Characterisation of the cold-shock response in *Mycobacterium smegmatis.*

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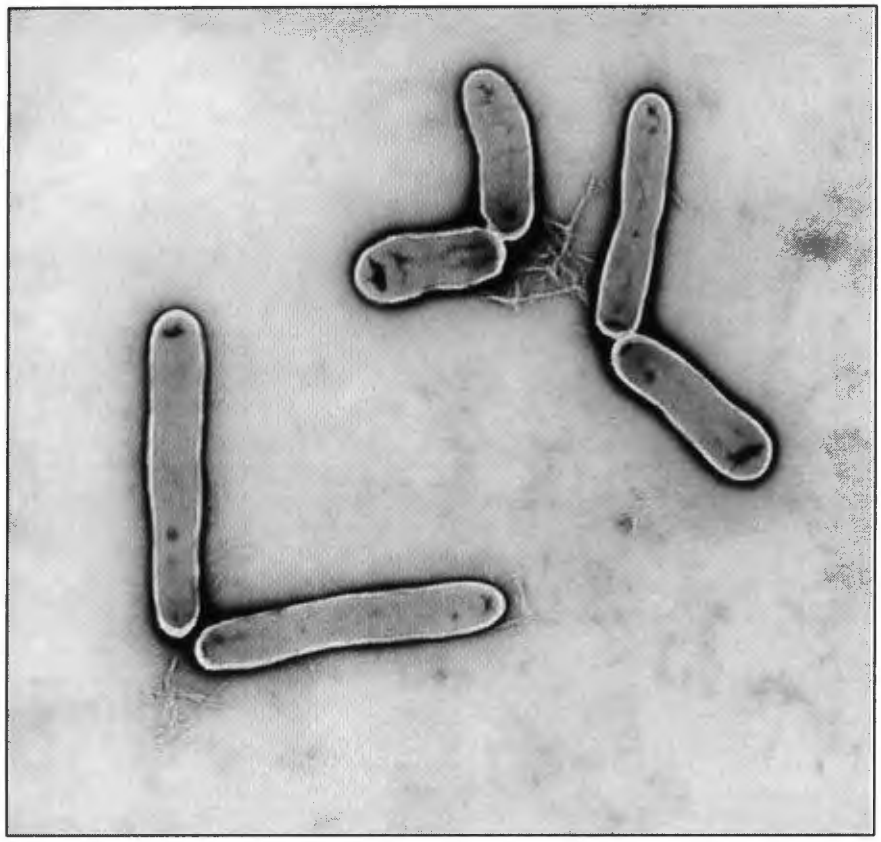

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BRIEF CONTENTS

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ABBREVIATIONS

aa	: Amino acids
ADC	: Albumin dextrose catalase
ATP	: Adenosine triphosphate
BCG	: Bacille Calmette-Guérin
bp	: Base pairs
BSA	: Bovine serum albumin
C-	: Carboxy-
^{14}C	: Carbon – 14
°C	: Degrees centigrade
CAP	: Cold-acclimation protein
CFU	: Colony-forming units
Ci	: Curie
CIP	: Cold-inducible protein
cpm	: Counts per minute
CSP	: Cold-shock protein
dATP	: Deoxyadenosine 5'-triphosphate
DB	: Down-stream box
dCTP	: Deoxycytidine 5'-triphosphate
DEPC	: Diethyl pyrocarbonate
dGTP	: Deoxyguanosine triphosphate
DMSO	: Dimethyl sulphoxide
DNA	: Deoxyribonucleic acid
DNase	: Deoxyribonuclease
DTT	: Dithiothreitol
dTTP	: Deoxythymidine triphosphate
EDTA	: Ethylenediaminetetra-acetic acid (disodium salt)
g	: Gram
GRP	: Glycine-rich RNA-binding protein
^{3}H	: Tritium

HBHA : Heparin-binding hemagglutinin

HCl : Hydrochloric acid

HEPES : N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HIV : Human immunodeficiency virus

hr : Hour

IEF : Iso-electric focusing

IgG : Immunoglobulin G

INH : Isonizid

IPTG : Isopropyl -β -D-thiogalactopyranosid

kb : Kilobase

kDa : Kilodalton

LAM : Lipoarabinomannan

M : Molar

mA : Milliamperes

MALDI-TOF MS: Matrix-assisted laser desorption/ionisation time of flight mass spectrometry

MCS : Multiple cloning site

mg : Milligram

ml : Millilitre

mM : Millimolar

MOPS : Morpholinepropanesulfonic acid

mRNA : Messenger ribonucleic acid

N- : Amino-

NaAc : Sodium acetate

ng : Nanogram

NRP : Non-replicating persistent state

OD : Optical density

ORF : Open reading frame

Pab : Primary antibody

PAGE :Polyacrylamide gel electrophoresis

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

pg	: Picogram
pI	: Isoelectric point
POD	: Peroxidase
PVDF	: Polyvinylidene difluoride
PZA	: Pyrazinamide
RLU	: Relative light units
RNA	: Ribonucleic acid
RNase	: Ribonuclease
RNP	: RNA-binding domains
rpm	: Revolutions per minute
rRNA	: Ribosomal ribonucleic acid
SDS	: Sodium dodecyl sulphate
SDS-PAGE	: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	: Standard Error of the Mean
ssDNA	: Single-stranded deoxyribonucleic acid
t	: Time
TAE	: Tris-acetate EDTA
TB	: Tuberculosis
TBS	: Tris-buffered saline
TCA	: Tri-chloroacetic acid
TNF	: Tumor necrosis factor
Tris	: Tris(hydroxymethyl)aminomethane
µCi	: Microcurie
µg	: Microgram
µl	: Microlitre
µM	: Micromolar
UTR	: Untranslated region
UV	: Ultra-violet
V	: Volts
v	: Division rate

VBNC : Viable but non-culturable

v/v : Volume/volume

W : Watts

w/v : Weight/volume

X-gal : 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosid

YT : Yeast-tryptone

Amino acids:

Ala / A : alanine

Arg / R : arginine

Asn / N : asparagine

Asp / D : aspartic acid

Cys / C : cysteine

Gln / Q : glutamine

Glu / E : glutamic acid

Gly / G : glycine

His / H : histidine

Ile / I : isoleucine

Leu / L : leucine

Lys / K : lysine

Met / M : methionine

Phe / F : phenylalanine

Pro / P : proline

Ser / S : serine

Thr / T : threonine

Trp / W : tryptophan

Tyr / Y : tyrosine

Val / V : valine

ABSTRACT

The response of *Mycobacterium smegmatis* to a cold shock was investigated in order to gain insight into the stress responses of members of the genus *Mycobacterium*. *Mycobacterium smegmatis* cultures were shocked from 37°C to 30°C, 25°C, 15°C, and 10°C and the effects on both growth (ATP concentration, culture turbidity, colony-forming units) and metabolism (incorporation of ^{14}C-leucine and ^{3}H-uracil) were investigated. The magnitude of the cold-shock response was found to be dependent upon the degree of the cold shock. A cold shock to 10°C had the greatest effect and resulted in a "lag period " of 24 hours in both the growth and metabolism of the culture. The synthesis of proteins was reduced 20-fold during this period, indicating at block in translation. The cold-shock response in *Mycobacterium smegmatis* was an adaptive response with growth eventually being resumed at the colder temperature, but at a reduced rate. Using the techniques of one-dimensional sodium-dodecyl-sulphate polyacrylamide gel electrophoresis and two-dimensional protein gel electrophoresis, ^{35}S-methiononine-labelled proteins that were synthesised during the cold shock were analysed. At least fourteen radio-labelled proteins were induced during the first 24 hour period and these demonstrated two distinct patterns of cold-shock induced expression: transient and continuous. Depending upon the pattern of expression and size, the cold-shock proteins were classified as "cold-induced proteins", "cold-shock proteins" or "cold-acclimation proteins". CipM, a 27kDa protein, was identified as the major cold-shock protein through one-dimensional protein electrophoresis. From N-terminal sequence data generated from a protein (CipM.1) within this band, a corresponding degenerate DNA probe was used to isolate *cipM.1*. This gene was cold-inducible, with mRNA levels transiently increasing 5-7 fold after a 37°C to 10°C cold-shock. Homologues of this cold-shock gene are found in the genomes of *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The corresponding mycobacterial proteins showed homology at the N-terminus to the HUβ subunit of HU of *Escherichia coli* and possessed similar C-terminal proline, lysine and alanine degenerate repeats to the mycobacterial heparin-binding hemagglutinin. The response of several mycobacterial cold-shock gene homologues to a cold shock was also investigated, by northern-hybridisation and S1 nuclease analysis. The *cspA* homologue of *Mycobacterium smegmatis* demonstrated a 16-24 fold transient induction in mRNA levels following a 37°C to 10°C temperature-shift, while *gyrA* mRNA levels were maintained at a constant level throughout the cold shock. Although some similarities were demonstrated between the cold-shock response of *Escherichia coli* and *Mycobacterium smegmatis*, definite differences occur in the proteins that are involved in the adaptive stages of the response.

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SECTION A:

UNDERSTANDING THE RELEVANCE OF THE MYCOBACTERIAL COLD-SHOCK RESPONSE

CHAPTER 1

Mycobacteria: Latency and stress

1.1: Latency and Dormancy.

1.1.1: Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), a debilitating disease that accounts for more than 3 million deaths annually (Parrish *et al.,* 1998). Although the disease was seemingly eradicated from the developed nations early in the 1950's, with the emergence of HIV and the increase in the number of immunocompromised patients in these areas, TB has again become a serious global issue.

M. tuberculosis is a member of the genus *Mycobacterium,* belonging to the *Actinomycete* branch of the Gram-positive bacteria. These bacteria are aerobic, non-motile, non-spore-forming, slightly curved or straight rods, that are characteristically acid-fast (Roberts *et al.,* 1991). *M. tuberculosis* is capable of causing both disease (pulmonary, pleural, genitourinary, skeletal, central nervous system, abdominal and pericardial TB) and asymptomatic latent infection. It is this latent infection (latency) that is one of the biggest problems in the treatment and eradication of TB, with over one third of the world's human population estimated to be "latently infected " with "dormant" tubercle organisms (Parrish *et al.*, 1998). Following infection, the host mounts an immune response, which if successful prevents disease and destroys most of the infecting organisms. However, even when disease is averted, a few organisms survive and persist in a "dormant state" that is not transmittable (latent infection). Patients with a latent infection have no clinical symptoms, but remain positive with the tuberculin skin test. The problem is that these dormant bacteria are not susceptible to the current drug regime and are capable of reactivation at any time, resulting in active TB. It has been postulated that the chances of developing TB from the reactivation of dormant organisms is 2-23% per lifetime. With the emergence of HIV, this risk of reactivation increases to 5-10% per year (reviewed in Parrish *et al.*, 1998).

1.1.2: Generation of dormant organisms

Where these dormant bacteria reside in the body or in what state they persist is poorly understood. The cell-mediated immune response to a mycobacterial infection is outlined below in Figure 1.1 and suggests at what stage the dormant bacteria are generated (Dannenberg and Rook, 1994; Chan and Kaufmann, 1994). Containing a mycobacterial infection is quite a complex task, due to the fact that these bacteria have developed several ways of avoiding the typical immune advances. Any disturbance or failure of this immune surveillance system results in the progression towards active TB disease.

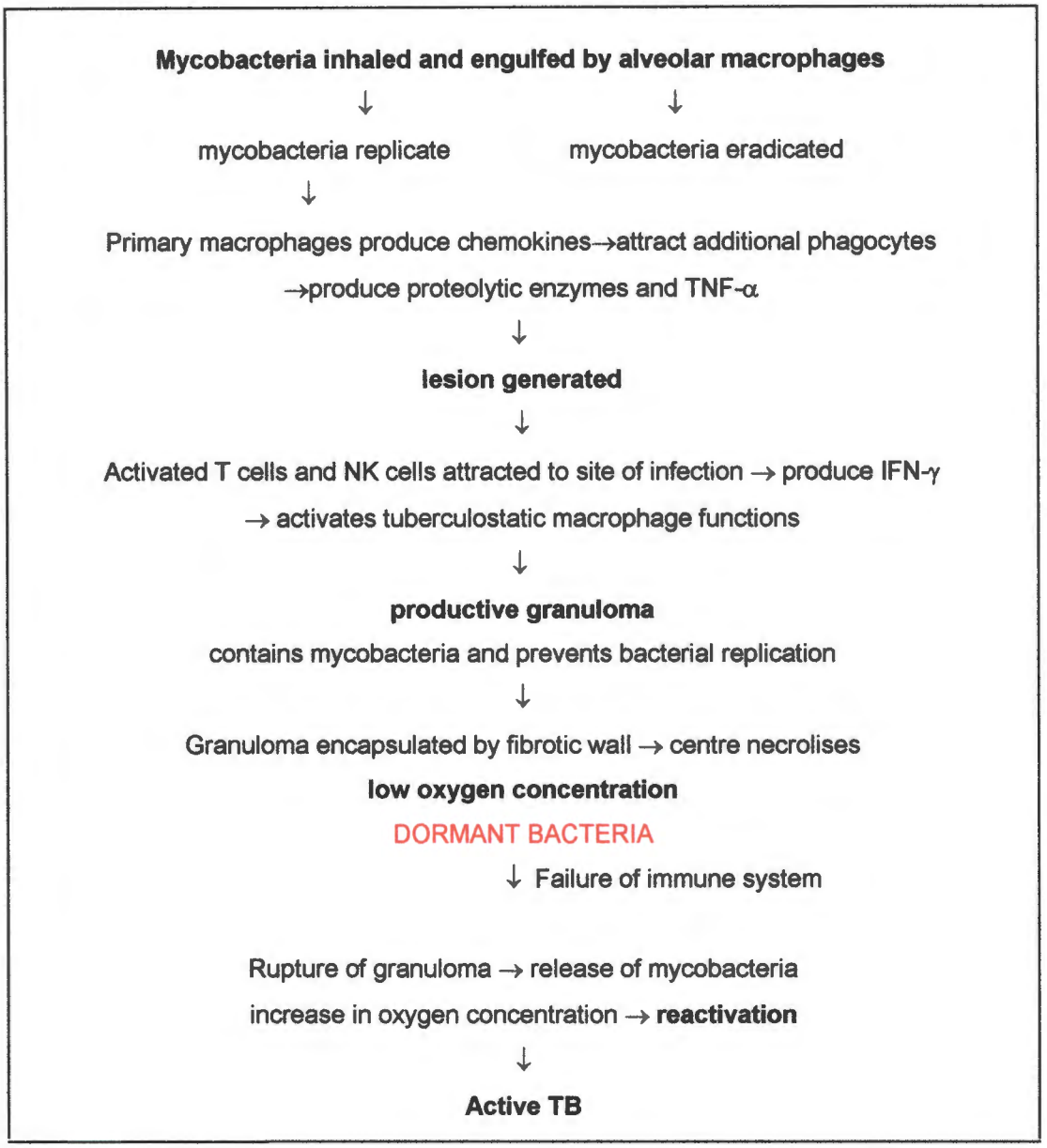


Figure 1.1: Cell-mediated protective immunity in response to a mycobacterial infection.

Diagrammatic representation of the human immune response to a *M. tuberculosis* infection, as described by Dannenberg and Rook, 1994; Chan and Kaufmann, 1994 and Rook and Bloom, 1994.

Within the macrophage, the bacteria are located in phagosomes. Not only are *M. tuberculosis* able to limit the degree of acidification within these phagosomes (reviewed in Clemens, 1996), but they also prevent the fusion between phagosome and lysosome, limiting exposure to proteolytic enzymes (reviewed by Clemens, 1996; Britton *et al.*, 1994). Once the macrophages have been activated and the granuloma formed, the bacteria are exposed to toxic oxygen free radicals and bactericidal nitric oxide. Although some bacteria are killed at this stage, most are just prevented from replicating. Again the mycobacterium has developed strategies for defeating the macrophage attack (Britton *et al.*, 1994). Cell wall glycolipids such as lipoarabinomannan (LAM) have been implicated in modulating the cytokine signals for macrophage activation as well as limiting the

action of free oxygen radicals (Britton *et al.*, 1994). Once the granuloma is encapsulated, the bacteria also have to contend with microaerophilic/anaerobic conditions, an acidic environment (following cell necrosis in the granuloma) and severe nutrient depletion, all of which are unfavourable for bacterial survival, yet some survive. It has been proposed that the remaining bacteria in the granulomas are the dormant bacteria that are capable of reactivation, when the immune system becomes impaired (Rook and Bloom, 1994). Reactivation has been associated with protein-calorie malnutrition, viral infections (including HIV) and treatment with glucocorticoids, all of which are linked with a reduction in T cell function (Britton *et al.*, 1994).

It is clear from the above description that the bacteria are exposed to severe stress inside the macrophage and granuloma, it has been proposed that the dormant-state represents a stress-induced protected state, possibly an extreme form of stationary phase (Young and Cole, 1993). Are these dormant organisms in a spore-like, metabolically inactive state, or are they in a state where growth rate and death rate are balanced, with the bacteria remaining metabolically active but not proliferating? Some evidence supports the first theory, with the presence of sporulation regulatory genes (*sigF*) in the *M. tuberculosis* genome and because these organisms are able to avoid immune detection for such long periods (Parrish *et al.* 1998). Structurally however, no evidence exists to support the idea of spore formation.

1.1.3: Models for latency/dormancy

It was more than 40 years ago that the existence of dormant bacilli ("persisters") was first demonstrated (McCune *et al.*, 1966a and 1966b). Using the "Cornell model", a mouse model of latency (Figure 1.2), McCune and co-workers (1966a and 1966b) elegantly showed the persistence of *M. tuberculosis* long after "effective" chemotherapy.

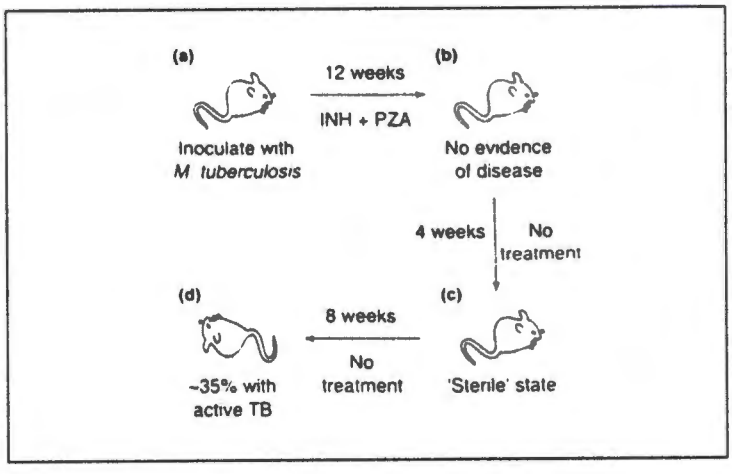


Figure 1.2: The Cornell mouse model of dormancy.

Schematic diagram of the Cornell model of dormant tuberculosis (Parrish *et al.*, 1998). **A)** Mice are inoculated with 10^5 colony-forming units of *M. tuberculosis* (H37Rv). **B)** After 12 weeks treatment with isoniazid (INH) and pyrazinamide (PZA), there is no evidence of disease. **C)** Four weeks after the withdrawal of the INH/PZA treatment, no evidence of culturable tubercle bacilli can be demonstrated (dormant organisms). **D)** Eight weeks later however, 35% of the mice develop culture-positive, active TB.

As mentioned earlier, it is possible that the dormant bacilli persist inside closed lesions (encapsulated granulomas) where they would experience oxygen deprivation. Using this theory, Wayne proposed another model to study dormant organism, an anaerobic model of persistence, where the bacteria are stressed by lowering the oxygen concentration (reviewed by Wayne, 1994a). As *M. tuberculosis* cells are exposed to gradual oxygen depletion, they pass through two "non-replicating persisting states" (NRP) (Wayne and Hayes, 1996). NRP1 occurs when the bacteria are exposed to an oxygen concentration of 1%. These bacteria are not actively dividing, but the cells are still metabolically active, with cell wall synthesis and RNA synthesis observed. NRP2 occurs when the atmosphere becomes truly anaerobic. These bacteria no longer replicate, yet they remain viable for several weeks (Wayne, 1976). If oxygen is reintroduced, the cells begin synchronised growth, suggesting that the organisms are not halted in a random state. At the initiation of replication in this oxygenated environment RNA synthesis is immediate, followed by the synchronised cell division. There is a definite lag period before DNA synthesis is seen. The hypothesis is that the persistent forms of the bacillus are actually diploid, having synthesised a full DNA complement during the settling process, but enzyme induction is required before cell division can occur (Wayne, 1977). Persistent organisms in this anaerobic model display a different drug resistant pattern to exponentially growing cells, indicating a change in their metabolism and replication status (Wayne and Hayes,. 1996). This alteration of metabolism is further indicated by the changes seen in the concentrations of various enzymes: isocitrate lyase, glycine dehydrogenase, catalase, superoxide dismutase, pyruvate dehydrogenase and malic dehydrogenase, when comparing "dormant" bacteria to those that are actively replicating (Wayne and Lin, 1982). Although research into the protein composition of these bacteria is limited, Wayne and Sramek (1979) found one antigenic protein (URB-1) in dormant bacilli that was not present in well-aerated replicating cultures. Although its function is unknown, it is possible that it this protein has a protective effect against anaerobic conditions. At present, there is no direct evidence that the non-replicating bacilli found in the microaerophilic sediments are the same as those responsible for latent TB infection.

1.1.4: Dormancy in other bacteria.

Several other bacteria can enter a protected state, that is perhaps similar to the mycobacterial "dormant state", when exposed to severe stress (especially a temperature downshift). This state is referred to as the "viable but non-culturable" (VBNC) state. The bacteria are unable to undergo sustainable cell division in or on normal culture media, but tests for viability show that the cells are still alive and even metabolically active (Oliver, 1993). Several factors have been shown to induce the VBNC state: temperature, salt levels, nutrient levels, light intensity and aeration. Figure 1.3 shows the response of *Vibrio vulnificus* to a temperature shift to 5°C, where it enters the VBNC state. There is a considerable reduction in the number of colony forming units on agar, while direct viable cell count methods show no significant reduction in the number of cells. *Escherichia coli* has been shown to enter this state following severe salt stress (0.8M NaCl); *Campylobacter jejuni* when incubated in saline at 20°C; and *Vibrio cholerae* when cold-shocked at 4-6°C. In fact, at least

twenty different bacteria enter this state when challenged with a particular stress (reviewed by Olivier, 1993). Is it possible that the VBNC state is similar to the "non-replicating persistence", described by Wayne (1976)?

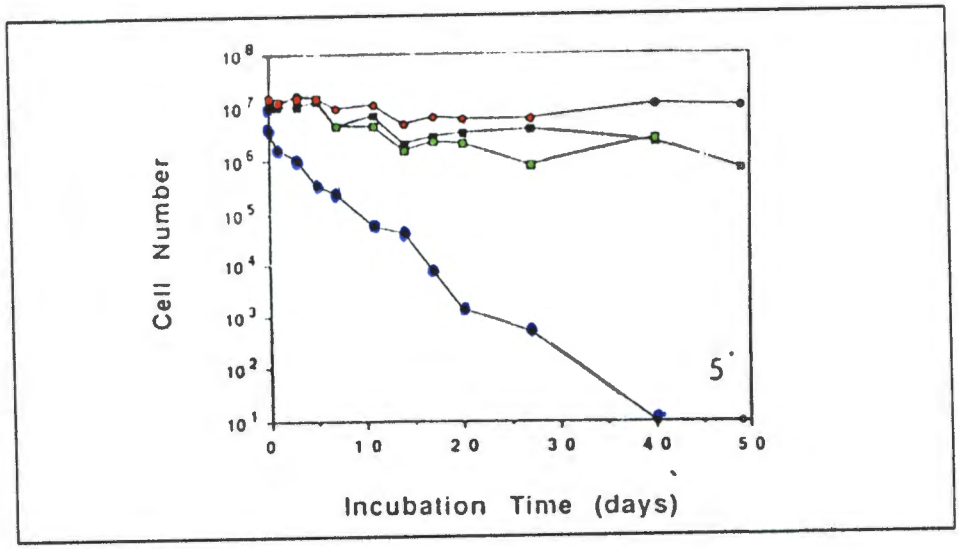


Figure 1.3: Entry of *V. Vulnificus* into the viable but non-culturable state.

Cells were incubated in an artificial seawater microcosm at 5°C. Cells were enumerated by acridine orange direct counts (●), direct viable counts by the method of Kogure *et al.* (1979) (■), direct viable counts by the INT method (p-iodononitrotetrazolium violet) (■) and plate counts on marine agar (●) (Olivier, 1993).

1.2: MYCOBACTERIAL STRESS RESPONSES.

1.2.1: Introduction

When *E. coli* cells enter stationary phase, they undergo a change in gene expression and are able to resist starvation and other environmental stresses (Kolter *et al.*, 1993; Siegele and Kolter, 1992). As mentioned earlier, it is possible that the dormant state of *M. tuberculosis* represents an extreme form of stationary phase, induced by the severe stress that the bacteria are exposed to when engulfed by the macrophage and encased within a granuloma (Young and Cole, 1993). Research into the genetic switches involved in inducing the stationary-phase and the effects of various stresses on mycobacteria has become more prevalent, in an attempt to understand the mycobacterial response to stress and possibly find a stress that mimics the switch into dormancy. Some of the key findings of this research are briefly discussed below.

1.2.2: Mycobacterial stationary phase.

Alternative sigma factors such as RpoS have been implicated in the stationary phase survival of both *E. coli* and *Salmonella* (Fang *et al.*, 1992, Siegele and Kolter, 1992). The role of alternative sigma factors of mycobacteria in stationary-phase survival and stress tolerance was investigated

by DeMaio *et al.* (1996). They were able to show the presence of a *sigF* (an alternative sigma factor) homologue in *M. tuberculosis*. The protein product of this gene shows significant sequence similarity to the SigF sigma factor, which is involved in sporulation in both *Bacillus subtilis* and *Streptomyces coelicor*. This *sigF* homologue was detected in all slow-growing pathogenic mycobacteria tested (*M. tuberculosis*, *Mycobacterium bovis BCG*, *Mycobacterium avium* and *Mycobacterium leprae*), but not in the fast-growing variants (*Mycobacterium smegmatis* and *Mycobacterium absecessus*). Maximal *sigF* expression in *M. bovis BCG* was seen following a cold shock (24 hours at 4°C), nitrogen depletion and upon entry into stationary phase. Transcription was also weakly stimulated during oxidative stress and alcohol shock, while not at all induced during exponential growth. DeMaio and co-workers (1996) concluded that mycobacterial SigF was a stationary phase/ stress response sigma-factor. They suggested that this sigma factor may be important in the survival of the bacterium outside the host. Understanding how the bacteria are able to survive outside the host is of great importance in controlling the spread of the disease.

Another gene that has been linked to stationary-phase persistence, is the *acr* gene. This gene encodes an α-crystallin protein homologue (16kDa) that is one of the major antigens recognised by the host immune system during a TB infection (Engers *et al.*, 1986). Not only is this protein the most prominent stationary-phase induced protein in *M. tuberculosis* (Yuan *et al.*, 1996), but it is also induced in response to a decrease in oxygen concentration (Yuan *et al.*, 1996) where it has a role in stabilising cell structures (Cunningham and Spreadbury, 1998). Acr has also been implicated in *M. tuberculosis* replication during infection of macrophages (Yuan *et al.*, 1998).

1.2.3: Mycobacterial heat-shock stress response.

M. tuberculosis has a well-defined heat-shock response, showing induction of at least twenty proteins and repression of a further eight, upon transfer of the culture from 37°C to 42°C (Lee and Howitz, 1995). The novel finding about several of these heat-shock proteins is that they are also major antigens (Young and Garbe, 1991), that are produced inside the macrophage and are then recognised by the host immune response (reviewed by Andersen and Brennan, 1994). These include the following proteins: 71kDa antigen, a homologue of DnaK of *E. coli* (heat-shock protein) (>50% sequence identity) (Young, 1988; Young and Mehlert, 1989); 65kDa antigen, a homologue of the GroEL heat-shock protein of *E. coli* (>50% sequence identity) (Young, 1988); 12kDa antigen, which is homologous to the *E. coli* GroES heat-shock protein. As mentioned earlier, α-crystallin (16kDa antigen) is also a major antigen. It shows limited homology to the small heat-shock family (Verbon *et al.*, 1992), but it is not heat-inducible (Yuan *et al.*, 1996). The 65kDa antigen is also heat-inducible in *M. smegmatis* (Shinnick *et al.*, 1988), along with the 71kDa antigen of *M. bovis BCG* (Young and Mehlert, 1989). Many heat-shock proteins act as chaperones, assisting in protein folding and removal of denatured proteins (reviewed in Buchner, 1996; Hendrick and Hartl, 1993). The mycobacterial heat-shock proteins, produced within the macrophage, may have a similar chaperone function to previously characterised heat-shock proteins, ensuring correct protein folding in the intracellular environment.

Lee and Horwitz (1995) identified a total of sixteen mycobacterial proteins that were induced inside the macrophage, which included the previously mentioned antigens. Eight of these proteins were also induced by either low pH, hydrogen peroxide or heat shock, confirming the importance of stress proteins within the macrophage environment. However, these authors clearly demonstrated that the situation within the macrophage is complex, with no single external stress producing the same protein profile as that produced by the intracellular mycobacteria.

Wu *et al.* (1997) identified a mycobacterial homologue to the SigE alternative sigma factor, a protein that has a role in the heat-shock stress response of *E. coli*. In *E. coli*, SigE is induced in response to the accumulation of abnormally folded outer membrane proteins and is involved in the transcriptional regulation of the periplasmic protease, DegP, and the heat-shock sigma factor Sig32 (Erickson and Gross, 1989). Wu *et al.* (1997) propose that the mycobacterial SigE may function in an analogous manner, after being induced by stress-induced changes in the number and conformation of membrane-bound proteins. By inducing the activity of DegP it may play a role in maintaining the appropriate levels of correctly–folded membrane proteins inside the macrophage.

1.2.4: Mycobacterial oxidative stress response

The best-studied mycobacterial stress response at the genetic level is the oxidative stress response. As already mentioned, mycobacteria within the macrophage are susceptible to a variety of host immune defence mechanisms, including reactive oxygen intermediates. The production of these oxygen radicals by cytokine-activated macrophages is limited to some extent by the action of mycobacterial cell wall glycolipids, such as LAM, which also play a role in scavenging these destructive molecules once they are synthesised (Britton *et al.*, 1994). However, for a pathogen to be successful, it needs to have mechanisms within the cell to prevent oxidative damage. In *E. coli* and *Salmonella typhimurium*, the central regulator of the peroxide stress response is OxyR (Christman *et al.,* 1985; Christman *et al.,* 1989). OxyR positively regulates at least nine genes including *kat*G (catalase-peroxidase), *ahpC* (alkyl hydroperoxide reductase), and *gorA* (glutathione oxireductase), all of which act together to neutralise the effects of the oxygen radicals (Toledano *et al.,* 1994; Christman *et al.*, 1985). The *oxyR* gene is present in most slow-growing mycobacteria, but there are mutations within the *oxyR* of *M. tuberculosis*, that render the gene inactive (Deretic *et al.*, 1995). It has been proposed that this lack of *oxyR* function is responsible for the high susceptibility of *M. tuberculosis* to isoniazid (INH), a "front-line" anti-tuberculosis drug (Deretic *et al.*, 1995; Zhang *et al.*, 1996), that in addition to affecting mycolic acid synthesis has also been shown to interact with KatG to produce reactive oxygen intermediates, which may have a general damaging effect on the bacteria (Shoeb *et al.*, 1985). With the emergence of multi-drug resistant strains of *M. tuberculosis*, research into this stress response is of vital importance and is currently the subject of a world-wide research effort.

1.2.5: Mycobacterial cold-shock stress response

Although the cold-shock response has not yet been defined at the protein level for any of the mycobacteria, some preliminary metabolic effects of a temperature-shift have been investigated. Takayama *et al.* (1978) demonstrated that a cold shock from 37°C to either 20°C or 16°C, resulted in the altered synthesis of fatty acids in exponentially growing cultures of *M. tuberculosis* H37Ra. Both the ratios of mycolic acids and unsaturated fatty acids decreased in relation to the total fatty acid synthesis, while the ratio of saturated fatty acids rose sharply. At the same time decreased cell viability was noted in the cultures incubated at both 20°C and 16°C. As discussed further in Chapter 2, one characteristic of cold-shocked bacteria is the increased synthesis of unsaturated fatty acids, which are important in maintaining membrane fluidity at the colder temperature and therefore ensuring bacterial viability (Marr and Ingraham, 1962). Takayama *et al.* (1978) proposed that the inhibition of mycolic acid and unsaturated fatty acid synthesis during growth at 20°C and 16°C may therefore explain the decrease in cell viability of *M. tuberculosis*.

Although no other information has yet been recorded about the effects of a cold shock on *M. tuberculosis*, this stress has already been used to generate "dormant" organisms. In pulmonary TB, rifampin is the most effective drug. It is thought that this may be due to rifampin having activity against the dormant bacilli in the lesions (Fox and Mitchison, 1975; Dickinson and Mitchison, 1976). This would imply that the dormant bacilli had periods of metabolic activity, that were long enough for the rifampin (which requires active cellular metabolism) to have an effect. Dickinson and Mitchison (1981) used a cold shock from 37°C to 8°C to generate "dormant" bacilli, with the cultures returned to 37°C for brief periods to induce active metabolism, in order to test the efficacy of rifampin under these conditions. Several interesting observations were made about the effects of the cold on the bacteria: 1) Contrary to previous findings (Takayama *et al.*, 1978), these authors showed that even in the cold, cell viability (according to colony-forming units) was maintained over a 5-week period. 2) Neither INH nor rifampin had any bactericidal effects on cultures maintained at 8°C. However, when the cultures were transferred to 37°C for short periods (1-6hrs), rifampin became more bactericidal than INH. 3) When transferred from 8°C to 37°C, logarithmic growth was resumed immediately (no lag period). Why these authors used this model for testing drugs against "dormant" organisms is not clear. The metabolic and replication status of the mycobacteria following a cold shock has not yet been established (not necessarily a persistent state) and how the bacteria would be exposed to "bursts" of metabolically-favourable conditions, is unclear.

Transmission of *M. tuberculosis* is caused by the inhalation of "droplet nuclei". These are produced following evaporation of the droplets of respiratory secretions, containing viable bacteria, that are expelled from the infected patient during a cough. These nuclei are about 10μm in diameter and can contain between 3 and 10 tubercle bacilli. The nuclei are extremely stable and can remain in the air for extended periods, settling at a speed of 12mm/min (reviewed by Smith and Moss, 1994). More importantly the bacteria remain viable within these droplets (viability decreasing by less than one log after 9 hours (Loudon *et al.*, 1969)), capable of causing infection

upon inhalation. Is it possible that the bacteria within these nuclei are also in a "dormant state" (non-replicating persistence), caused by the sudden drop in temperature, change in oxygen pressure and severe limitation of nutrients? Studying the effects of a cold shock on mycobacterial species would help elucidate the survival mechanism involved in this important part of mycobacterial disease.

1.3: AIMS OF THIS STUDY.

Clearly, significantly more research needs to be performed to understand the mechanism and genetics of dormancy. A greater understanding of the mycobacterial response to stress will however go a long way to achieving this aim. By establishing how a bacterium responds to a stress, genes that are essential for cell viability and even basic cellular mechanisms are elucidated.

In the past, mycobacterial dormancy has been associated with pathogenic, slow-growing strains. This however slows research, due to the precautions that need to be taken and the length of time required for these bacteria to replicate. Recently however, oxygen-deprivation in *M. smegmatis* was shown to produce characteristics of dormant *M. tuberculosis* (Hutter and Dick, 1998; Dick *et al.,* 1998), as defined by the anaerobic model (Wayne, 1977). *M. smegmatis* is a fast-growing, non-pathogenic variant, that is found in the environment (Wayne, 1984). No special containment facilities are required and the bacterium has a growth rate that is considerably faster than *M. tuberculosis* (3 hours compared with 3 days), making it a more suitable bacterium for research into mycobacterial stress and dormancy.

The aim of this study was to study the effects of a specific stress, cold shock, on *M. smegmatis*. Cold-shock stress has been associated with dormancy (VBNC) in other bacteria and has even been used as a model for generating dormant *M. tuberculosis*. Therefore, as well as providing valuable information about how mycobacteria respond to stress, it may also provide insight into the dormancy issue. Chapter 2 details what is currently known about the bacterial cold-shock stress response and the approach that was used for the evaluation of the effects of a temperature reduction on *M. smegmatis*.

CHAPTER 2

The bacterial cold-shock response

2.1: Introduction.

The effects of temperature on microbial growth can be expressed by an Arrhenius plot (Arrhenius, 1889). For most bacteria, linear growth occurs over a wide temperature range, spanning at least 30°C. Within this temperature range, the growth rate changes quite dramatically, but the protein composition of the cell remains fairly constant. Above or below this optimal temperature range, the response is no longer linear and cellular growth eventually ceases. Rapid temperature changes to the upper or lower limits of the growth range have pronounced effects on the physiology of the cell and specific stress proteins are induced, to facilitate the maintenance of basic functions within the stressed cell (Berger *et al.*, 1996).

The cold-shock response is an adaptive stress response, which occurs when the temperature is reduced to the lower temperature limits of cellular growth. It is characterised by the induction of a specific subset of proteins, cold-shock proteins, which enable the cells to adapt to the temperature down-shift. The best-studied example of this stress response is that of *E. coli*. This review details the cold-shock response for this bacterium and highlights the effects of a cold shock on other organisms.

2.2: Defining the cold-shock response.

In *E. coli*, the cold-shock response is characterised by an initial lag period in cellular activity (cell growth and metabolism), that follows a temperature downshift of at least 13°C. It is during this lag period that the synthesis of cold-shock proteins is induced (Jones *et al.*, 1987). The length of the lag period and the level of induction of the stress proteins is related to the degree of the temperature shift, with a change from 37°C to 10°C being associated with the longest lag period and highest level of protein induction (Jones *et al.*, 1992a). When bacterial cells are transferred from 37°C to 10°C, this lag period is 4-6 hours (NG *et al.*, 1962; Shaw and Ingraham, 1967). During this time, most protein synthesis is repressed, with only 30-40 proteins being synthesised. Twenty of these proteins show an induction in their rates of synthesis during this period and are referred to as the cold-shock proteins (Table 1) (Jones *et al.*, 1987; Jones and Inouye, 1994; Panoff *et al.*, 1998). Clear repression of heat-shock proteins (GroEL and DnaK) and continued synthesis of many of the proteins involved in transcription and translation, which are normally repressed during a period of low growth, also occurs during this lag period (Jones *et al.*, 1992a). Following the induced expression of the cold-shock proteins, cellular activity (cell growth, cell

division and synthesis of macromolecules) is restored and growth continues, but at a reduced rate (NG *et al.*, 1962; Shaw and Ingraham, 1967).

TABLE 1: COLD-SHOCK PROTEINS OF *E. COLI* (Panoff *et al.*, 1998).

Protein name:	Protein function:	Cold-shock induction:
CspA[1]	Transcriptional activator / RNA chaperone	>200 fold
CspB[2]	RNA chaperone	NQ
CspG[2]	RNA chaperone	NQ
GyrA[3]	Subunit of DNA gyrase - DNA supercoiling	NQ
Trigger factor[4]	Molecular chaperone	2 fold
NusA[5]	Termination and anti-termination	5-10 fold
H-NS[6]	global transcriptional repressor/activator	3- 4 fold
RecA[5]	DNA recombination / SOS response	2- 5 fold
Polynucleotide Phosphorylase[5]	Degradation of mRNA	5-10 fold
INF2α[5]	Transcriptional initiation factor	2- 5 fold
INF2β[5]	Transcriptional initiation factor	2- 5 fold
Pyruvate Dehydrogenase[5]	Decarboxylation of pyruvate	2- 5 fold
RbfA[7]	Ribosomal protein/ initiation of translation	NQ
CsdA[8]	Ribosomal protein/ unwinds ds RNA	NQ
Dihydrolipamide Acetyltransferase[5]		2- 5 fold
Hsc66[9]	Molecular chaperone	10-11 fold (mRNA)
F84.0[5]	Unknown function	10-20 fold
G41.2[5]	Unknown function	5- 10 fold
G55.0[5]	Unknown function	2- 5 fold
G74.0[5]	Unknown function	5-10

References: 1= Goldstein *et al.*, 1990; 2 = Bae *et al.*, 1997; 3 = Jones *et al.*, 1992b; 4 = Kandror and Goldberg, 1997; 5= Jones *et al.*, 1987; 6 = La Teana *et al.*, 1991; 7 = Jones and Inouye, 1996; 8 = Jones *et al.*, 1996; 9 = Lelivelt and Kawula, 1995.

NQ = not quantified.

In the case of heat shock, the major problem for the cell is the accumulation of incorrectly folded proteins. Induced heat-shock proteins act as molecular chaperones, which ensure correct protein folding and peptidases, which remove denatured proteins (reviewed in Buchner, 1996; Hendrick and Hartl, 1993). During cold shock, the cell experiences other problems: 1) decrease in membrane fluidity, which damages membrane-associated functions such as active transport and protein secretion; 2) stabilisation of secondary structures in RNA and DNA, which alter the efficiency of transcription, translation and DNA replication. The first problem may be solved by increasing the synthesis of unsaturated fatty acids, which are then incorporated into the membrane phospholipids and increase membrane fluidity. A change in the lipid composition of the cell, in response to temperature changes, have been demonstrated in *E. coli*, with a temperature reduction resulting in the increase in the percentage of unsaturated fatty acids during the first hour of the lag period (Shaw and Ingraham, 1965; Marr and Ingraham, 1962). The cold-shock proteins resolve the second issue. As seen in Table 1, most of the proteins are involved in maintaining the

structure and function of DNA (RecA, GyrA, H-NS, CspA) or RNA (CsdA, PNP, INF2α, INF2β, RbfA, NusA, CspA). For example, one of the functions of CspA, which is the major cold-shock protein of *E. coli*, is that of a RNA chaperone, facilitating the translation of mRNA by preventing secondary structures in the transcript (Jones and Inouye, 1994; Jones *et al.*, 1987; Yamanaka *et al.*, 1998)

2.3: Growth and metabolism during the cold-shock response.

NG *et al.* (1962) performed the initial growth analysis of the cold-shock response in *E. coli.* They characterised the response produced by the bacteria, when transferred from 37°C to 10°C, by measuring cell turbidity and the number of viable cells (colony-forming units). Following the cold shock, a 4-5 hour lag period was noted before the bacterial cell mass increased (culture turbidity), during which time the viable cell count also remained constant, indicating that no significant cell death occurred during this period. Increases in cell turbidity were noted after the 4-5 hour period, but 11 hours passed before the viable cell count increased (synchronous division occurred). Subsequent research by Shaw and Ingraham (1967) showed that this observed lag period in cell growth also extended to the synthesis of all macromolecules - DNA, RNA and protein. Figure 2.1 shows the results of their experiments. The cellular RNA and DNA content (Figure 2.1.A), as well as total protein (Figure2.1.B) were measured and revealed that the levels of these macromolecules remained fairly constant for a 4 - 7 hour period. These graphs illustrate that the lag in macromolecule synthesis corresponded with that seen in the dry weight and optical density measurements. The actual synthesis of proteins and RNA was measured by the incorporation of radiolabeled nucleotides (^{3}H-leucine and ^{14}C-uracil) into acid-precipitable material (Figure 2.1.C). Again the results showed little or no increased incorporation of radioactivity for the first 4 hours. Protein synthesis resumed after the 4-hour lag period, followed by the synthesis of RNA at 6 hours post temperature-shift. The simultaneous halting of the synthesis of all the macromolecules (in medium supporting growth) is unique to the cold-shock response, with most other stresses, such as starvation, resulting in the immediate stoppage of RNA and protein synthesis (amino acid starvation (Pardee and Prestidge, 1956) or DNA synthesis only (starvation of thymine) (Cohen and Barner, 1954).

As determined by cell mass measurements (dry weight), the normal exponential growth rate for *E. coli* at 37°C is 0.904hr^{-1} (specific growth rate constant, k). Following the 4-5 hour lag period observed in cell mass after a shift from 37°C to 10°C, there is a period of relatively rapid growth (k = 0.07hr^{-1}), which then slows to the steady-state growth rate at 10°C of 0.04hr^{-1} (NG et al., 1962; Shaw and Ingraham, 1967). Growth is therefore resumed once the cells have adapted to the temperature shift, but at a reduced rate (20-fold reduction).

Shaw and Ingraham (1967) suggested several reasons for the existence of the lag period: 1) sudden loss of permeability; 2) the need to destroy or dilute a toxic product produced; 3) the need

to synthesise a specific molecule for growth at 10°C. Experiments testing the permeability of the bacteria during a cold shock (using alpha-methyl-D-glucoside) showed that there was indeed an initial loss of permeability but this was restored within an hour, which was not long enough to explain the lag period. The respiratory activity of the bacteria was also reduced (1/4 respiration rate at 37°C), but remained constant throughout the cold shock (Shaw and Ingraham, 1967). At that time, the investigators could not distinguish between the other two possibilities, but from further work that has been performed in this regard, it is likely that the lag period is caused by the need to synthesise specific proteins (cold-shock proteins) (Jones and Inouye, 1996).

