IDENTIFICATION AND CHARACTERISATION OF PROTEASES IN *MYCOBACTERIUM TUBERCULOSIS*

by

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To my parents

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TABLE OF CONTENTS

ABSTRACT

Virulence determinants of *M. tuberculosis* remain largely unknown. Of key interest has been the ability of the bacterium to survive intracellularly within its host cell, the macrophage, and its ability to cause extensive tissue necrosis. Exported proteases are commonly associated with virulence in bacterial pathogens, yet their role in *Mycobacterium tuberculosis* has virtually not been studied. Preliminary experiments showed *M. tuberculosis* culture filtrates contained a proteolytic activity inhibited by mixed serine/cysteine protease inhibitors and activated by Ca^{2+}, features typical of some serine proteases, notably subtilisins, and possibly metalloproteases. Purification attempts were unsuccessful. A family of five genes that encode putative, secreted, serine proteases has recently been described in *M. tuberculosis*. These proteases share 36-47% sequence identity and are all encoded with putative signal peptides, suggesting that they are translocated across the cytoplasmic membrane. One member, *mycP1*, was selected for further study. The gene product, mycosin-1, was 30-35% identical to bacterial subtilisin-like serine proteases and contained the classic catalytic triad and oxyanion hole. Mycosin-1 also contained a typical signal peptide, a likely propeptide, and a C-terminal hydrophobic sequence with a high transmembrane potential. Topology analyses predicted mycosin-1 to be a type I ectoprotein. Consistent with this, expression of mycosin-1 in *M. tuberculosis* and in *Mycobacterium smegmatis* transformed with *mycP1* (*M. smegmatis*-P1) was limited strictly to the cell envelope, as seen by Western blotting, and immunogold electron microscopy. Only full-length, 50-kDa mycosin-1 was observed by Western blotting in broth-grown *M. tuberculosis* and *M. smegmatis*-P1 lysates, whereas a 40-kDa species was detected in 6-week *M. tuberculosis* culture filtrates. A similar 40-kDa immunoreactive band was also observed in lysates of macrophages infected with *M. tuberculosis*, consistent with robust transcription of the

mycP1 gene during growth in macrophages. Since putative mature mycosin-1 has a molecular weight of 38.6 kDa, the 40-kDa protein may represent activated mycosin-1 after propeptide cleavage. In conclusion, mycosin-1 is an exported, cell envelope-associated subtilisin homolog that is expressed during growth of *M. tuberculosis in vitro* and in macrophages.

INDEX OF TABLES

INDEX OF FIGURES

ABBREVIATIONS

AG	arabinogalactan
AIDS	acquired immunodeficiency syndrome
ALLM	N-acetyl-Leu-Leu-methional
ALLN	N-acetyl-Leu-Leu-norleucinal
ATCC	American Tissue Culture Collection
BAL	bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
BoNT	botulinus neurotoxin
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CMI	cell-mediated immunity
CnBr	cynogen bromide
cpm	counts per minute
CR	complement receptor
CTL	cytotoxic T cell
ddH_2O	distilled deionised water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DOTS	directly observed treatment, short-course
DTT	dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamido-[4-guanidino]-butane
EDTA	ethylenediaminotetraacetic acid
EM	electron microscopy
ETZ	electron transparent zone
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage-colony stimulating factor
GSH	Groote Schuur Hospital
GST	glutathione-S-transferase
h	hour/s
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]
HIV	human immunodeficiency virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobases
kDa	kilodaltons
LA	Luria agar
LAM	lipoarabinomannan
LB	Luria broth
L-J	Loewenstein Jensen
LOS	lipoologosaccharides
MB	Middlebrooks

MBP	mannose binding protein
MDR	multi-drug resistant
MHC	major histocompatibility complex
MR	mannose receptor
μCi	microcuries
μg	micrograms
μm	micrometers
min	minute/s
ml	millilitre
MR	mannose receptor
MW	molecular weight
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanograms
NSF	*N*-ethylmaleimide-sensitive fusion protein
PAGE	polyacrylamide gel electrophoresis
pBS	pBluescript
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGL	phenolic glycolipid
pI	isoelectric point
PIM	phosphatidyl inositol mannoside
PMSF	phenylmethanesulfonyl fluoride
psi	pounds per square inch
RbCl	rubidium chloride
RD1	deletion region 1
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
sdH_2O	sterile distilled water
SD	standard deviation
SDS	sodium dodecyl sulphate
SNAP	soluble NSF-attachment protein
SNARE	SNAP receptor
SOD	superoxide dismutase
SP-A	surfactant protein A
TACO	tryptophane aspartate-containing coat protein
TB	tuberculosis
TCA	trichloroacetic acid
TeNT	tetanus neurotoxin
TGF	transforming growth factor
TLCK	L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone HCl
TM	transmembrane
TNF	tumor necrosis factor
TPCK	L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone
Tris	tris(hydroxymethyl)aminomethane
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organisation

INTRODUCTION

As the 21st century approaches, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, remains the scourge of mankind, accounting for 8 million new cases of tuberculosis and 2.9 million deaths from the disease annually (Bloom *et al.*, 1992). It is estimated that one third of the world's population harbors *M. tuberculosis*, placing them at risk of developing the disease. Despite the significant morbidity and mortality rate caused by this disease, research into the pathogenesis of tuberculosis has been slow. This was partly due to the initial successes of First World countries in controlling this disease, and partly due to the difficulty in culturing and manipulating *M. tuberculosis*. The lack of research directed towards *M. tuberculosis* infection ensured that, in the mid to late 1980's, when there was an increasing incidence of tuberculosis worldwide, the numerous theories regarding the pathogenesis of tuberculosis remained unproven.

In response to the increasing global incidence of tuberculosis, new research programmes and international collaborations were established to study the physiology, biochemistry and molecular biology of *M. tuberculosis*, with the ultimate aim of identifying novel drug targets and developing an effective vaccine. Of key interest have been the ability of the *M. tuberculosis* bacillus to survive the microbicidal mechanisms of its host cell, the macrophage, and the ability of the bacillus to cause tissue pathology, characterised by cavity formation. However, despite the upsurge in tuberculosis research, mycobacterial virulence determinants remain largely unknown (virulence is defined as the competence of an infectious agent to produce pathological effects).

Proteases are produced by an array of pathogenic microorganisms. Many of these proteases are actively involved in the ability of the microorganism to establish disease, and

are especially prevalent in microorganisms that cause tissue destruction. At the outset of this study, little was known about protease activities in *M. tuberculosis* and, apart from a few preliminary reports, the subject received sparse attention. The hypothesis that *M. tuberculosis* produces proteases that actively contribute to the pathogenesis of tuberculosis formed the basis of the present work, and to that end, several approaches were taken to identify and characterise protease-encoding genes and proteolytic activities in this organism. In addition to increasing our knowledge of *M. tuberculosis* biochemistry and physiology, the discovery of proteases may provide novel drug targets that could facilitate the development of new therapies.

This thesis begins with two literature review chapters detailing essential aspects of *M. tuberculosis* (Chapter 1) and the roles of proteases in infectious diseases (Chapter 2). These chapters are followed by experimental chapters that describe the identification of proteolytic activity in *M. tuberculosis* culture filtrates (Chapter 3), as well as the initial characterization of mycosin-1, the first member of a novel family of putative subtilisin-like serine proteases (Chapters 4-7). The characterization of mycosin-1 includes an analysis of its predicted sequence (Chapter 4); PCR amplification of the *mycP1* gene, cloning and expression of *mycP1* in *E. coli* and *M. smegmatis*, and detection of mycosin-1 in *M. tuberculosis* (Chapter 5); localisation of mycosin-1 (Chapter 6); and, finally, detection of mycosin-1 expression *in vivo* (Chapter 7).

CHAPTER 1

Mycobacterium tuberculosis

1.1. INTRODUCTION

The origin of *M. tuberculosis* has been hotly debated for many years. The favoured view is that *M. tuberculosis* originated from a mutant of *M. bovis*, the causative agent of tuberculosis in cattle, other animals, and occasionally humans. Therefore, the first humans with tuberculosis likely contracted the disease through the consumption of infected beef or milk. This probably occurred in the Neolithic period (4000-7000 BC) when cattle first became domesticated (Manchester, 1984). Interestingly, this period

corresponds with the earliest evidence of human tuberculosis (Sager *et al.*, 1972). Most reports suggesting the presence of tuberculosis are based on the identification of tuberculous-like lesions in human skeletal remains. However, these reports should be cautiously interpreted as several other afflictions (for example, bacterial and fungal infections, trauma, and bone tumors) cause bone lesions resembling those of tuberculosis. Although there are reports indicating tuberculosis in humans dating back to 5000 BC, the earliest definitive evidence dates back to the Dynastic period (which started about 3500 BC), where evidence of pulmonary and vertebral tuberculosis has been found in Egyptian mummies (Zimmerman, 1979). In addition, microscopic examination of vertebral lesions in these mummies revealed the presence of acid-fast bacilli, presumed to be *M. tuberculosis* and *M. bovis*.

With the advent of the first chemotherapeutic agents for tuberculosis control in 1952, the incidence of tuberculosis began to steadily decline in First World Countries. However, since 1985, First World Countries have experienced a steady increase in the incidence of tuberculosis (Murray, 1989; Cantwell *et al.*, 1994). It has been suggested that this increase is linked to the burgeoning HIV epidemic and the emergence of multi-drug resistant strains of *M. tuberculosis* (Selwyn *et al.*, 1989; Bloch *et al.*, 1994). Developing countries, however, have not enjoyed any respite from this disease and continue to be plagued by the pathogenic effects of *M. tuberculosis* infection.

In 1991, in response to the increasing world incidence of tuberculosis, the World Health Organisation (WHO) recommended that each National Tuberculosis Programme institute tuberculosis control measures that work towards attaining two objectives by the year 2000. Firstly, to treat successfully 85% of detected smear-positive cases, and secondly, to detect 70% of all such cases. The vast increase in the global incidence of tuberculosis further prompted the WHO in March 1993 to declare tuberculosis a global emergency. In the same year, the WHO established a worldwide surveillance and

monitoring project and adopted a programme of directly observed treatment, short-course (DOTS) as its strategy for global tuberculosis control. The need to take 4 drugs (isoniazid, rifampicin, ethambutol, and pyrazinamide) for a period of 6 months has fostered a large amount of non-compliance with this medication in afflicted communities. Furthermore, non-compliance has resulted in the devlopment of partially treated tuberculosis and the emergence of drug-resistant *M. tuberculosis* strains. The DOTS strategy seeks to bypass the non-compliance problem by ensuring that trained personnel directly observe tuberculosis patients to be taking their medication. Initial success (cure rates of 80-90%) was achieved with using DOTS in areas of China and Africa (Murray *et al.*, 1991; China Tuberculosis Control Collaboration, 1996). However, implementation of DOTS by national programmes has been slow, and in those countries where DOTS has been used, only a small proportion of tuberculosis patients have access to the programme. Despite these initial problems, DOTS has achieved a measure of success (summarised in Table 1.1).

Table 1.1. Key findings of the 1999 WHO Tuberculosis Report

1) 76% of the global population, encompassing an estimated 85% of tuberculosis patients, were living in countries that had adopted DOTS. However, only 35% of the global population (2.1 out of 5.8 billion), and 35% of tuberculosis cases, lived in areas where DOTS was available.
2) 12% of all estimated tuberculosis cases, and 16% (10% in 1995) of all smear-positive cases, were detected under DOTS. Despite these low detection rates, the detection of smear-positive cases using DOTS has been increasing at about 100 000/year since 1994. At this rate the global target of 70% case detection would be reached by 2015. However, by adding 250 000 extra cases/year (10% of the unnotified cases living in DOTS countries), this target could be reached by 2005.
3) Treatment success rates under DOTS varied from 58% in the African region to over 80% in the American, Eastern Mediterranean and Western Pacific regions. Overall success rates (1994-1997) were 77-79% under DOTS and 54-57% worldwide.

Although the benefits of DOTS are beginning to be appreciated, an epidemiological model of tuberculosis projects that even with optimal uptake and

implementation of DOTS the morbidity and mortality from tuberculosis in the next three decades will be enormous (Murray *et al.*, 1998). This model predicts a total of 225 million new cases and 79 million deaths from tuberculosis between 1998 and 2030. In addition, Murray *et al.* (1998) suggest that active case-finding by mass miniature radiography could save 23 million lives in the same time period. This is in direct contrast to the WHO recommendation of passive case-finding as the primary strategy for detecting tuberculosis.

In 1908 Calmette and Guerin began work on a virulent bovine strain of *M. tuberculosis*. After 13 years and 230 subcultures this strain [termed the bacillus of Calmette and Guerin (BCG)] lost its virulence for animals (Grange *et al.*, 1983). The resulting vaccine was first used to immunise a child in 1921 and subsequently has been used to immunise more than 1 billion children in more than 182 countries (Huebner 1996). There has been much speculation on the BCG vaccine as the many clinical trials set up to evaluate its efficacy have provided variable results (Fine & Rodrigues, 1990; Bloom & Fine, 1994; Colditz *et al.*, 1994; Huebner 1996). However, there is general consensus that BCG vaccination protects children from disseminated disease and from tuberculous meningitis (Bloom *et al.*, 1992; Rodrigues *et al.*, 1993). With increasing age the degree of protection wanes resulting in little or no protection after 10-15 years (Styblo & Meijer, 1976; Rodrigues *et al.*, 1993). Most clinical trials show a wide variation in efficacy of the BCG vaccine in adults (i.e., 0-77%), suggesting that childhood vaccination may be ineffective in the prevention of tuberculosis in adults (Bloom et al., 1992; Huebner, 1996). Murray *et al.* 1998 suggest that a new vaccine with 50% efficacy could lower the incidence of tuberculosis by 36 million cases and mortality by 9 million cases over the next 30 years. Thus, extensions to the DOTS programme are required if the morbidity and mortality produced by *M. tuberculosis* are to be curtailed over the next 30 years.

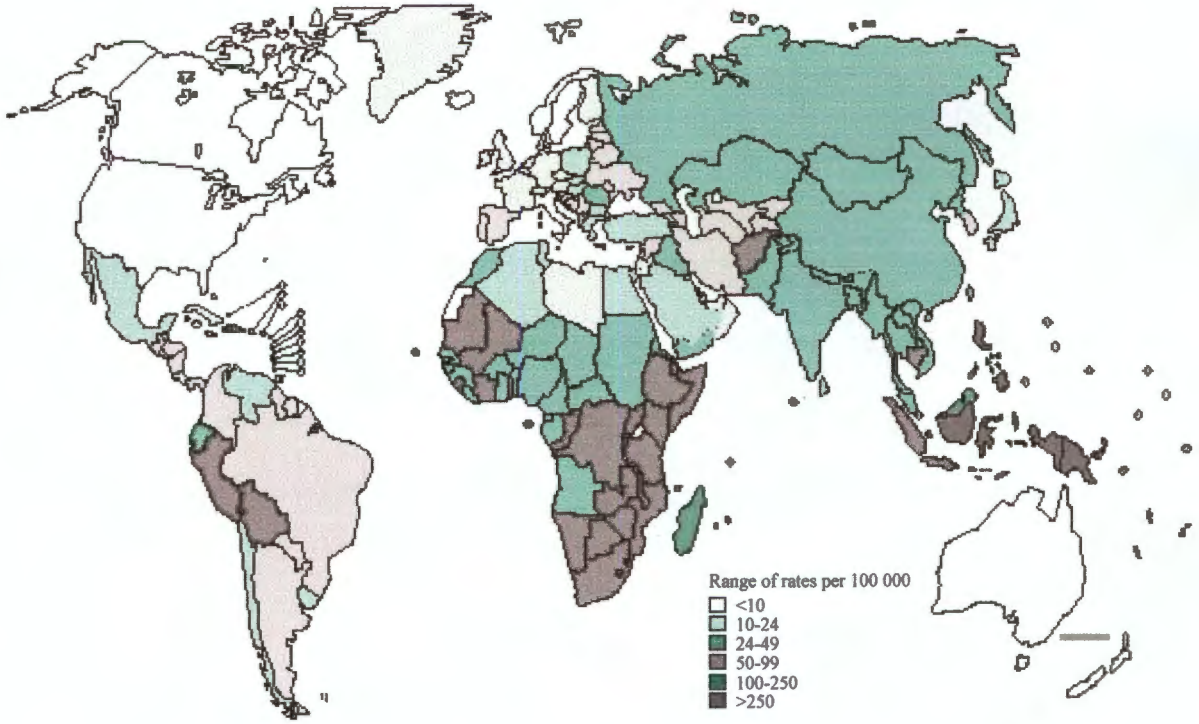
Clearly, the predicted morbidity and mortality figures resulting from *M. tuberculosis* infection indicate that a cohesive and co-ordinated world collaboration directed towards the eradication of tuberculosis from all communities is urgently required.

In this chapter, the essential features of the *M. tuberculosis* bacillus are reviewed, focusing on the mechanisms used to overcome host defenses and how it establishes disease. In addition, recent advances in the understanding of various aspects of *M. tuberculosis* biochemistry, physiology and molecular biology are discussed.

1.2. INCIDENCE

In the past, data reflecting the world-wide incidence of tuberculosis has been inaccurate and notification rates poor. Therefore, National Programmes and the scientific community relied on estimates. Now, through the endeavours of the WHO Tuberculosis Programme, accurate data and increasing notification rates are emerging that paint a gloomy picture, particularly in the Developing World. History has proven that the brunt of the tuberculosis burden falls on the disadvantaged communities of the world, and some suggest that it has become a useful barometer of the standard of living and equity in any society (Coovadia & Benatar, 1991). This is confirmed by the data recently released by the WHO Tuberculosis Programme that indicates a higher incidence of tuberculosis in Third World Countries and countries in which a significant proportion of the population are disadvantaged. Those countries most affected by the tuberculosis epidemic are to be found in Africa (including South Africa, Tanazania, Kenya, and Uganda), Asia (including Vietnam, Cambodia, India, and China), South America (including Peru), the Phillipines, and Indonesia (Figure 1.1A). The highest rates of HIV/TB co-infection are found in sub-Saharan Africa, with significant co-infection rates also present in the rest of Africa, India, Indonesia, and Peru (Figure 1.1B). First World Countries have also

A



B

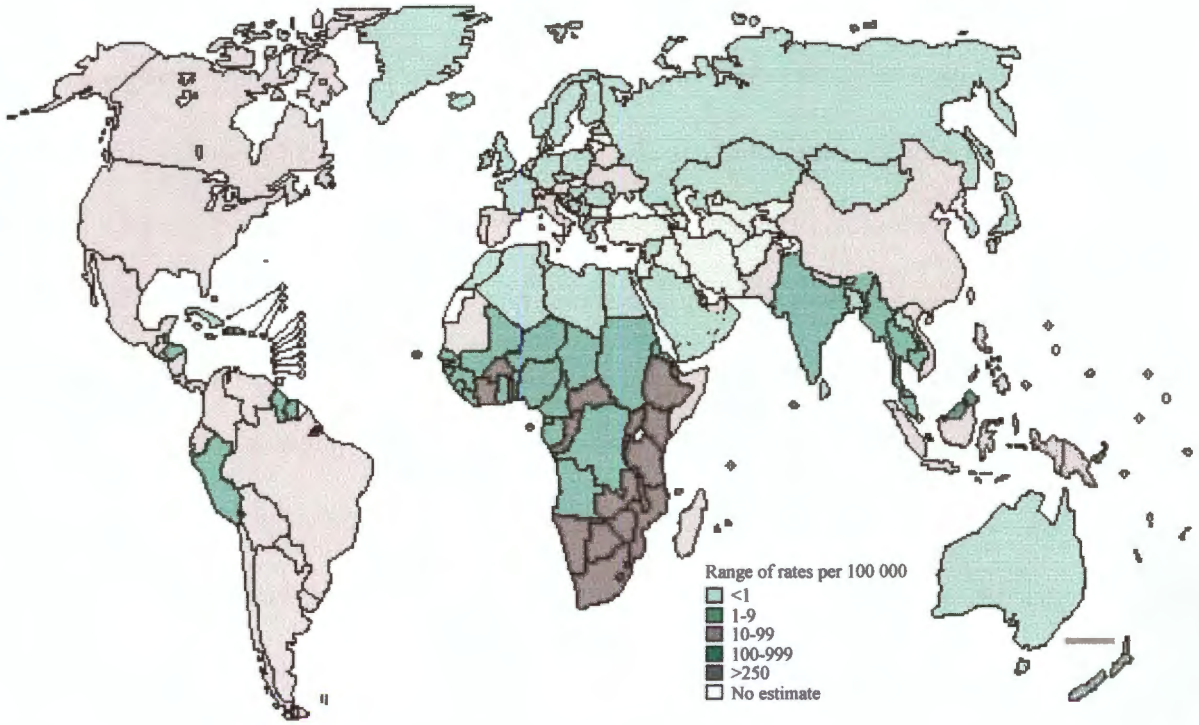


Figure 1.1. Global incidence of tuberculosis. A) Global incidence of tuberculosis and B) Global incidence of HIV/tuberculosis co-infection. Both maps were constructed with data from 1997 and are obtained from the 1999 WHO Tuberculosis Report.

experienced a steady increase in the number of tuberculosis patients and *M. tuberculosis* is now a significant player in the pathogenic microbial world of these countries.

Amongst the reasons suggested for the increase of tuberculosis in First World Countries are unemployment, increasing numbers of immigrants from Third World Countries, the global increase in HIV infection, drug abuse, and the emergence of drug-resistant strains (Bloom & Murray, 1992).

The global increase in HIV infection has had a major influence on the rise of tuberculosis, especially in the Developed World (Bloom *et al.*, 1992). Since cell-mediated immunity is essential for controlling *M. tuberculosis* infection, tuberculosis is frequently the first indication of HIV infection which characteristically has a lowered CD4 count resulting in a dampened cell mediated immune response (Barnes *et al.*, 1991; Jones *et al.*, 1999). HIV-infected tuberculosis patients are more likely to present with extrapulmonary disease (i.e., miliary TB, meningitis, and lymphadenitis) (Bloom et al., 1992) and are more likely to develop life-threatening manifestations of tuberculosis (Barnes *et al.*, 1991). Furthermore, evidence is accumulating suggesting that tuberculosis may accelerate the course of HIV infection (Barnes & Modlin, 1996). With the rampant spread of HIV infection through the Developing World it becomes evident that to prevent a logarithmic increase in the number of deaths from HIV and/or tuberculosis strict control measures as well as novel therapies are urgently required.

1.3. THE MYCOBACTERIAL CELL ENVELOPE

Traditionally the mycobacterial cell envelope has generated much interest and excitement as it was thought to be unique and appeared to have immunogenic components. Accordingly, over many years, the mycobacterial cell envelope has been the target of enthusiastic research as investigators were hoping it contained the secret to the pathogenic success of *M. tuberculosis*. However, the true contribution of the

mycobacterial envelope to the virulence of these pathogens remains uncertain despite enormous efforts to understand its structure and function. Although most of the core components are not unique to mycobacteria, the arrangement nevertheless is such that it forms a structure that is unusual and characteristic for its genus. The cell envelope consists of the plasma membrane, cell wall and an outer loosely attached 'capsule'. To date, the capsule has only been demonstrated in pathogenic mycobacterial species, however it is unknown whether or not the capsule is restricted to pathogenic mycobacteria (Daffé & Etienne, 1999).

1.3.1. Molecular composition

1.3.1.1. Plasma membrane

The plasma membrane is a typical bacterial cytoplasmic membrane and within the mycobacteria genus all are similar (Akamatsu *et al.*, 1966; Kumar *et al.*, 1979; Silva & Faccioli, 1983). It consists of polar lipids that are assembled, in association with proteins, into a lipid bilayer. The main lipids are phospholipids, of which phosphatidylinositol mannoside (PIM), phosphatidylglycerol, cardiolipin and phosphatidylethanolamine predominate. Small amounts of phosphatidylinositol and menaquinones (2-methyl-polyprenol-1,4-naphthoquinone) are also present (Goodfellow & Minnikin, 1984). Ornithine lipids and the carotenoid, leprotene, have been found in *M. tuberculosis* cultures, but, there appears to be uncertainty about their precise location (David, 1984; Laneelle *et al.*, 1990). The fatty acid components of the phospholipids consist mainly of octadecenoic (stearic), octadecenoic (oleic), and tuberculostearic (10-methyl stearic) fatty acids (Okuyama *et al.*, 1967). The glycosylphosphopolyprenols, phosphodecaprenol, and phosphooctahydroheptaprenol, are thought to be plasma membrane associated (Takayama & Goldman, 1970; Besra & Chatterjee, 1994).

Another molecule thought to be plasma membrane associated is lipoarabinomanan (LAM). LAM, a polysaccharide with broad immunological activity, is composed of arabinose and mannose units and is covalently linked to a phosphatidylinositol group (Hunter & Brennan, 1990). Although its location is unknown it is presumed that the phosphatidylinositol group anchors it in the plasma membrane. Two recently suggested models show LAM spanning the cell wall skeleton or linking the plasma membrane and cell wall skeleton (Daffé & Draper, 1998). Most of the arabinan termini are capped with oligomannosyl units (ManLAMs) in the slow growers (for example, *M. tuberculosis* and *M. bovis*) and inositolphosphate groups in the fast growers (for example, *M. smegmatis*) (Chatterjee *et al.*, 1992; Venisse *et al.*, 1993; Khoo *et al.*, 1995). Lipomannan, structurally related to LAM but lacking the arabinan segment, is probably also anchored in the cell membrane (Hunter & Brennan, 1990).

To date, few proteins have been found associated with the plasma membrane. Compounding this has been the difficulty in subfractionating *M. tuberculosis* envelope components and separating out contaminating polysaccharides. Lee *et al.* (1992) have shown a 16-kDa protein with some similarity to low-molecular-weight heat shock proteins to be the major membrane protein in *M. tuberculosis* Erdman. The recent completion of the *M. tuberculosis* genome sequence has indicated the presence of numerous putative membrane proteins that await experimental study.

1.3.1.2. Cell wall

The cell wall consists of a covalently linked cell-wall skeleton that is non-covalently associated with a variety of glycolipids (Table 1.2), glycopeptidolipids and proteins. The cell-wall skeleton consists of peptidoglycan, arabinogalactan and mycolic acids that are all covalently linked.

The peptidoglycan layer (made up of alternating glucosamine and muramic acid units) forms the backbone and although similar to the commonest bacterial peptidoglycan (i.e., Alγ), it differs in that the muramic acid is N-glycolylated instead of N-acetylated and the crosslinks include bonds between two diaminopimelic acid residues as well as between diaminopimelic acid and D-alanine (Adam *et al.*, 1969; Wietzerbin *et al.*, 1974).

The next layer is comprised of arabinogalactan (AG) which is attached to the peptidoglycan layer by an oligosaccharide linker, galactofuranosyl, that is in turn attached through a phosphodiester bond to a muramic acid residue of peptidoglycan (McNeil *et al.*, 1990). AG is a polysaccharide consisting of a backbone of galactose units with three chains of arabinose units branching off from near its reducing end (Daffé *et al.*, 1990, 1993). The non-reducing termini of the arabinan chains consist of pentaarabinosyl residues to which the mycolic acids are attached by an ester link to arabinose (Azuma *et al.*, 1968; Kanetsuna *et al.*, 1969). These mycolic acids are clustered in groups of four and are present on two thirds of the pentaarabinosyl units in *M. tuberculosis* (McNeil *et al.*, 1991). The pentaarabinosyl units in other mycobacterial species are less mycoloylated. The mycolic acids (long-chain α-branched β-hydroxy fatty acids) make up the final layer of the cell wall skeleton and have long carbon chain lengths (50 to 60 carbon atoms with a branched α-side chain containing another 24 carbon atoms). This confers wax-like properties, hence contributing to the hydrophobicity and impermeability of the envelope.

1.3.1.3. Capsule

The presence of a mycobacterial capsule has been a topic of debate for some time (reviewed in Daffé & Etienne, 1999). Technical artefacts cast doubt over the electronmicroscopic detection of an electron transparent zone (ETZ) outside the bacterial wall but surrounded by the phagosomal membrane. However, as electron microscopic

techniques have been improved, the existence of a capsule now has greater credibility. This has been mainly due to improved electron microscopy sample preparation afforded by embedding mycobacteria using the freeze-substitution technique (Graham & Beveridge, 1990; Paul & Beveridge, 1992, 1994). This technique has improved the ultrastructural visualisation of the outer layer of the envelope, by maintaining the integrity of hydrated structures and minimising lipid extraction, thereby stabilising cell structures.

Using both physical (glass-bead agitation) and detergent extraction techniques, the components of the capsule have been recently characterised and demonstrated to consist mainly of polysaccharides and protein (Ortalo-Magné *et al.*, 1995). There is inter-species variation in the ratio of these 2 components with polysaccharides the predominant constituent in pathogenic species (*M. tuberculosis*, *M. kansasii*, and *M. gastri*) and protein in non-pathogenic species (*M. smegmatis* and *M. phlei*) (Ortalo-Magné *et al.*, 1995; Lemassu *et al.*, 1996). The major polysaccharides are a 100-kDa D-glucan (the major capsular polysaccharide in *M. tuberculosis*), 13-kDa D-arabino-D-mannan (in slow-growing mycobacteria the D-arabinan segment is capped on the non-reducing ends by oligomannosides), and a 4-kDa D-mannan. Small amounts of uncharacterised oligo- and polysaccharides are also present.

There appears to be a mixture of secreted, cell envelope-associated, or cytoplasmic membrane-anchored proteins. Examples, in *M. tuberculosis*, of the many surface-exposed proteins are alanine dehydrogenase (Andersen *et al.*, 1992), the 19-kDa and 38-kDa lipoproteins (Garbe *et al.*, 1993), and the 30/31-kDa fibronectin-binding proteins (Wiker & Harboe, 1992). Raynaud *et al.* (1998) demonstrated the presence of 8 enzymes in the culture filtrates and/or cell surfaces of pathogenic mycobacteria (*M. tuberculosis* and *M. bovis*). In non-pathogenic mycobacteria (*M. smegmatis* and *M. phlei*) these enzymes were present deeper in the cell envelope.

Ortalo-Magné *et al.* (1995) have argued against the common belief that the capsule is predominantly made up of lipid. They have shown that lipid only comprises 2-5% of outer capsular constituents. Most lipid (Table 1.2) is in the inner part of the capsule with the acyl trehaloses [dimycolyl trehalose (cord factor), sulfatides] predominating. Some phospholipids (e.g., PIM), species-specific lipids (e.g., dimycocerosate of phthiocerol), type-specific lipids [e.g., phenolic glycolipids (PGL), lipooligosaccharides (LOS)] and glycopeptidolipids (glycoconjugates unique to mycobacteria and not found in PGL-containing species) are present in the outer layer of the capsule (Ortalo-Magne′ *et al.*, 1996b).

Table 1.2. Distribution of glycolipids in mycobacteria

Compound	Pathogenic species	Non-pathogenic species
Trehalose dimycolate (TDM)	*M. tuberculosis*, *M. bovis*, *M. avium*, *M. kansasii*, *M. parafortuitum*, and others	*M. smegmatis*, *M. phlei*, others
Sulfatides	*M. tuberculosis*	
Polyphthienoyl trehalose	*M. tuberculosis*	
Polyphleoyl trehalose		*M. smegmatis*, *M. phlei*
2,3 diacyltrehalose (DAT)	*M. tuberculosis*, *M. africanum*, *M. bovis*, *M. fortuitum*	
Lipooligosaccharides	*M. tuberculosis*, *M. kansasii*, *M. malmoense*, *M. szulgai*, *M. gordonae*, *M. paratuberculosis*, *M. fortuitum*, *M. mucogenicum*, and others	*M. smegmatis*, *M. gastri*
Glycopeptidolipids (GPL)	*M. paratuberculosis*, *M. simiae*, *M. fortuitum*, *M. lepraemurium*, *M. chelonae*, *M. peregrinun*, *M. porcinum*, *M. senegalense*, *M. abscessus*, MAIS complex	*M. smegmatis*
Phenolic glycolipids (PGL)	*M. tuberculosis*, *M. leprae*, *M. bovis*, *M. marinum*, *M. kansasii*, *M. ulcerans*, *M. haemophilum*	*M. gastri*
Phosphotidylinositol mannosides (PIM)	All mycobacterial species	

Adapted from Vergne & Daffé (1998)

Most of the polysaccharides and proteins present on the cell surface were also detected in *M. tuberculosis* culture filtrates. This suggests that *in vitro* the capsule is shed into the medium (Ortalo-Magne′ *et al.*, 1995). Therefore, Daffé & Draper (1998)

suggest that in an intracellular environment this shed material (predominantly polysaccharides and proteins) could be retained by the phagosomal membrane and may contribute to the 'electron transparent zone' seen on EM sections of pathogenic mycobacteria within host cells.

1.3.2. Ultrastructure

The three components of the cell wall skeleton are covalently linked forming a semi-rigid supramolecular structure (Figure 1.2).

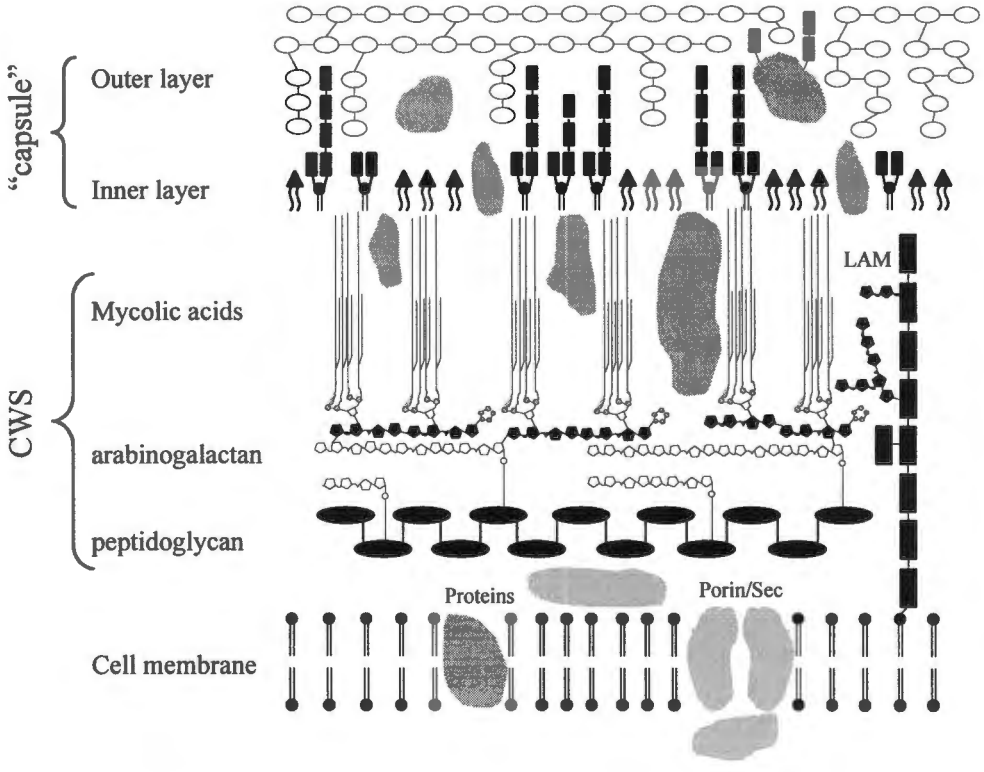


Figure 1.2: Ultrastructure of the *M. tuberculosis* cell envelope. Schematic representation of the structural components making up the cell envelope, i.e., cell membrane, cell wall skeleton (CWS), and capsule. Key : , phospholipids; , protein; , glucose, glucosamine, and muramic acid; , galactose; , arabinose; , galactofuranosyl linker; , pentaarabinosyl units; , mannose; , glycolipids; , polysccharide.

The hydrophobic mycolic acids are thought to be arranged perpendicular to the arabinogalactan-peptidoglycan layer and are covered by an outer leaflet of free wall lipids interdigitating between them (Minnikin, 1982). Hence, the whole structure forms an asymmetric lipid bilayer. The presence of this lipid bilayer is consistent with X-ray diffraction studies (Nikaido *et al.*, 1993) and electron microscopy (Draper, 1982). In addition, by freeze-fracture microscopy, Rastogi *et al.* (1991) revealed the presence of two distinct cleavage planes. The inner cleavage plane incorporates the cell membrane and it is presumed that the outer cleavage plane incorporates the mycolic acids and free wall-lipids. The outer lipid bilayer is covered, in turn, by the capsule composed of polysaccharides, protein, and some lipid. The mycolic acid layer and the outer leaflet of glycolipids are together responsible for the extreme lipophilicity and waxiness of the envelope.

1.3.3. Envelope components and virulence

Although there are no definitive data yet, it is likely that the mycobacterial cell envelope contributes to the pathogenicity of the bacterium. The structure of the envelope ensures that there is an impermeable barrier between the bacillus and the host, and the envelope contains components that are resistant to host defence mechanisms (Daffé & Etienne, 1999). In addition to affording passive protection, some envelope components have been implicated in active processes. Many believe that the tissue pathology characteristic of advanced tuberculous disease is the consequence of a maladjusted immune system. In this regard some envelope components have been implicated in immune system modulation. LAM, cord factor, and sulpholipid 1 can stimulate tumor necrosis factor (TNF)-α release from macrophages (Silva & Faccioli, 1988; Moreno *et al.*, 1989; Brozna *et al.*, 1991); LAM and sulpholipid 1 are able to inhibit activation by

interferon (IFN)-γ (Pabst *et al.*, 1988; Hunter *et al.*, 1988); phenolic glycolipids and arabinomannan are immunosuppressive (Ellner *et al.*, 1979; Fournié *et al.*, 1989); and PIM is able to inhibit proliferation of peripheral blood monocytes. Furthermore, LAM can induce IL-1, IL-6 and IL-10 production (Barnes *et al.*, 1992); inhibit induction of MHC Class 2 genes (Chan *et al.*, 1991); inhibit T-cell proliferation (Moreno *et al.*, 1989); and inhibit IL-2, IL-5, and GM-CSF gene transcription (Chujor *et al.*, 1992). Apart from immunomodulation, sulpholipid 1 has been implicated in inhibition of phagosome-lysosome fusion (Goren *et al.*, 1976), and suppression of protein kinase C and the oxidative activity of phagocytes (Brozna *et al.*, 1991).

The spectrum of potentially pathogenic effects of LAM extend to the scavenging of reactive oxygen intermediates (Chan *et al.*, 1991), stimulation of nitric oxide (Adams *et al.*, 1993), induction of macrophage early response genes (Roach *et al.*, 1994), and inhibition of protein kinase C (Adams *et al.*, 1993). The multitude of potentially pathogenic effects of LAM have ensured it much attention as the prime candidate for a virulence factor. However, the debate regarding the location of LAM and the fact that LAM is present in both pathogenic and non-pathogenic strains have made the interpretation of this data, in the context of it as a virulence factor, confusing. In advanced mycobacterial disease, envelope components from dead or dying bacilli have been shown to be toxic and highly antigenic (Goren, 1982; Bloch, 1995). This may be responsible for the intense cell-mediated reaction that is characteristic of advanced disease and which may be partly responsible for the observed tissue destruction.

The contribution by the cell envelope to intracellular survival of *M. tuberculosis* is discussed further in section 1.6.2.

The evidence reviewed here and in the literature is probably sufficient to implicate the mycobacterial cell envelope in the pathogenicity of *M. tuberculosis*. However, it is not sufficient to completely explain the difference in virulence between

the pathogenic and non-pathogenic mycobacterial species, whose cell envelopes essentially share the same structure and major components. Thus, by itself, the mycobacterial cell envelope cannot account for all the differences in virulence between pathogenic and non-pathogenic mycobacteria, and additional factors must play a significant role.

1.4. METABOLISM AND NUTRITION

The hallmark of *M. tuberculosis* growth is its slow doubling time of 12-20 hours compared to the 20 min of *E. coli* and the 1-2 hours of fast-growing members of the mycobacterium genus (*M. smegmatis*, *M. fortuitum*, *M. chelonei*) (Wheeler & Ratledge, 1988). This difference in growth rate incited researchers to suggest a vastly different metabolism in *M. tuberculosis* and a possible association with virulence. However, no fundamental difference in the metabolism of *M. tuberculosis* and other bacteria has been uncovered and no explanation for this phenomenon was given (Ratledge, 1982; Wheeler & Ratledge, 1988). In addition, slow growth does not correlate with virulence as *M. tuberculosis* shares this characteristic with avirulent *M. bovis* BCG and low-virulence mycobacteria such as *M. avium*, *M. intracellulare*, *M. scrofulaceum* and *M. kansasii* (Pascopella *et al.*, 1992). Attempts to explain the phenomenon of exceptionally slow growth rate on the basis of substrate limitation imposed by the thick cell wall and/or the scarcity of ribosomal RNA genes (1 in *M. tuberculosis* vs 7 in *E. coli*) are not convincing in the face of large variations of growth rates among mycobacterial species that share these properties (Hiriyanna & Ramakrishnan, 1986; Wheeler & Ratledge, 1988).

Although the *in vitro* nutritional requirements of *M. tuberculosis* are rather simple [simple carbon source (e.g., glycerol), inorganic nitrogen (NH_4^+), inorganic elements and trace elements] the *in vivo* scenario is likely to be quite different. Residence inside a

macrophage or a granuloma is likley to impose some nutritional restrictions on the bacillus. It is noteworthy then that the recent completion of the *M. tuberculosis* genome sequence has revealed the presence of a diverse array of genes potentially involved in the *M. tuberculosis* metabolic machinery (Cole *et al.*, 1998). Genes required for the synthesis of all the essential amino acids, vitamins and enzyme co-factors have been identified, as well as the enzymes necessary for glycolysis, the pentose phosphate pathway, and the tricarboxylic acid and glyoxylate cylces. Furthermore, sequence information suggests that *M. tuberculosis* can metabolise carbohydrates, hydrocarbons, alcohols, ketones, and carboxylic acids.

Due to the variety and quantity of lipids available in mammalian cells and the tubercle, it has been suggested that *in vivo M. tuberculosis* is likely to be lipolytic (splitting up of fat) rather than lipogenic (forming fat) (Wheeler & Ratledge, 1994). Indeed the genome sequence has indicated the presence of ~ 250 distinct enzymes involved in fatty acid metabolism (compared to the ~50 enzymes in *E. coli*) (Cole *et al.*, 1998). Furthermore, fatty acid transporter proteins have recently been found in *M. tuberculosis* (Hirsch *et al.*, 1998).

Another source of carbon could be the degradation of proteins and in this regard proteases may play a significant role. Although not much is known about *M. tuberculosis* proteases, the *M. tuberculosis* genome sequence has indicated the presence of in excess of 20 putative protease genes, some of them encoding secreted proteases. In addition, a gelatinase has been detected in pathogenic mycobacterial species but not in nonpathogenic members (Kannan *et al.*, 1987) and a Ca^{2+}-dependent protease with elastolytic (splitting up of elastin) activity has been detected in stationary phase *M. tuberculosis* culture filtrates (Rowland *et al.*, 1997). Further evidence favouring the ability of *M. tuberculosis* to degrade macromolecules has been the demonstration of 22 extracellular enzyme activities in the culture filtrates and/or surface-

located material of *M. tuberculosis* (Raynaud *et al.*, 1998). Of these, the location of 8 was unique to pathogenic strains whereas in nonpathogenic strains these 8 enzymes were located more deeply in the cell envelopes. The ability to break down macromolecules extracellularly would necessitate the ability to transport the breakdown products into the bacterium. In this regard a number of transport proteins and putative transport proteins have been identified. Apart from the fatty acid transporter protein already mentioned, *M. tuberculosis* possesses a 38 kDa phosphate-binding protein [present in the cell envelope and its expression is increased by phosphate-starvation (Espitia *et al.*, 1992)] and iron-transport proteins. In addition, scrutiny of the *M. tuberculosis* genome reveales the presence of a number of putative transport proteins.

An intracellular residence may also limit the bacterium's access to a nitrogen source. Again, *M. tuberculosis* appears to possess an array of enzymes (for example, alanine dehydrogenase, glutamine synthetase, urease, aminopeptidases, and amidases) potentially involved in nitrogen acquisition. At least two of these enzymes, alanine dehydrogenase and glutamine synthetase, are located on the surface and present in higher quantities in the culture supernatants of pathogenic mycobacteria than their nonpathogenic counterparts (Andersen *et al.*, 1992; Harth *et al.*, 1994; Raynaud *et al.*, 1998). The *M. tuberculosis* urease was shown to be active over a wide pH range and expression of this enzyme was increased 10-fold upon nitrogen-deprivation (Clemens *et al.*, 1995b). This would suggest that urease plays an important role in nitrogen acquisition, possibly also under adverse conditions.

Iron acquisition is deemed essential for *in vivo* growth of bacteria and *M. tuberculosis* is again able to meet the challenge by producing two high-affinity iron binding molecules (siderophores), mycobactin (cell-associated) and exochelin (secreted). These proteins are also present in other members of the mycobacteriaceae family and, although there is substantial inter-species variation in the amount of siderophore

production, it cannot be correlated with virulence (Barclay & Ratledge, 1988). *M. tuberculosis* has 4 mycobactins which are present in both virulent and avirulent strains but no structural analysis has been published on these proteins (Barclay & Ratledge, 1988). The exochelins bind iron in the extracellular environment, transfer it to the mycobactins which then facilitate the transport of iron through the plasma membrane to the interior of the bacterium. Although structural information on the exochelins is sparse, the preliminary data suggest that those from slow-growing mycobacteria are structurally distinct from those of rapidly growing mycobacteria (Gordon & Andrew, 1996). Furthermore, *M. tuberculosis* exochelins are able to remove iron from the human iron-binding proteins, transferrin and lactoferrin (Gobin & Horwitz, 1996), suggesting that the ability of *M. tuberculosis* to acquire iron from various sources may be related to virulence. However, definitive evidence linking iron acquisition to virulence is lacking and will require generation of the appropriate mutants.

