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***Identification and isolation of  
growth-phase specific proteins of  
Mycobacteria***

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*Thesis presented for the Degree of  
MASTER of SCIENCE in Medicine  
in the Department of Medical Microbiology  
Faculty of Medicine  
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***PER TE LEONE***

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# ABSTRACT

The aim of this project was to identify growth phase –specific heat shock proteins of *Mycobacterium smegmatis* LR222.

A growth curve was constructed using the ATP assay. This method was shown by B. A. Ntolosi to be the most accurate indicator of when the organism entered the various phases of growth. It was possible to determine that *M. smegmatis* LR222 entered the exponential phase of growth after a short lag phase of 4 to 8 hours and persisted in this phase for 20 to 22 hours. It then reached the stationary phase, which lasted for 40 to 46 hours. Protein heat shock assays were performed on growth phase-specific samples. This allowed the identification of a 43-46 kDa in molecular weight stationary phase protein on one-dimensional SDS-PAGE. The protein was induced in cells entering the stationary phase of growth and not by heat shock as it was induced under both the control and the heat shock temperatures. The protein was further characterised by two-dimensional gel electrophoresis, which demonstrated resolution into two, strongly age-associated, proteins. Subtractive RNA hybridisation was attempted in order to obtain a subtraction cDNA probe from a stationary phase RNA sample depleted of sequences common in both the exponential and the stationary phase samples. Ribosomal RNA was removed from the total RNA by the process of photobiotinilation. The mRNA was then used as a template to synthesise with reverse transcriptase single stranded cDNA. cDNA/mRNA denatured hybrids were hybridised to the exponential phase RNA sample. The new hybrids were “subtracted” by chemical cross-linking with DZQ and the unique cDNA used to produce by random primers a radioactively labelled probe. A *M. smegmatis* library was probed but unfortunately no signal was observed. Further adjustments and improvements to this technique are required before it can be used effectively.

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# CHAPTER 1

## LITERATURE REVIEW

Dubos and Dubos (1992) state that the current tuberculosis epidemic accelerated due to the social conditions in which poor people lived in during the Industrial Revolution in Europe. Cities were crowded, people had poor diets and worked long hours in unhealthy environments. In "The White Plague" they write: "Tuberculosis was, in effect, the social disease of the 19<sup>th</sup> century, perhaps the first penalty that capitalistic society had to pay for the ruthless exploitation of labour".

Tuberculosis (TB) continues to remain a major communicable disease, particularly in developing countries, where socio-economic underdevelopment is associated with this disease and has made its control difficult (Ehlers, 1993). The incidence of TB has also increased in developed countries due to: poor living conditions, immigration, poverty, the current HIV epidemic, the increased occurrence of drug-resistant strains of *Mycobacterium tuberculosis* and the lack of an effective vaccine (Weiss, 1992). Despite predictions of its eradication, more than three million people die of TB each year and this situation is expected to continue into the next century. In 1993, the gravity of the situation led the World Health Organisation (WHO) to declare TB a global emergency, in an attempt to heighten public and political awareness.

Concerned global efforts to curb TB's impact on human health have been enormous, nevertheless *M. tuberculosis* remains one of the leading causes of death from infectious diseases. From the WHO 1998 report, in 1997 3.7 million people died of TB, 2.5 million of diarrhoea, 2.3 million of HIV/AIDS and between 1.5 and 2.7 million of malaria.

In the words of the WHO "Not only has Tuberculosis returned, but it has

upstaged its horrible legacy” (Wilkinson and Grover, 1996).

### **1.1.HISTORY OF TUBERCULOSIS**

Tuberculosis is an ancient disease. Skeletal deformities characteristic of tuberculosis have been found in paleolithic and neolithic fossilised fauna and it is thought that a TB-like disease afflicted animals before the advent of mankind (Mayor, 1932). Hindu literature, dating back to 2000 BC, mentions a “slow wasting fever” in elephants, and the ancient Egyptians report about phthisis among domesticated cattle. Phthisis (from the Greek “wasting away”), the most destructive of cattle diseases, became such a scourge amongst animals in Europe that it led to intermittent human famine from the Middle Ages through to the 18<sup>th</sup> century.

Evidence of human TB can be seen in ancient artwork, and mummies from pre-Dynastic Egypt and pre-Columbian Peru have been depicted with Pott’s disease.

It was the Greek philosopher Hippocrates (460-370 BC), “the father of Medicine”, who described phthisis as a condition associated with wasting and pulmonary disease, and Galen (AD 131-201), characterised clinical phthisis as a bleeding ulceration of the lungs (Burke, 1955). He also regarded the disease to be of contagious aetiology and advised reduced contact with consumptives. However, until the 16<sup>th</sup> century all of the great pathologists believed the disease to be constitutional, a form of tumour or abnormal gland, rather than infectious (Burke, 1955).

Ideas, however, had been changing in the late 1400s, partly thanks to the invention of printing and the advent of modern anatomy, new principles of human dissection were established. In the “De Contageone”, Fracastorius (1483-1553), states the modern theory of contagion by micro-organisms. He postulated that contagion resulted from exhaled particles from diseased lungs, and he first applied the term “fomites” to infected clothing and utensils.

A pathological entity associated with the disease was not recognised until 1882, when Robert Koch (1843-1910) identified the tubercle bacillus (Grange

and Bishop, 1982). He demonstrated at the Berlin Physiologic Society on March 29, that this microorganism caused consumption by fulfilling his own postulates: the organism was constantly present in the tissues of the infected host; it could be grown artificially in pure culture and after many subcultures; it could reproduce the disease when inoculated into susceptible animals (Rees, 1988).

Thanks to Koch's work, new approaches to TB therapy appeared together with the sanatorium concept, for which Edward Trudeau is credited (Ryan, 1992).

## **1.2. EPIDEMIOLOGY OF TUBERCULOSIS**

TB displays the principal characteristics of a global epidemic disease. However, compared to the classic epidemics of acute communicable disease, the TB epidemic cycle is not easily perceived because the disease is disseminated slowly, is ubiquitous and in many countries has taken several generations to evolve and complete its cycle (Des Prez *et al.*, 1990; Fine, 1984).

TB has devastating effects in developing countries, where 95% of cases occur. Nearly 80% of these cases occur in persons who are in their reproductive years, that is between 15 and 59 years of age (Kochi, 1991).

From 1953, when national surveillance began, through to 1984, the United States experienced a significant decline in TB cases. This was due to improved sanitary conditions and to the development of new drugs such as streptomycin (1945), isoniazid (1952) and rifampicin. This apparent defeat of the disease led to the closing down of the sanatoria and to the decline in research for new drugs and vaccines. And yet, just a couple of decades later the situation has drastically changed with the incidence of TB surging world-wide. In other industrialised nations TB was an uncommon disease but in recent years several countries have reported a rising incidence (Dooley, 1994; Garrett, 1994).

For the ten year period 1990-1999 the WHO estimates that 88.2 million

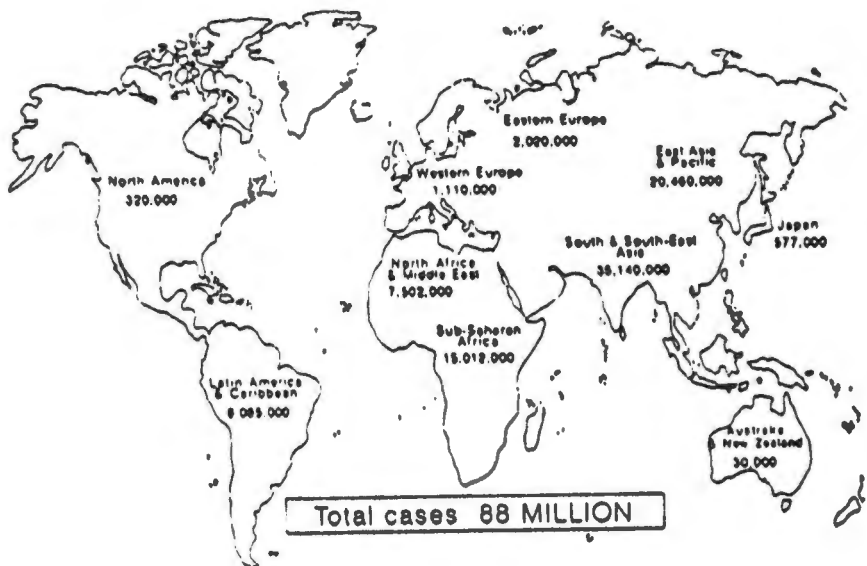


Fig. 1.1. World map showing estimated TB cases, 1990-1999 (From Dolin *et al.*, 1994).



Fig. 1.2. World map showing estimated TB deaths, 1990-1999 (From Dolin *et al.*, 1994).

### **1.2.1. HIV-TB interactions**

Since the mid-1980s it has been clear that the HIV/AIDS pandemic is having a devastating effect on the prevalence of TB (John, 1993). It is estimated that more than three million people are dually infected with TB and HIV in the world, 2.4 million in Sub-Saharan Africa alone (Kochi, 1991). Chretien (1990a) has termed the combination of these two infections the “cursed duet”. Thanks to its ability to destroy the immune system, HIV has emerged as the most significant risk factor for progression of dormant TB infection to clinical disease and at the same time, TB has emerged as the most severe opportunistic disease associated with HIV infection (Chretien, 1990a; Chretien, 1990b). Because HIV infected persons have a higher frequency of extrapulmonary TB, the disease is difficult to diagnose and the infected individual may spread the disease further.

Sadly, as the association between TB and HIV infection becomes more widely known, the diagnosis of TB will begin to carry an additional social stigma.

### **1.2.2. Transmission, pathogenesis, and clinical manifestation**

An American scientist, William Wells, was one the first to study the transmission of TB in the 1930s. Wells made a distinction between organisms associated with dust and organisms associated with infectious droplet nuclei produced in the respiratory tract of humans. Through some elegant experiments, he demonstrated that droplet nuclei can carry tubercle bacilli directly to lung alveoli. In the late 1960s, more evidence on airborne transmission between people was provided when an outbreak of TB was recorded on the naval vessel “Richard Byrd” (Reichman, 1993). This added further insight into the importance of airborne transmission and direct contact. The principal risks for acquiring TB were, therefore, breathing, coughing, sneezing and speaking, all actions that would release organisms-bearing particles.

In healthy persons infection with *M. tuberculosis* is more likely to be

asymptomatic (Grange, 1980; Grange, 1988). The risk of developing clinical TB after becoming infected is low, approximately 10%, with 90% possibility of the infection remaining dormant. Persons with dormant TB become tuberculin skin test positive, this being the only way of proving the presence of infection. *M. tuberculosis* does not produce toxins, its pathogenicity for humans is, therefore, linked to its capacity to survive and multiply inside the human macrophage. The immune reactions of the infected host contribute to the pathogenesis of the disease (Havlin *et al.*, 1991). It is important with a communicable disease, such as TB, to distinguish between infection and disease. To initiate infection, virulent bacilli must reach the alveolar epithelium and may in immunocompetent individuals, be carried up the mucociliary escalator, swallowed and eliminated thanks to the highly acidic environment of the stomach. The droplet nuclei can also be phagocytosed by an activated alveolar macrophage, which will usually kill the few ingested bacilli. The 1 to 2  $\mu\text{m}$  droplet nuclei that reach the alveoli contain two to three tubercle bacilli and  $10^3$  to  $10^4$  bacilli are required to initiate cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH). Of great importance at this stage are the efficacy of the macrophage to kill the bacilli, the bacilli's virulence and the site of involvement. If the bacilli are highly virulent and are ingested by a macrophage with low microbicidal power, then the bacilli will not be killed and will proliferate. The macrophage will eventually lyse, releasing the bacilli, which will be phagocytosed by other alveolar macrophages and by non-activated monocytes from the blood. The inactivated (immature) macrophage from the blood stream will initiate a lesion called a "tubercle". Dannenberg and Rook (1994) cite Lurie as referring to a symbiotic relationship between the macrophages and the bacilli, the first not being activated and therefore incapable of killing the invader, and the second incapable of injuring the macrophage because the host has not yet developed tuberculin-type hypersensitivity (PPD<sup>+</sup>). On the long run the tuberculous lesion, however, will become bigger due to further accumulation of macrophages containing bacilli. In the meantime, the bacilli will multiply inside the macrophage, without affecting it. When multiplication stops and the host has become tuberculin positive, the lesions undergo caseous necrosis. In the resistant host, and if

the number of bacilli is low, the primary tubercle lesion will regress and will become walled off arresting the disease. However, if the number is large, the tubercle will enlarge and local lung tissue will be destroyed. Cavities form, allowing large numbers of bacilli to spread to the outside through coughing. The solid necrotic lesion, possibly as a result of released hydrolytic enzymes (probably proteases, nucleases, and lipases) from inflammatory cells, may liquefy, creating a rich medium for the proliferation of bacilli (Bloom and Murray, 1992). The pathologic and inflammatory processes produce the characteristic weakness, fever, weight loss, chest pain and, when a blood vessel is eroded, bloody sputum.

### **1.2.3. Mycobacterial survival in macrophages**

*M. tuberculosis* is an intracellular organism. Together with other intracellular organisms such as *Listeria* spp. and *Salmonella* spp., the tubercle bacillus has evolved sophisticated mechanisms that enable it to survive and live inside the hostile environment of the macrophage (Abshire and Neidhardt, 1993; Riley, 1995).

Mechanisms of uptake of *M. tuberculosis* by the macrophage include several specific receptors such as integrins and CR3 receptors (Ferguson *et al*, 1997). Usually, following bacterial phagocytosis, the phagosome fuses with the lysosome and inactivation of the invading organism occurs. Other antimicrobial functions of macrophages include: generation of reactive oxygen intermediates (ROI) by oxidative burst and production of reactive nitrogen intermediates (RNI) (McDonough, 1993).

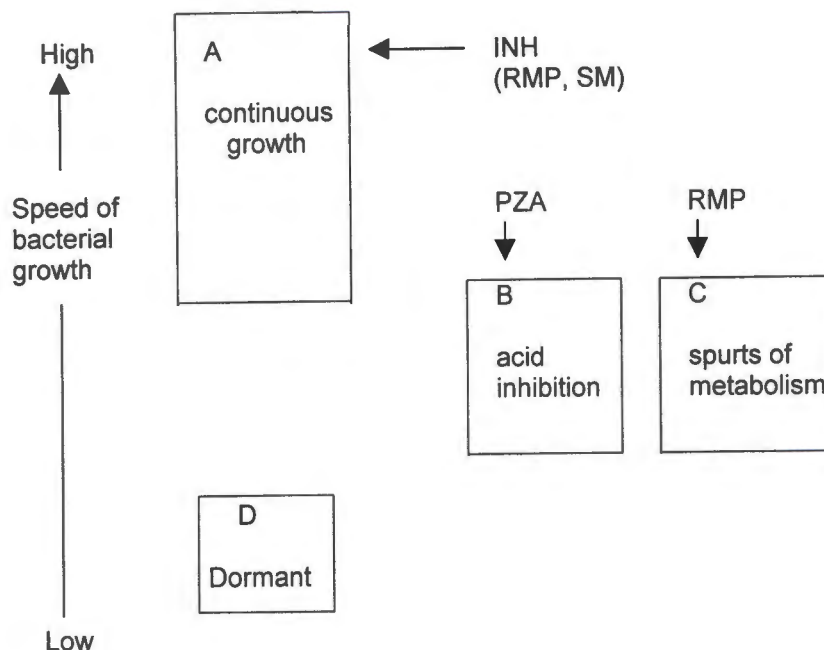
Gordon and colleagues (Clemens, 1996) have demonstrated that *M. tuberculosis* is capable of blocking phagosome-lysosome fusion by producing ammonia. Of crucial importance is the specialised cell wall of mycobacteria and their ability to produce key stress proteins i.e. heat shock proteins, that allow the bacillus to survive and thrive in the macrophage (Mudayoor and Shinnick, 1994).

#### **1.2.4. Antibiotic therapy**

The discovery of streptomycin in 1945, and later of isoniazid (INH) and rifampicin (RIF), rendered TB a disease that could be cured (Cannetti *et al.*, 1968; Bloom and Murray, 1992). Over the decades there have been many treatment regimens and two principles have proved to be important.

