The *Mycobacterium smegmatis* 'Proteome': Effect of Growth Phase on Total Protein Synthesis and on the Response to Heat Shock

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Medical Microbiology

Faculty of Medicine

UNIVERSITY OF CAPE TOWN

August 1998

For the African Renaissance…

Acknowledgements

I wish to thank my supervisor, Prof. Lafras Steyn, for having the foresight to initiate studies on Mycobacterial 'Proteomics' - an area that has now become the focus of attention in the field of tuberculosis research; for providing me with opportunities to attend both local and international conferences, thus enabling me to keep up with developments in relevant research fields; and for his constructive input and optimism throughout the duration of this project. I would also like to thank Dr. Harold Zappe for his advice during the preparation of this thesis.

I wish to thank Glaxo-Wellcome Pharmaceuticals (*Action TB* Research Initiative) without whose financial assistance this work would not have been possible. I would also like to express my gratitude to the South African Foundation for Research and Development for awarding me the Prestigious Postgraduate Scholarship for financial assistance during these studies.

My sincere gratitude to several researchers whose actions have demonstrated the true nature of collaborative research: Dr. Joe Lutkenhaus (for providing the anti-FtsZ antiserum); Dr. Morten Harboe (for the anti-Ag85 antiserum); Joanna Betts (Glaxo-Wellcome, for mass-spectrometric sequencing of proteins); and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (for supplying monoclonal antibodies and the *Mycobacterium smegmatis* LR222 strain).

To my parents, my brothers - Bomi and Zola, your unconditional support and your pride in my achievements has been an inspiration. Penny and Nomfuzo - thank you for the faith you displayed in my abilities, particularly when the going got tough. Matiwaza-T, thank you for your advice and encouragement throughout this journey.

Brief Contents

Abstract

As an initial step towards characterisation of the molecular processes that define the phenotype of the mycobacterial stationary phase, the effect of growth phase of *Mycobacterium smegmatis* on total protein synthesis and on the heat shock response was investigated. *De novo* protein synthesis was monitored by labelling with ^{35}[S]methionine and the protein expression profiles analysed using one- and/two-dimensional polyacrylamide gel electrophoresis, autoradiography, and/or immunoblot analysis. The ATP content of the culture was found to be a more accurate indicator that cells were entering stationary phase than the number of colony forming units (CFU). A plateau in the ATP growth curve preceded several stationary phase-induced events: a transitory cessation in the increase in number of CFU; a decrease in the rate of accumulation of the cell division protein FtsZ; inhibition of the synthesis of 58, 30.5, and 20 kDa exponential phase proteins; induction of the 48, 46, 32, 31, 25, and 20 kDa stationary phase (post-exponential phase) proteins; and the highest induction of the 95 kDa, 75 kDa (DnaK), 66 kDa (GroEL), and ~17 kDa (doublet) proteins in response to heat shock. Identification of the stationary phase-induced proteins should enable their roles in the multigenic processes that occur during transition into stationary phase to be determined.

The amino acid sequence of one of the ~17 kDa heat shock proteins (with an apparent molecular weight of 16.8 kDa, named Hsp17-2) showed significant homology to open reading frame 28 of *M. tuberculosis* cosmid MTCY01B2. This is the first time a functional characteristic has been assigned to this open reading frame, and it remains to be seen if Hsp17-2 represents a new family of heat shock proteins. Synthesis and secretion of the antigen (Ag)-85 complex proteins was demonstrated for the first time in *M. smegmatis*. Heat shock resulted in increased release of Ag85A and Ag85B but not of Ag85C in *M. smegmatis*. No heat-induction of the Ag85 complex could be demonstrated in *Mycobacterium bovis* BCG. Whereas heat shock resulted in increased release of the 19 kDa lipoprotein antigen in both *M. bovis* BCG and *M. tuberculosis* H37Rv, its presence in *M. smegmatis* could not be demonstrated. This study presents an experimental approach which may prove useful in investigating the effect of various environmental stresses on the profile, and hence the function of secreted proteins.

Summary

Mycobacterium tuberculosis, the causative agent for tuberculosis, is capable of causing both active disease and latent forms of infection. The genetic regulatory mechanisms which facilitate the conversion of *M. tuberculosis* from active growth to the dormant or persistent state have not yet been characterised. Available genetic evidence regarding the physiological state of persisting bacilli points to the possible existence of either a metabolically active state or if they are in a spore-like, metabolically inactive, form awaiting a signal to resume division. To complement available genetic evidence and to provide information on if and when the predicted gene products are translated, the total protein output encoded by the genome (i.e. the proteome) during different stages was investigated under both normal growth conditions and in response to heat shock stress in *Mycobacterium smegmatis*.

The number of colony forming units (CFU), the adenine triphosphate (ATP) content of the culture, and the optical density at a wavelength of 600nm were used to monitor the growth of cultures of *M. smegmatis* over a period of 72 hours. The optimum conditions for monitoring *de novo* protein synthesis were found to consist of a 1-hour incubation at either 37°C (total protein synthesis) or 48°C (heat shock) in the presence of ^{35}[S]methionine for protein labelling. Both the cellular (cell lysate) and the secreted proteins (supernatant) were analysed by one- and/or two-dimensional polyacrylamide gel electrophoresis followed by autoradiography and/or immunoblot analysis. Densitometric analysis was used to quantify the intensity of the signals in autoradiographs.

The ATP content of the culture was found to be a more accurate indicator of stage of transition of *M. smegmatis* from exponential phase to stationary phase. A plateau in the ATP curve (from 28 hr onwards) preceded the following stationary-phase induced events: a momentary cessation in the increase in number of CFU (between 30 and 36 hr); a decrease in the rate of accumulation of the cell division protein FtsZ (from 36 hr); inhibition of the synthesis of exponential phase proteins of 58, 30.5, and 20 kDa (from 36 hr); a 3-fold reduction in pre-induction (37°C) levels of the 95 kDa (Hsp90),

66 kDa (GroEL), 42 kDa (DnaJ), and ~17 kDa heat shock proteins (from 36 hr onwards) - while the levels of the 75 kDa (DnaK) heat shock protein remained constant throughout growth; the induction of stationary phase specific (post-exponential phase) proteins of 48 kDa (from 40 to 72 hr), 46 kDa (from 32 to 48 hr), 32 kDa (from 28 to 36 hr), 31 kDa (from 44 to 72 hr), 25 kDa (from 44 to 72 hr), and 20 kDa (from 48 to 72 hr); a plateau in the number of CFU (from 40 hr onwards); and the highest induction of the 75 kDa (28 hr), 95 kDa (44 hr), 66 kDa and ~17 kDa (40 hr) proteins in response to heat shock. It appears that attempts to define the mycobacterial stationary phase would benefit from the identification and characterisation of proteins that are induced during the transition into stationary phase.

The heat-inducible ~17 kDa doublet of *M. smegmatis* was resolved into four strongly heat-inducible radioactive spots by two-dimensional polyacrylamide gel electrophoresis. One of the peptides (Hsp17-2, with an apparent molecular weight of 16.8 kDa) was purified from silver-stained gels and the amino acid sequence determined using matrix-assisted laser-desorption/ionisation (MALDI) mass spectroscopy. The sequences of two peptide fragments from Hsp17-2 were found to be identical to internal fragments of a 16.2 kDa protein of unknown function that is encoded by open reading frame 28 of the *M. tuberculosis* H37Rv cosmid MTCY01B2. The nucleic acid sequence of the *M. tuberculosis* H37Rv gene was used to design primers for amplifying the Hsp17-2 gene from the genome of *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv. The gene encoding Hsp17-2 was successfully amplified from *M. bovis* BCG and *M. tuberculosis*, but not in *M. smegmatis*. The PCR amplification products were cloned and their identity confirmed by restriction digestion analysis and DNA sequencing. The sequence of the gene encoding *M. bovis* BCG Hsp17-2 was found to be 100% and 82% identical to the sequence of *M. tuberculosis* H37Rv cosmid MTCY01B2.28 and *M. leprae* cosmid B1133, respectively. This is the first time that a functional characteristic has been assigned to this open reading frame and the existence in *M. smegmatis* of at least two other as yet unidentified heat inducible proteins of a similar size suggests that these proteins may belong to a new family of heat shock proteins. An increase in the severity of the heat shock stress resulted in increased synthesis of the *M. smegmatis* ~17 kDa doublet (i.e. protein levels at 50°C > 48°C > 42°C), suggesting that these proteins may function

during conditions of severe stress, perhaps under the control of the σ^E subunit of RNA polymerase. Further studies will be required to establish whether Hsp17-2 is immunodominant; has molecular chaperone activity; is a member of a new class of heat shock proteins; or if it is induced by other stresses.

The effect of heat shock (48°C for 1 hour) on protein secretion was investigated in the presence of ^{35}[S]methionine. *De novo* synthesis and release of proteins with apparent molecular weights of 53, 46, 32, 30, and 22 kDa was observed. Heat shock resulted in 2.2-, 1.7-, and 4.1-fold increase in the release of the 32, 30 and 22 kDa proteins, respectively. Immunoblot analysis was used to confirm that the 32 and 30 kDa proteins were members of the Ag85 complex - a class of proteins with fibronectin-binding and mycolyl -transferase activities. Heat shock resulted in increased release of Ag85A and Ag85B, but not of Ag85C in *M. smegmatis*. No heat-induced released of the Ag85 complex proteins could be demonstrated in *M. bovis* BCG. The demonstration of active synthesis and release of the Ag85 complex proteins into the supernatant (short-term culture filtrate) of *M. smegmatis* is in contrast to a previous report which, based on the inability to detect the Ag85 complex in the culture filtrate of *M. smegmatis*, suggested that the proteins were not secreted. In agreement with the previous studies, no Ag85 complex proteins could be detected in the long-term culture filtrates of *M. smegmatis*. These results suggest that the *M. smegmatis* Ag85 complex is secreted into the medium, but unlike in *M. tuberculosis* or *M. bovis* BCG where the proteins accumulate with increasing age of culture, the proteins may become degraded in long term culture filtrates of *M. smegmatis*. The significance of the differences between slow growers and fast growers in the secretion of the Ag85 complex awaits further investigation. Immunoblot studies using the HYT6 monoclonal antibody that is specific for the 19 kDa lipoprotein antigen demonstrated no reactivity in the both the pellet (cell lysate) and supernatant fractions of *M. smegmatis*, whereas in *M. tuberculosis* H37Rv and *M. bovis* BCG, HYT6 reacted with a 23 kDa protein in the supernatants of heat-shocked (48°C) cells but not with the supernatants from the corresponding controls (37°C). Further studies will be required to achieve a better understanding of the role of heat shock in the mechanisms involved in the release of lipoproteins from the mycobacterial cell wall.

Contents

Chapter 1

Literature Review

Chapter 2

Construction of *Mycobacterium smegmatis* Growth Curves and Standardisation of Heat Shock Conditions

Chapter 3

The effect of Growth Phase of *Mycobacterium smegmatis* on Total Protein Synthesis and on the Response to Heat Shock.

Chapter 4

Identification of a Novel 17 kDa Heat Shock Protein (Hsp17-2) in *Mycobacterium smegmatis.*

Chapter 5

The effect of Heat Shock on the Synthesis and Release of Proteins in *Mycobacterium smegmatis.*

Chapter 1

Literature Review

1. General Background

1.1. The Genus *Mycobacterium*

Members of the genus *Mycobacterium* are widespread in nature, ranging from harmless inhabitants of soil and water to being agents of such devastating diseases as tuberculosis and leprosy. The name *Mycobacterium* was given to this genus by *Lehmann* and *Newmann* in 1896 on account of the mould-like appearance of strains when grown as pellicles on nutrient broth (Grange, 1980). Mycobacteria are obligate aerobes, have a slow rate of growth *in vitro*, and the pathogenic species are usually intracellular parasites (Tsukamura, 1981). They belong in the *Actinomycete* branch of Gram-positive bacteria, a group that includes *Nocardia*, *Corynebacterium* and *Streptomyces*, and morphologically are intermediate between *Corynebacterium* and *Streptomyces* (Shinnick and Good, 1994).

Several classification schemes have been used to differentiate the more than 70 species assigned to the genus (Shinnick and Good, 1994). One of the more useful schemes was proposed by Runyon. He divided the mycobacteria into groups as follows: Group I - slow-growing photochromogens (which produce a yellow pigment when grown in light); Group II - slow-growing scotochromogens (which produce an orange pigment when grown in light or dark); Group III - slow-growing nonchromogens (non-pigmented under any circumstances); and group IV - rapid-growers (Runyon, 1959). The rapid-growers (which divide every 3 hours) show visible growth from dilute inocula within 7 days, and the slow-growers (with doubling times of about 18 hours) require more than 7 days for visible growth (Wayne, 1976; Shinnick and Good, 1994). Phylogenetic relationships inferred using the highly conserved 16S rRNA sequences have also reflected a division between rapid- and slow-growing mycobacteria (Stahl and Urbance, 1990). The natural division between

slow- and fast-growing species has also been reflected by rRNA gene copy number: one genomic copy among the slow growers (e.g. *Mycobacterium tuberculosis, Mycobacterium bovis and Mycobacterium intracellulare*) and two copies amongst the rapid growers (e.g. *Mycobacterium smegmatis, Mycobacterium phlei*), (Bercovier *et al.*,1986). It has been hypothesised that the low rRNA copy number in mycobacteria may be one of the reasons why these bacteria grow slower than other prokaryotes. *Mycobacterium leprae* has so far not been cultured *in vitro*, and as a result cannot be classified according to growth rate. Most mycobacteria have large genomes (in the range of 2.2 to 4.5×10^9 Da) compared with most other prokaryotes. Within the mycobacteria, pathogenic organisms tend to have smaller genomes: *M. leprae*, 2.2×10^9 Da; *M. tuberculosis*, 2.5×10^9Da; *M. bovis* BCG, 2.8×10^9 Da; compared with for example, non-pathogenic *M. smegmatis* with 4.2 to 4.5×10^9 Da. Mycobacteria have a G+C content of between 64 and 70% in their DNA with two exceptions - *M. leprae* (58%) and *M. lufu* (61%) (Clark-Curtiss, 1990)

M. tuberculosis and other mycobacteria have been regarded as part of the Gram positive group, and are in addition, defined by their ability to withstand discoloration with acid alcohol (i.e. acid-fastness) after routine staining with auramine-O or by the Ziehl-Neelsen protocol (Tsukamura, 1981). A distinguishing feature between acid-fast and other Gram-positive bacteria is that the molecules attached to or associated with the peptidoglycan layer (a cross-linked polymer of amino sugars and amino acids that determines the shape of the bacterial cell) are predominantly lipids in mycobacteria rather than the proteins and polysaccharides found in other bacteria (Lederer *et al.*,1975). The mycobacterial envelope consists of the plasma membrane which provides a semi-permeable barrier, and the cell wall which provides mechanical support and protection. The cell wall consists of an inner layer of moderate electron density thought to contain peptidoglycan; a wider electron transparent layer which appears to be the arabinogalactan layer linked through ribose residues to mycolic acids intercalated with hydrocarbon chains of lipids and glycolipids to form a regular layer; and an outer electron-opaque layer of variable appearance and thickness believed to form the capsule of the mycobacterial cell. The polysaccharide PIM is believed to be anchored on the plasma membrane (Brennan and Draper, 1994). Khoo *et al* (1995) have provided evidence which suggests that the acylated form of PIM is a common

lipid anchor for the multiglycosylated forms of PIM, i.e. LAM (which traverses the entire cell wall) and LM. Acylation of PIM to form triacylated mannophosphoinositide has been postulated to be a key regulatory step in the biosynthesis of the LAM and LM (Khoo *et al.*, 1995). It is believed that the mycolic acid-rich regular layer forms a pseudo-outer membrane analogous to the outer membrane of Gram-negative bacteria. Thus it may be more appropriate to consider the acid-fast organisms as a distinct group as far as cell wall architecture is concerned, that should command a staining status equivalent to that of Gram-positive and Gram-negative organisms.

1.2. History and Epidemiology of Tuberculosis

1.2.1. Origins of M. tuberculosis *infection in humans*

The burden of ill-health caused by tuberculosis makes it one of the most important diseases in the history of humankind. When and where tuberculosis first afflicted humanity predates written records. The inability of *M. tuberculosis* to infect cattle and the close genetic similarities between *M. tuberculosis* and *M. bovis* (the organism which causes tuberculosis in cattle, other animals and in humans), has led to the speculation that cattle may have been the original source of tuberculosis in humans, and that *M. tuberculosis* is a mutant of *M. bovis* (Clark *et al.*,1987; Steele and Ranney, 1958; Grange and Collins, 1987). Widespread domestication of cattle in the north-eastern Mediterranean basin and Middle East between 5000 and 7000BC is thought to have started the infection of the human population with *M. bovis.* (Clark, 1962; Manchester, 1983, 1984). The earliest evidence of tuberculosis in humans comes from Egypt, where skeletal remains and ancient art, spanning the period 3400 to 1000 BC, depict a hunchback deformity resulting from bone lesions that are normally characteristic of skeletal tuberculosis. It has been speculated that *M. bovis*, instead of *M. tuberculosis*, may have been responsible for the bone lesions since it is a more common cause of skeletal tuberculosis and rarely causes pulmonary tuberculosis (Hare, 1967). Extensive studies of Egyptian mummies have not provided much evidence of pulmonary tuberculosis since the lungs and other organs are rarely well

preserved even when the body is mummified (Morse, 1961). *M. tuberculosis* was first demonstrated as the causative agent for tuberculosis by Robert Koch in 1882 (Koch, 1932).

1.2.2. The first tuberculosis epidemic

In early history, tuberculosis probably occurred as a sporadic and unimportant disease. The industrial revolution which accelerated in Britain during the eighteenth century, led to the rapid growth of towns, and often to severe overcrowding (Dubos and Dubos, 1953). As a result of the poor living conditions, tuberculosis infection started to increase in the 1700s and early 1800s, reaching epidemic proportions of 700 cases per 100 000 people in Western Europe (Bloom and Murray, 1992), and 400 to 750 deaths/100,000 in the United States (Grigg, 1958). In 1885-1890, an American physician, Edward Livingstone Trudeau, performed a classic study which clearly demonstrated the important role of the environment in modifying an individual's susceptibility to tuberculosis. He demonstrated that rabbits, which had been inoculated with virulent *M. tuberculosis* and were allowed to eat and roam freely, survived tuberculosis better than rabbits confined to damp, sunless conditions and fed on a poor diet (Meade, 1972).

1.2.3. The second tuberculosis epidemic

As Europeans continued to move around the world through migration and colonisation, the tuberculosis epidemic followed them, thus initiating the second tuberculosis epidemic in these areas. From about 1830, sunny, dry climates and high altitudes were promoted as good therapy for tuberculosis. This led to large scale influx of tuberculous immigrants initially to different parts of Europe, and subsequent to the development of European colonies, to North America, Africa and Australia (Thompson, 1889; Weber, 1885; Thomas and Gandevia, 1959). Colonisation brought about changes in the demographic distribution of indigenous populations. For example, the Indians of Qu'Appelle Valley in Canada who had been nomadic buffalo

hunters, were forced to settle in reservations. Their confinement resulted in tuberculosis death rates as high as 9000/100,000 by 1886 (Ferguson, 1955). In southern Africa, the development of the mining industry also played a significant role in spreading the disease. The over-crowding and poor working conditions in the mines favoured the spread of infection between miners. The practice of employing migrant mine workers and then repatriating those who developed tuberculosis played a key role in disseminating the disease to rural areas. The hut tax led to overcrowding in rural areas and facilitated spread of tuberculosis among families of returning mine workers (Packard, 1989; Strebel and Seager, 1991). The promotion of dry, sunny places such as South Africa, as health resorts resulted in a large influx of tuberculosis sufferers from Europe into towns in the Western Cape region, with Cape Town being the main port of entry. The Western Cape region currently has the highest incidence of tuberculosis in the country (McDonald, 1984; Yach, 1988; Metcalf, 1991). Recent genetic typing studies by van Helden (personal communication) have shown that one of the predominant strains of *M. tuberculosis* in the Western Cape is genetically related to the Asian strains, suggesting that that Asians who were brought in as slaves may have also been an important source of tuberculosis in this region.

It has been suggested that within a particular population in a defined geographical area, the tuberculosis epidemic reaches its peak within 50 to 75 years after its beginning and then slowly declines, possibly as the more resistant survivors propagate (Daniel *et al.*,1994). By the early 1900s, the first tuberculosis epidemic had reached its peak and the mortality rate had declined to 150 deaths/100 000 in England and Wales (Fig.1.1) and 188/100,000 in the United States (Fig. 1.2). Evidence suggests that, even before the chemotherapeutic era, which began with the discovery of streptomycin in 1944 (Schatz *et al.*,1944), tuberculosis in developed countries had been declining for at least 40 years (Fig. 1.1 & 1.2). On the other hand, as late as the beginning of the 20th century, tuberculosis remained essentially unknown in sub-Saharan Africa (Cummins, 1920). The reported incidence of tuberculosis in South Africa has increased progressively since the 1920s, reaching its highest peak of 372 cases/100 000 of the total population in 1963 (Fig. 1.3). By 1990, the reported incidence had diminished to approximately 237 cases/100 000 (Glatthaar, 1982). The peak of the tuberculosis epidemic in sub-Saharan Africa coincided with the introduction of

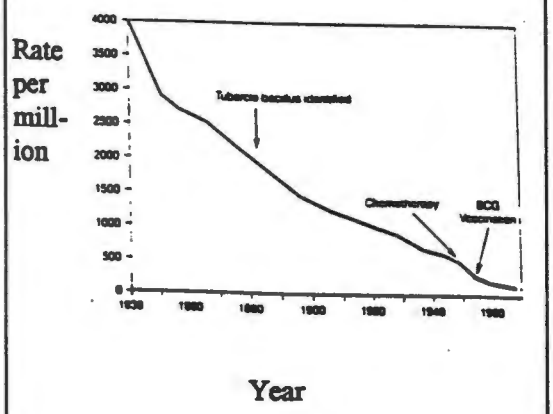


Fig. 1.1.: The decline in respiratory tuberculosis death rates in England and Wales since 1838. Standardised to the 1901 population (Source: McKeown, 1979).

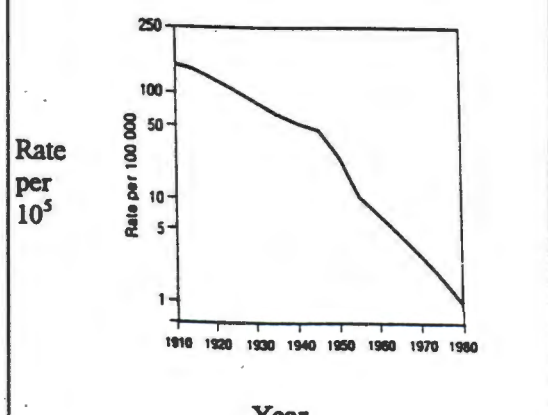


Fig. 1.2.: The decline in tuberculosis death rates in the United States from 1910 to 1980 (Source: Comstock, 1982).

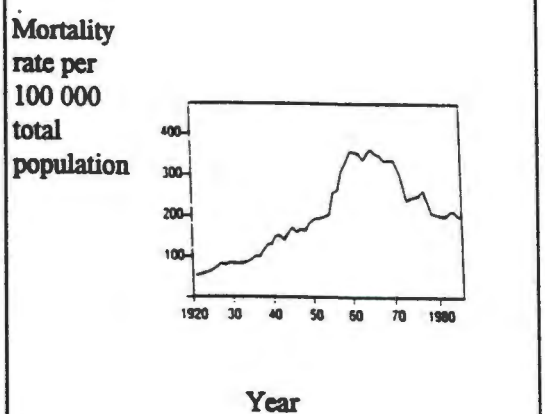


Fig. 1.3.: Annual tuberculosis notification rate in South Africa, 1921-1985 (Source: Epidemiological Comments, 1985).

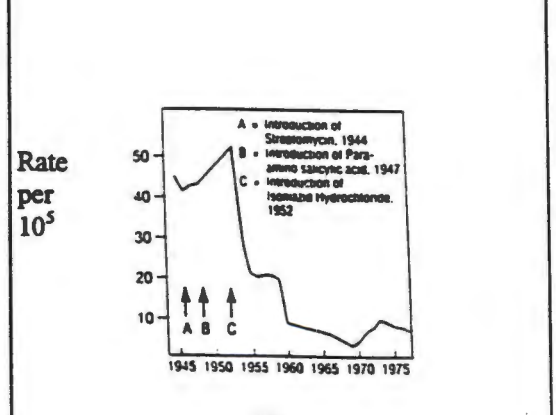


Fig. 1.4.: Notified annual tuberculosis death rate, South Africa, 1945-1977 (Source: Kustner, 1979)

chemotherapy from in 1946 (Fig. 1.4) and probably as a result thereof, the mortality rates were never as high as they had been in the developed countries. The influence that chemotherapeutic intervention has had on the development of natural resistance to tuberculosis in communities that experienced the second tuberculosis epidemic still remains to be seen.

Perhaps improvement in socio-economic conditions in developed countries played a more important role than natural resistance in the observed tuberculosis decline in the pre-chemotherapeutic era. For example, the tuberculosis prevalence rates in South Africa have been reported to be between 1.8 and 3.7 times higher in coastal areas (e.g. Western Cape) when compared with inland areas (Fourie and Knoetze, 1986), and that incidence rates were strongly associated with race and geographical origin, namely 17/100 000 for Whites, 62/100 000 for Asians, 240/100 000 Blacks (Africans), and 626/100 000 for Coloureds (Department of National Health and Population Development, 1990). It remains to be seen whether the higher incidence in the Coloured population, which resides largely in the Western Cape coastal region, and generally has better socio-economic conditions than the African population, is due to environmental or genetically determined factors specific to this population group. HIV is unlikely to be the reason for the high incidence of tuberculosis in the Western Cape since this region has the lowest HIV incidence in the country.

1.2.4. Genetic factors: contribution to Mycobacterium tuberculosis *infection*

In addition to environmental conditions, genetic factors have also been implicated in the susceptibility to tuberculosis infection and hence the incidence or spread of disease. Despite earlier studies which suggested that there were no racial differences in susceptibility to tuberculosis between black and white Americans within a similar environment (Katz and Kunofsky, 1960), there is evidence to suggest that genetic factors do play a role in determining susceptibility to tuberculosis. One example is the tragic incident in Lubeck in 1927 where infants were inadvertently immunised with a virulent *M. tuberculosis* strain (Anonymous, 1935). A marked variation in disease outcome ranging from death to recovery led to the speculation that genetic differences

may have been responsible for the observed differences. In addition, studies amongst twins show a higher degree of concordance of tuberculosis among monozygotic than dizygotic twins (Comstock, 1978). Another example of a racially determined susceptibility to tuberculosis is the finding that black Americans converted to tuberculin positive status more frequently than whites following the same exposure to *M. tuberculosis*. The study suggested that the alveolar macrophages of blacks may be less effective in destroying inhaled bacilli than those of whites (Stead *et al.*,1990). It has also been suggested that a person's resistance to infection tends to correlate with the region of his or her ancestry, that is, ancestors of persons in the more resistant group tended to derive from densely populated areas and cities once rife with tuberculosis, whereas the ancestors of persons in the more sensitive group tended to derive from areas once free of tuberculosis (Stead, 1990).

Recent studies on why some individuals escape HIV infection despite being at high risk, and why certain people who contract the virus progress to AIDS unusually slowly, have uncovered a genetic trait that protects against AIDS (O'Brien and Dean, 1997). People carrying standard alleles of the gene for CCR5 protein - a macrophage receptor that binds gp120 molecule of HIV particles which, in conjunction with the CD4 molecules on macrophages, allow HIV to infect macrophages. In contrast, people who possess deletion mutants of the CCR5 gene resist infection, possibly because the protein made from the mutant gene is not displayed on macrophage surface. The study also showed that the frequency of the mutant allele was higher in Caucasian Europeans (10%) and Caucasian Americans (11.1%) compared to the low incidence of 1.7% in African Americans and 0% in Native Americans, Africans and East Asians. Based on this evidence, it would be of interest to investigate if the tendency for blacks to convert to tuberculin positive status more frequently than whites is a result of differences in the mechanisms involved in the uptake of bacilli into macrophages.

Available experimental evidence in support of a role for genetic determinants in susceptibility to tuberculosis comes from animal studies. The *Bcg*^{r} gene has been shown to confer resistance to *M. bovis* BCG infection in mice (Schurr *et al.*,1991). The *Bcg*^{r} gene, encoding Nramp protein has been shown to be homologous to a eukaryotic nitrate transporter (Vidal *et al.*,1993), leading to suggestions that resistance

to *M. bovis* BCG infection may be mediated through regulation of trafficking of reactive nitrogen intermediates (RNI) within macrophages (Gros *et al.*,1983; Chan *et al.*,1992; Flesch and Kaufmann, 1991). Recent evidence, however, indicates that members of the NRAMP family function as divalent cation transportes of broad specificity (Gunshin *et al.*, 1997). Homologues of NRAMP have also been identified in a wide variety of organisms, including humans, insects, plants and yeasts (Cellier *et al.*, 1995, 1996). Two isotypes of NRAMP have been identified in mammals: NRAMP1, which is restricted to cells of the macrophage or monocyte lineage, and NRAMP2, which is expressed ubiquitously (Cellier *et al.*, 1996). The demonstration of NRAMP homologues in the intracellular pathogens *M. leprae and M. tuberculosis,* have led to a model which suggests that competition for divalent cations, which are required for the synthesis of enzymes (superoxide dismutases and catalases) required for defence against macrophage killing mechanisms, plays a pivotal role in the interaction between the host macrophages and the parasite (Agranoff and Krishna, 1998).

1.2.5. Tuberculosis and HIV: the cursed duet

Evidence suggests that, irrespective of its magnitude, the tuberculosis problem in developed countries has been decreasing for the last 40 years (Frost, 1937; Styblo, 1980). For example, in the Netherlands, the annual risk of infection in 1985 was 0.012%. On the other hand, in developing countries tuberculosis continues to be a major problem and there appears to have been virtually no tendency for tuberculosis to eliminate itself in the absence of extensive control measures (Murray *et al.*,1990). Sub-Saharan Africa, with an annual risk between 1.5 to 2.5% in 1985, is estimated to have the highest risk of tuberculosis infection in the world (Cauthen *et al.*,1988). A 1% annual risk of infection corresponds, on average, to an prevalence of 50 smear-positive cases per 100 000 population (Murray *et al.*,1990). During the 1980s, the numbers of reported cases of tuberculosis started to increase in both developed and developing countries. The number of reported tuberculosis cases in the United States increased by 20% between 1985 and 1992 (Centre for Disease Control and Prevention, 1993), while in the same period, the annual number of cases in Zambia

nearly tripled, that in Malawi more than doubled, and those in Tanzania and Burundi increased by about 70 and 40%, respectively (Narain *et al.*,1992). It has since been established that the sudden increase in tuberculosis incidence was linked to the emergence of HIV infection.

Chretien (1990) has referred to the association of tuberculosis and HIV infections as a 'cursed duet' as they combine to yield an original and specific couple with mutually aggravating interactions. Compared to the 5 to 10% lifetime risk of developing later tuberculosis among HIV-negative people with primary tuberculosis lesions, HIV-positive people have an 8% annual risk of tuberculosis (Selwyn *et al.*,1989). HIV infection worsens the tuberculosis situation by increasing reactivation of latent tuberculosis as well as by favouring rapid progression of new tuberculosis infections (Narain *et al.*,1992). Both diseases affect cell-mediated immunity: HIV infection predominantly destroys CD4+ Th1 lymphocytes but also affects alveolar macrophages, while *M. tuberculosis* is able to multiply inside alveolar macrophages (Chretien, 1990). Studies of HIV infected individuals show that Th1 cells are progressively lost, shifting the ratio to a Th2-dominant population (Clerici and Shearer, 1993). Th2 cells have a negative regulatory effect on Th1 cells (Mosmann and Coffman, 1989). Current research efforts have focused on elucidating the molecular mechanisms involved in the interaction between the immune response, HIV and *M. tuberculosis*. Recent studies include characterisation of the effects of the micro-environment (cytokine profile) created by tuberculosis on HIV infection of T lymphocytes (Garrait *et al.*,1997); the distinct actions mediated by different TNF_{α} receptors on HIV replication in human macrophages (Herbein *et al.*,1997); and studies on proteins promoting attachment of *M. tuberculosis* to alveolar macrophages during HIV infection (Downing *et al.*,1995).

In industrialised countries, 80% of individuals infected with *M. tuberculosis* are aged 50 years or more, while in developing countries, 75% of infected persons are less than 50 years old (Kochi, 1991). In industrialised countries, the younger generation, which is at a higher risk of HIV infection, is almost free of tuberculosis infection, and as such, the overall proportion of people with tuberculosis and AIDS is <10% (De Cock *et al.*,1992). However, in developing countries where tuberculosis affects mainly the

younger generation, the high annual risk of infection and high prevalence of primary tuberculosis means that each of these subjects may develop tuberculosis disease particularly if they become HIV positive. Due to the aggravating nature of HIV and tuberculosis on each other in dually infected persons, populations such as those in sub-Saharan Africa, who already have a high prevalence of tuberculosis, account for 75% of the world's proportion of people with dual HIV and tuberculosis infection (World Health Organisation, 1993). The level of risk of tuberculosis infection and its trend in the group affected by HIV infection are therefore important factors which will determine the impact of HIV infection on the future of tuberculosis.

