *M. smegmatis* strain, mc^2155::pUSHδB, derived from mc^2155, contained an inactivated *rnhA* allele integrated at the *rnhA* locus (Figure 25). The construct pUSHδB contains a truncated *rnhA* gene with a frameshift mutation at the internal *Bam*HI site, as well as the hygromycin resistance marker gene (*hyg*), and the *B. subtilus sacB* gene encoding levansucrase. The gene *sacB* is lethal to mycobacteria in the presence of 5-10% sucrose, and is used as a marker for positive selection of gene-replacement events into mycobacteria (Pelicic *et al.*,1996). pUSHδB is not able to replicate in mycobacteria and therefore must undergo recombination with the chromosome in order for transformants to be hygromycin resistant (Hyg^R) It became integrated into the chromosome by means of a single cross-over (SCO) event, which results in the tandem arrangement of the two alleles in the chromosome, to give mc^2155::pUSHδB. This strain was constructed in an attempt to inactivate the *M. smegmatis rnhA* gene by introducing an unmarked frameshift mutation (Dawes, 1998). If a second cross over event occurs within the gene, indicated as II in Figure 26, this would result in allelic replacement of the wild type (WT) gene by the frameshift-inactivated allele (DCO mutant). However, if the recombination event occurs on the same side of the frameshift mutation, indicated as I in Figure 26, a DCO can also occur such that the WT phenotype is restored. In previous work (Dawes, 1998) a DCO mutant (Km^R, Hyg^S, Suc^R) was unobtainable and a WT genotype was only ever recovered from attempts to knock out the *rnhA* allele. This suggested that the *rnhA* might be essential in *M. smegmatis*. In this study attempts were made to ascertain whether provision of the *M. tuberculosis rnhA in trans* might facilitate recovery of the *rnhA* DCO mutant in *M. smegmatis* by complementation. Such an

outcome would strongly suggest that the *M. tuberculosis rnhA* encodes functional RNase HI activity.

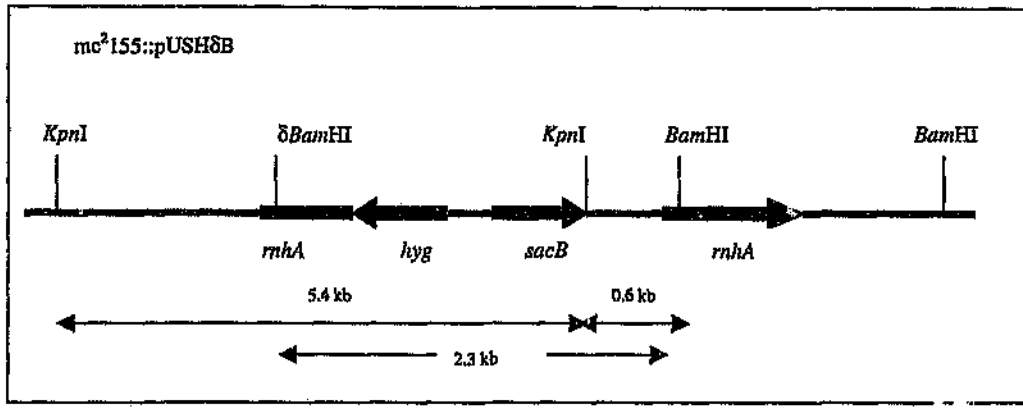


Figure 25: Restriction map of mc²155::pUSHδB

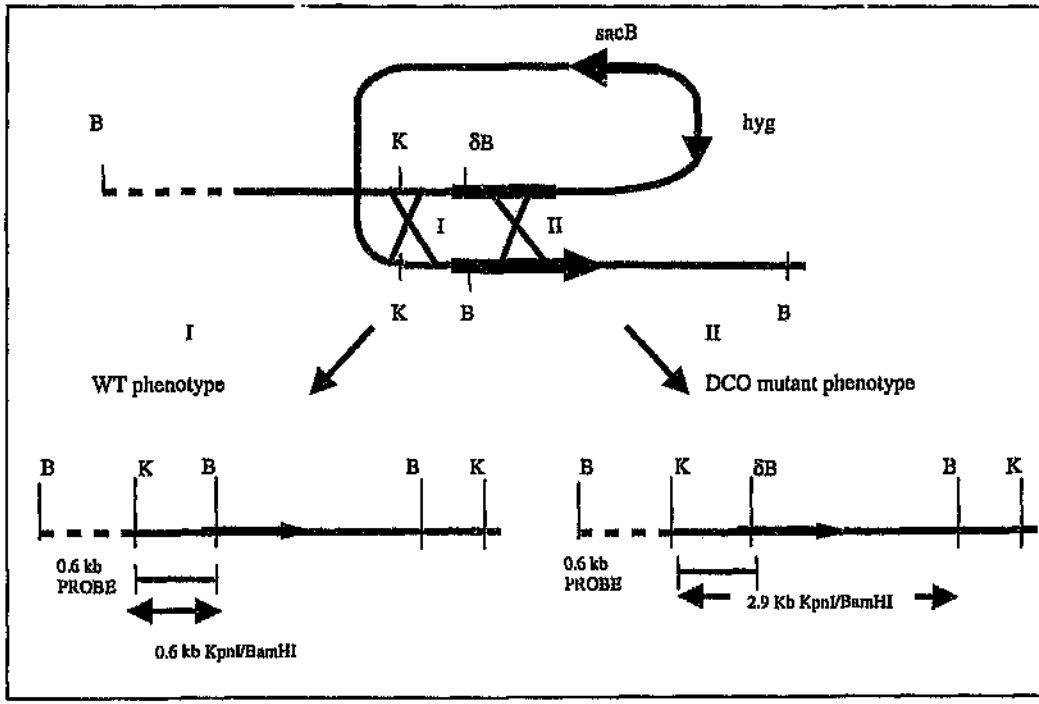
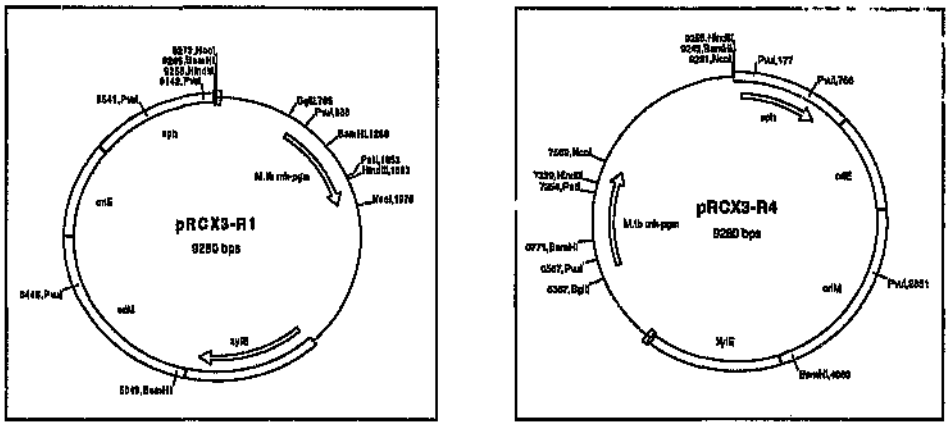


Figure 26: Possible types of double cross overs that can occur to give (I) WT phenotype and (II) DCO *rnhA* mutant.