In addition to the presence of numerous metabolic pathways, the metabolic versatility of *M. tuberculosis* is highlighted further by its ability to survive starvation. This bacterium has been shown to be able to remain viable without any access to nutrients, suggesting that, in some unknown way, it is able to shut down its metabolism (Nyka, 1974). This "starved-state" is, however, associated with some morphological changes in that the starved bacillus loses its acid fastness and is resistant to any form of staining (chromophobicity) (Nyka, 1974; Barclay & Ratledge, 1989). Chromophobic bacilli have been isolated from chronic, caseous lesions (Nyka, 1974).

In summary, the above data describe a highly adaptable organism, one that grows in a metabolically active, acid-fast form when conditions are favourable, and rapidly switches to an inactive chromophobic state under adverse conditions of starvation and hypoxia. The latter state would be consistent with its predicted *in vivo* behaviour where

it appears to be able to remain dormant for many years only to reactivate and cause disease.

1.5. MACROPHAGE RECEPTORS USED BY *M. TUBERCULOSIS*

The ability of *M. tuberculosis* to establish disease appears to be critically dependant on its ability to invade and survive within host mononuclear phagocytic cells (namely, macrophages). *M. tuberculosis* gains entry via a number of receptors present on the surface of the macrophage. Some investigators have proposed that the intracellular fate of the bacillus may be determined, in part, by the receptor to which it binds. Consequently, this has become an area of active research. *In vitro* the bacillus is able to bind opsonically to a wide range of receptors that includes complement receptor (CR) types 1, 3 and 4, the mannose receptor (MR), surfactant protein A receptor (SFA-r), CD14 (on microglial cells), macrophage scavenger receptors, and the F_c receptor. In addition, *M. tuberculosis* can bind directly (non-opsonically) to CR3, CR4, the mannose receptor, and CD14. The preferred receptor/s *in vivo* are unknown.

CR3, a prinicipal phagocytic receptor on monocytes and neutrophils, has been the focus of avid research as it is used by a diverse group of intracellular pathogens (Falkow *et al.*, 1992; Isberg & Tran Van Nhieu, 1994). Furthermore, as a member of the β_2-integrin family, it is likely to be involved in signal transduction pathways and cytoskeletal rearrangements. It expresses a broad ligand specificity that includes complement component C3bi (the best documented ligand), various extracellular proteins, bacterial proteins, and microbial oligo- and polysaccharides (Ehlers & Daffé, 1998). *In vitro*, *M. tuberculosis* is able to bind opsonically (CR3 is the dominant receptor for complement-opsonised particles) and non-opsonically to CR3 (Schlesinger *et al.*, 1990; Hirsch *et al.*, 1994a; Cywes *et al.*, 1996). The former is usually to the C3bi

binding site while the latter is to the lectin site (Cywes *et al.*, 1997). Binding to the lectin site is probably mediated by envelope polysaccharides, including a D-glucan.

The ability of *M. tuberculosis* to bind opsonically depends on the availability of C3bi. Apart from activating the alternative pathway of complement activation, *M. tuberculosis* is able to generate C3bi by recruiting complement component C2a to form a C3 convertase on the surface (Schorey *et al.*, 1997). This results in cleavage of C3 to C3b, which is then deposited on the bacterial surface. Although C3b binds to CR1, cleavage of C3b by factor I (synthesised in the lung) generates C3bi, thus promoting binding to CR3. Furthermore, this C2a-mediated C3 cleavage is only present in pathogenic mycobacteria, suggesting that it may be a virulence factor. Direct binding to the β-glucan lectin site results in rapid phagocytosis by unactivated mononuclear phagocytes. This is in direct contrast to C3bi-opsonised particles that are able to bind, but without the presence of inflammatory mediators no internalisation occurs (Sampson *et al.*, 1991). Thus, direct binding may provide an efficient way of promoting bacillus uptake by mononuclear phagocytes in the uninflamed lung. In addition, entry via this receptor may be beneficial for intracellular survival as it has been shown to result in a diminished or absent respiratory burst (Wright & Silverstein, 1983; Berton *et al.*, 1992). However, direct proof that CR3-mediated entry is beneficial for intracellular survival is still needed.

CR4 is also a member of the β_2-integrin family and has been shown to be the predominant integrin receptor on the macrophage (Zaffran *et al.*, 1998). *M. tuberculosis* can bind both directly and opsonically to this receptor (Schlesinger *et al.*, 1990; Hirsch *et al.*, 1994a; Zaffran *et al.*, 1998; Schlesinger *et al.*, 1990). In addition, binding to this receptor resulted in rapid tyrosine phosphorylation of a major 60 kDa macrophage protein, presumed to play a role in *M. tuberculosis* phagocytosis. The ligand and binding-site involved in direct binding are unknown.

The mannose receptor (MR) is a C-type lectin that is only present on mature macrophages. It mediates attachment to glycoconjugates terminating in mannose, fucose and N-acetylglucosamine. This receptor is able to promote the uptake of virulent *M. tuberculosis* (for example, Erdman and H37Rv), but not avirulent *M. tuberculosis* H37Ra, by human monocyte-derived macrophages (Schlesinger, 1993). Since it is strongly downregulated by inflammatory mediators, including mycobacterial antigens, the MR probably plays an important part in promoting uptake of *M. tuberculosis* by alveolar macrophages in the uninflamed lung (Mokoena & Gordon, 1985; Schreiber *et al.*, 1993). Its role after the initial infection is probably minor. Although its ligand is unknown, some researchers consider mannose-capped LAM to be a leading candidate (Schlesinger *et al.*, 1996). The problem with this is that the location of LAM is unknown and may not be on the bacterial surface (Ortalo-Magné *et al.*, 1995; Ortalo-Magné *et al.*, 1996a). Hence, it may not be available for binding to MR. There are other possible candidates, including neutral, nonacylated arabinomannan and phosphatidylinositol mannoside (PIM).

Surfactant protein A (SP-A), present in alveolar fluid, may play a role in the pathogenesis of tuberculosis as it is present at the site of initial inoculation. This protein, together with the mannose binding protein (MBP) and complement component Clq, is a member of the collectin family (C-type lectins with an added collagenous region). These molecules are able to bind to cell surface receptors, probably mediated by the collagen-like domain. SP-A has been shown to bind to at least two distinct receptors: a) $C1qR_p$ – a 126-kDa protein that is expressed on monocytes, macrophages, neutrophils and endothelial cells (Nepomuceno *et al.*, 1997); and b) SPR210 – a 210-kDa that is expressed on type II pneumocytes and macrophages (Chroneos *et al.*, 1996). CR1 is able to bind C1q with high affinity, and therefore, although it remains to be demonstrated, it may be an additional receptor for SP-A (Klickstein *et al.*, 1997). Opsonisation with SP-

A enhances macrophage binding and uptake of *M. tuberculosis* (Downing *et al.*, 1995), and opsonisation with MBP enhances binding of *M. tuberculosis* to endothelial cells, fibroblasts, and type II pneumocytes (Hoppe *et al.*, 1997). Thus this may be a mechanism utilised by the bacterium to enter both phagocytic and non-phagocytic cells. The advantage conferred on the organism by using this mode of entry remains to be elucidated.

CD14 (a phosphatidylinositol glycan-linked membrane protein) can bind LAM of *M. tuberculosis* H37Ra and induce the production of interleukin (IL)-8 (Pugin *et al.*, 1994). In addition, microglial cells have been shown to be able to bind *M. tuberculosis* using CD14 (Peterson *et al.*, 1995). Although this is followed by internalisation, it is unclear whether CD14 is assisted by another protein.

Scavenger receptors (class A) on human mononuclear phagocytes have been shown to bind *M. tuberculosis* Erdman (Zimmerli *et al.*, 1996). The likely ligands may be sulfatides as they have been demonstrated to compete with other ligands for class A scavenger receptor binding (Ernst, 1998). Whether these receptors are able to activate the cytoskeleton or whether they achieve internalisation through the use of other receptors remains to be elucidated.

1.6. PATHOGENESIS

1.6.1. Overview

The ability of *M. tuberculosis* to establish disease appears to be critically dependant on its ability to invade and survive in host mononuclear phagocytes, primarily macrophages. The extent to which this is possible is a consequence of the balance between the inherent microbicidal power of the host macrophage and the dose and virulence of the invading bacilli (Dannenberg, 1991; Dannenberg & Rook, 1994). The

degree of damage appears to be a direct result of the interplay between a vigorous tissue-damaging cell-mediated immune response and ill-defined bacterial virulence factors.

M. tuberculosis commonly enters the body via the inhalation of small aerosol droplets (1-2 μm) that pass into the lower respiratory tract. In humans, the inhalation of *M. tuberculosis* is followed by one of four potential outcomes: 1) all bacilli are killed by the host's immune response with no potential for tuberculosis in the future; 2) the bacilli may grow, multiply and cause clinical disease (referred to as primary infection); 3) the bacilli may become dormant and never cause disease (referred to as latent infection), in which case the only sign of exposure is a positive tuberculin skin test; and 4) the latent organisms may replicate again and cause clinical disease (referred to as reactivation).

Although *M. tuberculosis* has been shown to invade non-phagocytic cells *in vitro*, no evidence has yet been presented that this happens *in vivo* (Hoppe *et al.*, 1997; Birkness *et al.*, 1999) . Thus, the contribution of epithelial and other non-phagocytic cells lining the respiratory tract is unknown. It has been universally accepted that the alveolar macrophage represents the first line of the host defense in the airway. These cells are constitutively phagocytic, and are able to rapidly phagocytose the inhaled bacilli. *M. tuberculosis* gains entry into alveolar macrophages via a number of receptors present on the surface of the macrophage (reviewed in section 1.5). It has been suggested that receptor choice may determine the intracellular fate of the phagocytosed bacilli, however this remains to be conclusively demonstrated.

The microbicidal activity of the macrophage ensures that the majority of tuberculosis infections are arrested at this initial stage. Only 10% of individuals exposed to *M. tuberculosis* will develop clinical disease and those with latent infection have a 5-10% lifetime risk of developing disease, indicating how effective the host's defense mechanisms are in preventing disease (Israel *et al.*, 1994). In these individuals the only signs of infection may be a positive tuberculin skin test or a small, calcified lung lesion

seen on a chest X-ray. In some instances, however, the bacilli are able to resist the microbicidal activity of the alveolar macrophage and survive.

The intracellular survival strategies utilised by *M. tuberculosis* (reviewed in section 1.6.2) are incompletely understood at this time but have been suggested to hinge on the ability of the bacillus to avoid a respiratory burst upon phagocytosis, to effectively neutralise toxic oxygen and nitrogen intermediates, and to avoid phagolysosome fusion (Wright & Silverstein, 1983; Berton *et al.*, 1992; Chan *et al.*, 1992; Armstrong & Hart, 1971). The bacilli that are not killed by the alveolar macrophage multiply, cause lysis of the cell and are released. The released bacilli are then further phagocytosed by alveolar macrophages and nonactivated macrophages (Dannenberg, 1991). The latter cells are blood monocytes that have been attracted by released bacilli, cellular debris and chemotactic factors (C5a, cytokines, etc). As this lesion develops, the incoming immature macrophages begin to play a more dominant role as they are able to get to the center of the lesion where the bacilli reside, whereas the alveolar macrophages tend to be more peripheral. Dannenberg (1991) refers to this as the symbiotic stage (occuring 7-21 days postinfection) as neither bacillus nor macrophage is able to inflict any damage to one another. During this time logarithmic growth of the bacilli occurs unhindered, as the macrophages are not activated and are therefore unable to kill the multiplying bacilli.

Thus, increasing numbers of macrophages and bacilli accumulate in the lesion. The cytoplasm of unactivated macrophages appears to be a good growth medium for *M. tuberculosis* and active growth occurs (Danenberd & Rook, 1994). The host attempts to remove this favourable environment by destroying the cells in which the multiplying bacilli reside. This is evident by the development of caseous necrosis. Removal of the favourable intracellular environment results in exposure of the bacilli to the unfavourable conditions of solid caseous material (low pH, anoxia, nutrient depletion), inhibition of growth and phagocytosis of bacilli by activated macrophages with antimycobacterial

activity (Poole *et al.*, 1970). The development of caseous necrosis stops logarithmic growth of intracellular bacilli, suggesting a potential benefit to the host. However, the host achieves this through the sacrifice of some of its tissue (Canetti, 1955; Poole *et al.*, 1970).

The cause of caseous necrosis has become an area of contention. Dannenberg (1991) suggests that necrosis is a consequence of the development of delayed-type hypersensitivity to bacillus components that are released upon macrophage lysis. Orme (1998), however, disagrees and postulates that the centers necrose because the concentration of cytokines reaching these cells is too low or absent, thus allowing subsequent degeneration. He bases this on the observation that, after several weeks, epithelioid cells in the center of granulomas in rabbits and guinea-pigs begin to degenerate and become necrotic whereas in mice this does not occur. The granulomas in rabbits and guinea-pigs are surrounded by a mantle of lymphocytes whereas mice have rafts or wedges of lymphocytes that penetrate through extensive sections. Thus, in rabbit and guinea-pig granulomas, it is likely that insufficient cytokines will reach the central cells, these cells will not become activated and will eventually degenerate. In contrast, the presence of lymphocytes through large sections of mice granulomas ensures sufficient cytokines reaching cells, thus preventing degeneration. Keane *et al.* (1997) propose that apoptosis of *M. tuberculosis*-infected macrophages also plays a role in the development of caseous necrosis. They showed that *M. tuberculosis* infection of macrophages *in vitro* induced apoptosis and that this appeared to be mediated by TNF-α. In addition, there was evidence of apoptosis in sections from active tuberculous granulomas, but not quiescent lesions.

Whatever the cause of caseous necrosis, it appears that the host attempts to kill the invading pathogens at the expense of its own cells and tissues. The purpose of the tissue-damaging component of the immune response is to localise the bacilli to the

caseous center thus preventing disease progression. It is usually achieved when there are a small number of bacilli; however, when large numbers are present extension of the caseous focus is possible. Many unactivated macrophages and lymphocytes accumulate around the caseous center. Only activated macrophages have mycobactericidal activity, so the host's ability to prevent disease progression now rests on its ability to activate the surrounding macrophages. This is usually accomplished by T-lymphocytes, attracted and activated by macrophage-produced cytokines, that, in turn, produce lymphokines that activate macrophages. There is evidence that the cytokines interleukin (IL)-8, tumor necrosis factor (TNF)-α and IL-1β play a role in attracting and activating T-lymphocytes. Alveolar macrophages obtained by bronchoalveolar lavage (BAL) in patients with tuberculosis have been shown to release elevated levels of IL-8 (Law *et al.*, 1996a), TNF-α and IL-1β (Law *et al.*, 1996b). In addition, intact mycobacteria, their cell wall components (e.g., LAM) and secreted proteins (e.g., 20 kDa and 46 kDa proteins) stimulate the release of these compounds from macrophages (Zhang *et al.*, 1995).

TNF-α plays a central role in the host's ability to restrict *M. tuberculosis* infection. Experimental data (from both animals and humans) have revealed the importance of TNF-α in the following: inducing granuloma formation in BCG infection (Kindler *et al.*, 1989); enhancing the antimycobacterial activity of macrophages (Flesch & Kaufmann, 1990); mouse survival of *M. tuberculosis* infection (Flynn *et al.*, 1995); and growth inhibition of *M. tuberculosis* in human alveolar macrophages (Hirsch *et al.*, 1994a). In addition, Silver *et al.* (1998) showed that production of TNF-α by mononuclear phagocytes is greater with increasing strain virulence. However, apart from its potential protective function, TNF-α (and IL-1β) has also been implicated in the weight loss, night sweats and tissue necrosis characteristic of active tuberculosis (Takashima *et al.*, 1990). Macrophages infected with virulent *M. tuberculosis* are more

susceptible to TNF-α-induced cytotoxicity than those infected with attenuated or avirulent strains (Filley *et al.*, 1999), prompting some investigators to question whether the ability of *M. tuberculosis* to stimulate TNF-α production serves primarily to promote pathogenesis rather than protection. These cytokines, however, probably play a critical role in attracting and activating T-lymphocytes, essential for protection against *M. tuberculosis*.

The importance of the $CD4^+$ helper T cell subset has now become well-established; however, evidence is mounting implicating $CD4^+$ and $CD8^+$ cytotoxic T cells (CTLs), γδ T cells and $CD4^-CD8^-$ T cells in the host's immune response (reviewed in section 1.7). The immune system networks involved in host defense against *M. tuberculosis* are complex and, as such, the literature contains conflicting experimental data. This is likely a result of different experimental methodologies and the difficulties experienced in attempting to approximate *in vivo* conditions. Despite these difficulties, the greater body of work supports a T_H1 response (T_H1/T_H2 cells are subsets of T helper cells differentiated *in vitro* according to the blends of cytokines they produce) to *M. tuberculosis* infection (Schluger *et al.*, 1998; Ellner, 1999). Therefore, in the context of *M. tuberculosis* infection, it appears that the $CD4^+$ helper T cells amplify the immune response by activating macrophages (helped by $CD8^+$ T cells, and also γδ T cells and $CD4^-CD8^-$ T cells) and recruiting new immune cells to the site of the lesion (Stenger & Modlin, 1999). The $CD4^+$ and $CD8^+$ CTL's lyse unactivated macrophages laden with *M. tuberculosis* bacilli releasing them for uptake by activated macrophages that have antimycobacterial activity. In addition, lysis by $CD8^+$ CTL's can result in direct killing of *M. tuberculosis* (Stenger *et al.*, 1998). The γδ T cells and $CD4^-CD8^-$ T cells control inflammation by lysing T cells, thereby dampening the immune response and preventing extensive tissue injury.

The extent of disease depends on the balance between an exuberant tissue-damaging immune response and a more focussed antimycobacterial cell-mediated immune (CMI) response in inhibiting *M. tuberculosis* multiplication. In sensitive hosts the bacilli that are released at the edge of the caseous center are taken up by unactivated macrophages and the tissue-damaging response is continued in order to stop intracellular bacillary multiplication (Lurie, 1964; Dannenberg, 1991). Thus the caseous center enlarges and, as more and more local lung tissue is destroyed, there is increased potential to erode into the lymphatics and bloodstream with dissemination to extrapulmonary sites. In the resistant host, the strong CMI ensures that there are enough activated macrophages surrounding the caseous center to phagocytose and destroy released bacilli. With time the primary tubercle becomes walled off, the caseous center is resorbed, fibrous tissue is deposited and the disease is arrested. Dystrophic calcification may occur at the site of caseous necrosis. The bacilli that were able to disseminate are either destroyed at their metastatic site or reside there in a dormant state (Dannenberg & Rook, 1994).

Sometimes, even with a strong CMI response, progressive disease occurs resulting in liquefaction of the caseous center and ultimately cavity formation (Canetti, 1955; Lurie, 1964; Dannenberg & Rook, 1994). The factors that cause liquefaction are unknown. The liquefied material is a good growth medium for *M. tuberculosis* and unhindered extracellular growth occurs (Dannenberg, 1991; Dannenberg & Rook, 1994). This material can drain into neighbouring bronchi and alveoli, causing a broncho- or lobar pneumonia and/or allowing a large number of bacilli to be spread with coughing to the outside (Bloom & Murray, 1992). Erosion into blood vessels can result in haemoptysis and/or haematogenous dissemination to extrapulmonary sites.

An effective chemotherapeutic regimen comprising at least 3-4 antibiotics usually results in clinical improvement and cure of active disease. However, the prolonged duration of such chemotherapy (usually a minimum of 6 months) fosters non-

compliance, making relapse of active disease a common feature, especially in Third World Countries. Clearly, an effective vaccine would be optimum for the control of tuberculosis in these communities. Since the concept of dormant bacilli encompasses a bacterium in which the metabolism has been down-regulated, it is likely then that these bacteria will not be affected by current chemotherapeutic agents, whose modes of action are directed at various metabolic pathways. Thus, dormant bacilli remain resident in human tissues for many years and retain the ability to cause active disease. The conditions required to reactivate these bacilli are unknown but may include various clinical states that involve some degree of immunosuppression. Clinicians suggest that most adult tuberculous disease is a consequence of reactivation rather than new or re-infection (Coovadia & Benatar, 1991).

1.6.2. Intracellular survival

Intracellular survival strategies have been an area of active research, generating data which have yielded some insights into the possible mechanisms, but no definitive conclusions have been reached.

1.6.2.1. Barrier

The unique structure of the mycobacterial cell envelope may promote intracellular survival by presenting a barrier that is impermeable and impervious to microbicidal macromolecules (for example, degradative enzymes and bactericidal peptides) and contains structures that are resistant to degradation by host enzymes (Daffé & Draper, 1998).

1.6.2.2. Respiratory burst

An important part of the microbicidal activity of the macrophage is the

respiratory burst, which generates reactive oxygen intermediates (OH^-, H_2O_2, O_2^-) that kill invading bacteria and other pathogens. It is in this context that envelope/capsular components may play an important role. Entry into a macrophage via the CR3 receptor does not elicit a respiratory burst. Therefore, by expressing a surface ligand for CR3, *M. tuberculosis* may be able to enter the alveolar macrophage without precipitating a respiratory burst (Wright & Silverstein, 1983; Berton *et al.*, 1992). In addition, some envelope components have been shown to either inhibit the production of reactive oxygen intermediates [e.g., sulfatides (Pabst *et al.*, 1988; Brozna *et al.*, 1991)] or inactivate them [(e.g., LAM (Chan *et al.*, 1991)].

Superoxide dismutase (SOD) and catalase/peroxidase (*katG*) are both able to inactivate reactive oxygen intermediates and are present in mycobacteria (Middlebrook & Cohn, 1953; Andersen *et al.*, 1991; Balaji & Boom, 1998). In pathogenic strains these enzymes are surface-exposed but are cytosolic in non-pathogenic strains (Escuyer *et al.*, 1996; Raynaud *et al.*, 1998). A similar distribution of SOD in other bacteria has been associated with virulence in those strains containing surface-exposed enzymes (Beaman *et al.*, 1983; Beaman & Beaman, 1990). Presumably, surface-exposed enzymes have direct access to and can neutralise reactive oxygen intermediates before significant damage to the bacterium can occur. The presence of catalase/peroxidase in *M. bovis* and *M. tuberculosis* species has been associated with enhanced virulence of these bacteria in the guinea-pig (Wilson *et al.*, 1995; Li *et al.*, 1998).

1.6.2.3. Nitrogen intermediates

Despite the data presented above, a sizeable amount of work is accumulating suggesting that reactive oxygen intermediates have a limited role to play in host defense. Investigators now support the idea that reactive nitrogen intermediates (NO, NO_2, HNO_2) may play a more significant role (Denis, 1991; Chan *et al.*, 1992). This has been

relatively well studied in the murine model, where invesigators have shown that *M. tuberculosis* is able to replicate much faster in a mouse lacking the ability to produce inducible nitrogen oxide synthase (iNOS) than in the wild-type mouse (MacMicking *et al.*, 1997). In other experiments, an interferon regulatory factor-1 (transcription enhancer of the iNOS gene) knockout mouse, whose macrophages are unable to release NO, was unable to clear infection with BCG and succumbed (Kamijo *et al.*, 1994); similarly, mice treated with iNOS inhibitors succumb to infection with *M. tuberculosis* (Chan *et al.*, 1995). However, the involvement of reactive nitrogen intermediates in host defense in humans remains a contentious issue. This initially rested on the inability to demonstrate iNOS in human macrophages and the finding by Kuo *et al.* (1996) that alveolar macrophages from patients with tuberculosis have a higher capacity for the generation of reactive oxygen intermediates compared with cells obtained from normal control subjects. However, since then, Nicholson *et al.* (1996) have demonstrated upregulation of iNOS in alveolar macrophages from patients with tuberculosis, and Rockett *et al.* (1998) showed that 1,25-dihydroxyvitamin D_3 can induce iNOS and inhibit *M. tuberculosis* growth in a human macrophage-like cell line.

Reactive nitrogen intermediates induce the production of a set of proteins, termed Nox (Garbe *et al.*, 1996). Recently, Nox16 was found to be the major protein induced upon exposure of *M. tuberculosis* to reactive nitrogen intermediates (Garbe *et al.*, 1999). This protein was found to be identical to the α-crystallin homolog, sHsp16 (16 kDa small heat shock protein), which has been implicated in biologic states associated with low metabolic rates and dormant conditions in other organisms (Liang *et al.*, 1997). The role of Nox16 in the *M. tuberculosis* infectious process remains to be elucidated.

1.6.2.4. Inhibition of phagosome-lysosome fusion

Phagosome-lysosome fusion with the release of lysosomal hydrolases that digest

and kill ingested microbes is an integral part of the macrophage defense against invading pathogens. Not only is it possible that the *M. tuberculosis* envelope may protect it from these hydrolases, but it is now well documented that *M. tuberculosis* is able to inhibit phagosome-lysosome fusion (Armstrong & Hart, 1971). This appears to be linked to the exclusion of the proton ATPase from the phagosomal membrane (Sturgill-Koszycki *et al.*, 1994), thereby preventing phagosome acidification and arresting maturation of the phagosome at an early endosome stage (Clemens & Horwitz, 1995) [this occurs at the rab7 stage, a small GTP-binding protein specific for late endosomes (Via *et al.*, 1997)]. In contrast, cytokine-activated macrophages, when stimulated with interferon (IFN)-γ and lipopolysaccharide, are able to acidify mycobacterial phagosomes more readily (Schaible *et al.*, 1998; Via *et al.*, 1998).

Although inhibition of phagosome-lysosome fusion is not unique to *M. tuberculosis* – *Legionella pneumophila*, *Toxoplasma gondii* and *Chlamydia psitacci* also reside in phagosomes that do not fuse with lysosomes – it does appear that the *M. tuberculosis* phagosome reacts very differently with the endosomal-lysosomal pathway. The *L. pneumophila* phagosome does not contain any endosomal or lysosomal markers, suggesting that its interaction with the endosomal-lysosomal pathway is minimal (Clemens, 1996). This is in direct contrast to the *M. tuberculosis* phagosome that contains endosomal markers (MHC class II and transferrin receptor molecules) (Clemens & Horwitz, 1995) and modest amounts of lysosomal membrane glycoproteins (CD63, LAMP-1) (Xu *et al.*, 1994; Clemens & Horwitz, 1995). By retaining interactions with endosomes, *M. tuberculosis* resides in a phagosome in which the intracellular contents are constantly restored, presenting one way in which the bacterium may obtain nutrients for intracellular growth and multiplication. Thus, phagosome manipulation may be intimately linked to the intracellular growth of virulent mycobacteria. Although

the molecular mechanisms underlying this process are unknown some researchers have suggested the following:

1) The size and surface characteristics of viable mycobacteria can promote close phagosome membrane apposition and hence preclude membrane tubule formation required for vesicular trafficking and maturation (De Chastellier & Thilo, 1997).

2) Receptor choice my dictate the subsequent phagosomal development, as uptake via the Fc receptor does not support phagosome manipulation and results in an acidified phagosome (Armstrong & Hart, 1975). Furthermore, nonopsonic FimH-mediated binding and uptake of *E. coli* into macrophages produces tight-fitting phagosomes whereas antibody-mediated uptake produces loose, spacious phagosomes (Baorto *et al.*, 1997).

3) Intracellular growth of *M. tuberculosis* results in the specific induction of an array of proteins that may interfere with host cell signalling events (Lee & Horwitz, 1995).

Recently, a phagosomal coat protein, tryptophane aspartate-containing coat protein (TACO), has been implicated in the inhibition of *M. bovis*- and *M. bovis* BCG-containing phagosomes fusing with lysosomes (Ferrari *et al.*, 1999). This protein prevented phagosomes containing live organisms, but not dead organisms, from fusing to lysosomes. In macrophages lacking TACO, the mycobacterial-containing phagosomes fused with lysosomes resulting in degradation of the organisms. Moreover, expression of TACO in nonphagocytic cells prevented lysosomal delivery of mycobacteria and prolonged their intracellular survival. TACO was shown to belong to the Trp-Asp (WD) repeat protein family whose members are involved in a variety of processes, including signal transduction and cytoskeletal organisation. Ferrari *et al.* (1999) suggest that TACO may be involved in influencing the actin/tubulin cytoskeleton and/or micro-tubule regulation, although this remains to be elucidated.

Although the inhibition of phagosome-lysosome fusion may be significant, occasionally fusion has been noticed (Armstrong & Hart, 1975; McDonough *et al.*, 1993; Bloom *et al.*, 1994). When phagosome-lysosome fusion does occur, *M. tuberculosis* is able to bud off from the phagolysosome and multiply in a newly formed vesicle (McDonough *et al.*, 1993). Although the avirulent H37Ra strain behaves in a similar manner, it is not able to multiply in this vesicle. These observations are in direct contrast to those of Armstrong & Hart (1975) and Clemens & Horwitz (1995) who found no evidence that live *M. tuberculosis* can enter a lysosomal compartment. Instead, they found that only dead bacilli enter a lysosomal compartment, suggesting that active processes exerted by the mycobacteria are required to prevent transport to endosomal/lysosomal organelles.

1.6.2.5. Escape into the cytoplasm

Shigella flexneri and *Listeria monocytogenes* escape into the cytoplasm to successfully evade the microbicidal mechanisms of the macrophage (Sansonetti *et al.*, 1986; Bielecki *et al.*, 1990). Conflicting data regarding the ability of *M. tuberculosis* to escape into the cytoplasm have been published, making this concept an area of contention. Myrvick *et al.* (1984) and McDonnough *et al.* (1993) have observed *M. tuberculosis* H37Rv in the cytoplasm. In addition, they noted that avirulent *M. tuberculosis* H37Ra and *M. bovis* BCG could not escape into the cytoplasm, suggesting that this process was related to virulence. However, Clemens & Horwitz (1995) did not find any evidence for *M. tuberculosis* Erdman in the cytoplasm. Despite these conflicting observations, a contact-dependant cytolysin expressed by *M. tuberculosis* (King *et al.*, 1993) has been identified, suggesting that *M. tuberculosis* has the ability to form pores in membranes, possibly allowing escape into the cytoplasm. This cytolysin was not present in avirulent *M. bovis* BCG and was expressed at a lower

level in avirulent *M. tuberculosis* H37Ra. Membrane-bound cytolysins play an important role in the ability of intracellular bacteria (for example, *L. monocytogenes* and *S. flexneri*) to enter and exit host cells (Welch, 1991; Sansonetti, 1993).

1.6.2.6. Secreted proteins

M. tuberculosis bacilli are likely to induce or suppress genes in response to the changing intracellular milieu. Furthermore, growth within macrophages leads to a marked induction in the activity of the mtrA gene, which is part of an *M. tuberculosis* two-component regulator system that may transduce signals relating to environmental changes accompanying intracellular growth (Via *et al.*, 1996). Investigators have confirmed the presence *in vivo* of proteins that are not present *in vitro* (Lee & Horwitz, 1995). These proteins are presumed to be required for intracellular survival and are likely to include various cell-surface and secreted proteins, although the identities of these are unknown. Some of these cell-surface and secreted proteins may be proteases. Furthermore, these proteases may be part of the *M. tuberculosis* arsenal that helps the bacillus survive intracellularly within a hostile phagocytic cell (discussed in detail in Chapter 2). Thus, the intracellular phenotype of *M. tuberculosis* may be quite different.

1.6.3. T cell immune response

There is compelling clinical evidence that a vigorous and controlled host immune response is essential for the elimination of *M. tuberculosis* bacilli from an infected host. Furthermore, the successful elimination of *M. tuberculosis* depends mainly on the efficient interaction between infected macrophages and antigen-specific T cells. This part of the chapter reviews current evidence on the contribution of the various T cell subsets to protection from *M. tuberculosis*.

1.6.3.1. $CD4^+$ helper T cells

Involvement of the $CD4^+$ helper T cell subset has now become well-established. This is a result of experiments that revealed an aggravation of disease in CD4-depleted mice (Podrazzini *et al.*, 1987), the ability of $CD4^+$ T cells from immunised mice to protect nonimmune mice against *M. tuberculosis* (Orme, 1987), and a dramatically increased susceptibility of AIDS patients to tuberculosis (Barnes *et al.*, 1991; Jones *et al.*, 1999). The $CD4^+$ T cells are thought to protect against *M. tuberculosis* infection by the antigen-specific production of cytokines which activate infected macrophages to kill their intracellular bacillary load. $CD4^+$ T cells recognise antigen that is derived from the phagocytic pathway and which is presented by an antigen presenting cell (primarily macrophages and dendritic cells) in the context of major histocompatibility (MHC) class II molecules.

The $CD4^+$ helper T cells are further differentiated into two phenotypes, T_H1 and T_H2. The driving force behind this differentiation is unknown but may include a specific cytokine/lymphokine milieu or some endocrine factors. T_H1 cells produce IFN-γ and IL-2 which activate inflammatory and phagocytic cells. T_H2 cells predominantly secrete IL-4, but also IL-5 and IL-10, which not only drives the immune system to a humoral response but also inhibits the cell mediated response. IL-3, lymphotoxin and granulocyte-macrophage-colony stimulating factor (GM-CSF) are secreted by both T_H1 and T_H2 cells. Evidence to date points to a T_H1-type response to *M. tuberculosis* infection. Perhaps the most convincing data were obtained by Barnes *et al.* (1993) who demonstrated higher levels of the T_H1 cytokines IFN-γ and IL-2 in pleural fluid than in blood, in patients with tuberculous pleuritis. In addition, there was a lower level of the T_H2 cytokine IL-4 in pleural fluid than in blood, and lymphocytes from pleural fluid produced more IFN-γ and IL-2 when stimulated with *M. tuberculosis* than did peripheral

blood lymphocytes. From these data it was concluded that there is a T_H1-type response at the actual site of disease. Further supporting a T_H1 response is the finding of increased levels of IL-12 in pleural fluid and BAL cells from patients with tuberculosis (Zhang *et al.*, 1994), and that IL-12 is released by macrophages upon phagocytosis of *M. tuberculosis* (Fulton *et al.*, 1996; Ladel *et al.*, 1997). Since IL-12 is known to produce a T_H1-type response it may be a possible regulator of T cell phenotypes in tuberculosis.

1.6.3.2. γδ T cells

M. tuberculosis antigens are also able to activate human γδ T cells (especially $V\gamma9^+$ and $V\delta2^+$) which recognise mycobacterial antigens in a non-MHC-restricted manner, distinct from $CD4^+$ T cells, and have been shown to respond to small phosphate-containing antigens (Balaji & Boom, 1998). A role for these cells has been demonstrated in murine models (Ladel *et al.*, 1995; Li *et al.*, 1996) and γδ T cells from healthy tuberculin-positive individuals produce a T_H1-type cytokine profile and have cytolytic activity for *M. tuberculosis*-infected macrophages (Tsukaguchi *et al.*, 1995). However, in humans, there is no evidence for the accumulation of these cells at the site of tuberculous disease, and it has been suggested that they may be involved in the early immune response (Tazi *et al.*, 1991). Recent studies have shown that engagement of a mycobacterial antigen with a γδ T cell receptor induces the upregulation of FasL on chronically infected γδ T cells (Manfredi *et al.*, 1998). Interaction with Fas on immigrating monocytes results in activation-induced cell death of the γδ T cells. Therefore, the envisioned role for γδ T cells is that they participate in the early containment of disease and, as the disease progresses, Fas-FasL apoptosis removes the majority of *M. tuberculosis*-reactive γδ T cells (Li *et al.*, 1998). Stenger & Modlin (1999) suggest that the reduction in γδ T cells may limit inflammation and inhibit tissue

damage, but perhaps at the expense of the development of chronic infection (Boom *et al.*, 1992; Gately *et al.*, 1992).

1.6.3.3. $CD4^+$ and $CD8^+$ cytolytic T cells (CTLs)

It is becoming increasingly recognised that a cytotoxic T cell response also plays a significant role in protection against *M. tuberculosis*. There is evidence implicating both $CD4^+$ and $CD8^+$ CTLs (Kaufmann, 1988; Ottenhof & Mutis, 1990; Tan *et al.*, 1997). $CD4^+$ CTL activity is greatly enhanced at the site of disease compared to that of peripheral blood cells, suggesting that they play a role in the local immune response. However, Tan *et al.* (1997) demonstrated that alveolar macrophages are more resistant to $CD4^+$ CTL-mediated cytolysis than are blood monocytes. It is unknown whether the reduced cytolysis is mediated by *M. tuberculosis* thereby shielding itself from host microbicidal mechanisms, or whether this is a host mechanism that prevents the spread of released bacilli.

As the major cytolytic pathway used by $CD4^+$ CTLs is FasL-induced apoptosis, Oddo *et al.* (1998) assessed the effect of adding recombinant FasL to *M. tuberculosis*-infected macropahges. This induced apoptosis and also led to a decrease in *M. tuberculosis* viability. TNF-α-induced apoptosis produced similar results; however, nonapoptotic complement-induced cell death had no effect on the viability of *M. tuberculosis*. Furthermore, *M. tuberculosis*-infected macrophages were more resistant to FasL-induced apoptosis and this correlated with reduced levels of surface Fas expression. Thus, the ability of *M. tuberculosis* to modulate surface molecules may be yet another dimension of its ability to escape host defense mechanisms. However, Stenger & Modlin (1999) caution that the above experiment reflects the effects of endogenously added FasL, and whether this is the contact-dependent pathway utilised by

$CD4^+$ CTLs to effect cytolysis remains to be determined. Lewinsohn *et al.* (1998) show that $CD4^+$ CTL clones can also induce apoptosis in a FasL-independent manner.

The contribution by $CD8^+$ cytotoxic T cells is uncertain. Although there is strong evidence implicating these cells in protective immunity in animal models (Flynn *et al.*, 1992), there is, unfortunately, conflicting data from human studies. Confusion arose from earlier studies that revealed human *M. tuberculosis*-specific cytolytic T cells to be $CD4^+$ not $CD8^+$ (Ottenhof & Mutis, 1990), $CD8^+$ T cells in tuberculosis patients are not concentrated at the site of disease (Barnes *et al.*, 1989), and that the severity of tuberculosis in HIV-infected individuals is unaffected by the $CD8^+$ T cell count (Jones *et al.*, 1999). Nevertheless, more recent studies indicate involvement of $CD8^+$ T cells in the human immune response to tuberculosis. $CD8^+$ T cells recognise antigen that has been processed in the cytosol and that is presented in the context of MHC class I molecules. Tan *et al.* (1997) showed $CD8^+$ *M. tuberculosis*-reactive CTLs in the lungs of tuberculosis patients and Lalvani *et al.* (1998) found IFN-γ secreting $CD8^+$ T cells in the peripheral blood of tuberculosis patients. In addition, cloning of peripheral blood mononuclear cells from these patients yielded T cells which recognized ESAT6, an early secretory antigen of *M. tuberculosis* (Mohagheghpour *et al.*, 1998). CD 8^+ T cells are potent producers of IFN-γ and probably afford protection from *M. tuberculosis* infection by complementing $CD4^+$ T cells as a source of IFN-γ. Tascon *et al.* (1998) have shown that, in their mouse model, IFN-γ production by $CD8^+$ T cells was the method of protection against *M. tuberculosis* infection and not the ability of $CD8^+$ T cells to lyse *M. tuberculosis*-infected cells.

Recently, a subset of $CD8^+$ T cells have been shown to recognise nonpeptide antigens of *M. tuberculosis* presented in the context of human CD1 molecules, further adding to the complexity (Gong *et al.*, 1998). This subset is able to recognise and lyse *M. tuberculosis*-infected macrophages. In addition, some *M. tuberculosis*-specific human

$CD8^+$ T cells can recognise antigen not restricted by MHC class I molecules or CD1. This suggests a novel nonpolymorphic MHC class Ib antigen presenting pathway involved in immunity to tuberculosis (Lewinsohn *et al.*, 1998). Recent studies have demonstrated that $CD8^+$ T cells lyse infected cells via the granule-exocytosis pathway and, in so doing, can stimulate the release of molecules that can directly destroy the intracellular microbe (Stenger *et al.*, 1998). One of the candidate molecules, granulysin, can directly kill extracellular *M. tuberculosis*. Furthermore, experiments using infected cells in the presence of granulysin and perforin (a pore forming molecule that colocalises with granulysin in cytotoxic granules) revealed that granulysin was able to kill intracellular *M. tuberculosis* only in the presence of perforin (Stenger *et al.*, 1998). In addition to its cytolytic role, $CD8^+$ T cells can also produce large amounts of IFN-γ, contributing to the role played by $CD4^+$ T cells in activating macrophages.

1.6.3.4. $CD4^-$ $CD8^-$ T cells

Double-negative ($CD4^-$ $CD8^-$) T cells recognise antigen in a CD1-restricted manner (Porcelli, 1995) and have been demonstrated to be able to lyse cells pulsed with mycobacterial lipids (Beckman *et al.*, 1994) and glycolipids (Sieling *et al.*, 1995). In addition they can also release IFN-γ. More recently they have been shown to be able to lyse *M. tuberculosis*-infected macrophages via the Fas-FasL pathway but this does not result in killing of the bacteria (Stenger *et al.*, 1997; Gong *et al.*, 1998). This is in direct contrast to $CD4^+$ and $CD8^+$ CTLs, and the role of these CD1-restricted double-negative T cells has been suggested to be related to controlling inflammation by dampening the host's immune response and preventing extensive tissue injury (Stenger & Modlin, 1999).

1.6.3.5. T cell hyporesponsiveness

Newly diagnosed tuberculosis patients have hyporesponsive T cells, emphasising the importance of an effective T cell response for protection from *M. tuberculosis* infection (Onwubalili *et al.*, 1985; Toossi *et al.*, 1986; Torres *et al.*, 1994). There is evidence that the immunosuppressive cytokines, TGF-β and IL-10, play a role in T cell hyporesponsiveness (Zhang *et al.*, 1995). In addition, Hirsch *et al.* (1999) have recently shown that T-cell apoptosis may also be involved.

TGF-β, produced mainly by monocytes and macrophages, has profound anti-inflammatory effects, part of which is the deactivation of macrophages and the downregulation of IFN-γ, TNF-α and IL-1 (Ruscetti *et al.*, 1993). Experimental data supporting the involvement of TGF-β has shown that this cytokine is able to block phagocytosis and growth inhibition of *M. tuberculosis* by mononuclear phagocytes (Hirsch *et al.*, 1994b; Hirsch *et al.*, 1997). Furthermore, TGF-β is increased in mononuclear phagocytes and lung granulomas from patients with pulmonary tuberculosis (Toossi *et al.*, 1995).

IL-10 has been shown to be increased by *M. tuberculosis* infection of human and murine macrophages (Orme *et al.*, 1993). In addition, IL-10 transgenic mice are more susceptible to disseminated *M. bovis* BCG infection whereas wild-type mice are able to restrict the infection (Murray *et al.*, 1997).

Hirsch *et al.* (1999) have shown an increased propensity for apoptosis of T cells (CD4 and non-CD4) from tuberculosis patients when stimulated with *M. tuberculosis*. In addition, they also showed that there is a higher percentage of spontaneous apoptosis in tuberculin-positive healthy individuals and in patients with active pulmonary tuberculosis than in tuberculin-negative individuals. Data suggesting that activated T cells are predisposed to apoptosis (Ju *et al.*, 1995; Munn *et al.*, 1999) led the authors to suggest

that the increased susceptibility of T cells from patients with active tuberculosis to apoptotic cell death is a consequence of activation *in vivo*. The mechanism of T-cell hyporesponsiveness appears to be an interplay between immunosuppressive cytokines and apoptosis. Since TGF-β has been shown to induce B-cell apoptosis (Chaouchi *et al.*, 1995) and arrest the growth of activated T cells (Ahuja *et al.*, 1992), it is posssible that this cytokine is involved in T cell apoptosis.

1.7. CONCLUSION

M. tuberculosis is a complex and insidious organism that for centuries has baffled and bewildered clinicians and scientists alike. In this introductory review, an attempt was made to highlight the enormous progress achieved in the past decade to understand the mechanisms used by *M. tuberculosis* to establish disease. Despite recent advances, however, few virulence factors of *M. tuberculosis* have been clearly defined. The increasing global incidence of tuberculosis, and in particular of multi-drug resistant strains of *M. tuberculosis*, necessitates the discovery of novel drug targets and an effective vaccine. Global collaboration in the implementation of TB control programmes is required if we are to stem the tide. The potential for 60-80 million lives to be lost from tuberculosis during the next 2-3 decades clearly justifies intensive research directed towards the identification of potential virulence determinants of *M. tuberculosis*. The rising tide of tuberculosis ensures that *M. tuberculosis* will enter, yet again, a new millennium as "The Captain of All These Men of Death"[1] – this presents a challenge to all scientists to guarantee that this will be the last millennium in which the *M. tuberculosis* bacillus enjoys the privilege.

[1] "The captain of all these men of death that came against him to take him away, was the consumption, for it was that brought him down to the grave." John Bunyan (1680), *In* The Life and Death of Mr Badman (Dent, London, 1928), p. 282

CHAPTER 2

Proteases

2.1 INTRODUCTION

Proteases are enzymes that catalyse the hydrolysis of peptide bonds in proteins and/or peptides. They are distributed throughout all living organisms, where they participate in many physiological functions. These enzymes are increasingly implicated in the pathophysiology of a number of diseases, including infectious, neoplastic and inflammatory diseases, justifying the enormous amount of research that has focused on them. Notably, proteases are emerging as potential candidates in the pathogenesis of a number of infectious (and non-infectious) diseases and drugs designed to inhibit them have been associated with an improved clinical outcome (Travis *et al.*, 1995). For example, the four separate HIV-1 protease inhibitors (saquinavir, ritonavir, indinavir, and

nelfinavir) that inhibit proteases involved in processing the human immunodeficiency virus *gag-pol* polyprotein are responsible for a decrease incidence in clinical progression and mortality (Deeks *et al.*, 1997), and angiotensin-converting enzyme inhibitors (designed to inhibit the metalloprotease angiotensin-converting enzyme) heralded a major breakthrough in the treatment of hypertension and congestive cardiac failure (Opie, 1999). Thus, proteases are emerging as novel drug targets in pathologically diverse diseases.

