The Cold shock response of *Mycobacterium vaccae*

Kirstie Jane Norgate

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Department of Medical Microbiology
Royal Free and University College London Medical School

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Abstract

Bacteria respond to a sudden decrease in temperature with a response, which involves the induction of a specific set of proteins, the cold shock proteins (Csps). The major Csp, cold shock protein A (CspA), of the extensively studied Escherichia coli (E.coli), is highly homologous to the cold shock domain (CSD) of the eukaryotic Y-box proteins. This high degree of conservation indicates that the Csps may also have a role in autoimmunity as has been implicated for the conserved heat shock proteins.

This thesis attempts to characterize the cold shock response of the non-pathogenic mycobacterium, Mycobacterium vaccae (M. vaccae), to identify its Csps, and to determine the presence of Csp antibodies in the sera of individuals with vascular disease.

The response of M. vaccae to cold shock was investigated by monitoring changes in both growth and cellular protein composition. Cold shock resulted in a 90 to 100 fold increase in the generation time. The synthesis of at least 12 proteins was induced during the first 24h of cold shock; the major M. vaccae Csp was identified as a putative CspA homologue. The M. vaccae cspA gene was identified by Polymerase chain reaction (PCR), with high homology to the other mycobacterial cspA genes. Gene sequence analysis revealed that the mycobacteria and E.coli share a number of cspA expression control features. Notably, an unexpected decrease in cspA expression was noted on cold shock.

Evaluation of antibody binding to cellular lysates from control and cold-shocked M. vaccae, using immunoblotting, revealed a potential predilection of binding to a putative Csp in the sera of individuals with vascular disease.

In conclusion, this study has started to characterize the M. vaccae cold shock response and the humoral immune response to Csps, which indicates that immune responses to Csps may have a role in vascular disease development, potentially through an autoimmune pathway.
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Abbreviations

1° primary
2° secondary
ACP Acyl carrier protein
ADB Anti-downstream box
APB Amersham Pharmacia Biotech
ATP Adenosine triphosphate
bp Base pair
BSA Bovine serum albumin
Cap(s) Cold acclimation protein(s)
cDNA copy DNA
CIRP Cold-inducible RNA binding protein
CSD Cold shock domain
CsdA Cold shock DEAD box helicase
Csp(s) Cold shock protein(s)
CspA Cold shock protein A
CspB-l Cold shock protein B-l
CSR cold shock response
DB Downstream box
Dbp DNA-binding protein
DEAD box helicase Type of ATP dependent helicase
DIG-labelled Dioxygenin-labelled
DNA Deoxyribonucleic acid
ds Double-stranded
ECF Extracytoplasmic function
ECL enhanced chemiluminescence
ELISA Enzyme-linked immunosorbant assay
FA’s Fatty acids
FRGY1/2 Frog Y-box protein 1/2
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
Gnd 6-phosphogluconate dehydrogenase
gRNA guide RNA
Grp Glycine-rich protein
GyrA DNA gyrase subunit A
HAS Human serum albumin
Hsc Heat shock protein cognate
Hsp(s) Heat shock protein(s)
Hsp60/65 Heat shock protein 60/65
Hsp70 Heat shock protein 70
Hsp90 Heat shock protein 90
IgG/H/A Immunoglobulin G/H/A
IPG Immobilised pH gradient
kDa kilo Daltons
LJ slope Lowënstein Jensen slope
mRNA Messenger ribonucleic acid
MRSa Methicillin resistant Staphlococcus aureus
NCIMB National collection of industrial and marine bacteria
NCTC National collection of type cultures
NTU  Nephelometer unit
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PVD  Peripheral vascular disease
RA  Rheumatoid arthritis
RACE  Rapid amplification of cDNA ends
RbfA  Ribosomal factor A
Rbp  RNA binding protein
RBP16  gRNA binding protein
Rf  relative mobility
RNA  Ribonucleic acid
RNP1  Ribonucleoprotein binding motif 1
RNP2  Ribonucleoprotein binding motif 2
RP  Raynaud's phenomenon
RT-PCR  Reverse transcription PCR
SDS-PAGE  SDS polyacrylamide gel electrophoresis
SigA  Sigma Factor A
SLE  Systemic lupus erythematosus
Ss  Single-stranded
TBS  Tris buffered saline
TCA  Tricarboxylic acid
TopA  Topoisomerase A
UTR  Untranslated region
YB1  Y-box protein 1
IEF  Isoelectric focusing
OD  Optical density
1D  one-dimensional
2D  two-dimensional

Amino acids
Arg  Arginine    Glu  Glutamine    Gly  Glycine
His  Histidine   Ile  Isoleucine   Leu  Leucine
Lys  Lysine      Phe  Phenylalanine  Ser  Serine
Thr  Threonine   Trp  Tryptophan   Tyr  Tyrosine
Val  Valine
Chapter 1

1.0 Introduction to Mycobacteria

The genus mycobacterium consists of saprophytic and pathogenic bacteria. The most famous are the disease causing organisms of tuberculosis and leprosy, *Mycobacterium tuberculosis* (*M.tb*), and *Mycobacterium leprae* (*M.leprae*), respectively, and *M.bovis*, a strain of which, the Bacillus Calmette-Guérin strain, forms the basis of the anti-tuberculosis vaccine, BCG.

Mycobacteria have probably existed since the very earliest times of biological life on this planet (Ratledge and Stanford, 1982). Ancient mycobacterial DNA, *M.tb* and *M.leprae*, have been identified from skeletal and mummified tissues, from as early as 140 AD (Donoghue et al., 1998; Haas et al., 2000; Konomi et al., 2002; Rafi et al., 1994; Zink et al., 2002). Moreover, DNA from mycobacteria of the *M.tb* complex, which consists of *M.tb*, *Mycobacterium bovis*, *Mycobacterium africanum*, and *Mycobacterium microti*, has been identified in the bone of an extinct long-horned bison, approximately 17000 years before the present day (Rothschild et al., 2001). Tuberculosis and Leprosy are still important diseases in the twenty-first century. Currently over a third of the world's population are infected with the tuberculosis bacillus, and 750000 people were registered as undergoing treatment for leprosy at the beginning of 2000.

1.1 Mycobacterial Taxonomy

Mycobacteria belong to the order of Actinomycetes, a diverse group of Gram-positive eubacteria, with a mycelial growth habit. Actinomycetes produce filaments (hyphae) that form a loose network (mycellium); the amounts of mycelial activity vary between the genera.

Within the actinomycetes, the mycobacteria are contained within the Nocardioform group, which contains four closely related genera with a distinctive and complex cell wall. The cell wall consists of peptidoglycan cell
wall, like the Gram positive bacteria, which is covalently linked to a second polysaccharide polymer (arabinogalactan) made up of arabinose and galactose (Lambert, 2002; McDonnell and Russell, 1999). These components form the basic structural skeleton of the cell wall. Mycolic acids, long chain length fatty acids, are covalently linked/esterified to the arabinogalactan polymer, forming the inner region of the thick waxy coat that surrounds the peptidoglycan/arabinogalactan skeleton. Mycolic acids account for up to 60% of mycobacterial cells dry weight (Lambert, 2002). The outer surface of the waxy coat contains complex lipids and waxes; glycopeptidolipids, lipopolysaccharides, and proteins, including porin channels, through which hydrophilic molecules diffuse into the cell (Lambert, 2002; McDonnell and Russell, 1999). Between 30% and 60% of the cell envelope has been reported to consist of lipids (Minnikin, 1982). The large quantities of lipid in the cell wall can significantly reduce the permeability of the walls of this group of bacteria, making them difficult to stain, and giving them their outward characteristics.

Mycobacteria are very hydrophobic, and are resistant to chemical injury by acids, alkalis and many other disinfectants used to kill other bacteria (McDonnell and Russell, 1999). Insufficient quantities of the hydrophilic biocides are able to penetrate the mycobacterial cell wall to produce a lethal effect (McDonnell and Russell, 1999). Moreover the high lipid content of the cell wall is a major barrier to the penetration of antimicrobial agents (Draper, 1998; Jarlier and Nikaido, 1994; McDonnell and Russell, 1999). In some cases resistance also extends to the killing mechanisms of macrophages, the slow growing mycobacteria M. tb, M. bovis, and M. leprae and others are intracellular pathogens that can survive and grow within host macrophages (Li et al., 2001; Monahan et al., 2001). It is likely that the thick cell wall prevents the lytic enzymes within the phagosome destroying the mycobacteria (Draper, 1998). Furthermore they can survive long periods of starvation or aridity without dehydration. This thick, lipid rich, hydrophobic cell wall allows their identification through one of their most important characteristics, their acid fastness. The imperviousness of the cell walls means that mycobacteria can be stained with a number of small molecular basic dyes however they cannot be decolourised.
with subsequent acid washes. This allows the rapid identification of mycobacteria particularly in pathological samples or those cases of suspected mycobacterial disease.

The actinomycetes were originally believed to be mid-way in the development between bacteria and fungi (Ratledge and Stanford, 1982). However, mycobacteria are no different from other groups of bacteria in their essential physiological and biochemical characteristics. Nevertheless fundamental differences exist for mycobacteria to be assigned their own genus.

Another distinctive feature of mycobacteria is their very slow growth rates. The genus itself be can be divided into two sub-genera based on growth rate; slow growers and fast growers. Although the faster growing species still have slower generation times than many other bacteria. The rapid growers consist of those mycobacterial species, which under optimal conditions yield, from dilute inocula, colonies on solid media that are easily visible within less than 7 days. The slow growers however require 7 days or more, and at least one species, *M. leprae*, cannot be cultured *in vitro*. The slow growers contain the pathogenic species including *M. tuberculosis* (*M. bovis*, *M. africanum*), and *M. microti* of the *M. tuberculosis* complex, and perhaps *M. leprae*. The fast growers consist of saprophytic organisms that are widely distributed on both terrestrial and aquatic habitats. There are currently 60 well-defined species of mycobacteria within the Mycobacterium genus, plus many others under critical analysis for possible inclusion into the genus.

**1.2 Immunogenicity of Mycobacteria**

Mycobacteria are very immunogenic, they elicit a strong response from the immune system, whether humoral or cell-mediated. Mycobacteria were discovered, by accident, to be ‘adjuvants’. An adjuvant can be defined as a substance that enhances the humoral or cell-mediated immune response to antigens injected simultaneously with it or within a period of time closely spaced to the injection of the adjuvant (Stewart-Tull, 1983). The injection of Guinea pigs with *M. bovis* shortly followed by a suspension of sheep erythrocytes, resulted in an increased level of anti-sheep erythrocyte antibodies in the
*M. bovis* infected Guinea pigs compared to the uninfected guinea pigs (Stewart-Tull, 1983). The *M. bovis* injection had increased the normal response to sheep erythrocytes. Adjuvant research led to the production of Freund’s complete and incomplete adjuvant. Freund’s adjuvant consists of heat-killed *M. tb* bacteria emulsified in white mineral oil, where as in the incomplete adjuvant the bacteria are omitted. The two adjuvants are still in common use in immunological research with animals (Allen et al., 2003; Eisenberg et al., 2003; Mauri et al., 2003), although *M. tb* is predominantly replaced by the killed fast-growing *M. butyricum*. Components of the mycobacterial cell that have been found to have adjuvant activity include, the cell wall component peptidoglycan, mycobacterial RNA (Stewart-Tull, 1983), and heat shock protein 70 (Hsp70) (Harmala et al., 2002).

In addition to their adjuvant activity mycobacteria also have antigenic activity. Antigens are substances that are capable of inducing an immunological response to them, and may be proteins or carbohydrates, combined together, or in combination with lipids. Mycobacteria possess antigens of all of the above types, including components of the cell wall, cytoplasmic membrane, cytoplasmic matrix, ribosomes, stored substances, metabolites and enzymes. Indeed immune responses have been detected to cell wall constituents including trehalose containing glycolipids (Munoz et al., 1997) and lipopolysaccharides (Hunter et al., 1983), and cellular proteins including heat shock proteins 65 and 70 (McKenzie et al., 1991; Young, 1990a). Humoral immune responses have been noted to between 50 and 60 mycobacterial antigens (Axelsen et al., 1974). This number will be greater as the cell-mediated immune system is likely to respond to a different set of antigens. The bacterial antigens are important in the cause and prevention of disease and in the induction of protective immune mechanisms.

Mycobacterial antigens can also be used to identify a particular species. The antigens of mycobacteria have been classed into 4 groups. Group i consists of at least 4 antigens that are present in all mycobacteria and nocardia. At least 2 of these antigens are also shared by the other genera in the nocardioform
group, rhodococci and corynebacteria (Stanford et al., 1975). The group ii antigens are shared between all species of slow growers, and the majority of fast-growers share the group iii antigens. The group iv antigens are specific for the individual mycobacterial species, some of these are shared by all the strains of the species whereas others are indicative of particular subspecies. Interestingly *M. leprae* and *M. vaccae* only possess the group i antigens, and their own group iv antigens they are missing the group ii and iii antigens, respectively. A taxonomic relationship was suggested between *M. leprae* and certain fast-growing species, notably *M. vaccae* and *M. nonchromogenicum*, based on immunological studies (Navalkeret al., 1980; Stanford, 1983), though other studies link *M. leprae* with *M. tb*.

1.3 *Mycobacterium vaccae*

Very slow growing mycobacterial pathogens such as *M. tb* are difficult to grow and manipulate. The long generation times mean that a single experiment can take weeks, or months to complete. So experimenters have relied on the relatively fast growing species *M. smegmatis*, which is particularly useful in molecular cloning experiments as mycobacterial genes are poorly expressed from their own promoters in *Escherichia coli* (E.coli). Recently another fast growing non-pathogenic mycobacterium *M. vaccae* has been suggested as an alternative cloning host for the study of mycobacterial genetics (Medeiros et al., 2002). Moreover, it has been developed as a vaccine. The vaccine SRL172 is a heat-killed preparation of the NCTC11659 strain of *M. vaccae*. The vaccine was originally developed to be an adjunct to the treatment of tuberculosis and leprosy. The vaccine has also been used in clinical trials against lung and other cancers, asthma and hayfever, and has been found to be a safe vaccine for human use, with no serious adverse side effects (Balagon et al., 2000; Eaton et al., 2002; Lehrer et al., 1998; O'Brien et al., 2000; von Reyn et al., 1997).

Identification of important mycobacterial antigens in disease, whether they are associated with mycobacterial or immune-mediated disease, allows the possibility of modulating this existing safe vaccine for prophylaxis or treatment of these diseases. For this reason my work on the mycobacterial cold shock
response has been carried out with two strains of *M. vaccae*; Gm27 (NCTC 11659) and Job5 (NCIMB 11807, ATCC 29678), the second strain is used commercially for steroid production. *M. vaccae* is a saprophytic mycobacterium that has been isolated from terrestrial and aquatic locations. Many strains are scotochromogenic, producing coloured pigments under all light conditions, although some strains produce little pigment without photostimulation. Both strains of *M. vaccae* I have used produce pigments of different colours.
Chapter 2

2 Introduction to the Cold Shock Response

All living organisms respond to potentially damaging environmental stresses, such as nutrient deprivation, temperature changes, and exposure to toxic chemicals. This response is brought about through the increased synthesis of a family of proteins collectively known as the stress proteins (Young and Elliott, 1989). The most well known and best characterised of these proteins are the heat shock proteins (Hsps). The stress response is interesting in itself, and there is increasing evidence that implicates stress proteins, in particular Hsps, in autoimmune disease. More recently a cold shock response has been discovered in prokaryotes. The knowledge of cold shock proteins (Csps) has been established in the rapidly growing Escherichia coli (E.coli) and bacillus species. This knowledge forms the basis of this thesis into the cold shock response of the more slowly growing mycobacteria.

2.1 Heat Shock Proteins (Hsps)

The heat shock response is universal and its components are among the most conserved genetic elements presently known, involving recognisable homology across the boundaries of the prokaryotic, eukaryotic and archaeabacterial kingdoms (Neidhardt et al., 1984). It is a cellular response to stress characterised by the increased expression of a class of proteins, the Hsps. Many of the proteins upregulated during the stress response have tremendous structural and functional conservation. Three major heat shock protein families were identified, Hsp60 (Hsp65/GroEL), Hsp70 and Hsp90, which have greater than 40% between the prokaryotic and eukaryotic counterparts (Becker and Craig, 1994). Functionally, the Hsps were found to be involved in protein biogenesis, and were identified as molecular chaperones, preventing deleterious protein misfolding and aggregation (Becker and Craig, 1994).

Polypeptide chains must be correctly folded, processed and localised in order that they may perform their biological function (Becker and Craig, 1994). Under
normal conditions proteins are prone to misfolding and aggregation on translation and translocation, due to their reactive hydrophilic and hydrophobic groups (Ang et al., 1991) as a result proteins termed chaperonins are needed to maintain the unfolded state. Under conditions of stress, such as heat shock, the structural integrity of proteins is challenged, as usually hidden interactive domains are exposed (Becker and Craig, 1994). The Hsps prevent heat denaturation of proteins by binding to these interactive domains and stabilising them. Alternatively, if misfolding or aggregation has occurred, binding by the Hsps allows disaggregation and refolding of the proteins into an active form, or putatively targets these deleterious proteins for degradation (Ang et al., 1991). Some Hsps have been shown to be proteases themselves, such as \textit{E.coli} clpP proteases (Kroh and Simon, 1990), or indirectly contribute to proteolysis by aiding in the presentation to proteases the polypeptides destined for degradation (Ang et al., 1991).

The Hsps are constitutively expressed and perform essential cellular functions. Hsp60 and Hsp70 are essential for \textit{E.coli} survival (Becker and Craig, 1994). Furthermore cystosolic Hsp90 in vertebrates forms an important component of steroid receptors.

2.2 The Cold Shock Response

Hsps were induced when bacteria and other organisms were subjected to sudden increases in temperature. In the last 20 years scientists have investigated the opposite scenario, cold shock. A cold shock response, analogous yet distinct to the heat shock response, has been discovered in bacteria, principally \textit{Escherichia coli} (\textit{E.coli}), when bacteria are exposed to a 13°C or greater drop in temperature (Jones et al., 1992a). Generally the larger the temperature shift the greater the cold shock response (Jones et al., 1992a). The cold shock response (CSR) is defined as the sum of all cellular reactions required for efficient adaptation to a sudden decrease in environmental temperature (Weber and Marahiel, 2002). The CSR manifests itself as a change in protein expression in \textit{E.coli} and \textit{Bacillus subtilis} (\textit{B.subtilis}) (Graumann et al., 1996; Jones et al., 1987). In \textit{E.coli} some proteins are
induced (cold shock proteins\(^1\) (Csps)) or continue to be synthesized at preshift levels, while others, including the Hsps, are severely repressed (Jones et al., 1987). The overproduction of GroEL/ES (the *E.coli* Hsps) or Hsps generally while protective against high temperatures reduces the viability of cells at 4°C, indicating a reason for the suppression of the Hsps expression on cold shock (Kandror and Goldberg, 1997). The induction of Csps is believed to be an adaptive response, enabling the cell to cope with the lower temperature, and allowing growth to continue (Jones et al., 1992a).

The cold produces two distinct problems for the cell. The membrane fluidity decreases as the temperature decreases, as a result membrane-associated functions such as active transport, and protein secretion are severely affected (Yamanaka et al., 1998). In addition, the secondary structures of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are stabilised, which is believed to affect the efficiencies of transcription, translation and DNA replication (Yamanaka et al., 1998). The biochemical requirements for thermotolerance and cold-tolerance are therefore distinct and mutually exclusive (Kandror and Goldberg, 1997).

\(^1\) – Cold shock proteins: proteins whose expression is transiently increased on cold shock, which then decrease to a lower basal level.

2.2.1 Cold shock in *Escherichia coli*

2.2.1.1 Membrane composition

Membranes are normally in a liquid crystalline form and will undergo a reversible transition to a gel state when the temperature drops (Thieringer et al., 1998; Yamanaka, 1999). Many organisms have developed mechanisms to compensate for the reduced fluidity of the membrane, they increase the level of unsaturated fatty acids (FAs) within the membrane. This process of altering the fatty acid composition of the membrane, homoviscous adaptation (Sinensky, 1974), is also seen when temperatures are increased. Phospholipids with
unsaturated FAs have lower melting points and a greater degree of flexibility than saturated FAs (Thieringer et al., 1998).

A number of mechanisms are employed by different organisms to increase the unsaturated FA composition of the membrane. *E.coli* increases the amount of cis-vaccenic acid and decreases the amount of palmitic acid incorporated into the membrane phospholipid (Marr and Ingraham, 1962). When *E.coli* were cold shocked from 42 to 24°C an increased ratio of unsaturated to saturated FAs was noted within 30sec of cold shock (Garwin and Cronan, 1980). The increased unsaturated FA synthesis was not affected by the inhibition of RNA or protein synthesis, indicating that response was not brought about by the increased synthesis of an enzyme (Garwin and Cronan, 1980). The enzyme β-ketocayl-Acyl Carrier Protein (ACP) Synthase II plays a key role in the change in FA composition, it catalyses the elongation of palmitoleic acid to cis-vaccenic acid (Garwin et al., 1980). This enzyme’s synthesis is not induced on cold shock however it’s enzyme activity is increased at low temperature (Garwin et al., 1980; Garwin and Cronan, 1980). So *E.coli* has an efficient system to increase the unsaturated FA composition of its membrane without the need to synthesize an enzyme.

Other organisms have different systems to bring about changes in their membrane FA composition; for example *B.subtilis* induces transcriptionally the synthesis of a membrane-bound fatty acid desaturase, which desaturates saturated FAs (Grau et al., 1994).

### 2.2.1.2 *E.coli* cold shock proteins

Cold shocking *E.coli* from 37 to 10°C results in a lag period of 4h before growth resumes at a new growth rate, with a generation time of 24h (Jones and Inouye, 1994; Shaw and Ingraham, 1967). During the 4h lag/acclimation period there is no net synthesis of total DNA, RNA, or protein (Shaw and Ingraham, 1967). However, a number of proteins expression was upregulated on cold shock.
Jones et al (1987) identified 12 proteins that were detectable at 37°C, and were synthesised at levels 2 to 10 fold higher on cold shock than at 37°C. A 13th novel protein was identified that was undetectable at 37°C, and was synthesised rapidly on shifting to 10°C, reaching a maximum of 13% of the total protein synthesis an hour after cold shock (Jones et al., 1987). This protein has been called Cold Shock Protein A (CspA), and is the major cold shock protein of *E.coli*. The 12 proteins that are also made at 37°C include polynucleotide phosphorylase, NusA, initiation factor 2α and 2β, RecA, dihydrolipoamide acetyltransferase, pyruvate dehydrogenase (lipoamide) (Jones et al., 1987), DNA Gyrase (Jones et al., 1992b), H-NS, and CsdA (Jones et al., 1996), the cellular functions of these proteins are described in Table 2.1.

The proteins are divided into two classes of cold shock proteins based on their expression, Class I and Class II. Class I cold shock proteins are expressed at an extremely low level at 37°C but are dramatically induced upon cold shock (>10 fold), and include CspA. Whereas the class II proteins are expressed at a certain level at 37°C and are moderately induced on cold shock (Thieringer et al., 1998). The Class I proteins are all associated with the ribosome or translation.

The cold shock proteins have functions within the processes of:

- **Energy generation**, two of the cold shock proteins are components of the pyruvate dehydrogenase complex, which is essential for the conversion of pyruvate to acetyl Coenzyme A (acetyl CoA). Acetyl CoA is a precursor metabolite for a number of biosynthetic pathways, however it is also an important substrate for the Tricarboxylic Acid (TCA) cycle, the major route of ATP generation. This indicates that the energy generation metabolic pathway is regulated upon cold shock (Yamanaka, 1999).

- **Chromosome condensation**, DNA gyrase (Jones et al., 1992b) and nucleoid-associated DNA-binding protein H-NS (Dersch et al., 1994) are upregulated on cold shock. Negative supercoiling of plasmid DNA of *E.coli* cells has
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold Shock Protein A (CspA)</td>
<td>RNA chaperone</td>
<td>(Jiang et al., 1997)</td>
<td>Class I</td>
</tr>
<tr>
<td>Polynucleotide phosphorlase (Pnp)</td>
<td>Putative mRNA RNase</td>
<td>(Donovan and Kushner, 1986) (Jones et al., 1987)</td>
<td>Class I</td>
</tr>
<tr>
<td>Transcriptional termination factor NusA</td>
<td>Involved in termination and antitermination of transcription</td>
<td>(Jones et al., 1987)</td>
<td>Class I</td>
</tr>
<tr>
<td>Ribosome binding factor A (RbfA)</td>
<td>Stabilises the ribosomes and allows translation of cellular mRNAs</td>
<td>(Jones and Inouye, 1996)</td>
<td>Class I</td>
</tr>
<tr>
<td>Cold-shock DEAD-box protein A (CsdA)</td>
<td>Major ribosomal associated protein, with RNA helix destabilising activity, increasing translational efficiency in the cold</td>
<td>(Jones et al., 1996)</td>
<td>Class I</td>
</tr>
<tr>
<td>Initiation factors 2α and 2β</td>
<td>Mediate binding of tRNA^{Met} to small ribosomal subunit for initiation of translation</td>
<td>(Jones et al., 1987)</td>
<td>Class II</td>
</tr>
<tr>
<td>Recombinant A (RecA)</td>
<td>Involved in recombination</td>
<td>(Jones et al., 1987) (Thieringer et al., 1998)</td>
<td>Class II</td>
</tr>
<tr>
<td>Dihydrolipoamide acetyltransferase</td>
<td>Components of pyruvate dehydrogenase complex, which converts pyruvate to acetyl CoA, an important substrate for the TCA cycle as well as Fatty Acid synthesis</td>
<td>(Yamanaka, 1999)</td>
<td>Class II</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gyrase A (GyrA)</td>
<td>α-subunit of the topoisomerase DNA Gyrase</td>
<td>(Sugino et al., 1977)</td>
<td>Class II</td>
</tr>
<tr>
<td>Nucleoid protein H-NS</td>
<td>High affinity DNA binding protein, associated with the bacterial nucleoid</td>
<td>(La Teana et al., 1991)</td>
<td>Class II</td>
</tr>
<tr>
<td>Trigger Factor (TF)</td>
<td>Molecular chaperone essential for cell viability at low temperatures</td>
<td>(Kandror and Goldberg, 1997)</td>
<td>Class II</td>
</tr>
<tr>
<td>Heat shock cognate 66 (Hsc66)</td>
<td>Molecular chaperone, Hsp70 homologue, induced on cold shock</td>
<td>(Leivelt and Kawula, 1995)</td>
<td>Class II</td>
</tr>
</tbody>
</table>

Table 2.1: *E. coli* cold shock proteins
been shown to increase upon cold shock (Goldstein and Drlica, 1984). Both DNA gyrase and H-NS proteins are believed to play an important role in the DNA supercoiling reaction (Yamanaka, 1999). Bacterial DNA is kept in a state of negative supercoiling, by the action of Gyrase and Topoisomerase I, which favours DNA unwinding, and transcription (Watson et al., 1987b). It is possible that Gyrase function is reduced with cold shock or that the DNA needs to be more negatively supercoiled to promote transcription. Therefore regulating the DNA superhelical tension may be important for the maintenance of DNA replication, transcription and recombination (Yamanaka, 1999).

- Protein Biogenesis, two molecular chaperones have been discovered to be cold shock proteins, Trigger Factor (TF), and Heat shock cognate 66 (Hsc66). Although both are class II proteins, their expression being upregulated late in the CSR, they have important functions in ensuring correct proteins folding and re-folding of cold damaged cells. TF in particular is important for viability at low temperatures (Kandror and Goldberg, 1997). *E.coli* cells lacking TF stored at 4°C have significantly reduced viability compared to normal cells (Kandror and Goldberg, 1997).

- Protein Translation – The majority of the proteins upregulated, including the major cold shock protein, are involved with the ribosome and protein translation.

### 2.2.1.3 Protein translational machinery

The biggest problem for *E.coli* cells cold shocked to low temperatures is the effect on translation. Shifting *E.coli* to subminimal temperatures resulted in a block in the initiation of translation, polysomal run-off, and the accumulation of 70S ribosomes and ribosomal subunits (Broeze et al., 1978; Friedman et al., 1971). Jones and Inouye (1996) reported that cold shocking *E.coli* from 37 to 15°C resulted in a temporary inhibition in the initiation of translation. A transient decrease in polysomes accompanied by an increase in 70S monosomes was seen (Jones and Inouye, 1996). In addition VanBogelen and Neidhardt (1990)
were able to induce the CSR when cells were incubated with a group of antibiotics, the C group, which affected ribosomal function.

Interestingly, the heat shock response could also be induced when *E. coli* cells were incubated with a different group of antibiotics, the H-group, which also affected the ribosome, and thus the translational capacity of the cell. VanBogelen and Neidhardt (1990) suggested that the ribosome was the sensor for the heat and cold shock responses, which were induced by different effects on the translatable capacity of the cell. Jones and Inouye (1996) supported this idea, their experiments indicated that shifting *E. coli* to a low temperature results in an inhibition in translation, which induces the cold shock response and increases the translational capacity of the cell.

The magnitude of the cold shock response was previously shown to be proportional to the extent of the temperature decrease (Jones et al., 1992a). Jones and Inouye’s (1996) experiments indicated it was more specifically proportional to the degree of inhibition of ribosomal function. Jones and Inouye (1996) proposed the ‘Cold-shock Ribosome Adaptation Model’. At high temperatures, the ribosome is translatable for cellular mRNAs. A shift to a low temperature results in a cold-sensitive block in the initiation of translation for most cellular mRNAs. However, the mRNAs for the cold-shock proteins can be efficiently initiated resulting in the induction of the cold shock proteins. Cold-shock proteins RbfA, CsdA, and initiation factors α and β associate with the ribosomal subunits and 70S monosomes resulting in efficient translation of cellular mRNAs. Furthermore the cold shock protein, RbfA, was found to be essential for the translation of non-cold shock proteins at low temperature, the overproduction of RbfA resulted in faster adaptation at low temperature (Jones and Inouye, 1996).

The synthesis of cold shock proteins and ribosomal proteins, despite the inhibition of protein translation, indicates that the mRNA’s are able to bypass the block in the initiation of translation, and form the initiation complex. The class I Csps have an unusually long 5' untranslated region (5'UTR), which are
believed to play an important role in their expression at low temperature (Mitta et al., 1997). Moreover the Csps mRNA contains a 14bp sequence located 12 bases downstream of the initiation codon, designated the downstream box (DB). This sequence is complementary to a sequence near the decoding region of 16S rRNA, called the anti-downstream box (ADB). The sequence had been shown to mediate efficient translation from the initiation codon even in the absence of the Shine-Delgarno sequence (a sequence complementary to the 3' hydroxyl end of the 16S rRNA component of the 30S ribosomal subunit, and is required for the initiation of translation) (Sprengart et al., 1996). Interestingly the class I Csps have >10 matching bases, whereas the class II Csps have 8 or more matching bases between the DB and ADB (Mitta et al., 1997). In addition the class II proteins do not have an unusually long 5'UTR. This may explain why the class I proteins are expressed at higher amounts. The DB is proposed to allow cold shock genes to bypass the requirement for cold shock inducible ribosomal factors at low temperatures (Mitta et al., 1997).

2.2.2 Cold Shock in Other Organisms

Analogous cold shock responses have been discovered in mesophilic, psychrotrophic and psychrophilic bacteria, including *Aquaspirillium articum* (*A.articum*) (Roberts and Inniss, 1992), *Pseudomonas fragi* (*P.fragi*) (Hebraud et al., 1994), *Arthrobacter globiformis* (*A.globiformis*) SI55 (Berger et al., 1996), *B.subtilis* (Graumann et al., 1996; Lottering and Streips, 1995), and *Enterococcus faecalis* (*E.faecalis*) JH2-2 (Panoff et al., 1997). The number of Csps induced does not appear to be dependent on the type of organism (mesophilic/psychrophilic), or the minimum temperature of growth. Csps have been discovered in yeasts, slime moulds, animals and plants (Phadtare et al., 1999). *E.coli* as well as other prokaryotic and eukaryotic organisms induce a specific pattern of gene expression upon an abrupt temperature downshift. The universality of Csps indicates that the cold shock response is present in many, if not all, organisms to cope with the detrimental affects of cold on cells (Graumann and Marahiel, 1996).
When bacteria are grown at low temperatures for extended periods of time, they express a particular set of proteins classified as cold acclimation proteins (Caps). There is considerable crossover between proteins classified as CspA, and those classified as caps (Bayles et al., 1996; Berger et al., 1996; Gumley and Inniss, 1996). Proteins required for surviving cold shock are also important for the continued survival of the bacteria at low temperatures.

2.2.3 Cold shock protein A
As stated above CspA is the major cold shock protein of E.coli. The protein has been characterised and was found to be a cytoplasmic 7.4kDa protein, consisting of 70 amino acids, with an isoelectric point (pl) 5.92. The protein is very hydrophilic, containing greater than 20% charged amino acids (Goldstein et al., 1990). CspA has been found to have striking similarity with a conserved domain, the cold shock domain (CSD), in the human DNA-binding proteins, DbpA, DbpB, and YB-1 (Wistow, 1990).

2.2.3.1 The Y-box proteins
The human DNA-binding proteins belong to the Y-box family of proteins. The first Y-box protein was discovered to be a transcriptional factor that bound to a motif, called the Y-box, within the promoter of a major histocompatibility complex (MHC) class II gene (Didier et al., 1988). The protein, termed Y-box 1 (YB1), had an absolute binding requirement for the sequence CCAAT, within the Y-box, and a relative specificity for the rest of the Y-box sequence (Didier et al., 1988). An inverted CAATT sequence (ATTGG) is found within the Y-box regulatory sequence in the promoters and enhancers of all the mammalian MHC class II genes (Wolffe et al., 1992). The Y-box proteins consist of 3 domains; a variable N-terminus, a highly conserved nucleic acid recognition domain (Weber et al., 2002), and a hydrophilic C-terminal domain, which contains alternating acidic and basic/aromatic regions (Matsumoto and Wolffe, 1998; Murray et al., 1992) (Figure 2.1). The CSD of YB1 is 44% identical to CspA of E.coli (Lee et al., 1994).
The Cold shock domain (CSD) homologous to the bacterial CspA is flanked by a variable N-terminal domain and a C-terminal domain which consists of alternating acidic and basic/aromatic regions.

The Y-box proteins are involved in transcriptional and translational regulation (Matsumoto and Wolffe, 1998). Y-box proteins have been recognised as transcriptional factors for a wide range of genes, resulting in the activation or repression of transcription (Ladomery and Sommervile, 1995). They are expressed tissue-specifically and are involved in the regulation of distinct sets of genes (Ladomery and Sommervile, 1995). Furthermore the Y-box proteins regulate the control of genes involved in cell proliferation (Ladomery and Sommervile, 1995).

Y-box proteins are also involved in translational control. Y-box proteins regulate the expression of maternal and paternal mRNA in non-dividing germ cells. *Xenopus laevis* has two Y-box proteins, FRG Y1 (Frog Y-box 1) and FRG Y2. FRG Y2 is found in the testes and immature oocytes (Tafuri and Wolffe, 1990). FRG Y2 has a role in the translational silencing of maternal mRNA, by 'masking' it from the translational machinery (Bouvett et al., 1995). The FRG Y2 protein is a major component of ribonucleoprotein storage particles (mRNPs) within which maternal mRNA is sequestered away (Bouvett et al., 1995). During oogenesis maternal mRNA is stored for release at oocyte maturation and early embryogenesis. Transcription is not initiated until after the 12th round of cell division in the zygote, thus all mRNAs and proteins required for the assembly of the first 4000 cells must be contained within the unfertilized egg (Tafuri et al., 1993). A similar situation has been discovered with paternal mRNA in spermatogenesis in mice (Tafuri et al., 1993).
2.2.3.2 Structure and function of CspA

The three dimensional structure of CspA has been elucidated, and reveals a protein composed of five antiparallel β-strands connected by loops (Schindelin et al., 1994) (figure 2.2). These β-strands form two β-sheets that make up a β-barrel structure (Schindelin et al., 1994). The three-dimensional structures of the homologous \textit{B. subtilis} CspB, \textit{Bacillus caldolyticus} CspB, and \textit{Thermotoga maritima} CspB, have also been obtained (Kremer et al., 2001; Mueller et al., 2000; Schindelin et al., 1993; Schnuchel et al., 1993), and the β-barrel structure is conserved in all the homologues. The hydrophobic residues of the proteins are located within the β-barrel forming a hydrophobic core (Schindelin et al., 1994; Schnuchel et al., 1993). In addition, the structure of the CSD of the human Y-box protein YB-1, has been elucidated and this also has a β-barrel structure (Kloks et al., 2002). The major differences between the prokaryotic Csps and the eukaryotic CSDs, is that the eukaryotic CSDs have a longer N-terminal and a longer loop between strands β3 and β4.

The proposed function of \textit{E.coli} CspA is as a transcriptional activator and RNA chaperone (Jiang et al., 1997; Jones et al., 1992b; La Teana et al., 1991). \textit{E.coli} CspA has been shown to preferentially bind to an oligonucleotide, derived from the \textit{gyrA} promoter, containing the CCAAT sequence, but doesn’t bind to the double-stranded (ds) DNA sequence (Schindelin et al., 1994). This binding has also been noted with the \textit{B. subtilis} CspB homologue, which preferentially binds to single stranded (ss) DNA containing the sequences CCAAT and ATTGG, but not dsDNA (Kremer et al., 2001). Furthermore \textit{E.coli} CspA transcriptionally activates the expression of the cold shock genes \textit{gyrA} (Jones et al., 1992b) and \textit{hns} (La Teana et al., 1991). The exact mechanism of transcriptional activation is unclear, however the presence of a CCAAT sequence within the promoter regions of both genes may have a role in the activation. The binding of \textit{E.coli} CspA to the CCAAT region within the \textit{gyrA} promoter sequence supports this mechanism. This sequence had been found in the promoters of several other cold shock genes including \textit{recA}, \textit{nusA}, and polynucleotide phosphorylase (Jones et al., 1992b). It has been suggested that \textit{E.coli} CspA maintains the open complex formed by RNA polymerase during transcription.
Figure 2.2: Three dimensional structure of *E. coli* CspA

A shows the location of the 5 β-strands and loops, and the N- and C- terminals. B shows the β-barrel structure. The structures were obtained from the CSDBase database (Weber, 2002).
The main function of the CspA proteins is as an RNA chaperone. Jiang et al (1997) revealed that CspA was able to cooperatively bind to ssRNA, without sequence specificity. The presence of two RNA binding motifs, RNP1 (ribonucleoprotein) and RNP2 are typical of RNA-binding proteins also supports its function as an RNA chaperone (Landsman, 1992; Nagai et al., 1990). The binding site for ss-nucleic acids has been proposed to be on one surface of the proteins (Schindelin et al., 1993; 1994). Positive charges are arranged mainly on one face of the proteins, which attract the negatively charged nucleic acids. The aromatic amino acids that form part of the RNP1 and RNP2 motifs, are also arranged on this surface, which are believed to interact directly with the bases and backbone of RNA (Schindelin et al., 1994). These motifs are highly conserved, and have been shown to be essential for binding of ss-nucleic acids through mutational analysis (Schroder et al., 1995).

CspA has been shown to bind mRNA and to increase its susceptibility to ribonucleases, indicating that the secondary structure of the mRNA was affected (Jiang et al., 1997). To be able to efficiently process, transport, store, translate and degrade mRNA, it must be in a single-stranded conformation, i.e. no secondary structures. Secondary structures in mRNA have a higher stability at lower temperatures (Graumann and Marahiel, 1998), therefore increased levels of CspA during cold shock, may be essential to counteract this stability (Graumann et al., 1997). CspA is believed to work in conjunction with the DEAD box-helicase, CsdA, which has the ability to unwind double-stranded RNA (Jones et al., 1996). CspA binds to ssRNA molecules stabilising them in a single-stranded structure, which increases translational efficiency, as mRNA in a linear form is essential for the initiation of translation (Graumann and Marahiel, 1998; Sommerville, 1999). It has been proposed that the cold shock response relies on the pools of mRNA present at the time of cold shock in the cell, the cold shock proteins being preferentially translated, which allows the efficient acclimatisation to a colder environment (Graumann and Marahiel, 1998).
2.2.3.3 *E. coli* possesses a family of CspA genes

A family of nine homologous CspA proteins is present in *E. coli* (CspA-CspI) (Phadtare et al., 1999; Yamanaka et al., 1998). The genes encoding these proteins, are understood to have arisen from a number of gene duplication events that resulted in them diversifying and developing different roles within the cell (Yamanaka et al., 1998). Proteins CspA, CspB, CspG, and CspI are cold-shock inducible (Jones et al., 1987; Lee et al., 1994), (Bae et al., 1999). CspC and CspE are expressed constitutively at 37°C (Yamanaka et al., 1994). CspE synthesis level is also transiently increased on dilution of stationary phase cells into fresh medium at 37°C (Bae et al., 1999).

CspDs expression is almost completely inhibited by cold shock (Lee et al., 1994), and was found to be induced by stationary phase growth (Yamanaka and Inouye, 1997). The expression of CspD was shown to be inversely dependent on growth rate and was induced upon glucose starvation (Yamanaka and Inouye, 1997). The function of CspH and F are unknown at present. The members of the CspA family are all believed to be RNA and ssDNA binding proteins, with different physiological functions within the cell, the exception being CspF and CspH. These proteins do not contain the well-conserved aromatic residues in the RNP1 and RNP2 motifs, which are essential for binding to RNA and ssDNA and it has been speculated that CspF and CspH may bind to DNA rather than RNA (Yamanaka et al., 1998).

There appears to be overlap between the function of the CspA family of proteins. In a *cspA* deletion mutant the levels of the cold-inducible CspB and G are increased and may compensate for the lack of CspA (Bae et al., 1997). In addition, high levels of CspA are found in *E. coli* cells deficient of *cspE* at 37°C, suggesting that the CspA protein takes over the function of CspE in it’s absence at 37°C (Bae et al., 1999).

Multiple gene families have also been noted in *B. subtilis* and *Lactococcus lactis* (*L. lactis*). *B. subtilis* has a 3-gene family, *cspB*, *cspC* and *cspD*, and a minimum
of one gene is essential for cell viability (Graumann et al., 1997). *L. lactis* has a family of 5 Csps, CspA-E, four of which, CspA-D, were cold shock inducible (Wouters et al., 1998).

### 2.2.3.4 Control of CspA expression

CspA is dramatically and transiently induced on cold shock, reaching 13% of the cells total protein synthesis 1 to 1.5h after a shift to 10°C, and subsequently drops to a basal level before the end of the acclimation phase (Goldstein et al., 1990). The cold-shock induction of *cspA* is controlled at the levels of transcription, mRNA stability and mRNA translational efficiency (Fang et al., 1997).

Cold shock results in the induction of CspA transcription (Jiang et al., 1993; Tanabe et al., 1992), and an AT-rich region sequence immediately upstream of the *cspA* promoter termed the UP element, enhances *cspA* promoter activity and transcription at low temperatures (Mitta et al., 1997) (Figure 2.3). Although cold shock induction of *cspA* expression is not dependent on it's own promoter, CspA expression was still cold shock induced when it's promoter was replaced with an alternative non-cold shock promoter (Brandi et al., 1996; Fang et al., 1997).

The *cspA* transcript is constitutively transcribed at any temperature (Fang et al., 1997). However, CspA production is barely detectable at 37°C, and this has been attributed to extreme instability of *cspA* mRNA at 37°C, which has an estimated half-life of approximately 10sec (Brandi et al., 1996; Goldenberg et al., 1996). On shifting to 15°C *cspA* mRNA becomes extremely stable, although this stability is transient as the mRNA's half-life decreases to 10min, after 90min at 15°C, the same degradation rate of mRNA from cells grown for prolonged periods at 15°C (Goldenberg et al., 1996). In addition, ribosomes obtained from cold shocked cells were able to more efficiently translate *cspA* mRNA, than those obtained from control cells, grown at 37°C (Brandi et al., 1996). The *cspA* mRNA stability plays a major role in the level of transcript available, and the cold shock response.
Figure 2.3: *cspA* gene structure; including the unusually long 5'-untranslated sequence (5'-UTR), the promoter UP element, and the cold box.

The arrow represents the transcription initiation point. SD, MET, and DB represent the Shine-Delgarno sequence, translation initiation codon, and the downstream box, respectively. ORF and 3'-UTR are abbreviations for the open reading frame and 3'-untranslated region, respectively.
The \( cspA \) mRNA, along with the mRNAs of the other class I cold shock proteins, has an unusually long 5’untranslated region (5’UTR) (Figure 2.3). Overproduction of the 5’UTR of \( cspA \) causes the prolonged synthesis of cold shock proteins CspA, CspB, CspG, and CsdA, and extended the acclimation phase, during which growth was halted (Jiang et al., 1996). In addition, deletion analysis of the \( cspA \) mRNA 5’UTR revealed that the 5’UTR is responsible for \( cspA \) mRNAs extreme instability at 37°C along with its transient induction during the acclimation phase (Mitta et al., 1997). The 5’UTR’s also contain a consensus sequence called the cold box, which is highly conserved in class I cold shock genes (Jiang et al., 1996), it is believed to be a transcriptional pausing site and is involved in the repression of \( cspA \) expression (Bae et al., 1997). The cold box is also thought to be the binding site for a transcriptional or translational repressor (Jiang et al., 1996).

CspA negatively regulates its own expression during cold acclimation (Bae et al., 1997; Jiang et al., 1997). CspA has been found to bind to its own mRNA, in particular the 5’UTR region (Bae et al., 1997; Jiang et al., 1997) destabilising existing secondary structures in \( cspA \) mRNA, and making it susceptible to RNAse digestion (Jiang et al., 1997). It is possible that CspA binds to the cold box within the 5’UTR (Bae et al., 1997).

CspA negatively regulates its own gene expression at the level of transcription and mRNA stability, and may also affect the expression of the other Class I CspS with long 5’UTRs. Moreover, CspE of \textit{E.coli} has been shown to specifically inhibit \( cspA \) expression, probably by interacting with the transcription elongation complex at the \( cspA \) cold box region (see below) (Bae et al., 1999).
2.2.3.5 Homologues of *E.coli* CspA

Proteins homologous to *E.coli* CspA have been found across the bacterial kingdom. To date greater than 90 CspA-like proteins have been identified in Gram positive and Gram negative, psychrophilic, psychrotrophic, mesophilic and thermophilic bacteria (Yamanaka, 1999). So far the only exceptions are *Helicobacter pylori* and *Mycoplasma genitalium* (Graumann and Marahiel, 1996). Homologues have been recently discovered in the earliest diverging bacterial branches of *Aquifex* and *Thermotoga* (Graumann and Marahiel, 1998; Kremer et al., 2001). The sequence identity is remarkably conserved across the kingdom, with *Aquifex* and *Thermotoga*’s proteins sharing 62-68% sequence identity with CspA proteins from Gram positive bacteria (Graumann and Marahiel, 1998).

Putative homologues to *E.coli* CspA have been found in the important pathogens *M.tuberculosis* (*M.tb*) and *M.leprae*. Although a cold shock response has not been reported in these organisms, a cold shock response was reported recently in the non-pathogenic species *M.smegmatis* (Shires and Steyn, 2001).

The presence of homologues to *E.coli* CspA in the *Aquifex* and *Thermotoga* genus demonstrated the evolutionary conservation of this protein. The universality, evolutionary conservation and the homology of the major cold shock proteins indicates their importance in maintaining the viability of these organisms.

Furthermore a homologous protein domain, the CSD, has been found in the eukaryotes, both plant and animal. A CspA homologue has been reported in Winter Wheat. The protein is a cold-regulated nucleic acid protein with an N-terminal CSD, and a glycine-rich region, interspersed with zinc fingers. The protein has ssDNA, dsDNA, and RNA binding capabilities (Karlson et al., 2002). The CSD as stated above is also present in the mammalian Y-box proteins. Figure 2.4 reveals the high degree of conservation of the CspA/CSD amino acid across the evolutionary divide. Consequently the major stress protein of bacteria has a highly homologous protein domain in mammals.
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<td>70</td>
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<tr>
<td>S.Clavuligerus SC7.0</td>
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<td>E.E.G.S.E.E.N.E.E.GHSP...Q.T.G.R.-...</td>
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<tr>
<td>H.sapiens YB-1</td>
<td>E.E.E.G.S.E.E.N.E.E.GHSP...Q.T.G.R.-...</td>
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**Figure 2.4: Comparison of E.coli CspA with some other CspA-like proteins.**

The amino acid sequence of *E.coli* CspA (Jones et al., 1994) was compared with CspB from *B.subtilis* (Winnicky et al., 1992); with SC7.0 from *Streptomyces clavuligerus* (Av-Gay et al., 1992); with CspA of *M.tb*; with the Cold shock domain (CSD) of WCSP of winter wheat (*Triticum aestivum*) (Karlson et al., 2002); and with the CSD of YB1 from *Homo sapiens* (Wistow, 1990; Wolfe et al., 1992). Amino acid residues entirely conserved with the *E.coli* CspA sequence are represented by a dot; gaps in the sequence are represented by a dash.
2.3 Stress Proteins and Disease

Stress proteins are frequently the targets of the humoral and cell-mediated immune response to infection (Lamb et al., 1989). The evolutionary conservation of the heat shock proteins (Hsps), and the more recently discovered cold shock proteins (Csps), makes them attractive targets for the immune system, as there is the potential for many bacterial and parasitic organisms to carry these highly homologous antigens.