Construction of complementation plasmid. The 3.6 kb *Xba*I-*Eco*RI fragment encoding the *rmhA-pgm* ORF was excised from pADM8, and cloned in the vector pRCX3, which is a low copy number mycobacterial-*E. coli* shuttle vector. It contains the aminoglycoside phoshotransferase (*aph*) gene, conferring kanamycin resistance (Km^R). Figure 28 shows restriction analysis of the constructs, designated pRCX-R1 and pRCX-R4. These plasmids carry the *rmhA-pgm* ORF in opposite orientations, as shown in their restriction maps (Figure 27).



(a) (b)

Figure 27: Restriction map of (a) pRCX-R1 and (b) pRCX-R4

The integrant, mc^2155::pUSH-δB was transformed to Km^R with pRCX3, pRCX-R1 and pRCX-R4. The presence of the plasmids was confirmed by electroduction into *E. coli*, followed by restriction analysis (data not shown). Prior to selecting on sucrose, transformants were cultured in liquid media with Hyg and Km antibiotic selection in order to maintain the SCO and ensure that the reversion to WT phenotype did not occur as an early event. Cultures were serially diluted and spread to single colonies on LA-Km/5% suc. Of the 30-50 suc^R colonies, 30 of each were picked and checked for

Hyg sensitivity by resuspension in *ca.* 10 µl of LB, and spotting 2 µl of each onto LA-Km/Hyg versus LA-Km plates in order to distinguish between true DCO strains and spontaneous Suc^R, *sacB* mutants. The experimental procedure of the complementation experiment is summarised in the flow diagram in Figure 29 and the results are shown in Table 7.

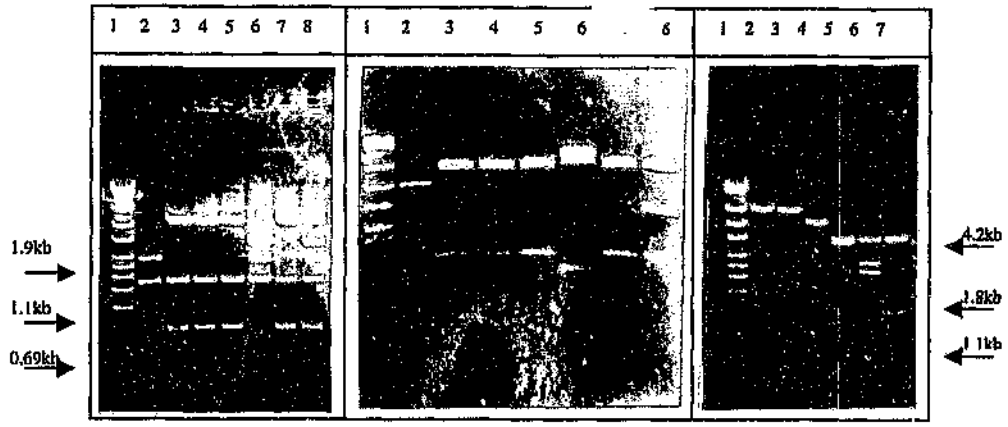


Figure 28: Restriction analysis of complementation vectors pRCX3, pRCX-R1, and pRCX-R4. (a) lane 1: DNA marker λIV; lane 2: pRCX3; lane 3: pRCX-R1; lane 4: pRCX-R2; lane 5: pRCX-R3; lane 6: pRCX-R4; lane 7: pRCX-R5; lane 8: pRCX-R6, all *Pvu*I digested. (b) lane 1: DNA marker λIV; lane 2: pRCX3; lane 3: pRCX-R1; lane 4: pRCX-R2; lane 5: pRCX-R3; lane 6: pRCX-R4; lane 7: pRCX-R5; lane 8: pRCX-R6, all *Nco*I digested. (c) lane 1: DNA marker λIV; lanes 2 and 5: pRCX-R1 *Hind*III, *Bam*HI- digested, respectively; lanes 3 and 6: pRCX-R4 *Hind*III, *Bam*HI-digested; lanes 4 and 7: pRCX3 *Hind*III, *Bam*HI-digested.

Table 7. Results of the DCO events

Plasmid	N°. Hyg^S	N°. screened	Phenotype
pRCX3	7/30	3	WT
pRCX-R4	13/30	12	WT
pRCX-R1	12/30	12	WT

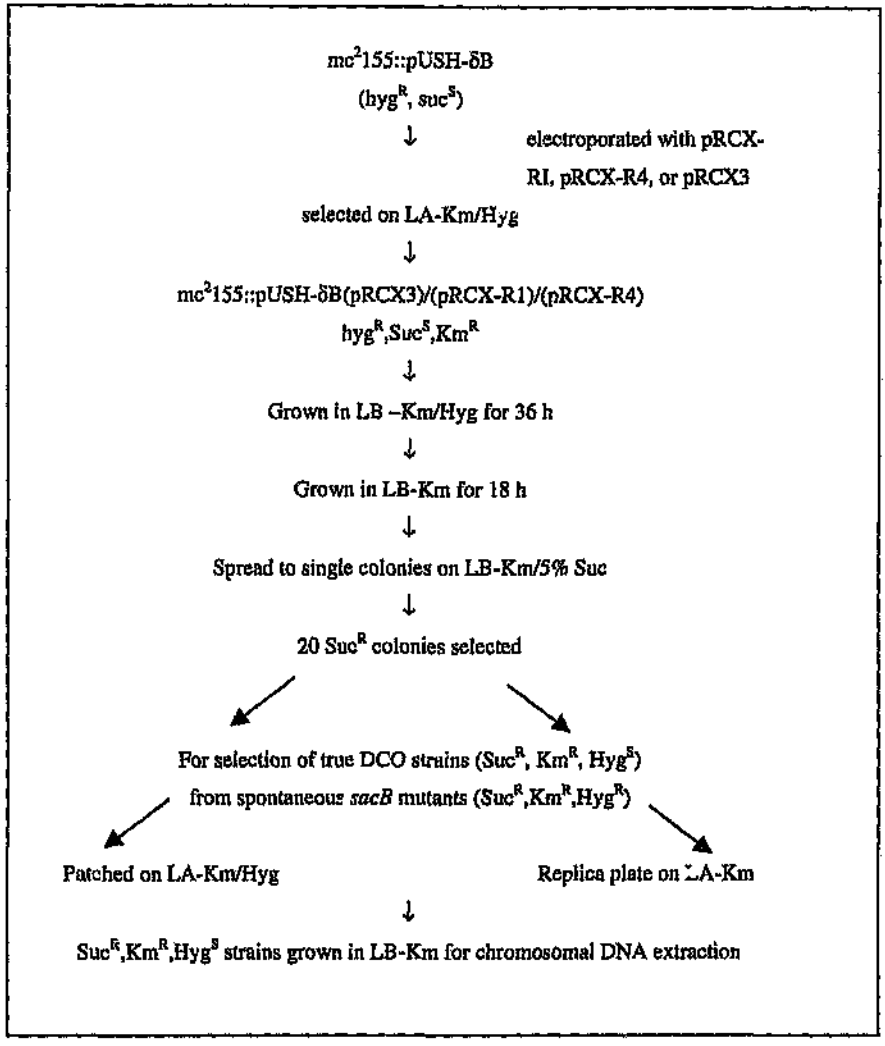
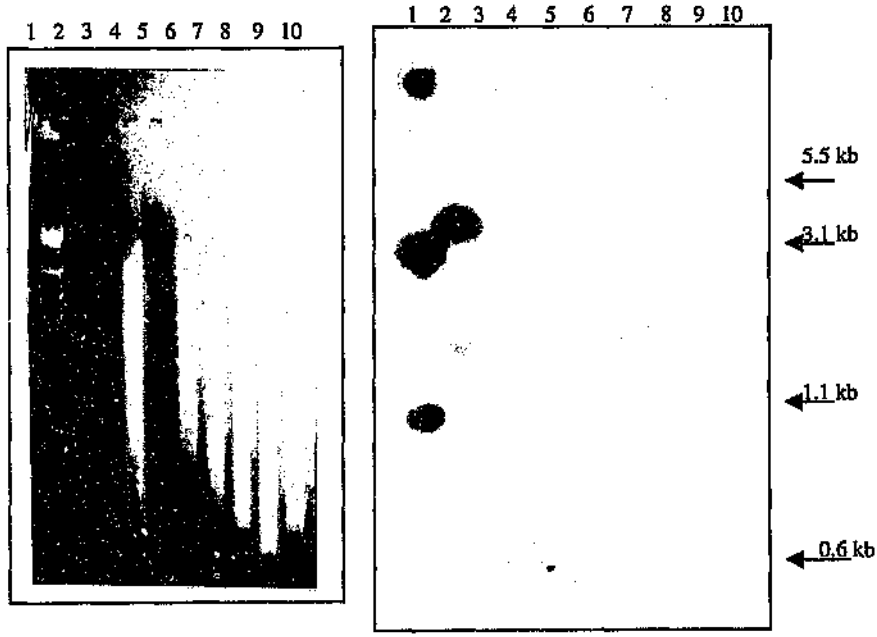