The first principle underlines that, in order for therapy to be successful, a minimum of two drugs must be administered. Monotherapy with even the most potent bactericidal drug such as isoniazid, may cause spontaneous drug resistance in a small number of bacilli, which may then select a resistant bacterial population. This results in treatment failure and acquired drug resistance.

The second principle is that treatment of TB must continue after amelioration of clinical disease. Prolonged chemotherapy is required to eliminate a small population of metabolically inactive organisms. Inadequate treatment could lead to relapse months or years after apparent cure. During the 1950s and 1960s, eighteen to twenty four months of therapy were required to ensure a cure. Today, with the multidrug therapy available, only six months are required. From these principles, TB therapy may be divided into an initial bactericidal therapy and a subsequent sterilising phase. Isoniazid is the most potent bactericidal drug available, whilst rifampicin and pyrazinamide (PZA) are sterilising drugs (Dickinson and Mitchison, 1981). Mitchison (1985) explained the effects of drugs used in the modern short-course chemotherapy (SCC) on the basis of different actions that they have on separate populations of tubercle bacilli. The diagram on page 13 (Figure 1.3.) shows that the largest population consists of extracellular bacilli that are metabolically active and are killed by the bactericidal action of isoniazid. The population of dormant bacilli, that have periodic bursts of activity, are eliminated by rifampicin. A third population consists of intracellular bacilli and of bacilli found in caseous material, which is an acidic environment. This population of bacilli is susceptible to the action of pyrazinamide.



**Fig. 1.3.** Mitchison' s bacterial population hypothesis: (A) actively growing bacilli killed mainly by isoniazid, (B) semidormant bacilli inhibited by an acid environment, killed mainly by pyrazinamide, (C) semidormant bacilli with spurts of active metabolism, killed mainly by rifampicin, (D) completely dormant bacilli not killed by drug. (From Mitchison, 1985).

All viable bacilli must be eliminated by chemotherapy in order to avoid relapses and the short-course chemotherapy seems to be effective in doing this. However, the therapy must be completed and too often non-compliance is a threat to a successful cure.

### 1.2.5. Drug-resistance

Drug resistance may develop during inadequate therapy of a patient (secondary or acquired resistance) or a person may become infected by a strain that is already resistant (primary resistance). Resistance may develop to one or more drugs and the most important forms of resistance are those affecting the drugs used in modern short-course therapy. Multi-drug resistance (MDR) is usually defined as resistance to isoniazid and rifampicin.

The incidence and trends of drug resistance may show considerable variation within a country.

Drug resistance surveillance programmes are essential for TB control programmes since primary resistance trends provide an indication of the effectiveness of treatment regimens, while acquired resistance rates can point out failures in the managerial system (Weyer and Kleeberg, 1992).

### **1.3. THE BCG VACCINE**

BCG remains the most controversial of all currently used vaccines, due to its perceived variation in protective efficacy. Yet it is the most used vaccine of all, having been administered to over three billion people since the 1950s (Stover *et al.*, 1991).

In the early 1900s, Calmette and Guerin at the Pasteur Institute attenuated a strain of *M. bovis* by passaging it two hundred and thirty one times on potato slices soaked in ox bile and glycerine. This process took thirteen years and resulted in the production of a strain that was unable to produce progressive tubercle lesions in experimental animals (Roche *et al.*, 1995).

It was first used as a vaccine in Paris in 1921, particularly for children at high risk of developing TB. It immediately gained popularity across Europe, even after the Lubeck (Germany) incident in which 72 children died after being immunised with a contaminated batch of BCG vaccine. The vaccine is particularly protective against disseminated TB and meningitis in children. It is puzzling that BCG's protective efficacy against pulmonary TB in adults can vary from 0 to 80% in different trials throughout the world (Bloom and Murray, 1992). This huge disparity can be due to several factors: quality of the vaccine; partial protection from non-tuberculous mycobacterial infection; route of vaccine administration; and dosage. Also, the type of vaccine strain used is of great importance. In fact, after the strain was attenuated by Calmette and Guerin, it was distributed to many laboratories where it was subcultured. This led to the production of a variety of daughter strains, which differ widely in their effects on humans and in animals. Thus, the characteristics of the

vaccine are not permanent, however the strain has never reverted to virulence.

BCG still remains one of the safest live vaccines in use, except with immunosuppressed individuals who may develop local or disseminated infection.

#### **1.4. THE MYCOBACTERIA**

The generic name *Mycobacterium* was introduced by Lehmann and Neuman in the first edition of their "Atlas of Bacteriology", published in 1896. At that time the genus only contained two species: *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Wayne and Kubica, 1986).

The name *Mycobacterium*, meaning fungus-bacterium, was derived from the way the tubercle bacillus grows, as mould-like pellicles on the surface of liquid media (Ratledge *et al.*, 1989). The non-culturable leprosy bacillus was included in the genus because it shares a staining property with the tubercle bacillus, that is: resistance to decolourising by weak mineral acids after staining with one of the arylmethane dyes. This property, called acid-fastness, is the basis of the Ziehl-Neelsen stain. Acid-fastness, although a useful distinguishing property, is not unique to the Mycobacteria: bacterial spores, for example, are often strongly acid-fast, and members of the related genus *Nocardia* are weakly acid-fast.

Shortly after the introduction of the generic name, acid-fast bacilli were cultured from birds and cold-blooded animals such as frogs, turtles and fish, and differences between tubercle bacilli isolated from man and cattle were observed. The variation of properties within the genus is extensive, and is reflected in the range of virulence, habitat, rate of growth, nutritional requirements and antigenicity. There are, in fact, relatively few properties that are common to all mycobacteria and yet these properties clearly distinguish this genus. Many of the unique characteristics of the mycobacteria are found in their complex lipid-rich cell walls.

Initially the mycobacteria were thought to have evolved from a group of Gram-positive aerobic rods, which includes the genera *Corynebacterium* and *Nocardia*. Mycobacteria are weakly Gram-positive, aerobic, non-sporing and non-motile organisms. They are characterised by a thick waxy cell wall of low permeability (Brennan and Nikaido, 1995). Mycobacteria are mainly free-living saprophytes and only a small minority has adapted to a dependence on a living host. The hydrophobic wall makes these bacilli resistant to many disinfectants, harsh environmental conditions and hostile host habitats. The observation that dead mycobacteria can persist within macrophages for more than a week after ingestion can be attributed to the durable, impermeable nature of the cell wall which affords great resistance to oxidative stress (Barry, 1996). Paradoxically their natural habitats are watery ones: mud, ponds, rivers, and estuaries. Mycobacteria are often found at air-water interfaces, this is why Grange (1987) have termed the tubercle bacilli “the ducks of the microbial world”. This situation allows them to derive oxygen from the atmosphere and nutrients from the water.

Mycobacterial cell walls are composed of a variety of soluble proteins, carbohydrates, lipids, and three insoluble macromolecules: arabinogalactan, peptidoglycan, and mycolic acids, together they constitute the mycolylarabinogalactan peptidoglycan (Besra and Brennan, 1997; Slayden *et al.*, 1996). The mycolic acids are the major component of the cell wall and are made up of long C<sub>60</sub>-C<sub>90</sub>  $\alpha$ -alkyl,  $\beta$ -hydroxy fatty acids (Lee *et al.*, 1995).

Members of the Mycobacteria are divisible into slow growers, rapid growers and those not yet cultivated *in vitro* (Segal, 1984; Grange, 1988). The slow growers include the *M. tuberculosis* complex - which comprises *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* -, *M. avium*, *M. intracellulare* and *M. kansasii* (Rogell *et al.*, 1990) Examples of the rapid growers are *M. smegmatis* and *M. fortuitum*. *M. smegmatis* was one of the first rapid growers to be described. Presumably this was, in part, a result of the desire to employ a rapidly growing model for the slowly growing tubercle bacillus. The not yet cultivated group is composed solely of *M. leprae*, which

is an obligate pathogen of man (Stewart-Tull, 1982).

#### **1.4.1. Mycobacterial genomics**

The mycobacterial genomes consist of a single chromosome with a size range of  $2.8 \times 10^9$  to  $4.5 \times 10^9$  base pairs (bp), and precisely 4.411.529 bp (Cole *et al.*, 1998). Interestingly its guanine (G) plus cytosine (C) content ranges between 64 and 70 % of the total base content, which is higher than that of other bacteria. It has been shown that *M. tuberculosis*, *M. bovis* and *M. africanum* have strong DNA homology of between 77 and 96%. Although these organisms have different cultural, biochemical and epidemiological characteristics, from a genetic point of view they can be regarded as varieties of the same species rather than distinct species (Wayne and Kubica, 1986).

#### **1.4.2. Nutrition and growth**

*M. tuberculosis* has two main growth characteristics: it grows slowly under the best conditions of culture, it has a mean generation time of 17-18 hours, with colonies only visible after two to four weeks of incubation on solid media; and it does not grow on ordinary culture media but only on enriched media containing an egg-potato base or a serum (albumin) base. On the other hand this organism does not require any particular growth factors or vitamins, even though various compounds potentiate its growth *in vitro*. Examples of such compounds are serum albumin, egg yolk and catalase, which probably absorb toxic moieties in the culture medium.

*M. tuberculosis* is considered to be an aerobe, that produces catalase, peroxidase and superoxide-dismutase, and its growth rate is highly dependent on the oxygen tension. When the oxygen tension is high, as in tuberculosis lung cavities, the tubercle bacillus multiplies freely; when it is much lower, as in caseous lung foci, it multiplies slowly or not at all (Wayne, 1976).

The tubercle bacillus is capable of oxidising a wide range of compounds. In

the laboratory the preferred carbon sources used for growth of *M. tuberculosis* are: glycerol, pyruvate or glucose. Ferric ammonium citrate, which is usually part of the culture medium, provides the organism with the source of iron for, and asparagine is usually used as the source of nitrogen. Four major inorganic elements are also essential for growing *M. tuberculosis* in the laboratory, these are: potassium, magnesium, sulphur and phosphorous.

### **1.5. BACTERIAL GROWTH**

Growth is an essential component of microbial functions, as any given cell has only a finite life span in nature and the species is maintained only as a result of continued cell growth and division. The bacterial cell is essentially a synthetic machine, which is able to duplicate itself (Ingraham *et al.*, 1983). The synthetic processes of bacterial cell growth involve as many as two thousand chemical reactions of a wide variety of types (Brook *et al.*, 1991).

A typical growth curve can be divided into several distinct phases called: the lag, the logarithmic (or exponential), the stationary and the death phases, respectively (Fishor *et al.*, 1995). When a culture in the stationary phase is transferred into fresh medium, growth usually does not begin immediately. Growth is delayed for a variable period of time, depending on the temperature, the particular nutrient or toxic product that limited growth in the original culture and the length of time in which the culture was held in the stationary phase of growth. During this period of time called the lag phase, the composition and size of the cells is adjusting to that characteristic of the exponential phase of growth. On the other hand, if an exponentially growing culture is inoculated into fresh medium, under the same conditions of growth, a lag phase is not seen and exponential growth continues at the same rate (Kjelleberg, 1993). Rates of exponential growth vary greatly; for instance *Salmonella typhi*, the aetiological agent of typhoid fever, grows very rapidly in culture, with a generation time of 20 to 30 minutes, whereas the tubercle bacillus, *M. tuberculosis*, grows slowly, with one doubling per day. The rate of exponential growth is also influenced by environmental conditions, such as

temperature, composition of culture medium, as well as by characteristics of the organism itself. Exponential growth can not occur indefinitely. Usually an essential nutrient of the culture medium is used up or some waste product of the organism builds up in the medium to an inhibitor level and exponential growth ceases. The culture is induced to enter the stationary phase. In the stationary phase there is no net increase or decrease in cell number (Zambrano and Kolter, 1996). However, even though no growth occurs during this phase, many cell functions may continue, including energy metabolism and some biosynthetic processes (Kolter, 1993; Zambrano *et al.*, 1993). If incubation continues after a population reaches this phase, eventually the cells will die. During the death phase the total count measured by direct microscopic count may remain constant but the viable count slowly decreases.

#### **1.5.1. Bacterial growth in vivo and environmental stresses**

The above description of bacterial growth, however, is only well defined *in vitro*. The growth cycle that bacteria undergo in their natural habitat is somewhat different due to nutrient limitation, variable temperatures and other natural influences. Because of these erratical conditions, micro-organisms frequently experience starvation and/or other stresses. To overcome and survive these periods of starvation, many bacteria have evolved specialised metabolic states that allow them to maintain cell viability during starvation and will be able to resume growth rapidly when nutrients become available. Some Gram-positive organisms such as *Bacillus subtilis* differentiate into spores as their survival strategy (Mathiopoulos and Sonenshein, 1989; Loewen and Hengge-Aronis, 1994). Other organisms will form multicellular aggregates and fruiting bodies, while some Gram-negative bacteria, such as *Escherichia coli*, *Salmonella* spp. and *Vibrio* spp., enter stationary phase and remain viable (Lange and Hengge-Aronis, 1991). During this phase significant physiological changes take place, which allow the cell to survive a wide variety of environmental stresses, heat and high salt concentration. Light microscopy,

for instance, reveals changes in cell morphology and *E. coli* cells and some marine bacteria are observed to become much smaller and spherical (Zhang, 1996). The fatty acid composition of many marine bacteria also changes and their surfaces become more hydrophobic and more adhesive. The topology of the chromosome becomes affected by starvation, and in *E. coli* changes in the negative super helical density of reporter plasmid are observed, and in *Vibrio* spp. the nucleoid becomes highly condensed (Smith, 1995).

The stationary phase was considered to be a period of metabolic inactivity, during which the cell waited for nutrients. It has, however, been shown that some level of endogenous metabolism is maintained in order to transport substrates into the cell. If this ability is impaired, the starved cell will be unable to resume growth when nutrients become available in its environment and will eventually die (DeMaio *et al.*, 1996).

New proteins are required during this period of resistance. These proteins seem to be involved in maintaining viability and are required to resume growth when the nutrients become available. In starved *E. coli*, the rate of protein turnover increases five fold, however, RNA stability decreases and 20 to 40% of total RNA is lost during the first several hours of starvation. DNA remains stable in most bacteria (Siegele and Kolter, 1992).

Adaptive responses to various stresses involve a series of genetic switches that control the metabolic changes taking place in the organism (Loewen and Hengge-Aronis, 1994).

A common regulatory mechanism involves the modification of sigma factors. Transcription in prokaryotes is mediated by the RNA polymerase holoenzyme, which is made up of two major components: the core RNA polymerase and the sigma factor. The first consists of two  $\alpha$  subunits and one each of  $\beta$  and  $\beta'$  subunits. The second, the sigma factor, binds to the core conferring promoter specificity (Predich *et al.*, 1995). Sigma factors have been found to be involved in sporulation in *B. subtilis* ( $\sigma^{43}$ ) and in the stress response and stationary phase in *E. coli* ( $\sigma^{32}$ ), suggesting that a sigma factor cascade may be involved in the stress response, stationary phase and dormancy of

mycobacteria (Young and Cole, 1993).

### **1.5.2. Bacterial heat-shock proteins**

Exposure of cultured cells or organisms to elevated temperature, results in induction of the synthesis of a small number of highly conserved proteins, the heat-shock proteins (HSP) (Young *et al.*, 1992). This response is universal. It has been observed in every organism in which it has been sought, from eubacteria to archebacteria, from mice to soybeans (Lindquist, 1986).

Studies of the heat shock response began in 1962 on *Drosophila* cells (Schlesinger, 1990). The initial studies on cloned heat-shock genes and purified proteins led to two important results: HSPs proved to be highly conserved among widely divergent organisms, i.e. the major heat-shock protein, hsp70, has about 50% its sequence conserved between *E. coli* and man, and some domains are 96% similar; the second important point was that several of the major HSPs are members of protein families that include proteins normally present and usually essential for cell function (Schlesinger, 1990). Studies in vitro have also shown that many other stimuli other than elevated temperature such as nutrient starvation, contact with heavy metals, oxidants, UV radiation and others, can induce the production of these proteins which are more often called stress proteins (Patel *et al.*, 1991).