Members of the Hsp70 and Hsp60 families represent major targets for the immune system in many infections involving helminths, protozoa, bacteria and viruses (Kaufmann, 1990). Antibodies against members of the Hsp70 family have been found in infections involving *Plasmodium falciparum* (malaria), *Trypanosoma cruzi* (Chagas disease), *Leishmania donovani* (visceral leishmaniasis), *Schistosoma mansoni* and *S.japonicum* (schistomiasis), *M.leprae* (leprosy) and *M.tb* (Kaufmann, 1990). In addition, T-cells reactive to Hsp65 have been identified in tuberculosis and leprosy patients.

Due to the high degree of homology between all the Hsps and the dominant antigenicity of the Hsps, there is a high possibility of developing antibodies and lymphocytes that are also reactive to self-proteins. Immune responses against stress proteins can be highly cross-reactive and can involve anti-self reactivity (Lamb et al., 1989; Young, 1990b; Young and Elliott, 1989). Increasing evidence implicates the Hsps in the development of several autoimmune diseases including in Rheumatoid arthritis (RA), insulin dependent diabetes mellitus in mice and humans, and in systemic lupus erythematosus (SLE) (Xu and Wick, 1996), although their involvement in these diseases is not fully understood. Furthermore, elevated antibody levels to heat shock protein cognates have been observed in several of the above diseases. Raised antibodies to Hsps have been detected in a number of autoimmune diseases, including Hsp65 in RA, Hsp90 in ankylosing spondylitis, and Hsp70 and Hsp90 in SLE (Kaufmann, 1990).
It is theorised that the body produces lymphocytes that recognise Hsps, as a result of bacterial/parasitic infection, that are capable of reacting with self-proteins (Lamb et al., 1989). Local accumulation of Hsps, for example due to viral infection, may result in the activation of auto-reactive lymphocytes, which trigger an immune response that results in the pathological damage associated with autoimmune disease (Lamb et al., 1989).

The most compelling evidence for the involvement of Hsps in the evolution of autoimmune disease comes from the development of atherosclerosis. The humoral and cellular immune systems are believed to have an essential role in the pathogenesis of this disease. Many factors have been implicated in the development of atherosclerosis including hypercholesterolemia (raised cholesterol levels), modified lipoproteins, smoking, hypertension, and diabetes mellitus. Atherosclerosis arises as a result of damage to the arterial cell wall e.g. by haemodynamic stress. An atherosclerotic lesion is characterised by the proliferation of the endothelium (intima) and the muscle cells of the arterial wall (myointimal hyperplasia), the accumulation of lipids, and the deposition of extracellular matrix components at the site of damage (Wick et al., 1995). The result is a thickening of the arterial wall that can lead to the artery becoming blocked. Although atherosclerotic lesions can occur anywhere in the body, sites subject to increased haemodynamic stress are particularly prone to lesion development (Wick et al., 1995). Van der Wal and co-workers (1989) reported the presence of activated T-cells and macrophages in human atherosclerotic lesions, indicating that an immune-mediated response was involved in their development.

Xu et al (1993) investigated the immune response to various antigens of the atherosclerotic lesion. They tried to induce atherosclerosis in rabbits with normal cholesterol levels either, by feeding them a high cholesterol diet or, by immunising them with various antigens. After 16 weeks, all the rabbits fed on the high cholesterol diet had developed atherosclerosis, along with those rabbits that had been immunized with preparation containing Hsp65, or recombinant Hsp65, suggesting that an (auto) immune response to Hsp65
initiated the development of atherosclerosis. When the rabbit's aortas were stained for Hsp65, there was increased staining within the atherosclerotic lesions, compared to normal sections of the aorta. Furthermore, expanded T-cell lines derived from the lesions had significantly higher Hsp65 activity than those developed from the peripheral blood of the same animal (Xu et al., 1993a). These experiments indicate a causal relationship between anti-Hsp65/60 antibodies and the development of atherosclerosis.

This theory has been supported further by studies that have revealed an increased expression of Hsp60 on human arterial endothelium (intima) with atherosclerotic lesions (Kleindienst et al., 1993). Furthermore, a significant correlation (p<0.01) between serum antibody levels to Hsp65/60 in subjects and the occurrence of carotid atherosclerosis, independent of other established factors (Xu et al., 1993b). Experiments in vitro revealed that stressed endothelial cells are destroyed by purified antibody to Hsp60 that has approximately a 60% amino acid homology with Hsp65 of bacteria (Schett et al., 1995). In addition, patients undergoing coronary angioplasty have raised levels of antibody to Hsp60. If these levels fall after the operation, the arteries remain unblocked (Mukherjee et al., 1996). If the levels remain high however, the arteries block again within three months. All of these experiments and observation reveal an important role for an anti-Hsp65/60 immune response in the pathogenesis of atherosclerosis.

It is now believed that whenever the endothelium (intima) of arterial walls is damaged, stress proteins are expressed (Hsp60), in the cellular cytoplasm but also on the cell surface. Circulating antibodies bind to the Hsps and the cell may be destroyed by complement action. This releases substances that do two things, 1) attract macrophages and T cells, 2) cause replication of the underlying smooth muscle cells, temporarily thickening the artery wall. Attracting T cells will result in the release of cytokines. In response to these there will either be further multiplication of smooth muscle cells, which may lead to myointimal hyperplasia, and the blocking of the artery, or the reduction of
smooth muscle cell replication. This is dependent on the T-cells attracted and the mix of cytokines released.

How the anti-Hsp60/65 antibodies are induced is unclear though it is probable part of the response to bacterial infection. The potential exists for an antiself response to occur as a result of post-infection boosting of the antibody through the homology between the Hsp of the infecting bacteria and the stress proteins of mitochondrial origin in tissue cells.

There is increasing evidence that the Hsps are involved in the evolution of several autoimmune diseases, probably due to the high sequence homology among all of Hsps. The discovery of the cold shock response and Csps, in particular the CspA family, with a similarly high homology between the prokaryotic organism, and the human Y-box proteins, allows the possibility of a similar involvement in autoimmunity.

Humoral and cell mediated immune responses, to the homologous CspA, have been reported in infections of humans and rodents with Methicillin resistant Staphlococcus aureus (MRSA) and M.tb, respectively (Lorenz et al., 2000; Weldingh et al., 2000) moreover previous reports have indicated that antibodies to Csps of the non-pathogenic mycobacterium, Mycobacterium vaccae (M.vaccae) exist in humans (Baker, 1998; Maynard, 1997; Nzula, 1996).

Limited work has been carried out in the cold shock response on mammalian cells. It has been of little interest, except in the areas of adaptive thermogenesis, cold tolerance, and storage of cells and organs (Fujita, 2000). However cold shock to mammalian cells appears to have a comparable effect to prokaryotes, changing the lipid composition of mammalian cell membranes, and reducing the rate of protein synthesis and cell growth (Nishiyama et al., 1997).

So far only one cold shock inducible protein, CIRP (cold-inducible RNA binding protein) has been identified in mammalian cells. CIRP belongs to a glycine-rich RNA-binding protein family (Danno et al., 1997), and is expressed constitutively.
in most tissues of adult mice (Nishiyama et al., 1998). Notably CIRP has no homology to the major cold shock protein of prokaryotes, CspA. Although, it appears to have a comparable function to that described for CspA. CIRP is believed to have a function as an RNA chaperone, as well as being involved in the spermatogonia development, and the promotion or maintenance of differentiation (Fujita, 2000; Nishiyama et al., 1998). Moreover its expression also appears to be induced by chemical that inhibit translation (Fujita, 2000), indicating that the cold shock effects translation in prokaryotic and mammalian cells. It has been suggested that the state of the ribosome is the physiological sensor for the induction of the cold shock response in mammalian cells (Danno et al., 1997; Fujita, 2000), and this was similarly suggested for prokaryotes (Jones and Inouye, 1994; VanBogelen and Neidhardt, 1990).

Interestingly two types of cold-inducible RNA binding proteins have been identified in plants. Both are Glycine-rich proteins, however the first type (Rbp-Grp) has homology to cyanobacterial cold-inducible RNA-binding proteins and mammalian CIRP (Carpenter et al., 1994; Nishiyama et al., 1997; Sato, 1994); the second type (CSD-Grp) contains a Cold shock Domain (CSD), and therefore has homology to the prokaryotic CspA-like proteins and the Y-box proteins (Karlson et al., 2002). A Rbp-Grp and CSD-Grp protein have been identified in Arabidopsis thaliana (Carpenter et al., 1994; Karlson et al., 2002). The Rbp-Grp is induced on cold shock, and is believed to be involved in circadian rhythms (Carpenter et al., 1994). Whereas a function for the CSD-Grp has not been identified yet, but a homologous protein is involved in the cold shock response of winter wheat (Karlson et al., 2002). It appears that plants possess two different types of RNA-binding protein that are involved in the cold shock response, which indicates that this may be the case for all eukaryotes. The search for potential mammalian Csps continues. So far cold shock expression of the Y-box proteins, the homologues to the prokaryotic major cold shock protein, CspA, has not been described. However the upregulation of plant proteins containing a CSD on cold shock indicates that the homologous Y-box proteins may be upregulated during cold shock in mammalian cells.
Interestingly, members of the Y-box protein family have been induced by stress-related stimuli including UV irradiation (Koike et al., 1997), anticancer agents (Ohga et al., 1998), and small affecter molecules such as thrombin and interleukin-2 (Olga et al., 2001; Sabath et al., 1990). Members of the Y-box family are involved in a wide variety of biological functions including the regulation of gene expression at transcriptional and translational levels, and in DNA repair (Marenstein et al., 2001). The latter function appears to be activated by conditions that cause DNA damage, such as UV radiation. YB-1 has been shown to stimulate human endonuclease III, which initiates base excision repair of pyrimidine bases (Marenstein et al., 2001).

Notably, nuclear expression of Y-box proteins has been noted in a number of cancers, including synovial sarcoma (Oda et al., 2003), ovarian cancer (Yahata et al., 2002), and breast cancer (Janz et al., 2002). Y-box proteins are normally located throughout the cell, with the greatest concentration in the cytoplasm (Koike et al., 1997). The differential expression of the Y-box proteins is a molecular marker of carcinoma. Moreover, the translocation of the Y-box protein into the nucleus appears to correlate with increased resistance to chemotherapy (genotoxic drugs) that cause DNA damage (Janz et al., 2002; Oda et al., 2003; Yahata et al., 2002). Furthermore, an increased expression of the Y-box protein, DbpB/YB1, has been noted in breast cancer (Rubenstein et al., 2002) and colon cancer (Ohga et al., 1996). DbpB was constitutively expressed in non-malignant tissue, and was overexpressed in breast and colon cancer cells (Rubenstein et al., 2002) (Ohga et al., 1996). Significantly, DbpB was also present on the cell surface of breast cancer cells, and antibodies to this protein were isolated from sera of patients with breast cancer (Rubenstein et al., 2002), indicating a potential role in the immune surveillance of malignant cells.

Thus it appears that the Hsps and Y-box proteins appear to have similar characteristics, their expression is increased during stress and both have been detected on the cell surface of chemically transformed tumour cells (Rubenstein et al., 2002; Ullrich et al., 1986). Moreover there is an indication that antibodies
to Y-box proteins are involved in the immune surveillance of transformed cells, and humoral and cell mediated immune responses, to the homologous CspA, have been reported on pathogen infection (Lorenz et al., 2000; Weldingh et al., 2000).

Other candidates for cold shock proteins include the DEAD box (ATP dependent) helicases. The DEAD box helicase, CsdA, has been shown to be an important protein in the cold shock response on *E.coli* (Jones et al., 1996), and the eukaryotes also have members of DEAD box family of RNA helicases (Luking et al., 1998). The potential for there to be other Csps conserved from bacteria to man still exists. Few of the proteins that constitute the bacterial cold shock response have been characterised in the prokaryotes examined. So far CspA is the only protein determined to be conserved across the bacterial kingdom, with eukaryotic homologues, but there may be others.

Thus there is evidence for the existence of a cold shock response in mammalian cells and tissues. There are several vascular diseases that result in a reduced blood flow, including Ischaemia, and Claudication. Chronic, long-treated, leprosy patients often show a progressive deterioration in peripheral blood flow, and nerve function. As a result the fingers of leprosy patients are disproportionately cold even at high ambient temperatures (Abbot et al., 2002). The cooler skin temperature is a result of the reduced blood flow in the digits. Many vascular diseases reduce blood flow that may result in a reduction in temperature in the surrounding cells and tissues, which may initiate a cold shock response. The potential for the expression of cold shock proteins homologous to prokaryotic Csps exists. These may be recognised by the immune system as non-self, to which antibodies and cell mediated responses may be raised. These immune components may be involved in the progression of vascular disease, or exacerbate existing conditions.

An immunotherapeutic vaccine has been developed from *M.vaccae*. Mycobacteria are highly antigenic and *M.vaccae* shares many proteins with the important pathogen *M.tb*. The immunotherapeutic vaccine has been found to
be effective in the treatment of several vascular diseases. The *M. vaccae* vaccine has been used in the treatment of Raynaud's phenomenon (RP). RP is a circulatory disease that is characterised by colour changes in the skin (white, blue or red). They also suffer cold induced numbness and occasional discomfort. The Raynaud's attacks are usually induced by cold, but can be induced by rapidly changing temperatures. Treatment of Raynaud's with *M. vaccae* immunotherapeutic has alleviated the symptoms of RP (personal communication J.Stanford). In addition, treatment of leprosy patients with the immunotherapeutic vaccine resulted in a significant increase in the finger circulation and skin temperature values, when compared with those on individuals treated with a placebo (Abbot et al., 2002).

The component of the immunotherapeutic vaccine that is responsible for these responses is unknown, although it is possible that it involves cold shock proteins. Reduced blood flow to limbs as a result of obliterative arterial disease (atherosclerosis, ischaemia, RP), results in a reduced oxygen supply to the limb, leading to anoxic shock, and the failure to maintain temperature leading to cold shock. If antibodies to Csps are present, theoretically they may cause further damage via the complement cascade. Investigation into the presence of Csps in a highly immunogenic organism such as *M. vaccae*, and the determination of circulating antibodies to these proteins, may uncover more information about the mechanism of these diseases, and determine whether an immune response to cold shock proteins is involved in circulatory disease.

The major aims of this project were: to characterise the cold shock response of *M. vaccae*, to determine the presence of a CspA homologue; to identify any *M. vaccae* cold shock proteins, and detect the presence of any circulating antibodies to these proteins in the sera of individuals with various vascular diseases.
Chapter 3

3 Preliminary Experiments

3.0 Introduction

The cold shock response has been studied in many different bacteria, and extensively in, the Gram negative bacterium *E.coli*. All of the published cold shock experiments were carried out with bacteria in mid-exponential phase, which were cultured in liquid media. Traditionally mycobacteria are cultured on solid media, making the ascertainment of growth dynamics impossible. Preliminary cold shock data has been obtained with *M.vaccae* cultures grown on solid media (Baker, 1998; Maynard, 1997; Nzula, 1996), however to provide any reproducibility the bacteria needed to be transferred into liquid culture. Homogenous cultures of *M.vaccae* and the determination of their growth dynamics were required.

Cold shocks of varying degrees have been published, Goldstein et al (1990) cold shocked exponentially growing *E.coli* cultures from 37 to 15 or 10°C, whereas Berger et al (1996) cold shocked *Arthrobacter globiformis* SI55 between 25 and 4°C. Previously it had been established that the greatest cold shock response from *M.vaccae* was seen when cold shocked from 32 to 6°C (Baker, 1998; Maynard, 1997; Nzula, 1996). The growth of many bacterial species has been reported to be monitored using Optical Density readings (Berger et al., 1996; Hebraud et al., 1994; Roberts and Inniss, 1992), to allow the growth of *M.vaccae* to be monitored a similar method needed to be developed.

*M.vaccae* strains Gm27 (NCTC 11659) and Job5 (NCIMB 11807) were cultured in liquid media, and the generation time of the cultures was determined by viable counts. A spectrophotometric method to measure the effect of cold shock on the growth of *M.vaccae* was created.
3.1. Materials and Methods

3.1.1 Mycobacterial culture and culture medium
Stock cultures of *M. vaccae* strains Gm27 and Job5 were maintained in non-antigenic Sauton’s media at 32°C, and on LJ slopes stored at -20°C (Appendix 1). Mycobacteria were cultured at 32°C in 50ml volumes of Sauton’s broth (Appendix 1), in 250ml Erlenemeyer flasks, in an orbital incubator (Gallenkamp) at 100rpm. A non-ionic surfactant, tyloxapol (Sigma) 0.5%v/v, was added to the cultures to prevent adherence to the side of the flasks and to reduce clumping in the cultures. The liquid cultures were subcultured every week, and the LJ slope cultures were routinely used to start new *M. vaccae* cultures to prevent genetic drift. The LJ slopes were thawed for 4-8h; a 10μl loop-full of *M. vaccae* was used to inoculate new *M. vaccae* cultures.

3.1.2 Mycobacterial Growth dynamics
The growth dynamics of *M. vaccae* strains Gm27 and Job5 in liquid broth were unknown, and in order to standardise the experiments; these were elucidated.

3.1.2.1 Determination of Bacterial concentration using Viable Counts
A viable count was performed; duplicate flasks for each strain were inoculated with 1ml of an established culture in stationary phase. The cultures were aseptically sampled, in triplicate, twice a day for 5 days. Each sample was subjected to a series of 1in10 dilutions in sterile 0.1%v/v tyloxapol, and 10μl was streaked onto individual 7H10 Middlebrook agar plates (Appendix 1), and incubated at 32°C until the bacterial colonies were clearly visible. Agar plates with greater than 100 colonies were counted, and the mean value from the triplicate data was calculated. This was done for both strains of *M. vaccae*, and linear regression analysis was applied to the exponential phase of the growth curves. The generation time of culture from the triplicate experiments was calculated and the mean generation time of each strain of *M. vaccae* determined.
3.1.2.2 Determination of Bacterial Concentration using Non-Culture Methods

The viable count gave an accurate value for the number of colony forming units in a culture, however the time taken to obtain a value was impractical for cold shock experiments. An alternative method was established.

A comparison was carried out between the viable count method and 3 methods of measuring bacterial concentration:

- absolute count by haemocytometer
- nephelometer measurement
- spectrophotometric absorbance measurement at 400nm ($A_{400\text{nm}}$)

**Total count**

A total count was performed using a Neubauer improved haemocytometer and a phase contrast microscope. The cultures were diluted in sterile deionised water containing 0.1% v/v tyloxapol, bacteria were disaggregated by vortexing in a sterile 1.5ml Eppendorf with four 1.5 to 2mm glass beads for 30s, 10µl was pipetted into a sealed haemocytometer. The bacteria were counted at x400 magnification. In order to ensure accuracy more than 100 bacteria were counted for each culture. Background was assessed, using sterile diluent, and subtracted from the sample counts.

**Nephelometer measurements**

Bacterial concentration was determined in 20ml volumes, in a turbidimeter (Merck), calibrated using 5 and 60 nephelometer unit (NTU) standards at 860nm, range 0-1000NTU. The cultures were diluted in Sauton's media, to bring the concentrations into the above range.

**Spectrophotometer measurements at 400nm**

The absorbance of cultures was measured in a UV/Visible spectrophotometer (LKB Biochem, Ultrospec II), in a 500µl volume quartz cuvette with a 1cm path length, at 400nm. The blank was 500µl of Sauton's media. Well-mixed culture (500µl) was added to the cuvette and the absorbance measured. The cultures
were diluted in Sauton's media to bring them the limits of absorbance, <3.000 absorbance units (A). The 400nm wavelength was chosen, as the orange and yellow colour of the two *M. vaccae* stains would interfere with the traditional 600nm wavelength.

### 3.1.3. Method Comparison

Four flasks of liquid Sautons media were inoculated on 4 separate days to produce a range of bacterial concentrations. For each culture a nephelometer (turbidity meter) reading, spectrophotometer reading (A<sub>400nm</sub>) was taken and a total count and viable count were performed. The results were compared to the viable count to determine the most efficient and accurate method of measuring the number of viable bacteria/ml.

### 3.1.4 Correlation of absorbance at 400nm and bacterial concentration

Viable counts and absorbance measurements were carried out concurrently on a culture of *M. vaccae* strains Gm27 and Job5. Each strain was inoculated into separate flasks to give an initial absorbance of ~0.1. The cultures were sampled regularly and the A<sub>400nm</sub> readings were taken, and viable count plates were set up. The A<sub>400nm</sub> and viable count values were transformed (log<sub>2</sub> and log<sub>10</sub> respectively), and plotted against each other in linear axes. A linear regression analysis was performed to calculate the linear relationship between the two methods.

### 3.1.5 Cold Shock Experiments

#### 3.1.5.1 Effect of cold shock on growth of *M. vaccae* Gm27 and Job5

The cold shock method was adapted from published experiments with other bacteria (Jones et al., 1987; Roberts and Inniss, 1992; Goldstein et al., 1990). Flasks, containing 50ml of media, were inoculated with approximately 9×10<sup>6</sup> bacteria of *M. vaccae* strains Job5 and Gm27, which corresponds to an initial absorbance of 0.1A at 400nm. For each strain two flasks were inoculated, a control and cold shock flask. Both flasks were incubated at 32°C in an orbital shaker at 100rpm to mid-exponential phase, 40h, (t=0). The cold shock flask
was transferred to a 6°C shaking waterbath (Grant OLS 200), 100 rpm. The
time taken to reach 6°C was recorded for the first experiment. The flasks were
sampled regularly and the A₄₀₀nm readings taken. Again the bacteria were
diluted with Sauton's media to bring the readings into the spectrophotometric
range <3.000 (A). The cultures were monitored for up to 350h.

3.2 Results

3.2.1 Mycobacterial growth dynamics
Agar plates with greater than 100 colonies were counted, omitting any
contaminated plates. The number of colony forming units (cfu)/ml was
calculated using the following equation.

\[
\text{Number of cfu/ml} = \frac{\text{Number of colonies counted}}{\text{(to multiply to a ml volume)}} \times 100 \times \text{dilution (x10°})
\]

The mean value from the triplicate data was calculated. A graph was plotted
with age of culture (h) against log cfu/ml a representative graph is illustrated in
Figure 3.1. The generation time was calculated from the linear portion of each
graph by linear regression analysis. The residuals were checked to ensure
equal variance and normal distribution. The mean generation time was
calculated for each strain of \textit{M. vaccae} (Table 3.1). The exponential phase was
determined to be between 5 and 60h. All the published cold shock experiments
were carried out with mid-exponential phase cultures and it was decided to cold
shock the cultures when they were 40h old.
Figure 3.1: Representative growth curve for *M. vaccae* strains Gm27 and Job5.

The mean of triplicate values is depicted on the graph, with standard error of the mean error bars. Where error bars are not visible they are contained within the symbol.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Generation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. vaccae</em> Gm27</td>
</tr>
<tr>
<td>1</td>
<td>8.55</td>
</tr>
<tr>
<td>2</td>
<td>7.90</td>
</tr>
<tr>
<td>3</td>
<td>8.57</td>
</tr>
<tr>
<td>Mean generation time</td>
<td>8.20</td>
</tr>
<tr>
<td>STDEV</td>
<td>0.381</td>
</tr>
<tr>
<td>STD Error</td>
<td>0.127</td>
</tr>
</tbody>
</table>

Table 3.1: the Calculated generation times for *M. vaccae* strains Gm27 and Job5
3.2.2 Determination of Bacterial Concentration by Non-Culture Methods

3.2.2.1 Method comparison

Cultures of differing ages, 0h, 24h, 48h and 72h, were sampled for method comparison. For each culture; a nephelometer and spectrophotometer (\(A_{400\text{nm}}\)) measurement was taken; a total count and a viable count performed. The results of each method were compared with the viable count result. The viable count and total count data was transformed by \(\log_{10}\), and the nephelometer and spectrophotometer readings were transformed by \(\log_2\).

All four measurements were plotted against the age of the cultures (Figure 3.2). The measurements were transformed to provide data with normal variance to allow linear regression to be carried out. Each method measurement was plotted against the mean \(\log_{10}\) viable count reading (Figure 3.3). The relationship between the viable count and each method was assessed.

The plot of total count against viable count (Figure 3.3A) produced a curved plot indicating that there was not a straight-line relationship between the measurements, so a linear regression analysis was not carried out. The plots of viable count against the nephelometer (Figure 3.3B) and spectrophotometer (Figure 3.3C) readings revealed what appeared to be straight-line relationships with the viable counts. Linear regression analysis was carried out to assess the strength of the linear relationship of the transformed data. The \(r^2\) values for the viable count versus nephelometer reading, and viable count versus spectrophotometer readings, are 0.9883 and 0.9912 respectively. The spectrophotometer readings have a stronger correlation with the viable count readings than the other methods of measurement. Figure 3.2 indicates that the spectrophotometer and nephelometer readings followed the viable count values the most closely.

The spectrophotometric \(A_{400\text{nm}}\) method was determined to be the most effective method for measuring the bacterial concentration. In addition it was a fast method, the bacterial concentration was could be determined very quickly. The
Figure 3.2: Measurements of *M. vaccae* Gm27 bacterial concentration using 4 different methods.

The bacterial concentration in 4 cultures of *M. vaccae* Gm27 of differing ages, 0h, 24h, 48h and 72h. The total count and viable count data were transformed by $\log_{10}$, and the nephelometer and spectrophotometer values were transformed by $\log_2$.

NTU – nephelometer unit, arbitrary unit.
Figure 3.3: Correlation of Viable count data with (A) total count data, (B) nephelometer and (C) spectrophotometer readings.

The linear regression line and 95% confidence intervals are depicted on graphs B and C, along with the $r^2$ value.
nephelometer also gave a good linear relationship with the viable counts, although not the strongest relationship. The method required 20ml volumes which were impractical, providing disposal problems.

The viable count and total count appeared to have a curvilinear relationship, of all the indirect methods this method seemed to be the most affected by non-viable bacteria. Further experiments were carried out to try to quantify the relationship between the $A_{400\text{nm}}$ and the viable count.

### 3.2.2 Correlation of absorbance at 400nm and bacterial concentration

The results of the viable count and the $A_{400\text{nm}}$ measurements were transformed and plotted against each other on a linear graph. To ensure that the variances were equal the viable count data was transformed by $\log_{10}$, the $A_{400\text{nm}}$ readings were transformed by $\log_2$. Figure 3.4 reveals the effect of transforming the $A_{400\text{nm}}$ by $\log_2$. The transformation ensures that the $A_{400\text{nm}}$ readings show the pattern of growth of the two strains of *M.vaccae* more closely.

Linear regression analysis was carried out on the viable count and $A_{400\text{nm}}$ data from both strains of *M.vaccae*. A linear relationship exists between the $A_{400\text{nm}}$ readings and the viable count results (Figure 3.4). However, the last absorbance reading, taken as the cultures were entering stationary phase, appeared to reduce the linearity of the relationship. By omitting the last value from the plots and repeating a linear regression analysis, the linear relationship increased, the $r^2$ values increase from 0.9705 to 0.9767, and 0.9767 to 0.9949 for Gm27 and Job5 respectively (Figure 3.5). The $A_{400\text{nm}}$ is an accurate method for measuring the bacterial concentration in a culture with the limitation that the cultures have not entered stationary phase, i.e. the cultures are not older than 70h.
Figure 3.4: The growth of *M. vaccae* Gm27 and Job5 at 32°C measured by *A*$_{400nm}$ and viable count.

Graphs A and B show the effect of transformation of the *A*$_{400nm}$ readings on the pattern of growth. The transformed *A*$_{400nm}$ readings closely follow the pattern of growth depicted by viable count. The mean values (± standard error of the mean) of duplicate values are represented.
Figure 3.5: Linear relationship between log_{10}cfu ml^{-1} and log_{A_{400nm}} for M. vaccae strains Gm27 and Job5.

Graphs A and C are the original data. Graphs B and D are modified graphs missing the final point, which illustrate the effect of entry in stationary phase on the linear relationship. The linear regression line, the $r^2$ values and the 95% confidence lines are depicted on each graph.
3.2.3. Effect of cold shock on the growth of *M. vaccae* Gm27 and Job5

The time taken for a culture growing at 32°C to reach 6°C on cold shock was recorded (Figure 3.6). The temperature of the water bath (6.2°C) was reached in 9min, and fell below 7°C in less than 6min. The rate of cooling is similar to that quoted for *Pseudomonas fragi* (*P. fragi*), cold shocked from 30 and 20°C to 5°C in 12min and 10min respectively (Michel et al., 1997).

The effect of a 32 to 6°C cold shock on the growth of mid-exponential phase *M. vaccae* cultures was determined by measuring the absorbance of cold shocked cultures at regular intervals. A control culture was also sampled throughout each experiment, to ensure that the 32°C cultures followed a typical pattern of growth (data not shown).

The 32 to 6°C cold shock significantly affects the growth of both strains of *M. vaccae*, the overriding effect was the significant increase of the generation time. However, the response to cold shock was variable (Figure 3.7). In all the cold shocked cultures a gradual increase was noted in the A$_{400nm}$ readings after cold shock. But, in two of the experiments, a sudden increase in the A$_{400nm}$ readings was noted, indicating a synchronous division of bacteria, between 85 and 150h after cold shock (Figure 3.7). The increase varied in time between two experiments, plus a difference in the time frame of the doubling was noted between two different cultures of *M. vaccae* Job5 in a single experiment (Figure 3.7C). The results indicated that the cold shock had synchronised the cultures.

To confirm that the absorbance readings from the cold shocked cultures corresponded to viable bacteria, the cold shock experiment was repeated and a viable count carried out concurrently with the absorbance readings. As the absorbance method of bacterial number is an indirect method, confirmation that the bacteria were still viable was required. In addition any changes in absorbance needed to be shown to be a bacterial increase and not the mycobacteria increasing in size, as has been reported by other experimenters.
Figure 3.6: Time taken for a 32°C culture to cool to 6°C

Figure 3.7: Effect of a 32 to 6°C cold shock on the growth of *M. vaccae* Gm27 and Job5.

A, representative growth curve where cold shock resulted in a gradual increase in cell number. B and C, growth curves of experiments where cold shock caused an apparent synchronous division. The mean of duplicate values (± standard error of the mean) are plotted.
(Shaw and Ingraham, 1967). Cultures of *M. vaccae* Gm27 and Job5 were subjected to viable counts concurrently with $A_{400\text{nm}}$ readings. The viable count revealed that the $A_{400\text{nm}}$ readings represented viable bacteria. Unfortunately a sudden increase in the $A_{400\text{nm}}$ was not noted during the viable count experiment. So it could not be determined whether the sudden increase in $A_{400\text{nm}}$ readings noted in some of the cold shock experiments, was due to the culture doubling or due to an increase in the size of the bacteria. Although the correlation of the $A_{400\text{nm}}$ readings with the colony forming units, suggest that the increase in $A_{400\text{nm}}$, was due to an increase in the number of bacteria.

The reason for the apparent culture doubling in some of the cold shocked cultures and not in the others, is unclear. Of the five cold shock experiments carried out, an apparent culture doubling was noted in two of them. The generation time for the growth in the cold of those cultures that did not undergo a synchronous division was calculated. The growth in the cold was subjected to regression analysis and the time taken for the culture to double was extrapolated. *M. vaccae* Gm27 and Job5 were found to have generation times of approximately 35 and 27 days, respectively, a 90 to 100 fold increase from the 32°C growth rate.

It is unclear why there is a differential response to cold shock. Although it is evident that *M. vaccae* is still able to grow, if very slowly, at 6°C.

### 3.3 Discussion

Prior to investigating the cold shock response, and identifying cold shock proteins in the mycobacterial species *M. vaccae* Gm27 and Job5, the generation time and exponential phase of these bacteria needed to be established. *M. vaccae* Gm27 and Job5 had generation times of 8h20 and 7h12 respectively. The exponential phase was established using data from the viable counts, from a stationary phase inoculum, and was found to be between 5 and 60h.

In order to monitor the growth of cultures in real-time, a method of measuring bacterial concentration that correlated with viable count data was required. The
viable count was compared with three indirect methods of measuring bacterial concentration; total count, spectrophotometry (absorbance at 400nm), and nephelometry.

The absorbance at 400nm was determined to be the most effective method for measuring bacterial concentration. This method was simple and provided good correlation with the viable count data, when transformed by log₂, if used before the culture entered stationary phase. The nephelometer provided similar results but the linear regression analysis produced a weaker linear relationship (Figure 3.3). The total count was time consuming and produced an inferior linear regression with the viable count values (Figure 3.3), possibly due to the lack of distinction between viable and non-viable bacteria by this method.

The relationship between the viable count and total count appeared to be a curvilinear relationship. The relationship between the total and viable count becomes less linear as the culture gets older, and is nearing stationary phase. The total count measures viable and non-viable bacteria, as the culture nears stationary phase the proportion of non-viable bacteria increases, so the total count will register more bacteria than the viable count.

All of the three methods compared with the viable count were indirect methods, and are therefore all affected by the presence of non-viable bacteria. However, the methods based on light scattering are less affected by the presence of non-viable bacteria, it may be that non-viable bacteria scatter light less effectively than viable bacteria, and therefore have less of an affect than on the total count. When a beam of light is passed through a suspension of bacteria the reduction in the amount of light being transmitted as a consequence of scattering is thus a measure of the bacterial mass present. The proportionality of light scattering is strict only at low values, the proportionality decreases with increases in light scattering (Stanier et al., 1993). As the cultures of *M. vaccae* Gm27 and Job5 near stationary phase, and the numbers of bacteria per ml increase to high absorbency values the linearity of the relationship decreases (Figure 3.5).
The $A_{400\text{nm}}$ appears to be the least affected by the presence of non-viable bacteria in the culture during log phase growth. The accuracy of the method appears to be limited to the log phase of the culture, as the cultures entered stationary phase the linear correlation between the viable count and absorbance readings appeared to weaken. The stationary phase would have seen an increase in the number of non-viable bacteria, which would affect the $A_{400\text{nm}}$ reading. Establishing the $A_{400\text{nm}}$ as the method of measuring culture concentration allowed the effect of the cold shock on the growth of *M.*vaccae strains Gm27 and Job5 to be determined.

Cold shocking two strains of *M.*vaccae (Gm27 and Job5) from 32 to 6°C had a dramatic effect on their growth rates. Comparable to the *B.*subtilis response to cold shock (Lottering and Streips, 1995), the mycobacteria continued growing at 6°C, with a greatly increased generation time (90 to 100 fold increase), and no apparent lag phase. This is contrary to *M.*smegmatis; a 37 to 10°C cold shock resulted in an 18 to 24h lag phase (Shires and Steyn, 2001).

The overriding response to cold shock in *M.*vaccae was a significant increase in the generation time of the two strains studied. For all the cultures examined a very gradual increase in $A_{400\text{nm}}$ was demonstrated. However the response to cold shock was variable, in two of the experiments a sudden increase in $A_{400\text{nm}}$ was also noted, which indicated that the cultures had been synchronised by the cold shock (Figure 3.7). Cold shock has previously been shown to synchronise cultures (Shaw and Ingraham, 1967), and temperature cycling is a method used for inducing culture synchrony (Stanier et al., 1993). The viability of the mycobacteria at 6°C, and the continued correlation between the viable count data and the $A_{400\text{nm}}$ readings was confirmed. Although, a sudden increase in the $A_{400\text{nm}}$ readings was not noted during this experiment, it was assumed that the sudden increase in $A_{400\text{nm}}$ was due to an increase in the number of bacteria. An increase in absorbance has previously been reported, during cold shock experiments with *E.*coli, which was the result of an increase in bacterial size (Shaw and Ingraham, 1967). During this experiment the steady increase in absorbance of the culture disguised a synchronous division of the bacteria.
However, the increase in absorbance in the *M.vaccae* cultures occurred over ~6h, (less than the generation time at 32°C) and the culture appeared to return to its previous growth rate, indicating that the sudden increase in $A_{400\text{nm}}$ was not due to an increase in bacterial size.

The time frame for the apparent synchronous division differed between experiments, and also between duplicate cultures within an experiment (Figure 3.7). It is unclear why synchronous divisions were noted with some cultures and not others, or why the time frame of division differed.

Notably, a cold shock of 37 to 10°C resulted in a 50-fold increase in the generation time of *M.smegmatis* (Shires and Steyn, 2001), from 3-4h to 7-8 days. The generation times of the two strains of *M.vaccae* (Gm27 and Job5) were around 7-9h at 32°C (Table 3.1). Generation times at 6°C were calculated for the cultures that did not undergo a synchronous division, and revealed that the rate of growth at 6°C was 90 to 100 fold slower than at 32°C. The generation times were 35 days and 27 days for Gm27 and Job5 respectively, these times are longer than the time period the cultures were studied for, and although an increase in the $A_{400\text{nm}}$ was noted it did not represent a division of the entire culture.

The response to cold shock of the two fast growing mycobacteria *M.vaccae* and *M.smegmatis* appears to differ. *M.vaccae* continues to grow at 6°C with a significantly increased generation time, while *M.smegmatis* enters a lag period, before growth resumes at a slower rate (7 to 8 days) than at 37°C (3 to 4h) (Shires and Steyn, 2001). In addition *M.vaccae* appears to be more sensitive to cold shock than *M.smegmatis*. The two species were cold shock over a comparable range of temperature, 15 and 16°C for *M.vaccae* and *M.smegmatis*, respectively. The magnitude of the cold shock response is dependent on the range of the temperature shift, rather than the minimal temperature; the larger the range of temperature shift, the greater the effect, and the response (Jones and Inouye, 1994; Shires and Steyn, 2001). However, it is possible that the 6°C cold shock temperature for *M.vaccae* was nearing the minimal temperature of
growth for this organism, and this may explain the slower growth rate of the 
*M. vaccae* strains. Moreover, the two organisms were grown in significantly 
different media. *M. vaccae* was cultured in a minimal Sautons’ media, whereas 
*M. smegmatis* was cultured in a rich media (7H9) (Shires and Steyn, 2001). The 
more stringent media may have had a significant affect on *M. vaccae*’s ability to 
respond to cold shock. A cold shock experiment was carried out with *M. vaccae* 
Gm27 cultured in 7H9 media. The culture had a faster generation time at 32°C 
and after cold shock to 6°C (data not shown), indicating that the lower cold 
shock temperature and more stringent culture conditions caused the slower 
growth rate in *M. vaccae*.

The stringent culture conditions and the cold shock temperature, potentially 
close to the minimal temperature of growth, may also explain the variable 
response to cold shock. These conditions may have made the cultures 
sensitive to minimal differences in media composition or minor fluctuations in 
temperature. A minor increase in temperature may have been enough to trigger 
a synchronous division, and may explain the variable response to cold shock by 
*M. vaccae*.

A 32 to 6°C cold shock has a profound effect on the growth of *M. vaccae*, and 
although it’s response to cold shock has been seen to be variable, the cold 
shock results in a dramatic increase in the generation time of the two strains of 
*M. vaccae* studied. The mycobacteria are still able to grow at 6°C, though very 
slowly.
Chapter 4

4 Materials and methods

4.1 Cold shock experiments

The *M. vaccae* strains were cold shocked for periods of 1h, 4h, 12h, and 24h. A stationary phase culture of *M. vaccae* was used to inoculate a flask of Sauton's liquid medium (50ml), to give an initial absorbance of ~0.1 (approximately 9x10^6 bacteria), for each time period. The cultures were incubated in an orbital incubator (Gallenkamp) at 32°C for 40h, and transferred to a 6°C shaking waterbath (Grant). This was denoted as time 0 for cold shock. A control flask was grown at 32°C for 40h.

4.2. Extraction of *M. vaccae* cold shock proteins

Control and cold shocked mycobacteria were harvested by centrifugation at 27200g for 20min, at 32 or 6°C depending on the experimental temperature. The supernatant was discarded, the pellet weighed, frozen in liquid nitrogen, and stored at -20°C until use.

To each thawed bacterial pellet 6 sterile glass beads (2.5-3mm) were added. The pellets were washed twice with 20ml of low ionic buffer, PGSK, (NaH₂PO₄·H₂O 0.52g/l, Na₂HPO₄·2H₂O 8.8g/l, NaCl 2.83g/l, KCl 0.372g/l, and glucose 11.0g/l) by resuspension and centrifugation at 27200 x g for 15 min, at the experimental temperature (32°C or 6°C). The pellets were resuspended in a volume of 20mM Tris pH7.0 containing protease inhibitors (Merck), at a ratio of 2ml per g of pellet, and a maximum of 1ml was added to a 2ml Eppendorf tube. Sterile glass beads (0.1mm) were added to the tube to approximately three-quarters full, and the tube was sealed. Bacteria were lysed with a mini-bead beater (Stratech Scientific) with 4 bead beating cycles of shaking for 80sec at full speed, followed by an 80sec rest on ice.
4.2.1 Protein harvesting

The lysed proteins were extracted by centrifugation from the rest of the cell debris. The base of each 2ml Eppendorf was cleaned with 70% ethanol to remove any foreign proteins, and a small hole was pierced in the bottom of each tube with a 1mm metal needle. This Eppendorf tube was placed into the top of a 1.5ml Eppendorf, and into a 50ml Beckman centrifuge tube. The tube was centrifuged at 27200 × g for 10min at 4°C. The protein containing supernatants were filtered through a 0.2μm low protein-binding filter (Millipore) to remove any remaining cell debris.

4.2.2 Protein concentration

The proteins were concentrated using Centricon filters YM3 (Millipore), which have a maximum 2ml volume, and a molecular weight cut off of 3KDa. The ultrafiltration membranes contain trace amounts of glycerine, which needed to be removed before use. Sterile deionised water (2ml) was washed through the membrane, at 5000 x g in a Beckman centrifuge, until at least half the water had passed through the membrane (ca. 30min). The filter was then inverted and centrifuged at 1000 x g for 2min to extract the water from the membrane. A maximum of 2ml of protein sample was added to the filter device and centrifuged at 5000 x g for 1h. Once the protein sample was concentrated to less than 500μl the filter was inverted and the protein sample recovered.

4.2.3 Evaluation of protein concentration

The protein concentration was determined using the Biorad detergent compatible protein assay, based on the Lowry protein assay. The standard microplate assay procedure was used, which measures 0.1-1.5mg/ml of protein. The standard curve utilised bovine serum albumin (BSA), at concentrations between 0.1-1.5mg/ml. The blank for each experiment was the protein buffer. The standard or sample (5μl) and 25μl of working reagent A were added to each well, and the plate gently mixed. Reagent B (200μl) was added to each well and mixed thoroughly on a Thermomax microplate reader (Molecular Devices). The plate was incubated at room temperature for a minimum of
15min, and the optical density (OD) was measured at 650nm using the microplate reader. The concentration of protein standards against OD$_{650}$ was plotted and the sample concentrations calculated from the standard curve. The proteins were then analysed by one-dimensional (1D) and two-dimensional (2D) gel separation techniques.

4.3 Protein Analysis of non-labelled proteins

4.3.1. 1D SDS-Page Separation

Proteins were separated using a discontinuous SDS-PAGE system, based on the method devised by Laemmeli (1970). A range of polyacrylamide gel strengths (6%, 10%, 15%, and 17.5%) were used to separate the proteins, the strength used is noted in the text. Gels were produced for the PROTEAN Xii electrophoresis tank, with a 40ml lower gel {appropriate % (v/v) polyacrylamide, 15mM Tris pH8.8, 0.01% (w/v) Sodium Dodecyl Sulphate (SDS), 0.00125% (v/v) TEMED, 0.01% (w/v) ammonium persulphate (APS)} and a 20ml 5% stacking gel (5% (v/v) polyacrylamide, 2.52mM Tris pH6.8, 0.01% (w/v) SDS, 0.01% (w/v) APS, 0.002% TEMED). A Tris-glycine-SDS running buffer was used (0.248M Tris, 2.5M glycine, 0.1% (v/v) SDS). Protein samples were mixed with 2x SDS protein loading buffer (100mM Tris pH6.8, 200mM dithiothreitol (DTT), 4% (w/v) SDS, 0.2% (w/v) Bromophenol blue, and 20% (v/v) Glycerol) in a 1:1 ratio, and boiled for 5 min. Proteins were loaded at the concentration indicated in the text, and the appropriate range molecular weight rainbow markers (Biorad) used. Electrophoresis was performed at 70-180 V, at 4°C, until the loading dye reached the bottom of the gel.

4.3.1.1 Staining

The gels were stained with Coomassie blue stain (2.5g/l Page Blue 83 stain (Merck), 45% (v/v) methanol, 10% (v/v) glacial acetic acid) until darkly stained, and destained with a solution of 50% (v/v) methanol/ 10% (v/v) acetic acid. The gel was mounted on 3MM Whatman grade filter paper, and dried on a vacuum gel dryer (Biorad) at 60°C until dry.
4.3.2 2D SDS-Page Separation – non-radiolabelled samples (National Institute of Medical Research)

Protein samples were separated by isoelectric point (pI) in the first dimension, and molecular weight in the second dimension. The first dimension, isoelectric focusing, was executed using Pharmacia Immobilized dry strips (IPG) {Amersham Pharmacia Biotech, (APB)}, which come in a variety of lengths and pH profiles. IPG 18cm strips, pH3-10, were used. The APB Multiphore system and the Investigator system (Genomic Solutions) were used to run the first and second dimensions, respectively. Powder-free low protein gloves were used throughout the 2D procedures to prevent contamination.

4.3.2.1 1st Dimension

The protein samples were filtered through a micro-ultrafiltration device (Millipore low protein binding 0.22μ Durapore filter unit), at 11600g for 10min, and diluted in solubilisation buffer [(7.7M urea (Genomic solutions), 2.2M thiourea (FLUKA), 4% CHAPS, 65mM DTE, 20mM Tris pH7.0, (Genomic Solutions)] to give 600μg of protein within 300μl of solution.

The IEF apparatus was allowed to cool to 20°C before use. Bromophenol blue (4μl) and IPG buffer (0.8%) (APB) were added to the protein samples. The IPG strips were re-swelled with 0.35ml of each sample. The IEF apparatus was assembled and run according to the APB instruction manual.
The IEF programme is displayed below.

<table>
<thead>
<tr>
<th>Voltage V</th>
<th>mA</th>
<th>Ohms</th>
<th>Time</th>
<th>Vh</th>
</tr>
</thead>
<tbody>
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</tr>
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<tr>
<td>100</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Break</td>
</tr>
</tbody>
</table>

4.3.2.2 The second dimension

All solutions were made with fresh deionised water. The plates were thoroughly cleaned before use, by soaking overnight in Microclean detergent (Genomic Solutions), washing with tap water, deionised water, and finally HPLC grade ethanol (Sigma). Polyacrylamide gels (12%) (Appendix 1) were poured in an Investigator casting system (Genomic Solutions). A solution of 1x running buffer (56mM Tris, 52mM glycine and 1% SDS) was used in the lower reservoir, and a solution of 2x running buffer in the top. The IPG strips were prepared for SDS-PAGE by equilibration in 3ml of 50mM Tris pH6.8, 6M urea, 30%(v/v) glycerol, 2% SDS and 2% DTE, for 12min, and in 3ml of 50mM Tris, pH6.8, 6M urea, 30%(v/v) glycerol, 2% SDS and 2.5% iodoacetamide, plus a trace of bromophenol blue for 5min. A 1% (w/v) low-melting point agarose was made and pipetted onto the top of the second dimension. The IPG strips were lowered into the agarose, which was allowed to set. Double strength tank buffer was used in the upper tank. Electrophoresis was performed at 1000mA (Glycosystems 2DE powerpack), and the gels stained with Hochstrass silver stain (see below).
4.3.2.3 Hochstrass Silver staining

- **Fixation** - the gels were washed individually in 500ml of deionised water for 5min, fixed in 40% (v/v) ethanol, 10% (v/v) acetic acid solution for 1h, and left in 5% (v/v) ethanol, 5%(v/v) acetic acid solution overnight.