2.2 CLASSIFICATION

Over 300 proteases have been discovered and are currently classified according to the reaction catalysed, the chemical nature of the catalytic site, and their evolutionary relationships (Barrett & Rawlings, 1991; Barrett, 1994).

a) Reaction catalysed

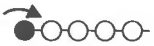
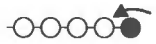
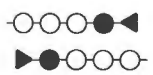
This is the mechanism by which the International Union of Biochemistry and Molecular Biology has classified enzymes. According to the *Enzyme Nomenclature 1992*, class 3 contains hydrolases, and subclass 3.4 contains the peptide hydrolases or peptidases (also referred to as proteases or proteinases). Using the principle of reaction catalysed, subclass 3.4 is divided into the exopeptidases (their action is restricted to the amino or carboxyl termini of proteins) and endopeptidases (cleave internal peptide bonds) (Table 2.1).

The exopeptidases are subdivided into: those acting at a free N-terminus and liberating a single amino acid residue, a dipeptide or a tripeptide (aminopeptidases, dipeptidyl–peptidases and tripeptidyl-peptidases, respectively), and those acting at a free C-terminus and liberating a single amino acid (carboxypeptidase) or a dipeptide (peptidyl-dipeptidase); those that are specific for dipeptides (dipeptidases); and those that

remove terminal residues that are substituted, cyclized, or linked by isopeptide bonds (peptide linkages other than those of α-carboxyl to α-amino groups) (omega peptidases).

Endopeptidases preferentially hyrolyse peptide bonds in the inner regions of proteins. A subclass of these, termed oligopeptidases, act on oligopeptide or polypeptide chains smaller than proteins.

Table 2.1. Classification of peptidases by type of reaction catalysed and catalytic type

Reaction catalysed	Catalytic type	Action	EC subsection
Exopeptidases			
Aminopeptidases			3.4.11
Dipeptidyl-peptidases			3.4.14
Tripeptidyl-peptidases			
Carboxypeptidases			
	Serine-type carboxypeptidases		3.4.16
	Metallocarboxypeptidases		3.4.17
	Cysteine-type carboxypeptidases		3.4.18
Peptidyl-dipeptidases			3.4.15
Dipeptidases			3.4.13
Omega peptidases			3.4.19
Endopeptidases			
	Serine endopeptidases		3.4.21
	Cysteine endopeptidases		3.4.22
	Aspartic endopeptidases		3.4.23
	Metalloendopeptidases		3.4.24
	Endopeptidases of unknown catalytic mechanism		3.4.99

Adapted from Barrett, (1994)

b) Catalytic type

There are 4 distinct types of catalytic mechanisms used by proteases that serve as the basis for another classification system (Hartley, 1960). Proteases are assigned to 5 different sub-groups according to the functional residue in the active site (Barrett, 1979; Barrett, 1994). These subgroups are: serine proteases, cysteine proteases, aspartic proteases, metalloproteases, threonine proteases, and proteases of unknown catalytic mechanism. Both the carboxypeptidases and endopeptidases are sub-classified in this way (Table 2.1). Inhibitors provide the most reliable way of establishing the catalytic mechanism (Table 2.2).

Table 2.2. Inhibitors used for the determination of protease catalytic type

Serine	Cysteine	Metallo	Aspartic
APMSF[i] Antithrombin III α1-Protease inhibitor Aprotinin[i] 3,4 dichloroisocoumarin[i] Pefabloc	Cystatin E-64[i]	Bestatin EDTA EGTA Phosphoramidon 1,10-phenanthroline	Pepstatin
Leupeptin PMSF[i] TLCK[i] (trypsin-like) TPCK[i] (chymotrypsin-like) Chymostatin ALLM ALLN			
α_2-macroglobulin[i]			

[i]irreversible inhibitors
Abbreviations: APMSF, 4-(amidinophenyl)methanesulphonyl fluoride; E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid; PMSF, phenylmethanesulphonyl fluoride; TLCK, L-1-chloro-3-[4-tosylamido]-7-amino-2 heptanonehydrocloride; TPCK, L-1-chloro-3-[4-tosylamido)-4-phenyl-2-butanone; ALLM, N-acetyl-Leu-Leu-methional; ALLN, N-acetyl-Leu-Leu-norleucinal

Unfortunately, there is some cross-reactivity of group inhibitors. For example, EDTA inhibits proteases that are dependent on Ca^{2+} for optimal activity. Although the majority of Ca^{2+}-dependent proteases are metalloproteases, the subtilisin family (serine proteases) and calpain family (cysteine proteases) also show augmented activity in the presence of Ca^{2+} and are inhibited by EDTA. Some serine proteases contain a cysteine residue close to the active site aspartate residue, consequently these proteases are activated by thiol-containing compounds and inhibited, to some degree, by cysteine protease inhibitors.

C) Evolutionary relationship

Based on their amino acid sequences, proteases in each subgroup are divided into different families (a group of proteases that are shown by their primary structures to be evolutionarily related) (Rawlings & Rawlings, 1993). All the members of a family are believed to have diverged from a common ancestral protein. Within each family there are occasionally sets of families that, although diverging from a common ancestral protein, have diverged so far that their relationship can no longer be proven. These sets of families are termed "clans". The clans are distinguished by the linear order of catalytic site residues, clusters of conserved amino acids around the catalytic residues, and the tertiary structures of the proteins (Barrett, 1994).

2.3 CATALYTIC TYPES

2.3.1 Serine proteases

These proteases are dependent on an active site serine residue for activity. The many proteases requiring a serine residue for activity are derived from separate evolutionary origins, consequently there are fundamental differences among them (Barrett & Rawlings, 1995). Amino acid sequence analysis indicates that there are about

Table 2.3. Families and clans of serine proteases

Clan	Family	Representative member	Known catalytic residues
PA	S1A	Chymotrypsin (*Homo sapiens*)	.....H.....D.....S.....
	S1B	glutamyl endopeptidase (*S. aureus*)	.....H.....D.....S...
	S1C	protease Do (*E. coli*)	.....H....D.....S....
	S1D	lysyl endopeptidase (*Achromobacter lyticus*)	.....H....D..S..
	S1E	streptogrisin A (*Streptomyces griseus*)	.....H.....D.....S.....
	S3	togavirin (*Sindbis* virus)	.....H.....D.....S.....
	S6	IgA1-specific serine-type prolyl endopeptidase (*N. gonorrhoeae*)	.....S.....
	S7	flavivirin (yellow fever virus)	.....HD....S.....
	S29	hepacivirin (hepatitis C virus)	.....H...D...S.....
	S30	P1 proteinase (plum pox potyvirus)	.....H..D...S.....
	S31	NS3 polyprotein peptidase (cattle viral diarrhea virus)	.....S.....
	S32	serine endopeptidase (equine arteritis virus)	.....H..D.....S...
	S35	36 kDa protease (apple stem grooving virus)	.....H.....D....S.....
	S43	porin D (*Pseudomonas aeruginosa*)	
SB	S8A	subtilisin (*Bacillus licheniformis*)	....D...H.....S.....
	S8B	kexin (*Saccharomyces cerevisiae*)	.....D.....H.....S.....
	S8C	tripeptidyl-peptidase II (*Homo sapiens*)	...D.....H.....S...
SC	S9A	prolyl oligopeptidase (Sus scrofa)	.....S..D...H.....
	S9B	dipeptidyl-peptidase IV (*Homo sapiens*)	.....S.....D.....H.....
	S9C	acylaminoacyl-peptidase (*Homo sapiens*)	.....S.....D.....H.....
	S10	carboxypeptidase C (*S. cerevisiae*)	.....S.....D...H....
	S33	prolyl aminopeptidase (*N. gonorrhoeae*	.....
	S9	Prolyl oligopeptidase	.....S...D..H...
	S15	X-Pro dipeptidyl-peptidase (*L. lactis*)	.....S.....
	S28	lysosomal Pro-X carboxypeptidase (*Homo sapiens*)	.....
	S37	PS-10 peptidase (*S. lividans*)	
SE	S11	D-Ala-D-Ala carboxypeptidase A (*B. stearothermophilus*)	.....SXXK.....
	S12	D-Ala-D-Ala carboxypeptidase B (Streptomyces sp.)	.....SXXK.....
	S13	D-Ala-D-Ala peptidase C (*E. coli*)	....SXXK.....
SF	S24	repressor LexA (*E. coli*)	.....S.....K.....
	S26A	signal peptidase I (*E. coli*)	.....S.....K.....
	S26B	signalase (*Saccharomyces cerevisiae*)	.....S.....K.....
	S41A	Tsp protease (*E. coli*)	
	S41B	tricorn core protease (Sulfolobus solfataricus)	
SH	S21	assemblin (human cytomegalovirus)	.....H.....S.....H.....
SK	S14	endopeptidase Clp (*E. coli*)	.....S.....H.....
	S49	protease IV (*E. coli*)	
SX	S16	endopeptidase La (Escherichia coli)	
	S18	omptin (*E. coli*)	
	S19	chymotrypsin-like protease (*Coccidioides immitis*)	
	S38	chymotrypsin-like protease PrtB (*Treponema denticola*)	

Adapted from the MEROPS web site (http://www.bi.bbsrc.ac.uk/Merops/Merops.htm) and Barrett & Rawlings, (1995).

D, aspartate; H, histidine; S, serine; K, lysine

37 evolutionary families (Rawlings & Barrett, 1993; Rawlings & Barrett, 1994), most of which can be further assigned to a specific clan (Table 2.3), making 8 different clans. Three-dimensional structures of members from four of the clans reveal that they are completely unrelated, suggesting that serine proteases have at least 4 separate evolutionary origins (Rawlings & Barrett, 1994). The classic catalytic triad containing serine (nucleophile), aspartate (electrophile), and histidine (base) is present only in clan A (chymotrypsin), clan B (subtilisin), and clan C (carboxypeptidase C). However, the order of these residues in the primary sequence is different for each of the 3 clans.

In contrast, the catalytic mechanism of clan E and clan F is different and includes catalytic residues –Ser-Xaa-Xaa-Lys- and a Ser / base dyad, respectively (Rawlings & Barrett, 1994). So far, only members of clan A and B have been demonstrated to be produced as inactive zymogens, requiring propeptide cleavage for activity.

2.3.2 Cysteine proteases

The activity of cysteine proteases is dependent upon a catalytic dyad of cysteine and histidine. These proteases are widely spread in nature from viruses to mammals. The primary sequence order of these residues differs among the 47 families that are incorporated into this group (Rawlings, 1994). Some families show identity, especially around the catalytic site residues, making it possible to further subdivide them into 6 clans (PA, CA, CD, CE, CF, and CH). Those families not yet assigned to any clan are grouped into clan CX. Cysteine proteases are usually active only (or optimally) in the presence of reducing agents, for example, dithiothreitol and/or cysteine.

2.3.3 Aspartic proteases

This group depends on aspartic acid residues for their catalytic activity. Initially it was thought that these proteases were confined to eukaryotes, but several examples

have now been found in microorganisms. This group incorporates 19 families, and some of these families can be attributed to a clan, making two clans (AA and AB). Those families that cannot be attributed to any specific clan are placed into clan AX. The microbial aspartic proteases are divided into pepsin-like enzymes (produced by *Aspergillus*, *Penicillium*, *Rhizopus* and *Neurospora*) and rennin-like enzymes (produced by *Endothia* and *Mucor* spp.) (Rao *et al.*, 1998). Most aspartic proteases show optimal activity at an acidic pH (pH 3 to 4) hence they are sometimes referred to as acidic proteases.

2.3.4 Metalloproteases

This group contains a diverse array of proteases that are united by their requirement for a divalent metal ion for activity, usually zinc. The amino acid signature, HEXXH, constituting the core of one of the two zinc binding sites has been identified as being highly conserved in most metalloproteases (Jongeneel *et al.*, 1989). However, this motif is also common in a variety of protein sequences that are not metalloproteases. Rawlings & Barrett (1995a) warn that only 20 % of protein sequences in the SwissProt database that share the HEXXH motif are metalloproteases. To increase specificity for metalloproteases, Jongeneel *et al.* (1989) have redefined the signature to *a*-*a*-H-E-*a*-*a*-H-*a*-*b*, where *a* represents an uncharged residue and *b* a hydrophobic residue. The two histidines participate in the co-ordination of the zinc atom, while the conserved glutamic acid is catalytic. About 57 families have been recognised and can be assigned to 10 different clans (Rawlings & Barrett, 1999). Clan MA contains proteases in which a glutamic acid residue (HEXXH + E) or a histidine residue (HEXXH + H) completes the Zn-binding site. The histidine residue is usually 8 residues after the HEXXH motif (Figure 2.1) (Rawlings & Barrett, 1995a, 1999). The other clans contain proteases that have the HEXXH motif but additional metal ligands are as yet unidentified (some in clan

MA and MX); proteases in which the metal atoms are bound at motifs other than the HEXXH consensus sequence (clans MC, ME and MJ); single zinc ion ligated to His, Asp and His (clan MD); two zinc ions ligated by Lys, Asp, Asp, Asp, Glu (clan MF); two cobalt or manganese ions ligated by Asp, Asp, His, Glu, Glu (clan MG); two zinc ions ligated by His (or Asp), Asp, Glu, Asp (or Glu), His (clan MH); and metalloprotease families not yet assigned to any clan (clan MX).

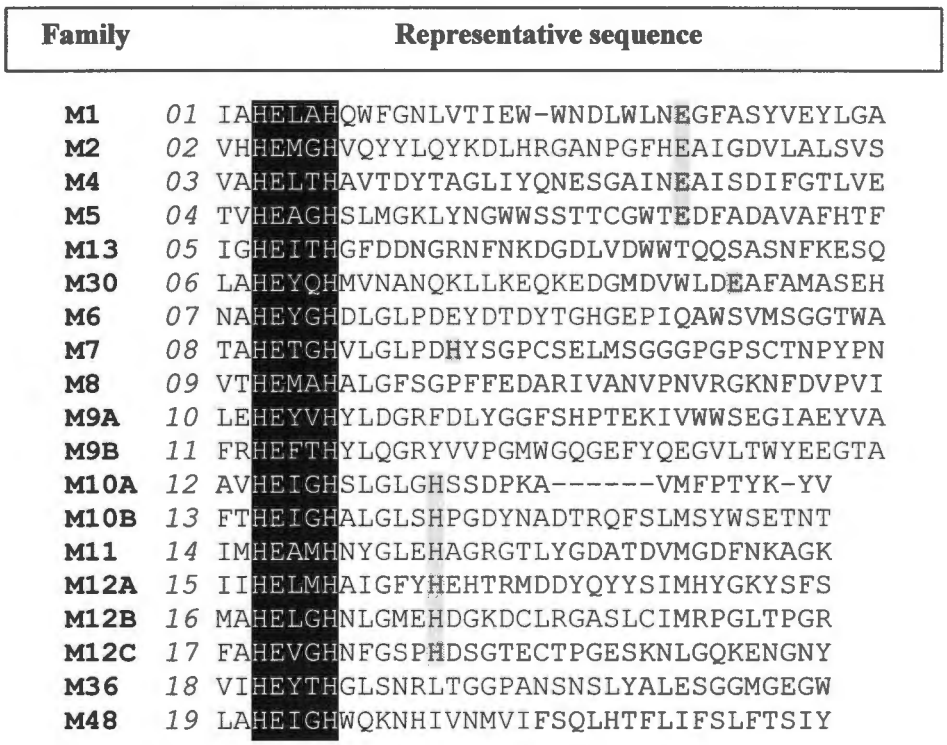


Figure 2.1. Clan MA of metalloproteases. Families are represented by one typical member. The HEXXH motif is demonstrated by white letters on a black background. The residue that is known to represent the third metal binding site is indicated by black letters on a grey background . Key to sequences : 1, human membrane alanyl aminopeptidase (P15144); 2, germinal dipeptidyl-dipeptidase A (P22966); 3, *Bacillus* sp. thermolysin (P06874); 4, *Streptomyces cacaoi* mycolysin (P20910); 5, human neprilysin (P08473); 6, *Staphylococcus hyicus* hyicolysin (Q08002); 7, *Bacillus. thuringiensis* immune inhibitor A (P23382); 8, *Streptomyces. lividans* snapalysin (P43162); 9, *Leishmania major* leishmanolysin (P08148); 10, *Vinbrio alginolyticus* microbial collagenase (P43154); 11, *Clostridium perfringens* microbial collagenase, class I (P43153); 12, human macrophage elastase (P39900); 13, *Serratia marcescens* serralysin (P23694); 14, *Chlamydomonas reinhardtii* gametolysin (P31178); 15, *Astacus fluviatilis* astacin (P07584); 16, *Crotalus adamanteus* adamalysin; 17, Bos taurus ADAM10 (Q10741); 18, *Aspergillus fumigatus* fungalysin (P46074); 19, *Streptomyces cervisiae* Ste24p endopeptidase (P47154). Adapted from Rawlings & Barrett (1999).

2.4 PHYSIOLOGICAL FUNCTIONS OF PROTEASES

Proteases are ubiquitous enzymes present in all organisms and play a critical role in essential metabolic and regulatory functions. The diverse array of essential processes in which they are involved are critical for fundamental cellular activities. An in-depth review of protease functions is beyond the scope of this chapter, but some essential functions will be highlighted.

Proteases are involved in the continuous, balanced degradation of proteins, which is essential for the maintenance of the amino acid pool, ensuring adequate nutrition and the synthesis of vital proteins. In mammalian hosts there are several protease/antiprotease systems that regulate the turnover of essential connective tissue proteins. Attention has focused on one of these systems, the elastase/antielastase system, which may contribute to the etiology of inflammatory or degradative diseases, such as emphysema, atherosclerosis, arthritis and bacterial lung infections (Werb *et al.*, 1982; Stockley, 1983; Umeki *et al.*, 1988). Endogenous host elastases - neutrophil elastase, macrophage elastase and tissue elastase - are regulated by serum protease inhibitors, most notably α1-protease inhibitor. An imbalance in the elastase/elastase inhibitor ratio results in increased elastolysis and possible pathology. Although the turnover of elastin fibres in adult mammals is very slow, some degradation does occur under physiologic circumstances, which includes growth, wound healing, tissue remodelling, and pregnancy (Werb *et al.*, 1982).

Proteases, and more specifically, lysosomal proteases, are an integral part of mammalian host defense against invading microbes. Phagocytic cells (for example, monocytes, tissue macrophages, and Kupffer cells) ingest foreign molecules, including invading microbes, by phagocytosis. The resulting phagocytic vacuole (phagosome) fuses with lysosomes, forming a phagolysosome, in which the entrapped foreign molecules are destroyed by a variety of lysosomal hydrolases (including lysozyme,

phospholipase A_2, ribonuclease, deoxyribonuclease, and proteases). Inhibition of phagosome-lysosome fusion, thereby avoiding the potentially toxic effects of lysosomal enzymes, forms part of the repertoire of strategies used by intracellular pathogens to survive within macrophages.

Additional homeostatic mechanisms in which proteases are involved include the regulation of gene expression (Roberts *et al.*, 1977; Kalisz, 1988); the regulation of many rate-controlling processes by activating the enzyme zymogens involved (generation of protein hormones, assembly of fibrils and viruses, blood coagulation, and fertilisation of ova by sperm) (Van de Ven *et al.*, 1990; Hutton, 1990); and the degradation of a variety of regulatory proteins (e.g., the heat shock response, the SOS response to DNA damage, and programmed cell death) (Gottesman Maurizi, 1992; Visser *et al.*, 1996).

Loss of homeostatic balance in the control of key proteolytic systems can lead to severe pathological consequences, including diseases such as emphysema, Alzheimer's disease, and rheumatoid arthritis (Stockley, 1983; Tetley, 1993; Koo *et al.*, 1999).

2.5 SECRETION MECHANISMS OF EXTRACELLULAR BACTERIAL PROTEASES

Although microorganisms elaborate proteases from all four catalytic types, bacterial extracellular proteases are most commonly of the serine or metallo catalytic type, and are involved in a wide spectrum of processes. These range from general "house-keeping functions" that are responsible for the homeostatic stability of the bacterium to pathogenic functions that in one or more ways contribute to the pathogenic success of the bacterium (discussed further in section 2.6). Secreted or membrane-bound bacterial proteases are more likely to be involved in disease pathogenesis, as these proteases have direct access to host tissues, inflammatory mediators, and immune molecules.

Gram-positive and Gram-negative bacteria secrete proteases into the extracellular medium. To prevent unwanted proteolysis, most extracellular bacterial proteases are first synthesised as inactive precursors that are then processed to the mature active protease (Wandersman, 1989).

In Gram-positive bacteria, secreted proteases are synthesised as preproenzymes containing an amino-terminal signal peptide followed by a propeptide and then the mature protein (Pugsley & Schwartz 1985; Wandersman, 1989). The signal peptide is usually required for translocation of the protease across the cytoplasmic membrane, where it is cleaved off by a signal peptidase, a component of the sec-dependent general export pathway; this generates the proenzyme form. The lengths of propeptides vary from protease to protease [for example, 8 amino acids in the *B. licheniformis* penicillinase (Chang *et al.*, 1982), 77 amino acid residues in the *B. amyloliquifaciens* subtilisin BPN' (Eder *et al.*, 1993), and 190 amino acid residues in the *B. subtilis* neutral protease (Yang *et al.*, 1984)], and the propeptide can be located anywhere in the precursor form. The commonest location is between the signal peptide and the mature protein; however, it can also be located as a carboxy-terminal extension of the mature protein. The function of the bacterial propeptide has not been completely elucidated, but it has been implicated in promoting the correct folding of the protease (Eder *et al.*, 1995), altering protease specificity (Wandersman, 1989), serving as a membrane anchor (Wandersman, 1989), playing a role in the secretion process (Pugsley & Schwartz, 1985); (Fujishige *et al.*, 1992; Braun & Tommassen, 1998), and inhibiting the premature intracellular activation of the protease (maintaining the zymogen in an inactive form to prevent unwanted proteolysis) (Baker *et al.*, 1992; Braun & Tommassen, 1998).

Apart from a signal peptide-dependent secretory pathway, Gram-negative bacteria are also able to secrete proteins in a signal peptide-independent manner (Pugsley, 1993). The signal peptide-dependent pathway is similar to that in Gram-positive bacteria and

examples of Gram-negative bacterial proteases secreted via this pathway are the IgA proteases (*Neisseria* spp., *Haemophilus influenzae*, and *Streptococcus sanguis*), *Pseudomonas aeruginosa* elastase (Kessler & Safrin, 1999), and the *Serratia marcesens* serine protease. Interestingly, most of these proteases have propeptides situated at the C-terminus of the mature protease. The *Erwinia chrysanthemi* proteases B and C are secreted via the signal peptide-independent pathway. These proteases are dependent on specific secretion functions that are encoded by genes adjacent to the protease structural genes (Delepelaire & Wandersman, 1989, 1991).

2.6 PATHOGENIC POTENTIAL OF BACTERIAL PROTEASES

It is becoming increasingly apparent that bacterial proteases have a far greater potential to contribute to pathogenesis than was generally recognised, and consequently they are now receiving greater attention. A review of the literature reveals the presence of virulence-associated proteases among diverse microbial pathogens, including Gram-positive and Gram-negative bacteria, viruses, parasites, fungi, and yeasts. In these microbes, proteases are involved in numerous processes, including potentially pathogenic functions (Travis *et al.*, 1995; Goguen *et al.*, 1995; Maeda & Yamamoto, 1996; Harrington, 1996). A number of areas in which microbial proteases are known to exert their pathological effects are discussed below.

2.6.1 Tissue destruction

a) *Direct*

Bacterial proteases can directly degrade proteins that are essential to tissue architecture, namely collagen, fibronectin and elastin, resulting in massive tissue destruction. Many bacterial diseases associated with tissue destruction have been linked to the ability of the bacterium to express proteases that degrade one or more essential

tissue proteins (Table 2.4). Prime examples are: corneal keratitis caused by *Serratia marcesens* (Molla *et al.*, 1986) and *Pseudomonas aeruginosa* (protease-negative strains of this species do not cause the disease) (Fisher & Allen, 1958); periodontitis caused by *Porphyromonas gingivalis* (Potempa *et al.*, 1995); gangrene caused by *Clostridium perfringens* (Rood & Cole, 1991; Matsushita *et al.*, 1994); and pneumonia caused by *Legionella pneumophila* (Baskerville *et al.*, 1986), *Aspergillus fumigatus* (Kolattukudy *et al.*, 1993; Kothary *et al.*, 1999), and *P. aeruginosa* (Galloway, 1991) (Table 2.4 contains a partial list of bacterial proteases involved in direct tissue destruction). Tissue degradation can benefit the bacterium by providing a source of amino acids that are essential for growth, breaking down tissue planes facilitating bacterial spread, and providing an environment suitable for growth, as is the case with anaerobic bacteria.

Table 2.4. Tissue destructive enzymes of bacterial pathogens

Organism	Disease	Substrate	Reference
Porphyromonas spp.	Periodontal disease	Collagen	Potempa *et al.*, (1995)
Serratia spp.	Corneal keratitis	Collagen	Molla *et al.*, 1986
Clostridium spp.	Necrotising diseases Pregnancy complications	Collagen	Bond *et al.*, (1984) Matsushita *et al.*, (1994)
E. coli	Pregnancy complications	Collagen	McGregor *et al.*, (1987)
Peptostreptococcus spp.	Breast abscess, diabetic foot necrotic pulp chambers	Collagen	Krepel *et al.*, (1991)
Enterococcus faecalis	Infections (wound, urinary tract, biliary tract), endocarditis, septicaemia intra-abdominal abscesses	Collagen	Su *et al.*, (1991)
Legionella pneumophila	Legionnaire's disease	Collagen	Baskerville *et al.*, (1986)
Pseudomonas aeruginosa	Corneal keratitis, Emphysema Cystic fibrosis infections	Collagen Elastin	Galloway *et al.*, (1991)
Aspergillus fumigatus	aspergillosis	Elastin	Kothary *et al.*, (1984); Kolattukudy *et al.*, (1993)
Staphylococcus aureus	pneumonia	Elastin	Potempa *et al.*, (1988)
Staphlococcus epidermidis	Perifollicular or macular atrophy	Elastin	Sloot *et al.*, (1992)
Vibrio spp.		Elastin Fibronectin	Smith *et al.*, (1982)
Serratia spp.		Fibronectin	Molla *et al.*, 1986

b) Indirect

Fibroblasts, neutrophils, and macrophages secrete proenzymes including latent connective tissue-degrading metalloproteinases. Microbial proteases acting on these

enzymes can activate collagenases and tissue plasminogen activator, both of which can cause tissue destruction and facilitate bacterial translocation (for example, the cysteine protease of *P. gingivalis* activates the complement system, neutrophils are attracted to the sight of inflammation, the dying neutrophils release elastase, Cathepsin G, proteinase 3, collagenase and gelatinase which then degrade connective tissue) (Uitto *et al.*, 1989; Matsumoto *et al.*, 1992; Sorsa *et al.*, 1992; Travis *et al.*, 1995).

2.6.2 Immune evasion

The complement system is an integral part of the host's defense against invading microbes. Part of the complement system's function is the recruitment of phagocytic cells (by generating chemotactic factors) and opsonisation (enabling recognition by phagocytic cells). Bacteria avoid this process by producing proteases that degrade complement components and immunoglobulins. This renders the host's immune system "blind" to infecting pathogens, contributing to both extracellular and intracellular survival. The *Serratia* 56 K protease and Group B *Streptococcus* C5a protease can both inactivate complement chemotactic component C5a (Hill *et al.*, 1988; Molla *et al.*, 1989), and the *Candida albicans* proteinase and *Yersinia pestis* Pla protease can degrade complement component C3 (Sodeinde *et al.*, 1992; Kaminishi *et al.*, 1995). In addition, proteases from a number of bacteria (for example, *P. aeruginosa*, *S. epidermidis*, *C. albicans*, and *Neisseria* spp) can degrade immunoglobulins, notably IgA and IgG, thereby disrupting opsonisation and evading phagocytosis (Plaut *et al.*, 1975; Galloway, 1991; Sloot *et al.*, 1992; Kaminishi *et al.*, 1995).

Bacterial metabolic products that act as chemotactic factors, for example formyl-Met-Leu-Phe peptide, are also substrates for some bacterial proteases. *Pseudomonas* elastase is able to degrade this peptide and destroy its receptor present on the surface of neutrophils (Ijiri *et al.*, 1994).

Some bacteria escape the host's immune response by interfering with T-cell activation and function. A *Legionella pneumophila* protease can degrade IL-2 and cleave CD4 on human T cells, suggesting a contribution by this protease to the ability of the bacterium to evade the host's immune mechanisms (Mintz *et al.*, 1993). Included in the diverse repertoire of the well-characterised *Pseudomonas* elastase is an ability to inactivate IFN-γ, thereby diminishing activation of macrophages and other inflammatory cells (Horvat, 1989).

2.6.3 Activation of the kallikrein-kinin cascade

The bradykinin-generating cascade plays an important part in the host's attempt to attract phagocytes to the site of infection. Physiological activation of this system results in vasodilation and increased vascular permeability, improving access of phagocytic cells to the area of infection. Inappropriate stimulation or upregulation of this system results in persistent vasodilation, hypotension and shock. In fact, Khan & James (1993) demonstrated that the activation of the kallikrein-kinin cascade is a critical factor in the development of septic shock. A large number of bacterial proteases are able to activate the bradykinin-generating cascade at one or more steps (Figure 2.2) (Molla *et al.*, 1989; Kaminishi *et al.*, 1990; Shibuya *et al.*, 1991; Maeda & Yamamoto, 1996). The resulting enhancement of vascular permeability ensures an adequate supply of nutrients to the site of infection, promoting bacterial growth. Some bacteria exploit the increase in vascular permeability to gain access to the systemic circulation, aiding dissemination (Sakata *et al.*, 1996). Activation of human plasma kallikrein can also lead to the activation of neutrophils (neutrophil proteases may contribute to tissue damage and dysregulation of physiological processes), the coagulation cascade, fibrinolytic system (aiding bacterial dissemination), and the complement system.

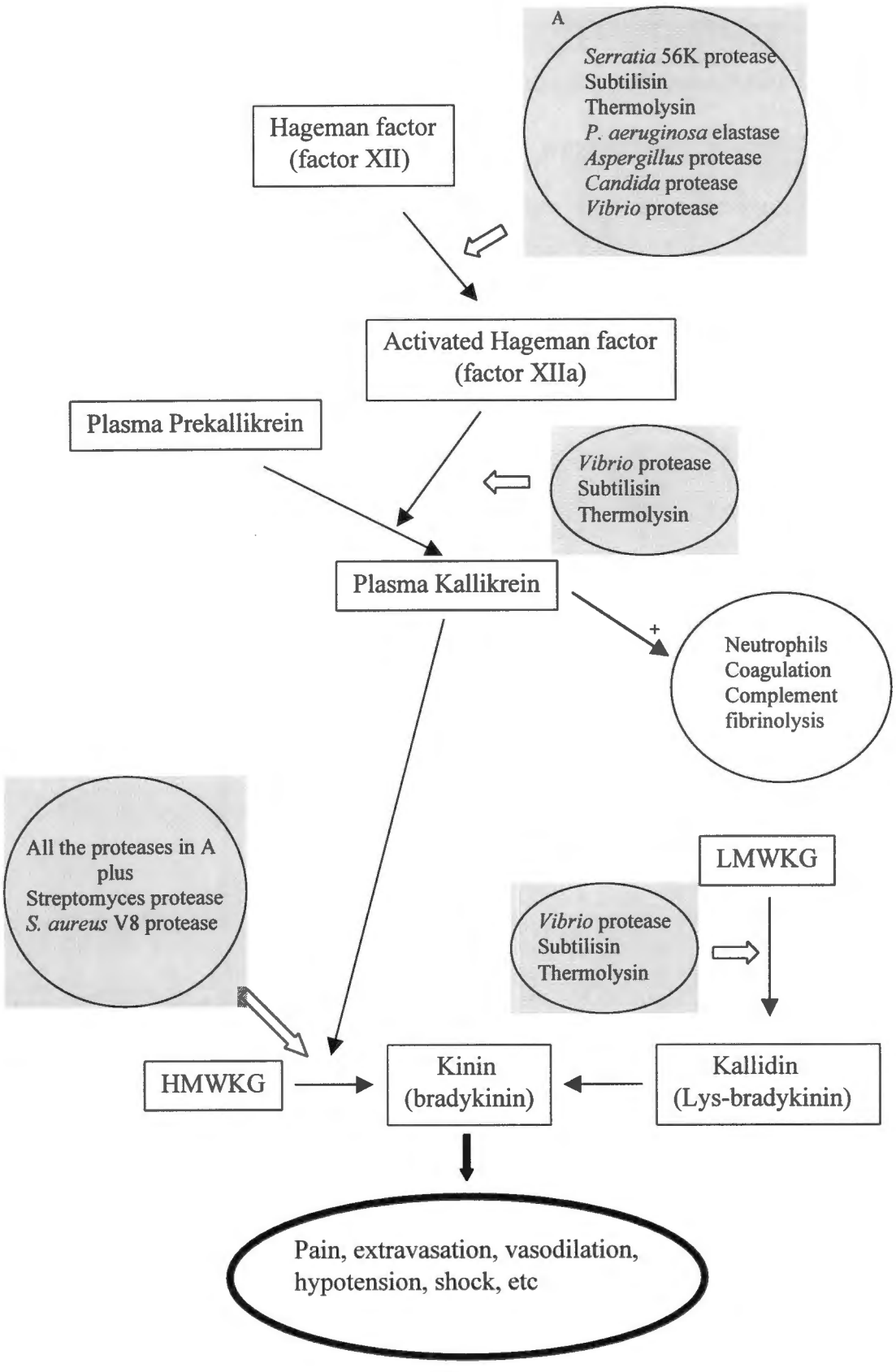


Figure 2.2. The role of bacterial proteases in activating the bradykinin generating cascade. The steps where bacterial proteases are known to be involved are highlighted in grey. Adapted from Maeda & Yamamoto (1996).

The host attempts to localise invading bacteria, thereby allowing it to focus bactericidal mechanisms on a specific area. Localisation is partly achieved by the coagulation cascade, which when activated, deposits fibrin, entrapping the bacteria and preventing dissemination. However, some bacterial proteases activate plasmin, dissolving fibrin clots and allowing dissemination (Lottenberg *et al.*, 1994). Although activation of the clotting cascade is a well-regulated universal host response to bacterial infection, dysregulation of this response by bacterial proteases can result in increased fibrin deposition (Kaminishi *et al.*, 1994). This, together with inhibition of antithrombin III by some microbial proteases, results in a hypercoagulable state or disseminated intravascular coagulation syndrome.

2.6.4 Cytotoxicity

Pathogenic bacteria have developed methods of targeting their proteases into the interior of host inflammatory cells. This involves a mechanism dependent on α_2-macroglobulin, an inhibitor of diverse proteases present in human serum. The α_2-macroglobulin-protease complex gains access to fibroblasts and macrophages via α_2-macroglobulin receptors present on their surface. Once inside lysosomes, partial degradation of this complex regenerates the activity of the protease, resulting in degradation of intracellular proteins and cell death (Maeda *et al.*, 1987). Both the *Serratia* 56 K protease and *Pseudomonas a*lkaline protease can induce cell death through this mechanism (Maeda *et al.*, 1987; Maeda & Molla, 1989).

2.6.5 Inactivation of plasma protease inhibitors

Host proteolytic systems are usually finely controlled by a number of plasma protease inhibitors. Bacterial proteases can dysregulate this homeostatic mechanism by degrading the inhibitors, allowing unregulated proteolytic activity by both bacterial and

host proteases. The pathological consequences of antithrombin III degradation have already been mentioned. Similarly, degradation of α1-protease inhibitor, complement component C1-inhibitor, and α2-macroglobulin, result in pathological consequences for the host. For example, the elastase/antielastase system in mammals is controlled by serum protease inhibitors, especially α1-protease inhibitor (Stockley, 1983). A decrease in this inhibitor may result in unopposed elastolysis resulting in tissue destruction. Examples of bacterial proteases that promote elastolysis include *Pseudomonas* elastase and *C. albicans* acid protease (Morihara, 1979; Kaminishi *et al.*, 1995). These proteases degrade α1-protease inhibitor (and in the case of the *C. albicans* acid protease also α_2-macroglobulin) thereby releasing normally inhibited host serine proteases (for example, neutrophil elastase), promoting tissue destruction. Similarly, the coagulation cascade, fibrinolysis, complement activation, and phagocytosis, all regulated by host-derived proteases, may also be affected.

2.6.6 Nutrition

Many bacteria (for example, *Pseudomonas*, *Serratia*, and *Bacillus*) produce proteases that degrade macromolecules as a nutrient source (Travis *et al.*, 1995). Involvement of these proteases in nutrient acquisition is demonstrated by the inability of protease-deficient strains to grow on media containing large peptides and few sources of nitrogen (Travis *et al.*, 1995); growth resumed once free amino acids or proteolytic enzymes were added. Organisms unable to adequately degrade host proteins may instead exploit the activities of phagocyte-derived proteases (Travis *et al.*, 1995).

2.6.7 Activation of bacterial toxins

Several bacterial toxins are activated by bacterial and host proteases, for example: diphtheria toxin, anthrax toxin, botulinum toxin, cholera toxin, and tetanus and botulinum neurotoxins (Gordon & Leppla, 1994). Furthermore, the botulinus and tetanus

neurotoxins (BoNT and TeNT, respectively) are now known to be proteases (Montecucco & Schiavo, 1993). These two neurotoxins are zinc metalloproteases that both act specifically on protein components of the neuroexocytosis apparatus (Montecucco & Schiavo, 1994). Söllner *et al.* (1993) proposed a hypothesis for general intracellular membrane fusion in all eukaryotic cells. This hypothesis requires NSF (*N*-ethylmaleimide-sensitive fusion protein) and SNAPs (soluble NSF-attachment proteins) as soluble factors for transport vesicle docking and fusion at multiple stages of the secretory and endocytic pathways. NSF interacts with SNAPs that have attached to SNAREs (SNAP receptors) present on transport vesicles (v-SNAREs) and target vesicles (t-SNAREs). Thus, each transport vesicle has its own specific v-SNARE that will attach to a cognate t-SNARE found only on the intended target membrane (Söllner *et al.*, 1993). VAMP (vesicle-associated membrane protein)/synaptobrevin, SNAP-25, and syntaxin are all SNAREs that are involved in the neuroexocytosis apparatus (Clary *et al.*, 1990; Whiteheart *et al.*, 1993). Both BoNT and TeNT cleave these SNAREs, thereby impairing the assembly of the neuroexocytosis apparatus and blocking neurotransmitter release (BoNT-B, -D, -F, -G and TeNT cleave VAMP/synaptobrevin, BoNT-A, -E cleave SNAP-25, and BoNT-C cleaves syntaxin) (Montecucco & Schiavo, 1994). The same kinds of vesicle membrane proteins likely regulate endocytosis and phagocytosis, thus it can be speculated that intracellular pathogens, such as *M. tuberculosis*, may elaborate proteases that degrade specific SNAREs, and thus promote intracellular survival by inhibiting membrane fusion events integral to host defense.

2.6.8 Other pathogenic mechanisms

Many bacterial proteases have a specific function, which is related either to the physiology of the bacterium or to the pathogenesis of disease. However, there are bacterial proteases that possess a diverse repertoire of functions that could contribute to

pathology in a number of ways. A typical example is the *S. pyogenes* cysteine protease (SCP). SCP releases biologically active proteins, such as M proteins and protein H, from the streptococcal surface (M proteins possess antiphagocytic properties and promote adherence; and protein H is an immunoglobulin-binding protein) (Berge & Björck, 1995). The release of M proteins promote bacterial dissemination, and the binding of protein H to immunoglobulin G (thereby forming immune complexes) contributes to the development of glomerulonephritis and rheumatic fever. SCP activates the streptococcal C5a peptidase, thereby blocking C5a-mediated granulocyte migration and prevents phagocytes reaching the site of infection (Cleary *et al.*, 1992). In addition, SCP also activates kinins and a human endothelial cell matrix metalloprotease (Herwald *et al.*, 1996; Burns *et al.*, 1996); it acts synergistically with other *S. pyogenes* products, further increasing tissue injury (Shanley *et al.*, 1996); and it enhances the ability of the bacterium to invade human respiratory epithelial cells (Tsai *et al.*, 1998).

Recently, a novel pathogenic mechanism of microbial metalloproteases was described. It is well known that various endogenous host membrane-bound metalloproteases are responsible for the release of effector molecules (for example, TNF-α and its receptor, angiotensin converting enzyme, interleukin-6 receptor (IL-6R), and the Fas ligand). Vollmer *et al.* (1996) showed that the *S. marcesens* metalloprotease and various bacterial supernatant samples (*S. aureus*, *P. aeruginosa*, *L. monocytogenes*, and *B. subtilis*) released biologically active IL-6R *in vitro*. The ability of bacteria to cleave cell surface recognition molecules or receptors for growth factors and cytokines could contribute to the virulence of the organism by disturbing inter-cell communications and cellular interactions. This may also result in the cells becoming refractory to cytokine stimulation.

Lastly, the ability of intracellular bacteria to manipulate the phagosome and preventing phagosome-lysosome fusion is presumed to be a potential virulence

mechanism employed by some bacteria to promote intracellular survival. At least one bacterial protease has been demonstrated to degrade an endosomal membrane protein preventing phagosome-lysosome fusion. The IgA1 protease of *Neisseria meningitidis* and *Neisseria gonorrhoeae* cleaves LAMP1, an integral membrane glycoprotein of late endosomes and lysosomes that is thought to play an important role in maintaining the stability of these compartments (Lin *et al.*, 1997). In addition, the ability to cleave LAMP1 has been associated with an enhanced survival in human epithelial cells. Since these proteases are only produced by pathogenic *Neisseria* spp., it is presumed that their function is related to virulence. If phagosome-lysosome fusion occurs, it is possible that bacterial proteases are capable of inactivating lysosomal hydrolases. *Leishmania mexicana amazonensis* depends on the surface membrane Zn-protease, gp63, for phagolysosomal survival (Seay *et al.*, 1996). The exact mechanism by which gp63 accomplishes this is unknown; however, it has been proposed that this protease is able to degrade lysosomal hydrolases, creating a hydrolase-free microenvironment around the parasite cell body.

2.7 PROTEASES IN MYCOBACTERIA

Very little is known about the diversity and functions of mycobacterial proteases. Until 1997 there were only four short reports of proteases in mycobacteria: three reports studying the enzymatic profile of *M. tuberculosis* detected extracellular protease activity (Reich *et al.*, 1981; Casal & Linares, 1984; Kannan *et al.*, 1987), and one report described the identification of a putative serine protease in *M. paratuberculosis* (Cameron *et al.*, 1994). This putative serine protease is homologous to HtrA proteases, which are stress proteins that are induced by heat shock and oxidative stress, thus suggesting a role for this protease in the *M. paratuberculosis* stress response.

More recently, candidate proteases have been reported in mycobacteria, including FtsH, two secreted serine proteases (MTB32A and MTB32B), and an unidentified elastolytic metalloprotease in *M. tuberculosis* (Rowland *et al.*, 1997; Raynaud *et al.*, 1998; Anilkumar *et al.*, 1998; Skeiky *et al.*, 1999); the 20S proteasome and the Lon protease in *M. smegmatis* (Knipfer & Schrader, 1997; Roudiak & Schrader, 1998; Roudiak *et al.*, 1998b) and the Clp protease ATPase subunit, ClpC, in *M. leprae* (Roquette *et al.*, 1998); but the data remain sparse. Amongst the paucity of research data on *M. tuberculosis* proteases there are two studies that provide compelling evidence for the expression and secretion of proteases by this organism. The first describes the identification of a zinc metalloprotease in *M. tuberculosis* H37Rv culture filtrates (Rowland *et al.*, 1997). This metalloprotease degrades elastin and was found to be most abundant in the stationary phase of the *M. tuberculosis* growth cycle. Since elastin is an integral component of the lung tissue architecture (30% of the lung is elastin), elastin degradation would result in severe lung tissue damage. It is possible that *M. tuberculosis*, in response to the nutritional stress present in the stationary phase of growth, secretes this metalloprotease that provides essential nutrients through the degradation of elastin but in so doing causes severe lung tissue damage. This may partly explain the lung tissue damage characteristic of tuberculosis. Unfortunately, the authors were unable to purify this protease so its substrate preferences and function are unknown.

The second report describes the identification of two putative secreted serine proteases in *M. tuberculosis* (Skeiky *et al.*, 1999). One of these proteases induced IFN-γ secretion from peripheral blood mononuclear cells isolated from only purified protein derivative (PPD)-positive individuals. Thus, the authors concluded that this protease may be part of the hosts immune response to *M. tuberculosis* and could be a candidate for inclusion in a subunit vaccine.

The few reports suggesting the presence of secreted proteases in *M. tuberculosis* have been verified by the *M. tuberculosis* genome sequence that revealed the presence of of more than 20 putative protease genes (a partial list is presented in Table 2.5), some of them containing putative signal peptides, suggesting that they may be secreted (Cole *et al.*, 1998). However, at present, little is known about the diversity of mycobacterial proteases, patterns of expression, substrate specificities, and their role in pathogenesis.