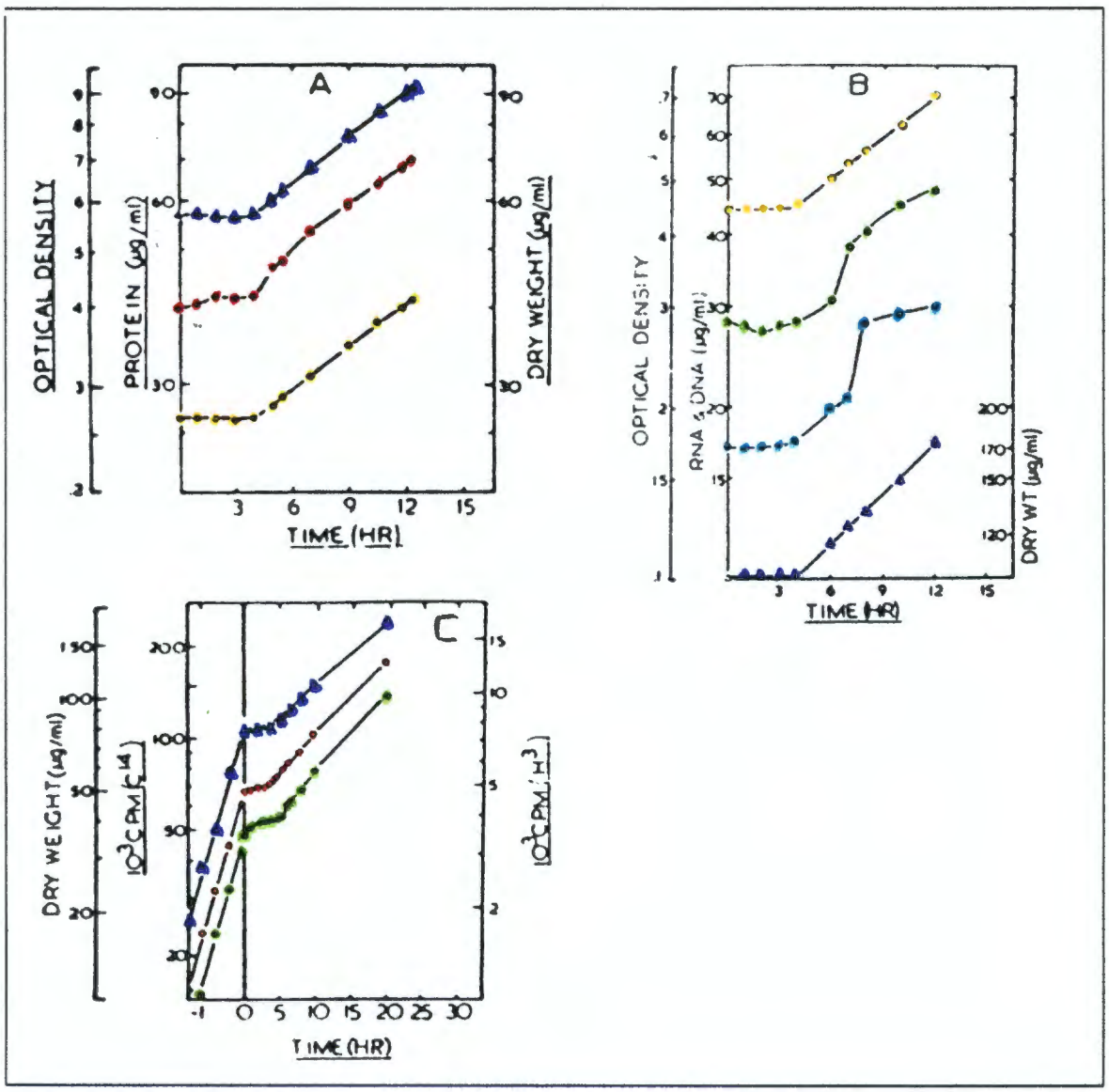


Figure 2.1: Growth and metabolic analysis of the cold-shock response.

Effect of a shift in temperature from 37°C to 10°C in E. coli ML30 on: (A) the synthesis of protein (●) and increase in optical density at 420nm (●) and dry weight (▲); (B) the synthesis of RNA (●) and DNA (●) in relation to the increase in optical density (●) and dry weight (▲); (C) on the incorporation of uracil-2-^{14}C (●) and L-leucine-4,5-^{3}H (●), compared to the increase in dry weight (▲) (Shaw and Ingraham, 1967).

2.4: Cellular sensors of cold shock.

2.4.1: Ribosomes as temperature sensors.

The mechanism by which the cold-shock response is activated is not completely resolved and may be a combination of several sensor molecules. Both heat-shock and cold-shock proteins are produced in response to a change in the translational capacity of the cell. In an attempt to investigate whether the ribosome, as part of the translational apparatus, was one of the sensors of temperature changes, VanBogelen and Neidhart (1990) tested a variety of antibiotics that target the ribosomes. Their aim was to see if they could elicit either a heat-shock or cold-shock response, marked by the production of specific proteins, by artificially altering the state of the ribosome. They found that the antibiotics could be divided into two groups: group H antibiotics (puromycin, kanamycin, streptomycin), which caused the induction of heat-shock proteins; and group C antibiotics (chloramphenicol, tetracycline, erythromycin), which induced cold-shock proteins and repressed heat -shock proteins - the protein profile of the cold-shock response. They also found that by altering the concentration of the antibiotics, a mild or severe temperature change could be mimicked. These results indicated that the ribosomes were involved in the cascade of events needed to trigger these two stress responses, and may even be the actual sensors.

Jones and Inouye (1996) suggested that the cold-shock trigger is more specifically the accumulation of "cold sensitive non-translatable ribosomes", and they have proposed a model to explain this response - the Cold-Shock Ribosome Adaptation Model (Figure 2.2).

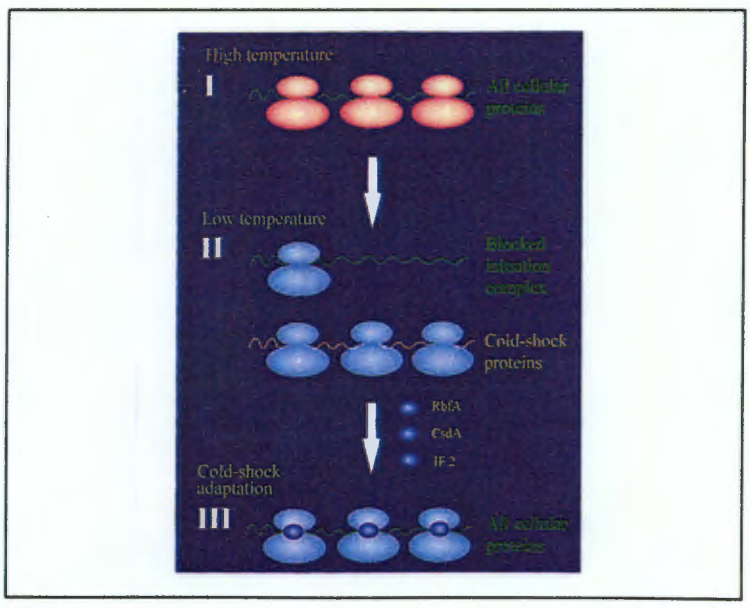


Figure 2.2: The Cold-Shock Ribosome Adaptation Model.

At high temperatures, the ribosome is translatable for cellular mRNAs (step I). A shift to a low temperature results in a cold-sensitive block in initiation of translation of most cellular mRNAs. However, the mRNAs for the cold-shock proteins can be efficiently initiated, resulting in the induction of cold-shock proteins (step II). Cold-shock proteins RbfA, CsdA and initiation factor 2 associate with the subunits and 70S monosomes, resulting in efficient translation of cellular mRNAs (step III) (Jones and Inouye, 1996).

Broeze *et al.* (1978) were the first to demonstrate that a cold shock (37°C→5°C) results in a block at the initiation of translation, that is characterised by the accumulation of 70S monosomes. This accumulation of 70S monosomes and 50S/30S subunits was also evident following a cold shock to 15°C or 10°C, indicating that a block at the initiation of translation is a general response to a cold shock (Jones and Inouye, 1996). Jones and Inouye (1996) refer to these ribosomal subunits as "cold-sensitive non-translatable ribosomes", and propose that their accumulation signals a change in the translational capacity of the cell (Figure 2.2). Cold-shock proteins are then produced in response to this signal, with their function being to convert the ribosomes to "cold-resistant translatable ribosomes" and thus increase the translational capacity of the cell. Some of these proteins associate directly with the ribosome such as RbfA, CsdA and initiation factors (INF2β and INFα). Others such as CspA have a more peripheral role. CspA can act as an RNA chaperone, preventing the formation of secondary structures in the newly transcribed mRNA, so that it is more readily translated. Once the translational capacity of the cell is restored (to some extent), cold-shock protein expression is again repressed.

The question that this model does not address is how the mRNAs for the cold-shock proteins are translated by the unadapted ribosomes. Mitta *et al.* (1997) have since located a "downstream box" (DB) in the coding region of several cold-shock genes (*cspA, cspB and csdA*), that is complimentary to the anti-DB of 16SrRNA. They propose that this sequence allows the mRNA molecules to form initiation complexes without the aid of the accessory proteins and thus become successfully translated (discussed further in section 2.5.1.B.iii).

2.4.2: (p)ppGpp as a modulator of the cold-shock response.

Another issue that deserves more attention is the mechanism by which the change in the translational capacity of the cell (accumulating ribosomal subunits) activates the induction of the cold-shock proteins. During stress such as amino acid starvation (stringent response) and carbon source deficiency, ribosomes are stalled by the decrease in the quantity of charged tRNA molecules, which causes a change in the translational capacity of the cell. In response to this, levels of guanosine 5' tri-phosphate-3' di-phosphate and guanosine 5' di-phosphate-3' di-phosphate ((p)ppGpp) are increased, which results in a decrease in the rate of synthesis of ribosomal proteins and RNA. The (p)ppGpp molecules can bind directly to the RNA polymerase (Reddy et al., 1995) and it has been proposed that this binding occurs early in the initiation of transcription, preventing the formation of an open-complex (Heinemann and Wagner, 1997). This binding also plays a role in "pausing" the RNA polymerase during elongation (Krohn and Wagner, 1996). In this way, components for the translation process are kept in a balanced state. Levels of (p)ppGpp are also found to vary upon temperature changes, with a cold shock resulting in a decreased level that is proportional to the magnitude of the temperature-shift (Mackow and Chang, 1983). Jones *et al.* (1992a) have shown that decreasing levels of (p)ppGpp play a positive role in activating the cold-shock response, and that increased levels actually inhibit the induction of several of the cold-shock proteins (NusA, PNP and RecA). As already mentioned, the translational

capacity of the cell is lowered during cold shock by the block in the initiation of translation, thus the proportion of charged tRNA molecules is higher than the functional translational apparatus. This causes a decrease in the levels of (p)ppGpp, which in turn activates the production of genes necessary to increase the quantity of translational machinery, thereby restoring the balance. The exact mechanism is not yet known. However, although decreasing levels of (p)ppGpp alone cannot account for the induction of the cold-shock response, they may modulate the activation of a cold-shock regulator (Jones *et al.*, 1992a).

2.5: Cold-shock proteins.

Cold-shock proteins, of *E.coli* and other bacteria, have recently been grouped into different categories based upon their size and the timing of their expression, leading to some complication in the cold-shock terminology. "CSPs" are cold-shock proteins less than 10kDa in size, that are induced during the initial shock phase of the response (lag period), while "CIPs" (cold-induced proteins) are cold-shock proteins greater than 10kDa in size (Graumann and Marahiel, 1996). The signature of a CSP/CIP is that its induction is transient, with levels declining once the cells have adapted. There is another group of proteins called "CAPs" (cold-acclimatisation proteins or cold-acclimation proteins), whose levels are induced during cold shock (not necessarily during the lag period), but they are continuously over-expressed while the bacteria remain at the lower temperature (Hebraud *et al.*, 1994; reviewed in Panoff *et al.*, 1998 and Graumann and Marahiel, 1996). At present CAPs have not been defined for *E. coli*, but have been demonstrated in *Arthrobacter globiformis* (Berger *et al.*, 1996) and *Enterococcus faecalis* (Panoff *et al.*, 1997).

So far, the key proteins in the *E. coli* cold-shock response are CspA, H-NS, GyrA, CsdA and RbfA, all of which are transiently induced. The function of these proteins and the manner by which they are regulated during the cold-shock response will be discussed in detail below. Two interesting molecular chaperones have also been identified as cold-shock proteins and these will also be discussed briefly.

2.5.1: Cold-shock protein A (CspA).

2.5.1.A: CspA protein function.

CspA is the major cold-shock protein of *E. coli.* It is transiently induced (200 fold) at cold-shock temperatures (Jones *et al.*, 1992a) and can constitute up to 13% of total cellular protein following a cold shock (250,000 molecules per cell) (Goldstein *et al.*, 1990). The protein itself is composed of 70 amino acids (Goldstein *et al.*, 1990), that are arranged in five anti-parallel strands, forming a β-barrel (NewKirk *et al.,* 1994; Schindelin *et al.,* 1994). CspA has the ability to bind to both RNA and single-stranded DNA (Newkirk *et al.*, 1994; Schindelin *et al.*, 1994; Jiang *et al.,* 1997). According to the three-dimensional structure of CspA, the residues involved in both RNA and DNA-binding are exposed on one face of the protein (lys^{10}, Trp^{11}, lys^{16}, Phe^{18}, His^{33}, Phe^{20}, Phe^{31}, Phe^{34}, Tyr^{42},

Lys^{60}) with eight of the nine aromatic amino acid residues of CspA forming an "aromatic ridge". It has been proposed that the binding of RNA, as well as single-stranded DNA is facilitated by the interaction between the aromatic rings of these amino acids with the aromatic bases of the nucleic acids (Newkirk *et al.*, 1994; Schindelin *et al.,* 1994). Several of these aromatic residues form part of two recognised RNA-binding motifs: RNP-1 (KGFGFI) and RNP-2 (VFVHF), which also contribute to the nucleic acid binding capability of CspA (reviewed in Yamanaka *et al.*, 1998).

CspA binds co-operatively, but weakly, to RNA in a non-sequence specific manner. Jiang *et al.* (1997) propose that CspA may act as an RNA chaperone, with its binding allowing the unfolding, or prevention, of secondary structures in the RNA (see reviews of Jones and Inouye, 1994; Yamanaka *et al.*, 1998). The binding is strong enough to prevent the formation of secondary structures in the newly synthesised transcript, yet the protein is easily removed as the ribosome moves along the mRNA transcript (Jiang *et al.*, 1997).

CspA is similar in structure and sequence (43% homology) to the DNA-binding domain (cold-shock domain) of the Y-box transcription factors (YB1 and FRGY2, specifically) (Wistow, 1990; Wolffe *et al.*, 1992). These proteins bind to the Y-box motif (CCAAT sequence element) located in some eukaryotic gene enhancers and promoters (Didier *et al.*, 1988). As mentioned above, CspA can bind to DNA and, like the Y-box proteins, binds to specific promotor sequences containing the Y-box motif. Thus far two cold-shock genes have been identified that possess the CCAAT motif and are positively regulated by CspA. These are *h-ns* (La Teana *et al.,* 1991; Brandi *et al.*, 1994) and *gyrA* (Jones *et al.*, 1992b), both of which are involved in the control of DNA supercoiling. Several other cold-shock genes also possess this motif, including *cspA* itself (Goldstein *et al.*, 1990), *recA* (Horii *et al.*, 1980), *nusA* (Granston *et al.*, 1990) and *pnp* (Regnier *et al.*, 1987). Qoronfleh *et al.* (1992) identified two other cold-inducible promoters that possess the CCAAT conserved motif. Further research should reveal if CspA is able to regulate the expression of these other cold-shock genes. CspA is actually regarded as the general activator or regulator of the cold shock response (La Teana *et al.,* 1991; Qoronfleh *et al.,* 1992). This is due to its rapid, transient expression, the fact that it is the major cold-shock protein and is produced prior to the increased synthesis of the other cold-shock proteins, and due to its ability to act as a positive transcriptional activator of at least two other cold-shock genes.

E. coli has a relatively large family of *csp* genes (*cspA* → *cspI*), identified through their homology to *cspA* (reviewed in Yamanaka *et al.*, 1998). Only three of these, *cspA* (Goldstein *et al.,* 1990), *cspB* (Lee *et al.,* 1994) and *cspG* (Nakashima *et al.,* 1996), have been shown to respond to cold shock. CspB and CspG are also putative RNA chaperones and are able to complement the functions of CspA, if it is absent (Bae *et al.*, 1997). Induction of *cspD* has been observed during stationary phase and is linked to nutrient deprivation (Yamanaka and Inouye, 1997).

Several bacteria other than *E. coli* possess CspA-like proteins. To date, at least fifty homologues have been identified (based on sequence homology and some functional studies) in a wide

variety of prokaryotes: thermophiles, mesophiles, pyschrophiles and pyschrotrophic bacteria (Yamanaka *et al.*, 1998). Some of the bacteria containing CspA homologues are noted here: *Streptomyces clavuligerus* (Av-Gay *et al.*, 1992), *S. typhimurium* (Craig *et al.*, 1998), *Salmonella enteritidis* (Jeffreys *et al.*, 1998), *Bacillus subtilis* (Willimsky *et al.*, 1992), *Bacillus cereus* (Mayr *et al.*, 1996), *Shigella flexneri* (Francis and Stewart, 1997) and *Klebsiella pneumoniae* (Francis and Stewart, 1997).

2.5.1.B: CspA protein expression and gene regulation.

As mentioned earlier, CspA levels are increased to the level of 13% of total cellular protein, and this is achieved within 60-90 minutes following a temperature reduction (Figure 2.3.A) (Goldstein *et al.*, 1990). The question posed is "at what level is this increase regulated?"

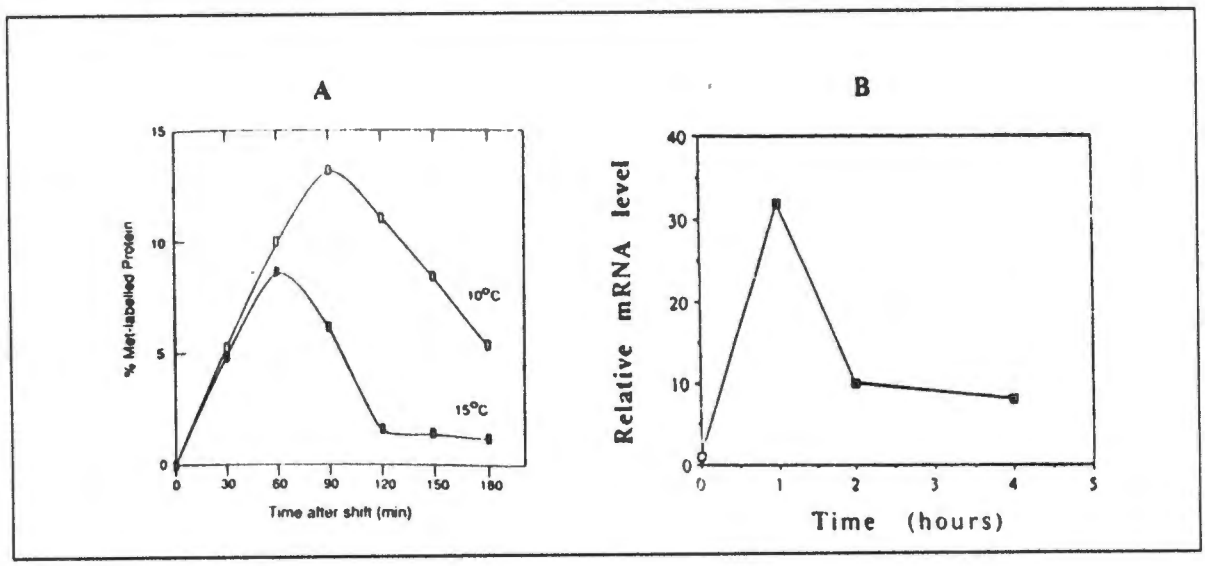


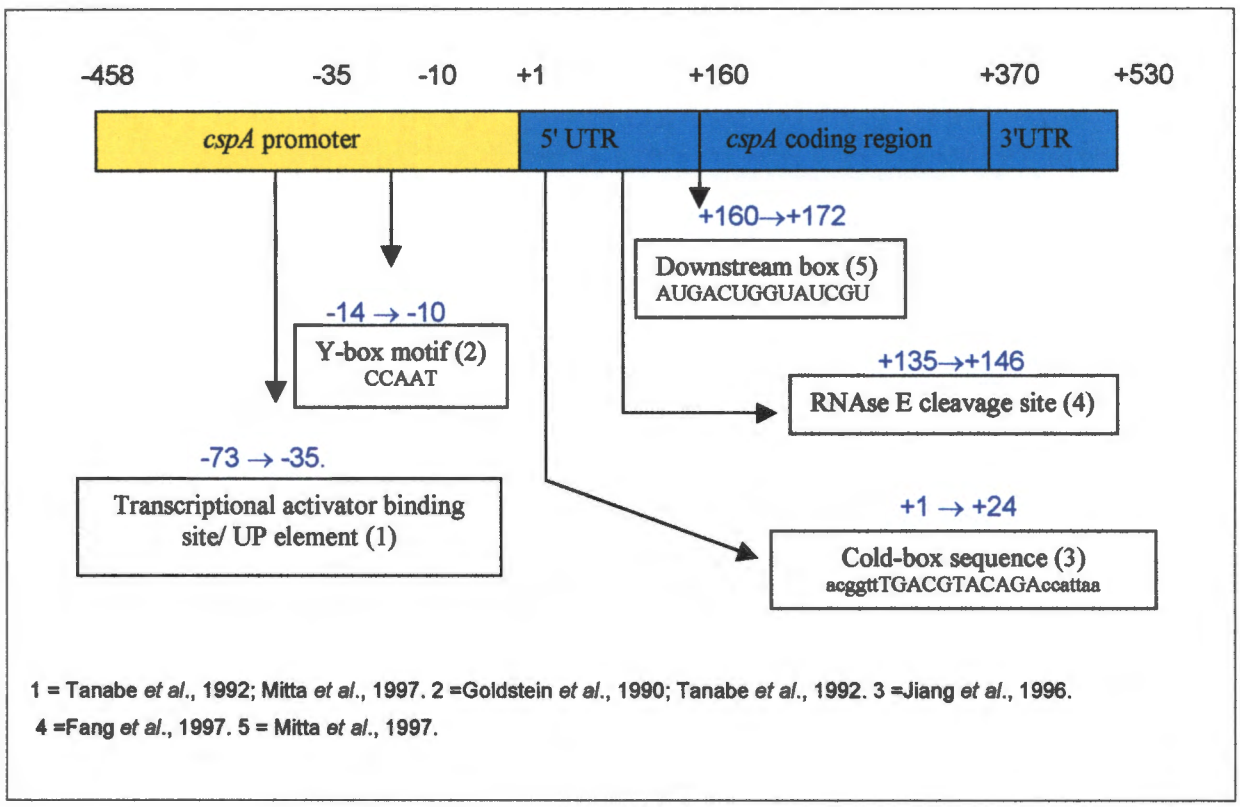
Figure 2.3: CspA expression - protein and mRNA induction.

A) Transient induction of CS7.4 (CspA) protein: Exponentially growing cultures of *E. coli* were transferred from 37°C to either 15°C or 10°C. Aliquots (1.1ml) were then pulse-labelled with 10μCi of L-[^{35}S]methionine for 30 minute periods and electrophoresised on a 17.5% SDS-polyacrylamide gel. The resulting autoradiograph was scanned and the percentage of methionine-labelled CS7.4 in the whole cell extract was calculated, after each 30-minute period (Goldstein *et al.*, 1990). B) Transient induction of *cspA* mRNA: Graphical presentation of the changes in the quantity of cspA mRNA, following the transfer of a culture of *E. coli* from 37°C to 15°C, as determined by primer extension analysis. Samples for RNA extraction were taken at 0hr, 1hr, 2hr, 3hr and 4hr after the temperature shift (Goldenberg *et al.*, 1996).

Several groups have demonstrated that following a cold shock there is a dramatic increase (30 fold) in the quantity of *cspA* mRNA, and that the pattern of mRNA expression mimics that of the CspA protein (Figure 2.3.B). This indicated that the control of CspA expression was at the transcriptional level (Tanabe *et al.*, 1992; Jiang *et al.*, 1993; Goldenberg *et al.*, 1996). Interestingly, the increase in levels of *cspA* mRNA could not be explained by increased promoter activity alone (only 3-4 fold increase), but rather by increased stability of the transcript at the lower temperature

(Goldenberg *et al.*, 1996). This increased stability was shown to be highly specific to the *cspA* mRNA and not a general cold-shock phenomenon. The *cspA* promoter is actually highly active at all temperatures, but at 37°C the half-life of the mRNA is only 10s, making it undetectable. At 15°C the transcript is stabilised 60-75 fold, resulting in an extended half-life of between 10 and15 minutes (Jiang *et al.*, 1993; Goldenberg *et al.*, 1996). This increased stability is however, only transient, with the transcript becoming unstable again when the cells have adapted, several hours after exposure to the cold (Goldenberg *et al.*, 1996). The dramatic changes in CspA protein levels are thus mainly controlled at the post-transcriptional level, by changes in transcript stability.

Much research has been performed to identify the elements of the promoter and transcript, that are required for this type of regulation. Figure 2.4 outlines the structure of the *cspA* gene (Goldstein *et al.*, 1990; Tanabe *et al.*, 1992) and areas that have been implicated in the regulation of gene expression.



1 = Tanabe *et al.*, 1992; Mitta *et al.*, 1997. 2 =Goldstein *et al.*, 1990; Tanabe *et al.*, 1992. 3 =Jiang *et al.*, 1996. 4 =Fang *et al.*, 1997. 5 = Mitta *et al.*, 1997.

Figure 2.4: Structure of the *cspA* gene of *E. coli*.

Diagrammatic representation of the *cspA* gene of *E. coli*, indicating areas of relevance to gene regulation as reported by the research groups 1-5 (discussed in following text). UTR = untranslated region.

2.5.1.B.i: Gene regulation through the control of cspA mRNA stability.

In *E. coli*, RNAse E (ribonuclease E) is a major controlling factor in the stability of mRNA. Recently an RNAse E consensus sequence was found in the 5'-untranslated region (UTR) of *cspA*. Alteration of the secondary structure at this site prevents cleavage and dramatically increases mRNA stability (Fang *et al.*, 1997). Secondary structures in both RNA and DNA are highly prevalent at low temperatures, thus the formation of a simple secondary structure may be the reason for the increased transcript stability at 10°C. A role for the 3' UTR in the control of mRNA stability has also been demonstrated (Goldenberg *et al.*, 1996, Brandi *et al.*, 1996), but how this is achieved still remains to be elucidated.

Another region of the 5' UTR, a putative repressor binding site (cold-box sequence), proved to be responsible for the destabilisation of the *cspA* mRNA during the adaptive period (Jiang *et al.*, 1996). Once levels of the cold-induced repressor are high enough, the repressor binds to the transcript, the mRNA is destabilised and transcription is negatively affected (Jiang *et al.*, 1996; Bae *et al.*, 1997). CspA, itself, has been implicated in modulating this repression activity. As mentioned earlier, CspA can bind to RNA molecules, and it is proposed that by binding to the *cspA* transcript it prevents the formation of secondary structures and allows RNase cleavage, thus destabilising the mRNA. How transcription would be affected in this model is unclear. Jiang *et al.* (1996) demonstrated that it was essential to repress *cspA* expression during the adaptive phase, as continual CspA synthesis prevented recovery of general protein synthesis and adaption of the bacteria to the cold. This cold-box sequence has been demonstrated in the 5'UTRs of several other cold-shock transcripts (*csdA* and *cspB*) and could represent a general mechanism for the transient nature of cold-shock gene expression.

2.5.1.B.ii: Transcriptional control of cspA gene expression.

A transcriptional activator-binding site was found in the promoter region of the *cspA* gene (Tanabe *et al.*, 1992) and was shown to be occupied by a cold-induced protein, during cold-shock conditions. This activator may be responsible for the slight increase in promoter activity seen by Goldenberg *et al.* (1996) at 15°C. Mitta *et al.* (1997) have since identified a UP element in this region. In conjunction with Goldenberg *et al.* (1997), they suggest that this AT rich section is important for the high activity of the *cspA* promoter seen at all temperatures.

The 5'UTR, that was previously discussed, is not only involved in controlling mRNA stability, but has also been implicated in down-regulating transcription at 37°C (region +27 → +143), possibly by attenuation (Mitta *et al.*, 1997; Goldenberg *et al.*, 1997).

2.5.1.B.iii: Translational control of cspA expression.

Brandi *et al.* (1996) demonstrated several other types of post-transcriptional regulation for CspA expression. They found that in addition to the increased stability of the transcript at the cold-shock

temperature, the protein itself, was 5-10 fold more stable at 10°C, and that the mRNA transcript was more efficiently translated at the lower temperature. CspA was found to be involved in this increased translational efficiency. Brandi *et al.* (1996) suggest that cold-shocked ribosomes preferentially translate *cspA* mRNA. In support of this theory, and as a possible explanation for why cold-shock genes are specifically induced during cold shock, a "downstream box" (DB) was discovered in the sequence of *cspA,* as well as several other cold-shock genes (Mitta *et al.*, 1997). As mentioned earlier, this sequence is complementary (10 out of 14 bases) to a region of 16SrRNA (anti-DB) and has been shown to be essential for mRNA translation during cold shock. Mitta *et al.* (1997) propose that this sequence, through the interaction with the 16srRNA molecule, allows for the formation of a stable initiation complex in the absence of the cold-shock ribosomal factors. Transcripts that do not possess this sequence can only be readily translated once the additional ribosomal proteins have been synthesised (RbfA and CsdA). The interaction between the DB and anti-DB sequences therefore allows the preferential expression of cold-shock proteins during the initial shock response. Because of this finding, Mitta *et al.* (1997) suggest that the most important control of the induction of CspA is at the translational level. They agree that elevated levels of transcript occur, but without the increased translational efficiency caused by the DB sequence, no increase in CspA protein is seen.

The induction of CspA is obviously complex, involving transcriptional, post-transcriptional and translational controls.

2.5.2: Gyrase A (GyrA).

In *E. coli*, the degree of DNA supercoiling is determined by the competing actions of DNA gyrase, which introduces negative supercoils and by DNA topoisomerase 1, which relaxes the DNA. The degree of DNA supercoiling has been shown to be temperature sensitive, reacting to both increases and decreases in temperature (reviewed by Tse-Dinh *et al.*, 1997). Mizushima *et al.* (1997) demonstrated that plasmid supercoiling in *E. coli* increases almost immediately following a cold shock (2 minutes) and then returns to its original state about 60 minutes later. This change could be attributed to activities of both DNA gyrase and HU, a major chromatin-associated protein. The increase in negative supercoiling, that occurred following a cold shock in *B. subtilis* DNA, was also linked to the activity of DNA gyrase (Grau *et al.*, 1994; Krispin and Allmansberger, 1995). The introduction of negative supercoils into the DNA chromosome provides free energy, making processes that require strand separation (initiation of transcription, replication) more favourable. This would definitely be advantageous in an environment where secondary structures are stabilised (cold temperatures).

Jones *et al.* (1992b) demonstrated that the A subunit of DNA gyrase (GyrA) was one of the cold-shock proteins of *E. coli*, induced during the adaptive phase of the cold-shock response. This induction was due to the interaction of CspA with the *gyrA* promoter, which contains three ATTGG (CCAAT) sequences that are the recognition sites for the binding of CspA. They also

demonstrated that GyrB is synthesised during the cold shock, at levels slightly higher than the other proteins, which suggests a concomitant increase in functional DNA gyrase enzyme during the cold shock. They proposed that the increase in DNA gyrase synthesis may be to compensate for the reduced enzyme activity experienced at the lower temperature, as it is vital to maintain the integrity of the DNA. It is thought that HU facilitates the action of DNA gyrase (Malik *et al.*, 1996), whether HU synthesis is also increased in the cold has not yet been demonstrated.

Another interesting function that has been ascribed to the action of DNA gyrase during cold shock (in *B. subtilis*) is the induction of the synthesis of unsaturated fatty acids (UFA) (Grau *et al.*, 1994). The genes responsible for UFA synthesis are only transcribed at temperatures lower than 20°C (Fulco, 1983), becoming activated by changes in supercoiling that are initiated by DNA gyrase (Grau *et al.*, 1994). As mentioned earlier, the retention of membrane fluidity is essential at low temperatures and this is achieved by increasing the proportion of UFAs in the membrane. DNA gyrase activity is therefore not only essential for DNA and RNA related functions, but for maintaining the integrity of the cell membrane as well.

2.5.3: Histone-like nucleoid structuring protein (H-NS).

2.5.3.A: H-NS function.

H-NS is a chromatin-associated protein that is found abundantly in enterobacteria (± 20 000 molecules per cell) (reviewed by Ussery *at al*, 1994 and Atlung and Ingmer, 1997). Like all DNA-binding proteins, this 15.4kDa (137aa) protein is rich in charged residues (Falconi *et al.*, 1988), although unlike other DNA-binding proteins, these residues are mainly acidic. The protein is composed of two domains, the N-terminus facilitating oligomerisation and the C-terminus containing the DNA-binding domain. This domain is unusual, as it does not contain a recognised DNA-binding motif. H-NS is able to bind to all forms of nucleic acid, but binds preferentially to double-stranded DNA (Falconi *et al.*, 1988). It is capable of binding non-specifically to DNA, as well as more specifically to AT rich regions that have a "curved or bent" conformation (Yamada *et al.*, 1990). Different protein complexes are formed when H-NS binds to DNA, with trimer and tetramer complexes involved in the specific binding to "curved" sequences, while non-specific binding involves H-NS dimers (Tippner and Wagner, 1995; Spurio *et al.*, 1997).

Genes with high homology to H-NS have been identified in several enterobacteria: *P. vulgaris, S. flexneri, E. coli* and *S. typhimurium* (Atlung and Ingmer, 1997), as well as in *Haemophilus influenza* (46% homology) *(Fleischmann* et al., 1995). Recently, a functionally homologous protein to H-NS was also identified in *Bordetella Pertussis* (Goyard and Bertin, 1997). However, using the techniques of the polymerase chain reaction (PCR) and Southern hybridisation, no homologue has yet been found in any Gram-positive organism. This could indicate that H-NS is only present in the enteric bacteria, or that it has diverged significantly in the other species. Functional assays, as performed by Goyard and Bertin (1997) could be used on DNA-binding proteins in these organisms to find functional counterparts to H-NS.

Depending on its mode of binding, H-NS has two possible roles in the cell. The first role is as a structural protein, which binds non-specifically to the DNA and condenses the chromosome. The second role, which involves the binding of H-NS at specific DNA sequences, is that of a transcriptional modulator which affects the expression of a large variety of genes (at least forty genes) (reviewed by Atlung and Ingmer, 1997). Many of these genes are affected by environmental signals such as temperature, osmolarity, anaerobiasis, pH or growth phase, all of which are known to alter DNA topology. The promoters of many of these genes have putative "curved sequences" and have been shown to be sensitive to DNA supercoiling (e.g: *proU* promoter (Higgins *et al.,* 1988)). Initially it was thought that due to the relatively non-specific binding capabilities of H-NS, genes were affected in a global manner. However, it has been demonstrated that H-NS acts by associating with a specific gene (Yoshida *et al.*, 1993), probably due to subtle changes in the topology of the DNA, such as increased curvature, that are induced by environmental signals. How this interaction affects transcription is not clear at this stage. H-NS binding sites in some genes (*proU, rrnB, virB*) overlap the promoter elements, thereby preventing the binding of RNA polymerase (Ueguchi and Mizuno, 1993). However, in other cases H-NS can effectively cause transcriptional repression from binding-sites as far away from the promoter as one kilobase (Fletcher and Csonka, 1995). In these instances it has been proposed that H-NS acts indirectly by "silencing" a region of the gene via changes in the DNA supercoiling (Goransson *et al.*, 1990). In this way the function of the transcriptional apparatus is affected. Many of the genes that are affected by H-NS are positively regulated by transcriptional activators such as Fis, VirB and Lrp (reviewed by Atlung and Ingmer, 1997). By inhibiting transcription of these activators, H-NS is capable of down-regulating many genes at once. Because of this indirect mode of action, H-NS is often referred to as a transcriptional-modulator rather than a repressor.

H-NS has been shown to be vitally important in the expression of virulence genes in several enteric bacteria: enteroinvasive *E. coli* (Colonna *et al.*, 1995), *S. typhimurium* (O'Bryne and Dorman, 1994, Harrison *et al.*, 1994) and *S. flexneri* (Porter and Dorman, 1994). Virulence genes of *S. flexneri* are regulated by both osmolarity and temperature, ensuring that virulence genes are only expressed inside the lower digestive tract of the host, which has a temperature of 30°C and high osmolarity conditions (Dorman *et al.*, 1990, reviewed by Hale, 1991). H-NS has been found to be the thermo-osmoregulator of these virulence genes, negatively affecting transcription when conditions are not ideal (Porter and Dorman, 1994, Hromockji *et al.*, 1992).

2.5.3.B: H-NS regulation and cold-shock expression.

The *hns* gene is autoregulated (Falconi *et al.*, 1993; Ueguchi *et al.*, 1993, Dersch *et al.*, 1993), with Fis (another nucleoid-associated protein) acting as the transcriptional activator (Falconi *et al.*, 1996). The Fis binding site overlaps the H-NS binding sites, in the *hns* promoter region, and achieves transcriptional activation by both stimulating expression and preventing the H-NS repression.

It is important to note that levels of H-NS remain fairly constant throughout normal growth. The levels of *hns* mRNA and DNA synthesis in the cell are linked. There is no expression of *hns* when DNA replication has halted. Thus the HNS:DNA ratio is kept constant (Free and Dorman, 1995). The only time that H-NS levels in *E. coli* vary considerably is following a cold shock, where levels increase 3-4 fold during the third hour of the lag phase (La Teana *et al.*, 1991). CspA was found to act as a transcriptional activator of the *hns* promoter under cold-shock conditions, binding to a 110bp element within the promoter (La Teana *et al.*, 1991, Brandi *et al.*, 1994). Gel-shift assays revealed that CspA not only binds to this region, but also stimulates the binding of RNA polymerase to the *hns* promotor. It was suggested that the interaction between RNA polymerase and CspA via protein-protein interactions permits the formation of an "open-complex, allowing CspA to act on the *hns* promotor (Brandi *et al.*, 1994). Interestingly, the research of Brandi *et al.* (1994) revealed that the conserved CCAAT element (Y-box motif) was not strictly needed for *hns* promotor activation. Although H-NS synthesis is not absolutely essential for bacterial survival in the cold (Dersch *et al.*, 1994), its absence severely impairs the ability of the bacteria to adapt to the cold.

As mentioned earlier, DNA supercoiling is affected by a temperature reduction. Whether increasing quantities of H-NS are produced to compact the chromosome during these conditions (structural role), or more specifically to control transcription of a number of genes (transcriptional modulator) is not yet clear.

2.5.4: D-E-A-D box protein (CsdA).

CsdA is a 70kDa (646 aa) protein that is induced during the cold-shock response of *E. coli*. It is the protein product of the *deaD* gene, which encodes a DEAD-box protein, a protein that contains the D E A D amino acid motif. Toone *et al.* (1991) originally isolated the *deaD* gene based on it's ability to suppress *rpsB* mutants. The *rpsB* gene encodes the ribosomal protein S2, which is involved in the initiation of translation (Laughrea and Moore, 1978). Upon sequence analysis of *deaD,* the predicted protein product was found to have significant homology to eIF4A (eukaryotic translation factor) and other members of the DEAD family of ATP-dependant RNA helicases, several of which are known to interact directly with ribonucleoproteins. Because of this similarity and it's functional ability to suppress a defect in one of the ribosomal proteins (S2), Toone *et al.* (1991) suggested that CsdA has some role to play in the function or assembly of the translational complex. Jones *et al.* (1996) have since confirmed that this protein, in its purified state, has the ability to unwind double-stranded RNA, but notably in the absence of ATP. Proteins that are able to unwind mRNA secondary structures are probably needed in the initiation complex and CsdA is a likely candidate.

After a temperature shift from 37°C to 15°C, CsdA was found exclusively in the ribosomal fraction of the cellular extract from *E.coli* and was clearly a ribosomal-associated protein, associated with

both the 30S and 50S subunits via ionic interactions (Jones *et al.*, 1996). It's function in the cold-shock adaptive response was investigated by completely deleting the gene from the chromosome and then analysing the protein content of the cell following a cold shock (37°C to 15°C). The expression of several proteins was affected, the most pronounced effect being the altered synthesis of heat-shock proteins. Normally, heat-shock protein synthesis is repressed during the initial cold shock and is then reactivated to allow renewed cellular growth at the lower temperature. In the absence of CsdA, reactivation of heat-shock protein synthesis is not seen and cellular growth is impaired (Jones *et al.*, 1996). From these observations it was suggested that CsdA is required to restore normal cellular functioning under cold-shock conditions by reactivating the expression of heat-shock proteins. This is probably achieved at the mRNA level, with CsdA destabilising cold-shock-induced secondary structures in the heat-shock protein transcripts, thereby allowing successful transcription by the ribosomes. This theory of CsdA activity agrees with one originally suggested by Toone *et al.* (1991). They proposed that CsdA acts as a translational accessory factor, specifically activating or enhancing the translation of specific genes through the denaturation of RNA secondary structures.

Both CsdA and RbfA (described below) are essential for ribosomal function during cold shock, in order to allow translation of non-cold-shock proteins. They are an important part of the "cold-shock adaption" model mentioned in section 2.4.A, where binding of these co-factors to the ribosome converts the ribosomes from "cold-sensitive" to "cold-resistant" ribosomes, capable of performing translation (Jones and Inouye, 1996).

2.5.5: Ribosome-associated binding factor (RbfA).

RbfA is involved in ribosomal maturation and/or the initiation of translation. It is a 15kDa protein that associates with the 30S ribosomal subunit at the 5'-terminal helix (Dammel and Noller, 1995). It was originally isolated as a multicopy suppressor of a cold-sensitive mutation located on the 5'helix of the 16SrRNA in *E.coli*, but has since been shown to be a cold-shock protein (Jones and Inouye, 1996). In fact its synthesis during cold shock is vital for the recovery of general protein synthesis and continued growth at low temperatures. When using a RbfA over-producing mutant, growth adaption to the lower temperature was much faster with a higher rate of protein synthesis. A *rbfA* deletion mutant constitutively expressed the cold-shock proteins and eventually ceased to grow (at 15°C) (Jones and Inouye, 1996). Following a cold shock, part of the physiological trigger for the cold-shock response is the block in initiation of translation (Broeze *et al.*, 1978; Jones and Inouye, 1996). It would therefore be imperative to induce the synthesis of a protein, like Rbfa, which is involved in restoring ribosome function to enable the cells to adapt.

2.5.6: Molecular chaperones.

Molecular chaperone functions are normally associated with heat-shock proteins, especially the Hsp70 family (reviewed by Hendrick and Hartl, 1993; Buchner, 1996). However, two cold-shock proteins have chaperone functions as well: Hsc66 and trigger factor.

Hsc66 is an interesting protein in that it demonstrates high similarity at the sequence level (62%) to the heat-shock protein DnaK (Hsp70 protein) and yet is not induced by heat shock, but rather by cold shock (Lelivelt and Kawula, 1995). Levels of *hsc66* (*hscA*) mRNA were shown to increase 11-fold after 3-4 hours at 10°C, with a definite increase seen at the protein level as well (exact protein levels were not determined). Judging by its homology to DnaK, it is assumed that Hsc66 has similar functions, acting as a molecular chaperone in the cold. Lelivelt and Kawula (1995) also demonstrated that the *hscB* gene, situated upstream of *hscA,* was also cold-inducible. This gene is a member of the *dnaJ* superfamily whose products act as co-factors of the Hsp70 family.

Trigger factor protein (48kDa) in *E. coli* was previously shown to function as a molecular chaperone. It associates with nascent polypeptides by binding to the ribosome and functions as a co-translational folding catalyst (Hesterkamp and Bukau, 1996). It has recently been demonstrated that the amino-terminus of the protein (118 amino acids) is responsible for binding to the ribosome (Hesterkamp *et al.*, 1997). Trigger factor also promotes protein degradation by enhancing the effects of GroEL (heat-shock protein) (Kandror *et al.*, 1997). These functions are normally associated with heat-shock proteins, but Kandror and Goldberg (1997) have shown that trigger factor is more important at low temperatures, enhancing cell viability in the cold. This is in contrast to heat-shock proteins, which lower cell survival when expressed at cold-shock temperatures. Kandror and Goldberg (1997) also demonstrated that trigger factor is a cold-shock protein, with levels increasing 2-fold following a cold shock (37°C→10°C) and that increased levels of this protein are observed even once growth has resumed. This pattern of expression could indicate that trigger factor is actually a cold-acclimation protein. Heat-shock proteins and trigger factor probably perform similar functions, but protect against different forms of temperature-induced damage. Trigger factor may be essential in repairing cold-damaged proteins or in assisting protein folding and translation. (Kandror and Goldberg, 1997).

2.6: The cold-shock response in other systems

2.6.1: Prokaryotes

The cold-shock response has been demonstrated in several groups of bacteria, but most investigators have concentrated on defining the proteins that are synthesised during the cold shock. Little research has been performed to elucidate the metabolism or the regulation of this stress response in these bacteria.

2.6.1.A: The cold-shock response in other mesophilic bacteria: *B. subtilis.*

When cultures of the Gram-positive soil bacterium *B. subtilis* are shifted from 37°C to 10°C, many cold-shock proteins are induced, but there is no significant lag period in culture growth (Graumann *et al.*, 1996). This phenomenon was also seen in the enteric mesophile *E. faecalis*, which when transferred to 8°C, undergoes an immediate decrease in generation time, with no obvious lag period (Panoff *et al.*, 1997).

Two-dimensional protein analysis revealed that a total of fifty-three polypeptides was induced in *B. subtilis* upon transfer from 37°C to 10°C, 15°, 20°C and 25°C (Lottering and Streips, 1995). Comparative analysis with the heat-shocked or salt-stressed *B. subtilis* revealed that several of the cold-shock proteins were actually general stress proteins, expressed during different types of stress (Lottering and Strieps, 1995; Graumann *et al.*, 1996). Several of the unique cold-shock proteins have been microsequenced and include proteins such as aconitase (Lottering and Strieps, 1995), CspB, CspC, CspD, ribosomal protein S6, triosephosphate isomerase and Gap (Graumann *et al.*, 1996).

As in *E. coli*, a family of cold-shock proteins exists in *B. subtilis*, the difference being that all the *B. subtilis* proteins are strongly induced by a cold shock and are prominent cold-shock proteins. All the homologues found in *B. subtilis* had conserved RNA-binding domains (RNP-1 and RNP-2) (Graumann *et al.*, 1996) and like CspA of *E. coli*, were able to bind to RNA and ssDNA (Graumann *et al.*, 1997). Mutation studies have shown that the Csp family is essential for adaptation to the lower temperature and for cell viability at all temperatures, and that mutations in one of the genes can be complemented by the actions of the other proteins (Graumann *et al.*, 1997). It has also been demonstrated that at least one of these proteins, CspB, can protect cells against freezing damage and has been implicated in cryotolerance, possibly serving a role as an antifreeze protein (Willimsky *et al.*, 1992). CspB is the major cold-shock protein in *B. subtilis*, involved in the synthesis of at least fifteen other cold-shock proteins (Graumann *et al.*, 1996). Willimsky *et al.* (1992) originally sequenced the protein and isolated the corresponding *cspB* gene. At both DNA and protein levels, CspB showed 60% identity with CspA of *E. coli*. This gene was found to be expressed weakly at 37°C, with increased levels of mRNA observed following the cold shock.