These stress proteins apparently serve multiple functions. Under normal conditions, they are mostly involved in the folding and assembly of newly synthesised proteins, they are known as molecular chaperones (McFadden, 1990). Almost all that is known about the regulation of the heat-shock response is derived from studies on *E. coli*. Seventeen HSPs seem to be present in *E. coli*, three major proteins of 94-, 70-, and 58-kDa in molecular mass. The 94-kDa HSP, the product of the *lon* gene, is a protease that is involved in the regulation of protein turnover in *E. coli*. The 70-kDa protein, product of the *dnaK* gene, has a heterogeneous array of functions which suggests that it catalyses specific inter- and intramolecular protein interactions (Guglielmi *et al.*, 1991). GroEL, the 58-kDa protein, has been

found to be fundamental to bacterial physiology; it is in fact essential in *E. coli* for growth at all temperatures and it probably plays a role in DNA replication and mRNA transcription. Several mycobacterial HSPs have been identified as homologs of major HSPs in *E. coli* (Shinnick *et al.*, 1988). It has been demonstrated that 65-, 71-, and 18-kDa antigens function as HSPs in *M. smegmatis*, *M. bovis* BCG, and *M. habana* respectively, as also for 90-, 71-, 65-, 45-, and 15-kDa proteins in *M. tuberculosis*.

The heat-shock response is closely linked to the survival of bacterial pathogens within the mammalian host (Lathigra, 1991). Studies on *Salmonella typhimurium* conducted by Buchmeier and Heffron (1990) have demonstrated that DnaK and GroEL are selectively produced following entry into the host's macrophages. Therefore, the characterisation of the heat-shock response in mycobacteria will provide an important model for studying the regulation of genes which may have a role in virulence and pathogenicity of mycobacteria in diseases such as TB.

## **1.6. LATENCY IN *M. TUBERCULOSIS***

Evidence from the natural history of TB in humans and from experimental animals has shown that *M. tuberculosis* is capable of adapting to prolonged periods of dormancy/latency in tissues, therefore causing latent TB (De Wit *et al.*, 1995).

Recurrent TB is usually the result of reactivation of latent bacilli rather than a new acquired infection. Dormant bacilli remain in a quiescent state in the host, the location in which they reside is still not known, they are not susceptible to antimycobacterial drugs and have a potential for revival.

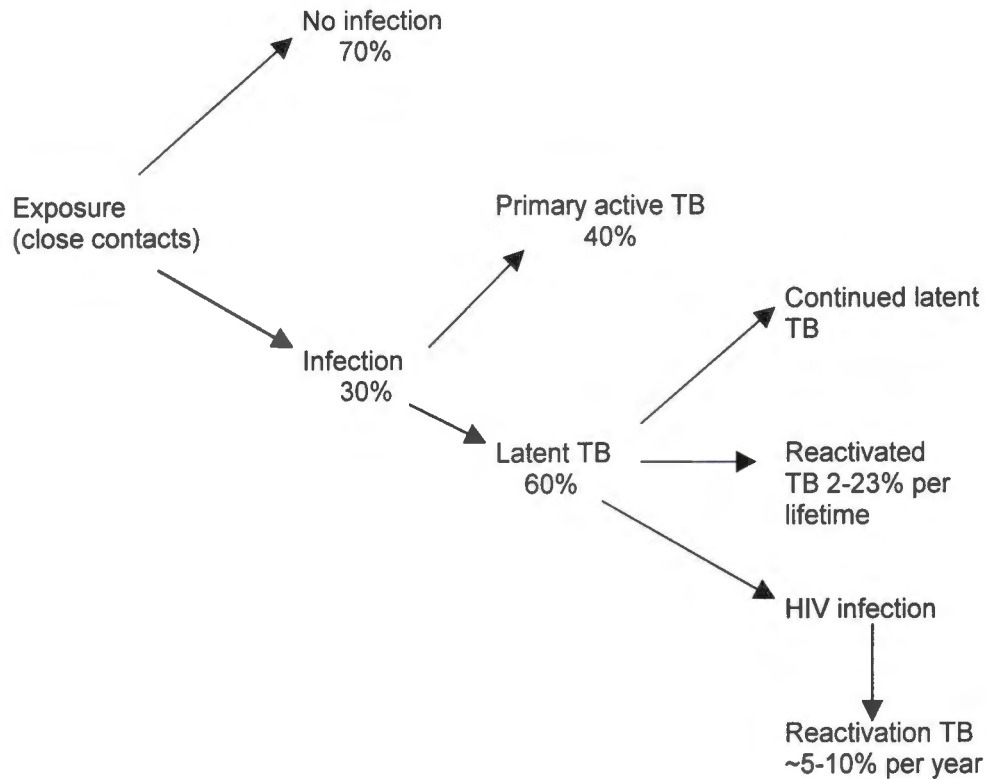
Parrish *et al.* (1998) define latency of tuberculosis as follows: "Latent TB is a clinical syndrome that occurs after an individual has been exposed to *M. tuberculosis*, the infection has been established, and an immune response has been generated to control the pathogen and forced it into a quiescent state."

Latent TB is a major problem world-wide, with one-third of the population

harbouring the tubercle bacilli. It has been estimated that individuals infected with dormant bacilli carry a 2 to 23% risk of reactivating the disease (Parrish *et al.*, 1998) (See Fig. 1.4). This group of individuals has become a major problem in TB control, in fact it would be ideal to develop an “anti-reactivation vaccine” rather than a conventional protective vaccine (Dhillon and Mitchison, 1994). Until a way to prevent latent TB or to kill dormant bacilli is found, the eradication of this terrible disease will remain but a hope.

Figure 1.4. on page 24 clearly shows the different pathways that infection with *M. tuberculosis* can take.

In 1933 Corper and Chon published a study in which they showed the ability of the tubercle bacillus to survive environmental stresses. They inoculated *M. tuberculosis* in a bottle which was stored at 37° C and twelve years later, when they examined the culture, they found that there were viable bacilli in the sediment of the bottle. The late 1940s, early 1950s was a period of intense surgical activity, involving resection of pulmonary lesions, an activity that became unnecessary thanks to the availability of effective antibiotics. Bacteriological examinations of human pulmonary lesions, representing different stages of development and arrest, were carried out. The majority of the old blocked lesions from resected lung tissues, i.e. lesions into which air could not penetrate due to the dense contents of the lesion itself, contained acid-fast bacilli that could be seen microscopically, but could not be cultured. These viable bacilli had persisted in some lesions for months or years. At least 20% of blocked lesions from TB patients, whose sputum had converted to negative status nine or more months prior to resectional surgery, were found to yield small numbers of colonies of *M. tuberculosis* when the specimen was cultured. This demonstrated that a potential for adaptation of organisms that had been considered to be obligate aerobes to survive under anaerobic conditions (Wayne, 1976).



**Figure 1.4.** Outcomes associated with exposure to *Mycobacterium tuberculosis*. Following close contact, 30% of individuals become infected, with about 40% of these individuals developing primary active tuberculosis (TB) and 60% developing latent infection. 2-23% of immunocompetent patients with latent TB will reactivate at a later date, while patients with HIV develop reactivation TB at a rate of ~5-10% per year. (From Parrish *et al.*, 1998).

### 1.6.1. Wayne 's model of dormancy

In the Wayne model of persistence, cultures of *M. Tuberculosis* are subjected to gradual oxygen withdrawal by incubation in unagitated sealed containers. Growth under such conditions leads to a balance between the replicating rate, the diffusion of the oxygen, and the settling of bacilli to the bottom of the flask (Mekalonos, 1992). As they settle, the cells pass through a gradient of decreasing oxygen concentration to an oxygen-poor environment, during which time they gradually adapt to survive under a non-replicating, persistent microaerophilic and later under an anaerobic state. The microaerophilic state is associated with a ten-fold induction of glycine dehydrogenase activity, whilst in the later anaerobic state the glycine dehydrogenase activity will

decline and alteration in drug susceptibility is observed (Wayne, 1977). The production of a unique antigenic protein named URB-1 has also been found only in dormant bacilli. When the switch to an anaerobic environment is sudden, however, the tolerance to the new environment is greatly reduced (Wayne and Lin 1982). When resumption of aeration is gradual three steps occur: production of RNA, synchronised cell division commences (Wayne, 1977), and a new round of synthesis of DNA initiates (Wayne, 1994). The adaptation to the gradual oxygen depletion *in vitro* may be analogous to the situation in host tissues where the bacilli switch from aerobic metabolism to microaerophilic or anaerobic pathways (Wayne, 1994).

This model has also proved the existence of differences in antibiotic susceptibility. During the non-replicating status *M. tuberculosis* is not affected by antibiotics that are effective during the “normal” viable status, suggesting that changes in the metabolism of the bacillus have occurred.

### **1.6.2. The Cornell Model of dormancy**

The existence of dormant bacilli after chemotherapy was shown by McCune and others in the 1950s, soon after the introduction of isoniazid. Their studies can be considered the key studies on mycobacterial dormancy and their relevance to TB chemotherapy has been invaluable. This study known as the Cornell model, together with similar studies conducted by Mitchison, Grosset and colleagues some twenty years later, demonstrated that rifampicin could kill tubercle bacilli even with short periods of contact, whereas other drugs such as isoniazid require about one generation time (Michison, 1985). In the original Cornell model, mice were infected with a heavy dose of a virulent strain of *M. tuberculosis* and the disease was allowed to progress for two weeks, at which time isoniazid and pyrazinamide were given in combination for several weeks. Treatment was then discontinued and lung and spleen cultures were performed at regular intervals. The organs became negative for tubercle bacilli soon after the treatment was initiated and maintained their negative status for some time. However, if no further treatment was given the

reappeared in these tissues. The resurgence of growth could also be hastened by immunosuppressive agents. This model of persistence also proved that the tubercle bacilli undergo drastic metabolic alterations, that protect them from the action of antituberculous drugs and allow them to survive during long periods of stress.

### **1.6.3. Further comments on latency**

As previously mentioned and as pictured in Fig. 1.4, several factors can influence the reactivation of dormant TB: HIV infection, drug use, cancer, malnutrition, diabetes, drug therapy and other factors that compromise the individual's immune system.

From the Cornell and Wayne 's models of dormancy, several hypothesis can be drawn on how tubercle bacilli remain latent. The two most intriguing hypothesis are that the bacilli enter an altered developmental state in which they lose their acid-fastness and therefore are not detected (Khomenko, 1987; De Wit and Mitchison, 1993). The other explanation is that the latent bacilli might be present in such low numbers that they are not detected microscopically.

An important question regarding the physiological state in which quiescent bacilli persist must still be answered. The bacteria could be present in an extreme form of stationary phase, in which there are low levels of metabolism. Individuals with latent TB have reduced risks of reactivating the disease when treated with rifampicin (Mitchison, 1985) proving that some level of metabolism must be present in order for the drug to be effective. Another explanation could be that these dormant bacilli are present in a spore-like state, thanks to which they become invisible to the immune system. Homologs of sporulation regulatory genes in *M. tuberculosis* have been found, showing that a similar physiological state could be present (Gaugadharam, 1995).

Not much is known about the location in which dormant bacilli reside. They could be hidden in the old pulmonary granulomas or in the lymph nodes of the

lungs that comprise the Ghon complex (calcified lesions which correspond to the initial site of pulmonary infection and a hilar lymph node). However, this explanation does not comply with the fact that about 15% of TB cases are extrapulmonary. It seems more likely, therefore, that dormant bacilli may reside in several sites, most probably where the infection was established.

### **1.7. AIMS OF THE PROJECT**

The aim of this study was an attempt to identify growth phase-specific proteins in *M. smegmatis* LR222, a fast-growing, non-pathogenic member of the mycobacteria.

The first step was to establish the growth curve of the organism using the ATP assay. From the growth curve it was possible to determine when the organism entered the exponential and the stationary phase of growth. Heat shock assays on protein samples prepared at various time points of growth were performed and SDS-PAGE carried out in order to screen whether new proteins were synthesised as the organism aged and entered stationary phase. Heat shock was an ideal stress condition especially because stationary phase cultures have been found to be more resistant to heat stress than growing cells, hence suggesting that the cell's response to heat shock is dependent of the growth phase (Young *et al.*, 1996). A 43-46 kDa stationary phase-specific protein, synthesised independently from the heat shock stress was observed on one-dimensional SDS-PAGE. The aim of the project focused, thereafter, on the characterisation and the sequencing of this age-specific protein. The methods of choice were two-dimensional electrophoresis and RNA subtractive hybridisation. The protein of interest demonstrated resolution into two spots on autoradiography, of pI values between 5 and 4.5. Unfortunately the novel RNA hybridisation method used in this study was not successful. A subtraction cDNA probe from a stationary phase RNA sample, depleted of sequences common in both exponential and the stationary phase samples, was used to probe a *M. smegmatis* library, however, no signal was observed.

# **CHAPTER 2**

## ***GROWTH CURVE IN Mycobacterium smegmatis***

### **2.1. SUMMARY**

The aim of the following experiment was to construct the growth curve of *Mycobacterium smegmatis* strain LR222. The method of choice was that of the Adenosine Triphosphate (ATP) assay. This method was shown by Ntolosi (PhD, 1998,) to be the most accurate indicator of when *M. smegmatis* LR222 enters stationary phase.

### **2.2. INTRODUCTION**

#### **2.2.1. Bacterial cell growth**

The process of bacterial cell growth can be divided into 4 phases: the lag phase, the exponential phase, the stationary phase and the death phase (Fishor *et al.*, 1995). If a sample from a stationary phase culture is inoculated into fresh medium, growth does not begin immediately. A short period called the lag phase, in which no growth is detected, precedes the exponential phase. During this period, cells are adjusting to their new environment and their composition and size changes to that characteristic of cells in the exponential phase of growth. The latter starts when cells begin to divide. The rate of this phase is influenced by several parameters such as temperature, medium in which cells are growing and aeration. Also, rates of exponential phase vary and depend on the characteristics of the microorganism itself.

Microbial populations enter stationary phase for several reasons, some of which include nutrient limitation and O<sub>2</sub> availability (Ingraham *et al.*, 1983; Kjelleberg, 1987; Kjelleberg, 1993). In stationary phase there is no increase nor decrease in cell mass, even though many cell functions continue to persist to allow bacterial viability. If incubation continues, the cells will eventually reach the death phase of growth.

## **2.3. MATERIALS AND METHODS**

### **2.3.1. Mycobacterial culture**

A single colony of *M. smegmatis* LR222 (J. Crawford, Centre for Disease Control, Atlanta, USA) was picked from Middlebrook 7H9 agar plates and inoculated in 20 ml Middlebrook 7H9 medium, supplemented with 10% albumine-dextrose-catalase (ADC) and 0.05% Tween 80, and incubated with continuous shaking on an orbital shaker at 37° C (180 rpm). After incubation for 48 hours, cells were subcultivated (1/100 dilution) in 400 ml of Middlebrook 7H9 medium, containing 10% ADC enrichment medium and 0.05% Tween 80. The culture was incubated on an orbital shaker at 37° C for 2 weeks. This was required in order to obtain a uniform population of cells, which was then divided into aliquots (200 µl) and stored in 15% (v/v) glycerol at – 70° C for future use as starter cultures.

### **2.3.2. Growth curve of *M. smegmatis* using the ATP assay**

*M. smegmatis* strain LR222 (0.4 ml) was inoculated in 400 ml of Middlebrook 7H9, supplemented with 10% ADC enrichment medium and 0.05% Tween 80, and incubated at 37° C with continuous shaking (180 rpm). Samples were taken, in duplicate, every 2-4 hours (specific times are shown in Figure 2.1). At each time point, only 2 ml of culture was removed to ensure that conditions of growth were not affected by sampling.