1.3. Pathogenesis of Pulmonary Tuberculosis

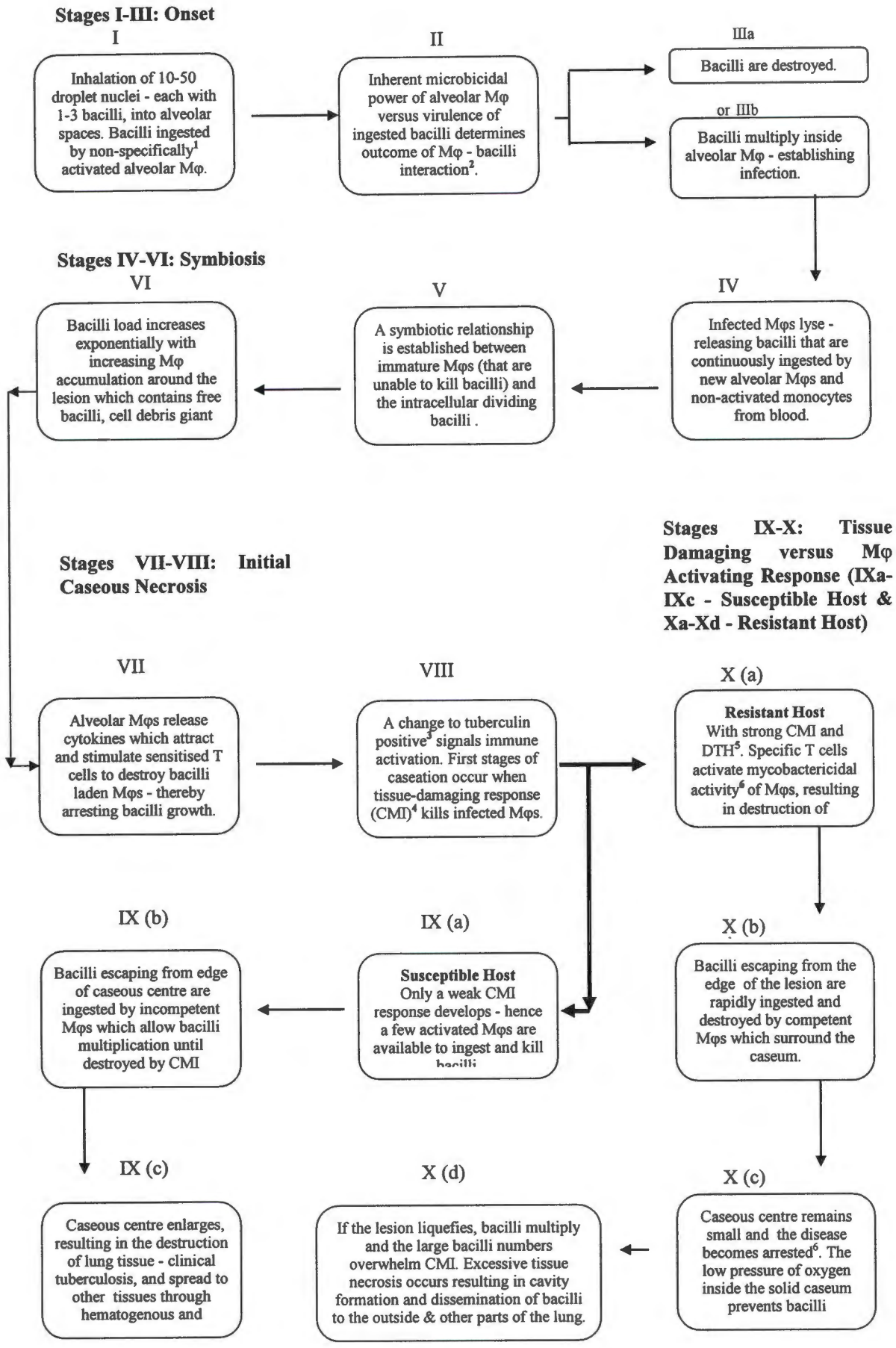
Tuberculosis pathogenesis is believed to be a result of an interplay between tissue-damaging and macrophage (Mφ) activating immune responses, which are mechanisms for controlling bacillary multiplication within the infected host. Extensive reviews have been written on the pathogenesis of pulmonary tuberculosis (Lurie *et al.*,1952; Dannenberg, 1991; Dannenberg and Rook, 1994; Chan and Kaufmann, 1994). The steps involved have been summarised in figure 1.5 (numbers 1-6 refer to the legend in the following page).

1.4. Control of Tuberculosis

1.4.1. Chemotherapeutic Intervention

Antimycobacterial chemotherapy began with the discovery and introduction of streptomycin in a controlled tuberculosis trial in 1948 (Schatz, 1944; British Medical Research Council, 1948). Subsequent studies led to the establishment by 1952 of a combined chemotherapy regimen that required the use of streptomycin, para-amino-salicyclic acid (PAS), and isoniazid (a potent drug that is only active against tubercle bacilli) for two years or more in the treatment of tuberculosis (British Medical Research Council, 1973). The introduction of the broad-spectrum antibiotic rifampicin

Fig. 1.5.: Pathogenesis of Pulmonary Tuberculosis



Pathogenesis of pulmonary tuberculosis (stages and numbers refer to figure 1.5)

- **Stages I-III: Onset**

1. Alveolar Mφs - non-specifically activated by various inhaled particles and general stimulation of the mononuclear phagocyte system - may or may not be able to destroy ingested bacilli
2. Crowle & Elkins (1990) have reported that alveolar Mφs from blacks permit significantly more replication of *M. tuberculosis* than those from whites, suggesting that resistance to establishment of infection is partly under genetic control.

- **Stages IV-VI: Symbiosis**
- **Stages VII-VIII: Initial Caseous Necrosis**

3. Following the same exposure to tubercle bacilli, blacks have been reported to convert to tuberculin positive (a status indicating infection) more frequently than whites, suggesting that the alveolar Mφs of blacks may be less effective in destroying bacilli than those of whites. However, among the converters, the incidence of clinical disease is the same (Stead *et al.* 1990).

- **Stages IX-X: Tissue Damaging versus M(Activating Responses: IXa-IXc: Susceptible Host**
  Xa-Xd: Resistant Host

4. DTH (delayed type hypersensitivity reaction) - tissue damaging response which kills infected Mφs. Mediated mainly by CD8+ cytotoxic T lymphocytes (α/β) which recognise bacilli specific antigens presented with MHC (major histocompatibility complex) class I on surfaces of infected cells. DTH response causes both caseous necrosis and cavity formation (pulmonary damage).

5. CMI (cell mediated immune) response - activates Mφs to kill intracellular bacilli. After engulfing bacilli, Mφs produce cytokines including: IL-1 (induces fever and is a growth factor for Th1 cells); IL-6 (induces mycobactericidal effects and activates B lymphocytes); IL-8 (chemotactic for T cells); IL-10 (inhibits cytokine production by monocytes and T cells); IL-12 (important in the development of Th1 cells; TGFβ (inhibits Mφ effector function and T cell proliferation); TNFα (essential for granuloma formation, synergises with IFNγ to stimulate mycobactericidal activity of Mφs, and my cause fever, weight loss and tissue necrosis); GM-CSF and $1,25(OH)_2$ Vit. D_3 which activate Mφ mycobactericidal activity. **T lymphocytes** that are recruited to the site of infection produce cytokines which include IL-2 (T cell growth factor), IL-10, IL-3,TNFα, IFNγ (a key endogenous agent triggering mycobactericidal effects of Mφs and inhibits Th2 proliferation) and GM-CSF - which are secreted by the memory **Th1 CD4+** cells; as well as IL-3, IL-4 (inhibits Th1 generation and induces Mφ antimycobacterial activity), IL-5 (promotes eosinophil growth and differentiation), IL-6, IL-10, TNFα, IFNγ -which are secreted by suppresser **Th2 CD4+** cells. **γ/δ T cells** play a role in the initial immune response - appearing at site of infection after the arrival of PMNG and prior to CD4+ and CD8+ T cells. They are CD4-/CD8- (only a minority has been found to be CD8+) and are thought to recognise MHC-like molecules and they produce IL-2, IL-4, IL-5, IL-10, TNFα, and IFNγ.

6. Antimycobacterial effector functions of activated Mφs include phagolysosome fusion, generation of reactive oxygen intermediates (ROI) by the oxidative burst and the production of reactive nitrogen intermediates via the L-arginine-dependent cytotoxic pathway. *M. tuberculosis* is thought to evade antimycobacterial activity by:- preventing acidification of bacilli laden vacuoles, thereby inhibiting phagolysosome fusion; and by inhibiting the oxidative burst through production of LAM and PGL-1. LAM is a major cell wall associated lipopolysaccharides which inhibits protein kinase C (a key enzyme in the oxidative burst), is a scavenger for ROI, can inhibit transcription of some INFγ activated genes, and may induce Mφs to secrete immunosuppressive cytokines such as IL-10. PGL-1 scavenges oxygen radicals and down-regulates ROI production.

Protective immunity is extraordinarily inefficient in terminating infection and, at the same time highly efficacious in preventing disease. Because the relationship between *M. tuberculosis* and host immunity is a labile one, any diminution of protective immunity, for example, the loss of Th1 cells due to HIV infection, will cause progression into clinical disease.

in 1966 formed the basis of what is now known as short-course chemotherapy - a six months regimen that is currently the most effective treatment against tuberculosis (Batten, 1969; Fox, 1977). In the initial intensive phase of treatment (which lasts for two months) tuberculosis patients are treated with a combination four front-line drugs (rifampicin, isoniazid, pyrazinamide and ethambutol or streptomycin) to prevent the emergence of mutants that are resistant to a single drug. During the continuation phase (i.e. the following four months), patients are treated with only rifampicin and isoniazid to kill any persisting organisms. Second-line drugs (amikacin, capreomycin, ethionamide, fluoroquinolones, kanamycin, para-amino-salicyclic acid, thiacetazone, and viomycin) are only weakly active against tubercle bacilli and have high levels of secondary side effects and as a result are mostly used in cases where *M. tuberculosis* develops resistance to the front-line drugs (Cole, 1994).

There are at least four populations of bacilli that have to be targeted by antimycobacterial drugs: one made up of actively multiplying bacilli at neutral pH in the liquefied caseous material lining cavity walls; two populations of slower growing organisms found within the acid pH of the macrophages and within solid caseous areas where, despite the neutral pH, low oxygen tension permits only intermittent multiplication; and a small population of dormant or persisting bacilli whose location still has to be established (Benatar, 1991; Dannenberg and Rook, 1994). Isoniazid, rifampicin, and streptomycin (in order of potency) act on actively dividing extracellular bacilli in aerobic cavity lesions, while rifampicin, isoniazid, and pyrazinamide (in order of potency) act on slowly dividing organisms within macrophages and in closed anaerobic caseous lesions (Grosset, 1980). Table 1.1 provides a summary of known sites of action of antimycobacterial agents and the mechanisms employed by bacilli for drug resistance (Cole, 1994; Young, 1994; Alcaide *et al.*, 1997). There is currently a need for the development of drugs which can be targeted against the dormant or persistent population of *M. tuberculosis*. The elimination of dormant bacilli will not only reduce the incidence of tuberculosis resulting from reactivation of latent infection, particularly in HIV infected individuals, but may also improve compliance by reducing the time required for treatment. In addition, the emergence of *M. tuberculosis* strains that are resistant to available

Table 1.1.: Sites of action of antimycobacterial agents and the mechanisms of drug-resistance.

Antimycobacterial Agents	Mechanisms of Action	Mechanisms of Drug Resistance
Ethambutol	Arabinogalactan or arabinomannan synthesis?	Mutation in *embB* gene which encodes arabinosyl transferase
Ethionamide	Mycolic acid synthesis?	Mutation in *inhA* which encodes a novel InhA product thought to be involved in cell wall biosynthesis.
Isoniazid (INH)	Mycolic acid synthesis? Converted to an active analogue by the *katG* encoded catalase-peroxidase. The active analogue is believed to interfere with nicotinamide (NAD) biosynthesis, leading to the production of iso-NAD.	Mutation in *inhA* (as above). Mutation in *katG* resulting in reduced levels or activity of catalase-peroxidase, and hence resistance of the bacilli to the effects of isoniazid.
p-amino-salicylic acid (PAS)	Inhibits biosynthesis of tetrahydrofolic acid, thus blocking the production of the purine and pyrimidine bases required for nucleic acid synthesis.	
Fluoroquinolones (ciprofloxacin, ofloxacin, sparfloxacin)	Disrupt the bacterial chromosome by inhibiting the supercoiling activity of DNA gyrase, a type II topoisomerase.	Mutations in the *gyrA* gene which encodes the A subunit of DNA gyrase.
Rifampicin	Inhibits transcription by interacting with the β subunit of bacterial RNA polymerase.	Mutations in the *rpoB* gene which encodes the β subunit.
Streptomycin	Disrupts bacterial protein synthesis by preventing translation of mRNA by the ribosomes.	Mutations in *rpsL* and *rrs* genes which alter the ribosomal protein S12 and the 16S rRNA, respectively.

antimycobacterial agents has also highlighted the need for increased effort in the development of new drugs.

1.4.2. Vaccination

BCG (Bacille Calmette *et* Guerin) has been in use since 1921, and is the only available vaccine for protection against tuberculosis. The BCG vaccine was developed by Calmette and Guerin using a strain of *M. bovis* which was rendered avirulent by a series of passages in a potato-gylcerin medium supplemented with ox bile (Calmette, 1927; Guerin, 1957). Despite its wide usage and its many advantages (i.e. it can be given at birth or any time thereafter; a single inoculation can produce long-lasting sensitisation; it is safe; it is relatively stable; it produces a scar which is useful for epidemiological surveillance; it is inexpensive; and it also provides protection against leprosy), BCG appears to consistently provide protection against primary, disseminated forms of tuberculosis, but its protective efficacy against pulmonary disease appears to vary widely in different parts of the world (Bloom and Fine, 1994; Rodrigues *et al.*,1993; Fine and Rodrigues, 1990). Factors which have been attributed to the variation in the efficacy of BCG include: methodological differences between different trials (Clemens *et al.*,1983); differences between BCG vaccines (Osborn, 1983; Comstock, 1988); genetic differences within and between populations (Schurr *et al.*,1991; Comstock and Palmer, 1966; Packe and Innes, 1988; Rotrigues *et al.*,1991); differences in virulence between *M. tuberculosis* strains (Mitchison, 1964; Hand *et al.*,1981); BCG protection against endogenous reactivation of long-standing infections but not exogenous re-infection (ten Dam, 1984; ten Dam and Pio, 1988; Bloom and Fine, 1994); and interference with or masking of protection by environmental mycobacterial infections (Palmer and Long, 1966; Edwards *et al.*,1969; Brown *et al.*,1985).

Several research strategies for the development of new and more effective vaccines against tuberculosis are currently being pursued, and these include: recombinant BCG vaccines (i.e. BCG expressing the appropriate *M. tuberculosis* protective antigens including antigens from other pathogens); attenuated *M. tuberculosis* vaccines (which

require the identification and deletion of genes responsible for virulence, with the hope that the deleted genes are not the basis for generating adequate protection); atypical mycobacterial vaccines (e.g. *M. vaccae* and *M. microti* - based on findings that certain environmental mycobacteria can provide protection against tuberculosis); killed or auxotrophic vaccines (which employ mutants that lack one or more of the enzymes essential for growth, and would therefore be safe for use in immunocompromised patients); and subunit or DNA vaccines (using clearly defined protective antigens such as the proteins secreted by live bacilli) (Bloom and Fine, 1994; Young and Duncan, 1995).

1.4.3. Chemoprophylaxis

The treatment of individuals who are known to be infected with *M. tuberculosis* with isoniazid (chemoprophylaxis or preventive therapy) has been shown to be effective in preventing subsequent progression to clinical tuberculosis (Ferebee, 1969). For example, treatment with isoniazid for 6 or 12 months reduced the incidence of reactivation tuberculosis by 65% or 90%, respectively (Comstock and Woolpert, 1972; Comstock *et al.*,1979). In addition to the direct benefit of reducing prevalence of disease for the individual, chemoprophylaxis is thought to have the indirect benefit of interrupting the transmission that an infectious case would cause in the absence of treatment (Murray, 1994). Despite its obvious benefits, chemoprophylaxis may not be beneficial to all individuals due to the risk of isoniazid-related hepatitis and the emergence of drug-resistant strains as a result of non-compliance (Snider *et al.*,1986; Moulding *et al.*,1989; Jordan *et al.*,1991; Murray, 1994). When considering cost-effectiveness, and the difficulties associated with identifying individuals who would benefit from chemoprophylaxis in TB endemic areas (i.e. those who recently converted to PPD+ status), it appears that chemoprophylaxis may only be feasible for high-risk groups such as HIV-infected individuals with tuberculosis infection (Pape *et al.*,1993).

1.5. Tuberculosis Research: Challenges for the Future

Tuberculosis continues to be increasingly a major problem in the world - not only because of its association with the AIDS pandemic, but in addition, due to overcrowding, social dislocations, poverty, the emergence of multiple- drug resistant strains, the lack of adequate tuberculosis control programmes. The host's protective immunity is extraordinarily inefficient in terminating *M. tuberculosis* infection and at the same time highly efficacious in preventing disease by walling off the focus of infection. There is still a need to explain the special ability of *M. tuberculosis* to survive in mammalian hosts and persist in a dormant state for long periods. In addition, studies on the immunopathogenesis of tuberculosis in the presence of HIV should lead to a better understanding of how HIV infection leads to the rapid progression of tuberculosis. It is hoped that a better understanding of the interaction between the host and tubercle bacilli will provide previously unimagined opportunities for developing new and effective drugs, improved diagnostic procedures, and vaccines that will ensure the effective control of tuberculosis globally.

2. Specific Background

2.1. Mycobacterial Protein Antigens

As outlined in figure 1.5, the interaction between the host's immune system and *M. tuberculosis* is complex and dynamic. To survive within the macrophage, *M. tuberculosis* must adapt to an intracellular environment that includes a powerful array of antimicrobial defences. Characterisation of the immunogenic protein antigens of the bacilli has been instrumental in promoting a better understanding of the host-parasite relationship. The initial selection of immunodominant antigens was based on identifying antibody defined antigens able to stimulate B cell proliferation (Young *et al.*,1985). It was also found that T cells played a major role in the defence against mycobacterial disease (Hahn and Kaufmann, 1981), and that antibody-identified antigens also stimulated T cells (Young, *et al.*,1988). Sequence analysis of several immunodominant mycobacterial antigens led to their identification as members of highly conserved heat shock protein families, related to the *Escherichia coli* proteins DnaK, GroEL, and GroES (Young, 1988). Mycobacterial protein antigens that have been identified in patients and animal models of mycobacterial infection have been classified according to common features such as function, subcellular localisation, and physical and chemical properties. The major groups of mycobacterial antigens fall under heat shock proteins, lipoproteins and secreted proteins (Young *et al.*,1992; Anderson and Brennan, 1994). The functional and structural characteristics of heat shock proteins and lipoproteins are summarised in tables 1.2 and 1.3, respectively.

2.2. The Heat Shock Response

Organisms as diverse as *E. coli* and humans when exposed to adverse stimuli, such as a sudden shift to a higher temperature, respond by inducing or accelerating the rate of synthesis of a set of proteins, called 'heat shock proteins' (Schlesinger *et al.*,1982). Heat shock protein families include Hsp110 (proteins > 100 kDa), Hsp90, Hsp70 (e.g. DnaK), Hsp60 (e.g. GroEL), and small Hsps (i. e. α-crystallin-like proteins) (Buchner,

Table 1.2.: Heat Shock Protein Families

Hsp Family	Functional and Structural Characteristics
Hsp60 (GroEL)	• Promotes folding of proteins *in vivo,* and is Involved in the modulation of the heat shock response (Mogk *et al.*,1997). • Functionally co-operates with a 10kDa heat shock protein - GroES which forms a single seven-subunit ring (Schmidt *et al.*,1994a, 1994b).) • Exists as a double ring of seven subunits each with a pronounced central cavity. Because the two ends of GroEL are structurally equivalent, one or two GroES rings can bind simultaneously to GroEL - forming complexes which appear as either an asymmetric 'bullet' or a symmetric 'football' under electron microscopy (Schmidt *et al.*,1994c; Buchner, 1996). • *M. tuberculosis* 65 kDa antigen exhibits 60% amino acid sequence identity with *E. coli* GroEL and 95% amino acid identity with *M. leprae* 65 kDa antigen (Young *et al.*,1988). • *groE* operons in eubacteria always contain only the *groES* and *groEL* genes in the order GroES- GroEL (Segal and Ron, 1996). • *M. tuberculosis, M. leprae and S. coelicolor* contain two *groEL* genes (Kong *et al.*,1993, Rinke de Wit, 1992; Mazodier *et al.*,1991). The mycobacterial 65 kDa antigen is encoded by a separate gene - *cpn60-2*, while the second *groEL* gene (*cpn60-1)* is arranged in an operon with the gene encoding the 10 kDa antigen (GroES homologue). The *cpn60-1* gene product (cpn60-1) displays 61% amino acid identity with the 65 kDa antigen (cpn60-2) and 53% and 41% identity with the *E. coli* GroEL protein and the human P60 protein, respectively. The *cpn60-1* gene is expressed as a polypeptide of about 60 kDa. Immunoblot studies using monoclonal antibodies directed against the 65 kDa antigen indicate no expression of cpn60-1 under heat shock conditions - perhaps due to uniqueness of recognised epitopes to the 65 kDa antigen.
Hsp70 (DnaK)	• Chaperones with diverse roles in protein metabolism: involved in the regulation of the heat shock response; stimulate protein export; facilitate degradation of abnormal proteins; and stabilise newly made proteins *in vivo* (Hendrick and Hartl, 1993). • *E. coli* has a single hsp70, DnaK, that is present constitutively at high levels and is inducible upon heat treatment or other metabolic stresses, while most eukaryotic cells carry both heat-inducible and constitutive (hsc70) forms of hsp70 (Hendrick and Hartl, 1993). • *M. tuberculosis* 71 kDa antigen (carboxy-terminal one-third) exhibits 40% amino acid sequence identity with *E. coli* DnaK (Young *et al.*,1988).

	• Consist of a highly conserved N-terminal ATPase domain (+/- 450 amino acids) and a less conserved C-terminal peptide binding domain (+/- 220 amino acids). • Co-operates with heat shock proteins DnaJ (41kDa) and GrpE (22kDa). DnaJ stimulates ATP hydrolysis by DnaK, thereby increasing concentration of high affinity ADP-DnaK while GrpE stimulates ADP-ATP exchange (Liberek *et al.*,1991; Palleros *et al.*,1991). • In eubacteria, the organisation of the *dnaK* operons has changed during evolution - resulting in addition and deletion of genes, and the order of genes within the operon is not conserved (Segal and Ron, 1996). • Organisation of *dnaK* operons in eubacteria: *orfA-grpE-dnaK-dnaJ* (found in most of the low G+C Gram-positive bacteria,); *dnaK-grpE-dnaJ-orfX* (found only in high G+C Gram-positive bacteria including mycobacteria); and *dnaK-dnaJ* (found in most of the α- and γ-purple proteobacteria) (Segal and Ron, 1996).
Hsp90 (Lon)	• A molecular chaperone with ATP-dependent protease activity. • In *E. coli*, production of Lon-protease from the *lon* gene is stimulated by the presence of abnormal proteins (Goff and Goldberg, 1985). . The abnormal proteins that are rapidly degraded by the ATP-dependent Lon protease are bound by DnaK and GrpE proteins (Sherman and Goldberg, 1991). It has been suggested that co-operation between the folding pathway and the protein degradation systems may be used as an effective control over the level of abnormal proteins in the cell (Hendrick and Hartl, 1993). • In eukaryotes, homologous proteins have been identified mostly in the cytoplasmic face of the endoplasmic reticulum. Found to be involved in steroid receptor activation and maturation of certain kinases by associating with a number of protein cofactors or effector proteins (e.g. Hsp70, DnaJ) in addition to non-native proteins (Buchner, 1996). Prevents irreversible molecular interactions of non-native proteins and appears to stabilise proteins against inactivation under heat shock conditions. Function seems to be ATP independent - suggesting the existence of chaperone pathways in which Hsp90 could bind non-native protein and DnaK/DnaJ would guarantee their effective folding (Jakob and Buchner, 1994).
LMW Hsp (α-crystallin-like proteins)	• Diverse family of proteins of 15 to 30 kDa - evolutionarily related to α-crystallin vertebrate eye-lens protein whose structural function is to maintain proper refractive properties and transparency of the eye lens (Ingolia and Craig, 1982). • Characterised by the presence of the highly conserved "α-crystallin domain" - a carboxy-terminal domain of 80 residues that is sometimes present

in duplicate, and is composed of two similar hydrophobic β-sheet-rich motifs connected to by a hydrophilic α-helical region (Caspers *et al.*,1995).

- C-terminal domain preceded by an N-terminal region of variable length with little or no similarity between the various branches of the family - which range from prokaryotic and fungal to plant and animal representatives (Casper *et al.*,1995).
- Tend to form large aggregates (mass +/- 149 kDa) of triangular shaped (trimer of trimers) oligomeric structures (Chang *et al.*,1996).
- Functionally involved as stable structural proteins and as molecular chaperones during normal development (Jakob *et al.*,1993; Horwitz, 1992; Landry *et al.*,1993) and induced under pathological and stressful conditions (Nene *et al.*,1986; Nerland *et al.*,1988; Verbon *et al.*,1992).
- Mycobacterial homologues include:
  - 18 kDa immunodominant antigen of *M. leprae* - shown to have 31% and 52% amino acid sequence identity with 17 kDa soybean and 18 kDa *Streptomyces albus* LMW Hsps, respectively (Nerland *et al.*,1988; Servant and Mazodier, 1995). Inferred to be a heat-shock protein based on the demonstration of cross-reactivity of a heat inducible 18 kDa protein of *M. habana* (now considered to be *M. simiae* serovar 1) with the "*M. leprae*-specific" monoclonal antibody L5 (Lamb *et al.*,1990).
  - 16 kDa immunodominant antigen of *M. tuberculosis* - with respectively 26.9, 28.9, and 31.2% amino acid identity with 17 kDa soybean, 18 kDa *M. leprae*, and 26 kDa yeast LMW Hsps (Verbon *et al*, 1992).
    - Can suppress thermal aggregation or denaturation of proteins under heat shock in an ATP-independent manner - but unable to restore activity, suggesting that other proteins may be needed to achieve the native state of folding (Chang *et al.*,1996; Yuan *et al.*,1996).
    - Accumulation of antigen observed in stationary phase cultures and following shift to oxygen-limiting conditions. Not induced by 10 mM H_2O_2, 5% ethanol, low carbon, spent medium or cold shock. Over-expression in *M. tuberculosis* results in slower decline in viability following end of log-phase growth and produces an extended lag phase in recombinant *M. smegmatis* (Yuan *et al.*,1996).
    - *M. tuberculosis* cultured under anaerobic conditions shown to develop a thickened cell wall outer layer (absent in aerobically cultured *M. tuberculosis* or in *M. smegmatis*), with the 16kDa antigen found associated with cell envelope fibrous peptidoglycan-like structures and intracellular and peripheral clusters. Suggested that bacilli may adapt to low oxygen (and hence dormant state) by cell wall thickening, with α-crystallin stabilising cell wall structures (Cunningham and Spreadbury, 1998).

	• α-Crystallin gene found to be limited to slow growing *M. tuberculosis* complex organisms - H37Rv, H37Ra, *M. bovis* BCG and a clinical isolate. Not found in *M. avium* and *M. smegmatis* (Yuan, *et al.*,1996).

Table 1.3.: Mycobacterial Lipoprotein Antigens

Lipoproteins	**Structural and Functional Characteristics**
	Presence of 19, 26, 27, and 38 kDa lipoproteins first demonstrated by (Young and Garbe, 1991b). Anchored on membrane or cell wall via the lipid moiety, thus exposing the protein on bacterial cell surface. N-terminal with lipoprotein signal peptide.
• 19 kDa	• Glycosylated - a feature normally associated with eukaryotes (Herrmann *et al.*,1996).
• 23 kDa	• MPT83/MPB83 - with respectively 61 & 76% identity to secreted MPB/T70. (Matsuo *et al.*,1996; Hewinson *et al.*,1996; Harboe *et al.*,1996; Vosloo *et al.*,1997).
• 26 kDa	• Lipoprotein (Young and Garbe, 1991b).
• 27 kDa	• Identified in expression libraries using sera from *M. bovis* infected cattle (Bigi *et al.*,1997).
• 38 kDa	• MPT47 (LI=5) - 38% homology to *E. coli* PstS/PhoS - protein involved in phosphate transport (Harboe and Wiker, 1992; Chang *et al.*,1994).

1996). Hsp110 has been characterised in detail only in eukaryotic systems where it is found in the nucleolus of both heat shocked and normal cells (Subjeck et al, 1983), and is postulated to protect ribosome production (Nover *et al.*,1986).

In viable cells, critical parameters of cellular metabolism or macromolecular structure must be monitored constantly to regulate adaptive regulons or alternative developmental programmes. Heat shock proteins play important roles in the adaptive response, ensuring maintenance of protein integrity through their role as molecular chaperones (Gething and Sambrook, 1992). The term "molecular chaperone" was coined to describe the function of nucleoplasmin, an abundant protein in *Xenopus* oocytes, that binds to histones and promotes nucleosome assembly by donating the bound histone to assembling chromatin (Laskey *et al.*,1978). The term was subsequently applied to a larger family of proteins which are expressed under both normal growth conditions and in response to stress, and whose function is to bind to and stabilise an unstable conformer of another protein and determine its fate *in vivo*: be it protein folding, oligomeric assembly, transport to a particular subcellular compartment and degradation (Ellis, 1987; Ellis and vanderVies, 1991; Hendrick and Hartl, 1993; Buchner, 1996). Heat shock proteins can also be collectively induced by and provide an adaptive response to stress evoked by heat (Lindquist, 1986); carbon starvation (Jenkins *et al.*,1991; Matin, 1991); stringent response (Grossman *et al.*,1985); amino acid analogues and unfolded proteins (Goff and Goldberg, 1985); DNA damaging agents (Krueger and Walker, 1984); alkaline shift (Taglicht *et al.*,1987); exposure to antibiotics (Neidhardt and VanBogelen, 1987); cold shock (VanBogelen and Neidhardt, 1990); and exposure to reactive oxygen and nitrogen intermediates (Garbe *et al.*,1996b).

2.2.1. Supervising Protein Folding: The Function of Molecular Chaperones

The molecular mechanisms by which chaperones influence protein folding processes is still unresolved. Significant progress has, however, been achieved in the understanding of some of the partial reactions of the chaperone folding cycles and in differentiating between functionally different chaperone families (Buchner, 1996).

Traditionally, studies on molecular chaperone systems have largely focused on discussing each class of molecular chaperones separately, giving the impression that these components act independently within the cell. Emerging evidence suggests that there is co-operation between the different chaperone families to create chaperone networks that contribute to the overall chaperone capacity of the cell. For example, in eukaryotic systems, aggregation of peptides that are being transported from the cytosol to mitochondria is prevented by hsp70 (the mitochondrial DnaK homologue) presumably in co-operation with DnaJ homologues. Subsequent folding in a number of cases seems to depend on the transfer of the newly imported peptide to hsp60, the mitochondrial GroEL homologue (Kang *et al.*,1990; Ostermann *et al.*,1989; Manning-Krieg *et al.*,1991; Mizzen *et al.*,1991; Koll *et al.*,1992). It has been suggested that the sequential action of hsp70 and hsp60 may apply more generally to the folding of newly synthesised polypeptides, not only in mitochondria but also in the cytosol. Observations from *in vitro* studies using homologous systems in *E. coli* led to the formulation of the hypothesis that DnaK/DnaJ/GrpE and GroEL/ES could co-operate in the folding of newly synthesised proteins in the cytosol of *E. coli* (Langer *et al.*,1992, Hendrick and Hartl, 1993). A summary of the functions of molecular chaperone during protein folding and the proposed co-operation between DnaK/DnaJ and GroEL/GroES systems is outlined in figure 1.6.

It is well documented that in addition to stress-induced synthesis, heat shock proteins are constitutively expressed under normal growth conditions. It has been calculated that the time required for a large folding polypeptide to sample all possible conformations on its way to the native state would be of the order of the age of the universe (Levinthal, 1968). This calls into question the view that newly synthesised proteins in the cell may fold in a largely spontaneous process that is specified by its amino acid sequence and supports a protein folding role for molecular chaperones under normal conditions. *In vitro*, most proteins can be spontaneously refolded if conditions of low protein concentration and temperature are chosen so that folding is favoured over aggregation which is the major competing reaction. Aggregation of the early folding intermediates results from the exposure of aggregation-prone hydrophobic residues that are normally buried within the native proteins (Jaenicke, 1987; Kuwajima, 1989). In actively growing cells, aggregation prone, newly

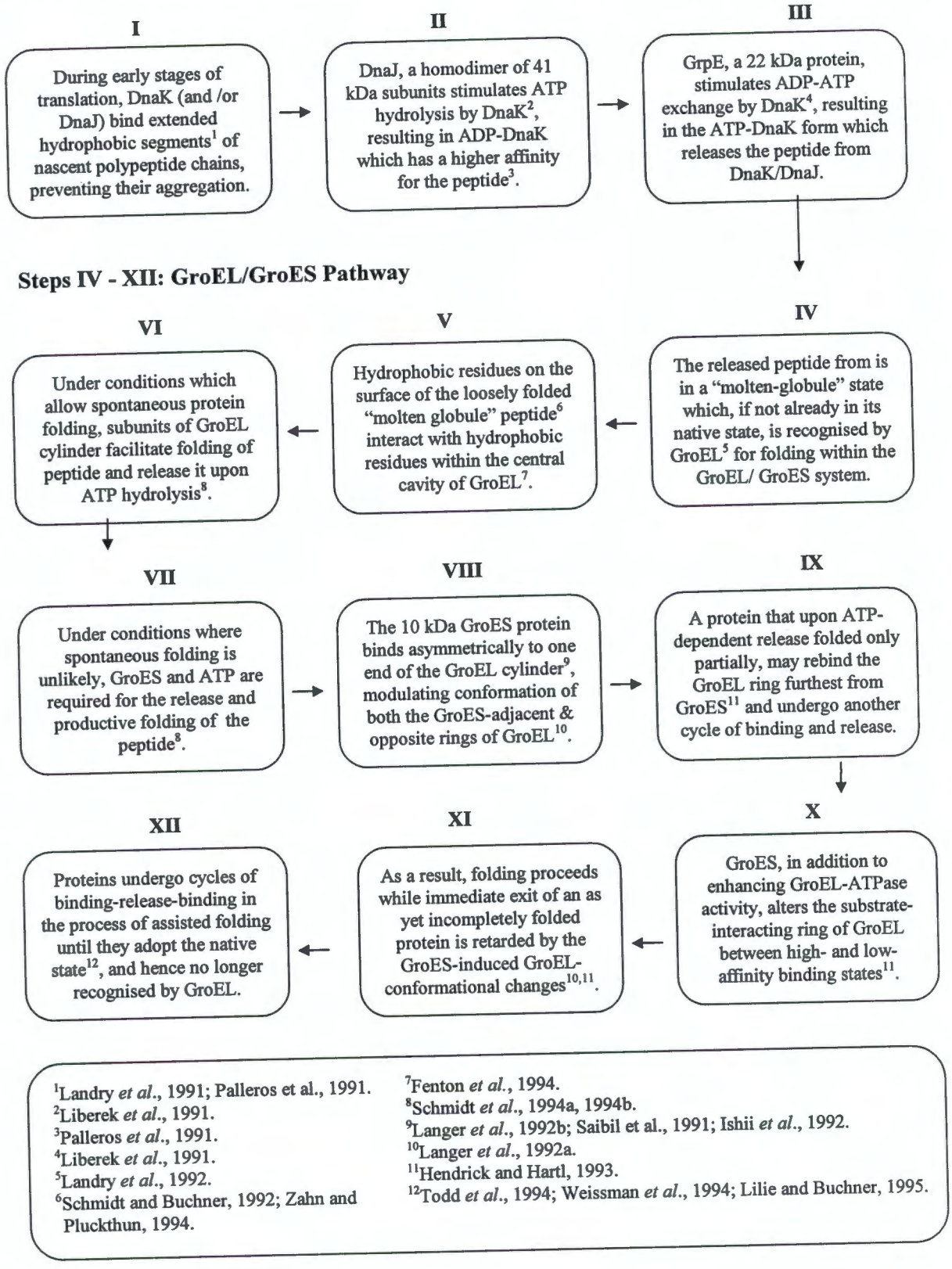
synthesised, polypeptides must be maintained in a folding-competent state at a wide range of temperatures and within the proteinaceous cytoplasm. Based on the concentration of ribosomes in a bacterial cell, the concentration of nascent polypeptides has been estimated to be as high as 50 μM (Darnell *et al.*,1986). As a direct consequence of the cellular conditions for protein folding, an important function of molecular chaperones is thought to be to prevent aggregation of partially folded nascent polypeptides and to ensure their proper folding (Hendrick and Hartl, 1993).