- **Sensitisation** - the gels were washed with water for 5min, and soaked in 0.5M sodium acetate (42.3g/l) 1% (v/v) glutaraldehyde for 30mins. Gels were washed 3 times in water for 10min; and twice in 0.05% naphthalene sulphonic acid, for 30min. Finally, gels were rinsed 4 times with deionised water for 15min.

- **Silver impregnation** - a silver stain was produced by rapidly mixing 80ml 7.5mM silver nitrate and 450ml 0.05%(v/v) ammonium hydroxide, followed by the addition of 4ml 10M NaOH. The solution was diluted to 2l with deionised water, and used to stain the gels.

- **Development** - gels were washed for 4min with deionised water 4 times, and developed with 0.005% (w/v) citric acid 0.1% (v/v) formaldehyde.

- **Stop solution** - gels were soaked for 5min in 5% (w/v) Tris base/5% (v/v) acetic acid, followed by 5% (w/v) Tris base/2% (v/v) acetic acid, for 1h. The spots developed very quickly, and the stop solution was added before they were fully developed, as the spot continued to develop after the stop solution was added. The gels were stored in 35% (v/v) ethanol/5% (v/v) glycerol.

After at least one hour in the storage solution, the gels were scanned with a Biorad gel scanner, and the image visualised with Quantity One Software (Biorad).
4.4 Radiolabeling experiments

4.4.1 Assimilation of radioactive $^{35}$S-methionine

To establish whether \textit{M. vaccae} continued to produce proteins after cold shocking the incorporation of radioactively labelled methionine ($^{35}$S) was studied. The departmental guidelines and disposal procedures for handling and disposing of radioactive materials were followed. A flask of Sauton’s liquid medium was inoculated with \textit{M. vaccae} Gm27, from a stationary phase culture ($A_{600nm}$ 0.1) and incubated for 40h at 32°C. At 40h, $t = 0$, the culture was sampled (4ml) into 6 screw-capped glass vials (15ml) (FGB-Anchor), one for the control (32°C) and the remaining 5 vials for cold shocks to 6°C of 1h, 2h, 4h, 8h, and 24h. The vials had been pre-incubated at the appropriate temperature. To the control and 1h cold shock aliquots, 7.5$\mu$Ci/ml of L-($^{35}$S)-methionine (\textit{in-vivo} cell labelling grade, APB SJ1015-500$\mu$Ci) was added, the control aliquot was returned to the 32°C incubator, and the cold shocked vials were placed in a shaking water bath at 6°C. After 1h, the control and 1h cold shocked culture aliquots, were transferred to two 2ml screw-capped eppendorfs, and harvested by centrifugation in a microfuge at 11600g for 10min. The supernatant was discarded, in a radioactive waste container, and the pellet washed twice with PGSK (1.5ml) by vortexing and centrifugation at 11600g for 10min. The supernatant was again discarded the tubes were weighed, the pellet weight calculated, the pellets were frozen in liquid nitrogen and stored at -20°C until required. Radioactive methionine (7.5$\mu$Ci/ml) was added to the remaining cold-shocked culture aliquot for the last hour of cold shock, the bacteria were harvested and washed as detailed above.

4.4.2 Lysis of radiolabelled bacteria

When all the samples had been collected, the pellets were thawed. Using the calculated pellet weights, the pellets were resuspended in 20mM Tris pH7.0 at a ratio of 40mg/100$\mu$l of cell pellet. The 2ml tubes were then filled with 0.1mm glass beads, the lids replaced and sealed with nescofilm. The bacteria were lysed using 4 cycles of 80sec at 500rpm and 80sec on ice. The proteins were
recovered as described in section (2.4.2 protein harvesting). The proteins were filtered using an amicon ultrafree MC centrifugal filter device (0.5ml, 0.22μm low-binding durapore membrane). The proteins were placed onto individual membranes and centrifuged at 11600g in a microfuge in the cold room (~6°C) for 40min. A Biorad DC protein assay was carried out on the proteins as described above.

### 4.4.3 Slot blot onto Hybond-C+ membrane

Each protein (50μg) was diluted with 20mM Tris pH7.0 to 200μl total volume. A BDH 36-well slot blotter consisting of two precision plates with 36 wells and a vacuum reservoir was utilised. A rectangle of Hybond-C extra and Whatmann 3MM filter paper were cut to fit the slot blotter, covering the desired wells and leaving enough clearance for the clamping screws. The filter paper was soaked with Protein dry transfer buffer [39mM glycine, 48mM Tris base, 0.037% SDS electrophoresis grade, 20% methanol (pH8.3)], and placed onto the second plate of the slot blotter. The Hybond-C extra membrane was wet with milliQ water, placed on top of the filter paper, and the second precision plate placed on top of it. The clamping screws were tightened gradually to seal the apparatus. A vacuum was attached to the plot via a Buchner flask to prevent any radioactive material entering the vacuum, and the vacuum switched on. A vacuum of 600mmHg/0.8Bar was sufficient vacuum pressure. Each sample was loaded into a well of the slot blotter; a sample was loaded into every other well. Once the sample had been drawn out of the wells and onto the membrane, the wells were rinsed with 100μl dry transfer buffer. The apparatus were then dismantled and decontaminated. The membrane was rinsed in dry transfer buffer and then left to dry on a piece of clingfilm. Once dry the blot was enclosed in a fresh piece of clingfilm and placed in the FLA3000 cassette with the IP plate. The plate was incubated with the blot before being scanned using the FLA3000 scanner (Raytek), according to the manufacturers’ instructions and the scan analysed.
4.4.4 Calculation of the amount of radioactivity in a sample

Each radiolabelled protein sample was precipitated with trichloroacetic acid (TCA) to remove any unbound radiolabel. A volume of each protein (generally 5μl) was mixed with an equal volume (5μl) of 10% w/v trichloroacetic acid in a 1.5ml flip-cap Eppendorf, and placed on ice for 30min. The precipitated proteins were pelleted by centrifugation in a microfuge at 11600g for 5min at room temperature. The supernatants were removed, care being taken not to disturb the pellet, and washed twice with 5% w/v TCA (10μl). The pellets were washed by resuspension in the 5% TCA by vortexing, and pelleted by centrifugation at 11600g for 5min. The washed pellets were resuspended in 10μl of 0.1M NaOH by vortexing.

In early radiolabelling experiments the resuspended proteins were diluted in 90μl of protein dry transfer and slot blotted onto Hybond-C extra membrane (described above). The membrane was rinsed in protein dry transfer buffer and allowed to dry. In later experiments the proteins were applied directly onto the Hybond-C extra membrane, without the use of the slot blotter, and allowed to dry.

A radioactivity standard curve was created by serially diluting (1 in 2) the radioactive methionine in 20mM Tris-HCl pH7.0. The dilution series varied, the standard curves ranged between a maximum of 5μCi and a minimum of 10pCi. A volume of radioactivity was sample from the stock and subjected to an initial dilution e.g. 1 in 10. The radioactivity was then diluted 1 in 2 in a total volume of 10μl for up to 1 in 4096 dilutions. Each dilution (5μl) was then pipetted onto a piece of Hybond-C extra membrane. The membrane was allowed to dry, the standard curve and sample membranes were then placed in a cassette with the IP plate, and left overnight. The IP plate was scanned the following morning using the FLA3000 scanner IP laser, and the amount of radioactivity in each sample was calculated, using the AIDA software.
4.5 Analysis of radiolabelled cold shock proteins

The radiolabelled proteins were separated by 1D and 2D SDS-Page separation.

4.5.1 Radioactive labelling of cold shock proteins

The method for radiolabelling cold shock proteins was developed from several published experiments on several bacteria (Gumley and Inniss, 1996; Hebraud et al., 1994; Morgan et al., 1986; Panoff et al., 1997; Roberts and Inniss, 1992; Whyte and Inniss, 1992). A flask of Sauton's liquid media was inoculated with a stationary phase culture of Gm27 to give an initial $A_{400nm}$ of ~0.1. The culture was grown at 32°C in an orbital incubator for 40h ($t=0$ for cold shock). A control and 1h cold shock sample (3ml) were taken from the culture and aliquoted into a pre-equilibrated (6 or 32°C) screw-capped glass vial (15ml). The lid was loosely attached to allow oxygen to enter the tubes. The control vial was returned to 32°C. Radioactive $^{35}$S-methionine (65μCi.ml) was added to the tubes, which were then placed in the Grant shaking water bath at 6°C. The rest of the culture in the Erlenmeyer flask was also placed in the shaking water bath at 6°C. The cold shocked sample was radiolabelled for 1h at 6°C. After 1h the bacteria were harvested by centrifugation as described above. The supernatant was decanted into radioactive waste and the bacterial pellet washed twice with PGSK (6°C) as described above. The pellet was weighed, frozen in liquid nitrogen, and stored at -20°C.

Radiolabelled cold shock proteins were obtained from 1h, 4h, 8h, and 24h time periods after cold shock. For each time point, the cold shocked culture was sampled (3ml) an hour before the end of each time point, i.e. 3h for the 4h sample, and 65μCi/ml of radioactivity added so the samples were radiolabelled for the last hour of cold shock. The control was labelled for 1h at 32°C the bacteria were then harvested and washed with PGSK (32°C) as with the cold shocked samples.

Once all the samples had been collected the pellets were thawed and resuspended in an appropriate amount of 20mM Tris-HCl, pH7.0 containing
protease inhibitors (Mini-complex protease inhibitors Boehringer Mannheim) at a ratio of 40mg:100μl. The bacteria were lysed as described in section (lysis of radiolabelled bacteria). The recovered protein solutions were filtered through an amicon ultrafree MC centrifugal device as described previously and the amount of radioactivity in each sample calculated.

4.5.2 1D SDS-Page separation
The radiolabelled proteins were separated using a discontinuous SDS-Page system as detailed above. The proteins were separated on gels that were poured in the laboratory, but also on 10-20% pre-poured gradient gels obtained from APB. The gels were stained as previously described with Coomassie blue stain. Gels that were to be laid down with X-ray film were soaked in a fluorographic reagent, Amplify, (APB) for between 15-30min. The reagent increases the sensitivity of detection by converting weak β-emissions to light which are efficiently recorded on film. All the radiolabelled gels were then dried as described above.

4.5.2.1 Detection of radiolabelled proteins

FLA3000 IP Plate
The dried gels of the radiolabeled proteins were placed into a cassette and exposed to the IP plate overnight. The following morning the IP plate was scanned using the FLA3000 scanner (Raytek) and the image analysed.

Autoradiography
The gels for autoradiography, which were soaked in amplify, for 15 to 30min before being dried, were exposed to X-ray film. In a dark room a piece of Hyperfilm MP (APB) was placed on top of radioactive gel in an autoradiography cassette. The cassette was placed in a -80°C freezer for varying amounts of time, varying from overnight to several weeks. Once a suitable amount of time had passed the cassette was opened under dark room conditions and the X-ray film developed using the automated developer (Xograph Imaging Systems, Compact X) according to the manufacturer's instructions.
4.5.3 Preparation of samples for 2D SDS-Page Separation

The radiolabelled proteins were separated in a different facility and with a different system from the non-radioactive proteins. The previous facilities were not set-up for radioactive samples so the radiolabelled work was carried out at the Ludwig Institute of Cancer research. As before the protein samples were separated by isoelectric point (pI) in the first dimension, and molecular weight in the second dimension. The isoelectric focusing was carried out using APB Immobilized Gradient dry strips (IPG), the profile used was the 18cm pH3-10 non-linear (NL) gradient. The APB Multiphore system was used to run the first dimension, and the APB Protean Xii system was used for the second dimension.

4.5.3.1 Preparation of 'cold' protein samples for 2D SDS-Page separation

Samples of non-labelled control and cold-shocked samples were produced to test in the new 2D-system to obtain the optimum experimental parameters before radioactivity was used. Control, 1h, 4h, 8h and 24h cold shocked proteins were produced. Unlabelled proteins were obtained using the method described in section 3.5.1, without the radioactivity. The bacteria were lysed using a modified bead-beating method; 5 bead-beating cycles of 20sec bead-beating at 5000rpm and 60sec on ice. The proteins were harvested, filtered through a 0.22um MC Ultrafree centrifugal filter, and subjected to a Biorad DC assay, as described previously.

The volume that contained 150μg of protein was calculated, and this was diluted in solubilisation buffer (7.7M Urea 2.2M Thiourea, 4% CHAPS, 65mM DTT, 20mM Tris-HCl pH7.0 and protease inhibitors), with 2% carrier ampholytes (pH3-10) and 7μl of bromphenol blue solution, to a total volume of 350μl.
4.5.3.2 Modification of protein lysis buffer

The effect of the addition of a detergent, Nonident-P40 (NP-40) to the lysis buffer, and dialysis of the lysed proteins on the quality of the 2D-gels produced was assessed.

A stationary phase culture of *M. vaccae* Gm27 was sampled into 2 sterile and preweighed 2ml screw-capped Eppendorf tubes. The bacteria were pelleted, washed with PGSK, and weighed as described above. The two bacterial pellets were resuspended in one of two lysis buffers; buffer 1, the original lysis buffer, 20mM Tris-HCl pH7.0, protease inhibitors; and buffer 2, 20mM Tris-HCl pH7.0, 1%NP-40, protease inhibitors. The bacteria were lysed using the bead-beating protocol detailed in section 3.5.3.1 and the proteins collected into a 1.5ml flip-capped Eppendorf tube. Each protein sample was passed through a 25-gauge needle, 4 times, and centrifuged at 11600g, for 10min at 4°C. The protein samples were then dialysed against MQ water to remove any salts etc, using Pierce slidealyzer cassettes (0.1-0.5ml, Molecular Weight cut-off 3500). The slidealyzer cassettes were rehydrated in MilliQ water for 30sec, and each protein sample injected into a cassette according to the manufacturer's instructions. The protein samples were dialysed against 2L of MilliQ water overnight. The following day the dialysed protein samples were recovered according to the manufacturer's instructions. The proteins were concentrated by drying the proteins in a speed vacuum drier for several hours. The proteins were then resuspended in 200µl of modified solubilisation buffer (7.7 Urea, 2.2M Thiourea, 4%CHAPS, 65mM DTT and protease inhibitors), the Tris-HCl buffer was omitted from the buffer, to reduce the salt concentration. A Coomassie plus protein assay (Pierce) based on the Bradford assay, was carried out on the protein samples, as the solubilisation buffer interfered with the Biorad DC protein assay. The assay was carried out using a microplate. Each sample (5µl) was pipetted into a well of the plate followed by 150µl of Coomassie plus reagent (ratio 1:30). Solubilisation buffer was used as the blank. The plate was shaken for 20sec on Thermomax microplate reader (Molecular Devices) and the absorbance at 565nm measured. The results were analysed and the amount of protein in each sample calculated. The proteins
were loaded onto a 12% mini-gel (Biorad) and run at 200V for 35min, the gel was then stained with Coomassie blue stain. The gel was used to assess the quality of the proteins before they were used in the 2D system.

Once the quality of the proteins had been determined to be good by 1D separation the proteins (150μg) were diluted in the modified solubilisation buffer, with 2% carrier ampholytes (pH3-10) and bromophenol blue solution (7.5μl), to a total volume of 350μl.

4.5.3.3 Radiolabelling of cold shock proteins for 2D SDS-Page separation

Having determined improved conditions for bacterial lysis and sample preparation the radiolabelling of the cold shock proteins was performed.

The 8h cold shocked sample was chosen to be compared with the control by 2D electrophoresis. A flask of Sauton's medium was inoculated with a stationary phase culture of *M. vaccae* Gm27 to give a starting A_{400nm} of ~0.1. The culture was incubated at 32°C for 40h, the culture was then aliquoted (3ml) into 10 screw-capped glass vials (15ml), 5 equilibrated at 32 and 6°C. The cold shock bottles were placed in the Grant shaking waterbath at 6°C, and the control bottles returned to the 32°C orbital incubator. The 8h cold shock bacteria were radiolabelled for the last hour of cold shock with 28.6μCi/ml of L-[^35S] in vitro cell-labelling mix (70% L-[^35S] methionine and 30% L-[^35S] cysteine) (APB). The control cells were radiolabelled for 1h at 32°C. The bacteria were harvested into preweighed, sterile 2ml screw-capped Eppendorf tubes, washed, weighed and frozen as described above. The pellets were resuspended in 20mM Tris-HCl with protease inhibitors, at a ratio of 40mg:100μl. The resuspended pellets for the 8h cold shock, and control were pooled into a single tube. Each tube was filled with 0.1mm glass beads, the lid replaced, and the tube sealed with nescofilm. The bacteria were lysed using 5 bead beating cycles of 20sec at 5000rpm and 60sec on ice. The proteins were recovered by centrifugation as described above (section 4.2.1). The proteins were filtered through a 0.45μm Ultrafree MC centrifugal unit, 11600g, 4°C for ~30min, the membranes were
rinsed with 30μl of modified solubilisation buffer, and centrifuged for an additional 10min, to collect any proteins retained by the filter. The proteins were then passed through a 28-gauge needle 3 times, and then centrifuged at 11600g, 4°C for 5min, to destroy and pellet any retained nucleic acids. Next, the proteins were dialysed in slidealyzer cassettes and dried using the speed vac, as described above (section 4.5.3.2). The dried proteins were resuspended in 200μl modified solubilisation buffer. The proteins were then subjected to a Bradford assay to obtain the protein concentration. The appropriate amount of each protein (160μg protein), was diluted in modified solubilisation buffer, with 2% carrier ampholytes, and bromophenol blue solution (7.5μl), to a final volume of 350μl.

4.5.4 2D SDS-PAGE Separation – Ludwig Institute

4.5.4.1 The first dimension
The IEF apparatus was allowed to cool to 18°C before use. The 350μl protein samples were pipetted into a single well of a level re-swelling tray (APB). The number of IPG strips required were removed from the -20°C freezer using forceps. The plastic backing was removed from each IPG strip with care. In turn, the strips were then placed face down into the protein solutions with care taken to limit the number of air bubbles under the strip; 3ml of dry strip cover fluid was then pipetted into the wells to prevent evaporation. The strips were left to re-swell overnight. The following day the multiphor system was assembled according to the manufacturer’s instructions. A strip was removed from the re-swelling tray using forceps and the dry strip cover fluid drained off the strip onto a piece of Whatmann 3MM filter paper. Care was taken to not touch the surface of the strip as this could destroy the gradient. The strip was placed into the strip aligner with the pointed end facing the anode (red) gel side up. The procedure was repeated for each strip. For each IPG strip, two 3cm electrode strips were cut and wet with 250μl MilliQ water and placed on the end of the strips, in line with the strips, with approximately 0.75cm of the end of the strip covered. The electrodes were then put into place, and pushed down to make contact with the strips. The tray was then filled with 150ml of dry strip
cover fluid. The electrodes were connected and the lid replaced, and the power pack (EPS 3501) programmed (APB).

A step-wise programme for the 1st dimension was used, as detailed below.

<table>
<thead>
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<th>mA</th>
<th>Ohms</th>
<th>Time</th>
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<tr>
<td>300</td>
<td>5</td>
<td>10</td>
<td>5h^?</td>
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</tbody>
</table>

1 – Allows a small residual current to flow if the program is not terminated manually

4.5.4.2 Second dimension

While the first dimension was running the second dimension was poured. Two gel types were poured; 9-16% gradient and 12% gels (Appendix 1).

All solutions were made with fresh MilliQ water. Clean plates (20x20cm and 20x22cm) were chosen, that had been washed mechanically and by hand, were chosen. The plates were polished with 70% ethanol. The gradient gels were poured in Biorad multigel tank, and the single percentage gels in the Biorad individual casting chamber (Appendix 1). A solution of 1x running buffer (25mM Tris 192mM glycine 1% w/v SDS) was used in the upper and lower tank reservoirs. Once the IPG strips had re-swelled, the Multiphor apparatus was dismantled and the strips removed using forceps, and placed gel-side up on a piece of Whatman 3MM grade filter paper. The excess dry cover strip fluid was drained off the strips, with care taken not to disrupt the strips' surface. The IPG strips were prepared for the second dimension by equilibrating them in 3ml of 50mM Tris pH6.9 6M Urea 30% v/v glycerol 2% SDS and 2% DTT, for 15min, in a re-swelling try. The equilibration of the strips was staggered by 3mins, so that they were equilibrated for the same time. A 1% (w/v) low-melting point agarose solution (in 1x running buffer) was made, once melted a quantity of bromophenol blue was added to create a visible blue colour was added. The 1x
running buffer was poured off the top of a second dimension gel and dried using a strip of Whatman 3MM filter paper. The 1% agarose solution was poured onto the top of the second dimension, to the top of the smallest plate. The equilibrated strip was cut down to 18cm, by measuring it against an 18cm line, and the excess gel was cut from the acid end of the strip. The strip was then placed in to the agarose, using forceps and a small spatula it was gently pushed through the agarose on to the top of the second dimension gel. This was repeated for each strip. Once the agarose had set, the gels were placed in biorad protean xii tanks, with care taken to ensure that the tanks were sealed, and there was no leak from the upper reservoir. Electrophoresis was carried out at 40mA per gel, until the bromophenol blue was evident in the polyacrylamide gel, the current was then turned down to between 10 and 20mA overnight. Once the bromophenol blue dye reached the bottom of the gels. The gel sandwiches were dismantled and the Agarose and IPG strip cut off the top of the gel. The gels were fragile and were handled with care, to try to prevent damage to the gels, e.g. tearing. The gels were then either stained with a modified Hochstrass silver stain, or blotted on to nitrocellulose membrane (radioactive gels).

4.5.4.3 Modified Hochstrass Silver staining

Each gel was stained in a separate staining box, which had been thoroughly cleaned and rinsed in MilliQ water. The stain development was carried out in glass containers that had been cleaned thoroughly. Between 300-500μl of each solution was used per gel. Care was taken throughout the staining process not to touch the gels, as fingerprints would show up on the stained gels, despite gloved hands. If the gels had to be touched they were touched at the very top of the gel, where there should have been no spots. The staining technique was very similar to that in section 4.2.2.3, however there were significant differences and these have been listed below.

- Fixation – as in section 4.2.2.3 except the gels soaked in 5% (v/v) ethanol, 5% (v/v) acetic acid for a minimum of 3h and up to 3 days.
• Sensitisation – the gels were washed in MilliQ water for 5min, and then soaked in a 2.5% glutaraldehyde solution for 30min. The gels were then washed extensively with MilliQ water, for 10 min, 3 times, and for 30min, 4 times.
• Silver impregnation – the silver stain was made as in section 4.2.2.3, however the gels were stained for 10min rather than 25min.
• Development – the gels were washed in MilliQ water for 5min, 3 times, and developed in 0.01% (w/v) citric acid, 0.1% (v/v) formaldehyde. Placing the gels in 5% (v/v) acetic acid stopped the stain development. The gels were soaked in stop solution for a minimum of 15min.
• Storage – the stained gels were stored in 7% (v/v) glycerol, 10% (v/v) ethanol solution.

A Biorad gel scanner was used to capture the gel images.

4.5.4.4 Electrophoretic protein transfer
The radioactive proteins were electrophoretically transferred to nitrocellulose membrane. A piece of Hybond-C membrane (APB), and two pieces of Whatman 3MM filter paper, slightly bigger than the gel, was cut for each gel. The gel, membrane, and filter paper were constructed into a sandwich in 1x transfer buffer. The sandwich was kept under the level of the buffer, to try to prevent air bubbles forming between the gel and membrane, until the sandwich had been sealed in a cassette. The cassettes were place in a Semi-Phor Transfer unit TE77 (APB), with the membrane facing the anode, and the gel facing the cathode. The gels were electrophoretically transferred at 60mA overnight. The following morning the apparatus was dismantled and the blots allowed to air dry. Once dry the blots were laid down with an imaging screen for a minimum of overnight, and a maximum of several weeks. The imaging screen were scanned using the Molecular Imager FX system (Biorad), and the gels analysed qualitatively for differences in protein expression.
4.6 Procedures for DNA extraction

4.6.1 DNA extraction for Mycobacterial liquid cultures

A 1.5ml aliquot of stationary phase culture was centrifuged in a 2ml screwcapped Eppendorf tube at 11600 x g for 5min in a Microcentaur benchtop centrifuge. The supernatant was discarded and the pellet washed twice in M1/15 Borate buffered saline (BBS) Na₂B₄O₇·10H₂O 3.63g/l. NaCl 6.19g/l Tween 80 0.0005% (v/v). The pellets were resuspended in 1.5ml of M1/15 BBS, boiled for 10min, and centrifuged at 11600g for 5min. The DNA-rich supernatant was decanted, aliquoted (200μl) into 0.5ml PCR tubes, and stored at -20°C.

4.6.2 DNA Extraction from Mycobacterial slope cultures

A 10μl loopful of the selected mycobacterial culture was suspended in 1ml of M1/15 BBS, in a screw-capped Eppendorf, and autoclaved at 15lbs for 15min. Tubes were centrifuged at 11600g for 5min and the supernatant decanted, aliquoted (200μl) into 0.5ml PCR tubes, and stored at -20°C.

4.7 RNA extraction

RNA was extracted from control and cold shocked M.vaccae Gm27 using the Qiagen RNeasy Mini Kit. The bacteria were cold shocked as described in the cold shock experiments. The RNeasy Mini protocol for Isolation of Total RNA from bacteria was followed, with the additional QiaShredder columns, and DNAse Set on column digestion. Using the A₄₀₀nm readings the volume that contained 5x10⁸ bacteria was calculated and harvested according to the protocol. The incubation time for the bacteria in the lysozyme-containing TE buffer (10mM Tris 1mM EDTA pH8.0) was extended from 10min to 15min. The homogenised cell lysates (in Buffer RLT, step 3) were frozen at -70°C until all of the samples had been collected and all processed together. In addition, the DNAse set digestion was increased from 15min at room temperature to 1h.
4.8 Preparation of complementary DNA (cDNA)

RNA sequences can be reverse transcribed to create complementary DNA (cDNA). This allows the amplification of specific mRNA sequences, which can be used to detect RNA molecules, or can be an alternative source of material for sequencing reactions. Reverse transcriptases, like other DNA polymerases, require primers for the initiation of DNA synthesis. The reaction is traditionally primed by an oligo(dT) primer which binds to the polyA tail of mRNA. However, the reaction can be primed by random hexamers and by a downstream PCR primer annealed to the RNA.

4.8.1 Preparation of cDNA for Real Time PCR

The mRNA contained within the total RNA extracted from the control and cold shocked cells was converted into single stand cDNA using the Qiagen Omniscript Reverse Transcriptase kit. Random Hexamers (Promega) were used to generate the cDNA. An RNAse inhibitor was added to the mix to prevent RNA degradation. The kit protocol was followed, although the volume of the components was altered to increase the amount of RNA that could be added to the reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Protocol Volume/reaction</th>
<th>Actual Volume/Reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Buffer RT</td>
<td>2.0μl</td>
<td>2.0μl</td>
<td>1x</td>
</tr>
<tr>
<td>dNTP Mix (5mM each dNTP)</td>
<td>2.0μl</td>
<td>2.0μl</td>
<td>0.5mM each dNTP</td>
</tr>
<tr>
<td>Primers (10μM)</td>
<td>2.0μl</td>
<td>2.0μl</td>
<td>1μl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1.0μl (10units/μl)</td>
<td>0.25μl (40units/μl)</td>
<td>10units</td>
</tr>
<tr>
<td>Omniscript Reverse Transcriptase</td>
<td>1.0μl</td>
<td>1.0μl</td>
<td>4 units</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>Variable</td>
<td>2.75μl</td>
<td>Up to 2μg</td>
</tr>
<tr>
<td>Template RNA</td>
<td>Variable</td>
<td>10μl</td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td>20μl</td>
<td>20μl</td>
<td></td>
</tr>
</tbody>
</table>

The cDNA reaction was carried out in a Phoenix thermocycler (Helena Biosciences). Extreme care was taken not to contaminate any of the kit components or the RNA with RNAses. The bench, icebox, and pipettes were...
thoroughly cleaned, and any racks used soaked in Chloros for a minimum of 10min. All procedures were carried out with gloved hands, as skin carries RNAses, and gloves were changed frequently to prevent contamination of any solutions.

4.8.2 Preparation of cDNA for Rapid Amplification of cDNA ends (RACE)
RNA extracted from a control culture of *M. vaccae* Gm27 was used to generate cDNA for the RACE reaction. The Omniscript Reverse transcriptase kit described above was used, however the primer used was an Oligo(dT) primer (10pmol/µl) consisting of 20 Thymidine bases, and not random hexamers.

4.9 Polymerase Chain Reaction (PCR) procedures
PCR leads to the amplification of specific DNA sequences by an enormous factor. PCR employs two oligonucleotide primers, 17-30 nucleotides in length that flank the DNA sequence to be amplified. The Template DNA e.g. *M. tuberculosis* DNA is heated to denature the DNA. The primers anneal to the denatured DNA at opposite sides of the target region, assuming the desired sequence is present. The primers are orientated to allow DNA synthesis to be performed. DNA polymerase extends the primers through the region between the two primers to give new strands of variable length. The extension reaction creates two double-stranded target regions, which can each be denatured ready for a second cycle of hybridisation and extension. By the third cycle two double-stranded molecules consisting of the target region have been created. By repeated cycles of heat denaturation, primer hybridisation, and extension, an exponential increase in the target regions occurs. By altering the temperature of the reaction mix the denaturation, hybridisation and extension reactions are permitted. Generally DNA denaturation occurs at 94°C. Primer annealing occurs between 50 and 71°C. The annealing temperature determines the specificity of the primer annealing, lower temperatures are more permissive and reduce the specificity of the annealing. The extension reaction occurs at a temperature of 70-72°C.
A number of different DNA polymerases can be used for the reaction. Taq polymerase from the thermophilic bacterium *Thermus aquaticus* is routinely used in PCR.

In all of the PCR reactions carried out extreme care was taken to prevent cross contamination, through the use of sterile, plugged pipette tips, regular glove changes, and regular cleaning of pipettes, racks and surfaces. In addition post-PCR work was carried out in a separate room, with specific pipettes and equipment. A negative control, containing all of the reaction components with Sigma Molecular Grade water or Sigma PCR water instead of DNA template, was used in every PCR, and where possible a positive control, known to work with the primers, was also included. Furthermore, the reaction components were kept on ice while the PCR reaction mix was compiled to prevent non-specific annealing before the PCR reaction was started.

### 4.9.1 Basic PCR

PCR was performed with a Phoenix thermocycler (Helena Biosciences). Reactions were carried out in 0.5ml thin-walled PCR tubes, in 50μl volumes, with PCR master mix, 1μl of each primer (10pmol/μl) and 3μl of template (DNA/cDNA). The pre- aliquoted 1.1x reaction mix, was provided from AB gene; working concentration (50μl) 1.5 units Taq polymerase, 75mM Tris-HCl pH8.8, 20mM (NH₄)₂SO₄, 1.5mM MgCl₂, 0.01%(v/v) Tween 20, 0.2mM each of dATP, dCTP, dGTP, and dTTP; or Promega, 2x PCR mastermix (50μl); working concentration (50μl) 1.25 units of *Taq* polymerase in a proprietary buffer pH8.5, 5μM each of dATP, dCTP, dGTP, and dTTP, and 1.5mM MgCl₂, the final volume was made up with nuclease free water.
Two basic PCR programmes were used. The basic 1 PCR programme was used for the initial PCR reactions, which was subsequently modified to the basic 2 PCR programme.

The basic 1 PCR programme:

<table>
<thead>
<tr>
<th>Segment</th>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>5min</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>94°C</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53°C</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>1min</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>72°C</td>
<td>3min</td>
</tr>
</tbody>
</table>

The basic 2 PCR programme:

<table>
<thead>
<tr>
<th>Segment</th>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>5min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53°C</td>
<td>2min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>2min</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>94°C</td>
<td>40sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53°C</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>20sec +1sec/cycle</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>72°C</td>
<td>1min</td>
</tr>
</tbody>
</table>

1 – the longer first cycle ensures complete strand separation and offsets any non-specific annealing that may have occurred between adding the DNA sample to the enzyme-containing mix and the heat cycle.

4.9.2 Touchdown PCR

A touchdown protocol was included in some PCR programmes where the optimum annealing temperature was not known. The touchdown programme was added to the basic PCR programme between segment 1 and 2. Several rounds of PCR, up to 10, were carried out with higher annealing temperatures,
the annealing temperature decreased by 1°C with every round of PCR. An example of a touchdown programme is detailed below.

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>94°C</td>
<td>40sec</td>
</tr>
<tr>
<td></td>
<td>63°C - 1°C/cycle</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>20sec</td>
</tr>
</tbody>
</table>

The touchdown programme increases the specificity of the PCR as specific annealing will be possible at higher annealing temperatures than non-specific annealing.

4.9.3 Hot Star Taq polymerase

During reaction setup and before the first denaturation step, low temperatures permit primers to bind non-specifically to the DNA template and allow primer-dimers to form. The hybrids can be extended during standard PCR to generate non-specific products. To try to prevent this the reaction mixes are stored on ice prior to standard PCR, however non-specific binding may still occur. To increase the specificity of the PCR and to HotStarTaq (Qiagen) was used with some PCR's. With HotStarTaq DNA polymerase the amplification is only started after an initial denaturation step (“hot start”), which prevents the extension of non-specifically annealed primers.

The HotStarTaq was provided in a 2x mastermix from Qiagen, with a final working concentration (50µl) of 1.25 units of HotStarTaq DNA polymerase in 1x Qiagen PCR buffer (1.5mM MgCl₂, and 200µM of each dNTP). The reaction mix was set up as in the basic PCR method. The enzyme is activated by a 15 minute, 95°C, incubation step before the amplification programme.

4.10 Inverse PCR

Inverse PCR allows the amplification of DNA flanking a region of known sequence. The DNA is cut with restriction endonucleases, and the fragments ligated intramolecularly to form circular molecules. Primers are designed so
that they extend outwards from a known core sequence, which amplify a linear fragment from a specific circular molecule. The amplified sequences are those that flank the core sequence in the genome, their lengths depend on the restriction sites either side.

A restriction digest map was obtained for *M. vaccae* Gm27 *cspA* (Appendix 2). Three restriction endonucleases that did not cut within the known sequence were chosen, Aat II, Bam HI, and Pst I. A restriction digest of *M. vaccae* Gm27 DNA was carried out with each enzyme in its appropriate buffer, and in the buffer for T4 ligase (Promega):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular grade water</td>
<td>5.5μl</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>1μl</td>
</tr>
<tr>
<td>Gm27 DNA</td>
<td>3μl</td>
</tr>
<tr>
<td>Restriction endonuclease</td>
<td>0.5μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10μl</strong></td>
</tr>
</tbody>
</table>

The DNA was incubated at 37°C overnight, and the enzymes heat inactivated at 65°C for 35min. Then 1 unit of T4 DNA ligase was added to each tube, which was then incubated at room temperature for 3h. The enzyme was heat inactivated by heating to 70°C for 10min. The DNA was then stored at 4°C until required. The DNA (3μl) was then subjected nested PCR, a Basic 2 PCR with an annealing temperature of 63°C, and 25 cycles of amplification was carried out with the outer primers, and annealing temperature of 57°C and 30 cycles of amplification with the inner primers. The inverse PCR primers are listed in the results, section 6.3.8.

4.11 Real Time PCR

Real Time PCR is a technique for quantitative reverse transcription-PCR (RT-PCR). RT-PCR allows the analysis of gene expression, as mRNA species are converted into cDNA. These species can then be detected through PCR, with primers directed against the cDNA's of interest. Real time PCR relies on the
detection of a fluorescent signal that is proportional to the amount of PCR product being produced. PCR products are measured in real time by the use of a double-stranded DNA binding dye (SYBR green), which cannot bind to single-stranded DNA or oligonucleotides (primers), and will specifically bind to the PCR product. The amount of PCR product can then be measured in real time using a fluorimeter. The quantification is based on the threshold cycle, the first PCR cycle with detectable fluorescence, which will be when the PCR amplification is exponential. The greater the quantities of a particular cDNA species present the smaller the threshold cycle. The amount of product is calculated by reference to a standard curve, which is amplified at the same time.

Real Time PCR was carried out with the ABI PRISM 7000 detection system with the Qiagen Quantitect SYBR Green PCR mastermix. The Qiagen Quantitect SYBR Green PCR protocol was followed adjusting the protocol for a 25μl reaction.

The real time PCR was carried out in PCR microtitre plates. Each sample was pipetted (1μl) into duplicate wells of the plate, along with the standard curve for the particular PCR. The number of negative controls added to the plate depended on the number of samples being subjected to real time PCR. If the number of samples filled the whole plate, 6 negative controls (nuclease free water) were pipetted out, this was reduced to 3 if only half the plate was used. Once the templates had been added to the plate, 24μl of the mastermix was pipetted into each well. The plate was sealed with a plastic cover and placed into the real time PCR machine. The standard real time PCR reaction is listed below.
A melting curve analysis was performed on the initial PCR's, to check for the presence of primer dimers and multiple products.

4.12 Rapid Amplification of cDNA ends (RACE)

RACE is a PCR-based technique that was developed to obtain full-length cDNA copies of low abundance of mRNAs, for cloning and sequencing. The technique achieves amplification of the region between a single short sequence in a cDNA molecule and its unknown 3’ or 5’ end. To find the 3’ end of the cspA gene 3’ RACE was employed. The technique employs two oligonucleotide primers, one specific for the sequence of interest, and a nonspecific primer targeted to the 3’ polyA-tail of mRNA molecules.

The cDNA (3μl), forward primer (1μl, 10pmol/μl) and oligo(dT)20 (1μl, 10pmol/μl) were added to 45μl of diluted promega mastermix (section 4.9.1 for details).

The cDNA was amplified in a Phoenix thermocycler. Two RACE reaction PCR programmes were used:

<table>
<thead>
<tr>
<th>Segment</th>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>94°C</td>
<td>40sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50°C</td>
<td>2min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>3min</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>72°C</td>
<td>15min</td>
</tr>
</tbody>
</table>
RACE reaction 2, based on the method by Ohara et al (1989).

<table>
<thead>
<tr>
<th>Segment</th>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>94°C</td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45°C</td>
<td>2min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>3min</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>72°C</td>
<td>5min</td>
</tr>
</tbody>
</table>

4.13 Analysis of PCR products

The PCR products were normally separated on 1.5% or 2.0% Agarose (Sigma) gels, initially made with Tris-borate-EDTA (TBE) buffer (Appendix 1), and subsequently with Tris-Acetic-EDTA (TAE) buffer (Appendix 1) (for ease of use), with the corresponding running buffer.

Gels were initially stained with 0.0002% (v/v) Ethidium bromide and the DNA bands visualised using UV illumination. Later SYBR green I (Molecular Probes) was used, which was diluted 1:10000 of stock solution in the agarose solution, and visualised using the FLA3000 scanner from Fujifilm (Laser 473nm, filter Y520).

PCR product (5μl) was mixed with 1μl 6x loading buffer, and loaded onto the agarose gel. Depending on the expected product size, a 100bp DNA ladder (Gibco BRL), or 1Kb DNA ladder (Gibco BRL) was used. Different gel sizes, 50ml, 100ml, or 200ml were used, and each were run in the appropriate gel tank (APB). The 50ml gels were electrophoresed at 90V, 60mA, for ~1h. The 100ml gels were run at 110V, 80mA, for 1-1.5h. The 200ml gels were run at 150V, 100mA for 1-1.5h.

4.13.1 Densiometric quantification of PCR product

The amount of PCR product in a sample was calculated by loading a known quantity of PCR product mixed with the appropriate volume of 6x loading buffer onto a 1.5/2% Agarose/TAE gel containing SYBR Green I (1:10000). A low
DNA mass ladder (Invitrogen) (2μl) was also mixed with 6x loading buffer and loaded onto the gel. The gel was electrophoresed and visualised using the FLA3000 system. A standard curve was created from the low DNA mass ladder using the AIDA software and the amount of DNA in each sample calculated.

4.13.2 DNA quantification by Picogreen assay

The PCR product concentration was determined using the PicoGreen dsDNA quantitation assay (Molecular Probes). DNA concentration between 25pg/ml and 1μg/ml is measured by the assay. A standard curve was made out of Calf-Thymus DNA in TE buffer (Appendix 1), at concentrations between 1ng and 100ng. The blank for each experiment was TE buffer. The standard or sample (10μl) was added in duplicate to a microplate with 90μl of TE buffer. The Picogreen reagent (150μl) was diluted in 30ml of TE buffer and 100μl added to each well. The plate was thoroughly mixed and incubated for between 2 and 5min at room temperature. The fluorescence was measured at fujifilm FLA3000 scanner, with the O580 filter and the 473nm wavelength laser (the dye has an excitation and emission maxima of ~500nm and ~520nm, respectively). A standard curve was created using the AIDA software and the amount of dsDNA in each sample calculated.

4.14 Purification of PCR product

4.14.1 Purification from PCR reaction mix

PCR products were purified from the PCR reaction mix using the Qiagen Qiaquick PCR purification kit using a microcentrifuge. The columns in the kit have a cut-off of 100bp. The appropriate protocol for extraction from PCR reaction mixes was followed. Multiple PCR reactions were carried out for each product to generate sufficient product for sequencing or labelling as a probe. A total of 10μg of DNA could be loaded onto a single Qiaquick column, so the products of replicate reactions were loaded onto one column. The PCR products were eluted in 30μl of Sigma PCR water and all the eluates for a single product were pooled.
4.14.2 Purification from low-melting point agarose gel

Products that could not be purified by the Qiaquick PCR purification kit were purified using the Qiaquick Gel Extraction Kit, the low molecular weight cut off of the columns, meant that occasionally primer dimers were retained. Products were mixed with an appropriate volume of loading buffer and loaded onto a 1.5% low-melting point agarose (Gibco Brl)/TAE gel, containing 0.0002% (v/v) Ethidium bromide. Depending on the volume of the product, the product was loaded into single or multiple wells. The gel was run at the appropriate voltage for its size (see above for details). The bands were visualised on an UV transilluminator, and excised from the gel using a clean scalpel. The excised band was placed in a pre-weighed 1.5ml flip-cap Eppendorf. The gel was exposed to minimal UV illumination, as it damages the DNA. Once all the bands had been excised and weighed, the amount of gel excised was calculated. The Qiaquick gel extraction protocol was then followed. The gel slices were incubated in buffer QG in a Hybaid oven. The optional wash steps in the protocol were included. The product was eluted from the Qiaquick column in 30μl of Sigma PCR water, and where multiple columns were used the eluates were pooled.

4.14.3 Preparation of PCR product for sequencing

PCR products were purified using the Qiaquick PCR purification or gel Extraction kits. The amount of product extracted was quantified. A total of 20ng/100bp of DNA were required per primer for sequencing. The required volume of each eluted product was pipetted into 1.5ml Eppendorfs and dried in a helavac freeze drier. The products were then sent to MWG Biotech with 10μl of the forward and reverse primers (10pmol/μl) that were used to generate each product.

4.15 Cloning of M.vaccae Gm27 partial cspA and sigA gene sequences

The process of molecular cloning involves isolating a DNA sequence of interest and obtaining multiple copies of it in an organism, usually a bacterium. Large quantities of the DNA molecule can be then isolated in pure form for detailed
molecular analysis. The PCR products of the partial cspA and sigA gene sequences were cloned to obtained large amounts of product to make a DIG-labelled DNA probe.

4.15.1 Transformation of E.coli

Competent cells were prepared by inoculating a single, freshly grown colony of *E.coli* XL1B (Stratagene) into 100ml Luria-Berani (LB) Medium (Appendix 1) and grown overnight in an orbital shaker at >200rpm at 37°C. The following morning the culture had reached late-log phase, and 100μl of this culture was inoculated into 100ml LB broth. The culture was grown for 2-4h until swirling opalescent bacterial growth could just be seen (OD<sub>600nm</sub> 0.5). The cells were stored on ice at this point. While the bacteria were growing the ligation reaction was set up according to the manufacturer's instructions (see below).

The bacteria were harvested by centrifugation at 3500rpm for 10min in a benchtop centrifuge, the supernatant was decanted, the tube inverted and allowed to dry on tissue paper. While the bacteria were being pelleted, 100mM CaCl<sub>2</sub> (Sigma) was made and placed on ice. The cells were pelleted in 15ml of 100mM CaCl<sub>2</sub> and the centrifugation repeated (3500rpm, 1min, 4°C). The supernatant was again decanted, the tube inverted on tissue paper and the pellet allowed to dry. Once dry the pelleted bacteria were stored on ice until used. Each pellet was resuspended in 2ml CaCl<sub>2</sub>, and left for 30min on ice.

The transformations were carried out in 15ml Falcon tubes. The bacteria (1ml) were added to each tube followed by the ligation mixture (10μl) and left on ice for 10-30min. A transformation control of 10μl of diluted plasmid was set up. The cell were transferred to a 42°C waterbath for exactly 90sec and then placed back on ice for 2min. Care was taken not to agitate the cells. To each tube 4ml of LB broth was added and then tubes were placed in a shaking incubator at 37°C for 20-60min. After this incubation the bacteria were pelleted (3500rpm, 10min, room temperature). The supernatant was decanted and the bacteria resuspended in the small volume of broth left in the bottom of the tube. The
resuspended bacteria were then used to inoculate 2 LB Agar ampicillin plates (Appendix 1) and incubated overnight at 37°C.

4.15.2 Ligation reaction

The Vector used for the cloning reaction was the pGEM-T Easy Vector System. A ratio of 3:1 insert DNA:vector ratio was used. Purified PCR products of cspA and sigA were used as the insert DNA. The amount of PCR product/μl was calculated, the concentration of cspA and SigA products was 8.51ng/μl and 5.86ng/μl, respectively. The amount of insert DNA required for both genes for a 3:1 ratio was 7.15ng of cspA product, and 25.20ng of sigA product. A maximum of 3μl of insert DNA could be added to the ligation reaction, so only 17.60ng of sigA DNA was added to its ligation reaction.

The pGEM-T Easy ligation reaction:

2X Rapid Ligation Buffer, T4 DNA ligase 5μl
pGEM-T Easy Vector (50ng) 1μl
PCR product variable (max. 3μl)
T4 DNA Ligase (3Weiss units/μl) 1μl
Deionised water to a final volume of 10μl

The ligation reactions were incubated at room temperature for 1h.

4.15.3 Identification of recombinant colonies

Recombinant colonies were identified by plasmid mini-preparation and restriction digestion. Colonies (12) were picked randomly off the plates for each ligation. Each colony was inoculated into 3ml of LB ampicillin (1mg/ml) broth (Appendix 1) and then incubated in an orbital shaker at 37°C overnight.

4.15.4 Miniprep of plasmid DNA

Following incubation, 1.5ml of each culture was transferred to a microfuge tube and centrifuged at 14000rpm for 1min, at room temperature. The supernatant
was aspirated using a needle attached to a 1ml syringe. Each of the following solutions were added to the tubes, sequentially, the solutions being thoroughly mixed by vortexing on the addition of each solution:

- 100μl solution 1 (50mM Tris-HCl pH7.5, 10mM EDTA, 100mg/ml RNase A)
- 100μl solution 2 (0.2M NaOH, 1% Triton X-100)
- 150μl solution 3 (3M KOAc pH4.8)

The tubes were then centrifuged for 3min (14000rpm, room temperature) and the pellet removed with a bent hypodermic needle. To the supernatant 500μl of isopropanol were added, the tube vortexed, and recentrifuged for 5min (14000rpm, room temperature). The supernatant was decanted and the DNA pellet washed with 70% ethanol, dried, and resuspended in 50μl of water containing 20μg/ml RNase A.

4.15.5 Restriction digest of mini-prep DNA

The mini-prep DNA was subjected to a restriction digest with EcoR1, for 1h at 37°C. The digest reaction is listed below.

- mini-prep DNA 3μl
- 10x buffer H 1μl
- EcoR1 enzyme 0.5μl
- Nuclease free water 5.5μl

A restriction digest was carried out with all of the mini-preps. The restriction digest reaction mixture (10μl) was mixed with 1μl of loading buffer (50% (v/v) glycerol, 1xTAE, 0.25% bromophenol blue) and loaded onto a 1% agarose/TAE gel (containing 0.0002% (v/v) Ethidium bromide). The gel was photographed and the number of recombinant bacterial cultures identified. One mini-prep culture was chosen for each insert, and 10μl inoculated into 400ml LB ampicillin broth (Appendix 1). The cultures were incubated in an orbital incubator (200rpm) at 37°C overnight.
4.15.6 Midi-prep of plasmid DNA

The cultures were prepared using the Gibco BRL high purity midi-prep kit. The kit protocol was followed to extract the plasmid.