Figure 29: Experimental approach used for complementation of mc²155::pUSHδB.

Colonies that were Km^R, Suc^R, and Hyg^S were picked and grown in 3 ml of LB-Km for chromosomal DNA extraction. Chromosomal DNA was digested with *Kpn*I-*Bam*HI and probed with a 0.6 kb (*Kpn*I-*Bam*HI) fragment from pPS (Dawes, 1998).

Southern blot analysis reveals that the DCO event in all of the transformants had resulted in a reversion to the WT phenotype. This is evident in Figures 30 and 31, where all strains tested positive for the 0.6 kb band, indicative of WT genotype (Figure 26). Upon *Kpn*I-*Bam*HI digestion, a DCO event resulting in an *mhA* mutant would be identified by the presence 2.9 kb band, and not a 0.6 kb band. Therefore no *mhA* mutants were obtained.



(a) (b)

Figure 30: (a) Agarose gel electrophoresis and (b) Southern blot analysis of *Bam*HI-*Kpn I* chromosomal digests of mc^{2}155::pUSHδB transformed with pRCX-R4. Lane 1: DNA marker λIV; lane 2: pPS plasmid control; lane 4: mc^{2}155::pUSHδB; lanes 6-10: mc^{2}155::pUSHδB(pRCX-R4). Blots were probed with 0.6 kb fragment excised from pPS, as shown in Figure 26.

The other background bands detected in the Southern blots were due to the presence of plasmid DNA, as is visible in the ethidium bromide stained agarose gel. This was confirmed by plasmid digestion of pRCX-R1 and pRCX-R4, which could explain bands of the following sizes; 4.2 kb, 1.3 kb, 3.1 kb and 2.3 kb 0.9 kb.

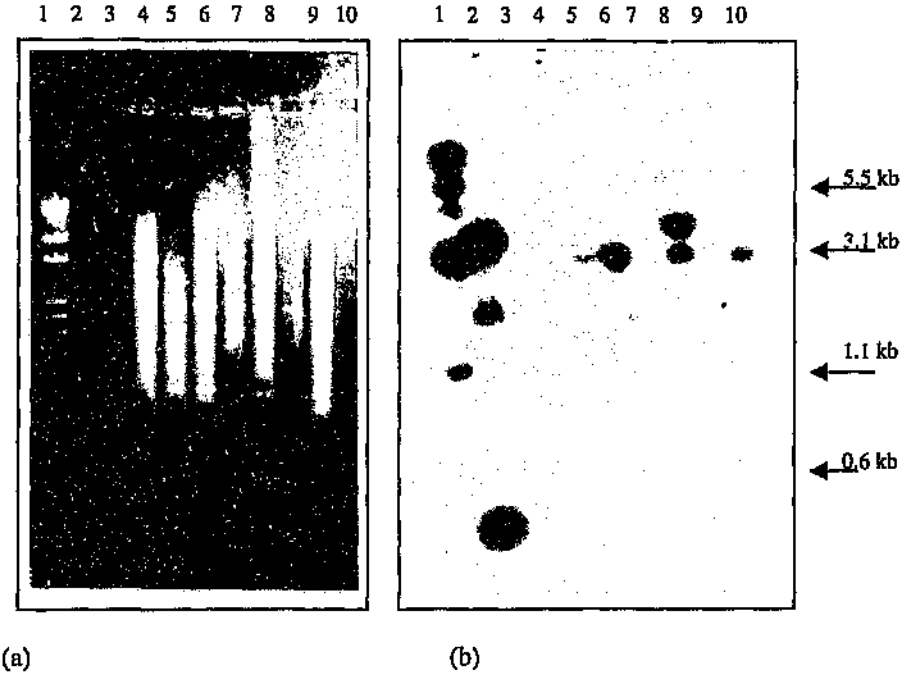


Figure 31: (a) Agarose gel electrophoresis and (b) Southern blot analysis of *Bam*HI-*Kpn* I chromosomal digests of mc^{2}155::pUSHδB transformed with pRCX-R1. Lane 1: DNA marker λIV; lane 2: pPS plasmid control; lane 4: mc^{2}155::pUSHδB; lanes 5-10: mc^{2}155::pUSHδB(pRCX-R4). Blots were probed with 0.6 kb fragment excised from pPS, as shown in Figure 26.

Based on the results of the complementation experiments, the ability of pRCX-R1 and pRCX-R4 to direct the expression of *M. tuberculosis rnhA-pgm*-encoded polypetides in *M. smegmatis* was assessed by Western blot analysis of mc^2155 and mc^2155::pUSHδB transformed with pRCX3, pRCX-R1, and pRCX-R4 respectively. The results of this experiment are shown in Figure 32. When probed with the anti-MBP::RNase HI antiserum, all extracts showed a common band at *ca.*37 kDa, and a new band, directed by pRCX-R4, was seen in both WT and SCO strains at *ca.* 54 kDa (lanes 3 and 7).