Due to the obvious importance of the *B. subtilis* cold-shock proteins (CspB, C and D) during optimal growth temperatures, as well as following a cold shock, Graumann *et al.* (1997) suggest that these proteins are required to prevent secondary structures in the mRNA transcript at all temperatures (chaperone function), allowing initiation of translation by the ribosomes. Higher levels of these proteins are required at lower temperatures (following a cold shock), as secondary structures are more readily formed in the mRNA at the lower temperature (see Figure 2.5). The obvious question then is, why are the cold-shock proteins (CspA, B and G) of *E. coli* not expressed during optimal growth temperatures? It has recently been demonstrated that the ribosomal protein S1 of *E. coli*, an important protein in the initiation of translation, is comprised of six domains that

share structural homology to CspA (Bycroft *et al.*, 1997). Thus during growth at 37°C, a protein similar to the cold-shock protein family is already involved in the initiation of translation. During a cold shock however, this function needs to be augmented, therefore the cold-shock protein family is induced (Graumann *et al.*, 1997). Although a putative homologue of the ribosomal protein S1 has been found in *B. subtilis* (Sorokin *et al.*, 1995), no S1 protein has been found to be associated with the ribosomes of *B. subtilis* (or any other Gram-positive bacteria investigated) (Muralikrishna and Suryanarayana, 1985). It is possible that during evolution, structural motifs may have been conserved, but different functions have developed for the S1 ribosomal proteins of Gram positive and Gram negative bacteria (Sorokin *et al.*, 1995). If this were the case, then the CspB, CspC and CspD proteins of *B. subtilis* may be fulfilling some of the functions of this ribosomal protein, explaining their expression at all temperatures (Graumann *et al.*, 1997).

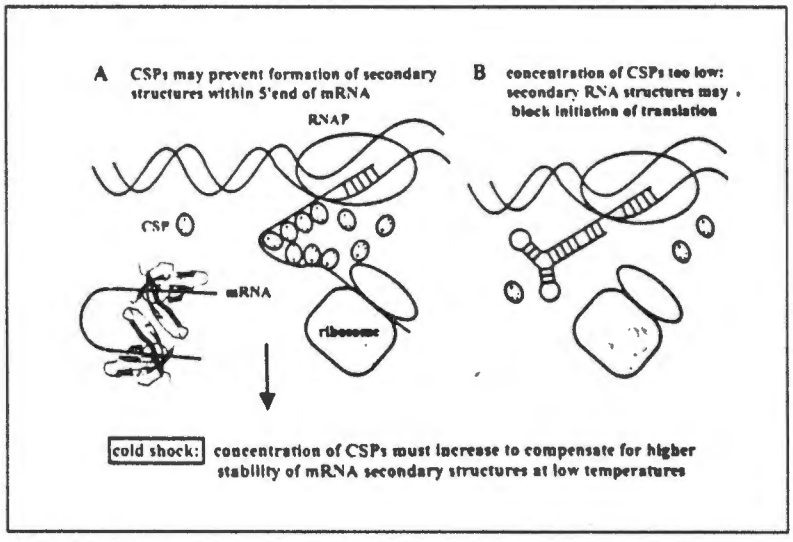


Figure 2.5: The role of Csps in the initiation of translation.

Hypothetical model for a function of bacterial cold-shock proteins (Csps) in coupling of transcription to the initiation of translation. RNAP = RNA polymerase (Graumann *et al.*, 1997).

As with *E. coli*, cold shock in *B. subtilis* results in an increase in the proportion of unsaturated fatty acids in the membrane. Aguilar *et al.* (1998) have recently shown that a fatty acid desaturase gene (*des*) from *B. subtilis* is one of the genes induced following a cold shock. This gene encodes a protein of 352aa, which functions as a delta 5 acyl-lipid desaturase, causing desaturation of membrane phospholipids. As mentioned earlier, the genes for the synthesis of unsaturated fatty acids were found to be controlled by the action of DNA gyrase (sensitive to supercoiling changes) (Grau *et al.*, 1994).

2.6.1.B: The cold-shock response in psychrotrophic bacteria.

In *E. coli*, the lag period following a cold shock is caused by a block in the initiation of translation, with the ribosomes unable to translate proteins (Broeze *et al.*, 1978; Jones and Inouye, 1996). During this lag period, specific ribosomal proteins are produced to relieve this blockage by converting the ribosomes into cold-resistant translatable ribosomes (cold-shock model (Jones *et al.,* 1996)). However, this may not be a general cold-shock phenomenon. When cultures of *Pseudomonas fluorescens* were cold shocked to 5°C, a 4 hour lag period in growth was seen, but this was not reflected in the rate of protein synthesis, which remained constant (Broeze *et al.*, 1978). This research demonstrated that the transient growth stoppage is this bacterium, was not due to a block in the initiation of translation, but that other mechanisms were involved in halting growth and initiating the cold-shock response.

The pyschrotrophic bacterium *A. globiformis* can grow in the temperature range of -5°C to 37°C, but grows optimally at 25°C. This bacterium experiences a cold-shock response at 4°C which results in the induced synthesis of twenty-eight proteins. Nineteen of these proteins are only transiently induced (cold-shock proteins), while the other nine are still over-expressed after growth has resumed at the lower temperature (cold acclimation proteins - CAPs) (Berger *et al*, 1996). The pattern of induction of the cold-shock proteins varies considerably depending upon the severity of the shock. The cold-shock proteins can therefore be grouped into different classes depending upon when they are induced: Group 1 - cold-shock proteins (transient expression) that are induced only after a large temperature shift; Group 2 - cold-shock proteins with optimal expression after mild temperature shocks; Group 3 - early CAPs (produced at all temperatures); Group 4 - late CAPs, expressed only once steady-state growth has been achieved at 4°C (Berger *et al.,* 1996). The protein A9 or CapA is the major cold-shock protein of *A. globiformis* (Berger *et al.,* 1996) and exhibits high homology to the CspA of *E. coli* and other organisms (Berger *et al.,* 1997). This protein is however, classified as a cold-acclimation protein, as it is still produced during prolonged exposure to 4°C (Berger *et al.*, 1996; Berger *et al.,* 1997).

A complicated pattern of cold-shock protein expression was also demonstrated in *Pseudomonas fragi* (Hebraud *et al.*, 1994), with different groups of cold-shock proteins being induced at different temperatures. The induction mechanism seen in these two bacteria (*A. globiformis* and *P. fragi*) is different from that of *E. coli*, where one set of proteins is induced at all cold-shock temperatures and it may explain how these bacteria can grow at such low temperatures.

The cold-shock response in *B. cereus* has been studied only at one temperature, but is nevertheless very different with only three proteins being induced upon transfer from 30°C to 7°C (Mayr *et al.,* 1996). The most abundant protein is a 7.5kDa protein, CspA. Using antibodies to this protein, six other proteins belonging to the cold-shock protein family were identified. Five of these were microsequenced and all five were shown to have significant homology to the cold-shock protein family of *E. coli,* and to contain the Y-box motif.

2.6.1.C The cold-shock response in thermophilic bacteria.

The optimal growth temperature of *Bacillus stearotherophilus* is 65°C. When shifted to a cold-shock temperature of 45°C a typical cold-shock response is seen. This is characterised by a lag period of 3 hours (as defined by the number of colony-forming units), during which time cold-shock proteins (L1-L5) are induced (Wu and Welker, 1991).

2.6.2: EUKARYOTES

2.6.2.A: The cold-shock response in Humans.

Eukaryotic systems also possess cold-inducible genes and are capable of reacting to cold-shock conditions. Recently two human cold-shock proteins have been identified: cold-inducible RNA-binding protein (CIRP) (Nishiyama *et al.*, 1997) and RBM3 (Danno *et al.*, 1997). The CIRP protein is a 18kDa member of the glycine-rich RNA-binding protein family (GRP). It has been shown to occur in a large range of cell types and increases in the levels of both CIRP mRNA and protein were demonstrated 12 hours following a 5°C temperature drop (37°C to 32°C). This protein appears to play a role in the cold-induced suppression of cell-proliferation. RBM3 is another member of the GRP family that responds to a cold shock (5°C temperature reduction), showing increased transcript levels after 24 hours of cold exposure (Danno *et al.*, 1997).

2.6.2.B: The cold-shock response in *Saccharomyces cerevissiae.*

When yeast cells are exposed to a drop in temperature from 30°C to 10°C, retarded membrane fluidity and splitting of vacuoles are but two of the physiological changes that can be seen. Interestingly though, Kaul *et al.* (1992) demonstrated, through cell viability studies, that prior cold-shock pre-treatment of cells protected the eukaryotic cells against the damage caused by freezing (cryotolerance). This increased cryotolerance after cold-shock pre-treatment has also been demonstrated in *B. subtilis* (Willimsky *et al.*, 1992) and *E. coli* (Goldstein *et al.*, 1990).

In yeast, the major cold-shock protein is nuclear localisation sequence recognition protein (NSRI), which is structurally related to mammalian nucleolin, a protein involved in ribosome synthesis. Kondo *et al.* (1992) investigated the expression of NSRI and showed that NSRI mRNA could be detected only during exponential growth at 30°C, but increased 3-fold after cold-shock (10°C) (protein levels increased 3-fold as well). Through the use of NSRI mutant cells it was established that NSRI is required for normal pre-rRNA processing and cell growth, and is essential for cells exposed to cold-shock temperatures. TIP1, TIR1 and TIR2 are three other cold-shock-induced genes of *Saccharomyces cerevissiae*. TIP1 and TIR2 are however also induced during heat shock and are therefore probably general stress proteins. All three are rich in serine and alanine residues and contain serine rich repeats. They have putative N-terminal signal peptides and hydrophobic C-terminals, indicating that the proteins may be membrane-bound. Mutant studies have revealed that

none of these cold shock proteins are essential for growth, further functions have yet to be elucidated (Kowalski *et al.*, 1995).

2.7: Conclusions.

In summary, the cold-shock response is a vital adaptive mechanism exploited by a wide variety of organisms in order to survive harsh temperature reductions. It seems that the mechanisms involved in inducing the response, as well as the metabolic characterisation of the response, may vary from one organism to another, but the induction of a specific set of proteins that allow adaptation to a cold environment is a universal property.

In at least three different species (*E. coli, B. subtilis*, homo sapiens), many of the cold-shock proteins that are induced are involved in RNA-binding. At present four different types of RNA-binding domains have been identified: RNP (Nagai *et al.*, 1995), KH (Musco *et al.*, 1996), dsRBD (Bycroft *et al.,* 1995) and the S1 RNA binding domain. This fourth domain was first identified by Subramanian (1983), who discovered that the ribosomal protein S1 consists of six copies of this 70aa motif. This ribosomal protein has been shown to play an essential role in the initiation of translation in Gram-negative bacteria. It is involved in binding the mRNA to the 30S subunit of the ribosome and also possesses RNA unwinding activity (ATP independent) (Steitz *et al.*, 1977; Szer *et al.*, 1976). An interesting feature of this S1 RNA binding motif, is that it is similar in three-dimensional structure to the cold-shock proteins (CspA, CspB, CspG) (Bycroft *et al.*, 1997) and occurs in many proteins involved in the initiation of translation and mRNA turnover. Many of the proteins that are induced during the cold-shock response of *E. coli* possess this motif: PNP, NusA, initiation factor, and CspA (CspB and CspG) (Bycroft *et al.*, 1997). In conjunction with this, the CsdA protein (cold-induced) also possesses similar functions to ribosomal protein S1 and can possibly functionally complement the S1 protein (Jones *et al.*, 1996). It is possible that the major role of these proteins induced during a cold shock, is to complement the actions of the ribosomal protein S1. By binding to the mRNA and preventing the formation of secondary structures, they may facilitate the initiation of translation. As already mentioned, Graumann *et al.* (1997) suggest that this may be the case for CspA, B and G, but could the S1 motif allow the other proteins to play a role in this as well? One thing is clear from the proteins that are induced, a cold shock causes abnormalities in the RNA structures and that these must be dealt with to allow the cell to function. Whether these RNA abnormalities are the sole cause of the block in translation needs to be confirmed. This is different from the heat-shock stress, where abnormalities at the protein level are the problem.

2.8: Aims of this study.

As mentioned in Chapter 1, the aim of this study was to investigate the effects of a cold-shock on *M. smegmatis*. The approach that was used was to define the cold-shock response in *M. smegmatis* as it has been in *E. coli*, so that a comparison of the effects of this stress on the two organisms could be made. This involved studying the cold-shock response from several angles.

Section B of this study details the general effects of a cold-shock, investigations of both the growth and metabolism (Chapter 3) of *M. smegmatis* and the changes in protein composition (Chapter 4) that occur following a temperature reduction. Section C describes the analysis of the cold-shock response at a more specific level, by investigating the effects of a cold-shock on the expression of mycobacterial homologues to known *E. coli* cold-shock genes (Chapter 5) and the characterisation of a mycobacterial cold-shock protein (Chapter 6).

SECTION B:

DEFINING THE COLD-SHOCK RESPONSE OF *M. SMEGMATIS*: METABOLIC AND PROTEIN ANALYSIS.

CHAPTER 3

Defining the growth and metabolic responses of M. smegmatis to a cold-shock

3.1: INTRODUCTION.

The cold-shock response in *E. coli* is an adaptive stress response, where a specific subset of proteins is induced to allow the bacteria to adapt to the temperature change and continue to grow, when the temperature is lowered by more than 13°C (reviewed by Jones and Inouye, 1994 and Panoff *et al.*, 1998). This response is characterised by an initial "lag" in cellular activities, such as cell division, protein synthesis, DNA and RNA synthesis, that occurs immediately after the temperature down-shift (NG *et al.*, 1962; Shaw and Ingraham, 1967). It is during this lag period, which lasts 4-6 hours following a temperature-shift from 37°C to 10°C, that the synthesis of specific cold-shock proteins is induced (Jones *et al.*, 1987; Jones and Inouye, 1994). Many of these cold-shock proteins are involved in relieving the block at the initiation of translation (Jones and Inouye, 1996, reviewed by Jones and Inouye, 1994 and Panoff *et al.*, 1998), that occurs as a result of the temperature reduction (Broeze *et al.*, 1978). Following the increased expression of these cold-shock proteins, cellular activity is restored and growth is resumed, but at a reduced rate.

The first step in characterising the cold-shock response of *M. smegmatis* was to establish if, as in *E. coli*, a temperature down-shift resulted in a lag in cellular activity, thereby giving a time-frame within which the induction of cold-shock proteins could be investigated (Chapter 4). This involved the monitoring of cell mass, cell division and metabolic activities such as protein and RNA synthesis. Culture turbidity is an accepted means of monitoring changes in cell mass in most bacterial cultures (Schlegel, 1986), however, in the case of mycobacterial growth studies, the formation of cell aggregates often gives misleading results (Wayne, 1994b). Calculations of the culture ATP (adenosine triphosphate) concentration have been used successfully as a rapid and sensitive alternative for studying the growth and viability of mycobacteria (Dhople, 1987; Dhople, 1989; Prioli *et al.*, 1985, Nilsson *et al.*, 1989), as well as a variety of other bacteria (Stanley, 1989). For a specific set of growth conditions, the intracellular ATP concentration remains constant for each cell, therefore these values can be directly related to cell mass and cell number (Stanley, 1989). In order to investigate the effects of a cold shock on the growth of *M. smegmatis*, culture turbidity (OD_{600nm}), the number of colony-forming units (CFU) and the ATP concentration were determined. At the metabolic level, the synthesis of protein and RNA was monitored following the temperature-shift.

3.2: MATERIALS AND METHODS.

The details of the commonly used buffers and solutions are listed in Appendix 1.

3.2.1: Culture conditions and cold shock.

3.2.1.A: Culture conditions.

M. smegmatis strain LR222 (Beggs *et al.,* 1995), was used throughout this study. Mycobacterial cultures were seeded (1/1000 dilution) from a frozen, stationary-phase (14 days), stock culture and grown in 200ml of Middlebrook 7H9 medium (Difco Laboratories), supplemented with 10% (v/v) ADC (albumin dextrose catalase - Appendix 1) and 0.05% (v/v) Tween 80 (polyoxyethylene-sorbitan monooleate) (Dubos and Davis, 1946), unless otherwise indicated. The cultures were incubated at 37°C with aeration (shaking incubator - 175rpm).

3.2.1.B: Cold-shock conditions.

Following 20-24 hours of aerated growth at 37°C (mid-exponential phase, OD_{600nm}= 0.1), the mycobacterial cultures were split into two separate culture bottles. One was retained at 37°C (control), while the other was transferred to an enclosed shaking-waterbath (180rpm) that was set to the cold-shock temperature (30°C, 25°C, 20°C, 15°C or 10°C). During growth at 37°C and at the cold-shock temperatures, cultures were continually assessed for contamination using the Ziehl-Nielsen mycobacterial stain (Heifets and Good, 1994).

3.2.2: Experimental design.

For all the experiments described in this section, three separate measurements were made at each time point and each experiment was repeated three times. The data from only one of the experiments is presented, with the error bars (calculated for each time point) indicating intra-experimental error. These curves are however, representative of the trend shown in all of the experiments.

3.2.3: Growth assessment.

3.2.3.A: Bacterial ATP concentrations.

The single-point bioluminescent assay for ATP was modified (reduced volumes) from that recommended by the manufacturers of the Bio-Orbit 1250 luminometer (Bio-Orbit (UK), Ltd), using the Promega Enliten™ Luciferase/Luciferin Bioluminescence detection reagent. Briefly, the bacterial ATP was extracted at each time point from 55µl of cell culture with the addition of 20% (w/v) trichloroacetic acid (TCA), to a final concentration of 1.5% (Lundin, 1984). This mixture was held on ice for 30 minutes. The acid was neutralised with 50µl of neutralising buffer (1M Tris-acetate pH 7.75) and diluted by the addition of 622µl of distilled water. This test sample solution was diluted 1/100, to give an ATP concentration in the 10^{-6}M - 10^{-11}M range. To measure the

background luminescence (B) of the assay components, 50µl of Luciferase/Luciferin reagent (Promega) was mixed with 347µl of 0.1M Tris-acetate/ 2mM EDTA buffer (pH 7.75) and the relative light units (RLU) were measured in the luminometer. An aliquot (100µl) of the diluted test sample was added to the luciferase/luciferin mixture and the RLU measured (C). To standardise the assay, 2.5µl of ATP standard (10^{-7}M) was added and the RLU measured again (I). To calculate the concentration of ATP in the original 55µl culture sample the following calculation was performed:

$$\text{ATP (M)} = [(C-B) \div I] \times 5 \times 10^{-10} \text{ (ATP standard concentration)} \times \text{sample dilution.}$$

3.2.3.B: Culture turbidity measurements.

Samples (1ml) of culture were removed at each time point and the absorbance at 600nm (OD_{600nm}) was recorded using a Beckman DU-40 spectrophotometer, using Middlebrook 7H9 medium as a blank.

3.2.3.C: Determination of the number of colony-forming units.

At each time point, 100µl of cells were removed from the cultures and diluted 10^3, 10^4 and 10^5 in PBS (pH 7.2). The cell samples were vortexed to disperse cell aggregates and 200µl of each dilution were plated, in duplicate, on Middlebrook 7H9 agar plates. The plates were incubated at 37°C for five days and the number of mycobacterial colonies (CFU) were counted. Results are presented as CFU / ml of cell culture.

To determine the generation time and growth rate (division rate) of the bacterium, the following equations were used (Schlegel, 1986):

$$g = V^{-1} \qquad V = [\log x - \log x_{-1}]\,[\log 2\,(t - t_{-1})]^{-1}$$

g = generation time
V = division rate
x = growth measurement at time t.

3.2.4: Metabolic assessment.

3.2.4.A: Quantification of RNA synthesis.

3.2.4.A.i: Bulk labelling of nucleic acids with ^{3}H-uracil.

[5,6-^{3}H]uracil (Amersham, 49Ci/mmol) and a solution of 20mM non-radioactive uracil (Sigma) were mixed at a ratio of 1:1 (^{3}H-uracil mix), to give a specific activity of 50mCi/mmol, and a final uracil concentration of 200µM, when added to the culture samples. At each time point, 1ml samples of culture were removed and incubated with 20µl of the ^{3}H-uracil mix (10µCi) for 10 minutes at the appropriate temperature. Following this labelling period, an equal volume (1ml) of ice-cold 10% (w/v) TCA was added and the mixture held on ice for 30 minutes. The acid-precipitable material

was collected by centrifugation (4000rpm for 10 minutes) and washed three times with cold 5% (w/v) TCA (4000rpm for 5 minutes). It was essential to ensure complete pellet resuspension during each wash step, to allow for the removal of all unincorporated radioactive material. The final pellet was dissolved in 1ml 0.1M NaOH and added to 9ml of scintillation fluid (Insta-gel II Plus, Packard). The radioactivity content (cpm) of the acid-precipitable material (bulk nucleic acids) was determined using a liquid scintillation counter (Beckman LS 7800). Nucleic acid synthesis was expressed as cpm / ml cell of culture.

The acid-precipitation of macromolecules and subsequent washing with 5% TCA was found to be more effective than the use of a filtration system (Wayne, 1977; Granozzi *et al.,* 1990) in removing unincorporated radioisotope, in these studies. This method resulted in background levels (no cells control) of less than 5% when labelling at 37°C.

3.2.4.A.ii: Determination of the DNA and RNA components of the ^{3}H-labelled nucleic acids..

To determine the amount of radioactivity incorporated into the separate nucleic acid components, the RNA component of the ^{3}H-radio-labelled nucleic acid bulk was degraded by alkaline hydrolysis (Wayne, 1977), with the remaining radioactivity representing radio-labelled DNA. Duplicate cell samples (1ml) were labelled with ^{3}H-uracil and one of the samples was processed as mentioned above. The duplicate sample was incubated with an equal volume of 0.6M KOH (final concentration: 0.3M KOH) for 24 hours at 37°C. Following this incubation period, the remaining DNA molecules were precipitated with an equal volume (2ml) of ice-cold 10% (w/v) TCA and processed as above.

3.2.4.B: Quantification of protein synthesis.

At each time point, 1ml samples of culture were removed and incubated with 10µl (0.5µCi) of L-[U-^{14}C]leucine (Amersham, 304mCi/mmol) for 10 minutes at the appropriate temperature. Following this labelling period, the proteins were precipitated by adding an equal volume (1ml) of 10% TCA and holding on ice for 30 minutes. The unincorporated radioactivity was removed and the radioactivity content of the final acid-precipitable material determined as described above (3.2.3.A). Protein synthesis was expressed as cpm/ml cell culture. As for the nucleic-acid labelling procedure, background radioactivity levels (no cells control) of less than 5% were achieved.

3.3: RESULTS.

3.3.1: Development of cold-shock analysis methodology.

3.3.1.A: Growth analysis of *M. smegmatis*.

All cold-shock experiments involving *B. subtilis* and *E. coli* have been performed on bacterial cells in the exponential-phase of growth, when the bacteria are able to respond rapidly to external stresses. The first step in the analysis of the cold-shock response of *M. smegmatis* was to establish a growth curve and evaluate the appropriate time for exposure to stress. This was achieved by monitoring the growth of the culture at 4-hourly intervals, during aerated incubation at 37°C. Two different methods of growth assessment were used: turbidity readings (OD_{600nm}) and ATP concentrations. Figure 3.1 shows the growth curve of *M. smegmatis* at 37°C, in Middlebrook 7H9 medium, supplemented with Tween 80 and ADC.

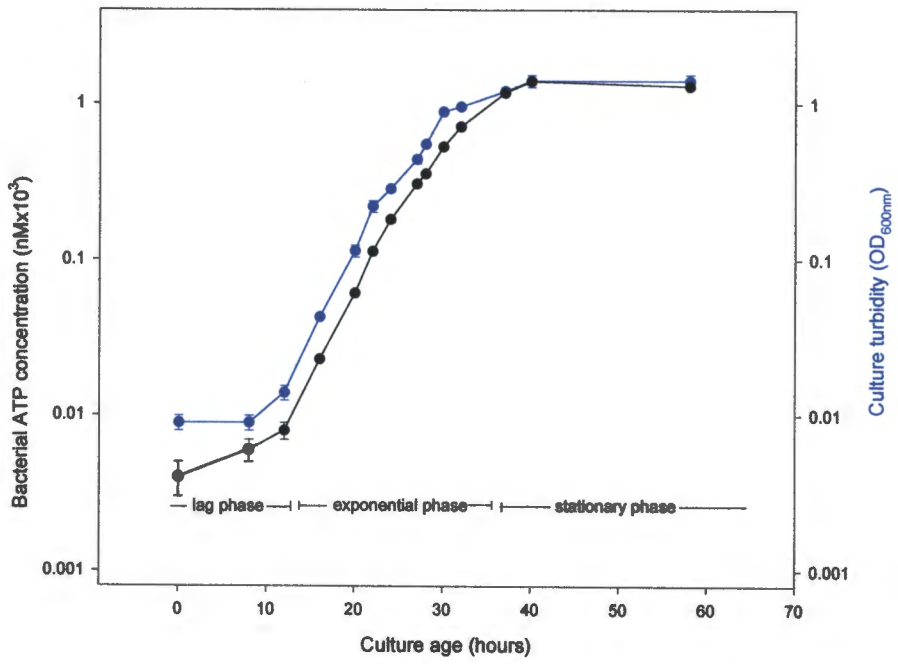


Figure 3.1: Growth curve of *M. smegmatis* (37°C).

A 200ml culture of *M. smegmatis* was grown with aeration at 37°C in Middlebrook 7H9 medium, supplemented with 0.05% Tween 80 and 10% ADC. At 4-hourly intervals the turbidity (OD_{600nm}) (black line) and the ATP concentration of the culture (blue line) were calculated. The error bars, calculated for each point, indicate intra-experimental error (Standard Error of the Mean – SEM).

Under the growth conditions used in these experiments, *M. smegmatis* LR222 demonstrated a generation time of 3-4 hours, with a division rate (v) of $0.25 \rightarrow 0.3 hr^{-1}$, as calculated from both the culture turbidity and ATP concentration data. The cultures appeared to enter exponential growth after 12 hours, with the exponential phase of growth spanning a 20-24 hour period. A culture of 20-24 hours was regarded as representing the mid-exponential phase of growth ($OD_{600mn} \approx 0.1$) and was chosen as the time-point for stressing the bacteria in the cold-shock studies.

3.3.1.B: Labelling of nucleic acids.

The DNA from *E. coli* can be efficiently and specifically labelled by the incorporation of extracellular thymine, while the RNA in these bacteria is labelled through the incorporation of radio-labelled uracil. However, in mycobacteria the situation is more complex. Mycobacteria (*M. tuberculosis* and *Mycobacterium phlei*) incorporate extracellular thymine very poorly into their DNA (Wayne, 1977) and as thymidine is rapidly converted into thymine (by the action of thymidine phosphorylase at the cell surface), this is not a suitable extracellular precursor either (Somogyi and Foldes, 1983). Instead it has been found that labelling the mycobacterial cells with radio-labelled uracil, results in the most efficient labelling of both mycobacterial DNA and RNA (Wayne, 1977; Somogyi and Foldes, 1983). A distinction can be made between the quantity of radioisotope incorporated into DNA from that of RNA, via the treatment of the labelled cells with KOH (Wayne, 1977). The cells are incubated with ^{3}H-uracil and the quantity of tritium contained within the acid-precipitable macromolecules is determined (total cpm). Following incubation with the radioactive base, a duplicate cell sample is treated with the alkaline solution prior to acid precipitation of the macromolecules. The KOH degrades the cellular RNA and the remaining tritium counts, in the acid-precipitable material, indicate the synthesis of DNA only (DNA cpm). The difference between the total cpm and the DNA cpm indicates the incorporation of ^{3}H-uracil into RNA.

In the present study, the synthesis of nucleic acids in *M. smegmatis* was analysed by the incorporation of ^{3}H-uracil into acid-precipitable material. Using the quantities of radioisotope recommended in previous nucleic acid studies on actinomycetes (Wayne, 1977; Somogyi and Foldes, 1983; Granozzi *et al.*, 1990), problems were encountered in labelling the nucleic acids so as to reflect the growth of the bacteria. As a consequence, a range of uracil concentrations (100nm to 200μM total uracil) and radioactivity concentrations (1μCi/ml → 10μCi/ml) were assayed. Various labelling times were also investigated, including the addition of ^{3}H-uracil at the beginning of the growth period, as performed by Wayne (1977), or pulse-labelling for shorter 10-minute or 30-minute periods. Labelling with 10μCi of ^{3}H-uracil at a specific activity of 50mCi/mmol for 10 minute periods, resulted in increases in ^{3}H cpm that reflected the growth of the organism as monitored by OD_{600nm} or ATP concentrations (Figure 3.2). Extending the incubation period to 30 minutes did not significantly improve the incorporation of radio-label into the bulk of the nucleic acids.

The treatment of the samples with 0.3M KOH revealed however, that this growth-related increase in ^{3}H CPM (total CPM) was due to the incorporation of ^{3}H-uracil into RNA only. Figure 3.2 clearly demonstrates that the amount of tritium present in the DNA fraction (post KOH treatment) remained constant, while the bacteria continued to grow (as measured by the ATP concentration). This indicated that under the conditions of this assay (short labelling period), labelling with ^{3}H-uracil reflected the increased synthesis of RNA (as cell numbers increased), but not DNA.

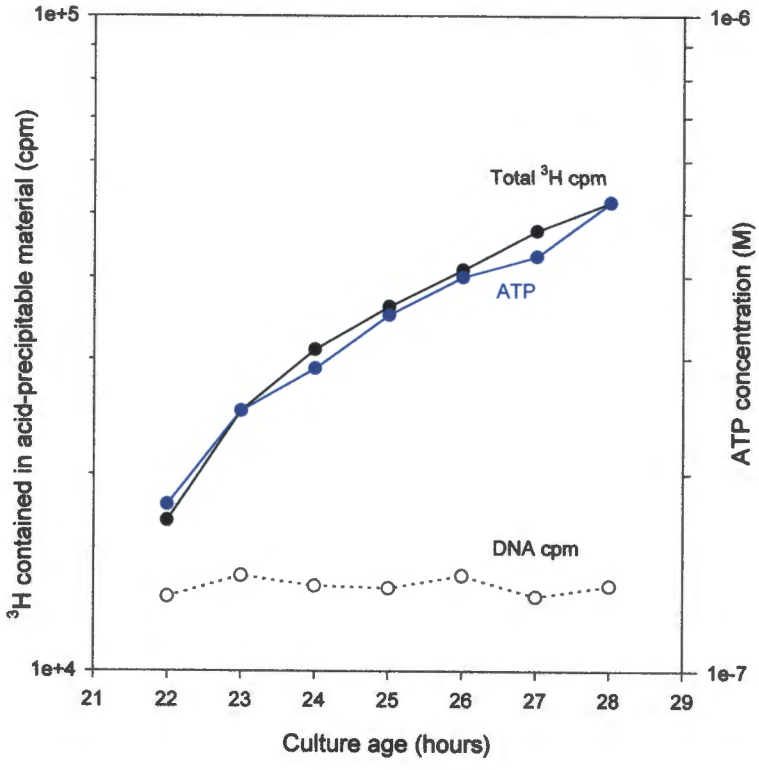


Figure 3.2: Radioactive labelling of mycobacterial nucleic acids.

M. smegmatis was cultured at 37°C in Middlebrook 7H9 medium, supplemented with 0.05% Tween 80 and 10% ADC. At the times indicated (culture age), duplicate cell samples (1ml) were incubated with 10μCi of ^{3}H-uracil (50mCi/mmol) for 10 minute periods. The total nucleic acid component of the one sample was precipitated with 1ml of 10% TCA and the radioactivity content of the acid-precipitable material was determined (total ^{3}H cpm). Following the labelling reaction, the duplicate sample was incubated with 1ml of 0.6M KOH for 24 hours at 37°C. The remaining nucleic acids were precipitated with 10% TCA and the radioactivity content determined (DNA cpm). The blue curve represents the ATP concentrations of the same bacterial culture as determined at each time point.

3.3.1.C: Influence of Tween 80 on growth assays.

When cultures of *M. smegmatis* were cold-shocked to temperatures below 25°C, the bacteria were found to aggregate to form clumps. The size of these clumps did not remain constant, increasing in size with longer incubation. It was not certain whether this clumping was due to changes in the bacterial cell walls, as a consequence of the cold shock, or whether this effect was extracellular.

Polyoxyethylene sorbate compounds such as Tween 80 are added to the mycobacterial growth medium not only to minimise aggregation of the mycobacterial cells, but also to enhance the growth of several mycobacterial species (Wayne, 1994b; Dubos and Middlebrook, 1948; Dubos and Davis, 1946). In the case of rapidly growing mycobacterial species such as *M. smegmatis*, the presence of a Tween 80-hydrolysing esterase enzyme (Tomioka et al., 1983) allows for the break down of the esterified detergent, releasing oleic acid, which is thought to act as a substrate for growth (Van Boxtel *et al.*, 1990; Schaefer and Lewis, 1965; Stinson and Solotorovsky, 1971).

In the present studies, 0.05% Tween 80 was found to be effective in maintaining cultures of *M. smegmatis* as single or loosely associated cells, when grown at 37°C. In comparison, *M. smegmatis* grown in the absence of Tween 80 formed small, tightly-associated groups of cells that were relatively constant in size throughout the growth cycle. Cultures of *M. smegmatis* were grown in the presence and absence of 0.05% Tween 80 and shifted from 37°C to 20°C, 15°C and 10°C. Microscope slides were prepared at 2-hourly intervals to monitor the formation of cell aggregates. No increased bacterial clumping was seen in the cultures without Tween 80, yet the increasing formation of large clumps was evident at cold-shock temperature, in cultures grown in the presence of Tween 80 (results not shown). It was therefore concluded that under cold-shock conditions, Tween 80 was no longer effective and the associated formation of clumps had the potential to interfere with growth assay measurements, such as turbidity readings and the determination of colony-forming units. The effects of the presence of Tween 80 and associated clumping on both metabolic and growth measurements, were assayed. Cultures of *M. smegmatis* were grown until mid-exponential phase in Middlebrook 7H9 medium, with and without Tween 80. The cultures were divided into two, with one being returned to 37°C and the other cold-shocked from 37°C to 20°C. The culture ATP concentrations, turbidity readings (OD_{600nm}), number of colony-forming units, incorporation of ^{3}H-uracil and ^{14}C-leucine were then assayed at 2-hourly intervals. Figure 3.3 shows how the presence of Tween 80 and the associated bacterial clumping gave misleading estimates of bacterial growth at cold-shock temperatures. The results for the cultures are slightly "offset" due to a one hour difference in the ages of the cultures at the point of cold shock.

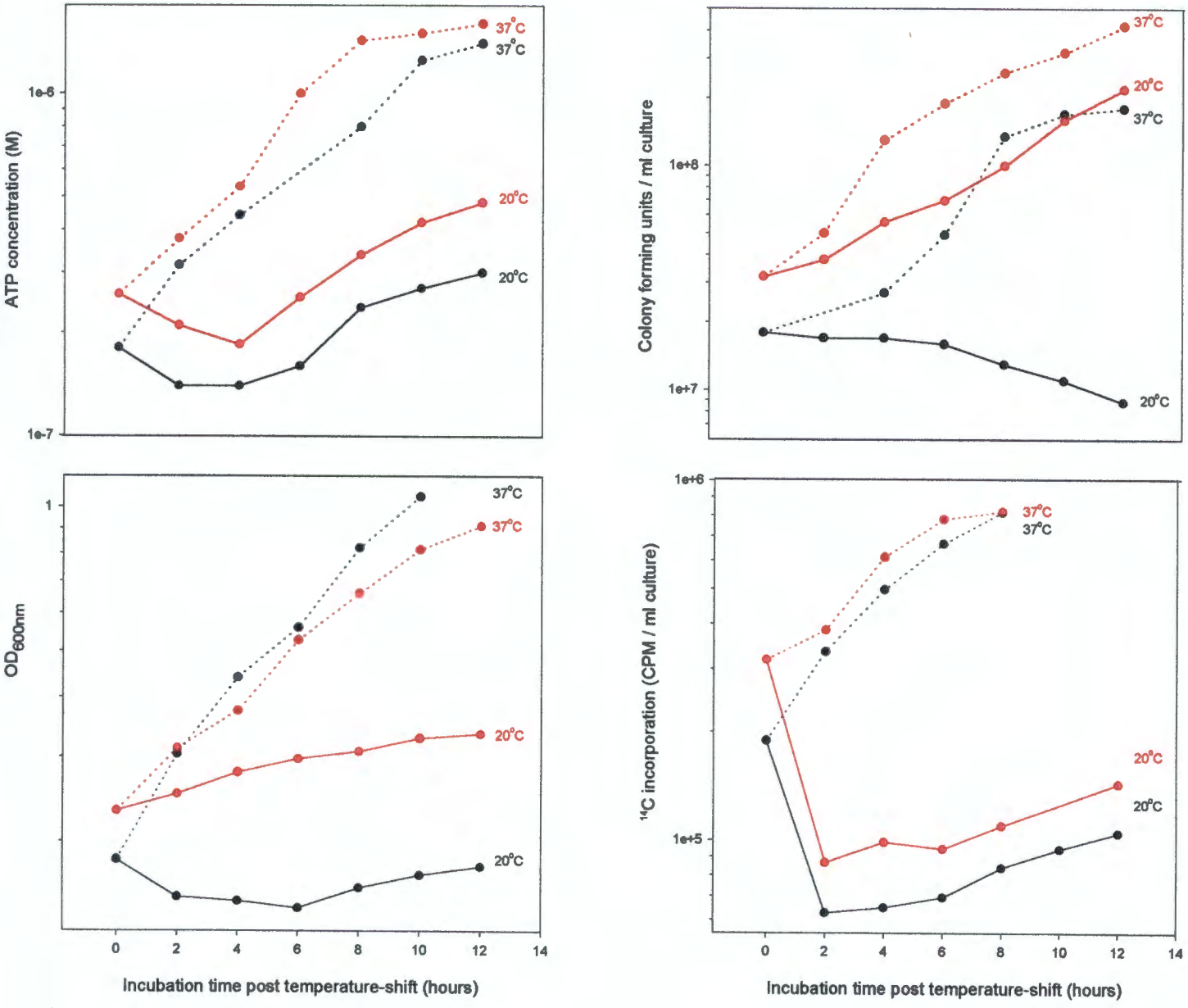


Figure 3.3: Effects of Tween 80 on cold-shock growth assays of *M. smegmatis*.

M. smegmatis was grown in Middlebrook 7H9 / ADC liquid medium (200ml), in the presence (black line) or absence (red line) of 0.05%Tween 80. Cultures were incubated with aeration at 37°C, for 20 hours (mid-exponential phase). At this point both cultures were divided into two, one half was returned to 37°C (control), while the other half was cold-shocked from 37°C to 20°C. At the times indicated (post temperature-shift), the ATP concentration, number of colony-forming units, culture turbidity (OD_{600nm}) and the synthesis of protein (^{14}C incorporation) was determined.

As observed from the data represented in Figure 3.3, the absence of Tween 80 had little or no observable effect on the growth of the mycobacterial cells during aeration at 37°C. Following the temperature-shift to 20°C, the measurements of bacterial ATP, incorporation of ^{14}C-leucine and ^{3}H-uracil (results not shown) were also not significantly affected by the presence or absence of the detergent, with the curves reflecting similar growth trends. The optical density readings and quantification of viable bacteria (colony-forming units) were, however, influenced by the presence of Tween 80 at the cold-shock temperature. The increasing formation of bacterial clumps in the presence of Tween 80 led to misleading data, such as the lag in the turbidity of the culture and the possible cold-induced cell death, that was indicated by the decreasing number of colony-forming units. Similar results were recorded when cultures were shifted from 37°C to other cold-shock temperatures. To avoid misinterpretation of the effects of cold shock on the growth of *M. smegmatis*, all studies that involved the determination of colony-forming units or optical density readings were performed on cultures grown in the absence of Tween 80.

3.3.2: The effects of cold shock on *M. smegmatis*.

3.3.2.A: Establishment of the cold-shock temperature range.

E. coli is able to grow at temperatures between 8°C and 42°C, with a cold-shock response occurring when the temperature is reduced by greater than 13°C, within this temperature range (Jones *et al.*, 1992a). The greatest induction of cold-shock proteins occurs when the temperature is reduced from 37°C to 10°C, a temperature at the lower limit of the growth range. This cold shock results in a lag in cellular activity of 4-6 hours (NG *et al.*, 1962; Shaw and Ingraham, 1967), with the synthesis of the cold-shock proteins induced during this period (Jones *et al.*, 1992a).

The temperature growth range of *M. tuberculosis* is fairly narrow, with growth observed at 37°C and 31°C, but not at 24°C or 45°C (Roberts *et al.*, 1991). Relatively rapid growth of *M. tuberculosis* was also observed when transferred from 37°C to 25°C (Takayama et al., 1978). *M. smegmatis* demonstrates rapid growth on solid medium over the temperature range 24°C to 45°C (Roberts *et al.*, 1991), but the effects of temperatures lower than 24°C have not been reported. As *M. smegmatis* is a soil-dwelling bacterium (Wayne, 1984), the lower limit of growth would be expected to be much lower than 24°C, perhaps as low as 8°C. Due to the possible similarity in growth temperature ranges of *E. coli* and *M. smegmatis*, a range of cold-shock temperatures was investigated that were previously tested on *E. coli*.

As an initial investigation, the ATP concentration of the culture was assayed following a cold shock from 37°C to 30°C, 25°C, 20°C, 15°C or 10°C. These preliminary experiments were performed to investigate the existence of a lag in cellular activity, similar to that of cold-stressed *E. coli* cells. Exponentially growing cultures (20-24 hours) of *M. smegmatis* (37°C) were split into two separate culture bottles, one was retained at 37°C (control), while the other was placed in a

shaking-waterbath set to the cold-shock temperature. Both the control and cold-shock cultures were aerated by shaking at a similar rate of 175-180rpm. The growth of the cultures was then monitored at 2-3 hourly intervals by measuring the ATP concentration (Figure 3.4).

Based on this ATP concentration determination (Figure 3.4), shifting the culture from 37°C to either 30°C or 25°C resulted in a slight decline in the growth rate and no lag was observed in the growth of the organism. This result compares well with the previously observed rapid growth of *M. smegmatis* on solid media at temperatures above 24°C (Roberts *et al.*, 1991). However, when the temperature was reduced by more than 12°C, a lag period in cellular growth immediately followed the temperature-shift. As with *E. coli*, the greater the temperature-shift, the longer the lag period before growth was resumed (Jones *et al.*, 1992a). Following a shift from 37°C to 20°C, this lag period lasted 4-6 hours. After a shift to 15°C, the lag period increased to 8-10 hours. Finally, a cold-shock from 37°C to 10°C resulted in the longest lag period, with the cells requiring 15-18 hours to recover from the 27°C temperature reduction.

3.3.2.B: Metabolic analysis of the mycobacterial cold-shock response.

The cold-shock response, as defined for *E. coli*, is a unique stress response in that all cellular processes are affected. A lag occurs in the synthesis of proteins, as well as both RNA and DNA (Shaw and Ingraham, 1967). This is in contrast to the starvation response, for example, where only the synthesis of RNA and proteins is affected immediately (Pardee and Prestidge, 1956).

The preliminary experiments described above (section 3.3.2.A), demonstrated that, as measured by the ATP concentration, a cold-shock from 37°C to 20°C, 15°C or 10°C resulted in a lag in the growth of *M. smegmatis*. The next step was to establish if, like *E. coli*, the synthesis of macromolecules was influenced by the cold shock. Cultures of exponentially growing cells were again cold-shocked from 37°C to 20°C, 15°C or 10°C, with the synthesis of RNA and protein being monitored at 2-3 hourly intervals via the incorporation of ^{3}H-uracil and ^{14}C-leucine, respectively. This analysis was coupled to the measurement of the ATP concentration, culture turbidity (OD_{600nm}) and number of colony-forming units, to further define the growth response. The results of this analysis are presented in Figures 3.5 to 3.8.

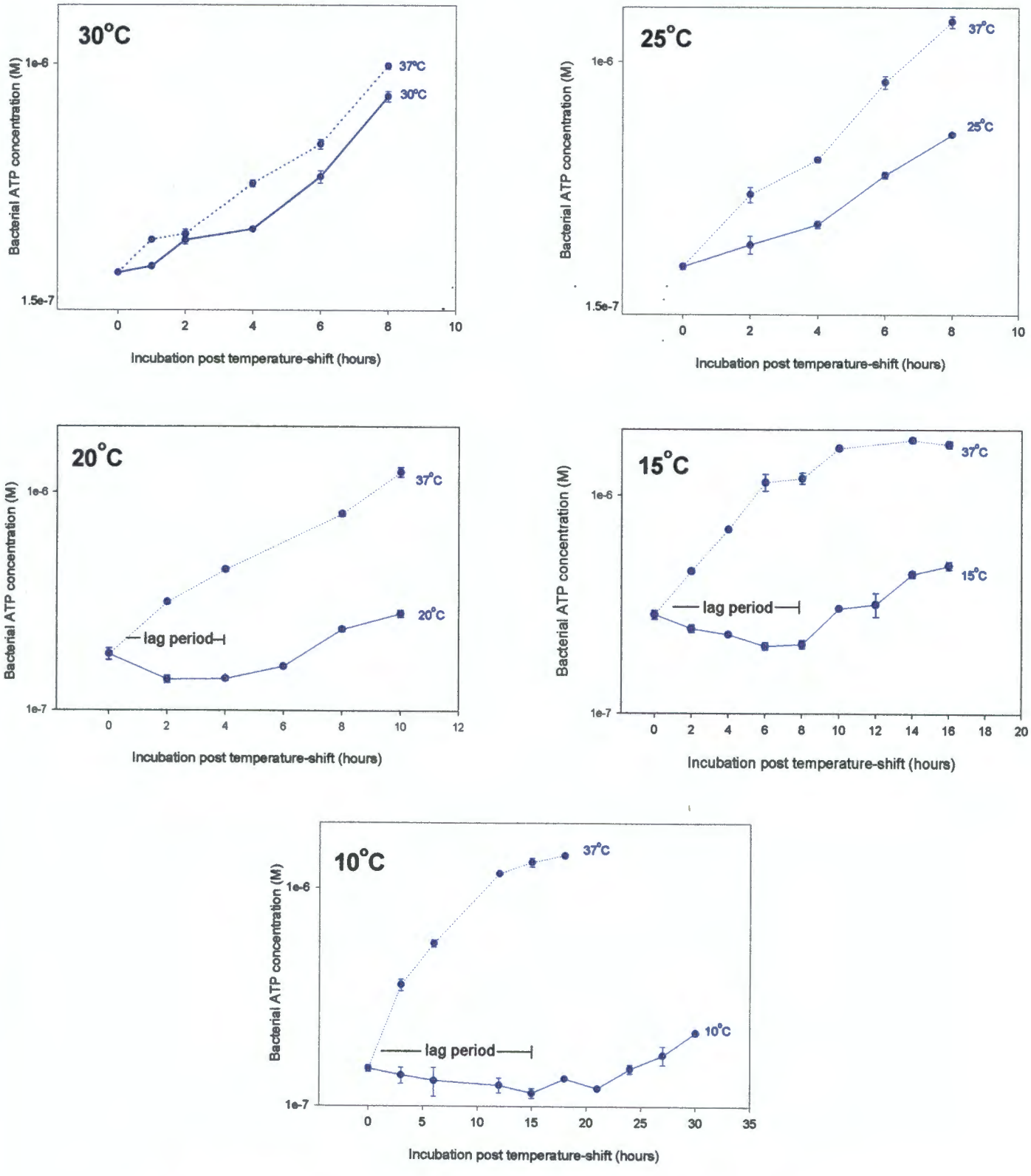


Figure 3.4: The effects of a temperature-shift on the growth of *M. smegmatis*.