A recent study on a quantitative assessment of the role of chaperonin proteins in protein folding *in vivo* presents an opposing view (Lorimer, 1996). Using values drawn from a number of *in vitro* studies on chaperonin functions of GroEL and GroES, together with the known rates of *in vivo* protein synthesis by *E. coli*, and the known quantities of GroEL and GroES in *E. coli*, an assessment of the general role of these proteins in protein folding was made. The quantitative evidence suggests that there is insufficient GroEL and GroES in *E. coli* to act as a global facilitator of protein folding. Instead, under natural stress-free conditions more than 95% of cellular proteins fold independently of the chaperonins. It still remains unclear therefore whether the function of molecular chaperones under normal conditions would be to assist folding of all or just some of the newly synthesised proteins. In addition, molecular chaperones are made up of polypeptides and as such should be as susceptible to heat denaturation as other cellular proteins. Available literature to date does not appear to address the question of how molecular chaperones are protected from denaturation. Perhaps there is an as yet unidentified pathway or characteristic feature that is responsible for stabilising molecular chaperones under adverse conditions.

2.2.2. Regulation of the Heat Shock Response

Almost all available knowledge about regulation of the prokaryotic heat shock response is derived from studies in *E. coli*. Exposure of bacterial cells to a higher

Fig. 1.6: Co-operation between DnaK/DnaJ and GroEL/GroES Chaperones



[1]Landry *et al.*, 1991; Palleros et al., 1991.
[2]Liberek *et al.*, 1991.
[3]Palleros *et al.*, 1991.
[4]Liberek *et al.*, 1991.
[5]Landry *et al.*, 1992.
[6]Schmidt and Buchner, 1992; Zahn and Pluckthun, 1994.
[7]Fenton *et al.*, 1994.
[8]Schmidt *et al.*, 1994a, 1994b.
[9]Langer *et al.*, 1992b; Saibil et al., 1991; Ishii *et al.*, 1992.
[10]Langer *et al.*, 1992a.
[11]Hendrick and Hartl, 1993.
[12]Todd *et al.*, 1994; Weissman *et al.*, 1994; Lilie and Buchner, 1995.

temperature results in a rapid and transient increase in the cellular level of σ^{32}, the sigma subunit responsible for transcription of heat shock genes in prokaryotes (Cowing *et al.*,1985; Fujita *et al.*,1987; Taylor *et al.*,1984). The heat-shock transcription factor (HSF) is responsible for transcription of heat shock genes in eukaryotes such as yeast, flies, mice, and humans (Wu, 1995). The 32 kDa sigma subunit (σ^{32}) is encoded by the *rpoH* gene (Neidhardt and VanBogelen, 1981; Yamamori and Yura, 1982; Grossman *et al.*,1984), and controls a regulon that includes genes for about 20 proteins (Neidhardt and VanBogelen, 1984). σ^{32} recognises -35 and -10 consensus sequences TNtCNCcCTTGAA and CCCCATtT, respectively (Lindquist, 1986). In addition to initiating transcription from heat shock genes, σ^{32} levels and activity play a critical role in regulating the heat-shock response (Yura *et al.*, 1993). Figure 1.7 outlines the different levels at which σ^{32} influences the heat shock response i.e. σ^{32} levels (synthesis, stability, and degradation) and σ^{32} activity.

Recent studies in *Bacillus subtilis* have identified an additional mechanism for regulating the heat shock response. An additional element, CIRCE (controlling inverted repeat of chaperone expression) that is located near the 5′ end of *dnaK* and *groESL* operon within the untranslated region, has been shown to act as a negative *cis* element of the *dnaK* operon (Zuber and Schumann, 1994). The CIRCE element consists of a highly conserved 9bp inverted repeat (IR) separated by a 9bp spacer (TTAGCACTC-N_9-GAGTGCTAA), and has been found in both *dnaK* and *groESL* operons of more than 30 bacterial species (Schmidt *et al.*, 1992; Wetzstein and Schumann, 1990; Segal and Ron, 1996). Both operons are negatively regulated at the level of transcription by the interaction of HrcA repressor with its operator, the CIRCE element. In *B. subtilis*, the HrcA negative regulator is encoded by the *hrcA* gene found within the heptacistronic *dnaK* operon: *hrcA-grpE-dnaK-dnaJ-orf35-orf28-orf50* (Schulz and Schumann, 1996; Homuth *et al.*,1997). While the DnaK/DnaJ chaperone system inhibits the heat shock response by physically interacting with σ^{32} subunit (see Fig. 1.7), the GroE chaperonin system has been shown to be a major modulator of the CIRCE heat shock regulon (Mogk *et al.*,1997). Mogk *et al.* (1997) propose that a thermal upshift generates an increase in the

Fig. 1.7: Regulation of the heat shock response: role of σ^{32} (adapted from Yura *et al.*, 1993)

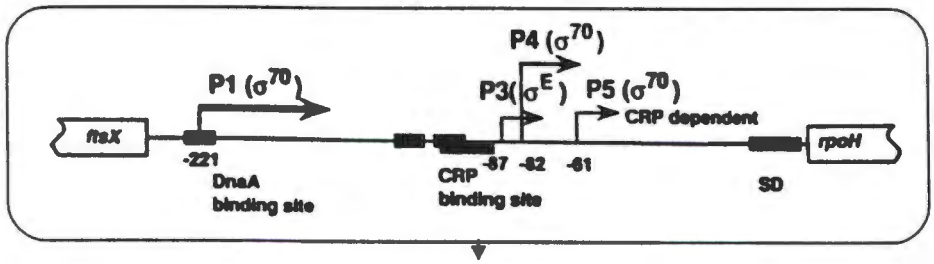
Mild Heat Shock (30°C–42°C)

- Initiates $E\sigma^{70}$ transcription from promoters P1, P4, P5. P1 (strongest promoter) & P4 responsible for transcription under most growth conditions (Erickson *et al.*, 1987; Fujita and Ishihama, 1987).
- P5 (weak promoter) - enhanced in absence of glucose & by addition of ethanol. Requires cAMP and CRP for *in vitro* function (Nagai *et al.*, 1990).

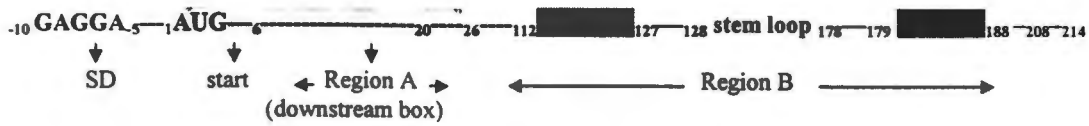
Lethal Temperature (50°C)

- Initiates $E\sigma^{E}$ (σ^{24}) transcription from P3 (Erickson and Gross, 1989; Wang and Kaguni, 1989).
- σ^{E} dependent genes may constitute a second heat-shock regulon whose function may be to strengthen or complement that of the σ^{32} regulon (Gross *et al.*, 1990).

***rpoH* gene**



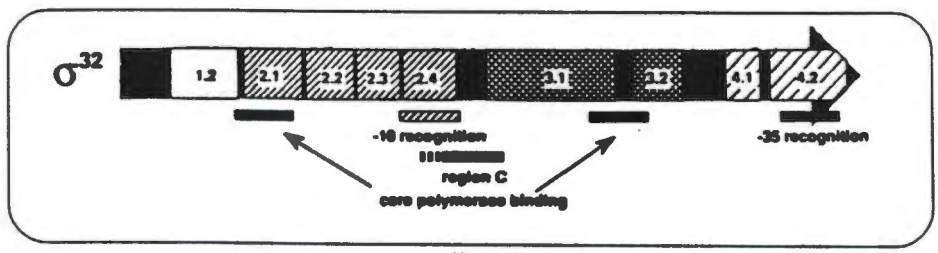
rpoH mRNA



Translational regulation of σ^{32} levels

Nucleotides 112 to 127 and 179 to 188 of region B, base pair with bases 26 to 12 and 11 to 1 of region A, respectively, thereby sequestering the initiation codon and inhibiting translation. Exposure to higher temperature may disrupt such a secondary structure, thus permitting higher rates of translation.

σ^{32} polypeptide



Regulation of σ^{32} level and activity

- Mild heat shock results in a 15- to 20-fold increase in σ^{32} levels within 5min, followed by a decline to new steady state level several fold higher than pre-shift level (Straus *et al.*, 1987). Increased σ^{32} level is due to de-repression at translation initiation (see above) and stabilisation of the usually unstable σ^{32} (half-life +/- 1 min under normal conditions). DnaK/DnaJ/GrpE chaperone is thought to be responsible for σ^{32} instability: either by binding σ^{32} and presenting it to an unidentified protein degradation system, or indirectly through their potential effect on protease activation (Gamer *et al.*, 1990; Liberek *et al.*, 1992; Yura *et al.*, 1993). DnaK/DnaJ is thought to bind σ^{32} through region C - a segment that is flanked by regions 2.1 and 3.1-3.2 which are responsible for binding to core RNA polymerase (Lesley and Burgess, 1989; Zhou *et al.*, 1992; Lonetto *et al.*, 1990), such that binding of RNA polymerase and DnaK/DnaJ may be mutually exclusive.
- An increase in temperature results in a sudden decrease in DnaK/DnaJ/GrpE owing to binding to unfolded/denatured proteins, thereby reducing its binding to σ^{32}, and facilitating association of σ^{32} with core RNA polymerase, and hence enhancing transcription of heat shock genes including DnaK/DnaJ. As the levels of DnaK/DnaJ become sufficiently high, they will resume binding to σ^{32}, repress or attenuate translation, destabilise σ^{32}, and prevent the association of σ^{32} with core RNA polymerase (Yura *et al.*, 1993).

concentration of non-native proteins, which temporarily depletes the free pool of GroE proteins. GroE proteins have been shown to be important for promoting renaturation of HrcA and preventing its spontaneous aggregation, thereby enabling HrcA to bind the CIRCE element and inhibit transcription from heat shock promoters. Depletion of GroE proteins leads to an increase in inactive HrcA, resulting in induction of heat shock genes due to lack of binding of HrcA to the CIRCE element. The *hrcA* gene has so far been sequenced from 10 species, and may be assumed to be present in all species where the CIRCE element has been described. Comparisons of the mechanisms involved in the regulation of *dnaK* and *groE* operons in different bacterial species demonstrate that in *E. coli*, both the *dnaK* and *groE* operons of are under σ^{32} control, while those of *B. subtilis* are both under HrcA control. Other bacterial species seem to inherit both mechanisms, i.e. the *dnaK* operon is under σ^{32} control while *groE* operon is controlled by HrcA (Mogk *et al.*,1997). The relative importance of both the σ^{32} and HrcA dependent regulatory mechanisms in controlling the overall heat shock response in different bacterial species remains to be clarified.

2.3. The Stationary Phase of Microbial Growth

One of the greatest challenges in designing more effective therapies for tuberculosis is to find drugs that will reduce the duration of treatment. Current antimycobacterial therapies kill actively growing cells within a few days, but need to be continued for long periods in order to eliminate dormant bacilli. Dormancy or stationary phase growth in *M. tuberculosis* is characterised by a state of non-replicating persistence with reduced metabolic activity and increased general stress resistance (Wallace, 1960; Wayne, 1976). The physiological and molecular changes that occur during differentiation of *M. tuberculosis* into the dormant state are still not clear. It is speculated that adverse factors such as low oxygen tension, oxygen radicals, and nutrient deprivation found within granulomas may induce the development of a dormant state (Dannenberg and Rook, 1994). Mechanisms which can inhibit the development of latency will not only shorten the duration of treatment, but will result in a considerable increase in patient compliance and hence reduce the incidence of drug-resistance.

2.3.1. Entry into Stationary Phase

Bacteria growing in batch culture eventually reach a point when growth rate decreases, indicating the onset of stationary phase. Exhaustion of any one of several essential nutrients may prevent growth (Groat *et al.*,1986; Spector *et al.*,1986; Nystrom *et al.*,1990). Starvation for a particular nutrient such as carbon, nitrogen, or phosphorus, results in the induction of specific regulons whose functions are designed to help the cell cope with that particular starvation. Each of these regulatory systems (cAMP/CRP for carbon sources, NtrB/NtrC/sigma-54 for nitrogen, or PhoR/PhoB for phosphorus) function via a sensor component that monitors the availability of a specific nutrient either directly or indirectly. For example, low NH_4^+ (monitored by NtrB protein) leads to the activation of the transcriptional regulator, σ^{54}, and transcriptional activator- NtrC, that turn on the expression of genes in the nitrogen regulon. The gene products of the nutrient starvation regulons, which involve 30 to 50 proteins, enable cells to perform functions which include high affinity uptake and metabolism of nutrients. When the induction of these regulons is insufficient to secure enough of the limiting nutrient, growth ceases and cells enter stationary phase, which is defined as the growth phase during which the number of bacteria ceases to increase.

The onset of stationary phase differs depending on the criteria used to define entry into stationary phase. For example, if optical density is used to measure cell growth, the onset of stationary phase is much earlier than if cessation of increase in cell numbers is used. This is because, as cells enter stationary phase, cell size (mass) decreases (hence the constant optical density), while the number of cells continues to increase. Entry into stationary phase therefore becomes a transition period beginning when cellular parameters cease increasing at proportional rates i.e. DNA, protein, and total cell mass no longer increase co-ordinately. It continues until no further increase in cell number is detected (Kolter *et al.*,1993).

2.3.2. Maintenance of Stationary Phase

2.3.2.1. Stationary Phase Regulons

While the proteins that are induced by each specific regulon vary widely depending on the conditions of starvation, a core set of 15-30 proteins is always induced in *E. coli* and has been designated as the stationary phase or 'Pex' (post-exponential) proteins (Matin *et al.*,1989). The existence of stationary phase specific regulons has been strengthened by the observation that blocking protein synthesis at the same time that cells are starved leads to decreased viability during stationary phase (Reeve *et al.*,1984). Stationary phase specific regulons may include gene products such as the *E. coli* DNA replication inhibitor whose promoter has been shown to be induced when cells enter stationary phase (Connell *et al.*,1987). A role in stress protection has been proposed for 'Pex' proteins since stationary phase cells are resistant to heat shock (>50°C) and high concentrations of H_2O_2 and NaCl (Matin, 1991; Jenkins *et al.*,1988; Jenkins *et al.*,1990). The multiple stress resistance found in cells that have never encountered a particular stress suggests that stationary phase cells may possess systems for DNA repair and the protection of membrane proteins that are not expressed in exponentially growing cells.

2.3.2.2. Morphological Differentiation

Physiological and molecular changes typical of stationary phase in *E. coli* include changes in cell morphology - where the rod-shaped cells become much smaller and almost spherical as a result of undergoing several cell divisions without an increase in cell mass (Ingraham *et al.*,1983; Siegele and Kolter, 1992). The cell envelope becomes covered with more hydrophobic molecules that favour adhesion and aggregation, with the membrane becoming less fluid due to the conversion of unsaturated membrane fatty acids to their cyclopropyl derivatives (Toumanen *et al.*,1988; Cronan, 1968; Kjelleberg *et al.*,1987). Chromosomal changes include the condensation of the nucleoid and an increase in negative DNA supercoiling consistent with reduced overall gene expression (Moyer and Morita, 1989; Baker *et al.*,1993).

An increase in the volume of the periplasm is accompanied by condensation of the cytoplasm (Reeve *et al.*,1984). The overall metabolic rate decreases with some endogenous metabolism maintained to enable the cell to take up nutrients and to resume growth when nutrients become available (Chapman *et al.*,1971; Kjelleberg *et al.*,1987).

Morphological differentiation in *Actinomycetes* such as *Streptomyces* involves the formation of physiologically distinct progeny. *Streptomyces* colonies growing on solid substrates undergo three principle stages of morphological differentiation: substrate growth, aerial mycelial growth and sporulation (Hopwood *et al.*,1973). *Streptomyces* undergoes two stationary phases during its growth cycle. The first stationary phase occurs in the middle of exponential phase (i.e. substrate growth) where a momentary cessation of growth is accompanied by the synthesis of antibiotic biosynthetic gene products (secondary metabolites) and a breakdown in the synthesis of macromolecules such as proteins and nucleic acids (Martin and Demain, 1980; Holt *et al.*,1992; Granozzi *et al.*,1990). The secondary stationary phase takes the form of spores whose characteristic feature is extreme resistance to a variety of environmental stresses, including resistance to the antibiotics they produce. Antibiotic secretion only occurs after the growth phase nears completion since antibiotic-producing species are sensitive to their own antibiotics during growth (Martin and Demain, 1990). The fact that even the naturally produced antibiotics that have been identified so far are only active against exponential phase cultures points out the enormity of the task of finding compounds that will be active against dormant mycobacteria. Of interest is the observation that the mechanisms of drug resistance adopted by various bacteria largely mimic those employed by antibiotic producers to avoid 'suicide' from their own antibiotics. These include modification (and thus detoxification) of the antibiotic by enzymes formed by the antibiotic producing strain, alteration of the antibiotic target in the producing cell, and a decreased uptake of the antibiotic (Demain, 1974). The recent finding that a homologue of the sporulation sigma factor SigF of *S. coelicolor* is present in slow-growing mycobacteria and that it is only strongly induced during stationary phase, nitrogen depletion, and cold shock (DeMaio *et al.*,1996), supports the possibility that dormancy in mycobacteria may represent differentiation into a spore-like state.

2.3.2.3. Regulation of the Stationary Phase Response

A major regulator of the general starvation response and an inducer of many genes in stationary phase has been identified in *E. coli* as the alternative sigma factor RpoS (σ^S), a product of a gene designated *rpoS* (Lange and Hengge-Aronis, 1991; Hengge-Aronis, 1996), and previously identified as *katF* or *appR* (McCann *et al.*,1991; Touati *et al.*,1986). Sigma S controls a regulon of 30 or more genes expressed in response to starvation and during the transition into stationary phase. The gene products have a diverse group of functions including protection against DNA damage, the determination of morphological changes, the mediation of virulence, osmoprotection, and thermotolerance (Loewen and Hengge-Aronis, 1994). Expression of the *katF* gene increases upon entry into stationary phase (Mulvey *et al.*,1990), but whether this is sufficient to account for the induction of stationary phase genes remains to be shown (Siegele and Kolter, 1992). The MysB sigma factor of *M. tuberculosis* was recently identified as a homologue of KatF and shown to reduce the growth rate when over expressed in *M. smegmatis* (Mulder, 1998). The observation that approximately 20 of the proteins induced by carbon starvation are KatF independent suggests that sigma S (KatF) is not the only regulator involved in the general starvation response (McCann *et al.*,1991). This group includes three heat shock proteins (DnaK, GroEL, and HtpG) whose induction during starvation is dependent on σ^{32} rather than KatF (Jenkins *et al.*,1991). Evidence from genetic analyses using transcriptional and translational fusions of *rpoS* and sigma S-dependent genes indicates that regulation of RpoS expression occurs at both transcriptional and post-transcriptional levels (Lange and Hengge-Aronis, 1991; Loewen *et al.*,1993; Mulvey *et al.*,1990; Hengge-Aronis *et al.*,1993), possibly in a multilevel regulation system reminiscent of regulation of σ^{32} during the heat shock response.

2.3.3. Resumption of Growth following Stationary Phase

Stationary phase, non-sporulating, bacteria only require the addition of nutrients to resume growth, while germination of bacterial spores is stimulated by activating agents such as heat, low or high pH, or reducing agents, before growth of the

germinated spore occurs (Foster and Johnstone, 1989). The rapid response to addition of nutrients suggests that uptake systems for nutrients such as glucose, mannitol, arginine, and leucine remain active during starvation (Albertson *et al.*,1990c; Davis and Robb, 1985; Faquin and Oliver, 1984; Marden *et al.*,1987). RNA synthesis restarts almost immediately after addition of nutrients while protein synthesis lags a short time (Albertson *et al.*,1990a). The initial protein synthesis is not inhibited by rifampicin, suggesting that it results from translation of mRNA already present within the cell (Jacobson and Gillespie, 1968). Cells start to increase in size while DNA synthesis lags, presumably reflecting the time needed for the cell mass to reach a critical level (Albertson *et al.*,1990b; Amy *et al.*,1983). At some point after resuming growth, cells lose their enhanced resistance to various environmental challenges (Albertson *et al.*,1990b). In *Vibrio* sp. S14, the synthesis of approximately 20 starvation-induced proteins is rapidly repressed and synthesis of at least three groups of temporarily regulated proteins is induced: i.e. group I, group II and group III proteins which are induced within the first 20 min, after 20 min and 80 min, respectively (Albertson *et al.*,1990b). In *E. coli*, Fis protein is induced during recovery from stationary phase and over-expression in stationary phase cells results in reduced viability (Ball *et al.*,1992).

There is still a need to understand the mechanisms governing the molecular and physiological changes that occur during stationary phase. A question raised by Siegele *et al.* (1992) asks if under stationary phase conditions, where only a fraction of the cells in a population survive what, if anything, is different about the survivors? Are the survivors a random sample of the population or do they represent a fraction of the population that entered stationary phase on a different pathway? Are they cells that were at a point in the cell cycle that enabled them to respond differently to the stationary phase-inducing stress? Kolter *et al.* (1993) suggest that the physiological processes that are so well characterised during exponential growth undergo interesting and varied changes during stationary phase and are still waiting to be explored.

2.4. Aims of the Present Study

The recently completed sequencing of the *M. tuberculosis* genome has made major strides in facilitating mycobacterial research. The available genetic information enables the prediction of the amino acid sequence of every protein in the genome. The prospect of correlating species-specific domains with phenotypic characteristic traits such as slow growth, virulence and dormancy may also become a reality. Where genetic analysis falls short however, is in providing information on when the predicted gene products are translated into functional proteins and at what concentrations, and hence their roles in polygenic processes and complex phenotypes occurring within the cell. To address this shortfall, attention is now being redirected towards studies on the 'proteome', i.e. the total protein output encoded by a genome under specified growth conditions (Humphery-Smith and Blackstock, 1997). This is the aim of the present study.

In an attempt to define the mycobacterial stationary phase or dormant state, and as an initial step towards characterisation of the global molecular and morphological changes that cells undergo during transition into stationary phase, protein expression profiles of cells at different stages of growth - from exponential to stationary phase - were compared to identify proteins and hence regulons that are specifically induced as cells enter stationary phase. It is believed that the toxic conditions found within granuloma in lungs of infected patients may act as a physiological stress that induces mycobacterial dormancy. Hence protein synthesis under both normal growth conditions and after heat shock stress was investigated. Heat shock provided an ideal stress condition for several reasons: (i) heat shock proteins account for a large fraction of the immunodominant mycobacterial antigens, and as such have been implicated in the interaction of bacilli with the host's immune system; (ii) in addition to heat shock induction, the heat shock regulon has been shown to be induced by other stresses such as DNA damaging agents and reactive oxygen and nitrogen intermediates, all of which mimic some of the immune defence mechanisms encountered by infecting bacilli; (iii) stationary phase cultures of *M. tuberculosis* have been shown to be more resistant to heat kill than actively growing cultures, suggesting a growth phase dependency in the cell's response to heat shock; and (iv) recent studies on heat shock protein synthesis in

S. coelicolor suggest that control of the heat shock regulon is growth phase dependent (Puglia *et al.*,1995). *Mycobacterium smegmatis*, a fast-growing non-pathogenic member of the genus, was used in the present study since it is much easier to grow and to radiolabel. In addition, unlike the slow growers which tend to form clumps of cells especially towards stationary phase, *M. smegmatis* grows as single cells and as such will enable transition into stationary phase to be monitored more accurately. It is hoped that information obtained from studies in *M. smegmatis* will enable comparisons to be made between pathogens which tend to be slow-growers and fast-growing non-pathogens. Such comparisons should assist in the elucidation of mechanisms that are specific to pathogens and hence the identification of potential virulence determinants.

Chapter 2

Construction of *Mycobacterium smegmatis* Growth Curves and Standardisation of Heat Shock Conditions

2.1. Introduction

A major problem with studies on the dynamics of growth of stirred or agitated cultures of *M. tuberculosis* is the tendency of the bacilli to form clumps, thereby making it difficult to follow growth quantitatively since it becomes difficult to measure the cells optically or to plate and count them accurately. The cell clumps are believed to contain bacilli in various stages of oxygen deprivation such that bacteria within a single culture represent a physiologically heterogeneous population (Wayne and Sramek, 1979). Addition of detergents such as polyoxyethylene sorbitan monooleate (Tween 80) largely overcomes clumping during exponential growth, but not in stationary phase cultures which tend to clump severely as the culture gets older. Cell clumping also presents problems in metabolic labelling studies where penetration of radioactive metabolites or stresses such as heat is not uniform to all cells, thereby making the interpretation of such results difficult particularly if exponential phase and stationary phase responses are to be compared.

For the present studies, a detailed analysis of total protein synthesis under normal growth temperature and in response to heat shock at different stages of growth, from exponential to late stationary phase, was required. To overcome the problems associated cell clumping when using *M. tuberculosis*, a non-clumping member of the genus, *Mycobacterium smegmatis* was used in these studies. It is hoped that since *M. tuberculosis* and *M. smegmatis* are members of the same genus, stationary phase specific regulons that may be identified in *M. smegmatis* will be similarly applicable in *M. tuberculosis*. If differences do exist, they may assist in identifying the cellular mechanisms which enable *M. tuberculosis* to be a virulent, slow-grower while *M.*

smegmatis is a non-pathogenic fast-grower. Growth curves for *M. smegmatis* have not been published previously, and as a result it was necessary to construct detailed growth curves using various methods of bacterial enumeration, i.e. optical density (OD) of culture, assay of bacillary adenine triphosphate (ATP), and the counting of colony forming units (CFU) on solid medium. The optimum experimental conditions (i.e. incubation period and heat shock temperature) required for induction of the heat shock response in *M. smegmatis* were also determined.

2.2. Materials and Methods

2.2.1. Bacterial Culture Conditions

Mycobacterium smegmatis LR222 (kindly provided by J. Crawford, Centers for Disease Control, Atlanta), was used throughout this study. Colonies of *M. smegmatis* were grown on Middlebrook 7H9 agar plates [made from Middlebrook 7H9 broth (Difco Laboratories) and 10% (w/v) bacteriological agar No. 1 (Biolab), supplemented with 5% (w/v) bovine serum albumin fraction V (Boehringer Mannheim), 2% (w/v) D (+) glucose, and 0.05% (v/v) Tween 80, hereafter referred to as ADT supplement]. A single colony was used to inoculate 20ml of Middlebrook 7H9 medium supplemented with ADT (Middlebrook 7H9/ADT medium) and incubated in an orbital shaker in a 37°C room for 48h. The cells were then sub-cultured into fresh medium (1/1000 dilution) and incubated under the same conditions in a tightly capped bottle (Schott) for 14 days to select for a uniform population of cells. Stocks of the 14 day old stationary phase cultures were frozen at -70°C in 15% (v/v) glycerol and were used to inoculate Middlebrook 7H9/ADT medium for growth curve and heat shock experiments.

2.2.2. Microscopic Examination of M. smegmatis

The Ziehl-Neelsen procedure (Heifets and Good, 1994) was used for staining cells for microscopic examination of changes in cell morphology during growth. The staining

procedure depends on the ability of mycobacteria to retain dye after treatment with an acid alcohol solution (acid-fastness). A heat-fixed smear of cells was flooded with carbol-fuschin stain (prepared by dissolving 0.3 g of basic fuschin in 10 ml of ethanol and then diluting to 100ml with aqueous 5% phenol). The smear was gently heated to steaming with the flame from a Bunsen burner for 5 min. The slides were washed in tap water and then flooded with 3% hydrochloric acid in 95% ethanol. The smear was washed with tap water to stop the action of the acid alcohol and then counter-stained by flooding with 3% aqueous methylene blue for 1 min. After rinsing with water, draining, and air-drying, the smear was examined under oil immersion objective (x100 magnification). Mycobacteria appeared as red-stained rods on a blue background.

2.2.3. M. smegmatis *Growth Curves*

Middlebrook 7H9/ADT medium was inoculated (1/1000 dilution) with 14-day old frozen aliquots of *M. smegmatis* cells. The volume of medium:culture vessel volume was 1:5 and the culture was incubated in a cotton-wool stoppered Ellenmeyer flask in a 37°C room with continuous shaking. Aliquots were taken from the shaking culture at two-hour intervals and used to enumerate bacterial cells by determining the number of colony forming units (CFU), the adenosine triphosphate (ATP) concentration of the culture, and the optical density (OD) at 600nm. The number of CFU/ml was determined by plating dilutions of aliquots on Middlebrook 7H9 agar plates. The ATP concentration ([ATP]) was quantified using the bioluminescence ATP Assay Kit (Promega). The principle of the ATP assay involved the use of the luciferase catalysed conversion of luciferin to the activated oxyluciferin in the presence of ATP. The amount of light emitted, given in relative light units (RLU), when oxyluciferin decayed to the ground state was equivalent to the ATP concentration of culture, and was detected by using a 1253 Luminometer (BioOrbit). ATP was extracted by mixing an aliquot of the growing culture with 1.4% trichloroacetic acid (TCA) followed by a 30 min incubation on ice. TCA releases cellular ATP by making the cell wall permeable (Stanley, 1989). The solution was neutralised with neutralising buffer [0.1 M Tris-acetate, pH 7.75] to avoid inhibition of the luciferase enzyme, and thereafter diluted 6.6-fold with ATP-free water. The ATP extract was added to a mixture of luciferin and

luciferase (L/L) (Promega) and the sample RLU determined. Thereafter, an internal ATP standard was added to a final concentration of $1x10^{-10}$ M ATP and the total RLU determined.

$$\text{ATP concentration of each sample} = \frac{(RLU_{sample} - RLU_{L/L})}{(RLU_{ATP\ std.} - RLU_{sample})} \times 10^{-10}\,M$$

The values obtained for ATP concentration, the number of CFU, and the OD_{600} were used to construct growth curves of *M. smegmatis* over a period of 72 hours.

2.2.4. Heat Shock and Protein Labelling

De novo protein synthesis was monitored by an adaptation of the methods described by Young and Garbe (1991) and Patel *et al.* (1991). To avoid affecting the growth dynamics of the culture, no more than 20% of the total volume of the culture was taken during sampling. Aliquots equivalent to $5x10^8$ cells ($5x10^{-6}$ M ATP) were taken at regular intervals during growth and centrifuged at 3000 rpm for 10 min at room temperature. The cell pellets were resuspended in 1 ml fresh Middlebrook 7H9/ADT medium pre-warmed to either 37°C (control) or heat shock temperature (as specified in section 2.3.5. under Results), and thereafter dispensed into microcentrifuge tubes. 10 μCi of ^{35}[S]L-methionine (specific activity 1000Ci/mmol; Amersham) was immediately added and the microcentrifuge tubes were incubated for the time intervals specified in Results section 2.3.5. below. At the end of each incubation period, non-radioactive methionine was added to a final concentration of 10 mM and the labelled cells collected by centrifugation at 13000 rpm for 3 min at room temperature. The cell pellets were kept frozen at -20°C until processed for protein extraction and electrophoretic analysis.

2.2.5. Cellular Protein Extraction

Cell pellets were washed twice in 1ml phosphate buffered saline (PBS) (pH 7) and resuspended in 3% sodium dodecyl sulphate (SDS) buffer [10% (v/v) glycerol; 3% (w/v) SDS, 0.0625 M Tris-HCl (pH 6.8)] before boiling at 100°C for 20 min to extract proteins. Cell debris were removed by centrifugation at 13000 rpm for 3 min and the supernatants (cell lysates) were transferred to clean tubes. The protein concentration of cell lysates was determined using a colorimetric assay based on the procedure of Lowry *et al.* (1951) as specified in the protocol for the Protein Assay Kit (Bio-Rad).