The relative amount of ATP produced by the cells was used as a measure of cell mass to plot a growth curve of the culture. The ATP levels were monitored by measuring the production of light when ATP, luciferin and oxygen were combined in the presence of the enzyme luciferase. The assay system was calibrated using an ATP standard of known concentration (Stanley *et al.*, 1989; and Prioli *et al.*, 1985). For each assay 1.4% (w/v) tricarboxylic acid (TCA), which releases cellular ATP, was added to the cells to a final volume of 60  $\mu\text{l}$ . The samples were held on ice for 30 minutes. The reaction was neutralised by adding 50  $\mu\text{l}$  of neutralising buffer (0.1 M Tris-acetate, pH 7.75, adjusted with acetic acid) and 622  $\mu\text{l}$  of ATP-free water. Promega Enliten luciferase luciferin (L/L) reagent was mixed with Enliten L/L reconstitution buffer, and 50  $\mu\text{l}$  of this mixture was incubated with 347.5  $\mu\text{l}$  of L/L buffer (0.1 M Tris-acetate, 2 mM EDTA, pH 7.75, adjusted with acetic acid). The background light emission ( $RLU_{L/L}$ ) from the mixture was measured in a Bio-Orbit 1253 luminometer. One hundred microlitres of cell sample was added and the light emission ( $RLU_{\text{sample}}$ ) measured. An aliquot (2.5  $\mu\text{l}$ ) of the  $10^{-7}$  M ATP standard was added to the cuvette (final concentration of  $1 \times 10^{-10}$ ) and the emission was measured ( $RLU_{\text{ATPstd.}}$ ). The molar concentration of ATP in the cells was calculated using the following formula:

$$ATP \text{ realised from each sample} = \frac{(RLU_{\text{sample}} - RLU_{L/L}) \times 10^{-10} \text{ M}}{(RLU_{\text{ATPstd.}} - RLU_{\text{sample}})}$$

The values were plotted on a logarithmic scale in order to obtain the growth curve of *M. smegmatis*. (Figure 2.1)

## 2.4. RESULTS AND DISCUSSION

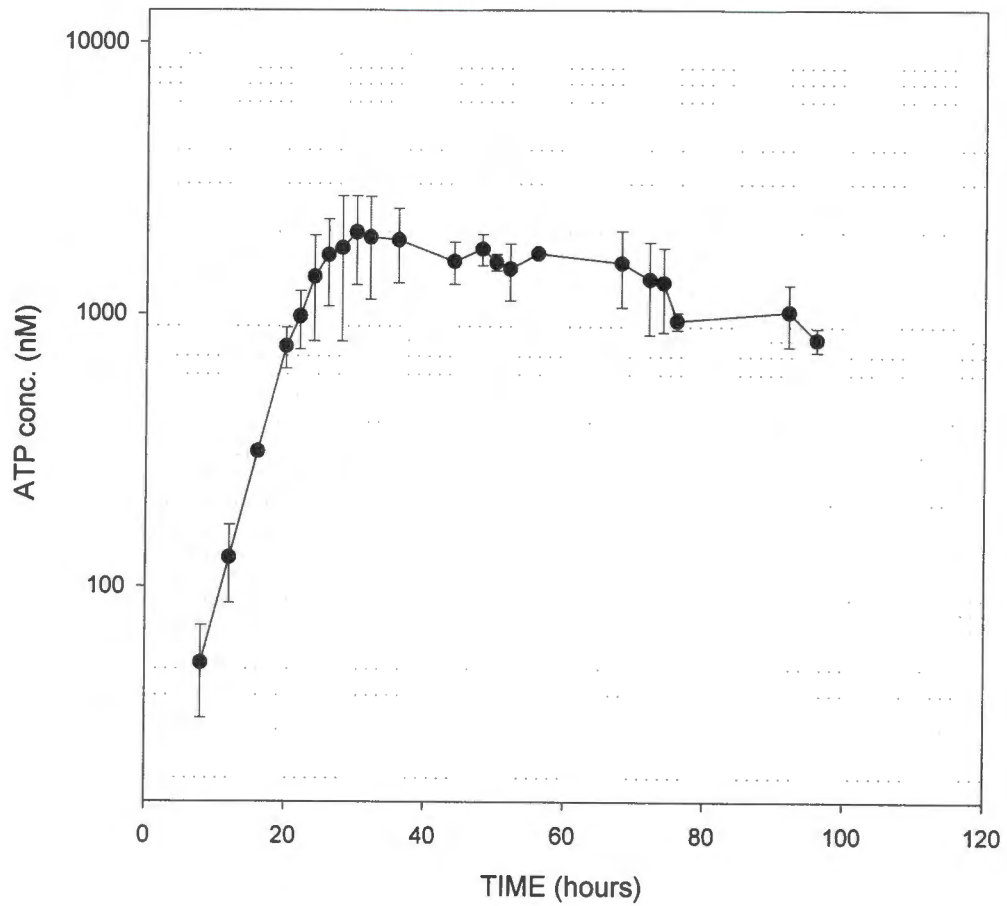
### 2.4.1. Growth curve of *M. smegmatis* using the ATP assay

The ATP assay is based on the quantitative measurement of a stable level of light produced as a result of an enzyme reaction catalysed by firefly luciferase. The enzyme reaction leading to light emission is the following:



Samples of *M. smegmatis* cells were taken at 2-4 hour intervals, up until the late stationary phase of the growth (96 hours). At these various stages of growth the concentration of ATP was assayed as a measure of cell mass; the values were plotted on a logarithmic scale and the growth curve extrapolated from it (Figure 2.1.). The exponential phase lasted approximately 20-22 hours. After a short lag phase of about 4-8 hours, the organism divided rapidly and then reached its plateau after about 28-30 hours. At this point it entered the stationary phase of the growth cycle, which lasted about 42-46 hours (Figure 2.1.)

The ATP assay proved to be a useful procedure in measuring cell biomass. In this study it was, therefore, possible to define when *M. smegmatis* LR222 entered the various phases of the growth curve.



**Fig. 2.1.** Growth curve of *M. smegmatis* LR222 using the ATP assay. The culture was grown in Middlebrook 7H9, supplemented with 10% ADC enrichment medium + 0.05% Tween 80, in an orbital shaker (180 rpm), for 96 hours. Samples were taken at 2 and 4 hours intervals until 76 hours, then at 82 and 96 hours to inspect whether the culture had entered the death phase of growth.

# **CHAPTER 3**

## **STRESS PROTEIN SYNTHESIS IN RELATION TO GROWTH IN *Mycobacterium smegmatis***

### **3.1. SUMMARY**

The aim of the following experiment was to perform protein heat shock assays in order to determine whether new heat shock proteins could be identified in relation to growth. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed to compare *M. smegmatis* strain LR222 protein samples from exponential phase and stationary phase of the growth curve (Westermeier, 1997). It was then observed that a certain protein of approximately 43-46 kDa in molecular weight was produced during stationary phase both at the control temperature of 37° C and under the heat shock temperature of 48° C.

The project thereafter focused on the identification of this stationary phase-specific protein.

### **3.2. INTRODUCTION:**

#### **3.2.1. The stress response**

Living organisms must be able to cope with rapid changes in their environment. These changes include exposure to elevated temperatures, heavy metals and toxins, oxidants and bacterial or viral infection (Morimoto *et al.*, 1994). They are able to adapt to these changes thanks to a rapid and often

dramatic change in patterns of gene expression. One inducible set of proteins is a family of heat-shock proteins (HSPs) (Puglia *et al.*, 1995). HSPs ensure cell survival under stressful conditions, which could lead to irreversible cell damage if left unchecked. These proteins have also been shown to be important in protein biosynthesis, specifically in the synthesis, transport and translocation of proteins and in the regulation of protein conformation from which they derive the common name of “molecular chaperones” (Young and Garbe, 1991; Jacobs *et al.*, 1995).

Members of the major HSP families, from bacteria to humans, share a very high degree of sequence identity. They make up a large fraction of the proteins within the cell, mounting to 5-10% of the total protein mass in cells growing under ambient conditions (Morimoto *et al.*, 1994).

The genes encoding HSPs can be induced in response to the above mentioned stress conditions. It is more appropriate to define these proteins with a more generic name such as “stress proteins”, due to the fact that they are induced by other stresses as well. These stress conditions may lead to the increased expression of constitutively expressed HSPs and to the induction of additional heat shock genes.

Most of the knowledge about the regulation of the heat shock response in prokaryotes has been obtained from studies with *Escherichia coli* (Fayet *et al.*, 1989; Patel *et al.*, 1991).

DnaK, GroEL, Gro ES, DnaJ and GrpE are the major HSPs produced by *E. coli* and were identified on the basis of their involvement in phage replication. For example DnaK, together with DnaJ and GrpE, disrupts the protein complex that forms at the origin of replication of phage lambda. This allows the release of the lambda P protein and initiation of DNA replication. HSPs have also been shown to play important roles in the metabolism of *E. coli*. Mutants with a deletion of the *dnaK* gene can survive at 30°C but show multiple phenotypic changes, including a defect in cell division. GroEL seems to have an important chaperone function in protein translocation in *E. coli* which is similar to that described for hsp70 in higher organisms (Shinnick *et al.*, 1988; McFadden, 1990).

HSPs are, therefore, clearly involved in metabolic functions in *E. coli*, including DNA replication, cell division and protein secretion. (Guglielmi *et al.*, 1991)

Several mycobacterial antigens have been identified as homologs of major HSPs in *E. coli* on the basis of gene sequence homology (McFadden, 1990). *Mycobacterium tuberculosis* has a 71-kDa protein homologous to DnaK and a 65-kDa protein homologous to GroEL of *E. coli*.

It has also been shown that HSPs may be involved in the regulation of virulence factors and other factors implicated in host-parasite interactions (Patel *et al.*, 1991). Studies with *Salmonella typhimurium* suggest that there might be a link between stress protein synthesis and survival of bacterial pathogens within the mammalian host during infection. It has, in fact, been observed that the most predominant proteins induced in *S. typhimurium*, following entry into the host macrophage in a tissue culture system, are DnaK and GroEL (Buchmeier and Heffron, 1990). Studies on the intracellular parasite *Listeria monocytogenes* suggest that factors important in host interactions may be induced by heat shock (Young and Garbe, 1991). A role for  $\sigma^{32}$ , the sigma unit responsible for transcription of heat shock genes, may also be involved in the regulation of expression of the cholera toxin (Young and Garbe, 1991).

Pathogenic mycobacteria, which are intracellular pathogens, as are *S. typhimurium* and *L. monocytogenes*, must also adapt to the environment within the host's macrophages. They must therefore adapt to the stress they may encounter in a host cell armed with an array of antimicrobial defences (Lee *et al.*, 1995).

Investigation of the stress response in mycobacteria will provide information in the understanding of fundamental aspects of mycobacterial genetics, physiology and of host-parasite interactions. Study of the regulation of stress proteins might uncover the way in which mycobacteria adapt to different environments in host tissues during infection.

### **3.3. MATERIALS AND METHODS**

#### **3.3.1. Growth of *M. smegmatis* and protein labelling**

*M. smegmatis* LR222 cells were grown as described in section 2.3.1. Aliquots were taken in duplicates after 24 and 28 hours (exponential growth phase), 32, 36, and 48 hours (stationary phase), and 72 and 96 hours (late stationary phase) of incubation. No more than 20% of the total volume was taken to avoid changing the growth parameters of the culture.

Each sample was labelled with 4  $\mu$ l  $^{35}$ [S]L-methionine (specific activity 1000 Ci/mmol; Amersham) and incubated for 1 hour at the control temperature of 37°C or at 48°C to deliver a heat shock. The pellets were harvested by centrifugation at 1300 rpm at room temperature for 5 minutes, and were kept at – 20° C until required for protein extraction. The pellets were washed 3 times in 1 ml phosphate buffer saline (PBS), dissolved in 3% sodium dodecyl sulphate (SDS) buffer and boiled for 15-20 minutes for protein extraction. The debris were removed by centrifugation at 1300 rpm for 5 minutes at room temperature, and supernatants containing the protein stored at – 4°C until required.

In order to monitor the growth curve, the ATP assay was performed, together with the Ziehl-Neelsen staining procedure, which allowed a direct observation of the bacilli. This microscopic examination permitted the observation of changes in the morphology of the *M. smegmatis* cells during the various growth phases. It also allowed a check of the purity on the culture (See appendix B for method).

#### **3.3.2. Protein assay**

The protein concentration of each sample was determined using the Bio-Rad DC protein assay, which is a colourimetric assay based on the procedure of

Lowry *et al.* (1951). All solutions were supplied with the assay kit (Appendix B).

### **3.3.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of growth-phase specific stress proteins**

The gel apparatus used and the electrophoresis analysis carried out in this experiment were according to the method of Laemmli (1970). Polyacrylamide gels were poured in vertical Hoefer gel pouring stands and electrophoresed using a Hoefer Electrophoresis Unit (Hoefer Scientific Instruments, California, USA).

The lower resolving gel of 12.5% acrylamide in 1.5 M Tris, 0.4% (w/v) SDS buffer was poured first and allowed to polymerise for at least 1 hour. The upper resolving gel (stacking gel) of 4% acrylamide in a 0.5 M Tris, 0.4% (w/v) SDS buffer was poured on top of the lower gel and allowed to polymerise overnight. A 15-well comb was inserted in the gel apparatus during the casting of the upper gel. A Tris-glycine based running buffer [0.3% (w/v) Tris, 1.4% (w/v) glycine, 0.1% (w/v) SDS] was used for electrophoresis which was carried out a constant current of 40 mA for 180-240 minutes. One tenth the volume of a solution of bromophenol blue (10%, w/v) and  $\beta$ -mercaptoethanol (50%, v/v) was added to each sample containing 20-30  $\mu$ g of protein and boiled for 5-10 minutes before loading. A molecular weight marker (Low Molecular Weight electrophoresis calibration, Pharmacia, Biotech, Sweden) was included into each electrophoresis run.

Following electrophoresis, the gel was stained by soaking in PhastGel<sup>®</sup> Blue R, prepared according to manufacturers' instructions, for 4-8 hours, and destained by washing with 30% (v/v) methanol/10% (v/v) acetic acid for 4-8 hours. The destained gel was soaked overnight in 10% (v/v) acetic acid/20% (v/v) methanol, containing 6% (v/v) glycerol prior to drying under vacuum at 65° C, to prevent the gel from cracking.

The dried gel was exposed in cassettes to Hyperfilm™-βmax and kept at – 70° C for about one week. The autoradiographs were then developed according to method (Appendix B).

### **3.4. RESULTS AND DISCUSSION**

#### **3.4.1. SDS-PAGE of *M. smegmatis* heat shock proteins**

This experiment was undertaken to establish by comparison when stationary phase proteins were induced and to determine whether the newly synthesised proteins were related to heat shock induced proteins. This was accomplished by radiolabelling *M. smegmatis* LR222 protein samples, taken at various time intervals (24, 28, 32, 36, 48, 72 and 96 hours), with <sup>35</sup>[S]L-methionine and incubating them at the control temperature of 37° C and at the heat shock temperature of 48° C. The time points corresponded to the exponential phase (24, 28 hours), the stationary phase (32, 36, 48 hours) and late stationary phase (72, and 96 hours) of cell growth.

Samples containing 20-30 µg of protein were separated by SDS-PAGE and visualised by autoradiography (Figure 3.1.). A 43-46 kDa protein, indicated in Figure 3.1 with arrow 1 seemed to be induced in late stationary phase. Interestingly, this protein did not seem to be affected by the temperature shift as it was synthesised both under heat shock and under the control temperature. Arrow 2 indicates another protein of about 70 kDa induced during late stationary phase. Arrow 3 indicates a heat shock protein of 17 kDa that was previously characterised by Ntulosi (1998), and the star indicates another heat shock protein of about 43 kDa.