Table 2.5. Putative protease genes detected in the *M. tuberculosis* genome

Gene number	Designation	Homology match
Rv2223c		Probable exported protease
Rv2224c		Probable exported protease
Rv2672		Probable exported protease
Rv3596c	*clpC*	ATP-dependent Clp protease
Rv3419c	*gcp*	Glycoprotease
Rv1223	*htrA*	Serine protease
Rv0125	*pepA*	Probable serine protease
Rv2213	*pepB*	Aminopeptidase A/I
Rv0800	*pepC*	Aminopeptidase I
Rv2467	*pepD*	Probable aminopeptidase
Rv2089c	*pepE*	Cytoplasmic peptidase
Rv2535c	*pepQ*	Cytoplasmic peptidase
Rv2782c	*pepR*	Protease/peptidase, M16 family
Rv2109c	*prcA*	Proteasome – type subunit 1
Rv2110c	*prcB*	Proteasome – type subunit 2
Rv0457c		Probable peptidase
Rv0983		Probable serine protease
Rv1977		Probable zinc metallopeptidase
Rv3668c		Probable alkaline seine protease
Rv3671c		Probable serine protease
Rv3886c		Protease
Rv0198c		Probable zinc metallopeptidase

2.8 CONCLUSION

The pathogenesis of tuberculosis comprises a number of critical processes in which proteases produced by *M. tuberculosis* could, in principle, be involved. The hallmark of an *M. tuberculosis* infection is the ability of the bacillus to evade the bactericidal mechanisms of the macrophage and to multiply intracellularly. Many

investigators believe that the inability of the *M. tuberculosis*-containing phagosome to fuse with lysosomes is a consequence of manipulation of the phagosome membrane by bacterial products. *M. tuberculosis* proteases could be involved in this process. In addition, the ability of the bacterium to grow and multiply intracellularly may depend on the proteolytic degradation of macromolecules thereby releasing amino acids and peptides as a nutrient source.

The ability of the host to limit an *M. tuberculosis* infection is dependent on an intense cell-mediated immune response. By elaborating proteases that degrade complement components, immunoglobulins, chemokines, and cytokines, T cell function would be severely compromised, enabling the bacillus to evade the immune response and promoting intracellular survival. The tissue destruction pathognomonic of active tuberculous lung disease has been attributed to delayed-type hypersensitivity to bacterial antigens. Although there is probably a large component of immune-mediated tissue destruction, it is possible that *M. tuberculosis* proteases could play a significant role.

Factors that promote dissemination of mycobacteria to other sites can be regarded as virulence factors. Post-primary reactivation disease is poorly understood but one possible scenario is escape of quiescent bacilli from fibrosed pulmonary foci, which in turn, may depend on the expression of proteases that degrade surrounding tissue proteins, aiding dissemination via the airways, lymphatics and bloodstream. As already discussed, such protease functions have been demonstrated in other bacteria.

In light of these considerations and the prevalence of proteases as virulence-associated factors in phylogenetically diverse bacterial pathogens, it is plausible that proteases may contribute to the pathogenic capacity of *M. tuberculosis*. Hence a search for and analysis of protease-encoding genes, and an investigation of the properties and patterns of expression of potential extracellular proteases, is warranted.

CHAPTER 3

Proteolytic activity in *M. tuberculosis* culture filtrates

3.1 INTRODUCTION

Microbial pathogens frequently produce extracellular proteases as virulence factors, which often contribute significantly to pathology (Travis *et al.*, 1995; Maeda *et al.*, 1996; Goguen *et al.*, 1995). There has been speculation on the mechanisms and molecules involved in the pathogenesis of the pathological sequelae (especially the pulmonary damage) characteristic of *M. tuberculosis* infection. The favoured view is that the observed pathology is a consequence of a robust host immune response. Although there is evidence linking the pathology to host factors, it remains possible that the observed pathology is bacterially mediated. The hallmarks of an *M. tuberculosis* infection are its ability to evade intracellular killing, to cause massive tissue destruction, and to cause reactivation disease after having remained dormant for many years. Other pathogenic bacteria are able to resist host immune attack and produce tissue destruction through the elaboration of proteases, thus making a search for *M. tuberculosis* proteases

warranted (Travis *et al.*, 1995; Harrington, 1996). At the start of this study, apart from three short reports suggesting the presence of proteolytic activity in *M. tuberculosis* (Reich *et al.*, 1981; Casal *et al.*, 1984; Kannan *et al.*, 1987), nothing was known about the diversity of *M. tuberculosis* proteases, patterns of expression, substrate specificity, and possible links to pathogenesis. This chapter describes the efforts made to induce and detect proteolytic activity in *M. tuberculosis* culture filtrates.

3.2 *M. TUBERCULOSIS* CULTURING METHODS

For years microbiologists have debated the wisdom of studying bacteria under laboratory conditions, necessitating cultivation in artificial media, as *in vitro* manipulation of bacteria may alter the gene expression profile compared to that driving *in vivo* propagation. *M. tuberculosis*, as a prime example, has been a constant challenge. In their quest to study the bacillus in its natural state, researchers have noticed that culture conditions influence the organisms' physiology, antigenicity, pathogenicity and chemical composition, which significantly influence our interpretation of the fate of tubercle bacilli within the host.

During the establishment of tuberculosis, the *M. tuberculosis* bacilli encounter, and overcome, a variety of host defense mechanisms. The bacilli are able to survive these initial salvos by the host defense and multiply, suggesting that they possess the machinery required to survive adverse conditions. Therefore, to accurately study *M. tuberculosis*, attempts are made to approximate *in vivo* conditions. However, to achieve this in the laboratory has proven difficult, and it has been the concern of many researchers that *M. tuberculosis* bacilli cultivated *in vitro* are not representative of *in vivo*-grown bacilli. To this end numerous studies have been performed comparing *in vitro*- and *in vivo*-propagated *M. tuberculosis* bacilli. Although no differences have been noted when comparing *in vitro* and *in vivo* grown bacilli with respect to morphology,

staining properties, colony characteristics and rate of growth, biochemical differences and differences in their pathogenicity for mice have been noted (Segal & Bloch, 1956 1957). Early experiments showed a significant difference in lipid content as well as total lipid concentration between *in vitro* and *in vivo* grown *M. tuberculosis* H37Rv (Sheehan & Whitwell, 1949). In addition, several chemical compounds (phthicol, tuberculostearic acid, phthioic acid, and polysaccharides) characteristic of *in vitro* grown *M. tuberculosis* bacilli could not be found in tuberculous lung tissue (Anderson *et al.*, 1943).

More recently, intracellular residence of *M. tuberculosis* bacilli in macrophages was shown to induce at least 16 and repress about 28 *M. tuberculosis* proteins (Lee *et al.*, 1995). When these bacteria were exposed to *in vitro* stress conditions - including heat-shock, low pH and H_2O_2 – about 10 of the macrophage-induced proteins were produced. Thus, at least 6 proteins were not detected during *in vitro* growth under both normal or stress conditions. Furthermore, the pattern of induced and repressed proteins was unique to each stress condition. From these experiments it is clear that *in vivo*-grown bacilli are dissimilar to the same bacilli grown *in vitro*.

Apart from the *in vitro* vs *in vivo* question other factors have been shown to be important in the culturing of *M. tuberculosis*. Much attention has focused on the level of aeration of *M. tuberculosis* cultures. This is based on the presumption that, in diseased tissues, the bacilli are primarily exposed to anaerobic conditions. Thus, some investigators conclude that aerating *M. tuberculosis* cultures will give rise to 'unphysiological' bacteria. Aeration fosters logarithmic growth (Volk & Myrvik, 1953) and can prevent early autolysis (Wayne & Diaz, 1967) giving rise to high yields of viable organisms. Smaller yields of viable bacilli are obtained under anaerobic conditions and these bacilli appear to behave differently *in vivo* after infection of mice. Collins *et al.* (1974) showed that as cultures aged, and especially under conditions of oxygen restriction, an increasing proportion of an intravenous challenge inoculum of

M. tuberculosis bacilli could be recovered from the lung compared with the spleen of an infected mouse. This suggests that varying the degree of aeration of *M. tuberculosis* cultures may alter the phenotype of the bacillus. Proof of an altered phenotype was obtained by the electron microscopic demonstration that anaerobically (or microaerobically) grown bacilli have a thicker cell wall outer layer and a reduced cell diameter compared to bacilli grown aerobically (Cunningham & Spreadbury, 1998). The altered phenotype, together with the presence of most of the enzymes required for anaerobic metabolism, and the induction of some proteins by reduced oxygen tension [e.g., the 16-kDa small heat shock protein (Cunningham & Spreadbury, 1998)], suggests *M. tuberculosis* has developed methods to survive anaerobic conditions. This may contribute significantly to its virulence as it encounters a large variation in partial oxygen pressures during the disease process and/or may play a pivotal role in the establishment of the dormant state.

The "aeration-effects" are influenced by the carbon source present in the culture medium. Growth of *M. tuberculosis* was inhibited under aerobic conditions when the medium contained glucose, but growth was stimulated when glycerol served as the source of carbon (Lyon *et al.*, 1961). Wayne (1994) suggests that the presence of glycerol may direct metabolism into a pathway that is not representative of the physiologic state of *in vivo* propagated bacilli and thus may be counterproductive in the *in vitro* study of *M. tuberculosis*. This notwithstanding, glycerol-containing medium can be beneficial as it has been reported to be able to maintain virulence of a strain of *M. tuberculosis* whereas there was decreased virulence (for the experimental mouse) when this strain was grown in detergent-containing medium (Wayne, 1994). This suggests that the addition of a detergent may influence the *M. tuberculosis* phenotype and would probably be counterproductive in attempting to approximate *in vivo* conditions. However, without a detergent in the culture medium, *M. tuberculosis*, due to its

hydrophobic nature, will grow as a waxy pellicle in a stationary culture and with extensive clumping in a stirred culture. As each bacterium within a clump has various degrees of access to oxygen and other nutrients, clumping will generate a physiologically heterogenous group of bacteria (Wayne, 1979). The presence of a detergent in the culture medium minimises the amount of clumping and ensures a more homogenous population of bacteria, but adds to the risk of potentially altering the *M. tuberculosis* phenotype.

In order to accurately study the biochemistry and physiology of *M. tuberculosis*, a culture containing bacilli that are at the same stage of their growth cycle (i.e., synchronised) is ideal. This is very difficult to achieve with *M. tuberculosis*. Nevertheless, bacilli that are propagated without rotation are more likely to yield synchronous cultures. As bacilli settle slowly through a natural oxygen gradient present in stationary cultures replication stops yet viability is not impaired for several weeks (Wayne, 1976). These bacilli arrive in the microaerophilic sediment in a uniform stage of their replication cycle and are thus synchronised (Wayne, 1977); approximately 8 h after reaeration the bacilli multiply in a synchronous manner.

Apart from the type of carbohydrate present, other compounds have been shown to affect the growth or biochemical nature of the bacilli. Two examples are, first, the absence of sulphur in the medium results in a decreased rate and extent of growth of *M. tuberculosis* (Youmans, 1946) and, second, phosphate starvation enhances the expression of P38, the immunodominant 38-kDa protein of *M. tuberculosis* (Espitia *et al.*, 1992).

3.3 MATERIALS AND METHODS

3.3.1 Bacterial stocks

Laboratory strains were *M. tuberculosis* H37Rv-HH (Cywes *et al.*, 1996) and Erdman (ATCC 35801). A clinical isolate of *M. tuberculosis* was GSH-3052 (from a pleural effusion) (Cywes *et al.*, 1997). The clinical isolate was obtained from the Bacteriology Laboratory, Department of Medical Microbiology, Groote Schuur Hospital, Cape Town, South Africa.

3.3.2 Culture media

Bacterial stocks were initially cultured on Lowenstein Jensen (L-J) agar slopes. An inoculum of the pure bacterial stock was then transferred into a liquid culture medium. To assess the effect of the type of culture medium on the production of proteolytic activity, the bacteria were grown in different media: Kirchner's (K^+), Sauton's (S) and minimal Kirchner's (Kirchner's without glycerol and asparagine; K^-) with or without an added protease substrate (either elastin, casein, BSA or haemoglobin).

Table 3.1. Comparison of Kirchner's and Sauton's medium

Kirchner's medium		**Sauton's medium**	
Na_2HPO_4	3.0 g	K_2HPO_4	0.50 g
KH_2PO_4	4.0 g		
		Citric acid	2.00 g
$MgSO_4$	1.07 g	$MgSO_4$	0.50 g
Tri-sodium citrate	2.50 g	$FeNH_3$-citrate	0.05 g
L-asparagine	5.00 g	L-asparagine	4.00 g
Glycerol	20.00 ml	Glycerol	40.00 ml
ddH_20	980.00 ml	ddH_20	960.00 ml

3.3.3 Culture of *M. tuberculosis*

All *M. tuberculosis* strains were initially grown on L-J slopes at 37°C for 2 weeks. The bacteria were then gently dislodged with a sterile cotton bud and transferred

into 2 ml Kirchner's medium. After passing this suspension through the tip of a 1 ml Gilson pipette to break up the larger clumps, it was then diluted 1:25 in the same medium. The liquid culture was incubated, shaking at 200 rpm, for 2 weeks at 37°C. Thereafter, 25 ml of the culture were inoculated into 75 ml of fresh Kirchner's medium and incubated, shaking at 200 rpm or standing, at 37°C for up to 4 weeks.

At 7 days, 14 days, 21 days and 28 days of incubation the cultures were harvested. After pelleting the bacteria by centrifugation at 3,000 x g for 20 min at 4°C, the supernatant was passed through a 0.22 μm low-protein-binding filter to remove residual bacteria that were not pelleted during centrifugation. To prevent adventitious microbial contamination, sodium azide was added to a final concentration of 0.02%, after which the culture filtrate was concentrated 20-fold using an Amicon concentration device containing a membrane with a 10-kDa cut-off. All of the procedural steps involving the preparation of the supernatant were carried out at 4°C to limit any proteolysis. Aliquots of the 20-fold concentrate of the culture filtrate were used to assay for proteolytic activity.

3.3.4 Preparation and iodination of fibrinogen

Fibrinogen (obtained from the MRC Liver Research Centre, Groote Schuur Hospital, Cape Town) was iodinated using IODO-GEN™ (Pierce and Warner, Cheshire, England) as an oxidising agent in the presence of 0.5 μCi [^{125}I]-Na/μg fibrinogen (Amersham International, England) (Shephard *et al.*, 1989; Fraker *et al.*, 1978). The iodinated protein is designated ^{125}I-fibrinogen.

3.3.5 Detection of proteolytic activity

i) ^{125}I-fibrinogen assay

Culture filtrate (20-fold concentrate; 200 μl) was added to a glass Kimble tube

containing 500 µl HEPES (100 mM, pH 7.3) and 3 µl ^{125}I-fibrinogen (1.5-2 x 10^6 cpm). A reaction without culture filtrate and a reaction without culture filtrate but containing 1 µl trypsin (1µg/ml) were run in parallel as the negative and positive control, respectively. The reactions were incubated (with constant agitation) at 37°C for 16-18 h. At various time-points, 50 µl of this reaction was used to measure degradation, quantitatively by 7.5% trichloracetic acid (TCA)-soluble peptide formation, and qualitatively by SDS-PAGE analysis and autoradiography. The TCA precipitation reaction mixture contained 300 µl PBS and 50 µl bovine serum albumin (5%) to which 50 µl protease assay was added. This reaction mixture was precipitated by the addition of 70 µl 50% (w/v) TCA at 4°C to result in a final concentration of 7.5%. After 1 h on ice, the precipitation reaction was gently mixed and 100 µl was transferred into a plastic Kimble tube (tube A), representing the total cpm. The rest of the reaction was centrifuged at 12,000 x g for 1 min and 100 µl of the supernatant was transferred into a second plastic Kimble tube (tube B), representing the TCA soluble peptide fraction. In all figures, percentage degradation refers to the percentage TCA solubility of ^{125}I-fibrinogen and was calculated using the following formula : % degradation= (tube B cpm/tube A cpm) x 100. For qualitative analysis using SDS-PAGE, 50 µl of the assay was mixed with an equal volume of SDS-PAGE loading buffer (2x), boiled for 5 mins and then subjected to electrophoresis under reducing conditions in a 7.5% SDS-polyacrylamide gel (Appendix A.5). Approximately 15,000 cpm were loaded per lane. Following electrophoresis, the gels were dried and the products visualised by autoradiography.

ii) Elastin-Congo red assay

Elastin-Congo red (Sigma) (10 mg) was added to 1 ml 30 mM Tris-HCl (pH 7.2) and immediately vortexed to prevent aggregation. A sample of the culture filtrate (500

µl) was added and the mixture was incubated at 37°C for 6 h. After centrifugation at 1,200 x g, the absorbance of the supernatant was read at 495 nm. A reaction containing sterile distilled water (sdH_2O) or 20 ng elastase (Sigma) instead of the culture filtrate sample served as the negative and positive control, respectively.

3.3.6 Characterisation of the proteolytic activity

i) Inhibitor profile

Inhibition of proteolytic activity was examined by incubation of the culture filtrate in the presence of various protease inhibitors (listed in Table 3.2) and ^{125}I-fibrinogen. The assay was then subjected to TCA precipitation and SDS-PAGE analysis, as described in section 3.2.5.

Table 3.2. Protease inhibitors used to establish the catalytic-type of protease activity

Inhibitor	Solvent	Working concentration
Aprotinin	H_2O	4 µg/ml
Leupeptin	H_2O	0.4mM
Pefabloc	H_2O	1mM
TLCK	HCl (1mM)	0.6mM
TPCK	methanol	0.6mM
Chymostatin	DMSO	0.2mM
PMSF	DMSO	1mM
3,4-dichloroisocoumarin (3,4-DCI)	DMSO	1mM
ALLN	DMSO	200 µg/ml
ALLM	DMSO	100 µg/ml
E-64	Ethanol: H_2O (1:1)	0.2mM
Pepstatin A	DMSO	0.07mM
1,10-phenanthroline	methanol	10mM
Phosphoramidon	H_2O	0.09mM
EDTA	H_2O	10mM

ii) pH and temperature optima

The effect of pH was examined by incubation of the culture filtrate with ^{125}I-fibrinogen under the standard assay conditions with exception of the buffer. The buffers

used were 100 mM sodium acetate (pH 4.5; 5.0), 100 mM tri-sodium citrate (pH 5.5-6.5), 100 mM HEPES (pH 7.0-8.0), and 100 mM sodium borate (pH 8.5-9.5).

The temperature dependence of the proteolytic activity was assessed using the standard assay conditions except that the assay was incubated at 4°C, 25°C and 37°C. Thermostability of the activity was determined at -70°C and 80°C. After exposure of the culture filtrate to these temperatures for 10 min, the samples were incubated under standard assay conditions at 37°C.

iii) Effect of metal ions

Culture filtrate samples were incubated under standard assay conditions in the presence of 2.5 mM $CaCl_2$ or 0.1 mM $ZnSO_4$.

3.3.7 Zymography

M. tuberculosis GSH-3052 culture filtrate (20 ml) was dialysed overnight against sdH_2O [dialysis tubing (Spectrapor, Los Angeles, USA) with a 12-14 kDa size cut-off], lyophilised, and resuspended in 40 µl sdH_2O and 40 µl non-reducing SDS-PAGE solubilisation buffer. The sample was not boiled. An aliquot (20 µl) was applied to a 10% SDS-polyacrylamide gel copolymerised with casein (1mg/ml). After electrophoresis at 4 °C (to prevent autodigestion), SDS was removed by successive washes in 2.5% (w/v) Triton X-100 in distilled water (dH_2O) for 20 min, 2.5% (w/v) Triton X-100 in 50 mM Tris-HCl (pH 7.4) for 20 min, and 50 mM Tris-HCl (pH 7.4) for 20 min. The gel was then incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.4), fixed in 10% methanol/10% acetic acid for 10 min, stained with Coomassie brilliant blue R-250 and then destained with 10% methanol/10% acetic acid. The gel was then viewed on a light box and dried.

3.4 RESULTS

3.4.1 Detection of proteolytic activity in *M. tuberculosis* culture filtrates

To test whether *M. tuberculosis* secretes proteases, two well characterised strains, H37Rv and Erdman, and a clinical isolate, GSH-3052, were grown in Kirchner's medium. The supernatants (equal volumes) were assayed for proteolytic activity using ^{125}I-fibrinogen as substrate. Proteolytic activity could be detected in the culture filtrates of all 3 strains, with the clinical isolate having the highest levels (56%) of degradation, followed by Erdman (44%), and then H37Rv (18%) (Figure 3.1).

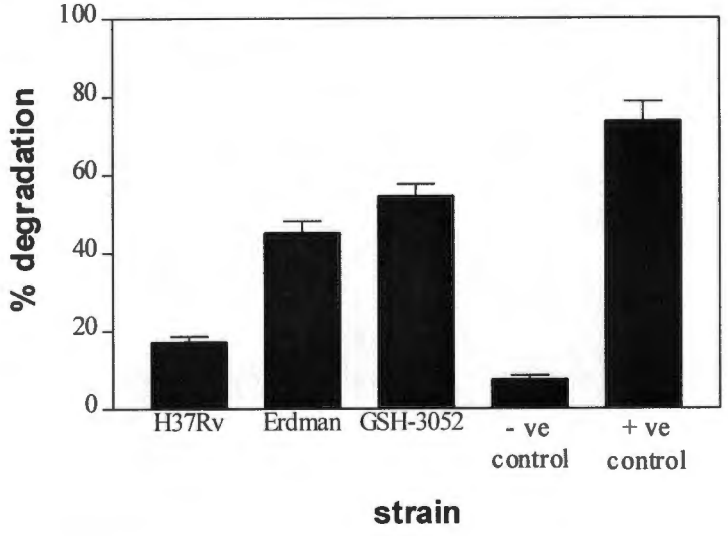


Figure 3.1. Detection of proteolytic activity in culture filtrates of 3 strains of *M. tuberculosis*. All strains were cultured in Kirchner's medium. The supernatants were filtered, concentrated and then assayed for proteolytic activity using ^{125}I-fibrinogen as substrate (equal cpm counts added per assay). The amount of degradation of fibrinogen was assessed by TCA precipitation (as described in Materials and Methods). Samples with no culture filtrate or trypsin (500 ng) were used as the negative (- ve) and positive (+ ve) controls, respectively. Data are the means and standard deviations of three samples from two independent cultures.

Although the proteolytic activity, as measured by TCA precipitation, was variable from experiment to experiment, SDS-PAGE analysis revealed a consistent pattern of degradation (Figure 3.3B). It appears that the observed fibrinogen degradation is

consistent with that described in the literature (i.e., initially the α chain is degraded, followed by the β chain, and finally the γ chain), however, since a time-dependent proteolytic assay was not performed the possibility of an alternative pattern cannot be excluded. Since no specific degradation products were obtained may be suggestive of non-specific proteolytic activity or the presence of more than one protease. Culture filtrates prepared after ~21-28 days of incubation showed maximal and most consistent levels of proteolytic activity (data not shown). However, to achieve the levels of proteolysis indicated, the culture filtrates required at least 20-fold concentration and an incubation period of between 16-18 hours. To assess the effect of the type of culture medium on the production of proteolytic activity, the bacteria were grown in different media, including minimal media with or without an added protease substrate (either elastin, casein, BSA or haemoglobin) (Figure 3.2).

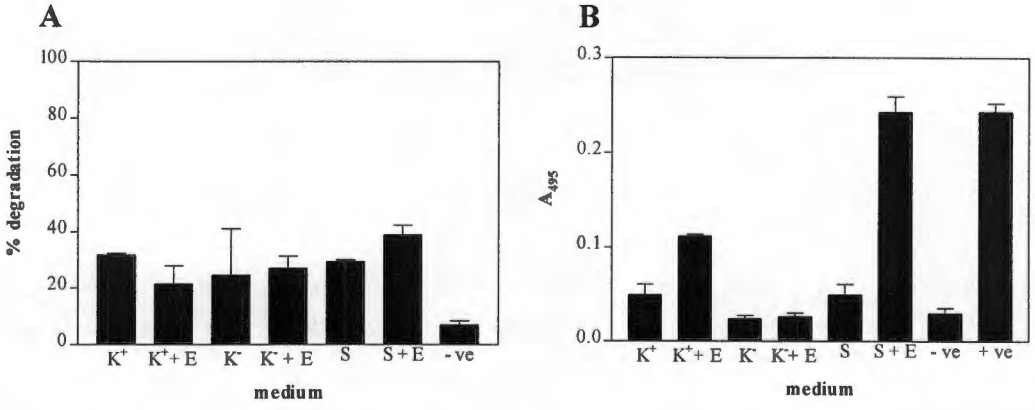


Figure 3.2. Growth of *M. tuberculosis* H37Rv in different media with or without elastin. Bacteria were grown in the indicated media with or without elastin (E) and the culture filtrates were then assessed for protease activity using (A) the standard ^{125}I-fibrinogen protease assay (fibrinogen degradation was assessed by TCA precipitation of the uncleaved products) and (B) elastin-Congo Red assay. A sample with no culture filtrate or with 20 ng elastase served as the negative (- ve) and positive (+ ve) controls, respectively. Data are the means and standard deviations from three samples of a typical experiment. Key: K^+, Kirchner's medium; K^-, minimal Kirchner's medium; S, Sauton's medium.

The use of minimal media was based on the premise that extracellular proteases may be induced under conditions of relative nitrogen limitation. In addition, a protease substrate (i.e., protein) was added to the different media in an attempt to induce protease production.

All cultures contained fibrinogen-degrading activity (Figure 3.2A). Although the levels of degradation were similar, the K^+ and S cultures produced the most consistent results. Cultures propagated in K^- or media supplemented with elastin produced inconsistent levels of degradation, as reflected by the error bars. Positive results using the elastin-Congo Red assay were obtained only for K^+ and S cultures. Highest levels of proteolytic activity were observed when both culture media were supplemented with elastin (Figure 3.2B). Differential protease expression was thus observed, in that the K^+ + E and the S + E culture filtrates contained both fibrinogen- and elastin-degrading activities, whereas the K^- + E culture filtrates only contained fibrinogen-degrading activity. The other protease substrates did not affect the proteolytic activity (data not shown). Although these results indicated differences in the proteolytic activity depending on the choice of culture medium, there was a high degree of interexperimental variability and the same results could not be consistently reproduced.

Since the literature suggests that aeration of *M. tuberculosis* cultures can effect the physiology and biochemistry of the bacterium, the bacteria were propagated rotating (aerated) or standing (non-aerated) and the resultant culture filtrates were assessed for proteolytic activity. Although there was slightly higher activity in the rotating cultures, it was not significant (data not shown). These experiments were done on both *M. tuberculosis* H37Rv and the GSH-3052 clinical isolate. In general, the most consistent results were obtained with *M. tuberculosis* GSH-3052, especially when grown in standard Kirchner's medium (K^+), and it was therefore used in all subsequent experiments.

3.4.2 Inhibitor profile of the protease activity

The effects of different protease inhibitors were investigated by incubating *M. tuberculosis* GSH-3052 culture filtrate samples in the presence of various standard inhibitors (Table 3.1). Fibrinogen degradation was assessed quantitatively by TCA precipitation of the soluble cleaved products (Figure 3.3A) and qualitatively by SDS-PAGE analysis (Figure 3.3B). The samples were compared to a control that contained no inhibitor and the TCA precipitation results are expressed as a percentage of this control. Strongest inhibition was obtained with chymostatin (77 ± 14%), ALLN (69 ± 20%), ALLM (66 ± 22%), and PMSF (66 ± 22%); moderate inhibition was obtained with 3,4-dichloroisocoumarin (38 ± 8%) and EDTA (33 ± 9%); whereas inhibition by other inhibitors was variable and generally less than 30%. Dimethylsulfoxide (DMSO) had no effect on the proteolytic activity. SDS-PAGE analysis (Figure 3.3B) shows that in culture filtrates containing chymostatin, ALLM, and ALLN, only the α-band is degraded while the β- or γ-bands are not degraded at all. In the presence of the remaining inhibitors the α-band is completely degraded with various levels of degradation of the β-and γ-bands. There were no significant differences in the inhibitor profile of the proteolytic activity in culture filtrates of *M. tuberculosis* H37Rv or GSH-3052 when propagated in K^+ or K^- media (data not shown). These data indicate that the predominant proteolytic activity is a serine protease, although there is also evidence for a metal-dependent activity.

3.4.3 The effect of pH, temperature, and metal ions on the protease activity

The effect of pH was examined by using ^{125}I-fibrinogen under the standard assay conditions, except for the buffer. Protease activity was detected over a broad pH range with two optima at pH 4.5 and 7.0 (Figure 3.4). Although this experiment was repeated on two separate occasions, low levels of proteolytic activity were obtained, making

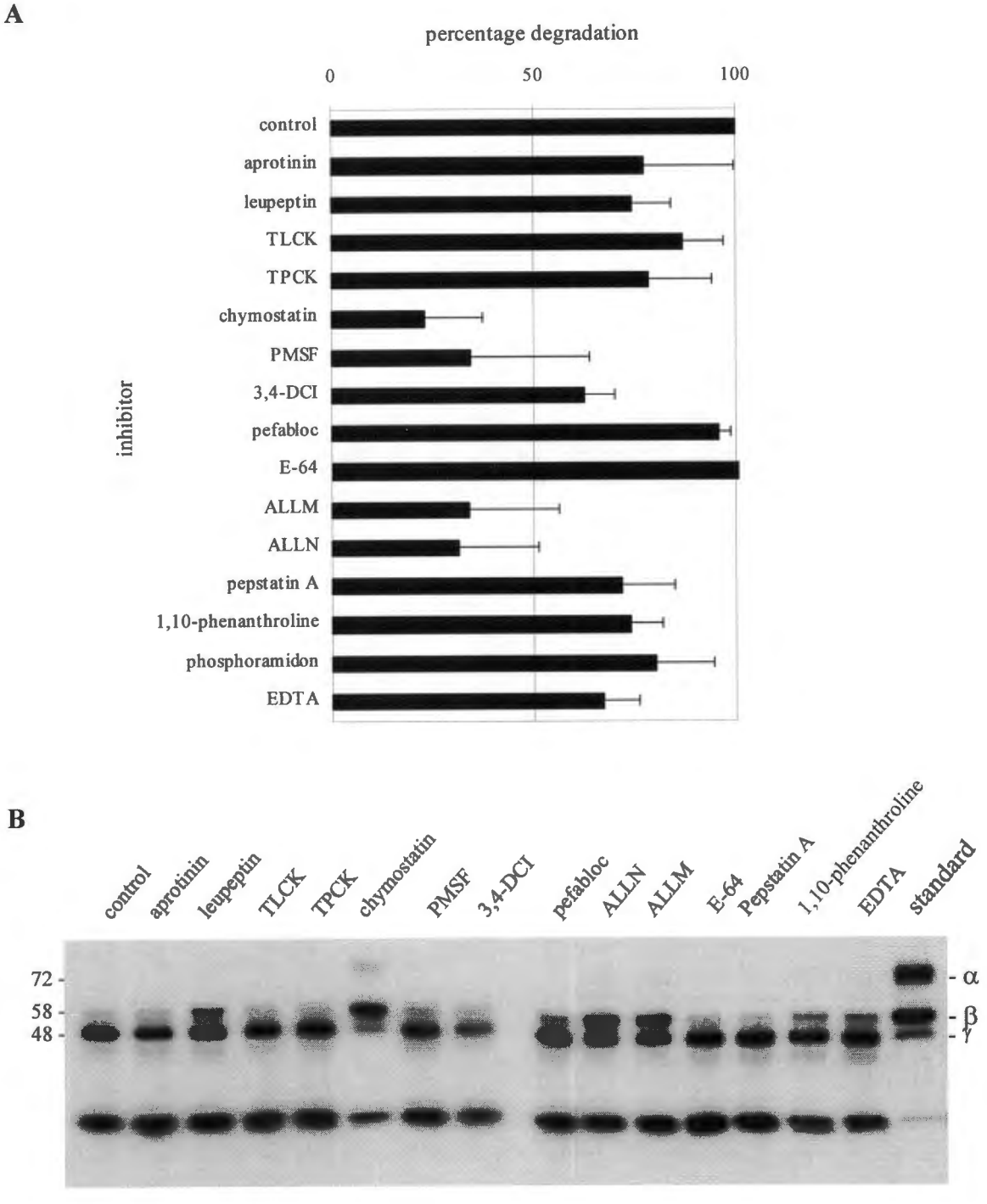


Figure 3.3. Inhibitor profile of the proteolytic activity in *M. tuberculosis* GSH-3052 culture filtrate. Aliquots of culture filtrate were incubated at 37°C for 16 h in the presence of ^{125}I-fibrinogen and the indicated inhibitor. Thereafter, degradation of ^{125}I-fibrinogen was determined by (A) TCA precipitation and (B) SDS-PAGE analysis, as described in Materials and Methods. The control consisted of culture filtrate with no inhibitor. All TCA results are expressed as a percentage of the control and represent the mean and standard deviation from six separate experiments.

interpretation difficult. However, the trend of two pH optima at 4.5/5.0 and 7.0/7.5 was consistent.

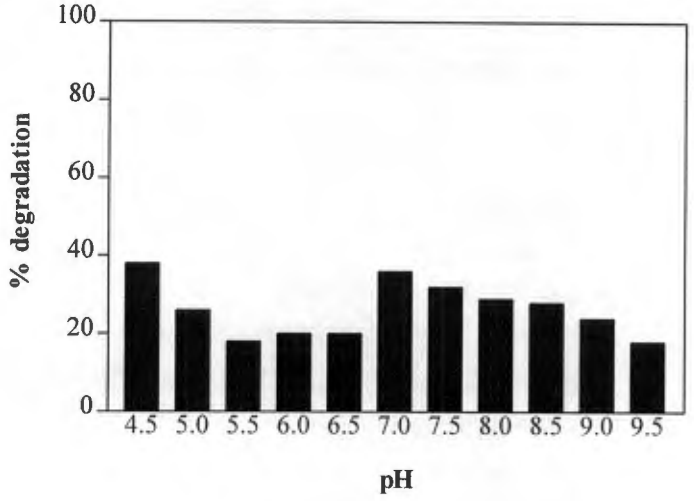


Figure 3.4. Effect of pH on the proteolytic activity in *M. tuberculosis* GSH-3052 culture filtrates. Culture filtrate samples were incubated with ^{125}I-fibrinogen under standard assay conditions, except for the buffer. A variety of buffers were used which provided a pH range of 4.5-9.5. After incubation at 37°C fibrinogen-degradation was assessed by TCA precipitation of uncleaved protein.

The effect of temperature on the proteolytic activity was assessed by incubating the culture filtrate at both standard and extreme temperatures, and then using the standard assay to assess the activity. Optimal activity was detected at 25-37°C but still present at 4°C (Figure 3.5A). Thermostability of the proteases was measured at -70°C and 80°C. The effect of temperature on the proteolytic activity was assessed by incubating the culture filtrate at both standard and extreme temperatures, and then using the standard assay to assess the activity. Optimal activity was detected at 25-37°C but still present at 4°C (Figure 3.5A). Thermostability of the proteases was measured at -70°C and 80°C. After exposure to these temperatures for 10 minutes the samples were incubated under standard assay conditions at 37°C. Activity was abolished by incubation at 80°C and was not affected by freezing at -70°C (Figure 3.5B).

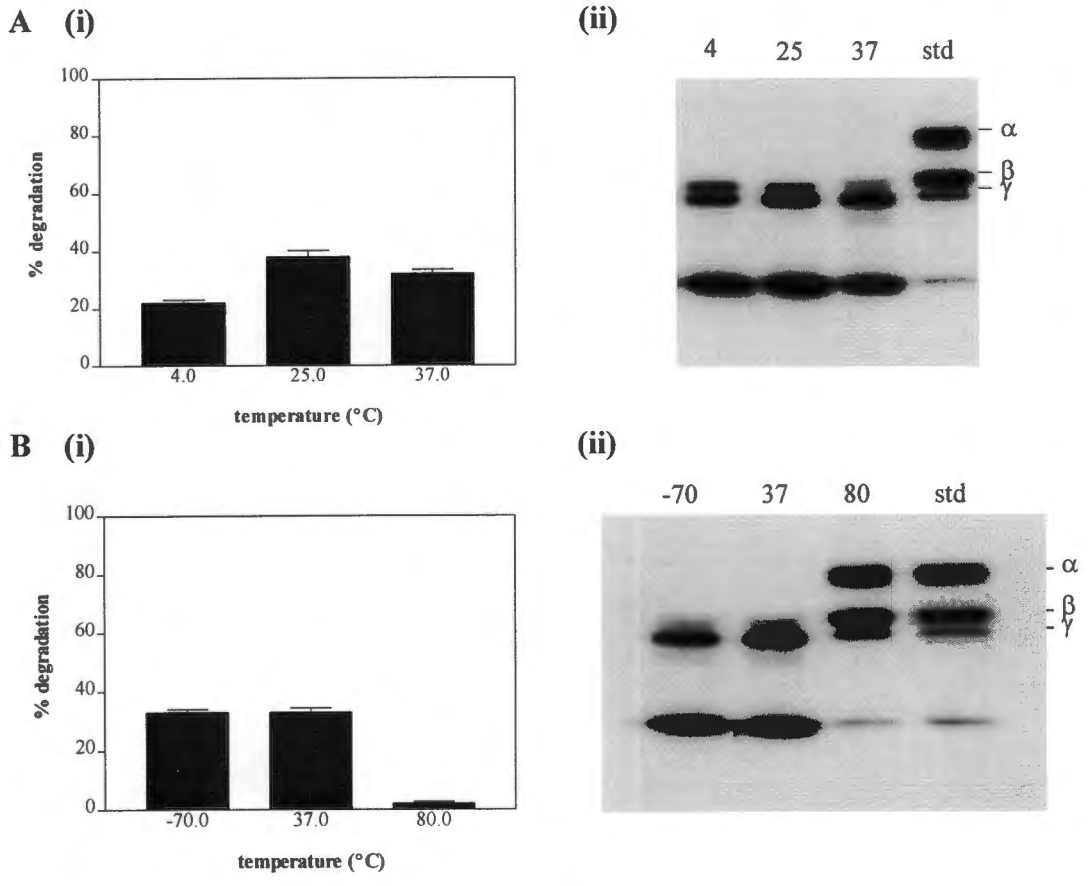


Figure 3.5. Effect of temperature on proteolytic activity in *M. tuberculosis* GSH-3052 culture filtrates. A) Samples of the culture filtrate were incubated with ^{125}I-fibrinogen for 16 h at the indicated temperatures. B) Samples of the culture filtrate were first exposed to the indicated temperatures for 10 min and then assessed for protease activity using the standard protease assay. Fibrinogen degradation was assessed by (i) TCA soluble peptide formation and (ii) SDS-PAGE analysis, as described in Materials and Methods. Data are means and standard deviations from three separate experiments.

As some inhibition was obtained with the metalloprotease inhibitors EDTA and 1,10-phenanthroline, the effects of two metal ions (calcium and zinc) were examined. Addition of calcium resulted in a 37.5% increase in protease activity (Figure 3.6) which was abolished by the addition of EDTA. EDTA alone inhibited the activity by 22%. Complete inhibition was not expected as this was a crude sample and probably contained more than one protease. Zinc had no effect on the proteolytic activity.

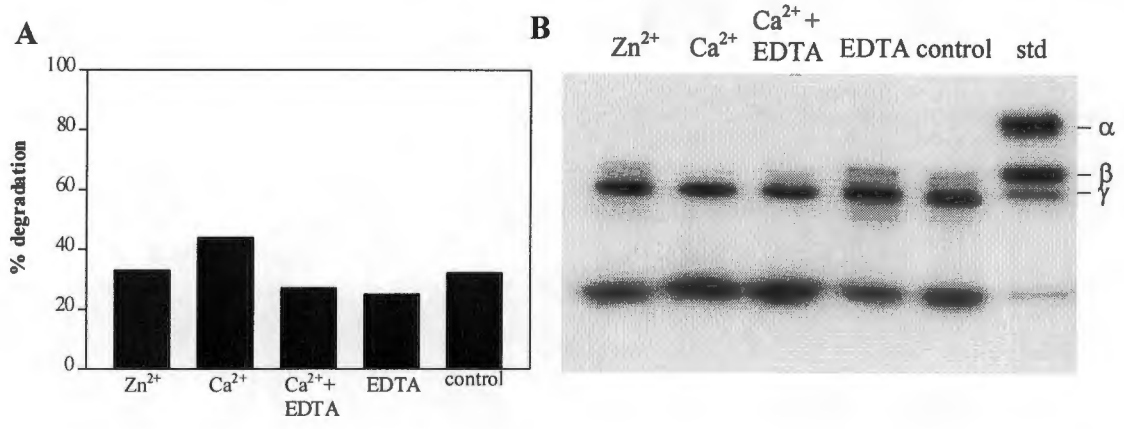


Figure 3.6. Effect of Ca^{2+} and Zn^{2+} on proteolytic activity in *M. tuberculosis* GSH-3052 culture filtrates. Samples of the culture filtrate were incubated under the standard assay conditions in the presence of $CaCl_2$ (2.5 mM) and $ZnSO_4$ (0.1 mM). Fibrinogen degradation was assessed by (A) TCA-soluble peptide formation and (B) SDS-PAGE analysis, as described in Materials and Methods. A sample with culture filtrate only was used as the control.

3.4.4 Zymography.

To determine the apparent molecular weight/s of the proteases in the culture filtrates, samples were resolved by electrophoresis in SDS-gels copolymerised with a protease substrate, casein. After incubating the gel overnight at 37°C in Tris-buffer (pH 7.4), the gels were stained and destained and assessed for zones of clearing. Caseinolytic activity was detected at ~ 44 kDa, ~ 46 kDa, and possibly at ~55 kDa (Figure 3.7).

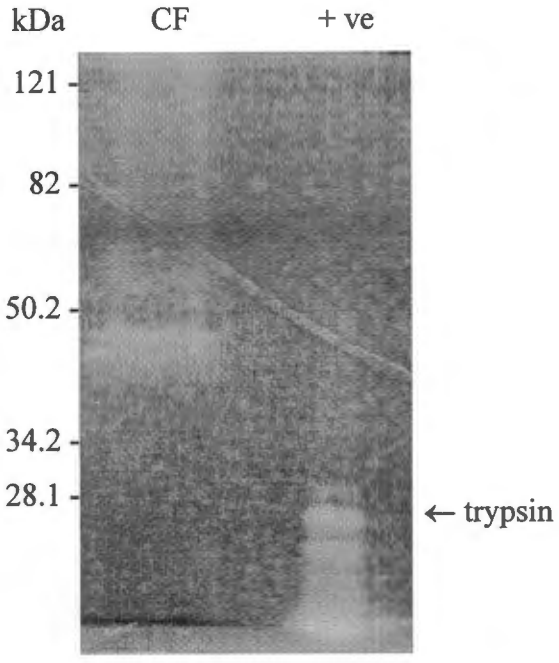


Figure 3.7. Zymogram of caseinolytic activity in the culture filtrate of *M. tuberculosis* GSH-3052. A 20 µl sample of concentrated culture filtrate (CF) was applied to a SDS-polyacrylamide gel copolymerised with casein (1 mg/ml). The gel was incubated overnight at 37°C, stained with Coomassie Brilliant Blue and destained. Caseinolytic activity is seen as a zone of clearing. Numbers on the left are molecular masses (in kilodaltons) of the markers. Trypsin (500ng; MW 23.5 kDa) was used as a positive (+ ve) control.

3.5 DISCUSSION

There has been little research into the potential of proteases as virulence determinants in *M. tuberculosis*. The current dogma (as espoused in textbooks of medicine and microbiology) that the bacterium is inert and that the pathology of tuberculosis is entirely a consequence of the host's immune response is a view that has been widely held for some time (Dannenberg, 1991; Toossi & Ellner, 1998; Orme, 1998). However, a wide range of microbes, especially those causing lung pathology, have been shown to possess proteases as potential virulence factors, thus making a search for *M. tuberculosis* proteases, and in particular secreted proteases, reasonable. In recent years, it has become evident that mycobacteria produce a surprising quantity and

diversity of soluble protein antigens (Andersen *et al.*, 1991; Andersen & Brennan, 1994). Moreover, *M. tuberculosis* possesses all of the components of the sec-dependent general export pathway (Chubb *et al.*, 1998). Therefore, it is possible that this organism expresses and secretes proteases.

To accurately assess, characterise, and purify proteolytic activity in bacterial supernatants requires a high yield of viable organisms. The slow growth rate of *M. tuberculosis* ensures that high yields are difficult to achieve with this bacterium. In addition, attempts to do so are usually at the expense of creating "unphysiological" conditions. This becomes important if approximation of *in vivo* conditions is of critical importance to the questions pursued. Furthermore, to accurately identify the stage of the growth cycle at which a specific protein is produced requires synchronised cultures. This, too, is difficult to achieve with *M. tuberculosis*, as *in vitro* culture conditions generate physiologically heterogeneous populations of bacilli. Growth of the bacilli without rotation would result in synchronous cultures and is probably closer to *in vivo* conditions, but the yields of bacteria are much lower than with rotating cultures. Similarly, higher yields of viable organisms are obtained from aerated cultures, but as the bacilli are more likely to be exposed to microaerobic or anaerobic conditions *in vivo*, it becomes a "juggling act" to approximate *in vivo* conditions and obtain cultures with high yields of viable organisms.

In this study, culture conditions were selected that would provide high yields of viable organisms and detectable levels of proteolytic activity but at the same time attempting not to deviate too far from *in vivo* conditions. Data are presented that show that *M. tuberculosis* culture filtrates contain proteolytic activity, albeit at a comparatively low and inconsistent level. The pattern of fibrinogen degradation (i.e., first the α chain then the β chain and lastly the γ chain is degraded) is consistent with the proteolytic degradation of fibrinogen reported in the literature. The α chain is most susceptible to

proteolytic cleavage, followed by the β chain, and then the γ chain. The increasing intensity of the γ band that is observed as more of the fibrinogen is degraded is probably due to the generation of a degradation product of the same molecular weight as the γ chain. This too has been observed previously (Kelly *et al.*, 1994; Jennings *et al.*, 1999). The low level of inter-experiment reproducibility and low activity may be explained by: (a) all extracellular microbial proteases are secreted as inactive zymogens with propeptides that require processing for activation (Wandersman, 1989), and *in vitro* culture conditions may not contain the necessary stimuli for activation; (b) extracellular proteases in *M. tuberculosis* may be induced and/or activated under specific conditions that remain to be identified; (c) exported proteases may not be truly soluble, and thus the secreted proteases may remain largely associated with the bacterial cell envelope; (d) *M. tuberculosis* may not produce exported proteases and the activities found are due to cytosolic proteases released from lysed bacteria; or (e) the preferred substrates remain to be identified.