2.2.6. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) analysis of the protein extracts in the presence of SDS was carried out using an adaptation of the method described by Laemmli (1970). The polyacrylamide gels were cast using the Vertical Slab Gel Electrophoresis Unit (Hoefer Scientific Instruments, California, USA). The stacking gel contained 4.5% acrylamide while the lower resolving gel had 15% acrylamide with an acrylamide to *N', N'*-methylene-bisacrylamide ratio of 30:0.8. Aliquots of cell lysates equivalent to 20μg of protein were mixed with one-tenth the volume of a solution of bromophenol blue (10% w/v) and β-mercaptoethanol (50% v/v). The samples were boiled for 5 minutes before loading on polyacrylamide gels. Electrophoresis was performed in electrophoresis buffer [0.3% (w/v) Tris, 1.4% (w/v) glycine, 0.1% (w/v) SDS], followed by staining of the gels in 0.1% Coomasie Blue R250 (Pharmacia) to visualise the molecular weight markers. After destaining in a solution of 30% (v/v) methanol and 10% (v/v) acetic acid, the gels were fixed overnight in a solution of 6% (v/v) glycerol and 20% (v/v) methanol, followed by drying under vacuum at 65°C and exposing to Hyperfilm β-max film (Amersham) at -70°C. Densitometric analysis of autoradiograms was carried out using a Shimadzu CS-9000 dual-wavelength flying spot scanner. Incorporation of label into each protein, and hence the amount of newly synthesised protein, was determined from the

density of each band and the area under the corresponding peak in the densitometric scan.

2.3. Results and Discussion

2.3.1. Culture of M. smegmatis

For the study of the dynamics of growth, it was necessary to perform bacterial cultivation under well defined conditions to produce accurate and reproducible results. Synchronisation of the cultures was achieved by maintaining cultures at stationary phase for a period of 14 days to select for a physiologically homogeneous stationary phase cell population. To avoid the physiological changes that would result if the culture was maintained by serial passage from culture to culture, the 14-day old culture was subdivided into small aliquots that were stored at -70°C and used directly to seed growth curve cultures. The culture conditions were designed in such a way that the same conditions could be used to support growth of both *M. smegmatis*, which grows as single cells, as well as bacilli such as *M. tuberculosis* which have a tendency to form clumps. Preliminary studies using *M. bovis* BCG grown in different mycobacterial media (i.e. Sauton, Middlebrook 7H9, and Kirchner) demonstrated that Middlebrook 7H9 medium supplemented with 0.05% Tween 80 (a polyoxyethylene derivative of sorbitan monooleate), provided a culture with the least amount of cell clumping. Tween 80 concentrations of 0.1% and above inhibited growth of *M. bovis* BCG. Tween 80 releases into the medium traces of free oleic acid which is toxic to mycobacterial cells (Wayne, 1994). The introduction of albumin into the medium overcomes the toxic effect by forming complexes with the oleic acid thereby enabling bacteria to grow.

2.3.2. M. smegmatis *Growth Curves*

Experimental methods that have been used for quantifying growth of mycobacterial cells in liquid culture include measurements of turbidity (absorbance or OD), plating and counting of colonies (CFU), and measurements of the 'energy' content of the cells ([ATP]). Growth curves obtained using OD_{600}, CFU and [ATP] were compared to analyse the dynamics of growth of *M. smegmatis*. A close correlation was observed between growth curves obtained by plotting ATP concentration or OD_{600} against time. Both curves increased exponentially up to 26 hours before plateauing (i.e. reaching stationary phase) from 28 hours (Fig. 2.1). In order to assess whether the plateau in the [ATP] curve was not due to saturation of enzyme or substrate in the ATP assay at high [ATP], up to 200-fold more ATP than that present in the internal ATP standard (with 7000-fold more RLU than a stationary phase cell extract) was added to the mixture of luciferin and luciferase and the RLU determined. Increasing amounts of ATP resulted in a proportional increase in the amount of light emitted from the ATP assay, demonstrating that the amounts of both luciferin and luciferase were not limiting at high ATP concentrations, and hence illustrating that the plateau observed in the ATP curve (from 28 hours onwards) was not due to saturation of enzyme or substrate.

The exponential increase in CFU curve followed a similar trend to the [ATP] and OD_{600} curves up to 28 hours. Thereafter, while [ATP] and OD_{600} plateaued, the CFU curve (after a four-hour lag between 32 and 36 hours) was followed by a second phase of exponential growth (36 to 40 hours), and hence only reached stationary phase 12 hours later. The growth curve obtained when plotting the number of CFU against growth phase gave a stepwise increase in cell number, i.e. with plateaus between 12 and 16 hours; 20 and 24 hours; and 32 and 36 hours (Fig. 2.1). At each step the cell concentration increased approximately seven fold.

Microscopic examination of Ziehl-Neelsen stained cells throughout growth demonstrated that the cell shape changed from the much larger hyphae-like bacilli shape observed during exponential phase to form smaller and more compact cells during stationary phase. A reduction in cell size has been reported as one of the typical morphological features of stationary phase cells, and has been attributed to several cell

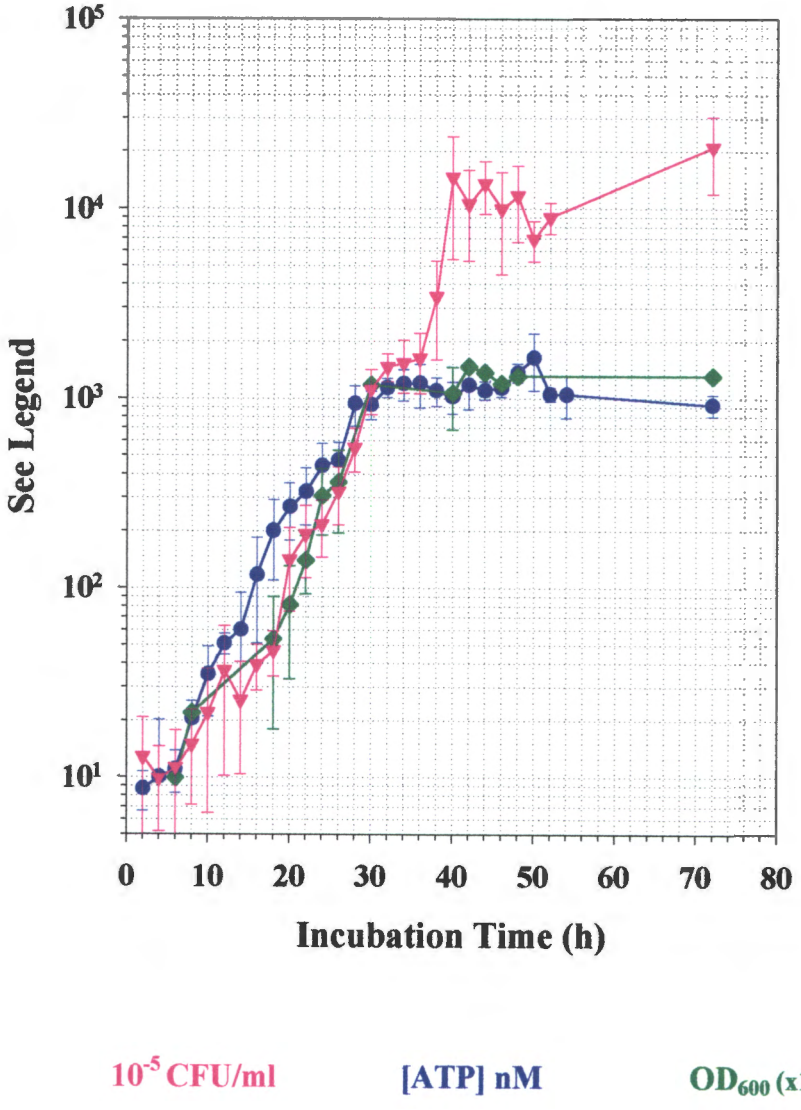


Fig. 2.1.: Growth curves of *M. smegmatis* grown with shaking in a liquid culture at 37°C. Different methods of bacterial enumeration, i.e. ATP concentration (nM), number of CFU/ml, and OD_{600} were used. A close correlation between the [ATP], OD_{600}, and CFU curves was observed from lag phase until late exponential phase (26 hours). Thereafter, the [ATP] and OD_{600} curves reached a plateau from 28 hours onwards while the number of CFU, after a transitory cessation in increase between 30 and 36 hours, followed by a second stage of exponential growth from 36 to 40 hours, plateaud 12 hours later i.e. from 40 hours. Data points (mean ± SEM) were obtained from three independent experiments. The difference between the stationary phases of the [ATP] and CFU curves (i.e. between 40 and 72 hours) was significant (P = 0.5, Students' t-test).

divisions occurring without an increase in cell biomass (Ingraham *et al.*,1983; Siegele and Kolter, 1992). It may be speculated that the second exponential increase in the number of CFU observed between 36 and 40 hours may be a result of cells undergoing further division to enable them to form the smaller spherical cells seen during stationary phase. The potential significance of the plateau between 32 and 36 hours in relation to the entry of cells into stationary phase is discussed further in section 2.3.4. below.

The ATP concentration was the method of choice for the regular monitoring of cellular growth for several reasons. The shapes of both the [ATP] and OD curves gave a more readily distinguishable point of cellular entry into stationary phase (from 30 hours) than the monitoring of cell size or the number of CFU. A shortfall in the use of OD for monitoring growth arises from the fact that at high cell concentrations (OD values greater than 0.2), a departure from linearity arises due to particle coincidence and necessitates correction. A correction curve which employs dilutions of barium sulphate suspension of known concentrations has to be used to allow for adjustment from observed to true OD values (Wayne, 1976). In addition, for mycobacteria that tend to form large clumps especially towards stationary phase, OD values tend to be inaccurate, necessitating the use of techniques such as sonication to disrupt the cell clumps. In the ATP assay on the other hand, both clumped and free cells are equally accessible to TCA and hence easily permeabilised to release ATP. The ATP content of culture is also useful in that it reflects their metabolic activity, and in addition gives an indication of cell size since the amount of ATP per cell is equivalent to cell mass (Stanley, 1989). The main advantage of the ATP assay is its speed (results obtained within half an hour) and sensitivity i.e. can detect and measure ATP below 1 picogram, which is equivalent to about 1000 'average' bacterial cells (Stanley, 1989).

2.3.3. A cessation in cell division precedes stationary phase

In the present studies, a cessation in the increase in number of CFU (i.e. between 30 and 36 hours) preceded entry into stationary phase (Fig. 2.1). This cessation in cell division differed from that observed during exponential phase (between 12 and 16

hours and between 20 and 24 hours) in that a 5-fold increase in the number of CFU during the subsequent cell division phase was achieved within 4 hours whereas it took 8 hours to achieve the same increase during exponential phase. It is conceivable that such a plateau may represent a stage during which gene products that regulate mechanisms which facilitate entry into stationary phase are expressed. It may be that DNA replication, and hence cell division, are inhibited to enable transcription of genes required for entry into and maintenance of stationary phase. Previous studies in *Streptomyces coelicolor* A3(2) demonstrated that a transitory cessation of growth at the end of vegetative mycelium formation preceded aerial mycelium formation (Granozzi *et al.*,1990). The cessation of growth, which was presumed to be induced by a physiological stress to the cells, was accompanied by a reduction in protein and nucleic acid synthesis which was only restored when morphological differentiation to form aerial mycelium resumed. Studies in *Streptomyces* have also shown that the mid-exponential phase plateau corresponds to a phase during which gene products for antibiotic synthesis are synthesised (Holt *et al.*,1992). In addition, homologues of the four major groups of heat shock proteins (GroEL1, GroEL2, Hsp90, DnaK, and DnaJ) are also synthesised at their highest levels during this phase (Pugllia *et al.*,1995). A possible scenario is that perhaps even in bacteria whose morphological differentiation is not as physiologically distinct as that of *Streptomyces*, a physiological stress may, for example, lead to the morphological differentiation normally associated with stationary phase cells (as discussed in chapter 1, section 2.3.2.2).

2.3.4. A decrease in the rate of accumulation of FtsZ precedes stationary phase

An analysis of cellular levels of the cell division protein - FtsZ was used to determine if cell division was inhibited during the lag in the increase in number of CFU between 32 and 36 hours (Fig. 2.1). FtsZ is an essential cell division protein in eubacteria and has been shown to be conserved in archaebacteria (Corton *et al.*,1987; Wang and Lutkenhaus, 1996). In *E. coli*, FtsZ is a tubulin-like 46 kDa protein that has GTPase activity and the ability to polymerise *in vivo*. It functions by forming a ring (Z-ring) at the division site and directs septation or cytokinesis by bringing about invagination of the cytoplasmic membrane (Bi and Lutkenhaus, 1991). A specific enzyme PBP3 (FtsI)

and other proteins (FtsQ, FtsA, FtsL, and FtsW) are responsible for co-ordinating in-growth of the peptidoglycan cell wall at this location. EnvA protein is required to split the resultant cross wall to form new cell poles (Donachie, 1993; Lutkenhaus and Addinall, 1997). Upon completion of division, FtsZ is not retained at the new cell pole, but is released into the cytoplasm (Lutkenhaus, 1993).

For analysis of cellular FtsZ levels in the present study, aliquots of cells were taken at regular intervals from mid-exponential phase (16 hours) to stationary phase (48 hours). Cellular protein extraction and electrophoretic analysis were carried out as described in sections 2.2.5 and 2.2.6 above, and were followed by immunoblot analysis as described in appendix I. A rabbit polyclonal antiserum against *E. coli* FtsZ (anti-FtsZ) was used at 1/1000 dilution in the immunoblot assay. Immunoblot analysis using anti-FtsZ polyclonal antiserum revealed a strong cross-reacting protein of approximately 46 kDa. The autoradiograph was scanned and the density of each band (arbitrary units) plotted against growth phase (Fig. 2.2). The levels of FtsZ (which were monitored from 16 to 48 hours) showed a progressive increase from 16 hours (exponential phase) until they peaked at 44 hours (stationary phase). The observed increase in FtsZ levels with increasing number of CFU during exponential phase may reflect the accumulation of FtsZ protein as cell division progresses and new cells continue to synthesise the protein. The decline in FtsZ levels observed between 44 and 48 hours may be a result of increased degradation such that the protein is maintained at a lower level in the absence of cell division during stationary phase, but in sufficient quantities to enable cell division to occur when growth resumes.

The levels of FtsZ were expected to plateau between 32 and 36 hours since no increase in FtsZ levels was expected in the absence of cell division during the lag in number of CFU between 32 and 36 hours. Instead of a plateau, the rate of accumulation of FtsZ was biphasic - increasing from a doubling time of 5 hours between 16 and 32 hours, to a doubling time of 14 hours between 36 and 44 hours. The accumulation of FtsZ without the corresponding increase in cell number (i.e. between 32 and 36 hours) may result in an increase in the FtsZ content of each cell. Such FtsZ accumulation may signal the initiation of a different phase of cell division, i.e. from cell division that is accompanied by an increase in cell mass seen during

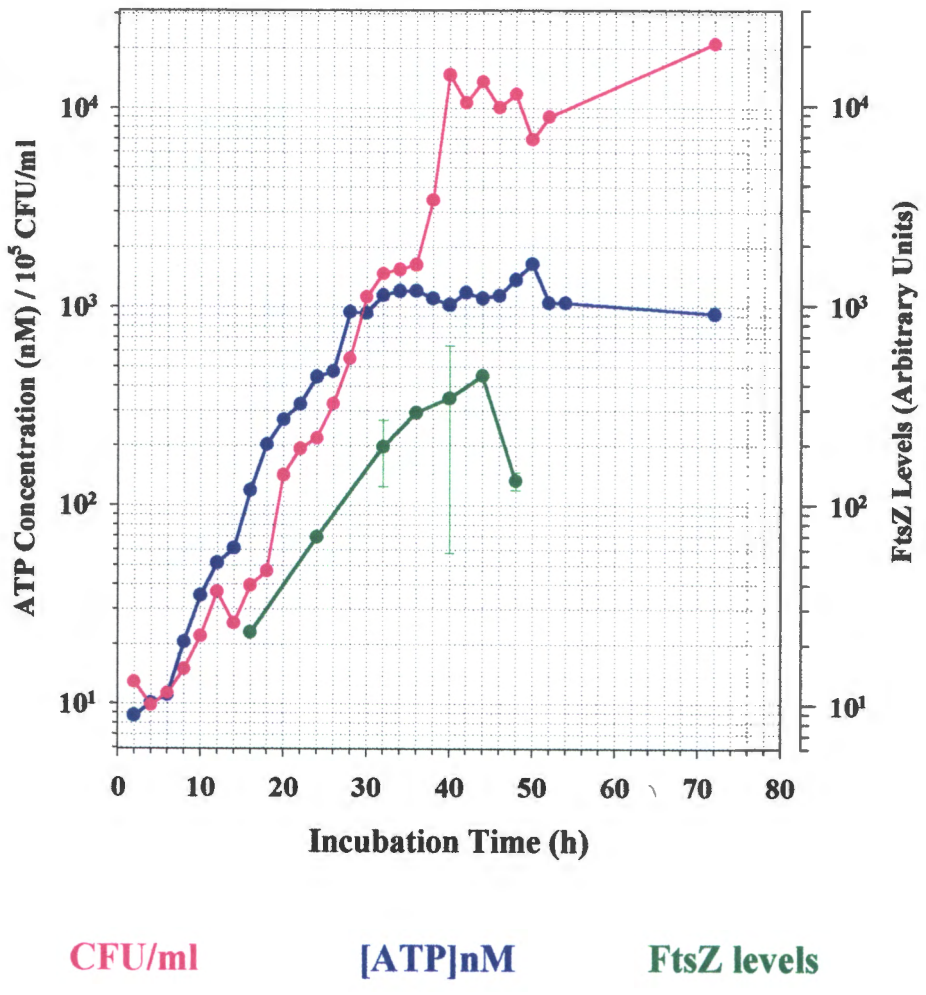


Fig. 2.2.: Cellular levels of FtsZ protein relative to growth phase of culture (i.e. intensity of bands from autoradiographs of immunoblots with 20μg total cellular protein per time point). The error bars represent standard error of the mean from two experiments. Data points for ATP and CFU curves are the same as in Fig. 2.1. FtsZ levels increased progressively from 16 to 44 hours before showing a decline by 48 hours. The cessation in cell division between 32 and 36 hours, in addition to coinciding with a plateau in the ATP curve, preceded a decrease in the rate of accumulation of FtsZ, i.e. from a doubling time of 5 hours (between 16 and 32 hours) to a doubling time of 14 hours (between 36 and 44 hours).

exponential phase, to division without accompanying mass increase which results in the smaller stationary phase cells. The level of FtsZ has been shown to control the frequency of cell division in *E. coli.* A two to sevenfold increase in the cellular level of the FtsZ protein has been reported to induce the formation of a minicell phenotype as a result of additional division events occurring at the poles in addition to the normal division site (Ward and Lutkenhaus, 1985). It is conceivable therefore that FtsZ may accumulate in high enough levels to enable additional cell division events which will result in the smaller stationary phase cells. A role for the FtsZ protein during the transition into stationary phase is supported by studies which showed a lowering of FtsZ over-expression in an *E. coli* mutant by the disruption of *rpoS* (which encodes the stationary phase sigma factor σ^S), suggesting that FtsZ over-expression was under σ^S control (Cam *et al.,* 1995). The σ^S-dependent over-expression of FtsZ probably occurs through an indirect mechanism since transcription from *ftsZ* promoters has been shown to be σ^S-independent (Smith *et al.,*1993). The fact that the transitory cessation in cell division coincides with a plateau in the ATP content of cells, and precedes the changes in cell morphology (e.g. cell size) that are observed during stationary phase, suggests that the cessation in cell division may represent an important stage during which genetic switches that enable cells to enter stationary phase occur. Attempts to elucidate mechanisms which enable *M. tuberculosis* to enter a dormant state may therefore benefit from a close scrutiny of the molecular changes that occur during this transitory cessation in cell division.

2.3.5. Standardisation of Heat Shock Conditions

Preliminary experiments were carried out to define the conditions to be used for heat shock experiments in terms of incubation temperature and incubation time. An aliquot taken from an exponential phase culture (24 hours) of *M. smegmatis* was incubated with [^{35}S]L-methionine at temperatures of 37, 42, 48, and 50°C, and each for a period of 30, 60, or 90 minutes as described in section 2.2.4. Cellular proteins were extracted and subjected to electrophoretic analysis (15% polyacrylamide gel) followed by autoradiography and densitometric analysis as described in sections 2.2.5. and 2.2.6. A

profile of newly synthesised proteins under different incubation temperatures and incubation periods is given in Fig. 2.3. The corresponding value of fold-induction of each heat-inducible band was obtained from a densitometric analysis of the autoradiographs and is given in Fig. 2.4.

Prominent bands of 95, 94, 75, 66, 42, and ~17 kDa (doublet) were induced in response to heat shock. Previous studies in *M. tuberculosis* and *M. bovis* BCG identified a single heat inducible band of 90 kDa which was shown to be more strongly induced at 45°C and 48°C than at 42°C (Young and Garbe, 1991; Patel *et al.*,1991). Possible homologues of the 90 kDa protein in *M. smegmatis* were the two heat inducible bands of 95 and 94 kDa. The 95 kDa protein was also more strongly induced at higher temperatures (48 and 50°C) than at the lower temperature of 42°C, and in addition gave a more dramatic induction in response to heat shock, i.e. with induction ranging from a minimum of 7-fold (90 min at 42°C) to a maximum of 88-fold (30 min at 50°C). The 94 kDa band on the other hand showed a more variable induction and only reached a maximum fold induction of 2.2 after 60 min at 48°C (Fig. 2.4). The significance of the existence in *M. smegmatis* of potentially two homologues of the 90 kDa heat shock protein will require further investigation. It is conceivable that due to the highly inducible nature of the 90 kDa heat shock proteins at extreme temperatures, 90kDa proteins may play a role in protecting cells against extreme temperatures. This suggests that in *M. smegmatis*, a free-living saprophyte, there may be a requirement for a different regulatory mechanism to cope with the more variable and extreme temperatures a free-living organism would be exposed to, and hence the possible requirement for two 90 kDa homologues.

The extreme temperatures of 48 and 50°C gave a stronger induction of 75 kDa (DnaK homologue), 42 kDa (DnaJ homologue) and ~17 kDa doublet compared to the mild temperature of 42°C. Similar responses have been reported for 75 and 42 kDa proteins in studies on *M. bovis* BCG and *M. tuberculosis*. No significant differences were observed in the induction of the 65kDa band (GroEL homologue) between the different heat shock temperatures. The fold-induction levels were generally much lower than those observed for the 95, 75, 42, and ~17 kDa proteins, suggesting that

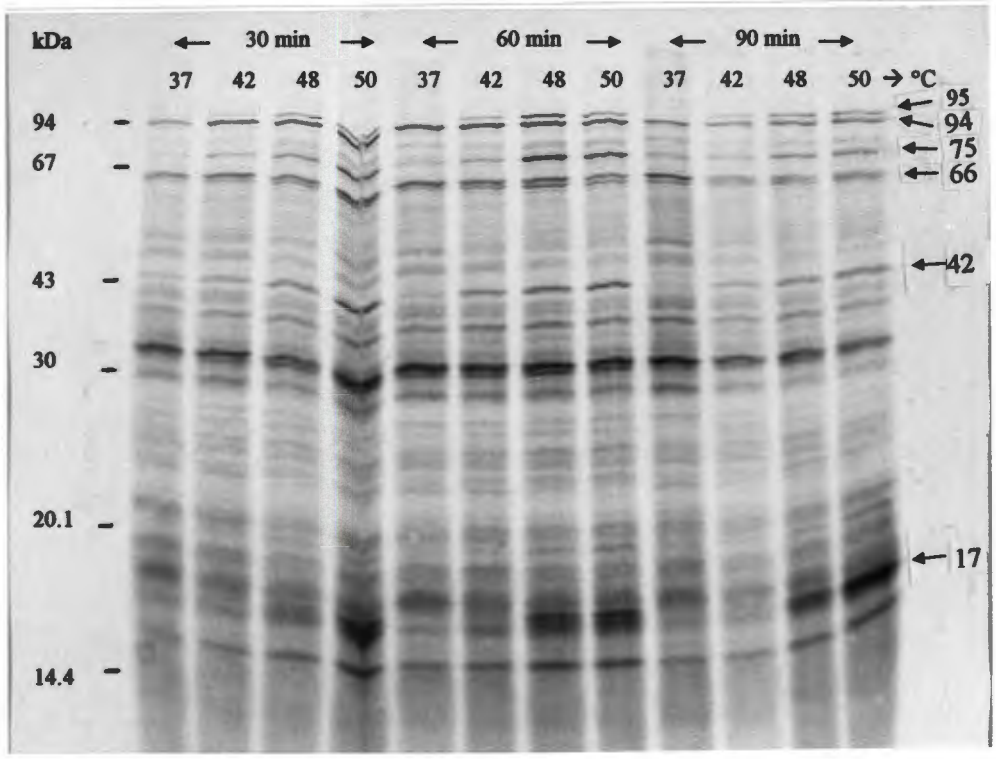


Fig. 2.3.: SDS-PAGE analysis of *M. smegmatis* heat shock response: the effect of different heat-shock temperatures (42, 48, 50°C) and different incubation times (30, 60, 90 min) on exponential phase cells (24 hours). Densitometric analysis of bands (see Fig. 2.4) revealed the induction of proteins of 95, 94, 75, 66, 41, and ~17 kDa (doublet) in response to heat shock. The molecular weights of the heat induced proteins of 75, 66, and 42 kDa proteins suggest that they may be homologues of *E. coli* DnaK, GroEL and DnaJ molecular chaperones, respectively. There was a more dramatic induction of the 95 kDa protein (ranging from a minimum of 7-fold induction to a maximum of 88-fold) in response to heat shock compared to that of the 94 kDa protein which showed a more variable response to heat shock. It seems likely that the 95 kDa band may represent the homologue of *E. coli* Hsp90. The ~17 kDa doublet was more strongly induced at higher heat shock temperatures (48 and 50°C) than at a lower temperature (42°C). It is unlikely that this is a homologue of the 16 kDa α-crystallin heat shock protein normally found in slow growing mycobacteria as its presence has not been demonstrated in *M. smegmatis*.

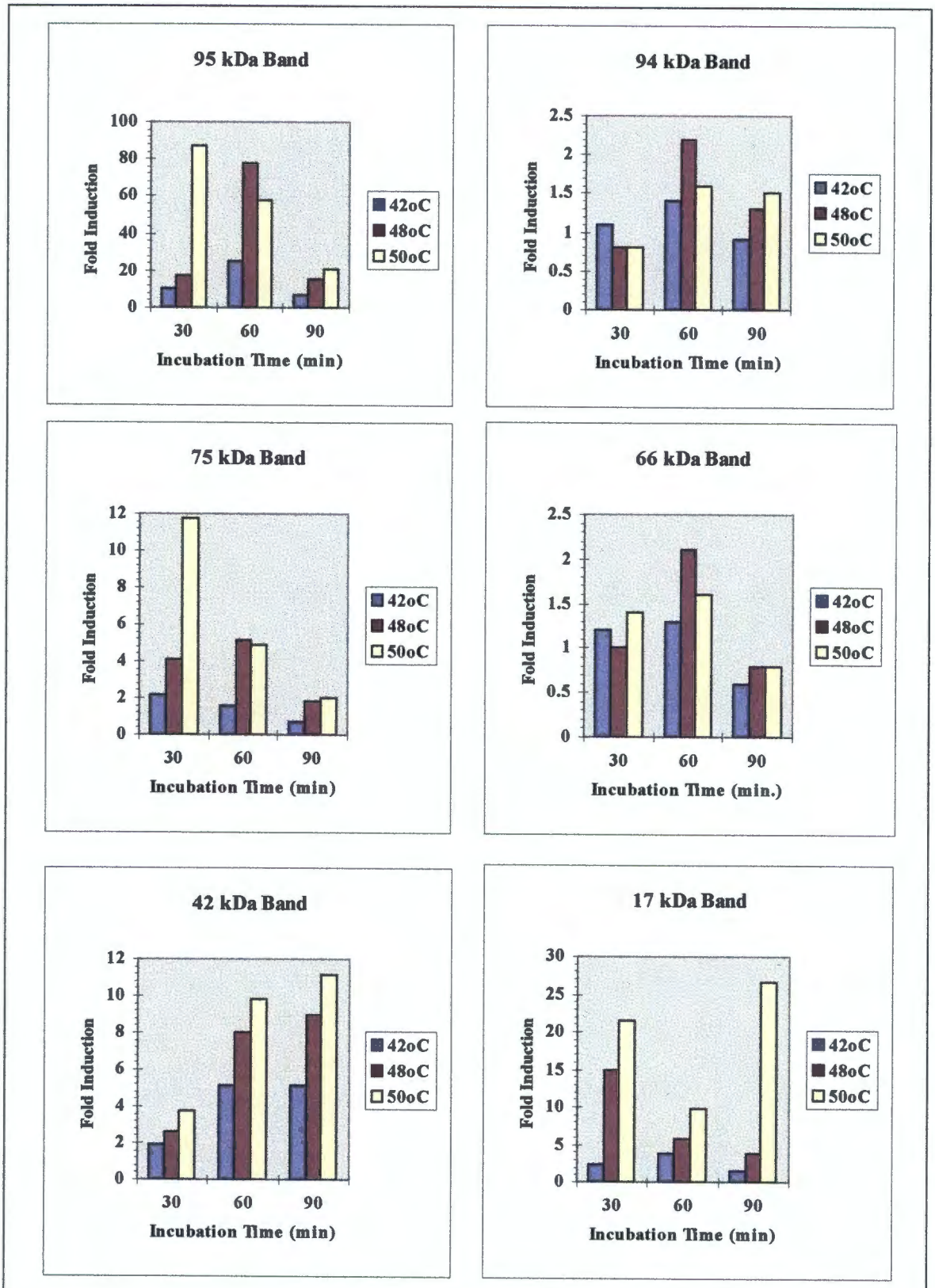


Fig. 2.4.: Densitometric analysis of the protein bands given in Fig. 2.3. Fold induction (above 37°C) of heat inducible proteins of 95, 94, 75, 66, 42, and 17 kDa after 30, 60, and 90 min incubation at 37, 42, 48 and 50°C. A fold induction of </=1 represents bands whose synthesis is inhibited / are not induced.

the intracellular levels of the 65 kDa protein under normal growth conditions are generally kept high, presumably to enable participation in other cellular functions. Studies in *M. tuberculosis* and *M. bovis* BCG showed a decline in the induction of GroEL at higher temperatures (45 and 48°C). This does not appear to be the case in *M. smegmatis* since incubation at 50°C appears to consistently give the highest fold induction of the 65 kDa protein. Both the 42 kDa and the ~17 kDa doublet gave a progressively higher fold-induction with increasing heat shock temperature, suggesting that their function may be more specific to the cell's response to heat shock. Apart from the 42 kDa band, whose induction showed a progressive increase with increasing incubation time, no significant relationship with incubation time was observed in the induction of the 95, 94, 75, 66, and ~17 kDa (doublet) proteins (Fig. 2.4).

The incubation conditions chosen for further studies on heat shock protein synthesis in relation to growth phase were the incubation time and temperature that gave the highest fold-induction of the 95, 94, 75, 66, 42, and ~17 kDa proteins (Fig. 2.4 and Table 2.1).

Table 2.1.: Summary of incubation times and temperatures giving the highest fold induction of heat-inducible protein bands.

Heat Induced Band	**Conditions Giving the Highest Fold Induction**	
(kDa)	**Incubation Temperature (°C)**	**Incubation Time (min.)**
95	50	30
94	48	60
75	50	30
66	48	60
42	50	90
17	50	90

When considering the highest fold induction values given in table 2.1., none of the incubation periods of 30, 60 and 90 min. gave a consistently high induction for all six

proteins. The second-highest fold induction for five out of the six proteins (see Fig. 2.4.) was obtained when using an incubation period of 60 min. A 60 min incubation at the heat shock temperature of 48°C gave good induction of all six proteins and were therefore the conditions of choice for use in subsequent heat shock experiments.

2.4. Conclusions

Detailed growth curves have for the first time been constructed for *M. smegmatis*. Comparisons between different methods of bacterial enumeration have led to the conclusion that the ATP content of culture is a more accurate indicator that cells are entering stationary phase than OD, CFU or cell morphology (Fig. 2.1). The ATP curve was the method of choice for monitoring growth in the studies of heat shock protein synthesis in relation to growth phase. A cessation (between 32 and 36 hours) in the increase in number of CFU coincided with a plateau in the ATP content of culture and preceded a reduction in the rate of accumulation of the cell division protein FtsZ (Fig. 2.2). The observation of a transitory cessation of growth which preceded stationary phase associated morphological changes in *M. smegmatis* suggested that this phase may be analogous to a similar cessation of growth that precedes stationary phase differentiation in *S. coelicolor* (Granozzi *et al.*,1990).

An SDS-PAGE analysis of protein extracts from cells that were incubated at 37, 42, 48 and 50°C for periods of 30, 60, or 90 minutes demonstrated a time-dependent and temperature-dependent induction of proteins of 94, 95 (Hsp90), 75 (DnaK), 66 (GroEL), 42 (DnaJ) and ~17kDa doublet (Fig. 2.3 and 2.4). The ~17 kDa doublet was more strongly induced under extreme heat shock (i.e. 37 to 48°C or 37 to 50°C) compared with mild heat shock (37 to 42°C). Amongst mycobacterial heat shock proteins, the 16 kDa α-crystallin heat shock protein has the closest molecular mass to the ~17 kDa doublet. Previous studies using immunoblot and Southern blot studies have not been able to identify an α-crystallin homologue in *M. smegmatis*. It would therefore be of interest to investigate the identity of the ~17 kDa doublet, particularly since it is strongly induced by severe heat shock. The experimental conditions chosen

for use in further heat shock experiments (see Chapter 3) consisted of a 60 min incubation at 37 or 48°C.

Chapter 3

The effect of Growth Phase of *Mycobacterium smegmatis* on Total Protein Synthesis and on the Response to Heat Shock

3.1. Introduction

Mycobacterium tuberculosis, the causative agent for tuberculosis (TB), is capable of causing both active disease and latent forms of the infection. While the active form of tuberculosis accounts for ~10 million new cases of TB per year world-wide, the latent form is estimated to affect about 1.7 billion people (one-third of the world's population), and each infected person has a 2 to 23% lifetime risk of reactivation (Enarson and Murray, 1996; Gedde-Dahl, 1952). The problem of latent infection is compounded by co-infection of individuals with HIV which increases the risk of reactivation of tuberculosis to 5 to 15% a year (Selwyn *et al.*,1989). Clinically, the latent form of TB is characterised by the presence of lesions in chest autoradiographs, a non-infectious state, a positive purified protein derivative (PPD+) status, and the absence of demonstrable acid-fast bacilli (Parrish *et al.*,1998). The physiological processes which define the latent state of the pathogen have not yet been characterised. Currently available antimycobacterial drugs kill actively dividing bacilli and as a result have to be administered over a period of at least six months in order to kill any persisting organisms (Cole, 1994). The elucidation of the mechanisms which enable *M. tuberculosis* to enter the latent state may lead to the development of novel antimycobacterial drug therapies which can be used for the elimination of latent bacilli. The availability of drugs that can be targeted against both dormant and actively dividing bacilli will not only reduce the duration of treatment, but will also have the added benefit of improving compliance and hence lead to a reduction in the incidence of multiple-drug resistant *M. tuberculosis* (Young and Duncan, 1995).