Cultures of *M. smegmatis* were grown at 37°C with aeration in Middlebrook 7H9 medium (ADC and Tween 80 additives), until mid-exponential phase (20-24 hours, OD = 0.1). At this time the cultures were divided into two, with one half retained at 37°C(control) and the other placed in a shaking water-bath set to 30°C, 25°C, 20°C, 15°C or 10°C (cold shock). The bacterial ATP concentrations were measured at 2-3 hourly intervals post temperature shift, for both the control and cold-shock culture components. The error bars represent intra-experimental error (SEM).

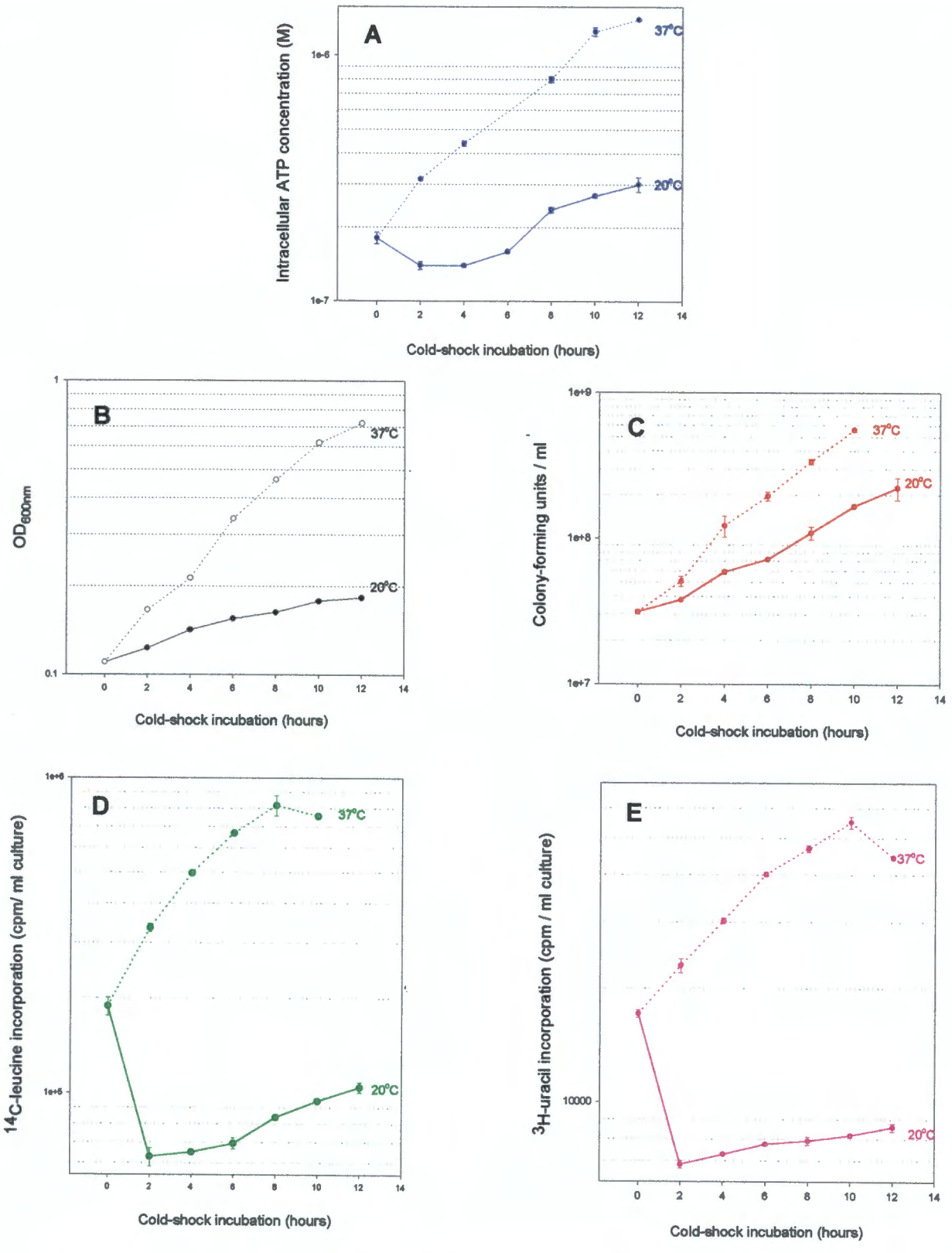


Figure 3.5: The effects of a 37°C→20°C cold shock on the growth of *M. smegmatis*.

An exponentially growing culture (20-24 hours) of *M. smegmatis* (Middlebrook 7H9, ADC, no Tween 80) was split into two separate cultures. One half was retained at 37°C, while the other was placed in a shaking water-bath set to 20°C. At 2-hourly intervals, post temperature-shift, the ATP concentration (A) and optical density of the culture (B), the number of colony-forming units (C) and the incorporation of ^{14}C-leucine (D) and ^{3}H-uracil into acid-precipitable material (E) was determined (as described in section 3.2). The error bars represent intra-experimental error (SEM).

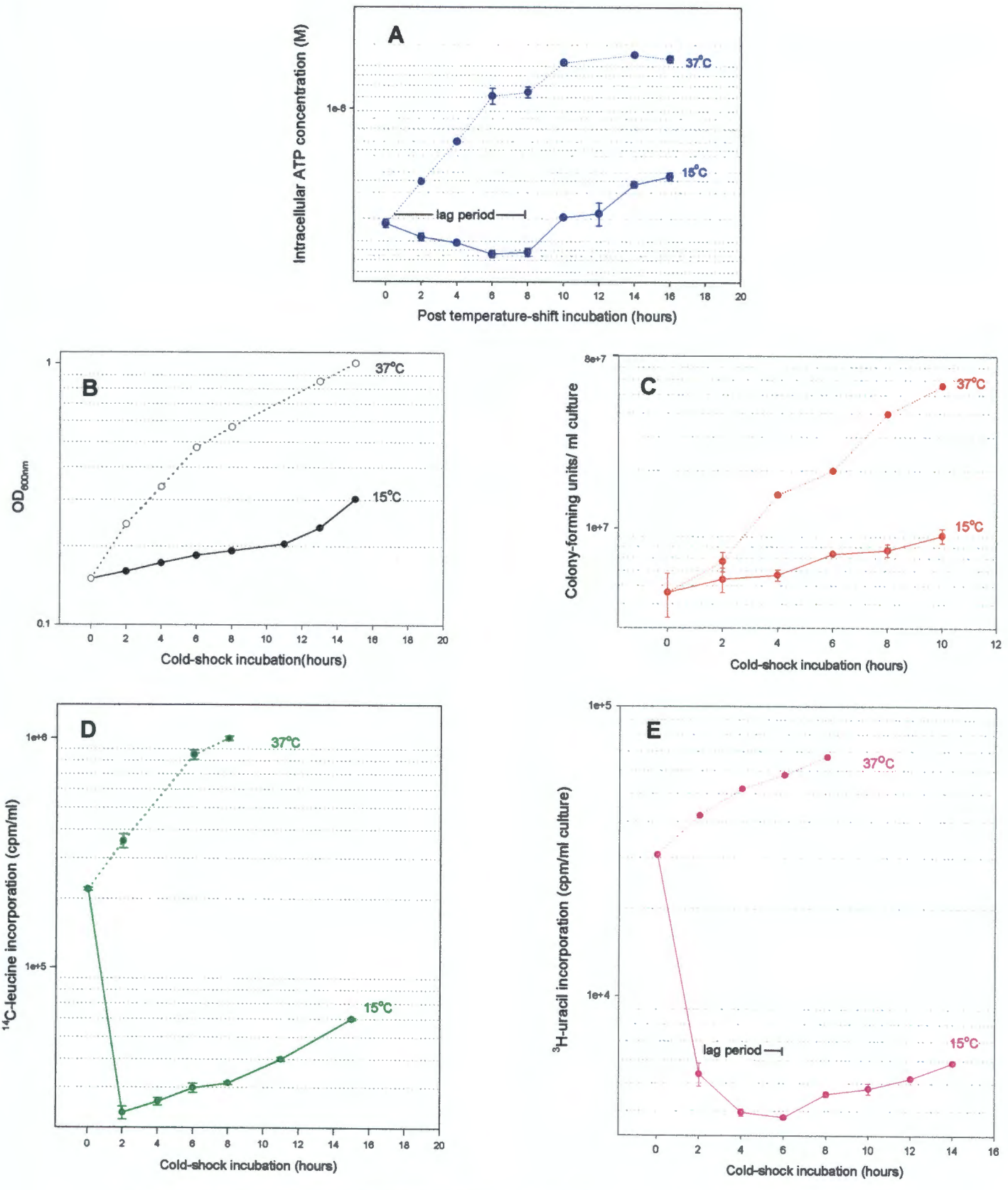


Figure 3.6: The effects of a 37°C→15°C cold shock on the growth of *M. smegmatis*.

An exponentially growing culture (20-24 hours) of *M. smegmatis* (Middlebrook 7H9, ADC, no Tween 80) was divided into two separate cultures. One half was retained at 37°C, while the other was placed in a shaking water-bath set at 15°C. At 3-hourly intervals, post temperature-shift, the ATP concentration (A) and optical density of the culture (B), the number of colony-forming units (C) and the incorporation of ^{14}C-leucine (D) and ^{3}H-uracil into acid-precipitable material (E) was determined (as described in section 3.2) for both cultures. The error bars represent intra-experimental error (SEM).

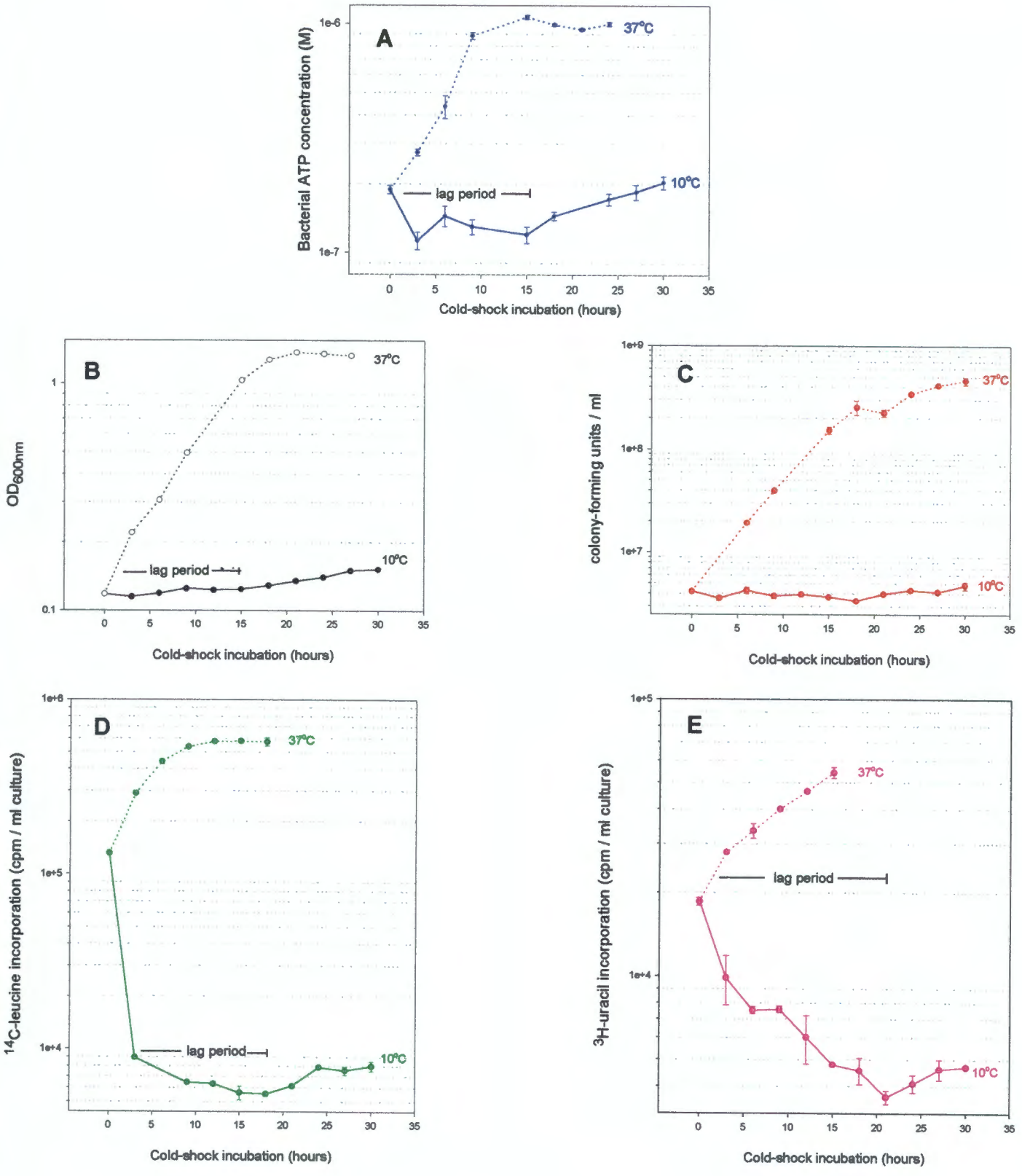


Figure 3.7: The effects of a 37°C→10°C cold shock on the growth of *M. smegmatis*.

An exponentially growing culture (20-24 hours) of *M. smegmatis* (Middlebrook 7H9, ADC, no Tween 80) was split into two separate cultures. One half was retained at 37°C, while the other was placed in a shaking water-bath set at 10°C. At 3-hourly intervals, post temperature-shift, the ATP concentration (A), optical density (B), number of colony-forming units (C), incorporation of ^{14}C-leucine (D) and ^{3}H-uracil into acid-precipitable material (E) was determined (as described in section 3.2). The error bars represent intra-experimental error (SEM).

3.3.2.B.i: Effects of a temperature reduction from 37°C to 20°C on growth and metabolism.

Following this detailed analysis of the cold-shock response, it became evident that although a cold shock from 37°C to 20°C resulted in a lag in the increase of bacterial ATP, it did not result in a lag of the synthesis of macromolecules (Figure 3.5). This 17°C temperature reduction did cause a rapid 3-fold reduction in the synthesis of both RNA and protein macromolecules (Figures 3.5.E and 3.5.D), but the cells immediately showed signs of recovery with no obvious lag in metabolic activity (as measured by the 2-hourly sampling schedule). The optical density of the culture (Figure 3.5.B) and the number of colony-forming units (Figure 3.5.C) demonstrated only a moderate reduction in the growth rate of the bacterium, again with no obvious lag.

3.3.2.B.ii: Effects of a temperature reduction from 37°C to15°C on growth and metabolism.

As with the cold-shock to 20°C, a temperature-shift from 37°C to 15°C resulted in the initial rapid reduction in the synthesis of both RNA and protein molecules (Figure 3.6), but the effect was more severe with the rates of synthesis being reduced by 6-fold and 10-fold, respectively. However, unlike the cold shock to 20°C, a period of 6-8 hours was required before the rate of RNA synthesis increased following the shift to 15°C (Figure 3.6.E). No lag was noted in the synthesis of protein molecules (Figure 3.6.D).

As previously mentioned, following this 22°C temperature reduction the ATP concentration did not increase for 8-10 hours (Figure 3.6.A). Although the cell turbidity did not remain constant during this period, an definite increase in the growth rate was indicated by the optical density readings after 12 hours of incubation at 15°C.

3.3.2.B.iii: Effects of a temperature reduction from 37°C to10°C on growth and metabolism.

The most dramatic changes in the cellular activities of *M. smegmatis* were observed when the temperature was reduced by 27°C, from 37°C to 10°C (Figure 3.7). Protein synthesis was substantially reduced during the initial stages of the cold-shock response (first 3 hours), demonstrating a 20-fold reduction (Figure 3.7.D). Although the response was not as immediate, the synthesis of RNA was also influenced, showing an overall 6-fold reduction during the first 21 hours of the cold shock (Figure 3.7.E). Analogous to the effects of a shift to 10°C on *E. coli* (Shaw and Ingraham, 1967), lag periods were noted in the synthesis of both of these macromolecules, where the levels of synthesis remained constant or steadily decreased for several hours at 10°C. An 18-21 hour lag was noted for the synthesis of protein, while an increase in the synthesis of RNA was only observed after 21-24 hours at 10°C.

This lag in metabolic activity was also reflected in the growth of the organism. In conjunction with the 15-18 hour plateau in ATP levels (Figure 3.7.A), at least 15 hours at 10°C was required before a notable increase was observed in the turbidity of the culture (Figure 3.7.B). During this period of

constant culture turbidity and ATP concentration, no obvious change was seen in the number of colony-forming units, indicating that no cold-induced cell death occurred.

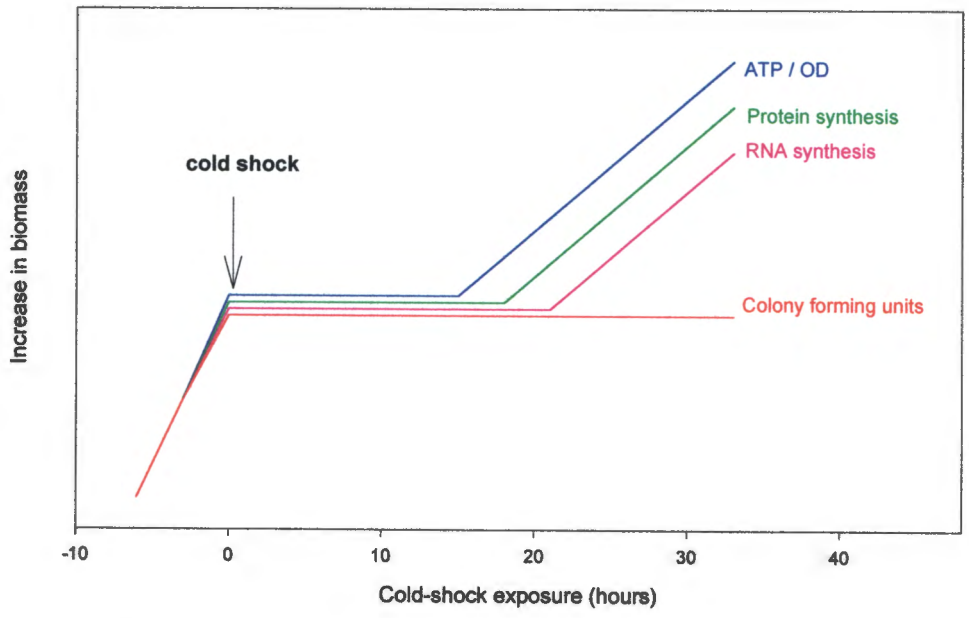


Figure 3.8: Diagrammatic representation of the unbalanced growth resulting from a cold shock in *M. smegmatis*.

Following the observed lag in cellular activity (18-24 hours), which is summarised in Figure3.8, the cells appeared to show an increase in metabolic activity. However, the new rate of growth at 10°C, as measured by the ATP concentration, ^{14}C-leucine incorporation and ^{3}H-uracil incorporation, was greatly reduced. The generation time, as determined by these growth indices changed from 3-4 hours ($v=0.25hr^{-1}\rightarrow 0.3hr^{-1}$) at 37°C, to 20→25 hours ($v=0.04hr^{-1}\rightarrow 0.05hr^{-1}$) at 10°C. Synchronised cell division (as indicated by a step-wise doubling in either OD or colony-forming-units) was not observed during this period of resumed metabolic activity (period: 21-30 hours post temperature-shift).

The growth recovery of *M. smegmatis* at 10°C, observed over a 30-hour incubation period (Figure 3.7), was confirmed by an extended eighteen day period of cold-shock analysis (Figure 3.9). Figure 3.9 demonstrates the rate of steady-state growth at 10°C, as measured by the various growth indices. The rates of growth determined during this longer period of re-growth analysis were considerably slower than those calculated for the first 10 hours of renewed growth at 10°C. As measured by the ATP concentration of the culture and cell turbidity readings (Figure 3.9.A and 3.9.B), the generation time for *M. smegmatis*, at 10°C, increased to 7-8 days ($v=0.0047\rightarrow 0.0057$).

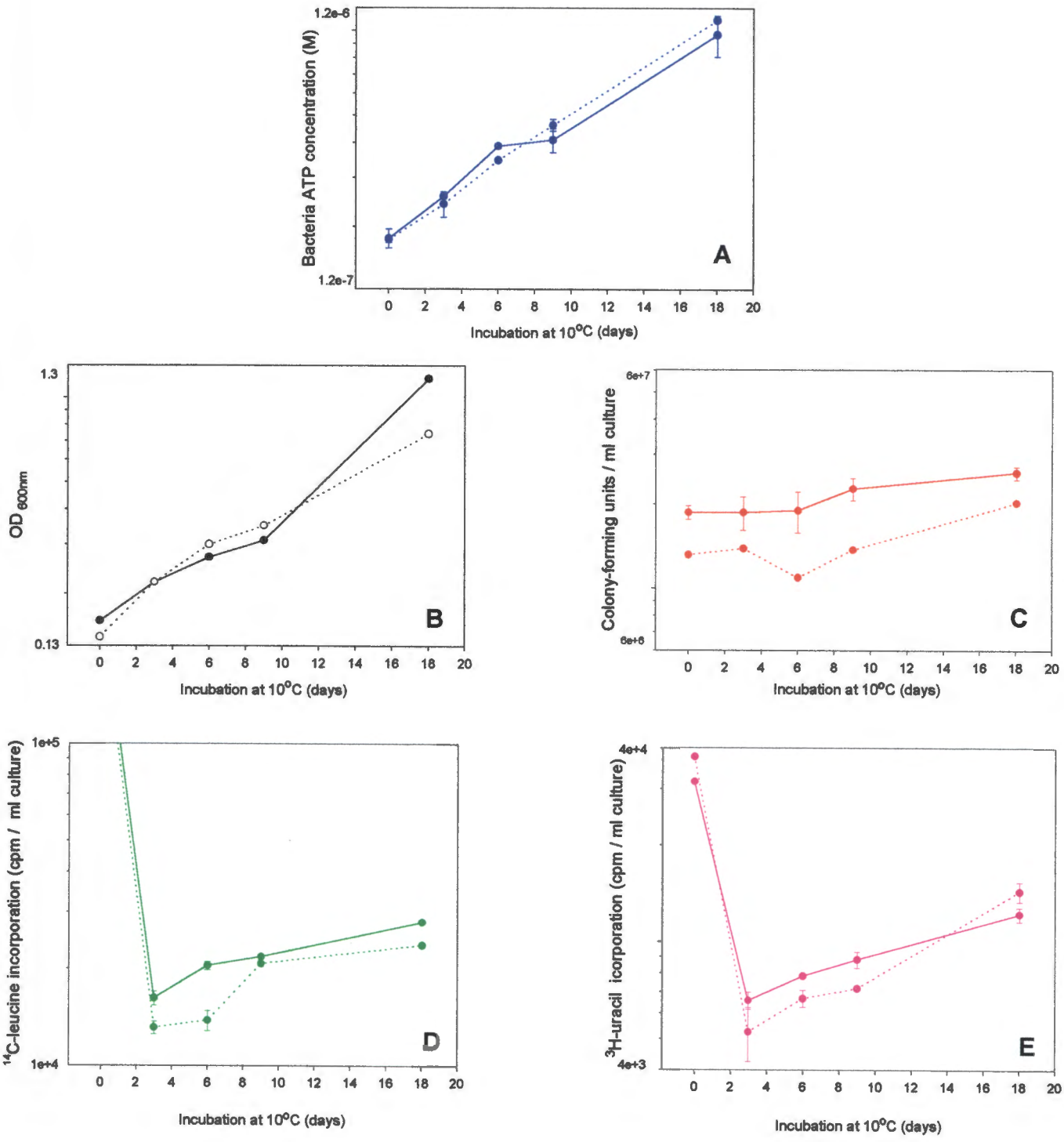


Figure 3.9: Growth recovery of *M. smegmatis* following a 37°C→10°C cold shock.

Two cultures of *M. smegmatis* were grown with aeration at 37°C in Middlebrook 7H9 (ADC, no Tween 80), until the mid-exponential phase of growth (20-24 hours). At this point the cultures were transferred to a shaking water-bath set to 10°C. The growth of both cultures was monitored at 3-day intervals, by determining the ATP concentration (A), optical density at 600nm (B), number of colony-forming units (C), incorporation of ^{14}C-leucine (D) and ^{3}H-uracil into acid precipitable material (E). The results obtained from each separate culture are represented, with the error bars indicating intra-experimental error (SEM).

An extended generation time of 12-13 days (v=0.0031→0.0035) was calculated by the observed increase in the synthesis of both RNA and protein. Although cell division did not appear to be synchronised, an increase in the number of colony-forming units was observed after 9 days incubation at 10°C.

3.4: DISCUSSION.

The cold-shock response in *E. coli* is characterised by a lag period in cellular growth and metabolism, that occurs immediately upon temperature downshift (temperature reduction of greater than 13°C). This lag is primarily caused by a block at the initiation of translation (*Broeze et al.*, 1978; Jones and Inouye, 1996), which leads not only to a lag in protein synthesis, but DNA and RNA synthesis, cell growth and cell division as well (NG *et al.,* 1962; Shaw and Ingraham 1967). Cold-shock proteins are produced during this lag period to relieve the translational blockage (Cold-shock Ribosome Adaptation model – Figure 2.2) (Jones and Inouye, 1996) and allow the bacteria to grow. When *E. coli* cultures are cold-shocked from 37°C to 10°C, a 4 hour lag is observed in the turbidity of the culture (NG *et al.*, 1962) as well as in the synthesis and quantity of total protein. Six hours of incubation are required before an increase in the quantity and synthesis of RNA is observed, with the DNA content of the culture remaining constant for 7 hours post temperature-shift (Shaw and Ingraham, 1967). After 11 hours at 10°C, synchronous cell division occurs (NG *et al.*, 1962; Shaw and Ingraham, 1967). Renewed growth at 10°C proceeds in an unbalanced fashion, with macromolecular synthesis occurring in a specific order: protein, RNA, DNA, before there is any evidence of cell division (Shaw and Ingraham, 1967). This is similar to the situation of a nutrient "up-shift", with RNA synthesis preceding protein synthesis (Mandelstam and McQuillen, 1968). A period of relatively rapid growth and metabolism follows the lag in cellular activity, before the steady-state rate of growth is established at 10°C (Shaw and Ingraham, 1967).

In order to investigate the cold-shock response of *M. smegmatis*, the effects of various temperature down-shifts were measured on both the growth and metabolism of the bacterium. The ultimate aim being the identification of the time period, during which cold-shock proteins would be induced. Not all bacteria respond to a cold shock as *E. coli* does (Chapter 2) and therefore it could not be assumed that *M. smegmatis* would respond as rapidly, or in the same way (block at the initiation of translation, lag in all cellular activities) as *E. coli* (4-6 hour lag period).

The preliminary experiments, which involved the measurement of culture ATP concentrations (Figure 3.4), revealed that a temperature-shift of greater than 13°C, resulted in a lag period in the growth of *M. smegmatis*. As in *E. coli*, the length of the lag period was related to the magnitude of the cold-shock, with a cold-shock from 37°C to 20°C resulting in a 4-6 hour lag period, 37°C to 15°C generating a 8-10 hour lag and a 37°C to 10°C temperature-shift causing a 15-18 hour lag period in growth. The effects of these three temperature down-shifts were investigated further, by monitoring the synthesis of protein and RNA, as well as the increases in cell division (number of colony-forming units) and cell mass (turbidity). These experiments (Figures 3.5, 3.6 and 3.7) demonstrated that only a cold-shock from 37°C to 10°C resulted in a lag in both the growth and metabolism of the bacteria (summarised in Figure 3.8). However, the synthesis of both protein and

RNA molecules was severely affected by the cold-shock at all three temperatures, with an initial drop in the rates of synthesis of both macromolecules observed during the first 3-hour period. The extent of the decreased synthesis was dependant upon the degree of the temperature-shift with protein synthesis decreasing by 3-fold at 20°C to 20-fold at 10°C.

After a temperature-shift from 37°C to 10°C, the ATP and culture turbidity levels remained constant for 15-18 hours, while a reduction was observed in the synthesis of both protein and RNA macromolecules, with the cells requiring 18-21 hours to show an increase in the levels of protein synthesis and 21-24 hours to show an increase in RNA synthesis. During this 24-hour period the number of colony-forming units remained constant. As with the recovery growth of *E. coli* at 10°C, a period of rapid unbalanced growth preceded the steady-state growth at 10°C (Shaw and Ingraham, 1967). The initial division rate (growth rate) at 10°C of $0.04 \rightarrow 0.05 hr^{-1}$, slowed to a steady-state rate of $0.0047 \rightarrow 0.0057 hr^{-1}$, with a predicted generation time of 7-8 days. An increase in the number of colony-forming units was noted after 9 days of incubation at 10°C, but the increase did not represent synchronous cell division. Shifting the bacteria from 37°C to 10°C resulted in a reduction of at least 50 fold in the growth rate of the bacteria (generation time at 37°C = 3-4 hours).

In *E. coli*, although there is an initial block at the initiation of translation (Broeze *et al.*, 1978) and a period of unbalanced growth follows the lag period, the RNA:protein ratio is not substantially altered between growth at 37°C and growth at 10°C (0.64 at 37°C, 0.67 at 10°C). This result implies that the macromolecular composition of the cell remains constant (Shaw and Ingraham, 1967). Some external stresses do however, result in a change in the composition of the cell. An example of this is seen in the nutrient "up-shift" experiments performed in *S. typhimurium* (Kjeldgaard *et al.*, 1958), where the RNA:DNA ratio increased for a period of time before cell division occurred. The 37°C to 10°C cold shock in *M. smegmatis* also led to a change in the macromolecular composition of the cell, with the RNA:protein ratio changing from 0.11 at 37°C to 0.5 at 10°C. As seen in Figure 3.7, the synthesis of protein was dramatically reduced by the temperature-shift , indicating a block in translation. Although synthesis was again resumed after 18-21 hours, the increase in the RNA:protein ratio (decrease in protein:RNA ratio), suggests that this blockage was not completely relieved, and that the cold-shock proteins of *M. smegmatis* are not as efficient as those of *E. coli* in counteracting the effects of the cold shock.

For further studies on the effects of a cold shock on the expression of proteins (Chapter 4) and specific genes in *M. smegmatis* (Chapters 5 and 6), a cold shock from 37°C to 10°C was used. As maximal protein and gene induction was found to occur within the cold-shock lag period in *E. coli*, the time period –1 to 24 hours post cold shock was concentrated upon in the studies in *M. smegmatis*.

CHAPTER 4

COLD-SHOCK PROTEINS OF M. SMEGMATIS

4.1: INTRODUCTION.

In *E. coli*, a distinct set of cold-shock stress proteins is synthesised during the lag in metabolic activity that follows a cold shock. These proteins are essential in restoring ribosome function, preventing the formation of secondary structures in the DNA and RNA and maintaining membrane fluidity (Jones and Inouye, 1994;Panoff et al., 1998). Depending upon the size and time of induction, these proteins can be grouped into different classes. CSPs, cold-shock proteins of less than 10kDa) and CIPs, cold-shock proteins greater than 10kDa, are transiently induced during the lag period (Graumann and Marahiel, 1996). CAPs, cold-acclimation proteins, are produced throughout the response and are required for continuous growth at the lower temperature (Panoff et al., 1998). Determining when these cold-stress proteins are induced helps to elucidate the sequence of events of the cold-shock response. In *E. coli*, the synthesis of at least twenty proteins is induced by a reduction in temperature from 37°C to 10°C, with individual induction levels varying from 2-200 fold (Panoff et al., 1998). The major cold-shock protein of this response is CspA. This small (7.4kDa, 70aa), acidic protein is involved in both DNA and RNA binding (Newkirk *et al.*, 1994; Schindelin *et al.*, 1994; Jiang *et al.,* 1997). Due to the rapid and transient increase in CspA levels (Goldstein *et al.,* 19990), it has been postulated that CspA is the primary regulator of the response, involved in the induction of other cold-shock proteins (La Teana et al., 1991., Jones et al., 1992b). With CspA levels increasing 200 fold at 60-90 minutes post temperature shift (Goldstein *et al.,* 1990), its induction is easily observed on one-dimensional sodium dodecyl sulphate (SDS) polyacrylamide gels, while the induction of the other proteins is only seen through the use of two-dimensional protein analysis.

As indicated in Chapter 3, a substantial period (24 hours) of reduced metabolic activity was demonstrated when a culture of *M. smegmatis* was transferred from 37°C to 10°C. This was shown to be comparable to the "lag period" shown by *E. coli* cells, when cold shocked from 37°C to 10°C, with a reduction in protein synthesis, RNA synthesis and cell division. The next step in the analysis of the mycobacterial cold-shock response was to determine if, like *E. coli*, a set of cold-shock stress proteins was produced during this metabolic lag period. Both one-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis techniques were utilised to analyse the proteins that were synthesised following a cold shock.

4.2: MATERIALS AND METHODS.

Details of the various buffers are listed in Appendix 1.

4.2.1: Culture conditions.

M. smegmatis, strain LR222 (Beggs *et al.*, 1995), was the only cell line used in this section of the study. Cultures of *M. smegmatis* were seeded (1/1000 dilution) from a frozen, stationary-phase, stock culture and grown in 200-1000ml volumes of Middlebrook 7H9 medium (Difco Laboratories), supplemented with ADC (see Appendix 1) and 0.05% Tween 80 (unless otherwise indicated). Following 20-24 hours of aerated growth at 37°C, mid-exponential phase cultures (OD_{600nm} = 0.1) were either placed directly in an enclosed shaking water-bath (180rpm) set at the cold-shock temperature (10°C), or split into two separate culture bottles. One of these was retained at 37°C (control), while the other was transferred to the cold-shock water-bath. The ATP concentration of the culture was measured to assess the growth of the cultures at 37°C and 10°C (section 3.2.2.A). Ziehl-Nielsen mycobacterial stains were performed regularly to exclude culture contamination.

4.2.2: One-dimensional SDS-PAGE.

4.2.2.A: Protein sample preparation.

Newly-synthesised proteins were labelled with L-[^{35}S] methionine. Culture samples (2ml), taken at each time point, were incubated with 1µl (15µCi) of L-[^{35}S] methionine (Amersham, 1000Ci/mmol) at the same temperature as the source culture for 60 minutes. Following the incorporation of the radio-labelled amino acid, the cells were collected via centrifugation (13000rpm, 10 minutes), washed with 0.5ml PBS (pH 7.2) and the cell pellet then resuspended in 100µl SDS loading buffer. These samples were boiled for 30 minutes to lyse the cells and denature the cellular proteins. Protein concentrations were determined using a colourmetric assay based on the method of Lowry *et al.* (1951) (Biorad DC protein assay kit), using dilutions of bovine serum albumin (1.5mg/ml – 0.180mg/ml) as standards.

4.2.2.B: Electrophoresis conditions.

Both glycine (Laemmli, 1970) and tricine (Schagger and von Jagow, 1987) buffer systems were used to separate the radio-labelled proteins. The details of the SDS-polyacrylamide gels (dimensions: 13x16x0.2cm) (12.5% Glycine and 16.5% Tricine) are listed in Appendix 1, along with the corresponding buffer systems. Acrylamide gels were stored at 4°C for 12-24 hours before being used, to ensure complete polymerisation (Weistermeier, 1993). Prior to loading, a 1/10th volume of 0.1% bromophenol blue / 50% β-mercaptoethanol solution was added to the protein samples (20µg), at which point the samples were again denatured at 100°C for 5 minutes. After sample loading, the proteins were electrophoresed at 20mA (constant current) for 30-60 minutes for protein stacking and then for a further 4-16 hours at 40mA (depending upon gel type). Proteins

contained in the Low Molecular Weight Calibration kit (Pharmacia) and the Rainbow™ coloured protein molecular weight marker kit (Amersham) were used as molecular weight standards. Following electrophoresis, the gels were stained with Coomassie® Brilliant Blue R-250 (Biorad), destained with 20% methanol/ 10% acetic acid and left to soak in 6% glycerol/ 20% methanol overnight. The gels were dried under vacuum and exposed to Hyperfilm βMax x-ray film (Amersham) for 3-7 days (-70°C).

4.2.3: Two-dimensional protein electrophoresis.

4.2.3.A: Protein sample preparation.

Twenty millilitre volumes of culture were removed at each time point and incubated with 10µl (150µCi) of L-[^{35}S] methionine, at the same temperature as the source culture for 60 minutes. The cell pellets were collected via centrifugation (13000rpm, 10 minutes), washed in 1ml PBS (pH 7.2) and then resuspended in 1ml PBS (pH 7.2). The bacterial cells were lysed using a Fast-Prep FR120 apparatus (Bio 101, Savant Instruments) (2 x 40 second cycles at speed setting 6, cooling on ice between cycles). Cell debris was removed by centrifugation (13000rpm, 10 minutes). Cellular proteins were then concentrated with the aid of a Centricon-3 filtration unit (Amicon), according to the manufacturer's specifications. Protein concentrations were determined as before (section 4.2.2.A), using the Biorad DC protein assay kit. An aliquot (5µl) of the concentrated protein sample was added to 5ml of scintillation fluid (Insta-gel II Plus (Packard)) and the radioactivity content (cpm) determined using a scintillation counter (Beckman LS 7800).

4.2.3.B: First dimension separation – iso-electric focusing (IEF).

The two-dimensional protein separation system used in this study was performed according to the methods developed by Görg *et al.* (1988 and 1991). Immobiline Drystrips (18cm), pH gradient 3-10 (non-linear) (Pharmacia) were used as the gel medium for the first dimension of IEF, separating proteins according to their iso-electric points. These strips were rehydrated for 6-16 hours at room temperature in rehydration solution before use. Samples containing 2 x 10^6 cpm (≈ 50µg of protein) were mixed with four volumes of 2-D sample buffer and loaded onto the rehydrated strip at the basic terminal. The electrophoresis conditions were as follows: 500V for 5 hours, followed by 3200V for a further 13-14 hours, using a Multiphor II electrophoresis unit (Pharmacia) set to 20°C. Once the IEF procedure was complete, the strips were wrapped in foil and stored at -70°C until needed.

4.2.3.C: Second dimension separation – SDS-PAGE.

Proteins contained within the Immobiline Drystrip were separated according to molecular weight on 10% Tricine SDS-polyacrylamide gels (dimensions: 25x20x0.1cm). The Immobiline Drystips were treated with equilibration solutions A and B (Appendix 1) and placed on top of the SDS-polyacrylamide gels at the anode terminal. The electrophoresis was executed in a Multiphor

II electrophoresis unit (Pharmacia) set to 15°C, using 3MM Whatman chromatography paper wicks soaked in cathode and anode buffer (Westermeier, 1993). The samples were electrophoresed at 20mA for 60 minutes and then the IEF strip was removed. Electrophoresis was continued for a further 8 hours at 40mA. Proteins contained in the Low Molecular Weight Calibration kit (Pharmacia) and the Rainbow™ coloured protein molecular weight marker kit (Amersham) were used as molecular weight standards. The SDS-polyacrylamide gels were silver-stained according to the method of Morrissey (1981), with the glutaraldehyde being omitted to enable mass spectrometric analysis. For sequencing purposes, silver-stained spots were cut out and freeze-dried in 2% acetic acid. To visualise the radioactive proteins, the gels were further soaked in 6% glycerol/ 20% methanol overnight, dried under vacuum at 50°C and exposed to Hyperfilm βMax X-ray film (Amersham) for 3-7 days (-70°C).

4.3: RESULTS.

4.3.1: One dimensional SDS-PAGE analysis of cold-shock proteins.

M. smegmatis proteins that were synthesised following a cold-shock from 37°C to 10°C were labelled by the incorporation of L-[^{35}S] methionine. This was performed to determine if a cold shock resulted in changes to the protein composition of the mycobacterial cell. The newly-synthesised proteins were initially analysed on one-dimensional SDS-polyacrylamide gels to obtain an overview of the cold-shock response. Two-dimensional protein analysis was then used to investigate the protein induction in more detail at specific time points.

The quantity of radioactive amino acid and duration of incubation required to label the mycobacterial proteins was determined experimentally, as the quantities of radioactivity (100-150μCi/1ml sample) and 5-30 minute labelling times used in *E. coli* studies were found to be unsuitable (Jones *et al.*, 1987; Jones *et al.*, 1996; Lelivelt and Kawula, 1995). As seen with the analysis of the rates of protein synthesis in Chapter 3 (section 3.3.2), a cold shock resulted in a rapid reduction in the rate of protein synthesis in *M. smegmatis*. This was found to have an affect on the radioactive labelling of the cold-shock proteins, with longer incubation times required to label the proteins sufficiently. Using a 2ml sample of culture, 15μCi of L-[^{35}S] methionine and 60 minutes incubation time (37°C and 10°C) were found to be sufficient to generate a signal that could be detected within a reasonable period of time (3-7 days).

4.3.1.A: Protein analysis of the 37°C to 10°C cold shock.

Protein synthesis was analysed at various time points, after the transfer of exponentially-growing cultures of *M. smegmatis* from 37°C to 10°C. Protein profiles were obtained hourly for the first 10 hours of the cold shock and also at 3-hourly intervals, to span the first 30 hours of the response. To monitor the long-term effects of the cold shock, protein synthesis was also investigated at 48, 72, 96, 120, 144 and 168 hours post-temperature shift. As controls, the protein profile of the culture was obtained prior to transfer from 37°C to 10°C (-1 → 0 hours) and during continued growth at 37°C (3-hourly intervals). The growth of the cultures were monitored by determining the cellular ATP concentration at each time point, to ensure that the culture was reacting to the cold shock as demonstrated in Chapter 3.

4.3.1.A.i: Protein analysis of the cold-shock lag period.

It was expected that *M. smegmatis* would react like *E. coli* and produce the most dramatic protein changes during the metabolic "lag period" following a cold shock. Because of this, most of the protein labelling experiments were concentrated on the period: 0 - 30 hours following the culture transfer to 10°C, to enable analysis of the lag period (24 hours) (section 3.3.2.B.iii) and several hours of recovery growth. Proteins were separated using both glycine and tricine buffer systems to

enable analysis of the entire protein profile (200kDa - 1kDa). Figure 4.1 shows the typical ^{35}S-methionine-labeled protein profile of cultures cold-shocked from 37°C to 10°C.

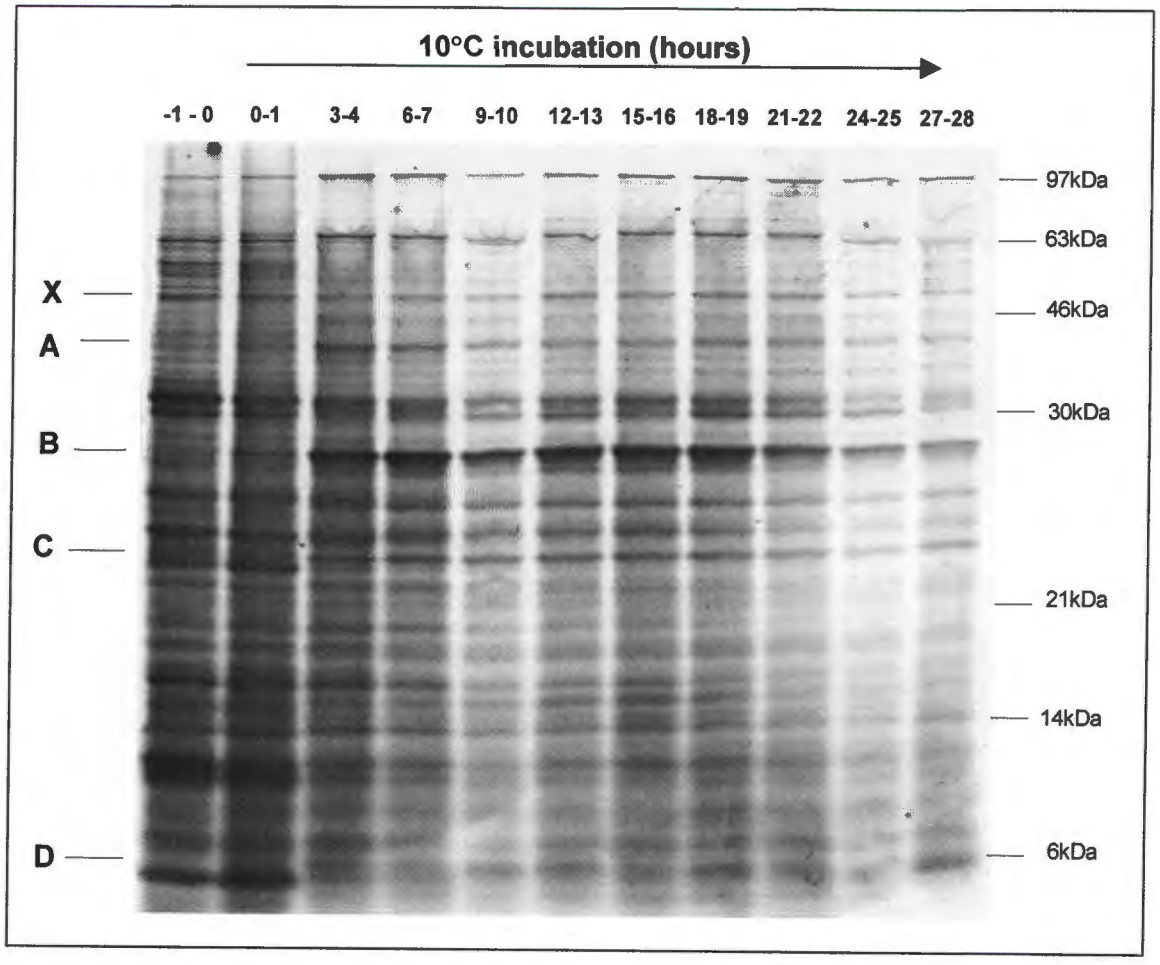


Figure 4.1: Cold-shock proteins of *M. smegmatis* (37°C→10°C).

Autoradiograph of a 16.5% SDS-polyacrylamide tricine gel, containing cellular proteins of *M. smegmatis* labelled with L-[^{35}S] methionine before (t=-1→0 at 37°C) and after a cold shock from 37°C to 10°C (0-28 hours). At the times indicated, aliquots of cell culture (2ml) were taken out of the bulk culture and incubated with 1µl (15µCi) of L-[^{35}S] methionine for 60 minutes. A-E represent the major cold-shock proteins, while X indicates a control band (no induction).

It was evident that although overall protein synthesis appeared lower following a cold shock, the number of proteins synthesised at 37°C and 10°C varied very little. Another interesting finding was that instead of a 6-8kDa protein band (CspA) being the dominant protein induced following a cold shock in *M. smegmatis*, the most obvious induction occurred in the synthesis of a 27kDa band, designated protein B.