Despite these problems the activity was partially characterised and found to be inhibited, to varying degrees, by both serine protease and metalloprotease inhibitors. As the class-specific cysteine protease inhibitor E-64 did not inhibit activity and most bacterial proteases belong to either the serine or metallo families, it is likely that inhibition by ALLM and ALLN (which are generally regarded as cysteine protease inhibitors) is due to their known cross-reactivity with some serine proteases. This usually arises when a serine protease (and sometimes a metalloprotease) contains a cysteine residue close to an active site residue, as it is then susceptible to inhibition by cysteine-type protease inhibitors (Barrett, 1994; Barrett & Rawlings, 1991).

Further characterisation of the activity revealed some enhancement by the addition of Ca^{2+}, but not of Zn^{2+}, suggesting that the culture filtrate activity could be partly attributed to a metal-dependant protease, consistent with moderate inhibition by

EDTA, although it was unlikely to be a true Zn-metalloprotease. It is worth noting that the activity of subtilisins and calpains are also augmented by Ca^{2+} and may therefore also show some inhibition with EDTA (Rawlings, 1994; Rawlings & Barrett, 1994). Thus, the metal-dependent activity detected in the culture filtrate may be consistent with the presence of a metalloprotease, subtilisin, or calpain.

Many bacteria produce proteases in response to nutrient deprivation. This occurs physiologically when bacteria enter their stationary phase of growth, as it is at this stage that bacteria usually encounter nutritional stress and many have been shown to secrete proteases (Gibb & Strohl, 1988; Roitsch & Hageman, 1983; Long *et al.*, 1981). Maximum activity of the proteolytic activity in the *M. tuberculosis* culture filtrates was detected after 4-5 weeks of growth, which likely coincided with the stationary phase of the *M. tuberculosis* growth cycle. To assess the effect of nutrient deprivation on the induction or repression of proteases, bacilli were cultured in nutrient-depleted media with or without a protein supplement (elastin, casein, BSA, or haemoglobin). Although there was no significant difference in the levels of activity, only complete media with or without elastin contained fibrinogen- and elastin-degrading activities, suggesting some nutritional influence on the induction of certain protease genes. This indicates that *M. tuberculosis* may utilise proteases for the breakdown of macromolecules, yielding amino acids required for protein synthesis and growth. Since elastin is an integral structural component of the lung (30% of lung tissue is elastin), detection of elastolytic activity may suggest a possible contribution of *M. tuberculosis* proteases to the tissue destruction characteristic of pulmonary tuberculosis. The presence of elastolytic activity in *M. tuberculosis* culture filtrates has been reported by Rowlands *et al.* (1997) who demonstrated the presence of an elastolytic calcium-dependent metalloprotease in culture filtrates of *M. tuberculosis* H37Rv grown in Sauton's medium. The assessment of the proteolytic activity produced in Sauton's medium that is presented in this chapter was

done partly in an attempt to reproduce the results obtained by Rowlands *et al.* (1997) as well as to assess the contribution of different culture media. The activity detected in the K^+ + E and S + E cultures was similar to that detected by Rowlands *et al.* (1997) in that both showed elastolytic activity. However, the protease activity detected by Rowlands *et al.* (1997) was completely inhibited by EDTA and retained activity after 10 min at 80 °C, whereas the activity detected in the K^+ cultures only showed 37% inhibition by EDTA and complete loss of activity by exposure to 80 °C for 10 min. This suggested that the activity detected in the K^+ culture filtrates was probably due to a protease that was different to that detected by Rowlands *et al.* (1997), motivating further characteristion of this activity.

Survival and growth of the bacilli in the host depend on its ability to overcome the numerous host defences and adverse conditions encountered. It is generally accepted that when bacilli are confined to the centre of well-organised granulomas they are subjected to bacteriostatic conditions of anoxia, acidity and nutrient deprivation. Frequently, the bacilli are not contained and the granulomas progressively enlarge with increasingly necrotic centres, often coalescing with neighbouring granulomas or eroding into adjacent tissues, producing large, necrotic, cavitating lesions. This sequence of events has been suggested to be entirely due to a self-defeating delayed-type hypersensitivity reaction on the part of the host. An alternative explanation could be that this kind of disease progression is partly due to the multiplication of virulent bacilli that, under adverse conditions of a well-organised granuloma, are able to synthesise and secrete proteases, that allow them to escape into a more favourable environment. To this end it is worth noting that the culture filtrate proteolytic activity is present over a broad pH and temperature range. This makes it tempting to suggest that the wide pH and temperature range of activity is indicative of the protease's potential role in benefiting the

bacillus under adverse conditions, but it may also indicate the presence of more than one protease.

Attempts to purify the protease/s – which included High Performance Liquid Chromatography (HPLC), affinity chromatography using a bacitracin column, and ion-exchange chromatography – were unsuccessful. This was probably due to the low expression levels and possibly to instability of the protease/s under the conditions used.

In conclusion, proteolytic activity has been detected in the culture filtrates of *M. tuberculosis*. The inhibitor profile suggests the presence of at least 2 proteases, one of which is a serine protease and the other possibly a metalloprotease. The low and inconsistent level of activity indicates that we are presently unable to reproduce the stimuli necessary for full induction or activation, or that the optimal substrate has not been found.

CHAPTER 4

Identification and sequence analysis of two putative *M. tuberculosis* protease genes.

4.1. INTRODUCTION

Intracellular residence of *M. tuberculosis* within macrophages induces the expression and secretion of specific proteins not evident during *in vitro* growth (Lee *et al.*, 1995). Although these proteins are presumed to play a role in invasion and intracellular survival, their precise contribution to the pathogenicity of *M. tuberculosis* is unknown. As an approach to identify *M. tuberculosis* secretory proteins, Mdluli *et al.* (1995) devised a signal sequence-detection strategy using vectors tailored to generate alkaline phosphatase (phoA) fusions to *M. tuberculosis* DNA. Using this strategy, a gene for a putative secreted serine protease, *Rv3883*, was identified. Homology matching of

this sequence with unannotated *M. tuberculosis* genomic DNA sequences deposited on databases at the Sanger Centre (Cambridge, UK) revealed a further 4 putative serine protease genes in the *M. tuberculosis* genome. The proteins encoded by these genes share 36-47% identity, and initial primary sequence analysis indicates that they are most homologous to members of the subtilisin family of serine proteases (Brown *et al.*, 1999). Furthermore, primary sequence analysis indicates that they constitute a novel subfamily of the subtilisins. For simplicity, these putative serine protease genes were designated *mycP1-5* (based on their sequential identification; *Rv3883* has been designated *mycP1*), and the proteases they encode mycosin-1-5, respectively. Each gene encodes a protein with a typical signal sequence and one or more hydrophobic sequences in the C-terminal half of the protein, suggesting that these proteins are likely secreted and anchored in the cytoplasmic membrane or lipid-rich regions of the cell envelope. It is possible that secreted proteins, and more specifically secreted proteases, may contribute to the virulence of *M. tuberculosis*, tempting one to speculate that this novel family of subtilisin-like proteases may play a role in the pathogenic lifestyle of this organism. Although these protease genes were identified prior to the annotation of the *M. tuberculosis* genome sequence, the recent publication of the genome sequence confirmed the presence of this family of proteases and indicated the presence of over 20 additional putative protease genes (Cole *et al.*, 1998). One of these additional genes, *Rv0419* (designated *mpr*1), encodes a putative secreted metalloprotease (designated Mpr1).

4.2. SUBTILISINS

The subtilisin family is the second largest family (clan SB, family S8) of serine proteases and is present in members from phylogenetically diverse classes of organisms, namely eubacteria, archaebacteria, eukaryotes, and viruses (Rawlings & Barrett, 1994). Almost all are endopeptidases, although there is also a tripeptidyl-peptidase. The prototypical member, subtilisin, is secreted into the medium by a wide variety of *Bacillus* species (Subtilisin BPN′ by *B. amyloliquifaciens*, subtilisin Carlsberg by *B. licheniformis*, subtilisin E from *B. subtilis* 168, subtilisin Savinase by *B. lentus*, and others). In addition, numerous subtilisin-like proteases are expressed and secreted by other bacteria.

A distinct subfamily of the subtilisins, the kexin subfamily, has been demonstrated in yeasts and animals and their main function is proprotein-processing. The subtilisins have been widely studied and their kinetics and mechanism of activity are well understood. Furthermore, over 50 X-ray structures have been determined (Wells & Estell, 1988).

4.2.1. Mechanism of action

Although the mechanism of action of subtilisin is identical to that of trypsin-like serine proteases, they are evolutionarily and structurally distinct. Their well-documented active-site involves the side chains of Ser-221, His-64, and Asp-32 (subtilisin BPN′ numbering).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}S \xrightarrow{k_2} E\text{-}Pr_2 + Pr_1 \xrightarrow{k_3} E + Pr_2$$

In the kinetic scheme for subtilisin, acylation is controlled by k_2 and deacylation by k_3, where E is enzyme, S is substrate, Pr_1 and Pr_2 are hydrolysed products, and E-Pr_2 is the acyl-enzyme complex (Carter *et al.*, 1991). In the rate-limiting acylation step for

hydrolysis of peptide bonds, His-64 accepts a proton from Ser-221 (Wells & Estell, 1988; Carter *et al.*, 1991). This results in a tetrahedral oxyanion intermediate (E-S) which is stabilised by at least two hydrogen bonds. Asn-155 provides one of the hydrogen-bonding groups in the oxyanion hole, and His-64 the other hydrogen-bonding group (Bryan *et al.*, 1986; Wells & Estell, 1988). As His-64 donates a proton to the leaving group amine, the tetrahedral E-S collapses to form an acyl-enzyme intermediate. The enzyme is released from this complex by hydrolysis, involving His-64 that removes a proton from a water molecule leading to nucleophilic attack upon the acyl-enzyme complex and finally to reprotonation of Ser-221 (Carter *et al.*, 1991). Therefore, in the overall mechanism, Ser-221 serves as a catalytic nucleophile; His-64 functions as a general acid-base; and Asp-32 positions the correct form of His-64 in the E-S complex and provides electrostatic stabilisation (Wells & Estell, 1988).

4.2.2. Secretion

All subtilisins are synthesised as a preproprotein with a signal peptide (~27-29 amino acids), and an N-terminal propeptide (~77-79 amino acids), which is followed by the mature protein. The signal peptide resembles a typical bacterial signal sequence and, in the case of the *Bacillus* spp. subtilisins, has a consensus signal peptidase cleavage site, Ala-X-Ala-Ala (Wong & Doi, 1986). The subtilisin propeptides have received a significant amount of attention as they appear to be intricately involved in the determination of the activity of subtilisin. The subtilisin propeptides are characterised by a highly homologous region at the C-terminal end, a common sequence, Tyr-Ile-Val-Gly-Phe-Lys, at the N-terminal end, and the presence of a large number of charged residues (~30%) (Jacobs *et al.*, 1985; Ikemura *et al.*, 1987). In addition, the predicted secondary structure and hydrophobic residue distribution of the propeptides show a high degree of

similarity among all subtilisins, even in regions where their primary structures are quite different (Jacobs *et al.*, 1985).

The full-length precursor form of subtilisin is associated with the cell membrane where it undergoes autocatalytic processing (Power *et al.*, 1986). The propeptide is essential for the correct folding of the protein and current evidence suggests that once the protein is folded the propeptide is autoprocessed hydrolytically by mature, active subtilisin (Eder *et al.*, 1993; Li *et al.*, 1995; Ikemura *et al.*, 1987). The cleaved propeptide is not released from the enzyme but remains noncovalently associated with the catalytic domain, acting as an autoinhibitor (Bryan *et al.*, 1995; Li *et al.*, 1995). Propeptides have been shown to be both substrates and strong slow-binding inhibitors of subtilisins BPN′ and Carlsberg (Hu *et al.*, 1996). The cleaved propeptide is necessary for activation of subtilisin but not for maintenance of activity. The driving force behind the autocatalytic processing is unknown. Only preprosubtilisin is detected when produced by coupled transcription-translation *in vitro,* suggesting that some structural, enzymatic or cofactor function is missing *in vitro* that is necessary to initiate processing (Power *et al.*, 1986).

There is some debate whether the autoprocessing/folding pathway is inter- or intramolecular. Denatured subtilisin is unable to refold by itself but refolding does occur with exogenously added synthetic propeptides or a prosubtilisin active-site mutant. Li *et al.* (1995) suggest that the exogenously added propeptide behaves as an intramolecular chaperone for folding and that its ability to perform this function is directly related to the affinity with which it can bind the mature active subtilisin to inhibit its activity. Since a prosubtilisin active-site mutant can direct the folding of denatured subtilisin, Hu *et al.* (1996) proposed a model that has the propeptide of one prosubtilisin behaving as a refolding template for the mature sequence of another. In this context the hydrolytic process would also be intermolecular. Although it has proven very difficult to

unequivocally distinguish intra- from intermolecular processing, the bulk of the evidence supports an intermolecular process. However, in saying this, there are some investigators that believe that both are feasible. Hu *et al.* (1996) suggest that when there is a low concentration of preprosubtilisin with little or no active enzyme around, then intramolecular processing or processing by a different protease may be a requirement. As more active mature subtilisin is produced, the intermolecular pathway probably takes precedent.

4.2.3. Kinetics, Inhibitors and Activators

The subtilisins exhibit typical Michaelis-Menten kinetics and have broad substrate specificity. They are usually active in the neutral to alkaline pH range and are stimulated by Ca^{2+}. Members of the kexin subfamily preferentially cleave after di-basic or multi-basic amino acids (Rawlings & Barrett, 1994). Some members of the kexin subfamily as well as some yeast subtilisin-like proteases are activated by thiol-containing compounds and inhibited by cysteine protease inhibitors (Rawlings & Barrett, 1994; Jany *et al.*, 1986). This has been attributed to the presence of a cysteine residue close to the active site histidine. Almost all bacterial subtilisins do not contain cysteine residues, the only exception being the subtilisin from *B. smithii* (Jany *et al.*, 1986). The subtilisins and subtilisin-like proteases are inhibited by serine protease inhibitors, notably PMSF, chymostatin, and others (Suzuki *et al.*, 1997). In addition, since they are stimulated by Ca^{2+}, EDTA also inhibits the activity of these proteases to some degree (Suzuki *et al.*, 1997). This is in contrast to the mammalian subtilisin-like proprotein convertases that, despite being serine proteases, are insensitive to typical serine protease inhibitors such as PMSF, TLCK, and TPCK.

4.3. IDENTIFICATION OF A PUTATIVE SUBTILISIN-LIKE PROTEASE GENE (*MYCP1*), AND SEQUENCE ANALYSIS OF ITS PREDICTED PRODUCT, MYCOSIN-1.

4.3.1. INTRODUCTION

The culture filtrate results presented in Chapter 3 are consistent with the presence of a serine protease and/or metalloprotease. The Ca^{2+}-stimulation and mild inhibition by EDTA is suggestive of the presence of a Ca^{2+}-requiring protease - either a subtilisin, metalloprotease or calpain. Furthermore, inhibition obtained with the cysteine protease inhibitors, ALLM and ALLN, is not uncommon with some members of the subtilisin family. Since these data are consistent with the discovery of a novel subfamily of putative subtilisin-like secreted serine proteases in the *M. tuberculosis* genome, it was decided to initiate the characterisation of the first member of this family, mycosin-1. The first part of this chapter details the sequence analysis of the *mycP1* protease gene and its predicted product, mycosin-1.

4.3.2. MATERIALS AND METHODS

4.3.2.1. Sequence analysis

All non-redundant sequence databases were scanned for sequences homologous to the *mycP1* gene and its predicted product, mycosin-1 (Table 4.1). In addition, various internet servers (Table 4.1) were used to further characterise the predicted mycosin-1 sequence: alignments with homologous sequences were optimised with Multalin v. 5.3.3; the signal peptide cleavage site was predicted using SignalP; transmembrane domain and topology predictions were determined with the Tmpred, TopPred2 and Sosui

programmes; a hydrophobicity plot was obtained using Tmpred; and secondary structure predictions were derived by the GOR v. 4 method.

Table 4.1. Internet servers used in the analysis of *mycP1* and *mpr1* protease genes and their products

Server	Description	URL
Database searches		
NCBI BLAST	National Center for Biotechnology Information; gappedBLASTP/BLASTN/ TBLASTN searches of all non-redundant peptide and nucleotide sequence databases	http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=0
Sanger Centre *M. tb* BLAST	BLAST searches of Sanger centre TB database	http://www.sanger.ac.uk/Projects/M_tuberculosis/ blast_server.shtml
MycDB BLAST	BLAST searches of Integrated *Mycobacterium* Database (Institut Pasteur)	http://kiev.physchem.kth.se/MycDB/ seqsearch_form.html
Sequence analysis tools		
ExPASy	Expert Protein Analysis System; general protein analysis tools	http://expasy.hcuge.ch/www/tools.html
TMPred TopPred 2 SOSUI	TM domain predictions; under ExPASy tools	
ExPASy compute MW	Molecular weight predictions; under ExPASy tools	http://expasy.hcuge.ch/ch2d/pi_tool.html
SignalP	Signal peptide motif and cleavage site prediction	http://www.cbs.dtu.dk/services/SignalP/
Multalin (version 5.3.3)	Multiple sequence alignment programme	http://www.toulouse.inra.fr/multalin.html

4.3.3. RESULTS

4.3.3.1. Sequence analysis of *mycP1* and its product, mycosin-1.

The *mycP1* gene is 1341 bp in length (Figure 4.1) and encodes a putative 446 amino acid protein (Figure 4.2). Using various internet databases (Table 4.1), *mycP1* was shown to have 77 % homology with a putative *M. leprae* serine protease gene (Y14967). Comparison of the deduced amino acid sequence of the mycosin-1 protein with sequences in databases was even more informative, revealing 79 % identity with the *M. leprae* serine protease (Y14967), 32.9% identity with an *S. coelicolor* serine protease

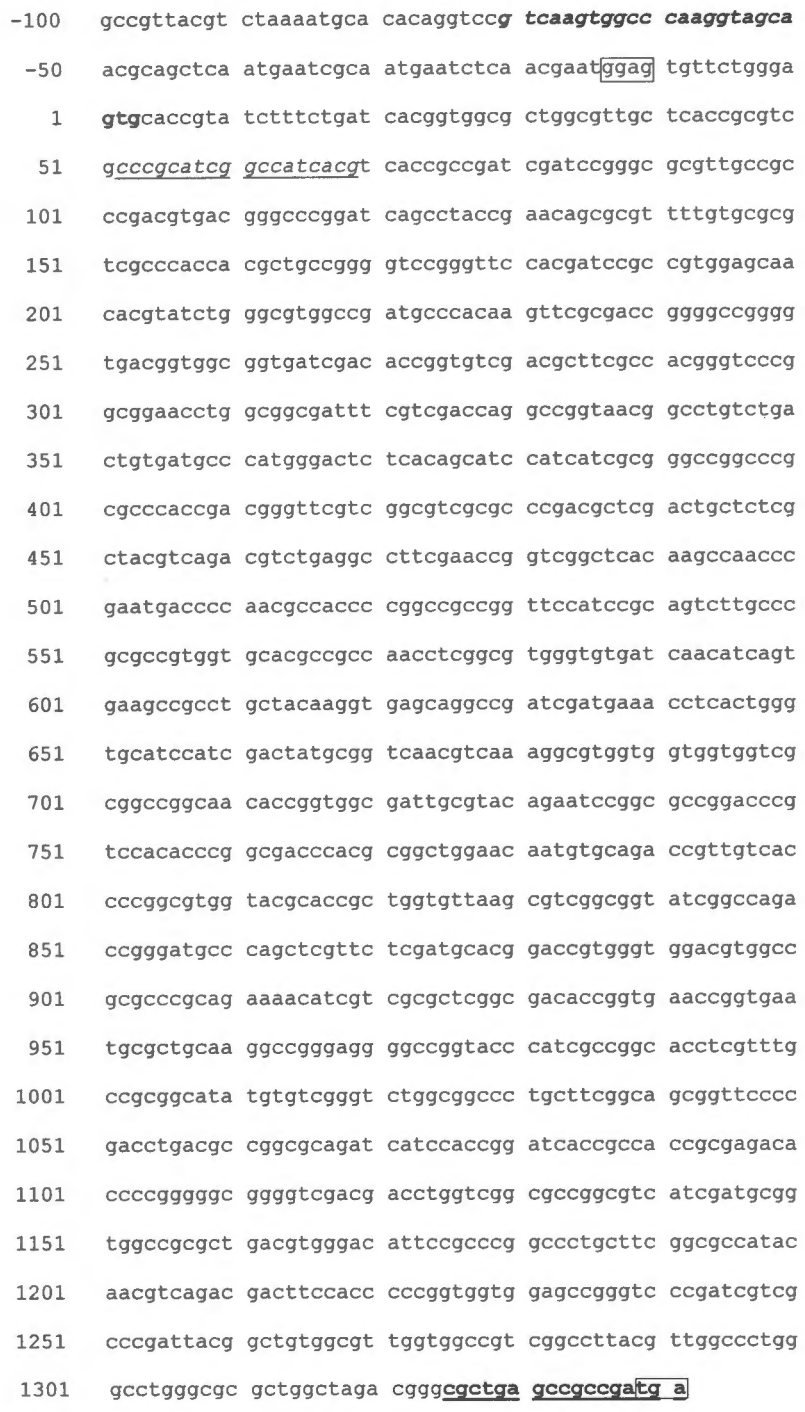


Figure 4.1. Nucleotide sequence of *mycP1* and its upstream flanking region. The translational start codon (boldtype letters) is preceded by a Shine-Dalgarno ribosome binding site (boxed letters). Also shown are the positions of the 3 primers used for PCR amplification of *mycP1*, i.e., p1mycf (***boldtype italic letters***), p1f (*underlined italics*), and p1r (**underlined boldtype letters**). The reverse primer (p1r) incorporates the translational stop codon (**boxed boldtype letters**).

(U33176), 33.2% identity with a *B. subtilis* serine protease, and 30.2% identity with *B. subtilis* subtilisin. The latter three proteases belong to the subtilisin subfamily (Family S8) of serine proteases. In addition, mycosin-1 showed various degrees of identity to other subtilisin-like serine proteases, especially around the active site residues (Figure 4.3). It is not unusual for bacterial proteases to have poor overall homology, but to reveal a high degree of sequence identity for the amino acids around the active site residues (Barrett, 1994; Barrett & Rawlings, 1995).

```
1           11                  31          41
VHRIFLITVA LALLTASPAS AITPPPIDPG ALPPDVTGPD QPTEQRVLCA
51          61          71      81          91
SPTTLPGSGF HDPPWSNTYL GVADAHKFAT GAGVTVAVID TGVDASPRVP
101         111                 131         141
AEPGGDFVDQ AGNGLSDCDA HGTLTASIIA GRPAPTDGFV GVAPDARLLS
151         161         171     181         191
LRQTSEAFEP VGSQANPNDP NATPAAGSIR SLARAVVHAA NLGVGVINIS
201         211         221     231         241
EAACYKVSRP IDETSLGASI DYAVNVKGVV VVVAAGNTGG DCVQNPAPDP
251         261         271     281         291
STPGDPRGWN NVQTVVTPAW YAPLVLSVGG IGQTGMPSSF SMHGPWVDVA
301         311         321                 341
APAENIVALG DTGEPVNALQ GREGPVPIAG TSFAAAYVSG LAALLRQRFP
351         361         371     381     391
DLTPAQIIHR ITATARHPGG GVDDLVGAGV IDAVAALTW IPPGPASAPY
401         411         421     431         441
NVRRLPPPVV EPGPDRRPIT AVALVAVGLT LALGLGALAR RALSRR
```

Figure 4.2. Deduced amino acid sequence of mycosin-1. The amino acid sequence was predicted fro the *mycP1* gene obtained from the *M. tuberculosis* database at the Sanger Centre (Cambridge, UK). The gene was translated using Protein translate (part of Expasy tools). Key: arrow, signal peptide cleavag site; boxed letters, active site residues.

The N-terminal sequence consists of an initial stretch of 5 polar amino acids (net positive charge), followed by 11 amino acids that are predominantly hydrophobic

residues (especially alanine and leucine), and which culminate in a stretch of 5 amino acids that are less hydrophobic and contain an alanine at the –1 and –3 positions of a potential signal peptidase cleavage site (marked by an arrow between amino acid 21 and 22 in Figure 4.2). This stretch of 21 amino acids complies with the criteria for a standard signal peptide (Pugsley *et al.*, 1985) and is consistent with what is predicted by the SignalP V1.1 internet server for signal peptide predictions. This suggests that mycosin-1 is likely translocated across the cell membrane.

Aligning mycosin-1 with the active site residues of various well-characterised subtilisins, the active site residues of mycosin-1 were predicted to be Asp-90, His-121, and Ser-332 (Figure 4.3).

Figure 4.3. Active site homology of mycosin-1 to subtilisin and subtilisin-like serine proteases. All amino acids homologous to mycosin-1 are indicated in boldtype letters. Those residues that are common to all are highlighted in black and those residues that are common in > 50% (and including mycosin-1) are highlighted in grey. **1**, *M. tuberculosis* mycosin-1; **2**, *S. coelicolor* serine protease (U33176); **3**, *B. subtilis* serine protease (L29506); **4**, *B. subtilis* thermostable alkaline serine protease (s50880); **5**, Thermus sp. serine protease (U17342); **6**, *B. subtilis* subtilisin (K01988); **7**, *B. subtilis* alkaline protease (D13157); **8**, *S. albogriseolus* subtilisin-like protease (D83672); **9**, *B. amyloliquifaciens* subtilisin BPN' (X00165); **10**, *B. licheniformis* subtilisin Carlsberg (X91262); **11**, *V. alginolyticus* alkaline serine protease (M25499)

* active site residue

All subtilisins and subtilisin-like proteases are produced as inactive zymogens with signal peptides (27-29 residues) and propeptides (77-79 residues). It is therefore

likely that mycosin-1 also includes a propeptide. Despite variable homology with the subtilisin propeptides, there appear to be 3 conserved amino acids, L-55, D-62, and P-63 (mycosin-1 numbering) (Figure 4.4) near the C-terminus of the propeptide. By aligning the predicted amino acid sequence of mycosin-1 with the above-mentioned conserved propeptide residues it appears likely that amino acids 22-63 comprise the mycosin-1 propeptide (Figure 4.4).

Figure 4.4. Propeptide homology. The putative propeptide of mycosin-1 was aligned with various subtilisin propeptides. Amino acids identical in all are highlighted in black and residues homologous to those in mycosin-1 are highlighted in grey. Key: mycosin-1, *M. tuberculosis* putative serine protease; Carl, *B. licheniformis* subtilisin Carlsberg (P00780); BPN, *B. amyloliquifaciens* subtilisin BPN′ (P00782); E, *B. subtilis* subtilisin E (P04189); NAT, *B. subtilis* var. natto subtilisin NAT (P35835); J, *B. stearothermophilus* subtilisin J (P29142); Amylo, *B. amylosaccharitícus* subtilisin Amylosaccharitícus (P00783); Y, Bacillus spp. Strain YAB, alkaline elastase YAB ((p20724); A, *B. alcalophilus* alkaline protease (P27693).

Furthermore, aligning putative mature mycosin-1 with three well-characterised mature subtilisins reveals that mature mycosin-1 likely starts with the amino acids proline-64 and tryptophan-65 (Figure 4.5). Therefore, prepromycosin-1 would have a molecular weight of 46.14 kDa and a pI of about 5, whereas mature mycosin-1 would have a molecular weight of 38.67 kDa and a pI of about 5.

The C-terminal half of mycosin-1 is predicted to be strongly hydrophobic, containing five hydrophobic regions (in addition to the N-terminal signal peptide), as

```
            64                                                    117
Mycosin-1   ....PWSNTY LGVADAHKFA .TGAGVTVAV IDTGVDAS.P RVPAEPGGDF VDQAGNGLSD
 Savinase   AQSVPWGISR VQAPAAHNRG LTGSGVKVAV LDTGI.STHP DLNIRGGASF VPGEPST.QD
     'BPN   AQSVPYGVSQ IKAPALHSQG YTGSNVKVAV IDSGIDSSHP DLKVAGGASM VPSETNPFQD
Carlsberg   AQTVPYGIPL IKADKVQAQG FKGANVKVAV LDTGIQASHP DLNVVGGASF VAGEAYN.TD

            118                                                   177
Mycosin-1   CDAHGTLTAS IIAGRPAPTD GFVGVAPDAR LLSLRQTSEA FEPVGSQANP NDPNATPAAG
 Savinase   GNGHGTHVAG TIAALNNSI. GVLGVAPSAE LYAVKVLGAS ..........  .......GSG
     'BPN   NNSHGTHVAG TVAALNNSI. GVLGVAPSAS LYAVKVLGAD ..........  .......GSG
Carlsberg   GNGHGTHVAG TVAALDNTT. GVLGVAPSVS LYAVKVLNSS ..........  .......GSG

            178                                                   237
Mycosin-1   SIRSLARAVV HAANLGVGVI NISEAACYKV SRPIDETSLG ASIDYAVNVK GVVVVVAAGN
 Savinase   SVSSIAQGLE WAGNNGMHVA NLS......L GSPSPSATLE QAVNSATS.R GVLVVAASGN
     'BPN   QYSWIINGIE WAIANNMDVI NMS......L GGPSGSAALK AAVDKAVA.S GVVVVAAAGN
Carlsberg   TYSGIVSGIE WATTNGMDVI NMS......L GGPSGSTAMK QAVDNAYA.R GVVVVAAAGN

            238                                                   297
Mycosin-1   TGGDCVQNPA PDPSTPGDPR GWNNVQTVVT PAWYAPLVLS VGGIGQTGMP SSFSMHGPWV
 Savinase   SGAG...... .......... ......SISY PARYAN.AMA VGATDQNNNR ASFSQYGAGL
     'BPN   EGTS...... .......... ..GSSSTVGY PGKYPS.VIA VGAVDSSNQR ASFSSVGPEL
Carlsberg   SGSS...... .......... ..GNTNTIGY PAKYDS.VIA VGAVDSNSNR ASFSSVGAEL

            298                                                   357
Mycosin-1   DVAAPAENIV ALGDTGEPVN ALQGREGPVP IAGTSFAAAY VSGLAALLRQ RFPDLTPAQI
 Savinase   DIVAPGVNVQ STYPGSTYAS .......... LNGTSMATPH VAGAAALVKQ KNPSWSNVQI
     'BPN   DVMAPGVSIQ STLPGNKYGA .......... YNGTSMASPH VAGAAALILS KHPNWTNTQV
Carlsberg   EVMAPGAGVY STYPTSTYAT .......... LNGTSMASPH VAGAAALILS KHPNLSASQV

            358                                                   417
Mycosin-1   IHRITATARH PGGGVDDLVG AGVIDAVAAL TWDIPPGPAS APYNVRRLPP PVVEPGPDRR
 Savinase   RNHLKNTATS LGS..TNLYG SGLVNAEAAT R.........  ..........  ..........
     'BPN   RSSLENTTTK LGD..SFYYG KGLINVQAAA Q.........  ..........  ..........
Carlsberg   RNRLSSTATY LGS..SFYYG KGLINVEAAA Q.........  ..........  ..........

            418                          446
Mycosin-1   PITAVALVAV GLTLALGLGA LARRALSRR
 Savinase   ..........  ..........  .........
     'BPN   ..........  ..........  .........
Carlsberg   ..........  ..........  .........
```

Figure 4.5. Alignment of putative mature mycosin-1 with mature subtilisin. The predicted amino acid sequence of mature mycosin-1 was aligned with mature subtilisin Savinase, BPN′ and Carlsberg. The alignment was optimised using Multalin v. 5.3.3. Mycosin-1 numbering

evidenced by the hydrophobicity plot (Figure 4.6B; sequences indicated with overlines in Figure 4.6A and labelled 1 to 5).

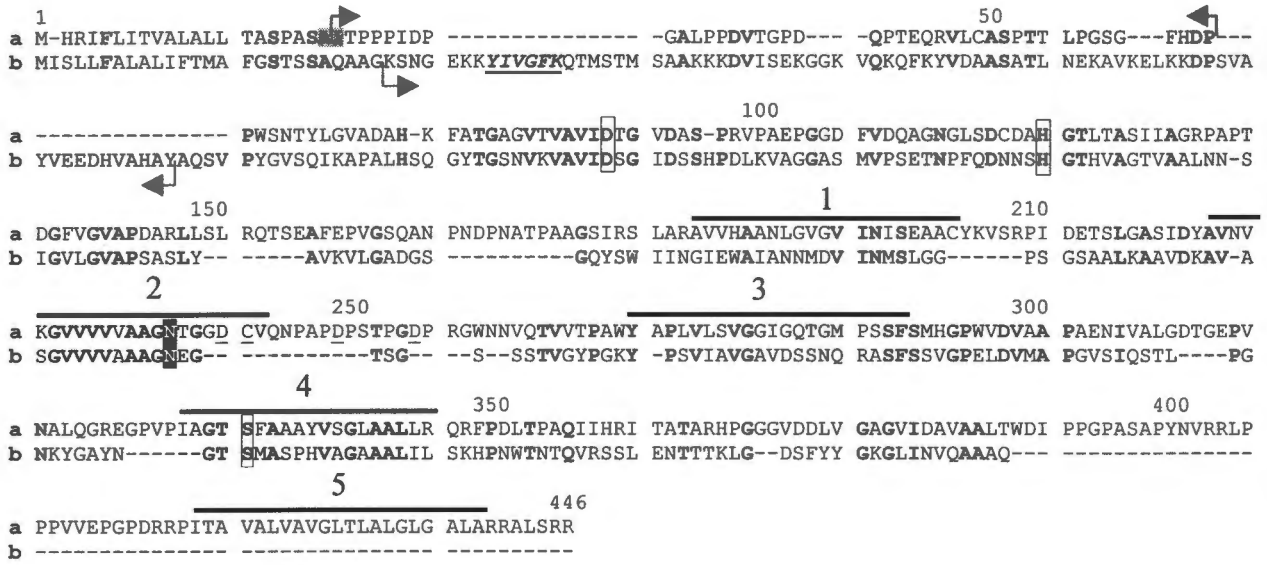
Three independent internet servers (SOSUI, TopPred 2, and Tmpred; Table 4.2) that predict potential transmembrane (TM) domains and preferred orientations of the amino and carboxy termini, were used to make some theoretical predictions for the potential TM topology of mycosin-1. The TMpred programme predicts membrane-spanning regions and their orientation by comparing the given sequence with a database (TMbase) of naturally occuring transmembrane proteins (Hofmann & Stoffel, 1993). This differs from the SOSUI programme which, using the physicochemical properties (i.e., hydrophobicity and charges) of the amino acid sequence, is able to predict the existence of a membrane protein, transmembrane helices and transmembrane helical regions with accuracies of 99%, 96% and 85%, respectively (Takatsugu *et al.*, 1998). Similarly, TopPred2 also uses physicochemical properties but in addition it uses the "positive-inside rule" (the observation that positively charged amino acids tend to be more prevalent in cytoplasmic than in extra-cytoplasmic segments in transmembrane proteins) to determine preferred orientations of each transmembrane segment (Von Heijne, 1992)

Table 4.2. Transmembrane segment predictions

TMpred		**TopPred 2**		**SOSUI**	
amino acid sequence	**preferred orientation**	**amino acid sequence**	**preferred orientation**	**amino acid sequence**	**preferred orientation**
4-22		4-22		1-23	
184-204	o-i				
223-243	i-o				
271-290	o-i	262-282	o-i		
327-345	i-o	325-345	i-o		
419-439	o-i	419-439	o-i	419-439	-

i, inside; o, outside

A



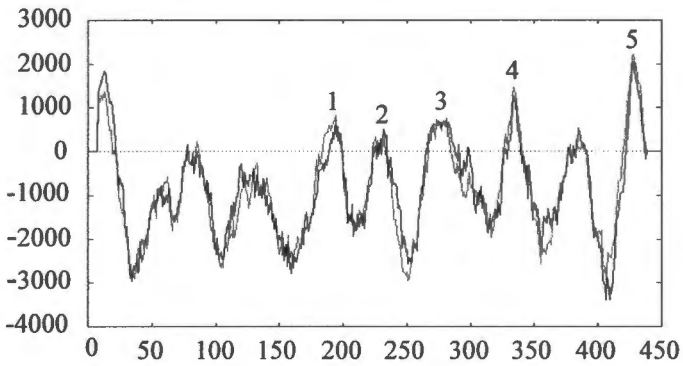


Figure 4.6. Sequence analysis of the *mycP1* gene product, mycosin-1. (A) Sequence alignment of mycosin-1 with the *B. amyloliquifaciens* subtilisin BPN′ protein. Single-letter amino acid code; conserved residues are in bold. The catalytic residues (D-90, H-121, and S-332; mycosin-1 numbering) are in open boxes; the oxyanion hole (N-237) is in a closed box. The mycosin-1 signal peptide cleavage site (A-21/I-22) is shaded. The arrows below the BPN′ sequence bracket the propeptide sequence; arrows above mycosin-1 indicate a presumed propeptide. Overlines (numbered 1-5) above the mycosin-1 sequence indicate hydrophobic regions and correspond to domains 1-5 shown in (B) and in Figure 4.7. (B) Hydrophobicity plot of mycosin-1 generated using the TMpred programme.

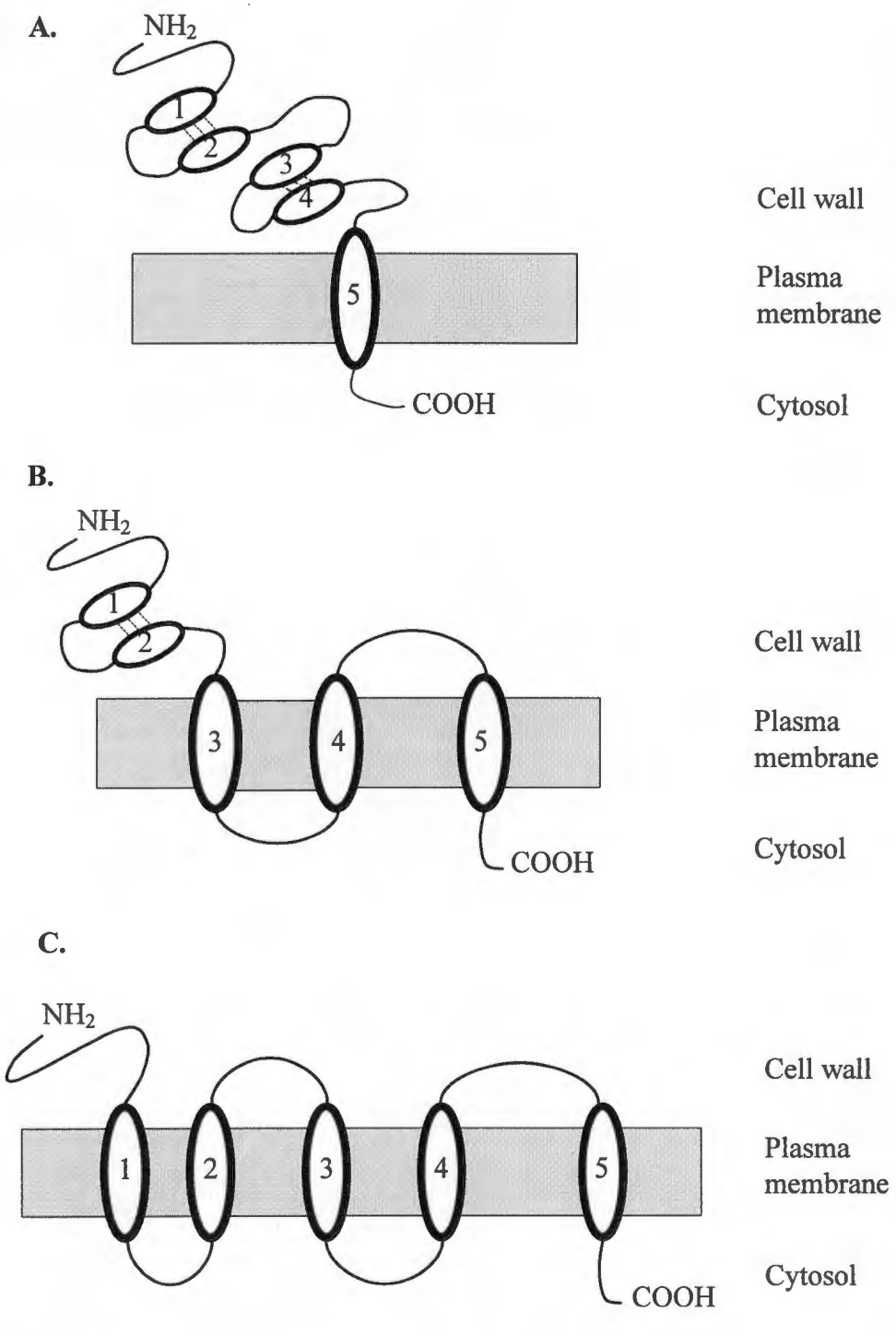


Figure 4.7. Transmembrane topology models of mycosin-1. Using three independent algorithms (Tmpred, TopPred2 and SOSUI, Table 4.1) predicting transmembrane domains and preferred orientations of the N- and C-termini, 3 transmembrane topological models were constructed. Regions 1-5 correspond to those depicted in Figure 4.6.

These three algorithms predict three possible models, with 1, 3 and 5 TM domains, respectively (Figure 4.7). Notably, each of the algorithms predicted an odd number of TM domains, which provides for an extracytoplasmic N terminus and an intracytoplasmic C terminus (i.e., a type I transmembrane orientation). Moreover, all 3 algorithms provided a strong consensus for region 5 (Figure 4.6A) as TM domain. This is consistent with its high hydrophobicity (Figure 4.6B), length (21 residues), and flanking arginine residues. Both TMpred and TopPred2 predictions have regions 4 and 5 in common, and significant overlap in region 3. However, the presence of active site residues in regions 2 and 4 reduce the likelihood that these sequences are true TM domains. Based on these results, Model A (Figure 4.7) would seem to be the most likely topological model for mycosin-1. This suggests that following translocation and cleavage of the signal peptide between alanine-21 and isoleucine-22 (Figure 4.6A), the bulk of the protein is extracytoplasmic and anchored in the cell membrane by a single, C-terminal TM domain, leaving a short 7-residue cytoplasmic tail. Secondary structure predictions support this model, indicating a strong likelihood of a α-helix for the entire segment 5, but only weak α-helical predictions for segments 1-4 (data not shown). Domains 1-4 may interact by hydrophobic interactions or hydrogen bonding (as shown in Figure 4.7A and 4.7B), but this is purely speculative.

4.3.4. DISCUSSION

Mycosin-1 shows various degrees of identity to subtilisins and subtilisin-like serine proteases. Although there is often poor overall homology between bacterial proteases, even if they are part of the same clan/family, the active site residues and flanking sequences are usually highly conserved. This is especially true for members of the subtilisin family that show considerable conservation of the amino acid sequence

around the catalytic triad Asp-32, His-64, and Ser-221 (subtilisin numbering) (Barrett & Rawlings, 1991). The catalytic triad of mycosin-1 (Asp-90, His-121, and Ser-332), similarly, shows a high degree of sequence identity with that of other subtilisin-like serine proteases. Furthermore, the residues around the catalytic site residues of mycosin-1 satisfy two out of the three consensus signatures for the catalytic site of subtilisins. If a protein includes at least two of the three active site signatures, the probability of it being a serine protease from the subtilisin family is 100% (Siezen & Brannigan, 1997).

All known subtilisin-like serine proteases and most extracellular bacterial proteases are produced as inactive zymogens with propeptides of varying lengths (Wandersman, 1989; Khan & James, 1998). These require processing and cleavage to produce the mature, active protease. It is likely that mycosin-1 is also produced as an inactive zymogen. By comparison with two well-characterised subtilisins, subtilisin Savinase and subtilisin BPN′, the region comprising amino acids 22 to 63 appeared to be the propeptide of mycosin-1. Should cleavage of the mycosin-1 propeptide occur via an intermolecular process (usually by another protease), then the presence of the candidate protease or the stimuli necessary for the induction of this protease would be essential for activation of mycosin-1. Alternatively, autocatalytic processing of the mycosin-1 propeptide would require the optimum pH, temperature, and co-factors necessary for optimal activity of mycosin-1. Failure to provide any of the parameters required for either inter- or intra-molecular processing would result in inactive mycosin-1. Most subtilisins require *in vivo* factors for activation and activity (Power *et al.*, 1986). If this is true for mycosin-1 then it may be very difficult to obtain active enzyme *in vitro* as approximation of *in vivo* conditions is often very difficult to achieve in the laboratory. This could explain why a low level of proteolytic activity was found in the culture filtrates.

Theoretical analysis of the deduced amino acid sequence of mycosin-1 reveals a 21 amino acid signal peptide, a 42 amino acid propeptide, active site residues that show homology to subtilisin-like serine proteases, 4 hydrophobic domains and a C-terminal region that may contain a hydrophobic transmembrane domain. The hydrophobic nature of this protein together with a C-terminal region that may be a membrane-anchor indicate that mycosin-1 is likely to be a cell-envelope associated protein. It is not uncommon for subtilisins or subtilisin-like proteases to contain membrane-anchoring sequences (Power *et al.*, 1986).

Mycosin-1 has 4 cysteine residues : the first (Cys-49) is in a region that is most likely to be the propeptide and will probably not be involved in the function of the enzyme; the second (Cys-118) is close to the catalytic histidine, which is also found in thermitase from *Thermoactinomyces vulgaris*, and in some eukaryotic homologues (proteinase K, yeast proteinase), and may be involved in catalysis; the last 2 residues (Cys-204 and Cys-242) are between the catalytic histidine and serine residues and may play a role in catalysis or stabilisation of the enzyme by disulphide bridge formation. It is unusual for bacterial subtilisins to contain any cysteine residues; however, there are some members of the subtilisin family, especially the eukaryotic homologues, that contain a single cysteine residue close to the catalytic histidine (as does mycosin-1, i.e. Cys-118). This residue has been shown to react with some thiol-reactive reagents, resulting in inhibition by cysteine protease inhibitors. In addition, Ca^{2+} has been shown to stimulate the activity of most subtilisins. This phenomenon of inhibition of some subtilisin-like proteases by cysteine protease inhibitors may help explain the culture filtrate results where, in addition to some Ca^{2+} stimulation, significant inhibition was obtained with calpain inhibitors I and II (i.e., ALLM and ALLN), inhibitors of calpain-like cysteine proteases. Since no putative cysteine protease genes have been found in the

M. tuberculosis genome sequence, it is possible that this inhibition reflects the inhibition of members of this newly identified subfamily of subtilisin proteases.