To fully characterise the inserts, including the direction of the inserts, several restriction digests were carried out. A map of the restriction sites within the two insert sequences was obtained. Using the Webcutter 2.0 software (www.firstmarket.com/cgi-bin/cutter/cut2.html) the restriction sites in the *M. vaccae* Gm27 cspA sequence, and the *M. tb* sigA sequence were determined (the *M. vaccae* sigA sequence is unknown) (Appendix 2). Using this information and the restriction site map for the pGEM-T Easy vector, multiple pairs of restriction enzymes that would cut the plasmid in 2-4 places, the pattern of which would enable the direction of the insert to be identified. The pairs of restriction enzymes were selected so that they would cut effectively in the same buffer. For each reaction 2μl of 10x buffer was added to a 1.5ml flip-capped Eppendorf, with 1μl of midi-prep DNA, 1μl of each enzyme and an appropriate volume of milliQ water to give a final volume of 20μl, in some incidences only one enzyme was needed. The digests were incubated in a 37°C incubator for 2h. The total digests were then mixed with 1μl of loading buffer and run on a 1.8% agarose/TAE gel (0.0002% ethidium bromide) and photographed.

<table>
<thead>
<tr>
<th>sigA restriction enzymes</th>
<th>cspA restriction enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamH1/Spe</td>
<td>Spe/Xmn</td>
</tr>
<tr>
<td>Nco/Sal</td>
<td>Spe/Dpn</td>
</tr>
<tr>
<td>PvuII/Sal</td>
<td>Nsi/Xmn</td>
</tr>
<tr>
<td>EcoR1</td>
<td>Nco/Xmn</td>
</tr>
<tr>
<td>NotI</td>
<td>EcoR1</td>
</tr>
<tr>
<td></td>
<td>NotI</td>
</tr>
</tbody>
</table>
4.15.7 Extraction of the cloned inserts

To obtain each construct, 10μl of midi-prep DNA was digested with 5μl of EcoR1 enzyme, 10μl of the 10x buffer H and 75μl of MilliQ water in a 1.5ml flip-capped Eppendorf, and incubated overnight at 37°C. The DNA was purified with a GFX PCR and DNA band purification kit (APB) according to the protocol. The DNA was resuspended in 10μl of Sigma PCR water. The purified DNA was mixed with 10x loading buffer and loaded onto a 1.5% low-melting point agarose/TAE gel containing 0.0002% (v/v) ethidium bromide. In addition a 3kb molecular weight marker was loaded onto the gel and the gel run at 50mA for 1h30. The desired bands were cut out of the gel with a sterile scalpel into weighed 1.5ml flip-capped Eppendorf tubes, and the DNA purified using the GFX PCR and DNA band kit, the protocol for purification of DNA from gel bands. The purified DNA was resuspended in 10μl of Sigma PCR water. The amount of each insert was calculated using the in gel quantification method. Several midi-prep extractions were carried out to obtain sufficient product for DIG-Labelling.

4.16 Dig-labelling of DNA probes

The cloned partial gene sequences of *M. vaccae* Gm27 *sigA* and *cspA* were obtained and labelled with digoxigenin (DIG) to be DNA probes to be used in southern and northern hybridisations. The partial gene sequences were labelled using the DIG High Prime DNA labelling and detection starter kit II.
The DIG high prime labelling procedure was followed. The procedure was designed to label 10ng-3μg of DNA. Approximately 100ng of each PCR product was added to the system. The DIG labelling reaction was carried out in 0.5ml thin-walled PCR tubes, in a phoenix thermocycler at 37°C for 21h. An incubation of 20h should have generated 1.5μg of labelled probe. The labelling efficiency of the high prime reaction was determined according to the kit protocol. All the buffers were made up with Diethyl pyrocarbonate (DEPC) treated water (Appendix 1). The probe concentration was optimised according to the DIG system user's guide for filter hybridisations (Roche).

4.17 Northern hybridization
Northern hybridization is a technique used for detection of a specific RNA molecule against a background of many other RNA molecules. The target RNA molecule is identified through the use of probes.

All of the equipment and working area was cleaned with RNase zap (Ambion) to prevent RNase degradation. All solutions were made up with DEPC treated water (Appendix 1).

The DIG Northern hybridization protocol was followed (Roche). Briefly, the RNA sample from control and cold shocked bacteria were pipetted (2μl) onto duplicate sheets of hybond N+ membrane (APB). The RNA was cross-linked to the membrane by placing the membrane on a sheet of Whatman filter paper that was wet with RNase free 10x SSC (1.5M NaCl, 150mM sodium citrate pH7.0), and UV cross-linked at 0.125J/cm² for 90sec. The membranes were prehybridised in an appropriate amount of DIG easy hybridization buffer (Roche) (20ml/100cm²). The membranes were prehybridized in separate tubes for at least 1h at 50°C. One of the duplicate membranes was incubated with a DIG-labelled cspA probe; the second was incubated with a DIG-labelled sigA probe (section 4.16), at the optimal probe concentrations (established according to the manufacturers protocol, Roche). The probes were hybridized to the membranes overnight in a hybaid oven at 55°C. The wash and detection
protocol was then followed (Roche). The hybridization was detected using chemiluminescent detection film (Lumi-film, Roche).

4.18 Southern hybridization

Similar to Northern hybridization, Southern hybridization allows the detection of specific DNA molecules, using a radioactive or digoxigenin labelled probe specific for the desired sequence. The original method was developed by Southern (1975,1979) for detecting fragments in an agarose gel that are complementary to a given RNA or DNA sequence.

Briefly, PCR products were separated in a 0.7% Agarose/TAE gel containing ethidium bromide. The gel was then soaked in 1.5M NaCl 0.5M NaOH for 45min on a rotary shaker, to denature the DNA, rinsed in milliQ water, and then soaked in 1M Tris pH7.4 1.5M NaCl for 30min, the buffer was changed after 15min. The blot was trimmed to remove the wells of the gel and placed in a container on top of 4 double sheets of Whatmann 3MM filter paper soaked in 20xSSC (3M NaCl, 75mM sodium citrate pH7.0). A piece of Hybond N+ membrane (APB) was cut to the same size as the gel and placed carefully on top of the gel to prevent air-bubble formation. The edges of the gel were surrounded with clingfilm to prevent a blotting by-pass. On top of this, 5 sheets of Whatmann 3MM filter paper, the exact size of the gel, were placed, followed by a large number of paper tissues. A glass platform and a 1Kg weight were then added. The construction was then left overnight to allow the DNA to blot onto the membrane.

The following day the sandwich was deconstructed and the DNA UV cross-linked to the membrane, at 0.125J/cm² for 90sec.

The DIG system Southern hybridization procedure was followed (Roche). Briefly, the membrane was pre-hybridized with DIG hybridization buffer in a small hybaid roller tube for at least 1h at 37°C. The membrane was then hybridized with the DIG-labelled cspA probe (section 4.16) in DIG Easy hybridization buffer (Roche) overnight at 37°C. The membrane was then
washed detected according to the manufacturers protocol (Roche) using Chemiluminescent detection film (Lumi-film, Roche).

4.19 Western blotting

Western blotting is a technique for analyzing and identifying protein antigens: the proteins are separated by electrophoresis in polyacrylamide gel, and then transferred ("blotted") onto a nitrocellulose membrane, where they bind in the same pattern as they formed in the gel. The antigen is overlaid with antibody, for example in sera, and then with anti-immunoglobulin conjugated with an enzyme. Bound antigens are identified by the addition of the substrate to the blot; a colour or fluorescent signal is then generated.

4.19.1 Extraction of proteins

Unlabelled proteins from control and 24h cold shocked protein samples were extracted following the extraction method used for radiolabelled protein samples (section 4.5.3.1).

4.19.2 SDS-Page separation of proteins

The concentration of the extracted proteins was measured using the Biorad DC protein assay. The selected proteins (7.5μg) were mixed with an equal volume of 2x SDS-loading buffer and loaded onto 10 or 12% precast polyacrylamide mini gels (Biorad), ECL™ protein molecular weight markers (APB). The gels were electrophoresed at 200V for 35min in a Biorad Ready Gel Cell.

4.19.3 Protein Dry-transfer

For each gel a piece of PVDF membrane (APB) was cut, to the same dimensions as the gel. In addition 6 pieces of Whatman 3MM filter paper the same dimensions as the gel were also cut. The membrane was prepared according to the protocol. Just before the dye had reached the bottom of the gel, the pieces of filter paper were soaked in protein dry transfer buffer (39mM Glycine, 48mM Tris base, 0.037% (w/v) SDS, and 20% (v/v) Methanol).
Once the gel electrophoresis had finished, 3 pieces of the soaked filter paper were placed on the bottom electrode of the dry transfer apparatus, followed by the PVDF membrane. The gel cassette was then dismantled the gel rinsed in MilliQ water and placed on top of the membrane followed by the remaining 3 pieces of filter paper. The lid of the apparatus containing the top electrode was replaced, and the apparatus attached to a powerpack. The dry transfer was carried out at 0.8mA/cm\(^2\) for 1h. The apparatus was then dismantled and the membrane placed in Phosphate buffered Saline (PBS) pH7.5 (Appendix 1). The lane containing the ECL (enhanced chemiluminescence) molecular weight markers (APB) was cut from the membrane, and the manufacturers protocol for development followed. The membranes were either subjected to a Western blot immediately or placed between two pieces of filter paper soaked in PBS, wrapped in clingfilm and placed in the fridge for a maximum of a 4 days. The membrane was checked regularly to ensure that it did not dry out.

4.19.4 Western blot

The ECL (enhanced chemiluminescence) western blotting detection kit (APB) protocol for western blotting was followed. Briefly the membrane was blocked in 5%(w/v) Marvel skimmed milk, in PBS for 1h at room temperature or overnight at 4°C. The membrane was rinsed briefly in PBS before being placed in the Mini protean II multiscreen apparatus (Biorad). A lane on a mini gel was covered by 2 wells in the multiscreen apparatus. The chosen sera (primary (1°) antibody) were divided 1/100 in blocking buffer, and 600µl was pipetted into a single well of the multiscreen apparatus, care was taken to ensure that no air bubbles were introduced to the system. The sera were incubated with the membrane for 1h at room temperature on a platform rocker (Ruckham, R100). The sera were removed using a 5ml syringe, and the well rinsed with 600µl of PBS. The apparatus was dismantled and the membrane washed 3 times for 5 minutes at room temperature. The membrane was then incubated with the secondary antibody (2° antibody), Goat antihuman IgG (Fc specific) peroxidase conjugate, which was diluted 1/3000 in blocking buffer. The optimal 2° antibody concentration was discovered by carrying out a western blot with a range of 2°
antibody concentrations with a single 1° antibody (serum). The membrane was incubated with the 2° antibody for 1h at room temperature, the antibody was then discarded the membrane rinsed in PBS, and then washed 3 times for 5min at room temperature with PBS. The ECL development protocol was then followed.
Chapter 5

5 M. vaccae Cold shock proteins

5.1 Introduction

Exposure of mycobacteria to sudden increases in temperature results in the induction of heat shock proteins (Hsps), which enable the organism to cope with the increased temperature (Patel et al., 1991). The heat shock response is universal and has been found in prokaryotic and eukaryotic cells, in animals and plants (Neidhardt et al., 1984). Exposure of bacteria to the opposite extreme, cold shock, has resulted in a response analogous yet distinct from the heat shock response. The cold shock response has been extensively studied in E. coli and B. subtilis. The response involves the upregulation of specific proteins, cold shock proteins (Csps). Preliminary experiments by previous investigators indicated that a cold shock response existed in M. vaccae (Baker, 1998; Maynard, 1997; Nzula, 1996) and such a response was recently reported in M. smegmatis (Shires and Steyn, 2001), confirming that the mycobacteria are able to elicit a cold shock response. The present study further investigates the cold shock response of M. vaccae.

5.2 Materials and Methods

M. vaccae strains Gm27 and Job5 were cultured at 32°C in Sauton's liquid medium. Mid-exponential phase cultures were cold shocked to 6°C as described in Chapter (preliminary experiments). The proteomes of control and cold shocked M. vaccae cultures were analysed by one-dimensional (1D) and two-dimensional (2D) SDS-polyacrylamide gel electrophoresis. The protocols are described in chapter 4. Analysis was carried out on unlabelled, and 35S-methionine labelled proteins. The radiolabelling of newly synthesized proteins is described in chapter 4.
5.3 Results

5.3.1 Preliminary analysis of *M. vaccae* cold shock proteins

5.3.1.1 1D SDS-PAGE analysis of *M. vaccae* cold-shock proteins

Previous experimenters reported the detection of *M. vaccae* cold shock proteins by one-dimensional SDS-Polyacrylamide gel electrophoresis (1D SDS-PAGE) of non-labelled samples (Baker, 1998; Maynard, 1997; Nzula, 1996). Therefore the effect of cold shock on protein expression was initially carried out by this method. In this study, proteins were extracted from two strains of *M. vaccae*, Gm27 and Job5, in non-shocked control and cold shocked cultures. The bacteria were subjected to cold shocks of 1, 4, 12, and 24h and the proteins were analysed using single percentage 1D SDS-PAGE gels. Subsequently the proteins were separated on 3 different percentage gels; 6%, 10% and 15%, with appropriate markers, to obtain the optimum separation for small, medium and large proteins (data not shown).

Initially the proteins were loaded onto the polyacrylamide gels with equal volumes of each sample. However this resulted in differences in the amount of protein loaded and made visualising differences in expression difficult. Therefore proteins were subjected to a Biorad DC assay to determine their concentrations. The optimum amount of protein for detecting bands was determined by gel electrophoresis, and 20μg and 100μg of protein were concluded to be optimal for visualising differences in strongly and weakly expressed proteins, respectively. No differences were noted in the protein profiles of control and cold shocked cultures of *M. vaccae* Gm27 and Job5, on all the different percentage gels employed. Further analysis of the Gm27 samples was carried out with a 17.5% polyacrylamide gel, which enabled the detection of differences in expression of small proteins, as the major cold shock protein of *Escherichia (E.) coli*, Cold Shock Protein A (CspA) is 7.4kDa in size. However, the technique failed to detect differential protein synthesis between the control and cold shocked cells.

To ensure that the failure to detect variation in protein synthesis was not due to the insensitivity of the Coomassie blue stain, a fluorescent stain was employed.
The control and cold shocked proteins of Gm27 and Job5 were separated on a 15% SDS-PAGE gel and stained with SYPRO Ruby stain (Molecular Probes). The proteins were detected using the FLA3000 scanner. The fluorescent stain was considerably more sensitive than Coomassie blue stain, as the small proteins at the bottom of the gel, were better differentiated (Figure 5.1). However, the increased stain sensitivity did not reveal any differences in protein expression.

5.3.1.2 2D SDS-PAGE analysis of M. vaccae cold shock proteins

The 1D SDS-PAGE analysis did not show de novo protein synthesis on cold shock. However, the separation of proteins by 1D SDS-PAGE is limited, as many proteins can share the same molecular weight, and only differ in their isoelectric point (pl). Thus a single band in a 1D gel, may represent several different proteins. To ascertain whether any changes in protein expression were being masked the unlabelled protein samples, from M. vaccae Gm27 only, were also separated by two-dimensional (2D) SDS-PAGE.

A control and 1h cold shocked sample were subjected to 2D separation. Qualitative analysis indicated the differential expression of a number of proteins between the control and 1h cold shocked sample (Figure 5.2). A number of protein spots had increased in size and intensity in the cold shocked sample, indicating an increased expression. In addition, there was also evidence of protein down regulation on cold shock. There was no evidence of any novel cold shock proteins. Furthermore, it was not clear whether a homologue to CspA, the major cold shock protein of E.coli was expressed (Jones et al., 1987). CspA is a small highly acidic protein, that was not expressed at 37°C in E.coli, and although several small acidic proteins could be identified, these were also detectable in the control samples.

The experiment was repeated, and the 2D analysis was carried out in duplicate: each sample was loaded onto two 2D gels, to ensure that any differences were reproducible (data not shown). The protein spots were analysed quantitatively.
Figure 5.1: Comparison of control and cold shocked protein profiles of *M. vaccae* Gm27 and Job5.

Picture of a 15% SDS-PAGE gel, stained with SYPRO Ruby, containing cellular proteins from a control non-shocked culture (C) (32°C) and cultures cold shocked to 6°C for the times indicated. The lanes containing the full-range molecular weight markers are indicated with M, and the positions and size of the marker proteins are indicated on the left.
Figure 5.2: Two-dimensional electrophoresis gels, stained with Hochstrasse silver stain, of *M. vaccae* Gm27 cellular proteins, from control and cold-shocked cells.

Total cellular extracts of (A) non-shocked control (32°C), and (B) 1 hour cold-shocked (6°C) cultures. Equal quantities of protein were subjected to IEF on pH 3-10 non-linear gradient IPG strips in the first dimension, and to SDS-PAGE on a 12% polyacrylamide gel in the second dimension. Proteins upregulated on cold shock are highlighted by a circle, proteins downregulated on cold shock are marked with a box.
using Melview software (Biorad), for the duplicate control and 1h cold shock gels. An increase in density of two-fold or more would be expected if a protein had been induced as a result of cold shock. However, there were no differences greater than 2-fold between any of the spots. The differences noted with the first 2D experiment were not reproducible and were likely to be a result of unequal silver staining.

5.3.2 Radiolabelling of \textit{M. vaccae} cold shock proteins

The failure to detect any cold shock proteins by SDS-PAGE suggested that the technique was not sensitive enough to detect changes in protein expression caused by cold shock. Many experimenters radiolabelled their csps with $^{35}$S-methionine and it was found that without selective labelling, proteins already present in the cell were masking any changes in protein expression. Hence radioactive ($^{35}$S) methionine was employed to detect any changes in protein expression, by labelling newly synthesized proteins in control and cold shocked cells.

5.3.2.1 Assimilation of $^{35}$S-methionine

To determine whether the cold shocked mycobacteria were still capable of utilising the radioactive methionine, the effect of cold shock on the assimilation of $^{35}$S-methionine by \textit{M. vaccae} (Gm27) was investigated. A culture of \textit{M. vaccae} Gm27 was cold shocked to 6°C. Samples of the culture were taken and newly synthesized proteins were radiolabelled for the last hour of cold shocks of 1, 2, 4, 8 and 24h. A control aliquot was radiolabelled for one hour at 32°C. The amount of $^{35}$S-methionine incorporated per µg of protein was calculated (Figure 5.3). Cold shock has a dramatic effect on the ability of \textit{M. vaccae} to assimilate $^{35}$S-methionine into its proteins. A 10-fold reduction was noted in the level of radioactive methionine incorporated into the proteins an hour after cold shock. This level remained fairly constant until 24h, when there appears to have been a 2-fold increase, above the cold shocked level, in the amount of $^{35}$S-methionine containing protein. The results indicate a cold-induced lag in protein synthesis in \textit{M. vaccae}, which ends between 8 and 24h after cold shock.
Figure 5.3: Incorporation of $^{35}$S-methionine by a mid-exponential phase culture of *M. vaccae* cold shocked to 6°C.

Newly synthesized proteins were radiolabelled for 1 h at each time point. The time 0 value, is the amount of radioactivity incorporated by *M. vaccae* cells at 32°C. The amount of radioactivity incorporated per μg of protein was calculated.
These results are comparable to the findings in *M. smegmatis*, where an increase in protein synthesis was noted after 18-21h (Shires and Steyn, 2001).

### 5.3.2.2 1D SDS-PAGE analysis of radiolabelled cold shock proteins

The ability of the bacteria to assimilate and incorporate $^{35}$S-methionine indicated that the bacteria were able to cope with the cold shock. Moreover, it indicated that protein translation was still occurring.

In contrast to *E. coli* and *M. smegmatis*, *M. vaccae* does not appear to have an obvious cold-induced lag in growth (Chapter 3), although *M. vaccae* does appear to have a lag in protein synthesis (Jones et al., 1987; 1992a; Shires and Steyn, 2001). The growth of *M. vaccae* was severely affected by the cold shock. The bacteria continue to grow with a greatly increased generation time (Chapter 3), a similar response to *B. subtilis* (Graumann et al., 1996). Thus the effects of cold shock on protein expression were initially monitored for the first 24h of cold shock.

Proteins were obtained from mycobacteria, in mid-exponential phase, which were cold shocked for 1, 4, 8 and 24h. Newly synthesized proteins were labelled by the incorporation of $^{35}$S-methionine for the last hour of cold shock. For the control, proteins were labelled for an hour at 32°C. The proteins were loaded onto 1D SDS-PAGE gels with equal quantities of radioactivity, (loading equal amounts of protein resulted in overexposure of the control or underexposure of the cold shocked proteins) and exposed to radiographic film.

Qualitative analysis of the autoradiographs indicated that a number of proteins were upregulated during the first 24h of cold shock. The cold shock period was subsequently extended to 96h, to determine the time period for cold shock induced changes of protein expression. Additional cold shock time points of 32, 48, 72 and 96h were examined. Again, the cells were incubated with $^{35}$S-methionine for the last hour of cold shock.
The majority of cold shock induced changes in the protein expression of *M. vaccae* occurred within the first 24h (Figure 5.4), comparable to *M. smegmatis*. The overall protein synthesis appeared to be significantly lower after cold shock to 6°C, although the number of protein synthesized at 32 and 6°C were similar, if not the same.

The protein translational machinery is obviously severely affected by cold shock, and this is reflected in the amount of each cold shock protein sample that had to be loaded. This was particularly evident with the 1h protein sample, as the width of this lane is significantly larger than the other lanes, indicating that the sample contains a large amount of non-labelled protein (Figure 5.4). The autoradiograph contradicts the $^{35}$S-methionine incorporation experiment, which indicated that the cold shocked bacteria assimilated $^{35}$S-methionine at approximately the same rate for the first 8h of cold shock. The reason for the discrepancy between the assimilation experiment and the autoradiograph is unclear. There may have been residual unbound $^{35}$S-methionine in the 1h cold shock protein sample in the assimilation experiment. Moreover, the profile for the 1h cold shock sample is very weak, indicating that the sample contains less radioactivity than the other samples. This is possibly due to unbound radioactivity not being removed from the sample during the Trichloroacetic acid precipitation step (Chapter 4).

Increased expression of a number of proteins was noted after cold shock, and quantitative analysis revealed that at least 4 proteins were upregulated 2 fold or more (Figure 5.4). These were determined to be *M. vaccae*’s cold shock proteins (Csps). Using the molecular weight markers to create a standard curve the size of the unknown proteins were calculated. The relative mobility ($R_i$) of each protein (markers and unknown) was determined, the $R_i$ of the molecular weight markers was plotted against $\log_{10}$ molecular weight, using linear regression analysis (Appendix 2) the equation of the standard curve was obtained, and the estimated molecular weights were calculated (Table 5.1).
Figure 5.4: Effect of a 32 to 6°C cold shock on the protein expression of *M. vaccae* Gm27.

Autoradiograph from a one-dimensional separation of $^{35}$S-methionine labelled proteins from a non-shocked control (32°C), and bacteria cold shocked for increasing periods of time, up to 96h. Newly synthesized proteins were radiolabelled for the last hour of cold shock. The control culture was radiolabelled for 1h at 32°C. Proteins were loaded with an equal amount of radioactivity (0.49 μCi) onto a 10-20% 1D SDS-PAGE gel. The position of the molecular weight markers are indicated on the left. Proteins induced on cold shock are indicated with arrows A-E, proteins downregulated on cold shock are indicated with arrows F and G. The autoradiographs were scanned with a densitometer and analysed using Quantity One software. The expression of each protein was expressed as a percentage of the total densitometric signal. A 2-fold or greater increase or decrease in the expression of a protein between the control and cold shock samples was taken as an upregulation or downregulation of expression, respectively.

From left to right, lane 1, 32°C control; lane 2, 1h cold shock; lane 3, 4h cold shock; lane 4, 8h cold shock; lane 5, 24h cold shock; lane 6, 32h cold shock; lane 7, 48h cold shock; lane 8, 72h cold shock; lane 9, 96h cold shock.
### Table 5.1: Putative *M. vaccae* cold shock proteins identified by 1D SDS-PAGE

<table>
<thead>
<tr>
<th>Unknown protein</th>
<th>Calculated molecular weight (kDa)</th>
<th>Expression pattern¹</th>
<th>Fold increase</th>
<th>Peak of increase (h)</th>
<th>Comparable protein in <em>M. smegmatis</em>²</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>44.1-46.7</td>
<td>Transient</td>
<td>2.0</td>
<td>32h</td>
<td>✓</td>
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<tr>
<td>B</td>
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<td>Continuous</td>
<td>2.3</td>
<td>48h</td>
<td>X</td>
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<tr>
<td>C</td>
<td>14.0-15.0</td>
<td>Transient</td>
<td>2.5</td>
<td>4h</td>
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<td>3.1</td>
<td>24h</td>
<td>✓</td>
</tr>
<tr>
<td>E</td>
<td>8.7-9.7</td>
<td>Continuous</td>
<td>4.0</td>
<td>24h</td>
<td>✓</td>
</tr>
</tbody>
</table>

¹ Transient, increased protein expression that reaches a peak during the cold shock period, and then falls to a basal level; continuous, increased expression throughout the cold shock, which reached a peak of expression but does not fall to a basal level.

² A comparable protein has the same molecular weight and expression pattern (Shires and Steyn, 2001).

Molecular weights were calculated from a standard curve, the values tabulated are the 95% confidence interval values.
Rf is given by:

\[ \frac{\text{Distance migrated by protein}}{\text{Distance migrated by solvent}} \]

Like *E.coli*, the *M.vaccae* response to cold shock appears to be instantaneous with differences obvious in the 1h cold shock profile (Figure 5.4). This response differs to that of *M.smegmatis*, in which the cold shock response was somewhat delayed, with no major changes being noted in the first hour of cold shock (Shires and Steyn, 2001). In *M.vaccae*, proteins E, D, and C, were upregulated within the first hour of cold shock, protein B within the first 4h of cold shock, and protein A was upregulated between 8 and 24h of incubation at 6°C (Figure 5.4). The analysis of the protein profiles from the longer 96h cold shock period did not reveal any additional proteins whose expression was increased after the initial 24h, indicating that the majority of protein expression changes occurred within the first 24h of cold shock.

There are two distinct patterns of expression of the *M.vaccae* cold shock proteins. Pattern one, the upregulation of the protein expression is transient, the proteins reach a maximum induction and then their expression decreases to a basal level. Pattern two, the upregulation of protein expression is continuous throughout the studied cold shock period (Table 5.1). These latter proteins should be assigned cold acclimation proteins (Caps), as they appear important for the survival of *M.vaccae* at 6°C.

A protein with an apparent molecular weight of ~9kDa was upregulated within the first hour of cold shock reaching a peak of expression after 24h. The protein was overexpressed for the rest of the cold shock. This protein is potentially a CspA homologue and contrary to *M.smegmatis*, this was found to be the major cold shock protein of *M.vaccae*, with a 4-fold increase in its expression. However the definition of a cold shock protein, is that it is a protein transiently induced during cold shock, its expression then returning to a basal level. The protein should be designated a Cold acclimatisation protein (Cap) as it was continually expressed throughout the cold shock period. It is possible that the
proteins expression decreases after 96h, the \textit{M. smegmatis} cold shock protein, CipMa, was expressed for at least 72h, but was not easily detectable after 120h of cold shock (Shires and Steyn, 2001).

A 27kDa protein, termed CipMa (cold-induced protein of mycobacteria), was found to be essential for cold acclimatisation of \textit{M. smegmatis} (Shires et al., 2002; Shires and Steyn, 2001). However, a Csp of comparable size was not identified in \textit{M. vaccae} (Table 5.1). Comparison of the \textit{M. smegmatis} and \textit{M. vaccae} Csps (Table 5.1) reveals that they have a number of Csps that are apparently the same size and have similar expression patterns, suggesting that they may share a number of Csps. Nevertheless, \textit{M. vaccae} does not appear to have a cold shock inducible CipMa homologue.

Similar to the cold shock responses of \textit{E. coli} and \textit{B. subtilis}, the downregulation of proteins was noted on cold shock in \textit{M. vaccae} (Figure 5.4). Qualitative analysis suggested that there were 4 proteins downregulated on cold shock, including a \~{}90kDa protein, however quantitative analysis revealed that only two proteins, E and F (Figure 5.4), were downregulated on cold shock. A 33-34kDa protein and a 37-38kDa protein were downregulated by 2.2 and 3.2 fold respectively.

\textbf{5.3.2.3 2D SDS-PAGE analysis of \textit{M. vaccae} cold shock proteins}

To further characterise the effect of cold shock on protein synthesis in \textit{M. vaccae} (Gm27) two-dimensional (2D) analysis was carried out. In order that the radiolabelled proteins could be analysed, a different 2D system that was equipped for radioactivity was used. The system was tested with non-labelled proteins to ensure that the 2D processes could be transferred between the system at the National Institute of Medical Research (NIMR) and the new system at the Ludwig Institute. Unfortunately the initial gels obtained at the Ludwig Institute were not of such good quality as at the NIMR (Figure 5.5). A lot of background and 'streaking' was noted on the gels, which indicated that the samples contained too high a salt concentration. The sample preparation
Figure 5.5: Effect of dialysis and addition of Nonident P40 detergent to the lysis buffer, to the two-dimensional separation of *M. vaccae* Gm27 proteins

Two-dimensional SDS-PAGE gels stained with Hochstrasse silver stain. Total cellular extracts of mid-exponential phase cultures of *M. vaccae* were separated by isoelectric focusing on pH3-10 non-linear IPG strips in the first dimension, and by SDS-PAGE on 12% polyacrylamide gels in the second dimension. **A**: proteins extracted using the original extraction procedure described in 4.5.3.1. **B**: proteins extracted using the modified lysis buffer, which contained the non-ionic chaotrope Nonident P40, and subjected to a dialysis step.
method was optimised, as the quality of the protein sample determines the success of the 2D experiment (Rabilloud, 1999). A dialysis step was added to the sample preparation procedure (Chapter 4, section 4.5.3.2) and a detergent Nonident P-40 (NP-40) was added to the lysis buffer to increase the solubilization of the proteins. The changes significantly increased the quality of the 2D gels obtained (Figure 5.5). Furthermore, the radioactively labelled amino acid was modified to a 70%:30% mix of $^{35}$S-methionine and $^{35}$S-cysteine. This increased the number of proteins radiolabelled, as not all of them contain methionine.

After optimisation of the two-dimensional system (Chapter 4, section 4.5.3.2), radiolabelled control and 8h cold shock were separated by 2D-electrophoresis and electrophoretically transferred onto PVDF membrane. Time constraints restricted the number of experiments carried out and the number of samples analysed. The 8h cold shock sample was chosen, as the 1D SDS-PAGE analysis indicated that the majority of changes in protein expression occurred within the first 24h, and that adequate radiolabel was incorporated into the newly synthesized proteins to create sufficient signal for autoradiographic detection. The previous two-dimensional analysis, with non-radiolabelled samples, had used the 1h cold shock sample. However, the dramatic reduction in the translation of new proteins after 1h cold shock, indicated that this sample would require significant optimisation to obtain a good autoradiograph.

The initial two-dimensional experiment was carried out with equal amounts of the control and cold shocked proteins (160μg), to try to identify the major cold shock proteins of *M. vaccae* (Gm27). However the autoradiographs (Figure 5.6) revealed that the translational machinery of the cell was so severely affected that insufficient radiolabel was incorporated into the cold shocked protein sample to allow the total effect of cold shock on the expression of proteins to be evaluated.
Figure 5.6: Synthesis of individual proteins following a cold shock from 32 to 6°C.

The autoradiographs from the two-dimensional separation of total cellular extracts of non-shocked control (32°C) and 8h cold-shocked (6°C) *M. vaccae* Gm27 cultures labelled with L-[³⁵S] in vitro cell-labeling mix (70% L-[³⁵S] methionine and 30% L-[³⁵S] cysteine). **A:** control autoradiograph, the proteins were radiolabelled for 1h at 32°C. **B:** 8h cold shock autoradiograph, the proteins were radiolabelled for the last hour of cold shock at 6°C. Equal quantities of protein (160µg) were subjected to IEF on pH3-10 non-linear gradient IPG strips in the first dimension, and to SDS-PAGE on a 12% polyacrylamide gel in the second dimension. Proteins induced by cold shock are circled and numbered U1-U11. Proteins down-regulated on cold shock are boxed and numbered D1-D7. The autoradiographs were analysed using Melview software. Although the signal was weak from the 8h cold shock autoradiograph, a number of proteins were paired between the two autoradiographs. Upregulation or downregulation of a protein was established as a 2-fold or greater increase or decrease in the percentage of the total densitometric signal of each paired protein on cold shock.
Nevertheless, despite the weakness of the 8h cold shock autoradiograph, a number of spots could be seen. The autoradiographs were scanned with adensitometer and the control and cold shock autoradiographs were analysed using the Melview software (GeneBio). Despite the weak signal, pairs of proteins between the control and cold shock gels were identified and changes in the expression of a number of the proteins was noted on cold shock. The amount of total densitometric signal each protein contained was calculated as a percentage of the total signal. The percentages for the paired spots were compared, and where a 2-fold increase or decrease in the signal of the proteins on the cold shock autoradiograph, as compared to the control autoradiograph, was noted, the protein was determined to be upregulated or downregulated, respectively, on cold shock. These results give limited information, as the proteins were not loaded on equal radioactivity. However, by normalising the densitometric signal against the total for the autoradiograph, the proportion of signal from each detected protein was calculated, which would be expected to change for upregulated or downregulated proteins on cold shock, giving a strong indication of the effect of cold shock on protein expression.

At least 12 proteins were found to be upregulated by 2 fold or greater on cold shock (Figure 5.6). In addition, at least 7 proteins were downregulated on cold shock. The electrophoretic transfer of proteins at the bottom of the 8h cold shocked gel was inadequate, hence there is no evidence of upregulation of small acidic proteins, or confirmation of the ~9kDa protein as the major cold shock protein of *M. vaccae*.

Time restraints meant that these experiments could not be repeated, or carried out with proteins that had been loaded with equal amounts of radioactivity. Thus the changes in the expression profile of the control and cold shocked samples could not be confirmed.

5.4 Discussion

In the current study the effect of a 32 to 6°C cold shock on the expression of proteins by *M. vaccae* was studied. The aim of the study was to determine
whether \textit{M. vaccae} had a cold shock response and to identify any cold shock proteins. In addition, to determine whether \textit{M. vaccae} contained a homologue to the Major Cold Shock protein of \textit{E. coli}, CspA. ID SDS-PAGE and 2D SDS-PAGE was used to monitor changes in protein expression, from native and \textsuperscript{35}S-methionine labelled cells.

It should be noted that the initial 1D protein analysis experiments were carried out with \textit{M. vaccae} Gm27 and Job5, however the remaining experiments were carried out with the Gm27 strain only. The 1D SDS-PAGE gels did not reveal any differences between the two strains, and it indicates that they respond to cold shock in the same way.

\textbf{The effect of cold shock on mycobacterial metabolism}

Cold shock has a significant effect on the metabolism of bacteria. When a culture of mid-exponential phase \textit{E. coli} was cold shocked from 37 to 10\textdegree C growth ceased for 4h (Jones et al., 1987; Shaw and Ingraham, 1967), no net synthesis was noted in DNA, RNA and protein. After 4h an increase was detected in protein synthesis, followed by RNA and DNA synthesis (Shaw and Ingraham, 1967). Although there was no net increase in protein, the induction of \textit{E. coli} transcriptional and translational, and cold shock proteins was noted (Jones et al., 1987; Jones et al., 1992a). A similar situation was noted in \textit{M. smegmatis}. A 37 to 10\textdegree C cold shock resulted in a cold-induced lag of 21-24h in all measured cellular activities (Shires and Steyn, 2001).

A 32 to 6\textdegree C cold shock resulted in a dramatic increase, 90 to 100 fold, of the generation time of \textit{M. vaccae} (Chapter 3), a similar response to \textit{B. subtilis} (Graumann et al., 1996). However there was no evidence of a cold-induced growth lag (Chapter 3), though cold shock of \textit{M. vaccae} induced a lag in protein synthesis. A 10-fold drop was noted in the amount of \textsuperscript{35}S-methonine incorporated on cold shock (Figure 5.3), the low level of incorporation remaining constant for the first 8h of cold shock, after which there was a 2-fold increase. Thus, there is a cold-induced lag in protein synthesis that ended between 8 and 24h after the start of cold shock.
The pattern of $^{35}$S-methionine incorporation in *M. vaccae* was similar to the incorporation of $^{14}$C-leucine in *M. smegmatis*. A 24-fold drop was noted in *M. smegmatis* in the amount of protein being synthesized, as determined by $^{14}$C-leucine incorporation (Shires and Steyn, 2001). An increase in the incorporation of $^{14}$C-leucine was noted after ~20h at 10°C, and *M. smegmatis* was determined to have a cold induced lag in protein synthesis of 18-21h (Shires and Steyn, 2001). These findings indicate a kinetically comparable cold-induced lag in protein synthesis, in both *M. vaccae* and *M. smegmatis*. Furthermore, these observations indicate that cold shock has a significant effect on the protein translational machinery of *M. vaccae*.

It is possible that the lag in the incorporation of $^{35}$S-methionine during cold shock is not due to the cold shock effect on the protein translational machinery but due to the active transport of the metabolite. The transport of metabolites was not the rate-limiting step during cold shock in *E. coli* (Breeze et al., 1978), and it seems that the same is true of mycobacteria. During the $^{35}$S-methionine incorporation experiments the amount of radioactivity in cell lysates and Trichloroacetic acid precipitated proteins differed (data not shown), with less $^{35}$S-methionine was incorporated into the proteins, indicating an excess of radioactive methionine in the cells. Moreover, translation in mycobacteria is very slow at normal growth temperatures (Winder, 1982), so it is likely that cold shock will have decreased the rate further.

The $^{35}$S-methionine incorporation results are confusing. The results indicate that approximately the same amount of $^{35}$S-methionine is incorporated per µg of protein, for the first 8h of cold shock. However, the analysis of the 1D SDS-PAGE autoradiograph, indicates that the amount of $^{35}$S-methionine incorporated into the 1h cold shocked sample is significantly less than it is into samples cold shocked for longer. The width of the labelled 1h cold shocked lane is greater than the other lanes indicating that the sample contains significantly more unlabelled protein (Figure 5.4). The reason for this discrepancy is unclear. It
may be a result of experimental error during the TCA precipitation; all of the unassimilated radioactivity may not have been removed.

**M. vaccae** cold shock proteins

The synthesis of *de novo* proteins on cold shock has been reported in *E. coli* and *B. subtilis*, and recently in *M. smegmatis* (Graumann et al., 1996; Jones et al., 1987; Jones et al., 1992a; Shires and Steyn, 2001). Analysis of non-labelled protein samples from control and cold shocked *M. vaccae* revealed no differences between the protein profiles, from 1D and 2D SDS-PAGE analysis (Figures 5.1 and 5.2). The findings reveal that no new proteins were synthesized *de novo* on cold shock. Furthermore, there did not appear to be any differences in the synthesis of any proteins between the control and cold shocked protein samples, and it seems likely that the changes in protein expression on cold shock were being masked by the proteins already present. The synthesis of protein macromolecules was shown to be significantly reduced on cold shock in *E. coli*, and more recently in *M. smegmatis*, probably due to a block in the initiation of translation (Shaw and Ingraham, 1967; Shires and Steyn, 2001). Therefore, any newly synthesized proteins would be a small proportion of those already present in the cell, making the detection of differential synthesis difficult.

During the *E. coli* cold shock-induced lag period the synthesis of proteins is dramatically reduced, and at least 20 cold-shock proteins were induced (Jones et al., 1987). 1D SDS-PAGE analysis of radiolabelled proteins from cold-shocked and non-shocked *M. vaccae* (Gm27) revealed that at least 5 cold shock proteins were induced during the first 24h of cold shock, and that no additional proteins were induced after this period (Figure 5.4). The number of cold shock proteins increased to 12 when analysed by 2D electrophoresis (Figure 5.5).

Cold shock causes a dramatic reduction in the number of proteins synthesized by *E. coli*, with only the cold shock proteins and those proteins involved in transcription and translation being produced (Jones et al., 1987; Jones et al., 1992a). In addition, the number of proteins synthesized by cold shocking
B. subtilis cells are significantly less than at 37°C, although the number produced is 3-fold greater than in cold shocked E.coli cells (Graumann et al., 1996). Unlike the cold shock responses of E.coli and B. subtilis, the number of proteins synthesised by M. vaccae, during cold shock, did not obviously decrease, although the level of protein synthesis decreased significantly. Similarly in M. smegmatis, cold shock proteins were induced against a backdrop of reduced total protein synthesis (Shires and Steyn, 2001).

The mycobacteria appear to have a pattern of protein expression comparable to those reported for the majority of psychrophilic and psychrotrophic bacteria studied. Cold shock has little effect on the number of proteins synthesized by the bacteria after cold shock. The synthesis of the majority of proteins is maintained after cold shock in the psychrotrophs Pseudomonas fragi (P. fragi) (Michel et al., 1997) and Arthrobacter globiformis (A. globiformis) (Berger et al., 1996). Although this response is not universal, cold shock significantly reduces the number of proteins synthesized by the psychrotrophic L. monocytogenes and L. innocua (Bayles et al., 1996; Phan-Thanh and Gormon, 1995). These observations indicate that the effect of cold shock on protein synthesis cannot be determined by the temperature range of growth i.e. whether the organism is psychrophilic, psychrotrophic or mesophilic (Berger et al., 1996). Nevertheless, the limited effect cold shock has on the number of proteins synthesized by A. globiformis, M. smegmatis and M. vaccae indicates that cold shock does not reduce the capability of the actinomycetes to synthesise the cells normal protein complement. Although it does affect the pattern of protein synthesis, some proteins are upregulated, and others downregulated on cold shock. Furthermore, the actinomycetes appear to have a comparable response to cold shock; the organisms overexpress the proteins that allow them to cope with the deleterious effect of cold shock.

The M. vaccae cold induced proteins had two patterns of expression, transient and continuous. Proteins that were rapidly but transiently overexpressed, which then decreased to a basal level, were designated cold shock proteins (Csp's), whereas proteins that were more or less rapidly induced but still overexpressed
during the 96h cold shock period were termed cold acclimatisation proteins (Caps). Like the findings in *E.coli*, changes within the protein profile of *M.vaccae* were noted within the first hour of cold shock. Although the 1h cold shock protein profile is weak, a number of proteins can be seen to be upregulated (Figure 5.4). This is contrary to the findings in *M.smegmatis* where the cold response was delayed, no major changes were observed in protein profile until the third hour of cold shock (Shires and Steyn, 2001). Interestingly, only one of the Csps, protein C, detected by 1D analysis reached its peak expression within the first 4h of cold shock. All the other proteins detected, whether Csp or Cap reached their peak of expression between 24 and 48h after cold shock. After 24h protein C had reached a level comparable to the control, suggesting that protein C has an important role in the immediate response of *M.vaccae* to cold.

The differences in the time frame for the cold shock response may be explained by the different culture conditions of the two mycobacteria. *M.vaccae* was cultured in minimal media with no antigenic components, whereas *M.smegmatis* was cultured in the rich Middlebrook 7H9 medium (Shires and Steyn, 2001). It’s possible that the more stringent conditions that *M.vaccae* was subjected to resulted in a different cold shock response, which enabled the mycobacteria to cope with the less favourable conditions. Thus, the more stringent conditions may make it more important for the *M.vaccae* to mount a faster response. Although, it could be argued that *M.smegmatis* would be more equipped to mount a faster cold shock response in the more favourable culture conditions.

Comparison of the *M.vaccae* cold-induced proteins (Cips) identified by 1D SDS-PAGE, and *M.smegmatis* Cips (Table 5.1) indicates that these mycobacteria may share a number of Cips. A number of the proteins of the two mycobacteria appear to be of a similar size, and have comparable expression patterns. Although it is not possible to say whether these are homologous proteins without further identification, it would appear that the two mycobacteria have a similar cold shock protein profile. Furthermore a similar number of cold shock proteins were identified by 2D electrophoresis, 12 and 14 for *M.vaccae* and
*M. smegmatis* respectively. These findings indicate that the two species share similar Cips. Although both sets of proteins would need further characterisation before the proteins could be determined to be the same.

Notably, there do appear to be significant differences between the Cips of the two species. A 27kDa protein, originally thought to be the major cold shock protein of *M. smegmatis*, termed Cold-Induced protein of Mycobacteria or CipMa, was upregulated on cold shock (Shires and Steyn, 2001). This protein is a histone-like protein (Shires and Steyn, 2001), and is essential for cold acclimatisation (Shires et al., 2002). Upregulation of a ~27kDa protein could not be detected on cold shock in *M. vaccae*. It appears that *M. vaccae* does not have a homologous cold shock protein. However, it should be noted that different 1D SDS-PAGE systems were used for the analysis of the *M. smegmatis* and *M. vaccae* cold shocked samples. Shires et al (2001) employed a Tris-Tricine SDS-PAGE system developed for the separation of proteins from 1 to 100kDa (Schägger and von Jagow, 1987) to analyse the Cips of *M. smegmatis*, whereas the traditional Tris-Glycine system (Laemmeli, 1970) was employed here. Moreover, proteins of 14 to 15kDa and 10.4 to 11.5kDa were identified in *M. vaccae* cold shocked samples by 1D SDS-PAGE however proteins of comparable size were only identified in *M. smegmatis* samples by 2D Electrophoresis. Further 2D SDS PAGE analysis of the *M. vaccae* Cips, may reveal a protein of a similar size, and should determine if the 1D-SDS PAGE system affected the detection of Cips. Nevertheless the findings indicate that a protein homologous to CipMa is not a *M. vaccae* cold shock protein.

A potential CspA homologue was identified in *M. vaccae*. CspA is the major Csp of *E. coli* and has an important role in the cold shock response (Goldstein et al., 1990; Jones et al., 1987). A protein with an apparent molecular weight of 8.7 to 9.7kDa was upregulated 4-fold on cold shock (Table 5.1, Figure 5.4), and continued to be overexpressed throughout the cold shock period examined. Comparable with *E. coli*, *B. subtilis* and *M. smegmatis* (Shires et al., 2002), this protein appears to be the major cold shock protein of *M. vaccae* as it was the protein most overexpressed on cold shock. Although this protein, on first sight,
appears to be too large to be a CspA homologue, *E.coli* CspA was originally identified with an apparent molecular weight of 10.6kDa, its real molecular weight was later determined to be 7.4kDa (Goldstein et al., 1990; Jones et al., 1987). In addition, CspA homologues of 8kDa and greater have been reported in *P.fragi* and *A.globiformis* (Berger et al., 1996; Michel et al., 1997). Therefore, it is plausible that the ~9kDa protein identified in *M.vaccae* is a cold shock induced CspA homologue. N-terminal sequencing, and immunoblotting against a peptide derived from the *cspA* gene sequence would determine whether this ~9kDa protein band is in fact a CspA homologue.

The putative CspA homologue appears to have a different expression pattern to that reported for *E.coli* CspA. Its expression remained high throughout the cold shock experiment, indicating that it a cold acclimation protein, rather than a cold shock protein. However, similar observations have been reported in other bacteria. In *B.subtilis*, changes in protein expression were noted within the first 30min of cold shock. However, CspB (CspA homologue) was not produced until the second 30min of the cold shock and continued to be synthesized at a high level throughout the growth at low temperature (Graumann et al., 1996). These findings indicate that, *B.subtilis* CspB is not transiently induced and is therefore also a Cap. Moreover, a CspA homologue was identified in *A.globiformis*, which was still overexpressed during prolonged growth at low temperature (Berger et al., 1996).