No major changes seemed to occur within the first hour of the cold shock, but after several hours at 10°C the increased synthesis of at least four proteins was evident. These included a 43kDa protein (A), a 27kDa protein (B), a 22kDa protein (C) and a 5-7 kDa protein (D), which were all induced within the first 3-4 hours of the response (Figure 4.1).

The cold-shock protein profiles obtained in this experiment were shown to be highly reproducible, with all four protein bands showing similar induction patterns in at least three separate experiments. It was also determined that these changes were not a result of culture ageing, as demonstrated by the synthesis of proteins during growth at 37°C (results not shown).

Figure 4.2 shows the induction profiles of the major cold-shock protein band, B (27kDa) and protein X, a control band whose synthesis was unaffected by the temperature shift. Protein B was induced as early as 2-3 hours post-temperature shift, with the rate of synthesis increasing until 9-12 hours of incubation at 10°C. At this point, this protein band comprised approximately 20 - 25% of the total protein synthesised at 10°C, as opposed to 1-2% of total protein at 37°C. This represented an increase of at least 10-12 fold. This substantial change suggests that this protein is important in the cold-shock response of *M. smegmatis*.

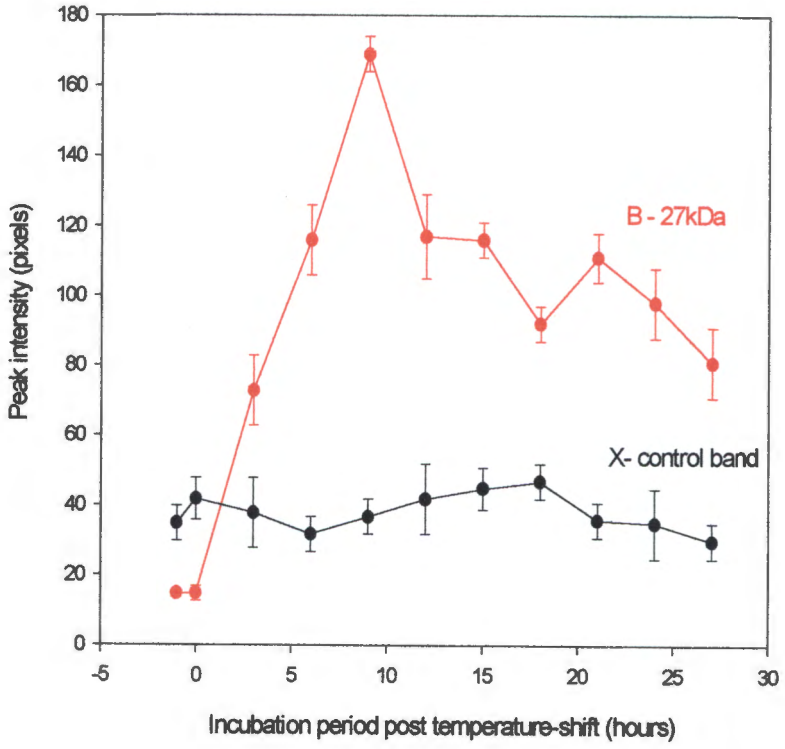


Figure 4.2: Induction profiles of the cold-shock protein B and control protein X.

Autoradiographs resulting from the analysis of L-[^{35}S] methionine-labelled proteins were scanned with a densitometer and the peak intensities (maximum intensity - background intensity) of protein bands B and X were calculated for each time point. The mean band intensity of three experiments was plotted at each time point. Errors bars represent the SEM.

In section 3.3.1.C, it was demonstrated that the presence of Tween 80 in the culture medium was associated with bacterial clumping. The protein-labelling experiments at 10°C were repeated with cultures grown in the presence and absence of Tween, to determine if the formation of clumps caused the observed protein changes. No differences were seen in the cold-shock protein profiles of + Tween / -Tween cultures. Due to the availability of Middlebrook 7H9 + Tween 80 medium, and because the presence of the Tween had no visible effect on the protein composition of the culture, all further studies were performed on cultures grown in the presence of Tween 80 (protein and RNA studies).

4.3.1.A.ii: Seven-day protein analysis of the cold-shock response.

The next step in the protein analysis was to confirm that the majority of the protein changes caused by the cold shock from 37°C to10°C were restricted to the period of reduced metabolic activity (0-24 hours). This question was addressed by performing a 7-day analysis of the proteins synthesised at 10°C. Proteins were radio-labelled prior to the 37°C→10°C transfer (-1→0 hours), as a control, and then at 24-hourly intervals after the cold shock (10°C incubation). The experiment was repeated three times, using glycine and tricine separation systems to enable analysis of the entire protein profile (200kDa - 1kDa). Figure 4.3 shows the results of one of the week-long analysis experiments, with the proteins separated on a 12.5% glycine-SDS polyacrylamide gel. Using these one-dimensional separation systems, no new protein bands or obvious induction could be seen when comparing the 24-hour cold-shock sample with the rest of the longer-term samples.

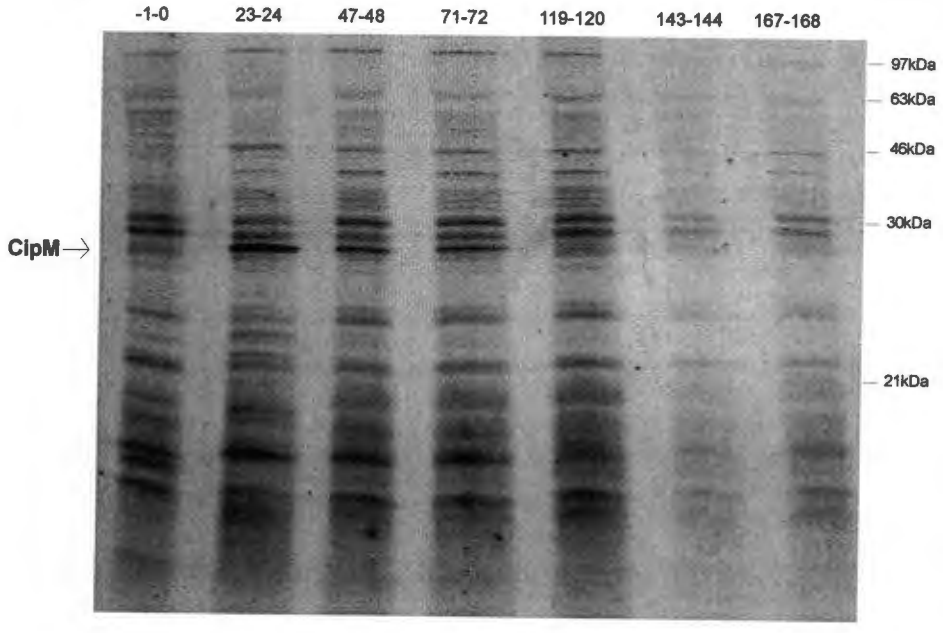


Figure 4.3: Seven-day cold-shock protein analysis.

Autoradiograph of a 12.5% SDS-polyacrylamide glycine gel, containing cellular proteins of *M. smegmatis* labelled with L-[^{35}S] methionine before (t=-1→0) and after a cold shock from 37°C to 10°C (0-168 hours - 24-hour intervals). At the times indicated, aliquots of cell culture (2ml) were taken out of the bulk culture and incubated with 1µl (15µCi) of L-[^{35}S] methionine for 60 minutes at 37°C (t=-1→0 hours) or 10°C (0-168 hours).

Some differences were however, observed when the culture was shifted from 37°C to 15°C. Figure 4.4 shows the protein profile of a 37°C→15°C temperature shift (Figure 4A) compared to that of a 37°C→10°C cold shock (Figure 4B). It is evident that the cold shock to 10°C gave the strongest protein response, with only CipM (protein B) being reproducibly induced by the 37°C→15°C temperature shift. The magnitude of induction of this protein was also greater at 10°C, as opposed to 15°C.

4.3.2: Two-dimensional analysis of cold-shock proteins.

Due to the limited separation capabilities of one-dimensional protein analysis by SDS-PAGE (separation based on molecular weight only), this technique is only useful in monitoring gross protein changes, like the induction of CspA (200-fold induction) in *E. coli*. Detecting more subtle changes (2-5 fold) in the protein composition of bacterial cells requires the complete separation of all the protein components. This can be achieved using two-dimensional protein electrophoresis, where the proteins are separated according to both their iso-electric points (pI) and molecular weights. Using this technique, the induced synthesis of at least fourteen different proteins was evident following a cold shock in *E. coli* (Jones *et al.*, 1987).

For the two-dimensional analysis of cold-shock proteins of *M. smegmatis*, key time points, which demonstrated significant protein changes, were selected for further analysis from the one-dimensional SDS-PAGE experiments. These included: -1→0 hours (37°C), 0→1 hours (10°C), 3-4 hours (10°C), 10-11 hours (10°C), 18-19 hours (10°C), 23-24 hours (10°C), 28-29 (10°C) and 48-49 hours (10°C).

4.3.2.A: Effects of a cold shock on protein synthesis in *M. smegmatis*.

The two-dimensional analysis of the time-course mentioned above, was performed twice (separate cultures), along with the selected analysis of two or more time points in several other cultures. The results of the two-dimensional analysis are shown in Figures 4.5 and 4.6. Although the first dimension separation (IEF) was performed over a pH range of 3-10, the majority (99%) of the mycobacterial proteins were detected in the pH 3-8 range, with no cold-shock proteins detected in the pH 8-10 range. These figures therefore only show the proteins present in the 3-8 pH range. Figure 4.5 supports the results shown in the one-dimensional SDS-PAGE analysis (Figure 4.1), indicating that few changes are seen in the overall protein composition of the cell during the first hour of the cold-shock response.

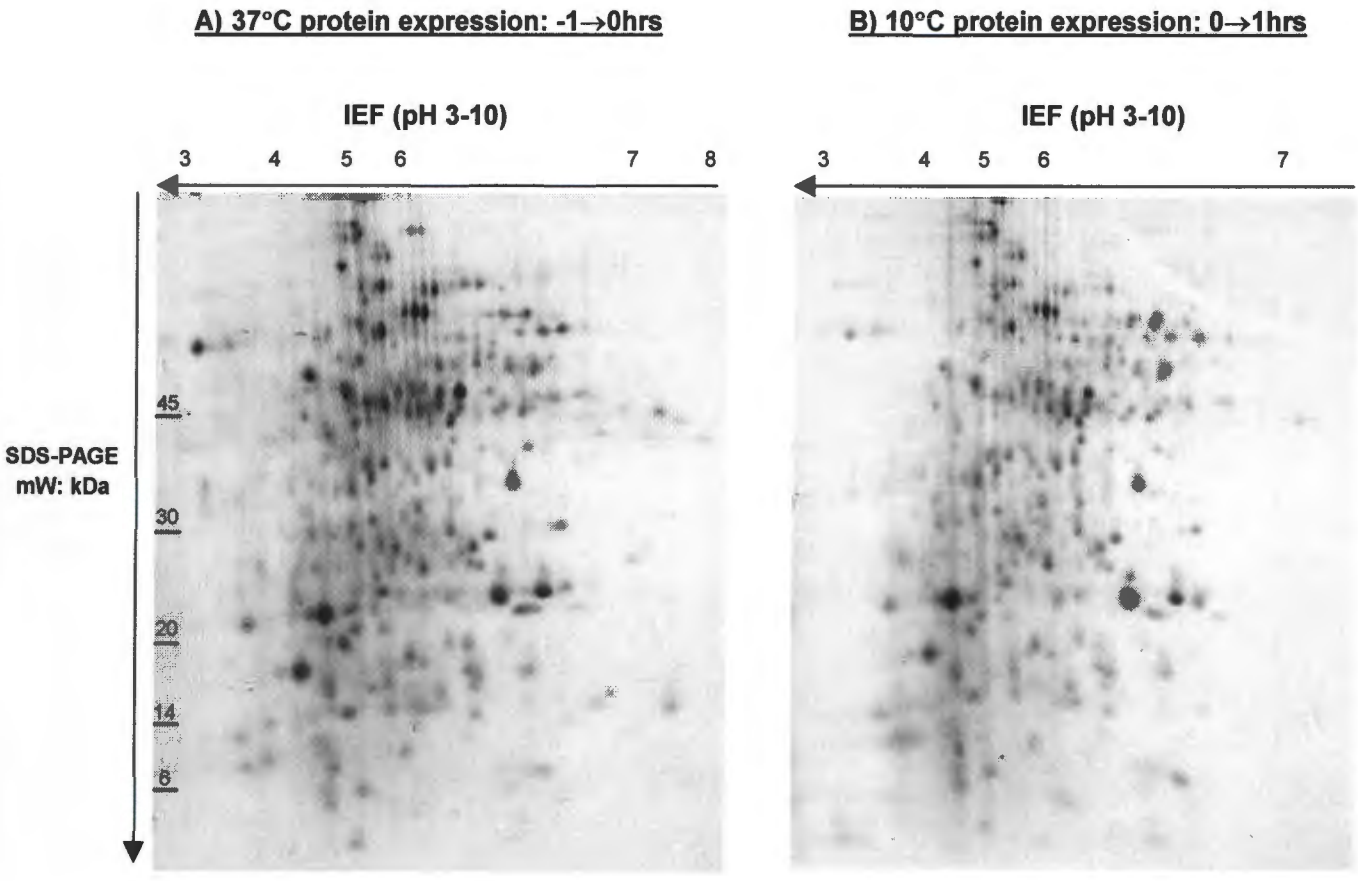


Figure 4.5: Two-dimensional analysis of the cold-shock proteins of *M. smegmatis*: first hour analysis.

Autoradiographs from the two-dimensional separation of protein extracts from L-[^{35}S] methionine - labelled *M. smegmatis* cells (pH 3-10 first dimension, 10% SDS-polyacrylamide tricine gel for the second dimension). **A)** Exponentially growing cultures of *M. smegmatis* were radiolabelled at 37°C for 60 minutes (-1→0hr), prior to the cold shock. **B)** Following the transfer of the same culture from 37°C to 10°C, the cells were immediately incubated with ^{35}S methionine for 60 minutes (0→1hr). Pre-stained standards (Amersham) were used as protein molecular weight markers.

Figure 4.6 shows the results of the two-dimensional separation of *M. smegmatis* proteins synthesised during the cold-shock time-course analysis (0-1hr, 10-11hr, 18-19hr and 28-29hr). Using this high resolution protein separation system, it became evident that the synthesis of a greater number of proteins was affected (by 10-11 hours at 10°C) than was originally indicated by the one-dimensional analysis. In addition to the large number of proteins whose synthesis was reduced by the temperature shift, the synthesis of at least fourteen proteins was reproducibly induced following the cold shock from 37°C to 10°C. These proteins were arbitrarily numbered 1-14, so that their synthesis could be specifically monitored during the cold shock.

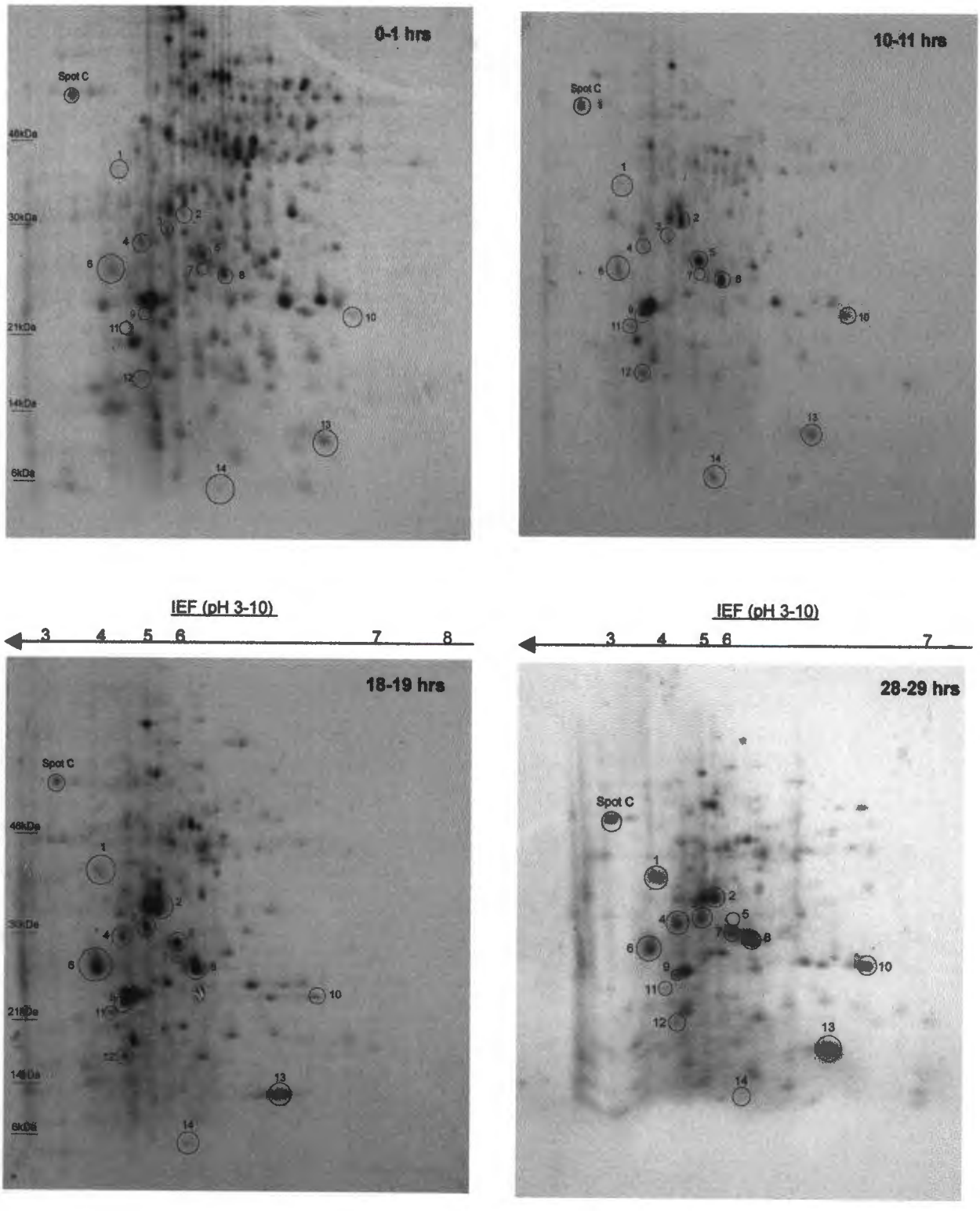


Figure 4.6: Two-dimensional analysis of the cold-shock proteins of *M. smegmatis*: Time-course analysis.

Autoradiographs from the two-dimensional separation of protein extracts from L-[^{35}S] methionine - labelled *M. smegmatis* cells (pH 3-10 first dimension, 10% SDS-polyacrylamide tricine gel for the second dimension). Exponentially growing cultures of *M. smegmatis* were shifted from 37°C to 10°C (t=0) and the proteins synthesised at various time points were analysed. At the post cold-shock times indicated 20ml culture volumes were incubated with 150μCi ^{35}S methionine for 60 minutes at 10°C. Proteins induced by the cold shock are circled and numbered 1-14. Spot C was used as a control for quantification purposes.

4.3.2.B. Classification of the cold-shock proteins of *M. smegmatis.*

To analyse the expression patterns and magnitude of induction of the cold-shock proteins, the protein spots were scanned and the densities calculated. To control for errors in sample loading, these densities were corrected according to the densities of an internal control spot, which was not obviously affected by the cold-shock. Figure 4.7 indicates the pattern of cold-shock expression of these proteins, as obtained from the spot densities of one complete time-course analysis. Similar patterns of expression were obtained when this time-course analysis was repeated.

Table 2 gives a summary of the nature of the cold-shock expression and magnitude of induction, as determined from the data presented in Figure 4.7.

Table 2: Classification of the cold-shock proteins of *M. smegmatis.*

Protein spot	Size (kDa)	Fold induction	Expression pattern[a]	Classification [b]
1	40-44	5-6	Transient	CIP
2	33-35	6-7	Transient	CIP
3	30-32	3-4	Continuous	CAP
4	28-30	4-5	Continuous	CAP
5	27-30	2-3	Transient	CIP
6	24-26	2-3	Continuous	CAP
7	26-28	11-12	Transient	CIP
8	24-26	4-5	Continuous	CAP
9	20-22	>7[c]	Transient	CIP
10	20-22	6-7	Continuous	CAP
11	19-21	>7[c]	Transient	CIP
12	15-17	6-7	Transient	CIP
13	10-12	4-5	Continuous	CAP
14	6-9	>7[c]	Transient	CSP

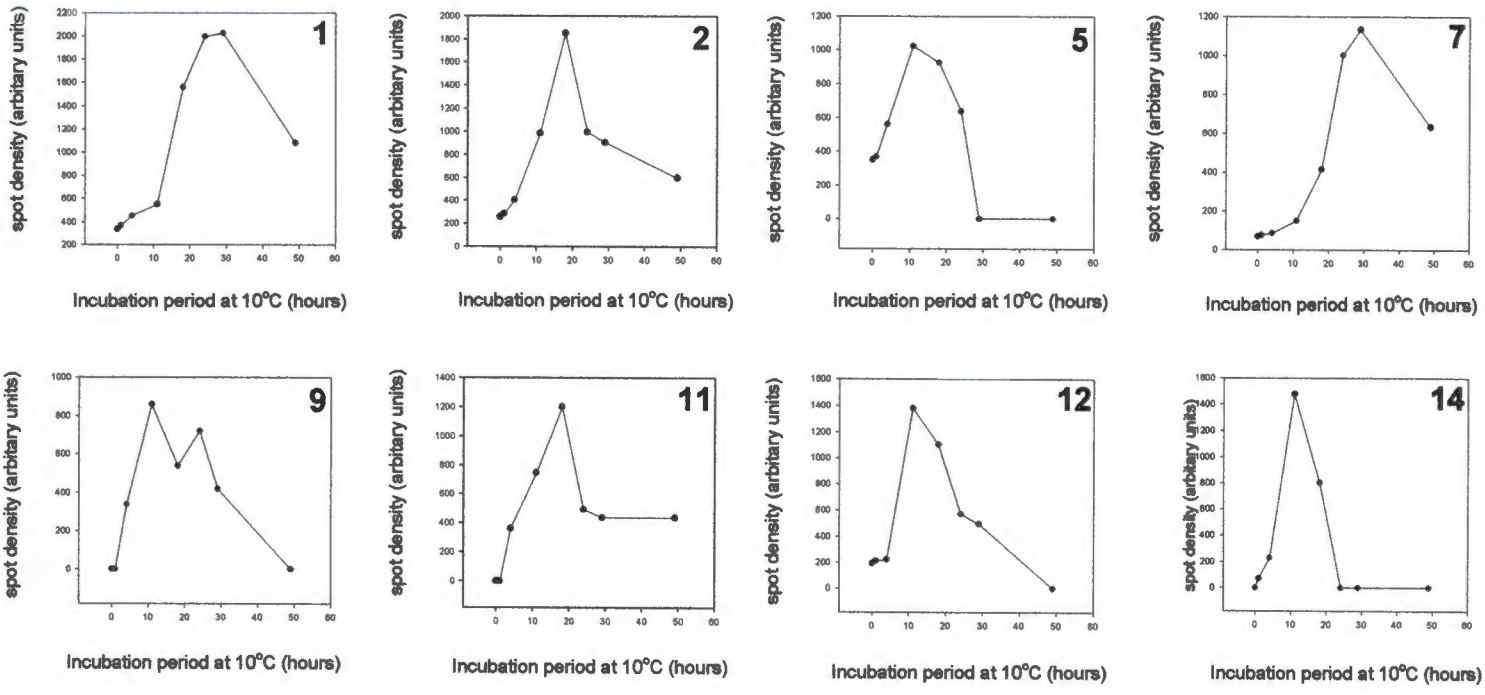
a: transient = peak expression during the 24 hour lag period; continuous= increased expression during re-growth period (28-29hrs and 48-49hrs).

b: CAP: continuous increased expression; CIP: transient expression, >10kDa; CSP: transient expression, <10kDa.

c: exact cold-shock induction levels could not be calculated due to the lack of a detectable signal at 37°C.

Most of the cold-shock proteins showed induction levels in the 2-7 fold range. However, four proteins showed increased levels of synthesis of greater than 7-fold and can therefore be classified as the major cold-shock proteins of *M. smegmatis*: proteins 7, 9, 11 and 14. Three of these proteins, 9, 11 and 14, were unique to the cold-shock response (not detectable at 37°C).

A) Transient induced expression: CSP / CIP



B) Continuous induced expression: CAP

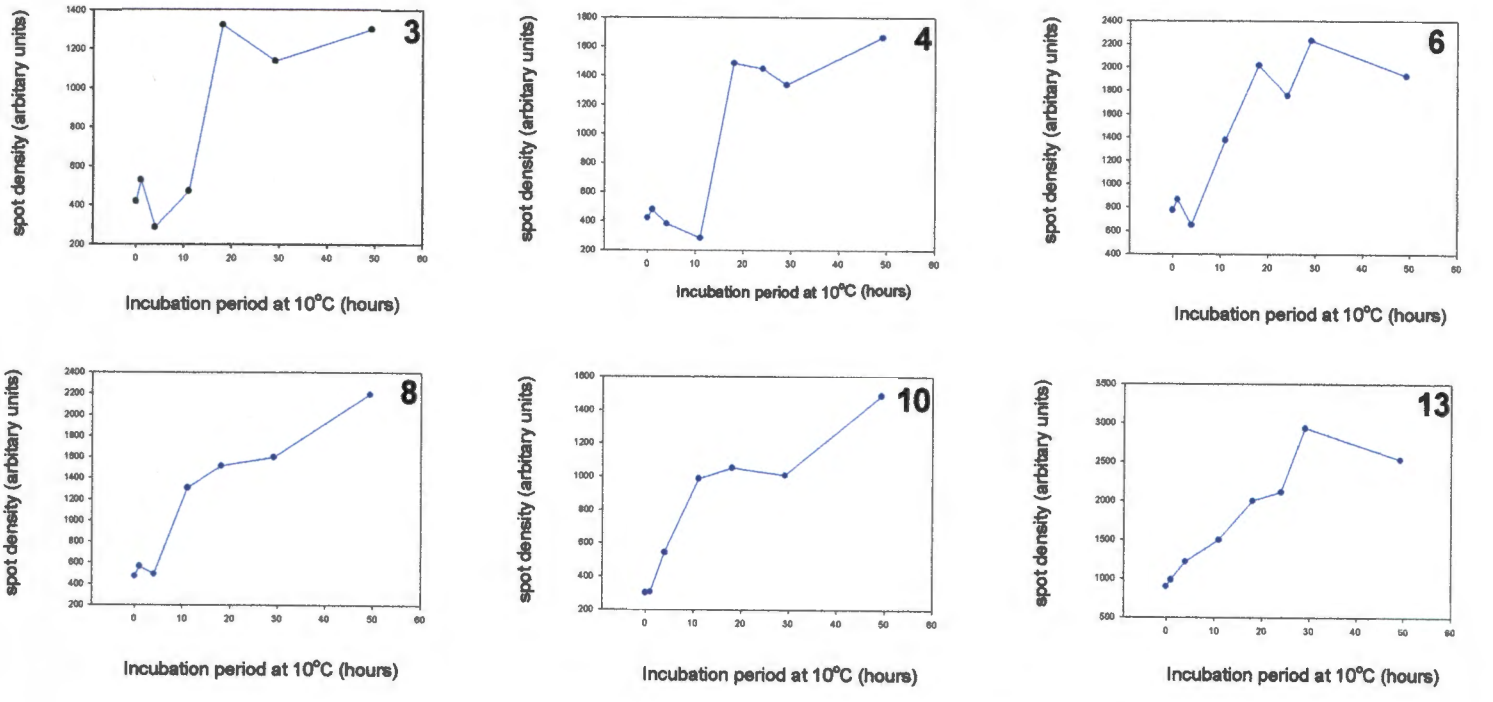


Figure 4.7: Induction-profiles of *M. smegmatis* cold-shock proteins.

Autoradiographs resulting from the two-dimensional analysis of cold-shock proteins, during the time-course analysis, were scanned using a densitometer and the values were corrected according to the density of control spot C (Figure 4.6). The spot densities were then plotted against incubation time at 10°C, after the 37°C to 10°C temperature-shift. Time 0hr = spot densities at 37°C, prior to the shift.

Two different patterns of cold-shock expression were demonstrated by these fourteen proteins (Table 2). Eight proteins (1, 2, 5, 7, 9, 11, 12, 14) exhibited transient induction, with their increased synthesis peaking during the putative metabolic lag period of 24 hours. This group of proteins could therefore be classified as cold-shock (CSP) or cold-induced proteins (CIP), depending upon their size (less than 10kDa or greater than 10kDa, respectively) (Table 2). The other six cold-shock proteins that were analysed (3, 4, 6, 10 and 13), demonstrated an expression pattern indicative of cold-acclimation proteins. All six showed elevated levels of synthesis during the first 24-hour period of the cold shock, but these proteins continued to be synthesised at elevated levels well into the growth recovery period. This result implies that these proteins are required for continuous growth at 10°C and can be designated as CAPs.

Although the number of time points was limited, the analysis of the 28-29 and 48-49 hour time points revealed that the cold-shock proteins of *M. smegmatis* were all induced within the first 24-hour period of the cold shock. No new cold-shock proteins were detected beyond 24 hours. This finding is in agreement with the results obtained with the one-dimensional analysis.

.4.3.2.C: Cold-shock induction of CspA.

Through the incorporation of L-[^{35}S] methionine, a 6-9kDa protein was identified as a transiently expressed cold-shock protein (Csp14). Its rapid, transient induction (Figure 4.7) was similar to that of CspA (7.4kDa) of *E. coli*, but its level of induction was clearly not as great as CspA, which constitutes up to 13% of the total protein synthesised during a cold shock (Goldstein *et al.*, 1990). Although the protein levels were increased, the final protein concentration of Csp14 was not sufficient to allow detection of the protein spot upon silver-staining. Attempts were made to use larger sample volumes for the two-dimensional analysis, but total protein concentrations of greater than 50μg gave distorted protein spots. The characterisation of Csp14 as a CspA homologue, through sequence analysis, could therefore not be achieved.

CspA of *E. coli* contains two methionine residues. The first is cleaved off during post-translational modifications (Goldstein *et al.*, 1990), but the presence of a second methionine residue enables this cold-shock protein to be labelled with L-[^{35}S] methionine. If *M. smegmatis* does in fact produce a CspA homologue that does not contain a second methionine residue, as is the case with the *Streptomyces* CspA homologue (Av-gay *et al.*, 1992), then it is possible that this protein would not be radioactively labelled.

To investigate the existence of a methionine-free CspA homologue in *M. smegmatis*, the second dimension SDS-polyacrylamide gels were silver-stained, revealing the complete set of bacterial proteins at any one time-point. Figure 4.8 shows the clear induction of a 8-11kDa protein (unlabelled). This protein (spot 15) was undetectable during growth at 37°C and during the first

hour of the cold shock, but was present in substantial quantities after 11 hours of incubation at 10°C. Protein 15 was detectable for approximately 8 hours (present at the 17-18 hour analysis), after which time it fell below detection levels. Due to the variability in sensitivity that was encountered with the silver-staining technique, accurate levels of induction could not be determined. However, as can be seen from Figure 4.8, the cold-shock induction of this protein was substantial.

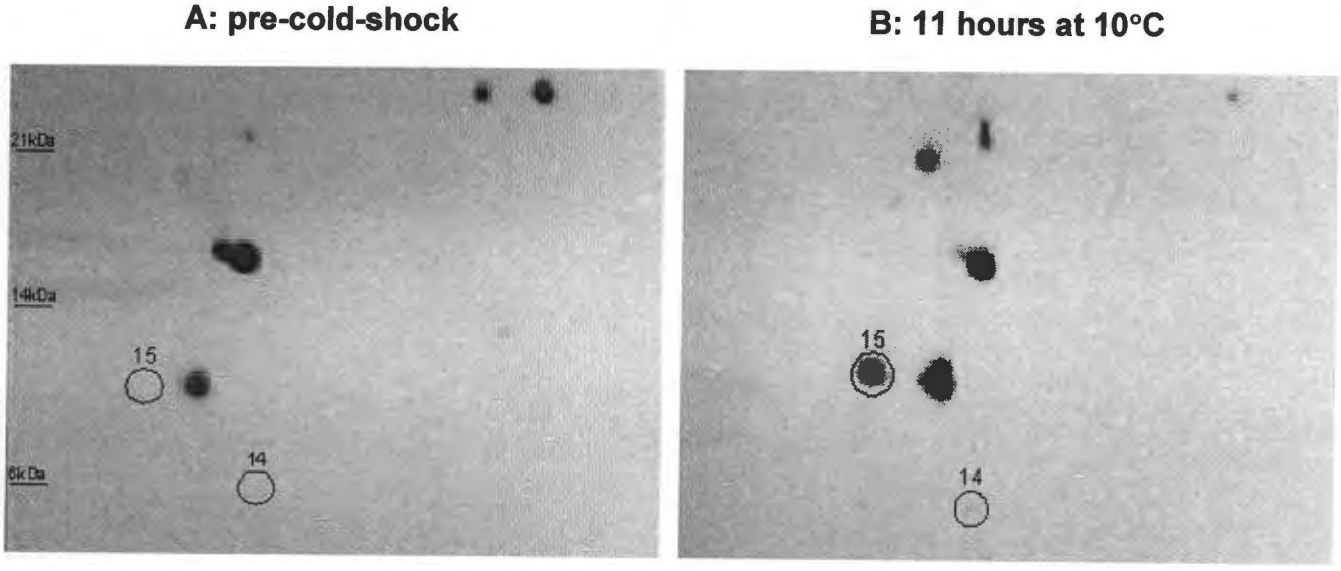


Figure 4.8: Cold-shock induction of a 8-11kDa protein (non-radio-labelled) in *M. smegmatis*.

(A) Photograph of the silver-stained cellular proteins (6-21kDa) of *M. smegmatis* present at 37°C (pre-cold-shock) and **(B)** after 11 hours incubation at 10°C (cold shock). Proteins were separated in the pH range 3-10 in the first dimension (IEF) and on 10% SDS-polyacrylamide tricine gels in the second dimension. The positions of the 8-11kDa protein (15) is indicated in relation to the position of the radio-labelled Csp14.

4.3.2.D: Identification of CipM.

According to the data presented in section 4.3.1, the major cold-shock protein of *M. smegmatis* was CipM, a 27kDa protein. Due to the separation limitations of the one-dimensional technique it was possible that this protein band contained more than one cold-shock protein. Two-dimensional analysis revealed that several proteins in the 25-30kDa size range were actually induced (4, 5, 6, 8), with no single protein showing the pattern of expression observed with CipM (Figure 4.2). It is therefore probable that the CipM band is composed of several of these proteins. As a means to identify the cold-shock proteins contained within this band, N-terminal sequence data was generated for one of the proteins contained within the one-dimensional CipM band. The details of this analysis are presented in Chapter 6.

4.4: DISCUSSION.

The analysis of the effects of a cold shock on the growth of *M. smegmatis* demonstrated that, like *E. coli*, a 37°C to 10°C temperature-shift resulted in a lag in the growth and metabolism of the bacteria (24 hours) (Chapter 3). In *E. coli*, the lag period is characterised by the induced synthesis of at least twenty cold-shock proteins (Jones *et al.,* 1992a; Panoff *et al.,* 1998). The results of the protein analysis of the cold-shock response in *M. smegmatis*, as described in this chapter, indicate that a set of at least fifteen cold-shock proteins is also induced during the lag period of this mycobacterial cold-shock response.

As previously described, as the magnitude of the cold shock is increased (37°C→25°C to 37°C→10°C), the metabolism of the *M. smegmatis* culture is more severely affected (section 3.3.2). This was shown to be the case with the induction of *M. smegmatis* cold-shock proteins as well (one-dimensional SDS-PAGE protein analysis). The cold shock from 37°C to either 25°C or 20°C did not noticeably change the protein composition of the cell, suggesting that these temperatures were well within the linear growth range of *M. smegmatis* (Berger *et al.*, 1996). The cold shock from 37°C to 10°C however, generated a significantly different protein profile, with the induction of a specific set of cold-shock proteins. As with *E. coli*, some of these cold-shock proteins were also induced at 15°C (one dimensional protein analysis), but the magnitude of the response was reduced. The amplitude of the cold-shock response of *M. smegmatis* (cold-shock protein synthesis and length of metabolic lag period) was therefore found to be dependent upon the degree of the cold shock, as is the case with the cold-shock response of *E. coli* (Jones *et al.,* 1987; Jones *et al.*, 1992a).

Both the one-dimensional and two-dimensional protein analysis showed the induction of several proteins, cold-shock proteins, during the first 24 hours of the cold shock from 37°C to 10°C. These proteins demonstrated elevated levels of synthesis against a backdrop of reduced global protein synthesis. No new cold-shock proteins were observed after this period. As indicated by the one-dimensional SDS-PAGE protein analysis, CipM, a 27kDa protein band, rather than a 6-8kDa protein (CspA, CspB - major cold-shock proteins of *E. coli* and *B. subtilis,* respectively) was preliminarily identified as the major cold-shock protein of *M. smegmatis*. This protein band comprised up to 25% of the total protein that was synthesised after 10 hours of incubation at 10°C. The pattern of expression demonstrated by this protein band indicated a transient cold-shock induction, hence this protein was labelled as cold inducible (cold-shock protein > 10kDa) (Graumann and Marahiel, 1996). Two dimensional protein analysis defined the induction of cold-shock proteins more clearly. Fouteen, ^{35}S-methionine-labelled, cold-shock proteins were shown to be reproducibly induced within the first 24 hour period after a 37°C to 10°C shift, with a further cold-shock induced protein, protein 15, identified through silver-stain analysis. The synthesis of the fourteen radio-labelled proteins was analysed further. Most of the proteins

demonstrated 2-7 fold cold-shock induction in their levels of synthesis, while four exhibited increases of greater than 7-fold, proteins 7, 9, 11 and 14 (Table 2), and were thus designated "major cold-shock proteins". As several cold-shock proteins migrated in the 25-30kDa size range, the major cold cold-shock protein, CipM, identified in the one-dimensional analysis, was probably composed of several of cold-shock proteins. The identification of one of the cold-shock proteins contained within this band is described in Chapter 6.

Using the technique of matrix-assisted laser desorption/ionisation – time of flight mass spectrometry (MALDI-TOF MS) attempts were made to sequence the cold-shock proteins from silver-stained, second dimension SDS-polyacrylamide gels. Silver-stained spots corresponding only to proteins 5, 8, 13 and 15 were identified and although protein spots were excised from five separate gels to provide ample protein, no sequence data could be generated by the mass spectrometry analysis (performed by Dr Betts, Glaxo-Wellcome, Stevenage, UK). The lack of data could be due to a combination of reasons including artefacts that are introduced by the staining technique and keratin contamination. Proteins of less than 15kDa also only generate a few peptides for mass spectrometry analysis, which makes analysis difficult (personal communication from Dr Betts, Glaxo-Wellcome).

Two patterns of induced expression were exhibited by the cold-shock proteins of *M. smegmatis* (Figure 4.7), transient and continuous, allowing the distinction between CSPs, CIPs and CAPs (Table 2). In *E. coli*, all of the cold-shock proteins that have been analysed thus far demonstrate transient cold-shock induction (CSP/CIP) (Graumann and Marahiel, 1996), with increased synthesis only observed during the lag period (4-6 hours). Many of these proteins are ribosome-associated proteins or RNA-binding proteins that play a role in restoring translational capacity to the cell, thereby allowing growth at the lower temperature (Jones and Inouye, 1994, Panoff *et al.,* 1998). In *B. subtilis* (Graumann *et al.,* 1996), *A. globiformis* (Berger *et al.*, 1996) and *E. faecalis* (Panoff *et al.,* 1997), however, some cold-shock proteins demonstrate continuous increased expression and are characteristic of growth at the lower temperature (CAPs) (Hebraud et al., 1994, Graumann and Marahiel, 1996) and are required for long-term survival at the lower temperature. It has been suggested (Berger *et al.,* 1996) that CAPs may function as proteases, removing denatured proteins whose accumulation would be deleterious to the cell or possibly be involved in the maintenance membrane fluidity, or the synthesis of antifreeze substances. Once the cold-shock proteins of *M. smegmatis* have been identified, a clearer understanding of their role in the cold-shock response can be established.

SECTION C:

GENETIC ANALYSIS OF THE COLD-SHOCK RESPONSE IN *M. SMEGMATIS*.

CHAPTER 5

Analysis of cold-shock gene homologues:cspA and gyrA

5.1: INTRODUCTION.

The protein data in Chapter 4 indicated that there was a defined cold-shock response in *M. smegmatis*, with at least fifteen proteins being induced in response to a temperature shift from 37°C to 10°C. In order to understand the mechanism behind this adaptive stress response, it is essential to identify these proteins. The approach that is often used to identify stress proteins is that of micro-sequencing, utilising the techniques of MALDI-TOF MS or N-terminal sequencing (Edman degradation) to obtain protein sequence data. Both of these techniques have been utilised in this study to attempt the identification of several cold-shock proteins of *M. smegmatis* (Chapters 4 and 6, respectively). Over the last couple of years, there has been an accumulation of data regarding the genome sequence of *M. tuberculosis* (Cole *et al.,* 1998). It is possible to use this sequence data to search for mycobacterial cold-shock gene homologues, based on their sequence homologies to the known cold-shock genes of *E.coli.* The cold-shock expression of these mycobacterial homologues can then be investigated, as an alternative method to identify cold-shock genes. This would not only lead to the identification of some of the genes and proteins involved in the stress response, but also give an indication of the mechanisms and sequence of events in cold-shock response, by comparison to the *E. coli* counterparts.

As mentioned earlier (Chapter 2), at least twenty cold-shock proteins have been identified in *E. coli (*reviewed by Jones *et al.*, 1994 and Panoff *et al.*, 1998), with CspA being the major cold-shock protein and the primary regulator of the response. This protein is induced rapidly following the cold shock (Goldstein et al., 1990) and has been shown to be a transcriptional activator of at least two other genes, *hns* and *gyrA* during the cold-shock response (La Teana *et al.*, 1991; Brandi *et al.,* 1994; Jones *et al.*, 1992b). The actions of both of these gene products have potentially widespread affects, altering the transcription of a large variety of genes (discussed in sections 2.5.2 and 2.5.3). CspA, H-NS and GyrA are thus pivotal to the co-ordination of the cold-shock response. Two other important cold-shock proteins that are induced later on in the response are RbfA (Jones and Inouye, 1996) and CsdA (Jones *et al.*, 1996). These are ribosomal proteins that are vital for restoring translational function to the cell, by converting the ribosome from a "cold-unadapted non-translatable state" to a "cold-adapted translatable state" (Jones and Inouye, 1996).

The aim of the following study was to establish the existence of mycobacterial equivalents of several cold-shock genes: *cspA*, *hns*, *gyrA*, *rbfA*, *csdA, recA, nusA* and *pnp,* and to determine the effects of a cold shock on the expression of *cspA and gyrA* in *M. smegmatis*.

5.2: MATERIALS AND METHODS:

The commonly used buffers and solutions discussed in this section are detailed in Appendix 1.

5.2.1: Culture conditions and cold shock.

5.2.1.A: Bacterial cell lines.

The following bacterial cell lines were used for this study: *M. smegmatis* strain LR222; *M. tuberculosis* strain H37rV (ATCC 27294); *M. bovis BCG* strain 172 (Tokyo); *E. coli K-12* strain DH5α.

As previously described, the mycobacterial cultures were grown with aeration at 37°C, in Middlebrook 7H9 medium (Difco Laboratories), supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80. Cultures of *E. coli* were grown with aeration at 37°C in 2XYT liquid medium. Ampicillin (50ug/ml) was added to the *E. coli* culture medium when the cells harboured pGEM-T plasmid constructs.

5.2.1.B: Cold-shock conditions.

For all cold-shock studies in this section, a cold shock refers to the transfer from 37°C to 10°C. Cultures of *M. smegmatis* were seeded (1/1000 dilution) from a frozen, stationary-phase, stock culture and grown in 200-1000ml volumes of Middlebrook 7H9 Tween 80/ ADC medium. As previously described, following 20-24 hours of growth at 37°C (mid-exponential phase), the cultures were split into two separate culture bottles. One was retained to 37°C (control), while the other was transferred to an enclosed shaking-waterbath that was set to 10°C (cold-shock culture). During incubation at 37°C and 10°C, growth was measured by determining the bacterial ATP concentration (section 3.2.3.A) and the cultures were continually assessed for contamination by the Ziehl-Nielsen "acid-fast" stain.

5.2.2: RNA analysis.

All solutions that were used in the extraction or analysis of RNA were pre-treated with diethylpyrocarbonate (DEPC) (200μl/100ml solution) before autoclaving, to inactivate RNases.

5.2.2.A: RNA extraction.

RNA was extracted, at various time points, from cultures (1000ml) of *M. smegmatis* growing at 37°C and following a cold shock to 10°C. Fifty millilitre volumes of culture were removed at each time point and the cells were collected by centrifugation (3000 rpm for 10 minutes). Total RNA was extracted according to the Fast-RNA kit-Blue (Bio 101), using the Fast-Prep FR120 apparatus (Bio 101, Savant Instruments) to disrupt the cells (2 x 40s cycles at speed setting 6, cooling on ice

between cycles). The RNA was dissolved in 30µl of SAFE water (DEPC-treated water) and stored at -70°C. Total RNA concentrations were calculated from optical density readings at 260nm, where 1OD unit = 40µg/ml of RNA.

5.2.2.B: Northern hybridisation.

5.2.2.B.i: Radioactive probe preparation.

Most of probes used in the northern hybridisation experiments were derived from purified PCR products (section 5.2.3.B.iii). If plasmid derived probes were used, these were first linearised with a suitable restriction enzyme. Linear DNA (100ng) was labelled with [α-^{32} P]dCTP (3000Ci/mmol, Amersham) via the process of random-priming, using the "Ready-to-go" dCTP DNA labelling beads (Pharmacia Biotech). The resulting double-stranded probe was denatured by boiling for 5 minutes and cooled on ice, prior to being added to the hybridisation solution.

5.2.2.B.ii: Northern transfer and DNA:RNA hybridisation.

The method used for northern transfer was that outlined by Fourney *et al.* (1988). Aliquots containing 40µg of total RNA were added to 15µl of electrophoresis sample buffer and denatured at 65°C for 15 minutes. Ethidium bromide (1µl of 10mg/ml solution) was added to each sample to allow visualisation of the RNA. Following electrophoresis in 1X MOPS buffer, on a 1.5% denaturing agarose/formaldehyde gel, the gel was photographed and the quality of the RNA assessed (clear 16S and 23S rRNA bands (Ausbel *et al.*, 1987)). The RNA was then transferred to a nylon membrane (Hybond N+, Amersham) via capillary blotting in a 10XSSC solution and cross-linked to the membrane with UV light (120 Joules).