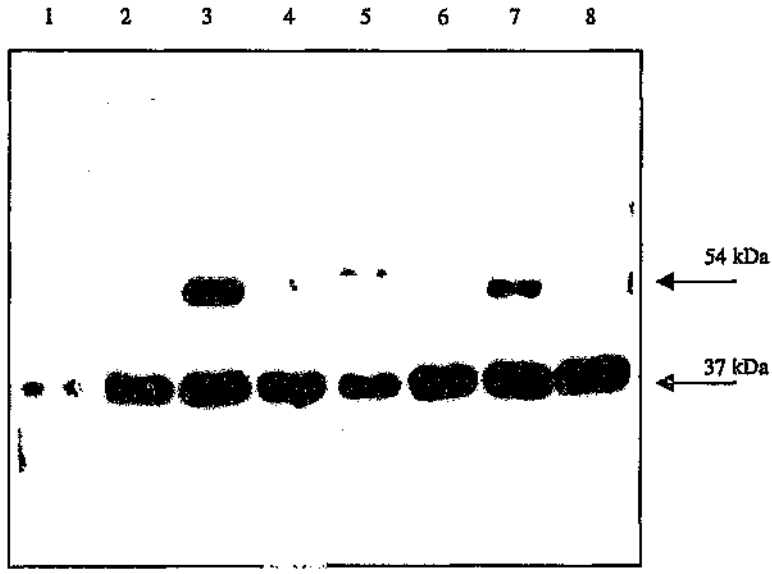


Figure 32: Western blot analysis of whole cell lysates of WT and SCO strains transformed with pRCX3, pRCX-R1, and pRCX-R4. Lysates were probed with Anti-MBP::RNase HI antisera (Serum 1,1:200 dilution). Lane 1: mc^2155; lanes 2-4: mc^2155 transformed with pRCX3, pRCX-R4, and pRCX-R1 respectively; lane 5: mc^2155::pUSHδB; lanes 6-8: mc^2155::pUSHδB transformed with pRCX3, pRCX-R4, and pRCX-R1 respectively.

Western blots, with Anti-MBP::RNase HI, were also performed against *E. coli* DH5α cell lysates carrying pRCX3, pRCX-R1, and pRCX-R4. Bands of *ca.* 76, 42, and 28 kDa were detected in each lysate, and were most likely due to non-specific binding of the antibodies. Therefore, the unique pRCX-R4-directed product only appears to be expressed in *M. smegmatis* (data not shown).

4. DISCUSSION

In this study, the *rnhA-pgm* ORF of *M. tuberculosis*, was cloned, and polypeptides overexpressed in *E. coli* for characterisation and analysis of this unusual gene fusion not previously associated with bacterial *rnhA*. As discussed in this section, new insights have suggested that the RNase HI-like domain, encoded by the N-terminal region of the gene, might have a function completely unrelated to that of RNase HI.

4.1 BIOCHEMICAL ANALYSIS

Cloning, gene induction, and protein purification. The *M. tuberculosis* sequences of interest, cloned in pMAL-c2 expression vector, were all shown to form translational fusions with the *malE* gene. The MBP::RNase HI recombinant fusion protein showed the highest yield after induction of gene expression and was purified to homogeneity after affinity chromatography. The MBP::RNase HI-PGM and MBP::PGM proteins showed lower yields than MBP::RNase HI following induction of gene expression. Low induction levels could be ascribed to instability of the mRNA transcript, or instability of the protein, rendering it susceptible to proteolysis by cellular proteases. Fractions of MBP::RNase HI-PGM and MBP::PGM proteins purified by column chromatography were contaminated with other polypeptides which were shown by Western blot analysis to correspond to degradation products. Degradation of MBP::RNase HI-PGM and MBP::PGM by *E. coli* proteases was investigated by including a cocktail of protease inhibitors in the lysis buffer. However, the protein obtained by purification in the presence of protease inhibitors was the same as that obtained in their absence, suggesting that the proteins were degraded by cellular proteases that were not inhibited by the protease inhibitor cocktail. Alternatively, these products may have been the result of prematurely

truncated polypeptides. Yields of recombinant proteins recovered by amylose affinity chromatography ranged from 10-30 mg/L. Based on the expression and recovery levels, it was therefore concluded that the proteins were predominantly located in the soluble cell extract. It has been reported that the typical yield of soluble fusion protein from an amylose column ranges between 10-40 mg/L, and variation in yield depends on the sequence cloned into the expression vector (NEB Protein Fusion and Purification System Manual).

Protease treatment. Upon treatment with Factor Xa, only partial cleavage of the recombinant proteins was observed, and no defined C-terminal products of the expected sizes corresponding to the *M. tuberculosis* polypeptides, were visible by Coomassie staining or Western blot analysis of SDS-PAGE gels. Factor Xa can cleave at non-canonical sites in some proteins and for some fusions, there is a correlation between instability of the protein of interest in *E. coli* and cleavage at additional sites. Presumably this cleavage activity at non-canonical sites depends on the three dimensional conformation of the fusion protein (NEB Protein Fusion and Purification System Manual). Similarly limited proteolysis of each of the recombinant fusion proteins with trypsin showed cleavage products, which were derived from the MBP domain. The inability to recover C-terminal products of the expected sizes might be due to complete degradation of the *M. tuberculosis* polypeptides, which may be accounted for by improper folding in *E. coli*.

Polyclonal antibodies and Western blot analysis. From the Western blot analysis it was evident that the MBP domain of the fusion proteins used for immunization was markedly more immunogenic than either the RNase HI or the PGM domains of the *M.*

tuberculosis protein. Anti-MBP::RNase HI antiserum detected a polypeptide of *ca.*37 kDa *in M. smegmatis* and *M. tuberculosis* cell extracts. Preliminary data showed that *M. smegmatis* does not contain a *rnhA-pgm* homologue in its genome, suggesting that this 37 kDa polypeptide does not correspond to the RNase HI-PGM fusion protein of *M.tuberculosis*. Although a second polypeptide of *ca.* 51 kDa was also detected in *M. tuberculosis*, its identity remains unknown. Antibodies raised against the MBP::RNase HI-PGM and MBP::PGM proteins, as well as the commercial anti-MBP antisera, detected a band of *ca.* 69 kDa in *M. tuberculosis* and *M. smegmatis* cell extracts. One could possibly ascribe this cross-reactivity to recognition of MBP-like or PGM-like proteins in *M tuberculosis* by the antisera. *M. tuberculosis* Rv1235 encodes an unknown lipoprotein, 468 aa in length, with high similarity (77.5%) to a protein in *M. leprae* which is putatively involved in maltose uptake. *M. tuberculosis* also contains several other phosphoglycerate mutase-like proteins encoded in its genome: (i) Rv0489 encodes for a 249 aa phosphoglycerate mutase 1 which is similar to PGM I of *E. coli*; (ii) Rv2419c is a probable phosphoglycerate mutase 2 of 223 aa, that shows homology to PGM2 of *E. coli*, and (iii) Rv3837c encodes a putative phosphoglycerate mutase 232 aa in length (Cole *et al.*, 1998). As is evident from the length of these polypeptides it is unlikely that any of the above candidates was responsible for the high molecular weight bands detected in the cell extracts.