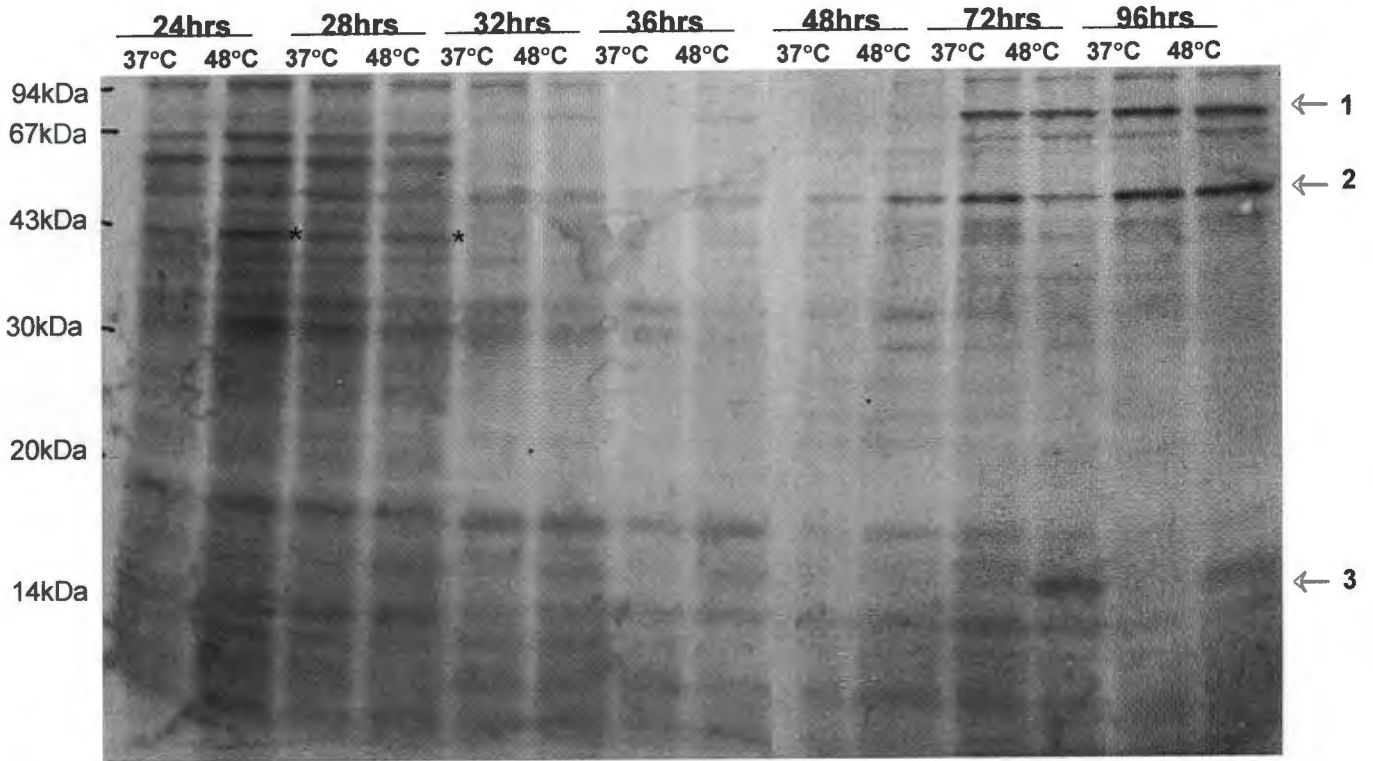
At each sampling time point the Ziehl-Neelsen staining technique was also carried out in order to monitor the purity of the culture and the morphological state of the cells. *M. smegmatis* cells appeared thin, filamentous-like rods when the sample was taken at stationary phase, and gradually changing with age to coccus-like rods, which in stationary phase formed small clumps. The

reduction in size has been described as one of the typical morphological features of stationary phase cells (Siegele and Kolt, 1992).

From this study two time points were chosen for the following experiment, that of 24 hours, which clearly denoted the exponential phase, and that of 48 hour, the stationary phase of growth.

The physical and metabolic changes which accompany stationary phase in mycobacteria are still largely unknown. Therefore, it would be of importance to concentrate research on the study of proteins produced during such period as they could be implicated in the maintenance of latency of mycobacteria.

## 1D-SDS-PAGE



**Fig.3.1.** Electrophoretic profile of heat-shock and time-induced proteins. This autoradiograph of an SDS-PAGE shows the protein profile of  $^{35}\text{S}$ [L]-methionine labelled *M. smegmatis* LR222 in response to heat at 48°C. The lanes labelled 37°C indicate the non-heat-shocked controls and those labelled 48°C the heat shocked samples. In each lane approximately 20  $\mu\text{g}$  of protein was used. On the left hand side the relative positions of the standard molecular weight markers are marked. The stationary-phase protein discussed in the results and discussion section is indicated by arrow 2. Arrow 1 indicates another protein of 70kDa that is also induced during late stationary phase. Arrow 3 indicates a heat-shock protein of 17kDa, that was previously characterised by Dr Ntolosi. The \* represents the exponentially-expressed heat-shock protein.

# **CHAPTER 4**

## **CHARACTERISATION OF A STATIONARY PHASE PROTEIN IN *M. smegmatis*.**

### **4.1. SUMMARY**

A stationary phase protein of approximately 43-46 kDa in molecular weight was observed on autoradiographs of SDS-PAGE in the previous Chapter. This protein appeared to be induced both under the control temperature of 37° C and under heat shock (48° C). Most importantly, it seemed to be induced when *M. smegmatis* LR222 entered stationary phase and persisted until late stationary phase.

In this study an attempt to characterise this protein by Two-dimensional gel electrophoresis is described (O'Farrell, 1975).

### **4.2. INTRODUCTION**

#### **4.2.1. Dormancy and Stationary phase**

Latent TB is a serious problem. The fact that one third of the entire human population may be latently infected with dormant TB bacilli underlines the enormity of this problem (Parrish *et al.*, 1998).

Initial infection with *M. tuberculosis* rarely leads to disease. The host's immune system is usually effective enough to eliminate most viable bacilli. However, if not

all the bacilli are removed, those that remain are capable of persisting in inactive state for decades and can reactivate when resistance is lowered by old age, immunosuppressants, AIDS and other factors (Bloom, 1994). Indicators that a person is infected with latent TB are the absence of clinical disease in a person with a positive skin test and a chest radiograph showing chest lesions (Parrish *et al.*, 1998).

The initial event in mycobacterial infection involves entry and multiplication within inactivated macrophages. Following this rapid growth, infected macrophages and their bacillary cargo are surrounded and walled off by newly recruited activated macrophages to form the characteristic caseous granuloma. Granuloma formation appears to create an environment uniquely capable of restricting the growth of mycobacteria through a process involving apoptosis of infected monocytes. It is within these primary granulomas and following exponential growth that bacterial dormancy probably occurs (Yuan *et al.*, 1996). Although the environment inside the granuloma is unknown, many factors, including oxygen deprivation, nutrient depletion, low pH, toxic oxygen species and other adverse conditions of this environment could potentially contribute to the induction and maintenance of mycobacterial dormancy.

The presence of these bacilli necessitates prolonged courses of chemotherapy - the short course chemotherapy lasts about 6 months - with problems such as cost, compliance and drug resistance arising (Gangadharam, 1995).

Yuan *et al.* (1996) have identified an  $\alpha$ -crystallin-like small heat shock protein of 16 kDa in molecular weight that becomes predominant at stationary phase. Overexpression of  $\alpha$ -crystallin in *M. tuberculosis* results in a decline in viability of the organism once it has entered the stationary phase. This protein has also been described in *M. smegmatis*. These findings suggest that mycobacterial  $\alpha$ -crystallin may play a role in enhancing long-term protein survival and therefore long-term survival of *M. tuberculosis*.

*M. smegmatis* LR222 stationary phase-specific protein observed in Chapter 3 on one dimensional SDS should be further characterised and then sequenced.

Understanding why the organism induces this protein while in stationary phase could help to elucidate the genetic mechanisms that allow *M. tuberculosis* to infect individuals and remain latent for prolonged periods.

Not much is known about these dormant/stationary phase organisms. It is therefore very important to understand their physiology, genetics and host-interactions in order to devise new drugs capable of killing these organisms or vaccines capable of preventing bacilli from attaining this dormant phase.

### **4.3. MATERIALS AND METHODS**

#### **4.3.1. Bacterial culture and protein preparation for two-dimensional electrophoresis**

*M. smegmatis* strain LR222 was inoculated in 400 ml Middlebrook 7H9 medium, supplemented with 10% ADC enrichment medium and 0.05% Tween 80, and incubated on an orbital shaker at 37° C (180 rpm). One aliquot (in duplicate) was taken at the exponential phase of cell growth (24 hours), and at stationary phase (48 hours). Each sample was radiolabelled with 10 µl of <sup>35</sup>[S]L-methionine and incubated at the heat shock temperature of 48° C, according to the method described in section 3.3.1. After 1 hour incubation, the samples were washed 3 times in PBS and transferred into Fast Prep microfuge tubes (BIO 101, Inc., California, USA) containing glass beads of 0.1 mm in diameter. The cells were lysed in a Fast Prep Instrument (BIO 101, Inc., California, USA) according to manufacturer's instructions. The cell lysates were recovered by centrifugation at 13000 rpm at room temperature for 5 minutes and transferred into fresh microfuge tubes.

The protein assay described in section 3.3.2. was performed to assess the protein content of the cell lysates.

### **4.3.2. Two Dimension Electrophoresis**

#### ***Rehydration of Immobiline DryStrips***

Immobiline DryStrips (Pharmacia, Biotech, Sweden) were used in this experiment. These are dried polyacrylamide gel strips, 110 mm in length, precasted on GelBond PAG plastic support films, with a pH range gradient of 3-10. A rehydration solution consisting of urea (8M), triton X-100 (0.5%), Pharmalyte 3-10 (0.5%), bromophenol blue (0.02%), and dithiothreitol (0.2%) was used to rehydrate overnight step (approximately 16 hours).

#### ***Sample preparation***

Sample solution consisting of urea (9M), triton X-100 (0.5%), Pharmalyte 3-10 (2%), bromophenol blue (0.02%), and dithiothreitol (1%) was added to an aliquot of the protein sample to a ratio of 1:4 [sample : sample solution (v/v)].

#### ***The first dimension: Isoelectric focusing (IEF)***

The rehydrated Immobiline DryStrips, which were aligned between the anode and the cathode electrodes, were placed on a Multiphor II Electrophoresis Unit. Once the strips were uniformly covered with silicone oil and the sample cups positioned on the strips, the samples were pipetted into the cups. Isoelectric focusing of the strips was carried out at an initial voltage of 500 V for 4 hours. The voltage was then increased up to 3000 V, for a total focusing time of 16 hours.

### ***Equilibration of the Immobiline DryStrips***

This was a two step process. In the first step, the strips were removed from the Multiphor II Electrophoresis Unit and placed into petri dishes containing 15 ml of equilibration solution, and incubated at room temperature, with gentle shaking, for 10 minutes. The equilibration solution consisted of urea (6M), 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] and for the first step it was supplemented with 0.5% (w/v) dithiothreitol (solution No 1).

In the second step, solution No. 1 was discarded and 15 ml of equilibration solution supplemented with 4.5% (w/v) iodoacetamide and 0.02% (w/v) bromophenol blue was added (solution No. 2). The strips were incubated with gentle shaking for 10 minutes. The equilibrated strips were then allowed to drain the excess liquid on a piece of moistened filter paper prior to loading them on a glycine gel for the second dimension run.

### ***The second dimension run: SDS-PAGE***

For second dimension electrophoresis, vertical Hoefer gel stands (section 3.3.3.) were used.

The lower resolving gel of 12.5% acrylamide in 1.5 M Tris, 0.4% (w/v) SDS buffer was poured first and allowed to polymerise for at least 1 hour. The upper resolving gel (stacking gel) of 4% % acrylamide in a 0.5 M Tris, 0.4% (w/v) SDS buffer was poured on top of the lower gel and allowed to polymerise overnight.

A two-welled comb was used during the polymerisation of the stacking gel. This comb was designed in such a way that it had a small well for running the molecular weight marker (Low Molecular Weight electrophoresis calibration, Pharmacia, Biotech, Sweden) and a big well for the horizontal placement of the Immobiline Drystrip. Once the marker and the strip were placed on the gel, electrophoresis was performed at a constant current of 20 mA, using a Tris-

glycine based running buffer [0.3% (w/v) Tris, 1.4% (w/v) glycine, 0.1% (w/v) SDS]. Once the blue dye had moved out of the strip, the voltage was increased to 40 mA. The total time to perform second dimension electrophoresis was between 180 and 240 minutes. The gels were stained by soaking in PhastGel® Blue R for 4-8 hours, destained by washing with 30% (v/v) methanol/10% (v/v) acetic acid overnight and soaked in 10% (v/v) acetic acid/20% methanol, containing 6% (v/v) glycerol prior to drying under vacuum at 65° C.

The dried gels were exposed in cassettes to Hyperfilm™-βmax and kept at – 70° C for about one week. The autoradiographs were then developed (Appendix B).

#### **4.4. RESULTS AND DISCUSSION**

The two-dimensional electrophoresis technique was chosen thanks to its characteristic of separating mixtures of proteins by pI and molecular weight on the 2D electropherogram. Cell lysates can be fractionated into individual proteins in order to obtain an overall picture of the protein composition and to enable location of individual proteins.

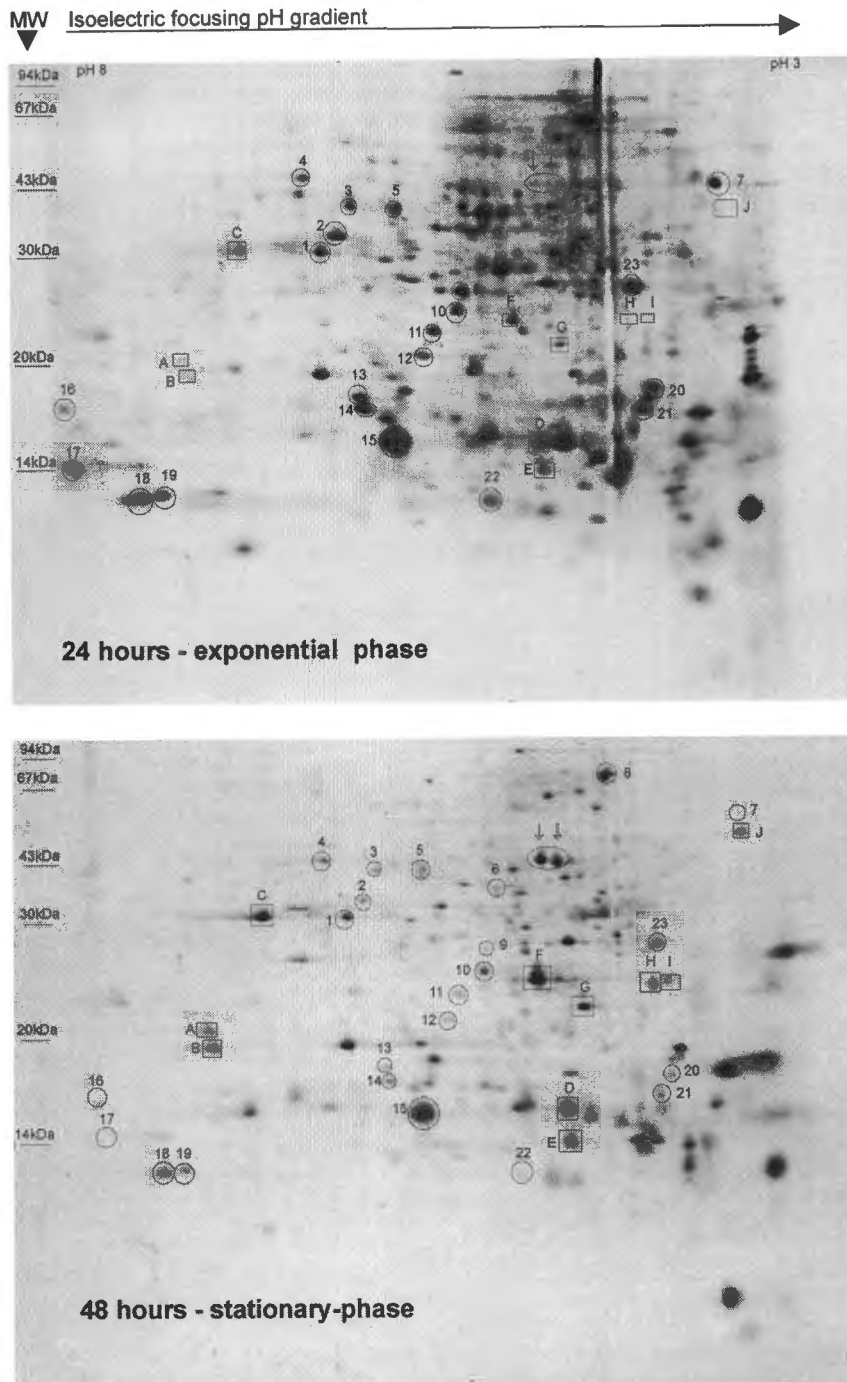
The result of the separation of *M. smegmatis* LR222 lysates is a pattern of spots that can be viewed in Figure 4.1. Equal amounts of radiolabelled protein (30 µg) from exponential phase (24 hours) and stationary phase (48 hours) samples were loaded onto each IEF strip. In the 24-hour old sample autoradiograph there is a higher induction of *de novo* synthesised proteins. There is a group of 6 proteins of 43-30 kDa with pI values between 4.5 and 6, indicated by numbered circles in Figure 4.1. exponential phase (1 to 6), which are strongly induced during the exponential phase of growth. One protein (numbered circle 7 in Figure 4.1.) of ~ 42 kDa and pI < 3 is also highly induced during exponential phase. Another protein (circle number 8) of about 67 kDa, pI .>4, can also be observed on the autoradiograph.