A major question concerning the physiological state of *M. tuberculosis* during latency is whether the bacilli are in a metabolically less active state or if they are in a spore-like, metabolically inactive, form awaiting a signal to resume division (Parrish *et al.*,1998). Genetic evidence for the existence of a spore-like state comes from the discovery of a homologue of the *Streptomyces coelicolor sigF* gene which encodes a sporulation sigma factor. The transcription of *sigF* has been shown to be induced by stationary phase (DeMaio *et al.*,1996). In addition, the ability of *M. tuberculosis* to remain in a dormant state for long periods during infection suggests that the bacilli may have the ability to exist in a spore-like state. The existence of a metabolically active stationary phase-like state has been suggested by a chemoprophylaxis study in which individuals meeting the criteria of latent TB were treated with isoniazid for 6 or 12 months, and were respectively shown to have a 65% or 90% reduction in the incidence of reactivation, suggesting that the latent bacilli were metabolically active (Comstock and Woolpert, 1972; Comstock *et al.*,1979). Recent genetic studies in *M. tuberculosis* have demonstrated the presence of the *katF* gene (Mulder, 1998), a homologue of the gene which encodes σ^S - a sigma subunit which functions as a global regulator of the molecular mechanisms that enable *Escherichia coli* to enter stationary phase (Siegele and Kolter, 1992; Loewen and Hengge-Aronis, 1994). Over-expression of KatF in *M. smegmatis* resulted in a reduction in the growth rate, suggesting a possible role for KatF in the reduced growth rate of stationary phase cells (Mulder, 1998).

Genetic studies alone are, however, unable to predict whether or not the identified genes are expressed, and if so under what conditions. In addition, the availability of the complete sequence of the *M. tuberculosis* genome now makes it possible to identify all the genes that are present in the genome and to predict the corresponding amino acid sequences. In order to complement the information obtained from genome sequencing initiatives, attention has now been redirected to proteome studies. The proteome is defined as the total protein output encoded by the genome under specified physiological conditions (Humphrey-Smith and Blackstock, 1997). Unlike genomic analysis, a knowledge of the proteome provides information on when predicted gene products are translated, their relative concentrations, and the extent of post-translational modification. The sequencing and characterisation of the expressed

proteins should enable the prediction of their involvement in the polygenic processes that occur within cells.

As an approach towards the identification of genes that are specifically induced when cells enter stationary phase, total protein synthesis at different stages of growth of *M. smegmatis* (from exponential to stationary phase) was investigated. The effect growth phase on the induction of heat shock proteins (which belong to Hsp90 (Lon), Hsp70 (DnaK), Hsp60 (GroEL), and LMW Hsp families - see Chapter 1, section 2.1) was also investigated. Mycobacterial heat shock proteins constitute one of the major groups of immunodominant antigens that are recognised by antibodies from the serum of TB patients (Young *et al.*,1985; Young, 1988). The immunodominance of heat shock proteins suggests that they may be actively involved in the interaction between bacilli and the immune system. The interaction may continue until the bacilli reach a stage where they switch to alternative developmental pathways such as those leading to the dormant state. Heat shock proteins function as molecular chaperones which mediate protein folding, assembly and membrane translocation of newly synthesised proteins within cells (Lindquist and Craig, 1988; Buchner, 1996). It is therefore conceivable that the stage during which such genetic switches occur may be accompanied by either a stimulation of the heat shock response such that heat shock proteins may assist in the folding of newly synthesised proteins, or the inhibition of the heat shock response if alternative stress resistance mechanisms have been activated. Determination of the effect of growth phase of *M. smegmatis* on protein synthesis and on the response to heat shock should not only result in the identification of proteins that participate in the molecular processes that enable mycobacterial cells to enter stationary phase, but may ultimately lead to the identification of the cellular processes which define the latent state of *M. tuberculosis*.

3.2. Materials and Methods

3.2.1. M. smegmatis *Culture, Heat Shock and Protein Analysis*

M. smegmatis was grown as described in Chapter 2, section 2.2.1. The ATP concentration of the culture (determined as described in section 2.2.2) was used for monitoring growth. Aliquots of cells were taken at four-hour intervals from the growing culture and used to determine *de novo* protein synthesis in the presence of ^{35}[S] L-methionine for protein labelling as described in section 2.2.4. The cells were incubated at 37°C for 1 hour (control) or under the optimum heat shock conditions (48°C for 1hour) determined in section 2.3.5 according to the method described in section 2.2.4. Labelled proteins were extracted and analysed by SDS-PAGE and the autoradiographs of the dried gels analysed by densitometry as described in sections 2.2.5 and 2.2.6. The density or fold induction of each heat-inducible proteins was plotted against the growth phase of the culture. For immunoblot analysis, the gels were processed as described in appendix I.

3.3. Results

3.3.1. Protein Synthesis as a Function of Growth Phase

Metabolic labelling with ^{35}[S]L-methionine was used to monitor protein synthesis in *M. smegmatis* under normal growth conditions and in response to heat shock at different stages of growth. The growth curves of the cultures were the same as in Figure 2.1. Aliquots of the growing culture were taken at 4 hour intervals over a period of 72 hours and labelled with ^{35}[S]L-methionine for 1 hour - either at 37°C or 48°C (heat shock). Equal amounts of the total cellular protein extract (20μg) were separated by SDS-PAGE, visualised by autoradiography (Fig. 3.1) and analysed by densitometry as described in section 2.2.4. Both quantitative and qualitative differences were observed in the synthesis of proteins during the different stages of

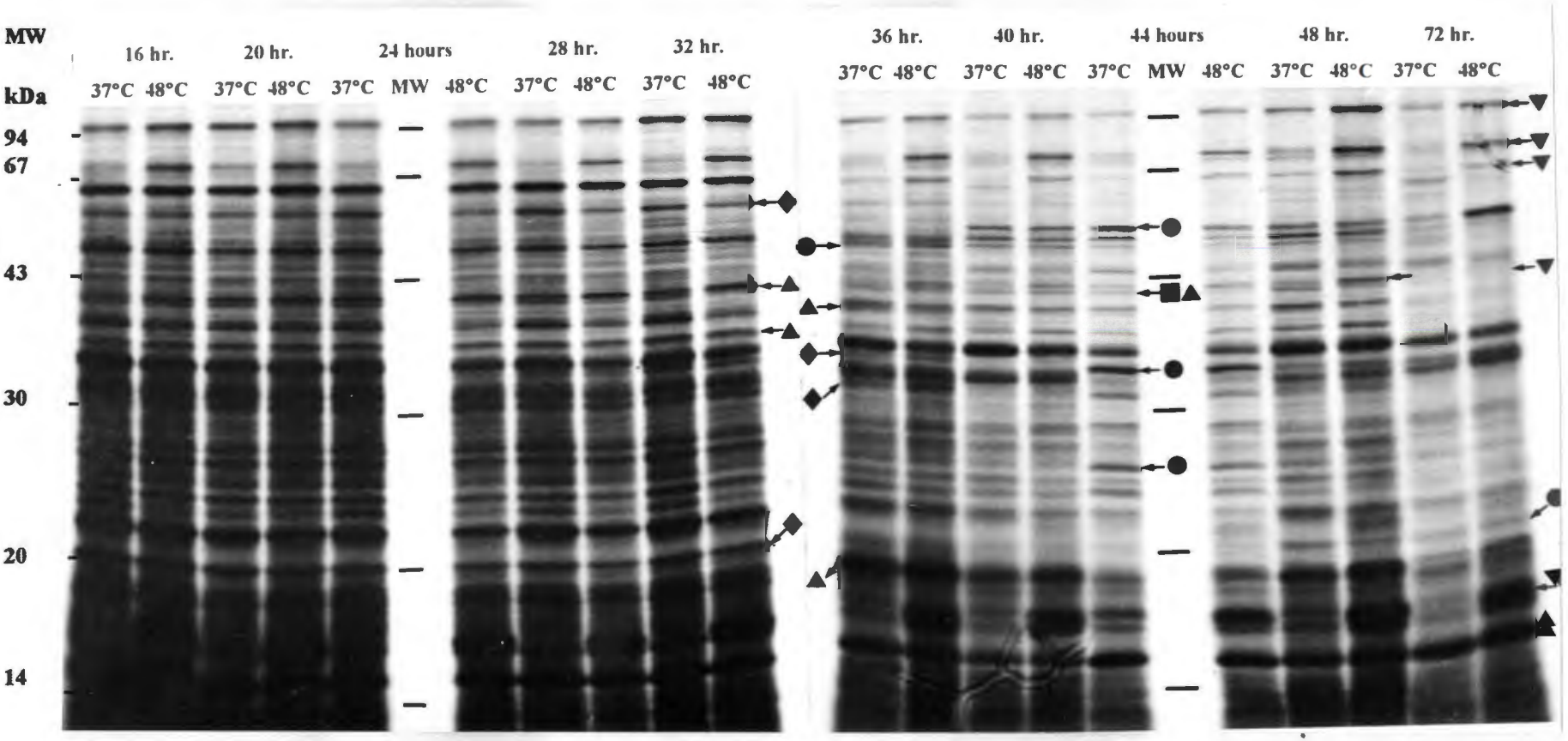
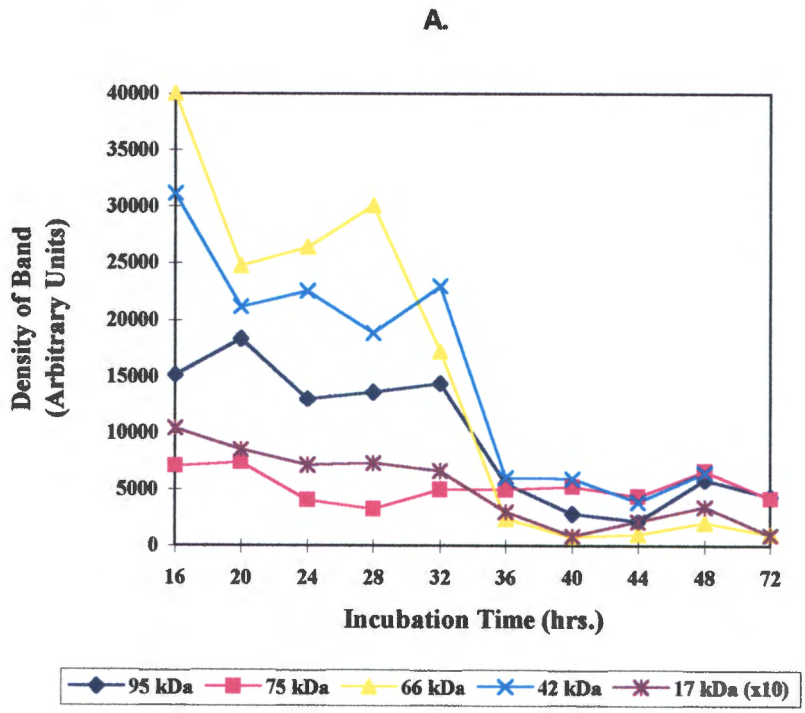


Fig. 3.1. SDS-PAGE analysis of samples (20 µg protein) labelled with ^{35}[S]methionine for 1 hr at 37°C or 48°C. Hours indicate growth phase as shown in Fig. 2.1. Stationary phase proteins (●) 46, 48, 25, 31, & 20 kDa (induced from 32, 40, 44, 44, & 48 hrs, respectively); exponential phase proteins (◆) 58, 32, 30.5 & 20 kDa (inhibited from 36 hr.); proteins absent from 72 hr (▲): 42, 40, 36, 34 & 19 kDa; heat induced proteins (▼): 95, 75, 66, 42, 17 kDa; heat inhibited protein (■) 40 kDa.

growth. Entry into stationary phase resulted in the induction of proteins of 46, 48, 25, 31, and 20 kDa (indicated by ● in Fig. 3.1), at 32, 40, 44, 44, and 48 hours, respectively, and the switching off of the 58, 32, 30.5, and 20 kDa exponential phase specific proteins (◆in Fig. 3.1) from 36 hours onwards. The 42, 40, 36, 34 and 19 kDa proteins (▲ in Fig. 3.1) were expressed during exponential phase and early stationary phase (up to 48 hours) but were absent from late stationary phase cells (i.e. after 72 hours). Heat shock resulted in the inhibition of synthesis of the 40 kDa protein (■ in Fig. 3.1) and the induction of proteins of 17 kDa (a double band of 16.5 and 16.8 kDa bands), 42, 66, 75, and 95 kDa (marked with ▼ in Fig. 3.1). Immunoblot analysis using the methods and antibodies described in appendix I was used to demonstrate that the 75, 66, and 42 kDa proteins were homologues of the *E. coli* DnaK, GroEL and DnaJ heat shock proteins, respectively (results not shown). The 95 kDa protein is presumably a homologue of the heat inducible *E. coli* Lon protease.

Densitometric analysis of heat inducible bands at 37 and at 48°C demonstrated a decline in the levels of the 95, 66, 42, and ~17 kDa proteins from 32 hours (i.e. when cells entered stationary phase), while the 75 kDa band remained relatively constant throughout (Fig. 3.2. A & B). After 40 hours, the levels of the 95 and ~17 kDa proteins at 48°C started to increase while the levels of the 42 and 66 kDa proteins remained low (Fig. 3.2.B). The magnitude of reduction of the stationary phase levels of the heat shock proteins was higher at 37°C than at 48°C. For example, the ~17 kDa protein, with the least reduction in levels, had a 4-fold reduction (i.e. from 10000 to 2500 units) at 37°C compared to 2-fold reduction (20000 to 10000) at 48°C, while the 66 kDa protein - with the highest magnitude of reduction in levels, had a 12-fold reduction (30000 to 2500) at 37°C compared to 7-fold (35000 to 5000) at 48°C (Fig 3.2.A & B). A plot of the total sum of the densities of all the radioactive bands in each lane against growth phase gave a profile similar to that of the 95 kDa band in Fig 3.2.A for both the 37°C and the 48°C samples (results not shown). A 3.2- and 3.5-fold reduction in total protein synthesis (incorporation of radioactivity) was observed from 32 hours for the 37°C and 48°C cultures, respectively.



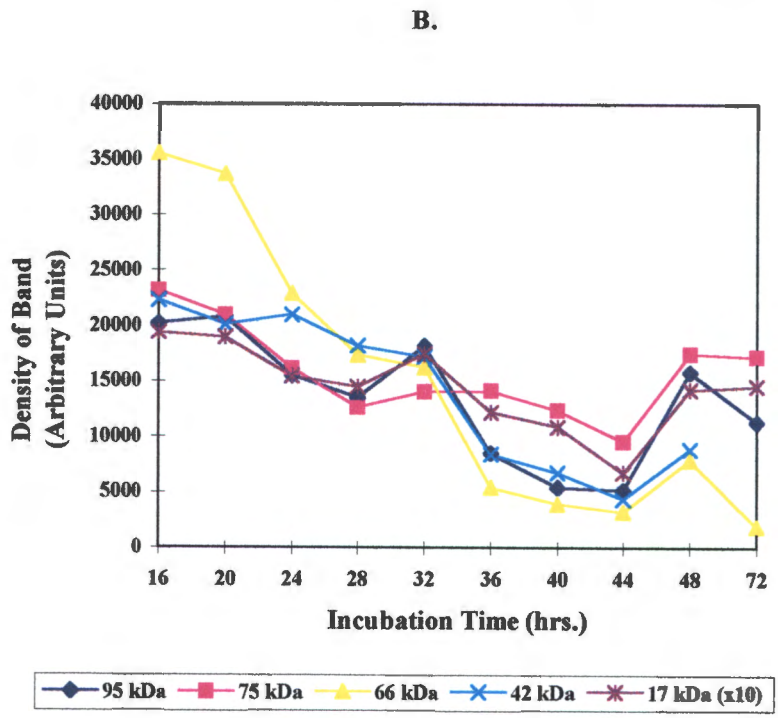


Fig.3.2.: Densitometric analysis of autoradiographs from the experiment shown in Fig. 3.1. Amounts of heat inducible protein bands after 1 hour of incubation at **A.** 37°C and **B.** 48°C in the presence of ^{35}[S]methionine. The profiles are representative of data from two other experiments. The plots of total protein (i.e. the sum of the density of radioactive bands in each lane) against growth phase for either 37°C or 48°C samples gave profiles similar to that of the 95 kDa band in Fig. 3.2.A (results not shown).

3.3.2. Induction of the Heat-Shock Regulon is Growth Phase Dependent

The magnitude of induction of heat-inducible protein bands was defined as the ratio of induced (48°C) to uninduced (37°C) bands, and was calculated by determining the density of bands in autoradiographs of SDS-PAGE gels. The mean fold induction from three experiments was plotted against growth phase (Fig. 3.3). The magnitude of induction of the 95, 66, and ~17 kDa proteins increased from about 1.8-, 1.5-, and 3.5-fold, respectively, during exponential phase (16 to 32 hours), to reach peaks of 3.5-, 6.5- and 9.5-fold between 40 and 44 hours, respectively, before declining to stationary phase levels. These were generally higher than the exponential phase levels (Fig. 3.3). On the other hand, the magnitude of induction of the 75 kDa protein was higher at the end of exponential phase (28 hours), while that of the 42 kDa protein showed no significant growth-phase dependency (Fig. 3.3). It is interesting to note that the DnaK (75 kDa) chaperone and its co-chaperone DnaJ (42 kDa) appear to be regulated differently from the other three major heat shock proteins, particularly since DnaK is involved in the regulation of the heat shock response (see discussion).

3.4. Discussion and Conclusions

Total protein synthesis and the response to heat shock in *M. smegmatis* demonstrated both qualitative and quantitative differences that were dependent on the growth phase of the culture. The different stages of growth of *M. smegmatis* were monitored by using growth curves constructed with either the number of colony forming units (CFU) or the ATP content of the culture as described in Chapter 2, section 2.3.2. Briefly, a 6-hour lag phase between 0 and 6 hours was followed by exponential growth from 8 to 28 hours. Thereafter, the ATP growth curve reached a plateau from 30 hours onwards, while the CFU growth curve - after a 4-hour lag between 32 and 36 hours, continued to increase until it reached a plateau from 40 hours onwards (see Fig. 3.3). A summary of the differences in the profiles of proteins synthesised during the different stages of growth of *M. smegmatis* in this study is given in Table 3.1.A.

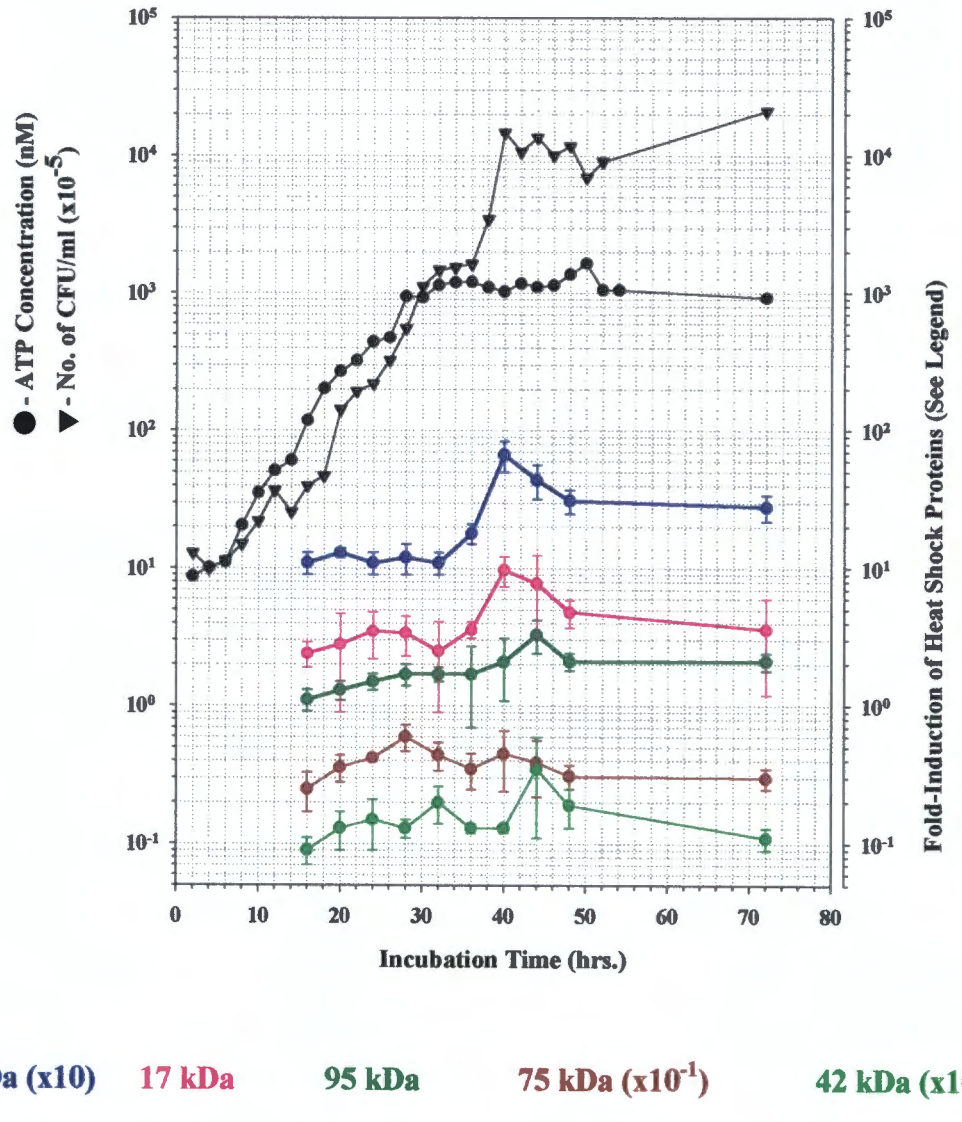
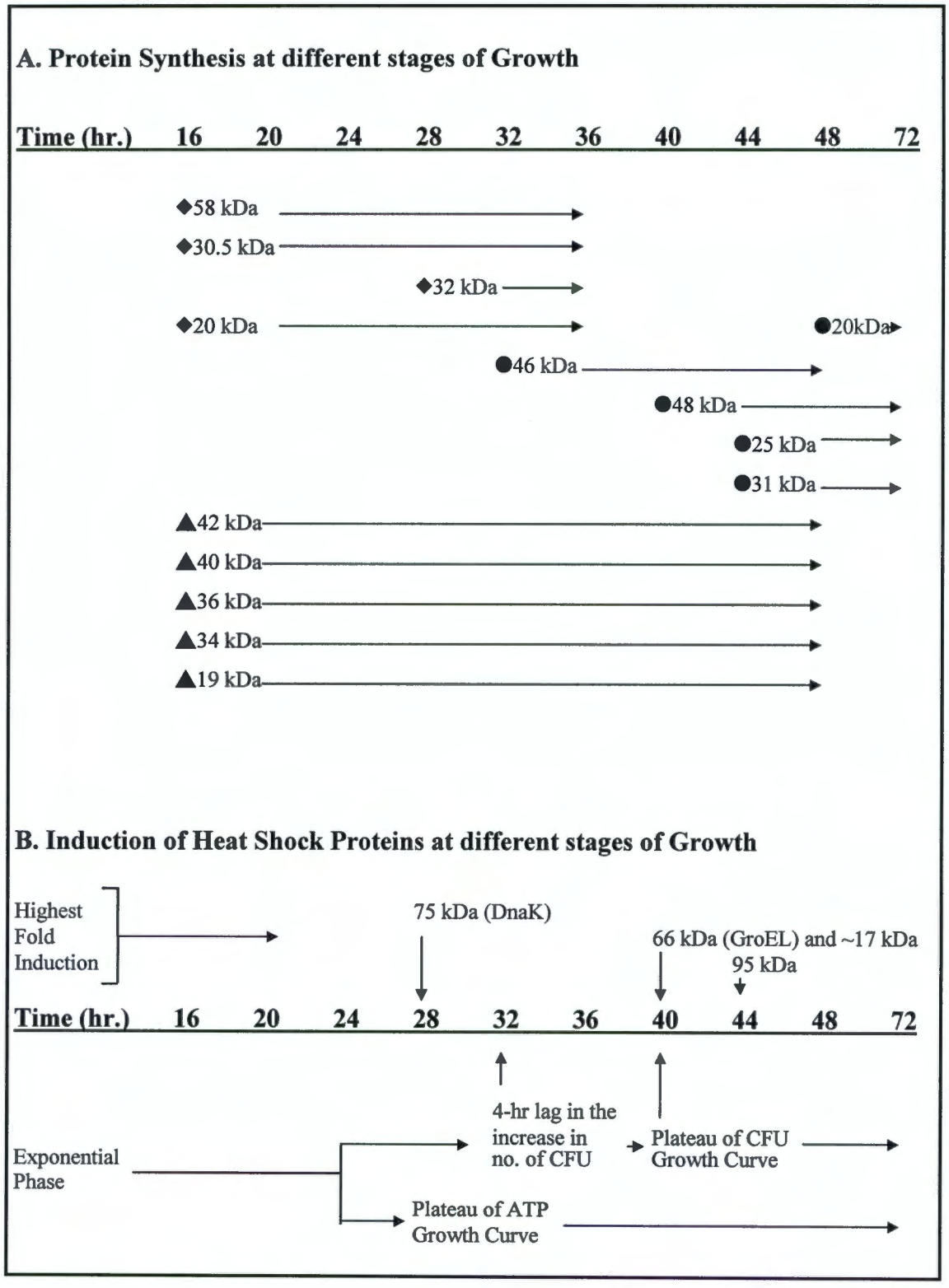


Fig. 3.3.: Fold induction (from 37 to 48°C) of heat inducible proteins of 95, 66, ~17, 75, and 42 kDa during different stages of growth - mean fold induction ± standard error of the mean from three experiments. The growth curves were constructed using either the number of CFU or the [ATP] of cells as previously described in Chapter 2 (Fig. 2.1). The highest induction of the 95, 75, 66, and ~17 kDa proteins was obtained at 44 hrs (3-fold), 28 hrs (6-fold), 40 hrs (7-fold) and 40 hrs (10-fold), respectively. The fold induction of the 42 kDa protein showed no growth phase dependency.

Table 3.1.: The sequence of events occurring during growth of *M. smegmatis*.



◆→ Exponential Phase Proteins
● →Stationary Phase Induced Proteins
▲→Proteins Absent from Late Stationary Phase (i.e. 72 hours)

The end of exponential phase growth (28 hours) coincided with the highest induction of the 75 kDa (DnaK) protein, while the highest induction of the 95, 66 and ~17 kDa heat shock proteins occurred 12 to 14 hours later - i.e. between 40 and 44 hours (Fig. 3.3). The induction of DnaK and GroEL during carbon-starvation induced stationary phase has been reported in *E. coli* (McCann *et al.*,1991), suggesting a role for heat shock proteins during entry into stationary phase. DnaK is thought to act as a negative regulator of the heat shock response by binding to RNA polymerase core enzyme such that the binding of σ^{32} (the sigma subunit which confers specificity for transcription of heat shock genes) to the core enzyme is inhibited (see Chapter 1, Fig. 1.6). It could therefore be speculated that the increased synthesis of DnaK at 28 hours may represent a stage during which DnaK levels are not only increased to respond to the heat shock, but in addition to cause the growth stage determined inhibition of the synthesis of the 95, 66, 42, and ~17 kDa heat shock proteins occurring from 32 hours until late stationary phase (Fig. 3.2.A & B).

The period between the end of exponential phase and the plateau in CFU (i.e. from 30 to 40 hours) saw several simultaneous events: a 4-hour lag the increase in number of CFU occurring between 32 and 36 hours (Fig. 3.3); a plateau in the ATP content of the culture from 30 hours onwards (Fig. 3.3); a decline in the rate of accumulation of the cell division protein FtsZ (Fig. 2.2); the transitory synthesis of proteins with apparent molecular weights of 32 kDa (◆) (from 28 to 36 hours) and 46 kDa (●) (from 32 to 48 hours) (Fig. 3.3); and the inhibition of synthesis of the 58, 30.5, and 20 kDa exponential phase proteins (◆). Differences in the kinetics of induction of proteins expressed during the onset of starvation-induced stationary phase have been reported in *E. coli* (Groat *et al.*,1986), *Salmonella typhimurium* (Spector *et al.*,1986; Spector and Cubitt, 1992), and *Vibrio* sp. (Nystrom *et al.*,1990). The profile of induced proteins was dependent on the inducing conditions. In *E. coli*, two-dimensional polyacrylamide gel electrophoresis demonstrated the presence of a core set of 15 to 30 proteins which was always induced regardless of whether it was starved of carbon, phosphorus, or nitrogen, and these have been designated the 'Pex' (postexponential) proteins (Groat *et al.*,1986). The identity of 'Pex' proteins is largely unknown. It is conceivable that there may be an overlap in the identities of 'Pex' proteins and the stationary phase-induced *M. smegmatis* proteins demonstrated in this study (see Table

3.1), and that proteins may be involved in the global morphological and metabolic changes that bacteria undergo during entry into stationary phase.

A general reduction in total protein synthesis, including the synthesis of the 95, 66, 42, and ~17 kDa heat shock proteins (▼) was observed from 32 hours onwards (Fig. 3.2). A 3-fold reduction in protein synthesis preceding differentiation to form aerial mycelium has been reported in *Streptomyces coelicolor* (Granozzi *et al.*, 1990). Unlike in *M. smegmatis*, where protein levels remained low until late exponential phase (Fig. 3.2.A), total protein synthesis in *S. coelicolor* resumed, nearly reaching the original values when aerial mycelium formation occurred. It appears therefore that genetic switches that result in the reduction in protein synthesis may be a prerequisite for the morphological differentiation that leads to the stationary phase events of aerial mycelium formation and sporulation in *Streptomyces*, or to the formation of the smaller, more compact cells typical of stationary phase in *M. smegmatis*. The synthesis of DnaK, the 19 kDa protein (▲), and a 15 kDa protein (indicated by ▲) remained constant up to 48 hours (Fig. 3.1 & 3.2), suggesting that their cellular levels may be under a different regulatory mechanism.

It appears that the inhibition of cell division between 32 and 36 hours (i.e. plateau in CFU) may not just be occurring to facilitate DNA synthesis in preparation for the next cycle of cell division (i.e. between 36 and 40 hours), but may also represent a stage during which genes required for entry into stationary phase are expressed. Sequencing and characterisation of the transiently induced 32 and 46 kDa proteins should enable their role during stationary phase differentiation to be determined. One of the functions of such proteins may be to enable cells to change from the mode of cell division that is accompanied by an increase in cell mass (as seen during exponential phase) to cell division that is not accompanied by mass increase - as illustrated by a plateau in the ATP curve while the number of CFU continues to increase between 36 and 40 hours (Fig. 3.3). One of the major aims of characterising the latent state of *M. tuberculosis* is to find novel sites for targeting drugs that could be used to kill dormant bacilli. An alternative approach may be to find drugs that could prevent bacilli from entering the dormant state. It is conceivable that prevention of the switching-off of the

exponential phase proteins (◆) may maintain bacilli in an exponential phase-like state against which current antimycobacterial therapies would still be active.

Stationary phase growth (i.e. plateau in the CFU curve from 40 hours onwards) was accompanied by the induction of a 48 kDa protein (●) from 40 hours; the highest induction of the 66, ~17, and 95 kDa heat shock proteins (▼) at 40 and 44 hours; and the induction of the 25 and 31 kDa proteins (●). Pre-induction levels of the 95, 66, and ~17 kDa proteins have been shown to be lower at stationary phase than at exponential phase (Fig. 3.2.A - 37°C). It may be that for the heat shock response to function efficiently, heat shock proteins have to be maintained above a certain minimum level. The increased efficiency of induction of heat shock proteins observed during stationary phase may therefore be a mechanism to compensate for the low pre-induction stationary phase levels of these proteins. Regulatory mechanisms that are specifically stimulated during stationary phase may facilitate an increase in the rate of expression of the heat shock genes to enable functional levels of the heat shock proteins to be achieved. This suggests that there may be a common regulatory mechanism which co-ordinates thermal and developmental induction of the heat shock regulon. A developmental control of the heat shock regulon has also been reported in *Streptomyces coelicolor* (Puglia *et al.*, 1995). However, in contrast to *M. smegmatis* where the highest induction of the 95 kDa (Lon), 66 kDa (GroEL), and ~17 kDa heat shock proteins occurred when cells entered stationary phase, studies in *S. coelicolor* have shown that the highest synthesis of GroEL1, GroEL2, DnaK and Lon occurred during the transitory cessation of growth (plateau) that precedes aerial mycelium formation and sporulation (Puglia *et al.*,1995; Granozzi *et al.*,1990). In addition, the transitory cessation of growth seen in *Streptomyces* has also been shown to coincide with the expression of genes for aerial mycelium formation and antibiotic synthesis (Maplestone *et al.*,1992; Holt *et al.*,1992). It is therefore conceivable that the peak in induction of heat shock proteins in *M. smegmatis* may be indicative of a stage during which genes that are required for stationary phase differentiation are expressed, as illustrated for example by the induction of the 48, 31, and 25 kDa stationary phase proteins (●). The significance of the highest fold induction of GroEL, Lon, and the ~17 kDa doublet occurring just as cells enter stationary phase is as yet unclear. It may be that heat shock proteins are required for their molecular chaperone function to

prevent heat denaturation of the newly synthesised stationary phase proteins (●). This is the first time that co-ordination between the thermal and developmental induction of the heat shock response has been reported in non-differentiating bacteria. Further studies in other non-differentiating bacteria will be required to determine if the developmental control of the heat shock regulon is a common mechanism to all bacteria or is unique to the actinomycetes (i.e. *Mycobacteria* and *Streptomyces*).