Other investigators have also attempted to raise polyclonal antibodies against other RNases HI, and have shown similar conflicting results (Crouch and Cerritelli, 1998). The *C. fasciculata* RNH1 gene predicts a protein of 494 aa in length, with a molecular weight of 54.7 kDa, while the *S. cerevisiae* RNH I encodes an RNase HI with an expected molecular weight of 39 kDa. Antibodies raised against the 54 kDa *C.*

fasciculata RNase H expressed in *E. coli*, react with two proteins of 65 kDa and 56 kDa, in Western blots of extracts of *C. fasciculata* (Campbell and Ray, 1993). A renaturation gel assay gave sizes of 38 kDa and 45 kDa (Ray and Hirres, 1995). Although the reason why these two methods of detecting the size of *C. fasciculata* RNase H give different results is unclear, it has been attributed to the antibodies detecting proteins unrelated to RNase H in the cell extracts. Polyclonal antibodies were also raised against RNase H of *S. cerevisiae*, but have not been able to detect any RNase HI reacting with these antibodies in Western blots of extracts from *S. cerevisiae* (Crouch and Cerritelli, 1998). This may be attributed to the levels of RNase HI present in *S. cerevisiae* being detectable by the RNase H renaturation activity assay, but below the detection limit of Western blot analysis. This might provide explanation for similar results obtained with studies of the *M. tuberculosis* putative RNase HI. In conjunction, these data suggest that these structurally related RNase HI proteins are very poorly immunogenic.

4.2 FUNCTIONAL ANALYSIS

Complementation of the *E. coli* host MIC3037 with *M tuberculosis rnhA* supplied *in trans* was unsuccessful. *E. coli* is a heterologous host, and the levels of RNase H needed to alleviate the requirement for the RecBCD protein is very low (Mizrahi and Itaya, 1998). Failure to complement may have been due to the sensitivity of this host strain to β-lactamase (Mizrahi and Itaya, 1998). All plasmids used for complementation carried the ampicillin resistance marker, and its production in the cell may have exceeded the tolerance levels of the MIC3037 host strain. Although the RNase HI of *M. smegmatis*, produced as an MBP recombinant fusion protein, displayed RNase HI activity *in vitro* (Dawes *et al.*, 1995), the effect of the MBP

domain on RNase HI function, *in vivo*, remains unknown and hence might be a significant factor contributing to the lack of complementation in *E. coli*.

Complementation of the mycobacterial host strain, mc^2155::pUSHδB, with the putative *M. tuberculosis rnhA* supplied *in trans* on pRCX-R1 and pRCX-R4, was also unsuccessful. One possible interpretation of the failure of this complementation experiment is that pRCX-R1 and pRCX-R4 did not express functional RNase HI. Upon completion of the genome sequence of H37Rv (Cole *et al.*, 1998), it became apparent that the *rnhA-pgm* ORF is actually situated in an operon. As shown in Figure 33, the operon comprises four genes, of which the *rnhA-pgm* ORF is the fourth. Intriguingly, the first gene of the operon, corresponds to *cobD* which encodes an aminotransferase protein involved in cobalamin (CBL; coenzyme B_{12}) biosynthesis (Cole *et al.*, 1998). Although the second and third genes have unknown function, it is tempting to speculate that the entire operon is involved in CBL biosynthesis. This overall genetic arrangement shown in Figure 33 is well conserved in *M. leprae*, although a number of the genes of *M. leprae* are possible pseudogenes.

The plasmid constructs pRCX-R1 and pRCX-R4 contain a 3.6 kb fragment from the *M. tuberculosis* cosmid, which encodes only partial sequence from the operon, and therefore does not contain its own promoter. From the Western blot analysis of WT and SCO strains, there appears to be expression of a 54 kDa protein in *M. smegmatis* directed by pRCX-R4. Although the size does not correlate to any specific protein expected from the operon, the data suggest that transcription is being directed from this plasmid, and that the translated protein is recognised by anti-MBP::RNase HI antisera. Transcription could arise from a fortuitous promoter formed as a cloning

artifact as a result of the juxtaposition of DNA sequence in the construct, giving rise to a polypeptide of 54 kDa. In light of the partial sequence encoded in the shuttle vectors, it was unlikely that RNase HI was actually expressed *in trans* in the *M. smegmatis* host, which might account for the negative complementation result.

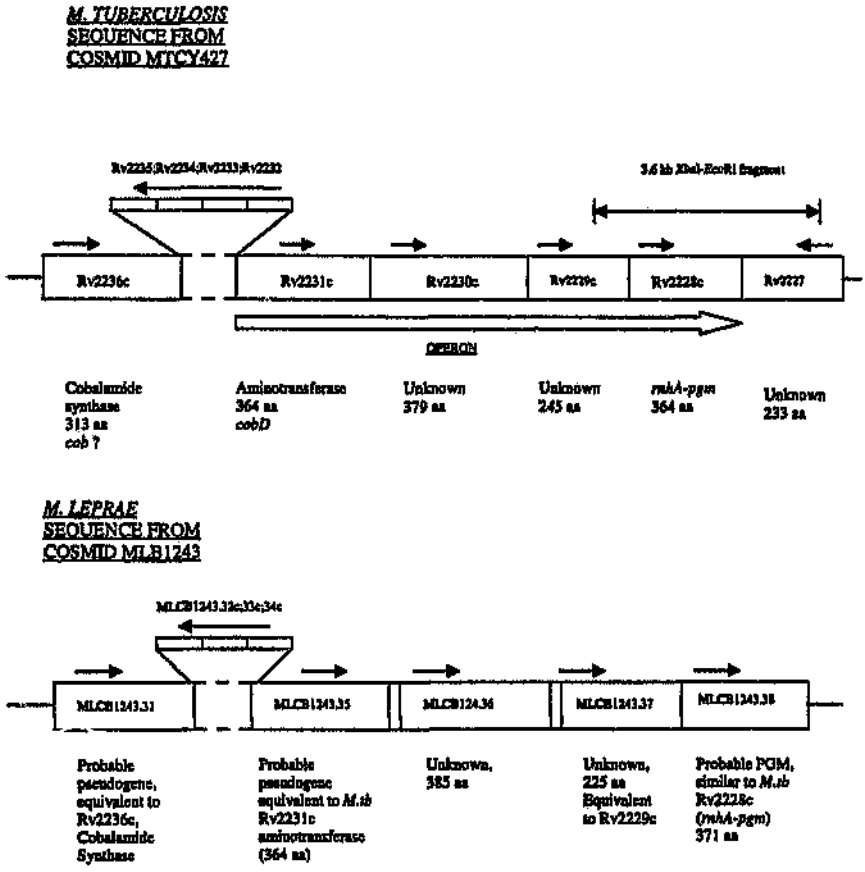


Figure 33: Structure of the *M. tuberculosis* operon containing the putative *rnhA-pgm* ORF. The 3.6 kb *Xba*I-*Eco*RI fragment which was cloned into pRCX3 is shown. The conserved genetic arrangement of this locus in *M. leprae* is also shown.