Proteins that are switched off at stationary phase, are those of 24, 23, 20, 17 kDa, and pI values of between 5 to 6.5 (numbered circles 9 to 12), 18, 19 kDa, pI

~6.5 (numbered circles 13 and 14), ~16 kDa, pI >6.5 (numbered circle 15), 16 and 15 kDa, with pI ~4 (numbered circles 20 and 21), 29 kDa, pI ~4 (numbered circle 23), ~17 kDa, pI ~8 (numbered circle 16), ~14 kDa, pI <8 (numbered circle 17) and below the 14 kDa (18 and 19), pI 5-4.5, and pI ~ 6.5 (22).

Comparison between the 2D-PAGE gel autoradiographs of the exponential and stationary phase samples gels demonstrated resolution of the 43-46 kDa protein of interest into two strongly age-associated proteins, with a pI between 5 and 4.5 (indicated in Figure 4.1. exponential phase with arrows). Other proteins strongly induced at stationary phase are of ~ 20 and ~ 19 kDa, pI <8 (squares marked with letters a and b), of ~ 30 kDa, pI ~ 7 (square c), of ~ 16 and ~14 kDa, pI of about 4.5 (squares d and e), ~46 kDa, pI <3 (square j) and a group of between 21-25 kDa in molecular weight, pI values between 5.5 and 4 (squares f to i).

## 2D-SDS-PAGE



**Fig.4.1.** Two-dimensional SDS-PAGE analysis of the proteins produced by *M. smegmatis*, during the exponential (24 hours) and stationary (48 hours) phases of growth, following a heat shock. A culture of *M. smegmatis* LR222 was grown, with continuous shaking, in 7H9 Middlebrook, supplemented with 10% ADC and 0.05% Tween 80. Proteins were labeled at the times indicated, with  $^{35}\text{S}$ [L]-methionine for 1 hour at 48° C. The numbers indicate heat-shock proteins induced during the exponential-phase, while the letters indicate stationary-phase-specific heat-shock proteins. The previously mentioned 43-45kDa stationary-phase protein resolves into two prominent spots, which are indicated by the arrows.

# **CHAPTER 5**

## **IDENTIFICATION OF UNIQUE STATIONARY PHASE GENES OF *M. smegmatis* THROUGH SUBTRACTIVE RNA HYBRIDIZATION**

### **5.1. SUMMARY**

The aim of this study was to obtain a subtraction cDNA probe, from a stationary phase sample, depleted of sequences common in both exponential and stationary phase RNA samples.

RNA was extracted from culture samples taken at the exponential and the stationary phase of growth of *M. smegmatis* LR222 cells. The 16S and 23S rRNA subunits were removed from the stationary phase RNA ("induced" RNA) by photobiotinilation. First strand cDNA synthesis was performed using reverse transcriptase and the photobiotinilated stationary phase mRNA. The cDNA/mRNA hybrids were then hybridised with the "uninduced" RNA (exponential phase RNA) in the subtractive hybridisation step. Once the "unique" cDNA had been obtained, it was radioactively labelled and used to probe a *M. smegmatis* library. This would allow to find the gene(s) encoding for the 43-46 kDa stationary phase protein observed on SDS-PAGE gels in Chapter 3 and further characterised by two-dimensional gel electrophoresis in Chapter 4. This experimental approach, however, failed and further investigation as to why it did not work is required.

## **5.2. INTRODUCTION**

A particular gene represents only a small part of a prokaryotic genome and to identify such a small part a specific probe, that reacts only with the particular sequence of interest, is needed. The aim of this technique is to differentiate genes that are only expressed under unusual conditions (i.e. dormancy) from those constitutively expressed (Kinger and Tyagi, 1993; Kikuta-Oshima *et al.*, 1994). The usual technique is to use a radiolabelled RNA or DNA probe, whose hybridisation with the gene is assayed by autoradiography. For the purpose of obtaining a DNA sequence that represents a particular protein, messenger RNA (mRNA), the template used to produce the protein *in vivo*, is used (Ghosh, 1996). However, it can be difficult to obtain the mRNA that represents a particular protein when the product is rare. There are several techniques for isolating a mRNA but a common problem is that intact, full-length RNA must first be purified. Rather than purify the RNA, a DNA copy of the RNA sequence is made. Reverse transcription makes it possible to synthesise a duplex DNA from any mRNA. The DNA can then be radioactively labelled, providing a powerful probe.

## **5.3. MATERIALS AND METHODS**

### **5.3.1. Preparation of plasmid DNA containing 16S RNA and 23S RNA fragments**

#### ***Large scale plasmid preparation***

The "Maxiprep" method used in this experiment is based on that described in Sambrook *et al.* (1989.)

Colonies of *Escherichia coli* LK111 containing either pUC19, with a 16S rRNA insert or pGEM-T, with a 23S rRNA insert were inoculated into a 5 ml 2YT Broth starter culture and incubated overnight at 37° C on an orbital shaker (180 rpm). One hundredth of the starter culture was then added to 500 ml of 2YT broth

containing 500 µl of Ampicillin (100 mg/ml) and incubated at 37° C with shaking (at 180 rpm), for approximately 16 hours. The cells were harvested by centrifugation at 4,000 rpm for 5 minutes. The pellets were resuspended in 8 ml of 1 M Tris-HCl, pH 8.0; 0.5 M EDTA, pH 8.0; of 20% w/v Glucose. To this, 16 ml of 10 N NaOH; 25% w/v SDS, were added, and left on ice for 5 minutes (the culture went clear and viscous as a consequence of this timed denaturing step). Twelve millilitres of pre-cooled of 5 M Potassium acetate; Glacial acetic (1 M) acid were added and the cultures kept on ice for 5 minutes (a white flocculate formed containing most of the chromosomal DNA, cellular protein and membrane fractions). These debris were recovered by centrifugation (13,000 rpm for 10 minutes at room temperature) and 0.6 ml of isopropanol was added to the supernatant fluid to precipitate plasmid. The samples were kept at room temperature for 10 minutes and the nucleic acids were recovered by centrifugation (13,000 rpm for 10 minutes, at room temperature) and washed in 70% ethanol before dissolving them in 8 ml of Tris-EDTA buffer (TE) (10 mM Tris, 1 mM EDTA, pH 7.6).

### ***Caesium chloride gradient centrifugation for purification of plasmid DNA***

Plasmid DNA was further purified by CsCl/ethidium bromide density gradients. To the 8 ml of DNA dissolved in TE, 8 g of CsCl and 0.8 ml of 10mg/ml ethidium bromide were added. The solutions were mixed, centrifuged (13,000 rpm for 10 minutes at room temperature) and the supernatants were recovered. Their refractive index was adjusted with a Refractometer to 1.390. The supernatants were then transferred into Beckman Vti65 ultracentrifuge tubes and centrifuged in a Vti65 ultracentrifuge at 50,000 rpm for 16 hours at 20° C.

Following centrifugation, the DNA was visualised under ultraviolet light (UV 310 nm). Two bands were visible: a distinct lower band corresponding to the covalently closed coiled plasmid DNA, and a less distinct higher band corresponding to chromosomal and/or nicked plasmid DNA. Closed coiled

plasmid DNA was eluted into a fresh microfuge tube. Ethidium bromide was removed from the plasmid DNA by adding an equal volume of salt saturated isopropanol. The upper phase containing the ethidium bromide was discarded, and 2 volumes of water were added. To the new volume, 1 total volume of water saturated butanol was added, the sample was mixed and kept at room temperature for 10 minutes. After centrifugation at 13,000 rpm for 15 minutes at room temperature, the supernatant was discarded and the pellets washed in 70% ethanol and dissolved in 0.5 ml of TE.

The DNA concentration was determined spectrophotometrically using a Beckmann DU-40 Spectrophotometer and calculated using the relationship  $1 \text{ OD}_{260} = 1 \text{ for } 50 \mu\text{g/ml of DNA}$ .

### ***Restriction enzyme digestion***

Plasmids pUC19(16S) and pGEM-T(23S) were linearised by digestion with restriction enzymes. The restriction enzyme *Pst*I (Boehringer Mannheim) was appropriate for the linearisation of both plasmids. The following was added to 35  $\mu\text{g}$  of pUC19(16S) and 65  $\mu\text{g}$  pGEM-T(23S): 10  $\mu\text{l}$  *Pst*I, 5  $\mu\text{l}$  buffer H (both Boehringer Mannheim), and 15  $\mu\text{l}$  of water. The samples were incubated at 37° C for 2 hours.

The linearisation of the plasmids was confirmed by agarose gel electrophoresis. An aliquot of digested DNA was separated on 3% agarose gel (1 x Tris-acetate EDTA buffer) together with a molecular weight at a constant voltage of 40 mA for approximately 120 to 180 minutes.

After separation, the DNA was visualised by placing the gel under UV light and was photographed.

### **5.3.2. RNA extraction from *M. smegmatis***

When working with RNA samples precautions against RNAase contamination were taken, these included wearing latex gloves at all times; buffers and water used in solutions were treated with 0.1% diethylpyrocarbonate (DEPC); pre-treatment of glassware and other instrument with DEPC-treated water and RNAase inhibitor (Boehringer Mannheim).

*M. smegmatis* strain LR222 was grown according to the method described in section 2.3.1. Culture samples (50 ml) were taken after 24 hours incubation (exponential phase of growth), and after 48 hours (stationary phase of growth). RNA was extracted from the samples using the FastPrep™ system (FastPrep™ FP120 instrument – Savant Instrument Inc., Farmingale, NY, U.S.A. - and FastRNA® Kit-Blue – Bio101 Inc., Vista, CA, U.S.A.) according to the manufacturers' instructions. RNA pellets were dissolved in 20 µl of DEPC-treated water. The RNA was quantitated by measuring spectrophotometrically the absorbance of a diluted sample at a wavelength of 260 nm, and the concentration was calculated using the relationship 1 OD<sub>260</sub>= 1 for 40 µg/ml of RNA.

The samples were stored at – 70° C until required.

### **5.3.3. Removal of 16S and 23S rRNA from stationary phase RNA preparations**

#### ***Photobiotinilation of plasmid DNA***

Plasmid samples containing the 16S rRNA (30 µg) and the 23S rRNA (60 µg) inserts were mixed at a ratio of 16S rRNA: 23S rRNA = 1:2, and water added to a final volume of 40 µl. The mixture was incubated at 100° C for 3-5 minutes to denature the plasmid DNA. In a darkroom, the samples of denatured DNA were placed 10 cm from a GE RSM 275W sunlamp and 1 µg/µl of freshly prepared

photobiotin acetate solution was added and the samples exposed to the light for 3 minutes. This activated the photobiotin, which allowed it to bind to the denatured DNA. After the photobiotinylation step, 70  $\mu$ l of water, 50  $\mu$ l of 0.1 M Tris, pH 9.0 and 200  $\mu$ l of water saturated butanol were added. The lower aqueous phase was recovered by centrifugation (13,000 rpm for 10 minutes, at room temperature) and to this 0.15 volumes of 2 M sodium acetate, pH5.0, and 2.5 volumes of 100% ethanol were added to precipitate the photobiotinylated DNA. Thereafter, the mixture was held on dry ice for 10-20 minutes. The pellet containing the DNA was obtained by centrifugation (13,000 rpm for 10 minutes at room temperature), washed in 70% ethanol and dissolved in 30  $\mu$ l of DEPC-treated water.

### ***Hybridisation of photobiotinylated plasmid DNA to total cellular RNA***

To the photobiotinylated DNA, 10-15  $\mu$ g of RNA from the stationary phase sample were added, together with 1  $\mu$ l of RNAase inhibitor to prevent RNA degradation. The nucleic acids were precipitated with 0.15 volumes of 2M sodium acetate, pH 5.0, and 2.5 volumes of 100% ethanol. The pellet was recovered by centrifugation (13,000 rpm for 10 minutes at room temperature) and dissolved in 16  $\mu$ l DEPC-treated water and 4  $\mu$ l of 5 x hybridisation buffer (2.5 M NaCl; 125 mM HEPES buffer, pH 7.5; 25 mM EDTA; 5% SDS). The sample was incubated at 100° C for 1 minute, to denature the DNA, then placed in a 68° C waterbath for 24 hours to allow hybridisation.

### ***Removal of double-stranded duplexes***

Streptavidin has the ability to bind to photobiotin, therefore, it is used to remove the photobiotinylated DNA, which has hybridised to the 16S rRNA + 23S rRNA molecules.

After the hybridisation step, the sample was incubated at 55° C for 5 minutes. To the hybridised sample 30 µl of 10 mM HEPES/10 mM EDTA buffer and 10 µl of 10 mg/ml streptavidin were added and the mixture was kept at room temperature for 10 minutes. To this were added 60 µl of phenol-chloroform and the sample centrifuged at 13,000 rpm for 2 minutes at room temperature. The aqueous phase, from which the DNA/RNA duplexes had been removed thanks to streptavidin, was recovered and the RNA obtained by adding 0.15 volumes of 2 M sodium acetate, pH 5.0, and 100% ethanol. The pellet was dissolved in 10 µl DEPC-treated water and stored at – 70° C.

To check that the 16S and 23S rRNAs had been removed from the bulk RNA, RNA gel electrophoresis was performed. RNA was separated using a 1.2% agarose/formaldehyde denaturing gel. A small 1.2% agarose RNA gel was prepared as follows: 0.75g of agarose, 38.5 ml of DEPC-treated water and 10 ml of 5 x gel running buffer (0.2 M morpholinopropanesulfonic acid (MOPS); 50 mM sodium acetate; 5 mM EDTA, pH 8.0) were added together and heated to allow the agarose to dissolve. Once the agarose had reached about 55-50° C, 2.55 ml of formaldehyde were added. The RNA samples were prepared as follows: 2 µl of RNA (20 µg) were added to 2.5 µl of DEPC-treated water, 5 µl 2 x gel running buffer, 3.5 µl of formaldehyde and 10 µl of formamide. The samples were incubated at 65° C for 15 minutes and then 2 µl of 10 x sample loading buffer (50% glycerol; 0.1 mg/ml bromophenol blue) and 0.5 µl of ethidium bromide were added before loading onto the gel. The gel was run at 200 volts for 180-240 minutes in 5 x gel running buffer. It was then visualised under UV light and photographed.

#### **5.3.4. First strand cDNA synthesis**

To a microfuge tube, 5-10 µg of “induced” stationary phase RNA, with rRNA molecules removed, and 2 µl 50 ng/µl hexamers (Boehringer, Mannheim) were

added and then incubated at 70° C for 10 minutes. The sample was subsequently held on ice for 5 minutes and the pellet recovered by centrifugation (13,000 rpm for 10 minutes at room temperature). To the pellet, 4 µl of 5x first strand buffer (Promega, Reverse Transcriptase buffer), 1 µl of 10 mM dNTP (Boehringer, Mannheim), 2 µl 0.1 M 1,4-Dithiothreitol (DTT) and 1 µl DEPC-treated water were added. The solution was gently mixed and incubated at 37° C for 2 minutes. To this, 5 µl of Superscript II (Promega, Reverse Transcriptase) were added and the sample was incubated at 37° C for 1 hour. Following this step, the hybrids cDNA/mRNA were denatured by incubating at 95° C for 3 minutes. The tube was then placed on ice for 5 minutes. To the sample, 1 µl of 5x first strand buffer (Promega, Reverse Transcriptase buffer), 0.5 µl 0.1 M DTT and 1.5 µl water were added and then incubated at 37° C for 2 minutes before adding 2 µl of Superscript II (Promega, Reverse Transcriptase) and incubating at 37° C for 1 hour.