During late stationary phase (72 hours), synthesis of the 42 kDa heat shock protein and other proteins (indicated by ▲ in Table 3.1.A) that were present during exponential phase and the early part of stationary phase (i.e. up to 48 hours) was inhibited, while a 20 kDa protein (●) was synthesised between 48 and 72 hours. The differences in protein synthesis between early and late stationary phase cells suggest that different regulons may be activated in late stationary phase cells. Such regulons may be responsible for the long-term survival *of M. smegmatis*, and as such may also be implicated in the latent state in *M. tuberculosis*. Based on migration positions, the 20 kDa protein stationary phase protein (●) appeared to be the same as a 20 kDa exponential phase protein (◆) whose synthesis was inhibited from 36 hours onwards. A possible reason for the synthesis of what appears to be an exponential phase protein during stationary phase may be to enable cells to prepare for the resumption of growth at the end of stationary phase (see Chapter 1, section 1.3.3).

In conclusion, it appears that attempts to define the mycobacterial stationary phase would benefit from the identification of proteins that are induced during the transition into stationary phase (as summarised in Table 3.1). Further analyses using two-dimensional polyacrylamide gel electrophoresis should enable more proteins to be identified and sequenced. The difficulties associated with working with *M. tuberculosis*, i.e. the clumping of cells especially towards stationary phase and the resulting difficulty in ensuring that the all the bacteria are at same stage of growth, make it difficult to perform as detailed a developmental investigation of protein synthesis as has been done for *M. smegmatis*. The availability of the *M. tuberculosis* genome sequence (and the corresponding amino acid sequences) makes it possible to identify the *M. tuberculosis* counterparts of proteins that are synthesised under specific growth stages in *M. smegmatis*. Differences in structural characteristics and

the mechanisms that regulate the expression of such genes will enable comparisons to be made between a pathogen (i.e. *M. tuberculosis*) and a non-pathogen (i.e. *M. smegmatis*) with the aim of identifying virulence determining mechanisms that are unique to the pathogenic mycobacteria.

Chapter 4

Identification of a Novel ~17 kDa Heat Shock Protein (Hsp17-2) in *Mycobacterium smegmatis*

4.1. Introduction

Cells from organisms as diverse as *Escherichia coli* and humans, when exposed to adverse stimuli such as a sudden shift to a higher temperature, respond by inducing or accelerating the rate of synthesis of a set of proteins called heat shock proteins (Schlesinger *et al.*,1982). Bacterial heat shock protein (Hsp) are classified into families, i.e. the Hsp90 (Lon) , Hsp70 (DnaK), Hsp60 (GroEL), and the low molecular weight (LMW) Hsp (α-crystallin) family. Heat shock proteins are also expressed under normal growth conditions and function as molecular chaperones whose primary function is to bind to and stabilise proteins and determine their fate *in vivo*: be it protein folding, oligomeric assembly, transport to a particular subcellular compartment or degradation (Hendrick and Hartl, 1993; Buchner, 1996). The heat inducible co-chaperones GroES and DnaJ/GrpE respectively assist in the functioning of GroEL and DnaK molecular chaperones (see Chapter 2, Table 2.1).

In the current studies, heat shock induction of homologues of Lon, DnaK, GroEL, and DnaJ with molecular weights of 95, 75, 66, and 42 kDa, respectively, was demonstrated in *M. smegmatis* (see Chapter 3, Fig. 3.1). The use of ^{35}[S]L-methionine for protein labelling failed to show incorporation of label into a heat inducible protein at about 12 kDa - the reported migration position for GroES in *M. tuberculosis*, presumably as a result of the absence of the initial methionine residue in the mature protein as has been reported in *M. tuberculosis* and *M. bovis* BCG (Yamaguchi *et al.*,1988; Young and Garbe, 1991a). In addition, a ~17 kDa doublet consisting of two strongly heat inducible (up to 10-fold) proteins with apparent molecular weights of 16.5 and 16.8 kDa was demonstrated in the presence of ^{35}[S]L-methionine (Chapter 2,

Fig. 2.2 & 2.4 and Chapter 3, Fig. 3.1 & 3.3). Based on size, the 16.5 and 16.8 kDa proteins appeared likely to be members of the LMW-Hsp α-crystallin family. The *M. tuberculosis* and *M. leprae* α-crystallin antigens have molecular weights of 14 and 18 kDa, respectively (Nerland *et al.*,1988; Verbon *et al.*,1992). The LMW Hsp family is a diverse family of proteins of 10 to 30 kDa which is evolutionarily related to the α-crystallin vertebrate eye-lens protein. Members of this family are characterised by the presence of the "α-crystallin domain" (Ingolia and Craig, 1982; Caspers *et al.*,1995). Although other members of the LMW-Hsp α-crystallin family have been shown to be induced by heat shock, only indirect evidence is available to support heat induction of the mycobacterial α-crystallin antigens. Using the *M. leprae* specific anti-α-crystallin monoclonal antibody - L5 (MabL5), Lamb *et al.* (1990) were able to show cross reactivity of MAbL5 with a heat inducible 18 kDa protein of *M. habana (M. simiae)*.

Previous studies have reported contrasting evidence regarding the presence of α-crystallin in *M. smegmatis*. Schoningh *et al.* (1990) reported the presence of α-crystallin in cell lysates of *M. smegmatis* when using the anti-α-crystallin F24-2 monoclonal antibody developed by Kolk *et al.* (1984) in an enzyme-linked immunosorbent assay (ELISA). On the other hand, Verbon *et al.* (1992) was unable to show reactivity against membrane-immobilised *M. smegmatis* lysates using the same monoclonal antibody. Despite the presence of α-crystallins in as diverse a range of organisms as bacteria, fungi, higher plants and animals (Caspers *et al.*,1995), recent Southern blotting studies using the *M. tuberculosis* α-crystallin gene as a probe could not demonstrate the presence of the gene in *M. smegmatis* and *M. avium* (Yuan *et al.*,1996). Low oxygen tension has been shown to induce cell wall thickening and the synthesis of α-crystallin in *M. tuberculosis* but not in *M. smegmatis*. The poor survival of *M. smegmatis* under low oxygen tension compared to *M. tuberculosis* led the authors to suggest that the induction of α-crystallin in *M. tuberculosis* may enable bacilli to survive the low oxygen tension conditions found in granulomas (Cunningham and Spreadbury, 1998). In the light of available evidence, it was therefore difficult to say whether or not the strongly heat inducible 16.5 and 16.8 kDa proteins were members of the α-crystallin family of proteins. The aim of the present

study was therefore to use peptide sequence analysis to identify the heat induced 16.5 and 16.8 kDa proteins of *M. smegmatis*.

4.2. Materials and Methods

4.2.1. Immunoblot Analysis

Immunoblot studies using the F24-2-3 anti-α-crystallin monoclonal antibody were carried out to determine whether or not an α-crystallin related protein was present in *M. smegmatis* and in addition to investigate if it was induced by heat shock. *Mycobacterium bovis* BCG, a member of the *M. tuberculosis* complex which has been shown to possess α-crystallin (Cunningham and Spreadbury, 1998), was used as a positive control for the immunoblot studies. *M. bovis* BCG was obtained from The State Vaccine Institute (Pinelands, South Africa) in single-dose vaccine vials. The cells were propagated as surface pellicles in Sauton medium. For the present studies, *M. bovis* BCG was grown by resuspending cells from one vaccine vial in 200 ml Middlebrook 7H9 medium (supplemented with 0.5% (w/v) albumin fraction V; 2% (w/v) D-glucose; and 0.05% (v/v) Tween 80). The cultures were incubated at 37°C in an orbital shaker for 7 days. Heat shock (at 42°C for 60 min) in the presence of ^{35}[S]L-methionine and electrophoretic analysis were carried out as described in Chapter 2, sections 2.2.4, 2.2.5 and 2.2.6. Immunoblot studies were performed according to the protocol described in appendix I.

4.2.2. Two - Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Sample Preparation

Heat shock and protein labelling of samples was carried out according to the method described in section 2.2.4 and the labelled cell pellets were washed twice in PBS (1 ml) before resuspending in PBS. Cell lysis was achieved by shaking the cell

suspension with glass beads (0.1 mm) in a FastPrep Instrument (BIO 101, Inc., California, USA) using the 2 ml tubes supplied by the instrument manufacturer. Cell debris was removed by centrifugation (13000 rpm for 3 min) and the supernatants (cell lysates) were transferred to fresh microcentrifuge tubes. The protein concentration of cell lysates was determined by using a colorimetric assay based on the procedure of Lowry *et al.* (1951) as specified in the protocol for the Protein Assay Kit (Bio-Rad). Two-dimensional gel electrophoresis was carried out according to the method developed by O'Farrell (1975), using protocols, chemicals and the electrophoresis system supplied by Pharmacia, Sweden.

First Dimension - Isoelectric Focusing (IEF)

Immobiline DryStrips (Pharmacia, Sweden) were used for IEF (first dimension). The Immobiline DryStrips consisted of dried polyacrylamide gel strips (110 mm long) cast by covalently polymerising a pH gradient (employing ampholytes in the pH range 3-10) on a GelBond PAG plastic film. A solution of urea (8M), triton X-100 (0.5%), Pharmalyte 3-10 (0.5%), bromophenol blue (0.02%), and dithiothreitol (0.2%) was used for the overnight (16 hours) rehydration of the Immobiline DryStrips. The rehydrated strips were 0.5 mm thick, 3 mm wide, and 110 mm long. An aliquot of the cell lysate (equivalent to 80μg of protein) was mixed in a 1:4 ratio with sample solution [urea (9M), triton X-100 (0.5%), Pharmalyte 3-10 (2%), bromophenol blue (0.02%), and dithiothreitol (1%)]. The samples (80μg of protein) were loaded on the cathodic side of rehydrated Immobiline strips which were aligned between the anode and cathode electrodes in a Multiphor II Unit (Pharmacia) as described in the manufacturer's instructions. IEF was carried out at an initial 500 Volts (V) for 4 hours, followed by overnight (15 hours) focusing at 3000V. The immobiline strips were subsequently either processed for the second dimension through a two-step equilibration process or stored frozen at -70°C prior to equilibration. For the first equilibration step, the strips were incubated for 10 min with gentle shaking in equilibration solution [6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, and 10% (v/v) Tris-HCl stock solution: 6.1% (w/v) Tris dissolved in distilled water and adjusted to pH 6.8 with 1 M HCl] supplemented with 0.5% (w/v) dithiothreitol. For the second 10

min equilibration step, the equilibration solution was supplemented with 4.5% (w/v) iodoacetamide and 0.02% (w/v) bromophenol blue. The isoelectric points (pI) of proteins were determined by direct comparison with the pI calibration markers (range pH 3-10) (Isoelectric focusing Kit, Pharmacia) separated using IEF.

Second Dimension - SDS-PAGE

For the second-dimension, SDS - polyacrylamide gels were cast using the Vertical Slab Gel Electrophoresis Unit (Hoefer Scientific Instruments, California, USA) as described in section 2.2.6. A two-well comb designed in such a way that it forms one wide well (122 mm) for placing the immobiline strip and a single well (5mm) for running the molecular weight marker was used during the casting of the stacking gel. After equilibration, the immobiline strip was placed horizontally in the wide well (filled with running buffer) and electrophoresis carried out at constant current (20mA) until the bromophenol blue dye had moved out of the strip into the stacking gel (4.5% acrylamide). Thereafter the current was increased to 40mA and electrophoresis continued until the tracking dye reached the bottom of the resolving gel (15 % acrylamide). For the autoradiographic detection of radioactively labelled proteins, the gels were stained in Coomasie Blue, fixed and dried as described in section 2.2.6. If proteins were to be recovered from the gel for MALDI-MS peptide sequencing, then the gels were stained with silver using the protocol described in appendix II. Gel pieces corresponding to peptides of interest were lyophilised for shipping purposes.

4.2.3. Mass Spectrometric Sequencing of Proteins

Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels was performed by Joanna Betts at Glaxo-Wellcome Pharmaceuticals (Stevenhage, United Kingdom) according to the protocols for matrix-assisted laser-desorption/ionisation time-of-flight - mass spectrometry (MALDI-TOF-MS) described by Patterson and Aebersold (1995) and Shevchenko *et al.* (1996). Briefly, the protein spot of interest is excised from a 2D-PAGE gel and subjected to in-gel trypsin

digestion. The released peptides are then deposited on the sample probe of the MALDI-mass spectrometer by co-crystallisation with an excess of matrix material (α-cyano-4-hydroxy-*trans*-cinnamic acid). Matrices are weak aromatic acids that are readily soluble in solvents commonly used for polypeptides, enhance protein and peptide solubility and strongly absorb the light of the laser used for ionisation. The crystals are then irradiated with a short-pulsed laser, resulting in the ionisation of the solid matrix followed by an energy transfer to the analyte, as well as the transfer of the generated intact ions into the gas phase. The laser pulse simultaneously triggers the clock used to measure the flight time of the ions to the detector. The time-of-flight is the time from triggering to striking the detector and is dependent upon the mass-over-charge ratio of the ions, i.e. smaller ions arrived at the detector faster than large ions. The mass of the released peptides is then determined. Protein databases, and DNA sequence databases translated into protein sequences, are assembled into a non-redundant sequence database. Peptide fragment masses (using specific cleavage rules, i.e. for trypsin, after arginine or lysine, but not when followed by proline) are computed from this database to generate a peptide mass database. The peptide mass database is then searched using the experimentally derived masses. A ranked output of the most likely candidates, based on the number of matches and the frequency of such matches occurring randomly in that mass range, is generated.

4.2.4. Polymerase Chain Reaction (PCR) Analysis

The *M. tuberculosis* 16.2 kDa protein gene (MTCY01B2.28, Fig. 4.4) was used to design two oligonucleotide primers for amplifying the corresponding genes from *M smegmatis, M. bovis* BCG and *M. tuberculosis* (positive control). Relative to the A residue of the initiator methionine codon ATG, the 5′ primer (5′-GAGCGCCTATAAGACCGTGG-3′) annealed to nucleotides +3 to +23, while the 3′ primer (5′-CTAGGTGGTGTGCACGATCAGC-3′) annealed to nucleotides +441 to +419. Genomic DNA was extracted from *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* using the protocol described in appendix III. In 0.5 µl microcentrifuge tubes placed on ice, mycobacterial genomic DNA (150 ng in 38.5 µl sterilised distilled

water) was mixed with 5 µl of 10X PCR Buffer (15 mM $MgCl_2$, 500 mM KCl, 100 m Tris-HCl, pH 8.3); 4µl deoxynucleotide triphosphate (dNTP) mixture (2.5 mM each of dATP, dCTP, dGTP, dTTP); 0.5 µl of each primer (100 pmol/µl); and 0.5 µl *Taq* polymerase (5 U/µl). The Taq polymerase, PCR buffer, and the dNTP mixture were supplied by TaKaRa Biomedicals, Shiga, Japan. The reaction mixtures were covered with a layer of paraffin oil (50 µl) before being subjected to a denaturation step at 95°C for 300 sec; followed by 30 cycles of 95°C for 60 sec, 70°C for 60 sec, and 72°C at 60 sec; and a final elongation step at 72°C for 300 sec in a JDI Model 8012 temperature profiler.

4.2.5. Cloning and DNA Sequencing

The phenol-extraction method (see appendix IV) was used to purify the PCR products from the agarose gels. Taking advantage of the template-independent addition of a single adenosine (A) to the 3′-end of PCR products by Taq DNA Polymerase (Ausubel, 1988), the PCR products were ligated to the pGEM-T vector (carrying a 3′-terminal T (thymidine) residue) using T4 DNA ligase (Promega, Madison, Wisconsin, USA). The pGEM-T Vector System (Promega) protocol was used for the ligation reactions; the transformation of pGEM-T constructs into competent *E. coli* strain DH5α cells; and for the screening of transformants for inserts. Competent DH5α cells were prepared using the calcium chloride based protocol described in appendix V. The blue/white X-gal (5-Bromo-4-Chloro-3-indolyl-β-D-galactoside) based procedure was used for the selection of transformants (see Sambrook *et al.*,1989).

The plasmid purification (mini-preparation) protocol described in Sambrook *et al.* (1989) was used to purify plasmids from the transformants for use in restriction enzyme digestions to screen for the clones containing the correct size inserts. The identity of the cloned inserts was subsequently confirmed by DNA sequencing of plasmids purified using the Nucleobond PC Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Endonuclease digestion and agarose electrophoresis were performed according to published protocols (Sambrook *et*

al.,1989). DNA sequence analysis was performed according to the primer extension dideoxy termination method of Sanger *et al.* (1977) using the protocol and reagents provided in the T7 Sequencing Kit (Pharmacia Biotechnology, Sweden).

4.3. Results

4.3.1. The ~17 kDa Heat-Inducible doublet (Hsp17) of M. smegmatis *is not α-crystallin, and the synthesis of α-crystallin in* M. bovis *BCG is not Induced by Heat Shock.*

The F24-3-2 anti-α-crystallin monoclonal antibody reacted with a band of 18.5 kDa band when used against the cell lysate of *M. bovis* BCG (Fig. 4.1). There was no significant difference in the levels α-crystallin between heat-shocked (42°C) and control (37°C) samples, demonstrating that α-crystallin was not induced by heat shock.

4.3.2. The ~17 kDa Heat-Inducible doublet is resolved into four peptides (Hsp17-1, Hsp17-2, Hsp17-3, and Hsp17-4) by two-dimensional PAGE.

In order to achieve a higher resolution of the ~17 kDa doublet (16.5 and 16.8 kDa) observed under SDS-PAGE (see Fig. 4.2), two-dimensional PAGE (2D-PAGE) analysis was performed on aliquots that were taken from the exponential phase (24 hours) of a culture of *M. smegmatis* and were labelled with ^{35}[S]L-methionine for 1 hour at either 37°C (control) or 48°C (heat shock). Equal amounts of protein (80 μg) were loaded onto each IEF strip and 2D-PAGE was carried out as described in materials and methods. An autoradiograph of the dried 2D-PAGE gels demonstrated a resolution of the ~17 kDa doublet into four strongly heat-induced peptides (indicated by numbered arrowheads (1 to 4) in Fig. 4.2).

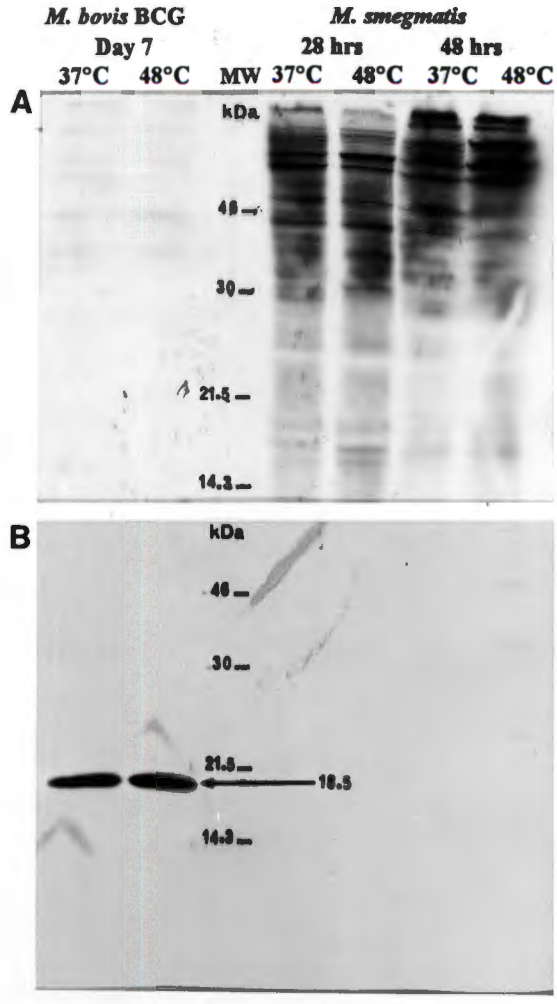


Fig. 4.1.: Detection of α-crystallin protein in *M. bovis* BCG and *M. smegmatis*: **A.** Autoradiograph of nitrocellulose-bound ^{35}[S]L-methionine-labelled proteins from *M. bovis* BCG and *M. smegmatis*. **B.** Immunoblot analysis of the same membrane probed with the F24-2-3 monoclonal antibody specific for the α-crystallin protein. F24-2-3 bound to a protein of 18.5 kDa in *M. bovis* BCG but not in *M. smegmatis*. The α-crystallin protein was not induced by heat shock in *M. bovis* BCG.

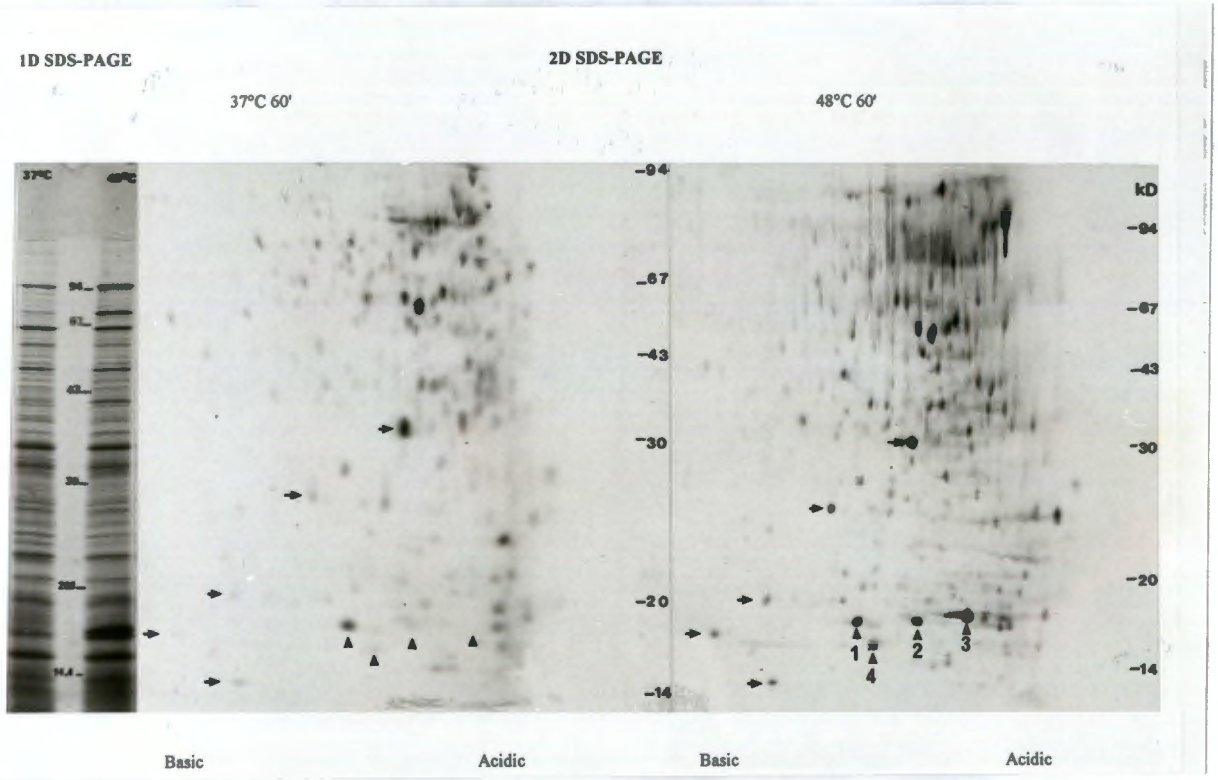


Fig. 4.2.: 1D and 2D SDS-PAGE analysis of *de novo* protein synthesis in samples taken from exponential phase cultures (24 hours) of *M. smegmatis* and incubated for 1 hour at 37°C or 48°C in the presence of ^{35}[S]methionine for protein labelling. Equal amounts of protein (40µg) were loaded onto the gels and the radioactive signals visualised by autoradiography. The heat induced doublet consisting of peptides of 16.5 and 16.8 kDa which was observed under 1D SDS-PAGE analysis (48°C), was resolved into 4 peptides (indicated by numbered arrowheads) with pI values between 4 and 6.5 when using 2D SDS-PAGE analysis.

4.3.3. The amino sequence of Hsp17-2 of M. smegmatis *is homologous to a hypothetical 16.2 kDa protein of unknown function from cosmid MTCY01B2.28 of* M. tuberculosis *H37Rv.*

In spite of the strong radioactive signals observed for the four heat induced peptides (Fig. 4.2), initial attempts to identify the proteins by the Edman degradation-based N-terminal sequencing failed as a result of the extremely low levels of proteins recovered from the gels, i.e. the protein spots remained invisible with Coomassie stain (with a detection limit of 50 to 100 ng). There was still insufficient protein for sequencing even after overloading the gels with up to 100 μg of lysate protein (the maximum limit of protein for the IEF strips) or after combining peptides from up to forty 2D gels. Recent developments in MALDI-TOF mass spectrometry have however made it possible to analyse down to picomole levels of isolated peptides (Vorm *et al.*,1994), and in combination with modifications to the more sensitive silver staining method (with a detection limit between 1 and 10ng), it has now become possible to visualise, and therefore purify and sequence peptides that are in the nanogram range (Shevchenko, 1996).

Silver staining of two-dimensional gels enabled the protein spots corresponding to the four strongly heat induced proteins to be visualised. Gel pieces corresponding to each spot were collected from five two-dimensional gels and subjected to MALDI-TOF - mass spectrometry. There was insufficient protein to enable the sequencing of peptides 1, 3, and 4 (Fig. 4.2). The sequencing of two peptide fragments from spot 2 resulted in sequences (VVGTDGSDS and LLGSVPANVAR) which demonstrated homology to internal fragments of a hypothetical 16.2 kDa protein (protein Rv1636) of unknown function that is encoded by open reading frame 28 on the *M. tuberculosis* cosmid MTCY01B2 (Philipp *et al.*,1996) (Fig. 4.3). Close homology to the proteins listed in Table 4.1 was obtained when using the *M. tuberculosis* amino acid sequence for database (Blast, NCBI, USA) searches.

MSAYKTVVVGTDGSDSSMRAVDRAAQIAGADAKLIIASAYLPQHEDARAADI
LKDESYKVTGTAPIYEILHDAKERAHNAGAKNVEERPIVGAPVDALVNLADE
EKADLLVVGNVGLSTIAGRLLGSVPANVSRRAKVDVLIVHTT

Fig. 4.3.: Deduced amino acid sequence of the coding region of the hypothetical 16.2 kDa protein from the *M. tuberculosis* cosmid MTCY01B2.28. Amino acid sequences of peptide fragments obtained from the MALDI-TOF mass spectrometry sequencing of peptide number 2, i.e. Hsp17-2 (Fig. 4.2) of *M. smegmatis* are underlined. The framed serine (S) residue is replaced by an alanine (A) residue in the sequence of *M. smegmatis*.

Table 4.1.: Proteins homologous to the amino acid sequence of the *M. tuberculosis* 16.2 kDa protein (146 amino acids).

Species	Amino acids	kDa	Identities (%)	Positives (%)	Characteristics
Methanobacterium thermoautotrophicum	149		29	47	Conserved protein (Acc. No. AE000865)
Methanococcus jannaschii	170		30	48	Conserved hypothetical protein (Acc. No. U67502)
Bacillus subtilis	148	15.9	30	46	Hypothetical protein (Acc. No. D31856)
Methanobacterium thermoautotrophicum	131		28	50	Conserved protein (Acc. No. AE000803)
Methanobacterium thermoautotrophicum	143		29	45	Conserved protein (Acc. No. AE000872)
Thermus aquaticus	137	14.6	28	48	Hypothetical protein (Acc. No. D87664)
Methanococcus jannaschii	162		31	62	Conserved hypothetical protein (Acc. No. U67502)

4.3.4. PCR amplification, cloning and DNA sequencing of Hsp17-2

The gene encoding Hsp17-2 was amplified from *M. smegmatis, M. bovis BCG* and *M. tuberculosis* H37Rv genomic DNA using the PCR primers designed from the *M. tuberculosis* gene as described under Materials and Methods. Ultra-violet (UV) visualisation of agarose electrophoresis separated PCR products demonstrated the amplification of specific bands of 439 base pairs (bp) from *M. bovis* BCG *and M. tuberculosis,* but not from *M. smegmatis* (results not shown). The bands were purified from the agarose gels, ligated into pGEM-T vector and then used to transform competent *E. coli* DH5α cells. *Nco*I and *Pst*I (which cut at positions that were 15 bp upstream and 20 bp downstream of the multiple cloning site, respectively) were used to digest the purified recombinant plasmids. The expected 474 bp fragment (35 bp vector sequence and 439 bp insert) was obtained from the clones of *M. bovis* BCG and *M. tuberculosis* PCR products (results not shown). The use of *Bgl*II (an enzyme which cuts at position +203 within MTCY01B2.28 gene of *M. tuberculosis*) to digest the 474 bp *Nco*I/*Pst*I fragment resulted in the expected bands of 256 bp and 218 bp (results not shown) - suggesting that the DNA amplification product of *M. bovis* BCG was similar to the *M. tuberculosis* MTCY01B2.28 gene. The cloned *M. bovis* BCG and *M. tuberculosis* PCR product were sequenced to confirm their identity. A total of 439 bases which demonstrated a 100% identity to gene 28 of the *M. tuberculosis* cosmid MTCY01B2 were obtained for both the *M. bovis* BCG and *M. tuberculosis* PCR products (Fig. 4.4). Database search using the nucleotide sequence of the *M. tuberculosis* Hsp17-2 gene revealed homology to the DNA sequences listed in table 4.2.

4.4. Discussion and Conclusions

In order to ensure the isolation of as pure a sample as possible for protein sequencing, two-dimensional electrophoresis was used for the separation of proteins from the cell lysates of heat-shocked (48°C) and control (37°C) *M. smegmatis* cells. Two-dimensional (2D) electrophoresis provides a powerful tool for the analysis and

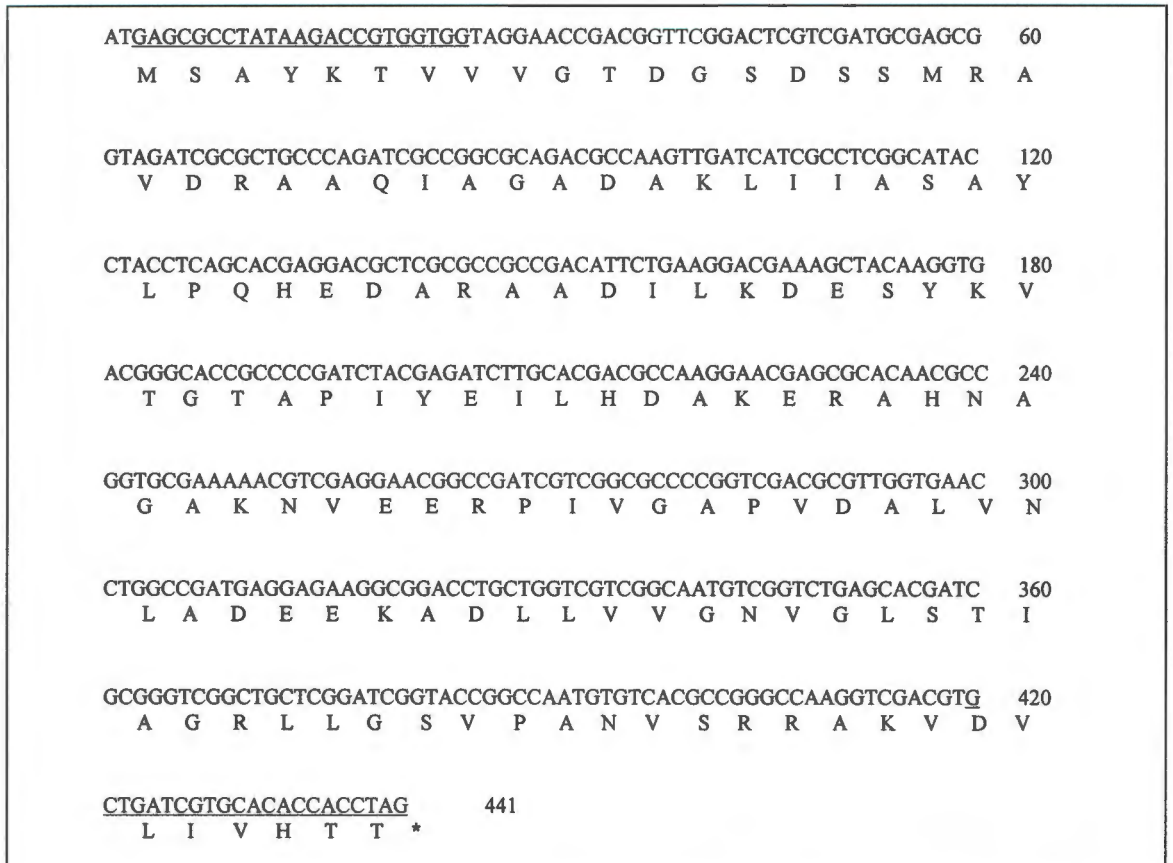


Fig. 4.4.: Amino acid and DNA sequences of *M. bovis* BCG Hsp17-2. The sequence was 100% identical to the corresponding gene from *M. tuberculosis* (cosmid MTCY01B2.28, Accession no. Z95554). The map position of the primers used for PCR amplification of the *M. smegmatis* gene are underlined.

Table 4.2.: DNA sequences homologous to the 441 bp gene (Fig. 4.4) encoding Hsp17-2 (16.8 kDa) of *M. smegmatis* or the 16.2 kDa protein of *M. tuberculosis*.

Species	Sequence	% Identity	Probability	Characteristics
Mycobacterium leprae *Acc. No. l78811*	Cosmid B1133 Locus MSGB1133CS	81%	2.2×10^{-106}	Unknown Function
Thermus aquaticus *Acc. No. D87664*		60%	0.082	Unknown Function

detection of proteins from complex biological sources due to its high resolution and sensitivity (O'Farrell, 1975). 2D-electrophoresis can resolve proteins differing in a single charge and is capable of detecting a protein containing as little as one disintegration per minute of either ^{35}S or ^{14}C by autoradiography (O'Farrell, 1975).