RNase HI functionality, as well as that of RNase HII and RNase HIII, has been investigated in several prokaryotic, eukaryotic, and archaeal species. An *rnhA* homologue, or the gene encoding a multidomain protein, in which the *rnhA*-like segment encodes one of these domains, has been found in *ca.* 20 organisms (Ohtani *et al.*, 1999). Similarly an *rnhB* homologue has been found in *ca.* 25 organisms. In *B. subtilis* a third RNase H encoded for by *rnhC*, was identified and designated RNase HIII (Ohtani *et al.*, 1999). *In vitro* evidence suggests that RNase HIII is functionally similar to RNase HI, although its primary structure is related to RNase HII. Evidence also suggests that the *rnhB* gene product (RNase HII) of *B. subtilis* may be functionally similar to *E. coli* RNase HII. The major RNase H activities detected in *E. coli* and *B. subtilis* cells may be ascribed to RNase HI and RNase HIII respectively. It has been proposed that the Mn^{2+}-dependent RNase HII is universally present in various organisms and Mg^{2+}-dependent RNase HIII, functions as a substitute for RNase HI in organisms which lack a functional *rnhA* gene (Ohtani *et al.*, 1999).

The availability of substantial microbial genome sequence information has suggested that bacteria, with the exception of the Mycoplasmas, all have two recognizable RNases H. In Mycoplasmas, only a single *rnhC*-like gene has been detected (Wenzel and Herrmann, 1989). The combination of *rnh*-like genes in bacteria varies in a nonobvious manner. Some organisms contain *rnhA* and *rnhB* genes (AB type), while others have *rnhB* and *rnhC* genes (BC type). As seen in Table 8 the combination of genes appears to be unrelated to the bacterial species.

Table 8: Types of RNases H in bacteria, eukarya, and archaea.

	Type of RNase H identified.*		
BACTERIA			
Mycoplasma	-	-	C+
Gram positives: *B. subtilis*	A-	B+	C+
E. faecalis	A+	?	?
M. tuberculosis	A?	B+	-
M. smegmatis	A+	B?	?
S. pneumoniae	-	B+	C+
Gram negatives: *E. coli*	A+	B+	-
A. aeolicus	-	B+	C+
EUKARYA	A+	B+	?
ARCHAE	-	B+	-

*Abbreviations: The presence of the letters A, B, or C indicates that the gene has been identified in the organism. Where no letter appears, the gene has not yet been identified in the organism; (–) indicates that the gene has no RNase H function; (+) indicates that the gene is probably functional; (?) indicates that the gene's presence and/or functionality is still undetermined.

The *rnhA* (*ypdQ*) gene of *B. subtilis* encodes an RNase HI homologue with 132 aa residues. This gene encodes a protein with no detectable RNase H activity *in vivo* or *in vitro* (Ohtani *et al.*, 1999). The proteins produced by the *B. subtilis*, *M. tuberculosis*, and *E. faecalis rnhA* homologues differ from other active RNases HI in two respects: (i) the replacement of Trp for His at the position, in which the active-site histidine residue is fully conserved (Kanaya *et al.*, 1990), and (ii) the absence of amino acids corresponding to the basic protrusion in the structure of *E. coli* RNase HI which is important for substrate binding (Keck and Marqusee, 1996). However in *M.*

tuberculosis, *B. subtilis*, and *E. faecalis* the four invariant acidic amino acid residues, which form the metal-ion binding site in RNase HI are conserved in the amino acid sequence (Cole *et al.*, 1998; Iwakura *et al.*, 1988; Bensing and Dunny, 1993). Conformational analysis of *B. subtilis* YpdQ/RNase HI suggested that this protein may fold into a structure related to that of *E. coli* RNase HI, and it is likely that the four invariant acidic residues do form a Mg^{2+} ion binding site in the *B. subtilis* homologue (Ohtani *et al.*, 1999). These data might suggest that an additional subunit may be required to supply the binding function provided by the basic protrusion in other RNases HI, or that the catalytic portion of RNase HI has been pirated, in effect, by another system, directing it to a completely different target molecule. A third possibility assumes that the protein has a function completely unrelated to the degradation of the RNA strand of an RNA-DNA hybrid.

The first gene of the operon in which the *rnhA-pgm* ORF is located, encodes the aminotransferase involved in the CBL biosynthetic pathway (Fig. 33). Five genes upstream from this operon is a gene encoding a probable cobinamide synthase (Cole *et al.*, 1998), also involved in CBL synthesis. Genes located together in an operon usually have related functions which are executed in a common biochemical pathway. Operonic genes are transcribed from a single promoter into a polycistronic mRNA molecule and will therefore also be co-ordinately regulated. The position of the *rnhA-pgm* ORF in an operon, including a gene involved in the biosynthesis of CBL, implies that *rnhA-pgm* might also be involved in this pathway. The *rnhA* catalytic portion may indeed be co-opted and used in this pathway for a totally unrelated function.

The *cobC* gene of *S.typhimurium* encodes a polypeptide of 26 kDa, which functions as a phosphatase specific for the CBL biosynthetic pathway in this organism (O'Toole *et al.*, 1994). Importantly, it has striking similarity to phosphoglycerate mutase, fructose-2,6-bisphosphatase, and acid phosphatase enzymes. *In vitro*, CobC dephosphorylates the CBL biosynthesis intermediate, N^1-(5-phospho-α-D-ribosyl)-5,6-dimethylbenzimidazole (α-ribozole-5'-P) to α-ribozole (N^1-α-D-ribosyl-5,6-dimethylbenzimidazole). The structure of α-ribozole-5'-P is shown in Figure 34. *In vivo*, the lack of CobC function blocks the synthesis of CBL from its precursors cobinamide and 5,6- dimethylbenzimidazole (DMB), and so prevents the assembly of the nucleotide loop of cobalamin (O'Toole *et al.*, 1994).

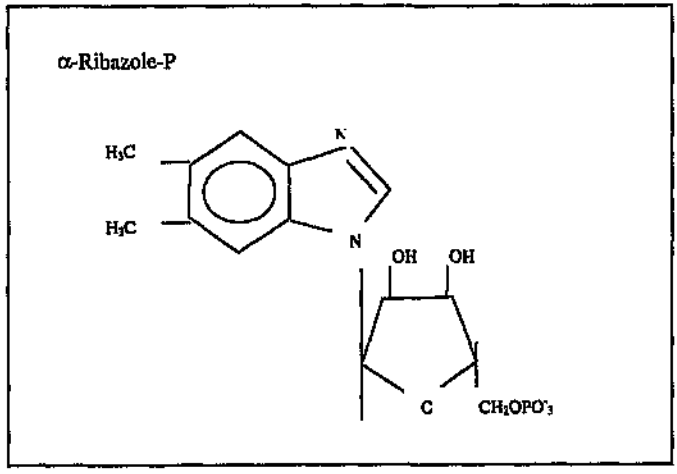


Figure 34: Chemical structure of the CobC substrate α-ribozole-5'-P.