To the cDNA/mRNA hybrids, 2 volumes of 1N NaOH were added and then incubated at 55° C for 15 minutes to hydrolyse the remaining RNA. To neutralise the mixture 1/10 volumes of 1 M HCl were added to the cDNA and then 16 µl of 10M ammonium acetate, pH 7.5, and 2 volumes of 100% ethanol were added to allow precipitation. The mixture was placed on dry ice and allowed to freeze. The pellet was recovered by centrifugation (13,000 rpm for 15 minutes at room temperature), washed in 70% ethanol and dissolved in 30 µl of DEPC-treated water.

### **5.3.5. Subtractive RNA hybridisation**

To the cDNA synthesised from the induced stationary phase RNA, 10-20 µg of “uninduced” RNA (exponential phase RNA) were added. Nucleic acids were co-precipitated with 0.1 volumes of 10 M ammonium acetate, pH 7.5, and 2 volumes of 100% ethanol. The pellet was retrieved by centrifugation (13,000 rpm for 10

minutes at room temperature) and dissolved in 5  $\mu$ l of water and 2  $\mu$ l of 5 x hybridisation buffer (2.5 M NaCl; 0.125 M HEPES buffer, pH 7.5; 25 mM EDTA; 5% SDS). The microfuge tube was placed at 68° C for 20 hours to allow hybridisation of the “uninduced” RNA to the single stranded DNA synthesised from “induced” RNA.

Once hybridisation had occurred, the nucleic acids were precipitated by adding 40  $\mu$ l of water, 3 volumes of 100% ethanol and were kept at – 20° C for 20 minutes. The cDNA/mRNA hybrids and unique single stranded cDNA were recovered by centrifugation (13,000 rpm for 15 minutes, at room temperature). To the dry pellet, 50  $\mu$ l of cross-linking buffer (25 mM of Tris-HCl, pH 7.0; 1 mM EDTA; 5% DMSO; 2 mM ascorbic acid) were added and the solution was incubated at 68° C for 3 minutes. The temperature was then lowered to 45° C and 1  $\mu$ l 10 mM 2,5-diaziridinyl 1-4-benzoquinone 1 (DZQ) was added and the microfuge tube incubated at 45° C for 20 minutes. After the cross-linking, 50  $\mu$ l of chloroform/isoamylalcohol (24:1) were added to the cDNA/hybrids. The aqueous phase, containing the unique cDNA molecules, was recovered by centrifugation (13,000 rpm for 10 minutes at room temperature) and nucleic acid recovered by adding 5  $\mu$ l 3 M sodium acetate, pH 5.6, 2 volumes of 100% ethanol, and placing the mixture at –20° C for 20 minutes. The pellet was then recovered by centrifugation (13,000 rpm for 15 minutes at room temperature), dried and dissolved in 20  $\mu$ l of water.

### ***5.3.6. Labelling the cDNA probe by random priming***

To a microfuge tube, the following were added: 1.2  $\mu$ l 50  $\mu$ g/ $\mu$ l hexamers (Boehringer Mannheim), 1  $\mu$ l 10 x PNK buffer (Boehringer Mannheim), 6.8  $\mu$ l [ $\gamma$ -<sup>32</sup>P] dATP (10  $\mu$ ci/ $\mu$ l, Amersham, 3000ci/mmol), 1  $\mu$ l T4 polynucleotide kinase (Boehringer Mannheim). The sample was incubated at 37° C for 45 minutes and then at 95° C for 3 minutes. To the cDNA the following was added: 6.4  $\mu$ l of

water, 2 µl of 0.5M dATP, 2µl of 0.5M dGTP, 2 µl of 0.5M dTTP, 0.5 µl of 0.5M dCTP (all Boehringer Mannheim), 10 µl [ $\gamma^{32}\text{P}$ ]-labelled hexamers, 3 µl [ $\alpha^{32}\text{P}$ ]-dCTP, 0.5 µl Sequenase II and 5 µl of Sequenase II buffer (both Amersham). The sample was kept at room temperature for 20 minutes and then placed at 65° C for 10 minutes.

The probe was stored at – 4° C until required.

### **5.3.7. Probing the *M. smegmatis* genomic library**

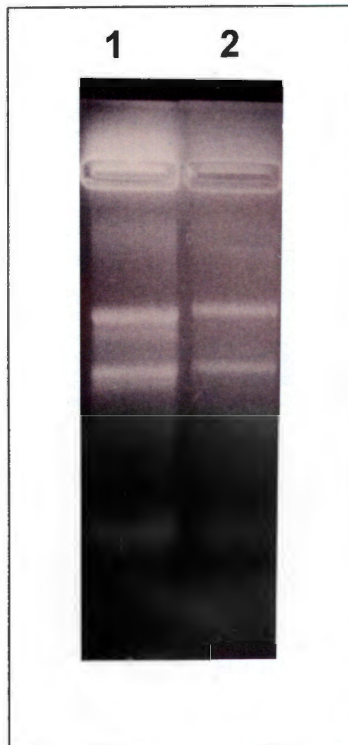
A gridded *M. smegmatis* gene library (by Dr Everett, Glaxo-Wellcome, Stevenage, UK) was used for probing. The membrane was pre-hybridized with 20 ml of pre-heated hybridisation solution (1 M KPO<sub>4</sub>, pH 7.4; 20 ml 20 x SSC; 50 x Denhardt's solution [2% (v/v) BSA; 2% (v/v) Ficoll; 2% (w/v) polyvinyl pyrrolidone (PVP)]; 10 mg/ml denatured salmon sperm DNA) for 2 to 3 hour in a hybridisation oven at 65° C. The cDNA probe was boiled and added to the pre-hybridised membrane, which was left to hybridise for a further 16 hours at 65° C. The membrane was washed once at 25° C for 15 minutes in 2 x SSC containing 0.1% SDS, sealed with a plastic sheath and exposed in a cassette to a Hiperfilm™ for 24 hours.

## **5.4. RESULTS AND DISCUSSION**

The RNA subtractive hybridisation technique performed in this study proved to be a lengthy and fastidious technique. Several steps had to be performed prior to obtaining a sufficient quantity on messenger RNA (mRNA), from which the 16S and the 23S rRNA subunit had been removed. Only then could the "clean" mRNA be used as a template for the synthesis of a [ $\gamma^{32}\text{P}$ ]-cDNA probe.

#### 5.4.1. RNA extraction from *M. smegmatis*

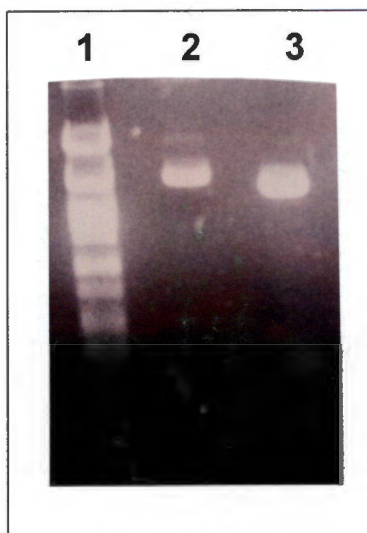
*M. smegmatis* strain LR222 was grown according to the method described in section 2.3.1. Two samples were taken, the first at 24 hours, the exponential phase sample, and the second one at 48 hours, the stationary phase sample. From these samples RNA was extracted using the FastPrep™ system and quantitated by measuring the absorbance at OD<sub>260</sub> ( 1 OD<sub>260</sub> = 1 for 40 µg/ml of RNA). Figure 5.1. shows a 1.2.% agarose/formaldehyde gel of the two samples. The gel was performed to check on the purity of the samples.



**Figure 5.1.** 1.2% RNA agarose/formaldehyde gel with samples of total RNA. The gel was performed to check on the purity of the RNA extracted. In lanes 1 and 2 show two bands which correspond to the 16S and 23S rRNA of the sample taken at 24 hours (lane 1) and of that taken at 48 hours (lane 2).

#### 5.4.2. Removal of 16S and 23S rRNA from stationary phase RNA

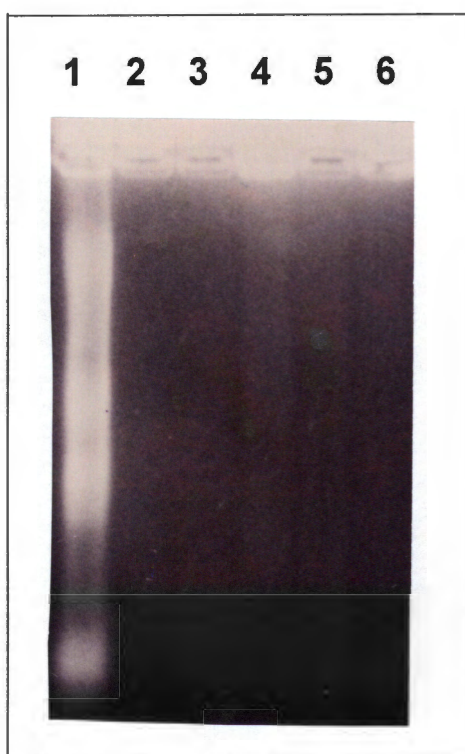
To remove ribosomal RNA, *E. coli* LK111 strains containing either the plasmid pUC19(16S) or the plasmid pGEM-T(23S), were used in this study. Plasmids were obtained using the “Maxiprep” plasmid preparation and then were further purified by CsCl/ethidium bromide density gradient. Plasmid pUC19 and pGEM-T were linearised by digestion with restriction enzyme *Pst*I. Figure 5.2. of a 1.2% agarose gel shows the linearised plasmids (lane 2 and 3).



**Figure 5.2.** 1.2% agarose gel of plasmid DNA (lane 1), linearised pUC19, with 16S rRNA insert (lane 2), and linearised pGEM-T, with 23S rRNA insert (lane 3).

The next step was that of photobiotinylation. The plasmids were denatured to allow the protein photobiotin to bind to the single stranded plasmid, once it had been activated by light. The photobiotinylated DNAs were hybridised to the stationary phase RNA for 24 hours. This allowed the formation of photobiotinylated plasmid DNA (containing either the 16S rRNA insert or the 23S

rRNA insert) and stationary phase RNA double stranded complexes. Streptavidin, which binds to photobiotin, was used to remove these complexes. The end result was stationary phase mRNA free of the 16S and 23S rRNA subunits. To confirm the success of this step, a 1.2% RNA agarose/formaldehyde gel was performed using total RNA as a control and mRNA. In line 1 of Figure 5.3. the 16S and 23S rRNA subunits of total RNA can be observed, whilst in lane 2-6 they are no longer present.



**Figure 5.3.** 1.2% RNA agarose/formaldehyde gel, showing in lane 1 total RNA (control) and in lanes 2-6 mRNA depleted of the 16S and 23S rRNA subunits.

### **5.4.3. Subtractive hybridisation**

Only at this point, with purified mRNA (See Figure 5.4., A), could the actual subtractive hybridisation technique begin.

Synthesis of first strand cDNA was performed by using the stationary phase mRNA as a template, which was randomly primed with hexamers and reverse transcriptase (Figure 5.4., B). The first strand synthesis was repeated to ensure that enough cDNA had been synthesised. After this step the remaining mRNA was removed by alkaline hydrolysis (Figure 5.4., C). The single stranded cDNA, synthesised from the stationary phase mRNA template, was hybridised to an excess of the exponential phase RNA (that does not produce the gene or genes of interest). Common cDNA/RNA hybrids formed after hybridising the mixture for 20 hours at 68° C (Figure 5.4., D). The hybrids were then chemically cross-linked with 2,5 daziranyl 1-4 benzoquinone 1 (DZQ) (Figure 5.4., E). The cross-linked hybrids containing sequences present in both the stationary phase RNA and the exponential phase RNA were removed by adding chloroform/isoamylalcohol, leaving the mRNA unique to the stationary phase sample. This was the actual subtractive hybridisation step (Figure 5.4., E).

### **5.4.4. Probing the *M. smegmatis* genomic library**

The cDNA was dually labelled by random priming using [ $\gamma$   $^{32}$ P]-hexamers together with dATP, dGTP, dTTP, dCTP and [ $\alpha$   $^{32}$ P]-dCTP and Sequenase II. The [ $\gamma$   $^{32}$ P]-labelled probe was used to probe the *M. smegmatis* library (Figure 5.3., F). The probe was boiled prior to adding to the pre-hybridised membrane, and hybridised in an oven at 65° C overnight (approximately 16 hours). The membrane was washed once in 2 x SSC + 0.1% SDS at 25° C for 15 minutes, sealed in a plastic sheath and exposed to Hyperfilm™ MP overnight. The autoradiograph was developed the following day but unfortunately did not show any probed sites on the gridded *M. smegmatis* library. The hybridisation

technique was repeated again, but this time the hybridisation temperature was lowered to 58° C, and hybridisation performed for 16 hours. The membrane was washed in 2 x SSC containing 0.1% SDS, sealed in a plastic sheath and exposed to Hyperfilm™ MP for 24 hours. The result was still a negative one and the technique revised and repeated with slight alterations. More RNA was extracted from both exponential and stationary phase samples. It was thought that with the removal of the 16S and 23S rRNAs, the bulk of the RNA, little mRNA was left to work with. Also, more care was taken while performing the photobiotinilating step. This step required to add photobiotin to several microfuge tubes in the complete absence of light, and only then was the light switched on to activate the protein.

Too many steps were required before reaching the actual subtraction one and the importance of having a substantial quantity of mRNA was essential to produce a subtracted probe by random primer labelling.

This technique was repeated several times, making sure that each step was carried out diligently, however, it was still not possible to obtain probed sites on the *M. smegmatis* membrane.

Due to time constraints it was not possible to improve the technique nor to find where the fault(s) lied.

A) mRNA isolated from cell producing gene or genes of interest



B) Synthesise first strand cDNA



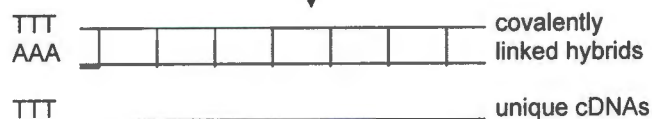
C) Hydrolyse parent RNA template



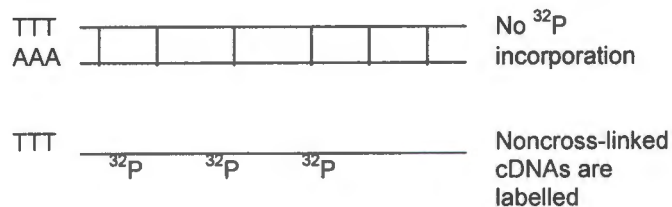
D) Hybridise to excess mRNA isolated from cells that does not produce gene or genes of interest



E) Chemically cross-link with DZQ



F) Random primer with [ $\alpha^{32}$ P]dCTP



USE AS A PROBE

**Figure 5.4.** Schematic representation of RNA subtractive hybridisation. (From Hampson *et al.*, 1994).

# CHAPTER 6

## GENERAL CONCLUSIONS

This study was an attempt to identify growth-specific proteins in *M. smegmatis* LR222. An *M. smegmatis* LR222 growth curve was determined using an ATP assay. This technique was chosen over others such as the measurement of turbidity (absorbance or optical density) and the plating and counting of colonies (CFU). In a previous study carried out by Ntolosi (1998), it was demonstrated to be the most accurate indicator of when *M. smegmatis* LR222 cells changed their phases of growth. The ATP assay is based on the quantitative measurement of light produced as a result of an enzymatic reaction catalysed by firefly luciferase. ATP was extracted from tricarboxylic acid (TCA) precipitated cells, which allowed total ATP extraction, including that from clumping cells. This gave a measure of cell biomass at the time of the sampling, since the amount of ATP per cell is proportional to the cell mass. This assay also proved to be a rapid technique and was performed when taking growth phase-specific samples for protein extraction.

Two approaches were taken: protein radiolabelling, which involved one-dimensional and two-dimensional electrophoresis, and RNA subtractive hybridisation.