The membrane was pre-hybridised in hybridisation solution for 4-6 hours (42°C) (Techne Hybridiser HB-1D), before the denatured radiolabeled probe was added. DNA:RNA hybridisation was allowed to proceed for 16-24 hours at 42°C. Following hybridisation, the membrane was washed as follows: two washes of 2XSSC / 0.1%SDS, for 20 minutes at 23°C; followed by two higher stringency washes of 1XSSC / 0.1%SDS, for 20 minutes at 42°C. The membrane was exposed to autoradiography film (Agfa CP-BU) at -70°C and the resulting bands were scanned using a densitometer (Shimadzu CS-9000).

5.2.2.C: S1 nuclease analysis.

5.2.2.C.i: Radioactive probe preparation.

*Bam*HI restricted DNA (5'end) was labelled with [α-^{32} P]dCTP using the 5'→ 3' polymerase activity of the Klenow fragment of DNA polymerase 1 ("fill-in" reaction) (Ausubel *et al.*, 1987). Restricted DNA (1-5µg) was added to 5 units of Klenow enzyme (Boehringer Mannheim), 1X Klenow buffer,

1.25 mM of dATP, dTTP, dGTP and 30μCi of [α-^{32}P]dCTP (3000Ci/mmol, Amersham) in a 20μl reaction volume. Following a 30 minute incubation at 37°C, the unincorporated [α-^{32} P]dCTP was removed through the use of a G-50 Micro-spin column (Pharmacia). The specific activity of the probe was calculated by counting 1μl of the prepared probe in 5ml of scintillation fluid, in a Beckman LS7800 scintillation counter. For each RNA sample, 100 - 200ng of probe, at a specific activity 10^7cpm/ug, was added.

5.2.2.C.ii: DNA:RNA hybridisation and S1 nuclease digestion.

The S1 nuclease analysis technique used for the quantification of specific mRNA, was that outlined by Ausbel *et al.* (1987). Following probe denaturation (100°C for 5 minutes), 200ng of probe DNA was incubated with 30μg of total RNA in 3X aqueous hybridisation solution (30μl total reaction volume) for an initial 10 minute period at 75°C, to denature the RNA, and then for a further 24 hours at 55°C to allow DNA:RNA hybridisation. S1 nuclease buffer (270μl) containing 200 units of S1 nuclease (Boehringer Mannheim) and 1mg of salmon sperm DNA was added to the DNA:RNA hybridisation mix and S1 nuclease digestion of single-stranded, non-hybridised probe allowed to proceed for 60 minutes at 37°C. The reaction was halted with the addition of 1/10th volume of 0.5M EDTA and the DNA:RNA were then hybrids precipitated with two volumes 100% ethanol (-70°C / 15 minutes). The resulting pellet was resuspended in 10μl 0.1M NaOH and 10μl loading dye. Following incubation at 100°C for 5 minutes, the remaining radioactive probe molecules were separated on a 6% acrylamide / 8M urea denaturing gel (1X NNB running buffer), the gel dried and then exposed to autoradiography film (Agfa CP-BU).

5.2.3: DNA analysis.

5.2.3.A: DNA extraction.

For use in both the polymerase chain reaction (PCR) and Southern hybridisation analysis, genomic DNA was extracted from turbid bacterial cultures, according to the method of Sambrook *et al.* (1989) for *E. coli* and Jacobs *et al.* (1991) for the mycobacterial strains. Bacterial genomic DNA was finally dissolved in distilled water and the DNA concentration calculated from optical density readings at 260nm, where 1OD unit = 50μg/ml.

5.2.3.B: Polymerase chain reaction (PCR).

The PCR primer sequences and the PCR cycling conditions, for each PCR primer set, are detailed in Appendix 2.

5.2.3.B.i: PCR reaction conditions.

All PCR reactions were composed of 0.1-0.5μg template DNA, 1X reaction buffer (50mM KCl, 10mM Tris.HCl (pH 8.3), 5mM $MgCl_2$, 20μg/ml gelatin), 100pmoles of each primer, 0.2mM of each dNTP (dATP, dTTP, dCTP, dGTP) and 5 units of Taq polymerase (Takara Biomedicals, Japan).

Additional dimethylsulphoxide (DMSO) was added to improve specificity where indicated (10%). As mentioned above, the PCR cycle conditions for each primer set are listed in Appendix 2, with 30 cycles used in each case. For each PCR amplification, a blank control (no DNA) and single primer reactions were performed, to ensure specificity of the reaction. The PCR products were separated on 1-2% agarose gels (1 X TAE running buffer), stained with ethidium bromide (10mg/ml) and visualised under UV light.

5.2.3.B.ii: Asymmetric cspA PCR.

For the amplification of *cspA* from *M. smegmatis* it was necessary to perform "asymmetric PCR", in order to amplify a larger DNA fragment. The reaction profile was modified from that described by Trueba and Johnson (1996): Linear amplification was performed on *M. smegmatis* genomic DNA with one CspA-specific primer only, **cspA1,** for 30 cycles at 60°C (high annealing temperature). The products of this reaction (5µl) were then used as the template for a second PCR reaction containing **cspA1** and an arbitrary, non-specific, degenerate primer (**msmegX**) (see Appendix 2). Thirty amplification cycles were performed at 54°C. The resulting double-stranded PCR products were separated on a 2% agarose gel and stained with ethidium bromide (10mg/ml), to allow visualisation.

5.2.3.B.iii: PCR product purification.

Following separation on an agarose gel the required amplification products were excised from the gel and purified using the "phenol-squeeze" technique (Seth, 1984). The gel slice was finely chopped and 0.5 - 1ml water-saturated phenol added. This mixture was frozen at -70°C for 30 minutes. Following centrifugation, the aqueous phase was extracted with an equal volume of phenol:chloroform:iso-amylalcohol (25:24:1 v/v), followed by an equal volume of chloroform:isoamylalcohol (24:1 v/v). The DNA was precipitated from the aqueous phase with 1/10th volume 3M NaAc pH 5.2 and one volume isopropanol (10 minutes at 13000 rpm). The DNA pellets were washed with 70% (v/v) ethanol and resuspended in 10-100µl of distilled water.

5.2.3.B.iv: PCR product cloning and sequence analysis.

To confirm the sequence of PCR amplified products, the DNA (100-500ng) was first ligated to the pGEM-T plasmid vector (Promega) using the pGEM-T Easy kit (Promega). Competent DH5α *E. coli* cells (Dagert and Ehrlich, 1979) were transformed with the ligation reaction and plated on 2XYT agar plates containing 50ug/ml ampicillin, 200µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid (X-gal) and 200µg/ml isopropyl-β-D-thiogalactopyranosid (IPTG) (Ausbel *et al.*, 1987). Clones with inserts (white colonies) were grown in 2xYT liquid media (50ug/ml ampicillin) and large-scale DNA extractions were performed using the Nucleobond AX extraction kit (Macherey-Nagel). This DNA was sequenced with an automated DNA sequencer, using the pUC19 sequencing primers (Boehringer Mannheim).

5.2.3.C: Southern hybridisation.

5.2.3.C.i: DNA preparation and Southern transfer.

Genomic DNA (10μg) was digested with 10 units of restriction endonuclease (Boehringer Mannheim), for 12-16 hours at 37°C (Sambrook *et al.*, 1989). The resulting DNA fragments were separated on 1.5% (w/v) agarose gels (1X TAE running buffer) and visualised by ethidium bromide staining. The DNA was transferred to a Hybond N+ nylon membrane (Amersham) according to the methods of Southern (1975) and Sambrook *et al.* (1989) and then cross-linked to the membrane with UV light (120 Joules).

5.2.3.C.ii: DNA:DNA hybridisation.

For radioactive hybridisations, the DNA probe was randomly labelled using the "Ready-to-go" dCTP DNA labelling beads (Pharmacia Biotech), as previously described in section 5.2.2.B. Membranes were pre-hybridised for 4-6 hours in hybridisation solution (42°C) and then incubated with the denatured probe (100ng) for a further 16-24 hours, at 42°C. Non-specifically bound probe was removed from the membranes with a series of SSC washes. Routinely, two washes of 2XSSC / 0.1%SDS, for 20 minutes at 42°C were followed by two washes of 1XSSC / 0.1%SDS, for 20 minutes at 55°C. For lower stringency conditions, all washes were performed with 2XSSC / 0.1%SDS, with two washes at 23°C, followed by two washes at 42°C. The membranes were exposed to autoradiography film (Agfa CP-BU) for 1-2 days at -70°C.

5.3: RESULTS.

5.3.1:Identification and expression of an *M. smegmatis cspA* homologue.

5.3.1.A: Identification of a *cspA* homologue.

5.3.1.A.i: Mycobacterial database analysis.

Homologues of *cspA* have been identified in a variety of bacteria including *B. subtilis* (Willimsky *et al.,* 1992), *S. typhimurium* (Craig *et al.,* 1998) and *S. clavuligerus* (Av-gay *et al.*, 1992), many of them identified through sequence homologies to *cspA* gene of *E. coli.* Prior to the completion and annotation of the *M. tuberculosis* genome sequence (Cole *et al.*, 1998), the available mycobacterial sequence database was searched for homologues to the *cspA* genes of both *E. coli* and *S. clavuligerus,* another member of the *Actinomycetes* genus. The cosmid, MTY15C10, of *M. tuberculosis* was found to contain an open reading frame (ORF) (position: 28425-28628, seq ID: g3261770 and accession number: z95436) with considerable homology (66% identity in 190bp overlap) to the *cspA* homologue of *S. clavuligerus* (Av-gay *et al.*, 1992). The predicted protein product of this mycobacterial gene also showed homology to several CspA homologues, including the CspA of *E. coli* (58% identity), and the cold-shock protein, CapA, of *A. globiformis (*70% identity) *(*Berger *et al.*, 1996; Berger *et al.,* 1997). This locus in the *M. tuberculosis* genome has since been annotated as a *cspA*-like gene (Cole *et al.*, 1998), with a similar counterpart (annotated as *cspB*) existing in the *M. leprae* genome (cosmid B2548, 94% identity to *cspA* of *M. tuberculosis)* (Eiglmeier *et al.*, 1993).

5.3.1.A.ii: PCR amplification of cspA - M. smegmatis.

PCR primers **cspA1** and **cspA2** (see Appendix 2) were designed to amplify the coding region of the *M. tuberculosis cspA* gene (247bp PCR product) (Dr R.Powles, personal communication) and were then used to amplify the putative *cspA* homologue in *M. smegmatis.* A PCR product of 155bp was amplified using these primers. However, following sequence analysis of this product it was evident that the *cspA* gene that had been amplified from the *M. smegmatis* genome was truncated at the 3' terminus.

To circumvent this problem and generate a full-length *cspA* PCR product, asymmetric PCR was performed, using one of the *cspA*-specific primers and a non-specific primer. Several products were amplified with this asymmetric approach, ranging from 200 - 1000bp in length. To confirm the amplification of *cspA*-specific products, PCR products larger than 300bp were used as templates in a PCR reaction using both of the *cspA*-specific primers, **cspA1** and **cspA2** (PCR product of 155bp). A 500bp PCR product from the asymmetric PCR amplification was found to contain the 155bp *cspA* fragment. This 500bp fragment was cloned into pGEM-T (Promega) and sequenced (two clones were sequenced in both directions).

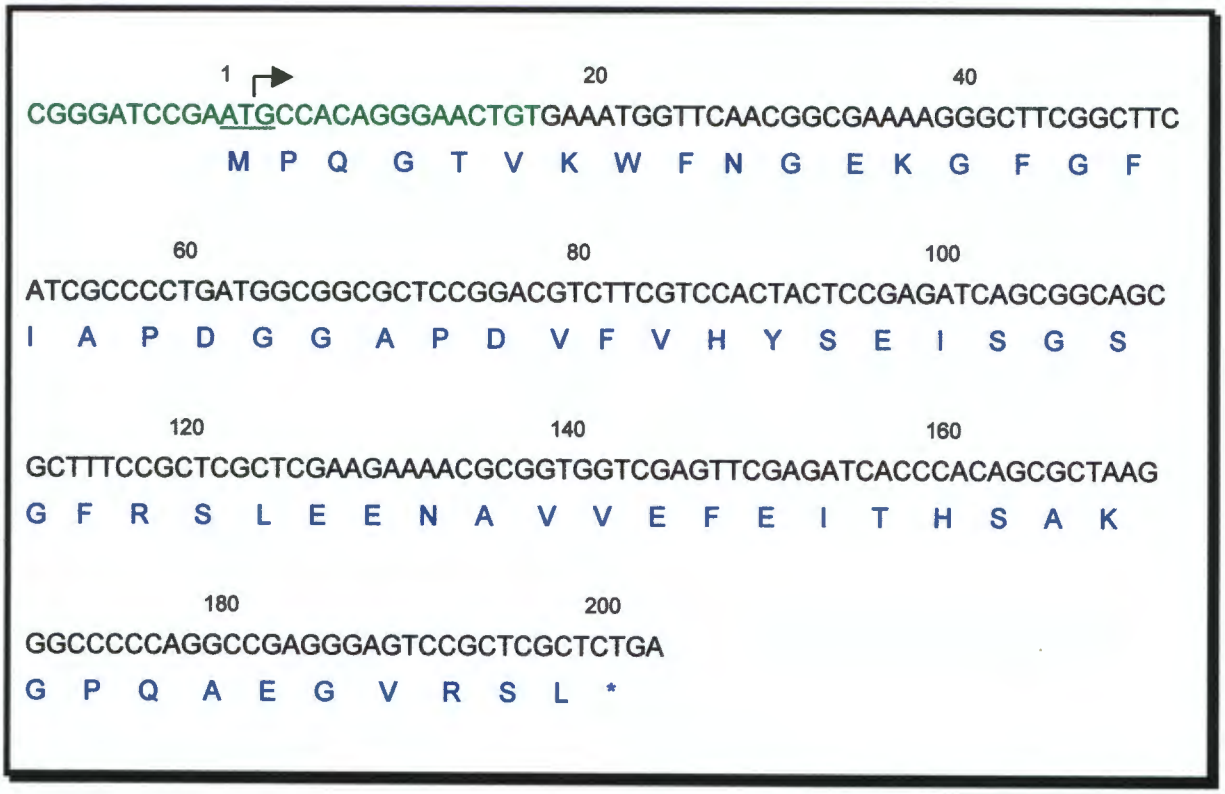


Figure 5.1: DNA sequence of the *M. smegmatis cspA* homologue.

The coding sequence for *cspA* is indicated in black, with the putative amino acid sequence indicated below in blue. The * symbol indicates the termination codon. The binding site for **cspA1** is indicated in green.

Sequence analysis (Figure 5.1) revealed that the *cspA* gene from *M. smegmatis* was 200bp in length and that it shared considerably homology (81%) with the *cspA* sequence from *M. tuberculosis*. This *M. smegmatis* gene encoded a putative protein product of 67aa in length, which is the same size as that encoded by *cspA* of *M. tuberculosis*, but slightly smaller than the CspA protein (70aa) of *E. coli*.

Figure 5.2 shows the homologies observed between the CspA protein of *E. coli* and the putative CspA homologues of *B. subtilis* (CspB), *S. clavuligerus*, *A. globiformis* (CapA), *M. tuberculosis* and *M. smegmatis*. The homologues of *B. subtilis* (Willimsky *et al.*, 1992), *A. globiformis* (Berger *et al.*, 1997) and *S. clavuligerus* (Av-Gay *et al.*, 1992) have all been shown to be cold-induced, with CspB of *B. subtilis* demonstrating similar functions to CspA of *E. coli* (Graumann *et al.*, 1996). The RNA-binding domains, as identified in *E. coli* and *B. subtilis*, as well as the residues that have been shown to be involved in binding to the CCAAT Y-box motif are also indicated

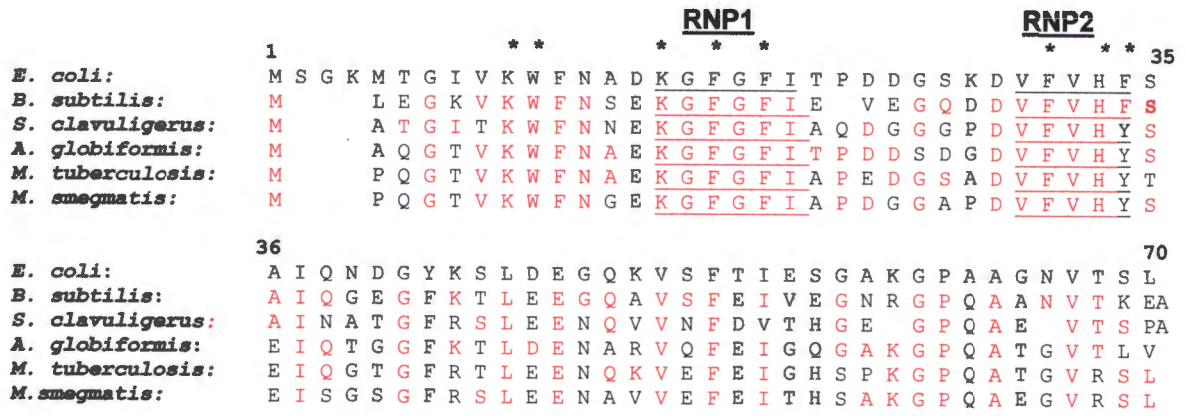


Figure 5.2: Comparative sequences analysis of CspA homologues.

The predicted amino acid sequences of several CspA protein homologues have been aligned and are compared to the amino acid sequence of CspA from *E. coli* (alignment as in Jones and Inouye, 1994). Amino acids that are identical to the *E. coli* sequence are indicated in red. The RNA-binding motifs (RNP) are under-lined and the conserved residues that are involved in binding to the cold-box are indicated by * symbols (Landsman, 1992; Newkirk *et al.*, 1994; Schroder *et al.*, 1995).

From the comparison shown in Figure 5.2, it is evident that a high degree of homology exists between the N-terminals (first 34 aa) of the mycobacterial CspA homologues and the other defined CspA-like proteins. It is within this region that the RNA-binding motifs and the residues that have been implicated in binding to specific promoter sequences (DNA-binding) are located. Apart from a conserved amino acid change at position 34 (phenylalanine→tryptophan), both mycobacterial proteins contain all the relevant amino acids for CspA function. The *M. smegmatis* CspA sequence showed 79% identity with the *M. tuberculosis* protein and 54% identity with the CspA protein of *E. coli*.

5.3.1.B: Expression of *M. smegmatis cspA* – northern hybridisation analysis.

A cold shock in *E. coli* results in a transient increase in the quantity of CspA protein (200-fold) (Goldstein *et al.*, 1990). This increase is mainly due to changes at the post-transcriptional level, with the stabilisation of the mRNA at 10°C (Goldenberg *et al.*, 1996; Jiang *et al.*, 1993). At 37°C, the transcript is so unstable that it is undetectable (10s half-life), while at 10°C the mRNA is stabilised and the detectable levels increase in a pattern similar to the CspA protein (Figure 2.3). In Chapter 4 of this study, two potential CspA protein candidates were identified (cold-shock induced and less than 10kDa in size). However, neither could be positively identified as CspA due to it the lack of protein sequencing data. If the *M. smegmatis cspA* is regulated in a similar manner to that of *E. coli*, then increasing levels of transcript should be seen during the lag phase of the cold-shock response of *M. smegmatis* and the expression of *cspA* can be directly assayed. This was investigated with the northern-hybridisation technique, using the 155bp *cspA* PCR product from *M. smegmatis* as a probe against RNA extracted during growth at 37°C and after a cold shock from 37°C to 10°C, according to the schedule shown in Figure 5.3.

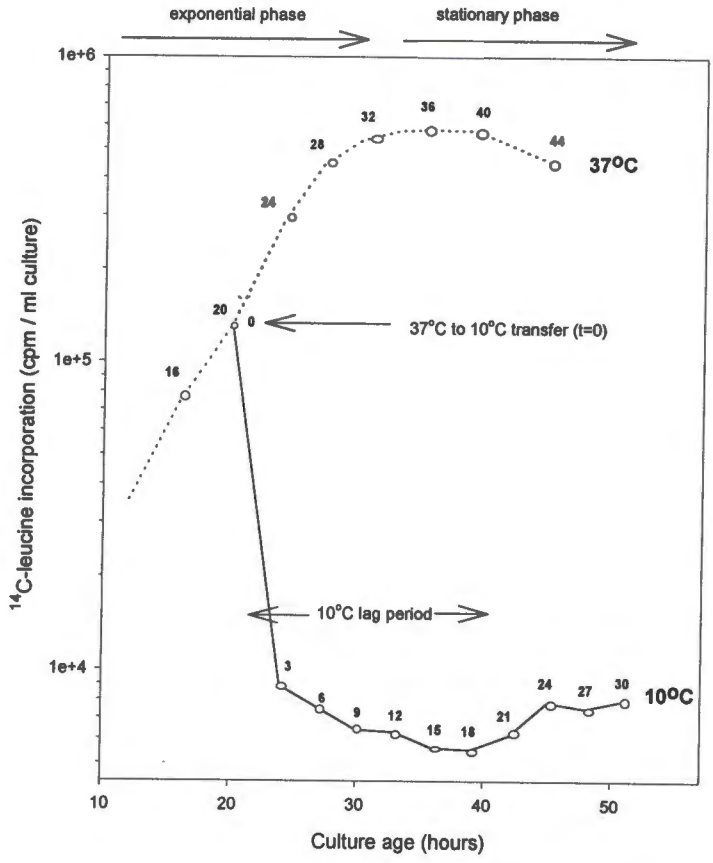


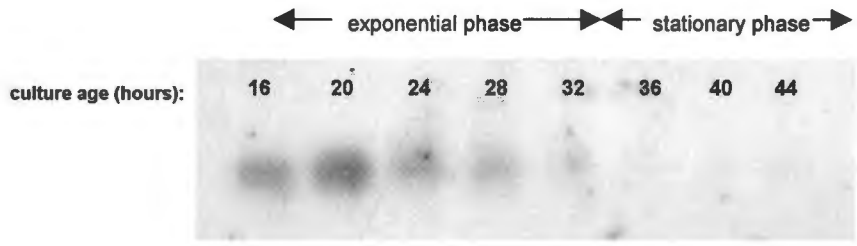
Figure 5.3: RNA extraction schedule.

Diagrammatic representation of the growth of *M. smegmatis* at 37°C and following a transfer from 37°C to 10°C (monitored by the incorporation of ^{14}C-leucine into protein), indicating the time points at which RNA was extracted. At 37°C the numbers indicate the culture age, while at 10°C they indicate the period after the temperature shift.

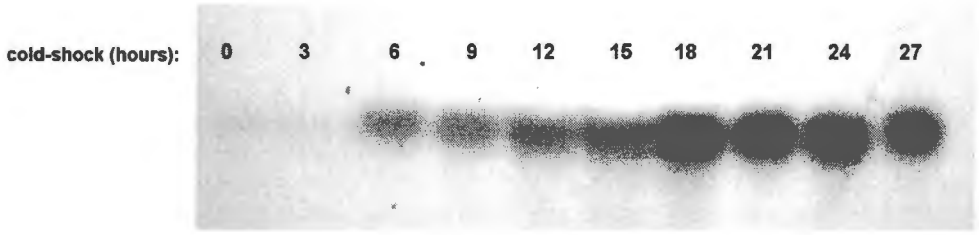
5.3.1.B.i: Expression of cspA during growth at 37°C.

The expression of *cspA* mRNA at 37°C and during a cold shock from 37°C to 10°C is shown in Figure 5.4. Firstly, the expression of this transcript during the normal phases of growth at 37°C was investigated (Figure 5.4.A). Contrary to the data obtained for *E. coli*, the *cspA* mRNA of *M. smegmatis* was detectable during growth at 37°C. This detectable expression was however, limited to the period of exponential growth (16 to 28 hours). The *cspA* mRNA levels decreased during the late exponential phase of growth and were at the limit of detection upon entry into stationary phase (36 hours post temperature-shift). These results were confirmed upon repeat analysis on a separate culture.

A: 37°C *cspA* expression



B: 10°C *cspA* expression - lag period analysis



C: 10°C *cspA* expression – 7 day analysis.

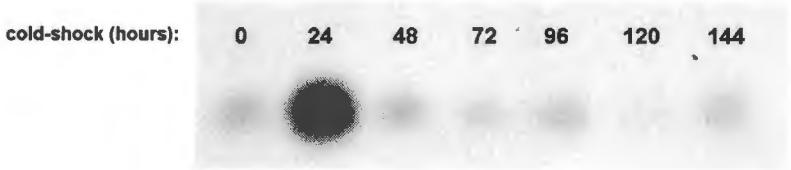


Figure 5.4: *cspA* expression in *M. smegmatis*.

A) RNA was extracted from cultures of *M. smegmatis* grown at 37°C, at the culture times indicated. This RNA (40μg) was electrophoresed on a 1.5% agarose/ formaldehyde gel and then transferred to Hybond N^+ nitrocellulose membrane via capillary-blotting. The picture shows the autoradiograph of the membrane following hybridisation with a [α-^{32}P]dCTP-labelled *cspA* 155bp PCR product (random-primed). **B)** RNA was extracted from *M. smegmatis* after the transfer of the culture from 37°C to 10°C, at the post-cold-shock times indicated (first 30 hours). This RNA was processed as described in (A) and the autoradiograph resulting from the hybridisation with the 155bp *cspA* probe is shown. **C)** *M. smegmatis* RNA was extracted at 24-hour intervals after the transfer of the culture from 37°C to 10°C. The RNA was processed as described above and the autoradiograph resulting from the hybridisation with the 155bp *cspA* probe is shown.

5.3.1.B.ii: Cold-shock expression of cspA (37°C to10°C transfer).

According to the data presented in Chapter 3, it was established that the "lag period" for the synthesis of macromolecules and growth, following a cold shock from 37°C to 10°C, spanned a 24-hour period in *M. smegmatis*. This corresponded with the protein data discussed in Chapter 4, which showed that most of the protein changes occurred within the first 24 hours. This time-frame was then selected to investigate the expression of *cspA* in *M. smegmatis* following a shift from 37°C to 10°C, time 0hr representing the state of the culture at 37°C. Figure 5.4.B clearly demonstrates the induction of *cspA* following the temperature shift (37°C→10°C). This mRNA analysis was repeated on four separate cultures, all of which showed definite increases in the levels of *cspA* mRNA, after several hours at 10°C. Two of the autoradiographs were scanned using a densitometer and the relative band intensities were plotted (Figure 5.5).

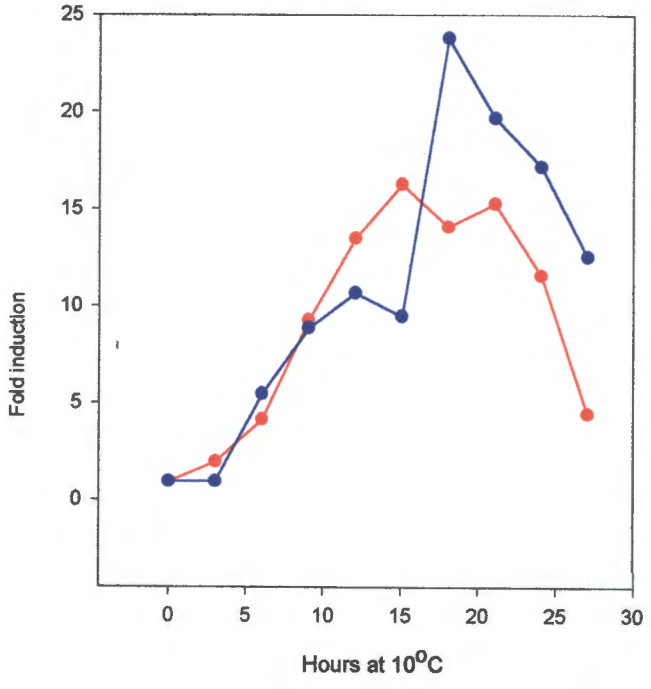


Figure 5.5: Induction of *cspA* mRNA following a 37°C→10°C cold shock.

Autoradiographs resulting from the northern analysis of *cspA* expression at 10°C were scanned and the band densities at 10°C compared to the band density obtained at 37°C (t=0). The two curves represent the relative increase in *cspA* band densities, over the cold-shock period (27 hours), from two separate experiments.

As can be seen in Figure 5.5, actual levels of induction varied between experiments. However, several key findings were consistently reproducible. Firstly, the induction was not immediate, with 3-6 hours of cold exposure required prior to an increase in *cspA* mRNA levels. Secondly, maximal induction of this transcript occurred between 15 and 18 hours at 10°C, with levels peaking at 16-24

fold higher than those seen at 37°C. Peak *cspA* expression was not maintained for long periods, with decreasing transcript levels already seen at 21 hours post-temperature shift.

This induction of *cspA* expression was not simply a consequence of an ageing culture, as Figure 5.4.A demonstrates that as the culture ages (exponential→stationary phase) *cspA* mRNA levels decline. The photographs of the agarose/formaldehyde gels prior to northern-transfer indicated equally loading of RNA samples (results not shown), which implied that the observed induction (16-24 fold) was not due to gross errors in RNA quantification.

In *E. coli*, the increase in *cspA* mRNA levels is transient and is limited to the lag period. From the results shown in Figure 5.4.B, it was suggested that the cold-induced, increased *cspA* expression in *M. smegmatis* was also transient, peaking towards the end of the lag period. To confirm this, RNA was extracted at 24-hourly intervals following the temperature shift, for a period of seven days and the levels of *cspA* mRNA were then determined. It is evident from Figure 5.4.C that increased levels of *cspA* mRNA were limited to the lag period which follows a cold shock, with mRNA levels returning to baseline by 48 hours. These results were reproduced in three independent experiments.

5.3.2: Identification and expression of a *gyrA* homologue in *M. smegmatis.*

5.3.2.A: Identification of a *gyrA* homologue.

Both subunits of DNA gyrase (GyrA and GyrB) have been characterised in *M. tuberculosis* (Takiff *et al.*, 1994) and *M. smegmatis* (Madhusudan and Nagaraja, 1995; Revel-Viravau *et al.*, 1996; Salazar *et al.*, 1996), with the GyrA proteins of these two mycobacterial species demonstrating 89% identity (Revel-Viravau *et al.*, 1996). The *M. tuberculosis gyrA gene* is located on cosmid MSGGYRAB , position: 2306-4822, accession number: L27512, (Takiff *et al.*, 1994) with the *M. smegmatis gyrA* gene located at position: 3334-5862 on cosmid MSGYRAB, accession number: X94224 (Revel-Viravau *et al.*, 1996).

At the time of the initiation of this project, the *M. smegmatis gyrA* had been identified (Madhusdan, 1994, direct Genbank data submission), but had not yet been fully characterised. PCR primers **gyrA1** and **gyrA2** (Appendix 2) were therefore designed to amplify the coding region of *gyrA* from *M. tuberculosis.* The resulting PCR product (2.5Kb) was cloned into pGEM-T (Promega) and sequenced to confirm the amplification of the mycobacterial *gyrA* gene. Southern-hybridisation demonstrated that an internal 500bp *Eco*RV/ *Bam*HI fragment of this PCR product detected only a single *gyrA* homologue in *M. smegmatis* (Figure 5.6). This mycobacterial *gyrA* PCR fragment was therefore suitable as a probe in the analysis of *gyrA* expression in *M. smegmatis.*

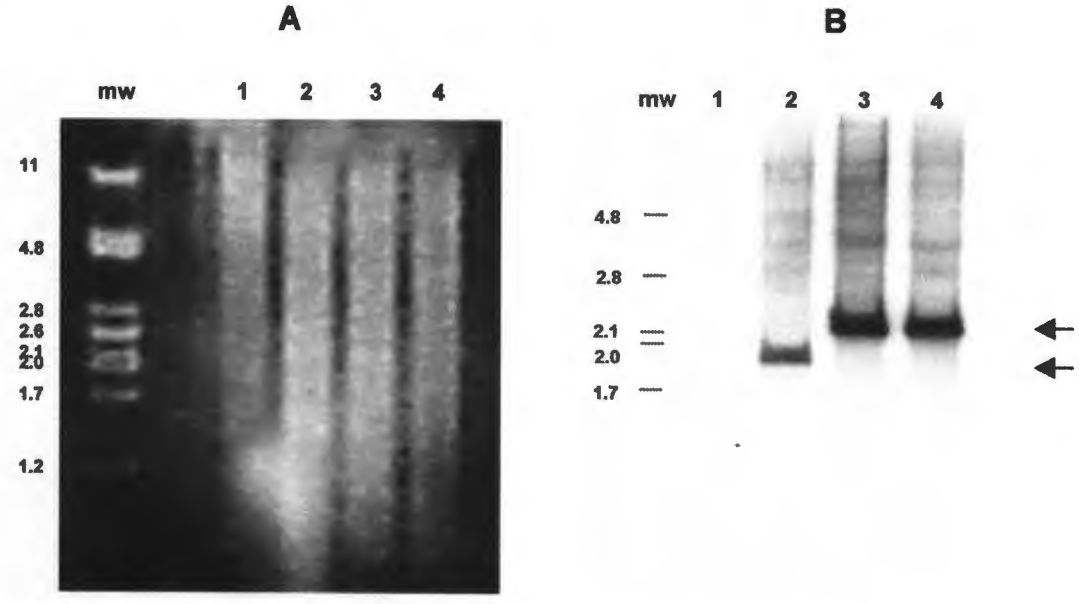


Figure 5.6: Mycobacterial homologues of *gyrA*.

Genomic DNA from *E. coli* (1), *M. smegmatis* (2), *M. tuberculosis* (3) and *M. bovis* BCG (4) was digested with *Bam*HI and electrophoresed on a 1.2% (w/v) agarose gel. Following transfer to a Hybond N^+ nylon membrane, the genomic DNA was probed with a [α-^{32}P]dCTP-labelled 500bp *Eco*RV / *Bam*HI *gyrA* PCR fragment from *M. tuberculosis*. **A)** Photograph of the ethidium bromide-stained agarose gel prior to transfer. **B)** Autoradiograph of the nylon membrane after hybridisation with the *M. tuberculosis gyrA* probe, mw = molecular weight marker, λ *Pst*I (kb).

5.3.2.B: Expression of *M. smegmatis gyrA* - S1 nuclease analysis.

GyrA was found to be one of the cold-shock proteins of *E. coli*, with protein levels increasing between the first and fifth hours of the cold shock from 37°C to 10°C. This increase was due in part to increased gene transcription, which was enhanced by the binding of CspA to the promoter of *gyrA* (Jones *et al.*, 1992b). In light of this, the cold-shock induction of *gyrA* transcription was investigated in *M. smegmatis*.

RNA was extracted during the growth of *M. smegmatis* at 37°C and following a cold-shock from 37°C to 10°C (Figure 5.3). The levels of *gyrA* mRNA were initially assessed using the northern hybridisation technique, using both the full length 2.5kb *gyrA* PCR product and the internal *Eco*RV/*Bam*HI fragments as radio-labelled probes. However, no defined *gyrA* mRNA bands could be detected during growth at 37°C or following the cold shock (results not shown). Due to the predicted length of the mRNA transcript (2546bp) it is possible that mRNA degradation may have occurred, thus preventing the detection of a well-defined signal. To circumvent this problem, S1 nuclease analysis was performed, where the size of the detectable transcript is determined by the size of the DNA probe. The use of a smaller internal probe allows the transcript to be detected even if the degradation process has begun.

5.3.2.B.i: S1 nuclease analysis - controls.

The internal 500bp *Bam*HI/ *Eco*RV *gyrA* fragment, from *M. tuberculosis*, was chosen as the probe for S1 nuclease analysis. For accurate quantification of mRNA via S1 nuclease analysis, several key controls need to be performed. The hybridisation time and temperature must be optimised, the probe must be present in excess, the quantity of S1 nuclease needed to degrade single-stranded probe must be determined and the signal intensity should be directly proportional to the quantity of RNA added. Figure 5.7 shows the results of these controls.

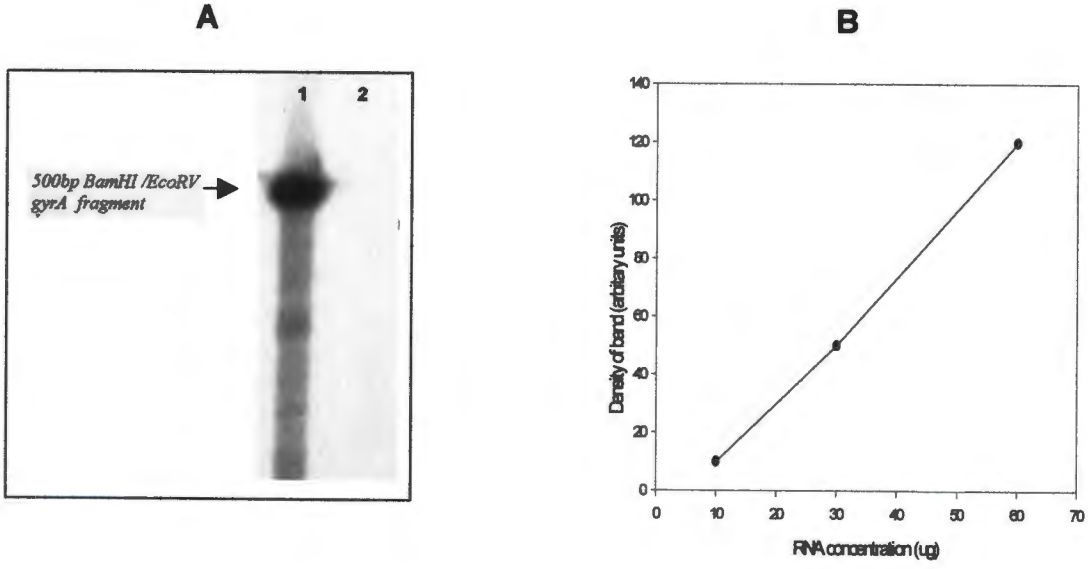


Figure 5.7: S1 nuclease controls for *gyrA* expression.

A) Efficiency of S1 nuclease: 200ng of ^{32}P-dCTP radiolabelled *Bam*HI / *Eco*RV *gyrA* probe was electrophoresed on a 6% acrylamide gel before (1) and after (2) incubation with 200 units of S1 nuclease (60 mins at 37°C). B) Proportional quantification of mRNA.: Varying quantities of total RNA (10 - 60μg) were incubated with the 200ng *gyrA Bam*HI / *Eco*RV radioactive probe (10^6 cpm) (55°C, 24 hours), digested with 200 units of S1 nuclease for 1 hour and then electrophoresed. The gel was dried and exposed to X-ray film for 1-2 days (-70°C) and the band intensities were then calculated using a densitometer (Shimadzu CS-9000).

Figure 5.7.A clearly demonstrates that sufficient S1 nuclease (200units) was being added to the samples to completely degrade the single-stranded probe, ensuring that a signal is only generated from protected *gyrA* DNA:RNA hybrids. By comparing the initial probe signal in this figure with that following a successful DNA:RNA hybridisation (Figure 5.8), it was also evident that at a concentration of 200ng DNA probe/30μg RNA, the probe was in excess. According to Figure 5.7.B, the conditions for the assay (55°C hybridisation for 24 hours, 200ng DNA (10^6 cpm) and 200units S1 nuclease / 60 minutes) were optimal, giving band intensities that were directly

proportional to the quantity of RNA added. The results from these controls confirmed that the conditions for the assay were sufficient to detect varying levels of *gyrA* mRNA.

5.3.2.B.ii: Expression of gyrA at 37°C and following cold-shock.

Total RNA was extracted during the growth of *M. smegmatis* at 37°C and following a cold shock from 37°C to10°C (Figure 5.3). The mRNA in these samples was hybridised with the *Bam*HI/ *Eco*RV *gyrA* fragment, treated with S1 nuclease and the resulting protected hybrids were then separated on a 6% acrylamide gel. The results from this analysis are presented in Figure 5.8.

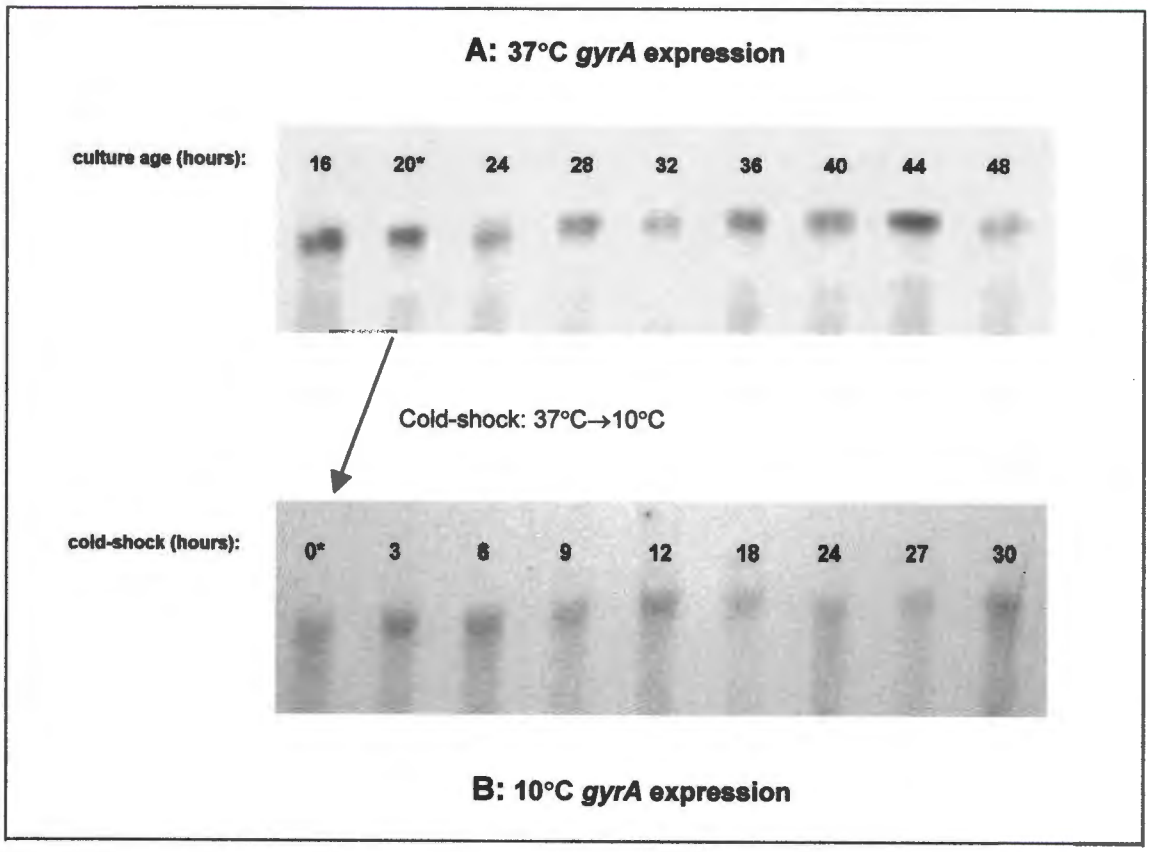


Figure 5.8: Expression of *gyrA* in *M. smegmatis* - S1 nuclease analysis.

Total RNA was extracted during the growth of *M. smegmatis* in Middlebrook 7H9 medium (Tween 80 and ADC additives), according to the schedule in Figure 5.3. This RNA was hybridised with a 500bp *Bam*HI/ *Eco*RV *gyrA* fragment ([α-^{32}P]dCTP end-labelled) from *M. tuberculosis*. Following treatment with S1 nuclease, gyrA-specific mRNA hybrids were separated on 6% acrylamide/ 8M urea denaturing gels. **A)** Autoradiograph of the S1 nuclease analysis of RNA extracted during growth at 37°C. **B)** Autoradiograph of the S1 nuclease analysis of RNA extracted after the transfer of the culture from 37°C to 10°C. ✳ = Time 0hr corresponds to *gyrA* transcript levels at the time of transfer of the culture to 10°C.

Expression of *gyrA* appeared constant during growth at 37°C, with no obvious changes in mRNA levels occurring upon entry of the culture into stationary phase (Figure 5.8.A). Surprisingly, no clear reproducible changes in the mRNA levels were seen upon transfer to 10°C either (Figure 5.8.B). As a further control for the experimental procedure, the expression of *gyrA* at 10°C was repeated, using a *Bam*HI/*Eco*RI fragment of a *cspA*-pGEM-T construct (155bp *cspA* PCR product) *as* a co-probe (Figure 5.9). While the *cspA* mRNA levels rose (consistent with previous results), *gyrA* mRNA levels again remained constant. This result demonstrated that the quantity and quality of the total RNA was sufficient to detect increasing levels of mRNA. Taken in conjunction with the other controls, it was apparent that a cold shock had no significant effect on *gyrA* transcript levels in *M. smegmatis*.

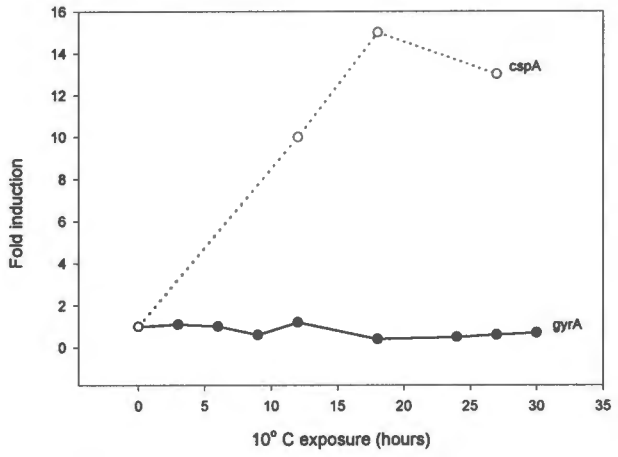


Figure 5.9: S1 nuclease analysis of *gyrA* and *cspA* expression in *M. smegmatis*.

RNA was extracted at 3-hourly intervals, after the transfer of an exponentially growing culture of *M. smegmatis* from 37°C to 10°C. This RNA was hybridised to both the *Eco*RV/*Bam*HI 500bp *gyrA* probe (*M. tuberculosis*) and a *Bam*HI/*Eco*RI fragment of a *cspA*-pGEM-T *construct (M. smegmatis)* ([α-^{32}P]dCTP-labelled). Following treatment with S1 nuclease, gyrA-specific mRNA hybrids and cspA-specific mRNA hybrids were separated on a 6% acrylamide/ 8M urea denaturing gel. The bands on the resulting autoradiograph were scanned using a densitometer and the band densities at 10°C were compared to the band density at time 0hr (37°C). The relative increase in band densities are represented here.

5.3.3: Identification of other *M. smegmatis* cold-shock gene homologues: *rbfA, hns, csdA, recA, pnp* and *nusA*.