A comparison of autoradiographs from heat-shocked and control samples enabled the protein spots corresponding to the heat induced doublet of ~17 kDa to be identified. Instead of two spots that would have been expected from the two bands (16.5 and 16.8 kDa) observed in 1D-SDS PAGE, four, strongly heat-induced, protein spots (Hsp17-1, Hsp17-2, Hsp17-3, Hsp17-4, respectively numbered 1 to 4 in Fig. 4.2) were identified. This is the first report of the existence of at least three, heat-inducible proteins with molecular weights of ~17 kDa in *M. smegmatis*. Young and Garbe (1991a) have reported the existence in *M. tuberculosis* of heat shock proteins of 15 and 20 kDa which appeared to be more strongly induced by severe temperature stress (37 to 48°C) than by mild stress (37 to 42°C). Using monoclonal antibodies directed against the 14 kDa α-crystallin protein (TB68) and the 19 kDa antigen (HYT6), the authors demonstrated that the 20 kDa protein did not overlap with the 19 kDa antigen, and that the 15 kDa protein did not overlap with α-crystallin. In the present studies, immunoblot analysis using the F24-2-3 anti-α-crystallin monoclonal antibody was unable to demonstrate the presence of α-crystallin in *M. smegmatis,* suggesting that α-crystallin was not one of the heat induced proteins (Fig. 4.1). In addition, the ~17 kDa doublet also showed an increase in induction with increasing heat shock temperature (see Chapter 2, Fig. 2.4). The increased induction of the *M. smegmatis* ~17kDa doublet and the 15 and 20 kDa heat shock proteins of *M. tuberculosis* in response to severe heat shock suggests that they may form part of a regulon that is specifically induced by severe stress. Peptide sequencing of the ~17 kDa doublet of *M. smegmatis* was therefore carried out to investigate whether the four protein spots were different forms of the same protein or if they represented different proteins which may be possible homologues of the 20 and 15 kDa proteins of *M. tuberculosis*.

Mycobacterium bovis BCG was incubated for 1 hour at either 37 or 48°C and the cell lysates used as positive controls for the binding of F24-2-3 monoclonal antibody to α-

crystallin in immunoblot studies. F24-2-3 reacted with a band of 18.5 kDa in *M. bovis* BCG, and no induction of the 18.5 kDa protein could be demonstrated in response to heat shock (Fig. 4.1). This is the first time that the absence of induction of α-crystallin in response to heat shock has been demonstrated in slow-growing mycobacteria. The inability of the mycobacterial α-crystallin protein to be induced by heat shock while showing induction in response to low oxygen tension suggests that the mycobacterial α-crystallin proteins might be under different regulatory mechanisms. Evidence to support this may come from studies on the effect of low oxygen tension on α-crystallin in other organisms. The significance of the absence of α-crystallin in *M. smegmatis* requires further investigation. It is conceivable that mechanisms such as mutations or truncations of genes which are responsible for inhibiting the expression of α-crystallin in *M. smegmatis* may be central to the understanding of how the pathogenic mycobacteria such as *M. tuberculosis* have adapted α-crystallin for functions specific to virulence.

Peptide sequence analysis of the *M. smegmatis* ~17 kDa heat shock proteins using MALDI-TOF mass spectrometry resulted in the identification of one of the four protein spots. Tryptic digests of the protein obtained from peptide spot 2 - subsequently named Hsp17-2 (Fig. 4.2) resulted in two fragments whose sequences showed homology to open reading frame 28 of the *M. tuberculosis* cosmid MTCY01B2. The *M. tuberculosis* open reading frame encodes a hypothetical protein of 16.2 kDa with no known function. The gene encoding the *M. bovis* BCG protein *(hsp17-2)* was cloned and shown to have 100% identity to the gene encoding the *M. tuberculosis* protein (Table 4.2). Failure to amplify the Hsp17-2 gene from *M. smegmatis* using the *M. tuberculosis* primers suggested that the nucleic acid sequences may be different. To overcome the possible nucleic acid sequence differences, cosmid or plasmid libraries of *M. smegmatis* will have to be used to clone and sequence the *M. smegmatis* Hsp17-2 gene. Due to lack of time, it was not possible to obtain the sequence of the *M. smegmatis* Hsp17-2 gene during the current studies.

Database (BLAST, NCBI) searches using the 146 amino acid sequence of *M. tuberculosis* demonstrated 28 to 31% identity to conserved proteins from

Methanobacterium thermoautotrophicum, Methanococcus jannaschii, Bacillus subtilis, Methanobacterium thermoautotrophicum, and *Thermus aquaticus* (Table 4.1). The presence of this protein in these bacteria has been identified largely through genome sequencing initiatives, and as a result not much effort has been made to characterise its cellular functions. The present study has therefore succeeded in assigning a functional characteristic to the protein - i.e. the protein has been shown to be induced by heat shock in *M. smegmatis*. Further studies will be required to over-express Hsp17-2 and use the purified protein to raise antibodies. Immunoblot studies with antibodies directed against Hsp17-2 should make it possible to determine whether or not the other three spots are different forms of the same protein. Synthesis of this protein at different stages of growth will also be investigated. In addition, the expression of the protein in other mycobacteria and under other conditions of stress will be assessed.

Attempts to use Northern blot analysis using the cloned *M. smegmatis* PCR products (randomly labelled with [α^{32}P] dCTP) to demonstrate heat-induction of Hsp17-2 messenger RNA in *M. smegmatis, M. tuberculosis,* and *M. bovis* BCG were unsuccessful. The gene encoding the L7/L12 ribosomal protein of *M. bovis* BCG (cloned and sequenced in this study) gave a strong positive signal when used as a probe against the same blot, thereby serving as a positive control for the procedure used for Northern blot analysis. Judging by the low levels of the protein in cell lysates (as observed by the inability to stain the protein with Coomasie Blue), it may be possible that the levels of the specific messenger RNA may be below the detection limits of the Northern blot assay. Characterisation of the mechanisms that control gene expression may enable comparisons to be made with Hsp17-2 and other heat shock proteins. It appears that Hsp17-2 may be part of a new group of heat shock proteins which may be specifically induced under conditions of severe heat shock, and may therefore be under the control of σ^E subunit (see Fig. 1.7). The highly conserved nature of Hsp17-2 and its presence in a diverse range of organisms suggests that it may form part of a cellular process that appeared early in evolution.

Chapter 5

The effect of Heat Shock on the Synthesis and Release of Proteins in *Mycobacterium smegmatis*

5.1. Introduction

The interest in mycobacterial culture filtrate proteins began as early as 1891 with Koch's discovery that a heat-inactivated concentrate of the constituents present in a glycerol-containing broth that had supported growth of *M. tuberculosis* for 8 weeks, was able to elicit a characteristic reaction 24 to 48 hours after subcutaneous injection in tuberculous animals and humans (Koch 1891, 1897). The product was named Koch's tuberculin and the skin reaction is referred to as a delayed-type hypersensitivity (DTH) reaction. Seibert and Munday (1932) later demonstrated that the active component consisted of the ammonium sulphate-precipitable protein fraction of the culture filtrate - the tuberculin purified protein derivative (PPD). PPD remains a useful diagnostic tool for tuberculosis. Various studies have since demonstrated that live bacilli were more effective in inducing protective immunity than killed bacilli, leading to the suggestion that viable mycobacteria may be required for the active secretion of antigens that are essential for the induction of protective immunity (Bloch and Segal, 1955; Rook, 1980; Lovick and Closs, 1984). As a result, secreted proteins have become the focus of attention in the search for mycobacterial antigens that could be used as subunit vaccines (Pal *et al.*,1992; Horwitz *et al.*,1995; Harth *et al.*,1997). A summary of secreted mycobacterial antigens that have been identified to date is given in table 5.1.

Secreted proteins are synthesised in a precursor form with a signal peptide (or leader sequence) of 20 to 40 amino acids that is required for transport across the cell wall. The N-terminal two or three amino acids are followed by a stretch of hydrophobic amino acids which anchor the peptide on the membrane. Specific signal peptidases

recognise and cleave a conserved sequence within the precursor to release the mature protein (Oliver, 1985; Pugsley *et al.*,1990). Both signal peptide-independent secretion (i.e. dependent on secretion factors) and signal-peptide dependent secretion have been reported in mycobacteria (Andersen and Brennan, 1994). The localisation index (LI) developed by Wiker *et al.* (1991) enables the secretion efficiency of mycobacterial antigens to be determined by expressing the relative concentration of protein located intracellularly and extracellularly.

In vitro studies to characterise mycobacterial secreted proteins have largely been performed by analysing the culture filtrates of cultures growing under normal growth conditions. It has been shown for example that the profile and quantity of secreted proteins is dependent upon the growth phase of the culture (Andersen *et al.*,1991). No attempts have thus far been made to analyse the effect that different environmental stresses or incubation conditions would have on the profile of all the secreted proteins. It is conceivable that different environmental signals may result in the induction of specific responses that may ultimately alter the profile of secreted proteins. A good example under *in vivo* conditions is the increased expression of the *mig* gene which codes for a secreted protein upon uptake of *M. avium* by macrophages (Plum *et al.*, 1997). The aim of this study was therefore to investigate the effect of heat shock on the quality and quantity of secreted proteins in *M. smegmatis*.

5.2. Materials and Methods

5.2.1. Mycobacterial Culture and Heat Shock

Mycobacterium smegmatis was grown at 37°C in Middlebrook 7H9 medium supplemented with ADT (0.5% (w/v) albumin fraction V; 2% (w/v) D-glucose; and 0.05% (v/v) Tween 80) as described in section 2.2.1 and 2.2.3. The growth curve of the cultures was similar to Fig. 2.1. Aliquots equivalent to $2x10^9$ cells were taken from late exponential phase (28 hours) and stationary phase (48 hours) of the culture and the cells were collected by centrifugation at 3000 rpm. *M. bovis* BCG was

Table 5.1: Secreted Mycobacterial Antigens[ϒ]

• 88 kDa	• KatG catalase-peroxidase - identified within a protein cluster of 85 to 88 kDa recognised by monoclonal antibodies known to react with sera from patients with tuberculosis (Sonnenberg and Belisle, 1997).
• 58 kDa	• glutamine synthetase (Harth *et al.*,1994).
• 52 kDa	• α-hydroxysteroid dehydrogenase (Sonnenberg and Belisle, 1997).
• 45 kDa	• MPT32 (LI=90) (Dobos *et al.*,1996;).
• 40 kDa	• L-alanine dehydrogenase (Andersen *et al.*,1992)
• 31.5 kDa	• MPT59/Ag85C (LI=70) - member of the Ag85 complex with fibronectin binding and mycolyl transferase activities (Belisle *et al.*,1997; Content *et al.*,1991).
• 31 kDa	• MPB44/Ag85A (LI=150)- member of the Ag85 complex with fibronectin binding and mycolyl transferase activities (Belisle *et al.*,1997; Borremans *et al.*,1989).
• 30 kDa	• MPB45/Ag85B (LI=20) - member of the Ag85 complex with fibronectin binding and mycolyl transferase activities (Belisle *et al.*,1997; Matsuo *et al.*,198;, Nagai *et al.*,1991).
• 27 kDa	• MPT51 (LI=25) - with 60% homology to N-terminal of MPT59. Member of the Ag85 complex (Nagai *et al.*,1991).
• 25 kDa	• Corresponds to ORF 28c of *M. tuberculosis* cosmid MTCY1a11 (Sonnenberg and Belisle, 1997).
• 24 kDa	• MPT64 (LI=40) (Nagai *et al.*,1991; Yamaguchi *et al.*,1989)
• 23 kDa	• MPB70 (LI=1000) - major secreted antigen of *M. bovis* (Wiker *et al.*,1991)
• 22 kDa	• Superoxide dismutase (SOD) inactivates toxic superoxide radicals generated by activated macrophages - (Thangaraj *et al.*,1990; Anderson, *et al.*,1991; Zhang *et al.*,1991).
• 18 kDa	• MPB80 - consists of various forms found with masses of 22 to 25kDa; carbohydrate associated with purified protein.
• 18 kDa	• MPT63 (LI>150) - (Nagai *et al.*,1991).
• 16 kDa	• Third most abundant immunodominant antigen after Ag85A and Ag85B (Horwitz *et al.*,1995; Harth *et al.*,1997).
• 15 kDa	• MPT53 (LI>70) - (Nagai *et al.*,1991).
• 14 kDa	• MPT46 - L=1 - secreted or not? (Wiker *et al.*,1990).
• 6 kDa	• ESAT-6 (Sorensen *et al.*,1995).

ϒ MPB & MPT represent mycobacterial protein *M. bovis* and *M. tuberculosis*, respectively. The numerical suffix denotes relative mobility in native PAGE - 7.7% acrylamide - (Nagai *et al.*,1981). LI values were obtained from Wiker *et al.* (1991).

obtained from The State Vaccine Institute (Pinelands, South Africa) as single-dose, vaccine vials containing cells which were propagated as surface pellicles in Sauton medium. For the present studies, *M. bovis* BCG was grown by resuspending the contents of one vaccine vial in 200 ml Middlebrook 7H9 medium supplemented with ADT. A five-day old culture of *M. tuberculosis* H37Rv growing in Middlebrook 7H9 medium supplemented with ADT was used to inoculate (1/100 dilution) fresh medium and both the *M. tuberculosis* H37Rv and *M. bovis* BCG cultures were grown with continuous shaking at 37°C. Cells from fourteen-day old cultures of *M. bovis* BCG and *M. tuberculosis* were collected by centrifugation of 100ml aliquots at 3000 rpm at room temperature.

To enable the proteins secreted into the supernatant to be concentrated without interference from excess albumin from the medium, the *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* cell pellets were resuspended in 2 ml Middlebrook 7H9 medium supplemented with only D-glucose and Tween 80. For the determination of the effect of other stresses, i.e. hydrogen peroxide (H_2O_2), isonicotinic acid hydrazide (isoniazid - INH) and low pH on protein synthesis, the *M. smegmatis* cells were resuspended in Middlebrook 7H9 medium (with D-glucose and Tween 80) supplemented with either 10 mM H_2O_2; 1 to 15μg/ml INH or with the pH adjusted to pH 5 using acetic acid. *De novo* protein synthesis during heat shock (1 hour at 48°C for *M. smegmatis* and 42°C for *M. bovis* BCG and *M. tuberculosis*) and at 37°C (control) was monitored in the presence of ^{35}[S]L-methionine as described in Chapter 2, section 2.2.4. At the end of the incubation period, the bacteria were collected by centrifugation at 3000 rpm and the cellular proteins extracted and analysed by SDS-PAGE (12 or 15% acrylamide) as described in Chapter 2, section 2.2.5 and 2.2.6. The supernatants were collected and used for the preparation of secreted proteins as described below.

5.2.2. Preparation of Secreted Proteins, Electrophoresis, and Immunoblot Analysis

The supernatants described in section 5.2.1. above were filtered through a 0.2 μm filter (Millipore, France) to remove bacteria and the secreted proteins were concentrated 20-fold by passing the supernatant through a Centricon-3 column with a molecular weight cut-off of 3 kDa (Amicon, Massachusetts, USA). The concentration of proteins in the supernatants was determined. For SDS-PAGE analysis, the supernatants (20 μg protein) were mixed with an equal volume of sample buffer (10% (v/v) glycerol; 3% (w/v) SDS; 0.0625 M Tris-HCl (pH 6.8); 0.2% (w/v) bromophenol blue; 10% (v/v) β-mercaptoethanol) and incubated in a boiling water bath for 5 min. Electrophoretic analysis was performed as described in Chapter 2, section 2.2.6. For two-dimensional electrophoresis, the supernatants (20μg protein) were mixed in a 1:4 ratio with sample solution (see section 4.2.2, first dimension) and the electrophoresis was performed as described in section 4.2.2. After one- and two-dimensional electrophoresis, the proteins were either transferred to nitrocellulose membranes and subjected to immunoblot analysis using the monoclonal antibodies HYT27 (anti-α-crystallin) and HYT6 (anti-19 kDa lipoprotein antigen) and the polyclonal antiserum-K92 (anti-Ag85B) as described in appendix 1, or detected by autoradiography from dried gels as described in Chapter 2, section 2.2.6.

5.2.3. Preparation of Culture Filtrate Proteins

For the preparation of proteins secreted into the medium during growth (culture filtrate proteins), frozen aliquots of *M. smegmatis* (prepared as described in Chapter 2, section 2.2.1) were used to inoculate (1/1000 dilution) Middlebrook 7H9 medium (200 ml) supplemented with 2% (w/v) D-glucose and 0.05% (v/v) Tween 80 and no albumin. Growth was monitored by assaying the ATP concentration of the culture as described in Chapter 2, section 2.2.3. The bacterial cells were collected by centrifugation and processed as described in section 5.2.1 above. The supernatant (i.e. culture filtrate) was concentrated and analysed by SDS-PAGE as described in section 5.2.2.

5.2.4. Determination of Isocitrate Dehydrogenase (ICD) Activity

The amount of ICD activity detectable in the supernatants from heat shock or stress experiments was used to assess the extent of cell lysis during the period of incubation. ICD activity was assayed using the Isocitrate Dehydrogenase Procedure No. 153-UV Kit (Sigma Diagnostics, Missouri, USA). ICD catalyses the decarboxylation of L-isocitrate to 2-oxoglutarate by reducing nicotinamide adenine dinucleotide phosphate (NADP). The absorbance of the reduced form of NADP (NADPH) is read at 340nm and the activity of ICD is proportional to the concentration of NADPH formed. ICD activity was calculated according to the method described in the supplied protocol.

5.3. Results

5.3.1. The release of Mycobacterium smegmatis *proteins into the culture supernatant is Increased in Response to Heat Shock*

To enable secreted proteins to be concentrated and separated by polyacrylamide gel electrophoresis without interference from excess albumin in the medium, bacterial cells were resuspended in medium without albumin during the heat shock assay. The effect of incubating cells in medium without albumin during heat shock was investigated by comparing the profile of intracellular proteins that were synthesised in the presence or the absence of albumin. Increased synthesis of two proteins (with apparent molecular weights of 60 and 30 kDa) was observed in the absence of albumin at both 37 and 48°C (Fig. 5.1). Further studies on secreted proteins were therefore performed by resuspending cells in medium without albumin.

The effect of heat shock on protein secretion in *M. smegmatis* was investigated by monitoring protein synthesis in exponential phase cells that were resuspended in medium without albumin and incubated for 1 hour at 37°C (control) or 48°C (heat shock) in the presence of ^{35}[S]L-methionine. SDS-PAGE analysis followed by autoradiography demonstrated the synthesis and release of proteins with apparent

molecular weights of 53, 46, 33, 31, and 22 kDa during the 1 hour of heat shock (Fig.5.1). Densitometric analysis of supernatant proteins demonstrated a 2.2-, 1.7-, and 4.1-fold induction of the 33, 31, and 22 kDa proteins, respectively, while the 53 and 46 kDa protein remained constant. Autolysis was monitored by measuring the activity of isocitrate dehydrogenase in the supernatant and the cell lysate after heat shock. The results for isocitrate dehydrogenase activity are given in Table 5.2.

5.3.2. The Antigen-85 complex is present in Mycobacterium smegmatis *and the synthesis and release of Ag85B is increased by Heat Shock*

In immunoblot studies using antibodies specific for the Ag85A and Ag85B proteins, Harth *et al.* (1997) were only able to demonstrate the presence of Ag85A in the cell lysate *of M. smegmatis*, but were unable to show binding to any extracellular proteins. The presence of proteins with apparent molecular weights of 31 and 33 kDa in the supernatants of *M. smegmatis* suggested that they may be homologues of the Ag85 complex (Ag85A, Ag85B or Ag85C). Immunoblot studies using Ag85 complex-specific antibodies were therefore used to identify the secreted proteins of *M. smegmatis*.

The use of monoclonal antibody HYT27 (which cross-reacts with Ag85A, Ag85B and Ag85C) showed binding to a single band of 32 kDa which was induced 2.8-fold in response to heat shock at 48°C (results not shown). Several attempts to reproduce the HYT27 binding experiment were unsuccessful, presumably as a result of a conformational change (occurring during the protein preparation) in the epitope recognised by HYT27. A rabbit polyclonal antiserum (K92) which was raised against the *M. tuberculosis* Ag85B protein was therefore used for immunoblot analysis. The polyclonal antiserum reacted with two bands of 30 and 32 kDa which were present in both the cell lysates and the supernatants of *M. smegmatis* and *M. bovis* BCG (Fig. 5.2). Heat shock (48°C) resulted in the induction of the 30 kDa protein in the supernatant (1.5-fold) and the lysate of *M. smegmatis* (Fig. 5.2.A). A longer exposure of the blot demonstrated the presence of the 30 kDa protein in the lysate at 37°C

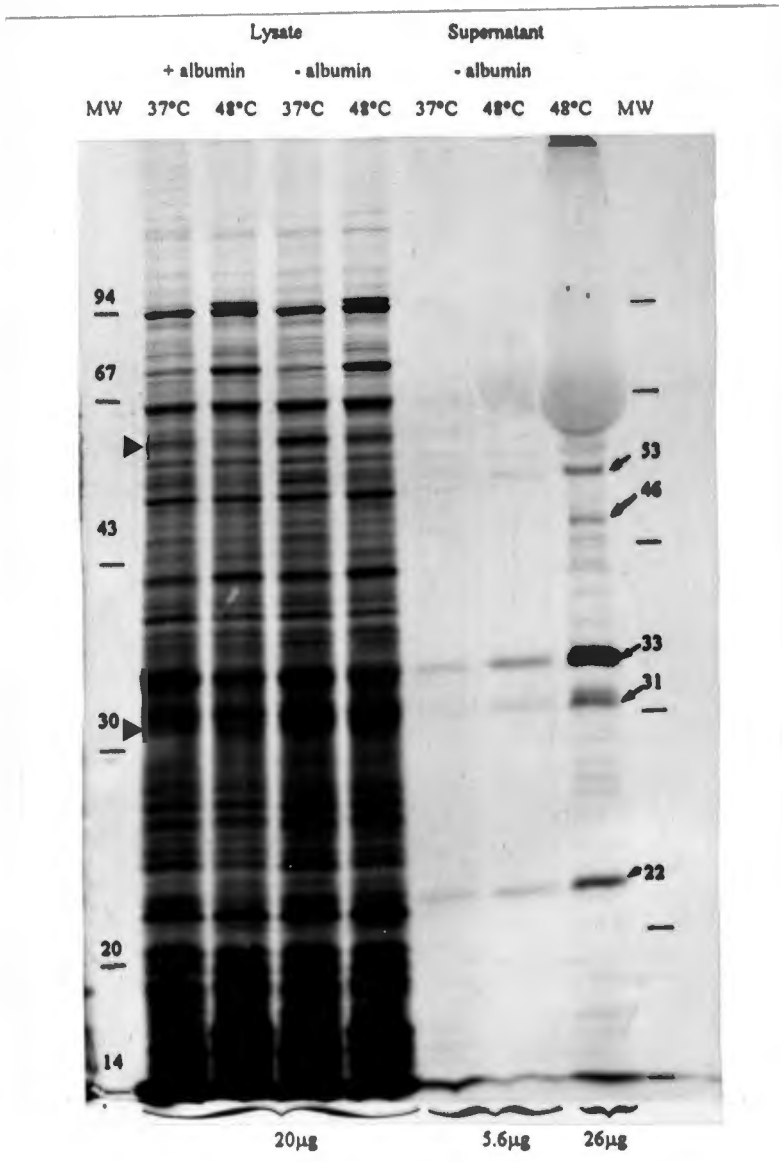


Fig. 5.1.: *De novo* protein synthesis in *M. smegmatis* incubated with ^{35}[S]methionine for 1 hr at 37 or 48°C. Intracellular protein synthesis (cell lysate) was monitored in medium with (+) or without (-) albumin. Proteins released into the supernatant after incubating cells for 1 hr at 37 or 48°C were monitored. Proteins (30 & 60 kDa) induced when cells were resuspended in medium without albumin are indicated with closed triangles. Protein sizes in kilodaltons (kDa).

Table 5.2: Isocitrate Dehydrogenase activity (U/ml)

Cell Lysate (Pellet)		Supernatant	
37°C	48°C	37°C	48°C
58700	69500	430	790

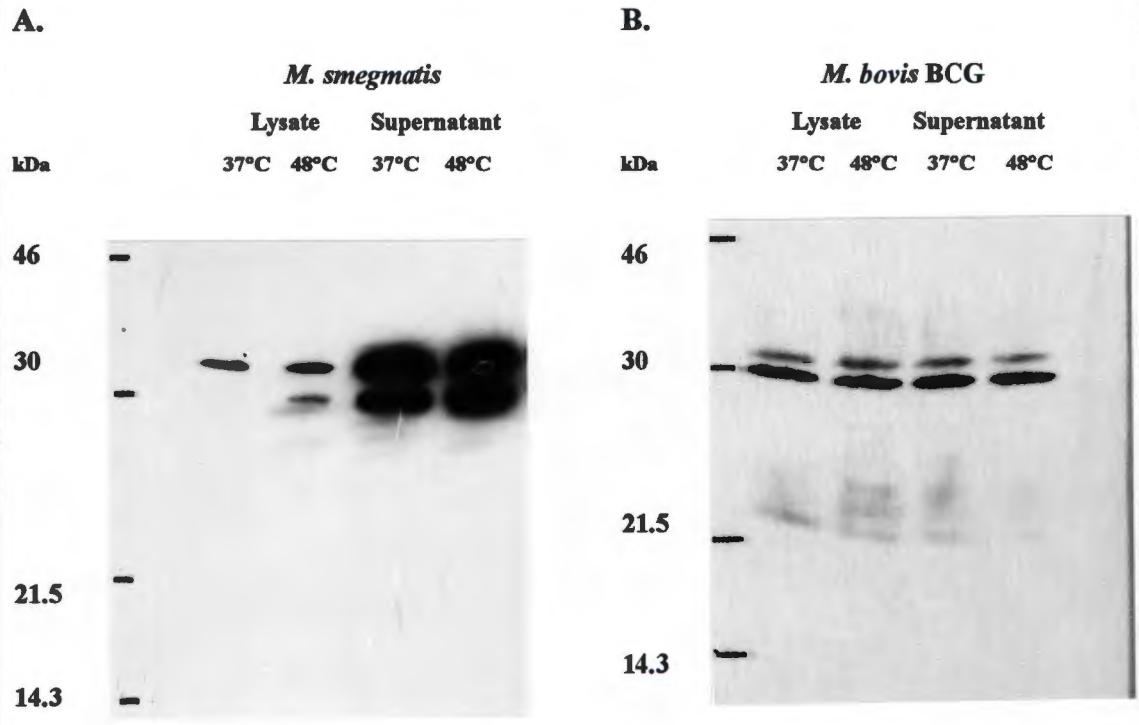


Fig. 5.2.: Immunoblot analysis of *M. smegmatis* (A) and *M. bovis* BCG (B) using anti-Ag85B antiserum. *M. smegmatis* and *M. bovis* BCG were incubated at 37 or 48°C for 1 hour. Equal amounts (20μg) of cellular proteins (lysate) and supernatant proteins were separated by SDS-PAGE.

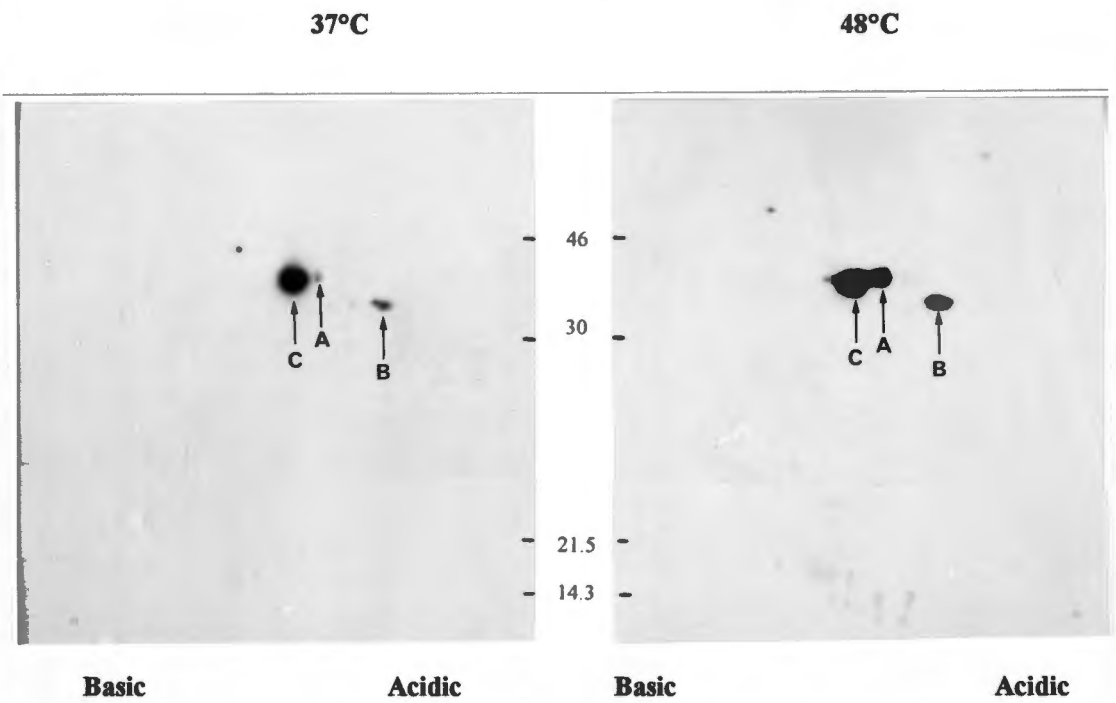


Fig. 5.3.: Two-dimensional PAGE analysis of proteins released by *M. smegmatis* after incubation at 37 or 48°C for 1 hour. Anti-Ag85B polyclonal antiserum reacted with three proteins - Ag85A, Ag85B, and Ag85C respectively indicated by A, B, and C, and with pI values between 4.6 to 6.

(results not shown). Heat shock did not have any effect on the lysate and supernatant levels of the 32 kDa protein of *M. smegmatis* (Fig. 5.2.A), nor on the 30 and 32 kDa proteins of *M. bovis* BCG. The secreted proteins were separated by two-dimensional PAGE and the gels processed for immunoblot analysis. The K92 polyclonal antiserum reacted with three spots at 37°C and at 48°C (Fig. 5.3). Using the two-dimensional map of secreted proteins created by Sonnenberg and Belisle (1997) for *M. tuberculosis,* the molecular weights and isoelectric points of the *M. smegmatis* proteins indicated that they correspond to the Ag85A, Ag85B and Ag85C proteins, respectively indicated by A, B, and C in Fig. 5.3. Heat shock resulted in an increase in the release of Ag85A and Ag85B.

5.3.3. The Ag85 complex proteins are not detectable in long-term culture filtrates of **Mycobacterium smegmatis.**

Protein secretion in the culture filtrate of a growing culture of *M. smegmatis* was investigated in an attempt to reproduce the experimental conditions under which Harth *et al.* (1997) were unable to demonstrate the secretion of Ag85 complex proteins in *M. smegmatis*. *M. smegmatis* was grown in Middlebrook 7H9 medium supplemented with only D-glucose and Tween 80 and with no albumin. The omission of albumin in the medium was to enable the collection of secreted proteins from the culture filtrate without the excess albumin. The growth of *M. smegmatis* in the absence of albumin was slow, only reaching late exponential phase levels (28 hours in Fig. 2.1) after 96 hours (4 days). The slow-growth was probably a result of oleic acid released by Tween 80 which, when not complexed to albumin, become toxic to mycobacterial cells. Equal amounts (20 μg) of culture filtrate proteins (i.e. proteins secreted into the medium during growth), cellular proteins (cell lysate), and supernatant proteins (i.e. proteins released into the medium during heat shock) were separated by SDS-PAGE and the presence of Ag85 complex proteins investigated by immunoblot analysis. K92 anti-Ag85B polyclonal antiserum reacted with the expected 30 and 32 kDa proteins in the lysate and supernatant fractions. There was only limited binding to a 32 kDa protein in the culture filtrate (Fig. 5.4). Whereas heat shock resulted in increased

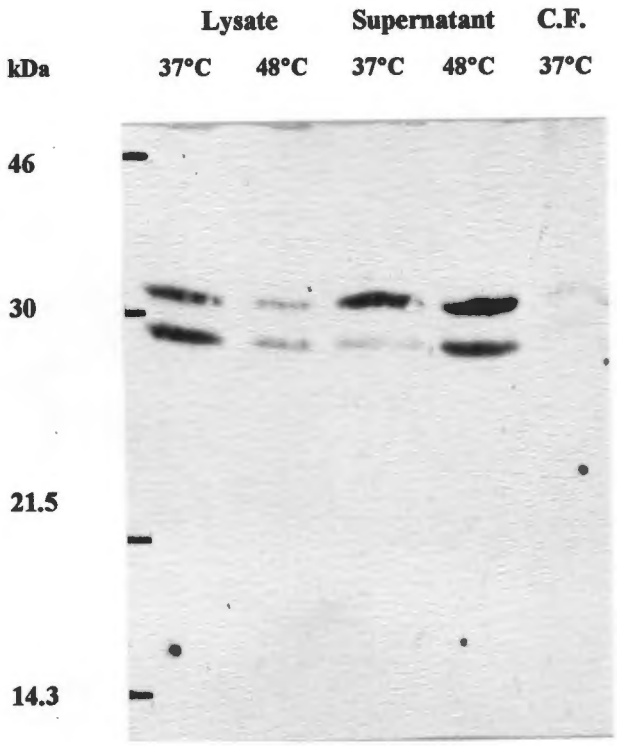


Fig. 5.4.: Immunoblot analysis (using K92 anti-Ag85B antiserum) of *M. smegmatis* grown in medium without albumin. 20μg of cell lysate, supernatant, and culture filtrate (C.F.) protein per lane.

release of the 30 kDa protein, the intracellular levels of both the 32 and the 30 kDa proteins were higher at 37°C than at 48°C. One of the proteins that were induced by incubating cells in medium without albumin was 30 kDa (see Fig. 5.1). It is therefore conceivable that growth of *M. smegmatis* in medium without albumin may have resulted in increased synthesis of the 30 kDa Ag85B protein.