Phosphoglycerate mutases (PGAM) and fructose-2,6-bisphosphatase enzymes have been demonstrated to have functional and structural homology, in addition to their amino acid sequence homology. Both classes of enzymes catalyse the removal of a phosphate moiety, and this activity is dependent on two highly conserved histidinyl

residues. The two conserved histidinyl residues are those thought to be critical in the active site of the phosphatases. The N-terminal histidinyl residue serves as an acceptor of the phosphate moiety, and the C-terminal histidinyl residue serves as a proton donor to the substrate molecule to complete the dephosphorylation reaction (Bazan *et al.*, 1989; Tauler *et al.*, 1990; Ostanin *et al.*, 1992). An alignment of *S. typhimurium* CobC to phosphoglycerate mutase, including the PGM of *M. tuberculosis*, and fructose-2,6-bisphosphatase enzymes is shown in Figure 35.

CobT has been demonstrated to be an N^1-α-phosphoribosyltransferase that catalyses the synthesis of α-ribazole-5'-P from DMB and nicotinic acid mononucleotide and mutants defective for CobT are, however, still proficient for CBL biosynthesis (Trzebiatowski *et al.*, 1994). CobC and CobT do not substitute for their respective activities *in vivo*, as thought initially, but the existence of other phosphatases can partially compensate for the lack of CobC function (O'Toole *et al.*, 1994). As evident from the alignment shown in Figure 35, the PGM domain encoded by *M. tuberculosis rnhA-pgm* contains the two catalytically essential histidinyl residues and shows sequence homology to CobC from *S. typhimurium*, making it a very likely candidate for being a CobC-like phosphatase responsible for carrying out the dephosphorylation of N'-(5-phospho-α-D-ribosyl)-5,6-DMB. The functional importance of the *rnhA-pgm* linkage in *M. tuberculosis* is unknown, but speculation may ascribe a metal chelating function to the RNase HI-like domain, for a dephosphorylation reaction. Assuming that, as in *B. subtilis*, the *M. tuberculosis* RNase HI domain adopts an *E. coli* RNase HI-like conformation, with the metal-ion binding site being conserved, the PGM domain may direct the RNase HI domain of *M. tuberculosis* to perform a function unrelated to RNase HI.

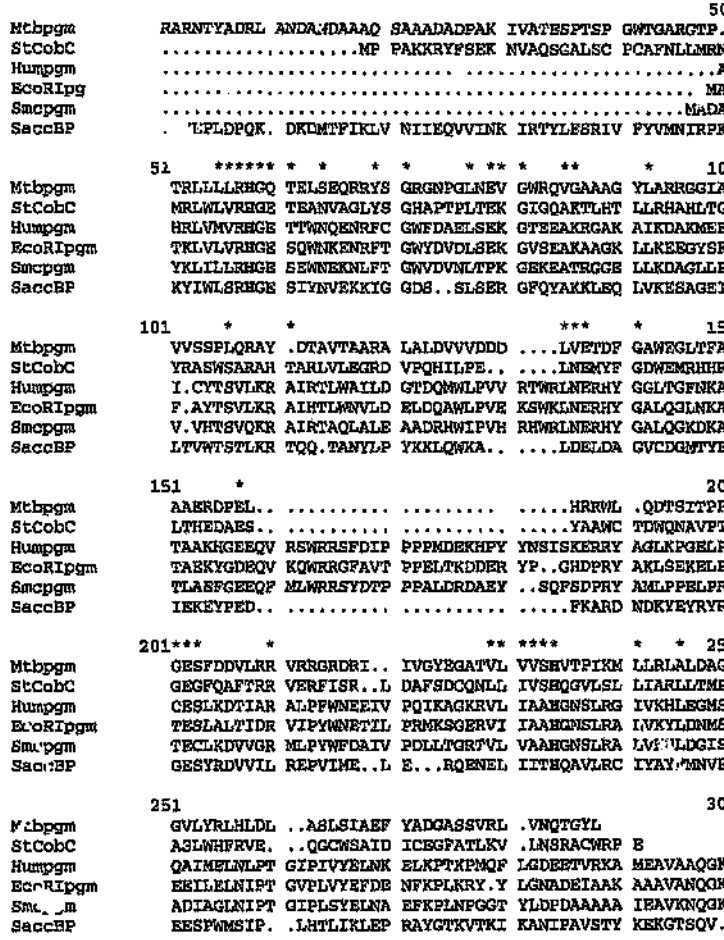


Figure 35: Homology of *S. typhimurium* CobC to phosphoglycerate mutase and fructose-2,6-bisphosphatase enzymes. The complete amino acid sequence of CobC is shown, whereas only the PGM-like domains of the other enzymes are shown. The amino acids absolutely or partially conserved are marked at the top of the alignment with an asterisk. Abbreviations: Mtbrp; PGM domain from *M tuberculosis* Rv2228c (Cole *et al.*, 1998); StCobC; CobC from *S. typhimurium* (O'Toole *et al.*, 1994); Humpgm: PGAM of *Homo sapiens* (Shanske *et al.*, 1987); Ecopgm: PGAM of *E. coli* (Davies and Davidson, 1982); Smcpgm: PGAM of *Streptomyces coelicolor* (White *et al.*, 1992); Saccbp; Fructose-2,6-bisphosphotase of *Saccharomyces cerevisiae* (Paravicini and Kretschmer, 1992). The two catalytic histidine residues are highlighted in bold.

Cobalamin biosynthesis has only been found to occur in prokaryotes, including aerobes, anaerobes, and facultative anaerobes of both the bacterial and archaeal domains, (Irion and Lungdahl, 1965; Krautler *et al.*, 1987; Mazumder *et al.*, 1986; Stuperich and Eisinger, 1988; Whitman and Wolfe, 1984; Wolf and Brey, 1986) and has been particularly well characterised in the enteric bacteria *S. typhimurium*, *E. coli*, and *P. denitrificans* (Blanche *et al.*, 1995; Roth *et al.*, 1993). Jeter *et al.* (1984) showed that *S. typhimurium* synthesizes cobalamin *de novo* only under anaerobic conditions. Two pathways for corrin ring formation, which is a main component of CBL, have been found. These include an aerobic pathway, in *P. denitrificans*, and an anaerobic pathway in *P. shermani* and *S. typhimurium*. It has been suggested that the CBL synthetic pathway may have evolved to allow anaerobic fermentation of small molecules in the absence of an external electron acceptor (Roth *et al.*, 1996). In contrast, very little is known about the role of CBL biosynthesis in mycobacteria. Studies on the synthesis of CBL by five reference strains of mycobacteria revealed that all five species synthesized CBL (Karasseva *et al.*, 1977). An optimum accumulation of CBL by BCG and *M. phlei* was observed on the 30th day of cultivation, addition of cobalt markedly enhanced the production of CBL in these two strains. CBL levels in *M. smegmatis*, *M. fortuitum*, and *M. asiaticum* were lower than those of BCG and *M. phlei* (Karasseva *et al.*, 1977). Genes for the biosynthetic pathway of CBL have been identified in *M. bovis* and *M. leprae*, and *M. tuberculosis*. *M. tuberculosis* contains all the genes needed for the CBL biosynthetic pathway, except for *cobF* and *cobO*, which are involved in corrin ring assembly (Cole *et al.*, 1998). The *cob* genes, in *M. tuberculosis* have not, however, been functionally characterised.