Homologues of mycosin-1 have been identified in various mycobacterial species. It has 79% identity to an *M. leprae* serine protease and 100% identity to a gene in the flanking region of deletion region 1 (RD1) of *M. bovis* BCG. RD1, and two other regions RD2 and RD3, represent regions of DNA that are absent from most *M. bovis* BCG strains, but present in most virulent *M. bovis* and *M. tuberculosis* strains. Of these three regions, RD1 has received much attention as it appears to be potentially involved in the regulation of multiple genetic loci (Mahairas *et al.*, 1996). To assess the expression of mycosin-1 in *M bovis* BCG, Western Blot experiments using affinity-purified anti-mycosin-1 antiserum as the primary antibody were performed on cell extracts of *M. bovis* BCG, *M. smegmatis*-P19 (negative control), *M. smegmatis*-P1 and *M. tuberculosis* H37Rv (the latter two served as positive controls). Both positive controls revealed a single immunodominant band corresponding to mycosin-1. In contrast, nothing was observed in the cell extracts of the negative control or *M. bovis* BCG. This experiment was repeated using anti-mycosin-2 or anti-mycosin-3 antiserum as the primary antibody. Using these antibodies an immunodominant band was observed in the cell extract of *M. bovis* BCG. In addition, only mycosin-3 was detected in the cell extract of *M. smegmatis*. Thus, it was concluded that mycosin-1 is the only member of this family that is not expressed in *M. bovis* BCG. Since *mycP1* is in the flanking region of RD1, it is tempting to suggest that RD1 contains regulatory elements essential for the expression of mycosin-1, although this remains to be proven. The contribution of mycosin-1 to the virulence potential of *M. tuberculosis*, *M. bovis or M. leprae* remains to be elucidated. However, the presence of this subtilisin subfamily (of which mycosin-1 was the first member to be identified) mainly in virulent mycobacterial species [only *mycP3* was detected in avirulent *M. smegmatis* (Brown *et al.*, 1999)], may suggest that it plays a role

in disease pathogenesis. This may include critical house-keeping functions, processing of secreted and/or extracellular proteins, immune system modulation, phagosome modulation, or other pathological processes. In addition, if the mycosins are anchored in the membrane then it is likely that they are embedded in the arabinogalactan layer of the cell wall and their function may be related to processing of cell wall components. However, until the substrate specificities and expression patterns of these proteases are determined the above suggestions remain speculative.

4.4. SEQUENCE ANALYSIS OF A PUTATIVE METALLOPROTEASE GENE, *MPR1*, AND ITS PREDICTED PRODUCT, MPR1

4.4.1. INTRODUCTION

The culture filtrate data presented in Chapter 3 indicated the presence of a metal-dependent proteolytic activity. Although this activity may be due to a member of the newly identified subfamily of subtilisin, it is possible that the activity may also be due to a metalloprotease. Many bacteria produce metalloproteases and the *M. tuberculosis* genome sequence has revealed the presence of at least three putative metalloprotease genes. Hence, it was decided to attempt the expression of one of these genes in parallel with that of mycosin-1. This part of the chapter details the sequence analysis of a putative metalloprotease gene, *mpr1*, and its predicted product, Mpr1.

4.4.2. MATERIALS AND METHODS

Sequence analysis of *mpr1* and its predicted product, Mpr1, was performed as described for mycosin-1 in section 4.2.3.1.

4.4.3. RESULTS

4.4.3.1. Sequence analysis of mpr1 and its product, Mpr1

The *mpr1* gene is 1497 bp in length (Figure 4.8) and encodes a putative 498 amino acid protein (Figure 4.9) with a predicted molecular weight of 52.95 kDa and a pI of 5.20. The N terminus consists of a polar region followed by a strongly hydrophobic stretch that ends in Ala-Gly-Cys which is a potential signal peptidase cleavage site with

-100 tgccaaaccg tgatccgggc ctgatctcgc cactgacccc gcaccgaccg

-50 atctagaatg ggat***ttcctt*** ***ggtgcatgcc*** ***g***ggcgggacg gggttaggag

1 **atg**catggtc gcgggcggta tcgacctctg gtccgctgtg ttcgccctcg

51 ccgggtggcc gcgtcggtgc ggaccccgat cgcctgtcta gcggcggtgg

101 tcgtgatagc *cggctgcacg* *accgtc*gtcg acgggcgggc gctgtccatc

151 ctcaacgacc cgttccgggt gggggggtctg cccgcgacca acggtccgag

201 cggcgcccgc cccgacgcac cggctgcgtc gggcacggtg atcaacacca

251 acaacggagc gatcgacaag ttgtcgttgt tgtcggtcaa cgacatcgag

301 gactactgga tggcggtcta cagcgaatcg ctgaagggca ccttccggcc

351 ggtcggcaag ctggtgtcct acgattccaa cgacccaagt agtccgatcg

401 tctgccacat tgacacctat cagctcgtca acgccttttt cagctctcgg

451 tgcaacttga ttgcctggga tcgaggggtc ttcatggcgg tcgcgcaaga

501 atacttcggc gacatgtccg tcaatggtgt gctggcacac gaattcgggc

551 atgctctgca agtgatggcg aatttggtta ccaggaaaga tcccaccatc

601 gtccgcgagc agcaagcgga ttgcttcgcc ggggtctatc tgtggtgggt

651 ggccgaaggt aagtcgacac gctttacgct gagcaccgcg gacgggctcg

701 accacgtgct cgccggcatc atcaccaccc gagacccggt gatggaagcc

751 gatgcggaaa acgacgacga acatgggtcg gccttggatc gggtcagcgc

801 gttccagctg ggcttcatca acggcacgcc ggcgtgcgcg gcgatcgacg

851 aggacgaagt cgagcggcgc cgcggtgacc tgccgacggc gttgcgggtc

901 gatgccagcg gcaacccaga gaccggcgag gtcggaatca acgaagagac

951 cctctcgacg ttgatggagt tgatgggcaa gatcttctcg ccgaagaatc

1001 cgcccacgct gtcctaccag ccggccggtt gcccagacgc caagcccagc

1051 ccaccggccg cctactgtcc ggccaccaac accatcgtgg tcgacctgcc

1101 cgccctggcg aggatgggca aggtggcctc ggcagcggaa cacagcctgc

1151 cgcagggcga tgacacgtcg ttgtcgattg tgatgtcgcg gtacgcgttg

1201 gcggtgcagc acgaacgcgg gctgccgatg cagagcccgt ggaccgcctt

1251 acggacggcg tgcctgaccg gcgttgcgca ccgcaagatg gccgtgccca

1301 tcgacctgcc ctccggccag caactcgtac ttaccgcggg tgatctcgac

1351 gaagcggttt ccgggttgct gaccaaccgc atggtcgcca gtgacgccga

1401 cggtgtcagc gttccggccg gtttcactcg gatagccgcg ttccgtgccg

1451 gcgtgggcgg cgacatggac gcatgctatg cc***cggtatcc*** ***gggatag***

Figure 4.8. Nucleotide sequence of *mpr1* and its upstream flanking region. The transcription initiation site (boldtype letters) is preceded by a Shine-Dalgarno ribosome binding site (boxed letters). Also shown are the positions of the 3 primers used for PCR amplification of *mpr1* i.e. p8mycf (boldtype italic letters), p8f (underlined italics) and p8r (underlined boldtype italic letters)

features consistent with those of a lipoprotein signal peptide (Pugsley, 1993). Furthermore, the sequence HEFGH (amino acids 180-184) is characteristic of the HEXXH motif of Zn-metalloproteases of the endopeptidase class (Vallee and Auld, 1990). These data suggest that Mpr1 is a Zn-metalloendopeptidase that is secreted but may remain anchored in the cell membrane as a lipoprotein with a lipid anchor.

```
1           11          21          31 ↓        41
MHGRGRYRPL VRCVRPRRVA ASVRTPIACL AAVVVIAGCT TVVDGRALSI
51          61          71          81          91
LNDPFRVGGL PATNGPSGAR PDAPAASGTV INTNNGAIDK LSLLSVNDIE
101         111         121         131         141
DYWMAVYSES LKGTFRPVGK LVSYDSNDPS SPIVCHIDTY QLVNAFFSSR
151         161         171         181         191
CNLIAWDRGV FMAVAQEYFG DMSVNGVLAH EFGHALQVMA NLVTRKDPTI
201         211         221         231         241
VREQQADCFA GVYLWWVAEG KSTRFTLSTA DGLDHVLAGI ITTRDPVMEA
251         261         271         281         291
DAENDDEHGS ALDRVSAFQL GFINGTPACA AIDEDEVERR RGDLPTALRV
301         311         321         331         341
DASGNPETGE VGINEETLST LMELMGKIFS PKNPPTLSYQ PAGCPDAKPS
351         361         371         381         391
PPAAYCPATN TIVVDLPALA RMGKVASAAE HSLPQGDDTS LSIVMSRYAL
401         411         421         431         441
AVQHERGLPM QSPWTALRTA CLTGVAHRKM AVPIDLPSGQ QLVLTAGDLD
451         461         471         481         491
EAVSGLLTNR MVASDADGVS VPAGFTRIAA FRAGVGGDMD ACYARYPG
```

Figure 4.9. Deduced amino acid sequence of Mpr1. The signal peptide cleavage site is marked by an arrow; the HEXXH motif characteristic of metalloproteases is shown (underlined boldtype italic letters).

Analysis of published bacterial metalloprotease sequences reveals that there appear to be two major groups that are differentiated by the conservation of the residues around the HEXXH motif and the spacing between the second and third zinc ligands (Figure 4.10). One group has 13 residues between the 2nd and 3rd zinc ligands and the other 19. A

lineup of Mpr1 with other bacterial metalloproteases shows that it belongs to the second group, of which the prototype is thermolysin. However, apart from some homology around the HEXXH motif, Mpr1 did not have any significant homology with any known proteins.

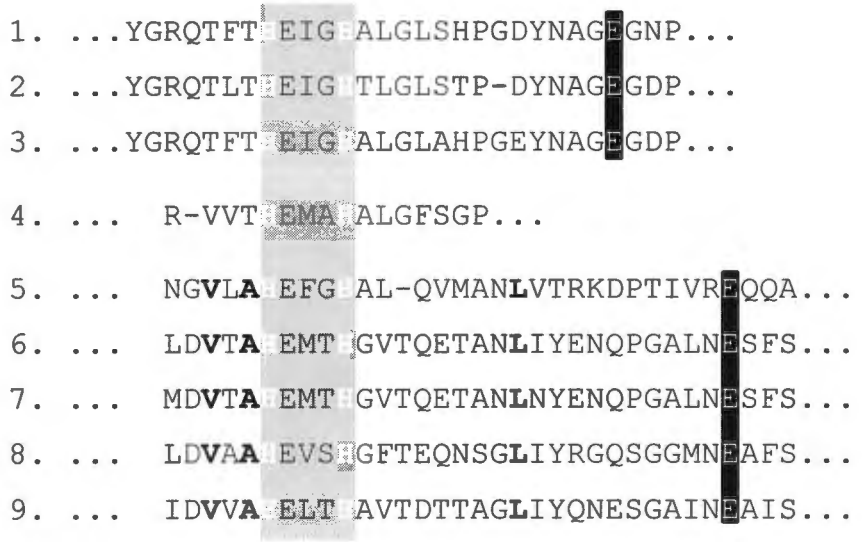


Figure 4.10. Comparison of the primary structure of Mpr1 with known metalloproteases. Conserved amino acid sequences around the HEXXH motif (grey background; the two His residues in bold white constitute the first and second Zn ligands) and the third Zn ligand (Glu : black background) are placed into two groups, based on the degree of sequence homology and on the spacing between the second and third Zn ligands (sequences 1-3 and 5-9). Sequence 4 partly fits with the first group (1-3), even though, surprisingly, it is not a bacterial protein, but derives from the surface glycoprotein gp63 of *Leishmania*. Sequences 9 and 10 show no homology other than the HEXXH motif. Sequences : 1) *Serratia* neutral protease, 2) *P. aeruginosa* alkaline protease, 3) *E. chrysanthemi* extracellular metalloprotease B, 4) *Leishmania* gp63, **5) *M. tuberculosis* Mpr1**, 6) *B. subtilis* neutral protease, 7) *B. amyloliquefaciens* neutral protease, 8) *P. aeruginosa* elastase, 9) *B. thermoproteolyticus* thermolysin, 10) *V. alginolyticus* collagenase, 11) *S. cacaoi* neutral protease.

Hydropathy plots show that this protease does not have any likely TM domains and is most probably secreted into the medium (not shown), unless it is a lipoprotein as predicted from the signal peptide cleavage site, and remains membrane-bound via a lipid anchor.

4.4.4. DISCUSSION

Theoretical analysis of the deduced amino acid sequence of Mpr1 reveals an N-terminal lipoprotein signal peptide and a HEXXH motif characteristic of the metalloprotease family (Jongeneel *et al.*, 1989). There are no significant hydrophobic regions, making it unlikely to be a transmembrane protein. However, since it is likely a lipoprotein, it will probably remain membrane-bound and not be secreted. Rowland *et al.* (1997) have described the presence of a calcium-dependent Zn-metalloprotease in stationary phase culture filtrates of *M. tuberculosis* H37Rv. In addition, this protease exhibited elastolytic activity, hinting at a potential role in the tissue destruction characteristic of pulmonary tuberculosis (30% of lung tissue is elastin). Notably, bacterial metalloproteases often possess collagenolytic and elastolytic activity, and have, therefore, been implicated in the pathogenesis of a number of disease processes (Harrington, 1996; Häse *et al.*, 1993). Although a definitive role for bacterial matrix-metalloproteases in disease pathogenesis has not yet been established, their association with diseases characterised by host tissue damage warrants further study of these enzymes.

The *M. tuberculosis* genome sequence contains at least three putative metalloprotease genes (Rv0419, Rv0198, and Rv1977) (Cole *et al.*, 1998). If the activities of these *M. tuberculosis* metalloproteases are consistent with the activities of other microbial metalloproteases, they could contribute to the tissue-destructive potential of *M. tuberculosis*. In addition, degradation of fibrotic capsules or connective tissue may provide an escape route for latent *M. tuberculosis* bacilli, thus aiding dissemination and disease progression.

However, for the purpose of this study, attention was focused on mycosin-1, as it was the first member of a novel family of subtilisin-like serine proteases to be detected. We have hypothesised that this family plays a significant role in the pathogenic potential

of *M. tuberculosis* (Brown *et al.*, 1999). In this study, Mpr1 was used as a negative control in the cloning and expression experiments involving mycosin-1, but its potential involvement in the pathogenic lifestyle of *M. tuberculosis* warrants future investigation.

CHAPTER 5

Cloning and expression of *mycP1* in *E. coli* and *M. smegmatis*, and detection of mycosin-1 in *M. tuberculosis*

5.1 HOSTS AND VECTORS USED IN MYCOBACTERIAL GENETICS

5.1.1 Introduction

For decades tuberculosis has been endemic in Third World Countries. Despite this, molecular analysis of mycobacteria has lagged behind that of other pathogens. This was partly a consequence of a lack of interest in First World Countries, due to successes in controlling this disease. Thus, 15 years ago, when tuberculosis cases began increasing world-wide, investigators found themselves challenged by the virtual absence of mycobacterial molecular biology, as bacterial molecular techniques had not been applied to this pathogen. Steadily, however, systems have been developed over the past decade that are suitable for the study of mycobacterial genetics and now, through the concerted effort of researchers around the world, the scientific community is becoming more familiar with the genetics of this genus, and especially *M. tuberculosis*. The recent publication of the *M. tuberculosis* genome sequence represents a great triumph in this regard, and has paved the way for the study of a diverse array of *M. tuberculosis* genes and proteins.

Proteins that are secreted or released by *M. tuberculosis* are thought to play a significant role in the ability of the bacterium to establish disease. Moreover, these proteins are becoming recognised as primary candidates for vaccine development and as potential drug targets. Nevertheless, despite the advances in mycobacterial genetics, the study of these proteins has been hampered by the difficulties experienced in manipulating *M. tuberculosis*. Its slow growth, infectious potential, and peculiar biochemical composition have made identification and purification of cellular constituents difficult. In addition, established techniques for the introduction of foreign DNA into bacteria provided disappointing results, necessitating the development of novel methods.

5.1.2 Alternative host species

To circumvent the problems associated with the study of *M. tuberculosis*, researchers have sought alternative host species that allow expression of *M. tuberculosis* genes. Although *E. coli* is able to translate some mycobacterial genes and produce active proteins (Leão *et al.*, 1995), including examples with N-terminal signal peptides (Hewinson *et al.*, 1993), expression is generally characterised by a low yield of soluble, correctly processed recombinant protein (Garbe *et al.*, 1993; Harth *et al.*, 1997). It has been suggested that high yields of protein can be obtained if the expressed proteins are highly conserved, for example, the *M. tuberculosis* heat shock proteins that are present in eukaryotes and prokaryotes (Mehlert & Young, 1989). Expression of proteins that are destined for the cell membrane has been shown to interfere with the function of the host membrane. Hence, some investigators have expressed these proteins without their signal peptides, thus directing the proteins into the cytoplasm (Pullen *et al.*, 1995; Schmid *et al.*, 1996). However, these proteins were subsequently found to be insoluble and inactive; in some cases, refolding and reactivation were possible.

The high GC content (~65%) of mycobacterial DNA suggests that uncommon transcription and translation signals may be used. It is likely, therefore, that bacteria with a similar GC content may be useful hosts for the expression of mycobacterial genes. *Streptomyces lividans*, a Gram positive bacterium belonging to the Actinomycetes order, as do mycobacteria, has a high GC content (~73%) and has been shown to recognise a variety of prokaryotic promoters. This organism has been used successfully for the expression of genes from *M. bovis* BCG, *M. tuberculosis*, and *M. leprae*, using their own promoters (Kieser *et al.*, 1986; Lamb & Colston, 1986). However, the non-pathogenic relatives of *M. tuberculosis* seem the most likely hosts for the successful expression of *M. tuberculosis* genes. *M. bovis* BCG, a sister-species of *M. tuberculosis*, seems an obvious choice as a host for *M. tuberculosis* genes. However, *M. bovis* BCG is a slow-

growing organism that tends to grow in clumps, making working with this organism tedious. *M. smegmatis* grows in clumps that are more tractable, it is fast growing, and heterologous mycobacterial genes have been successfully expressed in this organism.

The gene encoding the superoxide dismutase enzyme of *M. tuberculosis* was expressed from its own promoter in *M. smegmatis* and active enzyme was obtained (Zhang *et al.*, 1991). In contrast, when this enzyme was expressed in *E. coli* an exogenous promoter was essential for expression and the recombinant protein was functionally inactive. In addition, Harth *et al.* (1997) obtained high levels of expression and secretion of four major extracellular proteins of *M. tuberculosis* using *M. smegmatis* as host. All 4 proteins were expressed from their own promoters and were indistinguishable from their native counterparts. Attempts to obtain high-level expression and secretion of these proteins in *E. coli* or *B. subtilis* were unsuccessful. Both *M. smegmatis* and *M. aurum* have become the preferred host species as they are both members of the fast growing group of mycobacteria, are less prone to clumping, and methods have been developed that improve transformation efficiencies (Hermans *et al.*, 1990; Jacobs *et al.*, 1991).

5.1.3 Vectors

Numerous vectors have been developed for the shuttling of mycobacterial DNA between strains and/or species. The construction of these vectors has rested on the ability of the plasmid to replicate in a variety of organisms, in order to overcome the need to construct vectors with different replication functions for different hosts. Initial *E. coli*/mycobacterium shuttle vectors were derivatives of an *M. fortuitum* plasmid, pAL5000 (Hermans *et al.*, 1996). Additional vectors were constructed using pMSC262 from *M. scrofulaceum* (Goto *et al.*, 1991) and pNG2 from *Corynebacterium* (Radford & Hodgson, 1991). Most of the early vectors coded for kanamycin-resistance for the selection of transformants. This was not ideal as kanamycin was used for the treatment of

tuberculosis and there was some concern that kanamycin-resistant *M. tuberculosis* bacilli could develop. Thus, alternative selection markers were sought and hygromycin became the marker of choice. The majority of vectors contained mycobacterial sequences as those vectors lacking mycobacterial sequences were unable to replicate in some mycobacterial species.

Mycobacteriophages have also been used in the construction of vectors that are able to replicate in both *E. coli* and mycobacteria. It appears that incorporation of parts of the phage-specific replication mechanism stabilises plasmids in mycobacteria (Hermans & De Bont, 1996). Therefore, vectors have included either the whole phage (phAE1 from mycobacteriophage TM4, phAE15 from mycobacteriophage L1) (Jacobs *et al.*, 1987; Snapper *et al.*, 1988), the phage attachment site and integration genes (pMV361 and pMH5 from mycobacteriophage L5) (Stover *et al.*, 1991; Lee *et al.*, 1991), or a phage origin of replication (pRM64 using the mycobacteriophage D29 origin of replication) (Lazraq *et al.*, 1991), and in each case have been cloned into *E. coli* plasmids. These vectors stably insert foreign genes by a site-specific integration into the mycobacterial chromosome.

A further set of vectors was constructed based on homologous recombination. An example being the vector pY6002 (containing a copy of the *M. smegmatis pyr*F gene) that integrates itself into the chromosome of *M. smegmatis* (Husson *et al.*, 1990). Insertion of a foreign gene into the vector's copy of *pyr*F results in the integration of the gene into the chromosome thereby inactivating the host's *pyr*F gene. Finally, some investigators have constructed "artificial transposons" (e.g., pUS701, pUS702) whereby foreign sequences are inserted adjacent to a selection marker gene, introduced into the host, and integrated into the chromosome in the same way as a natural transposon (Dellagostin *et al.*, 1993).

5.2 CLONING AND EXPRESSION OF *MYCP1*⁻ IN *E. COLI* AND PRODUCTION OF AN ANTI-MYCOSIN-1 ANTIBODY IN RABBITS

5.2.1 INTRODUCTION

Mycosin-1 is a putative membrane-bound protease of *M. tuberculosis*. Although functional *M. tuberculosis* proteins have been expressed in *E. coli* (Hewinson & Russell, 1993), mycosin-1 expression in *E. coli* may present three potential problems. First, *E. coli* does not accurately process some *M. tuberculosis* signal peptides, leading to the expressed product being located intracellularly, making it prone to degradation by host proteases and subjecting it to an environment that may not support its function. Second, difficulties have been reported when expressing foreign membrane-active proteins in *E. coli*, as they may interfere with host membrane function (Senaratne *et al.*, 1998). Attempts to negate this have rested on removing the signal peptide that would direct the protein to its intramembrane location. However, this may lead to the production of an expressed protein that is insoluble and that will require detergents and/or denaturants to restore it to its native, active state. Third, expression of *M. tuberculosis* genes in *E. coli* is often characterised by a low yield of expressed product (Harth *et al.*, 1997), compromising detection and purification.

Mycobacterial species have been shown to accurately process *M. tuberculosis* signal peptides (Harth *et al.*, 1997). Therefore, it was decided to amplify *mycP1* without the signal sequence for expression in *E. coli*, and with a signal peptide and potential ribosome binding site for expression in *M. smegmatis*. Although expression in *E. coli* has potential disadvantages, it often provides a good expression system generating sufficient quantities of protein for the purpose of purification and production of antibodies. As will be described, this strategy provided sufficient protein for the production of an anti-mycosin-1 antibody. This part of the chapter describes the cloning of *mycP1*⁻ into

pGEX-2T, expression in *E. coli*, purification of the recombinant protein, and generation of an anti-mycosin-1 antibody in rabbits.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Transformations of cosmids Y15F10 and Y22G10 into *E. coli*

Lyophilised samples of *M. tuberculosis* H37Rv cosmids Y15F10 and Y22G10 were obtained from the Pasteur Institute, Paris, France. Sixteen nanograms of each cosmid was transformed into competent *E. coli* XL1-Blue cells (Appendix A.3) and plated onto Luria agar (LA) plates containing ampicillin (50 μg/ml). After an overnight incubation at 37°C, ampicillin-resistant colonies were used to inoculate 5 ml Luria broth (LB), incubated overnight at 37°C and the cosmid DNA was extracted using a shortened mini-prep protocol (Appendix A.4). LB (50 ml) containing ampicillin (50 μg/ml) was inoculated with cosmid-containing clones, incubated overnight at 37°C and preparative amounts of cosmid DNA were isolated using the Qiagen mini-prep DNA kit (Qiagen GmbH, Hilden, Germany), as per manufacturer's instructions. The amount of DNA was quantitated spectrophotometrically and by agarose gel electrophoresis.

5.2.2.2 PCR amplification of *mycP1* and *mpr1* protease genes.

Primer design was based on cosmid DNA sequences generated by the *M. tuberculosis* genome project (Sanger Center, UK); cosmid Y15F10, accession no. Z94121, for Rv3883 (designated *mycP1*), and cosmid Y22G10, accession no. Z84724, for Rv0419 (designated *mpr1*). Amplification was performed with the Expand™ High Fidelity PCR System (Boehringer Mannheim) using 100 ng of cosmid *M. tuberculosis* DNA as a template.

a) The *mycP1* gene was amplified using one reverse primer (p1r), 5′- AA AGA ATT CTG CAG TCA TCG GCG GCT CAG CG-3 (including *Eco*R1 and *Pst*I sites at the 5′ end) and one of two different forward primers. The first forward primer (p1f), 5′- CGT TCT AGA GGA TCC CCC GCA TCG GCC ATC ACG-3′ (including a *Xba*I and *Bam*H1 site at the 5 ′ end), corresponded to nucleotides 52-69 (Figure 4.1) and generated a product without the signal sequence (*mycP1*$^-$) (Figure 5.1). The second forward primer (p1mycf), 5′- ACA GGA TCC GTC AAG TGG CCC AAG GTA GCA-3′ (including a *Bam*H1 site at the 5′ end), corresponded to nucleotides –71 to –51 (Figure 4.1) and generated a product (*mycP1*$^+$) which incorporated 71 nucleotides upstream of the annotated translational start site thus containing the putative ribosomal binding site and signal sequence (Figure 5.1).

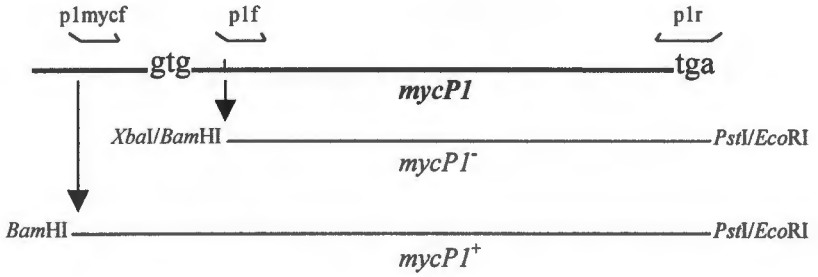


Figure 5.1. Schematic diagram of the PCR amplified fragments of *mycP1*. Fragment *mycP1*$^-$ was amplified using primers p1f and p1r and represents *mycP1* without the signal sequence (*). Fragment *mycP1*$^+$ was amplified using primers p1mycf and p1r, and represents *mycP1* plus some sequence upstream of the putative translational start site (gtg) which likely contains the ribosome binding site. For ease of cloning, appropriate restriction endonuclease sites were included at the 5′-end of each primer.

b) The *mpr1* gene was also amplified using one reverse primer (m1r), 5′- AAT CAA TTG CTG CAG CTA TCC CGG ATA CCG-3′ (including *Mun*I and *Pst*I sites at the 5′ end) and one of two forward primers. The first forward primer (m1f), 5′- GGA TCC TCT AGA GGC TGC ACG ACC GTC-3′ (including *Bam*H1 and *Xba*I sites at the 5′end), corresponded to nucleotides 112 to 126 (Figure 4.8) and generated a product without the

signal sequence (*mpr1*$^-$) (Figure 5.2). The second forward primer (m1mycf), 5′- ATT GGA TCC TTC CTT GGT GCA TGC CG-3′ (including a *Bam*H1 site at the 5′ end), corresponded to nucleotides –20 to –36 (Figure 4.8) and generated a product (*mpr1*$^+$) which incorporated 36 nucleotides upstream of the annotated translational start site and thus contained the putative ribosomal binding site and signal sequence (Figure 5.2).

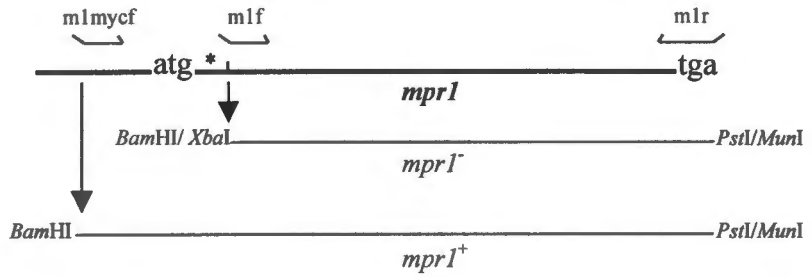


Figure 5.2. Schematic diagram of the PCR amplified fragments of *mpr1*. Fragment *mpr1*$^-$ was amplified using primers m1f and m1r and represents *mpr1* without the signal sequence (*) . Fragment *mpr1*$^+$ was amplified using primers m1mycf and m1r, and represents *mpr1* plus some sequence upstream of the putative translational start site (atg) which likely contains the ribosome binding site. For ease of cloning, appropriate restriction endonuclease sites were included at the 5′-end of each primer.

5.2.2.3 Cloning of *mycP1*$^-$ into pGEX-2T and transformation into *E. coli*

pGEX-2T DNA (2μg) (Pharmacia) and *mycP1*$^-$ DNA (2μg) were each digested with *Bam*HI and *Eco*RI (Appendix A.1). The purified products were ligated (Appendix A.2; designated pGEX-P1) and transformed into *E. coli* XL1-Blue rendered competent by the rubidium chloride method (Appendix A.3) (designated *E. coli*-P1). Vector DNA only was also transformed into competent *E. coli* cells (designated *E. coli*-pGEX; served as the negative control). Each transformation mixture (100 μl) was plated onto LA plates containing ampicillin (50 μg/ml) and, after an overnight incubation at 37°C, ampicillin-resistant colonies were used to inoculate 5 ml LB. After another overnight incubation at 37°C, the recombinant pGEX-P1 construct was extracted using the shortened mini-prep

protocol (Appendix A.1). To confirm the presence of the *mycP1*⁻ insert, pGEX-P1 DNA was digested with *Bam*HI and *Eco*RI. Glycerol stocks (25%) were prepared from *E. coli*-P1 and stored at -70°C (Sambrook *et al.*, 1989).

5.2.2.4 Expression and purification of recombinant mycosin-1

The GST-fusion protein was expressed and purified essentially as described by Frangioni *et al.* (1993). Briefly, *E.* coli-P1 cells were grown in LB to an OD_{600} of 0.8, induced with 0.1 mM IPTG for 2 h at 37°C and pelleted by centrifugation at 1,000 x g for 10 min. After washing with STE [10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA], the bacteria were gently resuspended in 0.075 x vol of STE containing lysozyme (final concentration 100 μg/ml). After a 15 min incubation on ice, dithiothreitol (DTT) was added to a final concentration of 5 mM and the mixture was left standing on ice for 15 min. N-laurylsarcosine (Sarkosyl) was added to a final concentration of 1.5% and the mixture was gently mixed for 10 min. The cells were then disrupted by sonication (Transsonic 310 water bath sonicator, Elma, Germany, 35 kHz) on ice-cold water for 2 min. The sonicate was centrifuged at 12,000 x g for 20 min and the supernatant recovered. Triton X-100 was added to the supernatant to a final concentration of 4% before the mixture was passed over a GST-agarose affinity column (0.5 ml/min). The fusion protein (GST-mycosin-1) was eluted with glutathione elution buffer [75 mM HEPES, 150 mM NaCl, 20 mM reduced glutathione, 5 mM DTT, 2% (w/v) N-octylglucoside].

Samples (100 μl) of *E. coli*-P1 and *E. coli*-pGEX pre- and post-induction of the GST-fusion protein, as well as a sample of purified products, were mixed with an equal volume of SDS-PAGE solubilisation buffer. After boiling the samples at 100°C for 5 min they were resolved on a 10% SDS-polyacrylamide gel and subjected to Western blot

analysis using an anti-GST mouse monoclonal antibody (1:1,000) as the primary antibody and peroxidase-labelled anti-mouse IgG as the secondary antibody (Appendix A.5).

5.2.2.5 Polyclonal antibody production

Blood was taken from each of two New Zealand White rabbits, centrifuged at 3,000 x g for 10 min, and the serum fraction (referred to as pre-immune serum) was removed and stored at -70°C in 1 ml aliquots. Purified GST-mycosin-1 fusion protein (100 μg) resuspended in incomplete Freund's adjuvant was injected subcutaneously into each rabbit. Three additional booster injections were administered at 3-week intervals. Blood (10 ml) was taken from each rabbit at 1 and 2 weeks after the last booster injection, centrifuged at 3,000 x g for 10 min, and the serum fraction (referred to as anti-mycosin-1 antiserum) was removed and stored at -70°C in 1 ml aliquots.

5.2.2.6 Affinity purification of the pre-immune serum and anti-mycosin-1 antiserum

LB (1 litre) containing ampicillin (50 μg/ml) was inoculated with *E. coli*-pGEX cells. After an overnight incubation at 37°C, GST production was induced by the addition of 0.1 mM IPTG for 2 h at 37°C. The bacteria were pelleted by centrifugation at 3,000 x g for 10 min and resuspended in 100 ml 0.1 M sodium borate/1 M NaCl, (pH 8.0). Lysozyme (2 mg/ml) was added and the mixture incubated at room temperature. After 20 min, DNAse I (10 μg/ml) and Triton X-100 (0.005%) were added and the mixture again incubated at 4°C for 1 h. After centrifugation at 8,000 x g for 20 min at 4°C, the supernatant was recovered and the pH adjusted to 9.0 with 1 M NaOH.

Two grams CnBr-activated Sepharose 4B (Sigma) was washed 4 times with 1 mM HCl and then equilibrated with coupling buffer [0.1 M sodium borate/0.5 M NaCl, (pH 9.0)]. The equilibrated sepharose was added to the supernatant (containing *E. coli* proteins

and GST) and incubated rotating at 4°C overnight. The uncoupled protein was removed by washing with coupling buffer. A sample was retained for OD determination to predict the coupling efficiency. To block remaining active sites, sepharose with bound protein was incubated with 0.2 M glycine (pH 8.2) at room temperature for 2-3 h and then washed with alternating coupling buffer and acetate buffer [0.1 M sodium acetate / 0.5 M NaCl (pH 4.0)]. Two columns, each containing 2 ml of sepharose, were prepared and washed with 10 column volumes of PBS. Pre-immune serum and anti-mycosin-1 antiserum were each passed 4 times over a column. Between each passage the column was regenerated with alternating washes of 0.1 M Tris/0.5 M NaCl (pH 8.5) and 0.1 M NaAc/0.5 M NaCl (pH 4.0). After the last passage the resultant eluent, containing pre-immune serum and anti-mycosin-1 antiserum depleted of antibodies to *E. coli* proteins and GST, was stored at 4°C. After a final wash with alternating 0.1 M Tris/0.5 M NaCl (pH 8.5) and 0.1 M NaAc/0.5 M NaCl (pH 4.0), the columns were washed with 3 column volumes of PBS and then stored at 4°C in PBS containing 0.02% sodium azide.

5.2.3 RESULTS

5.2.3.1 Transformation of cosmids

The cosmids, Y15F10 and Y22G10, containing *mycP1* and *mpr1*, respectively, were transformed into competent *E. coli* XL1-Blue cells and incubated overnight at 37°C. DNA extraction from ampicillin-resistant colonies from each plate confirmed the presence of the cosmids and 25 μg of each cosmid was prepared.

5.2.3.2 PCR amplification of *mycP1* and *mpr1*.

Amplification of *M. tuberculosis* H37Rv cosmid DNA with primers based on the genome sequence generated four fragments : a) a 1318-bp fragment (*mycP1*$^-$) and a 1386-

bp fragment (*mpr1*$^-$) representing the genes without their signal sequences (Figure 5.3A) and b) a 1434-bp fragment (*mycP1*$^+$) and a 1557-bp fragment (*mpr1*$^+$) which represent amplification of both genes to yield products incorporating their signal peptides and potential ribosome binding sites (Figure 5.3B). The sequence integrity of the PCR amplified DNA fragments was confirmed by automated DNA sequencing (ALF Express Sequenator).

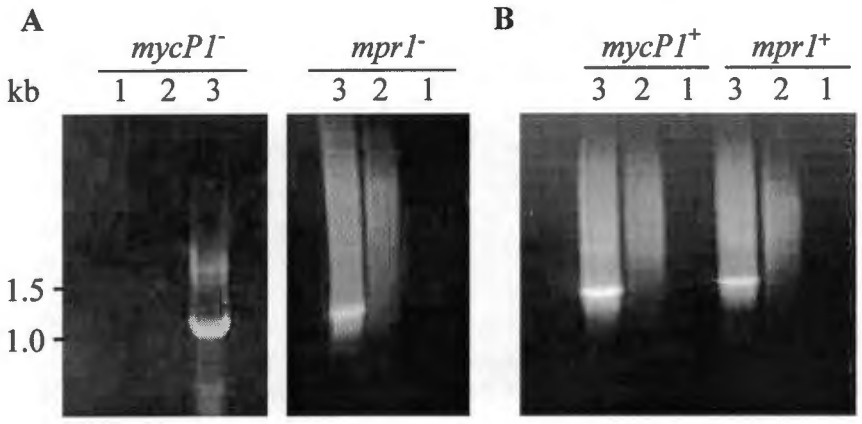


Figure 5.3. PCR amplification of *mycP1* and *mpr1* protease genes. Both protease genes were amplifie by 30 cycles of PCR. Template DNA was *M. tuberculosis* H37Rv cosmids Y15F10 and Y22G10 for *mycP1* and *mpr1*, respectively; 10 µl of each reaction was electrophoresed on a 0.8% agarose gel and then stained with ethidium bromide. Lane 1, PCR negative control (primers but no template DNA); lane 2, PCR negative control (template DNA but no primers); lane 3, PCR reaction (template DNA and primers). The numbers on the left are molecular weight markers in kb. **A)** *mycP1* and *mpr1* without a signal sequnce; **B)** *mycP1* and *mpr1* with a signal sequence and putative ribosome binding site

5.2.3.3 Cloning and expression of *mycP1*$^-$ in *E. coli*

For ease of cloning, *mycP1*$^-$ was amplified with *Xba*I and *Bam*H1 restriction endonuclease sites at the 5′ end and *Pst*I and *Eco*RI restriction endonuclease sites at the 3′ end. This 1318-bp fragment was cloned into the *Bam*HI and *Eco*RI restriction endonuclease sites of pGEX-2T in frame with the GST gene. Since the fragment lacks the *mycP1* signal sequence, a fusion protein of ~73 kDa (27-kDa GST protein fused to an expected 46-kDa mycosin-1) was expected to accumulate intracellularly. Both pGEX-P1

and pGEX-2T (negative control) were transformed into *E. coli* XL1-Blue. Numerous ampicillin-resistant colonies were obtained and the presence of both plasmids was confirmed by isolation of plasmid DNA followed by restriction enzyme analysis using *Bam*HI and *Eco*RI (Figure 5.4).

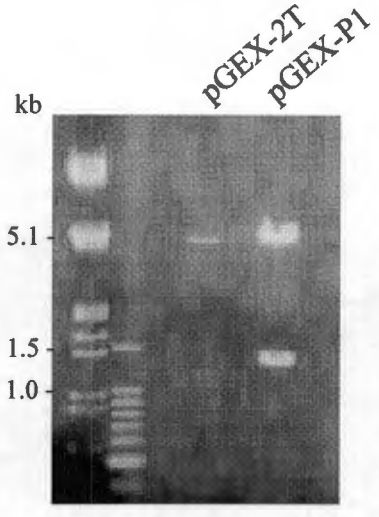


Figure 5.4. Agarose gel of pGEX-2T and the pGEX-P1 construct. *E. coli* XL1-Blue cells transformed with pGEX-2T and pGEX-P1 were grown at 37°C, pelleted, and plasmid DNA extracted (Appendix A.4). The presence of *mycP1*⁻ was confirmed by digestion of the DNA with *Bam*HI and *Eco*RI. Lanes 1 and 2 contain molecular weight markers and the numbers on the left are molecular weights in kb.

After growth at 37°C and induction with IPTG, whole cell lysates of uninduced and induced bacteria, as well as products purified on a GST-agarose affinity column, were analysed by SDS-PAGE and Western blotting (Figure 5.5). As expected, a 27-kDa protein corresponding to GST was observed in the induced and purified fractions of *E. coli*-pGEX (*E. coli* transformed with the vector pGEX-2T). The induced and purified fraction of *E. coli*-P1 (*E. coli* transformed with pGEX-P1) revealed the presence of a new band at ~73 kD, likely representing the GST-mycosin-1 fusion protein, based on cross-reactivity with an anti-GST monoclonal antibody (Figure 5.5).

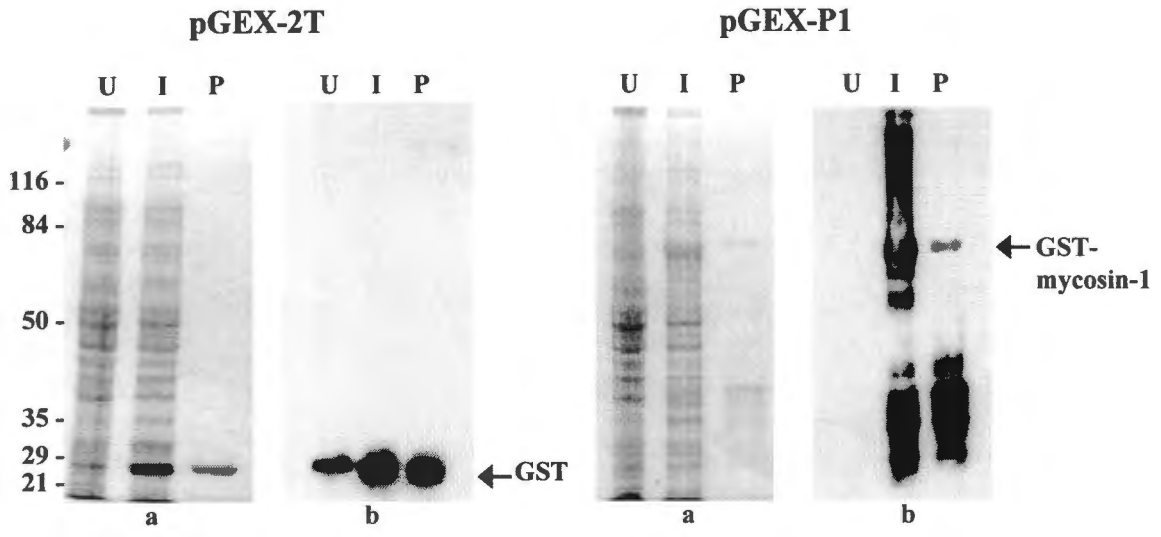


Figure 5.5. Expression of *mycP1*⁻ in *E. coli*. *E. coli* XL1-Blue cells transformed with pGEX-2T and pGEX-P1 were grown at 37°C and fusion protein production was then induced with 0.1 mM IPTG for 2 h. Thereafter, the bacteria were pelleted and sonicated. GST and the fusion protein were purified using a GST-agarose affinity column. Whole cell extracts of uninduced (U), induced (I) and purified (P) fractions were analysed by (a) SDS-PAGE and (b) Western blotting using an anti-GST monoclonal antibody as the primary antibody.

Most GST-mycosin-1 was recovered from the insoluble (cell lysate) fraction (data not shown) and significant amounts were lost during purification, consistent with the high degree of hydrophobicity predicted from the amino acid sequence. Addition of detergents to solubilise the fusion protein increased the amount of GST-mycosin-1 in the soluble fraction and improved purification yields, but significant amounts were still lost to the insoluble fraction.

The SDS-PAGE and Western blot of purified GST-P1 revealed additional lower molecular weight products, possibly representing degradation products of the fusion protein (Figure 5.5). The use of protease inhibitors, alternative *E. coli* hosts, or shorter expression times did not significantly reduce GST-mycosin-1 degradation (not shown).

5.2.3.4 Production of an anti-mycosin-1 antibody

Western blot analysis of cell fractions of *E. coli*-pGEX and *E. coli*-P1 revealed that the pre-immune serum only recognised some *E. coli* antigens (Figure 5.6).

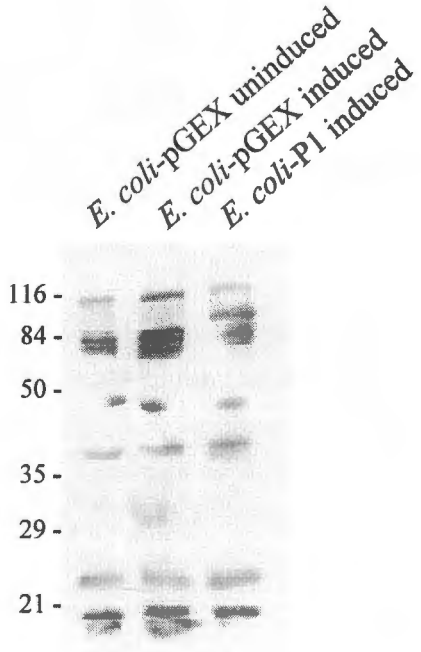


Figure 5.6. Detection of *E. coli* proteins using pre-immune serum. Whole cell extracts of uninduced and induced fractions of *E. coli* transformed with pGEX-2T and an induced fraction of *E. coli* transformed with pGEX-P1 were resolved on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and then subjected to Western blotting (Appendix A.5) using the pre-immune serum as the primary antibody (1:1,000). The numbers on the left are molecular weight markers in kDa.