Newly synthesised proteins were metabolically labelled with [<sup>35</sup>S]L-methionine in cells incubated either at a control temperature or at a heat shock temperature. The aim of this experiment was to identify proteins synthesised *de novo* at various phases of growth and to determine whether their induction was related to the heat shock stress or to the aging of the organism. This was

accomplished by comparing autoradiographs of labelled proteins on one-dimensional SDS-PAGE. From the autoradiographs it was possible to observe a 43-46 kDa protein which was strongly induced during stationary phase at both incubation temperatures. The one-dimensional SDS-PAGE proved to be a good screening technique to detect regulated proteins, however, it did not allow the determination of whether the "band" of interest corresponded to one or more proteins.

For two-dimensional electrophoresis two time points on the growth curve of *M. smegmatis* LR222 were chosen, that corresponded to the exponential phase and the stationary phase. Protein samples were taken at these time points and prepared for two-dimensional electrophoresis. This technique allows the separation of the protein by pI in one dimension and then molecular weight in second dimension. The 43-46 kDa stationary phase-specific protein was resolved into two spots with a pI value of 4.5-5.

The technique of RNA subtractive hybridisation was used to obtain a cDNA probe from a stationary phase sample of *M. smegmatis* LR222, from which sequences common to both the exponential and the stationary phase had been removed. Genes uniquely expressed under the stress conditions alone should be present. Unfortunately this technique was not successfully implemented. An *M. smegmatis* library was probed with the labelled subtracted cDNA but no signal was observed on autoradiography. The method described here was novel and attempted to constitute a one-tube reaction based on specific chemical cross-linking of cDNA-RNA hybrids. Previous methods relied on the physical separation of common cDNA-RNA hybrids from unique single stranded cDNA by using hydroxyapatite or avidin-biotin. This approach suffered from incomplete separation of these components and significant loss of probe. In the "new" method, RNA samples were taken at both exponential and stationary phase of the growth culture. Single stranded cDNA was synthesised by reverse transcriptase from stationary phase mRNA, from which the 16S and the 23S rRNA subunits had been removed by photobiotinilation. Common cDNA-RNA hybrids were then cross-linked with 2,5 daziridiny 1-4 benzoquinone 1 (DZQ) and removed. The

subtracted probe was produced by random primers labelling of the unique single-stranded cDNA using Sequenase II.

Several reasons for the failure to isolate the specific probe by this method are possible: i) once the ribosomal RNA had been removed, too little messenger RNA was left to construct the probe, or maybe ii) more labelled hexamers were required in priming the cDNA, iii) the main difficulty lied in the number of steps that had to be performed without having control points. The technique could be checked only until the removal of the ribosomal RNA, thereafter the technique was carried out solely in one test tube. Further investigation as to why this method was not successful is required, as a one-tube reaction protocol of subtractive hybridisation would be ideal.

The sequence and the functional characteristics of this 43-46 kDa stationary phase-specific protein and its role in permitting *M. smegmatis* LR222 to remain in stationary phase should be further investigated. This could be carried out by protein sequencing, either by N-terminal sequencing or by Matrix Assisted Laser-Desorption/Ionisation Time-Of-Flight (MALDI-TOF) and, thereafter, to screen the database for homologous proteins.

These findings could help in elucidating the genetic mechanisms that allow *M. tuberculosis* to persist in a dormant state.

# ***LIST OF ABBREVIATIONS***

2D	Two dimensional
$\alpha$	Alpha
A <sub>260</sub>	Absorbance at 260 nm
AIDS	Aquired immune deficiency syndrome
APS	Ammonium persulfate
ATP	Adenosine 5-triphosphate
BC	Before Christ
BCG	Bacille-Calmette-Guerin
bp	Base pairs
BSA	Bovine serum albumin
$\beta$	Beta
°C	Degrees Celsius
CDC	Centre for disease control
CFU	Colony forming units
Ci	Curie
dATP (A)	Doxyadenosine triphosphate
dCTP (C)	Doxycytidine triphosphate
dGTP (G)	Doxyguanosine triphosphate
dTTP (T)	Doxythymidine triphospahte
dH <sub>2</sub> O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	1.4-Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium Bromide
g	Gram
HEPES	N-2-hydroxyethylpiperazine-N <sup>1</sup> -2-ethanesulfonic acid
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus

Hrs	Hours
HSP	Heat shock protein
INH	Isoniazid
kb	Kilobase pair
kDa	Kilodalton(s)
LMW	Low molecular weight
M	Molar
mg	Milligrams
ml	Millilitres
μl	Microlitre
mM	Millimolar
MOPS	Morpholinepropanesulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
nm	Nanometres
OD <sub>600</sub>	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RNAase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
Sec	Second/s
SSDNA	Salmon sperm DNA
TAE	Tris-acetate EDTA buffer
TB	Tuberculosis
TCA	Tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
Tween 80	Polyoxyethylene sorbitan monooleate
YT	Yeast triptone
UV	Ultraviolet

V	Volt
v/v	Volume in volume
w	Watt
WHO	World Health Organisation
w/v	Weight/volume

# APPENDIX A

## A.1. BACTERIAL STRAINS

The bacterial strains used in this project were:

BACTERIAL STRAIN	GENOTYPE	REFERENCE
<i>M. smegmatis</i> LR222	High frequency transformation mutant	Beggs <i>et al.</i> , 1995
<i>M. tuberculosis</i> H37Rv	Virulent laboratory strain ATCC 27294	Steenken <i>et al.</i> , 1934

ATCC= American Type Culture Collection (Rockville, Md.)

## A.2. ENZYMES AND KITS

Buffer H	Boehringer Mannheim
FastRNA™ Kit, BLUE	BIO 101
<i>Pst</i> I	Boehringer Mannheim
Reverse transcriptase	Promega
Reverse transcriptase buffer	Promega
5 x first strand buffer	Promega
RNAase	Sigma
Sequenase II	United States Biochemicals
T4 polynucleotide kinase	Pharmacia
10 x PNK buffer	Boehringer Mannheim

### **A.3. CHEMICALS AND REAGENTS**

Acetic acid	BDH
Acrylamide	BDH
Agarose	Techcomp Ltd.
Ammonium acetate	BDH
APS (Ammonium persulfate)	Sigma
Ascorbic acid	Sigma
Bis (N,NI-methylene bis acrylamide)	Sigma
Bovine albumin	Sigma
Bromophenol blue	Sigma
Buthanol	Merck
Cesium cholride	Schwartz/Mann Biotech
Catalase	Boehringer Mannheim
Chloroform	Merck
Deoxynucleotide triphosphates	Boehringer Mannheim
DEPC (Diethyl pyrocarbonate)	Aldrich
DMSO (Dimethyl sulphoxide)	BDH
DTT (1.4-Dithiothreitol)	Boehringer Mannheim
DZQ (4-benzoquinone1)	Sigma
EDTA (Ethylenediaminetetra acetic acid)	Boehringer Mannheim
Ethidium bromide	Merck
Ethanol	BDH
Formamide	BDH
Glycerol	BDH
Glycogen	BDH
GTG agarose	BDH
HEPES (N-2-hydroxyethylpiperazine-N <sup>1</sup> - 2-ethanesulfonic acid)	Sigma
Hydrochloric acid	BHD
Hyperfilm-βmax	Amersham
Hyperfilm™ MP	Amersham
Iodoacetamine	Sigma
Isoamyl alcohol	Merck

Isopropanol	BDH
2-Mercaptoethanol	Merck
MOPS (Morpholinepropanesulfonic acid)	Sigma
Phenol	BDH
Photobiotin	Sigma
Potassium acetate	Merk
Salmon sperm DNA	Boehringer Mannheim
SDS (Sodium dodecyl sulphate)	Sigma
Sodium acetate	BDH
Sodium chloride	BDH
Sodium hydroxide	BDH
Streptavidin	BDH
TEMED (N,N,N',N'-tetramethyl-ethylenediamide)	Sigma
Tris(hydroxymethyl)amino-methane (Tris)	Boehringer Mannheim
Triton X-100	BDH
Tween 80	Sigma

#### **A.4. CULTURE MEDIA**

The culture media used in this study were prepared as follows:

##### **A.4.1. Middlebrook 7H9 medium**

Middlebrook 7H9 (Difco) 4.7 g

Tween 80 0.5%

Make up to 900 ml with dH<sub>2</sub>O and autoclave

##### **A.4.2. ADC enrichment medium**

BSA Fraction V 5 g

Glucose 2 g

Catalase 3 mg

Make up to 100 ml with dH<sub>2</sub>O and filter sterilise.

#### **A.4.3. 2 x Yeast Tryptone (YT)**

Bacto-tryptone	16 g
Bacto-yeast extract	10 g
NaCl	5 g

Make up to 1 l with dH<sub>2</sub>O and autoclave.

### **A.5. BUFFERS AND REAGENTS FOR THE BIOLUMINESCENT ASSAY OF ATP**

#### **A.5.1. Neutralising Buffer**

Tris-Acetate	0.1 M
--------------	-------

Adjust pH to 7.75 with acetic acid.

#### **A.5.2. Luciferin/Luciferase Buffer**

Tris-Acetate	0.1 M
EDTA	2 mM

Adjust pH to 7.75 with acetic acid.

#### **A.5.3. ATP-free water**

Deionised, distilled and autoclaved.

### **A.6. BUFFERS AND SOLUTIONS FOR SDS PAGE**

#### **A.6.1. SDS sample buffer**

SDS	3% (w/v)
β-mercaptoethanol	5% (v/v)
glycerol	10%
Tris-HCl (pH 6.8)	0.625 M
Bromophenol blue	0.01% (w/v)

#### **A.6.2. 10 x β-mercaptoethanol, bromophenol blue**

β-mercaptoethanol	50% (v/v)
-------------------	-----------

bromophenol blue            0.1% (w/v)

### **A.6.3. Acrylamide**

acrylamide                    30%

bis-acrylamide                0.8%

Keep in dark bottle and at 4 ° C for no longer than a month.

### **A.6.4. APS**

Ammonium persulfate        10%

Make up fresh daily in distilled water.

### **A.6.5. TEMED**

Use undiluted.

### **A.6.6. Lower gel buffer**

Tris                              1.5 M

SDS                                0.4%

Make up to 1 l with dH<sub>2</sub>O and adjust to pH 8.8 with concentrated HCl.

### **A.6.7. Upper gel buffer**

Tris                                0.5 M

SDS                                0.4%

Make up to 1 l with dH<sub>2</sub>O and adjust to pH 6.8 with concentrated HCl.

### **A.6.8. SDS running buffer (10 x stock solution)**

Tris-HCl                         30.3 g

Glycine                          144.1 g

SDS                                10 g

Make up to 1 l with dH<sub>2</sub>O.

### **A.6.9. Staining solution**

PhastGel<sup>®</sup> Blue R, as per manufacturers' instructions.

#### **A.6.10. Destaining solution**

Acetic acid                      10%

Methanol                         30%

Make up to 1 l with dH<sub>2</sub>O.

#### **A.6.11. Fixing solution**

Methanol                         20%

Mlycerol                         6%

Make up to 1 l with dH<sub>2</sub>O.

#### **A.6.12. PBS**

NaCl                                8 g

KCl                                 0.2 g

Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O                2.29 g

Make up to 1 l with dH<sub>2</sub>O, adjust to pH 7.2 and autoclave.

### **A.7. BUFFERS AND REAGENTS FOR RNA HYBRIDISATION SUBTRACTIVE LIBRARY**

#### **A.7.1. 5 x Hybridisation buffer**

NaCl                                2.5 M

HEPES buffer                    125 mM

EDTA                                25 mM

SDS                                 5%

DEPC treat over night and autoclave.

#### **A.7.2. Cross-linking buffer**

TrisHCl, pH 7.0                 25 mM

EDTA                                1 mM

DMSO                                5%

Ascorbic acid                    2 mM

### **A.7.3. Hybridization solution for probing**

KPO <sub>4</sub> , pH 7.4	1 M (1 ml)
20 x SSC	
50 x Denhart's solution	4 ml
denatured salmon sperm DNA	10 mg/ml

## **A.8. BUFFERS AND REAGENTS FOR DNA AGAROSE GEL ELECTROPHORESIS**

### **A.8.1. Tris-acetate EDTA buffer**

Tris-acetate	40mM
EDTA	1mM

## **A.9. BUFFERS AND REAGENTS FOR RNA AGAROSE GEL ELECTROPHORESIS**

The buffers and reagents used in this technique all contained DEPC (200 µl/100 ml) and were sterilised by autoclave. When preparing these reagents gloves were worn at all times.

### **A.9.1. 5 x gel running buffer**

MOPS	41.9 g
Sodium acetate	6.8 g
EDTA	1.9g (5mM)
Make up to 1 l with DEPC-treated H <sub>2</sub> O.	

### **A.9.2. RNA agarose gel**

GTG agarose	1.2%
5 x running buffer	8.36 ml
DEPC-treated H <sub>2</sub> O	38.5 ml
Dissolve agarose by boiling. Allow to cool to approximately 55 ° C.	
Formaldehyde	2.55 ml

### **A.9.3. Sample loading buffer**

EDTA (pH 8.0) 0.5 M (20 ml)

Bromophenol blue 0.025 g

Glycerol 5 ml

Make up to 10 ml with DEPC-treated water

### **A.10. SUPPLIERS**

Amersham Little Chalfont. Buckinghamshire, England.

BDH Poole, BH15 ITD, England.

Boehringer Mannheim GmbH Biochemica, PO Box 310, D-6800,  
Mannheim31, Germany.

Merk Frankfurter Strasse 250, D-6100,  
Darmstadt, Germany.

Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

Promega Corp Madison, WI, 53711-5399, USA.

Sigma PO Box 14508, St Louis MO 63178, USA.

United States Biochemicals PO Box 22400, Cleveland, Ohio,  
44122, USA

# **APPENDIX B**

## **B.1. PROTEIN ASSAY**

The Bio-Rad *DC* Protein Assay is a colourimetric assay for protein concentration following detergent solubilisation. The reaction is similar to the assay described by Lowry *et al.* (1951) but with the following improvements: the reaction reaches 90% of its maximum colour development within 15 minutes, and the colour changes not more than 5% in 1 hour.

The assay is based on the reaction of protein with alkaline copper tartrate solution and Folin reagent. There are two steps which lead to colour development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Colour development is primarily due to the amino acid tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

From the DC Protein Assay kit (Bio-Rad), solution A<sup>1</sup> was prepared by adding 20  $\mu$ l of solution S to every 1 ml of solution A. The Bio-Rad bovine serum albumin protein standard at a concentration of 1.5 mg/ml was diluted to concentrations of 0.75 mg/ml, 0.325 mg/ml and 0.1875 mg/ml in the same buffer as the protein sample whose concentration was to be determined. For each assay, 125  $\mu$ l of solution A<sup>1</sup> was mixed with 25  $\mu$ l of each protein sample and 1 ml of solution B was added. The samples were left at room temperature for approximately 15 minutes. The optical density, OD<sub>750</sub>, of each sample was read using the dilution buffer as a blank. The OD<sub>750</sub> of the Bio-Rad protein standards were plotted against concentration to give a linear graph and the concentration of protein in the samples was read from the graph using their

OD<sub>750</sub> values.

## **B.2. ZIEHL-NEELSEN STAIN**

A loop-full of mycobacterial cells was heat-fixed on a glass slide and thereafter flooded with carbon fuchsin (1g basic fuchsin, 10 ml absolute alcohol, 5% phenol in 100 ml distilled water). The smear was heated at intervals for 5 minutes and then allowed to cool. The carbon fuchsin was washed off with tap water and the slide flooded with 3% acid alcohol (3% hydrochloric alcohol, 95% absolute alcohol). The smear was rinsed with tap water and counterstained with methylene blue (0.5g methylene blue in 100 ml distilled water). After 30 seconds the dye was rinsed off with tap water and the slide allowed to air-dry and then examined under an oil immersion objective (x 100 magnification). Mycobacteria would normally appear as red stained bacilli, however, *M. smegmatis* LR222, which does not possess a waxy cell wall stained blue/pink.

## **B.3. DEVELOPMENT OF AUTORADIOGRAPH**

The X-ray Film was submerged in the developer solution (Polycon A, Champion Photochemistry, South Africa) for 5 minutes. After rinsing with water, or draining the excess developer solution, the reaction was stopped by submerging the autoradiograph in the stop solution (20% acetic acid) for 2 minutes. The autoradiograph was drained and submerged in the fixing solution (Super Amfix, Champion Photochemistry, South Africa) for 5-10 minutes. It was then rinsed thoroughly and air dried.

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