The putative *M.vaccae* CspA protein was detectable at 32°C, which contrasts the CspA expression in *E.coli* and *M.smegmatis*, in which CspA expression was undetectable at pre-shift temperatures. This may be a result of several proteins being contained within the same band on the 1D SDS-PAGE gel, as was found to be the case for *M.smegmatis* CipMa (Shires and Steyn, 2001). Or it may indicate that *M.vaccae* CspA has an important role during normal growth, as is the case for the homologous CspB in *B.subtilis* (Graumann et al., 1997), and for *E.coli* CspC, one of the family of nine CspA homologues in *E.coli* (Yamanaka et al., 1994; Yamanaka et al., 1998). Further analysis by 2D SDS-PAGE should reveal if this is the case.
Downregulation of *M. vaccae* proteins on cold shock

The cold shock response in *E. coli* is characterised by the increased synthesis of the cold shock proteins, the continued synthesis of the proteins involved in transcription and translation, and the downregulation of the heat shock proteins (Jones et al., 1987; Jones and Inouye, 1994). Downregulation of heat shock proteins has also been reported in *B. subtilis* (Graumann et al., 1996) and *P. fragi* (Berger et al., 1996). 1D SDS-PAGE analysis of proteins from *M. vaccae* cold shocked to 6°C revealed that the majority of proteins continued to be synthesized. Along with the increased expression of a number of Csps and Caps, the downregulation of 2 proteins was observed by 1D SDS-PAGE (Figure 5.4). Calculation of the molecular weights revealed proteins of 33.4 to 34.3kDa, and 36.7 to 38.1kDa. Moreover qualitative analysis indicated that a ~90kDa protein was downregulated on cold shock, indicating that it could be a heat shock protein. However, quantitative analysis revealed that the protein was not downregulated by 2-fold, the benchmark for changes in protein and gene expression. The downregulation of heat shock proteins and general stress proteins has been reported in *B. subtilis* and *E. coli* (Graumann et al., 1996). The ~34 and ~37kDa proteins, may be minor heat shock proteins of *M. vaccae*. Moreover, the downregulation of 7 proteins was noted on the 8h cold shock 2D SDS-PAGE autoradiograph (Figure 5.6). These findings indicate that *M. vaccae* also downregulates the synthesis of a number of proteins, including potentially some heat shock proteins, on cold shock. The heat shock and cold shock response have been reported to be antagonistic in *E. coli* and *B. subtilis*, with the expression of Hsps being repressed during cold shock and vice versa. The same situation may be true for *M. vaccae*.

Expression patterns of *M. vaccae* cold induced proteins

The induction of Csps, and Caps, and the presence of a putative CspA homologue indicates that *M. vaccae* has an analogous cold shock response to those extensively studied *E. coli* and *B. subtilis* (Graumann et al., 1996; Jones et al., 1987). Furthermore, comparison of the cold shock response reported for
\textit{M. smegmatis}, indicates that \textit{M. vaccae} has a comparable cold shock response. They potentially share the same major cold shock protein, CspA, (Shires et al., 2002), along with \textit{E. coli} and \textit{B. subtilis}, and also share a number of other proteins of similar size (Table 5.1). However, there are noticeable differences (Shires and Steyn, 2001). \textit{M. vaccae} does not appear to have a homologue to \textit{M. smegmatis} CipMa, a 27kDa protein essential for the acclimatisation of \textit{M. smegmatis} to the cold (Shires et al., 2002). A protein of comparable size was not observed to be upregulated on cold shock. Although this may be due to different analysis systems, the cold-induced proteins of \textit{M. vaccae} would need further characterisation and identification before the presence or absence of this protein could be confirmed.

Interestingly, the strength of the response to cold shock appears to differ between the two species. An increase in expression of >7-fold was noted for the 4 cold shock proteins originally identified in \textit{M. smegmatis}. Of the 5 Cips identified in \textit{M. vaccae}, the major cold shock protein was only induced by 4-fold, and the other proteins between 2 to 3 fold. Although 2D analysis indicated that larger increases in protein expression occurred on cold shock (data not shown). Again, the difference may be due to the different 1D SDS-PAGE systems used. It could also be a result of the differences in culture conditions. The stringency of the culture conditions may have made it harder for \textit{M. vaccae} to mount a stronger response. The \textit{M. vaccae} Cips need further investigation to determine if a difference in cold shock protein expression exists between \textit{M. vaccae} and \textit{M. smegmatis}.

The control of the cold shock response
The presence of a putative CspA homologue, which is the major cold shock protein in \textit{M. vaccae}, would indicate that the control mechanism for the cold shock response is similar to \textit{E. coli}. However, the effect of cold shock on the protein translational machinery in \textit{M. vaccae} does not appear to be as detrimental as it is for \textit{E. coli}. \textit{M. vaccae} is still capable of synthesizing the majority of its proteins, though at a significantly lower level, whereas \textit{E. coli} is only able to synthesise around 28 proteins during the first 4h of cold shock.
(Jones et al., 1987). These proteins are the Csps, and are involved with the transcriptional and translational machinery (Jones et al., 1987; Jones et al., 1992a). Many are ribosomal associated proteins or ribosome-binding proteins are believed to allow a block in the initiation of translation to be overcome, restoring the translational capacity of the cell (Jones and Inouye, 1994; Panoff et al., 1997). The Csps are overexpressed until the translational capacity has been restored, the synthesis of the majority of proteins is then resumed, and the cold shock proteins are downregulated.

Berger and co-workers (1996) suggested that cold shock has a deleterious effect on the control mechanism that regulates the expression of CspA. They suggested that the overexpression of CspA blocks the synthesis of other proteins. Moreover, that the mesophilic cold shock response was the result of a deregulated adaptive mechanism that allowed psychrotrophs to acclimatise to the cold. This may be the case for E.coli, however it appears not to be the case for the mycobacteria, as the continued expression of proteins has been observed in M.vaccae and also in M.smeegmatis (Shires and Steyn, 2001). Furthermore, the apparent inability of the psychrotrophic bacteria Listeria monocytogenes and L.innocua (Bayles et al., 1996) to synthesize the majority of their proteins immediately after cold shock, would suggest that not all psychrotrophs are so well adapted.

It is possible that two different cold shock response mechanisms exist, in terms of the changes in protein expression. One set of bacteria, including E.coli, B.subtilis, L.monocytogenes and L.innocua, significantly reduce the number of proteins they synthesize at the beginning of cold shock. The bacteria divert their translational machinery to produce a specific set of proteins, which includes the cold shock proteins and ribosomal proteins, later the synthesis of the rest of the bacteria's protein complement resumes (Graumann et al., 1996; Jones et al., 1987). In the second set of bacteria, including A.globiformis (Berger et al., 1996), Pseudomonas fragi (Hebraud et al., 1994), M.smeegmatis (Shires and Steyn, 2001), and M.vaccae, the rate of translation is significantly reduced, however the number of proteins produced during cold shock is...
comparable to those produced under pre-shift conditions. The type of cold shock response elicited does not appear to be linked to the presence of a growth lag phase on cold shock. A 37 to 10°C cold shock results in a 4h growth arrest for *E.coli* (Jones et al., 1987), whereas a 37 to 10°C cold shock results in the increased generation time of *B.subtilis* with no apparent growth lag (Graumann et al., 1996). Yet both organisms significantly reduce the number of proteins they produce on cold shock.

The initiation of translation may be more sensitive to cold shock in *E.coli* and the *Listeria* species, than are the mycobacteria and the majority of the psychrotrophs and psychrophiles. Although the protein translational machinery is severely affected in all of the bacteria in which cold shock experiments have been reported. The majority of these bacteria apparently do not need to divert the translational machinery to produce a limited number of proteins. Their CspS are overexpressed above the basal level of protein production. In *E.coli* cold shock is believed to cause a block in the initiation of translation. The synthesis of the cold shock proteins allows this block to be overcome. Whether cold shock results in a block in the initiation of translation in other bacteria needs to be determined. The continued synthesis of the total protein complement in many bacteria would suggest that a ‘block’ does not occur, but that the initiation of translation is severely affected by cold shock, significantly reducing the rate of translation. The cold shock proteins may help to increase the efficiency of this translation initiation step.

Homologues of the major cold shock protein of *E.coli* CspA have been found in over 50 bacteria, and appear to be conserved across the bacterial kingdom. These homologous proteins are significantly overexpressed in most of the cold shock responses reported, indicating that the CspA-like proteins have an important role in the cold shock response. Although, none of the CspA homologues of other bacteria are overexpressed by as many fold, >200 fold, as in *E.coli* (Jones et al., 1987), which indicates that they may not have such an important role in cold shock. This immense overexpression, may indicate the extent of the deleterious effect cold shock has on *E.coli* compared to other
organisms, but it may also be a result of the family of the nine homologous proteins in *E. coli*, which appear to have their own essential roles. Other bacteria contain between one and three copies of CspA, and these may perform many if not all of the functions of the nine proteins of the CspA family in *E. coli*. This may explain why they are not upregulated by such a large degree on cold shock. *E. coli* CspA is undetectable at 32°C, whereas CspA homologues have been detected at pre-shift temperatures in other bacteria, including *B. subtilis* (Graumann et al., 1996), *P. fragi* (Hebraud et al., 1994), *Enterococcus faecalis* (Panoff et al., 1997) and *A. globiformis* (Berger et al., 1996).

The conservation of other Csps has yet to be determined, as few experimenters have characterised and identified all of the Csps expressed during the cold shock response. Further investigation of the Csps of *M. vaccae* will allow the conservation of Csps other than CspA to be studied, and to further characterise the differences in the response between *E. coli* and mycobacteria. The results of this study reveal that *M. vaccae* is able to mount a cold shock response analogous to the response of *E. coli*, *B. subtilis*, and comparable to the response of *M. smegmatis*. Furthermore, *M. vaccae* has a major cold shock protein that is a putative CspA homologue, which appears to play an important role in its cold shock response.
Chapter 6

6.0 Detection of Cold shock Protein A (cspA) gene by Polymerase Chain Reaction

6.1 Introduction

The cold shock responses of the prokaryotes *E.coli* and *B.subtilis* involve the induction of homologous proteins CspA and CspB, respectively. These homologous proteins have a crucial role in the cold shock response of these organisms. CspA is the major cold shock protein of *E.coli*, when cold shocked from 37 to 10°C, this protein reaches 13% of the cells total protein synthesis 1h after cold shock (Goldstein et al., 1990). It is believed to be an RNA chaperone (Jiang et al., 1997), and is thought to be involved in the transcription of at least two other cold shock genes, *gyrA* (Jones et al., 1992b) and *hns* (La Teana et al., 1991). *E.coli* contains a family of 9 cspA homologues, genes cspA to cspI, of which cspB, cspG and cspI are also cold-shock inducible (Bae et al., 1999; Jones et al., 1987; Lee et al., 1994). Furthermore, *B.subtilis* contains a family of 3 genes, cspB, cspC and cspD, homologous to *E.coli* cspA, and at least one of which is essential for *B.subtilis* viability (Graumann et al., 1997). In addition, over 90 CspA-like proteins have been identified across the bacterial kingdom, including in *M.tb*, *M.leprae* and recently in *M.smegmatis*.

PCR provides an alternative method to determine whether *M.vaccae* contains a homologue to the major cold shock protein of *E.coli* and *B.subtilis*. In addition it would allow the effect of cold shock to be studied at the transcriptional and translational level, as well as studying the effect on the proteome.

6.2 Materials and Methods

6.2.1 DNA samples

DNA was extracted from liquid cultures of two strains of *M.vaccae* Gm27 and Job5. Furthermore, DNA was obtained from *M.smegmatis* (liquid culture), *M.tb* HRV37 (slope culture kindly extracted by G.McIntyre), and *M.leprae*
(generously donated by H. Donoghue) for use as positive controls. The DNA extraction and PCR procedures are described in Chapter 3. A number of primers and techniques were used to try and amplify the \(cspA\) gene in \(M.\) *vaccine* and these are described in the following results. As with all PCRs, where possible a known positive control was amplified to ensure efficacy. Furthermore, a negative control containing all the reagents, with molecular grade water (Sigma) substituted for DNA was included, to ensure there was no contamination. The \(M.\) *vaccine* DNA was added to the PCR reaction neat, without any dilution, or with a \(10^{-1}\) dilution. \(M.\) *tb* DNA was always used as a positive control, and where necessary \(M.\) *lepra* and \(M.\) *smegmatis* DNA were used, and this is noted in the text.

Each PCR was carried out on single samples, however any PCR that generated a positive result was repeated at least once, to confirm.

PCR products that required sequencing were purified as described in Chapter 3. Sequencing was carried out by MWGBiotech, using their “comfort read” service.

Any sequence obtained was compared with those in the GenBank and Entrez Genome databases of the National Centre for Biotechnology information.

6.3 Results

6.3.1 Initial PCR’s

The cold shock gene was primarily sought using primers designed from the published \(cspA\) (gi15610784) gene in the \(M.\) *tuberculosis* (\(M.\) *tb*) H37Rv genome. A region upstream and downstream of the gene was chosen (Figure 6.1), labelled \(cspa1\) (forward primer) and \(cspa\) 3 (reverse primer), and the primers synthesised (MWGBiotech). Table 6.1 contains the primer sequences and PCR programme used.
Figure 6.1: Location of cspA PCR primers.

Pairs of forward and reverse primers are labelled in the same colour. The start codon, ATG, and stop codon, TGA, are underlined in red and green, respectively.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Primer Sequence 5'-3'</th>
<th>Product size (bp)</th>
<th>PCR Programme</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3.1 Initial PCR</td>
<td>cspa1 : GAG GTA TGG CCG CCG CAG C</td>
<td>297</td>
<td>Basic (1) PCR Tm 60°C</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>cspa3 : GCC ATG TGG CCG GCA GAC G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3.2 PCR integrity</td>
<td>TB 11 : ACC AAC GATG GTG TGT CCA T</td>
<td>439</td>
<td>Basic (1) PCR Tm 60°C</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>TB 12 : CTT GTC GAA CCG CAT ACC CT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3.3 UMCSP PCR</td>
<td>CSPU5 : CCG AAT TCG GTA (ACT)AG TAA AAT GGT T(CT)A AC(GT) C</td>
<td>~200</td>
<td>Basic (1) PCR Tm 50°C</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>CSPU3 : CCC GGA TCC GGT TAC GTT A(GC)C (AT)GC T(CT)(GC) (ACT)GG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3.4 Primers from conserved</td>
<td>cspa5 : GAT CTC IAA CTC GAC CTT C</td>
<td>143</td>
<td>Basic (1) PCR Tm 53°C</td>
<td>30</td>
</tr>
<tr>
<td>regions</td>
<td>cspa6 : TGT GAA GTG GTT CAA CGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3.5 Primers designed from the 5' and 3' ends of the M.tb cspA gene</td>
<td>cspaF1 : GAC GTT GCG G(C)C GCG GTT C +</td>
<td>486, 536, 398</td>
<td>Touchdown PCR (Tm 70.0°C)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>cspaR1 : GCT G(AG)C TCA C(AC)(CT)C (AC)C CGA CAG TAA GCC,</td>
<td></td>
<td>then, basic (2) PCR Tm 65°C</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>cspaR2 : CCG CGA CCG CGG GAG GTA CTG,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cspaR3 : GTG GC(CT) AG(GC) GG(GC) CCC TT(AG) GGG C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cspaF2 : CCT GCG AGG TTC T(CT)G TGC C G +</td>
<td>413, 525, 387</td>
<td>Touchdown PCR (Tm 69.9°C)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>cspaR1, cspar2, cspar3</td>
<td></td>
<td>then, basic (2) PCR Tm 59.9°C</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>cspaF3 : CGG GTG AAG TTC CGG CGA CTC +</td>
<td>353, 465, 327</td>
<td>Touchdown PCR (Tm 70.7°C)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>cspaR1, cspar2, cspar3</td>
<td></td>
<td>then, basic (2) PCR Tm 65.7°C</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>CSPAF1, CSPAF3 + CSPA5</td>
<td>293, 235</td>
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</tr>
<tr>
<td></td>
<td>CSPAR6 + CSPAR2</td>
<td>380</td>
<td>Basic (2) PCR Tm 55°C</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 6.1: Some of the cspA primer sequences and PCR programmes used in the discovery of M. vaccae cspA.
Forward and reverse primers are listed first and second, respectively. Where several primer combinations were carried used the forward primer is listed followed by a + and then the reverse primers it was combined with. Degenerate bases are contained within brackets. I represents the base inosine.
The initial PCR failed to generate products with any of the DNA species. The lack of product in *M. tb* indicated that there was a problem with the PCR itself as a product would be expected since the primers were designed from the *M. tb* genome. However the primers could have formed secondary structures and this may be the reason for the lack of PCR product with *M. tb* DNA.

### 6.3.2 Checking the integrity of the PCR reaction

A PCR product could not be generated with the cspa1 and cspa3 primers, the lack of amplification of *M. tb* DNA suggested that there was either a problem with the primers, possibly secondary structure formation, or a problem with the PCR reaction. A PCR was carried out with primers TB11 and 12 (Telenti et al., 1993) which are based on the 65kD heat shock protein gene (Table 6.1). The primers were used to test the PCR system. Products were generated with all of the DNA species indicating that the previous PCR failure was due to problems with the primers (data not shown).

### 6.3.3 PCR using the universal major cold-shock protein primer

Francis and Stewart (1997) designed primers from the major cold shock protein (CspA) genes found in numerous bacteria. They based the primers on conserved regions within these genes, and were able to amplify 200bp sequences from more than 30 diverse Gram positive and negative bacteria. The published primers were used to search for the *cspA* gene in the two strains of *M. vaccae* (Table 6.1).

The Francis and Stewart (1997) method was not followed exactly. They lysed the bacteria by selecting a single colony and adding it to the PCR mix and heating at 95°C for 5min. DNA extraction by boiling is not an efficient method for mycobacteria, as they are difficult to lyse, due to the mycolic acids of the cell wall, and it was unlikely that sufficient DNA would be released from the cells. DNA was extracted as described in the chapter 4, section 4.6. The DNA species used were *M. tb* and *M. vaccae* both Gm27 and Job5 strains. Unfortunately there was not a positive control for this PCR, Francis and Stewart
had not carried out their PCR with mycobacteria, and a non-
mycobacterial DNA was not available.

The PCR failed to generate products with any of the DNA species employed. It is unclear why the PCR failed, analysis of the *E.coli*, *B.subtilis* and *M.tb* cspA sequences and the CSPU5 and CSPU3 primers, indicate that the degenerate primer sequence matched sufficiently for the primers to bind to *M.tb* DNA, and potentially the other mycobacterial genes (Figure 6.2). The CSPU5 and CSPU3 had an EcoR1 and a BamH1 restriction digest motif added on to the 5' end of the primers, respectively. It is possible that these motifs, which did not correspond to the cspA sequence, affected the primer binding efficiency, although mis-matches at the 3' end of the primers tend to have a more significant effect on primer binding.

### 6.3.4 PCR using primer designed from conserved regions

A BLAST search was carried out with the *M.tb* cspA gene to determine any regions of homology within the gene. Two distinct regions were identified. The first had homology with many bacterial species including *Micrococcus luteus* and *Arthrobacter globiformis* and from this region the forward primer, cspa6, was designed. The region encodes a number of important amino acids that have been shown to be involved in the structure and function of CspA (Schindelin et al., 1994; Schnuchel et al., 1993; Schroder et al., 1995). The second region's homology was restricted to *M.tb* and *M.leprae*, from which the reverse primer, cspa5, was designed (Table 6.1, Figure 6.1). An inosine was added to the cspa5 primer to create degeneracy, as there was a mis-match in one of the bases between the *M.tb* and *M.leprae* sequences in the region chosen for the primer. Inosine is a purine residue, which naturally occurs in tRNAs, that can form base pairs with cytidine, thymidine and adenosine.
Figure 6.2 – Location of the Major cold shock protein universal primers (Francis and Stewart 1997) in the E.coli, B.subtilis, and M.tb major cold shock protein gene sequences.

The location of the primer sequences within the major csps is highlighted. Degenerate bases are contained within brackets, and the added restriction enzyme sequences are underlined. An upper case letter indicates that the base is contained within all the sequences, and a lower case indicates that it is present in 2 of the sequences.
A PCR was set up with the new primers, cspa6 and 5 with \textit{M.tb} DNA (10^{-2} dilution) as the positive control (Table 6.1).

A PCR product was generated with all 3 DNA species (Figure 6.3). The expected product size was 143bp, a product halfway between 100 and 200bp was noted, indicating that the correct product was generated. The bands reveal that a gene homologous to \textit{M.tb} \textit{cspA} exists in \textit{M.vaccae} Gm27 and Job5. In addition it supports the idea that Gm27 and Job5 will have a cold shock response. The \textit{M.vaccae} PCR products were purified and sent for sequencing. The sequencing was successful and a 143bp sequence was obtained for \textit{M.vaccae} Gm27 and Job5 (Figure 6.4).

6.3.5 PCR to obtain the 5' and 3' ends of the cspA gene

The \textit{M.smegmatis} cspA gene sequence (AF281675) had been recently published in the GenBank database (Benson et al., 2002). Using the \textit{M.tb}, \textit{M.leprae}, and \textit{M.smegmatis} gene sequences, several upstream and downstream primers were designed. The \textit{M.smegmatis} published sequence consisted of the coding region only, whereas the \textit{M.tb} and \textit{M.leprae} genome projects provided the flanking sequences. The upstream sequences were conserved, although not identical, between \textit{M.tb} and \textit{M.leprae}, and four upstream primers cspaF1, F2, and F3 were designed from this region (Table 6.1). Three downstream primers were designed, cspaR1, R2, and R3, from the 3' end of the gene and the 3' flanking region (Table 6.1). The 3' end of the gene is not well conserved between the species, both in the gene and in the flanking sequences and this is reflected in the number of degeneracies in the reverse primers. Figure 6.1 reveals the location of these primers within the \textit{M.tb}, \textit{M.leprae} and \textit{M.smegmatis} gene sequences. All of the forward primers were combined with each reverse primer so that every primer combination was tested by PCR (Table 6.1).

Unfortunately PCR products were only generated with the positive control, \textit{M.tb} (10^{-2} dilution) DNA, from the primer combinations cspaF1 and F3 with cspaR2.
Figure 6.3: Conserved primer PCR products (section 6.3.4)

1.5% Agarose/TAE gel stained with SYBR Green. From left to right; lanes 1 and 6, 100bp DNA ladder: lane 2, negative control; lane 3, positive control *M. tb* DNA (10<sup>2</sup> dilution); lane 4, *M. vaccae* Gm27 DNA (neat); lane 5, *M. vaccae* Job5 DNA (neat).
Figure 6.4: Partial cspA sequence from *M. vaccae* Gm27 and Job5, obtained from multiple PCRs.

Section 1, obtained with primers cspa6 and cspa5; section 2, obtained with primers csoaF1 and cspa5; section 3, obtained with primers cspaF1 and deg1. Identical nucleotides between the sequences of the two species are depicted with a dot, any differences are marked with the alternative nucleotides. Gaps in the sequence are represented with a dash. The start codon, ATG, is underlined.
There were no PCR products generated from the degenerate reverse primers that were created from the homologous regions of *M.tb* and *M.leprae* cspA sequences. In addition, the primers that did generate PCR products appeared to be *M.tb* specific.

### 6.3.5.1 cspaF1 and F3 with cspa5

The primers (cspaF1-4 and cspaR1-3) designed from upstream and downstream regions of the *M.tb* and *M.leprae* cspA sequences, had not been used in combination with the primers cspa5 and 6, which had successfully generated a PCR product with *M.vaccae* Gm27 and Job5 DNA. The forward primers cspaF1 and cspaF3 that generated PCR products with *M.tb* DNA these were combined, in a PCR, with the reverse primer cspa5 (Table 6.1).

PCR products were generated with the *M.tb* positive control DNA from both sets of primers. The cspaF1 and cspa5 PCR reaction generated products with *M.tb*, Gm27 and Job5 DNA, with no non-specific binding (Figure 6.5). The cspaF3 and cspa5 PCR generated a product with *M.vaccae* Job5 and *M.tb* DNA only. The cspaF1 and cspa5 PCR was repeated, the product purified and sent to MWG Biotech for sequencing.

The sequencing was successful (Figure 6.4) and the first 15bp of the *M.vaccae* cspA gene from strains Gm27 and Job5 were obtained, along with ~140bp of upstream sequence.

### 6.3.5.2 cspaR2 and cspa6

The cspaR2 primer also generated a PCR product with *M.tb* DNA, and this was combined with the forward primer cspa6 in a PCR (Table 6.1) to try to obtain products from *M.vaccae* Gm27 and Job5 that contained the 3'end of cspA gene. Unfortunately a PCR product was only generated with the *M.tb* DNA. It is likely that the cspaR2 primer is too specific for the *M.tb* cspA flanking sequence.
Figure 6.5: PCR products generated with primers cspaF1 and cspa5

The products are separated on a 2% Agarose/TAE gel stained with SYBR green. From left to right; lane 1, 100bp DNA marker, lane 2, negative control; lane 3, M.tb DNA (10⁻² dilution); lane 4, M. vaccae Gm27 DNA (neat); lane 5, M. vaccae Job5 DNA (neat).
6.3.6 PCR to find the 3' end of the gene using primers designed from the \textit{M.smegmatis cspA} gene sequence

Sequence comparison between the mycobacterial species revealed that the \textit{M.smegmatis cspA} gene was most similar to the partial \textit{M.vaccae cspA} gene sequences already obtained. A primer (cspa9) was designed from the sequence at the 3' end of the gene (Table 6.2, Figure 6.1). The cspa9 primer was combined with cspa6 and cspaF1 forward primers in a PCR (Table 6.2). \textit{M.smegmatis} DNA (10^{-1} dilution) was used as an additional positive control along with \textit{M.tb} (10^{-1} dilution) DNA.

Both primer combinations generated PCR products with \textit{M.smegmatis} DNA, of the expected sizes. Unfortunately there were no products generated with the other mycobacterial species, (\textit{M.tb} and \textit{M.vaccae}), indicating that the primer was too specific for the \textit{M.smegmatis cspA} gene.

6.3.7 PCR to discover the 3' end of the gene using a downstream gene sequence

The genes downstream from the \textit{cspA} gene in \textit{M.tb} and \textit{M.leprae} were examined. By designing a primer against a downstream gene, and using a primer within the \textit{cspA} gene it was hoped that the 3' end of the gene could be obtained. Figure 6.6 illustrates the genes surrounding the \textit{cspA} gene in the \textit{M.tb} and \textit{M.leprae} genomes. A genome of unknown function and the Topoisomerase A gene, are located downstream from the 3' end of the \textit{cspA} gene. The genome sequences from \textit{M.tb} and \textit{M.leprae} were examined and a homologous sequence was identified in the gene of unknown function, from which a primer was designed (downstream 1) (Figure 6.6, Table 6.2). The cspa5 reverse primer sequence (Table 6.1) was reversed and complemented, to provide a forward primer (cspa5F) (Table 6.2) at the end of the known \textit{cspA} sequence, and reduce the size of the PCR product size that would be generated with the downstream primer.
Figure 6.6 - Location of the cspaSF and downstream 1 primers.

The cspA gene and surrounding genes in *M. tb* and *M. leprae* are illustrated. The gene sequences for primer cspa5F and downstream 1 are in bold, and bold and underlined, respectively. The arrows indicate whether the direction of the gene, whether it is on the positive or negative strand.

**Genes**
- ML0197 - ATP-dependent DNA/RNA helicase/pseudogene
- ML0198 - cspA
- ML0199 - Similar to *M. tb* Rv3647c
- ML0200 - putative DNA topoisomerase I/A

**M. leprae genome**

```
5'-AACCAGAAGGTCGAGTTTGAGATCGGCCACAGCCC-///-TAGCGAGGCTGGTTTGGTACCCGAGATTTCT-3'
3'-AACCAGAAGGTCGAGTTCGAGATCGGCCACAGCCC-///-CCAGGAGGCTGGTTTGGTACCCGAGGTCGCC-5'
```

**M. tb genome**

```
topA
Rv3646c
Rv3647c cspA
Rv3648c
Rv3649
```
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<thead>
<tr>
<th>Experiment</th>
<th>Primer Sequence 5’-3’</th>
<th>PCR programme</th>
<th>No. of cycles</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>6.3.6 - <em>M. smegmatis</em> cspA primer</td>
<td>cspa6, cspaF1 + cspa9: CAG ATG GTC CGG ACA CCC GTG G, cspaF2: GAA GGT CGA GTT IGA GAT C</td>
<td>Basic (2) PCR, Tm55°C and 64°C respectively</td>
<td>30</td>
<td>178, 331*</td>
</tr>
<tr>
<td>6.3.7 - Downstream primers</td>
<td>cspa5F: GAA GGT CGA GTT IGA GAT C</td>
<td>Basic (2) PCR, Tm57°C</td>
<td>30</td>
<td>375</td>
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<tr>
<td>6.3.8 - Inverse PCR</td>
<td>cspa5F: GAA GGT CGA GTT IGA GAT C</td>
<td>Basic (2) PCR, Tm57°C</td>
<td>30</td>
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<tr>
<td></td>
<td>Downstream 1: CTC GGG TAC CAA ACC AGC CTC</td>
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<td>25</td>
<td>-</td>
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<td>Outer reaction: Inverse 1: GCA GTC GCC TCG GCA GGA G</td>
<td>Basic (2) PCR, Tm57°C</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Inverse 2: CCG AAG GCG TGG GAC CTT C</td>
<td>Basic (2) PCR, Tm57°C</td>
<td>30</td>
<td>-</td>
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<tr>
<td></td>
<td>Nested reaction: Inverse nested 1: CAG GAG CCC CCG CTA CTT C</td>
<td>Basic (2) PCR, Tm57°C</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
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<td>Inverse nested 2: CCT TCC AGC TCA AGC TCT AG</td>
<td>Basic (2) PCR, Tm57°C</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>6.3.10 - Degenerate PCR</td>
<td>cspaF1 + deg1: CTT (ATGC) GG GCT (GC) TG GCC GAT CTC, deg2: CTG (GC) GG (GC) CC CT (ATGC) GG GC, deg3: G(AT)(GC) CG (AG) A C (AT) C (ATGC) G TGG CCT G, deg4: TCA GA (ATGC) (GC) G (AT) (GC) GC (AG) AC (AT) CC (ATGC) G</td>
<td>Basic (2) PCR, Tm64°C</td>
<td>30</td>
<td>306, 315, 332, 339*</td>
</tr>
</tbody>
</table>

Table 6.2: Additional primer sequences and PCR programmes used in the discovery of *M. vaccae cspA*.

Bracketed bases within the primer sequence highlight positions of degeneracy, with the alternative bases enclosed. Forward and reverse primers are listed first and second respectively. Where multiple combinations of primers were used, multiple forward or reverse primers are separated by a comma, the primer they are combined with is indicated by a +.

*Multiple product sizes are listed in the order the primers are listed.*
The downstream primer was combined with the cspa5F primer in a PCR (Table 6.1), with *M.tb* DNA (10^{-2} dilution) and *M.smegmatis* DNA (10^{-1} dilution) positive controls.

A PCR product was only generated with *M.tb* DNA. The downstream 1 primer was combined with the forward primer cspa6 to try to obtain products with the other species. However, again a PCR product was only generated with *M.tb* DNA.

To confirm whether the downstream 1 primer was too specific for *M.tb* DNA, *M.leprae* DNA was obtained from Dr H.Donoghue. The cspa5F and downstream 1 PCR was repeated with the *M.leprae* DNA as an additional positive control. The concentration of the *M.tb* and *M.leprae* DNA needed optimisation and after several PCR’s, products of the expected size were generated with *M.tb* and *M.leprae* DNA. Figure 6.7 reveals bands of ca 400bp in the *M.tb* and *M.leprae* lanes, the size of the products from the *M.tb* and *M.leprae* DNA differ slightly due to differences in the length of the intergenic sequences. Primer dimers are evident in several of the lanes. A very weak band of around 800bp was present in the JobS lane, which could have been a dimer of the expected PCR product (Figure 6.7).

The PCR was repeated to determine whether the band in the Job5 lane was reproducible. However, repetition of the PCR and close scrutiny of the gel revealed the 800bp band was part of a primer dimer ladder, and not a genuine PCR product.

### 6.3.8 Inverse PCR to try to obtain the 3'end of the cspA gene

The 3' end of the cspA gene was not obtained using basic PCR with primers derived from other mycobacterial cspA genes. The method of Inverse PCR was employed. Primers were derived form the *M.vaccae* Gm27 sequence already obtained; Inverse 1, Inverse 2, Inverse nested 1, Inverse nested 2 (Table 6.2).
Figure 6.7: Downstream PCR products (section 6.3.7)

Products were separated on a 2% Agarose/TAE gel stained with SYBR green. From left to right: lane 1, 100bp DNA marker; lane 2, negative control; lane 3, positive control *M.tb* DNA (10⁶ dilution); lane 4, *M.leprae* DNA (Neat); lane 5, *M.smegmatis* DNA (10¹ dilution); lane 6, *M.vaccae* Gm27 DNA (Neat); lane 7, *M.vaccae* Job5 DNA (Neat). The location of the ~800bp band generated with Job5 DNA is highlighted with the arrow.
Two sets of primers were designed, so that a nested PCR could be carried out to increase the sensitivity and specificity if the PCR.

A restriction digest map was generated of the known Gm27 cspA sequence (Appendix 2). Restriction enzymes (BamH1, Pst1, Aat1) that did not cut within this sequence were selected. An Inverse PCR was carried out as described in chapter 4, section 4.10. The inverse PCR failed to generate any products with the original or nested PCR. It was not possible to have a positive control for any of the reaction steps. The technique was abandoned for other techniques as the technique required considerable optimisation and it was felt that other methods would be more productive with the limited time left.

6.3.9 Rapid Amplification of cDNA ends

6.3.9.1 Initial RACE reaction
To try to discover the 3' end of the cspA gene 3'RACE was employed. Until recently it was believed that prokaryotic mRNA, unlike eukaryotic mRNA, did not have polyA tails. However researchers have suggested that a varying proportion of prokaryotic mRNA have polyA tails (Nilima Sarkar, 1997). The Qiagen Omniscript Reverse Transcriptase kit was used to generate cDNA from mRNA extracted from M.vaccae Gm27 bacteria growing at 32°C in exponential phase. The cDNA was created using an oligo(dT)$_{20}$mer primer (10pmol/ul) rather than random hexamers (Chapter 4, section 4.8.2.). To determine whether the mRNA of the desired gene had been converted into cDNA a PCR was carried out with primers cspa5 and 6 (Table 6.1, section 6.3.4). A weak band was obtained with the cDNA indicating that the cDNA contained the gene sequence of interest (Figure 6.8).

A RACE PCR was carried out based on the method by Frohman et al (1988) (Chapter 4, section 4.12, RACE reaction 1, Table 6.3). Forward primers cspa6 and cspa5F were combined with an oligo(dT)$_{20}$mer reverse primer. A smear of PCR product was generated with both specific forward primers (cspa6 and cspa5F) and the non-specific oligo(dT) (Figure 6.9).
A PCR was carried out with primers cspa5 and 6 to check the cDNA integrity and that the cDNA contained the transcript of interest. The expected product size was 143bp. The PCR products were separated on a 2% Agarose/TAE gel stained with SYBR green. From left to right, lane 1, 100bp DNA marker; lane 2, PCR negative control; lane 3, reverse transcription negative control; lane 4, PCR with *M. vaccae* Gm27 cDNA. A product of the expected size is evident in lane 4, indicating that the cDNA contained the transcript of interest.

**Figure 6.9: 2% Agarose/TAE gel of initial RACE reaction (section 6.3.9.1)**

Gel stained with SYBR Green. From left to right; lane 1, 100bp DNA marker; lanes 2 and 3 RACE with cspa6 specific primer; lane 2, negative control; lane 3, RACE PCR *M. vaccae* Gm27 cDNA; lanes 4 and 5 RACE PCR with cspa5F specific primer; lane 4, negative control; lane 5, RACE PCR *M. vaccae* Gm27 cDNA.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Primer sequence 5'-3'</th>
<th>PCR programme</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
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<td>6.3.9.1- Initial RACE</td>
<td>oligo(dT)$_{20\text{mer}}$:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT</td>
<td>cDNA generation$^1$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>cspa5f, cspa6 + oligo(dT)$_{20\text{mer}}$</td>
<td>RACE reaction $^2$</td>
<td>30</td>
</tr>
<tr>
<td>6.3.9.2 - Nested RACE</td>
<td>Outer reaction - cspa6 + oligo(dT)$_{20\text{mer}}$</td>
<td>RACE reaction $^2$</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Inner reaction - cspa5f + oligo(dT)$_{20\text{mer}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3.9.3 - RACE optimization</td>
<td>Outer reaction - cspa6 + oligo(dT)$_{20\text{mer}}$</td>
<td>Hotstart PCR - 95°C x 15min, then RACE reaction $^2$</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Inner reaction - cspa5f + oligo(dT)$_{20\text{mer}}$</td>
<td>Basic 2 PCR T$_{m}$ 45°C</td>
<td>30</td>
</tr>
<tr>
<td>6.3.9.5 - Modified RACE</td>
<td>Adapter oligo(dT):GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTTTTTTTTT</td>
<td>cDNA generation$^1$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>cspa6 + Adapter:GACTCGAGTCGACATCG</td>
<td>95°C 5min, 53°C 2min, 72°C 40min then Race reaction $^2$</td>
<td>40s</td>
</tr>
</tbody>
</table>

Table 6.3: Primer sequences and PCR procedures used during the RACE experiments

$^1$ The method for cDNA generation is listed in chapter 4, section 4.8.2. The adapter oligo(dT) was used instead of oligo(dT)$_{20\text{mer}}$ to primer the first strand cDNA synthesis.

$^2$ The RACE reactions are detailed in chapter 4, section 4.12.
6.3.9.2 Nested RACE

Ohara et al (1989) suggested that RACE PCR with a single specific primer combined with oligo (dT) would generally create a non-unique product. However carrying out a nested PCR with a second internal specific primer would result in the amplification of a unique sequence. To try to reduce the size range of the products generated, a nested RACE PCR using primer cspa6 and cspa5F, as the outer and inner specific primers, respectively, was carried out (Table 6.3). A negative control was set up for each PCR. The negative control for the outer PCR was used as a nested negative control. Both PCRs generated smears of product. The nested PCR apparently failed to generate a uniform product as a smear and not a single band was produced with the inner primer (Figure 6.10). In fact, the nested product smear was larger than the outer PCRs smear. The negative controls of both PCRs were clear, however the nested negative control also contained a smear of PCR product. The PCRs were repeated several times however the smear in the nested negative control remained despite extreme care being taken to prevent contamination.

6.3.9.3 RACE optimization

To try to decrease the size range of the PCR products generated, the PCR was optimised. Hot start PCR was tried (Table 6.3). By adding Hotstar Taq to the PCR it was hoped that any mis-priming that might have occurred at room temperature would be prevented. The PCR programme was also modified to try to increase the specificity; the denaturing, annealing and extension times were shortened (Table 6.3). In addition nested PCR was carried out with both the optimisations to try to further increase the specificity of the reaction, and reduce the range of products generated.

Unfortunately, the modified PCR programme and HotStar Taq did little to increase the specificity of the RACE PCR. In both PCR's the nested negative control was contaminated (data not shown). In addition the optimisations did not reduce the length of the product smear generated, they just altered the amount of product generated.
Figure 6.10: Nested RACE reaction PCR products (section 6.3.9.2)

2% Agarose/TAE gels stained with SYBR green.

Gel A: Outer RACE PCR products, generated with cspa6 and oligo(dT)$_{20}$mer. From left to right; lane 1, 100bp marker; lane 2, negative control; lane 3, outer RACE PCR, *M. vaccae* Gm27 cDNA.

Gel B: Inner RACE PCR products generated with primers csap5F and oligo(dT)$_{20}$mer. From left to right; lane 1, 100bp marker; lane 2, negative control; lane 3, nested negative control (negative control-outer PCR negative control); lane 4, Inner RACE PCR (template-outer PCR product).
6.3.9.4 Southern blot of the RACE PCR products

The PCR optimisations did little to increase the specificity of the PCR or generate a more uniform PCR product i.e. a single band. To confirm that the desired sequence was contained within the PCR products generated by the RACE PCR, a Southern blot was carried out with the PCR products generated in section 6.3.9.1. The Southern blot was probed with the DIG-labelled partial cspA (M. vaccae Gm27) sequence (Chapter 4, section 4.18). The 143bp product was generated using the primers cspa5 and 6, and the probe was expected to bind to the products generated with the cspa6 primer. The probe contains the sequence for primer cspa5 at one end, and therefore some binding was expected to products generated with the cspa5F primer. Although, only weak binding was expected as the cspa5 sequence was only a small proportion of the probe.

It was hoped that the smear seen in the nested negative control lane would be shown to be due to non-specific binding of the primers. The autoradiograph in figure 6.11 reveals that the first RACE PCR carried out with primer cspa6 and oligo (dT)\textsubscript{20mer} generated a smear of products which contained the sequence of interest. In addition a weaker smear was seen at the bottom of the nested PCR lane, indicating that there was some non-specific binding in this PCR, but that the sequence of interest was generated. There is a very weak smear in the nested negative control lane and the negative control lane, however they are barely detectable, and could be due to non-specific hybridisation. They indicate however that the PCR product smear seen in the nested negative control was due to non-specific binding.

The initial RACE PCR with the primers cspa6 and oligo(dT) was repeated, products purified and sent to MWG Biotech for sequencing. Unfortunately the sequencing reaction was unsuccessful.
Figure 6.11: Southern blot of nested RACE PCR products (section 6.3.9.2)

A - 2% Agarose/TAE gel of nested PCR reaction. The PCR reactions were carried out with a specific primer combined with oligo(dT)_{20}mer in the RACE reaction. Outer reaction carried out with primers cspa6 and oligo(dT)_{20}mer. Inner nested reaction carried out with cspa5F and oligo(dT)_{20}mer. From left to right, lanes 1 and 7, 100bp DNA marker; lane 2, outer PCR negative control; lane 3, outer RACE PCR; lane 4, inner PCR negative control; lane 5, nested negative control; lane 6, inner RACE PCR.

B - Southern blot of RACE PCR products. PCR products electrophoresed on a 0.7% Agarose/TAE gel were transferred to Hybond N+ nitrocellulose membrane via capillary blotting. Gel A shows the distribution of the PCR products. The picture shows the fluorograph of the membrane after hybridisation with a DIG-labelled cspA 143bp PCR product (chapter 4, section 4.18). From left to right, lane 1, cspa6 RACE PCR negative control; lane 2, cspa6 RACE PCR; lane 3, cspa5F RACE PCR negative control; lane 4, cspa5F RACE PCR nested negative control; lane 5, cspa5F nested RACE PCR.
6.3.9.5 Modified RACE reaction

Frohman and co-workers (1988) modified the oligo dT primer used to make the cDNA, they added an adapter sequence that contained the recognition sites for the restriction enzymes Xho I, Sal I, and Cla I. This added a unique sequence at the unknown end of the cDNA. A PCR reaction was then carried out using primers directed to the specific sequence of the gene of interest and a primer consisting of the adapter sequence only (Frohman et al., 1998) (Table 6.3). The RACE protocol used here was subsequently modified, an oligo (dT)$_{17}$-adapter primer was used in a reverse transcription reaction to generate cDNA (Table 6.2). A PCR reaction was then carried out based on the method by Frohman et al (1988) (Table 6.2). Primers cspa6 and the adapter primer were used to amplify the 3' end of the cspA gene. An extension step (40min at 72°C) was added to the first round of amplification to encourage cDNA second strand formation.

The PCR generated two distinct bands, one slightly larger than 200bp and one slightly smaller (Figure 6.12). The cspA coding region is expected to be 201bp (the length of the coding region in the other mycobacterial cspA genes) so PCR product bands of around 200bp indicated that the products contained the desired gene sequence. PCR optimisation did not improve the sharpness of the bands or the amount of product generated. The presence of two product bands was unexpected, it was possible that the bands represented the presence and absence of the 3' untranslated region (3'UTR) of the mRNA. Both bands were purified for sequencing, however the sequencing reaction failed to generate any meaningful sequence. It was possible that the bands generated were PCR artefacts, time constraints prevented southern blot analysis. In addition it was possible that the cDNA created was not of sufficient quality. It is not immediately clear why the sequencing reaction did not work.

6.3.10 PCR using degenerate primers

Using the known protein sequence of M.tb, M.leprae and M.smegmatis CspA degenerate primers were designed to obtain the 3' end of the M.vaccae cspA.
Figure 6.12: 2% Agarose/TAE gel of the PCR products of the modified RACE reaction (section 6.3.9.5)

The two PCR products generated with cspa6 and the adapter oligo(dT)$_{17}$mer, around 200bp in size, are highlighted with arrows. From left to right, lane 1, 100bp DNA marker; lane 2, PCR negative control; lane 3, RACE PCR with M. vaccae Gm27 cDNA.
gene. Degeneracies within the primers was determined by the conservation of an amino acid, and by the degeneracy in the codons for individual amino acids. Primers (4) were designed that spanned the distance between the end of the known \textit{M.vaccae cspA} sequence and the end of the gene as determined from the known mycobacterial sequences (Table 6.2, Figure 6.1). Each primer had overlapping sequence with the primer before and after it where appropriate, due to the lack of homology between the 3' end of the mycobacterial \textit{cspA} genes; multiple PCR products containing the same sequence would ensure that the sequence obtained was correct. Each degenerate primer was combined with primer \textit{cspaF1}, a PCR was carried out for each primer combination (Table 6.2). \textit{M.tb} DNA was used as the positive control.

PCR products for \textit{M.vaccae} Gm27 and Job5, were only generated for the \textit{cspaF1} and \textit{deg1} primer. Unfortunately the \textit{cspaF1/deg3} and \textit{cspaF1/deg4} combinations only generated products with \textit{M.tb} DNA, whereas the \textit{cspaF1/deg2} combination failed to generate a product with any of the DNA species used (data not shown). The product obtained with \textit{deg1} was sequenced; the PCR generated an additional 16 bases (Figure 6.4). It is unclear why the remaining primers failed to generate products with the \textit{M.vaccae} DNA. The \textit{deg 2} primer contains at least two regions where there are greater than three Gs or Cs in a row, it is possible that these primers would form secondary structures or bind non-specifically to the genomic DNA.

In addition, it is possible that the primers were not degenerate enough. Many amino acids are encoded by 4 codons, the variation occurring in the last base of the codon. To reduce the degeneracy of the primers, where only 2 codons for an amino acid were evident in the mycobacterial \textit{cspA} sequences, the equivalent degenerate base was added to the primer, rather than adding a base that would bind to any of the codons for that particular amino acid.
6.4 Sequence analysis

6.4.1 Identification of a *M. vaccae* cold shock protein A (*cspA*) gene

A gene for a homologue to the *E.coli* Major Cold shock protein, Cold shock protein A (CspA) was identified in two strains of *M. vaccae*, Gm27 and Job5. The first 171bp of the expected 201bp *cspA* coding region (the length of the coding region of the other mycobacterial *cspA* genes), along with 140bp of the upstream gene sequence, was obtained for the two strains (Figure 6.4). The gene was highly conserved between the two strains, with 4 base differences out of 171bp (none of which resulted in a change in the encoded amino acid), the variability was contained within the multiple codons for a single amino acid. It was not possible to obtain the last 30bp of the *cspA* gene from either strain of *M. vaccae*.

6.4.2 Mycobacterial *cspA*

Homologous genes have been reported in the pathogenic mycobacteria *M. tb* and *M. leprae*, and recently in the non-pathogenic *M. smegmatis* (Benson et al., 2002). Comparison of the mycobacterial *cspA* gene sequences (Figure 6.13), including the 5'upstream gene sequences, revealed a high degree of conservation of the sequence within the gene sequence and the 5' non-coding sequence. A minimum of 84% sequence identity exists between all of the mycobacterial *cspA* coding sequences (Table 6.4), although the sequence conservation decreases towards the 3' end of the gene. The partial *cspA* gene sequences of *M. vaccae* Gm27 and Job5 are most closely related to the *cspA* gene sequence of *M. smegmatis*, although the genes have a 90% and greater sequence identity with *M. tb* and *M. leprae cspA*. Notably, the sequence identity between the *M. smegmatis* and the *M. tb* and *M. leprae cspA* genes is significantly lower than this. However, determination of 3' end of the gene may reveal that the *M. vaccae* genes are less closely related to those of the slow-growing species, as the sequence variation increases towards the 3' end of the gene. It will be interesting to see, if the high homology of *M. vaccae cspA* to the genes of slow growing mycobacterial species is retained when the 3' end of the gene is elucidated.
Figure 6.13: Sequence alignment of the mycobacterial *cspa* gene sequences, including the 5'UTR sequences.