Until a few years ago, little genomic information was available for any of the mycobacterial species, however a concerted effort has now been made to generate sequence data for the slow-growing, pathogenic varieties, which are of medical significance (*M. leprae, M. avium* and *M. tuberculosis*). The recent completion of the *M. tuberculosis* genome sequencing project (Cole *et al.*, 1998) has provided a large database of ORFs, some of which have been annotated according to their overall homologies to known bacterial proteins or due to the presence of specific motifs

that characterises a group of proteins. The function of most of these putative proteins has yet to be established. Unfortunately, only a few genes have been characterised in the fast-growing, non-pathogenic species such as *M. smegmatis*. Some of these include genes that have a possible role in the cold-shock response, as implicated by their *E. coli* counterparts: *gyrA* (Revel-Viravau *et al.*, 1996; Salazar *et al.*, 1996), and *recA* (Papavinasasundaram *et al.*, 1997; Durbach *et al.*, 1997).

The mycobacterial database was searched for gene homologues to *rbfA, hns, csdA, pnp* and *nusA*, other known cold-shock genes of *E. coli*. The results of this search are detailed in Table 3. Some of these mycobacterial genes have been annotated as such according to homologies to genes in bacteria other than *E. coli*, therefore the percentage homology to the *E. coli* cold-shock genes are also given (DNAman sequence alignment program). As limited sequence data was available for *M. smegmatis*, it was necessary to probe the *M. smegmatis* genome directly to identify putative homologues. Using specific PCR primers (Appendix 2) the appropriate genes were amplified from *E. coli* or *M. tuberculosis* and used as probes in the Southern-hybridisation analysis of the *M. smegmatis* genome. The results of this analysis are detailed in Table 3, along with the details of the mycobacterial homologues of *cspA, gyrA* and *recA*.

The mycobacterial equivalents to *rbfA*, *nusA* and *pnp* demonstrated poor homology to their *E. coli* counterparts and are probably not functionally homologous. Although the mycobacterial *csdA* gene showed higher homology, the presence of an *M. smegmatis* equivalent could not be demonstrated through Southern hybridisation. For these reasons the cold-shock expression of these genes was not investigated.

Table 3: Mycobacterial cold-shock gene homologues

Gene	Mycobacterial gene Accession number:	*E. coli*: mycobacteria Protein homology	DNA homology	*M. smegmatis* gene Southern hybridisation[a]
rbfA	z81331 - *M. tuberculosis*	<25%	<25%	Negative [b]- *EC*
hns	None[c]	–	–	Negative – *EC*
csdA/deaD	z77137 - *M. tuberculosis*	46.7%[d]	39%	Negative - *MTB*
nusA	z95207 - *M. tuberculosis*	31%	<25%	not performed
pnp	z95558 - *M. tuberculosis*	<25%	<25%	not performed
recA	x99208 - *M. smegmatis*	66%	64%	Positive – *MTB*
cspA	z95436 - *M. tuberculosis*	58%	56%	Positive – *MTB*
gyrA	x94224 - *M. smegmatis*	45%	37%	Positive – *MTB*

a = Probes for the Southern hybridisation were specific PCR products from either *E. coli* (EC) or *M. tuberculosis (MTB)*.
b = For all of the Southern hybridisation experiments, the same set of bacterial genomic digests were used (*M. tuberculosis, E. coli, M. smegmatis*), with the ethidium-stained gel showing equal quantities of digested DNA (10μg) (see Figure 5.6). For all negative results, the control lanes gave positive reactions (*E. coli* or *M. tuberculosis*), yet even when washing stringency was lowered no specific bands could be detected in the *M. smegmatis* genome..
c= "None" refers to the lack of a mycobacterial gene with the appropriate annotation and no significant homology (< 30%) to the *E. coli* gene sequence.
d = This mycobacterial DEAD-box protein contains the ATP-dependant helicase signature (position 163-166:DEAD) and the ATP/GTP-binding site (position: 57-64) (Cole *et al.*, 1998).

5.4: DISCUSSION.

In *E. coli,* the induced synthesis of several cold-shock proteins is a result of increased levels of mRNA, either by stabilisation of the mRNA (CspA) (Jiang *et al.*, 1993; Goldberg *et al.*, 1996) or by increased gene transcription (H-NS, GyrA) (La Teana *et al.*, 1992; Jones *et al.*, 1992b). As an alternative method for identifying cold-shock proteins of *M. smegmatis*, homologues of several *E. coli* cold-shock genes were identified in *M. smegmatis*. The expression of two of these genes (*cspA* and *gyrA*) was also investigated by monitoring the levels of mRNA, following the transfer of *M. smegmatis* from 37°C to 10°C. This investigation demonstrated that a *cspA* homologue in *M. smegmatis* was cold-induced, while the *gyrA* gene was unresponsive to a cold-shock. The results described in this chapter indicate that there are significant differences between the cold-shock responses of *M. smegmatis* and *E. coli*, not only concerning the genes involved in the response, but also the cold-shock gene expression.

5.4.1: *M. smegmatis cspA* homologue.

CspA is the major cold-shock protein of *E. coli* (Jones *et al.*, 1987; Goldstein *et al.,* 1990) and judging by its rapid, transient induction and effect on other cold-shock genes (transcriptional activator), is probably the primary regulator of the response. Using the sequence data from the previously identified *cspA* gene of *M. tuberculosis* (Dr R.Powles, personal communication), a *cspA* homologue was identified and characterised in *M. smegmatis*. The putative protein product of this gene showed 54% identity to CspA of *E. coli* and 79% identity to the *M. tuberculosis* counterpart. This mycobacterial protein product also contained the conserved amino acids that have been implicated in both RNA and DNA-binding, in the CspA of *E. coli* and CspB of *B. subtilis* (Landsman, 1992, Schindelin *et al.*, 1994; Newkirk *et al.*, 1994; Schroder *et al.*, 1995).

In *E. coli*, the control of CspA expression is vital for cell survival and adaptation at the lower temperature. It is essential to have early, transient induction of this protein during a cold shock. Prolonged production of this protein prevents the resumption of general protein synthesis and growth at the cold-shock temperature (Jiang *et al.*, 1996). It is still a controversial issue at this stage, as to which level of control (transcriptional or translational) is the most important for increased production of CspA. However, it is agreed that increased levels of *cspA* mRNA are seen following a cold shock and that this is mainly due to increased stability of the transcript (Tanabe *et al.,* 1992; Jiang *et al,* 1993; Goldenberg *et al.*, 1996). Thus investigation of the expression of *cspA*, during a cold shock in *M. smegmatis*, could be performed at two levels, mRNA and protein.

At the mRNA level, a series of northern hybridisations revealed the cold-shock induction (16-23 fold) of *cspA* mRNA. This induction was shown to be transient, with increased levels seen only during the first 24-hour period. This cold-shock induction at the mRNA level suggested that the *M. smegmatis* CspA homologue was a cold-shock protein, like the homologues identified in *B. subtilis*, *S. clavuilgerus* and *A. globiformis*. These results did not, however, indicate whether this

increase in mRNA was due to changes at the transcriptional or post-transcriptional level. Although the transient induction of this transcript was not as short or as rapid as that of *E. coli* (Figure 2.3), the induction was similar to that of the *E. coli* gene in that it was limited to the lag in metabolic activity (24 hours) (section 3.3.2.B.iii), that was observed following the cold shock. The level of mRNA induction was also comparable with that of *cspA* from *E. coli*, showing a 16-23 fold peak induction compared to the 30 fold induction in *E. coli* (Goldberg *et al.*, 1996). However, the *cspA* mRNA levels of *M. smegmatis* peaked towards the end of the lag period (18-21 hours), suggesting that CspA may not be the primary regulator of the cold-shock response of *M. smegmatis*.

Another difference that was observed between the expression of cspA in *M. smegmatis* and *E. coli* was its expression during exponential growth at 37°C. In *M. smegmatis*, *cspA* mRNA is detectable during exponential growth at 37°C, while in E. coli, although *cspA* mRNA is transcribed, it is so unstable that it is not able to be detected or translated, providing an effective regulatory mechanism (Jiang *et al.*, 1993; Goldenberg *et al.*, 1996). In *M. smegmatis*, the presence of a detectable transcript at 37°C implies that the protein product is required during normal growth conditions, as well as cold stress. This pattern of expression is similar to that of *cspB* in *B. subtilis* (Willimsky *et al.*, 1992) and it has been demonstrated that expression of the cold-shock protein family (CspB, CspC, CspD) in *B. subtilis* is essential for survival at all temperatures (Graumann *et al.*, 1997). Graumann *et al.* (1997) suggest that these proteins may function in Gram positive organisms in an analogous manner to the ribosomal protein S1 of *E. coli (*Gram negative), preventing secondary structures in the mRNA and allowing initiation of translation. A homologue of the *E. coli* ribosomal protein S1 (40% identity) has been identified in two mycobacterial species, *M. leprae* (Fsihi and Cole, 1995) and *M. tuberculosis* (Cole *et al.*, 1998). Although it has not yet been fully characterised, a ribosomal-associated protein with some of the S1 characteristics has also been demonstrated in *M. smegmatis* (Yamada, 1982). However, the role of this protein in the initiation of translation or RNA binding was not established. As *M. smegmatis* shares some characteristics of Gram positive organisms like *B. subtilis*, it is possible that this mycobacterial S1 protein homologue may not function like its *E. coli* counterpart (Sorokin *et al.*, 1995; Muralikrishna and Suryanarayana, 1985). If this is the case, then it is possible that the CspA protein of *M. smegmatis* has similar functions to those proposed for CspB of *B. subtilis* and is essential for the survival of the bacteria at all temperatures.

The protein analysis of the cold-shock response in *M. smegmatis* (Chapter 4) led to the identification of two potential CspA candidates, due to their size of less than 10kDa and their rapid, transient, cold-shock expression. Neither could be positively identified as a CspA homologue, due to the lack of protein sequence data. As can be seen from Figure 5.10, the observed cold-shock pattern of *cspA* mRNA expression was also significantly different from that observed for either of the putative CspA protein candidates. An expression system for the purification of CspA is currently being constructed (Dr R. Powles, our laboratory), which will then be used to generate polyclonal antibodies, for the investigation of *cspA* expression in *M. tuberculosis*. Due to the high

degree of homology (79% identity) between the predicted CspA protein sequences of *M. smegmatis* and *M. tuberculosis*, these antibodies can be used in future work *to* unequivocally identify this cold-shock protein in *M. smegmatis* and study its expression.

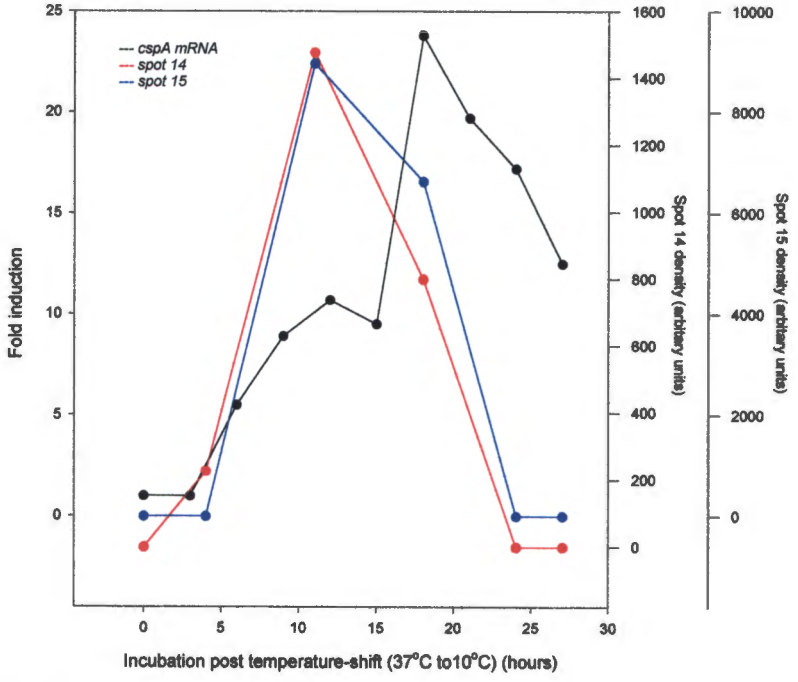


Figure 5.10: Comparison of *cspA* mRNA expression to that of two putative CspA protein homologues, following a cold shock.

The induction profile for *cspA* mRNA levels (section 5.3.1.B.), following a temperature shift from 37°C to 10°C, is compared to the cold-shock expression profiles of two putative CspA homologues, spot 14 in red and spot 15 in blue, as indicated by their spot densities (spot 13 = autoradiograph spot; spot 14 = silver-stained spot).

5.4.2: *M. smegmatis gyrA* homologue.

The GyrA subunit of DNA gyrase is of particular interest in mycobacteria for two major reasons. Firstly, mutations (substitutions) in the *gyrA* gene have been linked to mycobacterial resistance to fluoroquinolone (74-94% of resistance cases), one of the anti-tuberculosis drugs (Acaide and Telenti, 1997). Secondly, the mycobacterial GyrA is one of the proteins that contain an "intein", an internal sequence that is spliced out during post-translational modifications to produce an active protein. The GyrA intein (1260bp insertion) is a putative homing endonuclease (Fsihi *et al*, 1996). Surprisingly, this intein is not present in all mycobacterial *GyrA* species. It has been demonstrated in *M. leprae M. kansasii, M. malmoense*, *M. marinum, M. ulcerans, M. xenopi* (Sander *et al*,

1998) *M. flavescens* and *M. gordonae* (Fsihi *et al*, 1996), but it is not found in *M. tuberculosis* or *M. smegmatis*. The significance of this intein has not yet been elucidated.

The *gyrA* gene of *E. coli* is also one of the genes that has been shown to be directly affected by the induction of CspA during a cold shock. The *gyrA* promoter region possesses several recognition sites for the binding of CspA molecules and transcription has been shown to be positively affected by this protein interaction (Jones *et al.*, 1992b). Due to the impressive induction of *cspA* mRNA in *M. smegmatis* following a cold shock and because *gyrA* had already been shown to exist in mycobacteria, the induction of gyrA expression following a cold shock was investigated in *M. smegmatis*

S1 nuclease analysis was used to investigate the expression of *gyrA* in *M. smegmatis*, during normal growth at 37°C and following a cold shock from 37°C to 10°C. Unlike the *gyrA* of *E. coli* (Gomez-Gomez *et al.*, 1996), *gyrA* mRNA levels were found to be independent of the stage of growth of the cell, showing continuous, constant expression throughout the growth cycle of the bacterium. Under the experimental conditions described (S1 nuclease analysis), the *gyrA* gene was also unaffected by the cold shock to 10°C, again showing constant levels of expression. This implies that *gyrA* expression is not affected at the transcription level by a cold shock. However, the fact that the mRNA is still synthesised during the metabolic lag period, where the rate of overall RNA synthesis has been shown to drop more than 6-fold (section 3.3.2.B.iii), indicates that the protein product is essential for cell viability and adaptation at the lower temperature. This again shows similarity with the cold-shock response of *B. subtilis*, during which *gyrA* mRNA levels are also unaffected by the drop in temperature (Krispin and Allmansberger, 1995). The *B. subtilis* GyrA protein does however, play a crucial role in the cold-shock response, by activating the genes involved in desaturation of the cell membrane (Grau *et al.*, 1994).

5.4.3: *M. smegmatis* homologues of *hns, rbfA, csdA, pnp and nusA.*

Analysis of the database for homologues to the cold-shock genes of *E. coli*, showed that although mycobacterial equivalents have been identified for *rbfA, csdA (deaD), pnp* and *nusA,* the homology is quite low (20-35%) and these may not therefore, be functionally homologous to their *E. coli* counterparts. Southern-hybridisation indicated that *rbfA* and *csdA* were not present in the *M. smegmatis* genome A mycobacterial homologue to *hns* was not identified. Proteins such as RbfA and CsdA play significant roles in the cold-shock response of *E. coli* (Cold-shock Adaptation model) (Jones and Inouye, 1996) and therefore the existence of functional homologues in *M. smegmatis* would be worth further investigation.

CHAPTER 6

Characterisation of "CipM.1"

6.1: INTRODUCTION.

One-dimensional SDS-PAGE analysis, of proteins synthesised during a cold shock from 37°C to 10°C (Chapter 4), revealed the significant induction (10-11 fold increase) of a 27kDa protein, designated CipM (Figure 6.1). Several other protein bands showed some induction, but this was the most obvious and potentially contained the major cold-induced protein of *M. smegmatis.* Although it was understood that this protein band might have contained more than one protein, attempts were made to sequence the cold-induced protein from the one-dimensional SDS-PAGE gel, while the technique of two-dimensional SDS-PAGE was being established in the laboratory. This chapter details the identification of one of the proteins contained within the CipM protein band, CipM.1.

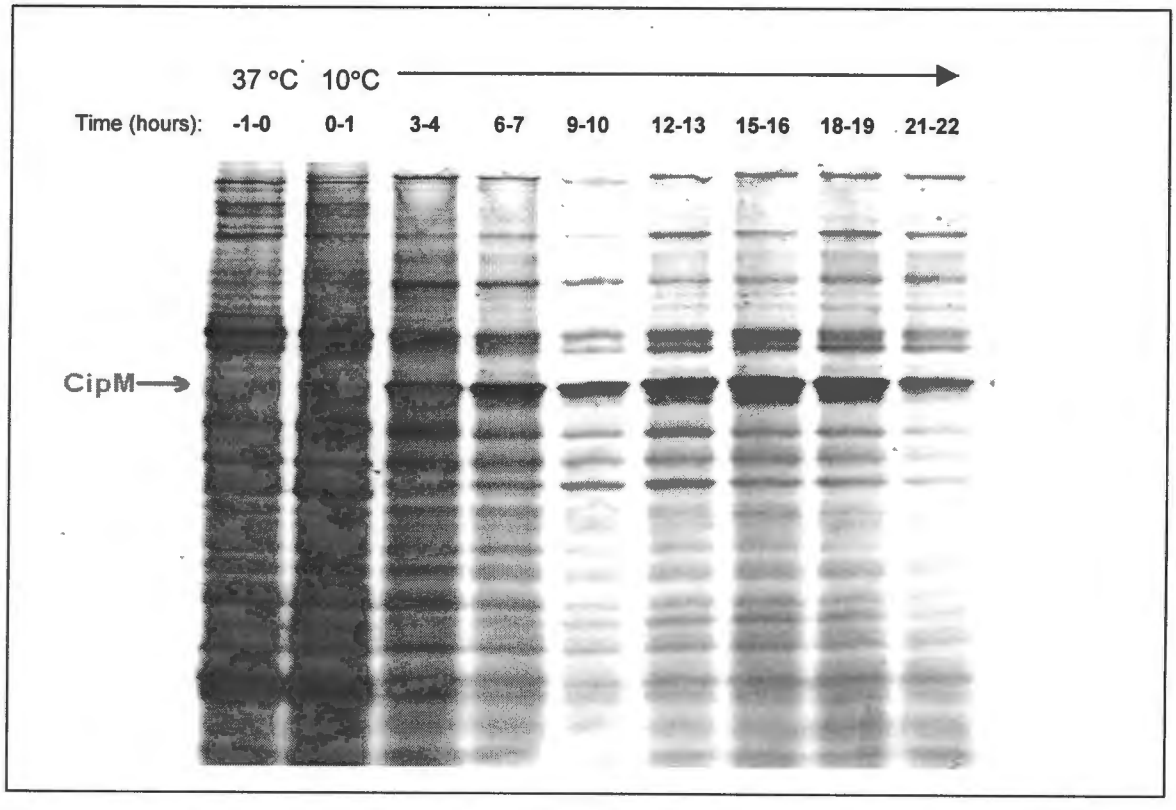


Figure 6.1: Cold-shock proteins of *M. smegmatis.*

Autoradiograph of a 12.5% SDS-polyacrylamide tricine gel, containing cellular proteins of *M. smegmatis* labelled with L-[^{35} S] methionine, before (t=0) and after a cold shock from 37°C to 10°C. Aliquots of cell culture (2ml) were incubated with 1µl (15µCi) of L-[^{35}S] methionine for 60 minutes at the cold-shock times indicated.

6.2: MATERIALS AND METHODS.

The DNA and RNA techniques referred to in this chapter are detailed in section 5.2. Preparation of radio-labelled protein samples and SDS-PAGE electrophoresis conditions were the same as those described in section 4.2.

6.2.1: Screening of *M. smegmatis* genomic library.

6.2.1.A: Genomic library preparation.

The *M. smegmatis* genomic library was prepared by Dr M. Everett at Glaxo Wellcome Inc. (Stevenage, UK). Briefly, *M. smegmatis* (LR222) genomic DNA was partially digested with *Sau*3A restriction enzyme and the fragments ligated to *Bam*HI restricted pBluescript II SK^+. These constructs were used to transform *E. coli* (XL1-Blue) competent cells. The individual clones were then imprinted upon Hybond N^+ (Amersham) nylon membranes in a specific sequence, using a Genetix Q-bot automated gridder/picker. The colonies were lysed and the plasmid DNA UV cross-linked to the membrane, as described for colony hybridisations (Ausbel *et al.*, 1987).

6.2.1.B: Probe preparation and hybridisation.

The DNA probe used to screen the genomic library described above, was a 33 base single-stranded DNA primer (see section 6.3.1.B), end-labelled with [γ-^{32}P]dATP. This was achieved with the following reaction: 100ng (10pmoles) DNA primer, 10 units of Polynucleotide kinase (Boehringer Mannheim), 1X polynucleotide kinase buffer (Boehringer Mannheim) and 4µl (40µCi) [γ-^{32}P]dATP (3000Ci/mmol) in a 20µl reaction volume, for 60 minutes at 37°C. The unincorporated, radioactive nucleotides were removed using a Microspin G25 Sephadex column (Pharmacia Biotech). Following 4-6 hours of pre-hybridisation at 42°C in hybridisation solution (Appendix 1), the membrane was incubated with the radioactive probe at 42°C for a further 16-24 hours (Techne hybridiser HB-D1). The membrane was then washed twice with a 2 X SSC / 0.1% SDS solution for 20 minutes at 23°C, followed by a further two washes with 0.2 X SSC / 0.1% SDS for 20 minutes at 42°C, to remove non-specifically bound DNA. The specific clones were detected by exposure (6-12 hours) of the membrane to autoradiography film (Agfa CP-BU).

6.2.2: Western hybridisation analysis.

Protein samples were prepared and separated by one-dimensional SDS-PAGE, as previously described in section 4.2.2. Following electrophoresis, the proteins were transferred from the polyacrylamide gel to a Hybond-C Super nylon membrane (Amersham), with the aid of stacks of 3MM Whatman chromatography paper soaked in transfer solution (see Appendix 1) and a semi-dry electroblotter, running at 200mA for 1-2 hours (Towbin *et al.*, 1979). Specific proteins were then detected on the membrane through the use of the Boehringer Mannheim Chemiluminescence Blotting Substrate (POD) kit. Briefly, the membrane was blocked for 16-24 hours (4°C) in 1% (v/v) blocking solution (10% stock supplied with the kit, diluted with TBS (see

Appendix 1)) and then incubated for a further 16-24 hours (4°C) with the primary antibody, PabCipM (1:200 dilution in 0.5% blocking solution). The membrane was washed in TBS (no tween) (2 X 10 minutes) and then in 0.5% blocking solution (2 X 10 minutes). The secondary antibody, POD-labelled anti-rabbit IgG (Boehringer Mannheim), was diluted 1:5000 (40mU/ml) in 0.5% blocking solution and incubated with the membrane for 60 minutes at room temperature. Following four 10 minute washes with TBS (no tween), the specific proteins were detected using the detection solution supplied with the kit. The membrane was exposed to autoradiography film (Agfa CP-BU) for varying lengths of time (60 seconds – 30 minutes), depending upon the signal intensity.

6.2.3: Protein preparation for sequence analysis.

The induction of CipM was not clearly demonstrated on coomassie-stained protein gels. To correctly identify this cold-induced protein band, cold-shock radio-labelled protein samples were used to "spike" the protein samples. Total protein (100μg) was obtained from cultures (200ml) that had been exposed to 10°C for periods of 1 hour (non-induced control) and 10 hours (highest level of protein induction) (Figure 4.2). These samples were then mixed with radioactive proteins that were labelled with L-[^{35}S] methionine (section 4.2.2.A) during the cold-shock period: 0→1 hours and 10→11 hours at 10°C. Following electrophoresis on a 15% SDS-polyacrylamide glycine gel (section 4.2.2.B), the proteins were transferred to polyvinylidene difluoride (PVDF) nitrocellulose membrane (Amersham), as described in section 6.2.2, using a semi-dry electroblotter (200mA for 1-2 hours). The membrane was then stained with Coomassie Brilliant-Blue (Biorad). After exposure of the membrane to Hyperfilm βmax X-ray film (Amersham) the cold-induced protein band (CipM) was identified and the corresponding Coomassie-stained band (27kDa) was excised.

6.2.4: Generation of strand-specific DNA probes (RNA transcripts).

Using the **cipM1-cipM2** PCR product (1kb) cloned into pGEM-T as the DNA source, strand-specific RNA probes were generated by means of either SP6 and T7 RNA polymerase activity. The DNA was first linearised with either *Pst*I (for the T7 polymerase reaction) or *Sp*HI (for the SP6 polymerase reaction) and then purified by phenol/chloroform extraction and NaAc/isopropanol precipitation (section 5.2.3.B.iii). RNA transcripts were produced from 0.5μg of DNA in the following reaction mixture, as recommended by Boehringer Mannheim: 10nmol of each dATP, dCTP, dGTP, dUTP, 1X transcription buffer (Boehringer Mannheim), 20 units RNase inhibitor (Boehringer Mannheim), 30μCi [α-^{32}P]dCTP (3000Ci/mmol) (Amersham) and 20 units SP6 or T7 polymerase (Boehringer Mannheim), in a 20μl reaction volume incubated at 37°C for 20 minutes. The template DNA was digested with 20units of DNase I (15 minutes at 37°C) and the unincorporated nucleotides were removed using G-50 Micro-spin columns (Pharmacia). The generation of radio-labelled RNA probes was confirmed by the electrophoresis of 2μl of the reaction mixture on a 1.5% denaturing agarose/formaldehyde gel and exposure to autoradiography film (Agfa CP-BU) .

6.3: RESULTS.

6.3.1: Identification of CipM.1.

6.3.1.A: Protein sequencing.

N-terminal protein sequencing was used as the first step in the identification of CipM.1, a protein within the CipM protein band. Unlike the technique of MALDI-TOF MS, relatively large amounts of protein (0.3 - 1μg of protein) are required to obtain sequence data by N-terminal sequencing, in fact the protein band must be visible upon Coomassie-staining. Coomassie-stained protein gels did not clearly show the induction of CipM, so to circumvent this problem radioactive samples were used to spike the protein preparations, as described in section 6.2.3. Following the transfer of the CipM protein band from a one-dimensional SDS-polyacrylamide gel to a nitrocellulose membrane (PVDF), the proteins were subjected to automated gas-phase Edman degradation, generating N-terminal protein sequence data. One dominant N-terminal amino acid sequence (Figure 6.2) was obtained courtesy of Dr Per-Ingvar Ohlsson at Umea University (Umea, Sweden), which represented the N-terminal sequence of CipM.1.

Met Asn Lys Ala Glu Leu Ile Asp Val Leu Thr Lys Lys Met Asn

Figure 6.2: N-terminal amino acid sequence of CipM.1.

The "CipM" band was transferred to a PVDF nitrocellulose membrane and the proteins subjected to Edman degradation. A, dominant N-terminal amino acid sequence was generated, representing the protein CipM.1.

This short amino acid sequence was used to search the available databases (Genbank, Sanger) for proteins with homologous N-terminal sequences. Initially, no significant homology was found to any listed protein sequence. However, following the recent completion of the *M. tuberculosis* genome sequencing project (Cole *et al.*, 1998), this short amino acid sequence demonstrated 85% identity to the *hupB* gene product of *M. tuberculosis* and the corresponding histone-like DNA-binding protein of *M. leprae* (discussed in section 6.3.1.B.ii).

6.3.1.B: DNA analysis.

6.3.1.B.i: Use of a degenerate DNA primer.

The next step in the identification process was to isolate and characterise the *M. smegmatis* gene encoding this N-terminal protein sequence. As no homology was initially found to any known protein sequence, PCR primers could not be designed to amplify up a relevant gene that could then be used to probe a *M. smegmatis* genebank. Instead, using the codon bias for mycobacteria (Dale and Patki, 1990), a degenerate primer was designed to correspond to the N-terminal protein sequence data for CipM.1. The first four amino acids of the sequence were not used to design the primer shown in Figure 6.3, due to possible sequencing errors caused by impurities.

(Met Asn Lys Ala)	Glu	Leu	Ile	Asp	Val	Leu	Thr	Lys	Lys	Met	Asn
msmegX:	**GAG**	**CTC** **G**	**ATC**	**GAC** **T**	**GTG** **C**	**CTG** **C**	**ACG** **C**	**AAG**	**AAG**	**ATG**	**AAC**

Figure 6.3: Degenerate primer (msmegX)

Using the codon bias for mycobacteria, the N-terminal amino acid sequence of CipM.1 (top line) was converted into a degenerate DNA sequence, **msmegX**, which was used to create the degenerate primer.

This degenerate primer (**msmegX**) was end-labelled with [γ-^{32}P]dATP and used as a probe to screen a *Sau*3A restriction fragment library of the *M. smegmatis* (LR222) genome. The DNA from at least sixteen clones hybridised to this probe. The plasmid DNA from eleven of these clones (pMSA - pMSK) was analysed. Nine were found to contain mycobacterial DNA inserts that hybridised to **msmegX** upon re-screening. The complete *M. smegmatis* genomic library was present in several copies on the gridded membrane and so the finding that some of the plasmid inserts (A, C, D) showed identical banding patterns, upon digestion with restriction enzymes, was not unexpected (results not shown). Insert DNA from plasmids pMSA, pMSE, pMSG, pMSH, pMSI, pMSJ and pMSK was used to probe *Bam*HI digested genomic DNA from *E. coli*, *M. smegmatis*, *M. tuberculosis* and *M. bovis BCG*. The insert DNA from most of the plasmids (A, G, H, I, J) gave identical banding patterns, indicating that one gene had been highlighted in the *M. smegmatis* genome by *msmegX*. This gene (insert A) was detected in several mycobacterial species, but not in *E. coli* (Figure 6.4).

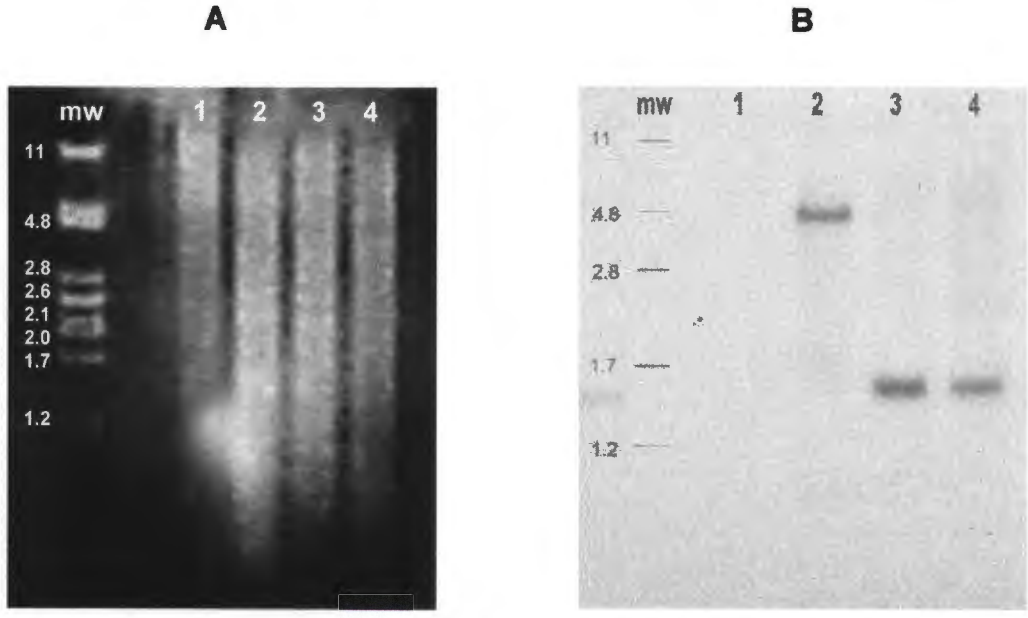


Figure 6.4: Southern hybridisation analysis of *M. smegmatis* genomic fragments: A, G, H, I and J.

Genomic DNA from *E. coli* (1), *M. smegmatis* (2), *M. tuberculosis* (3) and *M. bovis* BCG (4) was digested with *Bam*HI and electrophoresed on a 1.2% (w/v) agarose gel. Following transfer to a Hybond N^+ nylon membrane, the genomic DNA was probed separately with the *M. smegmatis* *Sau*3A DNA fragments A, G, H, I and J ([α-^{32}P]dCTP random-labelled). **A)** Photograph of the ethidium bromide-stained agarose gel prior to transfer. **B)** Autoradiograph generated after probing the genomic digests with the *Sau*3A fragment A (same pattern as G, H, I and J). mw = molecular weight marker λ *Pst*I (kb).

All further analysis was performed on insert A DNA, a 2.3kb *Sau*3A fragment contained within pMSA. Restriction analysis of the insert produced the following map (Figure 6.5), with **msmegX** hybridising to the *Eco*RI/*Bam*HI 1.5kb fragment, following Southern hybridisation of the restriction fragments.

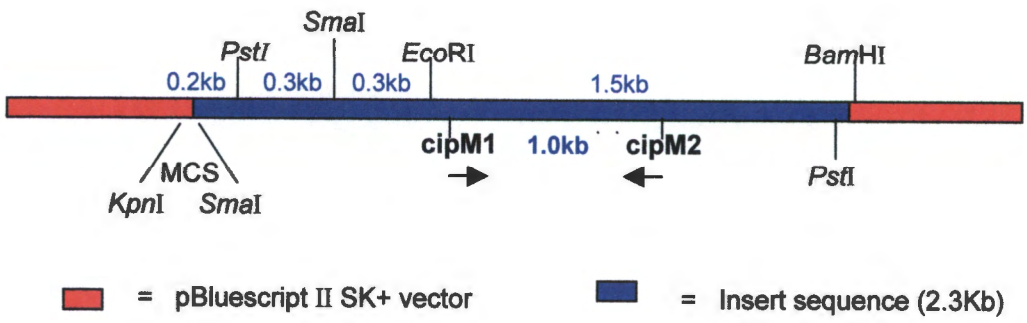


Figure 6.5: Restriction map of pMSA.

Plasmid pMSA was digested with a variety of restriction enzymes and the fragments separated on a 1.2% agarose gel. Fragment lengths were determined by comparison with λ *Pst*I and DNA marker VI standards (Boehringer Mannheim). Fragment lengths are given in kilobases (kb). MCS=plasmid multiple cloning site. **cipM1** and **cipM2**: *cipM.1* specific PCR primers

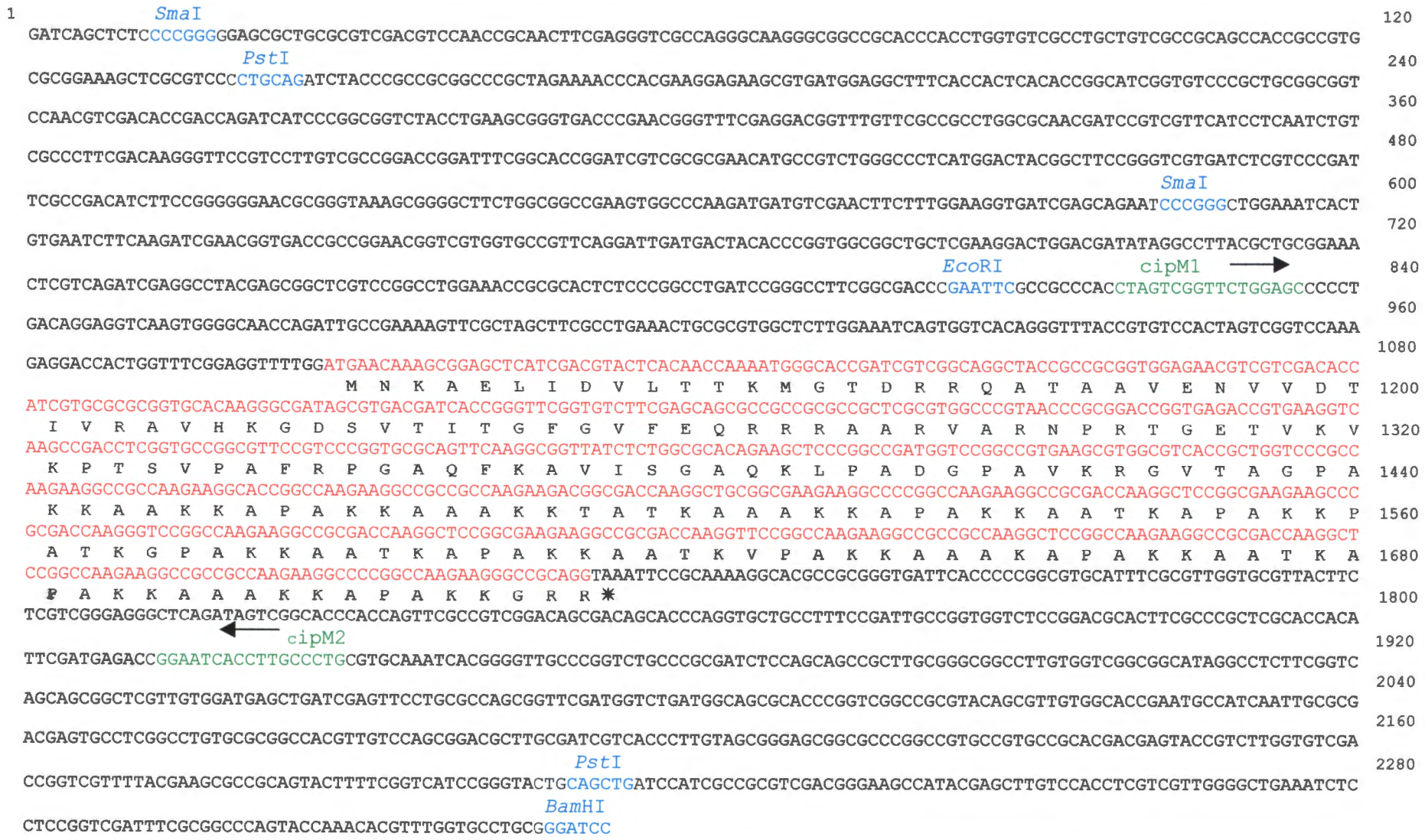


Figure 6.6: DNA sequence of the 2333bp mycobacterial DNA insert from pMSA (insert A).

Enzymes used in the analysis of this fragment of *M. smegmatis* genomic DNA are shown in blue. The positions for the binding of PCR primers **cipM1** and **cipM2** are highlighted in green, which generate a 1011bp product (between the arrows). The DNA sequence for ORF 1 is highlighted in red, with the predicted amino acid sequence given below this sequence.

6.3.1.B.ii: Sequence analysis of insert A.

Sequence data was obtained from the intact 2333bp DNA insert, a sub-clone of the 1.5Kb *Eco*RI/*Bam*HI restriction fragment and a PCR amplified product (using PCR primers **cipM1** and **cipM2**) (Appendix 2, for positions see Figure 6.5 and 6.6). Figure 6.6 details the sequence data obtained for the 2.3Kb insert. Using the GENEPRO™ Version 6.10 sequence analysis program, the positions of the ORFs within this fragment of mycobacterial DNA were located (Figure 6.7).

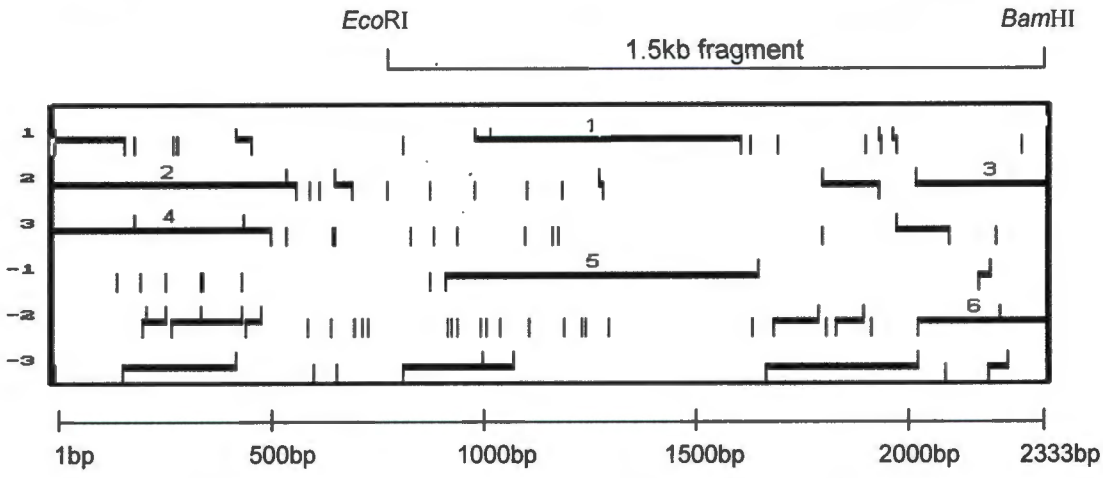


Figure 6.7: Putative open reading frames in insert A.

Using the GENEPRO™ DNA analysis program, putative ORFs were located in the 2.3Kb insert of pMSA (insert A). ORFs that could potentially encode proteins of greater than 15kDa (400bp) in size are numbered 1-6. The position of the *Eco*RI/*Bam*HI 1.5kb restriction fragment is also indicated.

Several putative ORFs were identified, within this 2.3kb *Sau*3A restriction fragment of *M. smegmatis* genomic DNA, that could potentially encode the CipM.1 protein. Through Southern hybridisation of the restriction fragments it was demonstrated that the degenerate probe, **msmegX**, hybridised to a sequence located within the 1.5kb *Eco*RI/*Bam*HI restriction fragment (results not shown). Closer analysis of the DNA sequence of this 1.5kb restriction fragment revealed a single position at which the **msmegX** primer could potentially hybridise (greater than 50% homology) - the N-terminus of ORF 1, an ORF of 208 amino acids. The degree of homology between the DNA sequences at the 5' end of this putative ORF and the degenerate probe was 76%. However, the degree of homology between the N-terminal amino acid sequence of ORF 1 and the N-terminal protein sequencing data was greater, demonstrating 87% identity (Figure 6.8). Based upon this sequence homology, the protein encoded by ORF 1 was designated CipM.1.

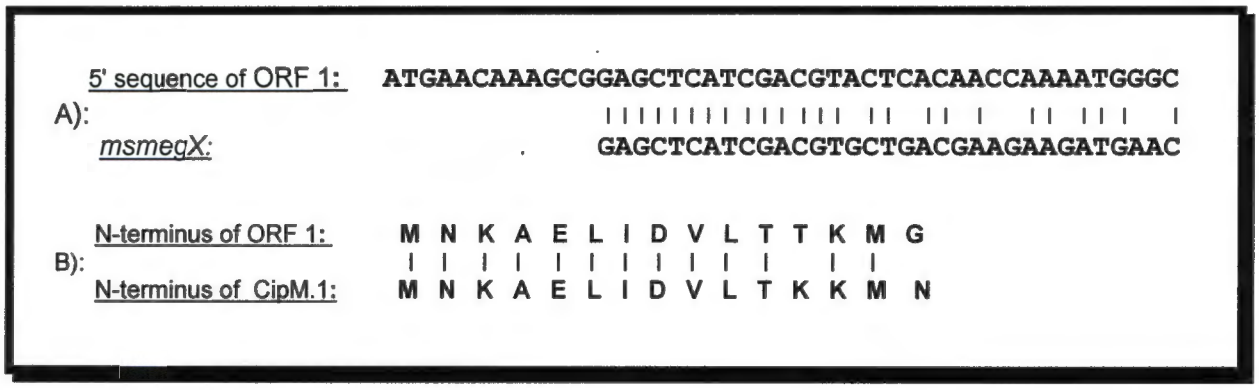


Figure 6.8: Comparison of the predicted sequence of CipM.1 versus the sequence of ORF 1.

A) Homology between the 5' DNA sequence of ORF 1 and the degenerate primer *msmegX* (76%). B) Homology between the predicted N-terminal amino acid sequence of ORF1 and the N-terminal sequence data obtained from CipM.1 (protein sequencing) (87%). Lines indicate identity.

A Blast homology search (Genbank database) (Altschul *et al.*, 1990) using the DNA sequence for ORF 1 (CipM.1), revealed high homology to a gene present in both *M. leprae* (83% identity over N-terminal 376bp) (accession number: Z99263, gene: MLCB637.34, position: 38644 - 39246) (Eiglmeier *et al.*, 1993) and *M. tuberculosis* (82% identity at N-terminal 400bp) (accession number: Z83018, gene: MTCY349.01, position: 274-918) (Cole *et al.*, 1998). Both genes encode putative mycobacterial proteins that possess a PS00045 bacterial histone-like DNA-binding protein signature. They also both show homology to the N-terminal region of the histone-like DNA binding protein II of *B. subtilis* (HB) (48.3% identity in 89aa overlap), as well as to the eukaryotic G777718 histone H1 protein (48.8% identity in 162 aa overlap). It has been suggested that this gene could represent the mycobacterial *hupB* gene, which encodes the HUβ subunit of the HU DNA-binding protein (Cole *et al.*, 1998). The lysine-rich C-terminus of these proteins, which consists of degenerate repeats of lysine, alanine, valine, proline and threonine, is unique to these two mycobacterial proteins (Eiglmeier *et al.*, 1993; Cole *et al.*, 1998).

The N-terminal amino acid sequence (109 aa) of CipM.1 of *M. smegmatis* showed 90% identity to the predicted N-terminus of the histone-like DNA-binding protein of *M. leprae* (protein: g2414560) and 93% identity to the N-terminal amino acids of the corresponding *hupB* gene product of *M. tuberculosis* (protein: g1694845). The C-terminus of CipM.1 (99 aa) consisted mainly of proline, lysine, alanine, valine and threonine residues, which was similar to the unique C-terminus found in the other two mycobacterial proteins. Although there was very little exact sequence identity between the three proteins at the C-terminus, they shared similar proportions of alanine, lysine, proline and threonine residues. Figure 6.9 demonstrates the degree of conservation between these mycobacterial proteins.