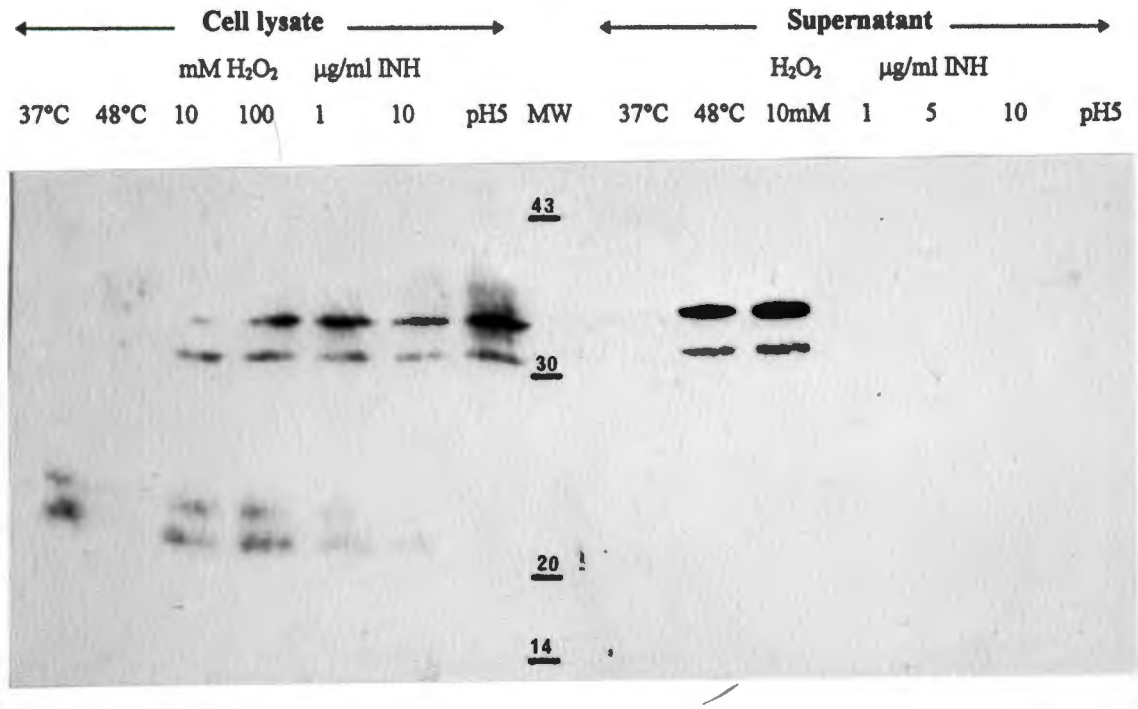
5.3.4. The Effect of Various Stress Conditions on the release of the Ag85 complex in Mycobacterium smegmatis.

The exposure of *M. smegmatis* to low pH resulted in the inhibition of the release of the Ag85 complex despite its continued synthesis within the cells as demonstrated by the detection of a 30/32 kDa double in the cell lysate (Fig. 5.5. A & B). The release of the Ag85 complex in response to INH and H_2O_2 in *M. smegmatis* were influenced by growth phase. While INH (5 μg/ml) resulted in increased release of the Ag85 complex in stationary phase culture, H_2O_2 (10 mM) only induced Ag85 complex release in late exponential phase culture. Despite the growth phase dependent variations in Ag85 complex secretion in response to INH and H_2O_2, intracellular proteins continued to be synthesised under all the stress conditions tested. The anti-Ag85B antiserum also cross reacted with a protein of ~23 kDa, a possible homologue of MPT51 - the 27 kDa member of the Ag85 complex.

5.3.5. The 19 kDa Lipoprotein Antigen is not expressed in M. smegmatis *and its release from* M. tuberculosis *H37Rv and* M. bovis *BCG is increased by Heat Shock*

Lipoproteins are normally anchored on the membrane or cell wall via their lipid moiety, thereby exposing the protein on the bacterial surface. The *M. tuberculosis* 19 kDa lipoprotein was identified by Young and Garbe (1991) and has recently been shown to be glycosylated - a feature normally associated with eukaryotic cells (Herrmann *et al.*,1996). While attempting to identify the heat-induced ~17 kDa doublet of *M. smegmatis* discussed in chapter 4, the HYT6 monoclonal antibody which is specific for

A. 28 Hours



B. 48 Hours

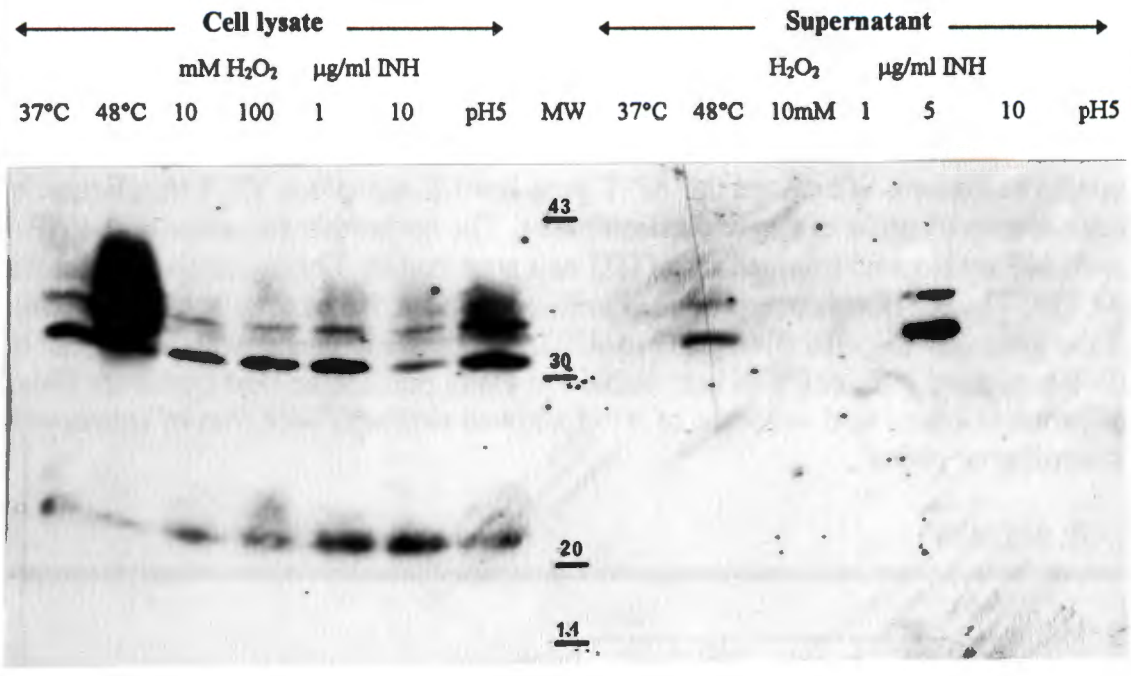


Fig. 5.5: Synthesis and release of *M. smegmatis* Ag85 complex proteins in response to heat shock, H_2O_2, INH and low pH (pH5) during: **A.** late exponential phase (28 hr), and **B.** stationary phase (48 hr). Immunoblot analysis was performed using anti-Ag85B antiserum (K92), and equal amounts (20µg) of protein were loaded in each lane. The molecular weights (MW) are in kilodaltons (kDa).

the *M. tuberculosis* 19 kDa lipoprotein antigen was also tested for its reactivity against the ~17 kDa proteins.

Monoclonal antibody HYT6 failed to show binding to both the cell lysate (results not shown) and the supernatant of *M. smegmatis* (Fig. 5.6). HYT6 did however demonstrate binding to a protein with an apparent molecular weight of 23 kDa when used against the supernatants from heat-shocked *M. tuberculosis* H37Rv and *M. bovis* BCG (Fig. 5.6). Glycosylation of the 19 kDa lipoprotein may account for the increase in the apparent molecular weight of the protein bound by the HYT6 antibody. The 23 kDa supernatant protein of *M. bovis* BCG and *M. tuberculosis* was only observed after heat shock (42°C). In view of the fact that the 19kDa antigen is normally anchored on the bacterial wall, it appears that heat shock may activate a mechanism which results in the protein being released into the medium, hence its presence in the supernatant of heat-shocked cells.

5.4. Discussion and Conclusions

The effect of heat shock on protein secretion in *M. smegmatis* was investigated. *De novo* protein synthesis monitored using radioactive ^{35}[S]L-methionine demonstrated the synthesis and release of proteins with apparent molecular weights of 53, 46, 33, 31, and 22 kDa, with the 33, 31, and 22 kDa proteins demonstrating a 2.2-, 1.7-, and 4.1-fold increase in release in response to heat shock (Fig. 5.1). The observed heat-shock induced increase in the release of some proteins while the levels of others remain constant suggests that the profile of secreted proteins may be influenced by the environmental stress conditions that the bacteria are exposed to. It is anticipated that such stress-specific changes in the profile of secreted proteins may enable the functions of secreted proteins under specific conditions to be elucidated.

The presence of isocitrate dehydrogenase (ICD) in the supernatant was used to investigate whether or not autolysis was the reason for the increased release of proteins

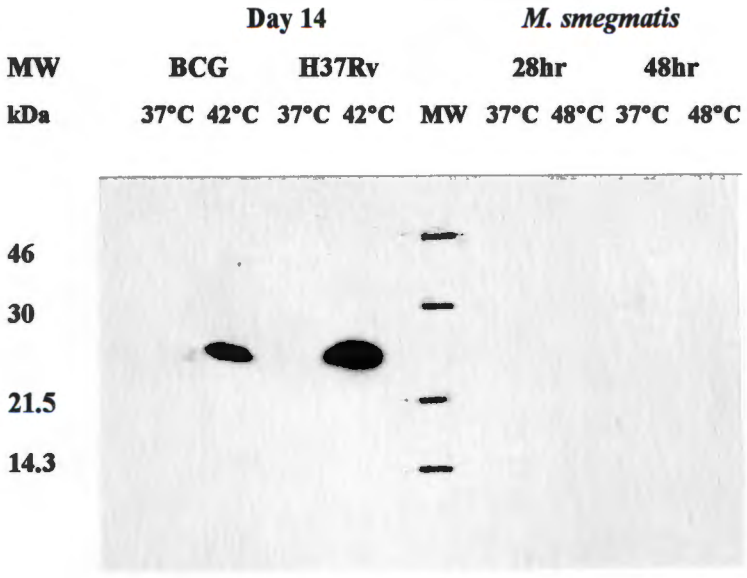


Fig. 5.6.: Immunoblot analysis (using HYT6 anti-19kDa antigen monoclonal antibody) of supernatants from heat-shocked (1 hour) *M. bovis* BCG (42°C), *M. tuberculosis* H37Rv (42°C), and *M. smegmatis* (48°C). A 1-hour incubation at 37°C was used as the uninduced control for each culture. HYT6 reacted with a 23 kDa protein in *M. tuberculosis* H37Rv and *M. bovis* BCG, but not in *M. smegmatis*. An autoradiograph of the above blot gave radioactive band signals that had similar intensities to those illustrated in Fig. 4.1.A .

during heat shock. ICD activity was detectable in the supernatants from cells incubated at 37 or 48°C, with the 48°C sample having higher ICD activity than the 37°C supernatant. It is unlikely that the higher activity of ICD in the 48°C supernatants was due to heat-induced damage to the cell wall since the activity in the cell lysate was also higher at 48 than at 37°C. It may be that the higher temperature (48°C) resulted in an increase in the rate of synthesis of isocitrate, thereby resulting in the higher levels of measurable NADPH. It is also unlikely that the observed activity of ICD in the supernatant is indicative of autolysis since none of the low molecular weight radioactively labelled proteins that are present in the cell lysate could be seen in the concentrated supernatant (Fig. 5.1). It was therefore concluded that the radioactively labelled proteins that were detected in the supernatant were actively synthesised and released during the 1 hour of heat shock.

The antigen-85 complex consists of a group of closely related secreted proteins with molecular weights of 31.5, 31, and 30 kDa, named Ag85C, Ag85A and Ag85B, respectively (see Table 5.1). During 1D-SDS-PAGE analysis, Ag85C and Ag85A are not properly resolved and as a result the pattern on immunoblot analysis usually reveals two bands at 30 and 32 kDa (Wiker and Harboe, 1992). The presence of proteins of 31 and 33 kDa in autoradiographs of *M. smegmatis* supernatants suggested that they may represent homologues of the Ag85 complex proteins. Harth, *et al.* (1997) had presented evidence to suggest that the Ag85 complex in *M. smegmatis* was not released into the culture filtrate. Studies using anti-Ag85 complex antibodies were therefore undertaken to identify the 31/33 kDa secreted protein doublet of *M. smegmatis*. A polyclonal antiserum (K92) that was raised against the *M. tuberculosis* Ag85B protein reacted with proteins of 30 and 32 kDa in the pellet (cell lysate) and supernatant of *M. smegmatis*. Immunoblot analysis of 1D-SDS-PAGE separated samples demonstrated heat-induction of only the 30 kDa protein (Ag85B) in both the pellet and the supernatant fractions (Fig. 5.2). Two-dimensional SDS-PAGE analysis of proteins *M. smegmatis* released into the supernatant showed binding of antibody to three peptides, which, based on previously published isoelectric point and molecular weight positions, corresponded to Ag85A, Ag85B and Ag85C (Fig. 5.3). The peptides corresponding to the Ag85A and Ag85B showed increased release in response to heat shock whereas Ag85C declined. It appears that the reason why the heat-induced

release of the Ag85A could not be demonstrated by immunoblot analysis of supernatant proteins separated using 1D-SDS-PAGE was a result of the increase in Ag85A being masked by the reduction in the levels of the more abundant Ag85C.

In attempt to reproduce the experiments where Harth *et al.* (1997) were unable to demonstrate the presence of Ag85 complex proteins in the culture filtrate of *M. smegmatis,* the culture filtrate of a late exponential phase culture (grown in 200ml of medium without albumin) was concentrated and analysed for the presence of the Ag85 complex. The cells were resuspended in fresh medium and incubated for 1 hour, and the corresponding cell lysate and supernatant proteins were subjected to immunoblot analysis. The polyclonal antiserum reacted with a doublet of about 30 kDa in both the cell lysate and the short-term supernatant (i.e. supernatants taken after 1 hour of incubation of cells) but not in the culture filtrate despite having been concentrated 20-fold (Fig. 5.4). It appears that *M. smegmatis* Ag85 complex is released into the medium, but unlike in *M. tuberculosis* where the proteins accumulate in the supernatant with increasing age of culture (Andersen *et al.*,1991), the proteins may be degraded in the long-term culture filtrates of *M. smegmatis*. This raises the question of whether or not the mechanism of action of the Ag85 complex in slow-growing pathogens is different from that of the fast-growing non-pathogens. Additional evidence for the existence of such differences is illustrated by the fact that heat shock did not increase the synthesis or release of Ag85B in *M. bovis* BCG (Fig. 5.2.B). It would be of interest to further investigate the implications of such differences, particularly in view of the role of the Ag85 complex in the binding of fibronectin which is thought to facilitate the uptake of mycobacteria by eukaryotic cells.

Preliminary studies on the effects of isoniazid, hydrogen peroxide and low pH (pH5) on the release of the Ag85 complex from *M. smegmatis* were conducted. Low pH inhibited the release of Ag85 complex from cells in both the late exponential phase and stationary phase cultures despite the proteins being detectable in the cell lysate. Whereas the Ag85 complex continued to be synthesised intracellularly in exponential phase and stationary phase cultures in the presence of both isoniazid and hydrogen peroxide, the release of Ag85 complex in response to hydrogen peroxide was only detectable in stationary phase cells while isoniazid only induced protein release in late

exponential phase cells. Isoniazid-induced increase in the secretion of the Ag85 complex has been reported in *M. tuberculosis* (Garbe *et al.*,1996a) and has led the authors to hypothesise that INH may act by inhibiting the synthesis of an intermediate which acts as a negative feedback regulator of Ag85 complex synthesis, thereby resulting in the up-regulation of Ag85 complex proteins. More studies will be required to analyse the inhibitory or inducing effect of various stresses on the secretion and hence the function of Ag85 complex proteins.

In conclusion, this study has been able to demonstrate that heat shock can influence the quality and the quantity of proteins released into the supernatants of *M. smegmatis* cultures. It has been shown that *M. smegmatis* possesses and secretes the Ag85 complex (i.e. Ag85A, Ag85B, and Ag85C), and that the release of Ag85A and Ag85B was induced by heat shock. The 19 kDa lipoprotein could not be identified in *M. smegmatis*, but was shown to be released from the cell walls of *M. tuberculosis* and *M. bovis* BCG in response to heat shock. This study presents an experimental approach that may be useful in investigating the functional role of protein secretion in the interaction of mycobacterial cells with their environment.

Chapter 6

General Conclusions

Mycobacterium tuberculosis, the causative agent for tuberculosis, is capable of causing both active disease and latent forms of infection. The genetic regulatory mechanisms which facilitate the conversion of *M. tuberculosis* from active growth to the dormant or persistent state have not yet been characterised. Available genetic evidence regarding the physiological state of persisting bacilli points to the possible existence of cells in either a metabolically less active state of stationary or unusually slow growth or a spore-like metabolically inactive form awaiting a signal to resume division. In an attempt to define the phenotype of the mycobacterial stationary phase and to provide information on if and when the predicted gene products are translated, the total protein output encoded by the genome (i.e. the proteome) during different stages of growth was investigated under normal growth conditions and in response to heat shock stress.

A major problem with studies on the dynamics of growth of stirred or agitated cultures of *M. tuberculosis* is the tendency of the bacilli to form clumps, particularly as the culture gets older. Cell clumps are believed to contain bacilli in various stages of oxygen deprivation such that bacteria within a single culture represent a physiologically heterogeneous population. This therefore makes it difficult to ensure that all the cells are in the same growth phase, and in addition, it presents problems in metabolic labelling studies where penetration of radioactive metabolites or stresses such as heat shock may be not be uniform to all cells. To avoid these problems, a non-clumping member of the genus, *Mycobacterium smegmatis*, was used in these studies.

The growth of cultures of *M. smegmatis* was monitored by using both the number of colony forming units (CFU) and the adenine triphosphate (ATP) content of the culture. The transition of *M. smegmatis* into stationary phase was found to be a co-ordinated process involving both morphological and molecular changes. The ATP content of culture was found to be a more accurate indicator of the stage during which changes associated with entry into stationary phase were initiated. Following

exponential phase growth, the transition into stationary phase commenced with a plateau in the ATP content of cells (biomass) which coincided with a momentary cessation in the increase in number of CFU between 32 and 36 hours. This was followed by a second exponential increase in CFU (from 36 to 40 hours), which differed from the first exponential phase increase (i.e. from 6 to 28 hours) in that a 5-fold increase in the number of CFU was achieved within 4 hours compared to the 8 hours it took to achieve the same increase during the first exponential phase. Thereafter, the number of CFU reached a plateau, i.e. 10 hours later than the ATP growth curve. Despite the continuing increase in number of CFU between 36 and 40 hours, the rate of accumulation of the FtsZ protein decreased from a doubling time of 5 hours (between 16 and 32 hours), to a doubling time of 14 hours between 36 and 44 hours, perhaps signalling the initiation of a different phase in the cell division process. It appears that the momentary cessation in the increase in CFU (i.e. inhibition of cell division) may occur to enable the expression of genes that allow cells to switch from the cell division that is accompanied by mass increase (as seen during exponential phase) to the pre-stationary phase type of cell division which occurs without an increase in mass. This was further supported by microscopic examination of Ziehl-Neelsen stained cells which showed a reduction in cell size during stationary phase.

Growth phase-dependent qualitative and quantitative differences in protein synthesis were observed. The period commencing with the plateau in the ATP curve (28 hours) and ending when the number of CFU ceased to increase (40 hours) was characterised by several events: a 3-fold reduction in pre-induction levels of the 95 kDa (Lon), 66 kDa (GroEL), 42 kDa (DnaJ), and ~17 kDa (doublet) heat shock proteins, while the levels of the 75 kDa (DnaK) heat shock protein remained constant throughout growth; inhibition of the synthesis of exponential phase proteins with apparent molecular weights of 58, 30.5, and 20 kDa; and the induction of at least six stationary phase specific (post-exponential phase) proteins with apparent molecular weights of 48, 46, 32, 31, 25, and 20 kDa. The sequencing and functional characterisation of the stationary phase induced proteins should enable the identification of their roles in the polygenic processes that take place during the transition of mycobacterial cells into stationary phase growth. The elucidation of such molecular processes may ultimately

enable the phenotype of the mycobacterial stationary phase to be elucidated, and hence lead to the characterisation of the persistent state of *M. tuberculosis*.

The magnitude of induction of heat-inducible proteins demonstrated a growth phase-dependency. Whereas the 75 kDa (DnaK) heat shock protein showed the highest magnitude of induction during late exponential phase (28 hours), the highest induction of the Lon, GroEL, and the ~17 kDa heat shock protein doublet was observed between 40 and 44 hours (stationary phase). The magnitude of induction of DnaJ in response to heat shock showed no growth phase dependency. The significance of the highest induction of heat shock proteins occurring over the period during which stationary phase proteins are induced will require further investigation. It is conceivable that heat shock proteins may be required for their molecular chaperone function to assist in the folding of the newly synthesised post-exponential phase proteins.

The previously uncharacterised heat-induced ~17 kDa doublet of *M. smegmatis* was resolved into four strongly heat-inducible peptides by two-dimensional polyacrylamide electrophoresis. Mass spectrometric sequencing of one of the silver-stained gel purified peptides (Hsp17-2, with an apparent molecular weight of 16.8 kDa) led to the identification of two fragments which were identical to internal fragments of a 16.2 kDa protein of unknown function that is encoded by open reading frame 28 of the *M. tuberculosis* cosmid MTCY01B2 (Accession No. Z95554). Homologues of Hsp17-2 have also been identified in *M. bovis* BCG (cloned and sequenced in this study), *M. leprae* (cosmid B1133), *Thermus aquaticus, Methanobacterium thermoautrophicum, Bacillus subtilis,* and *Methanococcus janachii.* This is the first time that a functional characteristic has been assigned to this open reading frame and the existence in *M. smegmatis* of at least two other as yet unidentified heat induced proteins of a similar size suggests that these proteins may belong to a new family of heat shock proteins. An increase in the severity of the heat shock stress resulted in increased synthesis of the *M. smegmatis* ~17 kDa doublet (i.e. protein levels at 50°C > 48°C > 42°C), suggesting that these proteins may function during conditions of severe stress, perhaps under the control of the σ^E RNA polymerase subunit. Further studies will involve the use of antibodies to investigate if Hsp17-2 in *M. tuberculosis* is induced by heat shock or other stresses, and also to

determine if the other three ~17 kDa radioactive peptide spots represent members of a family of proteins, or if they are different proteins. Further studies will be required to establish if Hsp17-2 is immunodominant or has molecular chaperone activity

The effect of heat shock (48°C for 1 hour) on protein secretion (i.e. supernatants) was investigated in the presence of ^{35}[S]L-methionine. *De novo* synthesis and release of proteins with apparent molecular weights of 53, 46, 32, 30, and 22 kDa was observed under one-dimensional PAGE. Heat shock resulted in 2.2-, 1.7-, and 4.1-fold induction of the 32, 30 and 22 kDa proteins, respectively. The anti-Ag85 complex (85A, 85B, & 85C) polyclonal antiserum reacted with both the 32 kDa (a doublet made up of Ag85C and Ag85A) and the 30 kDa (Ag85B) proteins under one-dimensional PAGE, but only the 30 kDa protein showed increased release in response to heat shock. Immunoblot studies on supernatant samples separated using two-dimensional PAGE demonstrated the heat induction of Ag85A and Ag85B but not of Ag85C. Increased release of Ag85A in response to heat shock could not be demonstrated when using one-dimensional PAGE, possibly as a result of its signal being masked by the more abundant Ag85C protein with which it migrates. The anti-Ag85 antiserum also reacted with a 30/32 kDa doublet (1D-PAGE analysis) in *M. bovis* BCG, but no induction of either band could be demonstrated in response to heat shock. In agreement with a study by Harth et al. (1997), the Ag85 complex proteins could hardly be detected in culture filtrates obtained from long-term cultures of *M. smegmatis*. It appears that in *M. smegmatis*, the Ag85 complex is secreted into the medium (i.e. short-term supernatants), but unlike in *M. tuberculosis* where the proteins accumulate with increasing age of culture (Andersen *et al.*,1991), the proteins may become degraded in long term culture filtrates of *M. smegmatis*. An understanding of the differences between *M. smegmatis* and *M. tuberculosis* in the mechanisms involved in regulating the stability of the Ag85 complex may lead to a better understanding of the role played by Ag85 proteins, be it fibronectin binding or cell wall synthesis (i.e. mycolyl transferase activity).

Immunoblot studies using the 19 kDa lipoprotein antigen-specific HYT6 monoclonal antibody demonstrated no reactivity in the both the pellet (cell lysate)and supernatant fractions of *M. smegmatis*. On the other hand, while HYT6 reacted with a 23 kDa

protein in the supernatants of heat-shocked (48°C) *M. tuberculosis* H37Rv and *M. bovis* BCG, no reactivity was observed in the supernatants from the corresponding control (37°C) samples. It is possible that the heat-induced release of the 19 kDa antigen may be a result of cleavage of chemical bonds due to the increased temperature. On the other hand, it is also conceivable that release of the 19 kDa antigen may originate from a signal pathway that is associated with the mycobacterial stress response. The apparent molecular weight of the released protein was 4 kDa larger than expected. This may be due to glycosylation of the peptide which could alter its mobility on polyacrylamide gels. Further studies on the mechanisms involved in the heat-induced release of the 19 kDa lipoprotein antigen and the potential implications during the stress response await further investigation.

Abbreviations

μ	micro
1,25$(OH)_2$ Vit D_3	1,25-dihydroxyvitamin D_3
1D	one-dimensional
2D	two-dimensional
A	adenine
Ag	antigen
AIDS	auto immune deficiency syndrome
Ala	alanine
ATP	adenine triphosphate
BCG	Bacille Calmitte et Guerin
bp	base pairs
C	cytosine
cAMP	cyclic- adenine monophosphate
cAMP	cyclic adenine monophosphate
CFU	colony forming units
CIRCE	controlling inverted repeat of chaperone expression
CMI	cell mediated immunity
cpn	chaperonin
CRP	cAMP receptor protein
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
FN	fibronectin
G	guanine
GM-CSF	granulocyte macrophage colony-stimulating factor
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HSF	heat shock transcription factor
Hsp(s)	heat shock protein(s)
ICD	isocitrate dehydrogenase
IEF	isoelectric focusing
IFN	interferon
IL	interleukin
INH	isoniazid (isonicotinic acid hydrazide)
IR	inverted repeat
kDa	kilodaltons
L/L	luciferin/luciferase
LAM	lipoarabinomannan
LM	lipomannan
LMW	low molecular weight
m	milli
M	Molar
Mφ(s)	macrophage(s)
MAb	monoclonal antibody

MALDI-TOF	matrix assisted laser-desorption/ionisation time-of-flight
mol.	mole
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
NADP	nicotinamide adenine dinucleotide phosphate
Nramp	natural resistance-associated macrophage protein
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGL-1	phenolglyocolipid 1
pI	isoelectric point
PIM	phosphatidylinositol mannoside
PIM	Phosphatidyl-*myo*-inositol mannoside
PMNG	polymorphonuclear granulocytes
RNA	ribonucleic acid
rRNA	ribosomal RNA
rRNA	ribosomal RNA
S	serine
SDS	sodium dodecyl sulphate
T	tyrosine
TB	tuberculosis
TCA	tricarboxylic acid
TGF	transforming growth factor
TNF	tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
Tween 80	polyoxyethylene sorbitan monooleate
YT	Yeast tryptone

Appendix

I. Immunoblot Analysis

After SDS-PAGE, the gel were soaked in transfer buffer [4.8g Tris and 22.4g glycine dissolved in 1600ml H_2O and made up to 2000ml with methanol] for 20 min. The proteins were electroblotted at 200mA for 1 hour onto Hybond-C+ Super nitrocellulose membrane (Amersham) using the Dri-Blot Unit (Hoefer Scientific, California, USA). Antibody detection of proteins was performed using the Chemiluminescence Blotting Substrate - Horseradish Peroxidase (POD) Kit (Boehringer Mannheim) according to the manufacturer's instructions. In principle, the assay involves detection of the membrane bound protein by a specific primary antibody which is in turn detected by a POD-labelled, secondary antibody. In the presence of hydrogen peroxide, POD catalyses the oxidation of luminol (a diacylhydrazide) to an activated intermediate which decays to the ground state by emitting light. The light signal is enhanced by 4-iodophenol and detected by autoradiography. The intensity of each band was quantified by using a Shimadzu CS-9000 dual-wavelength flying spot scanner as described in chapter 2, section 2.2.6.

The following antibodies were used according to the suppliers' recommended dilutions: *Escherichia coli* FtsZ polyclonal antiserum (donated by Joe Lutkenhaus, Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Centre, Kansas City, Kansas, USA); monoclonal antibodies HAT-3 (71 kDa antigen; DnaK), IIH9 (65 kDa antigen; GroEL), HYT27 (Ag85 complex antigens), HYT6 (19 kDa antigen), CS-01 (10 kDa antigen; GroES), F24-2-3 (α-crystallin) (all supplied by UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Centre for Disease Control, Atlanta, USA); *M. tuberculosis* Ag85B polyclonal antiserum (K92) (donated by Morten Harboe, Institute of Immunology and Rheumatology, University of Oslo, Norway); and polyclonal antiserum SPA-410 (41 kDa antigen; DnaJ) from StressGen, USA.

II. Silver Staining

Fix I: Ethanol (40% v/v)
Acetic Acid (10% v/v)
Fix gel by soaking for 1 hour or overnight (16 h) at room temperature

Fix II: Ethanol (30%v/v)
Potassium tetrathionate (0.25% w/v)
Sodium acetate (6.8% w/v)
Sensitise gel by soaking for 1 hour
Wash for 4 x 15 min in distilled water

Stain: Silver nitrate (0.2% w/v)
37% Formaldehyde (0.025% v/v)
Soak for 30 min
Remove silver nitrate solution and wash for 1 min in distilled water

Developer: Potassium carbonate (3% w/v)
37% Formaldehyde (0.015% v/v)
Sodium thiosulphate (0.0008% w/v)
Soak gel in developer until the desired stain intensity is achieved (maximum 30 min)

Stop: Tris (5% w/v)
Acetic acid (2% w/v)
Soak gel for 10 min and discard solution
Wash gel in acetic acid (5%) for 5 min and store in 1% acetic at 4°C

The solutions are made up in distilled water, and the staining procedure is performed at room temperature with shaking. Glutaraldehyde (1% v/v) interferes with mass spectrometric analysis by binding to, and altering the molecular mass of the peptide and should therefore only be included in fix II if the stained gels are not going to be used for mass spectrometric amino acid sequence analysis

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III. Preparation of Mycobacterial Genomic DNA

Cell lysis

Exponential phase cultures (10 ml) of *M. smegmatis*, *M. bovis* BCG or *tuberculosis* were incubated overnight at 37°C with D-cycloserine at a final concentration of 1mg/ml. The cells were collected by centrifugation at 4000 rpm for 15 min at room temperature, and were resuspended in 400 µl in Tris/EDTA buffer (10 mM Tris and 1 mM EDTA, adjusted to pH 8.0 with concentrated HCl) with 0.3 M sucrose. Lysozyme was added to the cell suspension to give a final concentration of 3 mg/ml before incubating at for 90 min. Proteinase K, SDS, and EDTA were added to final concentrations of 0.2 mg/ml, 0.1%, and 50 mM, respectively, and the solutions incubated at 55°C for 90 min. The reagents were supplied by Sigma, USA.

DNA extraction

An equal volume of phenol [prepared by dissolving 500g phenol, 0.6g 8-hydroxyquinoline, 2 M sodium hydroxide (7.5 ml), 6ml of 1 M Tris-HCl (pH 7.0) in 130ml water] was mixed with each suspension and the solutions were centrifuged at 13 000 rpm for 5 min at room temperature. The aqueous layer was transferred to a sterile microcentrifuge tube and the DNA extracted by adding an equal volume of phenol:chloroform (at the ratio of 1:1). The extraction procedure was repeated twice. A further extraction was performed with an equal volume of chloroform. The aqueous phase transferred to a sterile tube and 0.1x volume of 3 M sodium acetate (pH 7.0) followed by 2 volumes of chilled (-20°C) absolute ethanol were was added. The DNA was collected by centrifugation at 4°C for 10 min, and the pellet was resuspended in 100 µl of a solution of 50 mM Tris (pH 8.0), 100 mM sodium acetate and 100 µg/ml RNase. After incubating at 37°C for 15 min, the DNA was extracted with phenol:chloroform (as described above), followed by overnight precipitation with 2 volumes of ethanol at 4°C. The DNA was collected by centrifugation at 13 000 rpm for 15 min at 4°C, and thereafter resuspended in Tris/EDTA buffer.

IV. Purification of DNA fragments from Agarose Gels

(Adapted from Seth, 1984)

1. Cut out the DNA fragment of interest from the low melting point agarose gel using UV-illumination.
2. Chop the gel slice into small pieces using a scalpel blade and transfer to a microcentrifuge tube.
3. Mix with an equal volume of phenol [prepared by dissolving 500g phenol, 0.6g 8-hydroxyquinoline, 2 M sodium hydroxide (7.5 ml), 6ml of 1 M Tris-HCl (pH 7.0) in 130ml water] and homogenise by vortexing.
4. Freeze the homogenate at -70°C for 30 min.
5. Spin the frozen homogenate at 12 000 rpm for 10 min. to separate the phases.
6. Transfer the top aqueous phase containing the DNA to a sterile microcentrifuge tube and mix with an equal volume of phenol:chloroform in the ratio of 1:1.
7. Spin at 12 000 rpm for 2 min and remove the aqueous phase.
8. Mix with 0.6x volume of isopropanol and incubate at room temperature for 2 min.
9. Spin at 12 000 rpm for 4 min to pellet the DNA and resuspend in distilled water.

V. Preparation of Competent Cells

$CaCl_2$ Shock Procedure - adapted from Dagert and Ehrlich (1979)

1. Grow *E. coli* DH5α (1/200 dilution) overnight (16 hours) in 50 ml of 2 x YT (yeast tryptone) medium in a 200ml flask.
2. Collect cells by centrifugation at 6000 rpm for 10 min at 4°C.
3. Keep the cells ice cold in all further steps.
4. Resuspend the cells in ½ culture volume of 0.1 M $CaCl_2$. Hold on ice for 1 to 2 hrs.
5. Collect cells as before and gently resuspend in 0.1x culture volume of 0.1 M $CaCl_2$.
6. Mix competent cells with ice-cold glycerol to a final concentration of 10% (v/v) and leave on ice for 30 min. Store at -70°C.
7. For use, remove cells from -70°C storage and place them in an ice bath until just thawed. Mix by gently flicking the tube.

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