Residues 100% conserved are colour coded with white writing on a black background, residues conserved more than 80% are colour coded with white writing on a dark grey background, and residues conserved more than 60% are colour coded with black writing on a light grey background. A black arrowhead depicts the start of the coding region. The locations of the two predicted promoters are boxed, although only the −35 region of the extended promoter is highlighted. The extended motif is underlined. * indicates the putative transcriptional start site. The site of the potential inversion event is highlighted with ☆.
<table>
<thead>
<tr>
<th></th>
<th><em>M. tb</em> cspA</th>
<th><em>M. leprae</em> cspa</th>
<th><em>M. smegmatis</em> cspa</th>
<th><em>M. vaccae Gm27</em> cspa</th>
<th><em>M. vaccae Job5</em> cspa</th>
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<td>90%</td>
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<td>97%</td>
<td>100%</td>
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Table 6.4: Sequence identity between the cspA genes of *M. tb*, *M. lepra*, *M. smegmatis*, and the partial gene sequences of *M. vaccae Gm27* and Job5
It appears that there may have been an inversion in the \textit{M.vaccae} \textit{cspA} gene sequence. Towards the 3’ end, \textit{M.tb}, \textit{M.leprae} and \textit{M.smegmatis} have the sequence cac, cac, and cag, respectively, which encodes the amino acid histidine (His) and glutamine (Glu), \textit{M.vaccae} has the sequence acc, which encodes the amino acid threonine (Thr) (Figure 6.13).

### 6.4.3 Identification of \textit{cspA} promoter

A promoter region could not be identified in the ~140bp sequence upstream of the coding sequence of the \textit{cspA} genes identified in the two strains of \textit{M.vaccae} studied (Figure 6.13). Alignment with the \textit{M.tb} and \textit{M.leprae} upstream sequences revealed the presence of two putative promoters in the \textit{M.tb} and \textit{M.leprae} sequences further upstream of the sequences obtained for \textit{M.vaccae} Gm27 and Job5 (Figure 6.13). The first promoter sequence has a –10 region that matches the \textit{M.tb} T80 promoter (Bashyam et al., 1996) (Figure 6.14). It is difficult to identify the –35 region of the promoter, as the majority of mycobacterial promoters do not carry a sequence that closely resembles the typical prokaryotic –35 region consensus sequence, TTGACA (Bashyam et al., 1996). Bashyam et al (1996) analysed 14 \textit{M.smegmatis} and 10 \textit{M.tb} promoters and were unable to identify a conserved sequence for the –35 regions of the promoters. Indeed, the –35 regions of the putative promoters of the \textit{M.tb} and \textit{M.leprae} \textit{cspA} genes have no similarity with the –35 region of the \textit{M.tb} T80 promoter (Figure 6.14).

The second identified promoter sequence contains an extended –10 region, a TGN motif immediately upstream of the –10 region, similar to the \textit{M.tb} T101 promoter (Bashyam and Tyagi, 1998) (Figure 6.13 and 6.15). The TGN motif is believed to increase transcriptional strength in mycobacteria as has been reported for \textit{E.coli} (Bashyam and Tyagi, 1998).
Figure 6.14: Comparison of *M. tb* T80 promoter region and the putative *M. tb* cspA promoter region.

The –10 and –35 regions are underlined. The T80 promoter sequence was obtained from Bashyam et al (1996). The conserved hexameric –10 sequence for *M. tb* was 80%T, 90%A, 60%Y, 40%g, 60%A and 100%T. Capital letters indicate bases conserved by 50% or more, and letter Y indicates a pyrimidine base. The –35 region of mycobacterial promoters have little if any homology with the *E. coli* -35 consensus sequence, TTGACA (Bashyam et al, 1996).

Figure 6.15: Comparison of *M. tb* extended –10 promoters and the putative extended promoter of *M. tb* cspA

Extended –10 promoters have a TGN motif immediately upstream of the –10 region. The –10 region of the promoters, and the putative –35 region of cspA gene are underlined; the TGN motif is highlighted in bold. The *M. tb* sequences were obtained from Bashyam et al (1998).
TGN motifs have been identified for homologous cspA genes in *B. subtilis* (Willimsky et al., 1992), *Lactococcus lactis* (*L. lactis*) MG1363 (Wouters et al., 1998), and *Salmonella typhimurium* (*S. typhimurium*) LT2 (Horton et al., 2000), indicating an important characteristic of the cspA promoter. Moreover Bashyam and co-workers (1998) suggested that the TGN motif might facilitate transcription initiation in mycobacteria at low temperatures.

The *E. coli* cspA promoter was found to contain an AT rich UP-element (a target for the C-terminal domain of the α-subunit of RNA polymerase) at position −47 to −38, upstream of the −35 region, which has been shown to enhance promoter activity between 2 and 22 fold (Ross et al., 1993). However, such an element could not be identified upstream of the −35 region of the putative extended mycobacterial promoter (Figure 6.13).

The real promoter will need to be identified experimentally however the presence of a TGN motif in the cspA promoter of *E. coli*, and other bacteria indicates that the extended promoter is the true mycobacterial promoter, and further analysis was carried out with this as the cspA promoter.

6.4.4 Mycobacterial Cold shock proteins contain an unusually long 5'UTR

It appears that the mycobacterial cold shock proteins mRNA possess an unusually long ~140bp 5'UTR, comparable to those reported for the mRNAs of the *E. coli* and *B. subtilis* major cold shock protein genes, cspA and cspB, respectively.

The 5'UTR of *E. coli* cspA mRNA has an important role in the cold shock response. *E. coli* cspA mRNA is extremely unstable at 37°C (half-life; <12sec) (Brandi et al., 1996; Fang et al., 1997; Goldenberg et al., 1996), the mRNA immediately becomes stabilised upon cold shock (half −life; >20min) (Brandi et al., 1996; Fang et al., 1997; Goldenberg et al., 1996). The 5'UTR is believed to be responsible for this instability.
Overproduction of cspA 5'UTR, without the cspA coding region, resulted in a prolonged expression of the cold shock genes, and a prolonged lag period of cell growth (Jiang et al., 1996). The overproduction of the 5'UTR resulted in derepression of the cold shock gene expression including cspA. The derepression was attributed to the first 25 bases of the 5'UTR that contains an 11-base common sequence (cold box) found in the 5'UTR of cspA, cspB and csdA mRNAs (Jiang et al., 1996). The overproduction of cspA 5'UTR along with CspA resulted in a normal cold shock response (Jiang et al., 1996). Jiang et al (1996) proposed that a putative repressor bound to the cold box of cspA and repressed its expression. In addition they suggested that CspA either directly or indirectly promoted the repressor function. Moreover, Bae and co-workers (1997) revealed that CspA negatively regulates its own expression by binding to the unusually long 5'UTR of its own mRNA and destabilising its 2° structure (Jiang et al., 1997).

The highly conserved cold box is presumed to be a transcriptional pausing site, as the 5'UTR of *E.coli* cspA has a stem-loop structure that resembles the structure of transcriptional pausing sites and the rho-independent intrinsic terminators (Bae et al., 1997). The cold box is also believed to be involved in the repression of cspA expression (Bae et al., 1997).

Analysis of the mycobacterial cspA 5'UTRs indicated a potential cold box sequence at position +7 to +17 (Figure 6.16A). The *M.vaccae* sequences matched 7 of the 11 bases reported in the cspA genes of *E.coli* (Jiang et al., 1996) (Figure 6.16B), and at two further positions an adenine base had been exchanged for a thymidine, and a guanine residue for a cytosine, indicating a conserved substitution in the sequences. The *M.tb* and *M.leprae* putative cold box sequences had lower matching's to the *E.coli* cspA sequence, however this may reflect the GC rich nature of these organisms genomes. Moreover the putative sequence appears to be highly conserved between the mycobacteria.
Figure 6.16: Location of a putative cold box in the 5'UTR of mycobacterial cspA mRNA

A, alignment of the 5'upstream sequences of the mycobacterial cspA genes. The putative transcriptional start site is highlighted with an star, and the translational start codon is underlined. Identical residues are marker with a dot; differing nucleotides are marked with the appropriate letter; gaps are represented with a dash.

B, alignment of the putative cold box sequences of the Mycobacteria and the cold box of the E.coli cold shock genes, cspA, cspB, cspG, cspi and csdA, and the cold box sequence of Salmonella typhimurium cspA. The M.vaccae Job5 sequence has 7 of 11 nucleotides matching the E.coli cspA cold box.
Full characterization of the mycobacterial cspA promoters, the identification of the transcriptional start point, and carrying out protection experiments will help determine if this is indeed a transcriptional pausing site.

6.4.5 *M. vaccae* cspA mRNA contains a DB-like sequence

Mitta and co-workers identified (1997), within *E. coli* cspA mRNA, a translational enhancement element known as the downstream box (DB), located 12 bases downstream of the initiation codon. The DB is believed to enable the cold shock induced block in the initiation of translation to be overcome. The DB was originally thought to promote initiation in the absence of a Shine-Dalgarno sequence and has been shown to enhance the expression of various mRNA constructs, (Sprengart et al., 1996; Sprengart and Porter, 1997). The DB is complementary to bases 1469-1483 within the *E. coli* 16S rRNA, known as the anti-downstream box (ADB) sequence.

The region homologous to the *E. coli* 16S rRNA anti-downstream box (ADB), nucleotides 1469-1483, was identified in the *M. vaccae* VM0588 16S rRNA sequence (Accession number gi23955535), nucleotides 1422-1436. The first 40 bases of the mycobacterial (*M. tuberculosis*, *M. leprae*, *M. smegmatis*, and *M. vaccae*) cspA mRNA sequence are entirely conserved, as are the mycobacterial anti-DB 16S rRNA sequences. Thus a comparable DB sequence was sought in the *M. vaccae* Gm27 sequence.

In *E. coli* functional DBs were found close to the initiation codon (Ito et al., 1993) so the *M. vaccae* cspA sequence was analysed for a complementary sequence close to the initiation codon (Figure 6.17). RNA molecules are able to form unusual base-pairings, in addition to the expected A:U and G:C pairings, G:U pairs are also observed (Adams et al., 1986; Ito et al., 1993). Ito et al (1993) reported that a minimum number of 6 of 15 AU and GC matches, or 7 of 15 if GU matches were allowed, for a functional downstream box. Multiple (five) putative DB sequences were identified in *M. vaccae*, which complemented the putative ADB sequence (Figure 6.17). The most probable DB sequence is
Figure 6.17: Possible complementarity between the putative downstream box (db) of *M. vaccae* cspA mRNA and the putative 16S rRNA anti-downstream box (ADB).

Complementary bases (AU/GC) are in bold, GU pairs are in blue. Multiple alignments are shown. Alignments with 7 or more matching bases are highlighted with black arrows. The red arrow highlights the sequence with 6 GC/AT matchings and 1 GT match. The start codon (AUG), and a putative alternative start codon (GUG) are underlined.

*M. vaccae* 16S rRNA sequence: gi|23955535|gb|AF544639.1| *M. vaccae* isolate VM0588
sequence 5, which has 4 GC and 2 AT matches, and only 1 GU match, whereas the remaining sequences have significantly more GU matches, which are weaker interactions than the standard base pair interactions (Figure 6.17).

DB sequences were identified in all the cold shock proteins of *E.coli*. The DB sequences were identified at the 5th codon for *cspA*, *cspB* and *cspG*, whereas the DBs of the remaining cold shock proteins predominantly originated from the start codon (Mitta et al., 1997). Furthermore a second initiation codon was identified at the beginning of the DB sequence of *E.coli cspA*, *cspB* and *cspG*. A potential second start codon, GUG, exists at codon six in the *M.vaccae cspA* mRNA (Figure 6.17). Although AUG is the dominant start codon in the mycobacterial species, 33% of start codons in *M.tb* are GUG (Rocha et al., 1999). Putative DB sequences 1 and 2 contain part of the putative second start codon. Although the sequences are not in the same reading frame as this start codon, O'Connor and co-workers (1999) revealed that the DB enhancer activity is not dependent on the codons that make up the DB, rather on the DB RNA sequence itself.

The presence of a second start codon at the beginning of the *E.coli cspA* DB sequence would indicate a conserved feature of the major cold shock protein genes. Nevertheless, the higher proportion of GU matches in *M.vaccae* putative DB 1 and 2 would indicate less favourable interaction with the anti-DB than sequence 5, that originates from the start codon, AUG. A potential third start codon exists at bases 21 to 24 (GUG), however it is not in frame with the principle start codon, and is therefore unlikely to be a true start codon, as initiation from this point would result in a completely different protein sequence.

A DB has not been reported in the *B.subtilis cspB* mRNA. Analysis of the first 30 bases of the sequence, however, revealed the presence of a putative DB at base 19 (Figure 6.18, sequence 3), with 10 matchings to the putative ADB sequence, 8 GC/AU and 2 GU matchings, a similar complementarity level to *E.coli cspA* (Figure 6.19A). Although there is no obvious additional start codon in *B.subtilis cspB* mRNA.
Figure 6.18: Possible complementarity between the first 30 bases of *B. subtilis* cspB mRNA and the putative ADB of *B. subtilis* 16S rRNA.

Complementary bases, GC and AU, are highlighted in bold, GU complementary bases are in blue. Multiple alignments are shown. Alignments with 7 or more matching bases are highlighted with arrows. The start codon AUG is underlined. The putative DB sequence of *B. subtilis* cspB is highlighted with a red arrow. The *B. subtilis* ADB sequence was obtained from Moll et al 2001.
### Table: No. of matching

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<td>B.subtilis</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>M.vaccae</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

A

E.coli ADB : CUUUGUGAUAUCUGA : 15
L.lactis ADB : AGGUAAAGACGGAUGA : 15
B.subtilis ADB : AGGUCCGACAGAUGA : 15
M.vaccae ADB : AGGUGGAUGAGGCA : 15

B

E.coli DB : AUGACUGGUUACGGU- : 14
B.subtilis DB : UAAAAUGUUUCACU : 15
M.vaccae DB : AUGCCACAGGGAACU : 15

C

Figure 6.19: The DB and anti-DB (ADB) sequences of a number of prokaryotic species.

Nucleotides that are 100% conserved are highlighted with a black background, those 80% and 60% conserved are highlighted with a dark grey or light grey background respectively.

(A) The number of potential base pairs between the DB and anti-DB sequences in (B) and (C).
(B) Alignment of the putative DB sequences of *E.coli*, *B.subtilis*, and *M.vaccae* (Gm27 and Job5).
(C) Alignment of the putative ADB sequences of *E.coli*, Lactococcus *lactis*, *B.subtilis*, and *M.vaccae* VMO588. *L.lactis* and *B.subtilis* sequences were obtained from Moll et al (2001).
Comparison of (putative) anti-DB sequences from a number of prokaryotic organisms (Figure 6.19B) suggest that the anti-DB sequence is not highly conserved between the species, although a number of nucleotides appear to be conserved, there are considerable differences between the anti-DBs. There is no obvious consensus sequence, only a limited number of bases are entirely conserved between the sequences. The \textit{M.vaccae} cspA anti-DB sequence is more closely related to the \textit{L.lactis} anti-DB, with 66% sequence identity, than \textit{B.subtilis} and \textit{E.coli} anti-DB. Moreover, analysis of the putative DB sequences from the major cold shock protein mRNAs of a number of prokaryotes (Figure 6.19C), reveals that, like the anti-DB, the DB sequence is not conserved between the species, again there is no obvious consensus sequence. The conservation of a DB sequence in the major cold shock protein gene sequences indicates an important role in the cold shock response.

The presence of a putative cold shock box, and putative DB and anti-DB sequences suggests that the control mechanism for the expression of \textit{M.vaccae} cspA is similar to that reported for \textit{E.coli}.

6.4.6 \textit{M.vaccae} Cold Shock protein A

The 171bp \textit{M.vaccae} gene sequence obtained encodes 57 of an expected 67 amino acid protein. Comparison with the other published mycobacterial CspA proteins, reveals a strong conservation of the protein sequence (Figure 6.20). Only one difference occurs in the first 50 amino acids of the \textit{M.tb}, \textit{M.leprae}, \textit{M.smegmatis}, and \textit{M.vaccae} proteins, and this is a conserved substitution. The \textit{M.tb} and \textit{M.vaccae} proteins have a threonine (Thr) residue at position 37, whereas the \textit{M.leprae} and \textit{M.smegmatis} proteins have a serine (Ser) residue, both residues are neutral polar amino acids. Analysis of the complete protein sequences reveals differences in only 5 amino acid residues in total, 4 of which occur in the last 7 amino acids of the protein, indicating that the C-terminal is not so important in the function of CspA.

Interestingly the partial \textit{M.vaccae} protein sequence appears to most closely resemble the \textit{M.tb} protein, with 98% sequence identity (a difference of one
amino acid), compared to 94% with *M. smegmatis* (a difference of 3 amino acids). Although it should be noted that two of the differences in *M. smegmatis* are conserved substitutions. It’s surprising that the *M. smegmatis* and *M. vaccae* proteins are not more closely related as they both belong to the fast-growing mycobacterial sub-genus, particularly as the *M. vaccae* and *M. smegmatis* gene sequences are more closely related than *M. vaccae* and *M. tb* gene. The identification of the remaining amino acid residues will determine the reality of this unexpected relatedness.

Comparison of the mycobacterial protein sequences with other prokaryotic CspA homologues, and eukaryotic CSDs reveal that the proteins are most closely related to the Csp of *Arthrobacter globiformis* (*A. globiformis*) (Figure 6.20) with 70% sequence identity. As *A. globiformis* belongs to the same group (Actinomycetes) as the Mycobacteria this is not unexpected. The majority of residues are identical between the proteins, and of those that are not, many are conserved substitutions. In addition the mycobacterial proteins are marginally more closely related to CspB of Gram positive *B. subtilis* (~59% sequence identity), than CspA of Gram negative *E. coli* (~56% sequence identity).

### 6.4.7 Structure Cold shock protein A

Crystallography and nuclear magnetic resonance imaging (NMR) have revealed the three dimensional structure of *E. coli* CspA and *B. subtilis* CspB (Schindelin et al., 1993; Schindelin et al., 1994; Schnuchel et al., 1993), and more recently the structures of *Bacillus caldolyticus* and the hyperthermophilic bacterium *Thermotoga maritima* homologous cold shock proteins (Mueller et al., 2000) (Kremer et al., 2001). All the crystal structures have revealed that the cold shock proteins are β-barrel proteins, which are formed from 5 β-strands, connected by loops (Kremer et al., 2001; Mueller et al., 2000; Schindelin et al., 1993; Schindelin et al., 1994; Schnuchel et al., 1993). The proteins consist of two antiparallel β-sheets that are packed together at approximately 90° to give the molecules an asymmetric L-shape, called a Greek key formation (Schindelin et al., 1993).
Figure 6.20: Alignment of prokaryotic and eukaryotic cold shock domain. Conserved residues involved in DNA binding and conserved hydrophobic residues are marked with red arrows and black arrows, respectively. Gram negative bacteria are labelled blue, Gram positives dark red, Actinomycetes bright red, and the eukaryotic sequences green. Residues conserved 100% are coloured black; more than 80%, dark grey; more than 60%, light grey. The consensus sequence is located at the bottom of the alignment. Capital letters and small letters represent residues present in 100%, and >80% of the sequences, respectively. Numbers represent the presence of an amino acid from a similarity group: 1 - Aspartic acid (D) or Asparagine (N); 2 - Glutamic acid (E) or Glutamine (Q); 3 - Serine (S) or Threonine (T); 4 - Lysine (K) or Arginine (R); 5 - Phenylalaine (F), Tyrosine (Y) or Tryptophan (W); 6 - Leucine (L), Isoleucine (I), Valine (V) or Methionine (M). Abbreviations: B.caldolyticus and B.stearothermophilus - Bacillus species; C.perfringes - Clostridium perfringes; L.monocytogenes and L.innocua - Listeria species; L.lactis - Lactococcus lactis; P.fragi and P.putida - Pseudomonas species; S.aurantiaca - Stigmatella aurantiaca; S.aureus and S.epidermidis - Staphylococcus species; S.coelicolor - Streptomyces coelicolor; S.flexineri - Shigella flexineri; S.pyogenes - Streptococcus pyogenes; T.maritima - Thermotoga maritima; V.vulnificus - Vibrio vulnificus; Y.pestis - Yersinia pestis. FRGY2 CSD - Xenopus oocyte Y-box protein FRGY2 Cold shock domain; Human YB1 CSD - Human Y-box protein 1 Cold shock domain.
The 3D structure of *E.coli* CspA and *B.subtilis* CspB are very similar, although there are differences, they are mainly restricted to loop regions (Schindelin et al., 1994).

CspA has been conserved from bacteria to man and the recent crystallisation of the human YB1-Cold shock domain (CSD) revealed that the prokaryotic Csps and the eukaryotic CSD had structural as well as sequence homology. The eukaryotic CSDs consist of a closed 5-stranded anti-parallel β-barrel capped by a long flexible loop (Kloks et al., 2002). The major differences between the prokaryotic cold shock proteins and the eukaryotic CSDs, is that eukaryotic CSDs have a longer N-terminal and a longer loop between strands β3 and β4.

An interactive internet-embedded research platform CSDBase (Weber et al., 2002) has been developed, which provides information on proteins containing the CSD. CSDBase contains a database of computer generated structure models from known crystallographic and NMR structures. CSDBase contains the theoretical 3D structure of the mycobacterial cold shock proteins from *M.tb*, *M.leprae*, and *M.smegmatis*. Figure 6.21 (A and B) shows the theoretical mathematically modelled structure of CspA *M.tb*, which reveals that the mycobacterial CspA proteins also form a β-barrel structure consisting of 5 β-strands. The mycobacterial proteins appear to have the greatest similarity to *B.subtilis* CspB. Although, *B.subtilis* CspB differs from the mycobacterial and *A.globiformis* proteins in the loop 2 region, where it is missing a residue at position 22, present in the other proteins (Figure 6.20). The *B.subtilis* CspB protein has been used as the template to assign the positions of the β-strands for these proteins (Figure 6.21C).
Figure 6.21: Structure of *M. vaccae* CspA

A and B theoretical three-dimensional structure of *M. tb* CspA (Weber et al. 2002). View A highlights the β-barrel structure of the protein. B highlights the position of the β-strands and loops. C Hypothetical location of β-strands in Mycobacterial CspA proteins, *B. subtilis* CspB was used as the template (Schindelin et al. 1994; Schnuchel et al. 1993). The grey arrows represent the β-sheets and the lines represent the interconnecting loops. RNP1 and RNP2, ribonucleoprotein motifs 1 and 2.
6.5 Discussion

6.5.1 *M. vaccae cspA*

The major cold shock protein of *E. coli*, cold shock protein A (CspA), is highly conserved across the bacterial kingdom, and so far homologues have been identified in over 50 bacterial species. For the first time an homologous gene was identified in two strains of *M. vaccae*, Gm27 and Job5. Of the expected 201bp coding region (the length of the coding region of the other mycobacterial cspA genes) 171bp were obtained, along with ~140bp of the upstream gene sequence. The gene was highly conserved between the two strains, and in *M. tb*, *M. leprae* and *M. smegmatis* (Table 6.4). There is a minimum of 84% sequence identity between the species, and the sequence conservation decreases towards the 3’ end of the gene. Interestingly, the *M. smegmatis cspA* gene has the least homology with the other mycobacterial cspA genes. Moreover, the partial *M. vaccae* genes have greater homology with *M. tb* and *M. leprae cspA* than *M. smegmatis* does. However it should be noted that the 3’ end of the *M. vaccae cspA* genes has not yet been identified and this may reveal that the sequence identity for the complete gene is significantly less.

The decrease in sequence conservation towards the 3’ end of the gene may explain the inability to obtain the 3’ end of the *M. vaccae cspA* gene using primers designed from the other mycobacterial cspA genes. The differences in the 3’ end of the cspA gene sequences mean that primers directed against a particular mycobacterial cspA are only likely to generate products with that species, as the different sequences would result in mis-matches between the primer and the sequence, and prevent primer binding.

Furthermore, it is possible that the arrangement of genes around the cspA gene is different in the fast growing and slow growing mycobacteria. The published genome sequences of *M. tb* and *M. leprae* revealed that cspA is located between two hypothetical protein genes, one in the opposite transcriptional orientation, and the second in the same transcriptional orientation, which is followed by the
topoisomerase I/A gene, also in the same transcriptional orientation (Figure 6.6). There has been a recombination event in one of the species, as the cspA gene is orientated in opposite directions in M.tb and M.leprae. The gene is contained within a section of approximately 50000 bases that appear to have been relocated in one of the species, although it is unclear in which one.

The hypothetical protein gene between cspA and topA has homology between the two slow growing species, and from this homologous gene a primer was designed to try to obtain the 3' end of the cspA gene in M.vaccae. The primer failed to generate a PCR product with the DNA from the fast growing species, M.smegmatis or M.vaccae (section 6.3.7). It is possible, that the primer was designed from a section of the gene that was not well conserved in M.smegmatis and M.vaccae.

It is also feasible that there has been a recombination event in the fast growing species, and that the organisation of the cspA gene locus is not conserved between the fast growing and slow growing mycobacteria. The differential organisation of a conserved gene locus between fast and slow growing mycobacteria has been reported for the ECF (extracytoplasmic function) sigma factor H (Fernandes et al., 1999). The ECF sigma factors are a diverse family of proteins within the σ^70 class of bacterial RNA polymerase sigma subunits. The organisation of the mycobacterial chromosome, around the sigH locus, differs substantially between M.tb and M.smegmatis (Figure 6.22). The genes immediately 5' and 3' to the sigH gene differ between the two species, although the genes further 3' to the sigH gene are the same. The transcriptional orientation of the cspA gene differs in M.leprae and M.tb indicating a recombination event in one of these species, and the recombination event occurred close to the cspA gene (Figure 6.6). Thus, it is conceivable that a similar reorganization has occurred in the chromosome surrounding the cspA gene, in the fast and slow growing mycobacteria.
Figure 6.22: The sigH loci of *M. smegmatis* and *M. tuberculosis*.

The *sigH* genes are highly conserved, but there are significant differences in the organisation of the surrounding genes. The transcriptional orientation of the genes is indicated by the black arrows. Matching genes have the same pattern in both loci. The figure was taken from Fernandes et al (1999).

*Orf* = open reading frame
6.5.2 Regulation of cspA expression

The expression of *E.coli* cspA has been extensively analysed and is currently considered to be controlled at the level of transcription, mRNA stability and translation (Yamanaka, 1999).

6.5.2.1 cspA promoter

The *E.coli* cspA gene has a strong promoter, considered to be one of the strongest in *E.coli* (Yamanaka, 1999), which has an UP element as well as an extended −10 region (with a TGN motif) (Fang et al., 1997; Goldenberg et al., 1996; Mitta et al., 1997).

Analysis of the 5' non-coding sequence of the *M.vaccae* cspA gene failed to identify the promoter sequence. However comparison with the other published mycobacterial cspA genes (Figure 6.13) revealed a putative extended promoter with a TGN motif in the upstream sequence of *M.tb* and *M.leprae* (Figure 6.13 and 6.16). The TGN motif has been reported to increase the transcriptional strength of the promoter in *E.coli*, and the same is believed to be the case in mycobacteria (Bashyam and Tyagi, 1998).

Extended promoters have also been identified in the homologous cspA genes in *B.subtilis* (Willimsky et al., 1992), *L.lactis* MG1363 (Wouters et al., 1998) and *S.typhimurium* LT2 (Horton et al., 2000), indicating that this is an important characteristic of the cspA promoter. Moreover Bashyam and co-workers (1998) suggested that the TGN motif might facilitate transcription initiation in mycobacteria at low temperatures.

However, an UP element could not be identified in the *M.tb* and *M.leprae* sequences. UP elements have been identified in the major cold shock protein genes of *B.subtilis* (Willimsky et al., 1992), *L.lactis* (Wouters et al., 1998), and *S.typhimurium* (Horton et al., 2000), indicating that the cspA promoter in mycobacteria, in particular, slow-growing bacteria is not as strong as those reported for other bacteria. It should be noted however that the *E.coli* cspA
promoter is active at 37°C and 15°C, and is not necessary for cold shock induction (Fang et al., 1997), implying that control of the expression of CspA on cold shock is not at the level of transcription. These findings reduce the importance of the promoter strength, as CspA expression is under minimal transcriptional control.

6.5.2.2 Mycobacterial cspA mRNA has a long 5' untranslated sequence

The identification of this putative promoter revealed that the mycobacterial cspA mRNAs have a long 5' untranslated region (5'UTR) of approximately 150bp, comparable to those reported for the major cold shock protein genes cspA and cspB of E.coli and B.subtilis, respectively (Goldstein et al., 1990; Graumann et al., 1997; Jiang et al., 1996). The 5'UTR of E.coli cspA mRNA has an important role in the cold shock response. Increased mRNA stability is believed to be involved in the massive increase in CspA expression on cold shock. E.coli cspA mRNA is extremely unstable at 37°C, and is immediately stabilised up on cold shock to 15°C, this instability is attributed to the 5'UTR (Brandi et al., 1996; Fang et al., 1997; Goldenberg et al., 1996). The presence of a long 5'UTR in the mycobacterial cspA mRNA indicates that the 5'UTR also has an important role in the mycobacterial cold shock response.

Furthermore, the 5'UTR of E.coli cspA contains an 11-base common sequence called the cold box, which may form a stable stem loop structure (Jiang et al., 1996) reminiscent of a transcriptional pausing site (Bae et al., 1997). The cold box is believed to have an important role in autoregulation of cspA expression (Yamanaka, 1999).

The cold box sequence has also been identified in the 5'UTR of S.typhiumurium (Horton et al., 2000). Analysis of the 5'UTR of mycobacterial cspA revealed a putative cold box sequence at position +7 to +17 (Figure 6.16A and B). Interestingly the cold box has not been reported in other bacteria to date, and B.subtilis cspB and cspC mRNA were found to contain two conserved sequences, cs box 1 and 2 (Graumann et al., 1997) with no similarity to the E.coli cold box. However these sequences appeared to have a similar role in
autoregulation of expression in B.subtilis as the cold box sequence does in E.coli.

Characterization of the mycobacterial cspA promoters, the identification of the transcriptional start point, and the secondary structure of cspA mRNA 5'UTR will help to determine if this in indeed a transcriptional pausing site. Moreover, B.subtilis CspB, CspC and CspD have been shown to preferentially bind to the cs box 1 sequence (Graumann et al., 1997), which indicates a self-regulation mechanism, similar to that of E.coli, may also exist in B.subtilis. Determination of any preferential binding to the putative transcriptional pausing site sequence, will help determine if an autoregulatory mechanism for cspA expression also exists in mycobacteria, and if an autoregulatory mechanism for cspA expression is present in all bacteria.

6.5.3.3 M.vaccae cspA mRNA contains a DB-like sequence

When E.coli are cold shocked from 37°C to 10°C, growth ceases for 4h (Jones et al., 1987). During this time protein synthesis is blocked most probably at the initiation of translation (Broeze et al., 1978; Yamanaka, 1999). However the synthesis of cold shock proteins is able to by-pass this translational block. Mitta et al (1997) identified a translational enhancement element, known as the downstream box (DB), within the mRNA of E.coli cspA and a number of other cold shock genes. The DB is believed to enable the cold shock induced block in the initiation of translation to be overcome. The DB is complementary to bases 1469 – 1483 within the E.coli 16S rRNA, known as the anti downstream box sequence (ADB).

Sprengart et al (1996) speculated that the translational enhancement was due to the formation of a duplex between the DB sequence of the mRNA and the anti-DB sequence of the 16S rRNA. Deletion of the DB sequence from the E.coli cspA prevented the cold shock induction of CspA (Mitta et al., 1997). In addition, insertion of the DB sequence into a β-galactosidase gene made it cold shock inducible (Mitta et al., 1997). These results indicate a crucial role for the DB sequence in cold shock induction. Mitta et al (1997) proposed that the DB is
involved in the formation of a stable translational initiation complex at low
temperature, before the induction of cold shock translational factors, allowing
the block in the initiation of translation to be overcome. DB sequences were
identified in all the cold shock protein genes in *E.coli*, including RbfA and CsdA,
required for the formation of the translation initiation complex at low temperature
in the absence of cold inducible ribosomal factors (Mitta et al., 1997).

To determine whether a similar enhancement element existed in the
mycobacterial genes, the ADB was identified in the *M.vaccae* VM0588 16S
rRNA sequence (nucleotides 1422 – 1436). Analysis of the first 30 bases of
*M.vaccae cspA*, from the start codon, revealed a complementary sequence to
the ADB with 7 out of 11 matches with the ADB (Figure 6.17). Moreover, a DB
sequence was also identified in *B.subtilis cspB* (Figure 6.18). The degree of
translational enhancement by the DB is dependent on the complementarity
between the DB and ADB (Etchegary and Inouye, 1999b). The DBs of *E.coli
cspA* and *B.subtilis cspB*, had a higher degree of complementarity than the DB
of *M.vaccae cspA*, indicating that it is a weaker, but functional, translational
enhancer (Figure 6.19A) (Ito et al., 1993).

The mechanism of translational enhancement by the DB sequence is
controversial. The DB sequence was suggested to form a duplex with the ADB
of the 16S rRNA, small ribosomal subunit, near the decoding region (Sprengart
et al., 1996), enhancing and stabilising the interaction of initiating ribosomes
with mRNAs, by base pairing with the anti-DB (O'Connor et al., 1999). However
a number of workers dispute the base paring between the DB and anti-DB.
Structural evidence indicates that the ADB would not be available for base-
pairing with the DB (Moll et al., 2001), or that simultaneous DB-ADB binding
and translation initiation would not be possible (O'Connor et al., 1999).

Nevertheless, 16S rRNA subunits have been found to bind to DB-like
sequences (Etchegary et al., 1998; Ringquist et al., 1995), and increased
complementarity between DB and anti-DB increases the translational
enhancement (Etchegary et al., 1998). In addition, the ribosomal proteins S1
and S2 have been implicated in the translational enhancement. S2 is believed to cause structural changes in the 16SrRNA that allows the association of DB and the anti-DB (Etchegary and Inouye, 1999b). Whereas it’s postulated that S1 facilitates the interaction between mRNA and ribosomes by recognising DB like sequences, indicating a role in the formation of the initiation complex (Etchegary et al., 1998; O'Connor et al., 1999). It should be noted that such a mechanism may be restricted to the Gram-negative prokaryotes as homologues to the S1 ribosomal protein are absent in the Gram-positive prokaryotes (Rocha et al., 1999).

Despite the speculation over the mechanism of action, the DB in the mRNAs of E.coli cold shock protein plays an important role in translational efficiency (Etchegary and Inouye, 1999b). Furthermore a DB has been shown to be crucial for the heat shock induction of rpoH mRNA (Nagai et al., 1991). This suggests the DBs have essential roles in the translational efficiency of mRNAs under stress conditions (Mitta et al., 1997). The presence of putative DB sequences in the major cold shock protein mRNAs of mycobacteria and B.subtilis, suggests a similar role in promoting the translational efficiency of these organisms during cold shock. Although the requirement of a DB would need to be investigated, possibly through mutational analysis as was the case in E.coli cspA mRNA (Mitta et al., 1997).

The presence of an extended promoter, a long 5'UTR, and the presence of a putative transcriptional pausing site, and a putative DB sequence indicates that the expression of mycobacterial CspA is controlled by a similar mechanism to E.coli CspA. Furthermore, it suggests that this mechanism is conserved across the bacterial kingdom as these bacteria are not close evolutionarily.

6.5.4 Mycobacterial cold shock protein A

The partial M.vaccae cspA gene sequence encodes 57 of an expected 67 amino acid protein. The protein is highly conserved within the mycobacteria (Figure 6.20). Only one of the first 50 amino acids differs between the proteins
of \textit{M.tb}, \textit{M.leprae}, \textit{M.smeagmatis} and \textit{M.vaccae}, and this is a conserved substitution (Figure 6.20). These 50 residues contain the ribonucleoprotein (RNP) motifs, RNP1 and RNP2, which are important for RNA-binding. Indeed, \textit{E.coli} CspA is believed to be an RNA chaperone (Jiang et al., 1997). It also indicates the importance of these residues for the function of mycobacterial CspA. The C-terminal of the protein appears to be less important functionally as the remaining 4 differences occur in the last 7 amino acids of the protein.

The CspA protein appears to be conserved across the Actinomycetes, as the CspA of \textit{A.globiformis} (Figure 6.20) is the most closely related to the mycobacterial CspA proteins of all the proteins examined here.

\subsection{The structure and function of CspA}

As mentioned in section 6.4.7 the structure of the CspA proteins has been elucidated from a number of organisms. Moreover, the homologous cold shock domain (CSD) of the human Y-box protein, YB-1 has also been crystallised. These proteins/protein domains all have a \(\beta\)-barrel structure formed from 5 \(\beta\)-strands, with interconnecting loops. The theoretical structure of the mycobacterial proteins from \textit{M.tb}, \textit{M.leprae}, and \textit{M.smeagmatis}, was obtained (Figure 6.21), and revealed that these proteins also have a \(\beta\)-barrel structure. The position of the \(\beta\)-strands of the mycobacterial proteins was estimated using the \textit{B.subtilis} CspB protein as the template (Figure 6.21C).

The \(\beta\)-barrel structure in all of the crystallised proteins is made from two \(\beta\)-sheets. In \textit{B.subtilis} CspB \(\beta\)-sheet 1 is formed from \(\beta\)-strands 2 and 3, along with half of \(\beta\)-strand 1, the rest of \(\beta\)-strand 1, \(\beta4\) and \(\beta5\), from \(\beta\)-sheet 2. A cluster of 7 aromatic residues from a total of 9 in CspB is located in \(\beta\)-sheet 1. Of these, 6, Tryptophan\(^8\) (Trp), Phenylalanine\(^{15}\) (Phe), Phe\(^{17}\), Phe\(^{27}\), His\(^{29}\), Phe\(^{30}\), are located on the solvent exposed side of \(\beta\)-sheet 1 (Schnuchel et al., 1993).

In addition to these aromatic residues, a cluster of basic residues, which consists of Lysine\(^7\) (Lys), Lys\(^{13}\), His\(^{29}\), and Arginine\(^{56}\) (Arg), is found on the
solvent face of the molecule. These basic residues are believed to allow the
highly acidic CspB protein, to overcome the charge repulsion of a negatively
charged DNA molecule. The basic residues along with the solvent exposed
aromatic residues are believed to provide a site for DNA-binding, and possibly
RNA binding (Schindelin et al., 1994; Schnuchel et al., 1993). Some of these
aromatic residues belong to the RNA-binding motifs RNP1 and RNP2 (Figure
6.20). Substitutions of Phe^{15}, Phe^{17}, and Phe^{27} with alanine, and the
substitution of His^{29} with glutamine, all of which are located in the RNP1 and
RNP2 motifs, abolished the nucleic-acid binding activity of CspB (Schroder et
al., 1995). In addition, substitutions of the conserved basic residues Lys^{7}, Lys^{13},
and Arg^{56}, as well as the aromatic residues Tryptophan^{8} (Trp) and Phe^{30}
significantly reduced the binding activity, and implicated the side chains of these
amino acids in nucleic acid binding (Schroder et al., 1995).

The equivalent amino acid residues have been entirely conserved in the
mycobacterial and \textit{A. globiformis} Csps (Figure 6.20), except for Phe^{30}, which
has been replaced with a Tyrosine (Tyr) residue, and Tyr^{42} with a Phenylalanine
residue in the mycobacterial proteins. In both these instances one aromatic
residue has been replaced by another indicating a functional conservation.
Lys^{7}, Trp^{8}, Phe^{17}, Phe^{27} are entirely conserved among the protein sequences
studied (Figure 6.20). Lys^{13} is entirely conserved in the cold shock proteins, but
not the Y-box proteins (Schindelin et al., 1994). A Tyr or a Phe residue is found
at positions 15, and 49. A Valine (Val) or Isoleucine (Ile) residue is found at
position 63, and a Lys or Arg residue is located at position 56. In addition, the
His^{29} residue is entirely conserved in all the proteins examined except \textit{L. lactis}
CspA, which also lacks a conserved aromatic residue at position 30 (Figure
6.20). Furthermore, more than 80% of the cold shock proteins contain a Tyr or
Phe residue at position 30; this has been exchanged for a Glycine (Gly) residue
in the Y-box proteins. The conservation of these residues indicates their
importance in the function of the Csp proteins.

Schindelin co-workers (1993) identified a hydrophobic core of residues in
\textit{B. subtilis} CspB that included residues: Leucine^{2} (Leu), Val^{6}, Phe^{9}, Ile^{18}, Val^{20},
Val$^{26}$, Val$^{28}$, Ile$^{33}$, Leu$^{41}$, Val$^{47}$, Phe$^{49}$, Ile$^{51}$, and Val$^{63}$. Residues equivalent to Val$^{6}$, Ile$^{17}$, Val$^{26}$, Val$^{28}$, Val$^{47}$, Phe$^{49}$, Ile$^{51}$, and Val$^{63}$ were found to be structurally important in the CspA homologue of *T. maratima* (Kremer et al., 2001). The equivalent residues have been entirely conserved in the mycobacterial and A.globiformis proteins. The residues are also highly conserved among the other proteins studied. Val$^{6}$, Ile$^{18}$, Val$^{26}$, Val$^{47}$, Phe$^{49}$ and Val$^{63}$ are entirely conserved in the cold shock proteins and the Y-box proteins (Figure 6.20). Val$^{28}$ is conserved in over 85% of the sequences studied. Furthermore an Ile or Val residue is present in position 51 in all the sequences analysed except for *P.putida* CspA. The conservation of these residues highlights their structural and therefore functional importance to the CspA proteins.

The conservation of this protein across the bacterial kingdom indicates its importance in the cold shock response, and would suggest that it has a similar function in every bacteria. Indeed, Graumann and Marahiel (1997) expressed the major cold shock protein of *B.subtilis*, CspB, in *E.coli* at 37°C. The protein had a strong influence over the pattern of protein synthesis in *E.coli* (Graumann and Marahiel, 1997), both decreases and increases in the rates of synthesis of specific proteins was noted. Notably, the proteins induced by *B.subtilis* CspB, did not match those induced on cold shock. However, the proteins downregulated on CspB expression, were very similar to those downregulated on cold shock. Moreover, CspB expression induced the transcription of the *hns* gene in *E.coli* (Graumann and Marahiel, 1997), the transcription of which is also increased on CspA expression on cold shock (La Teana et al., 1991). These findings indicate that CspA homologues have a similar function in all bacteria.

### 6.5.6 Conservation of CspA

The mycobacterial CspA proteins are highly conserved within the mycobacterial genus, and contain many amino acid residues that are important in the structure and function of these proteins from bacteria to man. The sequence conservation attests the importance of these proteins in prokaryotic and eukaryotic cells.
The high degree of sequence conservation between the bacterial cspS and the eukaryotic CSDs shows an evolutionary relationship. The genome of the bacterial parasite *Rickettsia prowazekii* (*R. prowazekii*) has been recently elucidated. This organism is considered to be more closely related to mitochondria than any other organism studied so far (Andersen et al., 1998). Furthermore it contains a csp-like gene, which encodes a 70 amino acid residue protein, with around 57.6% homology with the cold shock protein A of *Streptomyces clavuligerus* (Andersen et al., 1998). It has been speculated that the ancestor of the eukaryotic CSD may have come from the ancestor of mitochondria, which may have been, or still be a bacterial parasite (Yamanaka, 1999). It has been proposed that the CSD gene was transferred from the mitochondria to the nucleus, and by gene duplication, arrangement, insertion and other recombination mechanisms, diverged (Yamanaka, 1999). This theory has been given strength by the discovery of a mitochondrial Y-box protein in the eukaryotic protozoan *Trypanosoma brucei* (*T. brucei*) (Haymann and Read, 1999).

Trypanosomes have a unique RNA processing mechanism, called RNA editing. Small RNA molecules, guide RNAs (gRNAs) carry the information for the RNA editing (Haymann and Read, 1999). A gRNA binding protein was identified, RBP16, which was found to be a Y-box protein. RBP16 was isolated from the organisms mitochondria, however the gene for the protein was located in the nucleus. The protein has a mitochondrial import sequence, an N-terminal CSD, and a C-terminal arginine- and glycine-rich region (Haymann and Read, 1999). RBP16 is the first mitochondrial Y-box family protein to be identified.

The Y-box proteins are composed of a highly conserved N-terminal CSD followed by a more variable C-terminal domain (Graumann and Marahiel, 1998). The CSD of RBP16 has a higher sequence identity to the prokaryotic Csps (43-46%) than to any eukaryotic CSDs (33-38%) (Andersen et al., 1998). The presence of protein resembling the eukaryotic Y-box protein, with a CSD that has a stronger homology to the prokaryotic Csps supports the theory of the eukaryotic CSDs arising from the prokaryotic Csps. The presence of the
RBP16 gene in the nuclear DNA of T.brucei and a mitochondrial location indicates that genetic transfer has occurred between the mitochondria and nuclear DNA. RBP16 has 37% sequence identity with the deduced CspA-like protein of R.prowazekii, 30% identity with the CSD of human YB1, 38% identity with FRGY2. It seems that a potential evolutionary route from the prokaryotic Csps to the eukaryotic Y-box proteins exists. The presence of apparent evolutionary intermediates in R.prowazekii and T.brucei further support this theory.

Interestingly a second csp-like gene has been identified in M.tb and M.leprae. The genes denoted cspB, appears to be a eukaryotic Y-box homologue, as along with a CspA-like sequence, they contain a C-terminal extra domain, which contains alternating acidic and basic domains, similar feature to the C-terminal domain of the vertebrate Y-box proteins (Yamanaka, 1999). These proteins contain eight alternating regions of basic/aromatic amino acid residues, BA islands, and acidic amino acid residues (Murray et al., 1992). The C-terminal domain of M.tb CspB is significantly shorter than that of human YB1, 72 amino acids as opposed to 199 of YB1, however it does appear to contain what seems to be BA islands (Figure 6.23).

The mycobacteria CspB proteins have limited sequence identity with the major cold shock protein homologue CspA. The CSD of M.tb CspB has 41% sequence identity with M.tb CspA. Furthermore is has significantly less sequence homology, than M.tb CspA, with other prokaryotic CspA-like proteins.

It shares 43% sequence identity to the A.globiformis CspA protein, whereas the M.tb CspA has 70% sequence identity (see above). In addition the CSD of human YB1 and M.tb CspB share 27% sequence identity. Nevertheless M.tb CspB does appear to contain an additional C-terminal extra domain reminiscent of the C-terminal domain of the vertebrate Y-box proteins.

The presence of a potential mycobacterial Y-box protein is interesting functionally as well as evolutionary, particularly as the invertebrate Y-box
Figure 6.23: Basic Aromatic islands of the C-terminal domains of the eukaryotic Y-box proteins and *M. tb* CspB.

A – BA islands of the C-terminal domains of human Y-box protein 1, and *Xenopus laevis*, frog Y-box protein 2 (FRGY2).

B – putative BA islands of *M. tuberculosis* CspB. The arrows highlight the position of the BA islands that are interspersed with the acidic residues. Basic residue, histidine (H), arginine (R), and lysine (L) have are highlighted with a blue background and white text. The aromatic residues phenylalanine (P), tyrosine (Y) and tryptophan (W) are highlighted with a light green background and orange text. The acidic residues, aspartic acid (D) and glutamic acid (E) are highlighted with a red background and white text.
proteins do not appear to have alternating acidic and basic regions (Thieringer et al., 1997; Yamanaka, 1999). Although given the low sequence homology it seems unlikely that a direct transfer has occurred between the mycobacteria and vertebrates.

The Y-box like proteins have so far only been identified in the slow-growing mycobacteria \textit{M.tb} and \textit{M.leprae}. However, a search of the BLAST database revealed homologous proteins in \textit{Streptomyces coelicolor A3} (2), and \textit{Corynebacterium effeciens} and \textit{Corynebacterium glutamicum} (ATCC13032), which belong to the same order (Actinomycetes) as the mycobacteria. It is interesting to speculate what role these proteins may have. Indeed, if these proteins also have the ability to sequester mRNA until needed, as in the case of the \textit{X.laevis} Y-box protein, FRGY2, it is possible that they may have a role in dormancy of Mycobacteria, Streptomycetes and Cornebacteria, and also in sequestering mRNA during spore formation in the Streptomycetes.

In summary, a gene for the homologue to the major cold shock protein of \textit{E.coli} was identified in two strains of \textit{M.vaccae}. Moreover analysis of the coding and non-coding 5' upstream sequence of the mycobacterial genes led to the identification of a putative extended promoter, and a number of elements involved in the control of the expression of \textit{E.coli} \textit{cspA}. These findings indicate that the control of \textit{cspA} expression is similar in \textit{E.coli} and mycobacteria, and suggests a similar control mechanism exists across the bacterial kingdom.
Chapter 7

7. The effect of cold shock on the expression of cspA mRNA

7.1 Introduction

CspA is the major cold shock protein of E.coli reaching 13% of the cells total protein synthesis an hour after a cold shock from 37 to 10°C (Jones et al., 1987). This protein plays an important role in the cold shock response of E.coli and is thought to be involved in the transcription of at least two other cold shock genes gyrA (Jones et al., 1992b) and hns (La Teana et al., 1991). A homologous protein was discovered in B.subtilis, CspB, which is induced several fold on cold shock (Graumann et al., 1996). Over 50 homologous proteins have been discovered across the bacterial kingdom, and a homologous protein was recently reported in M.smegmatis (Benson et al., 2002), which was upregulated 7-fold on cold shock (37 to 10°C) (Shires and Steyn, 2001).