The polyclonal anti-mycosin-1 antiserum, however, recognised the fusion protein, as well as GST and several *E. coli* proteins (Figure 5.7a). To improve the specificity of the antisera both were affinity-purified by passage over a CnBr-activated Sepharose 4B column bound to proteins from an induced fraction of *E. coli* transformed with pGEX-2T. After 2-3 passages over the column, all cross-reacting *E. coli* and GST antibodies were removed. The resulting affinity-purified polyclonal anti-mycosin-1 antiserum only recognised the fusion protein and degradation products (Figure 5.7b).

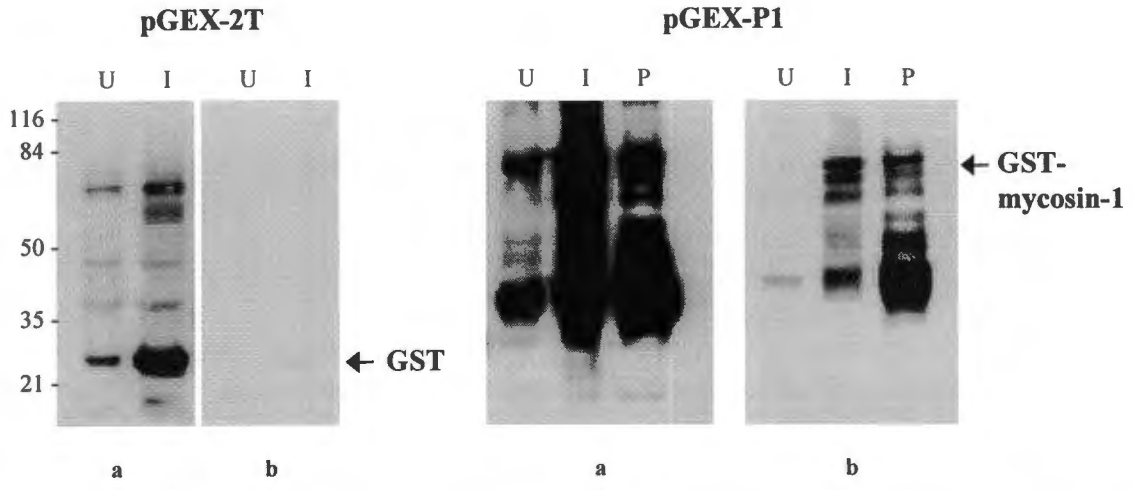


Figure 5.7. Detection of GST-mycosin-1 using anti-mycosin-1 polyclonal antiserum. Whole cell extracts of uninduced (U), induced (I) and purified (P) fractions of *E. coli*-pGEX and *E. coli*-P1 were resolved on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and then subjected to Western blotting using (a) polyclonal anti-mycosin-1 antiserum and (b) affinity purified polyclonal anti-mycosin-1 antiserum as the primary antibody. The numbers on the left ar molecular weight markers in kDa.

5.2.4 DISCUSSION

This chapter describes the recombinant expression of the *mycP1* gene product, mycosin-1, in *E. coli*. Since mycosin-1 is likely to be an exported, membrane-anchored protein, it was produced as a recombinant protein without its signal sequence to minimise the disturbance in the host membrane. Without the signal sequence mycosin-1 would not be translocated across the plasma membrane but would accumulate intracellularly. However, this, together with its hydrophobic nature, led to the production of recombinant GST-mycosin-1 that was insoluble. In addition, due to its intracellular residence there was a significant amount of degradation. Although the use of detergents in the purification procedure ensured that sufficient soluble protein was obtained to induce a useful antibody response in rabbits, a significant amount of recombinant protein remained in the insoluble fraction. Various strategies were attempted to prevent degradation - altering expression

times and temperatures, using protease deficient strains of *E. coli*, and using protease inhibitors – all unsuccessful.

The activity of some denatured proteins can be restored after renaturation. However, this is especially difficult to achieve with subtilisins. As mycosin-1 is predicted to be a subtilisin-like protease and part of our objective was to obtain an active recombinant protein, purification procedures were selected that would lead to as little denaturation as possible of the expressed product. This, unfortunately, was at the expense of losing a significant amount of the fusion protein to the insoluble fraction. For a number of technical reasons it was decided not to cleave off the GST portion but to assess activity of the fusion protein instead : a) Since the mycosin-1-GST fusion protein was mostly degraded it was felt that there would probably be insufficient amounts of undegraded mycosin-1 to purify and assess for activity; b) During the purification procedure further amounts of mycosin-1 would be lost; c) Separating thrombin (used to cleave the GST portion) from mycosin-1 would present a technical challenge. Although fusion to GST may render mycosin-1 inactive, there are reports indicating that despite fusion to GST, the activity of the heterologous enzyme can be retained. Activity of the GST-mycosin-1 fusion protein was assessed using ^{125}I-fibrinogen as the substrate (see section 3.2.5). However, no activity was detected. There are a number of possible explanations for this: the expression of some mycobacterial proteins in *E. coli* can yield inactive proteins; the GST fusion partner may be affecting activity; the choice of substrate may be incorrect; and propeptide cleavage may be required for activity.

The purified recombinant protein and the degradation products were used to generate an anti-mycosin-1 antibody. The resulting polyclonal antiserum reacted with mycosin-1 but also cross-reacted with GST and some *E. coli* proteins. This cross-reactivity was removed by affinity chromatography using a sepharose column containing bound GST and *E. coli* proteins.

5.3 EXPRESSION AND DETECTION OF MYCOSIN-1 IN TRANSFORMED *M. SMEGMATIS* AND WILD-TYPE *M. TUBERCULOSIS*.

5.3.1 INTRODUCTION

The slow growth and pathogenicity of *M. tuberculosis* have made working with this organism both tedious and hazardous. This has encouraged investigators to search for alternative host species for the study of *M. tuberculosis* genes. Since it is likely that all mycobacteria respond to the same DNA transcription and translation signals, several mycobacterial species have been investigated as hosts for *M. tuberculosis* genes. *M. smegmatis* and *M. aurum* have become the surrogate hosts of choice due to the fact that they grow faster and are less prone to forming clumps compared to other members of this genus. In addition, efficient transformation procedures have been developed for these organisms. It is the opinion of some investigators that for high protein expression and secretion of relatively unconserved mycobacterium-specific proteins, a mycobacterial host is required.

As mycosin-1 expressed in *E. coli* was degraded and inactive, an alternative approach was sought and *M. smegmatis* was the host of choice. It was likely that *M. smegmatis* would recognise the transcription and translation signals of *M. tuberculosis* genes, and thus the complete *mycP1* gene was amplified, incorporating 71 bp of upstream sequence that were likely to contain the ribosome-binding site and promoter sequences (designated *mycP1*$^+$). The amplified fragment was cloned into p19Kpro (a gift from Koen De Smet, Imperial College Medical School at St Mary's, London, UK), a derivative of pOLYG, downstream of the constitutive *M. tuberculosis* 19 kDa antigen promoter. This part of the chapter describes the cloning of *mycP1*$^+$ and *mpr1*$^+$ into p19Kpro, expression of *mycP1*$^+$ in transformed *M. smegmatis*, and detection of mycosin-1 in *M. tuberculosis*.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Cloning of *mycP1*$^+$ and *mpr1*$^+$ into pBluescript^{SK} (pBS)

pBS DNA (3 µg) (Stratagene) and *mycP1*$^+$ DNA (3 µg) were each digested with *Bam*HI and *Eco*RI, and *mpr1*$^+$ DNA (3 µg) was digested with *Bam*HI and *Mun*I (Appendix A.1). All products were separated by electrophoresis in a 0.8% agarose gel and purified using NucleoSpin Columns (Macherey Nagel, Germany), as per manufacturer's instructions. The purified products were quantitated by agarose gel electrophoresis and spectrophotometry. An equal amount of *mycP1*$^+$ DNA (150 ng) and *mpr1*$^+$ DNA (150 ng) was ligated to 300 ng pBS DNA (Appendix A.2). The ligated products are designated pBS-*mycP1*$^+$ and pBS-*mpr1*$^+$, respectively.

5.3.2.2 Transformation of pBS-*mycP1*$^+$ and pBS-*mpr1*$^+$ into *E. coli*

Of each ligation reaction, 10 µl (approximately 150 ng plasmid DNA) was transformed into competent *E. coli* XL1-Blue cells (Appendix A.3). Several white colonies were each inoculated into 5 ml LB, incubated overnight at 37°C, and plasmid DNA was extracted using a shortened mini-prep protocol (Appendix A.4). The insert size was confirmed by digestion with *Bam*HI and *Eco*RI for *mycP1*$^+$, and *Bam*HI and *Eco*Rv for *mpr1*$^+$. The insert-containing clones were then used to generate preparative amounts of DNA using the Qiagen mini-prep DNA kit (Qiagen GmbH, Hilden, Germany), as per manufacturer's instructions.

5.3.2.3 Subcloning of *mycP1*$^+$ and *mpr1*$^+$ into p19Kpro and transformation into *E. coli*

pBS-*mycP1*$^+$ DNA (3 µg), pBS-*mpr1*$^+$ DNA (3 µg), and p19Kpro DNA (3 µg) (Figure 5.8) were each digested with *Bam*HI and *Eco*Rv (Appendix A.1). The purified products were quantitated by agarose gel electrophoresis and spectrophotometry. One

hundred nanograms of *mycP1*$^+$ and *mpr1*$^+$ DNA was ligated to 200 ng p19Kpro (Appendix A.2), transformed into competent *E. coli* XL1-Blue cells (Appendix A.3), and plated onto LA plates containing hygromycin B (200 μg/ml). After an overnight incubation at 37°C, hygromycin-resistant colonies were inoculated into LB containing hygromycin B (200 μg/ml) and incubated overnight at 37°C.

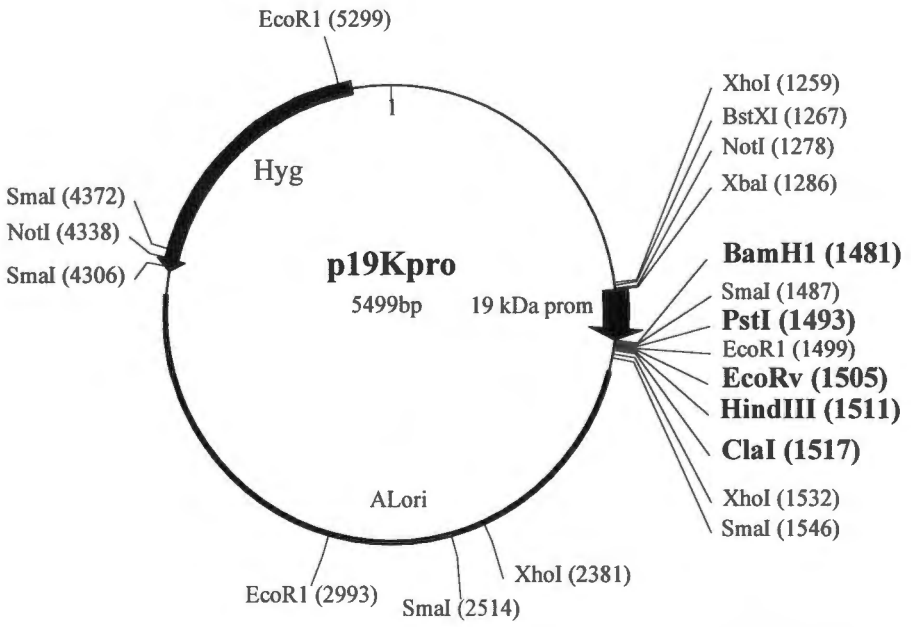


Figure 5.8. Restriction enzyme map of p19Kpro. This vector contains a hygromycin resistance cassette (Hyg) and the 19 kDa protein promoter has been cloned upstream of the multiple cloning site. All restriction enzyme sites that are in bold represent unique sites. This vector was a gift from Koen De Smet, Imperial College Medical School at St Mary's, London, UK.

Plasmid DNA was extracted using a shortened mini-prep method (Appendix A.4). Preparative amounts of p19Kpro-*mycP1*$^+$ (designated p19K-P1) and p19Kpro-*mpr1*$^+$ (designated p19K-M1) DNA was prepared from those clones containing *mycP1*$^+$ or *mpr1*$^+$ insert DNA, using the Qiagen mini-prep DNA kit (Qiagen GmbH, Hilden, Germany), as per manufacturer's instructions.

5.3.2.4 Electroporation of p19K-P1 and p19K-M1 into *M. smegmatis*

Middlebrook's (MB) medium (100 ml) supplemented with OADC (0.05% oleic acid, 5% BSA, 2% dextrose, 0.004% catalase, 0.085% NaCl) was inoculated with *M. smegmatis* MC^2155 bacteria (1 x 10^6). After 20 h growth at 37°C, the culture was placed on ice for 1.5 h, the bacteria were pelleted by centrifugation at 3,000 x g for 10 min, washed 3 times in ice-cold sdH_2O, and then resuspended in 0.5 ml ice-cold 10% glycerol. The electroporation procedure is very sensitive to the concentration of salts present in the DNA sample, therefore each DNA sample was cleaned with chloroform/isoamyl alcohol (24:1) and phenol, then re-precipitated with 100% ethanol. Aliquots (50 µl) of the bacteria prepared above were mixed with 50 ng p19K-P1 DNA, 50 ng p19K-M1 DNA, 50 ng p19Kpro DNA or sdH_2O (the latter two serving as the positive and negative control, respectively) and placed on ice for 1 min. These reactions were then added into an electroporation cuvette (0.2 cm electrode gap; BioRad) which was placed in the pulser (BioRad). After ensuring that all air bubbles had been removed, the cuvette was exposed to 2 pulses (2500 V, 25 µF, 1,000 ohms); 800 µl MB medium was added to each cuvette and the contents were transferred to a 6-well tissue culture dish. The dishes were incubated at 37°C for 4 h to allow for full expression of the hygromycin-resistance gene resident on p19Kpro. Thereafter, the contents of each well were plated onto MB agar plates containing hygromycin B (50 µg/ml) and incubated at 37°C for 4 days. Hygromycin-resistant colonies were transferred into 20 ml MB medium supplemented with OADC and hygromycin B (50 µg/ml), incubated for 20 h at 37°C and plasmid DNA was isolated (described below).

5.3.2.5 Isolation of plasmid DNA from transformed *M. smegmatis*.

Five millilitres of the above cultures were centrifuged at 2,500 x g for 20 min. The pelleted bacteria were resuspended in 150 µl GTE (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) containing 10 mg/ml lysozyme, and incubated overnight at 37°C, after which 300 µl 0.2 M NaOH/1% SDS was added and the mixture incubated on ice for 20 min. After adding 225 µl 5 M potassium acetate, the mixture was incubated on ice for a further 30 min. The suspension was then centrifuged at 12,000 x g for 45 min, the supernatant was retained, and 300 µl chloroform/isoamyl alcohol (24:1) and 300 µl phenol were added, mixed well, and centrifuged at 12,000 x g for 10 min. The aqueous phase was recovered and the plasmid DNA was precipitated by the addition of 1,200 µl ice-cold 100% ethanol. After a 1-h incubation at -70°C, the DNA was pelleted by centrifugation at 12,000 x g for 10 min, the pellet washed with 70% ethanol and air-dried. The plasmid DNA was resuspended in 50 µl sdH_2O and quantitated spectrophotometrically.

An aliquot from each plasmid preparation was digested with *Bam*HI and *Eco*Rv to confirm the presence of *mycP1*$^+$ and *mpr1*$^+$ insert DNA. *M. smegmatis* transformed with p19Kpro, p19K-P1 and p19K-M1 have been designated *M. smegmatis*-P19, *M. smegmatis*-P1 and *M. smegmatis*-M1, respectively.

5.3.2.6 Expression and detection of mycosin-1 in *M. smegmatis*.

Wild-type *M. smegmatis* and transformed *M. smegmatis* (*M. smegmatis*-P19, *M. smegmatis*-P1 and *M. smegmatis*-M1) were grown in MB medium supplemented with OADC and hygromycin B (50 µg/ml). The cultures were incubated rotating (200 rpm) at 37°C for 24-44 h, centrifuged at 3,000 x g for 10 min, and the pellet washed twice with PBS before being resuspended in the same buffer. The suspension was sonicated (W-385 sonicator, Heat Systems-Ultrasonics, Inc; output level 3; 80% duty cycle) on ice for 3

bursts of 4 min each, separated by 2-min cooling intervals. SDS was added to the sonicate to a final concentration of 2% and proteins extracted at 60°C for 2 h. The extract was centrifuged at 8,000 x g for 10 min to remove insoluble material, the supernatant mixed with an equal volume of 2 x SDS solubilisation buffer, and boiled at 100°C for 5 min. All fractions were subjected to SDS-PAGE and Western blotting analysis (Appendix A.5) using polyclonal anti-mycosin-1 antiserum (1:1,000) as the primary antibody and peroxidase-labelled anti-rabbit IgG as the secondary antibody.

5.3.2.7 Detection of mycosin-1 in wild-type *M. tuberculosis*.

M. tuberculosis GSH-3052 was inoculated into 100 ml Kirchner's medium and grown rotating (200 rpm) at 37°C for 1, 2, 3, 4 and 6 weeks. The bacteria were pelleted by centrifugation at 3,000 x g for 30 min, resuspended in PBS and heat-killed at 85°C for 45 min. The heat-killed bacteria were disrupted by sonication (W-385 sonicator, Heat Systems-Ultrasonics, Inc; output level 3; 80% duty cycle) on ice for 3 bursts of 4 min each, separated by 2-min cooling intervals. SDS was added to a final concentration of 2% and the proteins extracted by incubation at 60°C for 2 h. Insoluble material was pelleted by centrifugation at 8,000 x g for 10 min. An aliquot of the supernatant was mixed with an equal volume of 2 x SDS solubilisation buffer and boiled at 100°C for 5 min. In addition, the culture supernatants were filtered (0.22 μm; low-protein binding) to remove any remaining *M. tuberculosis* bacilli, dialysed against sdH_2O, and lyophilised. Equal amounts of protein (determined using the BioRad assay, as per manufacturer's instructions) from each week's filtrate was dissolved in an equal volume of sdH_2O and 2 x SDS solubilisation buffer, and boiled at 100°C for 5 min. The SDS-extract fractions and the culture filtrate fractions were subjected to SDS-PAGE and Western blotting analysis (Appendix A.5) using the polyclonal anti-mycosin-1 antiserum (1:1000) as the primary

antibody and peroxidase-labelled anti-rabbit IgG as the secondary antibody. In the following text and chapters of this thesis, whenever *M. tuberculosis* is mentioned it refers to wild-type *M. tuberculosis*.

5.3.2.8 Affinity purification of the pre-immune serum and anti-mycosin-1 antiserum

M. smegmatis-M1 bacteria were inoculated into MB medium supplemented with OADC and hygromycin B (50 μg/ml), incubated at 37°C for 44 h, pelleted by centrifugation at 3,000 x g for 10 min, the pellet was washed in PBS, and the bacteria were then sonicated (W-385 sonicator, Heat Systems-Ultrasonics, Inc; output level 3; 80% duty cycle) on ice for 3 bursts of 4 min each, separated by 2-min cooling intervals. After removing the cell debris by centrifugation at 3,000 x g for 10 min, the sonicate containing whole cell proteins of *M. smegmatis*-M1 was added to 2 g CnBr-activated sepharose-4B. The proteins were attached to the sepharose as described in section 5.2.2.4.

5.3.3 RESULTS

5.3.3.1 Cloning of *mycP1*$^+$ and *mpr1*$^+$ into pBS and p19Kpro

Both *mycP1*$^+$ and *mpr1*$^+$ were PCR amplified using primers designed to introduce restriction endonuclease sites at the 5′ and 3′ ends of the template genes. For the *mpr1*$^+$ fragment, a *Mun*I site (generating *Eco*RI-compatible ends) was constructed instead of a *Eco*R1 site, as the fragment contained an internal *Eco*R1 site. For ease of cloning it was decided to use the *Bam*HI and *Eco*Rv sites of p19Kpro. Therefore, both fragments were first cloned into the *Bam*HI and *Eco*RI sites of pBS, excised with *Bam*HI and *Eco*Rv, and then subcloned into the corresponding sites of p19Kpro. After transformation of p19K-P1 and p19K-M1 into *E. coli*, hygromycin-resistant colonies were randomly selected from

each transformation and their plasmid DNA isolated (Appendix A.1). Digestion with *Bam*HI and *Eco*Rv confirmed the correct size vector and insert DNA in all clones (Figure 5.9).

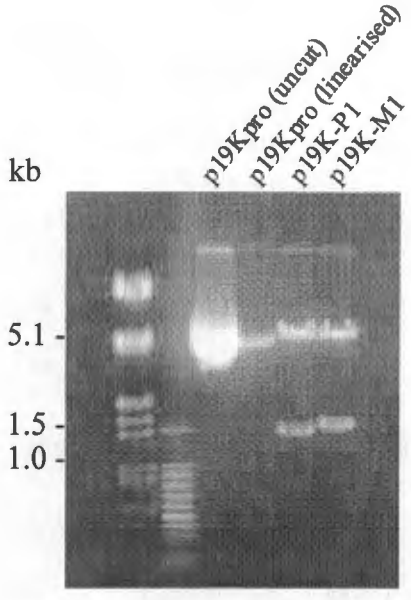


Figure 5.9. Ligation of *mycP1*$^+$ and *mpr1*$^+$ into p19Kpro. Both protease inserts were excised from pBS-*mycP1*$^+$ and pBS-*mpr1*$^+$ using *Bam*HI and *Eco*Rv and ligated into the corresponding sites in p19Kpro. The constructs were transformed into competent *E. coli* XL1-Blue cells, cultured at 37°C and plasmid DNA was isolated. The DNA was assessed for the presence of *mycP1*$^+$ and *mpr1*$^+$ insert DNA by digestion with *Bam*HI and *Eco*Rv. The digested products were resolved in a 0.8% agarose gel and then stained with ethidium bromide. The numbers on the left represent molecular weights in kb.

Preparative amounts of p19K-P1 and p19K-M1 DNA for electroporation into *M. smegmatis* were prepared using the Qiagen mini-prep DNA Kit (Qiagen GmbH, Hilden, Germany), as per manufacturer's instructions.

5.3.3.2 Electroporation of p19Kpro, p19K-P1, and p19K-M1 DNA into *M. smegmatis*

After electroporation of p19Kpro, p19K-P1, and P19K-M1 into *M. smegmatis*, numerous hygromycin-resistant colonies were obtained on the plates of *M. smegmatis* transformed with the plasmid constructs. No hygromycin-resistant colonies were obtained

on the plates when the plasmid DNA was replaced with H_2O (negative control for DNA contamination). Ten colonies were harvested from each electroporation event, plasmid DNA was isolated, and digested with *Bam*HI and *Eco*Rv. All colonies were shown to contain the correct constructs.

5.3.3.3 Detection of mycosin-1 in transformed *M. smegmatis* and in *M. tuberculosis.*

Dense cultures of *M. smegmatis* transformed with p19K-P1, p19K-M1, and p19Kpro (the latter two were used as negative controls) were harvested after 24-44 h, and the bacterial cell pellets were subjected to sonication and SDS extraction of whole cell proteins. Although SDS extraction is likely to denature mycosin-1, it was thought that due to the insoluble nature of mycosin-1, SDS would solubilise enough mycosin-1 for detection. Since the aim of this experiment was to detect the presence of mycosin-1 and not its activity, denaturation of mycosin-1 was not a concern. Proteins were extracted from wild-type *M. tuberculosis* (after 3 weeks growth) in a similar manner as described for *M. smegmatis.* The only difference was that the *M. tuberculosis* bacteria were heat-killed at 85°C for 45 minutes prior to sonication and extraction of whole cell proteins.

SDS-PAGE analysis of the SDS extracts from transformed *M. smegmatis* and wild-type *M. tuberculosis* revealed a large number of proteins but no dominant band or significant difference between the samples (Figure 5.10A). Analysis of the extracts by Western blotting using the preimmune antiserum and polyclonal anti-mycosin-1 antiserum as the primary antibody revealed cross-reactivity with numerous mycobacterial proteins (Figure 5.11A and C). There was, however, an immunodominant band at about 50 kDa (mycosin-1 is expected to be 46 kDa) in whole cell protein extracts of *M. smegmatis*-P1 and *M. tuberculosis* when using the anti-mycosin-1 antiserum.

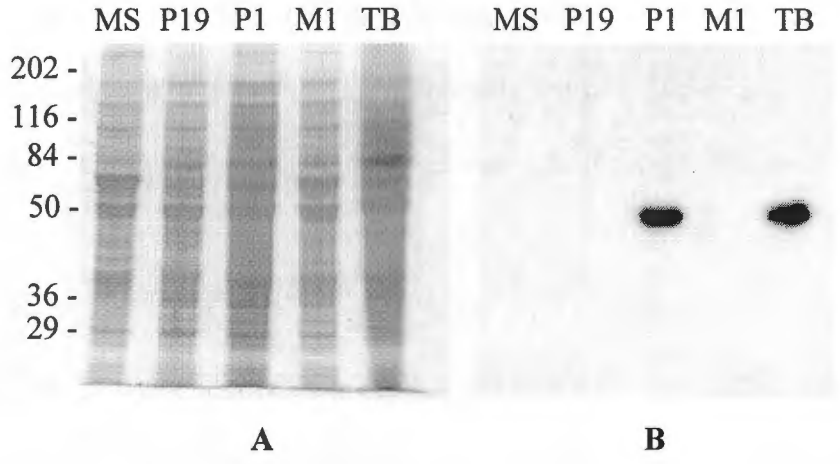


Figure 5.10. Detection of mycosin-1 in transformed *M. smegmatis* and wild-type *M. tuberculosis*. Transformed *M. smegmatis* and wild-type *M. tuberculosis* were grown at 37°C, the bacterial cells were pelleted, sonicated, and the proteins extracted with SDS. The extracts were resolved by (A) 10% SDS-polyacrylamide gel electrophoresis and transblotted onto nitrocellulose membranes. (B) Western blots were performed using affinity purified anti-mycosin-1 antiserum as the primary antibody. An equal amount of protein was added to each lane. The numbers on the left represent molecular weights in kDa. Key: MS, *M. smegmatis*; P19, *M. smegmatis*-P19; P1, *M. smegmatis*-P1; M1, *M. smegmatis*-M1; TB, wild-type *M. tuberculosis*.

The preimmune antiserum and anti-mycosin-1 antiserum were further affinity purified by passage over a CnBr-activated Sepharose 4B column with bound *M. smegmatis* proteins. Western blot analysis using the antisera affinity-purified of antibodies to *E. coli* and *M. smegmatis* proteins revealed no immunoreactive band with the preimmune serum (Figure 5.11B) and a 50 kDa immunodominant band in whole cell protein extracts of *M. smegmatis*-P1 and *M. tuberculosis* (Figure 5.10B and 5.11D). Whole cell protein extracts of *M. smegmatis*-P19 and *M. smegmatis*-M1, both serving as negative controls, did not show any immunoreactivity. In addition, wild-type mycosin-1 expressed by *M. tuberculosis* and heterologous, recombinant mycosin-1 expressed by *M. smegmatis* appeared to have the same molecular weight (Figure 5.10B). In all subsequent experiments the antiserum that was used was affinity-purified of antibodies to *E. coli* and *M. smegmatis* proteins and it is subsequently referred to as affinity-purified anti-mycosin-1 antiserum.

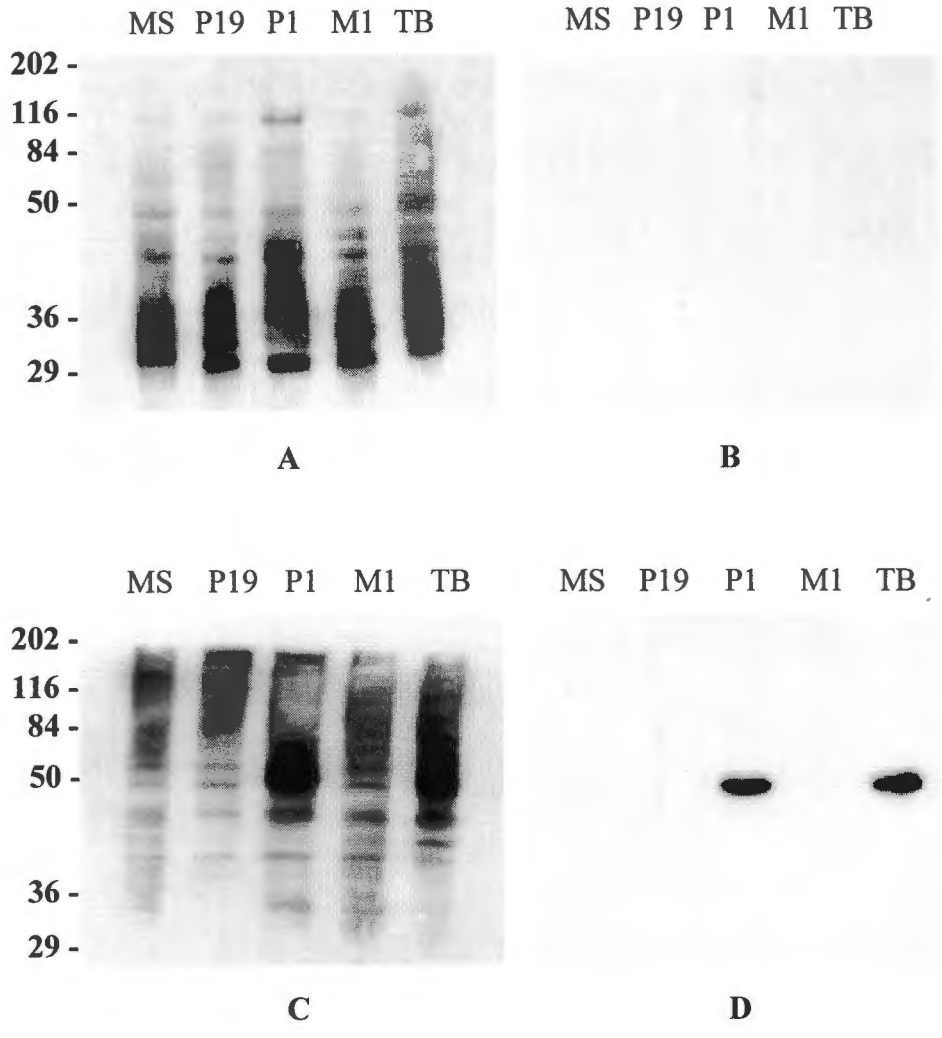


Figure 5.11. Detection of mycosin-1 in transformed *M. smegmatis* and in *M. tuberculosis*. Whole cell protein extracts from transformed *M. smegmatis* and *M. tuberculosis* were resolved by 10% SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis using (A) preimmune antiserum (B) affinity-purified preimmune antiserum (C) anti-mycosin-1 antiserum, and (D affinity-purified anti-mycosin-1 antiserum. An equal amount of protein was added to each lane. Key: MS, *M. smegmatis*; P19, *M. smegmatis*-P19; P1, *M. smegmatis*-P1; M1, *M. smegmatis*-M1; TB, *M. tuberculosis*.

5.3.3.4 Detection of mycosin-1 in *M. tuberculosis* culture filtrates

In Chapter 4 the likely presence of a serine protease in *M. tuberculosis* culture filtrates was described. To assess whether mycosin-1 is present and could possibly contribute to the serine protease activity detected in *M. tuberculosis* culture filtrates, whole cell protein extracts and filtrate proteins were analysed by Western blotting using affinity-

purified anti-mycosin-1 antiserum as the primary antibody. In addition, whole cell protein extracts and filtrate proteins from various stages of the *M. tuberculosis* growth cycle were assessed for the presence of mycosin-1, enabling some prediction on the stage of the growth cycle at which mycosin-1 may be produced. Western blot analysis revealed that mycosin-1 was present in *M. tuberculosis* whole cell protein extracts throughout the growth curve, with the highest expression levels detected in 1-week cultures (Figure 5.12a).

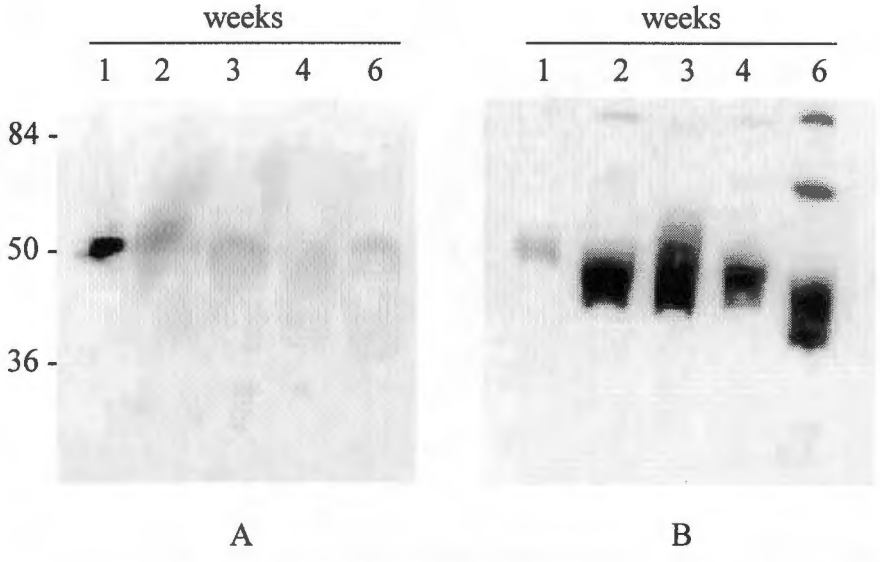


Figure 5.12. Detection of mycosin-1 in whole cell protein extracts and culture filtrates of *M. tuberculosis*. Proteins were isolated from (A) *M. tuberculosis* bacteria and (B) culture filtrates at various stages of growth. An equal amount of protein was added to each lane and separated by electrophoresis in a 10%SDS-poyacrylamide gel, transblotted onto a nitrocellulose membrane and subjected to Western blot analysis using affinity-purified anti-mycosin-1 antiserum as the primary antibody. The numbers on the left represent molecular weights in kDa.

Culture filtrates prepared from samples taken throughout the growth cycle contained mycosin-1, with a marked increase after the 2nd week of growth (Figure 5.12b). A decrease in molecular weight of mycosin-1 was noted in the filtrates from 2-week and 3-week cultures and a further decrease in the 6-week culture.

5.3.4 DISCUSSION

Expression of *M. tuberculosis* genes in *M. smegmatis* has met with great success. Investigators have demonstrated that *M. smegmatis* is able to recognise *M. tuberculosis* transcription and translation signals and can express *M. tuberculosis* proteins that are identical to their homologously expressed counterparts (Harth *et al.*, 1997). Furthermore, these proteins have been shown to be functionally active (Zhang *et al.*, 1991). Expression of mycosin-1 in *M. smegmatis* was consistent with this trend and the expressed protein was identical in size to the native protein in *M. tuberculosis*. However, despite repeated attempts using a variety of potential activation conditions - including treatment with acid, heat and trypsin - proteolytic activity could not be detected. Since most bacterial extracellular proteases, and especially subtilisins, express prodomains that must be cleaved for activation, this is likely true also for mycosin-1. However, the necessary conditions required for this cleavage have not been established.

The subtilisin protease family exhibit a wide spectrum of substrate specificity, ranging from the bacterial subtilisins that generally possess a broad substrate specificity to the mammalian subtilisin-like proprotein convertases (for example, human furin) that show specificity for multi-basic sites (Siezen *et al.*, 1994). In addition to preferring specific substrate cleavage sites, the convertases are usually insensitive to the typical serine protease inhibitors TLCK and TPCK, and their activity is augmented by Ca^{2+} (Hatsuzawa *et al.*, 1992). This was also a feature of the proteolytic activity detected in *M. tuberculosis* culture filtrates, suggesting that the filtrate activity may stem from a subtilisin-like protease that is between the primitive bacterial subtilisins and the highly diversified eukaryotic convertases in the evolutionary process. Thus, mycosin-1 may be a highly specific protease for which the appropriate substrate has not yet been identified, providing an alternative explanation for the inability to detect activity.

It has been well documented that during growth *M. tuberculosis* sheds envelope components into the medium (Ortalo-Magné *et al.*, 1995). Thus, the presence of mycosin-1 in *M. tuberculosis* culture filtrates is likely due to shedding rather than active secretion, although the precise mechanism remains to be elucidated. Interestingly, the molecular weight of mycosin-1 decreases from ~50 kDa in 1-week filtrates to ~45 kDa in 2-4-week filtrates to ~40 kDa in 6-week filtrates. Although there is a smear of protein in each lane in Figure 5.12, this is likely a result of overloading. When less protein was added a discrete band at ~50 kDa and ~39 kDa was observed (see wk6 sample in Figure 7.2). As mycosin-1 without the putative propeptide has a calculated molecular weight of ~39 kDa, the 40-kDa band may represent cleaved, active protease. Consistent with this was the detection of a proteolytic activity in *M. tuberculosis* culture filtrates that was inhibited by serine protease inhibitors. Furthermore, the proteolytic activity detected in *M. tuberculosis* culture filtrates increased after 3 weeks of growth. If activation of mycosin-1 is autocatalytic and intramolecular, which would be similar to the *B. subtilis* subtilisins (Power *et al.*, 1986), then it is possible that by 6 weeks of growth, as more and more active mycosin-1 accumulated, the majority of mycosin-1 would be present in its mature active form, as is suggested by the decreasing size of the mycosin-1 immunoreactive band on Western blots of filtrates from older *M. tuberculosis* cultures. Since attempts to purify proteases from *M. tuberculosis* culture filtrates were unsuccessful, the source of the serine protease activity remains to be elucidated.

In conclusion, mycosin-1 has been expressed in *M. smegmatis* and detected in *M. tuberculosis* whole cells and culture filtrates. Both recombinant and wild-type mycosin-1 are the same molecular weight. To date, no activity has been demonstrated for the 50-kDa forms of both recombinant and wild-type mycosin-1. The appearance of a 40-kDa form of mycosin-1 in 6-week *M. tuberculosis* culture filtrates may represent propeptide cleavage and activation. Interestingly, this corresponds to the appearance of a

serine protease activity in *M. tuberculosis* culture filtrates, although it could not be definitively demonstrated that mycosin-1 was responsible for this activity. Earlier attempts at purifying proteases from *M. tuberculosis* culture filtrates were unsuccessful.

However, in light of the data presented in this chapter, renewed attempts at purifying these proteases are currently underway.

CHAPTER 6

Localisation of mycosin-1

6.1 INTRODUCTION

Localisation of molecules within *M. tuberculosis* has been hampered by the complex structure of the cell envelope. The high lipid and carbohydrate content of the mycobacterial cell envelope has challenged conventional visualisation techniques, necessitating improved protocols for accurate visualisation of structures.

Electron microscopy has assisted researchers in appreciating the structural elements of the mycobacterial cell envelope. However, the various techniques have provided contradictory images, further adding to the complexity and mystique surrounding the ultrastructural features of the mycobacterial cell envelope.

Conventional electron microscopy techniques make use of dehydration steps that result in the collapse of structures, especially those containing carbohydrates. Since carbohydrates are integral components of the mycobacterial cell envelope, conventional electron microscopy provides distorted images (Daffé & Draper, 1998). The solvents used in section preparation solubilise and remove components of the cell envelope, further distorting the image. In addition, stains providing electron density for the major components of the mycobacterial cell envelope, namely carbohydrates and lipids, are few and their specificities are uncertain.

Preparation of electron microscopy samples using the freeze-substitution technique has vastly improved the ultrastructural study of the mycobacterial cell envelope (Graham & Beveridge, 1990). This technique results in minimal extraction of material by various solvents and maintains the integrity of structures.

Other techniques, including negative staining and freeze-fracture have contributed to the ultrastructural analysis of the mycobacterial cell envelope, but image distortion and questionable specificity are both potential shortcomings. Immunostaining is increasingly being used for the localisation of molecules within the mycobacterial cell envelope (Espitia *et al.*, 1992; Harth *et al.*, 1994). However, poor resolution and a limited range of available antibodies are current drawbacks of this technique.

It is very difficult to separate out the different components of the mycobacterial cell envelope, namely the intracellular, cell membrane, cell wall, and capsular fractions. Furthermore, currently there are no molecules that can accurately be localised to only one of the cell envelope fractions. Thus, assessment of the purity of each fraction after subfractionation is very difficult. Current subfractionation techniques employ differential centrifugation to separate out the different cell envelope fractions. This chapter describes the attempts made to localise mycosin-1, using differential centrifugation and electron microscopy techniques.

6.2 MATERIALS AND METHODS

6.2.1 Subcellular fractionation

The subfractionation technique is an adaptation of conventional techniques used in previous studies (Hirschfield *et al.*, 1990; Lee *et al.*, 1992; Ortalo-Magné *et al.*, 1995).

a) M. smegmatis-P1

M. smegmatis-P1 was grown in MB medium supplemented with OADC and hygromycin B (50 μg/ml). After 44 h growth at 37°C, the bacteria were pelleted by centrifugation at 3,000 x g for 10 min, washed twice in PBS, and then resuspended in PBS. Part of the pellet was used for electron microscopy (section 6.2.2a). The remaining part was sonicated (W-385 sonicator, Heat Systems-Ultrasonics, Inc; output level 3; 80% duty cycle) on ice for 3 periods of 4 min each, separated by 2-min cooling intervals. The sonicate was centrifuged at 1,000 x g for 30 min to remove debris and bacteria that resisted sonication. DNAse I (final concentration 0.2 mg/ml) and RNAse (final concentration 0.2 mg/ml) were added and the sonicate was left overnight at 4°C. The mixture was centrifuged at 20,000 x g for 30 min and the resulting pellet containing the cell walls was retained. The supernatant was then centrifuged at 100,000 x g for 3 h to separate the cell membranes from the intracellular components. The pellets containing the cell walls and cell membranes were resuspended in 0.5 ml 2% SDS and heated at 60°C for 2 h, after which insoluble components were removed by centrifugation. The intracellular fraction (the supernatant resulting from the 100,000 x g centrifugation) was concentrated 2-3 fold (Amicon spin columns; 10-kDa cut-off). All fractions were subjected to SDS-PAGE and Western blotting analysis using the affinity-purified anti-mycosin-1 antiserum (1:1,000) as the primary antibody (Appendix A.5).

b) *M. tuberculosis* GSH-3052

M. tuberculosis GSH-3052 was grown in Kirchner's medium, rotating at 37°C. After 3 weeks of growth the bacteria were pelleted by centrifugation at 3,000 x g for 20 min, washed twice in PBS, heat-killed at 85°C for 45 min and then resuspended in PBS. Sonication and subfractionation was performed as for *M. smegmatis*-P1 (section 6.2.1a). All samples were subjected to SDS-PAGE and Western blotting analysis using affinity-purified anti-mycosin-1 antiserum (1:1,000) as the primary antibody (Appendix A.5).

6.2.2 Electron microscopy

a) M. smegmatis

A portion of the bacterial cell pellet generated in section 6.2.1a was resuspended in PBS, incubated with 1% BSA in PBS for 2 min and then with affinity-purified anti-mycosin-1 antiserum (diluted 1:30 in PBS containing 1% BSA) overnight at room temperature. After two washes in PBS, the bacteria were fixed overnight in 2% paraformaldehyde/0.05% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). After fixing, the bacteria were embedded in 2% low melting point agarose, sucrose infiltrated by serial passage through a 50% (w/v) to 100% (w/v) sucrose gradient and then cryosectioned at -100 to -110°C. Ultrathin sections were placed onto formvar-coated nickel grids and each grid was floated on a droplet of PBS to allow for the diffusion of sucrose. Non-specific binding was blocked by floating the grids on droplets of 0.02 M glycine (3 x 1min) and then 1% BSA in PBS (5 x 1 min). After blocking, the grids were incubated with goat anti-rabbit IgG conjugated to 5nm-diameter gold particles at room temperature for 2 h. Thereafter, the grids were washed by floating on droplets of 1% BSA (10 x 1min) and PBS (5 x 1 min) and postfixed by floating on a droplet of 1% gluteraldehyde for 5 min. After washing with PBS (2 x 5 min) and sdH_2O (5 x 1 min), the sections were stained with

2% uranyl acetate/methyl cellulose (1:9) for 2 x 5 min. The grids were retrieved with a wire loop, gently dragged across Whatman filter paper to remove the staining solution, and air-dried for 10 min. They were then viewed by transmission electron microscopy. *M. smegmatis*-P19 bacteria were prepared as described above and were used to control for non-specific binding to anti-mycosin-1 antiserum (negative control).

b) *M. tuberculosis*

A portion of the bacterial cell pellet generated in section 6.2.1b was incubated with 1% BSA in PBS for 2 min and then incubated with affinity-purified anti-mycosin-1 antiserum (diluted 1:30 in PBS containing 1% BSA) overnight at room temperature. After washing with PBS, the bacteria were fixed in 2% paraformaldehyde/0.05% gluteraldehyde overnight at room temperature. Cryosectioning, immunogold-labelling and staining were performed as in section 6.2.2a. *M. tuberculosis* GSH-3052 bacilli were also labelled with affinity-purified pre-immune antiserum prior to fixation and were used to control for non-specific binding (negative control).

6.3 RESULTS

6.3.1 Subcellular fractionation

Subfractionation of *M. smegmatis*-P1 and *M. tuberculosis* cell lysates into wall, membrane and intracellular fractions indicated that mycosin-1 was predominantly present in the cell wall fraction (Figure 6.1). Minimal mycosin-1 was present in the membrane fraction of *M. smegmatis*-P1, but equivalent amounts were present in the membrane and wall fractions of *M. tuberculosis*. This result supports the prediction from the primary sequence that mycosin-1 may be a cell-envelope protein (contains multiple hydrophobic regions and a possible C-terminal transmembrane domain with a membrane anchor).