4.3 FUTURE RESEARCH PROSPECTS

The arguments presented above strongly support a functional role for the *rnhA-pgm* gene in *M. tuberculosis* in the CBL biosynthetic pathway. Further work to investigate this hypothesis is currently underway, which involves an assessment of phosphoribosyltransferase activity of the *M. tuberculosis* proteins by the TLC method described by O'Toole *et al.* (1994). Ideally this would involve developing a construct to produce a non-fusional recombinant protein from the *rnhA-pgm* ORF. Knockout constructs for inactivating the *rnh-pgm* of *M. tuberculosis* by allelic exchange have also been constructed in order to assess whether the *rnhA-pgm* mutants are auxotrophic for CBL. An interesting area of investigation would involve determining the conditions under which CBL might be essential to the survival of *M. tuberculosis.*

APPENDIX I

MEDIA

All media were sterilised by autoclaving at 121°C for 20 minutes.

Luria-Bertani Broth (LB) 10 g Tryptone; 5 g Yeast extract; 10 g sodium chloride per litre

L uria-Bertani Agar Plates (LA) 10 g Tryptone; 5 g Yeast extract; 10 g sodium chloride; 15 g agar, per litre.

2TY Broth 16 g Tryptone; 10 g Yeast extract; 5 g sodium chloride, per litre.

Minimal medium 1 x M9 salts; 1 mM magnesium sulphate; 0.1 mM calcium chloride; 1 mM thiamine.HCL; 0.2% glucose, per litre.

Middelbrook-Tween broth 4.7 g Middelbrook 7H9 broth base; (2 ml glycerol; 0.85 g sodium chloride)-autoclaved separately; 2.5 ml 20% Tween 80- filter sterilised and added after autoclaving.

Middlebrook Agar plates 18 g Middlebrook 7H10 agar powder; 10 ml glycerol-autoclaved and cooled to ca. 55°C; 100 ml OADC enrichment added.

OADC Enrichment 5 g/100 ml Bovine albumin, fraction V; 2 g/100 ml glucose; 0.85 g/100 ml sodium chloride; filter sterilised. Stored at 4°C.

GENERAL SOLUTIONS

10 x M9 salts 128 g $Na_2HPO_4.7H2O$; 30 g KH_2PO_4; 5 g NaCl; 10 g NH_4Cl.

Denhardt's Reagent (50x stock) 10 g/L Ficoll (Type 400; Pharmacia); 10 g/L polyvinylpyrrolidone; 10 g/L bovine serum; Albumin, fraction V (Sigma); Filter sterilise and store at –20oC.

Hybidisation Solution 0.5% SDS; 6X SSC; 5X Denhardt's Reagent; 50% deionised formamide.

Phosphate buffered saline (1 L) 8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4; pH 7.4

Rubidium Chloride Buffer 1 (RF-1) 12 g/L $RbCl_2$; 9.9 g/L $MnCl_2.4H_2O$; 1.5 g/L $CaCl_2.2H_2O$; 30 ml of 1M potassium acetate stock (pH7.5); 150 g/L glycerol; pH to 5.8 with 0.2 M acetic acid, filter sterile.

Rubidium Chloride Buffer 2 (RF-2) 20ml of 0.5 MOPS (pH6.8); 1.2 g/L $RbCl_2$; 11 g/L $CaCl_2.2H_2O$; 150 g/L glycerol; pH to 6.8 with NaOH, filter sterilise.

20X SSC 175.3 g/L NaCl; 88.2 g/L sodium citrate; pH adjusted to 7.0 with NaOH.

40% Acrylamide stock solution for PAGE (100 ml) 38 g acrylamide; 2 g bis-acrylamide; (deionised with 5 g Amberlite resin and filtered through Whatman 3MM filter paper. Stored at 4°C.

6% PAGE/7 M Urea solution (500 ml) 75 ml 40% acrylamide stock solution; 210 g Urea; 100 ml 5 x TBE buffer.

30% acrylamide stock for SDS-PAGE (100 ml) 30 g acrylamide; 0.8 g bis-acrylamide. Stored at 4°C

SDS-PAGE stacking gel 6% acrylamide; 0.176 M Tris pH6.8; 0.1% SDS; 0.035% TEMED; 25 mM ammonium persulphate.

SDS-PAGE resolving gel 12% acrylamide; 0.38 M Tris pH6.8; 0.1% SDS; 0.035% TEMED; 25 mM ammonium persulphate.

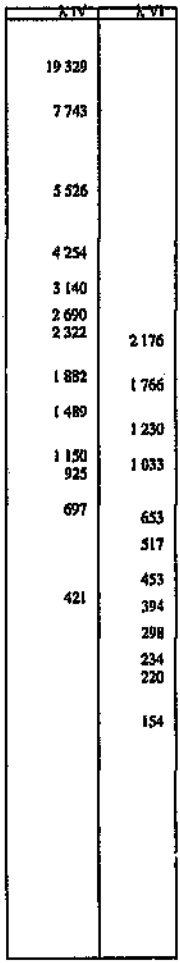
10X SDS-PAGE Running buffer (1 L) 30.3 g Tris base; 144 g glycine; 10 g SDS

8X Resolving buffer (100 ml) 36.3 g Tris base; 48 ml of 1 M HCl; pH 8.8

4X stacking buffer (100 ml) 6 g Tris base; 48 ml of 1 M HCl; pH6.8

APPENDIX II

DNA marker sizes



APPENDIX III

LIST OF ABBREVIATIONS

aa	amino acids
Amp	ampicillin
aph	gene encoding Km^R
BCG	bacille Calmette-Guèrin
bla	gene encoding Amp^R
bp	base pairs
CBL	cobalamin
cobC	gene encoding a phosphatse enzyme in CBL boisynthesis
cobD	gene encoding an aminotransferase enzyme in CBL biosynthesis
DCO	double cross over
DMB	dimethylbenzimidazole
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid
HIV	Human Immunodeficiency Virus
hyg	gene encoding Hyg^R
Hyg	hygromycin B
IPTG	isopropyl-α-D-galactopyranoside
kb	kilobases
Km	kanamycin
LA	Luria-Bertani agar
LB	Luria-Bertani broth
MBP	maltose binding protein
MDR	multi-drug resistant
NEB	New Enland Biolabs

OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGM	phosphoglycerate mutase
^{R}	Resistance
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
rnhA	gene encoding RNase HI
sacB	gene encoding levansucrase
SCO	single cross over
SDR	stable DNA replication
SDS	sodium dodecyl laurel sulphate
ss	single stranded
Suc	sucrose
TB	tuberculosis
TEMED	N,N,N',N',-tetramethylethylenediamine
Tween	polyoxyethylene sorbitan monooleate
X-gal	5-bromo-4-chloro-3-indolyl-α-D-thiogalactopyranoside
WHO	World Health Organisation
WT	wild type
ypdQ	*rnhA* homologue in *B. subtilis*

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Author:Thomsen ML
Name of thesis:Structure-Function Studies of a Putative Ribonuclease HI from mycobacterium

PUBLISHER:
University of the Witwatersrand, Johannesburg
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