The aim of the study was to characterise the cold shock response of M.vaccae and to identify its cold shock proteins. To further characterise the cold shock response, and to determine whether the highly conserved cspA gene was overexpressed on cold shock, the effect of cold shock on the expression of cspA was studied.

7.2 Materials and Methods

7.2.1 Cold shock experiments

M.vaccae strain Gm27 was cultured at 32°C in Sauton’s liquid media. Mid-exponential phase cultures were cold shocked to 6°C, as described in Chapter 3. Total RNA was extracted from control (non-shocked) and cold-shocked cultures and the level of cspA mRNA in the control and cold shocked samples was determined by Northern hybridization and Quantitative real time PCR (Chapter 4, sections 4.17 and 4.11, respectively).
7.2.2 PCR

DNA was extracted from liquid cultures of two strains of *M. vaccae*, Gm27 and Job5. DNA was also obtained from *M. smegmatis* (liquid culture), *M. tb* HRV37 (slope culture kindly extracted by G. McIntyre), for use as positive controls. The procedures for DNA extraction are described in Chapter 4, section 4.6.

A basic 2 PCR programme was used for all of the PCRs, and where necessary a touchdown programme was added to the PCR (Chapter 4, section 4.9.2). The primer sequences and PCR programmes are included in the results. As with all PCRs, a positive control was amplified in each reaction, to ensure each PCR’s efficacy, along with a negative reaction control.

7.3 Results

In order to analyse the effect of cold shock on cspA mRNA expression a housekeeping gene needed to be established. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) product is routinely used as the housekeeping gene in eukaryotic experiments, as the level of GAPDH mRNA stays constant in eukaryotic cells. However, an equivalent gene has yet to be routinely used in prokaryotes.

Mangan et al. (1997) reported the use of a PCR generated mycobacterial 16SrRNA probe to confirm equal loadings of total RNA on a Northern hybridisation blot. The 16SrRNA gene was chosen to be the housekeeping gene for the mRNA expression experiments.

7.3.1 PCR of partial 16SrRNA gene sequence

The cspA product was 143bp so only a partial gene sequence was needed to obtain a product of comparable size. Forward primer RAC106 was combined with the reverse primer RAC8 (Table 7.1), to generate a product of 275bp. Sigma PCR water was used to make up any solutions and the negative control as the 16SrRNA gene is present in every bacterium, and mycobacteria frequently contaminate water supplies. The prevalence of the 16S rRNA gene.
<table>
<thead>
<tr>
<th>Chapter section</th>
<th>Target sequence</th>
<th>Primer sequence (5' - 3')</th>
<th>Product size (bp)</th>
<th>PCR cycle condition</th>
<th>No of cycles</th>
</tr>
</thead>
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<tr>
<td>7.3.1</td>
<td>16S rRNA</td>
<td>RAC106 : ACT CGA GTG GCG AAC GGG TGA G RAC8 : TAT TCC CCA CTG CCT CCC GTA GGA GT</td>
<td>275</td>
<td>Touchdown PCR (T_m 70°C) then, a basic (2) PCR</td>
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<td>T_m 62°C</td>
<td>35</td>
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<td>7.3.2</td>
<td>GND</td>
<td>GND1 : ATG GTC CAC AAC GGC ATC G GND2 : AGG CCA CIA TCT TGG AGG C</td>
<td>452</td>
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<td>T_m 57.9°C</td>
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<td>Sigma factor A</td>
<td></td>
<td>SIGA1 : CAC CGG CCG (GC)GG CAT GGC SIGA2 : GTC TTG GA(CT) TCG ATC TGG C</td>
<td>501</td>
<td>Touchdown PCR (T_m 71.5°C) then, a basic (2) PCR</td>
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<td>T_m 58°C</td>
<td>30</td>
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<td>T_m 65.5°C</td>
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<td>Sigma factor A</td>
<td>SIGA6 : CTC CGC GCC CAG GTC CTG C</td>
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<td>SIGA8 : CCC TCG TCG CCG ATG CTC TGG</td>
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<td></td>
<td>SIGA3, SIGA6, and SIGA8,</td>
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<td>T_m 62.0°C</td>
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<td></td>
<td>SIGA7 : GTG CGG TCG AGA AGT TCG AC +</td>
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<td>Basic (2) PCR</td>
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<tr>
<td></td>
<td>SIGA3, SIGA6, and SIGA8</td>
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<td>30</td>
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<td></td>
<td>T_m 60°C</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 7.1: Primer sequences and PCR programmes used in the study of the effect of cold shock on cspA expression
The PCR procedures are listed in Chapter 4. The forward primer is listed first. Brackets represent degenerate residues and contain the alternative nucleotides. I represents the inosine base.
meant that the PCR was particularly sensitive to cross-contamination. The negative controls of initial PCR reactions were contaminated despite vigilance over glove changing to prevent glove tip contamination. Everything was meticulously cleaned to try to prevent contamination. The benches, door, drawer, fridge and freezer handles, ice buckets, tip boxes, pens, and pipettes were all cleaned with Flash and milliQ water, followed by 70% ethanol. The racks were soaked in Chloros (2500 parts per million available chlorine) for a minimum of 10 mins, to destroy any DNA, after every PCR reaction. Gloves were changed frequently. Furthermore, the DNA was not removed from the freezer until the primers had been distributed. Extreme care was taken to prevent glove tip contamination. Two negative controls were set up, one with and one without Sigma PCR water. These controls were used to determine whether any contamination originated in the Sigma PCR water or in the primers and PCR reaction mix. A successful PCR with clear negative controls was obtained (Figure 7.1).

It was subsequently realised the 16S rRNA did not remain constant on cold shock. Indeed, a 30% reduction in the transcription of rRNA was noted when *E.coli* was cold shocked from 37 to 20°C (Afflerbach et al., 1998). Following advice, the 6-phosphogluconate dehydrogenase (*gnd*) was investigated as a potential housekeeping gene. It had been routinely used in this capacity in experiments with *M.tb* (Dr P. Jenner, personal communication).

### 7.3.2 PCR to obtain a partial sequence of the 6-phosphogluconate dehydrogenase (*gnd*)

The *M.tb gnd* gene sequence was obtained from the Tuberculist web site ([www.genolist.pasteur.fr/Tuberculist](http://www.genolist.pasteur.fr/Tuberculist)). The *M.tb gnd* gene consists of 1455bp. The sequence was subjected to a BLAST search to identify regions of homology within the gene. A reverse primer was created from a region homologous to many species of bacteria, whereas the forward primer was created from a homologous region in *M.tb* and *M.leprae*. A basic 2 PCR was carried out (Table 7.1).
Figure 7.1: 2% Agarose/TAE gel of the 16SrRNA products

From left to right, lane 1: DNA marker; lane 2, *M.tb* DNA (10^-4 dilution), lane 3, *M. vaccae* Gm27 DNA; lane 4, *M. vaccae* Job5 DNA.
Unfortunately, a PCR product was only generated with the *M.tb* DNA, and the product was overamplified. It appears that the primers were too specific for *M.tb* despite choosing regions of homology within the *gnd* gene. There was no evidence that the *gnd* gene expression would not fluctuate with cold shock, and the primer sequences would need optimising before a primer was found that would generate a product with *M.vaccae* DNA. So another housekeeping gene was sought.

### 7.3.3 PCR to obtain a partial Sigma factor A gene product

Others have recently reported the use of a Sigma factor gene as a housekeeping gene, Sigma Factor A (*sigA*) (Frota et al., 2001). The Sigma Factor A gene is presumed to encode the major mycobacterial sigma factor due to its sequence, and because its *M.smegmatis* homologue has been shown to be indispensable (Gomez et al., 1998; Manganelli et al., 1999). *M.smegmatis* Sigma factor A levels were constant during exponential growth (Gomez et al., 1998). In addition the *sigA* transcript of *M.tb* was the same under different stress conditions including: exposure to hydrogen peroxide exposure, 0.5% SDS, and low pH (5.0); heat shock; mild cold shock, and incubation in an icebath (Manganelli et al., 1999). The *sigA* transcript levels were not constant after incubation in water, entry into stationary phase and with low culture aeration (Manganelli et al., 1999). The constant level of *sigA* transcript during most stresses and cold shock indicated that the *sigA* gene was a suitable candidate for the housekeeping gene.

**PCR with primers SigA1 and SigA2**

The *M.tb* *sigA* gene sequence was obtained and subjected to a BLAST search, by which the *M.smegmatis* *sigA* gene was acquired. Primers (*sigA1* and *sigA2*) were designed (Table 7.1) from a region of homology between the *sigA* genes from the two species. A PCR was carried out with Gm27 and Job5 DNA (Table 7.1, Figure 7.2). The PCR failed to generate a product with any of the DNA species indicating that there was a problem with the primers.
Figure 7.2: Location of sigA housekeeping gene primers.

Primers designed from a homologous region of the M.tb H37Rv and M.smegmatis sigA genes (MTU10059 and MSU09821 respectively). SigA1, SigA5, and SigA7 are forward primers. SigA2, SigA3, SigA6 and SigA8 are reverse primers.
PCR with primers SigA1 and SigA3

A second reverse primer SigA3 was designed and combined with the forward primer SigA1 (Table 7, Figure 7.2). Products of the expected size (500bp) were obtained with \textit{M.\textit{tb}} and Gm27 DNA, but not Job5 DNA. The PCR was repeated and products obtained for \textit{M.\textit{tb}}, Gm27 and Job5. However non-specific binding was noted with \textit{M.\textit{tb}} and Job5 DNA, several bands were noted in the Job5 lanes, above and below the expected product size. To try to optimise the PCR primer concentration was halved to 5pmol/\textmu l, and the annealing temperature was increased to 57.5°C, with the touchdown programme altered accordingly, and compared with a PCR with the original reaction conditions. However neither PCR generated any products with any of the DNA species. The PCR products initially produced could not be repeated despite returning to the original conditions and using freshly extracted \textit{M.\textit{vaccae}} DNA (in case of DNA degradation) (data not shown).

Magnesium chloride screen with primers SigA1 and SigA3

A magnesium chloride (MgCl\textsubscript{2}) screen was carried out to try to reproduce the SigA1/A3 PCR products, and to determine if the MgCl\textsubscript{2} concentration was too restrictive. For each DNA species a PCR reaction was set up with a MgCl\textsubscript{2} concentration of 1.5, 2.0, 2.5 and 3.0mM. Promega double strength (25\textmu l) mastermix with a MgCl\textsubscript{2} concentration of 1.5mM was used. The MgCl\textsubscript{2} concentration was increased by the addition of 50mM MgCl\textsubscript{2} (GibcoBRL). An additional positive control of \textit{M.\textit{smegmatis}} DNA was added to the PCR as its sigA sequence, along with the \textit{M.\textit{tb}} sequence, was used to design the primers. In addition, the \textit{M.\textit{smegmatis}} cspA gene had the closest sequence identity to the partial gene sequence of both \textit{M.\textit{vaccae}} strains. Thus, a primer that produced amplicons with \textit{M.\textit{smegmatis}} DNA was more likely to generate PCR products with \textit{M.\textit{vaccae}} DNA. The \textit{M.\textit{smegmatis}} DNA was used as a positive control in the following Sigma Factor A PCR reactions.

The MgCl\textsubscript{2} optimisation did not improve the PCR, either no products were generated, or multiple non-specific products were produced. Rather than continuing optimisation with these primers, new primers were designed.
PCR with primers SigA6 to A8

Two new forward (SigA5 and 7) and reverse primers (SigA6 and 8) were created. A PCR was set up with every combination of the old and new primers to try to obtain a PCR product with *M. vaccae* Gm27 and Job5 DNA (Table 7.1, Figure 7.2). The forward primers (SigA1, 5 and 7) had the lowest annealing temperatures, so these determined the annealing temperatures of the 3 PCR reactions.

The PCRs with the SigA1 primers failed to generate any products (data not shown). All of the new primers generated products with the positive controls, however only the SigA5 and A3 combination produced amplicons from both *M. vaccae* Gm27 and Job5 DNA. The remaining combinations generated products with either Gm27 or Job5 DNA. In addition there appeared to be some non-specific binding with the SigA5 with SigA8 and SigA3 combinations, and overamplification was noted in several of the reactions.

PCR with SigA5 and SigA8/A3 primer combinations

To try to increase the specificity of the PCR, a touchdown programme (10 cycles, starting Tm72°C) was added to increase the specificity of the PCR, and prevent non-specific binding the SigA5/A8 and SigA5/A3 primer combinations were repeated. However the specificity of the PCR was now too specific as a product was only generated with *M. tb* DNA, and was overamplified. The PCR was again repeated but without the touchdown programme, however the PCR product obtained with Job5 DNA could not be replicated, and limited amplification was obtained with Gm27 DNA.

PCR with freshly aliquoted primers

Reproducible amplification was proving difficult, the same PCR result was not obtained with repeat PCRs. This was discovered to be attributable to the PCR tubes in which the primers were stored, DNase and RNase free PCR tubes (MBP). The primers were subsequently stored in sterile PCR tubes (MBP). After the realiquoting the primers, the SigA5/A3, SigA5/A8, and SigA7/A3 PCRs
were repeated. PCR products were generated with all of the DNA species with the primer combination SigA5/A3 and SigA7/A3. These amplicons were reproducibly generated in repeated PCR reactions (Figure 7.3). The product sizes of SigA5/A3 and SigA7/A3 amplicons were 646 and 504bp, respectively. The SigA7/A3 primer combination was chosen to represent the housekeeping gene, as it was the smallest PCR product obtained with the *M. vaccae* DNA species, and closer to the size of the *cspA* PCR product.

### 7.3.4 The effect of cold shock on the expression of *M. vaccae* *cspA*

#### 7.3.4.1 Northern hybridisation of *M. vaccae* *cspA* and *sigA* mRNA

The effect of cold shock on the expression of *cspA* mRNA was studied by Northern hybridisation. The hybridisation experiments were carried out with RNA from *M. vaccae* Gm27 only. Time restraints prevented the analysis of both *M. vaccae* strains.

**Cloning of the housekeeping genes**

To ensure that a sufficient quantity of PCR product was obtained for northern hybridisation, the *sigA* (504bp) and *cspA* (143bp) PCR products (partial gene sequences) for *M. vaccae* Gm27 were cloned into the pGEM-T Easy Vector system as described in chapter 4, section 4.15. The *sigA* PCR product was generated from primers SigA7 and SigA3, and the *cspA* PCR product was amplified using primers cspa5 and cspa6 (Table 7.1).

*E. coli* XLI-Blue Competent cells were transformed with the ligated vectors. Kanamycin resistance was used to select transformed bacteria. Successful recombination was determined by plasmid mini-preparation and restriction digestion, as described in methods. Colonies (12) were picked for each insert. EcoR1 restriction digestion determined which recombinations had been successful. The appearance of ~500 and ~150bp bands corresponding to the *sigA* and *cspA* partial gene sequences were evidence of a successful
Figure 7.3: sigA PCR products from primer combinations SigA5/A3 and SigA7/A3

1.5% Agarose/TAE gel stained with SYBR green. From left to right, lane 1, 100bp DNA marker; lanes 2 to 6 SigA5/A3 PCR; lane 2, negative control; lane 3, M.tb DNA (10^{-2} dilution); lane 4, M.smegmatis DNA (10^{-1} dilution); lane 5, M.vaccae Gm27 DNA (10^{-1} dilution); lane 6, M.vaccae Job5 DNA (10^{-1} dilution); lanes 7 to 11 SigA7/A3 PCR; lane 7, negative control; lane 8, M.tb DNA (10^{-2} dilution); lane 9, M.smegmatis DNA (10^{-3} dilution); lane 10, M.vaccae Gm27 DNA (10^{-1} dilution); lane 11, M.vaccae Job5 DNA (10^{-1} dilution).

The expected product size for the SigA5/A3 PCR and SigA7/A3 PCR are 646bp and 504bp, respectively. The PCR in lane 8 is overamplified so that the M.tb product appears to be smaller than expected.
recombination (Figure 7.4). The products were separated on a 1% Agarose/TAE gel, the ~500bp product is clearly visible in on the gels, however the ~150bp band is weakly visible, and is present in at least 10 of the recombinants. A single mini-prep culture was chosen for each insert to be subjected to a plasmid midi-preparation.

To fully characterise both the inserts, restriction digests with a number of different enzymes were carried out. Figure 7.5 reveals the midi-prep restriction digests. The restriction digest patterns for a forward and reverse insert for both inserts are displayed in figures 7.6 and 7.7. Several of the digests produced unexpected patterns, particularly with the sigA insert. The digests revealed that the cspA product had been inserted in the forward position. The sigA digests were inconclusive, and would need sequencing to determine the direction of the insert. However, the plasmid was not sequenced, as this was not essential for the insert to be obtained. Sufficient quantity of each insert was obtained by plasmid maxi-preparation from the cultures chosen for the midi-preparation.

Northern Hybridisation of control and cold shocked total RNA
Cold shock experiments were carried out and total RNA extracted from control, and 1h, 4h, 8h, and 24h cold shocked bacteria. Total RNA (100ng) from each control and cold shock sample was pipetted on to two pieces of nitrocellulose membrane (Hybond N+). Each membrane was hybrised with a DIG-labelled probe for sigA or cspA transcript, as detailed in the methods. Despite repeated attempts no signal could be detected from the sigA or cspA probed blot. The lack of signal may have been due to a lack of RNA, the DIG system could detect levels of RNA at 0.3pg/μl. However, the transcripts may not have been present in such amounts as a large proportion of the RNA detected would have been 16S and 23S rRNA bands. The lack of signal may also have been due to the presence of RNAses that had not been eradicated, although considerable efforts were taken to prevent RNase contamination. Restrictions of time prevented further optimisation of this technique.
Figure 7.4: EcoR1 restriction digest of the recombinants transformed with cspA and sigA PCR products

Lanes 1, 14, 15 and 28, 1kb marker; lanes 2 to 6, 8 to 13, and 21, are cspA recombinants; lanes 7, 16 to 20, and 22 to 27 are sigA recombinants. The location of the 3kb pGem-T vector is marked with the top arrows in the two rows, and the bottom arrow in each row, marks the position of the 143bp cspA insert, and 504bp sigA insert, respectively. The size of each band in the marker is not marked as they are too close together.

Figure 7.5: Restriction digests of midi-preparations of cspA and sigA constructs

From left to right, lanes 1 and 13, 1kb marker; lanes 2 to 6 cspA construct digests; lane 2, Spe/Xmn digest; lane 3, Spe/Dpn digest; lane 4, Nsi/Xmn digest; lane 5, Nco/Xmn digest; lane 6, EcoR1 digest; lane 7, Not1 digest; lanes 8 to 12, sigA construct digests; lane 8, BamH1/Spe digest; lane 9, Nco/Sal digest; lane 10, PvuII/Sal digest; lane 11, EcoR1 digest; lane 12, Not1 digest. The sigA digest in lane 9 failed to digest properly, no banding pattern in evident in this lane.
Figure 7.6: Restriction digest patterns of pGEM-T Easy Vector - cspA construct.

The location of the restriction sites in the Vector and the insert are illustrated at the top, and the pattern of digest fragments for each enzyme pair is illustrated at the bottom. The pattern for the cspA gene product being inserted in a forward or reverse position are shown.
Figure 7.7: Restriction digest patterns of pGEM-T Easy Vector - sigA construct.

The location of the restriction sites in the Vector and the insert are illustrated at the top, and the pattern of digest fragments for each enzyme pair is illustrated at the bottom. The pattern for the sigA gene product being inserted in a forward or reverse position are shown.
7.3.4.2 Real Time PCR of sigA and cspA

To measure the transcript levels of cspA of *M. vaccae* a quantitative RT-PCR assay was developed. In this method, as in traditional RT-PCR, total RNA is used as a template to synthesize cDNA. The amount of cDNA produced for a specific transcript is proportional to the amount present in the original RNA sample, and this is measured by quantitative PCR. In this assay the amount of product is determined in real time using a fluorescent dye, SYBR Green I, which binds double-stranded DNA. SYBR green I dye binds every new copy of double-stranded DNA generated during amplification, an increase in fluorescence intensity is therefore proportional to the increase in PCR product. In real-time PCR, the reaction is characterised by the point in time during the cycling when the amplification of the target is first detected (the threshold cycle), rather than the amount of product generated after a fixed number of cycles. The higher the starting copy number of the transcript the sooner an increase in fluorescence is observed, and the threshold cycle is reached.

Total RNA was extracted from control and cold shocked (1h, 4h, 8h and 24h) *M. vaccae* Gm27 cells. To assess the quality of the RNA, a small aliquot of each RNA was electrophoresed on a non-denaturing agarose gel. The presence of bands corresponding to the 23S and 16S rRNA indicates the integrity of the RNA (Sambrook et al., 1989) Figure 7.8. In addition, to confirm there was no detectable DNA, a basic PCR was carried out with primers for the cspA gene, cspa5 and 6 (see Chapter 6, section 6.3.4, for details). No products were detected with any of the samples. The total RNA was converted into cDNA using random hexamers.

To study the effect of the cold shock on the expression of mRNA, the cDNA was subjected to real time PCR. Standard curves were created for the cspA and sigA transcripts using the control cDNA. A basic PCR was carried out with the sigA primers, SigA3 and A7, and the cspA primers, cspa6 and cspa5. After confirmation that single PCR products had been amplified, the amount of each PCR product was measured. The number of copies of the cspA and sigA transcripts in the cDNA sample were calculated (Table 7.2). The PCR products
Figure 7.8: 23S and 16S rRNA bands of RNA separated on a 2% Agarose/TAE gel

RNA was obtained from control and cold shocked *M. vaccae* Gm27 cells. The presence of the rRNA bands indicated that the extracted RNA was of good quality. From left to right; lane 1, control RNA; lanes 2 to 5 RNA extracted from cold shocked *M. vaccae* cells for; lane 2, 1h; lane 3, 4h; lane 4, 8h; and lane 5, 24h.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length of product (bp)</th>
<th>Average molecular weight of a nucleotide</th>
<th>Relative Molecular mass (rmm)</th>
<th>Amount of PCR product (μg/μl)</th>
<th>n</th>
<th>Avagadros number</th>
<th>copies/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>sigA</td>
<td>504</td>
<td>660</td>
<td>332640</td>
<td>0.04312</td>
<td>1.296 x 10^{-7}</td>
<td>6.02 x 10^{23}</td>
<td>7.81 x 10^{10}</td>
</tr>
<tr>
<td>cspA</td>
<td>143</td>
<td>660</td>
<td>94380</td>
<td>0.01564</td>
<td>1.657 x 10^{-7}</td>
<td>6.02 x 10^{23}</td>
<td>9.98 x 10^{10}</td>
</tr>
</tbody>
</table>

Table 7.2: The number of copies of the *cspA* and *sigA* transcripts in the cDNA sample used to make the real time PCR standard curves.

PCRs were carried out with primers for the two genes of interest, using a cDNA sample generated from control RNA. The amount of PCR product each PCR generated was measured, and the number of copies/μl of each transcript was calculated. Each PCR product was then diluted to 1 x 10^8 copies/μl and then subjected to 1 in 10 serial dilutions to create standard curves for the two genes.
were diluted to a concentration equivalent to \(1 \times 10^8\) copies/\(\mu l\), which was then subjected to serial 1 in 10 dilutions to create a standard curve from \(1 \times 10^8\) – \(1 \times 10^3\) copies/\(\mu l\). The standard curves were linear over four logs of copy number/\(\mu l\) (Figure 7.9 A and B). The control and cold shocked samples were subjected to real time PCR with primers to detect \(\text{sigA}\) and \(\text{cspA}\) transcripts (Table 7.1, Figure 7.10), and the number of copies/\(\mu l\) of each transcript in each sample calculated.

A dissociation protocol was added to the initial real time PCR with both transcripts, to confirm that no primer dimers were being produced, and that a single product was generated (Figure 7.9 C and D). A dissociation protocol consists of a melting curve analysis. The temperature is raised slowly to the melting point of the duplex DNA and the fluorescence monitored. Since SYBR Green I only binds double stranded DNA, the fluorescent signal decreases as the \(T_m\) of the DNA duplex is reached. The gel electrophoresis confirmed the \(\text{sigA}\) and \(\text{cspA}\) real time PCR products were of the expected size.

The \(\text{cspA}\) copy number values were normalised against the corresponding \(\text{sigA}\) values for each sample. Each real time PCR reaction was carried out in duplicate, and the cold shock reaction was repeated.

The \(\text{cspA}\) mRNA expression decreased on cold shock (Figure 7.11). A \(\sim 1.6\) fold reduction in \(\text{cspA}\) transcript was noted within the first hour for cold shock. This level further decreased to a 2-fold reduction after 4h. There appeared to be a slight increase in the level of transcript at 8h, however the \(\text{cspA}\) mRNA level fell to \(\sim 4.5\) fold lower than the 32°C level after 24h.
Figure 7.9: Real time PCR standard curves and dissociation curves for the cspA and sigA transcripts.
Standard curves of the cspA (A) and sigA (B) real time PCR. The dissociation curves for cspA (C) and sigA (D) real time PCR. At the end of each real time PCR, the products are heated very slowly from a low temperature (e.g. 65°C) to a high temperature (e.g. 95°C). At low temperatures, all PCR products are double stranded, so SYBR Green binds to them and fluorescence is high, whereas at high temperatures, PCR products are denatured, resulting in rapid decreases in fluorescence.
Figure 7.10: cspA and sigA real time PCRs, with cDNA made from control and cold shocked M. vaccae Gm27 mRNA.

A, cspA real time PCR, B sigA real time PCR. cspA PCR carried out with primers cspa6 and cspa5. sigA PCR carried out with primers sigA7 and sigA3.

Real-Time PCR is the ability to monitor the progress of the PCR as it occurs (i.e., in real time). Data is collected throughout the PCR process, rather than at the end of the PCR. The Real-Time PCR reactions are characterized by the point in time during cycling when amplification of the target is first detected, rather than the amount of target after a fixed number of cycles. The threshold is represented by the green line in the graphs, and is located in the log-linear phase. The threshold cycle is the cycle at which the amplification plot crosses the threshold, i.e., at which there is the first clearly detectable increase in fluorescence.

Delta Rn – fluorescence, it is the magnitude of the signal generated by the given set of PCR conditions.
Figure 7.11: Effect of cold shock on the level of cspA mRNA

The amount of cspA transcript was determined by real time PCR. The number of cspA transcripts was normalised against the amount of sigA housekeeping gene transcripts. The experiment was carried out in duplicate. The error bars represent the standard error of the mean.
7.4 Discussion

The effect of cold shock on the expression of the level of cspA mRNA transcript was studied by Northern hybridisation and quantitative real time reverse transcriptase (RT) polymerase chain reaction (PCR). To allow the normalisation of the real time PCR data, and to ensure that equal amounts of RNA were loaded onto the Northern hybridisations, an internal control was looked for. By using an internal standard, sample to sample variation is corrected. Unfortunately prokaryotes do not contain glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin, standard housekeeping genes in eukaryotes. So an equivalent housekeeping gene was searched for.

A number of potential genes were examined, including the 16SrRNA gene, which had been used to ensure equal loading of RNA for Northern hybridisations (Mangan et al., 1997; Patel et al., 1991). However, after attending the Acid Fast Club and having conversations with members from other mycobacterial laboratories, it was suggested that the 16SrRNA gene might not be the best housekeeping gene for cold shock. As there was some indication that the mycobacterial RNA genes are affected by cold shock (personal communication Dr P. Jenner). Indeed Afflerbach et al (1998) report a 30% reduction in the transcription of rRNA within 15min of a cold shock form 37 to 20°C. This indicated that the level of 16S rRNA in M. vaccae would not remain constant through cold shock. So an alternative housekeeping gene was sought. A second gene that had been used as a housekeeping gene with experiments in M.tb (personal communication, Dr P. Jenner) also proved inappropriate. Finally, the gene for the major sigma-70 (σ70) factor, Sigma factor A (sigA) was determined to be a suitable housekeeping gene for cold shock experiments. As the M. smegmatis sigA transcript levels were found to be constant during exponential growth (Gomez et al., 1998), and were also constant under different stress conditions, including mild cold shock in M.tb (Manganelli et al., 1999).
To investigate the effects of cold shock on the expression of cspA the level of cspA mRNA was quantified by real time RT-PCR and Northern hybridisation for the first 24h of cold shock. The changes in mRNA expression were studied for the same time frame as the cold shock protein experiments. As changes were noted in the protein expression profile within the first 24h, particularly in the expression of a putative CspA protein (Chapter 5). Unfortunately the Northern hybridisation experiments failed to generate any results, probably due to insufficient amounts of RNA.

By quantitative real time RT-PCR, the copy number per μl of cspA and sigA mRNA were obtained for control and cold shocked samples. The cspA value was normalised against the housekeeping gene (sigA) values to compensate for any sample to sample variation. Unexpectedly a decrease in the level of cspA was noted on cold shock (Figure 7.11). An immediate decrease in the cspA transcript was noted, which further decreased during the 24h period studied. These findings are contrary to those in E. coli, B. subtilis, and more recently in M. smegmatis. In E. coli, a 30 to 50 fold increase in cspA mRNA levels within the first hour of a 37 to 15°C cold shock (Goldenberg et al., 1996). Similarly an increase in the mRNA transcripts of the B. subtilis major cold shock protein genes, cspB and cspC, was noted on cold shock (Kaan et al., 1999; Willimsky et al., 1992). In addition, a 16 to 24 fold cold shock inducing an increase in cspA mRNA was reported in M. smegmatis (Shires et al., 2002). The reason for the difference in the expression pattern in M. vaccae is unclear.

Notably, the experiments carried out with B. subtilis and M. smegmatis were normalised against total RNA (Kaan et al., 1999; Shires and Steyn, 2001), and the E. coli experiments were normalised against the ribosomal RNA gene, rrn (Goldenberg et al., 1996). Shires found that the sigA gene was an unreliable housekeeping gene, as the level of its transcript fluctuated during cold shock experiments with M. smegmatis (personal communication). Furthermore, when the level of sigA transcripts, under different stress conditions in M. tb (including cold shock), was measured, only a single measurement was taken (Manganelli et al., 1999). So, any fluctuations in sigA mRNA would not be detected. Thus,
the decrease in *M. vaccae* cspA mRNA can only be taken as a decrease relative to the sigA level, and may not be a true reflection of what happens under cold shock. This may explain the different expression pattern for cspA in *M. vaccae* and *M. smegmatis*.

Interestingly, no increase in the *M. tb* cspA transcript was apparent on cold shock, however, cspA was maximally expressed during stationary phase (Shires et al., 2002). It is possible that the pattern of cspA expression in *M. vaccae* is similar to *M. tb*, although the overexpression of a putative CspA homologue on cold shock suggests that this is not the case (Chapter 5). The protein, was clearly detectable during exponential growth at 32°C, and was upregulated within the first 4h of cold shock to 6°C. The increase in the transcripts of the major cold shock protein genes in *E. coli* and *B. subtilis* were shown to match the increase in the expression of the protein, indicating that the increase in the protein expression was the result of an increase of the gene transcript (Graumann et al., 1996; Jiang et al., 1993; Kaan et al., 1999). There was no evidence of translational regulation. Furthermore, no significant induction in the level of *M. tb* CspA was noted on cold shock, which matched the pattern of cspA cold shock expression (Shires et al., 2002). Thus, the detected increase in the *M. vaccae* CspA protein was expected to be the result of an analogous increase in the cspA transcript. Although it is possible that the putative CspA protein is not a CspA-like protein but another small protein induced by cold shock. Confirmation of this protein as a CspA-like protein would determine whether the gene and protein expression profiles were contradictory.

The expression of the major cold shock protein genes in *E. coli*, *B. subtilis* and *M. smegmatis* appear to be regulated at the post-transcriptional level. The *E. coli* cspA gene is constitutively transcribed at all temperatures, however its expression at 37°C is prevented by the destabilisation of its mRNA (Fang et al., 1997). The half-life of *E. coli* cspA at 37°C is approximately 10sec, upon a shift to 15°C the mRNA becomes highly stable with a half-life of ~10min (Goldenberg et al., 1996). The increased level of mRNA on cold shock, is predominantly
caused by the mRNA stabilisation, although there is a minor increase in the transcription of \( \text{cspA} \) (Goldenberg et al., 1996). A similar situation exists in \( B.\text{subtilis} \), the \( \text{cspB} \) and \( \text{cspC} \) mRNAs are dramatically stabilised after a 37 to 15°C cold shock (Kaan et al., 1999). Although, like the mycobacterial CspA proteins, and unlike \( E.\text{coli} \) CspA, \( B.\text{subtilis} \) CspB is present in substantial amounts at 37°C (Graumann and Marahiel, 1996; 1999). The \( \text{cspB} \) and \( \text{cspC} \) transcripts have a longer half-life, of 1 min, at 37°C than \( E.\text{coli} \) \( \text{cspA} \) mRNA, which increased to around 30 min on cold shock (Kaan et al., 1999). Furthermore, Shires and co-workers (2002) revealed that the \( M.\text{smegmatis} \) \( \text{cspA} \) transcript had a greater stability on cold shock, indicating a post-transcriptional regulation of \( \text{cspA} \) expression. It is feasible that the transcription of \( \text{cspA} \) decreased on cold shock but that the half-life of the \( M.\text{vaccae} \) \( \text{cspA} \) transcript increased, enabling the increase in CspA expression. Indeed Kaan et al (1999) noted a 30-fold increase in the stabilisation of \( \text{cspB} \) and \( \text{cspC} \) transcripts, but only a 4-fold increase in the level of the \( \text{cspB} \) and \( \text{cspC} \) mRNA was recorded. They stated that the transcription of \( \text{cspB} \) and \( \text{cspC} \) had decreased, not increased on cold shock. It is possible that the severe effect on the translational machinery means that sufficient transcript is present to allow the overexpression of CspA. It would be interesting to compare the mRNA levels of a heat shock protein gene and \( \text{cspA} \) on cold shock on \( M.\text{vaccae} \). Heat shock proteins have been reported to be downregulated on cold shock, and the level of \( B.\text{subtilis} \) heat shock gene \( \text{groEL} \) transcript decreases on cold shock (Kaan et al., 1999). Comparison of the \( M.\text{vaccae} \) \( \text{cspA} \) expression with that of a heat shock protein would determine whether the decrease in \( \text{cspA} \) expression was due to a decrease in total transcription on cold shock, or the reduced transcription of \( \text{cspA} \). A greater fall in the Hsp gene transcript on cold shock would be expected if the fall in \( \text{cspA} \) mRNA was due to a downregulation of transcription.

The apparent discrepancy in the expression of \( \text{cspA} \) in \( M.\text{vaccae} \) and \( M.\text{smegmatis} \) is difficult to explain. Shires and co-workers used the same conditions as their initial investigation into the cold shock response of \( M.\text{smegmatis} \) (Shires and Steyn, 2001)(personal communication Dr. K. Shires),
and the culture conditions were less stringent than those employed in the experiments described here with *M. vaccae*. The more stringent conditions may have had a greater effect on transcription in *M. vaccae*. Furthermore, although the drop in temperature between *M. vaccae* and *M. smegmatis* was comparable, (27°C), the actual cold shock temperature was lower in the *M. vaccae* experiments (6°C compared to 10°C). It’s feasible that this temperature is closer to the minimum temperature of growth for mycobacteria, and has a greater detrimental affect on transcription and this may have affected the levels of *sigA* transcript. Moreover, the growth rate of *M. vaccae* was found to slow by 90 to 100 fold on cold shock (Chapter 3), compared to 50 fold for the comparable *M. smegmatis* experiments (Shires and Steyn, 2001).

Revaluation of the RNA extraction process (RNeasy kit (Qiagen)) indicated that it was not the best method to obtain mRNA species that represented transcription under non-shocked and cold-shocked conditions. The extraction method used relied on a process of enzymatic cell lysis, which has been reported to profoundly alter the gene expression (mRNA levels) (Mangan et al., 1997). Furthermore the extraction process was probably too slow as the half-life of bacterial mRNA is generally extremely short (average one and a half minutes) (Watson et al., 1987a). A cell disruption method with a reagent RNAzol™ B (Biogenesis) containing guanidium thiocyanate and phenol, was previously used to obtain pure undegraded RNA, however inexplicably this method stopped being effective, and undegraded RNA could not be obtained, despite considerable investigation into the problem. So an alternative method was sought, and although it appeared that good quality RNA could be obtained with this method, it seems likely that the mRNA species obtained were not a true reflection of the situation within the control and cold shocked cells.

Interestingly the major cold shock protein genes, *cspB* and *cspC*, of *B. subtilis* are induced during cold shock and stationary phase (Graumann and Marahiel, 1999). Graumann and Marahiel (1999) proposed that the inactivation of ribosomes is the main trigger for the post-transcriptional induction of the CspA-like proteins in bacteria. They stated that the relative increase of CspA-like
proteins allowed the continuation of transcription/translation at a basal level. So it appears that mycobacteria induce the expression of CspA under conditions that cause the inactivation of ribosomes, although it would appear that the fast-growing species induce CspA expression under conditions of cold shock, whereas the slow growing species induce CspA expression during stationary phase. The reason for this differential expression is unclear. *E.coli* has a family of nine CspA-like proteins, with different functions. Proteins CspA, CspB, CspG and CspI are cold-shock inducible, whereas protein CspD is induced during stationary phase (Yamanaka et al., 1998; Yamanaka, 1999). *M.tb* contains a second CspA-like gene, with an apparent C-terminal tail attached to the CspA-like protein. It's possible that this protein is induced under cold-shock conditions. However, the protein has low homology with the other CspA genes in mycobacteria, and the rest of the prokaryotes.

The expression of *M.vaccae cspA* appears to be downregulated on cold shock. However the RNA extraction system, housekeeping gene, and culture conditions need further investigation before it can be determined whether cold shock results in a *M.vaccae cspA* expression profile contrary to that of *M.smegmatis* and comparable to *M.tb*. Furthermore, elucidation of the expression of *M.vaccae* CspA on entry into stationary phase would further resolve this issue.
Chapter 8

8. Evaluation of Immunoglobulin G (IgG) response to *M. vaccae* cold shock proteins in healthy and vascular diseased individuals

8.1 Introduction

The heat shock proteins are one of the most conserved genetic elements, with considerable homology between bacterial and eukaryotic proteins, including those of man (Neidhardt et al., 1984). The conservation of the heat shock proteins makes them an attractive target for the immune system, as many bacterial and parasitic organisms carry these highly homologous proteins. Indeed members of the hsp70 and hsp60 heat shock protein families are major targets for many parasitic, viral and bacterial infections (Kaufmann, 1990).

Immune responses to heat shock proteins (Hsps) have been reported in autoimmune disease. Circulating antibodies to hsp90 have been reported in systemic lupus erythematosus (SLE) (Lamb et al., 1989). In addition, IgG and IgA antibodies that bind mycobacterial hsp70 and hsp65 have been reported in the peripheral blood and synovial infiltrates of patients with RA, but not in normal individuals (Young, 1990b). Increasing evidence implicates the heat shock proteins in the development of atherosclerosis, the development is believed to have an autoimmune component (Wick et al., 1995; Wick et al., 1997; Xu and Wick, 1996). Similarly there is increasing evidence that the Hsps are involved in the evolution of several autoimmune diseases, probably due to the high sequence homology among the Hsps.

The discovery of the cold shock response, and the cold shock proteins, one of which (CspA) has also been found to be conserved from bacteria to man, opens the possibility that a comparable situation exists i.e. that CspS may also be involved in the development or progression of a number of autoimmune diseases. Several vascular diseases result in a reduced blood flow, which may result in a reduction in temperature in the surrounding cells and tissues, where
potentially a cold shock response could occur. So the presence of circulating antibodies to *M. vaccae* Csps in sera from individuals suffering a number of vascular diseases was investigated. Please refer to Chapter 2, section 2.3 for a more detailed introduction.

### 8.2 Materials and Methods

The Western blotting technique was employed to ascertain the presence of immunoglobulin G (IgG) to mycobacterial cold shock proteins in the sera of healthy individuals, and those suffering a range of vascular diseases. Antibody binding was detected using a chemiluminescent detection system. The experimental procedures are detailed in Chapter 4.

#### 8.2.1 Mycobacterial antigens

Cell lysates were obtained from a mid-exponential phase *M. vaccae* Gm27 culture. Half the culture was cold shocked to 6°C for 24h, as the analysis of the cold shock proteins revealed that the most changes in protein expression were noted in the 24h sample (Chapter 5). The remaining culture was retained at 32°C as the control. The cell lysates were obtained from the control and cold shocked samples, and the extraction procedure is described in the methods chapter (section 4.5.3.1).

#### 8.2.2 Sera

In total 100 patients' sera were obtained (Appendix 2). These sera were kindly donated by Dr Y. Chan and Professor G. Stansby of St. Mary's Hospital, London. In addition a number of control sera were obtained within the Medical Microbiology department. Five patient groups were represented. These were:

1. **Claudicants** – patients with intermittent claudication of the legs. Intermittent claudication is characterised by pain and weakness (lameness) of the leg brought on by walking.
2. **Ischaemicals** – patients with critical ischaemia of the legs, needing urgent surgical intervention. Blood taken pre-operation. Ischaemia, is a low
oxygen state usually due to obstruction of the arterial blood supply or inadequate blood flow leading to hypoxia in the tissue.

3. Aneurysms – patients with abdominal aortic aneurysms. An aneurysm is a sac formed by a weakness of the wall of an artery, a vein, or the heart causing a dilation of the vessel with a thinner wall. The blood was taken pre-operation.

4. Peripheral vascular disease (PVD) – progressive occlusive disease of the arteries that supply the extremities, the arms and legs.

5. Controls – healthy individuals free from vascular diseases.

8.3 Western blot optimisation

Considerable optimisation was required for the Western blot technique. A high background was repeatedly noted. Extensive experimentation was carried out to eliminate the high background.

The literature reports that a high background could be the result of insufficient blocking of non-specific binding sites. The blocking solution used in this study was 5% non-fat milk in PBS. Different blocking agents, bovine serum albumin (BSA), and human serum albumin (HAS) were investigated at different concentrations (0.5 to 10%), and in different buffers; PBS, PBS-Tween, Tris buffered saline (TBS) and TBS-Tween (Appendix 1 for all buffer compositions and concentrations of individual blocking agents). However the 5% non-fat milk appeared the best blocking agent. Higher concentrations of this blocking agent (10%) prevented the detection of any antibody binding.

Non-specific binding of the primary (1°) and secondary (2°) antibody to the membrane was studied. Small squares of the membrane were subjected to the western blot detection method; one square was incubated with the 1° and 2° antibody, while the second square was incubated with the 2° antibody only. There was no binding detectable on either square, which indicated that non-specific binding by the 1° and 2° antibody to the membrane was not the problem. The 1° antibody incubation was carried out in the multiscreen
apparatus (Biorad), which was thoroughly cleaned between each blot to ensure that it was not the source of the high background.

To determine whether the high background was due to a component of the SDS-PAGE loading buffer, the loading buffer was analysed by 1D SDS-PAGE. The 2x SDS-loading buffer was loaded onto a 10% mini-gel, along with a protein sample extracted from a non-shocked control culture of \textit{M. vaccae} grown at 32°C. The gel was electrophoresed, the proteins transferred to PVDF membrane, and subjected to a western blot. Non-specific binding was evident in the lane that consisted of 2x SDS-loading buffer only. Indicating that the 1° and 2° antibodies were binding to a component of the loading buffer.

To ascertain the component of the loading buffer causing the high background, fresh 2xSDS-Page loading buffer was made up with Sigma molecular grade water, in case the MilliQ water was contaminated with mycobacteria. In addition the SDS-PAGE reducing agent was analysed. The reducing agent routinely used for SDS-PAGE, dithiothreitol (DTT), was compared with the alternative, β-mercaptopethanol. SDS-PAGE loading buffer is made up as a stock solution and the reducing agent is added to an aliquot immediately before use. The ‘old’ and ‘new’ loading buffers were made up with both reducing agents, and loaded onto a polyacrylamide gel, along with control protein, the gel was electrophoresed and the proteins electrophoretically transferred to PVDF membrane and western blotted. To examine the loading buffers the ‘old’ loading buffer and control protein were run next to each other repeatedly. There was no evidence of any bands in any of the loading buffer only lanes, except were it had been run on the gel between two control protein lanes. This indicated that the non-specific binding was not due to the loading buffer and that there may have been some overlap between lanes when the multiscreen apparatus was placed on the gel.

It was possible that the serum samples had undergone some degradation, and that the degradation products were non-specifically binding to the \textit{M. vaccae} cell lysates. The sera had been aliquoted into 200µl amounts after collection to
prevent freeze-thaw denaturation. An aliquot of each chosen serum was defrosted when required for the blot, and no serum was defrosted more than twice. It was decided to filter the sera to see if it removed any degradation products, and reduced the background. A control serum was filtered before use. The required amount of serum for the blot was diluted 1 in 2 with PBS and then placed in a 0.22μl centrifugal unit (Amicon) and centrifuged at 4°C at 11600g for ~10min until all the sera had been filtered through the membrane. Figure 8.1 is the first blot with filtered sera; bands are clearly visible on the western blot. The filtering appeared to have removed the background problem. However despite keeping the conditions constant the backgrounds were still very variable.

8.4 Results

Each of the 100 sera was subjected to western blotting with control and cold shocked cell lysates. Each serum was assigned a number and the sera for each western blot chosen by a random number generator. A limited number of western blots were successful because of the problem with high background, bands could only be detected in a limited number of blots, above the background. Nevertheless, antibody binding was detected with 24 sera, and each of the study groups was represented; 3 out of 24 control, 2 out of 13 Aneurysm, 6 out of 19 Claudicant, 6 out of 19 PVD, and 7 out of 24 Ischaemic sera, were found to bind to antigens from \textit{M.vaccae} extracts. Binding was not detectable with the rest of the sera from each group because of high backgrounds, and so only those sera in which any binding could be detected were included in the results, and subjected to statistical analysis.

No differences were noted between the binding of antibodies to control and cold shocked \textit{M.vaccae} lysates. This was not unexpected, as no novel cold shock proteins were detected on cold shock (Chapter 5). In fact, the cold shock proteins were upregulated above the level of expression in the control and therefore the control and cold shocked lysates contained the same protein profile i.e. they contained the same proteins. Furthermore, a selective method of labelling of the newly synthesized proteins was necessary to detect the
Figure 8.1 – Effect of serum filtration on Western blot background.

Western blot of human sera against *M. vaccae* Gm27 control proteins. Two dilutions of each serum $1/50$ and $1/100$ were filtered through a $0.2\mu m$ filter and incubated with the blot. The secondary antibody was an alkaline phosphatase conjugated Goat anti- human IgG ($1/3000$ dilution). Bound antibodies were detected with ECL chemiluminescent detection kit (Amersham Pharmacia Biotech).

From left to right; lanes 1 and 2, sera JH (Control); lanes 3 and 4, sera AR (Ischaemic); lanes 5 and 6, sera AS (Control); lanes 7 and 8, sera LMM (Claudicant); lanes 9 and 10, sera MK (Ischaemic).
upregulation in Csps, so it was unlikely that antibodies would have been able to
detect such small changes in the levels of the cold shock proteins.

The size of the antigens bound by the serum IgGs was calculated. The position
of each ECL (enhanced chemiluminescence) molecular weight marker was
marked on the fluorograph, and the relative mobility ($R_f$) of each protein was
calculated. The $R_f$ of the markers was plotted against $\log_{10}$ molecular weight,
and using linear regression analysis (Appendix 2) the equation of the standard
curve was obtained. This was used to calculate the molecular weight of the
bound antigens. A standard curve was generated for each blot.

$R_f$ is given by:

\[
\frac{\text{Distance migrated by protein}}{\text{Distance migrated by solvent}}
\]

For a number of sera it was difficult to identify the size of the antigen being
bound, as a smear was seen on the fluorograph rather than a band (evident in
figure 8.1). In addition, not all the ECL markers were detected on 1 blot, which
made the 95% confidence intervals for molecular weight calculations
significantly larger, than for the other standard curves.

IgG antibodies from all of the study groups bound antigens of a wide range of
molecular weights (Figure 8.2). Unfortunately, as so few western blots yielded
positive results it was difficult to determine any binding propensity of the
antibodies of the vascular disease sera, for specific M.vaccae antigens. In
particular, only three of the 24 control sera blots showed any antibody binding.