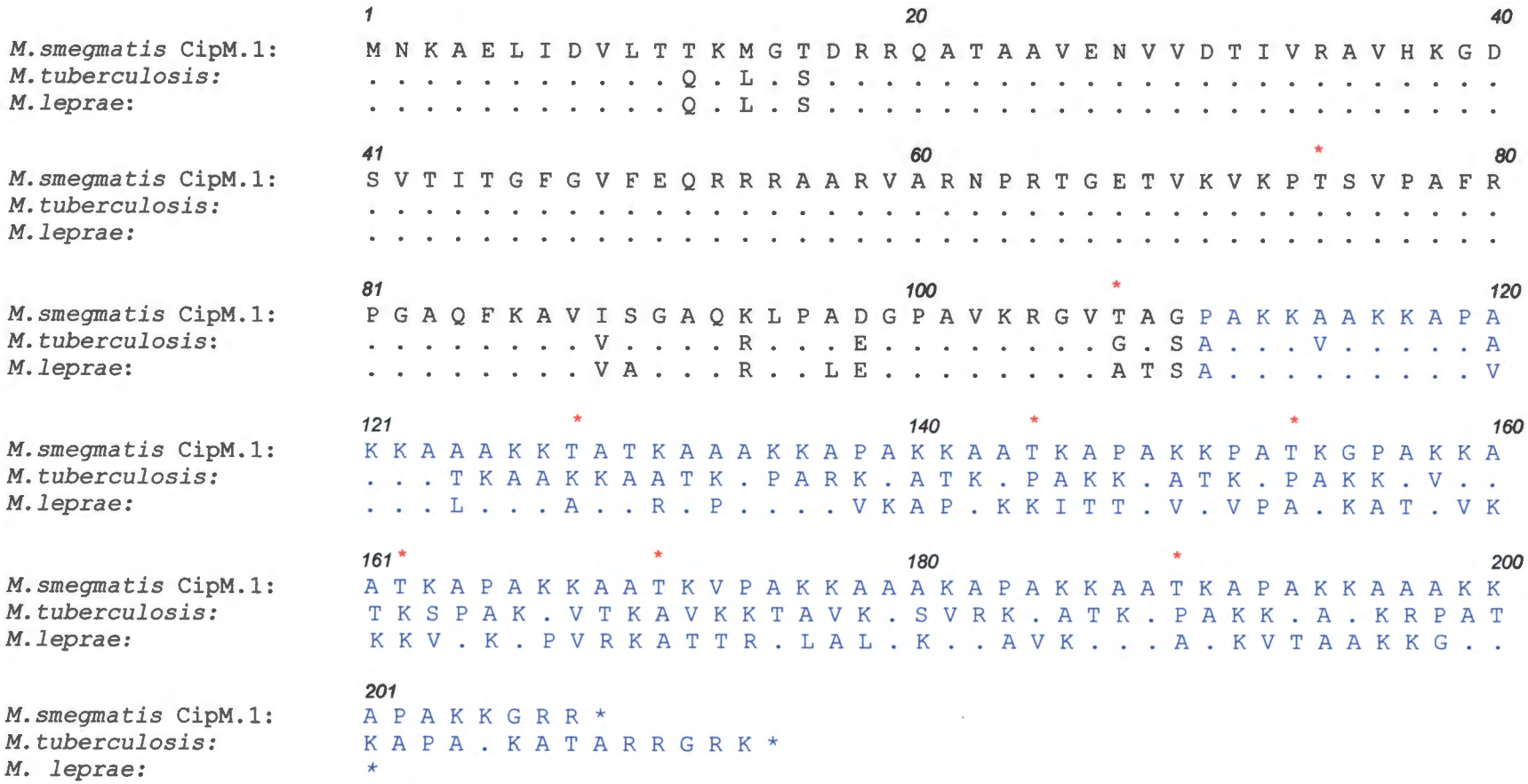


Figure 6.9: Mycobacterial histone-like DNA binding proteins.

Comparison of the predicted amino acid sequences of the putative histone-like DNA-binding proteins from *M. leprae* and *M. tuberculosis* with CipM.1 (ORF 1) of *M. smegmatis*. The amino acid sequence for *M. smegmatis* CipM.1 is written in full, while (.) symbols indicate identical sequence and the letters indicate sequence variations in the predicted protein sequences of the other two mycobacterial proteins. The lysine rich C-termini are indicated in blue. Putative O-glycosylation sites as predicted by NetOGlyc 2.0 (Center for Biological sequence analysis) are indicated by * symbols.

Using the predicted amino acid sequence of CipM.1, the Genbank database was again searched (utilising BlastP (Altschul *et al.,* 1997)) for other proteins sharing homology with this putative mycobacterial histone-like DNA-binding protein. The HU DNA-binding proteins of several bacterial species, including *E. coli*, *S. coelicolor*, *Bacillus stearothermophilus, B. subtilis* and *Vibrio proteolyticus* showed 30-50% identity to the N-terminus (90 aa) of CipM.1. The integration host factor proteins of several bacterial species (*E. coli*, *S. typhimurium*, *Serratia marcescens*) also demonstrated a degree of homology (30-35% identity) to the N-terminus of CipM.1 from *M. smegmatis.*

6.3.2: Cold-induced expression of CipM.1 – protein analysis.

6.3.2.A: Two-dimensional SDS-PAGE time-course analysis of CipM.1 expression.

According to the two-dimensional protein analysis described in Chapter 4, several cold-induced proteins were potentially present within the CipM protein band. To aid the identification of the specific CipM.1 protein spot following two-dimensional protein analysis and to then analyse its pattern of expression, the pI of CipM.1 was calculated using the predicted amino acid sequence (ORF 1). Due to the lysine rich C-terminus of this protein, the predicted pI of 12.13 was not surprising. The other histone-like DNA-binding proteins of *M. leprae* and *M. tuberculosis* gave predicted pI values of 12.2 and 12.4, respectively. Two programs were used for this calculation, DNA Man (Lynnon-Biosoft, Canada) and ExPaSy pI / Mw tool (Bjellqvist *et al.,* 1993), both programs giving similar values for these three mycobacterial proteins (pI range 11.9 - 12.5). Due to the highly basic nature of CipM.1, the first dimension separation of isoelectric focusing in the pH range 3-10, which is the widest separation system currently commercially available, would not have been sufficient to focus this protein. As a result, either the protein would have migrated collectively with other proteins having a pI of greater than 10, or it would have been electrophoresised off the Immobiline Drystrip. Neither result would have generated a defined CipM.1 spot following the second dimension SDS-PAGE analysis. Some progress has been made in producing immobilised gel gradients which span the pH 10-12 range (Bossi *et al.*, 1994; Görg *et al.*, 1997), but these are not yet commercially available and still do not efficiently separate mixtures of basic proteins without major modifications (depending upon protein mixture) (Herbert *et al.*, 1997).

6.3.2.B: Analysis of CipM.1 expression using polyclonal antibodies.

Through the use of CipM.1-specific antibodies it would be possible to determine the pattern of CipM.1 expression using a one-dimensional separation system. As an alternative to generating large amounts of purified, full-length protein, it is possible to generate specific antibodies using a peptide of 15-20 aa in length (Hancock *et al.,* 1998). This peptide approach was chosen to generate CipM.1-specific antibodies. For the purpose of future studies in other mycobacterial species, a peptide was selected from the conserved N-terminal amino acids of CipM.1. The

peptide shown in Figure 6.10 was chosen as a suitable candidate based on its predicted hydrophobicity, high antigenicity index and the presence of a beta turn (suggested by Dr R. Harris, Commonwealth Biotechnologies INC (CBI), Richmond, USA). CBI synthesised this peptide and then conjugated it to a known immunogenic peptide (maleimide activated keyhole limpet hemocyanin) to enhance the immunogenicity of the CipM.1 peptide.

59 76

Val Ala Arg Asn Pro Arg Thr Gly Gln Thr Val Lys Val Lys Pro Thr Ser Val

Figure 6.10: CipM.1 antigenic peptide.

From the N-terminal 109 aa of CipM.1, this peptide was predicted to be the most antigenic, based on its hydrophobicity and high antigenicity index. This peptide represents amino acids 59 - 76 in the intact CipM.1 polypeptide.

A portion of this peptide (1.25mg) was mixed with Freund's complete adjuvant and used to immunise two rabbits. A total of four injections were given to each rabbit, over a 49 day period. Three blood samples (primary antibody solution) were taken from each rabbit: day 44, day 63 and day 68 post initial inoculation, to assess the antibody response to the peptide (as recommended by Dr Harris, CBI). The antibodies (PabCipM) were tested against protein extracts from *M. smegmatis* cells before (37°C) and after several hours of exposure to 10°C. These protein extracts were separated on 16.5% tricine-SDS polyacrylamide gels and transferred onto Hybond-C Super nylon membrane. The Chemiluminescence Blotting substrate kit supplied by Boehringer Mannheim was used to detect the specific CipM.1-antibody interaction. Initial western hybridisations using the manufacturers' specifications did not yield a reaction in the 20-30kDa molecular weight range. To improve the sensitivity, a range of primary antibody solution dilutions were assayed (1:200 - 1:5000), with the membranes being incubated with the primary antibody solution for extended periods (24 hours at 4°C). All washing steps were performed without tween. Again, no specific antibody binding was seen in the 20-30kDa protein molecular weight range (results not shown).

6.3.3: Expression of *cipM.1* - mRNA analysis.

Although the N-terminal protein sequencing data suggested that ORF 1 encoded the CipM.1 protein, further protein analysis could not confirm that this putative protein or corresponding gene (*cipM.1*) was cold-inducible, or indeed if the protein was actually synthesised. Northern hybridisation analysis was used to investigate the expression of this ORF at the transcriptional level. Total RNA was extracted during growth at 37°C and after transfer to 10°C, according to the schedule shown in Figure 5.3. Following transfer to a Hybond N^+ membrane, this RNA was probed

with several [α-^{32}P]dCTP labelled DNA fragments (random-primed) from insert A (Figure 6. 11). These included the intact 2.3kb insert, the 1.5kb *Eco*RI/*Bam*HI restriction fragment, the double-stranded PCR product (1011bp) generated using **cipM1** and **cipM2**, and RNA probes produced from both the + and – DNA strands of the PCR product, to distinguish between the expression from ORF 1 and ORF 5 (section 6.2.4).

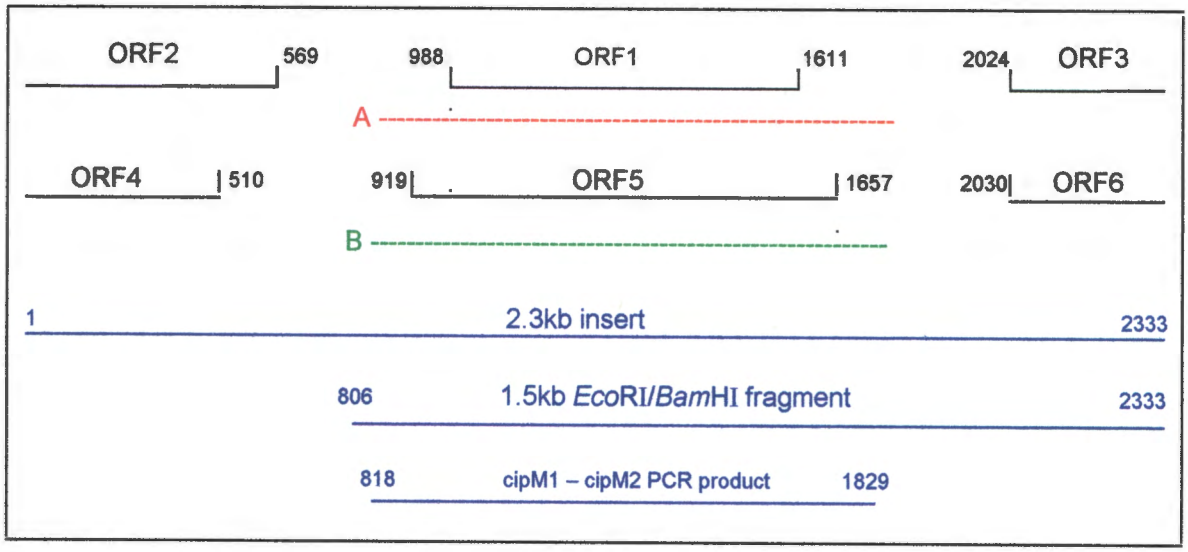


Figure 6.11: Probes used in the analysis of *cipM.1* expression.

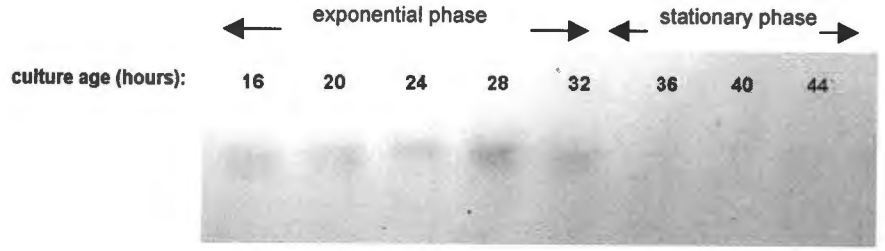
Schematic representation of the putative ORFs encoded by insert A (pMSA) and the probes used to locate transcriptionally active areas. The blue lines show the relative positions of the double-stranded DNA probes used in the northern hybridisation analysis. To distinguish between transcription from ORF 1 and ORF 5, RNA probes were generated from both the + and – strands of the cloned 1011bp PCR product, using the T7 and SP6 promoters of pGEMT, respectively. The + RNA (B), indicated in green will specifically hybridise to mRNA generated from ORF 5, while the – RNA (A), indicated in red, will hybridise to mRNA generated from ORF 1, in northern hybridisation analysis.

An identical transcript signal (size and intensity) was generated when using all of the double-stranded DNA probes in the northern analysis described below, with only the RNA probe complimentary to the ORF 1 transcript (SP6 promoter) generating a detectable radioactive signal (results not shown). These results indicated that only one of the putative ORFs in insert A was being transcribed – ORF 1. Using the 16S, 23S and 5S rRNA bands as markers, the size of the transcript was determined to be between 600bp and 700bp. A transcript of this size would encode a protein of 200-233 amino acids, which includes the size of the putative protein product of ORF1, 208aa. This data confirmed that ORF 1 (*cipM.1*) was being transcribed.

6.3.3.A: Growth-related expression of *cipM.1*.

The expression of *cipM.1* during growth at 37°C and 10°C is shown in Figure 6.12. Expression at 37°C (Figure 6.12.A) was shown to be similar to that of *cspA*, with mRNA being produced during the exponential growth phase of the culture (16-32 hours) and then levels declining upon entry of the cells into stationary phase (36 hours).

A: 37°C *cipM* expression



B:10°C *cipM* expression – lag period analysis

C: 10°C *cipM* expression – 7 day analysis

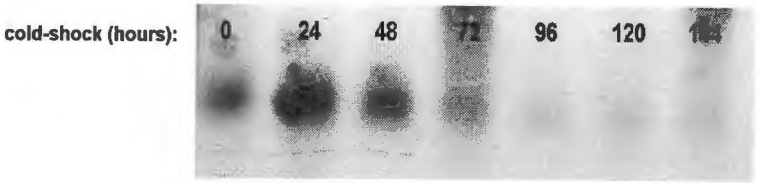


Figure 6.12: Expression of *cipM.1*- northern hybridisation analysis.

A) RNA was extracted from cultures of *M. smegmatis* grown at 37°C, at the culture ages indicated. This RNA (40μg) was electrophoresed on a 1.5% agarose/ formaldehyde gel and then transferred to Hybond N^+ nitrocellulose membrane via capillary-blotting. The picture shows the autoradiograph of the membrane following hybridisation with a [α-^{32}P]dCTP-labelled *cipM.1* 1011bp PCR product (random-primed). **B)** RNA was extracted from *M. smegmatis* after the transfer of an exponentially-growing culture (20-24 hours) from 37°C to 10°C, at the post-cold-shock times indicated (first 30 hours). This RNA was processed as described in (A) and the autoradiograph resulting from the hybridisation with the 1011bp *cipM.1* probe is shown. **C)** *M. smegmatis* RNA was extracted at 24-hour intervals after the transfer of the culture from 37°C to 10°C. The RNA was processed as described above and the autoradiograph resulting from the hybridisation with the 1011bp *cipM.1* probe is shown.

The RNA used in this experiment was the same (batch and quantity) as that used in the S1 nuclease analysis of *gyrA* expression at 37°C, where levels of *gyrA* were found to be constant throughout growth. Although it may be indirect, the constant *gyrA* levels do act as a control for the quality and quantity of the RNA used in this *cipM.1* RNA analysis. The photograph of the ethidium bromide stained gel, prior to transfer, also showed no major differences in the levels of total RNA (not shown).

6.3.3.B: Cold-shock expression of *cipM.1*.

Figure 6.12.B demonstrates the expression of *cipM.1* following a shift from 37°C to 10°C (first 30 hours). Although the induction of *cipM.1* mRNA was not as dramatic as that of *cspA* mRNA (16-24 fold induction) (section 5.3.1.B.ii), a definite increase in the quantity of mRNA could be seen after several hours at 10°C (time 0 = expression at 37°C, pre-cold shock). This result suggests that *cipM.1* is a cold-shock gene of *M. smegmatis*. The mRNA analysis was repeated three times, with all experiments showing an increase in the quantity of *cipM.1* mRNA after exposure to 10°C. The bands on the autoradiographs were scanned using a densitometer (Shimadzu CS-9000) and the relative band densities were plotted versus exposure time to 10°C (Figure 6.13).

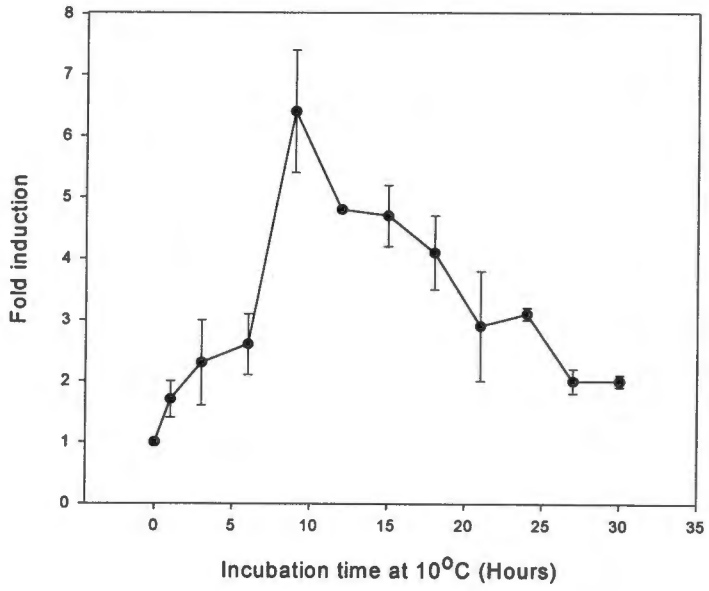


Figure 6.13: Expression of *cipM.1* during a cold shock (37°C→10°C).

Autoradiographs resulting from the northern hybridisation analysis of *cipM.1* expression were scanned using a densitometer (Shimadzu CS-9000). The band densities at 10°C were compared to the band obtained pre-cold shock and the relative increases in density were plotted. The error bars represent the standard error of the mean of three experiments.

The pattern of expression shown in Figure 6.13 demonstrates the transient cold-shock induction of *cipM.1*. Increasing levels of *cipM.1* mRNA were detected almost immediately after transfer to 10°C, with a 2-3 fold induction seen after 3 hours. Following 9-12 hours of cold exposure, levels of *cipM.1* mRNA reached a peak induction of 5-7 fold and then declined again slowly.

Analysis of *cipM.1* mRNA levels during a prolonged cold shock confirmed that increased expression was limited to the lag period (Figure 6.12.C). Following 48 hours incubation at 10°C, when the mycobacterial cells were showing signs of growth recovery (section 3.3.2.B.iii), *cipM.1* mRNA levels had returned to pre-cold shock levels. Further incubation at 10°C resulted in a decline in the quantity of detectable *cipM.1* mRNA. These results were confirmed following RNA analysis in a further two independent experiments (results not shown).

6.4: DISCUSSION.

As described in Chapter 4, the one-dimensional SDS-PAGE analysis of cold-induced proteins (Figure 6.1) showed that a 27kDa protein band, CipM, was the major cold-induced protein band of *M. smegmatis*. According to the results presented in this chapter, one of the proteins (CipM.1) contained within this protein band was identified as a histone-like DNA-binding protein. The cold-shock induction of the corresponding gene, *cipM.1*, suggests that this protein is one of the cold-induced stress proteins of *M. smegmatis*.

6.4.1: *cipM.1* gene identification.

Using the N-terminal protein sequencing data obtained from the analysis of the CipM protein band, a degenerate DNA probe (**msmegX**) was generated which hybridised to a specific DNA fragment in the *M. smegmatis* genome. Following isolation and sequencing of this fragment (insert A), it was established that the probe hybridised at the N-terminus of ORF 1, a putative open reading frame of 208aa. The N-terminal amino acid sequence of this ORF shared 87% homology with the protein sequence generated from the N-terminal protein sequencing of CipM.1. Northern hybridisation analysis confirmed that a transcript was generated from this ORF. From this data it was concluded that ORF 1 of insert A (pMSA) encoded CipM.1.

The DNA sequence of *cipM.1* shared considerable homology with a mycobacterial gene found in two other mycobacterial species: *M. leprae* and *M. tuberculosis* (Eiglmeier *et al.*, 1993; Cole *et al.*, 1998). This mycobacterial gene has been labelled as a putative *hupB* homologue, the gene that encodes one of the subunits (HUβ) of the HU protein, a histone-like DNA-binding protein of *E. coli* (Kano *et al.*, 1986; Kano *et al.*, 1988). This *hupB* classification was based upon sequence homology between the N-terminus of the two mycobacterial proteins and that of several other bacterial HU proteins, as well as an eukaryotic histone protein. Up until recently, the C-terminus of CipM.1 and the two other mycobacterial histone-like DNA-binding proteins, which consist of degenerate repeats of proline, lysine and alanine residues, was unique (Cole *et al.*, 1998). However, another mycobacterial protein, heparin-binding hemagglutinin (HBHA) (mycobacterial adhesin), has recently being sequenced (*M. tuberculosis* and *M. bovis BCG*) which possesses a C-terminal region of 40aa that is also rich in proline, lysine and alanine residues (Menozzi *et al.*, 1998) (Accession number: AF074390). This region shares 87% identity to several sections of the *M. smegmatis* CipM.1 C-terminal sequence. No homology (less than 10% identity) was noted between the N-termini of these two proteins.

An anomaly was noted in the electrophoretic size of CipM.1. The predicted molecular weight of this protein was calculated to be 21.3kDa (208aa), yet it migrated within a 27kDa protein band. This aberrant electrophoretic mobility is typical of highly charged proteins (i.e: histones and ribosomal proteins) and proteins rich in proline residues (Herbert *et al.*, 1997). As already

mentioned, the C-terminus of CipM.1 is indeed proline rich and the presence of numerous lysine residues contributes to the predicted highly basic pI of 12.13. Gel retardation can also be caused by the presence of carbohydrate moieties. Although bacterial glycoproteins are rare, several glycosylated mycobacterial proteins have already been identified (Dobos *et al.,* 1995; Espitia and Mancilla, 1989). Several putative glycosylation sites were identified on CipM.1, through computer analysis (Figure 6. 9). It is interesting to note that the HBHA protein described above also demonstrates an unexpected electrophoretic mobility. The predicted size of this protein is also 21.3kDa, yet it migrates at 28kDa when analysed by SDS-PAGE. The mobility shift in this case was found to be attributed to both the lysine/proline-rich repeats and to the presence of a carbohydrate moiety (Menozzi *et al.,* 1998).

6.4.2: *cipM.1* cold-shock expression.

Northern hybridisation analysis of *cipM.1* mRNA clearly demonstrated that the gene encoding ORF 1 was responsive to a cold shock, showing a 5-7 fold induction in mRNA levels after 9-12 hours of exposure to 10°C. Its cold-shock induction was, however, transient, with increased levels only noted during the first 24-hour period. Whether this induction was due to increased gene transcription or increased mRNA stability (as for *cspA* of *E. coli*) could not be determined from this set of experiments. Although this induction was not as dramatic as that of *cspA* of *M. smegmatis* (16-24 fold induction), the response was more rapid. This implied that the *cipM.1* gene product was required before that of *cspA.*

Increasing levels of *cipM.1* mRNA should result in the increased production of CipM.1, confirming that this protein, contained within the "CipM" protein band, was a cold-induced protein of *M. smegmatis.* From the transient nature of the cold-shock induction of the gene and from the size of the protein product (>10kDa), the classification of this protein as a cold-induced protein (Cip) is still valid. Due to the very basic nature of this protein (pI = 12.13), two-dimensional protein analysis was not possible, using the commercially available separation systems. Therefore in order to define the cold-shock expression pattern of this protein attempts were made to generate CipM.1-specific antibodies, which could then be used on proteins separated in a one-dimensional gel separation system. A peptide contained within the conserved N-terminus of the protein was used to generate polyclonal antibodies. However, a CipM.1-specific antibody reaction could not be demonstrated. Due to the similarity (48%) of the N-terminus of this protein with the eukaryotic histone H1 G777718 protein, it is possible that the CipM.1 peptide was not recognised as being of foreign origin and an immune response was not generated. Antibodies could be generated from the C-terminal lysine rich region, but these would not necessarily clarify the cold-shock expression pattern of CipM.1 if the HBHA mycobacterial protein characterised in *M. tuberculosis* (Menozzi *et al.,* 1998) is also present in *M. smegmatis.* Due to the similarity between these two proteins at the C-terminus, the CipM.1-specific polyclonal antibodies would probably cross-react with this protein.

With a migration size difference of less than 1kDa (HBHA = 28kDa) the results would be difficult to interpret. (Menozzi *et al.,* 1996, Menozzi *et al.,* 1998).

6.4.3: CipM.1 function.

The most obvious question is "what function does CipM.1, a putative HU subunit (histone-like DNA-binding protein), play in the cold-shock response of *M. smegmatis*?" In *E. coli*, HU and H-NS are the major chromosomal-associated proteins. As discussed in Chapter 2, H-NS, which is a cold-shock protein of *E. coli*, has a variety of functions including acting as a transcriptional modulator, repressing and activating a variety of genes which are responsive to environmental changes. HU, on the other hand, is mainly responsible for maintaining the integrity and stability of the bacterial chromosome (Rouviere-Yaniv *et al.,* 1979), with mutations in both of the HU genes resulting in reduced cell division and cell viability (Wada *et al.,* 1988). HU has also been shown to play a role in the initiation of DNA replication (Skarstad *et al.,* 1990), DNA breaking and rejoining in transposition and inversion reactions (Lavoie and Chaconas, 1993), as well as homologous recombination and recombinational repair of UV damaged DNA (Dri et al., 1992; Li and Waters, 1998). Although HU has not yet been classified as one of the cold-shock proteins, several lines of evidence suggest that it may play a role in the cold-shock stress response of *E. coli*. Malik *et al.* (1996) proposed that HU facilitates the action of DNA gyrase, which is one of the cold-shock proteins of *E. coli* (Jones *et al.,* 1992b) and both DNA gyrase and HU have been associated with the increase in negative supercoiling that occurs following a cold shock in *E. coli* (Mizushima *et al.,* 1997). HU mutants, mutated in both the *huA* and *huB* genes, are cold-sensitive, showing reduced viability following a cold-shock (Wada *et al.,* 1988). Finally, HU has also been directly linked with heat-shock stress, showing an involvement in the re-supercoiling of DNA. Ogata *et al.* (1997) propose that HU maintains the negative supercoiling of DNA during the stress and contributes to the thermotolerance of the bacteria. HU may therefore play a similar role (direct or indirect) in the cold-shock response of *E. coli,* maintaining the integrity of the chromosome structure. Due to the similarity of CipM.1 with HU and the highly charged nature of the C-terminus, it is possible that CipM.1 of *M. smegmatis* has a role in DNA or RNA-binding. This protein could therefore function in an HU-like capacity, condensing the chromosome and maintaining DNA structure during the cold shock. It could also function as a RNA-associated protein, preventing the formation of secondary structures in the mRNA transcripts and aiding translation during cold shock, like CspA and CsdA of *E. coli*.

As previously mentioned, only the N-terminus of CipM.1 shows homology to the HUβ subunit, with the C-terminus bearing no resemblance to HUα, the other subunit of HU. According to the analysis of Menozzi *et al.* (1998), the lysine/proline-rich C-terminus of HBHA, which shows identity to the C-terminus of CipM.1, is responsible for the cellular adhesion properties exhibited by this protein. It has been shown that an increasing number of respiratory pathogens produce heparin-binding adhesins that interact with sulphated carbohydrates on the surface of epithelial cells (Rostand and

Esko, 1997), as an initial step in a bacterial infection. The finding that the HBHA C-terminus is involved in binding to sulphated polysaccharides and therefore in binding to epithelial cells, suggests an important role for HBHA and possibly the mycobacterial histone-like DNA-binding proteins (CipM.1) in the initial stages of a mycobacterial infection.

Recently, an anaerobic model for dormancy was demonstrated in *M. smegmatis* (Dick *et al.,* 1998), with the oxygen-depleted cells exhibiting all of the characteristics of "dormant" *M. tuberculosis* (Wayne, 1977). A protein sequence, which is indicated as being upregulated in "dormant" *M. smegmatis* has since been deposited in the Genbank database by the same research group (Lee *et al.,* 1998). This protein, a histone-like protein (accession number: AF068138), has a sequence with 205 of the 208aa showing identity to CipM.1 (identical protein). This finding suggests that Cipm.1 is not only important during stress (cold shock), but that it plays a role in dormancy as well. This supports the idea that dormancy is a stress-induced state (Young and Cole, 1993) and that the study of mycobacterial stress responses may help to elucidate the proteins important for bacterial survival during dormancy.

CHAPTER 7

General conclusions and discussion

7.1: THE *M. SMEGMATIS* COLD-SHOCK RESPONSE.

The aim of the current project was to investigate the effects of cold-shock stress on *M. smegmatis,* a fast-growing mycobacteria that has recently been shown to exhibit characteristics of dormant *M. tuberculosis* under anaerobic conditions (Dick *et al.,* 1998). As well as providing information about the ability of mycobacteria to survive harsh environmental stresses, cold-shock survival is relevant in the transmission process of *M. tuberculosis*. Expelled bacteria are able to survive in the atmosphere, in droplet nuclei, for extended periods and yet maintain viability, causing infection upon inhalation (Loudon *et al.,* 1969). It is possible that these bacteria are suspended in a stress-induced persistent-state after being exposed to the temperature-reduction, the change in oxygen pressure and severe depletion in nutrients. The approach was to investigate this mycobacterial stress response along similar lines to the research into cold-shock stress in *E. coli.* Initially, the effects of temperature-reduction on growth and metabolism were investigated, to establish if the characteristic lag period, of *E. coli*, also occurred following the cold-shock in *M. smegmatis*. Following this analysis, the induction of cold-shock proteins was studied. After establishing the general effects of the stress, attempts were then made to identify some of the individual components of the mycobacterial cold-shock response. Using both the mycobacterial database and the knowledge of cold-shock induction of various *E. coli* genes, the cold-shock expression of several mycobacterial gene homologues was investigated. N-terminal protein sequencing was also employed to help characterise one of the mycobacterial cold-shock proteins.

M. smegmatis demonstrated similar characteristics to *E. coli*, when exposed to a cold shock. The magnitude of the cold-shock response was dependant upon the degree of the temperature reduction, as reflected in the length and extent of the lag in cellular activity and in the number of induced cold-shock proteins. The temperature-shift from 37°C to 10°C resulted in the most dramatic metabolic changes in *M. smegmatis*, with a 24 hour lag in metabolic activity occurring after the temperature reduction. Also, as with the cold shock in *E. coli*, both the synthesis of RNA and protein macromolecules were affected. In conjunction with a lag in the synthesis of these molecules, there was also a notable initial decrease in the rates of synthesis, which was found to become more apparent as the temperature was decreased. A 3-fold decrease in protein synthesis occurred upon the transfer of the culture to 20°C, as opposed to a 20-fold reduction at 10°C. The initial rapid decline in protein synthesis could indicate a possible cold-induced block in the translational machinery of the mycobacteria. Growth recovery at 10°C occurred in an unbalanced manner, with an increase in protein synthesis noted before an increase in RNA synthesis and cell

division. However, unlike the adaptation of *E. coli* to the colder environment, cell division at 10°C in *M. smegmatis* was not synchronous.

During the first 24 hours (lag period) of the cold-shock in *M. smegmatis*, changes at both the protein and mRNA levels were found to occur. At least fifteen cold-shock proteins were induced during this time period and these could be grouped into different classes depending upon their size and cold-shock expression: CSP, CIP and CAP. Three of these proteins appeared to be unique to the cold-shock response, as they were not expressed during growth at 37°C prior to the cold-shock. The majority of the proteins that were analysed exhibited transient induced expression and are probably required for the adaptation of the bacteria to the lower growth rate. Six of the proteins however, were expressed at higher levels during the adapted growth at 10°C and are probably necessary for continued cell viability at the lower temperature. Unlike the cold-shock response of *E. coli*, no single protein was induced to the same extent as CspA (200-fold induction). Following the identification of a *cspA* homologue in *M. smegmatis*, the cold-shock expression of this gene was investigated. A 16-24 fold induction in the quantity of *cspA* mRNA occurred within 15-18 hours of the response. However, this induction was not immediate and it peaked relatively late in the response, indicating that although CspA may indeed play a role it was not necessarily the primary regulator of the mycobacterial cold-shock response, as it is in *E. coli*. Several cold-shock proteins in *M. smegmatis* were transiently induced within the first 3-4 hours of the cold shock, making it difficult to identify a single protein that may be the primary or universal regulator of the response. Several other well-defined elements of the *E. coli* cold-shock response were also different in *M. smegmatis*, notably the constant levels of *gyrA* mRNA during a cold shock and the lack of homologues to RbfA and CsdA, key proteins in the cold-shock ribosome-adaptation model. Thus although the gross changes induced by the temperature reduction may be similar in *E. coli* and *M. smegmatis* (effects on growth, metabolism and protein composition), the actual genes and proteins involved in the response may differ. Due to these differences it is difficult to predict the role of the mycobacterial cold-shock proteins in relation to the effects of a cold shock on *E. coli*.

With the aid of N-terminal sequencing, one of the mycobacterial cold-shock proteins, CipM.1, was identified. This protein shows homology to two interesting proteins namely the HUβ subunit of the HU DNA-binding protein of *E. coli* (N-terminal homology) and the HBHA mycobacterial protein (C-terminal homology). Although HU has not yet been shown to be induced by cold exposure in *E.coli*, several lines of evidence suggests that it plays a role in maintaining cell viability during the stress. This is probably achieved through its ability to condense the chromosome and maintain the integrity of the DNA. Due to the highly charged nature of CipM.1 and this similarity to HU, it is possible that this protein has a DNA or RNA-binding role during the adaptive phase of the response. Proteins that bind to both DNA and RNA and prevent the formation of secondary structures are essential in the cold-shock response of *E. coli*. The similarity to HBHA has interesting connotations as it has been proposed that the C-terminus of this protein is involved in

cellular adhesion, an important event in the early infection process of respiratory pathogens (Rostand and Esko, 1997, Menozzi *et al.*, 1998). Furthermore, a group researching the effects of anaerobic stress in *M. smegmatis* recently identified *cipM.1* as one of the genes induced during dormancy in *M. smegmatis* (Lee *et al.*, 1998).

7.2: DORMANCY AND POTENTIAL DRUG TARGETS.

The cold-shock response in *M. smegmatis* is an adaptive stress response as it is in *E. coli* and although the bacteria take longer to adapt to the stress, they are able to regain cellular activity and establish cell division. In this regard this stress does not mimic the mycobacterial state of dormancy or non-replicating persistence. However, as previously stated one of the putative dormancy proteins of *M. smegmatis* has now also been shown to be induced by a cold shock. This supports the theory that dormancy is a stress-induced state.

Other proteins that have been identified through this cold-shock study may also have relevance to long term bacterial survival in a stressed environment, namely the cold-acclimation proteins. These proteins are characteristic of growth at the lower temperature and it has been proposed that in bacteria like *B. subtilis* and *A. globiformis* they are essential for cell viability at the lower temperature (Graumann *et al.*, 1996). Thus in conjunction with CipM.1, these CAPs may be useful anti-mycobacterial drug targets, aimed at the prevention of transmission by limiting survival in the external environment. Although it may not have a role in long term survival in a stressed environment, the pattern of expression of *cspA* suggests that the corresponding protein may have a role in cell viability at all temperatures, possibly being involved in the initiation of translation. This gene may therefore be another useful anti-tuberculosis drug target.

7.3: FUTURE WORK.

Establishing the importance of these *M. smegmatis* stress proteins in the stress responses of *M. tuberculosis* will be the first step in judging the relevance of the genes as potential drug targets. Gene knock-outs generated through homologous recombination and the use of anti-sense molecules to alter gene transcription could then be employed to establish the role of these proteins in cell viability under various conditions. Attenuated strains of *M. tuberculosis*, altered through gene knock-out technology may well provide an alternative vaccine candidate.

APPENDIX 1: BUFFERS AND SOLUTIONS.

Chemicals used in this study were supplied by Melford Laboratories, Biorad laboratories, BDH Chemicals Ltd, Sigma Chemicals, Fluka Biochemicals and Merck.

Bacterial growth:

2X YEAST TRYPTONE (YT) LIQUID MEDIA:

16g Bacto-tryptone
10g Bacto-yeast extract
5g Sodium chloride
Add water to 1l and autoclave.
For agar plates: add 15g agar to the above mixture and autoclave.

MIDDLEBROOK 7H9 medium:

2g/l Asparagine
0.5g/l Casitone
2.5g/l Sodium phosphate
1g/l Potassium phosphate
50mg/l Ferric ammonium citrate
10mg/ml Magnesium sulphate
0.5mg/ml Calcium chloride
0.1mg/l Zinc sulphate
0.1mg/l Copper sulphate

For liquid media: add 4.7g of the above medium (Difco) to 900ml distilled water and autoclave.
For agar plates: add 15g agar to the liquid media and autoclave.

ADC (albumin dextrose catalase):

5g Bovine serum Albumin (BSA) - fraction V
2g Glucose
3mg Catalase

Add water to 100ml and filter sterilize (Millipore filter - 20μm)
Use 100ml ADC per 900ml Middlebrook 7H9 (10%).

DNA analysis:

10X NNB (1l):

162g Tris

27.5g Boric acid

9.3g EDTA

10X Klenow buffer:

0.1M Tris.HCl pH 7.6

0.1M Magnesium chloride

0.5M Sodium chloride

10mM DTT

0.5mg/ml BSA

Distilled water

50X TRIS ACETATE BUFFER (TAE) (1l):

242g Tris

57.1ml Glacial acetic acid

100ml 0.5M EDTA pH 8.0

Use 1X TAE when running agarose gels.

20X SSPE (stock) (1l):

174g Sodium chloride

27.6g Sodium dihydrogen phosphate

7.4g EDTA, pH 7.4.

50X DENHARDTS SOLUTION (stock):

1% Ficoll

0.1% Polyvinylpyrrolidine

1% BSA fraction v.

HYBRIDIZATION BUFFER:

50% Formamide

5XSSPE

0.1%SDS

5X Denhardts solution

0.1% SDS

100μg/ml Herring sperm DNA.

RNA analysis:

S1 NUCLEASE BUFFER:

0.28M Sodium chloride

0.05M Sodium acetate pH 4.5

4.5mM Zinc sulphate

10X MOPS buffer (1l):

41.8g MOPS (Morpholinepropanesulfonic acid)

800ml DEPC-treated water

Adjust to pH 7.0

16.6ml 3M DEPC-treated sodium acetate

20ml 0.5M DEPC-treated EDTA (pH 8.0)

20X SSC (stock) (1l):

175.3g Sodium chloride

88.2g Sodium citrate

Adjust to pH 7.0

LOADING DYE (S1 nuclease):

0.3% Bromophenol blue

0.3% Xylene cyanol

10mM EDTA pH 7.5

97.5% Formamide

ELECTROPHORESIS SAMPLE BUFFER (RNA):

0.75ml Formamide

0.15ml 10X MOPS/EDTA buffer

50mM Sodium acetate, pH 7.0.

0.1ml Glycerol

0.08ml Bromophenol blue (10%)

0.1ml DEPC-treated water.

3x AQUEOUS HYBRIDIZATION SOLUTION:

3M Sodium chloride

0.5M HEPES pH 7.5

1mM EDTA pH 8

2X S1 NUCLEASE BUFFER:

0.56M Sodium chloride

0.1M Sodium acetate pH 4.5

9mM Zinc sulphate

HYBRIDIZATION BUFFER:

50% Formamide

5XSSPE

0.1%SDS

100ug/ml Herring sperm DNA

5X Denhardts solution

0.1% SDS

100μg/ml herring sperm DNA.

1.5% DENATURING AGAROSE/FORMALDEHYDE GEL (100ml):

1.5g Agarose

10ml 10X MOPS buffer

87ml DEPC-treated water

5.1ml 37% Formaldehyde

6% ACRYLAMIDE/ 8M UREA DENATURING GEL (100ml):

48g Urea

39ml Distilled water

15ml 40% (w/v) Acrylamide/ 2% (w/v) bis-acrylamide

10ml 10X NNB

To polymerise the acrylamide add 350μl 13% (w/v) ammonium persulphate and 80μl of N,N,N'N'-tetramethyl-ethylenediamine (TEMED)

Protein analysis:

TRANSFER SOLUTION (2l):

4.8g Tris

22.4g Glycine

400ml Methanol

TRIS-BUFFERED SALINE (TBS):

50mM Tris

150mM Sodium chloride

Adjust pH to 7.5

PHOSPHATE-BUFFERED SALINE (PBS) (pH 7.2):

137mM Sodium chloride

2.7mM Potassium chloride

4.3mM Di-sodium hydrogen phosphate

1.4mM Potassium di-hydrogen phosphate

SDS LOADING BUFFER:

3% (w/v) SDS

5% (v/v) β-mercaptoethanol

10% (v/v) Glycerol

0.625M Tris.Cl (pH 6.8)

0.01% (w/v) Bromophenol blue

GLYCINE LOWER GEL BUFFER (Stock) (500ml):

90.87g Tris

2g SDS

Adjust to pH 8.8

GLYCINE UPPER GEL BUFFER (stock) (500ml):

30.29g Tris

2g SDS

Adjust to pH 6.8

Tricine gel buffer (stock) (100ml):

36.3g Tris

0.3g SDS

Adjust to pH 8.45

12.5%T/ 3%C GLYCINE-SDS POLYACRYLAMIDE GEL:

Separating gel (50ml):

12.5ml Glycine Lower gel buffer

24.5ml Distilled water

12.5ml Acrylamide (Stock: 49.5% (w/v) acrylamide/ 0.5% bis-acrylamide)

100µl Ammonium persulphate (Stock: 10% solution (w/v))

10µl TEMED

Stacking gel (30ml):

7.5ml Glycine Upper gel buffer
18ml Distilled water
4.5ml Acrylamide stock
100µl Ammonium persulphate (Stock: 10% solution (w/v))
10µl TEMED

10X running buffer(1l):

30.3g Tris
144.1g Glycine
10g SDS
Use at 1X strength.

16.5% T/ 3%C TRICINE-SDS-POLYACRYLAMIDE GEL:

Separating gel (30ml):

10ml Acrylamide (Stock: 49.5% (w/v) acrylamide/ 0.5 % bis-acrylamide)
10ml Tricine Gel buffer
3.2ml Glycerol
6.8ml Distilled water
100µl Ammonium persulphate (Stock: 10% solution (w/v))
10µl TEMED

Spacer gel (20ml):

6.1ml Acrylamide stock
10ml Tricine Gel buffer
13.9ml Distilled water
100µl Ammonium persulphate (Stock: 10% solution (w/v))
10µl TEMED

Stacking gel (12.5ml):

1ml Acrylamide stock
3.1ml Tricine Gel buffer
8.4ml Distilled water
100µl Ammonium persulphate (Stock: 10% solution (w/v))
10µl TEMED

1X Cathode running buffer (1l):

2.42g Tris
3.6g Tricine
0.2g SDS

1X Anode running buffer (1l):

24.2g Tris

Adjust to pH 8.9

10%T / 3%C TRICINE -SDS POLYACRYLAMIDE GEL:

Separating gel (30ml):

6.1ml Acrylamide (Stock: 49.5% (w/v) acrylamide/0.5% bis-acrlamide)

10ml Tricine Gel buffer

3.2ml Glycerol

6.8ml Distilled water

100µl Ammonium persulphate (Stock: 10% solution (w/v))

10µl TEMED

Stacking gel and cathode/ anode buffers: As for 16.5%T/ 3%C Tricine gel.

(No spacer gel was used).

2-D SAMPLE BUFFER (25ml):

13.5g Urea

250mg DTT

0.5ml Ampholine 3-10 (Pharmacia)

0.13ml Triton X-100

Few grains of bromophenol blue.

REHYDRATION SOLUTION (25ml):

12g Urea

0.13ml Triton x-100

0.13ml Ampholine 3-10 (Pharmacia)

50mg DTT

EQUILIBRATION SOLUTION A (100ml):

36g Urea

30ml Glycerol

1g SDS

10ml 1M Tris.Cl pH 6.8

0.5g DTT

EQUILIBRATION SOLUTION B (100ml):

36g Urea

30ml Glycerol

1g SDS

10ml 1M Tris.Cl pH 6.8

4.5g Iodacetamide

Few grains of Bromophenol Blue

APPENDIX 2: PCR PRIMERS

Name	Sequence (5' → 3')	Reaction conditions
cipM1 **cipM2**	CTAGTCGGTTCTGGAGC (66°C)[1] CAGGGCAAGGTGATTCC (66°C)	95°C = 240s 93°C = 60s 60°C = 60s 72°C = 90s 72°C = 300s 30 cycles
csdA1 **csdA2**	GTGTTTCCGCCGTGGTTTGC (61.4°C) CTGCGA CGTTTGCTGACCTG (61.4°C)	95°C = 240s 93°C = 60s 58°C = 60s 72°C = 90s 72°C = 300s 30 cycles
cspA1[2] **cspA2**	CGGGATCCGAAATGCCACAGGGAACTGT (58°C) CGCAAGCTTGGTAACTCAGAGCGAGCGGA (67°C)	95°C = 300s 93°C = 60s 54°C = 60s 72°C = 90s 72°C = 300s 30 cycles
gyrA1[3] **gyrA2**	GACACGACGTTGCCGCCTG (68°C) CCCAGATTCATCAACCGAACACC (67°C)	95°C = 240s 93°C = 60s 65°C = 60s 72°C = 120s 72°C = 300s 30 cycles
hns1 **hns2**	CAACATCCGTACTCTTCGTGC (52.3°C) GCTTGATCAGGAAATCGTCG (52.2°C)	95°C = 240s 93°C = 60s 60°C = 60s 72°C = 60s 72°C = 300s 30 cycles
rbfA1 **rbfA2**	TAGTCCTCCTTGCTGTCGTC (59.4°C) CGAAAGAATTTGGTCGCC (59°C)	95°C = 240s 93°C = 60s 54°C = 60s 72°C = 60s 72°C = 300s 30 cycles
msmegX	GAGCTCATCGACGTGCTGACGAAGAAGATGAAC (G) (T) (C) (C) (C) (70°C)	

1 = predicted melting temperature (Tm)
2 = add 10% DMSO to reaction mixture
3 = use Expand High Fidelity Taq polymerase (Boehringer Mannheim) in the reaction mixture

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