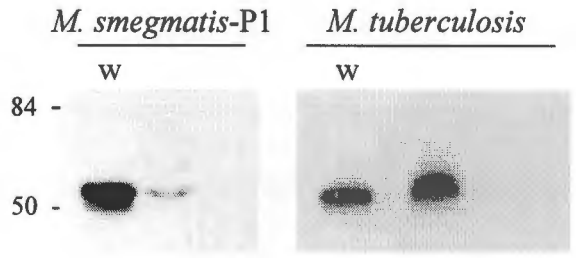


Figure 6.1. Subcellular fractionation of *M. smegmatis*-P1 and *M. tuberculosis*. Whole cell lysates were subfractionated into wall (w), membrane (m) and intracellular (i) fractions by differential centrifugation. The fractions were resolved on a 10% SDS-polyacrylamide gel, transblotted onto a nitrocellulose membrane and subjected to Western blotting analysis using affinity-purified anti-mycosin-1 antiserum as the primary antibody. The numbers on the left represent the molecular weights in kDa.

6.3.2 Electron microscopy

Using the cryosection immunogold technique abundant specific staining for mycosin-1 was detected in the cell envelope in *M. smegmatis*-P1 (Figure 6.2B) and to a lesser degree in *M. tuberculosis* (Figure 6.2D). Poor labelling was obtained when the bacteria were incubated with anti-mycosin-1 antiserum after fixation suggesting that fixative may alter the antigenicity of mycosin-1. To overcome this potential problem, whole bacteria were first labelled with anti-mycosin-1, then fixed and the cryosectioned. This way, mycosin-1 present in the cell envelope only would bind to anti-mycosin-1. Thus, any intracellular gold-labelling was considered to be non-specific. Clumping of *M. tuberculosis* bacilli presented major technical difficulties, hence mycosin-1 was mainly detected on bacilli at the edge of a clump. The negative controls, *M. smegmatis*-P19 pre-labelled with affinity-purified anti-mycosin-1 antiserum (Figure 6.2A) and *M. tuberculosis* pre-labelled with pre-immune antiserum (Figure 6.2C), were both negative.

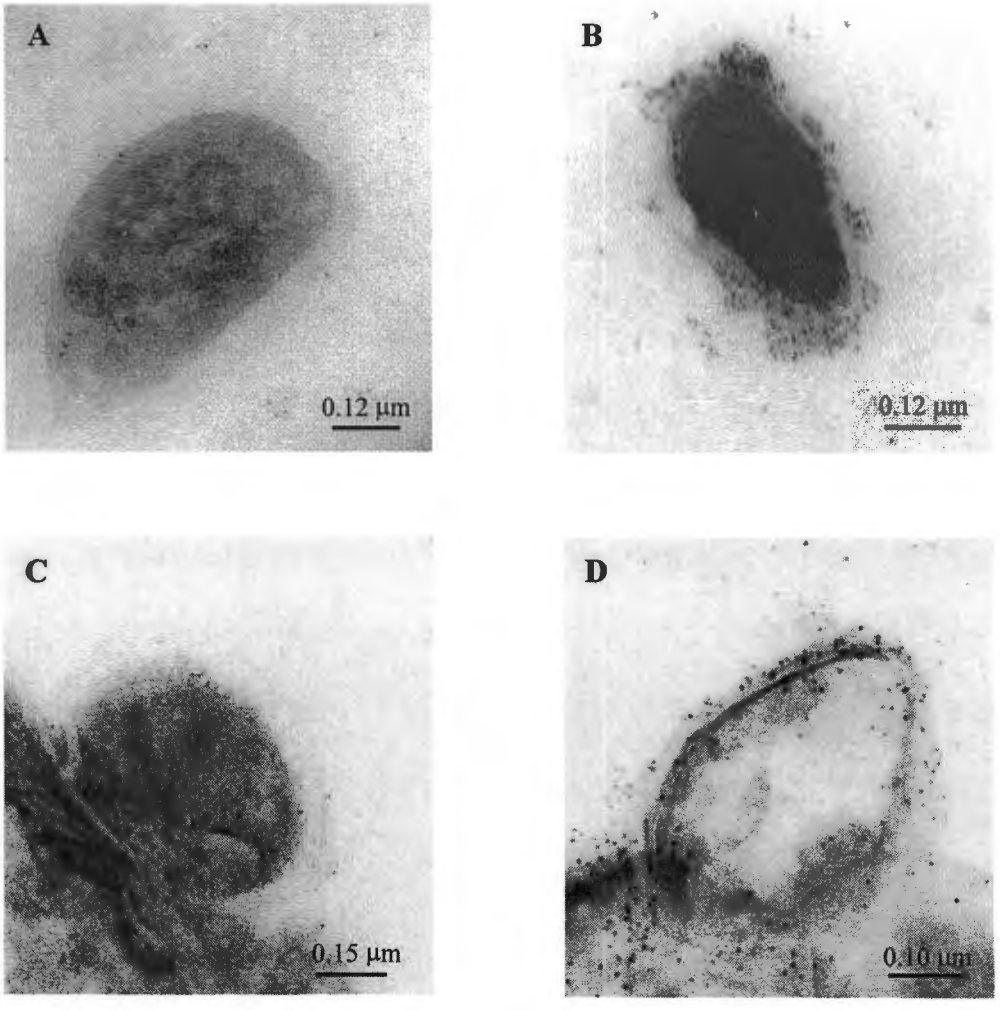


Figure 6.2. Localisation of mycosin-1 using electron microscopy. A) *M. smegmatis*-P19 (magnification x 49,500), B) *M. smegmatis*-P1 (magnification x 49,500) and D) *M. tuberculosis* (magnification x 100,000) were labelled with affinity-purified anti-mycosin-1 antiserum and then fixed. Thin sections, prepared using cryoelectronmicroscopy techniques, were labelled with goat anti-rabbit IgG conjugated to 5 nm-diameter gold particles. After staining, the sections were viewed by transmission electron microscopy. C) *M. tuberculosis* (magnification x 100,000) labelled with preimmune serum serve as a negative control.

6.4 DISCUSSION

Knowing the location of a protease may assist in postulating its possible functions. For example, proteases located on the cell surface are more likely to be involved in

virulence as it is from this position that they have direct access to host proteins and an intracellular protease is more likely to be involved in "house-keeping"/regulatory functions than a secreted protease. Therefore, an analysis of the subcellular location of mycosin-1 was undertaken. Cell wall, membrane and intracellular fractions of *M. smegmatis*-P1, *M. smegmatis*-P19 and *M. tuberculosis* were prepared using a combination of chemical extraction and centrifugation steps. These fractions were analysed by Western blotting, which revealed that in both *M. smegmatis*-P1 and *M. tuberculosis* mycosin-1 was localised to the cell envelope and was undetectable in the cytoplasm. In *M. tuberculosis* there appeared to be an equal distribution of mycosin-1 in the cell wall and cell membrane, although the methods used were not quantitative. Interestingly, in *M. smegmatis*-P1, mycosin-1 was almost entirely localised to the cell wall. This may suggest, firstly, that the native protein is processed and transported in a different manner to the recombinant protein; secondly, it may be a reflection of a more advanced stage in its transport, possibly a consequence of *M. smegmatis* being a faster grower than *M. tuberculosis*; and finally, it may reflect a greater tendency for the recombinant protein to be shed from the cell membrane in this organism

Electron microscopy techniques often provide distorted pictures. For this study ultrastructural clarity was not of prime importance. Rather, techniques providing good immunolabelling were considered. Preparation of EM sections using resin was not considered as resin may block epitopes, resulting in poor immunogold-labelling. In addition, the solvents and chemicals used in the sample preparation result in structural collapse and are not associated with good immunolabelling. The favoured technique for immunolabelling is cryoelectron microscopy. Using this technique, specific labelling of mycosin-1 was observed in the cell envelope of *M. smegmatis*-P1 and *M. tuberculosis*. In *M. smegmatis*-P1, intense staining was found in a loose outer layer of the envelope, which may be equivalent to the capsular layer that has been recently characterised (Daffé and

Draper, 1998). However, the resolution achieved with the immunogold technique used here is not sufficient to localise mycosin-1 immunoreactivity to a particular envelope layer with any degree of confidence. Significantly, no labelling was present in the control samples, *M. smegmatis*-P19 labelled with affinity-purified anti-mycosin-1 antiserum and *M. tuberculosis* labelled with affinity-purified pre-immune serum.

During the subfractionation technique it is often useful to use markers specific for the various fractions as an indication of the purity of each fraction. This is often achieved by detecting an enzyme activity specific for a certain fraction or using an antibody that binds to a protein specific for a certain fraction. However, for *M. tuberculosis* this has proven difficult. There are a few *M. tuberculosis* enzymes that can be used to assess the purity of the cytoplasmic and cell envelope fractions. Unfortunately, this approach could not be used with *M. tuberculosis* in this experiment as the bacilli were heat-killed at 85°C for 45 min, thus denaturing most enzymes and rendering them inactive. In addition, to date, there is a shortage of antibodies that are directed towards a protein specific for a certain fraction.

Nevertheless, the subfractionation and electron microscopy data are consistent with the presence of an export-directing signal sequence at the N-terminus of mycosin-1, as well as the presence of multiple hydrophobic domains in the C-terminal half, one or more of which could serve as membrane anchors. Hence, it is reasonable to conclude that mycosin-1 is an exported cell envelope-associated protein that performs an extracytoplasmic function. The nature of that function is unknown. It is intriguing that mycosin-1 is one member of a family of five highly homologous subtilisin-like proteases each with similar hydrophobic N- and C-terminal domains. It is tempting to speculate that these putative proteases perform a vital subcellular function that requires five related but presumably non-redundant proteins.

CHAPTER 7

Detection of mycosin-1 *in vivo*

7.1 INTRODUCTION

The wisdom in studying *M. tuberculosis* shaking (or stationary) in artificial media in the laboratory has long been debated. It has been recognised that *in vivo* conditions are likely to be very different, suggesting that experimental data obtained *in vitro* may not be applicable to the *in vivo* scenario. Early studies showed a biochemical difference between *in vivo* and *in vitro* cultivated *M. tuberculosis* bacilli (Segal & Bloch, 1956). In addition, there was also a difference in their pathogenicity for mice, suggesting that *in vivo* factors may be required for the expression of some virulence determinants (Segal & Bloch, 1957). More recent studies have shown that at least six proteins are only expressed *in vivo*, emphasising the necessity for the study of *in vivo* expression patterns of proteins (Lee & Horwitz, 1995). However, the study of *M. tuberculosis in vivo* using an animal model requires specialised equipment and facilities, both of which are costly and require expertise and regular maintenance. Therefore, many laboratories make use

of cultured cells infected with *M. tuberculosis* to obtain *in vivo* data. Whether this truly reflects the scenario within tuberculous tissues is debatable. However, it does provide a vehicle that approximates *in vivo* conditions, thus allowing for more accurate speculation on expression patterns within *M. tuberculosis*-infected tissues.

Mycosin-1 is likely expressed with a propeptide that requires cleavage for the generation of the mature active protease. Propeptide cleavage may be autocatalytic, as is the case with the *B. subtilis* subtilisins (Power *et al.*, 1986), or via another protease, either bacterially derived or from the host. It has been suggested that proteases are expressed in an inactive proform to regulate proteolysis and, possibly, to direct activity to a specific location. Activation occurs when the protease activity is required or when the protease reaches the correct location. Since it appears that mycosin-1 is inactive *in vitro*, it is possible that the factors required for activation are to be found *in vivo*. If this were the case, it would be similar to the *B. amyloliquifaciens* subtilisin that was only present as a preproprotein when produced by coupled transcription-translation and it was thus suggested that *in vivo* factors may be rquired to initiate processing and production of active enzyme (Power *et al.*, 1986).

This chapter details the attempts made to express and detect active mycosin-1 *in vivo*, using cultured macrophage-like cells and a mouse-model.

7.2 MATERIALS AND METHODS

7.2.1 Culture of P3889D_1 macrophage-like cells

P3889D_1 cells, a mouse macrophage-like cell line, were cultured as described (Haylett & Thilo, 1986). Briefly, P3889D_1 cells were grown and maintained at 37°C in 5% $C0_2$ and in medium (RPMI-1640) supplemented with 10% heat-inactivated FCS and penicillin/streptomycin. The cells were grown to confluency in tissue culture flasks (175 cm^2, Nunc flat bottom flasks) and the medium was changed every 48 h. After 4 days of growth, the medium, containing floating P3889D_1 cells from 3 confluent tissue culture dishes, was collected. The cells were pelleted by centrifugation at 1,200 x g, resuspended in fresh medium, and replated into 6-well tissue culture plates at a cell density of 4 x 10^6 cells/well. The plates were incubated overnight to allow for adherence of the cells to the bottom of the well.

7.2.2 Preparation of *M. tuberculosis* GSH-3052 and H37Rv

M. tuberculosis GSH-3052 and H37Rv bacteria were prepared as detailed in section 4.2.3. The bacteria were harvested after 3 weeks growth, pelleted by centrifugation at 3,000 x g for 20 min, and resuspended in PBS to a turbidity corresponding to McFarland's standard 3 (9 x 10^8 bacteria/ml).

7.2.3 RT-PCR detection of *mycP1* RNA in macrophage-grown *M. tuberculosis*.

J774.2 macrophages, grown and maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), were synchronously infected (Schlesinger *et al.*, 1990) with non-opsonized *M. tuberculosis* H37Rv at a ratio of 10 bacilli/cell. After 4 h, infected monolayers were washed three

times with Hanks' balanced salt solution to remove non-phagocytozed bacteria, refed with fresh growth medium, and incubated for 15 h at 37°C in 5% CO_2. Intracellular bacteria were then harvested by differential lysis using guanidine thiocyanate (Butcher *et al.*, 1998). Control bacteria consisted of *M. tuberculosis* bacilli grown in Middlebrook 7H9 medium supplemented with 10% OADC and 0.02% (v/v) Tween-80. RNA was extracted from the intracellular and broth-grown bacteria by the method of Mangan *et al.* (1997). RNA samples were confirmed to be free of contaminating DNA by performing polymerase chain reaction (PCR) amplification on the samples using primers that amplify a 275-bp fragment of the *M. tuberculosis dna*K gene (Patel *et al.*, 1991). The RNA samples were then subjected to reverse transcriptase (RT)-PCR amplification using primers (5′-TAC GCA CCG GAG AAC AGT-3′ and 5′-TAA CGG ACC TCC GAA GTG-3′) that amplified a 238-bp fragment of the *mycP1* gene (encoding amino acid residues 149-226, see Figure 4.2). In each case, 1 μg of RNA was reverse transcribed with Superscript RNase H-RT (Life Technologies, Paisley, United Kingdom) according to the manufacturer's instructions, 1/10 of the cDNA was then PCR amplified under standard conditions, and the products were resolved by electrophoresis on a 1% agarose gel. A Southern blot of the gel was performed by transfer onto a nylon membrane and hybridization with a PCR-generated probe (using the RT-PCR primers, above) labeled with the ECL detection system (Amersham, UK); hybridization, washing, ECL substrate reaction, and autoradiography were performed as specified by the manufacturer.

7.2.4 Infection of P3889D_1 macrophage-like cells

P3889D_1 cells prepared in section 7.2.1 were washed with PBS, and fresh RPMI 1640 medium supplemented with 10% heat-inactivated FCS was added. No antibiotic

was added. *M. tuberculosis* GSH-3052 bacteria [225 µl of a McFarland's standard 3 dilution (9×10^8 bacteria/ml)] were added to each well to obtain a bacteria:cell infection ratio of 50:1. The tissue culture plates were gently agitated to evenly distribute the bacteria, and incubated at 37°C in 5% CO_2 for 5 h. The cells were then gently washed with PBS to remove non-adherent bacteria, fresh medium (with no antibiotic) was added, and the plates were incubated at 37°C in 5% CO_2 for 3 days. Fresh medium was added and the plates reincubated for a further 2-3 days.

7.2.5 Detection of mycosin-1 in P3889D1 cells infected with *M. tuberculosis* GSH-3052

After 5-6 days incubation, the cells were detached in 5 mM EDTA, sonicated (W-385 sonicator, Heat Systems-Ultrasonics, Inc; output level 3; 80% duty cycle) on ice for 3 bursts of 4 min each, separated by 2-min cooling intervals, and centrifuged at 100,000 × g for 3 h. The pellet (containing bacterial envelope components, bacterial debris, cell membrane components, and cellular debris) was resuspended in SDS loading buffer. The supernatant (containing intracellular components) was concentrated 2-3 fold and SDS loading buffer was added. The pellet and supernatant samples were boiled at 100°C for 5 min, resolved on a 10% SDS-polyacrylamide gel (Appendix A.5), and then analysed by Western blotting using affinity-purified anti-mycosin-1 antiserum (1:1,000) as the primary antibody (Appendix A.5). Controls included uninfected P3889D_1 cells (negative control) and *M. smegmatis*-P1 lysates (positive control for mycosin-1 detection by Western blotting, prepared in section 5.3.2.6).

7.2.6 Infection of mice with *M. tuberculosis* GSH-3052

BALB/c mice were infected intravenously with 1 x 10^6 *M. tuberculosis* GSH-3052 bacilli. After 14 days, 21 days, 28 days and 60 days of infection, the mice were euthenased using a mixture containing ketamine (10%)(44mg/kg) and xylazine (0.25%)(5mg/kg) in sterile water. For each time-point an uninfected mouse was also euthenased. The lungs, liver and spleen from each mouse were harvested and each organ was divided in half. One half was rapidly frozen in liquid nitrogen and stored at -70°C, and the other half was fixed in 10% neutral buffered formalin for 48 h and then processed through to wax using standard methods. Sections 3 μm thick were cut, mounted on APES-coated slides and incubated at 56°C for 1 h. For histological assessment, the sections were stained with the standard stains of hematoxylin and eosin (H & E) and Ziehl-Neelsen (ZN). For immunohistochemical assessment, standard techniques were followed using affinity-purified anti-mycosin-1 antiserum as the primary antibody. Detection systems used were avidin-biotin (DAB was used as substrate) and FITC-labelled goat anti-rabbit IgG. To control for non-specific binding, samples were also labelled with affinity-purified preimmune serum.

7.3 RESULTS

7.3.1 Expression of the *mycP1* gene during growth of *M. tuberculosis* in macrophages.

Following a 4-h infection and 15 h of growth in J774 macrophage-like cells, intracellular *M. tuberculosis* bacilli were harvested and the extracted RNA was subjected to RT-PCR with *mycP1*-specific primers. By agarose gel electrophoresis, an expected 238-bp product was clearly observed for the RNA derived from the intracellular bacteria

but not for the broth-grown bacteria (Figure 7.1A). The 238-bp product was specific for the *mycP1* gene, as determined by Southern blotting, which also revealed faint bands in the lanes of broth-grown bacteria (Figure 7.1B). Although these data were suggestive of upregulation of *mycP1* expression during intracellular growth, this could not be formally concluded as this was not a quantitative RT-PCR assay.

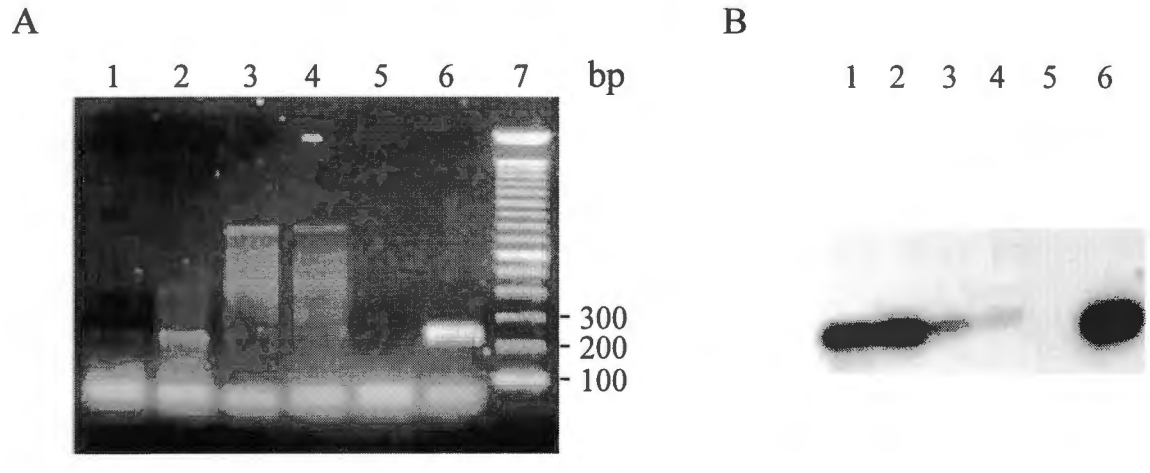


Figure. 7.1. Expression of the *M. tuberculosis mycP1* gene during growth in macrophages. Intracellular bacteria were harvested from infected J774 macrophages, bacterial RNA was extracted and subjected to RT-PCR with *mycP1*-specific primers, and the 238-bp products were resolved by agarose gel electrophoresis (A) and analyzed by Southern blotting (B) with an ECL-labeled probe. Lanes: 1, RNA from intracellular bacteria; 2, RNA from intracellular bacteria, diluted 1:2; 3, RNA from broth-grown bacteria; 4, RNA from broth-grown bacteria, diluted 1:2; 5, negative PCR control (no DNA); 6, positive PCR control (*M. tuberculosis* genomic DNA); 7, molecular weight marker.

7.3.2 Detection of mycosin-1 in *M. tuberculosis*-infected P3889D$_1$ cells

Equal amounts of protein [determined using the BioRad protein assay (BioRad Laboratories, Richmond, California), as per manufacturer's instructions] from the pellet and supernatant samples were resolved by 10% SDS-PAGE, transblotted onto nitrocellulose membranes, and probed with affinity-purified anti-mycosin-1 antiserum.

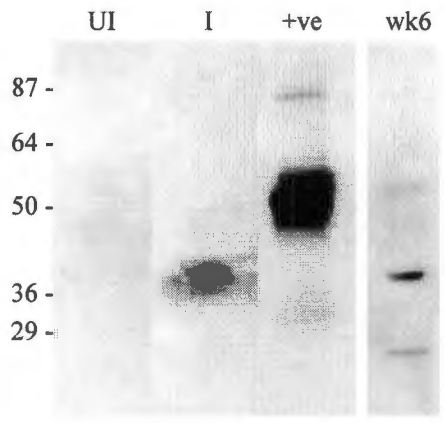


Figure 7.2. Expression of mycosin-1 during growth of *M. tuberculosis* in macrophages. Lysates of infected P3889D$_1$ cells were centrifuged at 100,000 x g and the pellets were resuspended in SDS buffer, resolved by 10% SDS-polyacrylamide gel electrophoresis, and analysed by Western blotting using affinity-purified anti-mycosin-1 antiserum as the primary antibody. Lanes: UI, uninfected macrophages; I, infected macrophages; +ve, *M. smegmatis*-P1 lysate (positive control); wk6, 6-week *M. tuberculosis* culture filtrate. The numbers on the left are molecular weights in kDa. This figure is a composite from two separate experiments.

A 40-kDa immunodominant band was observed in whole cell protein extracts of *M. tuberculosis*-infected P3889D$_1$ cells (Figure 7.2). This corresponded to an immunodominant band observed in 6-week *M. tuberculosis* culture filtrates. Furthermore, no bands were observed in whole cell extracts of uninfected P3889D$_1$ cells as well as high-speed supernatant samples (containing intracellular contents) of uninfected and infected P3889D$_1$ cells.

7.3.3 Infection of mice with *M. tuberculosis* GSH-3052

ZN staining of lung and liver tissue from infected mice revealed numerous acid fast bacilli (Figure 7.3B and 7.4B), confirming a significant level of infection. Histological examination of the *M. tuberculosis*-infected lung tissue from 14-21 days infection showed areas of non-specific inflammation (data not shown). After 21 days of infection there was an increasing number of inflammatory cells and occasional ill-defined

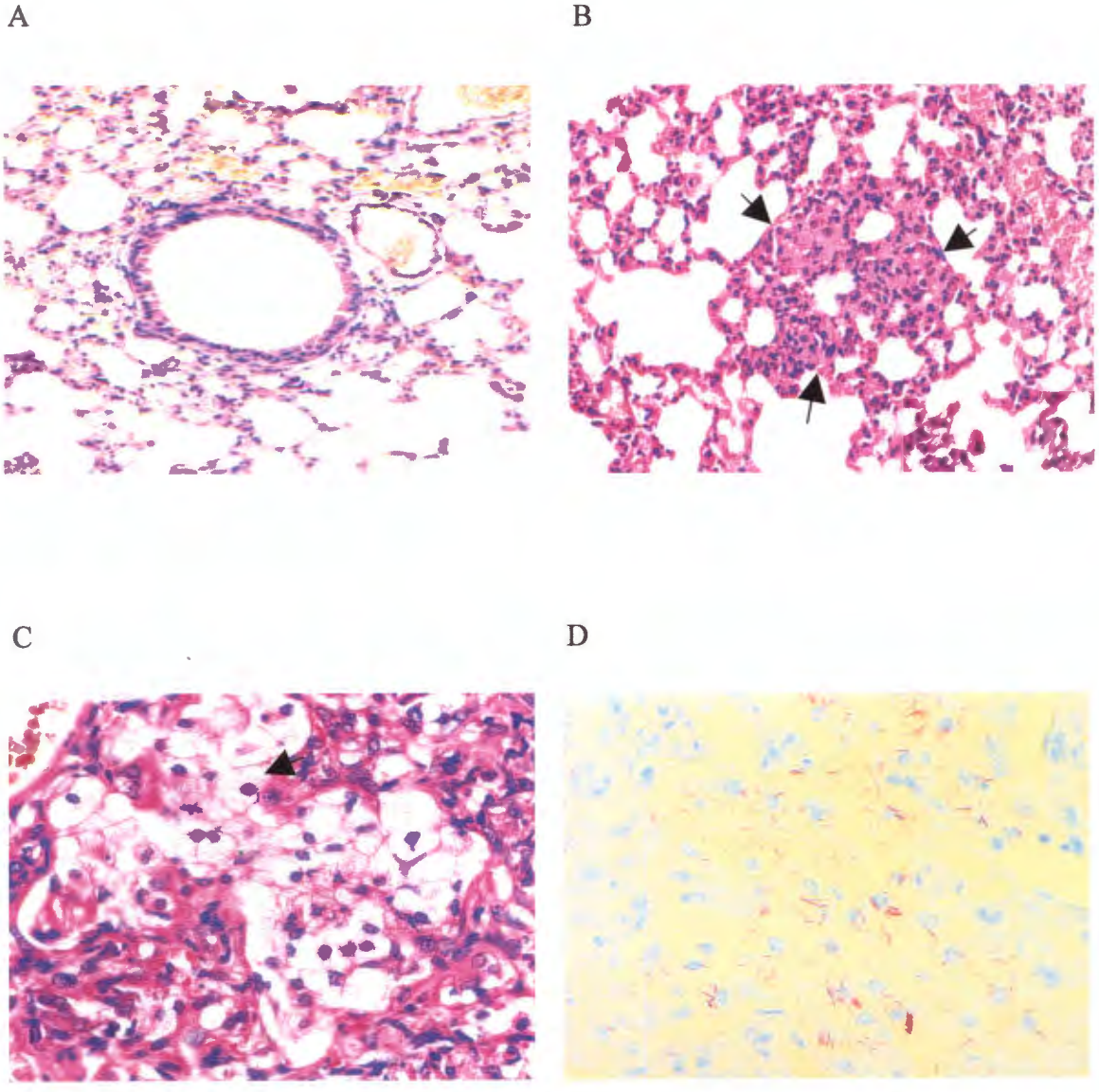
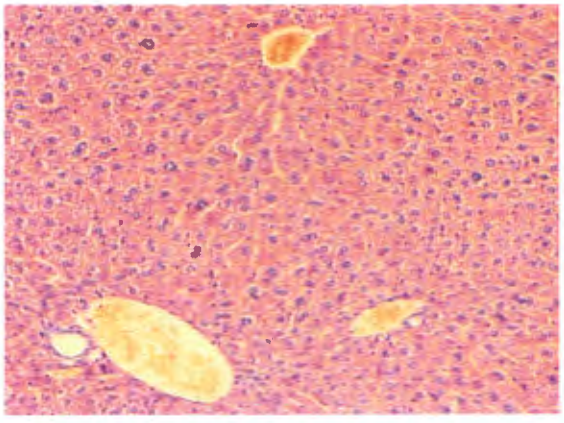
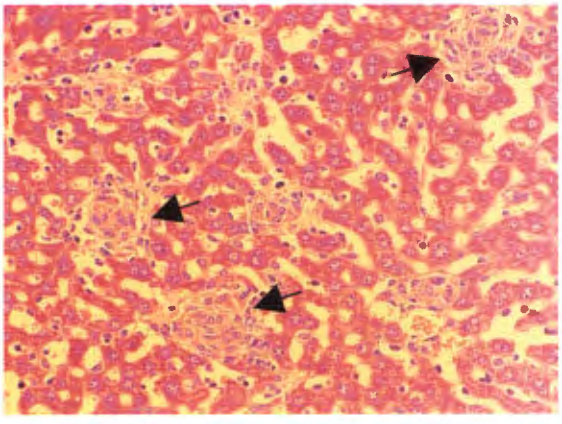


Figure 7.3. Lung tissue sections from control and *M. tuberculosis*-infected mice. BALB/c mice were infected intravenously with *M. tuberculosis* GSH-3052 and euthenased after 28 days of infection. A) Section of lung tissue from a control (uninfected) mouse showing normal tissue morphology. H & E stain, 200 x magnification. B) Section of lung tissue from an *M. tuberculosis*-infected mouse showing an ill-defined non-caseating epithelioid granuloma (arrows) containing epithelioid macrophages surrounded by lymphocytes and inflammatory cells. H & E stain, 200 x magnification. C) Section of lung tissue from an *M. tuberculosis*-infected mouse showing an ill-defined granuloma containing aggregates of foamy macrophages and inflammatory cells. H & E stain, 400 x magnification. D) Section of lung tissue from an *M. tuberculosis*-infected mouse showing numerous acid-fast bacilli (pink) that are scattered through large sections of tissue. ZN stain, 200 x magnification.

A



B



C

Figure 7.4. Liver tissue sections from control and *M. tuberculosis*-infected mice. BALB/c mice were infected intravenously with *M. tuberculosis* GSH-3052 and euthenased after 28 days of infection. A) Section of liver tissue from a control (uninfected) mouse showing normal tissue morphology. H & E stain, 200 x magnification. B) Section of liver tissue from an *M. tuberculosis*-infected mouse showing typical non-caseating epithelioid granulomas (arrows) containing epithelioid macrophages surrounded by lymphocytes and inflammatory cells. H & E stain, 200 x magnification. C) Section of liver tissue from an *M. tuberculosis*-infected mouse showing numerous acid-fast bacilli (pink) that appear to be localised to discrete lesions. ZN stain, 200 x magnification.

epithelioid granulomas (arrows in Figure 7.3B). At 60 days of infection numerous ill-defined non-caseating epithelioid granulomas were seen containing aggregates of foamy macrophages surrounded by numerous lymphocytes and inflammatory cells (Figure 7.3C). The formation of ill-defined granulomas is typical of *M. tuberculosis*-induced lung pathology in mice, especially when infected with an intravenous inoculum (North, 1995; Rhoades *et al.*, 1997). In contrast, the *M. tuberculosis*-infected liver tissue revealed typical non-caseating epithelioid granulomas (arrows in Figure 7.4B). Histological examination showed epithelioid macrophages surrounded by numerous lymphocytes and other inflammatory cells. No Langhans giant cells were seen. There was no caseous necrosis in any sections of the *M. tuberculosis*-infected mice tissue, consistent with the findings presented in the literature (North, 1995; Rhoades *et al.*, 1997). ZN staining of spleen tissue sections from each infected mouse revealed numerous acid-fast bacilli and histological examination showed typical non-caseating epithelioid granulomas (data not shown). Thus, the 3 organs examined showed features of active tuberculous disease.

7.3.4 Detection of mycosin-1 in *M. tuberculosis*-infected mice

Tissue sections as above were probed with anti-mycosin-1 antibodies and binding of these antibodies to mycosin-1 was detected using the avidin-biotin detection system or FITC-labelled goat anti-rabbit IgG. Faint staining for mycosin-1 was noted in the infected tissues, but also in the uninfected tissues (although less than in the infected tissues). A variety of antigen retrieval methods, including enzymatic digestion with trypsin, heat (HMAR), pressure cooking, etching with hydrochloric acid, and various combinations of the above, were unsuccessful in conclusively ruling out non-specific staining.

7.4 DISCUSSION

M. tuberculosis-infected macrophage-like cells were used to detect the intracellular expression of mycosin-1. Initial experiments (performed in collaboration with the Molecular Mycobacteriology Research Group, Division of Immunity and Infection, University of Birmingham, UK) on RNA isolated from broth-grown *M. tuberculosis* H37Rv and J774 macrophage-like cells infected with *M. tuberculosis* H37Rv revealed expression of *mycP1* in broth-grown *M. tuberculosis* H37Rv bacteria, as well as in the infected macrophages. In addition, these data were suggestive of upregulation of *mycP1* expression during intracellular growth, but these results were not unequivocal as the methods used were not quantitative.

The *B. amyloliquefaciens* subtilisin is only present as a preproprotein in *in vitro* transcription-translation experiments, suggesting that some structural, enzymatic, or cofactor function is missing *in vitro* that is necessary to initiate processing (Power *et al.*, 1986). Since it is well documented that intracellular residence of *M. tuberculosis* induces protein expression not evident during *in vitro* growth (Lee *et al.*, 1995), it is possible that a protein only expressed during *in vivo* growth, or other *in vivo* factors, may be responsible for the activation of mycosin-1. To address this hypothesis, it was decided to look for mycosin-1 expression in *M. tuberculosis*-infected macrophages using anti-mycosin-1 antibodies. The presence of mycosin-1 with a molecular weight of 38.67 kDa would be suggestive of signal peptide and propeptide cleavage (see section 4.2.4.1) and may suggest activation.

Mycosin-1 was only detected in whole cell lysates of *M. tuberculosis*-infected P3889D_1 cells and its molecular weight was noted to be ~40 kDa. Interestingly, this size corresponds to putative mature mycosin-1 after signal sequence and propeptide cleavage. In addition, the 40-kDa form of mycosin-1 was also observed in 6-week *M. tuberculosis*

culture filtrates. This smaller form has not been observed in any *M. smegmatis*-P1 fractions or in cell lysates of broth-grown *M. tuberculosis*. This likely suggests mycosin-1 activation (i.e., propeptide cleavage) *in vivo*, and also in stationary phase culture filtrates.

The presence of a potentially active form of mycosin-1 in stationary phase culture filtrates is in agreement with the proteolytic activity described in Chapter 3. Activity suggestive of a subtilisin-like protease was detected in low levels after 3 weeks of growth.

The likelihood for the *in vivo* expression of active mycosin-1 prompted a search for the presence of mycosin-1 in tuberculous tissue. To achieve this, and to confirm the virulence potential of the *M. tuberculosis* strain selected for use in this study (GSH-3052), mice were infected with *M. tuberculosis* GSH-3052 by intravenous inoculation and euthenased after various time periods of infection. The lungs, liver and spleen were harvested, and sections were prepared for histological assessment as well as for the detection of mycosin-1 expression in the infected tissues. Using standard staining techniques, all three organs were demonstrated to contain the pathological features of active tuberculosis, confirming the virulence of *M. tuberculosis* GSH-3052. After confirming that each organ contained active disease, it was decided to look for mycosin-1 in these infected tissues. As described previously (section 7.3.3), standard techniques as well as additional antigen retrieval techniques failed to show unequivocal evidence for the presence of mycosin-1 in the infected tissues. Altered antigenicity is a potential problem with most fixatives, especially formalin. In these experiments formalin was selected for its fixative qualities as well as its antimycobacterial effects. In addition, the mice organs were exposed to formalin for a lengthy period (48 hours). Perhaps the use of an alternative fixative with better antigen preservation qualities may improve the

detection of mycosin-1 in infected tissues. However, the use of formalin represents one of the many factors that may have to be altered in order to detect mycosin-1. The numerous technical difficulties inherent to the immunohistochemical techniques used, coupled with the difficulties experienced in working with *M. tuberculosis* and *M. tuberculosis*-infected tissues, precluded further work in this direction. However, this work serves as a foundation for future studies.

In conclusion, this chapter provides intriguing evidence for the processing and possible activation of mycosin-1 *in vivo*. Although the 40-kDa form of mycosin-1 detected within macrophages has not been demonstrated to be active, it is tempting to suggest that this is indeed the case. In this regard experiments have been planned to purify *in vivo* expressed mycosin-1 for detection and characterisation of activity as well as for N-terminal sequencing. It is unfortunate that unequivocal evidence for the expression of mycosin-1 in infected tissues could not be presented. However, future experiments have been planned to attempt to overcome the problems experienced. Although these experiments are potentially difficult and challenging, the unambiguous demonstration of active mycosin-1 in infected tissues would allow further speculation on the involvement of this protease in the tissue destruction characteristic of active tuberculous disease, and would provide a model system in which the effects of *M. tuberculosis* strains harboring mutated *mycP1* could be assessed.

Definitive factors conferring an intracellular survival advantage on *M. tuberculosis* have alluded scientists for many years. There is wide consensus that modulation of the phagosome is key to the survival and growth of pathogenic mycobacteria in macrophages. Maturation of the phagosome to a late compartment competent for fusion with lysosomes is inhibited, and hence pathogenic mycobacterial phagosomes do not acquire the characteristics of phagolysosomes (Clemens & Horwitz, 1995; De Chastellier

& Thilo, 1997; Russell *et al.*, 1997). It is unknown how this is accomplished, but it is presumed to involve both physico-chemical properties of the mycobacterial envelope and secreted factors (De Chastellier & Thilo, 1997; Russell *et al.*, 1997). It is not unreasonable to speculate that phagosome manipulation depends in part on proteolytic processing of bacterial or host proteins at the interface between the mycobacterial cell wall and the phagosome membrane. The mycosins and other *M. tuberculosis* proteases may be good candidates for further investigation in this regard.

CONCLUDING REMARKS AND FUTURE STUDIES

This study represents an initial characterisation of the first member of a new family of putative exported, membrane-associated, subtilisin-like serine proteases in *M. tuberculosis*. Mycosin-1 is an extracellular protein that is membrane and cell wall associated, as well as shed into the culture supernatant. The protein is expressed after infection of macrophages and is apparently subjected to proteolytic processing.

Thus far, proteolytically active mycosin-1 could not be produced recombinantly, but a serine protease activity consistent with a subtilisin-like enzyme was detected in *M. tuberculosis* culture filtrates. However, whether mycosin-1 contributes to the activity detected in *M. tuberculosis* culture filtrates remains to be elucidated. Although this study is not the first to detect protease activity in *M. tuberculosis*, it does provide the first definitive evidence for expression of a protease homologue *in vitro* and *in vivo*, and represents the first characterisation of a subtilisin homologue in *M. tuberculosis*.

Proteases are poorly studied in *M. tuberculosis*, which is surprising, considering that extracellular proteases frequently serve as virulence factors in bacterial pathogens. Moreover, a hallmark of pulmonary tuberculosis is extensive tissue necrosis, to which bacterial proteases could make an important contribution. Apart from direct tissue damage, *M. tuberculosis* proteases could be involved in a number of activities that are thought to be required for intracellular survival, including phagosome membrane modulation, intracellular nutrient acquisition, and immune system modulation; additionally, activation of host-derived matrix-metalloproteases could also be a mechanism contributing to tissue damage.

Whether the mycosins or other *M. tuberculosis* proteases are involved in virulence is unknown. However, their potential as virulence factors and novel drug targets warrants

further investigation. Since *M. bovis* BCG contains a homolgue of *mycP1* that is not expressed, it would be interesting to assess whether *M. bovis* BCG harbouring p19K-P1 is able to survive intracellularly and whether it would be able to cause disease in mice. In addition, future studies on mycosin-1 should include the determination of activation conditions, characterisation of the active protease (including substrate preferences), assessment of activity and N-terminal sequencing of the *in vivo* produced 40-kDa form, and assessment of the virulence of *M. tuberculosis* bacilli harbouring wild-type versus mutated *mycP1*.

Finally, the ability of *M. tuberculosis* to establish disease appears to be multifactorial. In addition, many microbes produce proteases that have been shown to be intimately involved in the ability of the microbe to establish disease. Hence, it seems likely that amongst the diversity of putative protease genes revealed in the *M. tuberculosis* genome sequence at least one will be shown to encode a protease that contributes to the pathogenicity of *M. tuberculosis*. Since definitive virulence factors have alluded *M. tuberculosis*-researchers for many years, it would seem that novel and relatively unstudied *M. tuberculosis* virulence targets should be sought. Proteases in *M. tuberculosis* are a prime example, and it is hoped that this study will trigger enthusiasm for the future study of many of the putative protease genes present in the *M. tuberculosis* genome.

APPENDIX A

A.1 Restriction endonuclease digestion

The methods used were an adaptation of those described by Sambrook *et al* (1989). Briefly, restriction endonuclease digests were performed in an eppendorf containing the appropriate restriction enzyme (3 u/μg DNA), the suggested restriction enzyme buffer (Boehringer Mannheim), plasmid DNA, and made up to a total volume of 20 μl with sdH_2O. The reactions were incubated at 37°C for 1-3 h. The digested products were separated by electrophoresis in a 0.8% agarose gel and then purified using NucleoSpin Columns (Macherey Nagel, Germany), as per manufacturer's instructions.

A.2 DNA ligation reactions

Ligation reactions were performed in a total volume of 20 μl per reaction. Each reaction contained: 1-2 units T4 DNA ligase (Boehringer Mannheim) per μg purified DNA, 10 x ligation buffer (Boehringer Mannheim), vector and insert DNA, and sdH_2O. The total concentration of plasmid DNA was 15 pmol/ml and contained an insert:vector ratio of 2:1. The ligation reactions were performed at room temperature for 9-16 h. To control for ligase activity, a sample of single-cut plasmid DNA was included as a positive control and double-cut (using two different restriction enzymes) plasmid DNA was included as a negative control.

A.3 Preparation and transformation of competent *E. coli* cells

The method used was a modification of the method described by Hanahan (1983). Briefly, one viable colony of *E. coli* XL1-Blue was picked from a freshly streaked agar plate, inoculated into 5 ml LB and incubated overnight at 37°C. This starter culture (1 ml) was used to inoculate 50 ml pre-warmed LB which was incubated at 37°C until an OD_{600} of 0.5. The bacteria were pelleted by centrifugation at 2,500 x g for 5 min at 4°C. To maintain the integrity of the cells all subsequent steps were performed at 4°C. The bacterial pellet was resuspended in ice-cold TFB 1 (100mM RbCl, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% (v/v) glycerol) and incubated on ice for 1-1.5 h. The bacteria were again pelleted by centrifugation at 2,500 x g for 5 min at 4°C and resuspended in ice-cold TFB 2 (10 mM MOPS, pH 7.0, 10 mM RbCl, 75 mM $CaCl_2$, 15% (v/v) glycerol). This mixture was divided into 100 µl aliquots and stored at -70°C.

Plasmid DNA (50-200 ng) was added to a 100 µl aliquot of competent cells, incubated on ice for 10 min, heat-shocked at 42°C for exactly 2 min and then placed directly on ice for 2 min. LB (400 µl) was added and the mixture incubated at 37°C for 60 min to allow for expression of the selective antibiotic. The transformed cells were then plated onto LA plates containing the appropriate selective antibiotic and incubated overnight at 37°C. Two additional samples containing competent cells with no added plasmid DNA and competent cells with uncut plasmid DNA were included to control for spurious transformation results and to monitor transformation efficiency, respectively.

A.4 Small-scale isolation of *E. coli* plasmid DNA

Plasmid DNA was extracted using an adaptation of the Ish-Horowicz and Burke method (1981). Briefly, one viable colony of transformed *E. coli* was grown at 37°C in 5 ml LB containing the appropriate selective pressure. Bacteria from 1.5 ml of this

culture were pelleted by centrifugation at 12,000 x g for 3 min. The pellet was resuspended in 50 µl TE buffer, 100 µl phenol/chloroform was added and the mixture vortexed for 3 min. Proteins were separated from the suspended DNA by centrifugation at 12,000 x g for 5 min and 50 µl of the clear top layer was carefully aspirated and transferred to a clean eppendorf tube. Eighteen microlitres NH_4Ac (7.5 M) and 140 µl ice-cold ethanol (100%) were added and the mixture incubated at –20°C for 15 min, to precipitate the DNA. The DNA was pelleted by centrifugation at 12,000 x g for 10 min, washed in 70% ethanol, air-dried for 15 minutes and resuspended in 25 µl TE buffer containing 1 µl Rnase (10 mg/ml).

A.5 SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gels were prepared and electrophoresed according to the method described by Laemmli (1970), using a mini-gel apparatus (BioRad Laboratories, Richmond, California). Equal amounts of protein (determined using the BioRad Protein Determination Kit, as per manufacturers instructions) was added to each lane. Electrophoresed proteins were transblotted (100 V) onto a hybond-C nitrocellulose membrane for 1 h at 4°C. All subsequent steps were performed at room temperature. The membrane was incubated in blocking buffer [PBS, 5% (w/v) skim milk, 0.1% (v/v) Tween-20] for 1-2 h, followed by a 1 h incubation with the primary antibody (diluted in blocking buffer). After washing in washing buffer [PBS, 0.1% (v/v) Tween-20] for 20 min, the membrane was incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG (ECL Western blotting kit, Amersham; 1:2,000 dilution in blocking buffer) for 1h and washed again in washing buffer for 15 min. The washed membranes were then treated with ECL detection reagents and exposed to photographic film, as recommended by the manufacturer.

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