Comparison of the M.vaccae Csps, and the antigens bound by serum
antibodies, revealed that a number of the antigens shared the same molecular
weight as the cold shock proteins, and were potentially Csps (Table 8.1).

However binding of antibodies by multiple sera was only detected for the 42-
44kDa antigen, see below.
Figure 8.2: *M. vaccae* proteins bound by Immunoglobulin G antibodies in the sera of healthy controls, and individuals with a number of vascular diseases.

The vascular disease column contained the pooled data from all the vascular disease groups. The arrowheads mark apparent clusters of antibody binding; the T-bars mark putative heat shock proteins. PVDs = peripheral vascular disease.
Table 8.1 - Comparison of the putative Csps of *M. vaccae* and the antigens bound by IgG antibodies from the sera of healthy controls and individuals with vascular diseases.

*sera from the PVD, Claudicant and Aneurysm sera groups contained antibodies to this antigen.

Note only those sera from which antibody binding could be detected are included.

The vascular disease groups, Claudicants, and PVDs, appeared to have a marker antigen, which was bound by a proportion of the group sera (Figure 8.2). Half the sera (3 out of 6) from those individuals with PVDs bound an antigen of 42-44kDa. Of the Claudicant sera, 4 out of 7 sera bound to an antigen with a calculated molecular weight of 49-51kDa. The results were subjected to a Fischer exact test analysis (Appendix 2). The presence of antibodies in the PVD and claudicant sera to the 42-44kDa and 49-51kDa antigens, respectively, was not significant. Nevertheless, as the background problems allowed the detection of antibody binding in such a small number of sera for each disease group, considerably more sera would need to be tested to determine whether the antibodies to these antigens, were true markers of the diseases.

Pooling the results for the disease sera revealed that the antibodies to the 42-44kDa protein also exist in the other vascular disease sera, and suggests that this antigen may be a marker for vascular disease in general (Figure 8.1). Statistical analysis (Fischers exact test, Appendix 2) however revealed that this antigen binding was not statistically significant. Although it should be noted that the low number of control sera (3), in which antibody binding could be detected above the background level, significantly affects the reliability of the statistical analysis. Interestingly, it's possible that this antigen is a cold shock protein. Cold shock proteins were identified in *M. vaccae* (Chapter 5) with estimated molecular weights of 41.4 – 43.5kDa and 44.1 – 46.7 kDa. It would appear that
the antibodies were binding to the smaller of the two proteins, however it remains possible that they were binding to both. A Csp of ~50kDa was not been identified during these experiments, which would indicate that the Claudicant marker antigen may not be a Csp.

Antibody binding was also detected in some control and vascular disease sera to antigens of molecular weights around 65 and 70kDa (Figure 8.1), the size of two of the major cold shock protein families.

8.5 Discussion

The western blotting indicated that there were humoral immune responses to *M. vaccae* cold shock proteins. A propensity of binding to two *M. vaccae* antigens, with apparent molecular weights of 42 - 44kDa and 49 – 51kDa, was identified in 50% of the sera of the PVD and Claudicant groups, respectively. However, the binding by the Claudicant sera and the PVD sera, was not statistically significant (Appendix 2). Moreover, pooling of the vascular disease sera results revealed that the 42 – 44kDa antigen was bound by 6 of the 21 (28.6%) sera in which binding was detected. This was not found to be statistically significant (Appendix 2), although the low number of results from the control group (3 out of 24) limits the value of the statistical results. The analysis of more sera is needed to determine if an antibody to this antigen, is a marker for vascular disease. It is possible that this protein is a common mycobacterial antigen that many individuals have antibodies to.

It is interesting to note that the bound 42-44kDa may be a cold shock protein (Csp) of *M. vaccae*. This suggests that an antibody to a potentiaial *M. vaccae* Csp may be a marker for vascular disease. Whether the presence of a Csp-antibody bears a causal relationship in vascular disease formation would need considerable further investigation.

Interestingly antibody binding to proteins of ~65 and ~70kDa were noted in the vascular disease and the control sera. It is possible that the sera may be binding to two of the major heat shock proteins of *M. vaccae* (Figure 8.1),
although these proteins have yet to be identified as Hsps. The Hsps have been reported to be the major targets of the humoral and cellular immune system (Lamb et al., 1989; Young, 1990b), so it would not be surprising if these antibodies were found to be directed against heat shock proteins. Especially as elevated levels of hsp70 antibodies have been recorded in the sera of individuals with various vascular diseases including, Ischaemia, Claudication and Aneurysms (Chan et al., 1999).

Eukaryotic organisms contain multiple hsp70 family members, whereas prokaryotes had previously been reported to contain a single hsp70 protein (Seaton and Vickery, 1994). Notably a cold inducible hsp70/DnaK homologue (Hsc66) has been reported in E.coli (Lelivelt and Kawula, 1995; Seaton and Vickery, 1994). Heat shock protein homologues that are constitutively expressed and not upregulated on heat shock, are called heat shock protein cognates (Ang et al., 1991). The E.coli hsc gene, encoded a protein (~66kDa) with ~40% identity to DnaK, which did not contain the heat shock promoter consensus sequence (Seaton and Vickery, 1994).

Hsc66 is induced approximately 11-fold, 3h after a 37 to 10°C cold shock (Lelivelt and Kawula, 1995). It has been speculated that Hsc66 acts as a molecular chaperone aiding the cell in recovery from cold shock stress (Lelivelt and Kawula, 1995). Moreover, additional hsp70 proteins involved in cold acclimation have been reported in Spinach (Spinacia oleracea) (Anderson et al., 1994), and two hsp70 cognates important for growth at low temperatures have been reported in the yeast Saccharomyces cerevisiae (Craig and Jacobsen, 1985). Furthermore, the expression of these two proteins was repressed on heat shock, supporting the idea that they may be cold shock proteins. The heat shock and cold shock responses have been found to be antagonistic in bacteria. The expression of heat shock proteins is repressed on cold shock, and the expression of cold shock proteins is downregulated on heat shock (Jones et al., 1987; Jones and Inouye, 1994).
It's possible that *M. vaccae* contains a cold shock inducible Hsp70 cognate. Although, only a single Hsp70 protein has been reported in *M. tb*. Nevertheless, the study into the cold shock response in *M. vaccae* has not revealed an upregulation in ~70kDa proteins (Chapter 5). Although, the characterisation of the Csps was incomplete, the response needs further analysis by 2D-electrophoresis. It would be interesting to note, whether antibodies to Hsp70 were cross-reactive to cold-induced heat shock protein cognates. Circulating antibodies to Hsp70 have been detected in a number of diseases including systemic lupus erythematosus (SLE) (Minota et al., 1988), and vascular disease (Chan et al., 1999). It is not clear as to which Hsp70 epitopes these antibodies bind, whether they are specific for heat shock proteins only, or whether they are shared with the non-heat shock inducible heat shock protein cognates. It is possible that antibodies circulating to Hsp70, are directed against heat shock protein cognates, which opens the possibility that a cold shock inducible protein, and not a heat shock inducible protein is being targeted. However, antibodies raised against *E.coli* DnaK/hsp70 could not detect Hsc66 on western blots, indicating that the major epitopes present in DnaK were not conserved in Hsc66 (Seaton and Vickery, 1994).

No antibody binding to proteins less than 15kDa in size was recorded, showing that there were no antibodies to the small Csps identified in *M. vaccae* (Chapter 5), in particular there appeared to be no antibodies to the CspA homologue, the major cold shock protein of *E.coli* and other prokaryotes. This may be due to limitations in the SDS-PAGE separation, and western blotting technique. The gel percentage employed was suboptimal for small protein separation. Furthermore, these proteins would have been located at the base of the gel and in the blotting apparatus employed they may not have been exposed to the sera during immunoblotting. In addition, although it is unlikely, it is conceivable that the sera may contain Immunoglobulin (Ig) M or IgA antibodies directed against CspA, which were not looked for in this study. It was thought that the conservation of CspA across the bacterial kingdom would make CspA an attractive target for the immune system. The heat shock proteins are major targets for the immune system. In fact the “Common antigen”, an
immunodominant protein shared by most bacteria was determined to be hsp60 (Shinnick et al., 1988). No immune responses to a putative CspA-like protein were identified during this study. However, further investigation is required to confirm whether humoral responses to CspA are present in the sera of individuals with vascular disease.

Fascinatingly, humoral and cellular immune responses to CspA of Methicillin resistant *Staphylococcus aureus* (*S.aureus*) (MRSA), and *M.tb* have been reported, respectively, from animals and humans infected with these organisms. Antibodies (IgM, G and A) to *S.aureus* CspA have been recovered from the sera from a number of individuals suffering MRSA sepsis, these antibodies were absent in the sera of healthy individuals and those individuals only colonized, not infected, with the organism (Lorenz et al., 2000). *M.tb* CspA was recognised by T-cells of infected mice and guinea pigs, but not by T-cells of naïve animals (Weldingh et al., 2000). These findings indicate that immune responses to CspA from *M.tb* and MRSA are generated on infection. The presence of anti-CspA antibodies and T-cells in MRSA and *M.tb* infections, respectively, and the high conservation of the CspA protein in prokaryotes indicates that this protein may be an important target for the immune system.

The process by which CspA becomes available to the immune system is unclear. Lorenz et al (2000) noted that MRSA CspA has a cytosolic location, though it is also found as an MRSA extracellular protein (Lorenz et al., 2000). Moreover, *M.tb* CspA is found in the short-term culture filtrate, although it lacks a signal peptide sequence for secretion (Weldingh et al., 2000). It has been suggested that this protein appears in the culture filtrate because of autolysis, inevitable in bacterial culture, and that the use of a more sensitive technique allowed its detection (Weldingh et al., 2000). However, it is possible that CspA has an alternative secretory pathway.

Of the 100 sera subjected to western blotting, antibody binding could only be detected above the background with 24 of these sera, and these blots were said to be successful. The reason for the low success rate, and the source of the
high background is still unclear, despite repeated attempts at optimisation. It is possible that the sera had become degraded, and that degradation products within the sera were non-specifically binding to the *M. vaccae* cell lysates. It is also feasible that the cell lysates contained glycolipids, and other cellular components, which caused non-specific antibody binding. The difficulty in reproducing western blots would also indicate that there was a problem with the electrophoretic transfer process, although the protocol remained the same throughout the study. As the cold shock proteins of *M. vaccae* had not been identified and purified the screening of sera against the entire proteome seemed the most appropriate way to detect antigens to potential Csps. A more efficient screening method is necessary to allow the humoral response to *M. vaccae*’s Csps to be studied.

The study revealed antibody binding from the sera of healthy controls, and individuals with various vascular diseases to a wide-range of *M. vaccae* proteins. Antibody binding was detected to two antigens with sizes matching the identified Csps of *M. vaccae* (Table 8.1, Chapter 5). This indicates the presence of circulating antibodies to mycobacterial/*M. vaccae* Csps in sera from healthy controls, and individuals with vascular diseases. Antibody binding from multiple vascular disease sera was found for only one of the Csps. Binding to this antigen was detected in 29% of the vascular disease sera with successful western blots, indicating a trend in the antibody binding. However the limited success of the western blotting means further analysis is needed to determine if there is a true binding propensity. The study was inconclusive, and needs expansion to ascertain whether an immune response to cold shock proteins is involved in circulatory disease. Identification and purification of the *M. vaccae* cold shock proteins will enable the presence of cold shock protein antibodies to be thoroughly investigated.
Chapter 9

9.0 Conclusions, speculations and future work

9.1 Effect of cold shock on the growth of *M. vaccae*

All organisms respond to potentially damaging conditions by producing a stress response. The most well known and best characterized of these responses is the heat shock response. More recently the focus has turned to the cold shock response. A cold shock response has been reported in a wide range of bacteria, and has been extensively studied in *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*). In addition a response has been recently reported in *Mycobacterium smegmatis* (*M. smegmatis*). To further characterize the mycobacterial cold shock response, the effects of a cold shock (32°C to 6°C) on liquid cultures of *Mycobacterium vaccae* (*M. vaccae*) were studied. The *M. vaccae* cold shock response was comparable to the response of *M. smegmatis* and analogous to that of *E. coli* and *B. subtilis*.

The *M. vaccae* cold shock response was studied by monitoring the effect of cold shock on the growth and protein profile of the organism. The response was variable, there was some evidence that the cold shock synchronised the cultures, however, the overall response was a dramatic increase in the generation time of the two strains of *M. vaccae*, Gm27 and Job5, studied. Both strains continued to grow at 6°C with estimated generations times 90-100 fold longer than at 32°C (Chapter 3). A similar increase, 50 fold, was noted when *M. smegmatis* was cold shocked from 37°C to 10°C (Shires and Steyn, 2001). Notably, there was no evidence of a lag in growth as reported for *E. coli* (Jones et al., 1987) and *M. smegmatis* (Shires and Steyn, 2001). Comparable to the response of *B. subtilis* (Graumann et al., 1996), growth continued but with an increased generation time.

The effect of cold shock on the growth of *M. vaccae* needs further investigation, due to its variable response. Moreover the study period needs to be extended to confirm the estimated generation times. These were calculated by
extrapolating from the growth noted during the study period. Notably, the generation time of *M. smegmatis* increased with increasing time in the cold (Shires and Steyn, 2001), and it would be interesting to discover if a similar response occurred with *M. vaccae*.

9.2 Cold shock proteins of *M. vaccae*

A drop from 32°C to 6°C, had a profound effect on protein translation in *M. vaccae*. Similar to *E. coli* (Shaw and Ingraham, 1967), cold shock resulted in a rapid reduction in protein synthesis and a 10-fold reduction in the amount of $^35$S-methionine incorporated into newly synthesised proteins (Chapter 5). Moreover, a lag in protein synthesis of between 1 and 3h was observed following cold shock. These results were similar to those found with *M. smegmatis* (Shires and Steyn, 2001) and are perhaps equivalent to the lag in growth recorded for *E. coli*.

The bacterial cold shock response, including that of *E. coli*, *B. subtilis* and *M. smegmatis* (Graumann et al., 1996; Jones et al., 1987; Jones et al., 1992a; Shires and Steyn, 2001), is characterized by the induced synthesis of a number of cold shock proteins. The results of the 1D and 2D SDS-PAGE protein analysis of the cold shock response in *M. vaccae* revealed that at least 12 proteins were upregulated within the first 24h of cold shock (Chapter 5). These proteins demonstrated a minimum of 2-fold cold shock induction in their synthesis levels. Unlike *E. coli* and *B. subtilis*, and similar to *M. smegmatis* and *Arthrobacter globiformis* (*A. globiformis*) (Berger et al., 1996), the number of proteins synthesised by *M. vaccae* did not decrease on cold shock, although, there was a significant reduction in total protein synthesis. Moreover, no novel proteins were detected on cold shock.

The induced proteins could be divided into two groups by the nature of their expression, namely, transient and continuous. Those proteins transiently induced were termed cold shock proteins, whereas those proteins that were continually induced throughout the cold shock period, which were apparently
essential for continued growth in the cold, were termed cold acclimatisation proteins (Caps) (Chapter 5).

The molecular weight of the Csps identified by 1D SDS-PAGE was established and the major Csp of \textit{M. vaccae} was determined to be a putative CspA homologue (Chapter 5), comparable to the major Csps of \textit{M. smegmatis} and \textit{E. coli} (Jones et al., 1987; 1992a; Shires and Steyn, 2001). Comparison of the Csps of \textit{M. vaccae} and \textit{M. smegmatis} indicated that they are similar. However, there was one notable exception, a 27kDa protein, CipMa, was upregulated on cold shock in \textit{M. smegmatis} and was shown to be crucial for cold shock adaptation. A protein of a similar size was not upregulated in \textit{M. vaccae}, indicating that there are differences between the cold shock responses of these two fast growing mycobacteria.

The 2D SDS-PAGE analysis yielded limited information. Time restraints prevented the full analysis of the protein expression. Insufficient radioactivity was incorporated into the proteins of the cold shocked sample, making identification of differences difficult. The radiolabelling technique would need to be modified to allow the incorporation of more radiolabel into the proteins, to ensure that the full cold shocked protein profile could be identified. Moreover, only one time point was analysed; to fully characterize the \textit{M. vaccae} cold shock response more time points need to be studied. Analysis of the cold shock response by 2D SDS-PAGE revealed a different time frame of Csp induction to that indicated by 1D SDS-PAGE in \textit{M. smegmatis} (Shires and Steyn, 2001).

Interestingly the CspA protein of \textit{M. tb} and Methicillin resistant \textit{Staphylococcus aureus} (MRSA) was found in culture filtrates (Lorenz et al., 2000; Weldingh et al., 2000). Analysing the \textit{M. vaccae} culture filtrates from control and cold shocked cultures may reveal secreted Csps that are upregulated on cold shock and missed in this analysis.

This study has started to characterise the Csps of \textit{M. vaccae}. Confirmation that the \textit{M. vaccae} major Csp is a CspA homologue is required, and further
characterisation of the remaining Csps is needed, through N-terminal sequencing, or Western blotting with antibodies to known proteins, mycobacterial or otherwise. This will help to determine if there are really differences in the Csp complement of the two fast growing mycobacteria. Moreover, identification of the cold shock proteins will provide a better understanding of their role in the cold shock response. Many of the cold shock proteins of *E.coli, Enterococcus faecalis* and *B.subtilis* are involved in restoring the translational capacity of the cell, they are ribosome associated or RNA-binding proteins. It should be noted that mycobacteria have a number of putative homologues to *E.coli*'s Csps. A BLAST search by Baker (1998) revealed that *M.tb* had a number of genes that were apparent homologues to a number of the *E.coli* Csp genes, suggesting that a number of the bacterial cold shock proteins are conserved across the bacterial kingdom, as well as CspA.

Characterizing the mycobacterial cold shock response provides an alternative experimental model with which to study environmentally regulated gene expression. It has been suggested that virulence genes may be coregulated as part of the heat shock regulon (Patel et al., 1991), and it would be interesting to determine if the same were true of cold shock. A cold shock regulon has been suggested in *E.coli*, although unlike the heat shock response a specific sigma factor does not appear to be necessary for cold shock protein induction (Yamanaka, 1999). Transcription of *E.coli cspA* did not require any *de novo* protein synthesis upon cold shock (Etchegary and Inouye, 1999a).

### 9.3 Identification and characterization of the *M.vaccae cspA* gene

For the first time a homologous gene to the *E.coli* major cold shock protein, CspA, was identified in *M.vaccae* (Chapter 6). The gene has also been identified in *M.tb, M.leprae,* and *M.smegmatis* (Benson et al., 2002). The gene was identified using PCR methodologies, and 85% of an expected 201bp gene was obtained. However, to obtain the full gene sequence it may need to be cloned.
9.3.1 Identification of conserved control elements

In addition to the coding sequence, ~140bp of the 5’upstream sequence was obtained. Analysis of the coding and non-coding sequence of the \textit{M.vaccae} and other mycobacterial genes revealed that the sequences shared a number of features with \textit{E.coli cspA} (Chapter 6).

Like \textit{E.coli cspA}, the mycobacterial \textit{cspA} genes were found to have a putative extended promoter with a TGN motif (Yamanaka, 1999), an unusually long 5’UTR, a potential cold box sequence, and a putative downstream box (Chapter 6). The presence of these elements indicates that mycobacteria have a similar control mechanism for the expression of \textit{cspA}. Although, it should be noted that there was no evidence of a second feature of the promoter of \textit{E.coli cspA}, an UP element upstream of the –35 region (Mitta et al., 1997) (Chapter 6).

The UP element and TGN motif enhance the promoters transcriptional strength, making the \textit{cspA} promoter one of the strongest in \textit{E.coli} (Yamanaka, 1999). The promoters of the major Csps of \textit{B.subtilis}, \textit{Lactococcus lactis} (\textit{L.lactis}) (Wouters et al., 1998), and \textit{Salmonella typhimurium} (\textit{S.typhimurium}) (Horton et al., 2000) have also been found to contain an UP element, and a promoter with a TGN motif. The reason for the absence of the UP element in mycobacteria is unclear. The \textit{E.coli cspA} promoter was found to be active at 37°C and 15°C. Thus although the strength of the promoter will determine the amount of transcript produced, it has little control over the expression of CspA at 15°C. UP elements have not been reported in mycobacteria, and it is possible that the lack of similarity between the –35 region of \textit{E.coli} and mycobacterial promoters (Bashyam et al., 1996) may indicate that other elements associated with the –35 region, such as the UP element, are also not conserved. The mycobacterial \textit{cspA} promoter needs to be determined by primer extension analysis, to confirm that the putative promoter identified is the actual mycobacterial promoter.

The stability of \textit{cspA} mRNA plays an important role in the expression of CspA, the control of the mRNA stability has been attributed to the 5’UTR of the mRNA (Brandi et al., 1996; Fang et al., 1997; Goldenberg et al., 1996). The
identification of a putative promoter for the mycobacterial cspA gene indicated that mycobacterial cspA mRNA also had an unusually long 5'UTR, indicating that an increase in mRNA stability could also be responsible for the expression of mycobacterial CspA. Indeed, *M. smegmatis* cspA mRNA increased in stability on cold shock (Shires et al., 2002). Determination of the half-life of *M. vaccae* cspA mRNA at 32°C and 6°C will reveal whether the same mRNA stabilization is involved in the upregulation of *M. vaccae* CspA on cold shock.

A translational enhancement element, the downstream box (DB), was identified in *E. coli* cspA mRNA, which is believed to allow the cold shock induced block in the initiation of translation to be overcome (Mitta et al., 1997). In fact, deletion of the DB sequence from *E. coli* cspA prevented the cold shock induction of CspA (Mitta et al., 1997).

The DB is complementary to bases 1469-1483 within the *E. coli* 16S rRNA, known as the anti-downstream box (ADB) sequence. Identification of the ADB in *M. vaccae* 16S rRNA and analysis of the first 30 bases, from the translational start point, revealed a putative DB sequence (Chapter 6). In addition, a putative DB sequence was also identified in *B. subtilis* cspB (Chapter 6). Notably, the DB of *M. vaccae* cspA appears to be a weaker translational enhancer than the DBs of *E. coli* and *B. subtilis*. The degree of translational enhancement is dependent on the complementarity between the DB and ADB (Etchegary and Inouye, 1999b), and the complementarity was lower for *M. vaccae* than *E. coli* and *B. subtilis* (Chapter 6).

Notably, a DB sequence has also been identified in the rpoH mRNA (gene product for RNA polymerase containing σ^{32}), which is essential for the heat shock induction of rpoH expression (Nagai et al., 1991). Heat shock and cold shock both reduce the translational capacity of cells. The DB sequences may therefore play a crucial role in the translational efficiency of mRNA under numerous stress conditions in which ribosomal function become impaired (Mitta et al., 1997).
Mycobacterial cspA appears to have several of the control elements reported for *E.coli*, leading to the conclusion that the control of cspA expression is similar. It also suggests that the control mechanism for cspA expression is conserved across the bacterial kingdom.

### 9.3.2 Mycobacterial cspA gene

The mycobacterial cspA gene is highly conserved within the mycobacterial genus, with a minimum of 84% sequence identity between the genes of *M.tb*, *M.leprae*, *M.smegmatis* and *M.vaccae* (Chapter 6). The genes encoded proteins with a minimum of 92% sequence identity, revealing that the majority of the gene sequence differences were contained within the genetic code for individual amino acids. The sequence conservation decreased towards the C-terminal end of the protein, indicating that this end of the protein was less important for the structure and function of CspA.

The crystal structure of *E.coli* CspA and *B.subtilis* CspB have been elucidated, as have the structures of the homologous proteins in *Bacillus caldolyticus*, and *Thermotoga maritima*, and the homologous Cold shock domain (CSD) of the human Y-box protein. All of the proteins/protein domains are composed of a β-barrel structure, composed of 5 β-stands connected by loops. The variation between the structures is confined to the loop regions. A theoretical model of CspA of *M.tb*, *M.leprae* and *M.smegmatis* (Weber et al., 2002) (Chapter 6), revealed that the mycobacterial CspA proteins also have this β-barrel structure.

Comparison of the mycobacterial CspA proteins with a number of homologues across the bacterial kingdom and the CSDs of the eukaryotic Y-box proteins, revealed the conservation of a number of amino acid residues crucial for their structure and function (Chapter 6).

Notably, there appears to be an evolutionary relationship between the bacterial CspS and the eukaryotic CSDs (Chapter 6). It seems that the eukaryotic CSDs are of mitochondrial origin. A bacterial parasite *Rickettsia prowazekii* has been identified as the organism more closely related to mitochondria than any other
organism so far studied (Andersen et al., 1998). \textit{R. prowazeki} contains a csp-like gene, which encodes a protein with 56.7\% homology with CspA of \textit{Streptomyces clavuligerus} (Andersen et al., 1998). It has been proposed that the CSD gene was transferred from the mitochondria to the nucleus, and diverged through recombination mechanisms (Yamanaka, 1999). Moreover, a mitochondrial Y-box protein was identified in the eukaryotic protozoan \textit{Trypanosoma brucei} (Haymann and Read, 1999).

Of interest, a second CspA-like protein (CspB) has been identified in the genomes of \textit{M. tb} and \textit{M. leprae} (Chapter 6). Yamanaka (1999) suggested that these proteins were eukaryotic Y-box protein homologues, as along with a CspA-like sequence, they contain an extra C-terminal domain with putative basic/aromatic (B/A) islands, a feature of the C-terminal domain of the vertebrate Y-box proteins (Chapter 6).

There is little sequence identity between \textit{M. tb} CspA and the CspA-like section of CspB suggesting a different function in the mycobacterium. It would be interesting to determine the function of this protein in mycobacteria, and whether the fast growing mycobacteria also have this protein. Homologous proteins were identified in the Actinomycetes \textit{Streptomyces coelicolor A3}, and \textit{Corynebacterium efficiens} and \textit{C. glutamicum} (ATCC13032), indicating that the protein has an important function in this order of bacteria.

### 9.4 Expression of \textit{M. vaccae cspA}

A 37°C to 15°C cold shock results in a 30-50 fold increase in \textit{cspa} mRNA levels in \textit{E. coli} (Goldenberg et al., 1996). A 23-fold increase in \textit{cspa} mRNA levels was recently reported in cold shocked \textit{M. smegmatis} (Shires et al., 2002). Analysis of the expression of \textit{M. vaccae cspa} after a 32°C to 6°C cold shock by quantitative real time RT-PCR detected an unexpected decrease in the \textit{cspa} mRNA level. A \(-1.6\) fold decrease was noted in the first hour that decreased further by \(-4.5\) fold after 24h (Chapter 7). These results contradict those reported for \textit{cspa} expression in \textit{M. smegmatis} (Shires and Steyn, 2001). Moreover, a putative CspA homologue was found to be the major cold shock
protein of *M. vaccae* reaching 11% of the cells total protein synthesis on cold shock (Chapter 6), which would be unexpected if the level of transcript had decreased. It should be noted that the results could not be confirmed by Northern hybridisation, most probably due to insufficient RNA.

The quantified real time PCR values for cspA were normalised against the values for the chosen housekeeping gene, Sigma factor A (SigA). The level of *sigA* was found to be constant through exponential phase in *M. smegmatis* (Gomez et al., 1998). Furthermore, the level of *M. tb sigA* transcript was found to be practically the same in mid-exponential phase, after a mild cold shock, and after a 2h incubation in an ice bath (Manganelli et al., 1999). However, the validity of the sigma factor A as a housekeeping gene was brought into doubt, as the levels were found to fluctuate on cold shock in *M. smegmatis* (Personal communication, K. Shires). Furthermore, the RNA extraction procedure may have affected the levels of cspA. The method employed had an enzymatic digestion step, which has been shown to profoundly alter gene expression (mRNA levels) (Mangan et al., 1997). The expression of cspA needs further investigation, to determine if the apparent downregulation is real or an experimental artefact. In addition, the *sigA* housekeeping gene needs to be looked at, and possibly exchanged for an alternative.

Interestingly, no increase in cspA/CspA expression was detected on cold shock in *M. tb*, although induction was noted on entry into stationary phase (Shires et al., 2002). The major cold shock protein genes, *cspB* and *cspC*, of *B. subtilis* are induced during cold shock and stationary phase (Graumann et al., 1996; 1997; Graumann and Marahiel, 1999). Moreover, one of the family of nine homologous CspA proteins (CspA to CspI) in *E. coli*, CspD is induced during stationary phase growth (Yamanaka and Inouye, 1997), indicating a role for CspA-like proteins in stationary phase.

Graumann and Marahiel (1999) proposed that the inactivation of the ribosome is the main trigger for the induction of CspA-like proteins in bacteria. The relative increase of CspA-like proteins allowed the continuation of
transcription/translation at a basal level. Thus it appears that mycobacteria induce the expression of CspA under conditions that cause ribosomal inactivation. Although it appears that fast growing and slow growing bacteria induce CspA under the different stress conditions of cold shock and stationary phase, respectively. It would be interesting to determine whether CspA is upregulated in *M. vaccae* and *M. smegmatis* during stationary phase. The effect of incubation of mycobacteria, both fast and slow growing, with the antibiotic chloramphenicol, which inhibits ribosome function, would also determine if ribosome inactivation was the trigger for mycobacterial CspA expression. Chloramphenicol has been shown to induce a cold shock-like response in *E. coli* (VanBogelen and Neidhardt, 1990), and in *B. subtilis* (Graumann et al., 1997) including production of the major cold shock proteins.

It would also be interesting to determine if CspA is crucial for cold shock adaptation and for transition into stationary phase in fast-growing and slow growing bacteria, through the creation of knockout mutants. In addition, it would be interesting to find out if CspA affects the expression of any other proteins.

Notably, Weber and co-workers (2001) have suggested a role for the *B. subtilis* major cold shock proteins, CspB and CspC in the coupling of transcription with translation. The Csps are found in the same cellular location as ribosomes, and are believed to influence the structure of the nucleoid. It has been proposed that the coupling of transcription and translation with membrane protein insertion and transport across the cell membrane pulls DNA towards the membrane, decondensing the nucleoid (Weber et al., 2001; Woldringh et al., 1995). The Csps are believed to bind to mRNA during transcription preventing the formation of secondary structures and allowing the initiation of translation (Weber et al., 2001). While there is active transcription the Csps were found to be located around the nucleoid, however when transcription ceased the proteins were found throughout the cytoplasm (Weber et al., 2001). Under conditions that affect the translational capacity of the cell, such as cold shock, the onset of stationary phase, and the incubation with the antibiotic chloramphenicol, it may be that the increased expression of CspB occurs because transcription is
proceeding at a faster rate than translation, as it may not be affected to such a large degree by cold shock. The Csps are needed to prevent secondary structures of the mRNAs being produced, until the translational capacity is restored, and in the case of cold shock, the block in the initiation of translation is overcome.

9.5 Comparison of mycobacterial cold shock responses

The cold shock responses of *M. vaccae* and *M. smegmatis* were similar in some aspects, however there were notable differences in the growth response and in the protein complement identified in the two species. The different cold shock conditions and media in which the experiments were carried out might explain the majority of these differences.

Experiments by previous workers with *M. vaccae* on solid culture, revealed that a 32°C to 6°C cold shock produced the greatest cold shock response (Baker, 1998; Maynard, 1997; Nzula, 1996). This temperature was used during the cold shock experiments here, however the significant effect on the growth rate and the protein translational machinery (Chapter 3 and Chapter 5), would suggest that the experimental temperature was too low. A cold shock of 37°C to 10°C was used for experiments in *M. smegmatis* (Shires and Steyn, 2001), this is a 27°C cold shock compared to the 26°C cold shock used for *M. vaccae*. Although the magnitude of the cold shock was comparable, it’s possible that 6°C was reaching *M. vaccae*’s minimal temperature of growth. This would explain why the protein translational machinery and growth rate was so dramatically affected, and may explain the different responses to cold shock seen between the two species.

Moreover, Sauton’s minimal media was employed during these experiments, so that immunological experiments could be carried out. Rich media contains bovine serum albumin (BSA) and other components would interfere with immunological screening of sera, through methods such as western blotting. Without this intention, the *M. smegmatis* experiments were carried out in the rich medium Middlebrook 7H9. The stringency of Sauton’s media may also have
affected *M. vaccae*’s ability to cope with the cold, and may further explain the differences noted between the two species. Thus it is possible that the fast-growing mycobacterial species respond to cold shock in a comparable way, despite the different experimental findings.

### 9.6 Immunological responses to *M. vaccae* cold shock proteins

Immune responses to the highly conserved heat shock proteins have been implicated in the development of several autoimmune diseases, including Rheumatoid arthritis, Systemic lupus erythematosus (SLE) and Insulin dependent diabetes mellitus. The high conservation of Hsps from bacteria to man allows for the development of cross-reactive antibodies, which recognise ‘self’ proteins. The discovery of the cold shock response in bacteria, and the identification of a cold shock protein with a homologous protein domain (cold shock domain, CSD) in the Y-box proteins of eukaryotes, opened the possibility of an involvement of antibodies cross-reactive to bacterial Csps and eukaryotic proteins. The major heat shock and cold shock proteins in man are encoded in the DNA of mitochondrial origin.

Furthermore an autoimmune component has been identified in the development of the vascular disease atherosclerosis. Many vascular diseases result in the occlusion of blood vessels, and reduced blood flow in the area of the occlusion. Reduced blood flow results in a cooling of the surrounding tissue, as is evident in leprosy sufferers with reduced blood flow to their fingertips and a cooler skin temperature (Abbot et al., 2002). The potential for a cold shock response in cooled cells and tissues exists, and it was possible that antibodies cross-reactive to human and bacterial cold shock proteins were involved in the development or progression of vascular diseases. The study described here (Chapter 8) sought humoral immune responses to the cold shock proteins of the non-pathogenic mycobacterium *M. vaccae*, in the sera of individuals suffering from a range of vascular diseases, including Ischaemia, claudication, peripheral vascular disease and aneurysms, which were absent in healthy controls. The study identified antibody binding to a putative *M. vaccae* cold shock protein, indicating a propensity of binding within the vascular disease sera.
The study was inconclusive, and a potential involvement of an autoimmune response to conserved cold shock proteins remains unproven. The study requires further investigation. Further identification and purification of the *M. vaccae* Csps will aid in the exploration of the humoral immune response to cold shock proteins. Problems of high background were routinely experienced with the Western blotting technique employed in the study. Fractionation of the *M. vaccae* cell lysate, by size, may reduce the background problems, by reducing the number of proteins being screened at one time. Furthermore, carrying out Western blots with two-dimensionally separated proteins, may help identify individual proteins of interest. As bands within one-dimensional electrophoresis gels, can contain several proteins of the same size. Moreover, an electroelution technique has been developed to allow the purification of individual protein species, identified by 2D gel electrophoresis (Weldingh et al., 2000). Purification of the individual *M. vaccae* csps means that they could be used to coat microtiter plates, and Enzyme-linked immunosorbant assays (ELISA) could be used to screen for immunological responses to these antigens. Furthermore, cloning the gene of the desired protein into an expression vector would allow large amounts of the protein to be obtained, and this again could be used to screen antibody responses through ELISA.

The eukaryotic Hsps and the eukaryotic Y-box proteins appear to have similar characteristics, their expression is increased during stress and both have been detected on the cell surface of chemically transformed tumour cells (Rubenstein et al., 2002; Ullrich et al., 1986). Moreover there is an indication that antibodies to Y-box proteins are involved in the immune surveillance of transformed cells (Rubenstein et al., 2002). Plus, humoral and cell mediated immune responses to the homologous bacterial CspA have been reported in infections of humans and rodents with Methicillin resistant *Staphylococcus aureus* (MRSA) and *M. tb*, respectively (Lorenz et al., 2000; Weldingh et al., 2000). This suggests that like the Hsps, CspA is a marker for bacterial infection. Moreover, the Hsps and the CSD of the Y-box proteins are both highly conserved in bacteria and man. Whether the Y-box proteins, or other conserved cold shock proteins have a
comparable role in autoimmune disease, particularly vascular disease, remains to be seen.

9.7 Cold shock, stationary phase, and immunotherapy

The upregulation of the CspA homologues has been reported in a number of bacteria, including *B. subtilis* (Graumann and Marahiel, 1999) and *M. tb* (Shires et al., 2002) during stationary phase. So far no other Csps have been reported to be upregulated during stationary phase, although this has so far not been fully investigated, and the possibility exists that they are.

If Csps build up during stationary phase, it’s highly likely that they will be contained in immunotherapeutic agents. Even Robert Koch’s “brown fluid” (Koch, 1890) was prepared from heat killed tubercle bacilli that had gone into stationary phase in a liquid Sauton’s-like medium. Spahlinger regularly prepared his tubercle bacilli by shutting liquid cultures in a dark cupboard for his immunotherapy. Although Spahlinger certainly knew about heat shock proteins, he did not appreciate the occurrence of cold shock proteins. Spahlinger used heat-shocked tubercle bacilli for the production of horse serum that he claimed was especially beneficial for the treatment of ‘surgical tuberculosis’ (specifically bone and joint disease) rather than pulmonary tuberculosis. Spahlinger’s method of tubercle bacilli preparation means that the bacterium would be in stationary phase (Orme, 2001). Moreover they may have also undergone cold shock, when stored in the cupboard, thus the immunotherapeutic may well have contained cold shock proteins. Friedmann prepared his *M. chelonei* for immunotherapy also as stationary phase cultures (Stanford et al., 2003). Furthermore, the immunotherapeutic vaccine derived from *M. vaccae*, SRL172, and used in clinical trials for the immunotherapy of a whole range of diseases (Hrouda et al., 1998; Lehrer et al., 1998; O’Brien et al., 2000), is a heat killed preparation of stationary phase *M. vaccae* grown at 32°C.

Thus although it remains uncertain whether csps are important for immunotherapy, each of the major successful immunotherapeutics employed
stationary phase organisms, which may have included constituents of csps in them.

The study has started to characterise the cold shock proteins of the fast growing mycobacterium, *M. vaccae*, and to identify any humoral immune responses to these proteins. The cold shock proteins may still have considerable clinical importance whether in autoimmunity, or as constituents of a number of immunotherapies obtained from mycobacteria. Further investigation of the cold shock response of *M. vaccae* may give significant insights into the development of vascular disease, and the important components of a number of successful immunotherapeutics. Furthermore with the recent discovery of a potential involvement of the Y-box proteins in cancer biology the relationship between the bacterial CspA-like proteins and the CSD of the Y-box proteins becomes more interesting and exciting. The existence of a safe immunotherapeutic based on *M. vaccae*, which potentially could be manipulated to be rich in a protein with homology to the Y-box proteins, opens the possibility that the immune response to the Y-box proteins could be modulated in diseases such as cancer, and this could be an important avenue for future investigation.
**Project aims**

- **Characterise the cold shock response in the liquid culture**
  The effect of cold shock on the growth of two strains of *M. vaccae* was determined, although the response was variable and needs further study to confirm the generation time of both strains at 6°C. The effect on protein synthesis was also determined.

- **Identify the *M. vaccae* cold shock proteins**
  A number of cold shock proteins were identified in *M. vaccae*.

- **Determine whether *M. vaccae* had a homologue to the highly conserved Cold Shock Protein A**
  A homologous gene to *E. coli cspA* was identified in *M. vaccae*, and sequenced, although the protein could not definitively be identified.

- **Determine the presence of humoral immune responses to the identified *M. vaccae* cold shock proteins in the sera of healthy controls and individuals with various vascular diseases.**
  The study identified an antibody to a putative cold shock protein, which may have a potential involvement in vascular disease.
References


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Jiang,W., L.Fang, and M.Inouye (1996). The Role of the 5'-End Untranslated Region of the mRNA for CspA, the Major Cold-Shock Protein of Escherichia coli, in Cold-Shock Adaptation. Journal of Bacteriology 178:4919-4925


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Appendix 1

Culture media

1. Sauton's Agar
For 1L; in 200ml of deionised water dissolve by heating, 3% v/v glycerol (Merck), 45mM asparagine (Sigma), 9.5mM citric acid (Merck), 8.6mM di-potassium orthophosphate (Merck), 1.0mM hydrated magnesium sulphate (Merck), 0.005% w/v ammonium ferric citrate (Merck). Allow to cool, make up to 980ml and pH to 6.8 with ammonium solution (Merck). Dissolve 1.2% agar (DIFCO) and autoclave at 121°C, 15lbs for 15min. Cool to 50-56°C, and add 20ml sterile 50% glucose (Merck), filtered through a 0.2μm filter. Distribute 25ml in 100ml sterile plastic containers, and slant.

1.2 Sauton's broth
Repeat the above omitting the agar, and distribute 49ml solution into 250ml Erlenmeyer flasks, add 0.5% v/v tyloxapol, seal with aluminium foil, and autoclave at 121°C, 15lbs for 15min. On cooling add 1ml of sterile 50% glucose to each flask.

2. Middlebrook 7H10 agar
2.1 Basal Medium
For 1L, suspend 19g of Bacto Middlebrook 7H10 agar (DIFCO) in 900ml deionised water containing 5ml of Glycerol (Merck) and boil to dissolve completely. Autoclave at 121°C, 15lbs for 15min. When cooled to 50-55°C, aseptically add 100ml of sterile Middlebrook OADC enrichment.

2.2 Middlebrook OADC Enrichment
Dissolve 0.5g oleic acid8, 50g bovine serum albumin fraction 5, 20g dextrose, 0.04g catalase (Beef), 8.5g sodium chloride in 1l of deionised water. Sterile filter through 0.2μm filter, aliquot into 10-20ml volumes, and incubated at 32°C to determine any infection.
*the oleic acid needs to be dissolved in small volume of sodium hydroxide.
3.1 Luria-Bertoni (LB) Broth
For 1l:
To 950ml of deionised water 10g of Bacto-tryptone (DIFCO), 5g of Bacto-yeast extract (DIFCO), and 10g of NaCl (Merck), was added. The flask was shaken until the solutes have dissolved. Adjust the pH to 7.0 with 5M NaOH (~0.2ml). Then adjust the volume of the solution to 1l with deionised water. Sterilise by autoclaving for 20min at 15lb/ inch², at 122°C.

3.2 LB Agar
To make 1l the method for LB broth was followed, with the addition of 15g of bacto-agar after the solution was pH'd and before the solution was made up to 1l. The agar was sterilised by autoclaving at 15lbs, 121°C for 20min.

3.3 LB ampicillin medium
LB ampicillin broth or agar was made according to the above methods. Once the medium had been autoclaved it was allowed to cool to 50°C before the thermolabile ampicillin was added at a concentration of 100µg/ml.

3.4 Ampicillin stock solution
Ampicillin was dissolved in deionised water at 100mg/ml concentration, filter sterilised and stored at -20°C.

Western blotting solutions
1. Phosphate buffered saline (PBS) pH7.5
For 1l, 11.5g disodium hydrogen orthophosphate anhydrous (80mM) (Merck), 2.96g sodium dihydrogen orthophosphate (20mM) (Merck), and 5.84g sodium chloride (100mM) (Merck) were dissolved, the pH checked, and the volume made up to 1l.
2. Tris buffered saline (TBS) pH7.6
For 1l, 2.42g Tris base (20mM) (Sigma), 8g sodium chloride (137mM) (Merck), and 3.8ml 1M hydrochloric acid (Merck) were dissolved, the pH checked, and the volume made up to 1l.

3. PBS Tween (PBS-T) and TBS Tween (TBS-T)
For 1l, dilute 1ml of Tween 20 (Sigma) in the corresponding buffer to give a concentration of 0.1% Tween 20.

4. Blocking agents and concentrations used

<table>
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<tr>
<th>Blocking agent</th>
<th>Concentrations tested in PBS/PBS-t</th>
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</thead>
<tbody>
<tr>
<td>Low fat milk (marvel)</td>
<td>5% 10%</td>
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<tr>
<td>Bovine serum albumin (BSA)</td>
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</tr>
<tr>
<td>Human serum albumin (HSA)</td>
<td>0.5% 1.0% 1.5% 2% 3%</td>
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</table>

Two-dimensional polyacrylamide gels NIMR

1.0 12% Investigator system polyacrylamide gels, NIMR

1.1 Acrylamide/PDA

Make up a 30% v/v Acrylamide (Pharmacia), 0.8% piperazine diacrylamide (PDA) (Biorad) solution with deionised water. Degas with mixed bed resin TMD-8 (Sigma) and filter out under vacuum through a 0.8μm filter.

1.2 12% polyacrylamide gel
For 720ml (6 gels) mix, 180.48ml 1.5M Tris-HCl pH8.8 (Sigma), 288ml Acrylamide/PDA, 243ml deionised water, and 2.76ml 20% sodium thiosulphate, degas by vacuum filtration and add; 369μl TEMED and 5.54ml 10% ammonium persulphate (APS) (Sigma).

Two-dimensional polyacrylamide gels Ludwig Institute

1. 6-16% gradient gels
Gels were poured in the investigator system, with a gradient maker (Amersham Pharmacia Biotech). For 6 gels, two solution of 9% and 16% acrylamide were made, and placed in the two separate containers of the gradient maker, the
lowest percentage closest to the valve as the investigator system fills from the bottom. The gradient maker is used according to the manufacturers instructions (Amersham Pharmacia Biotech).

<table>
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<tr>
<th>Component</th>
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<tr>
<td>40% acrylamide (Amersham Pharmacia Biotech)</td>
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<td>1.5M Tris pH8.8 (Severn Biotech Ltd)</td>
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<td>MilliQ water</td>
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<td>10% Ammonium persulphate* (Genomic Solutions)</td>
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<tr>
<td>TEMED* (Sigma)</td>
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* the APS and TEMED were added to each chamber just before use.

2. Single percentage (12%) gels

Single percentage gels were poured using the casting chamber for the Biorad Protean ii system. For 120ml (2 gels) mix; 30ml 1.5M Tris pH8.8, 48ml 30% acrylamide (Acrylamide/Bisacrylamide 37.5:1) (AMRESCO), 42ml milliQ water, 0.5ml 10% w/v Ammonium persulphate and 80μl TEMED.

DEPC water

Diethyl Pyrocarbonate (DEPC) (sigma) is an effective nuclease inhibitor. A 0.1% solution is used to inactive RNase (1ml/l solution). Each solution to be treated with DEPC was made up in advance. The appropriate amount of DEPC to give a 0.1% solution, was added to each solution. The solutions were stirred until all the DEPC had dissolved, there was no evidence of globules. The lids were then placed on the containers and the solutions autoclaved for 15min, at 15lbs and 121°C.
Restriction enzyme sites in *M. vaccae* cspA partial gene sequence used in the cloning experiments, for Materials and Methods section 4.15.6

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<td>BstUI</td>
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<tr>
<td>AccII</td>
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The following endonucleases were selected but don't cut this sequence:

sigA - 504 base pairs

Restriction enzyme sites in *M. tb* sigA partial sequence, the equivalent *M. vaccae* was used in the cloning experiments, for Materials and Methods section 4.15.6
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The following endonucleases were selected but don’t cut this sequence:

2.0 Molecular weight standard curves for radiolabelled cold shock proteins (Chapter 6)

Standard curves generated from broad range molecular weight markers (Amersham pharmacia biotech), separated on a 10 to 20% gradient gel. The relative mobility of each protein was calculated and plotted against the log10 Mwt for each standard. As it was a gradient gel, there was not linear relationship for all the markers, so the markers were split into low (A) (75, 50, 35, 30, 25, 15, 14.3, 10kDa) and high (B) (250, 160, 105, 75, 50kDa) molecular weight. To create a graph with normal distribution the Rf value for the high mwt markers was transformed by log10.
### 3.0 Appendices chapter 8

### 3.1 Sera used in the Western blotting experiments

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Western blot standard curves generated with ECL labelled molecular weight markers (Amersham pharmacia biotech). The relative mobility of each standard was calculated and plotted against the log10mwt of each marker. A linear regression analysis was carried out on each curve, and the equation of the line used to calculate the mwt of the antigen bound on the western blots.
3.3 Fishers exact test analysis of antibody binding in the sera of patients with vascular disease

All statistical analysis was carried out with Graph Pad In Stat software

Significance of binding by Peripheral Vascular disease patient's sera to a 42-44kDa antigen of \textit{M. vaccae}

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Two-sided P value = 0.4643, binding not significant

Significance of binding by Claudicant patient's sera to a 49-51kDa antigen of \textit{M. vaccae}

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Two sided P-value = 0.5055, binding not significant

Significance of binding of all the Vascular disease sera to a 42-44kDa antigen of \textit{M. vaccae}, a putative cold shock protein.

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Two sided P-value = 1.000, binding not significant